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Whole blood transcriptional variations between responders and non-responders in asthma patients receiving omalizumab

Running Title: Transcriptional profiles of omalizumab responders and non-responders

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ABSTRACT

Background: Anti-IgE (omalizumab) has been used for the treatment of moderate-to-severe asthma that is not controlled by inhaled steroids. Despite its success, it does not always provide patients with significant clinical benefits.

Objective: To investigate transcriptional variations between omalizumab responders and non-responders and to study the mechanisms of action of omalizumab.

Methods: The whole blood transcriptomes of moderate-to-severe adult asthma patients (N=45: 34 responders and 11 non-responders) were analyzed over the course of omalizumab treatment. Non-asthmatic healthy controls (N=17) were used as controls.

Results: Transcriptome variations between responders and non-responders were identified using genes significant (FDR<0.05) in at least one comparison of each patient response status and time point compared to control subjects. Using gene ontology and network analysis, eight clusters of genes were identified. Longitudinal analyses of individual clusters revealed that responders could maintain changes induced with omalizumab treatment and become more similar to the control subjects, while non-responders tend to remain more similar to their pre-treatment baseline. Further analysis of an inflammatory gene cluster revealed that genes associated with neutrophil/eosinophil activities were upregulated in non-responders and, more importantly, omalizumab did not significantly alter their expression levels. The application of modular analysis supported our findings and further revealed variations between responders and non-responders.

Conclusion & Clinical Relevance: This study provides not only transcriptional variations between omalizumab responders and non-responders, but also molecular insights for controlling asthma by omalizumab.

Keywords: asthma; transcriptome, omalizumab, anti-IgE, biomarkers

Abbreviations: ACT: asthma control test, AS: asthma patient, C: cluster, F: female, FDR: false discovery rate, FEV1: forced expiratory volume in 1 sec, FVC: forced vital capacity, HC: healthy control, IPA: ingenuity pathway analysis, M: male, N/A: not assessable, n.s.: not significant, NR:

non-responder, PALO: present in at least one sample, QuSage: quantitative set analysis of gene expression, R: responder, RNA: ribonucleic acid

1 INTRODUCTION

Asthma is a chronic lung disease characterized by airflow obstruction, bronchial hyper-reactivity and inflammation of the airways. The clinical benefits of anti-IgE (omalizumab, Xolair®) therapy in asthma demonstrates the fundamental roles of serum IgE and its high-affinity receptor, FcεRI, in the pathogenesis of allergic asthma¹.

Unlike other anti-IgE antibodies that crosslink FcεRI-bound IgE and can thus cause mast cell and basophil activation, omalizumab, is not anaphylactogenic. It binds the Cε3 domain of free IgE, preventing IgE binding to FcεRI^{2,3}, without binding FcεRI- or CD23 (FcεRII)-bound IgE⁴. By neutralizing free IgE, omalizumab can downregulate surface expression of FcεRI on mast cells and basophils as well as antigen presenting cells (APCs)^{5,6}. IgE-free FcεRI is unstable and is quickly internalized for degradation^{7,8}; as such, FcεRI expression levels can correlate with serum IgE levels⁹. Omalizumab thus limits IgE-mediated mast cell and basophil activation, which leads to reduced degranulation and secretion of pro-inflammatory mediators from those cells¹⁰⁻¹⁴. A decrease of FcεRI expression on APCs^{5,6} could also limit IgE-bound allergen uptake and presentation followed by decreased allergen-specific Th2-type immune responses.

Omalizumab is currently used in the United States for moderate-to-severe persistent asthma (severe persistent asthma in the European Union) that is uncontrolled despite treatment with inhaled corticosteroids and long-acting β-agonists. Studies have shown that omalizumab can reduce asthma exacerbations, symptoms, oral corticosteroid doses, and can improve quality of life^{4,15,16}. However, only a portion of asthma patients respond to omalizumab treatment with estimates ranging from 50 to 90 percent^{4,17,18}. Furthermore, the mechanisms of action of omalizumab have not been fully understood.

In this study, we assessed whole blood transcriptional profiles of individual asthma patients (N=45) before (weeks -1 and 0) and during 26-weeks of treatment with omalizumab (weeks 6, 14, and 26). Using network and pathway analyses, we uncovered variations between omalizumab responders and non-responders both before the initiation of treatment and over the course of treatment. Data from

this study will help in understanding the mechanisms of action of omalizumab and may also help in the determination of predictive biomarkers for omalizumab treatment response in the future.

2 METHODS

2.1 Study subjects

All patients (N=45) (Table 1) had asthma that was uncontrolled despite treatment with inhaled corticosteroids and/or long-acting β -agonists. Patients as well as non-asthmatic healthy controls (Table 1) were recruited under protocols approved by the Institutional Review Board. Informed consent, including consent for the use of patient samples in this study, was obtained. This work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Disease severity was defined based on a combination of asthma control test (ACT) score, low lung function (as defined by the forced expiratory volume in 1 sec, FEV₁, score of less than 80% of predicted), and by their frequency of symptoms, including total number of days with symptoms per

week and of nighttime sleep disruption (more than once per month). Patients were excluded if they were pregnant, under the age of 18 or recently on omalizumab.

2.2 Study design

Patients were prescribed omalizumab (Xolair®, Genentech) based on their physician's recommendation, and were dosed as per the manufacturer's dosing table (based on serum IgE and body weight). Patient blood was collected one week before the initiation of treatment and again on the day of initial treatment as baselines. Blood was then collected on week 6, week 14 and week 26 after the initiation of treatment. At each blood draw, patients filled out the ACT questionnaire and an evaluation form for total numbers of symptoms per week, short-acting β -agonist usage per week, and nighttime awakenings per week. Patients were also asked to perform the lung spirometer test.

2.3 Determination of responders versus non-responders

Response to omalizumab was defined based on improvements in asthma control, with non-responders still exhibiting uncontrolled asthma, as previously described¹⁶⁻²⁰. Asthma was considered uncontrolled based on a combination of factors including an ACT score less than 19, asthma symptoms and short-acting β -agonist usage at least twice per week and nighttime awakenings at least twice per month, a lack of change in asthma control medication, and indications of lack of asthma improvement by physicians, similar to other studies¹⁶⁻²⁰. Since lung function has not been shown to be an indicator of response to omalizumab²¹ it was not included in our responder classification. Table 1 summarizes the characteristics of responders and non-responders both before (baseline) and at week 26 of treatment.

2.4. RNA extraction and processing

Total RNA was isolated from whole blood using the MagMax Nucleic Acid Isolation Kit (Ambion, Texas) according to the manufacturer's instruction. Following extraction, an Agilent 2100 Bioanalyzer (Agilent) was used to attain the RNA integrity numbers (RIN) for each sample. All samples with RIN values of >5.5 were retained for further processing. There was no specific pattern on the patients on RIN value >5.5 . The RNA yields were attained using a NanoDrop 8000 (NanoDrop

Technologies). Both the RIN and yield data were managed using a laboratory information management system (LIMS) for quality control and sample tracking. After RNA extraction and quality control analysis, globin mRNA was depleted from a portion of each total RNA sample using the GLOBINclear-Human 96-well format kit (Ambion). This was then followed by another round of RIN and yield determinations for quality control purposes. All samples passing quality control were then amplified and labeled using the Illumina TotalPrep-96 RNA amplification kit (Ambion). Amplified/labeled RNA (1500 nanograms) was hybridized overnight to Illumina HT-12 V4 BeadChips (Illumina). Following hybridization, each chip was washed, blocked, stained, and scanned on an Illumina BeadStation 500, according to the manufacturer's protocols.

2.5 Microarray data processing and analysis

Each sample was background subtracted and scaled to the average of all samples. Raw values less than 10 were set to 10 and the data was $\log_2$ transformed. Probes were then filtered to select transcripts that were present in at least one sample (PALO) using an alpha of 0.01 for the signal detection, thus eliminating background. Out of approximately 47,000 probes, approximately 28,000 passed this criterion. The probes were then additionally filtered by removing probes with the lowest variability using a standard deviation of less than 0.344. This left approximately 20,000 probes for differential analysis. Principal variance component analysis (PVCA) was then conducted to assess and correct for chip-to-chip variation among samples. All processing steps, including batch correction, were implemented in JMP Genomics software (SAS, North Carolina)²².

Differential gene expression analysis was performed using Welch's *t*-test for comparisons between responder status groups at each time point in addition to each group versus non-asthmatic healthy controls at each time point. Replicates of non-asthmatic healthy controls were averaged for comparison to asthma patients. Paired *t*-tests were used for transcriptional changes within each group over time during treatment. The Benjamini-Hochberg false discovery rate (FDR) procedure was applied to adjust for multiple testing. Gene set analysis was conducted using the QuSAGE algorithm²³. QuSAGE was applied using both Baylor-derived modules as well as 8 gene clusters defined in this study.^{24,25}

Direct comparisons between responders and non-responders did not provide sufficient gene lists after multiple testing corrections. In order to characterize the signature differences between responders and non-responders, we adopted a strategy using gene clusters or modular level type analyses^{24,25,26}. Gene clusters were generated by first considering only transcripts that were statistically significant ($FDR < 0.05$) in at least one comparison between the responders or non-responders at any time point versus non-asthmatic healthy controls. Unsupervised hierarchical clustering was then performed on the mean expression values for each combination of response status and time point group. The Cubic clustering criterion, a measure of between relative to within cluster variability, was then used to select an optimal number of clusters for annotation. Qusage was also applied to these manually curated gene sets. The clustering procedure was conducted using JMP Genomics software. Clusters were annotated based on a combination of Ingenuity pathway analysis (IPA, QIAGEN Inc., <https://qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) and a comparison with the correspondence of gene clusters relative to the Module annotations^{24,25}. Modular analysis of transcriptional profiles were previously described²⁶. Briefly, we used a framework of 260 modules of transcripts co-expressed in blood across various immunological conditions. This was done to reduce data dimensionality while increasing interpretability of results. These modules were previously annotated using knowledge-based and data-driven approaches^{24,25}. Network and pathway analyses were generated using IPA (QIAGEN Inc., <https://qiagenbioinformatics.com/products/ingenuity-pathway-analysis>).

3 RESULTS

3.1 Assessment of global gene signatures of asthma patients

Baseline global gene signatures for asthma patients were assessed by comparing all patient samples (GEO accession number GSE134544) to those of non-asthmatic healthy control subjects. After conducting a Welch's t-test with $FDR < 0.05$, we identified 3,867 differentially expressed genes between non-asthmatic healthy controls (N=17) and patients (N=45) at baseline (Figure 1A). Of 45 patients, 34 patients were responders and 11 patients were non-responders (Table 1). Results of unsupervised clustering showed that there was no clear separation of responders, non-responders or controls (Figure 1B). However, 15 out of 34 responders were clustered with the majority of control subjects (16 out of 17) at baseline. The difference between responders and non-responders was subtle. Exclusion of past smokers did not result in significant changes in the clustering (data not shown).

We next examined profiles over time with omalizumab treatment from baseline (week 0) to week 26. Unsupervised clustering analysis of the 3,867 genes at each time point revealed that there was no clear separation between responders and non-responders (Figure 1C). The same analysis performed with samples from week 26 is presented in Figure S1 in the Supporting Information. Taken together, we were able to identify 3,867 differentially expressed genes (based on $FDR < 0.05$) between non-asthmatic healthy controls and patients, but comparisons of these global genes did not allow us to distinguish responders from non-responders at any time points tested.

3.2 Comparison of responders and non-responders using clustering analysis

To get a better overview of the effects of omalizumab treatment on patient transcriptional profiles, we generated parallel plots of all gene expression in both responders and non-responders relative to non-asthmatic healthy controls over time (Figure 2A). This revealed differences in the overall pattern of transcriptional activity between responders and non-responders. First, a subset of genes was highly expressed in the non-responders compared with controls over time. Second, responders become similar to non-asthmatic healthy control subjects over time by week 26, while non-responders initially become similar to controls especially at week 6, but by week 26 of treatment, revert back to their original pattern.

To examine signature differences between responders and non-responders, we conducted hierarchical clustering to stratify 8 similarly expressed transcript clusters, using genes significant ($FDR < 0.05$) in at least one comparison of each patient response status and time point versus controls at each time point. Gene ontology and network enrichment analysis were used to annotate the 8 clusters (Figure 2B and Table 2) that contain different gene sets. Figure 2B shows gene expression with respect to control subjects in both responders and non-responders over the course of treatment. By clustering similarly expressed genes, we were able to more easily view the changes in gene expression over time and compare this change between responders and non-responders. Cluster annotation was achieved by analyzing top canonical pathways and upstream regulators in each cluster (Table 2), as previously described²⁷.

We further assessed longitudinal activities of the 8 clusters by measuring their average fold-changes relative to controls (Figure 2C). It was of note that in all clusters, both responders and non-responders (except for cluster 7 in responders and cluster 6 in non-responders) became similar to non-asthmatic healthy controls during the initial 6 weeks of treatment. This trend of cluster activity was relatively well sustained in responders through week 26. However, non-responders exhibited cluster activity (except for clusters 4 for cell cycle control/proliferation and 6 for monocytes) that deviated from the control baseline by week 14 or week 26 of treatment. Such different cluster activities in responders versus non-responders were further emphasized by our observations that responders showed significant differences between baseline and week 26 in all clusters, while non-responders did not. Non-responders showed significant differences only between baseline and week 16 (except for

clusters 6 and 7). It was also interesting to note that there were no clusters in which patients had a significant change between baseline and week 6.

By comparing the differences in fold change between the two groups at each time point, we were also able to determine which clusters were significantly different ($p < 0.05$) using the gene set enrichment algorithm QuSAGE²³. We found that there was a significant difference at baseline (week 0) in clusters 2 (T cell/NK cell/cytotoxicity), 3 (hematopoiesis), 7 (glucose metabolism) and 8 (inflammation) between responders and non-responders (Figure 2C). At week 6, there was still a significant difference between responders and non-responders in clusters 2, 3 and 7 in addition to a lesser difference in cluster 5 (T cell regulation and activation). At week 14, only clusters 3 and 7 were significantly different; while at week 26, only cluster 8 was significantly different. We thus concluded that there were significant differences between responders and non-responders in the activities of selected gene clusters. Importantly, clusters 2, 3, 7, 8 were differentially expressed in responders and non-responders on week 0, although global gene analysis (Figure 1) did not permit us to distinguish responders and non-responders at any time points tested in this study. However, average fold changes in individual gene clusters do not necessarily reflect pathway activity.

3.3 Genes related to neutrophil activation and cytotoxicity are enriched in non-responders

We next examined gene expression changes from each cluster between responders and non-responders (see Figure S2 for clusters 1-7 in the Supporting Information and Figure 3A for cluster 8). The global visualization of cluster 8 (Figure 3A) indicated that there was a subset of genes statistically highly expressed in the non-responders compared with non-asthmatic healthy controls. From cluster 8, we isolated genes that had a fold change ≥ 1.25 in non-responders compared with control subjects. Of the 25 genes identified, 19 were annotated (Figure 3B). The majority of the identified genes were associated with the cytotoxicity or activation of neutrophils. These include α -defensins (*DEFA1*, *DEFA1B*, *DEFA3*, *DEFA4*)²⁸⁻³⁰ and the neutrophil azurophilic granule proteins cathepsin G (*CTSG*), elastase (*ELANE*), myeloperoxidase (*MPO*), bactericidal/permeability-increasing protein (*BPI*) and azurocidin 1 (*AZU1*). CEACAM6 (CD66c) and CEACAM8 (CD66b) are also known to activate neutrophils and eosinophils³¹⁻³³.

These genes were further analyzed for regulation and enrichment of biologically relevant networks by IPA. The predicted regulatory pathway was represented as a circular network with the transcripts on the circumference and the transcription factors and cytokines enriched by *in silico* IPA network growth in the center (Figure 3C). The predicted regulatory pathway indicates that these upregulated genes were associated with granulocyte development (*GFII*, *CSF3*, *IL3*, *N4BP2L2*, *NOTCH1*)³⁴⁻³⁸ as well as the pathogenesis of asthma and Th17/Treg dysregulation³⁹.

Network analysis also revealed the involvement of pro-inflammatory cytokines via NFκB, ERK, and P38 MAPK activation as well as mucin production and FcεR1α, IgE and IgG signaling (Figure 3D and E). Upregulation and activation of eosinophilic cationic protein (ECP)⁴⁰, RNase 3 (Figure 3B and E), were also observed in non-responders. Taken together, non-responders showed stronger activities of genes related to both neutrophil and eosinophil activation. From cluster 2, we could isolate 8 genes (*LOC651894*, *LOC728453*, *MPI*, *LOC100134301*, *SLAMF6*, *MRPS18B*, *RSL1D1*, and *LOC442727*) that had a fold change ≥ 1.25 in non-responders compared with control subjects. *LOC651894*, *MRPS18B*, and *RSL1D1* are genes for ribosomal proteins. *SLAM6* is a CD2 subfamily of immunoglobulin superfamily and this encoded protein is expressed on T, NK, and B cells.

3.4 Module-based analysis of transcriptional profiles

To further assess differences between responders and non-responders, we applied our modular analysis across all time points and compared variations in module activity patterns. As shown in Figure 4A, we were able to compare responders and non-responders with respect to non-asthmatic healthy controls and chart their changes over time with omalizumab treatment. The largest number of variations between asthma patients and non-asthmatic healthy controls occurs before treatment (week 0), and this is slowly decreased with omalizumab treatment. However, non-responders tend to exhibit a greater number of differentially expressed modules compared with controls than responders do, even at week 26. One of the strongest signatures exhibited by both patient groups is M5.15 (neutrophil module, indicated with an arrow). Although there was a slight decrease of neutrophil signatures in responders on week 6, they were relatively well maintained in both groups even with omalizumab treatment. We also observed that both groups of asthma patients exhibited increased inflammation modules, such as M3.2, M4.2 and M4.13 (indicated with dotted arrows) compared with

non-asthmatic healthy controls at baseline. However, with treatment, this signature tends to be decreased in responders but remain unchanged or subtle decreases in non-responders. Responders and non-responders similarly had a decreased cytotoxic/NK cell signature compared with healthy controls, as shown by modules M3.6 (indicated with an arrow). Non-responders also had a decreased T cell signature compared to non-asthmatic healthy controls and responders, as shown by M4.15 (indicated with an arrow), further explaining the activity of gene cluster 2 in Figure 2C over time. No circle indicates that no more than 10% of transcripts within the module were differentially expressed for the given labeled comparison.

To further uncover variations between responders and non-responders, we isolated modules that differed between non-responders and non-asthmatic healthy controls, but not responder and control or vice versa. As shown in Figure 4B, there were a large number of modules only present in non-responders versus controls before treatment. The majority of these modules were under-expressed in non-responders compared with controls. There were also a smaller number of modules that are only present in responder versus control. One of these modules, M3.1 (erythrocytes, indicated with an arrow), was under-expressed in responders compared with non-asthmatic healthy controls, but overexpressed in non-responders. It was also of note that non-responders showed over-expressions of M1.1 (platelet/coagulation cascade, indicated with an arrow) until week 14. In addition, many of the modules under expressed in non-responders were involved in cell cycle/proliferation and T cells, of which 24.8% of the transcripts contained in those modules overlap with cluster 2 (T cell/NK cell/cytotoxicity) (Figure 2).

We next analyzed the numbers of red blood cells (RBCs, erythrocytes), platelets, lymphocytes, monocytes, neutrophils, eosinophils, and basophils (see Figure S3). The number of RBCs was higher in non-responders than control subjects, but there was no difference between the two groups of patients. We also found no significant difference between responders and non-responders in platelets, lymphocytes, and monocytes although both patient types exhibited an increased number of monocytes compared with non-asthmatic healthy controls. The numbers of neutrophils, eosinophils, and basophils were not significantly different in the two groups of patients. These results indicate that the differences observed in Figures 1-4 were mainly due to the changes in gene expression, but not in cell numbers in the whole blood of responders and non-responders.

3.5 Pathway analysis comparisons between responders and non-responders

To identify networks and pathways that contribute to the outcomes of omalizumab treatment, we compared IPA canonical pathways and gene ontology biological processes^{41,42} between responders and non-responders before treatment (Figure 5). The predicted pathways are presented as circular networks with the transcripts on the circumference and the transcription factors, cytokines and transmembrane receptors enriched by *in silico* IPA network growth in the center. The 293 transcripts over-expressed in responders were connected to molecules involved in Th2 (*CSF3*, *IL4*, *IL5*, *IL18*, and *SPII*, red circles) and Th1 responses (*STAT1*, *STAT4*, *IL2*, and *SMARCR4*, blue circles) and their regulation (*CEBPA*, purple circle)⁴³ (Figure 5A). The 496 transcripts under-expressed in responders were connected to molecules involved in the suppression of inflammation (*TWIST1*, *FOXO1*, *FOXO3*, *TP53*, *CTNNB1*, and *SIM 1*, red circles) (Figure 5B), which is in line with the data in Figures 2 and 4.

In comparison, the 361 transcripts over-expressed in non-responders showed connections similar to responders to molecules involved in the Th2 responses (*CSF3*, *IL4*, *IL5*, red circles) and Th1 responses (*IFNG*, *STAT4*, and *SMARCR4*, blue circles). However, non-responders also showed connections to molecules that contribute to the promotion of allergic inflammation (*OSM*, *STAT3*, and *TNF*, purple circles) and that could be the outcome of inflammation (*TNFSF 11*) (Figure 5C). More different were the connected molecules under-expressed in non-responders, including those related to cellular differentiation (*E2F1*, *IL15*, *KMT2D*, *MYCN*, red circles) and T cell responses (*IL15*, *KLF3*, *SOX4*, blue circles) (Figure 5D).

Both patient groups shared many of the same enriched pathways (Figure 5E and F), particularly downregulated pathways compared with healthy (including EIF2 signaling, mTOR signaling⁴⁴, eIF4 and p70S6K signaling^{45,46}, natural killer cell signaling, diphthamide biosynthesis and mismatch repair in eukaryotes which correspond to cluster 1 (protein synthesis) in Figure 2. There were fewer pathways that were similarly overexpressed in non-responders compared to healthy. These included TREM1 signaling and IL-8 signaling that correspond to cluster 8 (inflammation) in Figure 2. TREM1 is known to be associated with allergic lung inflammation^{47,48}. TREM-1 is also known to co-operate with TLR signaling⁴⁹ and several pro-inflammatory mediators directly induced by TLR2 and TLR4, such as IL-8, CCL4 (MIP-1 β), IL-1 β , IL-6, and TNF α . Pathways that were uniquely upregulated in

responders include the production of nitric oxide and reactive oxygen species in macrophages, death receptor signaling, germ cell-Sertoli cell junction signaling, RANK signaling in osteoclasts, acute myeloid leukemia signaling, the role of NFAT in cardiac hypertrophy, Rac signaling⁵⁰ and GNRH signaling (Figure 5E). Many of these pathways are involved in cell adhesion and migration as well as NF- κ B activation⁵¹⁻⁵⁵.

Non-responders showed an upregulation in the role of JAK2 in hormone-like cytokine signaling, role of JAK family kinases in IL-6-type cytokine signaling, role of macrophage, fibroblasts and endothelial cells in rheumatoid arthritis, role of JAK1 and JAK3 in γ c cytokine signaling, oncostatin M signaling, Fc γ receptor-mediated phagocytosis in macrophages and monocytes, STAT3 pathway and the role of tissue factor in cancer (Figure 5F). These pathways are largely involved in cytokine signaling, inflammation and hematopoiesis⁵⁶⁻⁶¹. Overall, this highlights the differences in the underlying immune responses between responders and non-responders, with responders showing increased cell motility and non-responders showing increased cytokine signaling and inflammation networks.

4 DISCUSSION

Not all asthma patients respond equally to omalizumab treatment^{4,17,18}. Therefore, it is important to understand the mechanisms of action of omalizumab and to find potential transcriptional variations between omalizumab responders and non-responders, which might be used as potential predictive biomarkers in future. Thus far, baseline total serum IgE level¹⁷, bronchial galectin-3 concentration⁶², a history of severe asthma⁶³, and FeNO, blood eosinophils, and serum periostin¹⁶ have been reported for defining patients that would respond better to anti-IgE treatment.

In this study, we profiled the whole blood transcriptomes of a longitudinal cohort of asthma patients receiving anti-IgE therapy. However, analysis of heatmaps of differently expressed genes was

not powerful enough to reveal differences between responders and non-responders, and direct comparisons of responders with non-responders yielded no transcripts that passed FDR correction (Figure 1). In addition to the heterogeneity of asthma, common medications prescribed to moderate-to-severe asthma patients, including steroid and β -agonist, could also interfere with the genes induced by omalizumab^{64,65}. To overcome this challenge, we analyzed the variances in the expressions of groups of genes, using cluster and module analysis. This not only revealed differences before treatment, but also showed how responders and non-responders react differently over time before, during, and after anti-IgE treatment.

Interestingly, responders adopted a signature similar to non-asthmatic healthy controls over the course of treatment. It also appeared that responders were more similar to non-asthmatic control subjects than non-responders even before the initiation of treatment (Figure 1B). Non-responders showed a much different response to omalizumab by week 26 and tended to have expression patterns more similar to their baseline than to controls (Figure 2A). This was particularly even more evident in the levels of individual gene clusters (Figure 2B and 2C). Similar observations were also made in the modular analysis (Figure 4); non-responders had a strong module signature at baseline, which persisted even at week 26.

Further analysis of the changes of 8 gene clusters revealed that responders and non-responders had significantly different expression levels of clusters 2, 3, 7, and 8 before treatment (Figure 2B and 2C). Both clusters 2 (T cell/NK cell/cytotoxicity) and 7 (glucose metabolism) were higher in responders, while clusters 3 (hematopoiesis) and 8 (inflammation) were higher in non-responders, suggesting that clusters 2, 3, 7, and 8 could comprise predictors of response. In particular, clusters 3 and 7 could serve to identify response early on (significant at weeks 0, 6 and 14); and perhaps cluster 8 could help identify patients who have a consistent response (significant at weeks 0 and 26). In addition, responders exhibit a strong upregulation in pathways involved in cell mobility, while non-responders display a strong upregulation in pathways involved in cytokine signaling and inflammation. We also found 19 genes (in cluster 8) were higher in non-responders than responders before and after omalizumab treatment (Figure 3A). They, including genes encoding defensins^{29,30,66} elastase⁶⁷ CD66b³³ were highly associated with neutrophil and eosinophil activation and cytotoxicity (Figure 3B). In addition, neutrophil signatures were relatively well maintained in both groups even with

omalizumab treatment and this was not due to a discrepancy in cell counts. This suggests that omalizumab could be more effective for non-neutrophilic asthma than neutrophilic asthma. However, this needs to be further investigated in future.

To better understand the underlying differences between responders and non-responders that may influence response to anti-IgE treatment, we compared pathway analysis at baseline between both groups of patients (Figure 5). Responders and non-responders shared most of the downregulated pathways (Figure 5E and 5F). EIF2 signaling and protein synthesis were particularly downregulated, and this may also reflect corticosteroid administration⁶⁸. Therefore, the upregulated pathways might also reflect alterations in asthma pathogenesis. Non-responders also exhibited a strong signature of platelet activation (Figure 4B), as shown by the upregulation of tissue factor signaling and in the modules related to platelets. Platelets promote allergic asthma, partly by promoting Th2 responses, suppressing regulatory T cells⁶⁹ and airway remodeling⁷⁰. Platelet activation in responders compared with non-responders may warrant further investigation and potentially could be an additional biomarker for response.

Although both responders and non-responders show an increase in inflammation, this signature is stronger in non-responders (Figures 2, 3, and 4). While there is no difference in asthma control or severity between responders and non-responders before treatment, the increased inflammation in non-responders might also be associated with a greater degree of airway remodeling⁷¹⁻⁷³, which would not be susceptible to IgE neutralization.

We also observed a variation in the kinetics of anti-IgE effects on responder and non-responder transcriptional profiles. While responders showed a significant difference between baseline/week 0 and week 26 for each gene set cluster, there were no clusters for which non-responders had a significant difference (Figure 2C). However, in almost all clusters, non-responders were significantly different between baseline and week 14. There were also no clusters, either in responders or non-responders, which were significantly altered between baseline and week 6, which indicates that changes in gene signatures by anti-IgE take more than 6 weeks to become evident. The altered kinetics in non-responders compared with responders highlights the variance in asthma pathogenesis between these two groups and may signify that an IgE-independent mechanism is being asserted, particularly between week 14 and week 26 of anti-IgE treatment. Responders and non-responders

show a high degree of difference in cluster 7 (glucose metabolism). Glucose metabolism has recently come into the spotlight for its involvement in regulating T cell activation, differentiation and function⁷⁴. Non-responders tend to share a glucose metabolism signature more similar to non-asthmatic healthy controls, which may indicate a lack of T cell involvement in their asthma pathogenesis. This is supported by their low T cell signature shown in cluster 2 (Figure 2B and 2C). In addition, the increase of neutrophil signatures further supports an important role of neutrophils in the pathogenesis of asthma especially in non-responders. The alteration in cluster 3 (hematopoiesis) in non-responders compared with responders again indicates a variance in immune composition between responders and non-responders. It is quite clear, however, that non-responders exhibit a greater inflammatory signature than responders, as shown multiples times in our analysis. Interestingly, this inflammatory signature is significantly different between responders and non-responders only at baseline and week 26 (Figure 2C). This shows that non-responders may initially exhibit signs of response to anti-IgE, but this response cannot be sustained.

In summary, this study provides new insights for the regulation of asthmatic inflammatory responses by omalizumab treatment. In addition, selected gene clusters and modules that are differentially expressed by responders and non-responders could be considered as potential predictive biomarkers, although each of these gene clusters and modules still need to be further verified in future studies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Figure Legends

FIGURE 1 Allergic asthma blood transcriptional profiles with omalizumab treatment. (A) Hierarchical clustering. (B) Unsupervised analysis of differentially expressed transcripts (N=3867) between patients and non-asthmatic healthy controls (HC) at baseline (FDR<0.05). Samples are broken into non-asthmatic healthy controls (green), omalizumab non-responders (NR, blue) and omalizumab responders (R, red). Data are presented as log₂ expression normalized to control. (C) Unsupervised analysis of differentially expressed transcripts (N=3867) between patients and non-asthmatic healthy controls (HC) at baseline (FDR<0.05) shown over the course of omalizumab treatment. Samples are broken into HC (green), omalizumab NR (blue) and omalizumab R (red). Weeks of treatment are denoted as baseline/week 0 (gray), week 6 (aqua), week 14 (orange) and week 26 (purple). Data are presented as log₂ expression normalized to non-asthmatic HC.

FIGURE 2 Clustering of mean expression profiles reveals variations to treatment between responders (R) and non-responders (NR). (A) Parallel plots of all differentially expressed genes between patients (R and NR) and non-asthmatic healthy controls (HC) at baseline over the course of treatment. (B) Genes with significant changes between asthma patients and HC were hierarchically clustered, and k-means (k=8) were used to group genes with similar expression profiles across the time points. IPA pathway analysis was used to annotate each cluster. (C) Cluster average fold change compared with HC was calculated at each time point. Color coding for each cluster matches that presented in the heat map in panel B. R/HC comparisons are denoted by a dashed line and NR/HC comparisons are denoted by a solid line. Significance for panel B was set to $p < 0.05$ using the Qusage gene set algorithm.

FIGURE 3 Analysis of inflammation-related genes (cluster 8) reveals that non-responders (NR) show a neutrophilic phenotype. (A) Parallel plots of the genes associated with inflammation (cluster 8) in responders (R) and non-responders (NR). (B) List of the genes from cluster 8 with a fold change of 1.25 or more in the NR/non-asthmatic healthy control (HC) comparison. Cell types associated were found in the Human Protein Atlas (proteatlas.org) and GeneCards human gene database (genecards.org). (C) IPA network analysis for the predicted regulatory pathways and (D and E) the predicted networks.

FIGURE 4 Modular analysis indicates non-responders (NR) are more different from non-asthmatic healthy controls (HC) than responders (R). (A) Blood modular fingerprint of R and NR compared with HC over time. (B) Analysis of modules with difference in expression between R and NR.

FIGURE 5 Pathway analysis at baseline differentiates responders (R) and non-responders (NR). (A) IPA analysis for the 293 transcripts over-expressed in R and (B) the 496 transcripts under-expressed in R at baseline. (C) IPA regulatory pathway for the 361 transcripts over-expressed in NR, and (D) 475 transcripts under-expressed in NR compare with non-asthmatic healthy controls (HC) at baseline. Bar charts present the IPA canonical pathway enrichment in R (E) and NR (F) with HC. Gene enrichment was set to $FDR < 0.05$.

Table 1. Characteristics of anti-IgE (omalizumab) responders and nonresponders.

	Baseline				Week 26 of Treatment		
	Responder (n=34)	Non-Responder (n=11)	Responder vs non- responder <i>p</i> -value	Healthy Controls	Respo nder	Non- Responder	<i>p</i> -value
<u>Demographic</u>							
Age (years)	56.88 (19-76)	56.36 (28-68)	0.9222	53.41 (26-63)	N/A	N/A	
Sex (M/F) (%M)	11/23 (32)	4/7 (36)	>0.9999	6/11 (35)	N/A	N/A	
Total IgE (IU/mL)	356.1 (±501.6)	244.7 (±272.9)	0.5883	N/A	N/A	N/A	
Past Smoker, n(%) [^]	9 (26)	4 (36)	0.7036	N/A	N/A	N/A	
<u>Race</u>							
Caucasian, n(%)	32 (94)	9 (82)		13 (76)	N/A	N/A	
Black, n(%)	1 (3)	2 (18)		3 (18)	N/A	N/A	
Asian, n(%)	1 (3)	0 (0)		1 (6)	N/A	N/A	
<u>Clinical</u>							
ACT Score	12.41 (±4.61)	11.73 (±4.45)	0.6805	N/A	20.88 (±3.02)	15.82 (±5.33)	**0.0021
Oral Corticosteroid Use (%)	4 (11.8)	3 (27.3)	0.3368	N/A			
Inhaled Corticosteroid Use (%)	30 (88.2)	10 (90.9)	>0.9999	N/A			
Leukotriene Inhibitor Use (%)	19 (55.9)	5 (45.5)	0.7302	N/A			
<u>Asthma-related symptoms - no. of days in previous wk</u>							
Total symptoms	5.49 (±2.24)	5.09 (±2.35)	0.6859		1.66 (±1.83)	3.96 (±3.00)	*0.0184
β-agonist usage	7.71 (±5.64)	6.50 (±5.27)	0.5743		2.22 (±2.94)	4.23 (±3.96)	0.0712
Nighttime sleep disruption	2.96 (±2.36)	3.36 (±2.01)	0.5767		0.15 (±0.49)	2.00 (±2.37)	***0.0002
<u>Lung Function</u>							
FEV ₁ (% of predicted)	71.65 (±16.01)	76.73 (±18.39)	0.3921		76.74 (±13.4 6)	78.09 (±17.28)	0.9158
FVC (% of predicted)	77.53 (±14.62)	76.82 (±14.99)	0.8707		83.03 (±12.9 7)	80.45 (±14.23)	0.6567
FEF25-75 (% of predicted)	62.76 (±31.63)	76.55 (±36.37)	0.2677		65.23 (±27.9 4)	73.73 (±32.51)	0.4411

Values represent mean with range (age) or standard deviation, where applicable.

Oral steroid was not prescribed to patients for 6 months.

[^]Incomplete data

ACT: asthma control test, F: female, FEF25-75: forced expiratory flow during the mid (25-75%) of the FVC, FEV1: forced expiratory volume, FVC: forced vital capacity, M: male, % of pred: percent of predicted for age, sex and height

*P<0.05, **P<0.01, ***P<0.001. Significance was determined utilizing Mann-Whitney test or a Fischer's exact test where appropriate.

Table 2. Summary of pathway analysis to identify networks enriched in each cluster^a

	Canonical Pathways	score -log(p-value)	Upstream Regulators	score -log(p-value)
Cluster 1: Protein Synthesis (n=678)	EIF2 Signaling	13.90	MYCN	9.68
	Regulation of eIF4 and p70S6K Signaling	6.02	HNF4A	4.20
	mTOR Signaling	4.18	FUBP1	4.05
	Estrogen-mediated S-phase Entry	3.09	DOT1L	3.04
	Diphthamide Biosynthesis	3.02	FOXO3	2.92
	Natural Killer Cell Signaling	2.79	RBL1	2.91
	Telomerase Signaling	2.41	CBFB	2.82
	Cell Cycle: G1/S Checkpoint Regulation	2.24	ZBED1	2.78
	Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	2.11	RFX3	2.74
			SOX11	2.73
Cluster 2: T cell/NK cell/ cytotoxicity (n=451)	EIF2 Signaling	5.60	E2F1	3.64
	T Cell Receptor Signaling	2.44	MYCN	3.46
	Natural Killer Cell Signaling	2.20	HNF4A	2.70
	Purine Nucleotides De Novo Biosynthesis II	2.03	TWIST1	2.59
			KMT2D	2.14
Cluster 3: Hematopoiesis (n=195)	Glutamine Biosynthesis I	2.31	HOXA10	3.69
	Polyamine Regulation in Colon Cancer	2.28	TP73	3.49
			ATN1	3.37
			HDAC1	3.10
			ID3	2.82
			TRIM28	2.80
			NKX3-1	2.75
			MYC	2.56
			CHD4	2.45
			WBSCR22	2.33
Cluster 4: Cell cycle control/proliferation (n=439)	Protein Ubiquitination Pathway	4.09	HNF4A	5.08
	EIF2 Signaling	2.71	STAT5A	4.46
	Hypoxia Signaling in the Cardiovascular System	2.63	MYCN	3.17
	Triacylglycerol Biosynthesis	2.45	SIRT6	3.09
	Natural Killer Cell Signaling	2.09	MEN1	2.70
			NPAT	2.42
			TBX2	2.42
			E2F1	2.40
			FOXO3	2.24
			ERG	2.23
Cluster 5: T cell regulation and activation (n=368)	GNRH Signaling	3.16	HNF4A	3.42
	Nur77 Signaling in T Lymphocytes	2.81	TFDP2	2.74
	Calcium Signaling	2.28	SATB1	2.73
	B Cell Receptor Signaling	2.18	PPARGC1B	2.32
	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.17	ONECUT1	2.30
	Neuropathic Pain Signaling In Dorsal Horn Neurons	2.15	CUX1	2.26
	Cell Cycle Control of Chromosomal Replication	2.14	FOXP3	2.19
	IL-15 Production	2.14	CTNNB1	2.18
	RAR Activation	2.12	PLAG1	2.17
	NGF Signaling	2.09	SNAI1	2.14
	Isoleucine Degradation I	2.67	CBX5	3.68
	Semaphorin Signaling in Neurons	2.63	STAT5A	2.74
	Valine Degradation I	2.45	SPIB	2.26
Cluster 6: Monocytes (n=120)	Histamine Degradation	2.40	PRMT5	2.05
	Inflammasome pathway	2.31		
Cluster 7: Glucose metabolism (n=345)	GDP-mannose Biosynthesis	2.55	HDAC5	3.23
	Glycolysis I	2.30	BCOR	3.07
	UDP-N-acetyl-D-galactosamine Biosynthesis II	2.00	NUPR1	3.06
			SIN3B	2.69
			MYC	2.55
			ATF6	2.29
			MSGN1	2.25
Cluster 8: Inflammation (n=785)	Acute Phase Response Signaling	5.12	ESR1	6.28
	TREM1 Signaling	4.27	TP53	6.24
	IL-8 Signaling	4.26	CEBPA	6.15
	Role of JAK family kinases in IL-6-type Cytokine Signaling	4.22	SPI1	5.82
	Phosphatidylglycerol Biosynthesis II (Non-plastidic)	4.12	ECSIT	5.45
	Germ Cell-Sertoli Cell Junction Signaling	3.85	HIF1A	4.86
	Role of JAK1 and JAK3 in γ c Cytokine Signaling	3.73	NKX2-3	4.81
	STAT3 Pathway	3.64	PRDM1	4.75
	Acute Myeloid Leukemia Signaling	3.56	STAT1	4.65
	IL-6 Signaling	3.53	GFI1	4.49

^a For each cluster, the top 10 canonical pathways and upstream regulators were identified. The size of each gene list is displayed under the total. Based on an interpretation of significant pathways, each cluster is annotated where appropriate.

Figure 1

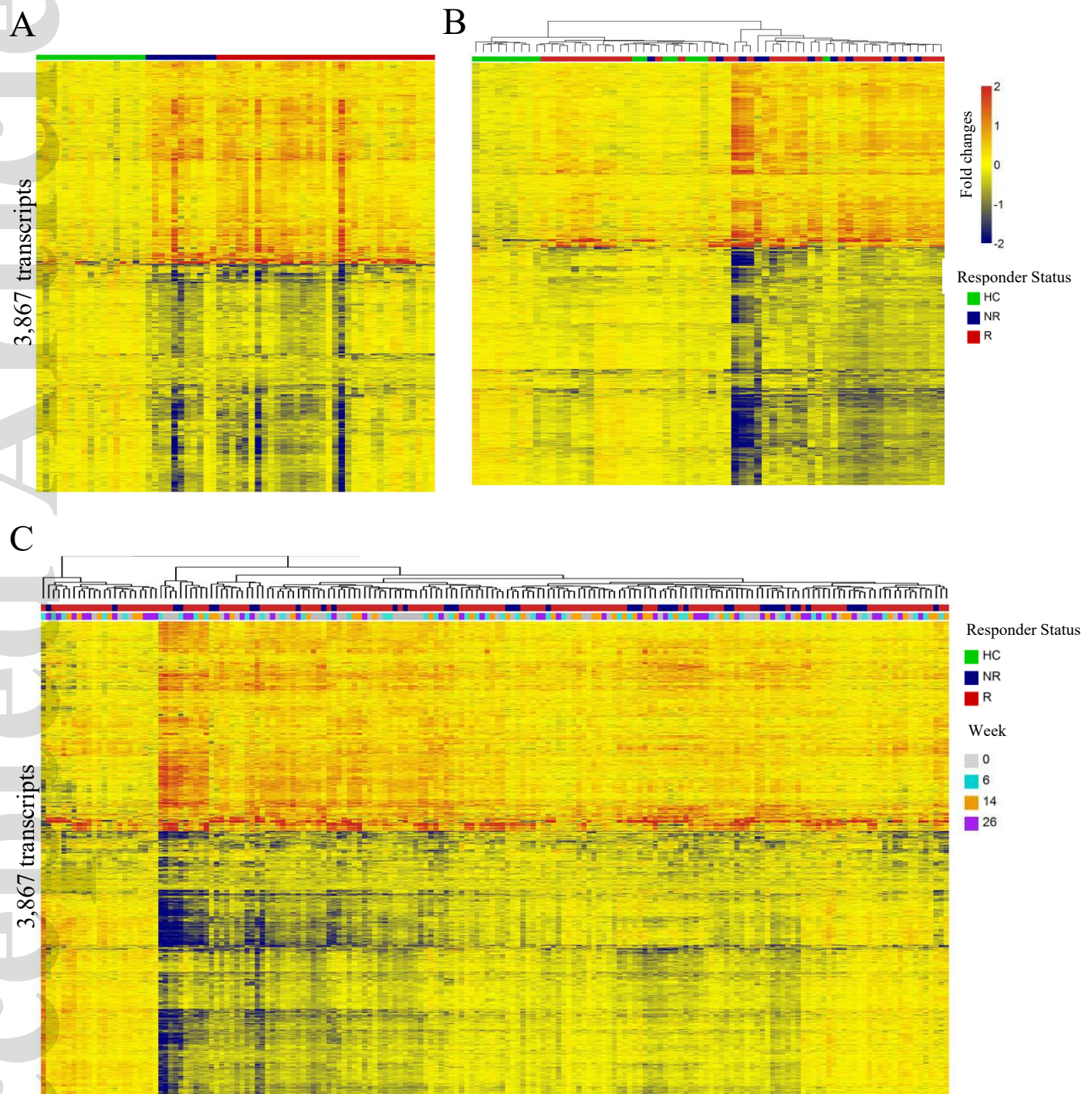


Figure 2

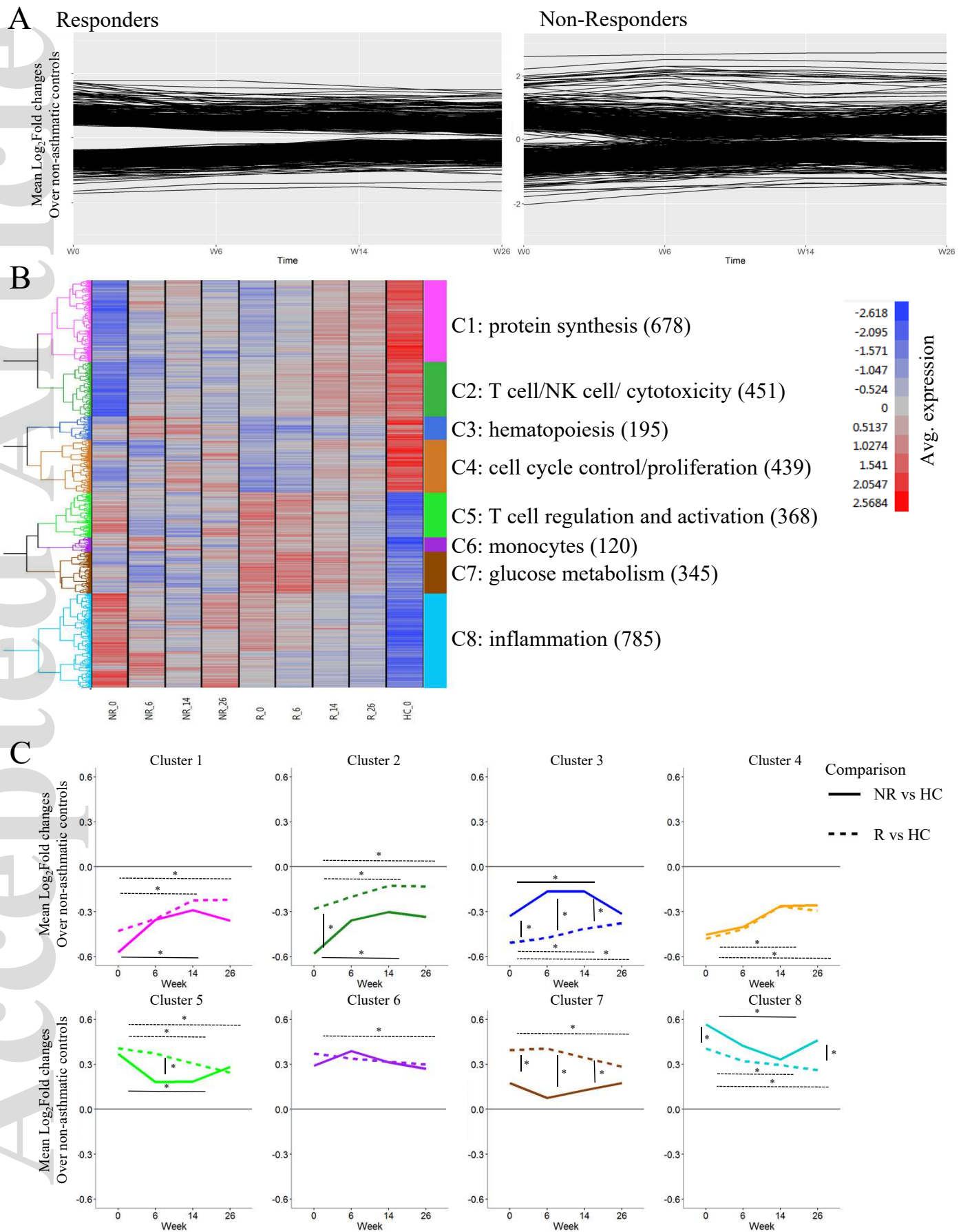
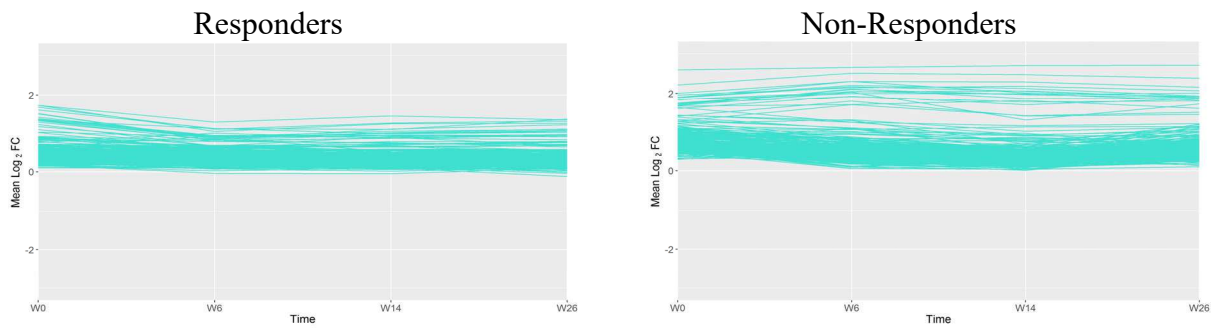


Figure 3

A C8: inflammation (785)



B

Gene Symbol	Description	Cell types associated
FKBP9L	FK506 binding protein 9-like	
TFF3	trefoil factor 3	Goblet cells
MPO	myeloperoxidase	Neutrophils/Eosinophils/MO
RNASE3	RNase A	Neutrophils
AZU1	azurocidin	Neutrophils
OLR1	LOX-1	B cells
	bactericidal/permeability-increasing protein	Neutrophils
BPI	defensin alpha 4	Neutrophils
DEFA4	defensin alpha 4	Neutrophils
CEACAM6	CD66c	Neutrophils/Eosinophils/B cells
ELANE	neutrophil expressed elastase	Neutrophils/MO
CEACAM8	CD66b	Neutrophils/Eosinophils/B cells
ZDHHC19	zinc finger	
DEFA1B	defensin alpha 1B	Neutrophils/ B cells/MO
DEFA1	defensin alpha 1	Neutrophils/ B cells/MO
DEFA3	defensin alpha 3	Lymphocytes
CTSG	cathepsin G	Neutrophils/B cells/MO/DC
OLFM4	olfactomedin 4	Neutrophils
DEFA1B	defensin alpha 1B	

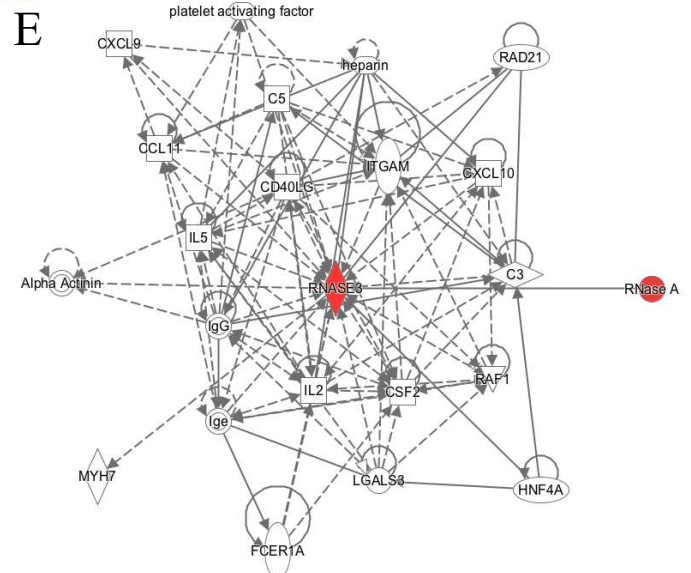
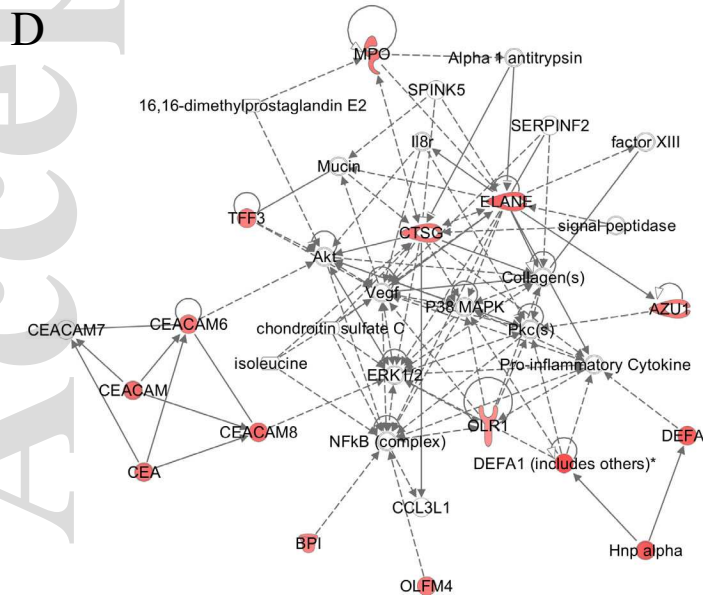
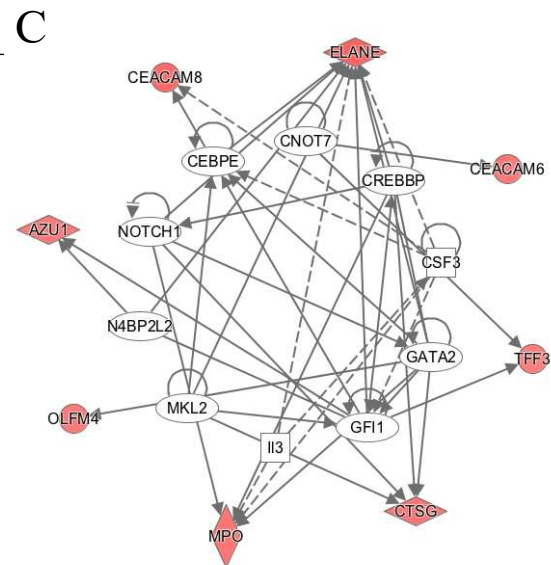


Figure 4

A

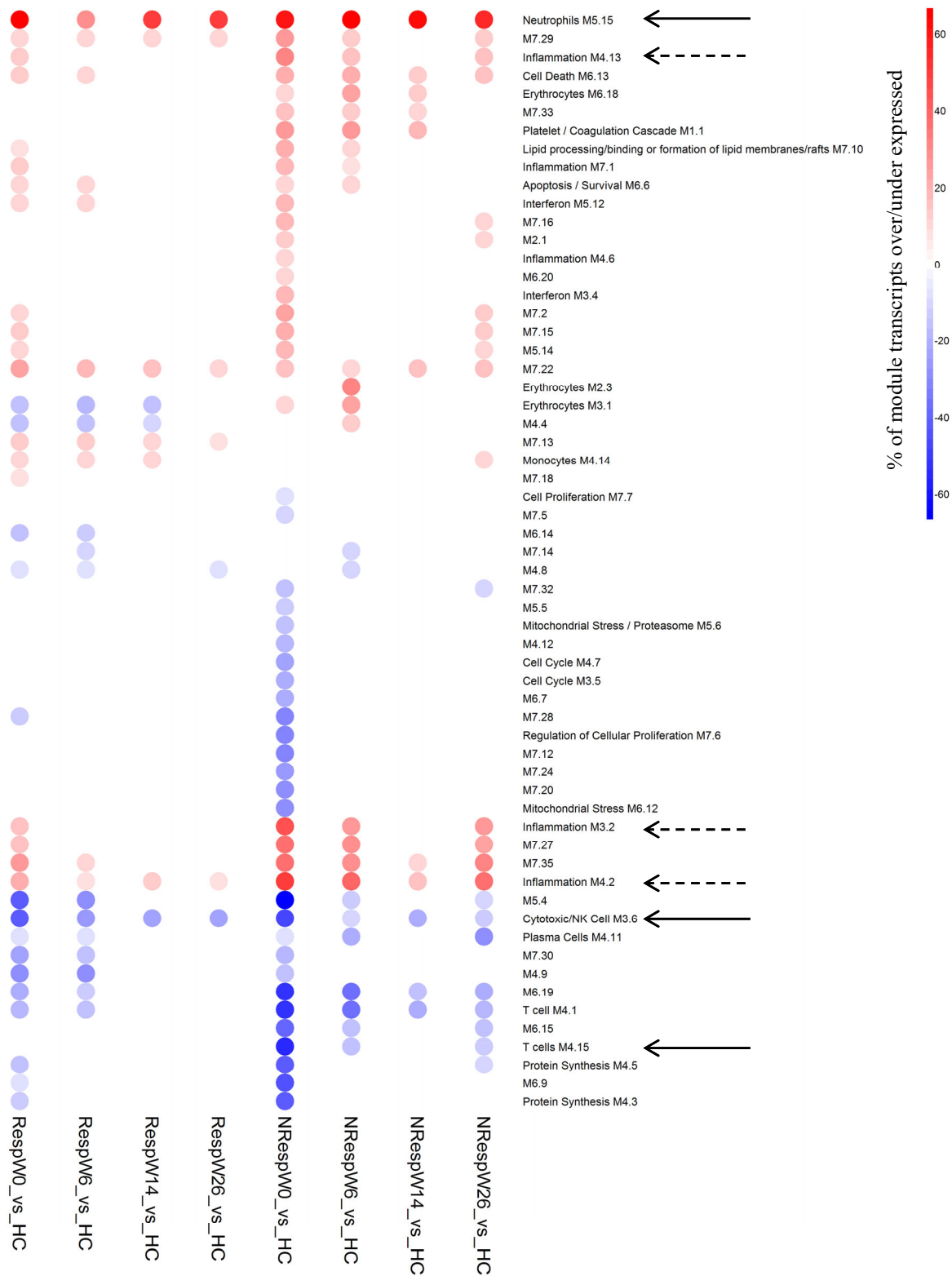


Figure 4 (continued)

B

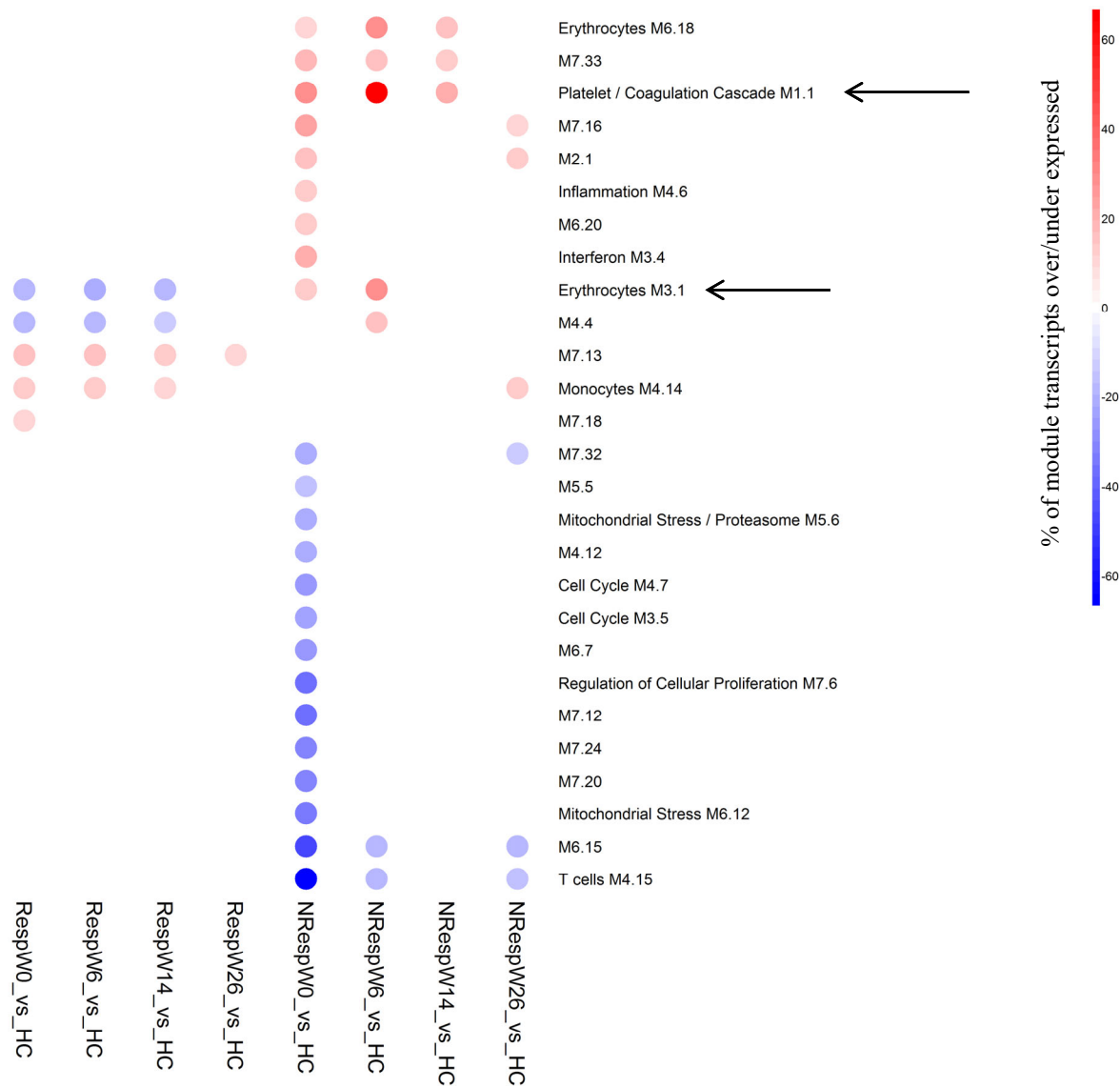
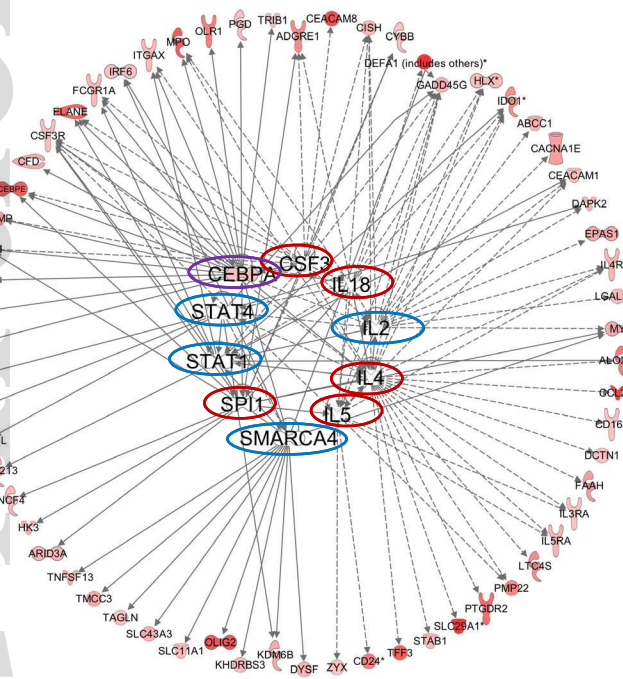


Figure 5

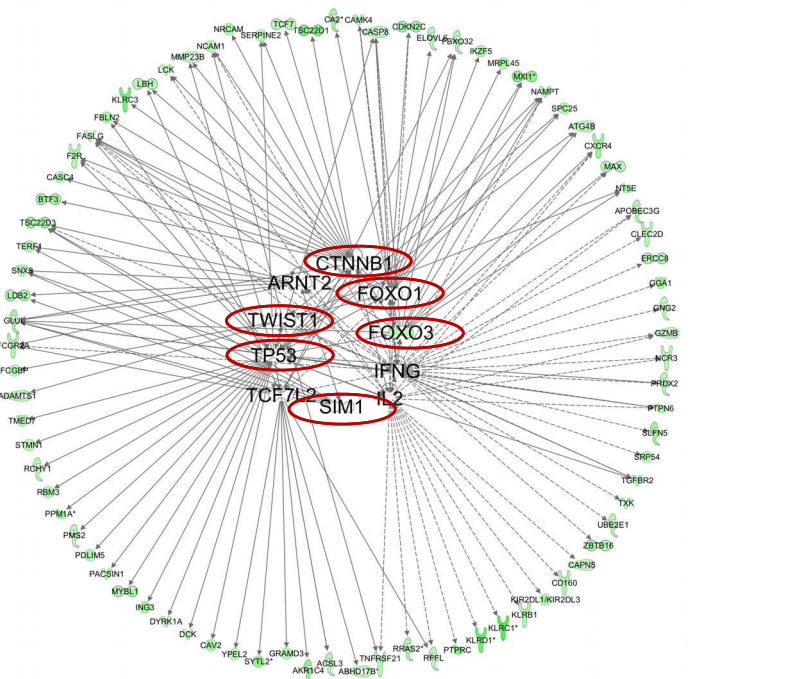
A

Up in R vs HC W0 (293)



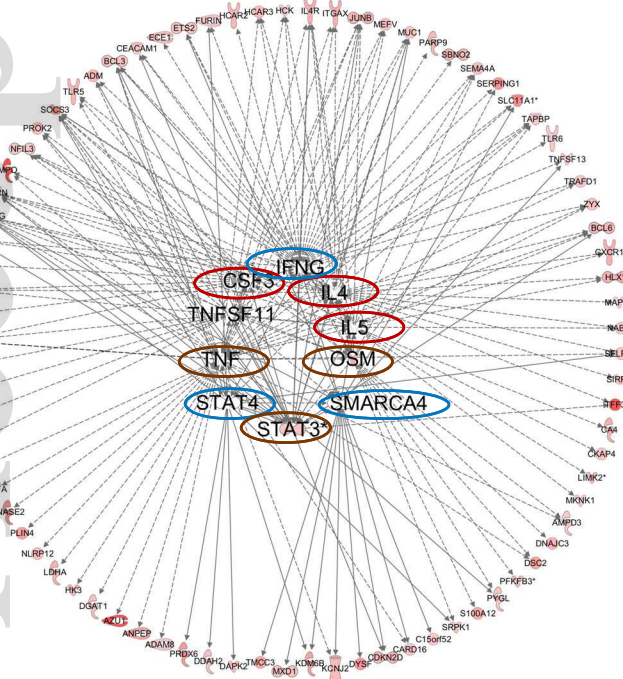
B

Down in R vs HC W0 (496)



C

Up in NR vs HC W0 (361)



D

Down in NR vs HC W0 (475)

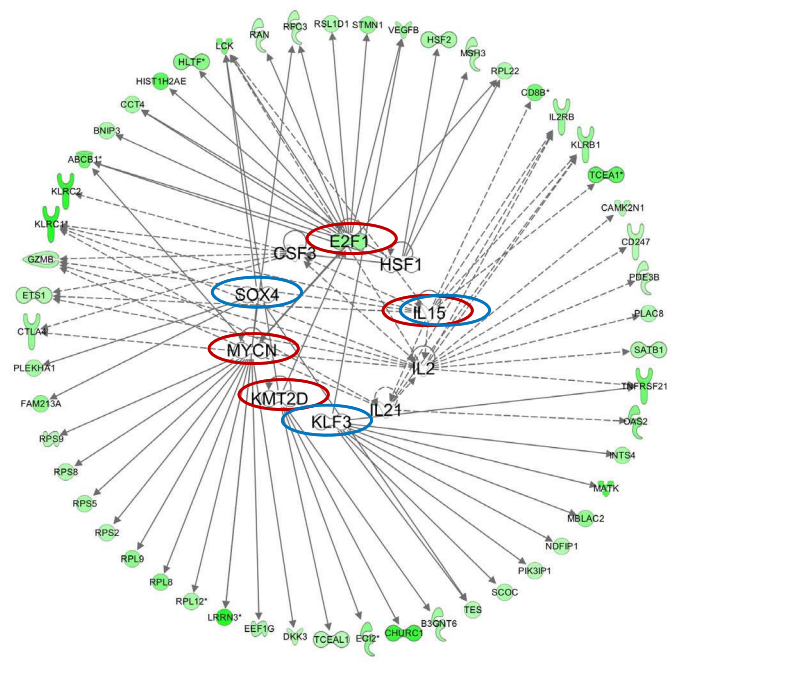
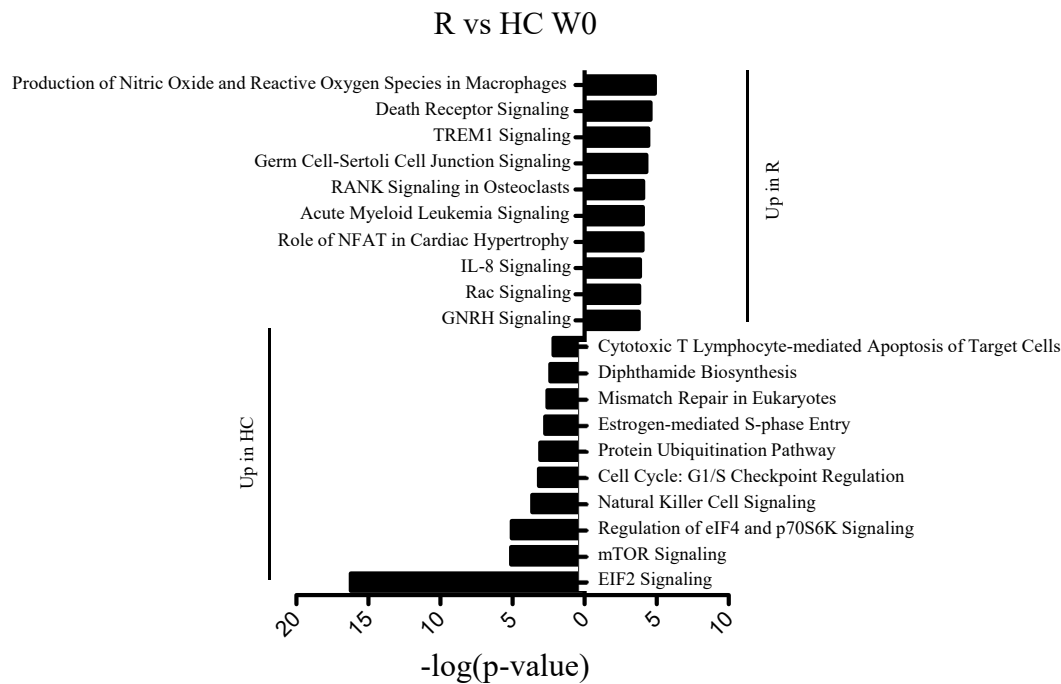
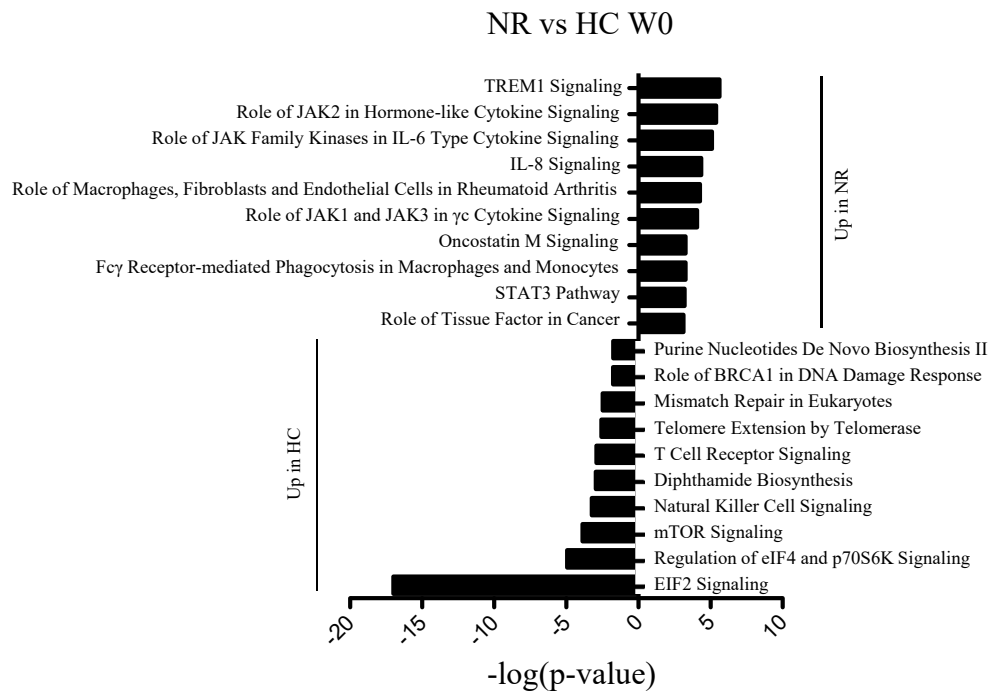


Figure 5 (continued)

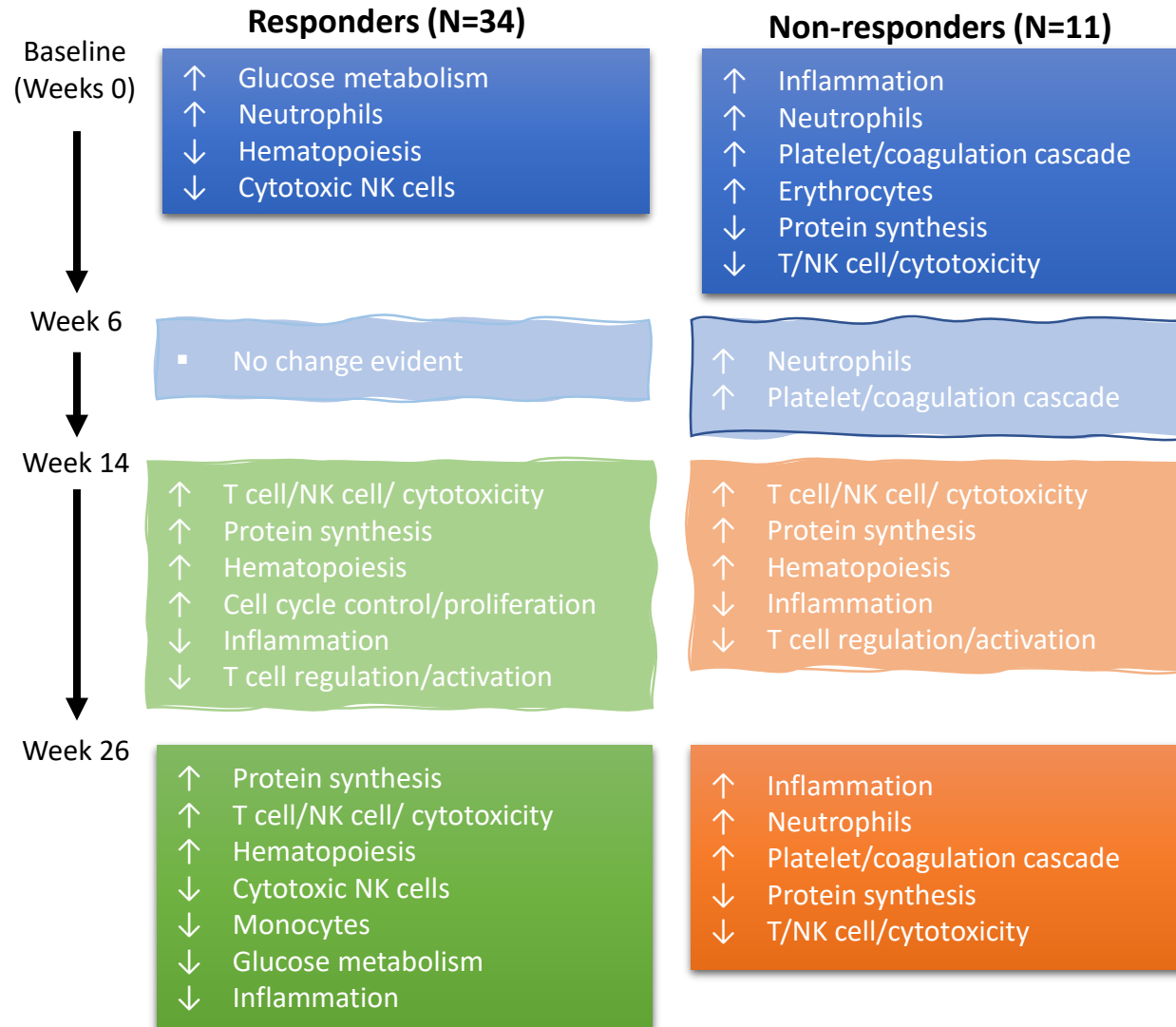
E



F



Gene cluster activity changes during and after omalizumab treatment



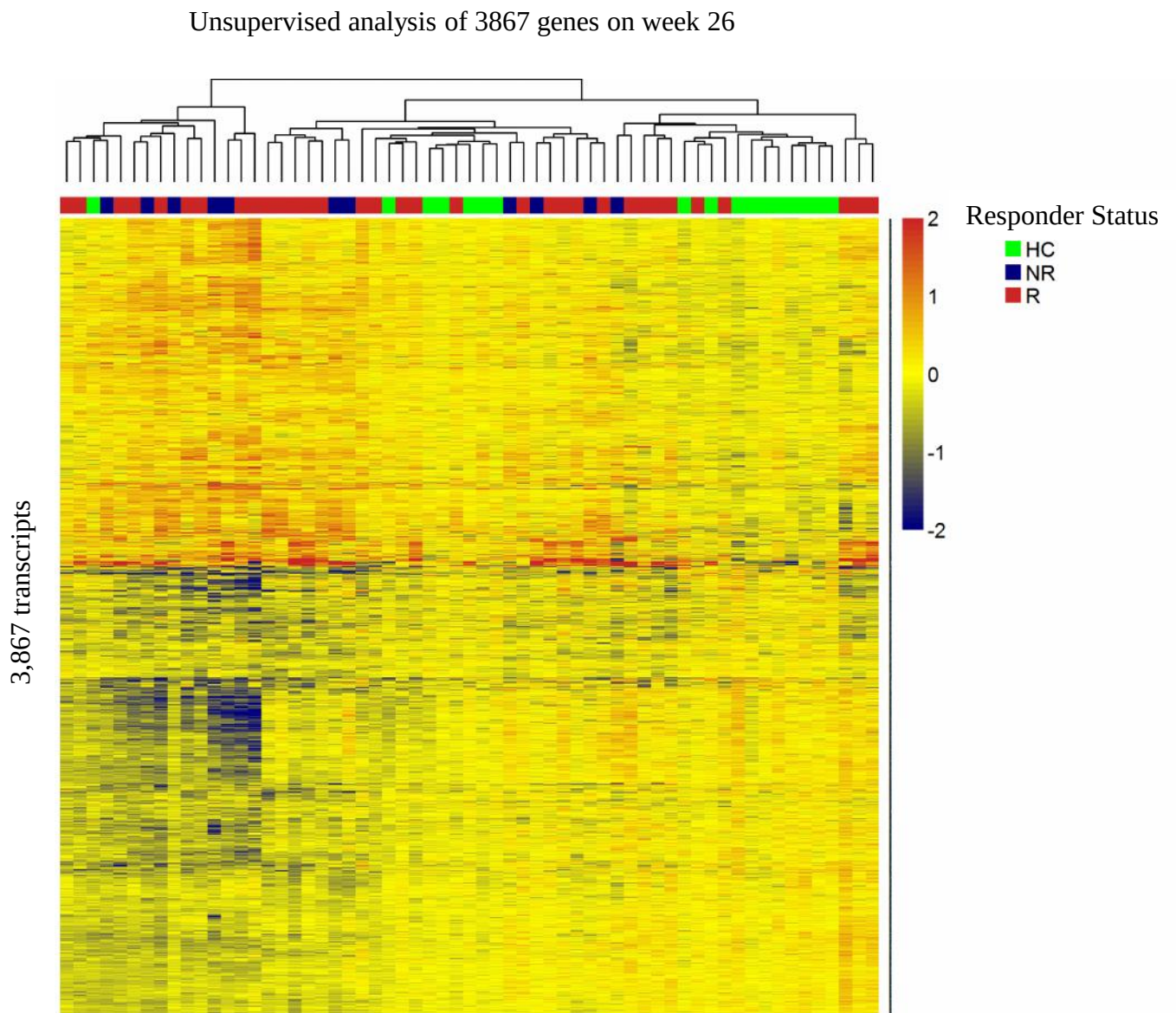


Figure S1. Transcriptional profiles of asthma patients treated with omalizumab for 26 weeks. Unsupervised analysis of the differentially expressed transcripts (N=3867) between patients (responders: R and non-responders: NR) and non-asthmatic healthy controls (HC) from baseline (FDR<0.05) at week 26 of omalizumab treatment. Samples are broken into HC (green), omalizumab NR (blue) and omalizumab R (red).

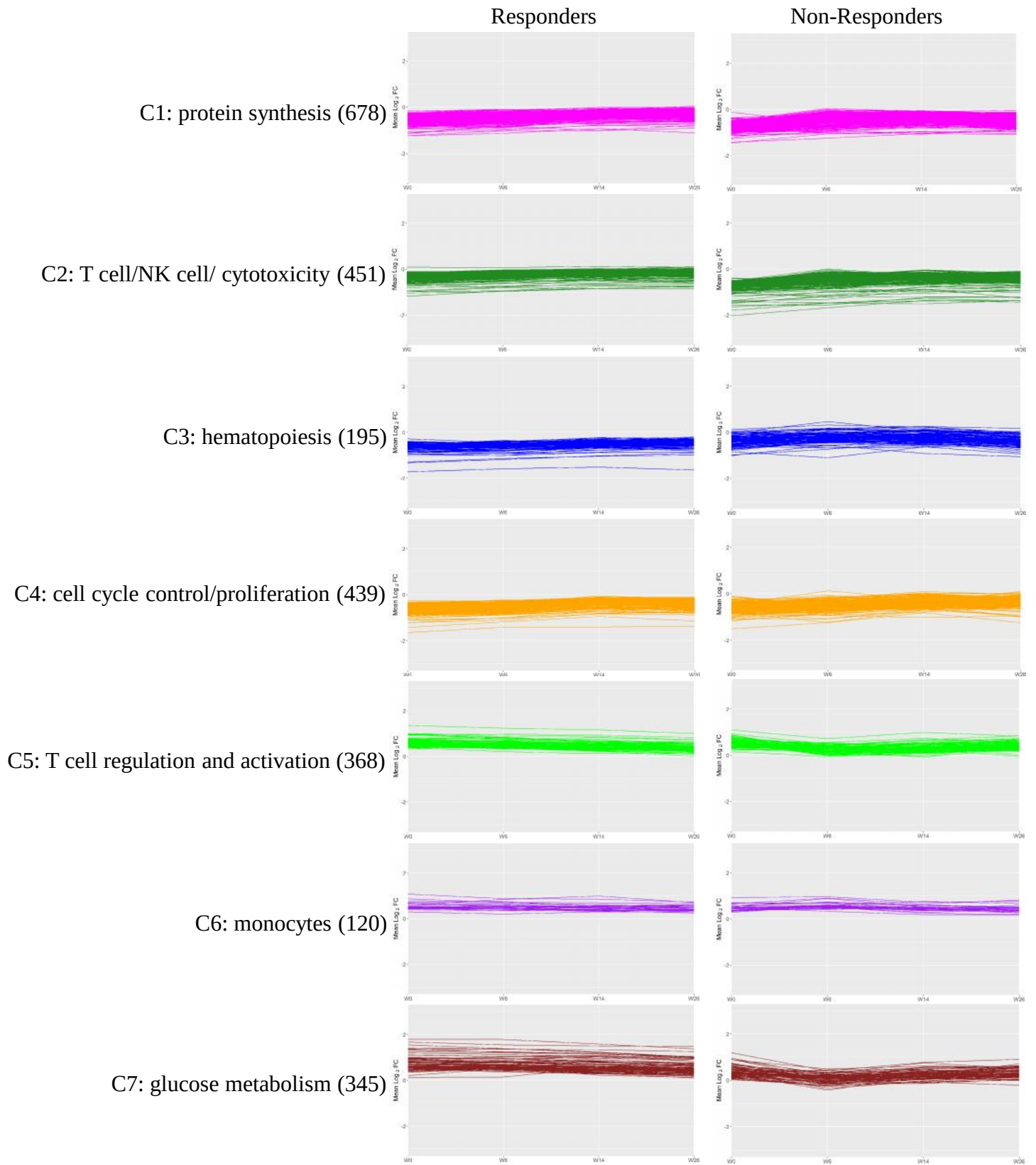


Figure S2. Parallel plots of the genes of 7 clusters (Figure 2B and Table III) in responders and non-responders.

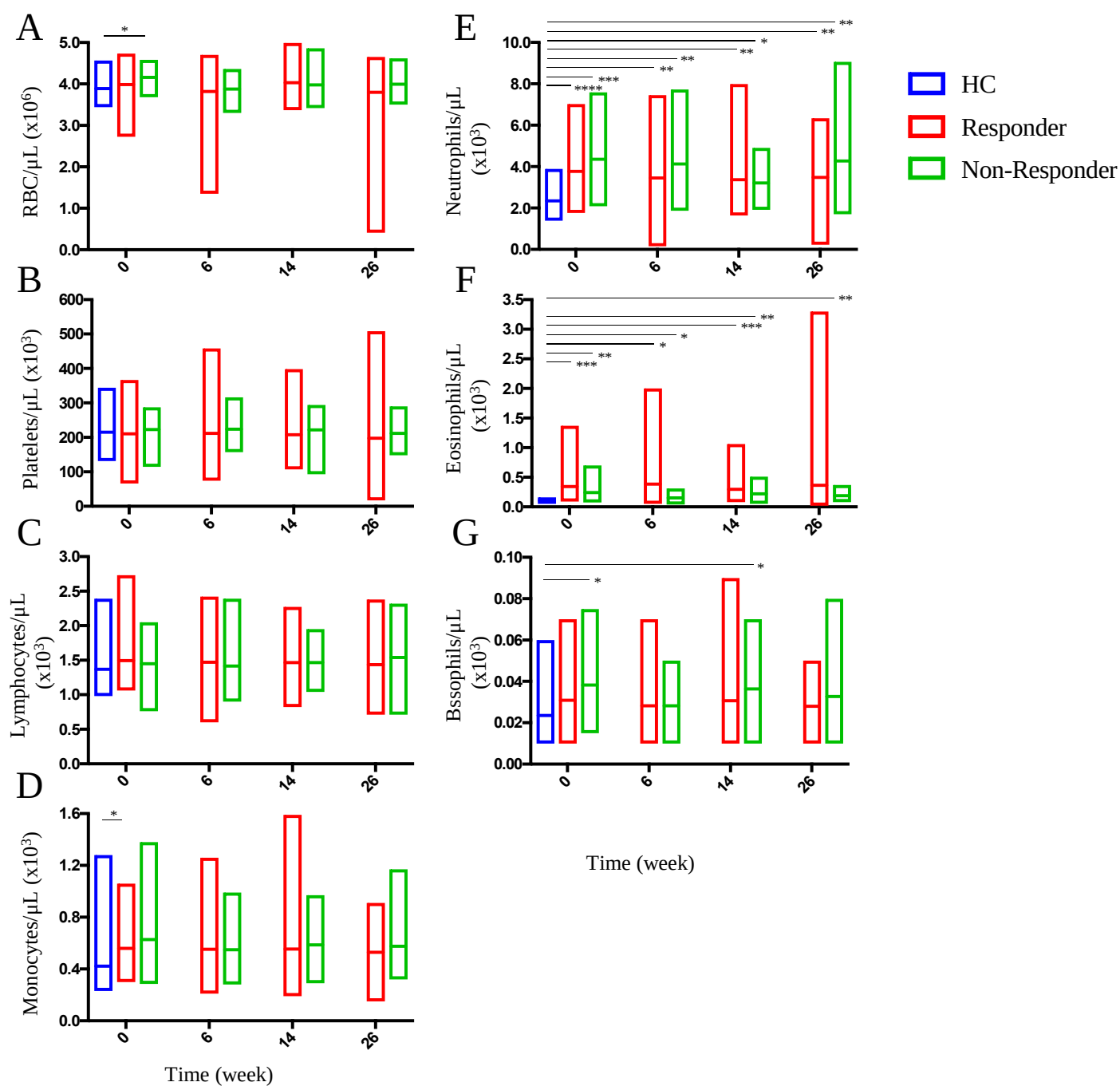


Figure S3. Cell counts acquired by CBC for (A) red blood cells (RBC), (B) platelets, (C) lymphocytes, (D) monocytes, (E) neutrophils, (F) eosinophils, and (G) basophils as cells per μL of whole blood. CBC data is incomplete for some donors. Statistical analysis done with unpaired t-test. * $P < 0.05$, ** $P < 0.01$. Bars represent mean with range.