Interleukin-10 from CD4+ follicular regulatory T cells promotes the germinal center response

Brian J. Laidlaw, Yisi Lu, Robert A. Amezquita, Jason S. Weinstein, Jason A. Vander Heiden, Namita T. Gupta, Steven H. Kleinstein, Susan M. Kaech, Joe Craft

CD4+ follicular regulatory T (Tfr) cells suppress B cell responses through modulation of follicular helper T (Tfh) cells and germinal center (GC) development. We found that Tfr cells can also promote the GC response through provision of interleukin-10 (IL-10) after acute infection with lymphocytic choriomeningitis virus (LCMV). Sensing of IL-10 by B cells was necessary for optimal development of the GC response. GC B cells formed in the absence of Tfr cell–derived IL-10 displayed an altered dark zone state and decreased expression of the transcription factor Forkhead box protein 1 (FOXP1). IL-10 promoted nuclear translocation of FOXP1 in activated B cells. These data indicate that Tfr cells play a multifaceted role in the fine-tuning of the GC response and identify IL-10 as an important mediator by which Tfr cells support the GC reaction.

INTRODUCTION

The germinal center (GC) response is essential for the production of memory B cells and class-switched, long-lived plasma cells that produce high-affinity antibodies (1). Understanding the mechanisms regulating the GC response is of interest because of the potential to harness this knowledge to bolster or, in the cases of autoimmunity and B cell lymphomas, restrain the GC reaction (2). Follicular helper T (Tfh) cells are a specialized subset of effecter CD4+ T cells that express the transcription factors Bcl6 and Aiol2, and the B cell follicle-homing chemokine receptor CXCR5, and reside within the GC (3, 4). Tfh cells regulate the GC response through secretion of cytokines [e.g., interleukin-21 (IL-21), IL-4, and interferon-γ (IFN-γ)] and expression of surface ligands such as CD40L that signal to GC B cells and promote their maturation (5). Competition for Tfh cell help regulates B cell selection within the GC because high-affinity B cells preferentially interact with Tfh cells and receive signals promoting the further proliferation and somatic hypermutation of their immunoglobulin (Ig) genes (6, 7).

A subset of effector Foxp3+ regulatory CD4+ T (Treg) cells that express CXCR5 and Bcl6 was recently described (8–10). These cells, known as follicular regulatory T (Tfr) cells, originate from thymic-derived Foxp3+ cells or naïve cells and reside within the follicles and GC in mice and humans, where they serve to modulate the magnitude and quality of the GC and Tfh cell responses (8–13). Tfr cells express the inhibitory co-receptor CTLA4, which is essential for their restraint of the GC response (14, 15). CTLA4 suppresses the latter response by modulating B cell expression of B7-2 (CD86) outside GCs (15) or by acting on GC B cells, either dependently or independently of B7-1 (CD80) and B7-2 (14–16). It may also function to control Tfh cell generation directly by altering CD28 engagement (17). Although Tfr cells deficient in CTLA4 have impaired suppressive ability in vivo, it is likely that both Tfr cells and follicular nonresident Treg cells act through CTLA4 to restrain the GC response (14, 15).

Tfr cells may also modulate the GC response through pathways independent of CTLA4. Treg cells regulate immune cells through control of IL-2 availability, surface expression of ectoenzymes (CD39 and CD73), inhibitory receptors (e.g., CTLA4 and Lag3), and secretion of cytokines such as IL-10, IL-35, and TGF-β (transforming growth factor–β) (18). During influenza infection, Treg cells indirectly promote Tfh cell differentiation and, subsequently, the GC response by limiting T cell exposure to IL-2 (19). IL-2 signaling through signal transducer and activator of transcription 5 (STAT5) and via Akt and mechanistic target of rapamycin (mTOR) can impair Tfr cell differentiation by suppressing Bcl6 expression and up-regulating that of Blimp1 (20). Whether Tfr cells may directly regulate the GC response through similar mechanisms remains ill defined.

Treg cell production of IL-10 is essential for the regulation of inflammation at environmental interfaces, such as the colon and lung, and can enhance the development of functional memory CD8+ T cells during acute lymphocytic choriomeningitis virus (LCMV) infection through suppression of inflammation in the spleen (21–23). IL-10 can also promote B cell proliferation, survival, and differentiation into antibody-secreting plasma cells (24–28), although it is not known whether Tfr cell–derived IL-10 can modulate the GC response. Here, we demonstrate that it does modulate the GC response during acute viral infection. These findings reveal that Tfr cells play a multifaceted role in the fine-tuning of the GC response and are capable of promoting or suppressing the GC response depending on the signals provided and the context in which these signals are received.

RESULTS

Treg cell–derived IL-10 promotes B cell differentiation and GC development

To examine IL-10 production by Tfr cells, we infected 10B10 IL-10 reporter mice acutely with the Armstrong strain of LCMV and assessed Tfr cell phenotype and kinetics. These reporter mice have a bacterial artificial chromosome transgene containing the Il10 gene locus, with the Thyl.1.1 complementary DNA (containing a stop codon) replacing the endogenous coding segment of exon 1 of the Il10 locus such that
Tfr cells

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in Foxp3-Il10 gated as in fig. S1, A and B, and as previously described (31–35). The ratio of Tfr cells to Tfh cells or GC B cells peaked at day 5 after infection and progressively declined on days 8 and 12 as the increase in Tfh cell and GC B cell numbers outpaced that of Tfr cells (Fig. 1B) (9, 10). There was an increased percentage of Thy1.1+ cells within the Tfr cell population relative to non-Tfr Treg cells at days 5 and 8 after infection, suggesting that IL-10 secretion may be a mechanism by which Tfr cells regulate the emerging GC response within the follicle (Fig. 1C). Detection of Thy1.1+ Treg cells within the GC by immunofluorescence was impeded because of technical complications related to the disrupted splenic architecture at days 5 and 8 after infection and the dimness of Thy1.1 expression. Only a small percentage of Tfh cells or GC B cells were competent to express IL-10, with their percentages declining over time (fig. S1C). They also made less of this cytokine on a per-cell basis relative to Tfr and non-Tfr Treg cells, suggesting that these cells, in comparison with Tfr or Treg cells, are not an important source of IL-10 for the GC response.

To evaluate the importance of Treg cell–derived IL-10 in the GC response, we generated mice that specifically deleted Il10 in Foxp3-expressing Treg cells, as previously described (29). Il10f/f Foxp3-Cre mice displayed no sign of overt disease at steady state and did not show any consistent trend toward differences in basal percentages of GC B cells, Tfh cells, or Tfr cells (fig. S2). There was also no difference in the Tfr-to-Tfh and Tfr-to-GC B cell ratios in the spleen, peripheral and mesenteric lymph nodes, and Peyer’s patches. We evaluated the B cell response after acute LCMV infection of these animals in comparison with IL-10 intact ones. Although this response appeared similar in the two groups at day 8 after infection, a statistically significant decrease in the percentage and number of B cells with an activated phenotype (IgDlo) and of those cells with a GC phenotype (GL7+CD95+) emerged over time in mice in which Treg cells lacked Il10 expression, with this difference peaking at day 15 after infection (Fig. 2A and fig. S3A). These mutant mice also displayed reduced GC size as determined by confocal imaging of splenic sections (Fig. 2B). We found that mice lacking Treg cell–derived IL-10 had a reduced percentage and number of plasmablasts (CD138+B220+ cells) compared with their IL-10 intact counterparts, albeit a similar number of cells with a GC-dependent memory B cell phenotype (B220+IgDloGL7+CD38+CD95+ cells) (Fig. 2C) (36, 37). They also had reduced serum titers of LCMV-specific IgG2a and IgG1 (Fig. 2D), with a similar number of LCMV-specific memory B cells (Fig. 2E). No defects in GC B cell or plasmablast numbers were evident in mice immunized with NP-OVA [(4-hydroxy-3-nitrophenyl)acetyl-ovalbumin] in complete Freund’s adjuvant (fig. S3B). There were also no apparent defects in GC B cell or plasmablast numbers in LCMV-infected IL-10−/− mice or in mice treated with an anti–IL-10 antibody, suggesting that IL-10 production by different cell types may play opposing roles in the regulation of the GC response (fig. S4, A and B) (38). Together, these data indicate that IL-10 specifically produced by Treg cells is important in promoting plasmablast differentiation and the development of the GC after viral infection.

**Tfr cell–derived IL-10 promotes the GC response**

These findings did not distinguish between the roles of Tfr cell–derived IL-10 and IL-10 produced by Bcl6+ Treg cells in regulating the GC response. Follicular nonresident Treg cells can modulate the GC response as early as day 3 after immunization and likely serve as precursors for Tfh cells (14). To discriminate between these possibilities, mixed bone marrow chimeras (mBMCS) were generated, in which Il10 was specifically ablated in Bcl6-expressing Tfh cells via generation of 50:50 Il10f/f Foxp3-Cre:Bcl6f/f Foxp3-Cre and control 50:50 Il10f/f:Bcl6f/f Foxp3-Cre chimeras (Fig. 3A) (12). In the absence of Tfr cell–derived IL-10, there was a significantly reduced percentage of IgDlo B cells at day 15 after LCMV infection and reduced percentages of GC B cells and plasmablasts (CD138+B220+ cells) in comparison with control chimeras. The importance of Tfr cell–derived IL-10 was further tested using

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*Fig. 1. Tfr cells robustly secrete IL-10 after acute viral infection. Analysis of the Treg cell response after LCMV infection in IL-10 reporter (10BIt Thy1.1) mice. (A) Quantification of the number of Tfr cells, non-Tfr Treg cells, Tfh cells, and GC B cells at days 0, 5, 8, and 12 after infection. Populations are defined as follows: Tfr cells, CD4+Ly6C−PSGL1hiCXCR5hiPD1+Foxp3+; non-Tfr Treg cells, CD4+CXCR5loPD1−Foxp3+; Tfh cells, CD4+Ly6C−PSGL1hiCXCR5hiPD1+Foxp3+; and GC B cells, B220+IgDloGL7−CD95+. (B) Quantification of the ratio of Tfh cells to Tfr cells or GC B cells at days 0, 5, 8, and 12 after infection. (C) Representative plot of IL-10 expression (assessed as Thy1.1) by Tfr cells (left) and non-Tfr Treg cells (middle) from mice as described in (A). Right: Frequency of Thy1.1+ cells in Tfr and non-Tfr Treg cells at days 0, 5, 8, and 12 after LCMV Armstrong infection. Statistical analyses were performed using unpaired two-tailed Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001). Data are from two experiments representative of four experiments with three to six mice per time point after LCMV Armstrong infection.*

Fig. 2. Regulatory CD4+ T cell–derived IL-10 is important for B cell differentiation and the GC response. Analysis of the B cell response in Il10f/f and Il10f/f Foxp3-Cre mice 15 days after LCMV infection. (A) Top: Representative plots of the B cell responses in Il10f/f or Il10f/f Foxp3-Cre mice. Bottom: Frequency and number of cells as indicated by the gates shown above. (B) Representative confocal images of the GC from Il10f/f or Il10f/f Foxp3-Cre mice. (C) Left: Frequency of plasmablasts and memory B cells defined by the expression of surface markers. Right: GC sizes as measured by ImageJ. The sections were taken from four mice of each genotype. Scale bar, 100 μm. (D) Enzyme-linked immunosorbent assay quantification of LCMV-specific IgG2a and IgG1 antibody levels, indicated by arbitrary units (a.u.). All the analyses of the GC response were performed 15 days after acute LCMV Armstrong infection. n.s., not significant. (E) Enzyme-linked immunospot quantification of the number of LCMV-specific IgG2a and IgG1 memory B cells. All the analyses of the GC response were performed 60 days after acute LCMV Armstrong infection. Statistical analyses were performed using unpaired two-tailed Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001). Data for (A) to (C) are from one experiment representative of three experiments with three to five mice per group carried out 15 days after LCMV Armstrong infection and from four experiments with three to five mice per group carried out 12 days after LCMV Armstrong infection. Data for (D) are pooled from two experiments carried out 12 or 15 days after infection. Data for (E) are pooled from two experiments with five to seven mice per group carried out 60 days after infection.
another approach in which mBMCs were generated using SAP-deficient (SAP−/−) mice in place of Bcl6f/fFoxp3-Cre (Fig. 3B). SAP is required for Treg cell differentiation (8, 9). Again, we found that loss of Treg cell–derived IL-10 resulted in decreased percentages of IgDlo B cells, GC B cells, and plasmablasts. These data indicate that Treg cell–derived IL-10 supports B cell differentiation and the GC response.

**IL-10 signals via B cells to promote the GC response**

There are multiple pathways by which Treg cell–derived IL-10 could modulate the GC response, including acting directly on B cells or indirectly through Tfh cells or dendritic cells (DCs) (39). The dominant GP 66–77 epitope was similar between the two groups, with 84 genes up-regulated and 54 genes down-regulated in the Il10f/fFoxp3-Cre mice compared with controls (Padj < 0.1) (Fig. 5A and fig. S6A). Among the DEGs were a number of genes used to distinguish dark zone and light zone GC B cells, including Cxcr4 and Ccl13 (6, 41). Gene set enrichment analysis (GSEA) was used to detect genome-wide changes and identified a significant enrichment for a light zone GC B cell signature in GC B cells isolated from Il10f/fFoxp3-Cre mice (Fig. 5, B and C, and fig. S6B). The dark zone is the proliferative compartment of the GC and the predominant site of Ig gene somatic hypermutation, in contrast to the light zone where GC B cells have a largely nonproliferative state and compete for the T cell help necessary to induce dark zone reentry (42–44). GC B cells isolated from Il10f/fFoxp3-Cre compared with control mice were also highly impaired in gene sets associated with translational activity (Fig. 5C). Together, these findings suggest that Treg cell–derived IL-10 promotes GC B cells to adopt a dark zone phenotype.

Fig. 3. Treg cell–derived IL-10 is important for B cell differentiation and the GC response. (A) Analysis of the B cell response at day 15 after acute LCMV Armstrong infection in 50:50 II10αf/fBcl6f/fFoxp3-Cre or II10αf/fFoxp3-Cre/Bcl6f/fFoxp3-Cre mBMCs. Left: Schematic for the experiment. Right: Data are pooled from two experiments with four to five mice per group carried out 15 days after LCMV Armstrong infection. (B) Analysis of the B cell response at day 15 after acute LCMV Armstrong infection in 50:50 II10αf/fSAP−/− or II10αf/fFoxp3-Cre/SAP−/− mBMCs. Left: Schematic for the experiment. Right: Data are from one experiment representative of two experiments with four to nine mice per group carried out 15 days after LCMV Armstrong infection. Statistical analyses were performed using unpaired two-tailed Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001).

seven mice per group carried out 15 days after LCMV Armstrong infection. Mice for the control 
dependent crosses used to generate the experimental groups.

(Fig. 4. IL-10 acts on B cells to regulate the GC response. (A) GC B cells and DCs, but not Treg cells, are responsive to IL-10. Analysis of IL-10 responsiveness in GC B cells, DCs (CD11c<sup>hi</sup>MHCII<sup>+</sup>), and Tfh cells. Splenocytes isolated from mice at day 12 after LCMV infection were stimulated for 30 min with IL-10, and pSTAT3 levels were determined. Data are from one experiment representative of three experiments with four mice per group. (B) Analysis of the B cell response in Il10<sup>−/−</sup>, Il10<sup>−/−</sup>Cd19-Cre, Il10<sup>−/−</sup>Cd11c-Cre, and Il10<sup>−/−</sup>Cd4-Cre mice 15 days after LCMV infection. Top: Representative plots of B cell response. Bottom: Quantification of B cell response shown above. Statistical analyses were performed using unpaired two-tailed Student’s t test (*P < 0.05; **P < 0.01). Data are from one experiment representative of two to three experiments with three to seven mice per group carried out 15 days after LCMV Armstrong infection. Mice for the control Il10<sup>−/−</sup> group were pooled from littermate controls produced from the independent crosses used to generate the experimental groups.

To more directly test this hypothesis, we determined the dark zone and light zone phenotypes of GC B cells from control mice and those lacking T<sub>reg</sub> cell–derived IL-10. In agreement with our RNA-seq data, there was a small but significant decrease in the percentage of dark zone GC B cells and an increase in that of light zone GC B cells in GC B cells from Il10<sup>−/−</sup>Foxp3-Cre mice (Fig. 5D). Aligning with previous results, we did not detect a difference in GC B cell proliferation or survival between the groups (fig. S7) (43, 44). These data support a model in which T<sub>reg</sub> cell–derived IL-10 acts on light zone GC B cells to skew them toward a dark zone phenotype. These data do not discount the possibility that T<sub>reg</sub> cell–derived IL-10 acts on B cells during the early stages of the GC response to influence their ability to later adopt a dark zone phenotype.

T<sub>reg</sub> cell–derived IL-10 promotes dark zone phenotype through induction of nuclear FOXO1

We next analyzed our RNA-seq data using Integrated Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com). This analysis revealed that eukaryotic initiation factor 2 (eIF2) signaling was the most significantly dysregulated pathway in Il10<sup>−/−</sup>Foxp3-Cre mice compared
Fig. 5. GC B cells in mice lacking regulatory CD4^+ T cell–derived IL-10 display an enhanced light zone GC B cell gene signature. (A) RNA-seq analysis of select DEGs among mRNA isolated from GC B cells pooled from Il10^f/f and Il10^−/−Foxp3-Cre mice 12 days after LCMV Armstrong infection presented as expression (log_2) in Il10^−/−Foxp3-Cre cells relative to that in Il10^f/f cells (key below; columns indicate paired replicates). (B) GSEA of light zone and dark zone signatures in GC B cells, based on published gene sets (6, 41). A positive enrichment score (ES) signifies enrichment in the Il10^−/−Foxp3-Cre sample relative to the Il10^f/f condition of a given gene set, that is, more highly expressed. (C) Normalized ES (NES) for select pathways identified from the Reactome Pathway Database (R), Kegg Pathway Database (K), and published gene sets (G), where dot size represents P values adjusted by the family-wise error rate (FWER). All gene sets attain significant enrichment (false discovery rate (FDR) < 0.001), with the exception of the dark zone gene set (FDR = 0.5). Data are from three independent experiments with three mice per group pooled for each sample. (D) Analysis of the light zone and dark zone GC B cell response in Il10^f/f and Il10^−/−Foxp3-Cre mice 15 days after LCMV infection. Representative plots (left) of GC B cells as gated in fig. S1B. The numbers of the outlined area indicate dark zone (top left) and light zone (bottom right) based on the expression of the surface markers CXCR4 and CD86. Middle: Frequency of GC B cells from light zone gate defined by CXCR4^+CD86^− and dark zone gate defined by CXCR4^−CD86^+ from Il10^f/f or Il10^−/−Foxp3-Cre mice. Right: Expression of CXCR4 in GC B cells. Statistical analyses were performed using unpaired two-tailed Student’s t test (P < 0.05). Data are from one experiment representative of two experiments with at least four mice per group carried out 15 days after LCMV Armstrong infection. MFI, mean fluorescence intensity.
context in which B cells are exposed to IL-10, cells were isolated from mice at day 5 after LCMV infection and cultured with or without IL-10. Day 5 coincides with the peak expression of IL-10 by Tfr cells, a major source of IL-10 within the inner follicle (Fig. 1C and fig. S1C), and allowed us to assess FOXO1 nuclear translocation in cells that have not yet had prolonged exposure to IL-10. Using Amnis ImageStream analysis, we observed an increase in the percentage of nuclear translocated FOXO1 in activated B cells (Fig. 6D). IL-10 did not induce nuclear translocation of FOXO1 in follicular B cells, despite robust pSTAT3 signaling (fig. S8C), suggesting that IL-10 acts in concert with other signals received by activated B cells, such as B cell receptor (BCR), costimulation, and cytokines, to promote FOXO1 activity.

In addition to regulating dark zone reentry, FOXO1 is also important in promoting Ig affinity maturation (42, 43). To assess affinity maturation, GC B cells from control Il10f/f and experimental Il10f/fFoxp3-Cre mice were sorted at day 15 after LCMV infection and a large number of the Ig heavy chain variable region (VH) genes were cloned and sequenced using a pooled primer approach, as previously described (47). The overall mutation frequency in the complementarity-determining region (CDR) was similar between groups (Fig. 6E). However, we identified a reduction in the degree of positive selection within the CDR in the Il10f/fFoxp3-Cre mice, which was absent in the framework region (FWR) (Fig. 6E). The trend was observed in two independent experiments but did not reach statistical significance because of the limitation of the number of clones obtained. We further examined the amino acid physicochemical properties of CDR3 because diversity in this region is a key determinant of antigen binding. Mice that lacked Treg cell–derived IL-10 displayed a trend toward a higher grand average of hydrophobicity (GRAVY) score, mean side-chain bulkiness, and abundance of aromatic residues in this region, consistent with the notion that less selection is occurring in these cells because positively selected cells typically display reduction in these metrics (fig. S9) (48).

**DISCUSSION**

Understanding the signals regulating GC B cell differentiation is critical for the development of targeted therapies that can modulate GC output. Here, we describe a role for Tfr cells in promoting the GC response through local production of IL-10 during viral infection. IL-10–secreting Treg cells act on B cells to drive their differentiation into plasmablasts and GC B cells. Both IL-10 expression and the Tfr to-GC B cell ratio peak at day 5 after infection, suggesting that Tfr cell–derived IL-10 may be predominantly acting early to drive B cell differentiation. Tfr cell–derived IL-10 promotes expression and activity of FOXO1, thereby facilitating adoption of a dark zone phenotype by GC B cells and potentially enhancing affinity maturation.
These results illustrate the complex role of T<sub>fr</sub> cells in the regulation of the GC response and the need to consider both the suppressive and stimulatory roles of T<sub>reg</sub> cells in control of humoral immunity. How do T<sub>fr</sub> cells balance their suppression and promotion of GC output? IL-10–competent T<sub>fr</sub> cells express high levels of CTLA4, so it is unlikely that IL-10–secerting and CTLA4-expressing T<sub>fr</sub> cells represent unique populations. One possible explanation is that suppression by T<sub>fr</sub> cells is reliant on cell contact (e.g., CTLA4-CD80/86 interactions). In such a scenario, light zone GC B cells engaged in productive major histocompatibility complex (MHC)–peptide–T cell receptor (TCR) exchange with T<sub>fr</sub> cells would be less available for this type of interaction, thus allowing IL-10 secretion by nearby T<sub>fr</sub> cells to stimulate GC B cell differentiation and dark zone reentry. However, GC B cells not engaged with T<sub>fr</sub> cells would be available to interact with T<sub>fr</sub> cells, where the suppressive function of CTLA4 could dominate the stimulatory role of IL-10. In this manner, T<sub>fr</sub> cells could promote the continued differentiation of affinity-matured, antigen-bearing GC B cells while suppressing those cells with low antigen affinity. Alternatively, B cells engaged in productive interactions with T<sub>fr</sub> cells, including before GC formation, might be rendered more sensitive to IL-10 signaling (or less susceptible to inhibition by CTLA4), providing signals that initiate dark zone polarization of these cells or their growth advantage. The finding that T<sub>fr</sub> cells restrict the outgrowth of non–antigen-specific GC B cells supports these models and suggests an important role for T<sub>fr</sub> cells in restraining the expansion of autoreactive GC B cells (9). It will be important for future work to more directly test these ideas and explore other mechanisms by which T<sub>fr</sub> cells may modulate the GC response.

Distinguishing between the function of T<sub>fr</sub> cells and follicular non-resident T<sub>reg</sub> cells in modulating the GC response is critical. Ablation of CTLA4 expression on T<sub>reg</sub> cells impairs CD86 expression on B cells before the formation of T<sub>fr</sub> cells, indicating that follicular nonresident T<sub>reg</sub> cells influence the GC response (14). It is likely that there are multiple mechanisms by which T<sub>reg</sub> cells could exert this effect, including regulation of T<sub>fh</sub> or B cell avidity and the activation state of DCs (22, 23, 49). Here, we found that depletion of T<sub>fr</sub> cells–derived IL-10 led to an impaired GC response. This finding does not rule out a contribution of IL-10–producing follicular nonresident T<sub>reg</sub> cells on the regulation of the GC response. It also does not distinguish between the roles of T<sub>fr</sub> cells inside and outside of the GC, as well as those cells present within the follicle before GC development.

T<sub>fr</sub> cell–derived IL-10 likely contributes to the GC B cell response through multiple mechanisms. Our work reveals its promotion of FOXO1 expression, which is required for dark zone formation because of its essential role in gene program instruction, including the up-regulation of the chemokine receptor CXCX4 required for GC B cell migration into the dark zone (42–45). However, although regulation of FOXO1 activity may explain the dysregulated dark zone phenotype and affinity maturation of GC B cells from mice lacking T<sub>reg</sub> cell–derived IL-10, it does not explain the decrease in GC B cell number and output. GC B cell proliferation and somatic hypermutation typically occur in the dark zone, but these processes are maintained even in cells that are unable to access the dark zone (42–44). Therefore, it is likely that T<sub>fr</sub> cell–derived IL-10 functions in a FOXO1-independent manner to regulate B cell differentiation and GC development.

The IL-10–STAT3 pathway has been implicated as a promoter of diffuse large B cell lymphoma (DLBCL) and systemic lupus erythematosus (50–53). A high level of circulating IL-10 and active intracellular STAT3 are associated with clinically aggressive cases of DLBCL (51, 52). Constitutively active STAT3 promotes cell proliferation and survival, and anti–IL-10R antibody treatment induces cell cycle arrest and apoptosis in DLBCL cell lines (50, 52). Among known STAT3 targets that are inhibited by IL-10R blockade are genes involved in cell cycle progression, antiapoptotic factors, and proto-oncogenes, including Cnrd1, Mcl1, Junb, and cMyc (50, 52). We found a robust impairment in pathways associated with RNA translation in mice lacking T<sub>reg</sub> cell–derived IL-10, suggesting that IL-10–driven STAT3 may regulate GC B cell proliferation and differentiation by inducing the translation of mRNAs encoding proliferation-promoting proteins or proteins involved in differentiation (54). It will be important to elucidate which genes IL-10–driven STAT3 is acting upon to drive B cell proliferation and differentiation during viral infection and the extent that this pathway overlaps with disease progression in individuals with DLBCL. Therapies designed to modulate the stimulatory potential of T<sub>fr</sub> cells could prove an effective method to bolster humoral immunity or restrain the expansion of autoimmune or malignant B cell clones.

**MATERIALS AND METHODS**

**Study design**

The aim of this study was to characterize the role of T<sub>fr</sub> cell–derived IL-10 in the regulation of the GC response after acute viral infection. Most of the experiments consisted of enumerating population frequencies by flow cytometry in different genetic mouse models, analysis of GCs by immunofluorescence, analysis of RNA-seq data, and quantification of nuclear localized transcription factors. Littermate comparisons were used for all experiments where possible. Control and experimental groups were age- and sex-matched. The investigators were not blinded. Experimental replications are indicated in the figure legends.

**Mice**

C57BL/6 mice were purchased from the National Cancer Institute or the Jackson Laboratory. B6.129P2-Il10<sup>tm1Cgn</sup>/J (Il10<sup>−/−</sup>), B6.129(Cg)-Floxp<sup>3</sup>tm3(DTR/GFPI)Ayr<sup>tf</sup>/j (Floxp<sup>3</sup>GFPI-DTR), B6.129S(FVB)-Bcl6<sup>tm1.Dntn</sup>/j (Bcl6<sup>−/−</sup>), B6.129S6-Sh2d1a<sup>tm1Pls</sup>/j (SAP<sup>−/−</sup>), and B6.129(Cg)-Floxp<sup>3</sup>tm4(YFPcre)Ayr<sup>tf</sup>/j (Floxp3-Cre) mice were purchased from the Jackson Laboratory. 10BiT mice (29), Il10<sup>−/−</sup> mice (55), and Cd4-Cre, Cd19-Cre, and Cd11c-Cre mice have been described. Il10ra<sup>−/−</sup> mice were generated by the Flavell laboratory, as previously described (22). All animal experiments were done with approval of the Yale Institutional Animal Care and Use Committee.

**Infection and treatments**

Mice were given intraperitoneal administration of 2 × 10<sup>5</sup> plaque-forming units of LCMV Armstrong. Diphtheria toxin was reconstituted according to the manufacturer’s instructions (Sigma). Mice were given diphtheria toxin at a dose of 50 μg per kilogram of body weight on days 4 and 5 (two doses) after infection, as described (56). For IL-10 blockade, an anti–IL-10 monoclonal antibody (0.25 mg/ml, JES5-2A5 clone, provided by J. M. M. den Haan, VU University Medical Center, Amsterdam, Netherlands) was administered intraperitoneally every other day. For NP-OVA in complete Freund’s adjuvant immunization, mice were given 100 μg of NP-OVA mixed with an equal volume of complete Freund’s adjuvant.

**Statistical analysis**

Results represent means ± SEM, unless indicated otherwise. Statistical significance was determined by paired or unpaired Student’s t test.
Statistical analyses were performed using Prism GraphPad software version 6.0 (*P < 0.05; **P < 0.01; ***P < 0.001).

SUPPLEMENTARY MATERIALS

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Supplementary Methods

Fig. S1. Flow cytometric gating strategy for Thp, pre-Thp, GC B, Tnop, and Th cells, with IL-10 expression by these populations.

Fig. S2. Mice lacking regulatory CD4+ T cell–derived IL-10 do not have defects in steady-state expression by these populations.

Fig. S3. Temporal development of the GC B cell response in mice lacking regulatory CD4+ T cell–derived IL-10.

Fig. S4. Systemic IL-10 is not required for the GC response.

Fig. S5. Regulatory CD4+ T cell–derived IL-10 is not required for effector CD4+ T cell differentiation.

Fig. S6. Heat map of DEGs based on RNA-seq.

Fig. S7. GC B cells in mice lacking regulatory CD4+ T cell–derived IL-10 display similar levels of proliferation and death.

Fig. S8. IL-10 does not induce FOXP1 nuclear translocation in IgD+ B cells.

Fig. S9. The VH CDR3 region of GC B cells in mice lacking regulatory CD4+ T cell–derived IL-10 displays altered amino acid physiochemical properties.

Table S1. Tabulated data for Figs. 1 to 6 and figs. S1 to S9.


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Interleukin-10 from CD4⁺ follicular regulatory T cells promotes the germinal center response

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Probing the functions of follicular regulatory T cells

CD4⁺ follicular regulatory T (Tfr) cells express key molecules that are associated with regulatory T cell and follicular helper T cell functions. Although it is known that Tfr cells produce interleukin-10 (IL-10), it has been unclear whether IL-10 production by these cells regulates germinal center (GC) responses in vivo. By specifically ablating IL-10 expression in murine Tfr cells, Laidlaw et al. demonstrate that Tfr cell–derived IL-10 does support GC responses in the context of acute viral infection. They found dendritic cells and B cells in the GCs to be IL-10–responsive and showed that IL-10 promoted GC B cells to adopt a dark zone phenotype.