Poly-γ-Glutamic Acid Encapsulation of *Bacillus anthracis* Inhibits Human Dendritic Cell Responses

Tanya M. Jelacic, Wilson J. Ribot, Steven A. Tobery, Donald J. Chabot and Arthur M. Friedlander

*ImmuHo* 2021, 5 (2) 81-89
doi: https://doi.org/10.4049/immunohorizons.2100004
http://www.immunohorizons.org/content/5/2/81

This information is current as of March 28, 2021.

**References**  This article *cites 53 articles*, 25 of which you can access for free at: http://www.immunohorizons.org/content/5/2/81.full#ref-list-1

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://www.immunohorizons.org/alerts
Poly-γ-Glutamic Acid Encapsulation of Bacillus anthracis Inhibits Human Dendritic Cell Responses

Tanya M. Jelacic,* Wilson J. Ribot,* Steven A. Tobey,* Donald J. Chabot,* and Arthur M. Friedlander*†

*United States Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702; and †Department of Medicine, Uniformed Services University of Health Sciences, Bethesda, MD 20814

ABSTRACT

The capsule of Bacillus anthracis is composed of a D isomer poly-γ-glutamic acid polymer, which is especially nonstimulatory to dendritic cells, even more so than similar mixed D, L isomer polymers from nonpathogenic Bacillus species. Capsule is an essential virulence factor for B. anthracis, protecting the bacilli from phagocytosis by innate immune cells. In this study, we demonstrate that encapsulation provides a further pathogenic advantage by shielding more inflammatory Ags on the bacillus surface, thereby reducing dendritic cell responses. We exposed human immature dendritic cells (DCs) to increasing multiplicities of infection (MOIs) of killed B. anthracis bacilli from the fully encapsulated wild-type Ames strain (WT) and an isogenic capsule-deficient strain (capA mutant). Both strains elicited robust cytokine responses, but IL-23, TNF-α, and IL-10 were significantly reduced in response to the encapsulated WT compared with capA mutant up to an MOI of 15. capA mutant bacilli could induce phenotypic maturation of immature DCs with upregulation of MHC classes I and II, CD83, and CCR7 at an MOI of 3.75, whereas encapsulated WT bacilli still did not induce significant upregulation of MHC classes I and II at an MOI of 15. DCs exposed to capA mutant bacilli (MOI 3.75) exhibited CCR7-dependent chemotaxis that was comparable to that of LPS-stimulated controls, whereas DCs exposed to encapsulated WT bacilli exhibited significantly less chemotaxis. We conclude that capsule shields more inflammatory surface Ags, delaying development of an adaptive immune response by reducing TNF-α, thereby inhibiting DC maturation. ImmunoHorizons, 2021, 5: 81–89.

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, is a highly virulent pathogen due to the toxins encoded on plasmid pXO1 (1) and the capsule encoded on plasmid pXO2 (2, 3). Capsule is a highly effective virulence factor. B. anthracis strains that are cured of pXO1 but retain pXO2 remain virulent in A/J and CBA/J mice (4, 5). In addition, toxin-null mutants have been shown to have comparable lethality to the parent strain in a murine pulmonary anthrax model (6), and a toxin negative strain is still virulent in guinea pigs (7). Furthermore, strains lacking the capsule, such as the Sterne strain, are highly attenuated and used throughout the world as veterinary vaccines for anthrax (8), and similar vaccines have been used in humans in countries of the former Soviet Union (9). One reason the capsule is such an effective virulence factor is because encapsulated bacilli are resistant to phagocytosis by immune cells (10–13). However, our present study shows that evasion of phagocytosis is not the only protection that encapsulation affords. B. anthracis capsule is a homopolymer that consists of D isomer glutamic acid residues connected by γ linkages (14, 15). The poly-γ-glutamic acid polymer is covalently linked to peptidoglycan on the bacillus surface to form the capsule (16–18). Capsule is a poorly immunogenic thymus-independent type 2 Ag (19, 20) and is resistant to degradative enzymes (12, 21, 22). In a recent study, we demonstrated that free capsule released by the bacilli elicits fewer cytokines in smaller amounts from human

Received for publication January 25, 2021. Accepted for publication January 25, 2021.

Address correspondence and reprint requests to: Dr. Tanya M. Jelacic and Dr. Arthur M. Friedlander, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702. E-mail addresses: tanya.m.obreiter.ctr@mail.mil (T.M.J.) and arthur.friedlander3.civ@mail.mil (A.M.F.)

ORCID: 0000-0002-1584-5300 (T.M.J.); 0000-0002-9691-4004 (W.J.R.); 0000-0003-4925-5779 (D.J.C.).

This work was supported by Defense Threat Reduction Agency Grant CBM.VAXBT.03.10.RD.015, Plan 921175.

Abbreviations used in this article: BMDC, bone marrow–derived DC; DC, dendritic cell; iDC, immature dendritic cell; mDC, mature DC; MFI, mean fluorescence intensity; MHC I, MHC class I; MHC II, MHC class II; MOI, multiplicity of infection; WT, wild-type Ames strain.

This article is distributed under the terms of the CC BY 4.0 Unported license.

Copyright © 2021 The Authors

https://doi.org/10.4049/immunohorizons.2100004

ImmunoHorizons is published by The American Association of Immunologists, Inc.
innate immune cells and is a much poorer ligand for human TLR2 than similar mixed d, l isomer poly-γ-glutamic acid polymers produced by nonpathogenic Bacillus species (22). This led us to hypothesize that encapsulation provides a shield for more inflammatory Ags on the surface of the bacillus to reduce the inflammatory response when B. anthracis bacilli interact with innate immune cells.

To test the idea that encapsulation reduces the innate immune response to B. anthracis, we exposed human immature dendritic cells (iDCs) to killed B. anthracis bacilli from the fully encapsulated wild-type Ames strain (WT) and an isogenic capsule-deficient strain (capA mutant) at varying multiplicities of infection (MOIs). We used killed bacilli to exclude the effects of the toxins without having to use a nontoxicogenic strain and so that we could ensure exposure to equivalent numbers of bacilli from both strains; an earlier study demonstrated that bacilli from an encapsulated strain of B. anthracis proliferated at a substantially higher rate than an isogenic nonencapsulated strain when cocultured with human monocyte–derived DCs, which likely also resulted in higher levels of secreted toxins (23). Both strains elicited robust cytokine responses, but there were notable decreases in certain cytokines in response to the encapsulated bacilli. IL-6 was noticeably reduced in response to the encapsulated WT, IL-23, TNF-α, and IL-10 significantly so. These reduced responses were consistent across MOIs ranging from 0.937 to 15. When cell surface marker expression was assessed, DCs exposed to capA mutant bacilli demonstrated a mature phenotype with expression of MHC class I (MHC I) and MHC class II (MHC II), CD83, and CCR7 comparable to that of LPS-stimulated controls at an MOI of 3.75, whereas DCs exposed to encapsulated WT bacilli did not. Indeed, DCs exposed to capA mutant bacilli showed significant upregulation of the maturation markers CD83 and CCR7 at an MOI of 0.937, whereas DCs exposed to encapsulated WT bacilli did not show significant upregulation of those markers until an MOI of 18. Comparing CCR7-dependent chemotaxis, we found that DCs exposed to capA mutant bacilli at an MOI of 3.75 had chemotaxis comparable to the LPS-stimulated positive control, whereas those exposed to encapsulated WT bacilli had significantly reduced chemotaxis. These results indicate that although encapsulation does not completely circumvent activation of DCs, it may effectively delay it until bacterial numbers are high. Reduction of TNF-α release, and, consequently, reduction of DC maturation, suggests that initiation of an adaptive response will likewise be reduced and delayed, enabling B. anthracis to replicate and disseminate throughout the host.

**MATERIALS AND METHODS**

**B. anthracis strains**

B. anthracis Ames and the isogenic capsule-deficient strain B. anthracis capA mutant (24) were from the United States Army Medical Research Institute of Infectious Diseases collection. Bacilli were inactivated by exposure to 4% formaldehyde.

**Human cells**

Leukopacks were obtained from the Department of Transfusion Medicine (Clinical Center, National Institutes of Health, Bethesda, MD). Human monocytes were purified and cultivated as before (24). Cultures were maintained for 5–7 d to allow for differentiation into iDCs and then were stimulated with 100 ng/ml LPS from Escherichia coli 055:B5 (Sigma-Aldrich, St. Louis, MO), encapsulated WT–killed bacilli, or capA mutant–killed bacilli or were maintained in medium alone. Two days later, media samples were removed for cytokine analysis, and the cells were used for flow cytometry or a CCR7-dependent chemotactic activity assay.

Research involving human subjects adhered to the principles identified in the Belmont Report (1979) and, unless certified as exempt, was conducted in accordance with an institutional review board–approved protocol and in compliance with Department of Defense, Federal, and State statutes and regulations relating to the protection of human subjects. Research involving human subjects certified as exempt was conducted in accordance with the conditions specified in connection with an exemption certificate.

**Cytokine measurement**

Duplicate wells of media samples were assayed for human IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-2, IFN-γ, GM-CSF, and TNF-α using human proinflammatory 9-plex platform Ultra-Sensitive plates (Meso Scale Discovery, Gaithersburg, MD). The plates were analyzed using a Sector Imager 2400 (Meso Scale Discovery) according to the manufacturer’s protocol. IL-23 was measured using the Human IL-23 ELISA Ready SET Go! kit (eBioscience, San Diego, CA) following the manufacturer’s protocol. Cytokine data are presented as the geometric mean picograms per milliliter ± geometric SD. Statistical significance of the data was determined by paired t tests of the log-transformed data (GraphPad Software, La Jolla, CA). A p value < 0.05 was considered significant.

**Flow cytometry**

Cells were stained with fluorescently labeled Abs specific for human MHC I (clone DX17, PE format), MHC II (clone G46-6, FITC format), CD83 (clone HB15e, PE format), or CCR7 (clone 150503, FITC format) or with isotype control Abs purchased from BD Pharamingen (San Jose, CA). Flow cytometry experiments were run on a FACSCalibur (BD Biosciences, Billerica, MA). Gating was forward scatter by side scatter. The data were analyzed using Cell Quest Pro software (BD Biosciences). Statistical significance of the data was determined by two-tailed t tests comparing average mean fluorescence intensities (MFI) for the various treatments to the untreated control (GraphPad Software).

**Chemotactic activity assay**

DCs were resuspended in RPMI 1640 medium supplemented with 1% BSA at 6 × 10⁵ live cells/ml and run in a CCR7-dependent chemotactic activity assay as described previously (24). Fluorescence was read at 480 nm/520 nm in a SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA).
Chemotactic index was calculated by dividing the relative fluorescence units observed in the presence of the CCR7 ligands by the relative fluorescence units observed in the absence of ligand. Statistical significance was determined by comparing the means of the chemotactic indices for the cells exposed to LPS, encapsulated WT-killed bacilli, or capA mutant–killed bacilli to the mean chemotactic index for the naive iDC control (GraphPad Software).

RESULTS

Human iDCs release smaller amounts of IL-23, TNF-α, and IL-10 in response to encapsulated bacilli

The capsule forms the outermost surface of encapsulated bacilli, completely covering the S-layer (25). Our previous work has shown that the poly-γ-glutamic acid capsule polymer produced by B. anthracis provokes very weak responses from human innate immune cells (26) and is significantly less stimulatory than similar poly-γ-glutamic acid polymers produced by other nonpathogenic Bacillus species (22). We hypothesized that the capsule shields more inflammatory Ags on the surface of the bacilli and that immune cells would respond less robustly to encapsulated bacilli than to nonencapsulated bacilli. To test this, we compared the cytokine responses of human iDCs to killed B. anthracis bacilli from a fully encapsulated WT and an isogenic capA mutant strain. The capA mutant strain produces 98% less capsule than WT (24) and has very little capsule on the cell surface (Fig. 1C). This leaves most of the surface of the bacilli exposed to the environment. This is demonstrated by the fact that, unlike fully encapsulated WT bacilli, capsule-deficient capA mutant bacilli cannot exclude India ink (Fig. 1B, 1D). This means that iDCs encountering encapsulated WT bacilli will initially sense only capsule, whereas iDCs encountering capA mutant bacilli will sense a variety of surface Ags. Human iDCs were exposed to encapsulated WT– and capA mutant–killed bacilli at MOIs of 0.937, 3.75, and 15 for 2 d. Media samples were then assayed for IL-8, IL-6, IL-10, IL-1β, IL-12p70, TNF-α, IL-2, IFN-γ, and IL-23. Five experiments were run with cells from five donors. Fig. 2 shows the ranges of the individual cytokine responses in picograms per milliliter in tabular form, and the mean cytokine results in graphic form. The iDCs responded robustly to both strains. Mean IL-8 was roughly equivalent across the board, with neither the strain nor the MOI having an effect (Fig. 2B), indicating that the response was saturated for both strains at an MOI of 0.937. In contrast, mean IL-23 was dramatically and significantly reduced in response to encapsulated WT bacilli compared with capA mutant bacilli at MOIs of 0.937 (p < 0.05), 3.75 (p < 0.001), and 15 (p < 0.001, Fig. 2C). Although not statistically significant, mean IL-6 responses to encapsulated WT bacilli were noticeably reduced compared with capA mutant bacilli (Fig. 2D). Mean TNF-α responses to encapsulated WT bacilli were reduced compared with capA mutant bacilli (Fig. 2E), and the differences were significant at MOIs of 3.75 (p < 0.01) and 15 (p < 0.05). Likewise, mean IL-10 responses were reduced in response to encapsulated WT bacilli compared with capA mutant bacilli (Fig. 2F), and the differences were significant at MOIs of 3.75 (p < 0.05) and 15 (p < 0.001). In contrast, mean IFN-γ and IL-12p70 responses to the two strains were roughly equivalent at all three MOIs (Fig. 2G, 2H). Mean IL-1β responses were only significant at an MOI of 15 for both strains and were not significantly different (Fig. 2I), whereas IL-2 was not elicited in significant amounts by either strain [data not shown]. Taken together, these results suggest that encapsulation does shield more inflammatory surface components of the bacilli in interactions with human iDCs.

Upregulation of cell surface markers by iDCs is decreased in response to encapsulated bacilli

TNF-α release was significantly reduced in DCS exposed to encapsulated WT–killed bacilli compared with capA mutant–killed bacilli even at an MOI of 15. TNF-α can be used as part of a cytokine mixture with IL-6, IL-1β, and PGE2 or on its own to induce maturation of human iDCs in vitro (26, 27). Thus, we hypothesized that the reduction in TNF-α release might correspond to reduced maturation of iDCs in response to encapsulated WT–killed bacilli compared with capA mutant–killed bacilli. To test this, we exposed human iDCs to encapsulated WT– and capA mutant–killed bacilli at MOIs of 0.937, 3.75, and 15 and assessed cell surface marker expression by flow cytometry. Negative control cultures were maintained in medium alone so that they would retain the iDC phenotype, whereas positive control cultures were exposed to 100 ng/ml LPS to induce a mature DC (mDC) phenotype. Two days later, the cells were analyzed by flow cytometry for expression of MHC I and MHC II.

https://doi.org/10.4049/immunohorizons.2100004

FIGURE 1. Capsule-deficient capA bacilli have very little capsule on the cell surface and do not exclude India ink like fully encapsulated WT bacilli.

(A and C) WT (A) and capA mutant (C) bacilli were stained with FITC-labeled anti-capsule Ab and examined by fluorescence microscopy [original magnification ×1000]. (B and D) WT (B) and capA mutant (D) bacilli were examined by India ink and phase-contrast microscopy [original magnification ×1000].
and CD83 and CCR7, markers associated with the mDC phenotype (28, 29). Five experiments were run with cells from five donors. Fig. 3A shows the average MFI for each of the markers for each treatment and the corresponding $p$ values for each treatment compared with the untreated iDC control. Fig. 3B–E shows the histograms from a single representative donor for the iDC control (Fig. 3B), the LPS-stimulated positive control (Fig. 3C), capA mutant–killed bacilli–treated (MOI 3.75, Fig. 3D), and encapsulated WT–killed bacilli–treated (MOI 3.75, Fig. 3E). As expected, exposure to LPS resulted in significant increases in expression of MHC I ($p < 0.01$), MHC II ($p < 0.05$), CD83 ($p < 0.01$), and CCR7 ($p < 0.01$). Exposure to capA mutant–killed bacilli resulted in significant increases in CD83 and CCR7 at all three MOIs and significant increases in MHC I and MHC II at MOIs of 3.75 and 15 (Fig. 3A). In contrast, exposure to encapsulated WT–killed bacilli resulted in significant mean increases in CD83 and CCR7 only at an MOI of 15, and although mean expression of MHC I and MHC II increased with increasing MOI, the increases were NS (Fig. 3A). Mean expression of MHC I, CD83, and CCR7 in capA mutant–killed bacilli–treated cells was nearly always significantly greater than in encapsulated WT–killed bacilli–treated cells (Fig. 3F). These data indicate that encapsulation inhibits DC maturation even at an MOI of 15.

**DCs exposed to encapsulated bacilli demonstrate decreased CCR7-dependent chemotaxis**

Expression of CCR7 in mDCs is critical for initiation of an adaptive immune response because it enables both chemotaxis toward and entry into lymph nodes (30). DCs exposed to capsule-deficient, capA mutant–killed bacilli at an MOI of 3.75 demonstrate upregulated expression of MHC I and MHC II, CD83, and CCR7 that are comparable to those induced by exposure to 100 ng/ml LPS. Even at an MOI of 0.937 capA mutant–killed bacilli, exposed DCs express mean levels of CCR7 that are comparable to those induced by exposure to LPS and that are greater at an MOI of 15 ($p < 0.05$). In contrast, expression of CCR7 in response to fully encapsulated WT–killed bacilli was significant only at an MOI of 15 and was consistently less than in capA mutant–killed bacilli–treated cells.
bacilli–treated cells (Fig. 3F). Thus, iDCs exposed to capA mutant bacilli at an MOI of 3.75 demonstrated a fully mature phenotype, whereas those exposed to the same number of encapsulated WT–killed bacilli did not. As a functional test of maturation, we assessed DCs exposed to capA mutant– or encapsulated WT–killed bacilli at an MOI of 3.75 for CCR7-dependent chemotaxis in a chemotactic activity assay using CCL19 and CCL21 as chemoattractants. Untreated iDCs and LPS-stimulated DCs served as negative and positive controls, respectively. Six experiments were run using cells from six donors. Consistent with the flow cytometry results, the LPS and capA mutant–killed bacilli–treated cells demonstrated functional maturation with comparable chemotaxis toward both CCR7 ligands (Fig. 4). The encapsulated WT–killed bacilli–treated cells also demonstrated modest chemotaxis toward both CCR7 ligands, but it was significantly less than that demonstrated by the capA mutant–killed bacilli–treated cells, consistent with the lower levels of CCR7 expression detected by flow cytometry. These results indicate that encapsulation inhibits functional maturation of DCs.

**DISCUSSION**

In this study, we explored the effects of encapsulation of *B. anthracis* on the innate immune response by comparing the responses of human DCs to killed bacilli from the fully encapsulated WT and the isogenic capsule-deficient capA mutant strain. Both strains elicited robust cytokine responses that were remarkably similar, with the notable exceptions of reduced release of IL-23, TNF-α, and IL-10 in response to the encapsulated WT. However, when DC maturation was examined in terms of cell surface marker expression and CCR7-dependent chemotaxis, it became clear that encapsulation greatly inhibited DC maturation.

In our previous work, we showed that cytokine responses to free *B. anthracis* capsule by human monocytes are comprised of small amounts of IL-8, IL-6, and TNF-α, whereas those of human DCs are limited primarily to IL-8 and sometimes IL-6 (22, 24). Yet, the cytokine responses to the encapsulated WT–killed bacilli by DCs were quite robust and included significant amounts of IL-23, TNF-α, IL-10, IFN-γ, and IL-12p70 in addition to IL-8 and IL-6 at most of the MOIs.

![Table](https://doi.org/10.4049/immunohorizons.2100004)
tested. We have shown that cytokine responses to free capsule are dependent upon both dose (24) and m.w. because reducing the m.w. of free capsule reduced the magnitude of the cytokine response of human monocytes (22). The capsule on the surface of the bacilli is in the more stimulatory high m.w. form. Furthermore, it would be sensed as very highly concentrated on the surface of the bacilli. Thus, the increased cytokine response to the encapsulated WT bacilli compared with the free capsule is likely a function of both the high density and high m.w. encountered by the DCs.

IL-8 was robustly elicited by both strains, and the response appeared to be saturated for both strains at an MOI of 0.937. As noted above, IL-8 is the primary cytokine elicited by free *B. anthracis* capsule from human iDCs, and it is also the cytokine released in the largest amount in response to free capsule by human monocytes (24), so it is unsurprising that this response should be so robust for both. Saturation likely explains why there was no difference observed between the two strains. IL-6 is the next most common cytokine released by human monocytes and iDCs in response to free *B. anthracis* capsule (22, 24), so it is not surprising that this response to the encapsulated WT bacilli is high as well. Although IL-6 was consistently lower in response to encapsulated WT–killed bacilli compared with capA mutant, the differences were not significant.

In contrast to IL-8 and IL-6, the IL-23, TNF-α, and IL-10 responses were dramatically and significantly reduced in response to encapsulated WT–killed bacilli compared with the capA mutant at MOIs ranging from 0.937 to 15. In our recent work, we demonstrated that capsule is a poor ligand for human TLR2 and TLR4, even more so than a similar poly-γ-glutamic acid polymer produced by the nonpathogen *B. licheniformis* (22). This especially poor stimulation of TLR2 may underlie the substantially reduced release of IL-23, TNF-α, and IL-10 because these cytokines have all been demonstrated to be released upon sensing of pathogens by TLR2. IL-23 release by human THP-1 monocytes in response to the intracellular protozoan parasite *Toxoplasma gondii* has been demonstrated to be dependent on TLR2 (31), as has IL-23 release by murine bone marrow–derived DCs (BMDCs) in response to *Streptococcus pneumoniae* (32). Expression of the IL-23 specific subunit p19 in response to *Mycobacterium tuberculosis* in mice has also been shown to be dependent on TLR2 (33). Other experiments with *M. tuberculosis* have shown reduced release of TNF-α and IL-10 from TLR2−/− murine BMDCs (34). Interestingly, release of IL-12p70 was not reduced in TLR2−/− murine BMDCs in response to *M. tuberculosis* (34), nor was expression of the IL-12p70–specific subunit p35 in TLR2−/− mice (33). We likewise did not observe a decrease in IL-12p70 with the encapsulated WT, suggesting that this cytokine is released via a mechanism other than TLR2 in response to both pathogens.

Our findings of reduced release of IL-23, TNF-α, and IL-10 in response to encapsulated bacilli are largely consistent with the findings of an earlier study by Hahn et al. (23) that compared the cytokine responses of human monocyte–derived DCs cocultured with encapsulated and nonencapsulated *B. anthracis* bacilli. In that study, vegetative bacilli were introduced to DC cultures, allowed to replicate for 3 h, and then killed with ciprofloxacin. Cytokines were measured 24 h after the addition of the bacilli. Hahn et al. (23) reported decreases in IL-12p40, IL-6, and TNF-α in response to encapsulated compared with nonencapsulated bacilli. We likewise observed decreases in TNF-α and consistent, although NS, decreases in IL-6. IL-12p40 is a subunit common to IL-12p70 and IL-23; we observed inhibition of IL-23, but no inhibition of IL-12p70. We also observed inhibition of IL-10, which was not reported in the study of Hahn et al. (23). IL-10 was released in smaller amounts than the other affected cytokines, so perhaps there was not enough to detect because of the shorter time course in the Hahn et al. (23) study. Thus, our results confirm those of Hahn et al. (23) and expand on them by demonstrating specific inhibition of IL-23 and revealing inhibition of IL-10. It is possible that some of the effects observed by Hahn et al. (23) were due to
toxins secreted during the 3-h replication period because the encapsulated strain replicated to a greater extent than the nonencapsulated strain or to bacterial degradation products, resulting from the killing with ciprofloxacin. TNF-α can be used as a part of a cytokine mixture with IL-6, IL-1β, and PGE2 or on its own to induce maturation of human iDCs in vitro (26, 27). Indeed, TNF-α has been shown to be essential for DC maturation in a study with TNF-α−/− mice (35). In addition to driving maturation of DCs, TNF-α has also been shown to support the survival of human DCs, especially iDCs (36–38). Thus, the reduction in TNF-α doubly impacts the initiation of the adaptive immune response by reducing maturation rates of DCs and by reducing viability of DCs. Evasion of the adaptive immune response by circumventing DC maturation has been observed with other pathogens, notably Brucella suis and Coxiella burnetii. Billard et al. (39) observed a failure to mature in human monocyte-derived DCs infected with B. suis. Similar to our results, they observed a dramatic decrease in release of TNF-α compared with positive control DCs infected with E. coli and no release of IL-10. They also observed decreased upregulation of MHC I and MHC II, CD83, and CCR7 as we did. In contrast to our results, they also observed a decrease in IL-12p70. Shannon et al. (40) observed a dramatic decrease in TNF-α release in response to a virulent strain of C. burnetii with full-length LPS compared with an avirulent isogenic strain with truncated LPS. They proposed a scenario much like ours, with full-length LPS in the place of capsule acting as a shield to prevent access to more inflammatory cell surface Ags that drive DC maturation.

Both anthrax toxins have been shown to impair DC function (41–46). Multiple studies have shown that pretreatment of human or murine DCs with purified lethal toxin reduces the release of TNF-α and IL-6 in response to various inflammatory stimuli (41–46). The reduced IL-6 observed by Hahn et al. (23) described above could be due to greater amounts of lethal toxin being secreted by the encapsulated bacilli before being killed with ciprofloxacin. Similarly, pretreatment of human or murine DCs with purified edema toxin has been shown to reduce release of TNF-α and IL-12 in response to inflammatory stimuli (42–44, 46). Our present study shows reduced IL-23, TNF-α, and IL-10 release in response to encapsulated B. anthracis bacilli compared with nonencapsulated bacilli. Thus, all three major virulence factors of B. anthracis inhibit release of TNF-α. The redundancy of this effect ensures that DC maturation will be impaired regardless of whether a DC first encounters encapsulated bacilli or is exposed to toxins. IL-23 was released in large amounts that were significantly reduced in response to encapsulated WT bacilli compared with capsule-deficient capA mutant bacilli at all three MOIs. Consistent with our results, Harris et al. (47) reported IL-23 release by human monocyte-derived iDCs in response to a nonencapsulated B. anthracis strain and subsequent stimulation of a Th17 response by autologous CD4+ T cells. Although IL-23 does not stimulate differentiation of Th17 cells, it does support their survival and promotes their production of IL-17 (48, 49). Our results suggest that the Th17 response would have been inhibited had they used an encapsulated B. anthracis strain. A study of inhalational anthrax in mice by Garraud et al. (50) demonstrated that IL-17 signaling in neutrophils was critical to surviving exposure to a nonencapsulated B. anthracis strain. Interestingly, although IL-17RA−/− mice were more susceptible than wild-type mice to a nonencapsulated B. anthracis strain, there was no difference between the IL-17RA−/− mice and the wild-type mice’s survival of infection with an encapsulated B. anthracis strain. Garraud et al. (50) did not assay for IL-23, but our results suggest that there is no change in susceptibility to the encapsulated strain in wild-type mice because the IL-17 axis is already inhibited because of a reduction in IL-23. Lending weight to this notion is a study of pulmonary Aspergillus fumigatus infection in mice in which optimal IL-17A production by neutrophils was shown to be dependent on IL-23 (51).

Shed capsule is resistant to degradation by human enzymes (22) and has been found to accumulate in the blood of infected animals (14, 52, 53). Shed capsule may also contribute to pathogenesis. In an earlier study with free capsule purified from broth cultures of B. anthracis, we demonstrated that exposure to capsule during differentiation results in DCs that have an impaired maturation response (24). Upregulation of CCR7 expression and CCR7-dependent chemotaxis were both reduced. Notably, IL-23, TNF-α, and IL-10 release were also reduced. Our current results showing reduced upregulation of CCR7 expression, CCR7-dependent chemotaxis, and IL-23, TNF-α, and IL-10 release upon exposure to encapsulated WT bacilli are consistent with these previous findings.

Encapsulation with D isomer poly-γ-glutamic acid, a particularly ineffective TLR2 ligand, greatly reduces the TNF-α and IL-23 responses of human DCs to B. anthracis. Reducing TNF-α release severely inhibits DC maturation and survival, whereas reducing IL-23 inhibits a Th17 response. The combined reduction of these two cytokines is a very effective means of promoting pathogenesis by inhibiting an adaptive immune response. DC maturation, the first step in the initiation of an adaptive immune response, occurred at a much lower MOI with the capsule-deficient capA mutant strain than with the fully encapsulated WT. Because higher numbers of encapsulated bacilli are required to stimulate DC maturation, the initiation of an adaptive response would be delayed until vegetative bacilli numbers are high, giving the bacilli more time to secrete toxins and shed capsule to further impair the immune response. The initial unproductive encounter between encapsulated bacilli and DCs gives the pathogen a very valuable advantage against the host. These results highlight the impact of capsule as a critical virulence factor and the importance of targeting the capsule for prophylactic and therapeutic medical countermeasures.

DISCLOSURES

The authors have no financial conflicts of interest.

ACKNOWLEDGMENTS

We thank Anna Trivett and Debra Tress for purifying the monocytes and Michael W. West for running the multiplex cytokine assays.
REFERENCES


