

Inflammatory Activity of Epithelial Stem Cell Variants from Cystic Fibrosis Lungs Is Not Resolved by CFTR Modulators

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Abstract

Rationale: CFTR (cystic fibrosis transmembrane conductance regulator) modulator drugs restore function to mutant channels in patients with cystic fibrosis (CF) and lead to improvements in body mass index and lung function. Although it is anticipated that early childhood treatment with CFTR modulators will significantly delay or even prevent the onset of advanced lung disease, lung neutrophils and inflammatory cytokines remain high in patients with CF with established lung disease despite modulator therapy, underscoring the need to identify and ultimately target the sources of this inflammation in CF lungs.

Objectives: To determine whether CF lungs, like chronic obstructive pulmonary disease (COPD) lungs, harbor potentially pathogenic stem cell “variants” distinct from the normal p63/Krt5 lung stem cells devoted to alveolar fates, to identify specific variants that might contribute to the inflammatory state of CF lungs, and to assess the impact of CFTR genetic complementation or CFTR modulators on the inflammatory variants identified herein.

Methods: Stem cell cloning technology developed to resolve pathogenic stem cell heterogeneity in COPD and idiopathic pulmonary fibrosis lungs was applied to end-stage lungs of

patients with CF (three homozygous CFTR:F508D, one CFTR F508D/L1254X; FEV₁, 14–30%) undergoing therapeutic lung transplantation. Single-cell–derived clones corresponding to the six stem cell clusters resolved by single-cell RNA sequencing of these libraries were assessed by RNA sequencing and xenografting to monitor inflammation, fibrosis, and mucin secretion. The impact of CFTR activity on these variants after CFTR gene complementation or exposure to CFTR modulators was assessed by molecular and functional studies.

Measurements and Main Results: End-stage CF lungs display a stem cell heterogeneity marked by five predominant variants in addition to the normal lung stem cell, of which three are proinflammatory both at the level of gene expression and their ability to drive neutrophilic inflammation in xenografts in immunodeficient mice. The proinflammatory functions of these three variants were unaltered by genetic or pharmacological restoration of CFTR activity.

Conclusions: The emergence of three proinflammatory stem cell variants in CF lungs may contribute to the persistence of lung inflammation in patients with CF with advanced disease undergoing CFTR modulator therapy.

Keywords: cystic fibrosis; CFTR modulators; lung inflammation; lung stem cells; neutrophils.

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Cystic fibrosis (CF) is a genetic condition that impacts lung, pancreas, liver, and gastrointestinal tract function (1, 2). Although pancreatic insufficiency, diabetes, and growth retardation are early manifestations of CF, the decline of lung function is by far the main cause of morbidity and mortality in these patients. From a pathogenic standpoint, biallelic mutations in CFTR (cystic fibrosis transmembrane conductance regulator) result in defective chloride and bicarbonate transport that, in turn, leads to dehydrated and hyperviscous secretions from the affected organs (3–7). In the pancreas, for instance, CFTR mutations prevent the flow of digestive enzymes to the gastrointestinal tract, which both interferes with nutrient processing and leads to pancreatitis and attendant diabetes (7). In the lung, dehydrated mucus, together with a more acidified extracellular fluid, is believed to favor the bacterial colonization of the airways (4–7). The persistence of microbes in the CF lung seemed to explain the chronic infiltration of neutrophils and the resulting chronic inflammation that drives lung deterioration in these patients (1, 2). However, the precise relationship between infection and inflammation in CF lungs is complex and the subject of intense investigation (8–10). Experiments with genetically engineered ferrets and pigs to model CF argue for a disconnect between lung inflammation and bacterial infections in this condition (11–13). For instance, although CFTR-mutant ferrets raised from birth on broad-spectrum antibiotics showed a prolonged lifespan compared with their symptomatically treated littermates, the lungs from both groups displayed CF-associated elevations in cytokines such as IL-8, TNF- α ,

and IL-1 β , and both groups eventually displayed mucus obstruction, neutrophil-mediated inflammation, and bronchiectasis (11, 12). Studies in the CF porcine model support an inflammatory component of this disease that is detached from infection (13). Persistent inflammation has also been observed in children with CF who have undergone successful treatment to eradicate *Pseudomonas* infections (14). Together, these data support a narrative that some form of “inappropriate” inflammation, either from lung epithelial cells or immune cells themselves, is driving the progressive lung deterioration in CF.

The notion of inappropriate inflammation in progressive CF lung disease is especially germane to enormous efforts to improve the activity of mutant CFTR function in these patients. The new class of CFTR modulator drugs (15, 16) arising from these efforts, including combinations of agents that enhance the trafficking of mutant CFTR to the plasma membrane and the gating of the mutant channel, effectively restore CFTR function and lead to significant improvements in body mass index and lung function (15–27). Despite these revolutionary advances, neutrophils and inflammatory cytokines remain high in the lung fluids of patients with established CF lung disease treated with CFTR modulator therapy (28, 29), underscoring the need to identify and target the sources of this inflammation to counter progressive lung disease.

A recent analysis of lungs of patients with chronic obstructive pulmonary disease (COPD) has revealed a disease-linked pattern of stem cell heterogeneity, including variants that promote mucus hypersecretion, myofibroblast activation linked to fibrosis, and neutrophilic inflammation (30), all

pathologic features of COPD lungs. As CF lung pathology overlaps that of the COPD lung (31–32), we asked in the present study if the CF lung displays a stem cell heterogeneity that might contribute to the inappropriate inflammation in these patients. Some of the results of these studies have been previously reported in the form of abstracts (33–35) and in preprint form (https://papers.ssrn.com/sol3/papers.cfm?abstract_id=4425298).

Methods

Human Subjects

This study assessed lung stem cell heterogeneity in wedge biopsies from four CF lungs marked by severe obstructive disease (FEV₁, 0.58–1.13 L, 14–30%; age range, 32–64 yr) obtained from the lung transplant service at the University of Iowa Health and Clinics (institutional review board [IRB]# 199507432) and four control lungs (age range, 42–59 yr) without obstructive lung disease from the University of Connecticut Health Center (IRB# 08-310-1) and the University of Texas Health Sciences Center, Houston (HSC-MS-08-0354/HSC-MS-15-1049) (see Table E1 in the online supplement). For cellular morphometrics studies, we obtained histological sections from an additional six CF lungs obtained from lung transplant services at the Houston Methodist Hospital and six control donor lungs without obstructive lung disease (University of Texas Health Sciences Center, Houston; HSC-MS-08-0354/HSC-MS-15-1049) (Table E2). Lung tissue from fetal demise cases was obtained under informed consent as deidentified material from the Brigham and Women’s Hospital, Boston, Massachusetts (2009P002281).

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Author Contributions: Experimental design and conception were done by W.X., F.D.M., J.F.E., K.R.P., M.V., A.S., C.P.C., and B.F.D. Human airway cell cloning and analysis *in vitro* and in xenografts were performed by W.X., Melika Khorrami, Melina Khorrami, A.H., and S.N. S.W., B.L., and J.L. performed informatics and molecular genetics analyses. F.Y., G.N.G., and S.C. generated the CFTR lentivirus and performed the biophysics analyses. M.L.M., C.P.C., K.R.P., H.J.H., H.K.-Q., and R.C.B. acquired consented biopsy materials and lung tissues. W.X., J.F.E., and F.D.M. wrote the manuscript with input from all other authors.

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue’s table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the

Subject: The progressive lung disease in patients with cystic fibrosis (CF) is marked by microbial colonization, chronic inflammation, and structural remodeling. Although lung inflammation would be a natural consequence of persistent infections, multiple observations in ferret and porcine CF models suggest lung inflammation in the absence of microbial infection. The sources of this “inappropriate” inflammation, and their dependence on CFTR (cystic fibrosis transmembrane conductance regulator) activity, are unknown, but compelling clinical data suggest that lung inflammation continues in patients with advanced disease despite effective CFTR modulator therapy.

What This Study Adds to the

Field: The present study addresses potential sources of inappropriate inflammation in the lungs of patients with CF and tests the potential of CFTR gene therapy or CFTR modulator drugs to mitigate this inflammation. Using single-cell cloning technology that detailed stem cell heterogeneity in lungs from patients with chronic obstructive pulmonary disease, we identified five stem cell variants common to lungs of patients with advanced CF, including three that show hyperinflammatory gene expression profiles in a constitutive manner and that drive neutrophilic inflammation when transplanted to mice. The constitutive inflammatory state of these three variants suggests that they contribute to the inappropriate inflammation detected in advanced CF lungs. Importantly, we assess the relationship between CFTR activity and the inflammatory properties of these three proinflammatory stem cells harbored by the advanced CF lung. We show that neither genetic complementation of the mutant CFTR gene nor triple combinations of CFTR modulators suppress the inflammatory gene signatures or the neutrophilic properties of these variant stem cells.

Results

Stem Cell Heterogeneity in End-Stage CF Lungs

This study assessed lung stem cell heterogeneity in wedge biopsies from four CF lungs marked by severe obstructive disease (FEV₁, 0.58–1.13 L, 14–30%; age range, 32–64 yr) obtained from lung transplant surgeries at the University of Iowa Health and Clinics (IRB# 199507432) and four control lungs (age range, 42–59 yr) without obstructive lung disease from the University of Connecticut Health Center (IRB# 08-310-1) and the University of Texas Health Sciences Center, Houston (HSC-MS-08-0354/HSC-MS-15-1049) (Table E1). These lung tissues were processed to patient-specific stem cell libraries using technology (30) that selectively supports the growth of clonogenic epithelial cells (Figure 1A and Table E1). Libraries from CF lungs and from control lungs without obstructive lung disease were processed in the same manner. In brief, lung tissue was enzymatically dissociated to single cells and plated onto lawns of irradiated 3T3-J2 murine embryonic fibroblasts (30). Over a 10-day period, colonies of epithelial cells formed at a rate of 1:1,000 to 1:3,000 of E-cadherin-positive epithelial cells plated. Single-cell RNA sequencing (scRNA-seq) profiles of these CF libraries revealed six major clusters of cells marked by distinct gene expression in contrast to control libraries that were dominated by a single major cluster (Figures 1B, 1C, E1, and E2). Cluster 1, which corresponds to the single dominant cluster comprising 60–85% of clonogenic cells of control adult (30) or fetal lungs, accounted for 6–30% of the cells in CF libraries.

Given the ability of xenografts generated from clonogenic stem cell variants in COPD to monitor the proinflammatory and profibrotic activity of these libraries relative to control libraries (30), we asked if the CF stem cell libraries would promote similar pathogenic features in xenografts. One million cells from each of four CF libraries and control libraries were injected subcutaneously into highly immunodeficient NOD-scidIL2g^{null} (NSG; 36) mice (Figures 2A–2C). These xenografted cells form polarized epithelia in discrete nodules that can be histologically assessed at 2 weeks. Unlike control library xenografts, those of CF libraries showed Muc5AC (mucin 5AC)

hypersecretion, extensive neutrophilic infiltration, and submucosal fibrosis in the form of Masson’s trichrome-positive fibrils (Figure 2C). Taken together with the heterogeneity of the CF libraries relative to control libraries revealed by scRNA-seq profiles, we anticipated that the major variant clusters in these libraries were linked to one or more of the pathologic phenomena observed in these xenografts.

Aberrant Fate Commitment in CF Stem Cell Variants

To decipher the cluster complexity apparent from the scRNA-seq of the CF libraries, we used fluorescence-activated cell sorting (FACS) to place single cells into 384-well plates for clone generation (Figure 3A). We sampled clones for proliferative expansion and whole-genome expression profiling by RNA sequencing. Guided by overlap between genes identified in the scRNA-seq profiles of each cluster and the RNA-seq profiles of sampled clones (Figure E3A), we identified two or more clones representative of each cluster. Stem cell clones corresponding to the six distinct clusters showed high expression of p63 (Figure E3B), a p53-related transcription factor essential for the long-term proliferative potential of stem cells of all stratified epithelia, including the epidermis, mammary and prostate glands, and the upper airways and lung (37–39). Moreover, each clone type displayed high (55–60%) rates of clonogenicity across 25 serial passages representing nearly 1 year of continuous growth (Figure E3C). The strong p63 expression, high clonogenicity, and extensive proliferative potential of these six “variants” support the concept that these clones possess stem cell properties. To further probe the features of these clonogenic variants, we assessed their lineage fate upon *in vitro* differentiation in air–liquid interface (ALI) cultures (Figures 3B and 3C). Variant 1 (hereafter CFv1), the dominant clone type in control lung, as well as CFv2, differentiated in ALI cultures to epithelia that express markers of terminal bronchial and alveolar epithelia, including Aqp4 and Sftpb (39) (Figures 3C and E3D). CFv3 and CFv4 differentiated to goblet cell metaplasia (GCM) marked by robust MUC5AC expression (Figures 3C and E3D). In contrast, both CFv5 and CFv6 formed squamous cell metaplasia (SCM) marked by IVL (involucrin) and by expression of multiple KRT (keratin) genes (Figures 3C and E3D). Gene expression

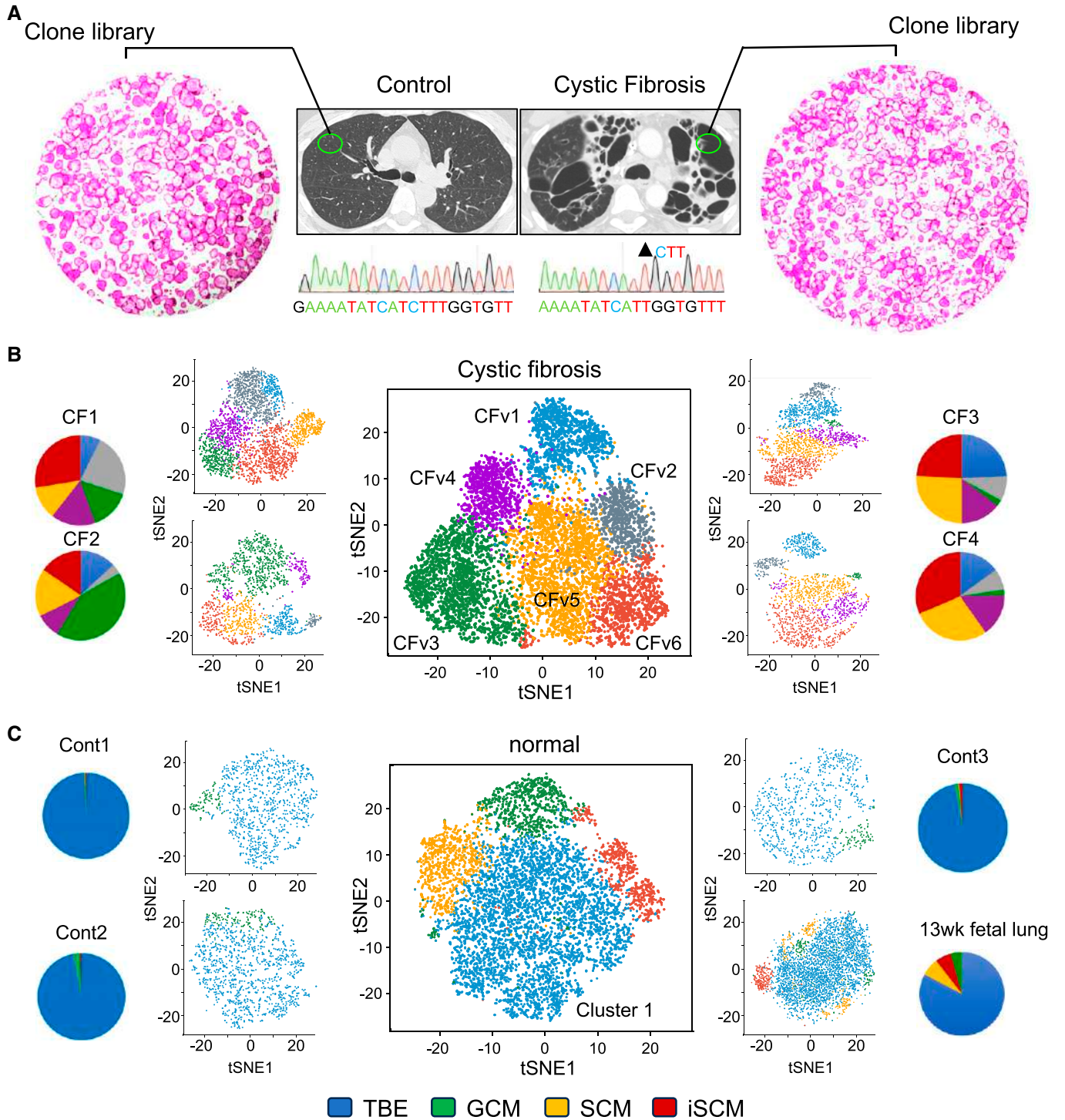


Figure 1. Stem cell libraries from cystic fibrosis lung. (A) Schematic of generation of libraries of epithelial stem cells from control and cystic fibrosis (CF) lungs. CF lungs show cystic bronchiectasis by computed tomography scan and a biallelic loss of phenylalanine 508 by DNA sequencing. Culture plates show colonies formed by clonogenic epithelial cells on lawns of irradiated feeder cells. (B) Single-cell RNA sequencing (scRNA-seq) cluster profiles of stem cell libraries of four CF lungs derived from transplant surgery together with pie charts quantifying the distribution of cells in the various clusters, and the merging of these profiles to detail CF epithelial variants 1–6 (CFv1–CFv6). (C) scRNA-seq analysis of three control adult lungs and one fetal lung, corresponding pie charts detailing variant clusters (TBE, blue; GCM, green; SCM, orange; iSCM, red), and a merged profile highlighting these clusters. GCM = goblet cell metaplasia; iSCM = inflammatory squamous cell metaplasia; SCM = squamous cell metaplasia; TBE = terminal bronchial epithelia; tSNE = t-distributed stochastic neighbor embedding.

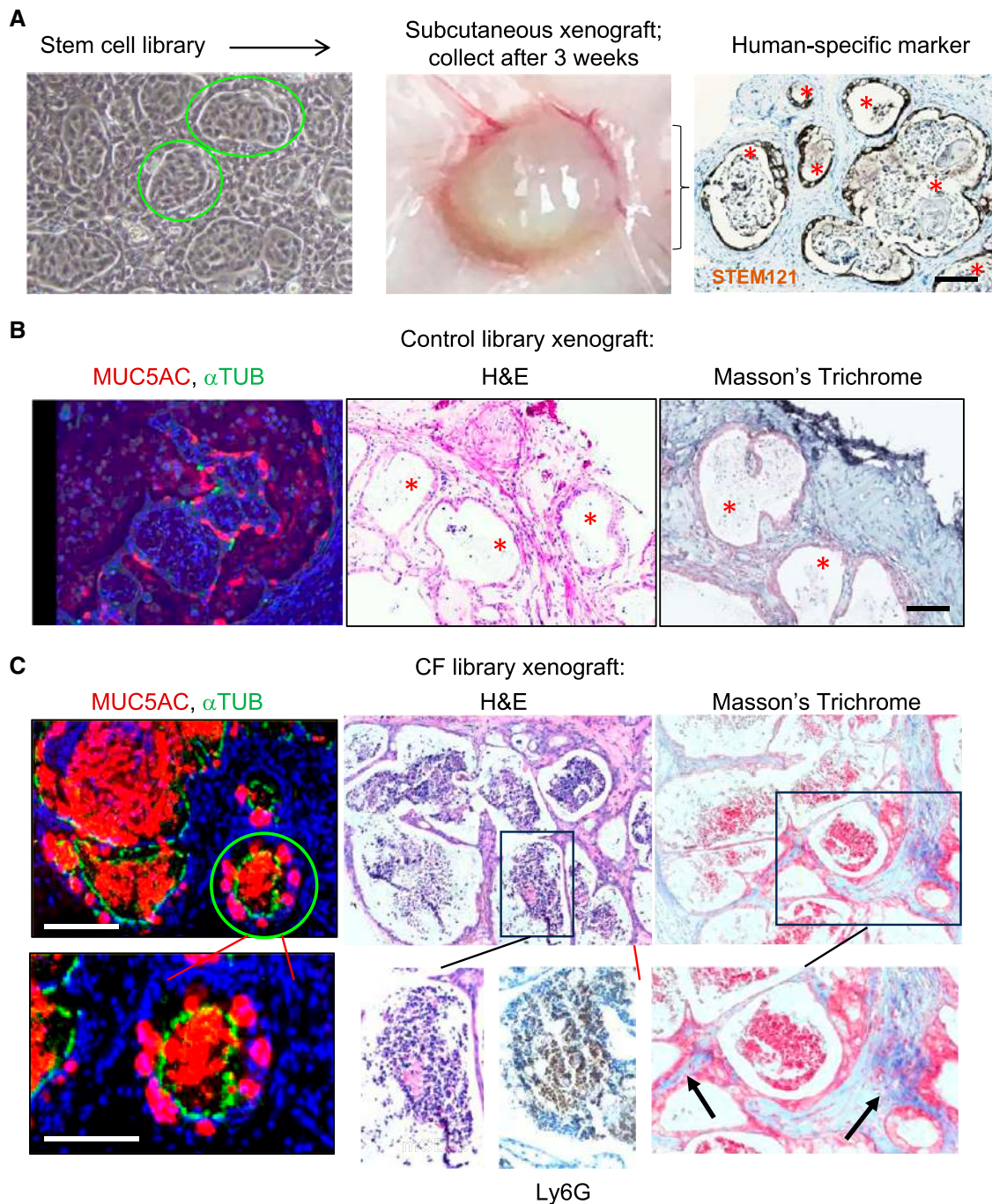


Figure 2. Xenografts of cystic fibrosis (CF) stem cell libraries display pathogenic features. (A) Schematic of xenograft model for assessing the pathologic properties of stem cell libraries involving the expansion of stem cell colonies (in green circles) on 3T3 feeder cells (left), the subcutaneous injection of library epithelial cells in immunodeficient mice to generate nodules (middle), and the analysis of self-assembled epithelial cysts (red asterisks) marked by the human-specific monoclonal antibody STEM123 (right). Scale bar, 200 μ m. (B) Histological assessment of xenograft nodules formed by stem cell libraries from patients without obstructive disease by Muc5AC immunofluorescence (left), hematoxylin and eosin (H&E, middle), and Masson's trichrome staining (right). Scale bar, 200 μ m. (C) Histological assessment of xenograft nodules formed from CF libraries by Muc5AC and α Tub immunofluorescence (left), H&E (middle) revealing cell infiltrates in epithelial cysts, and Masson's trichrome staining showing fibrosis adjacent to cysts (arrows, right). Scale bar, 200 μ m. Insets: Magnification of Muc5AC-positive goblet cells (left), immunohistochemistry of cellular infiltrates with antibodies to the neutrophil marker Ly6G (middle), and submucosal fibrosis via Masson's trichrome staining (blue). Scale bar, 200 μ m.

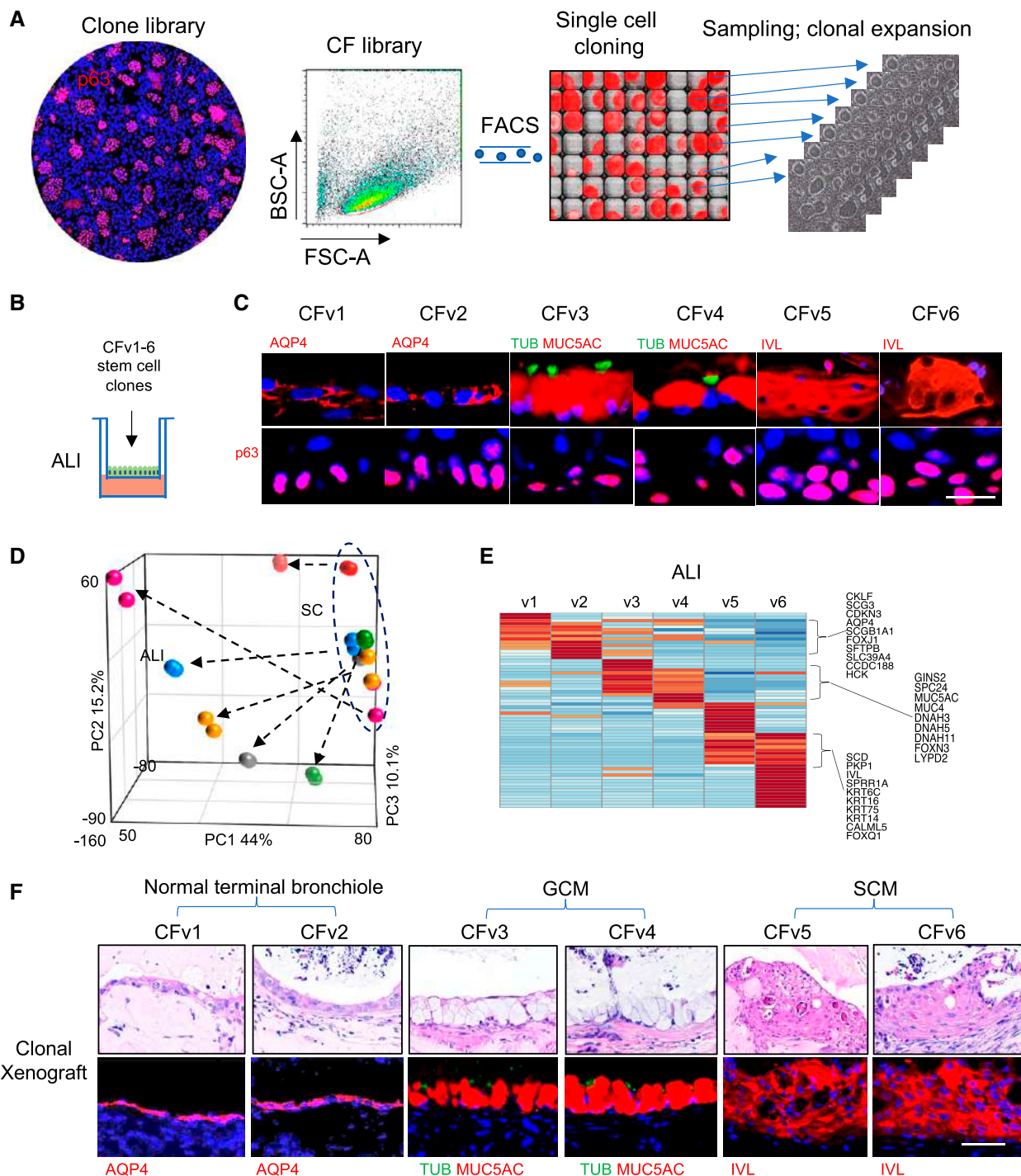


Figure 3. Clonogenic analysis of cystic fibrosis (CF) libraries reveals disease-linked fate profiles. (A) Schematic for isolating discrete clones representative of the cluster heterogeneity of CF stem cell libraries involving single-cell sorting to 384-well plates and clonal expansion. (B and C) *In vitro* differentiation of clones representative of each cluster (CF epithelial variants 1–6 [CFv1–v6]) evident in the single-cell RNA sequencing (scRNA-seq) analysis in air–liquid interface (ALI) differentiation cultures and marker immunofluorescence analysis of histological sections of differentiated epithelia by antibodies to Aqp4 (alveolar), Muc5AC (goblet cell metaplasia [GCM]), and IVL (involucrin) (squamous cell metaplasia [SCM]). (D) Principal component analysis of differential gene expression by CFv1–v6 clones as stem cells (SC) and their ALI-differentiated counterparts (ALI). (E) Heatmap of differential gene expression by epithelia derived from *in vitro* differentiation of CFv1–v6 clones showing typical markers of terminal bronchial epithelia (CFv1, CFv2), GCM (CFv3, CFv4), and SCM (CFv5, CFv6). Scale bar, 20 μ m. (F) Histology and indicated marker immunofluorescence of sections of nodules formed by xenografting CFv1–v6 clones. Scale bar, 200 μ m.

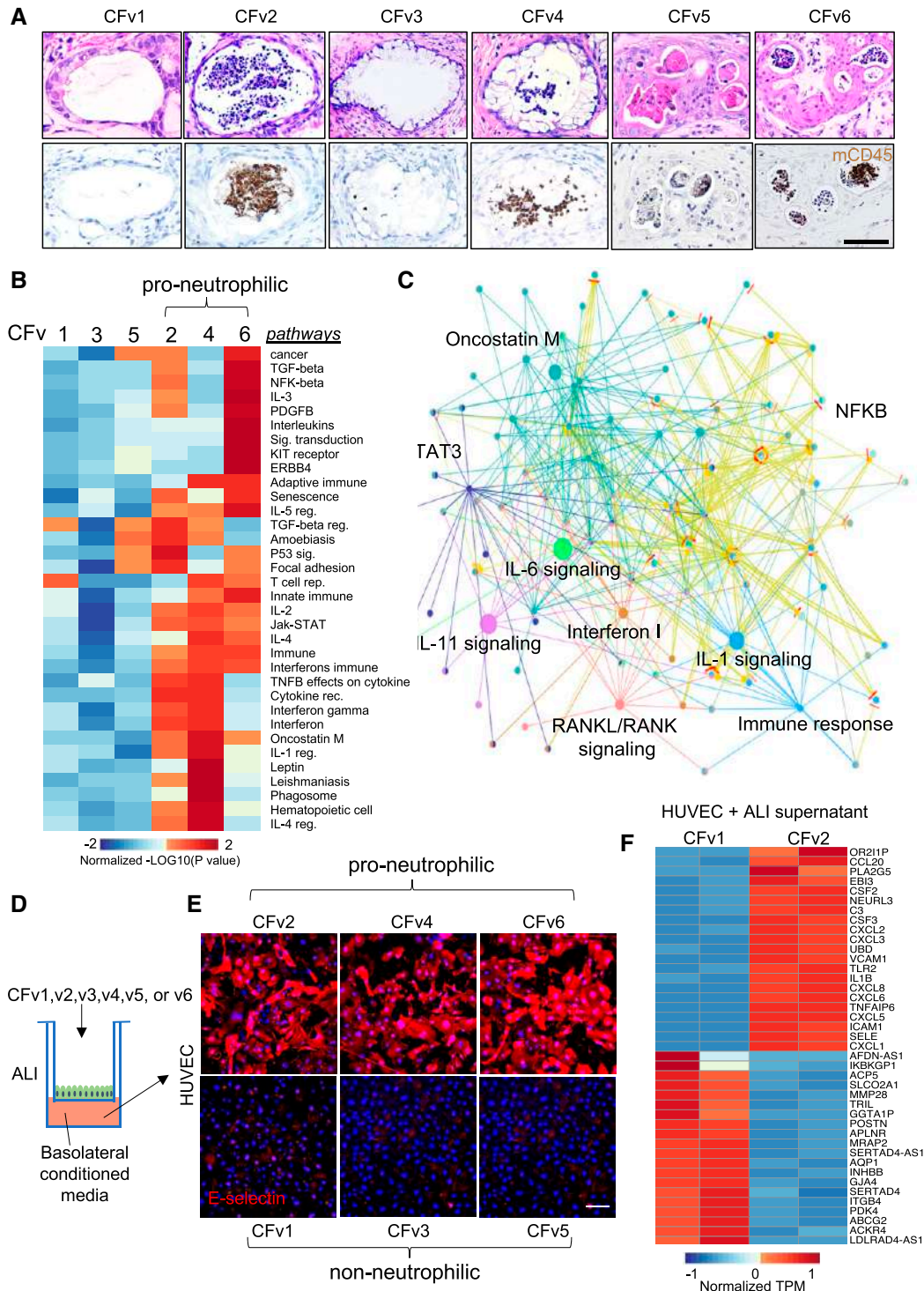


Figure 4. Three proinflammatory variants dominate cystic fibrosis (CF) lung. (A) Histological sections (upper panel) and CD45 immunohistochemistry (lower panel) depicting transepithelial accumulation of neutrophils in nodules of xenografted stem cell clones representative of CF epithelial variant 2 (CFv2), v4, and v6. Scale bar, 100 μm . (B) Heatmap of differential expression of inflammatory genes across CFv1–v6 clones. (C) Network analysis of inflammatory genes expressed in aggregate by CFv2, CFv4, and CFv6 stem cell clones. (D) Schematic for collecting basolateral media conditioned by air–liquid interface (ALI) differentiated CFv1–CFv6 stem cell clones for human vascular endothelial cell (HUVEC) activation assays. (E) Immunofluorescence micrographs of E-selectin expression on HUVEC cells after 48-hour exposure to conditioned media from ALI-differentiated clones representative of CFv1–v6. Scale bar, 30 μm . (F) Heatmap of differential gene expression in HUVEC cells exposed to media conditioned by differentiated CFv1 or CFv2 clones. TPM = transcripts per million.

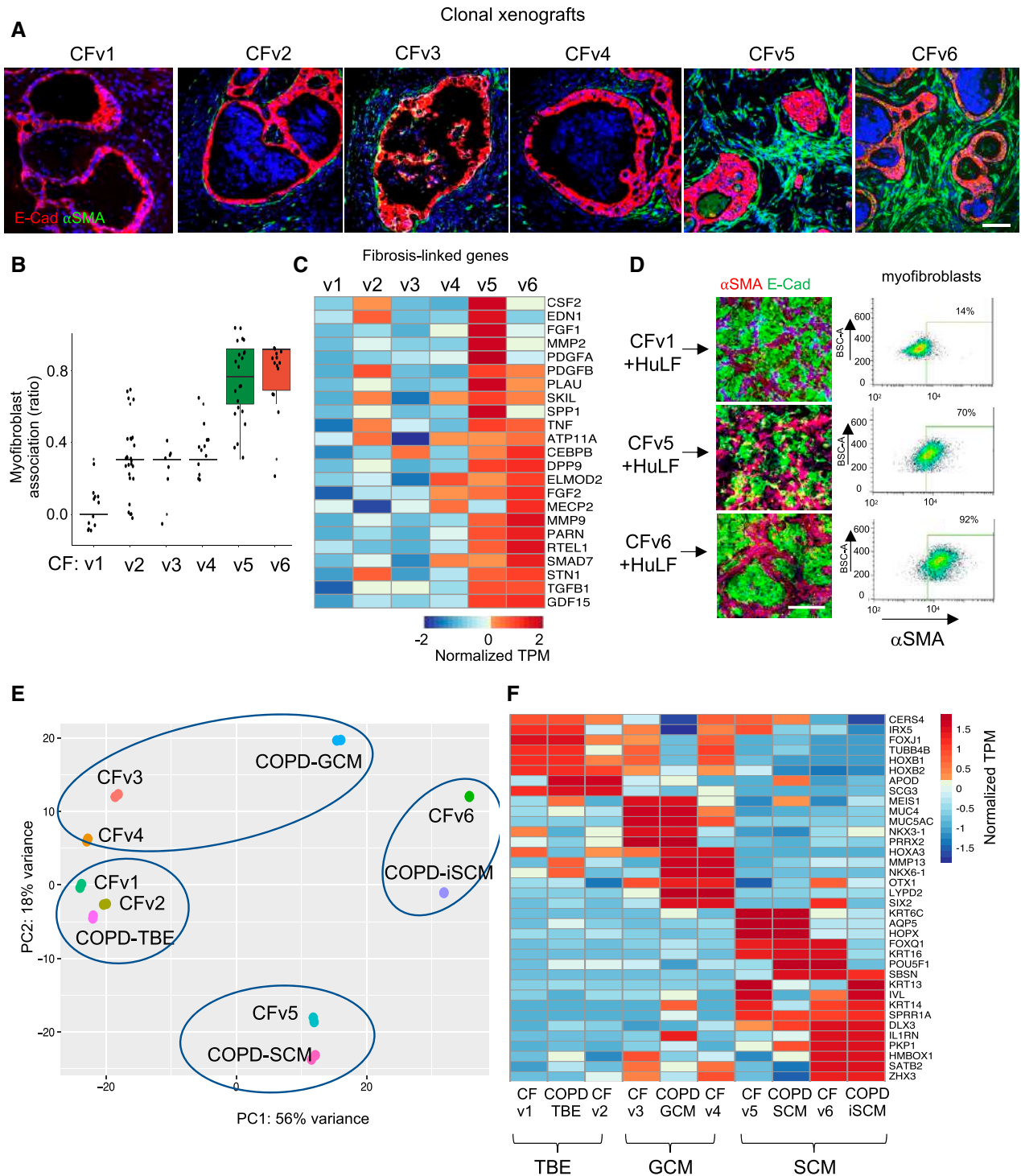


Figure 5. Overlap between cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) variant epithelial cells. (A) Immunofluorescence micrographs of histology sections of CF epithelial variants 1–6 (CFv1–v6) xenograft nodules stained with the epithelial marker E-cadherin (red), the myfibroblast marker α SMA (green), and the DNA dye DAPI (blue). Scale bar, 200 μ m. (B) Quantification of ratio of α SMA+ myfibroblasts bordering epithelial cysts in xenograft nodules formed by the indicated CF variant clone. (C) Heatmap of differential expression of genes linked to fibrosis by CF variant stem cell clones. (D) Immunofluorescence images and fluorescence-activated cell sorting analysis of cocultures of the indicated clones with normal HuLFs with antibodies to the epithelial marker E-cadherin (green) and the myfibroblast marker α SMA (red). Scale bar, 50 μ m. (E) Principal component analysis of whole-genome expression profiles of CFv1–v6 clones with those dominating in COPD lung (21). (F) Expression heat map of selected marker genes for TBE, GCM, and SCM in CFv1–v6 and those identified in COPD lung. HuLFs = human lung fibroblasts; GCM = goblet cell metaplasia; SCM = squamous cell metaplasia; TBE = terminal bronchial epithelia; TPM = transcripts per million.

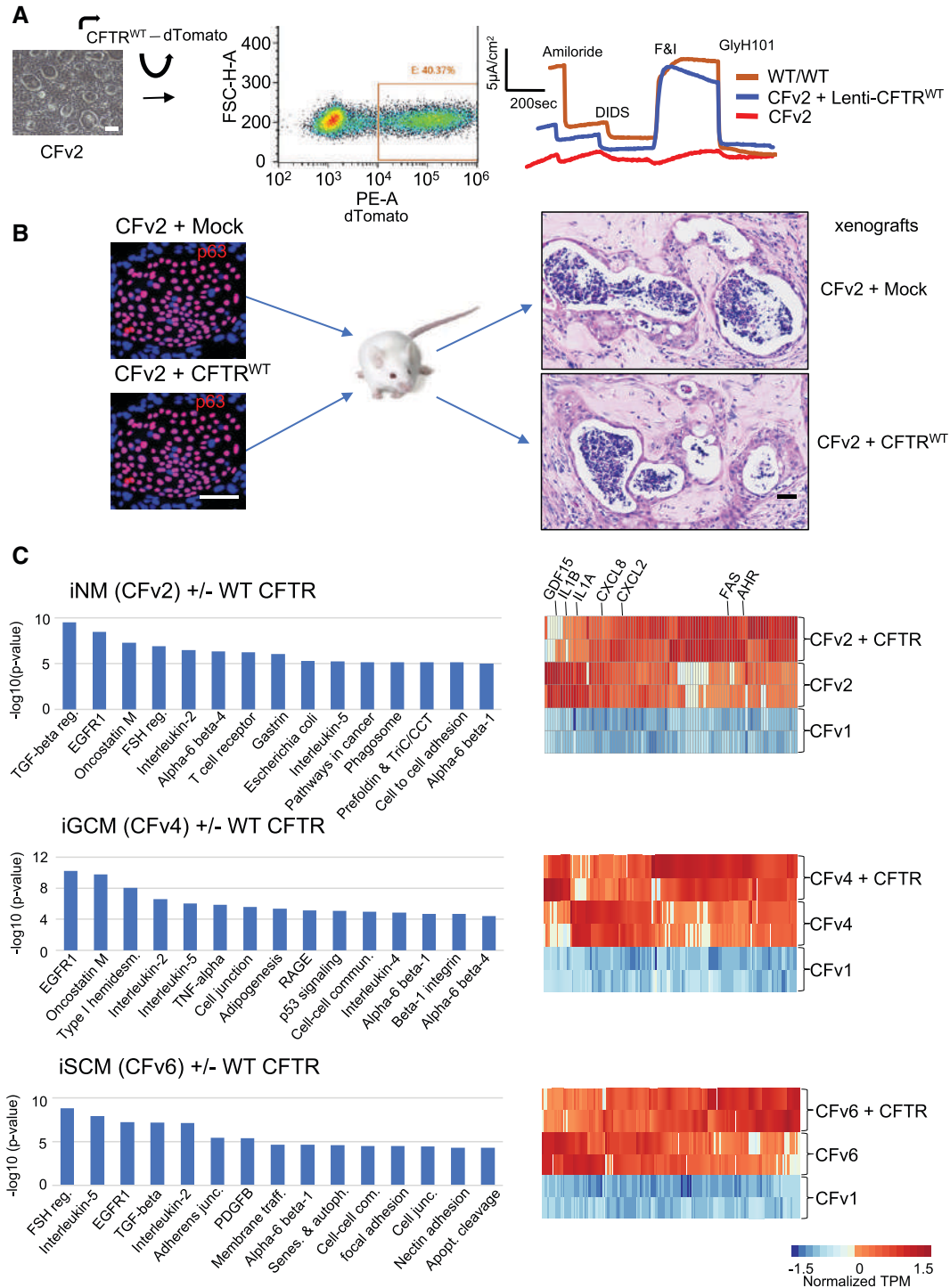


Figure 6. Inflammatory phenotype of cystic fibrosis (CF) variants independent of CFTR (cystic fibrosis transmembrane conductance regulator) activity. (A) Left: schematic of lentiviral transduction of the wild-type (WT) CFTR cDNA and the fluorescence-activated cell sorting of d-Tomato+ cells for clonal expansion. Scale bar, 100 μ m. Right: short-circuit current measurements of normal terminal bronchial epithelial stem cells (WT/WT), CF epithelial variant 2 (CFv2) (Δ F508/ Δ F508), and CFv2 transduced with a lentivirus driving WT CFTR (CFv2 + Lenti-CFTR^{WT}). See METHODS in online supplement. (B) Neutrophilic inflammation in xenograft nodules arising from transplanted CFv2 and CFv2 expressing transduced, WT CFTR. Scale bars, 50 μ m. (C) Histogram of gene sets common to CFv2 and CFv2 + WT CFTR, CFv4 and CFv4 + WT CFTR, or CFv6 and CFv6 + WT CFTR (left), and corresponding heatmaps of differentially expressed inflammatory genes listed in Figure E10. DIDS = anion exchange inhibitor 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid; F&I = forskolin and 3-isobutyl-1-methylxanthine (IBMX); FSC-H-A = forward scatter height and area; iGCM = inflammatory goblet cell metaplasia; iNM = inflammatory normal; iSCM = inflammatory squamous cell metaplasia; PE-A = phycoerythrin area.

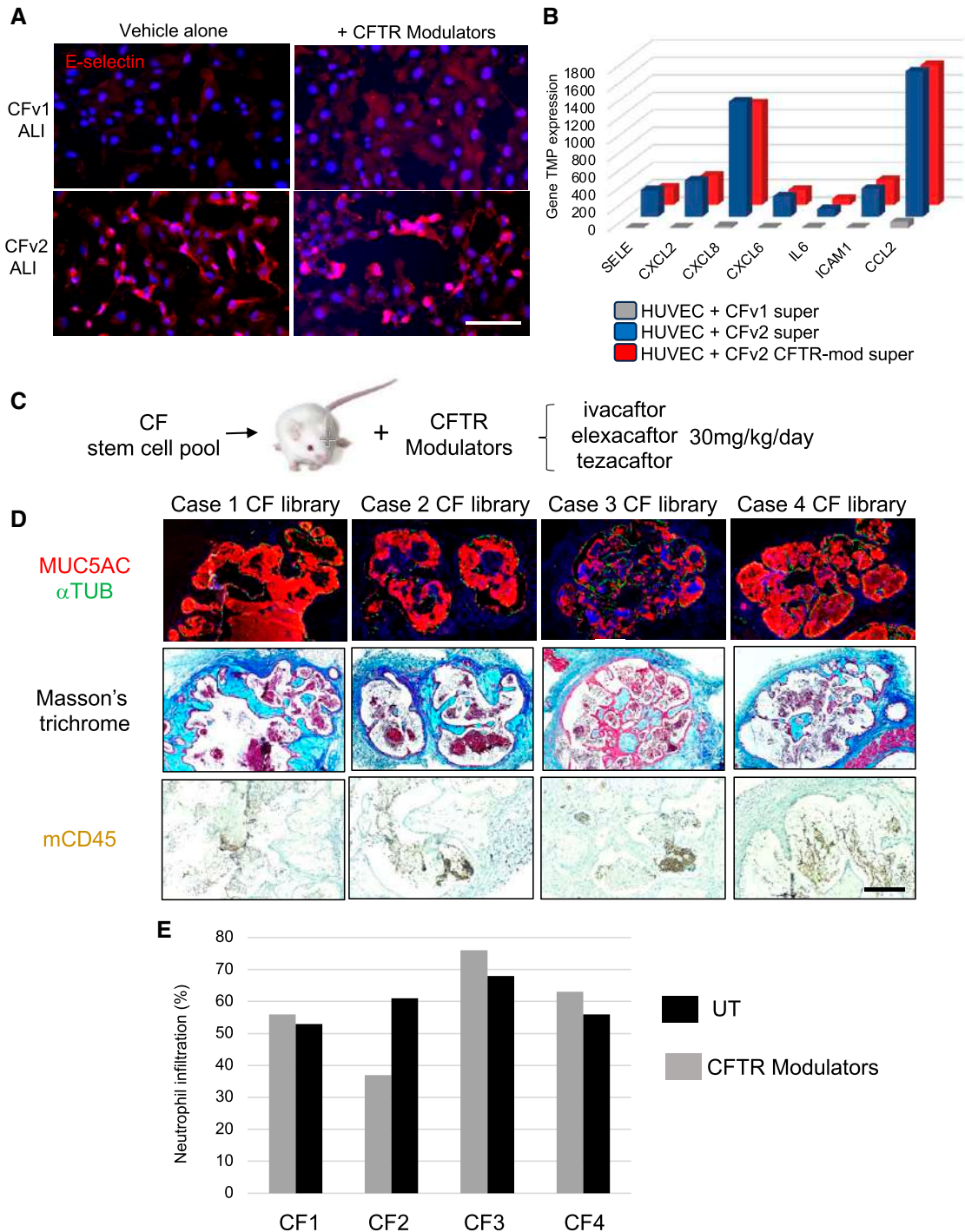


Figure 7. Impact of CFTR (cystic fibrosis transmembrane conductance regulator) modulators on pathologic features of cystic fibrosis (CF) library xenografts. (A) E-selectin immunofluorescence of human vascular endothelial cell (HUVEC) cultures exposed to media conditioned by CF epithelial variant 1 (CFv1) and CFv2 cells differentiated in the absence (left) and presence (right) of triple combinations of CFTR modulators (ivacaftor [3 μ M], elxacaftor [3 μ M], and tezacaftor [4 μ M]). Scale bar, 30 μ m. (B) Histogram of expression of key genes in HUVEC activation in cells treated with media conditioned by CFv1, CFv2, or CFv2 in the presence of triple combination of CFTR modulators. (C) Schematic of CF stem cell library transplantation to immunodeficient mice and systemic treatment of mice with CFTR modulator cocktail ivacaftor, elxacaftor, and tezacaftor (30 mg/kg/d). (D) Histological sections of xenograft nodules stained with antibodies to Muc5A/C and α -Tubulin (top), stained with Masson's trichrome to detect fibrosis (middle), and immunohistochemistry of antibodies to the murine hematopoietic marker mCD45 (brown). Scale bar, 200 μ m. (E) Morphometric quantification of neutrophilic inflammation in xenograft nodules of stem cell libraries from each of four CF stem cell libraries with and without systemic treatment by CFTR modulator cocktail ivacaftor, elxacaftor, and tezacaftor (30 mg/kg/d). TPM = transcripts per million; UT = untreated.

profiling of these variants supports the conclusion that CFv1 and v2 share fate commitments of terminal bronchial epithelial stem cells, CFv3 and v4 are committed to GCM, and CFv5 and v6 are committed to SCM (Figures 3D, 3E and E3D).

We also tested the fate of these variants *in vivo* using subcutaneous xenografts of each of the CF variant clones in NSG mice, in which these stem cells formed polarized epithelia within discrete nodules (c.f. Figures 2A and E4A). Consistent with the ALI differentiation patterns, transplanted CFv1 and v2 clones yielded ftpb1- and qp4-positive epithelia typical of terminal bronchial epithelia, CFv3 and v4 formed GCM expressing Muc5AC, and CFv5 and v6 differentiated to SCM expressing IVL (Figures 2F and 2G). Importantly, all six CF variant stem cells maintained their respective fate commitments even after 25 serial passages *in vitro* over approximately 250 days (Figure E4A), all the while showing minimal genetic changes, indicated by the absence of chromosomal copy number variation events and the paucity of nonsynonymous single-nucleotide variation events (Figures E4B and E4C).

Last, we asked how the profound stem cell heterogeneity of the CF lung was related to the known histological abnormalities associated with the lung remodeling that marks this disease (1, 2). In a morphometric analysis of p63 expression in epithelia in lung tissue from six CF cases and six control subjects (Table E2), we found that GCM dominated the epithelia in the CF lungs (69% vs. 9% of controls, $P = 0.0002$) and that SCM was also overrepresented (10% vs. 3% of controls, $P = 0.04$; Figure E5A and E5B). Using markers of the specific variants or sets of variants, including *Agr2* (CFv1), *Ccl2* (CFv2), *PscA* (CFv3, v4), *Upk1B* (CFv5), and *Cxcl8* (CFv2, v6), we could identify these variants in the remodeled CF lung and approximate their biased distribution in this disease (Figures E5C, E5D, E11, E12, and Detailed Methods in the online supplement).

Three CF Variants Independently Drive Inflammation

Xenografts of epithelial stem cell libraries from COPD lungs were marked by intense neutrophilic infiltration of nodules not seen in xenografts of control libraries (30). As xenografts of stem cell libraries from all four CF libraries showed a similar recruitment of

murine neutrophils marked by CD45 and Ly6G expression (c.f. Figures 2C and E6A–E6C), we asked whether any of the six CF variant clones could autonomously promote this neutrophilic response in xenograft nodules. Remarkably, clonal xenografts of three CF variants (CFv2, v4, and v6) showed robust neutrophilic infiltration of xenograft nodules, whereas the CFv1, v3, and v5 did not (Figures 4A and E7A). Given the neutrophilic response triggered by CFv2, CFv4, and CFv6, we asked if their respective gene expression profiles would reflect inflammatory signatures. Consistent with their proinflammatory activity, CFv2, v4, and v6 gene expression profiles were associated with inflammatory signaling pathways (31, 32, 40) previously linked to COPD and CF, including *Oncostatin M*, *TNF- α* , *Interleukins 1, 2, 4, and 5*, as well as the response to pathogen infections such as *Leishmaniasis* and *Amoebiasis* (Figures 4B and E7B). The association of these proinflammatory pathways, and the genes that comprise them (e.g., Figures 4C, E8A, and E8B), such as *CCL20*, *CXCL8*, and *CXCL1*, remain differentially high in the proinflammatory variants across months of serial passaging *in vitro* (Figures E8C and E8D) and are likely enforced by stable epigenetic mechanisms.

As neutrophil transepithelial migration is believed to rely on prior endothelial extravasation (41, 42), we asked if any of the CF variants secreted factors that promote endothelial cell activation (43). Medium conditioned by basolateral secretions of ALI-differentiated CF variant clones was applied to cultures of human vascular endothelial cells (HUVECs), which were monitored after 48 hours for E-selectin (SELE) expression by immunofluorescence (Figures 4D and 4E). Consistent with their neutrophil transepithelial migration status, supernatants from the proinflammatory variants v2, v4, and v6 triggered robust activation of SELE expression on endothelial cells, whereas those of CFv1, CFv3, and CFv5 did not (Figure 4E). Whole-genome expression profiling of HUVECs after exposure to CFv1 and CFv2 supernatants showed a broad set of genes linked to endothelial cell activation by CFv2, including *SELE*, *VCAM1*, *ICAM1*, and multiple chemokines and cytokines, including *IL1beta*, *CCL20*, *CXCL2*, *CXCL3*, *CXCL6*, and *CXCL8* (IL-8) linked to neutrophil chemotaxis (41–43) (Figure 4F).

CF Stem Cell Variants Overlap Those in COPD

Lung disease in CF and COPD share many features (1, 2, 6, 31, 32, 40), including chronic bronchitis, mucin hypersecretion, recurrent infections, and pathologic lung remodeling. Given these similarities, as well as the presence of proinflammatory and mucin-producing variant stem cells in both conditions, we asked whether any of the CF variants promoted an α SMA (α -smooth muscle actin)-positive myofibroblast response akin to those produced by two dominant variants in COPD (COPDv3, COPDv4) marked by SCM fate (30). Among xenografts of each of the six CF variants, those generated by CFv5 and CFv6 showed strong submucosal accumulation of α SMA + myofibroblasts (Figures 5A and 5B). As α SMA + myofibroblasts are widely considered an early indicator and essential factor in the development of tissue fibrosis (44), we refer hereafter to the lung variant stem cells that trigger α SMA + myofibroblast accumulation in xenografts as profibrotic. Consistent with the profibrotic activity of CFv5 and v6, these variants expressed high levels of genes linked to disease-associated fibrosis, including *FGF1*, *PDGFB*, *PLAU*, *TGFR1*, and *GDF15* (Figure 5C). In addition, the coculture of CFv5 or CFv6 cells with normal human lung fibroblasts was sufficient to convert these fibroblasts to α SMA-expressing myofibroblasts (Figure 5D). The profibrotic properties of CFv5 and CFv6, coupled with the SCM fate commitment of these clones, suggests their equivalence to the profibrotic variants that dominate the COPD lung and those present as minor variants in control lungs (30). These functional relationships between individual variants in COPD, CF, and normal lung are supported by similarities in gene expression profiles (Figures 5E and 5F). Two of the three inflammatory CF variants, CFv2 and CFv4, did not correspond to any of the major variants identified in COPD and may represent features specifically relevant to the CF lung. Taken together, these data suggest that CF and COPD show considerable overlap in their stem cell variant profiles in fate commitment, gene expression, and the ability to drive neutrophilic inflammation. These findings are consistent with the reported lung histopathology of these two conditions, which are marked by GCM, SCM, and mucosal inflammation (1, 2, 31, 32).

Variant Inflammation Is Independent of CFTR Activity

Prospective gene therapies for CF include genetic complementation of the mutant CFTR or correction of mutant alleles of this gene via gene-editing technologies (45). We asked whether restoring CFTR activity to proinflammatory CF variant stem cells would mitigate their pathogenic features. We transduced CFv2, v4, and v6 proinflammatory variant cells with a lentivirus co-expressing wildtype CFTR (46) and the fluorescent protein dTomato-positive and sorted individual dTomato+ cells by FACS to 384-well plates for clonal expansion (Figure 6A). The expression of wild-type CFTR transcript in these cells was confirmed by RT-PCR amplification and Sanger sequencing. Lentivirus-mediated expression of wild-type CFTR efficiently restored cAMP-inducible and GlyH101-inhibited chloride conductance in differentiated airway epithelium from these variants, as assessed by short-circuit current measurements (46) (Figures 6A and E9). The transplantation of CFTR-transduced CFv2, CFv4, and CFv6 clones to immunodeficient mice yielded xenograft nodules marked by neutrophilic infiltration similar to that of the CFTR-deficient CFv2 clone (Figure 6B). Consistently, wild-type CFTR activity did not appreciably alter the proinflammatory gene expression profiles of the parent clones (Figures 6C and E10).

We next tested whether CFTR modulators could suppress the production of factors by CFv2, v4, or v6 that mediate the paracrine activation of human endothelial cells. Treatment of ALI cultures with a triple combination of CFTR modulators (ivacaftor [3 μ M], elxacaftor [3 μ M], and tezacaftor [4 μ M]) failed to block the activation of HUVECs *in trans* by CFv2, v4, or v6 cells, as judged by E-selectin immunofluorescence and RNA-seq expression profiling of HUVEC cells linked to activation (41–43), including SELE, ICAM1, IL6, and CXCL8 (Figures 7A and 7B). We also probed the impact of the triple combination (30 mg/kg each daily for 1 wk) on the pathologic features evident in xenografts of stem cell libraries from all four CF cases, including neutrophilic inflammation, mucin hypersecretion, and myofibroblast activation. Again, this combination regimen had no obvious impact on neutrophil infiltration, the extent of fibrosis as judged by Masson's trichrome

staining, or the Muc5AC expression of xenograft nodules (Figures 7C–7E).

Discussion

We have identified a discrete set of major lung stem cell variants in patients with CF with advanced disease. The pathogenesis of these variants is suggested by their stable commitment to known histological abnormalities of the CF lung including GCM and SCM (1, 2, 32) and findings that tie one or more of these variants to neutrophilic inflammation, endothelial cell activation, mucin hypersecretion, and myofibroblast conversion, which in aggregate mirror elements of advanced lung disease in these patients. Four of the six variants that dominate the CF lung share fate commitment, gene expression, and pathogenic functions with the four major variants displayed by COPD lungs (30). The pathogenic variants in CF (CFv2–CFv6) were also found in control lungs and in fetal lungs, albeit at low ratios to the “normal” variant 1 basal cell, and likely represent a dynamic retinue of cells devoted to innate immune responses (30).

The heterogeneity of dominant stem cell variants in the CF lung may be germane to understanding how CFTR mutations lead to CF and strategies to address this disease via restoration of CFTR function. Despite the well-established benefits of new CFTR modulator drugs for patients with CF (15–29), it is less clear that they suppress lung inflammation in patients with advanced disease (28, 29). Relevant to those concerns, we found that CFTR-modulating drugs did not suppress the proinflammatory activity or gene expression of the three CF variants (v2, v4, or v6) that drive neutrophilic inflammation. Moreover, restoration of CFTR activity by gene complementation did not impact the nominally pathogenic features of any of the CF variants, including GCM or SCM, mucin hypersecretion, myofibroblast conversion, or endothelial cell activation. Taken together, these data support well-established roles of CFTR mutations in favoring bacterial colonization as an initiation of lung disease and a second wave of chronic inflammation driven by these variants that may be largely independent of CFTR activity.

The limitations of this study include the severity of CF lung disease examined and the emerging technology (30, 37–39) for cloning basal cells of the lung. All four CF cases assessed for clone heterogeneity underwent lung transplantation at the University of Iowa and thus represent a cohort of very advanced lung disease. How the uniform profile of variants seen in these advanced cases relates to patients with CF at earlier stages of the condition was not addressed in this study. We also cannot be certain that culture conditions for generating stem cell libraries, which were formulated around the clonogenicity of normal distal airway stem cells, will capture the plurality of stem cell variants linked to CF or any other lung condition. In addition, our studies were restricted to the epithelial component of CF and thus do not address the impact of CFTR mutations on the activity of hematopoietic or stromal lineages (47). We should also note that our phenotypic analyses of the variant stem cell in CF is limited to transcriptome profiling and interactions with host cells upon xenografting to immunodeficient mice and would be considerably strengthened by knowledge of underlying epigenetic and RNA stability mechanisms linked to inflammation (48, 49). Ascribing a proinflammatory phenotype to the three (CFv2, v4, and v6) variant stem cells was supported by a broad spectrum of differentially expressed genes associated with inflammation as well as the robust recruitment of host neutrophils in xenograft nodules formed by these cells. This conclusion is bolstered by the demonstration that conditioned media of these cells induced the activation of human endothelial cells (41–43, 50) *in vitro*. Given the absence of most hematopoietic lineages in the NSG (NOD^{scid} IL2R^{γnull}) mouse (36), our model was unable to assess whether the proinflammatory variants in CF also recruit other hematopoietic lineages, such as macrophages, B or T cells, or natural killer cells or other innate lymphocyte subsets absent from NSG mice. Apart from these limitations, the inability of CFTR activity restoration to impact the proinflammatory properties of these variant cells in CF highlights the need for additional therapeutics targeting these variants in patients with advanced lung disease. In this regard, the absolute commitment of the stem cell variants to distinct pathogenic fates

suggests underlying epigenetic control mechanisms whose interdiction could have therapeutic effects. Ongoing molecular analyses of epigenetic mechanisms operating in the CF variant stem cells, as well as in their counterparts in COPD (30) and idiopathic pulmonary fibrosis (IPF) (51), will likely reveal the basis of coordinate expression of inflammatory and fibrotic genes by these cells.

Presented with the staunch irreversibility of chronic lung diseases, the early application of CFTR modulators or gene therapy offers the potential to forestall or obviate the onset of CF lung pathology. If the pathogenic stem cell variants described here in CF lungs and elsewhere for COPD (30) and IPF (51) indeed drive and maintain these chronic lung conditions, their selective elimination, coupled with regenerative

medicine (39, 52), might synergize with the profound advances brought by CFTR modulator therapy. ■

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