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T follicular regulatory cells and IL-10 promote food antigen–specific IgE

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Food allergies are a major clinical problem and are driven by IgE antibodies (Abs) specific for food antigens (Ags). T follicular regulatory (Tfr) cells are a specialized subset of FOXP3+ T cells that modulate Ab responses. Here, we analyzed the role of Tfr cells in regulating Ag-specific IgE using a peanut-based food allergy model in mice. Peanut-specific IgE titers and anaphylaxis responses were significantly blunted in Tfr cell–deficient Foxp3-Cre Bcl6fl/fl mice. Loss of Tfr cells led to greatly increased nonspecific IgE levels, showing that Tfr cells have both helper and suppressor functions in IgE production in the germinal center (GC) that work together to facilitate the production of Ag-specific IgE. Foxp3-Cre Ptenfl/fl mice with augmented Tfr cell responses had markedly higher levels of peanut-specific IgE, revealing an active helper function by Tfr cells on Ag-specific IgE. The helper function of Tfr cells for IgE production involves IL-10, and the loss of IL-10 signaling by B cells led to a severely curtailed peanut-specific IgE response, decreased GCB cell survival, and loss of GC dark zone B cells after peanut sensitization. We thus reveal that Tfr cells have an unexpected helper role in promoting food allergy and may represent a target for drug development.

Introduction
Antibodies (Abs) that bind to antigen (Ag) with high affinity and help to eliminate pathogens and foreign substances are generated in the germinal center (GC) reaction (1–3). A specialized subset of differentiated CD4+ T cells, T follicular helper (Tfh) cells, are required in the GC reaction to help B cells generate high-affinity Abs (4). Tfh cells control the initiation as well as the overall outcome of the GC B (GCB) cell response (5, 6). Although Tfh cells are required to produce protective Abs, the deregulation of Tfh cells can also lead to autoimmunity, since Tfh cells can help B cells to produce self-reactive Abs (6–8). Ab responses are also modulated by T follicular regulatory (Tfr) cells, which develop from FOXP3+ Tregs and localize to the GC as a result of CXCR5 expression (9–15). Tfr cells, like Tfh cells, are dependent on the transcriptional repressor protein Bcl6 for their development, but unlike Tfh cells, Tfr cells also express the canonical Treg master regulatory transcription factor FOXP3 (9–14). Tfr cells have usually been found to act as repressor cells in the GC (9–11, 14). More recently, a Tfr-deficient mouse model, in which the Bcl6 gene was specifically deleted in FOXP3+ T cells (Bcl6KO Foxp3-Cre, referred to hereafter as Bcl6FC mice), was used by us and others to study Tfr cell function (12, 16–19). This mouse model has helped to show that Tfr cells can have a helper function in the GC and Ab response (15).

The increase in atopic and allergic diseases over the past 30 years is of epidemic proportions (20, 21). IgE mediates immediate hypersensitivity to allergens in atopic patients (22, 23). Despite its importance in allergic disease, how IgE responses develop in the GC is not well understood, and there is controversy over the specific pathways involved (24–28).

Recent studies have revealed that IL-4–secreting Tfh cells are absolutely required for the development of Ag-specific IgE (29, 30). The discovery of Tfh cells has expanded our knowledge of allergic immune responses, but much remains unclear about the regulation of allergen-specific IgE production in the GC. Furthermore, the role of Tfr cells in the IgE pathway is not known. Here, we investigated the role of Tfr cells in IgE production using a mouse model of food allergy and found, unexpectedly, that Tfr cells positively regulated the GC and Ag-specific IgE production, in part through the production of IL-10.

Results
Production of Ag-specific IgE is dependent on both Tfr cells and the GC reaction. To study the role of Tfr cells in regulating IgE production and IgE responses, we used the Bcl6FC mouse model, in which Tfr cells do not develop (12), as well as a widely used model of food allergy induced by intragastric (i.g.) sensitization with peanut protein plus cholera toxin (PCT) as an adjuvant (31–33). In this model (Figure 1A), high levels of peanut-specific IgE are induced and maintained in the circulation for weeks (31–33), and 4 weeks after the sensitization period, control (WT) mice produce substantial levels of peanut-specific IgE (Figure 1B). In Bcl6FC mice, the peanut-specific IgE response was almost undetectable after 4 weeks (Figure 1B). Peanut-specific IgG1 was present but significantly decreased in the Bcl6FC mice (Figure 1B). At the same time, we found that total IgE responses were significantly increased in Bcl6FC mice, showing a repressive effect of Tfr cells on nonspecific IgE responses (Figure 1B and Supplemental Figure 1;
Figure 1. Lack of Tfr cells in a food allergy model leads to loss of peanut-specific IgE and decreased anaphylaxis responses. Peanut allergy was induced with 2 i.g. doses of PCT given 7 days apart, and mice were bled at various time points after sensitization. (A) Schema showing the 36-day timeline, in which serum was tested 28 days after the last sensitization for peanut-specific Abs. (B-D) Control Foxp3-Cre mice (WT) and Bcl6FC mice were sensitized as in A, and day-36 serum was tested for peanut-specific IgE, IgG1, and total IgE (B) or at various time points during and after sensitization as indicated (red arrows in C) (C and D). Data for A and B are from 1 representative experiment of 4 experiments with 4–5 mice per group. Data for C and D are from 1 representative experiment of 2 experiments with 4–5 mice per group. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-way ANOVA with Holm-Šidák multiple comparisons test (B, D, and F) or 2-way ANOVA with Tukey’s multiple comparisons test (E and G).
The Journal of Clinical Investigation

RESEARCH ARTICLE

The levels were significantly lower in Bcl6FC mice (Figure 1D). To assess the physiological relevance of peanut-specific IgE levels, we induced anaphylaxis by giving a systemic challenge of peanut protein alone 4 weeks after mice were peanut sensitized. We found that a strong anaphylaxis response was induced in WT mice, whereas anaphylaxis was notably weaker in Bcl6FC mice (Figure 1E). The lower anaphylaxis response in Bcl6FC mice is not explained by a weaker affinity of the peanut-specific IgE, as the affinity of IgE from Bcl6FC mice for the peanut protein Ara H 3 is similar to that in WT mice.

We then analyzed the time course of serum peanut-specific IgE and IgG1 induction and maintenance after PCT sensitization (Figure 1, C and D). We observed that in WT mice, peanut-specific IgE was induced to high levels 1 week after the second PCT sensitization (day 15) and then slowly decreased over several weeks. However, in Bcl6FC mice, the initial induction of peanut-specific IgE was much weaker and then faded to undetectable levels 2 weeks after PCT sensitization. Peanut-specific IgG1 was strongly induced and maintained at high levels for weeks after the PCT sensitization in both WT and Bcl6FC mice, though the levels were significantly lower in Bcl6FC mice (Figure 1D). To assess the physiological relevance of peanut-specific IgE levels, we induced anaphylaxis by giving a systemic challenge of peanut protein alone 4 weeks after mice were peanut sensitized. We found that a strong anaphylaxis response was induced in WT mice, whereas anaphylaxis was notably weaker in Bcl6FC mice (Figure 1E). The lower anaphylaxis response in Bcl6FC mice is not explained by a weaker affinity of the peanut-specific IgE, as the affinity of IgE from Bcl6FC mice for the peanut protein Ara H 3 is similar to that in WT mice.

Figure 2. Tfr cells are required for normal Tfh and GCB cell numbers in a food allergy immune response. WT and Bcl6FC mice were sensitized with PCT as in Figure 1, and then on day 36, mesenteric LNs and SPs were analyzed for Tfh cells (A) and GCB cells (B) by flow cytometry. (C) Time course of the Tfr, Tfh, and GCB responses after PCT sensitization. Data for C were pooled from naive mice (2 experiments), day 15 (2 experiments), day 22 (2 experiments), and day 36 (8 experiments), with 4–5 mice per group (n = 8–10). Day 15 (D15) = 7 days after sensitization; day 22 (D22) = 14 days after sensitization; day 36 (D36) = 28 days after sensitization. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-way ANOVA with Holm-Šidák multiple comparisons test (B) or 2-tailed Student’s t test (C).
Tfr cells are required to maintain GC responses over time. We wondered whether the loss of peanut-specific IgE was explained by a loss of B cells in the GCs of Bcl6FC mice and thus examined GCB, Tfh, and Tfr cells in mesenteric lymph nodes (LNs) and spleens (SPs) to see if there was a defect in the GC reaction. In this model, both Tfh and Tfr cells from WT mice were over 90% CD45RB+, but Tfr cells expressed roughly half as much CXCR5 as Tfh cells (Supplemental Figure 5). Lower CXCR5 expression on Tfr cells compared with expression on Tfh cells has been observed previously with human cells (35). As expected, we found that Tfr cells were almost completely absent in Bcl6FC mice despite a robust Tfr response in WT mice on day 36 of the PCT sensitization (Supplemental Figure 6A). Unexpectedly, we observed a significant decrease in Tfh cells in Bcl6FC mice after PCT sensitization (Figure 2A) and an even larger loss of GCB cells (~70% decrease) in Bcl6FC mice after the PCT sensitization (Figure 2B). This loss of Tfh cells in Bcl6FC mice was not due to aberrant or “leaky” deletion of Bcl6 in FOXP3-YFP-negative Tfh cell precursor cells, as we did not detect significant levels of FOXP3-Cre activity in FOXP3-YFP-negative CD4+ T cells (Supplemental Figure 6, B–E). Next, we examined the time course of the GC response and noted that in WT mice, Tfr, Tfh, and...
Figure 4. Augmented Tfr cell development promotes higher IgE and correlates with increased GC responses after food allergy sensitization. WT and PtenFC mice were sensitized with PCT as in Figure 1. On day 36 of the sensitization system, (A) serum was tested for peanut-specific Abs, and (B–D) SPs and mesenteric LNs were analyzed for Tfr, Tfh, and GCB cells by flow cytometry as in Figure 2. Representative contour dot plots for each cell staining are shown along with graphs indicating the average percentage of cells as a fraction of the parental cell population and the total yield of cells. Data for A are from 1 representative experiment of 2 experiments with 3–5 mice per group. Data for B–D were pooled from 2 experiments with 3–4 mice per group (n = 6–10). *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-way ANOVA with Holm-Šidák multiple comparisons test (A) or 2-tailed Student’s t test (B–D).
Figure 5. IL-10 promotes GCB cell levels and peanut-specific IgE, and therapeutic blockade of IL-10 during food allergy sensitization leads to loss of IgE. (A) WT and MB1-Ill10ra−/− mice were sensitized with PCT. On day 36, GCB cells from LNs and SPs were stained and analyzed by flow cytometry, and GCB cells from LNs and SPs were stained and analyzed by flow cytometry. Representative contour dot plots of GCB cell staining are shown along with graphs indicating the average percentage of GCB cells and total yield of cells. (B) Peanut-specific IgE and IgG1 titers from day-36 serum of WT and MB1-Ill10ra−/− mice sensitized with PCT. Data for A and B are from 1 representative experiment of 3 experiments with 3–5 mice per group. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-tailed Student's t test (A) or 2-way ANOVA with Holm-Šidák multiple comparisons test (B and D).
GCB cell percentages all increased over time and remained high 4 weeks after the last sensitization, on day 36 (Figure 2C). The Tfh and GCB cell response was statistically normal at the early stages in Bcl6FC mice but was decreased at later stages of the analysis, with a sharp decrease in GCB cells observed on day 36 of the response. GCB cells from WT mice continued to expand up to day 36, but in Bcl6FC mice lacking Tfr cells, GCB cell growth leveled off by day 15. The frequency of peanut-specific GCB cells was similar between WT and Bcl6FC mice (Supplemental Figure 7), indicating that the loss of peanut-specific IgE and IgG1 was due to the loss of absolute numbers of GCB cells and not to a loss in the frequency of peanut-specific B cells. The normal Tfh/GCB cell response at early time points is further evidence that the later decline in the Tfh/GCB response in Bcl6FC mice was not due to abnormal deletion of Bcl6 by FOXP3-Cre in Tfh cell precursors. We confirmed these trends in a more robust PCT priming model involving 8 PCT sensitization doses rather than our normal 2 PCT sensitization doses, showing that Tfr cells were still required even for a much stronger allergic induction (Supplemental Figure 8).

Tfr cells actively promote Ag-specific IgE. Although our experiments showed that Tfr cells were necessary for peanut-specific IgE responses in the PCT model, our data did not indicate whether Tfr cells were simply required at some minimal level to help the GC

**Figure 6.** Altered GCB cell cycling and increased apoptosis in the absence of Tfr cells. (A) WT and MB1-II10ra−/− mice were sensitized with PCT. On day 36, GCB cells from SPs were stained and analyzed by flow cytometry for light zone (LZ) (CD86) and dark zone (DZ) (CXCR4) marker expression. Representative contour dot plots of GCB DZ/LZ cell staining are shown along with graphs indicating the average ratios of GCB LZ to GCB DZ cells. (B) WT and Bcl6FC mice were sensitized with PCT. On day 36, GCB cells from SPs were stained and analyzed by flow cytometry for LZ and DZ marker expression as in A. Representative contour dot plots of GCB DZ/LZ cell staining are shown along with graphs indicating the average ratios of GCB LZ to GCB DZ cells. Data for A and B are from 1 representative experiment of 2 experiments with 4–5 mice per group. WT and Bcl6FC (C) and MB1-II10ra−/− (D) mice were sensitized with PCT. On day 36 GCB cells from LNs were stained and analyzed by flow cytometry for viability using eBioscience Fixable Viability Dye. Representative viability stains are shown along with graphs indicating the average percentage of GCB cell death. Data for A and B are from 1 representative experiment of 2 experiments with 4–5 mice per group. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-tailed Student’s t test (A–D).
after as PtenFC mice; ref. 37) to test this idea. We sensitized Pten-FC mice with PCT and analyzed the resulting immune response. As shown in Figure 4A, peanut-specific IgE and IgG1 were strongly increased in PtenFC mice compared with levels in WT mice.

response or whether Tfr cells actively promoted peanut-specific GCB cell responses. We therefore used a mouse model previously shown to develop augmented Tfr responses due to a specific deletion of Pten expression in Tregs (Foxp3-Cre Ptenfl/fl, referred to hereafter as PtenFC mice; ref. 37) to test this idea. We sensitized Pten-FC mice with PCT and analyzed the resulting immune response. As shown in Figure 4A, peanut-specific IgE and IgG1 were strongly increased in PtenFC mice compared with levels in WT mice.

Figure 7. Blimp1-controlled Tfr cell–derived IL-10 is required for peanut-specific IgE production. (A) Il10 mRNA levels in Tregs or Tfr cells isolated from naive or PCT-sensitized WT mice on day 0 (naive) and day 15 as in the model shown in Figure 1. (B) Design for WT/Bcl6FC and Bcl6FC/Blimp1FC BM chimeras. (C–E) Mice generated as in B were sensitized with PCT. Then on day 36, (C) Tfh and Tfr cells were isolated by FACS, and Il10 expression was analyzed by qPCR. (D) Tfh, Tfr, and GCB cells from LNs were stained and analyzed by flow cytometry as in Figure 2; and (E) peanut-specific IgE and IgG1 titers from day-36 serum obtained from mice after PCT sensitization were measured. Data for A were pooled from 3 different cell sorts with 2–4 mice per sort (n = 6–10). Data for C–E are from 1 representative experiment of 2 experiments with 3–5 mice per group. *P < 0.05, **P < 0.01, and ***P < 0.001, by 2-way ANOVA with Tukey’s multiple comparisons test (A, C, and G) or 2-tailed Student’s t test (D).
We confirmed that Tfr cells were significantly augmented in the PCT-sensitized PtenFC mice (Figure 4B) and observed that this increase was associated with an increase in both Tfh and GCB cells (Figure 4, C and D). These data directly challenge the notion that Tfr cells act as suppressors of the GC response, as is standard dogma for Tfr function. Instead, our data showed that Tfr cells actively drove GC responses in this food allergy model. This idea is supported by a linear correlation analysis between the numbers of Tfr cells and other cells in the GC (Supplemental Figure 10A). As expected, the numbers of Tfh and GCB cells were tightly correlated, but Tfr cells also positively correlated with both GCB and Tfh cells to a highly significant degree ($P < 0.0001$). This finding, coupled with the diminished GCB cell response in Bcl6FC mice, indicates that Tfr cells acted as true helper cells of the Ab response in the PCT food allergy model. We did not observe abnormal expression of Il4, Il21, or Ifng by Tfh cells or Tfr cells from PtenFC mice (Supplemental Figure 10B), suggesting that the results we observed following PCT challenge in these mice were not due to aberrant expression of those key Tfh cytokines.

**IL-10 is critical for Ag-specific IgE responses.** Tfr cells produce IL-10 that can augment the GC and Ab response (16). We wondered whether Tfr cell-derived IL-10 was playing a similar helper role in our food allergy model. Since Bcl6FC mice lack Tfr cells, the smaller GC responses in Bcl6FC mice are consistent with loss of Tfr cell–derived IL-10 acting on GCB cells. To specifically test the role of IL-10 in regulating peanut-specific IgE in our food allergy model. Since Bcl6FC mice lack Tfr cells, the diminished GCB cell response in Bcl6FC mice, indicates that Tfr cells acted as true helper cells of the Ab response in the PCT food allergy model. We did not observe abnormal expression of Il4, Il21, or Ifng by Tfh cells or Tfr cells from PtenFC mice (Supplemental Figure 10B), suggesting that the results we observed following PCT challenge in these mice were not due to aberrant expression of those key Tfh cytokines.

**Tfr cell-derived IL-10 promotes Ag-specific IgE responses.** To further link Tfr cell-derived IL-10 to peanut-specific IgE, we first examined $Il10$ mRNA expression from FACS-isolated Tfr cells after PCT sensitization (Figure 7A). On day 15 after sensitization, we observed a significant increase in $Il10$ produced by WT Tfr cells compared with WT Tregs, which correlated with the appearance of IgE in the serum. Tfr cells from PtenFC mice produced less $Il10$ mRNA than did control Tfr cells, however, the difference was not statistically significant (Figure 7A). We next conducted experiments to link IL-10 made by Tfr cells directly to IgE production. First, we used a bone marrow (BM) chimera approach, in which we introduced either WT plus Bcl6FC BM or Blimp$^{−/−}$ Foxp3-Cre (BlimpFC) plus Bcl6FC BM into irradiated Rag1$^{−/−}$ mice (Figure 7B). Blimp1 is essential for IL-10 transcription in Tregs (39, 40). Gene profiling of Tfr cells from WT and Blimp1FC mice showed that the major Treg and Tfr suppressor genes, with the exception of $Il10$, were expressed normally in Blimp1FC mice (Supplemental Figure 12), and a decrease in $Il10$ transcripts in Tfr cells from Blimp1FC plus Bcl6FC chimeras was confirmed by quantitative PCR (qPCR) of FACS-isolated Tfr cells (Figure 7C). The data in Figure 7C also show that Tfr cells expressed at least 7 times more $Il10$ than did Tfh cells. Chimeras with WT plus Bcl6FC BM will develop WT Tfr cells (Figure 7B), and after PCT sensitization of these mice, we observed substantial levels of Tfr, Tfh, and GCB cells in LNs from these mice (Figure 7D), as well as a robust peanut-specific IgE and IgG1 response (Figure 7E). Chimeras with Blimp1FC plus Bcl6FC BM will only develop Blimp1$^{−/−}$ Tfr cells (Figure 7B), and after PCT sensitization, these mice had augmented Tfr cell development, as expected (41), as well as a significant increase in GCB cells (Figure 7D). Strikingly, Blimp1FC plus Bcl6FC BM chimeras had severely attenuated peanut-specific IgE and IgG1 responses (Figure 7E). These data show that Tfr cells incapable of producing normal IL-10 levels cannot drive the Ag-specific IgE response. Additionally, we found that anti–IL-10R Ab inhibited the peanut-specific IgE response in WT control mice to the level seen in Bcl6FC mice, whereas anti–IL-10R Ab did not inhibit the IgE response in Bcl6FC mice (Figure 7, F and G, and Supplemental Figure 13). These 2 lines of evidence indicate that production of IL-10 by Tfr cells was required for peanut-specific IgE production.

**Discussion**

Here, we show that Ag-specific IgE responses induced in a food allergy sensitization system were strikingly dependent on Tfr cells and, further, that Tfr cells played an active helper role in the production of Ag-specific IgE. Our data also point to a key role of IL-10 produced by Tfr cells in promoting the development of Ag-specific IgE-secreting cells from the GC. The data presented here fit into an emerging helper model of Tfr cell function that both refines and redefines the standard view of Tfr cells.

Initial studies of Tfr cells described them as suppressors of the GC and Ab response, however, more recent studies have indicated that Tfr cells can have a helper function. Our initial work...
with Bcl6FC mice showed an unexpected loss of Ag-specific IgG after immunization (13). Laidlaw et al. analyzed Tfr function in a virus infection model and showed that Tfr cells produce IL-10 within the GC to facilitate GCB cell growth and Ag-specific IgG production (16). Here, in a food allergy model, we show a strong positive regulation of Ag-specific IgE by Tfr cells and IL-10 and, further, that Tfr cells were critically required for the allergic IgE response to develop.

Strikingly, the augmented Tfr cells in PtenFC mice drove dramatically higher levels of peanut-specific IgE compared with that seen in WT mice, strongly supporting a positive helper role for Tfr cells in this model, in contrast to the common view that Tfr cells act as suppressors of the GC response (9–11, 14, 15). We further note that Pten-deficient Tfr cells strongly promoted a Th2-type IgE response despite being linked previously to aberrant Th1-type inflammatory disease (37). Indeed, in our model, Tfh and Tfr cells from PtenFC mice did not overproduce IFN-γ (Supplemental Figure 10), as was previously reported (37). These data suggest that Tfr and Tfh cell cytokine expression can be influenced by the type of immune response.

Relatively little work has been done on the regulation of IgE by Tfr cells. In 2014, Wing et al. published data that Treg deletion and specific deletion of Ctla4 in Tregs led to a large increase in NP-specific IgE after NP-OVA-alum immunization (36). One interpretation of these studies is that Tfr cells, which were affected in these model systems, strongly suppress IgE. However, since these experiments globally affected Tregs, a different and equally feasible explanation is that the IgE responses were enhanced by the loss of Tregs or Treg-expressed CTLA4 at the early stages of the immune responses, leading to greater inflammation, stronger Tfh and GCB cell responses, and subsequent higher production of Ag-specific IgE. Thus, we propose that an amplified early T cell expansion effectively overshadows the subtler effects of Tfr cells on the GC response. More recently, using an alternative strategy to delete Tfr cells in “Tfr-DTR” mice, Clement et al. showed that Tfr cells could suppress Ag-specific IgE in a house dust mite–induced (HDM-induced) airway inflammation model (42). This result suggests that either Tfr cells regulate Ag-specific IgE differently after airway challenge than after gut challenge, or that in the Tfr-DTR mouse system, global Treg responses are also affected, leading to greater inflammation from defective early Treg-mediated suppression. In support of this latter interpretation, overall lung inflammation was significantly increased in the Tfr-DTR mice (42).

Although some groups have shown that Tfr cells function as strong suppressors of B cell responses using in vitro culture systems, such cultures are unlikely to recapitulate the highly organized structure of the GC in vivo and thus may not reflect normal Tfr functions (15). Studies of Tfr cells in vivo are therefore key to understanding Tfr cell functions. Since mouse models are typically used to study in vivo Tfr cell function, little is known about human Tfr cell function in vivo. Furthermore, there is controversy about how to define human Tfr cells within lymphoid tissue. Although CD4+CXCR5+PD-1+BCL6+FOXP3+ Tfr cells are readily detected in mouse lymphoid tissues after immunization, analogous cell populations have been hard to find in human lymphoid tissues that contain GCs (43–45). Whether tissue-resident human Tfr cells uniformly express FOXP3, PD-1, or CD25 remains to be clarified (43–45). Human Tfr-like cells defined as CD4+CXCR5+PD-1+CD25+FOXP3+ can suppress B cell and IgE responses in vitro via IL-10 secretion (43), but as noted above, this may not reflect the in vivo setting. Whether there are very rare Tfr cells in humans that are clear counterparts to mouse Tfr cells or whether human Tfr cells consist of a variety of related follicular T cell populations that are distinct from mouse Tfr cells remains to be determined. Circulating Tfr cells have been examined extensively in humans (35, 43, 46–48), however, the relationship of these cells to tissue-resident Tfr cells is not known. Much work remains to be done on human Tfr cells.

One question that emerges from our study is why Ag-specific IgE responses appear to be much more sensitive to the loss of Tfr cells than Ag-specific IgG responses. The answer may reflect the unique nature of IgE as an Ag receptor for B cells in the GC. Specifically, IgE+ switched GCB cells have altered Ag receptor signaling, differentiate more readily into plasmablasts, display increased apoptosis, and are less efficiently selected than are GCB cells expressing other Ig isotypes (25, 27, 28, 49–51). Furthermore, inhibition of apoptosis in the GC helps to promote IgE responses (52). We hypothesize that Tfr-derived IL-10 is essential to maintain the survival and clonal expansion of IgE+ GCB cells by promoting entry into the GC dark zone, where they can proliferate rather than differentiate or undergo apoptosis.

The strong decrease in Tfh cells in Bcl6FC mice probably relates to the role of Tfr cells in maintaining the GC reaction via IL-10. We show here that IL-10 from Tfr cells promoted GCB cell survival, and in the absence of this IL-10, we observed fewer GCB cells. Since Tfh cells depend on GCB cells for stimulation, Tfh cell levels necessarily correlate with GCB cell levels, so that fewer GCB cells leads to fewer Tfh cells (53, 54). Recently, a Tfh cell subset, termed TFH13 for their expression of IL-13, was shown to drive the generation of high-affinity IgE Abs (55). To test whether Tfr cells affect the development of TFH13 cells, we isolated Tfh cells from WT and Bcl6FC PCT-sensitized mice and analyzed cytokine expression by qPCR (Supplemental Figure 14). We found no clear difference in the expression of Il4 or Il13 between WT and Bcl6FC Tfh cells, suggesting that defects in Tfh13 cell development did not explain the loss of IgE in Bcl6FC mice.

One interesting question is why we observed larger GCs in our Bcl6FC plus Blimp1FC BM chimeras, if Blimp1FC-deficient Tfr cells are deficient in IL-10 production. In other words, if Tfr cell–derived IL-10 is critical for GC size, then we should have seen fewer GCB cells in the chimeras. There are 3 potential explanations for our results of larger GCs in the Bcl6FC plus Blimp1FC chimera. First, the Tregs in the chimeras produced less IL-10. Second, lower levels of IL-10, leading to larger overall GCs. Third, lower levels of IL-10 may have been amplified from lower levels of IL-10, leading to larger overall GCs. Second, lower levels of IL-10 on average from Tregs amplified early T cell activation after PCT sensitization, leading to overall larger GCs. Third, loss of Blimp1 in Tregs led to a lack of repression of Bcl6, and thus Tfr cell differentiation occurred at a higher rate, as shown in Figure 7D. We propose that Tfr cells have additional B cell helper activity from factors besides IL-10 that is manifested in these chimeras due to the higher rate of Tfr cell development. Indeed, as shown in Supplemental Figure 12, Tfr cells produced Il4, Il21, and Tnfsf13b (also known as B cell–activating factor [BAFF]), cytokines which can all
help promote B cell responses. We did not observe an increased Tfh cell response in the chimeras (Figure 7D), indicating that there was no general increase in immune reactions. These data support the third explanation that Tfr cells have additional GCB cell helper activity besides that from IL-10. We should also note that, recently, Wang et al. published a study indicating that Blimp1-deficient Tfr cells are unstable and can differentiate into IL-17-producing cells that can produce IL-4 and IL-21 and promote larger GCs (56). Although this model could explain the discrepancy of larger GCs despite depleted IL-10 production from the Tfr cells, we found no evidence in our RNA-Seq data of higher Il17 in Blimp1-deficient Tfr cells (Supplemental Figure 12). This discrepancy could be due to the gut immune challenge model we used versus the OVA-Alum i.p. immunization the authors used, as well as other factors such as the microbiome, mouse housing, and genetic variations.

We also note that our study shows that Tfr cells were also required to repress non–peanut-specific IgE responses. We hypothesize that the accumulation of large numbers of nonspecific IgE GCB cells in Tfr-deficient responses leads to consumption of the available IL-10 in the GC and deprives the peanut-specific IgE GCB cells of critical IL-10, thus weakening the peanut-specific IgE GCB cell response. Another explanation for the role of Tfr-derived IL-10 in the Ag-specific IgE response is that GCs induced in Th2-type responses are inherently weaker than GCs induced in Th1-type responses because of a lack of strong inflammatory signals (57) and are more dependent on IL-10 from Tfr cells to maintain the response.

IL-10 has recently become recognized as a positive factor in Ab responses and particularly GCB cell responses (16, 58–60), and our study shows the positive regulation of food Ag IgE responses by IL-10. Since IL-10 is a positive mediator of IgE responses, this finding could be exploited to inhibit IgE-mediated allergy. We show that blocking IL-10 signaling after initiation of the peanut allergy sensitization can potently inhibit the production of peanut-specific IgE. These data have significant implications for therapies designed to treat food allergies and other IgE-mediated allergic diseases. Importantly, IL-10 is also a key cytokine involved in the suppression of immune responses and is part of the mechanism of allergen-specific immunotherapy (61–67). IL-10 produced by Tregs, Bregs, Tr1 cells, and other immune cell subsets has been shown to play an important role in suppressing undesired immune responses (61–67). Thus, there is an apparent dichotomy in the role of IL-10 in allergic regulation. Our hypothesis is that IL-10 acts as a general immune-suppressive factor at the early stages of T cell activation in the allergic immune response, but once GC reactions are formed, IL-10 has a unique role in promoting the GC and IgE-expressing B cells. Clearly, any therapy that involves manipulation of IL-10 signaling must take these dual roles into account. We observed that blocking IL-10 after immunization could block the development of peanut-specific IgE responses. Further work on the timing of in vivo IL-10 blockade in the development of food allergy will be important. Although IL-10 typically acts as an immune-suppressive factor when tested in vitro, IL-10 functions are also known to be pleiotropic (68), and recent work including the present study supports the idea of IL-10 pleiotropy in vivo (16, 58).

Last, we note that patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome often have elevated IgE levels and food allergy (69–71). These patients have mutations in the FOXP3 gene and thus defective Treg and Tfr cell function. The elevation of IgE in these patients fits with our results, in which disruption of Tfr cells or Treg function led to strongly increased total serum IgE. How patients with IPEX syndrome develop food allergy or food Ag–specific IgE is not known, but the severe immune dysregulation in this syndrome appears to be able to overcome the loss of Tfr cells for the production of IgE that is reactive with food Ags. Further research is required to understand the development of food allergy–promoting IgE in humans.

Overall, our study increases our understanding of the regulation of IgE responses and elucidates the complex function of Tfr cells in the GC.

Methods

Mice. All mutant mice were on a C57BL/6 background. Foxp3<sup>Foxp3<sup>+</sup>Cre</sup> (WT), Foxp3<sup>Foxp3<sup>+</sup>Cre</sup> Bcl6<sup>fl/fl</sup> (Bcl6FC), Bcl6<sup>fl/fl</sup>, and Cd4-Cre Bcl6<sup>fl/fl</sup> (CD4-BCL6-cKO) mice were described previously (12, 34). B6.129 (Cg-Foxp3tm3UKTmGtc/Aay/Fpr1<sup>–/–</sup>) (FOPX3-3DTR), B6.SJL-Ili0ra<sup>Ili0ra</sup>/J (Ili0ra<sup>Ili0ra</sup>), B6.129s7-Rag1tm1Mom/j (Rag1<sup>Rag1</sup>), B6.129P2-Ferc1<sup>Ili0ra</sup>/B<sup>Fl</sup>R1<sup>–/–</sup>), and WT C57BL/6J mice were obtained from The Jackson Laboratory. Foxp3<sup>Foxp3<sup>+</sup>Cre</sup> Pten<sup>fl/fl</sup> (PtenFC) mice were previously described (37). Mbi-Cre Bcl6<sup>fl/fl</sup> (MBI-Bcl6<sup>–/–</sup>) mice were obtained from Marion Pepper (University of Washington, Seattle, Washington, USA). Ili0ra<sup>Ili0ra</sup> mice were backcrossed with MB1-Cre mice to generate Mbi-Cre Ili0ra<sup>Ili0ra</sup> (MBI-Ili0ra<sup>Ili0ra</sup>) mice. Ili0ra<sup>Ili0ra</sup> mice were also backcrossed with Cd4-Cre mouse to generate Cd4-Cre Ili0ra<sup>Ili0ra</sup> (CD4-Ili0ra<sup>Ili0ra</sup>) mice. For Bcl6FC or PtenFC mice, only FOXP3-YFP-CRE mice were used as WT controls. For MBI-Ili0ra<sup>Ili0ra</sup> or CD4-Ili0ra<sup>Ili0ra</sup> mice, only Ili0ra<sup>Ili0ra</sup> mice were used as controls. Only Bcl6<sup>fl/fl</sup> mice were used as controls for CD4-BCL6-cKO mice. All mice, including conditional knockout mice, were on a C57BL/6 background. Six- to 10-week-old male and female mice were used for most experiments. For anaphylaxis assays, female mice were used, as they produced stronger responses. Mouse littermate comparisons were used whenever possible. Control and experimental mouse cohorts were age and sex matched. Mice were bred under specific pathogen-free conditions at the laboratory animal facility of the Indiana University School of Medicine.

Peanut sensitization. On the indicated dates, the mice were starved for 2 hours, and then each mouse was fed 300 μL 1.5% NaHCO3, water (i.g.). A half hour later, each mouse was sensitized with 1 mg peanut extract (Greer Laboratories) or OVA (MilliporeSigma) together with 10 μg cholera toxin (MilliporeSigma) (31–33). The mice were sacrificed on the indicated days, serum was collected, and the mesenteric LNs and SPs were harvested. For serum time course analyses, approximately 0.3 mL blood was collected from each mouse from the submandibular vein on the indicated dates.

Assessment of anaphylaxis. To assess anaphylaxis, 2 mg peanut extract protein without cholera toxin was administered i.p. per mouse 4 weeks after the second immunization (day 36), similar to previous studies (31–33). Mice were monitored for 50 minutes after challenge for a change in rectal (core) body temperature (Braintree Scientific). After 50 minutes, whole blood was collected into EDTA-coated tubes (BD Microtainer), and hematocrit values were determined with an Element HT5 Veterinary Hematology Analyzer (Heska). Clinical scores were evaluated 20–30 minutes after challenge as reported previously (31–33). Briefly the animals were scored as follows: 0, no clin-
Author contributions

MMX performed the experiments and generated most of the data shown, with experimental assistance from QC, BK, and HW. ALD and MMX jointly planned and conceived of the experiments and cowrote the manuscript.

Acknowledgments

We would like to thank Caleb Corn for help with mouse genotyping and Baohua Zhou and Charlie X Dong for critical evaluation of the data. We thank Tony Sinn for assistance with mouse irradiation and BM transplantation. We thank Marion Pepper for guidance with the B cell tetramer protocol. We would also like to thank Gowthaman Uthaman and Stephanie Eisenbarth for sharing their antibody affinity measurement protocol. This work was supported by NIH grants R01 AI132771 (to ALD) and R01 AI057459 and R01 AI129241 (to MHK). MMX was supported by a Careers in Immunology Fellowship from the American Association of Immunologists. Core facility usage was also supported by Indiana University Simon Cancer Center Support grants P30 CA082709 and U54 DK106846. Support was provided by the Herman B Wells Center in part from the Riley Children’s Foundation.

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Increased circulating CD4+CXCR5+FoxP3+ follicular regulatory T cells correlated with severity of systemic lupus erythematosus patients. *Int Immunopharmacol*. 2018;56:261–268.


