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Bcla As An Adjunct To Current Bacillus Anthracis Vaccination And Therapy Protocols

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BCLA AS AN ADJUNCT TO CURRENT *BACILLUS ANTHRACIS* VACCINATION AND THERAPY PROTOCOLS

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A DISSERTATION

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BCLA AS AN ADJUNCT TO CURRENT *BACILLUS ANTHRACIS* VACCINATION PROTOCOL

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ABSTRACT

Bacillus anthracis (Ba) is a Gram,-positive zoonotic bacterium that causes inhalational, cutaneous and intestinal Anthrax Disease. Although the infectious form of Ba is its endospore (spore), only the tripartite toxin-producing vegetative bacteria causes disease pathology. While current vaccination strategies target the Protective Antigen (PA) component of this toxin, vaccine-elicited immunity to the spore form of Ba is lacking. However, vaccines targeting spore components of Ba have the potential to neutralize the infectious form of Ba. We sought to determine if antibodies elicited through immunization with the main antigenic component of the spore surface- Bacillus collagen-like protein of Anthracis (BclA)- can mediate immunity and protect against lethal Ba infection.

The study of Ba is problematic due to its inherent virulence and bio-safety concerns. Many laboratories utilize the Sterne strain, which only require a BSL II facility, but limits researchers to the susceptible A/J or similar mouse strains. In our initial studies we confirm that C5 deficiency renders C57BL/6 mice susceptible to this strain. Because the majority of genetically manipulated mice are on the C57BL/6 background, the use of C5 deficient mice ($C5^{-/-}$) in the C57BL/6 background broadens our ability to study Ba-host interactions under a variety of experimental conditions.

Intranasal immunization with recombinant BclA and cholera toxin provided significant protection from lethal intratracheal Ba challenge to complement component 5-deficient ($C5^{-/-}$) C57BL/6 mice. Importantly, passive transfer of BclA-specific monoclo-

nal antibodies (mAbs) was similarly able to protect from lethal Ba challenge, and significantly increase spore uptake by macrophages and lead to enhanced spore-destruction *in vitro*. The effects of BclA-specific mAbs are mediated by Fc receptors, which promoted endo-lysosomal fusion, increased acidification of the endosomal compartment, and subsequently promoted spore destruction within macrophages.

Collectively, these results indicate that vaccine-elicited antibodies targeting the Ba spore component BclA can lead to protection from infectious Ba spores by promoting their uptake and destruction. BclA-targeted immunity could be a major supplement to current toxin-based vaccines. Furthermore, BclA-specific mAbs represent an important addition to current antibiotic regimens used to treat Ba, and may be of highly significant value in the treatment of multiple antibiotic-resistant Ba strains or bioengineered strains.

DEDICATION

To mom, dad, Mateo, and Jose.

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INTRODUCTION

Historical Context of Anthrax

A Plague since Antiquity

There are several historical references that suggest Anthrax Disease has been present throughout human history, highlighting the important role this disease has played in human events. Historians believe one of the first mentions of Anthrax occurs in the ninth book of Exodus as the fifth plague of Egypt (1500 B.C.) -manifested in the death of all of Pharaoh's livestock- (respiratory form of anthrax)[1-3]:

> "The hand of the Lord will bring a terrible plague on your livestock in the field—on your horses, donkeys and camels and on your cattle, sheep and goats." (Exodus 9:3, New International Version)

The sixth plague (plague of boils)-manifested as boils in the skin of Egyptians- (cutaneous anthrax)[1-3] is also hinted at in the same chapter:

> "So they took soot from a furnace and stood before Pharaoh. Moses tossed it into the air, and festering boils broke out on people and animals. The magicians could not stand before Moses because of the boils that were on them and on all the Egyptians."(Exodus 9:10-11)

Early Greece was familiar with anthrax as shown by the historic descriptions of the disease "burning wind of plague" by Homer (1230 B.C.) that Apollo inflected as punishment in the Iliad [4]:

> "Pack animals were his target first, and dogs but soldiers, too, soon felt transfixing pain from his hard shots, and pyres burned night and day."

The disease was also known in Hindu literature (500 B.C) as well as ancient Rome, as the poet Virgil (70-19 B.C.) went on to describe in detail how the murrain (plague, disease) of Noricum spread from animals to humans as well as the hardiness of the disease (probably referring to the spore) in his work *Georgics* [5]:

> "For neither might the hides be used, nor could one cleanse the flesh by water or master it by fire. They could not even shear the fleeces, eaten up with sores and filth, nor touch the rotten web. Nay, if any man donned the loathsome garb, feverish blisters and foul sweat would run along his fetid limbs, and not long had he to wait ere the accursed fire was feeding on his stricken limbs."

Repeated epidemics were described in post-Rome Europe and Asia. Hippocrates coined the term "anthracites" which described the coal-like characteristic black eschars of cutaneous anthrax. The "Black Bane" as it was known in Europe during the Middle Ages [6] had major outbreaks in Ireland (1050 A.D.), Germany (1250 A.D.), Italy (1552 A.D.). In China, Ge Hong and Ch'ao Yaun Fang described the disease (500 A.D.) as a disease common in animal husbandry [4].

Anthrax in Victorian England

Epizootic diseases occurred in Europe in large and small scale outbreaks [7]. Due to the apparent confusing clinical presentation of anthrax, little research had been done up until this time. Nicole Fournier was the first physician that classified (1769) *charbon malin* ("malignant charcoal") [7] in 1769. Fournier also recognized that anthrax was

transmitted through rags and wools, although Chabert made the first clinical and pathological description of anthrax in 1780[7-9]. This sudden interest in zoonotic diseases that could be transmitted to humans was the imperative drive towards the creation of several veterinary schools in Europe.

In 1823 Éloy Barthélémy demonstrated that transmission of blood and materials from lesions from an infected dead horse to a healthy horse caused the disease[10]. Brauell, Pollender, Davaine and Rayer all separately demonstrated the presence of rod-shaped bodies in the blood of infected animals and not in healthy animals[11]. Davaine was the first to show that anthrax affected several species via subcutaneous inoculation. These observations strongly suggested these bodies as the causative agent of the disease.

In Victorian England, John Bell (1877) was the first to describe "ragpicker's" or "woolsorter's" disease -inhalational or pulmonary anthrax- and recognized it as one of the first occupational hazards caused by a microorganism [12]. Recognition of anthrax as an occupational hazard led to some of the first recommendations and guidelines that eventually became official policy of the British Home Office.

These findings paved the understanding and subsequent findings of Robert Koch. Koch verified that the rod-shaped bodies found in sick animals were responsible for the disease and was able to culture these bacilli from the blood of infected animals on blood agar[3, 13] and, using the cultured organism was able to infect healthy animals. He further described in detail the complete life cycle of *B. anthracis* via suspended-drop culture methodology, that is, the ability of *B. anthracis* to sporulate (during "starvation conditions") and the ability of the spore survive long periods *in vitro*. He further demonstrated the ability of spores to cause anthrax and subsequently transmit the disease

to healthy animals. More importantly, he conclusively demonstrated that a specific bacterium was responsible for a specific disease, providing the foundation for his postulates on the transmission of infectious diseases[3]. In short, he postulated the four criteria to establish a causative relationship between a pathogen and a disease: the organism must be found in the host, be isolated in culture, be used from culture to cause disease in a healthy host, and re-isolated and cultured from the now sick host[13, 14]. William Smith Greenfield (1880) continued to further these studies[14]:

> "If we once admit that each distinct contagious disease is dependent on a lowly organism, we are led almost irresistibly to conclude that each of these organisms or contagia is a separate species… we may be able to isolate the organism upon which each disease depends, but we may be able to cultivate it under suitable conditions… how its growth may be checked… and thus lay the foundations of a true preventive medicine"

Greenfield successfully demonstrated that immunization of livestock against anthrax with a live, heat-attenuated strain of *B. anthracis* protected animals against disease[14]. These findings preceded Louis Pasteur's by a mere few months. However, it was Louis Pasteur that took credit for proving the germ theory of disease by inoculating a group of 25 cattle against anthrax with a vaccine consisting of live, attenuated bacilli. These animals and a control group were later infected with virulent anthrax and only those that received the attenuated vaccine survived the infection, while those that did not receive the immunization died. These experiments provided the proof for establishing the germ theory, which marked the development of modern medicine and microbiology as we know them today.

Anthrax in the Americas

Although anthrax has been in the Americas since first human migrations from Asia through the Bering Strait[15], it is believed these original strains were limited to the local bison populations. The first known site of an anthrax outbreak in the Americas occurred in Saint-Domingue (present day Haiti) in 1770. This is the disputed site of one of the largest anthrax outbreaks in history, which resulted in the death of 15,000 people due to gastrointestinal form of anthrax[7] after an earthquake befell the population. The poor sanitary conditions and the lack of food were the perfect catalysts for this epidemic. It is believed that anthrax had arrived via to Saint-Domingue via infected cattle from French settlements in Louisiana.

Anthrax was first reintroduced to colonial North America during the French settlement of Louisiana in the early 1700s and quickly spread in livestock throughout the United States [16]. There is evidence that anthrax had spread to domestic animals and far as California and New York in the second half of the 1800s[16], with several human cases in Texas, California, New York and Wisconsin. From Saint-Domingue, anthrax spread to the French West Indies and rest of the Americas[7]. Anthrax is now considered to be endemic in the United States, with a major incidence in the "anthrax belt" of the Great Plains.

Anthrax in the 20th and 21st Century

Worldwide incidence of human anthrax decreased significantly during the 20th century, with a major incidence occurring in workers closely associated with animal fibers (textile and mill workers)[6, 17]. Although the vaccine developed by Pasteur

afforded significant protection to those vaccinated, preparation of attenuated vegetative bacterium vaccine was problematic and at times it was either insufficiently attenuated (leading to death of the vaccinated animal) or too attenuated (unable to induce protection)[18]. The development of the Sterne-based spore vaccine in the late 1930s[19] by Max Sterne brought a sharp decline in animal and human cases[9, 20]. Until the introduction of the veterinary vaccine, there were 130 cases of human anthrax annually in the late 1950s in the United States. Since then, only 235 incidences of human anthrax were recorded between 1955 and 1994, with an incidence of < 1 cases per year between 1975 and 2000.

Even with those advances, the largest recorded anthrax epidemic took place in Zimbabwe in 1979-1980. There were over 9,400 cases of anthrax, most of them cutaneous that resulted in 182 fatalities. The effects of this epidemic are largely attributed to the civil war waging during this time period [21, 22]. This epidemic, plus recent resurgences in anthrax animal cases due to lack of experience recognizing and treating this disease, the knowledge that Iraq had considered using bioweapons during the first Gulf War[23], and the bioterrorist anthrax attacks of 2001 -in which four envelopes containing *B. anthracis* spores were mailed to government officials and news media representatives resulted in five fatalities out of 22 total cases[24]- brought this disease front and center as a major concern for terrorist attacks.

Anthrax as a Bioweapon

World War I

The first use of anthrax as a bioweapon occurred during World War I, when Germany targeted horses of countries to supply to Germany's enemies (Budapest in 1916 and France in 1917) with deliberate infection with anthrax[25]. It is believed that at this time Germany had developed a biological weapons program that included cholera, glanders and anthrax with the goal to infect livestock (specifically horses) and animal feed of enemy countries[6, 17].

The rationale for using *B. anthracis* as a bioweapon is varied and justified from a military standpoint: Biological aerosols are silent, odorless and invisible. In addition, inhalational anthrax has a 95-100% mortality rate when untreated, and with aggressive and early antibiotic therapy (within 24 hours of infection), the mortality rate is still close to 50%[24]. However, since the initial symptoms closely resemble influenza, antibiotic treatment is usually delayed. The hardy nature of *B. anthracis* spores and the low infective dose make them relatively easy to weaponize compared to other infectious agents. Weaponized spores are 1-5 microns in size, ideal for inhalation into the lower respiratory tract. It also has a low infective dose and the spore can stay within the host without causing disease for up to several months [26]. At the behest of Poland, anthrax and other biological weapons were included in the Geneva Protocol in 1925 and resulted in the banning of these agents[27].

World War II

Japan explored the use of anthrax as a bioweapon during the Sino-Japanese war and eventually used anthrax as a bioweapon from 1932 until the end of World War II. More than 10,000 prisoners died either due to infection with *B. anthracis* or executed after experimentation. The Japanese government conducted biological attacks on several cities in China that included contamination of water and food supplies to the dispersion of agents in bombs[6, 25].

Under the auspice of self-defense, the United States, Canada, Britain, France, Germany, Poland, Italy, Hungary, USSR developed programs for the study of *B. anthracis* and other biological agents as weapons. England in particular started anthrax studies in 1942 and focused on the viability and "range of spread" of spores when delivered with a conventional bomb[25]. The United Sates also developed a bioweapons program that included the large-scale production of *B. anthracis* "munitions." In 1971, the United States declared the end of its offensive biological weapons program

After World War II

The Soviet Union carried extensive research and development of biological weapons program. Research focused on optimization of lethality as well as antibiotic resistance, even after the signing of the Biological Weapons Convention Treaty of 1972. The anthrax epidemic in Sverdlovsk in 1979 was reported to have caused approximately 96 fatal cases of human gastrointestinal anthrax. Several years later, an American group of scientists found that an accidental release of aerosolized anthrax from a military

installation was the real cause of the deaths and that the mode of infection was not gastrointestinal but inhalational[28, 29].

In 1993, the religious group Aum Shinrikyo released a black mist from the rooftop of a building in Kameido, Tokyo, Japan. 41 residents reported foul smell, appetite loss, nausea, and vomiting to the local health authorities. However, no health hazards were identified during initial investigation. After evidence that Aum Shinrikyo was responsible for the sarin attacks in the subway Tokyo system of 1996, further investigation revealed that in the 1993 incident, the group released aerosolized spores of *B. anthracis.* Genetic analysis of the samples revealed that the group used Sterne strain to conduct the attacks. The use of the Sterne strain, low infective rate (10^4 spores/mL versus the optimal 10^9 spores/mL), high viscosity of the samples, and ineffective spray system contributed to the auspicious lack of human cases[30].

As previously discussed, the most recent use of anthrax as a weapon occurred in October and November of 2001. On September 9, Robert Stevens, a worker of the *Sun* tabloid received a letter containing a white powder. Mr. Stevens died on October 5, six days after first developing symptoms of the disease. Starting on October 12, seven news media workers and four postal employees acquired anthrax[31]. Although only 5 people died, it deeply traumatized the American public and spurred the study of new approaches for the identification, treatment and immunization to anthrax.

Biology of Bacillus anthracis

Clinical Features and Manifestations

Bacillus anthracis is a Gram-positive, non-motile, facultative anaerobic, large rod-shaped, spore-forming soil bacterium member of the Group 1 bacilli. Generally, the organisms in this group are non-fastidious, facultative anaerobic soil bacterium that can produce a resistant endospore in the presence of oxygen and include *B. cereus, B. thurangiensis, B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* [32-34]. Genetically, the members of this group are very similar, especially in sequence conservation and in gene synteny. Of this group, only *B. cereus, B. thuriangensis,* and *B. anthracis* are pathogenic. *B. anthracis* is the ethiologic agent of anthrax and is capable of causing infection through four routes: cutaneous, gastrointestinal, pulmonary and the recently described injectional anthrax[6, 31, 35]. If untreated, each form can progress to fatal disease, although the lethality is defined by route of exposure.

Pathophysiology of anthrax

Cutaneous anthrax is the most common form of the disease (95%)[36]. Cutaneous anthrax is prevalent in developing countries where animal and worker vaccinations are limited with an estimated 2000 cases worldwide. Cutaneous anthrax occurs when spores enter the body of a mammalian host via a cut, an abrasion in the skin or an insect bite[31, 37]. The head, neck and arms are the areas at greater risk of infection. Once in the subcutaneous layer of the skin, the spores germinate into the vegetative bacterium that produce localized edema. The primary skin lesion is a painless, pruritic papule, which then forms a vesicle that undergoes central necrosis and drying 3-5 days after

exposure[37]. Cutaneous anthrax is easily diagnosed and treated with a variety of antibiotics regimens and typically resolves without any complications or scarring as the eschar falls off seven days after infection. If severe local edema occurs, systemic infection can develop due to lymphatic circulation. Antibiotic treatment is recommended to decrease the possibility of malignant edema, which can have a mortality rate as high as 20 %[38].

Gastrointestinal anthrax is caused by the ingestion of vegetative bacterium or spores (usually ingestion of poorly cooked meats)[31]. Although it is believed to be extremely rare -as the incidence is unknown due to under reporting in rural areas-, two forms of gastrointestinal anthrax have been identified: oral-pharyngeal and lower GI. The oral-pharyngeal form is characterized with the development of an oral or esophageal ulcers followed by regional lymphadenopathy, edema, and sepsis[39]. Lower gastrointestinal anthrax is characterized by intestinal lesions in the terminal ileum or cecum. Initial symptoms of gastrointestinal anthrax include nausea, vomiting, and malaise[40] two to four days after ingestion, followed by bloody diarrhea, acute abdomen and sepsis. Intestinal perforation or toxemia leads to death with a mortality rate that approaches 100 %[41].

Inhalational anthrax human cases are also extremely rare and result after spores are inhaled into the lungs and reach the lower respiratory tract. It is the more severe form of anthrax and refractory to aggressive antibiotic therapy[42]. From 1901 until the anthrax attacks of 2001, only 18 cases of inhalational anthrax were recorded in the US. Resident macrophages phagocytose the spores, then translocate through the epithelial barrier and transport the spores to the mediastinal lymph nodes. It is believed that spores

germinate en route or once they reach the lymph nodes. Inhalational anthrax has a two-stage clinical presentation: Initially, patients present flu-like symptoms with cough, fever, and fatigue. The second stage is characterized by massive vegetative proliferation and toxin production that overwhelms innate defenses, causing rising fever, dyspnea, diaphoresis and shock with a mortality rate of 89 % [42]. Massive hemorrhagic mediastinitis is developed due to hemorrhage and pleural effusion into the mediastinal space. Respiratory and cardiovascular collapse leads to shock and death. Death occurs 3-5 days after the onset of symptoms[40, 43].

Injectional anthrax was first identified in 2009 in heroine users from Glasgow in the UK[44], which confirmed past observations made in 1988 India[45]. Tissue swelling and symptoms of soft tissue infection present 1-10 days after injection of heroine. Shock and organ failure were common in advanced stages of the infection. Excessive bleeding and edema were common findings during surgery on the sites of infection. Interestingly, unlike the cutaneous form of anthrax, there is no eschar formation in injectional anthrax.

Significance of the Endospore

B. anthracis has two distinct lifecycle stages; the vegetative form and the spore form, which is the infectious particle (in contrast to other Bacillus species) and is the form that most frequently exists in the environment due to their resistance to environmental stress[46, 47]. As previously discussed, sporulation is triggered by exposure to a highly aerobic environment and starvation of carbon, nitrogen and phosphorous–usually brought upon by the death of the host. Sporulation is an energy-intensive process that requires several hours to complete, and it is only initiated as a last

resort[48, 49]. In short, the vegetative bacterium will undergo an asymmetric cell division resulting in the formation of a mother cell and a forespore, each with a complete copy of the genome. The mother spore then utilizes all its resources to generate and deposit proteins that will compose a series of thin permeable (cortex, spore coat and exosporium) layers enclosing the tougher impermeable core. Once maturation is complete, the mother cell dies, lyses, and releases the spore into its surroundings. Fully formed spores are highly resistant to environmental insults [46, 47]that would rapidly and efficiently kill most bacterial species, including drying, extreme temperatures, ultraviolet light, ionizing radiation, disinfectants, hydrolytic enzymes and even prolonged periods of time.

The *B. anthracis* spore has an intricate structure composed of several layers[50]. The core is composed of small acid-soluble proteins (SASPs) and high calcium dipicolinic acid that protects the nucleoid from heat and radiation. The spore coat surrounds the cortex and it is followed by a peptidoglycan cortex that restricts[51] diffusion of molecules and maintain appropriately desiccated moisture levels within the core.

The outermost layer of the *B. anthracis* spore is the exosporium[50], which is in turn composed of two layers; the basal layer and the hair-like nap layer. The exosporium contains at least 20 proteins [52] and it is the major source of spore antigens [53]. The hair-like nap is composed of trimers of Bacillus collagen-like protein of Anthracis (BclA), the immunodominat protein of the exosporium[52]. BclA is anchored to the basal layer by an N-terminal domain[54], which is followed by a collagen-like region with multiple XXG domains[55]. A C-terminal globular domain promotes trimer formation[54].

Once the spore is exposed to multiple germinants (amino acids, ribonucleosides or peptidoglycan fragments) germination is triggered within the endospore. The spore has an influx of water, which rehydrates the core. The cortex and coat are rapidly shed and the bacterium resumes vegetative growth and is then able to produce the toxins associated with the disease.

Virulence Factors

The vegetative form of *B. anthracis* expresses three virulence factors: lethal toxin (LeTx), edema toxin (EdTx) and an anionic poly γδ-glutamyl capsule. Two large plasmids encode for these virulence factors; the toxins are encoded by the 183-kb pXO1 plasmid[43, 56-58], and the capsule is encoded by the *capBCADE* operon located on the 96-kb pXO2 plasmid[59, 60].

The capsule is the outermost layer of the vegetative bacterium, and is composed of repeating polymers of D-glutamic acid, which helps the capsule avoid phagocytosis and destruction by host immune cells[61], and more recently has been shown to suppress host immune response by delaying the maturation of immature dendritic cells[62]. Although the role the capsule plays in pathogenicity has not been fully understood, it has been shown that administration of capsule fragments enhanced cytotoxicity of lethal toxin in mouse macrophages. The Sterne strains lacks pXO2 and thus has lost the ability to produce capsule, hence it is used in veterinay vaccines and in most BSL2 laboratory studies.

Lethal and Edema Toxins

The tri-partite toxin encodeded by the pXO1 plasmid is classified as an A-B type toxin[63-67]. The protective antigen (PA), a cell-free secreted protein encoded by the gene *pagA*, serves as the B subunit for both toxins[63-67]. Toxin assemby is initiated when PA_{83} binds to tumor endothelial marker 8 (ANTXR1)[68] and capillary morphogenesis protein 2 (ANTXR2)[69]. ANTXR1 and ANTXR2 are surface associated proteins that may play a role in coagulation due to a von Willebrand factor A domain[70]. PA is responsible for for cell surface receptor binding and translocation of the pathogenic A subunits, Lethal Factor (LF) and Edema Factor (EF). In short, once PA_{83} binds to these receptors, a furin or furin-like protease cleaves it to form PA_{63}. PA_{63} then forms a heptamer (thus forming a prepore) that allows EF and LF to competitively bind to an exposed binding site on PA through a conserved domain on the N-terminal of each of the proteins [63-67]. The complex undergoes a clathrin-dependent endocytosis, and as the endocytic vesicle fuses with a cytoplasmic lysosome, acidification of the endosome leads to a conformational change that allows the pre-pore to insert into the endosomal membrane and allow release of EF and LF into the cell cytosol. Initially, it was thought that seven effector molecules could be translocated with each heptamer, but crystallography has shown only one molecule of EF or LF attached to the heptamer[71].

Edema factor (EF) is an 89 kDa molecule secreted as inactive adenylate cyclase during the bacterial vegetative state. Enzymatic activity of EF is calcium and calmodulin dependent. Once EF binds to calmodulin, there is a conformational change in the toxin that creates a pocket that allows the protein to bind a single calcium ion and a single molecule of 3'dATP, exposing and activating the catalytic site of the enzyme[72, 73]. EF

converts 3'dATP into cyclic adenosine monophosphate (cAMP). cAMP is a secondary cell messenger that participates in the regulation of many cellular processes. Elevation of cAMP triggers loss of water and ions from the cell, causing the characteristic edema found in anthrax infection[74].

Lethal factor (LF) is the major virulent factor responsible for the cytotoxicity and eventual death of the host. LF is a 90 kDa zinc-metalloprotease capable of cleaving and inactivating many cellular targets, specifically it targets for the cleavage and inactivation of the amino-terminus of mitogen activated protein kinase kinase (MAPKKs), disrupting cell signaling pathways causing apoptosis and rapid lysis of macrophages[75]. LT abolishes the expression of inflammatory cytokines. LT interrupts activation of tissue macrophages and reduces the expression and localization of co-receptors on antigen presenting cells. LT and ET inhibit and effective immune response and directly damage cells, suppress the host innate and adaptive immune response. LT and ET act synergistically to suppress early cytokine responses, inhibit neutrophil activation and chemotaxis, as well as interferon and NO production.

Cure and Prevention of Bacillus anthracis infections

B. anthracis provided the first scientifically reported example of antibiosis (antagonistic relationship between two microbes). In 1863, Pasteur injected laboratory animals with *B. anthracis* together with an unidentified bacterium, which prevented these animals from dying from anthrax[76]. This discovery inspired the work of Ernest Duchesne in 1897, who noted the rescue of animals infected with pathogenic bacteria after injection of supernatant of *Penicillum glaucum*. This observation paved the way by

the discovery of penicillin by Alexander Fleming in 1928[77, 78]. Although *B. anthracis* is sensitive to penicillin, the CDC recommends treatment with ciprofloxacin or doxycycline plus another antimicrobial for a period of no less than 60 days[79]. Initial therapy for localized or uncomplicated cases of naturally occurring anthrax includes oral antibiotic therapy, while those with advanced disease are treated with IV antibiotic treatment that includes an antimicrobial agent with CNS penetration[79].

Anti-toxin therapies

Several therapies that target the toxin components (specifically PA) are currently being investigated. Arthrivig (human anthrax immunoglobulin, or AIGIV) is derived from plasma of humans previously immunized with BioThrax (AVA) (Emergent BioSolutions Inc., Rockville, MD, USA) and is under development for the treatment of patients with manifest symptoms of anthrax disease. Although effective (89-100% protection in animal models) up to 12 hours post-exposure, the survival rate was only 39% if administered 24 hours post-exposure, highlighting the importance for early intervention[80]. Raxibacumab (ABthrax) (Human Genome Sciences; Rockville, MD, USA) is a human IgG1 monoclonal antibody that blocks PA binding to cell membrane receptors and has been approved by the FDA[35, 81]. Other anti-toxin approaches that target PA, LF and EF as well as DNA vaccination are currently under investigation[82-87].

Peptides

Antimicrobial peptides (AMPs) are short oligo- and poly-peptides (<100 amino acid) amphiphatic proteins with the ability to destroy microorganisms and inhibit their growth. The antibiotic activity of antimicrobial peptides is almost universally dependent upon interaction with the bacterial plasma membrane. This interaction is electrostatically driven, as the charge disparity between the cationic peptides and the negatively charged eukaryotic plasma membrane targets the bacteria, inducing membrane permeabilization on the pathogen[88]. Cathelicidins, a group of peptides characterized by conserved amino-terminal (cathelin-like) domain and a highly variable antimicrobial carboxy-terminal domain have been shown to *B. anthracis* spore killing activity in *in vitro*[89] and *in vivo*[90] models.

Vaccines

Vaccine induced protection with attenuated live bacteria was first recognized by W.S. Greenfield in 1880[14, 20]. Greenfield noted that continuous culture of *B. anthracis* "under specific conditions"[14] stripped the pathogen from virulence. However, it was Pasteur who established the vaccine schedule (two doses of heat-attenuated bacterium) adopted for use for animal vaccination over the next 50 years[20]. The vaccine was virtually unchanged until the 1920's introduction of glycerin and saponin. In 1938, Max Sterne identified an avirulent *B. anthracis* strain (34F2) that had lost its capacity to produce disease. This discovery led to the development of a live spore vaccine for veterinary use that has remained virtually unchanged until today[20].

In the 1930s and 1940s, a live spore vaccine (initially a combination of an acapsular strains STI-1 and NO 3) was developed in the USSR by Nikolai N. Ginsburg and made available for human consumption in the 1940s. This Live Anthrax Vaccine (LAV) can be administered via scarification or subcutaneous route, and aerosol immunization appears to be effective and safe[91-95]. This LAV PA^+ accelerated cell immunity that elicited protection 7-10 days after a single vaccination[94]. Although it appears to be well tolerated and protective to anthrax, it is consider unsuitable for human consumption in the Western world due to concerns of residual virulence. In the 1990's, PA adsorbed on Alum was added to LAV. These concerns led to the development of acellular vaccines in the UK and US that use PA to induce immunity[9, 20].

Anthrax vaccine, adsorbed (AVA or Biothrax® as is currently known) was developed in the US and is the only FDA-licensed anthrax vaccine in the US. It is prepared by aluminum hydroxide (alum) precipitation of *B. anthracis* Sterne strain, with PA as its main component. It has been shown that PA-based vaccines lower anthrax incidence among at-risk human populations[96]. Anthrax vaccine, precipitated (AVP), was developed in the UK and although uses the same key component as AVA, it has a lower PA concentration and higher concentration of EF and LT. Both vaccines require several doses that include annual booster to maintain efficacy. However, several studies have shown that the anti-PA humoral response is variable [97-99]. The intensive dosing regimen and concerns over the long-term efficacy, reactogenicity, practicality, and safety of both vaccines [20, 100, 101] have led to the search for improved vaccination strategies that might include a comprehensive approach that includes multiple targets for vaccination.

Among the new approaches investigated, new adjuvants that may increase immune response to PA are being considered. Newer adjuvants are reported to increase the immune response to PA vaccines as well as to Biothrax® and protect against infection in experimental models. PA combined with CpG Olygodeoxynucleotide (CpG ODN) –short, single-stranded DNA molecules that act as immunostimulants-, CpG ODN with the non-ionic block copolymer Pluronic F127, or the mast cell activator C48/90 increased the antibody response of animal models[102]. Conjugation of capsular proteins (as the capsule is the outermost layer of the vegetative bacterium) to protein carriers plus PA has shown induction to antibodies to PA and the capsule in mice[103].

Our Hypothesis

Vaccination with live toxinogenic, unencapsulated spores has been demonstrated to provide greater protection than PA based immunization[43, 104] which suggest that other antigens present in the spore may synergize with PA to provide better protection. Among prime candidates, BclA, the immunodominant protein in the exosporium, does not provide significant protection when administered alone in previous studies, but in combination with PA, increases protection against lethal challenge[105-107].

On the other hand, mucosal vaccination is one of the new anthrax immunization strategies being studied, as it theoretically offers the advantage of blocking the initial stages of infection when the initial spore-host contact occurs. This would prevent the germination of the spores, preventing or at least minimizing the production of toxins by the vegetative bacterium. However, data on the protection conferred by mucosal immunization has yielded ambivalent results[108, 109].

When we started these studies, we noted that passive administration of monoclonal antibodies to BclA provided A/J mice short-term protection against a lethal intratracheal anthrax spore challenge. We also noted that immunization with Complete Freund Adjuvant (CFA) and the C-terminus domain of BclA provided significant protection to subcutaneous anthrax challenge (Swiecki, unpublished results). Although previous studies that include DNA vaccination protocols that include plasmids encoding for BclA did not show protection[106, 107], recently, Köhler *et al.*, demonstrated that immunization with two plasmids (one encoding for BclA and another one for LF) offered 90% protection to lethal anthrax challenge. More importantly for our studies, immunization with plasmid encoding for BclA only, offered significant protection to lethal anthrax challenge [110]. In our studies, we sought to determine if antibodies to components of *B. anthracis* exosporium protects against a lethal intratracheal anthrax challenge.

Experimental Rationale

To test this hypothesis, we chose to focus on antibodies to BclA. We chose to use BclA as a vaccination target because it is the immunodominant and outermost component of anthrax spores (REF), and immunization with exosporium lacking both plasmids conferred protection to inhalational anthrax. Using a novel, three-time weekly intranasal immunization approach, we immunized $C5^{-/-}$ mice with recombinant BclA (rBclA) or exosporium in Cholera Toxin (CT). CT is a potent mucosal adjuvant [111-113] and promotes long-term immunological memory at mucosal surfaces[111, 114]. We chose a

mucosal adjuvant and a mucosal delivery method to target the spore during the initial stages of infection when the initial spore-host contact occurs and before toxins can be produced by the vegetative bacterium. Additionally, the development of highly specific mAbs to BclA would allow us to further elucidate the initial antibody-spore-host interactions. As our vaccination model, we chose mucosal vaccination

As our animal model we chose the complement 5 deficient ($C5^{-/-}$) C57BL/6 mice. *B. anthracis* animal model studies often use A/J and DBA/J mouse strains, because of their susceptibility to *B. anthracis* infection with the unencapsulated Sterne strain due to a defect in the mouse complement factor 5 (C5) locus [115, 116]. However, several issues inherent to the A/J and DBA/J strains [117] such as abnormal immune responses, breeding challenges, and lack of genetically manipulated strains renders A/J mouse an unwieldy model; it furthermore may be inefficient and imprecise as an *in vivo* model of *B. anthracis*:host interactions.

The well-defined C57BL/6 mouse is used as a background strain for a wide array of gene-targeted models, which makes C57BL/6 a highly suitable background for investigating the fine details of host requirements during interactions with *B. anthracis* spores. However, C57BL/6 mice are naturally resistant to inhalational Anthrax disease [115]. Because C57BL/6 mice can become susceptible to inhalational Anthrax by cobra-venom factor treatment with [116], mice gene targeted for specific complement components would likely also be susceptible without treatment. A complement-component gene-targeted model would lack the specific component without requiring prior systemic activation of the complement system, thus completely avoiding the nonspecific inflammatory consequences occurring in venom-treated mice. To address the

limitations of A/J strain-based Ba models, we utilized C57BL/6 mice rendered complement-component deficient ($C5^{-/-}$) in our Ba infection studies, and characterized that model within these studies.

In summary, we hypothesized that mucosal immunization with BclA using CT as adjuvant in a new mouse model would confer protection to a lethal intratracheal anthrax challenge. As described in the enclosed manuscript, we were able to demonstrate that mucosal immunization with exosporium components and CT provided protection to a lethal anthrax challenge. We were also able to demonstrate that in the presence of mAbs to BclA, there is a significant increase in spore uptake by phagocytes. We also demonstrated that the initial spore-mAb-macrophage interaction is mediated by Fc receptors, which promoted endo-lysosomal fusion, increased endosomal compartment acidification, and subsequently promoted spore destruction within macrophages. These results show that vaccine-elicited antibodies targeting BclA can lead to protection from infectious Ba spores by promoting their uptake and destruction. Furthermore, as others have shown, we demonstrate that BclA-targeted immunity could be a major supplement to current toxin-based vaccines, and that BclA-specific mAbs represent an important addition to current antibiotic regimens used to treat *B. anthracis*, and may be of highly significant value in the treatment of multiple antibiotic-resistant *B. anthracis* strains or bioengineered strains.

BCLA PROTEIN ANTIGEN-TARGETED IMMUNITY POTENTIATES PROTECTION AGAINST LETHAL *BACILLUS ANTHRACIS* INFECTION

by

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Running title: Anti-BclA antibodies promote lysosomal destruction of anthrax spores

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Abstract

Bacillus anthracis (Ba) is a Gram,-positive zoonotic bacterium that causes Anthrax Disease. Although the infectious form of Ba is its endospore (spore), only the tripartite toxin-producing vegetative bacterium causes disease pathology. Although current vaccination strategies target the Protective Antigen (PA) component of this toxin, vaccine-elicited immunity to the Ba spore form is lacking. Nonetheless, vaccines targeting spore components of Ba have the potential to neutralize the infectious form of Ba. Therefore, we asked if antibodies elicited by immunization with the main antigenic component of the spore surface, Bacillus collagen-like protein of Anthracis (BclA), can mediate immunity and protect against lethal Ba infection. Intranasal immunization with recombinant BclA and cholera toxin provided significant protection from lethal intratracheal Ba challenge in complement component 5-deficient ($C5^{-/-}$) C57BL/6 mice. Importantly, passive transfer of BclA-specific monoclonal antibodies (mAbs) similarly protected against lethal Ba challenge, significantly increased spore uptake by macrophages, and lead to enhanced spore-destruction *in vitro*. The effects of BclA-specific mAbs were mediated by Fc receptors, which promoted endo-lysosomal fusion, increased endosomal compartment acidification, and subsequently promoted spore destruction within macrophages. Collectively, these results indicate that vaccine-elicited antibodies targeting BclA can lead to protection from infectious Ba spores by promoting their uptake and destruction. BclA-targeted immunity could be a major supplement to current toxin-based vaccines. Furthermore, BclA-specific mAbs represent an important addition to current antibiotic regimens used to treat Ba, and may be of highly significant value in the treatment of multiple antibiotic-resistant Ba strains or bioengineered strains.

INTRODUCTION

Anthrax Disease is caused by the ubiquitous Gram-positive, spore-forming bacterium *Bacillus anthracis* (Ba). The disease presents in 3 forms: cutaneous, intestinal, and inhalational, with the latter usually being fatal and refractory to aggressive antibiotic therapy [6]. Although natural Anthrax Disease is uncommon, possible bioterrorism threats of, exemplified by the 2001 anthrax spore attacks, highlight the importance of developing improved vaccine and therapeutic strategies against this potential vector of biological warfare.

The vegetative stage of Ba is associated with production of a tripartite toxin that cause the pathologies associated with Anthrax. These toxins are produced by the vegetative bacteria when lethal factor (LF) and edema factor (EF) combine with a heptamer of protective antigen (PA) to form the Lethal (LTx) and edema (ETx) toxins[118]. Another important virulence factor produced by the vegetative bacteria is the Poly-γ-d-Glutamic capsule that allows the bacterium to evade phagocytosis[119-121]. The currently licensed anthrax vaccine, Anthrax Vaccine Adsorbed, targets PA, thereby restricting its efficacy to the Ba vegetative form . Herein, we investigated if a vaccination strategy targeting spore components could promote Ba neutralization at the initial point of spore-host interaction, thereby providing protection from vegetative Ba and the tripartite anthrax toxin. Bacillus collagen-like protein of Anthracis (BclA) is the major antigenic component in the Ba spore basal layer [52, 122], and as such, represents a prime candidate for vaccine development in strategies targeting the spore stage of Ba. In

these studies, we revisited how spore-specific antibodies affect the pathogenesis of Ba infection in mice [105, 123].

Ba infection studies often use A/J and DBA/J mouse strains, because of their susceptibility to Ba infection with the nonencapsulated Ba Sterne strain due to a defect in the mouse *complement factor 5* (*C5*) locus [115, 116]. However, several issues inherent to the A/J and DBA/J strains [117] such as breeding challenges , abnormal immune responses, and lack of genetically manipulated strains renders A/J mouse an unwieldy model; it furthermore may be inefficient and imprecise as an *in vivo* model of Ba:host interactions. Therefore, we explored other animal models for our studies. The well-defined C57BL/6 mouse is used as a background strain for a wide array of gene-targeted models, which makes C57BL/6 a highly suitable background for investigating the fine details of host requirements during interactions with Ba spores. However, C57BL/6 mice are naturally resistant to inhalational Anthrax disease [115]. Although C57BL/6 mice can become susceptible to inhalational Anthrax by cobra-venom factor treatment with [116], mice gene targeted for specific complement components would likely also be susceptible without treatment. importantly, a complement -component gene-targeted model would intrinsically lack the specific component without requiring prior systemic activation of the complement system, thus completely avoiding the nonspecific inflammatory consequences occuring in venom-treated mice. To address the limitations of A/J strain-based Ba models, we utilized C57BL/6 mice rendered complement-component deficient ($C5^{-/-}$) in our Ba infection studies, and characterized that model herein.

Using this model, we found that intranasal (i.n.) immunization with recombinant Bacillus collagen-like protein of Anthracis (rBclA) and Cholera Toxin (CT), as a mucosal

adjuvant, led to production of BclA-specific IgM and IgG antibodies, and provided significant levels of protection in a lethal i.t. Ba spore challenge. Importantly, immunization with whole exosporium led to even more enhanced protection from lethal spore challenge, illustrating the promise of spore-targeting vaccination strategies in host defense. We also show that passive transfer of BclA-specific monoclonal antibodies (mAbs) provides similar levels of protection to a lethal i.t. challenge as immunization. *In* vitro, BclA-specific antibodies significantly increased spore uptake by phagocytic cells, including the RAW264.7 cell line, bone marrow-derived macrophages (BMDM), and dendritic cells (BMDC). Although anti-BclA antibodies did not affect vegetative Ba outgrowth, they increased spore uptake five- to ten-fold, and significantly decreased intracellular bacterial survival.

Previous studies have suggested that Fc receptors participate in antibody-mediated protection against some intracellular organisms [124, 125]. In the current study, we show evidence that FcγR is at least partially responsible for the enhanced killing mediated by BclA-specific antibodies. Blocking anti-BclA/FcγR interactions with a FcγR-antagonist mAb = significantly reduced spore uptake by macrophages, however these effects appeared more pronounced for certain IgG antibody sub-types. To investigate how the anti-BclA/ FcγR interactions led to increased spore-killing, we assessed phagosomal-compartment acidification and phago-lysosomal fusion. We demonstrate here that anti-BclA Ab and FcγR synergize to more efficiently target Ba spores to the lysosomal compartments, subverting the mechanisms that allow Ba to otherwise escape lysosomal destruction[121], and reducing the intracellular burden of viable Ba bacteria. Overall, these results suggest that BclA-specific humoral immunity offers protection from

inhalational anthrax infection and highlight the potential to augment protection by incorporation of BclA into the vaccination regimen.

Materials and methods

Mice

6- to 8-week-old, male and female C57BL/6 and A/J male and female mice were purchased from the Jackson Laboratory. C5-deficient mice ($C5^{-/-}$) on C57BL/6 background were kindly provided by Dr. Alex Szalai (UAB, Dept. of Medicine). Mice were housed under specific pathogen-free conditions and used according to protocols approved by the University of Alabama at Birmingham (Birmingham, AL). $C5^{-/-}$ and A/J mice were used for inhalational virulence studies owing to their susceptibility to death by spore administration.

Spore preparation

Attenuated *B. anthracis* ($pX01^{+}/pX02^{-}$) Sterne 34F2 spores were obtained from Dr. Charles Turnbough, Jr. (UAB) and prepared as previously described [52, 122]. Briefly, sporulation was induced in bacterial cultures with Difco sporulation medium, then harvested spores were purified from vegetative remnants using a series of thorough washes with distilled (DI) water and a 50% Renografin (Bracco Diagnostics) gradient. Purified spores were suspended in DI water and stored at 4°C, protected from light. Spores and bacilli were quantified microscopically with a Petroff-Hausser bacterial counting chamber for all experiments unless otherwise noted.

Intra tracheal (i.t.) challenge and survival studies

Groups of 6-8 A/J, C57BL/6, and $C5^{-/-}$ C57BL/6 mice were anesthetized with Isoflurane (Nova Plus). Anesthetized mice were infected i.t. with 10^3-10^9 Sterne spores (when establishing LD50) in 50μl of PBS, or with a lethal dose of 10^7 for lethal challenges via plastic tip pipette. Animals were monitored for morbidity and mortality every four hours daily starting 2 days post-infection until moribund, and tissues were collected at days 3 and 5 to enumerate bacterial burden by colony plating as described below.

Tissue culture:

The RAW 264.7 cell line (TIB-71 ATCC) was grown in DMEM medium with 10% heat-inactivated fetal bovine serum (FBS). Bone marrow-derived macrophages and dendritic cells from C57BL/6 mice were prepared by culturing mouse bone marrow cells in RPMI with 10% heat-inactivated FBS plus 10 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) for 8 days.

Spore Uptake and Flow Cytometry:

Phagocytic cells (bone marrow derived macrophages or dendritic cells) were seeded at $5x10^4$ cells per well in 48-well plates (Costar) in 10% heat-inactivated FBS DMEM, then incubated overnight at 37°C. Approximately $2x10^9$ spores were labeled with Alexa Fluorochromes according to instructions (Molecular Probes/Invitrogen, Carlsbad, CA), and several different spore preparations (either labeled or unlabeled) of each spore type were used as indicated. Alexa labeling does not affect spore viability nor does it appear to influence spore-cell interactions[126]. Fluorescently labeled spores were incubated with

10 μg/ml anti-BclA or an isotype control mAb in 1% BSA/PBS for 30 mins on ice to promote opsonization. Cells were infected with opsonized spores in triplicate wells at an MOI of 10:1. Plates were centrifuged for 5 mins at 1200 RPM to promote spore-cell interaction, and incubated for 2 hrs at 37°C. Cells were washed three times with PBS, removed by mechanical pipetting, or by trypsinization, then stained with propidium iodide to distinguish intact viable from non-viable cells. Spore uptake within the viable cells was assessed by flow cytometry, and differentiated from spore adhesion via Trypan blue-mediated quenching of surface-bound exposed fluorescence of Alexa Fluor 488-labeled organisms [126]. Flow cytometry was performed with a FACSCalibur (BD Biosciences, San Diego, CA), and data were analyzed with FlowJo software from Tree Star (Ashland, OR) unless otherwise noted.

Fc Receptor-blockade:

FcγR in RAW cells and other cell lines were blocked for 30 mins at 4°C with a mixture of 1μg/ml each anti-mouse CD16/32 antibody (2.4G2, BD Pharmigen) and anti-mouse CD16/32 Ab93 (Affymetrix, eBioscience). After blocking, opsonized spores were added to cells, and spore uptake was and measured in the same manner as the spore uptake assay described above.

CFU studies

CFU studies on RAW cells were performed by methods used elsewhere with some modifications. One day prior to the experiment, cells were seeded into 24-well plates at $\approx 5 \times 10^5$ cells/well. 10^6 spores/well were pre-opsonized for 30 mins at 4°C with 10μg/ml

anti-BclA or an isotype control. Spores were added to RAW264.7 macrophages, and centrifuged for 1 min at 1200 RPM prior to incubation at 37°C for 30 minutes. Cells were washed three times with PBS and 50μg/ml gentamicin to remove non-internalized spores and destroy extracellular bacteria. Cells were washed three times to remove gentamicin and resuspended in serum-free DMEM, and subsequently incubated for 0, 1, 3 and 5 hour timepoints, at which point cells were scraped and lysed. The resulting lysates were plated in brain heart infusion agar plates (BHI) for colony counting. Data are presented as colony forming unit (CFU) per macrophage and the percent survival of spores within macrophages at each subsequent timepoint compared to the sample taken at 0 hr. CFUs were counted 24 hr after plating.

Acidification germination studies

Wild type Sterne spores were incubated in 10% BHI media (Difco) PBS at 37°C for 8 hr at pH 5.7, 6.0, 6.4, 6.9, 7.2, or 7.9. Samples were removed at various timepoints, and kept on ice. Samples were washed and stained with anti-BclA (JC8-5-PE) and/or an anti-cell wall mAb (EAII-FITC) for 20 mins on ice. Samples were washed and analyzed by flow cytometry. Dot plots and histograms showing spore and vegetative populations were generated with FlowJo.

Serum Antibody Analysis

Quantitative ELISAs were performed as previously described (Swiecki, 2006). Briefly, high-binding 96-well EIA/RIA plates (Costar) were coated overnight with recombinant BclA, BxpB at 1 μg/ml or exosporium (at 1 μg/ml of rBclA) in 0.1 M borate saline at

4°C. Plates were washed four times with PBS using an automatic plate washer. Wells were blocked for 30 min at room temperature with 1% BSA in PBS, and then washed four times. Then, 100 µl/well of diluted mouse sera (1 µl sera/ml) or purified anti-BclA Ab standards (IgM, IgG1, IgG2a, IgG2b, and IgG3) were added to the first row of 96-well plates. Samples and standards were then serially diluted 1/4. Plates were incubated at 37°C for 2 h and then washed four times. Dilutions of 1/500 of goat anti-mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 coupled with alkaline phosphatase (AP)[3] from Southern Biotechnology Associates were added to the plates (50 µl/well). Plates were incubated for 2 h at 37°C and then washed 4 times. AP-substrate (Sigma-Aldrich) was dissolved in AP-substrate buffer (1 mg/ml) and added to wells (50 µl/well) for 5–10 min at room temperature. Reactions were stopped by adding 25 µl/well of 5 N NaOH. Optical density was read at 405 nm with a Titertek Multiskan Plus ELISA reader (Flow Laboratories), and concentrations of serum Ig specific for BclA, BxpA, and BxpB were determined by Delta Soft ELISA analysis software (Bio-Tek). Sera, purified Abs, and secondary Abs were diluted in 1% BSA in PBS.

Intranasal immunization

Three groups of 6-to 8 –week-old $C5^{-/-}$ mice (10 mice per group) were immunized intranasally (i.n.) three times with (1µg) CT alone, with either 5µg of rBclA in exosporium or 5µg of recombinant BclA on days 0, 7 and 14. Prior to immunizations, mice were sedated with ketamine. 5µl of the vaccine preparation or the (CT-only control were pipetted directly into one nostril per mouse. After 3 min., 5µl of the vaccine preparation was pipetted directly into the other nostril for a total of 10µl per mouse.

Intraocular blood was collected on days 0, 10 and 20 to measure anti-BclA. Immunized mice were challenged i.t. with a lethal Ba dose (10^7). Morbidity and mortality were monitored 2 days after challenge.

Histology

Lungs: To assess lung morphology, lungs from 6- to 8-week-old C5+/+ C57BL/6, $C5^{-/-}$ and A/J mice were collected, then inflation-fixed for immunostaining, and light microscopy as previously described [127]. Briefly, 1ml of a 1:1 PBS: OCT mixture was injected into the lungs and blocked with OCT in 2-methylbutane (Sigma-Aldrich) submerged in liquid nitrogen. The lungs were cut into 4-μm thick sections with a cryostat at -18°C (IEC Minotome; International Equipment) and kept at -70°C until staining after fixation with acetone. Slides with these frozen and fixed sections were blocked for 20 min. at room temperature with PBS plus 10% horse serum, then stained for 20 min. at room temperature with fluorescently-labeled antibodies.

Gross pathology and histopathology: Groups of 3 mice per strain (C5+/+ C57BL/6, $C5^{-/-}$, A/J) were euthanized on days 3 and 5 post-i.t. challenge with 10^7 spores. Lung, blood, liver, and spleen were collected. Tissue samples were preserved in 10% neutral-buffered formalin for 24 hrs prior processing. Samples were paraffin embedded, processed to slides and stained with hematoxylin and eosin (HE).

ImageStream Colocalization Studies.

RAW264.7 cells were seeded at $1x10^6$ cells per well in 48-well plates (Costar) in 10% heat-inactivated FBS DMEM. Approximately $1x10^7$ spores per well were labeled with

Alexa Fluorochromes according to instructions (Molecular Probes/Invitrogen, Carlsbad, CA). Alexa labeling does not affect spore viability nor does it appear to influence spore-cell interactions [ref]. Fluorescently labeled spores were incubated with 10 μg/ml anti-BclA or an isotype control mAb in 1% BSA/PBS for 30 mins on ice to promote opsonization. Cells were infected with opsonized spores in triplicate wells at an MOI of 10:1. Plates were centrifuged for 5 mins at 1200 RPM and incubated for 2 hrs at 37°C. Cells were washed three times with PBS, removed by mechanical pipetting. Cells were permeabilized with BD Cytofix/Cytoperm kit according to instructions. Lysosomes were labeled with a commercially available antibody to Lamp1 for 30 mins and spore uptake and colocalization with lysosomal compartments within cells was assessed by ImageStream. Data were analyzed with ImageStream software unless otherwise noted.

Statistical Analysis

Differences in percent survival and mean-time-to-death (MTD) for mice challenged subcutaneously with Sterne spores following different immunization schemes were determined by Fisher's Exact Test and Kaplan–Meier Analysis, respectively. Differences in antibody titers generated by immunization were determined by one-way analysis of variance (ANOVA) followed by Tukey's pairwise post hoc comparisons. Fisher's Exact Test was used to analyze differences in outcomes for mice immunized with PA alone versus PA plus additional antigen(s) among low PA responders. To compare differences in germination rates among strains, changes in log10 counts of bacteria from *t*0 to *tx* were analyzed by a repeated measures ANOVA followed by one-way ANOVA and Tukey's pairwise post hoc comparisons. Differences in spore uptake by macrophages and

differences in percent survival of phagocytosed spores following incubation with different antibodies were compared by one-way ANOVA followed by Tukey's pairwise post-hoc comparisons.

Results

C5-deficient C57BL/6 mice display prolonged disease compared to A/J mice

As discussed above, C57BL/6 mice are naturally resistant to inhalational Anthrax disease [115]; however this strain offers a wide array of gene-targeted models that make it an otherwise highly suitable background for investigating the fine details of host requirements during interactions with Ba spores. It is therefore advantageous to develop methodologies for using this genetic background in inhalational Ba studies. To this end, we hypothesized that $C5^{-/-}$ C57BL/6 mice may be more phenotypically similar to the naturally C5-deficient A/J mice in the context of inhalation anthrax disease. Initially, we assessed the vulnerability of $C5^{-/-}$ C57BL/6 mice to intratracheal (i.t.) Ba challenge, and established the LD50 for an i.t. model of infection using our preparations of Ba Sterne spores. We administered between 10^3-10^8 spores i.t. in log-increments to $C5^{-/-}$ C57BL/6 mice. Although $C5^{-/-}$ C57BL/6 mice were resistant to infection at as high of a dose as 10^5 spores, we observed mortalities at 10^6 spores, and $\geq 10^7$ resulted in 100% mortality. We found that the LD50 for our i.t. Ba spore challenge was approximately 3.2×10^6 spores (Figure. 1A), and opted to use 10^7 spores, or ~3xLD50 for our immunization studies.

We next characterized the disease course of $C5^{-/-}$ C57BL/6 mice relative to $C5^{+/+}$ C57BL/6 and the commonly utilized A/J strain with our established 3xLD50 Sterne-spores dose. As previously reported, C5-sufficient C57BL/6 mice proved resistant to

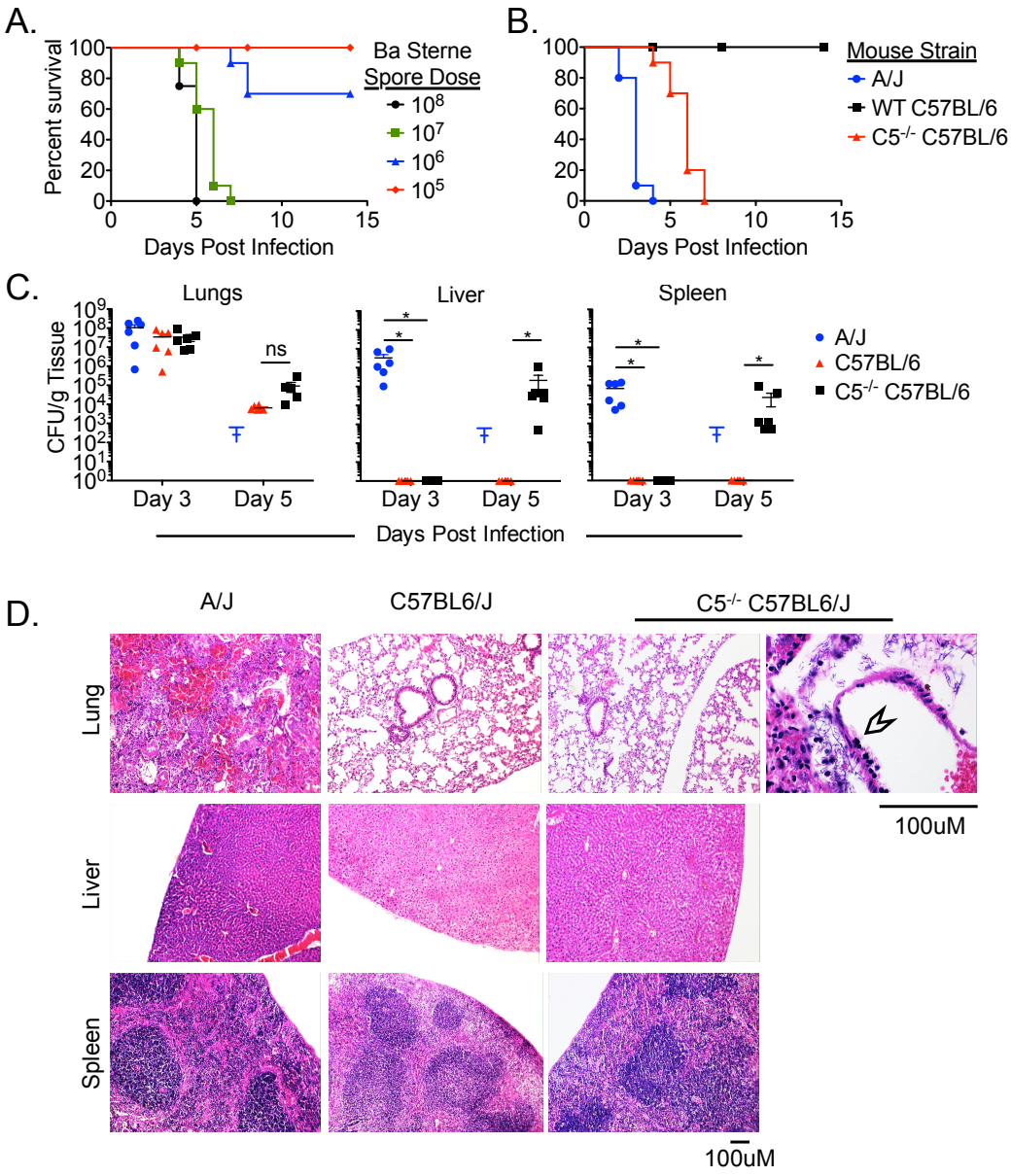

Figure 1. C5-deficient C57BL/6 mice are a suitable model for Anthrax studies and display bacterial dissemination similar to the classic A/J model but with protracted kinetics. (A) Survival curve of $C5^{-/-}$ C57BL/6 mice after i.t. infection with Sterne strain Ba spores. (B) Survival curves comparing the relative susceptibility of different mouse strains to i.t. Ba challenge and the infectious pathology of $C5^{-/-}$ C57BL/6 mice with the classically used A/J mouse strain and C5-competent C57BL/6 mice. (C) Bacterial load in tissues after respiratory Ba infection. (D) H/E staining after lethal i.t. Ba challenge 3 and 5 d.p.i.

inhalational infection [115], whereas A/J mice died 2-3 days post-infection. $C5^{-/-}$ C57BL/6 displayed protracted disease kinetics relative to A/J mice, but eventually died 5-7 days post-infection (Figure 1B). There was a statistically significant difference in mean time of death (MTD) between $C5^{-/-}$ C57BL/6 (5.5 days) and A/J (3 days) mice. We speculate that the difference in MTD results from the previously mentioned intrinsic deficiencies in A/J mice.

Previous reports suggest the cause of death in inhalational Ba in A/J mice is systemic spread and bacterial overgrowth [116]. Therefore, we examined whether $C5^{-/-}$ C57BL/6 mice die in a similar manner, by measuring Ba burden in lungs, as well as in the spleen and liver as a measure of dissemination, following lethal i.t. challenge with Sterne spores. We next compared the pathological features in the lung, spleen, and liver of A/J, $C5^{-/-}$ and $C5^{+/+}$ C57BL/6 mice after i.t. lethal challenge with Ba Sterne. Tissues were collected 3 and 5 and evaluated for bacterial out growth by colony forming unit enumeration and histological analysis. These post-infection time points were chosen for collection due to differences in MTD between A/J mice (~3 days) and $C5^{-/-}$ C57BL/6 mice (5-6 days), because they represent terminal stages of inhalational anthrax in each strain; tissues from A/J mice could not be collected at the 5 d.p.i. time point and all animals had died by day 4. At day 3, we observed high numbers of both vegetative bacteria and spores (10^8 for A/J, 10^7 for C57BL/6) in the lungs (Fig. 1c). This bacterial burden is likely due to spore uptake by resident macrophages and subsequent bacterial germination and dissemination throughout the host, possibly returning the bacterium to the initial infection site. Higher CFU counts at this time point in A/J mice compared to $C5^{-/-}$ and $C5^{+/+}$ C57BL/6 mice suggest less clearance that may be due to intrinsic immune

defects in A/J mice. At 3 d.p.i., $C5^{-/-}$ C57BL/6, $C5^{+/+}$ C57BL/6, and A/J mice all showed perivascular rod-shaped bacteria contained in the alveolar spaces of their lungs, consistent with macroscopic signs of pneumonia, and specifically, Ba-mediated disease, including fibrin deposits, edema, hemorrhage, and necrosis in alveolar spaces (Figure 1D).

Lethally challenged A/J mice also displayed significant vegetative bacteria and spore burden in the liver (~10^6/g, Figure 1C) and spleens (~10^5/g, Figure 1C) by 3 d.p.i., coinciding with the rapid development of morbidity and subsequent mortality in this group. At this time point, A/J mice showed significant microscopic alterations in splenic architecture [128, 129] including fibrin, with lymphoid depletion that includes nuclear fragmentation, as well as the presence clusters of rod-shaped bacteria. Clusters of bacilli were surrounded by neutrophils, and were readily noted in all structures of A/J mouse livers, as was neutrophil infiltration, congestion, and fibrin deposits. On day 3 after infection $C5^{-/-}$ C57BL/6 and $C5^{+/+}$ C57BL/6 mice showed normal splenic structure., without signs of infection. Consistent with these observations, neither vegetative bacteria nor spores were detectable in the liver or spleen of $C5^{-/-}$ or $C5^{+/+}$ C57BL/6 mice (Figure 1C). By 5 d.p.i however, $C5^{-/-}$ C57BL/6 mouse livers and spleens had high counts of vegetative Ba (10^5, 10^4 respectively), coinciding with development of anthrax-disease symptoms, whereas spleens and livers of $C5^{+/+}$ C57BL/6 mice remained free of Ba (Figure 1d,e). On day 5 $C5^{-/-}$ C57BL/6 spleens showed rod-shaped bacteria, fibrin and lymphoid depletion, similar to the pathology observed in the spleens of A/J mice 3 d.p.i.., The livers of $C5^{-/-}$ C57BL/6 mice additionally developed neutrophil infiltration,

congestion, fibrin deposits and hemorrhage, although neither liver nor spleens from $C5^{+/+}$ C57BL/6 spleens showed any abnormalities at either time point.

Collectively, these observations suggest that the emergence of mortality in both the A/J and $C5^{-/-}$ C57BL/6 mice coincides with Ba dissemination in the host. MTD correlates with detection of vegetative bacteria in the spleen and liver, and histological analysis demonstrates pathologies associated with bacterial spread and overgrowth in both strains. Furthermore, by 5 d.p.i, CFU in the $C5^{-/-}$ C57BL/6 lungs had started to decrease, consistent with effective bacterial clearance in the C57BL/6 strain. These results demonstrate that $C5^{-/-}$ mice did not die of pneumonia, but instead similarly to the A/J mice, bacteremia was the mode of death, suggesting that $C5^{-/-}$ C57/BL6 mice are an effective mouse model for inhalational anthrax studies.

Intranasal immunization with rBclA or exosporium confers limited protection to lethal i.t. challenge

BclA is the major antigenic component on the Ba spore basal layer; however adding rBclA as an antigenic component to current vaccination protocols has been disputed, with some groups claiming immunization with rBclA shows either no effectiveness as a booster to PA vaccination [130] or limited but significant protection [110]. To help understand what immune reactions might correlate with and be responsible for these contradictory outcomes, we asked whether immunization with recombinant BclA (rBclA) or purified exosporium produces a measurable immune response and antibody-mediated protection. Using a novel, three-time weekly intranasal immunization approach, we immunized $C5^{-/-}$ mice with rBclA or exosporium in CT. CT

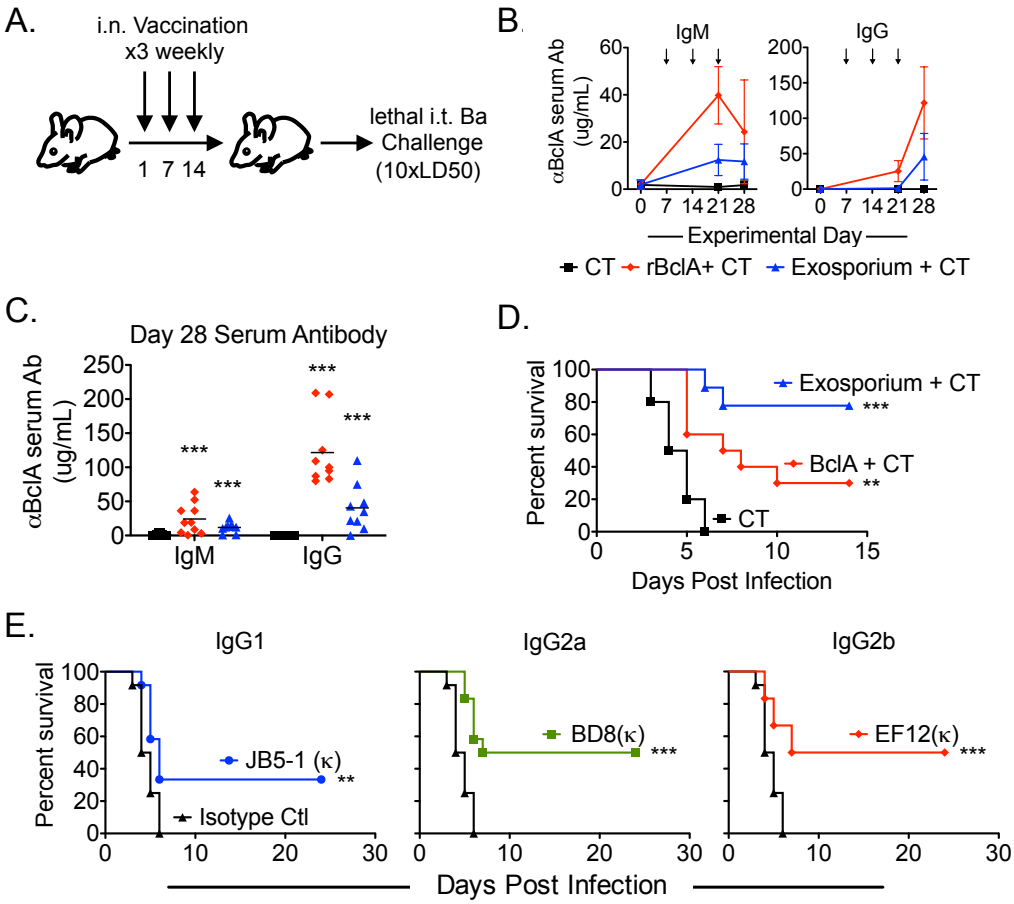

Figure 2. Antibodies induced by intranasal immunization with rBclA in CT, or passive transfer of various BclA-specific monoclonal antibodies confers protection from lethal Ba challenge. (A) Schematic diagram depicting immunization time course of $C5^{-/-}$ C57BL/6 mice. (B) BclA-reactive serum antibody concentrations measured by ELISA demonstrates that mice immunized with either whole exosporium or rBclA generate measurable levels of BclA-specific IgM and class-switched IgG antibodies. (C) Day-28 BclA-reactive serum antibody. (D) Survival curves of actively and passively immunized $C5^{-/-}$ C57BL/6 mice challenged i.t. with Ba. (E) Passive transfer of various anti-BclA IgG antibodies (200 μg) provide protection to lethal i.t. B.a. challenge.

is a potent mucosal adjuvant and promotes long-term immunological memory at mucosal surfaces.

We used $C5^{-/-}$ C57BL/6 mice for out BclA immunization studies, as we demonstrated they are a suitable surrogate for inhalational anthrax studies. We collected serum from all mice one day prior to the first immunization, 7 days after the second immunization (day 21), and 7 days after the third immunization (day 28) (Figure 2A). We then measured anti-BclA IgM and IgG serum antibodies by ELISA. Mice receiving rBclA+CT had high amounts of detectable IgG and IgM anti-BclA at our first serum-collection time point (7 days after the second immunization) and these levels continued to rise after the third immunization (Figure 2B). Notably, anti-BclA IgG and IgM levels were higher in rBclA-immunized mice compared to exosporium-immunized mice. One potential explanation for this difference in anti-BclA antibody levels may result from differences in glycosylation between the native BclA protein present in the exosporium and the non-glycosylated rBclA. Overall, these results demonstrate that i.n. immunization with exosporium or antigen-subunit vaccine containing the exosporium component BclA mounts a strong antigen-specific Ab response.

Intranasal immunization with rBclA or passive transfer of BclA-specific mAbs confer limited protection against lethal i.t. Ba Sterne spore challenge

Immunization through different routes with inactivated exosporium or exosporium components have shown various degrees of protection against inhalational anthrax [105, 130]. The exosporium used in these previous studies consisted of formalin-inactivated spores adjuvanted with alhydrogel. We have shown strong responses elicited

by i.n. immunization with exosporium adjuvanted with CT (Figure 2B,C). To test if this immunization strategy provides protection from inhalational anthrax challenge, we challenged $C5^{-/-}$ C57BL/6 mice intra tracheally with 3xLD50 following three consectuive i.n. immunizations with rBclA or exosporium in CT. Consistent with the notion that antibodies generated following i.n. immunization with rBclA are protective against inhalation anthrax disease, $C5^{-/-}$ C57BL/6 mice that were immunized with CT+rBclA showed partial protection (30% survival) whereas immunization with exosporium provided 80% protection in this model. None of the mice that received CT-alone survived the challenge (Fig. 2D). These results show that i.n. immunization with rBclA or exosporium adjuvanted with CT provide protection against lethal Ba challenge, and suggest that exosporium and exosporium components function as vaccine adjuvants.

Because the vigorous production of anti-BclA serum antibodies following after i.n. immunization of $C5^{-/-}$ C57BL/6 with rBclA (Figure 2A, B) was accompanied by partial protection from lethal Ba challenge (Fig. 1D), we next tested the ability of passively administered anti-BclA mAbs [53] to provide protection in our model of inhalation anthrax infection. We selected several hybridoma-derived mAbs of differing isotype subtypes: JB5-1 (IgG1κ), BD8 (IgG2a) and EF12 (IgG2b). 200 μg of each mAb was administered i.p. 24 hrs preceding a lethal i.t. challenge of 10^7 Ba spores. BclA-specific mAbs of the IgG2 subclass proved slightly superior in preventing Ba dissemination and outgrowth- administration of either BD8 or EF12 resulted in protection of ~50% of animals. The IgG1 mAb JB5-1 also protected a significant portion of animals, corresponding to ~35% survival (Figure 2E). This result shows that antibodies against BclA confer limited protection against inhalational anthrax, and suggest a contribution of

exosporium components as adjuncts for current vaccination therapies and treatment in anthrax infections.

Anti-BclA mAbs promote spore uptake by the RAW macrophage cell line, by BMDM, BMDC,as well as by alveolar and peritoneal macrophages.

To begin dissecting how anti-BclA Abs confer protection from infectious Ba spores *in vivo*, we studied the effects of anti-BclA mAb opsonization of Ba spores in isolated phagocytes. Briefly, we tracked the uptakeke of fluorescently-labeled spores to by phagocytic cells through *in vitro* flow cytometric phagocytosis assays. We also quantified the effects of BclA-specific mAbs on the uptake of the labeled spores. Representative flow cytometric plots from these phagocytosis assays are shown in Figure 3A; phagocytosis of Alexa-488 labeled spores resulted in an easily resolvable 2-log increase in fluorescence. We observed that basal levels of spore uptake In the presence of IgG isotype-matched control of approximately 20% of RAW264.7 macrophages were spore positive. Challenge of RAW264.7 macrophages with anti-BclA-opsonized spores resulted in significantly increased spore uptake ranging from 40-80%, depending on the mAb clone used (Figure 3B). Interestingly, degree of spore uptake appeared to segregate with IgG antibody subclass. Although IgG2b antibodies performed poorly in this assay, Ig2a mAbs were the strongest, and the IgG1 antibody JB5-1 was an intermediate performer (Figure 3c). Intriguingly, in passive transfer model, EF12 was highly effective at promoting mouse survival, suggesting that complement, which was irrelevant in these *in vitro* assays, may contribute to *in vivo* protection.

Careful examination of the flow cytometric data from these assays reveals discrete segregation of the spore-positive macrophage population into distinct populations of varying fluorescent intensity- corresponding to the number of phagocytosed spores. Importantly, macrophages challenged with anti-BclA opsonized spores reached higher maximum and mean spore-fluorescence values, representing significant increases in not only the percent of phagocytes that had ingested spores, but the number of spores that they had ingested as well. Interestingly, in the presence of IgM mAbs, there was no significant increase in spore-bearing cells suggesting the significant involvement of the complement system in IgM antibody-mediated opsonophagocytosis. We noted similar effects of anti-BclA antibodies mediating increased spore uptake in bone marrow-derived macrophages (BMDM), bone marrow-derived dendritic cells (BMDC) (Figure 3E,F), as well as peritoneal and alveolar macrophages, although these cell sources were less phagocytic than RAW cells. Overall, these results suggest that protection conferred by anti-BclA antibodies may result from increased spore uptake by phagocytic cells.

Fc receptors are necessary for increased anti-BclA mediated spore uptake.

Fcγ receptors (FcγR) on phagocytes are activated by pathogen-bound antibodies, then trigger phagocytes to ingest and destroy pathogens that otherwise escape phagocytosis mediated by innate cell-surface receptor recognition. Fc receptors also been contribute to host protection in antibody-mediated uptake of intracellular pathogens [124, 125]. Our laboratory has previously shown that Mac-1 integrin interaction with BclA mediates and redirects spore uptake by phagocytes, stongly suggesting that this protein in

directs phagocytic recognition of Ba [131, 132]. Correspondingly, our experiments presented here show a significant increase in anti-BclA antibody-mediated spore uptake (Figure 3). We thus hypothesized that enhanced Ba spore uptake by anti-BclA antibody is mediated by Fc receptors, and we tested this hypothesis by blocking FcγR with CD16/32 specific mAbs in our *in vitro* phagocytosis assay.

We observed significant decreases in the number of spore-bearing macrophages in the presence of FcγR blockade, relative to non-blocked cells (Fig. 4A). Interestingly, spore-fluorescence levels in anti-CD16/32 treated macrophages challenged with anti-Bcla Ab-opsonized spores was still higher than spore-fluorescence levels in macrophages spores treated with isotype control mock-opsonized spores. This observation suggests either incomplete receptor blockade, or additional means of phagocytosing IgG-opsonized spores. Indeed, the degree of spore-uptake inhibition accomplished by FcγR blockade was not uniform for all BclA-specific mAbs, and varied depending on the mAb isotype used. IgG1 and IgG2b BclA-specific mAbs were particularly notable for their dependency of FcR-mediated phagocytosis. Whereas IgG2a-mediated uptake was also significantly decreased by blocking FcRs, the results were not as dramatic as for the IgG1 mAbs. These results were subsequently replicated in BMDM and primary peritoneal cavity macrophages (Figure 4B, C). These results show that FcγRs significantly contribute to anti-BclA-mediated spore uptake, and that these effects may be particularly important for certain IgG subclasses of BclA-specific antibodies.

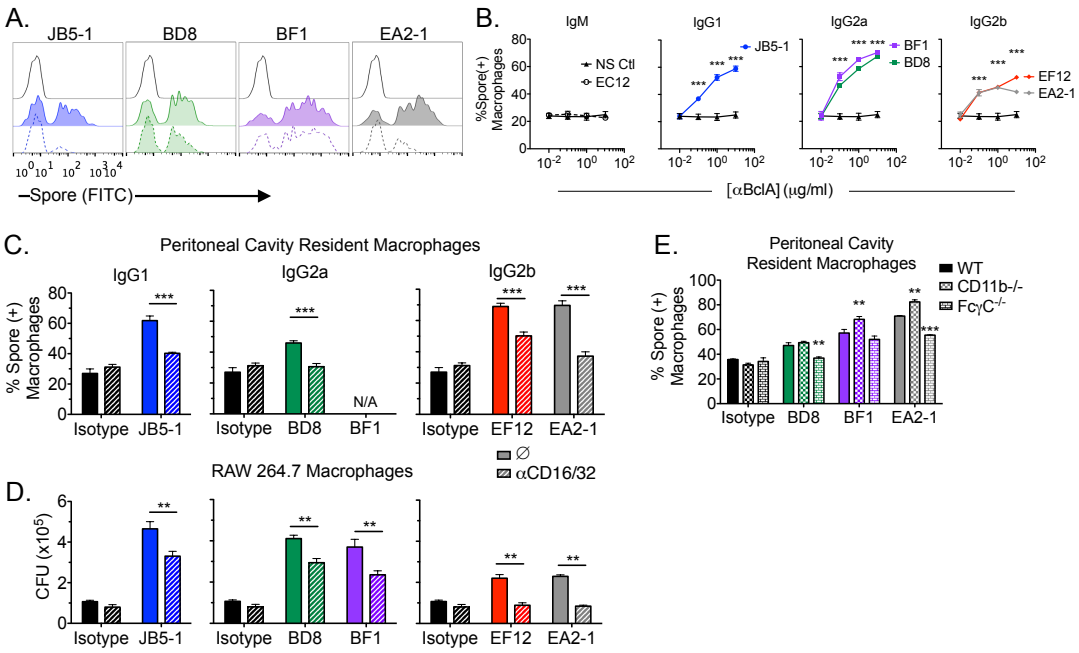

Figure 3. BclA-specific mAbs mediate Ba spore uptake that is partially mediated by FcγR. (A) Representative flow cytometric analysis of *in vitro* phagocytosis assay of Ba Sterne spores and RAW264.7 Macrophages following 2-hr coincubation. (B) Preopsonization of Sterne spores with titrated (0.001-10 **μ**g/mL) amounts of BclA-specific mAbs leads to increases in levels of spore-positive RAW264.7 macrophages. (C) Blockade of FcRγC with antagonistic mAbs results in significantly suppressed effects of BclA-specific mAbs on spore phagocytosis in Peritoneal Cavity resident macrophage cells (C), and RAW264.7 macrophages (D). (E) Peritoneal cavity resident macrophages derived from FcRγC$^{-/-}$ animals display similar defects in BclA-specific mAb dependent increases in spore uptake

Anti-BclA-mediated spore uptake increases spore destruction by RAW cells.

In these studies, we have shown that immunization with rBclA or passive transfer of anti-BclA mAb offers partial protection to mice challenged with a lethal dose of Ba spores, an effect that correlated with the ability of the same antibodies to significantly increase spore uptake by cultured phagocytic cells. We investigated whether anti-BclA Ab-mediated spore uptake increases spore destruction, in-turn inhibiting associated

bacterial survival, outgrowth, and inhalational anthrax disease pathology. To test this hypothesis, we measured viable spore CFUs from the intracellular fraction of macrophages following *in* vitro challenge with anti-BclA mAb opsonized spores. To account for the significant increase in spore uptake in the presence of anti-BclA mAbs *versus* a control, we calculated a ratio of Ba per cell/CFU count. In the presence of anti-BclA mAbs, the ratio of cells/CFU was initially significantly elevated (Supplemental Figure 1) until it normalizes to the level of an isotype control mAb, at approximately 3 hours post-infection,.

Because anti-BclA-mediated spore uptake is at least partially mediated by FcγR, we investigated whether these interactions contribute to increased spore killing. By measuring CFU derived from the intracellular fraction of RAW264.7 cells that had been challenged with spores in the presence of FcγR blockade, we evaluated the contributions of FcγR to the enhanced spore-killing effects of BclA-specific mAbs. When FcγR were blocked in the presence of anti-BclA, the ratio of Ba per cell/CFU was significantly decreased. However, the bacterial counts in the presence of FcR blocking FcγR was not as high as those from isotype control-treated cells. These results indicate that anti-BclA mAb-mediated spore uptake increases Ba spore destruction, suggesting that Fc receptor engagement may facilitate overcoming the immunoevasion tactics of Ba, leading to decreased bacterial dissemination, and reduced mortality during lethal Ba challenge.

Anti-BclA-FcγR spore targeting modifies lysosomal milieu leading to spore disposal by professional phagocytes.

Protective effects of antibodies against pathogenic bacteria have been generally ascribed to either neutralization or complement activation [124, 125]. Recently, Joller, et al. described another protection mechanism in which antibodies against lysosome-escaped intracellular pathogens target these pathogens to lysosomal compartments, and eventual destruction via an Fc-mediated process. Ba pathology, as currently understood, can be explained by Ba spores having accessed the phagolysosomal compartment, subvert microbicial activities, and utilize several mechanisms to allow the escape of very few bacteria from the lysosomal compartment. In the current studies, we have shown that anti-BclA antibodies increase spore uptake and killing by phagocytic cells, and these effects are at least partially attributable to FcγR-interactions. We therefore chose to investigate the effects of BclA-specifi mAbs on phagolysosome maturation following the uptake of Ba spores.

Initially, we chose to assess how anti-BclA-FcγR interactions affect lysosomal acidification. To this end, we adopted a method using spores dually labeled with an acid-labile fluorochome (FITC) and an acid-resistant fluorochrome (AF-647). We then measured the ratio of FITC/AF-647 fluorescence, using chloroquine (CQ) as a lysosomotropic agent that prevents endosomal acidification to establish baseline values [133]. In these initial studies, we noted that opsonization with anti-BclA mAbs led to

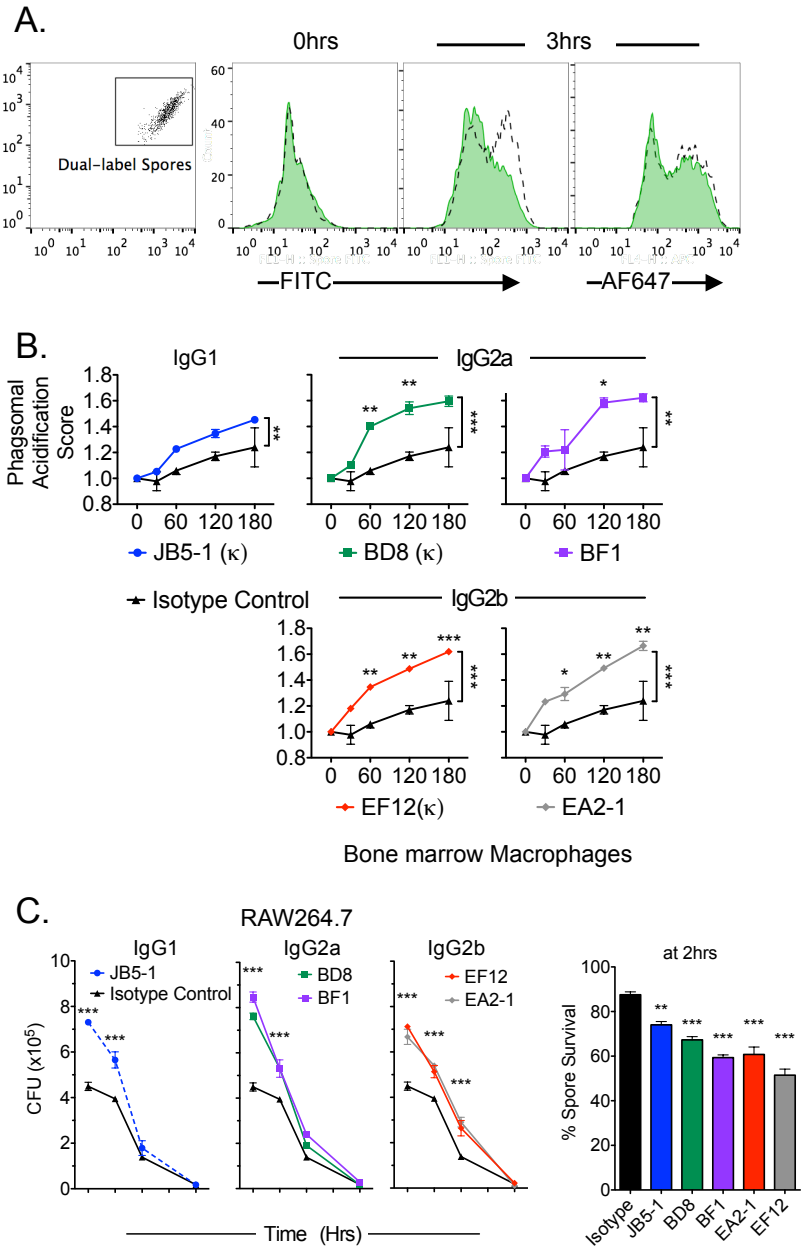

Figure 4. BclA-specific IgG antibodies induce phagosomal acidification and reduce colony burden in macrophages. (A) Dually-fluorescently labeled Ba spore track spore fate within macrophages following phagocytosis. (B) Phagosomal acidification scores following Ba spore uptake for preopsonized Ba spores with BclA-specific IgG mAbs. (C) Analysis of intracellular macrophage CFUs following Ba spore pulse, and determination of viable vegetative Ba bacteria following two-hour incubation.

significantly enhanced loss of FITC fluorescence, and exaggeration of the FITC/AF-647 ratio, demonstrating that BclA mAbs drive changes in the microenvironment of the phagosomal compartments following phagocytosis of Ba.

To further test the hypothesis that anti-BclA/ FcγR interactions are modifying the phagosomal compartments, we compared localization of fluorescently labeled spores to the lysosomal compartment using the Amnis Image Stream which combines microscopy and flow cytometry for high-throughput analysis of subcellular particle distribution. Following our *in vitro* phagocytosis assay, we stained macrophages with fluorescence-conjugated mAbs for the surface marker CD11b (MAC-1), and intracellularly for the early lysosomal marker Lysosome-Associated Membrane Protein-1 (LAMP-1). LAMP-1 staining, together with fluorescence signatures of labeled Ba spores, enables visualization and enumeration of spore-containing phagolysosomes (Figure 5). Utilizing the Bright Detail Similarity (BDS) feature, an algorithm available through this technology, we evaluated the colocalization of Ba spores and LAMP-1-positive lysosomal compartments in thousands of cell images on a pixel-by-pixel basis, allowing for accurate quantitative measurements of the efficiency of spore targeting to lysosomes. Analysis of our *in vitro* spore phagocytosis assay was significantly enhanced by the ImageStream technology. Although our acquired data supported our earlier observations that the degree of spore uptake was significantly increased under conditions of BclA-specific mAb opsonization, we were able to enumerate the number of spores per macrophage. Opsonization of Ba spores with BclA-specific mAbs led to 3- to 5-fold increases in spore number per macrophage. Importantly, evaluation of spore targetting to lysosomes by BDS revealed that, in agreement with our data on phagosomal acidification, the BDS of anti-BclA

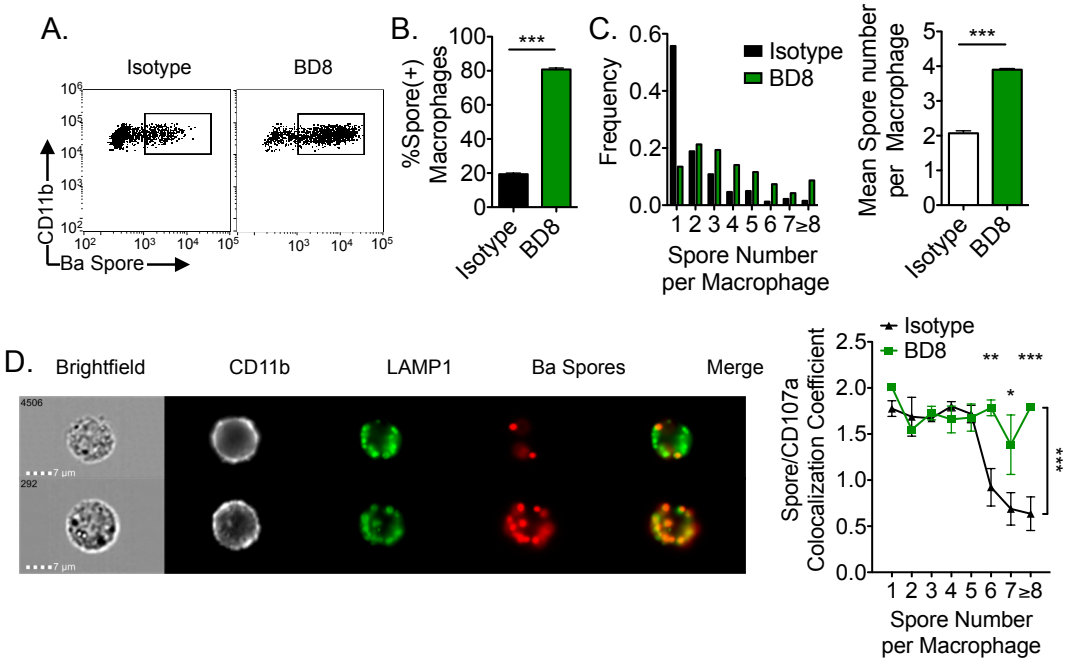

Figure 5. BclA-specific IgG antibodies improve targeting of Ba spores to lysosomal compartments and reduce vegetative bacteria inside macrophages. (A) Graphical gating strategy to identify and measure RAW cell uptake of spores and colocalization of fluorescently labeled spores with lysosomal compartments. (B) Gating strategy to measure uptake of spores. (C) Spore count per RAW cell 2 hours after infection with pre-opsonized spores. (D) Increased normalized frequency of colocalization of spores in RAW cells' lysosomal compartments of pre-opsonization with anti-BclA. (E) Increased % pre-opsonized spore + cells. (F) Increase in number of pre-opsonized spores per macrophage. (G) Bright detail similarity coefficiency increase with pre-opsonized spores.

preopsonized spores with the lysosomal compartment was significantly increased compared to control mAb opsonized spores. These results show that enhanced Ba-spore killing *in vitro*, and likely the improved survival in rBclA-immunized or anti-BclA passive transfer recipients in lethal i.t. Ba challenge result from enhanced targeting of Ba spores to lysosomal compartments. Overall, these results suggest that the FcγR-anti-BclA interaction affects the lysosomal milieu by arresting the full germination of spores

through more efficient acidification of the lysosome, providing the phagocyte more time to destroy Ba spores and suggest this mechanistic model as the explanation of the *in vivo* protective effects these mAbs and those induced by BclA immunization.

DISCUSSION

The current human vaccination protocol for *Bacillus anthracis* in the United States targets the toxins produced by the vegetative bacteria form of Ba, specifically the protective antigen (PA). However, other strategies for vaccination therapy are being investigated [134], including the use of spore components. We propose that targeting the Ba spore and preventing germination of the spore into the toxin-producing vegetative bacteria would provide another level of protection against anthrax, and represents a significant potential addition to the human-vaccination protocol.

In these studies, we have confirmed the observations of others that mouse C5-deficiency confers susceptibility to Ba Sterne, and have characterized a new mouse model of anthrax with significant advantages over the current widely used model. Most anthrax studies of initial spore-host interactions use A/J mice with Sterne spores, largely due to their susceptibility to infection with Sterne, the safely of use of Sterne under BSL II conditions, and the resulting low-cost associated animal care. This susceptibility has been speculated to result from a complement component deficiency [116]. In the current studies, we have confirmed that complement-deficient C57BL/6 mice, in particular complement factor 5-deficient ($C5^{-/-}$) mice, are also susceptible to Sterne spores. The susceptibility of $C5^{-/-}$ C57BL/6 mice has several advantages over several inherent drawbacks associated with the A/J strain that limit the latter's use in anthrax

investigation. Furthermore, the $C5^{-/-}$ C57BL/6 mice can be bred with the vast array of genetically modified C57BL/6 mice to further inquire which host factors are required the for the various outcomes that typically result upon anthrax exposure. Notably, there was a marked difference in the mean time of death (MTD) between Anthrax Sterne-infected A/J and $C5^{-/-}$ C57BL/6 mice (3 and 6 days, respectively). We speculate that this difference results from other immunological deficiencies intrinsic to A/J mice background -[135] JAX mice database- and would need to be studied further.

As a target for spore-specific immunization, we selected BclA: the main exosporium glycoprotein and immunodominant antigen [52, 54, 122]. Current toxin-based vaccination standards target the vegetative bacteria instead of the infectious form of Ba, and has a cumbersome schedule that includes three subcutaneous injections at 0, 2, and 4 weeks, and three booster vaccinations at 6, 12, and 18 months. To maintain immunity, the manufacturer recommends an annual booster injection. We hypothesized that immunization with exosporium, or a recombinant form of the main exosporium component BclA would provide protection against inhalational anthrax by specifically targeting the infectious form (Ba spore). To address this question, we immunized $C5^{-/-}$ C57BL/6 mice intra nasally with Cholera Toxin (CT) adjuvanted rBclA or exosporium three consecutive times in weekly intervals. We selected CT because it is a potent mucosal adjuvant that provides long-term memory via oral, intranasal, and even parenteral routes, using conventional and non-conventional pathways to induce these responses [136].

Immunization with rBclA or the exosporium induced a significant production of serum anti-BclA IgG and IgM in $C5^{-/-}$ C57BL/6 mice. Mice vaccinated with CT alone

showed no measurable response to BclA. Notably, higher levels of anti-BclA were measured in mice that received rBclA compared to those that received exosporium. We speculate that this effect is due to differing fine-specificities of these antibodies, and that antibodies elicited by rBclA more readily recognized the rBclA used to coat ELISA plates compared with antibodies to BclA in its native form (exosporium). Further glycosylation differences between rBclA and native BclA of the exosporium could also contribute to these differences in antibody titer readouts.

We further examined whether immunization with whole exosporium or rBclA confers immune protection, by challenging these mice with a lethal (3xLD50) i.t. Ba spore challenge. Immunization with rBclA using CT as adjuvant showed limited, yet significant protection (30% survival) compared to the control group (0% survival). Immunization with purified exosporium however, showed remarkable protection (80% survival). We speculate that the improved protection in the mice that received the exosporium *versus* the protection conferred by rBclA is due to the development of anti-BclA antibodies against the native form found in the exosporium; however it is possible that antibodies against other targets on the exosporium could also contribute to spores neutralization. To directly investigate the protective potential of anti-BclA monoclonal antibodies, we passively transferred our highly specific mAbs against BclA [53] of different IgG antibody subclasses to $C5^{-/-}$ C57BL/6 mice i.p. 24hrs preceding lethal challenge. Similarly to active immunization, passive transfer of anti-BclA mAbs offered partial, yet significant, protection against the lethal i.t. challenge.

To begin understanding how anti-BclA antibodies confer protection in our model, we developed a flow-cytometric phagocytosis assay using fluorescently labeled Ba Sterne

spores to quantitate and study spore clearance. We demonstrated that opsonization of Sterne spores with IgG mAbs against BclA leads to a 3.5-fold higher percentage of spore-bearing phagocytes compared with basal levels. Likewise, there were markedly higher CFU counts in the presence of anti-BclA, suggesting that apart from spore-bearing cells, there is an increase in the number of spores each macrophage phagocytoses. These findings contrast with the long-proposed "Trojan Horse" effect in which spores germinate within macrophages migrating to the draining lymph node. Once the spore has germinated into a vegetative bacterial cell, the bacterium can produce both the capsule (which allows it to escape targeting by other phagocytes), and the tripartite toxin (PA + LF + EF) associated with anthrax morbidity. This effect might still occur in the mice that succumb to infection; however, the partial protection seen with anti-BclA mAbs as well as BclA active immunization suggest that there is an Ab-mediated protection mechanism in our experimental model.

Because there is an increase in the number of spores per macrophage as well as an increase in the number of spore-bearing cells, we hypothesized that antibodies against spore components increased spore uptake and improve targeting of spores leading to decreased spore survival. To test this hypothesis, we extended our phagocytosis assay to assess phagosomal acidification following spore phagocytosis. In these experiments, we observed more loss of acid-labile fluorescence of dually labeled spores opsonized with BclA-specific mAbs than with control Abs, suggesting that anti-BclA IgG was enhancing phagolysosomal fusion. We then further evaluated later time points in our intracellular-CFU studies, in the presence of anti-BclA or an isotype control. Our results show that after initial spore-cell contact and subsequent cell lyses, the survival percentage of Ba in

relation with the initial spore uptake is diminished in comparison to an isotype control. We further demonstrated, using ImageStream technology, that in the presence of anti-BclA mAbs, there is enhanced targeting of spores to lysosomal compartments, by way of mapping spore- and lysosomal compartment-fluorescence signatures. We propose that increased acidification while the spore is in the process of germination allows for the destruction of the bacteria in the lysosomal compartments, accounting for the protection observed in our lethal i.t. challenge models.

These results show that antibodies against BclA could be an important adjunct to current vaccination therapies as others have noted [105, 110, 130, 137], although these studies have usually needed PA as a vaccination target in order to show protection. Furthermore, incorporation of BclA-specific monoclonal antibodies into current antibiotic regimens used to treat anthrax disease could provide novel microbicidal activity against potentially antibiotic-resistant or weaponized anthrax strains. Such passive immunotherapy could provide specific, neutralizing therapy to anthrax-exposure victims, and would additionally initiate their own productive immunity, as was the case in the passive immunotherapies given to Ebola virus victims in 2015.

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CONCLUSIONS AND FUTURE PERSPECTIVES

The use of *B. anthracis* as a biological weapon against the United States by an unknown entity in 2001, as well as the discovery that during the Iraq War of 1990 there was a distinct possibility of the use of weaponized anthrax against our military prompted the reevaluation of the threat of this agent and the development of new strategies against it. The possibility of the development of genetically modified strains that could bypass the current human vaccination protocol for *B. anthracis* that currently targets protective antigen (PA) prompts for the development of a multi-targeted approach. The use of antibiotics, although effective, has to be started shortly after infection, and the development of drug resistant *B. anthracis*[138, 139] indicates that other treatment and prevention strategies must be explored. We believe that targeting of the spore as a strategy for vaccination therapy would prevent germination of the spore into the toxin-producing vegetative bacteria and provide another level of protection against anthrax.

Among the many hurdles of anthrax studies is the cost associated with the use of Bio Safety Level III (BSL III) when using fully virulent *B. anthracis.* To circumvent this problem, studies that explore the initial spore-host interaction utilize the A/J mice in conjunction with Sterne spores. A/J mice are susceptible to infection with the unencapsulated Sterne strain, which can be safely used under BSL II conditions, with the added advantage of low-cost associated animal care. In these studies, we have confirmed the observations of others that mouse C5-deficiency confers susceptibility to *B. anthracis*

Sterne strain, and have characterized a new mouse model of anthrax with significant advantages over the current widely used model. In these studies, we have confirmed that complement-deficient C57BL/6 mice, in particular complement factor 5-deficient ($C5^{-/-}$) mice, are also susceptible to Sterne spores. The susceptibility of $C5^{-/-}$ C57BL/6 mice has several advantages over several inherent drawbacks associated with the A/J strain that limit the latter's use in anthrax investigation. Furthermore, the $C5^{-/-}$ C57BL/6 mice can be bred with the vast array of genetically modified C57BL/6 mice to further inquire which host factors are required the for the various outcomes that typically result upon anthrax exposure.

As a target for spore-specific mucosal immunization, we selected BclA: the main exosporium glycoprotein and immunodominant antigen [52, 54, 122]. Current toxin-based vaccination standards (Biothrax® immunization) are recommended to at-risk personnel only (veterinarian staff, members of the military) and only target the production of toxins by the vegetative bacteria. Other disadvantages of the vaccine are the cumbersome schedule that includes three subcutaneous injections at 0, 2, and 4 weeks, and three booster vaccinations at 6, 12, and 18 months with an annual booster to maintain immunity, the manufacturer recommends an annual booster injection. We hypothesized that immunization with exosporium, or a recombinant form of the main exosporium component BclA would provide protection against inhalational anthrax by specifically targeting the infectious form of the bacterium. To address this question, we immunized $C5^{-/-}$ C57BL/6 mice intra nasally with Cholera Toxin (CT) adjuvanted rBclA or exosporium three consecutive times in weekly intervals. We selected CT because it is a potent mucosal adjuvant that provides long-term memory via oral, intranasal, and even

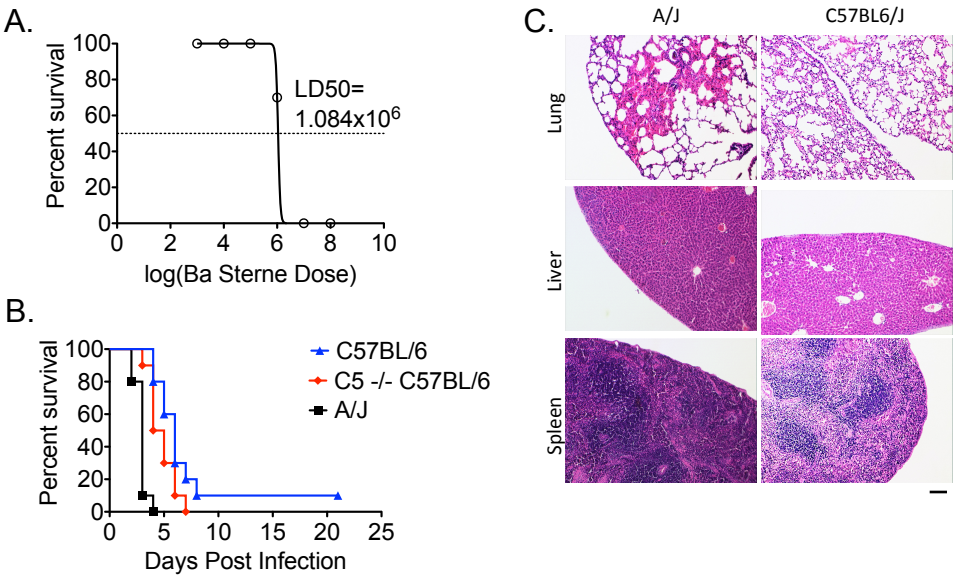
parenteral routes, using conventional and non-conventional pathways to induce these responses [136]. This immunization regimen with induced a significant production of serum anti-BclA IgG and IgM in $C5^{-/-}$ C57BL/6 mice. Immunization with rBclA using CT as adjuvant showed limited, yet significant protection (30% survival) to a lethal intratracheal challenge, while immunization with purified exosporium showed remarkable protection (80% survival). We speculate that the improved protection in the mice that received the exosporium *versus* the protection conferred by rBclA is due to the development of anti-BclA antibodies against the native form found in the exosporium; however it is possible that antibodies against other targets on the exosporium could also contribute to spores neutralization.

To further elucidate how anti-BclA antibodies confer protection in our model, we developed a flow-cytometric phagocytosis assay using fluorescently labeled *B. anthracis* Sterne spores to quantitate and study spore clearance. We demonstrated that opsonization of Sterne spores with IgG mAbs against BclA leads to a 3.5-fold higher percentage of spore-bearing phagocytes compared with basal levels. We also used CFU studies to analyze the survival of spores after opsonization and phagocytosis by RAW264.7 cells. There were markedly higher CFU counts once spores were opsonized with anti-BclA, suggesting that apart from an increase in spore-bearing cells, there is an increase in the number of spores each macrophage phagocytoses. These findings contrast with the long-proposed "Trojan Horse" effect in which spores germinate within macrophages migrating to the draining lymph node. The increase in the number of spores per macrophage as well as the number of spore-bearing cells, led us to investigate if antibodies against spore components increased spore uptake and improve targeting of spores, leading to decreased

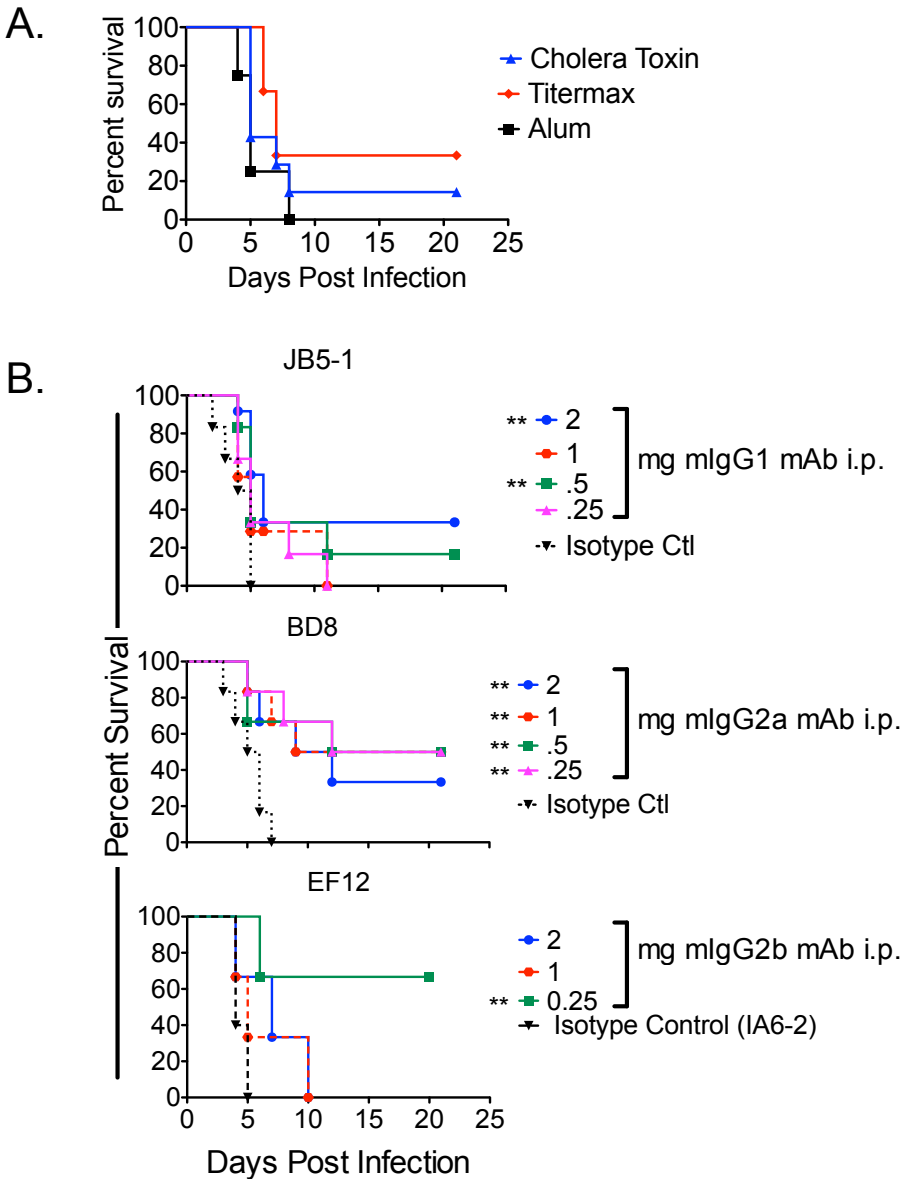
spore survival. To test this hypothesis, we extended our phagocytosis assay to assess phagosomal acidification following spore phagocytosis. In these experiments, we observed more loss of acid-labile fluorescence of dually labeled spores opsonized with BclA-specific mAbs than with control Abs, suggesting that anti-BclA IgG was enhancing phagolysosomal fusion. Curious as to the eventual fate of the spore, we evaluated later time points through intracellular-CFU studies. Our results show that after initial spore-cell contact and subsequent cell lyses, the survival percentage of *B. anthracis* in relation with the initial spore uptake is diminished in comparison to an isotype control. We further demonstrated, using ImageStream technology that, in the presence of anti-BclA mAbs, there is enhanced targeting of spores to lysosomal compartments, by way of mapping spore- and lysosomal compartment-fluorescence signatures. We propose that increased acidification while the spore is in the process of germination allows for the destruction of the bacteria in the lysosomal compartments, accounting for the protection observed in our lethal i.t. challenge models.

Our results show that mAbs against BclA could be an important adjunct to current vaccination therapies as others have previously noted [105, 110, 130, 137], although these studies have usually needed PA as a vaccination target in order to show protection. However, recent findings by Kohler et al. suggest that vaccination with BclA confers limited protection to a lethal anthrax challenge. Furthermore, incorporation of BclA-specific monoclonal antibodies into current antibiotic regimens used to treat anthrax disease could provide novel microbicidal activity against potentially antibiotic-resistant or weaponized anthrax strains. Such passive immunotherapy could provide specific, neutralizing therapy to anthrax-exposure victims, and would additionally initiate their

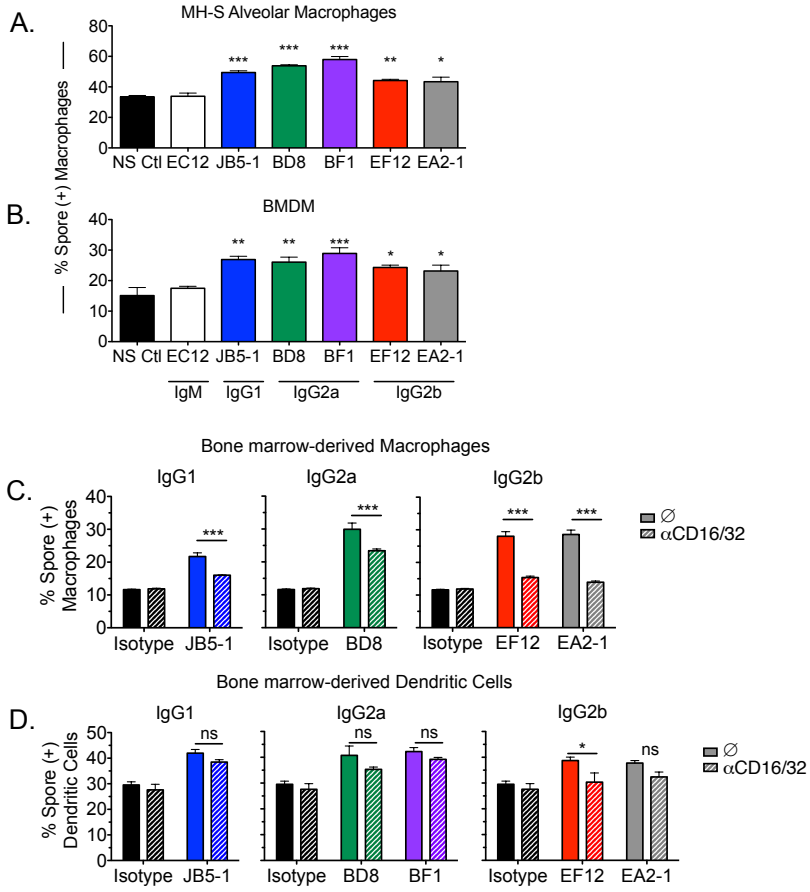
own productive immunity, as was the case in the passive immunotherapies given to Ebola virus victims in 2015.



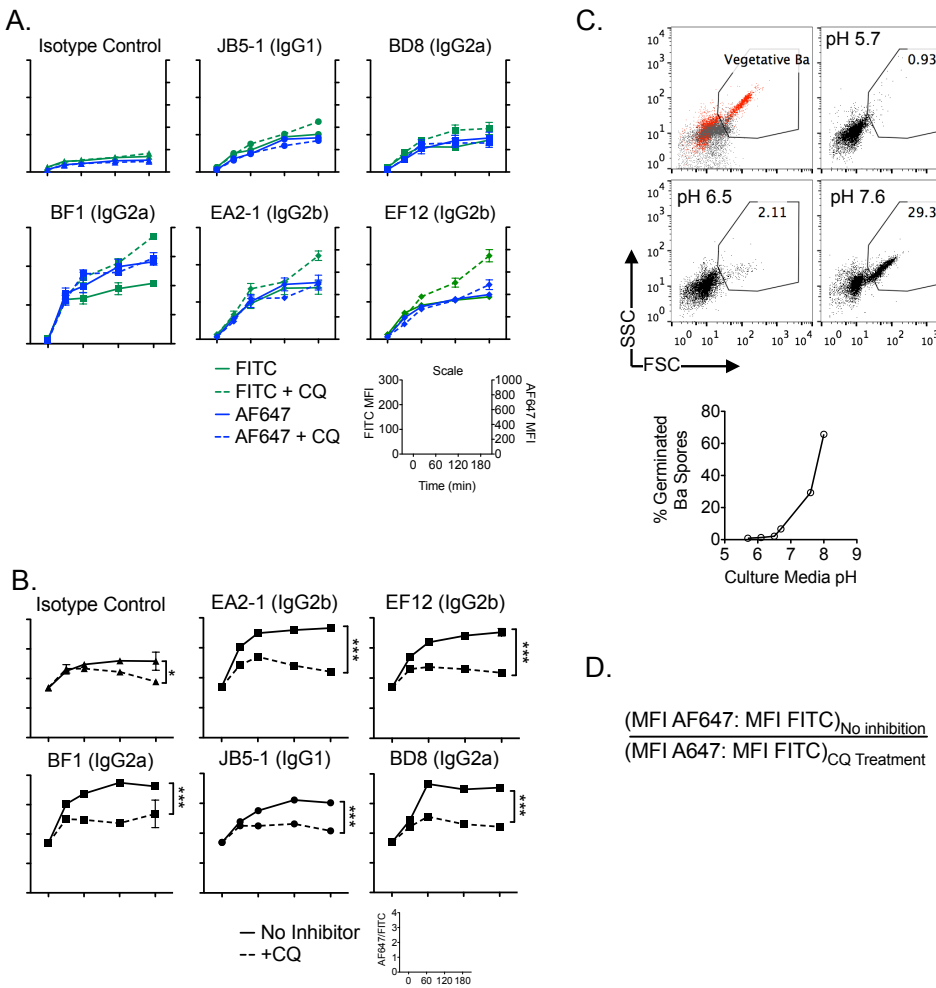
Supplemental Figure 1. Calculation of LD50 for i.t. Ba Sterne spore challenge, and demonstration of $C5^{-/-}$ C57BL/6 mouse to subcutaneous Anthrax infection.



Supplemental Figure 2. Comparison of protection conferred by different adjuvant formulations of recombinant BclA vaccine antigen, and titration of BclA-specific mAbs used for passive protection studies.



Supplemental Figure 3. Different phagocyte populations display different phagocytic activities and dependency on antibody opsonization.



Supplemental Figure 4. Raw and relative MFI data corresponding to acid-labile fluorochrome-dependent analysis of phagosomal maturatio

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APPENDIX A

IACUC APPROVAL FORM

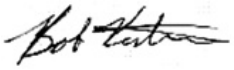
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: April 10, 2014

TO: JOHN F KEARNEY, Ph.D.
SHEL-410
(205) 934-6557

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Immunobiology of Bacillus Anthracis Spore-Host Interactions
Sponsor: NIH
Animal Project_Number: 140408755

As of April 18, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	25
Mice	C	640

Animal use must be renewed by April 17, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140408755 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

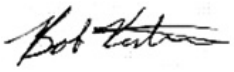
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: April 10, 2014

TO: JOHN F KEARNEY, Ph.D.
SHEL-410
(205) 934-6557

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on April 10, 2014.

Title: Immunobiology of Bacillus Anthracis Spore-Host Interactions
Sponsor: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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