Published in final edited form as:

Eur J Immunol. 2014 March ; 44(3): 752–762. doi:10.1002/eji.201343784.

The IL-23/Th17 axis is involved in the adaptive immune response to *B. anthracis* in humans

Kristina M. Harris¹, Girish Ramachandran^{2,3}, Subhendu Basu^{2,3}, Sandra Rollins¹, Dean Mann¹, and Alan S. Cross^{2,3}

¹Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, USA

²Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD, USA

³Department of Medicine University of Maryland School of Medicine, Baltimore, MD, USA

Summary

The neutralization of toxins is considered essential for protection against lethal infection with *B. anthracis* (BA), a select agent and bioterrorism threat. However, toxin neutralizing activity (TNA) alone would not be expected to provide sterile immunity. Therefore, we hypothesized that the development of an adaptive immune response against BA is required for bacterial clearance. We found that human monocyte-derived dendritic cells (hDCs) kill germinated BA bacilli, but not non-germinated BA spores. hDCs produce IL-1 β , IL-6, IL-12 and IL-23, and these cytokines are differentially regulated by germination-proficient versus germination-deficient BA spores. Moreover, the IL-23 response to BA spores is regulated by IL-1R-mediated signaling. hDCs infected with germinating BA spores stimulated autologous CD4⁺ T cells to secrete IL-17A and IFN- γ in a contact-dependent and antigen-specific manner. The T-cell response to BA spores was not recapitulated by hDCs infected with germination-deficient BA spores, implying that the germination of spores into replicating bacilli triggers the proinflammatory cytokine response in hDCs. Our results provide primary evidence that hDCs can generate a BA-specific Th17 response, and help elucidate the mechanisms involved. These novel findings suggest that the IL-23/Th17 axis is involved in the immune response to anthrax in humans.

Keywords

Bacillus anthracis spores; human dendritic cells; IL-1 β ; IL-23; Th17

Corresponding author: Alan S. Cross, M.D., Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore St., Baltimore, MD 21201, Ph: +1-410-706-5328, Fax: +1-410-706-6205, across@medicine.umaryland.edu.
Current Addresses: Kristina Harris, Immune Tolerance Network, 3 Bethesda Metro Center, Suite 400, Bethesda, MD 20814, kharris@immuntolerance.org; Subhendu Basu, Emergent BioSolutions, 2273 Research Blvd., Suite 400, Rockville MD 20850, basus@ebsi.com

Conflict of Interest

The authors declare no financial or commercial conflict of interest. S. Basu was an Assistant Professor of Medicine at the University of Maryland, Baltimore at the time these studies were conducted. He is currently employed with Emergent BioSolutions, which manufactures and markets the FDA-licensed anthrax vaccine BioThrax[®] (Anthrax Vaccine Adsorbed).

Introduction

Given the relative ease of production and dissemination, as well as the lethality of respiratory infection, *B. anthracis* (BA) remains a leading bioterrorism threat agent. In addition to its status as a select agent, this bacterial pathogen presents unique challenges to the host. The environmentally-acquired spore form of BA must germinate within host phagocytes to vegetative bacilli that express highly active toxins that mediate many clinical manifestations of disease. Protective antigen (PA) combines with either lethal factor (LF) or edema factor (EF) to form the potent toxins, lethal toxin (LT) or edema toxin (ET), respectively. The LT interferes with intracellular signaling pathways, which initiate the host pro-inflammatory cytokine response [1]. While LT directly activates the inflammasome leading to IL-1 β expression and pyroptosis, the BA spore activates the inflammasome by a different, IFN- β -dependent pathway. The IL-1 β generated by this latter pathway is required for host defenses against BA [2].

Current and next generation vaccines target the PA protein by which the LT and ET bind and enter host cells. Vaccine-induced serum toxin neutralizing activity (TNA) is considered a measure of vaccine efficacy. While neutralization of BA toxins is an essential element in protection against lethal BA infection, there is an ongoing risk of anthrax disease unless there is clearance of bacteria from the host. Few studies have characterized the mechanisms by which the host generates an adaptive immune response to infection with BA spores, especially in humans. Such studies must address how APCs such as hDCs process BA so that its antigens are presented to the T cells in order to generate a protective adaptive immune response.

Our laboratory has been interested in the early host response to BA and measures to enhance host anti-BA defenses. We now describe novel mechanisms by which infection of BA spores in human DCs initiates a Th17 adaptive immune response, which may lead to bacterial clearance and host protection.

Results

Human monocyte-derived DCs engulf and kill *B. anthracis*

To study their ability to phagocytose and kill BA spores, we challenged human monocyte-derived DCs (hDCs) with germination-proficient or germination-deficient (*gerh*) BA spores in vitro and enumerated the number of viable intracellular bacteria within the hDCs over time. As seen by electron microscopy, BA spores were visible within hDCs as early as 30 minutes post-infection at a MOI of 1:1 (Fig. 1A). The uptake of BA spores by hDCs was not uniform and some hDCs had multiple spores (Fig. 1Ai) whereas others had two spores (Fig. 1Aii) or no spores at all (data not shown).

Murine macrophages kill BA when infected with germination-proficient BA spores, but not mutant spores that are defective in their ability to germinate [3]. Therefore, we examined the capacity of hDCs to kill BA by infecting them with germination-proficient BA spores and BA spores (*gerh*), the latter with a deletion in the germination operon that results in delayed germination. We found that at early time points post-infection (16 hours), hDCs

efficiently killed BA that had germinated into vegetative bacilli, but were incapable of killing *gerh* spores (Fig. 1B). Notably, non-germinated spores could be recovered from viable hDCs up to 48 hours following infection with *gerh* spores as evidenced by their resistance to heat (65°C for 30–45 minutes). In contrast, infection with increasing doses of germination-proficient BA spores resulted in a marked decrease in the viability of hDCs (data not shown). This suggests that hDC viability is adversely affected when increasing numbers of intracellular BA spores germinate into viable BA bacilli.

BA infection elicits Th17- and Th1-promoting cytokines from human moDCs

Upon encountering microbes or microbial products, DCs produce cytokines and chemokines, and upregulate surface expression of HLA and costimulatory molecules that direct the adaptive immune response. The cytokine profile of responding DCs largely dictates the type of T helper cell response that will ensue. Thus, it is paramount that DCs exposed to bacterial pathogens, such as BA, produce proinflammatory cytokines that drive powerful Th17 and Th1 responses considered essential for host protection.

Increasing evidence identifies IL-1 β , IL-6, and IL-23 as key mediators in the development and maintenance of human Th17 responses [4–8]. This relatively newly described Th-cell subset, which produces IL-17 cytokines as well as IL-22 and/or IFN- γ , has been implicated in a number of inflammatory diseases [8–10], but is also considered critical for protection against bacterial and fungal pathogens [11–15]. Given the importance of IL-23 in Th17 activation and host protection from bacterial infections [16;17], we looked to see if BA spores stimulate hDCs to produce IL-23 and other proinflammatory mediators reported by Pickering et al.[18].

hDCs from healthy donors were infected with increasing doses of germinating BA spores or mutated *gerh* spores and culture fluids analyzed for IL-23, as well as IL-1 β , IL-6 and IL-12 proteins the following day. We found that BA spores triggered hDCs to secrete IL-23 in a dose-dependent manner (Fig. 2A). Consistent with previous reports, IL-1 β , IL-6, and IL-12 were also increased in culture fluids from hDCs infected with both germination-proficient and -deficient BA spores (Fig. 2B), and DC activation and maturation markers were upregulated on the surface of BA-infected DCs (Fig. 2D) [18–20]. The percent of CD80 and CD83-expressing hDCs were increased, whereas the densities of HLA-DR and CD40 molecules were augmented on the surface of hDCs infected with BA.

Somewhat surprisingly, germinating BA spores and mutated *gerh* spores stimulated hDCs to secrete similar amounts of IL-1 β and IL-23, whereas germinating BA spores induced superior production of IL-6 and IL-12 (Fig. 2B). These intriguing results suggest that two different mechanisms trigger production of IL-1 β /IL-23 and IL-6/IL-12 in hDCs, which has also been reported for hDCs infected with the intracellular gram-negative bacteria, *Chlamydia trachomatis* [21]. Given that IL-12 and IL-23 responses to agonists of PRRs are regulated differently in monocyte-derived hDCs [22–25], one could speculate that mutated *gerh* spores lack additional signaling components found in germinating spores that augment IL-6 and IL-12 responses. It is also possible that the spore form induces IL-1 β and IL-23, but that only the bacillary form induces IL-6 and IL-12.

We were the first to show that IL-1 β controls IL-23 production in human monocytes [26]. Since germination-proficient BA as well as *gerh* spores stimulated hDCs to produce similar levels of these two cytokines (Fig. 2B), we predicted that the IL-23 response of hDCs to BA spores was regulated by IL-1 receptor-mediated signaling. Addition of the naturally occurring IL-1 receptor antagonist (IL-1ra) to hDCs infected with BA spores, significantly inhibited IL-23 production (Fig. 2C), indicating that the IL-23 response of hDCs to BA spores is, at least partly, mediated by IL-1 receptor signaling. Thus, IL-1 β may play a critical role in the immune response to BA in humans at both the level of DC activation i.e. autocrine cytokine production and Th17-cell differentiation.

hDCs loaded with germinating BA spores trigger contact-dependent and antigen-specific Th17 responses

The Th17 pathway contributes to host protection against extracellular bacterial and fungal infections [27]. As such, patients with defects in genes involved in Th17 development suffer from recurrent extracellular microbial infections [28;29]. The cytokines IL-1 β , IL-6 and IL-23 are important mediators of Th17 (and memory Th1) responses [4;5;8;9;30], whereas IL-12 drives differentiation of Th1 cells [22;31]. Since hDCs infected with germinating BA spores produced all of these cytokines, we tested the hypothesis that BA-infected hDCs stimulate BA-specific Th17 and Th1 responses.

Highly pure CD4⁺ T cells (>92%) were obtained from PBMC of healthy individuals using a negative selection kit from Stemcell Technologies (Fig. 3A). Th17 cells, which express IL-1RI, IL-23R and CCR6 could be identified in a portion of circulating CD4⁺ T cells isolated from all healthy individuals tested (Fig. 3A). CD4⁺ T cells were then cultured with autologous hDCs infected with no spores, *gerh* or BA spores in the presence of recombinant human IL-7 and IL-15 for seven days to facilitate induction of a primary T-cell response. Semi-permeable transwell inserts were used to prevent CD4⁺ T cells from contacting BA-infected hDCs, as a means of assessing contact-dependent versus contact-independent, cytokine-induced T-cell activation. At the end of the week long co-culture, cell-free supernatants were collected and analyzed for secreted IL-17A and IFN- γ proteins, the hallmark cytokines of Th17 and Th1 cells, respectively.

As anticipated, hDCs infected with BA spores stimulated IL-17A and considerable quantities of IFN- γ in co-cultures with autologous CD4⁺ T cells (Fig. 3B). Importantly, IL-17A and IFN- γ responses were significantly inhibited when cellular contact between CD4⁺ T cells and BA-infected hDCs was prevented using 0.4 μ m semi-permeable transwell inserts (Fig. 3B). These results provide primary evidence that BA-infected hDCs induce IL-17A and IFN- γ , suggesting that the responses are likely contact-dependent and not merely the result of soluble mediators released by BA-infected hDCs.

Although IL-17A and IFN- γ responses tended to be augmented in co-cultures containing hDCs infected with germinating BA spores compared with those of co-cultures with hDCs infected with germination-deficient *gerh* spores, the differences were not consistent for all individuals tested. This finding is somewhat unexpected, given the disparate production of IL-6 and IL-12 we observed in hDCs infected with BA spores compared with that of *gerh* mutants (Fig. 2B). However, it is highly possible that during the week long co-culture

necessary for T-cell priming, the mutated *gerh* spores germinated, thereby negating the distinct cytokines responses we detected the day following infection with germinating BA spores or *gerh* mutants (Fig. 2B). Nevertheless, these findings indicate that both IL-17-producing Th17 and IFN- γ -producing Th1 cells respond to BA in humans, and in addition, raise a series of questions regarding the cell subset, specificity, and recall potential of the CD4⁺ T-cells responding to BA-infected hDCs.

To address these central issues, we conducted the following experiments. Cells from the primary studies described above that were primed with hDCs infected with no spores, *gerh* mutants, or BA spores, were harvested, washed to remove cytokines, counted and re-plated in fresh culture media for an additional two days with a second preparation of autologous hDCs infected with no spores, *gerh* mutants, germinating BA spores, or BA antigens LF and PA. Secreted IL-17A and IFN- γ proteins were quantified in the culture supernatants by ELISAs and the cell source of these cytokines identified by intracellular cytokine staining and flow cytometry.

T cells primed (1° challenge) and re-stimulated (2° challenge) with BA-infected hDCs elicited nearly 2-fold greater IL-17A and IFN- γ responses compared with i) T cells primed with BA-infected hDCs and re-stimulated with uninfected, *gerh*-, LF- or PA-infected hDCs, ii) T cells primed in the absence of spores and re-stimulated with BA-infected hDCs or ii) T cells primed with *gerh*-infected hDCs and re-stimulated with BA-infected hDCs (Fig. 4A). Notably, hDCs secondarily loaded with i) mutant *gerh* spores, ii) LF, or iii) PA were incapable of recapitulating the secondary IL-17A and IFN- γ responses elicited by hDCs infected with germination-proficient BA spores (Fig. 4A). In addition, the neo-antigen keyhole limpet hemocyanin (KLH) was not effective at re-stimulating T cells primed with BA-infected hDCs and vice versa (Fig 4B, left and right panels). Taken together, these results strongly suggest that optimal BA-specific, proinflammatory CD4⁺ T-cell responses are elicited by BA spores that germinate into replicating bacilli, and the immunogenic epitope(s) are distinct from LF and PA antigens previously described by others [32–34].

IL-17A and IFN- γ can be produced by separate CD4⁺ T cells or within the same CD4⁺ T cell. Consequently, cells that produce IL-17 alone or together with IFN- γ are recognized as Th17 cells, whereas sole producers of IFN- γ are generally considered Th1 cells [35;36]. Since both of these anti-microbial cytokines were produced in a BA-specific manner, we wanted to determine whether distinct or overlapping populations were the cell source in our in vitro system. Intracellular cytokine staining was performed on T cells primed and re-stimulated with hDCs infected with germinating BA spores, while T cells primed with uninfected hDCs (no spores) and re-stimulated with BA-infected spores served as a control. To optimize intracellular cytokine detection, PMA and ionomycin were added to both conditions in the presence of monensin for the last five hours of the two day re-stimulation period, and the cells analyzed by multi-parameter flow cytometry.

CD4⁺ T-cell populations producing IL-17A alone, IFN- γ alone, or these two cytokines simultaneously comprised both culture conditions. However, the proportion of CD4⁺ T cells producing both IL-17A and IFN- γ (double producers) increased more than 2-fold in T-cell cultures primed and re-stimulated with BA-infected hDCs compared with that of T cells

primed with uninfected hDCs and re-stimulated with BA-infected hDCs (Fig 4C). These studies demonstrate, for the first time, that hDCs infected with germinating BA spores activate T helper cells that simultaneously produce IL-17A and IFN- γ , a profile indicative of pathogen-induced Th17 cells [35–37].

Discussion

While the generation of a toxin-neutralizing antibody response is considered essential for conferring protection from lethal anthrax infection, an adaptive immune response by which APCs present BA spores and/or bacilli to T lymphocytes is likely to be required for clearance of bacteria by the host. Our studies demonstrate that for the induction of a human adaptive immune response, hDCs must ingest the BA spore and efficiently kill live bacilli that germinate for presentation to T cells in a BA antigen-specific and contact-dependent manner. The hDCs, however, are unable to kill the non-germinated BA spores. hDCs, therefore, play an important role in initiating the immune response to BA and are not simply vectors for the dissemination of BA spores within the host from the site of entry [40;41].

Multiple studies report that purified anthrax toxins impair the functional activity of hDCs and T cells [42–44], yet Ingram et al. [34] found a robust T-cell response in patients previously infected with cutaneous anthrax, and Paccani et.al. provided evidence that low concentrations of anthrax ET promoted Th17 responses in human CD4⁺ T-cells primed with anti-CD3 mAb in the absence of APC-derived cytokines [45]. Therefore, we speculate that the Th17 response to hDCs loaded with germinated BA bacilli is induced by APC-derived IL-1 β , IL-6, and IL-23, and likely other biological molecules that directly or indirectly enhance T cell levels of cAMP before the anthrax toxin-induced dysfunction is detected.

A few studies, primarily in mice, have addressed the role of T cells and/or IL-17A in the immune response to BA. A CD4⁺ T-cell response in mice has been implicated in protection against capsulated, non-toxigenic spores [46]. Two compelling studies in mice recently identified a critical role for IL-17A in protection from inhalation anthrax [47;48]. However, neutrophils, and not Th17 cells, were determined to be the primary source of IL-17 in one report [48] and the Th17 response required the adjuvant activity of cholera toxin in the other [47]. In humans, PA-specific, MHC II-restricted CD4⁺ T cells were detected in individuals immunized with the AVA vaccine [32;33;49], but the T-cell response to infection with BA spores was not examined. Natural exposure to cutaneous anthrax provided long-lasting T-cell immunity in humans to anthrax toxin epitopes distinct from those that induced T-cell immunity following immunization [34], but the mechanisms involved are unclear. The adenylate cyclase toxin of *B. anthracis* was recently shown to promote Th17 development of human CD4⁺ T-cells primed with anti-CD3 mAb [45], but it is not known if this mechanism is involved in the physiological context of BA antigen presentation.

We now demonstrate that infection of hDCs by viable BA spores induces the production of IL-23, a cytokine critical to the induction of a Th17 response, in an IL-1 β -dependent manner. These findings recapitulate in human cells our observation in the murine system of the complex and critical role of IL-1 β in the immune response to BA [2]. The present studies indicate that IL-1 β plays an essential role in the proinflammatory cytokine response to BA

spores during both phases of the immune response, as evidenced by innate cell production of IL-23 and BA antigen-specific T helper cell production of IL-17A. This is not surprising given that IL-1R signaling mediates IL-23 responses in human monocytes [26].

Our findings suggest that IL-23 and a Th17 response may play a crucial role in the immune response to anthrax infection, similar to what has been reported for *M. tuberculosis* [16;50;51] and other infections [11–15]. Co-culture of BA-infected hDCs with autologous T cells for 7 days followed by a re-exposure to BA-loaded hDCs, generates a Th17 response characterized by IL-17A and IFN- γ secretion that is contact-dependent and antigen-specific.

While the identification of specific BA antigens involved in the adaptive immune response was beyond the scope of this study, our studies suggest that antigens other than PA or LF, antigens examined by Ingram et al. [34], are required, since hDCs loaded with these BA proteins did not recapitulate the adaptive immune response (Fig. 4A). The in vitro adaptive immune response required germination of vegetative bacilli in that ingestion of the germination-deficient *gerh* was unable to elicit a recall response to cells that were primed with germination-proficient BA (Fig. 4A). The observation that infection with viable, but not irradiated spores, elicited a cytokine response supports our finding [52]. Of interest, recent reports indicate that APCs sense the presence of live intracellular bacteria through the expression of either bacterial mRNA or di-cyclic-GMP, both expressed by live bacteria [53–55]. These bacterial products likely are elaborated by replicating bacteria within the intracellular environment or escape into the cytosol during their intracellular killing. Our data are consistent with the findings in these recent reports.

In summary, infection with BA presents the host unique challenges and elicits a complex array of host responses. Although acquired from the environment as a spore, the host cannot kill BA until the spore germinates within myeloid-derived cells. At the moment of early outgrowth, however, there begins a competition between the vegetative bacilli's ability to generate its toxins, which destroy essential host defenses, and the ability of the host to efficiently kill the toxin-producing bacilli. The present studies show that IL-1 β plays an integral role in generating a protective Th17 adaptive immune response through its ability to induce high levels of IL-23 from hDCs in addition to direct activation of Th17-related genes in Th cells. We speculate that neutralization of anthrax toxin, a measure of immunity to BA, may provide the host sufficient time to generate an adaptive immune response that can clear the bacteria and/or it may also facilitate the development of an adaptive immune response by enhancing the uptake and killing of BA spores and the presentation of BA antigens by APCs to BA-specific Th cells.

Materials and Methods

B. anthracis (BA) spores and antigens

The BA Sterne strain, 34F2, was originally obtained from Dr. Les Baillie (Cardiff, Wales), and the germination-deficient *gerh* strain of Sterne 34F2 was obtained from the laboratory of Dr. Phillip Hanna (Ann Arbor, MI). BA protective antigen (PA) and lethal factor (LF) were purchased from List Biological Laboratories, Inc. (Campbell, CA).

Cell isolation

PBMCs were obtained from the peripheral blood of healthy human donors under a protocol approved by the University of Maryland IRB. PBMC were isolated by density gradient centrifugation in Ficoll-Hypaque according to manufacturer's instructions. Highly pure (>90%) lymphocytes and monocytes were obtained from healthy donors as above followed by countercurrent centrifugation elutriation. Cells were viably frozen using an automated step-down freezer, and stored in liquid nitrogen until used. Upon thawing, cell viability was determined by trypan blue exclusion. Untouched CD4⁺ T cells (>95% pure) were purified from lymphocytes by magnetic cell separation using the EasySep Negative Selection Human CD4⁺ T cell Kit (Stemcell Technologies). For some experiments, monocytes were isolated from thawed PBMC by magnetic cell separation using the EasySep Negative Selection Human Monocytes without CD16 Depletion Kit (Stemcell Technologies).

Generation and infection of hDCs with BA spores

To generate immature, monocyte-derived hDCs, human monocytes were cultured in cRPMI (RPMI-1640, 10% FBS, 1% L-glutamine, 1% Pen-Strep and 20 mM Hepes) (Invitrogen) supplemented with 50 ng/ml GM-CSF plus 25 ng/ml IL-4 (both from R&D Systems) at 10⁶ cells per ml for 3 days. hDCs were collected, washed, counted, and phenotyped for DC markers CD1a, CD11c, CD40, CD80, CD83, CD86, and HLA-DR by flow cytometry. To infect hDCs, the cells were washed and resuspended in serum free X-Vivo 10 media (Lonza, MD) without any antibiotics and put into sterile 5ml polystyrene tubes at 10⁶ cells per ml per tube. BA spores (WT or *gerh*) were added to hDCs at different multiplicities of infection (MOIs) (BA:hDC::1:1, 0.5:1, 0.1:1) and incubated at 37°C with 5% CO₂ for 30 minutes. After 30 minutes, 50 µg/ml of gentamicin was added to hDCs, and incubated for an additional 30 minutes to kill any extracellular vegetative bacilli. hDCs were washed twice with 1 ml of X-vivo 10 media to remove the antibiotic and any un-internalized spores. In some experiments, live or heat killed BA bacilli were added to hDCs at an MOI of 1:1. hDCs were then used as described in the following functional assays.

hDC killing of WT and *gerh* BA spores

Following the second wash, hDCs were lysed with 1ml of sterile distilled water to release the intracellular bacteria. Viable bacteria were enumerated by plating samples on LB-agar plates and counting the number of colonies formed at time 0. For the other time points (6 and 20 hours), hDCs were incubated in X-vivo 10 with gentamicin throughout the incubation period, washed twice with 1 ml of X-vivo 10 media to remove the antibiotic, lysed with 1ml of sterile distilled water and plated on LB-agar plates. The killing was calculated by subtracting the number of bacteria at different time points from the initial number at time zero, generating a log kill value. As a negative control for killing, hDCs were infected with *gerh* BA spores, which do not germinate for extended periods of time, and therefore are not killed [2].

Cellular responses of hDC to BA spores

hDCs infected with and without BA spores (WT or *gerh*), were resuspended at 10⁶ cells per ml in X-Vivo 10 with gentamicin, and incubated at 37°C with 5% CO₂ for 20 hours.

Cellular responses were determined by secretion of pro-inflammatory cytokines by ELISAs and increased expression of activation and maturation markers on the surface of hDCs by flow cytometric analysis.

Primary and secondary T cell responses to BA

The in vitro human T cell assay was adapted from standard assays conducted for the study of human immune responses [56]. Briefly, hDCs from healthy human donors were infected with BA spores (WT or *gerh*) at a M.O.I. of 1:1, or were left untreated. 10^5 control or infected hDCs were then cultured with 10^6 autologous CD4⁺ T cells per ml in cRPMI supplemented with 5 ng/ml IL-7 and 5 ng/ml IL-15 (both from R&D Systems) in 24 well plates for 7 days (primary). In some experiments, 0.4µm semi-permeable transwell inserts (Sigma) were used to prevent cellular contact between CD4⁺ T cells and BA-loaded hDCs. To assess secondary recall T-cell responses to BA, cells from the 7 day co-culture were harvested, washed, counted and re-plated at a hDC:TC ratio of 1:10 in 24 well plates for 48 hours with a second preparation of autologous hDCs that were infected with BA spores (WT or *gerh*) at a M.O.I. of 1:1, 2.5 µg/ml PA, 1 µg/ml LF, KLH (10 µg/ml) or left untreated.

Cytokine ELISAs

Cell-free culture fluids from control and BA infected hDCs were analyzed for secreted IL-1β, IL-6, IL-12p70 and IL-23 heterodimer by ELISAs (eBioscience) following the manufacturer's protocols. Supernatants from primary and secondary exposure of T helper cells to uninfected and infected hDCs were analyzed for IL-17A and IFN-γ by ELISAs (eBioscience) following the manufacturer's protocols.

IFN-γ ELISpot

hDCs were infected at a MOI 1:1 with BA spores or mock-infected, then washed and co-cultured with autologous lymphocytes for 7 days (primary) as described above. Cells from the 7 day co-culture were harvested, washed, counted and re-plated at 10^4 cells/100ul/well with no spores, BA spores, or KLH in 96 well ELISpot plates (Millipore) coated with anti-human IFN-γ (Mabtech) capture antibody. Cell cultures were incubated at 37°C in 5% CO₂ for 48 h and developed per manufacturer's instructions (Mabtech). Plates were stored overnight in the dark at room temperature, and spots were counted using a VersaScan microplate reader (Velocity 11, Palo Alto, CA). The mean totals of IFN-γ spot-forming cells (SFC) in triplicate wells were determined and expressed as numbers of SFC per 10^4 lymphocytes.

Surface and intracellular flow cytometric analysis

Surface expression of CD1a, CD11c, CD14, CD40, CD80, CD83, CD86, and HLA-DR (all conjugated mAbs from BD Pharmingen) was monitored on hDCs pre- and post-BA infection by flow cytometry. The purity of CD4⁺ T cells and proportion of CD4⁺ T cells expressing CD45RO (BD Pharmingen), CCR6, IL-1RI and IL-23R (all from R & D Systems) at the cell surface was also determined by flow cytometry. 10,000 live cells were acquired on a Canto flow cytometer (BD Pharmingen) and analyzed using FlowJo software and appropriate conjugated immunoglobulin isotype controls (BD Pharmingen).

To detect intracellular cytokines produced by CD4⁺ T cells re-exposed to BA-infected hDCs, 50 ng/ml PMA and 750 ng/ml ionomycin were added to hDC:TC co-cultures for the last 6 hours of the 48 hour re-challenge. 10 µg/ml monensin was also added to these cocultures for the last 4 hours. Cells were labeled with anti-human CD3 and CD4 mAbs for detection of surface antigens, then fixed and permeabilized with BD Cytofix/Cytoperm solution and labeled with anti-IFN-γ, anti-IL-17A mAbs (both from eBioscience) or appropriate immunoglobulin isotype controls in BD Perm/Wash Buffer following the manufacturer's instructions. 200,000 CD3⁺ cells were acquired on a BD Canto flow cytometer and analyzed by FlowJo software.

Statistical analyses

Results are displayed as individual data and means $\pm$ SD. Statistical significance was determined using two-tailed paired Student's *t* test. *p* values < 0.05 were considered statistically significant.

Acknowledgments

This work was supported by Contract Agreement #4027 of the Maryland Industrial Partnerships Program and NIH grant U54 AI 057168, Middle Atlantic Regional Center for Excellence in Bioterrorism and Emerging Infectious Diseases to ASC.

Abbreviations

BA	<i>Bacillus anthracis</i>
LF	lethal factor
PA	protective antigen
LT	lethal toxin
ET	edema toxin
TNA	toxin-neutralizing activity
<i>gerh</i>	germination-deficient BA spore
KLH	keyhole limpet hemocyanin

References

1. Agrawal A, Lingappa J, Leppla SH, Agrawal S, Jabbar A, Quinn C, Pulendran B. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature*. 2003; 424:329–334. [PubMed: 12867985]
2. Kang TJ, Basu S, Zhang L, Thomas KE, Vogel SN, Baillie L, Cross AS. *Bacillus anthracis* spores and lethal toxin induce IL-1β via functionally distinct signaling pathways. *Eur J Immunol*. 2008; 38:1574–1584. [PubMed: 18493980]
3. Kang TJ, Fenton MJ, Weiner MA, Hibbs S, Basu S, Baillie L, Cross AS. Murine macrophages kill the vegetative form of *Bacillus anthracis*. *Infect Immun*. 2005; 73:7495–7501. [PubMed: 16239551]
4. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol*. 2007; 8:942–949. [PubMed: 17676045]

5. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity*. 2009; 30:576–587. [PubMed: 19362022]
6. Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, Soumelis V. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. *Nat Immunol*. 2008; 9:650–657. [PubMed: 18454150]
7. McGeachy MJ, Chen Y, Tato CM, Laurence A, Joyce-Shaikh B, Blumenschein WM, McClanahan TK, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells in vivo. *Nat Immunol*. 2009; 10:314–324. [PubMed: 19182808]
8. Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. *J Clin Invest*. 2006; 116:1218–1222. [PubMed: 16670765]
9. Ishigame H, Nakajima A, Saijo S, Komiyama Y, Nambu A, Matsuki T, Nakae S, et al. The role of TNFalpha and IL-17 in the development of excess IL-1 signaling-induced inflammatory diseases in IL-1 receptor antagonist-deficient mice. *Ernst Schering Res Found Workshop*. 2006:129–153. [PubMed: 16329650]
10. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature*. 2008; 453:1051–1057. [PubMed: 18563156]
11. Jaffar Z, Ferrini ME, Girtsman TA, Roberts K. Antigen-specific Treg regulate Th17-mediated lung neutrophilic inflammation, B-cell recruitment and polymeric IgA and IgM levels in the airways. *Eur J Immunol*. 2009; 39:3307–3314. [PubMed: 19830731]
12. Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med*. 2006; 203:2271–2279. [PubMed: 16982811]
13. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity*. 2008; 28:454–467. [PubMed: 18400188]
14. Zelante T, De LA, D'Angelo C, Moretti S, Romani L. IL-17/Th17 in anti-fungal immunity: what's new? *Eur J Immunol*. 2009; 39:645–648. [PubMed: 19283705]
15. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest*. 2009; 119:1899–1909. [PubMed: 19509469]
16. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, Shen F, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol*. 2007; 8:369–377. [PubMed: 17351619]
17. Henry CJ, Grayson JM, Brzoza-Lewis KL, Mitchell LM, Westcott MM, Cook AS, Hiltbold EM. The roles of IL-12 and IL-23 in CD8+ T cell-mediated immunity against *Listeria monocytogenes*: Insights from a DC vaccination model. *Cell Immunol*. 2010; 264:23–31. [PubMed: 20483409]
18. Pickering AK, Osorio M, Lee GM, Grippe VK, Bray M, Merkel TJ. Cytokine response to infection with *Bacillus anthracis* spores. *Infect Immun*. 2004; 72:6382–6389. [PubMed: 15501768]
19. Brittingham KC, Ruthel G, Panchal RG, Fuller CL, Ribot WJ, Hoover TA, Young HA, et al. Dendritic cells endocytose *Bacillus anthracis* spores: implications for anthrax pathogenesis. *J Immunol*. 2005; 174:5545–5552. [PubMed: 15843553]
20. Hahn AC, Lyons CR, Lipscomb MF. Effect of *Bacillus anthracis* virulence factors on human dendritic cell activation. *Hum Immunol*. 2008; 69:552–561. [PubMed: 18662733]
21. Gervassi A, Alderson MR, Suchland R, Maisonneuve JF, Grabstein KH, Probst P. Differential regulation of inflammatory cytokine secretion by human dendritic cells upon *Chlamydia trachomatis* infection. *Infect Immun*. 2004; 72:7231–7239. [PubMed: 15557648]
22. Gerosa F, Baldani-Guerra B, Lyakh LA, Batoni G, Esin S, Winkler-Pickett RT, Consolaro MR, et al. Differential regulation of interleukin 12 and interleukin 23 production in human dendritic cells. *J Exp Med*. 2008; 205:1447–1461. [PubMed: 18490488]
23. Giacomini E, Iona E, Ferroni L, Miettinen M, Fattorini L, Orefici G, Julkunen I, et al. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J Immunol*. 2001; 166:7033–7041. [PubMed: 11390447]

24. Roses RE, Xu S, Xu M, Koldovsky U, Koski G, Czerniecki BJ. Differential production of IL-23 and IL-12 by myeloid-derived dendritic cells in response to TLR agonists. *J Immunol.* 2008; 181:5120–5127. [PubMed: 18802116]
25. Harris KM. Monocytes differentiated with GM-CSF and IL-15 initiate Th17 and Th1 responses that are contact-dependent and mediated by IL-15. *J Leukoc Biol.* 2011; 90:727–734. [PubMed: 21724805]
26. Harris KM, Fasano A, Mann DL. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. *J Immunol.* 2008; 181:4457–4460. [PubMed: 18802048]
27. Romani L. Immunity to fungal infections. *Nat Rev Immunol.* 2011; 11:275–288. [PubMed: 21394104]
28. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, Fulcher DA, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med.* 2008; 205:1551–1557. [PubMed: 18591410]
29. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, Migaud M, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science.* 2011; 332:65–68. [PubMed: 21350122]
30. Chung Y, Dong C. Don't leave home without it: the IL-23 visa to T(H)-17 cells. *Nat Immunol.* 2009; 10:236–238. [PubMed: 19221553]
31. Manetti R, Gerosa F, Giudizi MG, Biagiotti R, Parronchi P, Piccinni MP, Sampognaro S, et al. Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones. *J Exp Med.* 1994; 179:1273–1283. [PubMed: 7908322]
32. Kwok WW, Yang J, James E, Bui J, Huston L, Wiesen AR, Roti M. The anthrax vaccine adsorbed vaccine generates protective antigen (PA)-Specific CD4+ T cells with a phenotype distinct from that of naive PA T cells. *Infect Immun.* 2008; 76:4538–4545. [PubMed: 18678674]
33. Laughlin EM, Miller JD, James E, Fillos D, Ibegbu CC, Mittler RS, Akondy R, et al. Antigen-specific CD4+ T cells recognize epitopes of protective antigen following vaccination with an anthrax vaccine. *Infect Immun.* 2007; 75:1852–1860. [PubMed: 17283103]
34. Ingram RJ, Metan G, Maillere B, Doganay M, Ozkul Y, Kim LU, Baillie L, et al. Natural exposure to cutaneous anthrax gives long-lasting T cell immunity encompassing infection-specific epitopes. *J Immunol.* 2010; 184:3814–3821. [PubMed: 20208010]
35. Boniface K, Blumenschein WM, Brovont-Porth K, McGeachy MJ, Basham B, Desai B, Pierce R, et al. Human Th17 cells comprise heterogeneous subsets including IFN-gamma-producing cells with distinct properties from the Th1 lineage. *J Immunol.* 2010; 185:679–687. [PubMed: 20511558]
36. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, Monticelli S, et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature.* 2012; 484:514–518. [PubMed: 22466287]
37. Sallusto F, Zielinski CE, Lanzavecchia A. Human Th17 subsets. *Eur J Immunol.* 2012; 42:2215–2220. [PubMed: 22949319]
38. DeChatelet LR, Mullikin D, Shirley PS, McCall CE. Phagocytosis of live versus heat-killed bacteria by human polymorphonuclear leukocytes. *Infect Immun.* 1974; 10:25–29. [PubMed: 4210332]
39. Tomita T, Blumenstock E, Kanegasaki S. Phagocytic and chemiluminescent responses of mouse peritoneal macrophages to living and killed *Salmonella typhimurium* and other bacteria. *Infect Immun.* 1981; 32:1242–1248. [PubMed: 6788707]
40. Cleret A, Quesnel-Hellmann A, Vallon-Eberhard A, Verrier B, Jung S, Vidal D, Mathieu J, et al. Lung dendritic cells rapidly mediate anthrax spore entry through the pulmonary route. *J Immunol.* 2007; 178:7994–8001. [PubMed: 17548636]
41. Shetron-Rama LM, Herring-Palmer AC, Huffnagle GB, Hanna P. Transport of *Bacillus anthracis* from the lungs to the draining lymph nodes is a rapid process facilitated by CD11c+ cells. *Microb Pathog.* 2010; 49:38–46. [PubMed: 20188814]

42. Alileche A, Serfass ER, Muehlbauer SM, Porcelli SA, Brojatsch J. Anthrax lethal toxin-mediated killing of human and murine dendritic cells impairs the adaptive immune response. *PLoS Pathog.* 2005; 1:e19. [PubMed: 16254597]
43. Cleret-Buhot A, Mathieu J, Tournier JN, Quesnel-Hellmann A. Both lethal and edema toxins of *Bacillus anthracis* disrupt the human dendritic cell chemokine network. *PLoS One.* 2012; 7:e43266. [PubMed: 22937027]
44. Cleret A, Quesnel-Hellmann A, Mathieu J, Vidal D, Tournier JN. Resident CD11c+ lung cells are impaired by anthrax toxins after spore infection. *J Infect Dis.* 2006; 194:86–94. [PubMed: 16741886]
45. Paccani SR, Benagiano M, Savino MT, Finetti F, Tonello F, D'Elia MM, Baldari CT. The adenylate cyclase toxin of *Bacillus anthracis* is a potent promoter of T(H)17 cell development. *J Allergy Clin Immunol.* 2011; 127:1635–1637. [PubMed: 21310477]
46. Glomski IJ, Corre JP, Mock M, Goossens PL. Cutting Edge: IFN-gamma-producing CD4 T lymphocytes mediate spore-induced immunity to capsulated *Bacillus anthracis*. *J Immunol.* 2007; 178:2646–2650. [PubMed: 17312104]
47. Datta SK, Sabet M, Nguyen KP, Valdez PA, Gonzalez-Navajas JM, Islam S, Mihajlov I, et al. Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax. *Proc Natl Acad Sci USA.* 2010; 107:10638–10643. [PubMed: 20479237]
48. Garraud K, Cleret A, Mathieu J, Fiole D, Gauthier Y, Quesnel-Hellmann A, Tournier JN. Differential role of the interleukin-17 axis and neutrophils in resolution of inhalational anthrax. *Infect Immun.* 2012; 80:131–142. [PubMed: 22025514]
49. Shinn AH, Bravo NC, Maecker HT, Smith JW. TNF-alpha detection using a flow cytometric assay to determine cellular responses to anthrax vaccine. *J Immunol Methods.* 2003; 282:169–174. [PubMed: 14604550]
50. Dheda K, Chang JS, Lala S, Huggett JF, Zumla A, Rook GA. Gene expression of IL17 and IL23 in the lungs of patients with active tuberculosis. *Thorax.* 2008; 63:566–568. [PubMed: 18511642]
51. Scriba TJ, Kalsdorf B, Abrahams DA, Isaacs F, Hofmeister J, Black G, Hassan HY, et al. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol.* 2008; 180:1962–1970. [PubMed: 18209095]
52. Loving CL, Osorio M, Kim YG, Nunez G, Hughes MA, Merkel TJ. Nod1/Nod2-mediated recognition plays a critical role in induction of adaptive immunity to anthrax after aerosol exposure. *Infect Immun.* 2009; 77:4529–4537. [PubMed: 19620350]
53. Sander LE, Davis MJ, Boekschoten MV, Amsen D, Dascher CC, Ryffel B, Swanson JA, et al. Detection of prokaryotic mRNA signifies microbial viability and promotes immunity. *Nature.* 2011; 474:385–389. [PubMed: 21602824]
54. Sauer JD, Sotelo-Troha K, von MJ, Monroe KM, Rae CS, Brubaker SW, Hyodo M, et al. The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to *Listeria monocytogenes* and cyclic dinucleotides. *Infect Immun.* 2011; 79:688–694. [PubMed: 21098106]
55. Blander JM, Sander LE. Beyond pattern recognition: five immune checkpoints for scaling the microbial threat. *Nat Rev Immunol.* 2012; 12:215–225. [PubMed: 22362354]
56. Schlienger K, Craighead N, Lee KP, Levine BL, June CH. Efficient priming of protein antigen-specific human CD4(+) T cells by monocyte-derived dendritic cells. *Blood.* 2000; 96:3490–3498. [PubMed: 11071646]

Figure 1. hDCs uptake and kill germinating BA spores

(A) hDCs fixed and imaged by TEM 30 minutes after infection with BA spores are shown. Orange arrows indicate hDCs that had internalized BA spores within 30 minutes.

Magnification: **i)** 3200 $\times$, **ii)** 4400 $\times$, **iii)** 11000 $\times$, **iv)** 15000 $\times$. (B) hDCs were infected with Sterne 34F2 BA germination-proficient or -deficient (*gerh*) spores and killing of BA was determined at 16 hours post-infection by viable colony counts. Data are shown as mean $\pm$ SD of triplicate samples from one experiment representative of five independent experiments.

Figure 2. Germination-proficient BA spores stimulate hDCs to secrete IL-1 β , IL-6, IL-12 and IL-23 and upregulate activation markers

(A) hDCs were infected without or with decreasing doses of BA spores and cultured overnight for 18 hours. Secreted IL-23 protein was determined by ELISA. Data are shown as the mean $\pm$ SD of duplicate wells pooled from one individual tested in three independent experiments. (B) hDCs were infected with no spores (media), BA or $\Delta gerh$ spores (M.O.I. of 1:1) and cultured overnight for 18–20 hours. Cell-free culture fluids were collected and secreted IL-1 β , IL-6, IL-12 and IL-23 evaluated by ELISA. Data are shown as mean + SD of duplicate wells pooled from 5–6 independent experiments. Statistical significance determined by the paired two-tailed Student's t-test. (C) hDCs uninfected or infected with BA spores were incubated overnight with and without IL-1ra (100 μ g/ml), and IL-23 secretion was quantified by ELISA. The percent reduction of IL-23 with IL-1ra was calculated from BA- or $\Delta gerh$ - infected hDC without IL-1ra. Data are the mean of duplicate wells from one individual tested in three independent experiments. (D) hDCs were infected as described in panel B. Surface expression of activation (CD40, CD80, CD86, HLA-DR) and maturation (CD83) markers was determined by flow cytometry. Black line histograms are the surface antigen, and red line histograms are appropriate immunoglobulin isotype controls. The mean fluorescence intensity (top) and % positive cells (bottom) are shown. Histograms are representative of 5–6 different healthy donors tested. * p <0.05 between BA and BA + IL-1ra conditions.

Figure 3. BA-infected hDCs stimulate IL-17A and IFN- γ responses from autologous CD4⁺ T cells in a contact-dependent manner

(A) Highly pure human CD4⁺ T cells were isolated from PBMCs of healthy individuals by negative selection using commercially available kits. The purity and expression of surface markers associated with Th17 cells in humans (IL-1RI, IL-23R, CCR6) was determined by flow cytometry. (B) Human CD4⁺ T cells were cultured with autologous hDCs infected with no spores, BA or $\Delta gerh$ spores (M.O.I. of 1:1) at a hDC:TC ratio of 1:10 in cRPMI supplemented with IL-7 and IL-15 for 7 days. 0.4 μ m semi-permeable transwell inserts were used to prevent cellular contact between CD4⁺ T cells and hDCs infected with BA spores (BA + transwell). After the 7 day co-culture, culture fluids were collected and analyzed for primary IL-17A and IFN- γ responses by ELISA. Cells were harvested for additional experiments presented in Figure 4. Data are shown as mean + SD of 4–5 independent experiments assayed in duplicate. * $p < 0.05$ as determined by the paired two-tailed Student's t-test were considered statistically significant.

Figure 4. Optimal Th17 responses to BA are elicited by hDCs infected with germinating BA spores

(A) Cells from the 7 day co-culture were harvested, washed, counted and re-plated at a hDC:TC ratio of 1:10 for 48 hours with a second preparation of autologous hDCs that were infected with no spores, BA or *gerh* spores, or BA antigens PA or LF. Culture fluids were collected and analyzed for secondary IL-17A and IFN- γ responses by ELISA. Data are shown as mean + SD of duplicates of one donor of three tested. * $p < 0.05$ as determined by the paired two-tailed Student's t-test. (B) hDCs were infected with BA spores, washed and co-cultured with autologous lymphocytes for 7 days (primary challenge). Cells from the 7 day co-culture were harvested, washed, counted and re-plated with no spores, BA spores or KLH (10 μ g/ml) for 48 hours. Concentrations of secreted IFN- γ were then measured by ELISA and ELISpot assays. Data are shown as mean + SD of duplicate wells from one experiment representative of three independent experiments. (C) Intracellular cytokine staining was used to determine the cell source of IL-17A and IFN- γ secreted in primary co-cultures re-exposed to hDCs infected with no spores or BA spores. PMA and ionomycin were added to hDC:TC co-cultures in the presence of monensin for the last 6 hours of the 48 hour re-stimulation. 200,000 live cells were collected and intracellular detection of FITC-IFN- γ and PE-IL-17 was determined within the CD4⁺CD3⁺ gate by flow cytometric

analysis. Numbers are percent positive cells as determined by appropriate intracellular immunoglobulin isotype controls. Contour plots are representative of 3 different donors tested.