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 and poorly regenerate 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+ T cell depletion restores club cell reparative responses and prevents OB. In addition, ex-vivo analysis reveals a specific role for alloantigen-primed effector CD8+ T cells in preventing club cell proliferation and maintenance. Taken together, we demonstrate a vital role for club cells in maintaining lung transplant tolerance and propose a new model to identify the underlying mechanisms of OB.
An Obligatory Role for Club Cells in Preventing Obliterative Bronchiolitis in Lung Transplants

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**Abstract**

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$^+$ T cell depletion restores club cell reparative responses and prevents OB. In addition, ex-vivo analysis reveals a specific role for alloantigen-primed CD8$^+$ T cells in inhibiting club cell proliferation and maintenance. Taken together, we demonstrate a vital role for club cells in maintaining lung transplant tolerance and propose a new model to identify the underlying mechanisms of OB.
**Introduction**

For over three decades lung transplantation has remained the only viable option for many types of end-stage pulmonary diseases. Despite advances in surgical and post-operative 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 (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 non-ciliated epithelial secretory cells that are highly prevalent in the pulmonary distal bronchioles in both human and mice (3). They are often identified by the expression of *Sgrb1a1*, which encodes for the club cell secretory protein (CCSP, CC10), a 16 kDa peptide with reported anti-inflammatory properties that coats airway surfaces (4). Club cells help maintain small airway homeostasis through detoxifying xenobiotic and oxidizing molecules, secretion of anti-microbial 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 injury (6, 7). Curiously, many non-alloimmune stressors of airway 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 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 adaptive immune responses that are coupled to BOS risk. Additionally, we find that CD8+ 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 mice bearing the following genes: a reverse tetracycline activator gene driven by the club cell secretory protein (CCSP) promoter, Cre recombinase gene under control of the reverse tetracycline activator, 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 diphtheria toxin expression specifically in CSSP-expressing cells, resulting in their depletion and consequential injury to bronchiolar epithelium (6). Because triple transgenic 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 à B6 lung recipients, we administered CD40L (CD154) neutralizing antibodies (Abs) and the CD80/86 antagonist CTLA4Ig (Fig. 1A), which we have previously demonstrated induces established tolerance in the mouse OLT model three days after transplant (16). To implement club cell depletion, 3T B6 à B6 (syngeneic) and 3T FVB à B6 (allogeneic) recipient mice ingested doxycycline between post-operative days (POD) 7 and POD 9.5. Immunohistochemical analysis of syngeneic and allogeneic transplants on POD 11 revealed small airways denuded of CCSP+ cells, but with the preservation of cells that expressed acetylated α-tubulin (Ac-tubulin), a marker for ciliated cells (Fig. 1B, C). By POD 16, however, syngeneic recipients had repaired their bronchiolar epithelium as evident by reconstituted club and ciliated cell luminal monolayers that resembled pre-injured bronchioles. In contrast, allografts contained club cells predominantly arranged in non-luminal 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 (Sup. Fig. 1). Histological assessment of POD 16, 3T FVB → B6 recipients that underwent club cell ablation revealed high grade inflammatory bronchiolar injury and severe obliterative disease (Fig. 2A-C). In contrast, analogously treated syngeneic lung transplants had at most mild graft inflammation, lacked OB or peribronchial lesions up to POD 30 (Sup. Fig. 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 higher degree in BOS patients when compared to stable lung recipients (17). Hydroxyproline accumulation was significantly greater in allografts with OB when compared to syngeneic transplants that underwent club cell ablation (Fig. 2D). Masson’s trichrome staining of allograft OB lesions also revealed collagen deposition within transluminal fibrotic lesions (Fig. 2E). Finally, we did not observe the generation of OB lesions in allograft recipients following treatment with naphthalene (Sup. Fig. 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⁻ CD34⁻ CD31⁻ EpCAM⁹⁹ CCSP⁹⁹ 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 (Fig. 3A-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 remaining club cell compartment, the number undergoing replication was several-fold higher in syngeneic grafts when compared to allografts. Additionally, on POD 16, syngeneic grafts had nearly three-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 diphtheria gene and therefore do not develop OB (Sup. Fig. 4).

3T and 2T FVB allograft recipients had comparable numbers of intragraft CD4+ T cells, Foxp3+ and IFN-γ+ CD4+ T cells on POD 16 (Fig. 4A, B). However, club cell ablation led to greater accumulation of IL-17A+ CD4+, total CD8+ and IFN-γ+ CD8+ T cells within 3T FVB allografts. Additionally, specifically within airspaces of 3T FVB allografts, there was greater than a 3-fold increase of IFN-γ+ CD8+ T cells (Fig. 4C). The development *de novo* donor antigen-specific antibodies (DSA) is a reported risk factor for BOS development (21). We therefore asked if club cell ablation promotes the accumulation of antibodies that recognize FVB antigens (Fig. 4D).

When compared to recipients of 2T FVB allografts, there were significantly greater amounts of DSA to FVB antigens in the peripheral blood of 3T FVB allograft recipients.

Because BOS progression is reported coupled to the loss of tolerance to alloantigens and the pulmonary self-restricted antigens, Collagen V (ColV) and K-α1 tubulin (Kα1T) (22-24), we next assessed T cell antigen specificity through performing antigen recall assays on CD4+ and CD8+ T cells isolated from POD 16 lung transplants (Fig. 5A). Challenge of 3T FVB allograft CD4+ T cells with donor antigen presenting cells (APCs) generated significantly higher amounts of IL-17A but not IFN-γ production relative to CD4+ T cells from 2T FVB allografts. Syngeneic APCs bearing ColV or Kα1T peptides also stimulated higher
amounts IL-17A from 3T FVB allograft CD4\(^+\) T cells when compared from 2T FVB allograft CD4\(^+\) T cells. Despite the lack of overall expansion of intragraft IFN-\(\gamma\) \(^+\)CD4\(^+\) 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\(^+\) 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 (Fig. 5B-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\(^+\) 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 antibody-mediated T cell depletion (Fig. 6A-C, Sup. Fig. 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 (Fig. 6D). Additionally, club cell proliferative responses were sharply elevated over recipients that received control Abs (Fig. 4E, F). Because we observed the high accumulation of airspace-resident effector CD8\(^+\) T cells in lung allografts with OB we next tested the effects of CD8-depleting antibody administration to recipients (Fig. 7A-F). Similar to pan-T cell depletion, CD8\(^+\) T cell depletion improved club cell regenerative responses and prevented OB lesion generation. In contrast, OB development was unaffected by antibody-mediated CD4\(^+\) T cell depletion (Sup. Fig. 6). To further examine the effects of CD8\(^+\) T cells on
epithelial injury we co-cultured club cells with alloantigen-primed, unprimed allogeneic or syngeneic CD8+ T cells (Fig. 8A-C). Only alloantigen-primed CD8+ 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+ T cells (26), we asked if a neutralizing antibody that recognizes one of three MHC Class I alleles (H-2Kd) expressed by FVB mice (27) prevents CD8+ T cell-mediated inhibition of club cell maintenance (Fig. 8D). Blockade of H-2Kd significantly improved club cell maintenance indicating that direct interactions between airway-infiltrating CD8+ T cells and club cells prevent bronchial epithelial repair in lung allografts.
Discussion

In a new model of mouse orthotopic lung allograft transplantation 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. 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 a 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 metabolism 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 antibody production by plasma cells (19). Of note, in a new mouse orthotopic lung transplant 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 three-fold lower in allografts when compared to syngeneic grafts indicating there is a critical threshold of regenerative capacity required to adequately repair the bronchiolar epithelium.

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 Th17-mediated immunity (24), the enhanced recognition of Kα1T and ColV (23), the development of DSA (21, 22), and high numbers of effector CD8+ T cells in the airway (32, 33).

We also made new insights into effector T cell activation during OB development. To the best of our knowledge, this is the first report of the existence of Kα1T and ColV-specific effector CD8+ T cells in lung allografts. We also observed that increases in intragraft IL-17A+ CD4+ T cells occurs without significant changes in the overall number of graft-resident CD4+ T cells. This finding suggests possible de novo generation of T_h,17 cells within graft tissue through the recruitment of an uncommitted or naive CD4+ T cell pool. We have previously demonstrated that the targeted depletion of lung allograft-resident Foxp3+ CD4+ T cells abrogates established tolerance (34). Recent reports have also linked the loss of peripheral blood Foxp3+ CD4+ T cells with increased risk for CLAD (35, 36). Although we did not quantitate peripheral circulating Foxp3+ CD4+ 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+ CD4+ 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+ T cell numbers, this ratio sharply was sharply lower in allografts with OB. The development of Foxp3+ CD4+ 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+ CD4+ T cell abundance and function in OB pathogenesis.

Club cell ablation promoted elevated ColV mRNA and protein expression, which was especially evident within the epithelial basement membranes and OB lesions. Of note, IL-17 has been previously reported to promote ColV overexpression and the development of obliterative bronchiolitis (39). In line with this previous observation we demonstrated that CD4+ T cells isolated from allografts with OB made significantly more IL-17A when challenged with ColV peptides when compared to CD4+ T cells isolated from tolerant allografts. Despite increased T cell-mediated recognition of Kα1T we did not detect changes in its expression following club cell ablation. One possible reason for these differences comes from a recent finding that Kα1T protein is highly enriched within exosomes released from human solid organ allografts, including chronically rejecting 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 Kα1T 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 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 → B6 orthotopic lung transplant recipients immunosuppressed with cyclosporine and steroids at between one and three 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 likely due to observations that several CD4+ and CD8+ T cell subsets are required for the induction of immunosuppression-mediated lung allograft tolerance (16, 42, 43). Accordingly, we chose to antibody-deplete T cells 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+ or CD4+ T cells promote OB generation. CD8+ 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+ T cell depletion had no effect on OB lesion development possibly due of the elimination of regulatory CD4+ T cells, which are reported to inhibit effector CD8+ T cell function in allografts (49, 50). We further explored the role of CD8+ T cells in OB by co-culturing CD8+ T cells with club cells. Consistent with our observations of high numbers of airway resident effector CD8+ T cells in
lung allografts with OB, alloantigen primed CD8+ T cells inhibited club cell maintenance and proliferation. Additionally, in line with previous findings that airway-resident allogeneic CD8+ T cells recognize apical MHC Class I expressed on airway epithelium (26), we observed that neutralizing antibodies to H-2Kb improved club cell maintenance. Whether alloantigen-primed CD8+ 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 novel model of orthotopic lung transplantation 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 impact club cell regeneration and differentiation to identify new therapeutic targets for the prevention of BOS.
Materials and Methods

Mice and Orthotopic Lung Transplantation

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-BAXSM; Charles River). Mouse left lung orthotopic transplantation was conducted with 8-12 week donor and recipient mice as previously described by our group (42). To induce allograft acceptance, recipients received i.p. 250 µg of CD40L antibodies (Abs, MR1) on post-operative day (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) from POD 7 to POD 9.5. T lymphocyte cell depletion was accomplished by intraperitoneal injection of CD4 (500 µg GK1.5; Bio X Cell) or CD8 (500 µg 53-6.7; Bio-X-Cell) Abs or by using both aforementioned Abs for pan-T cell depletion. We used either Rat IgG2b (clone LTF-2; Bio-X-Cell), Rat IgG2a (clone 2A3; Bio-X-Cell) or both Abs for isotype controls for CD4+, CD8+ or pan-T depletion, respectively. Antibodies were administered on POD 6 and 11.

Histological Analysis

Harvested grafts were formaldehyde-fixed, paraffin-embedded and stained with hematoxylin-eosin 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 ungradable BX. 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 mins. Sections were then stained with 1:500 polyclonal rabbit anti-mouse/rat CCSP (Seven Hills Bioreagents) and mouse anti-Acetylated 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 antibody, 1:1000 (Thermo Fisher) and goat anti-rabbit Alexa Fluor 555-labeled secondary antibody, 1:1000 (Cell Signaling Technology). For ColIV and Ka1T immunohistological analysis 1:100 polyclonal rabbit anti-Collagen V (Abcam), 1:200 polyclonal rabbit anti-Kα1-Tubulin (Thermo Fisher) or polyclonal rabbit IgG Isotype (Abcam) Abs dilutions were used in conjunction with 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) where total pixels of stain were assessed outside nuclear areas. For intragraft collagen measurements we used a Hydroxyproline Assay Kit (Sigma-Aldrich) in accordance with manufacturers recommendations. Briefly, lung tissue was extracted for protein with 6.0 N HCl for 3 h 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 a RPMI 1640 solution with Type 2 collagenase (0.5 mg/mL) (Worthington Biochemical) and 5 units/mL DNAse (Sigma) for 90 min at 37 °C and then filtered through a 70-um cell strainer (ThermoFisher) 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) and Ki-67 (16A8; Biolegend) and CCSP (Seven Hills Bioreagents) was conducted with intranuclear Transcription Factor Staining Buffer Kit (Invitrogen) in accordance with manufacturers recommendations. For IFN-γ and IL-17A expression cells were first stimulated with 1 µM ionomycin (Sigma) and 20 ng/ml PMA (Sigma) for 3.5 h with 2 µM Golgi Plug (BD Biosciences) added for last 3 h of stimulation and stained with IFN-γ (XMG1.2; eBioscience) and IL-17A (TC11-18H10.1; Biolegend) using a Cytofix/Cytoperm kit (BD Biosciences) in accordance with manufacturers recommendations. For antigen specificity measurements, T cells were fractionated by positive selection using CD4+ or CD8+ immunomagnetic bead labeling kits (Miltenyi) from allograft cell suspensions and co-cultured in a 3:1 ratio with irradiated T cell-depleted FVB or B6 cell splenocytes for 96 hours pulsed with 0.5 µg/ml K-α1 tubulin and Collagen V (obtained from T. Mohannakumar, St. Joseph’s Hospital, Phoenix AZ). IFN-γ and IL-17A were measured with uncoated ELISA kits from Invitrogen in accordance with manufacturer’s recommendations.
**Semi-quantitative RT PCR**

Lung tissues were extracted for RNA with RNAeasy kit (Qiagen) and reverse transcribed with the high capacity cDNA reverse transcription kit (Thermo Fisher). Transcripts were semi-quantified with a PrimePCR Syber Green Assay (Bio Rad) kits for col5a1 (qMmuCED0046279), tuba1b (qMmuCED0040564) and atcb (qMmuCED0027505) in accordance with manufacturers recommendations.

**Club cell enrichment and culture with CD8+ 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 MS column (Miltenyi Biotec) mediated-positive selection. Enriched club cell fractions were resuspended in MTEC/Plus medium (52) and seeded at $3.3 \times 10^4$ cell per well in flat bottom 96 well tissue culture plates (Thermo Fisher) 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) prior to seeding. CD8+ T cells were isolated from B6 or FVB resting mouse spleens using CD8+ immunomagnetic bead labeling kits (Miltenyi) in accordance with manufacturers recommendations. To prime CD8+ T cells with alloantigen, T cell-depleted CD45.1+ FVB splenocytes were co-cultured with B6 CD8+ T cells at a 3 to 1 respective ratio with 100 ng/ml LPS for 72 h, which was used to augment alloantigen presentation. Alloantigen-primed CD8+ 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. 10^5 CD8^+ T cells were added to each club cell culture well for 24 h. For proliferation analysis, CD8^+ T cells were co-cultured with CFSE-labeled club cells for 36 h. 4 or 5 wells were used for each test condition. For MHC class I blockade experiments 10 µg/ml of neutralizing antibodies to H-2K^q (clone Y-3, Bio-X-Cell) or IgG2b isotype control (clone MPC-11, Bio-X-Cell) was added to cultures 1h prior to the placement of CD8^+ T cells. Club cells were released from plates using 0.05% trypsin (Thermo Fisher), washed, and then analyzed on a Zombie^- CD45^- CD31^- CD34^- CD326^+ CCSP^+ gate using Countbright beads (Thermo Fisher) for quantitative FACS analysis.

Statistical Analysis

Unless indicated a Mann–Whitney two-tailed U test was performed to assess statistical relevance using GraphPad Prism version 7.0 (GraphPad Software). P < 0.05 was considered significant.

Study Approval

Animal experiments were conducted in accordance with an approved Institutional Animal Care and Use Committee protocol (Protocol Number: 20160212).
Authors Contributions

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References


Figure 1

A.

Donor 3T(FVB) → Left Lung Transplant → Donor 3T(B6)

Recipient B6

Tolerance Induction

CD40L Ab

CTLA4lg

Club Cell Ablation

DOX

Analysis

Post-Operative Days (POD)

Day 0

Day 2

Day 7

Day 9.5

Day 16

B.

Prior to Transplant

3T(B6) vs. 3T(FVB)

CCSP Ac-tubulin

C.

3T(B6) > B6

3T(FVB) > B6

Day 7

Day 11

Day 16

CCSP Ac-tubulin
Figure 1. Lung allograft recipients are unable 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. The scale bar represents 50 µm. Data shown are a representative result from at least 3 independent experiments.
Figure 2

A. Trichrome

B. B Score

C. C Score

D. Hydroxyproline (µg/ml)

E. Trichrome
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 treatment 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). Bar plots represent mean score ± standard error (S.E.) of individual data obtained from 5 to 10 transplanted mice per group. (D) Hydroxyproline content and (E) trichrome staining of indicated transplant tissue at POD16. Data shown in (D) represent means ± S.E. (N ≥ 4/group: **p < 0.01). (A, E) is representative histology from at least 5 transplants where scale bars represent 500 µm or 50 µm.
Figure 3

A.

B.

C.

D.

E.

F.

G.

H.
Figure 3. Club cell regenerative responses are suppressed in lung allografts. (A) FACS gating strategy for the identification for club cells. Data shown are contour plots form a 3T FVB donor lung just prior transplantation. 3T B6 and 3T FVB transplants analyzed at indicated time points and assessed for club cells percent abundance and proliferation (Ki67) where (B) are representative contour blots and histograms from at least 4 transplants where (C) is the percent reduction of club cell abundance assessed on POD 11 calculated relative to levels prior to ablation on POD 7. (D) Percent recovery of the club cell compartment assessed on POD 16 calculated relative to levels prior to ablation on POD 7 and (E) is the total number of intragraft club cells on POD 16. Club cell proliferation is shown as percent abundance on (F) POD 11, (G) POD 16 or as a total number on (H) POD 16. Bar graphs represent means ± S.E. (N ≥ 4/group; n.s.; not significant,**p < 0.01, *** p < 0.001)
Figure 4

A.

2T (FVB) > B6

3T (FVB) > B6

B.

2T (FVB) > B6  ○
3T (FVB) > B6  ●

T cells

CD4

CD8


C.

2T (FVB) > B6

3T (FVB) > B6

D.

DSA

Fold MFI

Serum Dilution

1:4 1:16 1:64 1:256

0 5 10 15 20 25

2T (FVB) > B6  ○
3T (FVB) > B6  ●
Figure 4. Club cell ablation promotes intragraft 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 intragraft T lymphocytes where (A) are representative contour plots from (B) where T lymphocyte numbers are shown for each transplant and as a mean per group ± S.E. (N = 6/group: *p < 0.05). (C) Percent abundance of airway effector T cells. Data shown is from representative experiment of 3 transplants per group. (D) POD 16 recipient serum from 2T and 3T FVB recipients was co-incubated with FVB thymocytes at indicated dilution ratios and assessed for DSA with anti-mouse specific 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 ± S.E. where **p < 0.01.
Figure 5

A. 

2T (FVB) > B6 ○
3T (FVB) > B6 ●

CD4+ T cells

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<tr>
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<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>ColV</td>
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</tr>
<tr>
<td>Kα1T</td>
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CD8+ T cells

B. 

col5a1 tuba1b

Normalized to actb

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<th>3T (FVB)</th>
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C. 

2T FVB > B6

3T FVB > B6

D. 

Area ColV (%)

Area Kα1T (%)

0 2 4 6 8 10 12

0 1 2 3

n.s.
Figure 5. Loss of allogeneic and pulmonary self-antigen tolerance following club cell ablation. (A) POD 16 intragraft CD4+ and CD8+ T cell recall assays to syngeneic (B6) APCs, allogeneic (FVB) APCs, and syngeneic APCs that bear Kα1T or ColV peptides as measured by IFN-γ and IL-17A production. Data points shown are responses from individual transplants and means per group ± S.E. (N ≥ 5/group; *p < 0.05, **p < 0.01, ***p < 0.001). (B) Semi-quantitative RT-PCR measurements of intragraft ColV (col5a1) and Kα1T (tuba1b) mRNA shown normalized to beta-actin (actb). Each data point represents a single transplant along with a group mean ± S.E (N=5/group; *p <0.05). Representative (N ≥ 3/group) POD 16 allograft ColV and Kα1T (C) immunochemistry 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 ± S.E (***p <0.001; two-tailed t test).
Figure 6

A. 

H&E

Control IgG

CD4/8 Ab

Trichrome

B.

C.

D.

CCSP

Ac-tubulin

E.

F.
Figure 6. Pan-T depletion inhibits OB and promotes club cell regenerative responses. 3T FVB→ B6 recipients that underwent club cell depletion received either Control Ig or CD4 & CD8 depleting Abs on POD 6 and POD 11 were analyzed on POD 16 for bronchiole injury by (A) H&E and 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 (G) total numbers and (H) proliferating numbers on POD 16. Results shown in (A) are representative stains used for grading in (B, C) which show mean scores ± S.E. (N = 7/group; **p < 0.01, ***p < 0.01). The insets are 400X with a scale bar representing 50 µm. Stains shown in (D) are representative of 7 transplants and (E, F) show mean numbers ± S.E. (N ≥ 4/group: *p < 0.05, **p < 0.01).
Figure 7

A. Control IgG vs. CD8 Ab for H&E and Trichrome staining.

B. B-Score comparison for Control IgG and CD8 Ab.

C. C-Score comparison for Control IgG and CD8 Ab.

D. Immunofluorescence images of CCSP and Act-tubulin staining for Control IgG vs. CD8 Ab.

E. Comparison of # Club cells x 10^4 for Control IgG vs. CD8 Ab.

F. Comparison of # Ki67+ Club cells x 10^4 for Control IgG vs. CD8 Ab.
Figure 7. CD8⁺ 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 were analyzed on POD 16 for transplant bronchiole injury by (A) H&E and 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 (G) total numbers and (H) proliferating numbers on POD 16. Results shown in (A) are representative stains used for grading in (B, C) which show mean scores ± S.E. (N =5/group; **p < 0.01, ***p < 0.01). The insets are 400X with a scale bar representing 50 µm. Stains shown in (D) are representative of 5 transplants and (E, F) show mean numbers ± S.E. (N = 4/group: *p < 0.05, ***p < 0.001).
Figure 8. Alloantigen-primed effector CD8+ T cells inhibit club cell maintenance and proliferation. Club cells isolated by lineage negative enrichment from FVB lungs were co-cultured without (Untreated) or with unactivated syngeneic FVB (Syn), unactivated allogeneic B6 (Unprimed Allo) or alloantigen-primed B6 (Primed Allo) CD8+ T cells, or with supernatant from alloantigen-primed B6 CD8+ T cells (Primed Allo Supe). 36 hours later club cell abundance was analyzed and shown as (A) a representative contour plot from at least 4 independent experiments or (B) total numbers per experiment with a group mean ± S.E. (N ≥ 4/group: **p < 0.01, ***p < 0.001). Club cells analyzed for proliferation in the presence of indicated CD8+ T cells by (C) CFSE dilution shown as a representative histogram from 5 independent experiments along with a group mean ± S.E. (**p<0.01). (D) Club cell numbers remaining after co-culture with indicated CD8+ T cells in the presence of Control IgG or a neutralizing antibody directed against the H-2K\(^d\) (FVB haplotype) MHC Class I protein. Each data point represents an individual determination from 4 independent experiments with a group mean ± S.E. where *p <0.05 and **p <0.01; unpaired t test, 2-tailed.
Sup. Figure 1: POD 16 3T FVB → B6 recipient allograft tissue were stained with CCSP and acetylated a-tubulin (Ac-tubulin) antibodies. Upper and lower panels represent typical bronchioles with low to undetectable luminal Ac-tubulin expression, respectively. Data are representative of three transplants.
Supplemental Figure 2

Sup. Figure 2: H&E histology of POD 30 graft tissue from 3T B6 → B6 recipient that received DOX between POD 7 and 9.5. Data are representative of 3 transplants.
Supplemental Figure 3

A. Vehicle  Naphthalene

H&E

B. Trichrome

C. CCSP  Ac-tubulin

Sup. Figure 4: POD 16 (A) H&E histology, (B) trichrome and (C) immunohistochemical stain of allograft tissue from FVB → B6 recipient with that received either vehicle or naphthalene on POD 7. Data shown is from a representative transplant of 3 recipients per group.
Sup. Figure 4: H&E histology of POD 16 graft tissue from 2T FVB → B6 recipient that received DOX between POD 7 and 9.5. Data are representative of 2 transplants.
Supplemental Figure 5

Sup. Figure 5: FACS analysis of intragraft CD4+ and CD8+ T cells in 3T FVB > B6 recipients just prior to (POD 6) and after (POD 16) administration CD4 and CD8 depleting Abs on POD 6 and 11. Data are representative of 3 transplants.
Sup. Figure 6: 3T FVB → B6 recipients received Control IgG or CD4 depleting Abs on POD 6 and 11. On POD 16 allograft tissue was assessed for inflammation by H&E and trichrome stain. Data are from a representative transplant from 3 recipients per group.