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Transcriptional changes in alveolar macrophages from adults with asthma after allergen challenge.

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To the Editor,

Under homeostatic conditions, macrophages are the most abundant immune cells in the lung. Pulmonary macrophages are a heterogeneous cell population that can be classified in at least two distinct subpopulations, i.e., interstitial macrophages, located within the lung parenchyma, and alveolar macrophages (AM) which reside in the airway lumen, allowing direct contact with the environment (e.g., allergens, particulate matter, and commensal bacteria)(1). In recent years, AM have been shown to play an important role in environmental allergen-induced airway inflammation in asthma(2). Elimination of resident AM resulted in enhanced type 2 airway inflammation in a mouse asthma model, while depletion of blood monocytes resulted in abrogation of newly formed AM after allergen challenge and a decreased type 2 immune response(3, 4). Knowledge of phenotypic alterations of AM in allergic asthma in humans is limited(1, 2). In this study, we investigated the effect of house dust mite (HDM) and lipopolysaccharide (LPS) challenge on the transcriptome of AM from patients with mild asthma. We have shown previously that intrabronchial HDM/LPS challenge induces a mixed eosinophilic and neutrophil airways inflammation in asthma patients(5). Therefore, we hypothesize that exposure of AM to HDM/LPS would upregulate genes associated with eosinophil and neutrophil signaling.

AM were harvested from asthma patients who participated in a double blind randomized placebo controlled trial investigating the effect of C1-inhibitor on allergen-induced airway inflammation(5). The Medical Ethics Committee of the Academic Medical Center, Amsterdam (the Netherlands), approved the study and all patients gave their written informed consent. For the current analysis twelve patients (treated with placebo) with intermittent-to-mild asthma, defined according to the criteria of the Global Initiative for Asthma guideline(6), and HDM allergy, confirmed by a positive skin prick and radioallergosorbent test, were challenged via bronchoscopy with saline (as internal control) in one lung segment and HDM extract (50 biological units, *Dermatophagoides pteronyssinus* origin; Allergopharma, Zeist, the Netherlands), combined with LPS (75 ng; from *Escherichia coli*, Clinical Center Reference Endotoxin, kindly provided by Anthony Suffredini, National Institute of Health, Bethesda, MD, USA) in the contralateral lung segment. The addition of LPS to HDM mimics a natural allergen exposure as LPS is a widespread pollutant and coexisting with HDM in house dust(7). After seven hours, a bilateral bronchoalveolar lavage (BAL) was performed. BAL fluid was pooled (per lung subsegment) and centrifuged at 4°C and 400g for 10 minutes. Cells were stained with CD45 phycoerythrin (PE)-CF594 and CD71 Brilliant Violet (BV)421 (both BD Biosciences, San Jose, CA, USA). AM, defined as cells expressing CD45 and CD71, were isolated by flow cytometry sorting using FACS Aria III (BD

Biosciences) with high purity (>95%; gating strategy see Fig. S1). This sorting strategy is a limitation of our study as it selects CD71 positive AMS and does not discriminate between different AM subsets. In addition, flow cytometry did not include other markers, precluding discrimination of macrophages subsets. Sorted AM were stored in RNA later (Qiagen, Venlo, Netherlands) at -80 °C until analysis. Total RNA was isolated using RNeasy isolation kit (Qiagen) and quantified by Qubit® 2.0 (Life Technologies, Carlsbad, CA). Paired samples (AM from saline and HDM/LPS challenged lung segments of one individual) from eight patients had a RNA integrity number > 6 and were selected for further analysis. RNA-seq libraries were prepared using the KAPA RNA HyperPrep with RiboErase (Roche) according to manufacturer's instructions and sequenced using the Illumina HiSeq4000 (Illumina, San Diego, CA) instrument. Sequences were evaluated for quality (FastQC version 0.11.5, Babraham institute, Babraham, Cambridgeshire, United Kingdom) and trimmed (Trimmomatic version 0.36)(8). The remaining reads were used to align against the Genome Reference Consortium human genome build 38 (GRCh38) using HISAT2 (version 2.1.0)(9) with parameters as default. The HTSeq and DESeq2 methods are standard bioinformatics methods used to calculate and compare sequence counts, respectively. Pathway analysis was done using Ingenuity Pathway Analysis (QIAGEN bioinformatics) software. To control the false discovery rate, Benjamini & Hochberg adjusted p-values < 0.05 demarcated significance(10). Sequence libraries are publicly available through the National Center for Biotechnology Information under the following accession number GSE144576. The baseline characteristics of these patients and the leukocytes differentiation in BAL fluid are depicted in Table 1. None of the patients had any symptom at the study day.

Principal component analysis of 24,555 transcripts (sequence counts ≥ 10) revealed clear separation of saline or HDM/LPS challenges (Fig. 1A), showing that HDM/LPS induced a marked transcriptomic response in AM. Comparing HDM/LPS challenge to saline identified 1013 significantly altered transcripts (fold expression ≤ -1.2 or ≥ 1.2), of which 485 were upregulated and 528 downregulated as consequence of HDM/LPS challenge (Fig. 1B). Pathway analysis of upregulated genes revealed predominantly pro-inflammatory responses such as inducible nitric oxide synthase, Toll-like receptor, p38 mitogen-activated protein kinase and interleukin (IL)-1, IL-8, IL-17 signaling (Fig. 1C). IL-1 signaling has been implicated in neutrophilic subtype of asthma, especially an increased IL-1 β (2). Likewise, IL-17 has been identified as an important cytokine in the pathogenesis of asthma due to its capacity to promote neutrophilic airway inflammation, which is associated with a severe asthma phenotype(11). In agreement, our data shows upregulation of *CXCL1*, a chemoattractant for neutrophils, suggesting that AM aid in neutrophil recruitment following HDM/LPS challenge (Fig. 1D). In line with these finding, HDM/LPS challenge induced a marked

neutrophil influx in the airways(5). Interestingly, genes associated with the anti-inflammatory IL-10 signaling pathway were also upregulated. This could be explained by heterogeneity among AM including both pro- and anti-inflammatory subpopulations and/or concurrent induction of both pro- and anti-inflammatory pathways in the same AM. This observation suggests a regulatory role for AM to maintain homeostasis by counteracting the pro-inflammatory processes inflicted by allergen challenge. Among downregulated transcripts were genes associated with cell cycle and growth (T cell receptor, role of NFAT in cardiac hypertrophy), DNA damage and repair PI3/PI4-kinase family, including *ATM*, *PIK3CB* and *PIK3CG* (Table S1) (breast cancer regulation by stathmin 1) and metabolic pathways (e.g., insulin receptor and leptin signaling, Fig. 1C and D). Network graphs of the most enriched genes are displayed in Fig. S2 and S3.

To the best of our knowledge, this is the first investigation to examine gene expression profiles in AM from asthma patients challenged with HDM and LPS *in vivo*. Using a well-controlled study design allowing paired analysis of saline and HDM/LPS effects in the same patient, we identified different transcriptional profiles in AM as consequence of local exposure to HDM and LPS. These findings may provide a better understanding of AM functions in (exacerbations of) allergic asthma.

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Figure legend

Fig 1. Genome-wide analysis of alveolar macrophage response to HDM/LPS challenge. (A) Principal component analysis (PCA) of the 24,555 transcripts showing explainable variances of PC1 20% and PC2 13% between saline or HDM/LPS samples (B) Volcano plot depicting the differences in gene expression following either saline or HDM/LPS exposure. Horizontal line represents the multiple-test adjusted significance threshold adjusted p-value ≤ 0.05 ; fold expression ≥ 1.2 and adjusted p-value ≤ 0.05 ; fold expression ≤ -1.2 (C) Ingenuity pathway analysis of the canonical pathway gene expression profiles in HDM/LPS samples relative to saline samples. Red bars, overexpressed pathways; blue bars, under expressed pathways (D) Heatmap plot of the top significant genes between saline and HDM/LPS samples.

Table 1 Baseline patient characteristics**General**

Number	8
Female/male	7/1
Age, years	23.7 (21.8 – 25.5)
BMI	24.4 (21.5 – 27.2)

Asthma related symptoms¶ %

Wheezing	75
Cough	75
Dyspnea	83.3
Chest tightness	50
Seasonal variability	75
Symptom progression at night	33.3
Eczema	58.3
Allergic rhinitis	75
Family history of atopy	58.3

Allergy

House dust mite %	100
Total IgE, kU/L	349.5 (126.7 – 587.7)
<i>D. pteronyssinus</i> IgE, kU/L	43.9 (15.7 – 77.3)

Lung spirometry

FEV1 L	3.76 (2.9 – 4.3)
FEV1 % of predicted	98.2 (90.2 – 110.8)
FVC L	4.8 (3.6 – 5.1)
FVC % of predicted	96.7 (59.7 – 122.3)
Methacholine PC20, mg/mL†	2.6 (1.7 – 4.5)

Leukocytes in BAL fluid, cells/mL†

Alveolar macrophages pre challenge	44695 (25510 – 68380)
Alveolar macrophages post challenge	45114 (8782 – 59633) ns
Eosinophils pre challenge	309 (128 – 1440)

Eosinophils post challenge	23530 (2156 – 98747) **
Neutrophils pre challenge	2473 (1602 – 13528)
Neutrophils post challenge	95186 (13496 – 195794) *
CD4 T-cells pre challenge	2859 (1524 – 4871)
CD4 T-cells post challenge	7913 (2040 – 8810) **

Data are presented as mean with 95% confidence interval unless otherwise stated. *D. pteronyssinus*:

Dermatophagoides pteronyssinus; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; PC20: provocative dose causing a 20% fall in FEV1; NA: not applicable. #: Global Initiative for Asthma stage I/II.¶

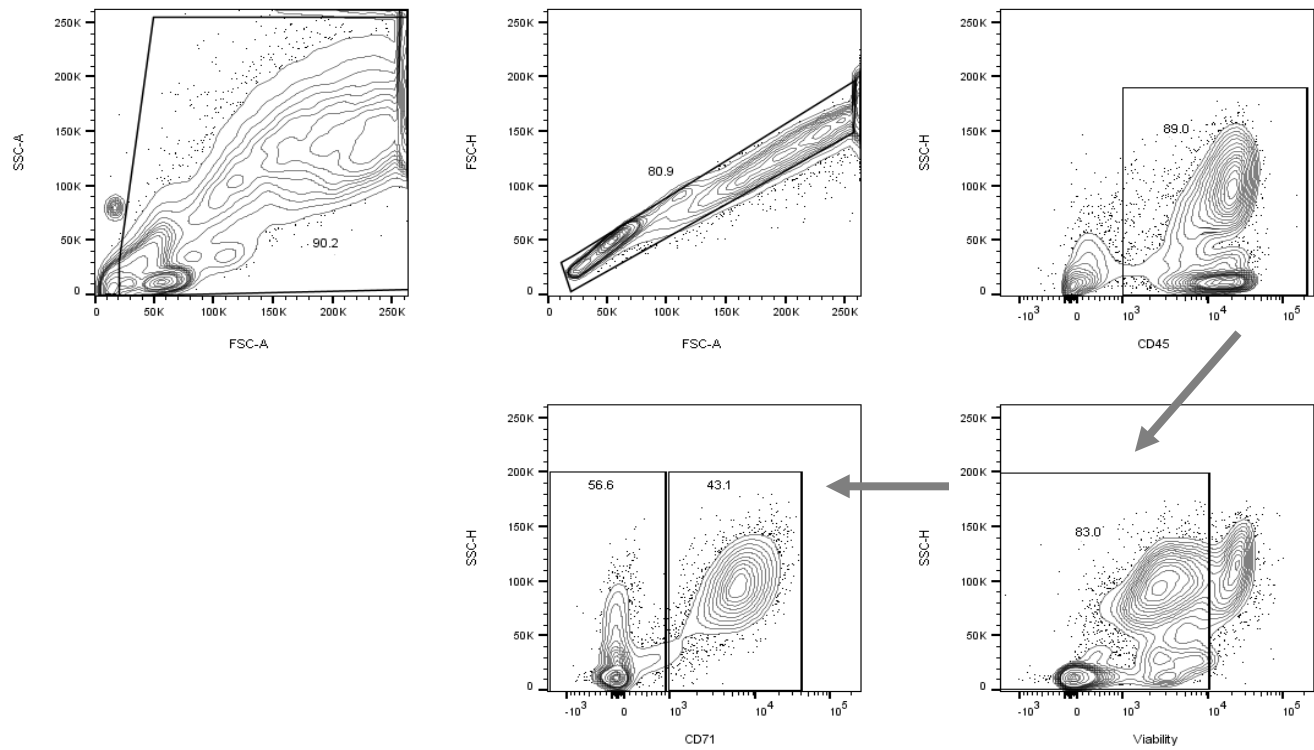
Symptoms occurred in last 6 months prior to study participation. † Data are expressed as median with interquartile range. ns: not significant, * $p < 0.05$, ** $p < 0.01$ compared to pre challenge. Data were analyzed by Wilcoxon signed rank test for comparison between groups.



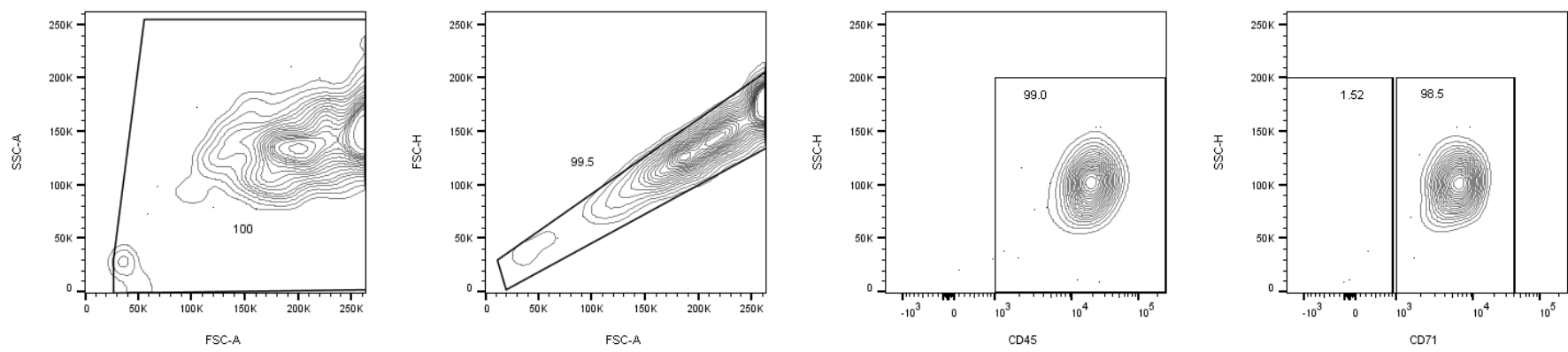
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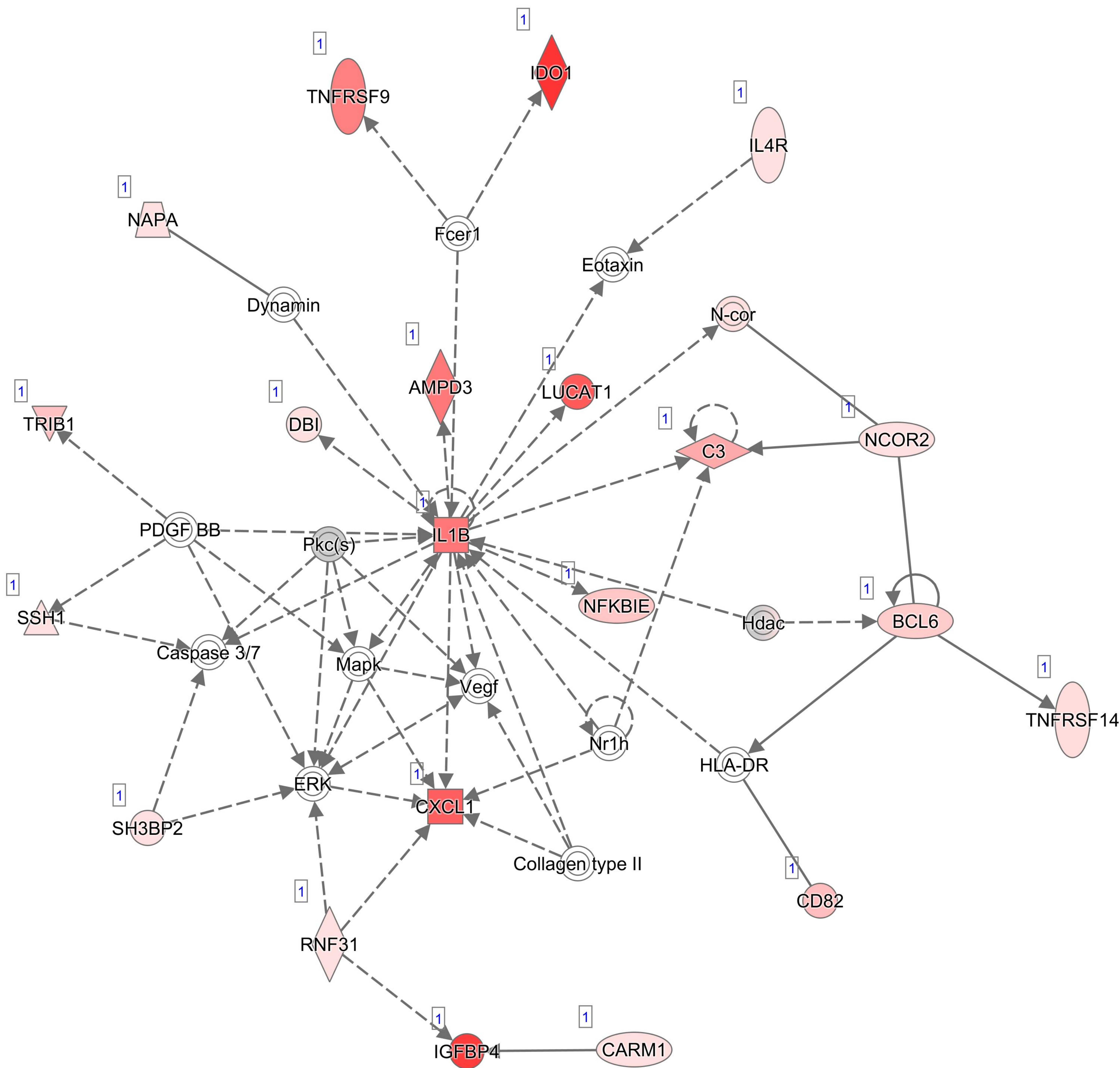
Fig S1.

A



B





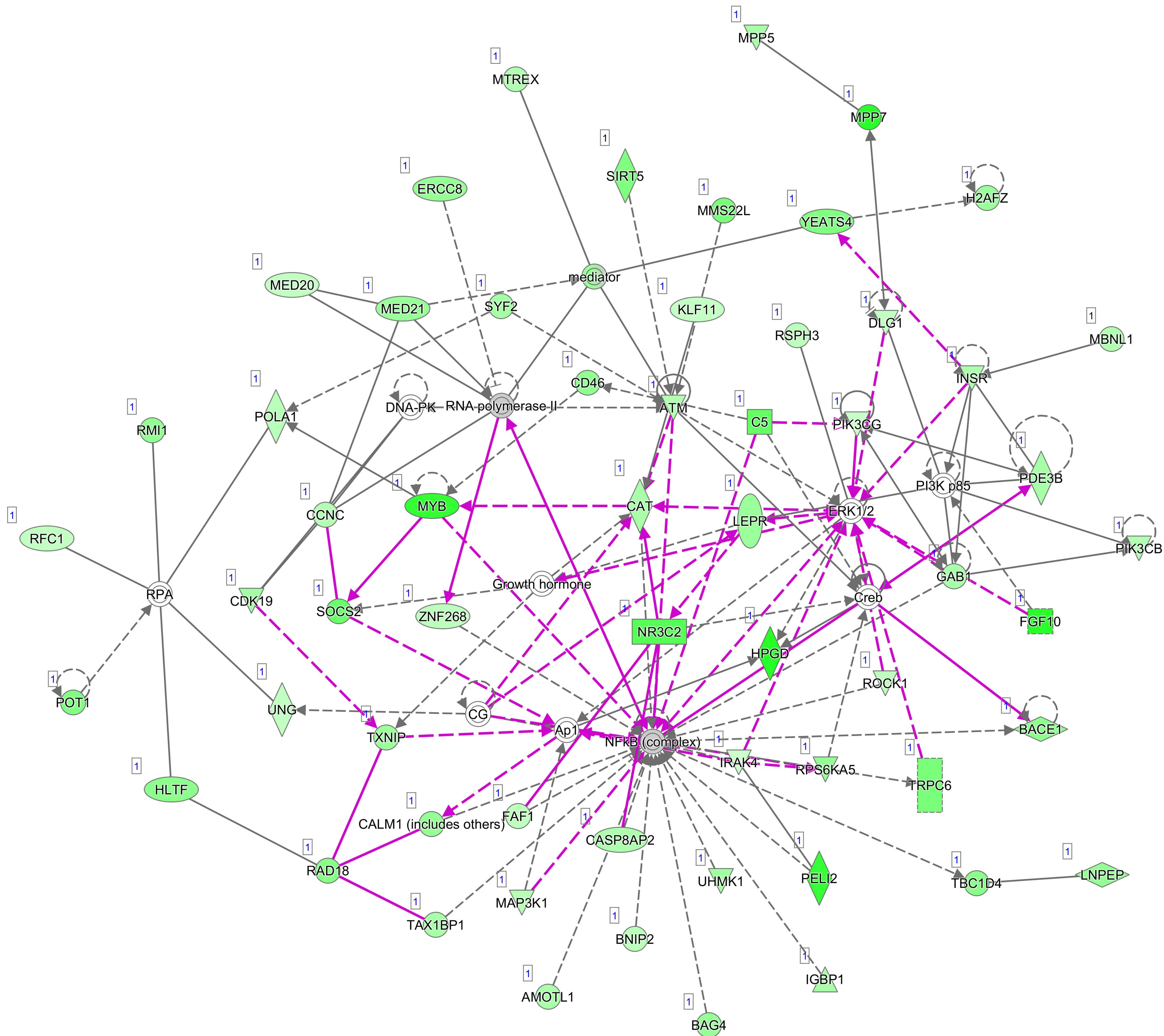


Table of enriched canonical signaling pathways

Upregulated Ingenuity Canonical Pathways	adjusted p-value	Genes
IL-10 Signaling	0.0166	FCGR2B, IL1A, IL1B, IL4R, MAP2K3, NFKB1, NFKBIB, NFKBIE
IL-1 Signaling	0.0166	GNA12, GNG2, IL1A, IRAK1, IRAK2, MAP2K3, NFKB1, NFKBIB, NFKBIE
iNOS Signaling	0.0166	HMGA1, IFNGR2, IRAK1, IRAK2, NFKB1, NFKBIB, NFKBIE
Colanic Acid Building Blocks Biosynthesis	0.0166	GALE, GMPPA, GMPPB, PMM2
GDP-mannose Biosynthesis	0.0166	GMPPA, GMPPB, PMM2
Remodeling of Epithelial Adherens Junctions	0.042	ACTB, ACTG1, HGS, TUBA1C, TUBB, TUBB4B, TUBG1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.043	CCR7, COL4A2, CSF1, ECE1, FLT1, IFNGR2, IGFBP4, IL1A, IL1B, IL4R, MYL9, NFKB1
TWEAK Signaling	0.043	BID, NFKB1, NFKBIB, NFKBIE, TNFRSF12A
IL-8 Signaling	0.048	CSTB, CXCL1, FLT1, GNA12, GNG2, IRAK1, IRAK2, MYL9, NFKB1, NFKBIB, PIK3R5, PLD2
Role of IL-17A in Arthritis	0.048	CXCL1, MAP2K3, NFKB1, NFKBIB, NFKBIE, PIK3R5
Toll-like Receptor Signaling	0.048	IL1A, IL1B, IRAK1, IRAK2, MAP2K3, NFKB1, TICAM1
p38 MAPK Signaling	0.048	DDIT3, IL1A, IL1B, IRAK1, IRAK2, MAP2K3, MYC, RPS6KB2, SRF
Downregulated Ingenuity Canonical Pathways	adjusted p-value	Genes
Breast Cancer Regulation by Stathmin1	0.032	ADCY9, ARHGEF6, ATM, CALM1, PIK3CB, PIK3CG, PPP1CC, PPP2R3A, PPP2R5A, PRKACB, PRKCI, RAP2A, RB1CC1, ROCK1, SOS2, UHMK1
Role of NFAT in Cardiac Hypertrophy	0.039	ADCY9, ATM, CALM1, CHP1, HDAC9, MAP3K1, PIK3CB, PIK3CG, PLCL2, PPP3CA, PRKACB, PRKCI, RAP2A, SOS2, TGFB2
Telomerase Signaling	0.039	ATM, ELF2, HDAC9, PIK3CB, PIK3CG, POT1, PPP2R3A, PPP2R5A, RAP2A, SOS2
Insulin Receptor Signaling	0.039	ATM, FOXO4, GAB1, INSR, PDE3B, PIK3CB, PIK3CG, PPP1CC, PRKACB, PRKCI, RAP2A, SOS2
T Cell Receptor Signaling	0.039	ATM, CALM1, CD3G, MAP3K1, PIK3CB, PIK3CG, PPP3CA, RAP2A, SOS2, TXK
Leptin Signaling in Obesity	0.046	ADCY9, ATM, LEPR, PDE3B, PIK3CB, PIK3CG, PLCL2, PRKACB

Supplemental figure legend

Fig. S1 Gating strategy for alveolar macrophages (AM) in BAL fluid. (A) single CD45 positive and viable cells expressing CD71 were sorted as AM. (B) The sorted cells were subsequently checked for its purity.

Fig. S2 Network graphs of genes involved in iNOS, IL-1 and IL-10 signaling (upregulated pathways)

Fig. S3 Network graphs of genes involved in breast cancer regulation (or DNA damage response), insulin and T cell receptor signaling pathways (downregulated pathways)