Supplementary File:

Detailed Methods:

Subjects:

Non-smoking (<5 packyears) Wake Forest subjects with asthma (ATS criteria) underwent comprehensive phenotypic characterization as approved by Institutional Review Board (IRB00021507); samples from sputum induction (N=116),[1,2,] and in a subset of subjects who additionally consented to undergo bronchoscopy, bronchoalveolar lavage (BAL) and biopsies (N=59)[3] were obtained. Criteria for designating "severe" asthma were taken from the ATS Workshop on Refractory Asthma (2000) which included ongoing daily symptoms, additional medications, low lung function, and increased healthcare resource needs despite treatment with either high dose inhaled or systemic corticosteroids. It should be noted that the subjects providing samples in our studies were examined during a stable period, without recent infection or exacerbation, which may alter mediator and receptor expression as observed following segmental allergen challenge[4].

Sputum:

The sputum induction and processing methods to separate fluid and cellular fractions were adopted from the Asthma Clinical Research Network[1,2,5,6]. Separate sputum cell cytospins were prepared for differential cell counts and for immunocytochemistry. Aliquots of sputum supernatant were stored at -80ºC until assayed for inflammatory cytokines for those subjects with acceptable sputum[2] and adequate volume of supernatant for cytokine assay.

Cytokine Assay:

Aliquots of sputum and BAL supernatant fluid were assayed with Milleplex Human Cytokine/Chemokine Panels I, II, and III (catalog numbers HCYTOMAG-60K, HCYP2MAG-62K, and HCYP3MAG-63K, respectively, Merck Millipore) per the manufacturer's instructions and as previously described[7].

Bronchoscopy Procedure and Sample Processing

Bronchoscopy with BAL and endobronchial biopsies that were examined for TRAIL and airway remodeling was performed in 59 subjects who consented to this extra procedure in SARP. The procedure has been described in detail[3,8], for a larger group of subjects, but not all subjects provided sufficient sample for the measures reported here, or, at the discretion of the bronchoscopist, for safety reasons the procedure was terminated prior to specific sample collection. Bronchoalveolar lavage fluid (BAL) was processed immediately to separate fluid and cellular fractions. Aliquots of BAL fluids were stored at -80°C until used for Millepore Luminex determination of cytokines (see below). BAL cell cytospins were prepared for differential cell counts and for immunocytochemistry. Endobronchial biopsies were formalin-fixed and paraffin-embedded. Staining of biopsy samples is described below.

Genotype Analyses

All TRAIL SNP genotypes (*TNFSF10*) were acquired from the Illumina HumanHap1M BeadChip from previous GWAS of these subjects[9]: rs11720451, rs1131535, rs3136601, rs3815496, rs3136597, rs16845759, rs4894559, rs231986, rs231983, rs2270418, rs6763816. TRAIL high and low groups were tested by Chi square for differences in genotype at each SNP; Trail level associations by genotype at each SNP were examined by ANOVA based on homozygous or heterozygous alleles.

Immunocytochemistry and Immunohistology:

Assessment of TRAIL receptors:

Duplicate subjects' sputum cell cytospins were separately immunostained for TRAIL receptors R1/DR4 and R3/DcR1. Not all sputum samples had sufficient cells, or quality (lower squamous cell contamination) to prepare cytospins in addition to those stained for differential count determination. Those samples with extra slides of sputum cells were immunostained as follows: blocking of biotin and avidin nonspecific sites, incubation with anti-TRAIL R1 and R3 antibodies (R&D MAB625 and MAB6301 mouse monoclonal antibodies, respectively for R1 and R3 receptors at equal concentrations; both were specified by manufacturer at equal concentrations to detect 1-2 ng of ligand); biotin-anti-mouse secondary antibody; streptavidin-alkaline phosphatase, and development with BCIP/NBT colorimetric substrate. All incubation steps with antibodies and developing reagents were kept to equal time intervals for the two different antibodies, and paired slides for these staining procedures were performed at the same time with the same buffers to reduce technical variability. The density of TRAIL R1/DR4 and R3/DcR1 staining was quantitated (Nikon Imaging System Elements) and averaged for at least 100 leukocytes for each subject. To assure that staining was equivalent for the two TRAIL receptor primary antibodies, background density of nonspecific staining on squamous epithelial cells was also assessed and found not to significantly differ ($p= 0.93$) between TRAIL R1/DR4 and R3/DcR1 stained slides.

Assessment of Airway Remodeling:

Sections from formalin fixed, paraffin embedded endobronchial biopsies were stained with eosin and hematoxylin to permit measures of epithelial basement membrane thickness, and proportion of smooth muscle area /biopsy area as characteristics of airway remodeling. Additional sections were immunostained with monoclonal antibody specific for collagen III by standard methods for

development with Vector Red substrate to permit measure of collagen deposition in the submucosal interstitium. Endobronchial biopsy samples were deparaffinized with Microclear twice and then washed with 100% ethanol. Tissues were rehydrated through decreasing concentrations of ethanol (95%, 70%, 50%, 30%) and washed with PBS. Following rehydration, samples were incubated in DAKO Target Retrieval Solution (S1699) at 95-99ºC for 20 minutes. Tissues were then blocked with fetal bovine serum (FBS), avidin and biotin (Vector Blocking kit). The 1º antibody was diluted in FBS, and slides were incubated overnight at 4ºC. After washing, sections were incubated in biotin-labelled species-specific 2º antibody, AB complex, and alkaline phosphatase substrate (Vector Red + Levamisole). Following development, Mayer's hematoxylin counterstain was applied and coverslips mounted. Images were captured and analyzed with the Nikon Elements Imaging System at either 4x or 40x magnification depending on the endpoint to be assessed; 4X was used for quantitation of collagen III density expressed as mean red per mm^2 and for measurement of smooth muscle area/total biopsy area in immunostained slides; 40X was used for quantitation of basement membrane thickness expressed as mean length in microns from at least 10 noncontiquous measurements, and for counts of immune cells/biopsy area in the subepithelial interstitium.

Statistics:

Demographic, and biomarker data are presented as means $\pm$ standard deviations, or medians (25%-75% quartiles) for continuous variables, and as %positive for categorical variables. Measures not meeting test for normal distribution were log, or square root transformed. Imputation was not performed for missing data. Continuous variables were tested by parametric; or non-parametric tests; categorical variables by Chi-square tests (SAS 9.2, or Sigmaplot 13). Variables found to have a p value <0.05 were accepted as significant.

Additional Results:

Table S1: Clinical characteristics including severity classification of subjects, medication use, and emergency visits, according to TRAIL Low or High groups are indicated. The TRAIL high and low groups did not differ for use of medications, or number of subjects with severe asthma, but had greater number of subjects with exacerbations provoked by physical activity.

Characteristic:	Low TRAIL (N=59)	High TRAIL (N=57)	P value
Severe asthma N (% positive)	15 (25.4)	9 (15.8)	0.293
Total Inhaled corticosteroid use N (% positive)	38 (64.4)	30 (52.6)	0.272
Total Long-acting b-agonist use N (% positive)	22 (53.7)	26 (45.6)	0.561
Systemic Steroid bursts N (% positive)	3 (7.3)	5 (8.8)	1.000
Emergency visit in past year for breathing problem N (% positive)	10 (17.2)	13 (22.8)	0.112
Exacerbations provoked by physical activity N (% positive)	31 (52.5)	41 (71.9)	*0.050*

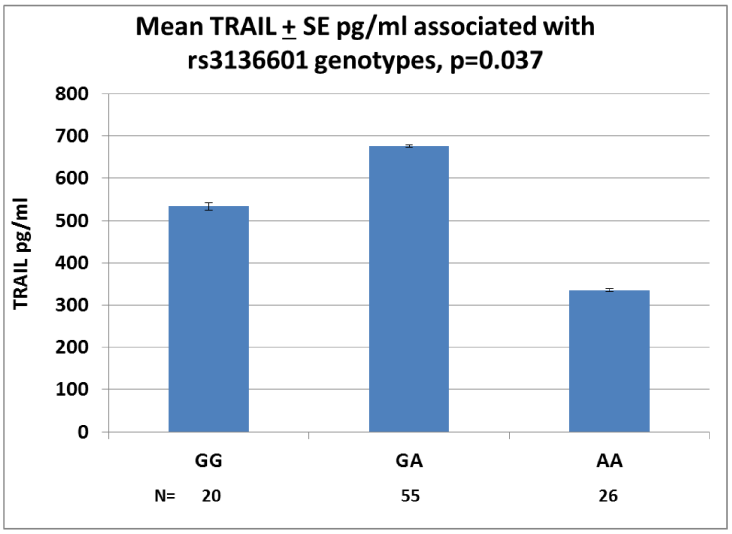
Table S2. Sum density levels were determined for leukocytes in sputum cell cytospins immunostained for TRAIL receptors R1/DR4 or R3/DcR1. The number of leukocytes analyzed are provided in parentheses. All cells in each field of view were analyzed to avoid selection of more densely stained cells, until at least 100 leukocytes had been assessed for each subject. The sum density for nonspecific background staining of squamous cells was also assessed and did not differ between TRAIL receptors R1 and R3 (p=0.933). The stain density for each subject's leukocytes were averaged and means for the two receptors compared.

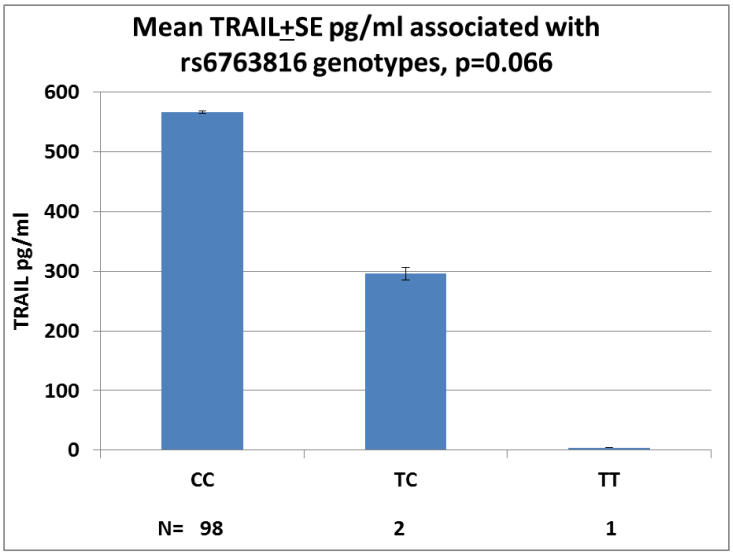
Subjects:	R1 sum density (N)	R3 sum density (N)	P value
0140R	211 (108)	451 (138)	
0217B	206 (121)	815 (100)	
0228F	200 (103)	348 (101)	
0258T	250 (101)	529 (134)	
0485Y	214 (110)	492 (104)	
0192R	132 (100)	189 (119)	
0280N	484 (131)	947 (100)	
Mean Sum Density for each Receptor:	242	539	**0.006**

Table S3. Inflammatory mediator levels associated with sputum granulocyte groups (Low Eos+Low Neu: <2%Eos+<40%Neu; Low Eos+High Neu: <2%Eos+≥40% Neu; High Eos+Low Neu: ≥2%Eos+<40%Neu; or High Eos+High Neu: ≥2%Eos+≥40%Neu as used for subject stratification in Hastie et al, JACI 2010; Moore et al, Allergy Clin Immunol 2013). Il-5, IL-13, CCL5, TGFα, TNFα had significantly highest levels in the subject group with both elevated sputum Eos and Neu. In contrast, IL-10, IL-17A and CCL20/MIP3α were highest in the subject group with Low Eos + High Neu; IFNγ did not reach statistically significant differences among the sputum granulocyte groups.

Cytokine/ Chemokine	**Low Eos + Low Neu**	**Low Eos + High Neu**	**High Eos + Low Neu**	**High Eos + High Neu**	**P value for 4 Eos+Neu groups**
IL-5	1.42 (1.12-2.43)	1.66 (1.11-2.62)	2.14 (1.57-5.56)	3.78 (1.29-6.71)	**0.023**
IL-13	1.61 (0.93-2.51)	1.95 (1.36-2.84)	2.06 (1.27-3.51)	2.28 (1.61-4.41)	**0.047**
CCL5/RANTES	2.55±4.97	9.04±4.48	4.13±5.05	9.75±2.44	**0.002**
TGFα	1.99±3.70	4.11±3.20	2.67±2.39	5.35±2.31	**0.007**
IL-10	2.42 (1.50-3.68)	3.78 (2.33-6.55)	2.63 (1.07-3.49)	2.44 (2.04-4.98)	**0.024**
IFNγ	2.22 (1.33-3.69)	4.11 (1.86-6.38)	2.00 (1.12-3.11)	3.63 (1.40-5.35)	0.056
TNFα	3.44±2.44	6.50±3.82	2.95±2.45	8.09±3.59	**0.004**
IL-17A	0.73±2.88	1.53±3.52	0.46±3.11	1.44±2.86	**0.002**
CCL20/MIP3α	79.3 (31-194)	195 (97-468)	95 (23-293)	143 (67-365)	**0.028**

Figure 1S. TRAIL levels (mean±standard error) for homozygous and heterozygous allelic groups at rs3136601 and rs6763816 are presented with number of subjects shown below.





Supplementary References:

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