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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 specific for food antigens. T follicular regulatory (TFR) cells are a specialized subset of FOXP3+ T cells that modulate antibody responses. Here we analyzed the role of TFR cells in regulating antigen-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 Bcl6-fl/fl mice. Loss of TFR cells led to greatly increased non-specific IgE levels, showing that TFR cells have both helper and suppressor functions on IgE production in the GC that work together to facilitate the production of antigen-specific IgE. Foxp3-cre Pten-fl/fl mice with augmented TFR cell responses had markedly higher levels of peanut-specific IgE, revealing an active helper function by TFR cells on antigen-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 GC B 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 germinal center (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 cell response (5, 6). While 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+ regulatory T cells (Tregs) and localize to the GC due to CXCR5 expression (9-15). TFR cells, like TFH cells, are dependent upon 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 are usually considered to act as repressor cells in the GC (9-11, 14). More recently, a TFR-deficient mouse model where the Bcl6 gene is specifically deleted in FOXP3+ T cells (Bcl6-fl/fl Foxp3-Cre or Bcl6FC mice) has been 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 last 30 years is of epidemic proportions (20, 21). Immunoglobulin E (IgE) mediates immediate hypersensitivity to allergens in atopic patients (22, 23). Despite its importance in allergic disease, how IgE responses develop in the GC are 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 regulate the GC and antigen-specific IgE production, in part through the production of IL-10.
Results

*Production of Antigen-specific IgE is dependent upon 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), and 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 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, total IgE responses were significantly increased in Bcl6FC mice, showing a repressive effect of TFR cells on non-specific IgE responses (Figure 1B, Supplemental Figure 1).

We then analyzed the time course of serum peanut-specific IgE and IgG1 induction and maintenance after PCT sensitization (Figure 1C, D). We observed that in WT mice, peanut-specific IgE is induced to high levels one week after the second PCT sensitization (day 15) and then slowly decreases over several weeks. However, in Bcl6FC mice, the initial induction of peanut-specific IgE is much weaker and then fades to undetectable levels at two 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. A strong anaphylaxis response was induced WT mice, while 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 to the peanut protein Ara H 3 was similar as the affinity from control mice (Supplemental Figure 2). To test if the strong anaphylaxis response was dependent on IgE, we tested FceR1-/- mice, which have Mast cells that cannot bind IgE, in our PCT-sensitization system. We observed that anaphylaxis symptoms were significantly lessened in FceR1-/- mice, indicating that IgE mediates much of the response to peanut challenge (Supplemental Figure 2). We then tested if the induction of peanut-specific IgE was dependent on the GC reaction by analyzing Cd4-cre Bcl6-fl/fl mice which lack TFH cells and cannot mount GC reactions (34).

After PCT sensitization, anti-peanut IgE and IgG1 responses as well as total IgE responses were completely ablated in these mice (Figure 1F), as well as in mice that cannot form GCs due to B cell-specific deletion of Bcl6 (Supplemental Figure 3). Cd4-cre Bcl6-fl/fl mice showed no signs of anaphylaxis when challenged systemically with peanut protein (Figure 1G), consistent with the loss of peanut-specific IgE in these mice. Similar results showing Ag-specific IgE dependence on GC responses and TFR cells were observed when ovalbumin was substituted for peanut protein in the food allergy model (Supplemental Figure 4), showing that these results were not unique to peanut as an Ag.

**TFR cells are required to maintain GC responses over time**

We wondered if 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 (LN) and spleens (SP) 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 on TFR cells compared to TFH
cells has been observed previously with human cells (35). As expected, TFR cells were almost completely absent in Bcl6FC mice despite a robust TFR response in WT mice at 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 see significant levels of FOXP3-CRE activity in FOXP3 YFP-negative CD4 T cells (Supplemental Figure 6B-E). Next, we examined the time course of the GC response and saw in WT mice, TFR, TFH and GCB cells all increased over time and remained high 4 weeks after the last sensitization (D36; Figure 2C). The TFH and GCB cell response is statistically normal at the early stages in Bcl6FC mice, but decreased at later stages of the analysis, with a sharp decrease in GCB cells at 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 at 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, not a loss in frequency of peanut-specific B cells. The normal TFH/GCB response at early timepoints is further evidence that the later decline in the TFH/GCB response in Bcl6FC mice is 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).
Treg cells are required for Antigen-specific IgE production in the food allergy model

We next wondered whether our results with Bcl6FC mice were unique to this mouse model of TFR deficiency. We therefore obtained Foxp3-diptheria toxin (FOXP3-DTR) mice where FOXP3+ Treg cells can be deleted by injection of diphtheria toxin (DT), causing loss of TFR cells (10, 36), and tested them in the PCT model as shown in Figure 3A. Similar to our results with Bcl6FC mice, deletion of total Tregs led to a dramatic loss of peanut-specific IgE and a significant but not complete loss in peanut-specific IgG1 (Figure 3B). We then confirmed that the DT treatment led to thorough deletion of Tregs and TFR cells at an early stage of the response in the Foxp3-DTR mice in both LN and SP (Figure 3C-D, Supplemental Figure 9A). These data support the idea that TFR cells derived from FOXP3+ Tregs are actively required for producing Ag-specific IgE. Also, consistent with results in Bcl6FC mice, total IgE levels were strongly elevated after Treg deletion (Figure 3B). Unlike Bcl6FC mice, overall TFH and GCB cell responses were increased after Treg deletion (Supplemental Figure 9 B-C), indicating that TFH and GCB cell levels are repressed by Treg cells and not by BCL6-dependent TFR cells.

TFR cells actively promote Antigen-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 if TFR cells were simply required at some minimal level to help the GC response or if 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 Treg cells (Foxp3-cre Pten-fl/fl or PtenFC mice (37)) to test this idea. We sensitized PtenFC 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 to WT mice. We confirmed 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 4C, 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 show that TFR cells actively drive GC responses in this food allergy model. This idea is supported by a linear correlation analysis between numbers of TFR cells and other cells in the GC (Supplemental Figure 10A). As expected, 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. This finding, coupled with the diminished GCB cell response in Bcl6FC mice, indicates that TFR cells act 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 (Supplementary Figure 10B), suggesting 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 Antigen-Specific IgE Responses**

TFR cells produce IL-10 that can augment the GC and Ab response (16). We wondered if 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, we used Mb1-cre IL-10Ra-fl/fl (MB1-Il10ra-/-) mice, where the IL-10 receptor alpha gene is deleted specifically in B cells and the B cells cannot respond to IL-10 signals. MB1-Il10ra-/- mice were sensitized with PCT and tested for GC and
peanut-specific Ab responses. As shown in Figure 5A-B, loss of IL-10 signaling in B cells led to significantly decreased GCB cell, peanut-specific IgG1 and peanut-specific IgE responses. Baseline TFH, TFR and GCB levels were normal in MB1-Il10ra/- mice, indicating no broad immune defect from loss of IL-10 signaling in these mice (Supplementary Figure 11A-C). Previously, neutralizing IL-10 Abs were shown to decrease IgE and gastrointestinal symptoms in an oral challenge model (38). We then wondered if we could block IgE production with anti-IL10R Ab in our peanut model. As shown in Figure 5C-D, repeated doses of anti-IL10R Ab in WT mice after PCT challenge led to a dramatic loss of peanut-specific IgE by day 29 with a significant drop in peanut-specific IgG1 at day 29. Significantly, anti-IL10R Ab treatment also strongly inhibited anaphylaxis after peanut challenge (Figure 5E), mirroring the loss of peanut-specific IgE. We tested the role of IL-10 signaling on CD4 T cells with Cd4-cre IL10Ra-fl/fl mice and the PCT sensitization system. While there was a slight decrease in TFH cell development in these mice, there was no difference in anti-peanut IgE and IgG1 and the GCB cell response (Supplementary Figure 11D-G). These data support the idea that IL-10 acts directly on GCB cells and not on TFH cells to promote the IgE response. We next examined the light zone and dark zone composition of GCB cells in MB1-Il10ra/- mice and found a large shift to light zone GCB cells in these mice (Figure 6A). These data are consistent with recent findings that IL-10 promotes entry of the GCB cell into the dark zone compartment of the GC (16), and that without IL-10 signaling, GCB cells accumulate in the light zone. We also saw a shift of GC B cells to the light zone in Bcl6FC mice (Figure 6B), consistent with a loss of TFR cell derived IL-10 acting on GCB cells. Furthermore, we observed a significant increase in apoptotic GCB cells in both MB1-Il10ra/- and Bcl6FC mice (Figure 6 C, D), which helps explains the loss of GCB cells in both strains of mice.
**TFR cell-derived IL-10 promotes Antigen-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). At day 15 after sensitization, there was a significant increase in *Il10* made by WT TFR cells compared to WT Tregs, which correlated with the appearance of IgE in the serum. TFR cells from PtenFC mice produced less *Il10* mRNA compared to 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, where we put either WT + Bcl6FC or *Blimp1-fl/fl Foxp3-cre* (Blimp1FC) + 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/TFR suppressor genes were expressed normally in Blimp1FC cells except for *Il10* (Supplementary Figure 12) and decreased *Il10* transcript in TFR cells from Blimp1FC + Bcl6FC chimeras was confirmed by QPCR of FACS isolated TFR cells (Figure 7C). The data in Figure 7C also show that TFR cells express at least 7 times more *Il10* than TFH cells. Chimeras with WT + 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 + 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 significantly increased GCB cells (Figure 7D). Strikingly, Blimp1FC + 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 find that anti-IL-10R Ab inhibits the peanut-specific IgE response in WT control mice to the level seen in Bcl6FC mice, while anti-IL-10R Ab does not inhibit the IgE response in Bcl6FC mice (Figure 7 F-G, Supplementary Figure 13). These two lines of evidence both indicate that production of IL-10 by TFR cells is required for peanut-specific IgE production.
Discussion

Here we show that Ag-specific IgE responses induced in a food allergy sensitization system are strikingly dependent on TFR cells and further that TFR cells play an active helper role in the production of Ag-specific IgE. Our data also point to a key role of IL-10 made 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 revises 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 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 are 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 to 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 do not over-produce Interferon-gamma.
(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 *Cita4* 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 stronger inflammation, stronger TFH and GCB cell responses and subsequent higher production of Ag-specific IgE. Thus, we propose that an amplified early TFH cell expansion effectively overshadows the more subtle 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 (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).

While 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 on 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. While CD4+CXCR5+PD-1++BCL6+FOXP3+ TFR cells are readily detected in mouse lymphoid tissues after immunization, analogous 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- cells 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 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 GC B cells have altered Ag receptor signaling, differentiate more readily into plasmablasts, display increased apoptosis, and are less efficiently selected than GC B 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+ GC B 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 likely relates to the role of TFR cells in maintaining the GC reaction via IL-10. We show here that IL-10 from TFR cells promotes GC B cell survival, and in the absence of this IL-10, we see fewer GC B cells. Since TFH cells depend on GC B cells for stimulation, TFH cell levels necessarily correlate with GC B cell levels, so that fewer GC B 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 if 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 did not find any 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 + Blimp1FC bone marrow 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 see fewer GC B cells in the chimeras. There are three potential explanations for our results of larger GCs in the Bcl6FC + Blimp1FC chimera. First, the Tregs in the chimeras will produce less IL-10 on average than Tregs from control mice. Thus, background immune responses may be amplified from less IL-10, leading to larger overall GCs. Second, less IL-10 on average from Tregs will amplify early T cell activation after PCT sensitization, leading to larger overall GCs. Third, loss of Blimp1 in
Tregs leads to lack of repression of Bcl6 and thus TFR cell differentiation occurs at a higher rate, which we observe 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 produce *Il4, Il21* and *Tnfsf13b* (BAFF), cytokines which can all help promote B cell responses. We did not observe an increased TFH cell response in the chimeras (Fig. 7D), indicating that there was no general increase in immune reactions. This data supports the third explanation that TFR cells have additional GC B cell helper activity besides IL-10. We should also note that recently, Wang et al published a paper 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). While this model could explain the discrepancy of larger GCs despite depleted IL-10 production from the TFR cells, we have found no evidence in our RNAseq data of higher *Il17* in Blimp1-deficient TFR cells (Supplemental Figure 12). This discrepancy could be due to the gut immune challenge model we use versus the Ova-Alum i.p. immunization they used, as well as other factors such as microbiome, mouse housing and genetic variations.

We also note that we show that TFR cells are also required to repress non-peanut-specific IgE responses. We hypothesize that the accumulation of large numbers of non-specific IgE GC B cells in TFR-deficient responses leads to consumption of the available IL-10 in the GC and deprives the peanut-specific IgE GC B 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 antigen-specific IgE response is that GCs induced in Th2-type responses are inherently weaker than GCs
induced in Th1-type responses due to 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 here 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 the
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
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 on the development of food
allergy will be important. While 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).
Lastly, we note that IPEX (Immune-dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome patients 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, where disruption of TFR cells or Treg function leads to strongly increased total serum IgE. How IPEX patients develop food allergy or food antigen-specific IgE is not known, but the severe immune deregulation in this syndrome appears to be able to overcome the loss of TFR cells for the production of IgE reactive with food antigens. Further research is required to understand the development of food allergy-promoting IgE in humans.

Overall, our work has increased our understanding of the regulation of IgE responses as well as helped us recognize the complex function of TFR cells in the GC.
Methods

Mice

All mutant mice were on a C57BL/6 background. Foxp3<sup>YFP-cre</sup> (WT), Foxp3<sup>YFP-cre</sup> Bcl6-fl/fl (Bcl6FC), Bcl6-fl/fl and Cd4-cre Bcl6-fl/fl (CD4-BCL6 cKO) mice were previously described (12, 34). B6.129(Cg)-Foxp3tm3<sup>DTR/GFP</sup>Ayr/J (Foxp3-DTR), B6(SJL)-Il10ratm1.1Tlg/J (Il10ra-fl/fl), B6.129S7-Rag1tm1Mom/J (Rag1-/-), B6;129P2-Fcer1g<sup>tm1Rav</sup>/J (FceR1-/-) mice and wild-type C57Bl/6J mice were obtained from Jackson Laboratories. Foxp3<sup>YFP-cre</sup> Pten-fl/fl (PtenFC) mice were previously described (37). Mbl-cre Bcl6-fl/fl (MB1-Bcl6-/-) mice were obtained from Dr. Marion Pepper (Univ. Washington, Seattle, WA). Il10ra-fl/fl mice were backcrossed to MB1-cre mice to generate or Mbl-cre Il10ra-fl/fl (MB1-Ill10ra-/-) mice. Il10ra-fl/fl mice were also backcrossed to Cd4-cre to generate Cd4-cre Il10ra-fl/fl (CD4-Ill10ra-/-) mice. For Bcl6FC or PtenFC mice, Foxp3-YFP-cre only mice were used as WT controls. For MB1-Ill10ra-/- or CD4-Ill10ra-/- mice, Ill10ra-fl/fl only mice were used as controls. Bcl6-fl/fl only mice were used as controls for CD4-BCL6 cKO mice. All mice including conditional knockouts were on the C57Bl/6 background. Male and female mice of 6-10 weeks old 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 at IUSM and were handled according to protocols approved by the IUSM Animal Use and Care Committee.
Peanut Sensitization

On indicated dates, mice were starved for two hours followed by feeding with 300 µl per mouse 1.5% NaHCO3 water intragastrically (i.g). After hour later, mice were sensitized with 1 mg per mouse of peanut extract (Greer Laboratories) or ovalbumin (Sigma-Aldrich) together with 10 µg per mouse of cholera toxin (Sigma-Aldrich)(31-33). Mice were sacrificed on indicated days and serum, mesenteric LNs and spleens were harvested. For serum timecourse analysis, about 0.3 ml blood was collected each mouse per time from the submandibular vein on indicated dates.

Assessment of anaphylaxis

To assess anaphylaxis, 2 mg peanut extract protein without cholera toxin was administered intraperitoneally per mouse four weeks post the second immunization (day 36), similar to previous studies (31-33). Mice were monitored for 50 mins after challenge for rectal (core) body temperature change (Braintree Scientific). After 50 mins, whole blood was collected into EDTA coated tubes (BD Microtainer) and hematocrit values were determined by an Element HT5 Veterinary Hematology Analyzer (HESKA). Clinical scores were evaluated at 20 to 30 mins after challenge as reported (31-33). Briefly: 0, no clinical signs; 1, scratching around the head and nose; 2, reduced activity with increased respiration; 3, wheezing and lying prone; 4, no response after prodding and convulsion; and 5, death.
Statistical Analysis.

All data analysis was done using Prism Graphpad software. Graphs show mean ± SEM. Unless otherwise stated, 2 tailed Student t tests or 2 way ANOVA with Tukey post hoc analysis were used. All ELISA panels were analyzed using 2 way ANOVA with Sidak's multiple comparison test. Only significant differences (P < 0.05) are indicated in Figures. The investigators were not blinded for the analyses. Experimental replicates and mouse numbers are indicated in specific Figure Legends.

Study Approval.

Animals were handled according to protocols approved by the Indianapolis campus Indiana University School of Medicine Institutional Animal Use and Care Committee (IACUC).

Additional methods and antibody clones used are in listed Supplemental Materials.
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**Author Contributions**

Markus M. Xie performed the experiments and generated most of the data shown, with experimental assistance from Qiang Chen, Byunghee Koh and Hao Wu. Hong Liu assisted with mouse breeding and genotyping. Kai Yang provided the PtenFC mice. Soheila Maleki and Barry Hurlburt provided Ara H 1 and Ara H 3 peanut proteins. Joan Cook-Mills provided FcεR1 KO mice. Mark Kaplan gave general advice and helped design the IgE affinity experiment. Alexander Dent and Markus M. Xie jointly planned and conceived of the experiments and co-wrote the manuscript.

**Competing Interests:** The authors declare no financial conflict of interest in this work.
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 two i.g. doses of peanut protein plus cholera toxin (PCT) 7 days apart, bled at different time-points after sensitization. (A) shows the day 36 (D36) time-line, where serum is tested 28 days after the last sensitization for peanut-specific Abs. (B-D) Control Foxp3-cre alone (WT) mice and Foxp3-cre Bcl6-fl/fl (Bcl6FC) mice were sensitized as in (A) and D36 serum tested for peanut-specific IgE, IgG1 and total IgE (B) or at various time-points during and after sensitization at the indicated time-points (red arrows) (C-D). Data for (A) and (B) are from one experiment representative of four experiments with four to five mice per group. Data for (C) and (D) are from one experiment representative of two experiments with four to five mice per group. (E) WT and Bcl6FC mice sensitized as in (A) were analyzed for anaphylactic responses at D36. Non-sensitized WT and Bcl6FC mice were negative controls. Data for (E) are pooled from two experiments with three to seven mice per group (N= 6-14). (F-G) Control Bcl6-fl/fl alone (WT) mice and Cd4-cre Bcl6-fl/fl (CD4-Bcl6 cKO) mice were sensitized as shown in (A). (F) D36 serum was tested for peanut-specific IgE, IgG1 and total IgE. (G) Mice tested for anaphylaxis as described in (E). Data for (F) are from one experiment representative of three experiments with four to five mice per group. Data for (G) are from one experiment representative of two experiments with three to five mice per group. Statistical tests: 2 way ANOVA Sidak’s multiple comparison test (B, D, F), 2 way ANOVA Tukey’s multiple comparison test (E, G). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.
**Figure A**

Day 0, 7, and 36

Peanut Cholera Toxin i.g.

**Figure B**

**Anti-Peanut Extract IgE**

- WT-PCT
- BCL6F-PCT

**Normalized OD450**

**Serum Dilutions**

- **Anti-Peanut Extract IgG1**

- **Total IgE**

**Figure C**

Day 0, 7, 15, 22, 29, 36, and 64

Peanut Cholera Toxin i.g.

**Figure D**

**Anti-Peanut Extract IgE**

- WT-PCT
- BCL6F-PCT

**Normalized OD450**

**Day: 0**

**Dilution Factor 1: 5**

**Anti-Peanut Extract IgG1**

**Time Course**

**Normalized OD450**

**Dilution Factor 1: 450**

**Figure E**

**Hypothermia**

- Naive WT
- Naive BCL6F
- WT-PCT
- BCL6F-PCT

**Temperature (°C)**

Time after challenge (min)

**Hemocrit (%)**

**Hematocrit (%)**

**Clinical signs**

**Score**

**Figure F**

**Anti-Peanut Extract IgE**

- WT-PCT
- CD4-BCL6cKO-PCT

**Normalized OD450**

**Serum Dilutions**

**Anti-Peanut Extract IgG1**

**Total IgE**

**Normalized OD450**

**Serum Dilutions**

**Serum Dilutions**

**Figure G**

**Hypothermia**

- Naive WT
- Naive CD4-BCL6cKO
- WT-PCT
- CD4-BCL6cKO-PCT

**Temperature (°C)**

Time after challenge (min)

**Hemocrit (%)**

**Hematocrit (%)**

**Clinical signs**

**Score**
Figure 2. TFR cells are required for normal TFH and GC B cell numbers in a food allergy immune response. WT and Bcl6FC mice were sensitized as in Figure 1 with PCT then at D36, mesenteric lymph nodes (LN) and spleens (SP) 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) are pooled from naïve (two experiments), D15 (two experiments), D22 (two experiments) and D36 (eight experiments) with four to five mice per group (N = 8-10). D15 = 7 days after sensitization, D22 = 14 days after sensitization, D36 = 28 days after sensitization. Statistical tests: 2 way ANOVA Sidak’s multiple comparison test (B), 2 way T-test (C). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.
Figure 3. Total Treg and TFR cells are required for antigen-specific IgE and IgG1 in a food allergy response. (A) FOXP3-DTR mice were treated with diphtheria toxin (DT) as indicated to deplete Treg cells, or given PBS as a control, sensitized with PCT as indicated, and bled for serum peanut-specific IgE and IgG1 Ab on D36 (B). Data for (B) are from one experiment representative of two experiments with four to five mice per group. (C-D) FOXP3-DTR mice were treated with DT or given PBS as a control, sensitized with PCT at days 0 and 7 as indicated, and then at day 9, draining mesenteric lymph node (LN) and spleen (SP) taken for analysis of CD4+FOXP3+PD-1+CXCR5+ TFR cells. TFR cells are quantitated as the percentage of FOXP3+ cells from CD4+CXCR5+PD-1+ T cells, and absolute number per LN or SP. Data for (D) are from one experiment representative of two experiments with four to six mice per group. Statistical tests: 2 way T-test (A, B), 2 way ANOVA Tukey’s multiple comparison test (C). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.
Figure 4. Augmented TFR cell development promotes higher IgE and correlates with increased GC responses after food allergy sensitization. WT and Foxp3-cre Pten-fl/fl (PtenFC) mice were sensitized as in Figure 1 with PCT. At D36 of the sensitization system, (A) serum was tested for peanut-specific Abs, and (B-D) spleens (SP) and mesenteric lymph nodes (LN) were analyzed for TFR cells, TFH cells and GCB cells by flow cytometry as in Figure 2. Representative contour dot plots for each cell staining are shown along with graphs showing average % of cells as a fraction of parental cell population and total yield of cells. Data for (A) are from one experiment representative of two experiments with three to five mice per group. Data for (B-D) are pooled from two experiments with three to four mice per group (N= 6-10). Statistical tests: 2 way ANOVA Sidak’s multiple comparison test (A), 2 way T-test (B, C ,D). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.
Figure 5. IL-10 promotes GC B 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-cre IL-10Ra-fl/fl (MB1-II10ra-/−) mice were sensitized with PCT and at D36, and GCB cells from LN and SP were stained and analyzed by flow cytometry. Representative contour dot plots of GCB cell staining are shown along with graphs showing average % of GCB cells and total yield of cells. (B) Peanut-specific IgE and IgG1 titers from D36 serum of WT and MB1-II10ra-/− mice sensitized with PCT. Data for (A) and (B) are from one experiment representative of three experiments with three to five mice per group. (C) Scheme for block of IL-10 receptor during PCT sensitization in female C57Bl/6 WT mice. Numbers indicate specific days for i.p. anti-IL10R Ab treatment, i.g. PCT gavage, blood sampling and anaphylaxis. Control mice received anti-HRP-IgG1 Ab. (D) Peanut-specific IgE and IgG1 titers from serum of control and anti-IL10R mice treated as described in (C) at the indicated timepoints. (E) Anaphylaxis response of control and anti-IL10R mice treated as described in (C). Anaphylaxis analysis was performed as in Figure 1. Data for (D) and (E) are from one experiment representative of two experiments with three to six mice per group. Statistical tests: 2 way T-test (A), 2 way ANOVA Sidak’s multiple comparison test (B, D). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.
Figure 6. Altered GCB cell cycling and increased apoptosis in the absence of TFR cells. (A) WT and Mb1-cre IL-10Ra-fl/fl (MB1-Ill10ra-/-) mice were sensitized with PCT and at D36, and GCB cells from SP 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 showing average ratios of GCB LZ to GCB DZ cells. (B) WT and Bcl6FC mice were sensitized with PCT and at D36, and GCB cells from SP were stained and analyzed by flow cytometry for LZ and DZ marker expression as in part A. Representative contour dot plots of GCB DZ/LZ cell staining are shown along with graphs showing average ratios of GCB LZ to GCB DZ cells. Data for (A) and (B) are from one experiment representative of two experiments with four to five mice per group. WT and Bcl6FC (C) or MB1-Ill10ra-/- (D) mice were sensitized with PCT and at D36, and GCB cells from LN were stained and analyzed by flow cytometry for viability using eBioscience™ Fixable Viability Dye. Representative viability stains are shown along with graphs showing average GCB cell death. Data for (A) and (B) are from one experiment representative of two experiments with four to five mice per group. Statistical tests: 2 way T-test (A-D). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.
A

B

C

D

Viability

CD38

B220+GL7+CD38

- WT

MBI-I10ra-/

LN-Dead GCB%

% of B220+GL7+CD38-cells

Viability
Figure 7. Blimp1 controlled-TFR cell derived IL-10 is required for peanut specific IgE production. (A) Il10 mRNA levels in Treg or TFR cells isolated from naïve or PCT sensitized WT mice on D0 (naïve) and D15 as in the model shown in Figure 1. (B) Design for WT/Bcl6FC and Bcl6FC/Blimp1FC bone marrow chimeras. (C-E) Mice generated as in (B) were sensitized with PCT then at D36, (C) TFH and TFR cells were isolated by FACS and Il10 expression analyzed by RT-QPCR, (D) TFH, TFR and GCB cells from LN were stained and analyzed by flow cytometry as in Figure 2, (E) Peanut-specific IgE and IgG1 titers from D36 serum of mice after PCT sensitization. Data for A was pooled from three different cell sorts with two to four mice per sort (N= 6-10). Data for (C - E) are from one experiment representative of two experiments with three to five mice per group after PCT sensitization. (F) Scheme for block of IL-10 receptor during PCT sensitization in WT and Bcl6FC mice. Numbers indicate specific days for i.p. anti-IL10R Ab treatment, i.g. PCT gavage, blood sampling and anaphylaxis. Control mice received anti-HRP-IgG1 Ab. (G) Peanut-specific IgE (D15) and IgG1 (D29) titers from serum of control and anti-IL10R mice treated as described in (F). Data for (F) and (G) are from one experiment representative of two experiments with three to four mice per group. Statistical tests: 2 way ANOVA Tukey’s multiple comparison test (A, C, G), 2 way T-test (D). P values are * p < 0.05, ** p < 0.01, *** p < 0.0001.