Gut dysbiosis breaks immunological tolerance toward the central nervous system during young adulthood

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Multiple sclerosis (MS) is an autoimmune disease targeting the central nervous system (CNS) mainly in young adults, and a breakage of immune tolerance to CNS self-antigens has been suggested to initiate CNS autoimmunity. Age and microbial infection are well-known factors involved in the development of autoimmune diseases, including MS. Recent studies have suggested that alterations in the gut microbiota, referred to as dysbiosis, are associated with MS. However, it is still largely unknown how gut dysbiosis affects the onset and progression of CNS autoimmunity. In this study, we investigated the effects of age and gut dysbiosis on the development of CNS autoimmunity in humanized transgenic mice expressing the M5-associated MHC class II (MHC-II) gene, HLA-DR2α, and T-cell receptor (TCR) genes specific for MBP87-99/DR2α that were derived from an MS patient. We show here that the induction of gut dysbiosis triggers the development of spontaneous experimental autoimmune encephalomyelitis (EAE) during adolescence and early young adulthood, while an increase in immunological tolerance with aging suppresses disease onset after late young adulthood in mice. Furthermore, gut dysbiosis induces the expression of complement C3 and production of the anaphylatoxin C3a, and down-regulates the expression of the Foxp3 gene and anergy-related E3 ubiquitin ligase genes. Consequently, gut dysbiosis was able to trigger the development of encephalitogenic T cells and promote the induction of EAE during the age window of young adulthood.

Significance

Multiple sclerosis (MS) is classified as an autoimmune disease of the central nervous system (CNS). Alterations of gut microbiota (gut dysbiosis) are frequently observed in MS patients. It is still unknown how gut dysbiosis contributes to development of MS. We report here that gut dysbiosis, which we attribute to expansion of enteric pathogenic bacteria, triggers and/or exacerbates the spontaneous development of experimental autoimmune encephalomyelitis, an animal model of MS. This occurs during the period of young adulthood by reducing development of Foxp3+ Treg cells and expression of E3 ubiquitin ligase genes involved in protection from autoimmune diseases. This study suggests that gut dysbiosis may play a pathological role in the initiation and/or progression of MS during a defined age window.


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Altered in the composition and function of the gut microbiota, referred to as dysbiosis, may be involved in the initiation and exacerbation of MS through the development of pathogenic T cells and suppression of Treg cells in the gastrointestinal (GI) tract and extra-GI organ systems (20–24). Dysbiosis can result from invasive intestinal pathogens, antibiotic treatment, physical damage to the mucosa, or host genetic factors, which lead to an overgrowth of harmful, minor microbial populations and a concomitant decrease in health-promoting bacteria (25). Imbalance of the gut flora has been implicated in the development of a variety of autoimmune diseases, including inflammatory bowel disease, type 1 diabetes, and MS, through the skewing of immune cells toward a proinflammatory phenotype (21–24). The “beneficial” enteric bacteria can promote the development of Treg cells (26–28); however, it has been suggested that a disturbed balance between beneficial and pathogenic enteric bacteria caused by gut dysbiosis may induce immune dysregulation and subsequently increase the risk of developing autoimmune diseases (29–31). However, it is still largely unknown how gut dysbiosis impacts CNS immune tolerance and whether gut dysbiosis may directly alter immune factors that disturb this process.

In this study, we show that gut dysbiosis breaks immunological tolerance to the myelin antigen, myelin basic protein (MBP), through the down-regulation of Foxp3 and E3 ubiquitin ligase genes during the period of adolescence and early young adulthood, after age-associated tolerance induction suppresses CNS autoimmunity after late young adulthood using humanized transgenic (Tg) mice that express MS-associated HLA-DR2a and T-cell receptor (TCR) genes specific for MBP87-99/HLA-DR2a (32).

**Results**

**Age-Dependent Development of Spontaneous Experimental Autoimmune Encephalomyelitis.** To examine how MBP-specific T cells can spontaneously differentiate into encephalitogenic T cells, we created Tg mice that express an MS-associated HLA-DR2a gene and TCR genes specific for MBP87-99/HLA-DR2a that were isolated from a 3A6 T-cell clone derived from an MS patient (referred to here as 3A6/DR2a Tg mice) (32). In our specific pathogen-free (SPF) facility at Rutgers–Robert Wood Johnson Medical School (Rutgers-RWJMS), ~26.5% of 3A6/DR2a Tg mice develop experimental autoimmune encephalomyelitis (EAE), with a wide clinical spectrum of disease severity (Table S1). Notably, EAE developed most frequently between 5 and 8 wk of age (Fig. 1A). Outside of the peak 5–8 wk of age, however, the frequency of EAE gradually declined, and EAE could not be observed after 18 wk of age. Furthermore, these Tg mice displayed chronic EAE as indicated by an invariant clinical score after the peak of disease (Fig. 1B). These data suggest that age is an important factor in the incidence of spontaneous EAE in 3A6/DR2a Tg mice.

**Age-Associated Induction of Central and Peripheral Tolerance in MBP-Specific T Cells.** Since CD4+ T cells play a prominent role in the initiation of EAE, we examined age-dependent changes in 3A6-TCR Tg CD4+ T-cell development in the thymus and spleen. We examined Tg mice at varying age groups: childhood (3–4 wk of age), adolescent and early young adult (5–10 wk of age), late young adult (11–20 wk of age), and middle-aged (21–35 wk of age) in non-EAE mice. Flow cytometric analyses of the thymus revealed that 3A6-TCR Tg CD4+CD8− T-cell (populace of Vβ5.1, CD3+CD4+CD8− cells) development is inefficient in 3- to 4-wk-old Tg mice but that it becomes efficient in 5- to 10-wk-old Tg mice (Fig. 2A and B). Furthermore, the development of 3A6-TCR Tg CD4+CD8− T cells is significantly decreased along with a massive reduction in CD4+CD8− double-positive T cells in 21- to 35-wk-old Tg mice (3.3%), and the population of MBP-specific CD4+ T cells (Vβ5.1+CD3+CD4+CD8− cells) within the CD4+CD8− T-cell compartment decreased in 21- to 35-wk-old mice (39.7%) (Fig. 2A). In the spleen, the number of Vβ5.1+CD3+CD4−CD8− T cells was low in 3- to 4-wk-old Tg mice but increased in 5- to 10-wk-old Tg mice, which reflects a similar observation in the thymus. However, cell number of Vβ5.1+CD3+CD4+CD8− T cells did not change in 11- to 20-wk-old Tg mice compared with 5- to 10-wk-old Tg mice, although thymocytes were reduced in 11- to 20-wk-old Tg mice. This could be because of homeostatic T-cell proliferation in the periphery (12, 13). However, 3A6-TCR Tg CD4+ splenic T cells were reduced in 21- to 35-wk-old Tg mice (Fig. 2).

We next assessed the effect of aging on cellular proliferation within the peripheral T-cell compartment in response to MBP87-99 peptide stimulation. Splenocytes isolated from non-EAE 3A6/DR2a Tg mice of various ages were cultured with varying concentrations of MBP87-99 peptide, and proliferation was measured by 3[H]-thymidine uptake. Proliferation in response to MBP87-99 peptide stimulation increased in 3A6/DR2a Tg mice over 95% of naïve and 65% of memory/effector splenic T cells of 21- to 35-wk-old mice, which reflects a similar observation in the thymus. However, cell number of Vβ5.1+CD3+CD4+CD8− T cells did not change in 11- to 20-wk-old Tg mice compared with 5- to 10-wk-old Tg mice, although thymocytes were reduced in 11- to 20-wk-old Tg mice. This could be because of homeostatic T-cell proliferation in the periphery (12, 13). However, 3A6-TCR Tg CD4+ splenic T cells were reduced in 21- to 35-wk-old Tg mice (Fig. 2).

**Fig. 1.** An age window exists for the development of spontaneous EAE. (A) Age-dependent onset of spontaneous EAE. Frequency of spontaneous EAE and disease severity are shown in Table S1. (B) Clinical EAE score after onset of disease. EAE score was examined for 6 wk after disease onset. Disease courses for mice with mild EAE (lower than 2.0 of mean score) and mice with severe EAE (higher than 2.0 of mean score) are shown. Mild EAE mice (n = 17) and severe EAE mice (n = 12). The data points are means ± SEM.

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**Fig. 2.** A, B: Age-dependent changes in the development of MBP-specific T cells. Time 0 is set to age 3 wk for all age groups. (A) Flow cytometric analysis of thymocytes from 3A6/DR2a Tg mice of various ages. (B) Flow cytometric analysis of splenocytes from 3A6/DR2a Tg mice of various ages.
Treg cells in the CD4 T-cell compartment and reduced T-cell compartment was high in 3- to 4-wk-old mice (Fig. 3F). Thus, 3A6-TCR Tg T cells respond to cognate MBP antigen more vigorously during the 5- to 10-wk-old period when spontaneous EAE develops most frequently, whereas these cells appear to have undergone anergy during the 21- to 35-wk-old period when EAE is not observed.

Young Adulthood Is the Most Risky Period for the Development of Pathogenic T Cells. We next examined the effect of aging on the development of Foxp3+ Treg cells in the periphery by flow cytometry analysis. Notably, Foxp3+ Treg cell development within the CD4+ 3A6-TCR Tg T-cell compartment was high in 3- to 4-wk-old mice and 21- to 35-wk-old mice but low in both 5- to 10-wk-old mice and 11- to 20-wk-old mice (Fig. 4A and B). Mice with already established EAE exhibited an even lower population of Foxp3+ Treg cells in the CD4+ T-cell compartment compared with 5- to 10-wk-old mice.

We next examined production of the proinflammatory cytokines GM-CSF, IL-17A, and IFN-γ. The splenocytes isolated from 5- to 10-wk-old Tg mice produced the highest levels of GM-CSF and IL-17A, similar to those of EAE mice, which declined with age (Fig. 4C). However, we could not observe a significant difference in IFN-γ production by splenic T cells isolated from 5- to 10-wk-old Tg mice, 11- to 20-wk-old Tg mice, and 21- to 35-wk-old Tg mice. Interestingly, 3- to 4-wk-old Tg mice barely produced any proinflammatory cytokines.

These data suggest that diminished CNS autoimmunity in 21- to 35-wk-old Tg mice could be caused by age-associated tolerance induction that is also mediated by an increased frequency of Foxp3+ Treg cells in the CD4+ T-cell compartment and reduced production of IL-17 and GM-CSF. Therefore, young adulthood is the most risky period for the development of CNS autoimmunity in 3A6/DR2a Tg mice.

Gut Dysbiosis During Young Adulthood Triggers CNS Autoimmunity. The next question was how immune tolerance to MBP is broken during young adulthood in this MS animal model. Since it has been suggested that microbial infection breaks immune tolerance (20, 33), 3A6/DR2a Tg mice were treated with antibiotics, and their development of spontaneous EAE was examined. Treatment of 3A6/DR2a Tg mice with antibiotics dramatically prevented the onset of spontaneous EAE (Fig. 5A). We next examined whether dysbiosis of the gut microbiota plays a role in the onset of EAE. IgM is produced in response to gut microbiota stimuli, elevated in instances of gut inflammation, and suggested to exclude expanding microbial communities from dissemination (34, 35). Therefore, IgM levels in the feces are likely to indicate a dysbiotic gut microbiome. To examine the association of fecal IgM with spontaneous EAE, fecal IgM levels were measured in non-EAE and spontaneous EAE mice. Interestingly, we found an association between high levels of fecal IgM production and the incidence of spontaneous EAE compared with non-EAE mice (Fig. 5B). To further assess the time course in production of fecal IgM and the development of EAE, 3A6/DR2a Tg mice were monitored for disease development through the ages of 3-15 wk old. Fecal IgM was highly elevated 1–2 wk before the onset of spontaneous EAE (Fig. 5C and D).

To then examine the effect of gut dysbiosis on the development of spontaneous EAE, we created dysbiosis-free 3A6/DR2a Tg mice. Since mouse colonies from The Jackson Laboratory have a healthy gut microbiota and are free of dysbiosis (36), IgM-low 3A6/DR2a (IgM-lo 3A6/DR2a) Tg mice were established by rederivation using C57BL/6J mice purchased from The Jackson Laboratory as foster mothers and housed in an isolator within our animal facility. Notably, fecal IgM levels were significantly low in...
the rederived 3A6/DR2a Tg mice compared with nonrederived mice (Fig. 5E), and none of the rederived IgM-lo 3A6/DR2a Tg mice developed EAE spontaneously (Fig. 5F). Taken together, these data suggest that an increase in fecal IgM levels is a biomarker of spontaneous EAE in 3A6/DR2a Tg mice.

Next, we examined the gut microbial composition in rederived (IgM-lo), IgM-lo, IgM-hi non-EAE, and IgM-hi EAE mice. Illumina 16S rRNA sequence analysis indicated that the family Bacteroidaceae was significantly higher in fecal IgM-hi non-EAE mice compared with rederived (IgM-lo) mice, and even higher in IgM-hi EAE mice.
Gut Dysbiosis Increases Gut Leakiness and Endotoxin Levels in the Periphery. Pathogen-associated molecular patterns (PAMPs), like endotoxin derived from microbes, can up-regulate the expression of complement C3, which is associated with autoimmunity (42, 43). We therefore evaluated the expression of the complement C3 gene in the spleens of 3A6/DR2a Tg mice. The expression of complement C3 was up-regulated in the spleens of IgM-hi non-EAE mice and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 7A). In accordance with increased intestinal permeability, blood endotoxin levels were markedly elevated in IgM-hi non-EAE and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 7B). These data suggest that gut dysbiosis can induce gut barrier leakiness.

**Gut Dysbiosis Up-Regulates the Expression of Complement C3 in the Periphery.** Pathogen-associated molecular patterns (PAMPs), like endotoxin derived from microbes, can up-regulate the expression of complement C3, which is associated with autoimmunity (42, 43). We therefore evaluated the expression of the complement C3 gene in the spleens of 3A6/DR2a Tg mice. The expression of complement C3 was up-regulated in the spleens of IgM-hi non-EAE mice and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 7A). In addition, the expression of complement C3 correlated with p-production of the complement C3, which is associated with autoimmunity (42, 43). We therefore evaluated the expression of the complement C3 gene in the spleens of 3A6/DR2a Tg mice. The expression of complement C3 was up-regulated in the spleens of IgM-hi non-EAE mice and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 7A). In accordance with increased intestinal permeability, blood endotoxin levels were markedly elevated in IgM-hi non-EAE and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 7B). These data suggest that gut dysbiosis can induce gut barrier leakiness.

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mice (Fig. 8C). These data suggest that gut dysbiosis can induce gut barrier leakiness and complement activation in the extra-GI immune system.

**Gut Dysbiosis Down-Regulates Expression of Foxp3 and E3 Ubiquitin Ligase Genes.** The down-regulation of E3 ubiquitin ligase genes is common in autoimmune diseases, including MS (46, 47). Therefore, we investigated the effect of gut dysbiosis on the expression of E3 ubiquitin ligase genes. The expression of the E3 ubiquitin ligase gene, Cbl-b, was significantly down-regulated in CD4+CD25− T cells of fecal IgM-hi non-EAE and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 8D). In addition, up-regulation of the complement C3 gene was inversely correlated with expression of the E3 ubiquitin ligase genes Cbl-b, Itch, and Grail (Fig. 8E). Particularly, down-regulation of Cbl-b was highly associated with up-regulation of complement C3.

Since the development of Foxp3+ Treg cells can be highly affected by the expression of E3 ubiquitin ligase and complement C3 genes (48–51), we next investigated the effect of gut dysbiosis on the development of Foxp3+ Treg cells. The development of Foxp3+ Treg cells was reduced in fecal IgM-hi non-EAE and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 9A and B). Interestingly, the down-regulation of Foxp3 genes in CD4+CD25+ T cells was inversely correlated with expression levels of the complement C3 gene (Fig. 9C). As a consequence of reduced Foxp3+ Treg development, the development of Th17 and GM-CSF Th cells was increased in fecal IgM-hi non-EAE and IgM-hi EAE mice compared with IgM-lo (rederived) mice (Fig. 9D and E). These data suggest that gut dysbiosis-mediated down-regulation of Foxp3 and E3 ubiquitin ligase genes could be involved in the breakage of immunological tolerance to CNS antigens.

**Discussion**

Although the etiology of MS is still unknown, multiple factors, including genetic, environmental, and aging factors, are mutually involved in the development of MS. Among genetic factors, HLA is the primary MS-associated gene. Therefore, we created Tg mice expressing the MS-associated HLA-DR2a gene and an MBP87–99/DR2a-specific 3A6 TCR gene isolated from an MS patient to investigate the aforementioned risk factors in CNS autoimmunity. We show here that gut dysbiosis breaks immunological tolerance to MBP through the up-regulation of complement C3 and subsequent down-regulation of Foxp3 and anergy-related E3 ubiquitin ligases genes during the age window of adolescence and young adulthood (5–10 wk old), thus highlighting a possible etiological role of gut dysbiosis in the development of CNS autoimmunity.

Age-associated thymic involution has been suggested to be involved in the development of autoimmunity. Although CD4+CD8− Fig. 6. Identification of enteric bacteria expanding in spontaneous EAE mice. Fecal IgM levels were examined by ELISA every week, and fecal DNA was isolated from rederived, fecal IgM-lo (<2.0 ng/mg total protein) non-EAE, fecal IgM-hi (≥2.0 ng/mg total protein) non-EAE, and IgM-hi EAE 3A6/DR2a Tg mice at different ages. Enteric bacterial families (A) and species (B) were identified by Illumina 16S rRNA sequence analysis: <5 wk old (n = 3–4), 5–10 wk old (n = 5–7), 11–20 wk old (n = 4–6), 21–35 wk old (n = 5–6).

**Fig. 7.** Gut dysbiosis promotes intestinal permeability and high endotoxin levels in blood circulation. (A) Increase in intestinal permeability in fecal IgM-hi non-EAE and IgM-hi EAE mice. FITC-dextran in the serum was measured 4 h after oral inoculation of FITC-dextran in fecal IgM-lo (rederived), fecal IgM-hi non-EAE, and IgM-hi EAE mice. **(P < 0.01; ***P < 0.001.

![Diagram](image-url)
naïve T cells can egress from the thymus and enter peripheral lymphoid organs, recent thymic emigrants are not fully functional until the age of adolescence (52, 53); therefore, the differentiation of autoreactive 3A6-TCR Tg T cells into encephalitogenic T cells is less efficient during childhood. Thymic involution begins during puberty (4–6 wk old) because of increased sex steroid hormones and consequently, leads to a decline in T-cell output to periphery from the thymus. However, the overlap peripheral T-cell population is largely maintained because of homeostatic T-cell proliferation in the periphery (12, 13). Indeed, cell number of Vβ5.1+CD3+CD4+CD8− T cells did not change in the spleens of 11- to 20-wk-old Tg mice compared with 5- to 10-wk-old Tg mice, even if thymocytes were reduced in 11- to 20-wk-old mice (Fig. 2B). However, 3A6-TCR Tg CD4+ splenic T cells were reduced in 21- to 35-wk-old Tg mice (Fig. 2B). This could be caused by massive T-cell deletion in the thymus and an age-dependent reduction of homeostatic proliferation as reported previously (54). Importantly, naïve T cells can differentiate into effector/memory T cells via homeostatic proliferation, which occurs in response to thymic involution and the reduction of T-cell egress from the thymus. Hence, these observations in combination with our findings of efficient 3A6-TCR Tg T-cell selection (Fig. 2), ability to proliferate in response to MBP (Fig. 3), and production of higher levels of proinflammatory cytokines (Fig. 4C) during adolescence/young adulthood suggest that this age window is the most risky period for encephalitogenic T-cell development. Likewise, the deletion of 3A6-TCR Tg T cells in the thymus after middle age (20 wk of age) (Fig. 2) and age-related anergy induction among surviving 3A6-TCR Tg T cells via...
the up-regulation of E3 ubiquitin ligase genes (Fig. 3F) also support adolescence/young adulthood as a high-risk period for encephalitogenic T-cell development. In addition, we observed an increase in Foxp3+ Treg cells with age (Fig. 4 A and B). Since Foxp3+ Treg cells are more resistant to thymic and peripheral deletion (55, 56), the Foxp3+ Treg compartment among CD4+ T cells increases after 20 wk of age. As a result, the efficiency of both intrinsic and extrinsic tolerance induction increases with age, given that the development of MBP-specific Th1, Th17, and Th GM-CSF is most efficient in 5- to 10-wk-old 3A6/DR2a Tg mice compared with IgM-lo (rederived) 3A6/DR2a Tg mice (39, 40) (Fig. 6B, Dataset S2, and Fig. S3). In addition, A. muciniphila, which is known to promote intestinal integrity and mucosal tolerance, was low in relative abundance in IgM-hi EAE mice compared with IgM-lo (rederived) 3A6/DR2a Tg mice (39, 40) (Fig. 6B, Dataset S2, and Fig. S3). Since immune tolerance is less efficient during adolescence and young adulthood in 3A6/DR2a Tg mice, gut dysbiosis during this period increases the risk of CNS autoimmunity. Interestingly, an exposure to environmental risk factors during or before adolescence has been reported to be associated with the development of MS (61).

Intestinal permeability is a well-known phenomenon caused by gut dysbiosis (41). A recent study also reported that adoptive transfer EAE with myelin-specific T cells induced a leaky gut (62), suggesting that activated myelin-specific T cells may also induce intestinal permeability and thus, affect gut dysbiosis. Since gut dysbiosis-free 3A6/DR2a Tg mice did not develop spontaneous EAE, gut dysbiosis could trigger CNS autoimmunity in 3A6/DR2a Tg mice through an increase in intestinal permeability and subsequent modulation of systemic immune functions. Indeed, we observed increased intestinal permeability in fecal IgM-hi mice that do not develop EAE (Fig. 7A). However, intestinal permeability was further increased on EAE development (Fig. 7A). Therefore, intestinal permeability could be initially induced
by gut dysbiosis and may be further worsened on EAE development in 3A6/DR2a Tg mice.

Complement C3 activation leads to the generation of its most potent effector molecules: the anaphylatoxins C3a and C5a. These anaphylatoxins can cooperate with Toll-like receptor signaling in the development of activated monocytes/macrophages and dendritic cells to promote the differentiation of encephalitogenic Th17 cells (63–65). Notably, complement C3 has been suggested to contribute significantly to the pathogenesis of EAE (66–68), and the activation and up-regulation of C5 in plaques, the cerebrospinal fluid, and systemic circulation have been shown in MS (69–71).

In this study, we show that dysbiosis-induced up-regulation of complement C3 in DCs outside of the GI tract (Fig. 8 B and C) and gut dysbiosis C5 up-regulation are correlated with a reduction of Foxp3+ Treg cells (Fig. 9 A–C). This reduction of Foxp3+ Treg cells could be caused by the down-regulation of Foxp3 gene expression through the binding of C3a to its associated receptor expressed on Foxp3+ Treg cells as reported previously (51). Therefore, the development of complement C3-hi DCs caused by gut dysbiosis may increase the risk of autoimmunity.

While it is unknown how gut dysbiosis up-regulates the expression of complement C3 in the immune system outside the GI tract, there are several possible mechanisms. Since DCs migrate into lymphoid organs outside of the gut under steady-state conditions (72), intestinal DCs expressing high levels of complement C3 caused by gut dysbiosis may migrate into lymphoid organs outside of the gut and promote proinflammatory Th cell differentiation or potentially skew unstable Foxp3+ Treg populations toward a proinflammatory Th phenotype (73, 74). Alternatively, the dysbiosis-induced overgrowth of pathobionts may promote intestinal inflammation, leading to intestinal permeability. Increased intestinal permeability is mainly caused by the disruption of tight junction proteins (75, 76) and leads to the passage of microbiota-derived PAMPs into the bloodstream, resulting in the activation of innate immunity and the complement pathway (63, 77). Indeed, intestinal permeability was increased and blood endotoxin levels were elevated in fecal IgM-hi non-EAE and IgM-hi EAE mice in our MS animal model (Fig. 7). Therefore, PAMPs derived from gut microbiota may up-regulate complement C3 in lymphoid organs outside of the GI tract on gut dysbiosis.

CBLB protein levels decrease significantly in the peripheral blood mononuclear cells of relapsing MS patients, and Cbl-b gene expression is correlated inversely with the frequency of MS relapses (47, 78). Furthermore, a decrease in Cbl-b and Itch expression is correlated with down-regulation of the Foxp3 gene in CD4+ CD25hi T cells in MS patients (46). Interestingly, we found that the expression of E3 ubiquitin ligase genes, Cbl-b and Itch, was down-regulated in CD4+CD25+ T cells on gut dysbiosis (Fig. SD). Since Cbl-b and Itch play an essential role in energy by ubiquitylating crucial T-cell signaling molecules, a reduction of E3 ubiquitin ligase can increase the risk of autoimmunity (79–82). In addition, as Cbl-b and Itch play an integral role in TGF-β signaling and the generation of Foxp3+ Treg precursors, a reduction in E3 ubiquitin ligase gene expression can also lead to a decrease in the development of inducible Foxp3+ Treg cells (48–50). Other than this, Itch expression in CD4+CD25− T effector cells is required for the suppressive action of CD4+CD25+ Tregs (50). Therefore, the dysbiosis-mediated production of anaphylatoxin C3a may affect the expression of Cbl-b and Itch genes. Since activation of NF-κB is involved in suppression of Cbl-b expression (83), NF-κB activation through the binding of anaphylatoxin C3a to its receptor may down-regulate the expression of the Cbl-b gene. Additional experiments are needed to explore the mechanism underlying the regulation of the E3 ubiquitin ligase genes by gut dysbiosis.

In summary, we show that gut dysbiosis during the age window of adolescence and young adulthood increases the risk of CNS autoimmunity by the up-regulation of complement C3, which may influence the down-regulation of Foxp3 and E3 ubiquitin ligase genes, and thereby suppresses tolerogenic mechanisms operating within the adaptive immune compartment. Importantly, inflammation-promoting stimuli that are driven by dysbiosis may be especially involved in autoimmune pathogenesis during the adolescent and young adulthood period, as this may be the age window wherein the development of pathogenic, self-antigen–specific adaptive immune responses is most efficient. Thus, our data suggest that gut dysbiosis during a young age window could play a pathological role in the initiation and progression of MS.

Materials and Methods
Animals. All experiments were carried out in compliance with Rutgers Institutional Animal Care and Use guidelines (Institutional Animal Care and Use Committee Protocol I12-007). 3A6/DR2a Tg mice were created using a 3A6 T-cell clone isolated from an MS patient as described previously (32). These mice were housed in an SPF facility within Rutgers-RWJMS; 3A6/DR2a Tg mouse breeding pairs were given ampicillin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), and vancomycin (0.5 mg/ml) in drinking water. Pups born from respective breeding pairs were further treated with the same antibiotic regimen until the age of 8–9 wk old. The development of EAE in the offspring was monitored until the age of 20 wk old.

Other materials and methods are described in SI Materials and Methods.

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