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Journal

The Journal of Immunology, 197(10)

ISSN

0022-1767

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Publication Date

2016-11-15

DOI

10.4049/jimmunol.1502676

Peer reviewed

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Author manuscript

J Immunol. Author manuscript; available in PMC 2022 March 09.

Published in final edited form as:

J Immunol. 2016 November 15; 197(10): 3771–3781. doi:10.4049/jimmunol.1502676.

Blomia tropicalis–Specific TCR Transgenic Th2 Cells Induce Inducible BALT and Severe Asthma in Mice by an IL-4/IL-13–Dependent Mechanism

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Abstract

Previous studies have highlighted the importance of lung-draining lymph nodes in the respiratory allergic immune response, whereas the lung parenchymal immune system has been largely neglected. We describe a new in vivo model of respiratory sensitization to *Blomia tropicalis*, the principal asthma allergen in the tropics, in which the immune response is focused on the lung parenchyma by transfer of Th2 cells from a novel TCR transgenic mouse, specific for the major *B. tropicalis* allergen Blo t 5, that targets the lung rather than the draining lymph nodes. Transfer of highly polarized transgenic CD4 effector Th2 cells, termed BT-II, followed by repeated inhalation of Blo t 5 expands these cells in the lung >100-fold, and subsequent Blo t 5 challenge induced decreased body temperature, reduction in movement, and a fall in specific lung compliance unseen in conventional mouse asthma models following a physiological allergen challenge. These mice exhibit lung eosinophilia; smooth muscle cell, collagen, and goblet cell hyperplasia; hyper IgE syndrome; mucus plugging; and extensive inducible BALT. In addition, there is a fall in total

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Disclosures

The authors have no financial conflicts of interest.

The online version of this article contains supplemental material.

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lung volume and forced expiratory volume at 100 ms. These pathophysiological changes were substantially reduced and, in some cases, completely abolished by administration of neutralizing mAbs specific for IL-4 and IL-13 on weeks 1, 2, and 3. This IL-4/IL-13–dependent inducible BALT model will be useful for investigating the pathophysiological mechanisms that underlie asthma and the development of more effective drugs for treating severe asthma.

The worldwide frequency of asthma has nearly doubled over the past 30 y and continues to rise in emerging economies (1). Drugs such as β agonists, corticosteroids, and, more recently, leukotriene antagonists have provided substantial clinical benefit for many asthmatics but are not effective in all patients (2) and struggle to control severe asthma in some patients (3, 4). Traditionally, OVA, an egg allergen, has been used in animal asthma models (5, 6) but have largely been replaced by respiratory allergens such as *Dermatophagoides pteronyssinus* (7–9), cockroach (10, 11), and *Blomia tropicalis* (12–14). Although these newer models are more closely aligned to human disease, they still do not recapitulate all of the features of asthma.

B. tropicalis is a storage mite that is widely present in homes, offices, and factories (15) in tropical or subtropical regions (16, 17). It is the major asthma allergen—comparable in importance to, or of greater importance than, *D. pteronyssinus*—in countries such as Colombia (18), Brazil (19), Singapore (20), China (21), and Venezuela (22), as well as in the southern United States (23). Blo t 5 is a 14-kDa protein that is the major *B. tropicalis* allergen (24). Recombinant Blo t 5 protein has been synthesized in bacteria (*Escherichia coli*) (24), yeast (*Pichia pastoris*) (20), and Chinese hamster ovary cells (25).

We have generated a novel TCR transgenic mouse (BT-II) whose CD4 T cells recognize a peptide (55–70) of Blo t 5. These cells use Vα11.1/11.2 and Vβ3 chains of the TCR that are coexpressed on a high percentage (>95%) of CD4 T cells. Polarization of these cells in vitro yielded Th2 cells that expressed GATA-3 (>90%), but not T-bet (<1%), and secreted large amounts of the Th2 cytokines IL-5 and IL-13. Transfer of these cells to naive mice, followed by thrice-weekly intranasal (i.n.) challenge with recombinant Blo t 5, induced airway inflammation and hyperresponsiveness. This response was restricted to the lungs, with 10 times fewer transgenic T cells migrating to the draining lymph node. The inflammatory pattern observed after a second week of exposure was severe, and mice exhibited respiratory symptoms within 15 min of in exposure to Blo t 5. Twenty-four hours after challenge, bronchoalveolar lavage (BAL) on week 3 shows that eosinophil levels average 4×10^6 per lung. This finding is paralleled by extreme pathological changes in the lung, akin to what is seen in patients who die of asthma, including mucus hypersecretion and plugging, formation of inducible BALT (iBALT), extensive airway restructuring, alveolitis, and increased collagen and smooth muscle deposition. The inflammatory response was still evident after 6 wk. Thus this transgenic mouse model may be useful to study the underlying pathogenesis of severe asthma and, by extension, to evaluate novel therapeutic modalities that have been developed for its treatment.

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Materials and Methods

Mice

Sex- and age-matched 8- to 10-wk-old specific pathogen–free C57BL/6J mice were bred at the Department of Comparative Medicine, National University of Singapore. Blo t 5 CD4 TCR transgenic mice (BT-II) were prepared from a Blo t 5–specific TCR cloned into a cassette provided by Diane Mathis, Harvard University, and generated by Level Biotechnologies, Taiwan. Cloning of the α β TCR and insertion into plasmids and generation of transgenic mice are detailed in Supplemental Figs. 1–4. Mice were maintained at a temperature between 20 and 26°C and 30–70% humidity, with a mean temperature of 23°C and 50% humidity. All experiments were conducted in accordance with institutional guidelines and were approved by the National University of Singapore Institutional Animal Care and Use Committee under protocol number 015/12.

Polarization of BT-II and OT-II CD4 T cells

Single-cell suspensions from BT-II or OT-II mouse lymph nodes were incubated with anti-CD4 MicroBeads isolated on a MACS LS column (Miltenyi Biotec, Singapore). Splenic dendritic cells (DCs) were digested with Liberase Cl (Roche, Switzerland) at 37°C for 30 min and isolated by centrifugation over OptiPrep Density Gradient Medium (Sigma-Aldrich, St Louis, MO), incubated with anti-mouse CD11c MicroBeads, and passed through a MACS LS column (Miltenyi Biotec). BT-II and OT-II CD4 T cells and splenic DCs were cocultured in 48-well plates with 10 μg/ml Blo t $5_{55–70}$ peptide (IIRELDVVCAMIEGAQ) or $OVA_{323–339}$, respectively (AnaSpec, Fremont, CA); 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ)1; 20 μg/ml anti-mouse IFN-γ; 20 μg/ml anti-mouse IL-12/IL-23 p40 (eBioscience, ImmunoCell, Singapore); and 20 μg/ml mouse IFN-γ R1/CD119 Ab (R&D Systems, Minneapolis, MN). Cells were restimulated on days 3, 7, and 11 with Blo t $5_{55–70}$, cytokines, and neutralizing Abs. Fresh DCs were added on day 7. Th2-polarized BT-II cells were harvested on day 14.

Adoptive transfer and i.n. Ag challenge

Th2-polarized BT-II cells (5×10^6 cells) were i.v. transferred into naive mice, and the next day 100μg recombinant Blo t 5 in 50μl sterile PBS was administered i.n. for three consecutive days. Mice were euthanized 1 d after the final challenge and blood, bronchoalveolar lavage (BAL), and lungs were harvested. Lungs were minced and digested in Liberase CI (Roche) at 37°C for 30 min and isolated over Ficoll-Paque (GE Healthcare Cleveland, OH).

Intracellular staining for transcription factors

Th2-polarized BT-II cells were stained with anti-mouse CD3 FITC (eBioscience) and anti-mouse CD4 PB (BD Biosciences, Franklin Lakes, NJ). After 30 min, cells were washed, then fixed and permeabilized by adding one part Fixation/Permeabilization Concentrate with three parts Fixation/Permeabilization Diluent for 1 h at 4°C, washed twice with 1× Permeabilization Buffer (eBioscience), and stained with anti-mouse GATA-3 eFluor 660 (BD Biosciences) and anti-mouse T-bet PE (eBioscience).

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CFSE labeling of BT-II Th2 cells

Th2 BT-II cells were labeled at 20 million cells/ml with CFSE (Life Technologies, Carlsbad, CA), diluted to 10 mM, at a 1:1 ratio at 37°C in the dark for 15 min, and the reaction was stopped with complete RPMI 1640 medium on ice for 5 min.

Recombinant Blo t 5 protein

The mRNA sequence of the gene encoding Blo t 5 is described in GenBank accession number U59102 (http://www.ncbi.nlm.nih.gov/nuccore/u59102). The construct was designed and codon optimized for expression in the *E. coli* bacteria by the OptimumGene algorithm (GenScript, Piscataway, NJ) and cloned into a pET28 expression vector (EMD Millipore, Billerica, MA). The Blo t 5 gene and the pET28 vector were digested with NcoI and BamHI, respectively. Following ligation and transformation into *E. coli* DH5α, colonies were screened by PCR and sequenced. Cells with DH5α and pET28-Blo t 5 were expanded and the plasmids purified using plasmid miniprep kits (QIAGEN, Germantown, MD) and transformed into *E. coli* BL21 for protein expression. *E. coli* cells transformed with pET28-Blo t 5 were grown in 1–1 cultures and induced to express recombinant Blo t 5 by the addition of isopropyl β-D-1-thiogalactopyranoside at the log phase. The soluble fraction of the bacterial lysate was precipitated with different concentrations of saturated ammonium sulfate separated on a MonoQ 5/50 GL anion exchange column (GE Healthcare).

Flow cytometric analysis of cells in BAL fluid and lungs

BAL was obtained using 3 × 0.7 ml cold PBS supplemented with 2% FCS (Biowest, Kansas City, MO) and 1% EDTA. Lungs were digested with Liberase CI (Roche). Both BAL and lung ceHs were stained with allophycocyanin anti-mouse Ly-6G (BioLegend, San Diego, CA), PE rat anti-mouse Siglec-F (BD Pharmingen), PetCP/Cy5.5 anti-mouse CDllc (BioLegend), and anti-mouse CD3 eEluor 450 (eBioscience). This staining panel distinguished neutrophils (Ly-6G^{+}CD11c) from eosinophils (Siglec-F^{+}CD11c^{-}) and macrophages (Ly-6G^{-}CD11c^{+}).

ELISAs

Cytokines IFN-γ, IL-5, IL-10, and IL-13 were measured using Mouse DuoSets (R&D Systems). IgE was measured using a Mouse IgE ELISA Set (BD Biosciences). The assays were carried out in accordance with the manufacturers' recommendations. Blo t 5–specific IgG1 was measured using microtiter wells coated with 100 μl recombinant Blo t 5 protein at a concentration of 5 μg/ml in carbonate buffer, pH 9.8, overnight at 4°C.

Lung function testing

Lung function was measured in two ways. In the first, it was measured as the change in airway resistance to increasing concentrations of nebulized methacholine (0.5–8.0 mg/ml) (Sigma-Aldrich). Mice were anesthetized, tracheostomized, and mechanically ventilated at a fixed breathing rate of 140 breaths/min using the FinePointe Series RC Sites (Buxco Research Systems, Wilmington, NC), and airway resistance and specific dynamic

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compliance were recorded. Results are expressed as a percentage of respective basal values in response to PBS.

The second used the Pulmonary Function Test Plethysmograph system (Buxco Research Systems) as recommended by the manufacturer (https://www.datasci.com/products/buxco-respiratory-products/pulmonary-function-testing). Mice were anesthetized with a mixture consisting of ketamine/medetomidine/saline. Sedation was ensured to be deep enough before the trachea was cannulatcd and connected to a built-in ventilator using a tracheal cannula. The data of breathing, airflow obstruction, and lung volumes were acquired using FinePointe software by measuring, and the parameters of total lung capacity (TLC) and forced expiratory volume were determined according to the manufacturer's recommendations. The results shown are TLC and forced expiratory volume at 100 ms (FEV100).

Effect of anti–IL-4/IL-13

To investigate the mechanism of the extreme inflammatory response, two neutralizing Abs specific for IL-4 (clone 1B11) and IL-13 (clone 1316H) (eBioscience) were administered as 50-μg doses i.v. and i.n. 1 h prior to the first i.n. Blot t 5 administration on days 2, 9, and 16.

Lung histology

Cardiac perfusion was performed with 20 ml PBS, and lungs were fixed in 4% paraformaldehyde (Sigma-Aldrich) for a week, dehydrated, and paraffin embedded. Sections (4 μm thick) were cut and stained with H&E, periodic acid–Schiff (PAS), and Masson’s trichrome (MTC) stains. To perform immunofluorescence staining, lung tissues were fixed with 2% paraformaldehyde in PBS with 30% sucrose at 4°C overnight and washed with PBS for 2 d at 4°C, then embedded in Optimum Cutting Temperature (OCT) compound (Sakura FineTek, Singapore); sections 5 μm thick were then cut on a cryostat (Leica, Singapore), air dried, and blocked with PBS containing 0.2% BSA. B cells were identified with rat anti-mouse B220 (1:200; eBioscience), T cells with Armenian hamster anti-mouse TcRβ (1:200; BD Pharmingen), and DCs with biotinylated Armenian hamster anti-CDllc (1:100; eBioscience) in PBS containing 1% normal mouse serum overnight at 4°C. Cy2-conjugated donkey anti-rat Ab (1:300; Jackson ImmunoResearch, West Grove, PA), Cy3-conjugated goat anti-hamster Ab, and donkey anti-avidin-labeled Ab (both 1:500; Jackson ImmunoResearch), respectively, were used for detection. Sections were counterstained with DAPI (KPL) and mounted with fluorescent mounting medium (Dako, Singapore) for analysis using a fluorescence microscope (AxioImager Z1, Zeiss, Singapore).

Lung scoring

Inflammation 0: normal (no inflammation); 1: <10%; 2: 10–29%; 3: 30–59%; 4: 60–89%; 5: 90–100% of the total number of airways in the whole section of lung are inflamed—also scored as a very severe disease state.

Airway remodeling 0: normal airways; 1: <10%; 2: 10–29%; 3: 30–59%; 4: 60–89%; 5: 90–100% of the total number of airways in the whole section of lung are remodeled—also scored as a very severe disease state.

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iBALT 0: normal (no iBALT) 1: <10%; 2: 10–29%; 3: 30–59%; 4: 60–89%; 5: 90–100% of the whole section of lung has iBALT.

Mucus (PAS) 0: normal (no mucus); 1: <10%; 2: 10–29%; 3: 30–59%; 4: 60–89%; 5: 90–100% of the total number of airways in the whole section of lung stain pink for mucus and 50% are mucus plugged—also scored as a very severe disease state.

Cell sorting and ex vivo assays

Lung cells were stained with anti-mouse CD3 allophycocyanin (eBioscience), anti-mouse CD4 PB (BD Pharmingen), anti-mouse Vα11.1, 11.2 FITC (BD Pharmingen), and anti-mouse Vβ3 PE (BD Pharmingen) and sorted into the transgenic (CD3CD4Vα11.1, 11.2^{+}V$\beta3^{+}$) and the nontransgenic (CD3CD4Vα11.1, 11.2^{-}V$\beta3^{-}$) subsets. Sorted cells were cultured with splenic DCs and recombinant Blo t 5 protein for 6 d, and supernatants assayed for IL-5, IL-10, and IL-13 by ELISA.

Statistical analysis

An unpaired two-tailed Student *t* test was used for comparison between two groups, and one-way ANOVA was used for comparison between multiple groups. Data are representative of at least two independent experiments, with three to four mice per group. Data are expressed as means ± SEM (*p* value range was indicated). Flow cytometric profiles and histological images are representations of repeated experiments.

Results

BT-II CD4 T cells assume an effector Th2 phenotype following polarization in vitro

Naive BT-II CD4 T cells were cultured with splenic DCs and Blo t $5^{55–70}$ for 14 d under Th2-polarizing conditions (IL-4, anti–IL-12, anti–IFN-γR, and anti–IFN-γ), as outlined in Fig. 1A. The genotype of the BT-II mice was examined by flow cytometry. Most (99%) of CD4 T cells coexpressed Vα11.1/11.2 and Vβ3 (Fig. 1B). Th2-polarized BT-II CD4 T cells expressed GATA-3 (92%), but not T-bet (0%) (Fig. 1C, 1D), and secreted large amounts of IL-5, IL-10, and IL-13 but little IFN-γ and no IL-17 (Fig. 1E). Th2-polarized BT-II cells were stained for CD62L, CD44, and CD45RB. More than 97% were $CD45RB^{Hi}$ and $CD62L^{Lo}$ (Fig. 1F), and 90% were $CD44^{Hi}$ and $CD62^{Lo}$ (Fig. 1G).

Adoptively transferred Th2-polarized BT-II CD4 T cells induce allergic lung inflammation and airway hyperresponsiveness

A total of 5×10^6 BT-II Th2 cells in sterile saline were transferred i.v. into C57BL6J mice that were restrained in a plastic tube and warmed with a heat lamp. Mice were subsequently challenged i.n. on 3 successive days with 100 μg recombinant Blo t 5, which induced significant eosinophilia in both the lung parenchyma and BAL (Fig. 1H). Challenge with incremental doses of methacholine increased airway resistance in Th2 cell–transferred and challenged mice (Fig. 1I), which also had increased numbers of peribronchial lymphocytes (Fig. 1J) and PAS-positive goblet cells (Fig. 1K).

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Adoptive transfer of BT-II Th2 cells followed by repeated i.n. exposure to recombinant Blo t 5 induced severe allergic asthma

Mice were transferred with 5×10^6 Th2-polarized BT-11 CD4 T cells and challenged i.n. with 100 μg Blo t 5 three times per week for 6 wk (Fig. 2A). In the second week, eosinophil numbers in BAL had increased to 2×10^6 per mouse and further increased to 4×10^6 per mouse at weeks 3 and 4 (Fig. 2B). This was paralleled by lung parenchymal eosinophils that rose to 12×10^6 per mouse on week 3 (Fig. 2C). Subsequently, BAL eosinophil numbers fell but were still >500,000 per mouse at week 6 (Fig. 2C). There was a smaller transient rise in neutrophils (Fig. 2D, 2E) that was not significant in the BAL and only significant in the lung on week 2. T lymphocytes in BAL also increased from week 1 to 3 when there were ~6×10^5 T lymphocytes per mouse (Fig. 2F). Serum IgE levels rose by 3 logs to 40 μg/ml (Fig. 2G), and IgG1 anti-Blo t 5 Abs rose to 40 μg/ml at week 2 (Fig. 2H). Both were still substantially elevated at week 6. CFSE-labeled adoptively transferred BT-11 Th2 cells were identified 24 h later in lung, spleen, and liver, with very few in lymph nodes (Fig. 2I), which is consistent with their effector memory phenotype (Fig. 1F, 1G).

In addition, on the first challenge of week 2, >80% of mice exhibited a lack of movement, a reduction in specific airway compliance (Fig. 2J), and a fall in body temperature (Fig. 2K) within 20 min of i.n. challenge. Thus mice transferred with BT-11 Th2 cells and i.n. challenged exhibited a severe allergic asthma–like airway response.

Both transgenic and nontransgenic T cell numbers rose 3 logs by week 2 but declined gradually after week 3 (Fig. 3A, 3B) and were still elevated at week 5. BT-11 T cells cultured ex vivo with DCs and Blo t 5 produced 8 ng/ml of IL-5 at week 2 (Fig. 3C). Nontransgenic T cells produced similar levels of cytokines (Fig. 3D). A similar pattern of response was seen for IL-10 (Fig. 3E, 3F) and IL-13 (Fig. 3G, 3H), which reached 3 and 50 ng/ml, respectively.

BT-II Th2 cells induced a severe asthma phenotype and iBALT formation upon repeated allergen exposure

H&E-stained sections obtained from the lungs of mice euthanized at week 3 revealed that the general alveolar architecture of the lungs was lost, with >80% of the whole section inflamed (Fig. 4A, 4B) and the lung wall altered (Fig. 4C, 4D). PAS-stained sections from these mice showed mucus plugging of the airways, and >20% of the airways were either mucus lined or mucus plugged (Fig. 4E, 4F). MCT-stained sections also indicated that more collagen was deposited around the airways of asthmatic than of naive mice (Fig. 4G).

Interestingly, extensive iBALT was observed around the airways and elsewhere, affecting >50% of the lung section. iBALT could be detected in the lung parenchyma (Fig. 5A) and around airways (Fig. 5B) and blood vessels (Fig. 5C). IBALT was examined in more detail using frozen sections. B cells ($B220^+$) were found in tight clusters and were closely associated with T cells ($TcR\beta^+$) (Fig. 5E) and DCs ($CD11c^+$) (Fig. 5F) as compared with their respective controls (Fig. 5G, 5H).

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OT-II Th2 cells induced a less severe and less persistent inflammatory response

To compare the response of an established CD4 TcR transgenic mouse that is specific for OVA, we polarized OT-II cells using the same protocol as for BT-II cells and transferred 5 $\times 10^6$ Th2-polarized OT-II CD4 T cells to naive C57BL6/J recipients that were challenged in groups of six thrice weekly for up to 6 wk (Supplemental Fig. 4A). Eosinophils in BAL increased to a maximum of 2.5×10^6 at week 2 but had declined to 2.5×10^5 by week 4 (Supplemental Fig. 4B). In contrast, the peak BAL eosinophil count (4×10^6) in the BT-II system occurred at week 4 (Fig. 2B). As for BT-II mice (Fig. 2D), there was no increase in BAL neutrophils after week 1 (Supplemental Fig. 4C). There was comparable expansion of BT-II (Fig. 2F) and OT-II (Supplemental Fig. 4D) T cells. As for BT-II mice (Fig. 2G), in which serum IgE increased from 100 ng/ml to 40 μg/ml, IgE levels in OT-II-transferred mice rose to 60 μg/ml (Supplemental Fig. 4E). However, the level of IgE fell much earlier in the OT-II–transferred mice. To examine the formation of iBALT in OT-II–transferred mice, lung sections were cut and stained at week 3 (Fig. 4A–C). iBALT was detectable compared with naive mice (Supplemental Fig. 4F, 4G) but to a much lesser extent than in the BT-II–transferred mice. Indeed, inflamation and airway remodeling were also much reduced in the OT-II–transferred mice.

Severely asthmatic mice are sensitive to treatment with mAbs to IL-4 and IL-13

To test the effect of neutralizing IL-4/IL-13 on the lung inflammatory response and the formation of iBALT, mice were transferred with 5×10^6 Th2-polarized BT-11 cells and challenged thrice weekly with 100 μg Blo t 5 i.n. for 3 wk. In addition, mice were administered 50 μg of anti–IL-4 and 50 μg of IL-13 i.n. and the same amounts i.v. before the first of the weekly challenges, as shown in Fig. 6A. Parameters of Th2 inflammation, including BAL (Fig. 6B) and lung eosinophilia (Fig. 6C), serum IgE (Fig. 6D), and Blo t 5–specific IgG1 (Fig. 6E), were substantially decreased in the mice treated with anti–IL-4/IL-13. BAL and lung neutrophilia (Fig. 6F, 6G), as well as BAL and lung T cells (Fig. 6H, 6I), were not significantly altered. Lung tissue sections from these mice were stained with H&E and PAS. Inflammation in the lungs of mice that received anti–IL-4/anti–IL-13 was reduced from >80 to <10% (Fig. 6J), airway remodeling from >80 to 10% (Fig. 6K), iBALT from 50 to <20% (Fig. 6L), and mucus secretion from 50 to <10% of the whole lung section (Fig. 6M). The alveolar architecture is preserved, although signs of lymphocyte infiltration still are evident with anti–IL-4/IL-13 treatment (Fig. 6N, 6O). Mucus plugging and goblet cell hyperplasia were substantially reduced in mice treated with anti–IL-4/IL-13 (Fig. 6P). Lung function was measured using the Buxco Pulmonary Function Test Plethysmograph system. The effect of neutralizing Abs on TLC (Fig. 6Q) and FEV100 (Fig. 6R) was determined and found to be reduced in sensitized and challenged mice. Treatment with anti–IL-4/IL-13 completely reversed this (Fig. 6Q, 6R). None of the mice treated with anti–IL-4/IL-13 exhibited respiratory or behavioral symptoms following challenge

Discussion

We have developed a mouse model of severe asthma by transfer of highly Th2-polarized respiratory allergen–specific transgenic CD4 T cells followed by repeated i.n. challenge with recombinant allergen. Key features of this response include highly elevated BAL and lung

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eosinophilia, mucus hypersecretion, extensive airway remodeling, bronchial plugging, and alveolitis. These changes are accompanied by peribronchial deposition of collagen fibers, smooth muscle hyperplasia, and iBALT formation. Transferred BT-11 Th2 cells expand >100-fold in the lung by week 2 and secrete large amounts of the Th2 cytokines IL-5, IL-10, and IL-13 when cultured ex vivo with Blo t 5 and DCs. Host lung CD4 T cells cultured in the same way secreted the same levels of these cytokines, indicating that the transferred T cells create conditions for priming endogenous CD4 T cells and polarizing them into Blo t 5–specific Th2 cells possibly through the formation of Th2 iBALT, although the direct effect of iBALT on the endogenous immune system has yet to be proved. Serum IgE and IgG1 Abs increased >1000-fold to 30–40 μg/ml. Neutralization with monoclonal anti–IL-4 and anti–IL-13 Abs administered at weekly intervals attenuated this response.

Adoptively transferred OVA-specific Th2 cells followed by OVA challenge has been shown to induce an asthma-like response in rats (26, 27) and mice (28–30). However, the magnitude of the allergic response is limited to ~4 γ 10^5 eosinophils in BAL and a modest increase in IgE. A limitation of asthma models using transferred T cells is that they fail to expand significantly in the adoptive host. Our study maximized polarization of Th2 cells by neutralizing Thl-inducing cytokines (IFN-γ and IL-12) and repeatedly stimulating the cells in the presence of IL-4. The BT-II Th2 cells generated expressed CD44 and CD45RB, but not CD62L. CD45RB is traditionally considered a marker of naive cells but has been described on Th2-polarized CD4 T cells (31–33) as well as on activated CD8 T cells (34). By week 2 the number of transgenic T cells had risen to 100,000 and by week 3 to 200,000–300,000 cells per lung. Such a dramatic in vivo expansion of Th2 cells has not previously been described and importantly was associated with extensive iBALT formation.

First described in rabbits (35, 36) and subsequently in mice (37), inducible iBALT has been reported in response to influenza infection and neonatal LPS (38). Furthermore, iBALT has been described in neonates and asthmatic children (39–41). Formation of iBALT is more frequently seen as a consequence of lung infection and may be dependent on IL-17 (42), although IL-17-independent iBALT has also been described (43). Regulatory T cells have been shown to inhibit iBALT (44) via a neutrophil-dependent process. In our study we observed extensive iBALT formation, with >50% of the airways in the whole lung sections affected. We observed iBALT proximal to airways and blood vessels but also seemingly independent of these in the lung parenchyma. Th2-associated iBALT has rarely been reported in asthma models (45), and there has been no investigation of its composition or the cytokines required to induce it.

A new therapeutic mAb to the common IL-4Rα-chain of the receptors for human IL-4 and IL-13 (Duplimab) has been reported to reduce inflammation in patients with atopic dermatitis (46–48) and those with moderate-to-severe asthma (49). We carried out experiments to inhibit IL-4 and IL-13 using two mAbs known to neutralize these cytokines and observed that mice that received Abs exhibited significantly decreased inflammation in all parameters except for the neutrophilia and the numbers of T lymphocytes. Histological examination of the lung sections from mice treated with anti–IL-4/anti–IL-13 revealed that these treatments were effective against eosinophilia, IgE and IgG1 anti-Blo t 5, airway

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remodeling, and iBALT formation. Thus it appears that in an allergic asthmatic response Th2 cytokines can cause iBALT formation.

In our study, we have developed a new mouse model of severe asthma that results in extensive lung inflammation, a strong adoptive and host Th2 response, airway remodeling, and the formation of IL4/IL-13-dependent iBALT. Mice transferred with highly polarized Th2 cells and i.n. exposed to the corresponding allergen exhibit respiratory symptoms when challenged i.n., a response, to our knowledge, that is hitherto unseen in previously described mouse asthma models. This study provides a new model of asthma that may help discriminate between drugs that do and do not work for severe and hard-to-treat asthma. In addition, our T cell transfer-challenge model sheds new light on the mechanism underlying Th2 iBALT formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

All gene-modified mice were bred under the Life Sciences Institute transgenic core breeding program. All flow cytometry and cell sorting were carried out in the Life Sciences Institute flow laboratory with assistance from Dr. P Hutchinson and G.H. Teo. Technical support was received from G.H. Soh, H.M. Wen, and L.H. Chua in the Department of Pediatrics, Yong Loo Lin School of Medicine, Singapore.

This work was supported by the National Medical Research Council (Grant NMRC 1321/2012), the Life Sciences Institute, the National University of Singapore (to D.M.K.), the Yong Loo Lin School of Medicine (to D.M.K., P.A.M., K.Y.C., Y.Z., W.S.P.W., and YA,), and the National Research Foundation National University of Singapore-Hebrew University of Jerusalem Programme for Inflammation (to D.M.K., H.S.W., and Q.Z.).

Abbreviations used in this article:

BAL	bronchoalveolar lavage
DC	dendritic cell
FEV100	forced expiratory volume at 100 ms
iBALT	inducible BALT
i.n.	intranasal(ly)
MTC	Masson's trichrome
PAS	periodic acid-Schiff

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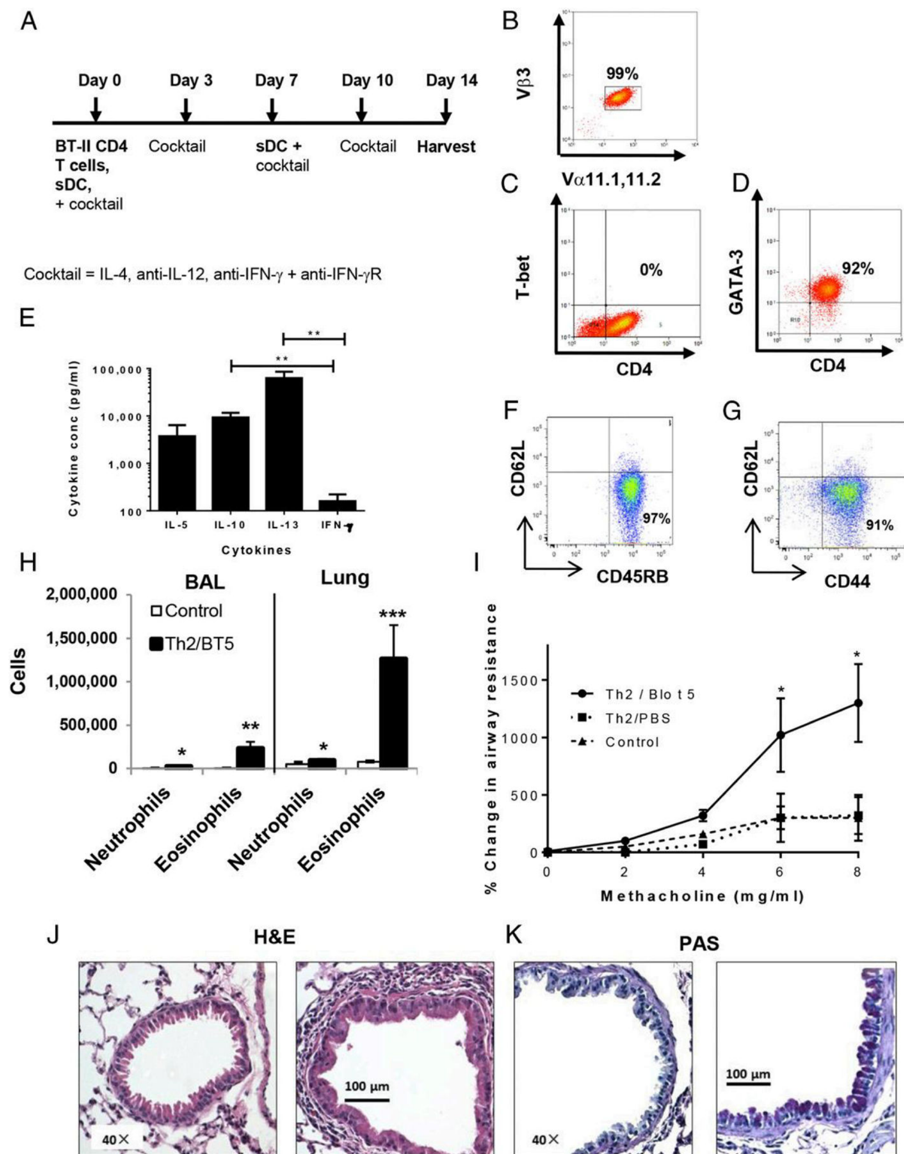

FIGURE 1.

Transfer of Th2-polarized BT-II CD4 T cells to C57BL6 mice, followed by allergen challenge, induced an asthma phenotype. (**A**) Experimental scheme. Mixture = anti-IFN-γ (20 mg/ml), anti-IFN-γR (20 mg/ml), anti–IL-12 (20 mg/ml), and IL-4 (10 ng/ml). (**B**) Expression of Vβ3, Vαil.1/11.2 by BT-II cells. Th2-polarized BT-II T cell expression of T-bet (**C**) and GATA-3 (**D**), and cytokines secreted by BT-II Th2 cells cultured with Blot t 5 (**E**). BT-II Th2 cells expressed low levels of CD62L and high levels of CD45RB (**F**) and CD44 (**G**). (**H**) BAL and lung eosinophils. The response of mice to methacholine challenge (**I**). Peribronchial mononuclear cell infiltration (**J**) and mucus secretion (**K**). The data shown are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student *t* test.

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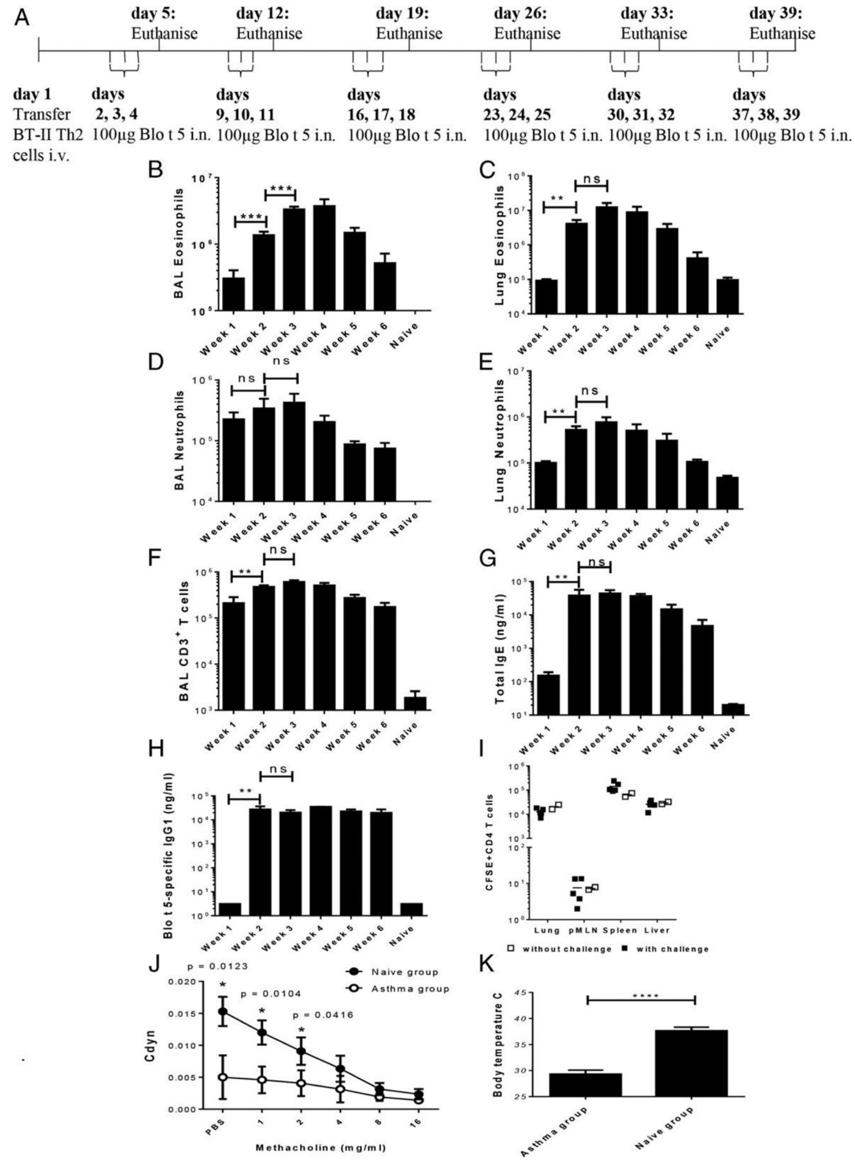

FIGURE 2.

Repeated challenge of Th2-polarized BT-II-transferred mice with Blo t 5 produced an extreme allergic response. Mice were transferred with Th2-polarized BT-IIT cells, as shown in the scheme (**A**). Eosinophils in BAL (**B**) and lung (**C**); neutrophils in BAL (**D**) and lung (**E**). T cells in BAL (**F**), total serum IgE (**G**), and serum Blo t 5-specific IgG1 (**H**) in BT-II Th2-transferred and Blo t 5-challenged mice. CFSE-labeled Th2-polarized BT-II CD4 T cells transferred 24 h earlier in lung, posterior mediastinal lymph node (pMLN), spleen, and liver (**I**). Specific compliance on week 3 (**J**) and body temperature 15 min after first challenge of week 2 (**K**). The data shown are representative of two to four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $p < 0.0001$, Student t test.

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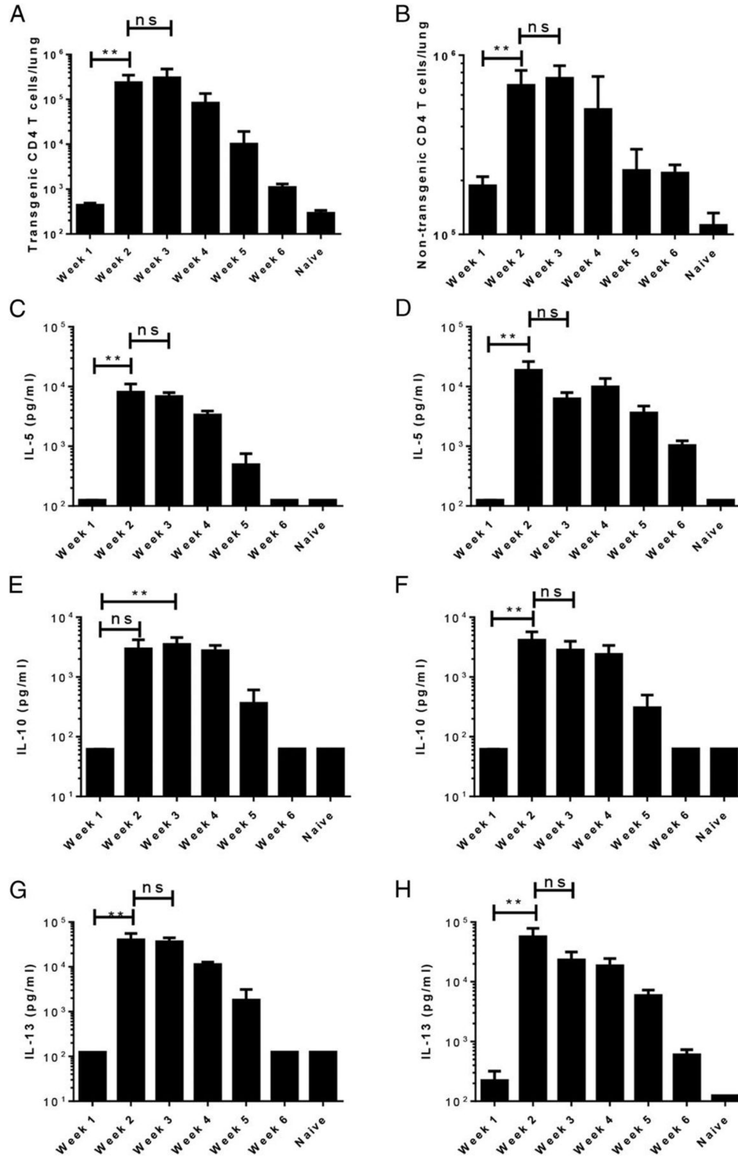

FIGURE 3.

Repeated allergen challenge to BT-II Th2-transferred mice expands transgenic and nontransgenic T cells that contribute equally to Th2 cytokine production. Transgenic (**A**) and nontransgenic (**B**) CD4 T cells in the lung. Cytokines secreted following 6-d culture with Blo t 5 and splenic CDllc^{+} DCs and both transgenic and nontransgenic CD4 T cells, IL-5 (**C** and **D**), IL-10 (**E** and **F**), and IL-13 (**G** and **H**). The data shown are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student *t* test. Cdyn, dynamic compliance.

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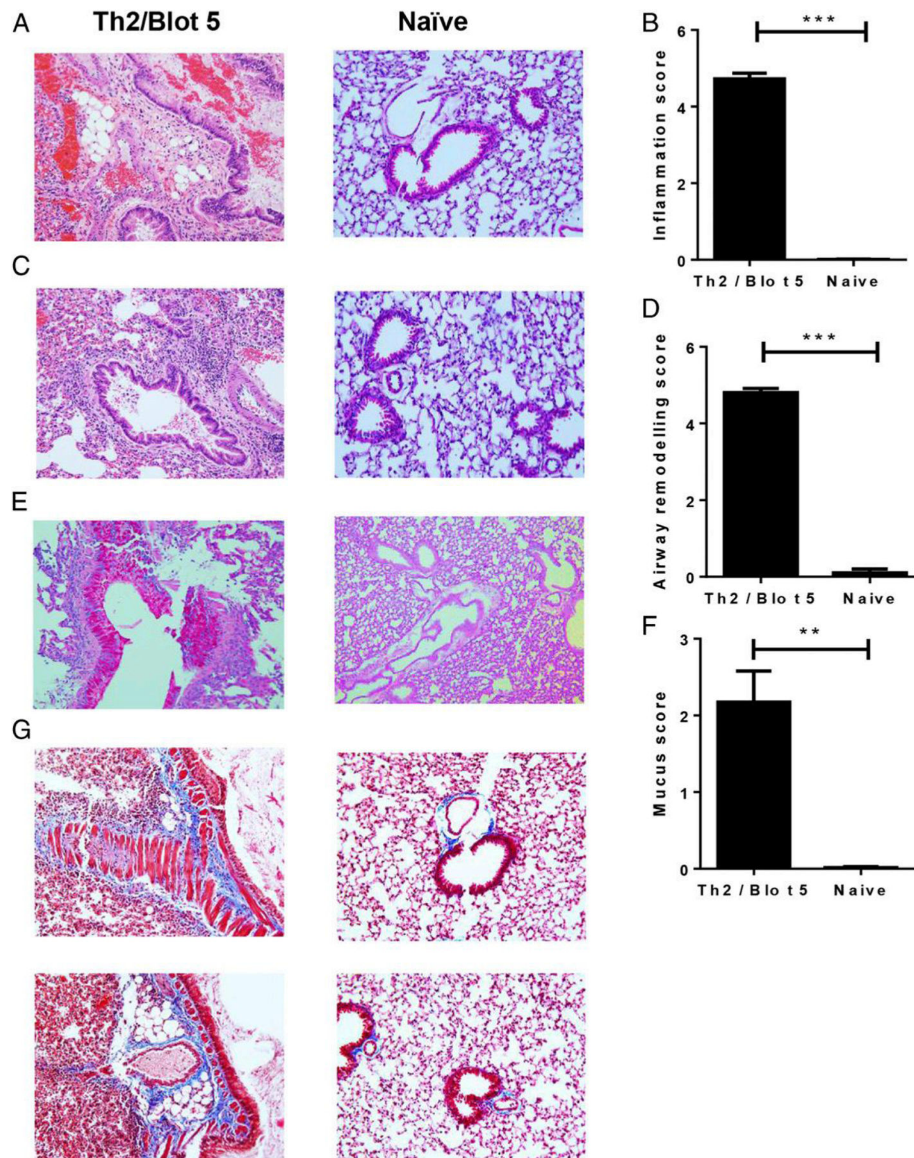

FIGURE 4.

Repeated weekly exposure to allergen-induced tissue damage, airway remodeling, mucus hypersecretion, and iBALT formation. Lungs from Th2 BT-II-transferred and allergen-challenged or naive mice were paraffin embedded, sectioned, and stained with H&E (**A** and **C**). Lung inflammation (**B**), airway remodeling (**D**), and mucus (**F**) were assessed blind by three independent observers. Mucus secretion was measured (**E**) by PAS staining and (**G**) collagen (blue) by MCT. Original magnification ×20 (A, C, E, and G). The data shown are representative of two independent experiments. $**p < 0.01$, $***p < 0.001$, Student t test.

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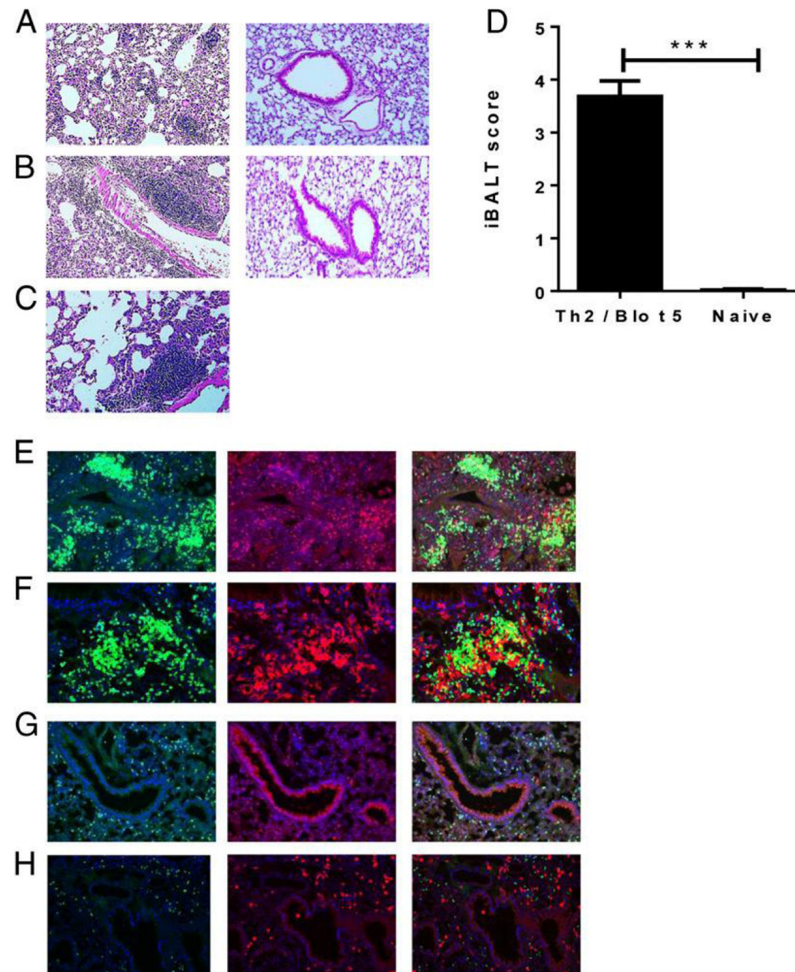

FIGURE 5.
Examination of iBALT. Lungs from Th2 BT-II-transferred and Blo t 5-challenged or naive mice were frozen and sectioned as described in *Materials and Methods*. Different patterns of iBALT included interstitial (**A**), peribronchial (**B**), and perivascular (**C**), which was scored by three independent observers of B cells (**D**). Frozen sections from BT-II Th2-transferred and challenged mice were stained with mAb to B220 (green) and T cells with mAb to TcRβ (red) (**E** and **G**). In addition, DCs from Th2 BT-II-transferred and allergen-challenged mice were stained (red) with mAb to CDllc and counterstained with anti-B220 (green) (**F** and **H**). Untreated mice that served as controls for (E) are shown in (G), and those that served as controls for (F) are shown in (H). For each panel the individual monoclonal staining is shown in the first two panels and the merged image in the third.

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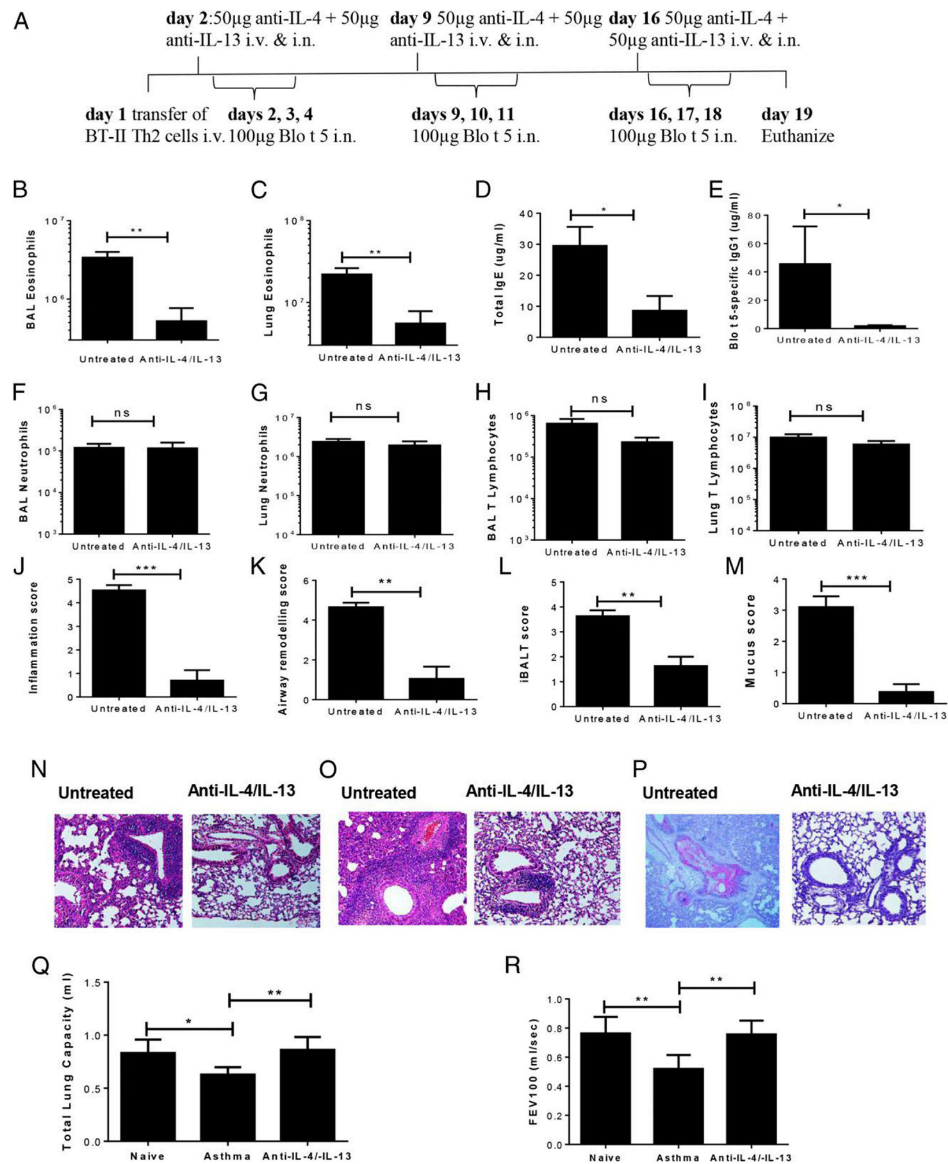

FIGURE 6.

Inhibition of asthma phenotype and lung pathological changes by anti–IL-4/IL-13. Monoclonal anti–IL-4 and IL-13 Abs were administered i.v. and i.n. 24 h before the first of three weekly challenges, and the mice were sacrificed on week 3 (**A**). BAL (**B**) and lung (**C**) eosinophils, total serum IgE (**D**) and Blo t 5 IgG1 (**E**), BAL (**F**) and lung (**G**) neutrophils, and BAL (**H**) and lung (**I**) T cells were measured in mice treated with anti–IL-4/IL-13 and compared with control animals. In parallel experiments, lungs were excised, fixed with paraformaldehyde, paraffin embedded, and stained with H&E. Lung pathological changes were compared with and without anti–IL-4/IL-13 and scored for inflammation (**J**), airway remodeling (**K**), iBALT formation (**L**), and mucus production (**M**) assessed by three independent observers. Examples of tissue sections stained with H&E (**N** and **O**) and PAS (**P**) are shown. (N–P) Original magnification ×20. Effect of neutralizing Abs on TLC (**Q**)

and FEV100 (**R**). Data are representative of two experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Student t test.

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