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

Kari M. Severson,1 Michael Mallozzi,2 Adam Driks, and Katherine L. Knight

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 Journal of Immunology, 2010, 184: 6782–6789.

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 requirements 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, 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 Peyers’ patches, bacteria promote B cell proliferation and the 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 appendixes 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 (VH or VL, respectively), and polyclonally 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 VHα and VHβ, and although the majority of B cells in wild-type (WT) rabbits of any age are VHα, the proportion of VHα and VHβ cells differs in ali/ali rabbits over time. At birth, the majority of B cells in ali/ali rabbits are VHβ, but as these rabbits age, a decline in the proportion of VHβ B cells is accompanied by an increase in VHα B cells, and in adult ali/ali rabbits VHα B cells predominate (12). VHβ and VHb B cells differ from each other by several amino acid residues in the FR of the VH domain (13). When these FR residues are modeled onto a three-dimensional ribbon diagram of the VH domain, they are positioned on two adjacent solvent-exposed strands of a β-pleated sheet and form a putative ligand binding site (10). Notably, VHα B cells have a higher proliferative capacity than VHβ B cells (14), and are positively selected in GALT by the intestinal

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Abbreviations used in this paper: fluo-3-AM, fluo-3-acetoxymethyl ester; FR, framework region; FSC, forward light scatter; I, induced; ILF, isolated lymphoid follicle; LB, Luria broth; scFv, single-chain Ab fragments containing the Ig VH and VL domains; SSC, side light scatter; U, uninduced; WT, wild type.

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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 H1a or V L 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 (bioMerieux, 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 exhaustation and purified over a renografin-50 gradient (Bracco Diagnostics, Princeton, NY) (15).

Ab reagents used were as follows: mouse anti-rabbit Fcy (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 IgM, anti-human IgG, anti-human Fc, FITC rabbit Fab anti-IgG, Cy2 goat Fab anti-mouse 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); E Bioscience 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; BioRad, Hercules, CA), and probed with 15–20 μg/ml scFv-Ig, followed by 2 μg/ml mouse anti-rabbit Fcy 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 H-SCFv-Ig construct was generated by PCR-amplifying VH and VL from a FITC-binding hybridoma 27.2-3 (17); the VL region was replaced with PCR-amplified FR1–FR3 from germline VH1a2 (18); VH and VL joined by a [Gly4Ser1]3 linker, were cloned into pcDNA3.1 (Invitrogen) containing the C2 and C3 domains of rabbit Fcy (Fig. 1A). V H-SCFv-Ig was generated by exchanging FR1–FR3 of the above construct with PCR-amplified FR1–FR3 of a VH1a-encoding gene, VH3033 (19). The VH4-SCFv-Ig containing VH4 and VL was obtained by substituting VH4 with VH4-amplified from bone marrow cDNA. The germline sequences of VH1a2, VH333, and VL were all confirmed by DNA sequence analysis (Supplemental Fig. 1). Chinese hamster ovary cells were transfected with scFv-Ig constructs using either CaPO4 or polyethylenimine (Polysciences, Warrington, PA), and stable transfectants secreting scFv-Ig were selected; scFv-Ig was purified using GammaBind G Sepharose columns (GE Healthcare, Pittsburgh, PA). Purity was assessed by SDS-PAGE.

Isolation of intestinal space-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.bcm.tamu.edu/).

Flow cytometry

Spores were stained with 15 μg/ml scFv-Ig, followed by 2.5 μg/ml mouse anti-rabbit Fcy 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 × 10⁶ spores and 1 × 10⁶ 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-EssK 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 anti-rabbit Fcy 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 Fab(’), anti-human IgG 1 or 10⁷ bclA mutant spores for 5 min.

All data were acquired with FACSCan or FACSCountII (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 Fcy 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 (IgMs, Biodiesel International, Saco, ME); IgMs, 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 Fc 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-IgM, 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 using a Leica DM IRB fluorescence microscope equipped with MagnaFire charge-coupled device camera.

The number of IgM Ki67 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 ExsI 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) pLyS 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, 10¹⁰ bacteria were introduced into the ligated appendices, and 3 wk later, the appendix was assayed for GALT development by immunohistochemistry. Rabbis 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. 1A) 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. 1B), 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. 1C). 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. 1D, Supplemental Table 2). These data suggested that a subset of intestinal bacteria, including Bacillus species, binds to VH or VL 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. 1E), and by Western blot scFv-Ig bound to proteins from Bacillus spore extracts (Fig. 1F, 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. 2A–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. 2B and 2C). 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. 2D). 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,

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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.
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. 3A, left). This band migrated similarly to that observed in spore extracts from Bacillus pumilus intestinal isolates (Fig. 1F). The same banding pattern was observed when we probed WT and cotO spore extracts with rabbit IgM (Fig. 3A, 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. 3B). 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 spectrometry 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. 3C, 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. 3D). 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 VH or VL. For example, protein A from S. aureus or protein L from Peptostreptococcus magnus binds to VH FR residues of VHIII gene family members, or VK FR, respectively (26–28). To test whether scFv-Ig binding to spores is mediated through VH or VL, we generated additional scFv-Ig proteins containing VH or VL domains encoded by different V genes (Fig. 4A, Supplemental Fig. 5), and analyzed their binding to spores. The original scFv-Ig contained VH1a2, a VH gene (encoding VHα-100-type-associated amino acid residues) that is expressed by 80–90% of B cells (18), and a VL gene from a FITC-binding rabbit hybridoma (17). To generate the second scFv-Ig, we replaced VH1a2 with the infrequently used VH1β33, a VH gene that does not encode VHα-100-type-associated amino acid residues, but instead encodes VHβ-associated amino acid residues. The third scFv-Ig retained VH1a2 and VL was replaced with an infrequently expressed VLγ gene, Vγ5. Modeling of the VH domain onto a ribbon diagram shows that the VHβ33-associated amino acid residues form a putative superantigen binding site (10), and we predicted that the mutant scFv-Ig derived from VH1β33 and VLγ would bind to spores, but that the scFv-Ig derived from VH1α2 and VLγ would not. Contrary to our prediction and similar to the binding observed with the original scFv-Ig, VH1β33-VLγ bound to spores (Fig. 4B and 4C), but replacing VLγ with VLγ greatly reduced spore binding (Fig. 4D, Supplemental Fig. 6). A human IgMκ myeloma protein also did not bind to spores as strongly as did a human IgMκ myeloma protein (Fig. 4G and 4H), suggesting that Vκ 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. 4E, 4F, and 4I). Taken together, these data suggest that Ig and IgG bind to spores via a superantigen binding site that is mediated largely through Vκ.

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. 5A), 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. 5B). 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. 6A). 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. 6B), the number of proliferating B cell follicles in these appendices was significantly decreased compared with WT (Fig. 6D). 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 VH gene during V(D)J gene recombination (18), giving rise to a population of B cells with highly homologous FR amino acid residues.

**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<sub>k</sub>) and human IgM (V<sub>λ</sub>) 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')<sub>2</sub> 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<sup>+</sup> Ki-67<sup>+</sup> 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.
Modeling of this V<sub>H</sub> domain onto a ribbon diagram showed that the conserved amino acid residues from V<sub>H</sub> 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 GALT B 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<sub>H</sub> or V<sub>L</sub> 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<sub>H</sub> domain encoded by V<sub>H</sub> genes belonging to the V<sub>H</sub>III 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<sub>L</sub> genes (27); and the glycoprotein, gp120, repetitively displayed on the surface of HIV virions, binds to V<sub>H</sub> domains encoded by genes of the human V<sub>H</sub>3 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<sub>H</sub> and V<sub>L</sub> domains, we determined that V<sub>C</sub>-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, EssK, that binds IgM and Fv. EssK was previously identified as a component of the exosporium (24, 25), and recently, we showed that EssK 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.

EssK 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 EssK 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<sub>H</sub> or V<sub>L</sub>), 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.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.
References


### TABLE S1: Bacterial strains used throughout this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10β</td>
<td>Cloning host</td>
<td>Knight Laboratory Collection</td>
</tr>
<tr>
<td>BL21 (DE3)/pLysS</td>
<td>Expression host</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Bacillus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KMS3</td>
<td>gerA::spec gerB::cmΩpcm::tet</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>gerK::erm; PS832 (B. subtilis)</td>
<td></td>
</tr>
<tr>
<td>ADL18</td>
<td>Wild type; PY79 (B. subtilis)</td>
<td>Driks Laboratory Collection</td>
</tr>
<tr>
<td>ADL831</td>
<td>Wild type; 569 (B. cereus)</td>
<td>Driks Laboratory Collection</td>
</tr>
<tr>
<td>RG1</td>
<td>Wild type; 34F2; pXO1⁺ pXO2⁻ (B. anthracis (Sterne))</td>
<td>P. Jackson</td>
</tr>
<tr>
<td>ADL2260</td>
<td>bclA::kan (B. anthracis (Sterne))</td>
<td>J. Bozue</td>
</tr>
<tr>
<td>MGM203</td>
<td>cotΩpMGM3 (B. anthracis (Sterne))</td>
<td>(21)</td>
</tr>
<tr>
<td>KMS2</td>
<td>exsKA::kan (B. anthracis (Sterne))</td>
<td>(15)</td>
</tr>
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</table>
FIGURE S1. Nucleotide and amino acid sequences of VH and VL germline genes used for scFv-Ig constructs. A, VH1a2 and VHy33 sequences. B, Vκ and Vλ sequences.

For details of scFv-Ig construction, refer to Materials and Methods.
### TABLE S2. Identity of scFv-Ig⁺ intestinal isolates from Fig. 1.

<table>
<thead>
<tr>
<th>scFv-Ig⁺ Isolate</th>
<th>Growth conditions</th>
<th>16S rRNA gene sequence identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LB, aerobic</td>
<td><em>Bacillus pumilus</em></td>
</tr>
<tr>
<td>2</td>
<td>Blood agar, aerobic</td>
<td><em>Bacillus subtilis, Bacillus ameloliquefaciens</em>¹</td>
</tr>
<tr>
<td>3</td>
<td>Phenylethanol, aerobic</td>
<td><em>Bacillus pumilus</em></td>
</tr>
<tr>
<td>4</td>
<td>Blood agar, aerobic</td>
<td><em>Bacillus subtilis subspecies</em></td>
</tr>
<tr>
<td>5</td>
<td>Blood agar, anaerobic</td>
<td><em>Bacteroides uniformis</em></td>
</tr>
<tr>
<td>6</td>
<td>Blood agar, anaerobic</td>
<td><em>Bacteroides ovatus</em></td>
</tr>
</tbody>
</table>

¹Based on the 16S rRNA gene sequence of the second isolate, we were unable to distinguish between *Bacillus subtilis* and *Bacillus ameloliquefaciens*. As a result, this isolate is designated by both names throughout the manuscript.
FIGURE S2. Electrophoretic analysis of extracts from intestinal *Bacillus* spores from Fig. 1F. To assess the efficiency of the spore protein extractions, lysates from the indicated intestinal *Bacillus* spores were analyzed by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining. While many proteins were extracted from spores, scFv-Ig bound to only a small number of proteins, demonstrating the specificity of scFv-Ig binding.
FIGURE S3. Flow cytometric analysis of *Bacillus* spores stained with scFv-Ig
(related to Fig. 2). Purified spores of the indicated *Bacillus* species were stained with
scFv-Ig followed by mouse anti-rabbit Fcγ and Dylight™ 649-conjugated goat Fab anti-
mouse IgG. The FSC vs SSC plots are depicted in the top rows, and the scFv-Ig
histograms are shown in the bottom rows. The scFv-Ig plots (unshaded histograms) were
compared to staining with indirect reagents alone (shaded histograms). A limited amount
of binding was observed to the surface of *B. subtilis* and *B. cereus* spores.
FIGURE S4. Western blot analyses of *B. anthracis* spore proteins (related to Fig. 3).

* A-C, *E. coli* lysates uninduced (U) or induced (I) to produce the indicated T7-tagged *B. anthracis* protein were probed with anti-T7-HP (A) or rabbit IgM from serum of a 6-day-old (B) or 3-week-old (C) rabbit. Arrows indicate IgM binding. These data suggest that although all four *B. anthracis* proteins were expressed, IgM bound only to ExsK.
FIGURE S5. Electrophoretic analysis of scFv-Ig proteins from Fig. 4. Coomassie blue-stained SDS polyacrylamide gels (left) and western blots (right) of purified scFv-Ig proteins probed with HRP-conjugated donkey anti-rabbit IgG. Proteins were either left unreduced (A) or reduced (B) prior to gel loading to assess the purity as well as the aggregation status. $V_{H1}$ encodes $V_{H\alpha}$; $V_{H33}$ encodes $V_{H\alpha n}$. These data suggest that the aggregation status of the scFv-Ig proteins does not affect binding to spores.
FIGURE S6. Flow cytometric analysis of bclA mutant B. anthracis spores stained with scFv-Igs from Fig. 4. 

A, FSC vs SSC dot plot spores. Gate indicates population used for staining analyses in B and C. 

B, Histograms of spores stained with V\textsubscript{H}1, V\textsubscript{\lambda}-scFv-Ig followed by mouse anti-rabbit Fc\textgreek{Y} and FITC-conjugated goat Fab anti-mouse IgG (unshaded) or secondary antibodies alone (shaded). 

C, Histogram overlay comparing V\textsubscript{H}1, V\textsubscript{\kappa}-scFv-Ig (from Fig. 2C) and V\textsubscript{H}1, V\textsubscript{\lambda}-scFv-Ig staining (from part B). These data confirm that V\textsubscript{H}1, V\textsubscript{\kappa}-scFv-Ig binds more intensely to spores than does V\textsubscript{H}1, V\textsubscript{\lambda}-scFv-Ig.