MICROBIOTA

B cell superantigens in the human intestinal microbiota

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IgA is prominently secreted at mucosal surfaces and coats a fraction of the commensal microbiota, a process that is critical for intestinal homeostasis. However, the mechanisms of IgA induction and the molecular targets of these antibodies remain poorly understood, particularly in humans. Here, we demonstrate that microbiota from a subset of human individuals encode two protein "superantigens" expressed on the surface of commensal bacteria of the family Lachnospiraceae such as *Ruminococcus gnavus* that bind IgA variable regions and stimulate potent IgA responses in mice. These superantigens stimulate B cells expressing human VH3 or murine VH5/6/7 variable regions and subsequently bind their antibodies, allowing these microbial organisms to become highly coated with IgA in vivo. These findings demonstrate a previously unappreciated role for commensal superantigens in host-microbiota interactions. Furthermore, as superantigen-expressing strains show an uneven distribution across human populations, they should be systematically considered in studies evaluating human B cell responses and microbiota during homeostasis and disease.

INTRODUCTION

At mammalian mucosal surfaces, a homeostatic barrier consisting of immunoglobulin A (IgA) antibodies, mucus, and antimicrobial peptides serves as a first line of defense against a complex array of antigens from the commensal microbiota, diet, and occasional enteric pathogens (*1*). IgA is produced by plasma cells that reside predominantly in the small intestinal (SI) lamina propria (LP), a large fraction of which arise in the absence of inflammation or immunization. These cells differentiate via T cell–dependent (TD) or T cell–independent (TI) mechanisms in mucosa-associated secondary lymphoid tissues such as Peyer's patches (PPs) or mesenteric lymph nodes (mLNs) (*2*–*4*). B cell activation in PPs and mLNs occurs in the presence of cytokines such as transforming growth factor–β, retinoic acid, and interleukin-10 that promote class switching to the IgA isotype and imprinting for intestinal homing (*4*). However, despite their abundance, the specificity and function of IgA antibodies remain poorly understood.

The specificity of IgA has been investigated using bacterial flow cytometry and 16*S* ribosomal RNA gene sequencing to visualize and identify members of the microbiota that are endogenously coated with IgA. These studies have revealed that a taxonomically distinct subset of microbiota in humans and mice is coated with IgA in vivo, whereas other microbes are not (*2*, *5*–*9*). IgA-coated microbiotas have been associated with inflammation and disease in humans with inflammatory bowel diseases or kwashiorkor malnutrition (*5*, *8*, *10*). Analysis of monoclonal antibodies (mAbs) derived from single murine SI IgA plasma cells has demonstrated that these cells typically produce polyreactive antibodies that individually bind multiple microbial taxa (*11*). Individual polyreactive IgA antibodies can bind to multiple structurally diverse antigens including lipopolysaccharides (LPSs), flagellins, DNA, and bacterial glycans in vitro (*11*). However, the microbiota-derived antigens recognized by IgA in vivo remain unknown. Moreover, microbial communities across human populations are exceptionally diverse (*12*), and it remains possible that distinct patterns of IgA reactivity might be observed in individuals with divergent microbial communities.

The functional consequences of IgA binding to microbiota remain poorly understood, and generalizable effects on commensal fitness in vivo have not been documented. Although numerous hypotheses have suggested that IgA may have detrimental effects on microbes by neutralizing surface determinants, promoting agglutination or enchained growth, altering gene expression, or by various other mechanisms (*13*–*16*), the constitutive presence of IgA-coated commensals at steady state suggests that any negative effects of IgA binding are not generally sufficient to drive extinction. By contrast, one recent study suggested that *Bacteroides fragilis* might express certain polysaccharides that attract IgA antibodies to enhance its colonization (*17*); however, the extent to which this phenomenon reflects active attraction of antibodies versus secondary changes in capsular composition that alter antibody accessibility to surface antigens remains unclear. Furthermore, members of the *Bacteroides* are not major targets of IgA in mice (*2*). Thus, it remains unknown whether commensals might express factors that allow them to attract or evade IgA antibodies. Identification of such factors might shed critical light on the enigmatic functions of IgA.

Whereas most microbial antigens stimulate only a tiny fraction of lymphocytes bearing specific B or T cell receptors, certain pathogenic microorganisms express superantigens that can stimulate large fractions of lymphocytes by binding to particular variable region gene families. Although numerous T cell superantigens have been characterized (*18*), only a handful of B cell superantigens have been described, including staphylococcal protein A (SpA) and peptostreptococcal protein L (Protein L) (*19*). Because of their highly potent immunostimulatory properties, superantigens have been associated exclusively with pathogenic organisms. *Staphylococcus aureus* colonization of the human nasopharynx and gastrointestinal tract represents the key risk factor for invasive disease (*20*, *21*), enabling this organism to become one of the deadliest known pathogens (*22*). Expression of the SpA superantigen is required for *S. aureus* persistence and virulence owing to its ability to stimulate nonspecific antibody responses and block the effector functions of the IgG Fcγ region (*23*–*26*). However, superantigens expressed by indigenous commensal, nonpathogenic bacteria have not been identified.

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Here, we report the identification and characterization of a class of B cell superantigens expressed by commensal bacteria of the family Lachnospiraceae that induce and bind a substantial fraction of IgA antibodies, thereby profoundly influencing intestinal B cell responses and IgA coating of commensals. These organisms and their superantigens are widespread, yet unevenly distributed across and within human populations around the world. Thus, superantigen-mediated stimulation and attraction of host IgA antibodies may represent a major mechanism of host-microbiota interaction in humans.

RESULTS

A subset of humans harbor microbiota with B cell superantigen–type properties

Our previous study of antibodies cloned from single murine SI LP IgA plasma cells indicated that these mAbs are commonly polyreactive and individually bind multiple bacterial taxa to coat a broad but defined subset of microbiota (*11*). To extend these observations, we tested whether murine IgA–derived mAbs might also bind human microbiota. We screened a panel of ~27 microbiota-reactive SI IgA mAbs, as well as two nonreactive negative control mAbs from naïve B2 cells (data file S1), by bacterial flow cytometry against fecal microbiota from 10 human infants that had been engrafted into germ-free (GF) mice as part of a separate study (*27*). We found that bacteria from 6 of 10 individuals showed a reactivity pattern consistent with that observed in mice, as expected, indicating that polyreactive murine IgA antibodies bind human microbiota (Fig. 1A). However, 4 of 10 individuals showed an atypical pattern in which the two negative control mAbs as well as a subset of SI IgA mAbs bound to microbiota with very high intensity (Fig. 1A). The mAb-binding microbes from these individuals were also highly coated with endogenous polyclonal IgA (Fig. 1A). Although our cohort included both healthy and cow's milk–allergic infant donors, this binding property did not segregate with allergic status (Fig. 1A). To further characterize this unusual pattern of reactivity, we examined binding of a larger panel of 53 SI IgA and 47 naïve B2–derived murine mAbs against these four individuals' microbiota (data file S1). This analysis revealed that virtually all mAbs that expressed VH5, VH6, or VH7 (VH5/6/7) family variable regions, regardless of their SI IgA or naïve B2 origin, bound strongly to these individuals' microbiota (Fig. 1B). This binding pattern was reminiscent of superantigens such as SpA, which, in addition to antibody Fc regions, binds particular variable region gene families irrespective of other variable elements (*24*, *28*). In contrast to human microbiota, we did not observe this superantigen-type binding in various mouse strains from several different colonies (fig. S1A), although it remains possible that the relevant microbes might be present in other strains or colonies of mice not examined here. These data indicate that a subset of humans harbor microbiota that exhibit superantigen-type binding to antibodies expressing murine VH5/6/7 variable regions.

To further characterize this atypical pattern of reactivity, we purified superantigen-expressing microbiota from one individual using a VH5 mAb (*11*) and isolated a strain of unclassified (UC) Lachnospiraceae, a member of the phylum Firmicutes and order Clostridiales, in pure culture. This strain showed strong binding to mAbs expressing VH5/6/7 variable regions in vitro (Fig. 1C). By contrast, non-VH5/6/7 mAbs generally lacked this reactivity, although a few appeared to bind with weaker intensity (Fig. 1C). On the basis of the phylogeny of this organism, we obtained and screened nine related strains for superantigen-type reactivity (fig. S1B). We identified two additional strains within the family Lachnospiraceae that showed similar superantigen-type binding patterns to the original isolate: *Ruminococcus gnavus* and *Coprococcus comes* (Fig. 1C and fig. S1B). By contrast, several other Lachnospiraceae members including *Clostridium scindens*, *Clostridium nexile*, and *Dorea formicigenerans* lacked this binding pattern, as did all members of the Firmicutes analyzed outside of this family (Fig. 1C and fig. S1B). These data indicate that expression of antibody-binding superantigens is frequent but not ubiquitous among different strains of the family Lachnospiraceae.

As the superantigen-type binding was identified in human but not in mouse microbiota, we reasoned that the superantigens would likely also bind to human antibodies. Staining with a panel of 26 fully human mAbs derived from anti-influenza responses revealed that strains of Lachnospiraceae exhibited superantigen-type binding to human VH3 variable regions (Fig. 1D and data file S1). Similar to our observations with mouse mAbs, non-VH3 antibodies usually did not show reactivity, although a handful of mAbs bound with lower intensity. We conclude that multiple members of the family Lachnospiraceae express superantigens that bind human VH3 and murine VH5/6/7 variable regions. Of note, these patterns are similar to the variable region-binding patterns exhibited by known superantigens such as SpA (*23*, *24*, *29*, *30*). However, these proteins lack the characteristic Fcγ-binding activity of SpA, as both reactive and nonreactive mAbs used in our study were expressed on a common human IgG1 backbone (Fig. 1, C and D). Similar to SpA, the superantigens were likely cell wall–anchored proteins, as our flow cytometry analyses suggested surface localization (Fig. 1, C and D).

Identification of the superantigens

We next sought to identify the superantigen(s). As a first step, we extracted total protein from the UC Lachnospiraceae or *R. gnavus* superantigen–expressing strains or a control *Escherichia coli* strain, and probed the lysates by Western blot with murine VH1 or VH5 mAbs. No reactivity was detected with the VH1 mAb, as expected, but the VH5 mAb identified three reactive bands of 50 to 75 kDa in the superantigen-expressing strains (Fig. 2A). Cell wall–targeted proteins in Gram-positive bacteria have well-characterized signals that direct sorting to this compartment, namely, an N-terminal signal peptide and a C-terminal LPXTG sortase motif (*31*). In addition, all known B cell superantigens including SpA and Protein L contain a series of 50- to 100–amino acid repeat domains that bind immunoglobulins and allow potent cross-linking of receptors on the B cell surface (*23*). By computational analysis of the publicly available *R. gnavus* genome sequenced for the human microbiome project (*32*), we identified ~17 predicted cell wall–targeted proteins. However, none of these proteins had a repeat-domain structure consistent with known superantigens. Further, cloning each of these proteins into *E. coli* followed by expression and purification with an N-terminal His tag failed to identify any factors that bound to VH5/6/7 family mAbs. Moreover, immunoprecipitation (IP) from *R. gnavus* lysates with a VH5 mAb followed by mass spectrometry analysis failed to identify any likely candidates or any proteins with predicted cell surface localization (fig. S2, A and B).

During the course of these studies, a new *R. gnavus* genome was published (*33*), which resolved a number of additional proteins that were absent from the 2007 genome by using Pacific Biosciences' long-read sequencing platform (*34*). Among these, we found two

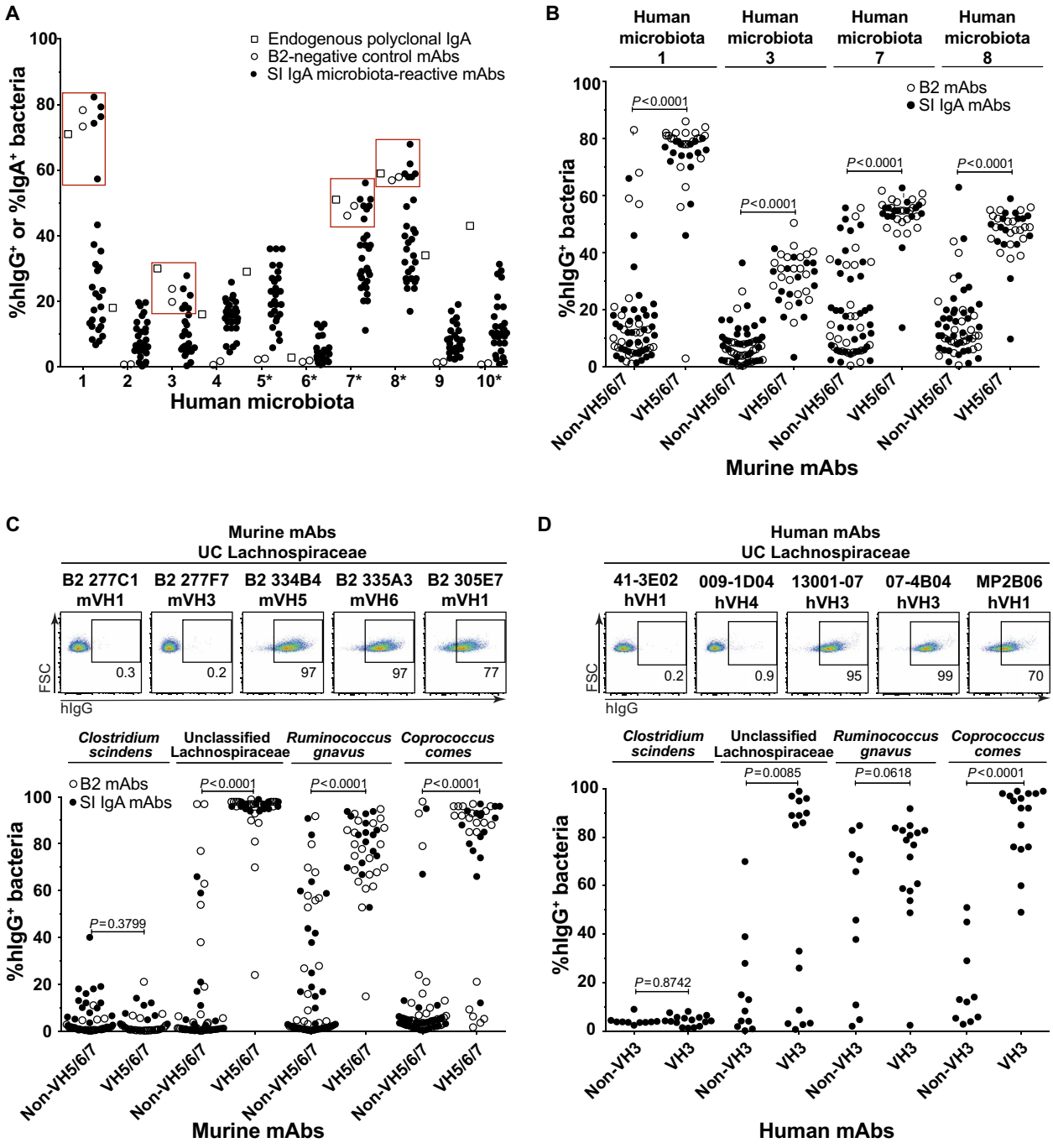

Fig. 1. A subset of human infants harbor microbiota with superantigen-type binding to mAbs expressing murine VH5/6/7 and human VH3 variable regions. (**A**) Bacterial flow cytometry analysis indicating the percent of fecal bacteria from 10 human donors grafted into GF mice that were coated with endogenous polyclonal IgA (open squares), or various negative control B2 mAbs (open circles), or microbiota-reactive SI IgA mAbs (closed circles) expressed with a human IgG1 backbone and detected with anti-hIgG reagents. hIgG, human immunoglobulin G; FSC, forward scatter. Each circle represents a distinct mAb. Red boxes highlight the four individuals with superantigen-type reactivity. Asterisks denote microbiota from cow's milk–allergic infants, and the remaining samples were from healthy infants. Data compiled from four independent experiments. (**B**) Bacterial flow cytometry analysis of the four human infants' microbiota from (A) that showed superantigen-type reactivity with a larger panel of 53 murine SI IgA–derived (closed circles) or 47 naïve B2–derived mAbs (open circles), grouped by heavy chain variable gene usage as indicated. (**C**) Representative flow cytometry plots (top) and **s**ummary of bacterial flow cytometry analysis (bottom) of indicated strains cultured in vitro and stained with 53 SI IgA– or 47 B2-derived mAbs, grouped by heavy chain variable gene usage as indicated. Data compiled from three independent experiments. (**D**) Representative flow cytometry plots (top) and summary bacterial flow cytometry analysis (bottom) of indicated strains cultured in vitro and stained with 26 fully human anti-influenza mAbs, grouped by heavy chain variable gene usage as indicated. Data compiled from three independent experiments. *P* values in (B) to (D) were calculated by unpaired *t* test.

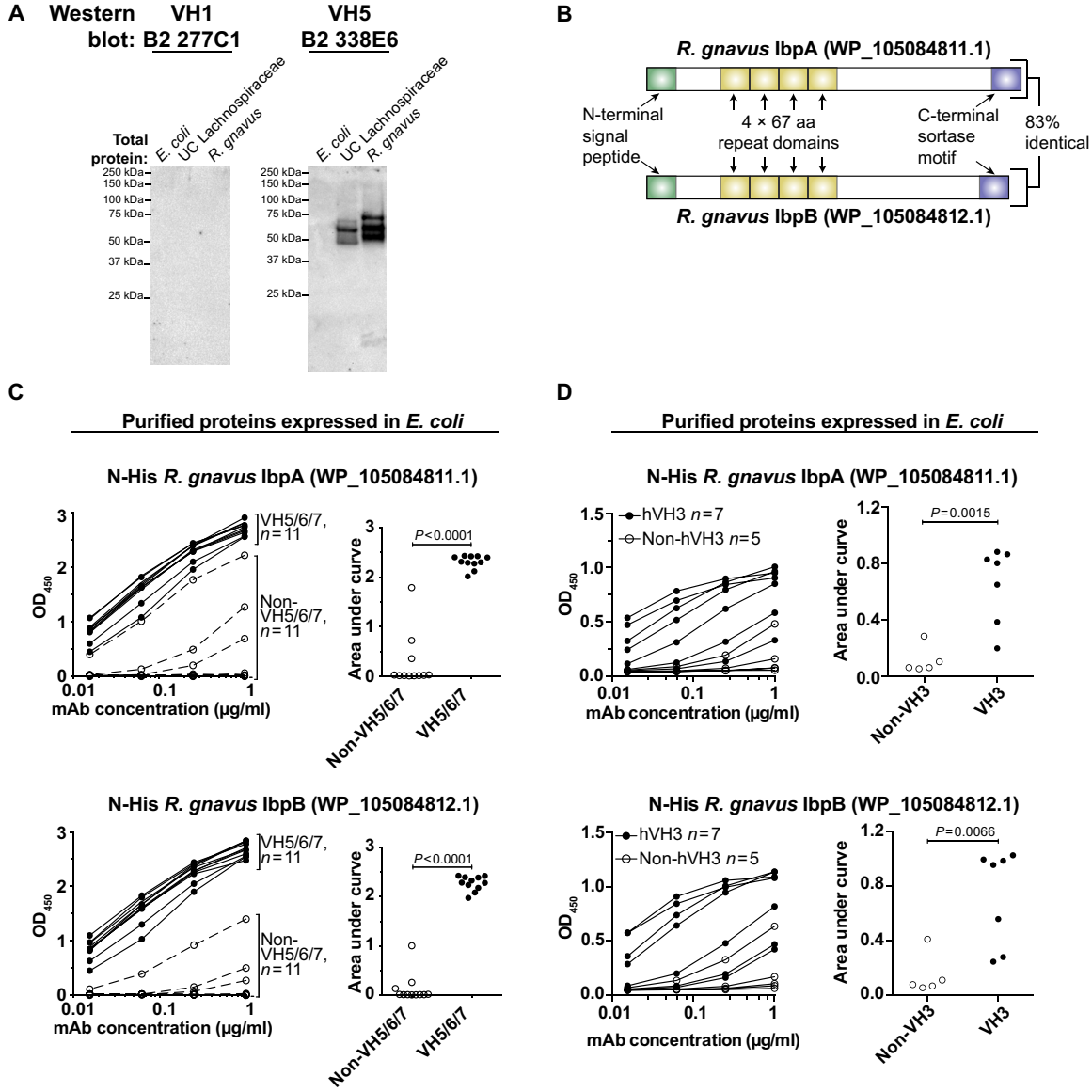

Fig. 2. Identification of the superantigens. (**A**) Western blot analysis of total protein lysates from indicated strains probed with nonsuperantigen-reactive VH1 mAb 277C1 or superantigen-reactive VH5 mAb 338E6. Representative of >3 independent experiments. (**B**) Diagram of the two superantigens. aa, amino acid. (**C**) ELISA analysis of purified *R. gnavus* proteins expressed in *E. coli* with an N-terminal His tag. Proteins were coated on plates and probed for reactivity against the indicated dose titration of 11 VH5/6/7 or 11 non-VH5/6/7 mAbs from naïve B2 cells, as indicated (left panels) or (**D**) against seven human VH3 and five non-VH3 antibodies. Right panels summarize the area under the curve for each antibody shown in the left panels. OD_{450}, optical density at 450 nm. Representative of two independent experiments. *P* values calculated by unpaired *t* test.

uncharacterized "hypothetical" proteins that bore features of known superantigens, including an N-terminal signal peptide, C-terminal sortase motif, four repeat domains of 67 amino acids each, and a predicted molecular weight consistent with that observed by Western blot (Fig. 2B). Reanalysis of mass spectrometry data against this new genome indicated that the two proteins were highly enriched after VH5 mAb IP (fig. S2B). The two proteins were located directly adjacent to one another in the genome and showed substantial similarity (Fig. 2B; ~83% nucleotide sequence identity), suggesting that they arose by gene duplication. Protein and nucleotide basic local alignment search tool (BLAST) analyses revealed no significant sequence homology to previously characterized superantigens or other known proteins.

To test whether these factors encode the mAb-binding superantigens, we cloned and expressed them with an N-terminal His tag in *E. coli* and analyzed the recombinant purified proteins by Western blot and enzyme-linked immunosorbent assay (ELISA). Both proteins bound strongly to mAbs expressing murine VH5/6/7 or human VH3 variable regions but rarely to mAbs expressing other variable regions, and they closely recapitulated the binding patterns observed by flow cytometry against whole bacteria (Fig. 2, C and D, and fig. S2C). These two proteins therefore likely account for the two lower–molecular weight bands observed by Western blot analysis of total lysates (Fig. 2A). The upper band likely represents a mobility shift of the same proteins due to residual attached peptidoglycan, a common

feature of sortase-anchored surface proteins (*35*), as mass spectrometry analysis of this band demonstrated the presence of the two superantigens but no other putative surface proteins (fig. S2B). On the basis of these analyses, we have designated these proteins as immunoglobulin-binding protein A (IbpA; WP_105084811.1) and IbpB (WP_105084812.1). We conclude that *R. gnavus* expresses IbpA and IbpB, two homologous immunoglobulin-binding superantigens, on its cell surface.

IbpA and IbpB stimulate murine and human B cells in vitro

We next sought to determine whether IbpA and IbpB were capable of directly stimulating B cells in vitro. We found that about 30% of murine B cells down-regulated surface IgM and CD19 (Fig. 3A) and up-regulated CD69 (Fig. 3B) after 6 hours of incubation in vitro with plate-bound IbpA or IbpB, consistent with potent B cell receptor–mediated cellular activation, and similar to that found in a majority of B cells after anti-IgM stimulation. To exclude the possibility that this activation resulted in part from contamination (e.g., LPS) during protein purification after expression in *E. coli*, we assessed the ability of IbpA and IbpB to activate B cells isolated from *Myd88*$^{-/-}$ mice that lack the ability to respond to LPS. IbpA and IbpB activated *Myd88*$^{-/-}$ B cells to a similar extent as wild-type B cells (Fig. 3, A and B). In contrast to IbpA and IbpB, purified LPS or CpG DNA up-regulated CD69 but failed to down-regulate IgM and CD19 in wild-type cells, and this response was lost in *Myd88*$^{-/-}$ cells, as expected (Fig. 3, A and B). Direct quantification of LPS contamination in Ibp protein preparations revealed a concentration of less than 5 endotoxin units (EU)/ml (data file S2). We conclude that IbpA and IbpB can directly activate a fraction of murine B cells in vitro, likely by binding the B cell receptor.

Using a similar flow cytometry assay, we next assessed whether IbpA and IbpB could activate human B cells purified from the peripheral blood of three individuals. IbpA and IbpB individually activated a large fraction of human B cells in all individuals, as indicated by down-regulation of surface IgM and CD19 (Fig. 3C) and up-regulation of CD83 and CD69 (Fig. 3D) after 6 hours of stimulation in vitro. Purified LPS or CpG DNA failed to elicit a similar pattern of response, as expected (Fig. 3, C and D). Together, these data suggest that both IbpA and IbpB can stimulate a substantial fraction of B cells in both mice and humans in vitro.

Superantigen-expressing strains induce and bind IgA antibodies in vivo

To determine whether these factors function as superantigens in vivo, we examined GF control mice or three groups of gnotobiotic mice colonized with (i) a control strain of Proteobacteria (UC Pseudomonadaceae) isolated from the fecal IgA^{+} fraction of a specific pathogen–free (SPF) mouse, which is bound by polyreactive IgA antibodies in vivo but lacks superantigen-type binding activity (*11*); (ii) the UC Lachnospiraceae superantigen–expressing strain; and (iii) the *R. gnavus* superantigen–expressing strain. Four weeks after colonization, fecal bacteria from all three groups were coated with IgA in vivo, as expected (fig. S3A). mAb staining demonstrated that fecal bacteria from mice colonized with the superantigen-expressing strains but not the UC Pseudomonadaceae bound a VH5 mAb, as expected, whereas none of the strains bound a control VH1 mAb (fig. S3B). In addition, bacteria from all three groups were bound to a lesser extent by a polyreactive murine VH3 antibody (fig. S3B). These data indicate that the superantigen-expressing strains can become IgA coated by both superantigen-type binding interactions as well as low-affinity natural polyreactive antibodies, although the relative contributions of each to their IgA coating in vivo remain unclear.

Recipients of both superantigen-expressing strains showed a 5- and 10-fold expansion of SI IgA plasma cells over recipients of UC Pseudomonadaceae or GF controls, respectively (Fig. 4, A and B). By contrast, the UC Pseudomonadaceae–colonized controls showed only a minor induction over GF (Fig. 4, A and B). SI IgA plasma cells in mice colonized with superantigen-expressing strains also showed reduced surface IgA staining relative to GF or UC Pseudomonadaceae–colonized controls (Fig. 4A). Although the mechanisms underlying this decreased surface IgA expression remain unclear, this observation further suggests that these commensal strains elicit different immune responses. In line with the observed increase in SI IgA plasma cells, luminal free IgA concentrations were substantially increased in the SI and colon of mice colonized with either superantigen-expressing strain relative to UC Pseudomonadaceae or GF controls (fig. S3C). There were no detectable differences in colonic LP IgA plasma cells between groups (fig. S3D). The observed increase in SI IgA plasma cells could be associated with preferential induction of TD IgA responses by these organisms rather than superantigen-driven interactions (*7*, *11*). However, repertoire analysis indicated that SI IgA plasma cells in all groups showed very low frequencies of somatic hypermutation (fig. S3E) normally observed in TD responses. Further, in all groups, mLN or PP germinal center B cells, T follicular helper cells, IgA class–switched B cells, or CCR6^{+} IgD^{+} precursors were similar to GF controls (fig. S3, F and G) (*36*) and differed from previous reports of preferential TD induction by other commensal bacteria (*37*). Although these data suggest that TI responses may be predominantly involved in the response to these superantigens, they do not rule out the possibility that both TI and TD responses are involved. We conclude that superantigen-expressing strains stimulate substantial SI IgA plasma cell accumulation in monocolonized mice.

To determine whether the expanded SI IgAs preferentially included superantigen-reactive variable regions, we analyzed the immunoglobulin repertoire of sorted SI IgA plasma cells from each group of mice. Both superantigen-expressing strains stimulated substantial accumulation of IgA plasma cells expressing VH5/6/7 gene families (Fig. 4C). By contrast, cells expressing VH1–47 or VH3–5 gene families were detected at similar numbers in all groups (Fig. 4C), indicating that, as expected, the observed IgA induction was not universal and preferentially involved the VH5/6/7 gene families. We conclude that superantigen-expressing UC Lachnospiraceae and *R. gnavus* polyclonally stimulate differentiation of IgA plasma cells expressing VH5/6/7 variable regions and bind their secreted antibodies, thereby increasing both free and bacteria-bound IgA in vivo.

R. gnavus and its superantigens are prevalent across human metagenomes

In this study, we observed microbiota with superantigen-type binding in 4 of 10 human infants; however, this analysis represented a limited cohort whose microbial communities were engrafted into GF mice. Having identified the genes encoding the superantigens, we therefore sought to determine their distribution across 424 healthy adult human metagenomes using publicly available data (Fig. 5, table S1, and data file S3) (*38*–*41*). Metagenomic read recruitment analysis revealed that *R. gnavus* and its superantigens were present in 42% of individuals from the United States and 43% from China, but

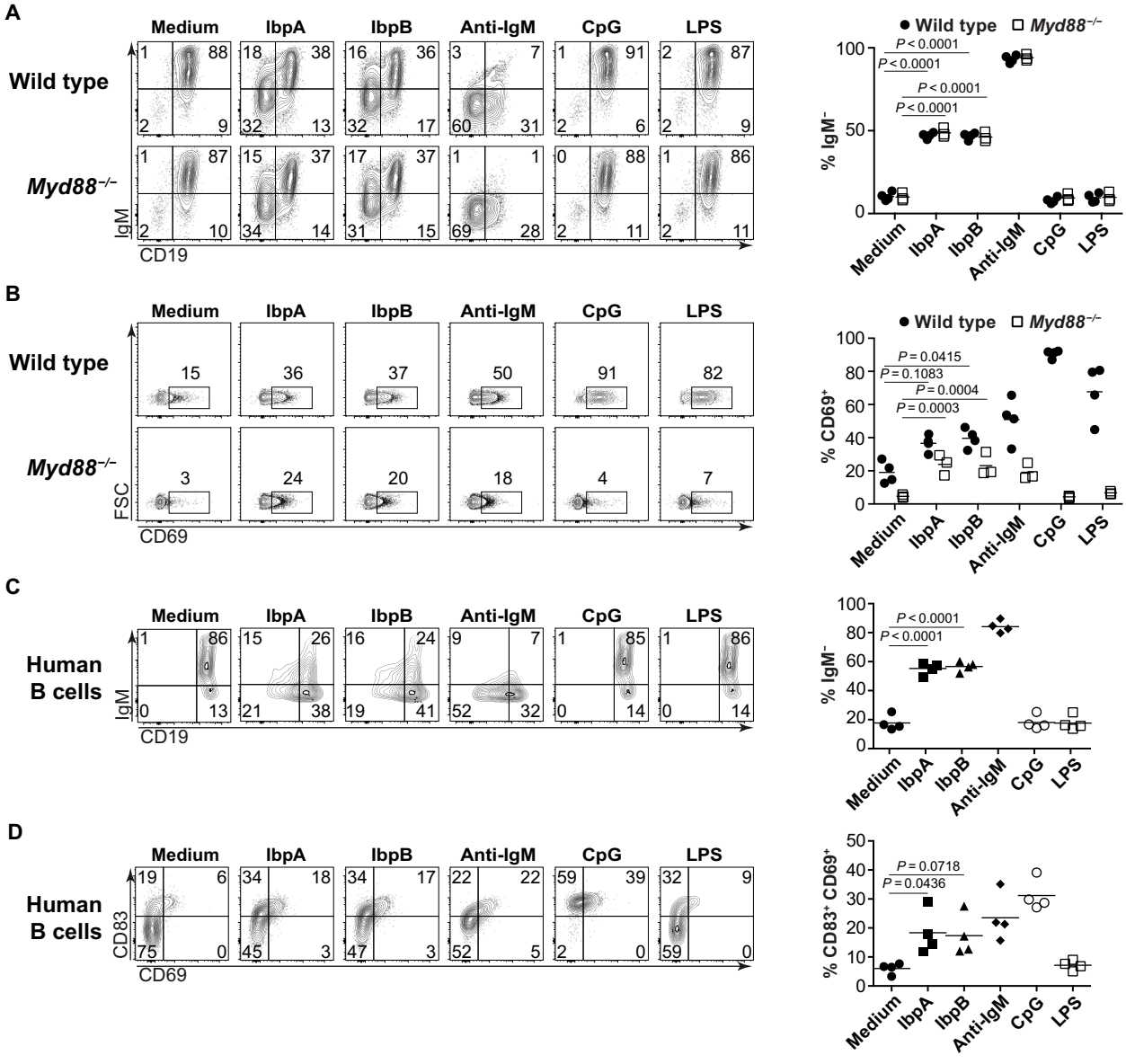

Fig. 3. IbpA and IbpB activate murine and human B cells in vitro. (**A**) Representative flow cytometry analyses and summary graphs depicting purified murine B cells from wild-type or *Myd88*$^{-/-}$ mice 6 hours after in vitro incubation with indicated stimuli and analyzed for surface expression of CD19 and IgM or (**B**) CD69. Data pooled from two independent experiments with a total of four wild-type and three *Myd88*$^{-/-}$ mice. (**C**) Representative flow cytometry analyses and summary plots depicting purified human B cells 6 hours after in vitro incubation with indicated stimuli and analyzed for surface expression of CD19 and IgM or (**D**) CD83 and CD69. Each data point represents one human sample. Samples were obtained from three individuals, and cells from one individual were isolated and analyzed in two independent experiments. Data pooled from two independent experiments. *P* values were calculated by one-way ANOVA.

rare among those from Fiji or Tanzania (7 and 0%, respectively; Fig. 5 and table S1). The two superantigens appeared to be core genes of *R. gnavus*, as both were detected whenever *R. gnavus* was present but not in its absence, and their coverage matched the rest of the genome (Fig. 5, table S1, and data file S4). These results likely underestimate the true prevalence of this class of superantigens, as related superantigen-expressing microbes such as the UC Lachnospiraceae and *C. comes* identified here may also contribute. However, a characterization of their prevalence will only be possible when full genome sequences for these strains become available. We conclude that superantigen-expressing strains of commensal bacteria are widespread, likely present in billions of individuals worldwide but variably distributed within and between human populations.

DISCUSSION

We describe here B cell superantigens expressed by commensal bacteria from the human gut microbiota. These factors are widespread but unevenly distributed across human populations and therefore may represent a common and fundamental mechanism of host-microbe interaction in the intestinal mucosa. We identified the superantigenic factors at the biochemical and molecular level and demonstrated that

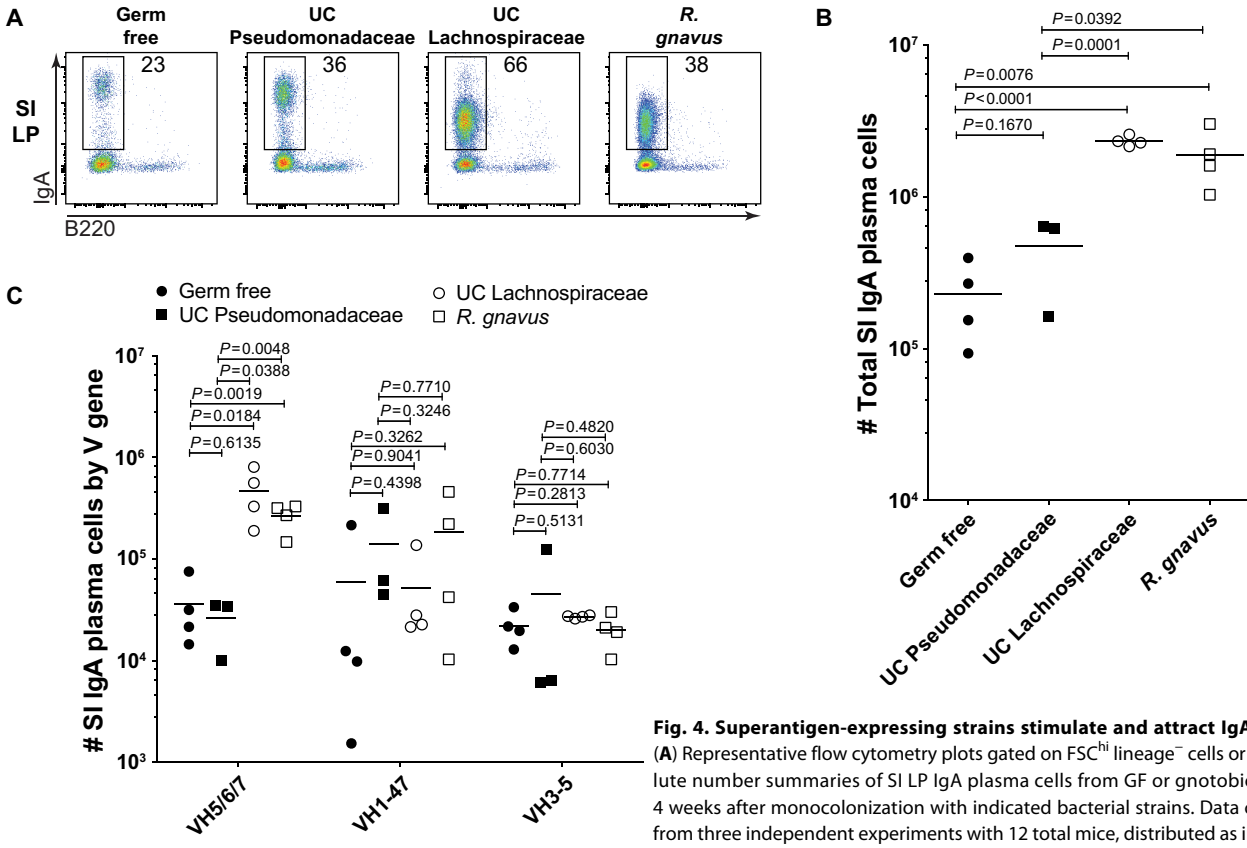

Fig. 4. Superantigen-expressing strains stimulate and attract IgA in vivo. (**A**) Representative flow cytometry plots gated on FSC^{hi} lineage^{-} cells or (**B**) absolute number summaries of SI LP IgA plasma cells from GF or gnotobiotic mice 4 weeks after monocolonization with indicated bacterial strains. Data compiled from three independent experiments with 12 total mice, distributed as indicated. (**C**) Absolute number of SI IgA plasma cells expressing indicated variable gene families. Calculated from the total cell number shown in (B) multiplied by the percent of the repertoire expressing indicated variable region genes as determined by sequencing of plasmids containing cDNA clones. One-way ANOVA *P* values were 0.0048 for VH5/6/7, 0.5156 for VH1-47, and 0.6958 for VH3-5. *P* values in (B) and (C) were calculated by unpaired *t* test.

they represent a class of proteins unrelated to other known B cell superantigens. Our data suggest that these proteins induce and bind IgA antibodies of the human VH3 and murine VH5/6/7 gene families with high affinity in vivo.

Ruminococcus was reported to be the defining taxon of one of three "enterotypes" that characterize human microbiota diversity across individuals (*12*). Although the precise classification of these enterotypes remains somewhat controversial (*42*), it is clear that many bacterial species including *Ruminococcus* are unevenly distributed across human individuals in the same geographic area and between populations in different locations (*43*). Distinct microbial communities have been associated with a variety of diseases including obesity, diabetes, autoimmunity, cancer, and inflammatory bowel diseases (*44*), but in most cases, it remains unclear how these communities mechanistically contribute to pathology in these complex disorders. Our findings suggest that the *Ruminococcus* enterotype is associated with a distinct immunological phenotype characterized by the presence of B cell superantigens. As the superantigen-targeted VH3 gene family is expressed by 50 to 60% of human B cells and intestinal plasma cells (*24*, *45*), it is possible that these microbial factors could substantially affect B cell responses in carriers and therefore alter the course of various diseases involving B cells and/or antibodies. A number of recent studies have identified enrichment of *R. gnavus* in the microbiota of humans with allergic disease (*46*), lupus nephritis (*47*), spondyloarthritis (*48*), and inflammatory bowel disease (*49*, *50*). Whether these relationships are correlative or causative remains unknown; however, our results suggest that detailed further investigations are warranted.

Recent studies have suggested that IgA coating may mark disease-associated members of the microbiota in inflammatory bowel diseases, autoimmunity, and kwashiorkor malnutrition (*5*, *8*, *10*). Although bona fide pathogens are likely to become IgA-coated in vivo, such studies are complicated by the presence of a variety of innocuous commensals that are also coated with IgA during homeostasis (*2*). Our findings emphasize that it will be essential to determine the presence of superantigen-expressing Lachnospiraceae strains in future studies of human IgA–coated microbiota, as these organisms may alter not only their own IgA coating but also the magnitude of IgA responses to other bacteria, particularly those that can be bound by polyreactive human VH3 antibodies (*11*).

A limitation of this study was the lack of methods for genetic manipulation of these commensal organisms, which would allow for further studies of the impact of B cell superantigens on host and microbiota. In particular, it remains unclear why these organisms would express superantigens. Although bacterial genomes are exceptionally plastic and readily adapt to maximize fitness (*51*), our analysis of wild *R. gnavus* strains across hundreds of human metagenomes indicated that the *ibp* superantigens are core components of the *R. gnavus* genome. Moreover, the presence of two proteins with highly similar functions suggests strong evolutionary pressure to express these factors. However, the precise mechanisms by which these superantigens affect the fitness of the commensals and/or the

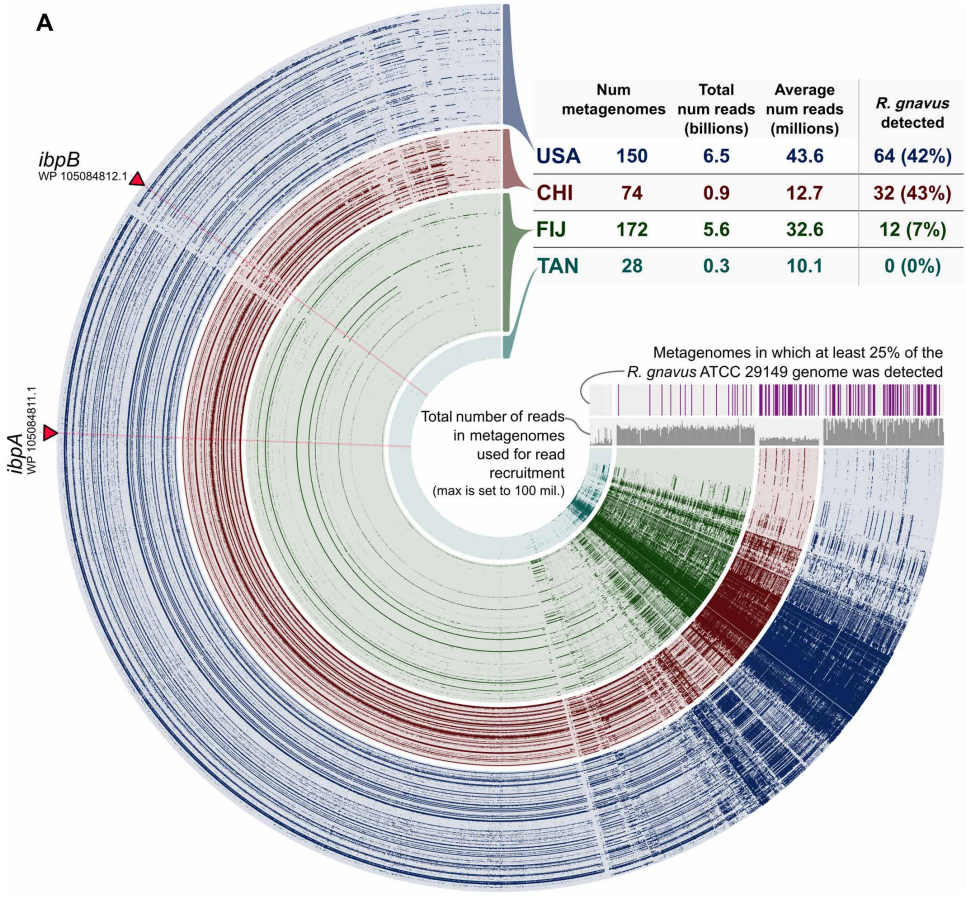

Fig. 5. Distribution of *R. gnavus* and its superantigens across human metagenomes. Dendrogram alignment of the *R. gnavus* ATCC 29149 genome to 424 human metagenomes (data files S3 and S4). Each spoke represents one gene in the *R. gnavus* genome, and each layer represents an individual human metagenome. The two superantigen genes are labeled. Intensity represents coverage of the open reading frame in the metagenome.

In summary, we report that commensal bacteria of the family Lachnospiraceae express potent, conserved B cell superantigens that stimulate IgA production and bacterial coating. Our observations are distinct from known mechanisms of IgA responses to commensals including polyreactive recognition by natural IgA antibodies (*11*) or high-affinity responses to pathogens or vaccines (*55*), and highlight an unexpected mechanism of host-commensal interaction previously associated only with pathogens. Our findings are in apparent contrast with well-known strategies for evasion of antibodies by bacteria (*56*) and suggest, instead, that certain commensal bacteria express factors that attract host antibodies.

MATERIALS AND METHODS

Study design

The objectives of this study were to assess the mechanisms of antibody recognition of the human microbiota and to define bacterial factors that regulate IgA targeting. All data, including outliers, were reported in relevant figures. Sample sizes were determined by reagent availability and were not prespecified. Randomization and blinding were not performed. Information regarding experimental replicates is reported in the figure legends. Primary data are reported in data file S2.

host remain unknown, and it is possible that they might have either beneficial or detrimental effects. For example, these organisms might attract IgA to promote intra- or interspecies agglutination, which could facilitate cooperative physiology (*52*), horizontal gene transfer (*53*), or antagonistic interactions via type VI secretion or other mechanisms (*54*). In addition, superantigen-mediated stimulation could amplify the host IgA response, which could lead to either enhanced or diminished ability to mount protective immune responses to pathogens. Our study did not assess whether Lachnospiraceae superantigens alter the human IgA repertoire in particular physiological conditions, such as during spontaneous colonization in infants, or in dysbiotic conditions, during which the abundance of these strains is altered.

Our study has several additional limitations. First, although we identified the superantigenic factors expressed by *R. gnavus*, the nature of the superantigenic reactivity observed in other strains of Lachnospiraceae remains unknown, and it is possible that this is achieved through either conserved or divergent mechanisms. Second, although we documented binding and activation of human B cells in vitro, we did not yet prove that these factors stimulate or modulate human immunity in vivo. Last, although we frequently identified the presence of the superantigen genes in human metagenomes, we did not determine whether these genes are expressed and functional in all individuals.

Mice

All mouse studies were performed according to guidelines approved by the University of Chicago Institutional Animal Care and Use Committee. *Myd88*$^{-/-}$ mice were purchased from the Jackson Laboratory. For non-gnotobiotic experiments, mice were housed under SPF conditions at the University of Chicago. In most experiments, mice were of the C57BL/6 strain background. In some experiments, fecal samples were collected and analyzed from 6-week-old male mice of C57BL/6, BALB/c, NOD (nonobese diabetic), or C3H/HeN strains 2 days after arrival from the Jackson Laboratory, Taconic Biosciences, or Charles River Laboratories. Colonization of mice with fecal samples from healthy or cow's milk–allergic infants was performed in the University of Chicago Gnotobiotic Animal Research Facility. C3H/HeN mice were colonized with 10 individual human infants' microbiota and maintained in individual gnotobiotic isolators, one per donor, as part of a separate study (*27*). The 10 individuals were Italian infants of mixed gender; informed consent was obtained, and experiments were approved by the University of Chicago Institutional Review Board. Detailed information on each individual was reported separately (*27*). Here, fecal samples from colonized mice were collected and analyzed for mAb reactivity. Experiments involving monocolonized gnotobiotic mice were performed in individual gnotobiotic isolators in the University of Chicago

gnotobiotic facility. Mice were 9 to 24 weeks old, with ages and genders randomly distributed across each experimental group and were analyzed 4 weeks after colonization.

Bacterial strains and culture

R. gnavus (29149), *C. comes* (27758), *D. formicigenerans* (27755), *C. nexile* (27757), *C. scindens* (35704), and *Clostridium ramosum* (25582) were purchased from the American Type Culture Collection (ATCC). The UC Lachnospiraceae strain was isolated as described below. *Listeria monocytogenes* was a gift from B. Jabri at the University of Chicago, and *Lactobacillus rhamnosus* was a gift from J. Alverdy at the University of Chicago. Microbial strains were grown on Schaedler blood agar (BD Biosciences) or chopped meat carbohydrate broth (BD Biosciences) in an anaerobic jar at 37°C with the exceptions of UC Pseudomonadaceae, which was grown on eosin methylene blue agar under aerobic conditions at 37°C and *Candidatus arthromitus* (segmented filamentous bacteria), which was propagated in monocolonized mice in gnotobiotic isolators and analyzed by staining of fecal samples.

mAb staining of fecal and cultured bacteria

For staining of fecal bacteria, one to two fecal pellets were homogenized by adding 1 ml of sterile phosphate-buffered saline (PBS) and vortexing horizontally for 5 min. Debris was pelleted by centrifugation at 400*g* for 5 min, and the supernatant was filtered through a sterile 70-μm strainer (Fisher Scientific) and transferred to a new 1.5-ml tube. Cells were centrifuged for 5 min at 8000*g* to pellet bacteria, and the supernatant was discarded. The bacterial pellet was resuspended in 3 ml of PBS 0.25% bovine serum albumin (BSA) with SYTO BC (1:7500; Life Technologies) for 15 min on ice.

For staining of cultured bacteria, 5-ml cultures were grown overnight under anaerobic conditions at 37°C and pelleted by centrifugation at 4700*g* for 15 min. Cells were resuspended in 1 ml of PBS 0.25% BSA with SYTO BC (1:7500) per milliliter of original culture and incubated for 15 min on ice.

For staining, 50 μl of bacterial suspension was transferred to a 96-well v-bottom plate. Fifty microliters of 2X mAb solution was added for a final concentration of 10 μg/ml and incubated for 20 min on ice. Cells were washed with PBS 0.25% BSA and centrifuged for 15 min at 4700*g*. Supernatant was discarded, and cells were resuspended in PBS 0.25% BSA 10% normal goat serum (Jackson ImmunoResearch) for 10 min on ice. Fifty microliters of 2X goat anti–hIgG-biotin (1:400 final concentration; Southern Biotech) and goat anti-mouse IgA–PE (phycoerythrin) (1:800 final concentration; Southern Biotech) was then added and incubated for 20 min on ice. For staining of cultured bacteria, the anti-mouse IgA reagent was not included. Cells were washed and centrifuged for 15 min at 4700*g*, and the supernatant was discarded. Cells were resuspended in 100 μl of PBS 0.25% BSA with streptavidin–APC (allophycocyanin) (1:800; BioLegend) and incubated for 20 min on ice. Cells were washed with PBS 0.25% BSA and centrifuged for 20 min at 4700*g*. Supernatant was discarded, and cells were resuspended in 200 μl of PBS 0.25% BSA with DAPI (4′,6-diamidino-2-phenylindole) (0.1 μg/ml; Life Technologies) before flow cytometry. Flow cytometry was performed with a low forward and side scatter threshold on an LSR II cytometer (BD Biosciences) to facilitate bacterial detection.

For purification and culture of mAb-binding bacteria, fecal pellets from mice colonized with human microbiota 1 (Fig. 1A) were homogenized and processed as described above. Bacterial suspension (250 μl) was added to a new 1.5-ml tube, and 250 μl of 2X mAb B2 334B4 in PBS 0.25% BSA was added (final concentration, 10 μg/ml) and incubated for 20 min on ice. Cells were washed and centrifuged for 5 min at 8000*g* and then resuspended in 250 μl of PBS 0.25% BSA 10% goat serum (Jackson ImmunoResearch) for 10 min on ice. Two hundred fifty microliters of 2X anti-human IgG-biotin (1:400 final concentration) in PBS 0.25% BSA was added and incubated for 20 min on ice. Cells were washed and centrifuged for 5 min at 8000*g* and then resuspended in 500 μl of streptavidin-APC (1:800 final concentration) in PBS 0.25% BSA for 20 min on ice. Cells were washed and centrifuged for 5 min at 8000*g* and then resuspended in 500 μl of PBS 0.25% BSA with anti-APC MACS (magnetic-activated cell sorting) beads (1:50; Miltenyi) for 20 min on ice. Cells were washed and centrifuged for 5 min at 8000*g* and then resuspended in 1 ml of PBS 0.2% BSA and run on an autoMACS separator using the Posselds program followed by a Qrinse. The positive fraction was then run a second time using the Posseld2 program. Eluate was serially diluted and plated on Schaedler blood agar (BD Biosciences) under anaerobic conditions at 37°C. Two days later, individual colonies were restreaked to obtain pure cultures. Two days later, these cultures were screened for VH5/6/7 mAb reactivity using the staining protocol described above.

Bacterial protein extraction

Saturated 5-ml cultures grown in chopped meat carbohydrate broth (BD Biosciences) under anaerobic conditions at 37°C were pelleted by centrifugation at 4700*g* for 15 min. The pellet was resuspended in 800 μl of lysis buffer from the NoviPure Microbial Protein Kit (Qiagen) and transferred to a bead beating tube. Cells were lysed by bead beating for 10 min with tubes taped horizontally on a vortex at room temperature. Tubes were centrifuged for 1 min at 12,000*g*, and supernatant was transferred to a new tube. Trichloroacetic acid (Sigma) was added to 10% v/v, and proteins were precipitated by incubation for 30 min on ice. Tubes were centrifuged for 10 min at 16,000*g*, and the supernatant was removed by aspiration. The pellet was washed by resuspension in 1 ml of ice-cold acetone and was centrifuged for 10 min at 16,000*g*. Supernatant was removed by aspiration, and the pellet was air dried for several seconds. The pellet was then resuspended in 0.5 M tris-HCl 4% SDS with Halt protease inhibitors (Thermo) and was solubilized by incubating for 30 min at room temperature followed by 3 min at 100°C. Protein was quantified by NanoDrop.

Coomassie gels and Western blots

Twenty micrograms of total protein was mixed with 2X Laemmli buffer (Bio-Rad) and 2.5% v/v β-mercaptoethanol (Sigma) and heated at 70°C for 10 min. The prep was left to cool for 2 to 3 min and was loaded onto a 10% precast polyacrylamide gel (Bio-Rad) and run for 1 hour at 100 V.

For Coomassie staining, gels were transferred to nanopure water and incubated for 15 min. Water was drained and replaced with Coomassie stain (Bio-Rad) for 45 min at room temperature. Gels were rinsed with water and then destained overnight with Coomassie destaining solution (Bio-Rad).

For Western blot, polyvinylidene difluoride membranes (Bio-Rad) were dipped in 100% ethanol and then membrane, gel, sponges, and filter paper were incubated in tris-glycine 20% methanol (Bio-Rad) for 15 min at room temperature. Transfer cassettes were assembled, and protein was transferred to the membrane with an electrophoretic

transfer apparatus (Bio-Rad) at 65 V for 2 hours. After transfer, the membrane was rinsed briefly in TBS-T (Thermo) and blocked in TBS-T 5% milk for 30 min at room temperature. Membranes were incubated overnight at 4°C with 8 ml of mAb at 1 μg/ml in TBS-T 5% milk. The next day, the membrane was rinsed three times for 5 min each with TBS-T and then incubated with 8 ml of goat anti-human IgG–HRP (horseradish peroxidase) (1:5000; Southern Biotech) in TBS-T 5% milk for 1 hour at room temperature. The membrane was washed three times with 5 ml of TBS-T for 5 min each and then incubated for 5 min with 7 ml of Clarity HRP substrate (Bio-Rad). The blot was removed from the solution and imaged with a ChemiDoc system (Bio-Rad).

Superantigen cloning and protein expression in *E. coli*

DNA from bacterial isolates was prepared using the PureLink Microbiome DNA Purification Kit (Thermo). For expression, proteins were cloned and expressed without their N-terminal signal peptide or C-terminal sorting signal. *R. gnavus* gene *ibpA* (WP_105084811.1) was purchased as a gBlock from Integrated DNA Technologies (IDT). The *ibpB* (WP_105084812.1) gene was cloned from genomic DNA using the primers tatgcatcatcatcatcatcacgtgGCAGAGCCAGTGGAAAAG and gctcgaatatcatcgatctcgagcgtcaATTTTTCTTCTTATCTTCTTTCTTAGTATCC with an initial denaturation of 98°C for 30 s followed by five cycles of 98°C for 10 s, 61°C for 30 s, and 72°C for 50 s. This was followed by 35 cycles of 98°C for 10 s and 72°C for 80 s. DNA bands were visualized by agarose gel electrophoresis and were cut and purified using a QIAquick Gel Extraction Kit (Qiagen). Fragments were incubated with Eco RI–digested Champion pET302 N-His vector (Thermo) and assembled by HiFi assembly (NEB). This mixture was transformed into XL10 Gold ultracompetent cells, and individual colonies were sequenced with forward primer TAATACGACTCACTATAGGG and reverse primer TAGTTATTGCTCAGCGGTGG. Colonies with appropriate inserts were grown in 35-ml cultures in Luria broth (LB) with ampicillin and plasmids purified using the HiSpeed Plasmid Midi Kit (Qiagen). Plasmids were then transformed into NiCo21 (DE3) competent *E. coli* (NEB) for protein expression. Twenty-milliliter cultures were grown overnight in MagicMedia *E. coli* expression media (Thermo), and His-tagged proteins were purified under native conditions using the Ni-NTA Spin Kit (Qiagen). Protein was quantified by NanoDrop.

ELISA analysis of mAb reactivity

R. gnavus proteins WP_105084811.1 and WP_105084812.1 were expressed in *E. coli* and purified as described above. Protein was then coated overnight at room temperature with 10 μg/ml, 50 μl per well carbonate buffer (Bethyl) onto 96-well ELISA plates (Thermo). The next day, plates were washed four times with TBS-T and incubated for 1 hour with 150 μl per well TBS 1% BSA blocking buffer at room temperature. Plates were washed four times with TBS-T and then incubated for 1 hour with mAbs at 1 μg/ml or four 1:4 serial dilutions in blocking buffer (50 μl per well) at room temperature. Plates were washed four times with TBS-T and then incubated for 1 hour at room temperature with 75 μl per well goat anti-human IgG-HRP (1:5000; Southern Biotech) diluted in TBS-T 1% BSA. were washed four times with TBS-T, and then 100 μl per well trimethylboron (TMB) HRP substrate (Bethyl) was added and incubated for about 5 min. The reaction was quenched with 100 μl of 0.18 M H_2SO_4, and the absorbance at 450 nm was read using an ELISA plate reader (BioTek).

Immunoprecipitation and mass spectrometry

mAbs were buffer exchanged into PBS using an Amicon 30K centrifugal filter and linked to agarose beads using the Pierce Direct IP Kit (Thermo). Protein lysate (1000 μg) was precleared by mixing with Pierce Control Agarose Resin according to the manufacturer's protocol and subsequently incubated with mAb-linked beads overnight at 4°C with rotation. The next day, the beads were washed three times with IP/lysis buffer and once with conditioning buffer as described by the manufacturer. Proteins were eluted in provided glycine pH 2.8 buffer and neutralized with 1 M tris. Eluate from 10 IPs was pooled and concentrated using a 0.5-ml Amicon 30K ultracentrifugal filter. The concentrate was then processed for SDS–polyacrylamide gel electrophoresis (PAGE) as described in the section on Coomassie gels and Western blots.

For mass spectrometry analysis, an SDS-PAGE gel run as described above was stained using the Pierce Silver Stain for Mass Spectrometry Kit (Thermo) according to the manufacturer's protocol. Bands were cut from the gel and destained per the manufacturer's protocol and then placed in 100 μl of nanopure H_2O and shipped to MS Bioworks LLC for mass spectrometry analysis. Common contaminants (i.e., keratins or trypsin) were removed from the analysis. Mass spectrometry data were analyzed using Scaffold (Proteome Software).

Human and mouse B cell purification and in vitro stimulation

For purification of untouched murine B cells, splenocytes from male and female wild-type C57BL/6 and female *Myd88*$^{-/-}$ mice were incubated with anti-mouse CD43 biotin (S7) and magnetically labeled with Streptavidin MicroBeads (Miltenyi) following the manufacturer's recommendations. Cells were separated using an autoMACS Separator with a purity of ≥90 to 95%.

For purification of human B cells, whole blood was carefully overlaid on Ficoll-Paque PLUS density gradient media (GE Healthcare) and centrifuged at room temperature for 30 min and 400*g* without brakes. Peripheral blood mononuclear cells were harvested from the interlayer, washed with PBS, and untouched B cells were purified (≥95% purity) using the Human B cell Isolation Kit (STEMCELL Technologies) following the manufacturer's recommendations. Whole blood was obtained from healthy volunteers with approval from the University of Chicago Institutional Review Board.

B cells were stimulated in 96-well plates for 6 hours at a density of 0.2×10^6 cells/ml in complete RPMI medium supplemented with 10% fetal calf serum (FCS) (Gibco). The final concentrations of soluble stimuli are as follows: 100 nM CpG (ODN2006 for human B cells and ODN1668 for mouse B cells, InvivoGen) and LPS (2 μg/ml) (Sigma). For stimulation with plate-bound stimuli, high-binding 96-well plates (Corning) were coated with 100 μl of 10 μg/ml IbpA, IbpB, and F(ab′)2-goat anti-mouse or F(ab′)2 anti-H IgM (Fisher) diluted in carbonyl coating buffer (Bethyl). To minimize endotoxin contamination of IbpA and IbpB produced from NiCo21 (DE3) *E. coli* lysates, we used High Capacity Endotoxin Removal Spin Columns (Fisher), and monitoring with Chromogenic Endotoxin Quant Kit (Fisher) showed <5 residual EU/ml.

Free IgA ELISAs

Fecal samples were weighed and resuspended at 0.1 mg/ml in PBS with protease inhibitors (Sigma) and then homogenized by vortexing horizontally for 5 min. Debris was pelleted by centrifugation at 400*g* for 5 min, and the supernatant was transferred to a new 1.5-ml

tube. This tube was centrifuged for 5 min at 8000*g*, and the supernatant was removed and saved for further analysis at −20°C. For analysis, 96-well ELISA plates (Thermo) were coated with 100 μl per well goat anti-mouse IgA (10 μg/ml; Bethyl) for 1 hour at room temperature in carbonate buffer (Bethyl). Plates were washed four times with TBS-T and then incubated with 200 μl per well TBS 1% BSA blocking buffer for 1 hour at room temperature. Plates were then washed four times with TBS-T. Samples (100 μl per well) were analyzed at 1:100 in TBS-T 1% BSA and four consecutive 1:10 serial dilutions and were compared against a standard curve generated using a mouse serum standard (Bethyl). Plates were incubated for 1 hour at room temperature and were washed four times with TBS-T. Goat anti-mouse IgA-HRP (100 μl per well) (1:50,000; Bethyl) diluted in TBS-T 1% BSA was added and incubated for 1 hour at room temperature. Plates were washed four times with TBS-T and then developed with 100 μl per well TMB HRP substrate for about 5 min at room temperature. The colorimetric reaction was quenched with 100 μl per well H_2SO_4, and the absorbance at 450 nm was read using an ELISA plate reader. Sample concentrations were determined by comparison to the standard curve fit to a four-parameter logistic curve.

Lymphocyte isolation from tissues

Mouse intestines were dissected, and fat and PPs were removed before processing. Intestines were opened by cutting longitudinally and were subsequently cut into ~1-cm pieces and placed into a 50-ml conical tube. Small intestines were cut in half and processed in two separate tubes, whereas colons were processed in a single tube. Ten milliliters of prewarmed RPMI 1% FCS 1 mM EDTA was added to the intestinal pieces, and they were incubated for 15 min at 37°C with shaking. Pieces were collected with a 100-μm cell strainer, and the supernatant was filtered into a new 50-ml tube. The pieces were then transferred back into the original 50-ml tube, and the strainer was washed and discarded. The intestinal pieces were again incubated with 10 ml of RPMI 1% FCS 1 mM EDTA for 15 min at 37°C with shaking and were collected with a 100-μm cell strainer. The supernatant from these first two washes was discarded as it contained predominantly epithelial cells and intraepithelial lymphocytes. Pieces were transferred to the original 50-ml tube, and 10 ml of prewarmed RPMI 20% FCS with collagenase (0.5 mg/ml) (Roche) and deoxyribonuclease (DNase; 0.1 mg/ml) (Sigma) was added. Pieces were incubated for 30 min at 37°C with shaking and were collected with a 100-μm cell strainer. The supernatant was collected, and the pieces were transferred back into the 50-ml tube. Ten milliliters of prewarmed RPMI 20% FCS with collagenase (0.5 mg/ml) (Roche) and DNase (0.1 mg/ml) (Sigma) was added, and pieces were again incubated for 30 min at 37°C with shaking and were collected with the same 100-μm cell strainer. Pieces were mashed using a disposable syringe plunger (BD), and the strainer was washed with 30-ml RPMI. Tubes were centrifuged for 5 min at 800*g*, and the supernatant was removed by aspiration. Cell pellets from the two SI fractions were combined, and they and the one colon fraction were each resuspended in 10 ml of 40% Percoll (Sigma) and transferred to a 15-ml conical tube. This tube was centrifuged for 20 min at 2100 rpm with no brake. Percoll was aspirated, and the pellet was washed with 1 ml of Hanks' balanced salt solution (HBSS) 0.25% BSA and centrifuged for 5 min at 800*g*. The pellet was then resuspended in HBSS 0.25% BSA with anti-CD16/32 (BioLegend) and stained for flow cytometry as described below.

Other tissues (spleens, mLNs, and PPs) were dissected and placed in HBSS 0.25% BSA and then transferred to and mashed on a 70-μm cell strainer with the plunger from a disposable 1-ml syringe (BD). The resultant cell suspension was transferred to a new tube and centrifuged for 5 min at 500*g* and then resuspended in HBSS 0.25% BSA with Fc block (BioLegend) and stained for flow cytometry as described below.

Lymphocyte flow cytometry

Single-cell suspensions were resuspended in HBSS 0.25% BSA with anti-CD16/32 (Fc Block; BioLegend) for 10 min on ice before staining with the following fluorophore or biotin-conjugated mAbs purchased from BioLegend, eBioscience, or BD unless otherwise indicated (clone in parentheses): B220 (RA3-6B2), CCR6 (29-2L17), CD4 (GK1.5 or RM4-5), CD8 (53-6.7), CD11c (HL3), CD19 (6D5), CD69 (1D4-C5), F4/80 (BM8), Gl7 (GL7), IgD (11-26c.2a), IgM (Il/41), NK1.1 (PK136), TER-119 (TER-119), Tcrb (H57-597), and goat anti-mouse IgA (Southern Biotech). Lineage cocktail for plasma cell staining included CD3, CD4, CD11c, F4/80, NK1.1, Tcrb, and TER-119. Cells were washed and centrifuged for 5 min at 500*g*. Supernatant was removed, and cells were resuspended in HBSS 0.25% BSA for analysis by flow cytometry using an LSR II cytometer (BD). Cell numbers were quantified using Spherotech counting beads.

After in vitro stimulation and incubation with a live/dead stain (Zombie Dye, BioLegend), human cells were first incubated with TrueStain FcX (BioLegend) for 20 min at 4° to 8°C and then stained with the following fluorophore-conjugated antibodies: CD19 (HIB1), CD69 (FN50), CD83 (HBI5e), or IgM (MHM-88). To prevent nonspecific binding of staining antibodies to IbpA and IbpB on the surface of in vitro–stimulated cells, cells were incubated with high-dose human IgG (1 mg/ml; Sigma) before the addition of fluorophore-conjugated antibodies. Gates were set based on the activation marker expression in unstimulated cells.

IgA repertoire analysis

SI IgA plasma cells (20,000 to 60,000) (lineage$^-$ B220$^-$ IgA$^+$) were sorted from each mouse using a BD Aria II cell sorter. Post-sort purity analysis verified the sorting efficacy of each sample (>97% purity and contaminating cells were nonplasma cells). Cells were centrifuged for 5 min at 800*g*, supernatant was removed, and the pellet was resuspended in 1 ml of TRIzol (Thermo) for 5 min at room temperature and then frozen at −80°C for later processing. Samples were thawed on ice, and 0.2 ml of chloroform was added; samples were shaken vigorously for 20 s and then allowed to settle at room temperature for 2 to 3 min. Tubes were then centrifuged at 10,000*g* for 15 min at 4°C. The aqueous (top) layer was removed with a pipette and transferred to a new sterile ribonuclease (RNase)–free tube. An equivalent volume of 100% ethanol was added, and the sample was mixed. The sample was then loaded onto an RNeasy column (Qiagen) in a collection tube and spun for 15 s at 8000*g*, and flow through was discarded. Buffer RW1 (700 μl; Qiagen) was added to the column, and it was centrifuged for 15 s at 8000*g*. The column was transferred to a new collection tube, and 500 μl of buffer RPE was added and then centrifuged for 15 s at 8000*g*. Flow through was discarded, and 500 μl of buffer RPE was added before centrifugation at 8000 g for 2 min. Flow through was discarded, and the column was centrifuged for 1 min at 8000*g*. The column was transferred to a new 1.5-ml collection tube, and 30 μl of RNase-free water was added and incubated for 1 to 2 min and then centrifuged for 1 min

at 8000*g* to elute the RNA. Complementary DNA (cDNA) synthesis was performed with the Superscript IV First Strand cDNA Synthesis Kit (Thermo) using poly-d(T) primers according to the manufacturer's protocol.

Immunoglobulin heavy chain variable regions were amplified from 2 μl of cDNA using Q5 Hot Start polymerase (NEB) and forward primer MsVHE GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG and reverse Cα primer GAAAGTTCACGGTGGTTATATCC. Thermal cycling conditions involved an initial denaturation at 98°C for 30 s followed by 35 cycles of 98°C for 10 s, 64°C for 20 s, and 72°C for 20 s, and a final elongation step of 72°C for 2 min. Polymerase chain reaction (PCR) fragments were visualized by agarose gel electrophoresis, and a ~500-nucleotide band was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). PCR fragments were cloned into vectors using the NEB PCR cloning kit, transformed into 10-beta competent *E. coli* (NEB), and plated on LB agar containing ampicillin. Individual colonies were picked, miniprepped, and sequenced using the forward primer ACCTGCCAACCAAAGCGAGAAC. Ninety-six colonies were picked per plate, which led to 80 to 90 sequences per mouse. Sequences were identified and mapped to variable region genes using IMGT (www.imgt.org). For somatic mutation analysis, mutations were identified using IMGT, and those found at the beginning or end of the sequence that were introduced by degenerate primers were discarded from analysis.

Characterizing the occurrence of *R. gnavus* across healthy human gut metagenomes

To estimate the abundance of *R. gnavus* ATCC 29149 genome and its superantigen genes across healthy human gut metagenomes, we used Bowtie2 v2.3.2 (*57*) with default parameters to recruit reads from publicly available gut metagenomes from healthy individuals. We used anvi'o v5.1 (*58*) to profile short metagenomic reads aligned to the *R. gnavus* ATCC 29149 genome, to estimate coverage and detection statistics per metagenome, and to visualize merged profiles in "gene mode" where the distribution of each gene of a genome is shown independently for accurate estimates of gene-level detection. To avoid overestimating "detection" as a result of nonspecific short read recruitment due to genomic regions conserved across multiple populations, we assumed that *R. gnavus* was detected in a given metagenome only if more than 25% of it was covered by at least 1X. We applied the same principle to identify metagenomes in which superantigens were detected.

Statistical analysis

Statistical analysis by unpaired student's *t* test or one-way analysis of variance (ANOVA) was performed using GraphPad Prism.

SUPPLEMENTARY MATERIALS

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Fig. S1. mAb reactivity to mouse microbiota and cladogram of individual bacterial strains analyzed.

Fig. S2. Immunoprecipitation, mass spectrometry, Coomassie, and Western blot analysis of purified proteins.

Fig. S3. Additional analyses of GF or gnotobiotic monocolonized mice.

Table S1. Distribution of superantigens across human metagenomes.

Data file S1. List of mAbs used in this study.

Data file S2. Primary data.

Data file S3. List of metagenomes analyzed.

Data file S4. Metagenomic recruitment analysis of *R. gnavus* genes including its superantigens.

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the experiments assessing in vitro activation by IbpA and IbpB. A.R.W. performed the computational analysis of human metagenomes supervised by A.M.E. C.H.P. and C.R.N. provided samples from gnotobiotic mice colonized with human microbiota. O.S. provided critical advice regarding identification of Gram-positive surface proteins. All authors reviewed and approved the final manuscript. **Competing interests:** C.R.N. is an inventor on provisional patent 62/755,945 submitted by the University of Chicago that covers the human microbiotas studied in Fig. 1. C.R.N. is the President and Cofounder of ClostraBio Inc. **Data and materials availability:** All data related to this study are present in the paper or Supplementary Materials.

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B cell superantigens in the human intestinal microbiota

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Bacterial superantigens spur IgA secretion

Mucosal IgA is abundant and interacts with the gut microbiome. To examine microbial induction of IgA in humans, Bunker *et al.* screened microbiota from infants against mouse and human IgA. Unexpectedly, they saw a subset of samples bound IgA in a way that indicated the presence of superantigens, which bind T cell receptors or B cell receptors outside of the typical antigen-binding region, leading to nonspecific activation. Putative superantigens were identified in commensal members of Lachnospiraceae, which activated human VH3-positive B cells and induced IgA production in mice. These data identify commensal superantigens and suggest that they may be dominant forces behind IgA production in humans.