University of Groningen

An airway epithelial IL-17A response signature identifies a steroid-unresponsive COPD patient subgroup

Christenson, Stephanie A.; van den Berge, Maarten; Faiz, Alen; Inkamp, Kai; Bhakta, Nirav; Bonser, Luke R.; Zlock, Lorna T.; Barjaktarevic, Igor Z.; Barr, R. Graham; Bleecker, Eugene R.

Link to publication in University of Groningen/UMCG research database

The Journal of Clinical Investigation

An airway epithelial IL-17A response signature identifies a steroid-unresponsive COPD patient subgroup

Stephanie A. Christenson, … , David J. Erle, Prescott G. Woodruff

Clinical Medicine **In-Press Preview** Immunology Pulmonology

BACKGROUND. Chronic Obstructive Pulmonary Disease (COPD) is a heterogeneous smoking-related disease characterized by airway obstruction and inflammation. This inflammation may persist even after smoking cessation and responds variably to corticosteroids. Personalizing treatment to biologically similar "molecular phenotypes" may improve therapeutic efficacy in COPD. IL-17A is involved in neutrophilic inflammation and corticosteroid resistance, and thus may be particularly important in a COPD molecular phenotype.

METHODS. We generated a gene expression signature of IL-17A response in bronchial airway epithelial brushings ("BAE") from smokers with and without COPD ($n = 238$), and validated it using data from two randomized trials of IL-17 blockade in psoriasis. This IL-17 signature was related to clinical and pathologic characteristics in two additional human studies of COPD: (1) SPIROMICS ($n = 47$), which included former and current smokers with COPD, and (2) GLUCOLD ($n = 79$), in which COPD participants were randomized to placebo or corticosteroids.

RESULTS. The IL-17 signature was associated with an inflammatory profile characteristic of an IL-17 response, including increased airway neutrophils and macrophages. In SPIROMICS the signature was associated with increased airway obstruction and functional small airway disease on quantitative chest CT. In GLUCOLD […]

Find the latest version:

1   Title: **An airway epithelial IL-17A response signature identifies a steroid-unresponsive**

2   **COPD patient subgroup**

3

4   **Authors:**

5   Stephanie A. Christenson[1], Maarten van den Berge[2], Alen Faiz[2], Kai Inkamp[2], Nirav Bhakta[1],

6   Luke R Bonser[1], Lorna T. Zlock[3], Igor Z. Barjaktarevic[4], R. Graham Barr[5], Eugene R. Bleecker[6],

7   Richard C. Boucher[7], Russell P. Bowler[8], Alejandro P. Comellas[9], Jeffrey L. Curtis[10], MeiLan K.

8   Han[10], Nadia N. Hansel[11], Pieter S. Hiemstra[12], Robert J. Kaner[13], Jerry A. Krishnan[14], Fernando

9   J. Martinez[13], Wanda K. O'Neal[7], Robert Paine III[15], Wim Timens[16], J. Michael Wells[17], Avrum

10   Spira [18], David J. Erle[1], Prescott G. Woodruff[1*]

11

12   **Affiliations:**

13   [1]Department of Medicine, University of California, San Francisco, CA

14   [2]University Medical Center Groningen, Department of Pulmonary Diseases and Research

15   Institute for Asthma and COPD (GRIAC), Groningen, The Netherlands

16   [3]Department of Pathology, University of California, San Francisco, CA

17   [4]Department of Medicine, University of California, Los Angeles, CA

18   [5]Department of Medicine, Columbia University Medical Center, New York, NY

19   [6]Department of Medicine, University of Arizona, Tucson, AZ 85724

20   [7]Marsico Lung Institute, University of North Carolina at Chapel Hill, NC

21   [8]National Jewish Health, Denver, CO 80206

22   [9]Department of Medicine, University of Iowa, Iowa City, IA 52242

23   [10]Department of Medicine, University of Michigan, Ann Arbor, MI 48103

24   [11]Department of Medicine, Johns Hopkins University, Baltimore, MD 21205

25   [12]Department of Pulmonology, University Medical Center, Leiden, the Netherlands

26   [13]Department of Medicine, Weill Cornell Medical Center, New York, NY 10065

27   [14]Breathe Chicago Center, University of Illinois at Chicago, IL 60608

28   [15]Department of Internal Medicine, University of Utah, Salt Lake City, UT

29   [16]University Medical Center Groningen, Department of Pathology and Medical Biology and

30   Research Institute for Asthma and COPD (GRIAC), Groningen, The Netherlands

31   [17]Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294

32   [18]Department of Medicine, Boston University School of Medicine, Boston, MA 02118

33

34   ***Corresponding Author:**

35   Prescott Woodruff: Department of Medicine, University of California, San Francisco, 513

36   Parnassus Ave, HSE 1355A, San Francisco, California 94143; prescott.woodruff@ucsf.edu,

37   415-476-4176

38

39   **Conflict of Interest:** SAC and PGW declare a patent pending related to this work.

40

43

44

45

46

47

48

49

50

51

52

Abstract

Background: Chronic Obstructive Pulmonary Disease (COPD) is a heterogeneous smoking-related disease characterized by airway obstruction and inflammation. This inflammation may persist even after smoking cessation and responds variably to corticosteroids. Personalizing treatment to biologically similar "molecular phenotypes" may improve therapeutic efficacy in COPD. IL-17A is involved in neutrophilic inflammation and corticosteroid resistance, and thus may be particularly important in a COPD molecular phenotype.

Methods: We generated a gene expression signature of IL-17A response in bronchial airway epithelial brushings ("BAE") from smokers with and without COPD (n=238), and validated it using data from two randomized trials of IL-17 blockade in psoriasis. This IL-17 signature was related to clinical and pathologic characteristics in two additional human studies of COPD: (1) SPIROMICS (n=47), which included former and current smokers with COPD, and (2) GLUCOLD (n=79), in which COPD participants were randomized to placebo or corticosteroids.

Results: The IL-17 signature was associated with an inflammatory profile characteristic of an IL-17 response, including increased airway neutrophils and macrophages. In SPIROMICS the signature was associated with increased airway obstruction and functional small airway disease on quantitative chest CT. In GLUCOLD the signature was associated with decreased response to corticosteroids, irrespective of airway eosinophilic or Type 2 inflammation.

Conclusion: These data suggest that a gene signature of IL-17 airway epithelial response distinguishes a biologically, radiographically, and clinically distinct COPD subgroup that may benefit from personalized therapy.

Trial Registration: ClinicalTrials.gov NCT01969344

Funding: Primary support from NIH/NHLBI. For others see below.

Word Count: 242

79 **Brief Summary** (25 words): A COPD subgroup displays an enhanced IL-17A airway epithelial

80 response associated with increased airway obstruction, neutrophilic inflammation, and a poor

81 response to corticosteroids.

82

83

84

85

86

87

88

89

90

91

92

93

94

95

96

97

98

99

100

Introduction

Personalizing treatment to "molecular phenotypes", *i.e.* to subsets of patients with shared

underlying biology, is an emerging strategy to guide therapeutic choices in chronic disease (1,

2). In respiratory disease, this strategy has particularly gained traction in severe asthma where

subgroups of patients with Type 2 and eosinophilic inflammation can be targeted using new

biologics (1-4). Much of the inflammation in chronic respiratory disorders, however, does not

respond to therapies directed against Type 2 inflammation. Identifying subgroups that display

enhanced non-Type 2 inflammatory pathways may lead to the repurposing of available biologics

indicated for other inflammatory disorders to target these subgroups.

Chronic obstructive pulmonary disease (COPD) is a highly prevalent respiratory disease, most

commonly associated with smoking. COPD is a major cause of morbidity and mortality

worldwide for which few interventions have been found that prevent disease progression (5).

Yet, molecular phenotyping has been less studied in COPD than in asthma and has focused on

eosinophilic and type 2 inflammation based on the previous work in asthma (2). Type 2

inflammation is likely relevant in only a minority of COPD patients (6). Nonetheless, this work

suggests that biologically distinct COPD subgroups exist and are clinically relevant. COPD

patients with high eosinophil counts or an airway epithelial genomic signature of Type 2

inflammation are more likely to respond to corticosteroids, and potentially to biologics targeting

eosinophils (6-9). These studies suggest the promise of molecular phenotyping in COPD, but

responses beyond Type 2 inflammation require further investigation.

The IL-17 family of cytokines includes six members that play various roles in mucosal host

defense and chronic inflammation (10). IL-17A stimulates the airway epithelium to produce

chemokines and other mediators which recruit and activate neutrophils and macrophages, cells

126   crucial to COPD pathogenesis (11). IL-17A is implicated in COPD-associated pathogenic

127   responses, including emphysema, lymphoid neogenesis, corticosteroid resistance, dysbiosis,

128   mucus hypersecretion, and ongoing inflammation despite smoking cessation (12-19). However,

129   many of these responses have not been investigated in human studies. By identifying the

130   COPD subgroup that manifests IL-17A associated inflammation (hereafter referred to as IL-17),

131   we hypothesize that we can distinguish a corticosteroid-unresponsive subgroup that may benefit

132   from anti-IL17 biologics. Anti-IL-17 biologics are now approved for the treatment of autoimmune

133   diseases, specifically psoriasis and psoriatic arthritis, and are being studied in COPD (20, 21).

134   Non-targeted trials of biologic therapies in COPD have failed to meet clinical endpoints,

135   suggesting the importance of directing therapy to appropriate subgroups (22).

136

137   Here we studied the transcriptional response of the airway epithelium to IL-17. We have found

138   that direct measurement of interleukin proteins, including IL-17A, can be difficult in human blood

139   and bronchoalveolar lavage fluid (23, 24). These challenges may contribute to the inconsistent

140   evidence for IL-17A protein levels being increased in COPD (25-30). Conversely, airway

141   epithelial cells have reproducible transcriptional responses to many interleukins. Thus our

142   general strategy has been to assay this epithelial response, which we have validated for IL-13

143   (24, 31, 32) and interferons (33).

144

145   We examined a genomic signature of the airway epithelial IL-17 response in three separate

146   human COPD studies in which bronchial airway samples were collected during bronchoscopy.

147   We first fit the IL-17 genomic signature, generated using bronchial epithelial cells exposed to IL-

148   17, to a cross-sectional study of ever-smokers with and without COPD (Bronchial Airway

149   Epithelial (BAE) dataset, n=237). We next established that the signature specifically identified

150   IL-17 associated inflammation by determining its response to other airway epithelial adaptive

151   immune responses (type 1 and 2) and to IL-17-directed biologic therapies in psoriatic skin

6

152 lesions. We then tested the associations between this IL-17 signature and clinical features in

153 two independent COPD studies which collected rich phenotypic data (GLUCOLD: n= 79 and

154 SPIROMICS: n=47, Study design in **Figure 1**). We hypothesized that our airway epithelial IL-17

155 genomic signature would be increased in a COPD subset, and associated with distinct clinical,

156 pathologic and radiographic characteristics.

157

158

159

160

161

162

163

164

165

166

167

168

169

170

171

172

173

174

175

176

177

7

178   **Results**

179   *Generation of an airway epithelial IL-17 associated gene expression signature in COPD*

180   We first characterized the airway epithelial response to IL-17 using whole transcriptome profiling

181   of IL-17 versus vehicle control-stimulated human bronchial epithelial cell (HBEC) cultures grown

182   at air liquid interface (ALI). The 100 genes most upregulated by $\log_2$ fold change in response to

183   IL-17 were studied as candidate IL-17 signature genes.

184

185   We examined these 100 genes in a previously generated bronchial airway epithelial

186   transcriptome profiling dataset derived from bronchoscopic brushing samples from ever-

187   smokers with (n=85) and without COPD (n=152, Bronchial Airway Epithelial (BAE) dataset,

188   demographics in **Table 1**). Candidate IL-17 signature genes were enriched amongst smokers

189   with COPD compared to those without (mean of the zero-centered $\log_2$ gene expression in

190   those with COPD=0.11 (±0.27) versus without= -0.60 (±0.19), p=6.10*10^{-6}, **Figure S1**).

191

192   Next, we generated a genomic signature of the IL-17 response specific to in vivo brushing

193   samples from smokers by restricting the 100 gene signature identified in the culture model to

194   those tightly correlated in the BAE dataset using an elastic net (34, 35). We took this additional

195   step because cell culture models cannot optimally reproduce the complexity of the in vivo

196   environment in which multiple mechanistic pathways impact gene expression, often with

197   disparate affect. This signature refinement process is based on the premise that highly inter-

198   correlated genes are co-regulated by the same molecular processes, a premise also used by

199   pathway analysis tools such as weighted gene co-expression analysis (36, 37). Starting with the

200   100 candidate genes as predictors, elastic net regression with leave-one-out cross validation

201   selected 10 genes highly correlated with a representative IL-17 related gene, *CCL20,* in the

202   BAE dataset (**Figure 2**). We chose *CCL20 a priori* to guide the elastic net gene selection to

8

203   specifically identify an IL-17/*CCL20*-associated response. *CCL20* was chosen for this role

204   based on: 1) biological relevance, as an epithelial gene known to be the only ligand for CCR6, a

205   chemokine receptor preferentially expressed by Th17 cells, and thus thought to be more specific

206   for an IL-17 response as compared to other adaptive immune responses (38), and 2) statistical

207   relevance, because it was highly upregulated ($\log_2$ fold change=2.92, FDR=0.0006) following IL-

208   17 stimulation in vitro. Importantly, this IL-17 associated gene was chosen to guide gene

209   selection as our goal was to retain co-associated genes due to their potential biologic relevance,

210   independent from outcomes of interest. We confirmed that the 10 genes selected by elastic net

211   and *CCL20* were all inter-correlated (**Figure S2**), verifying that the elastic net procedure

212   removed loosely correlated genes. Nearly all of the 10 genes have previously been shown to be

213   associated with IL-17 related inflammation (38-42). We thus used these 10 genes, along with

214   *CCL20,* to construct a gene signature of airway epithelial response to IL-17 using the mean of

215   their zero-centered $\log_2$ expression values.

216

217   We confirmed that the genes selected for the signature are measuring an IL-17 response not

218   just specific to *CCL20* in two ways. First, we evaluated the correlation between our IL-17

219   signature and a 5 gene airway epithelial IL-17 gene expression metric previously examined in

220   asthma (39). In the BAE dataset the two signatures were well-correlated (ρ=0.51, $p<2.2*10^{-16}$) in

221   COPD participants, **Figure S3, Table S1**). The signatures were also correlated in an additional

222   COPD dataset, GLUCOLD (demographics in **Table 1**), in which transcriptomic profiles from

223   endobronchial biopsies were obtained from 79 participants with COPD (ρ=0.49, $p=5.0*10^{-6}$,

224   **Figure S3, Table S1**). Second, we repeated the elastic net procedure using *SLC26A4*, the gene

225   most upregulated with IL-17 stimulation in cell culture also measured in the COPD array data

226   ($\log_2$ fold change=8.51, FDR=0), to guide the elastic net. The *SLC26A4*-based signature

227   incorporated 16 genes, 6 of which were also in the 11 gene *CCL20*-based signature, and was

228   highly correlated with the *CCL20*-based signature in the BAE and GLUCOLD datasets (ρ=0.97

9

229 and p<2.2*10^{-16}, ρ =0.87 and p<2.2*10^{-16}, respectively, **Figure S4, Table S1**). Thus, removal of

230 loosely associated genes from the 100 gene signature using *CCL20* to guide the elastic net

231 measured a response that does not appear to be exclusive to *CCL20*. However, we used the

232 *CCL20*-based signature for our subsequent analyses as it had clear advantages over the

233 others. The asthma signature was generated in a cell culture model and never fit to the in vivo

234 environment. *SLC26A4 is of* unclear significance in IL-17 biology, and thus we considered the

235 *CCL20*-based signature more biologically relevant.

236

237 *Validation of the IL-17 signature*

238 *IL-17 related gene expression confirmed in an additional Airway Epithelial Culture Dataset*

239 We validated the association between the 10 genes selected by elastic net and IL-17 stimulation

240 in another publicly available microarray dataset of HBECs grown at ALI and stimulated with IL-

241 17 for 24 hours (as opposed to the 7 day stimulation in our culture model) (GSE10240) (43).

242 Although two of the 10 genes (*SAA1* and *SAA2*) were poorly annotated on this array and could

243 not be measured, the rest were significantly upregulated after IL-17 stimulation in this validation

244 dataset (7 of 8 were within the top 50 genes by log$_2$ fold change) despite differences in cytokine

245 stimulation time.

246

247 *IL-17 related gene expression measures a response distinct from Type 1 and 2 immune*

248 *responses*

249 Only three of the 11 IL-17 signature genes were significantly altered after HBECs at ALI were

250 stimulated with interferon gamma, the main cytokine released from Th1 and Tc1 cells, and thus

251 indicative of a Type 1 (T1) response. Two of the genes were repressed and one induced with an

252 overall mean log$_2$ fold change of -0.19 (**Table S2**). None of the genes were significantly

253 upregulated in steroid-naïve mild-moderate asthmatics previously shown to have high type 2

254 (T2) gene expression (n=40) compared to asthmatics with low T2 expression (n=22) and

255 healthy controls (n=43) (**Table S3**).

256

257 *Decreased IL-17 signature expression following IL-17 blockade in psoriatic lesions*

258 To further validate that our IL-17 signature reflects an IL-17 response, we examined it in two

259 publicly available transcriptomic datasets of psoriatic skin lesions before and after controlled

260 treatment with anti-IL-17 biologics.

261

262 In the first dataset (GSE31652) (44), psoriatic skin lesion biopsies were taken at baseline and

263 after two weeks of Ixekizumab, an anti-IL-17 monoclonal antibody (n=6), or placebo (n=4). All

264 Ixekizumab-treated participants, but none of the placebo-treated, showed clinical improvement

265 of at least 75% at 6 weeks. The skin IL-17 gene signature decreased over 2 weeks in lesions

266 from Ixekizumab but not placebo-treated participants (p=0.003 for the interaction between

267 treatment and time, **Figure 3A and B**).

268

269 In the second dataset (GSE53552) (45), biopsies were taken from psoriatic skin lesions and

270 matched non-lesional skin at baseline (n=25). Psoriatic lesions were then sampled over 6

271 weeks after treatment with placebo (n=5) or a dose range of Brodalumab (n=20), an IL-17

272 receptor α-blocking monoclonal antibody. Psoriatic lesions showed higher IL-17 signature

273 expression compared to matched non-lesional skin (p=0.001, **Figure 3C-F**). The signature

274 decreased over time in psoriatic lesions in those who received 350 or 700mg compared to

275 placebo, but not in those who received 140mg (350mg: p=0.005 at 1 week, p=0.02 at 2 weeks,

276 and p=0.12 at 6 weeks, 700mg: p=0.002 at 2 weeks and 0.0006 at 6 weeks for the interaction

277 between treatment and time, **Figure 3C-F**). This was consistent with clinical treatment response

278 (all placebo-treated and three of four 140mg-treated participants showed no clinical treatment

279 response, all 700mg-treated and all but one 350mg-treated showed at least 70% clinical

11

280 improvement). The observation that our putative IL-17 signature tracked with clinical response

281 to an IL-17 inhibitor in two psoriasis clinical trials provides independent confirmation of its value

282 as a metric of IL-17 driven inflammation

283

284 *Characterization of the IL-17 signature in COPD transcriptional profiling datasets*

285 *Cross-sectional characterization of an IL-17 gene signature in the BAE dataset*

286 In the BAE dataset, our 11 gene IL-17 signature was higher in former smokers (mean of the

287 zero-centered $\log_2$ gene expression=0.29±0.46) compared to current smokers (-0.42±0.48,

288 $p<2.2*10^{-16}$, **Figure 4A Table S4**), and associated with older age (ρ=0.19, p=0.004). The

289 signature was increased in COPD compared to ever-smokers without COPD (i.e. those with

290 preserved lung function, 0.21±0.66 and -0.12±0.51 respectively, $p=1.34*10^{-5}$), even after

291 adjustment for smoking status and age ($p=6.2*10^{-6}$). The signature was also higher with

292 decreasing lung function (defined as the volume of air exhaled in the first second of a forced

293 expiratory maneuver, or FEV_1). Specifically, a higher gene signature was associated with lower

294 FEV_1 expressed as a percentage of the predicted value (FEV_1% predicted) across all

295 participants (1 unit increase in the IL-17 signature is associated with a 12 ml decrease in FEV_1,

296 $p=1.40*10^{-5}$) and amongst only COPD participants (associated with a 5.5 ml decrease in FEV_1,

297 p= 0.04), suggesting an association with increasing COPD severity (**Figure 4B**).

298

299 *Cross-sectional characterization in GLUCOLD and SPIROMICS*

300 We next studied baseline clinical characteristics associated with the IL-17 signature in

301 GLUCOLD and another COPD dataset, SPIROMICS (demographics in **Table 1**). GLUCOLD

302 included endobronchial biopsy transcriptomic profiles from steroid-naïve participants with

303 moderate to severe COPD (n=79). SPIROMICS included bronchial epithelial brushing profiles

304 from ever smokers with mild to moderate COPD (n=47). Similar to the BAE dataset, in both

305 GLUCOLD and SPIROMICS the IL-17 signature was associated with increasing age (ρ=0.24,

306 p=0.039 and ρ=0.20, p=0.046, respectively) and was higher in former compared to current

307 smokers (p=2.42*10^{-6} and 1.35*10^{-5} respectively, **Table S4**). We performed subsequent

308 analyses before and after adjustment for age and smoking status.

309

310 *Association with increased airway neutrophils and macrophages*

311 In GLUCOLD, the IL-17 signature was associated with increased airway biopsy neutrophil

312 (p=6.41*10^{-5}, **Figure 5A**) and macrophage counts (p=0.009, **Figure 5B**), but not eosinophils,

313 mast cell counts, or our previously described T2 genomic score (**Table 2**). Tissue cell counts

314 and the T2S score were not measured in SPIROMICS, but the T2S score was also not

315 associated with the IL-17 signature in the BAE dataset (**Table 2**). The IL-17 signature was

316 moderately associated with sputum neutrophil counts in both GLUCOLD (p=0.041, **Figure 5C**)

317 and SPIROMICS (p=0.033, **Figure 5D**) although this did not stand up to multiple comparisons

318 adjustment. There was no association with sputum eosinophil counts or any blood cell counts

319 (**Table 2**).

320

321 *Association with airway obstruction*

322 Similar to the BAE dataset, in SPIROMICS we found that a higher IL-17 signature was

323 associated with slightly greater airway obstruction in COPD (p=0.038 after adjustment for

324 smoking and age, **Figure S5, Table 3**), although this was not significant after adjustment for

325 multiple comparisons. In GLUCOLD we found a trend towards an association (p=0.06 before

326 and p=0.12 after adjustment for smoking and age, **Figure S5, Table 3).**

327

328 *Association with CT measurements of functional small airway disease*

329 In SPIROMICS, we obtained inspiratory and expiratory quantitative Chest CT scans at study

330 entry. We found that the IL-17 signature was associated with an increase in air-trapping in areas

331   devoid of emphysema (known as functional small airways disease (PRM^{fsad}) by parametric

332   response mapping (PRM) analysis (p=0.01, **Figure 6A**, **Table 3**) (46).The IL-17 signature was

333   not associated with PRM-measured emphysema (PRM^{emph}). However, almost all participants

334   who underwent bronchoscopy had mild disease with very few displaying significant emphysema

335   (**Figure 6B**).

336

337   *Association with decreased response to inhaled corticosteroids in GLUCOLD*

338   Following baseline bronchoscopy in GLUCOLD, 49 participants with available baseline biopsies

339   were randomized to treatment with 30 months of ICS-containing medication (n=33) or placebo

340   (n=16). A higher baseline IL-17 signature was associated with lack of improvement in post-

341   bronchodilator FEV$_1$ on ICS, whereas a lower IL-17 signature was associated with improvement

342   in FEV$_1$, as compared to placebo (p=0.028 for the interaction between treatment and time,

343   **Figure 7, Table 3**). We identified 28% of GLUCOLD participants as having high IL-17 gene

344   expression ("IL-17 high") by cluster partitioning (31% of COPD participants over all three

345   studies, including 33% of BAE and 34% of SPIROMICS participants, were "IL-17 high", **Figure**

346   **S6**). After categorization of participants based on this cluster partitioning, those with an "IL-17

347   low" designation were more likely to respond to ICS with an improvement in lung function while

348   "IL-17 high" was associated with lack of response to ICS at 30 months (p=0.047 for the

349   interaction between IL-17 status and percent change in FEV$_1$ after ICS compared to placebo).

350   We found that a high IL-17 signature is specific but not sensitive for steroid unresponsiveness.

351   Using the dichotimization into IL-17 high and low by cluster partitioning the specificity for steroid

352   unresponsiveness was 75% (**Table S5**). When the IL-17 high group is restricted to a slightly

353   higher cut-off at the top quartile of IL-17 signature values, the specificity increases to 94%

354   (**Table S6**).

355

14

356 The association between the IL-17 signature and change in FEV_1 amongst ICS-treated

357 participants was not due to those participants with low IL-17 signature expression reciprocally

358 exhibiting high Type 2 inflammation. The significance of the relationship between the IL-17

359 signature and ICS response persisted even after we adjusted for markers of steroid-responsive

360 Type 2 inflammation using either airway tissue eosinophils (p=0.027) or our previously identified

361 airway epithelial genomic signature of Type 2 inflammation (p=0.018, **Figure S7, Table 3**) (6).

362 The association also does not appear to be explained by IL-17 inflammation simply reflecting

363 tissue neutrophils or macrophages as adjustment for neutrophil or macrophage counts in the

364 model also did not change the relationship between the IL-17 signature and ICS response

365 (p=0.016 and 0.030, respectively, **Table 3**).

366

367 The IL-17 signature alone explained 23% of the variation in change in FEV_1 with corticosteroids

368 (r^2=0.23, **Table 3**). As expected given the low sensitivity of the IL-17 signature for steroid

369 unresponsiveness, the Area Under the Receiver Operator Characteristic Curve (AUC) was

370 modest (63%, **Figure S8**). However, there were no significant associations between other

371 biomarkers of inflammation (including sputum and blood cell counts) and change in FEV_1 in ICS

372 versus placebo-treated participants after adjustment for age and smoking status. Furthermore,

373 the AUCs for these other potential biomarkers (sputum eosinophils: 51%, blood eosinophils:

374 55%, sputum neutrophils: 52%, blood neutrophils: 45%) suggest that they lack any predictive

375 power for corticosteroid responsiveness in this dataset (supplemental **Figure S8**). Although

376 limited by small sample size, these proof-of-concept analyses suggest that our airway epithelial

377 signature of IL-17 response in COPD may mark FEV_1 response to ICS better than easily

378 measured cell differentials or other genomic markers of the adaptive immune response.

379

380

381

Discussion

382

383 In this study, we used three complementary human COPD studies to characterize the clinical

384 significance of the airway epithelial response to IL-17 in COPD. We showed that a signature of

385 IL-17 associated airway inflammation is upregulated in a subset of participants with COPD (31%

386 across studies), and is associated with distinct inflammatory, physiologic, and clinical features.

387 Increases in this signature are associated with an inflammatory profile characteristic of an IL-17

388 response, including increased airway neutrophils and macrophages but not eosinophils, Type 2

389 markers, or Type 1 gene expression. Decreases in the signature occur in response to

390 therapeutic blockade of IL-17 in psoriatic skin lesions, and this response corresponds to clinical

391 improvement in that disease. In COPD, the signature is further associated with more severe

392 airway obstruction and a novel CT biomarker of functional small airway disease that is predictive

393 of worsening airway disease over time (46, 47). Finally, higher IL-17 signature expression is

394 associated with a lack of response to ICS in COPD, whereas low expression may identify those

395 patients who benefit from ICS. This association does not simply appear to be due to reciprocal

396 alterations in Type 2 inflammation as the interaction between our IL-17 signature and treatment

397 was unaffected by adjustments for airway eosinophils or our Type 2 airway genomic signature.

398 Thus, our findings suggest that enhanced IL-17 inflammation characterizes a distinct subset of

399 COPD, and that identifying this subgroup may be important for therapeutic decisions.

400

401 In COPD, chronic exposure to smoking, microbial insults, and recurrent mucosal injury may all

402 contribute to immune activation with IL-17A producing T cells, supported by innate IL-17A

403 producing cells (17). This likely contributes to ongoing neutrophilic inflammation and

404 macrophage recruitment with subsequent airway remodeling and tissue destruction (48). We

405 found that our IL-17 gene expression signature is associated with increases in airway

406 neutrophils and macrophages, indicating an IL-17 response. It is related to worse clinical

407   outcomes across former and current smokers. These findings provide evidence for the

408   contribution of IL-17 inflammation to COPD pathology despite smoking cessation.

409

410   We found that the IL-17 response in COPD is heterogeneous, enhanced in a subgroup. Prior

411   studies found variability in IL-17-related inflammation within COPD (13, 25-30), and our data

412   suggest that this variability is clinically significant. Other studies have identified some

413   characteristics of IL-17 associated inflammation in COPD including more severe obstruction,

414   emphysema, and lymphoid neogenesis (13, 15). Here we comprehensively investigated the

415   associations between IL-17 driven inflammation and COPD patient characteristics. In addition to

416   an association with increased airway obstruction, we found associations with a novel CT

417   biomarker of functional small airways disease and corticosteroid unresponsiveness. COPD

418   phenotypes are heterogeneous and complex. Thus we hypothesize that multiple overlapping

419   molecular phenotypes underlie the complex clinical phenotypes we observe in chronic airway

420   diseases and that there will be an upper bound to the predictive power of any one biological

421   pathway (33, 49). However, a strength here is that we observe correlations that are reproducible

422   across our transcriptional datasets (for associations with neutrophils and FEV_1).

423

424   We had hypothesized that the IL-17 signature would be associated with increased emphysema,

425   as found in a previous study (13). We evaluated this using the recently developed PRM CT

426   analysis method (46). By matching inspiratory and expiratory scans, PRM improves the ability to

427   distinguish emphysema from functional small airway disease, both of which are associated with

428   low radio-density lung regions on expiration (i.e. air trapping). Our IL-17 signature is associated

429   with PRM[fsad] but not PRM[emph] in SPIROMICS. As the participants generally had mild to

430   moderate disease with minimal emphysema, the lack of association with PRM[emph] is not

431   surprising. The association with PRM[fsad] is of interest as the small airways are likely the main

432   site of airway inflammation in COPD, and small airway disease is thought to precede

17

433   emphysema (50). Studies using PRM have supported these findings. PRM^{fsad} is associated with

434   more rapid FEV$_1$ decline, particularly in mild to moderate disease (47). PRM^{fsad} is also the

435   greater contributor to radiographic abnormalities in mild to moderate COPD with both PRM^{fsad}

436   and PRM^{emph} contributing in severe disease (46). Thus, an association between our IL-17

437   signature and PRM^{fsad} in mild to moderate COPD does not preclude an association with

438   emphysema in more severe disease. In fact, it signifies an association with a more severe

439   phenotype amongst participants with milder airway obstruction and suggests that IL-17 related

440   inflammation may be a pathway on which to intervene to prevent the progression to emphysema

441   and severe airway obstruction.

442

443   Our IL-17 signature, when measured at baseline, is associated with a poor lung function

444   response to corticosteroids at 30 months. This corticosteroid responsiveness is not simply due

445   to participants with low IL-17 signature expression exhibiting low neutrophil counts or

446   reciprocally exhibiting high Type 2 inflammation. In murine models, Th-17 cell-mediated airway

447   inflammation has been shown to be corticosteroid resistant, in contrast to Th2 cell-mediated

448   inflammation (51). Here we show the association between an IL-17 inflammatory signature and

449   corticosteroid unresponsiveness for the first time in a longitudinal randomized controlled trial in

450   humans. Many patients with COPD do not respond to corticosteroids, and ICS are only

451   indicated in exacerbation-prone symptomatic COPD. However, corticosteroids are still used

452   broadly despite possible increases in adverse outcomes such as pneumonia (52). The

453   corticosteroid unresponsiveness finding suggests that a more easily measurable surrogate for

454   our IL-17 signature could serve as a biomarker for therapeutics in COPD. While it may be useful

455   to predict who will not respond to corticosteroids, it may be even more useful to predict who will

456   respond to therapies targeting IL-17 or associated inflammatory pathways as we found in

457   psoriatic lesions.

458

459　Our study relied on the airway epithelial gene expression response to IL-17, where the cytokine

460　induces a major effect, and the first line of defense against injury to the lung. Other studies have

461　relied on cell counts or immunoreactivity which are poorly correlated in the human lung (13).

462　Additionally, Th17 cells display a high level of plasticity, and are thus more unstable than Th1 or

463　Th2 cells (53), suggesting cell numbers may not represent cytokine response. Data is also

464　conflicting on whether IL-17+ cell counts are elevated in COPD and related to key pathologic

465　characteristics such as airway neutrophilia (28, 29). We, however, show that IL-17 signature

466　genes are not only upregulated in two separate experiments in which HBE cells were stimulated

467　with IL-17, but that our signature is associated with increases in airway neutrophils as well. We

468　also show that our signature is decreased in response to IL-17 blocking agents in psoriatic skin

469　lesions but distinct from airway epithelial Type 1 and 2 responses, further indicating that we are

470　marking an IL-17 specific epithelial response.

471

472　We acknowledge that fitting our IL-17 signature to *CCL20* could have limited its generalizability.

473　However, the signature generalized well in that: 1) it was highly correlated with two other IL-17

474　gene signatures (a signature previously studied in asthma (39) and a signature fit to *SLC26A4*,

475　the most significantly upregulated gene in our IL-17 stimulated HBE culture experiments) and 2)

476　our IL-17 signature was responsive to anti-IL17 therapy and reflective of clinical response in 2

477　randomized controlled trials in psoriasis. The advantage of fitting this gene signature to *CCL20*

478　is that it improved its "fit" to a more complex in vivo tissue environment rather than a simple cell

479　culture model. In a COPD patient this complex environment may be further compounded by

480　multiple airway insults (e.g. smoking, microbial colonization, exacerbations, medications) that

481　are not modelled well in culture. By retaining only tightly inter-correlated genes, a well-

482　established method for identifying genes in the same molecular pathway (36, 37), we removed

483　those genes that may be non-specific to an IL-17 response in vivo.

484

485 Our study has some potential limitations. For instance, some analyses were cross-sectional,

486 and those analyses can only show associations, not causality. Our longitudinal analyses were

487 limited by sample size. Thus, while we did find a strong association between our IL-17 signature

488 and lack of response to inhaled steroids over 30 months, an assessment of the predictive power

489 of the signature for corticosteroid responsiveness was quite limited. Furthermore, our definitions

490 of "high" and "low" for the IL-17 signature are highly dependent on the population in which they

491 were developed. Therefore, further studies will be needed to determine if the signature could be

492 used as a biomarker for steroid unresponsiveness, and to determine the best cut-off for IL-17

493 "high" and "low". We were also not powered to study the association between the signature and

494 exacerbation rates, which will be important to study in relation to therapeutic response. It was

495 not within the scope of this paper to identify the cause of the increased IL-17 response. We do,

496 however, see associations in current and former smokers, suggesting that more than just smoke

497 exposure is playing a role. The contributions of stimuli, such as alterations in the microbiome or

498 autoimmunity, to enhanced IL-17 related gene expression will require further study.

499 Furthermore, COPD phenotypes are heterogeneous and complex, and thus we hypothesize that

500 multiple overlapping molecular phenotypes underlie the complex clinical phenotypes we

501 observe in chronic airway diseases. Finally, future work will be needed to identify surrogate

502 biomarkers in more easily obtained specimens than airway brushings. This is similar to the

503 approach we took in our asthma studies in which we initially identified a Type 2 high asthma

504 molecular phenotype based on airway gene expression, and then expanded this work to identify

505 the best associated biomarkers (periostin, eosinophils, FeNO).

506

507 In summary, we show here that a signature of IL-17 associated airway inflammation is

508 upregulated in approximately a third of COPD participants and is associated with distinct

509 inflammatory, physiologic, and clinical features. Our findings suggest that the IL-17 signature

510 defines a molecular COPD phenotype that responds poorly to corticosteroid therapy, and which

511 could instead be the target of emerging therapies that interfere with IL-17 (44, 45, 48).

512

513

514

515

516

517

518

519

520

521

522

523

524

525

526

527

528

529

530

531

532

533

534

535

Materials and Methods

Transcriptomic Datasets:

Eight transcriptomic datasets were used for these analyses.

1. ***UCSF Human bronchial epithelial cell (HBEC) culture dataset:***

    Human bronchial epithelial cells obtained from the proximal airways of 6 lung donors

    rejected for transplant (5 without airway disease, 1 with asthma) were grown to confluence

    in an air-liquid interface culture (ALI) for 28 days as described previously (54). Some

    cultures were stimulated with IL-17A (10 ng/mL) for the final 7 days of culture or interferon

    gamma (IFNγ, 10 ng/mL) for the final 24 hours of culture. Matched cultures maintained in

    media without cytokine over the same time period were used as controls. Cultured cells

    were then harvested and underwent RNA isolation using the Qiagen miRNeasy kit (Qiagen

    Inc., Valencia, CA) as per manufacturer's protocol. RNA quality and quantity were assessed

    using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the

    NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Library

    preparation and multiplexing were done using the Illumina TruSeq Stranded Total RNA with

    Ribo-zero Human/Mouse/Rat kit (Illumina Inc, San Diego, CA) as per manufacturer's

    protocol at the UCSF Sandler Genomics Core Facility. 100 base pair paired-end sequencing

    was done on multiplexed samples via the Illumina HiSeq 2500 at the UCSF Genomics Core.

2. ***Bronchial Airway Epithelial (BAE) dataset****:* Bronchial epithelial brushings obtained from

    6th-8th generation bronchi of former and current smokers with a range of lung function

    (COPD=85, no COPD=152) were previously profiled by Affymetrix (Santa Clara, CA) HG 1.0

    ST Arrays (55). Spirometry was done in all participants. Raw microarray files may be

    downloaded from the Gene Expression Omnibus (GEO, accession: GSE37147) (54).

    Inclusion/exclusion criteria were previously published.

561

3. **Validation HBEC culture dataset:** Data were downloaded from GEO (GSE10240). Primary HBE cells provided by the Tissue Core Laboratory at the University of Pittsburgh or purchased from Cambrex (Lonza) were grown to confluence in ALI then stimulated apically and basolaterally with media control or IL-17A for 24 hours (3 replicates each) as previously described (43). Isolated RNA was profiled by Affymetrix HG U133A 2.0 Arrays.

4. *Asthma Dataset:* Bronchial airway epithelial brushings obtained by bronchoscopy from steroid-naive subjects with mild to moderate asthma (n = 62) and control subjects without asthma (n = 43) were previously profiled by Affymetrix HG U133 plus 2.0 Arrays (GSE67472) (24). Inclusion/exclusion criteria for this study were previously published (24). Subjects with asthma were divided into Type 2-high and -low subgroups (n = 40 and 22, respectively) using a validated standardized mean expression level of *POSTN*, *SERPINB2*, and *CLCA1* (24, 32). IL-17 associated genes were evaluated amongst those differentially expressed between Type 2-high asthma compared to Type 2-low asthma and healthy controls.

5. *Ixekizumab Psoriasis Dataset:* Data were downloaded from GEO (GSE31652). Biopsies of psoriatic skin lesions were taken at baseline and after treatment with two weeks of Ixekizumab (n=6) or placebo (n=4) and previously profiled by Affymetrix HG U133A 2.0 Arrays (44).

6. *Brodalumab Psoriasis Dataset:* Data were downloaded from GEO (GSE53552). Biopsies were taken from psoriatic skin lesions and matched non-lesional skin from 25 participants at baseline. The psoriatic lesions were then sampled over 6 weeks after treatment with

23

586   placebo (n=5) or a dose range of Brodalumab (140mg n=4, 350mg n=8, 700mg n=8). All

587   samples were previously profiled by Affymetrix HG U133 plus 2.0 Arrays (45).

588

589   **7.**   ***Gronigen and Leiden Universities study of Corticosteroids in Obstructive Lung***

590   ***Disease (GLUCOLD) dataset****:* Endobronchial biopsies from steroid-naïve participants with

591   moderate to severe COPD (n=79) were previously obtained by bronchoscopy and profiled

592   by Affymetrix HG 1.0 ST Arrays (GSE36221) (56). Blood collection, sputum induction, and

593   spirometry were done at the first study visit via previously described methods (57). A subset

594   of these participants was randomized to receive 30 months of placebo (n=16) or ICS with or

595   without long acting beta agonist (salmeterol, LABA) (n=33). Inclusion/exclusion criteria were

596   previously published (57).

597

598   8.   ***Subpopulations and InteRmediate Outcome Measures In COPD Study (SPIROMICS)***

599   ***dataset****:* A subgroup of participants in the SPIROMICS multi-center observational cohort

600   study underwent research bronchoscopy. RNA was obtained from bronchial epithelial

601   brushings from 3rd-4th generation bronchi of the right or left lower lobe of current and former

602   smokers with mild to moderate COPD (n= 47). RNA was used for profiling IL-17-associated

603   gene expression by two-step, nested-primer RT-qPCR as described previously (32). Primer

604   and probe sequences are listed in **Table S4**.

605

606   At least a 20 pack-year smoking history was required for inclusion, and participants were

607   classified as former smokers after one year of smoking cessation. Participants were

608   classified as having COPD based on spirometry, performed before and after four inhalations

609   each of albuterol (90µg dose per inhalation) and ipratropium (18µg dose per inhalation),

610   using the GOLD staging system (58). Full inclusion/exclusion criteria are included in **Table**

611   **S5**.

612

613   Blood collection, sputum induction, and CT scans were done at the first study visit. Sputum

614   induction was performed as previously described (59). Parametric response mapping (PRM)

615   of CT imaging was used to distinguish areas of normal lung (PRM^{norm}) from areas of

616   functional small airways disease (PRM^{fsad}) and emphysema (PRM^{emph}) as previously

617   described (46, 47). Briefly, PRM is a CT voxel-based imaging biomarker that utilizes

618   dynamic image registration to spacially align paired inspiratory and expiratory scans.

619   PRM^{fsad} is defined as areas of lung that are >-950 Hounsfield Units (HU) on inspiration and

620   <-856 HU on expiration. PRM^{emph} is defined as areas of lung that are <-950 HU on

621   inspiration and <-856 HU on expiration. PRM^{norm} is defined as areas of lung exceeding both

622   thresholds on inspiration and expiration.

623

624   ***Derivation of gene expression datasets:***

625   ***RNA-Seq (HBEC culture dataset):***

626   .fastq files were quality filtered and aligned to the human genome using STAR and the

627   ENSEMBL GRCh38 genome build (60, 61). Read counts were normalized and differential

628   expression analyses on matched samples were performed between 1) IL-17A stimulated

629   samples and controls, and 2) IFNγ-stimulated samples and controls using the DESeq2

630   package in R (62). Differential expression in DESeq2 is carried out using generalized linear

631   models following a negative binomial distribution. Results were trimmed to transcripts

632   indexed in the HGNC database and with a Ensembl gene biotype label of "protein_coding".

633   Multiple comparisons corrections were done using False Discovery Rate by the Benjamini-

634   Hochberg method (63).

635

636   ***Microarray (BAE, Asthma, GLUCOLD, Ixekizumab, and Brodalumab datasets):***

637   Each microarray dataset independently underwent background adjustment (without the use

638   of mismatch probes), quantile normalization, and probe summarization using the RMA

639   algorithm (affy package, Bioconductor, R) (64, 65). Entrez gene custom chip definition files

640   available for the appropriate microarray for each dataset at

641   http://brainarray.mbni.med.umich.edu were used for annotation. Batch effect was minimized

642   using Combat when appropriate (66).

643

644   ***qPCR (SPIROMICS dataset):***

645   Data were normalized to the mean of *PPIA*, *RPL13A*, *ACTB*, and *DNAJA1*, determined using

646   the SLqPCR package in R, as described previously (32, 67).

647

648   ***Derivation of the IL-17 genomic signature***

649 An IL-17 genomic signature specific to bronchial epithelial brushings from smokers was

650 generated using elastic net regression for feature selection in the BAE dataset. The 100 genes

651 most up-regulated in ALI models after IL-17A stimulation were used as candidate predictor

652 variables ("features"). Genes highly correlated with a representative IL-17 gene, *CCL20*, were

653 selected as features for inclusion into the IL-17 signature using elastic net regression via the

654 glmnet package in R with alpha=0.75 and leave-one-out cross-validation (68). Alpha was

655 selected at just below one to maximize sparsity (and thus limit feature selection) while allowing

656 for selection of closely correlated genes. *CCL20* and the 10 genes selected by elastic net

657 regression were used for generation of the IL-17 signature. The mean of the zero-centered log$_2$-

658 scale gene expression values of these 11 genes was used as the IL-17 airway epithelial

659 signature metric, a previously validated method (33, 39). To confirm that our IL-17 signature

660 was not just specific to *CCL20*, two alternative IL-17 signatures were generated. One was

661 generated using the above procedure with *SLC26A4*, the most upregulated gene following IL-17

662 stimulation in cell culture also measured in the COPD array data, guiding the elastic net. The

663 other was an IL-17 signature previously studied in asthma and was generated in the same way

664 as previously reported, using the mean value of the zero-centered gene expression of five IL-17

665 associated genes (39).

666

667 For the Ixekizumab and Brodalumab studies, four genes were excluded prior to deriving the IL-

668 17 signature metric: two genes that were poorly annotated in the microarray platform used

669 (*SAA1*, *SAA2*), and two genes that were not expressed above background (*CSF3*, *MTNR1A1*)

670 in these skin biopsies. As there was 100% concordance between the two psoriasis studies on

671 genes not expressed above background, we concluded that these genes were poorly expressed

672 in the resident skin cells. We did not, however, change the signature in any way based on

673 knowledge of the genes or relevance in psoriasis. The IL-17 skin signature was thus derived

674 using the mean value of the zero-centered $\log_2$-scale gene expression values of the remaining

675 seven genes (*CCL20*, *SLC26A4*, *TNIP3*, *CXCL3*, *CXCL5*, *CXCL6*, and *VNN1*).

676

677 ***Statistical analyses of the IL-17 genomic signature***

678 All regression analyses were performed using the limma package in R (69). For cross-sectional

679 analyses of the associations between the IL-17 signature and clinical variables (in the BAE,

680 GLUCOLD, and SPIROMICS datasets) linear or logistic regression were used, as appropriate.

681 Analyses were done before and after adjustment for age and smoking status. Race, gender, pack-

682 years, and inhaled corticosteroid use were evaluated as potential confounders as well. These

683 variables were, however, left out of the final models as they were not significantly associated with

684 IL-17 signature expression, and did not significantly alter the relationships between the IL-17

685 signature and outcomes beyond adjustments for age and smoking status. Data was transformed

686 when necessary for normal distribution. A P value less than 0.05 was considered significant.

687 However, multiple hypothesis testing was done using a false discovery rate when appropriate

688 (63). For the Ixekizumab and Brodalumab studies mixed effects models were used to relate the

689   IL-17 signature (as the outcome variable) to the interaction between treatment and time (fixed

690   effects) across participants (random effect).   For longitudinal analyses in GLUCOLD the

691   interaction between treatment (ICS or placebo) and the baseline IL-17 signature was related to

692   change in FEV_1 over 30 months. The ICS and ICS + long acting beta agonist groups were

693   combined to improve power as the long-term effects in these groups were comparable. In

694   secondary analyses, the interactions between the IL-17 score and A) tissue eosinophils, B) our

695   previously generated metric of Type 2 inflammation (the T2S score), or C) tissue neutrophils were

696   related to change in FEV_1 over 30 months amongst those GLUCOLD participants that received

697   ICS (6).

698

699   ***Clustering***

700   All clustering analyses were performed using euclidean distance with average linkage as the

701   distance metric. The NbClust package (R, bioconductor) was used to determine the best

702   participant clustering of the IL-17 signature genes, based on a majority vote of 30 indices that

703   evaluate partitioning (70). NbClust deals with the inherent variability in the many indices

704   available to determine the optimal number of clusters by requiring a consensus vote amongst

705   these indices on best partitioning. Participants with relatively high expression who clustered

706   separately from the majority of participants were considered "IL-17 High". Prior to determining

707   the best number of partitions the datasets were first stratified by smoking status given the large

708   effect of smoking on gene expression. Differences amongst indices in deciding best clustering

709   were generally due to separation of those with "IL-17 High" expression into one or more

710   categories, while those with low expression clustered together. The exception were two

711   participants in the SPIROMICS dataset with low expression that were partitioned into their own

712   groups. Six of 35 participants with relatively high IL-17 gene expression in the BAE dataset, 3 of

713   23 participants in the GLUCOLD dataset, and 5 of 18 participants in the SPIROMICS dataset

714 were partitioned out as the highest for IL-17 expression. For simplicity, all IL-17 high, including

715 these highest participants, were grouped together.

716

717 The IL-17 signature was then discretized into two categories: "IL-17 high" and "IL-17 low" using

718 two different methods to use as a categorical predictor for longitudinal analyses in GLUCOLD.

719 First, discretization was based on the best partitioning decided by NbClust, and then,

720 alternatively based on the top quartile of signature expression. Ten percent of samples with IL-

721 17 signatures closest to the partition were removed prior to discretization to diminish overlap.

722

723 ***Study Approval***

724 The included human studies were all approved by the institutional review boards at the

725 institutions involved in sample and data collection. All participants provided written informed

726 consent prior to inclusion in the study.

727

728

729

730

731

732

733

734

735

736

737

738

739

740

741   **Author Contributions:** SAC and PW contributed to the conceptualization of the study. LRB,

742   LTZ, and DJE carried out the HBEC culture experiments. SAC, MvdB, IZB, RGB, ERB, RCB,

743   RPB, APC, JLC, MKH, NNH, PSH, RJK, JAK, FJM, WKO, RP, WT, JMW, AS, and PGW were

744   involved in data collection and generation. SAC, MvdB, AF, KI, NB, and PGW contributed to

745   data analysis. All authors participated in critical manuscript writing and editing.

746

747

748

749

750

751

752

753

754

755

756

757

758

759

760

761

762

763

764

765

802

803

804

805

806

807

808

809

810

811

812

813

814

815

816

817

818 **References**

819 1. Lotvall J, et al. Asthma endotypes: A new approach to classification of disease entities within

820 the asthma syndrome. *J Allergy Clin Immunol.* 2011;127(2):355-360.

821 2. Woodruff PG, Agusti A, Roche N, Singh D, Martinez FJ. Current concepts in targeting chronic

822 obstructive pulmonary disease pharmacotherapy: Making progress towards personalized

823 management. *Lancet.* 2015;385(9979):1789-1798.

824 3. Shrimanker R, Pavord ID. Interleukin-5 inhibitors for severe asthma: Rationale and future

825 outlook. *BioDrugs.* 2017;31(2):93-103.

826 4. Pavord ID, et al. Mepolizumab for severe eosinophilic asthma (DREAM): A multicentre,

827 double-blind, placebo-controlled trial. *Lancet.* 2012;380(9842):651-659.

828 5. Adeloye D, et al. Global and regional estimates of COPD prevalence: Systematic review and

829 meta-analysis. *J Glob Health.* 2015;5(2):020415.

830 6. Christenson SA, et al. Asthma-COPD overlap. clinical relevance of genomic signatures of

831 type 2 inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.*

832 2015;191(7):758-766.

833 7. Brightling CE, et al. Sputum eosinophilia and short-term response to prednisolone in chronic

834 obstructive pulmonary disease: A randomised controlled trial. *Lancet.* 2000;356(9240):1480-

835 1485.

836 8. Pascoe S, Locantore N, Dransfield MT, Barnes NC, Pavord ID. Blood eosinophil counts,

837 exacerbations, and response to the addition of inhaled fluticasone furoate to vilanterol in

838 patients with chronic obstructive pulmonary disease: A secondary analysis of data from two

839 parallel randomised controlled trials. *Lancet Respir Med.* 2015;3(6):435-442.

840 9. Pavord ID, et al. Mepolizumab for eosinophilic chronic obstructive pulmonary disease. *N Engl*

841 *J Med.* 2017;377(17):1613-1629.

842 10. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the

843 expanding diversity of effector T cell lineages. *Annu Rev Immunol.* 2007;25:821-852.

844 11. Pridgeon C, et al. Regulation of IL-17 in chronic inflammation in the human lung. *Clin Sci*

845 *(Lond).* 2011;120(12):515-524.

846 12. Roos AB, et al. IL-17A and the promotion of neutrophilia in acute exacerbation of chronic

847 obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2015;192(4):428-437.

848 13. Roos AB, Sanden C, Mori M, Bjermer L, Stampfli MR, Erjefalt JS. IL-17A is elevated in end-

849 stage chronic obstructive pulmonary disease and contributes to cigarette smoke-induced

850 lymphoid neogenesis. *Am J Respir Crit Care Med.* 2015;191(11):1232-1241.

851 14. Yadava K, et al. Microbiota promotes chronic pulmonary inflammation by enhancing IL-17A

852 and autoantibodies. *Am J Respir Crit Care Med.* 2016;193(9):975-987.

853 15. Shan M, et al. Cigarette smoke induction of osteopontin (SPP1) mediates T(H)17

854 inflammation in human and experimental emphysema. *Sci Transl Med.* 2012;4(117):117ra9.

855 16. Kheradmand F, Shan M, Xu C, Corry DB. Autoimmunity in chronic obstructive pulmonary

856 disease: Clinical and experimental evidence. *Expert Rev Clin Immunol.* 2012;8(3):285-292.

857 17. Vanaudenaerde BM, et al. Innate and adaptive interleukin-17-producing lymphocytes in

858 chronic inflammatory lung disorders. *Am J Respir Crit Care Med.* 2011;183(8):977-986.

859   18. Zijlstra GJ, Ten Hacken NH, Hoffmann RF, van Oosterhout AJ, Heijink IH. Interleukin-17A

860   induces glucocorticoid insensitivity in human bronchial epithelial cells. *Eur Respir J.*

861   2012;39(2):439-445.

862   19. Chen Y, Thai P, Zhao YH, Ho YS, DeSouza MM, Wu R. Stimulation of airway mucin gene

863   expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem.*

864   2003;278(19):17036-17043.

865   20. Ritchlin CT, Krueger JG. New therapies for psoriasis and psoriatic arthritis. *Curr Opin*

866   *Rheumatol.* 2016;28(3):204-210.

867   21. Barnes PJ. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nature*

868   *reviews. Nat Rev Drug Discov.* 2013;12(7):543.

869   22. Durham AL, Caramori G, Chung KF, Adcock IM. Targeted anti-inflammatory therapeutics in

870   asthma and chronic obstructive lung disease. *Transl Res.* 2016;167(1):192-203.

871   23. Wanda K O'Neal, et al. Comparison of serum, EDTA plasma and P100 plasma for luminex-

872   based biomarker multiplex assays in patients with chronic obstructive pulmonary disease in the

873   SPIROMICS study. *J Transl Med.* 2014;12(1):9.

874   24. Woodruff PG, et al. T-helper type 2-driven inflammation defines major subphenotypes of

875   asthma. *Am J Respir Crit Care Med.* 2009;180(5):388-395.

876   25. Eustace A, Smyth LJ, Mitchell L, Williamson K, Plumb J, Singh D. Identification of cells

877   expressing IL-17A and IL-17F in the lungs of patients with COPD. *Chest.* 2011;139(5):1089-

878   1100.

879   26. Vargas-Rojas MI, Ramirez-Venegas A, Limon-Camacho L, Ochoa L, Hernandez-Zenteno R,

880   Sansores RH. Increase of Th17 cells in peripheral blood of patients with chronic obstructive

881   pulmonary disease. *Respir Med.* 2011;105(11):1648-1654.

882   27. Chang Y, et al. CD8 positive T cells express IL-17 in patients with chronic obstructive

883   pulmonary disease. *Respir Res.* 2011;12:43.

884   28. Di Stefano A, et al. T helper type 17-related cytokine expression is increased in the

885   bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol.*

886   2009;157(2):316-324.

887   29. Doe C, et al. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in

888   asthma and COPD. *Chest.* 2010;138(5):1140-1147.

889   30. Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway

890   hyperresponsiveness to methacholine. *Respir Med.* 2003; 97(6): 726-733.

891   31. Woodruff PG, et al. Genome-wide profiling identifies epithelial cell genes associated with

892   asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A.* 2007;

893   104(40): 15858-15863.

894   32. Bhakta NR, et al. A qPCR-based metric of Th2 airway inflammation in asthma. *Clin Transl*

895   *Allergy.* 2013;3(1):24.

896   33. Bhakta NR, et al. IFN-stimulated gene expression, type 2 inflammation, and endoplasmic

897   reticulum stress in asthma. *Am J Respir Crit Care Med.* 2018;197(3):313-324.

898   34. Sokolov A, Carlin DE, Paull EO, Baertsch R, Stuart JM. Pathway-based genomics prediction

899   using generalized elastic net. *PLoS Comput Biol.* 2016;12(3):e1004790.

900   35. Hughey JJ, Butte AJ. Robust meta-analysis of gene expression using the elastic net.

901   *Nucleic Acids Res.* 2015;43(12):e79.

902   36. Langfelder P, Horvath S. WGCNA: An R package for weighted correlation network analysis.

903   *BMC Bioinformatics.* 2008;9(1):559.

904   37. Tseng GC, Wong WH. Tight clustering: A resampling-based approach for identifying stable

905   and tight patterns in data. *Biometrics.* 2005;61(1):10-16.

906   38. Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-

907   mediated effector immunity. *J Allergy Clin Immunol.* 2015;135(3):626-635.

908   39. Choy DF, et al. TH2 and TH17 inflammatory pathways are reciprocally regulated in asthma.

909   *Sci Transl Med.* 2015;7(301):301ra129.

910   40. Al-Alwan LA, et al. Differential roles of CXCL2 and CXCL3 and their receptors in regulating

911   normal and asthmatic airway smooth muscle cell migration. *J Immunol.* 2013;191(5):2731-2741.

912   41. Anthony D, et al. Serum amyloid A promotes lung neutrophilia by increasing IL-17A levels in

913   the mucosa and gammadelta T cells. *Am J Respir Crit Care Med.* 2013;188(2):179-186.

914   42. Traves SL, Donnelly LE. Th17 cells in airway diseases. *Curr Mol Med.* 2008;8(5):416-426.

915   43. Kreindler JL, et al. IL-22 mediates mucosal host defense against gram-negative bacterial

916   pneumonia. *Nat Med.* 2008;14(3):275-281.

917   44. Krueger JG, et al. IL-17A is essential for cell activation and inflammatory gene circuits in

918   subjects with psoriasis. *J Allergy Clin Immunol.* 2012;130(1):154.e9.

919   45. Russell CB, et al. Gene expression profiles normalized in psoriatic skin by treatment with

920   brodalumab, a human anti-IL-17 receptor monoclonal antibody. *J Immunol.* 2014;192(8):3828-

921   3836.

922   46. Galbán CJ, et al. Computed tomography-based biomarker provides unique signature for

923   diagnosis of COPD phenotypes and disease progression. *Nat Med.* 2012;18(11):1711.

924   47. Bhatt SP, et al. Association between functional small airway disease and FEV1 decline in

925   chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2016;194(2):178.

926   48. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev*

927   *Immunol.* 2008;8(3):183-192.

928   49. Peters MC, et al. Plasma interleukin-6 concentrations, metabolic dysfunction, and asthma

929   severity: A cross-sectional analysis of two cohorts. *Lancet Respir Med.* 2016;4(7):574-584.

930   50. McDonough JE, et al. Small-airway obstruction and emphysema in chronic obstructive

931   pulmonary disease. *N Engl J Med.* 2011;365(17):1567-1575.

932   51. McKinley L, et al. TH17 cells mediate steroid-resistant airway inflammation and airway

933   hyperresponsiveness in mice. *J Immunol.* 2008;181(6):4089-4097.

934   52. Horita N, et al. Long-acting muscarinic antagonist (LAMA) plus long-acting beta-agonist

935   (LABA) versus LABA plus inhaled corticosteroid (ICS) for stable chronic obstructive pulmonary

936   disease (COPD). *Cochrane Database Syst Rev.* 2017;2:CD012066.

937   53. Muranski P, Restifo NP. Essentials of Th17 cell commitment and plasticity. Blood. 2013;

938   841 121(13): 2402-2414.

939   54. Bonser LR, Zlock L, Finkbeiner W, Erle DJ. Epithelial tethering of MUC5AC-rich mucus

940   impairs mucociliary transport in asthma. *J Clin Invest.* 2016;126(6):2367-2371.

941   55. Steiling K, et al. A dynamic bronchial airway gene expression signature of COPD and lung

942   function impairment. *Am J Respir Crit Care Med.* 2013;187(9):944-942.

943   56. van den Berge M, et al. Airway gene expression in COPD is dynamic with inhaled

944   corticosteroid treatment and reflects biological pathways associated with disease activity.

945   *Thorax.* 2013;69(1):14-23.

946   57. Lapperre TS, et al. Effect of fluticasone with and without salmeterol on pulmonary outcomes

947   in chronic obstructive pulmonary disease: A randomized trial. *Ann Intern Med.* 2009;151(8):517-

948   527.

949   58. Global strategy for the diagnosis, management, and prevention of chronic obstructive

950   pulmonary disease [homepage on the Internet]. http://www.goldcopd .org. Updated: 2016.

951   Accessed February 24, 2017.

952   59. Alexis N, Soukup J, Ghio A, Becker S. Sputum phagocytes from healthy individuals are

953   functional and activated: A flow cytometric comparison with cells in bronchoalveolar lavage and

954   peripheral blood. *Clin Immunol.* 2000;97(1):21-32.

955   60. Dobin A, et al. STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013;29(1):15-21.

956   61. Kroll KW, et al. Quality control for RNA-seq (QuaCRS): An integrated quality control

957   pipeline. *Cancer Inform.* 2014;2014(Suppl. 3):7-14.

958   62. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-

959   seq data with DESeq2. *Genome Biol.* 2014;15(12):550.

960   63. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful

961   approach to multiple testing. *J R Statist Soc B.* 1995;57:289-300.

962   64. Gentleman RC, et al. Bioconductor: Open software development for computational biology

963   and bioinformatics. *Genome Biol.* 2004;5(10):R80.

964   65. Irizarry RA, et al. Exploration, normalization, and summaries of high density oligonucleotide

965   array probe level data. *Biostatistics.* 2003;4(2):249-264.

966   66. Kitchen RR, et al. Correcting for intra-experiment variation in illumina BeadChip data is

967   necessary to generate robust gene-expression profiles. *BMC Genomics.* 2010;11(1):134.

968   67. Vandesompele J, et al. Accurate normalization of real-time quantitative RT-PCR data by

969   geometric averaging of multiple internal control genes. *Genome Biol.*

970   2002;3(7):RESEARCH0034

971   68. Friedman J, Hastie T, Tibshirani R. Regularization paths for generalized linear models via

972   coordinate descent. *J Stat Softw.* 2010;33(1):1-22.

973   69. Ritchie ME, et al. Limma powers differential expression analyses for RNA-sequencing and

974   microarray studies. *Nucleic Acids Res*, 2015;43(7):e47.

975   70. Charrad M, Ghazzali N, Boiteau V, Niknafs A. NbClust: An R package for determining the

976   relevant number of clusters in a data set. *J Stat Soft.* 2014;61(6):1-36.
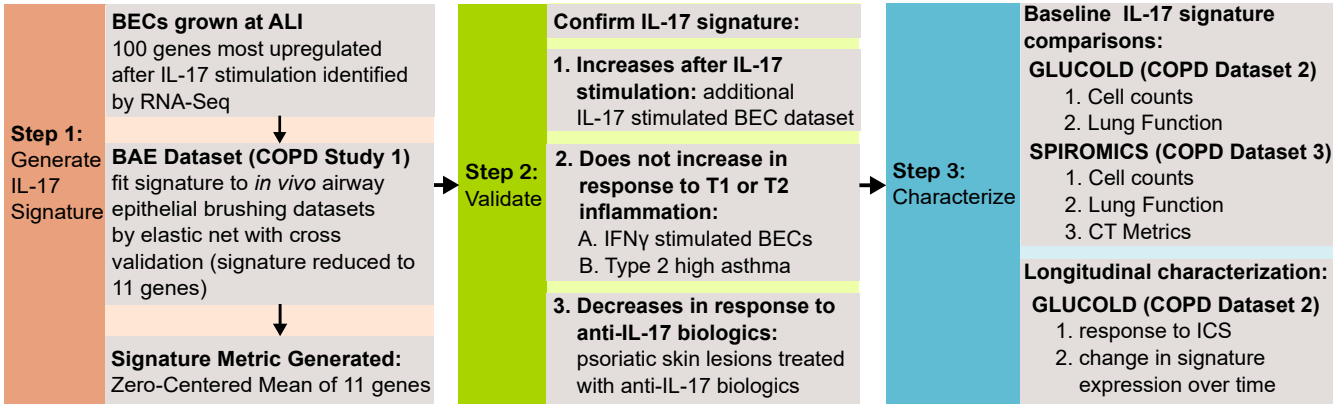
Figure 1. Study Design. Abbreviations: BEC: Bronchial Epithelial Cell, ALI: air-liquid interface, RNA-Seq: RNA Sequencing, BAE: Bronchial Airway Epithelial, T1: Type 1 Inflammation, T2: Type 2 Inflammation, IFNγ: Interferon Gamma, ICS: Inhaled Corticosteroid.
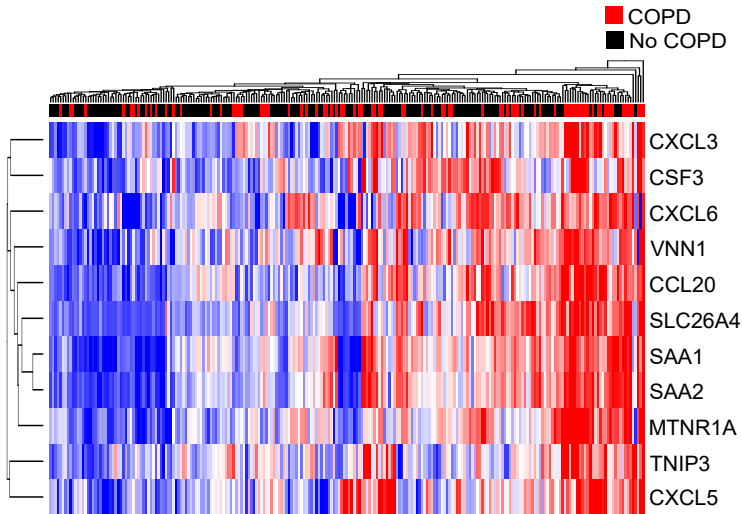
Figure 2. Hierarchical clustering of the 11 IL-17 signature genes in the BAE dataset (n=237). Signature genes shown in rows across participants in columns. Blue and red indicates low and high relative gene expression, respectively. Smokers with and without COPD are indicated by red and black in the above color bar, respectively. Clustering across participants and genes was done by Euclidean distance with average linkage.
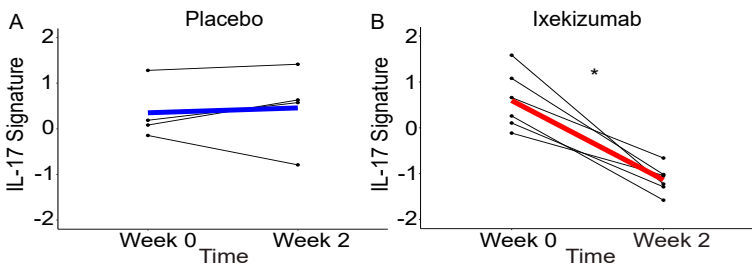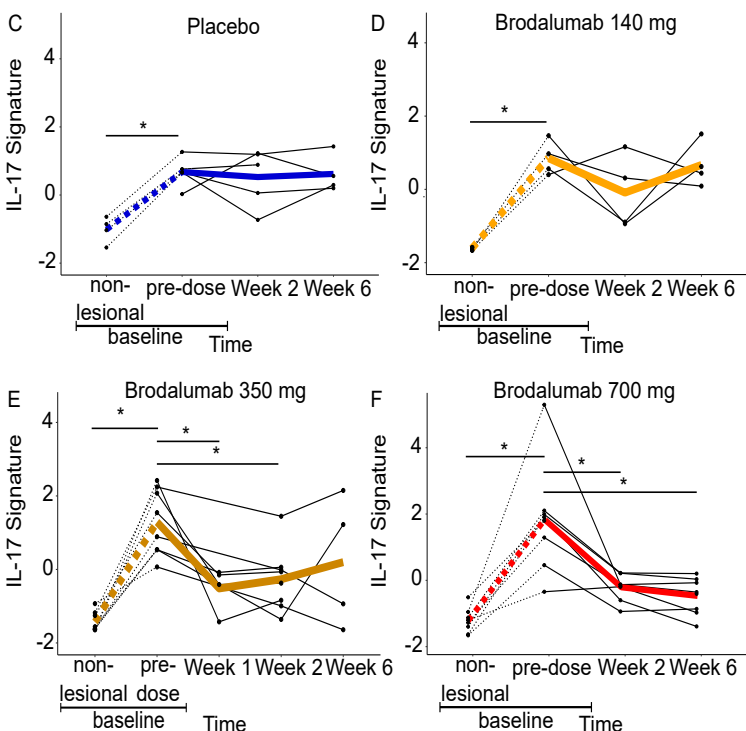
Figure 3. IL-17 blockade in psoriasis. Ixekizumab: The IL-17 signature was (A) decreased in psoriatic skin lesions (n=6) after 2 weeks of Ixekizumab compared to (B) placebo (n=4). Brodalumab: compared to (C) placebo (n=5), Brodalumab (n=20) at a dose of (D) 140mg did not, but (E) 350mg (at 1 and 2 weeks) and (F) 700mg (at 2 and 6 weeks) did result in a decrease in the IL-17 signature, consistent with clinical response. The IL-17 signature was higher in psoriatic lesions than matched non-lesional skin samples (C-F, dashed line). *p<0.05 using mixed effects models
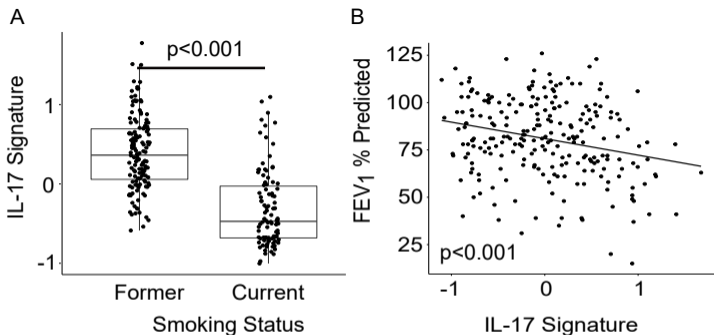
Figure 4. The IL-17 gene signature in the BAE dataset (n=237) is (A) increased in former (0.29±0.46) compared to current smokers (-0.42±0.48, p<0.001 by Wilcoxon Rank Sum test), and (B) associated with decreasing FEV$_1$% predicted (ρ=-0.23, p<0.001 by Spearman's correlation).
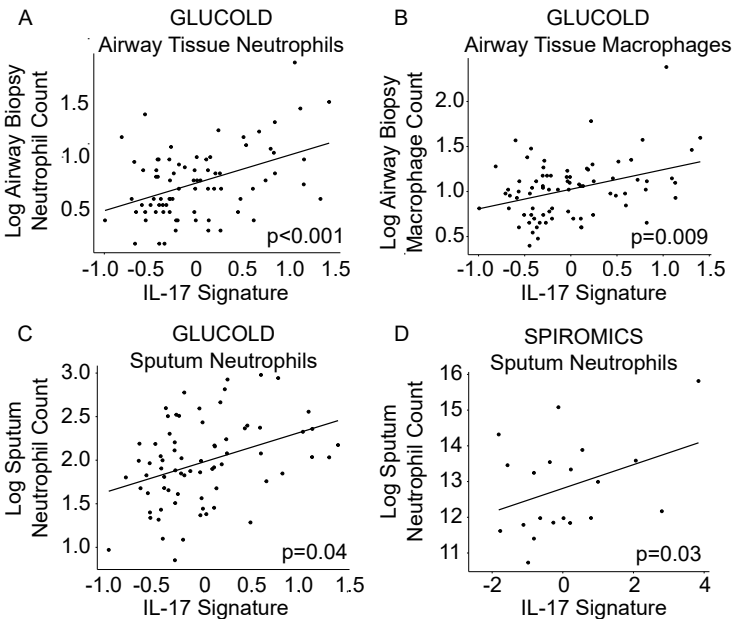
Figure 5. Airway neutrophils and macrophages. GLUCOLD (n=79): the IL-17 signature was associated with increasing log2 counts of (A) airway tissue neutrophils, (B) airway tissue macrophages, and (C) sputum neutrophils (n=72 with measured neutrophils). (D) SPIROMICS: the signature was associated with log2 sputum neutrophil counts (n=20). P-values shown for linear models adjusted for age and smoking status.
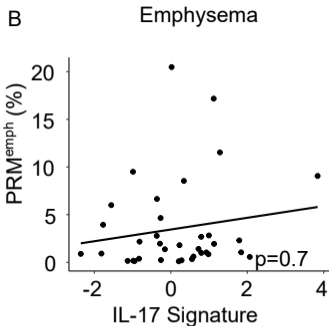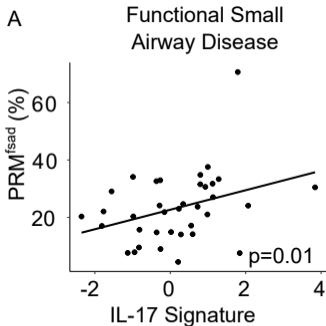
Figure 6. CT Biomarkers. The IL-17 signature was associated with increasing percent of lung area with (A) functional small airways disease (PRM^{fsad}) but not (B) emphysema (PRM^{emph}) by parametric response mapping of baseline CT scans (n=35). P-values shown for linear models adjusted for age and smoking status.
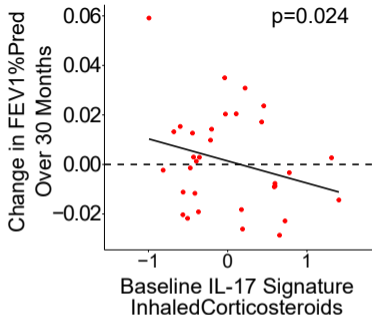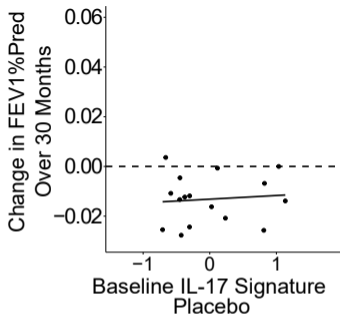
Figure 7. ICS response in GLUCOLD. An increased baseline IL-17 signature was associated with a greater decrease in percent change in FEV1 in the ICS ± long acting beta agonist group (n=33) compared to placebo (n=16) at 30 months (p=0.024 for the linear model interaction with adjustment for age and smoking status). Participants with low IL-17 signatures were more likely to show an improvement in FEV1 after ICS (greater than zero: above the dashed line).

Tables

Table 1. Demographic characteristics of the three datasets.

	BAE			GLUCOLD				SPIROMICS
	Current and Former Smokers ± COPD			Current and Former Smokers with COPD Subset randomized to placebo or inhaled steroids				Current and Former Smokers with COPD
	Smokers N=151	COPD N=87	p-val	All N=79	Placebo N=16	Steroid N=33	p-val	N=47
Age	65 (6)	64 (6)	0.22	61 (8)	58 (8)	61 (8)	0.22	63 (8)
Smoking Status								
Current	69	30	0.10	46	14	22	0.79	16
Former	82	57		33	7	15		29
Inhaled Steroid Use			0.0003					
Yes	7	18		0	0	0	NA	17
No	144	69		78	16	33		30
History of Asthma								
Yes	10	7	0.79	0	0	0	NA	11
No	141	80		78	16	33		33
$FEV_1\%$ Predicted	93 (13)	60 (14)	$<2.2*10^{-16}$	63 (9)	61 (9)	64 (9)	0.28	79 (18)

Means and (SD) or total counts are given for continuous and dichotomous variables respectively. For the BAE dataset p-values for differences between smokers with and without COPD by fischer's exact test or t-test as appropriate are given. GLUCOLD and SPIROMICS included only COPD participants, and point values are shown for all baseline participants. For GLUCOLD differences between those randomized to placebo or inhaled corticosteroid and the associated p-values are shown.

Table 2. Association between IL-17 metric and cell counts or T2S score.

	Unadjusted				Adjusted			
	R^2	Coefficient (SE)	p-val	FDR	R^2	Coefficient (SE)	p-val	FDR
Neutrophils								
GLUCOLD								
Endobronchial neutrophils	**0.19**	**0.27 (0.06)**	**$6.41*10^{-5}$**	**<0.001**	**0.20**	**0.26 (0.07)**	**0.0008**	**0.002**
Sputum neutrophils	**0.14**	**0.34 (0.10)**	**0.001**	**0.002**	**0.17**	**0.27 (0.13)**	**0.041**	**0.061**
Blood neutrophils (%)	0.002	-0.83 (2.00)	0.68	0.68	0.08	0.57 (2.26)	0.80	0.80
SPIROMICS								
Sputum neutrophils	**0.14**	**0.33 (0.19)**	**0.10**	**0.20**	**0.37**	**0.44 (0.19)**	**0.033**	**0.066**
Blood neutrophils	0.005	-0.02 (0.03)	0.63	0.63	0.09	0.03 (0.04)	0.43	0.43
Macrophages								
GLUCOLD								
Endobronchial macrophages	**0.13**	**0.22 (0.06)**	**0.0009**	**0.002**	**0.14**	**0.20 (0.07)**	**0.009**	**0.019**
Sputum macrophages	0.008	0.07 (0.09)	0.45	0.45	0.03	-0.02 (0.11)	0.84	0.84
SPIROMICS								
Sputum macrophages	0.008	0.05 (0.13)	0.71	N/A	0.02	0.06 (0.14)	0.68	N/A
Eosinophils								
GLUCOLD								
Endobronchial eosinophils	0.02	0.11 (0.10)	0.28	0.35	0.01	0.02 (0.12)	0.87	0.923
Sputum eosinophils	0.01	0.09 (0.08)	0.31	0.35	0.05	-0.01 (0.11)	0.92	0.923
Blood eosinophils	0.01	0.32 (0.34)	0.35	0.35	0.10	-0.21 (0.39)	0.59	0.923
SPIROMICS								
Sputum eosinophils	0.11	0.46 (0.40)	0.27	0.31	0.56	0.40 (0.41)	0.35	0.35
Blood eosinophils	0.02	0.07 (0.07)	0.31	0.31	0.07	0.12 (0.08)	0.15	0.30
Mast Cells								
GLUCOLD								
Endobronchial mast cells	0.007	-0.03 (0.04)	0.47	N/A	0.01	-0.02 (0.05)	0.67	N/A
T2S Score								
BAE	0.05	-1.49 (0.71)	**0.039**	N/A	0.10	-0.94 (0.85)	0.27	N/A
GLUCOLD	0.04	-1.01 (0.60)	0.096	N/A	0.14	-0.16 (0.67)	0.82	N/A

"Adjusted" models adjusted for smoking status and age. Count values for endobronchial tissue and sputum samples were logged prior to analysis. False discovery rates calculated on a cell type specific basis over each study.

FDR=False discovery rate, SE=standard error

Table 3. Association between IL-17 metric and clinical parameters in COPD participants.

	Unadjusted				Adjusted			
	R^2	Coefficient (SEM)	p-val	FDR	R^2	Coefficient (SEM)	p-val	FDR
Baseline								
BAE								
FEV1 % Predicted	0.01	-2.34 (2.28)	0.31	N/A	0.09	-5.52 (2.68)	**0.042**	**N/A**
GLUCOLD								
FEV1 % Predicted	0.04	-3.40 (1.81)	0.064	N/A	0.07	-3.23 (2.10)	0.129	N/A
SPIROMICS								
FEV1 % Predicted$^{\neq}$	0.03	-2.50 (2.1)	0.23	0.35	0.13	-5.24 (2.44)	**0.038***	**0.057**
CT PRM$^{emph\neq}$	0.03	0.61 (0.65)	0.35	0.35	0.08	0.78 (0.91)	0.40	0.40
CT PRM$^{fSAD\neq}$	0.12	3.40 (1.57)	**0.037**	**0.11**	0.26	5.70 (2.07)	**0.0097***	**0.029**
Longitudinal								
GLUCOLD: Change over 30 months after Inhaled steroid treatment compared to placebo (n=48)								
FEV$_1$ %	0.23	-0.014 (0.009)	0.11	N/A	0.36	-0.019 (0.008)	**0.024**	**N/A**
FEV$_1$ % also adjusted for airway tissue eosinophils	N/A	N/A	N/A	N/A	0.36	-0.020 (0.009)	**0.027**	**N/A**
FEV$_1$ % also adjusted for T2S score	N/A	N/A	N/A	N/A	0.38	-0.020 (0.008)	**0.018**	**N/A**
FEV$_1$ % also adjusted for airway tissue neutroophils	N/A	N/A	N/A	N/A	0.3	-0.022 (0.009)	**0.016**	**N/A**
FEV$_1$ % also adjusted for airway tissue macrophages	N/A	N/A	N/A	N/A	0.36	-0.019 (0.008)	**0.030**	**N/A**

"Adjusted" models adjusted for smoking status and age. Abbreviations: SE= Standard Error, FEV$_1$%Pred= Forced expiratory volume in one second percent predicited, PRM^{fSAD}= parameteric response mapping functional small airways disease biomarker, PRM^{emph}= parameteric response mapping emphysema biomarker, T2S= Type 2 Signature, N/A= Not Applicable, ≠ =parameters adjusted for multiple comparisons, *=p-value adjusted for FDR<0.1