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Chronic Follicular Bronchiolitis Requires Antigen-Specific Regulatory T Cell Control To Prevent Fatal Disease Progression

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To study regulatory T (Treg) cell control of chronic autoimmunity in a lymphoreplete host, we created and characterized a new model of autoimmune lung inflammation that targets the medium and small airways. We generated transgenic mice that express a chimeric membrane protein consisting of hen egg lysozyme and a hemoglobin epitope tag under the control of the Clara cell secretory protein promoter, which largely limited transgene expression to the respiratory bronchioles. When Clara cell secretory protein–membrane hen egg lysozyme/hemoglobin transgenic mice were crossed to N3.L2 TCR transgenic mice that recognize the hemoglobin epitope, the bigenic progeny developed dense, pseudo-follicular lymphocytic peribronchiolar infiltrates that resembled the histological pattern of follicular bronchiolitis. Aggregates of activated IFN-γ– and IL-17A–secreting CD4+ T cells as well as B cells surrounded the airways. Lung pathology was similar in Ifng−/− and Il17a−/− mice, indicating that either cytokine is sufficient to establish chronic disease. A large number of Ag-specific Treg cells accumulated in the lesions, and Treg cell depletion in the affected mice led to an interstitial spread of the disease that ultimately proved fatal. Thus, Treg cells act to restrain autoimmune responses, resulting in an organized and controlled chronic pathological process rather than a progressive disease. The Journal of Immunology, 2013, 191: 5460–5476.  

© D4+ CD25+ Foxp3+ regulatory T (Treg) cells act to control inflammatory responses at mucosal surfaces. The fatal autoimmune lymphoproliferative disease that develops shortly after birth in mice and humans deficient in Foxp3, the transcription factor that programs Treg cell–suppressive function, aptly illustrates their essential nature (1–5). Treg cells found in different anatomical locations within the same individual have unique TCR repertoires, variations in their cell surface phenotypes, and distinct gene expression profiles (6, 7). These findings are consistent with the notion that subsets of Treg cells exist, and that Treg cell–suppressive activity may be finely tuned to the microenvironment. For example, Treg cell expression of T-bet, IFN regulatory factor 4, and STAT3 contributes to the ability of Treg cells to control the associated Th1-, Th2-, and Th17-polarized inflammation, respectively (8–10). Among the different mucosal surfaces, the gastrointestinal tract has been the most extensively examined, as several studies have documented the molecular mechanisms underpinning Treg cell suppression of inflammatory responses in the gut (11). These include the production of IL-10 and the generation of induced Treg (iTreg) cells from the conventional T (Tconv) cell population (12–14). However, the role of Treg cells in controlling inflammatory and autoimmune responses involving other mucosal surfaces is less well characterized, and new disease models to dissect the role of Treg cells in different microenvironments are needed. The respiratory tree of the lung is continually exposed to diverse Ags during respiration. Consequently, the immune response at the mucosal interfaces in the lung must maintain a balance between protective immune responses to invading organisms and tolerance to harmless Ags, including both self-Ags and foreign inhaled Ags. The conducting and parenchymal tissue of the lung contains a complex, heterogeneous network of dendritic cells capable of sampling airway contents and migrating to the mediastinal lymph node (MdLN) to initiate immune responses (15). To control promiscuous activation of host defenses, the airways are equipped with several lines of defense, which include epithelial cell barriers, alveolar macrophages, and Treg cells (16). In other pulmonary diseases, including allergic inflammation and asthma, Treg cells were shown to control lung inflammation in both humans and mice (17). Specifically, a role for Treg cells in the development of tolerance to inhaled Ags and in the control of allergic airway inflammation has been documented (18–20). Although the mechanisms of disease development and regulation have been investigated...
in mouse models of lung disease that follow an interstitial pattern, no models exist to study autoimmune pulmonary disease primarily affecting the airways (21, 22).

Patients with underlying autoimmune conditions can develop a type of lymphocytic inflammation that consists predominantly of a peribronchiolar pattern. Collagen vascular diseases, particularly rheumatoid arthritis and Sjögren’s syndrome, are associated with the development of follicular bronchiolitis, a condition in which hyperplastic lymphoid follicles form in the BALT (23–25). These infiltrates typically involve B cells in the follicular areas and CD4+ T cells in the parafollicular sections (26). The etiology of follicular bronchiolitis is unknown, as an underlying disease may confound its presentation. Moreover, the role of Treg cells in the control of autoimmune peribronchiolar inflammation has not been investigated. Thus, the elucidation of the mechanisms both initiating and controlling disease at the mucosal interfaces of the lung may yield further insight into the complex immune regulation needed to maintain immunological homeostasis in this location.

In this report, we created and characterized a new model of chronic autoimmune lung inflammation containing organized and focal peribronchiolar infiltrates that were composed of both effector T (Teff) and Treg cells specific for the same lung Ag. Partial depletion of the Treg cells in affected mice led to an interstitial spread of the inflammatory infiltrate that ultimately proved fatal. Thus, Treg cells act to restrain autoimmune conditions, resulting in a well-controlled, chronic pathological process rather than a progressive disease.

**Materials and Methods**

**Mice**

Transgenic mice that express the N3.L2 TCR, a TCR utilizing Vα18 and Vβ8.3, which recognize Hb (64–76)/I-Ek, were previously described (27). We used a previously published strategy to generate lines of transgenic mice that expressed a membrane form of hen egg lysozyme (mHEL) containing Hb (64–76) as an epitope tag (mHEL/Hb) (28). To allow membrane expression of the chimeric protein, the HEL/Hb cDNA was joined in-frame with amino acids 76–88 of the CCSP promoter and mHEL/Hb coding region was electroeluted and was used to transfect NIH 3T3 cells. A 4.6-kb NotI to HindIII fragment containing the CCSP stalk, a transmembrane region, and a complete cytoplasmic domain (29). A 2.4-kb BamHI fragment from the plasmid pBHG/CSP-2.3 containing the Clara cell secretory protein (CCSP) promoter was cloned into pBluescriptSK+ (30). The resulting plasmid was co-transfected with Small and a 2.2-kb BamHI fragment from pBluescript containing the mHEL/Hb construct was cloned into the Small site. A 4.6-kb NotI to HindIII fragment containing the CCSP promoter and mHEL/Hb coding region was electroporated and used to electroporate the male pronuclei of fertilized B6.AKR oocytes. Three founders were obtained and screened for lung-specific transgene expression. The primers used to screen mice were 5′-GGA CGA TGT GAG CTG GCA GC-3′ (forward) and 5′-CTT CGC GCA GTT CAC GCT CGC-3′ (reverse).

Foxp3EGFP mice and the Foxp3RFP mice were generated and screened, as previously described, and the mice used in this study carried the Foxp3EGFP allele to mark Foxp3 expression (31, 32). cells were isolated from the spleens of 10- to 12-wk-old female NOD.CB.17-Scid/J (SCID) mice, as previously described (33).

Lung digest and isolation of lymphocytes

The lung digest protocol was modified from Grayson et al. (36). The lungs were flushed with 1 mL PBS via intracardiac injection and dissected away from the surrounding tissue. The lungs were diced and incubated in digest medium for 1 h at 37°C. Lung digest medium consisted of low glucose DMEM (Invitrogen) supplemented with 5% FCS, penicillin/streptomycin (Invitrogen), 10 mM HEPES (Invitrogen), 250 μM collagenase I (Workington Biochemical), 50 U/mL DNase I (Workington Biochemical), and 0.01% hyaluronidase (Sigma-Aldrich). EDTA was added at a final concentration of 2 mM during the last 15 min of incubation. After digestion, the back of a syringe plunger was used to macerate the cells through a 40 μM pore filter. The erythrocytes were removed with a RBC lysing buffer (Sigma-Aldrich).

**Abs and flow cytometry**

Cells collected from the spleen, peripheral lymph nodes, MDL, thymus, and lung were stained, as indicated. For flow cytometry, the anti-mouse Abs PE-conjugated anti-CD4 (H129.19; BD Pharmingen), Pacific Orange-conjugated anti-CD8 (H129.19; BD Pharmingen), allophycocyanin eFluor-780-conjugated anti-TCRβ (H57-597; eBioscience), and allophycocyanin-conjugated anti-CD62L (DTA-1; eBioscience) were prepared with the PE-Texas Red conjugated anti–CD25 (PC61; BD Pharmingen). Cells bearing the N3.L2 TCR were stained with a biotinylated clonotypic Ab (CAB) and subsequently stained with PE-Texas Red streptavidin (BD Pharmingen) (27). In some experiments, cells were stained with a biotinylated diphtheria toxin (DT) receptor (DTR) Ab (polyclonal goat IgG anti-human heparin-binding epidermal growth factor-like growth factor; R&D Systems), followed by PE-Texas Red streptavidin (BD Pharmingen). A four-laser custom LSRII was used to collect the data, and FlowJo software was used for analysis.

**Intracellular staining and cytotoxic analysis**

Intracellular cytokine staining was performed after a 5-h restimulation with PMA (5 ng/mL; Sigma-Aldrich) and ionomycin (0.5 μM; Sigma-Aldrich) in the presence of brefeldin A (1 μM; BD Biosciences). Surface staining of cells was performed using a modified FACs buffer containing 10 μg/mL brefeldin A. Cells were stained for 30 min with the primary anti-mouse Abs PE-conjugated anti-CD4 (H129.19; BD Pharmingen), Pacific Orange-conjugated anti-CD8 (H129.19; BD Pharmingen), allophycocyanin eFluor 780-conjugated anti-TCRβ (H57-597; eBioscience), and CAB followed with PE-Texas Red streptavidin (BD Pharmingen), then washed with the modified FACs buffer and fixed in 1% paraformaldehyde overnight at 4°C. After this incubation, cells were washed with 1 mL PBS and then permeabilized with 1 mL 0.1% saponin. Intracellular staining was performed for 30 min at room temperature with PE-Cy7–conjugated anti–IFN-γ (XMG1.2; BD Pharmingen), and Pacific Blue–conjugated anti–IL-17A (TC11-18H10.1; BioLegend) or with Pacific Blue–conjugated anti-Helios (22F6; BioLegend) and allophycocyanin-conjugated anti–CTLA-4 (UC10-4B9; BioLegend). A four-laser custom LSRII was used to collect the data, and FlowJo software was used for analysis. Serum cytokines were measured using the eBioscience FlowCytomix kit following the manufacturer’s recommendations.

**CellTrace violet analysis of proliferation**

CD3− splenocytes from B6.AKR mice were isolated by cell sorting, resuspended in R10 medium, and plated in a 96-well flat-bottom plate at 4.75 × 104 cells/well. All sorting was done on a FACSAria IIu (BD Biosciences). For the analysis of proliferation, the wells were supplemented with 2.5, 0.25, 0.025, or 0 μM exogenous Hb peptide in 50 μL R10. The peptides were synthesized, purified, and analyzed, as previously described (37). The peptide sequence, in single-letter amino acid code, is Hb (64–76)–GKKVITAFNEGLK, abbreviated N72. The following day, the spleen, lung, and peripheral lymph nodes of N3.L2 TCR transgenic or control mice were prepared and labeled with CellTrace Violet (1 μL/mL; Invitrogen) by incubating the cells at 37°C for 20 min. The percentage of cells bearing the N3.L2 TCR was determined using flow cytometry and then plated such that each well contained 2.5 × 104 responder CD4+ T cells in 50 μL R10. Cultures were maintained for 72 h at 37°C and then stained with allophycocyanin-conjugated anti-CD4 (RM4-5; BD Biosciences) and the Cab, followed by PE-Texas Red streptavidin.

In some experiments, the lungs of NOD.CB.17-Scid/J (SCID) mice were prepared and labeled with CellTrace Violet (1 μL/mL; Invitrogen) by incubating the cells at 37°C for 20 min. The percentage of cells bearing the N3.L2 TCR was determined using flow cytometry and then plated such that each well contained 2.5 × 104 responder CD4+ T cells in 50 μL R10. Cultures were maintained for 72 h at 37°C and then stained with allophycocyanin-conjugated anti-CD4 (RM4-5; BD Biosciences) and the CAB, followed by PE-Texas Red streptavidin.
Histology

Complete lungs were inflated with zinc formalin, fixed, processed, and stained with H&E using a histology core facility. Some sections were also stained with Mason’s Trichrome to evaluate collagen deposition. Blinded sections from the entire lung were examined by a pathologist (N.H.S.), and the extent of lung inflammation was evaluated on a 9-point scale that was developed based on a previously published scoring system (38). Three separate parameters were evaluated, as follows: bronchiolitis, follicular bronchiolitis, and alveolitis. Bronchiolitis was defined as prominent mixed, predominantly lymphocytic, and peribronchiolar cell accumulations. Follicular bronchiolitis was defined as dense nodular or follicular peri-bronchiolar lymphoid accumulations that expanded into the lung parenchyma. Alveolitis was defined as the accumulation of mixed inflammatory cells within the alveolar walls and spaces. Each parameter was blindly scored on a scale of 0–3 depending on the percentage of the tissue affected and the degree of inflammation observed for each parameter: 0, normal (very few or no inflammatory cells); 1, mild (<20%); 2, moderate (20–40%); and 3, severe (>40%). The total pathology score ranged from 0 to 9, a sum of all three scores.

Immunofluorescence

Whole lungs were inflated with a 1:1 Tissue-Tek O.C.T. compound (Sakura, Hayward, CA)/PBS mix. Lungs were frozen in Tissue-Tek O.C.T. compound in liquid nitrogen, and 7-μm sections were obtained with a cryostat. Sections were fixed with acetone and blocked with BSA and an avidin/biotin blocking kit (Vector Laboratories) per the manufacturer’s recommendation. Sections were stained with an anti-HEL Ab (F10.6.6) (39), followed by Alexa-488 (Invitrogen) or allophycocyanin-streptavidin (BD Pharmingen), and with a polyclonal rabbit anti-uteroglobin Ab (Abcam), followed by PE-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories). The coverslips were mounted with Prolong Gold antifade (Molecular Probes, Eugene, OR) to extract RNA. cDNA was synthesized using SuperScript III First Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) to extract RNA. cDNA was synthesized using Quantitect reverse transcription cDNA synthesis kit (Applied Biosystems, Foster City, CA). The following primers were designed to examine expression of the mHEL/HEb transcript: 5′-GACC ACC ACA CCT CGT CCA ATG ATG-3′ (coding) and 5′-GGC TCC AGG AAC CTC TCG AAC-3′ (noncoding). The following β-actin primers were used: 5′-CTA CGA GGG CTA GTC TCT CC-3′ (coding) and 5′-GCA TGC TGG CAT AGA GG-3′ (noncoding). Thymic tissue was isolated from mice expressing the mHEL/HEb construct under the MHC Eα promoter and used as a positive control for transgene expression (+Ctl) (40).

Real-time quantitative PCR

Splenocytes were isolated by standard methods. Lung cells were isolated, as described above. Cells were pooled from two N3.L2 × CCSP-mHEL/HEb × Foxp3EGFP mice, stained with allophycocyanin-conjugated anti-CD4 (RM4-5; BD Pharmingen), and sorted on a FACSAria IIu. CD4+ enhanced GFP (EGFP) Tconv cells and CD4+ EGFP Treg cells were sorted. RNA was isolated by RNAeasy Minikit per the manufacturer’s instructions. cDNA was synthesized using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen). Real-time quantitative PCR was performed using TaqMan primers for IL-10 (catalogue Mm00439616_m1), TGF-β (catalogue Mm00441724_m1), and GAPDH (catalogue Mm99999916_g1) on an Applied Biosytems StepOnePlus Real-Time PCR System. IL-10 and TGF-β were normalized to GAPDH by the ΔΔ cycle threshold method and expressed as relative units (41). Data shown are from three experiments.

Regulatory T cell depletion

A 50 μg/kg injection of DT was administered every other day to control B6.AKR × Foxp3EGFP mice or CCSP-mHEL/HEb × Foxp3EGFP mice. N3.L2 × CCSP-mHEL/HEb control mice, and N3.L2 × CCSP-mHEL/HEb × Foxp3DTR mice. All mice were sacrificed between 10 and 27 wk of age. The mice were weighed daily to monitor disease progression. When the mice became moribund or lost ∼20% of their baseline body weight, they were sacrificed. The calculated average time after the completion of two experiments was 17.4 d from the start of DT treatment, and the maximum number of injections given was 12. Control mice were sacrificed with paired experimental mice or after the average number of injections received by experimental mice (nine injections, calculated after two experiments).

Statistics

A linear random coefficient model was used to fit weight change over time. For the lung pathology scores, cell frequencies and numbers, and serum cytokine levels, a nonparametric Kruskal-Wallis test was used to compare the measurements between the groups. For two group comparisons, a Mann-Whitney U test was performed. Quantitative PCR data were analyzed with a paired t test. Data were analyzed using SAS (version 9.2) and Prism (version 5.0b) software.

Results

Lung-specific mHEL/HEb expression

In an effort to understand the mechanisms of disease pathogenesis and regulation affecting the Airways, we have created a preclinical mouse model of follicular bronchiolitis. Our transgenic system is built around two well-characterized antigenic proteins, an allelic variant of the minor β-chain of mouse Hb and hen egg lysozyme (HEL). The immunodominant peptide is Hbβ23−34 aα26−64, and Hb (64−76), and the response is I-Ek restricted. The Hb (64−76) determinant has been completely dissected, and the crystal structure of this peptide bound to I-Ek solved (42, 43). TCR transgenic mice have been developed that respond to either Hb (64−66)/I-Ek (N3.L2 line) or to HEL (46−61)/I-Ak (3A9 line), and CAb specific for each TCR have been developed (27, 44, 45). Our strategy for genetically developing both Hb (64−76) and HEL (46−61) epitopes into mice has been published (28). For this study, a 2.4-kb genomic DNA fragment containing the Clara cell secretory protein promoter was used to control transcription of the mHEL/HEb construct (HEL/HEb cDNA + the genomic Ld transmembrane region) (Fig. 1A). The CCSP promoter has been effective in expressing transgenes in Clara cells, a nonciliated epithelial cell population located at the junction of the conducting and respiratory Airways (30). Three founders were characterized, and in one founder line transcription of the mHEL/HEb construct was limited to the lung, as detected by RT-PCR (Fig. 1B). Thymic tissue was isolated from mice expressing the mHEL/HEb construct under the MHC Eα promoter and used as a positive control for transgene expression (+Ctl) (40). The transgenic mice were also bred to Foxp3EGFP mice to mark Treg cells for subsequent analysis (31). Clara cells, a nonciliated epithelial cell population located at the junction of the conducting and respiratory Airways, express the protein uteroglobin under the endogenous CCSP promoter (46). Transgenic mHEL/HEb protein expression colocalized with uteroglobin expression by immunofluorescence using a mAb specific for HEL (Fig. 1C). These data suggest that transgene expression is limited to lung Clara cells.

Self-reactive T cells escape central tolerance

To investigate the influence of lung mHEL/HEb expression in an autoimmunity setting, we crossed CCSP-mHEL/HEb mice with N3.L2 TCR transgenic mice that contain CD4+ T cells capable of reacting with the Hb (64−76) epitope. All of the mice analyzed were 8−16 wk of age unless otherwise indicated. There was a reduction in the percentage and number of CD4+ T cells isolated from the thymus of N3.L2 × CCSP-mHEL/HEb (bigenic) mice, as compared with N3.L2 control mice (data not shown). This reduction paralleled a reduction in the percentage and number of Cab+ T cells found in
the thymus (Fig. 2A). Similar results were seen in the spleens of bigenic mice (Fig. 2B). This decrease in Ag-specific T cells indicates the presence of the cognate Ag in the thymus, even though thymic expression of the mHEL/Hb construct was not detected by RT-PCR (Fig. 1B). Nevertheless, bigenic mice contained many CD4+ T cells that escaped thymic deletion and were recovered in both the thymus and the periphery.

To evaluate the potential for self-reactivity in bigenic mice, we first examined the ability of the unfractionated CD4+ T cell population to respond to stimulation by self-Ag. Both CD4+ EGFP+ Tconv cells and CD4+ EGFP+ Treg cells isolated from the lungs of bigenic mice proliferated when stimulated in vitro with the Hb (64–76) peptide (Fig. 2C). Similarly, cells isolated from the spleen and MdLN of N3.L2 and bigenic mice showed dose-dependent proliferation to peptide in vitro (Fig. 2D). However, in many cases, a lower percentage of the CD4+ EGFP+ Tconv population and CD4+ EGFP+ Treg population isolated from bigenic mice proliferated in response to Hb (64–76) peptide in vitro when compared with N3.L2 controls (Fig. 2C, 2D). Thus, bigenic mice retained autoreactive T cells that escaped central tolerance and maintained the ability to respond to antigenic stimulation ex vivo, albeit at a lower frequency than seen in controls. Because transgene expression largely was limited to lung Clara cells, a population of cells not known to present Ag, we examined the ability of lung APCs from CCSP-mHEL/Hb mice to present Ag to Ag-specific T cells from N3.L2 Rag1−/− mice. Lung CD11b+ CD11c+ cells were isolated using cell sorting and cultured with Ag-specific N3.L2 T cells. In the absence of exogenous peptide, N3.L2 T cells proliferated when cultured with freshly isolated lung CD11b+ CD11c+ cells from CCSP-mHEL/Hb mice, but not from B6.AKR control mice (Fig. 2E). No expression of the mHEL/Hb transcript was detected in CD11b+ CD11c+ cells from the lungs of CCSP-mHEL/Hb mice (Fig. 2F). Together, these data suggest that lung CD11b+ CD11c+ cells had indirectly obtained the mHEL/Hb epitope and thus the ability to activate Ag-specific T cells.

**Self-reactive T cells accumulate in the lungs**

Because N3.L2 × CCSP-mHEL/Hb mice expressed the mHEL/Hb Ag in the lungs and also contained Hb-specific T cells in the periphery, we examined the lungs of the bigenic mice for pathology. The lungs of the bigenic mice were infiltrated with increased numbers of CD4+ T cells, CD8+ T cells, and B220+ B cells, as compared with control and single-transgenic mice (Fig. 3A–C). There was an accumulation of Ag-specific CD4+ CD62L− T cells (Fig. 3D). An increased percentage and number of these CD4+ CD62L− T cells in the lungs of the bigenic mice were CD44+ CD62L−, indicating an activated effector/memory phenotype (Fig. 3E). There was a parallel decrease in the percentage of cells with an Ag-inexperienced phenotype (CD44− CD62L+) in the lungs of bigenic mice as compared with N3.L2 single-transgenic mice (Fig. 3F). The spleen did not show any significant increases in the number of CD4+ or CD8+ T cells, or B220+ B cells (Table I). A larger percentage of CD4+ CD62L− T cells in the spleen of bigenic mice had an activated effector/memory phenotype, which paralleled a decrease in the percentage and number of cells with an Ag-inexperienced phenotype (Table I). These data suggest that the inflammatory infiltration was focused on the lung, which expressed the cognate Ag. Similar increases in CD4+ and CD8+ T cells, B220+ B cells, CD4+ CD62L− T cells, and activated effector/memory cells were seen in the MdLN, the lung draining lymph node (Table II). Thus, self-reactive T cells in the bigenic mice migrated to the MdLN and lung and were activated.

Upon histological evaluation, we observed a mixed infiltrate that contained predominantly mononuclear cells, including numerous plasma cells, few macrophages, and occasional neutrophils. The infiltrate collected into dense peribronchiolar aggregates that affected small- to medium-sized airways, but largely spared the lung interstitium and alveolar septa (Fig. 3F). The disease penetrance was 100% for mice possessing both the N3.L2 and mHEL/Hb transgenes. To determine the degree and extent of the lung...
pathology, we modified a scoring system based on three parameters: bronchiolitis, follicular bronchiolitis, and alveolitis (see Materials and Methods) (38). Each parameter was scored 0–3 based on the degree and extent of inflammation, with 0 representing normal. The total pathology score ranged from 0 to 9, a sum of all three scores. The lungs of bigenic mice had an average total pathology score of 4, which was significantly increased over control and single-transgenic mice (Fig. 3G). We analyzed the lungs of N3.L2 and bigenic mice for collagen deposition using Masson’s Trichrome stain. A mild increase in collagen deposition was observed surrounding airways associated with inflammation in the bigenic mice (Fig. 3H). Immunofluorescence of the lungs from bigenic mice revealed an accumulation of CD3⁺ T cells and B220⁺ B cells in pseudo-follicular clusters surrounding airways expressing the mHEL/Hb transgene (Fig. 3I). Expression of the mHEL/Hb construct in the lungs of 21-wk-old bigenic mice was further demonstrated by RT-PCR, and was similar to mHEL/Hb expression in a CCSP-mHEL/Hb mouse (Supplemental Fig. 1). Together, these data show that Ag expression is sustained during the time frame that these experiments were conducted. Despite the emergence of a chronic, mixed lymphocytic infiltrate, bigenic animals survive (observed to age >6 mo) and breed.

Self-reactive CD4⁺ T cells produce proinflammatory cytokines

To further evaluate the nature of the inflammatory infiltrate that developed in the lungs of the bigenic mice, we isolated lymphocytes from the lung tissue and did intracellular cytokine staining. We found a 3-fold increase in the percentage and a 7-fold increase in the number of Ag-specific CD4⁺ CAb⁺ cells that were IFN-γ⁺ in the lungs of bigenic mice when compared with N3.L2 mice (Fig. 4A, 4B). A similar increase in Ag-specific IFN-γ-producing cells was also seen in the MdLN of the bigenic mice (Table II). There was

FIGURE 2. Self-reactive T cells escape central tolerance in N3.L2 × CCSP-mHEL/Hb mice. (A and B) Representative flow cytometry from the indicated 8- to 16-wk-old mice showing staining of CD4⁺ T cells with a CAb that recognizes the N3.L2 TCR. (A) CAb staining in the thymus (n = 9, 6, 11, 14; groups 1–4, respectively, at least six experiments). (B) CAb staining in the spleen (n = 15, 19, groups 3 and 4, respectively, at least six experiments). (C) Proliferation assay showing CD4⁺ CAb⁺ T cells from the lungs of N3.L2 and N3.L2 × CCSP-mHEL/Hb mice that were cultured for 72 h with Hb (64–76) peptide and CD3⁺ splenocytes from B6.AKR mice. FACs plots show CellTrace Violet Fluorescence versus EGFP. Numbers indicate the percentage of cells dividing in the CD4⁺ CAb⁺ gate. Numbers in parentheses indicate the percentage of cells dividing in the CD4⁺ CAb⁺ EGFP⁺ or CD4⁺ CAb⁺ EGFP⁻ gate, as indicated (three experiments). (D) Bar graph showing the proliferation of spleen (top panel) and MdLN (bottom panel) CD4⁺ CAb⁺EGFP⁺ or CD4⁺ CAb⁺ EGFP⁻ cells from the mice in (A). Error bars represent the SEM. (E) Proliferation assay showing CD11b⁺ CD11c⁺ cells isolated by cell sorting from the lungs of CCSP-mHEL/Hb–expressing mice (n = 3) and control B6.AKR mice (n = 2) that were cultured with N3.L2 T cells from N3.L2 Rag⁻/⁻ mice, plus or minus 10 μM Hb (64–76) peptide. FACs plots display CellTrace Violet Fluorescence for cells in the CD4⁺ gate (2–3 mice pooled per experiment.) (F) RT-PCR using RNA from CD11b⁺ CD11c⁺ cells isolated by cell sorting from the lungs of CCSP-mHEL/Hb–expressing mice (n = 3; three pooled mice). For these and all subsequent representative FACs plots, numbers indicate the mean percentage of cells in the quadrant. *p < 0.05, **p < 0.005. (+)Ctl, Positive control; MM, master mix control.
FIGURE 3. Self-reactive T cells accumulate in the lungs of N3.L2 × CCSP-mHEL/Hb mice. (A–C) Scatter plots showing the percentage (left) and number (right) of CD4⁺ T cells (A) and CD8⁺ T cells (B) (*n = 15, 6, 14, 19; groups 1–4, respectively) and B220⁺ cells (C) (*n = 13, 4, 13, 17, groups 1–4, respectively) found in the lung of 8- to 16-wk-old control and transgenic mice, as indicated (at least six experiments). (D) Scatter plots showing the percentage (left) and number (right) of CD4⁺ Cab⁺ T cells in the lung of the mice in (A) (*n = 14, 18; groups 3 and 4, respectively). (E) Representative flow cytometry showing expression of CD44 and CD62L on lung CD4⁺ Cab⁺ T cells from N3.L2 and N3.L2 × CCSP-mHEL/Hb mice (left). Scatter plots showing the number (right) of CD4⁺ Cab⁺ CD44⁻ CD62L⁻ T cells in the lung of the indicated mice (*n = 14, 17; groups 3 and 4, respectively). (F) Representative H&E-stained histological sections from the lungs of control and transgenic mice, as indicated, shown at original magnification ×10. Scale bar, 100 μm. (G) Total lung inflammation score from 8- to 18-wk-old mice where histology was obtained. The total pathology score (0–9) was based on the sum of three scores, ranging 0–3, for the following parameters: bronchiolitis, follicular bronchiolitis, and alveolitis. (H) Rep-
representative Masson’s Trichrome–stained histological sections from the lungs of the indicated transgenic mice, shown at original magnification ×10. Scale bar, 100 μm. (f) Frozen sections from the lungs of N3.L2 × CCSP-mHEL/Hb mice stained with an Ab specific for HEL (red), B220 (green), and CD3 (blue), shown at original magnification ×20. Scale bar, 100 μm. For these and all subsequent scatter plots, each symbol represents an individual mouse, and the horizontal bars represent mean values ± SEM. *p < 0.05, **p < 0.005.

To further investigate the role of IFN-γ and IL-17A in the development of lung pathology, we bred the N3.L2 × CCSP-mHEL/Hb mice onto an N3.L2 × CCSP-mHEL/Hb background or an IFN-γ−/− background partially reduced inflammation in the bigenic mice. This reduction was compensated by a reciprocal increase in cells producing either IFN-γ or IL-17A when one of the two was absent. This suggests that both IFN-γ and IL-17A contribute to the disease process, although polarization down either the Th1 or Th17 pathway is not essential for the development of disease.

Treg cells accumulate in N3.L2 × CCSP-mHEL/Hb mice

Although the N3.L2 × CCSP-mHEL/Hb mice developed severe lung infiltrates, the mice survived. When we examined the lungs of bigenic mice, we found an increased percentage and number of Ag-specific Cab+ EGFP+ Treg cells compared with N3.L2 single-transgenic mice (Fig. 6A, 6B). Importantly, there was a 4-fold increase in the percentage and 17-fold increase in the number of Cab+ Treg cells in the lungs of bigenic mice, compared with N3.L2 mice. The lungs of bigenic mice also contained more Cab− EGFP+ Treg cells compared with control and single-transgenic mice (Fig. 6C). Moreover, we saw an increase in the number of both Cab+ and Cab− Treg cells in the MLN of bigenic mice as compared with N3.L2 mice (Table II). The spleen of the bigenic mice did not show more Treg cells (Table I). When we crossed the N3.L2 and bigenic mice onto a Rag1−/− background, nearly all of the N3.L2 T cells found in the thymus of N3.L2 mice. The lungs of bigenic mice also contained more Cab− EGFP+ Treg cells compared with control and single-transgenic mice (Fig. 6D). Additionally, we saw an increase in the number of both Cab+ and Cab− Treg cells in the MLN of bigenic mice as compared with N3.L2 mice (Table II). The spleen of the bigenic mice did not show more Treg cells (Table I). When we crossed the N3.L2 and bigenic mice onto a Rag1−/− background, nearly all of the N3.L2 T cells found in the thymus of N3.L2 mice. The lungs of bigenic mice also contained more Cab− EGFP+ Treg cells compared with control and single-transgenic mice (Fig. 6D). Additionally, we saw an increase in the number of both Cab+ and Cab− Treg cells in the MLN of bigenic mice as compared with N3.L2 mice (Table II). The spleen of the bigenic mice did not show more Treg cells (Table I).
central tolerance and Ag-specific T cells escape to the lung in response to cognate Ag.

Next, we sought to characterize the cell surface phenotype of both the CAb+ and CAb− Treg cells found in the lungs of bigenic mice. About half of the lung Treg cells found in the lungs of bigenic mice was similar to that of Treg cells from the lungs of N3.L2 and control mice (Fig. 6F). Helios expression among the CAb+ Treg cells was intermediate, landing between the high and low peaks observed for the N3.L2 and wild-type EGFP+ cells. Helios expression in CAb+ Treg cells of the bigenic mice was similar to that of Treg cells found in the lungs of bigenic mice was similar to that of Treg cells from the lungs of N3.L2 and control mice (Fig. 6F). In summary, increased numbers of Treg cells found in the lungs of bigenic mice was similar to that of Treg cells from the lungs of N3.L2 and control mice (Fig. 6F).
FIGURE 5. N3.L2 × CCSP-mHEL/Hb mice deficient in IFN-γ or IL-17A. (A–C) Scatter plots showing the percentage (left) and number (right) of CD4⁺ T cells (A) (n = 19, 6, 11; groups 1–3, respectively), CD8⁺ T cells (B) (n = 19, 6, 11; groups 1–3, respectively), and B220⁺ B cells (C) (n = 17, 6, 11; groups 1–3, respectively) found in the lungs of 8- to 16-wk-old N3.L2 × CCSP-mHEL/Hb mice (group 1, included for comparison) compared with N3.L2 × CCSP-mHEL/Hb mice deficient in IFN-γ (group 2) or IL-17A (group 3) (four experiments). (D) Scatter plots showing the percentage (left) and number (right) of CD4⁺ CAb⁺ T cells in the lungs of the mice in (A) (n = 18, 6, 11; groups 1–3, respectively). (E) Scatter plots showing the percentage of CD4⁺ CAb⁺ CD44⁺ CD62L⁻⁻ T cells in the lungs of the indicated mice (n = 17, 6, 11; groups 3–5, respectively). (F) Representative H&E-stained histological sections from the lungs of the indicated transgenic mice, shown at original magnification ×10. Scale bar, 100 μm. (G) Total lung inflammation score from 8- to 20-wk-old mice where histology was obtained. (H) Representative intracellular cytokine staining of ex vivo stimulated cells isolated from the lungs of the indicated mice. Staining for IFN-γ and IL-17A in the CD4⁺ CAb⁺ gate is shown (n = 6, 6; 3–4 experiments). (I) Scatter plot showing the serum levels of IL-6 from the indicated mice (n = 8, 3, 3; groups 1–3, respectively). *p < 0.05, **p < 0.005, ***p < 0.0005.
FIGURE 6. Treg cells accumulate in N3.L2 × CCSP-mHEL/Hb mice. (A) Representative flow cytometry showing CAb versus Foxp3-EGFP fluorescence in the lung CD4⁺ T cells of control and bigenic mice (n = 14, 18; groups 3 and 4, respectively, at least 11 experiments). (B) Scatter plots showing the percentage (left) and number (right) of CD4⁺ cells that are CAb⁺ Foxp3⁺ from the lung of the mice in (A). (C) Scatter plots showing the percentage (left) and number (right) of CD4⁺ cells that are CAb⁻ Foxp3⁺ from the lung of the mice in (A) and control mice (n = 8, 6; groups 1 and 2, respectively, at least four experiments). (D) Flow cytometry showing CAb versus Foxp3-EGFP fluorescence in the thymus (top panels) and lung (bottom panels) CD4⁺ T cells of control and bigenic mice on the Rag²⁻/- background (n = 4, 6; left to right, three experiments). (E) Representative flow cytometry gated on CD4⁺ T cells showing Foxp3-EGFP fluorescence versus CD25 in the lungs of control and bigenic mice (n = 11, 11, left to right). (F) Representative flow cytometric staining of CD4⁺ CAb⁻ Foxp3⁺-EGFP⁺ (top) and CD4⁺ CAb⁺ Foxp3⁻-EGFP⁺ (bottom) T cells from the lung of N3.L2, and N3.L2 × CCSP-mHEL/Hb mice. Control B6.AKR mice CD4⁺ CAb⁻ Foxp3⁻-EGFP⁻ (black) and CD4⁺ CAb⁺ Foxp3⁻-EGFP⁺ (gray) staining is included for comparison, stained as indicated (n = 4, four experiments). **p < 0.005.
FIGURE 7. Kinetics of disease progression in N3.L2 × CCSP-mHEL/Hb mice. (A–C) Scatter plots showing the percentage (left) and number (right) of CD4⁺ T cells (A) \( n = 7, 4, 6, 6, 19 \); groups 1–5, respectively), CD8⁺ T cells (B) \( n = 7, 4, 6, 6, 19 \); groups 1–5, respectively), and B220⁺ cells (C) \( n = 6, 3, 5, 4, 17 \); groups 1–5, respectively) found in the lungs of control and transgenic mice that were 8 wk old, as indicated (at least three experiments). The values from 8- to 16-wk-old N3.L2 × CCSP-mHEL/Hb mice are included for comparison (group 5) in these and the following scatter plots. (D) Scatter plots showing the percentage (left) and number (right) of CD4⁺ CAB⁺ T cells in the lungs of the mice in (A) \( n = 5, 4, 18 \); groups 3–5, respectively). (E) Representative flow cytometry showing the expression of CD44 and CD62L on lung CD4⁺ CAB⁺ T cells from N3.L2 and N3.L2 × CCSP-mHEL/Hb mice <8 wk old (left). Scatter plot showing the number (right) of CD4⁺ CAB⁺ CD44⁺ CD62L⁻ T cells in the lungs of the indicated mice \( n = 5, 3, 17 \); groups 3–5, respectively). (F) Total lung inflammation score from <8-wk-old mice where histology was obtained. Scores from 8- to 16-wk-old N3.L2 × CCSP-mHEL/Hb mice are included for comparison. (G) Representative intracellular cytokine staining of ex vivo stimulated cells (Figure legend continues).
Treg cells localized to the lungs of bigenic mice and expressed Treg cell–associated markers.

Kinetics of disease progression

To analyze the kinetics of disease progression, we examined bigenic mice from the time of weaning (3 wk of age) up to 7 wk old. We compared bigenic mice <8 wk of age with age-matched control mice and with 8- to 16-wk-old adult bigenic mice. The lungs of young bigenic mice had increased numbers of CD4+ and CD8+ T cells and B220+ B cells as compared with age-matched control and single-transgenic mice (Fig. 7A–C). The increase in lymphocyte subsets was observed early, but these cell populations were similar in frequency and number when compared with adult bigenic mice (Fig. 7A–C). Young bigenic mice had a lower percentage but similar number of CD4+ CAb+ T cells as age-matched N3.L2 control mice. The expansion of CD4+ CAb+ T cells was first observed in adult bigenic mice (Fig. 7D). A higher percentage and number of the CD4+ CAb+ T cells had an activated effector/memory phenotype in young bigenic mice as compared with N3.L2 control mice. The number of activated effector/memory CD4+ CAb+ T cells was not further increased in the lungs of adult bigenic mice (Fig. 7E). These data suggest that the lung disease develops early and has minimal progression with age.

We also assessed the kinetics of the disease progression with histological sections and intracellular cytokine staining. An increase in the lung total pathology score was seen in young bigenic mice when compared with age-matched N3.L2 control mice. However, there was not a significant increase in the total pathology score of adult bigenic mice as compared with young bigenic mice (Fig. 7F). Compared with age-matched controls, bigenic mice <8 wk of age had increased numbers of CD4+ CAb+ cells that produced IFN-γ (Fig. 7G, 7H). Furthermore, the number of CD4+ CAb+ cells that were IFN-γ+ increased with age, as adult bigenic mice contained more IFN-γ-producing cells than young bigenic mice (Fig. 7H). Young bigenic mice also had an increase in the percentage and number of CD4+ CAb+ IL-17A+ cells compared with age-matched controls (Fig. 7G, 7I). Adult bigenic mice had a lower percentage of CD4+ CAb+ IL-17A+ cells than young bigenic mice, but had a similar number of CD4+ CAb+ IL-17A+ cells, suggesting that the IL-17A–producing cells may be involved early in the disease process (Fig. 7I). Serum levels of IL-6 and IFN-γ were not elevated in young bigenic mice compared with age-matched N3.L2 control mice. However, adult bigenic mice had an elevation in IL-6 and IFN-γ relative to young bigenic mice (Fig. 7J).

Next, we examined the accumulation of Treg cells with age. Importantly, there was an increased percentage and number of CAb+ Treg cells in the lungs of young bigenic mice as compared with age-matched control and single-transgenic mice (Fig. 7K). At this age, bigenic mice already have a ~7-fold increase in the percentage and ~12-fold increase in the number of Ag-specific Treg cells compared with N3.L2 mice. In fact, bigenic mice <8 wk of age had a higher percentage of lung CAb+ Treg cells compared with adult bigenic mice, suggesting that the drive for increased regulation is present early in the disease process (Fig. 7K). The number of CAb− Treg cells was also increased compared with control mice (Fig. 7L). In summary, the lung disease develops early, has minimal progression, and is accompanied by a substantial increase in Ag-specific Treg cells.

Treg cells control disease progression in N3.L2 × CCSP-mHEL/Hb mice

The bigenic mice have increased numbers of lung Treg cells, suggesting that Treg expansion is required to control the effector components of the infiltrate and limit disease progression. To test this hypothesis, we reduced the number of Treg cells in bigenic mice. N3.L2 × CCSP-mHEL/Hb mice were bred with transgenic Foxp3+DTR mice that express the DTR under the control of a Foxp3 promoter. In previous studies, we found that ~90% of the peripheral blood Treg cells are depleted with a single 50 μg/kg i.p. injection of DT into C57BL/6J × Foxp3DTR mice after 24 h (32). Analysis of control and bigenic mice bred to Foxp3DTR mice revealed that, at most, ~60% of Treg cells express the DTR (observed in lymph node; data not shown). Approximately 22% of lung Treg cells in bigenic mice express the DTR (data not shown). This allows for a partial Treg depletion and examination of the role of Treg cells in the disease process while avoiding other manifestations associated with the scurfy phenotype of Foxp3− deficient mice (2, 50). For Treg cell depletion, we administered a 50 μg/kg injection of DT every other day to B6.AKR × Foxp3DTR or CCSP-mHEL/Hb × Foxp3DTR control mice, N3.L2 × CCSP-mHEL/Hb control mice, and N3.L2 × CCSP-mHEL/Hb × Foxp3DTR mice. Mice were monitored for weight loss and were sacrificed when they became moribund or lost ~20% of their baseline body weight. Control mice were sacrificed with paired experimental mice or after the average number of injections received by experimental mice (nine injections).

Partial Treg depletion in bigenic mice led to weight loss, and all of the mice were sacrificed by 24 d after the initial DT injection. No significant weight loss was observed in the controls (Fig. 8A). With chronic depletion, DTR− Treg cells accumulate in B6.AKR × Foxp3DTR and CCSP-mHEL/Hb × Foxp3DTR control mice and repopulate the Treg cell compartment. Importantly, there was a reduced percentage of EGFP+ Treg cells in the lungs, MdLN, peripheral lymph nodes, and spleen of Treg cell–depleted bigenic mice (Fig. 8B). The frequency of lung Treg cells was reduced by ~43%, and the frequency of MdLN Treg cells was reduced by ~56%. Treg cell numbers were reduced in the MdLN and lymph node of DT-depleted Foxp3DTR bigenic mice (data not shown). Notably, this reduction in lung Treg cells reflected a significant reduction in the frequency of Ag-specific Treg cells (Fig. 8C). We then characterized the lung infiltrates in Treg-sufficient and -deficient bigenic mice and compared them with control DT-treated mice. As compared with their Treg cell–sufficient counterparts, there was an increase in the percentage of CD4+ and CD8+ T cells in the lungs of Treg cell–depleted bigenic mice, but this did not result in a parallel increase in the number of CD4+ or CD8+ T cells (Fig. 8D, 8E). Both the percentage and number of B220+ B cells were decreased in the lungs of Treg cell–depleted mice (Fig. 8F). The percentage and number of Ag-specific CD4+ CAb+ T cells in
FIGURE 8. Treg cells control disease progression in N3.L2 × CCSP-mHEL/Hb mice. (A) Linear regression analysis of the weight change over time following DT administration to control B6.AKR × Foxp3^{DTR} mice or CCSP-mHEL/Hb × Foxp3^{DTR} mice (gray, n = 8; two experiments), N3.L2 × CCSP-mHEL/Hb mice (black, n = 9, three experiments), and experimental N3.L2 × CCSP-mHEL/Hb × Foxp3^{DTR} mice (red, n = 7; three experiments). Dashed lines represent individual mice, and solid lines indicate a line fit to the data. DT was administered every other day. Weight loss was monitored, and mice were sacrificed when they became moribund or lost ∼20% of their baseline body weight (12-injection maximum). Control mice were sacrificed with paired experimental mice or after the average number of injections received by experimental mice (nine injections). (B) Bar graph showing the frequency of CD4^+ cells that are EGFP^+ in the lung, MdLN, peripheral lymph node (PLN), and spleen of the mice in (A). Error bars represent the SEM. (C) Scatter plots showing the percentage of CD4^+ cells that are CAb^+ Foxp3^+ from the lungs of the mice in (A) (n = 5, 7; groups 2 and 3, respectively). (D–F) Scatter plots showing the percentage (left) and number (right) of CD4^+ T cells (D), CD8^+ T cells (E), and B220^+ B cells (F) found in the lung (Figure legend continues)
the lungs of Treg cell–depleted mice were not significantly different from control DT-treated bigenic mice (Fig. 8G). When we examined the production of proinflammatory cytokines in the lungs, we found an increase in the percentage and number of CD4+ T cells that were IL-17A+ (Fig. 8H, 8J). This increase was not observed when we examined the CD4+ CD44+ T cells (Fig. 8I, 8J). The percentage and number of IFN-γ+ T cells were unchanged in Treg cell–depleted mice (Fig. 8H–J). Treg cell–depleted mice had increased serum levels of IL-6 as compared with control DT-treated bigenic mice (Fig. 8K).

Although no overt changes in the number of cells infiltrating the lungs were observed, when we examined the lungs from Treg-depleted mice we found extensive lung pathology. The infiltrates were no longer limited to a primarily peribronchiolar distribution, but now extended into the alveolar septa exhibiting an interstitial pattern (Fig. 8L). There was a significant increase in the overall lung pathology score of bigenic Treg cell–depleted mice, which was due to a significant increase in the alveolitis score (Fig. 8M). Notably, control mice did not develop interstitial lung disease with chronic Treg cell depletion (Fig. 8L, 8M). These data demonstrate that partial Treg cell depletion resulted in progression of the disease associated with a change in the disease pattern.

Treg cell–derived IL-10 is essential to mucosal tolerance and was likely to be important in this model (12). Quantitative PCR demonstrated increased Il10 expression in Treg cells isolated from the lungs of bigenic mice, as compared with Tconv cells from these same mice (Supplemental Fig. 2A). Breeding the N3.L2 × CCSP-mHEL/Hb mice on the Il10−/− background revealed a change of the disease pattern with extension of the peribronchiolar infiltrate into the surrounding alveolar septa (Supplemental Fig. 2B). This change was reflected by an increase in the alveolitis score that was similar to that seen with Treg cell depletion (Supplemental Fig. 2C). These data are consistent with the proposed role of Treg cells in the control of disease progression.

Discussion

The central finding of this report is that an increase of Ag-specific Treg cells in the lung was required to orchestrate control of autoimmune lymphocytic bronchiolitis and limit tissue damage. Partial Treg cell depletion decreased the ratio of Treg to Teff cells, leading to an interstitial spread of the inflammatory infiltrates that culminated in death. Thus, local Treg cell expansion in the setting of chronic autoimmunity prevents disease progression by promoting a focal, contained pathological process. These results have broad implications for a variety of human autoimmune diseases affecting the lungs and other target tissues. Chronic life-long autoimmune disease affects an estimated 23.5 million individuals nationwide, and the prevalence is rising (51). In many of these diseases, Treg cell numbers are increased in the affected tissues, implicating Treg cell expansion as a fundamental mechanism limiting disease progression (52). Our data support this hypothesis in a new model of autoimmune lung disease based on sustained expression of the target Ag. This model may not reflect conditions in which the target Ag is transiently expressed.

Multiple lymphocyte subsets are thought to contribute to the pathophysiology of follicular bronchiolitis (26). In our model, N3.L2 × CCSP-mHEL/Hb mice had increased numbers of IL-17A– and IFN-γ–producing Ag-specific Teff cells in the lungs. However, breeding the model onto the Ifng−/− or Il17a−/− background did not reduce lung pathology scores. Rather, in the absence of IFN-γ–producing Teff cells, IL-17A–producing cells expand, and vice versa. These data demonstrate that there is compensatory differentiation of self-reactive T cells into Th1 or Th17 cells and each cell type is sufficient to establish lung pathology. This is in accordance with other disease models, in which reciprocal increases in Th1 or Th17 cells occur when either cytokine is absent (53, 54). In addition to self-reactive T cells, a large number of B cells were recovered in the lungs of bigenic mice. Indeed, in an autoimmune TCR transgenic arthritis model, self-reactive T cells were shown to support autoantibody-producing B cells (55). The large numbers of B cells accumulating in the lungs and forming peribronchiolar clusters suggest that B cells may also contribute to the observed pathology.

Irrespective of the accumulation of B cells and proinflammatory cytokine-producing autoreactive T cells in the lungs, the N3.L2 × CCSP-mHEL/Hb mice survive. We have demonstrated that survival depends on the 4-fold increase in the frequency and 17-fold increase in the number of Ag-specific Treg cells that accumulated in the lungs as a counterbalance to the expansion of the self-reactive Teff cell population. However, the role of Treg cell Ag specificity during the resolution of other types of lung-specific inflammation is debated (56, 57). Antigenic stimulation of the TCR is required for Treg cells to exert suppressive function, but once activated these cells have been shown to suppress in an Ag-nonspecific manner in vitro (58). Other studies have suggested that Treg cell function is optimal when the Treg cells possess a diverse TCR repertoire (59, 60). Nevertheless, in TCR transgenic systems, such as ours, where there is likely to be lower TCR diversity, but also an abundance of cognate Ag, there may be an increased Treg cell suppressive potential (61). This hypothesis is consistent with our data that show half of all Ag-specific Treg cells divide when cultured with their cognate Ag, whereas only one-third of the Ag-specific Tconv cells divide under the same conditions. The increase in regulatory potential conferred by the transgenic system may be a key feature for disease control. This does not, however, preclude the notion that expanding the Treg TCR repertoire early on may reduce the extent of inflammation (32).

Treg cells possessing the transgenic TCR in bigenic mice had a similar phenotype to Treg cells that did not express a transgenic TCR. Notably, all of the Treg cells in the lungs of N3.L2 × CCSP-mHEL/Hb mice had low levels of CD62L expression. Similarly, in NOD mice, CD62Llow regulatory cells that were previously CD62L+ have been shown to reside within the inflamed pancreatic islets (62). Compared with the CD62Llow Treg subset, the CD62Lhigh Treg subset expresses different levels of chemokine receptors, and, in particular, higher levels of CCR7, which may in turn result in differential homing capabilities (63–65). A recent study looking at Treg cell control of a CD8+ T cell–mediated lung
disease found that in the setting of Treg cell depletion, a CD103⁺ CD62Llow Treg population accumulated in the lungs to prevent exacerbation of the disease (22). Previous studies have revealed conflicting results regarding the influence of CD62L expression on Treg cell suppressive function in vitro and in mouse models of graft-versus-host disease and diabetes (62, 63, 65–68). In this model, we identified CD62Llow Treg cells in the lung as the regulatory population necessary to contain autoimmune inflammation.

Numerous peribronchiolar pseudo-follicular aggregates that contained organized clusters of T and B cells formed in the lungs of bigenic mice. These structures were also associated with increased numbers of Treg cells, and diphertheria toxin–mediated depletion of lung Treg cells led to interstitial spread of the lymphocytic infiltrate and ultimately death. Similar tertiary lymphoid organs (TLOs), such as inducible BALT, can develop during chronic inflammation (69). TLOs have been observed in the target organs of many chronic autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, Sjögren’s syndrome, and ulcerative colitis (70). In particular, inducible BALT has been found in patients with rheumatoid arthritis and Sjögren’s syndrome that suffer from pulmonary complications (71). Previous work demonstrated that iTreg cells limit the formation of ectopic lymphoid tissue in a mouse model of chronic allergic inflammation (19). TCR transgenic mice specific for the H⁺/K⁺ ATPase developed ectopic lymphoid tissue in the stomach containing Treg cells. Transfer of CD25-depleted transgenic cells to a nude mouse resulted in diffuse lymphocytic infiltration and increased autoantibody production, also consistent with the hypothesis that Treg cells function to constrain the disease to organized ectopic lymphoid collections (72). In humans, follicular bronchiolitis can interconvert with lymphocytic interstitial pneumonitis, and the latter was associated with clinical deterioration (73). Together, these data suggest that the formation of organized ectopic lymphoid tissue requires Treg cell function.

The percentage of clonotype-negative CD4⁺ cells that produced IL-17A was increased in Treg cell–depleted mice, suggesting that clones with different Ag specificities are being activated. Treg cell depletion activates dendritic cells, which could enhance the presentation of subdominant or cryptic epitopes, leading to an altered disease phenotype through epitope spreading (50). Epitope spreading has been extensively demonstrated in mouse models of multiple sclerosis, arthritis, and type 1 diabetes, and evidence exists that this phenomena occurs in human autoimmune diseases as well (74). In a model of virus-induced autoimmunity, early CD8⁺ T cell responses to subdominant epitopes were enhanced with anti-CD25–mediated Treg cell depletion (75). Treg cells prevent intermolecular spreading of the immune response in a mouse model of Grave’s disease, thereby limiting lymphocytic infiltration of the thyroid and progression from hyperthyroidism to hypothyroidism (76). Treg cell depletion also leads to the expansion of germinal center T follicular helper cells, followed by a break in anergy and the production of Abs by previously anergic B cells (77). In our study, serum IL-6 levels were further increased in Treg cell–depleted bigenic mice, which may have implications for the development of Th17 cells, in addition to the increased numbers of Th17 cells observed. It will be of interest to examine the ability of Treg cells to control the response to subdominant epitopes and the generation of germinal center Th17 cells in this model.

In summary, we have created and characterized a new model of autoimmune follicular bronchiolitis that is driven by a steady production of Ag-specific T cells. Either IFN-γ or IL-17A–producing cells were sufficient to establish lung pathology, which were contained in TLOs by the expansion of Ag-specific Treg cells. Partial Treg cell depletion unleashed fatal disease progression involving extensive interstitial pneumonitis. Thus, in chronic autoimmune responses, Treg cells are required to convert progressive disease into a limited pathological process.

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References


