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INTERACTION OF BACILLUS ANTHRACIS EXOSPORIUM PROTEIN BCLA WITH COMPLEMENT FACTOR H AND SPORE PERSISTENCE IN THE LUNG

Sarah A. Jenkins

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A

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DOCTOR OF PHILOSOPHY

By

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Houston, TX

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DEDICATION

To my family for without their support, guidance, and love I would not be where I am today. Thanks Mom, Dad, Melissa, and Ryan.

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Thank you God for being with me throughout this journey in my life, for with you all things are possible.

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ABSTRACT

INTERACTION OF *BACILLUS ANTHRACIS* EXOSPORIUM PROTEIN BCLA WITH COMPLEMENT FACTOR H AND SPORE PERSISTENCE IN THE LUNG

Publication No---------

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Anthrax outbreaks in the United States and Europe and its potential use as a bioweapon have made *Bacillus anthracis* an interest of study. Anthrax infections are caused by the entry of *B. anthracis* spores into the host via the respiratory system, the gastrointestinal tract, cuts or wounds in the skin, and injection. Among these four forms, inhalational anthrax has the highest lethality rate and persistence of spores in the lungs of animals following pulmonary exposure has been noted for decades. However, details or mechanisms of spore persistence were not known. In this study, we investigated spore persistence in a mouse model. The results suggest that *B. anthracis* spores have special properties that promote persistence in the lung, and that there may be multiple mechanisms contributing to spore persistence. Moreover, recent discoveries from our laboratory suggest that spores evolved a sophisticated mechanism to interact with the host complement system. The complement system is a crucial part of the host defense mechanism against foreign microorganisms. Knowledge of the specific interactions that occur between the complement system and *B. anthracis* was limited. Studies performed in our laboratory have suggested that spores of *B. anthracis* can target specific proteins

such as Factor H (fH) of the complement system. Spores of *B. anthracis* are enclosed by an exosporium, which consists of a basal layer surrounded by a nap of hair-like filaments. The major structural component of the filaments is called *Bacillus* collagen-like protein of *anthracis* (BclA), which comprises a central collagen-like region and a globular C-terminal domain. BclA is the first point of contact with the innate system of an infected host. In this study, we investigated the molecular details of BclA-fH interaction with respect to the specific binding mechanism and the functional significance of this interaction in a murine model of anthrax infection. We hypothesized that the recruitment of fH to the spore surface by BclA limits the extent of complement activation and promotes pathogen survival and persistence in the infected host. Findings from this study are significant to understanding how to treat post-exposure prophylaxis and improve our knowledge of spores with the host immune system.

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ABBREVIATIONS

BclA	*Bacillus* collagen-like protein of *anthracis*
LF	Lethal factor
EF	Edema factor
NTD	N-terminal domain
CLR	Collagen-like region
CTD	C-terminal domain
fH	Factor H
SCR	Short complement regulators
BAL	Bronchoalveolar lavage fluid
CLCS	Crude lung cell suspension
NALT	Nasal associated lymphoid tissue
HiMS	Heat-inactivated mouse serum
HiNHS	Heat-inactivated normal human serum
MFI	Mean fluorescence intensity
CD	Circular dichroism
CFU	Colony forming units
I.N.	Intranasal inoculation
H&E	Hematoxylin and Eosin
Δ*bclA*	BclA deletion mutant
Δ*bclA*/BclAFL	BclA deletion mutant expressing full-length BclA
Δ*bclA*/BclACLR	BclA deletion mutant expressing the CLR of BclA
Δ*bclA*/BclACTD	BclA deletion mutant expressing the CTD of BclA
pDG1662	*B. subtilis* vector only control spores
pDG1662/BclAFL	*B. subtilis* spores expressing full-length BclA
pDG1662/BclACTD	*B. subtilis* spores expressing the CTD of BclA

CHAPTER ONE: INTRODUCTION

Bacillus anthracis, once serving as the prototype for Robert Koch's postulates regarding the causation of infectious disease, continues to generate concern as an agent of bioterrorism. The organism known to cause the disease, anthrax, is infrequent in the United States and other countries. Roughly 20,000 – 100,000 cases are reported worldwide. In December 2009, a previously healthy 24 – year old New Hampshire woman nearly died due to contact with contaminated drum skins [1]. In 2012, animal slaughter in an Indian tribal village led to human exposure and a fatality rate of 18% [2]. These cases and recent outbreaks of injectional anthrax in more than 50 heroin users in the United Kingdom and Germany [3-6] have sustained the importance of this potentially lethal infection in humans.

The natural hosts of *B. anthracis* are grazing herbivores. Human infections result from contact with *B. anthracis* via cuts or wounds in the skin, via the gastrointestinal tract, the respiratory system, and by injection. Among these four forms, inhalational anthrax is the most severe and has the highest lethality rate despite the appropriate antimicrobial agents and advanced supportive care available. The initial symptoms of inhalational anthrax resemble a common cold. Infected individuals can develop a mild fever, muscle aches, malaise, cough, and a sore throat. After a few days, severe breathing problems can develop leading to key pathological manifestations including edema, hemorrhage, mediastinal enlargement, and pleural effusions. Also, a key feature of inhalational anthrax

is the prolonged presence of spores in the host lung after the initial exposure. The ability of *B. anthracis* spores to persist in the lungs of animals including non-human primates and mice has been well documented [7-9]. This is the basis for the prolonged antibiotic regimen (60 days) recommended by the United States Department of Health and Human Services for patients with pulmonary exposure to *B. anthracis* spores. However, the underlying mechanism for persistence remains unknown.

***Bacillus anthracis* life cycle.** *B. anthracis* exists in two forms, endospores and vegetative bacilli. Spores of *B. anthracis* initiate anthrax infections. They are metabolically inactive and resistant to harsh conditions including ionizing radiation, extreme temperatures, detergents, and hydrolytic enzymes. Spores can survive in the environment for extended periods of time. *B. anthracis* strains are highly monomorphic due to the fact of endospores having prolonged periods of dormancy and reduced genetic variation. Electron micrographs of endospores reveal that the spore anatomy is composed of multiple layers (Fig. 1). Generally, spores consist of a dehydrated cellular core, which houses the spore DNA protected by dipicolinic acid (DPA) and small acid-soluble spore proteins (SASP) [10]. The inner membrane is where the germination receptors are located. The core is membrane-bounded and surrounded by a peptidoglycan cortex and a protective coat [10]. Some spore-forming pathogens, including *Bacillus cereus*, *Clostridium botulinum*, *Bacillus thuringeneis*, and *B. anthracis* contain an outermost layer called the exosporium. This structure is not observed in the nonpathogenic, *Bacillus subtilis* spore. It was recently discovered

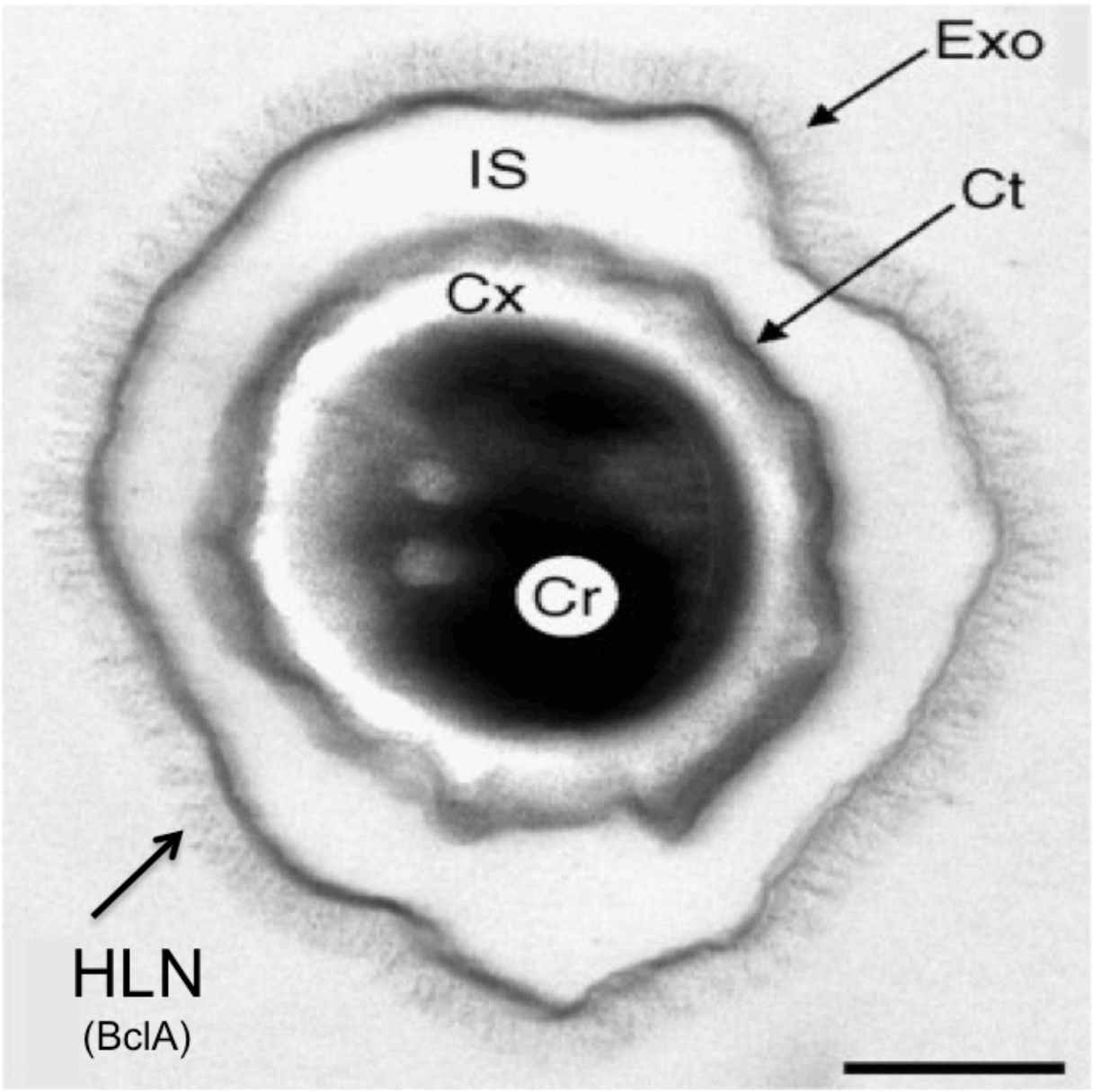


Figure 1. Endospore anatomy. Thin-section electron micrograph of *B. anthracis* Sterne strain spore. Core (Cr), Cortex (Cx), Coat (Ct), interspace (IS), Exosporium (Exo), and the Hair-like nap (HLN) composed of BclA protein is indicated. Image was obtained from Driks *et al* 2009 [10].

that the outermost layer of the *subtilis* spore is the spore crust composed of proteins CgeA and CotZ [11]. The exosporium constitutes about 2% of the mass of the spores containing 50% protein, 20% lipid, 20% neutral polysaccharides, and 10% other components [12]. Proteins comprising the exosporium of the *B. anthracis* spore have received considerable attention because these molecules are strong candidates for vaccines and ligands for spore detection. Scanning electron microscopy images have revealed that the exosporium contains a paracrystalline basal layer and a hair-like nap. Several basal layer proteins, ExsFA/BxpB, BxpA, BxpC and ExsY have been identified [12-14]. A single protein called *Bacillus* collagen-like protein of *anthracis* (BclA) was found to be the main component of the hair-like nap (Fig. 1) [15].

Germination is the process of spores becoming vegetative bacteria. In theory, the spore can remain in the spore state forever, until it encounters germinants, such as nucleosides and amino acids. Upon germination receptor activation, the core hydrates, causing an influx of water and an efflux of DPA, resulting in loss of heat resistance. Two lytic enzymes, CwlJ and SleB degrade the cortex. Eventually the coat and exosporium are displaced and growth of vegetative bacilli proceeds [16, 17]. Vegetative bacilli represent the pathogenic form of *B. anthracis*. Bacilli are rod-shaped (typically 5 – 7μm in length) and grow in long chains [18]. Vegetative bacilli have typical Gram-positive features, including a thick peptidoglycan cell wall, an S-layer, and a capsule composed of poly-γ-D-glutamic acid required for virulence [19]. These vegetative cells secrete a three-protein exotoxin composed of a cell binding

protein called protective antigen and two enzymes called lethal factor (LF) and edema factor (EF), which work together to impart their physiological effects in the host. EF is a calcium and calmodulin activated adenylate cyclase that converts intracellular adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). By increasing cAMP levels, EF acts to disrupt water homeostasis, impede balance of intracellular signaling pathways, and impair neutrophil function [20]. LF is a zinc metalloprotease that cleaves mitogen activated protein kinase kinases (MEKs), which respond to a diverse array of external signals and regulate various cellular responses, including inflammatory and macrophage activation. LF can interfere with macrophage release of chemokines and cytokines, such as IFNγ and TNF-α and cause lysis of these cells [20, 21]. LF is also known to inhibit antigen presentation by dendritic cells abrogating expression of co-stimulating molecules and can trigger apoptosis in endothelial cells [21]. When vegetative bacteria are nutrient depleted, they undergo sporulation, the process from vegetative bacilli to a spore. Sporulation begins with asymmetric cell division resulting in a mother cell and an endospore. The process is guided by the transcription factor Spo0A and two sigma factors σ^E and σ^F [22]. Eventually the mother-cell is shed and the resulting spore is capable of surviving harsh environments, such as high temperature, pH, salinity, and even time [22].

***Bacillus* collagen-like protein of *anthracis* (BclA).** BclA is the collagen-like surface glycoprotein of the *B. anthracis* spore. It was first isolated and identified as an exosporium structural protein by

Sylvestre *et al* in 2002. With the use of immunogold electron microscopy and a monoclonal anti-spore antibody, Sylvestre's group confirmed that BclA is a component of the hair-like projections on the exosporium [15]. The amino-terminal sequence of the purified protein led to identification of the corresponding gene in the *B. anthracis* genome sequence. The *bclA* gene encodes a 382 amino acid protein under the control of a sigma k dependent promoter [15] (Fig. 2, A). It is synthesized approximately 4 hours after the entry into sporulation and is absent in vegetative cells indicating that it is a spore-specific protein [15]. BclA is considered the immunodominant antigen on the spore surface because a large majority of anti-spore antibodies react with this protein [12].

BclA structure. BclA is comprised of three domains (Fig. 2, B). A short N-terminal domain (NTD), which is proteolytically cleaved and anchors BclA to the basal layer of the spore [23]. Its most striking feature is a collagen-like region (CLR) composed of strain-specific GXY repeats. It has been shown that the filament lengths of *B. anthracis* strains are directly proportional to the lengths of the CLRs [24]. The CLR region of BclA contains a 21-amino acid motif, $(GDT)_5(GDTGTT)$, which is repeated six times and contains different numbers of GPT repeats. The sequence GDTGTT present in the motif is unique to BclA [15] (Fig. 2, A). Two O-linked oligosaccharides have been identified in BclA, a 715kDa tetrasaccharide (anthrose) and a 234kDa disaccharide. Studies show that anthrose is attached to the CLR of BclA, but that the disaccharide might possibly be linked to another region [25]. Similar to mammalian collagen, the CLR of *anthracis* can form a triple helix. Glycine residues occupy every

A.

1 MSNNNYSNGLNPDESLSASAFDPNLVGPTLPPIPPFTLPTGPTGPTGPTGPTGPTGPTGP
61 TGPTGPTGPTGPTGPTGPTGDTGTTGPTGPTGPTGPTGPTGDTGTTGPTGPTGPTGPTGP
121 TGPTGPTGPTGPTGPTGDTGTTGPTGPTGPTGPTGPTGDTGTTGPTGPTGPTGPTGPTGP
181 TGPTGPTGPTGPTGPTGPTGPTGDTGTTGPTGPTGPTGPTGPTGDTGTTGPTGPTGPTGP
241 TGPTGPTGPTGATGLTGPTGPTGPSGLGLPAGLYAFNSGGISLDLGINDPVPFNTVGSQF
301 GTAISQLDADTFVISETGFYKITVIANTATASVLGGLTIQVNGVPVPGTGSSLISLGAPI
361 VIQAITQITTTPSLVEVIVTGLGLSLALGTSASIIIEKVA

B.

Figure 2. Protein sequence and schematic representation of BclA protein. (A) NCBI Reference Sequence YP_027402.1 for *B. anthracis* Sterne strain. Highlighted amino acids (aa) indicate sequences of N-terminus (blue), BclA-specific motifs (GDTGTT) in CLR (yellow), and C-terminus (green). **(B)** BclA protein consists of three domains. The N-terminal domain is 41aa in length. The mature form of the protein starts at residue 20. The CLR consists of strain specific (~70) GXY triplet motifs shaded in gray. The six 21-amino acid repeats $(GDT)_5GDTGTT$ are labeled 1-6. The sequence motif GDTGTT is unique to BclA protein. The C-terminal domain is 131aa in length.

third amino acid in the repeating GXY, which is a prerequisite for a triple helix formation [15]. The high content of prolines and threonines contribute to the stabilization of the triple helix. Furthermore, CLR sequences are more recently being described in prokaryotes, highlighting the importance of BclA protein in *B. anthracis*. The third domain of BclA is the C-terminal domain (CTD), which is the distal end of the filament. The CTD was shown to be important for trimer formation [26].

BclA functions. The exosporium is likely to play multiple roles in the interaction of the spore with its environment. For *B. anthracis*, BclA has been shown to mediate spore entry into host cells. It was reported previously that uptake of spores by phagocytes (*i.e.* macrophages, possibly dendritic cells) involved the interaction between integrin Mac-1 (Cd11b/CD18) receptor, co-factor CD14, and BclA [27, 28]. Our group showed that entry of spores into epithelial cells was mediated by BclA and host cell receptor integrin $\alpha 2\beta 1$ via a mechanism that required host complement protein C1q [29]. Other host cell interactions involving BclA or biological functions of BclA have not been described.

Host immune responses to *B. anthracis* spores. In general, interactions between exosporium proteins and host components influence the ways in which spores can survive in the infected host. BclA is a good vaccine and/or antibody target because it is the first point of contact with the host immune response. While the spore surface does not have any typical pathogen associated molecular patterns, such as lipopolysaccharide, lipoteichoic acid, peptidoglycan, or flagellin, a number of studies indicated that *B.*

anthracis spores are not "stealth" to host immune recognition. Spores are capable of activating TLR2 and MyD88 signaling [30] and inducing inflammatory cytokine production in both MyD88-dependent and independent manners [31, 32]. Interestingly, BclA did not play a role in the host immune recognition by spores [31]. Spores induced the secretion of IL-12 from $CD11b^{+}$ cells and IFN-γ by activating natural killer cells [31, 33, 34]. We previously showed that spores are capable of initiating the classical complement pathway via a direct interaction between BclA and C1q [35].

The complement system. The complement system is the major defense and clearance system against pathogens in the human host. It can be activated in three ways, classical, lectin, and alternative (Fig. 3) [36]. The classical pathway is antibody-dependent because it is initiated by IgM or IgG clusters. The main recognition molecule, C1q binds to immune complexes on microbial surfaces and initiates the classical complement system. In the lectin pathway, mannose-binding lectin and ficolins recognize carbohydrate patterns on microbial surfaces to activate complement. Unlike the other two pathways, the alternative pathway is initiated through the spontaneous hydrolysis of C3. All three pathways are able to form a C3 convertase, which is important for the activation of C3 to cleave into C3a and C3b. Cleavage of C3 exposes a reactive short-lived thioester moiety in C3b, which can covalently attach to amines and carbohydrate groups on targeted surfaces [36]. C3b deposition leads to opsonization for phagocytosis by phagocytes. In the presence of an additional C3b, the C3 convertase can function as a C5 convertase cleaving C5 into C5a and C5b. C5b associates with complement

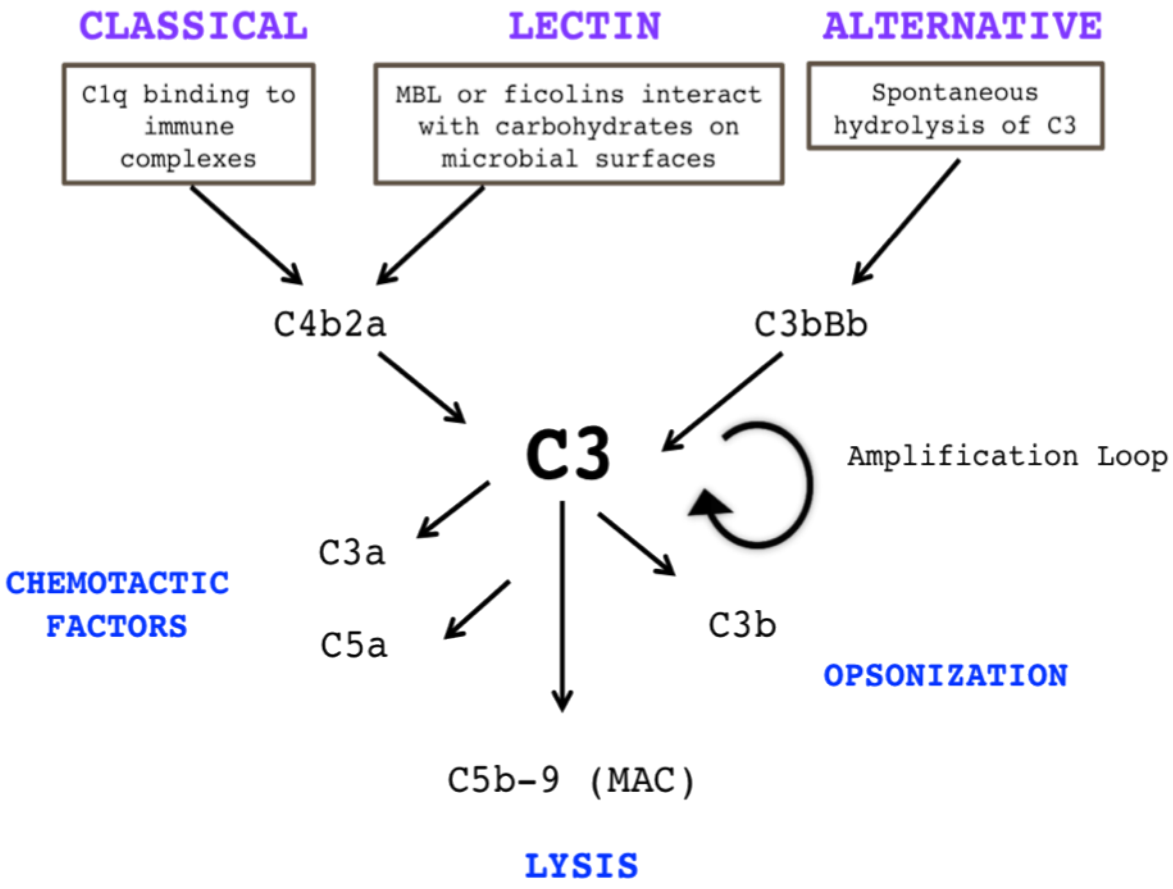


Figure 3. Activation pathways of the complement system. The Classical pathway is activated by the binding of C1q component of the C1 complex to its specific ligands (IgM or immune complexes). The Lectin pathway is initiated through mannose binding lectin (MBL) or ficolins that interact with carbohydrates on microbial surfaces. Both pathways generate a common C3 convertase (C4b2a). The Alternative pathway is initiated by the spontaneous hydrolysis of C3 ($C3_{H2O}$) which allows the binding of components Factor B and D to form C3bBb convertase that can cleave additional C3 molecules and initate the amplification loop. This pathway is constantly inhibited by Factor H and FHL-1 proteins. The C3 convertases of all pathways are important for the activation of C3 into C3a and C3b. C3b deposition on microbial surfaces leads to opsonization for phagocytosis. In the presence of an additional C3b, the C3 convertase functions as a C5 convertase cleaving C5 (C5b and C5a) and initiating the membrane attack complex (MAC) that leads to complement-mediated lysis. C3a and C5a are important chemotactic factors that are released. The illustration was adapted from Trouw *et al* 2011 [40].

components C6 – 9 to initiate the assembly of the membrane attack complex (MAC) that leads to complement-mediated lysis [36]. Importantly, the complement system releases anaphylatoxins (C3a and C5a) which are potent chemoattractants that mediate chemotaxis of neutrophils, monocytes, and macrophages to sites of complement activation, inflammation, and generation of cytotoxic oxygen radicals [36].

Soluble and cell-bound complement regulators help to control complement attack on host cells. In the alternative pathway, activation in solution is mainly controlled by factor H (fH) and its truncated homolog, factor H-like protein 1 (FHL-1) (Fig. 3). FH is encoded by a single gene (*HF1*) located on human chromosome 1q32 within the regulators of complement activation gene cluster (RCA) [37]. FHL-1 is the product of the alternative splicing of *HF1*. The liver constitutively produces fH, but synthesis can also occur in peripheral blood, lymphocytes, myoblasts, epithelial cells, endothelial cells, and many more cell types. FH plasma levels have been reported to vary from 110 – 615μg/ml [0.8 – 5μM] [37].

Factor H protein and structure. FH is a very large glycoprotein (155kDa). It is composed of 20 repetitive units called "short complement regulators" (SCRs; Fig. 4) [38]. Each SCR is encoded by a single exon, with the exception of SCR2, which is encoded by exons 3 and 4. SCRs are composed of 61 residues and joined by linkers that consist of 3 – 8 residues and are arranged in head-to-tail fashion [38]. Because of its large size, glycosylation, and presumed inter-SCR flexibility there is no high-resolution crystal structure for full-length fH. However, 14 of 20 SCRs have been resolved by

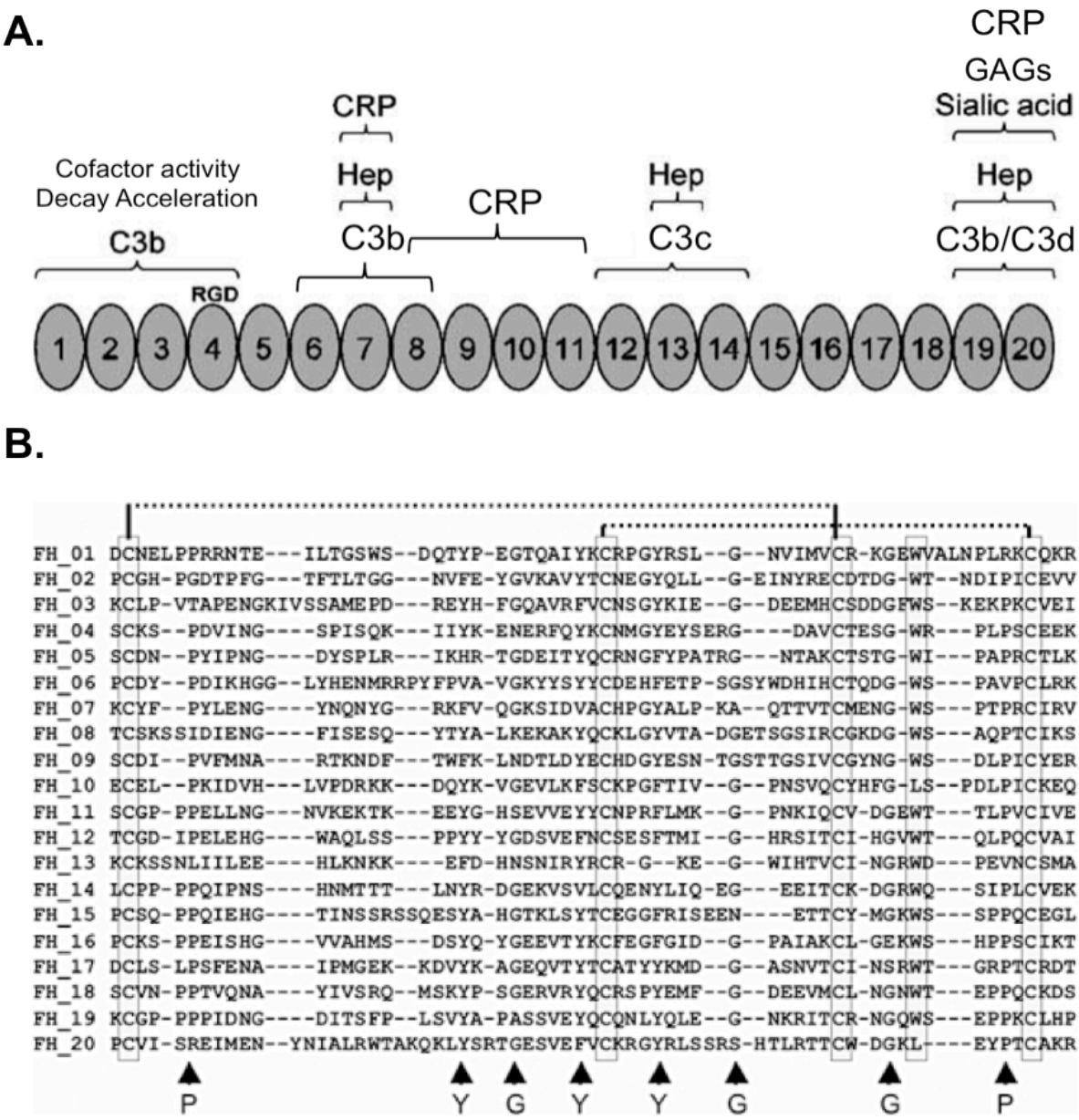


Figure 4. Factor H protein architecture and sequence of protein domains. (**A**) The factor H molecule is arranged like "beads on a string" composed of 20 repeat units called short complement regulators (SCRs). The location of binding sites for C3 fragments, polyanions (heparin, sialic acid, GAGs), and CRP are depicted above the diagram. The cofactor activity and decay acceleration functions of fH involve SCRs 1 – 4. The figure is adapted from Rodriguez de Cordoba *et al* 2004 [40]. (**B**) Sequence alignment of 20 SCRs of fH. Boxed inserts represent the 4 conserved cysteine residues and tryptophan. Arrows indicate well-conserved residues proline (P), tyrosine (Y), and glycine (G). Horizontal lines indicate disulfide bonds within the SCR modules. The figure is obtained from Schmidt *et al* 2008 [39].

crystallography and nuclear magnetic resonance. SCRs were reported to have a globular structure with six-stranded anti-parallel beta sheets connected with loops and turns [38]. Among the SCRs the conserved residues consist of four cysteines, two prolines, one tryptophan, and several other glycines and hydrophobic residues (Fig. 4, B). SCRs 1 – 4 are attributed to playing a role in cofactor activity and decay acceleration function of fH (Fig. 4, A). There are three binding sites for C3b (SCRs 1 – 4, 12 – 14, and 19 – 20) in addition to the three binding sites for heparin (SCRs 7, 13, and 19 – 20) [39, 40]. Sialic acid interacts at SCR 19 – 20. SCRs 7 – 11 and 19 – 20 engage with C-reactive protein [39, 40] (Fig. 4, A). The consensus sequence of fH SCRs are also seen in other members of the RCA family including C4b-binding protein (C4BP), complement receptor type I (CD35), decay accelerating factor (CD55), membrane cofactor protein (CD46), and fH-related proteins [39].

Factor H functions. FH mainly acts on C3 convertases (C3bBb) in the alternative pathway either by competing with Factor B for binding to C3b to accelerate the decay of the C3 convertase and preventing downstream cleavage events to occur or serves as a cofactor for Factor I-mediated cleavage of C3b to its inactivated form iC3b, which inhibits the amplification loop [41] (Fig. 3). Importantly, fH has the ability to recognize host-specific markers such as sialic acid or glycosaminoglycans, thereby contributing to self-recognition and prevention of self-attack. Microbes do not have these markers on their surfaces and therefore are typically more susceptible to complement attack.

Factor H deficiencies. There are a number of clinical implications that arise from aberrant fH activity due to the central role that fH plays in the regulation of complement. This is supported by clinical evidence showing that mutations or single nucleotide polymorphisms that affect fH recognition of host cells are associated with severe human diseases due to abnormal complement activation. In addition, observations in experimental animal models that have a deficiency in fH caused the animals to either die soon after birth or spontaneously develop diseases due to uncontrolled complement activation [37]. One such disease is called age-related macular degeneration (AMD), which is the leading cause of blindness in elderly people of European descent. A single amino acid variant in SCR 7 of fH has been strongly associated with a person's risk of developing AMD [42]. Other mutations in Factor B and C3 have also been attributed to the developmental risk of this disease. In particular, the mutation in fH reduces the affinity of fH for host markers (*e.g.* heparin and CRP); therefore decreasing the regulation of fH on critical surfaces, such as the back of the eye [42]. This mutation leads to an increase in inflammatory responses within the macula of the retina and leading to AMD. FH deficiencies can also lead to two rare but severe kidney diseases called membranoproliferative glomerulonephritis type II (MPGN II; dense deposit disease) and atypical hemolytic uremic syndrome (uHUS). Both diseases manifest at a very young age. Point mutations in either the conserved cysteine residues in SCRs or in particular SCRs 19 – 20 and autoantibodies that either neutralize fH or stabilize the C3 convertase lead to excessive complement activation and the

development of these diseases [42]. Current treatments for patients with these diseases include plasma infusion or plasma exchange therapy and renal transplantation.

Factor H-Ligand interactions. FH interacts weakly with its ligands (μM ranges). Many of the fH-ligand interactions reported exhibit a salt dependence because they are formed through opposing interactions. In general, fH is known to undergo 5 – 14% self-association in plasma if no other factors influence this equilibrium [38]. Evidence of self-association has been provided mainly from ultracentrifugation analyses, x-ray scattering concentration dependencies [43], and mass spectrometry, which revealed fH dimers and trimers [38]. Interactions between pairs of SCRs 6/8 and 16/20 are important for the formation of these fH oligomers. One of the most important fH-ligand interactions is fH and C3 fragments (C3b, C3c, and C3d). The interaction of fH with these C3 fragments is crucial for complement regulation. C3b binds to fH at SCRs 1 – 4, 6 – 8, and 19 – 20. C3c binds at SCRs 12 – 14 and C3d binds at SCRs 19 – 20 (Fig. 4, A). The affinity for binding to C3 fragments is about 0.59 – 2.9μM [38, 44]. Also, the interactions of fH with polyanionic molecules (e.g. heparin, glycosaminoglycans, and sialic acid; Fig. 4) are important. FH has a greater affinity to bind and attach to cells that express these specific markers. The affinity for binding is reported to be approximately 9.2nM [38, 44]. Other interactions of fH include binding to pentraxins (CRP and Pentraxin 3), apoptotic and necrotic cells (annexin-II, DNA, and histones), extracellular matrix proteins (fibromodulin, osteoadherin, and chondoradherin), and zinc, which causes fH to aggregate [44].

Recruitment of Factor H by bacterial pathogens. Given complement's central role as a host defense mechanism against invading microbes, it is not surprising that many microbes have evolved strategies to evade complement. Recent studies have reported microbial pathogens that are capable of recruiting fH [37]. The findings indicate that recruitment of fH is advantageous to the specific organisms. Potentially, microbes can recruit fH as a means to regulate and control the alternative pathway activation and evade the host defense mechanism. Binding of fH or FHL-1 proteins allows a direct and rapid inactivation of the newly formed C3b at the microbial surface. Generally, Gram-positive bacteria are protected from complemented-mediated lysis through their cell wall architecture, however; several Gram-positive pathogens, such as *Staphylococcus aureus* [45, 46], Group A streptococci [47-50], and *Streptococcus pneumoniae* [51-56] have all been shown to have the ability to bind fH. The best characterized is the PspC and Hic proteins of *S. pneumoniae*. Residues 38 – 158 of the N-terminus of the PspC protein are attributed to the interaction with SCRs 6 – 10, 13 – 15, and 19 – 20 on fH [52-54]. By interacting with fH, *S. pneumonia* has been shown to increase adhesion to host cells, resist complemented-mediated lysis, and suppress phagocytosis [54-56]. Two fH-binding proteins have been described for *S. aureus* (SdrE and Sbi). The binding regions for SdrE with fH have not been identified. The interaction of Sbi with fH involves domains III/IV on protein Sbi, SCRs 19 – 20 on fH and requires C3b/C3d for binding [45]. For *B. anthracis*, the ability to bind to fH has not been reported.

Knowledge of the dynamic interplay between pathogens and

components of the complement cascade are crucial to understanding the mechanisms of microbial invasion and persistence in an infected host. Despite the importance, knowledge of the specific interactions that occur between the complement system and *B. anthracis* is virtually non-existent. Likewise, the biological consequences of such interactions remain unknown. Moreover, the retention of spores observed from pulmonary anthrax exposure poses a problem with post-exposure prophylaxis. Despite this well-known phenomenon in inhalational anthrax, detailed information on how spores persist *in vivo* and factors contributing to persistence has been lacking.

In this dissertation, I examined the characteristics and spatial distribution of spores, as well as their association with different types of host cells. The results suggested potential mechanisms for spore persistence. Additionally, I investigated the basis of fH binding to the *B. anthracis* spore surface and defined the consequences of this interaction, particularly in relation to spore persistence, a critical aspect of *B. anthracis* pathogenesis in a host. I hypothesize that the recruitment of fH to the spore surface by BclA limits the extent of complement activation and promotes pathogen survival and persistence in the infected host.

If this model holds true, it will have significant contributions to the anthrax field. First, recruitment of fH by *B. anthracis* has not been reported. From the aspect of BclA-protein interactions, BclA is a novel ligand for fH. Second, fH is a major suppressor of complement activation. Hence, this study is likely to reveal a novel immune suppression mechanism of *B. anthracis*. Third, the idea that binding of fH and the presence of BclA is important

for persistent infections is a novel hypothesis and can have broad implications for many other pathogens, as well as vaccine development.

CHAPTER 2: *BACILLUS ANTHRACIS* PERSISTENCE *IN VIVO*

INTRODUCTION

One of the characteristic features of inhalational anthrax is the prolonged presence of spores in the lungs after an initial exposure. Decades ago this observation was reported by many researchers who were able to recover dormant spores from the lungs of non-human primates [7] and mice [8, 9] weeks or months after initial exposure. Additionally, studies by Heine *et al* reported that even with weeks of antibiotic treatments mice challenged with virulent *B. anthracis* spores still contained a substantial amount of spores in their lungs after discontinuation of the antibiotic regimen [8]. Pertinent studies in our laboratory showed that mice treated daily with antibiotics after a lethal challenge of *B. anthracis* spores, contained spores in the lung similar to mice treated without antibiotics. These studies suggested that spore persistence was observed independent of the use of antibiotics. Furthermore, our earlier experiments compared persistence in A/J mice, which are known to be more susceptible to the Sterne strain of *B. anthracis* and BALB/c mice, which are reported to be a resistant strain [57]. We found that host susceptibility is not a factor in spore persistence. Other studies have shown a delayed onset of anthrax infections (*e.g.*, 58 days after exposure) in exposed animals, and the persistence of spores in the lung was thought to be responsible for the delay [58]. Based on these studies, the current duration of antibiotic treatment recommended for people with pulmonary exposure to anthrax is 60 days.

Some microbial pathogens are able to thrive in the host despite the antimicrobial activities or host defense system causing a persistent infection. Unfortunately, our knowledge about the basis of bacterial persistent infections is not adequate to effectively prevent and treat such infections. There are several mechanisms that are described for bacteria that are able to do just that. These mechanisms include suppression of host immune responses [59], modulation of complement activation [60], resistance to phagocytic killing [61, 62], adaptation to the intracellular environment or to the mucosal surfaces [63, 64], biofilm formation [65], and evasion of host adaptive immunity [66]. For *B. anthracis*, the mechanism for spore persistence in the lung has not been investigated but has been largely attributed to the dormant nature and resilience of spores.

However, recent findings by our laboratory highlight the possibility that there may be factors and other properties of spores that contribute to spore persistence. Russell *et al* showed that spores were found inside epithelial cells in the mouse lung within hours after exposure to spores and that internalized spores survived inside lung epithelial cells [67, 68]. Thus the intracellular environment of lung epithelial cells can potentially be a niche for spores to persist. There has also been evidence for biofilm formation by *B. anthracis*, *B. subtilis* and other related species and the presence of spores within the biofilm [69]. Evasion of the host immune system by binding to complement regulators may potentially be one way spores may “hide” from the host defense system. Human pathogens, such as *Staphylococcus aureus* [45, 46], *Streptococcus pneumoniae [51]*, and *Streptococcus pyogenes* [49, 50] have all been

identified to bind complement regulator fH as an immune evasion tactic.

In this study, we investigated spore persistence in mice over a period of up to eight weeks. The spatial distribution of spores and their association with different host cells were examined. We also compared *B. anthracis* spores with spores from a non-pathogenic *B. subtilis* strain to determine whether "spores" in general can remain in a host for extended periods of time. The contribution of uptake by host cells and anthrax toxins to spore persistence was also examined. The results suggest that *B. anthracis* spores possess special properties that promote their survival and persistence in the host.

EXPERIMENTAL PROCEDURES

Bacterial strains and spore preparation. *B. anthracis* Sterne strain 7702, its isogenic toxin-deficient mutant strain (*Δlef*), plasmid-free *B. anthracis* strain 9131 derived from Sterne Strain 7702, and *B. subtilis* strain PY79 were provided by T. M. Koehler, UT Health, Houston, TX. Spores of these strains were prepared by culturing in PA media for 10 days at 30°C as described previously [67]. *E. coli* HB101 was grown in Luria Broth (LB) overnight at 37°C with shaking. The overnight cultures were washed three times with sterile Phosphate Buffered Saline (PBS) prior to inoculation into mice. Bacterial counts were determined by plating on LB agar plates and incubation overnight at 37°C.

Mouse infections. All animal experiments were carried out according to procedures approved by the Institutional Animal Care

and Use Committee, Texas A&M Health Science Center, Institute of Biosciences and Technology (IBT). BALB/c mice were originally purchased from the Jackson Laboratory and maintained in the IBT animal facility. For infection experiments, 6 – 8 weeks old and sex-matched mice were used. Mice were inoculated with spores or *E. coli* by intranasal instillation (i.n.) as previously described [29]. Briefly, mice were anesthetized with avertin (0.3mg/g) by intraperitoneal injection (i.p.). Mice were then inoculated i.n. with 20μls of a sub-lethal dose of spores (LD_{50} was determined in pilot studies to be ~1.7 x 10^8 cfu/mouse for BALB/c mice). Mice were monitored twice daily. Mice mostly appeared healthy with no physical signs of distress or illness throughout the experiments. To determine the bacterial burden in various organs mice were euthanized by avertin overdose. Lungs, kidneys, spleen, trachea and nasopharynx were collected at indicated time points. The tissues were homogenized in sterile cold PBS plus D-alanine, pH 7.4 (1 ml final volume) using a tissue homogenizer. Bronchoalveolar lavage (BAL) fluids were collected by lavaging the lungs with sterile PBS twice. The homogenates and BAL fluids were either directly diluted and plated to determine the number of total viable bacteria or heated at 68°C for 1 hour (hr) and dilution plated to determine the number of heat-resistant spores.

Isolation and immunofluorescence staining of crude lung cell suspension (CLCS). Lungs were harvested from mice at indicated time points and digested as previously described to obtain crude lung cells. The CLCS was stained using a previously described procedure with some modifications [67]. To stain for specific cell types and

spores, cells in the CLCS were allowed to attach to poly-L-lysine coated coverslips and then blocked with PBS containing 5% goat serum. Anti-BclA antibodies (rabbit polyclonal antibodies raised against recombinant BclA, a spore surface protein, 1:100) was added to the cells and incubated for 2 hrs followed by secondary antibodies conjugated to Alexa Fluor 594 to detect extracellular spores. Cells were then permeabilized, blocked and incubated again with anti-BclA antibodies followed by secondary antibodies conjugated to Alexa Fluor 488 to detect both extracellular and intracellular spores. Epithelial cells were detected with a pan epithelial mouse monoclonal antibody (Clone C-11, Chemicon, 1:250) recognizing different isoforms (4, 5, 6, 8, 10, 13, and 18) of cytokeratin, an epithelial specific marker, followed by secondary antibodies conjugated to Alexa Fluor 350. Anti-CD11c and anti-CD11b antibodies (1:250) were used to stain phagocytes in the CLCS and BAL fluids, respectively. Coverslips were mounted on slides using Fluorsave and viewed with a Zeiss Axiovert 135 microscope with the Axio Vision Software.

Histology and immunofluorescence staining of lung sections. This was done following a previously described procedure with slight modifications [67]. Briefly, lungs were harvested at indicated time points and fixed in 10% formalin. Embedding, sectioning and H&E staining were performed at the Breast Center Pathology Laboratory, Baylor College of Medicine, Houston, TX. To detect spores, lung sections were incubated in a solution containing anti-BclA antibodies (1:250) for 2.5 hrs followed by incubation with Alexa Fluor 594-conjugated secondary antibodies for 1 hr. Phalloidin Alexa

Fluor 488 was used to stain F-actin. DAPI was used to stain the nuclei. Some lung sections were also stained with wheat germ agglutinin Texas Red 595 (1:1000) to detect the plasma membrane. All dilutions were made in PBS pH 7.4 containing 2.5% fetal bovine serum. Five slides per mouse at each time point were examined. Lung sections were viewed with a Zeiss Axiovert 135 fluorescence microscope with the Axio Vision Software and an LSM 510 confocal laser scanning fluorescent microscope with the LSM 4.0 software (Zeiss).

Statistical analysis. Statistical analysis was performed using the two-tailed Student's *t*-test (Graph-Pad Prism 4.0).

RESULTS

The lung is the primary site for *B. anthracis* spore persistence. BALB/c mice were challenged intranasally (i.n.) with sub-lethal doses of spores (1.3×10^7 and 1.1×10^8) of *B. anthracis* Sterne strain 7702 ($pXO1^+$, $pXO2^-$). BALB/c mice are generally more resistant to the Sterne strain [57] and therefore provided us a model to assay for persistence of spores where mice can survive and remain physically healthy for weeks post-initial infection. The majority of mice in each group challenged appeared healthy and normal with no physical signs of distress or illness throughout the experiments. However, some mice died unexpectedly approximately a week after the initial exposure. To determine the condition of spores that went into the lungs for the i.n. challenge, we harvested the lungs of mice 1 hour after challenge and plated for spores and heat-sensitive vegetative bacilli (Time 0, Table 1). The results

showed that ≥90% of the recovered bacteria were spores, suggesting that most of the organisms that went into the lungs initially were spores.

Over a period of 8 weeks, the amount of vegetative bacilli and spores in different organs were evaluated. The results indicated that substantial amounts of bacteria were recovered from the lungs of mice at 2, 4 and 8 weeks post-inoculation (Fig. 5 and Table 1). Mice that were given ~10^8 spores contained on average 4.1 x 10^5 ± 9.7 x 10^4 colony forming units (cfu) and 9.2 x 10^4 ± 2.3 x 10^4 cfu per lung at 2 and 4 weeks post-inoculation, respectively (Fig. 5 and Table 1). These numbers correspond to approximately 0.3% and 0.09% of the initial inoculum. Furthermore, approximately 94% (2wks) and 50% (4wks) of the bacteria recovered from the lung were heat-resistant, indicating they were dormant spores. Additionally, mice inoculated with ~10^7 spores contained on average 3.7 x 10^5 ± 6.5 x 10^4, 2.3 x 10^4 ± 4.0 x 10^3, and 1.3 x 10^4 ± 6.0 x 10^3 cfu per lung at 2, 4, and 8 weeks post-inoculation, respectively (Fig. 5 and Table 1). The cfu numbers from mice infected with this dose represent 3.3%, 0.23%, and 0.09% of the initial inoculum retained in mice lungs over time. We found that on average 56, 70, and 81% of bacteria recovered from 2, 4, and 8 weeks post-inoculum were heat-resistant dormant spores. Heat-sensitive vegetative bacilli were also detected at all three time points, indicating that spore germination occurred in the lung although at a lower frequency. We observed decreases in the total bacteria and spore titers in the lung over the experimental period, suggesting a continuous host clearance process. The decrease in the lung bacterial burden from 2

Table 1. Bacteria and spore burden in the lungs of mice.

Inoculum (Spores/mouse)	Time (weeks)	Total Bacteria (cfu x 10^4/lung ± SEM)[b] Total Spores (cfu x 10^4/lung ± SEM)[c]	*p*-value[d] (vs. 2 wks)
1.3×10^7	0[a]	150 ± 0.1	–
		132 ± 0.2	
	2	37 ± 6.5	–
		20 ± 5.0	
	4	2.3 ± 0.4	<0.01
		1.6 ± 0.4	<0.05
	8	1.3 ± 0.6	<0.01
		1.1 ± 0.6	<0.05
1.1×10^8	0[a]	2600 ± 0.7	–
		2340 ± 0.4	
	2	41 ± 9.7	–
		39 ± 9.2	
	4	9.2 ± 2.3	<0.01
		4.4 ± 1.3	<0.01
	8	ND[e]	ND[e]

[a]Time at 0 weeks represents the average retained dose determined by harvesting the lungs 1 hour after intranasal inoculation and dilution plating the lung homogenates.
[b]The amounts of total bacteria in the lung as described in the Figure 5 legend are expressed as the mean ± SEM at 2, 4, or 8 weeks post-inoculation. Results are combined from two independent experiments.
[c]The amounts of total spores in the lung (shaded in gray) as described in the Figure 5 legend are expressed as the mean ± SEM at 2, 4, or 8 weeks post-inoculation. Results are combined from two independent experiments.
[d]Statistical analysis was performed using the two-tailed Student's t-test. *, $p < 0.05$; **, $p < 0.01$; compared to respective total bacteria and spore titers at 2 weeks.
[d]Not determined.

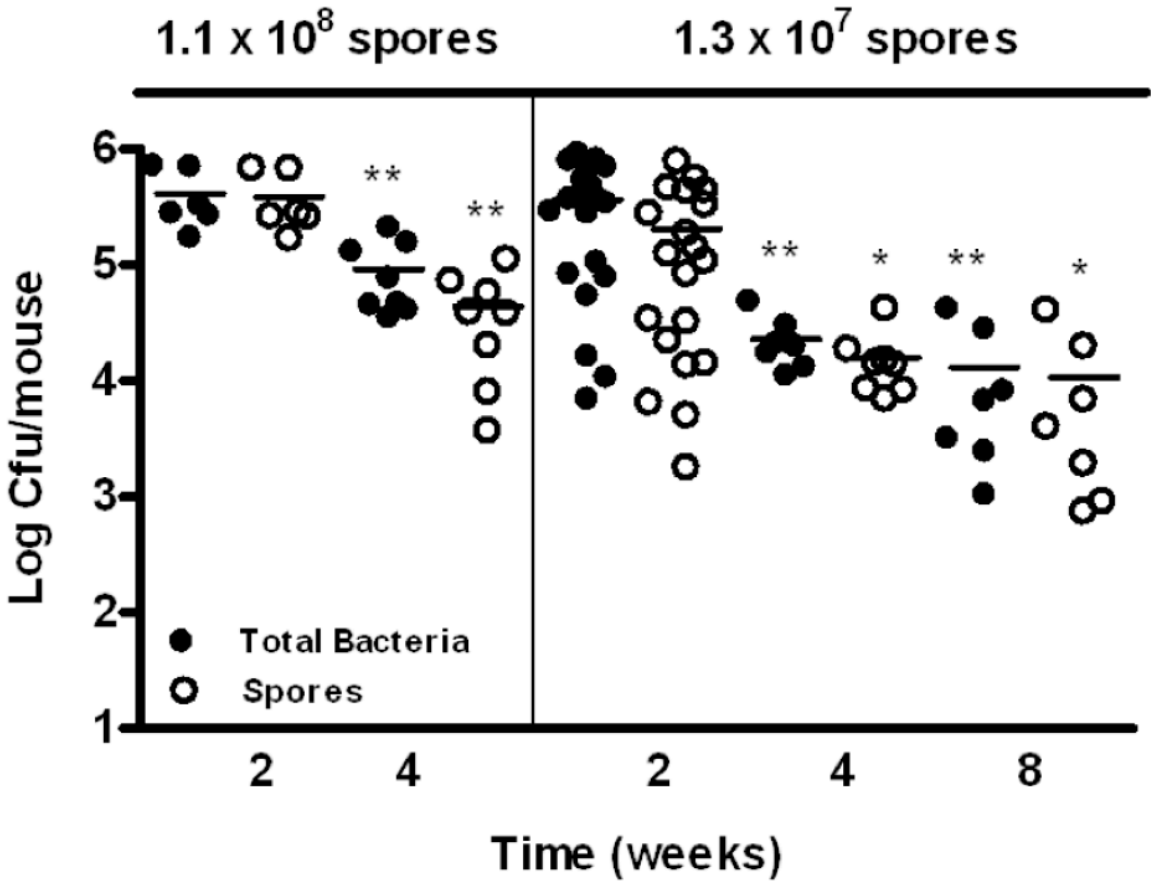


Figure 5. Bacterial and spore burden in the lungs of mice at 2, 4, and 8 weeks post-intranasal inoculation. Mice were inoculated i.n. with ~1.1 x 10^8 or ~1.3 x 10^7 spores/mouse. Lungs were harvested at 2, 4, and 8 weeks, homogenized, and dilution plated with or without heat treatment. The results were combined from at least two independent experiments. Closed circles represent total viable bacteria and open circles heat-resistant dormant spores. *, $p < 0.05$; **, $p < 0.01$; compared to respective total bacteria and spore titers at 2 weeks.

to 4 weeks was significant (~13 – 25 fold) and from 4 to 8 weeks was relatively moderate (Fig. 5 and Table 1).

To investigate if the lung was the major organ for spore persistence we examined the bacterial burden in other tissues over the experimental time frame. The bacterial titers in the spleen and kidney at 2 and 4 weeks were significantly lower (approximately 10^3 – 10^4 fold) than those in the lung (Fig. 6, A and B). Similarly, a low bacterial load was found in the spleen at 8 weeks (data not shown). Intranasal inoculation exposed the nasopharynx associated lymphoid tissue (NALT) to the spores. A previous report indicated that the NALT was among the first organs in which vegetative growth was observed following pulmonary exposure to spores [70]. We examined the bacterial burden in the nasopharynx, as well as, the trachea at 2 and 4 weeks post-inoculation. Less than 550 cfu were recovered from the trachea and less than 220 cfu from the nasopharynx at 2 weeks. Even lower numbers (240 and 17 bacteria) were recovered at 4 weeks from the trachea and nasopharynx, respectively (Fig. 6). These numbers were significantly lower than those recovered from the lung at both time points. Therefore, despite the fact that in acute anthrax infections bacterial growth is observed in multiple organs, including the ones examined in the above experiments, as expected, only the lung is the primary site for spore persistence.

***B. anthracis* has special properties that contribute to its ability to persist in mice.** We next investigated whether the ability of *B. anthracis* spores to persist in the lung was due to special properties of *B. anthracis* or if the lung tolerates metabolically inactive spores in general. Therefore, we examined if spores from

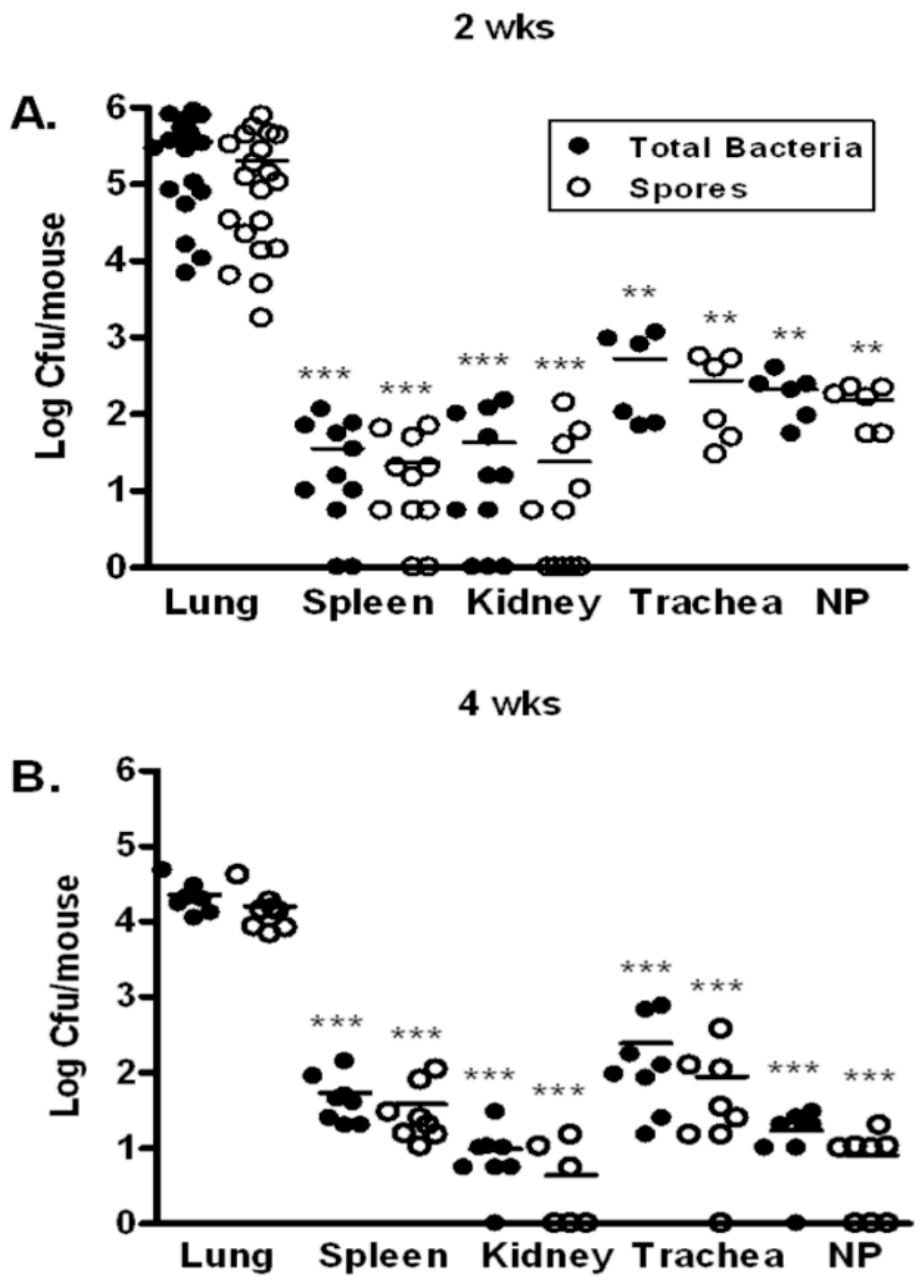


Figure 6. Bacterial and spore burden in various organs at 2 and 4 weeks post-inoculation. Mice were inoculated i.n. with ~1.3 x 10^7 spores/mouse. Bacterial burden in the lung, spleen, kidney, trachea, and nasopharynx (NP) at 2 (**A**) and 4 (**B**) weeks was determined as described in the experimental procedures. The results were combined from at least 2 independent experiments. Closed circles represent total viable bacteria and open circles heat-resistant dormant spores. **, $p < 0.01$; ***, $p < 0.001$; compared to respective total bacteria and spore titers in the lung.

Bacillus subtilis could persist in the lung equally well as those from *B. anthracis*. Mice were inoculated i.n. with approximately 1.3 x 10^7 7702 or *B. subtilis* strain PY79 spores. Lungs were harvested at 2 and 4 weeks post-inoculation and evaluated for total viable bacteria and spores. At 2 weeks post-inoculation, significantly more spores were recovered from the lungs of *B. anthracis*-infected mice (2 x 10^5 ± 5 x 10^4 spores/lung) compared to those from the lungs of *B. subtilis* (2 x 10^4 ± 7 x 10^3 spores/lung; Fig. 7). Similarly, at 4 weeks post-inoculation, mice inoculated with *B. anthracis* spores had approximately 13 fold more spores in the lungs compared to mice inoculated with *B. subtilis* spores (Fig. 7). These results suggest that *B. anthracis* spores are better at persisting in the mouse lung than *B. subtilis* spores. Mice were also inoculated with overnight cultures of *E. coli* HB101, a non-sporulating, non-pathogenic strain as a negative control. These mice had significantly fewer bacteria (<1250 bacteria at both time points) in the lung than those infected with *B. anthracis* and *B. subtilis*, respectively (Fig. 7). We also calculated the spore burden in the lung as a percentage of the initial inoculum. At 2 weeks, the percentages were approximately 1.7%, 0.25% and 0.01% for mice infected with 7702, PY79 and *E. coli*, respectively. At 4 weeks, the percentages were approximately 0.15%, 0.02% and 0.01% for the three strains, respectively. These results suggest that in addition to dormancy and resilience, *B. anthracis* spores may possess special properties (*i.e.,* exosporium), which are absent in *B. subtilis* that facilitate their persistence in the lung.

Association of spores with the lung epithelium, epithelial cells, and phagocytes. We next examined whether the persistent *B.*

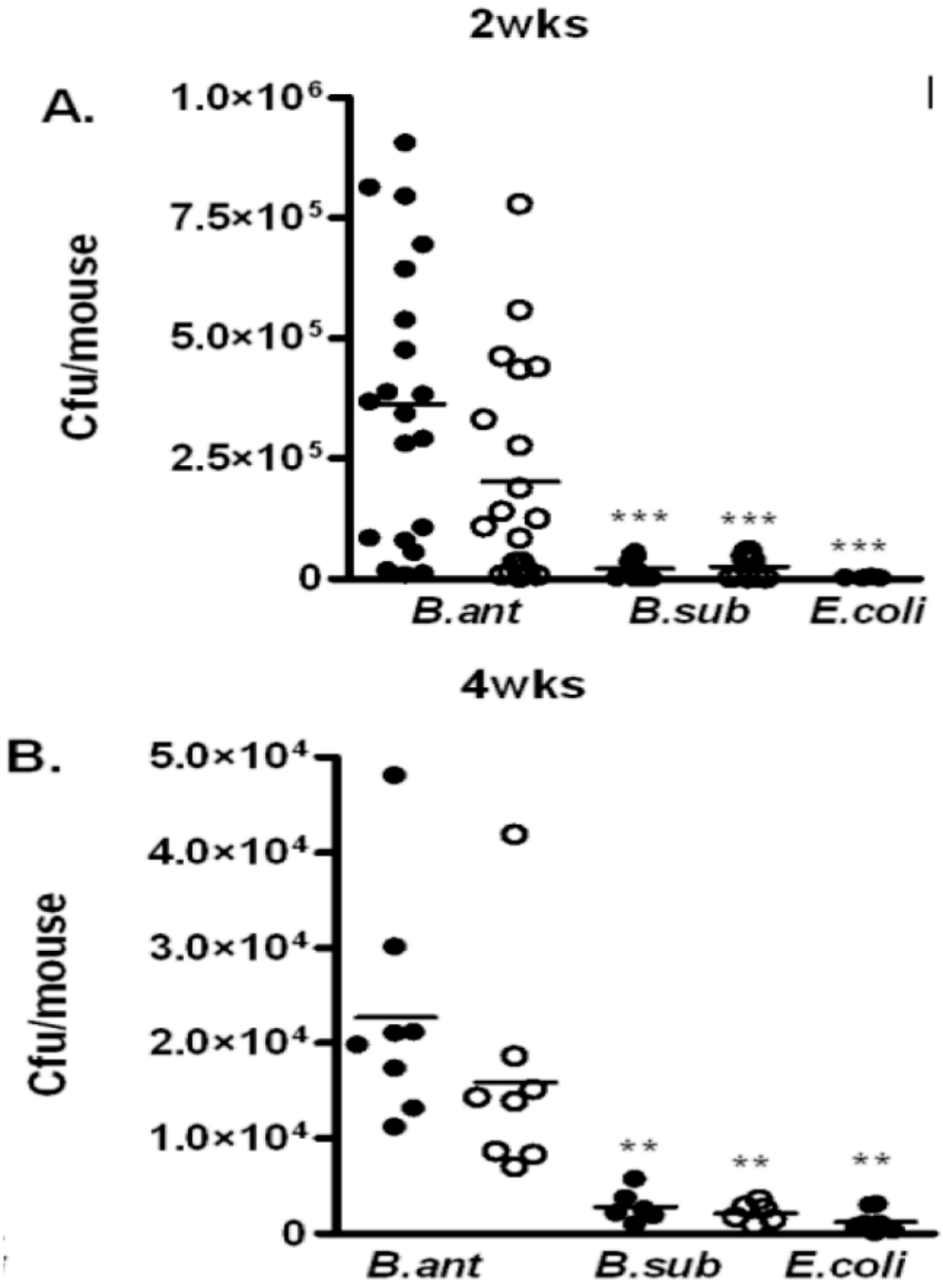


Figure 7. *B. anthracis* spores persisted in the lung significantly better than spores of *B. subtilis*. Mice were inoculated i.n. with 7702 spores (*B. ant*), PY79 spores (*B. sub*), or overnight cultures of HB101 (*E. coli*) at a dose of ~ 1.3 x 10^7 cfu/mouse. Lungs were harvested at 2 (**A**) and 4 (**B**) weeks post-inoculation, homogenized, and plated for total viable bacteria (closed circles) or heat-resistant dormant spores (open circles). The results were combined from at least 2 independent experiments. **, $p < 0.01$; ***, $p < 0.001$; compared to respective total bacteria and spore titers in *B. anthracis* infected lungs.

anthracis spores were tightly associated with the lung tissue or in the fluid lining the respiratory epithelium, the latter of which can be recovered in the bronchoaveolar lavage (BAL) fluid. The results showed that at both 2 and 4 weeks post-inoculation, there were significantly more total bacteria as well as spores in the lung tissues than in the BAL fluid, suggesting that spores preferentially associated with lung tissues (Fig. 8, A – D). The difference of spores recovered from the mouse lung tissue compared to the BAL in mice given ~10^7 spores was much more pronounced at 2 weeks post-inoculation (17 fold more cfu) than at 4 weeks (6 fold more cfu) (Fig. 8, A and B). Mice given ~10^8 spores had approximately 5 – 6 fold more cfu in their lung tissue than in their BAL at both 2 and 4 weeks post-inoculation (Fig. 8, C and D). Interestingly, at 8 weeks post-inoculation more bacteria were recovered from the BAL than the lung tissue (data not shown). These results show for the first time that dormant heat-resistant spores primarily associate with the lung tissue.

We further analyzed lung sections from infected mice to determine the location of spores in the lung. Lung sections were obtained from mice infected with *B. anthracis* spores at 2, 4, and 8 weeks. Hematoxylin and eosin (H&E) staining was performed to observe the general pathology in the lung. Overall, there was minimal pathology observed in the lung (Fig. 9). The alveolar and small airway epithelium as well as blood vessels appeared intact. For the most part of the lung, we observed no signs of inflammation or lesions (Fig. 9, A, B, D, E, G and H). We occasionally observed isolated foci that had inflammatory infiltrates at both 2 and 4

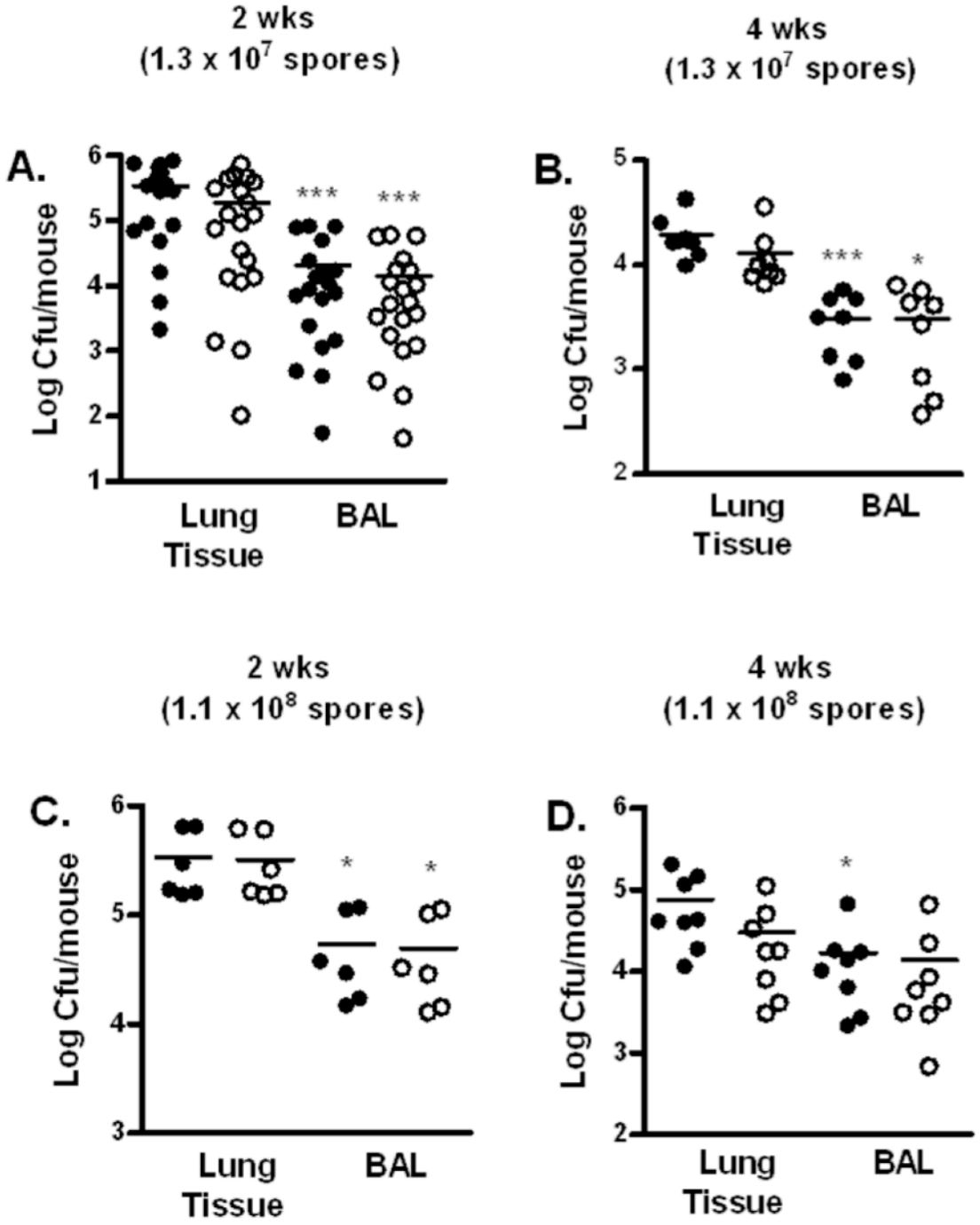


Figure 8. The majority of persisting spores associated tightly with the lung tissue. Mice were inoculated i.n. with ~1.3 x 10^7 spores/mouse (**A** and **B**) and ~1.1 x 10^8 spores/mouse (**C** and **D**). Lungs were lavaged with sterile PBS and collected. Total bacteria (closed circles) and spore (open circles) titers in the lung tissues and BAL fluids at 2 (**A** and **C**) and 4 (**B** and **D**) weeks were determined. The results were combined from at least two independent experiments. *, $p < 0.05$; ***, $p < 0.001$; compared to respective total bacteria and spore titers in the lung tissue.

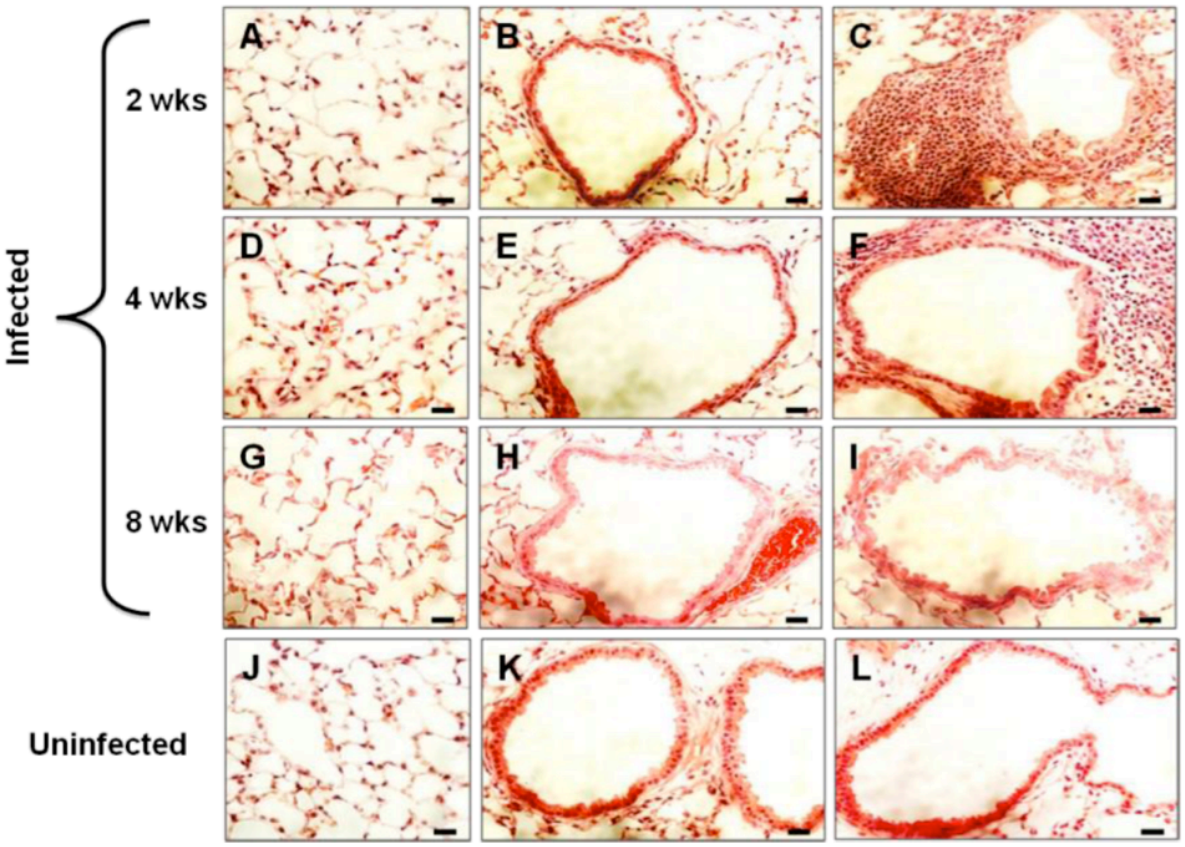


Figure 9. H&E stained lung sections of mice challenged with *B. anthracis* spores. Lungs from infected and control mice were collected at 2, 4, and 8 weeks post-inoculation, fixed and subjected to H&E staining. Representative images displaying the alveoli and the airway from mice infected for 2 (**A** – **C**), 4 (**D** – **F**), and 8 weeks (**G** – **I**), and uninfected control mice (**J** – **L**) are shown. Scale bars represent 20 µm.

weeks (Fig. 9, C and F). At 8 weeks, the lungs were indistinguishable from the uninfected lung (Fig. 9, G – L).

To detect spores in the lung, immunohistochemistry was performed. Lung sections were stained with antibodies specific for the exosporium protein BclA on spores. Cells were stained with phallodin and DAPI to visualize F-actin and the nuclei, respectively. Alternatively, cells were stained with wheat germ agglutinin conjugants to outline the plasma membrane. Microscopic examination of the stained sections revealed that spores were present in the lung at all three time points. Spores were observed associated with the alveolar and the small airway epithelium as single spores rather than in clusters (Fig. 10, A – F). Additionally, spores were seen distributed relatively evenly throughout the lung tissue similar to what was reported previously [67, 68]. Some of the spores that associated with the airway and alveolar epithelium appeared intracellular. To determine if the latter group of spores was indeed intracellular, we analyzed the Z-stacks of confocal images of 124 potential intracellular spores from 2 and 4 weeks. Of these, 101 (81%) were surrounded by F-actin (Fig. 11, A) or enclosed within the plasma membrane (Fig. 11, B) and therefore were likely to be intracellular. We also observed spores located within lymphoid-like tissues, although infrequently (Fig. 11, G). Positive antigen staining of either intact spores or spore fragments were observed in the alveolar space (Fig. 11, H), suggesting that inflammation was associated with the presence of dormant or germinating spores or spore fragments. Isolated foci displaying mild infiltration of lymphocytes were observed in the

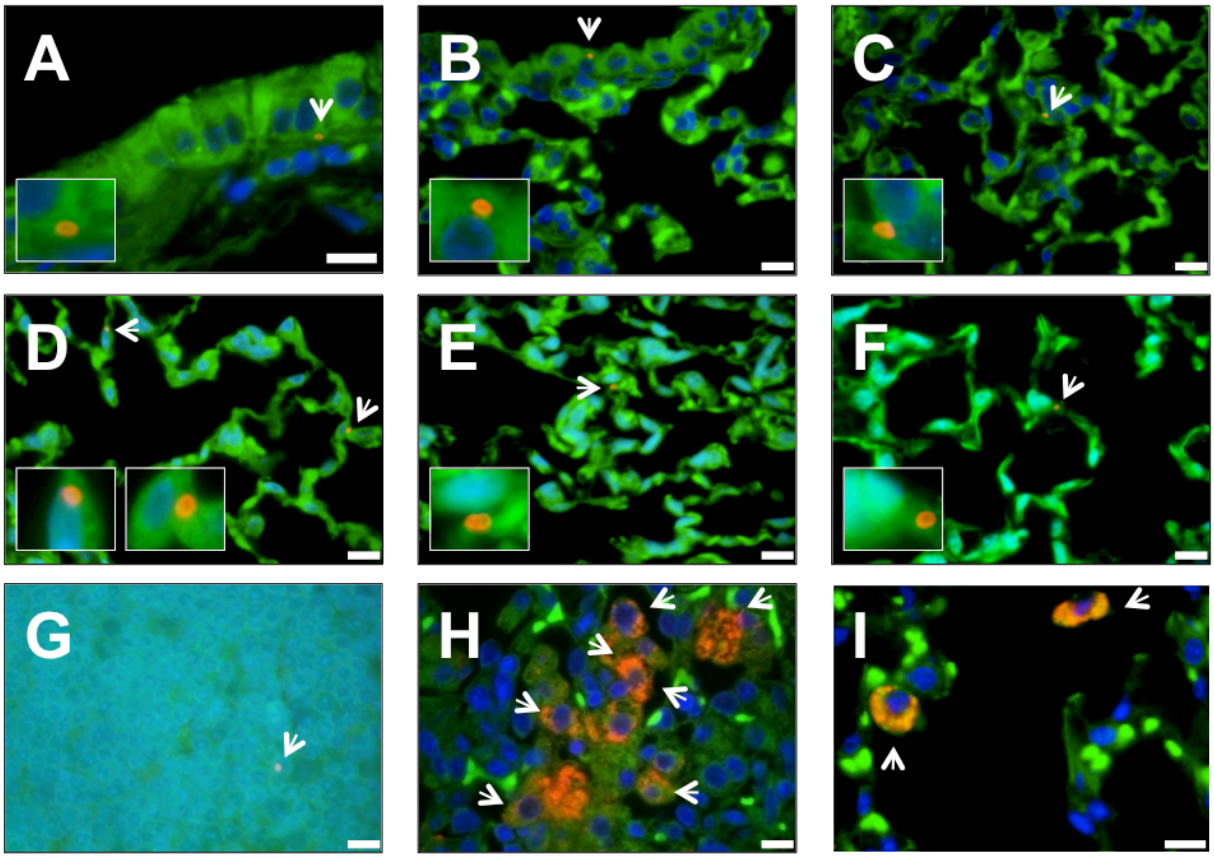


Figure 10. Representative images of immunofluorescently stained lung sections from mice challenged with *B. anthracis* spores. Mice were infected i.n. with ~ 10^8 spores/mouse. Lungs were harvested at 2 and 4 weeks, fixed, sectioned, and stained with anti-BclA antibodies and secondary antibodies conjugated to Alexa Fluor 594 (red), Alexa Fluor 488-conjugated phalliodin (green) and DAPI (blue), as described in the experimental procedures. Representative images are shown to indicate spore association with the small airway epithelium (**A** and **B**), the alveolar epithelium (**C** – **F**), lymphoid-like tissues (**G**), antigen staining (**H**), and macrophage association (**I**). Arrows indicate spores. The areas around those spores were enlarged and shown in boxed insets. Scale bars represent 10 μm.

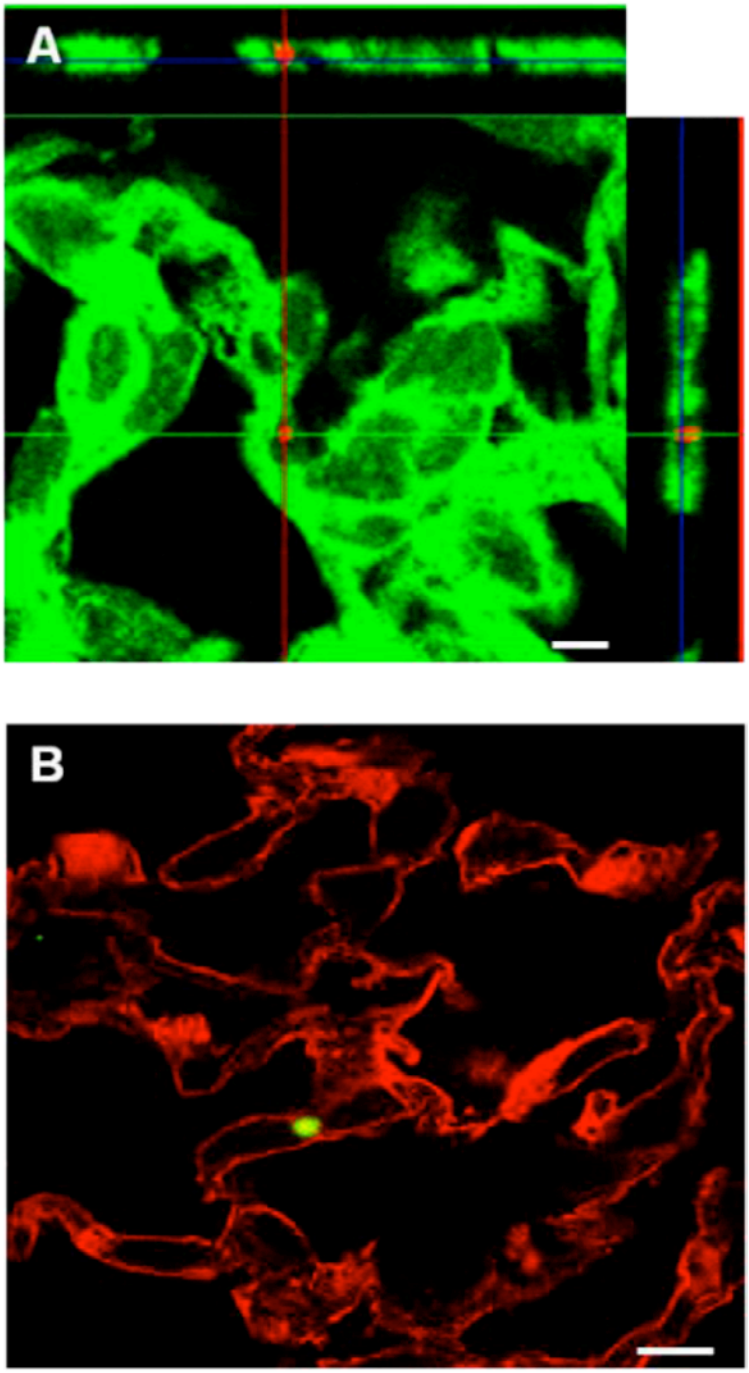


Figure 11. Confocal analysis of immunofluorescently stained lung sections. Lung sections were stained as described in the experimental procedures and analyzed by confocal microscopy. Representative images are shown. (**A)**, an image showing a spore (red) surrounded by F-actin (green). The projections show all planar views, including *xy* (center panel), *xz* (right panel) and *yz* (top panel)-stacks. (**B)**, the section was stained with wheat germ agglutinin to outline the plasma membrane (red) and anti-BclA antibodies to detect spores (green). Scale bars represent 10 μm.

immunofluorescently labeled lung sections, consistent with the H&E stained sections. Spore association with alveolar phagocytes was also observed (Fig. 11, I).

To investigate the specific cell types with which spores associated, crude lung cell suspensions (CLCS) were prepared from lungs harvested at 2 and 4 weeks post-inoculation. Epithelial cells in the CLCS were detected using anti-cytokeratin antibodies (Fig. 12, A). Approximately 23% of the CLCS cells were cytokeratin-positive, consistent with previous results [67]. Approximately 3.8% and 7.6% of epithelial cells examined were associated with spores at 2 and 4 weeks, respectively. On average, the percentage ratio of intracellular and extracellular adhered spores relative to cytokeratin positive epithelial cells was 0.95 ± 0.4% and 2.78 ± 1.1% at 2 weeks, and 1.9 ± 0.3% and 5.7 ± 1.1% at 4 weeks, respectively (Fig. 12, B). Thus, spores associated with lung epithelial cells both extracellularly and intracellularly with the majority being extracellular. The CLCS also contained lung dendritic cells and residual alveolar macrophages that were not removed by lavage. To determine spore association with these phagocytes we stained the CLCS using anti-CD11c antibodies. Multiple spores were often seen inside these phagocytes (Fig. 12, C). Approximately 4.1% and 3.3% of $CD11c^+$ cells contained spores at 2 and 4 weeks, respectively (Fig. 12, D). We also stained the BAL fluids with anti-CD11b antibodies and observed approximately 14.8% and 4.5% of $CD11b^+$ cells contained spores at 2 and 4 weeks, respectively (Fig. 12, E and F). Many of the phagocytes appeared to be packed with spores

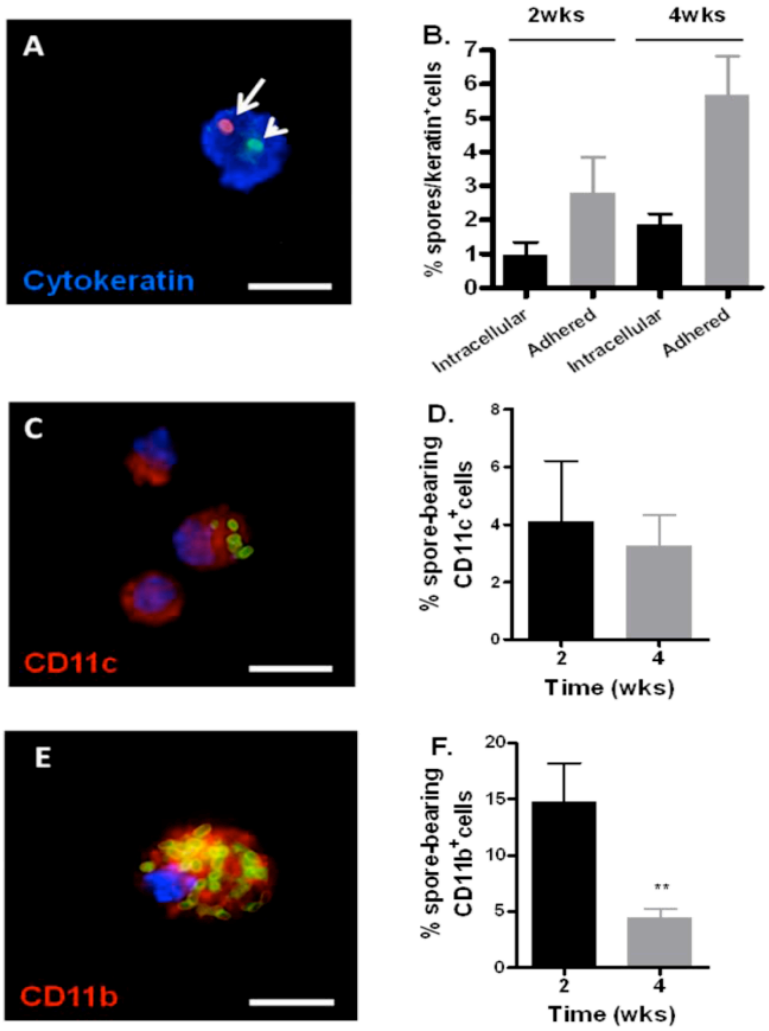


Figure 12. Spore association with epithelial cells and phagocytes in the lung. Mice were inoculated i.n. with ~ 1.1 x 10^8 spores per mouse. At 2 and 4 weeks post-infection, lungs were harvested and digested to obtain CLCS. Lungs were also lavaged to obtain BAL fluid. (**A)** A representative image of a lung epithelial cell stained with anti-cytokeratin antibodies (blue) containing an extracellular spore (red, long arrow) and an intracellular one (green, short arrow). (**B)** The percentage ratio of intracellular and extracellular adhered spores relative to the number of cytokeratin$^+$ cells counted at 2 and 4 weeks post-inoculation, expressed as the mean ± standard error of the mean (SEM). Approximately 500 – 600 cytokeratin$^+$ cells were counted per mouse. (**C** and **E**) representative images of cells stained positive with CD11c antibodies (**C**, red) and CD11b antibodies (**E**, red). Spores were stained green. Blue indicates DAPI staining for the nuclei. (**D** and **F)** the percentage ratio of spore-containing CD11c$^+$ and CD11b$^+$ cells relative to the total number of CD11c$^+$ and CD11b$^+$ cells counted at 2 and 4 weeks post-inoculation, respectively, expressed as the mean ± SEM. Approximately 100 CD11c$^+$ cells and 500 – 600 CD11b$^+$ cells were counted per mouse. Scale bars represent 10 µm. **, $p < 0.01$ comparison between 2 to 4 weeks % spore-bearing CD11b$^+$ cells.

however, making accurate enumeration of the intracellular spores difficult.

The anthrax toxins are not involved in spore persistence in the lung. Minimal inflammation observed in the lung from the H&E stained sections suggested that there was a subdued immune response in the presence of spores. *B. anthracis* lethal factor (LF) is a Zn^{2+}-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases (MEKs) and plays an important role in suppressing the host immune responses during anthrax infections [71]. We observed a low level of spore germination in the lung during the studies described above. LF was reported to be secreted shortly after spores germinate [72]. We investigated the possibility that LF secreted by the germinated spores suppressed the immune response and contributed to spore persistence in the lung. We compared the persistence of spores from the Sterne strain 7702 with that from the isogenic LF-deficient strain (Δ*lef*) [73] as well as a plasmid-free 7702 Sterne strain 9131, which does not produce any anthrax toxins. We did not observe any significant difference in either the total bacteria or the spore counts between the three strains at 2 or 4 weeks post-inoculation (Fig. 13, A and B), indicating that the anthrax toxins do not play a role in the persistence of spores in the mouse lung.

DISCUSSION

The ability to recover *B. anthracis* spores from the lungs of animal hosts weeks or months after the initial pulmonary exposure has been known for decades. For this reason, a 60-day antibiotic regimen is recommended for people with pulmonary exposure to spores

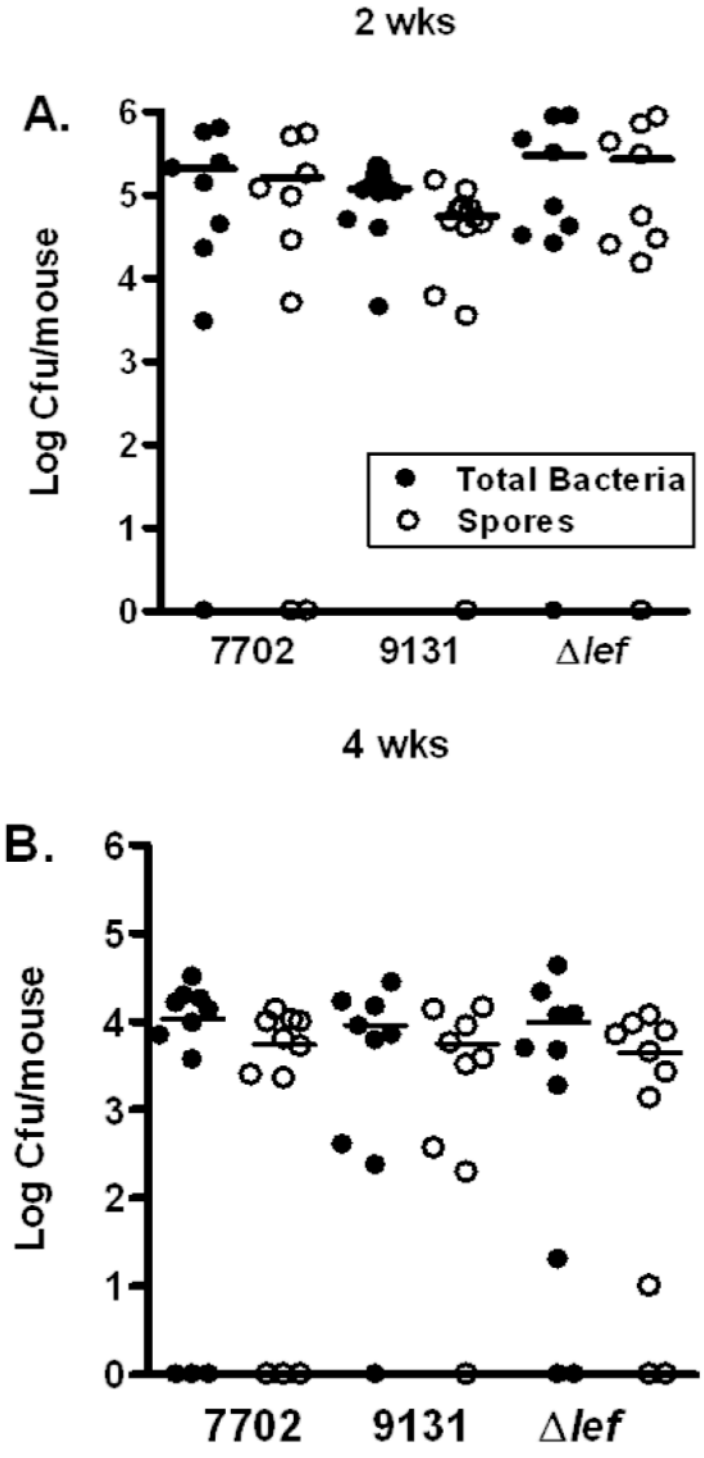


Figure 13. Bacterial and spore burden in the lungs of mice infected with toxin-deficient *B. anthracis* mutants. Mice were inoculated i.n. with ~ 1.5 x 10^7 spores. Lungs were harvested at 2 and 4 weeks post inoculation, homogenized and plated with or without heat treatment. The results were combined from two independent experiments. Closed circles represent total viable bacteria and open circles heat-resistant dormant spores.

[8, 9, 58]. However, beyond this knowledge detailed information on how spores persist *in vivo* and factors contributing to persistence was lacking. Here, we investigated the characteristics and spatial distribution of spores in mice as well as the association of spores with different types of host cells for up to 8 weeks. We also compared the persistence between spores of *B. anthracis* and *B. subtilis*. The results provided insights into the potential mechanisms underlying spore persistence, as discussed below.

It has been reported that during late stages of acute inhalational anthrax infections large amounts of vegetative bacilli of *B. anthracis* were present in multiple organs, including the liver, kidney, lymphoid tissues, and even the brain, presumably via hematogenous spread [9, 70]. Results from the current study suggest that chronic persistence of spores *in vivo* exhibits a different pattern of tissue distribution. The lung is the primary site for spore persistence. Interestingly, the number of spores in the NALT is orders of magnitude less than that in the lung despite that during a pulmonary exposure the NALT is also exposed to spores. Furthermore, previous studies in a mouse model for acute inhalational anthrax infections showed the detection of vegetative growth of *B. anthracis* in the NALT within hours of exposure to spores [70]. These results raise the possibilities that the lung appears to either “tolerate” spores better than the NALT, and/or discourage spore germination, and that the process of dissemination from the lung to other organs perhaps provides the necessary environmental cues or conditions for spores to germinate and become vegetative bacilli.

The majority of recovered bacteria in the lung were heat-resistant spores. Additionally, heat-sensitive bacilli were detected at all time points in the lung, but at significantly lower amounts compared to spore titers. This suggests that spore germination occurs although at a lower level during persistent infections. This can potentially be a cause for recurrent infections. In fact, a small number of mice succumbed to infection during the time course experiments; however these mice were not included in the analyses. It is possible that germination may occur inside host cells as a number of previous studies suggested intracellular germination using *in vitro* cultured cells [68, 74, 75]. The possibility that spores germinate extracellularly in the lung at a low frequency cannot be excluded either. In acute infections, different groups have investigated the site of spore germination after pulmonary exposure. Findings by Glomski *et al* [70] and Ross [76] indicated that spore germination occurred in organs other than the lung. However, studies by Sanz *et al* utilizing a Sterne strain that specifically expressed bioluminescence only upon germination reported germination proceeded in the lung [77]. The use of different bacterial/mouse strains and/or experimental methodologies utilizing different spore doses could have contributed to the different conclusions [70]. We also observed some spores in the other organs examined; however, the titers are orders of magnitude lower than those in the lung. These spores presumably spread to the distal organs via phagocytic cells.

The next question is where in the lung spores persist. The bacterial burden in the BAL fluids and in the lavaged lung tissues suggest that the majority of persistent *B. anthracis* spores appeared

to be tightly associated with lung tissues, as they could not be readily removed by lavage. Further examination of thin sections of the lung revealed that the spores adhered to the alveolar and airway epithelium and some were inside the cells lining the epithelium. This was further corroborated by immunofluorescence staining of lung cells harvested from the infected lungs. Differential immunofluorescence staining of lung epithelial cells indicated that there were more extracellularly adhered spores than intracellular ones. This suggests that adherence to the lung epithelium may be an important mechanism for spores to persist in the lung. Residing in an intracellular niche of lung epithelial cells may be a secondary mechanism. The results also indicated that epithelial cells were not the only cell type that spores associated with. We observed spores inside $CD11b^+$ and $CD11c^+$ cells at both 2 and 4 weeks, and most of the spores appeared intact. The results in Fig. 8 indicated that viable spores were recovered from the BAL fluids at 2 and 4 weeks. These results suggest that spores may be viable inside the phagocytes, consistent with previous reports that spores were able to survive inside phagocytic cells [78]. Therefore, it is possible that the intracellular environment of phagocytic cells is a niche for spore persistence. On the other hand, multiple groups with somewhat controversial results have investigated the fate of phagocytosed *B. anthracis* spores. Spores are thought to germinate inside macrophages. Some of the studies reported replication of vegetative bacilli inside macrophages [72, 75, 79], while others showed killing of germinated spores and vegetative bacilli by macrophages [80-82]. Therefore, recovery of viable spores from phagocytes does not

exclude the possibility of phagocytic killing of germinated spores and vegetative bacilli. The finding that total bacteria and spore titers in the lung significantly decreased over the experimental period, while the titers in other organs examined remained very low suggested a continuous host clearance process. Therefore, it is also possible that the spore-bearing $CD11b^+$ and $CD11c^+$ cells we observed reflect a part of the clearance process.

We investigated if the ability to persist in the lung could be a general phenomenon of *Bacillus* spores, *i.e.*, if spores from other species of *Bacillus* could also persist in the lung. The significantly lower bacterial/spore burden in the lungs of mice exposed to spores of a *B. subtilis* strain suggests that *B. anthracis* spores possess special properties that promote their survival and persistence in the lung. Moreover, it would be interesting to investigate if other exosporium-containing spores (*e.g. B. cereus*) could persist in the host in comparison to 7702 spores. *B. subtilis* spores are known to share a similar coat protein profile as that of *B. anthracis* spores but lack an exosporium which is present on the latter [83]. The exosporium is the outermost integument of *B. anthracis* spores and is composed of 20 — 30 proteins and glycoproteins [12, 14, 84]. It is possible that specific exosporium components contribute to *B. anthracis* spore persistence in the lung. BclA, a major component of the *B. anthracis* exosporium and absent in *B. subtilis* spores, was shown in a previous study to interact with surfactant protein C [85]; however the biological function of this interaction is unclear. It was previously shown that surface components on *B. anthracis* spores were sufficient to mediate spore

adherence and entry into epithelial cells [68] and that *B. subtilis* spores were much poorer at adherence or entry into host cells [86]. It was reported that entry of *B. anthracis* spores into epithelial cells was mediated by the spore surface glycoprotein BclA and host cell receptor integrin α2β1 via a novel mechanism that requires complement component C1q as a bridging molecule [29]. Spores from a BclA deletion mutant (*ΔbclA*) showed decreased entry into epithelial cells compared to spores from the isogenic parent strain [29]. However, other studies reported a different role for BclA that it reduces spore entry into non-phagocytic host cells [27, 87]. One of the factors contributing to the discrepancy may be the germination status of spores in the assays. It seems that if spores remain dormant, BclA plays an important role in host cell entry via the BclA-C1q-α2β1 pathway. However, if spores are allowed to germinate in the assay media, the presence of BclA appeared to be a disadvantage in host cell entry [29]. As the majority of spores remain dormant in the host lung, results from dormant spores are likely to be more relevant to the *in vivo* situation. Studies to investigate the role of BclA in spore persistence *in vivo* are currently underway in our laboratory.

Evans *et al*. reported that a bacterial lysate could induce innate resistance to infections caused by pulmonary exposure to *B. anthracis* spores. The authors further demonstrated that lung epithelial cells rather than macrophages or neutrophils were responsible for the induced resistance [88]. This demonstrates that the lung epithelium is an important player in the host defense against infections by *B. anthracis* spores. It is conceivable that in

addition to mediating adherence and entry, interactions between exosporium components and the lung epithelium may also influence the host immune responses in a way that favors the survival of spores in the lung. We are currently carrying out studies to investigate the precise role of specific exosporium components in spore persistence.

The very mild inflammation and pathology observed in the lung is also indicative of a subdued immune reaction to *B. anthracis* spores. While the spore surface does not have any typical pathogen-associated molecular patterns, such as lipopolysaccharide, lipotechoic acid, peptidoglycan, or flagellin, a number of studies indicated that *B. anthracis* spores are not "stealth" to host immune recognition. Spores are capable of activating Toll-like receptor 2 and MyD88-dependent signaling [30], inducing inflammatory cytokine production in both MyD88-dependent and independent manners [31, 32], activating natural killer cells [33, 34] and initiating the classical complement pathway via a direct interaction between the exosporium component BclA and C1q [35]. These findings from the literature suggest that the subdued immune response may be due to an active immune evasion/suppression mechanism of *B. anthracis* rather than passive inactivity of the spores. We demonstrated that the anthrax toxins, in particular the lethal factor, a major immune suppressor of *B. anthracis*, did not play a role in spore persistence in the lung. This implies that *B. anthracis* exosporium components may have immune suppression properties. We are currently investigating this possibility.

In summary, the results described in this study suggest that there are likely multiple mechanisms contributing to spore

persistence in the mouse lung. Association with the lung epithelium and immune suppression/evasion are two potential mechanisms. The results also suggest that spore surface components play important roles in mediating persistence. The work presented here has provided a foundation for further studies to elucidate the molecular mechanisms responsible for spore persistence. Understanding the mechanisms of persistence may potentially provide clues for developing more effective therapeutic regimens for anthrax infections and have implications for other persistent or chronic infections in general.

CHAPTER 3: BCLA MEDIATES FACTOR H BINDING TO THE SURFACE OF SPORES

INTRODUCTION

Factor H (fH) is a key suppressor of the alternative pathway of complement. It serves as the major cofactor for factor I, which mediates cleavage of C3b to its inactivated form (iC3b) and other smaller fragments. FH also accelerates the decay of C3 convertase (C3bBb) further inactivating complement activation and the amplification loop. The ability of fH to recognize specific markers on host cells is extremely important for limiting complement-mediated immune responses to self-surfaces. For microbial pathogens, which do not contain these specific markers, acquiring these host complement regulators on their surfaces is crucial for host immune evasion.

The number of bacterial pathogens that are capable of recruiting fH has been growing. Studies have identified specific fH binding proteins for several pathogens including, Sbi and SdrE protein in *Staphylococcus aureus* [45, 46], M-related proteins and Scl1 protein in Group A streptococci [49, 50, 89], PspC protein in *Streptococcus pneumonia* [51], CRASP and OspE proteins in *Borrelia* species [90-92], FhbB protein in *Treponema denticola* [93], and Yad A and Ail proteins in *Yersinia enterocolitica* [94]. Even proteins from fungi [95, 96], parasites [97, 98], and viruses [99-101] have been reported to recruit fH. For *B. anthracis*, interaction with fH has not been reported.

Spores of *B. anthracis* are generally thought to be resistant to innate immune defenses due to multiple extracellular layers and coat

proteins that likely react with and detoxify chemical agents [10]. In general, *B. anthracis* spores contain an outermost layer called the exosporium, which is comprised of a hair-like nap predominantly consisting of a single surface protein called, *Bacillus* collagen like protein of *anthracis* (BclA). BclA is a 19.7 – 37kDa protein that is encoded chromosomally [15]. BclA protein is organized into three domains. The N-terminal (NTD; 40 amino acids) and the C-terminal (CTD; 132 amino acids) domains are conserved among strains of *B. anthracis* [15]. Studies reported that cleavage of the NTD is required for efficient attachment of BclA to the exosporium basal layer [23]. The CTD is predicted to form an all beta structure with a jelly-roll topology; a similar fold as the TNF-like family of proteins (*e.g.* C1q, collagen VIII and X) [85]. In addition, the CTD contributes to trimer formation of the BclA protein and forms the distal end of each exosporium filament [26]. The third domain is the collagen-like region (CLR). The CLR is highly polymorphic among *B. anthracis* strains displaying a diverse number and organization of GPT repeats in the amino acid sequence [15]. The CLR, similar to mammalian collagen, forms a triple helix. By being the immunodominant antigen and the most external protein on the spore surface, BclA is likely to interact with the host environment.

Recent discoveries from our group indicate for the first time that spores of *B. anthracis* are not merely passive targets of the complement system. We have previously reported that BclA of *B. anthracis* can bind to the classical complement pathway recognition protein C1q [29, 35]. These findings indicated that spore interaction with C1q plays a central role in spore entry into both

phagocytic and non-phagocytic host cells. For phagocytic cells, the interaction results in classical complement pathway activation and opsonophagocytosis of spores in an IgG-independent manner [35]. For non-phagocytic cells, we reported that C1q acts as a bridging molecule between spore surface protein BclA and integrin α2β1 to mediate spore uptake in a complement activation-independent manner [29]. Recruitment of other components of the complement system by spores is less clear.

In this study, we investigated whether BclA directly interacts with complement regulator fH. We observed that the *B. anthracis* spore binds human and mouse fH via direct interaction with its exosporium protein BclA. Strains that expressed segments or full-length BclA protein bound fH significantly better than a deletion mutant of BclA. Both the CTD and CLR domains of BclA were involved in binding to fH. Interestingly, we hypothesize that *B. anthracis* spores appear to have developed a mechanism to suppress complement activation, by directly binding fH.

EXPERIMENTAL PROCEDURES

Bacterial strains and spore preparation. *B. anthracis* Sterne strain 7702, its isogenic deletion mutant strain (Δ*bclA*), *B. subtilis* strains PY79 and 168 were provided by T.M. Koehler, UT Health, Houston, TX. Complemented *B. anthracis* strains with full-length BclA (Δ*bclA*/BclAFL), the C-terminal domain (Δ*bclA*/BclACTD), and the collagen-like region domain (Δ*bclA*/BclACLR) were constructed by fusing the respective BclA segments to the BclA endogenous promoter and NTD and introducing the construct back into the Δ*bclA*

background as previously described by Tan *et al* [23]. Briefly, the constructs were cloned into an *E.coli-B. anthracis* pUTE583 shuttle vector: the region containing the 5′ non-coding sequence of BclA (to enable expression from the endogenous BclA promoter and ribosome binding site), the NTD (amino acid residues 1 – 38 of BclA, which is responsible for anchoring the protein to the exosporium [23]), and DNA fragments encoding the relevant regions of BclA or full-length BclA. The constructs were then introduced into the Δ*bclA* strain background [23]. *B. subtilis* 168 strains expressing full-length BclA (pDG1662/BclAFL) or the C-terminal domain (pDG1662/BclACTD) were constructed by taking the respective BclA segments (except the NTD) and fusing it to the C-terminus of CgeA [outermost surface protein on *B. subtilis* [11]. The fusion fragments were then cloned into vector pDG1662, which allows the ectopic integration of the fusion construct(s) at the non-essential amyE locus in the chromosome of *B. subtilis* [102, 103]. Immunofluorescence staining and flow cytometry using anti-BclA antibodies were performed to observe and quantify the expression levels of BclA on the surface of spores and the percentages of spores expressing the BclA protein. Spores of the *B. anthracis* strains were prepared by growing cultures on Luria Broth (LB) agar plates for 4 – 6 days in a 37°C incubator. The bacteria were scraped from the plates, washed three times with sterile cold Phosphate Buffered Saline (PBS) and heated twice for 30 minutes (min) at 68°C. *B. subtilis* spores were prepared by culturing in Difco sporulation (DSM) media for 5 – 7 days at 30°C. The spore suspensions were then filtered through a 3.0μM filter, counted, and

stored at 4°C. Bacterial titers were determined by plating on LB agar plates and incubation overnight at 37°C.

Serum, proteins, and antibodies. Normal Human Serum (NHS) was purchased from CompTech. Mouse serum (MS) was obtained by terminal blood collection from the posterior vena cava of the mice. Blood samples were allowed to clot for 2 hours (hrs) at room temperature (RT) and then centrifuged for 15 min. Sera was collected and stored immediately at -80°C until used. Heat-inactivated serum was prepared by heating NHS or MS at 56°C for 30 min. Purified human fH was purchased from CompTech. Mouse bronchoalveolar fluid (BAL) was obtained by making a small incision in the mouse trachea to allow the passage of a 23-gauge lavage tube into the trachea. Mice lungs were lavaged thrice using cold PBS. A pooled sample of BAL fluid was collected and concentrated. Heat-inactivated BAL was prepared by heating at 56°C for 30 min. Goat anti-human fH antibodies were purchased from CompTech. Sheep anti-human fH antibodies were purchased from Abcam. Secondary antibodies were purchased from Invitrogen. Purified human fH and C3b were also purchased from CompTech. Heparin (5000 IU/ml) was purchased from Baxter Health Care Corporation. Expression and purification of BclA was done as previously described [29] with slight modifications. Briefly, full-length and C-terminal domain BclA protein with an N-terminal His tag was expressed in *Escherichia coli* BL21 Rosetta 2 strain (Novagen). The recombinant protein was purified using Ni^{2+} affinity chromatography and ion-exchange chromatography in an AKTA prime plus FPLC system (GE Healthcare). The purified protein was analyzed by

SDS-PAGE and Circular dichroism to evaluate the purity and the proper folding of the recombinant protein.

Factor H binding to spores – Western blot analysis. Approximately 5 x 10^7 spores were incubated with 10µg/ml purified human fH, 10% heated NHS, 10% heated MS, or 100µl concentrated heated mouse BAL in the presence of 2.5mM D-alanine in PBS Buffer for 30 min at 37°C. Pellets were washed with cold PBS thrice. Equal amounts of spores were loaded onto a 12% SDS-PAGE and transferred to a nitrocellulose membrane for 50 min at 15V. The membrane was blocked with 5% milk in Tris-Buffered Saline with 0.05% Tween 20 (TBST) for 1 hr and then incubated with goat anti-human fH or sheep anti-human fH (1:1000 for each) in TBST overnight at 4°C. Membranes were thoroughly washed and incubated with rabbit anti-goat HRP or rabbit anti-sheep HRP-conjugated IgG (1:000 for each) for 1 hr.

Factor H binding to spores and recombinant proteins – Plate assay. Microtiter 2HB plates (Thermo Scientific) were coated with 10µg/ml purified human fH in Hepes-Buffered Saline (HBS) overnight at 4°C. Wells were washed to remove unbound protein with HBS with 0.05% Tween 20 (HBST) then blocked with 1% ovalbumin in HBST for 1 hr at RT. Control wells were blocked with block buffer only. Biotin-labeled spores with 2.5mM D-alanine were incubated at various dilutions for 30 min at 37°C followed by incubation with streptavidin-conjugated HRP (1:1000) for 1 hr. His-tagged recombinant proteins (rBclAFL and rBclACTD) were incubated with increasing concentrations of protein in blocking buffer at RT for 2 hrs followed by incubation with anti-his HRP (1:3000) for 1 hr. Plates were developed with SigmaFast OPD and read at 450nm.

Absorbance values were indicative of fH, with values from wells not incubated with spores or recombinant proteins subtracted as background.

Factor H binding to spores – Flow Cytometry. Approximately 5 x 10^7 spores were incubated with purified human fH (25µg/ml) in the presence of 2.5mM D-alanine for 30 min at 37°C. Samples were washed with sterile saline and fixed with 2% paraformaldehyde (MeOH free) for 20 min at RT. Spores were labeled with goat anti-human fH (1:400) in PBS with 2% BSA for 1 hr at RT followed by donkey anti-goat PE (1:400) in PBS with 2% BSA for another hr. Samples were analyzed with a flow cytometer (Accuri) using forward and side scatter parameters to gate on at least 20,000 spores. Results were compared using the mean fluorescence intensity (MFI).

Effect of Heparin, C3b, and NaCl on BclA-Factor H binding. Purified human fH or ovalbumin (10µg/ml) was adsorbed to microtiter plates and incubated with a fixed concentration of rBclA [2µM] with the addition of heparin (10 to 1000 IU/ml), C3b (5 to 50µg/ml), or NaCl (10 to 300mM) for 2 hrs at RT. After incubation, wells were washed thrice with HBST and incubated with HRP-conjugated anti-his antibodies (1:3000). Absorbance was measured at 450nm. Experiments were repeated at least three times in duplicates.

Statistical analysis. Statistical analysis was performed using the two-tailed Student's *t*-test (Graph-Pad Prism 4.0).

RESULTS

Factor H binds to the surface of *B. anthracis* spores. In order to analyze binding of host complement regulator fH to spores, the

Sterne strain 7702 ($pXO1^+$, $pXO2^-$) and non-pathogencic *B. subtilis* PY79 strain were incubated in heat-inactivated normal human serum (HiNHS). After extensive washing, cell lysates were separated by SDS-PAGE, transferred to a membrane, and analyzed by Western blot analysis using anti-human fH antibodies. One distinct band of ~150kDa was identified with 7702 spores, but was very weak with PY79 spores. A representative Western blot is shown in Fig. 14, A. Furthermore, we assessed the direct binding of fH to *B. anthracis* spores by incubating the spores with purified human fH. A similar band was detected for 7702 spores as with serum and a weaker band again was observed for PY79 spores (Fig 14, A). 7702 spores also showed binding to mouse fH when incubated with heated mouse serum (HiMS) or mouse bronchoalveolar lavage fluid (BAL) (Fig 14, A). *B. subtilis* spores weakly bound to mouse fH as seen with human fH. To further substantiate that *B. anthracis* spores bind fH directly, purified fH protein was adsorbed to the wells of a microtiter plate and incubated with various concentrations of 7702 or PY79 spores in an ELISA-type assay. 7702 spores bound fH in a dose-dependent manner, with >100-fold better binding than PY79 spores (Fig. 14, B). Additionally, flow cytometry analysis utilizing purified human fH indicated that 7702 spores significantly bound fH better than PY79 spores (Fig. 14, C). These results support that a dose-response relationship exists for fH binding to *B. anthracis* spores. Also, spores of *B. subtilis* had minimal binding to fH, suggesting that spores of *B. anthracis* possess a fH bacterial ligand on its spore surface that is either absent or distinct from that of *B. subtilis* spores.

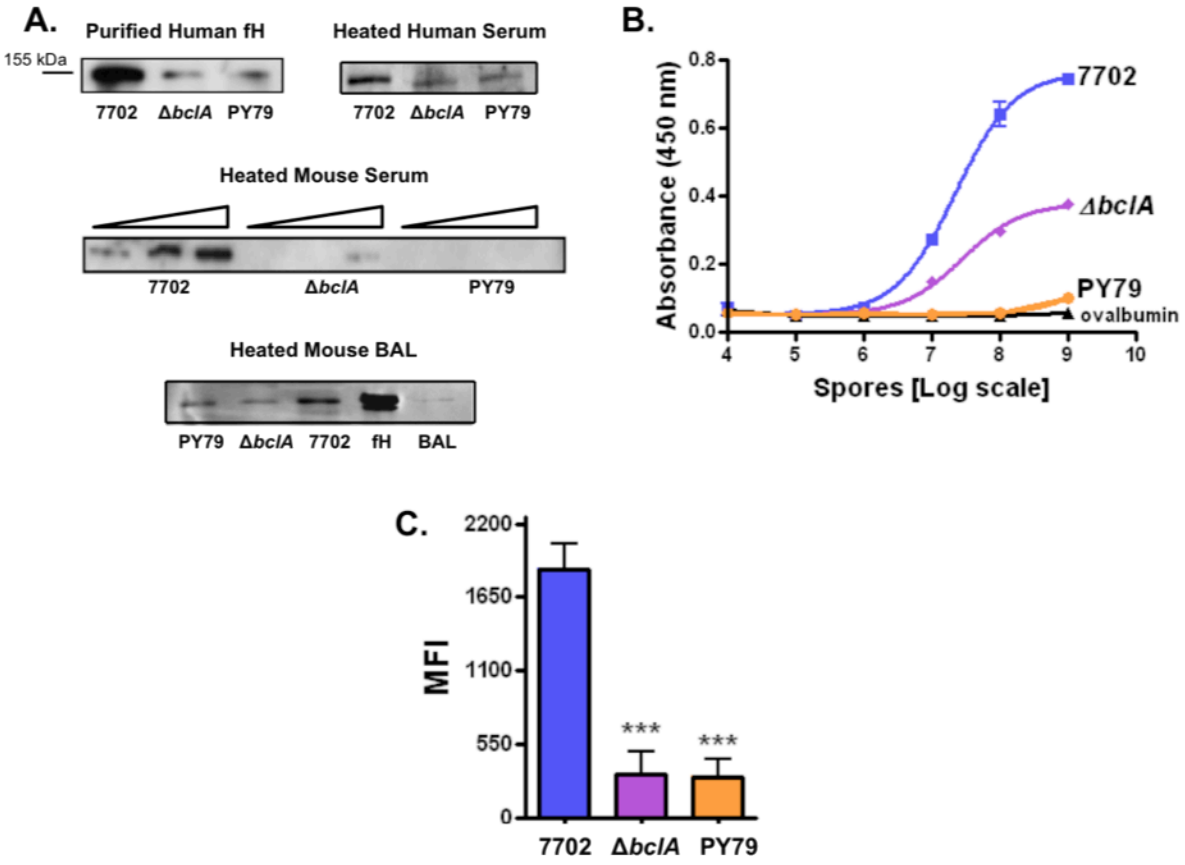


Figure 14. Human and mouse factor H bind to *B. anthracis* spores. (A) Approximately 10^7 spores (7702, *B. anthracis* Sterne strain; Δ*bclA*, *B. anthracis* Sterne strain BclA deletion mutant; PY79, *B. subtilis* strain) were incubated with purified human fH, heated NHS or MS and mouse BAL. Spores were washed, and bound proteins were separated by SDS-PAGE, and analyzed by Western blotting using anti-human fH antibodies. FH was detected as a 150kDa protein. (**B**) Purified fH or ovalbumin was adsorbed to a microtiter plate and incubated with various amounts of spores (10^4-10^9). Detection of fH binding was detected with anti-human fH antibodies. Binding curves are represented in log scale. **(C)** Binding of fH as assayed by flow cytometry. Approximately 10^7 spores were incubated with purified human fH and after extensive washing spores were incubated with anti-human fH antibodies. ***, $p < 0.001$; compared to 7702. Data represent the mean of at least three independent experiments ± SEM.

Factor H binds to spore surface protein BclA. In order to characterize the bacterial ligand mediating this interaction, we hypothesized that the *B. anthracis* BclA protein might represent the binding protein. BclA is the major surface protein and immunodominant antigen on *B. anthracis* spores [12]. Therefore, it is a likely target to interact with host complement proteins. To assess if BclA is important in fH binding, we utilized the *B. anthracis* BclA deletion mutant (Δ*bclA*) and compared binding of fH to our parent strain (7702). Spores were incubated in HiNHS, HiMS, mouse BAL, and purified human fH as indicated above. Mutant bclA spores revealed a weak band around 150kDa similar to *B. subtilis* PY79 spores (Fig. 14, A). Only 7702 spores revealed a strong band for fH. Additionally, we performed an ELISA-type assay and flow cytometry analysis using purified human fH to confirm that BclA is important for fH recruitment on spores. The results indicate that 7702 spores bound >10-fold better to fH than the deletion mutant on microtiter plates and significantly better in solution (Fig. 14, B and C).

We next complemented the BclA deletion mutant with full-length BclA (Δ*bclA*/BclAFL*)* as described in experimental procedures to investigate if the presence of BclA can increase the ability of Δ*bclA* spores to bind to fH. To verify BclA on the surface of spores we stained the spores with anti-BclA antibodies. Complemented spores stained positive for BclA protein similar to 7702 spores, but Δ*bclA* spores did not (Fig. 15, A – C). We also measured the expression levels of BclA on the surface of spores and quantitated the percentage of spores expressing BclA in our spore preparations by flow cytometry analysis (Fig. 16). Both 7702 and Δ*bclA*/BclAFL spores

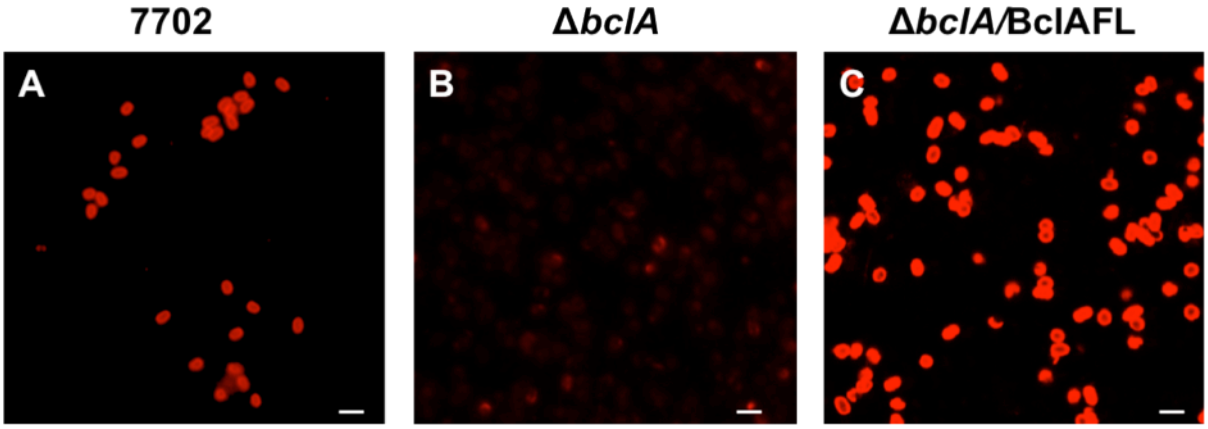


Figure 15. Representative images of immunofluorescently stained *B. anthracis* spores. (**A – C**) Immunofluorescence staining of spores (7702 parent strain, Δ*bclA* isogenic BclA deletion mutant, Δ*bclA*/BclAFL complemented full-length BclA strain) with anti-BclA antibodies and secondary antibodies conjugated to Alexa Fluor 594 (red). Scale bar represents 3μm.

had significantly higher BclA expression than Δ*bclA* spores (Fig. 16, D). In fact, Δ*bclA*/BclAFL spores had higher expression than 7702 spores suggesting that the complemented strain expressed greater levels of BclA protein on its surface. Approximately >65% of 7702 spore preparations expressed BclA protein on their surface and about >80% of the complemented strain preparations (Fig. 16, E). As expected, the deletion mutant did not express BclA protein on its surface. We then tested the Δ*bclA*/BclAFL spores in binding to fH. We used the previously described combination of Western blot, ELISA, and flow cytometry approaches to compare the binding of fH with these different spores. As shown in Fig. 17, Δ*bclA*/BclAFL spores had significantly greater fH binding with at least a 3-fold difference than the deletion mutant (Fig. 17, B and C). These results show that *B. anthracis* spores require BclA protein to bind to fH.

***B. subtilis* expressing BclA bound factor H.** To further examine the fH binding ability of BclA, we used *B. subtilis* spores expressing BclA. The *B. subtilis* genome does not encode BclA nor do these spores contain an exosporium [83]. We have also shown that spores of *B. subtilis* PY79 do not show direct binding to fH. Thus, *B. subtilis* spores can be used as a heterologous host to display BclA protein. Full-length BclA protein was expressed on the surface of *B. subtilis* spores as described in the experimental procedures. We performed immunofluorescence staining and flow cytometry with anti-BclA antibodies to verify BclA expression on the surface of these spores (Fig. 18, A – E). *B. subtilis* (pDG1662/BclAFL) was compared to the empty vector control *B. subtilis* (pDG1662) spores. *B. subtilis* spores expressing BclA (pDG1662/BclAFL) stained positive

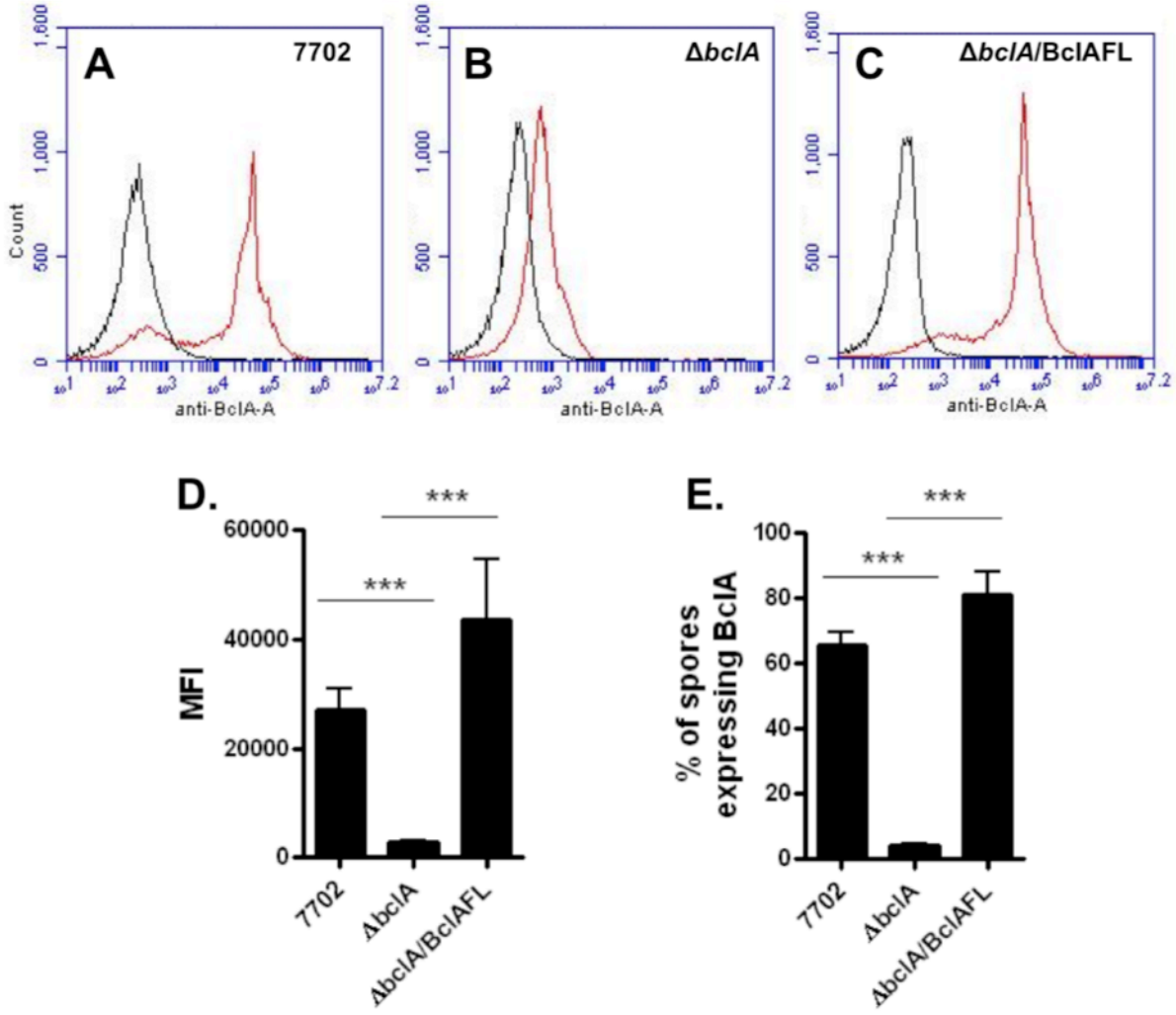


Figure 16. Flow cytometric quantification of BclA expression on surface of *B. anthracis* spores. Fixed spores were incubated with anti-BclA antibodies and secondary antibodies conjugated to PE. **(A – C)** Representative histograms of BclA staining on surface of spores. Black lines represent spores incubated with secondary antibody only and red lines represent spores incubated with anti-BclA antibody. BclA expression is observed by a shift to the right in the population of spores incubated with the anti-BclA antibody compared with spores incubated with secondary only antibodies. **(D)** Quantification of the expression level of BclA protein on the surface of spores measured in mean fluorescence intensity (MFI). **(E)** The percentage of spores expressing BclA protein in different spore preparations. Experiments were performed on at least three independent spore preparations and error bars represent the SEM. ***, $p < 0.001$ comparisons made between 7702 and Δ*bclA* and Δ*bclA* versus Δ*bclA*/BclAFL spores.

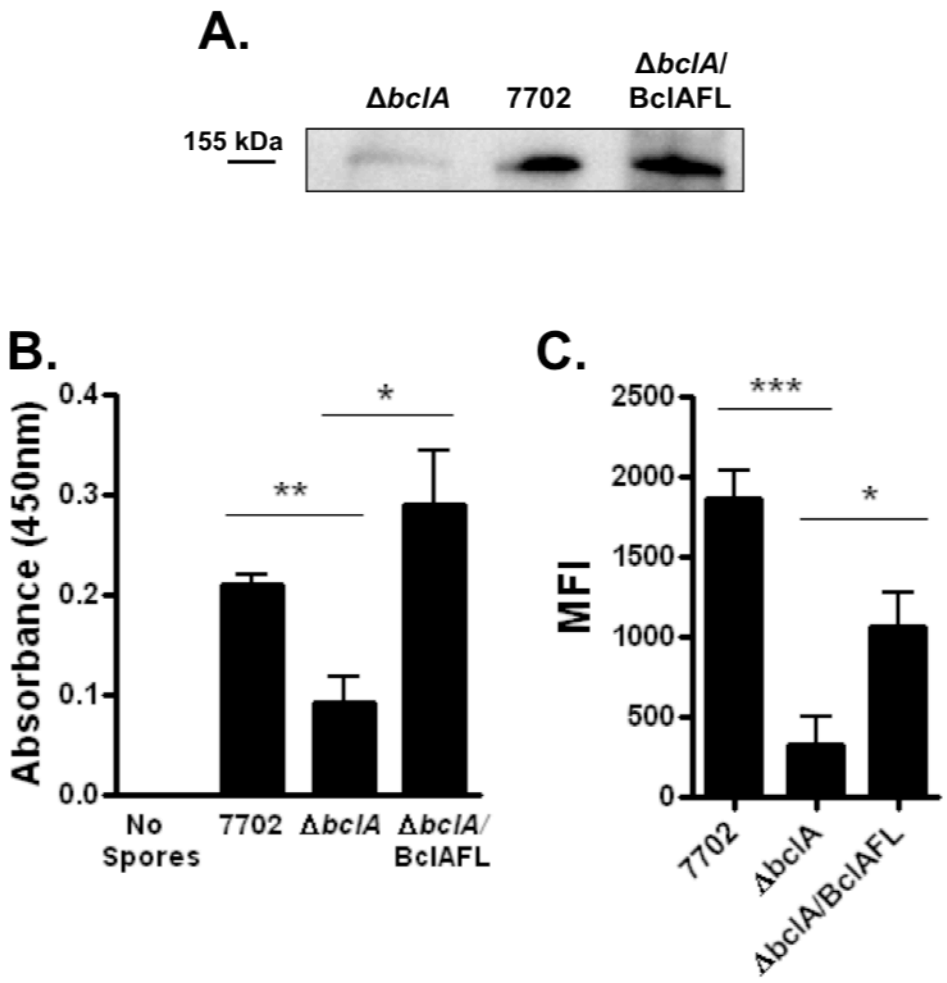


Figure 17. Complemented deletion mutant (Δ*bclA*/BclAFL) binds purified human factor H. (A) Approximately 10^7 spores were incubated with purified human fH (10µg/ml), washed, and bound proteins were separated by SDS-PAGE, and analyzed by Western blotting using anti-human fH antibodies. FH was detected as a 150kDa protein. **(B)** FH was adsorbed to microtiter plates and incubated with ~10^7 biotinylated spores or none as a negative control. FH binding was measured using anti-human fH antibodies. Experiments were performed on at least three independent occasions in triplicate, and the error bars indicate SEM. *, $p < 0.05$, Δ*bclA* vs. Δ*bclA*/BclAFL; **, $p < 0.01$, 7702 vs. Δ*bclA*. **(C)** Flow cytometric quantification of fH binding to spores. Spores were incubated with human fH using anti-human fH antibodies and gated on at least 20,000 spores. Results were compared using the mean fluorescence intensity (MFI). *, $p < 0.05$, Δ*bclA* vs. Δ*bclA*/BclAFL; ***$p < 0.001$, 7702 vs. Δ*bclA*. Experiments were performed at least three times, and the error bars indicate SEM.

for BclA protein and had significantly greater BclA expression compared to the empty vector pDG1662 (Fig. 18, F). Approximately >90% of pDG1662/BclAFL spores expressed BclA protein while there was no BclA protein detected for pDG1662 spores (Fig. 18, G). In fH binding assays, pDG1662/BclAFL spores bound 8-fold more fH than pDG1662 spores and the difference observed was significant (Fig. 19, A and B). Additionally, a fH band was detected with pDG1662/BclAFL spores compared to the pDG1662 spores in Western blot analysis (Fig. 19, C), confirming that the surface expression of BclA enhanced the binding of fH to *B. subtilis*.

Recombinant BclA binds purified factor H in a dose-dependent manner. To investigate the fH-binding ability of *B. anthracis*, we used the recombinant form of the BclA protein. Full-length BclA protein (BclAFL) was expressed and purified as a his-tagged recombinant protein. The recombinant protein formed a triple helix as shown by Circular dichroism (CD) spectra of a positive ellipticity at 220nm typical of a triple helix formation [26] (Fig. 20, A). Also, the recombinant protein was monitored by CD at 220nm from 6 – 50°C. Recombinant BclA protein had a melting temperature around 35°C and the helix was completely denatured at >40°C (Fig. 20, B). On an SDS-PAGE under reducing conditions rBclAFL migrated at about 70kDa and under non-reducing conditions migrated as an SDS-resistant trimer (>180kDa) as previously described [26] (Fig. 20, C). To verify the protein observed in the Coomassie stain was indeed our protein, we performed a Western blot using anti-his antibodies and detected a band at similar molecular weights as seen on the Coomassie stain (Fig. 20, D). To examine fH binding with rBclAFL,

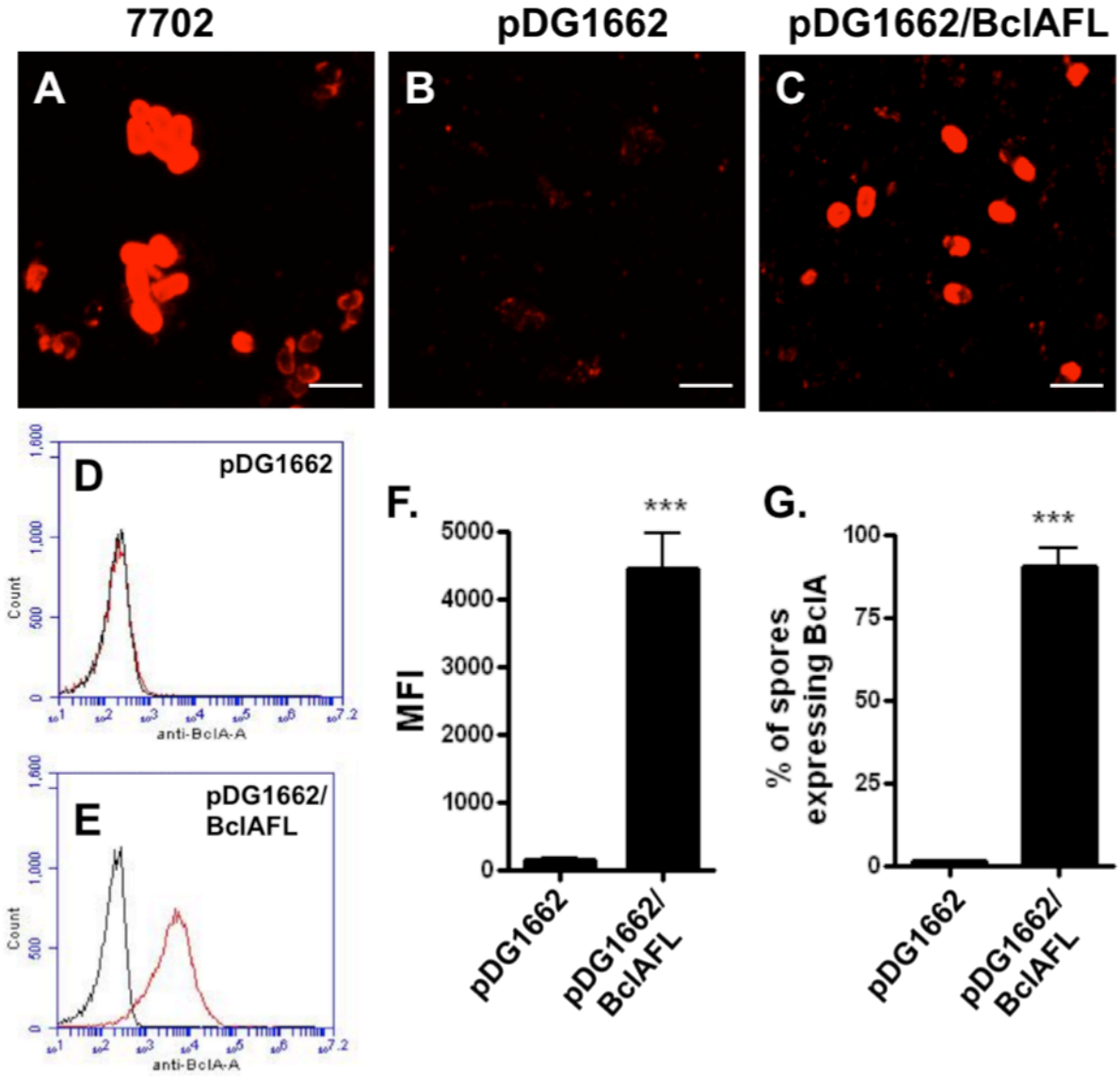


Figure 18. BclA expression on the surface of *B. subtilis* spores expressing full-length BclA. (A – C) Fixed spores (7702, *B. anthracis* strain; pDG162 *B. subtilis* 168 strain vector control; pDG162 *B. subtilis* 168 strain expressing full-length BclA) were probed with anti-BclA antibodies and secondary antibodies conjugated to Alexa Fluor 594 (red). A representative image of BclA staining is shown. **(D and E)** Representative histograms of BclA staining on surface of spores. Black lines represent spores incubated with secondary antibody only and red lines represent spores incubated with anti-BclA antibody. **(F)** Quantification of the expression level of BclA protein on the surface of spores measured in MFI. **(G)** The percentage of spores expressing BclA protein in different spore preparations. Experiments were performed on at least three independent spore preparations and error bars represent the SEM. Differences observed were significantly different ***, $p < 0.001$.

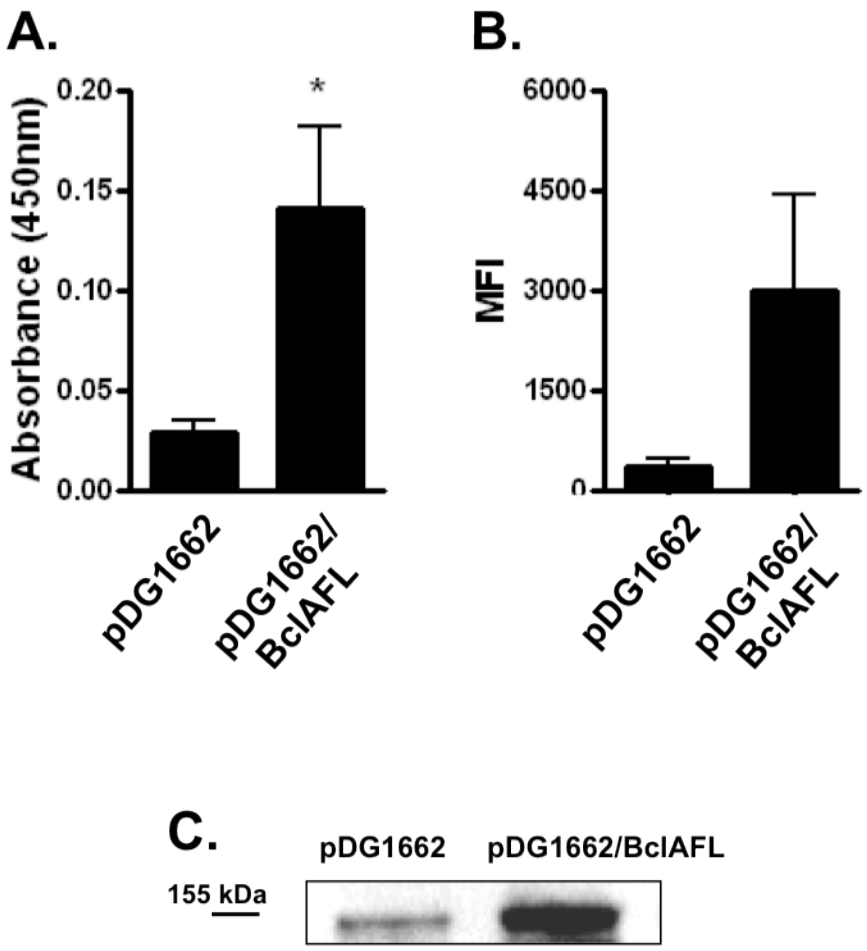


Figure 19. Factor H bound to *B. subtilis* spores expressing full-length BclA. (A) Purified human fH was adsorbed to microtiter plates and incubated with ~10^7 biotinylated spores. Absorbance values are indicative of fH binding. Differences observed were significant *, $p < 0.05$. Experiments were performed at least three times in duplicates. **(B)** Purified human fH was incubated with ~10^7 spores, washed, and subjected to flow cytometry evaluation using fH antibodies. Spore populations were gated on 20,000 counts and fH binding is represented as MFI. Differences observed were not significant. Experiments were performed at least three times. **(C)** Spores were incubated with purified human fH, washed, and bound proteins were subjected to Western blot analysis using fH antibodies. FH binding was indicated by a distinct band at ~150kDa.

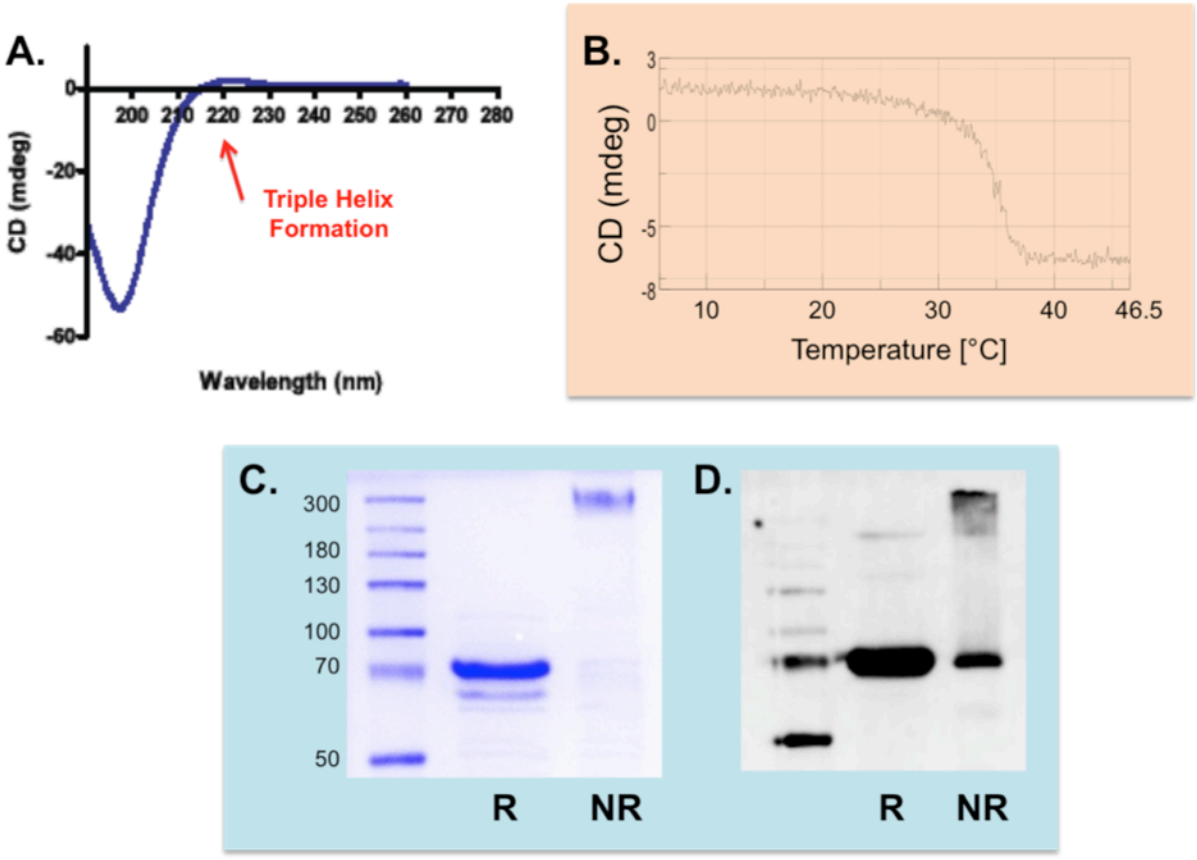


Figure 20. Characterization of full-length recombinant BclA protein. BclA of *B. anthracis* Sterne strain 7702 was recombinantly expressed in *E.coli* as a his-tagged protein and purified by affinity chromatography. The protein was analyzed by Circular dichroism **(A and B)**, Coomassie **(C)**, and Western blot using anti-his antibodies **(D)**.

purified human fH or control protein ovalbumin was adsorbed to microtiter plates and incubated with increasing concentrations of rBclAFL in an ELISA-type assay. As shown in Fig. 21, rBclAFL bound to fH in a dose-dependent and saturable manner, with a K_D of ~0.9 ± 0.45μM, which is >1000 fold more than ovalbumin. These results strongly suggest that BclA is indeed a fH binding protein of *B. anthracis*.

Mapping of BclA-Factor H binding domain. Recombinant protein and engineered spores expressing different domains of BclA were used to determine which region of BclA was responsible for the interaction with fH. First, we cloned the C-terminal domain of BclA (BclACTD) into the pBAD expression vector to generate a his-tag fusion protein and we purified the protein as described in experimental procedures. Analysis of the recombinant protein in CD spectra revealed an all beta structure similar to previous reports of its crystal structure [85] (Fig. 22, A). On a Coomassie stain rBclACTD migrated at about 17kDa and formed trimers under non-reducing conditions (~>40kDa; Fig. 22, B). Western blot analysis revealed a band at a similar molecular weight as seen in the Coomassie stain (Fig. 22, B). ELISA results showed that rBclACTD mediated binding to fH similar to rBclAFL (Apparent K_D for rBclAFL = 0.9 ± 0.45μM and rBclACTD = 2.1 ± 0.80μM, Fig. 23). To further assess the involvement of CTD in mediating binding to fH, *B. subtilis* spores were engineered to express the C-terminal domain of BclA. Expression of BclA on the surface of spores was again verified by immunofluorescence staining and flow cytometry (Fig. 24). Spores expressing the C-terminal domain (pDG1662/BclACTD) had higher levels

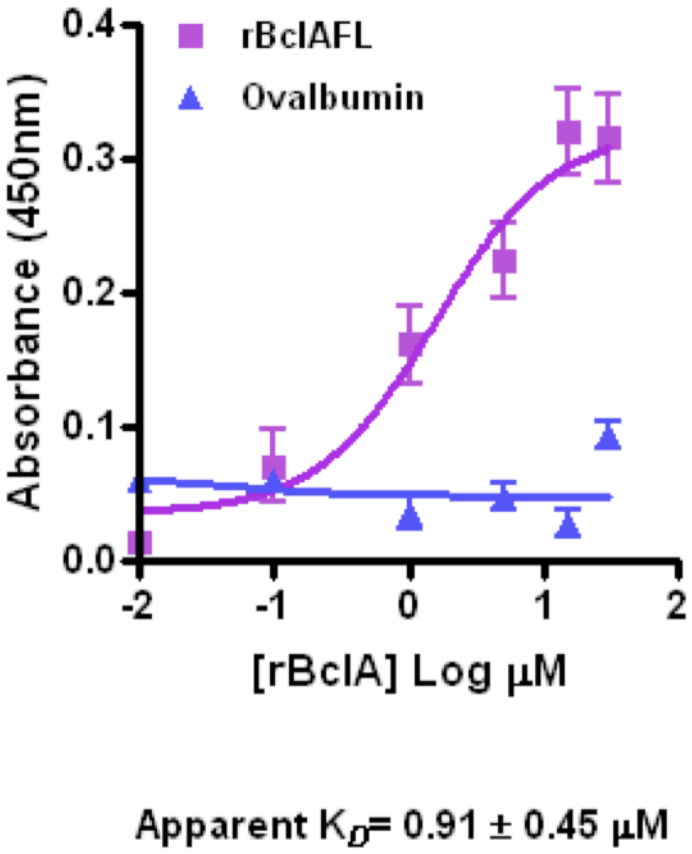


Figure 21. Factor H binds to recombinant BclA. Binding of rBclAFL to immobilized fH was analyzed by ELISA. Bound proteins were detected using anti-his antibodies. Ovalbumin was used as a negative control. Data shows the mean of at least three experiments, and SEM is indicated by error bars. Binding curves are shown in log scale.

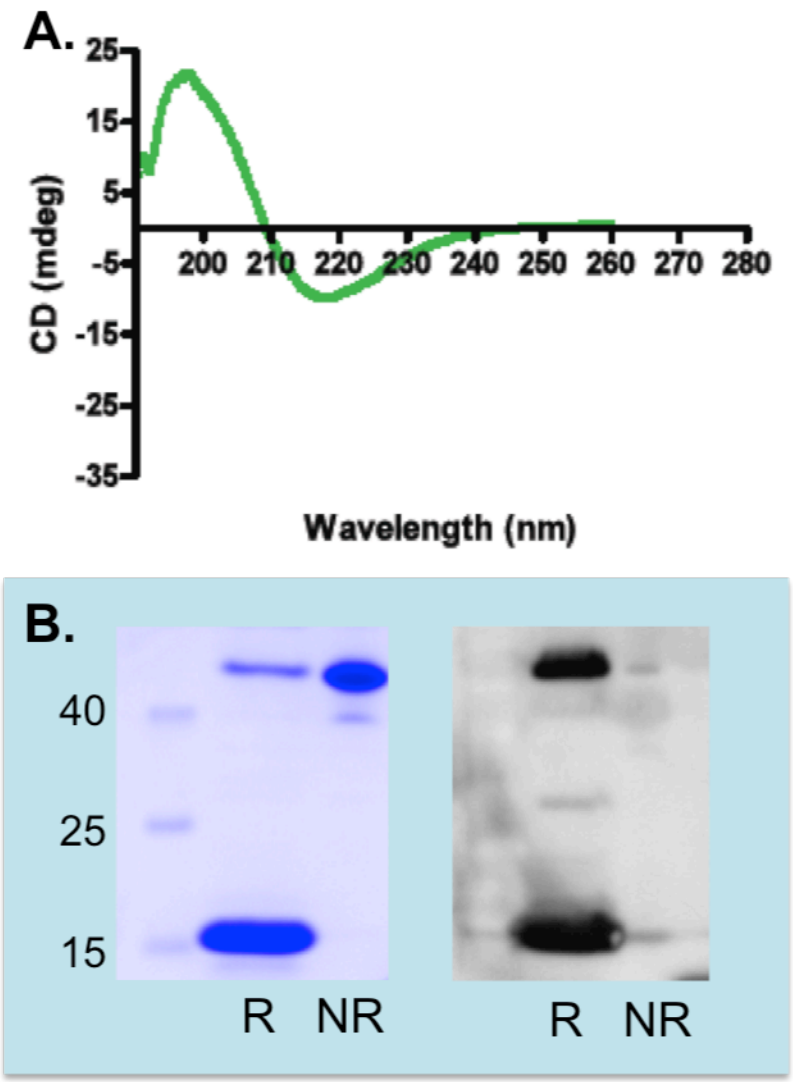
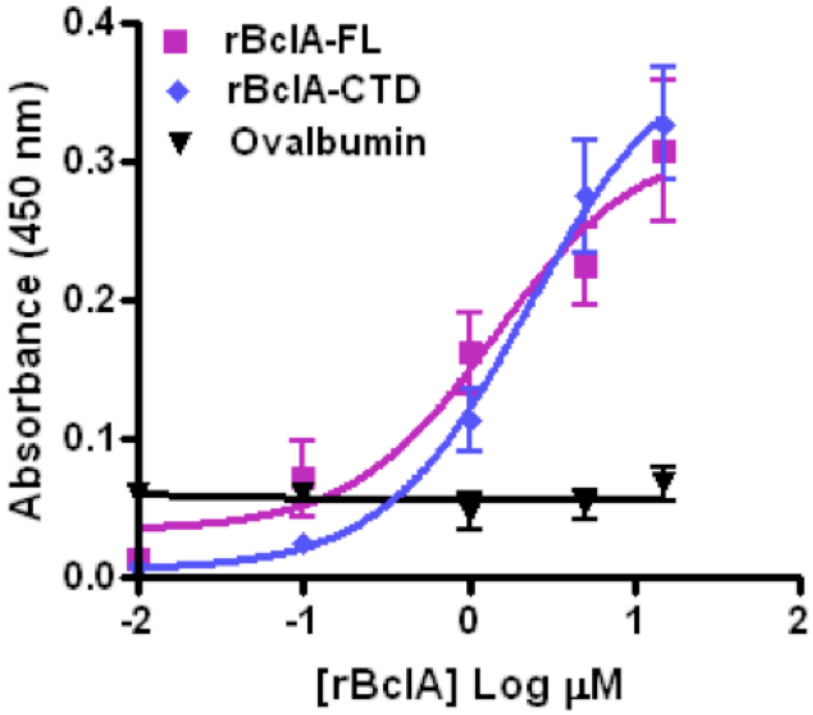


Figure 22. Characterization of C-terminal recombinant BclA protein. The C-terminal domain (CTD) of BclA of *B. anthracis* Sterne strain 7702 was recombinantly expressed in *E.coli* as a his-tagged protein and purified by affinity chromatography. The protein was analyzed by Circular dichroism **(A)** and Coomassie and Western blot using anti-his antibodies **(B)**.

of BclA expression compared to pDG1662/BclAFL and pDG1662 spores (Fig. 24, C). The percentage of spores expressing BclA was similar between pDG1662/BclAFL and pDG1662/BclACTD (Fig. 24, F). Western blot assays showed that pDG1662/BclACTD spores bound purified fH similar to pDG1662/BclAFL spores. A representative blot is shown in Fig. 25, A. ELISA and flow cytometry results indicated that pDG1662/BclACTD spores significantly bound to immobilized and soluble fH compared to pDG1662 spores (Fig. 25, B and C), suggesting that the CTD of BclA is involved in the interaction with fH. We were unsuccessful in generating a recombinant protein of the collagen-like region of BclA and engineering *B. subtilis* spores to express this specific region (BclACLR). However, we were able to complement *B. anthracis* Δ*bclA* spores with both the CTD and CLR regions of BclA. These spores were verified for BclA expression using anti-BclA antibodies as shown in Fig. 26, B and C. Δ*bclA*/BclACLR and Δ*bclA*/BclACTD spores had similar BclA expression levels compared to 7702 spores, but were lower than Δ*bclA*/BclAFL (Fig. 26, F). Also, the percentages of spores expressing BclA in different spore preparations were not significantly different among the different BclA-expressing spores (≥50% of spores expressed BclA; Fig. 26, G). We next examined fH binding with these complemented spore strains (Δ*bclA*/BclACLR and Δ*bclA*/BclACTD). Spores were incubated with purified human fH or HiNHS, washed, and cell lysates were prepared. Δ*bclA*/BclACLR and Δ*bclA*/BclACTD spores were able to bind to purified fH and serum fH (data not shown) as determined via Western blot. A representative Western blot is shown in Fig. 27, A. The band detected for the complemented fragment domains were stronger than



Apparent K_D (rBclA-FL) = 0.91 ± 0.45 µM
(rBclA-CTD) = 2.1 ± 0.80 µM

Figure 23. Factor H binds to recombinant C-terminal domain of BclA. Binding of rBclACTD to immobilized fH was analyzed by ELISA. Bound proteins were detected using anti-his antibodies. Ovalbumin was used as a negative control. Data shows the mean of at least three experiments, and SEM is indicated by error bars. Binding curves are shown in log scale.

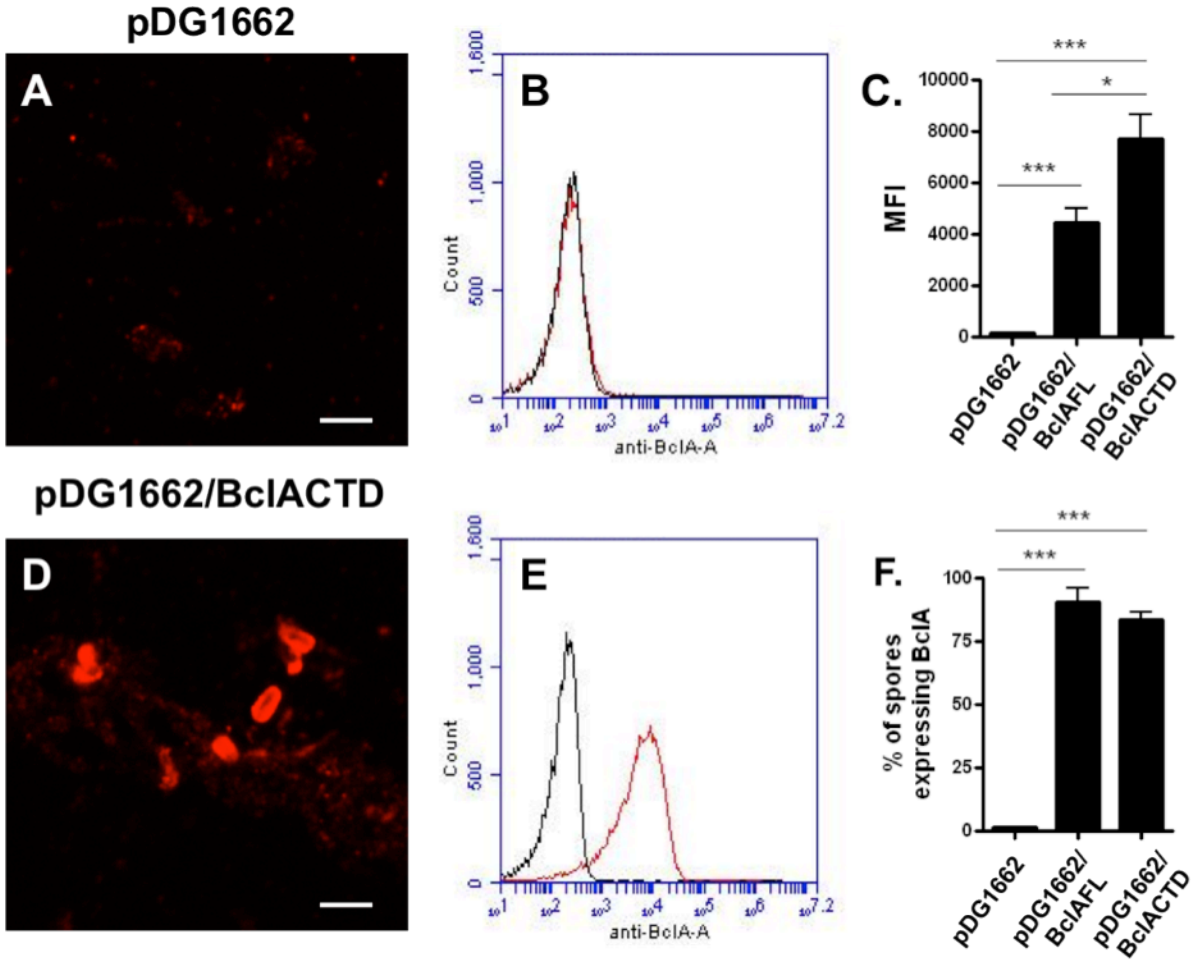


Figure 24. BclA expression on the surface of *B. subtilis* spores expressing C-terminal domain of BclA. (A and B) Fixed spores (pDG1662 *B. subtilis* 168 strain vector control and pDG1662 *B. subtilis* 168 strain expressing CTD of BclA) were probed with anti-BclA antibodies and secondary antibodies conjugated to Alexa Fluor 594 (red). A representative image of BclA staining is shown. **(C and D)** Representative histograms of BclA staining on surface of spores. Black lines represent spores incubated with secondary antibody only and red lines represent spores incubated with anti-BclA antibody. **(E)** Quantification of the expression level of BclA protein on the surface of spores measured in MFI. **(F)** The percentage of spores expressing BclA protein in different spore preparations. Experiments were performed on at least three independent spore preparations and error bars represent the SEM. Differences observed were significantly different *, $p < 0.01$; ***, $p < 0.001$.

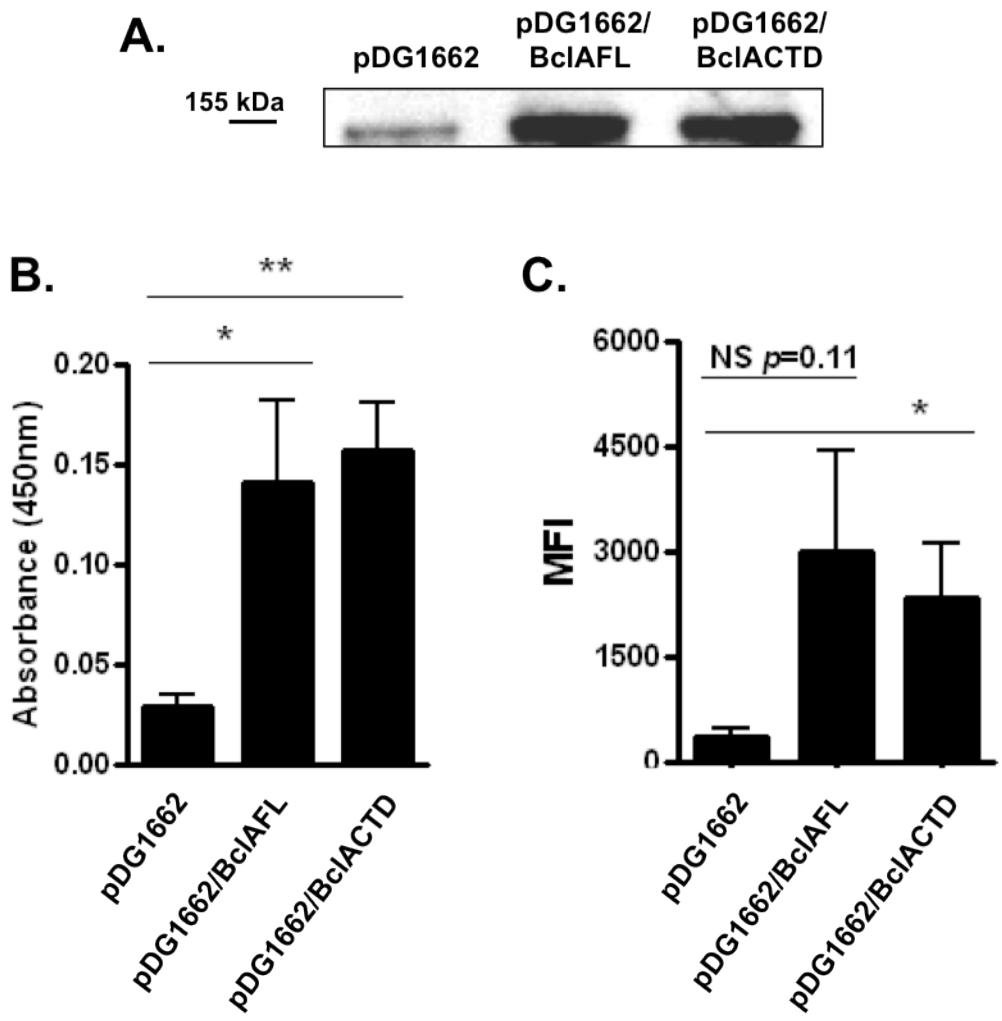


Figure 25. Factor H bound to *B. subtilis* spores expressing C-terminal domain of BclA. (A) Spores were incubated with purified human fH, washed, and bound proteins were subjected to Western blot analysis using fH antibodies. FH binding was indicated by a distinct band at ~150kDa. **(B)** Purified human fH was adsorbed to microtiter plates and incubated with ~10^7 biotinylated spores. Absorbance values are indicative of fH binding. Differences observed were significant *, $p < 0.05$. Experiments were performed at least three times in duplicates. **(C)** Purified human fH was incubated with ~10^7 spores, washed, and subjected to flow cytometry evaluation using fH antibodies. Spore populations were gated on 20,000 counts and fH binding is represented as MFI. Differences observed were not significant. Experiments were performed at least three times.

the band detected for 7702 and Δ*bclA*/BclAFL spores. A very weak band was detected for the deletion mutant (Δ*bclA*). Flow cytometry results indicated that Δ*bclA*/BclACLR and Δ*bclA*/BclACTD bound fH similarly and better than Δ*bclA*/BclAFL. These results suggest that both the CTD and CLR domains of BclA are involved in the ability of BclA to bind to fH.

Characteristics of BclA-Factor H interaction. Many other bacterial fH binding proteins (*e.g.* M6 protein of *S. pyogenes* [47], Ali of *Y. enterocolitica* [94], and OMPs of *H. influenza* [104]) have been reported to bind fH in the same regions in fH that have been described to bind host cell markers such as heparin. In order to deduce which site on fH is responsible for BclA binding, we first examined whether BclA binds to the heparin binding sites on fH (*i.e.* SCRs 7, 13, and 19 – 20) [44]. Using ELISA-type assays, purified human fH was adsorbed to microtiter plates and incubated with a fixed concentration of rBclA with or without increasing concentrations of heparin (10 – 1000IU/ml). Even at the highest concentration of heparin tested, rBclA binds to fH similar to when no heparin is present (Fig. 28, A). These results suggest that the binding site on fH for BclA does not involve the heparin binding sites.

C3b is also another important ligand for fH and the interaction is pivotal for fH complement regulation and host surface recognition. We next investigated whether BclA interacted on C3b binding sites (SCRs 1 – 4, 6 – 8, 12 – 14, and 19 – 20) [44]. ELISA results indicated that a range of 5 – 50μg/ml of C3b did not interfere with rBclA binding to fH adsorbed on microtiter plates

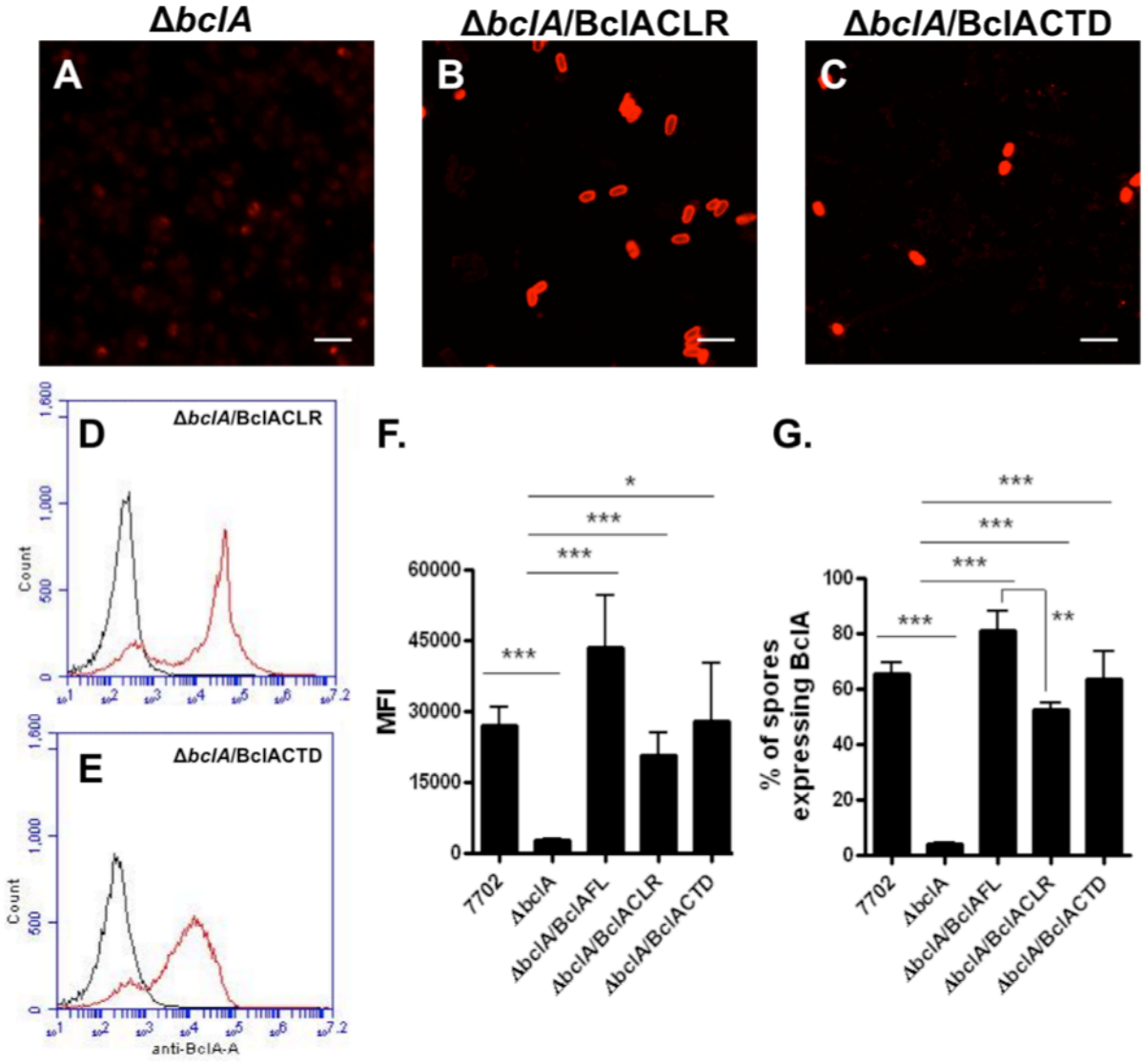


Figure 26. BclA expression on the surface of *B. anthracis* spores expressing the CLR and CTD domains of BclA. (A – C) Fixed spores were probed with anti-BclA antibodies and secondary antibodies conjugated to Alexa Fluor 594 (red). **(D and E)** Representative histograms of BclA staining on surface of spores. Black lines represent spores incubated with secondary antibody only and red lines represent spores incubated with anti-BclA antibody. **(F)** Quantification of the expression level of BclA protein on the surface of spores measured in MFI. **(G)** The percentage of spores expressing BclA protein in different spore preparations. Experiments were performed on at least three independent spore preparations and error bars represent the SEM. Differences observed were significantly different *, $p < 0.01$; ** $p < 0.001$, and *** $p < 0.0001$.

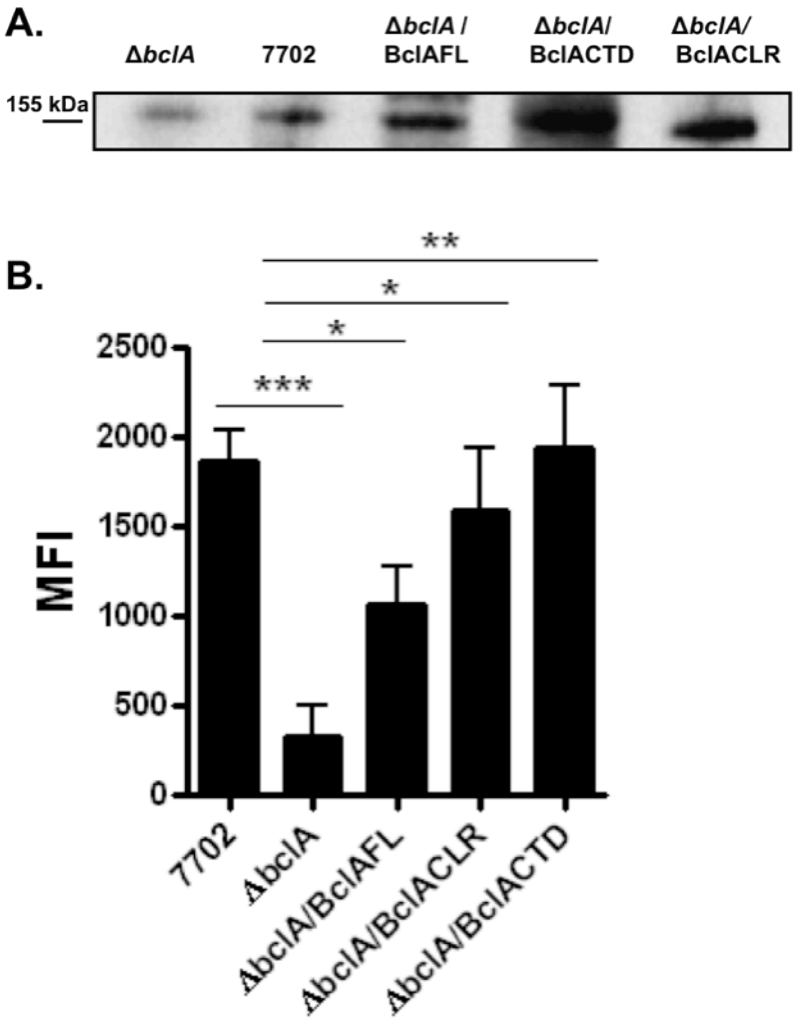


Figure 27. Factor H bound to *B. anthracis* spores expressing CLR and CTD domains of BclA. (A) Spores were incubated with purified human fH, washed, and bound proteins were subjected to Western blot analysis using fH antibodies. FH binding was indicated by a distinct band at ~150kDa. **(B)** Purified human fH was incubated with ~10^7 spores, washed, and subjected to flow cytometry evaluation using fH antibodies. Spore populations were gated on 20,000 counts and fH binding is represented as MFI. Experiments were performed at least three times. Differences observed were significant. *, $p < 0.01$; **, $p < 0.001$; Δ*bclA* vs. complemented strains; ***, $p < 0.001$; Δ*bclA* vs. 7702 spores.

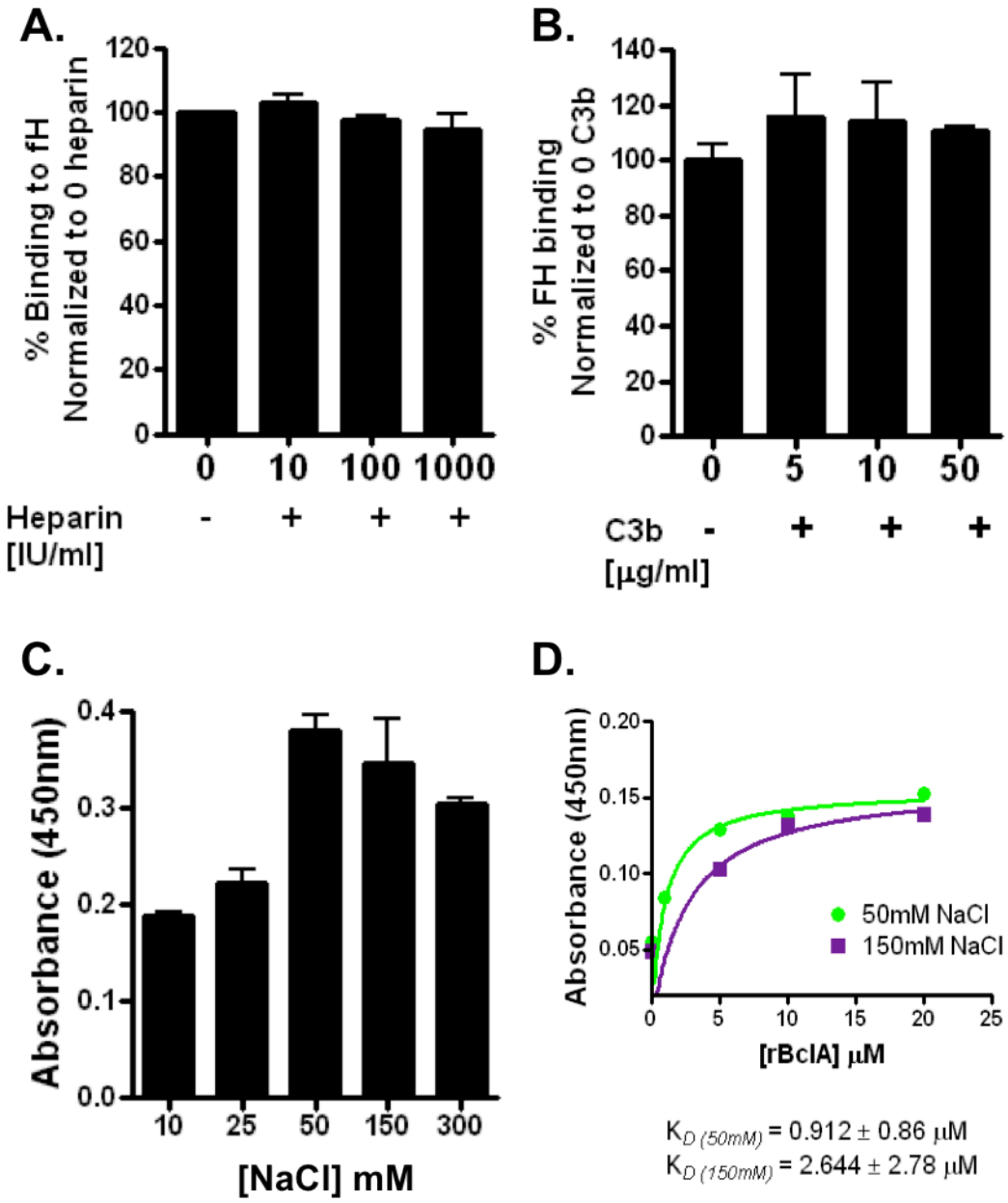


Figure 28. Characterization of factor H interaction with recombinant BclA. The effects of Heparin **(A)**, C3b **(B)**, and NaCl **(C and D)** on the binding of purified fH to rBclA protein are shown. A fixed concentration of rBclA was incubated with adsorbed purified fH (10µg/ml) on microtiter plates with various amounts of heparin (10 – 1000 IU/ml), C3b (5 – 50µg/ml), and NaCl (10 – 300mM). Following incubation, wells were washed and fH binding was detected with anti-his antibodies by ELISA. Data shown represent the mean of at least three experiments, and SEM is indicated by error bars. For A and B, data is normalized to 0 heparin or C3b.

(Fig. 28, B). Like heparin, C3b binding sites on fH at these concentrations do not seem to overlap with BclA binding sites on fH.

Lastly, fH recognition of polyanions as well as other fH ligands suggests that ionic interactions may be important for binding. We determined if BclA-bound fH was affected by different salt concentrations. To investigate this we tested if binding between fH and rBclA would be different if the salt concentrations in the buffer were increased from 10 to 300mM (Fig. 28, C and D). Our results showed that better binding between fH and rBclA occurred at concentrations >50mM. Additionally, we have shown throughout our experimental assays with spores that our buffers contain physiological concentrations of salt (150mM NaCl) and are able to interact specifically with fH. This data supports that ionic interactions may not be important for BclA binding to fH and that it is likely that BclA-fH interaction occurs *in vivo*.

DISCUSSION

Many pathogenic bacteria express proteins that interfere with the host defense mechanism, with complement evasion as a central strategy to their success in causing infection, survival, persistence, and disease in a host. As a highly successful pathogen, *B. anthracis* is no exception. *B. anthracis* spores are known to persist for long periods of time in infected hosts [7-9]. It is thus intriguing how spores overcome the host immune defenses. Our current knowledge of the ability of *B. anthracis* to subvert the complement system is limited and in the present study, we report that *B. anthracis* spores can recruit complement regulator protein fH to its

spore's surface, and this is specifically mediated by BclA. BclA is the outermost surface protein of *B. anthracis* spores, comprising the hair-like nap. It is known to mediate entry into phagocytes [27, 28] and non-phagocytic cells [29]. The first demonstration that BclA interacts with complement proteins was described by Gu *et al* in 2012 [35].

We identified BclA as a fH-binding protein using several techniques including Western blot, ELISA, and flow cytometry to provide insights into the interaction between fH and *B. anthracis* spores. We presented data showing enhanced binding of fH to 7702 spores compared to spores from a BclA deletion mutant and a non-pathogenic *B. subtilis* strain, which lacks the gene that encodes for BclA. Deletion of BclA significantly reduced binding to fH, but did not abolish it. We cannot rule out the possibility that other proteins exposed when BclA is not present can mediate fH binding on these spores. Also, it has been reported that Δ*bclA* spores bind more readily than the parental strain spores to extracellular matrix proteins, fibronectin and laminin [105], suggesting that other proteins on Δ*bclA* spores may play a role in *B. anthracis* immune evasion. However, additional experiments showed that complementation of the isogenic BclA mutant strain increased its ability to bind to fH, indicating that BclA is the major fH-binding protein on the surface of *B. anthracis* spores. Studies using the recombinantly expressed BclA validated the ability of this protein to bind to fH whether purified or in serum, with a dose-dependent relationship evident. The relative affinity of rBclA for fH appears to be in the similar range (0.59 – 2.9μM) as the affinity reported for fH

interaction with C3 fragments [38]. Also, the buffer used in the experimental assays contained physiological concentrations of salt (~150mM NaCl). This indicates that the interaction between BclA-fH is likely to occur *in vivo*. Furthermore, displaying heterologous proteins on the surface of *B. subtilis* spores has been used in many studies [106-111]. The principal approach used here was to fuse BclA protein to CgeA, a protein in the *B. subtilis* spore crust. The spore crust has been recently discovered as the outermost structure on the surface of *B. subtilis* spores [11]. Using a heterologous host, *B. subtilis*, we demonstrated that BclA expression on a bacterial surface significantly enhanced fH recruitment, which confirms our rBclAFL-fH binding data.

Moreover, we sought to determine the extent to which complement activation affects the binding of fH to *B. anthracis*. In heat-inactivated serum, which complement proteins become inactive and fH remains heat-stable, fH bound *B. anthracis* spores demonstrating that complement activation is not necessary for fH binding. This has also been reported for *S. aureus* strains [112]. In addition, our Western blot analysis suggest that *B. anthracis* may also bind to FHL-1 or fH-related proteins (data not shown), since we did observe faint bands at the respective molecular weights (~36 – 43kDa). Other pathogenic bacteria such as *S. pyogenes* [113], *S. auerus* [112], and *B. burgdorferi* [114] have also been shown to interact with these proteins.

We further examined which region of BclA was responsible for the interaction with fH. From our studies, we report that both the CTD and CLR domains of BclA can bind to fH. Interestingly, from flow

cytometry analysis we see that spores expressing CLR or CTD bind similar to fH suggesting that there may be two binding sites located on BclA for fH. Further clarification of the binding regions on BclA for fH binding will be determined in future experiments. Our results also provide insight into the regions on fH that are important for binding. The majority of fH binding proteins have been described to bind to domains 19 – 20 on fH [37]. A few have been described for interaction on heparin (SCR7) and C3b (6 – 8 and 19 – 20) binding sites, including M proteins and Fba of *S. pyogenes*, PorB 1A of *N. gonorrhoeae*, Ail of *Y. enterocolitica*, Tuf of *P. aeruginosa*, and Sbi of *S. aureus* [37]. For *B. anthracis*, our results indicate that BclA protein does not interact on heparin or C3b binding sites, suggesting that SCR domains (1 – 4, 6 – 8, 12 – 14, and 19 – 20) may not be involved in this binding interaction. Available sites that were not tested for BclA-fH binding include SCRs (5, 9 – 11, and 15 – 18). CRASP 1 protein of *Borrelia* species is known to interact at SCR 5 [115]. PspC protein of *S. pneumonia* has been described to interact at SCR 9 – 11 and 15 [52, 53]. No fH binding proteins have been described to interact at SCRs 16 – 18. However, it is possible that BclA may interact with the entire molecule of fH similar to what has been described for the fH binding protein of *Y. enterocolitica* (Yad A) [94, 116]. Future investigations for the fH binding regions will better clarify these conclusions.

In summary, the interaction between *B. anthracis* and complement proteins is only beginning to be explored. Our data clearly demonstrate that *B. anthracis* binds fH in a dose-dependent manner requiring neither complement activation, nor C3b or other serum

components. BclA is the major fH-binding molecule on the spore surface due to its ability to bind fH whether purified or in serum. To our knowledge, this is the first description of a *B. anthracis* spore surface protein that recruits a complement regulator. Future studies will address whether fH binding to spores via BclA suppresses complement activation on the spore surface and will survey the biological role of BclA-fH interaction in the development and chronic stages of anthrax infections.

Chapter 4: BIOLOGICAL FUNCTION OF BCLA-FACTOR H INTERACTION

INTRODUCTION

Our results in Chapter 3 suggest that *B. anthracis* spores have evolved a sophisticated mechanism to interact with the complement regulator protein fH via the spore surface protein BclA. Because fH is a major immune suppressor of complement, we wanted to know if the fH-BclA interaction could be an immune suppression mechanism important for spore persistence in a host (introduced and studied in Chapter 2). Spore persistence in the lungs of animals has been well known for many decades [7-9]; however the mechanisms that contribute to spore persistence is unclear. Our studies described in Chapter 2, demonstrated that anthrax toxins, in particular lethal factor, a major immune suppressor of *B. anthracis*, do not play a role in spore persistence in the lung. Also, one of the general theories regarding spore persistence is that spores are able to persist due to their dormant and resilient properties. Therefore, the host does not efficiently kill them. It is also possible that the lung is able to tolerate spores in general. However, we found that spores of *B. subtilis* did not persist in the lung as well as those of *B. anthracis*, suggesting that *B. anthracis* spores possess special properties that promote their survival and persistence in the lung. The exosporium is the outermost integument of *B. anthracis* spores, but is not present on *B. subtilis* [83]. The exosporium is composed of 20 – 30 proteins and glycoproteins [83]. It is possible that specific exosporium components may have immune suppression properties. Results described in Chapter 3 suggest that direct

interactions with complement inhibitor fH by *B. anthracis* spores via the exosporium protein BclA may play a role in mediating persistence in a host.

In this study, we sought to determine the biological function of *B. anthracis* spore interaction with fH. FH is abundantly present in plasma and is therefore a prime target for sequestration by pathogens where surface-bound fH can benefit their survival in a host. We used the spore persistence model that we previously developed (described in Chapter 2) to first evaluate the role BclA protein plays in spore persistence in the lungs of mice. And then to evaluate the role of binding of fH by challenging mice with spores that either were capable or incapable of binding to fH in our binding assays. To determine the significance of fH recruitment by BclA, we examined whether BclA-bound fH remained functionally active and able to act as a cofactor for factor I-mediated cleavage of C3b. We then determined whether the conversion of C3b to iC3b on the surface of spores occurs. We expected to find that fH binding leads to less complement activation on the spore surface and that there is a positive correlation between fH-binding and spore survival in persistent infections.

EXPERIMENTAL PROCEDURES

Bacterial strains and spore preparation. *B. anthracis* Sterne strain 7702, its isogenic deletion mutant strain (Δ*bclA*), and *B. subtilis* strains 168 were provided by T.M. Koehler, UT Health, Houston, TX. Complemented *B. anthracis* strains with full-length BclA (Δ*bclA*/BclAFL), the C-terminal domain (Δ*bclA*/BclACTD), and the

collagen-like region domain (Δ*bclA*/BclACLR) were constructed by fusing the respective BclA segments to the BclA promoter. The constructs were cloned into an *E.coli-B. anthracis* pUTE583 shuttle vector. The construct was then introduced into the Δ*bclA* strain background. *B. subtilis* 168 strain expressing full-length BclA (pDG1662/BclAFL) or the C-terminal domain (pDG1662/BclACTD) was constructed by taking the respective BclA segments (except the NTD) and fusing it to the C-term of CgeA. The fusion fragments were then cloned into vector pDG1662 and integrated into the *B. subtilis* 168 strain chromosome at the amyE locus. Immunofluorescence staining and flow cytometry using anti-BclA antibodies were performed to observe and quantify the expression levels of BclA on the surface of spores and the percentages of spores expressing BclA protein for each spore preparation. Spores of the different *B. anthracis* strains were prepared by growing cultures on Luria Broth (LB) agar plates for 6 – 8 days in a 37°C incubator. The bacteria were scraped from the plates, washed three times with cold sterile Phosphate Buffered Saline (PBS) and heated twice for 30 minutes (min) at 68°C. *B. subtilis* spores were prepared by culturing in Difco sporulation (DSM) media for 5 – 7 days at 30°C, washed with cold sterile PBS and heated twice for 30 min at 68°C. The suspension was then filtered through a 3.0μM filter, counted, and stored at 4°C. Bacterial titers were determined by plating on LB agar plates and incubation overnight at 37°C.

Mouse Infections. All animal experiments were carried out according to procedures approved by the Institutional Animal Care and Use Committee, Texas A&M Health Science Center, Institute of

Biosciences and Technology. BALB/c and C57Bl/6 mice were originally purchased from the Jackson Laboratory. $C1q^{-/-}$ mice [117] were obtained from Dr. Marina Botto, Imperial College London, UK. All mice were maintained in the IBT animal facility. For infection experiments, 6 – 8 week old and sex-matched mice were used. Mice were inoculated with spores by intranasal instillation (i.n.) as previously described [29]. Briefly, mice were anesthetized with avertin (0.3mg/g) by intraperitoneal injection (i.p.). Mice were then inoculated i.n. with 20µls of a sub-lethal dose of spores (1.5 x 10^7 spores). Mice were monitored twice daily over a two-week experimental period. Mice mostly appeared healthy with no physical signs of distress or illness throughout the experiments. Occasionally a few mice died within the experimental period and were not included in the data analysis. To determine the bacterial burden in the lungs, mice were euthanized by i.p. injection of avertin followed by exsanguination via the inferior vena cava. Lungs were collected at two weeks post-infection. The tissues were homogenized in sterile cold PBS plus D-alanine, pH 7.4 (1ml final volume) using a tissue homogenizer (Fisher Scientific). The lung homogenates were either directly diluted and plated to determine the number of total viable bacteria or heated at 68°C for 30 min and dilution plated in serial dilutions to determine the number of heat-resistant spores.

Deposition of iC3b on spores – Flow cytometry. Approximately 5 x 10^7 spores were incubated with 10% Normal Human Serum (NHS, CompTech) in the presence of 2.5mM D-alanine for various time points at 37°C. Samples were thoroughly washed with sterile saline and then fixed with 2% paraformaldehyde (MeOH free) for 20 min at room

temperature (RT). Spores were labeled with mouse anti-human iC3b (1:400; Quidel) in PBS with 2% BSA for 1 hour (hr) at RT followed by donkey anti-mouse 647 (1:400; SantaCruz) in PBS with 2% BSA for another hr. Samples were analyzed with a flow cytometer (Accuri) using forward and side scatter parameters to gate on at least 20,000 spores. Results were compared using the value for the mean fluorescence intensity (MFI).

Statistical analysis. Statistical analysis was performed using the two-tailed Student's *t*-test (Graph-Pad Prism 4.0).

RESULTS

BclA is an important factor for spore persistence in mice. To evaluate if BclA plays a role in *B. anthracis* spore persistence, BALB/c mice were challenged intranasally (i.n.) with 1.5 x 10^7 spores of *B. anthracis* Sterne Strain 7702 or its isogenic BclA deletion mutant (Δ*bclA*). The presence of vegetative bacilli and spores in the lungs was evaluated over a period of 2 weeks. The results indicated that significantly greater numbers of bacteria were recovered from mice infected with 7702 spores compared to mice infected with Δ*bclA* spores (Fig. 29, A). On average 7.8 x 10^5 ± 1.7 x 10^5 and 5.8 x 10^4 ± 2.5 x 10^4 colony forming units (cfu) were recovered from the mice infected with 7702 and Δ*bclA* spores, respectively. The majority of the bacteria (79%) recovered were heat resistant dormant spores similar to what was reported in our results in Chapter 2. To further evaluate BclA as an important factor in spore persistence, mice were challenged with the complemented strain Δ*bclA*/BclAFL. The number of bacteria recovered from the lungs of mice infected with Δ*bclA*/BclAFL

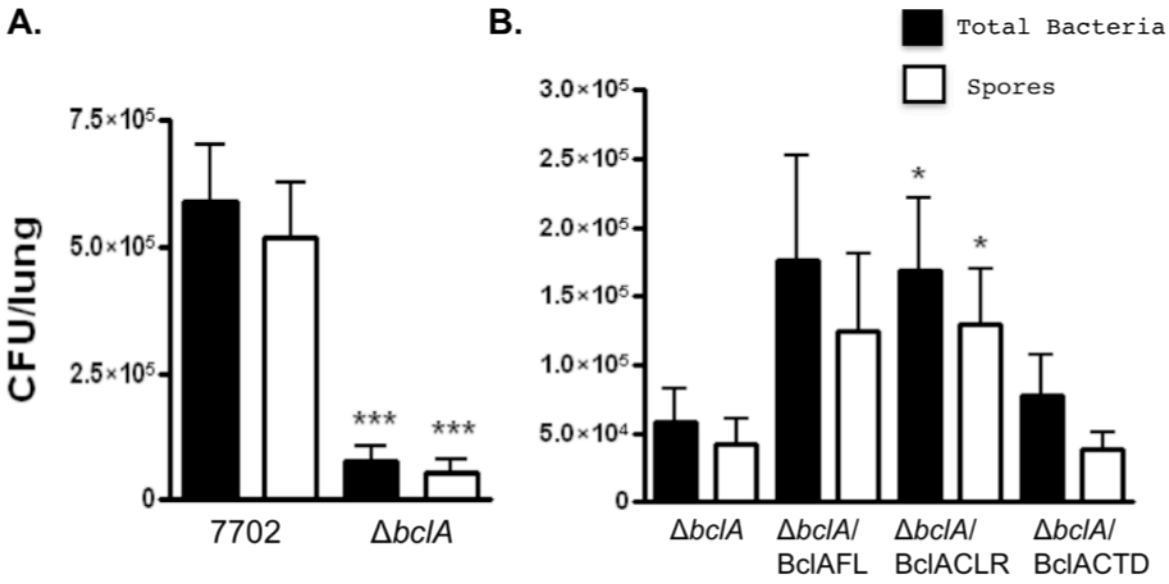


Figure 29. BclA expression contributes to *B. anthracis* spore persistence in the lung. Mice were inoculated i.n. with 7702, Δ*bclA*, and Δ*bclA*- expressing FL, CTD, and CLR of BclA protein **(A and B)** at a dose of ~1.5 x 10^7 cfu/mouse. Lungs were harvested at 2 weeks post-inoculation, homogenized, and plated for total viable bacteria (black bars) or heat-resistant dormant spores (white bars). The results were combined from at least 2 independent experiments. *, $p < 0.01$; Δ*bclA* vs. Δ*bclA*/BclACTD; ***, $p < 0.0001$; 7702 vs. Δ*bclA*; comparisons to respective total bacteria and spore titers in *B. anthracis* infected lungs.

was significantly greater than the number recovered from mice infected with Δ*bclA* (Fig. 29, B). These results suggest that the presence of BclA on the surface of *B. anthracis* spores is likely to contribute to their ability to survive and reside in the infected host for long periods of time.

We next determined if specific regions of BclA known to be important for fH-binding persisted in our mice model. BALB/c mice were infected i.n. with a sub-lethal dose (1.5×10^7 spores) of Δ*bclA*/BclACLR or Δ*bclA*/BclACTD spores. We observed that complementation with the CLR region of BclA resulted in a similar number of bacteria recovered in the lung as mice infected with BclAFL (Fig. 29, B). The number of spores recovered was significantly greater than the parental strain (Δ*bclA*). A different result was found with the complementation of the CTD region. Mice infected with Δ*bclA*/BclACTD had a slightly higher bacterial load in their lungs compared to mice infected with Δ*bclA* spores ($7.8 \times 10^4 \pm 2.9 \times 10^4$ vs. $5.8 \times 10^4 \pm 2.5 \times 10^4$ cfu) but lower than those infected with Δ*bclA*/BclACLR. We also challenged mice with *B. subtilis* spores expressing BclAFL and BclACTD. We observed that mice infected with both BclAFL and BclACTD spores had significantly greater numbers of cfu recovered from their lungs compared to mice infected with pDG1662 spores (Fig. 30). Collectively, the data indicate that spores that express BclA are better at persisting in the lung than spores that do not express BclA. Also, strains that have the ability to bind to fH (7702, Δ*bclA*/BclAFL, Δ*bclA*/BclACLR, Δ*bclA*/BclACTD, pDG1662/BclAFL, and pDG1662/BclACTD) are more likely to persist in the host than strains that do not (Δ*bclA* and pDG1662).

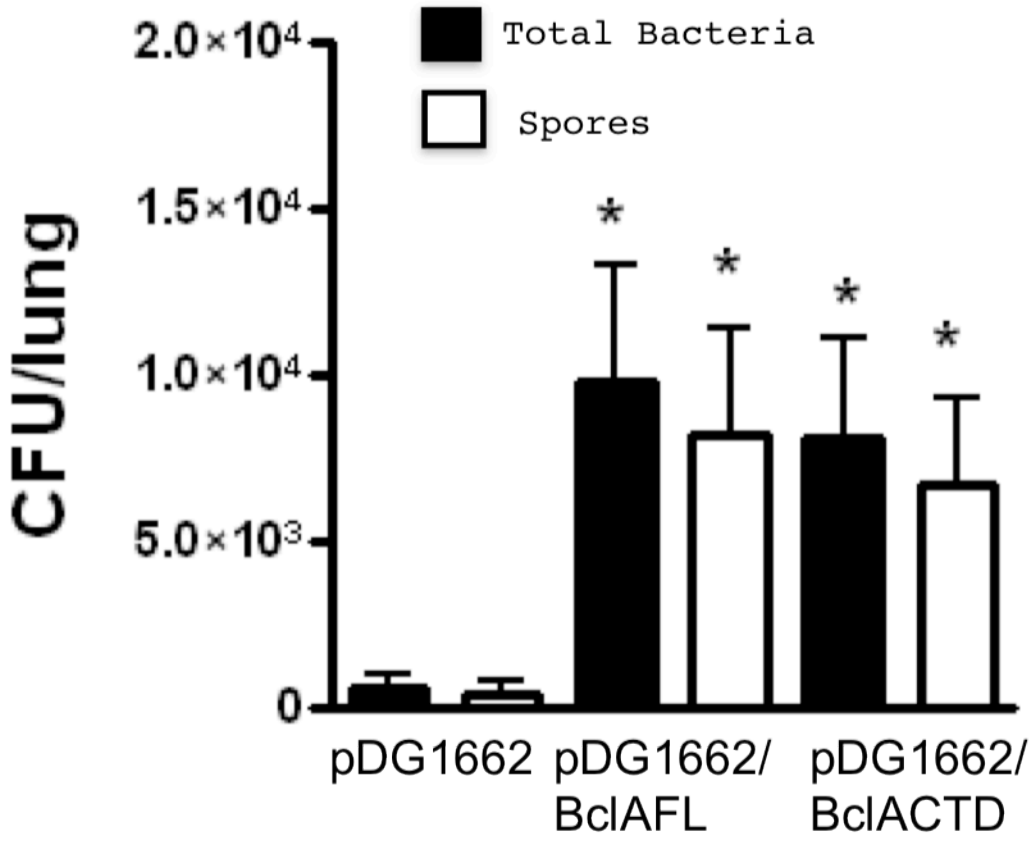


Figure 30. *B. subtilis* spores expressing BclA protein persist in the lung. Mice were inoculated i.n. with *B. subtilis* empty vector pDG162 spores and spores expressing BclA protein (pDG162/BclAFL; pDG162/BclACTD) at a dose of ~1.5 x 10^7 cfu/mouse. Lungs were harvested at 2 weeks post-inoculation, homogenized, and plated for total viable bacteria (black bars) or heat-resistant dormant spores (white bars). The results were combined from at least 2 independent experiments. *, $p < 0.01$; comparisons to respective total bacteria and spore titers to pDG162.

Spore persistence does not involve C1q. C1q is the ligand recognition subunit of the C1 complex of the classical complement pathway. It is able to interact with a variety of ligands, and functions in both a complement activation-dependent and –independent manner [118, 119]. We have shown that spores can initiate the classical complement pathway via a direct interaction between BclA and C1q [35] and we have demonstrated that C1q mediates spore entry into host epithelial cells using BclA and integrin α2β1 [29]. With respect to the direct interaction between *B. anthracis* spores and fH, we wanted to examine if the differences we saw in the persistence experiments described above were due to the ability of BclA binding to C1q. Therefore, we compared the lung cfu between wild-type (WT; C57Bl/6) and $C1q^{-/-}$ mice using all spore strains described above. The results demonstrated there was no detectable differences in the number of spores recovered from WT versus $C1q^{-/-}$ mice for any strain tested, indicating that C1q is not involved in spore persistence in a host (Fig. 31). This data further supports the theory that the fH-binding function of BclA is likely important for spore persistence.

BclA suppresses complement activation on the spore surface. FH negatively affects the formation of complement activating complexes (*i.e.* C3 convertase) and therefore is known to dampen the host immune response. We sought to examine the extent to which the surface expression of BclA affected the rate of iC3b conversion on the *B. anthracis* spore surface in order to determine whether BclA-bound fH is functionally active in its ability to provide cofactor

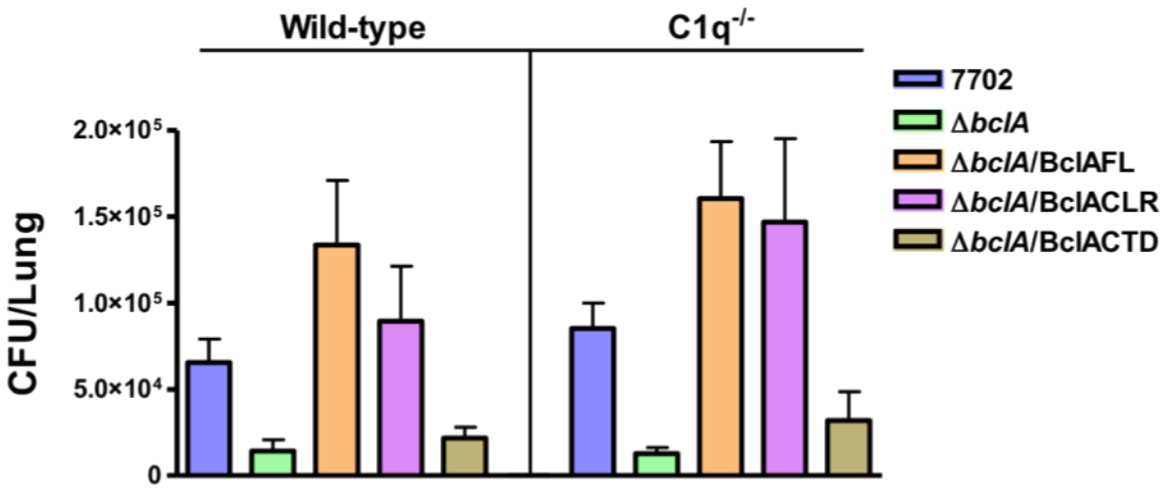


Figure 31. C1q does not play a role in *B. anthracis* spore persistence in the lung. Wild-type (C57Bl/6) and C1q$^{-/-}$ mice were inoculated i.n. with 7702, Δ*bclA*, and Δ*bclA* – expressing FL, CTD, and CLR of BclA protein at a dose of ~1.5 x 10^7 cfu/mouse. Lungs were harvested at 2 weeks post-inoculation, homogenized, and plated for total viable bacteria. The results were combined from at least 2 independent experiments. Differences between groups were not significant.

activity for factor I-mediated cleavage of C3b. 7702 and Δ*bclA* variants were incubated with NHS to allow complement activation via all pathways. The reactions were stopped at various time points, washed, and subjected to flow cytometry analysis using iC3b-specific antibodies. Over time, BclA-expressing strains had more iC3b deposited on the surface of their spores compared to the BclA deletion mutant (Fig. 32, A). We also performed a linear regression to calculate the slope, which represented the rate of iC3b over time. Similarly, BclA-expressing strains were faster at converting C3b to iC3b on their surfaces than the deletion mutant (Fig. 32, B). This data suggests that BclA is important for the conversion of C3b to iC3b on the surface of spores and this activity possibly involves its interaction with fH.

DISCUSSION

The most severe anthrax infections in humans occur via the inhalation of *B. anthracis* spores. Even with antibiotic treatment, inhalational anthrax is associated with high mortality and morbidity. A key feature of this form of anthrax is the persistence of spores in the lung of infected hosts for long periods of time. This is the basis for the prolonged antibiotic regimen (60 days) recommended for infected individuals. However, an underlying mechanism for spore persistence remained unknown. In Chapter 2, our results suggested that spore persistence is more than simply due to the dormancy of spores or hiding in a particular intracellular niche, rather there may be a general mechanism for avoiding and suppressing host immune recognition and clearance. The complement

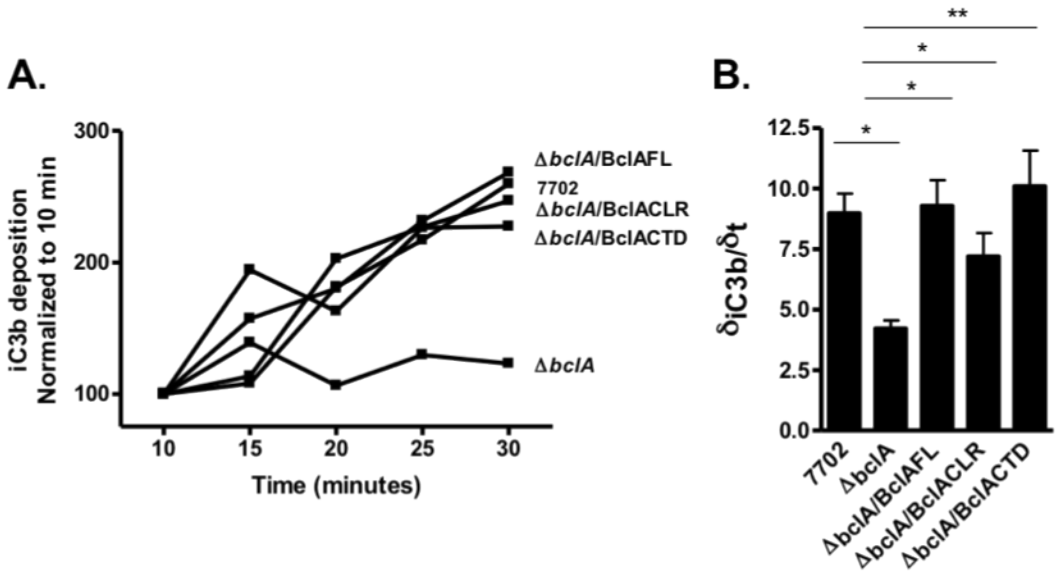


Figure 32. Kinetics of iC3b deposition on the surface of *B. anthracis* spores. **(A)** *B. anthracis* spores were incubated with 10% NHS for various times and subjected to flow cytometry analysis using iC3b-specific antibodies. iC3b deposition is shown normalized to 10 minutes. **(B)** Linear regression representing slope (rate of iC3b over time). Results are combined from at least 3 independent experiments. *, $p < 0.01$; 7702 vs. Δ*bclA*; *, $p < 0.01$; **$p < 0.001$; Δ*bclA* vs. complemented strains. Error bars represent SEM.

system is an important effector mechanism for host defense against microbial pathogens. There are several mechanisms that pathogens can use to circumvent innate immune killing through the complement system. Most commonly reported in the literature include the production of capsule to prevent complement recognition and the secretion of molecules that inactivate complement proteins. For example, the Staphylococcal complement inhibitor (SCIN) protein of *S. aureus* is a highly effective C3 convertase inhibitor that blocks conversion of C3 [120]. Another example is the cysteine protease SpeB of *S. pyogenes* that cleaves C3 and degrades C3b, thus escaping phagocytosis [121]. Alternatively, pathogens can recruit host complement regulators to their bacterial surface. We provided evidence in Chapter 3 that *B. anthracis* can bind fH and this interaction requires the spore surface protein BclA. Although fH has been shown by numerous other pathogens to subvert the complement system, this is the first report demonstrating its protective activity in *B. anthracis* spore survival in the host.

We first investigated whether the spore surface protein BclA was important for spore persistence. We previously determined a potential niche of spore persistence in the lung where primarily dormant spores were found to be closely associated with the lung epithelium for at least 8 weeks. Spore survival within the lung was much greater than those in other tissues indicating a potentially special circumstance of pathogen immune evasion and/or tissue tropism. It is likely that spore persistence in the lung is responsible for the ability of *B. anthracis* to cause disease recurrence. BclA, the outermost and very abundant protein of the

spore exosporium was a likely target to be involved in mediating spore persistence. We found that spores lacking BclA protein did not survive as well as spores that expressed full-length or fragments of BclA in mice. Among its other functions described for BclA (*i.e.* entry into host cells) this is the first demonstration that BclA on the surface of spores contributes to the persistence of spores in the host. To further evaluate the importance of BclA in this mechanism of persistence, we used a spore-forming heterologous host (*B. subtilis*) to display the same BclA variants on the spore surface. Our findings show that *B. subtilis* spores that expressed full-length BclA or fragments of BclA remained in the mouse lungs in greater numbers than spores that did not express BclA (pDG1662).

Moreover, *B. anthracis* spores expressing the CTD of BclA were not significantly different from the parental strain (Δ*bclA*) with regards to persisting in the mouse lung. It is possible that the proper display of the CTD on the surface of *B. anthracis* contributes to this result even though the expression level of BclA is similar to that of 7702 spores. We did observe *B. subtilis* spores expressing the CTD of BclA persisting in the mouse lung, so we cannot exclude that the C-terminal region of BclA is important in this mechanism of persistence. Further studies utilizing transmission electron microscopy are needed to validate the proper display of BclA on the surface of *B. subtilis* and *B. anthracis* spores.

FH deficient mice ($Cfh^{-/-}$) are known to have uncontrolled C3 activation resulting in C3 depletion from plasma. Therefore, $Cfh^{-/-}$ mice were not suitable for determining the role of fH during infections. To determine if *B. anthracis*-fH interaction is a

mechanism for persistence, the role of fH recruitment by BclA was inferred by using engineered spores that bound or did not bind to fH. Our results indicated that strains that are fH binding (BclA-expressing strains) were more likely to persist in the mouse lung than strains that did not express BclA (Δ*bclA* and pDG1662, non-fH binders). Determining the minimal binding region between BclA and fH and/or defining mutants that abolish fH binding would greatly strengthen the evidence for BclA-fH binding *in vivo*. Also, studies to determine the levels of intracellular/extracellular spores with the various BclA strains would provide clues on the survival potential of these spores within the lung. It would be interesting to see whether altering the surface display of the spores would change their specific location within the lung. Moreover, C1q was shown to not be involved, providing further validation that fH contributes to the persistence phenomenon. $C1q^{-/-}$ mice had similar numbers of bacteria and spores in the lungs of mice compared to wild-type mice, which excludes the direct BclA-C1q interaction.

With respect to the regulatory role of surface-attached fH, degradation of C3b was analyzed directly at the surface of *B. anthracis* spores after incubation with human serum. For all analyzed strains, iC3b deposition was more pronounced for strains that were BclA-expressing and had fH-binding activity. These specific spores were also faster at converting C3b to iC3b on the spore surface. Other bacterial fH-binding proteins (*e.g.* Yad A and Tuf) have been reported to promote factor I-mediated cleavage of C3b [94, 122]. Our unpublished data also reveals that iC3b coated spores survive better after phagocytosis than did non-coated spores (data not shown).

Collectively, these results indicate that the BclA-fH interaction likely contributes to an immune evasion strategy for the persistence of spores in the host.

Further insight into how BclA affects the longevity of *B. anthracis* spores in a host is that the presence or absence of BclA may affect the germination potential of spores and in turn affect their ability to survive in the lung. Brahmbhatt *et al* reported that the BclA deletion mutant germinated at a faster rate than the parent strain *in vitro* and *in vivo*, suggesting that BclA may slow spore germination *in vivo* [105]. Also, it has been known for many years that spores do not readily germinate in the alveolar spaces of the lung [123]. If this is true, then we would speculate that the slight delay in germination of *B. anthracis* spores may allow greater numbers of spores to survive (*i.e.* BclA is present longer, spores are able to recruit fH allowing for iC3b conversion to take place) and therefore spores are likely to remain longer in the host.

In summary, here we describe an uninvestigated area in *B. anthracis* research; that is a mechanism for spore persistence. The data presented highlights that fH-binding by BclA is a mechanism to limit complement activation on the spore surface and consequently promotes spore survival and persistence in the lung. We were able to demonstrate that there is a positive correlation between fH-binding strains (BclA-expressing strains) and spore survival in persistent infections. Future experiments will further confirm these conclusions and determine the general role of fH-binding in anthrax infections.

Chapter 5: DISCUSSION

More than a 100 years ago after its initial discovery to cause disease, *Bacillus anthracis* remains at the forefront of research because of its potential as a biological weapon. The significance of anthrax as a terror weapon was realized in 2001. Since then new research findings are improving our understanding of how *B. anthracis* causes disease and how to better prevent and treat it in case of a bioterrorism event.

One of the characteristic features of pulmonary anthrax is that spores could remain in the lungs for weeks, even months in infected animal hosts [7-9]. This retention of spores *in vivo* necessitated the prolonged use of prophylactic antibiotics for patients with pulmonary exposure to *B. anthracis* spores. The recommended antibiotic regimen is 60 days with an option for a 40-day additional treatment. However, the optimal duration of prophylaxis still remains unclear. Minimizing the duration of post-exposure antibiotic prophylaxis could be crucial to a successful defense against a large-scale bioterrorism attack. However, no investigations have been conducted to determine the characteristics and molecular mechanisms of spore persistence in an infected host, which is vital to understanding how to treat post-exposure prophylaxis.

Here, we developed a persistence model of inhalational anthrax in mice. The impact of our findings to *B. anthracis* pathogenesis is highly significant. We report that there are multiple mechanisms that contribute to spore persistence in the lung. One mechanism is the association with the lung epithelium. We demonstrated using

fluorescent microscopy that intranasal inoculation of mice with *B. anthracis* Sterne strain 7702 spores resulted in the observation that spores were inside lung epithelial cells *in vivo* and tightly associated with the alveolar epithelium up to 8 weeks post-infection. We report that ~8% of spores found in the lung were associated with cytokeratin-positive epithelial cells at 4 weeks post-infection. This percentage is similar to the spore association with alveolar macrophages and dendritic cells ($CD11b^+$ or $CD11c^+$) which was reported to be ~5%. Our results provided direct evidence that even in the presence of phagocytes, the lung epithelium is exploited by *B. anthracis* weeks post-infection. This data supports are previous *in vitro and in vivo* data that spores are internalized and survive in epithelial cells hours after infection [67, 68]. Findings that *B. anthracis* spores are found associated with the lung epithelium at a chronic stage of infection, suggests that spores internalized by epithelial cells may begin germination *in vivo*, which can lead to a recurrent infection. It was reported in mouse models, only a few organisms were required to cause infection if *B. anthracis* were introduced directly into the blood stream (LD_{50} 6 – 25 spores) [124]. Based on the percentage of intracellular spores we identified in epithelial cells, if even only a small number of them escape into the circulation, it would be sufficient to cause an infection.

The precise germination location *in vivo* remains poorly defined. Many groups have accepted that germination does not readily occur in the lungs [77, 123]. However, we have previously demonstrated in translocation assays using an *in vitro* model of the

alveolar epithelium that germinated spores/vegetative bacilli were recovered from the basolateral side of the epithelium despite the presence of germination inhibitors in the media, suggesting that germination occurred inside epithelial cells [68]. Also, findings by Sanz *et al* reported germination proceeded in the lung [77]. These studies raise the possibility that the lung epithelium not only acts as a portal for spore dissemination away from the lung and for spore persistence, but also provides a venue for spore germination. The use of germination defective mutants in this model would surely provide insight into the germination status of *B. anthracis* inside lung epithelial cells *in vivo*.

Collectively, these data provides us with the understanding that spores target so far three types of host cells (*i.e.* lung epithelial cells, macrophages, and dendritic cells) for spore persistence. The mechanisms of how *B. anthracis* spores are internalized by epithelial cells [29, 125], as well as phagocytes [27, 28] are known. Entry into epithelial cells and phagocytes is mediated by *B. anthracis* BclA protein via different receptors (*i.e.* $\alpha 2\beta 1$ and Mac-1, respectively) [27, 29]. In this study, we report that BclA-expressing strains are more likely to remain in the lung than spores that do not express BclA, suggesting that persistence is linked to the expression of BclA. This data and our finding that BclA mediates spore persistence have important implications to vaccine development.

Vaccination remains the best method available for combating anthrax infections. The current anthrax vaccine is based on protective antigen (PA) and requires 6 injections given over 18

months followed by annual boosters. Developing new and improved vaccines that require shorter administration time and more convenient administration routes is needed and is being pursued by multiple research groups. BclA is the major and the most exposed molecule on the surface of *B. anthracis* spores [15] and it has been a topic of investigation for many years. Studies have described its assembly process and timing [13, 26, 126], N-terminal cleavage events [23], covalent attachment to the basal layer by BxpB [127], and its association into large molecular weight complexes during exosporium assembly [128]. BclA belongs to a family of proteins called bacterial collagens, which also includes Scls in Group A streptococci [129] and PclA in *Streptococcus pneumoniae* [130]. Proteins containing collagen-like regions are recently being explored in prokaryotes and understanding the functions of BclA will have implications to other members of the bacterial collagen family. It seems that what we know about BclA is perhaps the crest of a whole new wave of information. Importantly, we want to understand how beneficial is the presence of BclA on the spore surface to the intracellular survival of *B. anthracis* spores *in vivo*. Other exosporium-containing spores, such as *B. cereus* contain BclA on its spore surface [131]. It would be interesting to determine if BclA protein on *B. cereus* or other similar bacterial collagen proteins (*i.e.* Sc1s) can mediate persistence of bacteria in mice similar to BclA from *B. anthracis*. Several animal studies have demonstrated that immunization with BclA either provided protection or improved the protective efficacy of PA against lethal challenges with *B. anthracis* spores [105, 132-134]. We question then, will BclA provide

better protection not only against acute infection but also spore persistence? The results from this study encourage future studies to determine the protective efficacy of recombinant fragments of BclA in long-term anthrax infections.

We also revealed from our model that spore persistence might involve an immune evasion/suppression mechanism. The interactions between the host's immune system and spores are also an important area of anthrax research. Therefore, our findings will surely improve our understanding of spores with the host immune system. We know that spores are capable of activating TLR2 and MyD88 signaling [30], inducing inflammatory cytokine production (*i.e.* IL-12 and IFNγ) [31, 32], activating natural killer cells [33, 34], and initiating the classical complement pathway via a direct interaction with BclA and Clq [35], but how these molecular pathways and host protein associations influence the ways in which spores survive longer in the infected host is not clear.

We report that the very mild inflammation and pathology observed in the lung is indicative of a subdued immune reaction to *B. anthracis spores*. These findings suggest that the subdued immune response may be due to an active suppression mechanism of *B. anthracis* rather than passive inactivity of spores. We demonstrated that the anthrax toxins, in particular lethal factor, a major immune suppressor of *B. anthracis* did not play a role in spore persistence in the lung. This implies that *B. anthracis* spore surface components might contribute to this mechanism. We speculate that the presence of BclA on spores may play a role in immune evasion, thus preventing spores from being efficiently cleared by the host. From our *in vitro*

data, BclA was identified as a factor H (fH) binding surface protein of *B. anthracis*. *B. anthracis* spores were able to recruit fH both from human and mouse serum and in a purified form, and this was mediated by BclA. We concluded that the *B. anthracis*-fH interaction was direct and independent of other serum proteins. It is this direct recruitment that may lead to the suppression of complement activation on the spores' surface, akin to fH binding to host self-surfaces.

In mapping the regions involved on BclA for fH binding, we identified two binding sites. Both the CTD and CLR domains of BclA bound equally well to fH using different approaches to determine fH binding. We believe it is possible to have multiple binding sites on BclA for fH interaction. *Borrelia hermsii* CRASP-1 protein was reported to require both N- and C-terminal domains for binding to fH [135]. *S. pyogenes,* Fba protein contained a major fH binding site (in the N-terminus coiled-coil domain), but also had a second weak binding site in the C-terminus [136]. In future work, inhibition assays utilizing rBclACTD and spores expressing the CLR of BclA will be evaluated to determine the relevance of two binding sites. As reported in the study, we were unsuccessful at synthesizing a gene for the CLR and therefore could not obtain a recombinant protein. However, near the end of this study we were able to obtain a commercially constructed CLR gene product, which will then be used to generate a recombinant protein of this region for future studies.

Furthermore, to have a better understanding about the regions of BclA that are important for fH binding, we performed sequence alignments with the sequence of BclA (CAD56870.1; *B. anthracis*

Sterne Strain) to that of several known fH-binding proteins that have been described for various microbial pathogens. From the sequence alignments we found that the BclA protein had no significant similarity to any of the following fH-binding proteins (Yad A, PspC, CRASP 1 and 2, SdrE, Sbi, Hic, LfhA, Por1A, Tuf, OmpA, FhBp, and FhbB). Also, no other sequence homologies between fH-binding proteins from different species have been reported. There have been only a few specific sequences identified for fH binding sites among the fH-binding proteins. In *S. pyogenes*, the major fH-binding site of Fba was determined to be YKQKIKTAPDKDKLLF in the N-terminus domain [136]. Similarly, a positively charged fH-binding site is found in the C-terminus domain of CRASP-3 of *B. burgdorferi* (LEVLKKNLK) [137]. A negatively charged binding site and hydrophobic interactions have been reported for PspC of *S. pnuemoniae* [53]. The Scl1 protein of Group A Streptococci, which also forms collagen triple helices similar to BclA, was reported to bind to fH [138]. However, a motif in the Scl1 V-region seemed to be involved in binding to fH and manipulation of the CLR did not alter the ligand-binding properties [138]. These studies and other reports suggest that there is so far no commonality (in particular, sequence motifs) among the regions on fH-binding proteins that are involved in fH binding. It is possible that the conformation and structure of the respective fH-binding proteins influence the way they associate with fH. Therefore, we expect that the regions/structure of BclA involved in this interaction will be unique to *B. anthracis*. In order to further elucidate the binding sites of BclA, future work will include the generation of truncated fragments of both the CLR and

CTD domains to define the residues that are important for binding to fH.

To further characterize this interaction we anticipate to map the region in fH recognized by BclA. Multiple groups have expressed fragments containing different SCRs or mutations as recombinant proteins. In general, the baculovirus expression system in the insect cell line Sf9 has been commonly used to produce fH fragments. Binding of fH fragments to rBclA and to spores expressing full-length or specific regions of BclA will be determined. The majority of fH-binding proteins have been described to bind to domains 19 – 20 on fH [37]. A few have been described for interaction on heparin (SCR7) and C3b (6 – 8 and 19 – 20) binding sites, including M proteins and Fba of *S. pyogenes*, PorB 1A of *N. gonorrhoeae*, Ail of *Y. enterocolitica*, Tuf of *P. aeruginosa*, and Sbi of *S. aureus* [37]. For *B. anthracis*, our results indicate that BclA protein does not interact on heparin or C3b binding sites at the concentrations tested, but further experiments are needed to exclude these sites. It is also possible that the interaction can occur with the entire length of fH. Studies to elucidate the regions on fH are currently underway in our laboratory.

The consequences of the interaction between BclA-fH are vital to our general understanding on how microbial pathogens evade the host immune system to cause infection. The more we acquire information about the specific details of these mechanisms of host evasion by pathogens, then the better we can treat, diagnose, and prevent infections. FH is one of the most important complement inhibitors. The ability of fH to recognize specific markers on host

cells is extremely important for limiting complement-mediated immune responses to self-surfaces. This is supported by clinical evidence showing that mutations or polymorphisms that affect fH recognition of host cells are associated with severe human diseases due to abnormal complement activation. The ability to recruit fH has been demonstrated in a wide range of microbial pathogens [37], including *B. anthracis*. This suggests that interaction with fH is a general microbial immune evasion strategy. For Gram-positive bacteria, the ability to bind complement inhibitors (*e.g.* fH and C4BP) is essential for evading opsonophagocytic killing by the complement cascade. Our findings indicate that BclA and most likely the fH-binding portion of BclA are important for survival and persistence of spores in the mouse lung. This suggests that even for resilient organisms as spores, binding to fH can offer a survival advantage.

Because BclA is only expressed on the surface of spores and is absent in vegetative cells [15], we focused on determining the effect of fH binding on spore persistence. The precise biological consequences of this interaction was investigated in this project by examining complement activation on the spore surface and spore persistence in the lungs of mice with spores that expressed BclA protein and were known fH-binders. In regards to the role fH plays in persistence, we showed that spores that can bind fH could remain in the lungs of mice significantly better than spores that were not able to bind fH. Furthermore, the activities of C1q were not involved in spore persistence. These findings highlight in an indirect way the possibility that the fH binding ability by BclA on these spores is contributing to the length of time these spores

remain in the mouse lung compared to spores that cannot bind to fH. Because this is the case, we believe that BclA recruitment of fH could allow immune evasion during early stages of infection (at least partially), which could potentially lead to increased persistence in the host. Unfortunately, we were unable to utilize $Cfh^{-/-}$ mice since they have uncontrollable C3 activation resulting in C3 depletion. In general, evaluating the role fH plays *in vivo* is difficult and providing direct evidence is a challenge. Nevertheless, one way to determine specifically the role of BclA-fH binding in spore survival and persistence in mice is to generate *B. anthracis* spores specifically expressing only the fH-binding function of BclA and spores that carry mutations in the respective binding sites. Comparison of results from mice infected with these isogenic mutants will provide us a better understanding of fH recruitment by these spores. Other ways may include utilizing exogenous fH or inhibitors of fH-binding sites and priming *B. anthracis* spore strains with these molecules to block their interaction *in vivo*. FH present on the *anthracis* surface at the time of infection may appear to promote a more pronounced bacterial persistence and/or blocking of fH-binding sites on spores may result in a greater level of bacterial clearance in the host. These studies would have to be carried out to further understand the biological significance of fH recruitment by *B. anthracis* spores.

Moreover, we found that BclA-expressing strains were able to suppress complement activation on their spore surface compared to a strain that lacked BclA protein. This data provided insight that BclA does not inhibit fH function and that BclA recruitment of fH

likely serves to suppress complement activation, similar to what has been reported for other microbial fH-binding proteins.

Our finding that BclA binds fH also has implications to vaccine development for anthrax infections. If BclA binding to fH is a mechanism for complement suppression and spore persistence in the lung, then we wonder if immunization with BclA, particularly the fH-binding region of BclA, can protect against both acute and persistent infections. Results from studies performed with *Neisseria meningitides* indicate that vaccines based on the fH-binding region have broad protective efficacy against meningococcal diseases [139-141]. This is encouraging and studies in our lab will be carried out to investigate the protective efficacy of BclA in anthrax infections.

The ability of *B. anthracis* spores to recruit fH warrants further attention because it is also likely that binding to fH influences the molecular pathways that enable *B. anthracis* to adhere and be phagocytosed by host cells. Besides its complement regulator activity, fH has been reported to play a role in the adhesion and entry into host cells by microbial pathogens. For example, *S. pneumoniae,* PspC protein recruitment of fH was shown to increase the attachment of pneumococci to host cells and increase the number of internalized bacteria [54]. In general, fH can bind via an RGD sequence of SCR4 to host cells [142]. Also, studies have reported that polymorphonuclear cells can bind immobilized fH via integrin CD11b/CD18 (CR3 receptor) [143]. This is interesting because *B. anthracis* spores are known to enter macrophages via the same receptor through BclA [27]. Studies from our group have shown that

spores expressing BclA or fragments of BclA are more likely to be phagocytosed by macrophages and survive phagocytosis than spores that lack expression of BclA (unpublished, Chunfang Gu). It is not clear if the fH binding function of BclA plays a role in phagocytosis (or entry into other host cells, such as epithelial) and in intracellular survival. This will be determined in future investigations. Further efforts to identify the roles of fH recruitment by *B. anthracis* spores will bring us closer to a better understanding of the versatile roles played by fH in both health and disease and that of *B. anthracis* spores in anthrax infections.

Importantly, we demonstrated that the deletion mutant of BclA had a low level of fH binding, suggesting that proteins exposed on the surface of these spores in the absence of BclA are likely to interact with fH. The exosporium is composed of >20 proteins. Using a systematic approach, we could eliminate each one of these proteins from spores and/or generate recombinant proteins and evaluate fH-binding. This will allow us to identify other proteins involved in fH binding on the BclA deletion mutant that would otherwise be masked by BclA. There are also several other complement proteins besides fH that will be evaluated for binding to spores. These include C4BP, an inhibitor of both classical and lectin pathways; FHL-1, an alternative splicing product of fH; fH-related proteins; and C3. The finding that spores may also bind to one or more of these proteins will surely expand our knowledge on the interactions between *B. anthracis* and the complement system.

In summary, this is the first report to determine mechanisms of *B. anthracis* persistence and the first demonstration that *B.*

anthracis spores can interact with host complement regulator fH (Model illustrated in Fig. 33). The data presented here have provided a step forward in understanding more about the persistence of spores adding to the theories that the asynchronous germination and/or the dormant and resilient properties of spores contributed to this persistent phenomenon. Medical literature on anthrax does not include any findings regarding long-term complications in infected individuals. Therefore, information gained in this study will be valuable to patients, military personnel, and health care workers, in addition to, the planning of the public health response in the event of a bioterrorist attack involving inhalational anthrax. The progress in our understanding of the factors involved in persistence and the interactions between *B. anthracis* and the host immune system (in particular the complement system) should greatly improve our ability to design safe and efficient vaccines. From a broader point of view, persistence is an important issue concerning a variety of microbial infections (*e.g.* uropathogenic *E. coli*, H. pylori, Group A streptococci [59, 63]). Persistence *in vivo* not only increases the number of hospital visits and health care costs, but also exposes the host body to prolonged stress and inflammation, raising the risk for other types of diseases, such as rheumatism and cancer. Moreover, by studying how microbial pathogens subvert the host defense system is critical to contemplating the fundamental properties of microbial pathogenesis and developing/improving on preventatives and therapeutics against these infections. Our mechanistic studies will have broad implications to a large number of clinically important microbial pathogens, as well as, our

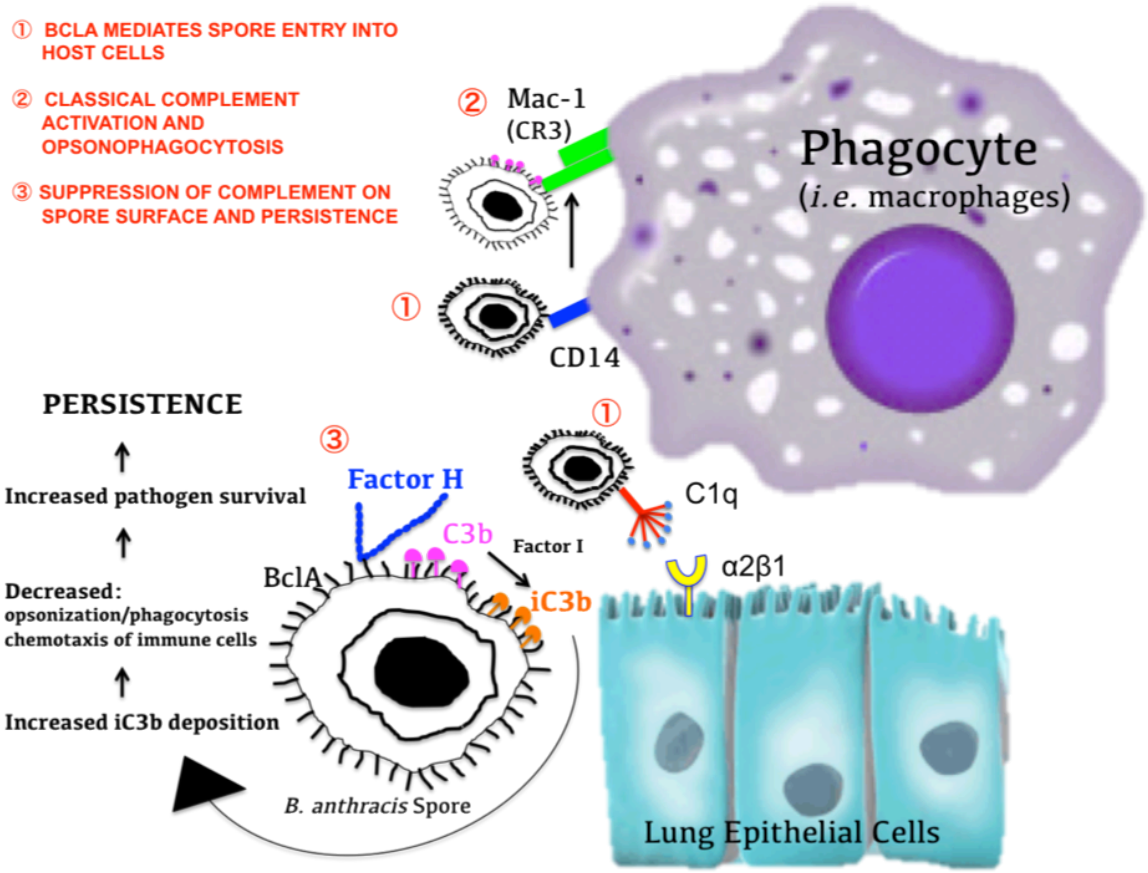


Figure 33. Model of an immune evasion strategy employed by *B. anthracis* spores for protection from complement and persistence in the host. *B. anthracis* spores are able to recruit fH to its spore surface via BclA. Recruitment of fH is crucial for the degradation of C3b to iC3b, which leads to suppression of complement activation. Inhibition of complement activation can potentially lead to increased pathogen survival for persistence in the host. Other possible mechanisms that contribute to persistence of spores are the association with the lung epithelium, association with phagocytes, and biofilm formation.

understanding of how the respiratory system functions under microbial challenge. Collectively, this work has provided precedence for future work related to the post-exposure prophylaxis regimens of *B. anthracis*.

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VITA

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