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

Ivonne Wollenberg,*,[†] Ana Agua-Doce,*,[†] Andrea Hernández,[‡] Catarina Almeida,*,[†] Vanessa G. Oliveira,*,[†] Jose Faro,[†,‡,1] and Luis Graca*,[†,1]

Follicular helper T (T_{FH}) 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 T_{FH} cells. We found that a proportion of follicular T cells, with phenotypic characteristics of T_{FH} 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. Adoptive 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 T_{FH} and regulatory T cells for the control of humoral immune responses. *The Journal of Immunology*, 2011, 187: 4553–4560.

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 (T_{FH}) 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 T_{FH} 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 T_{FH} cells show reduced numbers of germinal center B cells, as well as a reduction of Ag specific Ab (4). Besides direct B-T_{FH} contact, T_{FH} 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 T_{FH} cells to prevent overly intense GCRs and/or production of autoantibodies. A possible basis for

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Abbreviations used in this article: GC, germinal center; GCR, germinal center reaction; LN, lymph node; NA, numerical aperture; PD-1, programmed death 1; PNA, peanut agglutinin; T_{FH}, follicular helper T; $T_F$reg, follicular regulatory T cell; Treg, regulatory T cell.

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 T_{FH} 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), $Foxp3^{gfp}$ knock-in mice (generously provided by A.Y. Rudensky, Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York, NY), and $TCR\alpha^{-/-}$ mice (B6;129S2-$Tcra^{tm1Mom}$/J, 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 $Foxp3^{gfp}$ reporter mice) in a FACSAria (Becton Dickinson, Franklin Lakes, NJ), with doublet exclusion in all experiments. Unless stated otherwise in the text, 1×10^4 cells of the indicated cell population were injected intravenously into $TCR-\alpha^{-/-}$ 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 (GI191E), GITR (DTA-1), CD103 (2E7), CD69 (H1.2F3), 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 5×10^4 CD4 T cells were incubated for 3 d with 3 μg/ml plate-bound anti-CD3 (145-2C11; eBiosciences), 2 μg/ml soluble anti-CD28 (eBiosciences), and 5 ng/ml TGF-β (R&D Systems).

Suppression assay

Sorted populations of $Foxp3^{gfp+}$ T cells were cocultured in Terasaki plates (Greiner, Frickenhausen, Germany) with γ-irradiated APCs, and $Foxp3^{gfp-}$ 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 ^{3}H-thymidine (Amersham, 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 (Serotech), 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, anti-rat Ig-alexa633 (Invitrogen, Carlsbad, CA); and avidin-rhodamine (Vector) and streptavidin-DyLight488 from ThermoScientific (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.
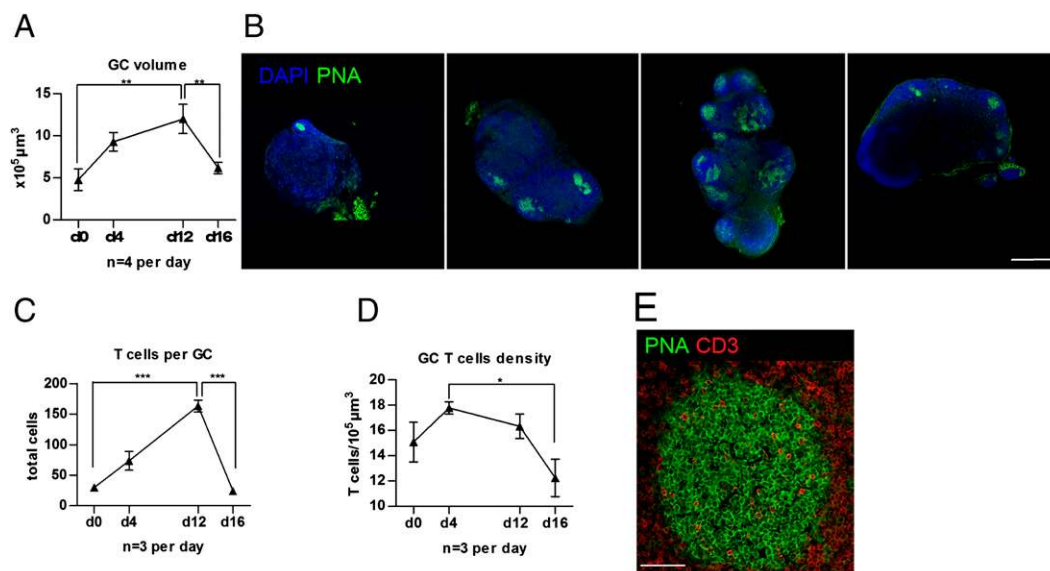
FIGURE 1. Kinetics of GCR and GC T cells. BALB/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed at the indicated time points, when cryosections of mesenteric lymph nodes were analyzed to follow the GCR. *A*, Average GC volume. GCs were identified by PNA staining and quantified by confocal 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_3Na 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-ethyl-benzothiazoline-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
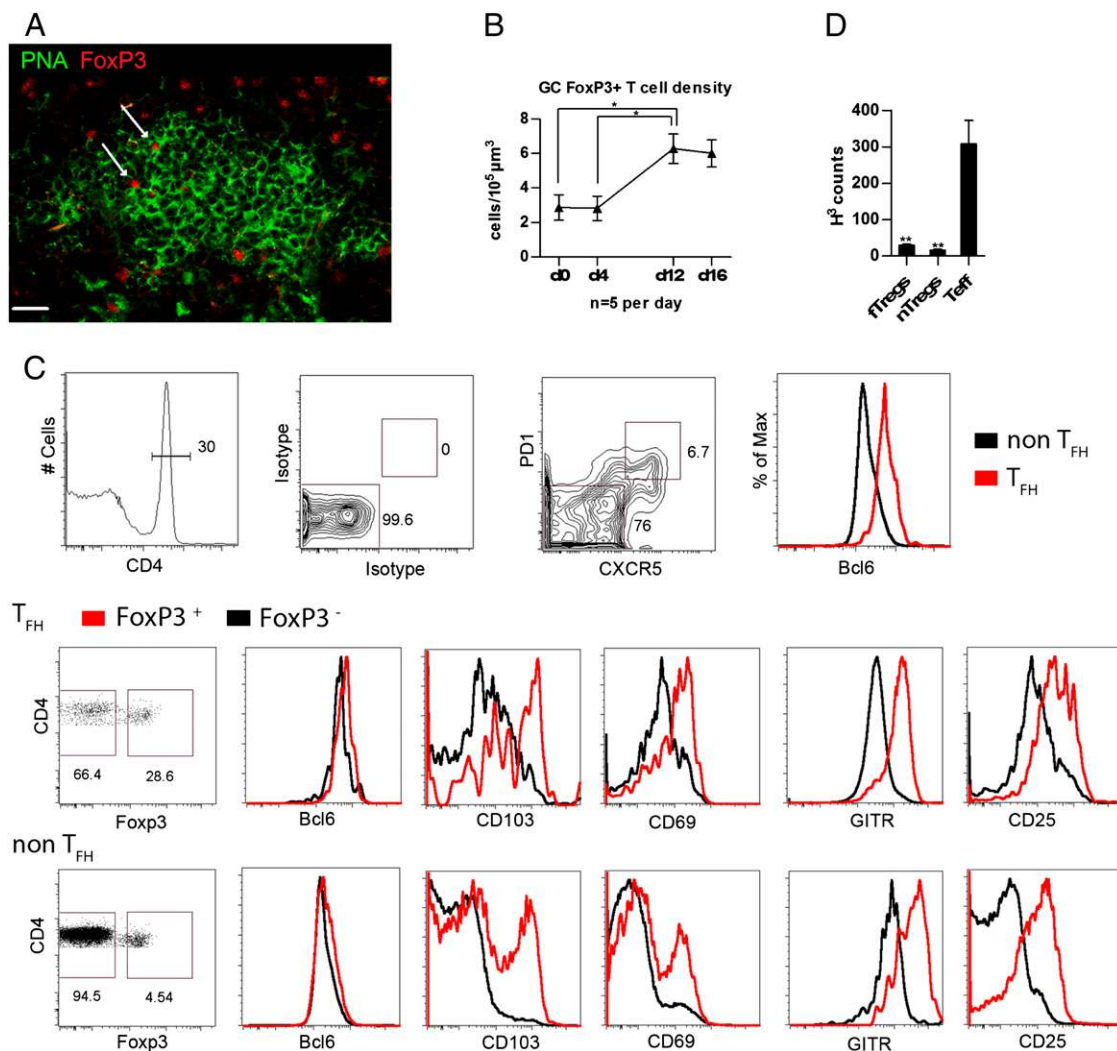

FIGURE 2. Foxp3^{+} T cells can be found within the GC and represent a subpopulation of follicular T cells. BALB/c mice were immunized with 20 μg OVA-alum i.p. and sacrificed at the indicated time points. *A*, Cryosections of LN were stained with PNA and anti-Foxp3 to identify Foxp3^{+} cells within the GC. Scale bar, 20 μm. *B*, The density of Foxp3^{+} T cells within the GC during the course of a GCR was quantified by confocal microscopy. The density corresponds to the total number of Foxp3^{+} cells within the PNA^{+} area divided by the measured GC volume. *C*, CD4^{+} lymphocytes from LNs of mice immunized with OVA-alum 12 d before were studied by flow cytometry. T_{FH} cells were defined as being CXCR5^{+}PD1^{+} as defined by the represented gate. The conventional extrafollicular T cells (non-T_{FH}) were defined as the CXCR5^{−}PD-1^{−} cells. T_{FH} cells had higher expression of Bcl-6 (*top right*). When we studied the T cells within the T_{FH} gate (*middle row*) and non-T_{FH} gate (*bottom row*), we found that both populations had a proportion of cells expressing Foxp3. We compared the expression of the indicated molecules within the Foxp3^{+} and Foxp3^{−} populations of T_{FH} and non-T_{FH} cells. *D*, Follicular Foxp3^{+} T cells (CD4^{+}CXCR5^{+}PD1^{+}Foxp3^{+}) and nonfollicular conventional Tregs (CD4^{+}CXCR5^{−}PD-1^{−}Foxp3^{+}) were sorted from the spleen and LNs of Foxp3[gfp] reporter mice. Tregs (2×10^3) were cocultured in triplicate with γ-irradiated APCs and Foxp3[gfp−] effector cells (1:3:1 ratio). Cultures were supplemented with 2.5 $\mu g/ml$ soluble anti-CD3 Ab for 3 d, and ^{3}H-thymidine was added in the last 12 h. Data are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$ (Mann–Whitney nonparametric, two-tailed test).

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 T_{FH} 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 T_{FH} 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
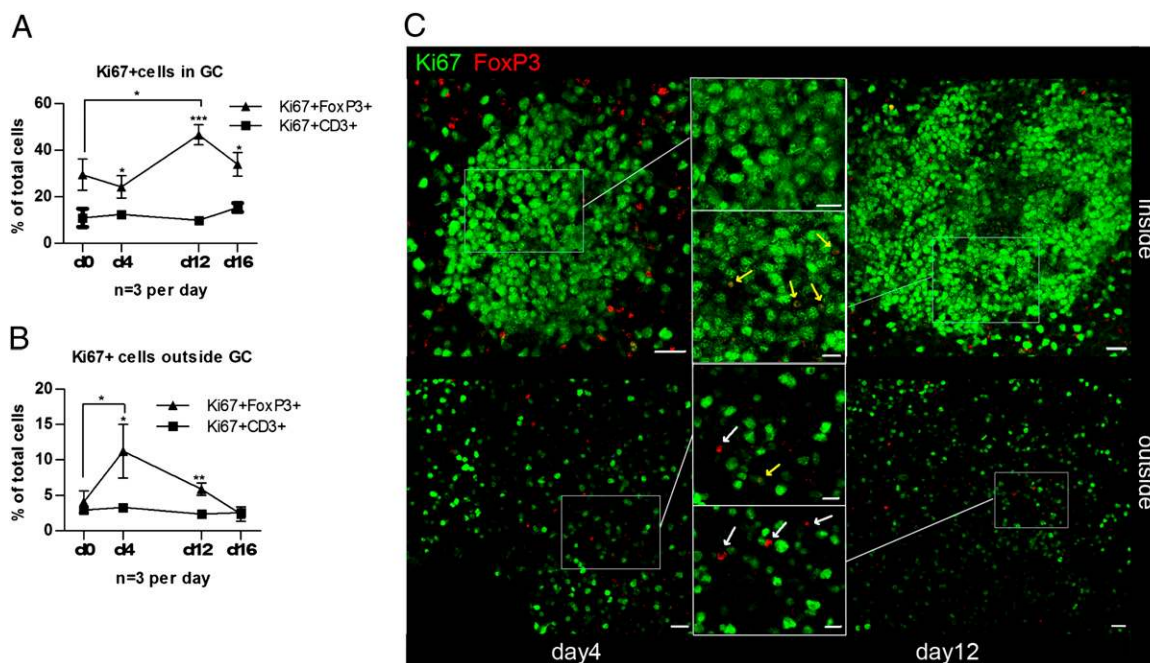
FIGURE 3. Kinetics of proliferation of T cells and Foxp3+ T cells during the GCR. BALB/c mice were immunized with OVA-alum as described in the text. The frequency of proliferating T cells was quantified by confocal microscopy based on Ki67 expression. *A*, Proliferating GC Foxp3+ cells and GC T cells were identified as Ki67+Foxp3+ cells and Ki67+CD3+ cells, respectively, within the PNA area and are depicted as a percentage of the total Foxp3+ or total CD3+ cells identified in the GC. *B*, A similar analysis was performed for T cells outside the GC. *C*, Representative stainings of the proliferation of GC T cells. Yellow arrows point to Ki67+Foxp3+ cells, white arrows to Ki67-Foxp3+ cells. Data are representative of two independent experiments. Scale bars, 20 μm (insets, 10μm). *p < 0.05, **p < 0.01 (Mann–Whitney nonparametric, two-tailed test).

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 Foxp3^{gfp} knock-in reporter mice to isolate a population of Foxp3^{gfp+} 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 Igs.

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$^+$ CD25^{bright} T cells (that we confirmed were predominantly Foxp3$^+$) from Foxp3^{gfp} 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 Igs (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 (T$_F$regs).

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$^-$Foxp3^{gfp+} natural Tregs into TCRα$^{-/-}$ 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 transferred 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 Foxp3^{gfp+} 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 Foxp3 expression (22, 23). We found that T$_{FH}$ cells are resistant to conversion toward a Foxp3$^+$ phenotype (Fig. 5E).

These data suggest that T$_F$regs 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
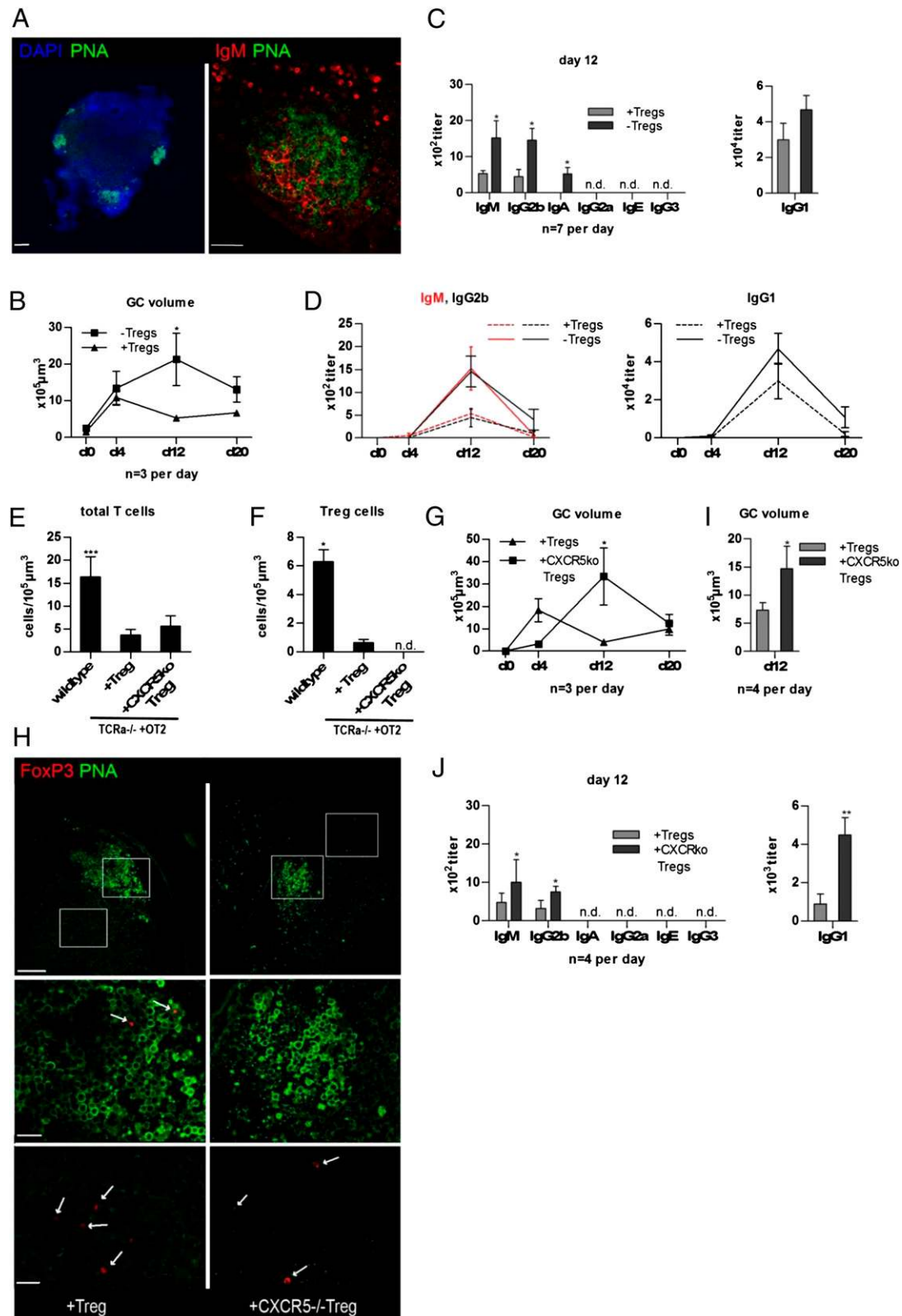
FIGURE 4. Follicular Foxp3^{+} T cells regulate the magnitude of GCR in vivo. *A*, TCRα^{−/−} mice were adoptively transferred with 10[4] OVA-specific T cells from OT2.Rag2^{−/−} mice. Some mice also received an equal number of Foxp3[gfp+] T cells. All mice were immunized with OVA-alum. The micrographs 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 (*F*) Foxp3^{+} Treg cells within GCs showing that, following adoptive cell transfers of defined T cell populations into T cell-deficient cells, 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*)
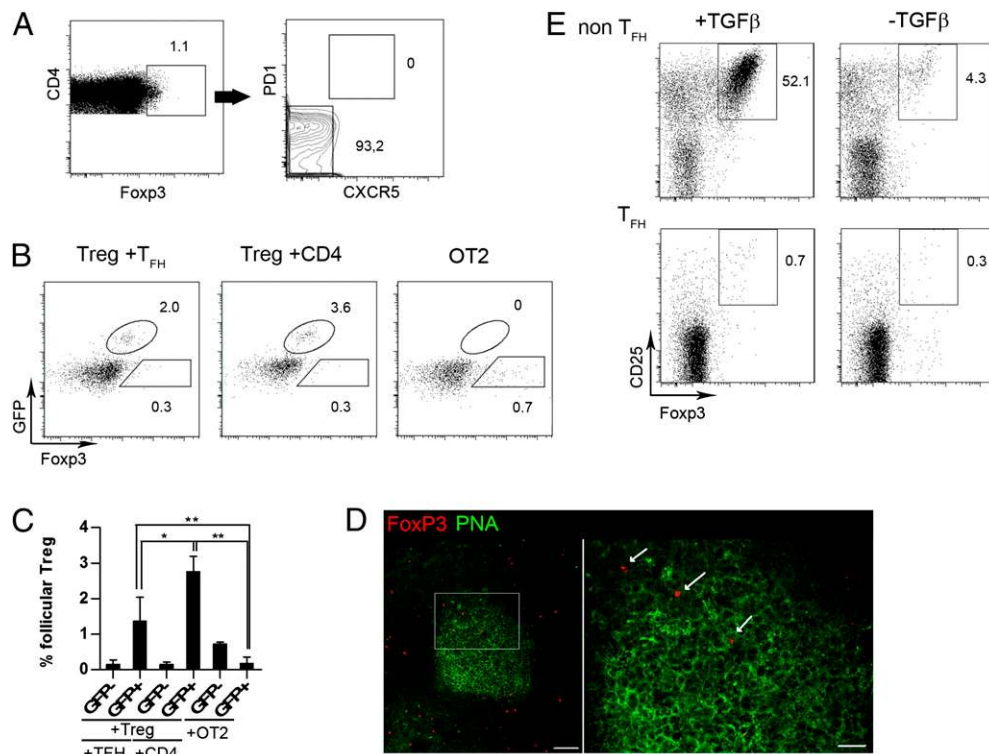
FIGURE 5. Follicular Foxp3$^+$ T cells originate from conventional Treg cells. *A*, Among CD4$^+$Foxp3$^+$ thymocytes, we could not find cells expressing CXCR5 and PD1. *B*, TCR-$\alpha^{-/-}$ mice were adoptively transferred with equal numbers of PD-1$^-$CXCR5$^-$Foxp3$^+$ Tregs sorted from Foxp3^{gfp} 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^{gfp} 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 μm (*left*), 20 μm (*right*). *E*, Foxp3^{gfp-} T$_{FH}$ (sorted as CD4$^+$PD-1$^+$CXCR5$^+$) and non-T$_{FH}$ 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).

hypothesis proposes that signaling from T cells is the responsible for GC termination (15). After observing an increase in T$_F$regs 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 T$_F$regs that control the GCR. Although our data do not prove unequivocally a role for T$_F$regs in the termination of the GCR, they clearly show that T$_F$regs affect the magnitude of this reaction from midterm on. To date, it is not clear whether this regulation operates by controlling effector T$_{FH}$ cells (16), and by that indirectly affecting GC B cells, or if that regulation acts directly on GC B cells or on both T$_{FH}$ and B cells (18).

A second outstanding question concerns the origin of T$_F$regs. We could not detect Foxp3$^+$ cells with a T$_{FH}$-like phenotype in the thymus. Furthermore, when we transferred OT2 cells into TCR$\alpha^{-/-}$ mice, we could not find in vivo conversion of those Foxp3$^-$ cells into T$_F$regs. When we transferred natural Tregs together with CD4 T cells, some of the natural Tregs (the only ones expressing Foxp3^{gfp} 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 T$_{FH}$ cells in the PP (17), some natural Tregs can also differentiate toward a T$_F$reg 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.

described in *A*. The GC volume was quantified as detailed in the text. Data are representative of two independent experiments. *H*, Representative micrographs showing that it was possible to identify Foxp3^{gfp+} T cells within day 12 GCs of mice adoptively transferred with Tregs, but not in mice transferred with CXCR5$^{-/-}$ Tregs, where Tregs remained outside the follicle. Scale bars, 100 μm (*top row*), 20 μm (*bottom two rows*). *I*, TCR-$\alpha^{-/-}$ mice were adoptively transferred with OT2 cells together with nonfollicular (CXCR5$^-$PD-1$^-$) Tregs sorted from wild type or CXCR5$^{-/-}$ mice and immunized with OVA-alum as described in *A*. The GC volume was quantified at day 12 as detailed in the text. *J*, Serum titers of OVA-specific Igs of different classes were determined by ELISA at day 12 after immunization. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Mann–Whitney nonparametric, two-tailed test). n.d., not detected.

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

1. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell* 67: 1121–1129.
2. Radbruch, A., G. Muehlinghaus, E. O. Luger, A. Inamine, K. G. Smith, T. Dörner, and F. Hiepe. 2006. Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat. Rev. Immunol.* 6: 741–750.
3. Good-Jacobson, K. L., C. G. Szumilas, L. Chen, A. H. Sharpe, M. M. Tomayko, and M. J. Shlomchik. 2010. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat. Immunol.* 11: 535–542.
4. Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325: 1001–1005.
5. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325: 1006–1010.
6. Kelsoe, G. 1995. In situ studies of the germinal center reaction. *Adv. Immunol.* 60: 267–288.
7. Dong, C., U. A. Temann, and R. A. Flavell. 2001. Cutting edge: critical role of inducible costimulator in germinal center reactions. *J. Immunol.* 166: 3659–3662.
8. Bergthorsdottir, S., A. Gallagher, S. Jainandunsing, D. Cockayne, J. Sutton, T. Leanderson, and D. Gray. 2001. Signals that initiate somatic hypermutation of B cells in vitro. *J. Immunol.* 166: 2228–2234.
9. Reinhardt, R. L., H. E. Liang, and R. M. Locksley. 2009. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat. Immunol.* 10: 385–393.
10. Ozaki, K., R. Spolski, C. G. Feng, C. F. Qi, J. Cheng, A. Sher, H. C. Morse, III, C. Liu, P. L. Schwartzberg, and W. J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science* 298: 1630–1634.
11. Morita, R., N. Schmitt, S. E. Bentebibel, R. Ranganathan, L. Bourdery, G. Zurawski, E. Foucat, M. Dullaers, S. Oh, N. Sabzghabaei, et al. 2011. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 34: 108–121.
12. Linterman, M. A., R. J. Rigby, R. K. Wong, D. Yu, R. Brink, J. L. Cannons, P. L. Schwartzberg, M. C. Cook, G. D. Walters, and C. G. Vinuesa. 2009. Follicular helper T cells are required for systemic autoimmunity. *J. Exp. Med.* 206: 561–576.
13. Crotty, S. 2011. Follicular helper CD4 T cells (TFH). *Annu. Rev. Immunol.* 29: 621–663.
14. Sims, G. P., H. Shiono, N. Willcox, and D. I. Stott. 2001. Somatic hypermutation and selection of B cells in thymic germinal centers responding to acetylcholine receptor in myasthenia gravis. *J. Immunol.* 167: 1935–1944.
15. Moreira, J. S., and J. Faro. 2006. Modelling two possible mechanisms for the regulation of the germinal center dynamics. *J. Immunol.* 177: 3705–3710.
16. Lim, H. W., P. Hillsamer, and C. H. Kim. 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J. Clin. Invest.* 114: 1640–1649.
17. Tsuji, M., N. Komatsu, S. Kawamoto, K. Suzuki, O. Kanagawa, T. Honjo, S. Hori, and S. Fagarasan. 2009. Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. *Science* 323: 1488–1492.
18. Lim, H. W., P. Hillsamer, A. H. Banham, and C. H. Kim. 2005. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J. Immunol.* 175: 4180–4183.
19. Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, et al. 2009. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31: 457–468.
20. Lindroth, K., M. Troye-Blomberg, M. Singh, F. Dieli, J. Ivanyi, and C. Fernández. 2002. The humoral response in TCR alpha⁻/⁻ mice. Can gammadelta-T cells support the humoral immune response? *Scand. J. Immunol.* 55: 256–263.
21. Read, S., V. Malmström, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
22. Oliveira, V. G., M. Caridade, R. S. Paiva, J. Demengeot, and L. Graca. 2011. Sub-optimal CD4+ T-cell activation triggers autonomous TGF-β-dependent conversion to Foxp3+ regulatory T cells. *Eur. J. Immunol.* 41: 1249–1255.
23. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
24. Keşmir, C., and R. J. De Boer. 1999. A mathematical model on germinal center kinetics and termination. *J. Immunol.* 163: 2463–2469.
25. Tarlinton, D. M., and K. G. Smith. 2000. Dissecting affinity maturation: a model explaining selection of antibody-forming cells and memory B cells in the germinal centre. *Immunol. Today* 21: 436–441.
26. Vinuesa, C. G., and M. C. Cook. 2001. The molecular basis of lymphoid architecture and B cell responses: implications for immunodeficiency and immunopathology. *Curr. Mol. Med.* 1: 689–725.
27. Linterman, M. A., W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner, M. Srivastava, D. P. Divekar, L. Beaton, J. J. Hogan, et al. 2011. Foxp3(+) follicular regulatory T cells control the germinal center response. *Nat. Med.* 17: 975–982.
28. Chung, Y., S. Tanaka, F. Chu, R. I. Nurieva, G. J. Martinez, S. Rawal, Y. H. Wang, H. Lim, J. M. Reynolds, X. H. Zhou, et al. 2011. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat. Med.* 17: 983–988.