

**The Nasal Mucosal Late Allergic Reaction to Grass Pollen involves Type 2
Inflammation (IL-5 and IL-13), the Inflammasome (IL-1 β) and Complement**

Authors: BR Leaker^{1,6}, VA Malkov^{2,6}, R Mogg², MK Ruddy², GC Nicholson¹, AJ Tan⁴, C
Tribouley², G Chen², I De Lepeleire³, NA Calder³, H Chung², P Lavender⁵, LN
Carayannopoulos² and TT Hansel^{4,7}

Affiliations:

¹Respiratory Clinical Trials Ltd., London, UK.

²Merck Research Laboratories, Rahway, NJ 07065, USA.

³MSD (Europe) Inc, Brussels, Belgium.

⁴Imperial Clinical Respiratory Research Unit (ICRRU), St Mary's Hospital, Imperial College,
London, UK.

⁵Department of Asthma, Allergy and Respiratory Science, King's College, London, UK.

⁶The first 2 authors contributed equally to this work

⁷Correspondence: TT Hansel (t.hansel@imperial.ac.uk)

One Sentence Summary: Nasal mucosal allergy involves multiple inflammatory pathways

Key words: late allergic reaction, type 2 inflammation, immune response, gene expression, corticosteroid

Key Messages

The nasal late allergic response (LAR) to grass pollen involves parallel pathways:

- type 2 inflammation and IL-4/5/13
- inflammasome involvement and IL-1 β
- complement
- circadian-associated regulators of gene expression

Capsule Summary

Non-invasive mucosal sampling (nasosorption and nasal curettage) was used following nasal allergen challenge with grass pollen in subjects with allergic rhinitis. The late allergic response (LAR) involves parallel changes in pathways of type 2 inflammation (IL-5 and IL-13), inflammasome-related (IL-1 β), complement and circadian-associated genes.

Abbreviations

allergic rhinitis (AR); nasal allergen challenge (NAC), late allergic response (LAR); total nasal symptom scores (TNSS)

ABSTRACT

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Non-invasive mucosal sampling (nasosorption and nasal curettage) was used following nasal allergen challenge with grass pollen in subjects with allergic rhinitis, in order to define the molecular basis of the late allergic reaction (LAR). It was found that the nasal LAR to grass pollen involves parallel changes in pathways of type 2 inflammation (IL-4, IL-5 and IL-13), inflammasome-related (IL-1 β), and complement and circadian-associated genes. ~~We used non-invasive mucosal sampling method to define the molecular basis of the LAR.~~ A grass pollen nasal spray was given to subjects with hay fever followed by serial sampling, in which cytokines and chemokines were measured in absorbed nasal mucosal lining fluid, and global gene expression (transcriptomics) assessed in nasal mucosal curettage samples. Twelve of nineteen subjects responded with elevations in IL-5, IL-13, IL-1 β and MIP-1 β /CCL4 protein levels in the late phase. In addition, in these individuals whole genome expression profiling showed upregulation of type 2 inflammation involving eosinophils and IL-4, IL-5 and IL-13; neutrophil recruitment with IL-1 α and IL-1 β ; the alternative pathway of complement (factor P and C5aR); and prominent effects on circadian-associated transcription regulators. Baseline IL-33 mRNA strongly correlated with these late phase responses; while a single oral dose of prednisone dose-dependently reversed most NAC-induced cytokine and transcript responses. This study shows that the late phase allergic response to grass pollen involves a range of inflammatory pathways and suggests potential new biomarkers and therapeutic targets. Furthermore, the marked variation in mucosal inflammatory events between different patients suggests that in the future precision mucosal sampling may enable rational specific therapy.

INTRODUCTION

Asthma and allergic rhinitis (AR) are increasing in prevalence in industrialized societies due to complex gene-environmental and microbial influences¹. Both asthma and AR are inflammatory disorders of the airways in which similar cell types and molecular pathways participate^{2, 3}. Furthermore, nasal and bronchial disease co-exist in many asthma patients, with similar immunopathology, underlying the “one airway” hypothesis⁴.

Mast cells are the main effector cells of acute/early allergic responses, through allergen cross-linking specific IgE molecules on the mast cell surface, and subsequent triggering of mast cell degranulation and release of vasoactive mediators⁵. Eosinophils are involved in acute and chronic mucosal inflammation and migrate into the nasal and bronchial mucosa during the late allergic reaction (LAR). Allergic inflammation is frequently described as “type 2 inflammation” and has a characteristic gene expression signature in bronchial epithelium⁶ and sputum of asthmatics⁷. IL-5 and IL-13 mediate many of the features of type 2 inflammation, being produced by T helper type 2 (Th2) cells and innate lymphoid cells of type 2 (ILC2 cells)⁸.

Access to nasal mucosal secretions and cells is required to understand biomarkers and molecular mechanisms associated with AR. Either lavage or nasosorption using strips of absorptive paper have been used to sample nasal mucosal lining fluid (MLF) mediators⁹⁻¹¹. More recently the nasosorption method has been refined by the use of synthetic materials, and since the approach is non-invasive it has proved ideal for sampling from children with allergic rhinitis¹² and infants¹³. Serial nasosorption samples have been used after nasal allergen challenge (NAC)¹⁴, including to assess the effects of a monoclonal antibody against anti-IL-13¹⁵. Nasal curettage is

a technique to obtain nasal epithelial cells without local anesthesia, and has been utilised for gene expression profiling after human rhinovirus infection ¹⁶, while nasal brushings have been used to define phenotypes of childhood asthma ¹⁷.

Detailed insights into molecular and cellular events in the airway mucosa are needed to define targets for new therapies, provide biomarkers for clinical trials, and to enable personalized medicine ^{2, 18}. This study examined the nasal mucosal late allergic reaction (LAR) by measurement of cytokines and chemokines in MLF, and assessment of gene expression patterns in nasal mucosal specimens. We used non-invasive mucosal sampling, obtaining serial nasosorption samples and nasal epithelial curettage sample at baseline and 8 hours after NAC. We compared the effects of single doses of oral prednisone with placebo on biomarkers of mucosal inflammation after NAC. In this manner we documented molecular events of the nasal mucosal LAR and the effects of corticosteroids within individual subjects.

RESULTS

Subjects and symptoms

Nineteen subjects with a confirmed history of seasonal allergic rhinitis and skin prick positive allergy to grass pollen were recruited and studied outside the London grass pollen season (Figure 1) (Table E1) (Figure E1). There were no significant adverse events and all subjects completed the study. NAC induced a prominent rise in early (1h) mean TNSS with a diminution of symptoms into the late phase (2-8h) (Figure E2). Participants were observed by clinical research staff to have allergic symptoms immediately after NAC, though the first TNSS after NAC were formally assessed at 1 hour. Symptoms consisted of sneezing for a few minutes, nasal discharge, and nasal and soft palate itching. Symptoms in the late phase (2-8h post NAC) are subjective, variable and of low magnitude: in our study reaching mean levels of approximately 2 out of 12, and being generally due to nasal obstruction. However, there were non-significant trends for IL-13 Responders (see definition in Methods - Statistical Analyses) to have higher early (1h) and late phase (2-8h) TNSS (Figures E2 and E3, with individual responses shown in Figure E4). Prednisone also showed a non-significant trend for decreased late phase (2-8h) TNSS in Responders, when compared to placebo TNSS. TNSS in the late phase (2-8h) correlated with those in the early phase (0 to 1h) in the placebo arm followed by NAC ($r=0.774$, $P=1.01E-04$) and in the prednisone at 10mg arm ($r=0.622$, $P=4.49E-03$), while approaching statistical significance in the prednisone at 25mg arm ($r=0.449$, $P=0.0536$). However, blood eosinophil counts, age and gender did not predict the late phase TNSS (2-8h) nor responder status.

Nasal MLF cytokines and chemokines

Overall, grass pollen NAC resulted in increases in type 2 cytokines (IL-5 and IL-13) and innate-

factors (CCL4/MIP-1 β and IL-1 β) from 2 to 8 hours, comprising a late-phase cytokine/chemokine response (Figure 2A and Figure E5) (Tables E2-E4). Single dose pretreatment with prednisone caused large and statistically significant dose dependent reduction of NAC-induced IL-5, IL-13, IL-1 β and CCL4 compared with placebo (Figure 2A). The degree of correlation (>0.9) between IL-5 and IL-13 fold changes under the different treatment regimens was remarkably high (Figure 2B). Inspection of individual patient IL-5 and IL-13 responses revealed significant between-subject variation that was used as the basis for defining responders (see Methods - Statistical Analyses) (Figure 2C and Figure E6). IL-1 β is present in unchallenged noses and levels increase slightly following NAC. However, corticosteroid given before NAC causes an apparent decrease in levels of IL-1 β that we speculate may be due to the effects of steroids and repeated sampling.

mRNA profiling

Prespecified analyses of mRNAs and cell type-associated signatures responsive to allergen and prednisone

Initial prespecified analysis of all participants' nasal mRNA using an absolute fold-change threshold of 1.5 and a Benjamini-Hochberg FDR set at $\leq 10\%$, yielded 2,693 and 136 probesets responsive to allergen and 25mg prednisone, respectively (Datafile S1). Among transcripts strongly increased by NAC, those of immunologic receptors and secreted products were among the most frequent. This included those associated with the expected allergen-activated type 2 inflammation elements such as eosinophils and Th2 cells (e.g. CLC/galectin-10, HRH/histamine receptor 4, CCR3/eotaxin receptor, CCL23, and CD69), as well as those associated with broader immune/inflammatory responses and innate immune effectors (e.g. IL1A, IL1B, OSM/oncostatin

M, CCL2, TGFB2, CD300, CLEC4A, and CD1C). In contrast, mRNAs with decreased levels following NAC include those of proteins participating in circadian-related signaling (e.g. PER1, PER3, NR1D1/REV-ERB α , and C1orf51/CIART) and other non-immune functions including epithelial growth and structure (e.g. MCAM/CD146, NRCAM, CYP1B1, LCE3D, LAMB1/laminin beta 1), cell division (e.g. NUSAP1/nucleotide and spindle associated protein, CDC25B, CDCA7, and SKP2). In general, prednisone pretreatment led to suppression of NAC-induced changes, with a few exceptions such as TGFB2. Figure 3A illustrates the strongly opposing effects of allergen and prednisone that were observed.

In addition to individual mRNA levels, the expression of prespecified multigene composite scores associated with eosinophils, neutrophils, and mast cells was also measured (Table E5). As shown in Figure 3B, eosinophil and neutrophil-related composite scores showed strong post-NAC average induction, and suppression by prednisone pretreatment.

Patterns of inter-individual gene expression: responders and non-responders

In order to identify natural patterns in NAC-response expression data, two unsupervised approaches were applied. In the first, hierarchical clustering of the 365 probe sets strongly responsive to allergen (Datafile S1) was performed using correlations between changes in expression (8 hours after NAC vs. study baseline) as the distance metric. In this analysis, IL-13 responders were more likely to be similar in terms of gene expression whereas non-responders had distinct and more heterogeneous patterns (Figure E7). To confirm this finding, a second approach - principal components analysis (PCA) - was applied. The first two principal components, encompassing 55% of the total variance, sufficed to separate IL-13 responders from

non-responders (Figure E8). Indeed, the first principal component alone, encompassing ~41% of the total variance, not only separates nasal type 2 cytokine responders from non-responders, but also correlates strongly ($r = \sim 0.8$, Figure 4A) with GMTWA_{2-8hrs} of type 2 cytokine secretion, suggesting commonality of the biology underlying IL-13 production and mRNA content changes following NAC.

Baseline gene expression and the IL-13 Response

Baseline gene expression was measured during the screening period, that was 1-3 months before subjects were given placebo and then subsequent NAC; the time gap across subjects differed based on randomization and exact period when placebo was given. . This baseline expression was examined to determine whether genes associated with asthma might also relate to the post-NAC IL-13 responses. Published GWAS has identified a number of genetic modifiers of asthma: CHI3L1, IL6R, DENND1B, IL1RL1, IL18R1, PDE4D, RAD50, IL13, IL33, SMAD3, ORMDL3, GSDMB, IL2RB and PYHIN1¹⁹. Accordingly, correlations of these gene transcript levels with IL-13 and IL-5 cytokine responses was measured. Of these correlations, only that between baseline IL33 mRNA and NAC IL-13/IL-5 response had Bonferroni-corrected significance below 0.05 (Figure 4B, Table E6). NAC late phase responders tended to have higher levels of pre-challenge IL33 nasal mucosal mRNA than did non-responders. The level of baseline IL33 mRNA expression showed a strong correlation with the magnitude of type 2 cytokine induction following NAC ($r \sim 0.7$, $p < 0.01$, Figure 4B) - suggesting a mechanistic basis for variation in magnitude of type 2 cytokine responses to allergen.

Further analysis was carried out for potential *de novo* gene signatures at baseline with a

reasonable FDR (i.e. <0.2) that would predict subsequent NAC Responders (Table E7). However, the study sample size was based on the primary end-point (PEP), and was too small to detect baseline prediction gene signatures.

A correlation matrix and dendrogram (Figure E9) demonstrated that Responder status is associated with certain parameters measured after NAC: IL-13 and IL-5 protein, the first principal components analysis (PCA1) across the allergen gene expression signature, the eosinophil gene signature, and gene expression for CCL23, IL-4, IL-13 and CD69. There is weaker correlation with a cluster for the neutrophil gene expression signature, IL1A and IL1B. The Responder group had only weak correlation with total nasal symptom scores (TNSS, 2-8h). Importantly, as expected given its strong correlation with IL-5 and IL-13 levels, IL-33 gene expression at baseline correlated with post-NAC IL-13 Responder status, (Figure E10). In addition, TNSS at 2-8h did not correlate with IL-5 and IL-13 protein or mRNA expression (Figure E11).

Pathways of inflammation genes and circadian-associated genes

Ingenuity Analysis was performed in IL-13 responders, with 1214 genes having a FDR of ≤ 0.05 , of which 41 genes were selected on the basis of their immune function (being cytokines, chemokines, soluble immunoregulatory factors and their receptors) and being involved in circadian-associated transcription regulation (Figure 5). Ingenuity Pathway Analysis was applied to the mRNA profiling data using the Core Analysis tool to identify canonical pathways enriched with genes determined to be differentially expressed (Figure 5A).

Increased gene expression after NAC involves 4 main inflammatory pathways: type 2 inflammation (IL-4, IL-5, IL-13 and CCR3); innate immunity mediated through IL-1 α , IL-1 β , IFNAR1 and the inflammasome; and the alternative pathway of complement activation (factor P and C5AR1) (Figure 5B). Significantly decreased gene expression was found for all 5 components of classical complement factor 1 (C1QA, C1QB, C1QC, C1R, C1S). Gene expression for circadian-associated mRNA was particularly modulated by allergen challenge. Figure 5C shows the effects of prednisone 25mg in NAC-induced changes: and this is approximately the opposite of the NAC effect, demonstrating the potent and broad effect of corticosteroids on the allergic inflammatory response. Table 1 (responses averaged over responders) and Figure 6 (individual patient responses) provide quantitative views of these observations.

Individuals responding to NAC with IL-5 and IL-13 in nasal MLF, also showed increases in eosinophil-related, type 2-like, neutrophil-related, IL-1-related, and circadian-associated gene expression (Table 1 and Figure 6). Prednisone pretreatment, as in the averaged transcript effects shown in Figure 3A, clearly suppressed allergen effects at the individual level for the analytes shown in Figure 6B. However, in some patients the complement signal was not inhibited by corticosteroid. Circadian-associated genes were differentially regulated either up or down by NAC; notably, in some patients these changes were not opposed by corticosteroid.

DISCUSSION

This study indicates that the nasal LAR involves gene and protein expression changes reflecting a set of inflammatory processes: type 2 eosinophilic inflammation associated with IL-4/5/13 release, inflammasome-type neutrophilic activation with IL-1 α/β , upregulation of the alternative pathway of complement involving factors P and D, and involvement of coagulation through the thrombin receptor (protease-activated receptor (PAR)-1). Stimulation of innate immune responses by grass pollen is consistent with house dust mite allergen having been shown to functionally mimic a component of the toll-like receptor (TLR) 4 complex and causing an innate immune response²⁰. In addition, grass pollen *Phleum P1* can directly stimulate IL-1/6/8 production by airway epithelial cells²¹, while whole grass extracts can stimulate bronchial epithelial cells to secrete cytokines²².

Serial non-invasive sampling was carried out from the nasal mucosa to provide a range of biomarkers that reflect molecular features of the reaction to allergen. We demonstrated the utility of this approach in relation to a single oral dose of corticosteroid, establishing NAC in the context of serial precision mucosal sampling from an accessible nasal site. There was a focus on establishing the features of the IL-5 and IL-13 response by immunoassay of MLF, and linking this response with gene expression profiling, and then identifying potential molecular biomarkers. Having studied actions of a corticosteroid, this type of sampling approach with NAC could also be useful in the context of new drug development in early clinical phase human studies. In this way by studying a novel drug in a NAC design in a small well-defined population, it is possible to evaluate biomarkers and establish a pharmacodynamic profile.

The nasal LAR is generally less distinct in terms of symptoms (TNSS) and physiological changes when compared to the late asthmatic reaction after inhaled allergen challenge, that can be precisely quantitated in terms of a decrease in forced expiratory volume in 1 second (FEV₁)²³. The early nasal allergic reaction involves rapid onset of a range of nasal symptoms, but the late nasal response is generally due to partial nasal obstruction. The occurrence of peak symptoms in the first 30 minutes after NAC, with low levels of TNSS in the late phase has been found by other investigators, who also found that a single dose of topical nasal corticosteroid was found to have a minimal effect on symptom scores²⁴. However, in our study there were consistent trends for IL-13 responders to have more symptoms in the late phase, and for a single dose of oral prednisone to decrease the level of these symptoms.

A weakness in our study is that TNSS and nasal function were not measured during the nasal early allergic reaction (EAR) within the first hour of NAC. Nasal function can be measured by peak nasal inspiratory flow (PNIF)¹⁴, acoustic rhinometry or magnetic resonance imaging (MRI)²⁵. In addition it would have been of interest to have measured mediators during the EAR, such as tryptase¹⁴, PGD₂ and IL-12/23p40²⁶. Hence it is not possible from the current study to assess the influence of symptoms, nasal function and mediators during the EAR on the subsequent LAR. Indeed, mast cells are known to be major players in the EAR and could have an important controlling influence on immune events in the LAR. Furthermore, it will be important to assess the clinical significance of having a LAR after NAC, both after NAC and in relation to natural exposure. However, given the weakness and variability of TNSS in the LAR, a larger sample size would be required for this endpoint.

Measuring the late nasal IL-13 response in MLF after NAC allowed the formal *post hoc* identification of responder subjects (12 of 19). Since a pre-specified primary endpoint (PEP) was the geometric mean time weighted average (GMTWA) for IL-13, “responder” status was defined as nasal IL-13 GMTWA being significantly elevated compared to baseline levels. Responders had a non-significant trend for an increase in symptoms in the late phase. In addition, the biologic validity of selecting responders was supported by the concordance of IL-13 responses with those of IL-5 and IL-1 β , as well as with the first principal component across the allergen mRNA signature, and post-NAC eosinophil-related gene signature levels.

A range of clinical studies have been carried out in severe eosinophilic asthma using biologics to inhibit type 2 inflammation. These have involved anti-IgE and monoclonal antibodies that block interleukins 4, 5 and 13¹⁸. In particular, recent studies with anti-IL-5 have selected patients for therapy based on a raised blood eosinophil count^{27, 28}. Our study in patients with allergic rhinitis illustrates that raised IL-5 in the LAR only occurs in some patients. This raises the question whether in patients with allergic rhinitis and allergic asthma there may also be considerable variation in levels of mucosal type 2 inflammation cytokines. In addition, we speculate that measurement of levels of cytokines in MLF, employing nasosorption and/or bronchosorption may assist in stratification of patients with a range of respiratory diseases, and selection of patients for therapy with specific biologics.

In patients with AR it is logical to measure IgE responses at the target organ, and much clinical research has been performed on local IgE production by the nasal mucosa²⁹. In our study we did not measure IgE responses in nasosorption samples, although IgE has been measured in nasal lavage and nasal aspirates in allergic rhinitis^{30, 31}. In addition, it has been found that NAC can

boost serum allergen-specific IgE at 4 weeks after a single NAC ³². Hence, following natural allergen exposure and after NAC with a range of natural and recombinant allergens, it would be of interest to measure nasal and serum total and specific IgE responses.

Following NAC we used gene expression in nasal curettage samples to show prominent increases in an eosinophil signature, but also a concomitant influx of neutrophils. Neutrophil involvement in allergic reactions is well described and occurs at an earlier stage than eosinophil recruitment. Neutrophils are associated with cytokines such as IL-1 α and IL-1 β , and their recruitment may be partially mediated through IL-8. Collectively, this might reflect the presence on epithelial cells of pattern-recognition receptors that can be activated by grass pollen, cause chemokine and cytokine production, and contribute to eosinophil and neutrophil recruitment in the LAR ³³. Production of IL-1 α and IL-1 β has been described in association with the nasal LAR ³⁴, following nasal lipopolysaccharide challenge ³⁵, these events may be associated with the inflammasome ³⁶. We were also interested to find elevation in IFNAR1 during the LAR, since this is an important regulator of IL-1 production and activation.

The alternative pathway of complement activation is a fundamental arm of the innate immune response to microbial pathogens ³⁷. The current study showed upregulation of genes for the alternative pathway (CFP, C5aR1) and down regulation for genes of the classical pathway (especially genes for components of C1) following NAC ³⁸. Complement C3 is expressed by bronchial epithelial cells ³⁹ as well as by mast cells ⁴⁰, and cleavage by mast cell tryptase can generate anaphylatoxins (C3a and C5a) that can bind to mast cell receptors and continue this

activation into the late phase⁴¹. Indeed, our findings are consistent with prior reports of C3a and C5a detection after NAC⁴², with increased nasal mucosal C3aR and C5aR in AR⁴³, and raised plasma C3a and C5a levels during allergen immunotherapy⁴⁴. Recently it has been proposed that C3a and C5a may regulate type 2 and Th17 immune responses to allergens⁴⁵. Furthermore, when peripheral blood mononuclear cells from patients with AR are cultured with pollen allergens, there is dysregulation in genes of the complement system⁴⁶.

NAC increases gene expression for protease-activated receptor PAR-1 (thrombin receptor), suggesting an influence on coagulation. Thrombin levels have been shown to be elevated after NAC⁴⁷. Together, these features of the LAR suggest that grass pollen extract may act as a “microbial mimic” and trigger a diverse innate mucosal inflammatory response involving IL-1 β production with complement and coagulation activation.

This study also showed that *baseline* IL-33 gene expression in nasal curettage specimens taken from AR subjects at screening correlated strongly with the intensity of the subsequent LAR in the placebo arm after NAC. IL-33 is elevated on immunoassay of nasal secretions from patients with allergic rhinitis⁴⁸, and polymorphisms in genes for the IL-33-IL-1 receptor-like 1 (IL1RL1) pathway have been associated with asthma^{19, 49}. Importantly, IL-33 is found preformed in epithelial cells as an alarmin, and is released by the nasal mucosa in response to stimuli such as mechanical contact¹⁰; and IL-33 may be important in activating ILC2 cells⁸.

The fold-change induction of nasal IL-5 and IL-13 measured by immunoassay in this study were strongly correlated, suggesting a common cell of origin, perhaps by allergen-specific T helper

type 2 (Th2) cells and/or ILC2 cells (27). In a recent study flow cytometry was performed on nasal curettage dispersed cells, these cells were obtained before and after NAC with grass pollen in allergic asthmatics⁵⁰. It was shown that allergic asthmatics had an enrichment of ILC2 cells at baseline, and these numbers significantly increased after NAC, and correlated with peak IL-5 levels after NAC. However, it is still important to quantitate the relative numbers of Th2 and ILC2 cells that are responding to NAC. Thus we hypothesize that baseline IL-33 expression reflects a rheostat-like mechanism that sets the response intensity of the mucosa to allergen exposure by regulating Th2 cells and/or ILC2 cells in the production of IL-5 and IL-13. If correct, this would extend the involvement of innate mechanisms in allergy, and may offer further therapeutic options⁸.

Unexpectedly, circadian-associated regulatory transcripts were strongly modulated during the LAR. Circadian influences control corticosteroid production, and molecular details of circadian-associated gene roles in immunity have been defined⁵¹. More specifically, an epithelial circadian clock has been shown in Clara cells to control pulmonary inflammation and glucocorticoid action⁵². In our gene expression analysis of the subjects with a LAR we noted a profound differential effect on genes of the BMAL-1-CLOCK complex: with up-regulation of genes for activating factors such as BMAL-1, RORA and RORC; while there was down-regulation of genes for repressing factors such as PER1-3, Rev-ERB α and CIART. However, the strong decreases seen in PER1, CIART, and NR1d1 are very likely of epithelial origin, since down-regulation in gene transcription by cells newly entering the sampled tissue are not likely to be reflected as strong mRNA reductions. Our clinical observations are consistent with recently described effects of epithelial circadian genes on inflammation in

murine airway irritant models ^{52, 53}. Circadian gene expression changes were relatively weakly affected by prednisone pretreatment, suggesting that these may be useful targets for new therapy to complement corticosteroids – a hypothesis requiring testing in studies controlling for diurnal factors.

This study provides insights into the molecular basis of the nasal LAR to grass pollen. Using serial mucosal sampling, parallel inflammatory pathways were demonstrated, and levels of inflammatory mediators were correlated with gene expression data. In addition, a dose response of a single dose of an oral glucocorticoid (prednisone) was demonstrated on cytokine and mRNA expression. The mixed immunopathology of the LAR has profound implications for therapy, since current anti-allergic medications tend to be based on inhibiting type 2 inflammation. However, our results suggest potential roles of the inflammasome, complement activation and circadian-associated genes in mediating immunopathology. A focus on these novel nasal mucosal pathways could potentially be used to select AR patients for rational specific therapy.

METHODS

Study design. We performed a randomised double blind 3-way cross-over study to compare the effects of 2 single doses of oral prednisone (10mg, 25mg) with placebo on biomarkers of mucosal inflammation and transcriptomics post NAC (Figure 1A and 1B). All recruitment and study procedures were timed to occur outside the summer grass pollen season. Subjects were required to have a history of seasonal allergic rhinitis (AR) to grass pollen, and also positive intra-epidermal skin prick tests to Timothy grass pollen extract. Patients with a history of other allergic diseases including asthma, eczema and food allergy were excluded from the study. Blood eosinophil counts were measured at baseline in all subjects. Total and specific serum IgE levels were not measured. The study was registered with ClinicalTrials.gov as NCT0082806, and was overseen by the Research Ethics Committee of Hounslow and Hillingdon (London, UK).

Nasal Allergen Challenge (NAC). Timothy grass pollen (Aquagen, *Phleum pratense*; ALK, Horsholm, Denmark) was administered using a nasal Bidose applicator (Aptar Pharma). A single nasal spray (100µL) was given to each nostril in each challenge (5000SQ-U/nostril = approximately 1µg/nostril of *Phleum* P5 (Phl p 5) allergen). Sampling occurred before NAC and hourly thereafter. This repeated nasal sampling does not cause spurious elevations in nasal cytokines and chemokines³⁵.

Total nasal symptom scores (TNSS). TNSS were recorded before NAC and hourly thereafter for eight hours. Nasal congestion, rhinorrhoea, itching and sneezing were scored from 0 to 3 (0=none, 1=mild, 2=moderate, 3=severe symptoms).

Nasosorption: collection of mucosal lining fluid (MLF). Synthetic absorptive matrix (SAM) was cut into 7 mm x 30 mm strips with rounded ends (Accuwik Ultra, Pall Life Sciences, Portsmouth, UK). Accuwik Ultra is no longer available, but a CE-marked nasosorption kit is available from Hunt Developments (Midhurst, West Sussex). Two SAM strips were carefully placed back to back and inserted into the right nostril against the inferior turbinate of the lateral wall of the nasal cavity. Nasosorption was performed for 2 minutes, with application of nasal clips to ensure good apposition on the mucosal surface of the nasal cavity. After withdrawal, SAM strips were placed into 300µL of pre-chilled (4°C) assay buffer (Millipore AB buffer: containing protein, azide and detergent, pH 7.4) preloaded into the upper chamber of a Spin-X cellulose acetate filtration device (Costar). Spin filtration was then performed at 4°C for 5 min at 16,000g. The eluate was collected and immediately stored at -80°C for later immunoassay.

Nasal lavage. This was performed using sterile normal saline (5.0ml) with flushing into the nasal cavity 20 times over ~1 minute⁵⁴. Recovered fluid was then centrifuged to obtain cells and a differential leukocyte count performed after staining a standard cytospin.

Measurement of chemokines and cytokines in MLF. Concentrations of CXCL5 (12.33), IL-1β (0.44), IL-4 (2.83), IL-5 (0.27), IL-8 (2.10), MCP-1 (1.24), MIP1α (20.9), MIP-1β (4.1), and RANTES (4.75) were determined using a Luminex 10-plex bead-based assay (R&D Systems); and those of IL-13 (0.25) using a single-plex plate-based assay (MesoScale Diagnostics). Respective lower limits of quantitation (LLOQ in pg/mL) appear in parentheses.

Nasal mRNA collection and measurement. Nasal scrapes were collected using Rhino-Probe®

plastic nasal cures (Arlington Scientific), with a baseline sample at screening and samples at 8h after each NAC (Figure 1). Cells were immediately lysed in Qiagen RLT buffer, and stored at -70°C until purification using the RNeasy system (Qiagen). RNA quality was assessed using an Agilent Bioanalyzer (RNA 6000 Pico Kit). All analyzed samples had 28S/18S rRNA ratios of >0.75 and <3 and an RNA Integrity Number of >5. Total RNA was amplified using the NuGEN Ovation Whole Blood Solution protocol (NuGEN, Inc., San Carlos, CA). Amplified biotin-labeled material was hybridized to a custom-designed Affymetrix array (Gene Expression Omnibus GEO reference GPL10379). All analyzed arrays had percent of detected probesets >30%. Expression level data were submitted to the US NCBI and are accessible at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67200>.

Statistical analyses. Geometric mean time weighted averages (GM TWA) across the 2 to 8 hour post-challenge time period were compared among the treatments using a mixed ANOVA model for a 3-period crossover design. The model included fixed factors for sequence, treatment, and period, and a random effect for patients within sequence. The between treatment fold differences were estimated as the exponent of the difference in least-square means from the model; 90% CI (one-sided alpha = 0.05) were also obtained from the model. The within treatment differences were estimated in a similar manner. Cytokine concentrations were analyzed after a log transformation, and thus assessed by a fold change relative to baseline.

Sample size was that required for >80% power to detect a 0.5 fold mean reduction in NAC-induced IL-5¹⁵. The R statistical programming environment was used for inferences, correlations, and plot generation involving cytokines and chemokines alone. SAS 9.2 was used for statistical inference, and mixed linear models on cytokines and mRNA data; while Matlab

7.11.2 (R2010b) with Statistics Toolbox was used for profiling-related plot generation, clustering, and PCA on profiling data.

Nasal cytokines and “Responder” status

The primary end-point for nasal cytokines was the fold change from baseline in the Geometric Mean Time-Weighted Average cytokine concentration measured by immunoassay at 2-8 hours (GMTWA_{2-8hrs}). Data in this LAR phase after NAC was log-transformed for analysis. To define participants who responded to NAC with IL-13 protein production (“Responders” and “Non-Responders”), a *post hoc* analysis was done to identify the magnitude of the nasal IL-13 response relative to baseline (-1h). A threshold for IL-13 was calculated from our data at baseline, below which 95% of fold changes in an unchallenged population would be expected to fall. Values of IL-13 greater than this threshold were used to identify “Responders”.

Prespecified mRNA expression analysis. Normalization was performed using RMA quantile normalization. Only probes with Affymetrix MAS5 $P > 0.05$ in $\geq 50\%$ of samples in any treatment group were analyzed. This left 31,892 probesets for analysis, of the 52,369 on the array. Generally, statistical analysis was restricted to probesets showing fold changes > 1.5 . Probeset expression levels were log-transformed prior to statistical analysis using a mixed model appropriate for a three-period crossover design. The model had terms for baseline (nasal scrape mRNA collection up to 28 days prior to starting treatment periods) as covariate, fixed treatment and period effects, and random subject effects. When multiplicity was explicitly addressed, Benjamini-Hochberg false discovery rate (FDR) was set to 10%.

Unsupervised clustering of mRNA expression. Hierarchical clustering of the 365 probe sets

strongly responsive to allergen in the prespecified analysis (Datafile S1) was performed using correlations between changes in expression (8 hours after NAC vs. study baseline) as the distance metric, with average linkage used for clustering. mRNA expression was compared to NAC-induced IL-13 expression. To minimize possible bias associated with fold change filtering, only MAS-5 filtering was used – resulting in 2,693 MAS5-present probesets for this analysis.

Identification of Eosinophil, Neutrophil and Mast Cell Gene Markers. A large database containing gene expression from over 1000 blood and 1000 respiratory tissue samples was analysed to identify clusters of genes consistently correlated with across samples. K-means unsupervised clustering based on Pearson correlation distance of oblique component was employed. Manual curation on the basis of r values and published functional biology of the various genes was used to develop non-overlapping gene sets for eosinophils, neutrophils and mast cells (Table E5). These gene sets could then be used to assess the presence and/or activity of a particular cell type.

Ingenuity Pathway Analysis. Gene expression changes after NAC were analysed using 52,369 probe sets corresponding to 31,892 genes. Multiplicity correction was based on Benjamini-Hochberg FDR. The fold change (+/-) was ranked by z-score, with a FDR cut-off of ≤ 0.05 .

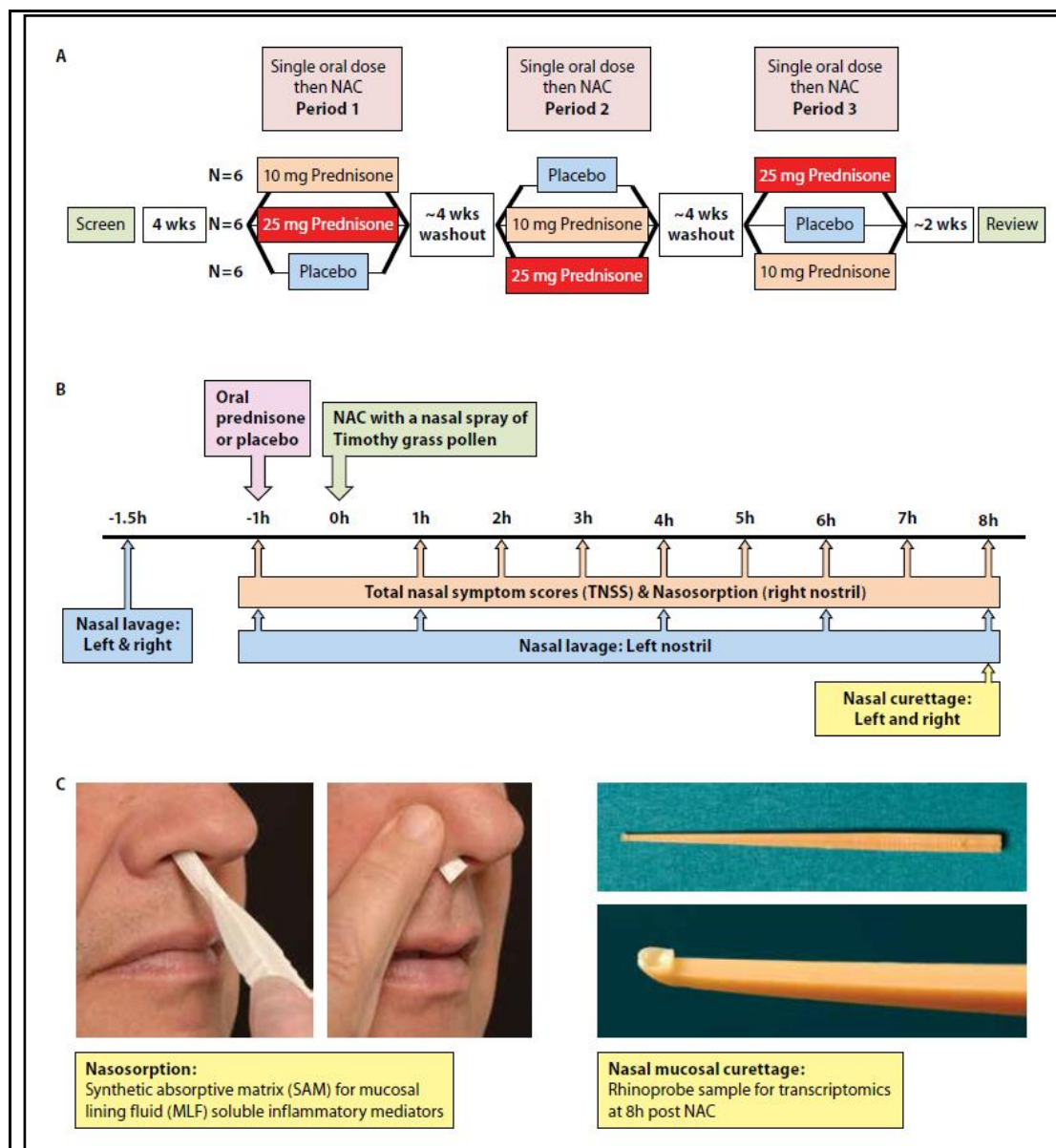
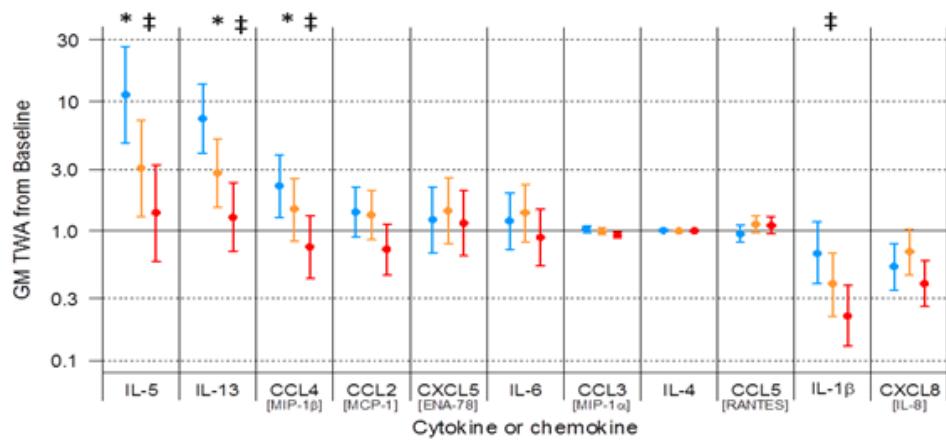


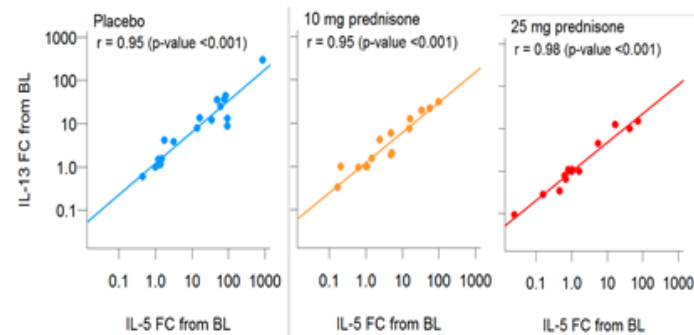
Figure 1. Study design and nasal mucosal sampling procedures.

(A) Randomized, double-blind, 3-way crossover study of the effects of a single oral dose of prednisone (10mg or 25mg) versus placebo given 1h before nasal allergen challenge (NAC). (B) Events taking place during NAC study visits. Both nostrils were lavaged, and the oral tablet given (prednisone or placebo). NAC was performed with Timothy grass pollen extract (nasal spray of 100 μ L, with Phl p 5 allergen at 1 μ g/nostril). Total nasal symptom scores and nasosorption sampling of the right nostril were carried out at hourly intervals to 8h; with lavages at 1,4,6,8h post NAC on the left nostril, and finally bilateral nasal curettage with a Rhinoprobe at 8h. (C) Nasal sampling techniques consisted of nasosorption with synthetic absorptive matrix and nasal curettage with a 10cm long plastic curette (screening and 8h post NAC). NAC = nasal allergen challenge.

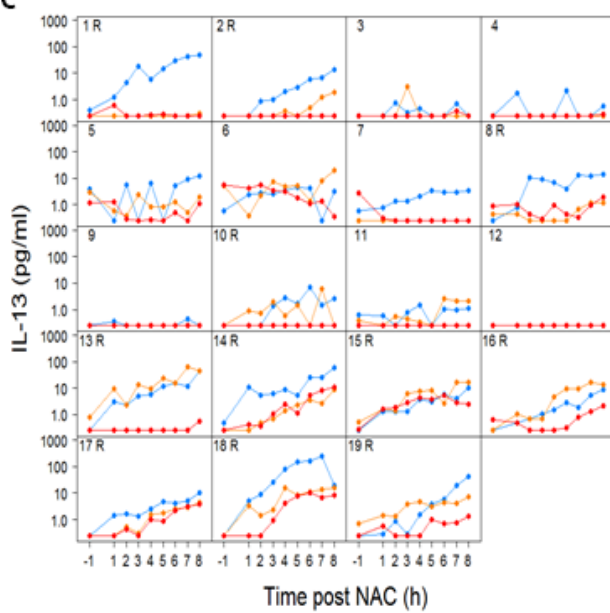
A



B



C



D

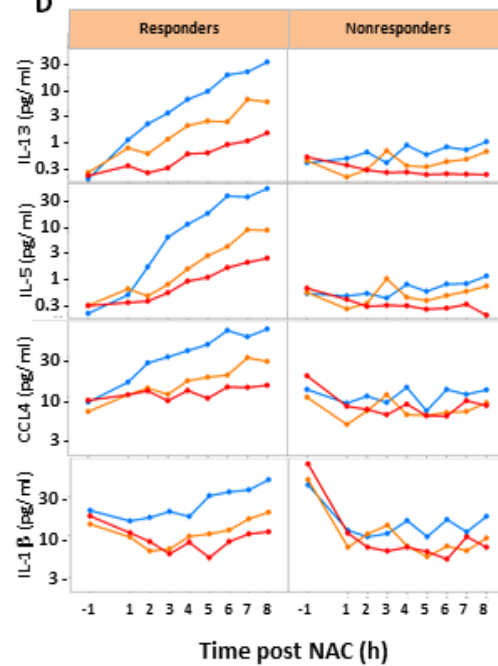
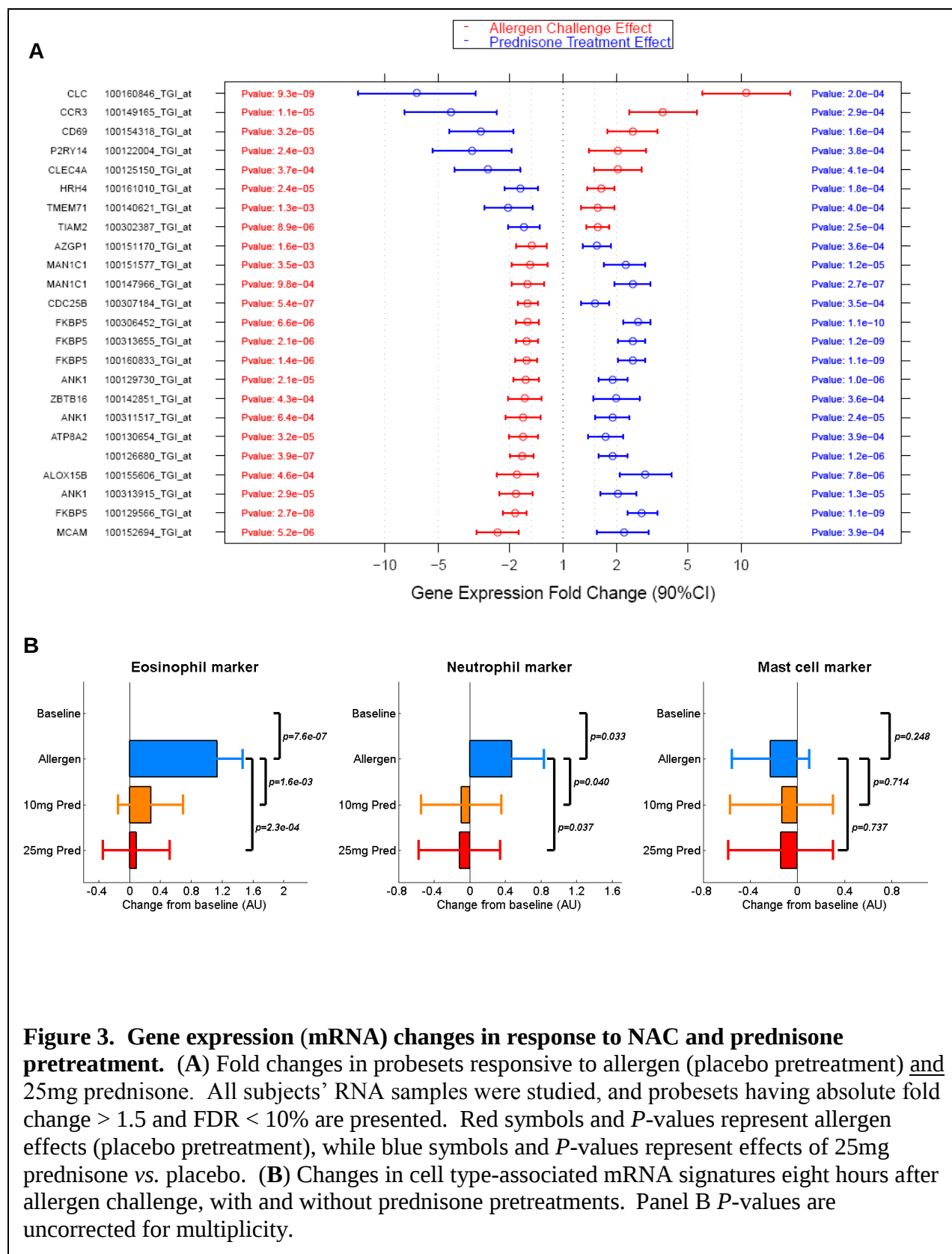


Figure 2. Cytokine and chemokine levels in nasal mucosal lining fluid (MLF).

(A) Geometric mean 2h-8h time weighted average (GMTWA_{2-8hrs}) fold change from baseline in cytokine or chemokine levels in response to NAC. Error bars represent 90% CI, but are not shown in part (D) for clarity. Asterisks signify allergen responses (placebo pretreatment before NAC) with $P < 0.05$. Double daggers signify dose responsive effects (versus placebo) of prednisone pretreatment with $p < 0.05$ for all comparisons. (B) Dot plot of IL-13 vs. IL-5 fold changes from baseline under each treatment. Pearson correlations appear in each panel. Solid lines represent least-squares regression lines. (C) Time courses of IL-13 production in individual participants. “R” refers to an IL-13 responder. (D) Time courses of geometric mean cytokine levels in IL-13 responders (left panels) and non-responders (right panels). N=18 subjects (one subject yielded insufficient nasal lining fluid for full analysis). For all panels blue, orange, and red symbols signify placebo, 10mg prednisone, and 25mg prednisone pretreatment, respectively.

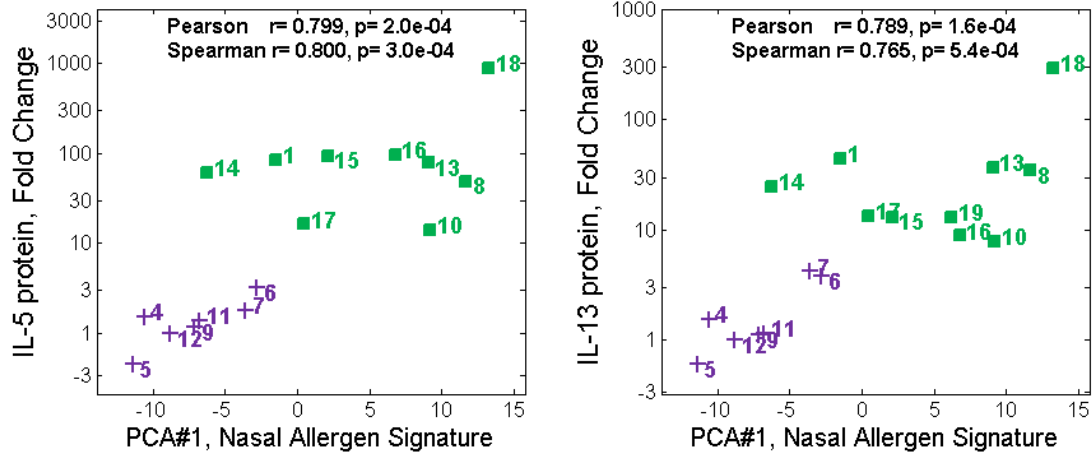
Abbreviations: BL = baseline, CI=confidence interval, FC= fold change, GMTWA= geometric mean time-weighted average, IL= interleukin, NAC = nasal allergen challenge



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A



B

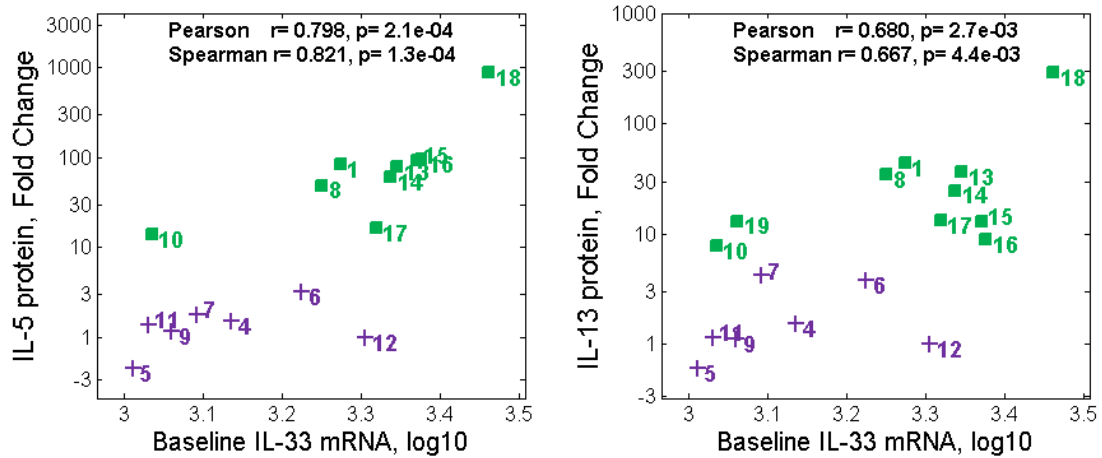


Figure 4. Gene expression (mRNA) changes in nasal curettage samples with respect to IL-5 and IL-13 protein levels. Dot plots of allergen-induced GMTWA_{2-8hrs} log fold change from baseline in nasal lining fluid IL-5 and IL-13 compared with (A) first principal component coefficient of nasal scrape gene expression for subjects pretreated with placebo, and (B) baseline nasal scrape IL-33 mRNA expression. Green squares and purple cross characters correspond to cytokine responders and non-responders respectively. Pearson and Spearman correlation coefficients for the relationships appear above the respective dot plots.

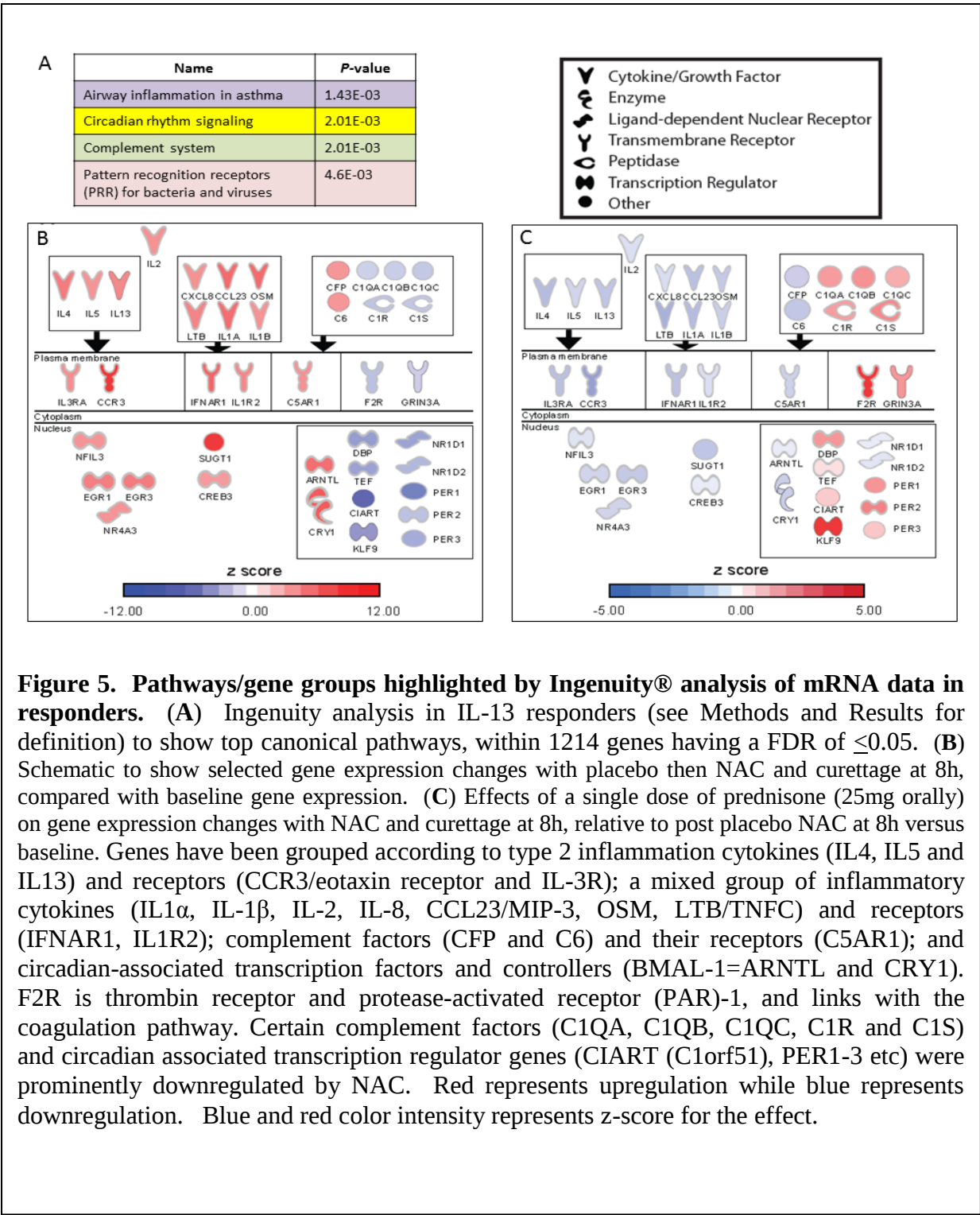


Table 1. Altered Gene Expression for Cytokines, Chemokines, Complement Factors and Circadian-Associated Genes: Nasal Allergen Challenge and the Effects of Prednisone.

IL-13 Responders only, FDR<0.04, selecting from 987 genes

Gene Probe set	Notes	Pre-NAC Treatment	Fold change (CI 90%)	p-value
IL-4 100136820_TGI_at NM_000590	interleukin-4	Allergen 10mg prednisone 25mg prednisone	1.846 (1.44, 2.35) 0.653 (0.47, 0.9) 0.557 (0.40, 0.77)	0.00022 0.03778 0.00624
IL-5 100135525_TGI_at NM_000879	interleukin-5	allergen 10mg prednisone 25mg prednisone	3.468 (1.90, 6.32) 0.568 (0.25, 1.25) 0.407 (0.1, 0.89)	0.0016 0.23341 0.0649
IL-13 100125222_TGI_at NM_002188	interleukin-13	allergen 10mg prednisone 25mg prednisone	1.748 (1.36, 2.23) 0.711 (0.52, 0.96) 0.627 (0.46, 0.84)	0.00069 0.06614 0.01519
CCR3 100149165_TGI_at NM_001837	eotaxin-1/2/3 receptor	allergen 10mg prednisone 25mg prednisone	8.056 (4.78, 13.5) 0.193 (0.09, 0.39) 0.154 (0.07, 0.31)	3.01E-7 0.00064 0.00015
IL-1 α 100163104_TGI_at NM_000575	interleukin-1 α	allergen 10mg prednisone 25mg prednisone	5.9 (2.80, 12.5) 0.351 (0.12, 0.97) 0.265 (0.0, 0.73)	0.00042 0.09366 0.03652
IL-1 β 100126016_TGI_at NM_000576	interleukin-1 β	allergen 10mg prednisone 25mg prednisone	10.69 (4.55, 25.1) 0.212 (0.06, 0.68) 0.128 (0.04, 0.40)	0.00007 0.03322 0.00655
OSM 100137287_TGI_at NM_020530	oncostatin-M IL-6-like cytokine	allergen 10mg prednisone 25mg prednisone	6.627 (3.42, 12.8) 0.256 (0.10, 0.64) 0.263 (0.1, 0.65)	0.00005 0.01779 0.01932
IFNAR1 100303602_TGI_at NM_000629	IFN- α receptor	allergen 10mg prednisone 25mg prednisone	1.352 (1.22, 1.49) 0.824 (0.71, 0.94) 0.79 (0.68, 0.91)	0.00003 0.02721 0.00894
C5AR1 100302987_TGI_at NM_001736	complement C5a receptor-1	allergen 10mg prednisone 25mg prednisone	1.968 (1.43, 2.0) 0.562 (0.36, 0.87) 0.581 (0.37, 0.90)	0.00118 0.03555 0.04556
CFP 100145758_TGI_at NM_002621	complement factor properdin	allergen 10mg prednisone 25mg prednisone	2.269 (1.57, 3.27) 0.38 (0.2, 0.64) 0.354 (0.21, 0.58)	0.00075 0.00367 0.00171
C1R 100145579_TGI_at NM_001733	complement factor 1 component r	allergen 10mg prednisone 25mg prednisone	0.487 (0.36, 0.65) 1.682 (1.11, 2.54) 1.557 (1.03, 2.34)	0.00034 0.0408 0.07687
C1QA 100159021_TGI_at NM_015991	complement factor 1 component q, a chain	allergen 10mg prednisone 25mg prednisone	0.577 (0.45, 0.72) 1.438 (1.04, 1.98) 1.411 (1.02, 1.94)	0.00041 0.06352 0.07637
CIART 100121748_TGI_at NM_144697	C1orf51, CHRONO circadian-associated repressor of transcription	allergen 10mg prednisone 25mg prednisone	0.2 (0.15, 0.26) 0.846 (0.61, 1.16) 1.003 (0.73, 1.37)	1.67E-14 0.37841 0.98593
ARNTL 100142502_TGI_at NM_001030273	BMAL1b, aryl hydro-carbon receptor nuclear translocator-like	allergen 10mg prednisone 25mg prednisone	1.411 (1.24, 1.59) 0.971 (0.82, 1.14) 0.933 (0.79, 1.09)	0.00007 0.76279 0.46334
CRY1 100144351_TGI_at NM_004075	PHLL1, cryptochrome circadian clock 1	allergen 10mg prednisone 25mg prednisone	1.53 (1.34, 1.75) 0.913 (0.78, 1.06) 0.818 (0.70, 0.95)	0.00001 0.32261 0.03696
PER1 100145189_TGI_at NM_002616	period circadian clock 1	allergen 10mg prednisone 25mg prednisone	0.365 (0.30, 0.44) 0.921 (0.71, 1.18) 1.32 (1.03, 1.69)	2.08E-9 0.57276 0.06239

Type 2 inflammation

Innate inflammation:

Complement factors

Circadian-associated genes related to transcription

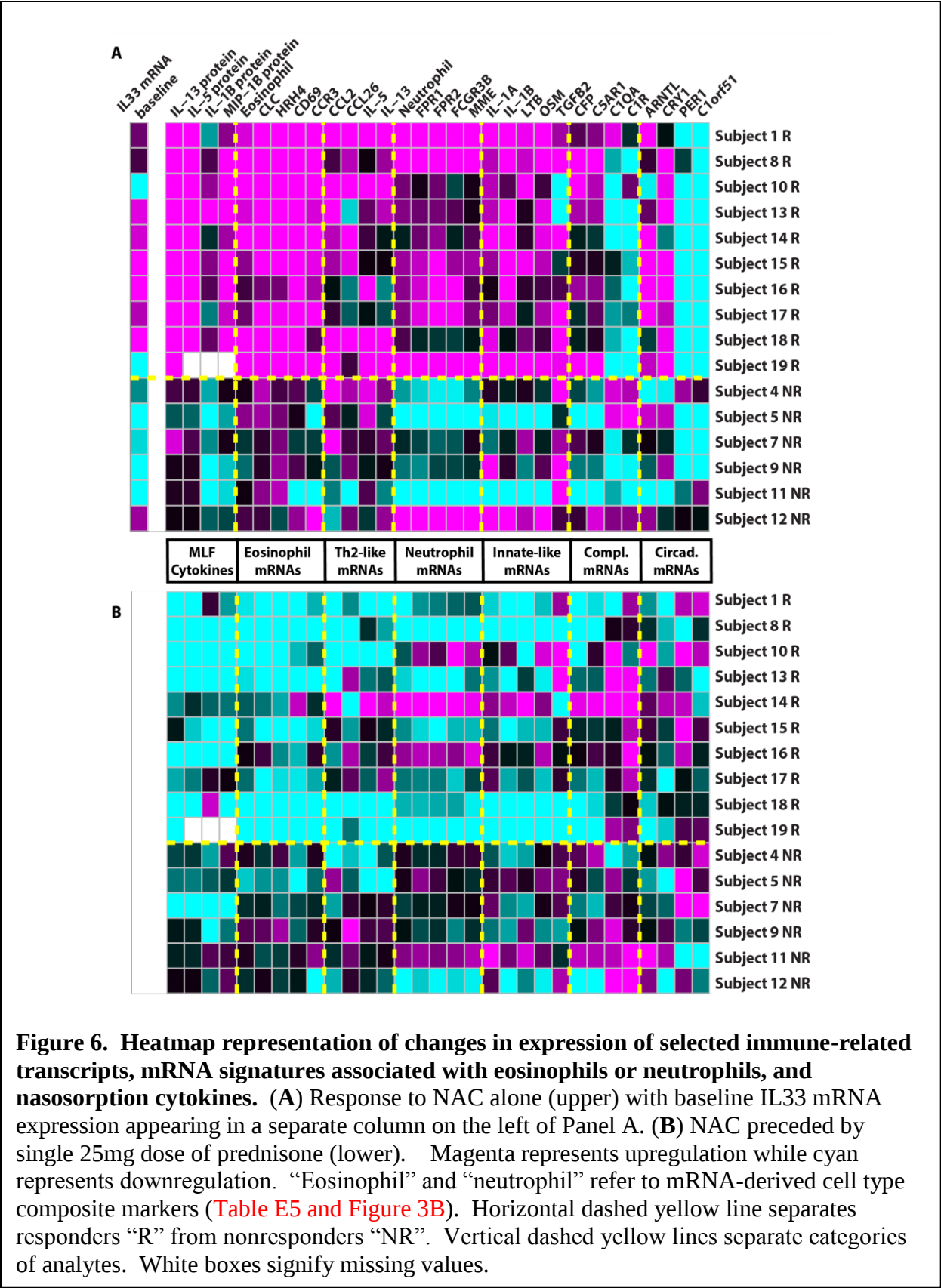


Figure 6. Heatmap representation of changes in expression of selected immune-related transcripts, mRNA signatures associated with eosinophils or neutrophils, and nasosorption cytokines. (A) Response to NAC alone (upper) with baseline IL33 mRNA expression appearing in a separate column on the left of Panel A. (B) NAC preceded by single 25mg dose of prednisone (lower). Magenta represents upregulation while cyan represents downregulation. “Eosinophil” and “neutrophil” refer to mRNA-derived cell type composite markers (Table E5 and Figure 3B). Horizontal dashed yellow line separates responders “R” from nonresponders “NR”. Vertical dashed yellow lines separate categories of analytes. White boxes signify missing values.

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Present affiliations: LNC and GC are currently with Celgene, RM is with Janssen R & D (Spring House, PA), MKR is with Alnylam, CT is with Novartis, NAC is with GSK, and CC is with Otsuka.

Author contributions: TTH, BRL and MKR wrote the protocol -- CC, NC, and IDL providing modifications; BRL and TTH were principal clinical investigators; NC, CC, and IDL provided Sponsor operational and compliance support, LNC and MKR were Sponsor's medical monitors, GCN and AJT performed patient and laboratory procedures; RM developed the statistical plan, and analyzed cytokine and symptom data; VM, GC, CT and TTH analyzed profiling data; PL and TTH provided Ingenuity analyses; GC, LNC, BRL, PL, VM, TTH, MKR, and CT interpreted the data; LNC and TTH wrote the first draft of the manuscript -- BRL, PL, VM & RM contributed to subsequent drafts and final manuscript. All authors reviewed the manuscript.

Competing interests: CC, LNC, GC, NC, IDL, RM, VM, MKR, and CT are or were MSD

534 employees and may own stock or stock options in Merck & Co. Inc. BRL and GCN are
535 employees of Respiratory Clinical Trials, Ltd. There are no patents arising from these data.

536 **Data and materials availability:** Microarray data obtained in this study are available at the
537 Gene Expression Omnibus (GEO) of the U.S. National Institutes of Health's National Center for
538 Biotechnology Information with accession number GDS67200.

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