Antibacterial Role for Natural Killer Cells in Host Defense to *Bacillus Anthracis*

**Running title**: NK cell role in innate immunity to *B. anthracis*

Christine M. Gonzales¹, Courtney B. Williams¹, Veronica E. Calderon², Matthew B. Huante³, Scott T. Moen¹, Vsevolod L. Popov², Wallace B. Baze⁵, Johnny W. Peterson¹, ³, ⁴, and Janice J. Endsley¹, ², ³, ⁴∗

Departments of Microbiology and Immunology¹, and Pathology², Sealy Center for Vaccine Development ³, Center for Biodefense and Emerging Infections⁴, University of Texas Medical Branch, Galveston, TX 77555, Department of Veterinary Sciences⁵, University of Texas, M.D. Anderson Cancer Center, Bastrop, TX 78602.

Keywords: Natural Killer Cell, Anthrax, *Bacillus anthracis*, Bactericidal, Innate immunity

*Corresponding author. Mailing address: University of Texas Medical Branch, 301 University Blvd, Galveston, TX, USA. Phone: (409) 772-3142. Fax: (409) 747-6869. Email: jjendsle@utmb.edu.
Natural killer (NK) cells have innate antibacterial activity that could be targeted for clinical interventions to infectious disease caused by naturally occurring or weaponized bacterial pathogens. To determine a potential role for NK cells in immunity to B. anthracis, we utilized primary human and murine NK cells, in vitro assays, and in vivo NK cell depletion in a murine model of inhalational anthrax. Our results demonstrate potent antibacterial activity by human NK cells against B. anthracis bacilli within infected autologous monocytes. Surprisingly, NK cells also mediate moderate antibacterial effects on extracellular vegetative bacilli but do not have activity against extracellular or intracellular spores. The immunosuppressive anthrax lethal toxin impairs NK IFN-γ expression but neither lethal nor edema toxin significantly alter the viability or cytotoxic effector function of NK cells. Compared to human NK cells, murine NK cells have similar, though less potent, activity against intracellular and extracellular B. anthracis. In vivo depletion of murine NK cells does not alter animal survival following intranasal infection with B. anthracis spores in our studies, but significantly increases bacterial load in the blood of infected animals. Our studies demonstrate that NK cells participate in the innate immune response against B. anthracis and suggest that immune modulation to augment NK cell function in early stages of anthrax should be further explored in animal models as a clinical intervention strategy.
INTRODUCTION

*B. anthracis* is a gram-positive, spore-forming, rod-shaped bacterium and the etiologic agent of inhalational, cutaneous and gastrointestinal anthrax infection. Spores from this agent have been used as a bioterrorism weapon and are considered particularly hazardous because of their ability to resist heat, dryness, sunlight, and other factors that limit viability of bacterial pathogens. The most severe form of the infection (inhalational) presents itself as a cold or flu lasting several days eventually leading to respiratory failure and death. The availability of preventive or therapeutic measures to combat anthrax in the general public is very limited (3, 8). Vaccination for anthrax in the U.S. involves a multiple injection dosing schedule with Anthrax Vaccine Adsorbed (AVA), also known as Biothrax®, and is only available to those with a high risk of exposure to *B. anthracis* (8). Annual booster injections with AVA, an aluminum hydroxide-adsorbed, sterile culture filtrate of *B. anthracis*, are required to maintain immunity. Additionally, post exposure antibiotics have been utilized as a prophylactic for individuals exposed to or potentially exposed to *B. anthracis* spores(3). The effectiveness of these antibiotics, however, is dependent on early administration after exposure and patient compliance for a prolonged period of therapy. Thus, there is a need to develop additional and more effective clinical interventions to prevent morbidity and mortality due to *Bacillus anthracis* infection.

The potential to target the innate immune system through clinical modulation is of interest for reducing infectious disease caused by many pathogens. Natural Killer (NK) cells are an important component of the innate immune system and have been extensively examined for contributions to tumor cell eradication and for roles in viral and parasitic clearance (21). More recently, the role and function(s) of NK cells in response to various bacterial infections has begun to be defined. NK cells, (which are potent producers of IFN-γ and cytotoxic granule proteins) have antibacterial activity and reduce the level of bacteremia or sepsis in bacterially infected hosts (9, 14, 16, 29). Following infection, NK cell action in the innate immune response
to tumors or virus occurs through lytic activity against the infected or altered host cells. A role for NK cells in clearance of bacterial pathogens is generally understood to occur via activation of infected cells (e.g., macrophages) by NK cell-derived IFN-γ or through cytotoxic attack of the infected cell (12, 27). Previous studies of the murine splenocyte responses to a laboratory strain of *B. anthracis* suggested activation of NK cell IFN-γ can occur (15), though the induction of IFN-γ in this model was sensitive to suppressive activity of *B. anthracis* lethal toxin (LT). Our recent work demonstrated that granulysin, an antimicrobial protein restricted to the granules of human NK cells and cytotoxic lymphocytes, had potent lytic activity against a highly pathogenic *B. anthracis* isolate (Ames) (10). To date, however, the immune contribution of human NK cells during *B. anthracis* infection has not been defined. Further, the role of murine NK cells in experimental models used to study human inhalational anthrax has also not been described.

In the current studies we demonstrate that human NK cells are not active against spores, but efficiently reduce CFU of *B. anthracis* when exposed to both infected autologous monocytes and extracellular bacilli. Further, anthrax lethal toxin (LT) suppresses NK cell expression of IFN-γ but does not impair proliferative responses, cytotoxic activity, or expression of the antibacterial protein granulysin. We further observe that murine NK cells also have antibacterial activity against *B. anthracis*, though the potency is less compared to human NK cells. Results from *in vivo* depletion of asialo GM1+ cells show that NK cells contribute to control of bacteremia in a murine pulmonary anthrax model. Overall, our studies demonstrate that NK cells can have an antibacterial role that contributes to host defense against *B. anthracis*, can be investigated further in a relevant animal model of inhalational anthrax, and could be a candidate mechanism to target by immune modulation for clinical intervention in cases of anthrax.
MATERIALS AND METHODS

**Bacteria.** Spores were prepared by inoculating *B. anthracis* (Ames strain) in Schaeffer’s sporulation medium (pH 7.0) with 16 g Difco Nutrient Broth. Prior to inoculation and after filter sterilization, 0.1% glucose, 1 mM Ca(NO₃)₂, 0.1 mM MnSO₄, and 1 µM FeSO₄ were added to the medium. Cultures were grown in 50-ml aliquots at 37°C with gentle shaking for 48 h. Subsequently, 100 ml of sterile distilled water was added to dilute the medium and to promote sporulation. After 10 days of continuous shaking, sporulation was confirmed using phase-contrast microscopy and a modified Wirtz-Conklin spore stain (19, 20). The spores were centrifuged in a sealed-carrier centrifuge at 4°C for 15 min at 630 x g. Spore pellets were then washed four times in Cellgro sterile water and finally re-suspended in sterile water. The spore suspension was layered onto a cushion of 58% Hypaque-76 at a ratio of 1:2.5 by volume. The tubes were centrifuged in a JA 25.50 rotor for 45 min at 4°C in an Avanti J-20XPI refrigerated centrifuge. The Hypaque supernatant was decanted and the pellet was washed with sterile water and resuspended in sterile water. Aliquots of the suspension were then stored at -80°C until used to challenge mice in a restricted-access Biosafety Level 3 animal facility registered with the Centers for Disease Control and Prevention and inspected by the Department of Defense and the United States Department of Agriculture. For in vitro studies, *B. anthracis* bacilli were cultured by plating 10 µL of the spore stock onto BBL™ Trypticase™ Soy Agar with 5% Sheep Blood plates (BD Franklin Lakes, NJ USA) overnight. A single colony from this plate was then inoculated into 2 mL of LB media (Sigma, St. Louis, MO) for 12 hours at 220 rpm 37°C. 100 µL of this stock was then taken and placed into 10 mL of fresh LB media and grown to log phase overnight (22, 23).

**Isolation of human blood monocytes and NK cells.** Peripheral blood was obtained by venipuncture from healthy individuals ages 21-49 as approved by the UTMB Institutional Review
Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Accuprep (Accurate Chemicals, Westbury, NY, USA). Monocytes (MN) were positively selected using CD14^+ MicroBeads (Miltenyi Biotech, Auburn, CA USA) with the AutoMacs system and cultured with 1400 U/mL GM-CSF as we have previously described (9). NK cells were negatively selected using an NK cell isolation kit (Miltenyi Biotech) and cultured with media alone or rhIL-15 (15 ng/ml, R&D Systems). In some experiments, monocyte-depleted PBMC were used to validate activity of *B. anthracis* toxins by measuring effects on T cells. T cells were activated using magnetic bead-bound antibodies to human CD3 and CD28 (Dynabeads®, Invitrogen) to activate T cells through engagement of the T cell receptor, as recommended by the manufacturer. Cells were maintained in complete culture media, RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and penicillin-streptomycin (Invitrogen). The determination of NK cell viability for non-toxin related experiments was performed by analysis of trypan blue exclusion. Assessment of cell viability due to toxin treatment was performed using the Annexin V-PE Apoptosis detection kit (BD Biosciences) and results read using a FACScanto flow cytometer (UTMB Flow Cytometry and Cell Sorting Core Facility).

**In vitro exposure to toxins.** Protective Antigen (PA), Lethal Factor (LF), and Edema Factor (EF) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) and stored at -80°C prior to use. Purified NK cells were treated with no toxin (NT), the PA component, LT, or edema toxin (ET) as previously described (5). Briefly, NK cells were incubated with PA (2.5 ug/ml), ET (2.5 ug/ml of PA and 0.625 ug/ml of EF), LT (1 ug/ml of PA and 0.2 ug/ml of LF), or both ET and LT for one hour prior to addition of media (control) or rIL-15. Similarly PBMC were exposed to toxins for 1 h prior to incubation with human CD3/CD28 bead-conjugated antibodies.
Flow cytometry. Purified NK cells or PBMC were harvested at the indicated times and labeled with antibodies (BD Biosciences) specific to human surface markers: CD56-PerCP/Cy5.5 and CD3-APC-Cy7. Cells were washed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) followed by labeling with antibodies (BD Biosciences) to intracellular granulysin-Alexa-488, Ki67-PE or perforin PE, and IFN-γ-APC. Lastly, cells were washed and resuspended in 400 µl of 2% ultrapure formaldehyde (Polysciences Inc.). A total of 50,000 cells were acquired on a FACS Canto (BD Biosciences) and compensation for spectral overlap was performed using FACS DIVA software (BD Biosciences). Isotype- and fluorochrome-matched non-specific control antibodies were used to determine background fluorescence. Analysis was performed using FCS Express version 3 (De Novo Software) as we have previously described (18). Data is presented as results of CD56+CD3- and CD3+CD56- gated events corresponding to NK cells and T cells, respectively.

Assessment of human NK cell antibacterial activity. Isolated MN were divided into aliquots in duplicate wells (10^5 cells per well) of flat-bottomed 96-well tissue culture plates (Corning) for CFU assessment for each treatment to be studied. Following overnight incubation to allow for adherence, supernatants were removed and cells were carefully washed three times with antibiotic free media. MN were then infected at a 1:1 bacterium-to-MN infecting ratio with B. anthracis Ames bacilli, in 50 µl of antibiotic-free cRPMI per well as we have previously described for assessing NK cell activity against mycobacteria (9). Plates were returned to a 37°C incubator for 1 h, at which time the supernatants were gently aspirated and each well was washed three times with RPMI 1640 with 10% fetal bovine serum and 50-µg/ml gentamicin to remove extracellular bacilli. Wells were then refilled with 100 µL of antibiotic-free cRPMI alone or containing NK cells from the same donor. Various ratios of NK cells (5:1, 1:1, 0.5:1, 0.1:1 0:1 NK/MN ratio and NK cell/bacteria ratio in 100mL) were added to autologous infected MN and to wells containing 10^5 CFU of B. anthracis (Ames) bacilli. Following 24 h or co-culture, the cells
were lysed with 250 µl of 1% Triton X-100 per well. CFU were determined by limiting dilution
growth on blood agar plates and counts were taken after 48 h growth as previously described
(22). These experiments were repeated using spores treated with d-histidine and d-alanine to
inhibit germination as previously described (19). Briefly, spores and monocytes were treated
with 2 mM d-histidine and 2 mM d-alanine (Sigma) for 1 h and spores were heated to 65 °C for
30 min to inactivate vegetative bacteria. Spores were used to infect monocytes 1:1 spore-to-MN
ratio of added directly to media. Media containing d-alanine and d-histidine was used throughout
the infection and culture period. Infected MN or free spores were cultured in the presence or
absence of a 5:1 E:T ratio of NK cells and enumeration of heat resistant CFU performed by
limiting dilution plating as described for enumeration of vegetative bacilli.

Assessment of human NK cell cytotoxic activity. Cytotoxic NK cell activity was measured
using the K562 human leukemia cell line (no. 45506; ATCC) as we have previously described
(9). K562 cells were maintained in cRPMI and one day prior to use, 10 µl/ml of DIOC (3,3’-
dihexyloxacarbocyanine iodide, Sigma) was added to fluorescently label K562 cells as
previously described (9). NK cells were treated with NT, PA, LT, ET, or LT + ET, for 1 hr and
then 15 ng/mL of IL-15 or carrier (media) was added. Following overnight incubation NK cells
were counted and placed in tubes at varying E:T ratios (10:1, 5:1, 1:1, 0.1:1, 0:1) with 20,000
DIOC-labeled K562 cell targets. Propidium iodide (PI) was then added to each tube, and tubes
were incubated at 37°C for 2 hrs. Following incubation, tubes were analyzed immediately by
flow cytometry and CTL activity was determined based on the % of non-viable (PI positive)
DIOC-labeled K562 target cells following incubation with NK cells compared to spontaneous
death during the 2 h culture period in the absence of NK cells.
**Scanning Electron Microscopy.** To visualize interactions between NK cells and extracellular *B. anthracis* (Ames), cultures were incubated as described above for 1 or 3 hrs. The samples were mounted on Thermanox coverslips coated with 1% poly-L-lysine, then fixed in a mixture of 2% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for at least 4-6 hours, post-fixed in 1% OsO4 in 0.1M cacodylate buffer, dehydrated in ethanol, processed through hexamethyldisilazane (HMDS) and air-dried. The coverslips were mounted onto the stubs and sputter-coated with iridium in Emitech K575x sputter coater (Ashford, Kent, England) at 20 mA for 20 sec. Samples were examined in a Hitachi S-4700 scanning electron microscope (Hitachi High Technologies America, Inc.) at 2 kV.

**Transmission Electron Microscopy.** NK cells and bacteria were cultured as described for performance of SEM. Cell/bacterial pellets were then fixed in a mixture of 2.5% formaldehyde, 0.1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2 containing 0.03% trinitrophenol and 0.03% CaCl2 and washed in 0.1 M cacodylate buffer pH 7.2. The pellets were post-fixed in 1% OsO4 in the same buffer, stained en bloc with 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in Poly/Bed 812 epoxy resin (Polysciences, Warrington, PA). Ultrathin sections were cut on a Reichert-Leica Ultracut S ultramicrotome, stained with lead citrate and examined in a Philips 201 or CM 100 electron microscope at 60 kV.

**Isolation of murine MN and NK cells.** Spleens were harvested from 6–8-week-old female Swiss Webster mice (Taconic Farms, Georgetown, NY) following sacrifice as approved by the Institutional Animal Care and Use Committee. Single cell suspensions were prepared from harvested spleens following tissue disruption as previously described (5) and PBMCs were isolated by density gradient centrifugation as described for human PBMC in the previous section. MN were obtained by allowing 2 h adherence to tissue culture treated flasks. Purified mouse NK cells were obtained by magnetic bead-based sorting using the DX5 antibody.
(Miltenyi). The isolated monocytes were then plated at 10^5/well in 96 well tissue culture plates and provided 1400 U/ml of recombinant GM-CSF. Antibacterial activity against free bacteria or infected monocytes was performed as described above for human NK cells using a 5:1 ratio of murine NK cells to B. anthracis-infected MN or B. anthracis bacilli.

**In vivo NK cell depletion.** Rabbit anti-asialo GM1 antibody (WAKO Chemical Company, Richmond, VA) was used to deplete NK cells as previously described (8). The commercial product was reconstituted with 2 mL of sterile water, and a 1:10 dilution was used for injection. Mice were injected with 200 µL (50 µg/mouse) of the anti-asialo GM1 antibody preparation or control rabbit serum (Sigma) via intraperitoneal (i.p.) route for two doses at 3 days prior to challenge and 2 days post challenge with B. anthracis Ames spores. Depletion was confirmed by flow cytometric detection of the mouse NK cells, at 2 and 5 days post depletion, in two uninfected mock-depleted and two uninfected NK-depleted animals. Animals were sacrificed and spleens were processed to single cell suspensions as previously described (5). Detection of murine NK cells was performed by labeling of total splenocytes with the DX5 marker, using a directly conjugated fluorescent (FITC) antibody by using flow cytometry procedures that have previously been described (18).

**Animal Infections.** Specific pathogen-free (SPF) 6–8-week-old female Swiss Webster mice were purchased from Taconic Farms and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Resources Center at UTMB. Mice were anesthetized with an 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 LD_{50} (approximately 5.6×10^4 CFU) of B. anthracis Ames strain spores in 40 µl 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. Two animals per treatment per time point were used in survival studies, while 5 animals from each treatment group were sacrificed at 18 and 36 h for evaluation of pathology and tissue and blood bacterial load. 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.

Assessment of tissue bacterial load. At 18 and 36 h post-infection, infected mice were anesthetized, followed by euthanasia using CO₂ narcosis. Cervical dislocation was subsequently performed to ensure death as approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee. 500 µl of blood was taken first from 5 mice via cardiac puncture and placed in 1.3 ml micro tubes coated with EDTA. After blood was collected, lungs were aseptically removed from 5 mice and placed in 1 ml of PBS in 50-ml Kendall tissue homogenizers (Kendell, Mansfield, MA). Following homogenization of the tissues in the biological safety cabinet in our CDC-approved biological safety level BSL-2 laboratory or animal BSL-3 facility, serial dilutions of blood and lung samples were prepared in PBS, and 100 µl of each dilution was plated on 5% sheep blood agar plates (BD Biosciences, Franklin Lakes, NJ). CFU were determined following 48 h growth as previously described (22). Additionally, an aliquot of blood and disrupted tissue was also used to inoculate liquid growth media to determine if bacteria were present. Samples were identified as positive or negative for bacteria following 48 h growth in liquid media and CFU enumeration on blood agar.

Pathology. At 18 and 36 h post-infection, aseptic collection of lungs was performed as described above. The tissues were fixed in 4% paraformaldehyde for 48 h and tested for viable bacteria by plating on blood agar plates. Tissue sections were routinely processed, embedded
in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) and evaluated by light microscopy. Tissue lesions were scored based on a severity scale of minimal, mild, moderate, and marked; the scale correlated with estimates of lesion distribution and extent of tissue involvement (i.e., minimal = 2-10%; mild >10-20%, moderate >20-50%, severe >50%). Acute inflammation indicated the presence of polymorphonuclear leukocytes (PMN). In some sections, bacteria consistent with *Bacillus anthracis* were present although bacterial special staining was not performed. This work was performed at the University Of Texas M.D. Anderson Cancer Center.

Statistics. Data are shown as the treatment mean ± SEM. Statistical differences were determined using a one-way ANOVA followed by a Dunnett’s multiple comparison test for group comparisons (GraphPad Software v5.0, San Diego, CA, USA). Statistically significant values are designated as follows: *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \).

RESULTS

**Human NK cells mediate killing of pathogenic B. anthracis bacilli.** To determine the potential for human NK cells to have a role in host defense against *B. anthracis*, peripheral blood NK cells were isolated from healthy human donors. Isolation of NK cells by magnetic bead-based procedures yielded highly purified populations (range of 95-99%) as shown by a representative flow plot in Fig. 1A. *B. anthracis* spores were not sensitive to effects of NK cells, either within infected monocytes or the extracellular media, as shown in Fig. 1B. However, NK cells were able to mediate killing of intracellular, and surprisingly, even extracellular *B. anthracis* in the vegetative form, within 24 h of co-culture as shown in Fig. 1C and D. NK cells in close contact with free *B. anthracis* bacilli could be observed by scanning and transmission electron
microscopy as shown in supplementary Fig. 1 and 2. NK cell reduction of CFU in infected monocytes was substantial, with a 3 log-fold reduction observed at an effector (E) to target (T) ratio of 5:1. A significant (p<0.05) reduction of intracellular bacteria was seen for E:T ratios of 5:1, 1:1, and 0.5:1 and for reduction of extracellular bacteria at E:T ratios of 1:1 and 5:1 (Fig. 1C). For subsequent experiments, a 5:1 E:T ratio of NK cells to infected MN or free bacteria was used.

*B. anthracis* toxins do not impair NK cell cytotoxic/antibacterial function. *B. anthracis* toxins, lethal toxin (LT) and edema toxin (ET) encoded by the pX01 plasmid, are major contributors to pathology during anthrax infection. Because previous studies have shown that these toxins can affect the functions of other immune cells (2, 5-7, 13), we sought to determine whether exposure to these toxins also had an effect on NK cells. To determine general effects of toxins on NK cell viability, IL-15 (an important homeostasis and growth factor for NK cells) was added to purified NK cells treated with toxins or left untreated. After 24 hours of exposure, the % of dead and apoptotic cells due to toxin treatment was compared to the spontaneous death/apoptosis in the NT group (Fig. 2A). Surprisingly, the toxin treated cells were moderately more viable than the non treated group, and this effect was significant with LT. As shown in Fig. 2, the antibacterial activity of NK cells against *B. anthracis* bacilli (Fig. 2B) and host cell targets (Fig. 2C) is not impaired due to exposure to anthrax toxins. Though NK cells treated with LT did not mediate a significant reduction of bacilli compared to the NT group (Fig. 2B), these effects were minimal and were similar to observations observed following treatment with the PA component. In all measurements, the effects of LT + ET were evaluated to determine the potential for additive or synergistic effects and none were noted (data not shown). Thus, viability and CTL function of NK cells are not impaired due to 24 h of exposure to LT, ET, or both.

**LT suppresses NK cell activation of IFN-γ.** Here we evaluated the potential for toxins to interfere with activation of NK cell proliferation and effector molecule expression in response to
positive stimuli. In these experiments human IL-15 was again used as positive stimuli to activate proliferation and effector molecule expression. The same measurements were simultaneously performed using human T cells from the same donor, as LT and ET have been previously shown to suppress murine T cell activation (5). T cells were activated with magnetic bead-conjugated antibodies to CD3 and CD28. As shown with representative donor data in Fig. 3A, T cells were strongly activated to proliferate (Ki67 cell cycle marker) and expressed increased levels of granulysin and IFN-γ, in response to TCR engagement. Exposure to LT reduced T cell expression of both granulysin and IFN-γ upon activation, while proliferation was not affected (Fig. 3A). ET did not have suppressive effects on the T cell parameters measured here, while treatment with LT + ET produced effects similar to those of LT (data not shown). Consistent with the responses observed in T cells, NK cells proliferated following activation with IL-15 and this response was not impaired by LT (Fig. 3B) or ET (data not shown). In contrast to T cells, NK cell expression of granulysin was not significantly impaired by LT though moderate suppression was observed in some donors (Fig. 3B, C). LT exposure suppressed IFN-γ expression by activated NK cells of several donors, and reached statistical significance with inclusion of 6 individual donors (Fig. 3B, D). Overall, our results show that similar to T cells, LT suppresses the capacity for NK cells to secrete IFN-γ upon activation. Expression of the antibacterial granulysin protein by activated NK cells, at least in response to IL-15, however, is not impaired by toxin exposure.

**Murine NK cells also have antibacterial activity against B. anthracis.** The antibacterial activity of murine NK cells against *B. anthracis* has not been described. To determine the potential to study the role of NK cells in a murine model of *B. anthracis* infection, we used DX5+ cells from isolated from splenocytes of healthy mice and repeated the antibacterial activity experiments that we performed with human NK cells. As illustrated in Fig. 4 A, B, murine NK cells were able to mediate killing of intracellular and extracellular *B. anthracis* within 24 h, at a 5:1 E:T ratio.
Depletion of NK cells does not impact survival or disease pathology of Swiss Webster mice following inhalational anthrax infection. Female Swiss Webster mice were dosed with 50 μg of anti-asialo GM1 to deplete NK cells 72 hrs prior to, and 48 h post infection with, 5 LD₅₀ of *B. anthracis* Ames spores. In the depleted group, DX5+ cells could not be detected in the spleen of animals as compared to the non-depleted group (Fig. 5A). Survival data of NK cell depleted and non-depleted Female Swiss Webster mice following intranasal challenge yielded no significant differences between the two groups (Fig. 5B). The mean time to death in the NK cell depleted group was 2 days vs. the non-depleted control group with a mean time to death of 2.5 days post challenge. This experiment was performed a total of 3 times (twice by intranasal route and once by aerosol distillation) with similar results.

After confirming the presence of *B. anthracis* in the lungs and blood, we evaluated the pathological consequences of infection in the lungs. The lungs were removed from both NK cell depleted and non-depleted mice infected with *B. anthracis* Ames spores at 18 and 36 h and processed for H&E staining. In general, lesions consistent with anthrax infection (edema, inflammation, presence of bacteria) were present in both NK cell depleted and non-depleted mice (Fig. 5C, D). The lesions were more frequent and severe at 36 h and not significantly different between the two groups of mice. Thus, depletion of NK cells does not alter the survival or pathology in this model of inhalational anthrax.

Depletion of NK cells increases bacteremia in murine model of inhalational anthrax. To determine the potential for NK cells to alter bacterial burden, we first evaluated the number of *B. anthracis* bacteria within the lung and blood at 18 and 36 h postinfection. Following intranasal infection with a 5 LD₅₀ dose (5.6×10⁶ CFU) of Ames spores, the lungs and blood were evaluated at 18 and 36 h post-infection for bacterial burden. As shown in Fig. 6B and C, at 18 h post-infection the lungs of control and NK-depleted animals contained measurable *B. anthracis* organisms while bacteria were not detected in the blood. By 36 h, the lungs and blood from both
treatment groups had measurable *B. anthracis* bacteria. In animals lacking NK cells, the lungs contained a non-significant increase in average bacterial numbers ($3.7 \times 10^6$ CFU/ml) compared to bacterial numbers ($1.8 \times 10^6$ CFU/ml) in non-depleted animals. In contrast, a dramatic increase in *B. anthracis* numbers was observed in the NK cell-depleted animals as compared to the control animals (Fig. 6 B, C). Thus, the absence of NK cells results in a significant increase in bacteremia by 36 hours.

**DISCUSSION**

The disease progression during inhalational anthrax is extremely rapid and occurs prior to development of an acquired immune response by the infected individual. Identifying avenues for augmentation of the innate immune system to complement antibiotic treatments, then, may lead to development of effective clinical interventions. NK cells are an important effector cell in the innate immune repertoire and have antibacterial activity that could contribute to host immunity early in infection. A role for NK cells has been identified for host defense to several bacterial pathogens including *M. tuberculosis*, *Listeria monocytogenes*, *E. coli*, and *Salmonella Typhimurium* (1, 4, 14, 16, 17, 24). The clinical potential for NK cell-based immune modulation is highlighted by results from a recent study, where targeted activation of NK cells was shown to generate protective innate immunity to pneumonic *Francisella tularensis* (26). Development of this type of strategy to augment innate immune mechanisms could reduce post-exposure morbidity and mortality from inhalational anthrax. In this study, we assessed the potential effector function of NK cells in the immune response to *B. anthracis* infection *in vitro* and sought to identify the significance of NK cells *in vivo* in a murine model of inhalational anthrax infection.

Our results provide the first demonstration that human NK cells have antibacterial activity against *B. anthracis* bacilli. The reduction of *B. anthracis* within infected monocytes that we
observed is consistent with other reports of antibacterial NK cell activity against bacterial pathogens that target the monocyte/macrophage cell lineage (4, 29). NK cells mediate antibacterial effects on infected host cells by direct cytolytic activity or though indirect action of secreted mediators (e.g. IFN-\(\gamma\)). NK cells are activated for cytotoxicity through separate and overlapping mechanisms that augment cytotoxic gene expression and activate degranulation against infected host cells (28). Ligation of natural cytotoxicity- or activating-receptors on NK cells by complementary ligands on the host cell triggers attack against target cells (21, 28). An interesting observation in our studies, however, is that NK cells also have significant antibacterial activity against extracellular \textit{B. anthracis}. The potency of the antibacterial activity against extracellular bacteria was less than that observed against infected monocytes; however, we still observed up to 50% reduction of bacterial CFU in cell culture media at a 5:1 E:T ratio. The mechanisms whereby NK cells would be activated to mediate direct damage of free bacteria are poorly understood. Natural cytotoxicity-receptors (NCR) are known to bind several viral as well as host cell proteins. An important member of the NCR family, NKp44, has been shown to directly bind ligands on the surface of \textit{Mycobacterium tuberculosis} and \textit{Pseudomonas aeruginosa} (11). Potentially, undefined \textit{B. anthracis} ligands could also be recognized by one of the NCR family of receptors expressed by NK cells. Identifying the molecules and receptors that mediate these interactions between NK cells and \textit{B. anthracis} or \textit{B. anthracis}-infected monocytes/macrophages may have relevance to design of effective immune modulation strategies.

Consistent with previous reports of anthrax toxin effects on murine cells (5, 15, 20), we observed suppression of human T cell and NK cell expression of IFN-\(\gamma\) due to LT exposure. This is important, as IFN-\(\gamma\) activates the host macrophage for enhanced intracellular clearance. We also provide the first report, to our knowledge, that LT inhibits activation of granulysin in human T cells. We have previously reported that granulysin, stored in the cytotoxic granules of NK cells
and cytotoxic T cells, has bactericidal activity against *B. anthracis* (10). Granulysin can directly damage bacterial membranes and can mediate both intracellular and extracellular bactericidal effects (25). In contrast to T cells, a significant effect of LT on NK cell granulysin expression was not observed in our study, though a significant effect may be realized upon inclusion of a larger donor pool given the non-significant decrease that we observed.

With the exception of IFN-γ, our studies show that anthrax toxins appear to have minimal effects on several other measures of NK cell effector function, including proliferation, viability, direct antibacterial activity, or cytotoxicity. LT and ET have well described immunosuppressive effects on several leukocyte populations, including T cells, B cells, NKT cells, and professional antigen presenting cells (2, 5, 13). LT has been shown to increase apoptosis and interfere with MAPK signaling resulting in decreased bactericidal activity (ROS) and cytokine production. In addition, LT can also cause a decrease in leukocyte proliferation and cellular activation as well as decrease B cell antibody production (5). In support of our findings, Joshi and colleagues recently reported a lack of LT effects on murine NK cells despite observing LT-mediated suppression of the NKG2D activation receptor on Natural Killer T cells (NKT) cells (20). The anthrax ET can act alone or synergistically with LT to amplify effects by also causing an increase in cAMP, such as occurs correspondent with decreased phagocytosis by toxin-exposed macrophages (2, 6). We did not observe any effects of ET on NK cell proliferation or function, despite use of both toxins at concentrations previously shown to impair leukocyte activation (5, 15, 20). Thus, NK cells may be less susceptible to the immunosuppressive effects of anthrax toxins and could potentially be targeted to clinically augment innate immune function.

Based on our observations of potent antibacterial responses of human NK cells against *B. anthracis*, we further explored the potential of these responses in vivo using a mouse model. The rabbit is the most relevant model for study of inhalational anthrax pathogenesis and for testing vaccine and therapeutics. Due to the availability of reagents, however, the mouse is the
model most often used for studying host defense against *B. anthracis*. Using isolated NK cells from healthy mouse spleen, we observed that, similar to human cells, murine NK cells also have potent bactericidal activity against extracellular and intracellular *B. anthracis*. Depletion of NK cells in a well-established murine model of inhalational anthrax, however, did not alter the course of infection or the pathology. This was not unexpected, as depletion of a single leukocyte subset in an immune competent mouse frequently does not significantly alter the survival outcome in infection models.

The primary route of infection during inhalational anthrax is the lungs. However, we did not observe differences in lung CFU or lung pathology due to NK cell depletion at 18 or 36 h post infection. We did however; see a dramatic and significant difference in the number of bacterial CFU in the blood of animals at 36 h post infection. Animals lacking NK cells had a significantly higher bacterial load in the bloodstream versus animals with a fully intact immune system. NK cell numbers in the lung are likely to be very limited in early infection at the time that rapid uptake and trafficking of spores is occurring. In contrast, NK cells are most abundant in the blood and would have plentiful opportunity to interact with circulating vegetative bacteria. Taken together, these finding suggests that NK cells play a complementary, rather than obligatory, role in innate immunity to *B. anthracis* in the murine model.

In summary, NK cells have antibacterial activity against *B. anthracis* bacilli or infected monocytes that may contribute to innate host defense. Anthrax LT suppresses human NK cell expression of IFN-γ, while many other NK effector functions are not significantly affected by either LT or ET. Depletion studies in a murine model of inhalational anthrax suggest a role for NK cells to control bacteremia. Augmentation of NK cell function, then, should be explored as a potential avenue for therapeutic intervention during *B. anthracis* infection.
ACKNOWLEDGMENTS

This research was supported by the NIH NIAID contract (NO1-AI-30065), the Department of Microbiology and Immunology (UTMB), the James W. McLaughlin Fellowship Fund (UTMB), and the NIH/NIAID Prematriculation Reinforcement Enrichment Program (1R25GM069285). We would also like to thank Mark Griffin from the Department of Microbiology and Immunology at UTMB for providing assistance in the core laboratory for flow cytometry studies.


FIG. 1. Antibacterial activity of human NK cells against *B. anthracis*. Natural killer cells were isolated by magnetic bead-based antibody from PBMC of healthy human donors. Shown in (A) flow cytometric analysis of NK cell purity, side scatter and forward scatter characteristics of isolated cells and % of isolated cells positive for the human NK marker CD56. The CFU of *B. anthracis* (Ames strain) was assessed following *in vitro* co-culture of primary human NK cells with (B) spore-infected autologous monocytes or extracellular spores, (C) *B. anthracis*-infected autologous monocytes, or (D) extracellular bacilli. An effector to target (E:T) ratio of 5:1 was used for determination of antibacterial activity by NK cells (effectors) against spores or spore-infected monocytes (targets). Activity of NK cells against vegetative bacteria within monocytes or growing in media was determined using E:T cell ratios of 0.25:1, 0.5:1, 1:1, and 5:1. Enumeration of CFU was performed by limiting dilution following 24 h of CD56+ NK cell exposure to infected monocytes, or extracellular vegetative bacteria or spores. Values shown are the means ± SEM of results from 4 individual donors, performed in duplicate. *p<0.05, ***p<0.00 indicate statistically significant differences in CFU numbers between control samples and samples cultured with NK cells.

FIG. 2. Effect of *B. anthracis* LT and ET on NK antibacterial and cytotoxic function. Natural killer cells were isolated by magnetic bead-conjugated antibody from PBMC of healthy human donors and cultured with rIL-15 (15 ng/ml). Shown in (A) summarized flow cytometric analysis of NK cell viability, displayed as % propidium iodide+annexin V+ (dead/apoptotic) NK cells following 24 h exposure to no toxin (NT), protective antigen (PA) component, lethal toxin (LT), or edema toxin (ET). Shown in (B), the CFU of *B. anthracis* (BA) bacilli following 24 h *in vitro* co-culture in media or with primary human NK cells (E:T ratio of 5:1) exposed to NT, PA, LT, or ET. Shown in (C), cytotoxicity of NK cells treated with NT, PA, LT, or ET against the classical NK cell target K562 cells, displayed as % PI positive K562 cells. Values shown are the...
means ± SEM of results from 4 individual donors. *p<0.05, indicate statistically significant differences due to treatment compared to NT (A) or BA (B).

FIG 3. *B. anthracis* LT suppresses activation of NK cell IFN-γ and T cell granulysin.

Purified NK cells or PBMC were rested (media) or exposed to PA (negative control), LT, or ET and activated with IL-15 (15 ng/ml) or antibodies to CD3 and CD28. Shown are flow cytometric results of (A), CD3^+CD56^- (T cell, n=4) and (B), CD56^+CD3^- (NK cell, n=6), events from a representative donor. The results displayed are the effects of LT on activation of T cell or NK cell proliferation (Ki67 proliferation marker), and expression of intracellular granulysin and IFN-γ activated by IL-15 or CD3/CD28. Shown in C and D are summarized flow cytometry results for expression of granulysin and IFN-γ by NK cells as affected by exposure to LT or ET. Summarized values shown in C and D are the means ± SEM of results from 6 individual donors. *p<0.05, indicate statistically significant differences due to treatment compared to the NT treatment.

FIG. 4. *In vitro* antibacterial activity of murine NK cells against *B. anthracis*. NK cells were isolated from single cell suspensions of spleens from Swiss-Webster mice by magnetic bead-based isolation using antibody to DX5. CFU of extracellular and intracellular *B. anthracis* was assessed *in vitro* following the addition of murine DX5^+ NK cells to (A) infected monocytes or (B) extracellular bacteria, at an E:T ratio of 5:1. The results indicate the mean and SD from 6 mice pooled in to 3 groups. Values are means ± SEM of these determinations. Enumeration of CFU was performed by limiting dilution following 24 h of NK cell exposure to infected monocytes, or extracellular vegetative bacteria or spores. Values shown are the means ± SEM of results from 6 animals. *p<0.05, **p<0.001 indicate statistically significant differences in CFU numbers between control samples and samples cultured with NK cells.
FIG. 5. In vivo depletion of NK cells does not alter survival of animals infected with *B. anthracis*. Female Swiss Webster mice were inoculated with 50 µg of control rabbit immunoglobulin serum or anti-asialo GM1 to deplete NK cells 72 h prior to, and 48 h post, infection with *B. anthracis* Ames spores. Shown in (A), flow cytometric analysis for NK marker DX5, confirming splenic depletion of NK cells in representative animals (n=2 per treatment group in each of 3 independent experiments), and (B) the mean time to death in the NK cell depleted and mock-depleted control animals post challenge, representative of one of three independent experiments. Shown in (C) is peribronchiar and perivascular edema with acute inflammation and bacilli in the lung of a mouse infected with *B. anthracis*, and (D) higher magnification of C showing edema, inflammatory cells, and bacilli present in the lung of this mouse.

FIG. 6. Depletion of NK cells increases bacteremia in murine model of inhalational anthrax. Female Swiss Webster mice were inoculated with 50 µg of control rabbit immunoglobulin or anti-asialo GM1 to deplete NK cells 72 h prior to, and 48 h post, infection with *B. anthracis* Ames spores. Mock- and NK cell-depleted Swiss Webster mice (5 animals per group per time point) were sacrificed at 18 and 36 h following intranasal infection with *B. anthracis* (Ames) spores. Enumeration of CFU from disrupted tissue was performed by limiting dilution plating on blood agar. Shown are bacterial CFU (A) in the lungs, and (B) in blood, at 18 and 36 h post infection. Shown in (C) is an assessment for the presence of blood bacteria following 48 h growth in selective liquid media, shown as the % of animals bacteremic, at 18 and 36 h post infection. ***p<0.001 indicate statistically significant differences in CFU numbers between mock and NK-cell depleted animals.
**Fig. 1**

**A**

B. anthracis spores

- SSC
- CFU/mL (log)
- 97%

**B**

B. anthracis spores

- CFU/mL (log)

<table>
<thead>
<tr>
<th>Effector to target ratio</th>
<th>0</th>
<th>0.5:1</th>
<th>1:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular spores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular B. anthracis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**C**

B. anthracis-infected monocytes

<table>
<thead>
<tr>
<th>Effector to target ratio</th>
<th>0</th>
<th>0.5:1</th>
<th>1:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore-infected monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK cells, ET of 5:1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

Extracellular B. anthracis

- CFU/mL (log)

<table>
<thead>
<tr>
<th>Effector to target ratio</th>
<th>0</th>
<th>0.5:1</th>
<th>1:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effector to target ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2

A

% NK cells, dead/apoptotic

NT PA LT ET

B

CFU (log) /ml

NT PA LT ET

NK, ET 5:1

C

% PI+ K562 targets

No Toxin
Protective Antigen
Lethal Toxin
Edema Toxin

Effector to target ratio
Fig. 4

- A. B. anthracis-infected monocytes
- B. Extracellular B. anthracis

<table>
<thead>
<tr>
<th>Effector to target ratio</th>
<th>CFU/ml (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>5:1</td>
<td>6</td>
</tr>
</tbody>
</table>

* ***

* *
Fig. 5

A

Mock-depleted

NK-depleted

DX5+ (NK) cells

B

% survival

Non-Depleted

NK Cell Depleted

Days post infection

C

D

NK Cell Depleted

Days post infection

% survival

Non-Depleted

NK Cell Depleted

DX5+ (NK) cells

Mock-depleted

NK-depleted

DX5+ (NK) cells

Mock-depleted

NK-depleted

DX5+ (NK) cells
Fig. 6

- Blood (CFU): CFU/ml (log)
  - ND: Non-Detect
  - Depleted

- Lung (CFU): CFU/ml (log)

- % Positive for bacteremia
  - 18 h, 36 h

- Blood (bacteremia): Depleted, Non-Depleted