Title: A transcriptomic method to determine airway immune dysfunction in T2-high and T2-low asthma.

Michael C. Peters*, M.D.[1], Lando Ringel[2]*, Nathan Dyjack[2], Rachelle Herrin[2], Prescott G. Woodruff, M.D.[1], Cydney Rios[2], Brian O'Connor[2] John V. Fahy, M.D.[1] and Max A. Seibold Ph.D.[2,3]

***** Authors contributed equally

ONLINE DATA SUPPLEMENT

Supplemental Materials and Methods:

Subjects and airway sampling:

We studied induced sputum samples stored in the Airway Tissue Bank at the University of California, San Francisco (UCSF). The biological samples were collected from research studies at UCSF from 2012-2015. The clinical characterization of participants and bio-specimen collection were performed according to standardized and uniform protocols(1,2).

Asthma subjects had a prior physician diagnosis of asthma and airway hyper-responsiveness (defined as a methacholine PC20 <8.0mg/ml off steroid or < 16mg/mL on steroids), or reversible airflow obstruction (defined as a post-bronchodilator increase in FEV1 of ≥12%) in subjects whose FEV1% predicted was < 60%. For asthmatic subjects who had been taking long-acting bronchodilators (LABA), with or without inhaled corticosteroids (ICS), the protocols included an initial visit to determine the safety of withholding LABA medication prior to the main characterization visit. Asthmatics assessed by a study physician to have stable disease were asked to discontinue LABA for 48h prior to characterization studies; others with unstable or severe asthma did not have LABA discontinued. Characterization studies included a physician directed history, asthma characterization questionnaire, asthma control test (ACT), spirometry, methacholine challenge, complete blood count with cell differential, and serum IgE levels. Subjects also underwent sputum induction and measures of nitric oxide in exhaled breath.

Healthy control subjects had no lifetime history of pulmonary disease and lacked airway hyper-responsiveness. Both asthmatic and healthy control subjects were excluded if they had a history of any lung disease other than asthma, any history of an upper or lower respiratory tract infection in the

4 weeks preceding the study, were taking beta-blocker medication, were actively smoking or previously smoked > 5 cigarettes per month and had a total pack year history >10 years, females who were pregnant or breast feeding,

All subjects had provided informed consent for the study they originally participated in, and they had also provided consent for their bio-specimens to be placed in the UCSF Airway Tissue Bank for studies in addition to the original protocol. All studies and airway tissue bank procedures were reviewed and approved by the UCSF Committee on Human Research.

Sputum induction and processing

Sputum induction and processing was done similarly in all protocols that contributed induced sputum samples to the tissue bank. Subjects inhaled nebulized 3% saline through a mouthpiece for 12 minutes, as previously described(1,2). Subject interrupted inhalation at two minute intervals to spit saliva into a saliva cup and induced sputum into a sputum cup. Saliva was discarded and induced sputum was processed within one hour. A 10% solution of Sputolysin (EMD Millipore) was added at a 1:1 g:ml (sputum weight : Sputolysin) ratio to the induced sputum, mixed using a serological pipette, and placed in a 37ºC shaking water bath for 15 minutes. Samples were removed at 5, 10, and 15 minute intervals for additional mixing with the pipette, and a portion of this sample was used to determine total and differential cell counts, as previously described. The sample was then centrifuged in the cold (4ºC) at 2000 rpm for 10 minutes. The remainder of the cell pellet was then resuspended in one 1mL of Qiagen RNAprotect Saliva Reagent.

RNA Sequencing

RNA was sequenced using the Ion PI template OT2 200 kit v3 for templating and the Ion PI sequencing 200 kit v3 kit for sequencing (ThermoFisher MA, USA). Barcoding allowed RNA samples to be combined and sequenced on PI sequencing chips and sequenced with an Ion Proton Sequencer using standard protocols. Read mapping was performed with the TMAP algorithm on the Proton server and read count table for each gene amplicon was generated using the Proton Ampliseq plugin. Read counts for gene amplicons across all runs were merged to generate the final raw expression data.

Table E1: Immune Cell Accession Numbers from GARVAN and IRIS databases.
(excel file)
Table E2: Gene Sets Specific for the Immune Cell Types
(excel file)
Table E3: 24 Weighted Gene Co-expression Networks (WGCNA) in sputum cells from asthma and healthy control subjects.
(excel file)
Table E4: Clinical Features of 27 healthy control subjects and 84 asthma subjects

Characteristic	Healthy (N=27)	Asthma (N=84)
Age (years)	37.9 (12.1)	41.0 (16.0)
Female sex - no. (%)	17 (63)	51 (61)
BMI (kg/m^2)	25.4 (6.5)	29.4 (6.8)
FEV1 % predicted	98.0 (12.7)	79.8 (15.0)
Blood Eosinophil (X 10^6/L)	122 (107)	295 (180)
Sputum Eosinophil %	0.3 (1.1)	3.0 (4.8)

Table E5: Immune Cell enriched WGCNA eigengene value differences between healthy control subjects (n=27) and T2 Low asthma subjects (n=35).

WGCNA Module	Mean Difference (Healthy-T2 Low)	p-value	FDR
Black	-0.036	0.081	0.162
Brown	0.014	0.542	0.645
Cyan	-0.053	0.036	0.108
Dark Green	<0.001	0.957	0.967
Dark Red	0.029	0.192	0.288
Green	0.052	0.032	0.108
Midnight Blue	-0.013	0.591	0.645
Red	0.030	0.191	0.288
Royal Blue	-0.063	0.002	0.029
Tan	-0.041	0.073	0.162
Turquoise	-0.025	0.267	0.356
Yellow	-0.055	0.032	0.108

Supplemental Figure E1: Multidimensional scaling of the top 500 most variant genes. Samples two standard deviation above the mean of the first principle component (x-axis) and the second principle component (y-axis) were removed (square points, n=11). The remaining points (circles, n=111) were analyzed. Red points indicate asthma samples blue points indicate healthy samples.

Supplemental Figure E2: Relationship between WGCNA modules and phenotypic features of asthma. A) Nine sputum WGCNA networks with associations to clinical traits. Each row corresponds to a network eigengen and each column to a clinical trait. Each cell contains the corresponding correlation and p-value. Red shading represents positive correlation and blue shading represents negative correlation. B) The cyan sputum WGCNA eigengene values were highly correlated to age C)

The royal blue sputum WGCNA eigengene values were significantly lower in obese patients and the green sputum WGCNA network was significantly higher in obese patients.

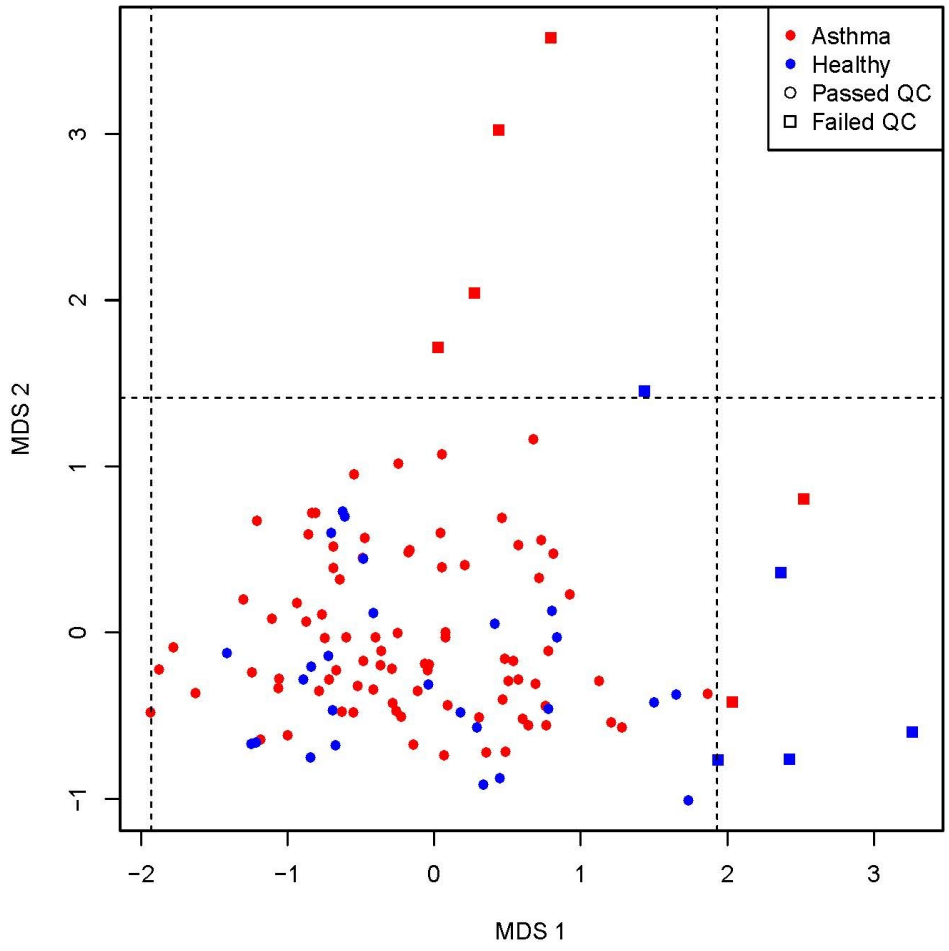
Supplemental Figure E3: Functional enrichment analysis of the black module. Gene ontology (GO) and KEGG mapping identify pathways of IL-2 production (red genes), activation of innate immune response and NF-KB (green genes), and IL-1 receptor activity (blue genes) in the black module.

Supplemental Figure E4: Royal Blue module is decreased in obese (BMI≥30) asthma subjects (n=31) compared to non-obese (BMI<30) asthma subjects (n=53). * Indicates significantly different $p<0.005$

Supplemental Figure E5: Royal Blue module negatively correlates to body mass index in patients on ICS and those not on ICS. Red dots indicate asthma subjects, blue dots indicate healthy control subjects.

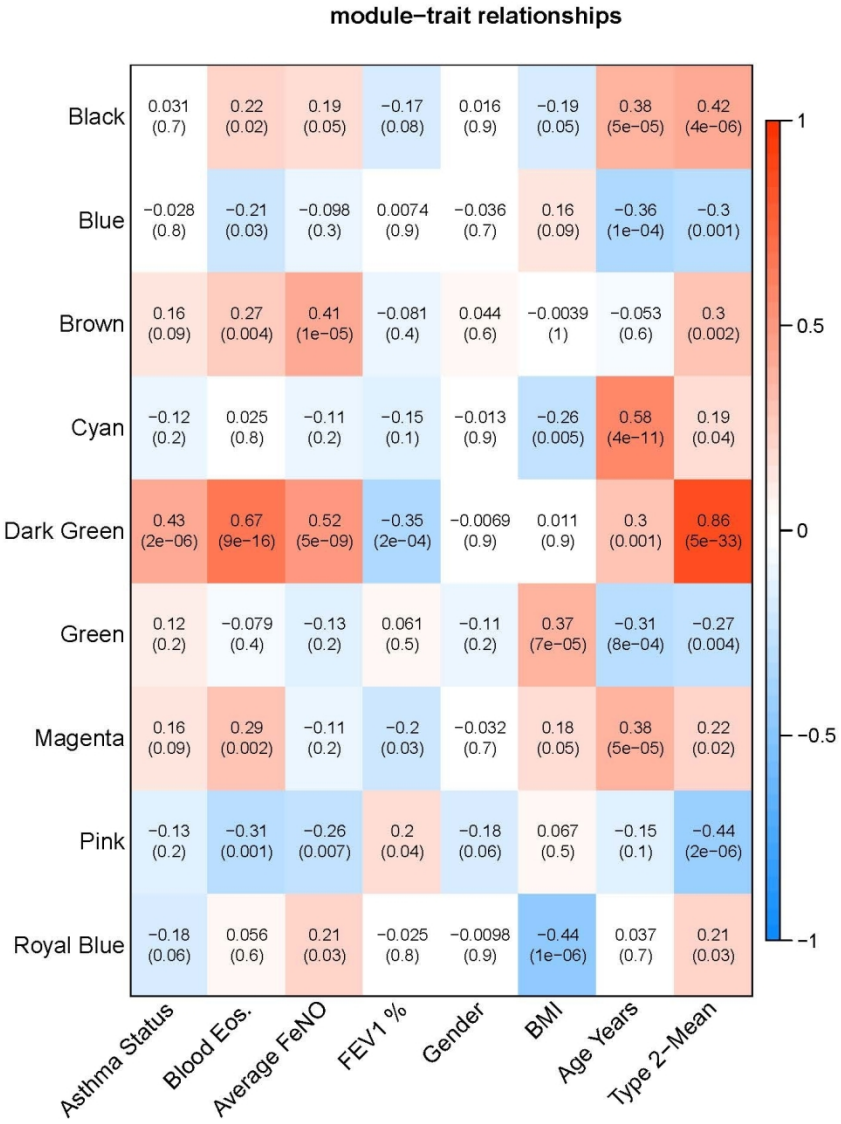
References:

1. Gershman NH, Wong HH, Liu JT, Mahlmeister MJ, Fahy JV. Comparison of two methods of collecting induced sputum in asthmatic subjects. Eur Respir J. 1996;9(12):2448–53.
2. Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. J Allergy Clin Immunol. 2014;133(2):388–94.



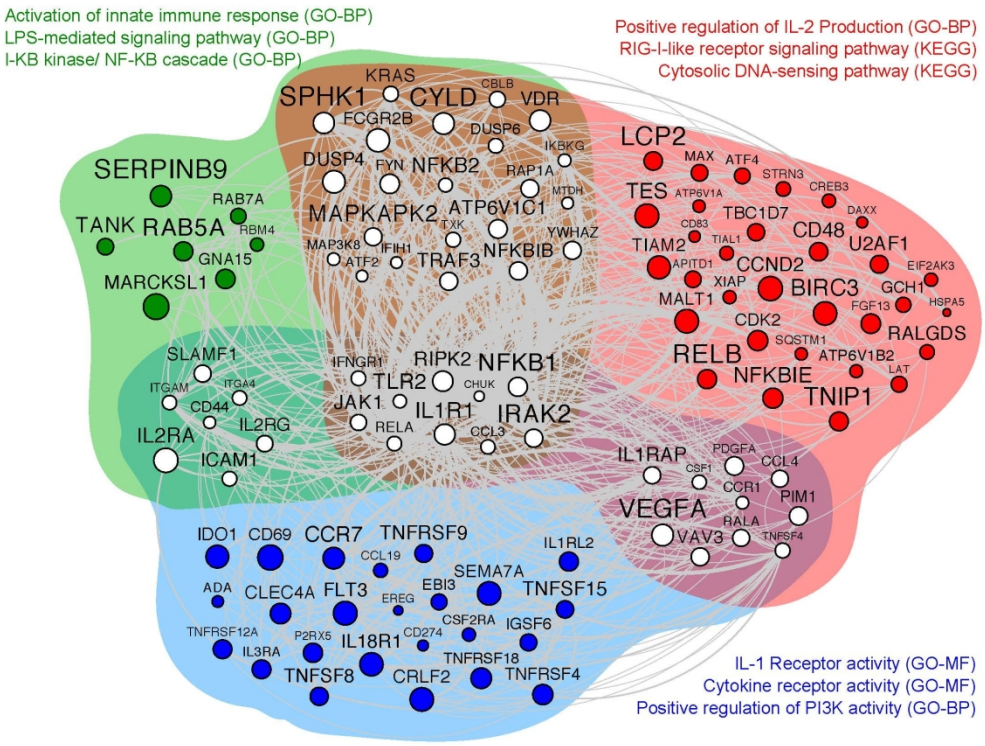
Supplemental Figure E1

177x177mm (200 x 200 DPI)



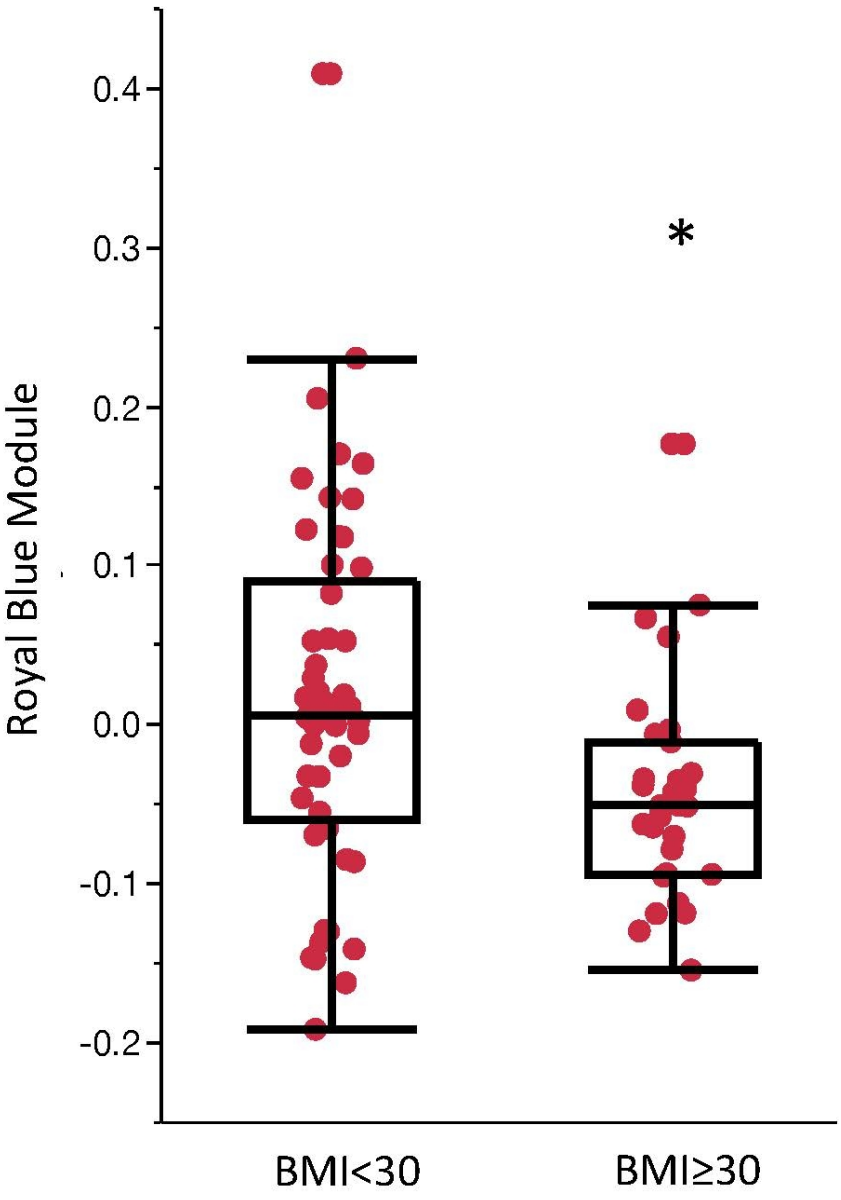
Supplemental Figure E2

203x254mm (200 x 200 DPI)



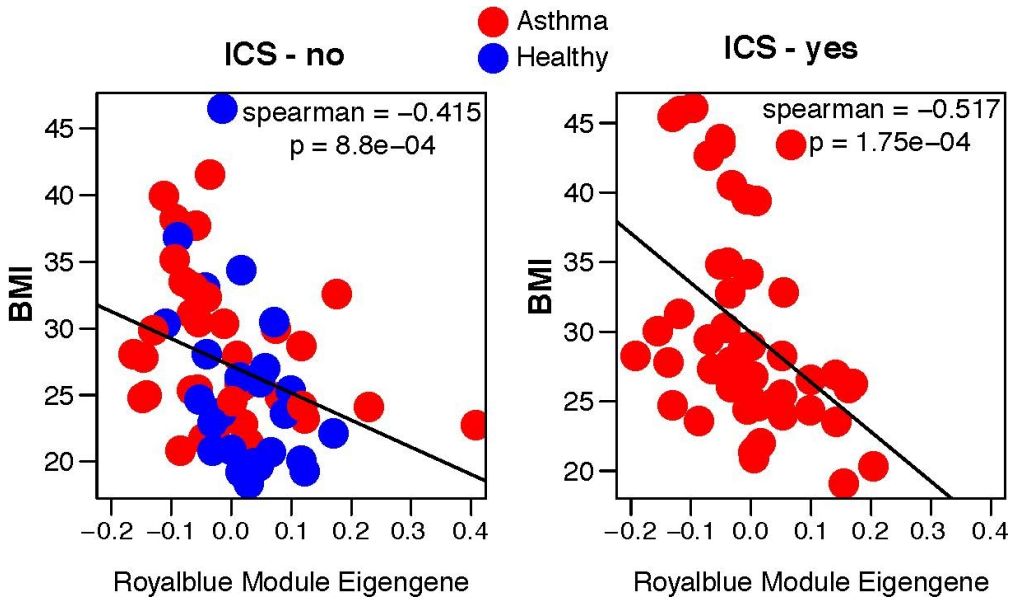
Supplemental Figure E3

279x215mm (200 x 200 DPI)



Supplemental Figure E4

117x163mm (200 x 200 DPI)



Supplemental Figure E5

154x91mm (200 x 200 DPI)