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Research Article

Immunology

Transplantation

Obliterative bronchiolitis (OB) is a poorly understood airway disease characterized by the generation of fibrotic bronchiolar occlusions. In the lung transplant setting, OB is a pathological manifestation of bronchiolitis obliterans syndrome (BOS), which is a major impediment to long-term recipient survival. Club cells play a key role in bronchiolar epithelial repair, but whether they promote lung transplant tolerance through preventing OB remains unclear. We determined if OB occurs in mouse orthotopic lung transplants following conditional transgene-targeted club cell depletion. In syngeneic lung transplants club cell depletion leads to transient epithelial injury followed by rapid club cell–mediated repair. In contrast, allogeneic lung transplants develop severe OB lesions that are largely devoid of club cells despite immunosuppression treatment. Lung allograft club cell ablation also triggers the recognition of alloantigens, and pulmonary restricted self-antigens reported associated with BOS development. However, CD8<sup>+</sup> T cell depletion restores club cell reparative responses and prevents OB. In addition, ex vivo analysis reveals a specific role for alloantigen-primed CD8<sup>+</sup> T cells in inhibiting club cell proliferation and maintenance. Taken together, our results demonstrate a vital role for club cells in maintaining lung transplant tolerance and propose a model to identify the underlying mechanisms of OB.

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# An obligatory role for club cells in preventing obliterative bronchiolitis in lung transplants

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Obliterative bronchiolitis (OB) is a poorly understood airway disease characterized by the generation of fibrotic bronchiolar occlusions. In the lung transplant setting, OB is a pathological manifestation of bronchiolitis obliterans syndrome (BOS), which is a major impediment to long-term recipient survival. Club cells play a key role in bronchiolar epithelial repair, but whether they promote lung transplant tolerance through preventing OB remains unclear. We determined if OB occurs in mouse orthotopic lung transplants following conditional transgene-targeted club cell depletion. In syngeneic lung transplants club cell depletion leads to transient epithelial injury followed by rapid club cell-mediated repair. In contrast, allogeneic lung transplants develop severe OB lesions that are largely devoid of club cells despite immunosuppression treatment. Lung allograft club cell ablation also triggers the recognition of alloantigens, and pulmonary restricted self-antigens reported associated with BOS development. However, CD8<sup>+</sup> T cell depletion restores club cell reparative responses and prevents OB. In addition, ex vivo analysis reveals a specific role for alloantigen-primed CD8<sup>+</sup> T cells in inhibiting club cell proliferation and maintenance. Taken together, our results demonstrate a vital role for club cells in maintaining lung transplant tolerance and propose a model to identify the underlying mechanisms of OB.

## Introduction

For over 3 decades lung transplantation has remained the only viable option for many types of end-stage pulmonary diseases. Despite advances in surgical and postoperative management techniques the medium survival after lung transplant significantly lags behind other solid-organ recipients at approximately 5.8 years (1). A major limitation to recipient survival is bronchiolitis obliterans syndrome (BOS), the most common form of chronic lung allograft dysfunction (CLAD) (2). BOS is diagnosed by the loss of forced expiratory volume to the exclusion of other causes of pulmonary dysfunction such as infection, acute rejection, or anastomotic complications. BOS severity is driven by the progression of obliterans bronchiolitis (OB), inflammatory fibrotic eruptions that partially or completely obstruct the distal bronchioles (1).

Club cells are nonciliated epithelial secretory cells that are highly prevalent in the pulmonary distal bronchioles in both humans and mice (3). They are often identified by the expression of *Scgb1a1*, which encodes the club cell secretory protein (CCSP, CC10), a 16-kDa peptide with reported antiinflammatory properties that coats airway surfaces (4). Club cells help maintain small airway homeostasis through detoxifying xenobiotic and oxidizing molecules, secretion of antimicrobial peptides, and promotion of mucociliary clearance. They also play a key role in bronchiolar epithelial repair through their ability to self-renew and differentiate into ciliated and goblet cells (5). In line with these observations, several studies have shown that targeted club cell ablation delays or prevents epithelial repair (6, 7). Interestingly, many nonalloimmune stressors of airway

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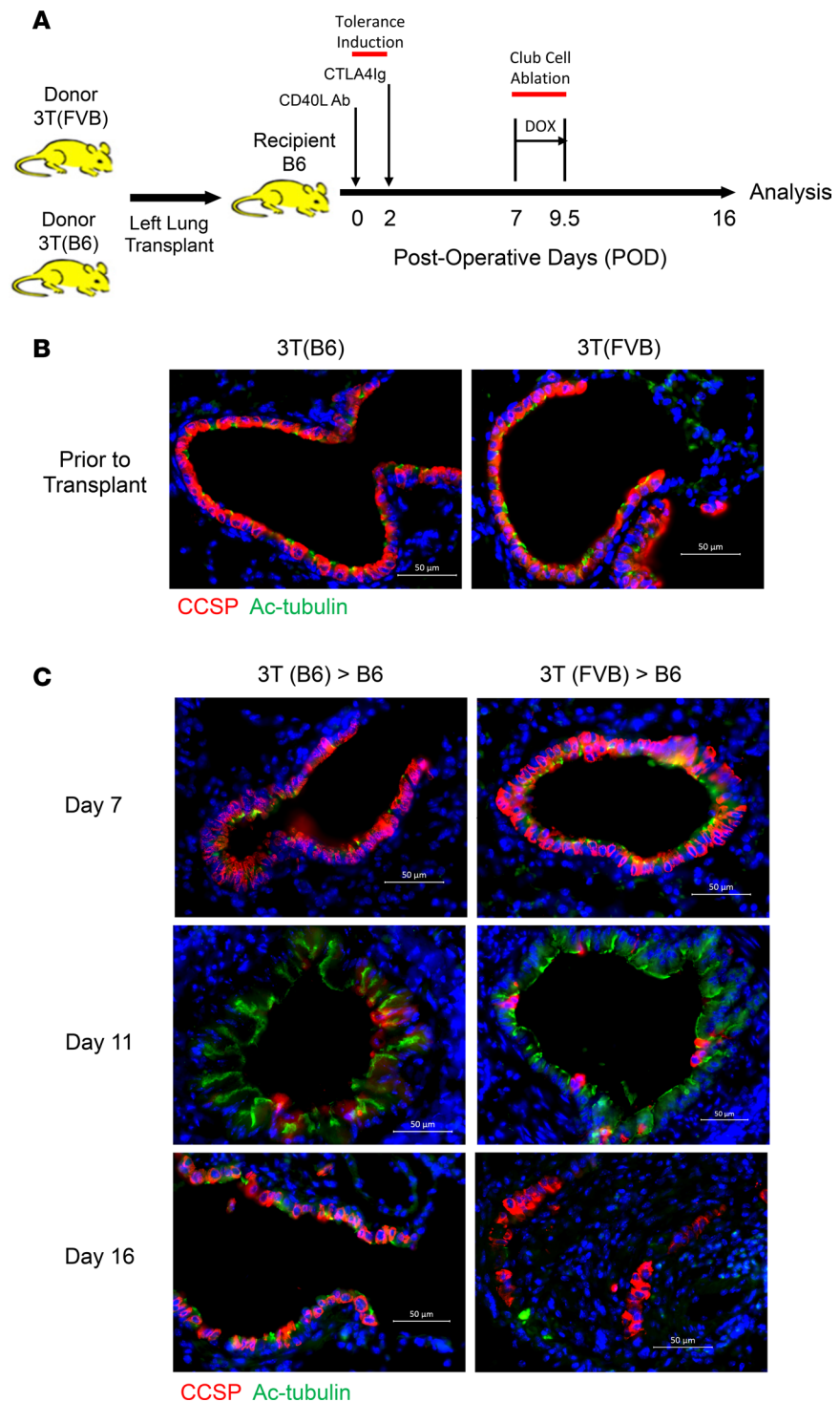
epithelium are risk factors for BOS development, which include primary graft dysfunction (8), *Pseudomonas aeruginosa* infection (9), community-acquired respiratory viral infections (10), and chronic aspiration of gastric acid (11, 12). There are also a few reports demonstrating club cell injury in lungs with BOS. For example, low CCSP levels in bronchiolar lavage fluid have been reported either as a risk factor for, or associated with, BOS development (13, 14). More recently, Palmer and colleagues demonstrated that patients with BOS have diminished CCSP expression in the airway epithelial cells of their terminal bronchioles (15). However, it remains to be investigated whether club cell loss is sufficient to trigger OB pathogenesis and promote immune responses known to be associated with BOS risk.

Here, we describe a mouse orthotopic lung transplant (OLT) model that generates OB lesions in response to bronchiolar epithelial injury generated through the conditional activation of transgenes that direct club cell ablation. Club cell loss leads to the augmentation of adaptive immune responses that are coupled to BOS risk. Additionally, we find that CD8<sup>+</sup> T cells play an important role in inhibiting club cell maintenance and proliferation.

## Results

*Club cell ablation triggers OB pathogenesis in lung transplant allografts.* To determine if the loss of club cells promotes OB, we utilized triple-transgenic (3T) mice bearing the following genes: a reverse tetracycline activator gene driven by the CCSP promoter, *Cre* recombinase gene under control of the reverse tetracycline activator, and a *lox-P*-activated diphtheria toxin A gene (DT-A). Ingestion of doxycycline by 3T mice induces *Cre*-mediated recombination of the *lox-P* DT-A locus that promotes DT expression specifically in CCSP-expressing cells, resulting in their depletion and consequential injury to the bronchiolar epithelium (6). Because 3T mice were originally developed on a mixed histocompatibility antigen background, we extensively backcrossed these mice with FVB and C57BL/6 (B6) mice to generate fully defined minor and major histocompatibility 3T FVB and 3T B6 strains for syngeneic and allogeneic transplantation. To induce allograft acceptance in 3T FVB → B6 lung recipients, we administered CD40L-neutralizing (CD40L is also known as CD154) antibodies (Abs) and the CD80/86 antagonist CTLA4Ig (Figure 1A), which we have previously demonstrated induces established tolerance in the mouse OLT model 3 days after transplant (16). To implement club cell depletion, 3T B6 → B6 (syngeneic) and 3T FVB → B6 (allogeneic) recipient mice ingested doxycycline between postoperative day (POD) 7 and POD 9.5. Immunohistochemical analysis of syngeneic and allogeneic transplants on POD 11 revealed small airways denuded of CCSP<sup>+</sup> cells, but with the preservation of cells that expressed acetylated  $\alpha$ -tubulin (Ac-tubulin), a marker for ciliated cells (Figure 1, B and C). By POD 16, however, syngeneic recipients had repaired their bronchiolar epithelium, as evident by reconstituted club and ciliated cell luminal monolayers that resembled preinjured bronchioles. In contrast, allografts contained club cells predominantly arranged in nonluminal monolayers without intervening ciliated cells. Additionally, many bronchioles had sharply reduced or completely absent luminal expression of Ac-tubulin, although scattered ciliated cells could be detected throughout the interstitium (Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.124732DS1>). Histological assessment of POD 16, 3T FVB → B6 recipients that underwent club cell ablation revealed high-grade inflammatory bronchiolar injury and severe obliterative disease (Figure 2, A–C). In contrast, analogously treated syngeneic lung transplants had at most mild graft inflammation, and lacked OB or peribronchial lesions up to POD 30 (Supplemental Figure 2). Total collagen deposition, which can be assessed by hydroxyproline assay or visualized by Masson's trichrome stain, is promoted by chronic airway injury and accumulates to a higher degree in BOS patients when compared with stable lung recipients (17). Hydroxyproline accumulation was significantly greater in allografts with OB when compared with syngeneic transplants that underwent club cell ablation (Figure 2D). Masson's trichrome staining of allograft OB lesions also revealed collagen deposition within transluminal fibrotic lesions (Figure 2E). Finally, we did not observe the generation of OB lesions in allograft recipients following treatment with naphthalene (Supplemental Figure 3), an agent that has been used to ablate club cells (18) but may possibly inhibit lymphocyte activation (19).

Previous work has demonstrated that club cells can be defined as pulmonary-restricted CD45<sup>+</sup>CD34<sup>+</sup>CD31<sup>+</sup>EpCAM<sup>hi</sup>CCSP<sup>hi</sup> cells (20). To further define the dynamics of bronchiolar epithelial repair in lung transplants we performed flow cytometric analysis to quantitate club cell abundance and proliferative responses following injury (Figure 3, A–H). In both syngeneic grafts and allografts there was an approximate 80% reduction in club cell abundance when measured 1.5 days after ablation. However, within the



**Figure 1. Lung allograft recipients are unable to repair their bronchiolar epithelium following club cell depletion.** (A) A mouse OLT model that generates OB in 16 days. B6 recipients of 3T FVB left lungs treated with CD40L Abs (POD 0) and CTLA4Ig (POD 2) to establish tolerance were fed doxycycline by food and water (DOX) from POD 7 to 9.5 and analyzed for graft inflammation on POD 16. 3T FVB and 3T B6 (B) donor lungs and (C) transplants stained with CCSP and Ac-tubulin Abs on POD 7, 11, and 16. Scale bars: 50 μm. Data shown are a representative result from at least 3 independent experiments.

remaining club cell compartment, the number undergoing replication was several-fold higher in syngeneic grafts when compared with allografts. Additionally, on POD 16, syngeneic grafts had nearly 3-fold more club cells relative to allografts. Therefore, club cell regenerative responses are sharply blunted in lung allografts that develop OB.

*Club cell ablation stimulates adaptive immune responses to lung transplants.* We next determined if club cell depletion leads to a loss of immune tolerance. To this end, we compared effector T cell responses in 3T FVB allografts to double-transgene (2T FVB) allografts that lack the lox-P-activatable DT-A gene and therefore do not develop OB (Supplemental Figure 4). 3T and 2T FVB allograft recipients had comparable numbers of intragraft CD4<sup>+</sup> T cells, Foxp3<sup>+</sup>CD4<sup>+</sup>, and IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells on POD 16 (Figure 4, A and B). However, club cell ablation led to greater accumulation of IL-17A<sup>+</sup>CD4<sup>+</sup>, total CD8<sup>+</sup>, and IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells within 3T FVB allografts. Additionally, specifically within airspaces of 3T FVB allografts, there was greater than a 3-fold increase of IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> T cells (Figure 4C). The development de novo donor-antigen-specific Abs (DSAs) is a reported risk factor for BOS development (21). We therefore asked if club cell ablation promotes the accumulation of Abs that recognize FVB antigens (Figure 4D). When compared with recipients of 2T FVB allografts, there were significantly greater amounts of DSA against FVB antigens in the peripheral blood of 3T FVB allograft recipients.

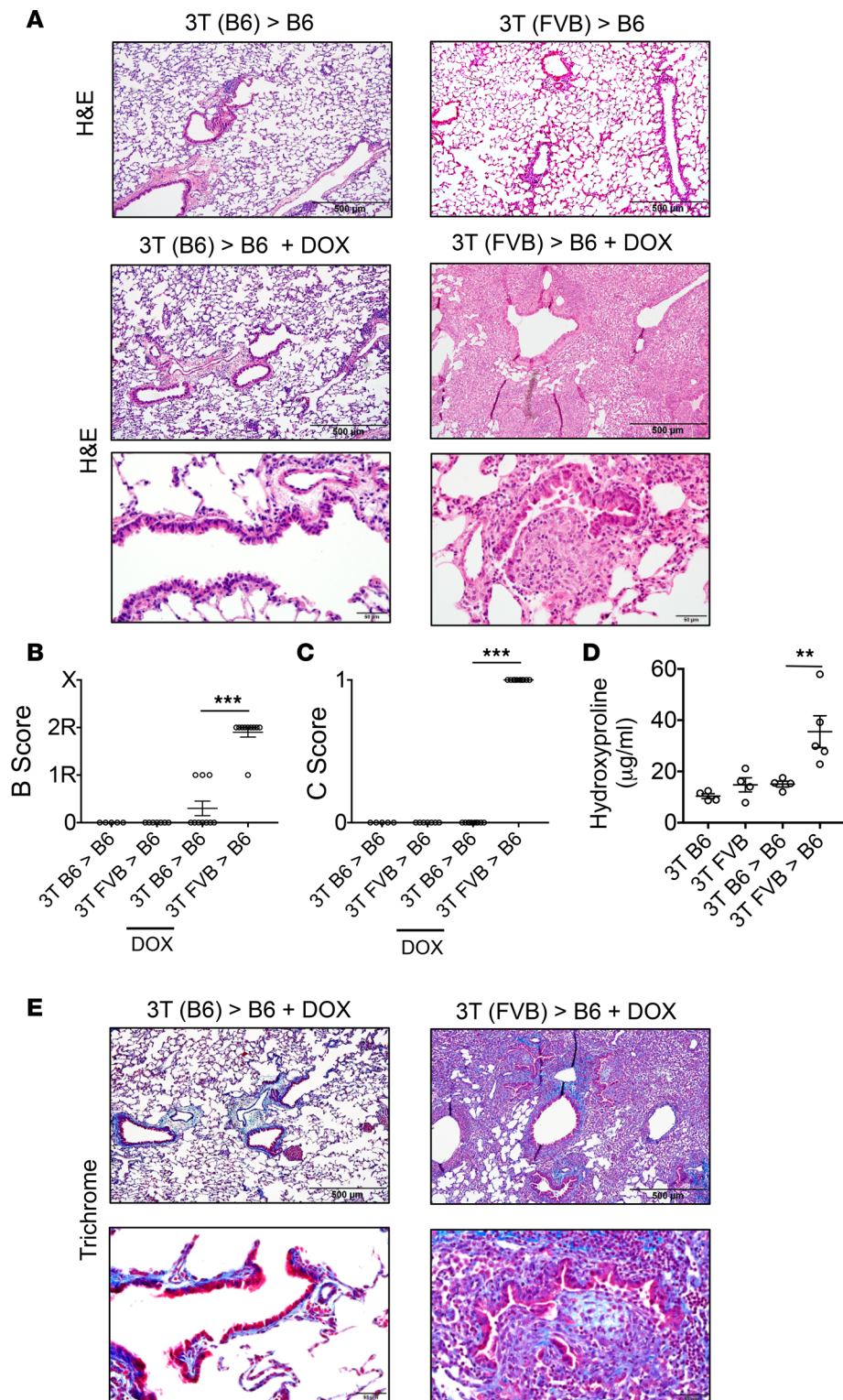
Because BOS progression is reportedly coupled to the loss of tolerance to alloantigens and the pulmonary self-restricted antigens, collagen V (ColV) and K- $\alpha$ 1 tubulin (K $\alpha$ 1T) (22–24), we next assessed T cell antigen specificity through performing antigen recall assays on CD4<sup>+</sup> and CD8<sup>+</sup> T cells isolated from POD 16 lung transplants (Figure 5A). Challenge of 3T FVB allograft CD4<sup>+</sup> T cells with donor antigen-presenting cells (APCs) generated significantly higher amounts of IL-17A but not IFN- $\gamma$  production relative to CD4<sup>+</sup> T cells from 2T FVB allografts. Syngeneic APCs bearing ColV or K $\alpha$ 1T peptides also stimulated higher amounts of IL-17A from 3T FVB allograft CD4<sup>+</sup> T cells compared with those from 2T FVB allograft CD4<sup>+</sup> T cells. Despite the lack of overall expansion of intragraft IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> T cells there was a small but significant augmentation of IFN- $\gamma$  production following ColV stimulation. In addition, club cell ablation led to more IFN- $\gamma$  production from 3T FVB allograft CD8<sup>+</sup> T cells following stimulation with donor antigens or self-antigens. In line with these observations, allografts with OB had accumulated higher intragraft ColV mRNA and protein along the epithelial basement membrane and within OB lesions (Figure 5, B–D). Together, these results demonstrate that club cell depletion induces the loss of tolerance to donor and pulmonary self-restricted antigens in lung transplants.

*Alloantigen-primed CD8<sup>+</sup> T cells inhibit club cell regenerative responses.* T cells are required for lung allograft rejection (25), but whether they regulate reparative responses by club cells remains unknown. Accordingly, we examined the role of T cells in our OB model by utilizing Ab-mediated T cell depletion (Figure 6, A–C, and Supplemental Figure 5). Pan-T cell depletion greatly reduced epithelial inflammation and eliminated OB lesions in 5 out of 7 allografts. Pan-T cell depletion also resulted in the repopulation of bronchiolar epithelium with club and ciliated cells (Figure 6D). Additionally, club cell proliferative responses were sharply elevated over recipients that received control Abs (Figure 6, E and F). Because we observed the high accumulation of airspace-resident effector CD8<sup>+</sup> T cells in lung allografts with OB we next tested the effects of CD8-depleting Ab administration to recipients (Figure 7, A–F). Like pan-T cell depletion, CD8<sup>+</sup> T cell depletion improved club cell regenerative responses and prevented OB lesion generation. In contrast, OB development was unaffected by Ab-mediated CD4<sup>+</sup> T cell depletion (Supplemental Figure 6). To further examine the effects of CD8<sup>+</sup> T cells on epithelial injury we cocultured club cells with alloantigen-primed, unprimed allogeneic, or syngeneic CD8<sup>+</sup> T cells (Figure 8, A–C). Only alloantigen-primed CD8<sup>+</sup> T cells inhibited club cell proliferation and maintenance. Noting our previous observations that apical surface MHC class I expression on airway epithelial cells is recognized by allogeneic CD8<sup>+</sup> T cells (26), we asked if a neutralizing Ab that recognizes 1 of 3 MHC class I alleles (H-2K<sup>a</sup>) expressed by FVB mice (27) prevents CD8<sup>+</sup> T cell-mediated inhibition of club cell maintenance (Figure 8D). Blockade of H-2K<sup>a</sup> significantly improved club cell maintenance, indicating that direct interactions between airway-infiltrating CD8<sup>+</sup> T cells and club cells prevent bronchial epithelial repair in lung allografts.

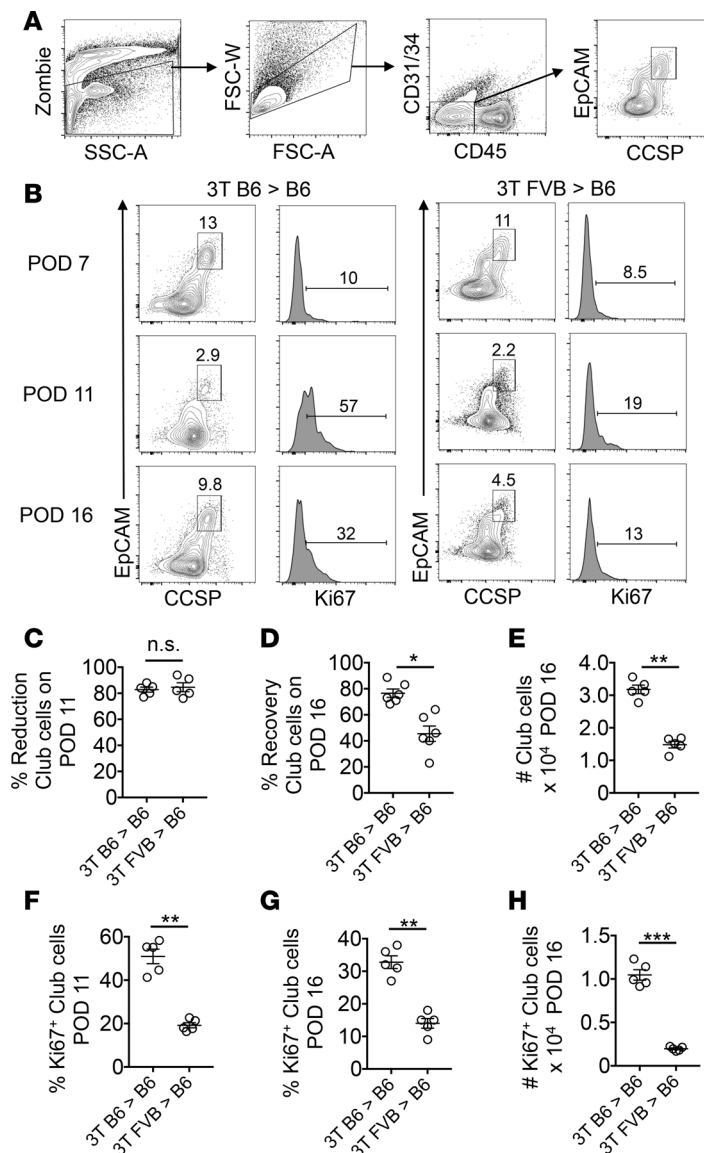
## Discussion

In the model of mouse orthotopic lung allograft transplantation reported in this study, we show that acute epithelial injury mediated by the ablation of club cells is sufficient to cause OB. The depletion and regeneration of the club cell compartment was assessed by immunohistochemistry and flow cytometric analysis.



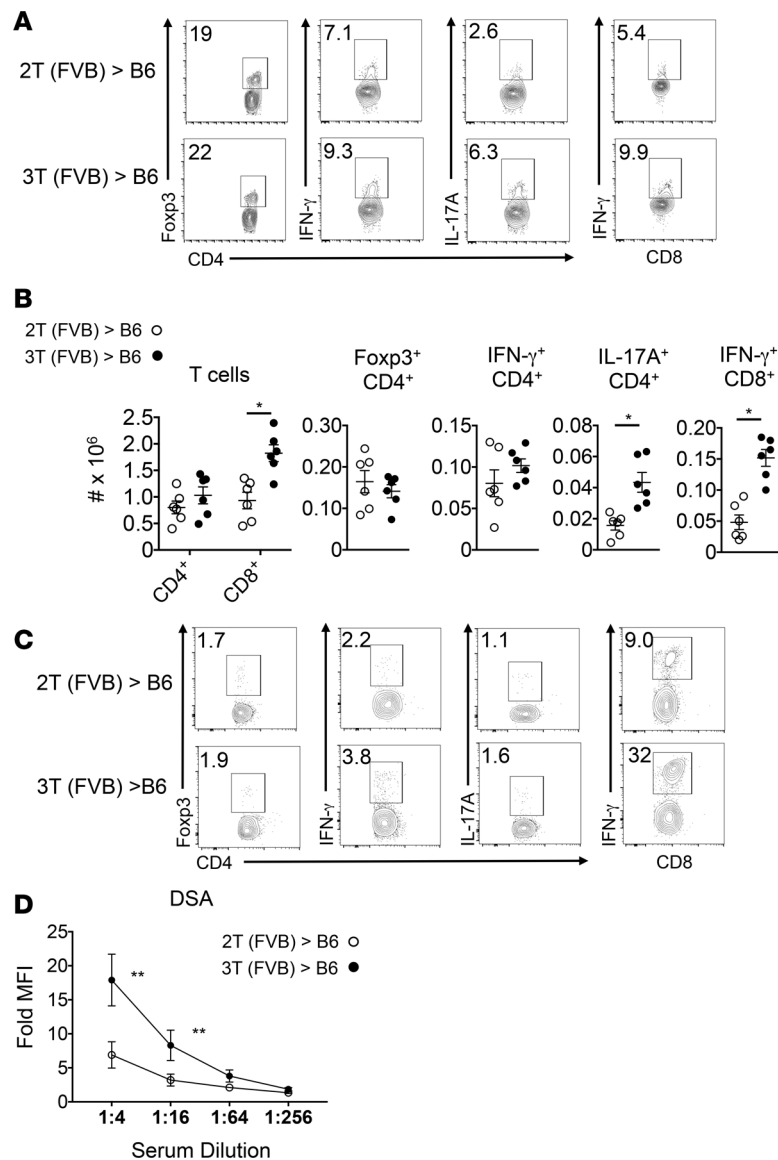


**Figure 2. Club cell ablation leads to severe OB lesions in lung allografts.** (A) H&E histological staining of indicated POD 16 transplants treated with or without DOX and blindly scored for (B) airway inflammation (B score) where 0 = none, 1R = low grade, 2R = high grade, and X = ungradable, and (C) the presence (1) or absence (0) of OB lesions (C score). Dot plots represent mean score  $\pm$  SEM of individual data obtained from 5 to 10 transplanted mice per group. (D) Hydroxyproline content and (E) Masson's trichrome staining of indicated transplant tissue at POD 16. Data shown in D represent mean  $\pm$  SEM ( $N \geq 4$ /group).  $**P < 0.01$ ,  $***P < 0.001$  by 2-tailed Mann-Whitney  $U$  test. Images in A and E are representative histology from at least 5 transplants. Scale bars: 500  $\mu$ m (upper images in both) or 50  $\mu$ m (lower images).



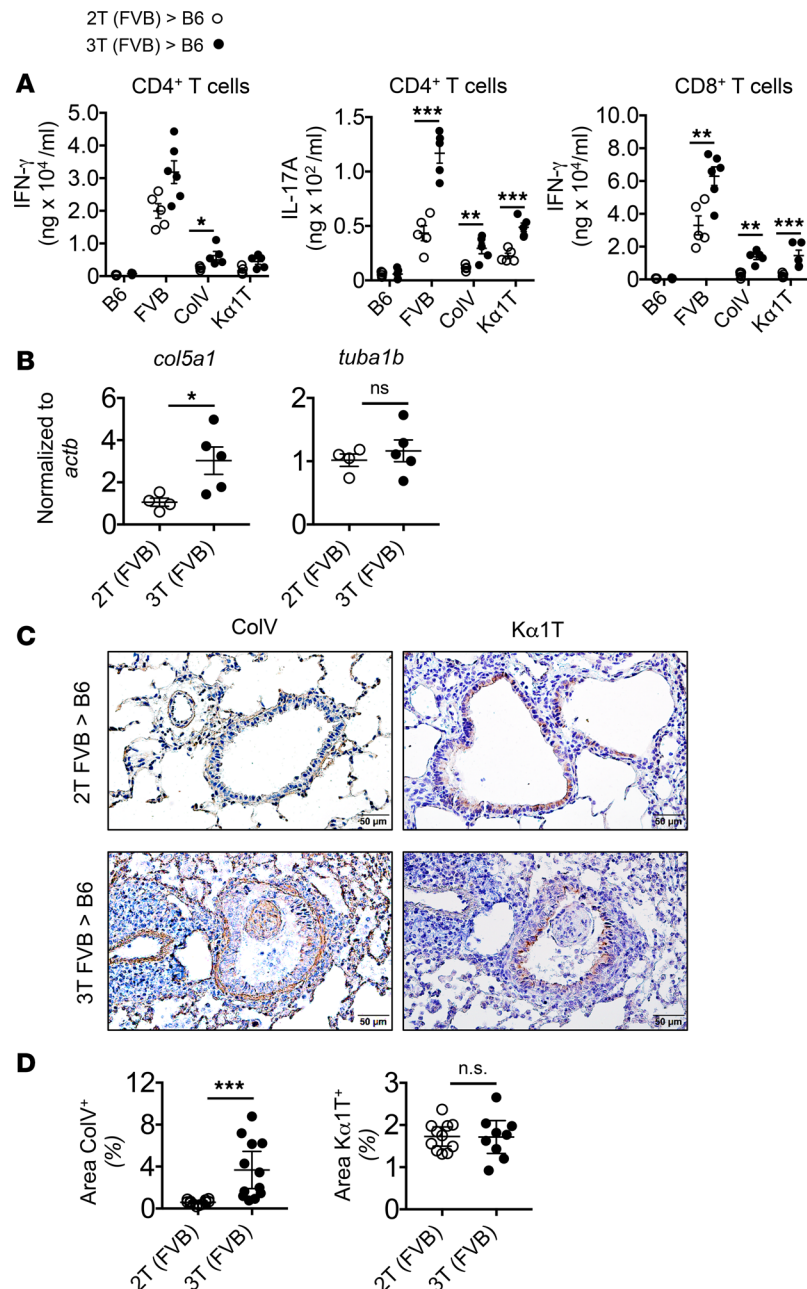
**Figure 3. Club cell regenerative responses are suppressed in lung allografts. (A)** FACS gating strategy for the identification of club cells. Data shown are contour plots from a 3T FVB donor lung just prior to transplantation. 3T B6 and 3T FVB transplants analyzed at indicated time points and assessed for club cell percentage abundance and proliferation (Ki67). **(B)** Representative contour blots and histograms from at least 4 transplants. **(C)** Percentage reduction of club cell abundance assessed on POD 11 calculated relative to levels prior to ablation on POD 7. **(D)** Percentage recovery of the club cell compartment assessed on POD 16 calculated relative to levels prior to ablation on POD 7. **(E)** Total number of intragraft club cells on POD 16. Club cell proliferation is shown as percentage abundance on **(F)** POD 11, **(G)** POD 16, or as a total number on **(H)** POD 16. Dot plots represent mean  $\pm$  SEM ( $N \geq 4$ /group). n.s., not significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by 2-tailed Mann-Whitney  $U$  test.

Given CCSP expression within epithelial progenitor pools, the latter approach allowed us to more precisely monitor the regenerative dynamics of club cells through the use of lineage-specific markers and intracellular CCSP expression (7, 20). Irrespective of whether the donor lung was on a B6 or FVB background we observed an average 80% reduction in club cells 1.5 days after doxycycline ingestion, indicating there was not a strain-specific induction of bronchiolar epithelial injury. Another approach to ablate club cells is through the systemic administration of naphthalene, which causes the production of cytotoxic metabolites via oxidation by cytochrome P450 2F2, a monooxygenase that is expressed in club cells (28). Naphthalene administration to our model was ineffective at producing OB lesions. Although the reasons for this are not clear, it has been reported that toxic naphthalene metabolites generated by the liver inhibit



**Figure 4. Club cell ablation promotes intra-graft effector T cell accumulation and DSA.** B6 recipients of 2T FVB and 3T FVB allografts that received DOX were analyzed by FACS on POD 16 for indicated intra-graft T lymphocytes. **(A)** Representative contour plots showing percentage abundance of graft-infiltrating effector T cells from **(B)** T lymphocyte numbers are shown for each transplant and as a mean per group  $\pm$  SEM ( $N = 6$  /group). \* $P < 0.05$  by 2-tailed Mann-Whitney  $U$  test. **(C)** Percentage abundance of airway-infiltrating effector T cells. Contour plots are a representative result from 3 transplants per group. **(D)** POD 16 recipient serum from 2T and 3T FVB recipients was coincubated with FVB thymocytes at indicated dilution ratios and assessed for DSA with anti-mouse IgM antibodies. Reactivity is shown as fold mean fluorescence intensity (MFI) relative to serum reactivity to syngeneic B6 thymocytes. Each data point represents a mean from at least 4 recipients per group  $\pm$  SEM. \*\* $P < 0.01$  by 2-tailed Mann-Whitney  $U$  test.

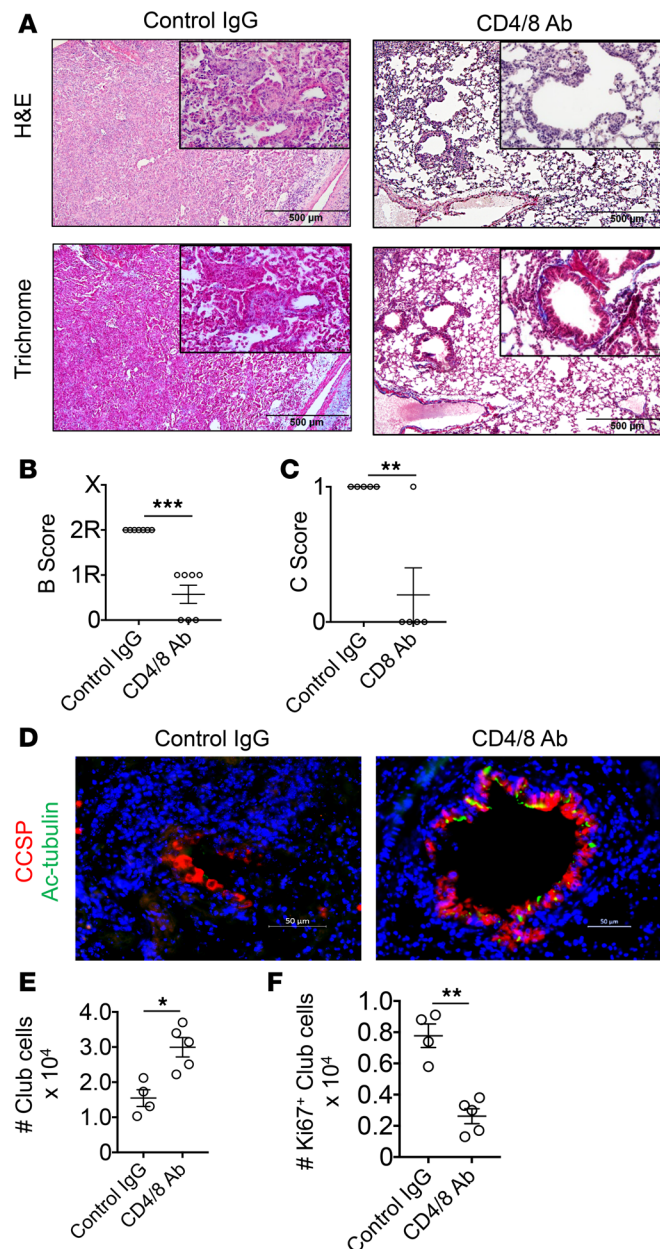
Ab production by plasma cells (19). Of note, in a new mouse OLT model of lymphocytic bronchitis, B cell deficiency protected allografts from club cell loss (29). Given that we observed DSA generation in our model, these observations collectively raise the possibility that naphthalene prevents a B cell-dependent response that promotes OB. Nevertheless, similar to reports of naphthalene-mediated club cell depletion, we observed the transient spreading of ciliated cells around denuded bronchioles and the retention of a population of injury-resistant club cells with high proliferative capacity (20, 30). This was most evident in the high number of proliferating club cells within transplants approximately 2 days after injury induction. Moreover, the relative abundance of proliferating club cells was nearly 3-fold lower in allografts when compared with syngeneic grafts, indicating there is a critical threshold of regenerative capacity required to adequately repair the bronchiolar epithelium.



**Figure 5. Loss of allogeneic and pulmonary self-antigen tolerance following club cell ablation.** (A) POD 16 intra-graft CD4<sup>+</sup> and CD8<sup>+</sup> T cell recall assays for syngeneic (B6) APCs, allogeneic (FVB) APCs, and syngeneic APCs that bear K $\alpha$ 1T or ColV peptides as measured by IFN- $\gamma$  and IL-17A production. Data points shown are responses from individual transplants and means per group  $\pm$  SEM ( $N \geq 5$ /group). (B) Semiquantitative RT-PCR measurements of intragraft ColV (*Col5a1*) and K $\alpha$ 1T (*Tuba1b*) mRNA shown normalized to  $\beta$ -actin (*Actb*). Each data point represents a single transplant along with a group mean  $\pm$  SEM ( $N = 5$ /group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by 2-tailed Mann-Whitney  $U$  test (A and B). Representative ( $N \geq 3$ /group) POD 16 allograft ColV and K $\alpha$ 1T (C) immunohistochemistry and (D) expression quantitation by staining area. In D, each histogram represents individual measurements from 3 or 4 randomly selected areas enriched for bronchioles for at least 3 transplants along with a mean per group  $\pm$  SEM. \*\*\* $P < 0.001$  by 2-tailed  $t$  test.

Club cell depletion led to the rapid loss of transplant-antigen-specific tolerance. The precise reasons for this observation remain unclear. Interestingly, Stripp and colleagues have shown that mice lacking CCSP expression have elevated Toll-like receptor 4 expression in pulmonary macrophages (31), suggesting that club cells could also play a regulatory role in preventing innate immune responses within lung transplants. Additionally, we observed a striking similarity between the pattern of adaptive immune responses following club cell ablation and clinical reports of lung transplant recipients with BOS. This included the augmentation of

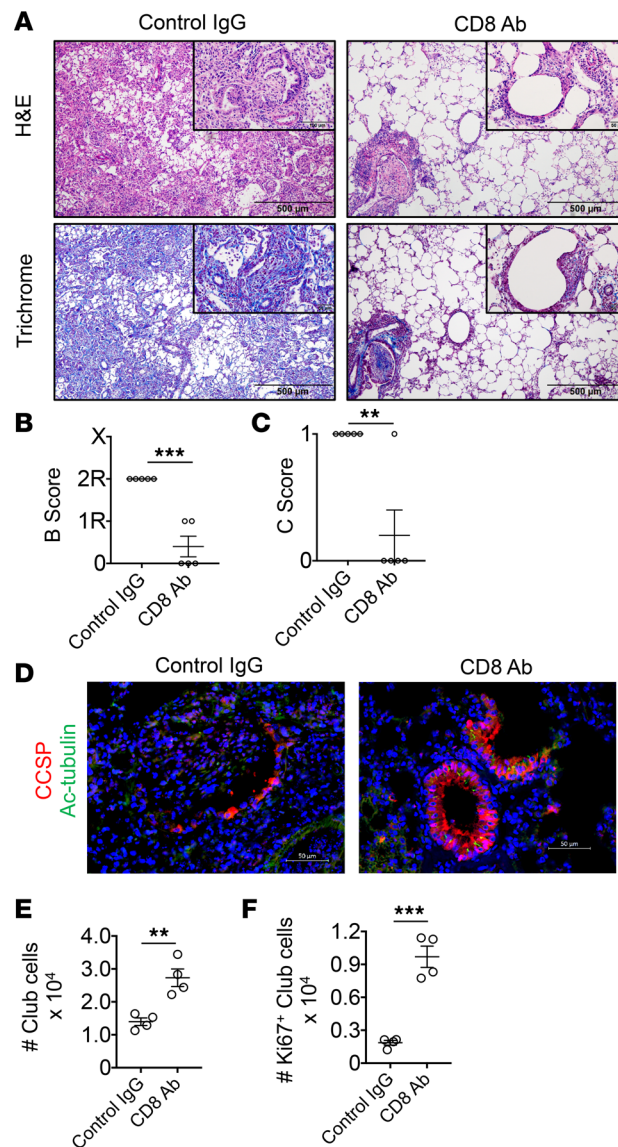




**Figure 6. Pan-T cell depletion inhibits OB and promotes club cell regenerative responses.** 3T FVB  $\rightarrow$  B6 recipients that underwent club cell depletion received either control Ig or CD4- and CD8-depleting Abs on POD 6 and POD 11 were analyzed on POD 16 for bronchiole injury by (A) H&E and Masson's trichrome stain, (B) airway inflammation, and (C) OB lesion score, or evaluated by immunohistochemistry for (D) bronchiolar epithelial repair with CCSP and Ac-tubulin Abs. Transplant club cell (E) total numbers and (F) proliferating numbers (Ki67<sup>+</sup>) on POD 16. Results shown in A are representative stains used for grading in B and C, which show mean scores  $\pm$  SEM ( $N = 7$ /group). The insets in A are  $\times 400$  (original magnification). Scale bars: 500  $\mu$ m (A) and 50  $\mu$ m (D). Stains shown in D are representative of 7 transplants and E and F show mean numbers  $\pm$  SEM ( $N \geq 4$ /group). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by 2-tailed Mann-Whitney  $U$  test.

Th17-mediated immunity (24), the enhanced recognition of K $\alpha$ 1T and CoIV (23), the development of DSA (21, 22), and high numbers of effector CD8<sup>+</sup> T cells in the airway (32, 33). We also made insights into effector T cell activation during OB development. To the best of our knowledge, this is the first report of the existence of K $\alpha$ 1T- and CoIV-specific effector CD8<sup>+</sup> T cells in lung allografts. We also observed that increases in intra-graft IL-17A<sup>+</sup>CD4<sup>+</sup> T cells occur without significant changes in the overall number of graft-resident CD4<sup>+</sup> T cells. This finding suggests possible de novo generation of Th17 cells within graft tissue through the recruitment of an uncommitted or naive CD4<sup>+</sup> T cell pool. We have previously demonstrated that the targeted depletion of lung allograft-resident Foxp3<sup>+</sup>CD4<sup>+</sup> T cells abrogates established tolerance (34). Recent reports have

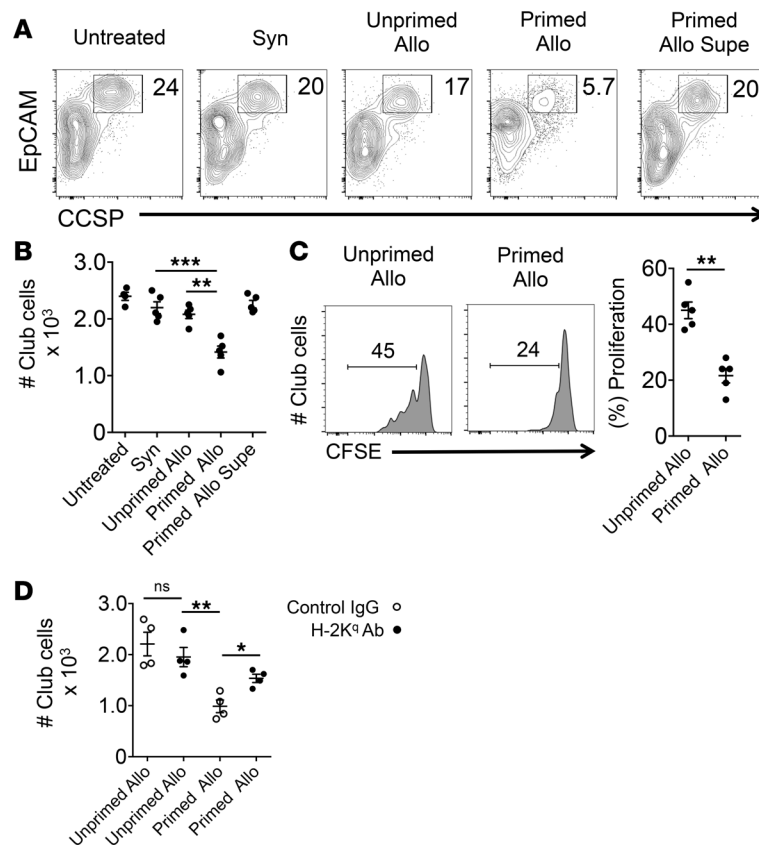




**Figure 7. CD8<sup>+</sup> T cell depletion inhibits OB and promotes club cell regenerative responses.** 3T FVB → B6 recipients that underwent club cell depletion received either control IgG or CD8-depleting Abs on POD 6 and POD 11 and were analyzed on POD 16 for transplant bronchiole injury by (A) H&E and Masson's trichrome stain, (B) airway inflammation, and (C) OB lesion score, or evaluated by immunohistochemistry for (D) bronchiolar epithelial repair with CCSP and Ac-tubulin Abs. Transplant club cell (E) total numbers and (F) proliferating numbers (Ki67<sup>+</sup>) on POD 16. Results shown in A are representative stains used for grading in B and C, which show mean scores ± SEM (N = 5/group). The insets in A are ×400 (original magnification). Scale bars: 500 μm (A) and 50 μm (D and insets in A). Stains shown in D are representative of 5 transplants and E and F show mean numbers ± SEM (N = 4/group). \*\*P < 0.01, \*\*\*P < 0.01 by 2-tailed Mann-Whitney U test.

also linked the loss of peripheral blood Foxp3<sup>+</sup>CD4<sup>+</sup> T cells with increased risk for CLAD (35, 36). Although we did not quantitate peripheral circulating Foxp3<sup>+</sup>CD4<sup>+</sup> T cells, there was no significant change in absolute numbers within graft tissues. One explanation for this discordant observation is that the ratio of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells to T cell effectors may be a more informative measure than is the assessment of absolute numbers (37). Indeed, due to increased Th17 and effector CD8<sup>+</sup> T cell numbers, this ratio was sharply lower in allografts with OB. The development of Foxp3<sup>+</sup>CD4<sup>+</sup> T cell regulatory dysfunction may also play an important role in allograft rejection (38). Thus, future studies will be required to better understand the role of Foxp3<sup>+</sup>CD4<sup>+</sup> T cell abundance and function in OB pathogenesis.

Club cell ablation promoted elevated ColIV mRNA and protein expression, which was especially evident within the epithelial basement membranes and OB lesions. Of note, IL-17 has been previously



**Figure 8. Alloantigen-primed effector CD8<sup>+</sup> T cells inhibit club cell maintenance and proliferation.** Club cells isolated by lineage-negative enrichment from FVB lungs were cocultured without (Untreated) or with either unactivated syngeneic FVB (Syn), unactivated allogeneic B6 (Unprimed Allo) or alloantigen-primed B6 (Primed Allo) CD8<sup>+</sup> T cells, or with supernatant from alloantigen-primed B6 CD8<sup>+</sup> T cells (Primed Allo Supe). Thirty-six hours later, club cell abundance was analyzed and shown as **(A)** representative contour plots from at least 4 independent experiments, or **(B)** total numbers per experiment with a group mean  $\pm$  SEM ( $N \geq 4$ /group). Club cells analyzed for proliferation in the presence of indicated CD8<sup>+</sup> T cells by **(C)** CFSE dilution shown as a representative histogram from 5 independent experiments along with a group mean  $\pm$  SEM.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  by 2-tailed Mann-Whitney *U* test (**B** and **C**). **(D)** Club cell numbers remaining after coculture with indicated CD8<sup>+</sup> T cells in the presence of control IgG or a neutralizing antibody directed against the H-2K<sup>a</sup> (FVB haplotype) MHC class I protein. Each data point represents a single determination from 4 independent experiments with a group mean  $\pm$  SEM.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$  by unpaired 2-tailed *t* test.

reported to promote ColV overexpression and the development of OB (39). In line with this previous observation we demonstrated that CD4<sup>+</sup> T cells isolated from allografts with OB made significantly more IL-17A when challenged with ColV peptides when compared with CD4<sup>+</sup> T cells isolated from tolerant allografts. Despite increased T cell-mediated recognition of K $\alpha$ 1T we did not detect changes in its expression following club cell ablation. One possible reason for these differences comes from a recent finding that K $\alpha$ 1T protein is highly enriched within exosomes released from human solid organ allografts, including chronically rejected lung transplants (40). As exosome protein cargo can be endocytosed and presented by dendritic cells (41), it remains plausible that T cells can be primed for recognition of K $\alpha$ 1T without increased evidence of expression within parenchymal tissues.

In our model we used a well-established costimulatory blockade regimen (16, 42, 43) to induce lung allograft tolerance, which interrupts both CD154/CD40 and B7/CD28 signaling in T cells. CD154 antagonism has recognized toxicity in humans due to CD154 expression on activated platelets (44). Also, in a mouse heterotopic heart allograft model, the reported mechanism by which costimulatory blockade induces acceptance is through the deletion of alloreactive T cells (45). In contrast, calcineurin inhibitors are thought to promote tolerance by largely inhibiting T cell activation (46). These observations suggest that the use of costimulatory blockade limits the clinical relevance of our model. However, OB lesions have been reported to form spontaneously in about half of BALB/c  $\rightarrow$  B6 orthotopic lung transplant recipients immunosuppressed with cyclosporine and steroids at between 1 and 3 months after engraftment (47).

Moreover, despite the use of costimulatory blockade, we could readily detect intragraft alloreactive T cells in 2T FVB recipients, suggesting such clones are not efficiently deleted in lung transplants.

How T cells regulate epithelial cell repair in lung transplants remains an understudied area. This is likely due to observations that several CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets are required for the induction of immunosuppression-mediated lung allograft tolerance (16, 42, 43). Accordingly, we chose to deplete T cells using Abs after the establishment of tolerance (16), but just prior to the induction of epithelial injury to study club cell responses. We observed that pan-T cell depletion largely reversed the loss of proliferating club cells, restored the club cell compartment, and largely eliminated OB lesions. However, we still noted some epithelial inflammation conceivably caused by the homeostatic expansion of residual intragraft alloreactive T cells (48). We extended these studies to determine whether CD8<sup>+</sup> or CD4<sup>+</sup> T cells promote OB generation. CD8<sup>+</sup> T cell depletion led to the promotion of club cell regenerative responses and protection from OB that was comparable to pan-T cell depletion. In contrast, CD4<sup>+</sup> T cell depletion had no effect on OB lesion development, possibly due to the elimination of regulatory CD4<sup>+</sup> T cells, which are reported to inhibit effector CD8<sup>+</sup> T cell function in allografts (49, 50). We further explored the role of CD8<sup>+</sup> T cells in OB by coculturing CD8<sup>+</sup> T cells with club cells. Consistent with our observations of high numbers of airway-resident effector CD8<sup>+</sup> T cells in lung allografts with OB, alloantigen-primed CD8<sup>+</sup> T cells inhibited club cell maintenance and proliferation. Additionally, in line with previous findings that airway-resident allogeneic CD8<sup>+</sup> T cells recognize apical MHC class I expressed on airway epithelium (26), we observed that neutralizing Abs against H-2K<sup>a</sup> improved club cell maintenance. Whether alloantigen-primed CD8<sup>+</sup> T cells kill or suppress the proliferation of club cells was not directly addressed by our studies, but is the subject of future investigation.

In summary, using a model of OLT we find that club cell ablation leads to development of OB and the induction of adaptive immune responses associated with BOS risk. Given that severe and reliable OB lesions are generated within 16 days after transplantation, this model should be useful for screening potential approaches to treating bronchiolar epithelial injury. To that end, our studies advocate for further defining T cell-dependent pathways that affect club cell regeneration and differentiation to identify new therapeutic targets for the prevention of BOS.

## Methods

**Mice and OLT.** C57BL/6 (B6) and FVB/N (FVB) were purchased from Jackson Laboratories. CCSP rtTA/TetOCre/DT-A (3T) mice were gifts from Jeffery Whitsett of The Children's Hospital of Cincinnati. 3T mice were backcrossed to greater than 99% FVB and C57BL/6 backgrounds, which was confirmed using microsatellite-assisted accelerated backcrossing (MAX-BAX, Charles River). Mouse left OLT was conducted with 8- to 12-week-old donor and recipient mice as previously described by our group (42). To induce allograft acceptance, recipients received i.p. 250 µg of CD40L Abs (MR1) on POD 0 and 200 µg of mouse recombinant CTLA4Ig on POD 2 (16).

**Club cell ablation and T cell depletion.** Club cell injury was triggered by doxycycline ingestion via food (625 mg/kg chow, ENVIGO) and water (2 mg/ml, Sigma-Aldrich) from POD 7 to POD 9.5. T lymphocyte cell depletion was accomplished by i.p. injection of anti-CD4 (500 µg GK1.5; Bio X Cell) or anti-CD8 (500 µg, 53-6.7, Bio-X-Cell) Abs or by using both aforementioned Abs for pan-T cell depletion. We used rat IgG2b (clone LTF-2; Bio-X-Cell), rat IgG2a (clone 2A3; Bio-X-Cell), or both Abs for isotype controls for CD4<sup>+</sup>, CD8<sup>+</sup>, or pan-T depletion, respectively. Abs were administered on POD 6 and 11.

**Histological analysis.** Harvested grafts were formaldehyde fixed, paraffin embedded, and stained with H&E or Masson's trichrome stain. Lung transplant histology was graded by a blinded pathologist using the 2007 revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection, where small-airway inflammation is scored Grade B0 (none), Grade B1R (low grade), Grade B2R (high grade), and BX (ungradable). OB is graded without regard to inflammation as C1 (present) or C0 (absent) (51).

Bronchiolar epithelium paraffin sections were first blocked with 5% goat serum and 2% fish gelatin (both from Sigma-Aldrich) at 25°C for 45 minutes. Sections were then stained with 1:500 polyclonal rabbit anti-mouse/rat CCSP (Seven Hills Bioreagents) and mouse anti- $\alpha$ -tubulin, 1:5000 (6-11B-1, Sigma-Aldrich) overnight at 4°C. For immunofluorescent visualization, we used goat anti-mouse Alexa Fluor 488-labeled secondary Ab, 1:1000 (Thermo Fisher Scientific) and goat anti-rabbit Alexa Fluor 555-labeled secondary Ab, 1:1000 (Cell Signaling Technology). For ColIV and  $\alpha$ 1T immunohistological analysis 1:100 polyclonal rabbit anti-ColIV (Abcam), 1:200 polyclonal rabbit anti- $\alpha$ 1T (Thermo Fisher Scientific),

or polyclonal rabbit IgG Isotype (Abcam) Ab dilutions were used in conjunction with a Vectastain Elite System kit (LS Bio) for detection in accordance with the manufacturer's recommendations. Quantitation of stain was conducted with ImageJ bundled with Java version 1.8.0 (NIH) in which total pixels of stain were assessed outside nuclear areas. For intragraft collagen measurements we used a Hydroxyproline Assay Kit (Sigma-Aldrich) in accordance with manufacturer's recommendations. Briefly, lung tissue was extracted for protein with 6.0N HCl for 3 hours at 120°C and then dried at 60°C overnight. Then, 10 µg of protein was assayed for reactivity for oxidized hydroxyproline with 4-(dimethylamino) benzaldehyde, which in turn generates colorimetric product that was read by absorbance at 560 nm on a BioTek Synergy HTX Microplate Analyzer.

*Flow cytometric analysis and antigen recall assays.* Lung tissue was minced and digested in RPMI 1640 with type 2 collagenase (0.5 mg/ml) (Worthington Biochemical) and 5 units/ml DNase (Sigma-Aldrich) for 90 minutes at 37°C and then filtered through a 70-µm cell strainer (Thermo Fisher Scientific) and treated with ACK lysing buffer (Worthington Biochemical). Live cell discrimination was conducted with the Zombie (Biolegend) fixable dye. Cell surface staining was conducted with the following Abs: CD45 (30-F11, eBioscience), CD45.2 (104, Biolegend), CD90.2 (53-2.1, eBioscience), CD4 (clone RM4-5, eBioscience), CD8α (53-6.7, eBioscience), CD31 (390, Biolegend), CD34 (HM34, Biolegend), and CD326 (G8.8, Biolegend). Staining for Foxp3 (FJK-16s, eBioscience), Ki-67 (16A8, Biolegend), and polyclonal rabbit anti-mouse CCSP (Seven Hills Bioreagents, catalog WRAB-3950) was conducted with an intranuclear Transcription Factor Staining Buffer Kit (Invitrogen) in accordance with the manufacturer's recommendations. For IFN-γ and IL-17A expression, cells were first stimulated with 1 µM ionomycin (Sigma-Aldrich) and 20 ng/ml PMA (Sigma-Aldrich) for 3.5 hours with 2 µM Golgi Plug (BD Biosciences) added for the last 3 hours of stimulation and stained with anti-IFN-γ (XMGI.2, eBioscience) and anti-IL-17A (TC11-18H10.1, Biolegend) using a Cytotfix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer's recommendations. For antigen specificity measurements, T cells were fractionated by positive selection using CD4<sup>+</sup> or CD8<sup>+</sup> immunomagnetic bead labeling kits (Miltenyi Biotec) from allograft cell suspensions and cocultured at a 3:1 ratio with irradiated T cell-depleted FVB or B6 cell splenocytes for 96 hours pulsed with 0.5 µg/ml Kα1T and ColV (obtained from T. Mohannakumar, St. Joseph's Hospital, Phoenix, Arizona, USA). IFN-γ and IL-17A were measured with uncoated ELISA kits from Invitrogen in accordance with the manufacturer's recommendations.

*Semiquantitative RT-PCR.* Lung tissues were extracted for RNA with an RNeasy kit (Qiagen) and reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Transcripts were semiquantified with PrimePCR Syber Green Assay (Bio-Rad) kits for *Col5a1* (qMmuCED0046279), *Tuba1b* (qMmuCED0040564), and *Atcb* (qMmuCED0027505) in accordance with the manufacturer's recommendations.

*Club cell enrichment and culture with CD8<sup>+</sup> T cells.* FVB lung tissue cell isolates were prepared as described for FACS preparation and incubated with biotin-conjugated Abs (all from eBioscience) specific for CD45.1 (clone A20), CD34 (clone RAM34), CD31 (clone MEC13.3), CD90.1 (clone HIS51), and CD15 (clone mc-480), washed, and then labeled with anti-biotin MicroBeads (Miltenyi Biotec) for negative selection on LS columns (Miltenyi Biotec). Remaining cells were then incubated with biotin-conjugated CD326 Abs (clone caa7-9G8, Miltenyi Biotec), washed, and then labeled anti-biotin MicroBeads for positive selection on an MS column (Miltenyi Biotec). Enriched club cell fractions were resuspended in MTEC/Plus medium (52) and seeded at  $3.3 \times 10^4$  cells per well in flat-bottom 96-well tissue culture plates (Thermo Fisher Scientific) coated with 50 µg/ml type I rat tail collagen (Becton Dickinson). For proliferation analysis, club cells were stained with 10 µM CFSE (Thermo Fisher Scientific) prior to seeding. CD8<sup>+</sup> T cells were isolated from B6 or FVB resting mouse spleens using CD8<sup>+</sup> immunomagnetic bead labeling kits (Miltenyi Biotec) in accordance with the manufacturer's recommendations. To prime CD8<sup>+</sup> T cells with alloantigen, T cell-depleted CD45.1<sup>+</sup> FVB splenocytes were cocultured with B6 CD8<sup>+</sup> T cells at a 3 to 1 respective ratio with 100 ng/ml LPS for 72 hours, which was used to augment alloantigen presentation. Alloantigen-primed CD8<sup>+</sup> T cells were removed from cultures by using immunomagnetic bead positive selection conducted with biotin-conjugated CD45.2 Abs (clone A20, eBioscience), anti-biotin MicroBeads, and LS columns. CD8<sup>+</sup> T cells ( $1 \times 10^5$ ) were added to each club cell culture well for 24 hours. For proliferation analysis, CD8<sup>+</sup> T cells were cocultured with CFSE-labeled club cells for 36 hours. Four or 5 wells were used for each test condition. For MHC class I blockade experiments 10 µg/ml of neutralizing Abs against H-2K<sup>a</sup> (clone Y-3, Bio-X-Cell) or IgG2b isotype control (clone MPC-11, Bio-X-Cell) was added to cultures 1 hour prior to the placement of CD8<sup>+</sup> T cells.



Club cells were released from plates using 0.05% trypsin (Thermo Fisher Scientific), washed, and then analyzed on a Zombie-CD45-CD31-CD34-CD326<sup>+</sup>CCSP<sup>+</sup> gate using Countbright beads (Thermo Fisher Scientific) for quantitative FACS analysis.

**Statistics.** Unless indicated, a 2-tailed Mann–Whitney *U* test was performed to assess statistical relevance using GraphPad Prism version 7.0. *P* < 0.05 was considered significant. n.s., not significant.

**Study approval.** Animal experiments were conducted in accordance with an approved Washington University Animal Care and Use Committee protocol (number 20160212).

## Author contributions

AEG, ZL, HJH, AKTP, JMG, and SLB designed the experiments. AEG, HJH, and AKTP conceptualized the model design. ZL, DS, MC, DLC, and HJH executed T cell studies. ZL performed immunohistochemical staining, developed the club cell assays, and made figures. FL performed the mouse orthotopic lung transplants. YF conducted the donor-specific antibody analysis. KNP validated and monitored the HLA haplotypes of the triple-transgenic donor strains. AEG, ZL, RH, DK, ASK, and SLB wrote and edited the manuscript.

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