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New Insights into Gastrointestinal Anthrax Infection

Jennifer L. Owen¹, Tao Yang^{2,3}, and Mansour Mohamadzadeh^{2,3}

¹Department of Physiological Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610, USA

²Department of Infectious Diseases and Pathology, University of Florida, Gainesville, FL 32608, USA

³Division of Gastroenterology, Hepatology & Nutrition, Department of Medicine, University of Florida, Gainesville, FL 32610, USA

Abstract

Bacterial infections are the primary cause of gastrointestinal (GI) disorders in both developing and developed countries, and are particularly dangerous for infants and children. *Bacillus anthracis* is the “archetype zoonotic” pathogen; no other infectious disease affects such a broad range of species, including humans. Importantly, there are more case reports of GI anthrax infection in children than inhalational disease. Early diagnosis is difficult and widespread systemic disease develops rapidly. This review highlights new findings concerning the roles of the gut epithelia, commensal microbiota, and innate lymphoid cells in initiation of disease and systemic dissemination in animal models of GI anthrax, the understanding of which is crucial to designing alternative therapies that target establishment of infection.

Keywords

Bacillus anthracis; B-1 cells; gastrointestinal anthrax; infectious enteritis; innate lymphoid cells; microbiota

Gastrointestinal infection

Infectious colitis is caused by a variety of bacterial, viral, and parasitic organisms. However, bacterial infections are the primary cause of gastrointestinal (GI) disorders, both in developing and developed countries [1], and diarrhea remains the second leading cause of death in children younger than five years of age, accounting for 1.3 million deaths every year worldwide [2, 3]. Infection by pathogenic bacteria causes disease either by disturbing

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Corresponding author: Mohamadzadeh M. (m.zadeh@ufl.edu).

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Conflicts of interest

The authors have no conflicts of interest to disclose.

the homeostatic balance between the host and the gut commensal microbes, and/or by systemic dissemination. The gut has been characterized as the “motor of critical illness” due to dysregulated crosstalk among the epithelia, immune system, and endogenous microflora of the gut [4], in which loss of balance between these tightly interrelated systems leads to the development of systemic manifestations of disease, reaching far beyond the intestine [5]. In this review, we focus on the current understanding of the synapses between GI anthrax infection and the protective innate populations of the GI tract. A better understanding of these interactions has important implications for the design of future studies and interventions that target establishment of infection by this deadly pathogen.

Anthrax infection

Bacillus anthracis is a sporulating Gram-positive bacterium that causes anthrax, an often fatal infection that occurs when endospores (see Glossary) enter the body through ingestion, inhalation, or abrasions in the skin. Regardless of the route of entry into the body, gastrointestinal, inhalational (pulmonary), and cutaneous infections can rapidly progress to fatal systemic anthrax [6]. *B. anthracis* is not considered to be an invasive organism; under normal circumstances, the healthy integument, intestinal mucosae, and respiratory cilia put up efficient barriers to entry of anthrax spores into the body [7]. Recently, a new form of anthrax has also been recognized, “injectional anthrax,” because of its association with the injection of *B. anthracis*-contaminated heroin in Scotland from 2009–2010 [8, 9]. During that time period, 47 patients had confirmed *B. anthracis* soft tissue infection, related to injection of contaminated heroin, with a fatality rate of 28%. Strikingly absent in most patients with injectional anthrax is the eschar formation typically associated with cutaneous *B. anthracis* infection. This lack of eschar development combined with the high fatality rate despite receipt of antimicrobial drugs, support the notion that the pathogenesises of injectional and cutaneous anthrax differ [10–12].

All forms of anthrax infection can quickly become systemic, characterized by a toxemia caused by the secretion of lethal toxin (LT) and edema toxin (ET), and septicemia, which is associated with the bacterium’s anti-phagocytic poly-D-glutamic acid capsule (Box 1) [13]. This anti-phagocytic capsule is produced by gene products encoded on the pXO2 plasmid and the tripartite exotoxin is encoded on the pXO1 plasmid (Figure 1) [14]. Disease initiating spores are highly resistant to environmental conditions, including chemical disinfectants, heat, desiccation, ultraviolet and ionizing radiation, and extreme pressure [6]. These physical properties also render the spores highly resistant to killing by the host’s immune system. Anthrax is the “archetype zoonosis;” no other infectious disease affects such a broad range of species, including humans [7]. This disease is zoonotic to most mammals, and grazing herbivores are considered most susceptible, as infectious anthrax spores can remain dormant in the soil for decades [15]. Herbivores are also thought to provide most of the human exposure risk for anthrax [16]. GI anthrax is considered to be the primary route of infection for livestock and can also occur in humans through the ingestion of contaminated food [17]. Ingested spores germinate within the herbivore host to produce the vegetative forms, which proliferate and produce their virulence factors (toxins and capsule) [6]. While anthrax has been well managed in developed countries, this disease

persists in areas of sub-Saharan Africa, Southeast Asia, and parts of the former Soviet Union with weakened public health systems [18].

Clinical signs of GI anthrax

GI anthrax can present clinically as either intestinal or, less commonly, oropharyngeal infection. The incubation period is typically between one to six days, and the means by which the bacteria establish infection at the mucosae are unclear. Both GI forms involve epithelial barrier breakdown and ulceration, and the mortality rate is variable, but may approach 100% depending on the outbreak [15], or 40% with appropriate antibiotic treatment [17]. Oropharyngeal anthrax is characterized by mucosal ulcerations, sore throat, enlargement of cervical lymph nodes, soft tissue edema, and dysphagia [16]. Intestinal anthrax is caused by infection of the stomach or bowel [19], and primarily manifests with ulceration of the ileum and/or cecum [20]; this should not be confused with the non-ulcerative hemorrhagic lesions associated with the septicemia that eventually results from anthrax infection [17, 21]. Illness begins with anorexia, nausea, vomiting, and fever, progressing to severe abdominal pain, hematemesis, melena, and/or frank blood in the stool [17].

There have been more case reports of GI anthrax in children than inhalational disease, and the clinical presentation can differ from that of adults. A review of cases from 1900–2005 found that none of the children presented with hematemesis, and only one case reported bloody stool. However, seven out of 20 children developed secondary meningoencephalitis, which is usually associated with inhalational anthrax in adults, likely from hematologic dissemination [22]. Locations endemic for anthrax exist on every continent that contains subtemperate or tropical regions, and GI anthrax fatalities have been reported in India, Iran, Turkey, Thailand, Uganda, Zimbabwe, and Gambia [7, 16]. During an outbreak in Uganda, 155 villagers became ill after consuming the meat of a zebu. Within 15 to 72 hours, 91% of the villagers had GI complaints, 9% had oropharyngeal edema, and nine victims, all children, died within 48 hours of disease onset [17, 23]. GI anthrax has been reported in the U.S. [24], but protection of the food supply has been credited for its rarity [17].

Cutaneous anthrax is the most common route of infection (95% of cases) and is easily diagnosed, as the black eschar lesions are always accompanied by significant edema. In contrast, disease onset can be insidious in the inhalational (5%) and GI (<1%) forms, with non-specific flu-like symptoms, fever, or mild gastroenteritis [6]. Thus, early diagnosis can be difficult, and widespread systemic disease develops rapidly, resulting in circulatory shock, respiratory failure, sepsis, and death [25]. This has led some investigators to propose that GI anthrax is underreported and underestimated, as this form of the disease tends to occur in rural areas of developing countries, and mild GI signs are non-specific and attract little suspicion, while severe infection can be fatal within several days, before the sick can reach medical attention [16]. A recent case report of GI anthrax in Turkey was linked to consumption of tainted meat and was only discovered after the patient developed pneumonia and sepsis secondary to GI infection [26].

Animal models of GI anthrax

The first published animal model of GI anthrax was reported in 2007 [13], and used a bioluminescent derivative of *B. anthracis* strain 9602P, which is derived from the highly virulent natural human isolate 9602 [27] and does not produce the protective antigen (PA) component of the toxins. Glomski *et al.* found that *B. anthracis* spores germinate and establish infections at the initial site of inoculation in both inhalational and cutaneous infections, and that mice initially develop infections in Peyer's patches upon intragastric inoculation of spores [13]. Bacilli, neutrophils, and hemorrhage were evident within the Peyer's patches, with systemic spread leading to similar lesions in the spleen and the lungs within 35 hours of inoculation. Vegetative bacteria were isolated from the feces four hours post-inoculation and spores were excreted for up to 48 hours post-inoculation. Abrasions in the mucosae were preferentially infected with spores; GI infection could also occur in areas of intact mucosae, but with slower kinetics. The authors inferred that in the absence of mucosal damage, *B. anthracis* may potentially invade the Peyer's patches via uptake by innate cells (e.g. microfold (M) cells and phagocytic cells) [13]. Indeed, it has been postulated that pre-existing mucosal lesions may also predispose humans to infection [28], and that *B. anthracis* outbreaks tend to occur after droughts due to oral mucosal abrasions caused by sharp, dried plant material [29].

Bacterial dissemination

During the fulminant stage of the disease, bacteria disseminate hematogenously to multiple target tissues and organs, which are characteristically highly vascularized [30]. As bacteria replicate in the bloodstream, LT and ET rapidly accumulate reaching a critical threshold level that will cause death even when the bacterial proliferation is restricted by antibiotics. During this final phase of infection, the toxins cause an increase in vascular permeability and a decrease in function of the heart, spleen, kidney, adrenal glands, and brain [30].

There are several theories on how *B. anthracis* is able to leave its primary location of infection (Box 2). Russell *et al.* demonstrated that non-phagocytic host cells may also play a role in the process of *B. anthracis* dissemination. Both spores and vegetative cells of *B. anthracis* Sterne strain were able to adhere to, and be internalized by, the cultured human fibroblast cell line, HT1080, and the human epithelial cell line, Caco-2 [31]. *B. anthracis* spores are taken up by lung epithelial cells *in vivo* soon after spores are delivered into the lung [32]. These findings suggest that *B. anthracis* may exploit non-phagocytic cells to disseminate *in vivo* [31], and may escape the lungs by several distinct mechanisms, including transcytosis across the epithelial barrier [33].

Role of the epithelia

The gut epithelial lining comprises a single layer of cells including absorptive enterocytes, enteroendocrine cells, goblet cells, and Paneth cells, all of which differentiate from epithelial stem cells located in the crypts of the villi [34]. These epithelial cell subsets are tightly associated by tight junctions to compose a semipermeable lining between commensal and pathogenic bacteria and the lamina propria [35, 36]. Goblet cells of the epithelium secrete heavily glycosylated mucins, which form the gelatinous layer of mucus that covers the

luminal surface of the gut. In the murine colon, the mucus consists of an outer diffuse layer and an inner layer that is tightly associated with the epithelial cells. A large majority of the commensal microbes are 'entrapped' in the outer layer; thus, the inner layer is virtually devoid of bacteria [34, 37]. The crucial role of the mucus layer in microbiota sequestration was shown using mice that lack the major intestinal secretory mucin and primary component of the mucus layer, MUC2. *Muc2*^{-/-} mice lack a mucus layer, allowing direct contact of commensal microbes with intestinal epithelial cells, which resulted in spontaneous colitis and colorectal cancer [34, 38, 39] (Figure 3).

Besides providing a physical barrier, epithelial cells sense the presence of pathogenic and commensal microbes through a variety of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and Nod-like receptors (NLRs) [40, 41]. Engagement of TLRs on epithelial cells by microbial associated molecular patterns on commensal bacteria provide the homeostatic signals required for the maintenance of the physical epithelial barrier and for the release of small antimicrobial peptides, including defensins, cathelicidins, angiogenins, secretory leukocyte protease inhibitor (SLPI), and the elastase-specific inhibitor, elafin [42, 43]. In addition to these antimicrobial proteins, epithelial cells also release retinoic acid (RA), a breakdown product of β -carotene, interleukin (IL)-33, IL-25, and other cytokines to modulate the function of different immune cells, including dendritic cells (DCs), and T and B cells [44]. In return, these immune cells provide signals to epithelial stem cells located at the base of the intestinal crypts needed for their proliferation and differentiation [45]. Epithelial cells and leukocytes are interdependent on each other, and deficiency in one cell type impacts the overall homeostatic balance and often results in a pathologic condition.

The mechanism(s) by which anthrax infection is initiated within the GI tract is not well understood. During initiation of GI anthrax infection, spores are ingested and germinate on or within the epithelium of the GI tract [17] in order to gain access to the draining lymphatics, including the Peyer's patches [13]. However, a mechanism of passage through the GI epithelia is presumably required, whether in the form of the primary lesion [13] or other means to bypass this barrier [46]. Cholesterol-dependent cytolysins (CDCs) [47] are a family of pore-forming toxins made by many infectious organisms, including the genera *Archanobacterium*, *Bacillus*, *Clostridium*, *Listeria*, and *Streptococcus* [48]. Recently, Bishop *et al.* evaluated the ability of the anthrax specific CDC, anthrolysin O (ALO), to disrupt GI epithelial barrier function by investigating the integrity of human Caco-2 brush-border expressor (C2BBE) cells, which form polarized monolayers with tight junctions and apical brush borders. Based on reduced transepithelial electrical resistance (TEER) and increased leakage of fluorescent dye [46], the barrier function was deemed to be compromised, likely due to tight junction dysfunction involving the tight junction protein occludin (Figure 3). The authors observed significant passage of vegetative anthrax bacteria across the C2BBE cells, which required ALO since ALO-deficient *B. anthracis* strains failed to induce monolayer dysfunction or to allow the passage of anthrax bacteria. Together these findings suggest a pivotal role for ALO in the establishment of GI anthrax infection and in the initial breach of the epithelial barrier [46].

Role of the gut microbiota in GI anthrax infection

The lower GI tract of healthy adults contains approximately 10^{14} symbiotic microorganisms composed of approximately 1000 species termed the gut “microbiota” [49, 50]. There is emerging evidence suggesting an important role for intestinal commensal microorganisms in the protection of the host mucosae against pathogen invasion. Commensal microbes maintain intestinal epithelial barrier function and inhibit pathogen attachment by stimulating epithelial cell production of mucus and antimicrobial peptides [51]. Commensal microbes also promote gut microbial diversity via competition for nutrient-rich niches, and modulate the innate and adaptive immune responses by influencing the development, differentiation, and effector function of different immune cell subsets [52]. In fact, normal development of regulatory cells and epithelial cell integrity are abolished in the absence of an intestinal flora, suggesting the need for certain microbial components to induce beneficial anti-inflammatory mechanisms [53].

The pathogenesis of GI anthrax was recently investigated in A/J mice, which are defective in complement factor 5 (C5), orally infected with toxigenic non-encapsulated *B. anthracis* Sterne strain (pXO1⁺ pXO2⁻) spores. In these studies, GI anthrax infection resulted in microbial dysbiosis with a significant decrease in species richness and changes in dominant phyla [54]. A significant decrease was noted in the relative abundance of *Bifidobacterium* with infection, while the relative abundance of Enterobacteriaceae was also decreased [54]. This was considered important as various strains of *Bifidobacterium bifidum* species have been reported to exert health benefits to the host, including antibacterial activities against pathogens (e.g. *Helicobacter pylori*), reduction of apoptosis of the intestinal epithelia of infants with necrotizing enterocolitis, modulation of host immune responses, and alleviation of certain chronic colonic dysfunctions [55] (Figure 2).

Intestinal epithelial integrity was disrupted with *B. anthracis* Sterne infection, as evidenced by decreased TEER in excised colonic tissue; therefore, the systemic presence of Enterobacteriaceae was examined. Indeed, members of this bacterial family were detected in the spleen, liver, and mesenteric lymph nodes (MLNs) of infected mice [54]. However, *Bifidobacterium* was not detected in these tissues, suggesting that its reduction in the colon may represent an actual depletion of this symbiotic bacterium, as it is potentially outcompeted for survival in the altered intestinal microenvironment resulting from infection. *B. anthracis* Sterne induced significant breakdown of intestinal barrier function and led to gut dysbiosis, resulting in systemic dissemination of not only *B. anthracis*, but also of commensals [54]. GI *B. anthracis* spore infection resulted in swift morbidity and mortality and was associated with pathogen dissemination throughout most visceral organs by induction of leakage in the intestinal barrier and significant changes in the gut’s microbial composition, all of which could orchestrate dysfunctional homeostasis and immune responses [54]. Thus, GI *B. anthracis* infection disturbs the fine-tuned balance of the composition of the gut microbiota, which in turn, likely induces uncontrolled pathogenic autoinflammation resulting in potential morbidity and mortality (Figure 4).

Innate lymphoid cells and intestinal infection

B lymphocytes are the only cell type in the host capable of producing antibodies [56]. In a healthy adult, 75% of the total immunoglobulin production (approximately 3–5 grams of IgA) is secreted at the mucosal linings [57, 58]. Naïve B cells are capable of producing IgM and IgD; however, with either T cell help or with the aid of certain soluble factors, they class switch to produce other antibody classes or isotypes. To maintain the composition of the microbiota and to combat pathogenic infiltration, two different types of B cells are located within the GI tract. An innate-like B cell population, known as B-1 cells, reside within the lamina propria of the intestine, are inefficient in generating high affinity antibodies, and produce germline encoded antibodies, especially IgM. Antibodies generated by these cells are evolutionarily conserved and are directed against antigenic components of commensal microbes and/or common pathogens that humans have encountered throughout evolution; therefore, unlike conventional B cells (B-2 cells), they act as a first line of defense [59].

B-1 cells are the major B cell subpopulation in the peritoneal and pleural cavities, and they continuously traffic to and from these body cavities through the omentum in a manner that requires CXC-chemokine ligand 13 (CXCL13), which is likely produced by resident macrophages [60]. B-1 cells express CD11b on their surfaces, a phagocytic receptor of myeloid lineage, which allows these cells to capture bacteria and act as an efficient antigen presenting cell [61]. Recognition, protection, and clearance of intestinal bacterial infection are critical functional properties of innate immunity, including B cells. Upon infection, high levels of various antibodies (e.g. IgA) can be produced in a T cell-independent manner by B cells, particularly by the B-1 sub-population [62, 63]. There are two different subsets of B-1 cells, B-1a and B-1b cells, depending upon the surface expression of CD5; B-1a cells are sIgM⁺ CD11b⁺ CD5⁺, whereas B-1b cells are sIgM⁺ CD11b⁺ CD5⁻ [61].

Innate lymphoid cells (ILC) are a recently discovered type of leukocyte that make up a small fraction of the total immune cell population in lymphoid organs, at epithelial barrier surfaces in the airways and in the GI tract, and in other tissues [64]. Their differentiation from a common precursor into the different ILC groupings is controlled by a combination of signature transcription factors [65]. ILCs lack productive antigen-specific receptor rearrangement, but they manifest remarkably similar transcription factor profiles and cytokine-producing capabilities to CD4⁺ T cells, indicating that ILCs may act as an innate equivalent to the CD4⁺ T helper cell arm of the adaptive immune system [66]. ILCs are classified into three major groups based on their cytokine secretion. Type 1 ILCs express the transcription factor T-bet and interferon γ (IFN γ) [67]. Type 2 ILC (ILC2) express the GATA-3 transcription factor, along with Kit, Sca1, and receptors that respond to IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) produced by other cells [68, 69]. Upon activation, these cells produce IL-5 and IL-13, which support the expansion of B-1 cells, especially CD5 expressing B-1a cells [70]. The cytokine profile of type 3 ILCs resembles that of Th17 cells, which express RAR-related orphan receptor γ t (ROR γ t) and the aryl hydrocarbon receptor (AhR) as their signature transcription factors, and are capable of secreting IL-22 and IL-17 [71].

Impaired colonic B cell responses with GI anthrax infection

An increase in the B-1a cell population was recently demonstrated in the lamina propria of A/J mice infected orally with *B. anthracis* Sterne spores within one day post-inoculation [72]. It was found that toxins secreted by *B. anthracis* impaired IgA and IgM secretion and surface receptor expression on B-1 cells. Interestingly, ILC2 that support the local expansion of B-1 cells [68] were reduced to 1/10 of that of uninfected mice (Figure 2). This reduction of ILC2 in the lamina propria three days post-infection was associated with reduced MAPK activity, which is consistent with MAPK being the central signaling molecule for ILC2 survival and function. The increase in B-1a cells in the GI anthrax infected gut was not due to increased proliferation, consistent with the observed decrease in ILC2, which aid in B-1a proliferation [72], suggesting an influx of B-1a cells into the lamina propria. Transcription of CXCL13, a critical chemokine involved in B-1a cell recruitment [73], increased more than ten-fold compared to the uninfected controls. These data suggest that GI anthrax infection results in increased CXCL13, which can recruit B-1a cells into the gut via its receptor, CXCR5 [72]. An increase in the transcription of potent chemoattractants for myeloid cells [74], CCL3, CXCL12, and CXCL14, was also seen with infection. A small increase in the transcription of CXCL10, which is implicated in T cell, natural killer (NK) cell, and monocyte recruitment, was also found.

Consistent with the acute nature of GI anthrax, increased germinal center (GC) B cell formation was not observed in infected mice; however, in mice that survived two-weeks postinfection, GC B cells accumulated within the Peyer's patches, and expression of IgA and IgM was not compromised [72]. A significant induction of B cell activation, as measured by downregulation of surface IgD, was also observed in the Peyer's patches of these survivor mice. Sera from mice five days post-infection failed to neutralize LT, despite the availability of circulating antigen for the potential induction of antigen-specific responses [72]. However, sera from surviving mice two weeks post-infection prevented cell death in the macrophage cell line, J774A.1 in a toxin neutralization assay. These findings demonstrate that survival from GI anthrax infection requires neutralizing antibodies by expansion of GC B cells in the Peyer's patches, and uncompromised function of B-1 cells and ILC2 in the gut [72], suggesting a functional correlation of B-1a cells and ILC2 that must be fine-tuned in order to resist anthrax challenge. Clearly, further studies are warranted to elucidate the role of other subtypes of ILC and their impact not only on B lymphocytes, but also on innate cells (e.g. DCs, macrophages) and T cell activation in GI anthrax infection.

Concluding remarks and future perspectives

Anthrax remains a major problem for both animals and human beings in developing countries [75]. Recent observations in animal models of inhalational anthrax have determined that the bacterial dissemination pattern during inhalational infection may be more similar to the GI form than previously thought [9]. GI anthrax has nearly the same mortality rate as inhalational anthrax, and can be fatal even with antibiotic therapy, especially in children. Importantly, prevalence of the GI form may be underreported because

of rapid disease progression and the difficulty in diagnosing this disease in rural areas and developing countries.

Gaps in our knowledge of GI anthrax pathogenesis can now be examined with modern systems approaches. Concomitantly, descriptive sequence-based studies are serving an important complementary role in defining priorities for pursuing mechanism-level understanding of this exceptionally complex interplay between resident microbial species that inhabit the GI tract and the resulting interactions that occur between these species and the host [76]. Moving forward, appropriate murine models such as A/J mice should prove useful in addressing questions regarding *B. anthracis* pathogenesis since mice offer a wide variety of immunologic and genetic tools that allow the determination of specific host factors involved in host–pathogen interactions [13]. It will be critical to identify mechanisms of bacterial dissemination of GI *B. anthracis* in animal models and to thoroughly study roles of the gut microbial composition and their bacterial products, intestinal epithelia, and mucosal immunity to advance development of potential treatments for this insidious and deadly disease, as well as to elucidate additional avenues (metabolomics, metagenomics) by which to control infection.

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Glossary

Anthrax toxin receptor 1 (ANTXR1)	a type I transmembrane protein that binds to PA. This protein is also called also known as tumor endothelial marker-8 (TEM8) because it is a tumor-specific endothelial marker that appears in be involved in colorectal cancer
Anthrax toxin receptor 2 (ANTXR2)	a type I transmembrane protein that binds to PA, also known as capillary morphogenesis gene 2 (CMG2)
B-1 cells	B-1 cells are considered innate immune cells that produce the majority of IgM and IgA, which are largely encoded by germline immunoglobulin genes. B-1 cells predominate during fetal and neonatal development, self-renew, and localize mostly to the peritoneal and pleural cavities. Subsets of B-1 cells can be delineated by differential expression of CD5
B-2 cells	Considered “conventional B cells,” B-2 cells are continually generated from bone marrow precursors, and circulate throughout the blood and secondary lymphoid tissues. B-2 cells can undergo class switching, somatic hypermutation, and generate memory cells

Cholesterol-dependent cytolysins (CDCs)	CDCs are a family of β -barrel pore-forming exotoxins that are secreted by Gram-positive bacteria. The presence of cholesterol in the target membrane is required for pore formation, but is not required by all CDCs for binding
Dysbiosis	a breakdown in the balance between protective intestinal bacteria versus harmful intestinal bacteria
Dysphagia	difficulty in swallowing
Edema factor (EF)	part of the three protein anthrax exotoxin that is a calmodulin-dependent adenylate cyclase that inhibits the immune response
Edema toxin (ET)	refers to the protein complex of edema factor (EF) bound to protective antigen (PA)
Endospores	dormant, hardy, non-reproductive structures produced by certain bacteria, especially Gram-positive bacteria
Eschar	piece of necrotic tissue that is sloughed from the surface of the skin; is characteristically thick, dry, and black dead tissue seen with cutaneous anthrax infection
Germinal center (GC) B cells	within germinal centers (GC) of lymphoid tissue, B cells proliferate, undergo affinity maturation of their B cell receptor genes, class switch for antibody production, and differentiate into longer-lived plasma cells or memory B cells
Hematemesis	vomiting of blood
Innate lymphoid cells (ILC)	innate lymphoid cells are a group of immune cells that belong to the lymphoid lineage but do not respond in an antigen specific manner. This relatively newly described group of cells has different subtypes with different physiological functions, some of them analogous to helper T cells, while also including cytotoxic natural killer (NK) cells
Lethal factor (LF)	lethal factor, part of the three protein anthrax exotoxin that inactivates neutrophils so they cannot phagocytose bacteria
Lethal toxin (LT)	refers to the protein complex of lethal factor (LF) bound to protective antigen (PA)
Melena	black, "tarry" stool that is associated with upper gastrointestinal bleeding
Meningoencephalitis	inflammation of the brain and the meninges, the membranes that surround the brain and spinal cord

Microbiota	the entire resident microbe population of a certain organ or organ system; often refers to the flora of the gastrointestinal tract.
Nod-like receptors (NLRs)	Nod-like receptors (nucleotide-binding oligomerization domain receptors) are pattern recognition receptors (PRRs) and play key roles in regulation of the innate immune response
<i>Nlrp1b</i>	gene encoding NACHT, LRR and PYD domains-containing protein 1, which is a component of the inflammasome pathway
Occludins	integral plasma-membrane proteins that together with the claudin group of proteins are the main component of tight junctions
pXO1	plasmid in <i>Bacillus anthracis</i> which controls the production of edema and lethal toxins, which are made of three proteins, EF, PA, and LF
pXO2	plasmid in <i>Bacillus anthracis</i> which codes for the capsule, a layer of polysaccharides outside of the cell wall that protects the bacteria against phagocytosis
Protective antigen (PA)	protective antigen is part of the three protein anthrax exotoxin that is a cell binding protein that binds to two surface receptors on the host cell
Peyer's patches	aggregated nodules of lymphatic tissue that play a central role in intestinal immunosurveillance. They are similar to lymph nodes in structure, except that they are not surrounded by a connective tissue capsule
Transepithelial electrical resistance (TEER)	transepithelial electrical resistance, an <i>in vitro</i> method for evaluating the permeability of epithelial cells by the measuring the electrical physical resistance
Toll-like receptors (TLRs)	Toll-like receptors play a critical role in the early innate immune response to invading pathogens by recognizing highly conserved structural motifs known as pathogen-associated microbial patterns (PAMPs), which are exclusively expressed by microbial pathogens
Transcytosis	the transport of macromolecules or bacteria from one side of a cell to the other side via the interior of the cell
Zoonosis	disease that can be passed from animals to humans or vice versa

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Highlights

- We briefly review all forms of anthrax infection
- We discuss molecular strategies of *B. anthracis* to initiate and sustain infection
- We focus on the current understanding of gastrointestinal (GI) anthrax infection
- We examine innate lymphocytes, the epithelia, and the microbiota with GI anthrax

Box 1**Anthrax toxins**

The tri-partite exotoxin of *B. anthracis* comprises a host cell receptor binding protein called protective antigen (PA) and two enzymatic proteins, lethal factor (LF) and edema factor (EF). LF and EF have no known activity on the host until they bind and are subsequently translocated into the target cell by PA, named for its efficacy in inducing protective immunity against anthrax. Intoxication of host cells begins when PA binds to either tumor endothelial marker-8 (TEM8; also known as anthrax toxin receptor 1 (ANTXR1)) [77] or capillary morphogenesis gene 2 (CMG2; also known as anthrax toxin receptor 2 (ANTXR2)) [78]. PA can also bind to $\beta 1$ integrins, which enhance uptake of PA by macrophages [79]. TEM8 and CMG2 are highly expressed within the vasculature, but studies have also described their expression on epithelial cells, particularly respiratory and intestinal epithelia, keratinocytes, and immune cells [14], as they bind collagen $\alpha 3$ (VI) and collagen IV/laminin, respectively [80–82].

Initially, PA is 83 kDa, however after binding to its cell surface receptor(s) it is cleaved by furin-like proteases to generate 63 kDa (PA63) and 20 kDa (PA20) fragments. PA63 then oligomerizes, enabling EF and/or LF to bind and be internalized into the target cell [82] (Figure 1). Once LF and EF are bound to PA, they are referred to as lethal toxin (LT) or edema toxin (ET) [14]. While the anti-phagocytic capsule is an essential virulence factor for the establishment of disease, the symptoms and lethality associated with anthrax are the result of LT and ET production following septicemia.

Anthrax toxins act during two distinct phases of infection. During the prodromal phase, which is often asymptomatic, the toxins act on immune cells to assist in establishment of infection. ET causes a steady elevation in cyclic adenosine monophosphate (cAMP), an important secondary messenger that is normally strictly controlled in mammalian cells, whereas LT cleaves most isoforms of mitogen-activated protein kinase kinases (MAPKKs) [83–85]. By subverting essential molecules common to the main signaling networks that control immune cell activation, effector function and migration, anthrax toxins systematically dismantle both the innate and the adaptive immune defenses of the host [84] (Figure 1). Lymphocytes and macrophages are some of the few cell types that can significantly be affected by exposure to LF, whereas EF is normally not cytotoxic to immune cells and appears to function primarily by altering their activation, migration, and/or production of cytokines [30, 84, 86].

Box 2**Antigen presentation and disease dissemination****The “Trojan horse model.”**

During inhalational anthrax infection, alveolar macrophages are reported to transport spores out of the lungs to regional lymph nodes (LNs); acting as a “Trojan horse” to carry *B. anthracis* into circulation [87]. Dendritic cells (DCs) have also been implicated in the swift transport of spores to the draining LNs [88–90]. Spores and virulence factors of *B. anthracis* affect murine bone marrow-derived DCs [91], mouse spleen-derived DCs [92], and human monocyte-derived DCs *in vitro* [93, 94], but currently, no data exist concerning the role of DCs in animal GI challenge experiments.

The “jailbreak model of dissemination.”

This model suggests that instead of spores requiring an intracellular mode of travel via antigen presenting cells in the LNs, spore germination and exotoxin release induce damage to epithelial barriers leading to dissemination from the LNs to the periphery [9]. Weiner and Glomski propose that spores enter the lumina of the airways, GI tract, or skin and germinate to become exotoxin-producing encapsulated vegetative bacteria at the site of initial entry into the host. Ongoing vegetative growth increases exotoxin and protease concentrations, leading to the breakdown of endothelial/epithelial barriers. Vegetative bacteria pass through the damaged barriers and traffic to regional draining LNs by bulk lymphatic flow without phagocytic transport. Bacteria in the LNs continue to produce virulence factors and replicate, eventually causing the loss of LN integrity. Bacteria then escape the LNs and enter the bloodstream, resulting in bacteremia and host death [9] (Figure 2).

This theory is supported by experiments showing dissemination from the lungs occurred in both BALB/c and SJL/J strains of mice depleted of macrophages via liposome encapsulated clodronate [95]. In fact, these mice succumbed significantly sooner to aerosol challenge with fully virulent, ungerminated *B. anthracis* Ames spores and had higher bacterial loads in the lungs than control mice treated with saline [95]. Conversely, mice made neutropenic by the administration of the chemotherapeutic agent, cyclophosphamide, or the rat anti-mouse granulocyte monoclonal antibody, RB6-8C5, had similar bacterial loads to control mice in low-dose aerosol challenge. Thus, the authors concluded that in their model, neutrophils played a minor role in the early host response, whereas macrophages played a more dominant role in early host defenses against infection by *B. anthracis* spores [95], and that there is a macrophage-independent route of spore germination and dissemination [32].

Figure 1. Pathogenesis of anthrax

A. Two virulent plasmids of *B. anthracis*, pXO1 and pXO2, encode edema factor (EF), lethal factor (LF), protective antigen (PA) and the anti-phagocytic capsule, respectively. **B.** PA attaches to the cell membrane via the interaction between PA83 (composed of PA63 and PA20) and ANTXR. **C.** PA83 is cleaved by membrane endoproteases from the furin family to form PA20 and PA63. **D.** Formation of the PA63 heptamer facilitates the translocation of EF and LF into target cells. EF promotes the production of cAMP, and LF cleaves MAPKKs to suppress signaling pathways.

Abbreviations: ANT XR, anthrax toxin receptors. cAMP, cyclic adenosine monophosphate. MAPKK, mitogen-activated protein kinase kinase.

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Figure 2. The jailbreak model of dissemination initiated within the gut

A. Homeostasis of the gut microbiota and physiological activation of the immune system within the gut. Red and green lines and blue ovals represent colonized commensal bacteria within the gut. **B.** Gastrointestinal infection with *B. anthracis* results in microbiota dysbiosis. Translocation of *B. anthracis* and commensal bacteria (e.g. *Enterobacteriaceae*) results in local inflammation in terms of suppression of dendritic cells (DCs) and T cells. A drastic reduction of cytokines and antibody production is accompanied by dysfunction of type 2 innate lymphoid cells (ILC2) and B cells. Filled downward arrows indicate decreased

quantities. Chains of black dashed lines indicate vegetative forms of *B. anthracis*. Red dashed lines around epithelial cells indicate damage or degradation.

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Figure 3. Damage of epithelial barrier upon *B. anthracis* infection

A. Interactions between commensal bacteria and epithelial cells maintain gut homeostasis. The inner mucus layer, composed of highly glycosylated mucin secreted by goblet cells, physically separates the majority of commensal bacteria from interacting with epithelial cells. Pathogen pattern receptors (PRRs), including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), recognize evolutionarily conserved motifs from microorganisms and endogenous danger signals, respectively, in the gut lumen and secrete retinoic acid and cytokines to activate the immune system during homeostasis. Epithelial stem cells at the base of the crypts maintain proliferation and differentiation in the presence of signals from immune cells to perpetuate the epithelia. Tight junction proteins, including those of the zona occludin family, claudin family and occludins, mediate cell-cell adhesion and act as barriers against the translocation of commensal bacteria from the gut lumen to the lamina propria. **B.** Decreased expression of tight junction proteins upon *B. anthracis* infection contributes to an increase in gut permeability and dissemination of bacteria into lamina propria. Vegetative forms of *B. anthracis* invade the gut lamina propria and suppress local immune responses. Chains of black dashed lines indicate vegetative forms of *B. anthracis*. Black dashed/dotted lines in middle panel indicate downregulated or degraded tight junction proteins.

Figure 4. Systemic immune suppression by GI infection with *B. anthracis*

GI infection with *B. anthracis* initiates immune responses at the gut lumen where germination and exotoxin release occur. Disruption of gut barrier function promotes the dissemination of bacteria into peripheral lymph nodes. Immune cells are suppressed locally, both in the gut and in the mesenteric lymph nodes. Invasion of *B. anthracis* pathogens into multiple organs (liver, lungs, kidneys) via blood circulation results in systemic suppression of immunity.