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Follicular Helper T (Tfh) Cells Mediate IgE Antibody Response to Airborne Allergens

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Abstract

Background—Type 2 helper T (Th2) cells have long been believed to play a pivotal role in allergic immune responses, including IgE antibody production and type 2 cytokine-mediated inflammation and pathology. A new T cell subset, T follicular helper cells (Tfh) cells, is specialized in supporting B cell maturation and antibody production.

Objective—To investigate the roles of Tfh cells in allergic immune responses.

Methods—Naïve mice were exposed to cytokines or natural allergens through the airways. Development of allergic immune responses was analyzed by collecting draining lymph nodes (LNs) and sera and by challenging the animals. Cytokine reporter mice and gene-deficient mice were used to dissect the immunologic mechanisms.

Results—We observed the development of IL-4-producing Tfh cells and Th2 cells in draining LNs following airway exposure to IL-1 family cytokines or natural allergens. Tfh cells and Th2 cells demonstrated unique phenotypes, tissue localization, and cytokine responses. Tfh cells supported the sustained production of IgE antibody *in vivo* in the absence of other T cell subsets or even when Th2 cell functions were severely compromised. Conversely, conditional deficiency of the master regulator *Bcl6* in $CD4^+$ T cells resulted in a marked reduction in Tfh cells and IgE antibody levels, but type 2 cytokine responses and eosinophilic inflammation in the airways remained unaffected.

Conclusion—Tfh cells play critical roles in the regulation of IgE antibody production. Allergic immune responses to airborne allergens likely involve two distinct subsets of IL-4-producing $CD4^+$ T cells, namely Tfh cells and Th2 cells.

Keywords

Follicular T cells; IL-4; IgE; allergy; allergens; Th2 cells

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INTRODUCTION

Type 2 immune responses to allergens and other environmental factors, including type 2 helper T (Th2) cell cytokine responses and the production of IgE antibodies, are key immunologic features of allergic diseases. Th2-type $CD4^+$ T cells and type 2 innate lymphoid cells (ILC2s) that produce interleukin (IL)-4, IL-5, and IL-13 have been considered major players in directing the pathophysiology of allergic disease, including airway eosinophilia, mucosal hyperplasia, and airway remodeling.[1, 2] However, our knowledge regarding the immunological mechanisms that regulate IgE antibody production is limited. Traditionally, Th2 cells are expected to play a critical role[3] because they produce IL-4, a key cytokine that regulates IgE class switching.[4]

Considerable heterogeneity exists in the clinical phenotypes and genetic contributions to allergic diseases. For example, not all patients with asthma develop IgE antibodies to allergens, and not all patients with detectable serum IgE antibody levels develop asthma.[5, 6] A genome-wide association study (GWAS) identified a link between asthma/airway eosinophilia and single nucleotide polymorphisms (SNPs) in *IL1RL1, IL18R, IL33, TSLP*, and *RORA*.[7] In contrast, associations between serum IgE levels and SNPs in *FCER1A, IL13*, *STAT6,* and *IL4R/IL21R* were identified, but very few genes commonly associated with both serum IgE level and asthma.[7] Thus, the fundamental question of whether Th2-type cytokine responses and IgE antibody production are regulated by the same immunological pathway remains unanswered.

T follicular helper (Tfh) cells are distinguished from other $CD4^+$ Th cells by their selective role in orchestrating germinal center (GC) responses and in promoting the development of memory B cells and long-lived plasma cells.[8, 9] In mice immunized systemically by intraperitoneal injection of ovalbumin (OVA) plus alum adjuvant, a positive correction between the development of IL-4-secreting Tfh cells and the production of IgE and IgG1 antibodies was observed.[10] Further, IL-4-secreting Tfh cells mediated class switching and affinity maturation of IgE and IgG1 in the lymph nodes of mice infected with the parasites *Leishmania major* and *Nippostrongylus brasiliensis*.[11] Other studies involving *Schistosoma mansoni* and *Heligmosomoides polygyrus* also demonstrated the production of IL-4 by Tfh cells.[12, 13] However, information regarding the contribution of Tfh cells to the regulation of IgE antibodies in allergic immune responses to airborne allergens remains limited.

Accordingly, to better understand the development of $CD4^+$ T cell subsets in response to inhaled allergens and to elucidate their roles in allergic immune responses, we utilized mouse models of airway allergen exposure. Our observations suggest a critical role for Tfh cells in the regulation of IgE antibody production. Hence, allergic immune responses might be mediated by Tfh and Th2 cells, which are two distinct subsets of IL-4-producing $CD4^+$ T cells.

MATERIALS AND METHODS

See the Methods section of this article's Online Repository for more details.

Mice

BALB/cJ, C.129-*Il4*^{tm1Lky}/J (4get), C57BL/6J, B6.129P2-*Tcrb*^{tm1Mom}/J (*Tcrb*$^{-/-}$), STOCK Tg(Cd4-cre)1Cwi/BfluJ (CD4-Cre), B6.129S7-*Il7r*^{tm1Imx}/J (*Il7r*$^{-/-}$), B6Cr.129S4-*Tnfsf4*^{tm1Sug}/Pgn (*Tnfsf4*$^{-/-}$), B6.129P2-*Icos*^{tm1Mak}/J (*Icos*$^{-/-}$), and B6.129S7-*Il1r1*^{tm1Imx}/J (*Il1r1*$^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME). The *Bcl6*$^{fl/fl}$ mice were previously described.[14] The procedures and handling of the mice were reviewed and approved by the Mayo Institutional Animal Care and Use Committee, Mayo Clinic, Rochester, MN.

Antigens, allergens and recombinant cytokines

Endotoxin-free OVA (< 0.5 EU/mg, verified by a Limulus amebocyte lysate assay; Wako Chemicals USA, Inc., Richmond, VA) was prepared using specific pathogen-free (SPF) chicken eggs (Charles River Laboratories, Wilmington, MA) under sterile conditions as previously described.[15]

Mouse airway exposure and challenge model

On days 0 and 7, naïve mice were lightly anesthetized with tribromoethanol or isoflurane and administered 100 μg OVA intranasally (i.n.) in the presence or absence of 100 ng or serial dilutions of IL-33 or IL-1β in 50 μl of endotoxin-free PBS. On day 11, the mice were euthanized by an overdose of pentobarbital, and mediastinal lymph nodes (mLNs) were collected. In some experiments, mice were exposed i.n. to *Alternaria* (50 μg), cockroach (50 μg), or HDM (400 μg) extracts, or bromelain (10 μg), or to *Alternaria* (50 μg) plus OVA (10 μg). Some mice were then challenged i.n. with 100 μg OVA using the protocols described in the figures and figure legends.

Plasma antibody levels

Plasma levels of OVA-specific IgE, IgG1, and IgG2a were measured by ELISA as previously described.[16] Total IgE, IgG1, IgM, and IgA levels were measured by ELISA in accord with recommended protocols using antibodies from BD Biosciences (San Jose, CA).

FACS analyses of mLN cells and lung cells

Anterior and posterior mLNs from each mouse were harvested and pooled. Single cell lung suspensions were prepared by digesting harvested lungs using a cocktail of collagenases in the presence of DNase as described previously.[15] The mLN cells and single cell lung suspensions were preincubated with Fc-receptor blockers for 30 min at 4°C and stained with combinations of antibodies and analyzed by gating on lymphocytic cells using a FACSCalibur™ flow cytometer (BD Biosciences Immunocytometry Systems). See the Methods section of this article's Online Repository for the list of antibodies and details regarding analysis strategies.

Gene expression analyses

Day 11 mLN cells from 4get mice exposed to OVA plus IL-33 were enriched for CD4$^+$ T cells (Negative Selection Kit, Stemcell Technologies, Vancouver, Canada) and stained as described above without fixation. CD4$^+$IL-4eGFP$^-$, CD4$^+$IL-4eGFP$^+$ ST2$^+$CXCR5$^-$, and

$CD4^+IL\text{-}4eGFP^+$ $ST2^-CXCR5^+$ populations were sorted using a FACSAria™ flow cytometer (BD Biosciences). Cells were lysed in Trizol (Invitrogen, Carlsbad, CA), and total RNA was extracted and purified using the PureLink® RNA Mini Kit (Thermo Fisher Scientific, Minneapolis, MN) followed by microarray and qRT-PCR analyses.

Cytokine production in vitro

Day 11 mLN cells were isolated, washed, and cultured in RPMI 1640 medium with 10% calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (complete RPMI) at a density of 4 x 10^5 cells/200 μl/well in a round-bottom 96-well tissue culture plate for 4 days. OVA was added at a concentration of 100 μg/ml. Alternatively, day 11 mLN cells were enriched for $CD4^+$ T cells and sorted for $CD3^+CD4^+ST2^-CXCR5^-$, $CD3^+CD4^+ST2^+CXCR5^-$, and $CD3^+CD4^+ST2^-CXCR5^+$ cell populations. B cells were isolated from day 11 mLN cells using a magnetic separation kit (Stemcell Technologies). T cells (20,000 cells/well) were cultured with B cells (100,000 cells/well) in complete RPMI medium (total volume 200 μl/well) with 100 μg/ml OVA for 7 days.

Adoptive transfer

$CD3^+CD4^+ST2^-CXCR5^-$, $CD3^+CD4^+ST2^+CXCR5^-$, and $CD3^+CD4^+ST2^-CXCR5^+$ cell populations from the donor C57BL/6 mice were sorted as described above. The recipient *Tcrb*$^{-/-}$ mice were administered OVA plus IL-33 i.n. on days 0 and 7, and injected with 75,000 sorted cells intravenously (i.v.) 1 day after the first exposure to OVA plus IL-33 (day 1). On days 21, 22, and 23, recipient mice were challenged i.n. with 100 μg OVA alone. Plasma was collected for analysis of specific antibodies on days 14, 24, and 49. In other experiments, similar cell populations were sorted and injected into *Il7r*$^{-/-}$ mice at a concentration of 150,000 cells/mouse. The recipient mice were exposed to OVA plus IL-33 on days 0, 3, and 6, and assessed for airway inflammation on day 8.

RESULTS

IL-33 promotes the development of two distinct subsets of IL-4-competent $CD4^+$ T cells in draining lymph nodes

Respiratory exposure to innocuous proteins generally induces immune tolerance.[17] Recently, airway epithelial cells that produce innate cytokines such as IL-1α, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), and that promote Th2-type immunity, were implicated in asthma and allergic diseases.[18] Further, members of the IL-1 family of cytokines have been shown to overcome suppression by regulatory T cells (Tregs) and to promote functional differentiation of T cells.[19]

To better understand the mechanisms involved in the development of Th2-type immunity to airborne allergens, we exposed the airways of naïve BALB/c mice to endotoxin-free OVA with or without IL-1β or IL-33 twice via intranasal (i.n.) administration (Figure 1A). Subsequently, we examined the production of type 2 cytokines in draining mediastinal lymph nodes (mLNs) on day 11 and plasma antibody levels on day 14. As expected, exposure to OVA alone resulted in negligible plasma anti-OVA antibody levels (Figure 1B). Similarly, the i.n. administration of IL-1β or IL-33 alone did not have a remarkable effect on

anti-OVA antibody levels (data not shown). In contrast, when animals were exposed to OVA in conjunction with IL-1β, plasma levels of anti-OVA IgE and IgG1, another Ig isotype related to Th2-type immunity,[20] increased significantly ($p<0.01$); however, no increases in the IgG2a isotype were observed. Likewise, the administration of OVA and IL-33 promoted the production of anti-OVA IgE and IgG1, but the levels were slightly lower than those induced by OVA plus IL-1β ($p<0.05$).

When cultured *in vitro* with OVA antigen, mLN cells from the mice exposed to OVA plus IL-1β produced IL-4 and IL-13, but only minimal IL-5 (Figure 1C). In contrast, mLN cells from the mice exposed to OVA plus IL-33 exhibited robust production of all three type 2 cytokines. Further, the concentrations of IL-13 produced by mLN cells from mice exposed to OVA plus IL-33 were significantly higher than the corresponding IL-13 levels produced by mLN cells from mice exposed to OVA plus IL-1β ($p<0.05$). However, the IL-4 concentrations produced by mLN cells from the mice exposed to OVA plus IL-33 and from mice exposed to OVA plus IL-1β were comparable. When mice previously exposed to OVA plus IL-33 were challenged i.n. with OVA alone, they developed marked airway eosinophilia and increased levels of IL-5 and IL-13 in bronchoalveolar lavage (BAL) fluids.[21] In contrast, mice previously exposed to OVA plus IL-1β produced modest airway eosinophilia and lower BAL levels of IL-5 and IL-13.

These findings led us to hypothesize that two or more subsets of Th2-like $CD4^+$ T cells with similar capacities to produce IL-4 but distinct IL-5 and IL-13 production might be involved. Th2 cells and Tfh cells are among the $CD4^+$ T cells that are known to produce IL-4.[11, 22] To characterize IL-4-producing cells, we exposed 4get IL-4-reporter mice[23] i.n. to OVA plus IL-1β or IL-33 using the same protocol as described in Figure 1A, and analyzed mLN cells on day 11. In 4get mice, an internal ribosomal entry site (IRES) and downstream enhanced green fluorescent protein (eGFP) are inserted at the 3' end of *Il4*, which allows the identification of cells that actively transcribe the IL-4 locus. Consequently, IL-4eGFP$^+$ $CD4^+$ T cells were clearly detectable in mLNs from mice exposed to OVA plus IL-1β or OVA plus IL-33 (Figure 1D and 1E).

The IL-33 receptor ST2 and the chemokine receptor CXCR5 are considered reliable markers for differentiated Th2 cells[24] and early and mature Tfh cells,[25] respectively. A majority (>80%) of $CD4^+$IL-4eGFP$^+$ cells in mLNs from mice exposed to OVA plus IL-1β were positive for $CXCR5^+$ but were negative for ST2 ($ST2^-CXCR5^+$). In contrast, $CD4^+$IL-4eGFP$^+$ cells from the mice exposed to OVA plus IL-33 consisted of two distinct populations that included $ST2^-CXCR5^+$ cells and $ST2^+CXCR5^-$ cells. Further, none of the IL-4eGFP$^+$ cells were positive for both ST2 and CXCR5.

To characterize the development of these two subsets of IL-4-competent $CD4^+$ T cells further, we examined the relationship between IL-33 dose and effect. Naïve mice were administered a fixed dose of OVA (100 μg) i.n. with various doses of IL-33. A small number of $CD4^+$ T cells consisting of $ST2^-CXCR5^+$ cells were IL-4eGFP$^+$ following administration of a low (1 ng) IL-33 dose (Figure 1F and 1G). Following the administration of an intermediate (10 ng) IL-33 dose, the number of IL-4eGFP$^+$ cells increased, and a majority (~80%) of the cells were $ST2^-CXCR5^+$. However, the frequency of $ST2^+CXCR5^-$ cells

relative to ST2$^-$CXCR5$^+$ cells increased significantly ($p<0.01$) following the administration of a higher (100 ng) IL-33 dose. Similarly, IL-33 at a range from 1 to 100 ng/dose induced production of anti-OVA IgE and IgG1 antibodies in a dose-dependent manner (Figure E1 of Online Repository). Kinetic studies showed that ST2$^-$CXCR5$^+$ cells were clearly detectable as early as 4 days after one exposure to OVA plus IL-33 (100 ng), while the development of ST2$^+$CXCR5$^-$ cells required more time and/or a second exposure to OVA plus IL-33 (Figure E2 of Online Repository). Thus, the types and doses of IL-1 family cytokines influenced the differential development of two subsets of IL-4-competent CD4$^+$ T cells in draining lymph nodes in the murine lung.

ST2$^-$CXCR5$^+$ CD4$^+$ T cells are bona fide Tfh cells

To investigate the identity of the CD4$^+$ T cell populations, we sorted IL-4eGFP$^+$ST2$^-$CXCR5$^+$ and IL-4eGFP$^+$ST2$^+$CXCR5$^-$ cells harvested from mice exposed to OVA plus IL-33, and analyzed polyA-containing RNAs by microarray using a pool of IL-4eGFP$^-$CD4$^+$ T cells as the control. The comparison of gene expression profiles revealed sets of genes that were unique to each population and not expressed by other populations (Figure 2A and 2B). For example, ST2$^+$CXCR5$^-$ cells expressed Th2 cell markers, including *Gata3*, *Il5*, *Il13*, and *Prdm1* (encoding Blimp-1), as well as *Il6*, receptors for certain chemokines and lipid mediators, and integrins (Figure 2C). Conversely, the ST2$^-$CXCR5$^+$ cells expressed Tfh cell marker genes, including *Bcl6*, *Pdcd1* [encoding programmed cell death protein 1 (PD-1)], and *Sh2d1a* [encoding signaling lymphocyte activation molecule (SLAM)-associated protein (SAP)], as well as a set of chemokine receptors and integrins that were distinct from those expressed by the ST2$^+$CXCR5$^-$ cells. *Tcf7* (encoding TCF-1), a recently identified upstream transcription factor for Tfh cells,[26] was expressed by ST2$^-$CXCR5$^+$ cells and IL-4eGFP$^-$ control cells. *Il4* and *Il21* were expressed by the ST2$^+$CXCR5$^-$ and ST2$^-$CXCR5$^+$ cells, whereas *Il5* and *Il13* were expressed only by ST2$^+$CXCR5$^-$ cells, which were verified by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Figure 2D). As well, qRT-PCR analyses also revealed that *Gata3* was most highly expressed by ST2$^+$CXCR5$^-$ cells, whereas *Bcl6* was most highly expressed by ST2$^-$CXCR5$^+$ cells.

To examine cytokine production, CD4$^+$ST2$^+$CXCR5$^-$ and CD4$^+$ST2$^-$CXCR5$^+$ T cells were isolated and cultured with OVA antigen and B cells from mLNs of sensitized mice, which served as antigen presenting cells (APCs; Figure 2E). We used the entire population of CD4$^+$ T cells as the source for cell sorting, so that we could obtain sufficient cell numbers for culture (Figure E3 of Online Repository). Although the ST2$^+$CXCR5$^-$ cells and ST2$^-$CXCR5$^+$ cells both exhibited robust production of IL-4, IL-5 was only produced by ST2$^+$CXCR5$^-$ cells (Figure 2F). As well, a significantly higher amount of IL-13 was produced by ST2$^+$CXCR5$^-$ cells than by ST2$^-$CXCR5$^+$ cells, but the reverse was the case for IL-21. Interestingly, when B cells isolated from the spleens of naïve mice were used as APCs, the ST2$^+$CXCR5$^-$ cells produced IL-4 and IL-13, but the ST2$^-$CXCR5$^+$ cells did not (Figure E4 of Online Repository). From these observations, we concluded that IL-4eGFP$^+$ST2$^+$CXCR5$^-$ cells were canonical Th2 cells and that IL-4eGFP$^+$ST2$^-$CXCR5$^+$ cells were Tfh cells specialized for the production of IL-4 and IL-21.

Isolated Th2 cells and Tfh cells preferentially mediate allergic airway inflammation and IgE antibody production, respectively

The stimulation of B cells by IL-4, together with other signals, leads to class-switch recombination to IgE.[20] Further, conventional models predict that Th2-type $CD4^+$ T cells serve as a major source of IL-4 for B cell production of IgE antibody.[3, 27] Therefore, to compare the capacity of Th2 and Tfh cells to mediate IgE antibody production, we chose an adoptive transfer approach. We isolated Th2 cells ($CD4^+ST2^+CXCR5^-$) and Tfh cells ($CD4^+ST2^-CXCR5^+$) from C57BL/6 mice previously exposed to OVA plus IL-33 and transferred them intravenously to naïve T cell-deficient *Tcrb*$^{-/-}$ mice (C57BL/6 background) with an intact B compartment[28] (Figure 3A). In the absence of T transferred cells, *Tcrb*$^{-/-}$ mice were unable to produce detectable levels of anti-OVA IgE or IgG1 antibody (Figure 3B). However, when Th2 cells from mice exposed i.n. to OVA plus IL-33 were transferred to *Tcrb*$^{-/-}$ mice, the mice produced anti-OVA IgE antibody, but the levels declined after day 24. When Tfh cells were transferred to *Tcrb*$^{-/-}$ mice, the mice exhibited robust production of anti-OVA IgE antibody, and the levels were maintained up to at least day 49. IgG1 anti-OVA isotype antibody levels in the mice continued to rise following the transfer of either Th2 cells or Tfh cells, but IgG2a antibody did not achieve detectable levels.

To compare the roles of Th2 and Tfh cells in airway inflammation, we used mice deficient in the IL-7 receptor alpha chain (*Il7r*$^{-/-}$) (C57BL/6 background) as recipients (Figure 3C). IL-33 can activate innate immune cells, such as ILC2s, which makes it difficult to interpret the contributions of T cells to airway inflammation, but *Il7r*$^{-/-}$ mice lack ILCs.[2] As expected, naïve *Il7r*$^{-/-}$ mice produced no detectable levels of cytokines when challenged i.n. with OVA plus IL-33 without the transfer of T cells. However, *Il7r*$^{-/-}$ mice that received Th2 cells and were challenged i.n. with OVA plus IL-33 produced marked levels of type 2 cytokines in bronchoalveolar lavage (BAL) fluids (Figure 3D) and developed profound airway eosinophilia (Figure 3E). Conversely, no inflammatory responses were observed in *Il7r*$^{-/-}$ mice that received Tfh cells.

To compare the anatomical location of Th2 cells and Tfh cells during type 2 immune responses, 4get mice previously exposed to OVA plus IL-33 were challenged i.n. with OVA antigen (Figure 4A). A large number of Th2 cells were localized in lung tissues and BAL fluids, and some were detectable in mLNs (Figure 4B and 4C). In contrast, Tfh cells remained in mLNs even after antigen challenge, whereas only a limited number were identified in lung tissues or BAL fluids. These findings suggested that Th2 cells were mobile and mediated type 2 cytokine production and inflammation in the airways. In contrast, the Tfh cells remained in the draining lymph nodes and likely contributed to the sustained production of IgE antibody.

Airway exposure to natural allergens induces Tfh and Th2 cells

The initial experiments employed IL-1 family cytokines as an adjuvant to induce the development and differentiation of $CD4^+$ T cells; however, the doses of these cytokines might have exceeded those observed in a physiologic setting. IL-4-competent Tfh cells have been previously reported in mice infected with helminth parasites.[11–13] Therefore, to examine the roles of Th2 and Tfh cells in allergic airway responses in a physiological

setting, we sought to use naturally occurring allergens implicated in human allergic airway diseases, including a fungus belonging to the genus *Alternaria*, the cockroach and house dust mite (HDM), and the cysteine protease bromelain as a generic model for protease allergens.[29–31]

Naïve 4get mice were exposed i.n. to allergen extracts twice without any adjuvants or exogenous cytokines (Figure 5A). IL-4-competent $CD4^+$ T cells (IL-4eGFP$^+$CD4$^+$ T cells) were clearly detected in mLNs of mice exposed to these allergens (Figure 5B). Among the allergens tested, the *Alternaria* extract induced the largest numbers of IL-4eGFP$^+$ cells (Figure 5C). Conversely, the cockroach extract yielded relatively small effects. A large proportion of IL-4eGFP$^+$ cells (up to 55%) demonstrated an ST2$^-$CXCR5$^+$ phenotype (Figure 5B and 5C), which suggested that airway exposure of naïve mice to natural allergens triggered Tfh cell development. ST2$^+$CXCR5$^-$ Th2 cells were also detected in mice exposed to *Alternaria*, HDM, or bromelain, but to a lesser extent than the detection of Tfh cells. Interestingly, bromelain induced a larger proportion of Th2 cells relative to Tfh cells, compared to the other allergen extracts, which suggested that the nature of the allergens affected the differential development of Tfh and Th2 cells.

Tfh cells are diminished by genetic depletion of Bcl6 in CD4$^+$ T cells

Because the *Alternaria* extract induced the largest numbers of both Tfh and Th2 cells among the allergens, we selected *Alternaria* as a model for subsequent experiments aimed at defining the roles of these cell types in allergic immune responses. The transcriptional repressor BCL6 is considered the master regulator for the Tfh cell lineage.[32, 33] However, germline *Bcl6* knockout mice developed spontaneous inflammation, most likely due to the pro-inflammatory phenotype of *Bcl6*-deficient myeloid cells.[34, 35]

To investigate the specific roles of Tfh cells, we used a floxed *Bcl6* mouse model in which *Bcl6* was conditionally depleted in $CD4^+$ T cells through the use of Cre recombinase expressed from the *Cd4* promoter [*Bcl6*$^{fl/fl}$*Cd4*-Cre mice[14, 36]]. These mice appeared normal, exhibited no apparent signs of disease (data not shown), and their thymus and spleen contained normal T cell numbers and CD4 and CD8 populations.[14] We exposed *Bcl6*$^{fl/fl}$*Cd4*-Cre mice i.n. to *Alternaria* extract spiked with 100 μg OVA (Figure 6A). OVA was included to allow us to monitor antigen-specific immune responses without relying on endogenous *Alternaria* proteins that are often unstable and are often present in variable concentrations in natural allergen extracts.[37] When exposed to *Alternaria* plus OVA, *Bcl6*$^{fl/fl}$ mice lacking CD4-Cre developed both ST2$^+$CXCR5$^-$ Th2 cells and ST2$^-$CXCR5$^+$ or PD-1$^+$CXCR5$^+$ double-positive Tfh cell populations (Figure 6B and 6C). Further, the Tfh cell population was abolished in *Bcl6*$^{fl/fl}$*Cd4*-Cre mice, but the Th2 cell population was not affected. The heterozygous floxed *Bcl6* mice (*Bcl6*$^{fl/+}$*Cd4*-Cre) exhibited an intermediate phenotype, suggesting that Tfh development was dependent on the copy number of *Bcl6*. The frequencies of B220$^+$ B cells within mLN lymphocytes were similar between *Bcl6*$^{fl/fl}$ mice and *Bcl6*$^{fl/fl}$*Cd4*-Cre mice (Figure 6D and 6E). Nonetheless, a marked decrease in the frequency and the total number of FAS$^+$PNA$^+$ germinal center (GC) B cells was observed in *Bcl6*$^{fl/fl}$*Cd4*-Cre mice.

To examine whether the antigen-specific effector functions of Th2 cells were affected by *Bcl6* deficiency, we restimulated mLN cells with the OVA antigen *in vitro*. Following stimulation with OVA, mLN cells from *Bcl6*$^{fl/fl}$ mice (no CD4-Cre) produced IL-4, IL-5, and IL-13 (Figure 6F). Conversely, *Bcl6*$^{fl/fl}$*Cd4*-Cre mice displayed increased spontaneous production of these cytokines without antigen stimulation. Further, when stimulated with OVA, *Bcl6*$^{fl/fl}$*Cd4*-Cre mice produced even greater amounts of IL-5 and IL-13 than *Bcl6*$^{fl/fl}$ mice. Thus, deletion of *Bcl6* in CD4$^+$ T cells resulted in striking decreases in both Tfh cells and GC B cells, but Th2 cell numbers were not affected. The data also suggested that the effector function of Th2 cells was enhanced in the absence of *Bcl6.*

Tfh cells are essential for the production of allergen-specific IgE antibodies

To investigate the roles of Tfh cells in allergic immune responses to inhaled allergens, *Bcl6*$^{fl/fl}$*Cd4*-Cre mice and their littermate controls (*Bc6*$^{fl/fl}$) were initially exposed i.n. to *Alternaria* extract with OVA, and then re-exposed i.n. to the OVA antigen alone (Figure 7A). As expected, *Bcl6*$^{fl/fl}$ mice that were only exposed to OVA did not develop antibodies at any time point (Figure 7B). When initially exposed to *Alternaria* extract plus OVA, *Bcl6*$^{fl/fl}$ mice developed anti-OVA IgE antibodies by day 14, which was sustained until at least day 35. In contrast, anti-OVA IgE antibodies were nearly undetectable in *Bcl6*$^{fl/fl}$*Cd4*-Cre mice up to day 35 ($p<0.01$). Similarly, the plasma anti-OVA IgG1 levels continued to increase in *Bcl6*$^{fl/fl}$ mice, but were undetectable in *Bcl6*$^{fl/fl}$*Cd4*-Cre mice. Further, the concentrations of total IgE and IgG1 in plasma were significantly reduced in *Bcl6*$^{fl/fl}$*Cd4*-Cre mice compared to *Bcl6*$^{fl/fl}$ mice; however, no differences were observed in plasma concentrations of total IgA and IgM.

To examine the production of type 2 cytokines and inflammation in the respiratory mucosa, mice were re-exposed to OVA i.n. on days 42 through 44. *Bcl6*$^{fl/fl}$ mice that were previously exposed to OVA alone and subsequently re-exposed to OVA did not exhibit increases in type 2 cytokines in the lung tissues (Figure 7C) or any signs of airway inflammation (Figure 7D). When initially exposed to *Alternaria* extract plus OVA and then re-exposed to OVA, *Bcl6*$^{fl/fl}$ mice displayed increased levels of IL-5 and IL-13 in the lungs and marked airway eosinophilia. *Bcl6*$^{fl/fl}$*Cd4*-Cre mice also showed comparable increases in these cytokines and airway eosinophilia, which suggested that Tfh cells are required for IgE antibody production, but unnecessary for airway inflammation.

To verify this concept, we used a different genetic model. The interaction between inducible costimulator (ICOS) and the ICOS ligand (ICOSL) plays a critical role in Tfh cell differentiation.[33, 38] Therefore, we subjected wild-type (WT) C57BL/6 mice or germline *Icos*-deficient mice (*Icos*$^{-/-}$) to an experimental protocol that was similar to the one described above for *Bcl6*$^{fl/fl}$*Cd4*-Cre mice (Figure E5A of Online Repository). Partial but significant decreases in ST2$^-$CXCR5$^+$ Tfh cells were observed in *Icos*$^{-/-}$ mice compared to WT mice ($p<0.05$, Figure E5B and E5C), but the number of ST2$^+$CXCR5$^-$ Th2 cells was not affected. Further, when CD4$^+$ T cells from *Icos*$^{-/-}$ mice were restimulated with OVA *in vitro*, the cells produced IL-5 and IL-13 (Figure E5D), and the cytokine levels were higher compared to those from WT mice. In contrast, the plasma levels of anti-OVA IgE and IgG1 antibodies were markedly reduced in *Icos*$^{-/-}$ mice (Figure E5E).

OX40L contributes to type 2 cytokine responses and airway inflammation but not to the development of Tfh cells or IgE antibody production

The prior observations led us to speculate that unlike a conventional model, the two key components of allergic immune responses, which are IgE antibody production and type 2 cytokine responses, could be independently regulated by two subsets of IL-4-producing $CD4^+$ T cells. To test this concept from a different angle, we sought a model in which the terminal differentiation of Th2 cells was impaired, but Tfh cells were unaffected.

OX40L, a tumor necrosis factor (TNF) family ligand that is the product of *Tnfsf4*, has been shown to play a role in the development of pathologic Th2 cells that are capable of mediating strong type 2 inflammatory responses in mice.[39] When exposed i.n. to the *Alternaria* extract plus OVA, the numbers of $ST2^-CXCR5^+$ Tfh cells and $ST2^+CXCR5^-$ Th2 cells developed by mice lacking *Tnfsf4* (*Tnfsf4*$^{-/-}$) were comparable to WT C57BL/6 mice (Figure 8A and 8B). Nonetheless, the production of IL-4, IL-5, and IL-13 by T cells from *Tnfsf4*$^{-/-}$ mice was minimal following *in vitro* stimulation with OVA, which suggested that the effector functions of Th2 cells were impaired (Figure 8C; $p<0.05$). As well, when challenged with OVA *in vivo* (Figure 8D), *Tnfsf4*$^{-/-}$ mice displayed significantly lower levels of IL-4 and IL-13 in the lungs compared to WT mice (Figure 8E; $p<0.05$ and $p<0.01$, respectively), and airway eosinophilia was nearly abolished (Figure 8F). In contrast, *Tnfsf4*$^{-/-}$ mice developed robust anti-OVA IgE and IgG1 antibodies compared to corresponding levels observed in WT mice (Figure 8G).

DISCUSSION

In the current study, we exposed the airways of naïve mice to cytokines and natural allergens and examined the development of adaptive type 2 immune responses. A novel concept derived from this study was that two major components of allergic immune responses, IgE antibody production and type 2 cytokine responses, were regulated separately by Tfh and Th2 cells *in vivo*. The conventional model suggests that Th2-type $CD4^+$ T cells play key roles in various features of allergic immune responses, including IgE antibody production, the production of type 2 cytokines, airway eosinophilia, and mucosal hyperplasia.[3, 27] Our findings not only suggested that Tfh cells were necessary for IgE antibody production, but also that Tfh cells were sufficient even when canonical Th2 cells were absent or their effector functions were compromised. In contrast, Th2 cells played major roles in type 2 cytokine production and eosinophilic airway inflammation even in the absence of Tfh cells or IgE antibodies, which suggested that Tfh cells and Th2 cells play distinct roles in allergic immune responses.

As shown in Figure 2, Th2 and Tfh cells were clearly distinguishable by their expression of key transcription factors and cell surface molecules, and by the production of IL-5, IL-13, and IL-21. Further, no overlap was detected between Th2 and Tfh cell populations when the phenotypes of IL-4eGFP$^+$ cells were examined by fluorescence-activated cell sorting (FACS) using ST2 and CXCR5 as markers (Figure 1). Further, Th2 cells were mobilized to the lungs and airways during allergic immune responses, whereas Tfh cells remained in draining lymph nodes (Figure 4). These observations were consistent with an earlier study involving a *Nippostrongylus brasiliensis* infection model that demonstrated distinctive tissue

localization of Th2 cells (expressing both *Il4* and *Il13*) and Tfh cells (expressing *Il13* but not *Il4*).[22] Therefore, the likelihood exists that allergic immune responses consist of two distinct subsets of IL-4-producing $CD4^+$ T cells, each of which demonstrates unique phenotype, localization, and effector functions as summarized in Figure E6 of Online Repository. By the extension of this concept, it is tempting to speculate that certain human allergic diseases may involve Tfh cells rather than Th2 cells. Examples would be allergic diseases involving demonstrated roles for IgE antibodies and minimal signs of mucosal inflammation, such as food allergies and anaphylaxis.

IL-4-producing Tfh cells were previously described in mice infected with *Leishmania major* or various helminth parasites,[11-13, 22] and in mice immunized systemically by injection of OVA plus alum.[10] Our observations contributed to this knowledge by demonstrating that exposure of the airways of naïve BALB/c or C57BL/6 mice to natural allergens, such as *Alternaria* and HDM, induced IL-4-production by Tfh cells. Further, the proportions of Tfh and Th2 cells generated were dependent on the allergen. Nonetheless, questions remain regarding the immunological mechanisms involved in the development of Tfh cells following exposure to allergens. Previously, IL-6 and IL-21 were shown to induce Tfh cells *in vitro*. Specifically, $CD4^+$ T cells cultured with IL-6 or IL-21 expressed BCL6 and/or CXCR5 mRNA in a STAT3-dependent manner.[33, 40] However, the mechanisms of Tfh cell development *in vivo* appear to be rather complex. *Il6*$^{-/-}$, *Il21*$^{-/-}$, and *Il21r*$^{-/-}$ mice, as well as *Stat3* deficient mice, exhibited no or minimal defects in Tfh cell development.[41–43] However, the blockage of IL-6 in *Il21*$^{-/-}$ mice and in *Il6/Il21* double knockout mice resulted in a partial decrease in Tfh cells.[44, 45]

In this study, we found that the combined intranasal administration of IL-33 or IL- 1β and OVA effectively induced IL-4-competent Tfh cells, which suggested an alternative method for the generation of Tfh cells *in vivo* in the absence of infectious agents. However, mice lacking specific receptors, including IL-1R1 (receptor for IL-1α and IL-1β) or IL-1RL1 (ST2, receptor for IL-33), developed levels of IgE antibody that were comparable to WT animals (data not shown). Hence, there appears to be considerable redundancy in the Tfh-development pathway. This speculation is further supported by the observation that although only IL-33 effectively induced Th2 cells (Figure 1), Tfh cells were induced by either IL-33 or IL-1β. Further, the concentration of IL-33 required to induce Tfh cells was approximately one-tenth or less the level needed for Th2 cell induction. The apparent redundancy and sensitivity in the system regarding the generation IL-4-producing Tfh cells, and the established extreme sensitivity of IgE- based recognition of allergens might have evolved to ensure the protection of the host against certain pathogens and avoidance of unfavorable environmental factors.[46]

At the molecular level, our mouse allergy models verified critical roles for BCL6 and ICOS in the development of Tfh cells *in vivo*. BCL6 is considered the master regulator for Tfh differentiation[32, 33] because it inhibits the differentiation of other $CD4^+$ T cell subsets through the repression of the transcription factor Blimp1.[32] Germline *Bcl6*$^{-/-}$ mice develop spontaneous inflammatory diseases.[34, 35] The use of *Cd4*-driven Cre recombinase in *Bcl6*-floxed mice allowed us to determine that the deficiency of *Bcl6* in the $CD4^+$ T cell compartment resulted in nearly complete depletion of Tfh cells *in vivo* (Figure 6), but it did

not induce systemic inflammation. The numbers of ST2-positive mature Th2 cells were not affected in these mice, but the production of type 2 cytokines was enhanced, which reflected the capacity of BCL6 to inhibit GATA3 expression and Th2 cell differentiation.[47]

ICOS has been shown to play an important role in the development and effector functions of Tfh cells and various other T cells subsets, including Th1, Th2, Th17, and Treg cells.[48] Mice deficient in ICOS signaling consistently displayed decreased serum levels of IgE and IgG1.[49–52] ICOS has also been shown to promote the expansion of Th2 cells,[53, 54] which provides a potential mechanistic basis for decreased levels of IgE, but the results are controversial.[50, 55] In this study, *Icos*$^{-/-}$ mice exhibited marked decreases in Tfh cells compared to WT mice when exposed to airborne allergens, which was consistent with previous findings.[11, 33, 38] However, these mice paradoxically exhibited enhanced Th2-type cytokine responses. The differences in our results and some previous observations could be explained by differences in the models, including adjuvants used and routes of antigen administration. Nonetheless, ICOS signaling is critical for *Bcl6* expression;[38] thus, it was expected that ICOS deficiency could result in a phenocopy of *Bcl6* deficiency in CD4^{+} T cells. Notwithstanding, BCL6 and ICOS likely served as a molecular switch and played a role in determining whether naïve CD4^{+} T cells differentiated into Tfh or Th2 cells.

It has been established that IL-4 and its downstream signaling molecules are required for the class-switch recombination of IgE antibody *in vitro* and *in vivo*.[56] In our model, Tfh and Th2 cells from day 11 mLNs were both capable of producing IL-4 when they were restimulated with antigen *in vitro*, despite the fact that the *Il4* regulatory mechanisms in Tfh and Th2 cells appear distinct.[10, 22] We also found that the adoptive transfer of either Tfh or Th2 cells induced the production of IgE and IgG1 antibodies (Figure 3). Importantly, the production of IgE antibody induced by transfer of Tfh cells was sustained for at least 4 weeks after the final antigenic stimulation; however, the IgE response induced by Th2 cells was relatively transient. Nonetheless, sustained increases in IgG1 antibody were observed following the transfer of either Tfh or Th2 cells. Previous studies involving the injection of mice with a goat antibody to mouse IgD indicated that T cell help exclusive of IL-4 production was important for the generation of an IgE response, but not for an IgG1 response.[57] Hence, Tfh cells might provide signals other than IL-4 for optimized and sustained IgE production, perhaps through a direct physical interaction with B cells or the release of additional soluble factors. IL-21, which is abundantly produced by Tfh cells,[9] could be a candidate factor. Nonetheless, the roles of IL-21 in IgE antibody production are rather controversial. For example, IL-21 induced [58] or inhibited [59, 60] IgE antibody production *in vitro*. In an *in vivo* study, IL-21R-deficient mice produced even higher levels of IgE. [61] Alternatively, any molecules that are identified by microarray analyses as unique to Tfh cells (Figure 2), such as PD-1, SAP, B and T lymphocyte attenuator (BTLA), or a combination of these molecules, could play a comparable role. Accordingly, additional studies aimed at identifying critical molecule(s) provided by Tfh cells, which are involved in the sustained production of IgE antibodies during allergic immune responses, are required.

Given our observations, the robust and unique characteristics of Tfh cells in the mediation of IgE antibody production might explain the pathophysiology of various IgE-dependent allergic diseases, such as food allergies and anaphylaxis. Recognizing the involvement of

Tfh and Th2 cells, which are two distinct subsets of IL-4-producing $CD4^+$ T cells (Figure E6 of Online Repository), could aid in redefining the mechanisms involved in allergic diseases. Nonetheless, achieving a better understanding of the molecular and immunological mechanisms involved in the differentiation and functions of Tfh cells and methods to disrupt these pathways will be of central importance in the development of new treatment and preventive strategies for allergic diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APCs	antigen presenting cells
BAL	bronchoalveolar lavage
BTLA	B and T lymphocyte attenuator
eGFP	enhanced green fluorescent protein
FACS	fluorescence-activated cell sorting
GC	germinal center
GWAS	genome-wide association study
HDM	house dust mite
ICOS	inducible costimulatory
ICOSL	ICOS ligand
IL	interleukin
ILC2	type 2 innate lymphoid cells
i.n	intranasal
i.p	intraperitoneal
IRES	internal ribosomal entry site
i.v	intravenous
mLN	mediastinal lymph node
LNs	lymph nodes

OVA	ovalbumin
PD-1	programmed cell death protein 1
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SAP	signaling lymphocyte activation molecule-associated protein
SNPs	single nucleotide polymorphisms
SPF	specific pathogen-free
Tfh	follicular helper T
Th2	type 2 helper T
TNF	tumor necrosis factor
Tregs	regulatory T cells
TSLP	thymic stromal lymphopoietin
WT	wild-type

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Key Messages

- IL-4-producing Tfh cells develop when mice are exposed to airborne allergens.
- Allergen-specific Tfh cells and Th2 cells possess distinct characteristics.
- The Tfh cells are necessary and sufficient for IgE antibody production.
- Allergic immune responses involve two subsets of IL-4-producing $CD4^+$ T cells.

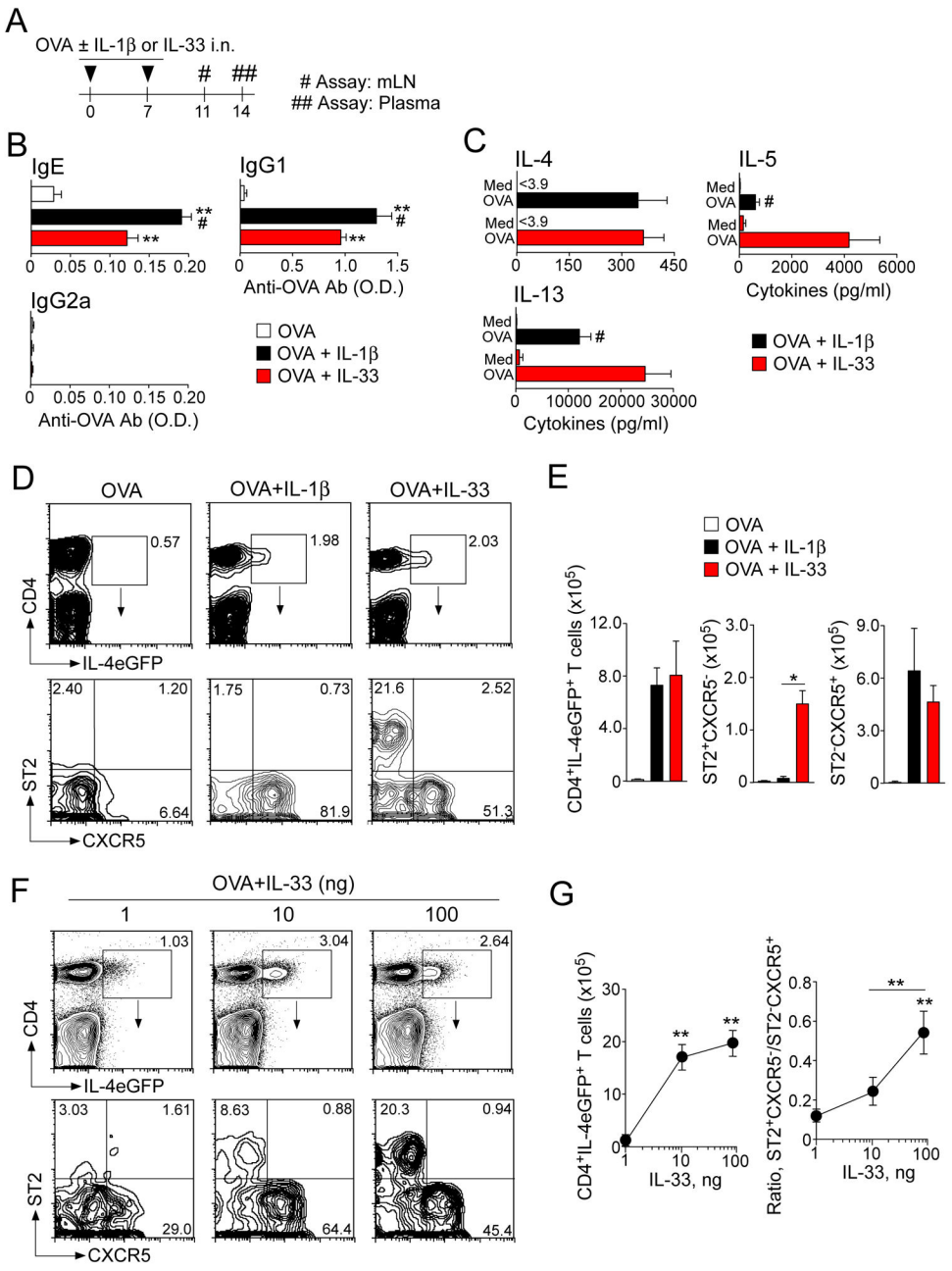

Figure 1.

IL-33 promotes the development of two distinct subsets of IL-4-competent CD4$^+$ T cells. (A) A schematic representation of a mouse airway sensitization model. Naïve 4get mice (BALB/c background) were exposed i.n. to OVA with or without IL-1β or IL-33. (B) On day 14, plasma was analyzed for anti-OVA antibodies by ELISA. * = $p<0.05$ and ** = $p<0.01$ compared to mice exposed to OVA alone. # = $p<0.05$ compared to mice exposed to OVA plus IL-33. Data are presented as the mean ± SEM (n = 4–5 in each group). (C) Day 11 mLN cells were cultured with or without OVA. The cytokine levels in the supernatants were analyzed by ELISA. # = $p<0.05$ compared to OVA plus IL-33. Data are presented as the

mean ± SEM (n = 4–5 in each group). (D–G) Day 11 mLN cells were gated on lymphocytes, and the expression of CD4, IL-4eGFP, ST2, and CXCR5 was analyzed by FACS. (D) Representative scattergrams showing the expression of CD4 and IL-4eGFP in a total lymphocyte population, and the expression of ST2 and CXCR5 in $CD4^+$IL-4eGFP^+ lymphocytes. (E) Numbers of each cell population are presented as the mean ± SEM (n = 4 in each group). * = $p<0.05$ between the groups indicated by horizontal lines. (F) Naïve 4get mice were exposed i.n. to OVA with different doses of IL-33 as described in Figure 1A, and day 11 mLN cells were analyzed. (G) Numbers and the ratio of each cell population are presented as the mean ± SEM (n = 4 in each group). ** = $p<0.01$ between the groups indicated by horizontal lines or as compared to 1 ng IL-33. (C, E, G) Data are a pool of two separate experiments.

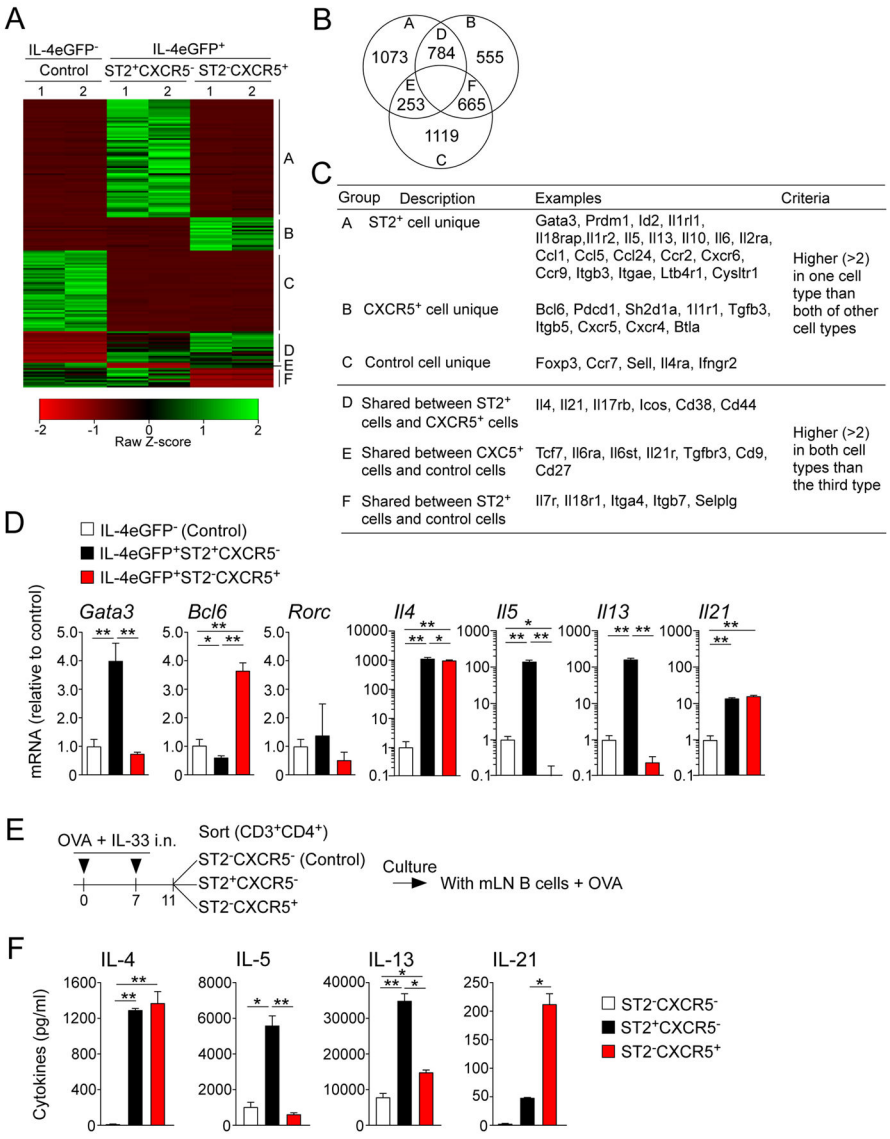

Figure 2.

ST2⁻CXCR5⁺ CD4⁺ T cells are bona fide Tfh cells. (A–D) Naïve 4get mice (BALB/c background) were exposed i.n. to OVA plus IL-33 as described in Figure 1A. The cell populations indicated ($CD4^+$IL-4eGFP$^-$, $CD4^+$IL-4eGFP$^+$$ST2^+CXCR5^-$, and $CD4^+$IL-4eGFP$^+$$ST2^-CXCR5^+$) were sorted from day 11 mLN cells, and mRNA was analyzed by microarray and qRT-PCR. (A) The heat map shows microarray gene expression data (genes selected for fold difference >10) based on cells isolated from two separate experiments. (B) The Venn diagram shows the number of gene probes in each gene expression group. (C) Representative genes in each group are presented using the criteria described in the Table. (D) mRNA expression of select genes was analyzed by qRT-PCR, and the results were normalized to 18s RNA levels and expressed as a ratio to the $CD4^+$IL-4eGFP$^-$ population (mean ± SEM, n = 4). * = $p<0.05$ and ** = $p<0.01$ between the groups indicated by horizontal lines. (E, F) Naïve BALB/c mice were exposed i.n. to OVA plus IL-33, as described in Figure 1A. Day 11 mLN cells were sorted into

$CD3^+CD4^+ST2^-CXCR5^-$, $CD3^+CD4^+ST2^+CXCR5^-$, and $CD3^+CD4^+ST2^-CXCR5^+$ cell populations and cultured with B cells isolated from mLNs in the presence of OVA. (F) Cytokine levels in the supernatants were analyzed by ELISA. Data are presented as the mean ± range from duplicate cultures. The figure is representative of two separate experiments.

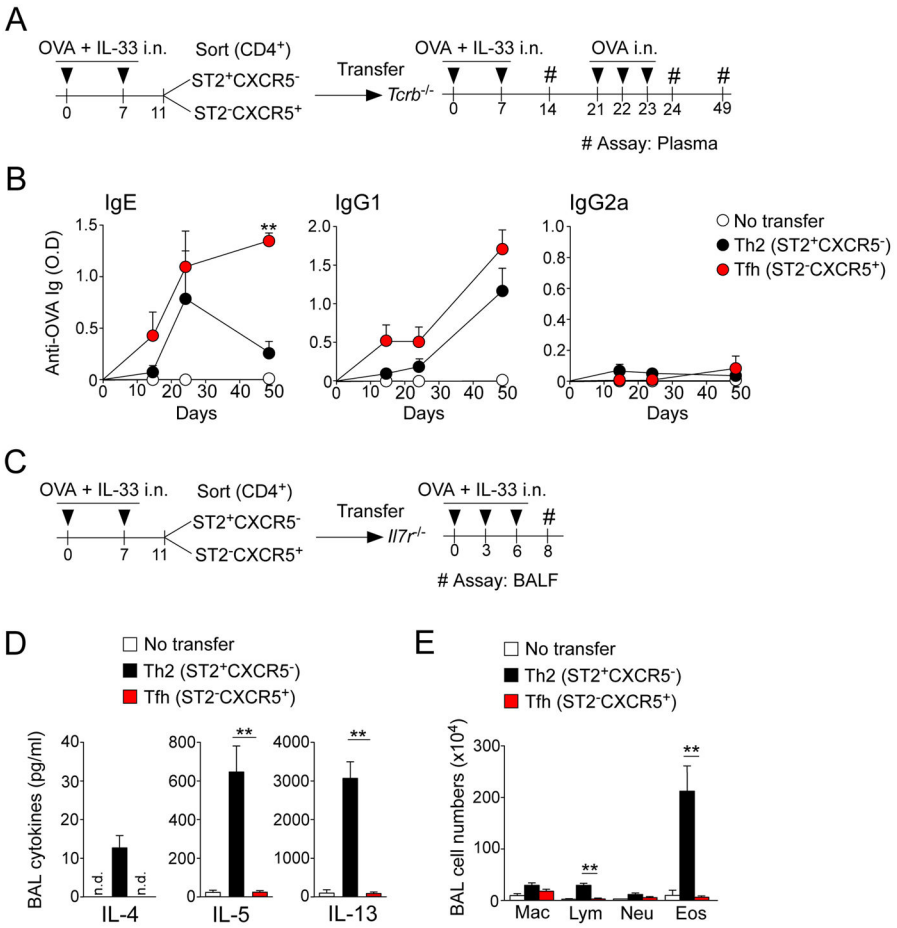

Figure 3.

Th2 and Tfh cells preferentially mediate allergic airway inflammation and IgE antibody production, respectively. (A) A schematic representation of an adoptive transfer model to *Tcrb*$^{-/-}$ mice (C57BL/6 background) (B) The plasma antibody levels were analyzed by ELISA. Results are the mean ± SEM (n = 3 in each group). ** = p<0.01 compared to mice that received Th2 cells. (C) A schematic representation of an adoptive transfer model to *Il7*$^{-/-}$ mice (C57BL/6 background). BAL fluids were analyzed for cytokine levels (D) and inflammatory cell numbers (E). Results are the mean ± SEM (n = 3 in each group). ** = p<0.01 between the groups indicated by horizontal lines. (B, D, E) Each figure is representative of two separate experiments.

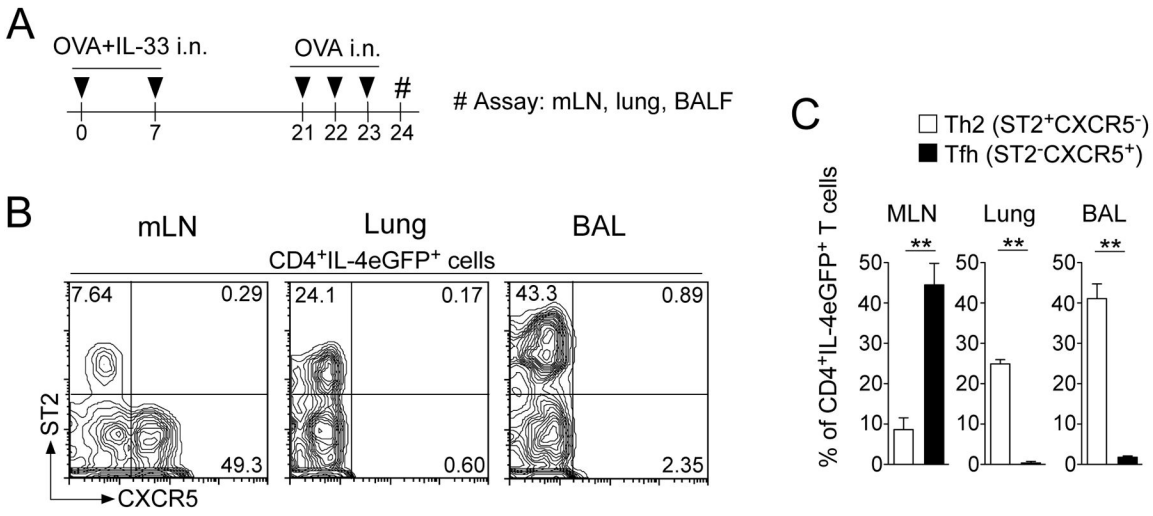

Figure 4.

Th2 and Tfh cells localize differently during allergic immune responses. (A) A schematic representation of a mouse airway sensitization and challenge model. (B) Representative scattergrams show the expression of ST2 and CXCR5 in the $CD4^+IL\text{-}4eGFP^+$ population. (C) Percentages of each cell population are presented as the mean ± SEM (n = 8 in each group). ** = $p<0.01$ between the groups indicated by horizontal lines. Data are a pool of two separate experiments.

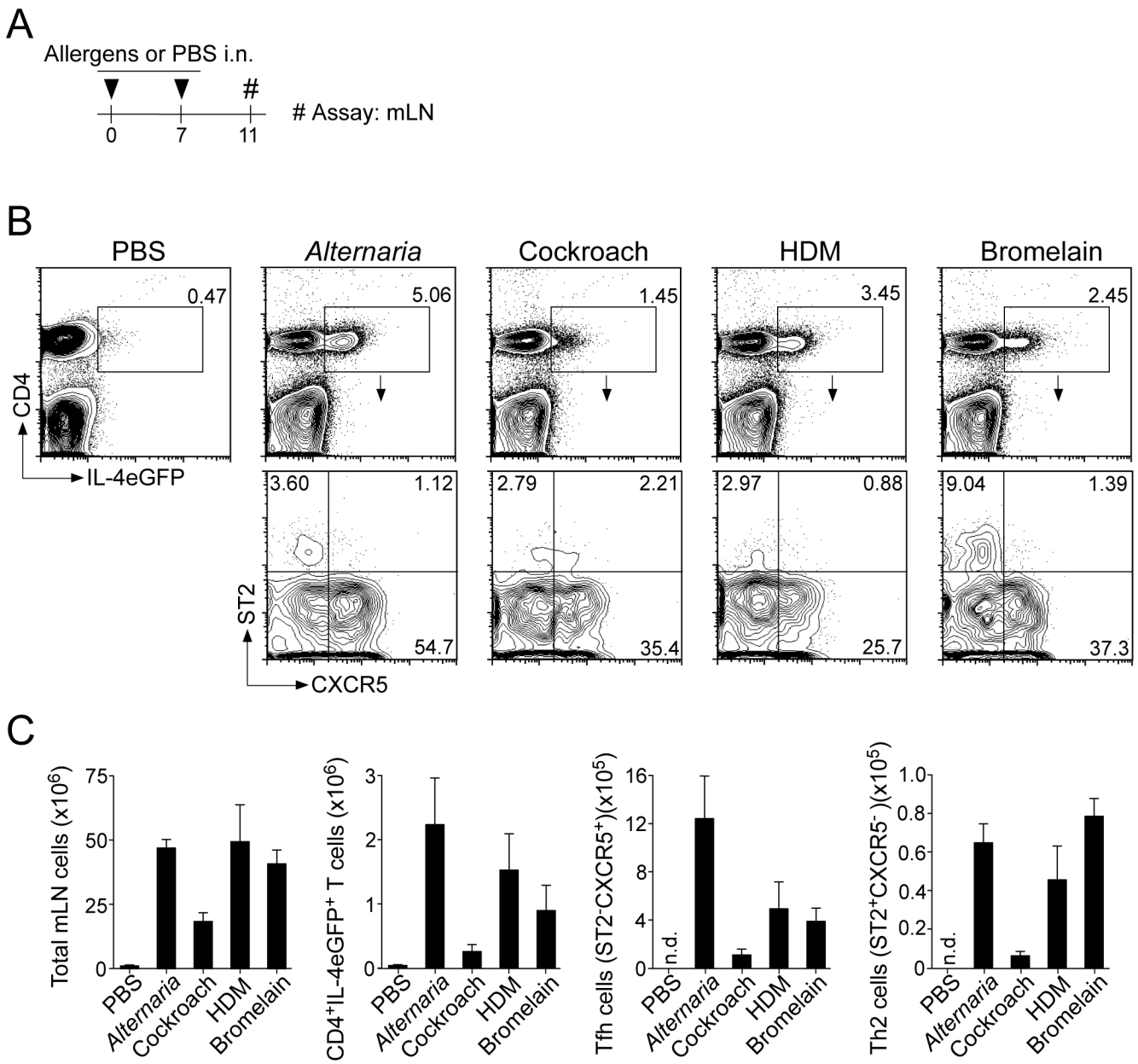

Figure 5.

Airway exposure to natural allergens induces Tfh and Th2 cells. (A) A schematic representation of the airway allergen exposure model. Naïve 4get mice (BALB/c background) were exposed i.n. to allergen extracts without any adjuvants. Day 11 mLN cells were gated on lymphocytes, and the expression of CD4, IL-4eGFP, ST2, and CXCR5 was analyzed by FACS. (B) Representative scattergrams are shown. (C) Numbers of each cell population are presented as the mean ± SEM (n = 3 in each group). Data are representative of two separate experiments, except for HDM, where the experiment was performed only once.

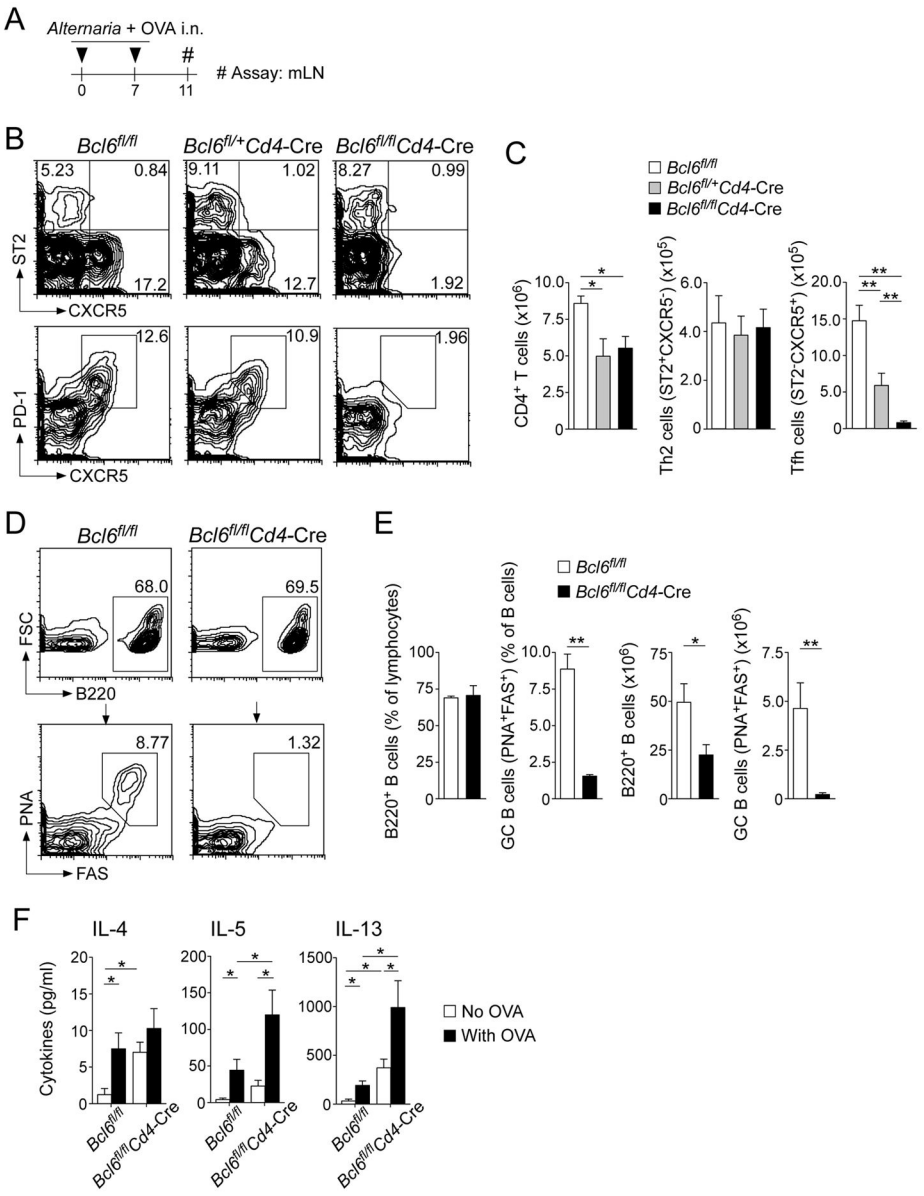

Figure 6.

Tfh cells are diminished by depletion of *Bcl6* in CD4$^+$ T cells

(A) A schematic representation of a mouse airway sensitization model. (B) Representative scattergrams show the expression of ST2, CXCR5, and PD-1 in the CD4$^+$ T cell population. mLN cells were analyzed by gating on CD4$^+$ T cells in the lymphocyte population. (C) Cell numbers in each cell population are presented as the mean ± SEM (n = 5–7 in each group). * = $p<0.05$ and ** = $p<0.01$ between the groups indicated by horizontal lines. Data are a pool of three separate experiments. (D, E) Expression of B220, PNA, and FAS in mLN lymphocytes was analyzed by FACS. (D) Representative scattergrams show the expression of B220 in the lymphocyte population and the expression of PNA and FAS in the B220$^+$ cell population. (E) Percentages and cell numbers in each cell population are presented as the mean ± SEM (n = 4 in each group). * = $p<0.05$ and ** = $p<0.01$ between the groups

indicated by horizontal lines. (F) Day 11 mLN cells were cultured with or without OVA, and the cytokine levels in the supernatants were analyzed by ELISA. Results are shown as the mean ± SEM (n = 4 in each group). * = $p<0.05$ between the groups indicated by horizontal lines.

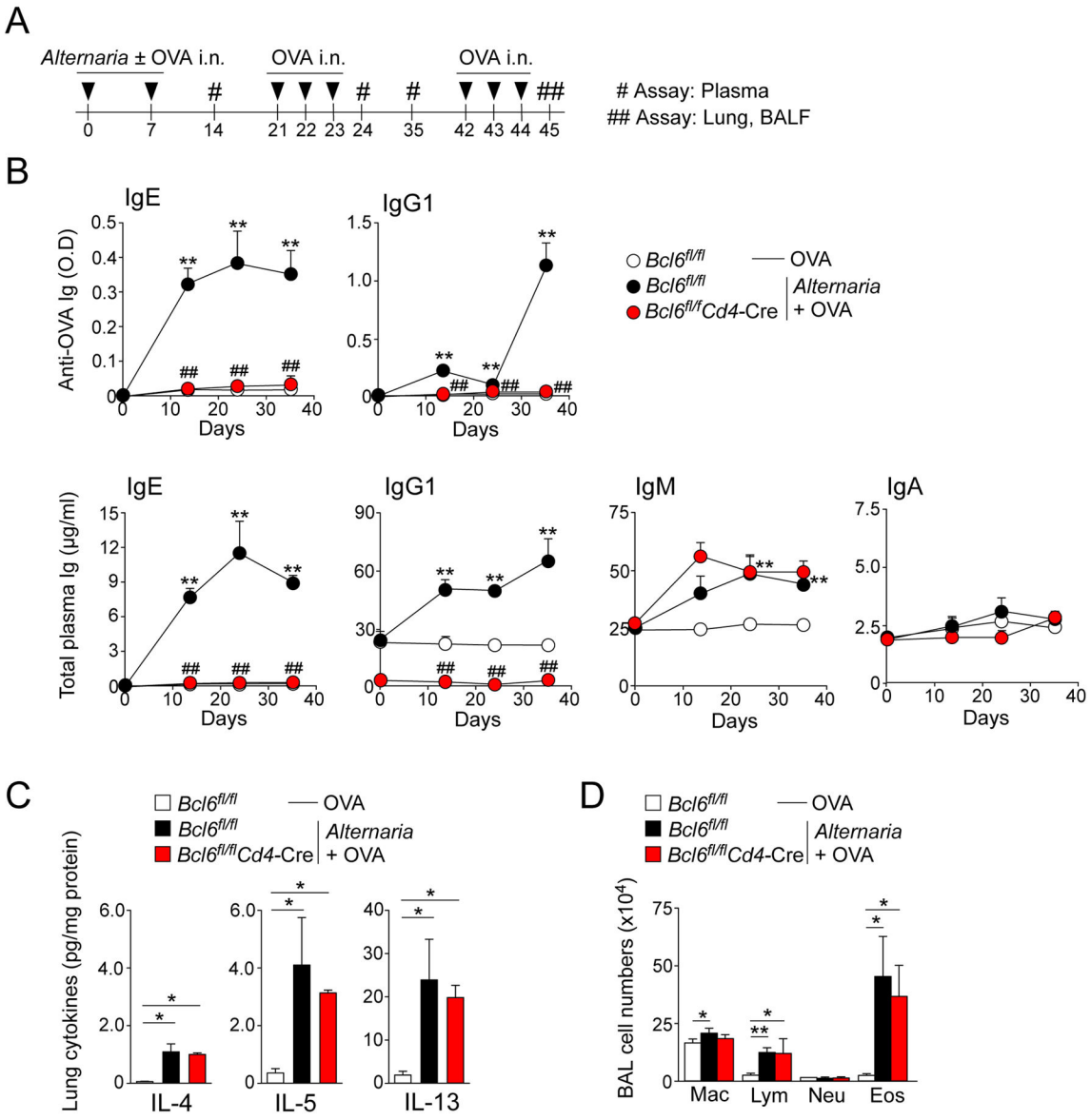

Figure 7.

Tfh cells are necessary for the production of allergen-specific IgE antibody. (A) A schematic representation of a mouse airway sensitization and challenge model. (B) Plasma levels of anti-OVA IgE and IgG1 antibodies and total IgE, IgG1, IgM, and IgA were analyzed by ELISA. ** = $p<0.01$ compared to *Bcl6* $^{fl/fl}$ mice previously exposed to OVA alone. ## = $p<0.01$ compared to *Bcl6* $^{fl/fl}$ mice previously exposed to *Alternaria* extract plus OVA. Data are presented as the mean ± SEM (n = 5 in each group). The cytokine levels in lung homogenates (C) and the numbers of inflammatory cells in BAL fluids (D) were analyzed on day 45. (C, D) Results are presented as the mean ± SEM (n = 5 in each group). * = $p<0.05$ and ** = $p<0.01$ between the groups indicated by horizontal lines. Data are representative of two separate experiments.

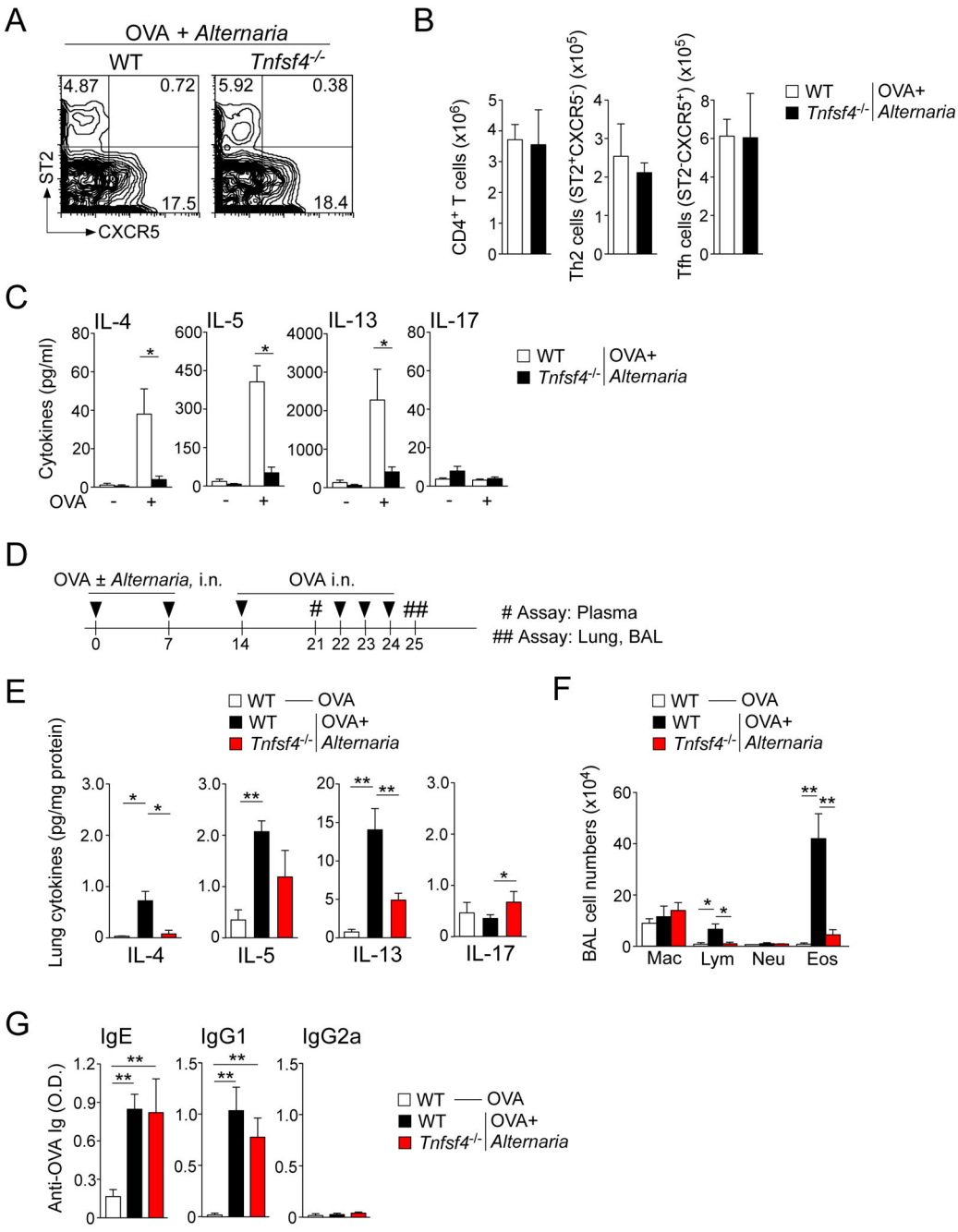

Figure 8.

OX40L contributes to type 2 cytokine responses and airway inflammation but does not affect the development of Tfh cells or IgE antibody production. (A–C) Naïve C57BL/6 (WT) and OX40L knockout (*Tnfsf4*$^{-/-}$) mice were exposed i.n. to OVA and *Alternaria* extract on days 0 and 7, and mLN cells were analyzed on day 11. (A) Representative scattergrams show the expression of ST2 and CXCR5 in the CD4^{+} T cell population. (B) Numbers of each cell population are presented as the mean ± SEM (n = 4 in each group). (C) mLN cells were cultured with or without OVA, and the cytokine levels in the supernatants were analyzed by

ELISA. Results are the mean ± SEM (n = 5–7 in each group). * = $p<0.05$ between the groups indicated by horizontal lines. Data are representative of two separate experiments. (D) A schematic representation of a mouse airway sensitization and challenge model. The cytokine levels in lung homogenates (E) and the numbers of inflammatory cells in BAL fluids (F) were analyzed on day 25. Results are the mean ± SEM (n=5–7 in each group). * = $p<0.05$ and ** = $p<0.01$ between the groups indicated by the horizontal lines. (G) Plasma levels of anti-OVA IgE, IgG1, and IgG2a antibodies were analyzed by ELISA. * = $p<0.05$ and ** $p<0.01$ between the groups indicated by horizontal lines. Data are presented as the mean ± SEM (n = 5–7 in each group). (C, E, F, G) Data are a pool of two separate experiments.