

Title: Th2 and Th17 inflammatory pathways are reciprocally regulated in asthma

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36 **One Sentence Summary:** Therapeutic perturbation in a murine model of asthma
37 reveals robust Th2/Th17 cross regulation, corresponding to the mutually
38 exclusive transcriptomic signatures we observe in human asthma, and makes a
39 case for concurrent blockade of both IL-13 and IL-17A for better disease control.

40

ABSTRACT

Increasing evidence suggests that asthma is a heterogeneous disorder regulated by distinct molecular mechanisms. Here, in a cross-sectional study of asthmatics of varying severity (n=51), endobronchial tissue gene expression analysis revealed three major patient clusters: Th2-high, Th17-high, and Th2/Th17-low. Th2-high and Th17-high patterns were mutually exclusive in individual patient samples, and their gene signatures were inversely correlated and differentially regulated by IL-13 and IL-17A. To understand this dichotomous pattern of Th2 and Th17 signatures, we investigated the potential of type 2 cytokine suppression in promoting Th17 responses in a preclinical model of allergen-induced asthma. Neutralization of IL-4 and/or IL-13 resulted in increased Th17 cells and neutrophilic inflammation in the lung. However, neutralization of IL-13 and IL-17 protected subjects from eosinophilia, mucus hyperplasia, airway hyperreactivity and abolished the neutrophilic inflammation, suggesting that combination therapies targeting both pathways may maximize therapeutic efficacy across a patient population comprising both Th2 and Th17 endotypes.

INTRODUCTION

Asthma is a chronic disorder, characterised by episodic airway hyperresponsiveness (AHR), and remodelling with variable degrees of eosinophilic and neutrophilic inflammation. Asthma causes significant morbidity and mortality(1-4), and approximately 10% of patients have disease that is resistant to current therapies(1, 4). This group consumes 50–60% of health care costs attributed to asthma, underscoring the necessity to discover new therapies(5).

The clinical expression of asthma is heterogeneous with several distinct phenotypes identified(6, 7). Identifying the molecular mechanisms driving subtypes of asthma has the potential to reveal drug targets, biomarkers to predict treatment response, and appropriately target therapy, as evidenced by recent clinical studies of Th2 cytokine antagonists(8)(9).

In addition to the Th2 pathway, attention has focused on Th17 cytokines as candidate alternative drivers of severe asthma pathophysiology(10). IL-17A and F can amplify selected NF- κ B dependent signalling pathways such as those induced by TNF α , a cytokine upregulated in asthmatic airways, and are further upregulated following allergen challenge and experimental rhinovirus infection (11-17). In particular, IL-17A may contribute to neutrophilic airway inflammation *via* upregulation of *CSF3* and *CXCL* chemokines(18-20), mucus gland hyperplasia, AHR(18, 21) and corticosteroid resistance(18, 22).

83 Therapeutic strategies targeting Th2 and Th17 inflammatory pathways are
84 currently under active investigation in asthma. However, the nature and extent of
85 the activity of these two pathways in individual patients is unclear. Th2 cytokines
86 can negatively regulate Th17 cytokine expression, and inhibiting Th2 cytokines *in*
87 *vitro* or *in vivo* has the potential to increase IL-17A production and IL-17A-
88 dependent airway inflammation(23, 24). The cross-talk between Th2 and Th17
89 pathways is therefore complex, and it has been proposed that targeting Th2
90 cytokines might promote corticosteroid resistant IL-17-dependent neutrophilic
91 airway inflammation(10, 24). Here we show that Th2 and Th17-related gene
92 expression signatures are mutually exclusive in the airways of asthma patients,
93 but both are associated with eosinophilic inflammation. In an *in vivo* preclinical
94 model, we show that therapeutic targeting of Th2 and Th17 cytokines,
95 respectively, can lead to amplification of activity of the opposing pathway.

RESULTS

IL-13 and IL-17A induce distinct gene expression patterns in bronchial epithelial cells

To define core sets of IL-13- and IL-17-inducible transcripts for analysis in bronchial tissue, we stimulated normal human bronchial epithelial cells (NHBE) cultured at air-liquid interface (ALI) with 10 ng/mL IL-13 or 10 ng/mL IL-17A +/- 10 ng/mL TNF α , and assessed the expression levels of transcripts associated with IL-13 and IL-17A signalling(19, 25, 26). We confirmed the IL-13-dependent expression of *POSTN*, *CLCA1*, and *SERPINB2* (**Figure 1**), which have been previously described as highly correlated with *IL13* and *IL5* expression and eosinophilic airway inflammation(25, 26) in asthmatic bronchial epithelial brushings. IL-17A and IL-17F induce the expression of the neutrophil hematopoietic factor *CSF3* and the neutrophil chemoattractants *CXCL1*, 2, 3, and *IL8*(19). The expression of *CSF3*, *CXCL1*, 2, 3, and *IL8* were modestly inducible by IL-17A alone or more robustly in combination with TNF α (**Figure 1**). This is in keeping with existing literature, and relevant to asthma where TNF α is expressed at elevated levels(11-17). Relative to unstimulated cells, IL-13 stimulation suppressed the expression of IL-17A-inducible transcripts. To a lesser degree, IL-17A suppressed the expression of IL-13-inducible transcripts.

Transcriptional analysis of bronchial biopsies based on Th2 and Th17 signatures identifies three distinct subgroups of asthma patients

To assess the relative levels and relationships between transcripts inducible by Th2 or Th17 cytokines, we analyzed previously reported gene expression microarrays of endobronchial biopsies from 51 asthma patients spanning a range of severity and corticosteroid use(27). Consistent with the *in vitro* stimulation experiments, the three IL-13-inducible genes comprising the Th2 signature, and the five IL-17-inducible genes comprising the Th17 signature, respectively, were highly inter-correlated (**Supplementary Figure 1**). Th2 and Th17 gene expression scores were calculated based on average values of zero-centered gene expression values. The expression levels of canonical Th2 and Th17 cytokines, *IL13* and *IL17A*, measured by qPCR were generally low as 93% (39/42) and 76% (32/42) of available samples respectively had undetectable levels (<LLOQ, **Supplementary Table 1**). However, Th2 and Th17 scores were significantly elevated (**Figure 2A and 2B**) in samples in which *IL13* or *IL17A* mRNA could be detected by qPCR.

There was a significant negative correlation between Th2 and Th17 gene expression scores (**Figure 2C**, Spearman's $\rho=-0.35$, $p=0.011$). In keeping with the modest reciprocal suppression of Th2 signature transcripts by IL-17A+TNF α and Th17 signature transcripts by IL-13 in NHBEs (**Figure 1**), Th2-high and Th17-high gene signatures in bronchial biopsies were mutually exclusive: there were no patients that were simultaneously Th2-high and Th17-high. Similarly, subjects for whom *IL13* mRNA could be detected had undetectable levels of *IL-17A* mRNA and vice versa (**Supplementary Table 1**).

Next we classified subjects on the basis of their Th2 and Th17 scores. We employed a two-step cluster analysis using the log likelihood method based upon their factor loading score for Th2 or Th17 scores. Fifteen models were evaluated using different numbers of clusters between 1 and 15. The best fitting model and thus the number of clusters was determined by first calculating the Bayesian Information Criterion (BIC). Based upon this approach we identified three clusters as optimally fitting the data structure that appeared to capture patients with either Th2 score dominant ("Th2-high" asthma), Th17 score dominant ("Th17-high" asthma) or Th2/Th17 score low ("Th2/17-low" asthma) (**Figure 2C**).

Th2-high and Th17-high molecular phenotypes are both associated with eosinophilic inflammation

Th2-high steroid-naïve asthma is associated with eosinophilic inflammation(25, 28), while Th17 signature genes include potent neutrophil chemoattractants(29, 30). We therefore hypothesized that molecular phenotypes of Th2 and Th17 inflammation would relate to physiologic measures of eosinophilic and neutrophilic inflammation, respectively. Unexpectedly, we observed evidence of eosinophilic inflammation in both Th2- and Th17-high asthma.

Fractional exhaled nitric oxide (FeNO) and sputum eosinophils were significantly increased in Th2-high asthma compared to Th17-high and Th2/17-low asthma (**Figures 3A and 3B**). Median blood eosinophil counts were significantly elevated in Th2-high versus Th2/17-low asthma, but were also

161 elevated in Th17-high asthma though not statistically significant versus Th2/17-
162 low asthma (**Figure 3C**). Lamina propria eosinophil counts in bronchial biopsies
163 were comparable in both Th2-high and Th17-high asthma, and both were
164 significantly elevated versus Th2/17-low asthma (**Figure 3D**). We assessed
165 serum periostin, a systemic biomarker of eosinophilic airway inflammation(31)
166 and IL-13 activity(32) in subjects for whom serum and bronchial biopsies were
167 collected contemporaneously. Unexpectedly, serum periostin concentrations
168 were highest in Th17-high asthma as compared to Th2-high and Th2/17-low
169 asthma, but not statistically significant. There were no significant differences or
170 associations between measures of neutrophilic inflammation in peripheral or
171 airway compartments and molecular phenotypes of Th2 or Th17 inflammation
172 (**Supplementary Figure 2**).

173 **The Th17-high molecular phenotype is associated with corticosteroid-** 174 **dependent moderate to severe asthma**

175 The clinical characteristics of the patients separated by molecular phenotypes of
176 Th2/17 inflammation are shown in Supplementary Table 2. We did not detect
177 significant differences in patient characteristics or clinical measures including
178 FEV1% predicted, FEV₁/FVC ratio, AHR, or exacerbation frequency. However,
179 mean values of FEV1 % predicted and FEV1/FVC ratio were lowest in the Th17-
180 high group.

181 While there was a range of doses of inhaled or oral corticosteroids across
182 all three Th2/17 molecular phenotypes, the Th17 signature was not observed in

steroid-naïve patients (**Supplementary Table 2**). Integrating this observation with the associations between Th2- and Th17-high asthma and eosinophilic inflammation (**Figure 3**), we have delineated subsets where molecular phenotypes of Th2- and Th17-high asthma are observed (**Figure 4**). Th2-high asthma was only observed in subjects with evidence of eosinophilic asthma defined as blood eosinophils $\geq 300/\mu\text{l}$ or sputum eosinophils $\geq 3\%$ or biopsy lamina propria eosinophils $\geq 10/\text{mm}^2$. Th2-high, Th17-high, and Th2/17-low phenotypes were observed among eosinophilic moderate-severe asthma in approximately equal proportions. The molecular phenotype of Th2/17-low asthma was observed among all subsets and was predominant among moderate-severe asthmatics using inhaled corticosteroids (ICS) who did not display any evidence of eosinophilia. Finally, Th17-high asthma was observed only in moderate to severe asthma patients; primarily in subjects who had evidence of eosinophilia and were taking ICS or ICS+OCS.

Therapeutic blockade of Th2 cytokines during experimental allergic asthma induces Th17 inflammation

Considering the mutually exclusive Th2 and Th17 signatures identified in human asthmatic airways, and the potential for IL-13 suppression to promote IL-17 production *in vitro*(23), we investigated the effects of IL-4 and IL-13 blockade, singly or in combination, in a murine model of allergic asthma. These studies consisted of 3 weeks of intranasal sensitization against house dust mite (HDM) extract and 4 weeks of biweekly intranasal HDM challenges with antibody

therapy targeting IL-4, IL-13, or both cytokines on the preceding day (**Figure 5A**). Anti-IL-4, anti-IL-13, and anti-IL-4/13 reduced airway inflammation (**Figure 5B**). Mice receiving anti-IL-13 and anti-IL-4/13 were significantly protected from arteriolar hypertrophy and fibrosis, whereas mice treated with anti-IL-4 were less protected from fibrosis and still had evidence of arteriolar hypertrophy (**Figure 5B and C**). The mucus response assessed by histological analysis and lung *Muc5ac* expression was significantly abrogated by anti-IL-13 or anti-IL-4/13 treatment, but less so by anti-IL-4 alone (**Figure 5D, Supplemental Figure 3A**). Anti-IL-4 and anti-IL-4/13 reduced lung *IL13* expression and CD4⁺IL-13⁺ cells in lung and lymph node (**Figure 5E, Supplemental Figure 3B**). All anti-Th2 interventions resulted in significantly increased tissue expression of *IL17* and CD4⁺IL-17⁺ cells in lung and lymph node, particularly anti-IL-4 (**Figure 5F, Supplemental Figure 3B**). Furthermore, downstream eosinophilic and neutrophilic infiltration reflected this shift in Th2/Th17 effector cytokines. Anti-IL-4 or anti-IL-4/13-treated mice had significantly reduced eosinophil numbers in tissue, whereas anti-IL-13 alone produced a significant, but less dramatic reduction (**Figure 5E and Supplemental Figures 3C**). Meanwhile, we found marked increases in neutrophil infiltration in lungs of all treated mice, most pronounced with anti-IL-4 (**Figure 5F**). Furthermore, expression of Th2-driven genes, *Cc1a3* (ortholog for human *CLCA1*) and *Cc11*, were substantially reduced with anti-IL-13 or anti-IL-4/13 treatment (**Figure 5E**). Despite effectively reducing markers of the Th2 response, anti-IL-13 did not affect tissue expression or CD4 staining for IL-13 and may have induced greater IL-13 expression. These observations are

consistent with previous reports that IL-13 serves as an important regulator of downstream Th2 effector functions rather than impacting Th2 cell differentiation, and may be explained in part by an altered balance in receptor complex availability for IL-4 in the context of IL-13 blockade(33-36). Importantly, IL-17 responsive genes *Cxcl1*, *Cxcl3*, and *Csf3* were increased in anti-IL-4 treated animals, and *Csf3* was increased by both anti-IL-13 and anti-IL-4/13 (**Figure 5F**). Notably, the increases in neutrophils, IL-17, and IL-17 responsive gene expression were modest or non-existent in untreated mice compared to saline controls, suggesting that a robust Th17 response only occurred with perturbation of the Th2 pathway.

Dexamethasone suppresses Th2 pathways but enhances Th17 pathways in the mouse asthma model

Given the association of corticosteroid use with patients exhibiting a Th17-high signature, we interrogated the murine HDM model to determine if corticosteroid therapy alone might contribute to features of Th17-high asthma. Dexamethasone treatment reduced pathophysiologic features including AHR, tissue fibrosis, eosinophil infiltration, and IL-4/13 expression. Dexamethasone treatment partially suppressed IL-17 mRNA expression but increased the frequency of neutrophils in the lung concomitant with marked upregulation in tissue expression of the IL-17-inducible chemokines *Cxcl3* and *Cxcl1* (**Supplemental Figure 4**). In addition to recapitulating observations from published studies that corticosteroid treatment effectively suppresses Th2-driven inflammation, but fails to significantly reduce

IL-17-driven inflammation(18), these data add that corticosteroid use may specifically contribute to creating an environment permissive to an enhanced neutrophilic response.

Combined blockade of IL-13 and IL-17 attenuates the Th17 signature observed during anti-IL-13 inhibition of the Th2 response

The reciprocal regulation of Th2 and Th17 responses, both *in vivo* and in epithelial cells *in vitro*, provides an explanation for the mutually exclusive Th2 and Th17 signatures in a large proportion of patients with asthma (**Figures 2 and 4**). This could be important clinically when targeting these cytokine pathways as efficacy-limiting compensation may occur if only a single pathway is blocked. Therefore, we tested whether combined IL-13 and IL-17 antibody blockade would reduce Th2-driven disease and concomitantly inhibit the Th17 response. Mice treated with either anti-IL-13 alone or anti-IL-13+anti-IL-17 had significantly reduced AHR and mucus response relative to control antibody treated animals or anti-IL-17 alone (**Figure 6A and B**). Importantly, the increased IL-17 production and neutrophil infiltration associated with anti-IL-13 treatment alone were partially and completely abrogated, respectively, in mice receiving dual anti-IL-13/anti-IL-17 therapy (**Figure 6C**). Furthermore, we detected increased IL-4 and IL-13 producing CD4⁺ T cells in the lungs of anti-IL-17 treated animals (**Supplemental Figure 5**), supporting dual cross regulation between the Th2 and Th17 cytokine networks.

DISCUSSION

Until recently, asthma was considered as a single disease entity associated with eosinophilic airway inflammation driven by type 2 inflammatory cytokines (IL-4/5/13)(37). However, asthma can occur in the absence of significant Th2/eosinophilic inflammation across the spectrum of severity(25, 38-42). There is increasing interest in the role of Th17-dependent pathways in asthma, but it is not known how Th2 and Th17 pathways interact in asthmatic airways. With the potential for counter-regulation already identified, it is important to understand the consequences of singly inhibiting Th2 and Th17 pathways.

In this study we used Th2-related and Th17-related gene signatures and individual gene expression data to measure the activity of Th2 and Th17 pathways in human asthmatic airways, and HDM-sensitised murine airways. The analysis of Th2/Th17-dependent gene transcription adds important value by providing evidence of relevant cytokine activity. This is exemplified by the previously described Th2 gene signature which is robustly expressed in a subset of asthmatic subjects(25, 26), correlates with airway eosinophilia, and has precipitated the development of periostin as a biomarker for predicting the response to Th2-targeted therapy(9, 31). Using this approach, we have validated a Th17-dependent gene signature (*CXCL1*, 2, and 3, *IL8*, and *CSF3*), and shown that the induction of these genes is further amplified by TNF α , an important mediator associated with IL-17 inflammation, that has been described to be upregulated in the airways in many studies of asthma (11-17, 43-46). We also

confirmed in NHBES that IL-13 and IL-17 reciprocally regulate each other's respective signatures. Using this approach we have shown that IL-17-related signalling is evident in the airway tissue of a subset of patients with moderate-severe asthma using corticosteroids. Intriguingly, Th2 and Th17 activity were inversely correlated, and clustering patients demonstrated that Th2-high and Th17-high disease were mutually exclusive. This suggests that there is reciprocal regulation of these two pathways *in vivo* in human asthma, which is consistent with the reciprocal relationship we found for Th2 and Th17 signature genes induced by IL-13 and IL-17A *in vitro*, and the ability for IL-13 to attenuate IL-17A production in human Th17 cells(23). However, this interpretation is limited by the inherent restrictions of studying human subjects cross-sectionally.

To examine this potential counter-regulation in more detail we turned to an *in vivo* HDM-driven murine asthma model. This demonstrated that with a strong Th2 stimulus, there is also IL-17 induction. However, the downstream consequences of IL-17 induction appeared to be relatively limited, as there were no changes in airway neutrophils and the IL-17-dependent cytokines *Cxcl1* and *Cxcl3* did not increase significantly. However, while IL-13 and IL-4/13 blockade in combination mitigated a wide array of pathological consequences to HDM exposure, these same interventions enhanced IL-17 expression, IL-17-dependent chemokine/cytokine expression, and lung neutrophilia. This confirms that Th2 cytokines are powerful suppressors of IL-17-driven inflammation, and also raises the possibility that Th2-targeted treatment in asthma may contribute to the emergence of an adverse Th17 permissive environment, limiting

therapeutic efficacy over time. Given recent interest in IL-17 as an alternative driver of asthma, and the proposed idea that targeting Th2 cytokines might promote corticosteroid resistant IL-17-dependent neutrophilic airway inflammation(10, 24), these observations highlight the importance of understanding the regulation and interplay of Th2 and Th17 responses for developing and optimizing therapeutic intervention strategies.

Our identification of mutually exclusive Th2 and Th17 expression may seem contradictory with reports of dual Th2 and Th17 cytokine-expressing CD4+ T cells(47, 48). However, the demonstration that IL-13 strongly represses IL-17A dependent genes in epithelial cells suggests that in the context of co-expression, IL-17 dependent transcription will be attenuated. Our data from human bronchial biopsies support this interpretation.

As the Th17 signature genes include potent neutrophil chemoattractants(29, 30), we hypothesized that Th17 signature expression in patients would relate to measures of neutrophilic airway inflammation, as we described previously for inflammatory dermatoses(49). As observed previously, Th2-high asthma was associated with eosinophilic inflammation(25, 28). Unexpectedly, Th17-high asthma was also associated with elevated numbers of lamina propria eosinophils, and there were no relationships in blood, sputum, or lamina propria neutrophils among Th2 or Th17 molecular phenotypes. However, tissue neutrophil counts are reported to be similar between health and asthma(27, 39, 50-52), and their activity may be more important than their

number. Nevertheless, these observations have important implications for the selection of patients in clinical trials of anti-IL-17 therapy. Indeed, anti-IL-17RA therapy in symptomatic moderate-severe asthmatics using ICS failed to demonstrate efficacy in a recent study, which may not have been appropriately stratified to assess subsets of patients with activity of the IL-17 pathway(53). An ongoing study of another anti-IL-17 therapy in moderate-severe asthma excludes patients with elevated blood eosinophils and, hence may exclude patients with the potential for benefit(54).

An important question arises as to the stability of the Th2 and Th17 phenotypes and whether patients can move from one to the other. Indirect evidence from the expression of periostin suggests that this is a likely scenario. Periostin gene expression is directly inducible by IL-13 but not IL-17A(42) (and figure 1), elevated serum periostin levels predict clinical benefit from IL-13 inhibition(9), and serum periostin concentrations in anti-IL13-treated moderate-severe asthma patients fall to levels observed in healthy non-asthmatic subjects(32). Furthermore, IL-13 induces *NOS2* expression, contributing to elevated FeNO in asthma patients(55), which also predicts benefit from and is decreased by anti-IL-13 treatment(9, 32). We observed elevated FeNO in Th2-high but not Th17-high asthma, suggesting that FeNO reflects airway IL-13 activity at a given point in time, whereas the turnover times for serum periostin and tissue eosinophils may be longer. Taken together, the elevated serum periostin and bronchial tissue eosinophilia but low FeNO in Th17-high asthma suggest that moderate-severe asthma may alternate between Th2-high, Th17-

high, and Th2/17-low states depending on recent exposures to immune stimulatory factors. This cannot be determined formally due to the cross-sectional nature of the present study but future studies should examine these patterns over multiple longitudinal samples and exposures.

We observed Th17-high asthma exclusively in corticosteroid-treated moderate-severe asthma (Figure 4), consistent with the demonstration that corticosteroids may promote IL-17 production in some patients(56, 57). In addition, dexamethasone intervention in the HDM model potentially inhibited disease and the Th2 response, but resulted in enhanced markers of Th17 inflammation despite reducing IL-17. This could be the result of experimental timing, or potentiated signalling from residual IL-17 due to deregulation of the Th2 inhibitory pathway. Taken together, our data suggest that inhibition of Th2 responses and the concomitant loss of IL-17 regulation resulting from corticosteroid exposure or selective Th2 inhibition in asthmatic airways may create a Th17 permissive environment. Subsequent exposure to frequently encountered exogenous stimuli such as allergen, infection, pollution and perhaps corticosteroids themselves may then enhance IL-17 expression. The same environment would exist in true Th2-low patients who should also be susceptible to IL-17 upregulation (summarised in **Supplementary Figure 6**). In our preclinical mouse model, combined treatment with anti-IL-13/17 ameliorated AHR, lung pathology and the Th2 response, and importantly, dramatically reduced the IL-17-dependent chemokine and neutrophil response observed with anti-IL-13 treatment alone. Thus optimal therapeutic benefit in asthma may be

387 achieved through simultaneous Th2 and Th17-pathway directed therapy.
388 However, while therapies targeting Th2 cytokines have shown efficacy in subsets
389 of patients with moderate-severe asthma(58), formal assessments of the impact
390 of IL-4, IL-5, and/or IL-13 inhibition on Th17 activity have not been described in
391 those studies and represent an area for future investigation.

392 There were limitations to this study that may serve as subjects of further
393 investigation or alternative interpretations. This *post-hoc* analysis was based on a
394 cross-sectional study and is unable to assess the longitudinal intra-patient
395 variability of Th2/17 signature classification. The number of asthmatics (n=51)
396 and the relative paucity of subjects who are “non-eosinophilic” limits our ability to
397 evaluate this population where current and emerging therapies are unlikely to
398 provide meaningful clinical benefit. While our designated “Th17 signature” is
399 consistent with the biological activity of IL17A and/or F, we cannot exclude the
400 possibility that other cytokines are contributing to it in vivo. While Th2 and Th17
401 signature expression was statistically significantly upregulated (Figure 2A and B)
402 in subjects in whom *IL13* or *IL17* respectively was expressed, caution must be
403 made in the interpretation of this data due to the high percentage of subjects with
404 undetectable cytokine levels. Furthermore, assessing this gene expression
405 pattern in endobronchial biopsies depends on invasive procedures and precludes
406 routine use, highlighting the need to identify and develop non-invasive
407 biomarkers of this Th17 pathway activity.

408 In summary, we have identified Th2-high, Th17-high and Th2-17-low
409 clusters of patients with asthma. We propose that with suppression of Th2

activity by targeted therapy or corticosteroids, or absent Th2 activity (true Th2-low), a Th17-permissive environment exists. Combined with data from a murine model of allergic asthma, we suggest that in a subset of patients, a direct relationship between Th17-high and Th2-high disease exists, whereby, through mutual cross regulation, Th17-high asthma may represent a transition or switch away from Th2-mediated disease. Our studies therefore suggest that combined targeting of IL-13 and IL-17 in patients expressing either a Th2 or Th17 signature could provide additional efficacy over single Th2 or Th17 inhibition.

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Materials and METHODS

Study Design

The overall goals of this study were to identify gene signatures associated with IL-13 and IL-17 driven inflammation and then utilize these signatures to characterize the regulation and interaction of these cytokines in asthma and their association with commonly studied biomarkers of asthmatic disease. Candidate signature associated genes were identified via cytokine stimulation of human bronchial epithelial cells *in vitro* and then a *post-hoc* analysis was conducted in patient bronchial biopsies. To evaluate these pathways in the context of therapeutic blockade, we used a chronic house dust mite model of murine asthma with therapeutic anti-cytokine antibody treatment. In the murine studies, sample sizes were determined on the basis of previous experience and prior statistical analyses of the model. In the murine studies, standard measurements were used to assess inflammation, gene expression and airway hyperreactivity. Murine histology was scored by blinded observers. Sample size and replicates for all mouse studies are included in the figures and legends.

NHBE culture and stimulation

Primary normal human bronchial epithelial cells (NHBE) were purchased from Lonza (Walkersville, MD). 6.5 mm diameter 0.4 μ M pore density Transwell plates from Corning Life Sciences, were collagen coated using 100 μ g/ml PureCol from Advanced BioMatrix. NHBE were seeded in Transwells and maintained in serum-free bronchial epithelial cell growth medium (BEGM, Lonza) for 96 hours or until

confluent. Thereafter the apical media was removed, and cells were fed basolaterally with Pneumacult complete air liquid interface (ALI) medium (Stem Cell) and differentiated for a period of 21 days. Differentiated NHBE were cultured alone in ALI culture media or stimulated for 24 hours with IL-13 (10 ng/ml), IL-17A (10 ng/mL), or TNF α (10 ng/mL), alone or in combinations thereof (n=3 technical replicates). Total RNA was extracted from NHBE using the Qiagen RNeasy Kit.

Gene expression analyses

RNA was isolated from homogenized bronchial biopsies and real-time PCR (qPCR) was performed as described previously(28). Whole genome expression microarrays from asthmatics (n=51)(27) were analyzed. Microarray based Th2 and Th17 score was calculated by case-wise averaging of zero-centered gene expression data after annotation based independent filtering(59), i.e. if multiple probes correspond to an Entrez gene, the probe with the highest Inter-Quartile Range was selected. *SERPINB2*, *CLCA1*, and *POSTN* were used as IL13-responsive Th2 signature genes as described previously(25, 26). *CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3* were used as IL17-responsive Th17 signature genes.

Gene expression analyses from *in vitro* NHBE stimulation experiments and bronchial biopsy samples were conducted utilizing TaqMan® Gene Expression Assays that were purchased and conducted per manufacturer's instructions for *CXCL1* (Hs00236937_m1), *CXCL2* (Hs00601975_m1), *CXCL3* (Hs00171061_m1), *IL8* (Hs00174103_m1), *CSF3* (Hs00738432_g1), *SERPINB2*

(Hs01010736_m1), *CLCA1* (Hs00976287_m1), *POSTN* (Hs00170815_m1), *IL13* (Hs99999038_m1) and *IL17A* (Hs99999082_m1). Target gene expression was normalized by housekeeping genes *GAPDH* (4333764F) and *HPRT1* (Hs02800695_m1).

Patients and assessments

Patients were recruited from two centres, Leicester and Belfast, and their clinical data and tissue have been used in previously published studies(27, 60). Asthma severity was defined by British Guideline on the Management of Asthma treatment steps(61). Of the 26 severe patients at step 4/5, 21 met the American Thoracic Society criteria for refractory asthma(1).

The study was approved by the Research Ethics Committees of both Institutions(04/Q2502/74, 06/NIR02/114). Written informed consent was gained from all participants prior to their involvement.

Physiologic measures are reported only for those derived during the bronchoscopic study. Consequently, FeNO, sputum eosinophil percentage, and serum periostin measurements are reported for Leicester subjects only.

Fibre-optic bronchoscopy

Subjects underwent bronchoscopy conducted according to British Thoracic Society guidelines(62). Bronchial mucosal biopsy specimens were taken from the right middle lobe and lower lobe carinae, fixed in acetone, and embedded in glycol methacrylate (GMA), as described previously(63). Biopsies were also

placed immediately in RNA preservative (RNAlater, Ambion) and prior to processing gene expression analysis.

Immunohistochemistry (IHC)

GMA-embedded tissue was cut and immunostained as described previously(63). Primary antibodies were used against the following antigens: eosinophil major basic protein and neutrophil elastase. Isotype controls were also performed.

Assessment and quantification of immunohistochemical staining

Epithelial and submucosal areas in sections were identified and measured using a computer analysis system (AnalySIS, Olympus). Numbers of positively stained nucleated cells in each compartment were counted blind. Cells staining in sequential sections were co-localised using computer analysis.

Serum Periostin

Serum periostin levels were measured with a proprietary sandwich ELISA with 2 mAbs capable of detecting all known splice variants of human periostin as previously described(64).

Murine House Dust Mite Model

6-8 week old female BALB/C mice obtained from Taconic Farms Inc were sensitized by intranasal inhalation with 200 micrograms of house dust mite extract in 30 microliters of sterile saline on day 0, and 100 micrograms on days 7 and 14. Challenges were repeated twice a week with 50 micrograms intranasally for four weeks starting on day 21. For therapies, 250 or 150 micrograms each of anti-IL-4 (11b11), anti-IL-13 (262A-5-1), anti-IL-17 (16H4.4F3) or control (10E7.1D2) antibody was injected intraperitoneally the day before intranasal

challenges starting on day 20. Mice were terminally anesthetized on day 46 with sodium pentobarbital. All animals were housed under specific pathogen-free conditions at the National Institutes of Health in an American Association for the Accreditation of Laboratory Animal Care-approved facility.

Ethics Statement: The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved all of the experimental procedures (protocol LPD 16E). The Program complies with all applicable provisions of the Animal Welfare Act ([http://www.aphis.usda.gov/animal_welfare /downloads/awa/awa.pdf](http://www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf)) and other federal statutes and regulations relating to animals.

Histopathology

Murine lung lobes were harvested and inflated/fixed with a Bouin's-Hollande solution. Fixed tissue was embedded in paraffin for sectioning, and stained with Wright's Giemsa for airway inflammation and cellular infiltration analysis and Periodic acid–Schiff (PAS) for assessing airway mucus. Inflammation, PAS staining and cellular infiltrate was scored by a blinded observer.

Fibrosis Quantification

Hydroxyproline was measured to determine collagen content by hydrolyzing a weighed lung lobe in 6N HCl at 110°C overnight before neutralization with 10 N NaOH. Quantification was performed by colorization compared to a standard curve of hydroxyproline.

Cell Isolation and Flow Cytometry

Approximately 75 mg of lung tissue was diced and incubated in 100 U/ml of collagenase (Sigma) at 37°C for an hour with rocking. Tissue was then passed through a 100-µm nylon filter to obtain a single cell suspension. Leukocytes were isolated on a 40% Percoll (Sigma) gradient, and treated with ACK buffer to remove erythrocytes. Lymph nodes were passed over a 100-µm filter and treated with ACK. For BAL samples, the trachea of anaesthetized mice was cannulated and lungs were lavaged with 1 ml of 5 mM EDTA sterile PBS. BAL samples were centrifuged and treated with ACK before fixation. Isolated cells from lung, lymph node and BAL were either immediately fixed for cellular analysis or stimulated with phorbol 12-myristate 13-acetate (10ng/ml) and ionomycin (1 µg/ml) in the presence of Brefeldin A (10 µg/ml) for three hours and fixed. Cells were permeabilized (Cytotfix/Cytoperm buffer; BD Biosciences) and stained for 30 minutes with antibodies for CD16/32, CD45, Siglec F, Ly6G, Ly6C, CD11b, CD4, IL-4, IL-13, TNF-α, IFN-γ, and IL-17.

Murine Gene expression analyses

Lung tissue was homogenized in TRIzol Reagent (Life Technologies) with a Precellys 24 (Bertin Technologies). Total RNA was extracted with chloroform using a MagMax-96 Total RNA Isolation Kit (Qiagen), and reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Life Technologies). Gene expression was quantified using Power SYBR Green PCR Master Mix (Applied Biosystems) by RT-PCR on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Gene expression is described relative to RPLP2 mRNA levels in saline challenged lung tissue.

556 **Murine Primer Sequences.**

Gene	Forward qPCR primer sequence	Reverse qPCR primer sequence
<i>Rplp2</i>	TACGTCGCCTCTTACCTGCT	GACCTTGTTGAGCCGATCAT
<i>Il13</i>	CCTCTGACCCTTAAGGAGCTTAT	CGTTGCACAGGGGAGTCT
<i>CCL11</i>	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
<i>Muc5ac</i>	CAGGACTCTCTGAAATCGTACCA	AAGGCTCGTACCACAGGGA
<i>Clca3</i>	AGGAAAACCCCAAGCAGTG	GCACCGACGAACTTGATTTT
<i>Il17</i>	CAGACTACCTCAACCGTTCC	AGCATCTTCTCGACCCTGAA
<i>Cxcl1</i>	CTGGGATTACCTCAAGAAC	GAAGCCAGCGTTCACCAGAC
<i>Cxcl3</i>	CCCCAGGCTTCAGATAATCA	TCTGATTTAGAATGCAGGTCCTT
<i>Csf3</i>	GTGCTGCTGGAGCAGTTGT	TCGGGATCCCCAGAGAGT
<i>IL4</i>	ACGAGGTCACAGGAGAAGGGA	AGCCCTACAGACGAGCTCACTC

557

558 **Murine Airway Hyperreactivity Measurement**

559 Mice were analysed for AHR on day 46 of the HDM exposure model by total
 560 body plethysmography (DSI-Buxco Research Systems). Mice were challenged
 561 with increasing doses of methacholine inhalation (3-50 mg/ml), measurements
 562 collected over the following 5 minutes were used to calculate Penh readouts.
 563 Data was analysed using FinePointe software (Buxco).

564 **Statistical analysis**

Prism (Version 6; GraphPad), R Project software, version 2.15.1 (<http://www.R-project.org>) and SPSS statistics (IBM, Version 20) was used for statistical analysis and graphing. Mann-Whitney or Kruskal-Wallis test was utilized for testing the dependence of continuous versus categorical variables. Spearman's rank correlation was utilized to test for dependence between two numeric variables. Fisher's exact test was utilized to test for dependence between categorical variables. Missing data was not imputed and treated as Missing Completely At Random. Murine data was compared with a two-tailed t-test, with Welch's correction when an F test comparing variances had a P value of <0.05, or a two-way ANOVA.

SUPPLEMENTARY MATERIALS

Figure S1. Inter-correlation between the three IL-13-inducible genes, and the five IL-17-inducible genes in endobronchial biopsies.

Figure S2. Neutrophilic inflammation in peripheral or airway compartments are not associated with molecular phenotypes of Th2 or Th17 inflammation.

Figure S3. Differential effects of Th2 blockade on mucous and inflammatory responses in allergic asthma.

Figure S4. Dexamethasone suppresses Th2 pathways but enhances Th17 pathways in the mouse asthma model.

585 **Figure S5.** Evidence of dual cross regulation between the Th2 and Th17

586 cytokine networks.

587 **Figure S6.** A summary of the potential interplay between Th2 (IL-4/13)- and IL-

588 17-dependent signalling in asthmatic airways.

589 **Table S1.** Mutual exclusivity of IL-17 and IL-13 expression in endobronchial

590 biopsies.

591 **Table S2.** Clinical characteristics of T_H2/17-low, Th2-high, and Th17-high asthma.

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Author contributions: TAW, KMH, PB, LCW, DFC, and JRA conceived the study; DFC, KMH, LAB, TRR, JGE, JA, PB designed experiments; DFC, KMH, LAB, KMV, JCS, RLG, TWT, SW, AS, ED, CAB, DRN, GJ, JJ performed experiments; DFC, KMH, LAB, TRR, PB, ARA, JRA, AS, SS analysed the data; CO, PB and LH performed bronchoscopy; BH recruited patients and performed the clinical assessments; DFC, KMH, TAW, PB, JRA and TRR wrote the paper. All authors reviewed the manuscript for intellectual content and approved the final version.

Competing interests: David F. Choy, Deepti R. Nagarkar, Guiquan Jia, Emma Doran, Alexander R. Abbas, Janet Jackman, Lawren C. Wu, Jackson G. Egen, and Joseph R. Arron are currently or were employees of Genentech, Inc. during the execution of this study. Peter Bradding and Liam Heaney received funding from Genentech, Inc towards this work.

FIGURE CAPTIONS:

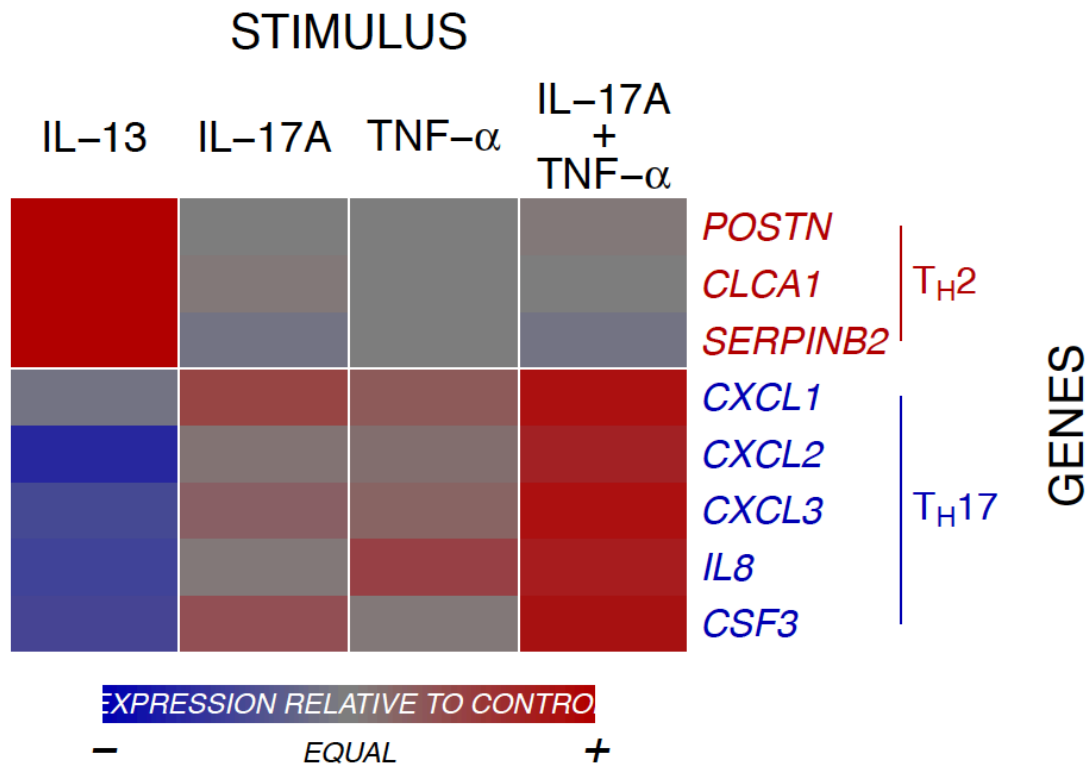


Figure 1. IL-13 and IL-17A induce Th2 and Th17 signature genes in Normal Human Bronchial Epithelial (NHBE) cells. NHBE cells were grown and differentiated at air liquid interface, then stimulated for 24 hours with IL-13 (10 ng/ml), IL-17A (10ng/mL), TNF- α (10 ng/mL), or IL-17A (10ng/mL) and TNF- α (10 ng/mL) (n=3 technical replicates). Gene expression was assessed by qPCR. Differential expression is represented by heatmap of averaged replicates of untreated control zero-centered and scaled values.

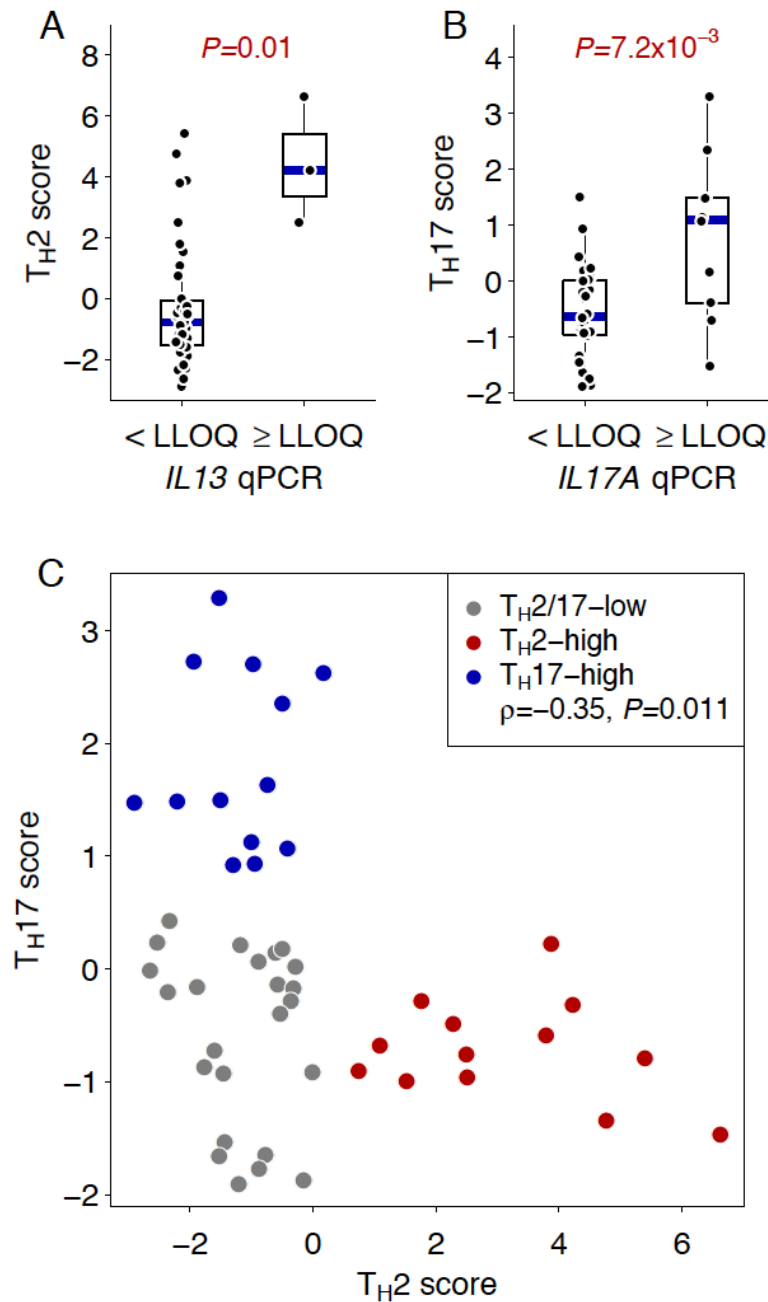


Figure 2. Three distinct asthma subgroups are defined by mutually exclusive Th2 and Th17 signature expression. The expression of endobronchial biopsy (A) Th2 and (B) Th17 signature scores are significantly elevated ($P < 0.05$, Kruskal-Wallis) in samples with detectable *IL13* and *IL17A* respectively. (c) Th2 and Th17 scores from 51 asthmatic bronchial biopsy

microarrays are plotted by scatterplot. Th2 and Th17 signature scores are inversely correlated (Spearman's $\rho = -0.35$, $P = 0.011$). Clustering Th2 and Th17 scores was utilized to classify subjects as Th2 score dominant (Th2-high, red), Th17 score dominant (Th17-high, blue), and Th2/17 score low (Th2/17-low, gray).

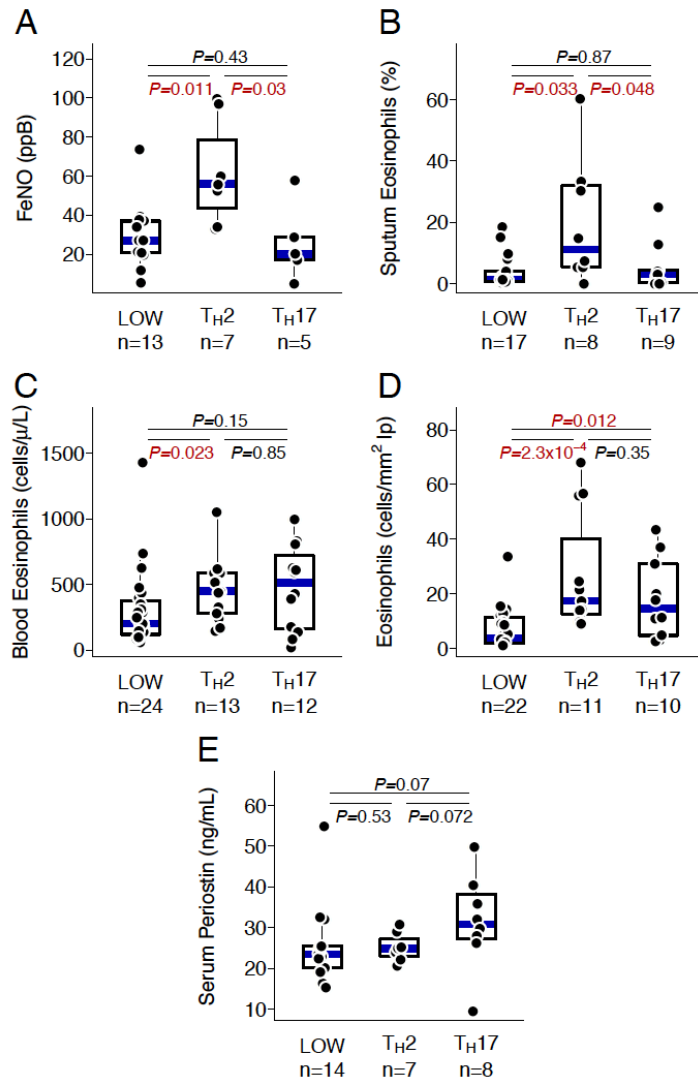
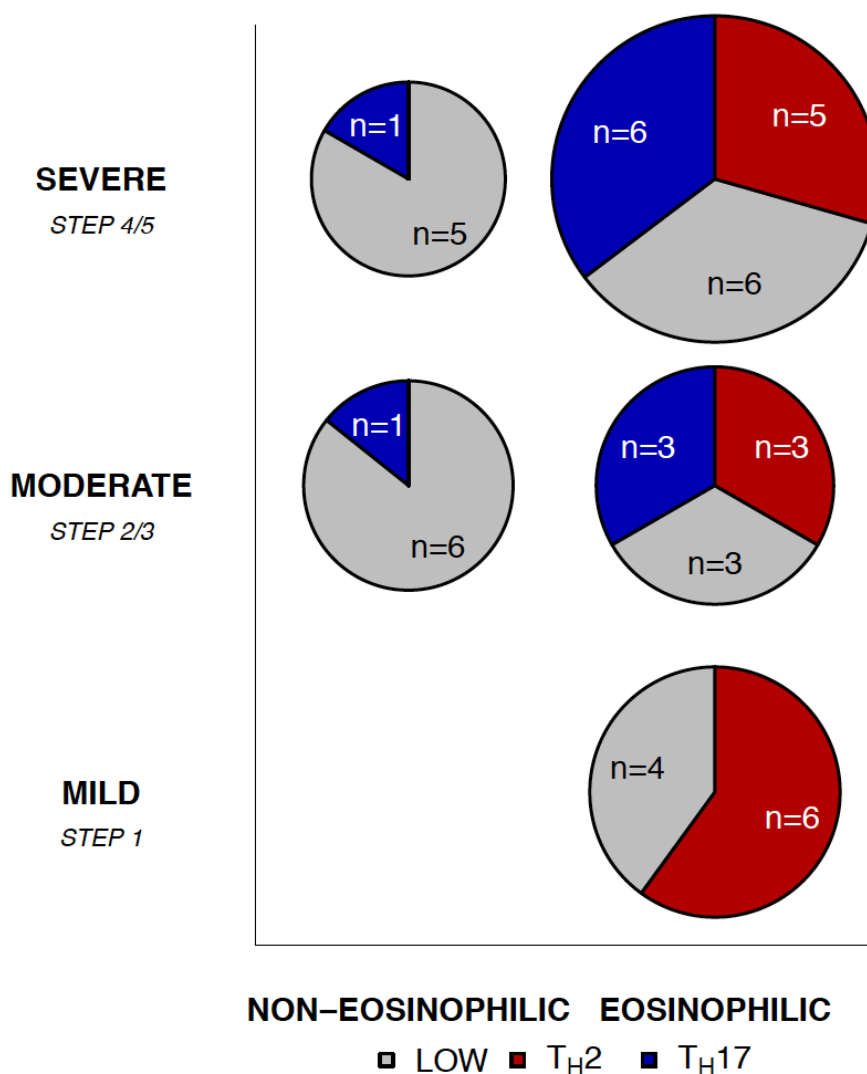


Figure 3. Th2 and Th17-high asthma are both associated with elevated levels of physiologic Th2/eosinophilic measures of inflammation. Pairwise comparisons (Mann-Whitney test) were made among Th2/17-low (LOW), Th2-high (TH2), and Th17-high (TH17) asthmatics subjects. Red font indicates $P < 0.05$. Th2-high subjects were associated with elevated (A) FeNO and (B) sputum eosinophil percentage as compared to low inflammatory and Th17-high asthma. (C) Blood eosinophils were elevated in Th2-high versus low-inflammatory asthma. (D) Lamina propria eosinophil counts were elevated in

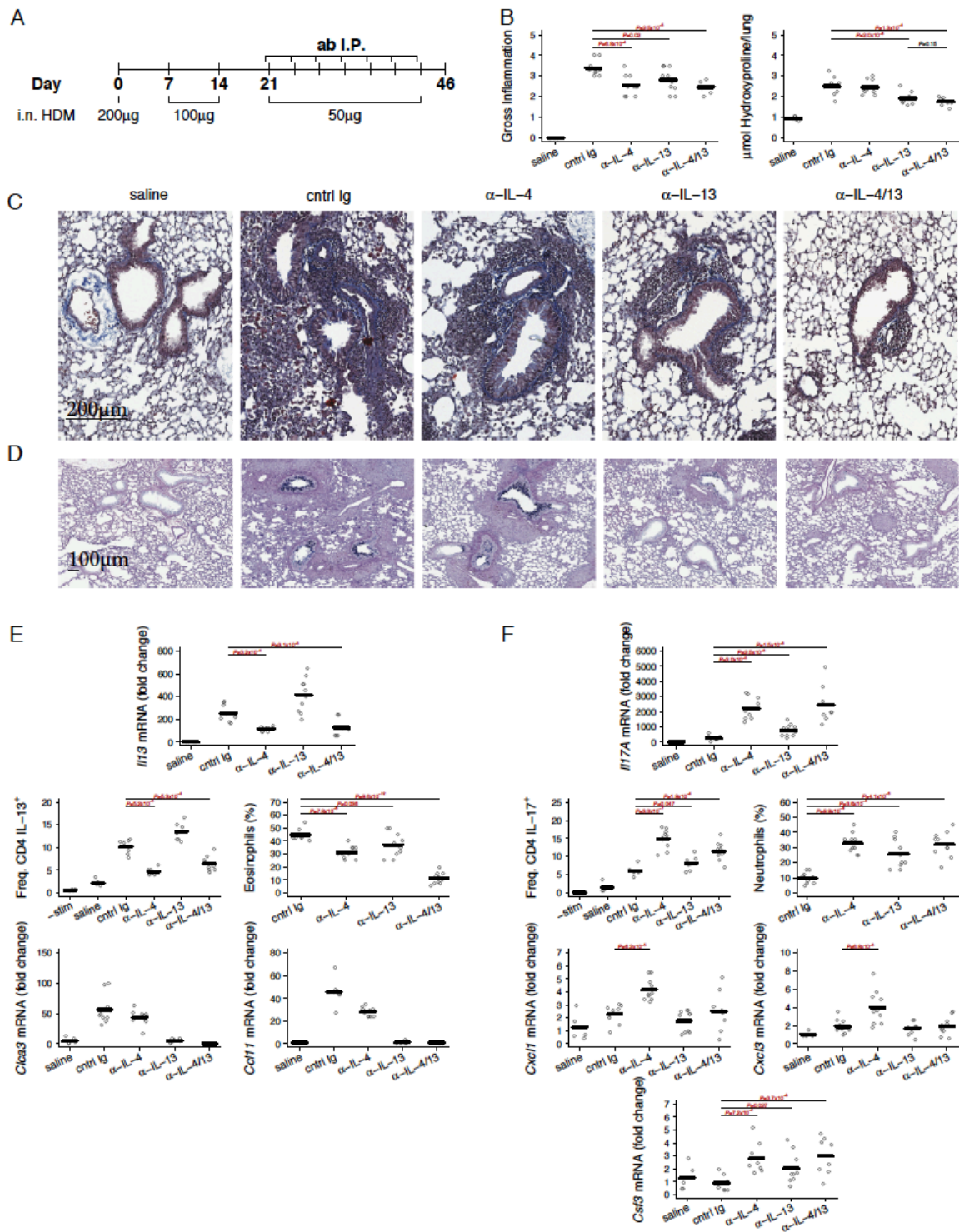
both Th2 and Th17-high versus low-inflammatory asthma. (E) Median levels of serum periostin was highest in Th17-high versus Th2-high and Th2/17-low asthma.



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954 **Figure 4. Proportions of Th2/17 molecular phenotypes of asthma by**
 955 **clinical evidence of eosinophilia and severity.** Asthmatics were classified
 956 based on evidence of eosinophilic asthma, defined as (blood eosinophil count \geq
 957 300 per microliter) *or* (sputum eosinophil percentage \geq 3) *or* (biopsy lamina
 958 propria eosinophil count \geq 10 per mm²) and clinical severity (BTS/SIGN treatment
 959 step). Pie charts represent the proportion of Th2/17-low, Th2-high (TH2), and

Th17-high (T_H17) asthmatic subjects per eosinophilic/severity category. The area of each pie chart is proportional to the number of subjects in that category.



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977 **Figure 5. Therapeutic Blockade of Th2 Cytokines During Experimental**
 978 **Allergic Asthma Induces compensatory Th17 Inflammation. Mice were**

intranasally sensitized against house dust mite extract for three weeks at 200,
100 and 100 micrograms each week, and subsequently challenged with biweekly
intranasal exposures for 4 weeks with or without targeted antibody therapy each
day prior to challenge against IL-4, IL-13 or both cytokines (250 micrograms i.p)
(**A**). Lung pathology was assessed by scoring for gross inflammation in Giemsa
stained histological sections(**B**), assaying for hydroxyproline content as a
surrogate for fibrosis (**B**), and Masson's trichrome staining of airways (**C**). Airway
mucus production was assessed by PAS staining (**D**). IL-13 (**E**) and IL-17 (**F**)
production was measured by gene expression and in restimulated CD4 T cells by
flow cytometry. The percent of eosinophils and neutrophils was quantified from
Giemsa stained histological lung sections (**E** and **F**). Whole lung tissue
expression of *C/cca3* and *Cc/11* Th2 markers, and *Cxc/3*, *Cxc/1*, and *Csf3* Th17
markers were assessed by quantitative PCR (**F**). (P values shown, Two-tailed T
tests, N=4-10)

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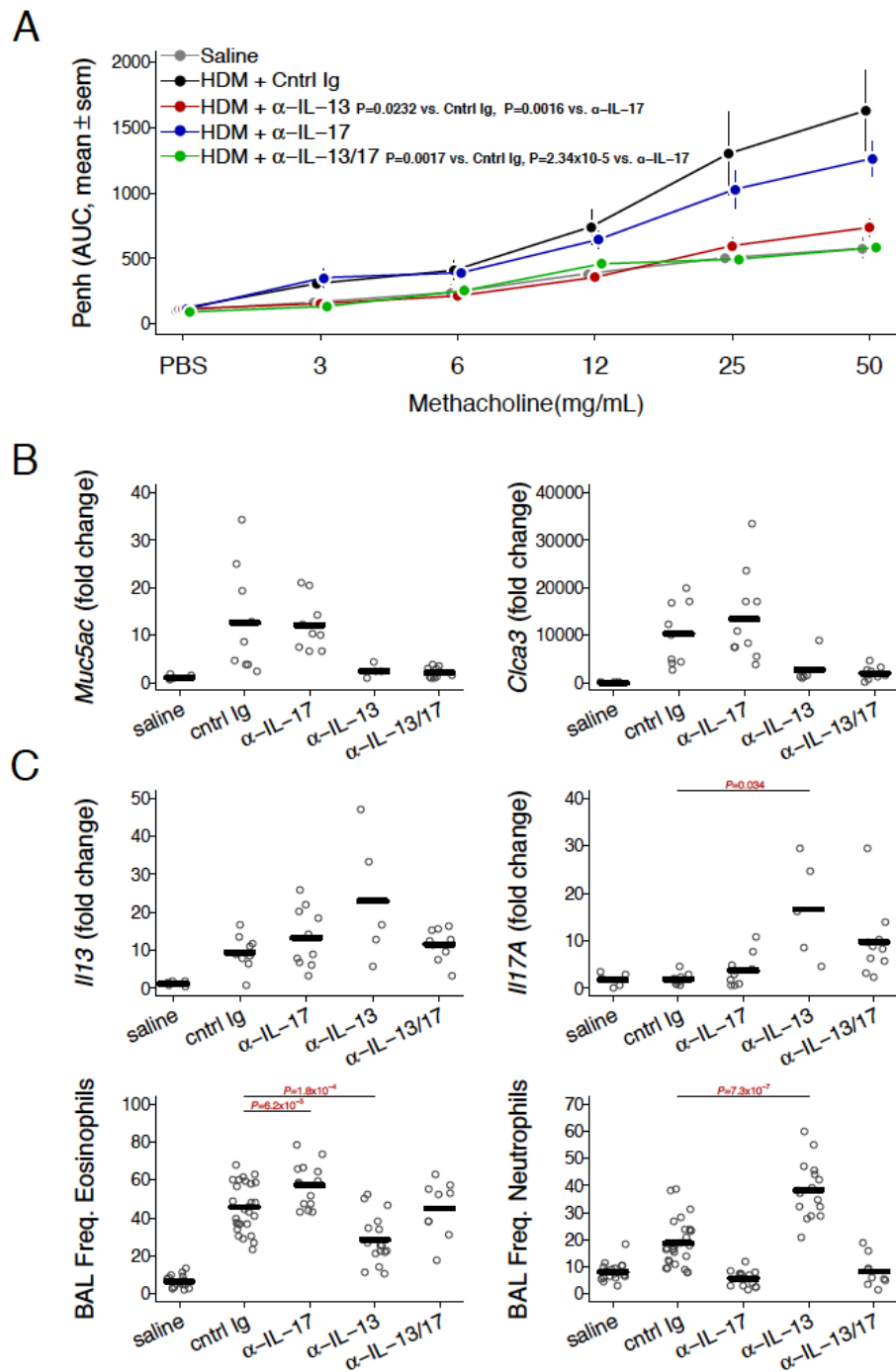


Figure 6. Dual therapeutic blockade of IL-13 and IL-17 prevents emergence of a Th17 signature induced by antibody inhibition of the Th2 response. Mice on the chronic house dust mite model of allergic asthma were treated a day

1003 prior to each challenge with anti-IL-17, anti-IL-13 or both (150 micrograms).
1004 Efficacy of IL-13/17 Dual blockade was assessed by airway hyperreactivity
1005 (AHR) (Two-way ANOVA, SEM shown, N=5-10) (**A**), and mucus response gene
1006 expression (**B**). Immune response was measured by gene expression analysis of
1007 IL-13 and IL-17, and flow cytometric analysis of the frequency of Siglec-F+
1008 eosinophils and Ly6G+ neutrophils in BAL (**C**). (P values shown, Two-tailed T
1009 tests, N=5-28)

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Title: Th2 and Th17 inflammatory pathways are reciprocally regulated in asthma

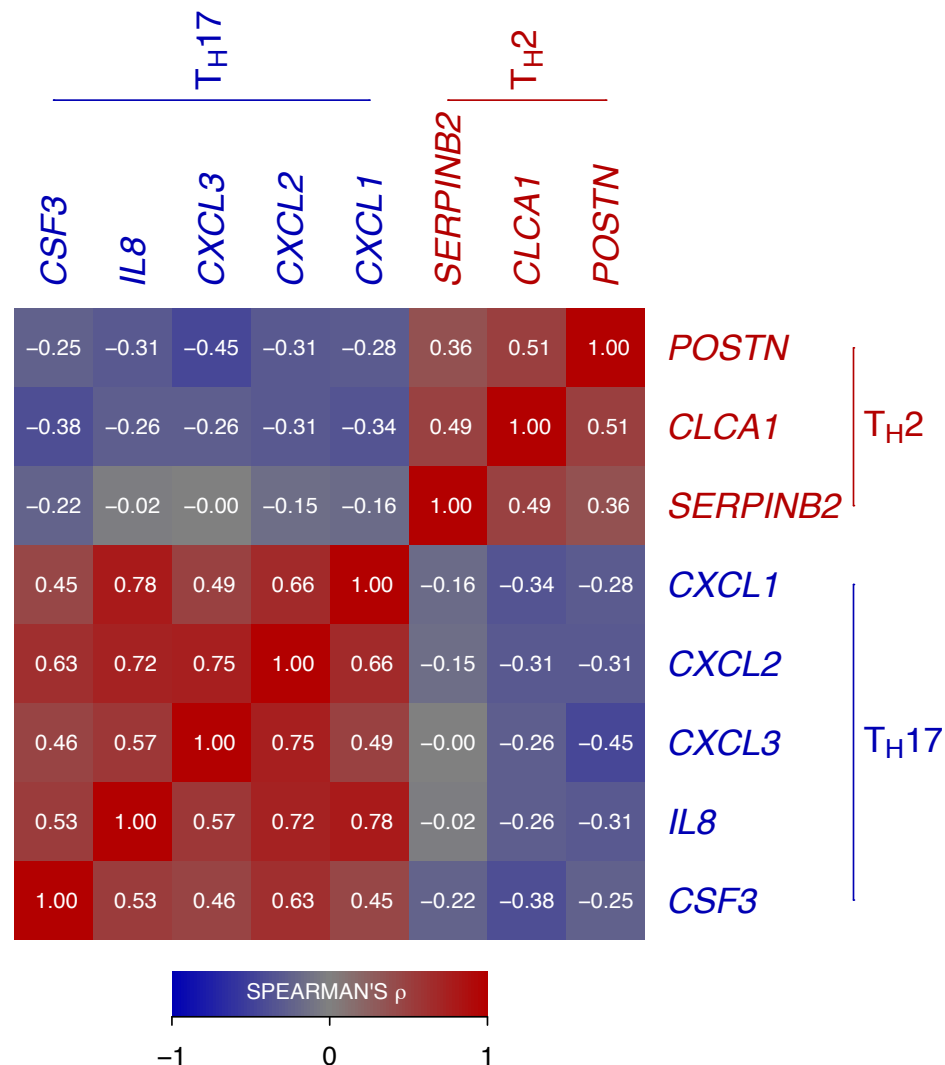
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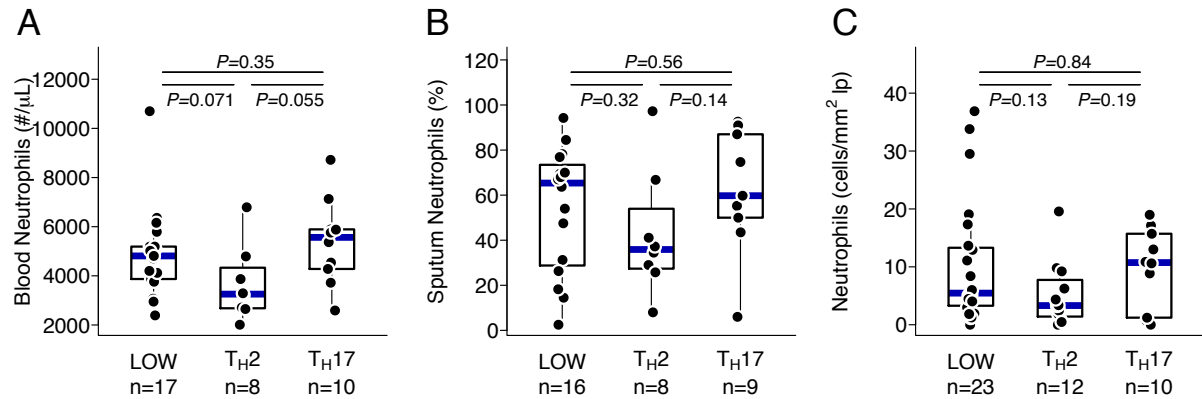
[†]JA, TW and PB are co-senior authors

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SUPPLEMENTARY MATERIALS



Supplementary Figure 1. Inter-correlation between the three IL-13-inducible genes, and the five IL-17-inducible genes in endobronchial biopsies. The correlation (Spearman's ρ) matrix of Th2 and Th17 signature gene expression of endobronchial biopsy microarrays from 51 asthmatics are represented by heatmap. Genes comprising the Th2 (annotated in red font) and Th17 (annotated in blue font) signatures are inter-correlated.



Supplementary Figure 2. Neutrophilic inflammation in peripheral or airway

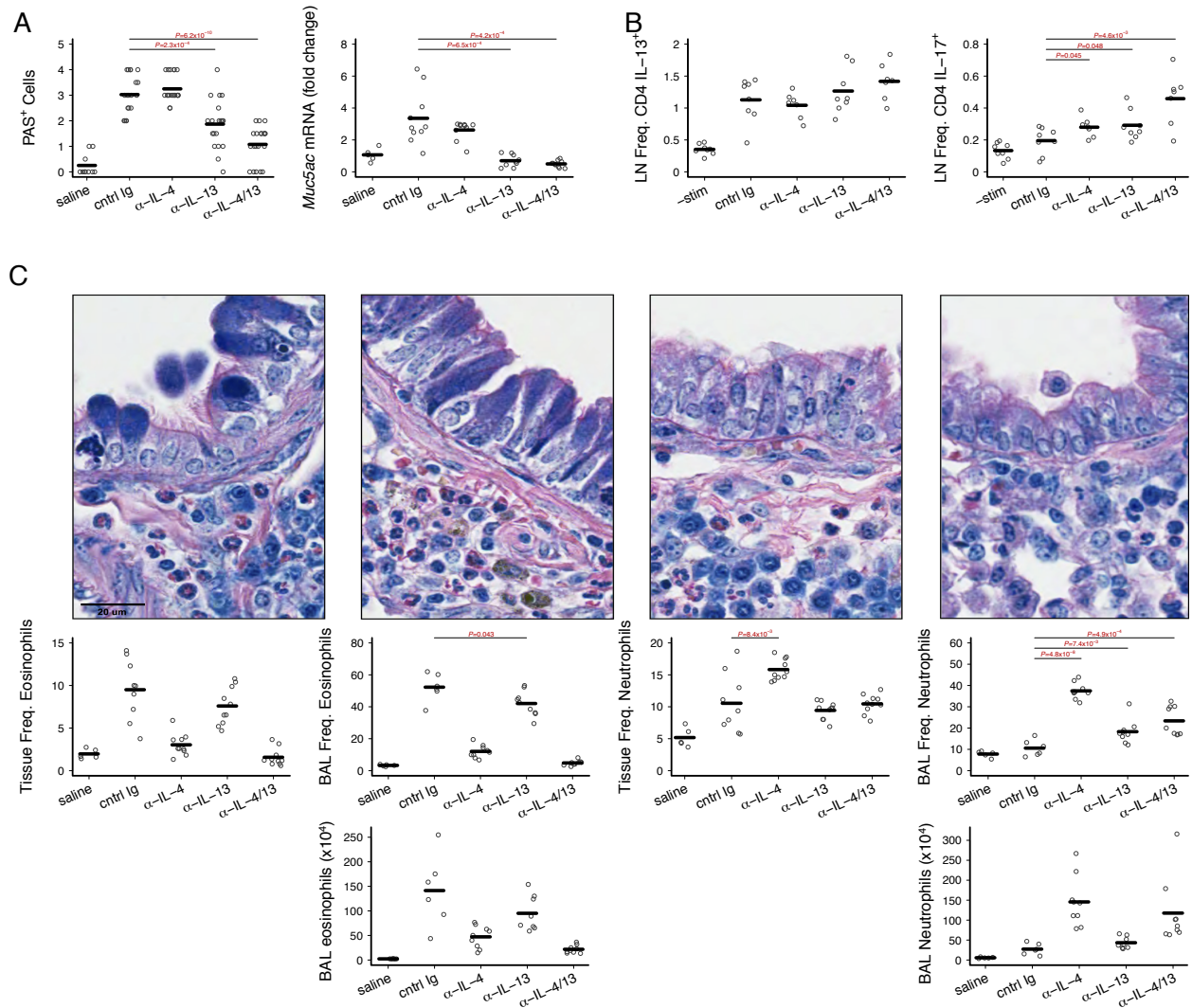
compartments are not associated with molecular phenotypes of Th2 or

Th17 inflammation. Blood (a), sputum (b), and lamina propria (c) neutrophils are

assessed pairwise among distinct clusters of Th2/17 score: Th2/17-low, Th2-

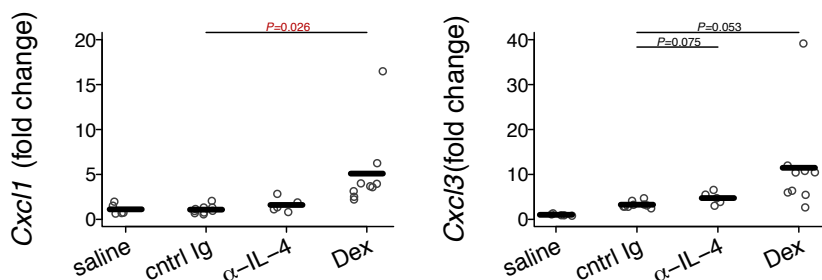
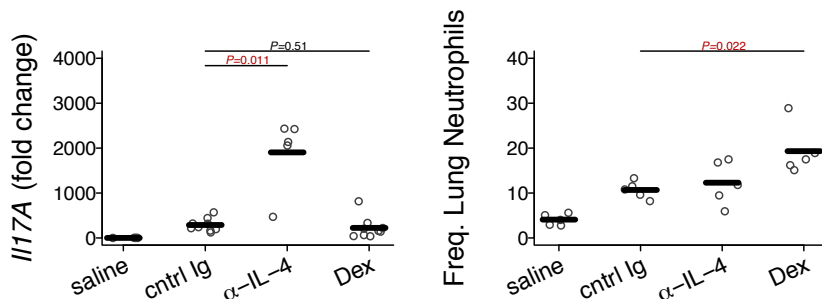
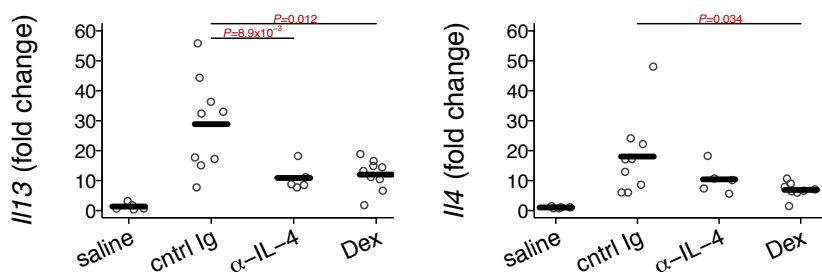
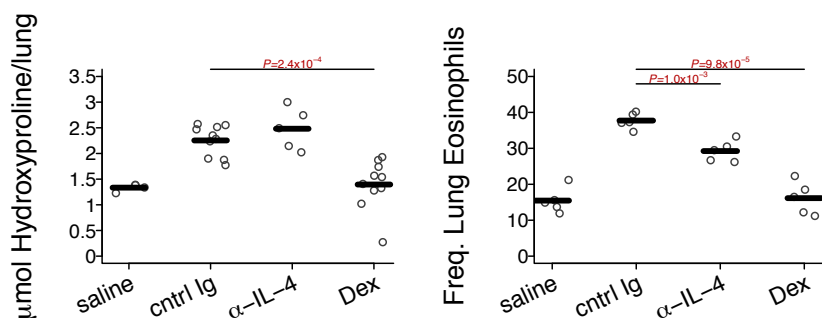
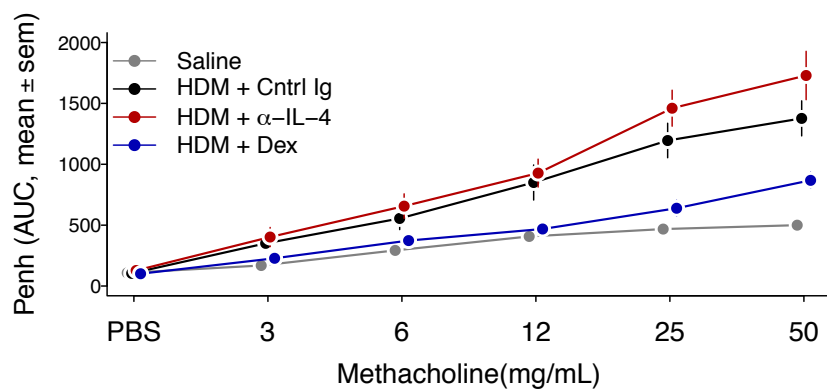
high, and Th17 high asthma. No statistically significant differences (Kruskal-

Wallis) were observed.

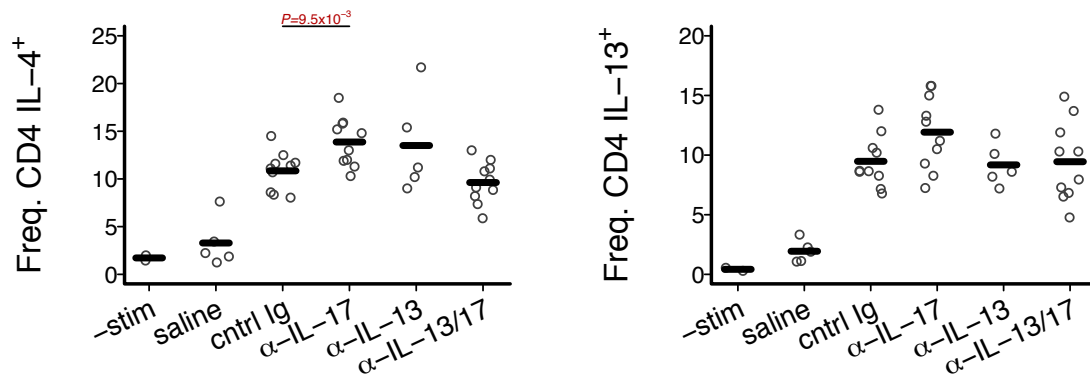


Supplementary Figure 3. Differential effects of Th2 blockade on mucous and inflammatory responses in allergic asthma. The mucus response was measured by quantifying PAS positive cells and measuring lung tissue expression of the mucin MUC5AC in anti-cytokine treated mice (a). Intracellular IL-13 and IL-17 staining of restimulated lymph node isolated CD4 cells (b.). Giemsa stained sections (c.) were used to quantify gross inflammation and percentages of infiltrating eosinophils and neutrophils in figure 5. Analysis of frequencies of Siglec-F positive eosinophils and Ly6G positive neutrophils

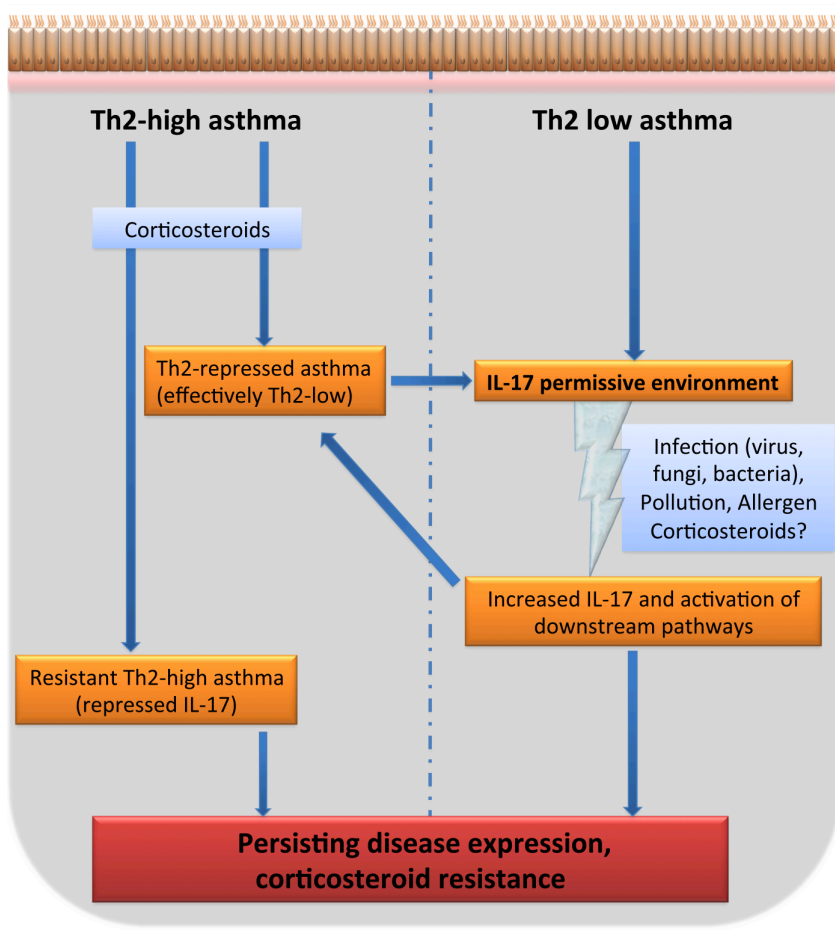
isolated from lung tissue and BAL and absolute numbers of neutrophils and eosinophils in BAL (c.). (P values shown, Two-tailed T tests, N=5-20)



Supplementary Figure 4. Dexamethasone suppresses Th2 pathways but enhances Th17 pathways in the mouse asthma model. Mice on the chronic house dust mite model of allergic asthma were treated 5 times a week with 2mg/kg dexamethasone i.p. or a day prior to each challenge with anti-IL-4 (150 micrograms). Airway hyperreactivity to methacholine challenge was measured in mice chronically exposed to HDM and treated with control antibody, anti-IL-4, or Dexamethasone. Allergic disease was assessed using hydroxyproline content as a surrogate of airway fibrosis, and quantifying Siglec-F+ eosinophils isolated from lung tissue. Airway inflammation was assessed by lung tissue expression of IL-13, IL-4, or IL-17A and by flow cytometric analysis of lung isolated Ly6G neutrophils. Th17 signature was assessed by expression analysis of CXCL3 and CXCL1 (c.). (P values shown, Two-tailed T tests, N=5-10)



Supplementary Figure 5. Evidence of dual cross regulation between the Th2 and Th17 cytokine networks. Frequencies of restimulated IL-4 and IL-13 producing CD4 T cells in the lung were assessed by flow cytometric analysis of intracellular staining from isolated lung leukocytes. (P values shown, Two-tailed T tests, N=2-10)



SUPPLEMENTARY FIGURE 6

Supplementary Figure 6. A summary of the potential interplay between Th2 (IL-4/13)- and IL-17-dependent signalling in asthmatic airways. We propose that with uncontrolled Th2 signalling, IL-17 and IL-17-dependent pathways are suppressed. In patients where Th2-dependent signalling is controlled by corticosteroid therapy, or absent as in true Th2-low asthma, an IL-17 permissive environment exists. In this environment, exposure to appropriate stimuli, which may include allergen, infectious agents, pollutants or even

corticosteroids, IL-17-dependent pathways are accentuated, and Th2-dependent pathways remain suppressed.

	<i>IL13</i> < LLOQ	<i>IL13</i> ≥ LLOQ
<i>IL17A</i> ≥ LLOQ	10	0
<i>IL17A</i> < LLOQ	29	3

Supplementary Table 1. Mutual exclusivity of IL-17 and IL-13 expression in endobronchial biopsies. Expression of *IL13* and *IL17A* was assessed by qPCR in available endobronchial biopsy samples (n=42) from the microarray study, and levels were generally low. The relationship of *IL13* and *IL17A* is expressed as the counts of samples with undetectable (<LLOQ) and detectable (>LLOQ) expression. No subject was observed to have detectable *IL13* and *IL17A* expression by qPCR.

	T _H 2/17-LOW	T _H 2-high	T _H 17-high	<i>P</i>
N	24	14	13	
Age (years)	38 ± 2.4	34 ± 3.1	43 ± 4.2	0.2
Age of onset (years)	22 ± 3.4	20 ± 3.3	19 ± 5.8	0.55
Sex (F/M)	11/13	7/7	3/10	0.33
FEV ₁ % predicted	88 ± 2.8	91 ± 5	77 ± 5.6	0.089
FEV ₁ /FVC (%)	73 ± 2	73 ± 4	65 ± 3.8	0.21
Methacholine PC ₂₀	0.9 [0.04,3]	2.5 [0.69,12]	0.23 [0.06,3.3]	0.27
Exacerbations (# prev yr)	2 [0,2.5]	0 [0,3]	1 [0,3]	0.53
Severity (Mild/Moderate/Severe)	4/9/11	6/3/5	0/4/9	0.076
ICS dose (BDP eq)	650 [300,800]	250 [0,1600]	800 [800,1600]	0.082
Oral CS (N/Y)	19/5	10/4	9/4	0.77
Atopic (%)	82	55	80	0.22
Sensitized to HDM (% of atopic+)	94	100	88	0.69

Supplementary Table 2. Clinical characteristics of T_H2/17-low, Th2-high, and Th17-high asthma. The counts of subjects in each class are represented in the top row (N). Normally distributed data are summarized as mean ± standard error (Kruskal-Wallis *P* values). Non-Gaussian distributed data are summarized as median [1st quartile, 3rd quartile] (Kruskal-Wallis *P* values). Categorical data is summarized as the counts of each level (Fisher's exact test *P* value).