Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis

Yun Kyung Lee[a,1], Juscilene S. Menezes[a,1], Yoshinori Umesaki[b], and Sarkis K. Mazmanian[a,2]

[a]Division of Biology, California Institute of Technology, Pasadena, CA 91125; and [b]Yakult Central Institute for Microbiological Research, Tokyo 186-8650, Japan

Edited by Jeffrey I. Gordon, Washington University School of Medicine, St. Louis, MO, and approved June 23, 2010 (received for review January 19, 2010)

Although the effects of commensal bacteria on intestinal immune development seem to be profound, it remains speculative whether the gut microbiota influences extraintestinal biological functions. Multiple sclerosis (MS) is a devastating autoimmune disease leading to progressive deterioration of neurological function. Although the cause of MS is unknown, microorganisms seem to be important for the onset and/or progression of disease. However, it is unclear how microbial colonization, either symbiotic or infectious, affects autoimmunity. Herein, we investigate a role for the microbiota during the induction of experimental autoimmune encephalomyelitis (EAE), an animal model for MS. Mice maintained under germ-free conditions develop significantly attenuated EAE compared with conventionally colonized mice. Germ-free animals, induced for EAE, produce lower levels of the proinflammatory cytokines IFN-γ and IL-17A in both the intestine and spinal cord but display a reciprocal increase in $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs). Mechanistically, we show that gut dendritic cells from germ-free animals are reduced in the ability to stimulate proinflammatory T cell responses. Intestinal colonization with segmented filamentous bacteria (SFB) is known to promote IL-17 production in the gut; here, we show that SFBs also induced IL-17A–producing $CD4^+$ T cells (Th17) in the CNS. Remarkably, germ-free animals harboring SFBs alone developed EAE, showing that gut bacteria can affect neurologic inflammation. These findings reveal that the intestinal microbiota profoundly impacts the balance between pro- and antiinflammatory immune responses during EAE and suggest that modulation of gut bacteria may provide therapeutic targets for extraintestinal inflammatory diseases such as MS.

Th17 cells | $Foxp3^+$ regulatory T cells | autoimmunity | multiple sclerosis | segmented filamentous bacteria

Immunologic dysregulation is the cause of numerous human disorders. Multiple sclerosis (MS) is an autoimmune disease that affects the CNS (1). Common early symptoms include numbness, pain, burning, and itching of the extremities. Cognitive problems include memory disturbances, decreased judgment, and inattention. In extreme cases, MS patients develop partial or complete paralysis and dementia. The inflammatory response in MS is directed to myelin, the protective coating of nerves. Disease involves uncontrolled autoreactive T cells and additional cells of the immune system that infiltrate the CNS and attack the myelin sheath (2). Although significant clinical and scientific efforts have been expended for decades, many aspects regarding the nature of MS are still unknown. However, it is apparent that disease progression involves genetic as well as environmental factors. Genome-wide association studies have revealed polymorphisms in several candidate genes that encode for cytokines and their receptors as well as immunoregulatory molecules associated with various autoimmune disorders (3). However, high rates of discordance in monozygotic twins, where concordance does not exceed 30% (4), indicate that environmental factors may play an equal or even more important role than genetics in the development of MS.

Experimental autoimmune encephalomyelitis (EAE) is an animal model that reproduces many of the features of MS (5). EAE is induced by immunization with CNS antigens, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or proteolipid protein (PLP) or their immunodominant peptides. Myelin-reactive T cells that induce EAE are only pathogenic in wild-type mice if activated with the self-antigen in the presence of bacterial adjuvants, such as *Mycobacteria tuberculosis* extracts and/or pertussis toxin. Furthermore, several reports have suggested that MS in humans is associated with microbial contact; paradoxically, some microorganisms seem to potentiate disease, whereas others seem to prevent MS (6). Therefore, altered microbial stimulation of the immune system may be a likely underlying environmental component of disease. Although infections may affect immune responses, encounters with pathogenic microbes are relatively rare and opportunistic. Conversely, environmentally exposed surfaces of mammals are colonized for life with 100 trillion indigenous bacteria, creating a diverse ecosystem whose contributions to human health seem to be profound (7). The gastrointestinal tract harbors the greatest numbers and complexity of microorganisms, known as the microbiota, which have evolved with their hosts for millions of years and regulate human nutrition, metabolism, and immune-system function. Furthermore, the intestinal microbiota contains both pro- and antiinflammatory products that modulate immune responses (8). Therefore, the community composition of the microbiota may have profound effects on the immune status of the host and may impact the development and/or progression of inflammatory diseases such as MS.

After lineage commitment in the thymus, naïve $CD4^+$ T cells enter the periphery where they sense environmental signals that further instruct their maturation and function. During responses to infectious disease, microbial and host signals at the site of infection provide cues to naïve T cells to induce their differentiation into various pro- and antiinflammatory subsets. For instance, infection by intracellular pathogens drives the development of T-helper 1 (Th1) cells, whereas responses to extracellular pathogens are predominantly of the Th2 and Th17 subset (9). These proinflammatory T-helper cells coordinate many aspects of the innate and adaptive immune response to effectively clear microbial invaders. Although T cells presumably evolved to control microbial infections, unrestrained and indiscriminate T cell responses lead to host destructive pathologies such as inflammatory bowel disease (IBD), type 1 diabetes (T1D), rheumatoid arthritis (RA), and MS.

This paper results from the Arthur M. Sackler Colloquium of the National Academy of Sciences, "Microbes and Health" held November 2–3, 2009, at the Arnold and Mabel Beckman Center of the National Academies of Sciences and Engineering in Irvine, CA. The complete program and audio files of most presentations are available on the NAS Web site at http://www.nasonline.org/SACKLER_Microbes_and_Health.

Author contributions: Y.K.L. and S.K.M. designed research; Y.K.L. and J.S.M. performed research; Y.U. contributed new reagents/analytic tools; Y.K.L., J.S.M., and S.K.M. analyzed data; and Y.K.L., J.S.M., and S.K.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Y.K.L. and J.S.M. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: sarkis@caltech.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1000082107/-/DCSupplemental.

A primary and dominant mechanism to prevent deleterious self-reactions is mediated by regulatory T cells (Tregs) (10). Various subsets of $CD4^+$ Treg cells control organ-specific autoimmunity and are also induced at the site of infection to dampen immune responses after pathogen clearance. Microbial signals and the immune environment that they create during infection modulate the peripheral function of T cells. A series of recent studies now reveals that noninfectious symbiotic microbes fine tune $CD4^+$ T cell responses (11). Therefore, how the microbiota influences the T-helper/Treg axis seems to be a critical component of various immune-mediated diseases.

Recent studies have begun to reveal the extraordinary diversity and complexity of the ecosystem that we provide to microorganisms. Advances in genomic technologies have shown that humans harbor dozens of bacterial species in our stomachs, hundreds on our skin and oral cavity, and thousands within our lower gastrointestinal (GI) tract (12–14). The magnitude of these interactions and the evolutionary forces that drive them must exert profound influences on the biology of both microbe and man. Although the contribution of the microbiota to GI function is well-documented, recent speculations propose that gut bacteria may control extraintestinal biological functions. Herein, we investigate the concept that commensal microbes can influence immune responses distant from mucosal surfaces by examining the role of the microbiota on the onset and severity of EAE. We show that germ-free animals, devoid of all microbial colonization, develop significantly reduced neuro-immune disease symptoms and pathology and express lower levels of inflammatory cytokines associated with EAE compared with mice colonized with a complete microbiota. During EAE induction, germ-free animals display higher levels of $Foxp3^+$ Treg cells in various lymphoid tissues. Furthermore, germ-free mice induced to develop EAE display reduced Th1/Th17 proinflammatory responses in the CNS. The reduction in proinflammatory T cell responses seems to involve a defect in the innate immune system, because dendritic cells (DCs) from germ-free animals do not prime MOG-specific T cell activation as efficiently as DCs from colonized animals. To determine if specific microbial species can drive inflammation in the CNS, we colonized germ-free animals with segmented filamentous bacteria (SFBs), which are known to induce Th17 cells in the gut. Remarkably, SFB-monoassociated animals showed increased Th17 cell responses in both the colon and small intestine as well as within the spinal cords of animals. Most importantly, animals colonized intestinally with SFBs developed EAE on immunization with self-antigens. Collectively, these studies suggest that the microbiota contains organisms that direct both pro- and antiinflammatory immune responses in the CNS. Understanding the role of the microbiota in directing host immune responses may be critical in the design of rational therapies for MS.

Results

Germ-Free Mice Are Highly Resistant to the Development of EAE. To evaluate the role of the microbiota on EAE induction, C57BL/6 mice maintained under sterile (germ-free; GF) or specific pathogen-free (SPF) conditions were immunized with MOG peptide in complete Freund's adjuvant (CFA). This procedure expands and activates an antigen-specific T cell response that leads to inflammation targeted to the myelin sheath. Animals were monitored for signs of EAE, and disease onset and progression between groups were assessed by a standard scoring system (5). After disease induction, SPF animals displayed a typical course of disease, where animals exhibited symptoms beginning at day 10–12 and regressing by day 35 postimmunization (Fig. 1*A*). Remarkably, GF mice showed significantly attenuated signs of disease as shown by their EAE scores compared with SPF animals. With our immunization protocol, most SPF animals developed EAE, whereas only some GF animals showed any signs of disease (Fig. 1*B*), and those that did develop EAE had reduced clinical scores, reflected by milder paralysis and shorter duration of EAE symptoms. We next assessed disease onset by examining tissues for both an inflammatory response and pathology. During EAE, immune cells migrate into

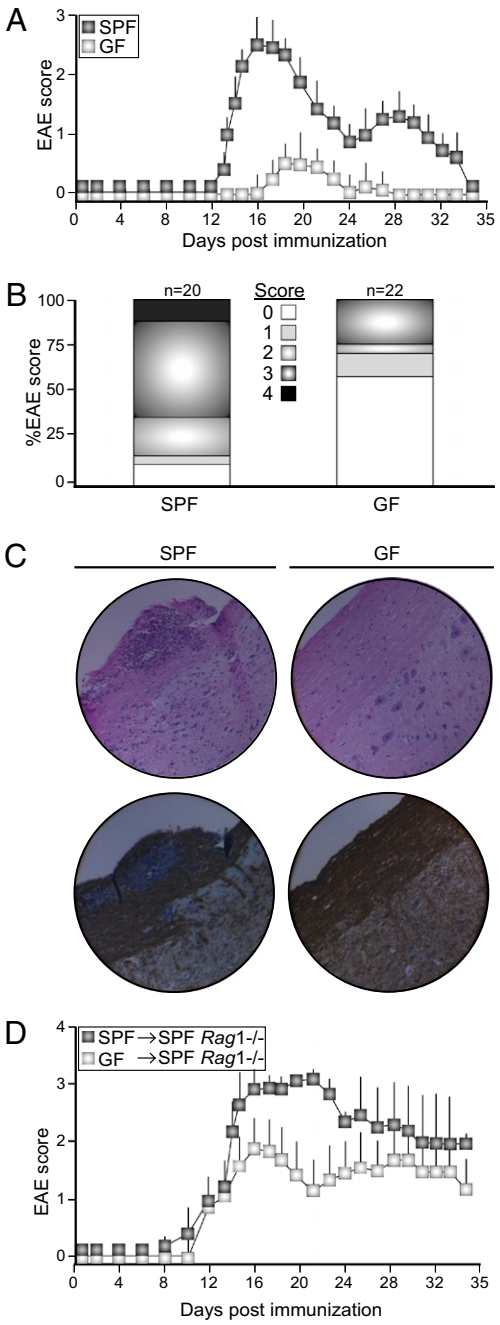

Fig. 1. GF mice develop less severe EAE than SPF mice. (*A*) Clinical EAE scores of SPF (dark symbols) and GF (light symbols) mice immunized with MOG peptide in CFA plus pertussis toxin. Data are representative of three independent experiments. Symbols represent the mean ± SD at each time point. (*B*) Proportions of SPF and GF mice that develop various degrees of EAE. Results are combined from four independent experiments, with a total number of animals per group of 20 for SPF and 22 for GF mice. (*C*) H&E (*Upper*) and myelin basic protein (*Lower*) staining of the spinal cord of SPF and GF mice at the onset of EAE on day 12. Each panel is representative of tissue analyzed from at least four mice. (*D*) EAE score of conventional $Rag^{-/-}$ recipient mice ($n = 4$) that were reconstituted with in vitro primed $CD4^+$ cells from SPF (black symbols) or GF (gray symbols) mice immunized with MOG/CFA and pertussis toxin. Data are representative of three independent experiments. Symbols represent the mean ± SD at each time point.

the CNS and mediate tissue destruction. Histological staining of the spinal cords of animals revealed that SPF mice had increased infiltration of leukocytes into the CNS and greater erosion of the myelin sheath compared with GF animals (Fig. 1*C*). Therefore, the basis for why GF animals do not develop disease seems to involve the absence of the inflammatory cascade in the CNS that is observed in SPF mice. These results indicate that the microbiota potentiates the induction of EAE.

CD4$^+$ T Cells from GF Animals Can Be Activated to Induce EAE. Because GF animals have deficits in the development of certain inflammatory T cell subsets (15, 16), the reduction in EAE development may reflect an inability to activate a proinflammatory self-reactive T cell response. To investigate whether self-reactive T cells from GF can induce EAE, lymphoid cells from MOG/CFA-immunized SPF or GF mice were harvested 8–10 d post-immunization and restimulated in vitro with MOG peptide to induce an antigen-specific inflammatory response. After in vitro stimulation with antigen, CD4$^+$ T cells were subsequently transferred into recombination activating gene (RAG)-deficient SPF mice (lacking T and B cells), and the development of EAE was monitored. Interestingly, CD4$^+$ T cells from GF animals retained the capacity to promote disease when stimulated under inflammatory conditions, albeit to a slightly lower degree than cells from SPF mice (Fig. 1*D*). Recipient animals adoptively transferred with CD4$^+$ T cells from GF mice developed EAE with kinetics similar to those of animals repopulated with T cells from SPF animals. However, the overall magnitude of disease in cells from GF donors was lower than that of SPF animals (although clearly increased from that of completely GF mice, as in Fig. 1*A*). Although the reason for this intermediate phenotype remains unclear, it may be because of incomplete microbial stimulation of cells by the SPF microbiota after transfer or an increased proportion of transferred Tregs from germ-free donor animals. In either case, our findings show that T cells from GF animals are not inherently unresponsive, suggesting that their inflammatory status is actively regulated by the microbiota. These results indicate that cells from GF mice can be reprimed on in vitro stimulation to induce EAE and that microbial interactions with the host immune system control inflammation in the CNS. Therefore, the microbiota dynamically and reversibly impacts the programming of pathogenic immune responses during autoimmunity.

Animals Resistant to EAE Display Reduced Proinflammatory Responses. EAE is driven largely by a proinflammatory T cell response that coordinates the infiltration of immune cells into the CNS. Various subsets of T-helper cells have been shown to potentiate disease, and both Th1 and Th17 reactions seem to be highly associated with the development of EAE. As mentioned earlier, infection by pathogens induce Th1 and Th17 immunity. These proinflammatory T-helper cells coordinate many aspects of the innate and adaptive immune response to effectively clear microbial invaders. Commensal bacteria also share the ability to activate these subsets of cells; however, it seems the microbiota activates/expands T-helper cells in a controlled manner without causing pathologic inflammation (17). If the microbiota augments our immune system by sustaining basal inflammatory T helper cells to better protect their host against infectious disease, would they also prime our self-immune system to autoimmunity? We, therefore, investigated if GF animals were reduced in their proinflammatory T cell responses on EAE induction. Animals were immunized with MOG/CFA, and cells were recovered from draining lymph nodes of SPF and GF mice before the onset of disease (day 8) and restimulated in vitro with MOG peptide. After 3 d of in vitro culture stimulation, the proinflammatory profile of CD4$^+$ T cells was assessed. Analysis of the canonical Th1 cytokine (IFN-γ) and the Th17 cytokine IL-17A by intracellular cytokine staining revealed increased proinflammatory T cells in SPF animals compared with GF mice (Fig. 2*A*). Accordingly, CD4$^+$IFNγ$^+$ T cell proportions are significantly decreased in GF mice on day 8 after immunization, before the initial signs of paralysis (Fig. 2*B*). ELISA analysis of cultured CD4$^+$ T cells from draining lymph nodes show that germ-free mice express lower levels of both IFNγ and IL-17A compared with animals with a complete microbiota (Fig. 2*C*). Similar results were found in the spleen during the onset of EAE (Fig. S1). Therefore, CD4$^+$ T cells from GF animals display a reduced systemic proinflammatory profile compared with cells from SPF animals. Cells cultured in the absence of MOG peptide under the same in vitro conditions resulted in no cytokine production, showing that the observed proinflammatory responses were antigen-specific. Lineage differentiation of Th17 cells is mediated by the orphan nuclear receptor retinoic acid–related orphan receptor (RORγt), a transcription factor that is required for IL-17A production. Furthermore, RORγt-deficient animals do not develop EAE, showing the importance of Th17 cells for the disease process (18). We, therefore, analyzed the expression of RORγt in the CNS of immunized SPF and GF animals. Consistent with the reduction in IL-17A levels, the transcript for RORγt is greatly decreased in CD4$^+$ T cells of the CNS of GF animals compared with SPF (Fig. 2*D*). Recent reports have shown that Th17 cells and RORγt expression are reduced in the GI tract of GF animals (19, 20); our data

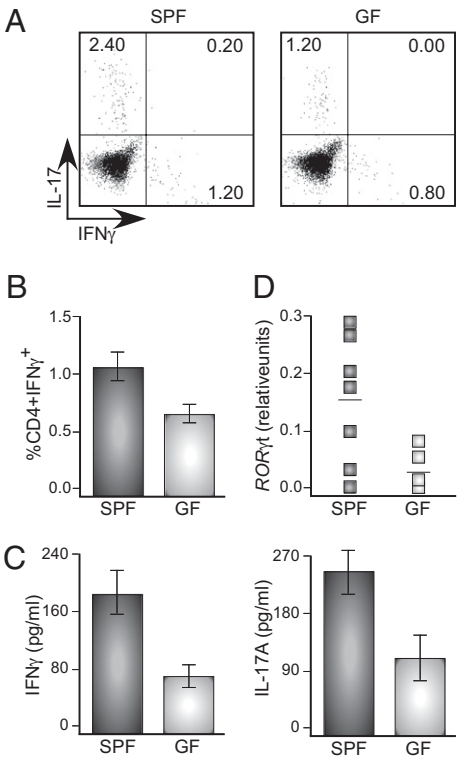

Fig. 2. Inflammation is attenuated in GF mice on EAE induction. (*A*) Lymphocytes were harvested from draining LN of treated SPF and GF mice at 8 d after immunization with MOG/CFA. Intracellular staining of CD4$^+$ T cell for IFNγ and IL-17A after 3 d in vitro culture with MOG peptide, restimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for the last 5 h. Numbers in each quadrant indicate percentage of cytokine-positive CD4$^+$ T cells. (*B*) Mean ± SD of the CD4$^+$IFNγ$^+$ T cell subsets from *A*. Data are representative of three independent experiments with at least four mice per group. (*C*) Real-time PCR of the transcription factor RORγt in cells isolated by Percoll gradient from spinal cords of SPF and GF mice at the onset of disease (day 8). Each symbol represents a single mouse. (*D*) IFNγ and IL-17A cytokine ELISA from cells harvested from draining LN of SPF and GF mice 8 d after immunization with MOG/CFA and cultured for 3 d in vitro with MOG peptide. Data are representative of three independent experiments, with mean ± SD of samples run in triplicate.

further these findings by revealing that both systemic and neuronal proinflammatory Th17 responses are reduced during EAE in the absence of the microbiota. Collectively, the diminished expression of Th1 and Th17 responses in GF mice provides a cellular explanation for the observation that microbial colonization of animals promotes EAE.

Increased Tregs During EAE Induction in GF Mice. A healthy immune system is designed to balance inflammatory responses to invading microbes with suppressive mechanisms that limit collateral damage to host tissues. Uncontrolled or excessive immune reactions to pathogens may lead to autoimmunity and other inflammatory diseases. Tregs provide a primary mechanism to suppress aberrant immune responses and promote protection from inflammatory disease (21). Many Tregs are marked by expression of CD4, CD25, and the transcription factor Foxp3 (Forkhead Box transcription factor for Treg differentiation). Very intriguingly, microbial influences on regulatory T cell function have emerged as a possible means by which beneficial bacteria may modulate host immune responses (8). Because $Foxp3^+$ Treg cells are able to suppress proinflammatory T-helper cells that drive autoimmunity such as EAE, we next investigated the levels of antiinflammatory Tregs during immune responses to MOG in GF and SPF mice. Cells from the draining lymph nodes and spleen were stimulated in vitro with MOG peptide 8 or 15 d after immunization, reflecting time points before and during the peak of EAE, respectively. Astonishingly, analysis by flow cytometry shows that proportions of $CD4^+CD25^+Foxp3^+$ Treg cells were increased in GF compared with SPF mice (Fig. 3). At both day 8 (Fig. 3 *A* and *B*) and day 15 (Fig. 3 *C* and *D*) after the induction of EAE, animals without microbial colonization contained significantly higher proportions of Treg cells. This phenotype seems once again to be antigen-specific, because no differences in $Foxp3^+$ Treg proportions were observed between groups when cells were cultured in the absence of MOG peptide (Fig. S2). These results show that although proinflammatory T cell responses to self-antigens are diminished, GF animals concomitantly display increased Treg cells in lymphoid organs during resistance to EAE.

Innate Immune Cells from GF Animals Are Defective in Activation of MOG-Specific T cells. Our results reveal that the microbiota influences gut–brain immune responses during EAE. We sought to determine the cellular nature for the inability of GF animals to mount autoreactive T cell responses. DCs are capable of priming various T-helper cell reactions in the gut (22). We, therefore, purified $CD11c^+$ DCs from the mesenteric lymph nodes (MLNs) of GF and SPF animals and cocultured these cells with MOG-specific T cells to measure IL-17A and IFNγ production in response to MOG peptide. Cytokine analysis revealed that after 3 and 5 d of culture, DCs from GF mice were defective in promoting IL-17A (Fig. 4*A*) or IFNγ (Fig. 4*B*) expression compared with DCs harvested from SPF animals. As expected, no cytokine production was detected from cocultures without MOG peptide stimulation, confirming specific T cell activation. Consequently, these results show that DCs from germ-free animals have a reduced capacity to induce both Th1 and Th17 cell response to self-antigens, consistent with reduced EAE development in animals without a microbiota.

Intestinal Colonization with SFB Promotes EAE. The absence of EAE in GF animals suggests that the microbiota stimulates proinflammatory immune responses that lead to inflammation and pathology in the CNS. Over the past few years, it has become evident that Th17 cells play a pivotal role in EAE development, once thought to be driven by Th1 cells only. Although the relationship between Th1 and Th17 cells during EAE is an active area of research, it seems reasonable to propose that members of the microbiota that promote Th17 cells could initiate the inflammatory cascade that ultimately leads to inflammation in the CNS. This notion has been supported by a series of reports that shows that GF animals are highly reduced in intestinal Th17 cells (19, 20). Furthermore, recent studies have now identified that specific members of the microbiota, SFBs, are uniquely able to induce Th17 cell differentiation in the small intestine (17, 23). We wondered if SFBs were also able to promote the Th17 cell lineage outside the gut and perhaps, restore the defect in EAE development found in germ-free mice. Groups of GF animals were either monocolonized with SFBs (GF-SFB) or left GF and were compared with conventionally colonized animals for EAE induction after immunization with MOG peptide. Remarkably, animals harboring intestinal SFBs alone were highly susceptible to EAE symptoms compared with GF mice (Fig. 5*A*). GF mice were delayed in the onset of disease compared with GF-SFB and conventionally colonized animals, and the severity of disease was significantly lower in GF mice relative to animals harboring SFBs. Although colonization with SFBs did not induce EAE to

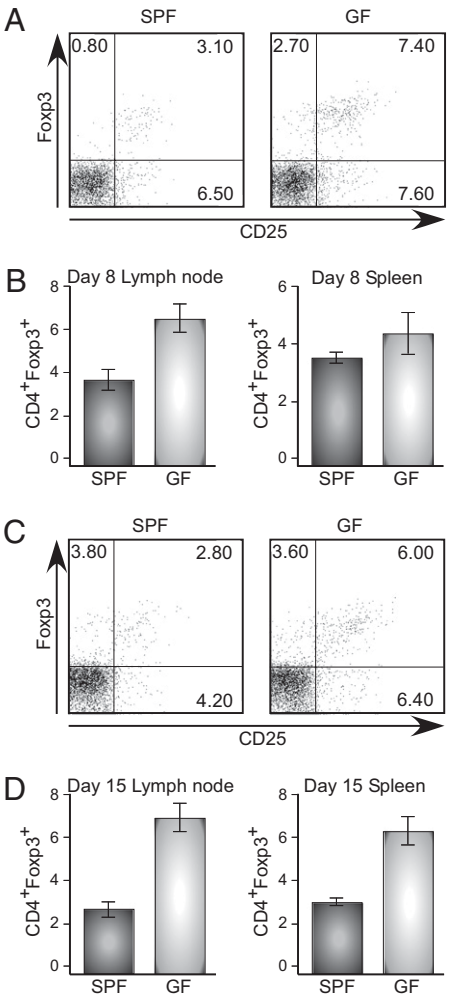

Fig. 3. Increase in $CD25^+Foxp3^+$ Treg cells after induction of EAE in GF mice. Intracellular staining of $CD4^+CD25^+Foxp3^+$ T cells cultured for 3 d with MOG peptide, restimulated with PMA/ionomycin for the last 5 h. Lymphocytes were harvested from draining lymph nodes (LN) and spleens of SPF and GF mice at 8 d p.i. (*A* and *B*) or 15 d p.i. (*C* and *D*) after i.v. immunization with MOG/CFA. In *A* and *C*, numbers in each quadrant indicate percentage of positive cells. In *B* and *D*, results are shown for mean ± SD of the $CD4^+$ subsets of the cells from draining LN and spleen on day 8 and day 15 post-immunization. Data are representative of three independent experiments with at least four mice per group.

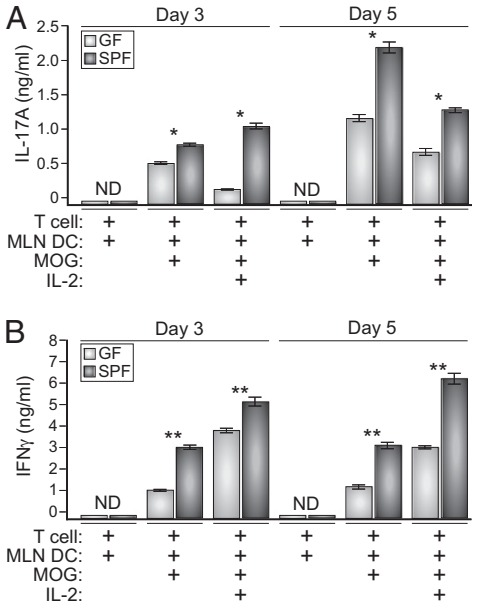

Fig. 4. DCs from GF animals are defective in inducing Th17 and Th1 responses from CD4+ T cells. Purified CD4+ T cells from MOG-Tg mice were cultured with MLN DCs from SPF or GF mice in the absence or presence of MOG peptide and IL-2. At day 3 and day 5 of culture, IL-17A (*A*) and IFNγ (*B*) cytokines were measured by ELISA. Data are representative of two independent experiments, with mean ± SD of samples run in triplicate. $*P < 0.05$; $**P < 0.005$.

the maximal level of SPF animals with a complex microbiota, GF-SFB animals showed pronounced disease compared with GF mice. These findings show that specific intestinal microbial species known to induce Th17 cells are sufficient to promote disease in the CNS. It is important to note that SFBs are not overt pathogens that cause intestinal inflammation and/or pathology (24). Therefore, our studies suggest that the immunomodulatory capacity of the microbiota extends to extraintestinal sites and highlights the importance of gut bacteria on immune responses throughout the body.

Microbiota Regulates Pro- and Antiinflammatory Responses in the Gut and CNS. To determine if colonization with SFBs results in restoration of T-helper cells that are missing in GF animals, we examined Th1 and Th17 cell phenotypes in the intestines and spinal cords of mice induced for EAE. Consistent with our findings above that Th1 and Th17 cells are reduced in the spleens of GF animals, we observed a significant decrease in IL-17A– and/or IFNγ-producing $CD4^+$ T cells in the small-intestinal lamina propria (SI LPL) in the absence of a microbiota (compare GF and SPF) (Fig. 5*B*). Remarkably, $CD4^+$ T cells harvested from the spinal cords of animals at the peak of disease also showed a decrease in IL-17A– and IFNγ-producing single-positive and double-positive cells. Thus, the microbiota influences immune responses in the CNS. Furthermore, monoassociation with SFBs (GF-SFB) resulted in a considerable increase in proinflammatory IL-17A and IFNγ production in the spinal cords (and intestines) of mice that succumbed to EAE. In agreement with previous studies that show that SFBs induce intestinal T-helper cell phenotypes during steady colonization (17, 23), these data show that IL-17A is also elevated in the gut during EAE. Furthermore, our findings reveal that intestinal colonization with SFBs promotes Th1, Th17, and double-positive T cells in the spinal cords of mice, providing evidence that gut-bacteria prime immune responses that extend to the CNS. We conclude that GF animals display highly reduced EAE symptoms because of a lack of immune stimulation by gut-bacteria.

We wanted to explore whether the balance between T-helper and Treg cells was also affected by SFBs. We have shown above that systemic proportions of $Foxp3^+$ Tregs are increased in GF animals, and others have shown a similar phenotype in gut tissues (19). Fig. 5*C* shows that, consistent with previous reports, $Foxp3^+$ Tregs are increased in the SI of GF mice compared with conventional colonization, and monoassociation with SFBs reduces the proportion of Tregs in the gut. However, when $CD4^+$ T cells of the CNS were examined during EAE, GF animals displayed a significant decrease in $Foxp3^+$ Tregs (as well as Th17 cells) (Fig. 5*C*, spinal cord). Colonization of GF animals with SFBs resulted in a Th17/Treg profile that was indistinguishable from conventionally colonized animals. It is still unclear whether colonized animals develop EAE because of increased T-helper cells (or their activity) or a deficiency in Treg function. However, during the peak of disease, GF animals have reduced pro- and antiinflammatory T cells in the CNS compared with colonized animals, revealing the profound effects of the microbiota immune responses in the CNS.

Numerous studies have shown that $Foxp3^+$ Treg cells prevent autoimmunity (25). It seems that microbial colonization may provide proinflammatory signals that affect the reciprocal development of T-helper and Treg cells. Moreover, because systemic and neuronal compartments of animals are devoid of microbes during normal commensal colonization, our findings support a model whereby microorganisms found at mucosal or environmentally exposed surfaces of the body affect immune responses that modulate inflammatory disease at extraintestinal anatomical locations. These studies reveal that the microbiota controls the T-helper/Treg axis outside the gut and suggest that immune stimulation by the microbiota may be a critical target for treatment of inflammatory diseases such as MS.

Perspective. Reflecting a growing medical crisis in Western societies, recent epidemiologic and clinical reports have revealed dramatic increases in the incidences of several immune disorders: IBD, asthma and allergies, T1D, RA, and MS (26). The hygiene hypothesis proposed over two decades ago speculated that these increases are the result of lifestyle changes and medical advances that reduce exposure to microbial pathogens. Microbial infections are, in fact, rare and opportunistic. In contrast, mammals are colonized for life with extraordinary multitudes of indigenous bacteria, and the contributions of this enormous and diverse ecosystem to human health remain poorly understood. Recent studies have launched a revolution in biology aimed at understanding how (and more importantly, why) mammals harbor symbiotic bacteria. We have recently shown that symbiotic intestinal bacterial molecules direct the development of the mammalian immune system and confer protection from intestinal disease (15, 27); thus, fundamental aspects of mammalian health are absolutely dependent on microbial symbiosis. For example, microbial diversity within the gut correlates to physiological states such as obesity and metabolic disease (28, 29). Furthermore, alterations in the community composition of the microbiota, known as dysbiosis, may be a critical factor in numerous immune-mediated diseases in humans (e.g., IBD, asthma, and allergies) (8).

It has been suggested for over a decade that microorganisms contribute to the pathogenesis of MS (30–35). However, findings have not been sufficient to establish an etiological or causal relationship between microbes and neurological inflammation. The role of gut bacteria in shaping GI immune responses is well-accepted, and studies have highlighted a key role for the commensal microbiota in the development of intestinal Th17 cells (11, 19, 20, 36). GF mice display very low numbers of Th17 cells within the LP of the intestine compared with SPF animals (19, 20). Moreover, specific species of bacteria and commensal bac-

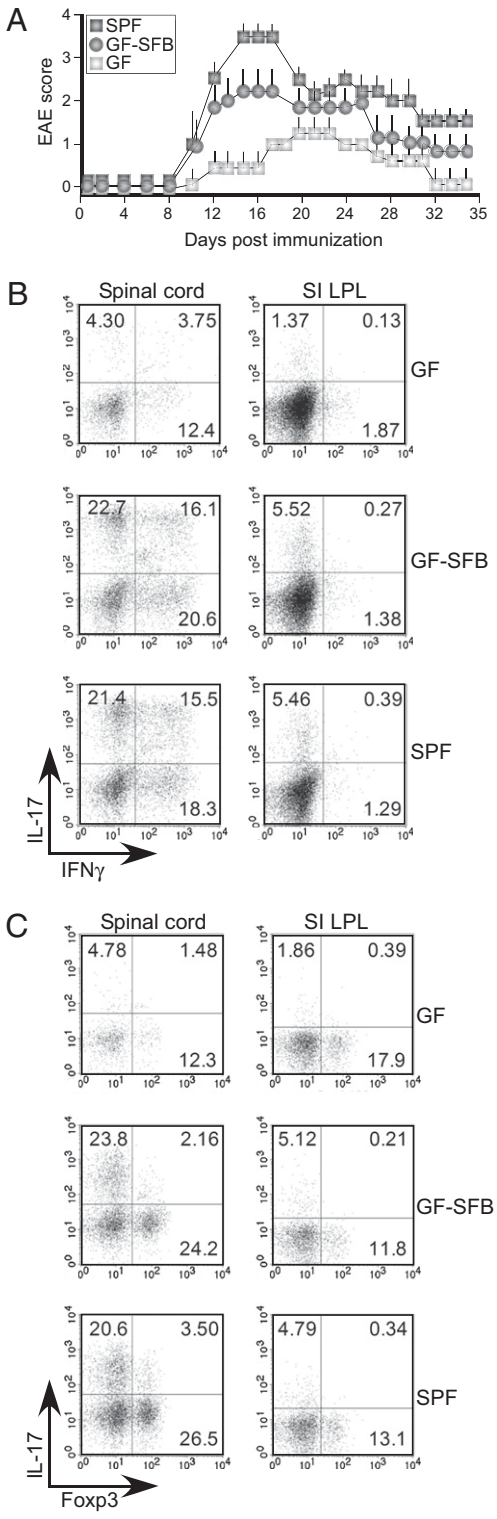

Fig. 5. SFBs promote proinflammatory T cell responses outside the gut during EAE. (*A*) Clinical EAE scores of SPF, GF, and GF-SFB colonized mice immunized with MOG-peptide in CFA plus pertussis toxin. GF mice were colonized with SFB for 3 wk before MOG/CFA immunization (GF-SFB). Data are representative of two independent experiments with at least four mice per group. Symbols represent the mean ± SD at each time point. (*B*) Representative FC plots of IL-17A– and IFNγ-producing $CD4^+$ T cells from LPLs of spinal cords and small intestines of SPF, GF, and GF-SFB colonized mice at day 15 after EAE induction. Numbers in each quadrant indicate percentage of cytokine-positive $CD4^+$ T cells. Data are representative of two independent experiments with at least four mice per group. (*C*) Representative FC plots of IL-17A– and Foxp3-producing $CD4^+$ T cells from LPLs of spinal cords and small intestines of SPF, GF, and GF-SFB colonized mice at day 15 after EAE induction. Numbers in each quadrant indicate percentage of positive $CD4^+$ T cells. Data are representative of two independent experiments with at least four mice per group.

terial molecules have been proposed to modulate Th17 differentiation (17, 19, 23, 37). Thus, the microbiota plays an active role in the development and function of inflammatory T-helper cell pathways in the gut. However, the direct affects of microbial colonization on extraintestinal inflammatory pathways remain largely unexplored. Recent reports show that the microbiota affects the onset of T1D (38) and RA (39) through mechanisms involving innate immune signaling by bacteria. These studies also reveal that GF animals display dramatically altered disease onset (for both T1D and RA) compared with SPF animals. We report herein that GF animals develop significantly reduced EAE, extending the cadre of autoimmune diseases that are modulated by the microbiota. Several studies have, however, reported the use of antibiotics in protecting animals from EAE (40–42). It is also noteworthy to mention that, in clinical trials studies, the antibiotic minocycline has shown initial promise as a therapeutic for human MS (43, 44). The target of the antibiotic (i.e., pathogen and/or commensal bacteria) is unknown. Ochoa-Reparaz et al. (42) have recently shown that oral (but not systemic) antibiotic treatment reduces EAE, specifically implicating gut bacteria in the disease process. The proposed mechanism involves the modulation of intestinal DC subsets that influence the function of $CD4^+CD25^+$ Treg cells. These studies show the dynamic nature of the interaction between gut bacteria and the immune system, because depletion of the microbiota can reprogram systemic immune responses. Although infections have been speculated for many years to play a role in human diseases (as posited by the hygiene hypothesis), increasing evidence now suggests that nonpathogenic microbes may profoundly affect several autoimmune diseases such as T1D, RA, and EAE.

We reveal that microbial signals from the gut promote inflammation in extraintestinal tissues. Remarkably, GF mice develop significantly less paralysis and histological signs of EAE, and concordantly, they display increased $Foxp3^+$ Treg proportions after induction of disease compared with SPF mice. Presumably, these $Foxp3^+$ Tregs suppress the increased Th1 and Th17 responses found in animals on colonization with commensal bacteria. The basis for why GF animals are resistant to EAE does not seem to be a defect in T-helper cell function, because $CD4^+$ T cells from GF animals can promote EAE after in vitro activation with self-antigen and subsequent transfer into naïve $RAG^{-/-}$ animals. Previously, it has been shown that autoreactive T cells (specific for PLP) exist in GF SJL mice; however, EAE development was never assessed in this study (45). This is consistent with our findings suggesting that the microbiota modulates the Th1/Th17 vs. Treg axis and may not influence the presence of autoreactive cells but rather modulates their immune status. Clearly, more work is needed to determine how autoreactive T cells escape negative selection in the thymus. However, once in the periphery, it seems that the microbiota plays a fundamental role in the activation state of T cells—even outside of the gut.

Because the balance between pro- and antiinflammatory responses is important during many diseases, understanding how the microbiota shapes immune responses (either in the intestine, the CNS, or elsewhere) is critical for human health. It has been well-documented that GF animals do not develop intestinal disease in animal models of colitis (8). This suggests that microbial stimulation is required for the immune responses during IBD, and specific (potentially pathogenic) members of the microbiota seem to activate a proinflammatory response that leads

to host pathology. However, it is also known that beneficial bacteria have evolved mechanisms to ameliorate intestinal inflammation and experimental colitis, and several probiotic therapies for IBD are in development (46). Perhaps, the same microbial influences reported for IBD may play a role during MS (i.e., specific microbes stimulate T-helper responses, whereas others prevent them). We propose the hypothesis that dysbiosis of the intestinal microbiota is an important factor in the development and/or severity of MS. Future studies must integrate the concept that immunologic responses outside the gut are influenced by microbial colonization, and accordingly, the design of therapies for MS may involve probiotic microorganisms that can modulate inflammation of the brain and CNS.

Materials and Methods

Animals. Female C57BL6/J, Rag$^{-/-}$, and MOG-Tg mice (Jackson Laboratory) were housed in a conventional or GF facility at Caltech. GF mice were maintained in sterile isolators and fed autoclaved food and water (Class Biologically Clean). Mice were screened weekly for contamination by bacterial plating and PCR as in ref. 15. GF mice were colonized with SFB by introduction of fecal pellets from SFB monocolonized mice (47). All procedures were performed according to guidelines of the Institutional Animal Care Committee at the California Institute of Technology.

Cell Transfer. Cells from spleen and lymph nodes (LN) of immunized mice were harvested at day 8 postimmunization (p.i.), and 5×10^6 cells were cultured with $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) and IL-12 for 48–72 h. The cultured cells were then separated into CD4$^+$ population using MicroBeads (Miltenyi Biotech) and analyzed for purity by flow cytometry (FC) before transfer (>98% CD4$^+$). Recipient mice were injected with 5×10^6 cells/mouse i.v. and monitored daily for clinical signs of EAE.

EAE Induction. Groups of SPF or GF mice at 8–10 wk of age were immunized s.c. with 150 μg $MOG_{35–55}$ peptide per mouse (Synthetic Biomolecules) and 200 μg of *Mycobacterium* tuberculosis H37Ra (Difco) divided among three sites on their backs. On the same day and 2 d later, they will be injected i.p. with 150 ng/mouse of pertussis toxin (List Biological) in 0.3 mL PBS. Disease scores were measured as follows: 1, tail paralysis; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hind and forelimb paralysis; 5, moribund/dead.

Cell Culture. Draining LN and spleen cells from groups of immunized SPF or GF mice were collected after immunization, resuspended in complete RPMI medium, and then incubated with media alone or the $MOG_{35–55}$ peptide.

Intracellular Cytokine Staining. Cells obtained from in vitro cultures were incubated for 4–5 h with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) plus brefeldin A (2 μg/mL; Sigma) for the final 2 h. Cell surfaces were stained with the appropriate fluorescence-labeled antibodies: anti-CD4 and anti-CD25. After surface staining, cells were washed and resuspended in Permeabilization-Fixation solution (BD Cytofix/Cytoperm kit; BD Pharmingen), and intracellular cytokine staining was performed with appropriate fluorescence-labeled antibodies: anti–IL-17A and anti–IFN-γ (eBioscience) according to manufacturer's protocol.

Cytokine ELISA. Supernatant obtained from in vitro cultures was analyzed for IL-17A and IFN-γ following the guidelines of the manufacturer's protocol (eBioscience).

Quantitative Analysis of mRNA. RNA was extracted using the RNEasy Qiagen kit following the manufacturer's protocol and was reverse-transcribed with oligo(dT) primer (BioRad) according to the manufacturer's protocol. The cDNA served as template for amplification of target genes as well as the housekeeping gene L32 by real-time PCR with universal PCR Master Mix (BioRad). Expression of target genes was calculated by comparison of relative levels after normalization to L32 expression.

CD4 T Cell Isolation and MLN DC Isolation. Spleens and LN were collected from SPF or GF mice, and single-cell suspensions were prepared by mechanical disruption. CD4$^+$ T cells were isolated using negative selection MicroBeads according to the manufacturer's protocol (Miltenyi Biotec). For ex vivo DC isolation, MLNs were digested with collagenase D (100 U/mL) and DNase I (20 μg/mL) for 30 min at 37 °C and then incubated for 5 min in PBS plus EDTA. CD11C$^+$ DCs from MLNs were enriched using CD11c MicroBeads according to the manufacturer's protocol (Miltenyi Biotec); 5×10^5 CD4$^+$ T cells were cultured with 10^5 MLN DCs in the absence or presence of 20 μg/mL $MOG_{35–55}$ peptide and 100 U/mL IL-2.

SI LPL Isolation. Intestines were dissected, Payer's patches were removed, and the epithelial layer was also removed by sequential incubation with HBSS containing DTT (154 μg/mL) followed by 2 μM EDTA. The remaining gut tissue was digested with 100 U/mL collagenase D and 20 μg/mL DNase I at 37 °C for 30 min with gentle agitation. Undigested tissue was removed by straining, cells were washed in HBSS, and total lamina propria cells were purified on 40/80% Percoll gradient.

Preparation of Spinal Cord Lymphocytes. Spinal cords were cut into pieces and digested for 30 min at 37 °C with collagenase D and DNase I followed by 40/80% Percoll gradient.

Statistical Analysis. *P* values were calculated by the two-tailed Student *t* test. Error bars in EAE score data represent the SD at each time point and were calculated using Prism software (GraphPad).

ACKNOWLEDGMENTS. We are grateful to members of the Mazmanian laboratory for their critical review of the manuscript. We thank Rochelle A. Diamond (Caltech) for help with cell sorting. S.K.M. is a Searle Scholar. This work is supported by funding from the California Institute of Technology, the Weston Havens Foundation, and the Edward Mallinckrodt Jr. Foundation (to S.K.M.).

1. Goverman J (2009) Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* 9:393–407.
2. Bhat R, Steinman L (2009) Innate and adaptive autoimmunity directed to the central nervous system. *Neuron* 64:123–132.
3. Becker KG, et al. (1998) Clustering of non-major histocompatibility complex susceptibility candidate loci in human autoimmune diseases. *Proc Natl Acad Sci USA* 95:9979–9984.
4. Willer CJ, et al. (2003) Twin concordance and sibling recurrence rates in multiple sclerosis. *Proc Natl Acad Sci USA* 100:12877–12882.
5. Stromnes IM, Goverman JM (2006) Active induction of experimental allergic encephalomyelitis. *Nat Protoc* 1:1810–1819.
6. Ascherio A, Munger KL (2007) Environmental risk factors for multiple sclerosis. Part I: The role of infection. *Ann Neurol* 61:288–299.
7. Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124:837–848.
8. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 9:313–323.
9. Bettelli E, et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235–238.
10. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T cells and immune tolerance. *Cell* 133:775–787.
11. Chow J, Mazmanian SK (2009) Getting the bugs out of the immune system: Do bacterial microbiota "fix" intestinal T cell responses? *Cell Host Microbe* 5:8–12.
12. Bik EM, et al. (2006) Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci USA* 103:732–737.
13. Gao Z, Tseng CH, Pei Z, Blaser MJ (2007) Molecular analysis of human forearm superficial skin bacterial biota. *Proc Natl Acad Sci USA* 104:2927–2932.
14. Gill SR, et al. (2006) Metagenomic analysis of the human distal gut microbiome. *Science* 312:1355–1359.
15. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 122:107–118.
16. Niess JH, Leithäuser F, Adler G, Reimann J (2008) Commensal gut flora drives the expansion of proinflammatory CD4 T cells in the colonic lamina propria under normal and inflammatory conditions. *J Immunol* 180:559–568.
17. Ivanov II, et al. (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139:485–498.
18. Ivanov II, et al. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121–1133.
19. Ivanov II, et al. (2008) Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4:337–349.
20. Atarashi K, et al. (2008) ATP drives lamina propria T(H)17 cell differentiation. *Nature* 455:808–812.
21. Sakaguchi S, et al. (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212:8–27.
22. Coombes JL, Maloy KJ (2007) Control of intestinal homeostasis by regulatory T cells and dendritic cells. *Semin Immunol* 19:116–126.
23. Gaboriau-Routhiau V, et al. (2009) The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. *Immunity* 31:677–689.

24. Talham GL, Jiang HQ, Bos NA, Cebra JJ (1999) Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun* 67:1992–2000.
25. Round JL, O'Connell RM, Mazmanian SK (2009) Coordination of tolerogenic immune responses by the commensal microbiota. *J Autoimmun* 34:J220–J225.
26. Bach JF (2002) The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 347:911–920.
27. Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 453:620–625.
28. Turnbaugh PJ, et al. (2009) A core gut microbiome in obese and lean twins. *Nature* 457:480–484.
29. Vijay-Kumar M, et al. (2010) Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. *Science* 328:228–231.
30. Brorson O, Brorson SH, Henriksen TH, Skogen PR, Schøyen R (2001) Association between multiple sclerosis and cystic structures in cerebrospinal fluid. *Infection* 29: 315–319.
31. Steiner G (1952) Acute plaques in multiple sclerosis, their pathogenetic significance and the role of spirochetes as etiological factor. *J Neuropathol Exp Neurol* 11:343–372.
32. Ahlqvist J, et al. (2005) Differential tropism of human herpesvirus 6 (HHV-6) variants and induction of latency by HHV-6A in oligodendrocytes. *J Neurovirol* 11:384–394.
33. Cohen JI (2000) Epstein-Barr virus infection. *N Engl J Med* 343:481–492.
34. Thacker EL, Mirzaei F, Ascherio A (2006) Infectious mononucleosis and risk for multiple sclerosis: A meta-analysis. *Ann Neurol* 59:499–503.
35. Stratton CW, Wheldon DB (2006) Multiple sclerosis: An infectious syndrome involving Chlamydophila pneumoniae. *Trends Microbiol* 14:474–479.
36. Zaph C, et al. (2008) Commensal-dependent expression of IL-25 regulates the IL-23-IL-17 axis in the intestine. *J Exp Med* 205:2191–2198.
37. Hall JA, et al. (2008) Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity* 29:637–649.
38. Wen L, et al. (2008) Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109–1113.
39. Abdollahi-Roodsaz S, et al. (2008) Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. *J Clin Invest* 118:205–216.
40. Yokote H, et al. (2008) NKT cell-dependent amelioration of a mouse model of multiple sclerosis by altering gut flora. *Am J Pathol* 173:1714–1723.
41. Melzer N, et al. (2008) A beta-lactam antibiotic dampens excitotoxic inflammatory CNS damage in a mouse model of multiple sclerosis. *PLoS ONE* 3:e3149.
42. Ochoa-Repáraz J, et al. (2009) Role of gut commensal microflora in the development of experimental autoimmune encephalomyelitis. *J Immunol* 183:6041–6050.
43. Zabad RK, et al. (2007) The clinical response to minocycline in multiple sclerosis is accompanied by beneficial immune changes: A pilot study. *Mult Scler* 13:517–526.
44. Zhang Y, et al. (2008) Pilot study of minocycline in relapsing-remitting multiple sclerosis. *Can J Neurol Sci* 35:185–191.
45. Anderson AC, et al. (2000) High frequency of autoreactive myelin proteolipid protein-specific T cells in the periphery of naive mice: Mechanisms of selection of the self-reactive repertoire. *J Exp Med* 191:761–770.
46. Sartor RB (2005) Probiotic therapy of intestinal inflammation and infections. *Curr Opin Gastroenterol* 21:44–50.
47. Umesaki Y, Okada Y, Matsumoto S, Imaoka A, Setoyama H (1995) Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol Immunol* 39: 555–562.