Published in final edited form as:

J Heart Lung Transplant. 2023 January ; 42(1): 53–63. doi:10.1016/j.healun.2022.09.018.

IL-33 mediates Pseudomonas induced airway fibrogenesis and is associated with CLAD

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Abstract

BACKGROUND: Long term outcomes of lung transplantation are impacted by the occurrence of chronic lung allograft dysfunction (CLAD). Recent evidence suggests a role for the lung microbiome in the occurrence of CLAD, but the exact mechanisms are not well defined. We hypothesize that the lung microbiome inhibits epithelial autophagic clearance of pro-fibrotic proteins in an IL-33 dependent manner, thereby augmenting fibrogenesis and risk for CLAD.

METHODS: Autopsy derived CLAD and non-CLAD lungs were collected. IL-33, P62 and LC3 immunofluorescence was performed and assessed using confocal microscopy. *Pseudomonas aeruginosa* (PsA), *Streptococcus Pneumoniae* (SP), *Prevotella Melaninogenica* (PM), recombinant IL-33 or PsA-lipopolysaccharide was co-cultured with primary human bronchial epithelial cells (PBEC) and lung fibroblasts in the presence or absence of IL-33 blockade. Western blot analysis

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Author contributions

N.S.S., M.M.B. developed concept and designed experiments; N.S.S., K.N.P., J.F, E.O.N collected the samples; N.S.S., M.M.B., M.A.K., A.K, S.B.R, A.S processed samples and performed experiments; N.S.S., K.P., S.S., S.B.R, analyzed data; N.S.S., S.B.R, and M.M.B. drafted the manuscript and N.S.S., H.G., D.R. A.C, A.S, A.W., D.R. H.R.M. performed critical review of the manuscript and revised accordingly.

Data statement

All data will be available to review upon request.

Conflict of interest

None.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.healun.2022.09.018.

and quantitative reverse transcription (qRT) PCR was performed to evaluate IL-33 expression, autophagy, cytokines and fibroblast differentiation markers. These experiments were repeated after siRNA silencing and upregulation (plasmid vector) of Beclin-1.

RESULTS: Human CLAD lungs demonstrated markedly increased expression of IL-33 and reduced basal autophagy compared to non-CLAD lungs. Exposure of co-cultured PBECs to PsA, SP induced IL-33, and inhibited PBEC autophagy, while PM elicited no significant response. Further, PsA exposure increased myofibroblast differentiation and collagen formation. IL-33 blockade in these co-cultures recovered Beclin-1, cellular autophagy and attenuated myofibroblast activation in a Beclin-1 dependent manner.

CONCLUSION: CLAD is associated with increased airway IL-33 expression and reduced basal autophagy. PsA induces a fibrogenic response by inhibiting airway epithelial autophagy in an IL-33 dependent manner.

Keywords

CLAD; IL33; microbiome; autophagy; fibrogenesis

Long-term survival of lung transplant recipients is limited by chronic lung allograft dysfunction (CLAD), commonly termed chronic rejection.[1,2] Although several risk factors such as post-transplant ischemia reperfusion injury (primary graft dysfunction), acute cellular rejection, lung infections, gastro-esophageal acid reflux and airway ischemia have been associated with CLAD, its exact pathogenesis remains poorly understood, representing a critical knowledge gap.[3–5] Emerging literature from several groups including ours suggest a role for lung microbial dysbiosis in CLAD.[6–9] Our recent study reported that CLAD airways have definable lung dysbiosis with altered respiratory pathogen and/or commensal ratios and increased abundance of *Proteobacteria*, and reduced *Bacteroidetes*.[9] In addition, we reported that airway microbiome induces fibrogenesis via the TGFβ/SMAD pathway.[9] Likewise, pro-inflammatory bacteria such as *Staphylococcus* and *Pseudomonas* are known to trigger genes encoding for catabolic remodeling, while commensals such as *Prevotella* and *Veilonella* correlate with activation of genes encoding for anabolic remodeling in the lung.[6] Although, several host-pathogen interactions have been reported, the exact mechanistic pathways linking microbiome to risk for fibrogenesis and subsequent CLAD are not well delineated.[5]

Autophagy is an adaptive stress response that is known to increase autophagosome dependent clearance of dysregulated pro-fibrotic proteins that can lead to pathological lung fibrosis.[10,11] Emerging literature suggests that basal autophagy is reduced in fibrosis and that correction of autophagy can reduce fibrotic burden.[11–13] Like chronic fibrotic lung diseases, human CLAD is characterized by dense fibrosis within small airways and/or parenchyma due to chronic epithelial injury.[14,15] IL-33 is an “alarmin” cytokine that is part of the IL-1 cytokine superfamily that can be induced by epithelial cells in response to microbial stimuli.[16,17] Some IL-1 cytokines such as IL-1α, IL-1β, IL-33 have been linked to reduction in macrophage and mast cell autophagy[11,18–21] but its impact on bronchial autophagy is not well understood. IL-33 is a pleotropic chromatin binding cytokine that can impact both innate and adaptive (Th-2) immune responses.[16,22] A recent study showed that

subjects with acute and chronic heart transplant rejection have an increased expression of cytokine IL-33 in the endomyocardial heart biopsies; wherein IL-33 was linked to favorable modulation of macrophage function.[23] Likewise, uptake in IL-33 expression has been found to be contributory in several chronic lung diseases such as IPF and asthma with a role in modulation of Th-2 responses.[24,25] However, the role of IL-33 in CLAD and its role in autophagy regulation leading to fibrogenesis has not been delineated. Likewise, the impact of the lung microbiome in alterations of the IL-33/autophagy axis and subsequent fibrogenesis in the lung have not been previously evaluated.

We hypothesize that pseudomonas aeruginosa (PsA) inhibits epithelial autophagic clearance of pro-fibrotic proteins in an IL-33 dependent manner, thereby augmenting fibrogenesis and risk for CLAD. In this study, we report the IL-33 and autophagy signatures in human CLAD lungs and elucidate the mechanistic link between PsA induced IL-33 and autophagy inhibition leading to increased fibrogenesis.

Methods and Materials

Human specimens

Bronchiolitis obliterans syndrome (BOS)-CLAD and non-CLAD lung tissue were procured from re-transplanted subjects with CLAD or from general autopsy from non-CLAD subjects. Both CLAD and non-CLAD specimens were processed by pathology core using a similar processing protocol. Institutional Review Board approval was obtained for the studies (University of South Florida IRB Pro # Pro00032158). Demographic details of the subjects are presented in Supplementary Table 1. CLAD (re-transplanted subjects), and non-CLAD transplant (who died of non-allograft failure issues) lung tissues were processed and used for immunofluorescence, quantitative polymerase chain reaction (qPCR) and Western blot analyses using published methods.[9] (Details including antibodies and primers used are provided in the supplementary Table 2&3 respectively). Normal lung tissues (uninvolved tissue from oncological surgeries) were also used to evaluate IL-33 expression in comparison to non-CLAD tissues (Supplementary Figure 1).

Cell culture and *bacterial* co-cultivation experiments

Normal human primary bronchial and/or tracheal epithelial cells (PBEC) (ATCC PCS-300-010) were used in this study and propagated per protocol. *Pseudomonas aeruginosa* strain PAO1 (ATCC BAA-47), *Streptococcus pneumoniae* strain (CIP 104225, ATCC), *Prevotella melaninogenica* strain VPI 2381 (ATCC, VA) were grown per provided instructions and inoculated in culture in MOI of 1:1 in AECB medium without antibiotics and plates were incubated overnight for 16 hours at 37°C in a CO_2 incubator per published protocol.[26–28] Isolation of RNA for quantitative RT-PCR or protein for ELISA or Western blot analysis was performed. For the autophagy related experiments, chloroquine (CQ), or Baflomycin A an autophagy inhibitor that interferes with lysosomal degradation was added two hours prior to harvest to adjudicate autophagic flux. Further details are provided in the supplementary text.

PBEC-fibroblast co-culture experiments

Cells were plated into a 35 mm 6-well plate and allowed to reach a confluency of approximately 80%. Lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* was procured from Millipore Sigma (Catalog No. L7018-100MG) and used at a final concentration of 100μg/ml of culture media for a period of 48 hours. Human lung Fibroblasts (CC-2512 Lonza) were grown in transwell inserts and grown until according to manufacturer's instruction, PBECs grown as above. LPS stimulation and ST2/IL-33R blockade experiments were started when both the fractions, PBECs in the lower compartment and fibroblasts in the upper compartment had reached a confluency of 80%. PBECs were grown in the lower compartment due to challenges in PBEC adherence to the transwell support in the upper compartment in the absence of collagen coating. Collagen coating was avoided due to possible inaccuracies with measurement of soluble collagen assays performed in Figure 7. Both PsA-LPS and ST2 blockade were performed in the PBEC compartment. PBECs with overexpressed Beclin and with siRNA mediated silencing with appropriate controls were also included. Soluble Collagen in the supernatant of fibroblast compartment was assayed by Sircoll assay (Biocolor life science assays) using manufacturer's instructions.

IL-33 blockade experiments

Blocking experiments were done by adding anti-ST2/IL-33R antibody (R &D Systems) at a concentration of 1 μg/ml to PBECs for a period of 4 hours followed by PSA/PSA-LPS exposure. Recombinant IL33 for accessing the effect of IL33 on autophagy was also procured from R & D Systems (Cat 3625-IL).

Beclin silencing and overexpression

The Beclin overexpression clone was procured from Origene (CAT#: RC201629). The said clone has human BECN1 cloned into pCMV6-Entry vector with a MYCDDK tag at its C terminal for the purpose of probing the exogenous protein. The overhang adds an additional 2.8kD to the exogenous Beclin (thus making its MM 62.8kD) and hence the two bands are seen in the Beclin blot in overexpression lane (the lower 1 being the endogenous Beclin and the upper 1 being the exogenous one). A mixture of 3 DsiRNA, procured from IDT (hs.Ri.BECN1.13.1, hs.Ri. BECN1.13.2, hs.Ri.BECN1.13.3) were used at a concentration of 12pM/well in 12 well trans well plates for silencing experiments.

Immunofluorescence and image acquisition

Unstained slides were prepared and deparaffinized and rehydrated followed by epitope retrieval using IHC-TEK Epitope retrieval steamer using established protocols.[29,30] After addition of appropriate primary and secondary antibodies per protocol. Fluorescent images were captured using an Olympus VS120 slide-scanning microscope at 40X. For cell type co-localization studies, images were captured using a Leica confocal microscope using a 63X oil immersion lens. Image processing and analysis was done in FIJI-ImageJ (for details please see supplement).

Statistical analysis

For statistical testing, normality for each data set was assessed by Shapiro-Wilk test followed by the appropriate statistical testing (*t*-test or non-parametric, two tailed, alpha cutoff significance <0.05). Details of individual tests used are provided in the figure legends. GraphPad Prism version 8.0 was used to analyze and plot the *in vitro* data. Illustration for Figure 7 was done using BioRender Illustrator.

Results

IL-33 expression is markedly increased in CLAD airway cells

We investigated the role of IL-1 superfamily of cytokines (IL-1α, IL-1β, IL-18, IL-33) in CLAD. Using a derivation cohort, we found that IL-33 gene expression was significantly increased in CLAD, while IL-1α had a non-significant increasing trend in CLAD (Figure 1A). To add rigor, we utilized a larger independent CLAD cohort to validate these initial findings. The results confirmed no difference in IL-1α expression and a marked increase in IL-33 expression in CLAD compared to non-CLAD lending credence to further investigate the role of IL-33 in CLAD (Figure 1B). To complement these findings, we performed immunofluorescence (Figures 1C and D, supplementary Figure 2A) and western blot (Supplementary Figure 3) that showed increased IL-33 protein expression in CLAD airway tissue sections compared to non-CLAD. To delineate whether the IL-33 staining was specific to airway epithelial cells alone, we performed co-staining with cytokeratin 5. In non-CLAD lungs, wherein, the tissue architecture was well preserved, IL-33 expression co-localized with cytokeratin 5. In contrast the CLAD tissue, IL-33 co-localized both with cytokeratin 5 and independent of it in the interstitium. Furthermore, IL-33 was noted to be intra-nuclear (Supplementary Figure 2B).

Basal autophagy is impaired in human CLAD

To adjudicate the basal autophagy, we evaluated the microtubule associated protein LC3 and downstream P62 expression in CLAD and non-CLAD lung tissue by immunofluorescence. Quantification of LC3 positive puncta is the gold standard to identify autophagosomes in cells.[31] We observed that CLAD airway sections contained significantly fewer LC3 positive puncta and higher P62 expression (Figures 2A, B and Supplementary Figure 4) compared to controls suggesting reduced basal autophagy. Likewise, Western blotting was performed to assess the autophagy markers microtubule-associated protein LC3B-I and 3B-II (LC3-I and LC3-II) showing reduced autophagy (decreased LC3B-II/LC3B-I/GAPDH) in CLAD compared to non-CLAD (Supplementary Figure 5), complementing our immunofluorescence findings.

Impact of microbial exposure on induction of IL-33 in PBECs

To determine the effects of the respiratory microbiome on IL-33 induction by PBECs we used three common respiratory bacteria found in the airways of lung transplant recipients; PsA (gram negative), SP (gram-positive) and PM (respiratory commensal). Exposure of PBECs to PsA and SP resulted in increase in IL-33 expression compared to non-exposed controls (Figures 3A and B, Supplementary Figure 6A, 6B, 6E), whereas PM exposure

did not alter IL-33 expression (Figure 3C and Supplementary Figure 6C). PBEC IL-33 mRNA expression with PsA exposure increased drastically with the length of PsA exposure (Supplementary Figure 6A). IL-33 and cytokeratin5 immunofluorescence of PBECs upon PsA-LPS exposure revealed mostly cytoplasmic co-localization of IL-33 (Supplementary Figure 6F). In contrast to IL-33, PsA-LPS exposure resulted in significant reduction in IL-1α mRNA expression compared to controls (Supplementary Figure 6D).

Impact of microbial exposure on PBEC autophagy

Next, we investigated the effects of the above bacteria on PBEC autophagy. Exposure of PBECs to PsA resulted in a reduction in autophagy (decreased LC3II/LC3I/GAPDH) (Figure 4A). To determine if this was related to a block in autophagy or decreased autophagic flux, we tested the effect of adding chloroquine (CQ), an autophagy inhibitor that interferes with the binding of autophagosomes to lysosomes. [32] Addition of CQ to PBECs alone resulted in accumulation of LC3-II confirming lysosomal degradation block and intact autophagic flux in the PBECs (Figure 4A). To confirm that the response seen was not exclusive to addition of CQ alone, we tested the effect of Baflomycin A, an autophagy inhibitor, showing a similar response to CQ (Supplementary Figure 7). Likewise, SP exposure resulted in autophagy block (Figure 4B), whereas PM did not alter PBEC autophagy (Figure 4C)

IL-33 blockade rescues PsA induced cellular autophagy inhibition in PBECs

To delineate whether the bacteria induced autophagy inhibition was IL-33 dependent, we utilized PsA as a representative respiratory bacterium to conduct further mechanistic investigations. PBECs were exposed to PsA with and without IL-33 receptor (ST2) blockade. ST2 blockade significantly recovered autophagy as assessed by LC3 turnover assay (Figure 5A). These data were complemented with P62 expression studies demonstrating suppression of P62 upon ST2 blockade suggesting restoration of autophagic flux (Figure 5B). Furthermore, PsA exposure resulted in a trend towards reduction of Beclin-1 expression and IL-33 blockade significantly rescued Beclin-1 expression in PsA exposed PBECs (Figure 5C) suggesting Beclin-1 as a potential mediator of PsA/IL-33 induced autophagy dysregulation.

ST2 blockade attenuates PsA induced myofibroblast differentiation

We investigated the effect of IL-33/ST2 blockade on PsA induced fibrogenesis using a human PBEC-fibroblast co-culture model. First, we adjudicated the impact of PsA-LPS on IL-33 and ST2 expression in this co-culture model. Similar to whole bacteria PsA, PsA-LPS exposure to the PBEC/fibroblast co-culture resulted in increased IL-33 expression in PBECs but no change in IL-33 expression in the fibroblasts (Supplementary Figure 8). Likewise, exposure of PsA-LPS to PBECs and fibroblasts separately in independent experiments showed similar results (Supplementary Figure 8). These data validate our model and suggest that PsA-LPS exposure does not impact fibroblast IL-33 expression. A non-significant trend towards increase in ST2 expression was noted in PBECs in co-culture and PBEC monoculture upon PsA-LPS exposure (Supplementary Figure 8), whereas no discernable trend in ST2 expression were noted in fibroblasts in co-culture or monoculture in independent culture upon PsA-LPS exposure. Next, we exposed PsA-

LPS to the co-culture model with and without ST2 blockade to assess its impact on fibrogenic signaling. PsA-exposure without ST2 blockade induced TGFβ1 expression in PBECs and activated myofibroblasts (assessed by α-SMA) in the fibroblast fraction (Figures 6A and B). ST2 blockade in the model abrogated PBEC TGFβ1 response and attenuated α-SMA expression in fibroblasts (Figures 6A and B). To assess if the effect of PsA on myofibroblast activation was, in part, mediated by PBEC-fibroblast crosstalk rather than its direct effect on fibroblast, we exposed fibroblasts alone and in co-culture with PBECs to PsA-LPS in separate experiments. The results showed that PsA-LPS exposure increased fibroblast α-SMA expression four-fold in PBEC/fibroblast co-culture compared to fibroblast monoculture (Supplementary Figure 9).

Beclin-1 regulates IL-33/microbiome mediated autophagy inhibition and downstream fibrogenesis

In the above experiments, IL-33 blockade rescued autophagy and abrogated myofibroblasts activation upon PsA exposure. To delineate whether these were two independent pathways that were activated upon PsA exposure or interlinked, we exposed the PBEC-fibroblast co-culture model to recombinant (r) IL-33 alone or in conjunction with PsA-LPS to test its effect on the pathway. rIL-33 exposure alone downregulated Beclin-1 and inhibited autophagy similar to PsA, whereas addition of rIL-33 in conjunction with PsA-LPS accentuated the autophagy inhibition further suggesting an additive effect (Supplementary Figure 10A, B, C, D, E). Next to illuminate the role of Beclin-1 in IL-33/microbiome mediated autophagy dysregulation, we utilized Beclin-1 siRNA and overexpression vector in our co-culture model. First, we confirmed Beclin-1 silencing and overexpression in the respective experiments by western blotting. Next, exposure of PsA-LPS to Beclin-1 silenced PBECs in our PBEC/fibroblast co-culture model showed a significant increase in fibroblast activation (increased αSMA in fibroblast) compared to controls (Figures 7A and B). In contrast, Beclin-1 overexpression attenuated the PBEC fibroblast activation (Figures 7A and B) suggesting a regulatory role for Beclin-1 in PsA-LPS induced autophagy dysregulation and downstream fibrogenesis. Furthermore, to assess the effect of the myofibroblast activation on extracellular matrix formation, we measured the newly synthesized collagen in the cell culture supernatant. Exposure of the model to PsA-LPS led to marked increase in new collagen synthesis whereas IL-33 blockade, or PBEC Beclin-1 overexpression dampened this response (Figure 7C).

Discussion

In this study, we report for the first time the IL-33 and basal autophagy signatures in human CLAD. Furthermore, we demonstrate the impact of respiratory pathogens and commensal on induction of IL-33 and autophagy dysregulation. Finally, we show the impact of PsA induced autophagy dysregulation on downstream fibrogenesis and the roles for IL-33 blockade in attenuation of autophagy dependent fibrogenesis. These represent the first clear mechanistic data linking microbiome with induction of IL-33 and its impact on autophagy and subsequent fibrogenesis in the lung. These data provide a new pathway of allograft injury, active in CLAD and lay the foundation for further *in vivo* studies to test the utility of the IL-33/autophagy axis as a therapeutic target in mitigation of allograft injury leading to

CLAD. Importantly, this new signaling pathway is likely operative in other chronic airway disorders such as idiopathic pulmonary fibrosis and other interstitial lung diseases.

Emerging studies have outlined a potential role for IL-33 in the pathogenesis of chronic lung diseases such as asthma, COPD and IPF.[24,33,34] In this study, we found airway IL-33 expression to be increased in CLAD compared to non-CLAD. These findings of increased expression likely reflect the array of injury responses mediated by IL-33 to various triggers such as cigarette smoke, allergens and infections that have been implicated in the pathogenesis of chronic lung diseases. IL-33 is constitutively expressed in the lung epithelium but can be induced to a higher level upon cellular stress[35] and in case of CLAD likely via chronic epithelial injury. In CLAD tissues, we found IL-33 to be majorly intra-nuclear but diffusely distributed; both co-localizing with cytokeratin5 (marker for airway epithelial cells) and extracellular in the interstitium, whereas in non-CLAD it was majorly localized with cytokeratin5. The CLAD tissue sections are characterized by significant loss of airway epithelium and architectural distortion due to fibrosis, collagen deposition in the interstitium. The interstitial IL-33 staining noted in CLAD may represent extracellular IL-33 released after epithelial cell damage and additional IL-33 expression in non-epithelial cell types. IL-33 is a nuclear cytokine which originally was thought to be only secreted extracellular by cell necrosis or injury.[36,37] Moreover, even extracellular IL-33 after cell necrosis was found to be chromatin bound.[38] More recently, IL-33 co-secretion from the intact cells has been identified via exosomes using the nSMase2-regulated endosome pathway.[39] In our *in vitro* model IL-33 expression upon PsA exposure was majorly noted in the cytoplasmic and peri-nuclear location. Furthermore, IL-33 was found to be significantly increased in the supernatant of PsA exposed PBEC cultures. Together these may possibly represent extracellular secretion of IL-33 from intact cells. A recent study showed that the effect of IL-33 on downstream gene induction was independent of its nuclear location.[40] Further studies need to be conducted to delineate the secretion mechanisms involved in IL-33 signaling and its relevance in CLAD.

IL-33 is a pleotropic “ alarmin” cytokine that can impact both innate and adaptive (th-2) immune responses.[22,41–43] IL-33 can upregulate FOXP3+ CD4 regulatory T cells to promote tolerance and reduce inflammation.[44] In contrast, IL-33 promotes release of pro-inflammatory mediators from mast cells and upregulation of Th-2 cytokine responses including ILC2s.[45,46] Likewise, IL-1 cytokines including IL-33 have been reported to have a crosstalk with autophagy[10,47]; an adaptive stress response that can lead to clearance of dysregulated proteins to modulate inflammation. Dysregulated autophagy has been linked to several chronic inflammatory lung diseases including pulmonary fibrosis.[13,48,49] Autophagy regulates the secretion of IL-1 cytokines such as IL-1α and IL-1β, by either transcriptional regulation or a direct autophagosome based sequestration of cytokine pro-forms, though it is unclear if IL-33 is regulated in a similar manner[50,51] Pharmacological or siRNA inhibition of autophagy increases IL-1α, IL-1β, IL-18 secretion, whereas these cytokines in turn can augment autophagy as part of a negative feedback loop to reduce inflammatory mediators.[52,53] However, the reports on the impact of IL-33 on cellular autophagy or vice-versa have been conflicting, wherein IL-33 inhibits neuronal cell autophagy but augments autophagy in the intestinal macrophages.[18,54,55] These contrasting results suggests that IL-33 may have variable impact on autophagy based on cell type and disease states. In

this study, we discovered that the basal autophagy in CLAD airway cells was reduced. Although, several factors could alter the basal autophagy, we hypothesized that the increased IL-33 expression seen in CLAD, in part, was contributory to the reduced basal autophagy. Bacterial infections/colonization's are known risk factors of CLAD and can also induce IL-33[17,56] To investigate this axis better, we tested the effect of bacteria as a trigger for IL-33 induction and investigated its impact on autophagy and downstream fibrogenesis.

Previously, it has been reported that airway macrophages exposed to PsA and *Staphylococcus* induces high levels of TNF and COX-2 gene expression in contrast to low level stimulation by *Prevotella* and *Streptococcus*.[6] However, in this current study, we observed that respiratory pathogens PsA and SP induced IL-33 and PBEC autophagy, whereas PM, a respiratory commensal, did not alter IL-33 expression or PBEC autophagy. It is likely that the differences in airway cell types that is, macrophages vs PBECs and divergent SP strains used in the two studies are contributory to the variation in the pro-inflammatory and/or fibrotic response seen due to SP. Additionally, in our model, exposure to recombinant IL-33 alone inhibited PBEC autophagy inhibition similar to that seen during exposure to PsA and SP. Whereas, recombinant IL-33 in conjunction with PsA-LPS accentuated autophagy inhibition further suggesting an additive effect. Interestingly, IL-33 receptor blockade in PsA exposed PBECs restored both Beclin-1 and PBEC autophagy suggesting that a PsA induced an IL-33 dependent autophagy inhibition via canonical the Beclin-1 autophagy pathway. We and others have previously reported that lung dysbiosis characterized by an imbalance of respiratory pathogens and commensals is associated with CLAD.[9,57,58] The divergent impact of the respiratory pathogens and commensal on the induction of IL-33 and dysregulation of autophagy in this study may be a potential mechanism involved in dysbiosis related tissue injury and fibrogenesis. Future studies investigating the role of polymicrobial dysbiosis in the activation of this tissue injury pathway are needed to delineate this better.

TGFβ is known to play a central role in lung fibrogenesis by activation of several downstream canonical and non-canonical fibrogenic pathways.[59] We have previously shown that PsA can induce fibrogenesis by activation of the TGFβ/SMAD signaling pathway.[9] This current study confirmed this observation and additionally found that IL-33 blockade attenuated PsA induced TGFβ and myofibroblast activation and/or collagen formation. These effects of PsA on fibrogenesis were autophagy dependent and autophagy augmentation (upregulation of Beclin-1) annulled the effect of PsA on fibrogenesis. Previous studies have reported that deficiency of Beclin-1 is linked to fibrogenesis and increased collagen deposition,[60] results that are aligned with our current findings. We speculate that cathepsins and matrix metalloproteases, that are inherent to lysosomal activity and known to impact TGFβ activity[61,62] may be involved in autophagy regulation of downstream fibrogenesis. Likewise, our results suggest that IL-33 is an upstream regulator of PBEC autophagy via Beclin-1 modulation and hence IL-33 blockade abrogates the PsA induced fibrogenic response. Our data shows that the effect of PsA on fibrogenesis is mediated by PBEC -fibroblast crosstalk rather than a direct effect of PsA on fibroblast activation. Furthermore, it is interesting that the PBEC IL-33 expression upon PSA-LPS exposure in co-culture of PBECs with fibroblasts versus PBEC monoculture was 5 times higher. Although the exact cause for this observation is unclear, it seems that presence of

activated fibroblasts or a metabolic mediator released by it due to PSA-LPS provides positive feedback for further IL-33 generation by PBECs. Our ongoing studies are actively investigating the mechanisms involved in bacterial induction of IL-33 and IL-33 modulation of Beclin-1 in this signaling pathway. Overall, these exciting observations shed light on a novel IL-33/autophagy axis that is activated by PsA and can impact airway fibrogenesis and potentially increase CLAD susceptibility (Figure 8). Further investigations into this novel pathway can help identify therapeutic targets to mitigate dysbiosis induced airway fibrogenesis and CLAD. Moreover, it is possible that other risk factors that predispose to CLAD such as acid reflux related epithelial injury, allo-immune dysregulation may also induce IL-33 and activate this signaling pathway.

Our study has uncovered a potential role for IL-33 in CLAD pathogenesis. However, given the cross-sectional nature of our human specimens, it is unclear whether IL-33 expression or reduced basal autophagy seen in CLAD is a cause or effect of CLAD. Our ongoing prospective studies evaluating airway IL-33 and PBEC autophagy signatures longitudinally will help in delineating this better. Although, we have focused on IL-33 in this study based on our human specimen findings, other IL-1 cytokine members such as IL-1α, IL-1β may have synergistic or antagonistic interactions with IL-33 and impact autophagy. Likewise, the in vivo effects of the IL-33 on epithelial cell autophagy and on activation of type 2 responses needs to be determined to assess their relative contributions to airway fibrogenesis and risk for CLAD. Finally, in our PBEC-fibroblast co-culture model, PBECs were not polarized due to the technical challenges with adherence of PBECs to transwell insert in the absence of collagen coating as described in the methods. Given that the secretory function of polarized epithelial cells can vary from non-polarized epithelial cells, it is possible that alternative IL-33 signaling pathways may be active in response to microbial exposure. Our future mechanistic studies will address this important technical aspect to determine the impact of polarized PBECs on IL-33 and/or autophagy axis and its cross talk with fibroblasts.

In conclusion, this study reports for the first-time increased IL-33 expression and reduced basal autophagy in CLAD compared to non-CLAD. We also describe a new mechanism illuminating the role of *Pseudomonas* in airway fibrogenesis via the IL-33/autophagy axis that may contribute to risk for CLAD. Future mechanistic studies using animal transplant models need to be conducted to study the in vivo effects of the IL-33/Autophagy axis on CLAD and investigate the therapeutic benefit of modulating this signaling pathway to prevent and/or treat of CLAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

NSS was funded by USF NIS award and Brigham and Women’s Hospital New investigator fund 1R01HL161620 to NSS.

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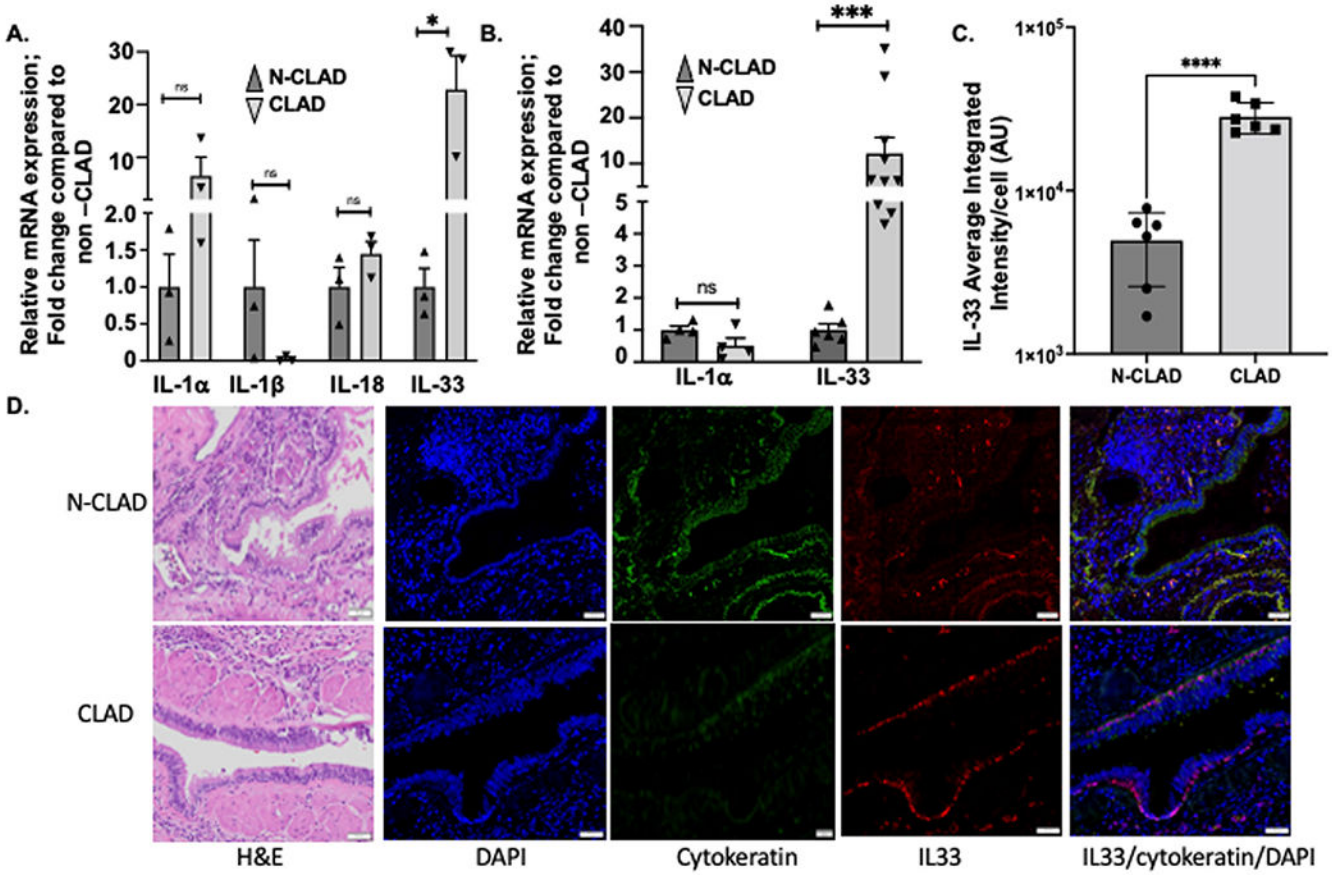

Figure 1.

IL-33 expression is markedly increased in CLAD airway cells. (A) Relative mRNA expression of IL-1 superfamily cytokines in CLAD and non-CLAD lung tissue shown as fold change compared to non-CLAD. ($n = 3$) (B) IL-1α ($n = 4$ each CLAD and non-CLAD) and IL-33 ($n = 10$ CLAD, $n = 6$ non-CLAD) relative mRNA expression in CLAD and controls in an independent validation cohort shown as fold change compared to control. All relative mRNA expressions have been normalized to GAPDH. (C) IL-33 average integrated intensity and/or cell (AU) in CLAD and non-CLAD tissue sections ($n = 6$ fields of view). (D) Representative immunofluorescence images showing H&E, DAPI (blue), cytokeratin 5 staining (green) and IL-33 (red) in CLAD and non-CLAD lung tissue. ****$p < .0001$, ***$p < .001$, *$p < .05$. *Error bars* show mean±SEM. All unpaired t test except for IL1β in A (Mann-Whitney test). (Color version of figure is available online.)

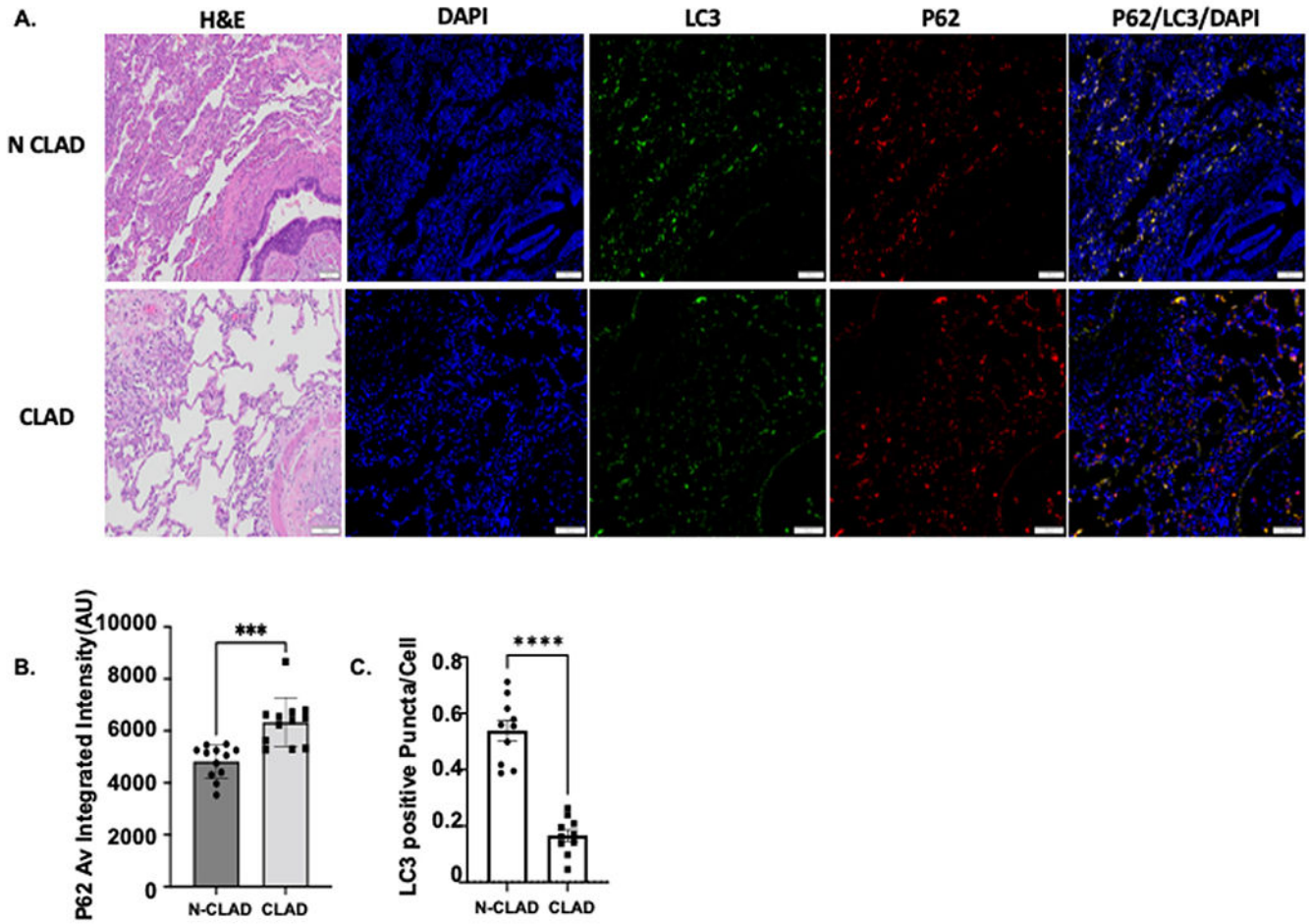

Figure 2.

Basal autophagy is impaired in human CLAD. (A)Representative immunofluorescence image showing LC3 (green), P62 (Red), DAPI (blue) expression in CLAD and non-CLAD lung tissue (B) P62 average integrated intensity (AU) in CLAD and non-CLAD (n = 12 fields of view) (C) Number of LC3 positive puncta and/or cells in CLAD and non-CLAD (n = 10 fields of view). ***p < .001, ****p < .0001, *Error bars* represent mean±SEM, unpaired t test. (Color version of figure is available online.)

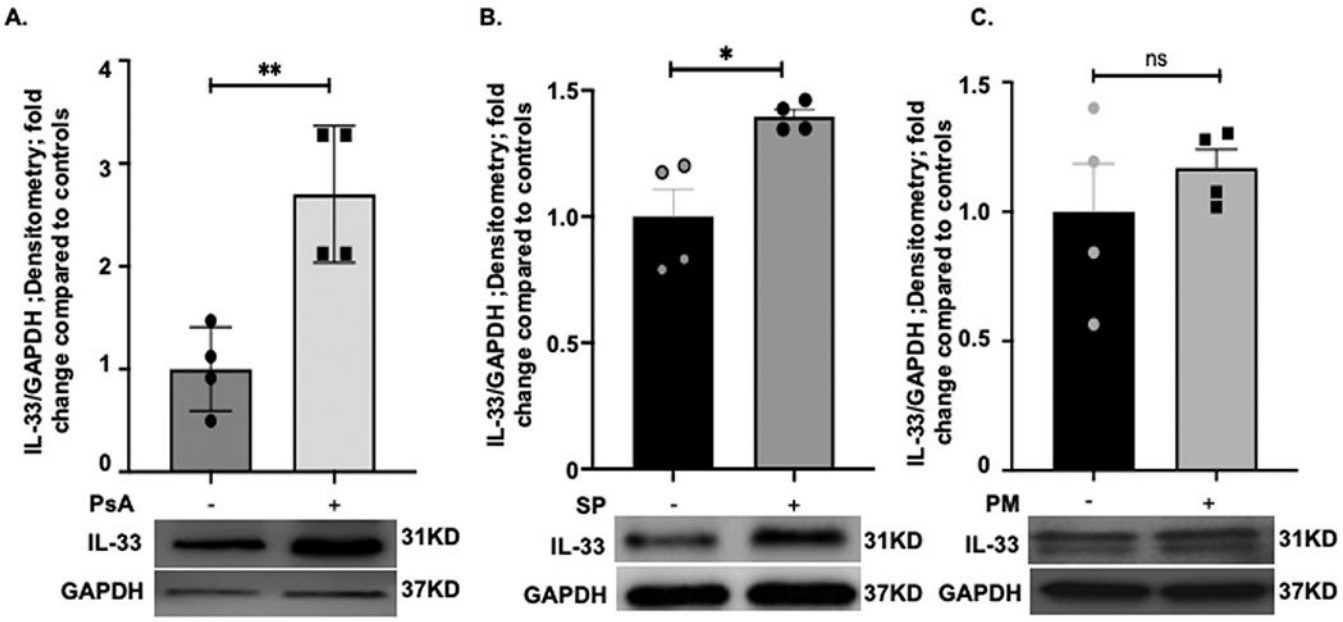

Figure 3.

Impact of microbial exposure on induction of IL-33 in PBECs. (A) Densitometric analysis of independent western blot experiments ($n = 4$) showing expression of IL-33 in PsA exposed PBECs. (B) Densitometric analysis of independent western blot experiments ($n = 4$) showing expression of IL-33 in SP exposed PBECs. (C) Densitometric analysis of independent western blot experiments ($n = 4$) showing expression of IL-33 in PM exposed PBECs. Representative western blot images are shown in inset. *Error bars* represent mean±SEM, P value, $^{**}p < .01$, $^{*}p < .05$, unpaired *t*-test.

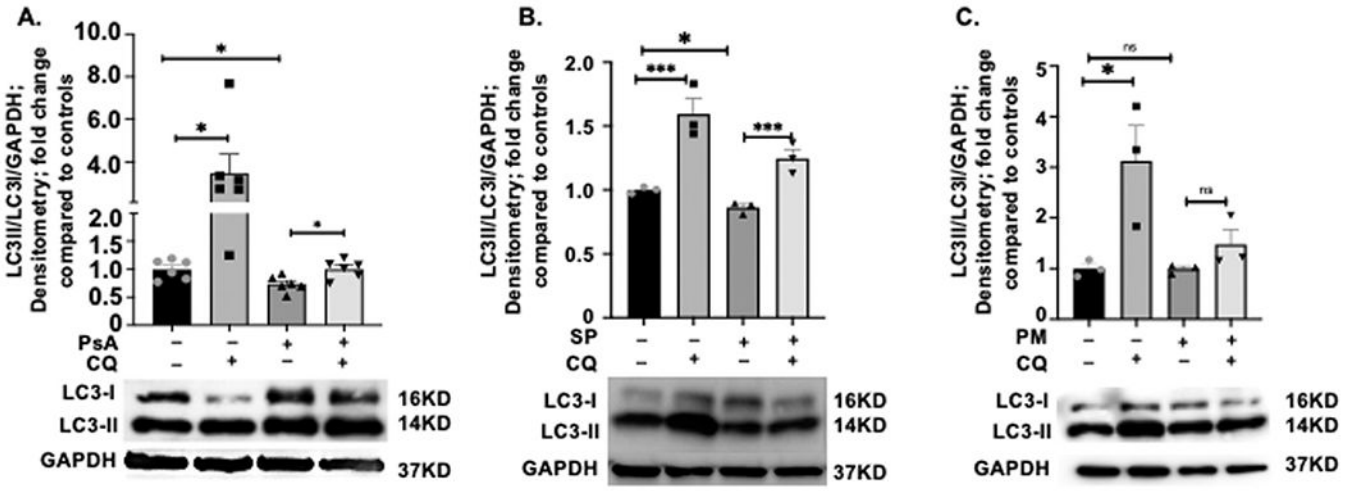

Figure 4.

Impact of microbial exposure on PBEC autophagy. Representative western blot and densitometric analysis of independent experiments showing LC3-II/LC3-I/GAPDH in (A) PsA exposed PBECs ($n = 6$), ***$p < .001$, Kruskal-Wallis 1 way ANOVA, with Benjamini Krieger Yukutieli step-up multiple testing correction, (B) SP exposed PBECs ($n = 3$), ***$p < .001$, 1 way ANOVA, Benjamini Krieger Yukutieli step-up correction (C) PM exposed PBECs ($n = 3$), *$p < .05$, one-way ANOVA, with Benjamini Krieger Yukutieli step-up correction. *Error bars* represent mean±SEM.

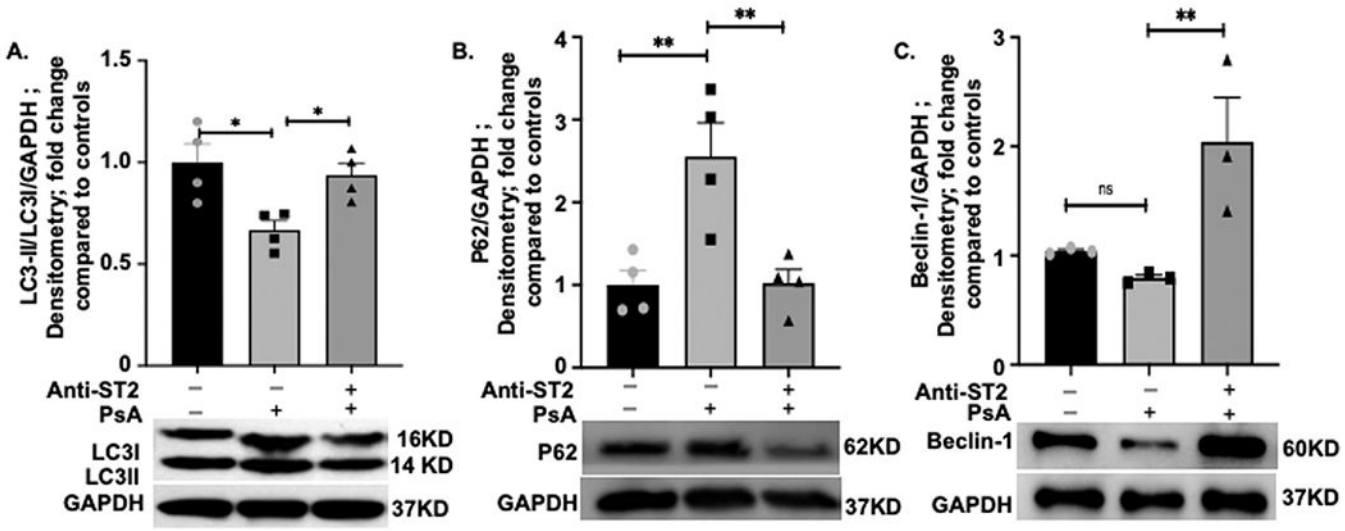

Figure 5.

IL-33 receptor (ST2) blockade rescues autophagy in PsA exposed PBECs. Representative western blots and densitometric analyses of independent experiments showing (A) LC3-II/LC3-I/GAPDH, *$p < .05$, one-way ANOVA, with Tukey's multiple comparison post-test (B) P62/GAPDH, **$p < .01$, 1 way ANOVA, with Tukey's multiple comparison post-test (C) Beclin-1/GAPDH with and without IL-33 receptor (ST2) blockade, *$p < .05$, 1 way ANOVA, with Tukey's multiple comparison post-test. *Error bars* represent mean±SEM (n = 3-4).

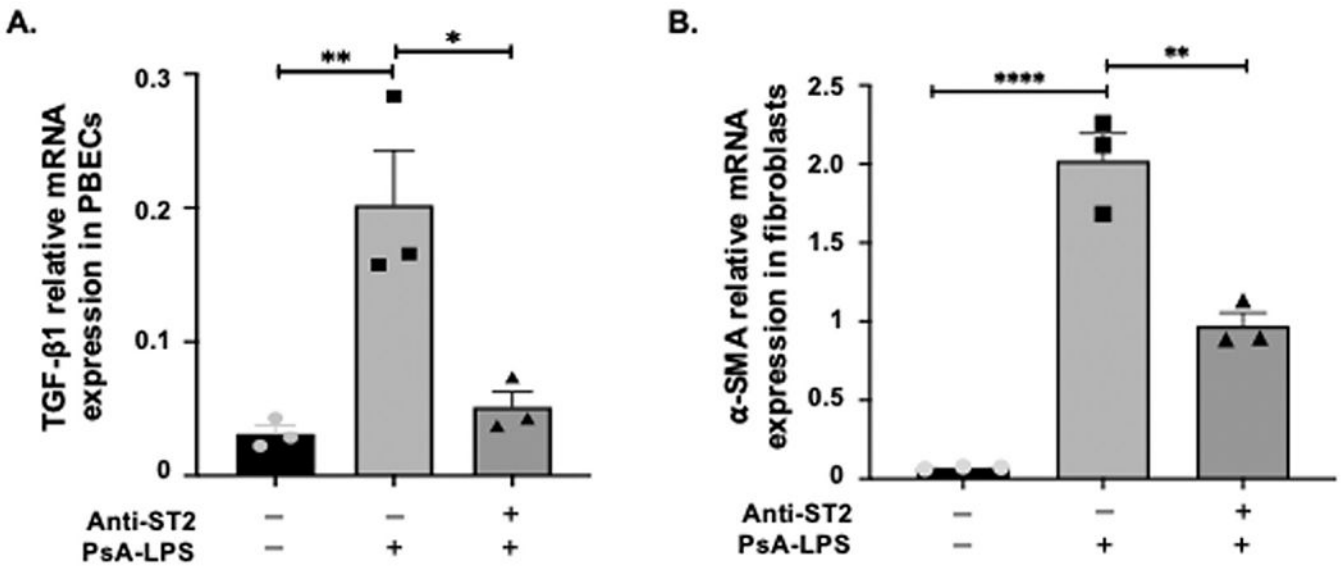

Figure 6.

IL-33/ST2 receptor blockade attenuates TGF β1 expression in PBECs and induces myofibroblast differentiation. PsA-LPS (100μg/ml) exposure with and without anti-IL33/ST2 blockade in PBEC-fibroblast co-culture model (A) Relative mRNA expression of TGF β1 in PBECs ($n = 3$), $^{**}p < .01$ 1 way ANOVA, with Tukey's multiple comparison post-test (B) Relative mRNA expression of α-SMA in fibroblasts ($n = 3$), $^{****}p < .0001$, one-way ANOVA, with Tukey's multiple comparison post-test. *Error bars* represent mean±SEM. Both PsA-LPS and ST2 blockade were performed in the PBEC compartment and mRNA expressions normalized to GAPDH.

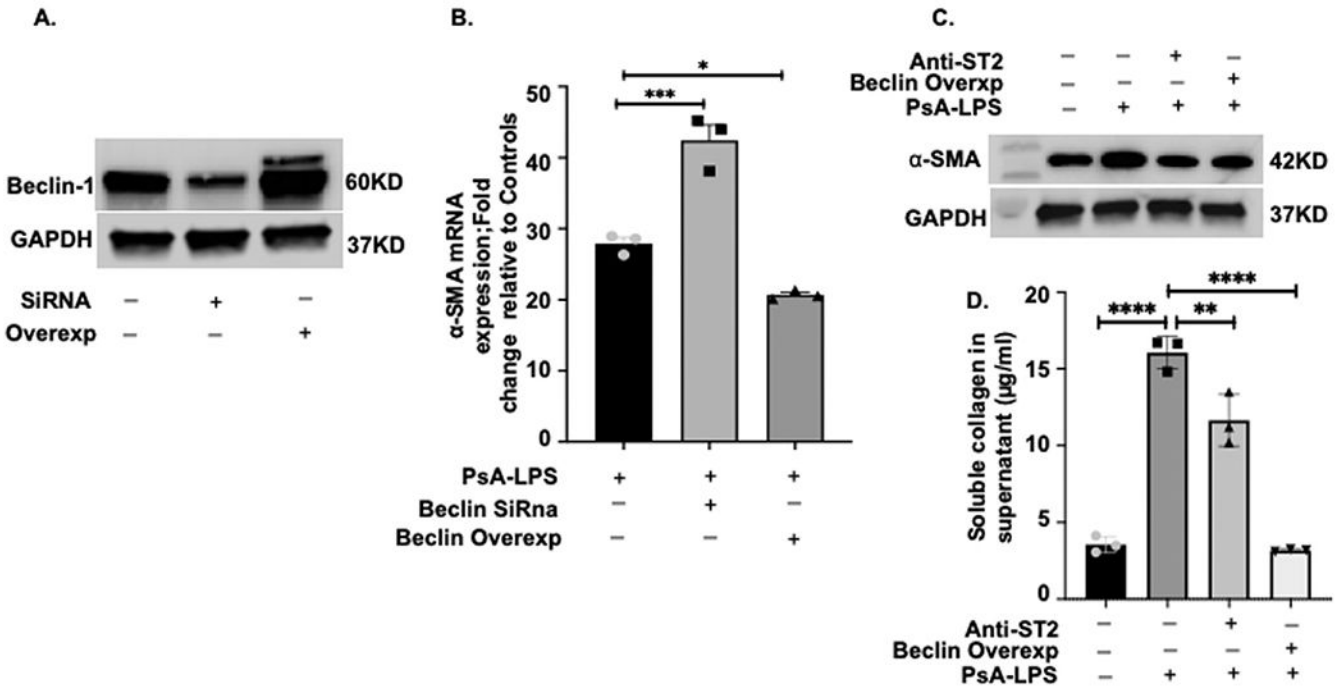

Figure 7.

Beclin-1 overexpression attenuates PsA-LPS/IL-33 induced fibrogenesis. (A) Representative western blot showing Beclin-1 silencing and overexpression (B) α-SMA mRNA expression expressed as fold change with respect to control in fibroblasts from PsA-LPS exposed PBEC and/or fibroblast co-culture model (with Beclin-1 silenced or overexpressed PBECs), ($n = 3$), mRNA expressions normalized to GAPDH, 1 way ANOVA, ****$p < .0001$ with Tukey's multiple comparison post-test (C) Representative western blot showing α-SMA protein expression in fibroblasts ($n = 3$) from above experiment (D) Sircoll assay showing soluble collagen content in the cell culture supernatants from the above experiments ($n = 3$). *Error bars* represent mean±SEM, one-way ANOVA, ****$p < .0001$ with Tukey's multiple comparison post-test.

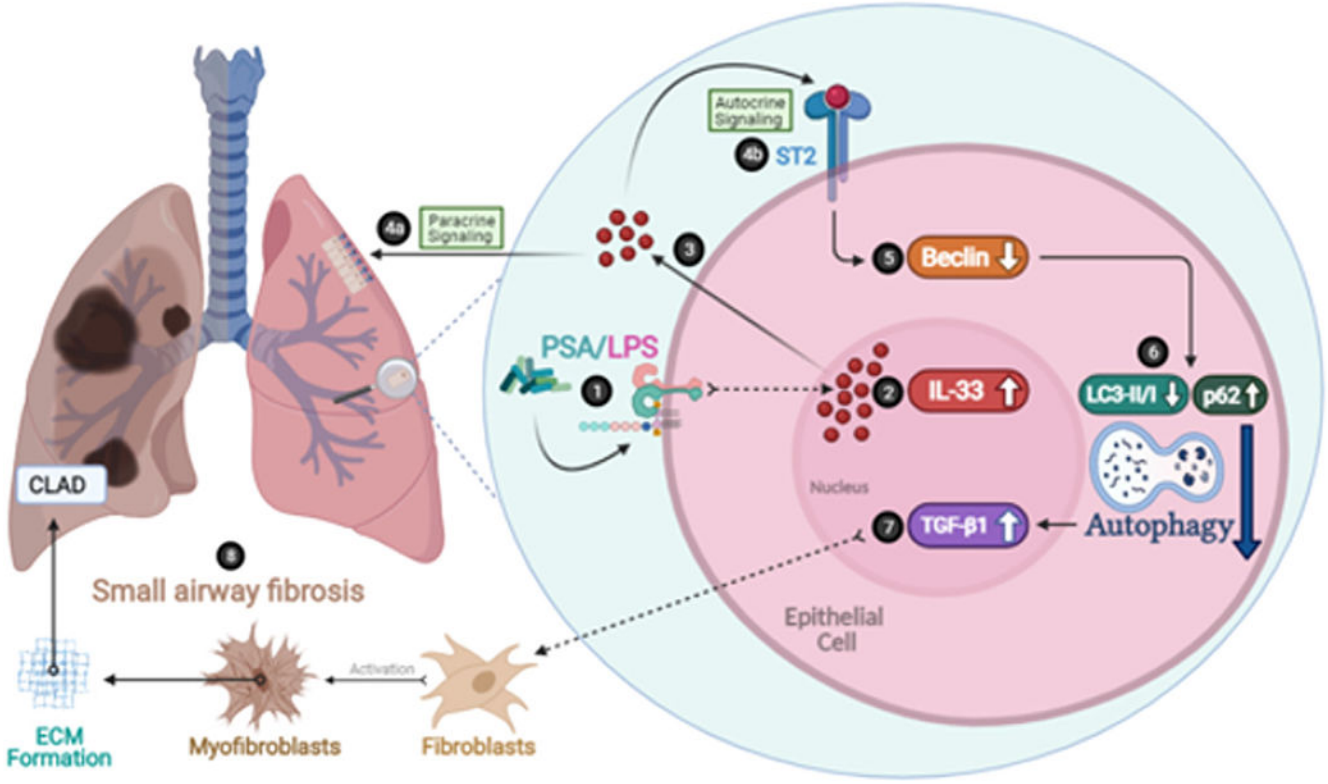

Figure 8.

Schematic diagram showing the pathways involved in lung microbiome induced IL-33/autophagy axis and downstream fibrogenesis. PsA/LPS induces IL-33 in bronchial epithelial cells (1 and 2), IL-33 is excreted to the extracellular space for cell signaling (3), IL33 activates IL33/ST2R receptor in autocrine and paracrine manner (4a and 4b), ST2R activation inhibits Beclin-1 (5), resulting in impaired autophagy (6), and increased TGF-β1 expression (7). This activates fibroblast differentiation and ECM synthesis (8), leading to small airway fibrosis and CLAD.