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

Sudhir K. Yadav[a,1], Sridhar Boppana[a,b,1], Naoko Ito[a], John E. Mindur[a,c], Martin T. Mathay[a,d], Ankoor Patel[a], Suhayl Dhib-Jalbut[a], and Kouichi Ito[a,2]

[a]Department of Neurology, Rutgers–Robert Wood Johnson Medical School, Piscataway, NJ 08854; [b]Department of Biological Sciences, Delaware State University, Dover, DE 19901; [c]Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114; and [d]Department of Genome Sciences, University of Washington, Seattle, WA 98105

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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 MS-associated MHC class II (MHC-II) gene, HLA-DR2a, and T-cell receptor (TCR) genes specific for MBP87-99/DR2a 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.

immune tolerance | aging | dysbiosis | complement | multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that involves inflammatory demyelination and axonal loss. Although the etiology of MS is still unknown, the activation and differentiation of myelin-specific Th cells into pathogenic Th1, Th17, and/or GM-CSF–producing Th cells has been suggested as an early disease process contributing to MS development (1). Under healthy conditions, T cell-mediated autoimmunity toward myelin antigens is largely prevented by thymic deletion (central tolerance) in addition to cell intrinsic and extrinsic peripheral tolerance mechanisms (2, 3). For example, as tissue-specific antigens (TSAs)—including myelin proteins—are expressed in the thymus, self-reactive TSA-specific T cells with a high avidity for TSA:MHC class II complexes undergo an apoptotic cell death termed "negative selection" in the thymus (3, 4). Thymic negative selection is incapable of eliminating all TSA-specific T cells. However, autoreactive T cells, which escape this process, may further undergo peripheral deletion or anergy (5). Nevertheless, a certain proportion of autoreactive T cells can still subvert such cell-intrinsic peripheral tolerance mechanisms and are thus kept under cell-extrinsic suppression by Treg cells, including forkhead box protein P3 (Foxp3) Treg cells (6). Taken together, it has been suggested that factors, which lead to dysregulated central and peripheral tolerance, greatly increase the risk of developing autoimmune diseases, including MS.

Genetic and environmental factors contribute to the dysregulation of immune tolerance (1, 7). Age is another risk factor for MS, most commonly affecting young adults (8–10). As thymic activity begins to decline after puberty, age-associated thymic involution has been suggested to be involved in the initiation of autoimmunity. In particular, thymic involution perturbs thymic negative selection, thereby permitting autoreactive T cells with high self-avidity to escape thymic negative selection (11). In addition, thymic involution reduces the egress of T cells and triggers the proliferation of naïve T cells in the periphery; this is referred to as homeostatic proliferation (12, 13). During homeostatic proliferation, certain populations of autoreactive naïve T cells can expand and differentiate into Th1 and Th17 memory/effector T cells, which may then participate in the initiation of autoimmunity and allograft rejection (14–16). Thus, aging can promote the development of pathogenic effector/memory T cells. However, the induction of age-associated tolerance has also been reported to suppress the activation of pathogenic autoreactive T cells by regulating both intrinsic (e.g., immunological ignorance, anergy, and peripheral deletion) and extrinsic peripheral tolerance (e.g., Treg-mediated immune suppression) (10, 17–19). Therefore, it seems that an imbalance between age-dependent pathogenic effector/memory T-cell development and age-related tolerance induction may serve as a risk factor for developing autoimmune diseases like MS.

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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[1]S.K.Y. and S.B. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: itoko@rwjms.rutgers.edu.

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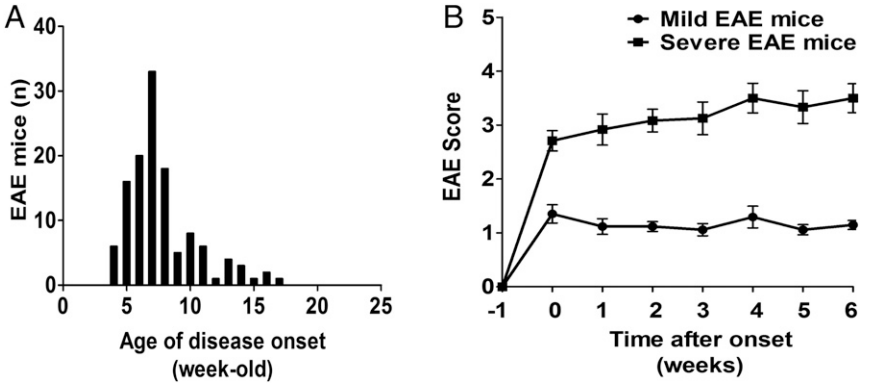

Fig. 1. An age window exists for the development of spontaneous EAE. (*A*) Age-dependent onset of spontaneous EAE. Frequency of spontaneous EAE and disease severity are shown in Table S1. (*B*) Clinical EAE score after onset of disease. EAE score was examined for 6 wk after disease onset. Disease courses 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.

Alterations 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, while 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. 1*A*). 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 invariable clinical score after the peak of disease (Fig. 1*B*). 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 (population of $V\beta5.1^+CD3^+CD4^+CD8^-$ 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. 2 *A* 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 ($V\beta5.1^+CD3^+CD4^+CD8^-$) within the $CD4^+CD8^-$ T-cell compartment decreased in 21- to 35-wk-old mice (39.7%) (Fig. 2*A*). In the spleen, the number of $V\beta5.1^+CD3^+CD4^+CD8^-$ T cells was low in 3- to 4-wk-old Tg mice but increased in 5- to 10-wk-old mice, which reflects a similar observation in the thymus. However, cell number of $V\beta5.1^+CD3^+CD4^+CD8^-$ 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 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 was increased in splenocytes isolated from 5- to 10-wk-old Tg mice compared with 3- to 4-wk-old Tg mice, and it gradually declined with age thereafter (Fig. 3*A*). Proliferation of splenocytes isolated from 21- to 35-wk-old Tg mice in response to MBP87-99 was much less than that from 5- to 10-wk-old Tg mice (Fig. 3*A*), and cell trace analysis also indicated that proliferation of 3A6-TCR Tg $CD4^+$ T cells in response to the MBP antigen was highly reduced in 21- to 35-wk-old Tg mice (Fig. 3 *B* and *C*). Importantly, over 95% of naïve and 65% of memory/effector splenic T cells of 21- to 35-wk-old Tg mice were found to be alive among splenocytes in support of anergy induction rather than cellular apoptosis (Fig. S1 *A* and *B*), and both naïve and memory/effector T cells appeared to undergo age-related anergy induction based on their inability to proliferate as vigorously in response to MBP87-99 (Fig. S1*C*). In accordance with the lack of response to cognate antigen, the phosphorylation of ERK was equally impaired in 21- to 35-wk-old

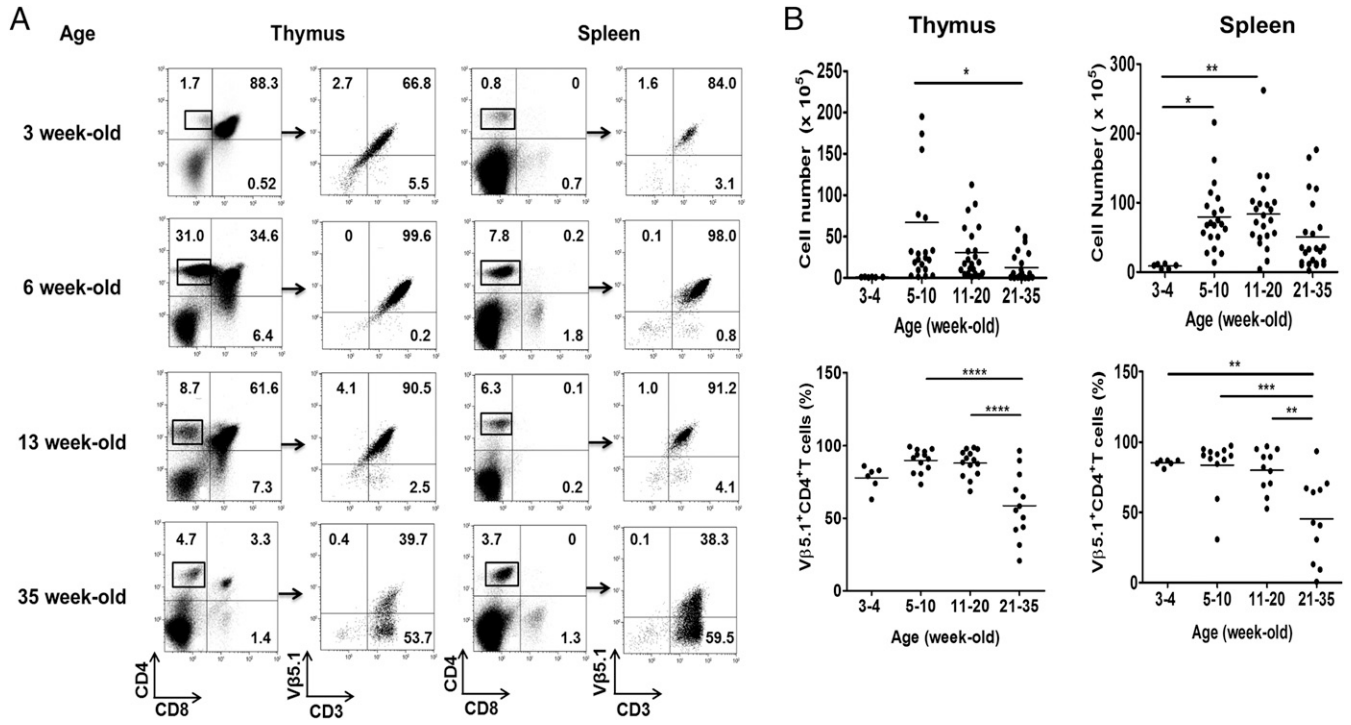

Fig. 2. Effect of aging on the development of MBP-specific Tg T cells. (*A*) Development of MBP-specific 3A6-TCR Tg T cells in the thymus and spleen. Thymocytes and splenocytes isolated from mice of different ages were stained with anti-CD4, anti-CD8, anti-CD3, and human TCR Vβ5.1 mAbs (the MBP-specific 3A6-TCR Vβ). $CD4^+CD8^-$ cells (4,000–20,000 events) were gated for the analysis of $V\beta5.1^+CD3^+$ T cells. (*B*) Cell numbers of $V\beta5.1^+CD3^+CD4^+CD8^-$ T cells in the thymus and spleen (*Upper*) and percentage of $V\beta5^+CD3^+CD4^+CD8^-$ T cells in the $CD4^+CD8^-$ T-cell compartment (*Lower*). Non-EAE mice of different ages were used for the above experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.001$.

Tg mice compared with 5- to 10-wk-old Tg mice (Fig. 3 *D* and *E*). Furthermore, the expressions of anergy-associated E3 ubiquitin ligase genes [*gene related to anergy in lymphocytes* (*Grail*), *casitas B-lineage lymphoma-b* (*Cbl-b*), and *Itch*] were also elevated in $CD4^+$ T cells isolated from 21- to 35-wk-old mice compared with those isolated from 5- to 10-wk-old mice (Fig. 3*F*). 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 then 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. 4 *A* 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. 4*C*). 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. 5*A*). 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. 5*B*). 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. 5 *C* 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

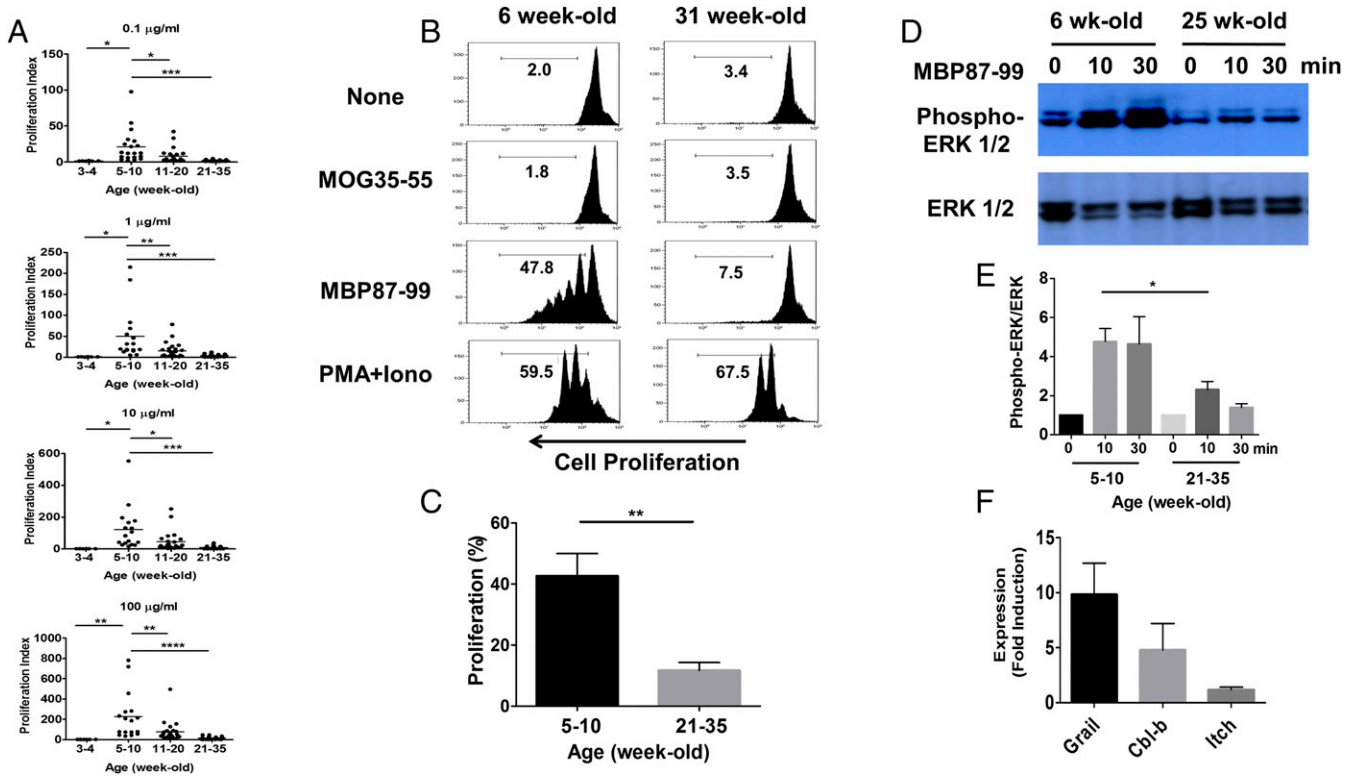

Fig. 3. Age-associated induction of anergy in MBP-specific Tg T cells. (*A*) Proliferation of splenocytes in response to MBP87-99 peptide. Splenocytes isolated from mice of different ages were cultured with varying concentrations of MBP87-99 (100, 10, 1, and 0.1 μg/mL) for 3 d and examined for proliferation by [^{3}H]-thymidine uptake. The stimulation index was calculated as described in *SI Materials and Methods*. (*B* and *C*) Proliferation of Vβ5.1$^+$CD4$^+$ T cells in response to MBP peptides. Splenocytes were stained with cell trace violet and cultured with 10 μg/mL MBP87-99 for 4 d. Vβ5.1$^+$CD4$^+$ T cells (20,000–30,000 events) were gated for cell division assay (*B*), and percentage of dividing cells is shown (*C*). Mean ± SEM (n = 7 in 5- to 10-wk-old mice and n = 8 in 21- to 35-wk-old mice). (*D* and *E*) Representative image of phosphorylation of ERK (phospho-ERK) in CD4$^+$ T cells between 5- to 10-wk-old and 21- to 35-wk-old Tg mice. CD4$^+$ spleen T cells isolated from 6- or 25-wk-old Tg mice were cultured with 10 μg/mL MBP87-99 for the indicated time and analyzed for the phospho-ERK and total ERK by Western blot (*D*). Quantification of the ratio of phospho-ERK to total ERK (*E*). Mean ± SEM (n = 3). (*F*) Up-regulation of E3 ubiquitin ligase genes with aging. CD4$^+$CD25$^-$ T cells were isolated from 5- to 10-wk-old and 21- to 35-wk-old Tg mice, and expressions of *Grail*, *Cblb*, and *Itch* were examined by quantitative PCR. Fold inductions of 21- to 35-wk-old Tg mice were normalized to those of 5- to 10-wk-old Tg mice. Mean ± SEM (n = 3). Non-EAE mice of different ages were used for the above experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.001.

the rederived 3A6/DR2a Tg mice compared with nonrederived mice (Fig. 5*E*), and none of the rederived IgM-lo 3A6/DR2a Tg mice developed EAE spontaneously (Fig. 5*F*). 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

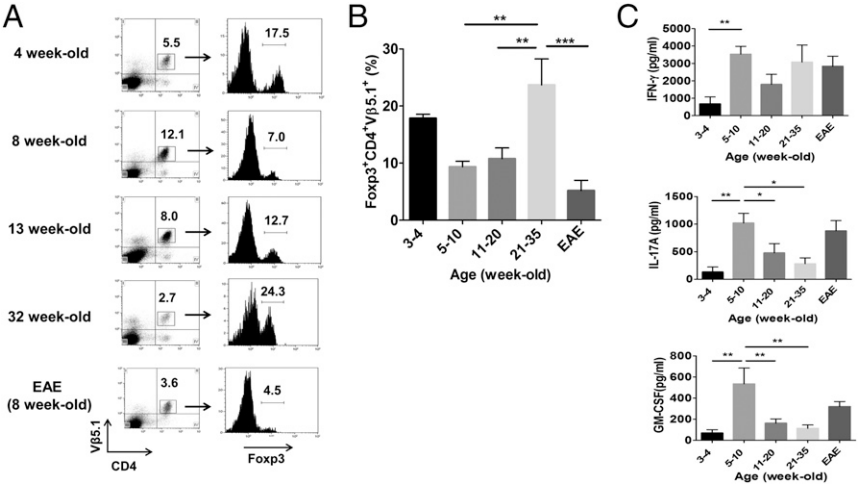

Fig. 4. Effect of aging on the development of Foxp3$^+$ Treg cells and pathogenic T cells. (*A* and *B*) Development of Foxp3$^+$CD4$^+$ T cells in the spleen. Splenocytes isolated from the mice of different ages were stained with anti-CD4, -Vβ5.1, and -Foxp3 mAbs, and Vβ5.1$^+$CD4$^+$ splenocytes (5,000–13,000 events) were gated for the analysis of Foxp3 expression (*A*). Frequency of Foxp3$^+$Vβ5.1$^+$CD4$^+$ T cells in the CD4$^+$ T-cell compartment is shown (*B*; n = 5–7). (*C*) Production of proinflammatory cytokines. Splenocytes isolated from 3A6/DR2a Tg mice of different ages were cultured with 10 μg/mL MBP87-99 for 3 d, and the production of IFN-γ, IL-17A, and GM-CSF was examined by ELISA. Mean ± SEM (n = 10–16). *P < 0.05; **P < 0.01; ***P < 0.001.

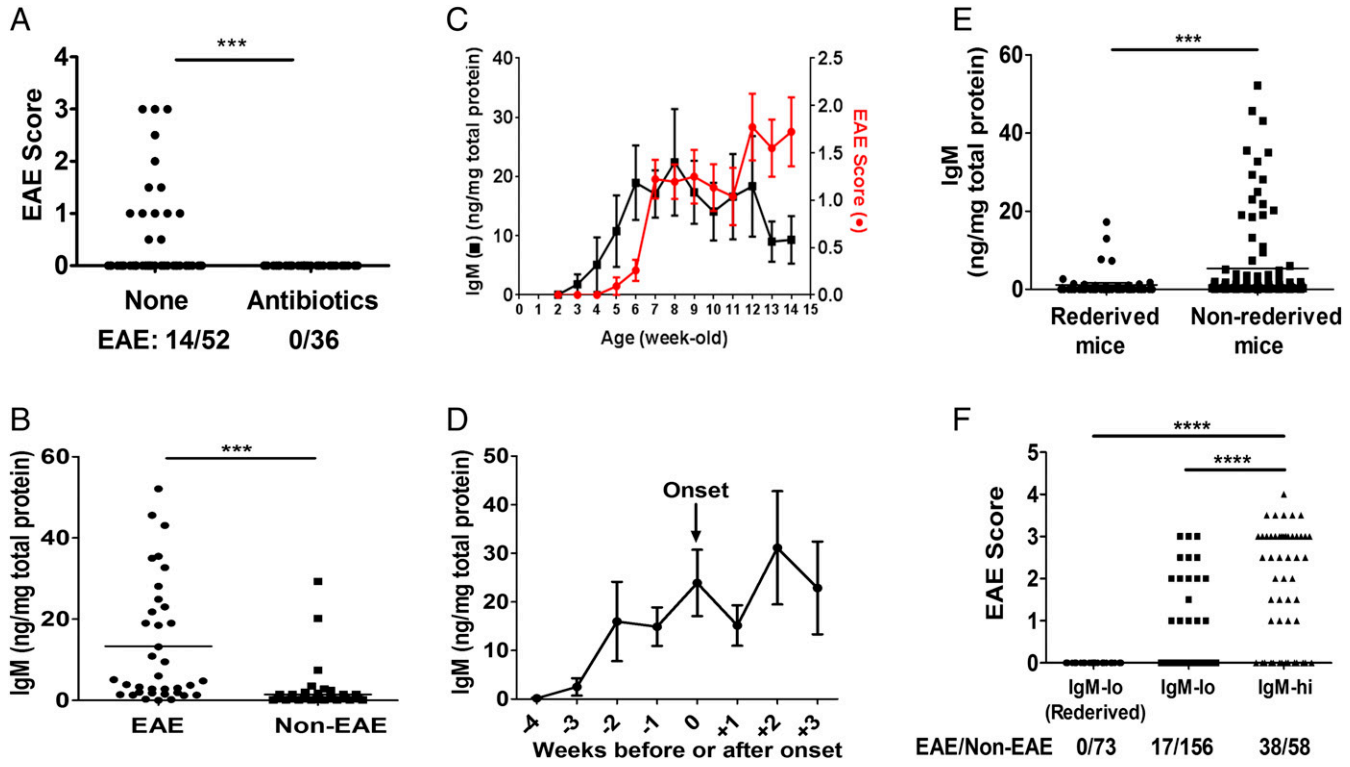

Fig. 5. Association of fecal IgM levels with the development of spontaneous EAE. (*A*) Antibiotic treatment effect on development of spontaneous EAE. Breeding pairs of 3A6/DR2a Tg mice were treated with a mixture of ampicillin (1 mg/mL), metronidazole (1 mg/mL), neomycin (1 mg/mL), and vancomycin (0.5 mg/mL), and their offspring were continuously treated with antibiotics. The development of EAE was examined until 20 wk of age. (*B*) Association of high production of fecal IgM with the development of spontaneous EAE. Feces were collected from EAE ($n = 37$) and non-EAE mice ($n = 74$), and fecal IgM and total protein were measured by ELISA and bicinchoninic acid (BCA) assay, respectively. Average fecal IgM concentration (nanograms per milligram of total fecal protein) during the period from 5 to 10 wk old is shown. (*C*) Time course in fecal IgM production (■) and development of spontaneous EAE (●). Mean ± SEM ($n = 26$). (*D*) Increase in fecal IgM before the onset of spontaneous EAE. Fecal IgM levels before and after clinical EAE onset are shown. Fecal IgM increased before disease onset in 85% of 3A6/DR2a mice. Mean ± SEM ($n = 26$). (*E*) Fecal IgM levels in rederived 3A6/DR2a mice. Fecal samples were collected from rederived ($n = 54$) and nonrederived mice ($n = 111$) during the age of 5–10 wk old, and IgM concentration was measured by ELISA. (*F*) Development of spontaneous EAE in fecal IgM-lo (rederived), fecal IgM-lo, and IgM-hi mice. IgM-lo and IgM-hi mice were determined by average fecal IgM concentration during the period from 5 to 10 wk old. IgM-lo mice: <2.0 ng/mg total fecal protein; IgM-hi mice: ≥2.0 ng/mg total fecal protein. 3A6/DR2a Tg mice ($n = 214$) were divided into fecal IgM-hi ($n = 58$) and fecal IgM-lo ($n = 156$) groups. Development of EAE was examined in fecal IgM-lo (rederived), fecal IgM-lo, and fecal IgM-hi mice. ***$P < 0.001$; ****$P < 0.0001$.

mice (Fig. 6*A*, Dataset S1, and Fig. S2). Analysis of bacterial species showed that *Bacteroides vulgatus*, *Bacteroides acidifaciens*, and *Bacteroides xylanisolvens* were expanding in IgM-hi EAE mice compared with rederived (IgM-lo) mice (Fig. 6*B*, Dataset S2, and Fig. S3). In contrast, the families of *Porphyromonadaceae*, *Lachnospiraceae*, *Verrucomicrobiaceae*, *Flavobacteriaceae*, and *Clostridiaceae* were high in relative abundance in the rederived (IgM-lo) mice compared with IgM-hi EAE mice (Fig. 6*A*, Dataset S1, and Fig. S2). Interestingly, analysis of bacterial species showed that *Parabacteroides goldsteinii*, *Akkermansia muciniphila*, and *Alkaliphilus crotonatoxidans* were significantly low in relative abundance in IgM-hi EAE mice compared with the rederived (IgM-lo) mice (Fig. 6*B*, Dataset S2, and Fig. S3). This microbial composition pattern was similar among 5- to 10-wk-old, 11- to 20-wk-old, and 21- to 35-wk-old groups. Notably, *B. vulgatus* is potentially involved in gut pathology and dysbiosis-mediated gut inflammation (37, 38), while *A. muciniphila* is known to promote intestinal integrity and mucosal tolerance (39, 40). Therefore, gut dysbiosis affecting the expansion of pathogenic gut microbiota and reduction of health-associated gut microbiota may trigger the development of spontaneous EAE.

Gut Dysbiosis Increases Gut Leakiness and Endotoxin Levels in the Periphery. Dysbiosis induces leakiness of the gut, resulting in the translocation of microbial products into systemic circulation (41). We examined gut barrier leakiness and blood endotoxin levels in fecal IgM-lo (rederived), IgM-hi non-EAE, and IgM-hi EAE mice. Mice with fecal IgM-hi levels show an increase in intestinal permeability compared with IgM-lo (rederived) mice, which was further exacerbated in IgM-hi EAE mice (Fig. 7*A*). 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. 7*B*). 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. 8*A*). In addition, the expression of complement C3 correlated with production of the complement C3 protein and the C3-derived cleavage product, anaphylatoxin C3a (Fig. S4). Of note, anaphylatoxin C3a was produced in dendritic cells (Fig. 8*B*) as reported previously (44, 45), and its production by dendritic cells was higher in fecal IgM-hi non-EAE and IgM-hi EAE mice compared with IgM-lo (rederived)

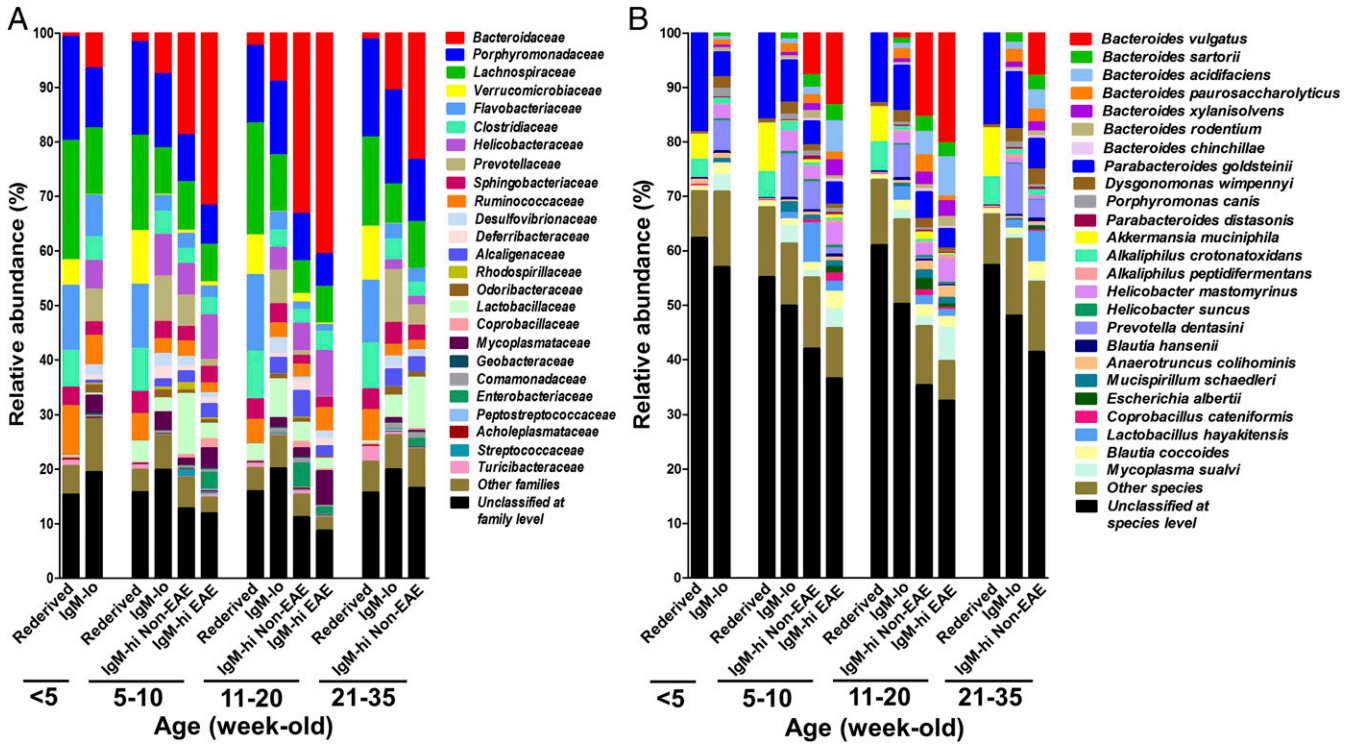

Fig. 6. Identification of enteric bacteria expanding in spontaneous EAE mice. Fecal IgM levels were examined by ELISA every week, and fecal DNA was isolated from rederived, fecal IgM-lo (<2.0 ng/mg total protein) non-EAE, fecal IgM-hi (≥2.0 ng/mg total protein) non-EAE, and IgM-hi EAE 3A6/DR2a Tg mice 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).

mice (Fig. 8*C*). 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. 8*D*). 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. 8*E*). 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. 9 *A* 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. 9*C*). 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. 9 *D* 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 $CD4^+CD8^-$

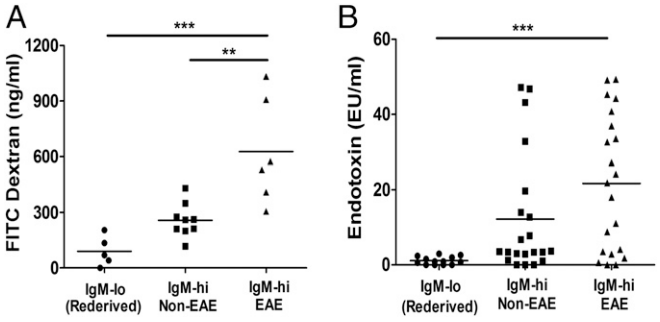

Fig. 7. Gut dysbiosis promotes intestinal permeability and high endotoxin levels in blood circulation. (*A*) Increase in intestinal permeability in fecal IgM-hi non-EAE and IgM-hi EAE mice. FITC-dextran in the serum was measured 4 h after oral inoculation of FITC-dextran in fecal IgM-lo (rederived),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

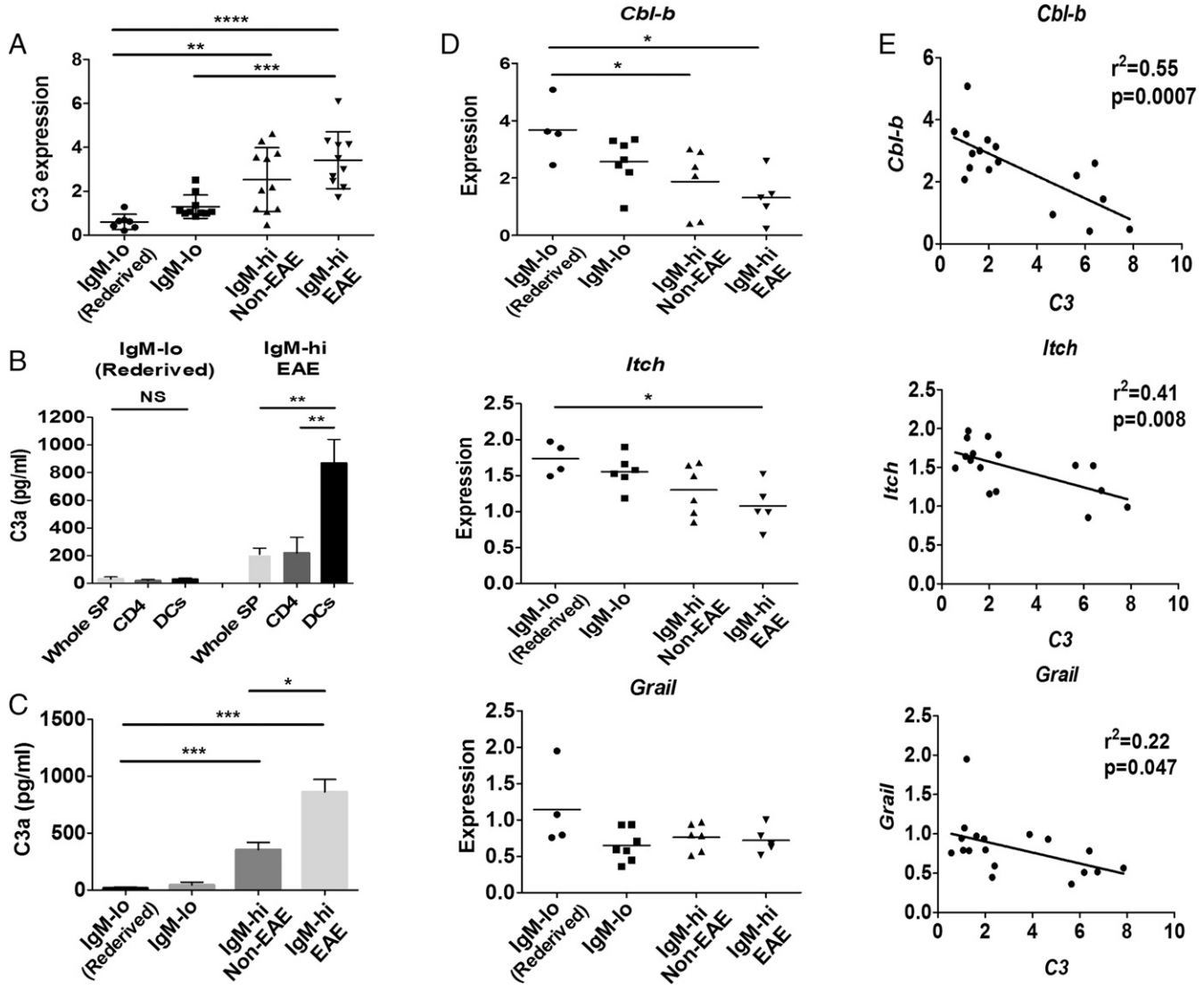

Fig. 8. Up-regulation of complement C3 and down-regulation of anergy-related genes by gut dysbiosis. (*A*) Expression of complement C3 gene in the spleen. Expression of complement C3 gene in the spleens isolated from fecal IgM-lo (rederived), fecal IgM-lo, fecal IgM-hi non-EAE, and IgM-hi EAE mice was measured by quantitative PCR. (*B*) Dendritic cells express complement C3. $CD4^+$ T cells and DCs isolated from the spleens of IgM-lo (rederived) or IgM-hi EAE mice were cultured for 3 d, and production of anaphylatoxin C3a in the culture supernatant was measured by ELISA. Mean ± SEM ($n = 3$). (*C*) Increase in anaphylatoxin C3a in the DCs isolated from fecal IgM-hi non-EAE and IgM-hi EAE mice. DCs isolated from the spleens of fecal IgM-lo (rederived), fecal IgM-lo, fecal IgM-hi non-EAE, and IgM-hi EAE mice were cultured for 3 d, and production of anaphylatoxin C3a in the culture supernatant was measured by ELISA. Mean ± SEM ($n = 3$–6). (*D*) Down-regulation of E3 ubiquitin ligase genes in $CD4^+CD25^-$ T cells isolated from fecal IgM-hi non-EAE and IgM-hi EAE mice. $CD4^+CD25^-$ T cells were isolated from the spleen of fecal IgM-lo (rederived), fecal IgM-lo, fecal IgM-hi non-EAE, and IgM-hi EAE mice and examined for the expression of *Cbl-b*, *Itch*, and *Grail* genes by quantitative PCR. (*E*) Down-regulation of E3 ubiquitin ligase genes in $CD4^+CD25^-$ T cells is correlated with the up-regulation of the complement C3 gene in whole-spleen cells. Expressions of E3 ubiquitin ligase (*Cbl-b*, *Itch*, and *Grail*) and complement C3 in $CD4^+CD25^-$ T cells and whole-spleen cells, respectively, were measured by quantitative PCR. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.001$; NS, nonsignificant.

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 overall peripheral T-cell population is largely maintained because of homeostatic T-cell proliferation in the periphery (12, 13). Indeed, cell number of $V\beta5.1^+CD3^+CD4^+CD8^-$ 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. 2*B*). However, 3A6-TCR Tg $CD4^+$ splenic T cells were reduced in 21- to 35-wk-old Tg mice (Fig. 2*B*). 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. 4*C*) 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

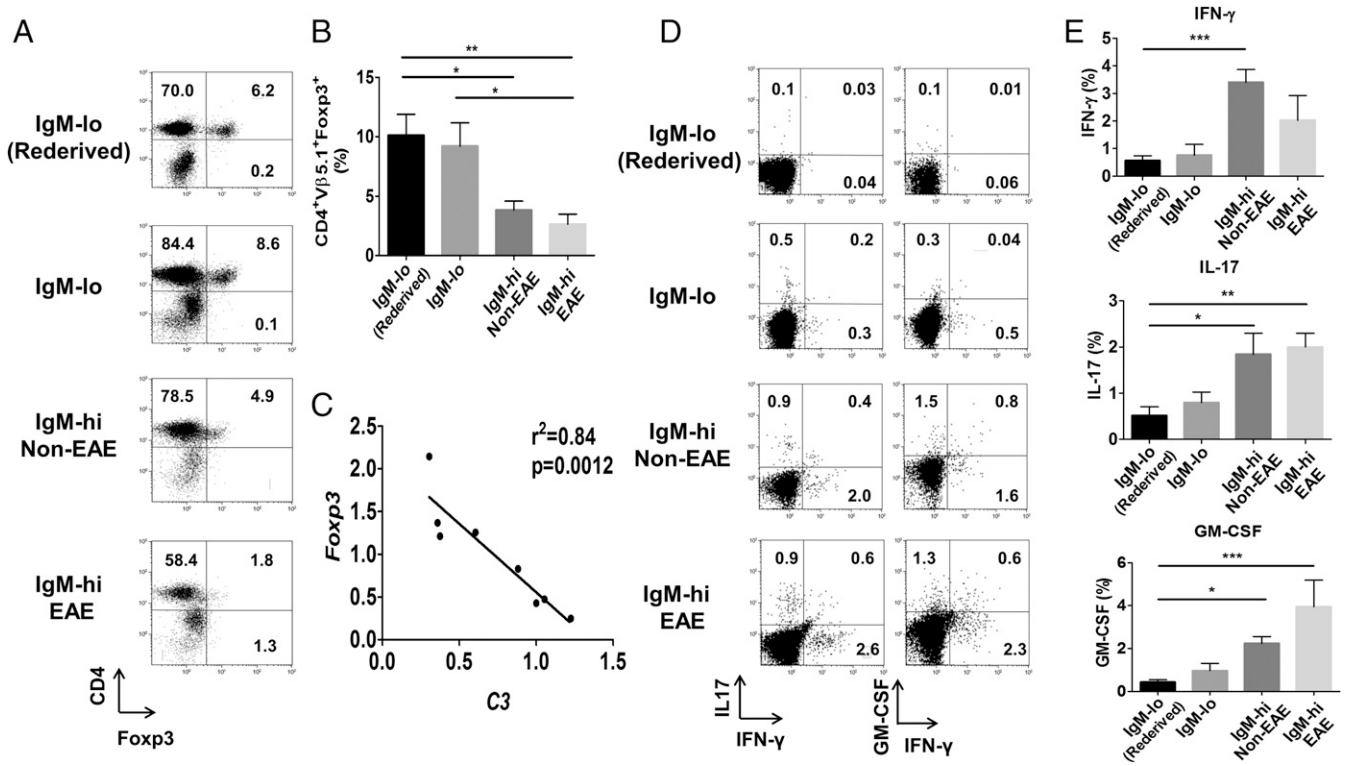

Fig. 9. Effect of gut dysbiosis on development of Foxp3$^+$ Treg cells and pathogenic T cells. (*A*) Inefficient development of Foxp3$^+$CD4$^+$ T cells in fecal IgM-hi non-EAE and IgM-hi EAE mice. Splenocytes isolated from 5- to 10-wk-old fecal IgM-lo (rederived), fecal IgM-lo, fecal IgM-hi non-EAE, and IgM-hi EAE mice were stained with anti-CD4, CD3, Vβ5.1, and Foxp3 mAbs, and Vβ5.1$^+$CD3$^+$ T cells (10,000–25,000 events) were gated for the expression of Foxp3 and CD4. (*B*) Frequency of CD4$^+$Vβ5.1$^+$Foxp3$^+$ T cells in the CD4$^+$Vβ5.1$^+$ T-cell compartment of 5- to 10-wk-old mice is shown. Mean ± SEM (n = 3–4). (*C*) Expression of the *Foxp3* gene in CD4$^+$CD25$^+$ T cells is inversely correlated with expression levels of the complement C3 gene in whole-spleen cells. Expressions of Foxp3 and complement C3 in CD4$^+$CD25$^+$ T cells and whole-spleen cells, respectively, were measured by quantitative PCR. (*D* and *E*) Increase in pathogenic T cells in fecal IgM-hi non-EAE and IgM-hi EAE mice. Splenocytes isolated from fecal IgM-lo (rederived), fecal IgM-lo, IgM-hi non-EAE, and IgM-hi EAE mice were stained with anti–GM-CSF, –IL-17A, and –IFN-γ mAbs after their cultivation with MBP87-99 at 10 μg/mL in the presence of Brefeldin A to examine the production of these cytokines in response to MBP antigen. Vβ5.1$^+$CD4$^+$ T cells (10,000–16,000 events) were gated for the analysis of production of IFN-γ, IL-17A, and GM-CSF cytokines (*D*). Percentage of T cells that produce IFN-γ, IL-17A, or GM-CSF in the Vβ5.1$^+$CD4$^+$ T-cell population is shown (*E*). Mean ± SEM (n = 4–6). *P < 0.05; **P < 0.01; ***P < 0.001.

the up-regulation of E3 ubiquitin ligase genes (Fig. 3*F*) 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 and decreases thereafter. Therefore, adolescence and young adulthood are the most risky periods for the development of pathogenic MBP-specific T cells in 3A6/DR2a Tg mice.

In this study, we investigated how autoreactive T cells specific for myelin antigens can differentiate into encephalitogenic T cells spontaneously in 3A6/DR2a Tg mice during adolescence and young adulthood. Since gut dysbiosis is frequently observed in MS patients and is a possible pathological event in the initiation and progression of disease (22–24), we examined whether a dysbiotic gut microbiota can affect the development of spontaneous EAE. Dysbiosis is characterized by the overgrowth or enrichment of a minor population of potentially harmful bacteria (pathobionts) that are normally kept at low levels (25). The gut ecosystem shifts to a state of dysbiosis through perturbations caused by antibiotics, diet, immune deficiency, or infection. The overgrowth of *Bacteroidaceae* has been shown to occur during intestinal dysbiosis in mice (57–59). Since IgM is produced in response to an overgrowth of pathobionts (34, 35) and an increase in fecal IgM levels was associated with the production of lipocalin-2, a marker of gut inflammation (60) (Fig. S5), we examined the association of fecal IgM levels with the development of spontaneous EAE. Interestingly, we observed that high fecal IgM levels and an overgrowth of pathobiont *B. vulgatus* were associated with spontaneous EAE development (Figs. 5 *B–D* and 6*B*, 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. 6*B*, 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. 7*A*). However, intestinal permeability was further increased on EAE development (Fig. 7*A*). 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 C3 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 complement C3 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$^+$ CD25^{high} 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. 8*D*). Since *Cbl-b* and *Itch* play an essential role in anergy 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 Treg cells (50). Therefore, the down-regulation of *Cbl-b* and *Itch* gene expression in CD4$^+$CD25$^-$ T cells reduces the development of inducible Foxp3$^+$ Treg cells and also renders CD4$^+$CD25$^-$ T cells more resistant to immune suppression by Foxp3$^+$ Treg cells. Although the mechanism detailing how the expression of *Cbl-b* and *Itch* is down-regulated by gut dysbiosis is still unknown, gut 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 ligases 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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