typically, Th17 cells show a greater degree of context-dependent plasticity[20,21] associated with a higher *in vivo* survival and self-renewal capacity.[22] Using fate-reporter animals, IFN-γ producing T cells that appeared in the CNS of EAE mice were shown to be almost exclusively derived from cells that formerly produced IL-17.[23] The conversion of IL-17-producing T cells into IFN-γ producers during EAE appears to confer an increased pathogenic phenotype to those T cells, accompanied by downregulation of RORγt and upregulation of T-bet. This suggests that the tracking of RORγt expression in T cells is not necessarily enough to identify ongoing pathogenic responses resulting from Th17 cells in CNS autoimmunity. In addition, the development of conventional Th17 cells and distinct T cell subsets producing IL-17 cells requires RORγt. Deletion of RORγt results in impaired differentiation of all types of IL-17-producing T cell subsets, as well as the impaired development of other newly-identified immune cell subsets, lymphoid tissue inducer (LTi) cells and a part of type 3 innate lymphoid cells (ILC3) that also express RORγt.[24,25] It is conceivable that not all of those RORγt-positive T cells producing IL-17 are necessarily involved in pathogenesis of CNS autoimmunity. Therefore, identification of novel molecular marker(s) that exclusively represent pathogenic IL-17-producing T cells is highly desirable.

EAE is a prototype autoimmune disease model that has greatly contributed to elucidating the pathogenesis of MS.[26] EAE can be induced in laboratory animals by active immunization with myelin antigens or by passive transfer of myelin antigen-reactive T cells. Th1 cells reactive to myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) are capable of inducing clinical and pathological manifestations of EAE after transfer into naive mice; thus, Th1 cells producing IFN-γ have long been believed to play a central role in the pathogenesis of MS. However, the "Th1 disease" dogma has been challenged by contradicting results showing that gene-targeted mice deficient in IFN-γ[27,28] or IFN-γ receptor, and mice deficient for IL-12 signaling, are still susceptible to EAE. Subsequently, it was shown that IL-23, and not IL-12, is essential for the development of EAE,[7] resulting in the identification of pathogenic IL-23-dependent Th17 cells that produce the unique inflammatory cytokine, IL-17.[29] Currently, it is widely accepted that Th17 cells play an important role in the development of inflammatory autoimmune diseases either independently or collaboratively with Th1 cells.

What is NR4A2?

NR4A2, also known as Nurr1, is a nuclear receptor family member that, to date has no known endogenous ligand (Fig. 1). The members of the NR4A subfamily (NR4A1/Nur77, NR4A2/Nurr1 and NR4A3/NOR-1) are mostly expressed at very low levels in a wide variety of metabolically-demanding and energy-dependent tissues, such as skeletal muscle, adipose tissue, heart, kidney, liver and brain.[30] On particular stimulation, high levels of NR4A expression are induced in these tissues, reminiscent of immediate early genes. The diversity of signals that lead to this expression suggests that NR4A2 functions in a manner highly dependent on cell type and context. NR4A2 is primarily expressed in the CNS, particularly in the cortex, ventral midbrain, brain stem and the spinal cord, and it appears to have important functions in both the development and specific responses of dopaminergic neurons.[31,32] Therefore, many studies regarding NR4A2 have focused on the functional analysis of NR4A2 and its relevance to the pathology of Parkinson's disease. Indeed, mutations in the NR4A2 gene are well known to be associated with familial Parkinson's disease, reflecting the essential role for NR4A2 in the development and survival of neuronal organization of substantia nigra.[33,34] In contrast, much less attention has been paid to the functional role of NR4A2 in T cells. More than a decade ago, NR4A1 and NR4A3 were shown to mediate apoptotic processes of mature and immature T cells.[35–37] However, these studies do not provide insight into the functional implications of upregulated expression of NR4A2 in T cells. Recently, NR4A2 has come into the spotlight as a pivotal pathogenic component for modification of inflammatory milieus of rheumatoid arthritis, atherosclerosis and cancer, which will be discussed later in more detail.[38,39] Conversely, NR4A2 expression has also been implicated in reducing immune responses, including a potential role in neuroprotection from inflammation and repression of matrix metalloproteinases in joint inflammation, suggesting diverse roles for this transcription factor that are altered in a cell-type and context-dependent manner.[40,41]

Nuclear receptors are composed of several conserved functional domains including DNA-binding domain (DBD) with two zinc fingers in the N-terminal region of the molecule and the ligand-binding domain (LBD) in the C-terminal region with a less conserved structure. In the absence of specific ligands, most of the nuclear receptors are inactive by

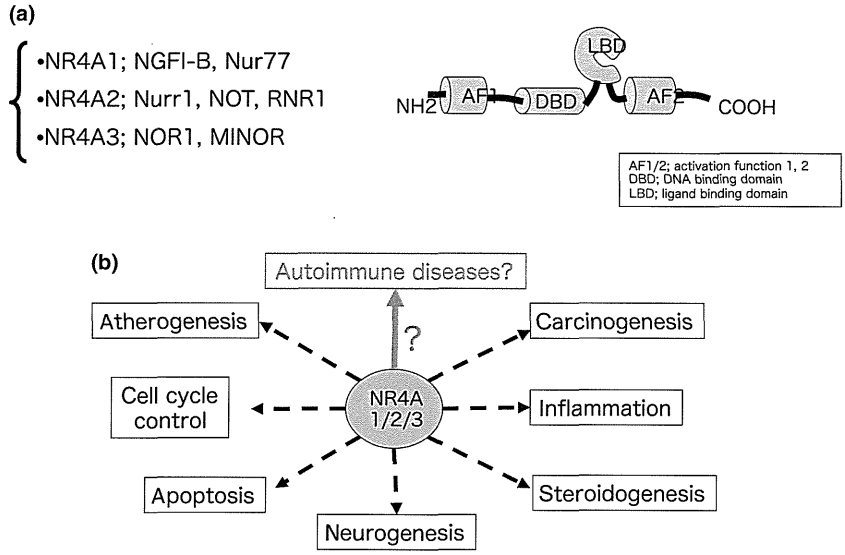


Figure 1 Versatile function of NR4A2 in a variety of biological and pathological responses. (a) Members of the NR4A family of nuclear receptors and their typical molecular structure. (b) Schematic summary of the organ and tissue-specific biological and pathological roles of the NR4A2.

interactions with co-repressor proteins. On ligand binding to a hydrophobic cleft in the LBD, a conformational repositioning occurs at the C-terminal amphipathic α-helix (H12) of the LBD that provides a well-defined surface (activation-function 2 [AF-2]) recognized by co-activator proteins, leading to the formation of multiprotein complex mediating gene activation, such as histone acetylation and chromatin modifications. However, NR4A2 encodes unusual and atypical LBD that lack canonical ligand binding properties.[42] Therefore, NR4A2 is believed to be a ligand-independent and constitutively active receptor, and its activity is tightly controlled at the level of transcription, post-transcriptional modification and multivalent complex formation with other molecules. The DNA-binding motif for the NR4A family members is the octanucleotide 5′-A/TAAAGGTCA (NGFI-B response element [NBRE]), where NR4A2 binds as monomers and homodimers. The pro-opiomelanocortin gene promoter contains another class of transcriptional targets for homodimers: Nur-responsive element (NurRE), with an inverted repeat, the NBRE-related octanucleatide, AAAT(G/A)(C/T)CA. NR4A1 and NR4A2 also bind as heterodimers with the retinoid X receptor (RXR) and bind a motif called DR-5. In addition, multivalent complex formation of NR4A2 with other transcription factors enables it to show non-canonical DNA binding.[43,44]

NR4A2-deficent neonates typically die at birth as a result of a severe defect in respiratory function despite having intact NR4A1/3 genes, suggesting a unique functional property for NR4A2.[45,46] Because of the selective expression of NR4A2 in the CNS, most of the target genes of NR4A2 known to date are limited to its role in this region. For example, NR4A2 is shown to play a role in the transcriptional activation of tyrosine hydroxylase involved in the synthesis of dopamine.[47,48] Another group of NR4A2 target genes reside in those relevant to bone formation, such as osteopontin and osteocalcin.[49,50] It is suggested that NR4A1 and NR4A3 are expressed in the thymus and mediate T cell receptor-mediated T cell apoptosis, but the distribution and function of NR4A2 in immune cells has not been extensively studied. Accordingly, a recent report showed that NR4A family proteins have essential roles in regulatory T (Treg) cell development by inducing promoter activity of the forkhead box P3 (Foxp3) gene;[51] however, NR4A2 itself was shown to be much less effective for transcription of the Foxp3 gene and subsequent Treg differentiation than other NR4A family members.[52] Meanwhile, a series of reports have suggested pivotal roles for NR4A family members, especially the NR4A2 subtype, in inflammatory responses, and they are aberrantly expressed in inflamed synovial tissue of patients with rheumatoid arthritis, psoriatic skin and atherosclerotic lesions. Therefore, NR4A receptors might contribute to the cellular processes that control inflammatory disorders including autoimmunity.

NR4A2 in MS

MS has an autoimmune pathology that is initiated by the development of autoimmune T cells reactive to myelin antigens, such as MBP, MOG and PLP. Immunologically, naïve T cells differentiate into

encephalitogenic T cells on encountering those with myelin autoantigens. Such encephalitogenic T cells must be preactivated in the periphery before they are able to penetrate into the CNS parenchyma.[53,54] Then, expansion of inflammatory processes within the CNS is triggered by pro-inflammatory cytokines and chemokines secreted by infiltrating autoreactive T cells after recognizing self-antigens in a major histocompatibility complex (MHC) class II-restricted manner in the CNS. Encephalitogenic T cells that generate the development of MS can be composed of both Th1 and Th17 cells, and the relative contributions of either of these distinct helper T cell populations might help explain the diversity of clinical and pathological manifestations, as well as the varying responses to therapy.[4] However, little is known about the helper T cell population responsible for the development of MS partly because of a lack of appropriate methodology to discriminate those encephalitogenic T cells. For example, although RORγt is a good marker to identify Th17 cells, the aforementioned complex behavior of RORγt expression in T cells, in addition to the fact that RORγt is also involved in lymphoid organogenesis, means that it is necessary to find new specific marker(s) that enable identification of pathogenic T cells in MS. Through comprehensive gene expression profiling analysis of RR-MS patients in remission, we previously showed that NR4A2 is selectively upregulated in peripheral blood T cells of MS patients.[12] Quantitative reverse transcription polymerase chain reaction analysis further revealed that NR4A2 expression in T cells from MS patients showed an approximately fivefold increase compared with healthy donors. We then applied an animal model of MS to further assess the role of the novel orphan nuclear receptor gene on T cell function.

NR4A2 in EAE

In EAE induced in C57BL/6 mice by immunization with a MOG_{35-55} peptide, NR4A2 was selectively upregulated in T cells of the peripheral circulation and those infiltrating into the CNS, but NR4A2 expression was not observed in T cells from secondary lymphoid organs, such as the spleen or draining lymph nodes.[13,14] In a kinetic analysis using reverse transcription polymerase chain reaction, we observed that NR4A2 expression in peripheral circulating T cells reached a maximum value 21 days after EAE induction, and the entire expression pattern of NR4A2 in peripheral blood T cells was well correlated with the clinical severity of EAE. Meanwhile, significant expression of NR4A2 was observed in the CNS-infiltrating T cells from day 9, when early signs of EAE become evident. These results suggest that NR4A2 expression was induced in T cells on induction of EAE, but the kinetics of expression significantly differs between peripheral blood T cells and CNS-infiltrating T cells. Recent studies have shown that autoimmune Th17 cells producing IL-17 play a central role in causing autoimmune inflammation,[55] and analysis of fate-reporter animals showed that those Th17 cells have a tendency to change their phenotype to become IFN-γ producing T cells in the CNS milieu of EAE mice.[23] Therefore, T cells accumulating in the CNS are characterized by massive production of those inflammatory cytokines along with significant expression of NR4A2. Accordingly, retroviral transduction of NR4A2 cDNA into splenic CD4+ T cells *in vitro* augmented production of IL-17 and IFN-γ on restimulation. Furthermore, NR4A2-expressing T cells in the CNS of EAE animals were accumulated in those producing IL-17 regardless of their secretion of IFN-γ (Fig. 2).[14] Therefore, NR4A2 might be considered as a useful marker to identify pathogenic Th17 cells in target organs or peripheral circulation. In addition, T cell expression of NR4A2 seems to have a strong link with exposure to a certain autoantigen, as *in vivo* induction of NR4A2 in T cells is observed only when immunized with self-peptide and not with a peptide of exogenous origin, such as ovalbumin. Interestingly, forced subcutaneous inflammation by intradermal injection of IL-23 causes upregulation of NR4A2 in peripheral blood T cells.[14] Therefore, T cell upregulation of NR4A2 emerges only after recognition of self-antigen that induces autoreactive IL-17-producing cells *in situ*.

It is noteworthy to point out that despite an apparent requirement for NR4A2 in Th17 differentiation in target organs, little NR4A2 expression was detected in secondary lymphoid tissues that are the proposed sites for T cell priming on encounter with self-antigens and the subsequent acquisition of a Th17 phenotype.[14] In addition, NR4A2 expression by CD4+ T cells was first observed in the target organ, much earlier than in peripheral blood T cells. Intriguingly, when autoreactive cells were induced by immunization with self-antigens in the absence of pertussis toxin administration, NR4A2 expression was not upregulated, possibly because of impaired access to target organs. Thus, the target organs, the CNS in the case of EAE, might represent the site of NR4A2 upregulation where pathogenic Th17 differentiation occurs *in vivo*, rather than during initial

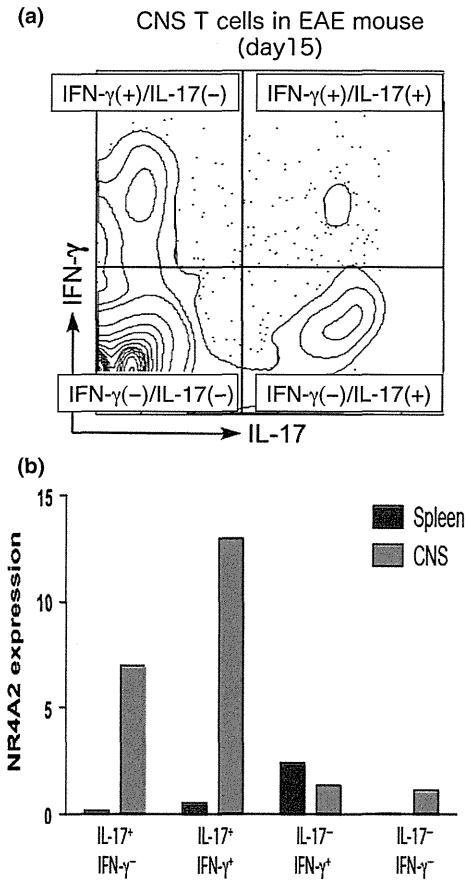


Figure 2 Distribution of cytokine-producing T cells in the central nervous system (CNS) of experimental autoimmune encephalomyelitis (EAE) mice and their expression of NR4A2. (a) There are distinct subsets of T cells accumulated in the CNS of EAE mice, composed of interleukin (IL)-17-producing T cells, interferon (IFN)-γ-producing T cells and double producers. (b) NR4A2 expression by subsets of cytokine-producing CD4+ T cells was analyzed using quantitative polymerase chain reaction at day 15 post-EAE induction for spleen and CNS-infiltrating cells.

T cell priming in the secondary lymphoid tissues. Cognate antigen interactions *in situ* are required to permit primed T cells moving from peripheral circulation to the CNS parenchyma.[53,54] Accordingly, primed encephalitogenic CD4+ effector T cells transferred into naive animals rapidly infiltrated CNS tissue, requiring MHC class II-dependent antigen presentation, whereas effector T cells specific for an irrelevant antigen did not enter CNS lesions.[54] Therefore, our data could suggest that active infiltration of encephalitogenic T cells primed in the periphery is not sufficient for generation of CNS autoimmunity despite the inflammatory potential of autoimmune responses and the potential T cell pathogenicity, and instead suggests that clinical induction of autoimmune disease is dependent on local reactivation of infiltrating T cells.[56] Furthermore, the differentiation processes of pathogenic T cells in the target organ under autoimmune conditions might enable the upregulation of NR4A2 in CD4+ T cells after reactivation by interactions with target organ antigen-presenting cells expressing CNS antigens, and the NR4A2-expressing T cells could fully represent activated pathogenic Th17 cells. Indeed, when NR4A2 expression is prevented by administration of NR4A2-specific siRNA *in vivo*, CNS-infiltrating T cells are still observed, albeit at lower numbers, but the Th17 responses in the target organ are markedly reduced with a reduction in clinical EAE. Again, although the importance of the activation of particular local responses has been previously suggested, our data shows that pathogenic Th17 responses in EAE result from a critical differentiation in the target organ.[11] The subsequent appearance of NR4A2 expression in the peripheral blood might represent T cells trafficking from, rather than to, the target organ and perhaps it is these T cells that are later reactivated after returning to the target organ and triggering disease relapses. Thus, NR4A2 might provide a cell marker identifying T cells, both in the target organ and circulatory systems, which have been reactivated during pathogenic inflammatory responses in the CNS. In addition, measurement and manipulation of NR4A2 could prove to be useful in clinical settings. As NR4A2 expression by peripheral blood CD4+ T cells is only observed after the initiation of inflammatory Th17 responses in the target organ, the presence of NR4A2 expression in the blood might be used to indicate when such responses have developed or that they are ongoing. It is conceivable that the status of immune activation in a target organ might be determined by measuring NR4A2 expression in a patient's blood. Thus, the use of NR4A2 as a biomarker for MS could indicate whether T cell infiltration into the target organ has been recently established, giving valuable insight into disease status.

Regulation of IL-21 by NR4A2 in autoimmunity

IL-21 is a pleiotropic cytokine primarily produced by activated T cells.[57] IL-21 plays a pivotal role in CD4+ T cell differentiation, the survival of both CD4+ and CD8+ T cells, and the effector function of cytotoxic T cells.[58–68] In addition, IL-21 is crucial for B cell survival and differentiation, leading to proper development of antibody-producing cells secreting mature immunoglobulins.[61] Intriguingly, IL-21 is shown to have a strong link to inflammation and autoimmune

diseases,[62] such as patients with systemic lupus erythematosus (SLE),[63,64] inflammatory bowel diseases (IBD)[65,66] and type 1 diabetes (T1D),[67,68] or in animal models for SLE,[69] IBD,[66] T1D[70] and rheumatoid arthritis (RA).[71] Although follicular helper T cells are known as professional IL-21-producing CD4+ T cells, IL-21 is also produced by Th17 cells.[72] The primary role for IL-21 in mouse Th17 cells is the expansion of developing Th17 cells.[17] IL-6 can induce the production of IL-21 by Th17 cells, and in turn, IL-21 acts in an autocrine manner to induce the expression of IL-23R on Th17 cells and further stabilize the Th17 phenotype.[73] The requirement of IL-21 for Th17 cell development *in vivo* is still controversial, as some reports show that Th17 cells can develop in the absence of IL-21,[74,75] whereas other reports show that the generation of Th17 cells is impaired in the absence of IL-21.[73,76,77] This is partly due to the redundant role of IL-6 and IL-21, in which Th17 cell differentiation *in vivo* under a strong inflammatory milieu with massive IL-6 production would conceal the effects of IL-21.[73,77] Interestingly, the amount of IL-21 production induced by Th17 differentiation is strongly reduced by transfection of NR4A2-specific siRNA into differentiating Th17 cells *in vitro*. We further showed that the sequential upregulation of IL-21 and c-Maf, followed by the induction of IL-23R and IL-17 transcripts during Th17 differentiation, was abolished when NR4A2 expression was prevented by siRNA treatment. Therefore, one consequence of preventing NR4A2 upregulation during Th17 differentiation *in vitro* is an absence of IL-21 secretion. The essential role of NR4A2 expression in Th17 differentiation through regulation of IL-21 is shown by the fact that the addition of exogenous IL-21 led to IL-23R upregulation, subsequently yielding normal IL-17 secretion. The precise mechanisms of the NR4A2-mediated regulation of IL-21 in Th17 cell development *in vivo* have not yet been elucidated, but it is postulated that NR4A2 might regulate T cell production of IL-17 *in vivo* by controlling signaling pathways intrinsic for effective Th17 cell differentiation (Fig. 3).

One obvious mechanism by which IL-21 drives autoimmunity is by supporting the expansion, promotion and survival of pathogenic helper T cell subsets. Accordingly, it is well known that autoimmune-prone mice produce more IL-21 compared with resistant strains,[59,68] and that the level of IL-21 production is well correlated to the progression of autoimmune diseases.[60] As aforementioned, IL-21 has a strong link to inflammation and organ-specific or systemic autoimmune diseases including SLE, IBD, T1D, and RA. Regarding the possible link of the

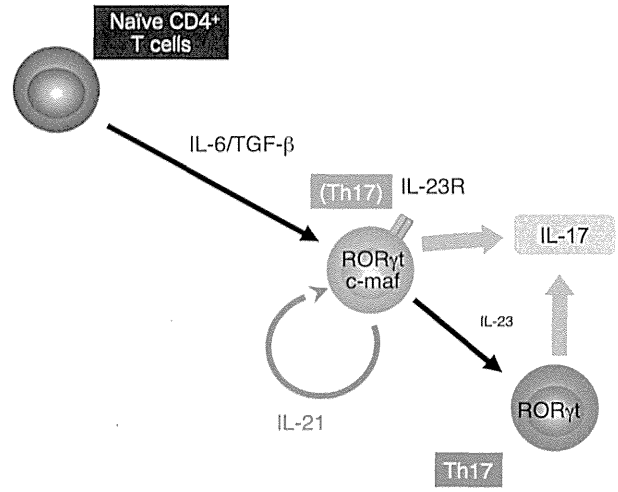


Figure 3 Multistep differentiation processes of T helper 17 (Th17) cells. T cell receptor stimulation of naive T cells in the presence of transforming growth factor (TGF)-β and interleukin (IL)-6 triggers initial Th17 cell differentiation. Th17 cells acquiring *c-Maf* expression produce IL-21, which augments Th17 cell amplification in an autocrine manner. IL-21 induces expression of the IL-23 receptor on the surface of differentiating Th17 cells that renders them responsive to IL-23. Exogenous IL-23 stabilizes the Th17 phenotype to secrete IL-17 and confers its effector function. RORγt, retinoic acid-related orphan receptor γt.

IL-21–Th17 axis to human CNS autoimmunity, such as MS, the proportion of memory Th17 cells and the IL-17 level are both shown to be much higher in patients with MS and neuromyelitis optica (NMO).[78] Accordingly, CNS-infiltrating cells expressing IL-21 were observed in both acute and chronic active white matter MS lesions in which IL-21 expression was restricted to CD4+ helper T cells.[6] Furthermore, therapeutic treatment with alemtuzumab causes secondary autoimmunity in a subset of MS patients who selectively show higher levels of serum IL-21, possibly through excessive T cells apoptosis and cell cycling after alemtuzumab-mediated lymphocyte depletion.[79] Interestingly, there are a couple of reports suggesting a significant correlation between IL-21 and NMO. First, production of IL-6 and IL-21 by CD4+ T cells *ex vivo* is shown to be directly associated with neurological disability in NMO patients.[80] In addition, higher concentrations of serum IL-21 were observed in NMO patients,[81] and concentrations of IL-21 protein in cerebrospinal fluid were significantly elevated in NMO patients, suggesting a positive correlation with humoral immunity.[82] Therefore, regulation of the IL-21–Th17 cell axis through NR4A2-mediated intervention holds considerable significance not only for MS, but also for NMO and other related neuroimmunological diseases.

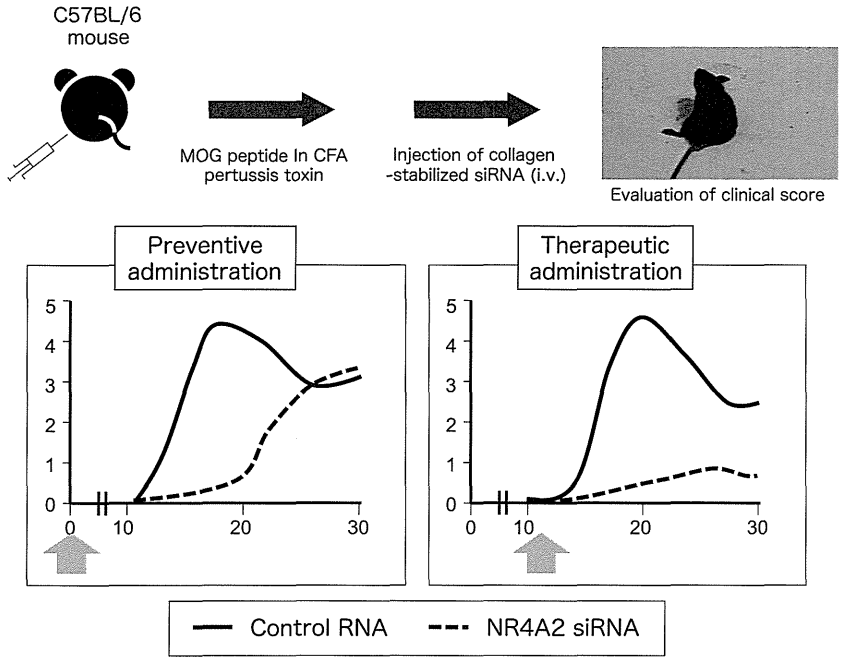


Figure 4 Effect of systemic administration of NR4A2-specific small interfering RNA (siRNA) on experimental autoimmune encephalomyelitis (EAE). NR4A2-specific or control siRNA was stabilized in a collagen matrix and administered intravenously to EAE mice either at the time of (preventive administration) or 10 days after (therapeutic administration) myelin oligodendrocyte glycoprotein (MOG) immunization. CFA, complete Freund's adjuvant.

NR4A2 as a possible target for MS therapy

NR4A2 expression is associated with the generation of autoimmune Th17 responses, and NR4A2 appears to control Th17 differentiation. We then aimed to prevent NR4A2 upregulation *in vivo* by systemically administering NR4A2-specific siRNA stabilized in a collagen matrix.[14] Both clinical EAE and NR4A2 expression in T cells were significantly reduced, with peak disease delayed by 10 days (Fig. 4). In addition, IL-17 production, but not IFN-γ production by CD4+ T cells infiltrating the CNS, was also reduced. NR4A2 siRNA-treatment prevented the accumulation of CD4+ T cells, particularly those secreting IL-17 into the CNS. Furthermore, equivalent numbers of IL-17-producing T cells in the CNS were observed between the control and the NR4A2 siRNA-treated mice at the delayed onset of clinical EAE. The late onset of EAE could be attributed to degradation of the siRNA, as administration of the siRNA at the onset of EAE significantly prevented the induction of clinical EAE. These findings suggest that the absence of NR4A2 expression during active autoimmune disease reduces clinical symptoms of EAE and Th17 responses. Therefore, NR4A2 might prove to be a potent therapeutic target for the treatment of MS and other Th17-mediated autoimmune diseases.

It is well known that methotrexate significantly suppresses expression of NR4A2 in patients with active psoriatic arthritis.[83] Accordingly, the expression level of NR4A2 after treatment with methotrexate is well-correlated to the disease activity score. Therefore, intervention of NR4A2 activity with chemical compounds might provide a potential strategy for future treatment of MS. In addition, the fact that NR4A2 mutations are associated with familial Parkinson's disease has led to significant interest in the identification of selective low-molecular-weight modulators that are helpful for analyzing the mode of action of the NR4A subfamily.[43] A growing number of NR4A2 modulators with unique chemical structures have also been described.[84–87] Furthermore, the antineoplastic and anti-inflammatory drug, 6-mercaptopurine, has been shown to activate NR4A2 through modulation of the cellular content of purine nucleotides.[88] A number of typical and atypical antipsychotic drugs, such as haloperidol, chlorpromazine, clozapine and so on, induce the transcription of NR4A2, even though they are all developed to augment NR4A2 activity.[89,90] Therefore, therapeutic application of NR4A2 inhibitors or NR4A2 modifiers converted from those NR4A2 activators through modification of chemical structure might be considered for possible future treatment of RR-MS.

Future perspective

EAE is a versatile experimental model useful for analyzing the immunopathological, neuropathological and therapeutic aspects of MS, including inflam-

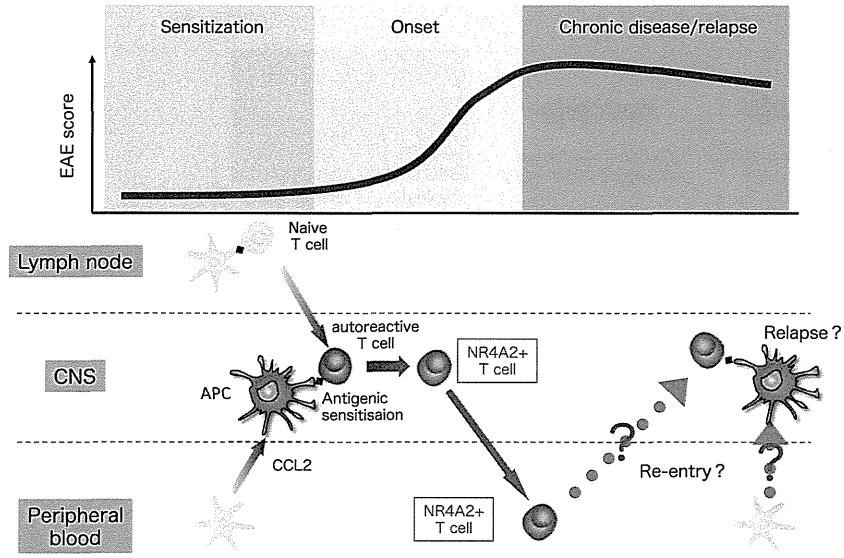


Figure 5 Possible behavior of NR4A2-expressing T helper 17 (Th17) cells during the course of multiple sclerosis/experimental autoimmune encephalomyelitis (EAE). On encountering the myelin antigen, naive T cells are primed to differentiate into effector T cells, such as Th17 cells in secondary lymphoid tissue. Then, those effector T cells are recruited to the central nervous system (CNS) and restimulated with antigen presented by local antigen presenting cells, resulting in upregulation of NR4A2 expression in pathogenic Th17 cells. Th17 cell-mediated local inflammation causes recruitment of inflammatory effector cells to the CNS, leading to immunopathogenic symptoms of multiple sclerosis/EAE. The subsequent appearance of NR4A2 expression in the peripheral blood could represent T cells trafficking from the target organ. At the later phase of EAE, T cells might egress the target organ to peripheral circulation and perhaps it is these T cells that are reactivated thereafter in the target organ triggering disease relapses. Therefore, NR4A2 could provide a good biomarker for identifying pathogenic T cells in both the target organ and in circulation. APC, antigen-presenting cells; CCL2, chemokine (C-C motif) ligand 2.

mation, demyelination, axonal damage, and after gliosis, the resolution of inflammation, remyelination and drug screening. Given the data showing that NR4A2 is selectively upregulated in the peripheral blood T cells of RR-MS patients, we have shown a strong link between NR4A2-expressing Th17 cells and their pathogenic role in CNS autoimmune inflammation through the analysis of EAE (Fig. 5), suggesting that NR4A2 represents a promising therapeutic target for MS and other autoimmune diseases. In addition, there are other inflammatory CNS diseases with distinct, but overlapping with the RR-MS, phenotype, such as NMO, progressive forms of MS and related demyelinating diseases.[91] Therefore, further analysis of RR-MS as an NR4A2-expressing Th17-mediated autoimmune disease will provide helpful clues for understanding the pathogenesis of CNS autoimmunity.

Acknowledgement

This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan (JSPS KAKENHI, Grant Number 24590497), and a research grant on "Super Special Consortia" for supporting the development of cutting-edge medical care from the Cabinet Office, Government of Japan.

Conflict of interest

None.

References

1. McFarland HF, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol.* 2007; **8**(9): 913–9.
2. Beecham AH, Patsopoulos NA, Xifara DK, et al. Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. *Nat Genet.* 2013; **45**(11): 1353–60.
3. Sawcer S, Hellenthal G, Pirinen M, et al. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature.* 2011; **476**(7359): 214–9.
4. Goverman J. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol.* 2009; **9**(6): 393–407.
5. Matusevicius D, Kivisakk P, He B, et al. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler.* 1999; **5**(2): 101–4.
6. Tzartos JS, Friese MA, Craner MJ, et al. Interleukin-17 production in central nervous system-infiltrating T cells

and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol.* 2008; **172**(1): 146–55.
7. Cua DJ, Sherlock J, Chen Y, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature.* 2003; **421** (6924): 744–8.
8. Komiyama Y, Nakae S, Matsuki T, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol.* 2006; **177**(1): 566–73.
9. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* 2005; **201**(2): 233–40.
10. McGeachy MJ, Cua DJ. The link between IL-23 and Th17 cell-mediated immune pathologies. *Semin Immunol.* 2007; **19**(6): 372–6.
11. Bailey SL, Schreiner B, McMahon EJ, Miller SD. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4+ T(H)-17 cells in relapsing EAE. *Nat Immunol.* 2007; **8**(2): 172–80.
12. Satoh J, Nakanishi M, Koike F, et al. Microarray analysis identifies an aberrant expression of apoptosis and DNA damage-regulatory genes in multiple sclerosis. *Neurobiol Dis.* 2005; **18**(3): 537–50.
13. Doi Y, Oki S, Ozawa T, Hohjoh H, Miyake S, Yamamura T. Orphan nuclear receptor NR4A2 expressed in T cells from multiple sclerosis mediates production of inflammatory cytokines. *Proc Natl Acad Sci USA.* 2008; **105**(24): 8381–6.
14. Raveney BJ, Oki S, Yamamura T. Nuclear receptor NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signalling. *PLoS One.* 2013; **8**(2): e56595.
15. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 2006; **441**(7090): 235–8.
16. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 2006; **126** (6): 1121–33.
17. Bauquet AT, Jin H, Paterson AM, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. *Nat Immunol.* 2009; **10**(2): 167–75.
18. Awasthi A, Kuchroo VK. Th17 cells: from precursors to players in inflammation and infection. *Int Immunol.* 2009; **21**(5): 489–98.
19. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. *Nature.* 2010; **467**(7318): 967–71.
20. Lee YK, Turner H, Maynard CL, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity.* 2009; **30** (1): 92–107.
21. Yang XO, Nurieva R, Martinez GJ, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity.* 2008; **29**(1): 44–56.
22. Muranski P, Borman ZA, Kerkar SP, et al. Th17 cells are long lived and retain a stem cell-like molecular signature. *Immunity.* 2011; **35**(6): 972–85.
23. Hirota K, Duarte JH, Veldhoen M, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol.* 2011; **12**(3): 255–63.
24. Buonocore S, Ahern PP, Uhlig HH, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature.* 2010; **464**(7293): 1371–5.
25. Eberl G, Littman DR. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. *Immunol Rev.* 2003; **195**: 81–90.
26. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol.* 2011; **164**(4): 1079–106.
27. Chu CQ, Wittmer S, Dalton DK. Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med.* 2000; **192**(1): 123–8.
28. Ferber IA, Brocke S, Taylor-Edwards C, et al. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol.* 1996; **156**(1): 5–7.
29. Korn T, Oukka M, Kuchroo V, Bettelli E. Th17 cells: effector T cells with inflammatory properties. *Semin Immunol.* 2007; **19**(6): 362–71.
30. Maxwell MA, Muscat GE. The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal.* 2006; **4**: e002.
31. Perlmann T, Wallen-Mackenzie A. Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. *Cell Tissue Res.* 2004; **318**(1): 45–52.
32. Wallen A, Perlmann T. Transcriptional control of dopamine neuron development. *Ann N Y Acad Sci.* 2003; **991**: 48–60.
33. Decressac M, Volakakis N, Bjorklund A, Perlmann T. NURR1 in Parkinson disease–from pathogenesis to therapeutic potential. *Nat Rev Neurol.* 2013; **9**(11): 629–36.
34. Le WD, Xu P, Jankovic J, et al. Mutations in NR4A2 associated with familial Parkinson disease. *Nat Genet.* 2003; **33**(1): 85–9.
35. Cheng LE, Chan FK, Cado D, Winoto A. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J.* 1997; **16**(8): 1865–75.
36. Liu ZG, Smith SW, McLaughlin KA, Schwartz LM, Osborne BA. Apoptotic signals delivered through the T-cell receptor of a T-cell hybrid require the immediate-early gene nur77. *Nature.* 1994; **367**(6460): 281–4.
37. Woronicz JD, Calnan B, Ngo V, Winoto A. Requirement for the orphan steroid receptor Nur77 in apoptosis of T-cell hybridomas. *Nature.* 1994; **367**(6460): 277–81.

38. Han YF, Cao GW. Role of nuclear receptor NR4A2 in gastrointestinal inflammation and cancers. *World J Gastroenterol.* 2012; **18**(47): 6865–73.
39. McMorrow JP, Murphy EP. Inflammation: a role for NR4A orphan nuclear receptors? *Biochem Soc Trans.* 2011; **39** (2): 688–93.
40. Mix KS, Attur MG, Al-Mussawir H, Abramson SB, Brinckerhoff CE, Murphy EP. Transcriptional repression of matrix metalloproteinase gene expression by the orphan nuclear receptor NURR1 in cartilage. *J Biol Chem.* 2007; **282**(13): 9492–504.
41. Saijo K, Winner B, Carson CT, et al. A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. *Cell.* 2009; **137**(1): 47–59.
42. Wang Z, Benoit G, Liu J, et al. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature.* 2003; **423**(6939): 555–60.
43. Mattes H. NR4A subfamily of receptors and their modulators. In: Ottow EWH, eds. *Nuclear Receptors as Drug Targets. Methods and Principles in Medicinal Chemistry* 39. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim: Wiley-VCH; 2008. 431–52.
44. Oki S, Raveney BJE, Doi Y, Yamamura T. Versatile orphan nuclear receptor NR4A2 as a promising molecular target for multiple sclerosis and other autoimmune diseases. In: Shibasaki MIM, Osada H, eds. *Chembiomolecular Science: At the Frontier of Chemistry and Biology.* Springer Japan, Tokyo: Springer; 2012. 193–200.
45. Castillo SO, Baffi JS, Palkovits M, et al. Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. *Mol Cell Neurosci.* 1998; **11**(1–2): 36–46.
46. Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T. Dopamine neuron agenesis in Nurr1-deficient mice. *Science (New York, NY).* 1997; **276**(5310): 248–50.
47. Baffi JS, Palkovits M, Castillo SO, Mezey E, Nikodem VM. Differential expression of tyrosine hydroxylase in catecholaminergic neurons of neonatal wild-type and Nurr1-deficient mice. *Neuroscience.* 1999; **93**(2): 631–42.
48. Sakurada K, Ohshima-Sakurada M, Palmer TD, Gage FH. Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development (Cambridge, England).* 1999; **126**(18): 4017–26.
49. Lammi J, Huppunen J, Aarnisalo P. Regulation of the osteopontin gene by the orphan nuclear receptor NURR1 in osteoblasts. *Mol Endocrinol.* 2004; **18**(6): 1546–57.
50. Pirih FQ, Tang A, Ozkurt IC, Nervina JM, Tetradis S. Nuclear orphan receptor Nurr1 directly transactivates the osteocalcin gene in osteoblasts. *J Biol Chem.* 2004; **279**(51): 53167–74.
51. Sekiya T, Kashiwagi I, Inoue N, et al. The nuclear orphan receptor Nr4a2 induces Foxp3 and regulates differentiation of CD4+ T cells. *Nat Commun.* 2011; **2**: 269.
52. Sekiya T, Kashiwagi I, Yoshida R, et al. Nr4a receptors are essential for thymic regulatory T cell development and immune homeostasis. *Nat Immunol.* 2013; **14**(3): 230–7.
53. Bartholomaus I, Kawakami N, Odoardi F, et al. Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature.* 2009; **462** (7269): 94–8.
54. Kawakami N, Nagerl UV, Odoardi F, Bonhoeffer T, Wekerle H, Flugel A. Live imaging of effector cell trafficking and autoantigen recognition within the unfolding autoimmune encephalomyelitis lesion. *J Exp Med.* 2005; **201** (11): 1805–14.
55. Aranami T, Yamamura T. Th17 Cells and autoimmune encephalomyelitis (EAE/MS). *Allergol Int.* 2008; **57**(2): 115–20.
56. Kawakami N, Lassmann S, Li Z, et al. The activation status of neuroantigen-specific T cells in the target organ determines the clinical outcome of autoimmune encephalomyelitis. *J Exp Med.* 2004; **199**(2): 185–97.
57. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. *Annu Rev Immunol.* 2008; **26**: 57–79.
58. Allard EL, Hardy MP, Leignadier J, et al. Overexpression of IL-21 promotes massive CD8+ memory T cell accumulation. *Eur J Immunol.* 2007; **37**(11): 3069–77.
59. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell.* 2004; **117**(2): 265–77.
60. McGuire HM, Vogelzang A, Ma CS, et al. A subset of interleukin-21+ chemokine receptor CCR9+ T helper cells target accessory organs of the digestive system in autoimmunity. *Immunity.* 2011; **34**(4): 602–15.
61. Parrish-Novak J, Dillon SR, Nelson A, et al. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. *Nature.* 2000; **408** (6808): 57–63.
62. Ettinger R, Kuchen S, Lipsky PE. Interleukin 21 as a target of intervention in autoimmune disease. *Ann Rheum Dis.* 2008; **67**(Suppl 3): iii83–6.
63. Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol.* 2004; **173**(9): 5361–71.
64. Bubier JA, Sproule TJ, Foreman O, et al. A critical role for IL-21 receptor signaling in the pathogenesis of systemic lupus erythematosus in BXSB-Yaa mice. *Proc Natl Acad Sci USA.* 2009; **106**(5): 1518–23.
65. Monteleone G, Monteleone I, Fina D, et al. Interleukin-21 enhances T-helper cell type I signaling and interferon-gamma production in Crohn's disease. *Gastroenterology.* 2005; **128**(3): 687–94.

66. Fina D, Sarra M, Fantini MC, et al. Regulation of gut inflammation and th17 cell response by interleukin-21. *Gastroenterology*. 2008; **134**(4): 1038–48.
67. Liu SM, Lee DH, Sullivan JM, et al. Differential IL-21 signaling in APCs leads to disparate Th17 differentiation in diabetes-susceptible NOD and diabetes-resistant NOD.Idd3 mice. *J Clin Investig*. 2011; **121**(11): 4303–10.
68. McGuire HM, Vogelzang A, Hill N, Flodstrom-Tullberg M, Sprent J, King C. Loss of parity between IL-2 and IL-21 in the NOD Idd3 locus. *Proc Natl Acad Sci USA*. 2009; **106**(46): 19438–43.
69. Herber D, Brown TP, Liang S, Young DA, Collins M, Dunussi-Joannopoulos K. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R.Fc reduces disease progression. *J Immunol*. 2007; **178**(6): 3822–30.
70. Spolski R, Kashyap M, Robinson C, Yu Z, Leonard WJ. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. *Proc Natl Acad Sci USA*. 2008; **105**(37): 14028–33.
71. Young DA, Hegen M, Ma HL, et al. Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in animal models of rheumatoid arthritis. *Arthritis Rheum*. 2007; **56**(4): 1152–63.
72. Liu SM, King C. IL-21-producing Th cells in immunity and autoimmunity. *J Immunol*. 2013; **191**(7): 3501–6.
73. Zhou L, Ivanov II, Spolski R, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*. 2007; **8** (9): 967–74.
74. Sonderegger I, Kisielow J, Meier R, King C, Kopf M. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo. *Eur J Immunol*. 2008; **38** (7): 1833–8.
75. Coquet JM, Chakravarti S, Smyth MJ, Godfrey DI. Cutting edge: IL-21 is not essential for Th17 differentiation or experimental autoimmune encephalomyelitis. *J Immunol*. 2008; **180**(11): 7097–101.
76. Korn T, Bettelli E, Gao W, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature*. 2007; **448**(7152): 484–7.
77. Nurieva R, Yang XO, Martinez G, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature*. 2007; **448**(7152): 480–3.
78. Li Y, Wang H, Long Y, Lu Z, Hu X. Increased memory Th17 cells in patients with neuromyelitis optica and multiple sclerosis. *J Neuroimmunol*. 2011; **234**(1–2): 155–60.
79. Jones JL, Phuah CL, Cox AL, et al. IL-21 drives secondary autoimmunity in patients with multiple sclerosis, following therapeutic lymphocyte depletion with alemtuzumab (Campath-1H). *J Clin Investig*. 2009; **119**(7): 2052–61.
80. Linhares UC, Schiavoni PB, Barros PO, et al. The ex vivo production of IL-6 and IL-21 by CD4+ T cells is directly associated with neurological disability in neuromyelitis optica patients. *J Clin Immunol*. 2013; **33**(1): 179–89.
81. Wang HH, Dai YQ, Qiu W, et al. Interleukin-17-secreting T cells in neuromyelitis optica and multiple sclerosis during relapse. *J Clin Neurosci*. 2011; **18**(10): 1313–7.
82. Wu A, Zhong X, Wang H, et al. Cerebrospinal fluid IL-21 levels in Neuromyelitis Optica and multiple sclerosis. *Can J Neurol Sci*. 2012; **39**(6): 813–20.
83. Ralph JA, McEvoy AN, Kane D, Bresnihan B, FitzGerald O, Murphy EP. Modulation of orphan nuclear receptor NURR1 expression by methotrexate in human inflammatory joint disease involves adenosine A2A receptor-mediated responses. *J Immunol*. 2005; **175**(1): 555–65.
84. Dubois C, Hengerer B, Mattes H. Identification of a potent agonist of the orphan nuclear receptor Nurr1. *Chem Med Chem*. 2006; **1**(9): 955–8.
85. Hintermann S, Chiesi M, von Krosigk U, Mathe D, Felber R, Hengerer B. Identification of a series of highly potent activators of the Nurr1 signaling pathway. *Bioorg Med Chem Lett*. 2007; **17**(1): 193–6.
86. Chintharlapalli S, Burghardt R, Papineni S, Ramaiah S, Yoon K, Safe S. Activation of Nur77 by selected 1,1-Bis (3′-indolyl)-1-(p-substituted phenyl)methanes induces apoptosis through nuclear pathways. *J Biol Chem*. 2005; **280**(26): 24903–14.
87. Morita K, Kawana K, Sodeyama M, Shimomura I, Kagechika H, Makishima M. Selective allosteric ligand activation of the retinoid X receptor heterodimers of NGFI-B and Nurr1. *Biochem Pharmacol*. 2005; **71**(1–2): 98–107.
88. Wansa KD, Muscat GE. TRAP220 is modulated by the antineoplastic agent 6-Mercaptopurine, and mediates the activation of the NR4A subgroup of nuclear receptors. *J Mol Endocrinol*. 2005; **34**(3): 835–48.
89. Beaudry G, Langlois MC, Weppe I, Rouillard C, Levesque D. Contrasting patterns and cellular specificity of transcriptional regulation of the nuclear receptor nerve growth factor-inducible B by haloperidol and clozapine in the rat forebrain. *J Neurochem*. 2000; **75**(4): 1694–702.
90. Maheux J, Ethier I, Rouillard C, Levesque D. Induction patterns of transcription factors of the nur family (nurr1, nur77, and nor-1) by typical and atypical antipsychotics in the mouse brain: implication for their mechanism of action. *J Pharmacol Exp Ther*. 2005; **313**(1): 460–73.
91. Karussis D. The diagnosis of multiple sclerosis and the various related demyelinating syndromes: a critical review. *J Autoimmun*. 2014; **49c**: 134–42.

Immunity
Article

Foxp3$^+$ T Cells Regulate Immunoglobulin A Selection and Facilitate Diversification of Bacterial Species Responsible for Immune Homeostasis

Shimpei Kawamoto,[1,6] Mikako Maruya,[1,6] Lucia M. Kato,[1,6] Wataru Suda,[4] Koji Atarashi,[2] Yasuko Doi,[1] Yumi Tsutsui,[1] Hongyan Qin,[1,5] Kenya Honda,[2] Takaharu Okada,[3] Masahira Hattori,[4] and Sidonia Fagarasan[1,*]
[1]Laboratory for Mucosal Immunity
[2]Laboratory for Gut Homeostasis
[3]Laboratory for Tissue Dynamics
Center for Integrative Medical Sciences (IMS-RCAI), RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi, Yokohama, 230-0045 Kanagawa, Japan
[4]Center for Omics and Bioinformatics, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwa-no-ha, Kashiwa, 277-8561 Chiba, Japan
[5]Department of Medical Genetics and Developmental Biology, 4th Military Medical University, Chang-Le Xi Street #17, Xi'an 710032, China
[6]Co-first author
*Correspondence: sidonia-f@rcai.riken.jp
http://dx.doi.org/10.1016/j.immuni.2014.05.016

SUMMARY

Foxp3$^+$ T cells play a critical role for the maintenance of immune tolerance. Here we show that in mice, Foxp3$^+$ T cells contributed to diversification of gut microbiota, particularly of species belonging to Firmicutes. The control of indigenous bacteria by Foxp3$^+$ T cells involved regulatory functions both outside and inside germinal centers (GCs), consisting of suppression of inflammation and regulation of immunoglobulin A (IgA) selection in Peyer's patches, respectively. Diversified and selected IgAs contributed to maintenance of diversified and balanced microbiota, which in turn facilitated the expansion of Foxp3$^+$ T cells, induction of GCs, and IgA responses in the gut through a symbiotic regulatory loop. Thus, the adaptive immune system, through cellular and molecular components that are required for immune tolerance and through the diversification as well as selection of antibody repertoire, mediates host-microbial symbiosis by controlling the richness and balance of bacterial communities required for homeostasis.

INTRODUCTION

The main function of the immune system is to protect the host against pathogens, such as bacteria or viruses. However, unlike the systemic immune system, the gut immune system does not eliminate microorganisms but instead nourishes rich bacterial communities and establishes advanced symbiotic relationships (Sutherland and Fagarasan, 2012). Not only are the gut bacteria essential for nutrient processing, production of vitamins, and protection against pathogens (through competition for space and nutrients), but the development and maturation of the immune system depends on these bacteria (Fagarasan et al., 2010; Geuking et al., 2011; Hooper et al., 2012; Sutherland and Fagarasan, 2012). The primary individual microbiota (Mb) composition probably reflects the maternal hand-over during or immediately after birth (Kau et al., 2011; Nicholson et al., 2012). However, the subsequent shaping of the microbial landscape is probably driven by complex interactions with the host immune system, through a network of regulatory components involving both the innate and adaptive immune system (Fagarasan et al., 2010; Hooper et al., 2012; Maynard et al., 2012).

Our previous studies demonstrated that the absence of immunoglobulin A (IgA) (the major effector molecule of the adaptive immunity in the gut) or the impaired IgA selection in germinal centers (GCs) due to deregulated T cell control severely affects the balance of gut bacterial communities, resulting in massive activation of the whole body immune system (Fagarasan et al., 2002; Kawamoto et al., 2012; Suzuki et al., 2004; Wei et al., 2011). The absence of a subset of Foxp3$^+$ T cells induced by bacterial antigens also modifies the composition of gut Mb by evoking mucosal T helper 2 (Th2) cell-mediated inflammation (Josefowicz et al., 2012). Interestingly, the Foxp3$^+$ T cells induce GC and IgA responses by generating GC T cells (Tsuji et al., 2009), and their depletion causes a rapid loss of specific IgA responses in the intestine (Cong et al., 2009). Together, all these observations point to the existence of a Foxp3-IgA axis in maintaining the balance of gut Mb. It remains unclear, however, how these specific arms of the adaptive immune system mediate host-microbial interactions in the gut.

We show that Foxp3$^+$ T cells, by acting in both GC-independent and -dependent manners, repress inflammation and support IgA selection in the GCs of Peyer's patches (PPs), resulting in diversification of gut Mb. Balanced and diverse Mb stimulates, in turn, the host immune system by promoting the expansion of Foxp3$^+$ T cells and induction of GC and IgA production in the gut through a symbiotic regulatory loop.

RESULTS

Reduced Diversity of Gut Microbiota in Immunodeficient Mice

We evaluated the impact of acquired immunity on gut Mb by analyzing various mice that partially or completely lack cellular and structural components of the adaptive immune system in gut. We found that mice lacking both B and T cells (*Rag1*$^{-/-}$), and as such having just rudimentary PPs, have considerably less diverse bacterial communities compared with *Rag1*$^{+/-}$ littermates or with wild-type (WT) mice raised in the same facility (Figure S1A available online). Not only the *Rag1*$^{-/-}$ mice, but also mice lacking only B cells (*Ighm*$^{-/-}$) or T cells (*Cd3e*$^{-/-}$), and thus lacking GCs, also had reduced bacterial diversity and different phylogenetic structures of bacterial communities compared with their heterozygous littermates or WT mice (Figures S1A and S1B). The results indicate that the adaptive immune system and its functionally organized follicular structures (i.e., PPs with GCs) facilitated diversification and influenced the structures of bacterial communities in gut.

Foxp3$^+$ T Cells Are Required for Maintenance of Diverse Microbial Communities in Gut

Given that the PPs are enriched in B cells that interact mainly with CD4$^+$ T cells, we next asked which CD4$^+$ T cell subset(s) contribute to diversity and composition of gut Mb. For this, we transferred distinct CD4$^+$ T cell populations (naive CD4$^+$ T cells and Foxp3$^+$ T cells) into *Cd3e*$^{-/-}$ mice, and the Mb were assessed 10–12 weeks after the injection. The transfer of naive CD4$^+$ T cells alone (isolated from the spleen and peripheral lymph nodes of WT mice) considerably decreased bacterial diversity even below that observed in *Cd3e*$^{-/-}$ mice (Figure 1A). The change was associated with gut inflammation caused by expansion of T cells with inflammatory properties (Figures 2A, 2B, and 2E). Therefore, in mice, similar to humans, the inflammatory environment due to deregulated T cell populations did not afford the maintenance of complex bacterial communities (Manichanh et al., 2006; Nishikawa et al., 2009; Ott et al., 2004). Furthermore, the transfer of naive CD4$^+$ T cells failed to increase bacterial diversity even in the absence of overt inflammation (i.e., in mice treated with anti-IL-12p40 that had considerably reduced expansion of inflammatory T cells and no signs of wasting disease or colitis) (Figures S1C–S1G). The cotransfer of naive CD4$^+$ T cells along with Foxp3$^+$ T cells led to reconstitution of the microbial diversity to levels observed in WT mice (Figure 1A). Not only the diversity but also the phylogenetic structures of bacterial communities become more similar to WT mice when naive CD4$^+$ T cells were transferred together with Foxp3$^+$ T cells (Figure 1B). In fact, the transfer of Foxp3$^+$ T cells alone increased bacterial diversity and modified the composition of Mb in *Cd3e*$^{-/-}$ mice almost to the degree found in WT mice (Figures 1A and 1B). Strikingly, the Foxp3$^+$ T cells facilitated the diversification of Firmicutes, particularly of nonpathogenic Clostridia belonging to cluster IV and XIVa, which were recently reported to be effective inducers of Foxp3$^+$ T cells in the gut (Figure 1C; Atarashi et al., 2011, 2013). Thus, not only can Firmicutes induce Foxp3 expression, but Foxp3$^+$ T cells can in turn feedback to Mb facilitating the maintenance and diversification of these major spore-forming bacteria. Taken together, the presence of Foxp3$^+$ T cells was required for the establishment of complex bacterial communities in both inflammatory and noninflammatory environments in the gut.

Requirement for Foxp3$^+$ T Cells Acting as Tfr Cells for Gut Microbiota Regulation

Foxp3$^+$ T cells could exert their regulatory effect on Mb in multiple ways. They could do it simply by preventing the expansion of Foxp3$^-$ T cells and their excessive production of cytokines and therefore by controlling inflammation in a GC-independent manner (Izcue et al., 2006; Josefowicz et al., 2012). Alternatively, the Foxp3$^+$ T cells could act through their regulatory roles on PP GCs and IgA synthesis, by becoming T follicular regulatory (Tfr) (CXCR5^{hi}PD1^{hi}Foxp3$^+$) and T follicular helper (Tfh) (CXCR5^{hi}PD1^{hi}Foxp3$^-$) cells, as previously reported (Chung et al., 2011; Cong et al., 2009; Linterman et al., 2011; Tsuji et al., 2009; Wollenberg et al., 2011). The transfer of Foxp3$^+$ T cells into *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice (which lack both T cells and antibodies others than IgM), failed to increase Mb diversity even though the cells expanded well and generated considerably more GCs, including Tfh and Tfr cells in PPs (Figures 1D and 1E). This observation strongly suggests that the Foxp3$^+$ T cells contributed to shape the Mb and supported the gut mutualism by regulation of IgA production in the intestine.

The generation of both Tfr and Tfh cells depends on activation and induction of Bcl6 expression (Chung et al., 2011; Johnston et al., 2009; Linterman et al., 2011; Nurieva et al., 2009; Yu et al., 2009). To discriminate between the regulatory functions outside and inside the GCs, we performed experiments with Foxp3$^+$ T cells sufficient or deficient for Bcl6 expression isolated from WT mice and *Bcl6*$^{yfp/yfp}$ mice, respectively, in which the function of Bcl6 was inactivated by yellow fluorescent protein (YFP) insertion (Kitano et al., 2011). Thus, naive CD4$^+$ T cells were cotransferred at a 1:1 ratio with CD4$^+$CD25$^+$ T cells (more than 98% of which expressed Foxp3) from WT mice (hereafter CD25$^+$WT T cells) or from *Bcl6*$^{yfp/yfp}$ mice (hereafter CD25$^+$ *Bcl6*$^{yfp/yfp}$ T cells) into *Cd3e*$^{-/-}$ mice. In the presence of naive CD4$^+$ T cells, both CD25$^+$ WT T cells and CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells had a similar expansion and maintenance of Foxp3 expression (Figure S2A). Furthermore, both CD25$^+$ WT T cells and CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells prevented the expansion of naive T cells in the gut and there were no signs of mucosal inflammation or wasting disease in these transferred mice (Figures 2A–2D). Accordingly, the production of cytokines like IFN-γ and TNF-α was reduced whereas that of IL-10 was increased in the presence Foxp3$^+$ T cells, regardless of their Bcl6 expression (Figure 2E). Both groups of mice remained protected from inflammation even 6 months after the transfer (Figures S3A–S3C). Therefore, in the gut, Foxp3$^+$ T cells controlled the expansion of naive CD4$^+$ T cells and their production of proinflammatory cytokines, independently of Bcl6 expression. However, CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells failed to normalize the bacterial diversity, which remained considerably lower than that observed in the presence of CD25$^+$ WT T cells (Figure 2F). The phylogenetic structures of bacterial communities in mice transferred with naive CD4$^+$ T cells together with CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells, although distinct from mice transferred with naive CD4$^+$ T cells alone, remained different from those in mice cotransferred with

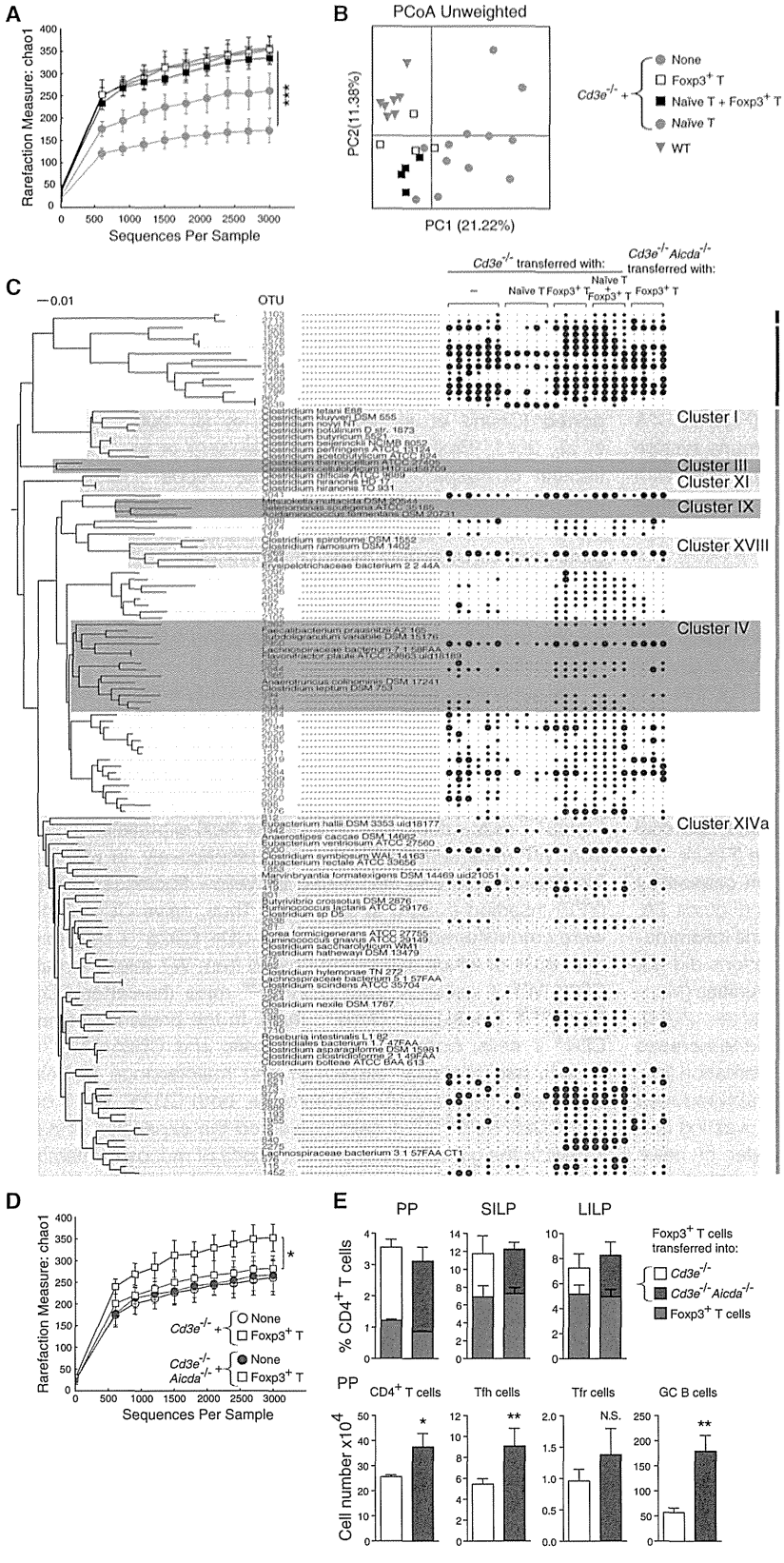


Figure 1. Increased Diversity of Firmicutes by Foxp3$^+$ T Cells

(A and D) Diversity of bacterial species (>97% identity) as indicated by Chao1 rarefaction measure based on 1–3,000 sequences.

(B) Unweighted UniFrac plot, comparing phylogenetic differences between microbial communities, clustered by principal coordinate analysis (PCoA).

(C) Phylogenetic analysis of OTUs associated with the presence of Foxp3$^+$ T cells. OTUs were defined and quantified by QIIME, followed by t test analyses between the Foxp3-deficient group (naive CD4$^+$ T cells transferred mice) and the Foxp3-sufficient group (Foxp3$^+$ T cells transferred alone or along with naive CD4$^+$ T cells). OTUs with statistically significant difference ($p < 0.05$) are shown. The results of nontransferred *Cd3e*$^{-/-}$ or *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice transferred with Foxp3$^+$ cells are shown in parallel. The 16S rRNA sequences of statistically different OTUs ($p < 0.05$) were used to construct the phylogenetic tree. The sequences of other Clostridia bacteria used for the tree were obtained from known genome sequences or ribosomal database project. The calculation was performed with the MEGA v.5.1 package and the neighbor-joining method with a bootstrap of 500 replicates. Circle size correlates with the relative abundance of each OTU. Large circles, medium circles, and small dots indicate ≥1%, <1%, and 0%, respectively.

(E) The percentage of CD4$^+$ T cells in the gut and the numbers of indicated cell populations in *Cd3e*$^{-/-}$ and *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice transferred with Foxp3$^+$ cells.

Mean ± SEM for four to seven mice per group. Two-tailed unpaired Student's t test was used to compare between (A) WT and *Cd3e*$^{-/-}$ + naive CD4$^+$ T cells and between (D and E) *Cd3e*$^{-/-}$ and *Cd3e*$^{-/-}$*Aicda*$^{-/-}$ mice transferred with Foxp3$^+$ cells; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, N.S., no significant difference. See also Figure S1.

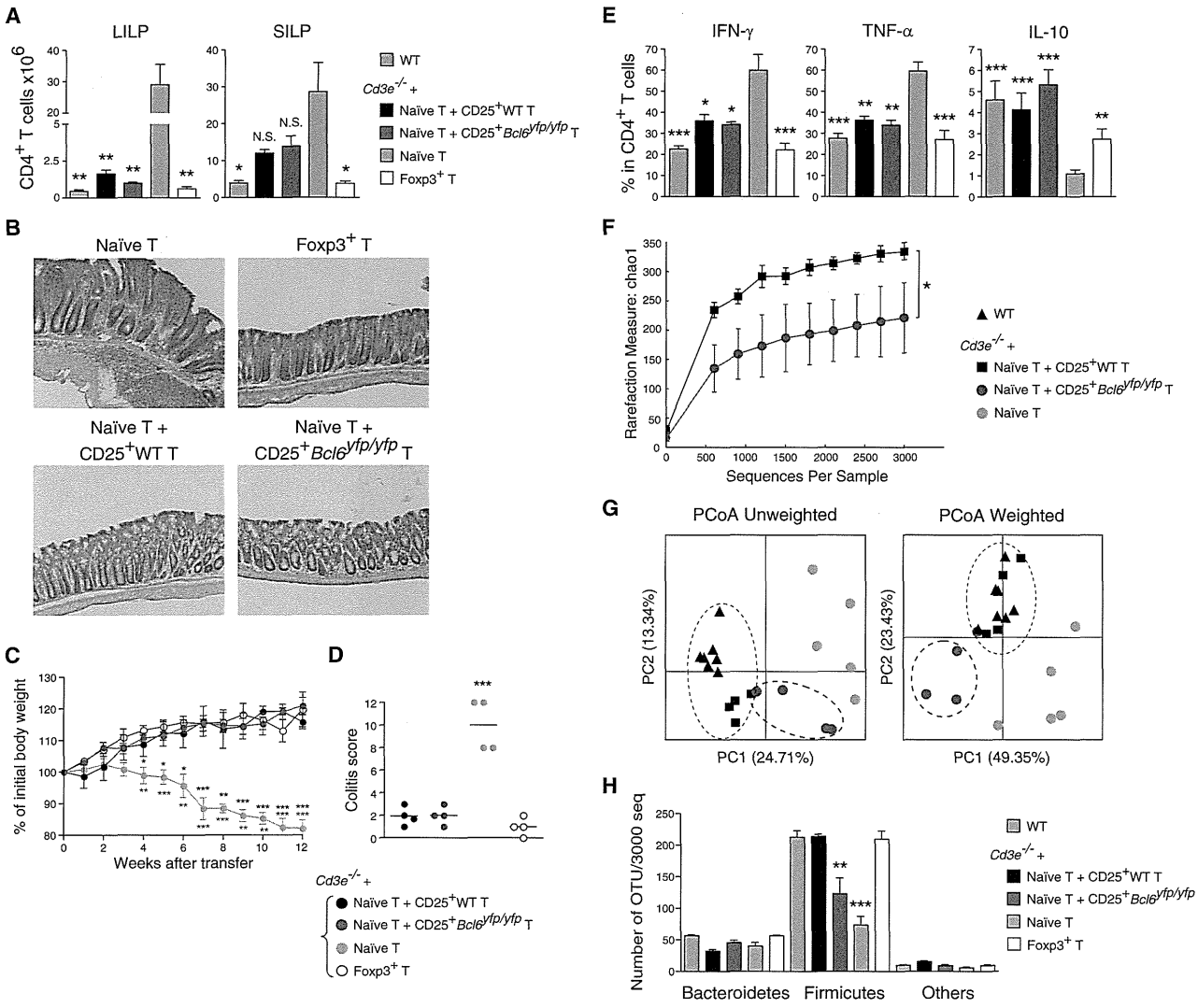


Figure 2. Foxp3$^+$ T Cell Migration into PP GCs Is Critical for Regulation of Microbiota

(A) Total numbers of CD4$^+$ T cells isolated from LILP and SILP.

(B) Hematoxylin-eosin staining sections of LI from *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cells. Note that CD25$^+$ T cells prevented the massive infiltration of inflammatory cells regardless of their Bcl6 expression. At least four mice per group were analyzed and representative data are shown.

(C) The change of body weight (presented as percent of original weight) of *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cells. Mean ± SEM for three to five mice per group.

(D) Colitis score for *Cd3e*$^{-/-}$ mice transferred with indicated T cells. Each point represents an individual mouse.

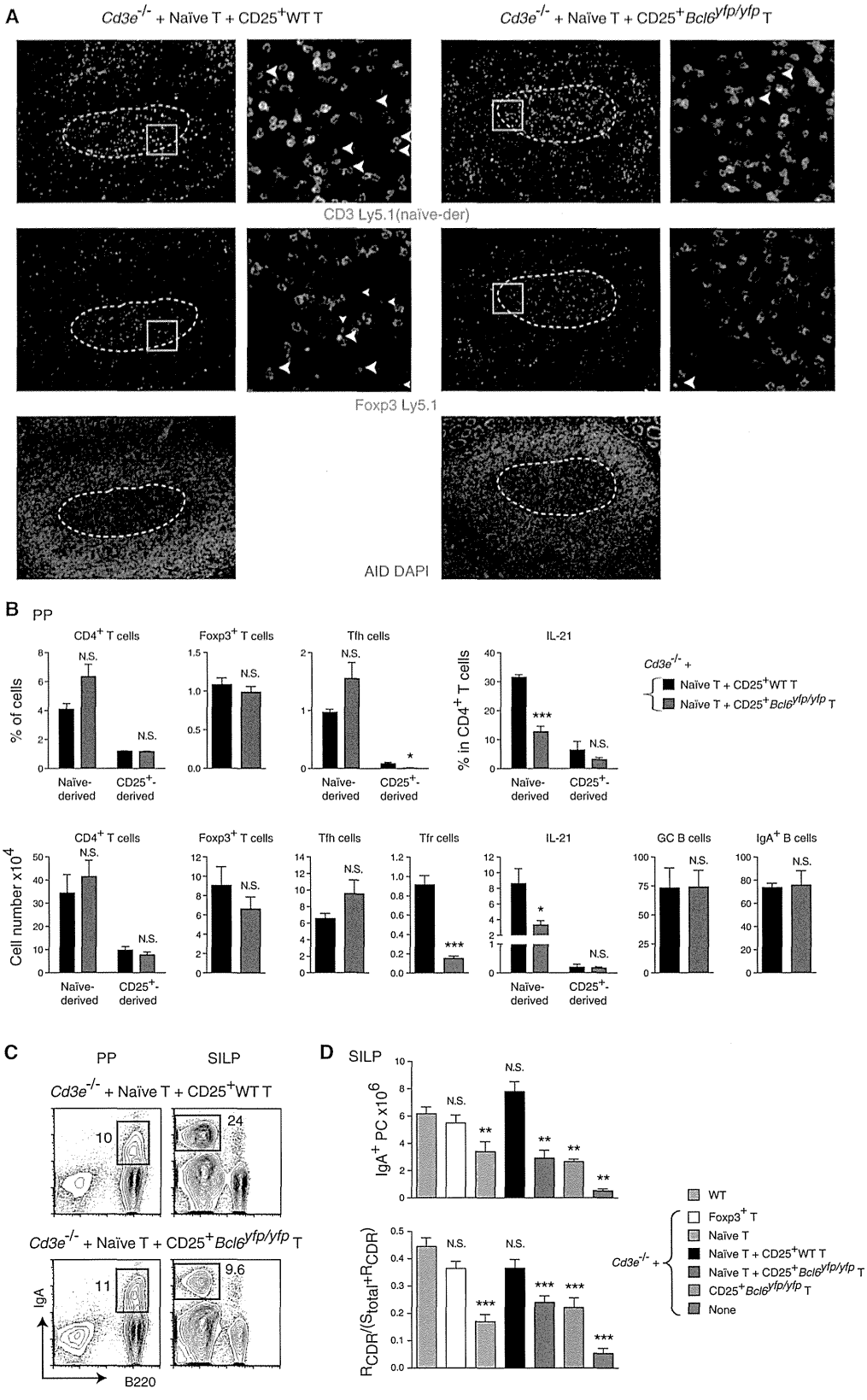
(E) Frequency of IFN-γ$^+$, TNF-α$^+$, and IL-10$^+$CD4$^+$ T cells from SILP of *Cd3e*$^{-/-}$ mice transferred with the indicated CD4$^+$ T cell subsets or from WT mice. Means ± SEM for four to six mice per group.

(F) Diversity of bacterial species as indicated by Chao1 rarefaction measure based on 1–3,000 sequences in *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cell subsets; four mice per group were analyzed.

(G) Communities clustered by principal coordinates analyses of the unweighted and weighted UniFrac distances (which measure qualitative and quantitative differences between microbial communities, respectively).

(H) Species diversity (numbers of OTUs/3,000 sequences) in cecal contents from WT or *Cd3e*$^{-/-}$ mice 10–12 weeks after the transfer of indicated CD4$^+$ T cell subsets. Mean ± SEM from four to seven mice per group.

Two-tailed unpaired Student's t test was used to compare between the indicated mouse groups and (A and E) *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells, (C) *Cd3e*$^{-/-}$ mice transferred with naive T cells along with CD25$^+$ WT T cells or CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells, (D) other transferred mouse groups, (F) *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells together with CD25$^+$ WT T cells, or (H) WT mice; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; N.S., no significant difference. See also Figure S2.



(legend on next page)

CD25+ WT T cells (Figure 2G). Thus, we observed a pronounced reduction in diversity of Firmicutes in *Cd3e*$^{-/-}$ mice cotransferred with CD25+*Bcl6*$^{yfp/yfp}$ T cells compared with mice cotransferred with CD25+ WT T cells or WT mice (Figure 2H). The decrease in Firmicutes diversity was mostly due to the excessive expansion of species belonging to *Lachnospiraceae*, which induced an overall shift in bacterial communities with an increase in the ratio of Firmicutes to Bacteroidetes (2.05 and 0.73 in mice cotransferred with CD25+*Bcl6*$^{yfp/yfp}$ T cells and CD25+ WT T cells, respectively) (Figures S2B and S2C). Taken together, the results indicate that the maturation and differentiation of Foxp3+ T cells into GC Tfr or Tfh cells, which depends on Bcl6 expression, was important for maintaining symbiosis with gut Mb.

Foxp3+ T Cells Regulate Both Quantities and Qualities of IgAs

The cotransfer of naive T cells with either CD25+*Bcl6*$^{yfp/yfp}$ T cells or CD25+ WT T cells equally induced GCs in PPs, but with very different features (Figure 3A). Thus, the mice that received naive CD4+ T cells and CD25+*Bcl6*$^{yfp/yfp}$ T cells almost completely lacked Tfr cells yet contained many Tfh cells mostly derived from the naive CD4+ T cells (Figures 3A and 3B). In contrast, the mice that received naive CD4+ T cells with CD25+ WT T cells had GCs with apparently more Tfr cells and fewer Tfh cells (some of them also generated from CD25+ WT T cells upon downregulation of their Foxp3 expression) (Figures 3A, top and middle, and 3B). Interestingly, the production of IL-21 by PP T cells was considerably reduced in mice cotransferred with CD25+*Bcl6*$^{yfp/yfp}$ T cells, suggesting that the presence of Foxp3+ T cells in GCs might regulate cytokine production by Tfh cells (Figure 3B).

Strikingly, although there were no obvious differences in IgA+ B cells in PPs (Figures 3B and 3C), the frequencies and numbers of IgA plasma cells in the small intestine lamina propria (SILP) were much decreased in mice cotransferred with CD25+*Bcl6*$^{yfp/yfp}$ T cells (Figures 3C and 3D). More importantly, the IgAs produced in the absence of Bcl6 expression by Foxp3+ T cells had a decreased affinity maturation index, suggesting defective selection in the GCs, in agreement with previous observations (Figure 3D; Linterman et al., 2011). In contrast, the CD25+ WT T cells cotransferred with naive CD4+ T cells reconstituted the SILP of *Cd3e*$^{-/-}$ mice with high numbers of apparently well-selected IgA plasma cells (Figures 3C and 3D). Thus, Foxp3+ T cell presence in the GCs was critical for regulating both the qualities and frequencies of IgAs in the gut.

Quality of IgAs Regulates Diversity of Microbiota

Because GC and IgA regulation by Foxp3+ T cells appeared to contribute considerably to shaping of gut Mb, we next evaluated the bacteria-coating properties of IgAs elicited in the presence or absence of Foxp3+ T cells. Therefore, we stained fecal bacteria-bound IgA with antibodies recognizing both IgA heavy (V_H) and light ($V\kappa$) chains and established a setting and acquisition mode that allowed a clear separation of IgA-coated bacteria (Figures 4A, S4A, and S4B). In the absence of Foxp3+ T cell regulation, the proportion of IgA-coated bacteria increased, as did the overall intensity of IgA staining (Figures 4A and S4B), a feature also observed in patients with inflammatory bowel diseases (IBD) (van der Waaij et al., 2004). This abundantly coated bacteria profile could reflect differences in the bacterial communities (i.e., large-size bacteria giving stronger signals) or differences in IgA qualities and their binding properties. To distinguish between these possibilities, we sequenced and analyzed bacteria that were sorted as IgA^{neg}, IgA^{int}, and IgA^{hi} from the feces of *Cd3e*$^{-/-}$ mice transferred with different populations of CD4+ T cells or from control (WT) mice (Figure S4A). The identified species from noncoated or coated bacterial fractions (we consider one operational taxonomic unit [OTU] as one species) were used for principal coordinate analysis (PCoA) (Figure 4B). WT mice and *Cd3e*$^{-/-}$ mice transferred with Foxp3+ T cells exhibited a similar profile in which all fecal bacteria or IgA^{neg} groups were substantially separated from IgA^{neg} or IgA$^{int/hi}$ groups or from cecal bacteria. In contrast, in the absence of Foxp3+ T cell regulation (i.e., naive CD4+ T cells transferred alone or along with CD25+*Bcl6*$^{yfp/yfp}$ T cells), there was no clear distinction among IgA^{neg}, IgA^{int}, and IgA^{hi} fractions (Figure 4B). This was confirmed by plotting in Venn graphs, in which the size of circles in the Venn graph reflects the diversity of bacterial species for each fraction (Figures 4C, S4C, and S4D). In mice with Foxp3+ T cell regulation, a smaller percentage of bacterial species overlapped among all the IgA^{hi}, IgA^{int}, and IgA^{neg} fractions, and IgA^{hi} fraction exhibited higher diversity, compared with those in mice without Foxp3+ T cell regulation (Figures 4C, 4D, and S4C).

The less-overlapping profiles correlated with a higher affinity selection index of IgAs, whereas the largely overlapping profiles associated with reduced affinity selection index of IgAs (Figure 3D). To test whether selected and nonselected IgAs differently coat the same bacteria, equal amounts of IgA elicited in the presence or absence of Foxp3+ T cells were tested for binding in vitro against several defined anaerobic bacterial strains isolated from mouse gut. As shown in Figures S4E and S4F,

Figure 3. Foxp3+ T Cell Migration into PP GCs Is Critical for IgA Selection

(A) Representative sections of PPs in *Cd3e*$^{-/-}$ mice transferred with naive CD4+ T cells together with CD25+ WT T cells or CD25+*Bcl6*$^{yfp/yfp}$ T cells stained as indicated and revealing GC B cells and T cells. T cells derived from naive CD4+ T cells appeared in yellow, and the CD25+-derived T cells are stained in green only. The arrows in upper panels indicate the presence of Foxp3-derived Tfh cells, while the arrows in middle panels indicate Tfr cells. Smaller arrows indicate reduced levels of Foxp3 by some Tfr cells.

(B) The percentage and total numbers of the indicated cells in PPs of *Cd3e*$^{-/-}$ mice transferred with naive CD4+ T cells together with CD25+WT T cells or CD25+*Bcl6*$^{yfp/yfp}$ T cells. Mean ± SEM for three to five mice per group.

(C) Flow cytometric profiles of cells from PPs and SILP. B220$^-$IgA+ gate represent plasma cells. Numbers on plots indicate the frequency of cells in the gate.

(D) Total numbers and affinity maturation index of IgA-producing cells from the SILP of WT, *Cd3e*$^{-/-}$, and *Cd3e*$^{-/-}$ transferred with indicated CD4+ T cell subsets. Two to three mice per group, around 50 sequences per mouse were analyzed. R_{CDR}, replacement in CDR1 and CDR2; S_{total}, silent mutations in both CDRs and in framework regions 1 to 3 (FWR1–3).

Two-tailed unpaired Student's t test was used to compare between the indicated mouse groups and (B) *Cd3e*$^{-/-}$ mice transferred with naive CD4+ T cells together with CD25+ WT T cells or (D) WT; ***$p < 0.001$; N.S., no significant difference. See also Figure S3.

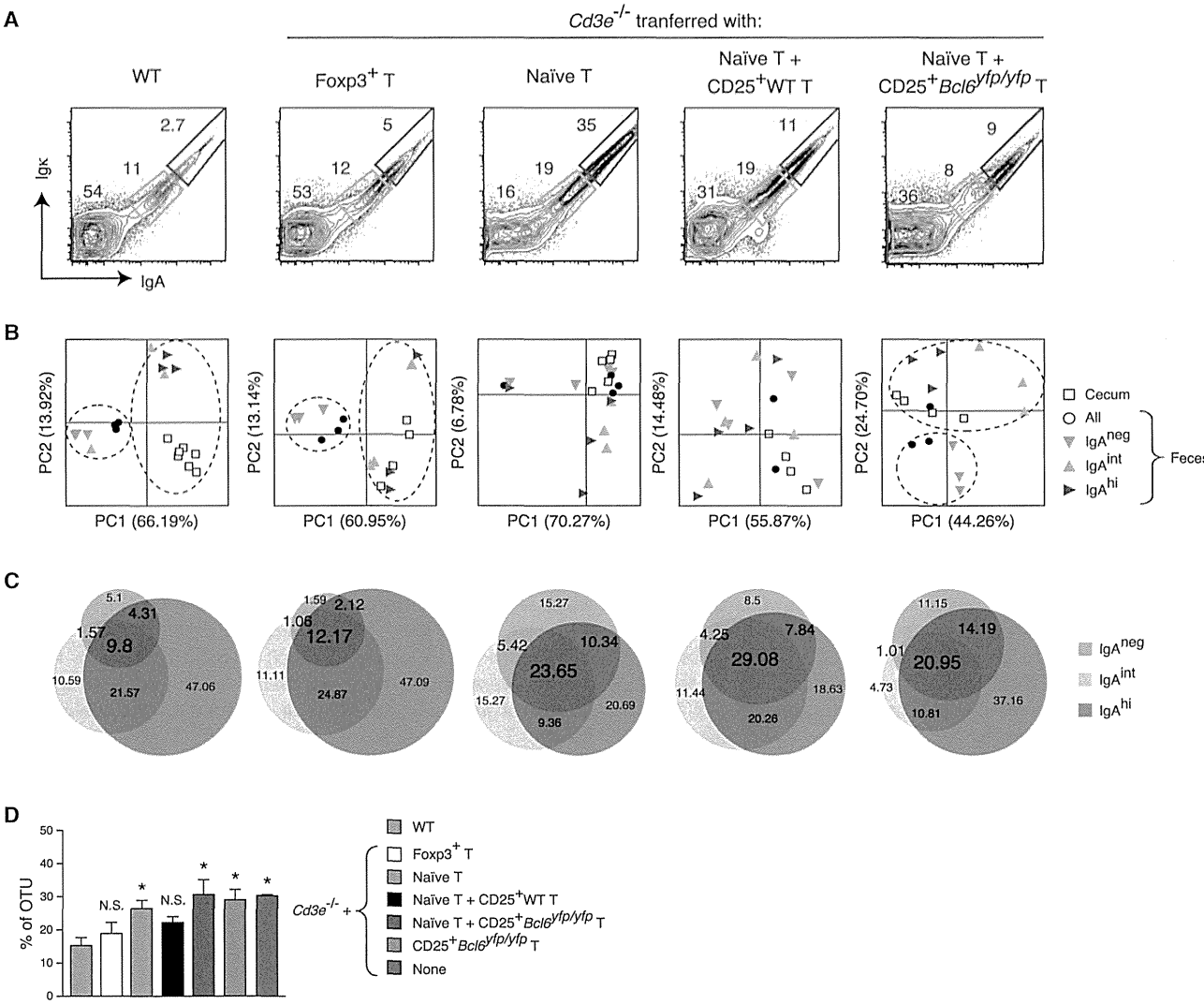


Figure 4. Specific IgA Coating Contributes Maintenance of Microbiota

(A) Representative flow cytometric profiles of fecal bacteria stained as indicated from WT or *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cell subsets. Sorting gates for IgA^{neg} (IgA$^-$Igκ$^-$), IgA^{int} (IgA^{int}Igκ^{int}), or IgA^{hi} (IgA^{hi}Igκ^{hi}) are indicated.

(B) Communities clustered by principal coordinate analyses of weighted UniFrac distance of 16S rRNA sequences from total cecal and fecal bacteria, and the IgA^{neg}, IgA^{int}, and IgA^{hi} sorted bacterial fractions from WT or *Cd3e*$^{-/-}$ mice transferred with indicated CD4$^+$ T cell subsets.

(C) Venn diagrams showing the frequencies of bacterial species (OTUs) from the IgA^{neg}, IgA^{int}, and IgA^{hi} sorted bacterial fractions from WT or *Cd3e*$^{-/-}$ mice 10–12 weeks after the transfer of the indicated CD4$^+$ T cell subsets. Data represent one of three to four experiments with consistent results. Numbers represent percentages.

(D) The frequency of OTU overlap among IgA^{neg}, IgA^{int}, and IgA^{hi} sorted bacterial fractions from WT or *Cd3e*$^{-/-}$ mice 10–12 weeks after the transfer of the indicated CD4$^+$ T cell subsets. Mean ± SEM from three to four mice per group. Two-tailed unpaired Student's t test was used to compare between WT and the indicated mouse groups; *$p < 0.05$; N.S., no significant difference.

See also Figure S4.

the nonselected IgAs from mice transferred with naive CD4$^+$ T cells or CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells had higher coating capacity than the IgAs from mice transferred with Foxp3$^+$ T cells. These results suggest that the IgAs generated and selected in the presence of GC Foxp3$^+$ T cells coated moderately and rather specifically a large diversity of bacterial species. This coating might contribute to maintenance rather than elimination of indigenous bacteria to keep the diversity.

Regulation of Immune System by Microbiota

We hypothesized that the presence of very diverse bacterial species would facilitate the perpetual induction of GCs and IgA and the maintenance of Foxp3$^+$ T cell pool in the gut. To test this hypothesis, we performed Mb transplantation experiments. Thus, germ-free (GF) mice were gavaged with Mb harvested from feces of *Cd3e*$^{-/-}$ mice transferred with Foxp3$^+$ T cells (hereafter Foxp3Mb) or naive CD4$^+$ T cells (hereafter Naive4Mb). Mb

harvested from nontransferred *Cd3e*$^{-/-}$ mice (CD3Mb) served as control. The mice were analyzed 2 weeks later. In the PPs, colonization with Foxp3Mb resulted in increase of B cells and T cells, and their activation and differentiation into GC B cells and Tfh cells, respectively (Figures 5A, 5C, S5A, and S5B). Interestingly, the Foxp3Mb induced preferential switching of GC B cells from IgM to IgA, the characteristic gut GC signature. In contrast, Naive4Mb or CD3Mb induced fewer GCs and Tfh cells, with the Naive4Mb supporting B cell class switching to IgG1 rather than to IgA, because of increased IL-4 production by CD4$^+$ T cells located in the PPs of these mice (Figures 5A and 5C). In the SILP, Foxp3Mb generated many more IgA-producing cells and Foxp3$^+$ T cells compared with CD3Mb or Naive4Mb (the latter inducing not Foxp3 but rather T cells secreting IL-4, IL-17, or TNF-α) (Figures 5B, 5D, and S5C). Importantly, the induction of these two characteristic gut homeostatic responses (IgA and Foxp3) by Foxp3Mb associated with a considerably higher diversity of bacterial species compared with mice transplanted with NaiveMb or CD3Mb (Figures 5E and 5F). Because most species correlating with the induction of IgA and Foxp3$^+$ T cells were Firmicutes (Figure S5D) and this phylum contains many spore-forming bacteria, we next performed gavage experiments with the spore fraction from Foxp3Mb (Spore^{Foxp3Mb}). Colonization of GF mice with Spore^{Foxp3Mb} induced GC B cells and Tfh cells that facilitated preferential switching to IgA in the PPs (Figures 5A, 5C, and S5B) and increased the IgA plasma cells compartment and favored the generation or expansion of Foxp3$^+$ T cells in the LP (Figures 5B, 5D, and S5C).

To further evaluate the link between Mb and gut Foxp3$^+$ T cells, we changed the experimental strategy slightly. We first inoculated GF *Cd3e*$^{-/-}$ mice with Foxp3Mb or Naive4Mb and 1 week later transferred Foxp3$^+$ T cells into these mice (Figure 6A). The T cell expansion and B cell responses were assessed 2 weeks after the cell transfer. As shown in Figures S6A and S6B, Foxp3Mb helped the expansion of Foxp3$^+$ T cells in MLN and LP of the SI and LI more vigorously than Naive4Mb. In the PPs, Foxp3Mb but not Naive4Mb facilitated the activation and differentiation of Foxp3$^+$ T cells into Tfh cells with GC and IgA-inducing properties (Figures 6A and 6B; Tsuji et al., 2009). Thus, Foxp3Mb promoted maturation of the gut immune system and exhibited robust Foxp3 and IgA-supportive properties in gut.

Dominant Immune-Regulatory Role of Foxp3Mb

To further evaluate the features of Foxp3Mb in more competitive settings, we performed experiments with specific-pathogen-free (SPF) young mice. Thus, 3-week-old WT mice were colonized by fur painting with Foxp3Mb. Adult WTMb, CD3Mb, or Naive4Mb mice were used for comparison. Strikingly, even in competitive situations, the Foxp3Mb (or a similarly complex and balanced adult WTMb) had IgA-inducing properties (Figure 7A). Indeed, compared with noninfected mice, the Foxp3Mb induced a substantial increase in frequencies and numbers of GCs and IgAs in PPs (Figures 7A and 7B). Naive4Mb or CD3Mb induced activation and IgA differentiation in PPs, but to a much more limited degree than Foxp3Mb or WTMb. Yet, the IgA production was increased when Naive4Mb was mixed with Foxp3Mb (Figures 7A and 7B). The results clearly demonstrated the prevalent IgA-inducing properties of Foxp3Mb. They also confirmed the potential of Mb selected and maintained by the immunocompetant host to dominantly regulate postnatal maturation of the immune system.

DISCUSSION

In this manuscript we have revealed that (1) differentiation of Foxp3$^+$ T cells into Tfr cells is required for the IgA's selection in GCs; (2) the amount and quality of IgAs directly influence the diversity and phylogenetic structure of bacterial communities; (3) rich and balanced Mb induce maturation of the gut immune system by promoting Foxp3$^+$ T cells and IgAs; and (4) in turn, the Foxp3$^+$ T cells and IgAs, through controlled diversification of stimulatory bacterial species, establish a self-regulatory loop mediating host-bacterial mutualism. Thus, it appears that the adaptive immune system contributes to the maintenance, rather than elimination, of complex microbial communities that probably enrich the genomic and metabolic capacity of the host, which is required for gut homeostasis and health.

Multiple studies revealed the importance of balanced Mb for the maintenance of gut barrier and immune homeostasis. Biased expansion of certain bacterial species impairs epithelial barrier and induces excessive activation of the immune system and generation of T cell subsets with inflammatory properties (Fagarasan et al., 2010; Kamada et al., 2013; Kawamoto et al., 2012; Littman and Pamer, 2011). Furthermore, reduced microbiome richness when accompanied by inflammatory phenotypes also associates with obesity, insulin resistance, and dyslipidemia (Karlsson et al., 2013; Le Chatelier et al., 2013; Qin et al., 2012). We demonstrated that the acquired arm of the immune system impacts considerably the diversity and phylogenetic structure of microbial communities in the gut.

We showed that Foxp3$^+$ T cell migration and differentiation into Tfr cells in the GCs is critical for IgA selection. The lack of Tfr cells associates with increased number of Tfh cells that license not only the mutated, presumably high-affinity, but also the germline and less mutated (and presumably poly- and/or self-reactive) B cells to emerge from the GCs, as previously reported (Baumjohann et al., 2013; Good-Jacobson et al., 2010; Kawamoto et al., 2012; Linterman et al., 2011; Vinuesa et al., 2013). In the absence of Tfr cell regulation, Tfh cells had skewed helper properties (e.g., due to production of different cytokines). The Tfh cell phenotype in GCs lacking Tfr cells may reflect not only the lack of direct suppressive effects of Tfr cells on Tfh cells, but also the downstream events resulting from deregulation of GCs.

Thus, it is highly likely that defective selection of IgAs leading to microbial changes would affect the generation of bacterial metabolic products that are required for induction of gut effector T cell subsets, like Foxp3$^+$, RORγt$^+$, or Foxp3$^+$RORγt$^+$ T cells (Atarashi et al., 2011, 2013; Ivanov et al., 2009; Lochner et al., 2008; Zhou et al., 2008). For example, short-chain fatty acids (SCFAs), especially butyric acid, derived from fermentation of dietary fibers by certain bacterial species (e.g., *Clostridia*), appear to facilitate the induction and expansion of Foxp3$^+$ T cells in gut through epigenetic changes (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). We also observed that a reduced bacterial diversity in *Cd3e*$^{-/-}$ mice coincided with lower intestinal amounts of SCFAs (including acetate, propionate, or butyrate)

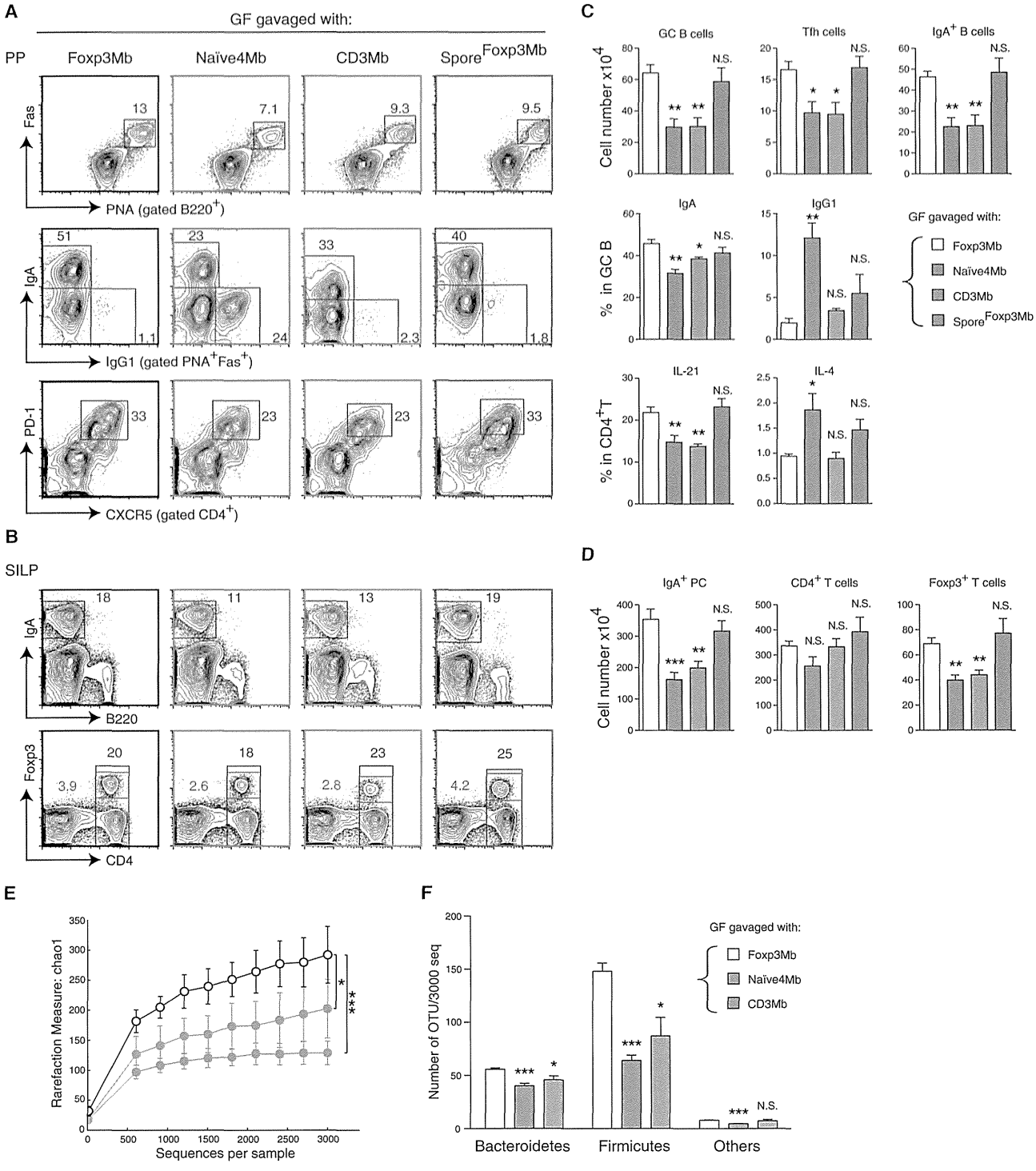


Figure 5. Foxp3-Regulated Microbiota Induces Maturation of Gut Immune System

(A–D) Flow cytometric profiles of (A) PP cells and (B) SILP stained as indicated and total numbers of indicated cell populations from (C) PPs and (D) SILP of GF mice gavaged with fresh microbiota obtained from *Cd3e*$^{-/-}$ mice transferred with naive CD4$^+$ T cells (Naive4Mb) or Foxp3$^+$ T cells (Foxp3Mb), or nontransferred *Cd3e*$^{-/-}$ mice (CD3Mb) as control. Gavage with spore fraction obtained from Foxp3Mb is also shown. Feces were obtained 10–12 weeks after T cell transfer. Mice were analyzed 2 weeks after bacterial transplantation. Data represent one of the three experiments with consistent results. At least four mice per group were analyzed.

(legend continued on next page)

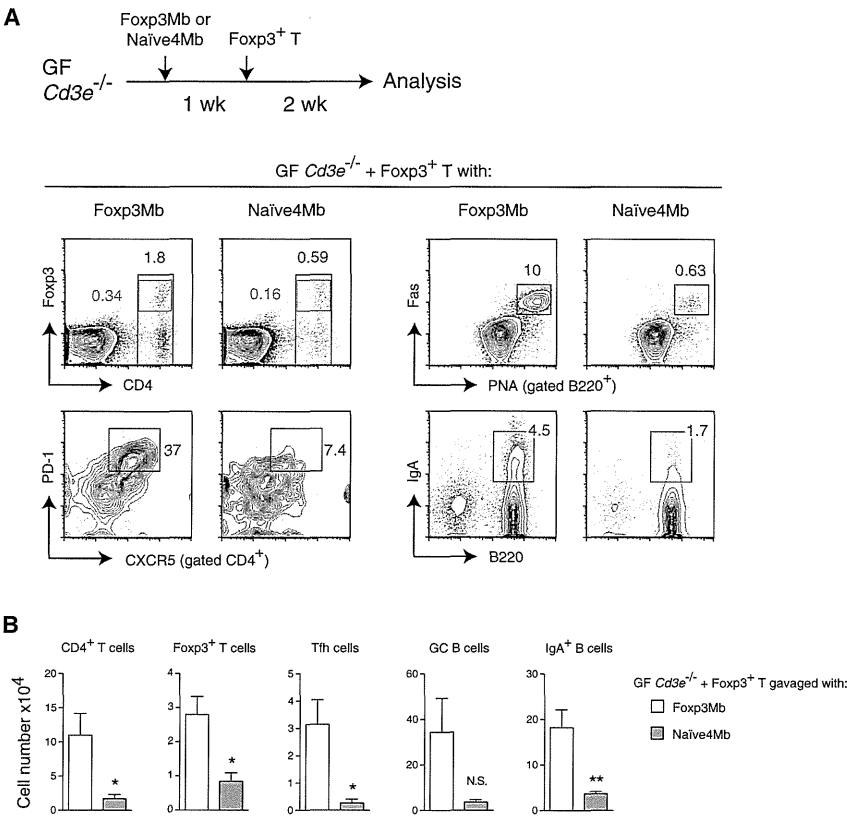


Figure 6. Foxp3-Regulated Microbiota Has Foxp3-Supportive Properties

(A) Scheme of the transfer experiment with GF *Cd3e*$^{-/-}$ mice and flow cytometric profiles of cells isolated from PPs of GF *Cd3e*$^{-/-}$ mice gavaged with fresh microbiota obtained from transferred mice and injected with Foxp3$^+$ T cells as indicated in scheme and stained for the indicated markers. Numbers indicate the frequency of cells in the gate. Data represent one of the two experiments with consistent results. At least four mice per group were analyzed.

(B) Total numbers of CD4$^+$ T, Foxp3$^+$ T, Tfh, GC B, and IgA$^+$ B cells in PP obtained from the indicated mice. Mean ± SEM in bar graphs for four mice per group. Two-tailed unpaired Student's t test was used to compare between these groups; $^{**}p < 0.01$; $^{*}p < 0.05$; N.S., no significant difference. See also Figure S6.

and that these levels recovered upon reconstitution of mice with Foxp3$^+$ T cells and normalization of Mb (data not shown). Additional microbial-derived metabolites are probably modulating other subsets of T cells, which upon activation and interaction with B cells could convert into GC Tfh cells with distinct helper characteristics (Hirota et al., 2013; Takahashi et al., 2012; Tsuji et al., 2009).

The Foxp3-IgA module probably involves complex feedback and feed-forward loops between Mb and immune cells that extend well beyond mucosal immune system (Fagarasan et al., 2002; Suzuki et al., 2004; Wei et al., 2011). For example, when Foxp3 control of GCs was missing (i.e., *Cd3e*$^{-/-}$ mice cotransferred with naive and CD25$^+$*Bcl6*$^{yfp/yfp}$ T cells), we observed a higher ratio of Firmicutes to Bacteroidetes even in the absence of overt inflammation. This shift associates with a considerable increased body weight, suggesting alterations in energy harvest and metabolism (Turnbaugh et al., 2006). In contrast, in the complete absence of Foxp3$^+$ T cells, there was a pronounced reduction of Firmicutes, which together with expansion of Proteobacteria could account for the decreased weight observed in such mice with overt inflammation (Elson and Cong, 2012).

We revealed that reduced diversification and affinity maturation of IgAs in the GCs associated with abundant coating of bacteria with largely nonspecific IgAs and reduced diversity and skewed gut Mb. Conversely, diversified and well-selected IgA repertoires in GCs associated not only with specific bacteria coating but also with rich and balanced bacterial communities. These observations suggest that bacteria coating by highly diversified and selected IgAs contributes to maintenance rather than elimination of indigenous bacteria, thus increasing the diversity and stability of Mb. It is accepted that IgA can control infection by coating pathogenic bacteria and preventing their contact to the gut epithelium, a process called immune exclusion (Strugnell and Wijburg, 2010). However, IgA coating of commensal bacteria might promote changes in the bacteria itself, such as modification in the bacterial gene expression (Peterson et al., 2007), influencing their metabolic processes as well as their biogeography, proliferation, and survival within the gut. There might be multiple mechanisms (which may work differently depending on the bacteria type, its growth stage and location, and available dietary components) by which IgA binding controls the Mb, but these remain to be elucidated in future studies.

Our study raises the question as to whether different types of Mb are "seen" differently by the immune system and, if so, whether they trigger distinct type of immune responses. We found that a complex and balanced Mb promptly elicits immune responses with typical mucosal characteristics, namely induction of GCs with IgA-supporting properties, and induction or expansion of CD4$^+$ T cells, especially of Foxp3$^+$ T cells. In contrast, a poor and skewed Mb provokes responses with mixed mucosal and systemic characteristics (i.e., GC

(E and F) Diversity of bacterial species (>97% identity) (E) and numbers of OTUs/3,000 sequences (F) in cecal contents from GF mice gavaged with the indicated microbiota; mean ± SEM from four to six mice per group. Two-tailed unpaired Student's t test was used to compare between the GF mice gavaged with Foxp3Mb and the indicated mouse groups; $^{***}p < 0.001$; $^{*}p < 0.05$; N.S., no significant difference.
See also Figure S5.