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Materials and Methods
Figs. S1 to S3
Tables S1 to S11
References (*19*–*34*)

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The Toll-Like Receptor 2 Pathway Establishes Colonization by a Commensal of the Human Microbiota

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Mucosal surfaces constantly encounter microbes. Toll-like receptors (TLRs) mediate recognition of microbial patterns to eliminate pathogens. By contrast, we demonstrate that the prominent gut commensal *Bacteroides fragilis* activates the TLR pathway to establish host-microbial symbiosis. TLR2 on $CD4^+$ T cells is required for *B. fragilis* colonization of a unique mucosal niche in mice during homeostasis. A symbiosis factor (PSA, polysaccharide A) of *B. fragilis* signals through TLR2 directly on $Foxp3^+$ regulatory T cells to promote immunologic tolerance. *B. fragilis* lacking PSA is unable to restrain T helper 17 cell responses and is defective in niche-specific mucosal colonization. Therefore, commensal bacteria exploit the TLR pathway to actively suppress immunity. We propose that the immune system can discriminate between pathogens and the microbiota through recognition of symbiotic bacterial molecules in a process that engenders commensal colonization.

Throughout our lives, we continuously encounter microorganisms that range from those essential for health to those causing death (*1*). Consequently, our immune system is charged with the critical task of distinguishing between beneficial and pathogenic microbes. Toll-like receptors (TLRs) are evolutionarily conserved molecules that promote immune responses, and TLR signaling by innate immune cells is indispensable for proper activation of the immune system during infections. T cells also express functional TLRs (*2*–*4*), and TLR signaling has furthermore been shown to restrain immune responses (*5*). As symbionts and pathogens produce similar molecular patterns that are sensed by TLRs, the mechanisms by which our immune system differentiates between the microbiota and enteric infections remain unknown.

Whereas the intestinal microbiota contains hundreds of bacterial species and is integral to human health (*6*), the mucosal immune system employs an arsenal of responses to control enteric pathogens. Germ-free mice lack proinflammatory T helper 17 (T_H17) cells in the gut (*7*, *8*) (Fig. 1A), and only select symbiotic bacteria can induce T_H17 cells (*9*, *10*). Most microbes express common TLR ligands (e.g., peptidoglycan, unmethylated CpG, and lipoproteins); therefore, how do symbionts avoid triggering intestinal immunity in their mammalian hosts? We examined the hypothesis that the human gut commensal *Bacteroides fragilis* evolved molecular mechanisms to suppress T_H17 responses during homeostatic colonization. As predicted previously (*7*, *10*, *11*), we found that *B. fragilis* mono-associated animals did not induce T_H17 cell development in the colon compared to germ-free controls (Fig. 1A). The beneficial contributions of *B. fragilis* require a single immunomodulatory molecule named polysaccharide A (PSA), which prevents and cures inflammatory disease (*12*–*14*). Colonization with *B. fragilis* in the absence of PSA (*B. fragilis*ΔPSA), however, resulted in significant T_H17 cell responses in the gut (Fig. 1, A and B). Colonic lamina propria (LP) lymphocytes from *B. fragilis*ΔPSA mono-associated animals displayed increased secretion of interleukin-17A (IL-17A) in vitro (fig. S1) and elevated transcription of *Il17a* and the T_H17-specific lineage differentiation factor RORγt (*Rorc*) (Fig. 1, C and D) (*15*). Differences in T_H1 responses were not observed during *B. fragilis*ΔPSA colonization (fig. S2). Cells from *B. fragilis* mono-associated animals produced low amounts of IL-17A during in vitro T_H17 skewing assays, whereas $CD4^+$ T cells from *B. fragilis*ΔPSA animals display elevated IL-17A production (fig. S3). Administration of purified PSA to *B. fragilis*ΔPSA mono-associated animals suppresses T_H17 immunity (Fig. 1E). Thus, *B. fragilis* actively restrains T_H17 cell responses during colonization.

Recent studies have shown that certain gut bacteria can promote regulatory T cell (T_{reg}) induction (*11*, *14*). T_{regs} expressing the transcription factor Foxp3 (forkhead box P3) suppress proinflammatory T_H17 cell reactions. To test whether T_{regs} prevent immune responses during *B. fragilis* colonization, we reconstituted germ-free $Rag1^{-/-}$ mice with bone marrow from Foxp3-DTR (diphtheria toxin receptor) donors, which allowed for specific ablation of T_{regs} by administration of diphtheria toxin (DT) (*16*). Mice were mono-associated with *B. fragilis* to induce T_{reg} development (Fig. 1F). DT treatment of mice resulted in depletion of $Foxp3^+$ T cells (Fig. 1F), with a concomitant increase in T_H17 responses (Fig. 1G and fig. S4), which suggests that $Foxp3^+$ T_{regs} are required for suppression of T_H17 cells during *B. fragilis* colonization.

PSA is an immunomodulatory bacterial molecule that shapes host immune responses (*17*). Induction of IL-10 and interferon-γ (IFN-γ) from $CD4^+$ T cells by PSA requires TLR2 signaling (*14*, *18*). We sought to determine the mechanism whereby *B. fragilis* suppresses T_H17 cell responses by testing whether PSA functions through TLR2 signaling by dendritic cells (DCs) and/or $CD4^+$ T cells. PSA elicited a significant increase in IL-10 and IFN-γ production from mixed cultures of wild-type DCs and wild-type $CD4^+$ T cells in vitro (Fig. 2A and fig. S5). When $Tlr2^{-/-}$ T cells were cocultured with wild-type DCs, however, PSA-induced IL-10 production was reduced, whereas IFN-γ expression was not affected (Fig. 2A and fig. S5), which indicated that PSA required TLR2 expression on T cells to promote IL-10 production. IL-10 responses to PSA were specific to T cells (fig. S6). Consistent with previous findings (*18*), proinflammatory IFN-γ production was dependent on TLR2 signaling by DCs (fig. S5); however, IL-10 production was unaffected in cultures containing wild-type $CD4^+$ T cells and $Tlr2^{-/-}$ DCs (Fig. 2A). Therefore, TLR2 expression by T lymphocytes is necessary for IL-10 production by PSA.

$CD4^+$ T cells produce IL-10 in response to PSA in the absence of antigen-presenting cells (APCs) (Fig. 2B). Moreover, PSA induces IL-10 expression from purified T cells in a dose-dependent manner, whereas other TLR2 ligands do not (fig. S7). TLR2 can function as either a homodimer or a heterodimer with TLR1 or TLR6 (*19*). PSA could induce high amounts of IL-10 from wild-type, $Tlr1^{-/-}$, and $Tlr6^{-/-}$ $CD4^+$ T cells; however, IL-10 production was lost only from $Tlr2^{-/-}$ $CD4^+$ T cells and T cells deleted in the TLR adapter molecule MyD88 (Fig. 2B). To determine T_{reg} suppression as a function of cell-

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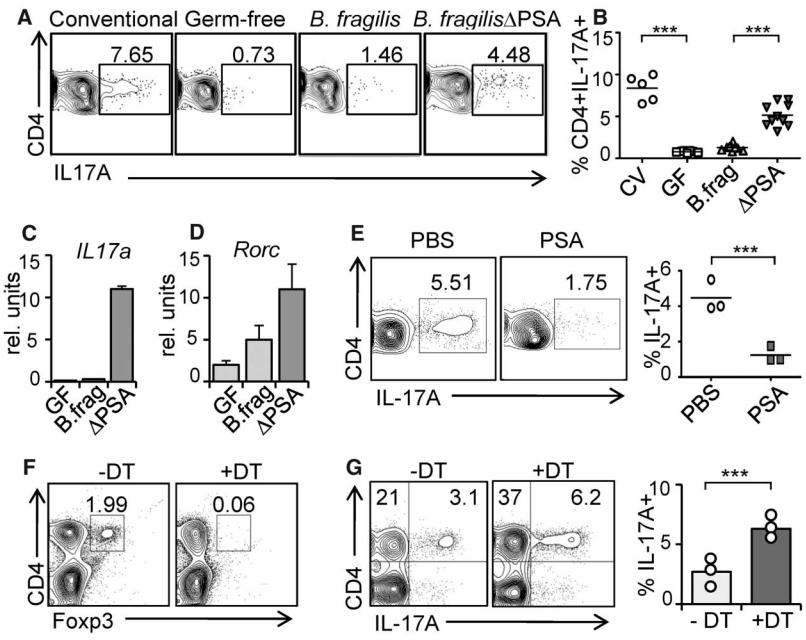

Fig. 1. PSA actively suppresses T_H17 cell development during *B. fragilis* colonization through Foxp3$^+$ T_{regs}. (**A**) Colonic lamina propria lymphocytes (LPLs) were harvested and stained with antibodies against CD4 and IL-17A and analyzed by flow cytometry. Numbers indicate the percentage of CD4$^+$IL-17A$^+$ (T_H17) cells. Conventional mice are specific pathogen free. (**B**) Compiled data from three independent experiments as in (A). CV, conventional; GF, germ-free; B.frag, *B. fragilis*; ΔPSA, *B. fragilis*ΔPSA. ***$P < 0.001$, two-way analysis of variance. (**C** and **D**) CD4$^+$ T cells were isolated from the mesenteric lymph nodes of the indicated animals. RNA was collected and used as a template to determine the relative levels of IL-17A (C) and RORγt (D) transcript. Error bars represent SDs from triplicate samples. The data are representative of three independent experiments. (**E**) *B. fragilis*ΔPSA mono-associated mice were treated with either phosphate-buffered saline (PBS) or PSA, and the LPLs were isolated and the percentage of CD4$^+$ IL-17A–producing cells was determined by flow cytometry. Each symbol represents an individual animal (n = 3 to 4 mice per group). ***$P < 0.001$. (**F** and **G**) Germ-free *Rag1*$^{-/-}$ animals were reconstituted with bone marrow from Foxp3-DTR mice and then mono-associated with *B. fragilis*. Animals were treated with either PBS (–DT) or diphtheria toxin (+DT) as described (*16*). Colonic LPLs were harvested after T_{reg} ablation and restimulated with phorbol 12-myristate 13-acetate (PMA)–ionomycin and brefeldin A. Cells were stained with antibodies to CD4, Foxp3, and IL-17A and analyzed by flow cytometry. (Right) Symbols represent T cell proportions from individual mice within a single experiment (n = 3 to 4 mice per group) and are representative of two independent trials. ***$P < 0.001$.

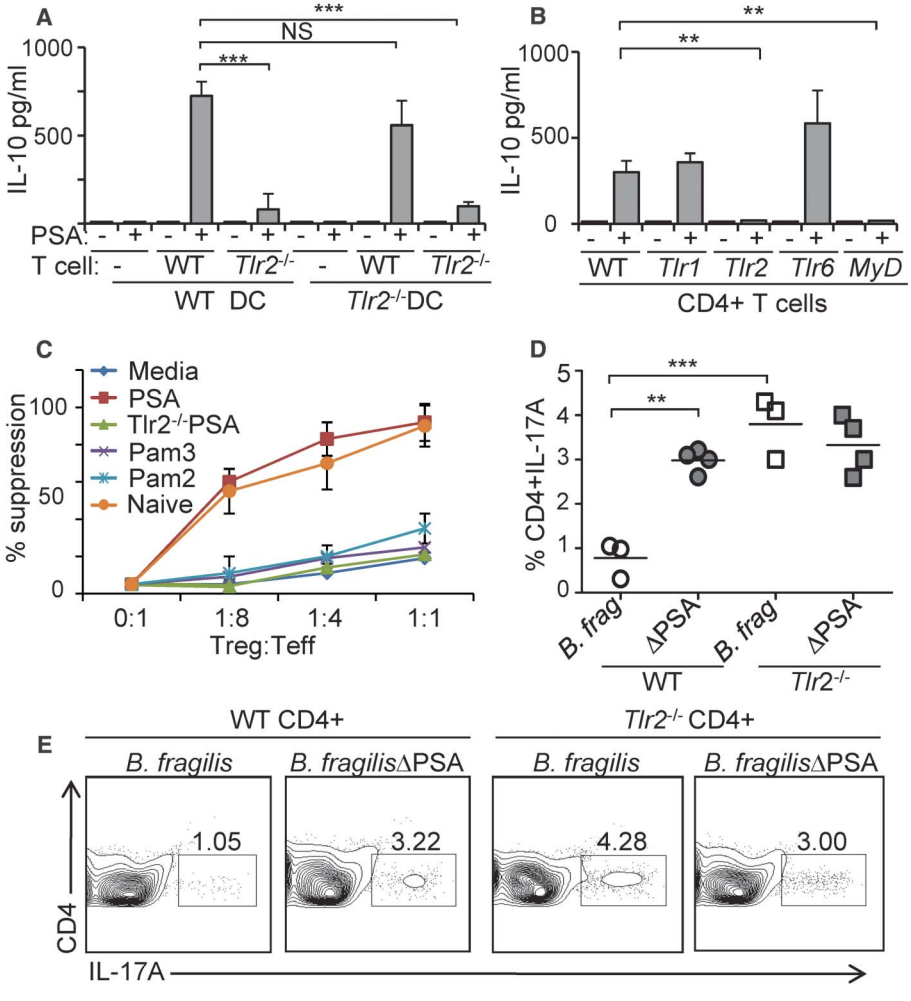

Fig. 2. PSA signals through TLR2 on CD4$^+$ T cells to suppress T_H17 cell responses. (**A**) Bone marrow–derived dendritic cells from wild-type (WT) or *Tlr2*$^{-/-}$ mice were incubated with splenic CD4$^+$ T cells. Amounts of secreted IL-10 were determined by enzyme-linked immunosorbent assay (ELISA). Error bars represent SDs from two independent assays performed in quadruplicate. ***$P < 0.001$; NS, not significant. (**B**) CD4$^+$ T cells were isolated from WT mice, and the indicated knockout mice and cells were stimulated as in (A). MyD is *Myd88*$^{-/-}$. IL-10 was assayed by ELISA. **$P < 0.01$. Error bars represent SDs for quadruplicate samples and are representative of two independent trials. (**C**) CD4$^+$Foxp3$^+$ T_{regs} were purified from Foxp3^{EGFP} mice (*26*) and *Tlr2*$^{-/-}$ X Foxp3^{EGFP} mice and stimulated with plate-bound antibodies against CD3 and recombinant TGF-β, with PSA or with the indicated TLR ligands. Equal numbers of live cultured T_{regs} were then incubated with CFSE (carboxyfluorescein diacetate succinimidyl ester)–pulsed responder cells (CD4$^+$Foxp3$^-$). Percent suppression is determined by the ratio of proliferating responder cells in each condition relative to proliferation in the absence of added T_{regs}. Error bars are SDs from a single experiment performed in duplicate and are representative of two independent trials. (**D** and **E**) Germ-free *Rag1*$^{-/-}$ animals were reconstituted with CD4$^+$ T cells from WT or *Tlr2*$^{-/-}$ mice and then mono-associated with either WT *B. fragilis* or *B. fragilis*ΔPSA. Colonic LPLs were isolated and analyzed for T_H17 cell proportions by flow cytometry. Plots are gated on CD4$^+$ cells. (D) Each symbol represents an individual animal (n = 3 to 4 mice per group), and data are representative of two independent trials. **$P < 0.01$; ***$P < 0.001$.

intrinsic TLR2 signaling, we measured proliferation of responder T cells after coculture with T_{regs} stimulated in vitro with PSA, or classical TLR2 ligands Pam3CysK or Pam2CysK. The proportion of $Foxp3^+$ T cells was equivalent under all conditions (fig. S8). PSA-treated T_{regs} displayed increased suppressive capacity compared to media or other TLR2 ligand–treated T_{regs} (Fig. 2C). IL-2 was not produced by T_{regs} during treatment with any of the TLR2 ligands, and IL-2 neutralization had no effect on in vitro suppression (fig. S9). Notably, the suppressive capacity of PSA-treated T_{regs} was lost when $Foxp3^+$ T cells were deficient in TLR2 (Fig. 2C). PSA likely directed the development of inducible T_{regs} by promoting the expression of IL-10, transforming growth factor–β2 (TGF-β2), and Foxp3 from purified $Foxp3^+$ T cells in a TLR2-dependent fashion (fig. S10). Collectively, these studies show that unlike other TLR2 ligands, PSA enhances T_{reg} function and gene expression in the absence of APCs through TLR2 signaling directly on $CD4^+Foxp3^+$ Treg cells.

We next determined whether PSA signals through TLR2 on $Foxp3^+$ T_{regs} during *B. fragilis* colonization of animals to suppress T_H17 cell responses. Germ-free *Rag1*$^{-/-}$ mice were reconstituted with purified $CD4^+$ T cells from wild-type or *Tlr2*$^{-/-}$ animals. Animals were subsequently mono-associated with either *B. fragilis* or *B. fragilis*ΔPSA. Unlike wild-type animals, IL-10–producing $Foxp3^+$ T_{regs} were not induced in the gut of *B. fragilis* mono-colonized animals that were reconstituted with *Tlr2*$^{-/-}$ $CD4^+$ T cells (fig. S11). Although we observed minimal T_H17 cell development in wild-type mice mono-associated with *B. fragilis*, T_H17 cell responses were significantly increased during colonization of animals that were reconstituted with *Tlr2*$^{-/-}$ $CD4^+$ T cells (Fig. 2, D and E). Furthermore, TLR2-deficient $CD4^+$ T cells from *B. fragilis* mono-associated mice produced more IL-17A compared to mice reconstituted with wild-type $CD4^+$ T cells (fig. S12). Collectively, these data demonstrate that *B. fragilis* requires TLR2 to induce $Foxp3^+$ T_{regs} during intestinal colonization and actively suppresses T_H17 responses through engagement of TLR2 specifically on T cells.

The intestinal microbiota occupies both mucosal and luminal niches during normal colonization; however, the biogeographic distributions of specific microbial species are poorly characterized. We reasoned that the functions of PSA were driven by an evolutionary impetus to prevent deleterious mucosal immune reactions to *B. fragilis*, enabling bacteria to associate with host tissue. Intriguingly, we discovered a population of bacteria that intimately associates with the intestinal epithelium (Fig. 3A). Whole-mount preparations of medial colons were probed for bacteria after labeling with *B. fragilis*–specific antisera (fig. S13). Three-dimensional reconstruction of confocal microscopic images revealed microcolonies of *B. fragilis* residing within colonic crypts (Fig. 3A). The amounts of *B. fragilis* associated with host tissue represented a fraction of total bacteria (Fig. 3, B and C), but likely are an important population that are in close proximity to the host immune system. We speculated that the amounts of tissue-associated bacteria would be sensitive to host immune responses such as T_H17 cell induction. Notably, animals colonized with *B. fragilis*ΔPSA displayed profoundly reduced numbers of tissue-associated bacteria when compared to animals colonized with wild-type *B. fragilis* (Fig. 3D). Treatment of *B. fragilis*ΔPSA–colonized animals with purified PSA corrected this defect and increased colonization of *B. fragilis*ΔPSA to wild-type bacterial levels (Fig. 3D). Only tissue-associated bacteria were affected, because no differences were observed in the amounts of bacteria in the gut lumen by either strain (fig. S14) (*17*). Collectively, these data identify a previously unappreciated mucosal niche for *B. fragilis* and reveal that PSA is required for maintaining host-bacterial symbiosis at the epithelial surface of the gut.

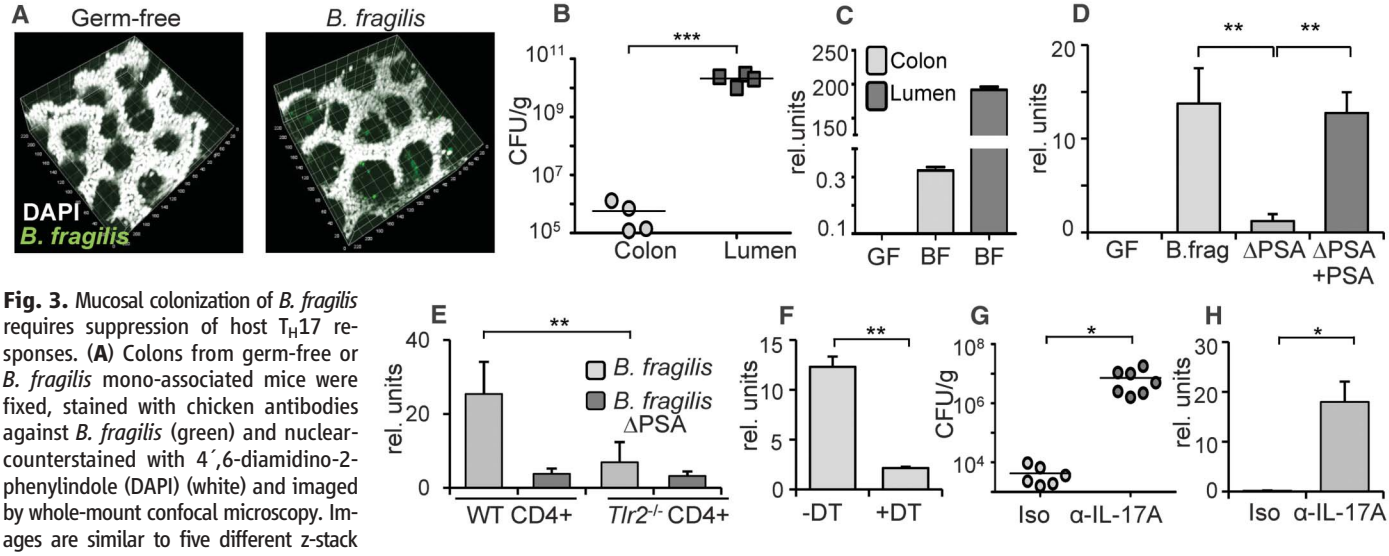

Fig. 3. Mucosal colonization of *B. fragilis* requires suppression of host T_H17 responses. (**A**) Colons from germ-free or *B. fragilis* mono-associated mice were fixed, stained with chicken antibodies against *B. fragilis* (green) and nuclear-counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (white) and imaged by whole-mount confocal microscopy. Images are similar to five different z-stack images per colon and representative of five mice. (**B**) Colon sections or luminal contents from *B. fragilis* mono-associated mice were homogenized and serially diluted to obtain live bacterial counts. CFUs (colony-forming units) per gram of tissue were determined after microbiologic plating. Each symbol represents an individual animal (n = 3 to 4 animals per group) and is representative of three independent trials. ***$P < 0.001$. (**C**) Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis was performed with *Bacteriodes*-specific primers on RNA extracted from colon tissue or luminal contents. GF, germ-free; BF, *B. fragilis*. Error bars represent SDs from individual mice in the same experiment and are representative of two independent trials. (**D**) qRT-PCR analysis for *B. fragilis* was performed on RNA extracted from colon homogenates from indicated animals. The bar furthest to the right shows colonization of *B. fragilis*ΔPSA in animals orally treated with purified PSA. GF, germ-free; B.frag, *B. fragilis*; ΔPSA, *B. fragilis*ΔPSA. Data are shown for four animals per group and are representative of two independent trials. **$P < 0.01$. (**E**) Germ-free *Rag1*$^{-/-}$ animals were reconstituted with *Tlr2*$^{-/-}$ or WT $CD4^+$ T cells and colonized with either WT *B. fragilis* or *B. fragilis*ΔPSA. Colons were prepared and analyzed as in (D). **$P < 0.01$. (**F**) Germ-free *Rag1*$^{-/-}$ animals were reconstituted with Foxp3-DTR bone marrow and colonized with *B. fragilis*. Two months after reconstitution animals were treated with either PBS (–DT) or with diphtheria toxin (+DT), and colons were prepared as described in (D). **$P < 0.01$. (**G** and **H**) Neutralization of IL-17A increases *B. fragilis* colonization. Germ-free animals were colonized with *B. fragilis*ΔPSA and treated with either an antibody that neutralizes IL-17A (α-IL-17A) or an isotype control (Iso). Colon homogenates were analyzed by live bacterial plating (G) or qRT-PCR (H) as described in (B) and (C). Each symbol in (G) represents an individual animal. Error bars in (H) show the SDs from individual animals and are compiled data from two independent trials with three or four animals per group. *$P < 0.05$.

Our findings suggest that PSA induces T_{regs} through TLR2 signaling to suppress T_H17 cell responses and promote mucosal colonization by *B. fragilis*. To test this model, we measured colonization levels of *B. fragilis* in *Rag1*$^{-/-}$ mice reconstituted with TLR2-deficient CD4$^+$ T cells. Tissue association by wild-type *B. fragilis* in the colon was reduced to the levels of *B. fragilis*ΔPSA in these mice (Fig. 3E and fig. S15). Moreover, Foxp3$^+$ T_{reg} ablation in *B. fragilis* mono-associated animals resulted in significantly reduced amounts of tissue-associated *B. fragilis* (Fig. 3F), without affecting bacterial numbers in the lumen of the gut (fig. S16). Finally, to functionally determine the role of IL-17 responses in mucosal association, we treated *B. fragilis*ΔPSA mono-associated animals with a neutralizing antibody to IL-17A. Whereas the amounts of *B. fragilis*ΔPSA in isotype control–treated animals remained low, neutralization of IL-17A resulted in a 1000-fold increase in tissue-associated bacteria (Fig. 3, G and H). These data indicate that IL-17 suppression by PSA is required by *B. fragilis* during association with its host. Therefore, unlike pathogens that trigger inflammatory responses through TLRs to clear infections, symbiotic colonization by *B. fragilis* is actually enhanced via the TLR pathway. We conclude that PSA evolved to engender host-bacterial mutualism by inducing mucosal tolerance through TLR2 activation of T_{reg} cells.

The gastrointestinal tract represents a primary portal for entry by numerous pathogens. Toll-like receptors recognize MAMPs (microbial-associated molecular patterns) expressed by bacteria and coordinate a cascade of innate and adaptive immune responses that control infections (*20*). Although TLRs have classically been studied on innate immune cells, recent reports have demonstrated their expression by T cells in both mice and humans (*4*, *21*–*23*). As bacteria contain universally conserved MAMPs, how do commensal microbes, unlike pathogens, avoid triggering TLR activation? It is historically believed that the microbiota is excluded from the mucosal surface (*24*). However, certain symbiotic bacteria tightly adhere to the intestinal mucosa (*9*–*11*), and thus immunologic ignorance may not explain why inflammation is averted by the microbiota. Our study provides new insight into the mechanisms by which the immune system distinguishes between pathogens and symbionts. The functional activity of PSA on T_{regs} contrasts with the role of TLR2 ligands of pathogens, which elicit inflammation, and thus reveals an unexpected function for TLR signaling during homeostatic intestinal colonization by the microbiota. Although engagement of TLR2 by previously identified ligands is known to stimulate microbial clearance of pathogens, TLR signaling by PSA paradoxically allows *B. fragilis* persistence on mucosal surfaces. These results identify PSA as the incipient member of a new class of TLR ligands termed "symbiont-associated molecular patterns (SAMPs)" that function to orchestrate immune responses to establish host-commensal symbiosis. On the basis of the importance of the microbiota to mammalian health (*25*), evolution appears to have created molecular interactions that engender host-bacterial mutualism. In conclusion, our findings suggest that animals are not "hard-wired" to intrinsically distinguish pathogens from symbionts, and that microbial-derived mechanisms have evolved to actively promote immunologic tolerance to symbiotic bacteria. This concept suggests a reconsideration of how we define self versus nonself.

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Supporting Online Material

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Figs. S1 to S16

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A Packing Mechanism for Nucleosome Organization Reconstituted Across a Eukaryotic Genome

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Near the 5′ end of most eukaryotic genes, nucleosomes form highly regular arrays that begin at canonical distances from the transcriptional start site. Determinants of this and other aspects of genomic nucleosome organization have been ascribed to statistical positioning, intrinsically DNA-encoded positioning, or some aspect of transcription initiation. Here, we provide evidence for a different explanation. Biochemical reconstitution of proper nucleosome positioning, spacing, and occupancy levels was achieved across the 5′ ends of most yeast genes by adenosine triphosphate–dependent trans-acting factors. These transcription-independent activities override DNA-intrinsic positioning and maintain uniform spacing at the 5′ ends of genes even at low nucleosome densities. Thus, an active, nonstatistical nucleosome packing mechanism creates chromatin organizing centers at the 5′ ends of genes where important regulatory elements reside.

Statistical positioning depends on the presence of a genomic barrier within a linear array of nucleosomes (*1*). Nucleosomes within the array will passively align at regular intervals from the barrier, independent of sequence or other external factors, rather than arrange randomly. Nucleosome organization in vivo displays patterns that are consistent with statistical

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