

Bcl6 and Blimp1 reciprocally regulate ST2⁺ Treg-cell development in the context of allergic airway inflammation

Byunghee Koh, PhD,^{a,b} Benjamin J. Ulrich, PhD,^b Andrew S. Nelson, PhD,^a Gayathri Panangipalli, BS,^c Rakshin Kharwadkar, MS,^d Wenting Wu, PhD,^e Markus M. Xie, PhD,^b Yongyao Fu, MS,^b Matthew J. Turner, MD, PhD,^f Sophie Paczesny, MD, PhD,^{a,b} Sarath Chandra Janga, PhD,^c Alexander L. Dent, PhD,^b and Mark H. Kaplan, PhD^{a,b}
Indianapolis, Ind

Background: Bcl6 is required for the development of T follicular helper cells and T follicular regulatory (Tfr) cells that regulate germinal center responses. Bcl6 also affects the function of regulatory T (Treg) cells.

Objective: The goal of this study was to define the functions of Bcl6 in Treg cells, including Tfr cells, in the context of allergic airway inflammation.

Methods: We used a model of house dust mite sensitization to challenge wild-type, *Bcl6*^{fl/fl} Foxp3-Cre, and *Prdm1* (Blimp1)^{fl/fl} Foxp3-Cre mice to study the reciprocal roles of Bcl6 and Blimp1 in allergic airway inflammation.

Results: In the house dust mite model, Tfr cells repress the production of IgE and Bcl6⁺ Treg cells suppress the generation of type 2 cytokine-producing cells in the lungs. In mice with Bcl6-deficient Treg cells, twice as many ST2⁺ (IL-33R⁺) Treg cells develop as are observed in wild-type mice. ST2⁺ Treg cells in the context of allergic airway inflammation are Blimp1 dependent, express type 2 cytokines, and share features of visceral adipose tissue Treg cells. Bcl6-deficient Treg cells are more susceptible, and Blimp1-deficient Treg cells are resistant, to acquiring the ST2⁺ Treg-cell phenotype *in vitro* and *in vivo* in response to IL-33. Bcl6-deficient ST2⁺ Treg cells, but not Bcl6-deficient ST2⁺ conventional T cells, strongly promote allergic airway inflammation when transferred into recipient mice.

Lastly, ST2 is required for the exacerbated allergic airway inflammation in *Bcl6*^{fl/fl} Foxp3-Cre mice.

Conclusions: During allergic airway inflammation, Bcl6 and Blimp1 play dual roles in regulating Tfr-cell activity in the germinal center and in the development of ST2⁺ Treg cells that promote type 2 cytokine responses. (J Allergy Clin Immunol 2020;■■■■:■■■-■■■.)

Key words: Interleukin-33, ST2, Bcl6, Blimp1, T follicular regulatory T cells, ST2-expressing regulatory T cells, allergy, asthma

Bcl6 is a transcriptional repressor that is a central regulator of immunity with functions in T cells, macrophages, and B cells.¹⁻⁷ Bcl6 is critical for the germinal center (GC) response, controlling both T follicular helper (Tfh) cells and GC B cells.¹⁻⁶ Bcl6 is also expressed in a population of cells coexpressing Foxp3, the lineage-defining factor for regulatory T (Treg) cells. Like Tfh cells and GC B cells, these cells, which are termed *T follicular regulatory (Tfr) cells*, colocalize to the GC.⁸⁻¹⁰

In the GC, Tfh cells help B cells to produce high-affinity antigen-specific antibodies.^{11,12} However, dysregulated Tfh cells promote B cells' production of self-reactive antibodies, leading to autoimmune disease.¹³⁻¹⁵ It had been proposed that Tfr cells regulate excessive GC reactions by suppressing proliferation of Tfh cell and self-reactive B cells.^{8-10,16,17} However, recent studies have reported that Tfr cell-derived IL-10 is important for maintenance of the GC response and provides helper functions for the antibody response.¹⁸ Thus, the regulatory and helper functions of Tfr cells might differ depending on the context of the immune response.

In the context of allergic disease characterized by production of T_H2 cytokines such as IL-4, IL-5, and IL13, and allergen-specific IgE, Bcl6 likely has multiple functions. Previous reports had shown increased allergic inflammation in mice that have germline deletion of Bcl6.¹⁹⁻²² Enhanced allergic inflammation was thought, at least in part, to be from effects of Bcl6 deficiency in Treg-cell populations that resulted in increased levels of T_H2 cytokines and expression of genes, including *Il1rl1*, which encodes the IL-33R ST2.²² In mice that lack Bcl6 in all CD4⁺ T cells and thus lack Tfh cells, there is no IgE production in response to allergen sensitization and an absence of IgE-dependent immune function.²³ In allergic pulmonary inflammation, how Bcl6 influences Treg-cell function is unclear.

In this study, we revealed that Tfr cells are critical for the allergen-specific humoral immune response during house dust mite (HDM)-induced airway inflammation. Tfr-cell deficiency

From ^athe Department of Pediatrics and Herman B. Wells Center for Pediatric Research Indiana University School of Medicine, Indianapolis; ^bthe Department of Microbiology and Immunology, ^cthe Department of Biochemistry and Molecular Biology, and ^dthe Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis; ^ethe Department of Biohealth Informatics, School of Informatics and Computing, Indiana University-Purdue University, Indianapolis; and ^fthe Department of Dermatology, Richard L. Roudebush VA Medical Center, Indianapolis. This work was supported by Public Health Service grants from the National Institutes of Health (grant R01 AI057459 to M.H.K. and grant R01 AI132771 to A.L.D.). B.J.U. was supported by grants T32 AI060519 and F30 HL147515. A.S.N. was supported by grant T32 HL007910. M.M.X. was supported by a Careers in Immunology Fellowship from the American Association of Immunologists. M.J.T. was supported by the Department of Veterans Affairs (grant IK2 CX001019 [VA CDA2]). Core facility use was also supported by Indiana University Simon Cancer Center Support Grant (P30 CA082709 and U54 DK106846). Support provided by the Herman B. Wells Center was obtained in part from the Riley Children's Foundation.

Disclosure of potential conflicts of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication August 8, 2019; revised February 12, 2020; accepted for publication March 3, 2020.

Corresponding author: Mark H. Kaplan, PhD, Department of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Dr, Room 420, Indianapolis, IN 46202. E-mail: mkaplan2@iu.edu.

0091-6749/\$36.00

© 2020 American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2020.03.002>

Abbreviations used

BAL: Bronchoalveolar lavage
 ChIP: Chromatin immunoprecipitation
 FACS: Fluorescence-activated cell sorting
 GC: Germinal center
 HDM: House dust mite
 iTreg: *In vitro*-cultured regulatory T
 mLN: Mediastinal lymph node
 PRDM1: Gene encoding Blimp1
 qRT-PCR: Quantitative real-time PCR
 RNA-seq: RNA sequencing
 Tconv: Conventional T
 Tfh: T follicular helper
 Tfr: T follicular regulatory T
 Treg: Regulatory T
 VAT: Visceral adipose tissue
 WT: Wild-type

increased total and antigen-specific IgE production and T_H2 responses contributing to more severe airway inflammation. The development of ST2⁺ Treg cells was reciprocally regulated by Bcl6 and Blimp1. Bcl6 deficiency promoted ST2⁺ Treg-cell development in mediastinal lymph nodes (mLNs) and lungs, and the increased pulmonary inflammation was ST2 dependent, defining a critical role for Bcl6 in restraining the development of proallergic ST2⁺ Treg cells.

METHODS**Mice**

All mice were of a C57BL/6 background, except as specified for BALB/cJ mice in strain comparisons (Jackson Laboratory, Bar Harbor, Me). BALB/cJ, C57BL/6, *Foxp3*-Cre^{YFP}, *Bcl6*^{fl/fl}*Foxp3*-Cre^{YFP}, *Bcl6*^{fl/fl}CD4-Cre^{YFP}, *Prdm1*^{fl/fl}*Foxp3*-Cre^{YFP}, *Il1rl1*^{-/-}, and *Il1rl1*^{-/-}*Bcl6*^{fl/fl}*Foxp3*-Cre^{YFP} mice were maintained under specific pathogen-free conditions. C57BL/6 × B6.SJL-Ptprc^a Pepc^b/BoyJ F1 mice were used as recipients for the adoptive transfer experiments. All experiments were done with 6- to 8-week-old mice. All experiments were performed with the approval of the Indiana University Institutional Animal Care and Use Committee.

Treg-cell culture

Naive CD4⁺CD62L⁺ T cells from mice were positively selected from the enriched CD4⁺ T cells from spleen and lymph nodes by using MACS beads and columns (Miltenyi Biotec, Somerville, Mass). Naive CD4⁺CD62L⁺ T cells were activated with plate-bound anti-CD3 (2 μg/mL; 145-2C11 [BioXCell, Lebanon, NH]) and soluble anti-CD28 (0.5 μg/mL; BD Pharmingen, San Diego, Calif) in complete culture media, RPMI 1640 (ThermoFisher Scientific, Waltham, Mass) containing 10% FBS (Atlanta Biologicals, Flowery Branch, Ga), 1% antibiotics (penicillin and streptomycin stock; 5000 μg/mL of penicillin and 5000 μg/mL of streptomycin), 1 mM sodium pyruvate, 1 mM L-glutamine, 2.5 mM of nonessential amino acids (stock; 100×), 5 mM HEPES (all from LONZA, Basel, Switzerland), and 57.2 μM 2-mercaptoethanol (Sigma-Aldrich, St Louis, Mo), to generate Treg cells (5 ng/mL of hTGF-β1, 10 U/mL of hIL-2, 10 μg/mL of anti-IFNγ, 10 μg/mL of anti-IL-4, or 1–10 ng/mL of IL-33 (all from PeproTech, Rocky Hill, NJ). For splenic Treg cell culture, YFP⁺ Treg cells were sorted from spleen of *Foxp3*-Cre^{YFP} mice and activated with plate-bound anti-CD3 (2 μg/mL) and soluble anti-CD28 (0.5 μg/mL) and hIL-2 (200 U/mL) with IL-33 (1 or 10 ng/mL) in complete culture media. Cells were grown at 37°C under 5% CO₂ and harvested on day 3 for analysis.

HDM extract-induced allergic airway inflammation

Mice were challenged intranasally with HDM (Greer Laboratories, Lenoir, NC) extract every other day for 16 days. HDM extract (25 μg) was diluted with PBS (25 μL) and administered via the nose. Mice were euthanized 1 day after final intranasal challenge. Bronchoalveolar lavage (BAL) cells were collected twice by lavaging the lungs with 1 mL of PBS. To prepare a single-cell suspension from lungs, lung tissues were chopped and incubated with 0.5 mg/mL of collagenase A (Roche, Basel, Switzerland) in Dulbecco's modified Eagle medium (ThermoFisher Scientific) at 37°C for 45 minutes. After the tissues had been ground with a mesh stainless steel strainer, red blood cells were removed by ammonium-chloride-potassium lysing buffer (LONZA). After the reaction had been stopped by adding buffer (PBS with 0.5 % BSA), the cells were washed with the buffer followed by filtration through 70-μm nylon mesh to remove debris. Single-cell suspensions were used for flow cytometry, RNA isolation, or CD4⁺ T-cell enrichment. For the adoptive transfer experiment, ST2⁺ Treg (CD4⁺ ST2⁺ Foxp3/YFP⁺) cells or Tconv (CD4⁺ ST2⁺ Foxp3/YFP⁻) cells were sorted from lungs of CD45.2⁺ *Foxp3*-Cre^{YFP} and *Bcl6*^{fl/fl}*Foxp3*-Cre^{YFP} mice (CD45.2), and 50,000 cells were transferred to naive F1 (CD45.1) recipient mice via tail injection. Following adoptive transfer, the recipient mice were challenged with HDM 3 times at 1 hour, 24 hours, and 48 hours after the adoptive transfer. Mice were euthanized 1 day after final intranasal challenge.

RT-PCR and qRT-PCR

Total RNA was extracted by using TRIzol reagent (ThermoFisher Scientific) and reverse-transcribed using qScript cDNA synthesis kit (Quantabio, Beverly, Mass). For quantitative real-time PCR (qRT-PCR), Taqman real-time PCR assay (ThermoFisher Scientific) was used for gene expression analysis. Gene expression was normalized to housekeeping gene expression (β₂-microglobulin). The relative gene expression was calculated by the change-in-threshold (2^{-ΔCT}) method. All experiments were performed in duplicate in 2 independent experiments; the results are presented as SEMs of biologic replicates. Taqman probes sequences are listed in Table E1 (in the Online Repository available at www.jacionline.org).

Flow cytometric analysis

For cytokine staining, lung or BAL cells were stimulated with phorbol 12-myristate 13-acetate (Sigma-Aldrich) and ionomycin (EMD Millipore, Burlington, Mass) for 2 hours followed by monensin (BioLegend, San Diego, Calif) for a total of 6 hours. After fixation with 1% formaldehyde for 10 minutes at room temperature, cells were washed 2 times with fluorescence-activated cell sorting (FACS) buffer (PBS with 0.5 % BSA). For transcription factor staining, cells were fixed with Foxp3/Transcription Factor Fixation Buffer (ThermoFisher Scientific) at 4°C in dark for 30 minutes or overnight. For cytokine staining and transcription factor combination staining, cells were fixed with 3.7% formaldehyde for 5 minutes at 4°C followed by fixation with Foxp3/Transcription Factor Fixation Buffer at 4°C for 3 hours. Fixed cells were permeabilized with permeabilization buffer (ThermoFisher Scientific) and stained for cytokines and transcription factors with fluorochrome-conjugated antibodies at 4°C in dark for 1 hour. Stained cells were washed 2 times with FACS buffer and resuspended in FACS buffer for flow analysis. The fluorescent antibodies for flow cytometric analysis are listed in Table E2 (in the Online Repository available at www.jacionline.org).

ELISA

IgE capture antibody (BD Bioscience; 553413) was coated on a 96-well plate (NUNC, Roskilde, Denmark) with coating buffer (dH₂O with 0.1 M NaHCO₃, pH 9) and incubated at 4°C overnight. After the plate had been washed 3 times with wash buffer (PBS with 0.05% Tween-20), ELISA buffer (PBS with 2% BSA) was added to the wells and incubated at room temperature for 1 hour. After the plate had been washed 3 times with wash buffer, 10-fold- to 100-fold-diluted samples and standard IgE were added and incubated at

4°C overnight. The next day, after the plate had been washed 3 times with wash buffer, the biotinylated secondary antibody (BioLegend; 406904) was added and incubated at room temperature for 1 hour. After the plate had been washed 3 times with wash buffer, wash buffer with 0.05% of avidin-alkaline phosphatase (Sigma-Aldrich) was added and incubated at room temperature for 1 hour. After the plate had been washed 3 times with wash buffer, substrate buffer with *p*-nitrophenyl phosphate (PNPP) (Sigma-Aldrich) was added to each well. The reaction was stopped by adding stop solution (1N NaOH) and measured with a Biorad Microplate 680 ELISA reader. For HDM-specific IgE and IgG1, ELISA kits (Chondrex, Redmond, Wash) were used.

ChIP

Splenic Treg cells (1×10^6) were stimulated for 3 days before being cross-linked for 15 minutes with 1% formaldehyde at room temperature with rotation. The reaction was quenched by addition of 0.125 M glycine and incubation at room temperature for 5 minutes. Cells were washed with ice-cold PBS 2 times. Fixed cells were lysed in cell lysis buffer on ice for 10 minutes. Chromosomal DNA was fragmented to a size range of 200 to 500 bp by using an ultrasonic processor (Vibra-cell, Newtown, Conn) (30% amplitude for 8 sets of 10-second bursts). After sonication, the debris was removed by centrifugation at 13,000 rpm for 10 minutes at 4°C and the supernatant was transferred to a new tube and diluted 10-fold with chromatin immunoprecipitation (ChIP) dilution buffer. After preclearing, the supernatant was incubated with the ChIP antibodies for H3K27ac (Abcam, Cambridge, United Kingdom) at 4°C overnight with rotation. The following day, immunocomplexes containing antibody, protein, and/or DNA were incubated with protein agarose A beads at 4°C for 2 to 4 hours. Immunocomplexes were washed with low-salt, high-salt, LiCl, and TE buffer. Complexes were incubated with elution buffer (0.1M NaHCO₃) at room temperature for 15 minutes with rotation. After centrifugation at 2000 rpm for 2 minutes at room temperature, the supernatant was transferred to new fresh tube. After the elution step had been repeated, 25 μ L of 4M NaCl was added to 500 μ L of supernatant to reverse the crosslinks at 65°C overnight. The next day, DNA was purified by using phenol-chloroform extraction and resuspended in nuclease-free water and analyzed by quantitative PCR. SYBR green master mix (Applied Biosystems, Foster City, Calif) was used to measure amplification of DNA with a 7500 Fast Real-Time PCR system (Applied Biosystems). After normalization to the input DNA, the amount of output DNA of each target protein was calculated by subtracting that of the IgG control. ChIP antibodies and primer sequences are listed in Table E3 (in the Online Repository available at www.jacionline.org).

RNA sequencing library preparation and sequencing

cDNA libraries were prepared with 1 ng of RNA per sample by using a Clontech SMART-Seq v4 Ultra Low Input RNA Kit (TaKaRa, Shiga, Japan). Libraries were sequenced with a 2×75 -bp paired-end configuration on a HiSeq4000 sequencer (Illumina, San Diego, Calif) by using a HiSeq 3000/4000 PE SBS Kit. A Phred quality score (Q score) was used to measure the quality of sequencing. More than 90% of the sequencing reads reached Q30 (99.9% base call accuracy).

RNA-seq alignment and analysis

The sequencing data were first assessed by using FastQC (Babraham Bioinformatics, Cambridge, United Kingdom) for quality control. All sequenced libraries were then mapped to the mouse genome (University of California Santa Cruz mm10) by using the STAR RNA-sequencing (RNA-seq) aligner.²⁴ The reads distribution across the genome was assessed by using bamutils (from ngsutils).²⁵ Uniquely mapped sequencing reads were assigned to mm10 refGene genes by using featureCounts.²⁶ Differential expression analyses were performed by using edgeR v3.22.3 implemented in the Bioconductor package²⁷ to identify differentially expressed mRNAs between ST2⁺ and ST2⁻ samples. Biologic coefficients of variation between the samples were estimated by using an empiric Bayes approach under the assumption

that the data follow a negative binomial distribution. We filtered out low-expression transcripts based on percentage of samples (<50%) and a count per million cutoff of 0.5. A total of 14,943 mRNAs remained after filtering and were used in the differential expression analysis by edgeR. Statistical significance was defined as a false discovery rate *P* value of .05 or less and a fold change of 2 or more comparing expression level between knockout cells and controls. The heatmap and locus-by-locus volcano plot were generated by using the R software package. Gene set enrichment analysis²⁸ was used for the whole genome functional enrichment analysis based on annotation files from the Molecular Signatures Database immunologic gene sets to identify biologic pathways that were significantly enriched after 3000 permutations.

Heatmap generation and functional enrichment

For visualizing the expression data obtained from the RNA-seq analysis, heatmaps were generated. Normalized counts for the most differentially expressed genes (*P* < .05 and false discovery rate of <0.05) between ST2⁺ and ST2⁻ Treg-cell subtypes were given as input to generate clustered heatmaps based on functional themes using the Morpheus platform²⁹ and heatmapmer.³⁰ Ontology analysis of genes was performed by using gProfiler³¹ to generate summaries of the enriched biologic processes.

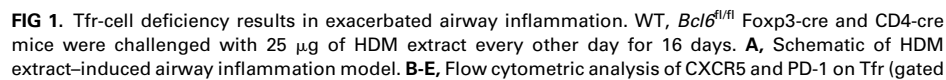
RESULTS

Tfr-cell deficiency results in exacerbated airway inflammation

To define the regulatory effects of Bcl6 deficiency on allergic airway inflammation, wild-type (WT) and *Bcl6*^{fl/fl} mice crossed to either Foxp3-cre or CD4-cre transgenic mice, which had previously been shown to lack Bcl6 in specific cell populations,^{2,32-38} were exposed to HDM extract every other day for 16 days (Fig 1, A). Tfr-cell populations were decreased in the mLN of the *Bcl6*^{fl/fl} Foxp3-cre mice, consistent with a previous report,³² in the absence of altered Treg-cell populations (Fig 1, B and C and see also Fig E1, A in this article's Online Repository at www.jacionline.org). Unexpectedly, the Tfh-cell and GC B-cell populations were also significantly decreased in *Bcl6*^{fl/fl} Foxp3-cre mice (Fig 1, D-G). Despite a marked decrease in GC B cells, *Bcl6*^{fl/fl} Foxp3-cre mice produced increased concentrations of HDM-specific IgE and total IgE compared with WT mice (Fig 1, H). *Bcl6*^{fl/fl} CD4-cre mice do not produce IgE concurrent with the lack of a GC response (Fig 1, D-H). The numbers of total cells, eosinophils, neutrophils, total CD4⁺ T cells, and T_H2 cytokine-producing T cells in the BAL were significantly increased in *Bcl6*^{fl/fl} Foxp3-cre mice compared with in WT and *Bcl6*^{fl/fl} CD4-cre mice (Fig 1, I and J and see also Fig E1, B and E). Similar to in the BAL, *Bcl6*^{fl/fl} Foxp3-cre mice showed increased total cells and T_H2 cytokine-producing T cells in lung tissue (Fig 1, K and see also Fig E1, C and E). However, the numbers of IL-10-producing T cells in the BAL and lungs were similar between the 2 conditional mutant strains (see Fig E1, D). Consistent with a previous report,²³ *Bcl6*^{fl/fl} CD4-cre mice had numbers of BAL cells comparable to those in WT mice, but increased inflammation in lung tissue (Fig 1, I and K) despite impaired IgE production. These data indicated that the lack of Bcl6 in Foxp3-expressing cells resulted in dysregulated type 2 responses and exacerbated airway inflammation.

Bcl6 deficiency in Treg cells leads to increased ST2⁺ Treg-cell development

The mechanism for increased airway inflammation in *Bcl6*^{fl/fl} Foxp3-cre mice was unclear. A previous report demonstrated



that Bcl6-deficient splenic Treg cells expressed more *Il1rl1*, encoding the IL-33 receptor ST2, than WT Treg cells did.²² Moreover, recent studies revealed that ST2⁺ Treg cells in the lung lost their suppressive function and promoted severe airway inflammation in response to IL-33 treatment.³⁹ Therefore, we hypothesized that *Bcl6*^{fl/fl} Foxp3-cre mice might have more ST2⁺ Treg cells that could be responsible for exacerbated airway inflammation. To test this, we analyzed ST2⁺ Treg cells in lungs and mLNs from HDM-challenged mice. Interestingly, both *Bcl6*^{fl/fl} Foxp3-cre and CD4-cre mice had significantly greater percentages of the ST2⁺ Treg-cell population in Treg-cell or total CD4⁺ T-cell populations than WT mice did (Fig 2, A and B). There was an increase in the overall Treg-cell population in the lung in *Bcl6*^{fl/fl} Foxp3-cre mice compared with in WT mice; this included an increase in the ST2⁺ Treg-cell population (Fig 2, C). Moreover, ST2 expression was considerably higher on ST2⁺ Treg cells from *Bcl6*^{fl/fl} Foxp3-cre mice than on those from WT mice (Fig 2, D). ST2⁺ Treg cells are highly activated, and ST2 expression is enhanced by *Gata3*.⁴⁰⁻⁴³ We confirmed higher *Gata3* expression in lung and mLN ST2⁺ Treg cells and an activated phenotype marked by increased expression of CD44 and decreased expression of CD62L compared with in ST2⁺ Treg cells (see Fig E2, A-C in this article's Online Repository at www.jacionline.org).

Consistent with data in Fig 1, J and K, the numbers of ST2⁺ conventional T cells (Tconv cells), which include T_H2 cells in both lungs and mLNs, were increased in *Bcl6*^{fl/fl} Foxp3-cre and CD4-cre mice (Fig 2, E and F). Increased ST2⁺ Treg-cell populations in the lungs and mLNs of *Bcl6*^{fl/fl} Foxp3-cre mice developed in the absence of altered ST2⁺ Tconv cells, and this effect of Bcl6 deficiency in Foxp3-expressing cells was also detected in unchallenged mice (see Fig E2, D). Importantly, in WT mice, Bcl6 expression is mutually exclusive to the ST2⁺ Treg-cell population (see Fig E2, E and F). *Bcl6*^{fl/fl} Foxp3-cre mice had increased ST2⁺ Treg-cell and ST2⁺ Tconv-cell populations in mesenteric lymph nodes compared with those in WT mice (see Fig E2, G), suggesting that the effects of Bcl6 deficiency on the ST2⁺ Treg-cell population were systemic. The majority of ST2⁺ Treg cells in lung express Helios, suggesting that most ST2⁺ Treg cells are thymus-derived Treg cells (see Fig E2, H). These data suggest that Bcl6 negatively regulates ST2⁺ Treg-cell development and that in the absence of Bcl6 there is an accumulation of ST2⁺ Treg cells in the lung during allergic airway inflammation.

Next, we tested whether ST2⁺ Treg-cell development varies with genetic background by comparing ST2⁺ Treg-cell generation in HDM-challenged C57BL/6 and BALB/c mice, the latter of which are more susceptible to allergic inflammation.^{44,45} Although the numbers of ST2⁺ Treg cells and Tconv cells in lung were significantly higher in BALB/c mice, the percentages of these cell types were similar between the groups (see Fig E3, A in this article's Online Repository at www.jacionline.org).

The numbers and percentages of ST2⁺ Treg cells and Tconv cells in mLNs were similar between the groups (see Fig E, B). In spite of the difference between the numbers of ST2⁺ Treg cells and Tconv cells in the lung, the fold changes in the numbers of total lung and mLN cells after HDM challenge were similar between the groups (see Fig E3, C), suggesting that the development and function of ST2⁺ Treg cells are independent of genetic background.

Proinflammatory transcriptional profile of ST2⁺ Treg cells in allergic airway inflammation

To further define the phenotype of ST2⁺ Treg cells in the mLNs that develop in the allergic airway model, we sorted Treg cells from mLNs of HDM-challenged WT mice based on the expression of YFP (Foxp3-reporter) and activation markers to identify Tfr cells (CXCR5⁺ PD-1⁺), ST2⁺ Treg cells (CD44^{high} and CD62L^{low} ST2⁺), ST2⁺ Treg cells (CD44^{high} and CD62L^{low} ST2⁺), and naive Treg cells (CD44^{low} and CD62L^{high}). In Tfr cells, there was a high ratio of *Bcl6* to *Prdm1* (encoding Blimp1) expression that was reversed in ST2⁺ Treg cells (Fig 3, A). We confirmed the Bcl6 gene expression pattern by protein expression (Fig 3, B). This is similar to the correlation between Blimp1 and ST2 expression in visceral adipose tissue (VAT) Treg cells.⁴⁶ Expression of *Bach2*, a repressor of inflammatory cytokine expression in Treg cells,^{47,48} was decreased in ST2⁺ Treg cells. However, the enhanced *Foxp3* expression in ST2⁺ Treg cells suggested that these cells are not intrinsically unstable. *Ccr7*, encoding a chemokine receptor for homing to secondary lymphoid organs,^{49,50} and *Cxcr3*, encoding a chemokine receptor for migrating to sites of type1 inflammation,^{51,52} were significantly decreased in ST2⁺ Treg cells compared with in ST2⁺ Treg cells (Fig 3, A). CCR8-expressing Treg cells are capable of migrating to sites of T_H2 cell-mediated inflammation,⁵³ and *Ccr8* mRNA was significantly enriched in ST2⁺ Treg cells. Importantly, T_H2 cytokine genes were enriched in ST2⁺ Treg cells (Fig 3, C). Although *Il4* expression was greatest in Tfr cells, ST2⁺ Treg cells still expressed *Il4* in greater amounts than other Treg-cell subsets did. Thus, ST2⁺ Treg cells acquired a profile associated with type 2 inflammation.

To further explore the ST2⁺ Treg-cell signature in allergic airway inflammation, we performed RNA-seq on sorted ST2⁺ and ST2⁺ Treg cells isolated from lung. We observed significant differences in the gene expression patterns between the 2 Foxp3⁺ populations (Fig 3, D). Among the significant differences in the ST2⁺ population were increased type 2 cytokine and transcription factor levels and decreased expression of genes encoding RORγt and T-bet (Fig 3, E [left]). We confirmed increased T_H2 cytokine production in ST2⁺ Treg cells at the protein level (see Fig E4, A in this article's Online Repository at www.jacionline.org). We observed similar patterns of differential gene expression when

CD4⁺ Foxp3⁺ cells and Tfr (CD4⁺ Foxp3⁺) cells in mLNs. Representative dot plots (B and D) and average percentage (C and E) of Tfr cells and Tfr cells. (F and G) Flow cytometric analysis of GL7 and Fas on B220 B cells in mLNs. Representative dot plot (F) and average percentage (G) of GC B cells. H, Concentration of HDM-specific IgE, IgG1, and total IgE in the serum, as measured by ELISA. I-K, Numbers of total cells; eosinophils; neutrophils (I); and IL-4-, IL-13-, or IL-17-producing CD4⁺ T cells and eosinophils in the BAL (J) and lung (K). BAL and lung cells were restimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 5 hours to measure cytokine production by using intracellular staining. Cells for flow cytometric analysis were gated for lymphocyte size and granularity and for the expression of CD4. Data are means ± SEMs for 8 to 10 mice per group and representative of 3 independent experiments. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

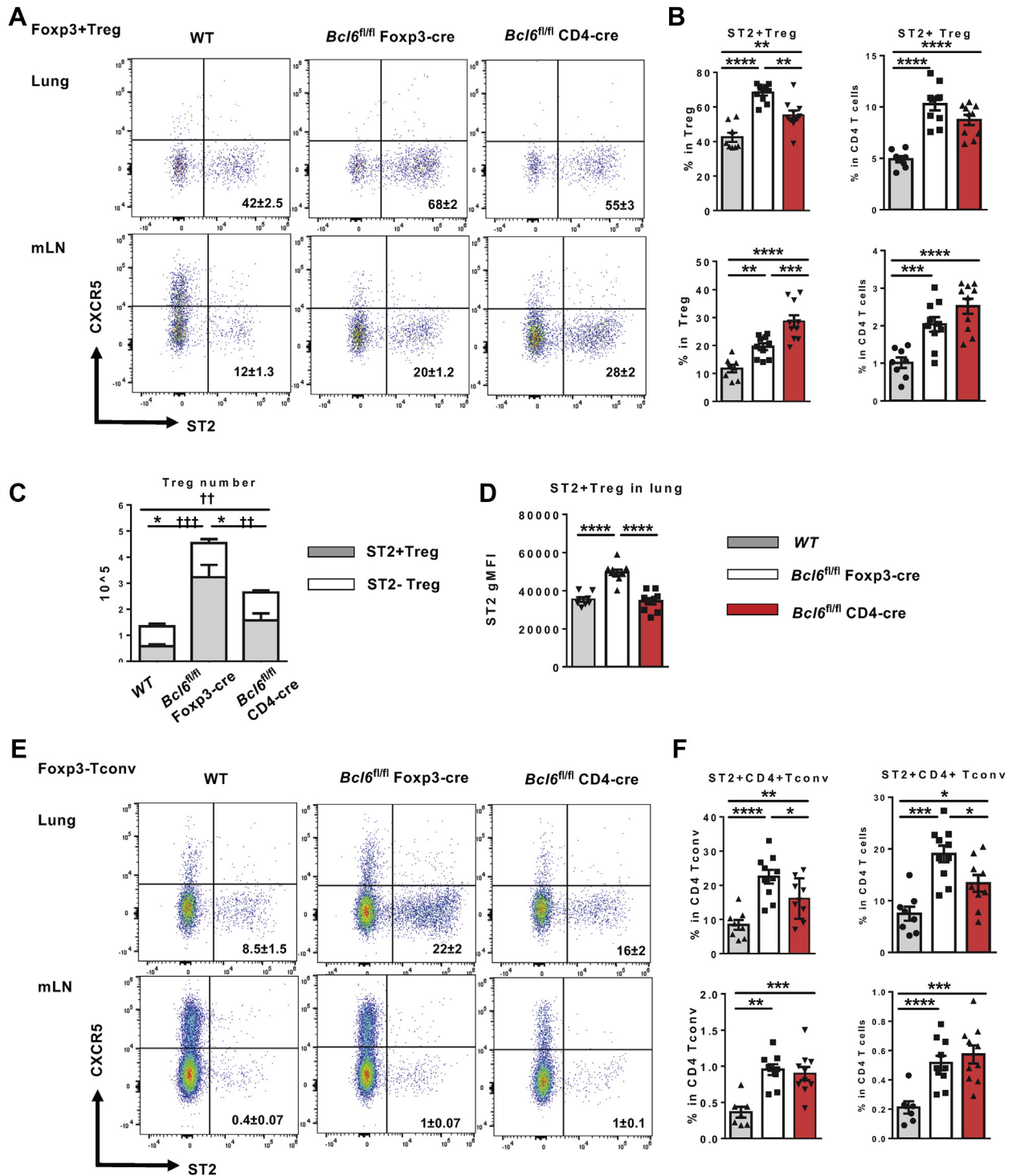


FIG 2. Bcl6 deficiency in Treg cells leads to increased ST2⁺ Treg-cell development. Mice were challenged as in Fig 1. **A** and **B**, Flow cytometric analysis of ST2⁺ Treg cells in lungs and mLNs. **C**, Number of ST2⁺ Treg cells (comparison marked by an asterisk) and ST2⁺ Treg cells (comparison marked by a dagger) in lung. **D**, ST2 geometric mean fluorescence intensity of ST2⁺ Treg cells in lung. **E** and **F**, Flow cytometric analysis of ST2⁺ Tconv cells in lungs and mLNs. Staining for CXCR5 and ST2 is shown on cells gated for lymphocyte size and granularity, CD4, and either Foxp3⁺ (**A** and **B**) or Foxp3⁻ (**E** and **F**) populations. Representative dot plots (**A** and **E**) and average percentages of ST2⁺ Treg cells in Treg cells (left) and in total CD4⁺ T cells (right) (**B**) or ST2⁺ Tconv cells in CD4⁺ Tconv cells (left) and in total CD4⁺ T cells (right) (**F**) are shown. Data are means ± SEMs for 8 to 10 mice per group and representative of 3 independent experiments. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001; ††*P* < .01; †††*P* < .001.

we analyzed ST2⁺ and ST2⁻ Treg cells isolated from the lung by qRT-PCR (see Fig E4, B-D). Gene set enrichment analysis identified the VAT Treg-cell signature in the ST2⁺ Treg cells (Fig 3, F). Indeed, there was a clear distinction in the transcriptional signatures of ST2⁺ and ST2⁻ Treg cells that respectively aligned with VAT Treg cells and peripheral Treg cells (Fig 3, E [right]). Ontology analysis of genes' enrichment in ST2⁺ Treg cells revealed that ST2⁺ Treg cells are more sensitive to environmental cytokines and produce effector cytokines during airway inflammation (Fig 3, G). Together, these data demonstrate that ST2⁺ Treg cells in the context of allergic lung inflammation have a pro-type 2 inflammatory phenotype and share a genetic signature with VAT Treg cells.

Bcl6 inhibits and Blimp1 promotes ST2 expression in Treg cells

To directly test whether Bcl6 is a negative regulator of ST2⁺ Treg-cell development, splenic Treg cells from WT and *Bcl6*^{fl/fl} Foxp3-cre mice were sorted and stimulated in the presence or absence of IL-33 for 3 days. Treatment with IL-33 significantly increased the frequency of ST2⁺KLRG1⁺ Treg cells in WT and Bcl6-deficient cultures (Fig 4, A-C). The induction of ST2 and KLRG1 on Treg cells by IL-33 is consistent with previous reports,⁴⁰⁻⁴³ and double-positive Treg cells were more prevalent in resting or IL-33-treated Bcl6-deficient cultures. Interestingly, IL-33 treatment decreased *Bcl6* expression while increasing *Prdm1* expression (Fig 4, D). Similar to *ex vivo* Treg cells, *in vitro*-cultured Treg (iTreg) cells cultured with IL-33 displayed increased *Il1rl1* expression and concomitantly decreasing *Bcl6* mRNA (see Fig E5, A and B in this article's Online Repository at www.jacionline.org). Bcl6-deficient iTreg cells expressed more *Il1rl1* than in WT iTreg cells under all conditions. We then tested whether the ST2⁺ Treg-cell population responded to exogenous IL-33 by intranasal challenge. In WT and *Bcl6*^{fl/fl} Foxp3-cre mice there was a significant increase in ST2⁺ Treg cells recovered from the lungs compared with untreated mice, as well as a significantly higher percentage in *Bcl6*^{fl/fl} Foxp3-cre mice than in WT mice (Fig 4, E). We then sorted ST2⁺ and ST2⁻ Treg cells from IL-33-treated WT mice and observed enriched *Gata3* expression in ST2⁺ Treg cells and enriched *Bcl6* expression in ST2⁻ Treg cells (see Fig E5, C). Bcl6-deficient splenic Treg cells did not produce T_H2 cytokines in the absence of IL-33 signaling despite expressing comparable levels of *Gata3* in the presence or absence of IL-33 signaling (see Fig E5, D). Treatment of *ex vivo* Treg cells with IL-33 increased the H3K27ac modification at the *Il1rl1* and *Prdm1* gene loci (see Fig E5, E and F). These data suggest that the IL-33/ST2 signaling pathway promotes ST2⁺ Treg-cell development by suppressing Bcl6 and promoting Blimp1 expression. Consistent with a previous report,⁴⁰ *Gata3* expression was not altered after IL-33 treatment but was increased in Bcl6-deficient Treg cells compared with in WT cells, irrespective of IL-33 treatment (Fig 4, D). IL-33 treatment of *ex vivo* Treg cells from BALB/cJ and C57BL/6 mice showed comparable ST2⁺ Treg-cell development (see Fig E5, G and H). Overall, these data suggest that Bcl6 negatively regulates ST2⁺ Treg-cell development by repressing the expression of the ST2⁺ Treg-cell promoting transcription factors *Prdm1* and *Gata3*.

Naive *Il33*^{-/-} mice have normal ST2⁺ Treg-cell numbers and populations in both lymphoid and nonlymphoid tissues.⁴¹ On

the basis of that data, IL-33 may be dispensable for generation of ST2⁺ Treg cells.⁴¹ Similarly, IL-33 failed to induce ST2 expression in ST2⁻ Treg cells.⁴² We then explored potential drivers of ST2⁺ Treg-cell development. The IL-2/STAT5 pathway is critical for development and maintenance of Treg cells⁵⁴⁻⁵⁸ and the IL-2/STAT5/Blimp-1 axis represses Tfr-cell development by inhibiting Bcl6 expression.⁵⁹ In addition to Bcl6 antagonistic functions,^{5,59} the preferential expression of Blimp1 in ST2⁺ Treg cells suggested that Blimp1 might be a key player. To directly test this, we stimulated splenic Treg cells from WT and *Prdm1*^{fl/fl} Foxp3-cre mice with or without IL-33 for 3 days. Interestingly, Blimp1 deficiency nearly eliminated the KLRG1⁺ population and significantly reduced the ST2⁺ population in basal and IL-33-treated conditions (Fig 4, F-H). Consistent with the *in vitro* results, Blimp1-deficient mice had reduced ST2⁺ Treg-cell percentages in naive mice and during HDM-induced airway inflammation (Fig 4, I). Moreover, Bcl6-expressing Treg cells in lung were increased in Blimp1-deficient mice (see Fig E5, I). We confirmed the positive effects of Blimp-1 deficiency on Tfr-cell development in mLNs² (see Fig E5, J and K). These data suggest that Blimp1 positively regulates ST2⁺ Treg-cell development, possibly by antagonizing Bcl6 expression.

Bcl6-deficient ST2⁺ Treg cells promote airway inflammation

To discriminate the effects of Bcl6 deficiency in Treg cells and Tconv cells (which are not Bcl6-deficient in *Bcl6*^{fl/fl} Foxp3-cre mice), we adoptively transferred sorted lung ST2⁺ Treg cells or Tconv cells from WT or *Bcl6*^{fl/fl} Foxp3-cre HDM-challenged mice to naive WT recipient mice. After 3 challenges with HDM, we analyzed the numbers of inflammatory cells in BAL and lung (Fig 5, A). Interestingly, although the total numbers of BAL cells were similar among groups, which is indicative of the acute challenge (Fig 5, B), the mice that received Bcl6-deficient ST2⁺ Treg cells had the greatest increases in the numbers of lung cells, neutrophils, eosinophils, and CD4⁺ T cells whereas the WT ST2⁺ Treg-cell recipient mice had cell numbers comparable with those of the PBS control (Fig 5, C-D). Transferred ST2⁺ Tconv cells did not induce inflammation. In Bcl6-deficient ST2⁺ Treg-cell recipient mice, the vast majority of ST2⁺ donor cells were Foxp3⁺, although a subpopulation lost Foxp3 expression, suggesting that Bcl6-deficient Treg cells might be unstable in this model (Fig 5, E).⁶⁰ These data suggest that Bcl6 plays important roles in regulating both development and function of ST2⁺ Treg cells, which directly mediate airway inflammation.

Although the mechanistic basis of the capacity of Bcl6-deficient ST2⁺ Treg cells to mediate inflammation is unclear, it is likely linked to greater expression of ST2 itself (Fig 2, D) and the effects of Bcl6 on expression of ST2⁺ Treg-cell effector molecules⁶¹ that include *Areg*, *Lilrb4*, and *Ccl3* but not type 2 cytokines such as *Il5* and *Il13* (Fig 5, F). This suggests that the ability of Bcl6-deficient Treg cells to promote inflammation, including neutrophils, is due to increased proinflammatory activity and chemokine production. We also observed that Bcl6 deficiency resulted in increased *Prdm1* in ST2⁻ Treg cells (Fig 5, F), suggesting that Blimp1 might be an important target in the acquisition of ST2 expression.

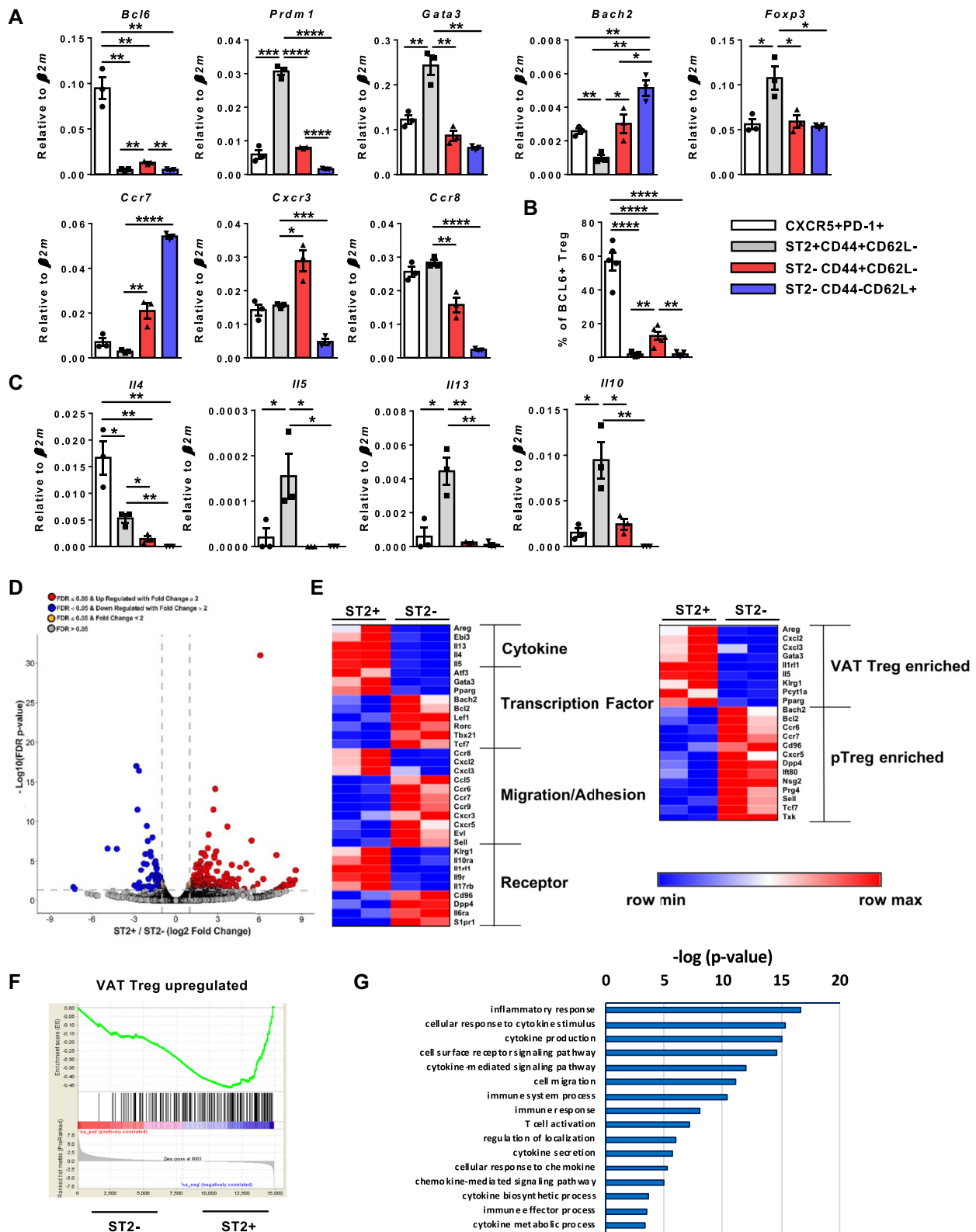


FIG 3. Proinflammatory transcriptional profile of ST2⁺ Treg cells in allergic airway inflammation. Treg cells in mLN of HDM-challenged WT mice were sorted on the basis of expression of CD4, Foxp3-YFP, and activation markers. **A**, TF gene expression was measured by using quantitative PCR. **B**, The Bcl6-expressing Treg-cell population was measured by using intracellular staining. **C**, T_H2 cytokine gene expression was measured by using quantitative PCR. All gene expression was normalized to $\beta 2m$ expression. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. **D-G**, ST2⁺ Treg cells and ST2⁻ Treg cells in lungs of HDM-challenged WT mice were sorted according to expression of

ST2 deficiency compensates for the lack of Bcl6 in Treg cells during allergic airway inflammation

In a previous study, *Il1rl1*^{-/-} mice showed attenuated allergic airway inflammation with impaired GATA3⁺ T_H2-like Treg-cell development.³⁹ The authors suggested that ST2 is indispensable for T_H2-like Treg-cell development. In parallel, our data indicate that ST2 is important for IL-33-mediated Bcl6 repression. Therefore, we questioned whether ST2-deficiency would limit the effects of Bcl6 deficiency in Treg cells during allergic airway inflammation. To answer this question, we generated *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice and induced HDM airway inflammation. As expected, ST2 expression was not detected on Treg cells or Tconv cells in either *Il1rl1*^{-/-} or *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice (see Fig E6, A in this article's Online Repository at www.jacionline.org). ST2-deficient mice also had a defect in the generation of Tfh-cell, Tfr-cell, and GC B-cell development (Fig 6, A). Importantly, ST2 deficiency did not compensate for the lack of Bcl6 in the Tfr-cell population, and the Tfr-cell population in *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice was similar to that in *Bcl6*^{fl/fl} Foxp3-cre mice, but significantly less than that in control mice (Fig 6, A). Accordingly, the increased total and antigen-specific IgE that resulted from Tfr cell deficiency was not compensated by simultaneous deficiency in ST2 (Fig 6, B). These results suggested that *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice maintain the effects of Tfr cell deficiency and provided the opportunity to assess the loss of IL-33 responsiveness on the increased allergic airway inflammation in *Bcl6*^{fl/fl} Foxp3-cre mice.

To test this, we analyzed airway inflammation and observed that *Il1rl1*^{-/-} and *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice have comparable numbers of cells in the BAL and lung to those in WT mice (Fig 6, C and D). The numbers of IL-13-producing CD4⁺ and GATA3⁺ Tconv cells were significantly decreased in *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice compared with those in *Bcl6*^{fl/fl} Foxp3-cre mice and similar to the levels observed in WT mice (Fig 6, E and F). Despite diminished inflammation and T_H2 cytokine production, lung Treg cells from *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice expressed more GATA3 than did Treg cells from WT mice (Fig 6, G), suggesting that Bcl6 is required for Gata3 repression. Importantly, the percentages of cells that were Foxp3⁺ KLRG1⁺ (a surrogate for ST2 because the majority of KLRG1⁺ Treg cells are also ST2⁺) were similar in the absence ST2 (see Fig E6, B and C). This suggests that the diminished inflammation in *Il1rl1*^{-/-} and *Il1rl1*^{-/-}*Bcl6*^{fl/fl} Foxp3-cre mice is due to the lack of ST2 but not to the lack of a Treg-cell compartment. Together, these data suggest that the absence of ST2 attenuates the exacerbated allergic airway inflammation that occurs in mice with Bcl6-deficient Treg cells and distinguishes the effects of Bcl6 in Tfr cells from the effects in Treg cells that control allergic inflammation.

DISCUSSION

Bcl6-expressing Tfr cells are important for the humoral immune response through regulation of GC reactions.⁸⁻¹⁰

Depending on the inflammatory milieu, Tfr cells can function as repressors or helpers of the GC reaction.¹⁸ Dysregulated functions of Tfr cells can lead to autoimmune diseases.⁶² Yet, the function of Bcl6 in Treg cells in the context of allergic airway inflammation is not fully understood. In this article, we have defined critical roles for Tfr cells in regulating the production of antigen-specific IgE in an HDM extract-induced airway inflammation model. This observation confirms similar observations regarding the function of Tfr cells in controlling IgE that were made in a recent report using a distinct genetic model.⁶³

Our article defines an additional role for the balance of Bcl6 and Blimp-1 activity in Treg cells. Bcl6 represses and Blimp-1 activates ST2 expression in Treg cells in a cell-intrinsic manner. The adoptive transfer studies highlight the cell-intrinsic nature of this phenomenon because Bcl6 is deleted only in Foxp3-expressing cells in this model and would not affect the function of Tconv cells. The data suggest that Bcl6 is important in ST2⁻ Treg cells to maintain the ST2⁻ phenotype. In the absence of Bcl6, Blimp-1 expression is increased and there is an increase in the percentage of ST2⁺ Treg cells, as well as an increase in the amount of ST2 expressed per Treg cell. The ST2^{high} Treg cells would be more sensitive to an allergic environment, and with increased expression of other effector molecules, Bcl6-deficient Treg cells are potent at promoting allergic inflammation. The ability of Bcl6-deficient Treg cells to recruit additional effectors of allergic inflammation further amplifies this activity. It is also possible that increased ST2 expression decreases the stability of the Treg cells and an inflammatory environment converts them into ST2⁺ Tconv cells that could also potentially affect the generation of inflammation.

There has been significant characterization of the ST2-expressing Treg-cell population in the colon and lung.^{40,41,43} ST2⁺ Treg cells are highly activated T_H2-like Treg cells that retain Foxp3 expression and have increased GATA3 and production of T_H2 cytokines in response to IL-33. IL-33 enhances the differentiation, accumulation, and maintenance of ST2⁺ Treg cells in inflamed tissues.⁴⁰⁻⁴² ST2⁺ Treg cells are effective in suppressing T_H1 or T_H17 cell inflammation in a colitis model and T-cell proliferation, but they fail to suppress, and potentially exacerbate, T_H2-type responses in an allergic airway disease model.^{39,40,61} In support of a key role for Bcl6 in controlling this Treg-cell population, Treg cells from germline *Bcl6*-deficient mice have increased ST2 expression, suppress T_H1/T_H17 cell-driven colitis, and inhibit T-cell proliferation but promote T_H2-type allergic airway inflammation.²² Human ST2⁺ Treg cells are found in the skin of patients with systemic sclerosis.⁶⁴ Similar to murine ST2⁺ Treg cells, these human ST2⁺ Treg cells and *in vitro*-cultured human Treg cells produce T_H2 cytokines in response to IL-33.^{64,65} Moreover, human Treg cells from HDM-sensitized children with asthma have suppressive function on T_H1 cells but fail to suppress allergen-specific T_H2 cytokine expression and amplify T_H2 cytokines in response to IL-33.⁶⁶

CD4, Foxp3-YFP, and ST2. **B-E**, Analysis of RNA-seq of sorted ST2⁺ and ST2⁻ Treg cells (each from 2 biologic samples) from lung tissues of HDM-challenged WT mice. **D**, Volcano plot comparing gene expression in ST2⁺ Treg cells with that in ST2⁻ Treg cells. **E**, Heatmaps showing differential expression of genes related to functions (*left*) and genes aligned with VAT Treg cells and peripheral regulatory Treg (pTreg) cells (*right*). **F**, Gene set enrichment analysis of ST2⁺ Treg cells versus ST2⁻ Treg cells and VAT Treg cells versus pTreg cells gene list. **G**, Ontology analysis of gene enrichment in biologic process in ST2⁺ Treg cells.

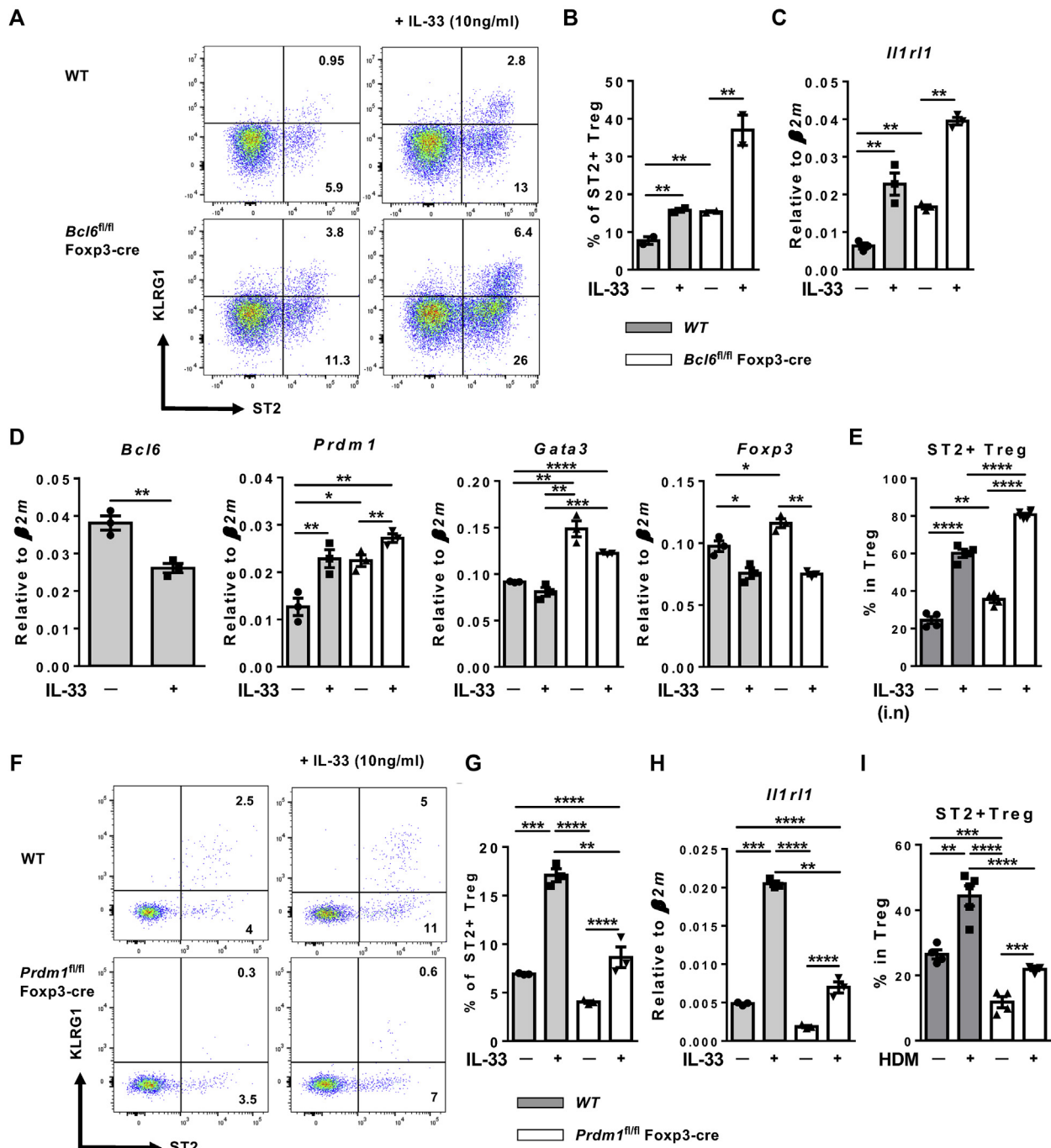


FIG 4. Bcl6 inhibits and Blimp1 promotes ST2 expression in Treg cells. Splenic Treg cells from WT and *Bcl6^{fl/fl}* Foxp3-cre mice were sorted and stimulated with anti-CD3, anti-CD28, and IL-2 with or without IL-33 (10 ng/mL) for 3 days. Representative dot plots (A) and average percentage (B) of ST2⁺ Treg cells. C and D, Quantitative PCR analysis of *Il1rl1* and transcription factor gene expression. E, WT and *Bcl6^{fl/fl}* Foxp3-cre mice were challenged intranasally with 0.5 μ g of IL-33 for 3 consecutive days. Average percentage of ST2⁺ Treg cells in lung. F-H, Splenic Treg cells from WT and *Prdm1^{fl/fl}* Foxp3-cre mice were sorted and stimulated with anti-CD3, anti-CD28, and IL-2 with or without IL-33 (10 ng/mL) for 3 days. Representative dot plots (F) and average percentages (G) of ST2⁺ Treg cells. H, Quantitative PCR analysis of *Il1rl1*. I, WT and *Prdm1^{fl/fl}* Foxp3-cre mice were HDM-challenged as in Fig 1. Average percentage of ST2⁺ Treg cells in lung. C, D, and H, Gene expression was normalized to $\beta 2m$ expression. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. Data are means $\pm$ SEMs for 3 to 5 mice per group and representative of 2 independent experiments.

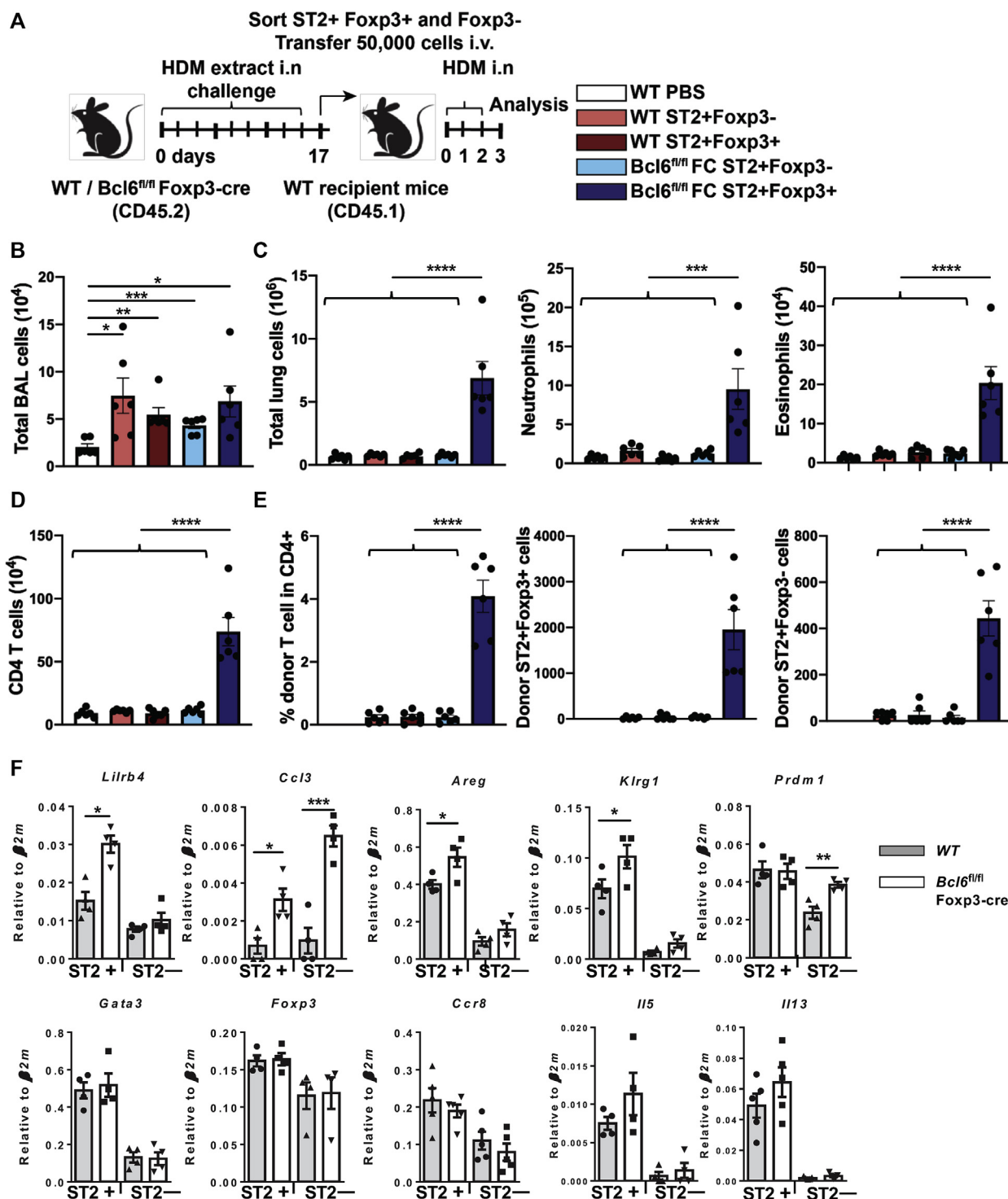


FIG 5. ST2⁺ Treg cells promote airway inflammation. ST2⁺ Treg cells and Tconv cells were sorted from the lungs of HDM-challenged WT or *Bcl6*^{fl/fl} Foxp3-cre and adoptively transferred to recipient mice. Following transfer, mice were challenged 3 times with HDM. **A**, Schematic adoptive transfer experiment. **B**, Total BAL cells. **C** and **D**, Total lung cells, neutrophils, and eosinophils (**C**) and total CD4⁺ T cells (**D**) in lung. **E**, Percentage of donor CD4⁺ T cells, ST2⁺ Treg cells, and ST2⁺ Tconv cells in lungs of recipient mice. **F**, ST2⁺ and ST2⁻ Treg cells in lungs of HDM-challenged WT or *Bcl6*^{fl/fl} Foxp3-cre mice were sorted on the basis of expression of CD4, Foxp3-YFP, and ST2. Gene expression was measured by using quantitative PCR and normalized to $\beta 2m$ expression. **B**, A Student unpaired 2-tailed *t* test was used to compare WT PBS with other groups. **C-E**, One-way ANOVA with a *post hoc* Tukey test was used to generate *P* values for multiple comparisons. ****P* < .001; *****P* < .0001. Data are means $\pm$ SEMs for 6 mice per group pooled from separate analyses. **F**, A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < 0.05; ***P* < .01; ****P* < .001; *****P* < .0001.

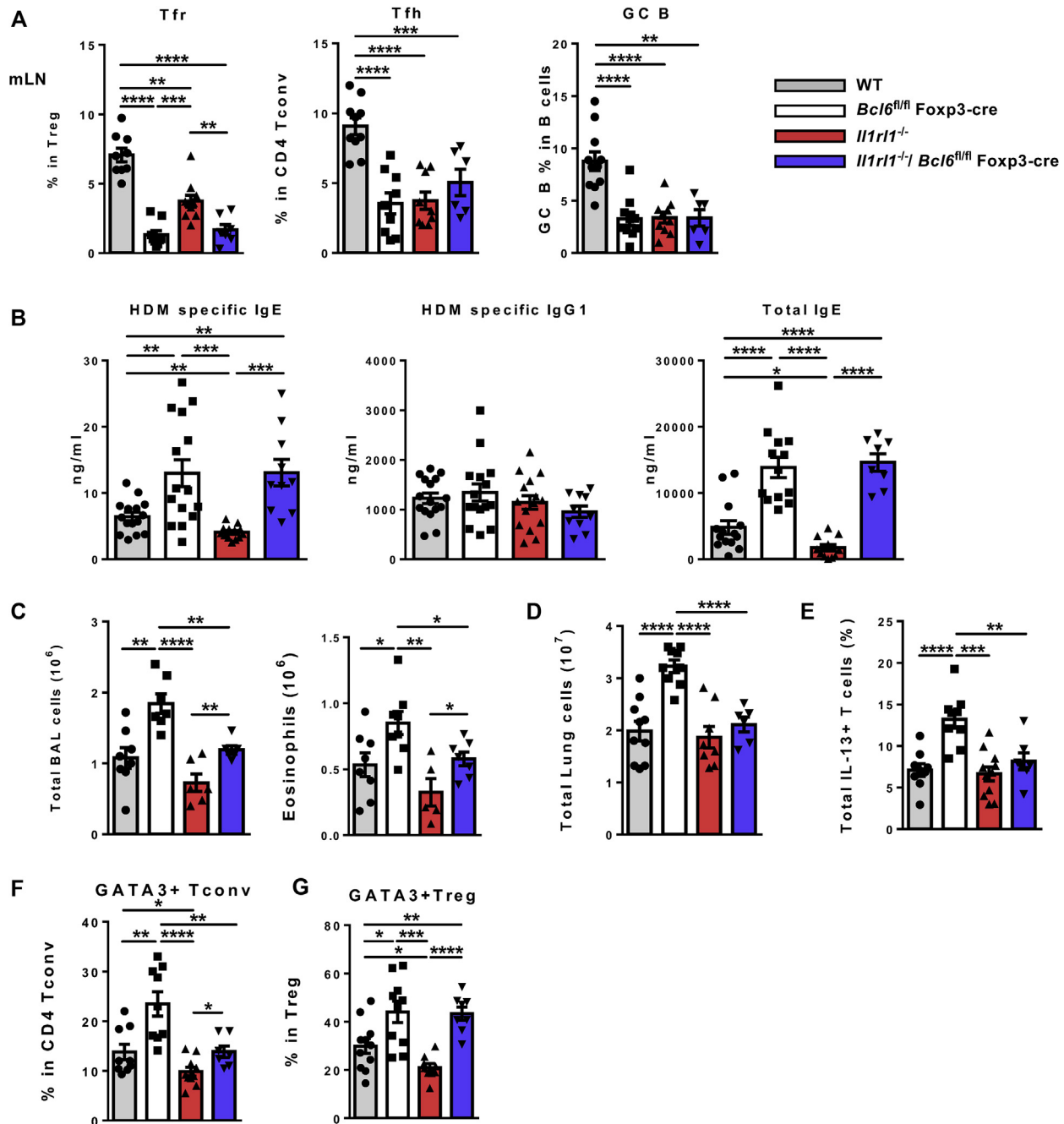


FIG 6. ST2 deficiency compensates for the lack of Bcl6 in Treg cells during allergic airway inflammation. WT, *Bcl6^{fl/fl}* Foxp3-cre, *Il1rl1^{-/-}*, and *Il1rl1^{-/-}* *Bcl6^{fl/fl}* Foxp3-cre mice were challenged as in Fig 1. **A**, Flow cytometric analysis of Tfr cells, Tfh cells, and GC B cells in mLN. **B**, Concentration of HDM-specific IgE and IgG1 and total IgE in the serum measured by ELISA. Number of total BAL cells and eosinophils (**C**), lung cells (**D**), percentage of IL-13-producing CD4⁺ T cells (**E**), and GATA3-expressing Tconv cells (**F**) in lung tissues. **G**, GATA3-expressing Treg cells in lung. Lung cells were restimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 5 hours to measure cytokine production by using intracellular staining. Cells for flow cytometric analysis were gated for lymphocyte size and granularity and for the expression of CD4. Data are means $\pm$ SEMs for 8 to 10 mice per group and representative of 3 independent experiments. The Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

However, the mechanisms controlling ST2⁺ Treg-cell development are still not fully defined.

In the RNA-seq analysis we identified a transcriptional signature associated with the ST2⁺ Treg cells in allergic lung inflammation. There is an enrichment of genes associated with

type 2 immunity in the ST2⁺ Treg-cell population, whereas genes associated with type 1 and type 17 immunity are enhanced in the ST2⁻ Treg cells. There is remarkable overlap between the transcriptional signature in the allergic ST2⁺ Treg cells and the VAT ST2⁺ Treg cells. Whereas VAT ST2⁺ Treg cells maintain

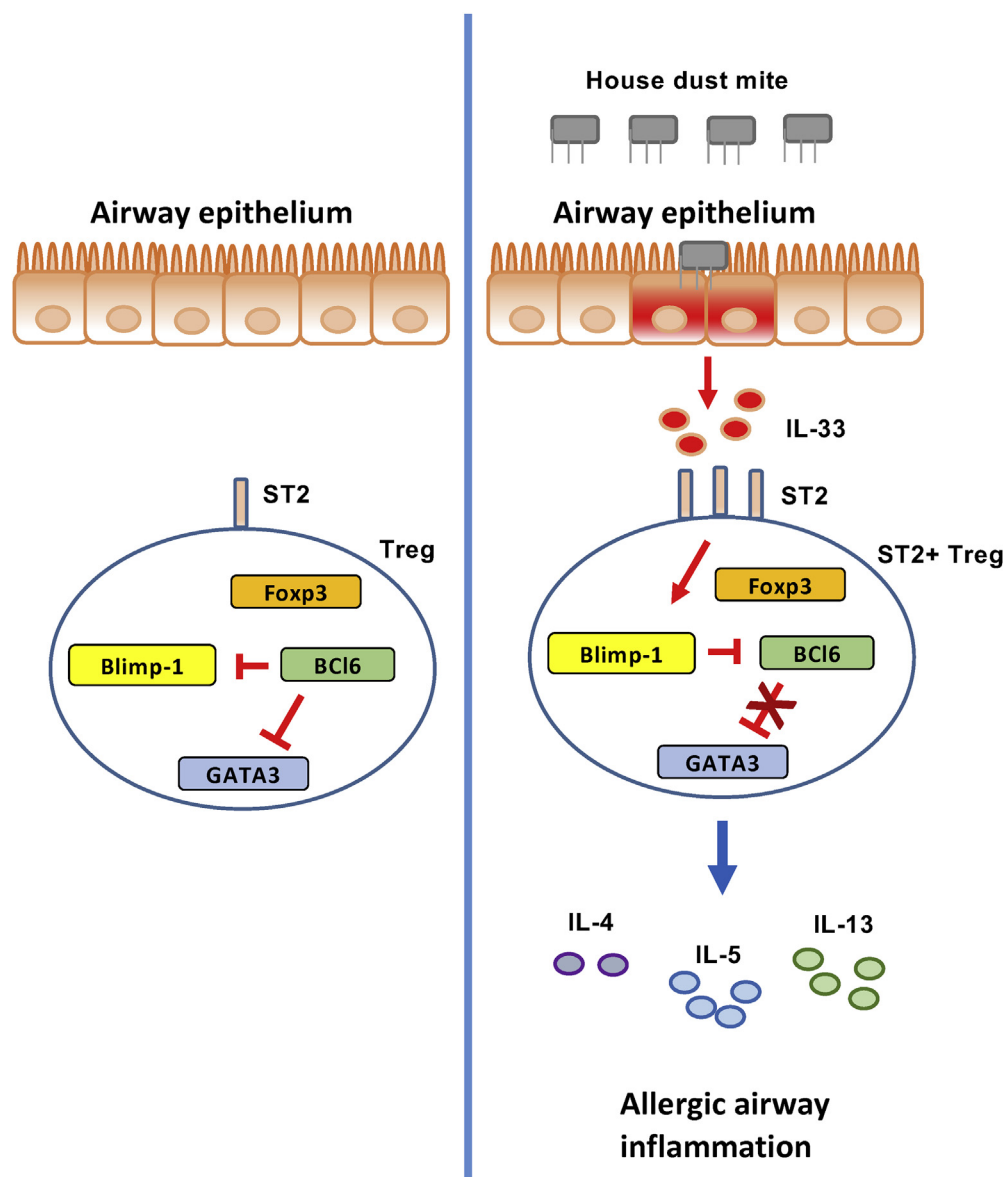


FIG 7. Schematic diagram to describe the roles of Bcl6 in the regulation of IL-33-mediated ST2⁺ Treg-cell development exacerbating allergic airway inflammation.

insulin sensitivity through control of inflammation in adipose tissue,⁶⁷ ST2⁺ Treg cells in lung promote allergic airway inflammation.³⁹ The function of VAT ST2⁺ Treg cells might be to promote modest type 2 inflammation, but we have not examined whether this might be amplified in the *Bcl6*^{fl/fl} Foxp3-Cre mice. Whether ST2⁺ Treg cells control cellular metabolism in the lung also remains to be tested. The development and accumulation of VAT Treg cells depend on PPAR- γ , which is regulated by the IL-33/ST2 pathway and mediated by IRF4 and BATF expression.⁴⁶ The IL-33/ST2 pathway subsequently activates GATA3, leading to expansion of ST2⁺ Treg cells and expression of T_H2 cytokines.^{40,41} However, naive *Il33*-deficient mice develop ST2⁺ Treg cells,⁴¹ suggesting that there are unidentified signaling pathways for ST2⁺ Treg-cell development. In this study, we defined Blimp1 as a key player for ST2⁺ Treg-cell development. ST2 is preferentially expressed in Blimp1⁺ Treg cells,⁴⁶ and we confirmed preferential expression of Blimp1 in ST2⁺ Treg cells.

Blimp1 deficiency impairs ST2⁺ Treg-cell development *in vitro* and *in vivo*. Although there is a decreased number of ST2⁺ Treg cells in *Prdm1*^{fl/fl} Foxp3-Cre mice, these mice have airway inflammation to comparable to that of WT mice (data not shown). This observation could be due to several factors, including Blimp1 playing a critical role in the functions of Treg cells⁶⁸ and the fact that T_H2 cytokine production is already very low in WT Treg cells and cannot be lowered further by the absence of Blimp1. Blimp1 is a well-known repressor of Bcl6 transcription, and increased Bcl6 expression in Blimp1-deficient Treg cells likely contributes to the decreased development of ST2⁺ Treg cells from Blimp1-deficient Treg cells. Bcl6 negatively regulates a subset of genes associated with the ST2⁺ Treg cell subset, including *Prdm1* and *Il1rl1*, both of which contribute to the ST2⁺ Treg-cell phenotype. Thus, Bcl6 and Blimp1 are reciprocal regulators of Treg-cell fate, similar to their reciprocal regulation of T_H-cell and B-cell differentiation.

The functions of Bcl6 in regulating type 2 responses are complex. One of the first observations in *Bcl6*^{-/-} mice was an increase in T_H2 responses.^{19,20} However, this occurs through Bcl6 function in a number of cell types. One potential mechanism is through the repression of GATA3, which has been observed in conventional and Treg cells.^{22,69} In our studies using mice that lacked Bcl6 specifically in Foxp3-expressing cells, we did not observe differences in GATA3 and ST2 expression in the ST2⁺ Treg-cell population. Rather, we observed expansion of the ST2⁺ Treg-cell population. Moreover, adoptively transferred Bcl6-deficient ST2⁺ Treg cells induced airway inflammation in recipient mice after limited challenges, whereas ST2⁺ Bcl6-deficient Tconv cells or either subset of WT ST2⁺ T cells did not. These data suggest that Bcl6 is important for the suppressive function of ST2⁺ Treg cells, and this study provides a first step to defining the mechanism. As deletion of ST2 normalized the increased T_H2 inflammation observed in the *Bcl6*^{fl/fl} Foxp3-Cre mice, it suggests that the IL-33/ST2 pathway represents a major Bcl6 target that contributes to the control of allergic inflammation.

The effects of ST2 deficiency can vary with the model examined. In our study, ST2 deficiency did not alter overall inflammation or T_H2 cells compared with that in control mice. In the Ova/Alum model, ST2-deficient mice developed attenuated airway inflammation that was linked to impaired DC activation.⁷⁰ In the HDM model, ST2-deficient mice showed varying phenotypes depending on the dose of HDM, sensitization protocol, and analysis time point after last challenge. In a chronic HDM model, reduced airway remodeling, airway hyperresponsiveness, and airway inflammation were observed in ST2-deficient mice.⁷¹ However, in another HDM model, TSLP and IL-25 compensate for the loss of ST2 and diminished T_H2 cells by expanding ILC2.^{72,73} Thus, the effects of IL-33 signaling are sensitive to the context of the response. Regardless, the lack of a phenotype of ST2 deficiency in our studies allowed us to examine the effects in the context of cell-specific Bcl6 deficiency. ST2 deficiency in our studies resulted in decreased GC responses. This could be linked to the effects of ST2 deficiency on DC function^{70,74} rather than to an intrinsic effect in Tfr cells.

Our studies further define the complex roles of Tfr cells in regulating antibody responses, particularly IgE. Tfr-cell deficiency did not alter influenza-specific IgG production in an influenza infection model.^{59,62} However, Tfr cells suppress antigen-specific B-cell function in several models.⁸⁻¹⁰ In our HDM-induced airway inflammation model, Treg-cell-specific Bcl6-deficient mice (*Bcl6*^{fl/fl} Foxp3-cre) similarly had reduced Tfr-cell numbers but produced higher concentrations of HDM-specific IgE and total IgE than did WT mice while producing comparable concentrations of HDM-specific IgG1. These data suggest that Tfr cells function as a repressor of IgE production. A recent report based on a study using an approach to delete Foxp3- and Cxcr5-expressing cells suggested that Tfr cells also limit allergic inflammation.⁶³ This raises another important aspect of these 2 models in comparing the deletion of Tfr cells versus the loss of Tfr-cell and other Treg-cell functions by genetic deletion of Bcl6. Importantly, the effects of Bcl6 in the Tfr-cell and Treg-cell compartments are distinguished by the effects of ST2 deficiency, as Tfr cells are ST2⁻. Comparing these and other models will be required to refine the distinct function of Tfr cells and ST2⁺ Treg cells.

In this article we have defined specific functions for Bcl6 in the Foxp3-expressing CD4⁺ T-cell subset (Fig 7). In the context of HDM-induced allergic airway inflammation, Bcl6 is required for Tfr cells to develop and repress IgE production. In Treg cells, Bcl6 represses ST2, Gata3, and Blimp1, and in the absence of Bcl6 there is an expansion of the ST2⁺ Treg-cell population, increased T_H2 cell development, and increased allergic airway inflammation. Importantly, codeficiency of ST2 and Bcl6 distinguishes these 2 activities of Bcl6 in Foxp3-expressing cells by normalizing the airway inflammation without having an effect on the production of IgE. The presence of the KLRG1⁺ Treg-cell population in the ST2-deficient mice suggests that the impact is specifically from IL-33 signaling rather than from a loss of the entire population. Each of these functions will be important to understand the detailed mechanisms of allergic airway inflammation.

Key messages

- Tfr cells limit IgE production in mice challenged by airway allergen.
- Bcl6 and Blimp1 reciprocally regulate ST2⁺ Treg-cell development.
- ST2⁺ Treg cells promote allergic airway inflammation.

REFERENCES

1. Basso K, Dalla-Favera R. BCL6: Master regulator of the germinal center reaction and key oncogene in B cell lymphomagenesis. *Adv Immunol* 2010;105:193-210.
2. Xie MM, Koh BH, Hollister K, Wu H, Sun J, Kaplan MH, et al. Bcl6 promotes follicular helper T-cell differentiation and PD-1 expression in a Blimp1-independent manner in mice. *Eur J Immunol* 2017;47:1136-41.
3. Hatzi K, Nance JP, Kroenke MA, Bothwell M, Haddad EK, Melnick A, et al. BCL6 orchestrates Tfh cell differentiation via multiple distinct mechanisms. *J Exp Med* 2015;212:539-53.
4. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, et al. Bcl6 mediates the development of T follicular helper cells. *Science* 2009;325:1001-5.
5. Johnston RJ, Poholek AC, DiToro D, Yusuf I, Eto D, Barnett B, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 2009;325:1006-10.
6. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 2009;31:457-68.
7. Yu RY, Wang X, Pixley FJ, Yu JJ, Dent AL, Broxmeyer HE, et al. BCL-6 negatively regulates macrophage proliferation by suppressing autocrine IL-6 production. *Blood* 2005;105:1777-84.
8. Wollenberg I, Agua-Doce A, Hernandez A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. *J Immunol* 2011;187:4553-60.
9. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat Med* 2011;17:975-82.
10. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, et al. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 2011;17:983-8.
11. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. *Nat Immunol* 2010;11:114-20.
12. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* 2009;30:324-35.
13. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 2009;206:561-76.
14. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. *J Exp Med* 2008;205:2873-86.

15. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* 2005;435:452-8.
16. Sage PT, Alvarez D, Godec J, von Andrian UH, Sharpe AH. Circulating T follicular regulatory and helper cells have memory-like properties. *J Clin Invest* 2014;124:5191-204.
17. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor CTLA-4 controls B cell responses by modulating T follicular helper, T follicular regulatory, and T regulatory cells. *Immunity* 2014;41:1026-39.
18. Laidlaw BJ, Lu Y, Amezcua RA, Weinstein JS, Vander Heiden JA, Gupta NT, et al. Interleukin-10 from CD4(+) follicular regulatory T cells promotes the germinal center response. *Sci Immunol* 2017;2.
19. Dent AL, Hu-Li J, Paul WE, Staudt LM. T helper type 2 inflammatory disease in the absence of interleukin 4 and transcription factor STAT6. *Proc Natl Acad Sci U S A* 1998;95:13823-8.
20. Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science* 1997;276:589-92.
21. Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, et al. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat Genet* 1997;16:161-70.
22. Sawant DV, Sehra S, Nguyen ET, Jadhav R, Englert K, Shinnakasu R, et al. Bcl6 controls the Th2 inflammatory activity of regulatory T cells by repressing Gata3 function. *J Immunol* 2012;189:4759-69.
23. Kobayashi T, Iijima K, Dent AL, Kita H. Follicular helper T cells mediate IgE antibody response to airborne allergens. *J Allergy Clin Immunol* 2017;139:300-13.e7.
24. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013;29:15-21.
25. Breese MR, Liu Y. NGSUtils: a software suite for analyzing and manipulating next-generation sequencing datasets. *Bioinformatics* 2013;29:494-6.
26. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 2014;30:923-30.
27. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* 2012;40:4288-97.
28. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;102:15545-50.
29. Starruss J, de Back W, Brusch L, Deutsch A. Morpheus: a user-friendly modeling environment for multiscale and multicellular systems biology. *Bioinformatics* 2014;30:1331-2.
30. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmaper: web-enabled heat mapping for all. *Nucleic Acids Res* 2016;44:W147-53.
31. Reimand J, Kull M, Peterson H, Hansen J, Vilo J. g:Profiler—a web-based toolset for functional profiling of gene lists from large-scale experiments. *Nucleic Acids Res* 2007;35:W193-200.
32. Wu H, Chen Y, Liu H, Xu LL, Teuscher P, Wang S, et al. Follicular regulatory T cells repress cytokine production by follicular helper T cells and optimize IgG responses in mice. *Eur J Immunol* 2016;46:1152-61.
33. Xie MM, Dent AL. Unexpected help: follicular regulatory T cells in the germinal center. *Front Immunol* 2018;9:1536.
34. Xie MM, Fang S, Chen Q, Liu H, Wan J, Dent AL. Follicular regulatory T cells inhibit the development of granzyme B-expressing follicular helper T cells. *JCI Insight* 2019;4.
35. Hollister K, Chen Y, Wang S, Wu H, Mondal A, Clegg N, et al. The role of follicular helper T cells and the germinal center in HIV-1 gp120 DNA prime and gp120 protein boost vaccination. *Hum Vaccin Immunother* 2014;10:1985-92.
36. Hollister K, Kusam S, Wu H, Clegg N, Mondal A, Sawant DV, et al. Insights into the role of Bcl6 in follicular Th cells using a new conditional mutant mouse model. *J Immunol* 2013;191:3705-11.
37. Xie MM, Liu H, Corn C, Koh BH, Kaplan MH, Turner MJ, et al. Roles of T follicular helper cells and T follicular regulatory cells in autoantibody production in IL-2-deficient mice. *Immunohorizons* 2019;3:306-16.
38. Wu H, Xie MM, Liu H, Dent AL. Stat3 is important for follicular regulatory T cell differentiation. *PLoS One* 2016;11:e0155040.
39. Chen CC, Kobayashi T, Iijima K, Hsu FC, Kita H. IL-33 dysregulates regulatory T cells and impairs established immunologic tolerance in the lungs. *J Allergy Clin Immunol* 2017;140:1351-63.e7.
40. Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* 2014;513:564-8.
41. Siede J, Frohlich A, Datsi A, Hegazy AN, Varga DV, Holeciska V, et al. IL-33 Receptor-expressing regulatory T cells are highly activated, Th2 biased and suppress CD4 T cell proliferation through IL-10 and TGFbeta release. *PLoS One* 2016;11:e0161507.
42. Matta BM, Reichenbach DK, Zhang X, Mathews L, Koehn BH, Dwyer GK, et al. Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD. *Blood* 2016;128:427-39.
43. Yang J, Ramadan A, Reichenbach DK, Loschi M, Zhang J, Griesenauer B, et al. Rorc restrains the potency of ST2+ regulatory T cells in ameliorating intestinal graft-versus-host disease. *JCI Insight* 2019;4.
44. Singh B, Shinagawa K, Taube C, Gelfand EW, Pabst R. Strain-specific differences in perivascular inflammation in lungs in two murine models of allergic airway inflammation. *Clin Exp Immunol* 2005;141:223-9.
45. Page K, Lierl KM, Herman N, Wills-Karp M. Differences in susceptibility to German cockroach frass and its associated proteases in induced allergic inflammation in mice. *Respir Res* 2007;8:91.
46. Vasanthakumar A, Moro K, Xin A, Liao Y, Gloury R, Kawamoto S, et al. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nat Immunol* 2015;16:276-85.
47. Kim EH, Gasper DJ, Lee SH, Plisch EH, Svaren J, Suresh M. Bach2 regulates homeostasis of Foxp3+ regulatory T cells and protects against fatal lung disease in mice. *J Immunol* 2014;192:985-95.
48. Roychoudhuri R, Hirahara K, Mousavi K, Clever D, Klebanoff CA, Bonelli M, et al. BACH2 represses effector programs to stabilize T(reg)-mediated immune homeostasis. *Nature* 2013;498:506-10.
49. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999;99:23-33.
50. Schneider MA, Meingassner JG, Lipp M, Moore HD, Rot A. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. *J Exp Med* 2007;204:735-45.
51. Hall AO, Beiting DP, Tato C, John B, Oldenhove G, Lombana CG, et al. The cytokines interleukin 27 and interferon-gamma promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* 2012;37:511-23.
52. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* 2009;10:595-602.
53. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature* 2009;458:351-6.
54. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity* 2008;28:100-11.
55. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, et al. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 2007;109:4368-75.
56. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* 2007;178:280-90.
57. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 2005;6:1142-51.
58. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 2005;6:1152-9.
59. Botta D, Fuller MJ, Marquez-Lago TT, Bachus H, Bradley JE, Weinmann AS, et al. Dynamic regulation of T follicular regulatory cell responses by interleukin 2 during influenza infection. *Nat Immunol* 2017;18:1249-60.
60. Sawant DV, Wu H, Yao W, Sehra S, Kaplan MH, Dent AL. The transcriptional repressor Bcl6 controls the stability of regulatory T cells by intrinsic and extrinsic pathways. *Immunology* 2015;145:11-23.
61. Delacher M, Schmidl C, Herzig Y, Breloer M, Hartmann W, Brunk F, et al. Rbpj expression in regulatory T cells is critical for restraining TH2 responses. *Nat Commun* 2019;10:1621.
62. Fu W, Liu X, Lin X, Feng H, Sun L, Li S, et al. Deficiency in T follicular regulatory cells promotes autoimmunity. *J Exp Med* 2018;215:815-25.
63. Clement RL, Daccache J, Mohammed MT, Diallo A, Blazar BR, Kuchroo VK, et al. Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. *Nat Immunol* 2019;20:1360-71.
64. MacDonald KG, Dawson NA, Huang Q, Dunne JV, Levings MK, Broady R. Regulatory T cells produce profibrotic cytokines in the skin of patients with systemic sclerosis. *J Allergy Clin Immunol* 2015;135:946-e9.
65. Liu Q, Dwyer GK, Zhao Y, Li H, Mathews LR, Chakka AB, et al. IL-33-mediated IL-13 secretion by ST2+ Tregs controls inflammation after lung injury. *JCI Insight* 2019;4(6).
66. Reubsaet L, Meerding J, Giezeman R, de Kleer I, Arets B, Prakken B, et al. Der p 1-induced CD4(+)FOXP3(+)GATA3(+) T cells have suppressive properties and contribute to the polarization of the TH2-associated response. *J Allergy Clin Immunol* 2013;132:1440-4.

67. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* 2009;15:930-9.
68. Garg G, Muschaweckh A, Moreno H, Vasanthakumar A, Floess S, Lepenietier G, et al. Blimp1 Prevents Methylation of Foxp3 and Loss of Regulatory T Cell Identity at Sites of Inflammation. *Cell Rep* 2019;26:1854-68.e5.
69. Kusam S, Toney LM, Sato H, Dent AL. Inhibition of Th2 differentiation and GATA-3 expression by BCL-6. *J Immunol* 2003;170:2435-41.
70. Besnard AG, Togbe D, Guillou N, Erard F, Quesniaux V, Ryffel B. IL-33-activated dendritic cells are critical for allergic airway inflammation. *Eur J Immunol* 2011;41:1675-86.
71. Zoltowska AM, Lei Y, Fuchs B, Rask C, Adner M, Nilsson GP. The interleukin-33 receptor ST2 is important for the development of peripheral airway hyperresponsiveness and inflammation in a house dust mite mouse model of asthma. *Clin Exp Allergy* 2016;46:479-90.
72. Vannella KM, Ramalingam TR, Borthwick LA, Barron L, Hart KM, Thompson RW, et al. Combinatorial targeting of TSLP, IL-25, and IL-33 in type 2 cytokine-driven inflammation and fibrosis. *Sci Transl Med* 2016;8:337ra65.
73. Verma M, Liu S, Michalec L, Sripada A, Gorska MM, Alam R. Experimental asthma persists in IL-33 receptor knockout mice because of the emergence of thymic stromal lymphopoietin-driven IL-9(+) and IL-13(+) type 2 innate lymphoid cell subpopulations. *J Allergy Clin Immunol* 2018;142:793-803.e8.
74. Matta BM, Lott JM, Mathews LR, Liu Q, Rosborough BR, Blazar BR, et al. IL-33 is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. *J Immunol* 2014;193:4010-20.

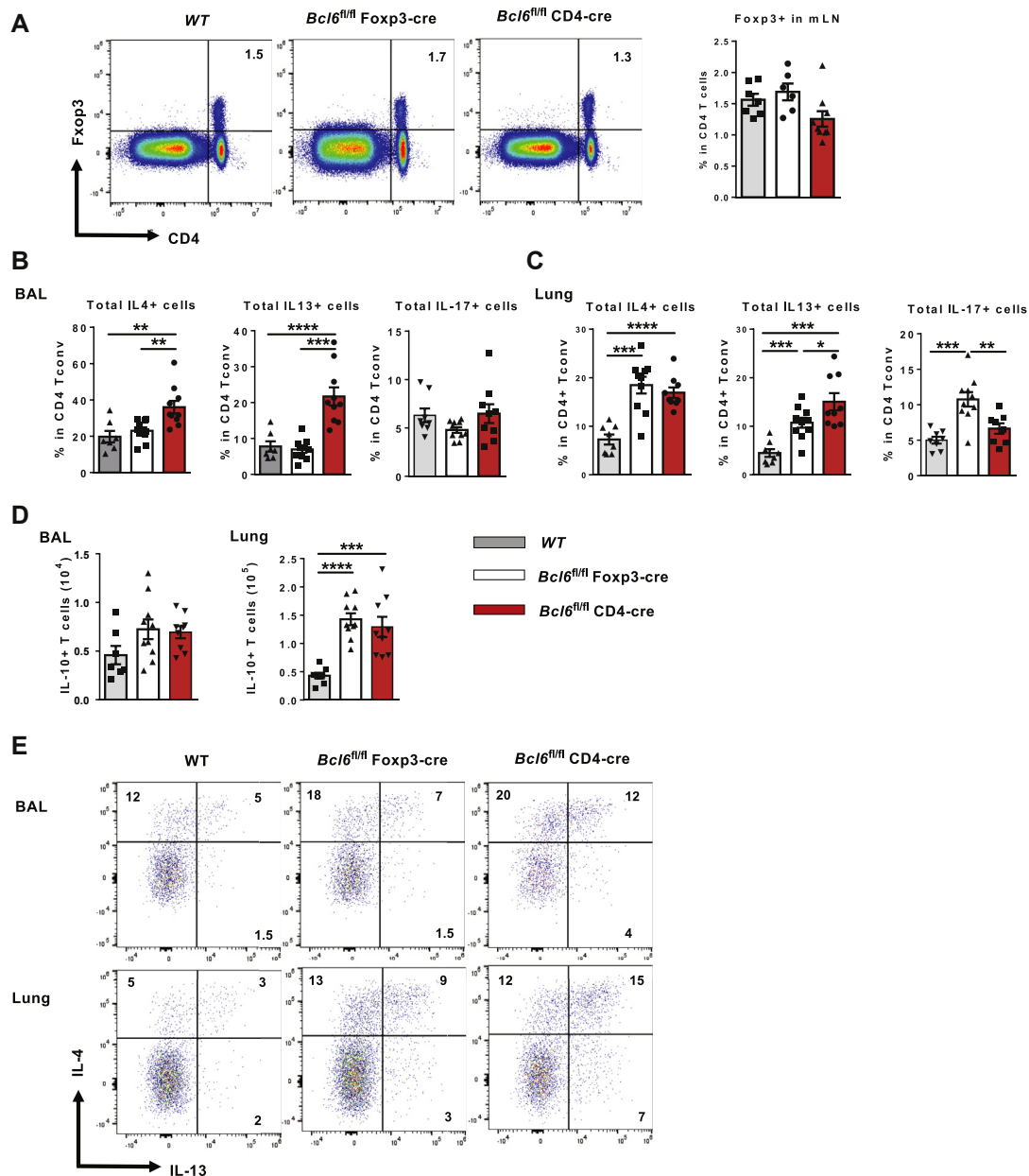


FIG E1. Tfr-cell deficiency results in exacerbated airway inflammation. Mice were sensitized and challenged with HDM as in Fig 1. **A**, Representative dot plot (left) and average percentage (right) of Foxp3⁺ Treg cells in CD4⁺ T cells in mLN. Percentage of IL-4⁺, IL-13⁺, or IL-17⁺-producing CD4⁺ T cells in BAL (**B**) and lung (**C**). **D**, Numbers of IL-10⁺-producing CD4⁺ T cells in BAL and lung. **E**, Representative dot plots of IL-4⁺ or IL-13⁺-producing CD4⁺ T cells. Data are means $\pm$ SEMs for 8 to 10 mice per group and representative of 3 independent experiments. A Student unpaired 2-tailed *t* test was used to generate *P* values. ***P* < .01; ****P* < .001; *****P* < .0001.

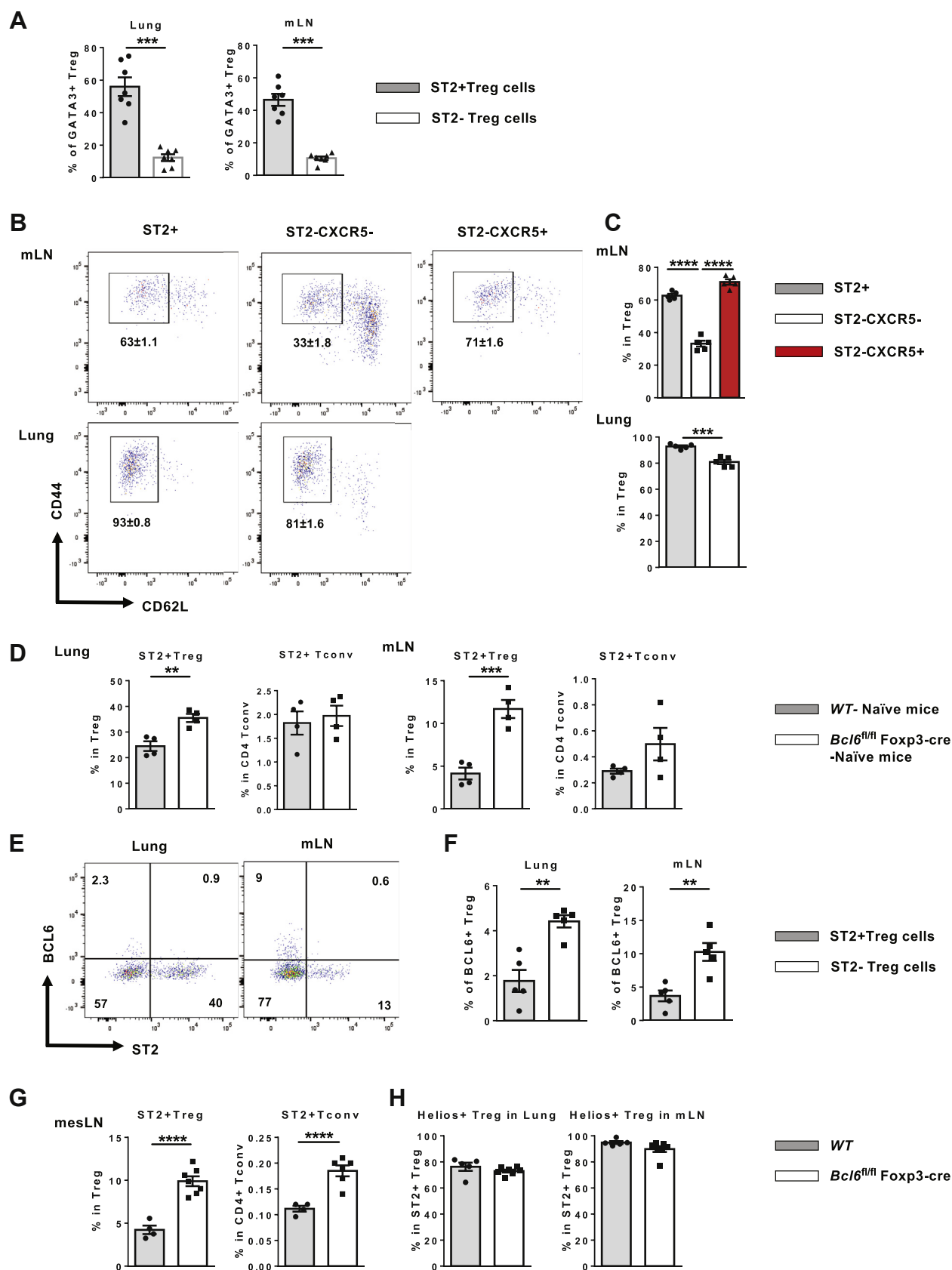


FIG E2. *Bcl6* deficiency in Treg cells leads to increased ST2⁺ Treg-cell development. Mice were sensitized and challenged with HDM as in Fig 1 (A-C and E-H). **A**, Average percentage of GATA3-positive cells in ST2⁺ or ST2⁻ Treg cells in lungs and mLNs of WT mice. **B** and **C**, Activation status, CD44^{high} and CD62L^{low}, of Treg

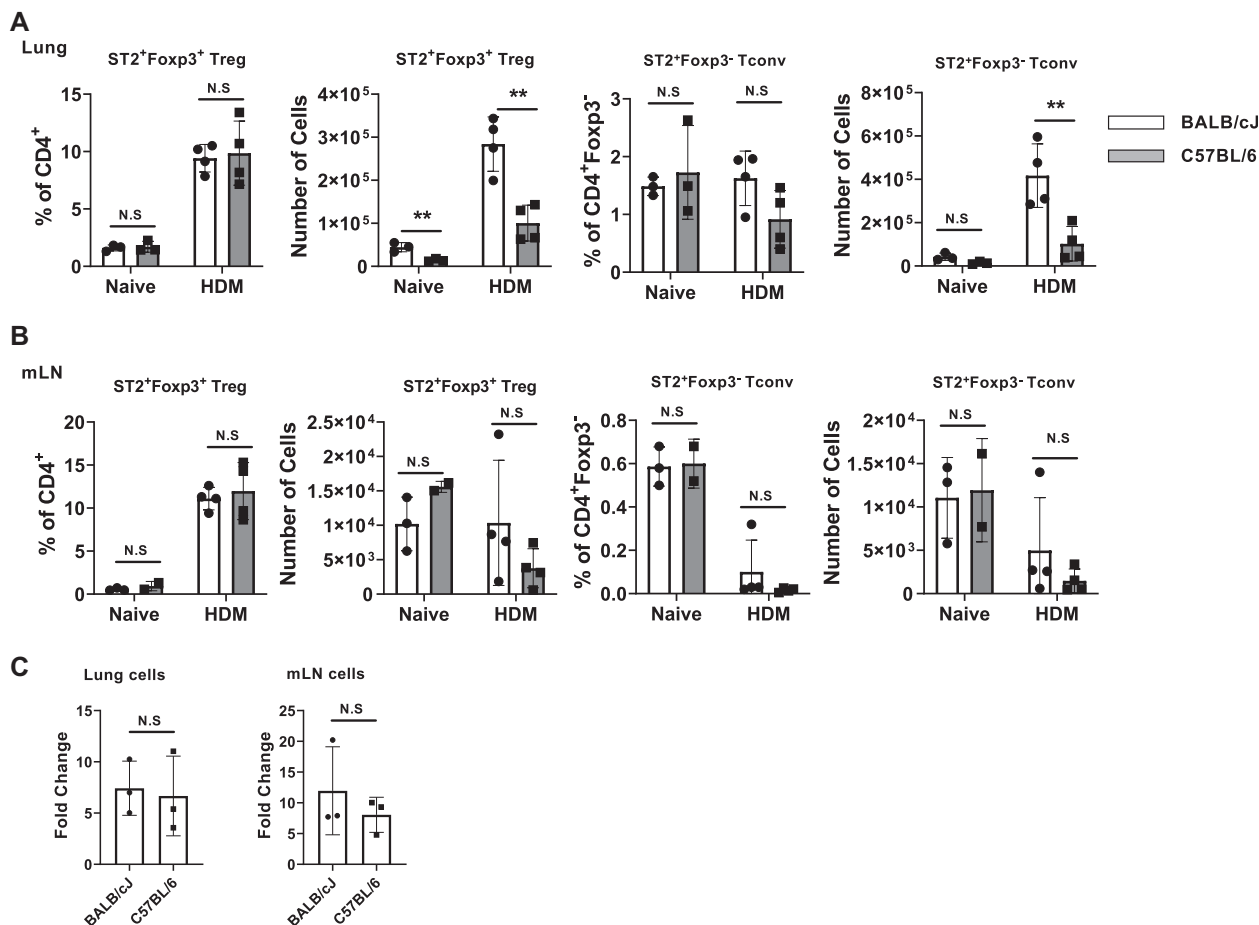


FIG E3. ST2⁺Foxp3⁺ Treg cells are present in multiple strains of mice. Mice were sensitized and challenged to HDM as in Fig 1. Flow cytometric analysis of ST2⁺ Treg cells and ST2⁺ Tconv cells in the lungs (A) and mLNs (B) of naive and HDM-challenged BALB/cJ and C57BL/6 mice. C, Increase in cellularity between naive and HDM-challenged mice represented as fold change for each strain tested. A Student unpaired 2-tailed *t* test was used to generate *P* values. ***P* < .01.

cells in mLNs and lungs. Representative dot plots (B) and average percentage (C) of activated Treg cells. D, Percentages ST2⁺ Treg cells and ST2⁺ Tconv cells in lungs or mLNs of naive WT and Bcl6^{fl/fl} Foxp3-Cre mice. Representative dot plots (E) and average percentage (F) of BCL6-expressing Treg cells in ST2⁺ or ST2⁻ Treg cells in lungs and mLNs of WT mice. G, ST2⁺ Treg cells and ST2⁺ Tconv cells in mesenteric lymph nodes (mesLNs). H, Percentage of Helios-positive Treg cells in lungs and mLNs. A-H, Data are means ± SEMs for 4 to 6 mice per group and representative of 2 independent experiments. A Student unpaired 2-tailed *t* test was used to generate *P* values. ***P* < .01; ****P* < .001; *****P* < .0001.

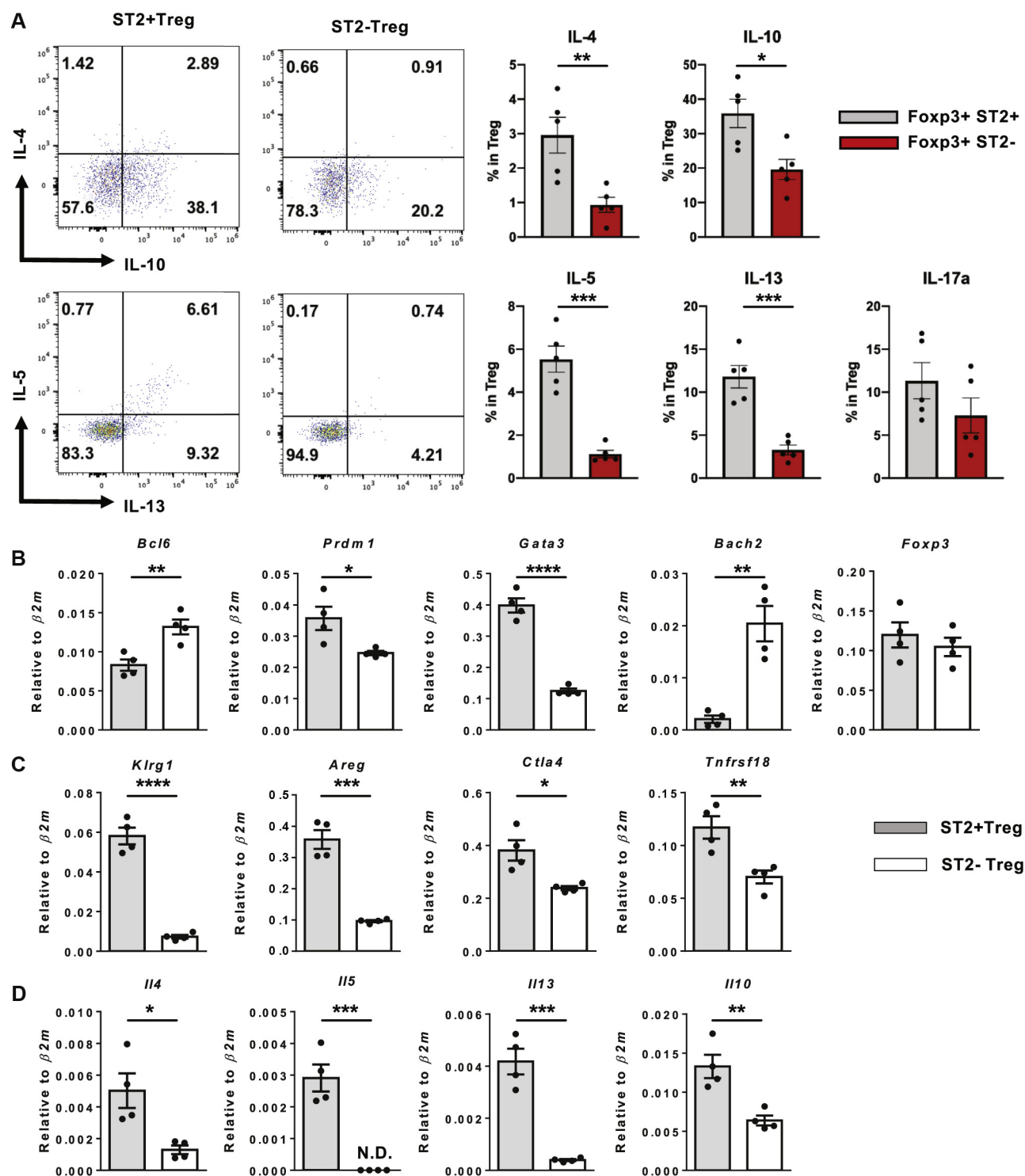
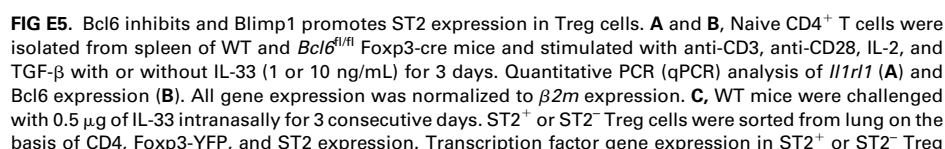


FIG E4. Proinflammatory transcriptional profile of ST2⁺ Treg cells in allergic airway inflammation. **A**, Representative dot plot (left) and percentage (right) of intracellular staining of T_H2 cytokines in ST2⁺ or ST2⁻ Treg cells. **B-D**, ST2⁺ and ST2⁻ Foxp3/YFP⁺ Treg cells were sorted from the lungs of HDM-challenged WT mice. Gene expression was measured by using quantitative PCR and normalized to $\beta 2m$ expression. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.



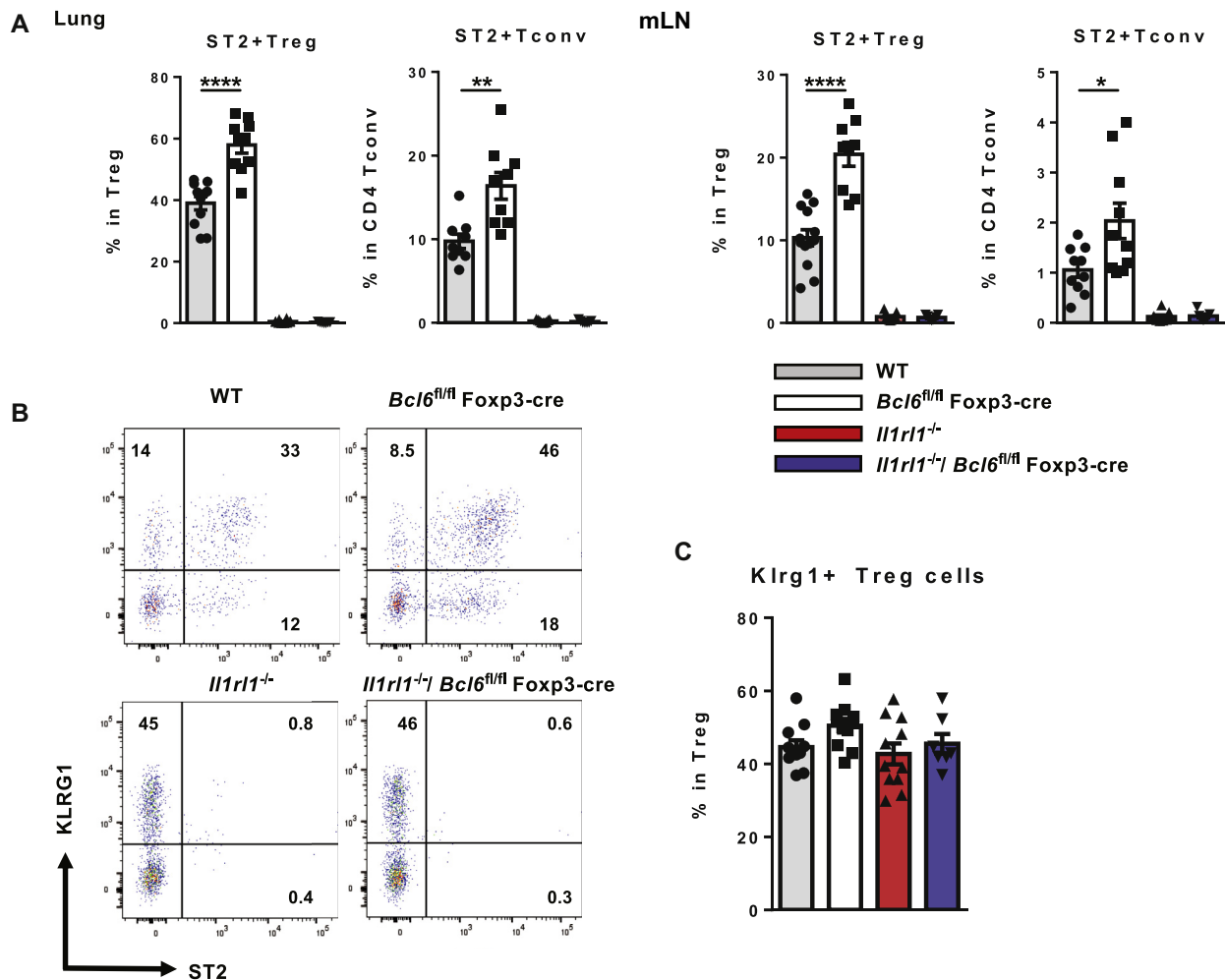


FIG E6. ST2 deficiency compensates for the lack of Bcl6 in Treg cells during allergic airway inflammation. **A**, Flow cytometric analysis of ST2⁺ Treg cells and Tconv cells in lungs and mLN. **B**, Flow cytometric analysis of ST2 and KLRG1 expression and total KLRG1-expressing Treg cells in lung (**C**). Data are means $\pm$ SEMs for 8 to 10 mice per group and representative of 3 independent experiments. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

cells measured by using qPCR and normalized to $\beta 2m$ expression. **D**, Splenic Treg cells from *Il1rl1^{-/-}* and *Il1rl1^{-/-}* *Bcl6^{fl/fl}* Foxp3-cre mice were sorted and stimulated with anti-CD3, anti-CD28, and IL-2 with or without IL-33 (10 ng/mL) for 3 days. *Il13*, *Il4*, and *Gata3* expression was measured by using qPCR. **E** and **F**, WT splenic Treg cells were sorted and stimulated with anti-CD3, anti-CD28, and IL-2 with or without IL-33 (10 ng/mL) for 3 days. On day 3, cells were harvested for ChIP analysis for H3K27ac at the *Il1rl1* (**E**) and *Prdm1* (**F**) gene loci. **G** and **H**, Splenic Treg cells from WT C57BL/6 and BALB/cJ mice were sorted and stimulated with anti-CD3, anti-CD28, and IL-2 with or without IL-33 (10 ng/mL) for 3 days. Representative dot plots (**G**) and average percentage (**H**) of ST2⁺ Treg cells. **I-K**, WT and *Prdm1^{fl/fl}* Foxp3-cre mice were sensitized and challenged to HDM as in Fig 1. **I**, Average percentages of BCL6⁺ Treg cells in lung. Representative dot plots (**J**) and average percentage (**K**) of Tfr cells in mLN. Data are means $\pm$ SEMs for 3 to 5 mice per group and representative of 2 independent experiments. A Student unpaired 2-tailed *t* test was used to generate *P* values. **P* < .05; ***P* < .01; *****P* < .0001.

TABLE E1. Taqman probes for quantitative PCR

Gene	Catalog No.
<i>Areg</i>	Mm00437583_m1
<i>β2m</i>	Mm00437762_m1
<i>Bach2</i>	Mm00464379_m1
<i>Bcl6</i>	Mm00477633_m1
<i>Ccl3</i>	Mm00441259_g1
<i>Ccr7</i>	Mm00432608_m1
<i>Ccr8</i>	Mm00843415_s1
<i>Ctla4</i>	Mm00486849_m1
<i>Cxcr3</i>	Mm00438259_m1
<i>Foxp3</i>	Mm00475165_m1
<i>Gata3</i>	Mm00484683_m1
<i>Il10</i>	Mm00439614_m1
<i>Il13</i>	Mm00434204_m1
<i>Il1rl1</i>	Mm00516117_m1
<i>Il4</i>	Mm00445259_m1
<i>Il5</i>	Mm00439646_m1
<i>Klrg1</i>	Mm00516879_m1
<i>Lilrb4</i>	Mm01614371_m1
<i>Prdm1</i>	Mm00476128_m1
<i>Tnfrsf18</i>	Mm00437136_m1

TABLE E2. Fluorescent antibodies for flow cytometric analysis

Antigen/name	Clone	Fluorochrome	Company	Catalog No.
B220	RA3-6B2	PerCP-Cy5.5	BioLegend	103236
BCL-6	K112-91	BV421	BD Biosciences	563363
CD11b	M1/70	PerCP-Cy5.5	eBioscience	45-0112-82
CD11c	N418	PE-Cy7	eBioscience	25-0114-82
CD4	GK1.5	PerCP-Cy5.5	BioLegend	100434
		APC-Cy7	BioLegend	552051
CD44	IM7	BV510	BD Biosciences	563114
CD62L	MEL-14	APC	BD Biosciences	553152
CXCR5	L138D7	PE-Cy7	BioLegend	145516
F4/80	BM8	FITC	BioLegend	123108
FAS (CD95)	SA367H8	PE	BioLegend	152608
Foxp3	MF23	FITC	BD Biosciences	560403
GATA3	L50-823	AF647	BD Biosciences	560068
GL7	GL7	APC	BioLegend	144617
IFN- γ	XMG 1.2	PerCP-Cy5.5	eBioscience	45-7311-82
IL-10	JES5-16E3	FITC	BioLegend	505006
IL-13	eBio13A	PE	eBioscience	12-7133-82
IL-17A	eBio17B7	PE-Cy7	eBioscience	25-7177-82
IL-4	11B11	AF647	BioLegend	504110
KLRG1	2F1	BV510	BD Biosciences	740156
		APC	BD Biosciences	561620
Ly6G	1A8	APC	BioLegend	127613
PD-1	29F.1A12	PerCP-Cy5.5	BioLegend	135208
SiglecF	E50-2440	PE	BD Biosciences	552126
ST2	U29-93	PE	BD Biosciences	566309
Fixable Viability Dye		eFluor 780	eBioscience	65-0865-14

TABLE E3. Sequence of ChIP primers

Primer	Forward (5'-3')	Reverse (5'-3')
<i>Il1rl1</i> enhancer	GCCAACCACAACAGCAGATGGGGAAA	ACTGAGATCCTGCCCTGGCTTCCT
<i>Il1rl1</i> promoter	TGGCCTCCTTGGAAGGCTTGGT	AGTGCAGGAGGGGCATGGAGATGA
<i>Prdm1</i> intron	TGCTTTCTCGGTTTCAGTTGA	GAGTGAGCTGCTTTGGAAGG
<i>Prdm1</i> promoter	CCAGTAGGCCTTTCATGGCT	TGCTCAGGTTGAGAAAGCAGT