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Regulation of the Germinal Center Reaction by Foxp3+ Follicular Regulatory T Cells

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Follicular helper T (TFH) cells participate in humoral responses providing selection signals to germinal center B cells. Recently, expression of CXCR5, PD-1, and the transcription factor Bcl-6 has allowed the identification of TFH cells. We found that a proportion of follicular T cells, with phenotypic characteristics of TFH cells and expressing Foxp3, are recruited during the course of a germinal center (GC) reaction. These Foxp3+ cells derive from natural regulatory T cells. To establish the in vivo physiologic importance of Foxp3+ follicular T cells, we used CXCR5-deficient Foxp3+ cells, which do not have access to the follicular region. Adaptive cell transfers of CXCR5-deficient Foxp3+ cells have shown that Foxp3+ follicular T cells are important regulators of the GC reaction following immunization with a thymus-dependent Ag. Our in vivo data show that Foxp3+ follicular T cells can limit the magnitude of the GC reaction and also the amount of secreted Ag-specific IgM, IgG1, IgG2b, and IgA. Therefore, Foxp3+ follicular regulatory T cells appear to combine characteristics of TFH and regulatory T cells for the control of humoral immune responses.

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Germinal centers (GCs) are temporary structures in secondary lymphoid organs in which Ag-specific B cells undergo extensive proliferation, class-switch recombination and somatic hypermutation (SHM), a unique process whereby they can acquire higher affinity for the Ag (1). Across several cell divisions, high-affinity GC cells are selected and differentiate eventually into either memory cells or long-lived plasma cells, providing the basis of a process called “affinity maturation of serum Abs”. Long-term humoral immunity, as it is achieved in many prophylactic vaccines, is provided by those plasma cells and memory B cells and is a critical component to protect the body during subsequent infection (2).

In the last 15 y, an important body of information concerning the role of so-called follicular helper T (TFH) cells in the GC reaction (GCR) has been obtained. This particular subpopulation of CD4+ T cells is characterized by expressing high membrane levels of CXCR5 (the receptor of the follicular chemokine CXCL13, essential for follicular homing) and programmed death 1 (PD-1; a key molecule for B cell survival and selection, presumably through interaction with PD-ligand 2 (3]) and high levels of the transcription factor Bcl-6 (4, 5). Productive interactions between GC B cells and TFH cells are mediated by cognate interactions through TCR and MHC class II–peptide interactions, CD40–CD40L, and ICOS–ICOS ligand (6). When CD40–CD40L interactions are prevented, the ongoing GCR stops and GCs dissolve within 24 h (6). Mice that are deficient in ICOS or ICOS ligand also have impaired GC formation and isotype switching (7). Triggering of the SHM process in GC B cells is also dependent on CD40–CD40L interactions (8). Immunized mice that lack TFH cells show reduced numbers of germinal center B cells, as well as a reduction of Ag specific Ab (4). Besides direct B-TFH contact, TFH cells also contribute to the GCR through soluble mediators like IL-4 and IL-21 (9, 10). A recent report claimed distinct subpopulations of blood CXCR5+ T cells can be found in humans producing cytokines characteristic of Th1, Th2, and Th17 responses (11).

Deregulation of proliferation, mutation, and differentiation in GCs can lead to detrimental outcomes, including oncogenesis and immunodeficiency (12, 13). Moreover, it has been shown that mutant B cells with self-specificity can be supported in GCs and contribute to autoimmunity (14). Therefore, regulation of the quality and quantity of plasma cells and memory B cell populations in GCs is important to prevent immunopathology.

How this regulation is achieved remains poorly understood. A potential mechanism for this could be the presence within the polyclonal follicular T cell population in GCs of some regulatory T cells with specificity for ubiquitous self-antigens. In agreement with that, theoretical modeling points to processes related to T cell proliferation as being the dominant steps in the GC dynamics (15). We thus propose the hypothesis that there are regulatory mechanisms affecting Ag-specific TFH cells to prevent overly intense GCRs and/or production of autoantibodies. A possible basis for
some of the required GC regulation would be the involvement of Foxp3+ regulatory T cells (Tregs) during the GCR.

Currently, little is known about Tregs in connection with GCs. Some Tregs are known to express high levels of CXCR5 and to display positive chemotaxis toward a CXCL13 gradient in vitro (16). Furthermore, CD25+ T cells (presumably Tregs) were already shown to be present in the GCs of human tonsils and to suppress in vitro T cell activation and Ig production by B cells (16). In contrast, it was shown that within Peyer patches, Tregs can originate Treg1 cells (17).

In this report, we show that in murine lymph nodes Tregs participate in the GCR, contributing to both the dynamics and the amplitude of the GCR. In addition, we show that those follicular Tregs have an effect on Ab production during a T-dependent immune response.

Materials and Methods

Mice and immunization

BALB/c, C57BL/6, CXCR5−/−, OT2.Rag2−/− mice (TCR transgenic, OVA-specific), Foxp3+ knock-in mice (generously provided by A.Y. Rudensky, Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York, NY), and TCRα−/− mice (B6;129S2-Tcell1m1Mom1, backcrossed to C57BL/6 background, from S. Tonegawa, RIKEN-MIT Center for Neural Circuit Genetics and RIKEN Brain Science Institute, Cambridge, MA) were maintained in specific pathogen-free facilities. Procedures were conducted in accordance with guidelines from the Animal User and Institutional Ethical Committees.

Unless otherwise stated in the text, animals were immunized i.p. with 20 μg OVA (Sigma, St. Louis, MO) previously run through a DetoxyGel column (Pierce, Rockford, IL) in 2 mg endotoxin-free aluminum hydroxide (alum; Alu-gel-S; Serva, Heidelberg, Germany).

Adoptive transfer

For adoptive cell transfers, single-cell suspensions from a pool of spleen and LNs were sorted based on expression of CD4, CXCR5, PD-1, CD25, and GFP (cells from Foxp3 reporter mice) in a FACS Aria (Becton Dickinson, Franklin Lakes, NJ), with doublet exclusion in all experiments. Unless stated otherwise in the text, 1 × 10^6 cells of the indicated cell population were injected intravenously into TCR-α−/− mice. In all transfer experiments, mice were immunized with OVA-alum 1 d after adoptive transfer.

Flow cytometry

Single-cell suspensions of draining LNs were analyzed by flow cytometry using mAb targeting: PD-1 (J43), CD4 (L3T4), Thy1.2 (53-2.1), Foxp3 (FJK-16s), Bcl6 (G191E), GITR (DTA-1), CD103 (H.1.2F3), CD25 (all from eBioscience), and anti-CXCR5 (2G8) and anti-Rat IgG2a (R35-95) (BD Bioscience, San Diego, CA). Foxp3 staining was performed using the Foxp3 Staining Set (eBioscience) following the manufacturer’s instructions.

In vitro conversion

Sorted populations of 5 × 10^5 CD4 T cells were incubated for 3 d with 3 μg/ml plate-bound anti-CD3 (145-2C11; eBioscience), 2 μg/ml soluble anti-CD28 (eBiosciences), and 5 ng/ml TGF-β (R&D Systems).

Suppression assay

Sorted populations of Foxp3+ T cells were cocultured in Terasaki plates (Greiner, Frickenhausen, Germany) with γ-irradiated APCs, and Foxp3+ effector cells (1:3:1 ratio). Cultures were supplemented with 2.5 μg/ml soluble anti-CD3 for 3 d, with the addition of 1 μCi ^3H-thymidine (Amer sham, Sunnyvale, CA) in the last 12 h.

Confocal microscopy

Cryosections fixed in acetone (20 μm; Sigma) and vibratome sections from PFA-fixed tissue (50 μm; Sigma) were obtained from draining LNs. For Foxp3 staining sections were permeabilized with the Fix/Perm buffer from the Foxp3 staining Set (eBioscience). Samples were stained with the following primary Abs: rabbit anti-CD3, anti-Ki67, anti-GFP, anti-OVA; and rat anti-CD3 (Abcam), anti-IgM-TxRd (Southern Biotech, Birmingham, AL), anti-KJ-FITC (Caltag, Carlsbad, CA), anti-CD4-alexa647 (Sero tech), peanut agglutinin (PNA)-FITC and PNA-bio (Vector, Burlingame, CA). We used the following as secondary Abs: anti–FITC-alexa488, anti-rabbit Ig-alexa488, anti-rabbit Ig-alexa647 (Invitrogen, Carlsbad, CA); and avidin-rhodamine (Vector) and streptavidin-DyLight488 from Thermo Scientific (Rockford, IL). Images were acquired using an LSM710 confocal microscope (Zeiss, Jena, Germany) equipped with a ×5 (0.16 numerical aperture [NA]; Zeiss), ×10 (0.30 NA; Zeiss), ×20 (0.80 NA; Zeiss) and a ×40 (1.30 NA; Zeiss) objective. Image analysis was performed using the LSM image browser.

FIGURE 1. Kinetics of GCR and GC T cells. BALB/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed at the indicated time points, when cryosections of mesenteric lymph nodes were analyzed to follow the GCR. A. Average GC volume. GCs were identified by PNA staining and quantified by confocal microscopy. The volume (in cubic micrometers) was calculated from the Z-stack, multiplying the area by the depth of the GC. B. Representative stainings from nonimmunized mice, and mice sacrificed on days 4, 12, and 16 after immunization. Scale bar, 500 μm. C. Total number of T cells, and (D) density of GC T cells during a GCR as analyzed by confocal microscopy. GC T cells were identified as CD3+ cells within the PNA area. E. Representative staining from an LN on day 12 after immunization. Data are representative of two independent experiments. Scale bar, 50 μm. ***p < 0.001 (Mann-Whitney nonparametric, two-tailed test).
Quantification of GC volume

The area of the ellipse circumscribing the GC was measured by the LSM image browser software. The volume was then calculated from the Z-stack, multiplying the area by the depth of the GC, and represented in cubic micrometers (i.e., a volume of 100,000 μm$^3$ is obtained in a structure of 100 × 100 μm and with a depth of 10 μm).

ELISA assay

Isotype-specific Ab titers in the serum were determined by ELISA on 96-well plates (BD Falcon) coated with 50 μg OVA (grade V; Sigma) in carbonate buffer (pH = 9.6) plus N$_2$Na 0.01%. Nonspecific binding was blocked with 1% BSA (AMRESCO, Solon, OH) in PBS plus Tween-20 0.05%. Sera were diluted 1:50 or 1:100 in PBST, and 3-fold serial dilutions of serum samples were incubated. Negative controls (normal mouse serum diluted 1:100) were included in all assays. The plates were washed, and 50 μl isotype-specific goat anti–mouse-HRP conjugates (Southern Biotech) was added. ELISAs were developed with ABTS (2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (Sigma-Aldrich). The OD at 405 nm was determined after 4, 5, and 6 min with an automatic ELISA plate reader (Envision Multilabel Reader 2104, Perkin Elmer) and the highest values were recorded.

Statistical analysis

Statistical significance was determined using the two-tailed nonparametric Mann–Whitney U test, and $p < 0.05$ was deemed significant (in the figures, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Results

Codevelopment of the GCR and GC T cells

To analyze GC T cells, BALB/c mice were immunized with OVA to trigger a GCR and sacrificed at days 0, 4, 12, and 16 to evaluate the initiation, peak, and decline of the GCR. Mesenteric LNs were
collected, and 20-μm sections were stained with PNA and anti-CD3 to identify GCs and T cells. After immunization, the GCR peaked at ∼day 12 or a few days before, as measured by the average GC volume using confocal microscopy (Fig. 1A, 1B).

The quantification of total T cells within the GCs revealed that the kinetics of T cell numbers parallels that of the global GCR (Fig. 1C). However, we calculated the density of T cells within the GC (i.e., number of T cells per unit of GC volume) because GC volume increases during GCR. We found that the T cell density was highest at day 4 after immunization (Fig. 1D), although the real peak is probably a few days later.

**Follicular T cells contain a Foxp3+ subset of suppressor cells**

Given the observed changes in the density of T cells within the GC during a GCR, we investigated the putative presence of an active regulatory mechanism. Although Tregs were previously observed in germinal centers (16, 18), the precise nature of follicular Tregs, their relationship with extrafollicular counterparts, and their in vivo functional significance has remained unknown. We therefore investigated whether follicular Tregs represent a significant subpopulation of the T cells found in the GC.

As illustrated in Fig. 2A, Foxp3+ cells were readily detectable in the GCs of mesenteric LNs. Interestingly, we found that the density of Foxp3+ T cells peaked at day 12 after immunization (Fig. 2B), at the time when the density of T cells was reduced (Fig. 1D) and the contraction phase of the GCs was initiated. We confirmed these data using Foxp3gfp knock-in mice (in which Foxp3 and GFP are fused together), showing a perfect overlap of Foxp3 staining and GFP expression (Supplemental Fig. 1A). By analyzing nonconsecutive sections from the same LN, we also established the reliability of the quantitative approach that we used (Supplemental Fig. 1B).

To further characterize the GC Foxp3+ cells that we observed by confocal microscopy, we analyzed follicular T cells of mesenteric LNs by flow cytometry. We confirmed the existence of a Foxp3+ subpopulation within follicular T cells (Fig. 2C). We found that follicular Foxp3+ T cells share several markers with conventional Tregs, such as high levels of CD25, GITR, or CD103, but also retain the expression of molecules characteristic of Tfh cells, such as CXCR5 and PD-1, and the lineage-specific transcription factor Bcl-6 (Fig. 2C). Furthermore, we found that FACS-sorted follicular Foxp3+ T cells (sorted based on CD4, PD-1, CXCR5, and Foxp3gfp expression) had similar immune suppressive potential as nonfollicular Foxp3+ T cells (Fig. 2D).

As a result, Foxp3+ T cells found in GCs and B cell follicles appear to represent a subpopulation of follicular T cells, bearing a distinctive phenotype from the extrafollicular conventional Tregs. Importantly, Bcl-6 was recently identified as the master transcription factor for the Tfh lineage, by turning on a widespread gene repressor program acting on key transcriptional regulators of other Th cell lineages, namely Tbet (Th1) and RORγt (Th17), as well as a large number of miRNAs (4, 5, 19). It is important to note, however, that a repressive function toward the Treg lineage, or its key regulator Foxp3, was not found.

**Increased density of Foxp3+ GC T cells correlates with their increased proliferation as the GCR develops**

We next assessed the proliferative behavior of GC Foxp3+ T cells by quantifying the number of cells positive for the proliferation marker Ki67. As shown in Fig. 3, the frequency of proliferating (Ki67+) Foxp3+ cells within the GC is greater than the frequency of proliferating Foxp3+ cells outside the GC. In addition, there is a significant increase of proliferation at day 12 after immunization—a time that, as discussed above, correlates with an increase in the density of Foxp3+ T cells.
in the frequency of Foxp3+ cells within the GC. It is noteworthy that at day 4 after immunization, the proliferation of Foxp3+ cells increases significantly outside the GC. On the contrary, the frequency of proliferating cells within total GC T cells does not change during the GCR.

**Foxp3+ GC T cells regulate the magnitude of GCR**

After observing an increased frequency of Foxp3+ GC T cells at day 12 after immunization, we assessed the functional significance of such cells for the regulation of GCR. For this purpose we used TCR-α−/− mice, which lack α/β T cells and Tregs, and reconstituted those mice with OVA-specific CD4 T cells from OT2. Rag2−/− mice (that also lack Foxp3+ T cells). The OT2 cells were transferred alone or with sorted Tregs. We took advantage of Foxp3gfp knock-in reporter mice to isolate a population of Foxp3gfp+ Tregs by flow cytometry. The recipient mice were immunized with OVA, and GCR was followed at different time points. We studied GC resolution at day 20, instead of day 16, to have better discrimination concerning a putative impact on the serum titer of switched Iggs.

Because TCRα−/− mice are known to show an impaired ability to develop GCs owing to their lack of α/β T cells (20), we first wanted to ensure that TCRα−/− mice could develop GCs after adoptive cell transfer of Ag-specific cells. Lymph node sections from TCR-α−/− mice transferred with OT2 cells (CD4+ T cells from OT2.Rag2−/− mice) and immunized with OVA-alum were analyzed for the presence of GCs by staining with PNA and anti-IgM. This study revealed the ability of OT2 cells to mount a GCR (Fig. 4A), with kinetics having a relatively high magnitude (Fig. 4B). However, cotransfer of Foxp3+ Tregs together with OT2 cells, significantly reduced the magnitude of the GCR (Fig. 4B). Consistent with this finding, we also found that the adoptive transfer of Foxp3+ Tregs had an effect on Ab production, leading to lower serum titers of secreted OVA-specific IgG1, IgM, IgG2b, and IgA (Fig. 4C). The Ab titres were reduced approximately 1 log by day 20, but with mice transferred with Tregs still showing lower serum titres (Fig. 4D).

However, given the known ability of Foxp3+ Tregs to suppress T cells responses, it was possible that the observed regulation was acting on the generation of helper T cells during their early stage of activation taking place in the T zone of the LN, and not within the GC. To address this issue we took advantage of CXCR5−/− mice. These mice provided the tool to discriminate between the in vivo physiologic effects of Tregs within and outside the follicle, because Tregs from CXCR5−/− mice have a function similar to wild type Tregs but cannot enter the follicle. Therefore, we repeated the experiment performing an adoptive transfer of OT2 cells into TCR-α−/− mice. Flow cytometry-sorted CD4+ CD25bright T cells (that we confirmed were predominantly Foxp3+) from Foxp3gfp or CXCR5−/− mice were transferred with the OT2 cells. As anticipated, Tregs from CXCR5−/− mice could not be found within the GC, while the effector OT2 cells could still be found in the GC (Fig. 4E, 4F). Moreover, the ratio of effector to regulatory cells observed in the GC of TCR-α−/− mice transferred with T cells was similar to that in wild type conditions (Fig. 4E, 4F), although with a smaller number of cells. The ratio of follicular Tregs to T effector cells is not too different from the ratio observed within conventional extrafollicular populations (i.e., ~10%). Remarkably, we were able to show the physiologic role of follicular Foxp3+ T cells, because we found that adoptively transferred CXCR5−/− Tregs could not reproduce the phenotype observed with wild type Tregs (Fig. 4G). Only mice transferred with Tregs able to express CXCR5 had detectable Tregs within the GC (Fig. 4H), whereas both groups of mice had transferred Tregs in locations outside the follicle.

To address whether follicular Foxp3+ T cells derive from nonfollicular precursors, we repeated the adoptive cell transfer described above, but now using nonfollicular (PD-1+ CXCR5+) Tregs sorted from wild type or CXCR5−/− mice. We found that at day 12 after immunization, mice transferred with Tregs from CXCR5−/− donors had greater GCs (Fig. 4I). Furthermore, mice transferred with Tregs deficient in CXCR5 had greater titres of serum Iggs (Fig. 4J). It should be noted, however, that this defect in regulation of isotype switch and Ab production by CXCR5−/− Tregs was not complete, probably representing a degree of extrafollicular regulation and possibly affecting the availability of T FH cells within the follicle.

Considering these results, it appears that CXCR5 expression by Treg cells is essential to endow them with the ability to control the GCR. As a result, we propose naming this Foxp3+ T cell subset from GCs that express PD-1, CXCR5, and Bcl-6, as regulatory follicular T cells (Treg).

**Follicular Foxp3+ T cells derive from conventional Treg cells**

We then investigated whether Foxp3+ follicular T cells derive from conventional T cells or from natural Tregs that acquire a follicular phenotype. We confirmed, as anticipated, that the thymus does not contain a population of Foxp3+ T cells with follicular characteristics (Fig. 5A), indicating that the follicular Foxp3+ T cells acquire their phenotype in the periphery.

To establish the origin of follicular Foxp3+ cells, we adoptively transferred sorted T FH or conventional CD25+ CD4+ T cells from wild type mice, together with PD-1+ CXCR5+ Foxp3gfp+ natural Tregs into TCR-α−/− mice, that were subsequently immunized with OVA-alum. Because adoptive transfer of a CD4 population devoid of Tregs leads to inflammatory bowel disease (21), control mice that did not receive Tregs were transfused with OT2 cells, as these TCR-transgenic T cells do not lead to autoimmunity in the absence of Tregs. We found in both cases that the majority of follicular Foxp3+ T cells recovered from the TCR-α−/− mice had derived from the transferred Treg population, where GFP was expressed together with Foxp3 (Fig. 5B–D). Intriguingly, that fraction was larger when T FH cells were transferred together with Foxp3gfp+ Tregs, suggesting a role for T FH cells in recruiting Foxp3+ follicular T cells, perhaps by promoting their proliferation. This issue remains to be further investigated.

Furthermore, we sorted T FH and conventional CD4 T cells that were stimulated in vitro with plate-bound anti-CD3 in the presence of anti-CD28 and TGF-β—conditions that are known to induce Fox3 expression (22, 23). We found that T FH cells are resistant to conversion toward a Foxp3+ phenotype (Fig. 5E).

These data suggest that T reg cells derive predominantly from natural Tregs, whereas T FH cells are not amenable to TGF-β-dependent conversion into Foxp3+ regulatory cells.

**Discussion**

Owing to the absence of experimental data, there are various mathematical models attempting to explain the regulation and termination of the GCR. One hypothesis suggests an increasing impairment of the engagement of B cell receptors and Ag through either Ag consumption (24) or Ag masking (25). Both of these concepts assume that GC termination is regulated by the amount of Ag presented within the GC by follicular dendritic cells. Another hypothesis suggests GC termination is caused by increased differentiation of GC B cells toward memory and plasma cells because of signals from follicular dendritic cells (15). A third
FIGURE 4. Follicular Foxp3+ T cells regulate the magnitude of GCR in vivo. A, TCRα−/− mice were adoptively transferred with 10⁶ OVA-specific T cells from OT2.Rag2−/− mice. Some mice also received an equal number of Foxp3gfp+ T cells. All mice were immunized with OVA-alum. The micrographs show GCs in mice that did not receive Foxp3+ T cells at day 12 after immunization. Scale bars, 200 μm (left), 50 μm (right). B, Quantification of the GC volume from LNs of mice immunized with OVA-alum in the presence or absence of adoptively transferred Foxp3+ T cells. GCs were identified as a PNA+ region within the follicle marked by IgM. C and D, Serum titres of OVA-specific Igs of different classes were determined by ELISA at different times after immunization. In the absence of Tregs, the mice produced significantly higher levels of IgM, IgG1, IgG2b, and IgA. E, Quantification of total CD4+ T cells and Foxp3+ Treg cells within GCs showing that, following adoptive cell transfers of defined T cell populations into T cell-deficient mice, there was a reduced number of both T cells and Tregs compared with wild type mice, although their ratio was roughly maintained. G, TCR-α−/− mice were adoptively transferred with OT2 T cells and Tregs sorted from wild type or CXCR5−/− mice and immunized with OVA-alum as (Figure legend continues).
hypothesis proposes that signaling from T cells is the responsible for GC termination (15). After observing an increase in TFreg numbers in the GC toward the end of the GCR, we suggest a wider scenario whereby this cell population has a role in controlling the magnitude and duration of the GCR.

The lack of Tregs in our transfer system profoundly modified the outcome of the GCR, as those mice showed larger GCs at day 12, and greater production of different classes of Ig than in the presence of Tregs. Moreover, using Tregs isolated from CXCR5\(^{+/−}\) mice, we created a system with Tregs that are unable to gain access to GC follicles. As a result, the data showed that it is specifically the TFregs that control the GCR. Although our data do not prove unequivocally a role for TFregs in the termination of the GCR, they clearly show that TFregs affect the magnitude of this reaction from midterm on. To date, it is not clear whether this regulation operates by controlling effector TFH cells (16), and by that indirectly affecting GC B cells, or if that regulation acts directly on GC B cells or on both TFH and B cells (18).

A second outstanding question concerns the origin of TFregs. We could not detect Foxp3\(^{+}\) cells with a TFH-like phenotype in the thymus. Furthermore, when we transferred OT2 cells into TCR\(^{α−/−}\) mice, we could not find in vivo conversion of those Foxp3\(^{−}\) cells into TFregs. When we transferred natural Tregs together with CD4 T cells, some of the natural Tregs (the only ones expressing Foxp3\(^{+}\)) in those mice) acquired access to the follicles and phenotypic follicular characteristics. Therefore, we believe it is likely that, in the same way that natural Tregs can originate TFH cells in the PP (17), some natural Tregs can also differentiate toward a TFreg phenotype in LNs and the spleen. This suggestion has important implications for understanding how the dynamics of T and B cells within GCs is controlled.

GC deregulation or malfunctions are linked to the onset of severe diseases, as cancer and autoimmunity (26). Our data show that follicular T cells include a new regulatory subpopulation, which by controlling the GCR may ultimately have an effect on preventing such pathologies.

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**FIGURE 5.** Follicular Foxp3\(^{+}\) T cells originate from conventional Treg cells. 

A. Among CD4\(^{+}\)Foxp3\(^{+}\) thymocytes, we could not find cells expressing CXCR5 and PD1. 

B. TCR-α\(^{−}\) mice were adoptively transferred with equal numbers of PD-1\(^{−}\)CXCR5\(^{−}\)Foxp3\(^{−}\) Tregs sorted from Foxp3\(^{+}\) knock-in mice and CD4\(^{+}\)CD25\(^{+}\) T cells (T\(_{FH}\); PD-1\(^{−}\)CXCR5\(^{−}\) or CD4: PD-1\(^{−}\)CXCR5\(^{+}\)) from B6 mice. Control mice were transferred with Foxp3\(^{−}\) OT2 cells alone. All mice were immunized with OVA-alum. The dot plots represent follicular CD4\(^{+}\) T cells, gated in the PD-1\(^{−}\)CXCR5\(^{+}\) region, analyzed for Foxp3 and GFP expression. Representative data are from four mice per group. 

C. The majority of Foxp3\(^{+}\) cells are also GFP\(^{+}\), indicating that they are derived from adoptively transferred Tregs (from Foxp3\(^{+}\)mice), n = 4; *p < 0.05, **p < 0.01, (Mann–Whitney nonparametric, two-tailed test). 

D. Micrograph showing Foxp3\(^{+}\) T cells in the GC of adoptively transferred mice (white arrows). Scale bar, 50 \(\mu\)m (left), 20 \(\mu\)m (right). 

E. Foxp3\(^{+}\) TFH (sorted as CD4\(^{+}\)PD-1\(^{−}\)CXCR5\(^{+}\)) and non-TFH cells (sorted as CD4\(^{+}\)PD-1\(^{−}\)CXCR5\(^{−}\)) were cultured for 3 d with plate-bound anti-CD3 in the presence or absence of 5 ng/ml TGF-β. The frequency of cells converted into Foxp3\(^{+}\) was analyzed at the end of the culture (n = 3, representative of two independent experiments).
Note added in proof. While our manuscript was being reviewed, two publications have reported the identification of follicular Foxp3+ regulatory T cells and their functional significance in regulating GCR (27, 28). Those reports complement the findings described in this study.

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Disclosures

The authors have no financial conflicts of interest.

References