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B Cell Development in GALT: Role of Bacterial Superantigen-Like Molecules

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Abstract

Intestinal bacteria drive the formation of lymphoid tissues, and in rabbit, bacteria also promote development of the preimmune Ab repertoire and positive selection of B cells in GALT. Previous studies indicated that *Bacillus subtilis* promotes B cell follicle formation in GALT, and we investigated the mechanism by which *B. subtilis* stimulates B cells. We found that spores of *B. subtilis* and other *Bacillus* species, including *Bacillus anthracis*, bound rabbit IgM through an unconventional, superantigen-like binding site, and in vivo, surface molecules of *B. anthracis* spores promoted GALT development. Our study provides direct evidence that B cell development in GALT may be driven by superantigen-like molecules, and furthermore, that bacterial spores modulate host immunity.

The mammalian intestinal tract harbors a large consortium of bacteria, called the intestinal microbiota, which consists of ~500–1000 different bacterial species. Mice raised under sterile, germfree conditions have provided insight into the requirement of the intestinal microbiota for the development and function of nearly every mammalian organ system, including the immune system. Although many studies have focused on the importance of the intestinal microbiota as a whole, only a handful of studies have addressed how individual bacterial species contribute to innate and adaptive immunity. For example, *Bacteroides thetaiotaomicron* promotes innate immune function by stimulating intestinal epithelial cells to produce antimicrobial peptides, which are known to limit bacterial translocation across the epithelial barrier to promote intestinal homeostasis (1, 2). Another *Bacteroides* species, *Bacteroides fragilis*, induces a systemic balance of Th1 and Th2 cells that modulates cell-mediated adaptive immune responses (3). Additionally, segmented filamentous bacteria have been shown to promote cell-mediated immunity (4), as well as induce development of the humoral adaptive immune system by promoting the production of secretory IgA (5). These studies illustrate that different bacterial species in the gut stimulate distinct components of the immune system.

In rabbit, development and expansion of a preimmune Ab repertoire are dependent on interactions between GALT and the intestinal microbiota (6). Additionally, in the appendix, sacculus rotundus, and Peyer's patches, bacteria promote B cell proliferation and the

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formation of B cell follicles (7), which, for the purpose of this study, we refer to as GALT development. Rhee et al. (8) previously demonstrated that not all bacterial species equally induce GALT development. Following the introduction of several intestinal bacterial species, either alone or in combination, into germfree appendices, *Bacillus subtilis* and *B. fragilis* together optimally promoted B cell proliferation and Ig gene diversification. Whereas *B. fragilis* alone did not promote GALT development, *B. subtilis* alone sometimes could, suggesting that of the two bacterial species, *B. subtilis* is the major contributor. The mechanisms by which *B. subtilis* promotes GALT development, however, remain largely unknown.

Ig gene repertoire analyses of B cells from the rabbit appendix suggest that the formation of a large B cell repertoire results from a polyclonal stimulation of B cells in GALT (9, 10). Interestingly, a known B cell superantigen, protein A from *Staphylococcus aureus*, when cointroduced into germfree appendices with *B. fragilis*, stimulated the formation of B cell follicles, suggesting that a bacterial-derived B cell superantigen may serve as a polyclonal inducer of GALT development. B cell superantigens are molecules that bind to surface Ig outside of the conventional Ag binding site, to conserved framework region (FR) amino acid residues in the V region of the H or L chains (V_H or V_L, respectively), and poly-clonally activate B cells (11).

Further support that B cells in GALT can be stimulated by superantigens was obtained from observations in *ali/ali* mutant rabbits. Rabbits contain two types of B cells known as V_Ha and V_Hn, and although the majority of B cells in wild-type (WT) rabbits of any age are V_Ha, the proportion of V_Ha and V_Hn B cells differs in *ali/ali* rabbits over time. At birth, the majority of B cells in *ali/ali* rabbits are V_Hn, but as these rabbits age, a decline in the proportion of V_Hn B cells is accompanied by an increase in V_Ha B cells, and in adult *ali/ali* rabbits V_Ha B cells predominate (12). V_Ha and V_Hn B cells differ from each other by several amino acid residues in the FR of the V_H domain (13). When these FR residues are modeled onto a three-dimensional ribbon diagram of the V_H domain, they are positioned on two adjacent solvent-exposed strands of a β-pleated sheet and form a putative ligand binding site (10). Notably, V_Ha B cells have a higher proliferative capacity than V_Hn B cells (14), and are positively selected in GALT by the intestinal microbiota (10). We hypothesized that one mechanism by which the intestinal microbiota promotes the formation of B cell follicles in GALT is through a superantigen-like mechanism.

In this study, we generated single-chain Ab fragments containing the Ig V_H and V_L domains (scFv) and tested whether they bind to bacteria through a putative superantigen binding site. We found that IgM and scFv containing either V_Ha or V_Hn bind to *Bacillus* spores via an unconventional Ag binding site and that spore surface molecules activate B cells in vitro and in vivo. Our data suggest that *Bacillus* spores stimulate GALT development through a superantigen-like mechanism.

Materials and Methods

General methods

Bacterial strains are shown in Supplemental Table 1. *Escherichia coli* and *Bacillus anthracis* vegetative cells were grown in Luria broth (LB). Gut bacteria were grown on LB agar, blood agar (bioMérieux, Marcy l'Etoile, France), phenylethanol agar (Difco [Becton Dickinson, Franklin Lakes, NJ]), or Difco sporulation medium (Difco [Becton Dickinson]) agar. *Bacteroides* strains were grown anaerobically on blood agar plates. *Bacillus* spores were generated by exhaustion and purified over a renografin-50 gradient (Bracco Diagnostics, Princeton, NY) (15).

Ab reagents used were as follows: mouse anti-rabbit Fcγ (clone C101–359), mouse anti-rabbit IgM (clone 367), biotinylated mouse anti-rabbit IgM (clone 367), and mouse anti-human Ki-67 (BD Biosciences, San Jose, CA); FITC goat Fab anti-mouse IgG, Dylight 649-goat Fab anti-mouse IgG, HRP donkey anti-mouse IgG (H + L), goat $F(ab')_2$ anti-human Ig, goat anti-human Fcγ, FITC rabbit Fab anti-goat IgG, Cy2 goat Fab antimouse IgG, and Cy3 streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA); rabbit IgM (hybridoma supernatant; Knight Lab, Maywood, IL) (16); HRP anti-T7 Tag (Novagen, Madison, WI); Alexa Fluor 568-goat anti-mouse IgG (Invitrogen, Carlsbad, CA; Molecular Probes, Eugene, OR); and anti-human IgM (clone SA-DA4; eBioscience San Diego, CA).

For Western blot analysis, spore extracts were prepared, as described (15); *E. coli* lysates were prepared according to the pET manual (Novagen). Proteins were separated by SDS-PAGE (15% for spore extracts and 10% for *E. coli* lysates), transferred to nitrocellulose (0.2-μm pore; Bio-Rad, Hercules, CA), and probed with 15–20 μg/ml scFv-Ig, followed by 2 μg/ml mouse anti-rabbit Fcγ and 160 ng/ml HRP donkey anti-mouse IgG (H + L); rabbit IgM (hybridoma supernatant or 1:100 dilution serum), followed by 2 μg/ml mouse anti-rabbit IgM and 160 ng/ml HRP donkey anti-mouse IgG (H + L); or HRP anti-T7 Tag (1:10,000). Blots were developed with ECL substrate (Thermo Scientific, Rockford, IL).

Generation of scFv-Ig proteins

The V_Ha–scFv-Ig construct was generated by PCR-amplifying V_H and V_κ from a FITC-binding hybridoma 27.2-3 (17); the V_H region was replaced with PCR-amplified FR1–FR3 from germline *V_H1a2* (18); V_H and V_κ, joined by a $[Gly_4Ser_1]_3$ linker, were cloned into pcDNA3.1$^-$ (Invitrogen) containing the C_H2 and C_H3 domains of rabbit Fcγ (Fig. 1*A*). V_Hn-scFv-Ig was generated by exchanging FR1–FR3 of the above construct with PCR-amplified FR1–FR3 of a V_Hn-encoding gene, *V_Hy33* (19). The V_Ha-scFv-Ig containing *V_H1* and V_λ was obtained by substituting V_κ with V_λ PCR-amplified from bone marrow cDNA. The germline sequences of *V_H1a2*, *V_Hy33*, V_κ, and V_λ were all confirmed by DNA sequence analysis (Supplemental Fig. 1). Chinese hamster ovary cells were transfected with scFv-Ig constructs using either $Ca_3(PO_4)_2$ or polyethylenimine (Polysciences, Warrington, PA), and stable transfectants secreting scFv-Ig were selected; scFv-Ig was purified using GammaBind G Sepharose columns (GE Healthcare, Pittsburg, PA). Purity was assessed by SDS-PAGE.

Isolation of intestinal spore-forming bacteria

Luminal contents were flushed from adult rabbit appendices with 1× PBS, 5% FCS (FACS buffer), and debris was removed by centrifugation (300 × *g*) for 1 min. Bacteria were pelleted by centrifugation at 2800 × *g* for 15 min and resuspended in 4 ml buffer; 50 μl of bacteria was stained with scFv-Ig (see below). scFv-Ig$^+$ and scFV-Ig$^-$ bacterial populations were sorted on the FACSAria (BD Biosciences) and plated on LB agar, blood agar, MacConkey agar (Difco), or phenylethanol agar; bacteria were subjected to Gram stain, phase-contrast microscopy, and flow cytometry. The identity of each bacterial isolate was determined by nucleotide sequence of 16S rRNA genes PCR amplified using pan primers; identities were determined using National Center for Biotechnology Information Microbes BLAST (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/).

Flow cytometry

Spores were stained with 15 μg/ml scFv-Ig, followed by 2.5 μg/ml mouse anti-rabbit Fcγ and 4 μg/ml FITC goat Fab anti-mouse IgG or Dylight 649-goat Fab anti-mouse IgG; or rabbit IgM (hybridoma supernatant), followed by 2.5 μg/ml mouse anti-rabbit IgM and 4 μg/ml Dylight 649-goat Fab anti-mouse IgG.

To assess spore binding to Ramos cells, 1×10^9 spores and 1×10^6 cells were incubated 30 min at 4°C in 100 μl of FACS buffer. Cells were washed twice, and spore binding was detected with rabbit anti-ExsK serum (1:4000; provided by J. Bozue, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD), followed by 2.5 μg/ml mouse antirabbit Fcγ and 4 μg/ml FITC goat Fab anti-mouse IgG. For calcium flux experiments, Ramos cells were labeled with fluo-3-acetoxymethyl ester (fluo-3-AM) and fura red (Invitrogen; Molecular Probes), according to the manufacturer's instructions. Calcium flux was analyzed in 500 μl total volume following addition of 8 μg/ml goat $F(ab')_2$ anti-human Ig or 1×10^9 *bclA* mutant spores for 5 min.

All data were acquired with FACSCanto or FACSCantoII (BD Biosciences) at the LUMC FACS Core Facility, and data were analyzed using FlowJo analysis software (Tree Star, Ashland, OR). For flow cytometric analyses, quadrants were set based on staining with secondary reagents alone.

Microscopy

Spores were stained and visualized by phase-contrast microscopy (15) using the following: 10 μg/ml scFv-Ig, followed by 2.5 μg/ml mouse anti-rabbit Fcγ and 10 μg/ml Alexa Fluor 568-goat anti-mouse IgG; rabbit IgM (hybridoma supernatant), followed by 2.5 μg/ml mouse anti-rabbit IgM and 10 μg/ml Alexa Fluor 568-goat anti-mouse IgG; human serum (1/10 dilution) or 20 μg/ml myeloma proteins (IgMκ, Biodesign International, Saco, ME; IgMλ, The Binding Site, San Diego, CA; of unknown Ag specificity), followed by 2.5 μg/ml mouse anti-human IgM and 10 μg/ml Alexa Fluor 568-goat anti-mouse IgG; or human serum (1:10 dilution), followed by 9 μg/ml goat anti-human Fcγ and 4 μg/ml FITC rabbit Fab anti-goat IgG. Gram staining was carried out using standard methods.

For immunohistochemical detection of IgM^+ $Ki67^+$ cells, 10 μm OCT frozen appendix tissue sections were fixed, blocked with 10% goat serum, and stained sequentially with the following: 10 μg/ml mouse anti-human Ki-67, followed by 15 μg/ml Cy2 goat Fab anti-mouse IgG, and 10 μg/ml biotinylated mouse anti-rabbit IgM, followed by 18 μg/ml Cy3 streptavidin. Following incubations with each of the above reagents, tissues were washed three times for 3 min each with 1× PBS. Samples were visualized with a Leica DM IRB fluorescence microscope equipped with MagnaFire charge-coupled device camera.

The number of $IgM^+Ki\text{-}67^+$ follicles was determined by fluorescence microscopy of four nonserial sections taken, when possible, from different regions of the appendix (tip, middle, and cecal-proximal). The number of double-stained follicles per section was counted; counts from multiple appendices receiving the same inoculum were averaged and the SEM was determined.

Transmission electron microscopy was performed, as described (20).

B. anthracis spore protein production

Spore proteins separated by SDS-PAGE were excised and submitted to Stanford University Mass Spectrometry for proteomics analysis. Proteins were identified by standard database searches. ExsK, ExsB, and ExsJ open reading frames were PCR-amplified from *B. anthracis* (Sterne) genomic DNA and expressed in *E. coli* using the pET expression system (Novagen) in frame with T7 and 6X-His tags. BL21 (DE3) pLysS *E. coli* were transformed, and protein expression was induced with 1 mM isopropyl β-D-thiogalactoside. Protein expression was confirmed with HRP-conjugated T7-Tag Ab (Novagen), and scFv-Ig (IgM) binding was assessed by Western blot.

Germfree appendix rabbits

The appendix of neonatal rabbits was ligated, as described (8). After 4 wk, 10^{10} bacteria were introduced into the ligated appendices, and 3 wk later, the appendix was assessed for GALT development by immunohistochemistry. Rabbits were housed in the Comparative Medicine Facility at Loyola University Medical Center under an experimental protocol approved by the Loyola University Medical Center Institutional Animal Care and Use Committee.

Results

Characterization of Fv binding to intestinal bacteria

Based on our hypothesis that the intestinal microbiota stimulates GALT development through a superantigen-like mechanism, we predicted that some intestinal bacteria would bind IgM at an unconventional Ag binding site. To test this hypothesis, we generated scFv-Ig with known Ag specificity (anti-FITC) (Fig. 1*A*) and analyzed its binding to total appendix lumenal contents by flow cytometry. We identified a small population of intestinal bacteria to which scFv-Ig bound (Fig. 1*B*), FACS sorted these scFv-Ig–binding commensals, and plated the bacteria on a variety of media under both aerobic and anaerobic conditions. We observed colonies of 11 different morphologies and confirmed scFv-Ig binding to 6 of the 11 isolates (Fig. 1*C*). By phase-contrast microscopy and 16S rRNA gene sequence analysis, we determined that 4 of the 6 scFv-Ig–binding isolates were spore-forming *Bacillus* species (Fig. 1*D*, Supplemental Table 2). These data suggested that a subset of intestinal bacteria, including *Bacillus* species, binds to V_H or V_L domains independent of Ag specificity.

Rhee et al. (8) previously showed that *B. subtilis* potently induces GALT development, but that a strain blocked at an early stage of sporulation (due to a mutation in the gene *spoIID*) did not promote B cell proliferation in the appendix. These data indicated that sporulation is required for *B. subtilis* to induce B cell follicle formation in GALT. We reasoned that the lack of GALT development in response to sporulation-deficient *B. subtilis* may either be because sporulation is required for *B. subtilis* to survive in the appendix environment or because the act of sporulating, or spores themselves, induces B cell proliferation. Consistent with the possibility that bacterial spores may stimulate B cells, by flow cytometry we found that scFv-Ig bound to a proportion of purified spores from intestinal *Bacillus* isolates (Fig. 1*E*), and by Western blot scFv-Ig bound to proteins from *Bacillus* spore extracts (Fig. 1*F*, Supplemental Fig. 2). The lack of scFv-Ig binding to the entire population of intestinal *Bacillus* spores suggests that some feature of the spore surface limits accessibility of the scFv-Ig–binding molecule(s). Furthermore, we investigated scFv-Ig binding to spores from other *Bacillus* species, and found that whereas scFv-Ig bound to only a small proportion of *B. subtilis*, *Bacillus cereus*, and WT *B. anthracis* (Sterne strain) spores, it bound to the entire population of *B. anthracis* spores bearing a mutation (in the *bclA* gene) that results in the lack of a major spore-surface structure, the hairlike projections (Supplemental Fig. 3, Fig. 2*A–C*) (21, 22). This finding is consistent with the possibility that an exosporium molecule from WT spores prevents Fv from binding; this molecule is absent from the surface of *bclA* spores, allowing Fv to bind. Due to the intense binding of scFv-Ig to *bclA* mutant *B. anthracis* spores, we used this strain for further binding analyses. We found that intact rabbit IgM also displayed greater binding to *bclA* mutant *B. anthracis* spores than to WT spores (Fig. 2*B* and 2*C*). As expected from the uniqueness of the composition of the spore surface, scFv-Ig did not bind to *bclA* mutant *B. anthracis* vegetative cells (Fig. 2*D*). Taken together, these data strongly suggested that Fv of IgM binds to a molecule(s) on the surface of *Bacillus* spores.

Identification of *Bacillus* spore proteins to which Fv binds

The interaction observed between scFv-Ig and *Bacillus* spores suggested that Fv binds to a molecule(s) on the surface of spores, and that this occurs through an unconventional superantigen-like binding site. To identify this spore-surface molecule, and to characterize the spore superantigen binding site on Fv, we took advantage of a *B. anthracis* strain lacking the outermost spore layer, the exosporium (due to a mutation in the gene *cotO*) (23). We used gel electrophoresis to separate proteins from WT and *cotO* mutant *B. anthracis* spore extracts, and probed the resultant bands with scFv-Ig by Western blot. We predicted that scFv-Ig would bind to a molecule from WT spores, but not from *cotO* mutant spores, because *cotO* mutant spores lack the exosporium. As predicted, scFv-Ig bound to an ~25-kDa molecule in WT, but not *cotO* mutant *B. anthracis* spore extracts (Fig. 3*A*, *left*). This band migrated similarly to that observed in spore extracts from *Bacillus pumilus* intestinal isolates (Fig. 1*F*). The same banding pattern was observed when we probed WT and *cotO* spore extracts with rabbit IgM (Fig. 3*A*, *right*), and the 25-kDa band from WT spore extracts stained darker with Coomassie blue than did the band from *cotO* mutant spore extracts (Fig. 3*B*). The 25-kDa regions from the WT and *cotO* mutant spore extracts were excised from the gel and submitted for mass spectrometry analysis to identify the protein composition of these bands.

From the several peptides identified by the mass spectometry analysis, we focused on three (ExsK, ExsB, and ExsJ) that are known or very likely to be present in the exosporium (24, 25), and that were present in the WT band, but absent from the *cotO* band. To determine which, if any, of these three proteins scFv-Ig binds, we induced the expression of each protein in *E. coli* and probed lysates with scFv-Ig by Western blot. ScFv-Ig and rabbit IgM bound to a protein from the lysates containing ExsK, but not to the uninduced lysate nor to lysates containing either of the other two proteins (Fig. 3*C*, Supplemental Fig. 4). These data indicated that scFv-Ig binds to ExsK, and suggested that ExsK is an IgM-binding protein. To test whether *B. anthracis* spores contain additional molecules that bind scFv-Ig, we probed *exsK* mutant *B. anthracis* spores with scFv-Ig by Western blot. Interestingly, scFv-Ig bound to a molecule in both WT and *exsK* mutant spores (Fig. 3*D*). We conclude that *B. anthracis* spores contain multiple protein species that bind to Fv, one of which is ExsK.

Identification of the spore binding site on Fv

Previously characterized B cell superantigens have been shown to bind to either V_H or V_L. For example, protein A from *S. aureus* or protein L from *Peptostreptococcus magnus* binds to V_H FR residues of V_HIII gene family members, or V_κ FR, respectively (26–28). To test whether scFv-Ig binding to spores is mediated through V_H or V_L, we generated additional scFv-Ig proteins containing V_H or V_L domains encoded by different V genes (Fig. 4*A*, Supplemental Fig. 5), and analyzed their binding to spores. The original scFv-Ig contained *V_H1a2*, a V_H gene (encoding V_Ha-allotype-associated amino acid residues) that is expressed by 80–90% of B cells (18), and a *V_κ* gene from a FITC-binding rabbit hybridoma (17). To generate the second scFv-Ig, we replaced *V_H1a2* with the infrequently used *V_Hy33*, a V_H gene that does not encode V_Ha-allotype-associated amino acids, but instead encodes V_Hn-associated amino acid residues. The third scFv-Ig retained *V_H1a2* and *V_κ* was replaced with an infrequently expressed V_L gene, *V_λ*. Modeling of the V_H domain onto a ribbon diagram shows that the V_Ha-associated amino acid residues form a putative superantigen binding site (10), and we predicted that the mutant scFv-Ig derived from *V_H1* and *V_λ* would bind to spores, but that the scFv-Ig derived from *V_Hy33* and *V_κ* would not. Contrary to our prediction and similar to the binding observed with the original scFv-Ig, V_Hy33-Fv bound to spores (Fig. 4*B* and 4*C*), but replacing *V_κ* with *V_λ* greatly reduced spore binding (Fig. 4*D*, Supplemental Fig. 6). A human IgMλ myeloma protein also did not bind to spores as strongly as did a human IgMκ myeloma protein (Fig. 4*G* and 4*H*), suggesting that V_κ

greatly contributes to the spore binding site. Furthermore, the binding of rabbit and human Ig was consistent with characteristics of a superantigen-like binding. Rabbit and human Ig bound to spores independent of H chain isotype and Ag specificity: polyclonal rabbit and human IgM and human IgG from serum each bound strongly to the spore surface (Fig. 4*E*, 4*F*, and 4*I*). Taken together, these data suggest that Fv and Ig bind to spores via a superantigen binding site that is mediated largely through V_{κ}.

B cell activation in response to *B. anthracis* spores

The binding of Fv and IgM to the surface of *Bacillus* spores through a superantigen binding site suggested that spores may function like superantigens, and therefore, should bind and activate B cells. To test this, we incubated human Ramos B cells (IgM-expressing lymphoma) with *B. anthracis* spores, and assessed spore binding and calcium flux by flow cytometry. As predicted, spores bound to Ramos B cells (Fig. 5*A*), and we observed an increase in fluorescence intensity following stimulation of fluo-3-AM and fura red-loaded Ramos B cells with *bclA* mutant spores, indicating that the cells had been activated (Fig. 5*B*). Although the fluorescence induced by spore binding was somewhat different from that observed after stimulation with anti-Ig, these data suggested that *B. anthracis* spores bind and stimulate B cells in vitro.

GALT development in response to *B. anthracis*

The intense binding of Ig to the surface of *B. anthracis* spores, the binding of spores to human B cells, and the calcium flux observed in human B cells in response to *bclA* mutant spores led us to test whether *B. anthracis*, like *B. subtilis* (8), stimulates B cells in GALT. To test this, we introduced WT *B. anthracis* cells into germfree appendices and analyzed GALT development by two-color immunohistochemistry. Using this approach, we identified large follicles with many proliferating B cells in response to *B. anthracis* (Fig. 6*A*). Because *Bacillus* spores stimulate B cells in vitro, and sporulation is required for GALT development in vivo (8), we tested whether the spore surface contributes to this development by introducing *cotO* mutant *B. anthracis* cells into germfree appendices. Although the *cotO* mutant promoted development of GALT (Fig. 6*B*), the number of proliferating B cell follicles in these appendices was significantly decreased compared with WT (Fig. 6*D*). Taken together, our data suggest that molecules on the surface of *Bacillus* spores stimulate B cell development in GALT.

Discussion

For years, it has been recognized that intestinal bacteria are essential for development and function of the innate and adaptive immune systems. There is growing interest in understanding not only how the intestinal microbiota as a whole promotes various aspects of innate and adaptive immunity, but also how individual bacterial species and individual bacterial molecules are involved. Rhee et al. (8) demonstrated that the combination of two bacterial species, *B. subtilis* and *B. fragilis*, is sufficient to stimulate B cell development in rabbit GALT, but the molecular mechanism by which either of these bacteria promote GALT development remains unclear.

Bacteria may stimulate GALT development by a number of different mechanisms, but accumulating evidence suggests that B cells are polyclonally stimulated in GALT (8–10). In general, B cells can be polyclonally stimulated by bacteria through the engagement of IgM by B cell superantigens, and several characteristics of rabbit B cells make them good candidates for stimulation by B cell superantigens. First, nearly all B cells use the same V_H gene during V(D)J gene recombination (18), giving rise to a population of B cells with highly homologous FR amino acid residues. Modeling of this V_H domain onto a ribbon

diagram showed that the conserved amino acid residues from V_Ha B cells, which are positively selected by the intestinal microbiota in GALT, form a putative superantigen binding site (10). Finally, introduction of protein A, a known B cell superantigen, into germfree appendices promotes B cell proliferation and follicle formation (8).

In this study, we searched for bacterial superantigens from the indigenous microbiota that may polyclonally stimulate GALTB cells, by first isolating bacterial species from the appendix lumen that bound to Fv containing the putative superantigen binding site on IgM. Two remarkable findings came from this experiment, as follows: most of the culturable Fv-binding bacteria were spore-forming *Bacillus* species, and furthermore, one of the intestinal isolates was *B. subtilis*, a bacterial species that we previously identified as a major contributor to GALT development (8). By examining spores from these gut-derived spore-forming bacteria, we found that Fv binds directly to spores, suggesting that the surface of intestinal *Bacillus* spores may harbor a B cell superantigen.

The surface of *Bacillus* spores possesses characteristics of known B cell superantigens, including repetitive subunits that render them oligovalent and mediate simultaneous binding to multiple V_H or V_L regions, resulting in the cross-linking of IgM and subsequent activation of B cells (11). For example, protein A contains five highly homologous extracellular domains that can each bind to a V_H domain encoded by V_H genes belonging to the V_HIII gene clan (26, 28); *P. magnus* protein L contains between four and six homologous domains that bind to conserved amino acid residues in the FRs encoded by several V_κ genes (27); and the glycoprotein, gp120, repetitively displayed on the surface of HIV virions, binds to V_H domains encoded by genes of the human V_H3 family (29). Similar to protein A, protein L, and HIV gp120, *Bacillus* spores are structurally repetitive, bind to Ig V region amino acid residues, and activate B cells. A number of proteins are repeatedly displayed on the spore surface, and by staining *B. anthracis* spores with Fv containing different V_H and V_L domains, we determined that V_κ-containing Fv binds strongly to the spore surface, and that *B. anthracis* spores bind and activate B cells in vitro. Additionally, B cell superantigens bind Ig independent of their Ag specificity and H chain isotype. We observed binding to *B. anthracis* spores by Fv with anti-FITC specificity and also by polyclonal rabbit and human IgM of unknown Ag specificity. Furthermore, both IgM and IgG bound to *B. anthracis* spores. These observations are consistent with the possibility that the surface of *B. anthracis* spores harbors a molecule with characteristics of a B cell superantigen.

In fact, using a proteomics approach, we identified a molecule from *B. anthracis* spores, ExsK, that binds IgM and Fv. ExsK was previously identified as a component of the exosporium (24, 25), and recently, we showed that ExsK is surface exposed, yet partially occluded by other molecules on the spore surface (15), much like the molecule(s) on *Bacillus* spores that binds Fv. The limited accessibility of Fv-binding molecules at the spore surface leads us to consider how these molecules can stimulate B cells in vivo. We suggest that superantigen-like molecules on the spore surface are most likely revealed to B cells in vivo either during sporulation, germination, or following modification of the spore surface by host proteases.

ExsK is not the only protein from *B. anthracis* spores that Fv binds, and also, Fv binds molecules from intestinal *Bacillus* spores that do not contain an exosporium, suggesting that spore molecules other than ExsK bind Fv in a superantigen-like manner. We also think it is unlikely that *Bacillus* species are the only group of intestinal bacteria that stimulate B cell development. We also isolated Fv-binding *Bacteroides* species from the gut, which suggests that the capacity to bind IgM is not limited to spores. Rather, we suggest that B cell proliferation in the appendix is promoted by multiple superantigen-like molecules from

several diverse bacterial species. Most likely, in the ecological niche of the gut, there is evolutionary pressure for multiple organisms to stimulate the immune system, as well as pressure for any given member of the microbiota to stimulate through multiple pathways. Further evaluation of Fv-binding molecules from the intestinal bacterial isolates will be required to determine the relatedness, if any, of these molecules, how specifically these molecules bind Fv (through V_H or V_κ), and if these molecules exhibit superantigen-like activity in vitro or in vivo.

Based on our findings, we propose that polyclonal B cell stimulation by a *Bacillus* superantigen is a plausible mechanism for GALT development. Although we believe that B cell superantigens may drive GALT development, we do not think that this mechanism operates independently, nor is the only mechanism by which B cells are stimulated in the appendix. Stimulation of B cells by superantigens traditionally leads to cell death and deletion of entire B cell populations for extended periods of time (30, 31). However, death induced by B cell superantigens has been shown to be inhibited by the presence of additional signals mediated by LPS (TLRs), CD40L, or IL-4 in vitro (32). These observations suggest that in addition to stimulation by a B cell superantigen, an additional signal provided by bacteria or another cell type (e.g., T cells, dendritic cells, or epithelial cells) is needed for B cell proliferation and survival in the appendix. In fact, V. Yeramilli and K.L. Knight (unpublished data) determined that GALT development requires CD40–CD40L interactions.

Bacteria may also stimulate B cells through receptors other than the BCR (e.g., TLRs) or by first engaging and activating another cell type in GALT, such as a dendritic cell or T cell, which upon activation produces molecules that activate B cells. Because previous data suggest that GALT development proceeds independent of B cell Ag specificity (8–10), we suggest that B cell proliferation in rabbit GALT occurs independent of cognate T cell help. T-independent mechanisms that could potentially stimulate B cell activation in GALT have been reported. For example, in response to TLR stimulation by the intestinal microbiota, human intestinal epithelial cells secrete a proliferation-inducing ligand (APRIL), which in turn promotes B cells to undergo class switch recombination and produce IgA (33). In mice, GALT consists mainly of Peyer's patches and isolated lymphoid follicles (iLFs), two tissues that serve as sites of IgA production (34). The formation of iLFs occurs after birth and requires the intestinal microbiota (35). The recognition of Gram-negative peptidoglycan by the innate immune receptor, NOD-1, expressed in intestinal epithelial cells, promotes the formation of iLFs (35). Either of these mechanisms may also be induced by the intestinal microbiota to promote the formation of B cell follicles in the appendix.

In conclusion, as was demonstrated a few years ago for T cell development, in which a single bacterial molecule, polysaccharide A from *B. fragilis*, can promote the balance of peripheral Th1 and Th2 cell subsets (3), our study reveals that bacterial B cell superantigen-like molecules promote B cell development in GALT. Our findings also uncover a previously unappreciated aspect of the intestinal microbiota, in which bacterial spores, as opposed to vegetative cells, harbor immunostimulatory molecules that promote development of the mammalian immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

fluo-3-AM	fluo-3-acetoxymethyl ester
FR	frame-work region
FSC	forward light scatter
I	induced
iLF	isolated lymphoid follicle
LB	Luria broth
scFv	single-chain Ab fragments containing the Ig V_H and V_L domains
SSC	side light scatter
U	uninduced
WT	wild type.

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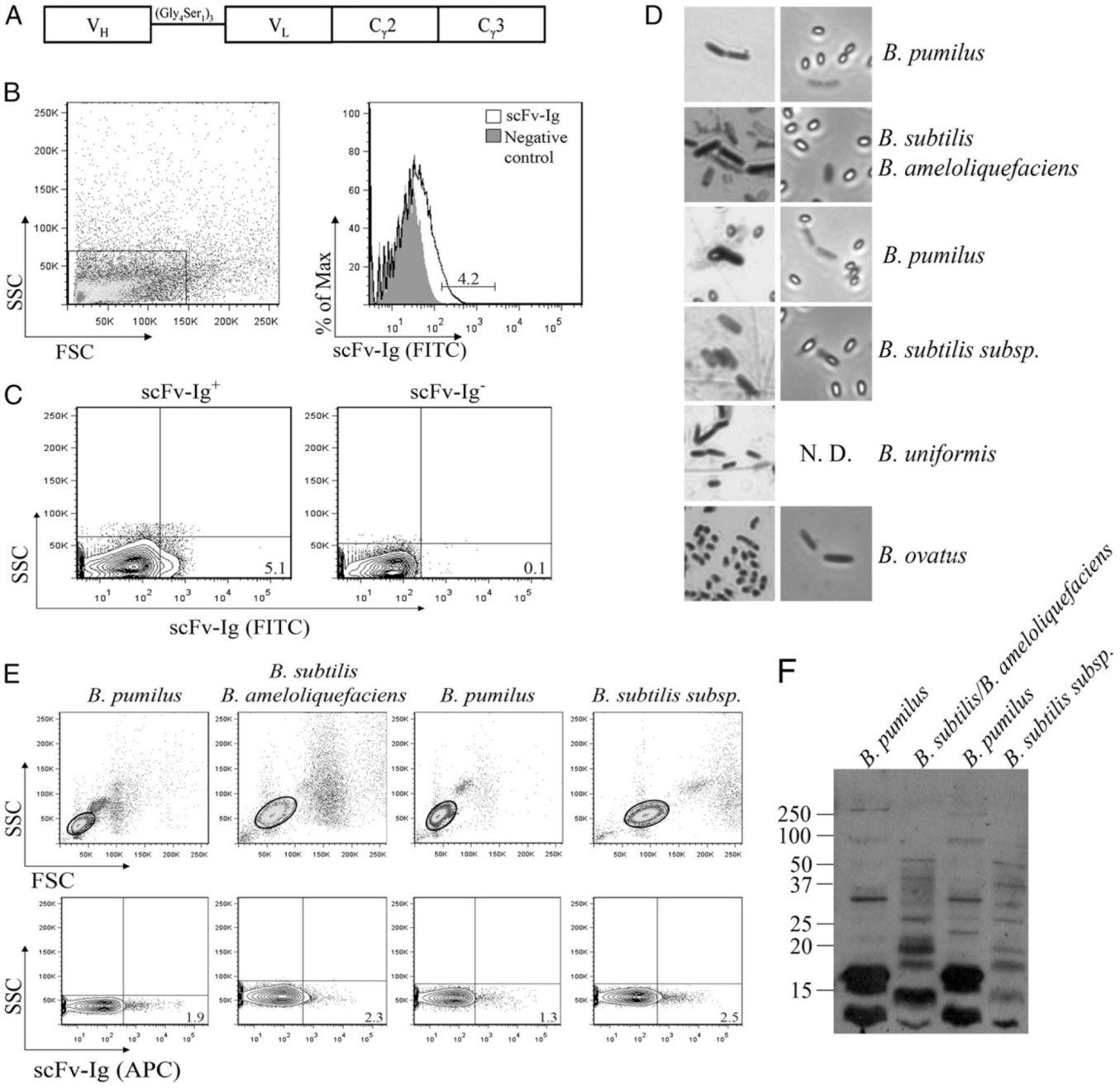

FIGURE 1.

Flow cytometric, morphological, and Western blot analyses of scFv-Ig binding to lumenal bacteria. *A*, Schematic representation of scFv-Ig with V_H (V_Ha), flexible linker ([Gly_4Ser_1]$_3$), V_κ, and Cγ2 and Cγ3 domains of Fcγ. *B*, Flow cytometry of appendix lumenal bacteria stained with scFv-Ig. *Left*, FSC versus SSC. Gate, population analyzed. *Right*, Staining with scFv-Ig (unshaded histogram); secondary Abs alone (shaded histogram). Bar and number indicate the percentage of scFv-Ig$^+$ events FACS sorted. *C*, FACS-sorted bacteria were grown in vitro and restained with scFv-Ig; one representative scFv-Ig–binding bacterial isolate (scFv-Ig$^+$, *left*) and scFv-Ig–nonbinding bacterial isolate (scFv-Ig$^-$, *right*) are shown. *D*, Gram stain (*left column*) and phase-contrast (*right column*) images of scFv-Ig–binding bacterial isolates (for phase-contrast microscopy, bacteria were

cultured on sporulation media). Intestinal isolates were identified by 16S rRNA gene sequence analysis (Supplemental Table 2). *E*, Flow cytometry of scFv-Ig binding to purified spores from *Bacillus* intestinal isolates. *Top*, FSC versus SSC. Gates indicate population used for scFv-Ig staining below; *bottom*, SSC versus scFv-Ig staining. The percentage of scFv-Ig^{+} spores is indicated in *bottom right quadrants*. *F*, Western blot of spore extracts from the intestinal *Bacillus* isolates probed with scFv-Ig. See also Supplemental Fig. 2. FSC, forward light scatter; SSC, side light scatter.

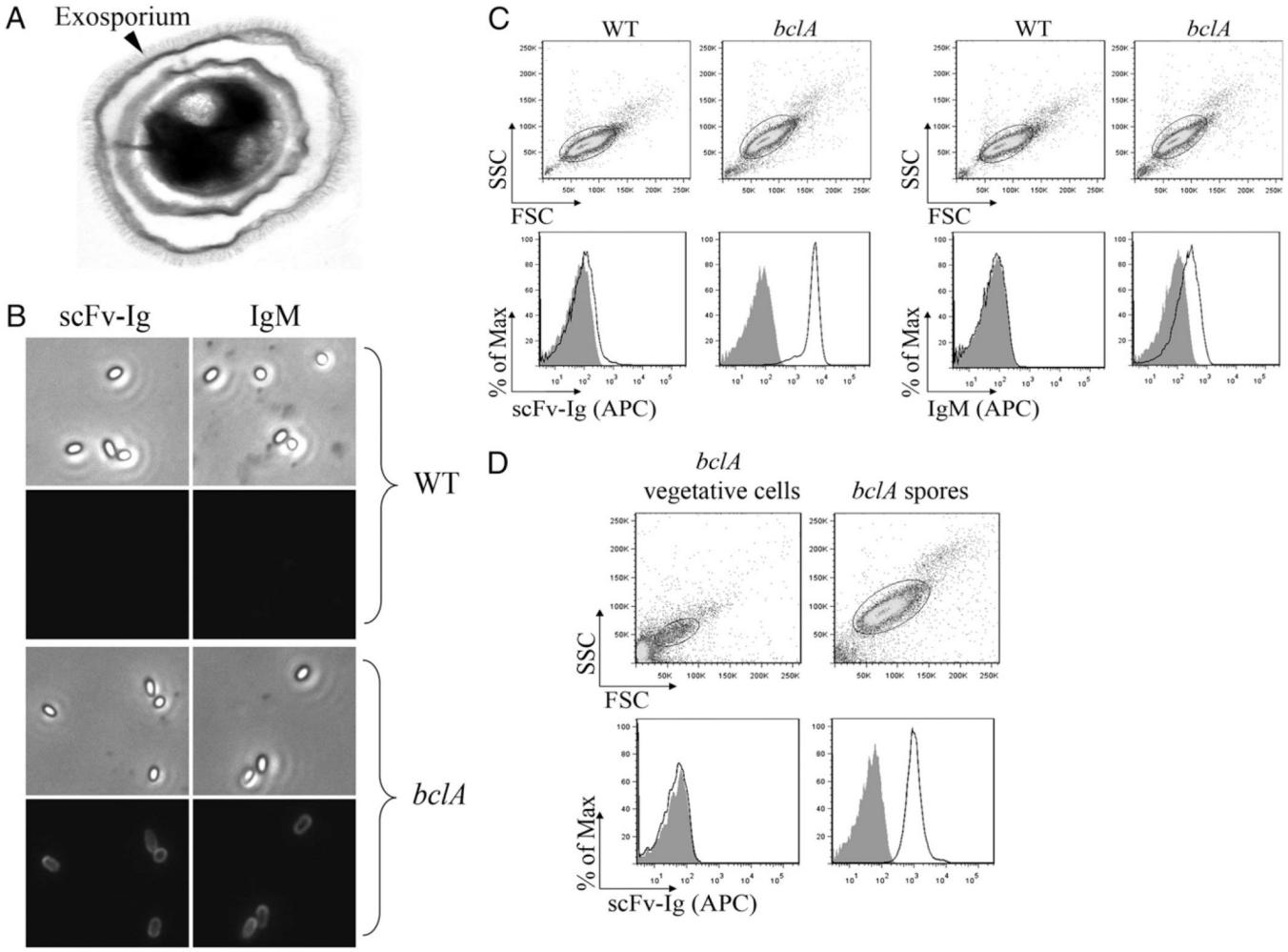

FIGURE 2.

Microscopic and flow cytometric analyses of *B. anthracis* spores. *A*, Transmission electron micrograph of a WT *B. anthracis* endospore. Exosporium marked with arrow. *B*, Phase-contrast (*top*) and fluorescence (*bottom*) images of WT and *bclA* mutant *B. anthracis* spores stained with scFv-Ig (*left*) or rabbit IgM (*right*). *C*, Flow cytometry of WT and *bclA* mutant *B. anthracis* spores stained with scFv-Ig (*left*) or rabbit IgM (*right*). *Top row*, FSC versus SSC. Gates, population for staining with scFv-Ig or IgM (unshaded histograms) and indirect reagents alone (shaded histograms, *bottom row*). See also Supplemental Fig. 3. *D*, Flow cytometry of *bclA* mutant *B. anthracis* vegetative cells (*left*) and spores (*right*) stained with scFv-Ig. scFv-Ig staining, unshaded histograms; indirect reagents alone, shaded histograms.

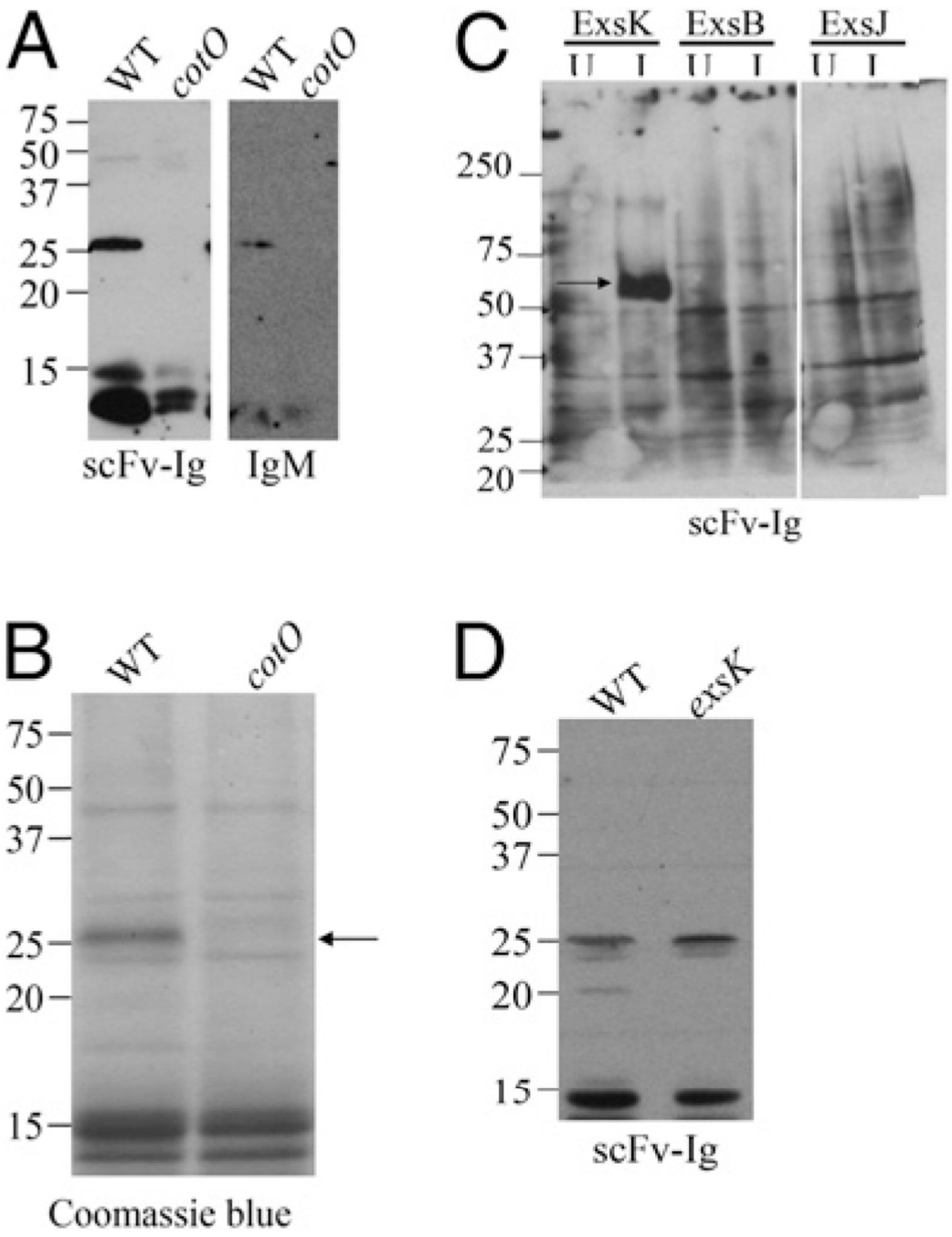

FIGURE 3.

Western blot analyses of *B. anthracis* spore extracts and *E. coli* lysates probed with scFv-Ig or rabbit IgM. *A*, WT and *cotO* mutant spore extracts probed with scFv-Ig (*left*) or rabbit IgM (hybridoma supernatant, *right*). *B*, Coomassie blue-stained proteins from extracts of WT and *cotO* mutant spores. Arrow, region excised for mass spectrometry analysis. *C*, Western blot of unreduced *E. coli* lysates U or I to produce the indicated *B. anthracis* protein, probed with scFv-Ig. Arrow indicates scFv-Ig binding. See also Supplemental Fig. 4. *D*, Western blot of WT or *exsK* mutant spore extracts probed with scFv-Ig. Molecular size markers are shown. I, induced; U, uninduced.

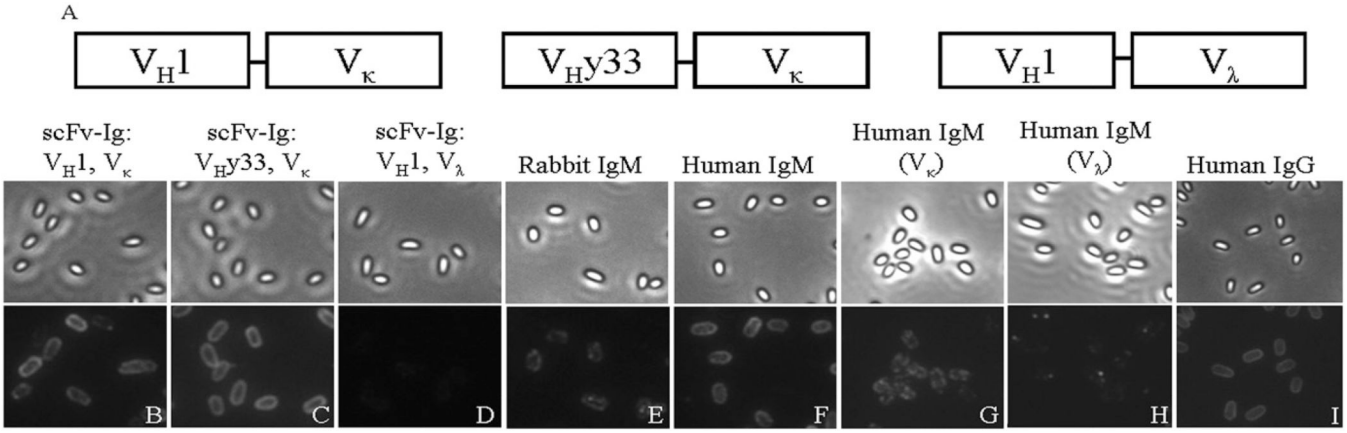

FIGURE 4.

Immunofluorescence of mutant scFv-Ig binding to *Bacillus* spores. *A*, Schematic diagrams of scFv-Ig proteins used for spore-binding experiments. See also Supplemental Fig. 5. *B–I*, Phase-contrast (*top*) and immunofluorescence (*bottom*) images of *B. anthracis bclA* mutant spores stained with the indicated Ig. Rabbit IgM, polyclonal Ig from serum; human IgM and human IgG are polyclonal Ig from serum; human IgM (V_κ) and human IgM (V_λ) are myeloma proteins. See also Supplemental Fig. 6.

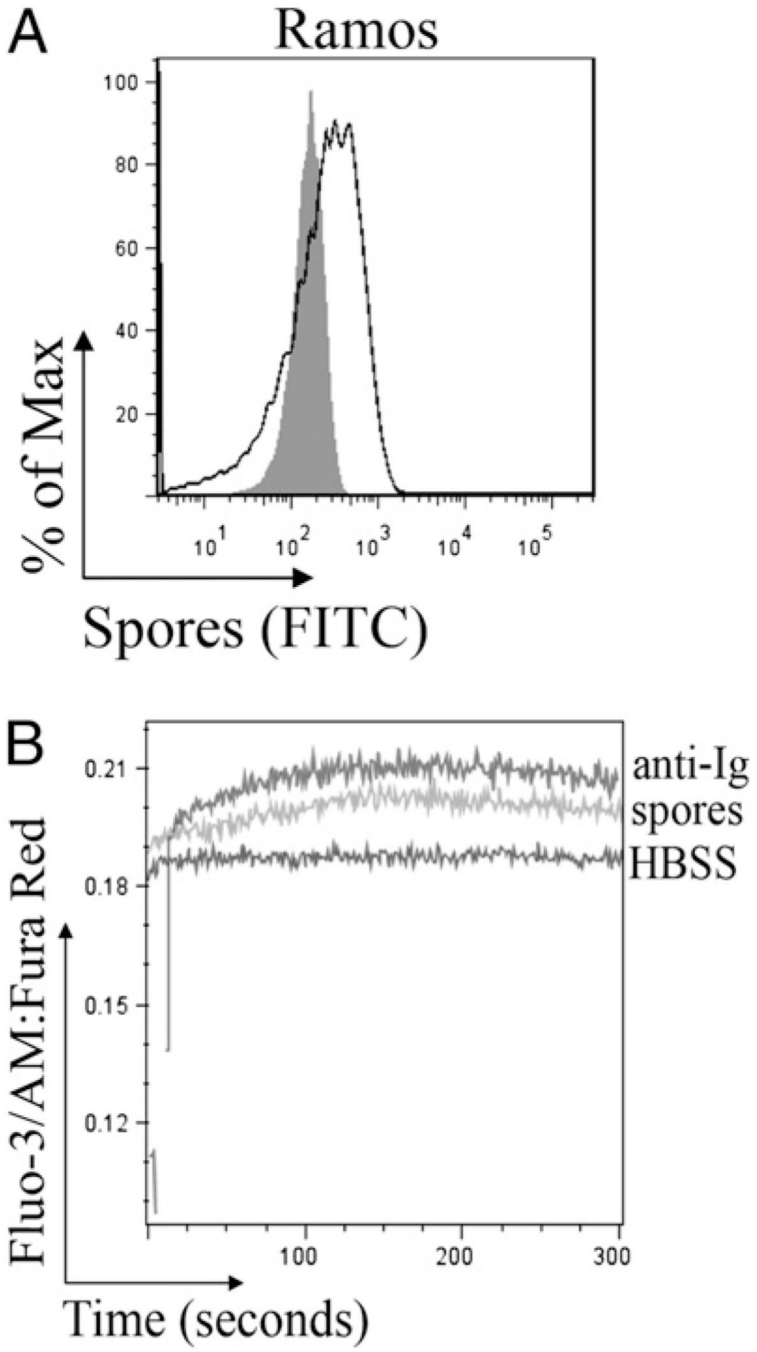

FIGURE 5.

Flow cytometric analyses of spore binding and calcium flux of human (Ramos) B cells incubated with *B. anthracis* spores. *A*, Histogram of human Ramos B cells incubated with spores (unshaded histogram) or without spores (shaded histogram; negative control) and then stained with FITC rabbit anti-ExsK. *B*, Histograms of fluo-3-AM:fura red fluorescence detected in Ramos cells following stimulation with goat $F(ab')_2$ anti-human Ig (*top line*), *bclA* spores (*middle line*), or HBSS buffer (*bottom line*).

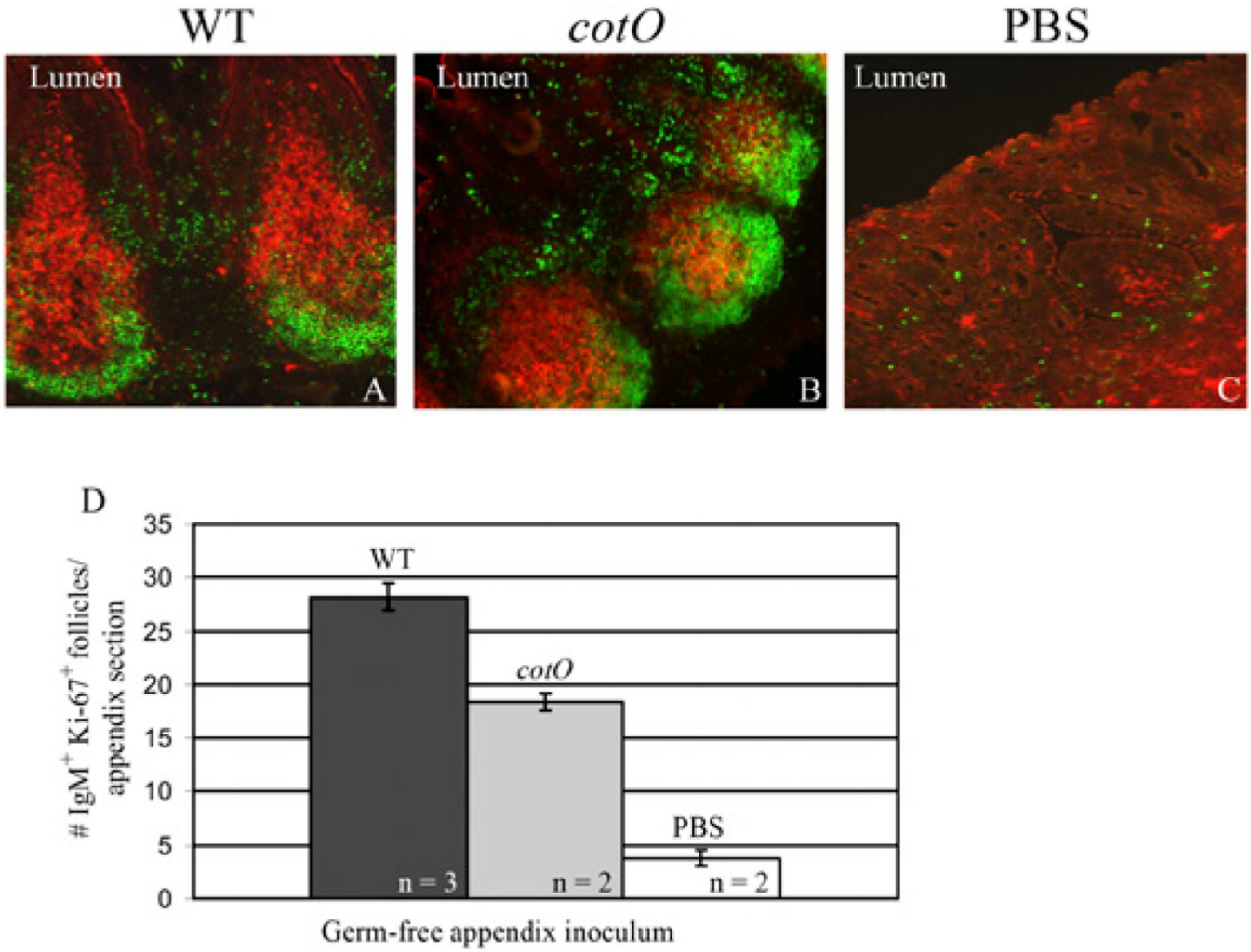

FIGURE 6.

Immunohistological analyses of appendix sections. *A–C*, Anti–Ki-67 (green) and anti-IgM (red) staining of appendix sections from rabbits injected with WT *B. anthracis* cells (*A*), *cotO* mutant *B. anthracis* cells (*B*), or PBS (*C*) at 4 wk of age and analyzed 3 wk later (original magnification × 100). *D*, Quantification of the average number of $IgM^+Ki\text{-}67^+$ follicles per appendix section observed in appendices from *A–C*. A minimum of four nonserial tissue sections was analyzed from each appendix. *n* is the number of appendices examined. Error bars, SEM.