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Airway Inflammation and IgE Production Induced by Dust Mite Allergen-Specific Memory/Effector Th2 Cell Line Can Be Effectively Attenuated by IL-35

Chiung-Hui Huang,^{*,1} Evelyn Xiu-Ling Loo,^{*,1} I-Chun Kuo,^{*} Gim Hooi Soh,^{*} Denise Li-Meng Goh,^{*} Bee Wah Lee,^{*} and Kaw Yan Chua^{*,†}

CD4⁺ memory/effector T cells play a central role in orchestrating the rapid and robust immune responses upon re-encounter with specific Ags. However, the immunologic mechanism(s) underlying these responses are still not fully understood. To investigate this, we generated an allergen (major house dust mite allergen, Blo t 5)-specific murine Th2 cell line that secreted IL-4, IL-5, IL-10, and IL-13, but not IL-9 or TNF- α , upon activation by the cognate Ag. These cells also exhibited CD44^{high}CD62L⁻ and CD127⁺ (IL-7R α ⁺) phenotypes, which are characteristics of memory/effector T cells. Experiments involving adoptive transfer of this Th2 cell line in mice, followed by three intranasal challenges with Blo t 5, induced a dexamethasone-sensitive eosinophilic airway inflammation. This was accompanied by elevation of Th2 cytokines and CC- and CXC-motif chemokines, as well as recruitment of lymphocytes and polymorphic mononuclear cells into the lungs. Moreover, Blo t 5-specific IgE was detected 4 d after the last intranasal challenge, whereas elevation of Blo t 5-specific IgG1 was found at week two. Finally, pulmonary delivery of the pVAX-IL-35 DNA construct effectively downregulated Blo t 5-specific allergic airway inflammation, and i.m. injection of pVAX-IL-35 led to long-lasting suppression of circulating Blo t 5-specific and total IgE. This model provides a robust research tool to elucidate the immunopathogenic role of memory/effector Th2 cells in allergic airway inflammation. Our results suggested that IL-35 could be a potential therapeutic target for allergic asthma through its attenuating effects on allergen-specific CD4⁺ memory/effector Th2 cell-mediated airway inflammation. *The Journal of Immunology*, 2011, 187: 000–000.

Allergen-specific CD4⁺ Th2 cells home to specific compartments and function through their production of Th2 cell-associated cytokines: IL-4, IL-5, IL-9, IL-10, and IL-13 (1). These Th2 cells play a pivotal role in the pathogenesis of allergic asthma (2). Th2 cells are essential for IgE production through the regulation of B cell class switching via the production of IL-4 and IL-13 (3). Cross-linking of high-affinity Fc ϵ Rs on mast cells and basophils leads to activation and degranulation of these cells. Mediators released from the degranulated mast cells and basophils trigger the development of IgE-mediated Th2-type allergic responses (4). Through the production of IL-5, lung-homing Th2 cells can recruit eosinophils that lead to the manifestation of tissue eosinophilia. In addition, Th2 effector cytokines, IL-4 and IL-13, can directly act on epithelial and smooth muscle cells to induce mucus production, goblet cell hyperplasia,

and airway hyperresponsiveness. IL-4, IL-13, and IL-25 also act on T cells, macrophages, and IL-5- and/or IL-13-producing non-B and non-T cells in lungs to induce and exacerbate allergic asthma (1, 2, 5).

It is well known that subpopulations of allergen-specific memory Th2 cells are generated during an immune response to allergens. The precise mechanisms underlying the generation of Th2 memory cells in vivo remain elusive, and allergic asthmatic patients maintain Th2 memory cells in their lungs during remission (6, 7). After recovering from a single episode of allergic asthma, allergen-specific memory Th2 cells reside within the lungs of mice for their remaining life span (8). Upon specific allergen exposure, these allergen-specific Th2-skewed memory T cells respond in a rapid and robust fashion, resulting in eosinophilic lung inflammation, mucus production, airway hyperresponsiveness, and allergen-specific IgE production (8). Moreover, repeated allergen exposure will further stimulate the memory Th2 cells to induce persistent airway inflammation and asthma exacerbations (9). It is conceivable that novel treatments targeted at suppressing or eliminating allergen-specific Th2 memory cells could be a promising approach to ameliorate diseases (10). Mouse models of allergic asthma are mostly induced by OVA as the prototype allergen (11, 12) or by the transfer of cytokines and blocking mAb-polarized T cells obtained from OVA peptide-specific TCR transgenic mice (13–15). Despite the robustness of OVA-based animal models (16), there are concerns that OVA is not an aero-allergen relevant to human disease. Furthermore, recent studies showed that the intrinsic property of allergens may contribute significantly to the development of allergic diseases in humans (17, 18). To the best of our knowledge, there are no published reports on the generation and characterization of a long-lived memory/effector Th2 cell line specific for a clinically relevant allergen involved in human allergic asthma.

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; EBI3, EBV-induced gene 3; IL-12p35, p35 subunit of IL-12; i.n., intranasal(ly); RACE, rapid amplification of cDNA ends; Treg, regulatory T cell.

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In this article, we describe the generation and characterization of a long-lived memory Th2 cell line expressing the TCR specific for an H-2^b-restricted epitope for a dust mite allergen, Blo t 5, which has been identified as the most important major allergen of *Blomia tropicalis* (19). These Blo t 5-specific Th2 effector cells were originally primed in mice sensitized epicutaneously with the Blo t 5 protein without adjuvant; oligoclonality was achieved after the long-term maintenance with the Blo t 5 protein in vitro. These Blo t 5-specific Th2 memory cells induced eosinophilic airway inflammation and Blo t 5-specific IgE production upon intranasal (i.n.) Blo t 5 challenges. These allergic responses were effectively attenuated by the newly identified regulatory cytokine IL-35.

Materials and Methods

Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the Centre for Animal Resources, Singapore. All mice were maintained in individually ventilated cages in a specific pathogen-free facility at the Satellite Animal Holding Unit, National University of Singapore. Sterilized food and water were provided ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee according to the National Advisory Committee for Laboratory Animal Research Guidelines, Singapore.

Proteins and peptides

Recombinant Blo t 5 and Der p 2 were prepared as described previously (19, 20). To generate large Blo t 5 fragments, the cDNA of three overlapping Blo t 5 fragments, corresponding to residues 1–45, 40–80, and 70–117, were amplified by PCR from the full-length Blo t 5 cDNA, cloned into the pGEX-4T vector, and transformed into *Escherichia coli* TG-1 cells. Protein expression was induced by isopropyl- β -D-thiogalactopyranoside, and GST-fused Blo t 5 fragments (GST-Blo t 5_{1–45}, GST-Blo t 5_{40–80}, and GST-Blo t 5_{70–117}) were purified (21). A panel of 16 residues-Blo t 5 peptides, with the offset of 3 residues, was synthesized for use in T cell epitope mapping (Mimotopes, Clayton, Victoria, Australia).

Generation and maintenance of Blo t 5-specific T cells

Mice were sensitized by epicutaneous patching with 50 μ g recombinant Blo t 5 on days 0, 14, and 28 (22). Sera was collected at weekly intervals, and amounts of Blo t 5-specific IgE and IgG1 were determined by ELISA. At day 35, mice were sacrificed, and the spleens were collected. Splenocytes were cultured in the presence of 10 μ g/ml Blo t 5 in RPMI 1640 (Hyclone Laboratories, Logan, UT), supplemented with 10% heat-inactivated FBS (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin), 1 mM sodium pyruvate (Hyclone Laboratories), and 5.5×10^{-2} mM 2-ME (Life Technology, Grand Island, NY). Recombinant mouse IL-2 (BD PharMingen, San Diego, CA) was added to cultures on days 3 and 5 to a final concentration of 10 U/ml. On day 7, the live cells were harvested by Ficoll-Paque (GE Healthcare, Piscataway, NJ) density-gradient centrifugation and restimulated with 10 μ g/ml Blo t 5 in the presence of mitomycin C (Roche Diagnostics, Mannheim, Germany)-treated splenocytes from naive mice as APCs (mitomycin C-APCs). To generate and maintain the Blo t 5-specific T cell line, the cells were stimulated weekly with Blo t 5, as described above.

Detection of Blo t 5-specific Igs and total serum Ig isotypes

ELISAs used to quantify the amounts of Blo t 5-specific IgE, IgG1, and IgG2c in mouse sera were described previously (20, 23). All mAbs were purchased from BD PharMingen, unless otherwise specified. Total serum IgE, IgG1, IgM, and IgA were determined by ELISA with capture mAbs (anti-mouse IgE [R35-72], anti-mouse IgG1 [A85-3], anti-mouse IgM [LO-MM-3] [Serotec, Oxford, U.K.] and anti-mouse IgA [C10-3]) and detection mAbs (biotin-conjugated anti-mouse IgE [LO-ME-3], biotin-conjugated anti-mouse IgG1 [LO-MG1-2], biotin-conjugated anti-mouse IgM [LO-MM-9] [all from Serotec] and biotin-conjugated goat anti-mouse IgA [Sigma-Aldrich]). Purified mouse IgE (IgE-3), IgG1 (MOPC-31C), IgA (M18-254), and IgM (G155-228) were used to generate standard curves. The levels of total serum IgG2c were determined with an IgG2c ELISA kit (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's protocol.

Quantitation of cytokines and chemokines

Cytokine concentrations were quantified by ELISA. Rat anti-mouse IL-4 (BVD4-1D11), IL-5 (TRFK5), IFN- γ (R4-6A2) (BD PharMingen), IL-10 (JES052A5), and IL-13 (38213) (R&D Systems, Minneapolis, MN) were used as capture Abs. Biotin-conjugated rat anti-mouse IL-4 (BVD6-24G2), IL-5 (TRFK4), and IFN- γ (XMG1.2) (BD PharMingen) and biotin-conjugated goat anti-mouse IL-10 and IL-13 (R&D Systems) were used as detection Abs. Recombinant mouse IL-4, IL-5, IFN- γ (BD PharMingen), IL-10, and IL-13 (R&D Systems) were used to generate standard curves for references. Chemokines, CCL2, CXCL1, CXCL5, CXCL9, and CXCL10, in BALF were measured using Millipore multiplex reagents (Millipore, Bedford, MA) and the Bio-Plex 200 suspension-array system (BioRad Laboratories, Hercules, CA).

T cell-proliferation assay

The Blo t 5-specific T cell-proliferation assay was performed by culturing 5×10^4 Blo t 5-specific T cells with 10 μ g/ml recombinant Blo t 5 or Der p 2 in the presence of 2×10^5 mitomycin C-APCs for 48 h. [³H]-thymidine (0.1 μ Ci/well) was added to each well and cultured for an additional 18 h. The cells were harvested, and [³H]thymidine incorporation was measured by liquid scintillation counting. To identify the epitope of the Blo t 5-specific T cell line, 0.7 μ M recombinant Blo t 5, GST-Blo t 5_(1–45), GST-Blo t 5_(40–80), GST-Blo t 5_(70–117), GST, or 3.5 μ M synthetic peptides were used together with mitomycin C-APCs.

Analysis of TCR V β and J β gene usage

To characterize the TCR V β gene usage of Blo t 5-specific T cells, the cells were stained with a panel of FITC-labeled V β -specific mAbs (BD PharMingen) and analyzed by flow cytometry. To analyze the usage of TCR J β gene, Blo t 5-specific T cells were stimulated with anti-CD3 and CD28 mAb for 24 h. Cells were harvested, and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA). Two micrograms of total RNA and 1 μ g oligo(dT)₁₈ primer were used to synthesize the first-strand cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) at 42°C for 1 h, according to the manufacturer's instructions. PCR was performed using the V β 3 gene-specific primer (sense) paired individually with 12 J β gene-specific primers (antisense) (Supplemental Table I). The sequences of these V β 3 and J β gene-specific primers were obtained from the international ImMunoGeneTics information system. PCR reactions were analyzed by gel electrophoresis to identify the gene usage.

Analysis of TCR V α gene usage

TCR V α gene usages of the Blo t 5-specific T cell line were analyzed by molecular cloning methods. The 5'-rapid amplification of cDNA ends (RACE) of TCR V α gene was performed using the SMART RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, CA). First-strand cDNA was synthesized using 1 μ g total RNA and the 5'-cDNA synthesis primer with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. 5'-RACE PCR was performed using the universal primer mix, a gene-specific primer for the C region of TCR α genes (5'-TGA ACT GGG GTA GGT GGC GTT GG-3') and advantage cDNA polymerase (Clontech Laboratories), according to the manufacturer's instructions. The PCR product was cloned into the pCR2.1-TA-cloning vector (Invitrogen) and transformed, and colonies were picked and DNA sequenced to identify the TCR V α gene.

Flow cytometric analyses and intracellular staining

The cell surface phenotypes, at rest and upon activation, were studied by flow cytometry with the following mAbs purchased from BD PharMingen. Biotin-conjugated anti-CD49b (DX5); FITC-conjugated anti-CD4 (RM4.5) and anti-CD278 (7E.17G9); PE-conjugated anti-CD4 (RM4-5), anti-CD8b.2 (53-5.8), anti-CD27 (LG.3A10), anti-CD62L (MEL-14), anti-CD127 (SB/199), anti-CD134 (OX-86), anti-CD137 (1AH2), and anti-CD152 (UC10-4F10-11); PerCP-conjugated anti-CD3e (145-2C11) and anti-CD8b.2 (53-5.8); and allophycocyanin-conjugated anti-CD4 (RM4.5), anti-CD25 (PC61), and anti-CD44 (IM7). For intracellular cytokine staining, cells were stimulated with immobilized anti-CD3e (145-2C11) and soluble anti-CD28 (37.51) (BD PharMingen) in the presence of 3 μ M monensin (Sigma-Aldrich, St Louis, MO) for 6 h. Cells were stained with surface markers, fixed, and permeabilized with Cytofix/Cytoperm solution and further stained with PE-conjugated anti-IL-4 (BVD4-1D11) and allophycocyanin-conjugated anti-IFN- γ (XMG-2.1) (BD PharMingen). Flow cytometry analyses were performed using the FACSCalibur flow

cytometer, and results were analyzed by CellQuestPro software (Becton Dickinson, Franklin Lakes, NJ).

Induction of airway inflammation by the adoptive transfer of Blo t 5-specific Th2 cells into naive mice

Long-term cultured Blo t 5-specific Th2 cells were routinely harvested 7 d after restimulation with Blo t 5 and washed with sterile PBS. For adoptive cell transfer, 2.5×10^6 cells in 150 μ l PBS were injected into recipient mice i.v. through the tail vein. In the experiment, for which CFSE (CalBiochem, San Diego, CA)-labeled cells were used, Blo t 5-specific Th2 cells were labeled with 10 μ M CFSE for 10 min at 37°C before the cell transfer. The control mice received an injection of 150 μ l PBS only. One day after the cell transfer, all mice were given daily i.n. challenge with 50 μ g Blo t 5 protein in 50 μ l PBS twice or thrice, as indicated in the figure legends. One or two days after the last i.n. challenge, mice were lavaged three times with 0.8 ml cold HBSS without calcium and magnesium (Sigma-Aldrich) through tracheostomy. The supernatants of the first 0.8 ml lavage fluid were collected and stored at -20°C for the analysis of cytokines and chemokines. The cells were collected from three lavages, and absolute cell counts were determined using a hemocytometer. Differential cell counts were performed by analyzing 500 cells/mouse based on standard hematological characteristics of macrophages, lymphocytes, neutrophils, and eosinophils.

Histochemistry

For histological examination, lungs were obtained 1 d after the last i.n. Blo t 5 challenge and were immediately fixed in 10% neutral buffered formalin. Paraffin-embedded tissues were cut into 4- μ m-thick sections and stained with H&E or periodic acid-Schiff.

Dexamethasone suppression

Dexamethasone was administered to mice by i.p. injection at dosages of 0.5 or 2 mg/kg (Rotexmedica, Trittau, Germany) 1 h before each i.n. Blo t 5 challenge.

Construction of pVAX-IL-35

The strategy to generate the single-chain IL-35-coding sequence was similar to the method described previously (24). EBV-induced gene 3 (EBI3) was amplified from the first-strand cDNA prepared from LPS-activated bone marrow-derived dendritic cells. The primers used were as follows: EBI3 sense: 5'-A AAA GGA TCC ATC CAA GGA ACA GAG CCA CAG AGC ATG TCC-3' and EBI3 antisense: 5'-AGA ACC ACC ACC AAG CTT GGG CTT ATG GGG TGC AC-3'. BamHI and HindIII sites were introduced into EBI3 sense primer and antisense primer, respectively. The p35 subunit of IL-12 (IL-12p35) was amplified from the first-strand cDNA prepared from splenocytes. The primers used were as follows: IL-12p35 sense: 5'-GGT GGT GGT TCT GAA TTC AGG GTC ATT CCA GTC TCT GGA CCT G-3' and IL-12p35 antisense: 5'-A AAA GCT AGC TCA GGC GGA GCT CAG ATA GCC CAT CAC CCT GTT G-3'. EcoRI and NheI sites were introduced into IL-12p35 sense primer and antisense primer, respectively. Two complementary oligonucleotides encoding for four repeats of GGGs linker were designed and annealed. These were linker sense: 5'-A ACC AAG CTT GGT GGT GGT TCT GGT GGT GGT TCT GGT GGT TCT GGT GGT TCT GGT GGT TCT GGA TCC GGT T-3' and linker antisense: 5'-A ACC GAA TTC AGA ACC ACC ACC AGA ACC ACC ACC AGA ACC ACC ACC AGA ACC ACC ACC ACC AAG CTT GGT T-3'. HindIII and EcoRI sites were included in the linker sense and linker antisense primers. Amplified EBI3, IL-12p35, and the double-stranded linker were digested with respective restriction enzymes and ligated into BamHI and NheI sites of the pVAX vector (Invitrogen). The correct recombinant single-chain IL-35 in the pVAX vector (pVAX-IL-35) was verified by DNA sequencing. Plasmid DNA was purified from *E. coli* strain DH5 α using the EndoFree Plasmid Giga Kit (Qiagen) and dissolved in sterile distilled water at 2.0 mg/ml for use in DNA immunization.

Intratracheal instillation of pVAX-IL-35 on the suppression of Blo t 5-specific Th2 cell line-induced airway inflammation

Mice were instilled intratracheally with 400 μ g pVAX or pVAX-IL-35 plasmid DNA on day 0. One day later, mice were given a transfer of 2×10^6 Blo t 5-specific Th2 cells i.v. The mice were challenged i.n. with 50 μ g Blo t 5 protein on days 2–4. On day 5, bronchoalveolar lavage fluid (BALF) was collected and analyzed as described above. Cytokines/chemokines in BALF were assessed by cytokine/chemokine multiplex assays (Millipore).

Intramuscular injection of pVAX-IL-35 on the suppression of Blo t 5-specific Th2 cell line-induced humoral responses

Mice were injected i.m. with 50 μ g pVAX or pVAX-IL-35 plasmid DNA with electroporation on days 0 and 14 (25). A total of 2×10^6 Blo t 5-specific Th2 cells was transferred i.v. into the mice on day 17. The first course of i.n. challenge (50 μ g Blo t 5 protein) was given on days 18–20. The second course of i.n. challenge (50 μ g Blo t 5 protein) was given on days 77–79. Sera were collected on days 30, 37, 44, 51, 84, and 91 for the quantification of Blo t 5-specific and total serum Igs by ELISA.

Statistical analyses

All animal experimental results are presented as mean $\pm$ SEM and were analyzed by the two-tailed Mann-Whitney *U* test. A *p* value < 0.05 was considered significant.

Results

Generation of the Blo t 5-specific Th2 cell line

We previously showed that epicutaneous sensitization with allergens could induce a prominent allergen-specific Th2 immune response (22). Compared with control mice, Blo t 5 epicutaneously sensitized mice produced significant levels of Blo t 5-specific IgE and IgG1 (Fig. 1A) but had no detectable Blo t 5-specific IgG2c (data not shown). Splenocytes from Blo t 5-sensitized mice showed elevated production of Th2 cytokines IL-4, IL-5, and IL-13, as well as Th1 cytokine IFN- γ (Fig. 1B). These splenocytes were repeatedly stimulated in vitro with Blo t 5 allergen for 1 y to establish a highly polarized Th2 cell line. These highly polarized Th2 cells proliferated specifically upon restimulation with Blo t 5 but not with the irrelevant allergen Der p 2 (Fig. 1C). They also secreted high levels of IL-4, IL-5, IL-10, and IL-13 but no detectable levels of IFN- γ (Fig. 1D) TNF- α , or IL-9 (data not shown). Hence, a Blo t 5-specific Th2 cell line had been

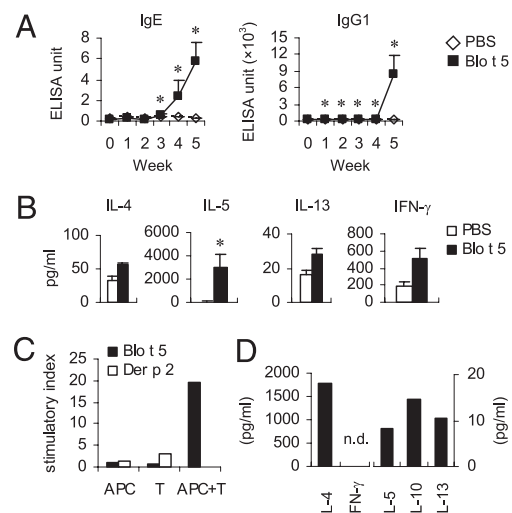


FIGURE 1. Generation of Blo t 5-specific Th2 cell line by epicutaneous patching with Blo t 5 protein or PBS of C57BL/6 mice ($n = 4$). *A*, The kinetics of Blo t 5-specific IgE and IgG1 production was determined by ELISA. *B*, ELISA cytokine profiles of culture supernatant from splenocytes at day 35. Harvested splenocytes were stimulated in vitro with Blo t 5 and again 7 d later. Culture supernatants were collected at 48 h. Results are presented as mean $\pm$ SEM. *C*, Proliferation assays. Specificity of Blo t 5-specific T cell line 1 y after in vitro culture. The Blo t 5-specific T cells (T) were stimulated with Blo t 5 or Der p 2 (control), in the presence (APC+T) or absence (T) of APCs. APC alone was used as the background control. Stimulatory index was calculated as (cpm of cells stimulated with Ag)/(cpm of cells without Ag stimulation). *D*, ELISA cytokine profiles of culture supernatants 3 d after Blo t 5 restimulation. Data are representative of three separate experiments. **p* < 0.05. n.d., not detectable.

established and maintained in vitro from in vivo sensitized and polarized allergen-specific Th2 cells.

Epitope mapping of Blo t 5-specific Th2 cell line

The specificity of the Blo t 5-specific Th2 cell line was further characterized by T cell epitope mapping using T cell-proliferation assays. The initial experiments on T cell-epitope mapping were performed using the three overlapping GST-fusion Blo t 5 fragments: GST-Blo t 5₁₋₄₅, GST-Blo t 5₄₀₋₈₀, and GST-Blo t 5₇₀₋₁₁₇. Blo t 5-specific Th2 cells only showed a positive T cell-proliferative response to the second GST-fusion Blo t 5 fragment, GST-Blo t 5₄₀₋₈₀. Fine T cell epitope mapping with 33 overlapping synthetic peptides showed that Blo t 5-specific Th2 cells reacted strongly to the peptide comprising residues 55–70 and reacted mildly to the peptide comprising residues 58–73 (Fig. 2A). These two peptides were encompassed within the second Blo t 5 fragment from residues 40–80.

TCR gene usage of Blo t 5-specific Th2 cell line

After 6 mo of in vitro culture, flow cytometric analysis of Blo t 5-specific Th2 cell line showed that the cells were positive for TCR Vβ3 (87%), TCR Vβ4 (9.8%), and TCR Vβ6 (0.8%) (Supplemental Fig. 1). After 12 mo of in vitro culture with repeated Ag restimulation, a homogeneous TCR Vβ3⁺ Blo t 5-specific Th2 cell line was obtained (Fig. 2B). PCR analysis of the joining (J) gene

usage of TCR Vβ3⁺ Blo t 5-specific Th2 showed exclusive usage of the TCR Jβ2.4 gene (Fig. 2C). These results demonstrated that the Blo t 5-specific Th2 cell line had become oligoclonal during the process of repeated Ag restimulation. The TCR Vα gene usage in this Th2 cell line at 12 mo was analyzed by molecular cloning analysis. The combination of TCR Vα11 with TCR Jα34 genes was observed in 20 of 22 sequenced clones. The combination of TCR Vα4 with TCR Jα27 genes, as well as TCR Vα8 with TCR Jα28 genes, was observed in the other two clones. Thus, the usage of TCR Vα genes was more heterogeneous than was the usage of TCR Vβ genes.

Phenotype of Blo t 5-specific Th2 cell line

Surface marker expression profiling showed that the resting Blo t 5-specific Th2 cells were negative for CD62L (CD62L[−]), had high levels of CD44 expression, and were positive for CD127 (IL-7Rα), therefore exhibiting the typical memory/effector T cell phenotype of CD44^{high}CD62L[−]CD127⁺ (26, 27) (Fig. 3). This profile is distinctly different from that of CD4⁺ T cells purified from naive C57BL/6 mice, which expressed varying degrees of CD62L and CD44. In addition, the resting Blo t 5-specific Th2 cells expressed higher levels of CD25 (IL-2Rα), CD134 (OX40), and CD278 (ICOS) but had a reduced level of CD27 compared with CD4⁺ T cells from naive mice. Upon polyclonal activation with anti-CD3 and anti-CD28 mAbs, Blo t 5-specific Th2 cells and naive CD4⁺ cells both showed upregulation of cell surface markers CD25, CD27, CD134, CD137 (4-1BB), CD278, and CD152 (CTLA-4), as well as downregulation of CD127 (Fig. 3B, Supplemental Fig. 2). The activated Blo t 5-specific Th2 cells maintained a higher CD25 expression than did activated CD4⁺ T cells from naive mice (Fig. 3B). Hence, the activation profile of the Blo t 5-specific Th2 cell line was not altered significantly after the long-term in vitro culture.

Adoptive transfer of Blo t 5-specific Th2 cell line induced rapid IgE production after in vivo Ag challenges

To elucidate the helper function of Blo t 5-specific Th2 cells in vivo, mice were adoptively transferred with resting Blo t 5-specific Th2 cells or PBS and then challenged i.n. with Blo t 5. The ability of B cells to produce Blo t 5-specific Abs was examined. Mice systemically sensitized by epicutaneous patching with Blo t 5 allergen were included for comparison. Mice adoptively transferred with Blo t 5-specific Th2 cells produced significant levels of Blo t 5-specific IgE on day 8 (Fig. 4A), and Blo t 5-specific IgG1 remained

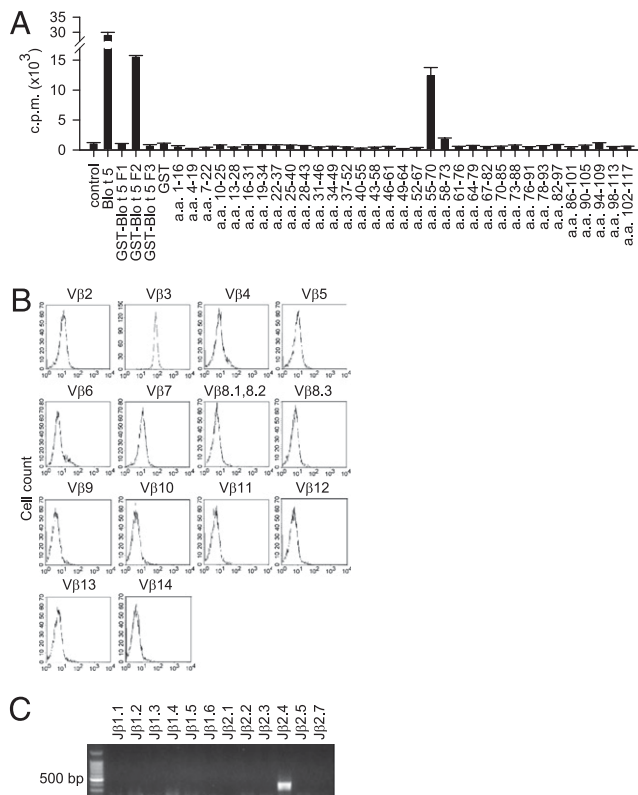


FIGURE 2. Characterization of the Blo t 5-specific T cells. *A*, Cell-proliferation assay measured by [³H]-thymidine uptake. Blo t 5-specific Th2 cell line was unstimulated (control) or stimulated with Blo t 5, GST-Blo t 5₁₋₄₅ (F1), GST-Blo t 5₄₀₋₈₀ (F2), GST-Blo t 5₇₀₋₁₁₇ (F3), GST proteins, or 16-residue synthetic peptides. Data are representative of two experiments represent mean ± SEM from triplicate wells. *B*, Flow cytometric analysis of TCR Vβ gene usage of the Blo t 5-specific Th2 cell line using a panel of Vβ-specific mAbs. *C*, PCR evaluation of Jβ usage of the Vβ3⁺ Blo t 5-specific Th2 cell line using a Vβ3 specific forward primer and a panel of 12 Jβ-specific reverse primers. Data are representative of two separate experiments.

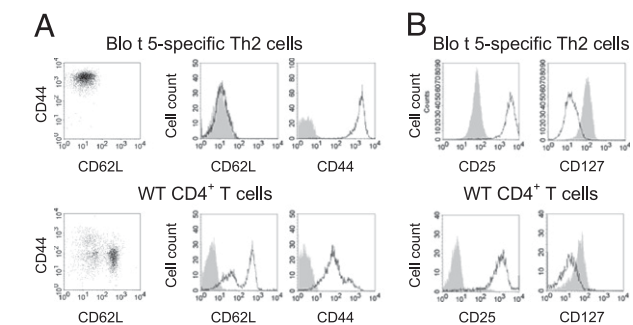


FIGURE 3. Flow cytometric analysis of cell surface phenotype of Blo t 5-specific Th2 cell line. *A*, CD62L and CD44 expression of resting Blo t 5-specific Th2 cells and purified CD4⁺ T cells from C57BL/6 mice (WT). Shaded graphs represent cells stained with isotype controls. *B*, CD25 and CD127 expression of Blo t 5-specific Th2 cells and CD4⁺ cells of C57BL/6 mice before (shaded graphs) and after (black lines) activation with mAbs against CD3ε and CD28. Data are representative of two separate experiments.

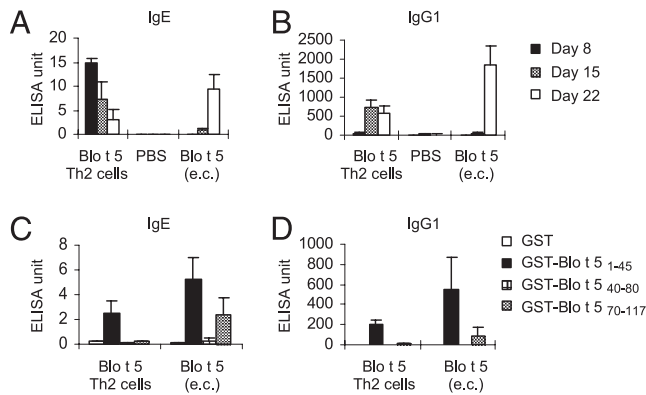


FIGURE 4. Blo t 5-specific Ab production induced by Blo t 5-specific Th2 cells. Kinetics of Blo t 5-specific serum IgE (A) and IgG1 (B) were measured by ELISA. Naive C57BL/6J mice ($n = 4$) were given i.v. transfer of Blo t 5-specific Th2 cells (Blo t 5 Th2 cells) or PBS on day 1, followed by three i.n. Blo t 5 challenges. A group of C57BL/6J mice was sensitized by epicutaneous patching (Blo t 5 e.c.). C and D, Epitope mapping of Blo t 5-specific IgE and IgG1. Blo t 5-specific IgE (C) and IgG1 (D) were detected against three GST-fused Blo t 5 fragments: GST-Blo t 5₁₋₄₅, GST-Blo t 5₄₀₋₈₀, and GST-Blo t 5₇₀₋₁₁₇. Sera from the adoptive transfer group (Blo t 5 Th2 cells) (day 8 for the IgE assay and day 22 for the IgG1 assay) and sera from the epicutaneous-sensitized group (Blo t 5 e.c.) (day 22 for both IgE and IgG1 assays) were analyzed by ELISA. Results are presented as mean $\pm$ SEM. Data are representative of three separate experiments.

at the basal level on day 8 and started to increase from day 15 onward (Fig. 4B). In contrast, mice epicutaneously sensitized with Blo t 5 developed significant levels of Blo t 5-specific IgE and IgG1 later (day 22). Furthermore, both groups of mice exhibited similar profiles of IgE and IgG1 reactivity to Blo t 5 fragments. There was a stronger Ab reactivity to the N-terminal fragment Blo t 5₁₋₄₅, a weak reactivity to the C-terminal fragment Blo t 5₇₀₋₁₁₇, and no reactivity to the middle fragment Blo t 5₄₀₋₈₀ (Fig. 4C, 4D). In summary, this long-term-cultured Th2 cell line retained its helper function for Blo t 5-specific Ab production by B cells, and its kinetics of Blo t 5-specific IgE production was faster than that of Blo t 5-specific IgG1 production.

Airway inflammation induced by Blo t 5-specific Th2 cell line

The ability of Blo t 5-specific Th2 cells to induce lung inflammation was studied by adoptive cell transfer of Blo t 5-specific Th2 cells or PBS, followed by i.n. Blo t 5 challenges. Mice that received adoptive transfer of Blo t 5-specific Th2 cells had a 20-fold increase in the total infiltrating cells in BALF compared with control mice. More than 60% of these infiltrated cells in BALF were eosinophils; numbers of lymphocytes, monocytes, and neutrophils were also significantly increased (Fig. 5A). In addition, histological examination demonstrated the existence of perivascular and peribronchial eosinophilic inflammation. Goblet cell hyperplasia and enhanced mucus production were also manifested in lungs of the recipient mice (Fig. 5B). Hence, the adoptive transfer of resting Blo t 5-specific Th2 cells could elicit the typical acute allergic lung inflammation upon i.n. Blo t 5 challenge.

Phenotypic analysis of the infiltrated lymphocytes showed that cellular infiltration into the lungs involved CD4⁺ T cells, as well as CD8⁺ T cells, NK cells (CD3⁺CD49b⁺), and NKT cells (CD3⁺CD49b⁺). There was ~2.5-fold more CD4⁺ T cells ($53.6 \pm 3.2\%$) than CD8⁺ T cells ($19.2 \pm 5.7\%$). Notably, the donor cells (CFSE⁺) accounted for only 3% of the total infiltrated CD4⁺ T cells (Fig. 6A), and there were similar numbers of NK ($9.7 \pm 0.9\%$) and NKT ($11.7 \pm 1.5\%$) cells infiltrated into the lungs of

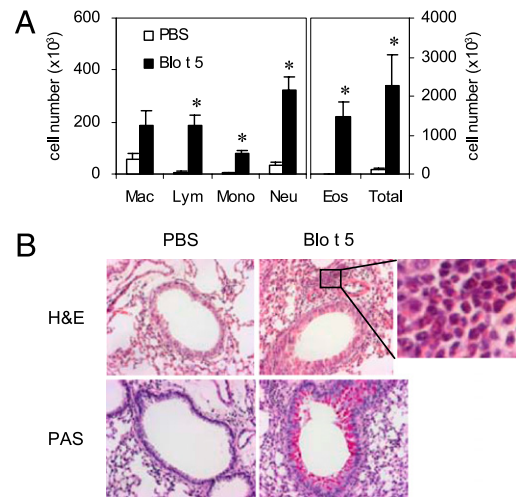


FIGURE 5. Induction of airway inflammation and lung histopathology by Blo t 5-specific Th2 cells. Naive C57BL/6J mice ($n = 4$) were given i.v. transfer with Blo t 5-specific Th2 cells (Blo t 5) or PBS on day 1, followed by 3 i.n. Blo t 5 challenges on days 2–4. BALF and lungs were collected on day 6. A, Differential cell counts for macrophages (Mac), lymphocytes (Lym), monocytes (Mono), neutrophils (Neu), and eosinophils (Eos) were calculated from cytospin preparations. Results are presented as mean $\pm$ SEM. * $p < 0.05$, compared with PBS group. B, Lung histopathology was assessed with H&E- or periodic acid-Schiff-stained paraffin-embedded sections (original magnification $\times 200$). The magnified microphotograph shows the infiltration of eosinophils into the perivascular area. Data are representative of three separate experiments.

these mice (Fig. 6B). Intracellular staining showed that the major IL-4-producing cells in BALF were the donor cells (CFSE⁺) ($7.8 \pm 1.2\%$), with a small contribution coming from nondonor CD4⁺ ($1.3 \pm 0.3\%$) and CD8⁺ ($2.7 \pm 0.2\%$) T cells. Approximately 13% of the infiltrated CD8⁺ T cells ($12.7 \pm 1.7\%$) expressed IFN- γ , whereas only 3% of infiltrated CD4⁺ T cells produced IFN- γ ($3.0 \pm 0.7\%$), suggesting that the CD8⁺ T cells could be the major IFN- γ -producing cells in BALF (Fig. 6C). The Th2 cytokines IL-4, IL-5, and IL-13, but not the Th1 cytokine IFN- γ , were elevated in BALF of recipient mice (Fig. 7B). The chemoattractants for polymorphic mononuclear cells (CXCL1/KC and CXCL5/LIX), lymphocytes (CXCL9/MIG, CXCL10/IP-10), and monocyte/macrophages (CCL2/MCP-1) were significantly increased in BALF of these mice (Fig. 7C). Dexamethasone significantly suppressed the Th2 cell-induced infiltration of eosinophils, lymphocytes, and monocytes but not neutrophils (Fig. 7A). Dexamethasone also significantly suppressed the Th2 cytokines and chemokines in BALF (Fig. 7B, 7C). A reduction in CXCL1/KC was observed, but this did not reach statistical significance. These results indicated that the allergic lung inflammation induced by Blo t 5-specific Th2 memory/effector cells was dexamethasone sensitive.

IL-35 attenuated Blo t 5-specific Th2 cell line-mediated allergic airway inflammation

To investigate whether IL-35, a relatively new regulatory cytokine, could attenuate the Blo t 5-specific Th2 cell line-induced lung inflammation, a plasmid DNA encoding recombinant single-chain IL-35 (pVAX-IL-35) was delivered into the airway by the intratracheal route. Mice administered pVAX-IL-35 (Fig. 8A) showed reduced numbers of infiltrated total cells, eosinophils, lymphocytes, and neutrophils (Fig. 8B), and significantly attenuated levels of Th2 effector cytokines, IL-4, IL-5, and IL-13, in BALF (Fig. 8C). Concomitantly with the suppressed Th2 cytokine

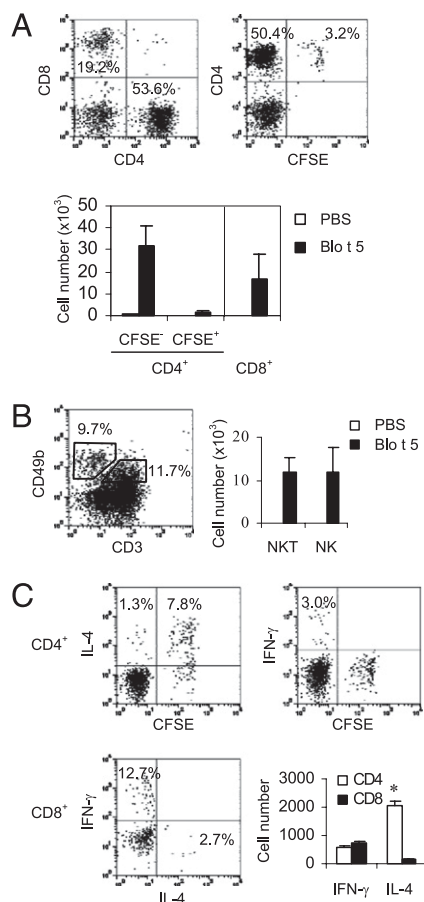


FIGURE 6. Cellular composition of infiltrated lymphocytes in BALF. Naive C57BL/6 mice ($n = 4$) were given i.v. transfer of CFSE-labeled Blo t 5-specific Th2 cells on day 1 and received two i.n. challenges with Blo t 5 or PBS on days 2 and 3. BALF was collected on day 5, and the infiltrated lymphocytes were analyzed by flow cytometry. **A**, Percentages of CD4⁺, CD8⁺, and CFSE⁺ cells among CD3⁺ cells. The numbers of infiltrated CD4⁺CFSE⁻, CD4⁺CFSE⁺, and CD8⁺ cells were calculated from the collected total cells in BALF. **B**, Percentages of NK (CD3⁻CD49b⁺) and NKT (CD3⁺CD49b⁺) cells were analyzed among the lymphocyte area, according to parameters of forward scatter versus side scatter. The numbers of infiltrated NK and NKT cells were calculated from collected total cells in BALF. **C**, Intracellular cytokine staining for IL-4- and IFN- γ -producing CD4 and CD8 T cells among CD3⁺ cells. The CD4⁺ T cells were further analyzed for CFSE⁺ and CFSE⁻ populations. The numbers of IL-4⁺ and IFN- γ ⁺ CD4 and CD8 cells were calculated from collected total cells in BALF. Results are presented as mean $\pm$ SEM. Data are representative of two separate experiments. $*p < 0.05$.

concentrations in BALF, CCL2/MCP-1, CXCL1/KC, CXCL5/LIX, CXCL9/MIG, and CXCL10/IP-10 concentrations in BALF were attenuated in mice treated with pVAX-IL-35 (Fig. 8D). Thus, locally expressed IL-35 suppressed Blo t 5-specific Th2 cell line-mediated lung inflammation by reducing Th2 effector cytokines and CC- and CXC-motif chemokines for the recruitment of polymorphic mononuclear cells and lymphocytes.

IL-35 suppressed Blo t 5-specific IgE and IgG1 and total serum IgE

To investigate whether systemic administration of IL-35 could suppress Blo t 5-specific Th2 cell-initiated Blo t 5-specific humoral responses, mice were injected i.m. with pVAX-IL-35 or pVAX twice before the adoptive transfer of Blo t 5-specific Th2 cells (Fig. 9A). Mice that received pVAX-IL-35 injection produced lower titers of Blo t 5-specific IgE on days 30, 37, and 84 (Fig. 9B)

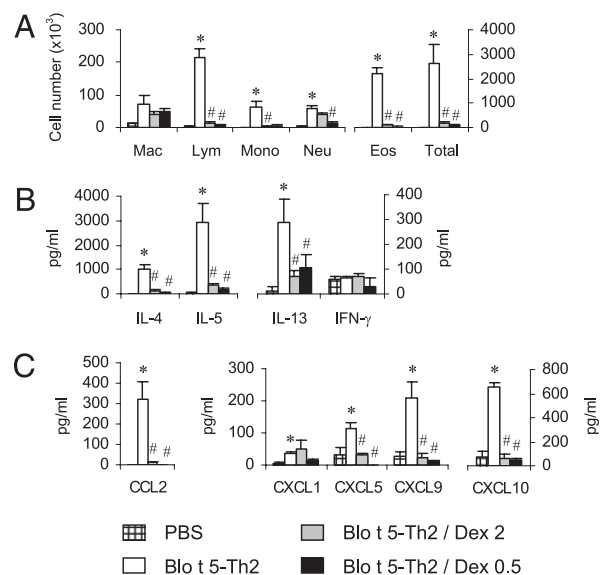


FIGURE 7. Dexamethasone suppression of the Blo t 5-specific Th2 cell-induced airway inflammation. Naive C57BL/6J mice ($n = 4$) were given i.v. transfer of Blo t 5-specific Th2 cells (Blo t 5-Th2) or PBS, followed by three i.n. Blo t 5 challenges. Dexamethasone was injected i.p. at doses of 0.5 mg/kg (Blo t 5-Th2/Dex 0.5) or 2 mg/kg (Blo t 5-Th2/Dex 2). **A**, Total and differential cell counts of infiltrated macrophages (Mac), lymphocytes (Lym), monocytes (Mono), neutrophils (Neu), and eosinophils (Eos) from BALF were calculated from cytospin preparations. **B**, ELISA cytokine profiles in BALF. **C**, Multiplex assay chemokine profiles in BALF. Results are presented as mean $\pm$ SEM. Data are representative of three separate experiments. $*p < 0.05$, compared with mice that received PBS; $\#p < 0.05$, compared with Blo t 5-Th2 group.

compared with pVAX-injected mice. Blo t 5-specific IgG1 production was persistently suppressed up to day 91 in the pVAX-IL-35-treated mice (Fig. 9C). Hence, systemic delivery of plasmid DNA encoding IL-35 provided the short-term suppression of Blo t 5-specific IgE production after the first course (on days 18–20) of i.n. Blo t 5 instillation, as well as the long-term suppression of Blo t 5-specific IgE production after the second course (on days 77–79) of i.n. Blo t 5 instillation, as evidenced by the persistence of significant suppression of Blo t 5-specific IgE at day 84 and IgG1 at both days 84 and 91.

To examine whether systemic administration of IL-35 had any effect on the production of Ig isotypes, total serum IgE, IgM, IgA, IgG1, and IgG2c were measured. There was a significant suppression of total serum IgE on days 84 and 91 in pVAX-IL-35-treated mice (Fig. 9D). In contrast, there was no significant effect on the levels of total serum IgG1 (Fig. 9E), IgM, IgA, or IgG2c for up to 3 mo (Supplemental Fig. 3). Therefore, the suppressive effect of IL-35 on the production of total serum IgE persisted into the second course of Blo t 5 i.n. challenges, which were performed on days 77–79. These results indicated that systemic IL-35 administration conferred a long-term suppression of Blo t 5-specific and total IgE production without exerting a pan immunosuppressive effect on other Ab isotypes.

Discussion

In the current study, we generated a long-term oligoclonal Th2 cell line from splenocytes obtained from mice epicutaneously sensitized with Blo t 5 allergen. Upon Blo t 5 activation, this cell line predominantly produced high levels of Th2 cytokines IL-4, IL-5, IL-13, and IL-10 (Fig. 1B) but not IL-9 or TNF- α (data not shown). Hence, this cell line was IL-10-secreting conventional Th2 cells not thymic stromal lymphopoietin-induced TNF- α –

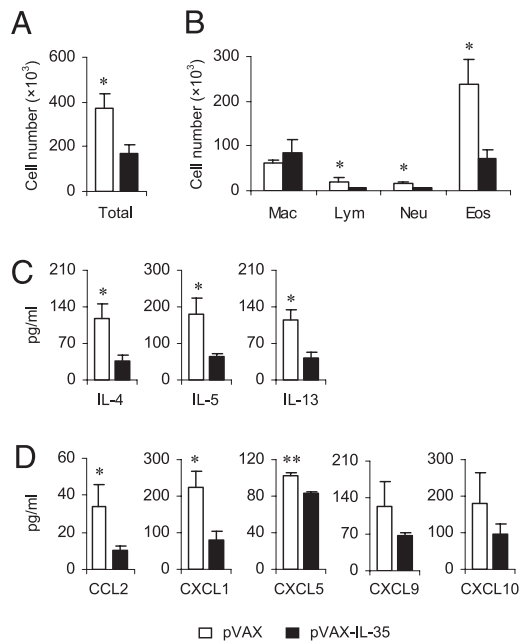


FIGURE 8. Suppressive effects of pVAX-IL-35 on Blo t 5-specific Th2 cell-induced airway inflammation. C57BL/6 mice ($n = 6-7$) were given an intratracheal instillation of pVAX control or pVAX-IL-35 prior to Blo t 5-specific Th2 cell transfer and Blo t 5 challenges. Total cell count (A) and differential cell counts for macrophages (Mac), lymphocytes (Lym), neutrophils (Neu), and eosinophils (Eos) (B) in BALF were analyzed. Cytokines (C) and chemokines (D) in BALF were analyzed by multiplex assays. Results are presented as mean $\pm$ SEM. Data are representative of two separate experiments. * $p < 0.05$, ** $p < 0.005$.

secreting inflammatory Th2 cells (28). It is known that in vivo activation of CD4⁺ T cells upon Ag exposure results in extensive proliferation and differentiation into effector cells (29, 30). Most of these cells die rapidly, but a small fraction of effector cells persist as memory cells to confer enhanced immune responses against the same Ag. The resting Blo t 5-specific Th2 cells expressed high levels of CD44 without the expression of CD62L, indicating that these T cells are a classical memory/effector phenotype of CD4⁺ T cells (Fig. 3A) (26, 27). Previous studies demonstrated that CD62L⁺ memory T cells recirculated primarily through nonlymphoid tissues and preferentially entered peripheral tissues to mediate inflammatory reactions (31). Hence, the adoptively transferred CD44^{high}CD62L⁺ Blo t 5-specific Th2 cells may be readily recruited to the lungs upon i.n. Blo t 5 challenge to induce a rapid and robust allergic airway inflammation and pulmonary pathophysiological changes in the recipient mice (Fig. 5).

IL-7R α is normally expressed at high levels on naive T cells, and it is rapidly downregulated upon T cell activation (32) and re-expressed by a small fraction of effector T cells destined to survive as memory cells (33). A recent study showed that IL-2 could promote the re-expression of IL-7R α and, therefore, enhance CD4⁺ T cell memory (34). The expression of IL-7R α was downregulated on Blo t 5-specific Th2 cells upon activation and subsequently re-expressed on resting Blo t 5-specific Th2 cells (Fig. 3B). These observations further confirmed the memory phenotype of the Blo t 5-specific Th2 cells. Notably, the Blo t 5-specific Th2 memory cells expressed high levels of CD25 (IL-2R α) at the resting stage, and the expression of CD25 was upregulated upon activation (Fig. 3B). The high CD25 expression by these cells may explain, in part, why these cells could be maintained by exogenous IL-2 without the addition of IL-7 in vitro. We cannot rule out the possibility that the survival of these Blo t 5-

specific memory T cells was dependent on the endogenous IL-7 present in the cell cultures. A recent report demonstrated that IL-7R α expression on CD4⁺ memory colitic T cells is essential for the development and persistence of colitis, suggesting that targeting the IL-7/IL-7R α signaling pathway in the colitogenic CD4⁺ T cells could be a useful therapeutic strategy for the treatment of autoimmune diseases (35). It would be worthwhile to investigate whether IL-7R α -expressing CD4⁺ memory Th2 cells could play a similar role in the pathogenesis of allergic asthma.

This Blo t 5-specific memory Th2 cell line was oligoclonal in nature, because it expressed limited numbers of TCR V α genes and only the TCR V β 3 gene (Fig. 2B), and it predominantly recognized residues 55–70 of the Blo t 5 protein (Blo t 5_{55–70}) (Fig. 2A). Cell-transfer studies indicated that these Blo t 5-specific Th2 cells could be activated in vivo upon exposure to Blo t 5 and were capable of helping B cells to produce Blo t 5-specific Abs (Fig. 4A, 4B). Moreover, the short-term cultured T cells derived from epicutaneously sensitized mice (i.e., the splenic T cells stimulated with Blo t 5 for two rounds in vitro) showed that such T cells predominantly recognized peptides Blo t 5_{55–70} and Blo t 5_{58–73} (data not shown), indicating that Blo t 5_{55–70} is likely to be a physiological and an immunodominant epitope of Blo t 5 in C57BL/6 mice. Thus, the Th2 cell line recognized a physiological epitope, rather than a cryptic epitope, of Blo t 5.

It is known that CD44^{high}CD62L⁺ memory T cells can provide efficient and fast help to B cells, resulting in the production of high-affinity Ag-specific Abs (27). However, it is intriguing to note that mice that received the Th2 cell line transfer showed a faster kinetics in Blo t 5-specific IgE production than in IgG1 production. The mechanism underlying this rapid production of Blo t 5-specific IgE has yet to be resolved. It is reasonable to propose that allergen delivery through the nasal mucosa route may induce IgE production by the local mucosa B cells (36, 37) via a possible mechanism that involves a direct IgE class switch from IgM driven by a permissive microenvironment with high levels of IL-4 and IL-13 secreted by the activated effector memory Th2 cells upon i.n. Blo t 5 challenge (38, 39). However, a more conventional mechanism that involves a sequential IgE class switch from IgM or IgG in the systemic lymphoid organs cannot be ruled out (40). Further studies examining the ϵ germline transcript, SeS μ DNA switch circles, I ϵ and C ϵ RNA are required to understand whether the memory/effector Th2 cells possess an intrinsic ability to induce direct IgE class switch systemically or locally.

The Blo t 5-specific Th2 cell line induced a highly eosinophilic allergic airway inflammation. In addition to eosinophils, it induced the recruitment of lymphocytes, monocytes, and neutrophils to the lungs. Further analysis of the lymphocyte composition in BALF showed that Blo t 5-specific donor Th2 cells contributed to only a small percentage of the total infiltrating lymphocytes, consisting of CD4⁺ T cells, CD8⁺ T cells, NKT cells, and NK cells. This increased number of nondonor lymphocytes after i.n. allergen challenges could be a consequence of bystander activation of CD4⁺ T cells as a result of cross-talk between an Ag-specific stimulated CD4⁺ memory T response and heterologous CD4⁺ T cells, which involves IL-2 and IL-7 (41, 42). Cross-talk can also occur among other cell types, including alveolar macrophages, epithelial cells, fibroblasts, basophils, and pulmonary DCs. This form of cross-talk in the airway environment can robustly amplify the Th2 responses in vivo through IL-4, IL-25, IL-33, and thymic stromal lymphopoietin (43). In view of this, we reasoned that the recruitment of nondonor lymphocytes, monocytes, and polymorphs, such as eosinophils and neutrophils, into the airways probably occurred through the conducive cytokine/chemokine

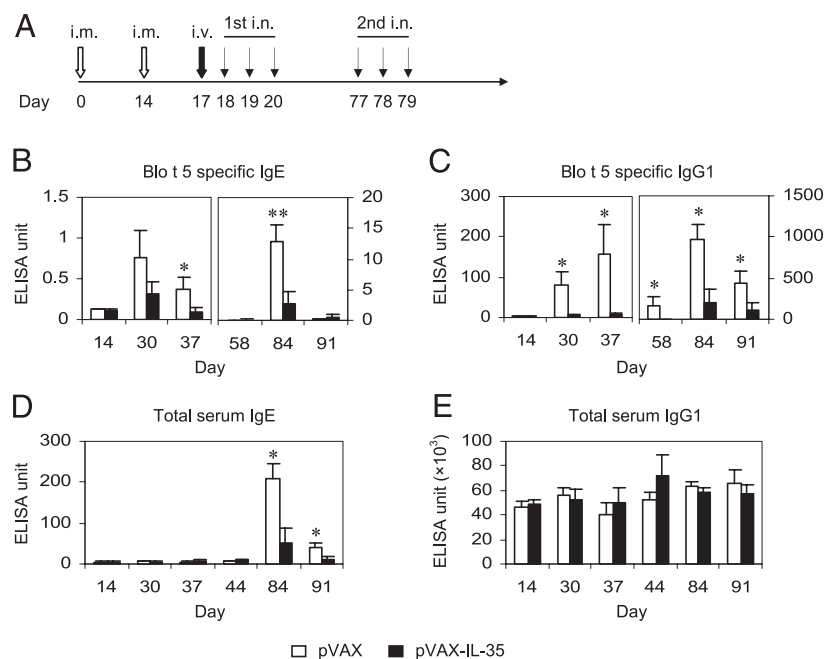


FIGURE 9. Effects of pVAX-IL-35 on humoral responses. A, Schematic diagram of animal experiment. Mice ($n = 7$) were given i.m. injection of pVAX or pVAX-IL-35 and received i.v. transfer of Blo t 5-specific Th2 cells, followed by two courses (1st and 2nd) of i.n. Blo t 5 challenges on the indicated days. The levels of Blo t 5-specific IgE (B) and IgG1 (C) and total serum IgE (D) and IgG1 (E) were assayed by ELISA. Results are presented as mean $\pm$ SEM. Data are representative of three separate experiments for results obtained until day 37 and two separate experiments for results obtained until day 91. * $p < 0.05$, ** $p < 0.001$.

microenvironment produced by the activated Th2 cells, eosinophils, or other innate and structural cells in lungs.

Development of new therapeutic strategies for the treatment of allergic asthma by targeting activation, differentiation, cell surface phenotype, migration capacity, effector function, and survival of Th2 memory cells has been explored (44). This Blo t 5-specific Th2 memory cell-induced mouse allergy model may serve as a useful system for exploration and evaluation of new therapeutics. In this study, this model gave us leverage to explore the feasibility of using IL-35 as a therapeutic cytokine for the treatment of allergic airway inflammation and suppression of allergen-specific IgE production. Recent studies revealed that IL-35 is a novel cytokine that is constitutively expressed in mouse CD4⁺CD25⁺ regulatory T cells (Tregs) and contributes to their suppressive function (45). The anti-inflammatory activity of IL-35 was evaluated in an experimental model of inflammatory bowel disease (45, 46). In addition to playing an immunosuppressive role in experimental inflammatory bowel disease, IL-35 was shown to attenuate the established collagen-induced arthritis in mice with concomitant suppression of IL-17 production (47, 48) and blocking of antitumor immunity (46). IL-35 seems to contribute to the regulatory microenvironment induced by Tregs. However, to our knowledge, there had been no prior study examining the effect of IL-35 on allergic diseases.

Studies on IL-35 have been hampered by the fact that production of purified rIL-35 is challenging, because the expression level is low, and the stability of the rIL-35 protein is problematic. This scenario prompted us to adopt both local and systemic plasmid DNA encoding IL-35 gene-delivery approaches to circumvent problems associated with the production of the rIL-35 protein. In this study, we demonstrated that pulmonary administration of pVAX-IL-35 effectively attenuated eosinophil and lymphocyte recruitment into BALF. Th2 effector cytokines, as well as the CC- and CXC-motif chemokines that were chemoattractants for polymorphic mononuclear cells and lymphocytes, were also reduced in BALF from mice that received pVAX-IL-35. It is known that IL-4 and IL-5 stimulate the production of CCL8/MIP-2 and CXCL1/KC, whereas IL-4 also induced higher CCL2/MCP-1 production (49). Hence, IL-35 may exert a suppressive effect in vivo by attenuating the levels of Th2 effector cytokines pro-

duced by the transferred Blo t 5-specific Th2 cell line, thus resulting in a decrease in the production of chemokines and, subsequently, reduced eosinophilia and cellular infiltration.

Mice that received the pVAX-IL-35 treatment prior to the transfer of the Blo t 5-specific Th2 memory cell line also showed a long-term suppression of allergen-specific IgE, IgG1, and total serum IgE production, without affecting concentrations of other Ig isotypes in sera (Fig. 9, Supplemental Fig. 3). This could be due, in part, to the long-lasting in vivo expression of IL-35 by the i.m. plasmid DNA delivery (50), hence providing a constitutive source of IL-35 and maintaining the long-term protection effect on Blo t 5-specific IgE and total IgE production even 2 mo later, upon the second course of i.n. challenges (Fig. 9B, 9D). An alternate possibility is that the initial IL-35 protein expression expanded the CD4⁺CD25⁺ Tregs or induced a new regulatory population of cells, iT_R35, that then conferred long-term protection on Blo t 5-specific IgE and total IgE production (46–48). Class switching from IgM to IgE is mainly regulated by the combined effect of two signals from Th2 cells. The first is IL-4/IL-13 and downstream STAT6 signaling, and the second is signaling from ligation of CD40L with CD40 on B cells (3). The suppressive effects of IL-35 on Blo t 5-specific IgE and total serum IgE may be through the inhibition of production of Blo t 5-specific Th2 effector cytokines IL-4 and IL-13, because these cytokine levels were reduced in BALF of pVAX-IL-35-treated mice (Fig. 8C). IL-13 also influences total serum IgE, as evidenced by a strong association between single nucleotide polymorphisms in the *IL-13* gene and total serum IgE in humans (51, 52). In addition, a single nucleotide polymorphism in the distal IL-13 promoter was demonstrated to have a functional effect on total serum IgE levels (53). Thus, the control of IL-13 levels may be directly or indirectly associated with total serum IgE.

In summary, a long-term Blo t 5-specific Th2 line has been established from in vivo polarized T cells. These cells were TCR V β 3⁺, secreted Th2 effector cytokines, and exhibited the effector memory phenotype. This Th2 cell line-induced allergic lung inflammation is reversible by dexamethasone treatment. Furthermore, administration of a plasmid encoding the new regulatory cytokine IL-35 exerted a potent suppressive effect on Th2 cell-mediated allergic lung inflammation, allergen-specific IgE and

IgG1 production, and total IgE production. These data suggested that IL-35 may be a potential therapeutic target for allergic airway inflammation. The current study opens new avenues to investigate the roles of CD62L⁺ memory/effector Th2 cells in the induction of airway inflammation and IgE production.

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Disclosures

The authors have no financial conflicts of interest.

References

- Lambrecht, B. N., and H. Hammad. 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity* 31: 412–424.
- Galli, S. J., M. Tsai, and A. M. Piliponsky. 2008. The development of allergic inflammation. *Nature* 454: 445–454.
- Bacharier, L. B., and R. S. Geha. 2000. Molecular mechanisms of IgE regulation. *J. Allergy Clin. Immunol.* 105: S547–S558.
- Gould, H. J., and B. J. Sutton. 2008. IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8: 205–217.
- Wang, Y. H., and Y. J. Liu. 2009. Thymic stromal lymphopoietin, OX40-ligand, and interleukin-25 in allergic responses. *Clin. Exp. Allergy* 39: 798–806.
- Larché, M., D. S. Robinson, and A. B. Kay. 2003. The role of T lymphocytes in the pathogenesis of asthma. *J. Allergy Clin. Immunol.* 111: 450–463, quiz 464.
- Yurovsky, V. V., E. J. M. Weersink, S. S. Meltzer, W. C. Moore, D. S. Postma, E. R. Bleeker, and B. White. 1998. T-Cell repertoire in the blood and lungs of atopic asthmatics before and after ragweed challenge. *Am. J. Respir. Cell Mol. Biol.* 18: 370–383.
- Mojtabavi, N., G. Dekan, G. Stingl, and M. M. Epstein. 2002. Long-lived Th2 memory in experimental allergic asthma. *J. Immunol.* 169: 4788–4796.
- Barnes, P. J. 2008. Immunology of asthma and chronic obstructive pulmonary disease. *Nat. Rev. Immunol.* 8: 183–192.
- McKinstry, K. K., T. M. Strutt, and S. L. Swain. 2010. The potential of CD4 T-cell memory. *Immunology* 130: 1–9.
- Das, A. M., R. J. Flower, P. G. Hellewell, M. M. Teixeira, and M. Perretti. 1997. A novel murine model of allergic inflammation to study the effect of dexamethasone on eosinophil recruitment. *Br. J. Pharmacol.* 121: 97–104.
- Janssen, E. M., M. H. Wauben, E. H. Jonker, G. Hofman, W. Van Eden, F. P. Nijkamp, and A. J. Van Oosterhout. 1999. Opposite effects of immunotherapy with ovalbumin and the immunodominant T-cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 21: 21–29.
- Walter, D. M., J. J. McIntire, G. Berry, A. N. J. McKenzie, D. D. Donaldson, R. H. DeKruyff, and D. T. Umetsu. 2001. Critical role for IL-13 in the development of allergen-induced airway hyperactivity. *J. Immunol.* 167: 4668–4675.
- Randolph, D. A., C. J. L. Carruthers, S. J. Szabo, K. M. Murphy, and D. D. Chaplin. 1999. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J. Immunol.* 162: 2375–2383.
- Lee, S.-C., Z. H. Jaffar, K.-S. Wan, S. T. Holgate, and K. Roberts. 1999. Regulation of pulmonary T cell responses to inhaled antigen: role in Th1- and Th2-mediated inflammation. *J. Immunol.* 162: 6867–6879.
- Kumar, R. K., C. Herbert, and P. S. Foster. 2008. The “classical” ovalbumin challenge model of asthma in mice. *Curr. Drug Targets* 9: 485–494.
- Hammad, H., and B. N. Lambrecht. 2008. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 8: 193–204.
- Trompette, A., S. Divanovic, A. Visintin, C. Blanchard, R. S. Hegde, R. Madan, P. S. Thorne, M. Wills-Karp, T. L. Gioannini, J. P. Weiss, and C. L. Karp. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588.
- Kuo, I. C., N. Cheong, M. Trakultivakorn, B. W. Lee, and K. Y. Chua. 2003. An extensive study of human IgE cross-reactivity of Blo t 5 and Der p 5. *J. Allergy Clin. Immunol.* 111: 603–609.
- Tan, L. K., C. H. Huang, I. C. Kuo, L. M. Liew, and K. Y. Chua. 2006. Intramuscular immunization with DNA construct containing Der p 2 and signal peptide sequences primed strong IgE production. *Vaccine* 24: 5762–5771.
- Yi, F. C., N. Cheong, P. C. Shek, D. Y. Wang, K. Y. Chua, and B. W. Lee. 2002. Identification of shared and unique immunoglobulin E epitopes of the highly conserved tropomyosins in *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. *Clin. Exp. Allergy* 32: 1203–1210.
- Huang, C. H., I. C. Kuo, H. Xu, Y. S. Lee, and K. Y. Chua. 2003. Mite allergen induces allergic dermatitis with concomitant neurogenic inflammation in mouse. *J. Invest. Dermatol.* 121: 289–293.
- Ding, Y., S. V. Seow, C. H. Huang, L. M. Liew, Y. C. Lim, I. C. Kuo, and K. Y. Chua. 2009. Coadministration of the fungal immunomodulatory protein FIP-Fve and a tumour-associated antigen enhanced antitumour immunity. *Immunology* 128(1, Suppl.):e881–e894.
- Collison, L. W., M. R. Pillai, V. Chaturvedi, and D. A. A. Vignali. 2009. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J. Immunol.* 182: 6121–6128.
- Wolfowicz, C. B., T. HuangFu, and K. Y. Chua. 2003. Expression and immunogenicity of the major house dust mite allergen Der p 1 following DNA immunization. *Vaccine* 21: 1195–1204.
- Bingaman, A. W., D. S. Patke, V. R. Mane, M. Ahmadzadeh, M. Ndejmbi, S. T. Bartlett, and D. L. Farber. 2005. Novel phenotypes and migratory properties distinguish memory CD4 T cell subsets in lymphoid and lung tissue. *Eur. J. Immunol.* 35: 3173–3186.
- Tokoyoda, K., S. Zehentmeier, A. N. Hegazy, I. Albrecht, J. R. Grün, M. Löhning, and A. Radbruch. 2009. Professional memory CD4⁺ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity* 30: 721–730.
- Zhu, J., H. Yamane, and W. E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annu. Rev. Immunol.* 28: 445–489.
- Garcia, S., J. DiSanto, and B. Stockinger. 1999. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity* 11: 163–171.
- Jenkins, M. K., A. Khoruts, E. Ingulli, D. L. Mueller, S. J. McSorley, R. L. Reinhardt, A. Itano, and K. A. Pape. 2001. In vivo activation of antigen-specific CD4 T cells. *Annu. Rev. Immunol.* 19: 23–45.
- Sallusto, F., D. Lenig, R. Förster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708–712.
- Park, J.-H., Q. Yu, B. Erman, J. S. Appelbaum, D. Montoya-Durango, H. L. Grimes, and A. Singer. 2004. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity* 21: 289–302.
- Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4: 1191–1198.
- Dooms, H., K. Wolslegel, P. Lin, and A. K. Abbas. 2007. Interleukin-2 enhances CD4⁺ T cell memory by promoting the generation of IL-7R alpha-expressing cells. *J. Exp. Med.* 204: 547–557.
- Shinohara, T., Y. Nemoto, T. Kanai, K. Kameyama, R. Okamoto, K. Tsuchiya, T. Nakamura, T. Totsuka, K. Ikuta, and M. Watanabe. 2011. Upregulated IL-7 receptor (α) expression on colitogenic memory CD4⁺ T cells may participate in the development and persistence of chronic colitis. *J. Immunol.* 186: 2623–2632.
- Cameron, L., A. S. Gounni, S. Frenkiel, F. Lavigne, D. Vercelli, and Q. Hamid. 2003. S epsilon10 S mu and S epsilon10 S gamma switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for direct as well as sequential class switch recombination. *J. Immunol.* 171: 3816–3822.
- Takhar, P., L. Smurthwaite, H. A. Coker, D. J. Fear, G. K. Banfield, V. A. Carr, S. R. Durham, and H. J. Gould. 2005. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. *J. Immunol.* 174: 5024–5032.
- Gauchat, J. F., D. A. Leberman, R. L. Coffman, H. Gascan, and J. E. de Vries. 1990. Structure and expression of germ-line epsilon transcripts in human B cells induced by interleukin 4 to switch to IgE production. *J. Exp. Med.* 172: 463–473.
- Punnonen, J., G. Aversa, B. G. Cocks, A. N. McKenzie, S. Menon, G. Zurawski, R. de Waal Malefyt, and J. E. de Vries. 1993. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acad. Sci. USA* 90: 3730–3734.
- Zhang, K., F. C. Mills, and A. Saxon. 1994. Switch circles from IL-4-directed epsilon class switching from human B lymphocytes. Evidence for direct, sequential, and multiple step sequential switch from mu to epsilon Ig heavy chain gene. *J. Immunol.* 152: 3427–3435.
- Di Genova, G., N. Savelyeva, A. Suchacki, S. M. Thirdborough, and F. K. Stevenson. 2010. Bystander stimulation of activated CD4⁺ T cells of unrelated specificity following a booster vaccination with tetanus toxoid. *Eur. J. Immunol.* 40: 976–985.
- Boyman, O. 2010. Bystander activation of CD4⁺ T cells. *Eur. J. Immunol.* 40: 936–939.
- Paul, W. E., and J. Zhu. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* 10: 225–235.
- Epstein, M. M. 2006. Targeting memory Th2 cells for the treatment of allergic asthma. *Pharmacol. Ther.* 109: 107–136.
- Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450: 566–569.
- Collison, L. W., V. Chaturvedi, A. L. Henderson, P. R. Giacomini, C. Guy, J. Bankoti, D. Finkelstein, K. Forbes, C. J. Workman, S. A. Brown, et al. 2010. IL-35-mediated induction of a potent regulatory T cell population. *Nat. Immunol.* 11: 1093–1101.
- Niedbala, W., X. Q. Wei, B. Cai, A. J. Hueber, B. P. Leung, I. B. McInnes, and F. Y. Liew. 2007. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur. J. Immunol.* 37: 3021–3029.
- Kochetkova, I., S. Golden, K. Holderness, G. Callis, and D. W. Pascual. 2010. IL-35 stimulation of CD39⁺ regulatory T cells confers protection against collagen II-induced arthritis via the production of IL-10. *J. Immunol.* 184: 7144–7153.
- Li, L., Y. Xia, A. Nguyen, Y. H. Lai, L. Feng, T. R. Mosmann, and D. Lo. 1999. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J. Immunol.* 162: 2477–2487.

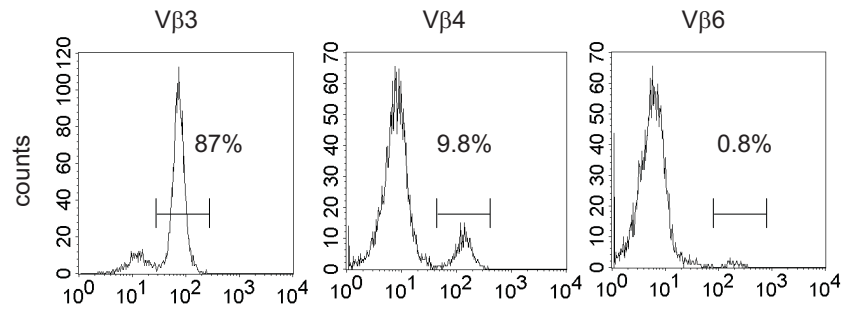
50. Chua, K. Y., I.-C. Kuo, and C.-H. Huang. 2009. DNA vaccines for the prevention and treatment of allergy. *Curr. Opin. Allergy Clin. Immunol.* 9: 50–54.
51. Graves, P. E., M. Kabesch, M. Halonen, C. J. Holberg, M. Baldini, C. Fritzsche, S. K. Weiland, R. P. Erickson, E. von Mutius, and F. D. Martinez. 2000. A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. *J. Allergy Clin. Immunol.* 105: 506–513.
52. Maier, L. M., J. M. M. Howson, N. Walker, G. P. Spickett, R. W. Jones, S. M. Ring, W. L. McArdle, C. E. Lowe, R. Bailey, F. Payne, et al. 2006. Association of IL13 with total IgE: evidence against an inverse association of atopy and diabetes. *J. Allergy Clin. Immunol.* 117: 1306–1313.
53. Kiesler, P., A. Shakya, D. Tantin, and D. Vercelli. 2009. An allergy-associated polymorphism in a novel regulatory element enhances IL13 expression. *Hum. Mol. Genet.* 18: 4513–4520.

Data Supplement

Supplementary Table 1. Primers for PCR amplification of the TCR β gene

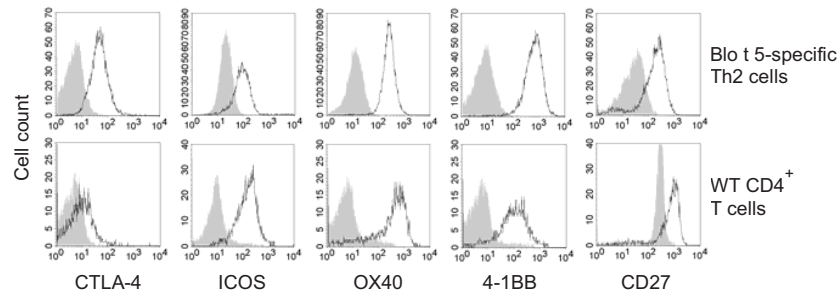
Name	Primer sequence (5' to 3')
TCR(V β 3.1)F	CGA <u>CTCGAG</u> GCC ACC ATG GCT ACA AGG CT
TCR(J β 1.1)R	CGC <u>ATCGAT</u> TTA CCT ACA ACT GTG AGT CTG GTT C
TCR(J β 1.2)R	CGC <u>ATCGAT</u> TTA CCT ATT ACC AAA AGC CTG GTC C
TCR(J β 1.3)R	CGC <u>ATCGAT</u> TTA CCT ACA ACA ATG AGC CGG CTT C
TCR(J β 1.4)R	CGC <u>ATCGAT</u> ATA CCC AGG ACA GAC AGC TTG GTT C
TCR(J β 1.5)R	CGC <u>ATCGAT</u> TTA CCT AGA ACA GAG AGT CGA GTC C
TCR(J β 1.6)R	CGC <u>ATCGAT</u> ATA CCT GTC ACA GTG AGC CGG GTG
TCR(J β 2.1)R	CGC <u>ATCGAT</u> TTA CCT AGG ACG GTG AGT CGT GTC
TCR(J β 2.2)R	CGC <u>ATCGAT</u> TTA CCC AGC ACT GTC AGC TTT GAG
TCR(J β 2.3)R	CGC <u>ATCGAT</u> TTA CCG AGA ACA GTC AGT CTG GTT C
TCR(J β 2.4)R	CGC <u>ATCGAT</u> TTA CCT AGC ACC GAT AGT CGG GTG
TCR(J β 2.5)R	CGC <u>ATCGAT</u> TCA CCT AAC ACG AGG AGC CGA GTG
TCR(J β 2.7)R	CGC <u>ATCGAT</u> TTA CCT AAA ACC GTG AGC CTG GTG

Supplementary Figure S1



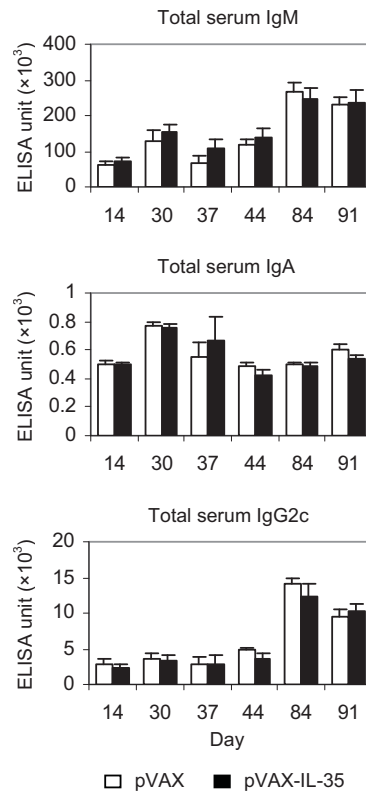
Supplementary Figure S1. Analysis of TCRVβ gene usage of the Blo t 5-specific Th2 cell line after six months of *in vitro* culture. The TCRVβ gene usage of the Blo t 5-specific Th2 cell line was determined by staining with a panel of Vβ specific mAbs and analyzed by flow cytometry. The Blo t 5-specific Th2 cell line was stained positive for mAbs against Vβ3, Vβ4 and Vβ6 at 87%, 9.8% and 0.8% respectively. All the rest mAbs showed no positive staining after Blo t 5-specific Th2 cells had been repeated re-stimulated with Blo t 5 and APCs for 6 months. These data are representative of two separate experiments on different cycles of T cell cultures.

Supplementary Figure S2



Supplementary Figure S2. Profiles of co-stimulatory cell surface markers on resting and activated Blo t 5-specific Th2 cell line. Blo t 5-specific Th2 cells and naive CD4⁺ T cells from C57BL/6 mice (WT) were activated with mAbs against mouse CD3 ϵ and CD28. The expression of cell surface markers CD152 (CTLA-4), CD278 (IOCS), CD134 (OX40), CD137 (4-1BB) and CD27 was analyzed by flow cytometry before (grey histograms) and after (black lines) T cell activation at 48 hours. Data are representative of three separate experiments.

Supplementary Figure S3



Supplementary Figure S3. Total serum immunoglobulins from mice treated with pVAX and pVAX-IL-35. The experimental protocol is the same as in Figure 9A. Sera were collected on indicated days and analyzed by sandwich ELISA. Results are presented as mean $\pm$ SEM ($n = 7$). Data are the representative of three separate experiments for results obtained within day 37 and two separate experiments for results obtained till day 91.