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An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System

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Summary

The mammalian gastrointestinal tract harbors a complex ecosystem consisting of countless bacteria in homeostasis with the host immune system. Shaped by evolution, this partnership has potential for symbiotic benefit. However, the identities of bacterial molecules mediating symbiosis remain undefined. Here we show that, during colonization of animals with the ubiquitous gut microorganism *Bacteroides fragilis*, a bacterial polysaccharide (PSA) directs the cellular and physical maturation of the developing immune system. Comparison with germ-free animals reveals that the immunomodulatory activities of PSA during *B. fragilis* colonization include correcting systemic T cell deficiencies and T_H1/T_H2 imbalances and directing lymphoid organogenesis. A PSA mutant of *B. fragilis* does not restore these immunologic functions. PSA presented by intestinal dendritic cells activates $CD4^+$ T cells and elicits appropriate cytokine production. These findings provide a molecular basis for host-bacterial symbiosis and reveal the archetypal molecule of commensal bacteria that mediates development of the host immune system.

Introduction

Immediately after a sterile birth, mammals are initiated into an organized and lifelong process of colonization by foreign organisms. Shaped by eons of evolution, some host-bacterial associations have developed into prosperous relationships creating diverse environments. No better example exists in biology than the astounding numbers of bacteria harbored by the lower gastrointestinal tract of mammals (Hooper et al., 1998). By young adulthood, humans and other mammals are host to $\sim 10^{12}$ viable bacteria per gram of colonic content, consisting of 500–1000 microbial species and outnumbering host cells by 100-fold (Hooper and Gordon, 2001). The magnitude of this interaction between commensal bacteria and mammals must predictably exert fundamental influences on the physiology of both. The most impressive feature of this relationship may be that the host not only tolerates but has evolved to require colonization by commensal microorganisms for its own development and health.

Autochthonous (indigenous) bacteria in the mammalian gut have long been appreciated for potential benefits to the host: provision of essential nutrients, metabolism of indigestible compounds, defense against colonization by opportunistic pathogens, and contributions to the development of the intestinal architecture (Hooper et al., 2000; Hooper et al., 2002). How and, more importantly, why does the immunocompetent gut environment allow the presence of multitudinous foreign organisms? Researchers have proposed that certain commensal bacteria have evolved to aid in the host's health; several organisms are being studied for probiotic (beneficial) potential (Guarner and Malagelada, 2003; Rastall, 2004). The "hygiene hypothesis" suggests that the appropriate bacterial constitution of the human microflora is a factor in protection from allergy and asthma (Umetsu et al., 2002; Von Hertzen and Haahtela, 2004). Investigations have shown that the interactions of commensal bacteria with Toll-like receptors are critical for intestinal homeostasis (Rakoff-Nahoum et al., 2004). The intimate relationships between commensal microorganisms and the host immune system are increasingly evident (Macpherson and Harris, 2004; Noverr and Huffnagle, 2004).

The mammalian immune system is a dynamic and remarkable organ. In recognizing, responding, and adapting to countless foreign and self molecules, the immune system is central to processes of health and disease. $CD4^+$ T cells, a major component of the immune system, are required for vital aspects of proper immune function, from reactions to infectious agents to control of autoimmune reactions and cancers (Janeway et al., 2001). Effector $CD4^+$ T cells are of two general subtypes, T helper 1 (T_H1) and T helper 2 (T_H2), each carrying out distinct and opposing activities. The proper balance between T_H1 and T_H2 immunologic responses is critical to overall human and animal health (Neurath et al., 2002; Sheikh and Strachan, 2004). A role for commensal bacteria in establishing this equilibrium has been postulated (Bowman and Holt, 2001; Rook and Brunet, 2002). We investigated the molecular contributions of specific autochthonous organisms to the cellular development of the host immune system.

Bacteroides fragilis is a ubiquitous and important Gram-negative anaerobe that colonizes the mammalian lower gastrointestinal tract. *Bacteroides* species are among the earliest-colonizing and most numerically prominent constituents of the gut microflora (Kononen et al., 1992). Although capsular polysaccharides are common in many bacterial species, *B. fragilis* elaborates an unprecedented eight distinct surface polysaccharides (Krinos et al., 2001). Several of these polymers have a novel zwitterionic structure, with both positive

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and negative charges in each repeating unit (Tzianabos et al., 1993). Zwitterionic polysaccharides (ZPSs) are unique T cell-dependent antigens that specifically mediate the proliferation of $CD4^+$ T cells in vitro (Brubaker et al., 1999; Tzianabos and Kasper, 2002). Adoptive transfer experiments show that responses to polysaccharide A (PSA), the most immunodominant ZPS of *B. fragilis*, are conferred by $CD4^+$ T cells, not by B cells or other T cells (Tzianabos et al., 1999). We have described the novel internalization and processing of PSA within endosomes of antigen-presenting cells (APCs) (Cobb et al., 2004). Subsequent presentation of processed polysaccharide by major histocompatibility complex class II (MHC II) molecules activates $CD4^+$ T cells and represents a previously undescribed pathway of antigen presentation. Thus, ZPSs appear to have evolved novel biological activities shaped by coevolution with the host immune system.

Herein we show that monocolonization of germ-free animals with *B. fragilis* is sufficient to correct several immunologic defects found in the absence of a bacterial microflora. The organism's immunomodulatory activity requires production of PSA, which mediates host immune-system development through specific cellular and molecular interactions. The significance of PSA's role in immune homeostasis lies in its ability to mediate establishment of a proper T_H1/T_H2 balance for the host, a fundamental aspect of healthy immunologic function. *B. fragilis* PSA is the first identified member of a novel class of molecules, referred to here as "symbiosis factors," that mediate the beneficial relationship between bacteria and mammalian hosts during mutualism.

Results

Monocolonization of Germ-free Animals with *B. fragilis* Results in $CD4^+$ T Cell Expansion

We investigated the effects of bacterial colonization on immune maturation in animals, exploring the role of the microbial flora in systemic T cell development. We used germ-free mice: animals born and raised in sterile isolators devoid of microbes. Initially, spleens were harvested from both conventionally colonized and germ-free mice and were analyzed for total $CD4^+$ T cells by flow cytometry (FC). All groups of mice had similar splenic total lymphocyte counts (average: 1×10^8). Consistent with seminal observations of a positive immunologic role of autochthonous bacteria (Dobber et al., 1992), the lymphocyte population purified from spleens of conventional SPF (specific-pathogen-free) mice with a diverse gut microflora contains a greater proportion of $CD4^+$ T cells than that of germ-free mice (Figure 1A). Previous studies have documented the beneficial role of commensal bacteria in intestinal development (Hooper, 2004). The observed alteration in $CD4^+$ T cell proportions of splenic lymphocytes highlights the profound effects of bacterial colonization of the gut on the systemic immune response.

SPF mice harbor a diverse and complex microbial flora. To stringently investigate the influence of specific bacterial constituents of the gut flora on the host immune system, we needed to colonize germ-free mice with a single bacterial species. This approach allows "real-time" measurements of responses to bacterial colonization in animals with a naive immune system. We chose the model microorganism *B. fragilis* because of its prominence in the normal microbial gut flora and its production of known immunomodulatory molecules (Kononen et al., 1992; Tzianabos and Kasper, 2002). In the absence of competing bacterial species, germ-free mice monoassociated with *B. fragilis* strain NCTC 9343 are readily colonized to high levels ($>10^{10}$ cfu/g of feces; Figure 1B). Flow cytometry of splenic lymphocytes from these mice shows a nearly complete restoration of $CD4^+$ T cells to conventional proportions (Figures 1A and 1C). Thus, *B. fragilis* monocolonization is sufficient to correct $CD4^+$ T cell deficiency in spleens of germ-free mice. No other bacterial species alone has been shown to correct lymphoid defects in germ-free animals (Cash and Hooper, 2005).

The Immunomodulatory Effects of *B. fragilis* Require Production of PSA

At least 2 of the 8 capsular polysaccharides of *B. fragilis* are ZPSs, a unique class of bacterial molecules with immunomodulatory properties (Tzianabos and Kasper, 2002). We wondered whether PSA, the most immunodominant and highly conserved ZPS, plays a role in splenic T cell expansion during *B. fragilis* commensalism. We used PSA-deficient *B. fragilis* ΔPSA to monocolonize germ-free mice (Coyne et al., 2001). The level of intestinal colonization by the mutant is indistinguishable from that by the isogenic parent strain, as assessed by fecal cfu counts (Figure 1B). Examination of splenic lymphocyte populations from mice colonized with *B. fragilis* lacking PSA but expressing all other antigens produced by this organism reveals an inability to correct $CD4^+$ T cell deficiencies in germ-free mice (Figure 1C). In pooled experiments (n = 4), the average proportions of $CD4^+$ T cells were: conventional, 17.82% ± 2.1%; *B. fragilis*, 18.05% ± 1.9%; *B. fragilis* ΔPSA, 10.95% ± 2.3%; and germ-free, 11.15% ± 1.5%. The effects were specific to $CD4^+$ T cells, as the proportions of $CD8^+$ T cells and $CD19^+$ B cells from splenic lymphocytes (Figure 1D) are indistinguishable between conventional and either monocolonized or germ-free mice, as previously observed (Pereira et al., 1986). Together, these results show that *B. fragilis* colonizing the gut of germ-free mice requires PSA production to correct host systemic $CD4^+$ T cell deficiencies during commensalism.

PSA Production by *B. fragilis* Directs Lymphoid Organogenesis

Commensal bacteria have long been appreciated for their positive impact on development of gut-associated lymphoid tissues (GALT, including Peyer's patches) and intraepithelial lymphocytes (IELs) and production of mucosal IgA (Hooper, 2004). We studied whether cellular immune maturation after bacterial colonization was also manifested in the morphological and ultrastructural development of peripheral lymphoid tissues. Germ-free animals have recently been reported to display defects in splenic structural development (Macpherson and Harris, 2004). We examined histological sections of spleens from germ-free mice colonized with wild-type *B. fragilis* or *B. fragilis* ΔPSA. Spleens from mice monocolonized with *B. fragilis* appear normal, with well-formed lymphocyte zones appearing as defined folli-

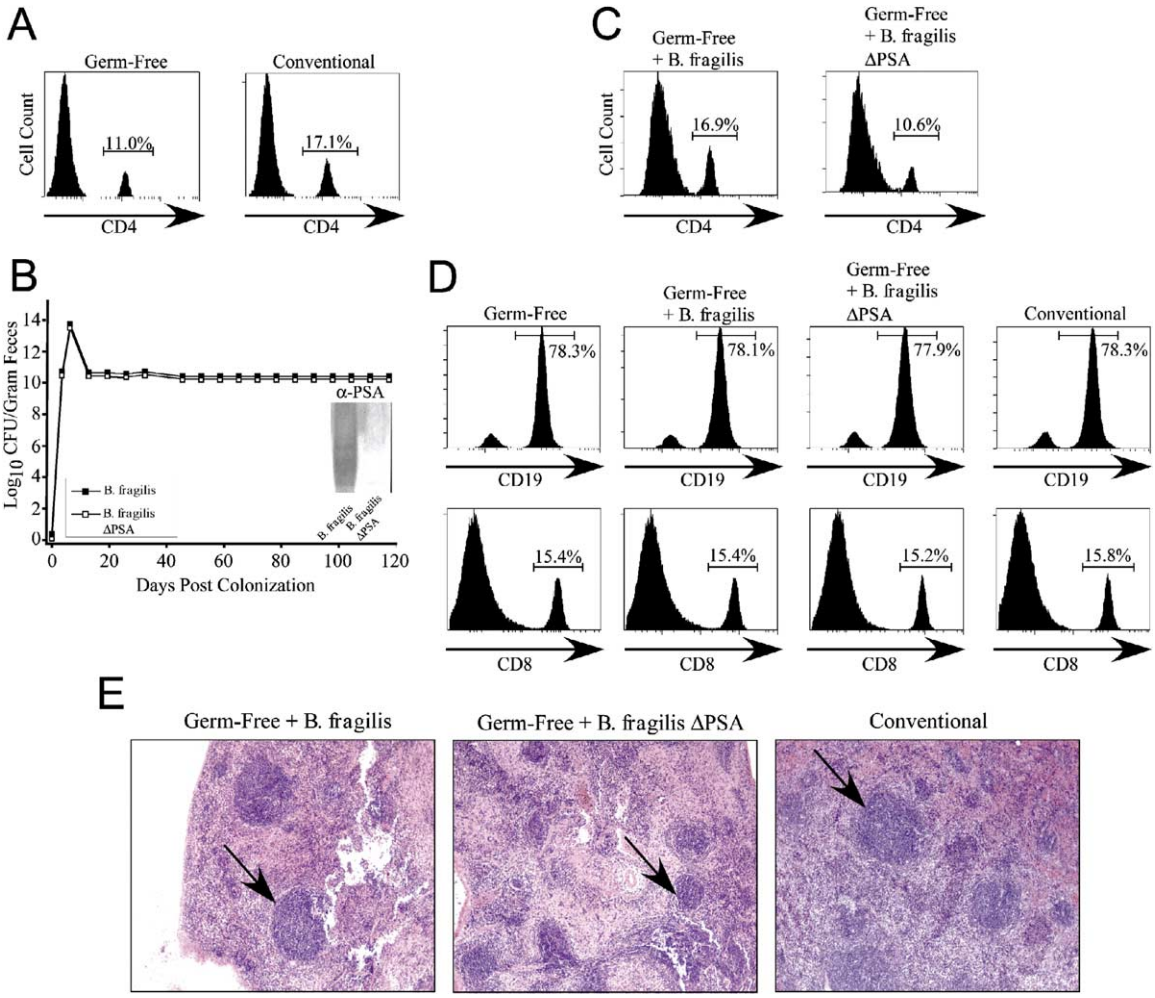

Figure 1. Cellular and Physical Immune Maturation in Germ-free Mice Requires PSA Production during Intestinal *B. fragilis* Colonization

(A) FC analysis of α-CD4-stained splenic lymphocytes from conventionally colonized (CNV) and germ-free (GF) mice reveals depletion of $CD4^+$ T cells in the absence of colonizing microflora in the GI tract. Results are representative of four experiments with pools of 3 to 5 mice.

(B) Quantitation of fecal cfu during monocolonization of GF mice with wild-type *B. fragilis* or an isogenic mutant deficient in PSA production (*B. fragilis* ΔPSA) reveals equivalent counts of viable bacteria from fecal pellets. Inset: Immunoblot of bacterial extracts with α-PSA after SDS-PAGE separation and electrotransfer to PVDF membranes shows the lack of PSA expression by *B. fragilis* ΔPSA.

(C) FC of α-CD4-stained splenic lymphocytes from GF mice shows that intestinal monocolonization of mice with *B. fragilis* results in complementation of $CD4^+$ T cells. In the presence of every other antigen produced during colonization with *B. fragilis* ΔPSA, the absence of PSA results in no increase in $CD4^+$ T cell counts. Results are representative of >10 experiments with single or pools of 3 to 5 mice.

(D) FC of splenic lymphocytes from CNV and GF mice colonized with wild-type *B. fragilis* or *B. fragilis* ΔPSA reveals no significant differences in $CD19^+$ B cells (upper panels) or $CD8^+$ T cells (lower panels) in the $CD4^-$ fraction. Results are representative of four experiments with pools of 3 to 5 mice.

(E) H&E-stained sections of spleens from CNV mice (right) and GF mice monocolonized with wild-type *B. fragilis* (left) and *B. fragilis* ΔPSA (center). White pulp containing lymphocytes appears as darker-staining follicular structures (arrows). The lack of large, well-defined follicles in mice colonized with *B. fragilis* ΔPSA is a measure of T cell depletion and reflects developmental defects in organogenesis. All images were taken at the same magnification.

cles (white pulp) similar to those in conventional mice with a complete gut flora (Figure 1E). The interspersed red pulp is densely packed with red blood cells and neutrophils. Spleens from germ-free mice colonized with *B. fragilis* ΔPSA show gross anatomical depletion of the lymphocyte zones similar to that in uncolonized germ-free mice (Macpherson and Harris, 2004). Follicles are smaller, less defined, and more fragmented than in germ-free mice colonized with wild-type *B. fragilis* or conventional mice (Figure 1E). The overall size and shape of spleens from all groups are comparable. This finding suggests a role for PSA specifically in lymphocyte development. Thus, changes in $CD4^+$ T cell expansion that are mediated by PSA produced by intestinal bacteria are consistent with the correction of physical and developmental defects in secondary lymphoid tissues. This observation reflects the importance of the beneficial relationship between commensal microorganisms and host physiology.

Purified PSA Is Sufficient to Expand T Cells in Germ-free and Conventional Animals

The inability of a PSA-deficient mutant to cause T cell expansion shows that the activity of this immunomodu-

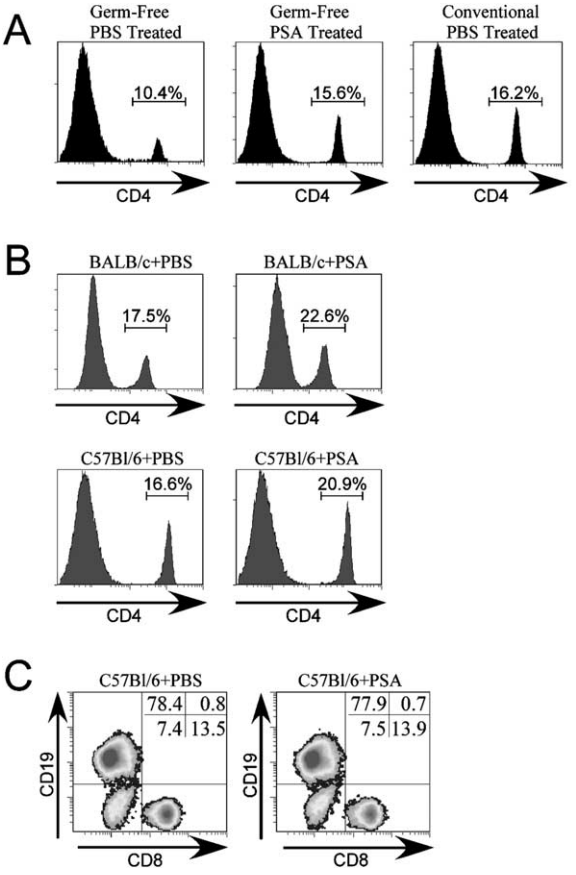

Figure 2. Purified PSA Treatment Is Sufficient for Expansion of CD4⁺ T Cells

(A) FC of α-CD4-stained splenic lymphocytes from GF mice treated intraperitoneally with PSA or PBS reveals PSA-dependent restoration of CD4⁺ T cells. Results are representative of two experiments with pools of four mice.

(B) FC shows that oral treatment of conventional C57Bl/6 and BALB/c mice with purified PSA (PSA) results in an increase in CD4⁺ T cell proportion among splenic lymphocytes over controls (PBS). Results are representative of four experiments with pools of 3 to 5 mice.

(C) FC shows that oral treatment of conventional mice with purified PSA does not affect proportions of CD8⁺ T cells or CD19⁺ B cells among the CD4⁻ splenic lymphocyte population.

latory molecule is required during *B. fragilis* colonization. Using chromatography to purify PSA extracted from the surface of *B. fragilis*, we investigated whether PSA alone is sufficient to counter the CD4⁺ T cell defects in germ-free mice. Purity was assessed by various methods, including H^1-NMR, spectroscopy, and gel electrophoresis. The preparation was devoid of contamination by protein, nucleic acid, and endotoxin (LPS). Uncolonized germ-free mice were treated intraperitoneally with purified PSA and then assessed for splenic T cell expansion. We found that purified PSA restores CD4⁺ T cell proportions among splenic lymphocytes in germ-free mice to conventional levels (Figure 2A).

To assess whether the route of administration to mice of various backgrounds affects PSA's immunomodulatory properties, both conventionally colonized C57Bl/6 and BALB/c mice received purified PSA intragastrically. This treatment recapitulates intestinal exposure of animals to PSA during colonization. Oral treatment led to a specific increase of splenic CD4⁺ T cells in conventional mice (Figure 2B), showing that PSA's effect is not exclusive to germ-free animals. CD8⁺ T cell and CD19⁺ B cell ratios were unaffected by PSA (Figure 2C) as formerly shown (Figure 1D). We found that specific recognition of purified PSA by host immune components in the intestines results in splenic CD4⁺ T cell expansion.

PSA Is Specifically Recognized by Dendritic Cells, with Consequent Cell Activation

All CD4⁺ T cell reactions require instruction to T cells by APCs (Kidd, 2003; Kapsenberg, 2003). PSA is recognized by APCs and then presented to T cells in vitro (Kalka-Moll et al., 2002). The cellular mechanism of PSA recognition in the intestine and subsequent signaling to expand splenic T cells is unknown. To characterize the APC responsible for PSA effects in animals, we fed mice fluorescently labeled PSA by gavage and then recovered cells from mesenteric lymph nodes (MLNs). As shown by flow cytometry, PSA specifically associates with CD11c⁺ dendritic cells (DCs; Figure 3A), and not with CD4⁺ T cells or CD19⁺ B cells (Figure 3A, data not shown) in the MLNs. The notion of in vivo DC recognition of PSA appears appropriate, as DCs are the only APCs known to sample luminal contents from the intestine and migrate to lymph nodes to initiate T cell reactions (Rescigno et al., 2001; Mowat, 2003). Consistent with this hypothesis, no PSA is recovered from spleens of orally treated mice (data not shown) despite splenic T cell expansion. In addition, confocal microscopy reveals that PSA is internalized by primary-cultured bone-marrow-derived DCs (BMDCs) and is subsequently displayed on the cell surface (Figure 3B). Thus it appears that DCs sample intestinal PSA by antigen uptake and migrate only as far as the local lymph nodes. These results agree with those of Macpherson and Uhr (2004), who observed that commensal bacteria are internalized by intestinal DCs that migrate to MLNs only.

We next assessed whether the association of PSA with BMDCs leads to cell activation and maturation, as only mature DCs can activate T cells. PSA-mediated maturation of DCs is measured by upregulation of MHC II among CD11c⁺ cells from 22% to 43% after PSA treatment (Figure 3C), a process required for efficient antigen display to the receptor on CD4⁺ T cells (Banchereau and Steinman, 1998; Thery and Amigorena, 2001). Treatment also increases expression of the costimulatory molecules CD80 (B7.1) and CD86 (B7.2) in a dose-dependent manner (data not shown and Figure 3D, respectively), a result further showing that PSA interacts with and induces maturation of DCs. These findings are consistent with the fact that DCs, not B cells, mediate intestinal antigen presentation to T cells after conventionalization of germ-free animals (Yamanaka et al., 2003).

PSA Induces T Cell Proliferation When Presented by DCs In Vitro

To further investigate cellular and molecular events underlying immune responses to PSA, we developed an

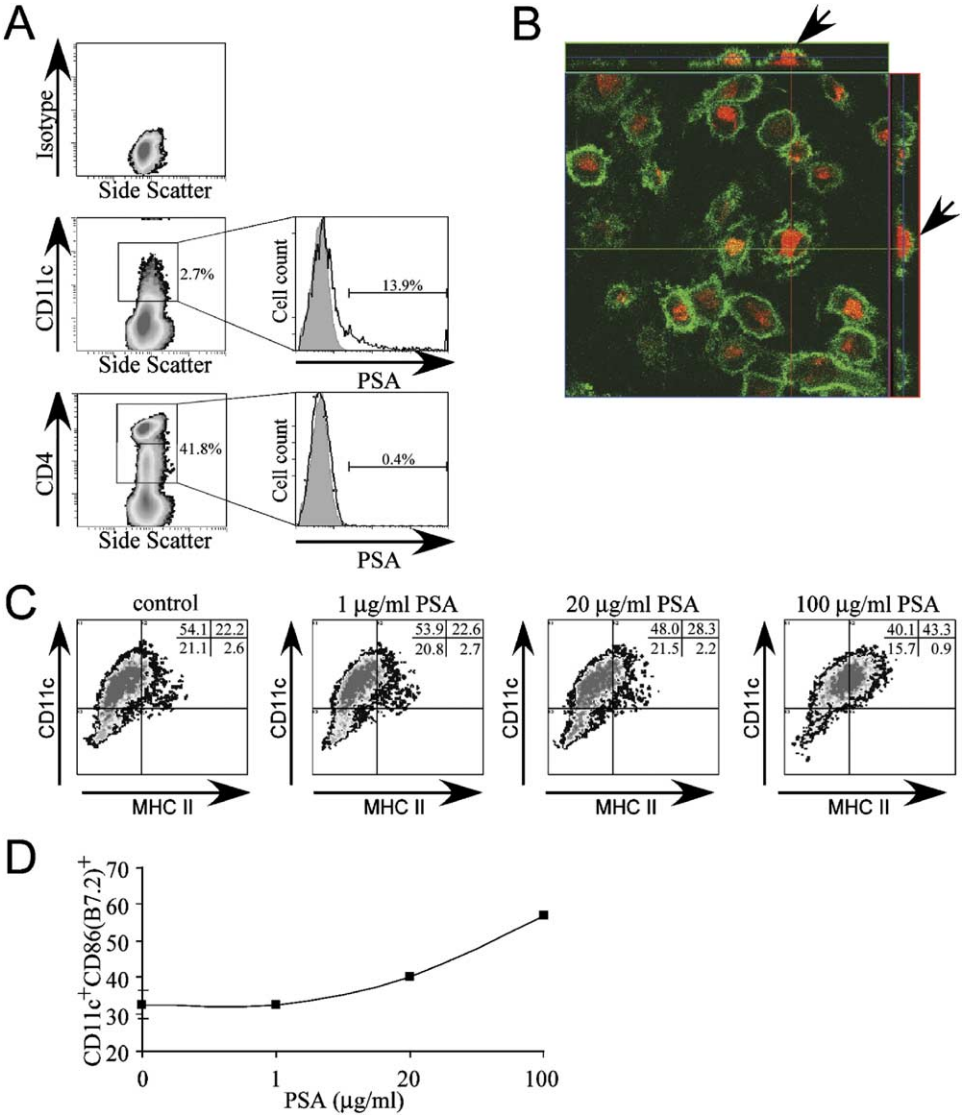

Figure 3. PSA Is Specifically Recognized by DCs in the GI Tract and In Vitro, with Consequent Cell Activation

(A) Oral treatment of mice with Alexa-594-labeled PSA results in antigen uptake by CD11c$^+$ DCs from MLNs. FC of CD11c$^+$ gated cells (boxed left panel, middle) analyzed for the presence of PSA (horizontal axis of right panels) shows colocalization of PSA with DCs. FC of isolated MLNs reveals no PSA associated with CD4$^+$ T cells from the same lymph nodes (boxed left panel, bottom). Isotype control is shown in the top left panel. Gray histograms represent unstained control, and thick black lines denote Alexa-594 signal from PSA on the horizontal axis. No PSA is detectable in splenic tissues (data not shown).

(B) Confocal microscopy of α-CD11c-labeled (green) and 24 hr PSA-treated (red) BMDCs in culture illustrates antigen in endosomes and surface display (arrowheads). Central image is the xy plane of a medial z section; upper and side panels are assembled z stacks.

(C) FC of BMDCs cultured for 24 hr with PSA shows activation by upregulation of MHC II (horizontal axis) among CD11c$^+$ cells (vertical axis).

(D) FC of CD11c$^+$ BMDCs cultured for 24 hr with PSA shows activation through upregulation of the costimulatory molecule CD86 (B7.2).

in vitro coculture system using primary cells to assess the ability of purified PSA to induce T cell proliferation and cytokine expression. As shown above, PSA treatment leads to CD4$^+$ T cell expansion in mice. Incubation of PSA with CD11c$^+$ BMDCs and naive splenic CD4$^+$ T cells leads to a dose-dependent increase in T cell proliferation (Figure 4A). Neutralization of the positive charge by chemical modification of PSA (N-acetylated [NAc] PSA) results in no incorporation of [^{3}H]thymidine—a marker for cell replication. This extends to DCs our observation that the zwitterionic structure of PSA is critical for biological activity (Kalka-Moll et al., 2002; Tzianabos and Kasper, 2002). Purified *E. coli* LPS does not induce T cell proliferation in mice (Figure 4A; LPS). PSA-mediated CD4$^+$ T cell proliferation requires both DCs and T cells; either cell type alone results in no incorporation of radiolabel (Figure 4A). Together, these results show that DCs can direct T cell proliferation in response to purified PSA in vitro.

PSA Induces T Cell Cytokine Production in Dendritic-Cell Cocultures

The two subtypes of effector CD4$^+$ T cells, T_H1 and T_H2, are defined by expression of the cytokines interferon γ

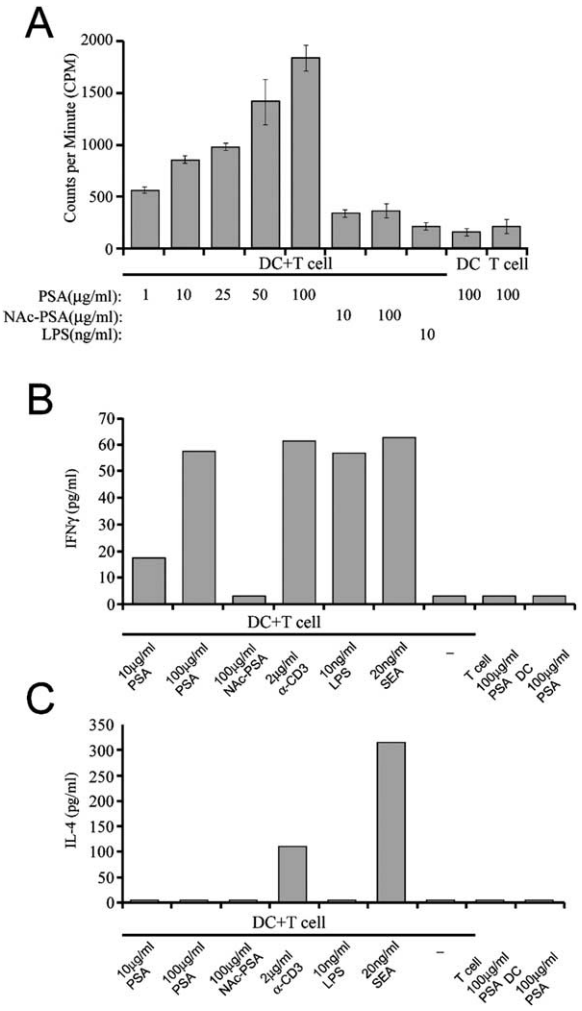

Figure 4. PSA Induces CD4+ T Cell Proliferation and T_H1 Cytokine Production In Vitro

(A) CD4+ T cell proliferation by [^{3}H]thymidine incorporation increases in response to irradiated BMDCs (1×10^6) incubated with PSA. Treatment of cocultures with NAc-PSA results in no increase in cell proliferation. LPS treatment or DCs or T cells alone do not support CD4+ T cell proliferation. Results are representative of three experiments. Error bars represent the ± SD from triplicate samples of a single experiment.

(B) PSA treatment stimulates IFNγ in DC-T cell cocultures, as measured by ELISA of culture supernatants after 48 hr of treatment. NAc-PSA treatment has no effect. Treatment with α-CD3, LPS, and SEA, all known stimulators of T_H1 cytokine expression, results in IFNγ expression. DCs or T cells alone treated with PSA do not support cytokine expression.

(C) PSA treatment does not stimulate expression of IL-4 in DC-T cell cocultures. Treatment with α-CD3 and SEA, known stimulators of T_H2 cytokine expression, results in IL-4 production.

(IFNγ) and interleukin 4 (IL-4), respectively (Janeway et al., 2001). As shown above, PSA induces CD4+ T cell expansion in *B. fragilis*-colonized mice and in vitro. To further characterize the effects of PSA-mediated T cell activation, we assessed cytokine profiles using purified cellular components. Coculture of DCs and CD4+ T cells in the presence of PSA yields dose-dependent upexpression of the T_H1 cytokine IFNγ (Figure 4B). The level of IFNγ production associated with PSA is comparable to that associated with several known potent IFNγ inducers (α-CD3, LPS, and staphylococcal enterotoxin A [SEA]) and requires both DCs and T cells (Figure 4B). Specificity is evidenced by the lack of T_H1 cytokine production after NAc-PSA treatment (Figure 4B).

T_H1 cytokine production suppresses T_H2 responses; conversely, T_H2 cytokine expression inhibits T_H1 responses. Normal immune responses require a controlled balance of these opposing signals. Examination of IL-4 expression in response to PSA treatment reveals no cytokine production by purified CD4+ T cells (Figure 4C; 100 μg/ml PSA). α-CD3 and the superantigen SEA are potent stimulators of both classes of cytokine (Figure 4C). As T_H2 cytokine production is a "default pathway" in many systems (Kidd, 2003; Amsen et al., 2004) and T_H1 cytokine production is antagonistic to T_H2 expression, the specific stimulation of IFNγ by PSA in vitro may provide a mechanism for establishing commensal-mediated homeostasis of the host immune system by balancing T_H1/T_H2 responses.

T_H1 Cytokine Production in Response to PSA Treatment Requires Signaling through the IL-12/Stat4 Pathway and MHC II Expression

A unique immunologic molecule, PSA is the only carbohydrate studied to date that is internalized into APCs and displayed by MHC II to T cells (Cobb et al., 2004)—a process previously reserved for protein antigens. We further characterized the molecular pathway for PSA-induced T_H1 cytokine production. Many of the molecular signaling events involving T helper cytokine expression are well characterized. The major pathway of IFNγ upregulation and T_H1 cell differentiation involves DC secretion of IL-12, which binds to the IL-12 receptor on T cells and signals to activate the T_H1-specific transcription factor Stat4 (Trinchieri, 2003). PSA stimulation of DC-T cell cocultures elicits a dose-dependent increase in IL-12 production (Figure 5A). DCs alone stimulated with PSA secrete IL-12 (Figure 5A, DC 100 μg/ml PSA), although at levels lower than those found for CD4+ T cells. This is the first bacterial polysaccharide shown to signal IL-12 secretion by APCs, as previously shown for classical protein antigens (Macatonia et al., 1995). NAc-PSA, lacking the essential positive-charge motif, does not stimulate cytokine production. To determine whether IL-12 is required for T_H1 cell differentiation, we measured IFNγ expression after PSA treatment in the presence of increasing concentrations of neutralizing antibody to IL-12 (Heufler et al., 1996). IL-12 neutralization abolishes PSA-mediated IFNγ secretion by CD4+ T cells in vitro (Figure 5B). Ablation of IL-12 signaling does not affect α-CD3-mediated IFNγ expression, as this signal is APC independent, acting directly on T cells. To determine whether IL-12 signaling that results in T_H1 lineage differentiation involves the Stat4 transcription factor, we incubated DCs from wild-type mice with CD4+ T cells from *stat4* knockout mice and measured IFNγ expression in response to PSA treatment. The absence of Stat4 greatly reduces T_H1 cytokine production (Figure 5C); thus, PSA specifically induces DCs to signal T cell differentiation through Stat4 transcriptional regulation.

To investigate whether antigen presentation of PSA

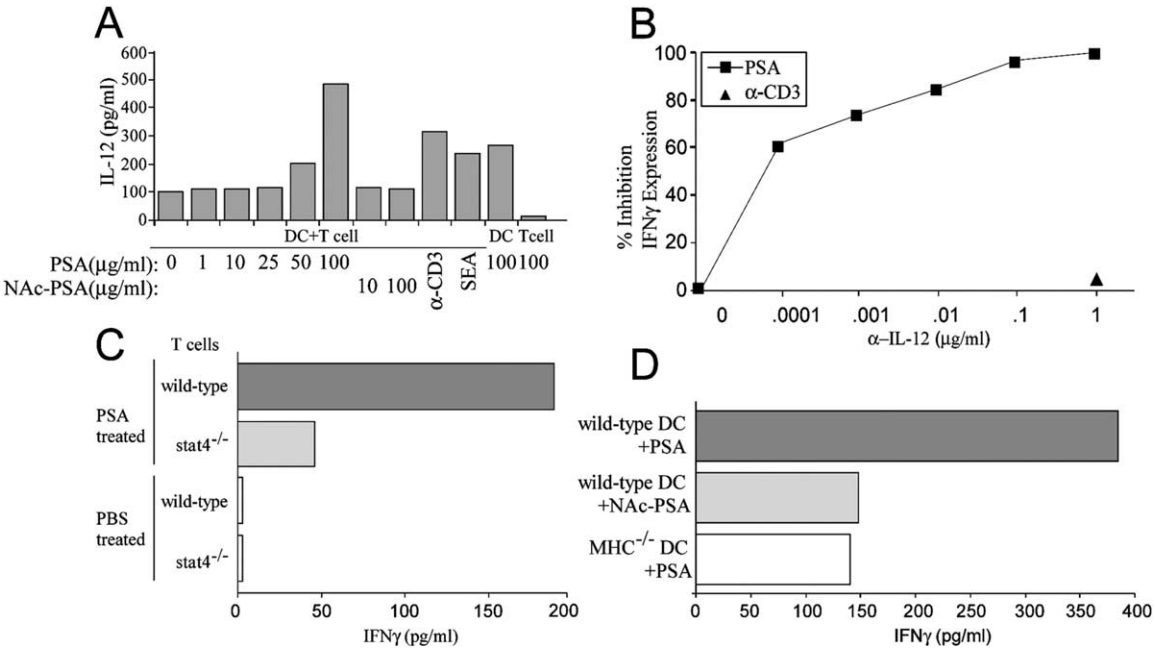

Figure 5. PSA Signals through the IL-12/Stat4 Pathway to Mediate T_H1 Cytokine Production, which Requires Presentation by MHC II

(A) PSA stimulates expression of IL-12, the T_H1 determining signal, in DC-T cell cocultures (1×10^6 of each cell type). NAc-PSA treatment has no effect. α-CD3 and SEA serve as controls for IL-12 expression by BMDCs. Results are representative of two experiments.

(B) IL-12 is required for PSA-mediated IFNγ production. IFNγ expression is abolished in DC-T cell cocultures treated with PSA (100 μg/ml) in the presence of neutralizing antibody to IL-12. Neutralization of IL-12 does not inhibit α-CD3-mediated IFNγ expression, which is IL-12 independent.

(C) PSA signals through Stat4 to induce IFNγ secretion by T cells. IFNγ expression from DC-T cell cocultures treated with PSA (100 μg/ml) is reduced when $CD4^+$ T cells are purified from spleens of *stat4* knockout rather than wild-type mice (Figure 4B).

(D) MHC II expressed on DCs is necessary for PSA-mediated IFNγ expression by $CD4^+$ T cells. IFNγ production by DC-T cell cocultures treated with PSA (100 μg/ml) is reduced when BMDCs are from MHC II knockout mice. NAc-PSA (100 μg/ml) treatment of wild-type DCs (MHC $II^{+/+}$) serves as the control for PSA-specific IFNγ expression.

is required for cytokine signaling, we purified DCs from MHC II-deficient mice and treated DC-T cell cocultures with PSA or NAc-PSA. The level of IFNγ expression is significantly higher in wild-type (MHC $II^{+/+}$) mice treated with PSA than in MHC II knockout mice (MHC $II^{-/-}$), which express amounts similar to a NAc-PSA control (Figure 5D). Together, these results demonstrate that T_H1 cytokine responses to PSA require MHC II expression by APCs and involve signaling through the IL-12/Stat4 pathway to induce T cell activation and proper cytokine expression.

PSA Is Required for Appropriate $CD4^+$ T Helper Cytokine Production during Colonization

A proper T_H1/T_H2 balance is critical for human and animal health; over- or underproduction of either response is associated with immunologic disorders. We investigated the effects of PSA on T_H1/T_H2 cytokine responses in colonized animals, again using germ-free mice. $CD4^+$ T cells from mouse spleens were purified and tested by ELISA for cytokine production. Figure 6A shows overproduction of the T_H2 cytokine IL-4 in spleens of germ-free mice compared with levels in conventional mice. This result is consistent with previous reports of the appreciably T_H2-skewed profile of mice devoid of bacterial contamination and reflects the human neonatal (precolonization) cytokine profile (Kirjavainen and Gibson, 1999; Prescott et al., 1998; Adkins, 2000; Kidd, 2003). This "default" T_H2 bias in the absence of bacterial colonization again highlights the profound contributions of the microflora to immune development and provides a model to test the effects of symbiotic bacteria on the establishment of appropriate host cytokine production.

Mice colonized with wild-type *B. fragilis* alone display a level of IL-4 production similar to that in conventional mice with a complex microflora (Figure 6A); this similarity shows the organism's sufficiency to correct systemic immune defects. Moreover, mice colonized with *B. fragilis* ΔPSA produce T_H2 cytokines at elevated levels similar to those in germ-free mice (Figure 6A). Thus, the expression of a single bacterial antigen allows *B. fragilis* to correct the IL-4 cytokine imbalance found in uncolonized animals.

Examination of IFNγ production by purified splenic $CD4^+$ T cells reveals that germ-free mice, which are T_H2-skewed, are deficient in production of this prototypical T_H1 marker when compared to conventional mice (Figure 6B). Colonization with wild-type *B. fragilis* alone is sufficient to correct the defect in IFNγ expression in germ-free mice, with levels nearly as high as those in conventional mice (Figure 6B). Lack of PSA

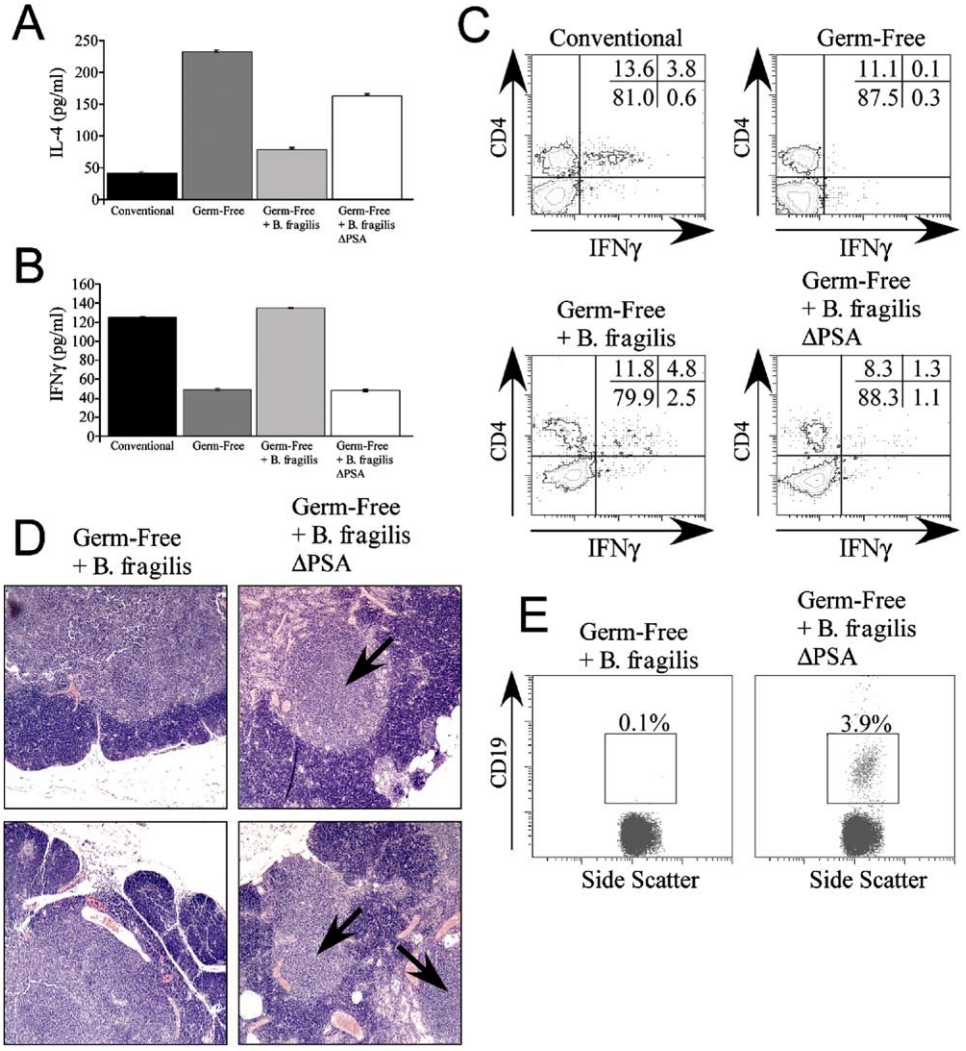

Figure 6. Colonization of GF Mice with PSA-Producing *B. fragilis* Corrects T_H1/T_H2 Imbalances Associated with Cytokine-Mediated Pathologies

(A) IL-4 production from splenic $CD4^+$ T cells stimulated in vitro with α-CD3/α-CD28 (2 μg/ml each) reveals that PSA is required to correct the T_H2 skew in GF mice. Compared with CNV mice, GF mice overproduce IL-4 (first and second bars). Intestinal colonization with *B. fragilis* (third bar) reduces the expression of IL-4 from levels in GF mice. *B. fragilis* ΔPSA colonization fails to correct the T_H2 skew (fourth bar). Results are representative of two experiments from pools of four mice. Error bars represent the ± SD from triplicate samples of a single experiment.

(B) IFNγ expression by splenic $CD4^+$ T cells during colonization indicates increased T_H1 cytokine production in CNV compared to in GF mice (first and second bars). PSA production by intestinal *B. fragilis* is required for the increase in IFNγ expression and immune homeostasis (third bar); homeostasis is not seen in the absence of PSA (fourth bar). Error bars represent the ± SD from triplicate samples of a single experiment.

(C) Intracellular cytokine staining and FC of in vitro-stimulated (500 ng/ml PMA, 5 μg/ml ionomycin) cultures of splenic $CD4^+$ T cells for 4 hr in the presence of brefeldin A show that IFNγ (horizontal axis) is produced specifically by $CD4^+$ T cells (vertical axis) during bacterial colonization. PSA production by *B. fragilis* is required for the specific increase in IFNγ expression to levels similar to those for CNV mice. GF and *B. fragilis* ΔPSA-colonized mice express low levels of T_H1 cytokines. Results are representative of three experiments with pools of 3 to 5 mice.

(D) Thymic histology of germ-free mice (H&E) colonized with wild-type or *B. fragilis* ΔPSA for over 1 year reveals follicles (arrows) within the inner medullary compartment in the absence of PSA. None of five *B. fragilis*-colonized compared to 3 of 5 *B. fragilis* ΔPSA-colonized mice displayed follicles.

(E) FC of thymic tissues recovered from groups of differentially colonized GF mice (ten per group) reveals the anomalous presence of $CD19^+$ B cells in *B. fragilis* ΔPSA-colonized mice, a condition likely resulting from increased T_H2 cytokine production in the absence of PSA.

production by the *B. fragilis* mutant during colonization of germ-free mice results in low-level production of T_H1 cytokines (Figure 6B). These results were corroborated by intracellular cytokine staining of splenic lymphocytes from each group, which confirms that IFNγ production is attributable to $CD4^+$ T cells (Figure 6C). The production of IL-2, another T_H1 cytokine, by $CD4^+$ T cells in gnotobiotic mice also requires PSA production (data not shown). Together, these results demonstrate that intestinal colonization of germ-free mice by

B. fragilis alone is sufficient to establish a proper systemic T_H1/T_H2 balance within the host—a fundamental aspect of the mammalian immune response.

Absence of PSA Production by *B. fragilis* during Colonization Is Associated with T_H2-Mediated Pathologies of the Thymus

Throughout our studies, specimens obtained at necropsy were subjected to histological examination. We noticed a rare pathology of the thymus exclusively in mice colonized with *B. fragilis* ΔPSA. Thymic tissues from germ-free mice colonized with wild-type *B. fragilis* appear normal, with a darker-staining outer corona and a uniform and homogeneous inner medullary compartment. Surprisingly, at >1 year of age, the majority of mice colonized with *B. fragilis* ΔPSA display the outgrowth of B cell-like follicles in the thymic medulla (Figure 6D). Flow cytometry shows that these tissues contain $CD19^+$ B cells (Figure 6E) not found in the normal thymus. This rare condition appears to be similar to human thymic hyperplasia, in which B cells are found in follicles of the medulla (Kasper et al., 2005). The latter condition is associated with numerous autoimmune disorders, most notably myasthenia gravis, a B cell-mediated pathology (Malhotra et al., 1992; Infante and Kraig, 1999). These disorders, as well as B cell outgrowths, are mediated by overproduction of T_H2 cytokines by $CD4^+$ T cells (Zhang et al., 1997; Janeway et al., 2001). It is compelling to speculate that the inability to restore proper T_H1/T_H2 balance in germ-free mice through appropriate commensal colonization results in an aberrant T_H2 response, which may lead to immune-mediated pathologies.

Discussion

PSA of Intestinal Bacteria as a Symbiosis Factor

The vertebrate brain and immune system are the only organs that require environmental interactions for development. We report here that maturation of the mammalian immune system requires the specific direction of an immunomodulatory molecule provided by symbiotic bacteria. *B. fragilis*, a ubiquitous constituent of the mammalian lower gastrointestinal microflora, elaborates a ZPS (PSA) that directs the development of $CD4^+$ T cells; the eventual result is the correction of immunologic defects found in the absence of bacterial colonization. Impaired systemic $CD4^+$ T cell maturation, aberrant T_H1/T_H2 lineage differentiation, and defective lymphoid-organ development are all corrected by PSA production during *B. fragilis* colonization. DCs in GALT apparently sample *B. fragilis* and/or PSA from the intestine and, after activation, migrate to lymphoid organs and signal T_H1 lineage differentiation through the IL-12/Stat4 pathway. As T_H1 cytokine production opposes the T_H2 default phenotype (Kidd, 2003), this process may contribute to protection from disease by creating appropriate cytokine balances in the immune system. The establishment of proper host immunologic function through responses to PSA identifies this molecule as a factor required for symbiosis.

The Timeless Struggle between Good and Bad (Bacteria)

The origins of microbiology lie in the study of bacterial pathogens. Louis Pasteur and Robert Koch were driven to identify the microbial etiologies underlying infections (Falkow, 2004). Investigation into pathogens has revealed microbial molecules that mediate aspects of the infectious process, many of which have been experimentally identified as virulence factors in animal models of disease (Monack et al., 2004). Countless molecular and cellular inflammatory responses to these molecules are well documented, and the results of some interactions lead to development of immunologic memory and resistance to reinfection. This acquired immunity reflects the immune system's ability to recognize and adapt to specific foreign molecules. However, bacterial infections by pathogens are rare and opportunistic. The vast majority of human encounters with bacteria involve harmless organisms found in our environment or as commensals in our bodies (Hooper and Gordon, 2001). These are the species with which we have coevolved. Unlike pathogens, certain commensals may be beneficial to human development and physiology.

The Gut Reaction to Symbiosis

How is the immunomodulatory signal provided by PSA in the intestine ultimately transduced to the rest of the immune system? DCs have received much attention as mediators of nonclassical T cell responses (Morelli and Thomson, 2003). Whereas other APCs provide proinflammatory instructions during infection, DCs uniquely signal protective and tolerance-inducing reactions by T cells (Colonna et al., 2004). This function may underlie DC positioning at mucosal and epidermal surfaces constantly encountering antigens that may not necessarily induce inflammation (e.g., antigens associated with commensal bacteria or food; inhaled antigens). DCs have evolved various mechanisms for sampling antigens from the gastrointestinal luminal compartment (Rescigno et al., 2001; Mowat, 2003). Concordantly, DCs are the APCs that mediate the generation and activity of regulatory T cells (Tregs), a $CD4^+$ T cell subset involved in suppression of immune reactions and direction of T_H1/T_H2 responses (Groux et al., 2004; Mills, 2004). We show here that PSA produced by an intestinal symbiont is presented to T cells by DCs, with consequent signaling, immune activation, and cytokine production. Neither *B. fragilis* nor PSA is detectable in spleens of colonized germ-free mice (data not shown), although we find PSA in MLNs. DCs internalize commensal bacteria in the gut and migrate only as far as MLNs to mediate immune responses (Macpherson and Uhr, 2004). As peripheral Tregs are known to traffic to sites of inflammation where they control T cell reactions (Kohm et al., 2002; Zou et al., 2004), T cell migration from lymph nodes to spleen may mediate the cellular signal-transduction mechanisms required for PSA-mediated immune development at extraintestinal sites.

Are Bacterial Symbionts the Victims of Good Hygiene?

In 1989, it was postulated that reduced exposure to infectious bacterial agents early in life due to improved

sanitation and antibiotic use explained increases in allergy among residents of industrialized countries, a concept termed the hygiene hypothesis (Strachan, 1989). In a proposed modified counterregulation model, improved hygiene limits exposure to immunomodulatory molecules of beneficial commensal bacteria that provide protection from unrelated immune diseases (Wills-Karp et al., 2001). Substantial evidence from the past 40 years indicates that incidences of atopy (allergy predisposition) and asthma have increased dramatically in Western nations but not in developing countries. Several epidemiologic studies indicate that atopic and nonatopic people differ in gut flora composition (Bjorksten et al., 1999; Kirjavainen et al., 2001; Kalliomaki et al., 2001). Thus, hygiene-associated deviations in gut microflora composition may be the environmental factor underlying development of atopy and asthma in genetically predisposed individuals.

"Does the microbiota regulate immune responses outside the gut?" (Noverr and Huffnagle, 2004). Asthma and allergies are nonintestinal immunologic disorders mediated by overproduction of T_H2 cytokines and of IgE—also a component of the T_H2 response (Umetsu et al., 2002). Here we show that colonization with the ubiquitous commensal *B. fragilis* corrects the systemic T_H2 bias found in the absence of bacterial colonization through specific production of T_H1 cytokines. Systemic expression of IFNγ, the characteristic T_H1 marker, inhibits T_H2 cytokine reactions and reduces IgE production (Kidd, 2003), providing a mechanism for establishment of immunologic cytokine balance. Germ-free mice and atopic patients with altered gut microflora have a highly T_H2-skewed cytokine profile (Shi and Walker, 2004). Colonization of germ-free mice with PSA-producing *B. fragilis* restores normal cytokine production. Probiotic studies with *Lactococcus* and *Bifidobacterium* species suggest that other prominent gut bacteria may also produce immunomodulatory molecules (Guarner and Malagelada, 2003; Rastall, 2004). Perhaps pervasive antibiotic use among children in industrialized countries leads to clearance of symbionts such as *B. fragilis* at an essential point in immune development and results in the absence of molecules with PSA-like activities. Aberrant immune development without specific direction by this class of immunomodulatory molecules may lead to a default pathway of T_H2 cytokine overproduction and the onset of atopic and asthmatic disorders. Future investigations must determine the function of immunomodulatory molecules of symbiotic bacteria in the critical balance between mammalian health and disease.

Experimental Procedures

Mice and Bacterial Strains

Conventional SPF mice of strains C57BL/6NTac, BALB/cAnNTac, B6.SJL-*Ptprc*^{a}/BoAiTac-H2-*Ab1*^{tm1GLM} N13 (MHC II$^{-/-}$), and B6.SJL-*Ptprc*^{a}/BoAiTac (MHC II$^{-/-}$ control) were purchased from Taconic Farms (Germantown, New York). C.129S2-*Stat4*^{tm1Gru} (Stat4$^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). These mice were housed in conventional cages at Harvard Medical School. Germ-free Swiss-Webster mice were from Taconic Farms and were housed in sterile isolators (Class Biologically Clean, Madison, Wisconsin). Mice were screened weekly for bacterial, viral, and fungal contamination. All procedures with mice were performed according to guidelines of the HMS Office for Research Subject Protection.

B. fragilis strain NCTC 9343 and its isogenic PSA deletion mutant have been described (Coyne et al., 2001). For mouse colonization, ~1 × 10^8 cfu of bacteria grown in BHI medium were spread on food and bedding. Mice were colonized for at least 60 days before examination. Results are for mice up to 8 months postcolonization (except thymic pathology, which requires aging over 1 year).

Lymphocyte Isolation from Tissues

Lymphocytes were isolated from tissues (Tzianabos et al., 2000). In brief, spleens, thymus, and MLNs were disrupted into single-cell preparations and enriched for lymphocytes over a Histopaque 1083 gradient (Sigma, St. Louis). Cells were washed with PBS and used directly or fixed with 0.5% PFA for 1 hr at 4°C.

Flow Cytometry, Fluorescence-Activated Cell Sorting, and Intracellular Cytokine Staining

Directly fluorochrome-conjugated monoclonal antibodies were used (BD Pharmingen, San Diego, California). For surface staining, lymphocyte preparations were washed twice in FC buffer (PBS with 2% FBS) and resuspended in 100 μl. 1 × 10^6 cells were incubated with antibodies at 2 μg/ml for 30 min at 4°C. For intracellular cytokine staining, cells were resuspended in 100 μl of Cytofix/Cytoperm buffer for 30 min at 4°C, washed with Perm/Wash buffer, and incubated with fluorochrome-conjugated anti-cytokine antibodies for 30 min at 4°C. Cells were washed and analyzed on a model FC500 Cytometer (Beckman Coulter, Fullerton, California), and all data were analyzed with RXP Analysis Software (Beckman Coulter). FACS was performed on a BD FACSAria, and cell purity was always >99%.

PSA Purification and Animal Treatment

PSA from *B. fragilis* NCTC 9343 was prepared (Tzianabos et al., 1992). For some studies, PSA was treated with acetic anhydride to neutralize the positively charged amino group (Tzianabos et al., 1994). Mice received 50 μg of PSA in 1.5% sodium bicarbonate/PBS intraperitoneally or intragastrically three times a week for 2 weeks. Rag2$^{-/-}$ mice received 30 μg of PSA (PBS controls) in alternating intragastric and subcutaneous treatments three times a week.

In Vitro Cytokine Stimulation and Proliferation Assays

For analysis of splenic cytokines, lymphocytes were isolated as above. CD4$^+$ T cells were purified with the MACS CD4 Sorting Kit (Miltenyi Biotec, Auburn, California). Cell purity was >97% CD4$^+$. The remaining lymphocytes were used as APCs after γ irradiation. 3 × 10^5 cells of each type were mixed in a 48-well plate, and α-CD3/α-CD28 (2 μg/ml) was added. Supernatants recovered after stimulation for 48 and 72 hr were analyzed by ELISA.

For cocultures, CD4$^+$ T cells were purified from splenic lymphocytes with a CD4$^+$ T Cell Subset Kit (R&D Systems). Cell purity was >95%. BMDCs from femurs of mice were purified. Cells were cultured for 8 days in C-RPMI-10 with GM-CSF (20 ng/ml; Biosource, Camarillo, California). Cells were >90% CD11c$^+$. 1 × 10^6 purified CD4$^+$ T cells were mixed with 1 × 10^6 purified CD11c$^+$ BMDCs and incubated at 37°C/5% CO_2.

ELISA plates were from precoated kits (Biosource). T cell proliferation assays were done with 1 × 10^5 cells of each type (APCs irradiated) after incubation for 96 hr. For the last 8 hr before harvest, wells were pulsed with [^{3}H]thymidine (1 μCi/well). Cells were washed, harvested, and counted by liquid scintillation (Wallac; now Perkin-Elmer, Boston). Data were expressed as mean cpm ± SD for triplicate wells.

Histological Tissue Analysis

Paraffin-embedded mouse tissues were stained with H&E. Sections were evaluated in blinded fashion by a single pathologist (R.T. Bronson, HMS).

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