

# Progress toward the Development of a NEAT Protein Vaccine for Anthrax Disease

Miriam A. Balderas,<sup>a</sup> Chinh T. Q. Nguyen,<sup>a</sup> Austen Terwilliger,<sup>a</sup> Wendy A. Keitel,<sup>a</sup> Angelina Iniguez,<sup>b</sup> Rodrigo Torres,<sup>b</sup> Frederico Palacios,<sup>b</sup> Celia W. Goulding,<sup>b,c</sup> Anthony W. Maresso<sup>a</sup>

Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas, USA<sup>a</sup>; Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California, USA<sup>b</sup>; Department of Pharmaceutical Sciences, University of California at Irvine, Irvine, California, USA<sup>c</sup>

*Bacillus anthracis* is a sporulating Gram-positive bacterium that is the causative agent of anthrax and a potential weapon of bioterrorism. The U.S.-licensed anthrax vaccine is made from an incompletely characterized culture supernatant of a nonencapsulated, toxigenic strain (anthrax vaccine absorbed [AVA]) whose primary protective component is thought to be protective antigen (PA). AVA is effective in protecting animals and elicits toxin-neutralizing antibodies in humans, but enthusiasm is dampened by its undefined composition, multishot regimen, recommended boosters, and potential for adverse reactions. Improving next-generation anthrax vaccines is important to safeguard citizens and the military. Here, we report that vaccination with recombinant forms of a conserved domain (near-iron transporter [NEAT]), common in Gram-positive pathogens, elicits protection in a murine model of *B. anthracis* infection. Protection was observed with both Freund's and alum adjuvants, given subcutaneously and intramuscularly, respectively, with a mixed composite of NEATs. Protection correlated with an antibody response against the NEAT domains and a decrease in the numbers of bacteria in major organs. Anti-NEAT antibodies promote opsonophagocytosis of bacilli by alveolar macrophages. To guide the development of inactive and safe NEAT antigens, we also report the crystal structure of one of the NEAT domains (Hal) and identify critical residues mediating its heme-binding and acquisition activity. These results indicate that we should consider NEAT proteins in the development of an improved antianthrax vaccine.

*Bacillus anthracis* is a Gram-positive, encapsulated, and sporulating bacterium notable as the causative agent of anthrax. The disease is most commonly reported in wild and domestic herbivores, but because of the ability of the spores to persist in the environment and be aerosolized, *B. anthracis* has been regarded as one of the most serious bioterrorism agents (1, 2). The spore, which exists in a metabolically inactive form, will germinate into a highly virulent vegetative cell upon entry into a host, where it encounters a niche rich in nutrients (3). It is believed that spores are engulfed by macrophages and are transported to draining lymph nodes where they germinate into vegetative cells, which then replicate and express a series of virulence factors, including anthrax toxin and a polyglutamic acid capsule (4–12). Disease is categorized according to the route of spore exposure. These routes include spore entry through the epidermis (cutaneous anthrax), spore entry through the alveolar epithelial surface (inhalational anthrax), spore entry through the gastrointestinal epithelium (gastrointestinal anthrax), or the most recently identified form contracted through injecting drugs contaminated with spores (injectional anthrax) (13, 14). Upon anthrax infection, vegetative bacilli proliferate in the initial site of inoculation and then spread to the lymphatic tissues and disseminate to other organs and, ultimately, the bloodstream (15, 16). The precise infectious dose of *B. anthracis* in humans by various routes is unknown, but it is believed that inhalational anthrax can develop in susceptible hosts after exposure to a relatively small number of spores (17).

*B. anthracis* vaccine development efforts have included the use of attenuated strains (18), heterologous expression hosts (19, 20), capsule conjugates (21, 22), inactivated spores (23, 24), and lipid-encapsulated DNA (25). The first vaccines against anthrax were developed in the 1880s by William S. Greenfield and Louis Pasteur using live attenuated cultures of *B. anthracis* (26, 27). Although

the vaccines were effective in livestock, the virulence of the vaccines varied, leading in 1939 to Max Sterne developing a live but attenuated vaccine from a nonencapsulated strain of *B. anthracis* that is the standard vaccine for livestock in the United States (18). Live attenuated vaccines have been linked with residual virulence leading to occasional animal casualties; thus, the vaccine was not regarded as safe for human use (28–30). Acellular vaccines against *B. anthracis* were sought. Growth of *B. anthracis* in chemically defined media (31, 32) and the identification of anthrax toxin and its components (33–37) led to the generation of the current licensed human anthrax vaccine, known as anthrax vaccine absorbed (AVA). AVA is a cell-free filtrate of cultures of an avirulent, nonencapsulated strain of *B. anthracis*. Evidence suggests that the principal component that elicits protection is protective antigen (PA), which is the cell-binding component of anthrax toxin. The mechanism of action of AVA seems to be due to anti-PA toxin neutralizing antibodies that provide protection against anthrax disease (38, 39). Historically, six immunizations over 18 months

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Address correspondence to Anthony W. Maresso, maresso@bcm.edu.

M.A.B. and C.T.Q.N. contributed equally.

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TABLE 1 Bacterial strains used in this study

Strain	Plasmid	Property	Reference(s)
<i>E. coli</i> BL21	pgst- <i>hal<sub>N</sub></i>	Encodes the NEAT domain (amino acids 29–152) of Hal <sub>N</sub>	53
<i>E. coli</i> BL21	pgst- <i>isdC</i>	Encodes the NEAT domain (amino acids 32–212) of IsdC	57
<i>E. coli</i> BL21	pgst- <i>isdX1<sub>N</sub></i>	Encodes the NEAT domain (amino acids 27–152) of IsdX1	49
<i>E. coli</i> BL21	pgst- <i>isdX2<sub>FL</sub></i>	Encodes the NEAT domains of IsdX2 (amino acids 30–859)	49
<i>E. coli</i> BL21	pgst- <i>bslK<sub>N</sub></i>	Encodes the NEAT domain (amino acids 46–165) of BslK	52
<i>E. coli</i> BL21	pgst- <i>hal<sub>N</sub>Y112A</i>	Encodes Hal <sub>N</sub> with an alanine substitution for tyrosine 112	This study
<i>E. coli</i> BL21	pgst- <i>hal<sub>N</sub>F116A</i>	Encodes Hal <sub>N</sub> with an alanine substitution for phenylalanine 116	This study
<i>E. coli</i> BL21	pgst- <i>hal<sub>N</sub>Y112A,F116A</i>	Encodes Hal <sub>N</sub> with alanine substitutions for tyrosine 112 and phenylalanine 116	This study
<i>B. anthracis</i> Sterne 34F2	pUTE657	Avirulent, attenuated, nonencapsulated strain (Spec <sup>c</sup> )	92, 93

have been recommended as a part of the preexposure AVA vaccination regimen, but evidence suggests that shorter inoculation intervals can be effective at eliciting sufficient levels of protective antibodies (40–42). Injection site and systemic adverse reactions (43), the undefined nature of AVA, the potential for batch variation (44), the extended vaccination regimen with recommended boosters (45), and the instability of recombinant PA preparations (46) have spawned efforts to determine if other antigens or measures can be used to produce a next-generation vaccine (46).

In this report, we describe progress toward the development of recombinant heme transporters as potential vaccine antigens. Five such transporters have been identified in *B. anthracis*, each possessing at least one conserved heme-binding motif referred to as the near-iron transporter (NEAT) domain. Secreted and surface-localized NEAT-containing proteins mediate the acquisition and import of heme from host heme sources such as hemoglobin (47–51). *B. anthracis* encodes five proteins that contain one or more NEAT domains: IsdC, IsdX1, IsdX2, BslK, and Hal (49, 52–55). IsdX1 and IsdX2 are secreted into the culture medium and actively acquire heme from hemoglobin (49, 56). IsdC is covalently anchored to the cell wall by a sortase-mediated mechanism and can receive heme from both IsdX1 and IsdX2 (54, 57). BslK is an S-layer protein that binds heme and can also transfer heme to IsdC (52). Finally, Hal is necessary for growth on hemoglobin and heme and is also thought to be attached to the bacillus cell wall (53). Two of these NEATs, IsdX2 and IsdC, were identified as being highly antigenic by a functional genomic-serologic screen, and Hal is immunoreactive with *B. anthracis* antisera (58). Guinea pigs infected with the fully virulent Ames strain of *B. anthracis* also produce antibodies to IsdX1 and IsdX2 (49). The NEAT-containing protein iron surface determinant B (IsdB) from *Staphylococcus aureus* was the basis of Merck's V710 vaccine. IsdB is a cell wall-anchored protein, conserved among diverse clinical isolates of *S. aureus*, that harbors two NEAT motifs and functions to assist with heme iron acquisition (59). It induced rapid antibody responses in macaques and increased survival after challenge in a murine *S. aureus* sepsis model (60). V710 phase I trials testing the formulation of the vaccine proved successful (61, 62); however, phase III trials evaluating the administration of the vaccine preoperatively to cardiothoracic surgery patients were halted, citing a higher frequency of deaths in the vaccine recipients than in the placebo recipients (63). Considering the general importance of iron uptake to the growth and replication of bacterial pathogens and attempts to construct a vaccine from such factors to prevent staphylococcal infection, we sought to determine if recombinant NEAT proteins can protect against anthrax disease.

## MATERIALS AND METHODS

**Bacterial strains and cloning.** The construction of pgst-*hal<sub>N</sub>*, pgst-*isdC*, pgst-*isdX1*, and pgst-*isdX2*, and pgst-*bslK<sub>N</sub>* was described previously and is summarized in Table 1. All strains were propagated in lysogeny broth (LB) with 100 µg/ml ampicillin (Caisson Labs, Logan, UT). Single- and double-alanine substitutions of the two aromatic residues in the heme-binding lip region of *hal* (Y112A, F116A, and Y112A,F116A) were generated by using QuikChange site-directed mutagenesis (Agilent, Santa Clara, CA) with the following primer pairs encoding the desired mutation (in bold-face type): forward primer 5'-CCC ACT TTA GGG **GCC** GAT AAG GAA TTC-3' and reverse primer 5'-GAA TTC CTT ATC **GGC** CCC TAA AGT GGG-3' for Hal<sub>N</sub>Y112A, forward primer 5'-GGG TAC GAT AAG GAA **GCC** AAA ATT CAG-3' and reverse primer 5'-CTG AAT TTT **GGC** TTC CTT ATC GTA CCC-3' for Hal<sub>N</sub>F116A, and forward primer 5'-CCC ACT TTA GGG **GCC** GAT AAG GAA **GCC** AAA ATT CAG-3' and reverse primer 5'-CTG AAT TTT **GGC** TTC CTT ATC **GGC** CCC TAA AGT GGG-3' for the Hal<sub>N</sub>Y112A,F116A double mutant. The Hal<sub>N</sub>Y112A and Hal<sub>N</sub>F116A single mutants were generated according to the manufacturer's specifications using plasmid pgst-*hal<sub>N</sub>* as the parent DNA containing the wild-type gene. This DNA template was then subjected to PCR amplification using the primer pairs mentioned above. PCR products were digested with 1 µl of DpnI (New England BioLabs, Ipswich, MA) at 10 U/µl for 2 h at 37°C. Plasmid DNA was then purified by using a QIAquick PCR purification kit (Qiagen, the Netherlands) and transformed into *Escherichia coli* DH5α. Mutations were verified by DNA sequencing. Single-mutation plasmid DNA was further manipulated with the Hal<sub>N</sub>Y112A,F116A double mutant primer set, subjected to PCR, DpnI digested, and transformed into *E. coli* DH5α. Once the double-alanine mutation was verified by sequencing, all of the resulting plasmids, pgst-*hal<sub>N</sub>Y112A*, pgst-*hal<sub>N</sub>F116A*, and pgst-*hal<sub>N</sub>Y112A,F116A*, were isolated and transformed into *E. coli* BL21 (New England BioLabs, Ipswich, MA). All NEAT proteins were expressed and purified as described previously (49). The concentration of each protein was measured by using the Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA; Fisher Scientific, Fair Lawn, NJ), used to generate a standard curve, and the homogeneity of the preparations was determined by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For NEAT preparations evaluated as recombinant vaccines, all samples were applied to Pierce HighCapacity Endotoxin Removal Resin columns (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions, and proteins were eluted with endotoxin-free buffer and stored at -20°C.

**Measurement of heme binding.** The Hal NEAT domains (~10 µM for each domain, including wild-type Hal<sub>N</sub>, Hal<sub>N</sub>Y112A, Hal<sub>N</sub>F116A, and Hal<sub>N</sub>Y112A,F116A) were individually incubated with hemin chloride (0 to 20 µM; Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at 25°C. Spectral analyses of all proteins from 280 nm to 480 nm, before and after incubation, were performed by using a DU800 spectrophotometer (Beckman Coulter, London, United Kingdom).

**Measurement of heme acquisition.** Purified glutathione S-transferase (GST)-NEAT (35  $\mu$ M for GST-Hal<sub>N</sub>, 20  $\mu$ M for Hal<sub>N</sub>Y112A, 20  $\mu$ M for Hal<sub>N</sub>F116A, and 35  $\mu$ M for Hal<sub>N</sub>Y112A,F116A) was individually immobilized on 2 ml of glutathione-Sepharose resin, washed with 40 ml of PBS (pH 7.4), and incubated with 1 ml of bovine methemoglobin (2.5  $\mu$ M; Sigma-Aldrich, St. Louis, MO) for 30 min at 25°C. After incubation, beads containing bound NEATs were centrifuged at 6,000  $\times$  g for 1 min, the supernatant (methemoglobin) was removed, and the beads were washed with 40 ml of PBS (pH 7.4). The GST-fused NEAT domains were then eluted from the beads with 0.5 ml of reduced glutathione (25 mM; Calbiochem, San Diego, CA) for 5 min, and the relative heme content was determined by comparing the intensity of the Soret band to that for a control reaction with GST-NEAT incubated with PBS only.

**Reconstitution and crystallization of Hal<sub>N</sub>.** The Hal NEAT (Hal<sub>N</sub>) domain of the *BAS0520* gene, encoding amino acids 26 to 155, was amplified out of plasmid pGEX2TK-0520\_NEAT (53) with Kod polymerase (EMD), using the following primers: forward primer 5'-GCC CAT GGA GAA TAT GGC TGT ACA AA GTC CAA AAA-3' and reverse primer 5'-CGC TCG AGT ACAA ATG TAC GCA TAT TCA CTT CAA ACT-3'. The forward and reverse primers contained the NcoI and XhoI endonuclease sites, respectively, for cloning into pET28a (Novagen) to produce a C-terminally His-tagged fusion protein. BL21-Gold(DE3) cells harboring pET28a-0520 were grown at 37°C in LB medium containing 30  $\mu$ g/ml kanamycin to an optical density at 600 nm (OD<sub>600</sub>) of 0.8, and protein expression was then induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) along with the addition of 1 mM 5-aminolevulinic acid hydrochloride (heme precursor; Sigma) for 4 h, after which the cells were centrifuged at 6,000  $\times$  g and resuspended in a solution containing 50 mM Tris-Cl (pH 7.4), 350 mM NaCl, and 15 mM imidazole. Cells were lysed by sonication with the addition of 5 mg egg hen lysozyme (Sigma) and 40  $\mu$ M phenylmethylsulfonyl fluoride (Sigma). The cell lysate was centrifuged at 30,000  $\times$  g for 20 min, and the supernatant was filtered by using a 1-mm glass fiber syringe filter (Pall). The supernatant was loaded onto a Ni<sup>2+</sup>-charged HisTrap column (GE Healthcare) and eluted with a linear imidazole gradient (15 to 500 mM imidazole). Fractions were analyzed by SDS-PAGE to determine Hal<sub>N</sub> purity. The final purification step was performed by using an S200 10/30 Superdex column (GE Healthcare) equilibrated with a solution containing 50 mM Tris (pH 7.4) and 150 mM NaCl to yield 100% homogeneous Hal<sub>N</sub>. The percentage of heme was determined by using the pyridine hemochrome and Lowry assays (Bio-Rad) (64, 65). To achieve fully heme-reconstituted Hal<sub>N</sub>, a hemin solution was added to a 1:1 molar ratio of heme to protein (Sigma) (64, 65). Crystals of heme-Hal<sub>N</sub> were grown at 90 mg/ml by a hanging-drop vapor diffusion method with a reservoir containing 0.2 M ammonium sulfate, 0.1 M Bis-Tris (pH 5.5), and 21% polyethylene glycol 3350 (PEG 3350) with a 1:1 (vol/vol) protein-to-reservoir drop. Crystals were harvested by using paratone oil as a cryoprotectant and frozen in liquid nitrogen. A native data set was collected at the Advanced Light Source at Berkeley National Laboratories (ALS Berkeley) on beamline 8.2.1 at a wavelength of 1.0 Å.

**Structure determination of heme-Hal<sub>N</sub>.** Data processing and scaling were carried out with the imosflm suite (66). Data processing statistics are summarized in Table S1 in the supplemental material. Initial models were obtained by molecular replacement in Phenix Phaser-MR (67) using search models of *B. anthracis* IsdX1 (PDB accession no. 3SZ6) (56), *B. anthracis* IsdX2<sup>NS</sup> (PDB accession no. 4H8P) (68), and *S. aureus* IsdA (PDB accession no. 2ITE) (69), followed by additional rounds of molecular replacement and autobuilding in Phenix MR-Rosetta (67). This was followed by manual building utilizing Coot (70) with iterative cycles of refinement with Phenix Refine (71). The final stereochemistry and geometry for each model were validated with MolProbity (72). The refinement parameters are summarized in Table S1 in the supplemental material. All molecular graphics were prepared by using PyMOL (73).

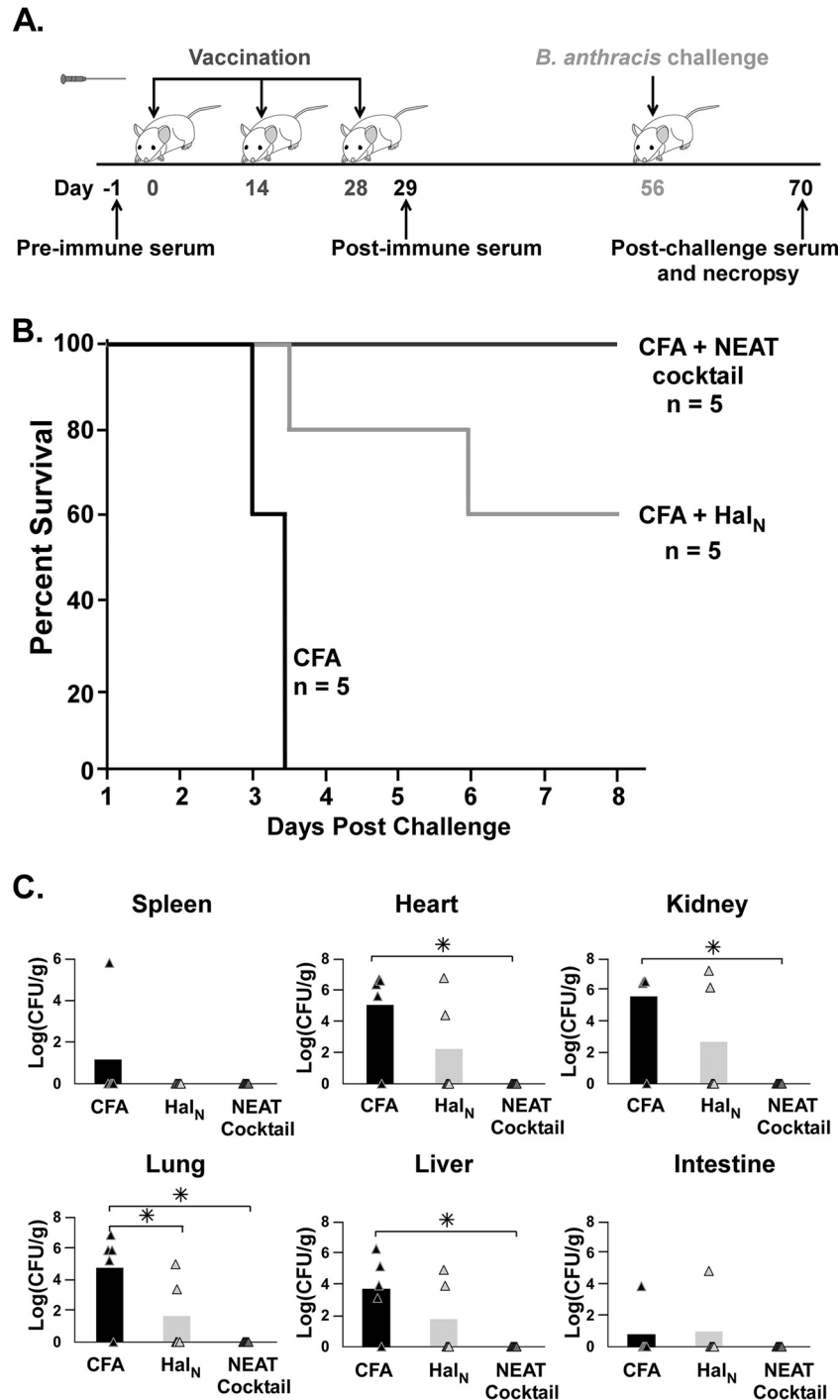
***B. anthracis* spore preparation.** *B. anthracis* Sterne 34F2 was propagated in 8 ml tryptic soy broth (TSB) and grown overnight at 250 rpm at 37°C. Cultures were then seeded into 400 ml of modified G medium

containing 0.2% yeast extract, 0.0025% calcium chloride dihydrate, 0.05% dipotassium phosphate, 0.02% magnesium sulfate heptahydrate, 0.005% manganous sulfate tetrahydrate, 0.0005% zinc sulfate heptahydrate, 0.0005% cupric sulfate pentahydrate, 0.00005% ferrous sulfate heptahydrate, and 0.2% ammonium sulfate at pH 7.1. Growth was maintained for 30 h at 30°C with shaking at 200 rpm to induce sporulation, and spores were centrifuged at 6,000  $\times$  g for 10 min, washed, and resuspended in 10 ml of water. Before use, spores were heat treated at 65°C for 1 h to kill any remaining vegetative cells, followed by serial dilution of the spore preparations on LB agar to determine the number of CFU per volume of spore preparation.

***B. anthracis* vaccinations and infections.** A group of 15 conventionally housed female A/J mice (Jackson Laboratory, Bar Harbor, ME) was used to determine the 50% lethal dose (LD<sub>50</sub>) of spores of *B. anthracis* strain Sterne 34F2. Each group, consisting of five mice, was challenged with a total volume of 200  $\mu$ l containing either 1  $\times$  10<sup>3</sup> spores, 1  $\times$  10<sup>4</sup> spores, or 1  $\times$  10<sup>5</sup> spores injected subcutaneously (s.c.) into the fatty tissue under the right hind leg. Prior to injection, spore preparations were heated to 65°C to kill any vegetative cells. The LD<sub>50</sub> was determined by using the method of Reed and Muench (74). For the vaccination studies, the regimen shown in Fig. 1A was followed. Six-week-old female A/J mice were vaccinated s.c. with either adjuvant alone or complete Freund's adjuvant (CFA; Sigma-Aldrich, St. Louis, MO) or aluminum hydroxide ("alum"; Thermo Scientific, Rockford, IL) in combination with each individual (4  $\mu$ g) or all ("cocktail" [4  $\mu$ g of each, for 20  $\mu$ g total]) recombinant NEAT domain preparations described above. After the first vaccine dose, mice given CFA were administered incomplete Freund's adjuvant for the second and third doses. After a total of three vaccinations were administered (on days 0, 14, and 28) and then 4 weeks after the third boost, the mice were challenged with either 5 or 10 times the LD<sub>50</sub> as described above. Mice were bled a day before the first vaccination (day -1) to attain a "preimmune" serum sample, on day 29 to attain a "post-vaccine" serum sample, and on day 70 or upon death to attain a "post-challenge" serum sample. Mice were monitored for morbidity by using a health index that tracks five disease characteristics (posture, activity level, coat appearance, skin turgor/tenting, and hyperpnea). Mild forms of each characteristic are scored as 0.5, while severe forms are scored as 1.0. Morbidity is achieved with a total score of 4.0, at which point the mice were euthanized and necropsied to isolate the lungs, liver, spleen, kidneys, heart, and intestine, and tissues were homogenized and plated onto LB agar to determine the number of CFU of *B. anthracis* per gram of tissue.

Animals were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* (75) and with approval by the Institutional Animal Care and Use Committee (protocol no. AN-5177:1) at Baylor College of Medicine.

**ELISA of mouse serum samples.** Enzyme-linked immunosorbent assays (ELISAs) were performed in triplicate by using Immulon 2HB 96-well microtiter plates (Thermo Scientific, Rockford, IL) coated overnight with 0.2  $\mu$ g/well of each purified NEAT domain (BslK<sub>N</sub>, Hal<sub>N</sub>, IsdC<sub>N</sub>, IsdX1<sub>N</sub>, and IsdX2<sub>PL</sub>) in 1  $\times$  PBS at 4°C. All reactions were carried out with a volume of 100  $\mu$ l. After the initial coating, each plate was washed three times with 200  $\mu$ l of 1  $\times$  PBS-0.05% Tween 20 (PBST) and blotted dry by inversion on clean paper towels. Plates were then blocked with 10% nonfat dry milk overnight at 4°C. Mouse sera diluted 1:2,000 from pre-immunized samples, postvaccination serum samples, and postchallenge serum samples were individually added to wells and then sequentially diluted 1:4,000, 1:8,000, and 1:16,000 in the microtiter plates and incubated for 2 h at 37°C with slow shaking at 20 rpm in a covered chamber. The plate was washed five times with PBST after each incubation, 100  $\mu$ l of 1:10,000-diluted peroxidase-conjugated anti-mouse IgG (product no. A8924; Sigma-Aldrich, St. Louis, MO) was then added to each well, and the plate was incubated for 1 h at 37°C with slow shaking at 25 rpm. Plates were washed five times with PBST and then developed with the 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine) ELISA substrate solution (Thermo Scientific, Rockford, IL), and the reaction was stopped with 2 M



**FIG 1** Subcutaneous vaccination with NEAT domains and CFA. (A) Schematic of the vaccination regimen and collection of serum from vaccinated mice. Animals were injected subcutaneously on day 0, 14, or 28 with either the NEAT domain of Hal (Hal<sub>N</sub>) (4  $\mu$ g) or the NEAT domains of IsdX1, IsdX2, IsdC, BslK, and Hal (4  $\mu$ g each, for 20  $\mu$ g total) mixed with CFA. Serum was drawn at day -1 (preimmune), day 29 (vaccinated), and day 70 (postchallenge) and tested for anti-NEAT antibodies by ELISAs (see Materials and Methods). (B) Survival curves for mice vaccinated with adjuvant (CFA) alone, adjuvant with Hal<sub>N</sub>, or adjuvant with the NEAT domain cocktail after subcutaneous challenge with  $10 \times LD_{50}$  of *B. anthracis* Sterne strain spores. For CFA versus the NEAT cocktail, the *P* value was 0.014 as determined by a log rank (Mantel-Cox) test, and the *P* value was 0.017 as determined by a Wilcoxon test. For CFA versus wild-type Hal, the *P* value was 0.111 as determined by a log rank (Mantel-Cox) test, and the *P* value was 0.074 as determined by a Wilcoxon test. (C) CFU of bacilli recovered from mouse organs from the experiments described above for panels A and B (asterisks denote statistically significant values [*P* < 0.01], as determined by one-way analysis of variance). For CFA versus the NEAT cocktail, *P* values were 0.186 for spleen, 0.061 for kidney, 0.074 for heart, 0.043 for lung, 0.082 for liver, and 0.186 for gastrointestinal tract. For CFA versus wild-type Hal, *P* values were 0.186 for spleen, 0.009 for kidney, 0.008 for heart, 0.008 for lung, 0.012 for liver, and 0.186 for gastrointestinal tract.

H<sub>2</sub>SO<sub>4</sub> according to the manufacturer's specifications. Assay plate results were recorded at an OD<sub>450</sub> with a BioTek Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT). Positive controls used included testing rabbit serum raised against individual NEAT proteins with peroxidase-conjugated anti-rabbit IgG (product no. A0545; Sigma-Aldrich, St. Louis, MO), and negative controls consisted of 1 × PBS in place of serum (background absorbance).

**ELISA data analysis.** Mean absorbance values at an OD<sub>450</sub> were calculated from measurements performed in triplicate and normalized by subtraction of the average background values with standard deviation values determined by applying a propagation-of-error equation (76). Antibody titers were calculated from a mouse IgG standard by coating Immulon plates with serial dilutions of an anti-mouse IgG Fab fragment (10, 1, 0.1, and 0.01 μg/ml) in duplicate for 16 h at 4°C with periodic shaking. Wells were washed twice with 200 μl of 1 × 0.05% Tween 20–PBS (wash buffer) and blocked with 2% nonfat milk and 100 μg/ml BSA overnight at 4°C. Wells were washed three times with wash buffer and incubated with 100 μl goat anti-mouse IgG-horseradish peroxidase (HRP) for 1 h at 37°C with periodic shaking. A colorimetric reaction was allowed to develop by using the 1-Step Ultra TMB ELISA substrate solution (Thermo Scientific) and 2 M H<sub>2</sub>SO<sub>4</sub>. A linear equation ( $y = mx + b$ ), where  $y$  is the OD value and  $x$  is the IgG concentration in micrograms per milliliter, was generated by plotting serial dilutions of mouse IgG versus the average absorbance values and solving for  $x$  to determine IgG levels.

**Assessment of the effect of anti-NEAT antibodies on *B. anthracis*.** Polyclonal anti-IsdC antiserum was generated in rabbits by using standard procedures at the Baylor College of Medicine Center for Comparative Medicine. Anti-IsdC IgG was purified from serum by using an Abcam antibody purification kit (catalog no. ab102704) according to the manufacturer's instructions and used to determine the effect of anti-NEAT antibodies on *B. anthracis* heme uptake and opsonin-mediated killing. For heme uptake, a blood serum mimic (BSM) (and 4 × BSM) was prepared, and bacillus growth in this medium was carried out as previously described (77), with 2,2-dipyridyl (500 mM; Alfa Aesar), iron sulfate (10 mM; J. T. Baker), ammonium sulfate (10 mM; BDH), and purified anti-IsdC antibody (0.3, 3, or 30 μg/ml) being added, as described in the legend to Fig. 5. Lysogeny broth (Life Technologies, Grand Island, NY) was prepared according to the manufacturer's instructions. For opsonin-mediated killing, MH-S alveolar macrophages (ATCC, Manassas, VA) were seeded at 1 day postinfection into 24-well plates at 1 × 10<sup>6</sup> cells/ml. Opsonization was initiated by adding a mixture of 30 μg/ml of anti-IsdC or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (negative control) (catalog no. ab8425; Abcam) with *B. anthracis* 7702 Sterne vegetative cells at a multiplicity of infection (MOI) of 5 for 30 min at 37°C with periodic shaking. The mixture was then added to MH-S cells to begin infection, and phagocytosis was synchronized by low-speed centrifugation for 1 min at 300 × *g*. At each designated time point, MH-S cells were washed with 1 × PBS and incubated with gentamicin (50 μg/ml) for 30 min at 37°C to eliminate extracellular bacteria. Cells were then washed with 1 × PBS and lysed with 50 μl of 1% Triton X-100. Intracellular bacterial counts were determined by serial plating of the lysed samples.

## RESULTS

**Vaccination with recombinant NEAT domains can protect against a lethal dose of *B. anthracis*.** We initiated experimentation to determine if vaccination with recombinant NEAT proteins could protect against infection with *B. anthracis*. The animal model of choice for these studies was the A/J mouse, a strain commonly used to assess anthrax disease, especially toxigenic forms of *B. anthracis* such as those used in this study (16). The reported LD<sub>50</sub> of *B. anthracis* Sterne strain 34F2 in the A/J model ranges from 10<sup>3</sup> to 10<sup>5</sup> spores (16, 30). To determine the LD<sub>50</sub> more precisely and narrow this range, we infected three groups of mice with 10<sup>2</sup>, 10<sup>3</sup>, or 10<sup>4</sup> *B. anthracis* spores and observed survival of

the mice over a 5-day period. All of the mice infected with 10<sup>4</sup> spores succumbed to infection by the fourth day, while mice challenged with 10<sup>3</sup> spores showed 80% survival by day 5, and all the mice treated with 10<sup>2</sup> spores survived (see Fig. S1 in the supplemental material). Based on these results, the LD<sub>50</sub> was calculated to be 3.2 × 10<sup>3</sup> spores (74).

We next purified each individual NEAT domain of IsdX1, IsdC, BslK, or Hal or all five NEAT domains as a polypeptide from IsdX2 from *E. coli* by GST affinity chromatography. Each preparation was assessed for purity by SDS-PAGE, the protein concentration was determined by using the Bradford method, and the ability to bind heme (an indication of a functional, folded protein domain) was assessed by using assays previously developed in our laboratory (68). Lipopolysaccharide (LPS) was removed by using a series of endotoxin-binding resins, and pure preparations were mixed with complete Freund's adjuvant (CFA). The vaccination regimen for all experiments in this study is presented in Fig. 1A, with three subcutaneous (s.c.) inoculations spaced 2 weeks apart. Serum samples were collected before dose 1 and 1 day after the last dose of the vaccine. Following vaccination and on day 56, mice were challenged with 10 times the LD<sub>50</sub> of *B. anthracis* strain Sterne, and mouse health was examined for 8 days postchallenge. While none of the mice receiving only CFA survived challenge, 60% of mice vaccinated with only the NEAT domain of Hal (Hal<sub>N</sub>) and all of the mice vaccinated with a cocktail of every NEAT survived a lethal dose of *B. anthracis* (Fig. 1B). At the time of necropsy, in mice given only CFA, the spleen, heart, kidney, liver, lung, and intestine generally contained high levels of *B. anthracis*, whereas mice vaccinated with Hal<sub>N</sub> or the NEAT cocktail showed reduced and no bacilli, respectively, in these samples ( $P < 0.01$ ) (Fig. 1C).

**NEAT-based protection from anthrax is associated with the production of anti-NEAT antibodies.** Pre- and postimmune serum samples from mice vaccinated with CFA, Hal<sub>N</sub>, or the NEAT cocktail were assessed for the presence of anti-NEAT antibodies by using ELISAs with purified NEAT domains from each protein. When all the components of the ELISA mixture were added, except for serum, little to no signal was observed in any of the ELISA reactions ("no-serum" group) (Fig. 2A to E). In addition, a weak signal was observed when serum from vaccinated individuals given only CFA was tested (Fig. 2, gray bars). Furthermore, little to no signal was observed prior to vaccination ("preimmune" group) (Fig. 2). Thus, the level of background in this assay was quite low. In contrast, mice vaccinated with the NEAT cocktail yielded a strong response that was observed only if the mice were vaccinated with the NEAT domains and was not observed prior to vaccination ("postimmune" group) (Fig. 2, blue and green bars). Antibody production was observed for all five components of the NEAT cocktail with titers that ranged from 1 to 4 ng/ml. In fact, for IsdX1, IsdX2, Hal, and BslK, positive antibody was observed even when serum was diluted 10,000-fold (not shown). Of particular importance was the observation that mice vaccinated with just Hal<sub>N</sub> produced antibodies that were reactive not only to Hal<sub>N</sub> but also to every other NEAT domain that was analyzed (Fig. 2A to E, blue bars). Indeed, with the exception of the NEAT domain from IsdC, the titers of anti-NEAT antibodies from Hal<sub>N</sub>-vaccinated mice were equivalent to those of antibodies from the cocktail-vaccinated animals (Fig. 2A to E, compare blue bars to gray bars). When considered in the context of the results from the challenge studies shown in Fig. 1, these data suggest that a 3-dose vaccination regimen using recombinant NEAT domains can sub-

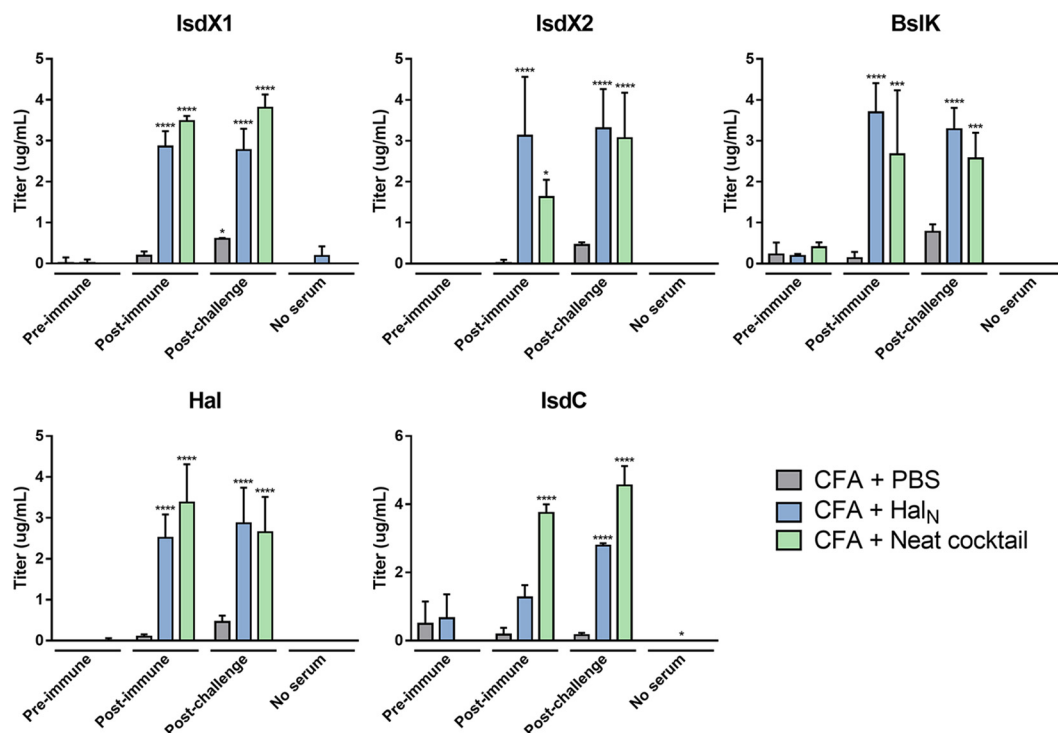


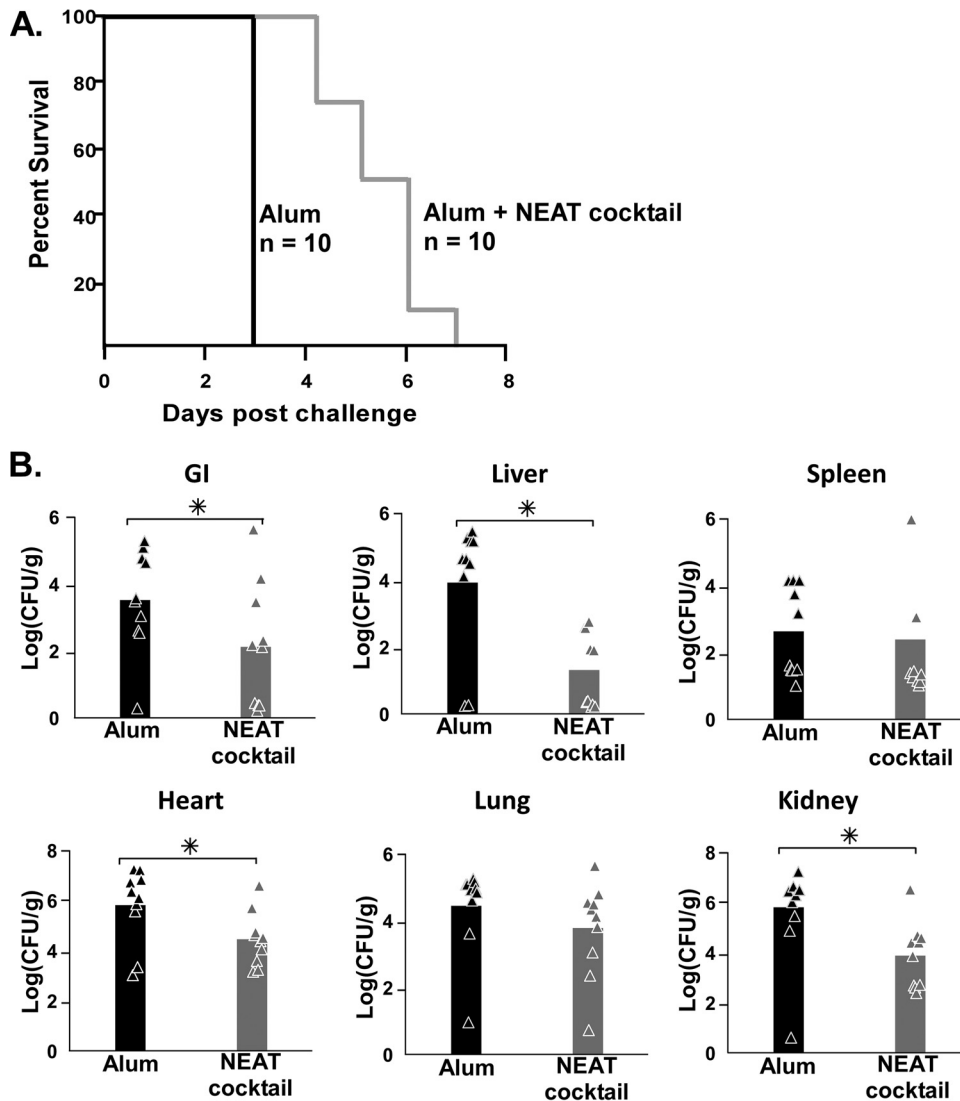
FIG 2 Reactivity of sera from mice vaccinated with the NEAT domains and CFA. Preimmune, postvaccine, and postchallenge sera from mice vaccinated with either CFA, Hal<sub>N</sub>, or the NEAT cocktail were evaluated for the presence of anti-NEAT antibodies by using an ELISA with purified NEAT domains from each NEAT protein: IsdX1, Hal, BslK, IsdC, and IsdX2. Asterisks represent results of Tukey's multiple-comparison test comparing CFA and Hal or CFA and the NEAT cocktail to the control (CFA plus PBS). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

stantially protect mice from a lethal dose of *B. anthracis* and that bacillus NEAT proteins are immunogenic, leading to the production of anti-NEAT antibodies. Furthermore, there is substantial cross-reactivity observed, in that vaccination with just a single NEAT domain (Hal<sub>N</sub>) produces antibodies that also bind the NEAT domains from the other four proteins.

**A NEAT cocktail is efficacious when combined with alum given intramuscularly.** Licensed vaccines, including AVA, generally use aluminum hydroxide (alum) as the adjuvant of choice because it is safe to use in humans (78). In addition, most alum-adjuvanted vaccines administered in the United States are given intramuscularly (i.m.). It has been demonstrated that injection site reactogenicity is increased when the s.c. route is used versus the i.m. route (79). Because of this, we conducted additional vaccination studies aimed at determining the effectiveness of a NEAT domain cocktail when alum was the adjuvant and the vaccinations were administered i.m., the preferred human vaccination protocol. Each recombinant NEAT domain was purified, 4  $\mu\text{g}$  of each NEAT domain was mixed together with alum, and the mixture was injected into the muscle of the right hind leg of mice ( $n = 10$ ) by using the general vaccination regimen and challenge described in the legend to Fig. 1. As shown in Fig. 3A, mice receiving the NEAT cocktail survived 4 days longer on average than their alum-only counterparts ( $P < 0.0001$ ). When organs were analyzed (following necropsy) for the number of CFU of bacilli, a significantly ( $P < 0.05$ ) lower level of *B. anthracis* was observed in the heart, kidney, liver, and intestine, but not the spleen or lungs, of mice given the NEAT cocktail than in the adjuvant-only controls (Fig. 3B). When serum was analyzed for the presence of anti-NEAT

antibodies, only the secreted hemophore IsdX2 yielded a detectable response upon ELISAs (see Fig. S2A and S2B in the supplemental material). No antibodies to any of the NEATs were detected in serum drawn before vaccination. Since IsdX2 harbors five nonidentical NEAT domains, while all of the other *B. anthracis* NEAT domain proteins have only one, these results suggest that the antibody response is dependent on the dose of the antigen, with higher titers being observed when more antigen is given. Taken as a whole, the data suggest that a NEAT protein cocktail, when used with a human-approved adjuvant and route of delivery, provides some level of protection against a lethal dose of *B. anthracis*, with the concomitant production of anti-IsdX2 antibodies albeit at lower titers. Partial protection and a significant reduction of the numbers of bacilli in organs indicate that even low levels of circulating anti-NEAT antibodies are sufficient to delay the development of anthrax disease.

**Anti-NEAT antibodies promote phagocyte-mediated killing of *B. anthracis*.** There are two main ways in which antibodies directed to NEAT domains may afford protection against *B. anthracis*. One way is through the inhibition of heme-iron import by blocking the ability of NEAT domains to bind hemoglobin, heme, or both. A second way is that the antibodies may act as an opsonin for recognition and eventual killing by phagocytic cells. We performed experiments aimed at differentiating between these possibilities by first testing if anti-NEAT antibodies prevent the growth of bacilli on heme as the only source of iron. *B. anthracis* strain Sterne 34F2 was grown in the presence or absence of hemoglobin (a source of heme) or  $\text{FeSO}_4$  (iron) under iron-limited conditions that are designed to mimic a blood-like environment (BSM) (77).

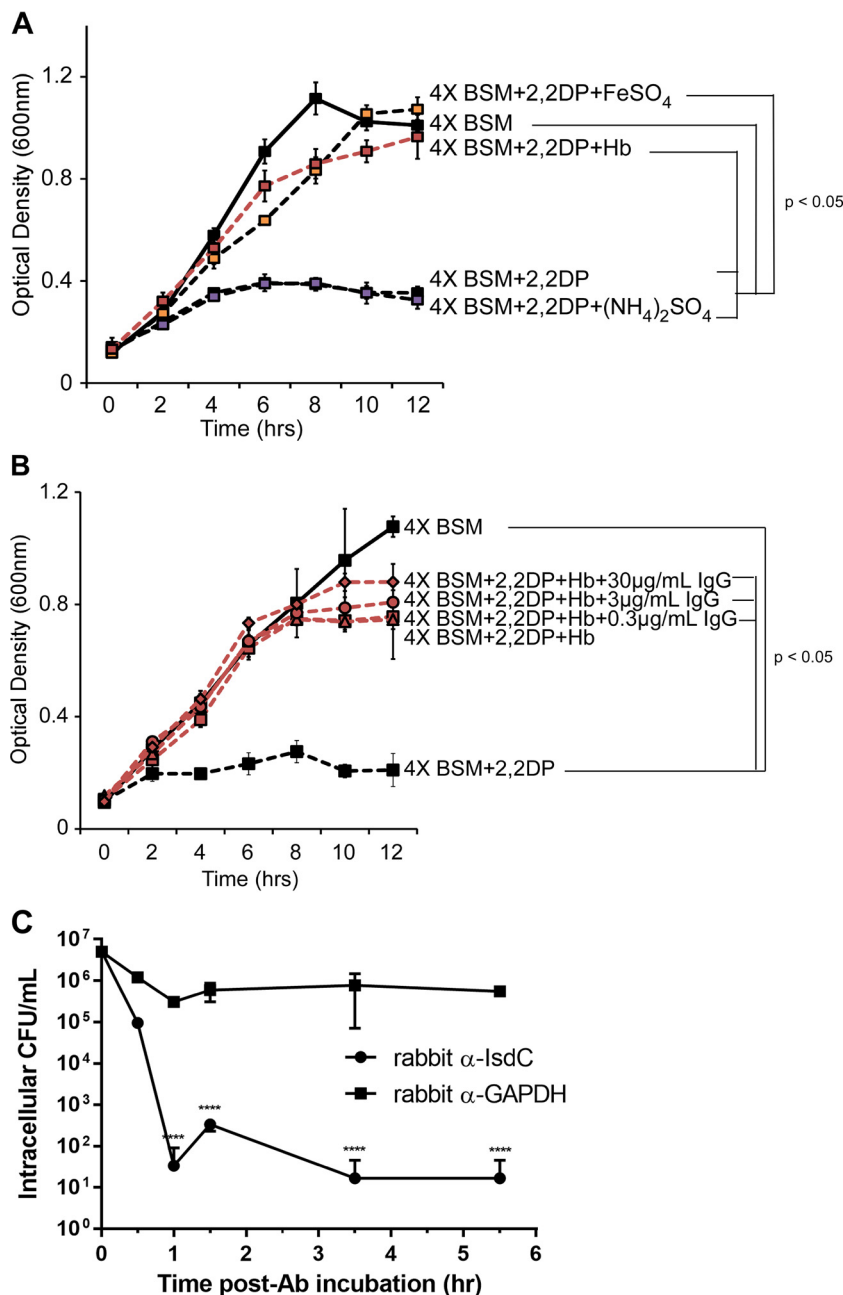


**FIG 3** Intramuscular vaccination with NEAT domains and alum. (A) Survival curves for mice vaccinated with adjuvant alone (alum) or alum plus a NEAT cocktail (4  $\mu\text{g}$  of each NEAT domain, for 20  $\mu\text{g}$  total) after challenge with  $5 \times \text{LD}_{50}$  of *B. anthracis* Sterne strain spores. For alum versus the NEAT cocktail, the  $P$  value was  $<0.0001$  as determined by a log rank (Mantel-Cox) test, and the  $P$  value was  $<0.0001$  as determined by a Wilcoxon test. (B) Comparison of the total *B. anthracis* CFU recovered from mouse organs (lungs, spleen, kidneys, heart, and gastrointestinal [GI] tract) treated with alum (black bars) alone versus alum with a NEAT cocktail (gray bars) after necropsy of animals on day 70. Asterisks indicate statistical significance ( $P < 0.01$ ) as determined by one-way analysis of variance.

A robust stimulation of growth was observed with the addition of iron or hemoglobin (iron in the form of heme) in the presence of an iron chelator (Fig. 4A, red, orange, and black squares). Little or no growth was observed without iron, suggesting that these conditions recapitulate an iron utilization response (Fig. 4A, purple squares and black circles). To test if antibodies that recognize NEAT proteins prevent heme-iron uptake under these conditions, we raised polyclonal antibodies to the NEAT protein considered to be the central conduit in the heme uptake cascade (IsdC) (55). Purified IgG antibodies, which recognize IsdC upon Western blotting (not shown), were next incubated with *B. anthracis* in BSM in the presence or absence of hemoglobin. No inhibition of bacillus growth was observed in the presence of IsdC antibodies, even at the highest concentration of IgG used (Fig. 4B).

To assess the effects of anti-NEAT antibodies serving as op-

sonins for alveolar macrophages and subsequent killing of *B. anthracis* once inside these cells, we cultured murine MH-S macrophages with bacilli that either were or were not preincubated with purified anti-IsdC antibodies (Fig. 4C). A decrease in the levels of bacilli of several orders of magnitude was observed for *B. anthracis*-incubated anti-NEAT antibody compared to the isotype-matched control group in the first 1 h of infection. In fact, a significant difference in bacterial survival under these two conditions was observed every hour for the entire 5.5-h infection. This difference was not due to an inhibition of the growth of bacilli in RPMI, the culture medium used for MH-S macrophages (not shown). Taken together, these two data sets suggest that anti-NEAT antibodies, at least those to IsdC, are not growth restrictive in iron-limiting environments for *B. anthracis*. Instead, such antibodies stimulate the killing of bacilli by alveolar macrophages, presum-



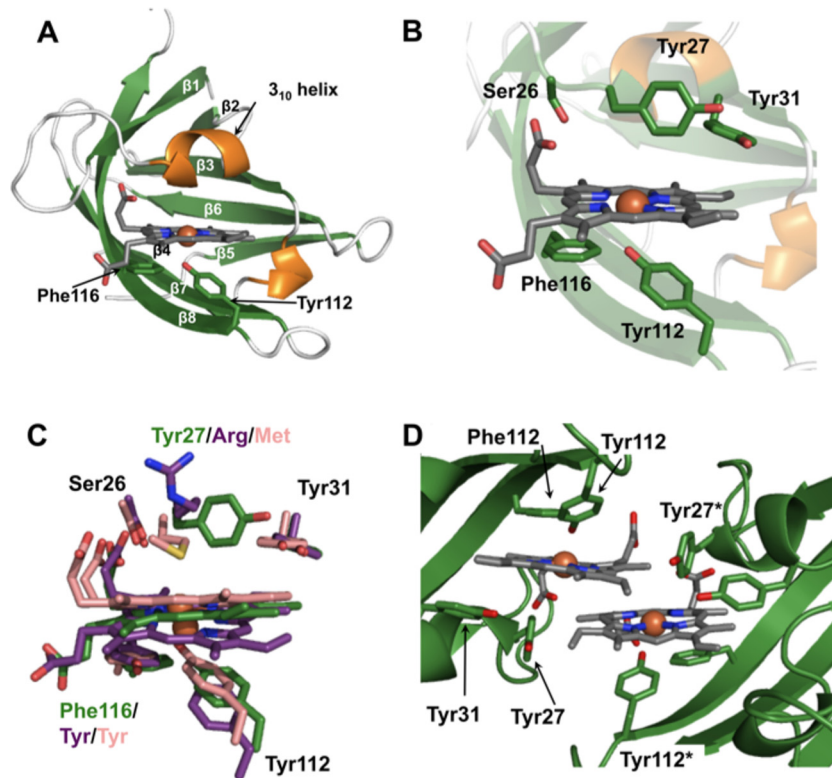
**FIG 4** Effect of anti-NEAT antibodies on iron intake or opsonin-mediated macrophage killing. (A and B) *B. anthracis* was cultured in 4× BSM (solid lines) or 4× BSM with 2,2-dipyridyl (2,2DP) (500 μM) (dashed lines) in the presence of iron sulfate (150 μM) (orange boxes), ammonium sulfate (150 μM) (purple boxes), and human hemoglobin (Hb) (15 μM) (red lines) (A) or hemoglobin with increasing concentrations of purified rabbit anti-IsdC (red symbols) (B), and growth was measured by absorbance spectroscopy (OD<sub>600</sub>). Each data point represents the average of results from three independent cultures ± standard deviations. (C) Purified rabbit anti-IsdC or a purified rabbit isotype control (anti-GAPDH), each at 30 μg/ml, was preincubated with *B. anthracis* strain Sterne for 30 min at 37°C, washed, and added to MH-S alveolar macrophages (MOI of 5), with phagocytosis being synchronized by low-speed centrifugation. At each designated time point, MH-S cells were washed with 1× PBS, incubated with gentamicin for 30 min to eliminate extracellular bacteria, and lysed with Triton X-100. Intracellular bacterial counts were determined by serial plating of the lysed samples. The values represent the means and standard deviations of data from three independent experiments. Data were analyzed by using an unpaired *t* test comparing the average values at each time point (\*\*\*\*, *P* < 0.00001). Ab, antibody.

ably through antibody-mediated opsonophagocytosis. By extension, these data may indicate that the protection afforded by NEAT vaccination in murine infection models may be more attributable to the phagocytosis of antibody-coated bacilli than to the inhibition of heme uptake through NEAT proteins. This may also explain why even a very low-level antibody response (alum

plus i.m. inoculation) (Fig. 3) can still induce a measurable level of protection from anthrax disease.

**Understanding the structural basis of NEAT activity for the design of a safer NEAT-based vaccine.** If NEAT proteins are to be evaluated or developed as potential vaccine candidates for the prevention of anthrax, it will be necessary to understand the molec-





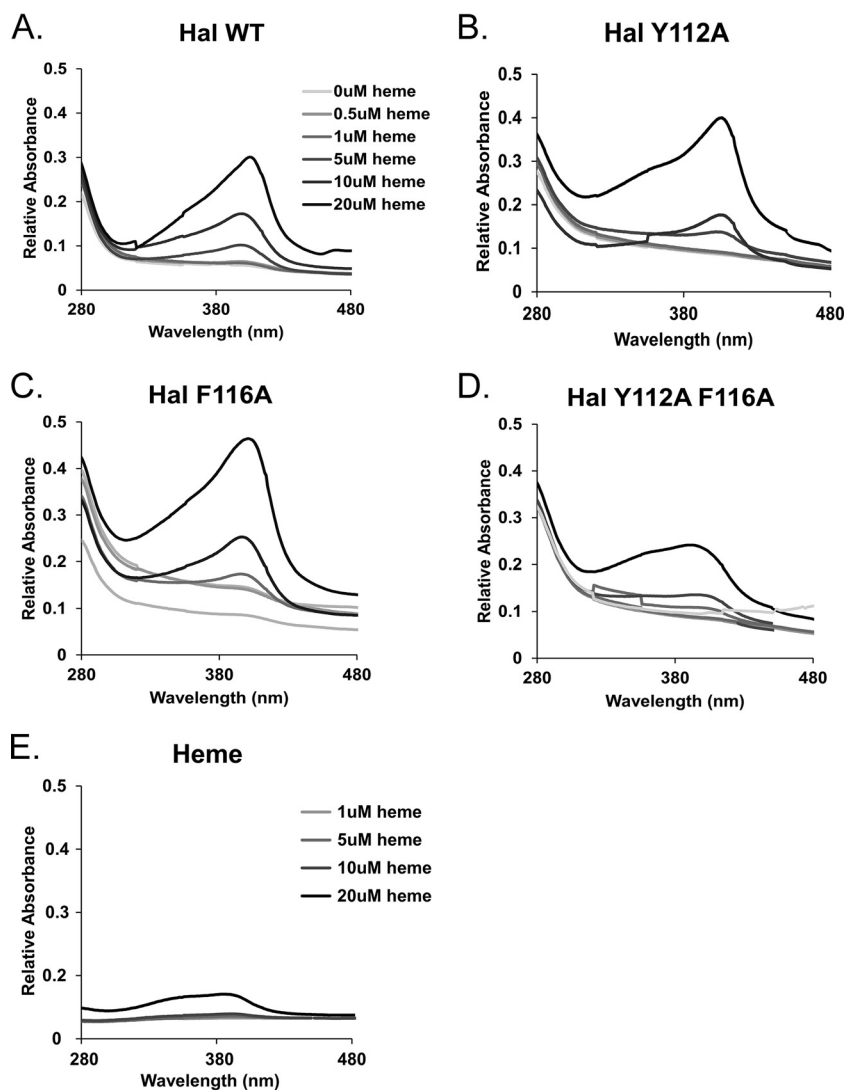
**FIG 5** Structure of heme-Hal<sub>N</sub>. (A) Ribbon representation of heme-Hal<sub>N</sub>, where  $\beta$ -strands and  $\alpha$ -helices are shown in green and orange, respectively. Important heme-binding residues are shown in stick representation with carbon, oxygen, and nitrogen atoms in green, red, and blue, respectively, and heme is also in stick representation with carbon atoms in dark gray. (B) Closeup of the heme-binding pocket with the same color scheme as that described above for panel A. (C) Superimposition of the heme-binding sites of Hal<sub>N</sub>, IsdX1, and IsdX2<sup>N5</sup>, where important heme-binding residues and heme are in stick representation, with carbon atoms of Hal<sub>N</sub> in green, IsdX1 in pink, and IsdX2<sup>N5</sup> in purple. (D) View of the dimeric interface of Hal<sub>N</sub>, which is mediated predominantly by the two heme molecules. “\*” denotes the second molecule in residue numbering (colored as described above for panel A).

ular basis for their function. This knowledge will help guide the design of recombinant forms that lack heme-binding or heme extraction activity, efforts that may reduce toxicity and make vaccines safer. As mentioned above, a version of the NEAT protein IsdB (V710) was evaluated in phase I and II clinical trials as a vaccine from *S. aureus*, but the trials were suddenly halted due to toxicity concerns (63). Because Hal is important in heme-iron utilization in *B. anthracis* (53), is homologous at the amino acid level to the other four NEATs from bacilli (55), and is a component of the NEAT cocktail used here, we investigated its structure and the molecular determinants of its activity to gain a better understanding of how to develop a safer NEAT-based vaccine.

All bacilli and staphylococcal NEAT domains studied thus far have a conserved heme-binding motif, YXXXXY (56, 68, 69, 80, 81). In these structures, the first tyrosine coordinates with heme-iron as the axial ligand, and the second tyrosine hydrogen bonds (H-bonds) to the axial tyrosine, stabilizing the interaction. As Hal<sub>N</sub> does not contain the characteristic heme-binding motif and has a phenylalanine in the fifth position (YDKKE) (53), we determined the structure of heme-Hal<sub>N</sub> to a 3.0-Å resolution to shed light on the mode of heme binding. The structure of Hal<sub>N</sub> consists of an immunoglobulin-like fold, as observed in multiple NEAT domain crystal structures (56, 68, 69, 80, 81), with eight  $\beta$ -strands arranged in two antiparallel  $\beta$ -sheets that form a  $\beta$ -sandwich (Fig. 5A). In the structure of Hal<sub>N</sub>, the predominately hydrophobic heme-binding pocket is formed by the long  $\beta$ -hairpin

(strands  $\beta 7$  and  $\beta 8$ , where  $\beta 8$  contains the heme binding motif) on the proximal side of the heme molecule and the  $3_{10}$ -helix region (SXXXXY) on the distal side, which is another conserved feature of NEAT domains (51, 56, 80, 81). On the proximal side of heme, the heme-iron is coordinated by the conserved Tyr112 via its hydroxyl group, with an Fe-O distance of 2.4 Å (Fig. 5A). This Fe-O distance reported for other NEAT domains is slightly shorter (2.0 to 2.2 Å) due to the increased electronegativity of the first Fe-coordinating Tyr H-bonding to the phenolate of the second Tyr within the heme-binding motif (80). In the NEAT domain of Hal, the second Tyr within this motif is replaced with a phenylalanine (Phe116). Thus, the coordinating Tyr112 is not as electronegative as other NEAT Fe-coordinating tyrosines, resulting in a slightly longer Fe-O coordinating distance. However, Phe116 further stabilizes the heme molecule by  $\pi$ -stacking interactions with the pyrrole ring (Fig. 5A). Within the  $3_{10}$ -helix region, the hydroxyl group of the conserved Ser26 H-bonds with the least-solvent-exposed heme propionate group (2.9 Å) together with the conserved Tyr31 that weakly  $\pi$ -stacks with the heme pyrrole ring (Fig. 5B).

The crystal structure of the NEAT domain of Hal helped guide mutational studies to assess the effect of some of these residues on the function of Hal. This is important because if NEAT proteins are to be studied or move forward as possible anthrax vaccines, the antigens that compose this vaccine should not contain any biological activity that can introduce toxicity or adverse reactions. Be-



**FIG 6** Analysis of heme-binding properties of Hal<sub>N</sub> mutants. Increasing concentrations of heme (inset) were incubated with wild-type (WT) Hal<sub>N</sub> (A), Hal<sub>N</sub>Y112A (B), Hal<sub>N</sub>F116A (C), Hal<sub>N</sub>Y112A,F116A (D), and heme alone (E) for 30 min, and spectral scans of the reaction mixtures were collected to assess the intensity of the Soret band, the spectral signal associated with heme binding.

cause there is structural evidence that Y112 and F116 are involved in heme coordination, we used site-directed mutagenesis to individually or collectively substitute an alanine for these residues and purified their corresponding recombinant domains. The heme-binding properties of the mutant domains were next assessed by measuring the intensity of the Soret band, an optical feature of heme complexed to protein, at ~400 nm. The titration of hemin, the oxidized form of heme, into a solution of wild-type Hal<sub>N</sub> resulted in the formation of a strong Soret maximum at 402 nm in a concentration-dependent manner (Fig. 6A), which was much more intense, red shifted, and defined in shape than the hemin-only control (Fig. 6E). Hal<sub>N</sub>Y112A and Hal<sub>N</sub>F116A, when mixed with hemin, demonstrated Soret intensities similar to those of the wild-type domain (Fig. 6B and C). However, the double mutant (Hal<sub>N</sub>Y112A,F116A) displayed features somewhat in between the wild-type profile and that of the hemin control, with a broad peak and lower intensity (Fig. 6D).

The compromised ability of the double mutant to bind hemin

compelled an examination of the relative heme acquisition activity from hemoglobin. Some NEAT domains, including the two secreted hemophores IsdX1 and IsdX2 as well as Hal, can extract heme from hemoglobin at rates that are higher than the rate of thermal disassociation of heme into solution (53). To determine if these mutant NEAT domains of Hal can acquire heme from hemoglobin, we employed a simple affinity chromatography technique whereby a known amount of the NEAT domain in question is purified as a GST fusion protein and bound to glutathione-Sepharose. Hemoglobin (in this case, bovine) is next added to the reaction mixture, the beads are extensively washed, the bound NEAT is eluted with excess glutathione, and eluants are assessed for heme transfer by Soret spectroscopy. When incubated with hemoglobin and analyzed in this manner, wild-type Hal<sub>N</sub> underwent an increase in the Soret band, which was suggestive of an acquisition of heme from hemoglobin (Fig. 7A). This was also observed for Hal<sub>N</sub>F116A, albeit at a somewhat lower level, when analyzed under the same conditions (Fig. 7C). However, a small

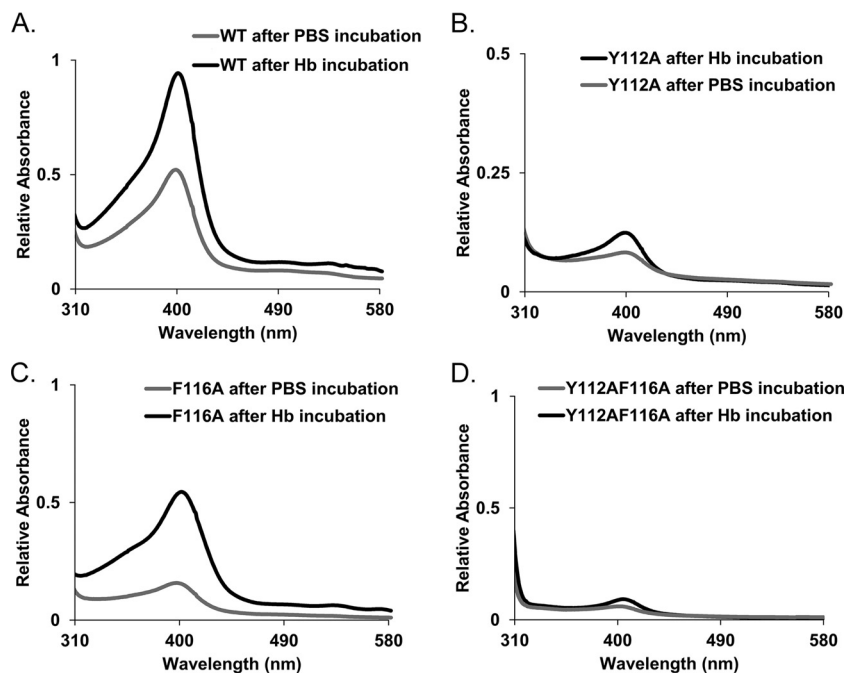


FIG 7 Analysis of the heme acquisition properties of Hal<sub>N</sub> mutants. Wild-type Hal<sub>N</sub> (A), Hal<sub>N</sub>Y112A (B), Hal<sub>N</sub>F116A (C), and Hal<sub>N</sub>Y112A,F116A (D) were incubated with hemoglobin (black lines) or buffer alone (gray lines) for 30 min at room temperature and assessed for their ability to actively scavenge heme from hemoglobin by the intensity of the Soret band.

increase in the Soret band was observed when Hal<sub>N</sub>Y112A was incubated with hemoglobin (Fig. 7B), with the Hal<sub>N</sub>Y112A,F116A double mutant being nearly identical in heme occupancy to the control sample that was incubated with PBS only (Fig. 7D). When considered as a whole and in combination with the heme-binding data shown in Fig. 6, these results suggest that Y112 and/or F116 is sufficient to bind heme but that Y112 is important for the acquisition of heme from hemoglobin. These findings also indicate that one should consider generating inactive NEAT domain antigens as anthrax vaccine components, especially if they retain the ability to elicit protective antibodies after immunization.

## DISCUSSION

The key findings of this study are that (i) a 3-dose vaccination regimen using NEAT domains as antigens is efficacious against a lethal challenge of *B. anthracis*; (ii) protection is correlated with both the production of anti-NEAT antibodies and overall lower levels of bacilli in organs and tissues; (iii) the Hal NEAT domain alone may also be efficacious albeit at lower levels; (iv) a NEAT vaccine delays the onset of anthrax disease with human-approved alum when delivered i.m.; (v) anti-NEAT antibodies against IsdC promote killing by alveolar macrophages but do not inhibit heme-dependent growth; (vi) the structure of the Hal NEAT domain has the conserved overall NEAT immunoglobulin-like fold, with F116 and Y112 coordinating the heme-iron, while Phe116 stabilizes the heme molecule by  $\pi$ -stacking with the porphyrin ring; and (vii) F116 and Y112 are important for heme binding, but Tyr112 in Hal seems to be important for heme acquisition from hemoglobin. These results provide a rationale to pursue additional efforts to develop NEAT proteins as a prophylactic vaccine for anthrax disease. This development may include using either wild-type or genetically modified NEATs that retain immunogenicity but are still

safe as well as combining the NEATs with other known protective antigens, such as anthrax toxin or protective antigen.

There are four main mechanisms by which antibodies against NEATs may work against a bacterial pathogen. The first mechanism is by inhibiting the binding of the NEAT to the major oxygen carrier protein in mammals, hemoglobin. Because hemoglobin coordinates heme-iron, it is a target for pathogenic bacteria. The staphylococcal NEAT protein IsdB as well as the two bacillus hemophores (IsdX1 and IsdX2) and the surface protein Hal all either stably or transiently bind hemoglobin for the purpose of releasing and acquiring its bound heme. By preventing direct access of the NEAT domain to hemoglobin, the rapid transfer of heme is inhibited, thereby decreasing the rate at which the growing pathogen can transfer heme to IsdC, the central conduit, which is thought to then relay heme and its bound iron into the cell. The second mode of inhibition is at the level of disrupting heme binding to the NEAT domain. Although the heme ligand is relatively small in comparison to the light and heavy chains of an antibody, the heme-binding site sandwiched between the 3<sub>10</sub>-helix and the  $\beta$ -hairpin encompasses a significant amount of surface area for this small ~15-kDa domain module. Steric hindrance blocking the entry of heme into this binding site would prevent all forms of heme scavenging, both free and direct forms of heme scavenging, thereby serving as an effective way to prevent heme-based iron uptake. Antibodies may also inhibit the transfer of heme between the NEAT domains, either from the secreted hemophores to surface NEATs (such as IsdC) or between surface NEATs themselves. Since NEAT-based heme uptake is regarded as a transfer cascade, this or a combination of all three modes of inhibition could produce a powerful mechanism to shut down this major iron uptake pathway. Torres et al. and Stranger-Jones and colleagues first reported that vaccination with staphylococcal surface proteins, one

of which was IsdB (heme receptor for human hemoglobin), was effective in preventing invasive infection with human isolates of *S. aureus* (59, 82). Indeed, Kim and coworkers reported that antibodies against IsdB could protect mice from *S. aureus* when passively transferred into mice and that the mechanism of protection was linked to the ability of these antibodies to block IsdB binding to hemoglobin and, thus, heme-iron uptake and not to the ability of these antibodies to promote opsonophagocytosis (83). Another explanation is that anti-NEAT antibodies recognize surface NEAT protein and promote the recognition of bacilli by phagocytes or complement.

An antibody response is produced upon vaccination with NEAT proteins. Purified polyclonal IgG raised in rabbits against the recombinant NEAT domain of *B. anthracis* IsdC, which is reactive to other bacillus NEAT domains on Western blots (not shown), does not prevent heme-iron uptake from hemoglobin under iron-limiting conditions. In contrast to what is observed for antibodies to *S. aureus* IsdB, antibodies against IsdC did not inhibit the growth of bacilli on hemoglobin as the only iron source, thereby precluding the first three mechanisms discussed above as the cause of the negative effect on bacilli. Instead, such antibodies promoted the opsonophagocytosis of bacilli by alveolar macrophages. The doses of the NEAT antigen and the adjuvant have not yet been optimized for this formulation, and so the level of antibody needed to induce this effect is currently unknown. However, because IsdX2 is the only NEAT to induce detectable antibodies in the alum formulation (IsdX2 contains five NEATs, compared to only one each for IsdX1, IsdC, BslK, and Hal), this suggests that we have not yet reached the threshold for the upper limit of delivered antigen in this system. In addition, it is encouraging that despite the low circulating levels of antibody in both the CFA and alum studies, a strong protective response against anthrax was still observed. This may mean that a robust antianthrax response is observed with low levels of circulating anti-NEAT antibodies. The unexpected finding that such antibodies may promote phagocytosis may thus prove useful in the development of a better anthrax vaccine. The current U.S.-licensed vaccine, AVA, is believed to work by stimulating the production of antibodies that neutralize anthrax toxin. However, this leaves open the question as to whether AVA targets replicating and growing bacilli. The addition of recombinant NEATs to such a vaccine may provide a powerful one-two punch that both inhibits toxin as well as promotes the clearance of bacilli.

This raises the question as to whether capsulated bacilli would be resistant to a vaccine strategy that targets surface proteins, especially if one of the properties of the vaccine is the ability to induce the phagocytosis of opsonized bacteria. It is thought that one of the functions of the capsule of pathogenic bacteria is to protect against the host immune system, perhaps by inhibiting phagocytosis (84). The poly- $\gamma$ -D-glutamic acid (PGA) capsule of *B. anthracis* is a poor immunogen; however, coupling PGA to a strong immunogen, including protective antigen, leads to anti-capsule antibodies that promote the killing and clearance of bacilli (25, 85, 86). It currently is not understood whether antibodies to NEAT domains would bind to capsulated bacilli. Whereas IsdX1 and IsdX2 are secreted proteins, IsdC is covalently attached to the cell wall (49). BslK is an S-layer protein and may be the most surface exposed of the five NEATs (52). The surface location of Hal is unknown, but it is likely upstream of the cell wall (53). Since heme-loaded hemophores and/or hemoglobin would be expected

to contact these downstream surface NEATs in the presence of capsule (otherwise, iron uptake in capsulated bacteria via heme might not occur), it may be possible for antibodies to also access these surface NEATs. Further studies will be needed to determine if the capsule represents a barrier to vaccine construction using this approach.

The suggestion that vaccines can be constructed from proteins involved in heme or iron acquisition is not unprecedented, and there already has been a major advance along these lines. IsdB also induced substantial production of IgG in rhesus macaques, suggesting that it also may do so in humans (60). In fact, the data were so convincing that Merck conducted three phase I and II clinical studies using a version of IsdB (termed V710) to determine how well it was tolerated in humans. Preliminary studies suggested that it was safe and induced an antibody response in humans (61, 62, 87). However, Merck abruptly stopped the study, citing a high rate of adverse vaccine-related events and multiorgan failure in another study (63). The details of these events have yet to be reported (88); however, in nearly all the subjects receiving V710, but not the placebo group, the prevaccination interleukin-2 (IL-2) and IL-17 levels were low, suggesting that some type of aberrant immune response contributed to an inability to resolve subsequent staphylococcal infections (89). Therefore, an understanding of the molecular properties of the NEAT domain can aid in the development of a safe anthrax vaccine.

Along these lines, our structural analysis of Hal is a step in this direction. The replacement of Y112 with an alanine indicates that Hal<sub>N</sub>Y112A can still bind heme but cannot accept heme from hemoglobin. This suggests that perhaps Y27 or Y30 may coordinate heme-iron in lieu of Y112, although the overall mechanism of heme acquisition from hemoglobin requires Y112. Thus, Y112 may act as an alternate ligand for heme-iron or may be required for the extraction and transfer of heme from hemoglobin, possibly at the level of the NEAT-hemoglobin interaction, as observed for R54 in IsdX1 (56). Even though Hal<sub>N</sub> has a phenylalanine (F116) substitution at the second tyrosine within the highly conserved heme-binding motif (YXXXY), heme-iron is still coordinated by the first tyrosine (Y112), as observed for all other *S. aureus* and *B. anthracis* NEAT domains (56, 68, 69, 80, 81), and F116  $\pi$ -stacks with the heme pyrrole ring. Interestingly, the second NEAT domain of the proposed hemophore from *Listeria monocytogenes* (Hbp2<sub>N2</sub>) also harbors an amino acid substitution at the second Tyr in the heme binding motif, to an alanine and not a phenylalanine, as observed for Hal<sub>N</sub>. The recently solved structure of Hbp2<sub>N2</sub> shows that unlike Hal<sub>N</sub>, the conserved first Tyr of the heme-binding motif does not form an axial ligand with heme-iron (90). Instead, a tyrosine on the adjacent  $\beta$ -strand ( $\beta$ 7) from the  $\beta$ -strand harboring the heme-binding motif ( $\beta$ 8) coordinates its hydroxyl group to heme-iron. Furthermore, Hbp2<sub>N2</sub> undergoes a major conformational change upon heme binding (90), which has not been observed for other NEAT domains, such as *B. anthracis* IsdX1 (56), *S. aureus* IsdA (69), and *S. aureus* IsdH (91). The authors of those studies suggest that this larger structural rearrangement of Hbp2<sub>N2</sub> is a product of the lack of both tyrosine residues within the heme-binding motif, since within NEAT domains with a conserved heme-binding motif, the two tyrosine residues form a noncovalent interaction that stabilizes the  $\beta$ -hairpin structural element. As Hal<sub>N</sub> also lacks the second tyrosine of the heme-binding motif, one may expect Hal<sub>N</sub> to have the same degree of structural plasticity as Hbp2<sub>N2</sub>. This may mean that any

inclusion of Hal or NEATs that are structurally similar to Hal in a vaccine may require multiple mutations at several residues in the heme-binding pocket to inactivate their heme uptake properties. Recombinant Hal containing a double substitution (Y112A and F116A), as guided by structural studies and confirmed to be inactive in functional studies, would seem to be a logical inclusion in an anthrax vaccine. Future studies will determine if vaccination with such NEATs still retains their protective effect.

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