



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Dysregulation of humoral immunity in Foxp3 conditional-knockout mice

Yuki Tai ^{a,1}, Kazuki Sakamoto ^{a,1}, Azumi Takano ^a, Katsura Haga ^a, Yohsuke Harada ^{a,*}^a Laboratory of Pharmaceutical Immunology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba, 278-8510, Japan

ARTICLE INFO

Article history:

Received 6 February 2019

Received in revised form

5 April 2019

Accepted 12 April 2019

Available online xxx

Keywords:

Humoral immunity

Regulatory T cells

T follicular helper

Cells

Germinal center

ABSTRACT

Foxp3⁺ regulatory T cells (Tregs) are crucial for maintaining tolerance to self-antigens and preventing autoimmune diseases. Loss of Foxp3 expression leads to autoimmunity and disrupts humoral immune responses, including hyperproduction of immunoglobulin E (IgE). Elucidation of how Tregs control antibody production can lead to the development of new therapies for autoimmune and allergic diseases. However, premature death of Foxp3-deficient mice makes it difficult to analyze the roles of Tregs in humoral immunity of adult mice. In this study, we developed Foxp3 conditional-knockout mice (*Foxp3*^{fllox} *R26*^{CreERT2}) in which the *Foxp3* gene was inducibly deleted by tamoxifen administration. After oral administration of tamoxifen, titers of immunoglobulins, particularly IgG2c and IgE, were increased in *Foxp3*^{fllox} *R26*^{CreERT2} mice compared with that in controls. Under these conditions, CD4⁺ T cells from *Foxp3*^{fllox} *R26*^{CreERT2} mice had increased expression of several activation markers, including inducible costimulator and CD40 ligand, as well as the cytokines interleukin 4 and interferon gamma. In addition, the proportions of T follicular helper (Tfh) cells and germinal center (GC) B cells were increased in *Foxp3*^{fllox} *R26*^{CreERT2} mice compared with those in controls. These results indicated that Tregs controlled excessive or pathogenic antibody production by suppressing Tfh cell differentiation and GC formation. Furthermore, these data suggested that *Foxp3*^{fllox} *R26*^{CreERT2} mice could be a useful tool for screening therapeutic agents.

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1. Introduction

Naive CD4⁺ T cells are driven by antigenic stimulation and cytokines into effector T cells with distinct immunoregulatory functions. Follicular helper T (Tfh) cells are a specialized helper T cell subset providing essential help to B cells in the germinal center (GC), where Tfh cells control immunoglobulin (Ig) isotype switching and somatic hypermutation by CD40 ligand (CD40L) and cytokines, such as interleukin (IL)-4, IL-21, and interferon gamma (IFNγ) [1]. Tfh cells exhibit a gene expression profile distinct from Th1, Th2, and Th17 cells and are defined by expression of C-X-C chemokine receptor 5 (CXCR5) and transcription factors, including B-cell lymphoma 6 (Bcl6). Tfh cells also express inducible

costimulator (ICOS) and programmed cell death-1 (PD-1), which are important for their migration to B-cell follicles and for providing signals for initiation and maintenance of B-cell GC responses [2].

Foxp3⁺ regulatory T cells (Tregs) are crucial for maintenance of immune homeostasis and prevention of autoimmunity and allergy [3]. In antibody responses, Treg deficiency causes elevated levels of serum immunoglobulins, including autoantibodies and IgE [4,5]. Treg-deficient mice display increased number of Tfh cells, resulting in aberrant GC reactions and producing large numbers of plasma cells [6–8]. In humans, immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), which is an autoimmune disorder caused by mutation of the *Foxp3* gene, shows large amounts of autoreactive antibodies [9]. Both Foxp3-deficient mice and patients with IPEX also exhibit hyperproduction of IgE [4,10], which may be caused by increased levels of IL-4 [11,12], an indispensable cytokine for IgE class switch recombination in B cells. Although understanding the control of antibody production by Tregs is essential for establishment of therapeutic strategies for these immune diseases, the underlying mechanisms through which

Abbreviations: GC, germinal center; Tfh, follicular helper T; CXCR5, C-X-C chemokine receptor type 5; ICOS, inducible costimulator; PD-1, programmed cell death 1; Bcl6, B-cell lymphoma 6; IL, interleukin; CD40L, CD40 ligand; IFNγ, interferon gamma.

* Corresponding author.

E-mail address: yohsuke@rs.noda.tus.ac.jp (Y. Harada).¹ These authors equally contributed to this work.<https://doi.org/10.1016/j.bbrc.2019.04.090>

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Tregs exert their regulatory functions for humoral immunity remain poorly understood.

Although both *Foxp3*-knockout and scurfy mice, in which Tregs are absent from birth, are valuable tools to evaluate the roles of Tregs in the immune system, it is difficult to determine how Tregs regulate humoral immune responses because these mice exhibit premature death [3]. Furthermore, it is also difficult to discriminate whether particular phenotypes observed in *Foxp3*-deficient mice are caused by direct effects of Treg deficiency or are secondary to the resulting systemic inflammation. *Foxp3*^{DTR} strains have been developed to overcome these problems [13–15]. In these strains, *Foxp3*⁺ Tregs express diphtheria toxin (DT) receptor, allowing selective depletion of Tregs by DT administration in adult mice. However, long-term ablation of Tregs is difficult using these mouse models because of DT toxicity and the emergence of anti-DT neutralizing antibodies [16,17].

In this study, we generated mice carrying a floxed allele of the *Foxp3* gene (*Foxp3*^{flox}). To conditionally delete the *Foxp3* gene, we crossed the mice with a mouse strain ubiquitously expressing a tamoxifen-inducible Cre recombinase-estrogen receptor 2 fusion protein (CreERT2) from the *ROSA26* locus (*R26*^{CreERT2}) [18]. After tamoxifen administration, the conditional *Foxp3*-knockout mice (*Foxp3*^{flox}*R26*^{CreERT2}) exhibited increased levels of serum immunoglobulins, including IgE and anti-double stranded (ds) DNA IgG, accompanied by increased proportions of Tfh cells and GC B cells. These data suggested that *Foxp3*⁺ Tregs controlled humoral immunity by suppressing Tfh cell differentiation and GC formation. These mice may be a useful tool for screening of therapeutic agents for immune disorders caused by Treg abnormalities.

2. Materials and methods

2.1. Mice

Foxp3^{flox} mice were generated by targeted insertion of loxP sites to flank exons 1–3 of the *Foxp3* gene (Fig. 1) by using the CRISPR/Cas9 system. To construct the donor plasmid, we subcloned the genomic DNA fragment of the *Foxp3* gene from the C57BL/6 background by polymerase chain reaction into pUC118. A loxP site was inserted between exons –1 and 1 and between exons 3 and 4. The pX330 plasmid (#42230; Addgene) was used as a CRISPR expression vector. The CRISPR target sequences (left side: 5'-TAGTCTTGTCTGATTCTACTGGG-3', and right side: 5'-TGACTGATAATAGCGATTGTGG-3') were selected for insertion of loxP sites. The target sequences were inserted into pX330. The pX330 *Foxp3* vector and donor plasmid pUC118 *Foxp3*^{flox} were co-injected into the pronuclei of fertilized oocytes obtained from C57BL/6J mice. *Foxp3*^{flox} mice were generated at the Laboratory Animal Resource Center, University of Tsukuba. *R26*^{CreERT2} mice [18] were generously provided by T. Ludwig. C57BL/6J mice were purchased from Sankyo Labo Service (Tokyo, Japan). Two- to 3-month-old mice were used in this study. All mice used in this study were maintained under specific-pathogen-free conditions, and animal care was in accordance with the guidelines of Tokyo University of Science.

2.2. Tamoxifen treatment

Deletion of the loxP-flanked allele of the *Foxp3* gene was induced by the oral administration of 2 mg tamoxifen (Cayman Chemical) in corn oil (Wako) once per day.

In the immunization experiments, mice were orally administered tamoxifen for 5 consecutive days prior to immunization.

2.3. Immunization

Mice were immunized intraperitoneally with 100 µg 4-hydroxy-3-nitrophenyl-acetyl (NP) conjugated ovalbumin (OVA; Biosearch Technologies) in alum on day 0. For the induction of pristane-induced lupus, mice received a single intraperitoneal injection of 0.5 mL pristane (Sigma-Aldrich) on day 4.

2.4. Flow cytometry

Single-cell suspensions of spleen or mesenteric lymph node cells were prepared in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS), 2 mM ethylenediaminetetraacetic acid, and 0.05% sodium azide and then stained with the following antibodies: anti-CD4 (RM4-5), anti-B220 (RA3-6B2), anti-ICOS (7E.17G9), anti-PD-1 (RMP1-30), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD40L (MR1), anti-cytotoxic T-lymphocyte antigen (CTLA)-4 (UC10-4B9), anti-IL-4 (11B11), and anti-IFNγ (XMG1.2) from BioLegend; anti-CXCR5 (2G8) and anti-GL7 (GL7) from BD Biosciences; and anti-Fas (15A7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD25 (PC61.5), anti-*Foxp3* (FJK-16s), and anti-Ki67 (SolA15) from eBioscience. For intracellular staining, cells were fixed and permeabilized using a *Foxp3* Staining Kit (eBioscience) according to the manufacturer's instructions. For intracellular cytokine staining, splenocytes were stimulated with 20 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1000 ng/mL ionomycin (Cayman Chemical) in the presence of Monensin (eBioscience) for 4 h at 37 °C, and cells were then fixed and permeabilized using BD Cytofix/Cytoperm according to the manufacturer's instructions. Data were collected using a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar).

2.5. Measurement of immunoglobulins by enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Thermo Fischer Scientific) were coated overnight at 4 °C with goat anti-mouse IgG1, IgG2b, IgG2c, IgG3, or IgM (Bethyl) or rat anti-mouse IgE (SouthernBiotech). Nonspecific binding was blocked with 1% bovine serum albumin in PBS for 1 h, and samples were incubated for 2 h at 37 °C. After washing, plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1, IgG2b, IgG2c, IgG3, or IgM (Bethyl) for 1 h at 37 °C. For the detection of IgE, plates were incubated with biotin-conjugated rat anti-mouse IgE (BD Biosciences) for 1 h at 37 °C followed by streptavidin-HRP (BD Biosciences) for 30 min at 37 °C. After washing, TMB Substrate (BioLegend) was added, and the plates were read at 450 nm with an Infinite F50R (Tecan). For the measurement of NP-specific antibodies, plates were coated with NP₂₃-BSA (Biosearch Technologies) and HRP-conjugated secondary antibodies were used for detection. For the detection of anti-dsDNA IgG, plates were precoated with 0.001% protamine sulfate (Nacalai Tesque) for 1 h at room temperature and were coated with salmon sperm DNA (Biodynamics) overnight at 37 °C. Nonspecific binding was blocked with 50% FCS in PBS with 0.05% Tween20 and 0.02% sodium azide, and samples were incubated for 1 h at room temperature. After washing, bound antibody was detected with HRP-conjugated goat anti-mouse IgG (Bethyl). For the assessment of anti-histone antibodies, plates were coated overnight at 4 °C with whole histones (ImmunoVision) in PBS. Nonspecific binding was blocked with PBS containing 10% FCS and 0.05% Tween20 (Wako), and samples were incubated for 2 h at 37 °C. After washing, bound antibodies were detected with HRP-conjugated goat anti-mouse IgG.

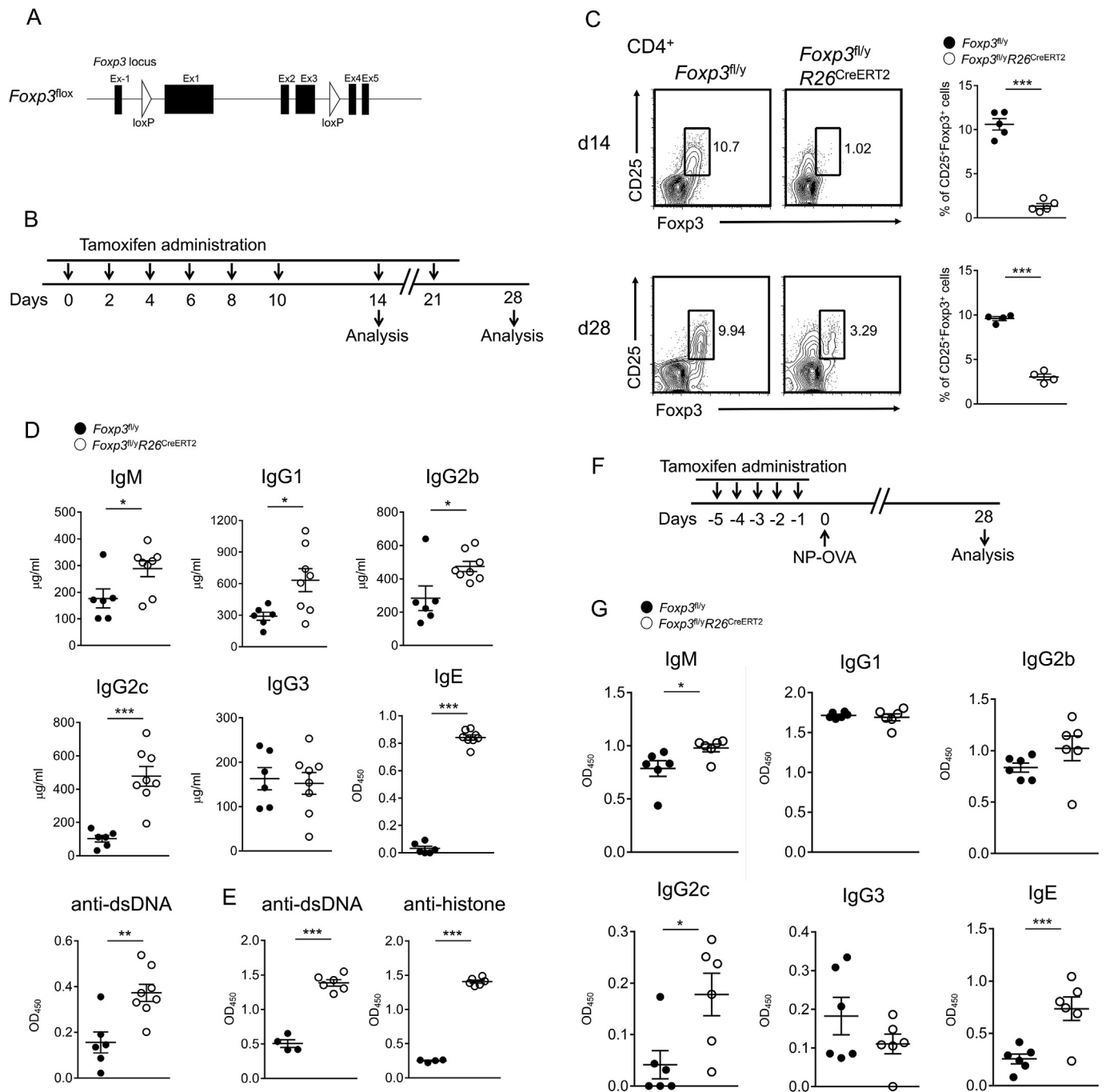
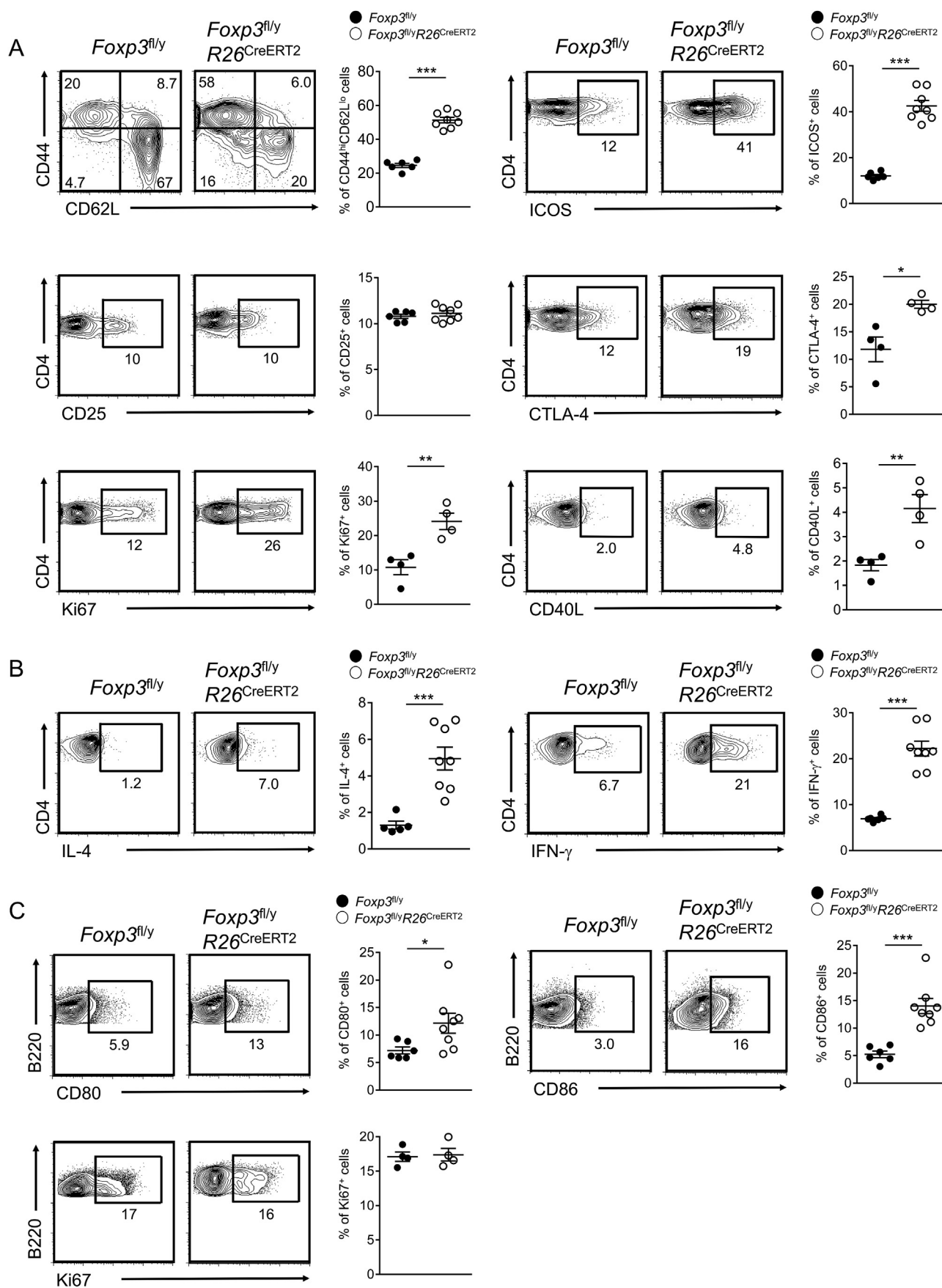


Fig. 1. Antibody responses induced by *Foxp3* gene deletion. (A) Schematic representation of the location of loxP sites in *Foxp3*^{fllox} mice. Exons 1–3 (black boxes) in the *Foxp3* gene were flanked by loxP sites (triangles). *Foxp3*^{fllox} mice were crossed with *R26*^{CreERT2} mice to generate *Foxp3* conditional-knockout mice. (B) Schematic of the experimental design. *Foxp3*^{fl/y} *R26*^{CreERT2} and *Foxp3*^{fl/y} control mice were orally administered tamoxifen once every 2 days until day 10 and then on days 14 and 21. Foxp3 expression was analyzed on days 14 and 28. Serum antibody titers were measured on day 28. (C) Tregs were analyzed by flow cytometry. Cells from spleens were stained for CD4 and CD25 on the cell surface, fixed, permeabilized, and then intracellularly stained for Foxp3. Representative flow cytometry plots (left) and quantification (right). The numbers in left panels indicate the percentages of CD25⁺Foxp3⁺ Tregs in CD4⁺ cells. (D) Serum levels of the indicated immunoglobulin isotypes and anti-dsDNA IgG were measured by ELISA. Data represent the concentration (IgM, IgG1, IgG2b, IgG2c, or IgG3) or the absorbance at 450 nm (IgE or anti-dsDNA IgG). (E) *Foxp3*^{fl/y} *R26*^{CreERT2} and *Foxp3*^{fl/y} control mice were orally administered tamoxifen, as shown in (B). Pristane was injected intraperitoneally on day 4. Serum antibody titers of anti-dsDNA IgG and anti-histone IgG were measured on day 46, and data represent the absorbance at 450 nm. (F) Schematic of the experimental design. *Foxp3*^{fl/y} *R26*^{CreERT2} and *Foxp3*^{fl/y} control mice were orally administered tamoxifen for 5 consecutive days and subsequently immunized with NP-OVA. Serum antibody titers were measured on day 28 after immunization. (G) NP-specific serum antibody titers were measured by ELISA. Data represent the absorbance at 450 nm. The data represent two (C, E, and G) or three (D) independent experiments. Each dot in the graphs represents an individual mouse, and horizontal lines represent means $\pm$ SEMs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's *t*-test).



2.6. Statistics

Statistical analyses were performed using two-tailed unpaired Student's *t*-tests. Differences with *p* values of less than 0.05 were considered significant.

3. Results

3.1. Generation of *Foxp3* conditional-knockout mice

Scurfy and *Foxp3*-knockout mice are known to die within 1 month after birth because of the development of fatal autoimmune lymphoproliferative disease [3], which preclude detailed analyses of Treg-mediated regulation of humoral immune responses in adult mice. To overcome this difficulty, we generated mice harboring a *Foxp3* allele flanked by loxP sites (*Foxp3*^{fl/y}; Fig. 1A). To conditionally delete the *Foxp3* gene, *Foxp3*^{fl/y} mice were crossed with the mice ubiquitously expressing tamoxifen-inducible CreERT2 from the ROSA26 allele (*R26*^{CreERT2}). The resulting *Foxp3*^{fl/y}*R26*^{CreERT2} mice, in which the *Foxp3* gene could be deleted after tamoxifen administration, allowed us to analyze the role of *Foxp3*⁺ Tregs in adult mice. To validate the *Foxp3* inducible deletion system, we administered tamoxifen six times at 1-day intervals and then on days 14 and 21. We examined *Foxp3* expression on days 14 and 28 by intracellular staining for *Foxp3* in CD4⁺ T cells (Fig. 1B). As shown in Fig. 1C, although about 10% of CD25⁺*Foxp3*⁺ cells were present in CD4⁺ T cells in control littermates, the percentage of CD25⁺*Foxp3*⁺ cells was decreased to about 1% in *Foxp3*^{fl/y}*R26*^{CreERT2} mice on day 14 after tamoxifen administration. The percentage of CD25⁺*Foxp3*⁺ cells was slightly increased to about 3% on day 28. These data indicated that the *Foxp3* gene was efficiently deleted in a time-specific manner and that the population of *Foxp3*⁺ Tregs was maintained at a low percentage for a long period of time in *Foxp3*^{fl/y}*R26*^{CreERT2} mice by this tamoxifen administration schedule.

3.2. Antibody responses induced by *Foxp3* gene deletion

To examine the effects of *Foxp3* gene deletion on antibody responses, we treated 3-month-old *Foxp3*^{fl/y}*R26*^{CreERT2} and control *Foxp3*^{fl/y} mice with tamoxifen as indicated in Fig. 1B and then measured serum immunoglobulin isotype levels on day 28 by ELISA. Consistent with previous observations obtained from other Treg-deficient mice [4,5,12], we found significant increases in serum IgM, IgG1, and IgG2b, as well as strong augmentation of serum IgG2c and IgE levels in *Foxp3*^{fl/y}*R26*^{CreERT2} mice compared with control *Foxp3*^{fl/y} mice (Fig. 1D). Anti-dsDNA IgG levels, a hallmark of systemic lupus erythematosus (SLE), were also significantly increased in *Foxp3*^{fl/y}*R26*^{CreERT2} mice compared with that in control *Foxp3*^{fl/y} mice (Fig. 1D). Similar increases in antibody titers were also observed in female *Foxp3*^{fl/y}*R26*^{CreERT2} mice (data not shown).

The increased levels of anti-dsDNA IgG in *Foxp3*^{fl/y}*R26*^{CreERT2} mice prompted us to further examine Treg-dependent autoantibody production in adult mice. To address this, we employed an

SLE-like autoimmune model induced by pristane, in which lupus autoantibodies against nuclear components, such as dsDNA and chromatin, are produced [19]. We found that titers of anti-dsDNA and anti-histone IgG serum antibodies were significantly elevated in *Foxp3*^{fl/y}*R26*^{CreERT2} mice compared with those in control mice 6 weeks after pristane treatment (Fig. 1E).

To assess antigen-specific antibody responses, we next immunized mice with NP-OVA and measured NP-specific antibody titers (Fig. 1F). Consistent with antibody responses observed in non-immunized mice, NP-specific antibody titers of IgM, IgG2c, and IgE in *Foxp3*^{fl/y}*R26*^{CreERT2} mice were significantly increased compared with those in control *Foxp3*^{fl/y} mice (Fig. 1G). We also observed increases in NP-specific IgG2b in *Foxp3*^{fl/y}*R26*^{CreERT2} mice, although the differences were not statistically significant. In contrast, there were no increases in titers of NP-specific IgG1 and IgG3 in *Foxp3*^{fl/y}*R26*^{CreERT2} mice. These data indicated that *Foxp3*^{fl/y}*R26*^{CreERT2} mice were a valuable mouse model for assessing Treg-mediated regulation of humoral responses in adult mice.

3.3. Effects of *Foxp3* gene deletion on T- and B-cell activation

Tregs control humoral immune response by suppressing CD4⁺ helper T-cell responses, which are important for B-cell activation [8]. Tregs also directly suppress B-cell responses. Thus, we next examined CD4⁺ T- and B-cell activation statuses by measuring the expressions of various activation markers. The percentage of activated CD44^{high}CD62L^{low} CD4⁺ T-cell population was increased in tamoxifen-treated *Foxp3*^{fl/y}*R26*^{CreERT2} mice compared with that in tamoxifen-treated control *Foxp3*^{fl/y} mice (Fig. 2A). CD4⁺ T cells from tamoxifen-treated *Foxp3*^{fl/y}*R26*^{CreERT2} mice had higher expression of T-cell activation markers, including ICOS and CTLA-4, but not CD25, than did cells from tamoxifen-treated control *Foxp3*^{fl/y} mice. The percentage of Ki67⁺ CD4⁺ T cells from tamoxifen-treated *Foxp3*^{fl/y}*R26*^{CreERT2} mice was also higher than that of cells from tamoxifen-treated control *Foxp3*^{fl/y} mice, indicating that *Foxp3* gene deletion induced the proliferation of CD4⁺ T cells (Fig. 2A). CD40L is a critical costimulatory molecule responsible for T cell-dependent B-cell activation. Thus, we determined the expression levels of CD40L in CD4⁺ T cells. CD4⁺ T cells from tamoxifen-treated *Foxp3*^{fl/y}*R26*^{CreERT2} mice showed significant increases in CD40L expression compared with those from tamoxifen-treated control *Foxp3*^{fl/y} mice (Fig. 2A).

Foxp3 gene deletion induced marked increases in serum IgE and IgG2c, as shown in Fig. 1. Class switch recombination to IgE and IgG2c is regulated by the cytokines IL-4 and IFN γ , respectively, produced by T cells. Therefore, we next examined whether CD4⁺ T cells in *Foxp3*-deleted mice had increased IL-4 and IFN γ production by intracellular staining for these cytokines. As expected, both IL-4 and IFN γ expression levels were significantly increased in CD4⁺ T cells from tamoxifen-treated *Foxp3*^{fl/y}*R26*^{CreERT2} mice compared with control *Foxp3*^{fl/y} mice (Fig. 2B). These data suggested that augmented IL-4 and IFN γ production from CD4⁺ T cells was responsible for increased IgE and IgG2c production in *Foxp3*-deleted mice.

Fig. 2. Effects of *Foxp3* gene deletion on T-cell and B-cell activation. *Foxp3*^{fl/y}*R26*^{CreERT2} and *Foxp3*^{fl/y} control mice were orally administered tamoxifen, as shown in Fig. 1B, and were then analyzed on day 28. CD4⁺ T cells and B220⁺ B cells were analyzed by flow cytometry. (A) Cells from spleens were stained for CD4, CD44, CD62L, ICOS, and CD25 on the cell surface. For the detection of CTLA-4, Ki67, and CD40L, cells were fixed, permeabilized, and then intracellularly stained for CTLA-4, Ki67, and CD40L. Representative flow cytometry plots (left) and quantification (right). The numbers in flow cytometry plots indicate the percentages of CD44^{hi}CD62L^{lo} (upper left), ICOS⁺ (upper right), CD25⁺ (middle left), CTLA-4⁺ (middle right), Ki67⁺ (lower left), and CD40L⁺ (lower right) cells in CD4⁺ cells. (B) Cells from spleens were stimulated with PMA and ionomycin in the presence of monensin for 4 h and then stained for CD4 on the cell surface. After cell-surface staining, cells were fixed, permeabilized, and then intracellularly stained for IL-4 and IFN γ . Representative flow cytometry plots (left) and quantification (right). The numbers in flow cytometry plots indicate the percentages of IL-4⁺ (left) and IFN γ ⁺ (right) cells in CD4⁺ cells. (C) Cells from spleens were stained for B220, CD80, and CD86 on the cell surface. For the detection of Ki67, cells were fixed, permeabilized, and then intracellularly stained for Ki67. Representative flow cytometry plots (left) and quantification (right). The numbers in flow cytometry plots indicate the percentages of CD80⁺ (upper left), CD86⁺ (upper right), and Ki67⁺ (lower left) cells in B220⁺ cells. The data represent three independent experiments. Each dot in the graphs represents an individual mouse, and horizontal lines represent means $\pm$ SEMs. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's *t*-test).

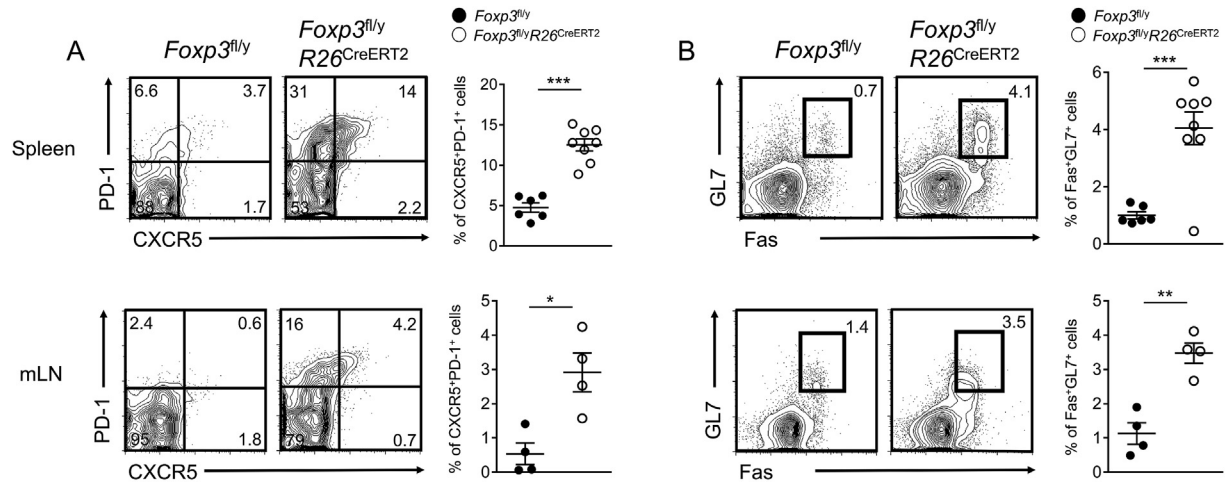


Fig. 3. Development of Tfh and GC B cells in Foxp3 conditional-knockout mice. *Foxp3^{fl/y} R26^{CreERT2}* and *Foxp3^{fl/y}* control mice were orally administered tamoxifen, as shown in Fig. 1B, and were then analyzed on day 28. Tfh cells and GC B cells were analyzed by flow cytometry. Cells from spleens (top) and mLN (bottom) were stained for CD4, CXCR5, and PD-1 (A) or for B220, Fas, and GL-7 (B). Representative flow cytometry plots (left) and quantification (right). Graphs indicate the percentage of CXCR5⁺PD-1⁺ Tfh cells in CD4⁺ cells (left) and the percentage of Fas⁺GL-7⁺ GC B cells in B220⁺ cells (right). Each dot in the graphs represents an individual mouse, and horizontal lines represent means $\pm$ SEMs. The data represent three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 (Student's *t*-test).

We next sought to examine B-cell activation status by evaluating the expression of CD80, CD86, and Ki67. Surface expression levels of both CD80 and CD86 on B cells from tamoxifen-treated *Foxp3^{fl/y} R26^{CreERT2}* mice were higher than those from tamoxifen-treated control littermates (Fig. 2C). However, there were no differences in the percentages of Ki67⁺ B cells between *Foxp3^{fl/y} R26^{CreERT2}* mice and control *Foxp3^{fl/y}* mice, indicating that B cells in Foxp3 conditional-knockout mice did not actively proliferate at this time point.

3.4. Augmented GC responses in Foxp3 conditional-knockout mice

Tfh cells, a distinct subset of Th cells expressing the chemokine receptor CXCR5 and the transcription factor Bcl6, migrate to B-cell follicles and promote the GC reaction. Given that depletion of Foxp3⁺ Tregs induced Tfh cell differentiation and the GC reaction [20], it is possible that the augmented humoral response shown in Fig. 1 was mediated by the GC response. Thus, we next asked whether Tfh and GC B-cell differentiation were augmented in Foxp3-deficient mice. Tamoxifen-induced deletion of the *Foxp3* gene resulted in a significant increase in CXCR5⁺PD-1⁺ Tfh cells and Fas⁺GL-7⁺ GC B cells in both spleens and mesenteric lymph nodes (mLNs) compared with that in control littermates (Fig. 3A and B). These data suggested that Tregs prevented inappropriate antibody responses by suppressing Tfh cell differentiation and GC formation.

4. Discussion

Elucidation of the Treg-mediated regulatory mechanisms affecting humoral immunity is essential for establishing therapeutic strategies for various autoimmune and allergic diseases. Therefore, in this study, we developed a new research tool, a Foxp3 conditional-knockout mouse, in which tamoxifen administration induced *Foxp3* gene deletion. *Foxp3* gene deletion in adult mice increased levels of serum immunoglobulins, including autoantibodies such as anti-dsDNA antibodies. Among immunoglobulin isotypes, IgE was sharply upregulated in Foxp3 conditional-knockout mice, which may affect the allergic symptoms observed in Treg-deficient mice [4]. Foxp3 deficiency in adult mice also induced T and B cell activation, particularly Tfh-cell and GC B-cell differentiation, which is critical for T cell-dependent humoral

immune responses. These data suggested that Tregs controlled excessive or pathogenic antibody production by inhibiting unnecessary Tfh differentiation and the subsequent GC response.

Our data suggested that the Tfh/GC axis was involved in the augmented antibody production induced by Foxp3 deficiency. However, humoral immune responses are not completely dependent on the GC reaction [21]. Bcl6-deficient mice have been shown to be defective in Tfh cell differentiation and GC formation [1]. As a result, they do not produce high-affinity antibodies, but still have T cell-dependent antibody responses after antigen immunization, albeit to a reduced extent. In addition, Th1 but not Tfh cells are required for influenza-specific IgG2 antibody responses [22], whereas Tfh but not Th2 cells are essential for IgE production induced by allergens [23], suggesting that distinct subsets of Th cells control isotype-specific antibody production. Given that Foxp3 deficiency induced various isotypes of antibodies, both Tfh-dependent and -independent antibody responses seemed to occur in Foxp3-deficient mice. Additional studies are required to determine which type of Th cells is responsible for humoral responses caused by Treg deficiency.

Recent reports have identified a new subset of Foxp3⁺ Tregs, termed Tfr (T follicular regulatory) cells [24]. These cells express Bcl6 and CXCR5, similar to Tfh cells, and are localized in B-cell follicles, where they control the expansion of Tfh and GC B cells. The increased antibody production in Foxp3-deficient mice may be attributed to defective Tfr function. However, we could not distinguish which cell type (i.e., Tregs or Tfr cells) suppressed antibody production because of deletion of the *Foxp3* gene in both cell types in our mouse model. Mice with Treg-specific Bcl6 deletion, in which Tfr-cell differentiation is compromised, showed no signs of aberrant antibody production until 8 weeks of age [25]; therefore, it was not likely that Tfr-cell deficiency caused the excessive antibody production observed in our Foxp3-deficient mice.

Foxp3⁺ Tregs are involved in various autoimmune and allergic diseases, in which pathogenic antibody production plays an essential role. Clarification of the mechanisms through which Tregs suppress unnecessary humoral responses is critical for establishment of therapeutic strategies. However, Scurfy and Foxp3-null mice, in which Foxp3⁺ Tregs are absent from birth, develop multiorgan inflammation, leading to early death, which makes it difficult to elucidate the Treg-mediated suppressive mechanism.

Thus, we believe that the Foxp3 conditional-knockout mouse model developed in this study will be a useful tool for analyzing the mechanisms through which Tregs regulate humoral responses and for screening agents for autoimmune and allergic disorders caused by Treg abnormalities.

Conflicts of interest

The authors claim no conflict of interest.

Acknowledgements

We thank M. Kurabayashi, J. Inagaki, R. Funato, R. Manabe, K. Koshida, and N. Sakamoto for technical support and animal maintenance. We thank S. Takahashi and S. Mizuno for generating Foxp3^{fllox} mice. We thank T. Ludwig for providing R26^{CreERT2} mice. This work was supported by a Grant-in-Aid for Scientific Research (C) (16K08247) to Y.H. This work was supported by the Hamaguchi Foundation and the Astellas Foundation for Research on Metabolic Disorders.

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