Published in final edited form as:

Microb Pathog. 2008 April ; 44(4): 293–310.

Transcriptional Profiling of Murine Organ Genes in Response to Infection with *Bacillus anthracis* Ames Spores

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Abstract

Bacillus anthracis is the gram positive, spore-forming etiological agent of anthrax, an affliction studied because of its importance as a potential bioweapon. Although *in vitro* transcriptional responses of macrophages to either spore or anthrax toxins have been previously reported, little is known regarding the impact of infection on gene expression in host tissues. We infected Swiss-Webster mice intranasally with 5 LD₅₀ of *B. anthracis* virulent Ames spores and observed the global transcriptional profiles of various tissues over a 48 hr time period. RNA was extracted from spleen, lung, and heart tissues of infected and control mice and examined by Affymetrix GeneChip analysis. Approximately 580 host genes were significantly over or under expressed among the lung, spleen, and heart tissues at 8 hr and 48 hr time points. Expression of genes encoding for surfactant and major histocompatibility complex (MHC) presentation was diminished during the early phase of infection in lungs. By 48 hr, a significant number of genes were modulated in the heart, including up-regulation of calcium-binding related gene expression, and down-regulation of multiple genes related to cell adhesion, formation of the extracellular matrix, and the cell cytoskeleton. Interestingly, the spleen 8 hr post-infection showed striking increases in the expression of genes that encode hydrolytic enzymes, and these levels remained elevated throughout infection. Further, genes involving antigen presentation and interferon responses were down-regulated in the spleen at 8 hr. In late stages of infection, splenic genes related to the inflammatory response were up-regulated. This study is the first to describe the *in vivo* global transcriptional response of multiple organs during inhalational anthrax. Although numerous genes related to the host immunological response and certain protection mechanisms were up-regulated in these organs, a vast list of genes important for fully developing and maintaining this response were decreased. Additionally, the lung, spleen, and heart showed differential responses to the infection, further validating the demand for a better understanding of anthrax pathogenesis in order to design therapies against novel targets.

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Keywords

Bacillus anthracis; anthrax; Affymetrix microarrays; murine model of infection

1. INTRODUCTION

Bacillus anthracis is a gram-positive, spore-forming bacterium of special interest to the biodefense community. The bacterium possesses two plasmids that are largely responsible for its virulence; the pXO1 plasmid harbors genes that code for anthrax toxins, and the plasmid pXO2 encodes genes for biosynthesis of a unique antiphagocytic capsule [1]. Both pXO1 and pXO2 plasmids have been studied to evaluate their potential role during inhalational anthrax using mouse, rabbit, and guinea pig models [2–5]. The plasmid pXO1 encodes three components of the protein anthrax toxins: lethal factor, edema factor, and protective antigen. Protective antigen (PA) binds either capillary morphogenesis gene 2 (CMG2) or tumor endothelial marker 8 (TEM8) on the surface of target cells, resulting in a conformational change of PA that enables the binding of either lethal factor (LF) or edema factor (EF) to PA and internalization of the AB-type toxin through a heptameric channel comprised of PA molecules [6]. The capsule, on the other hand, consists of poly-D-glutamic-acid [7], which has a negative net charge that resists phagocytosis by macrophages and dendritic cells [8]. Both toxins have been shown to have adverse effects on their target cells, while the capsule defends against bacterial phagocytosis and the subsequent display of *B. anthracis* immunopeptidome in a thymus-dependent manner [9].

Following the intentional release of *B. anthracis* in the United States in 2001 [10], significant anthrax-related research ensued and substantial progress was made regarding the understanding of how this organism elicits disease. Inhaled *B. anthracis* spores have been shown to reach as deep as the alveoli, where they are rapidly engulfed by alveolar macrophages and dendritic cells sampling the lung microenvironment [11]. A significant portion of the spores germinate into metabolically active, vegetative cells within the host phagocytes and begin multiplying. Transport to the mediastinal lymph nodes soon occurs, whereby the bacteria lyse the macrophage *via* an unknown mechanism. The organisms are then free to spread through the lymphatic and circulatory systems of the host. As the bacteria disseminate to the bloodstream, the two anthrax toxins (LeTx [LF+PA] and EdTx [EF+PA]) are secreted, causing massive edema and widespread hemorrhage. LeTx and EdTx have each been shown to possess unique strategies to disable the host immune response. LeTx cleaves the N-terminal region of mitogen-activated protein kinase kinases (MAPKKs), resulting in the disruption of a myriad of downstream signaling pathways [12]. This toxin also causes cytolysis of numerous cell types, including human and murine macrophages and endothelial cells [13,14]. Alternatively, EdTx is an adenylyl cyclase toxin capable of increasing cAMP levels within a vast array of host cell types [15]. More recently, it was reported that EdTx inhibits important functions of various immune cells [16–20]. For instance, EdTx impairs the phagocytic activity of human neutrophils [21], perturbs macrophage cytokine responses [20], and decreases macrophage and T-cell chemotaxis [22].

There is no data to-date, however, regarding the overall transcriptional responses of genes in different organ systems of animals after *B. anthracis* infection. As a result, it is still unclear how animals and humans succumb to infection [3]. Furthermore, few studies have utilized the Ames strain, a virulent form similar to what was used in the August 2001 attacks in the U.S., for studying *B. anthracis* and host interactions. Finally, the precise cause of lethality incurred by anthrax has yet to be described. We therefore chose to examine the specific effects of Ames spores on the transcriptional profiles of multiple organs in mice exposed by respiratory challenge to mimic inhalational anthrax.

For our animal model, we chose the Swiss-Webster mouse for several reasons. Outbred strains, such as this one, have advantages over inbred mice due to the heterozygosity of immune alleles [23]. Thus, the high genetic variation of Swiss-Webster mice mirrors that of the diverse human population [24]. Additionally, inbred mice possess many homozygous recessive alleles that may lead to detrimental phenotypes [23]. We utilized nasal instillation, as it is a commonly practiced method of simulating an aerosol challenge, and consequently, the inhalational route of infection. Tissue samples were cultured to detect bacteremia during the course of infection. To analyze the systematic immune effects of anthrax on the host, we utilized a 23-plex cytokine array profiling assay. Finally, in order to assess the effects of infection on mouse major organs, we examined tissue sections of the heart, spleen, and lungs for injury and also employed Affymetrix GeneChips to evaluate global transcriptional regulation of genes in these tissues.

The rationale for our choice of tissues was based on prior observations. The lung was chosen because of its role as the initial focus of infection during inhalational anthrax. Infection of the spleen is significant, because lymphoid homing receptors have been shown to be up-regulated on infected antigen-presenting cells [25]. Studying the transcriptional response of the spleen is also important, because understanding the effect of infection on mixed lymphocytes is essential for the creation of therapeutic regimens, such as effective vaccine strategies. Finally, the paucity of empirical indicators of murine anthrax infection observed in our laboratory and the previously reported negative effects of lethal toxin on hemodynamics in a lethal toxin susceptible rat model [26–28], warranted investigation on the transcriptional response of the heart.

2. RESULTS

2.1. Monitoring *B. anthracis* levels in the lungs, heart, and spleen during infection

We first evaluated the number of anthrax bacteria within each mouse organ of interest at various times during infection. The animals were infected by the intranasal route with a 5 LD₅₀ dose of Ames spores (5.6×10^4 cfu), and the lungs, heart, and spleen were extracted 8, 24, and 48 hr later. The organs of three animals were pooled at each time point, homogenized, serially diluted, and then plated onto blood agar plates. Results are expressed in Figure 1 as the number of anthrax organisms (cfu) calculated per each whole organ in 3 independent experiments. At 8 hr post-infection, only the lungs contained *B. anthracis* organisms (approximately 1.8×10^4 cfu). By 24 hr, both the heart and lung had anthrax bacteria (3×10^4 and 1×10^2 cfu, respectively). Forty-eight hours into infection, however, all organs showed dramatic increases in *B. anthracis* levels. For instance, over 7×10^5 cfu were recovered from the heart, and 1.3×10^6 cfu were detected in the spleen at 48 hr. After confirming the presence of *B. anthracis* in the organs of interest, we evaluated the pathological consequences of infection in these tissues.

2.2. Histopathological analysis of *B. anthracis*-infected tissues

The lungs, heart, and spleen were removed from both uninfected mice and those infected with Ames spores for 48 hr, and processed for H&E and Gram staining. Figure 2 illustrates the presence of *B. anthracis* in various organs of infected animals. In the lungs, bacilli were present within alveoli, alveolar walls, and small and large vascular lumens. Focally, small areas of perivascular/peribronchiolar edema were observed with numerous bacilli. Hemorrhage was also detected in some alveoli (Panel A). Although occasional bacilli were present within the myocardium, tissue damage to the heart was not observed (Panel B). On the other hand, the spleen displayed large numbers of bacilli accompanied by hemorrhage, congestion, fibrin, and edema. Approximately 75% of the red pulp architecture was effaced due to lymphoid depletion and to a lesser extent, lymphocytolysis. White pulp areas were reduced in size with cell loss, lymphocytolysis, and fibrin deposition mainly at the periphery (Panel C). Overall, these results

demonstrated *B. anthracis* bacilli infiltrating multiple tissue types and eliciting profound pathological consequences in the lungs and spleen.

2.3. *B. anthracis* infection elicits a biphasic pattern of cytokine secretion in the serum of mice

To further characterize the host response to *B. anthracis* infection, the systemic cytokine response of infected mice was evaluated. Blood samples were collected at 0, 8, 24, 36, and 48 hr post-infection, and the cytokine levels in sera were measured using a multiplex assay system. Pooled sera from three animals at each time point were assayed in duplicate.

Figure 3 shows that a biphasic response pattern was observed for IL-4, IL-6, G-CSF, KC, RANTES, and MCP-1 in sera of the *B. anthracis*-infected mice. In these terminally ill animals, anthrax infection induced an initial spike (8 hr), followed by a decline and then a second spike at 48 hr post-infection. IFN- γ showed a biphasic response, though not reaching statistical significance, with a second increase at 36 hr instead of 48 hr as displayed by the other cytokines which showed biphasic response. IL-12 (p40) levels were the highest among the bacteria-induced cytokines at all time points except 36 hr, with levels peaking at 275 pg/ml at 48 hr. TNF- α remained only slightly elevated except for a large increase to 450 pg/ml at 36 hr, making it the cytokine with the greatest concentration at this point. G-CSF showed a significant increase at 8 hr compared to basal levels, while KC significantly increased at 48 hr. No appreciable induction was noticed for IL-2, IL-3, and IL-1 β (data not shown).

2.4. Inhalational anthrax alters the expression of numerous murine genes in the lung, heart, and spleen at both early and late time points

After demonstrating anthrax bacilli infiltration and examining pathological consequences of infection in the lungs, heart, and spleen of infected mice, the host transcriptional response within these organs was investigated. RNA was isolated from the three organs at 8 or 48 hr following intranasal inoculation and subjected to Affymetrix GeneChip analysis. We performed three independent experiments with 3 animals per experiment and per time point. Further, for experiment, tissues from three animals were pooled.

Alterations in gene expression profiles found to be significant were compiled into lists by function and are summarized in Table 1. The complete list of altered genes is provided in the supplemental data (Tables I–VI). Specific examples of altered gene expression at 8 and 48 hr in the lungs, heart, and spleen are shown in Tables 2–7. The lungs and heart showed relatively few changes in gene expression at the early time point of 8 hr (15 and 11 altered genes, respectively), with example genes also listed in Tables 2 and 4. However, 48 hr into the course of infection, the transcriptional response of genes in these two organs increased (Tables 1, 3, and 5). In fact, the heart showed the greatest transcriptional response of genes of all organs at both 8 hr and 48 hr, with 298 genes either up- or down-regulated at the 48 hr time point (a list of selected genes are shown in Table 5). The spleen showed significant alterations in gene expression at both time points of 8 and 48 hr (Table 1), with a selected list of genes shown in Tables 6 and 7. The most notable was a dramatic increase in the expression of genes that code for pancreas-associated enzymes.

Although the list of altered genes for the lung at 8 hr post-infection was not large, several genes related to inflammation were up-regulated (i.e., clusterin and oxidized low density lipoprotein; Table 2). Examples of genes that were down-regulated included MHC class I receptor (H2-K1), surfactant associated protein C (Sftpc), and several genes encoding proteins related to cell proliferation. By 48 hr, the lung displayed more dramatic signs of response to infection, including up-regulation of genes involved in hypoxia or inflammation (Table 3). Down-regulated genes included those involved in cell growth, differentiation, and various other cellular processes.

In the heart at 8 hr, the majority of affected genes were down-regulated (Tables 1 and 4). Genes coding for molecules with vastly different functions were affected, such as those involved in nucleotide metabolism (guanine deaminase) and iron homeostasis (transferrin receptor). The receptor for transferrin remained down-regulated by 48 hr, but transferrin itself, as well as ferritin, became slightly up-regulated at this later time point (Table 5). By 48 hr, the heart responded tremendously with regard to transcriptional changes. Many key structural molecules important for cardiac function, including beta actin, collagen, vimentin, and fibronectin, were down-regulated. Expression of multiple genes related to cell adhesion and growth and development were also significantly decreased (i.e., cadherin 11, nuclear protein 1, and cyclin D2; Table 5).

Finally, splenic genes were also very responsive to infection with *B. anthracis* at both time points (Tables 6 and 7). The most profound effect on the spleen at both 8 and 48 hr post infection was the dramatic increase in expression of genes that coded for pancreatic enzymes such as amylase, elastase 3B, and pancreatic lipase. Interestingly, when serum amylase levels were analyzed (data not shown), no difference was observed in infected versus uninfected mice. Additionally, both time points revealed down-regulation of genes related to the adaptive immune response such as immunoglobulin heavy chains, T-cell receptor gamma (Tcrg-V4), and CD48 antigen (Tables 6 and 7).

2.5. Confirmation of *B. anthracis*-induced gene expression alterations in mice

In order to confirm the GeneChip findings, real-time RT-PCR was performed on eight genes that were chosen based on their involvement in inflammation, signaling, or physiological processes related to each respective organ (Table 8). RT-PCR experiments were performed in parallel, and fold-change values were determined after normalization of each gene to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression of genes encoding clustrin (Clu), angiopoietin-like 4 (Angptl4), and Retnl (expand; full name of this gene!!) was increased in the lung 2.1-, 3.7-, and 3.3-fold, respectively, as determined by GeneChip analysis. Similarly, RT-PCR of lung tissue samples revealed that these genes were up-regulated 3.98-, 2.81-, and 6.35-fold (Table 8). Two of the pancreatic enzyme-encoding genes that were up-regulated in the spleen by GeneChip analysis, amylase 2 (896.5-fold) and elastase 3b (403.9-fold; Table 6), were also shown to be up-regulated to an even greater extent by the RT-PCR data (3847.6- and 599.2-fold, respectively; Table 8). The transcriptional activities of two genes (Ptgds, Tfrc) from the heart at 8 hr were also in agreement with the RT-PCR data.

3. DISCUSSION

The hardiness and relative ease of aerosolization [29,30] of *B. anthracis* spores, together with their ability to cause significant mortality, [31] resulted in the use of this bacterium as a biological weapon in the United States in 2001. As a result of the intentional release of *B. anthracis*, five out of the eleven people infected with inhalational anthrax did not survive, despite antibiotic therapy [32]. This event prompted renewed interest in investigating the pathogenesis of this organism with the ultimate goal of developing novel treatments against infection with aerosolized *B. anthracis* spores.

Past publications have examined the global transcriptional responses of host cells to treatment with the anthrax toxins. For instance, GeneChip analysis of EdTx-treated murine macrophages revealed significant gene alterations that culminated in the modulation of various macrophage functions [20]. Similarly, transcriptional analysis of murine macrophages treated with LeTx [33] or infected with *B. anthracis* Sterne spores (vaccine strain) has been previously reported [34]. Although these studies exposed abundant macrophage genes seemingly important during interaction with spores or toxins, studies to date have neglected to examine the *in vivo* host response to *B. anthracis* spores. Here, we report for the first time, the *in vivo* global

transcriptional responses of genes in the lungs, heart, and spleen of mice intranasally infected with fully virulent *B. anthracis* Ames spores.

The infectious inoculum chosen for these studies was 5 LD₅₀ (56,000) Ames spores in order to ensure significant disease progression and pathological changes within the examined time frame. Preliminary experiments involved isolating RNA from mice infected 8, 24, 36, 48, and 60 hr post-infection and performing GeneChip analysis as described in the Materials and Methods section. Upon examination of the data, however, the time points showing the most striking gene expression changes occurred at earliest time point examined (8 hr) and at 48 hr post-infection. The transcriptional profiles between the 48 and 60 hr infection time points were very similar; thus, 48 hr was chosen as the final late time point for this study.

The lungs were analyzed because they are the primary route of infection for these experiments and in natural cases of inhalational anthrax. As expected, *B. anthracis* organisms were recovered from the lungs at all time points tested (8, 24, and 48 hr). Similar to previously reported findings [35–37], the number of organisms significantly increased over time. This supports the proposed model of anthrax infection, whereby spores engulfed by alveolar macrophages travel to the mediastinal lymph nodes, enter the bloodstream, and then disseminate to multiple tissue sites, including the return to the lungs [38,39]. Histopathological analysis of the lungs at 48 hr post-infection confirmed the presence of bacilli within the alveoli, alveolar walls, and small and large vascular lumens (Figure 2). Focally, small areas of edema and hemorrhage were observed with fibrin. Additionally, some, but not all, infected mice showed mixed cellular infiltrates of neutrophils and mononuclear cells. These findings are consistent with previous microscopic observations in the lungs of mice [40,41] and non-human primates [40] infected with *B. anthracis* Sterne spores.

Eight hours subsequent to intranasal inoculation of Ames spores, few transcriptional alterations were detected in the lungs (Table 2). However, the genes that were up-regulated indicated that the lung recognized the onset of an infection. One of the genes that responded with the greatest fold-change encodes clusterin, a molecule known to be elevated following inflammatory stress and localized lung damage [43]. Clusterin protects the lung against apoptotic signals, reactive oxygen species (ROS), and complement factors [44]. Likewise, lectin-like oxidized LDL receptor (Olr1), which was up-regulated 1.9-fold, is associated with inflammatory and endotoxin-induced stress and functions as a vascular tethering ligand for rolling leukocytes [45,46]. Alternatively, several genes involved in the immune response were down-regulated. The gene encoding MHC class I receptor (H2-K1) was down-regulated (2.5-fold) in the lung at 8 hr (Table 2), which may represent a bacterial mechanism of immune evasion by which the bacteria interfere with antigen presentation to host immune effector cells. Notably, the gene encoding surfactant protein C (Sftpc) was down-regulated (2.0-fold) by 8 hr. Sftpc, which normally functions to regulate lung surface tension and gas exchange, has been shown to be decreased in various animal models of inflammation and in response to TNF- α [47]. Sftpc and other surfactant proteins play a role in the innate host defense system of the lung by binding to pattern recognition molecules, such as CD14 [48]. Thus, lower Sftpc levels during anthrax infection may disrupt certain respiratory processes, as well as dampen innate immune defenses.

By 48 hr, the lung showed increased expression of genes related to inflammation, suggesting a more active participation of the immune response against infection (Table 3). This included CD14 antigen (3.5-fold increase), which is a receptor for the lipopolysaccharide (LPS)-binding protein/LPS complex. CD14 is also a cell-activating receptor for peptidoglycan [49], possibly explaining its alteration during infection with *B. anthracis*. Also, the expressions of the multi-functional metallothionein 2 (Mt2) and polymeric immunoglobulin receptor (pIgR) genes were up-regulated. Besides their ability to bind metal ions, metallothioneins become acute phase proteins during inflammation [50] and possess antioxidant properties that may protect tissues

against destructive inflammatory conditions [51,52]. Further, the Mt2 protein has been shown to be elevated during hyperoxia [53]. pIgR proteins are responsible for mediating the continuous delivery of polymeric immunoglobulins (IgA and IgM) to the mucosal epithelial surface and external secretions. Expression of pIgR is regulated by microbial products through toll-like receptor (TLR) signaling and by host factors, such as cytokines [54]. The elevation of CD14, Mt2, and pIGR gene expression likely assists the lungs in combating the infection and protecting itself from excessive damage resulting from inflammation.

The spleen was the second organ of interest during this study, mainly because it is the largest lymphoid organ in the body and filters the blood. As in other systemic bacterial infections, anthrax bacilli in the bloodstream are trafficked to the spleen and interact with a multitude of mixed lymphocyte populations. *B. anthracis* organisms did not appear in the spleens of Swiss-Webster mice until 48 hr post-infection. Other published data showed bacteria appearing as early as 20 hr post-inoculation by the intra-tracheal route in Balb/c mice [4,38]. This discrepancy is perhaps due to differences in the genetic background of the mice, the infection route, or inevitable variations caused by the complicated nature of the mechanism of dissemination employed by *B. anthracis*. Further, it is possible that organisms were present in the spleen of Swiss-Webster mice at 24 hr, but were not viable. Infected spleens displayed high numbers of bacilli with hemorrhage and edema (Figure 2). Major architectural damage to the red and white pulp regions was also observed. These observations correlated with what has been previously observed by others [37,41,55].

The spleen responded with a greater magnitude of transcriptional changes early in infection compared to the lungs and heart, with 86 genes altered at 8 hr post-infection (Table 1). Genes of multiple functional categories, such as cell growth, differentiation, and protein processing, were altered. However, the most striking effects involved those classified as digestive enzymes (Tables 6 and 7), including amylase 2 (896-fold at 8 hr, 1042-fold at 48 hr), elastase 3B (322-fold at 8 hr, 486-fold at 48 hr), and pancreatic lipase (186-fold at 8 hr, 116-fold at 48 hr). The extremely large fold-changes that were observed for these genes, along with very low levels of detectable fluorescence for uninfected splenic samples (data not shown), indicated that these transcripts were not present before infection and were induced in response to *B. anthracis*. Due to the increase in expression of genes for pancreas-associated enzymes, tissue sections of infected pancreata were examined. Although occasional blood vessels contained low numbers of bacilli, no significant lesions were observed (data not shown). Additionally, plating of pancreatic tissue homogenates also revealed the presence of *B. anthracis* by 48 hr (data not shown). Previous microarray studies by members of our group involving other bacterial pathogens did not reveal up-regulation of pancreatic digestive enzymes in the spleen of infected animals, suggesting this may be an anthrax-specific response (unpublished data). However, the splenic expression of so-called “pancreatic” enzymes is not a novel observance as amylase 2 and elastase 3b have both been observed in spleen [56] and alternative tissues such as the intestine, liver, and brain [57–59]. The peculiarity of its substantial increase is currently being examined by our laboratory. Another experimental replicate was performed utilizing the portions of spleen distal to the hilum with similar increases in one of the tested enzymes (amylase 2) based on real-time RT-PCR. The number of affected genes in the spleen increased to 124 at 48 hr post-infection (Table 1). The expression of cytokine-related genes, such as Cxcl1, Cxcl2, and IL-1 receptor type II were also increased (Table 7). Similar to findings in the lungs at 48 hr, CD14 expression was increased 2.6-fold. Pancreatitis-associated protein (PaP) is an anti-inflammatory protein that was increased 16.2-fold. PaP expression may be induced during inflammation through several pro- and anti-inflammatory cytokines or by itself. PaP then blocks NF- κ B activation and induces the expression of the anti-inflammatory factor suppressor of cytokine signaling 3 (SOCS3) [60] through the JAK/STAT3 (Janus kinase/Signal Transducers and Activators of Transcription 3)-dependent pathway [61].

Due to the high infectious dose of anthrax spores given, infected animals were close to death by 48 hr post-infection. Therefore, the time-frame was too short for an adaptive immune response to come into play. Even so, there was a noticeable reduction in multiple genes encoding molecules vital for mounting a full adaptive immune response (Table 7). This included immunoglobulin heavy chains and lambda chains, T-cell receptor gamma (Tcr γ -V4), and CD48 antigen. T-cell receptor gamma chain is expressed along with the delta chain on the surface of a subset of T lymphocytes. Unlike alpha-beta T-cells, gamma/delta T-cells directly recognize proteins and do not require the antigenic peptides to be presented in complex with the MHC [62]. CD48 has broad immunological roles, including cell adhesion [63] and innate responses to bacterial infection [64], and is required for proper CD4⁺ T-cell activation [65–67].

The effect of *B. anthracis* infection on the heart has not been previously addressed, and this was the third organ examined during this study. As a muscular organ with the task of distributing the host's blood supply, it was speculated that *B. anthracis* organisms would travel to this major organ at some point during infection. Indeed, samples of heart tissue removed at 24 hr and 48 hr post-infection showed the presence of *B. anthracis*. Histopathology confirmed the presence of anthrax bacilli in the myocardium; however, no significant lesions or tissue damage were noted in this organ (Figure 2). Although the load of *B. anthracis* organisms was lower in the heart compared to other tissues tested, this organ exhibited the most remarkable transcriptional response.

Nine of the eleven genes altered in the heart at 8 hr post-infection were down-regulated (Tables 1 and 4). Two notable genes include Csnk1, which is the catalytic α subunit of casein kinase II (CK2), and the receptor for transferrin (Tfrc)[68]. CK2 positively regulates Wingless-type MMTV (mouse mammary tumor virus) integration site family (Wnt) signaling [69,70], which is associated with tumorigenesis and has also been shown to participate in the inhibition of apoptosis *via* phosphorylation of the activity-regulated cytoskeletal (ARC) protein [71]. The receptor for transferrin remained down-regulated by 48 hr, but transferrin itself, as well as ferritin, was slightly up-regulated at this later time point. Transferrin is a glycoprotein that binds iron very tightly, but reversibly. Transferrin receptors on the surface of host cells bind transferrin and vesicle acidification releases the iron ions [72]. Most bacterial species require iron for efficient growth and possess specialized systems for iron expropriation. *B. anthracis* secretes siderophores to scavenge iron from the host [73,74]. Altering the expression of genes for transferrin, its receptor, and ferritin may represent a host strategy to prevent the acquisition of iron by *B. anthracis*.

The heart at 48 hr post-challenge showed the most dramatic change compared to the lungs and spleen in terms of the overall number of altered genes (298 genes; Tables 1 and 5). The receptor for the Fc fragment of IgG (Fc γ R2B) was increased 2.1-fold, along with GM-CSF (1.8-fold). Fc γ Rb is expressed on macrophages, neutrophils, mast cells, and B cells [75]. The alpha chain of the CD8 antigen was increased 1.8 fold. CD8-alpha molecules promote the survival and differentiation of activated lymphocytes into memory CD8 T-cells [76–79]. Additionally, two genes encoding antimicrobial proteins found in specific granules of neutrophils, cathelicidin (Camp) and neutrophilic granule protein (Ngp), were also up-regulated [80]. On the other hand, many genes important for host protection were down-regulated in the heart as a result of anthrax infection. Similar to what was found in the lung at 8 hr, the gene encoding MHC Class I receptor (H2-K1) was also down-regulated in the heart (2-fold).

A trend that appeared in the heart at 48 hr was a decrease in transcription of multiple genes involved in maintaining the underlying structure and cell-cell adhesion of the heart tissue. For instance, three genes involved in collagen formation were down-regulated. Collagens are a family of proteins that form triple-stranded, rope-like structures to strengthen and support many

tissues in the body, including cartilage, bone, tendon, and skin. The *Col1a1* and *Col1a2* genes (both decreased 2.3-fold) produce different components of type I collagen that combine to make a molecule of procollagen, which is then processed to form mature Type I collagen fibrils [81]. *Col5a2* (down 1.8-fold) functions in a similar manner to form Type V collagen fibrils [82]. Type V collagen also plays a role in assembling other types of collagen into fibrils in many connective tissues [83–85].

Cadherins, such as *Cdh11*, are cell surface glycoproteins that mediate Ca^{2+} -dependent cell-cell adhesion [86] and are thought to play an important role in development and maintenance of tissues through selective adhesion activity [87]. The expression of this gene was decreased 3.2-fold (Table 5). *CD44*, which was decreased 2.3-fold, is involved in many diverse processes such as lymphocyte activation and homing, and adhesion to the extracellular matrix [88]. *VCAM-1* is a cell surface glycoprotein expressed by cytokine-activated endothelium that mediates the adhesion of monocytes and lymphocytes during migration. During most inflammatory conditions, *VCAM-1* is up-regulated in the endothelium of postcapillary venules [89]. However, this gene was down-regulated 2.7-fold in anthrax-infected hearts.

Of particular interest, there are several genes that by their altered expression could quite possibly hamper cardiac function. For example, adrenomedullin (AM), which was decreased nearly 2-fold (Table 5), is a potent vasodilator peptide that has been shown to protect against cardiovascular damage by prohibiting the induction of oxidative stress [90]. A deficiency in AM, on the contrary, has been shown to increase ROS production resulting in exacerbated oxidative stress and marked tissue damage [91]. The expressions of metabolic genes, namely ATP citrate lyase and fatty acid synthase, were also profoundly decreased at the 48 hr time point in the heart (Table 5). ATP citrate lyase is the primary enzyme responsible for the synthesis of cytosolic acetyl-CoA, which is a crucial component involved in the regulation of carnitine palmitoyltransferase I activity and the rate-determination of mitochondrial fatty acid oxidation in the heart and other tissues [92]. Fatty acid oxidation serves as the principal mode by which energy is produced to maintain adequate heart contraction [93,94], and any negative shift could thus lead to a reduction in ATP generation within cardiac tissue and subsequently cardiac insufficiency. The decline in fatty acid synthase expression adds to this argument due to its involvement in the biosynthesis of long-chain fatty acids, the substrate used for fatty acid oxidation. Although many of the fatty acids used by the heart come from the circulatory system, a portion is synthesized and stored by the cardiac cells themselves. Lastly, the decline in the expression of tropomyosin 3 (Table 5) suggests possible dysfunction in the contraction of the myocardium, because tropomyosin plays a pivotal role in the actin/myosin machinery responsible for muscle contraction/relaxation.

An interesting pattern was observed in the IL-4, IL-6, G-CSF, KC, RANTES, IFN- γ and MCP-1 levels measured in the sera of anthrax-infected animals. A bimodal response (i.e., an increase at 8 hr, drop at 24 and 36 hr, and spike at 48 hr) was observed (Figure 3). Similarly, this trend was reported for two of these particular cytokines, IL-6 and MCP-1, by Firoved *et al.* [95] following administration of EdTx to mice. Excluding IL-4, the affected cytokines are pro-inflammatory and mediate recruitment of various immune cells such as neutrophils, basophils, T-cells, and monocytes. The spike at 8 hr may be indicative of the immediate immune response to inhalation of a foreign pathogen. For instance, murine cells are capable of recognizing dormant spores via the MyD88 receptor, and they respond with production of inflammatory cytokines [96].

Additionally, because alveolar macrophages rapidly engulf spores, by 8 hr these phagocytic cells are likely contributing with their own cytokine secretion. However, at later time points of 24 and 36 hr, large amounts of LeTx and EdTx are likely released into the bloodstream. Each of these toxins has unique immunomodulatory properties, including cytokine abrogation.

In both the mouse model and tissue culture systems, EdTx results in increased IL-6 secretion concomitant with impairment of the TNF- α response [95,97]. *In vitro*, LeTx decreases IFN- γ , IL-2, and IL-4 production in rats [25]. Additionally, LeTx has been shown to suppress the production of TNF- α and IL-1 β in murine macrophage cell lines [98]. Thus, the combined action of these two toxins may contribute to the observed drop in cytokine levels at 24 and 36 hr post-infection.

By 48 hr following inoculation with Ames spores, the mice were terminally ill. High titers of *B. anthracis* bacilli plagued the bloodstream and infiltrated multiple tissues as confirmed with histopathology. The second jump in certain cytokine levels (Figure 3) may result from host cells recognizing pattern-associated molecular products being shed by the bacteria, such as anthrolysin O [99] and peptidoglycan [100]. Perhaps this was the last attempt by the immune system to call in professional immune cells to try and control the infection. Analysis of the levels of selected biochemical components in the blood of infected mice was also performed. Although levels of three hepatic transaminases (alkaline phosphate, alanine aminotransferase, and aspartate aminotransferase) increased (2–3 fold) at 48 hr compared to normal mice, which is often indicative of liver injury or dysfunction [101,102], the increases were not statistically significant (data not shown). No significant changes were observed for the remaining parameters.

In summary, the use of virulent *B. anthracis* Ames spores and an intranasal infection route in mice allowed us to closely mimic conditions resembling the intentional release of weaponized, aerosolized anthrax spores. Stringent GeneChip analysis data revealed that the lungs, heart, and spleen of the infected mice underwent drastic transcriptional changes during early and late stages of the disease. Although numerous genes related to the host immunological response and various protection mechanisms were up-regulated in these organs, vast numbers of genes important for fully developing and maintaining this response were decreased. By identifying genes that play major roles during anthrax disease progression, we have attempted to provide investigators in the anthrax and biodefense fields with new information about transcriptional gene profiling during infection to advance the effort toward creating novel therapeutic strategies against this deadly disease.

4. MATERIALS AND METHODS

4.1. Mouse nasal inoculation

Specific pathogen-free (SPF) female Swiss Webster 7–8 week old mice were purchased from Taconic Farms (Georgetown, NY) and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Resources Center at UTMB. Mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine-HCl (90 mg/kg) and xylazine-HCl (10 mg/kg). After anesthesia, the mice were suspended by their front incisors to facilitate nasal inoculation. Mice were given 5 LD₅₀ (approximately 5.6×10^4 colony forming units [cfu]) of *B. anthracis* Ames strain spores in 40 μ l of phosphate buffered saline (PBS), after which the mice remained suspended for an additional 1–2 min to ensure a complete lung inoculation. Subsequently, the mice were returned to their cages for the appropriate incubation time. All animal experiments were performed in accordance with the regulations of the UTMB Institutional Animal Care and Use Committee and the NIH Office of Laboratory Animal Welfare.

4.2. Determining *B. anthracis* levels in mouse organs

At 8, 24, and 48 hr post-infection, infected mice were anesthetized, followed by euthanasia by CO₂ narcosis. Cervical dislocation was subsequently performed to ensure death. Lungs, hearts, and spleens were aseptically removed from 3 mice and placed in 1 ml of PBS in 50-ml Kendall

tissue homogenizers (Kendall, Mansfield, MA). Following homogenization of the tissues in the biological safety cabinet in our approved biological safety level (BSL)-2 or animal BSL-3 laboratories, serial dilutions of the samples were made in PBS and 100 μ l of each dilution was plated on 5% sheep blood agar plates (BD Biosciences, Franklin Lakes, NJ); which were incubated overnight at 37°C. In total, 3 independent experiments representing 3 biological replicates each were performed, and data were statistically analyzed using one-way ANOVA.

4.3. Tissue fixation and slide preparation

At 48 hr post-infection, aseptic collection of organs was performed as described above. The tissues were fixed in 4% paraformaldehyde for 48 hr and tested for viable bacteria by plating on blood agar plates. Tissue sections were routinely processed, embedded in paraffin, mounted on slides, and stained with hematoxylin and eosin (H&E), as well as the gram stain at the University of Texas, M.D. Anderson Cancer Center. Bacterial load and tissue architecture was evaluated and compared to that of control mice that were given only PBS.

4.4. Cytokine profiling

Infected mice were sacrificed at 8, 24, 36, or 48 hr post infection. A 22-gauge needle was used to perform a cardiac puncture and approximately 1 ml of blood was removed and placed in Microtainer serum separator tubes (BD Biosciences) at 4°C overnight. Blood was centrifuged at 1,000 $\times$ g for 10 min and the serum transferred to a new tube, filtered with a 0.22 μ m syringe filter, plated overnight to ensure sterility, and stored at -20°C until assayed. The sera of three mice from the control, 8, 24, 36, and 48 hr experimental groups were analyzed using a 23-plex Bioplex kit (Bio-RAD, Hercules, CA). Three replicates were performed and statistically parsed by one-way ANOVA. Whole blood from the aforementioned groups was used in conjunction with the ProChem V analyzer and a panel of biochemistry cuvettes (Drew Scientific Inc., Dallas, TX) to assess biochemical profiles. Analysis was performed as recommended by the manufacturer to measure the serum levels of amylase, alkaline phosphatase (AKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), bicarbonate, calcium, and glucose.

4.5. Affymetrix GeneChip analysis

Hearts, spleens, and lungs were aseptically removed from three anesthetized mice at 8 and 48 hr post-infection and placed in 50-ml tissue homogenizers with 1 ml RNALater (Ambion, Austin Inc., TX) on ice and immediately transferred to -80°C for storage. Due to the proximity of the pancreatic tail to the hilum of the spleen, great care was taken in isolating only the spleen during tissue extraction. The RNAqueous kit (Ambion) was used to purify RNA from the tissue homogenates. The RNA was allowed to precipitate overnight and then resuspended in a volume of 20 μ l diethylpyrocarbonate-treated water. Pellets were stored at -80°C. Subsequently, RNA samples were tested for sterility by streaking on 5% sheep blood agar plates. An Agilent chip was then used to analyze the purity of RNA samples, and the corresponding cDNA was applied to Affymetrix murine GeneChips (430 Plus 2; Affymetrix, Santa Clara, CA) for transcriptional profiling of genes in different organs.

Briefly, total RNA (25 μ g) was processed and hybridized to GeneChip arrays in the Molecular Genomics Core at UTMB. The cDNA synthesis, *in vitro* transcription, and labeling and fragmentation to produce the oligonucleotide probes were performed as instructed by the GeneChip manufacturer (Affymetrix). The probes were first hybridized to a test array (Affymetrix) and then to the 430 Plus 2 GeneChip, both performed using the GeneChip Hybridization Oven 640. The chips were washed in a GeneChip Fluidics Station 400 (Affymetrix) and the results visualized with a Gene Array scanner using the Affymetrix software. These experiments were performed in triplicate, and the data were analyzed separately using three different softwares: GeneSifter (VizX Labs, Seattle, WA), Significance Analysis of Microarrays (SAM; Stanford, CA) and Spotfire DecisionSite 9.0 (Spotfire Inc.,

Somerville MA). Changes in gene expression were considered significantly altered if the *p* value was less than 0.05, the fold-change value was at least 1.5, and alteration in gene expression occurred in all 3 experiments. Additionally, genes that were altered between any two uninfected control samples were disregarded, as these alterations most likely represented normal variations among mice. The data discussed in this publication will be deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and will be accessible through GEO series accession number prior to the publication of this manuscript.

4.6. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Real-time quantitative RT-PCR was performed in the Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems). RNA samples were first quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE) and qualified by analysis on an RNA Nano chip using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA). Synthesis of cDNA was performed using 1 µg of RNA in a 20-µL-reaction volume containing reagents from the Taqman Reverse Transcription Reagents kit (Applied Biosystems). Q-PCR amplifications were performed in triplicate, using 2 µl of cDNA in a total volume of 25 µl containing SYBR Green and 900 nM primers. Custom-designed primers used for real-time PCR amplification are listed in Table 9.

A typical protocol included reverse transcription at 50°C for 2 min and a denaturation step at 95°C for 10 min followed by 40 cycles with 95°C denaturation for 15 s and 60°C annealing/extension step for 1 min. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Negative controls containing water instead of RNA were concomitantly assayed to confirm that the samples were not cross-contaminated. Targets were normalized to reactions performed using 18S rRNA amplimers, and fold change was determined using the comparative threshold method [103].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by the U.S. Army Grant (DAMD 170210699), the NIH (U01 AI 5385802 and N01 AI 30065), and NIH/NIAID Western Regional Center of Excellence 1U54 AI057156-01. Cristi L. Galindo received support from an NIH cardiology fellowship, Cardiology Department, University of Texas Southwestern Medical Center. We would like to thank Dr. T. Wood and his staff from the department of Biochemistry and Molecular Biology at UTMB for providing facility of his core laboratory for microarray studies.

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Fig. 1.

B. anthracis infection in murine ($n = 3/\text{time point}$) lungs, heart, and spleen at 8, 24, and 48 hr post-infection. CFU/whole organ.

Fig. 2.

Histopathological analysis of anthrax-infected tissues. Lung (A), heart (B), and spleen (C).

Tissues were extracted 48 hr post-infection and stained with H&E. Bar size in the control and infected tissues (20X) = 200 μ m while the bar size in the control and infected tissue (40X) was 100 μ m.

Fig. 3. Cytokine array analysis of murine ($n = 3/\text{time point}$) sera at 0, 8, 24, 36, and 48 hr post-infection with Ames spores. Significant changes ($p < 0.05$) from 0 hr are indicated by asterisk.

summarizing the number of significantly altered genes in each tissue per time point, grouped by function

Table 1

	Heart 8 hr		Heart 48 hr		Lung 8 hr		Lung 48 hr		Spleen 8 hr		Spleen 48 hr	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Adhesion and Migration	-	-	5	11	-	-	-	1	3	-	4	1
Angiogenesis	-	-	-	2	-	-	-	-	-	-	-	-
Apoptosis	-	-	3	-	-	-	-	-	-	1	-	-
Cytoskeleton Reorganization	-	-	-	-	-	-	-	2	-	-	-	-
Digestive Enzymes	-	-	-	-	-	-	-	-	12	-	26	-
Electron Transport	-	-	-	-	-	-	-	-	2	-	1	-
Exocytosis	-	-	-	-	-	-	-	-	1	-	-	-
Growth, Differentiation, and Development	-	2	6	24	-	2	-	4	5	1	3	6
Immune and Stress Response	-	-	10	4	2	2	8	1	-	8	10	12
Iron Homeostasis	-	1	2	1	-	-	-	-	-	-	-	-
Metabolism	1	1	16	7	1	-	2	-	-	-	4	2
Nuclear Structure	-	-	-	-	-	-	-	-	2	-	-	-
Protein and RNA Processing	1	-	13	8	-	-	4	-	2	-	3	-
Signal Transduction	-	-	11	9	-	-	2	-	-	1	2	-
Transcriptional Regulation	-	1	10	9	-	2	-	2	-	1	6	4
Transport	-	-	8	3	1	-	-	2	-	-	2	2

Table 2Genes significantly altered in response to *Bacillus anthracis* infection in murine lungs at 8 hr

GenBank ID	Gene Name	Function	FC
Up-regulated in Response to <i>B. anthracis</i> Infection			
AV075715	Clusterin (Clu)	Stress response	2.1
NM_138648	Oxidized low density lipoprotein (lectin-like) receptor 1 (Olr1)	Inflammation; cell adhesion	1.9
Down-regulated in Response to <i>B. anthracis</i> Infection			
U96752	Major histocompatibility complex Q1b (H2-K1)	Antigen presentation	-2.5
BB428671	Platelet-derived growth factor, D polypeptide (Pdgfd)	Regulation of progression through cell cycle; proliferation	-2.1
AV169310	Surfactant associated protein C (Sftpc)	Regulation of liquid surface tension; regulatory gas exchange	-2.0

FC = fold-change

Negative sign (–) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 3Genes significantly altered in response to *Bacillus anthracis* infection in murine lungs at 48 hr

GenBank ID	Gene Name	Function	FC
Up-regulated in Response to <i>B. anthracis</i> Infection			
<i>Inflammation and stress response</i>			
NM_020581	Angiopoietin-like 4 (Angptl4)	Response to hypoxia or starvation; negative regulation of apoptosis; positive regulation of angiogenesis	3.7
NM_009841	CD14 antigen (Cd14)	Inflammatory response; Immune response to LPS	3.5
AA796766	Metallothionein 2 (Mt2)	Nitric oxide mediated signal transduction	6.0
NM_011082	Polymeric immunoglobulin receptor (Pigr)	Secretion of IgA and IgM (apical surface of epithelial cells)	1.9
<i>Metabolism</i>			
C85932	Crystallin, lamda 1 (Cry11)	Fatty acid metabolism	2.6
<i>Other</i>			
NM_020509	Resistin like alpha (Retnla)	Hormone signaling	3.3
Down-regulated in Response to <i>B. anthracis</i> Infection			
<i>Adhesion</i>			
BB477150	Protein tyrosine phosphatase, receptor type D (Ptpd)	Cell adhesion	-3.2
<i>Cytoskeletal organization</i>			
BM900139	Actin binding LIM protein family, member 3 (Ablim3)	Cytoskeleton organization and biogenesis	-1.8
<i>Growth and differentiation</i>			
BB428671	Platelet-derived growth factor, D polypeptide (Pdgfd)	Cell cycle regulation; proliferation	-2.1
NM_016686	Vascular endothelial zinc finger 1 (Vezf1)	Angiogenesis; endothelial cell development/differentiation; blood vessel morphogenesis	-2.5
<i>Signal transduction</i>			
BB216074	Protein kinase, cAMP dependent regulatory, type II beta (Prkar2b)	Inhibition of PKA catalytic subunits	-1.7
<i>Stress response</i>			
AB031049	REV3-like, catalytic subunit of DNA polymerase zeta RAD54 like (Rev3l)	DNA replication; DNA repair; response to DNA damage	-1.6
<i>Transcription regulation</i>			
AI639846	Transcription factor 4 (Tcf4)	Regulation of transcription, DNA-dependent; development	-1.7

FC = fold-change

Negative sign (–) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 4Genes significantly altered in response to *Bacillus anthracis* infection in murine hearts at 8 hr

GenBank ID	Gene Name	Function	FC
Up-regulated in Response to <i>B. anthracis</i> Infection			
AB006361	Prostaglandin D synthetase (Ptgds)	Conversion of PGH2 to PGD2; smooth muscle contraction/relaxation	1.7
Down-regulated in Response to <i>B. anthracis</i> Infection			
NM_007788	Casein kinase II, alpha 1 polypeptide (Csnk2a1)	Wnt receptor signaling pathway	-1.8
NM_011638	Transferrin receptor (Tfrc)	Iron ion homeostasis	-1.9

FC = fold-change

Negative sign (-) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 5
Genes significantly altered in response to *Bacillus anthracis* infection in murine hearts at 48 hr

GenBank ID	Gene Name	Function	FC
Up-regulated in Response to <i>B. anthracis</i> Infection			
<i>Apoptosis</i>			
AV228493	Interleukin-1 receptor-associated kinase 3 (Irak3)	Cytokine and chemokine mediated signaling pathway; apoptosis	1.7
NM_010177	Tumor necrosis factor (ligand) superfamily, member 6 (Tnfsf6); Fas ligand (Fasl)	Apoptosis; immune response	1.7
<i>Growth, differentiation, and development</i>			
AK004781	SRY-box containing gene 17 (Sox17)	Regulation of transcription, DNA-dependent; cell differentiation; cardiogenesis	2.2
<i>Immune response and inflammation</i>			
NM_009921	Cathelicidin antimicrobial peptide (Camp)	Defense response to bacterium	1.8
BB030365	CD8 antigen, alpha chain (Cd8a)	Immune response; cytotoxic T cell differentiation	1.8
BB530063	Fc receptor, IgG, low affinity Iib (Fcgr2b)	Negative regulation of type I hypersensitivity; phagocytosis; defense response	2.1
X03019	Granulocyte-macrophage colony stimulating factor (GM-CSF)	Immune response; myeloid dendritic cell differentiation	1.8
NM_008694	Neutrophilic granule protein (Ngp)	Defense response	2.3
<i>Iron homeostasis</i>			
BM237750	Transferrin (Trf)	Iron ion homeostasis	1.6
<i>Metabolism</i>			
BB503164	Carbonic anhydrase 12 (Car12)	One-carbon compound metabolism	2.4
AV236319	Carnitine palmitoyltransferase 2 (Cpt2)	Fatty acid metabolism	2.0
<i>Protein processing</i>			
NM_008456	Kallikrein 5 (Klk5)	Proteolysis	2.3
<i>Signal transduction</i>			
AV047570	Calmodulin 3 (Calm3)	G-protein coupled receptor protein signaling pathway	3.2
BC019382	Metallothionein 2A (Mt2a)	Phosphatidylinositol metabolism; Nitric oxide-mediated signaling	1.9
AV328460	Tachykinin receptor 3 (Tacr3)	G-protein coupled receptor protein signaling pathway; phosphatidylinositol-calcium second messenger system	2.0
AV246932	Regulator of G-protein signaling 2 (Rgs2)	Regulation of G-protein coupled receptor protein signaling pathway	1.65
<i>Stress response</i>			
BC015260	FK506 binding protein 5 (51 kDa) (Fkbp5)	Stress response; steroid signaling	3.2
<i>Transport</i>			
AV323698	ARP1 actin-related protein 1 homolog A (Actr1a)	Vesicle-mediated transport	1.9
Down-regulated in Response to <i>B. anthracis</i> Infection			
<i>Adhesion and migration</i>			
NM_009866	Cadherin 11 (Cdh11)	Cell adhesion	-3.2
BC005676	Cell surface glycoprotein CD44 (hyaluronate binding protein) (Cd44)	Cell adhesion	-2.3
BF227507	Procollagen, type I, alpha 2 (Col1a2)	Cell adhesion	-2.3
NM_007737	Procollagen, type V, alpha 2 (Col5a2)	Cell adhesion	-1.8
NM_011693	Vascular cell adhesion molecule 1 (Vcam1)	Cell adhesion	-2.7
<i>Angiogenesis</i>			
BB453314	Angiopoietin 1 (Angtpt1)	Angiogenesis; transmembrane receptor protein tyrosine kinase signaling pathway; development	-2.1
<i>Growth, differentiation, and development</i>			
BC004724	Fibronectin 1 (Fn1)	Cell morphogenesis; acute-phase response; cell-substrate junction assembly; cell adhesion	-2.2
NM_009627	Adrenomedullin (Adm)	Heart development; positive regulation of cell proliferation	-1.7
AI323506	Myelin basic protein (Mbp)	Myelination; cell differentiation	-1.9
C81601	Tissue inhibitor of metalloproteinase 2 (Timp2)	Negative regulation of cell proliferation; regulation of cAMP metabolism; regulation of MAPKKK signaling	-1.7
<i>Immune response and inflammation</i>			
L23495	MHC class I H-2K (H2-K1)	Antigen processing and presentation	-2.0
NM_018866	Small inducible cytokine subfamily B (Cys-X-Cys), member 13 (Scyb13) (Cxc113)	Chemotaxis; inflammation	-5.4
AY090098	Interferon, alpha-inducible protein 27 (Ifi27)	Defense response	-3.1
<i>Iron homeostasis</i>			
AK011596	Transferrin receptor (Tfrc)	Iron ion homeostasis	-1.7
<i>Metabolism</i>			
BB276877	ATP citrate lyase (Acly)	Acetyl-CoA biosynthesis; lipid metabolism	-3.4
AF127033	Fatty acid synthase (Fasn)	Fatty acid biosynthesis	-6.7

GenBank ID	Gene Name	Function	FC
NM_009127	Stearoyl-Coenzyme A desaturase 1 (Scd1)	Lipid biosynthesis; superoxide metabolism	-5.2
<i>Protein processing</i> AK009847	Protease, serine, 23 (Prss23)	Proteolysis	-2.5
<i>Signal transduction</i> BB746807	Adenylate cyclase 7 (Adcy7)	cAMP biosynthesis	-2.1
NM_022881	Regulator of G-protein signaling 18 (Rgs18)	Regulation of G-protein coupled receptor protein signaling pathway	-3.0
<i>Stress response</i> BC024835	Structure specific recognition protein 1 (Ssrp1)	DNA replication; DNA repair	-1.7
<i>Transcription regulation</i> BB124537	Myotrophin (Mtpn)	Regulation of transcription, DNA-dependent	-1.7
<i>Other</i> M12481	Actin, beta, cytoplasmic (Actb)	Structural constituent of cytoskeleton	-1.9
X53753	Tropomyosin 3, gamma (Tpm3)	Regulation of muscle contraction	-1.7

FC = fold-change

Negative sign (-) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 6Genes significantly altered in response to *Bacillus anthracis* infection in murine spleens at 8 hr

GenBank ID	Gene Name	Function	FC
Up-regulated in Response to <i>B. anthracis</i> Infection			
<i>Adhesion and migration</i>			
NM_025989	Glycoprotein 2 (zymogen granule membrane) (Gp2)	Cell adhesion	10.7
NM_008411	Integral membrane-associated protein 1 (Itmap1)	Substrate-bound cell migration, cell attachment to substrate	4.5
<i>Digestive enzymes</i>			
NM_009669	Amylase 2, pancreatic (Amy2)	Carbohydrate metabolism	896.5
NM_007919	Chymotrypsin C (caldecrin) (Ctcr)	Proteolysis	95.0
AV076302	Colipase, pancreatic (Clps)	Lipid catabolism	586.3
BI439550	Elastase 3B, pancreatic (Ela3)	Proteolysis	403.9
AI326372	Pancreatic lipase (Pnlip)	Lipid catabolism	186.2
<i>Electron transport</i>			
AK007743	Protein disulfide isomerase associated 2 (Pdia2)	Electron transport	8.9
<i>Exocytosis</i>			
AV050299	Syncollin (Syncn)	Calcium-sensitive regulation of exocytosis in exocrine tissues (putative)	56.4
<i>Protein processing</i>			
NM_009258	Serine protease inhibitor, Kazal type 3 (Spink3)	Endopeptidase inhibitor activity	10.3
<i>Stress response</i>			
AV060866	Phospholipase A2, group IB, pancreas (Pla2g1b)	Phospholipid metabolism; response to stress; cell proliferation	26.5
NM_009042	Regenerating islet-derived 1 (Reg1)	Stress response; association with pancreatic inflammation	89.3
Down-regulated in Response to <i>B. anthracis</i> Infection			
<i>Apoptosis</i>			
NM_009344	T-cell death associated gene (Tdag)	Apoptosis; FasL biosynthesis	-1.8
<i>Growth and differentiation</i>			
W77144	Inhibitor of DNA binding 4 (Id4)	Positive regulation of cell proliferation	-2.2
<i>Immune response</i>			
BE634960	CD48 antigen (Cd48)	T cell activation (putative)	-1.8
BC018402	Histocompatibility 2, D region locus 1 (H2-K1)	Antigen presentation	-2.4
BC025447	Immunoglobulin heavy chain (gamma polypeptide) (Igh-6)	Antigen processing and presentation; immunoglobulin mediated immune response; activation of MAPK activity	-1.8
M74124	Interferon activated gene 205 (Ifi205)	Immune response	-2.4
NM_011558	T-cell receptor gamma, variable 4 (Tcrg-V4)	Cellular defense response	-2.4
<i>Signal transduction</i>			
BB229616	G protein-coupled receptor 171 (Gpr171)	G-protein coupled receptor protein signaling pathway	-2.8
<i>Transcription regulation</i>			
D45210	Zinc finger protein 260 (Zfp260)	Regulation of transcription, DNA-dependent; development	-1.8

FC = fold-change

Negative sign (–) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 7
Genes significantly altered in response to *Bacillus anthracis* infection in murine spleens at 48 hr

GenBank ID	Gene Name	Function	FC
Up-regulated in Response to <i>B. anthracis</i> Infection			
<i>Digestive enzymes</i>			
NM_009669	Amylase 2, pancreatic (Amy2)	Carbohydrate metabolism	1,042
BI963522	Chymotrypsin C (caldecrin) (Ctrc)	Proteolysis	64.1
BI439550	Elastase 3B, pancreatic (ela3)	Proteolysis	485.8
AI326372	Pancreatic lipase (Pnlip)	Lipid catabolism	287.3
<i>Energy production</i>			
AK007743	Protein disulfide isomerase associated 2 (Pdia2)	Generation of precursor metabolites and energy	11.5
<i>Immune response and inflammation</i>			
NM_009883	CCAATenhancer binding protein (CEBP), beta (Cebpb)	Immune response; inflammation; regulation of macrophage activation/differentiation	2.6
NM_009841	CD14 antigen (Cd14)	Inflammation; response to LPS	2.6
NM_008176	GRO1 oncogene (Gro1) (Cxcl1)	Immune response; inflammation	4.5
NM_010555	Interleukin 1 receptor, type II (Il1r2)	Cell surface receptor linked signal transduction; inflammation	2.5
NM_011036	Pancreatitis-associated protein (Pap)	Acute-phase response; inflammation	16.2
NM_009140	Small inducible cytokine subfamily, member 2 (Scyb2) (Mip2)	Chemotaxis; inflammation	4.4
<i>Metabolism</i>			
BB068040	Glutamic pyruvate transaminase (alanine aminotransferase) 2 (Gpt2)	Metabolism	2.2
<i>Pancreatic functions</i>			
BC020534	Cholecystokinin A receptor (Cckar)	Pancreatic growth and enzyme secretion	6.0
AV050299	Syncollin (Syn)	Regulation of exocytosis	89.0
<i>Protein processing and proteolysis</i>			
NM_025989	Glycoprotein 2 (zymogen granule membrane) (Gp2)	GPI anchor binding	25.1
<i>Stress response</i>			
NM_019738	Nuclear protein 1 (Nupr1)	Cell growth in response to injury and proapoptotic stimuli (putative)	3.5
<i>Transcription regulation</i>			
AI467599	cAMP responsive element modulator (Crem)	Regulation of transcription, DNA-dependent	6.3
Down-regulated in Response to <i>B. anthracis</i> Infection			
<i>Growth and differentiation</i>			
BB770972	Growth arrest-specific 2 like 3 (Gas2l3)	Cell cycle arrest	-2.3
<i>Immune response and inflammation</i>			
NM_053200	Carboxylesterase 3 (Ces3)	Acyl-CoA metabolism; response to toxin	-1.9
BE634960	CD48 antigen (Cd48)	Immune response; T cell activation	-1.7
BC025447	Immunoglobulin heavy chain (gamma polypeptide) (Igh-6)	Activation of MAPK activity; immunoglobulin mediated immune response	-3.7
M94350	Immunoglobulin lambda chain (IgL) (Igl-V1)	Humoral immune response	-1.9
BM239828	Interferon-inducible GTPase (Iigp1)	Cytokine and chemokine mediated signaling pathway	-1.7
NM_011558	T-cell receptor gamma, variable 4 (Tcrg-V4)	Defense response	-1.6
<i>Metabolism</i>			
AW911807	Guanine deaminase (Gda)	Hydrolase activity	-3.0
<i>Stress response</i>			
AK004608	Heat shock 70kD protein 8 (Hspa8)	Regulation of progression through cell cycle; stress response	-2.0
BB442713	Mitogen-activated protein kinase kinase kinase 5 (Map4k5)	Stress response	-1.5
<i>Transcription regulation</i>			
D45210	Zinc finger protein (Zfp260)	Regulation of transcription, DNA-dependent	-1.8
<i>Transport</i>			
AI326108	Intersectin 2 (Itsn2)	Endocytosis; regulation of Rho protein signal transduction	-2.1

FC = fold-change

Negative sign (–) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 8
Real-time RT-PCR confirmation of altered genes identified by GeneChip analysis

Gene	Tissue	Time	Affy-metrix Fold Change	RT-PCR Fold Change	Function
Clusterin	Lung	8 hr	2.1	3.98	Molecular chaperone during stress-induced injury.
Angiopoietin-like Protein 4	Lung	48 hr	3.67	2.8	Protects vascularity during inflammation; maintains EC matrix.
Resistin-like Alpha	Lung	48 hr	3.32	6.34	Regulator of apoptosis and glucose uptake in adipose cells.
Amylase 2	Spleen	8 hr	896.46	3847.6	Pancreatic enzyme involved in starch metabolism.
Elastase 3	Spleen	8 hr	403.86	599.19	Pancreatic enzyme involved in breaking down elastin.
T-cell Gamma Variable 4	Spleen	8 hr	-2.4	-1.35	A variable region on the T-cell receptor.
Prostaglandin D2 Synthase	Heart	8 hr	1.7	2.23	Involved in muscle contraction and platelet aggregation.
Transferrin Receptor	Heart	8 hr	-1.87	-1.26	Receptor involved in iron uptake.

FC = fold-change

Negative sign (–) before fold-change value indicates down-regulation

Genes shown more than once were represented by more than one probe set on the array, and each was determined separately to be altered in response to *Bacillus anthracis* treatment

Table 9

Custom-designed primers used for confirmation of gene alterations induced by anthrax infection.

Genes encoding proteins	Forward Primer (5'–3')	Reverse Primer (5'–3')
Tfrc	AGAGACAGCAATTGGATTAGCAA	ATCCTCACAAAAACAAAAAGAACTG
Angpt14	CCCCCAATGGCCTTTCC	AAACCACCAGCCACCAGAGA
Clu	ATGAAGTTCTATGCACGTGTCTGC	GTCGCCGTTCATCCAGAAGT
Ela3	GGCCACTGCATCTCGACTTC	ATCACCTGTTCTTGGCCTTCCT
Ptgs	TGCAGCCCAACTTTCAACAA	TTGCACATATAACAATACAGCTTTCTTCTC
Retnla	TCCAGCTAACTATCCCTCCACTGTA	AGTCATCCCAGCAGGGCAG
Tcrg-V4	GGGAAGCCAACCTGGCA	AATTGATATTTCAGGTTGCTCCAAC
Amy2	GGACTTTAACGATAATAAATGTAATGGAGAA	CAAGTGCAAGATCCAGAAGGC