A novel CD4$^+$ T-cell dependent murine model of *Pneumocystis* driven asthma-like pathology

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Online Data Supplement

Methods

RNA sequencing pathway analysis

The FASTQ files from our previous study were uploaded to Illumina BaseSpace cloud-based software, aligned using TopHat version 1.0.0 and assembled with Cufflinks Assembly and DE, Version 1.1.0. Pathway analysis was then performed using iPathwayGuide, Version 1.4.0. KEGG and Disease pathways were analyzed for significance using a false discovery rate.

Purification of $CD4^+$ T-cells and adoptive transfer

Spleens from C57BL/6 mice were harvested, physically digested, and passed through a 70-µm filter. $CD4^+$ T-cells were isolated using a Stemcell™ EasySep™ Mouse kit (19852) and were subsequently enumerated. Each *Rag1*-/- mouse received 5 x 10^5 cells in 200 µL via intravenous injection. Mice were infected on the day of transfer as described above and sacrificed 6 weeks post infection.

ELISAs

96-well plates were coated overnight in a 9.2 pH carbonate buffer with 150 ng/well of *Pneumocystis* antigen, *Aspergillus fumigatus* antigen (GM3A01, Greer®), house dust mite extract (XPB70D3A2.5, Greer®), or a heat-killed *Streptococcus pneumonia* preparation. Plates were washed 5x with PBST and blocked in PBST with 5% milk for 2 hours at room temperature. Serum was diluted 1:64 (IgG assays) or 1:10 (IgE assays) in PBST with 5% milk and 100 µL was added to each well and the plate was incubated overnight at room temperature. Plates were then washed 5x with PBST and treated with

1:1000 goat anti-mouse IgG-HRP or 1:1000 goat anti-moues IgE-HRP (1030-05, Southern Biotech) diluted in blocking buffer. Following a one-hour incubation at room temperature, plates were washed 5x with PBST, developed with 1x TMB solution (BD OptEIA™, 555214), stopped with H_2SO_4 and read at 450nm. For human serum assays, bovine serum albumin was used in place of milk and rabbit anti-human IgG (A8792, Sigma®) and goat anti-human IgE (A18793, Life Technologies) were used as secondary antibodies.

Anti-PCAg Western blotting

PCAg (5 µg) or murine lung homogenate (10 µg) was denatured at 65° in LDS buffer with β-mercaptoethanol for 10 minutes and then added to a 4-20% tris-bis gel (Bio-rad) and run at 200V for 35 minutes. Following transfer to a nitrocellulose membrane (Bio-rad), the membrane was washed for 5 minutes in TBST and blocked at room temperature in TBST with 5% BSA for 2 hours. Serum samples were diluted 1:100 in TBST with 5% BSA, applied to the membrane, and incubated at 4° overnight. Membranes were washed three times with TBST for 5 minutes each, followed by addition of secondary antibody (described in ELISA) for one hour at room temperature. Membranes were then washed, developed using SuperSignal® West Pico Chemiluminescent Substrate (#34080, Thermo Scientific) and luminescence was measured for 1 second.

Flow cytometry

Whole lung cells were isolated prepared as described in the *ex vivo* whole lung cell stimulation methods. Briefly, cells were plated, centrifuged, and resuspended in surface antibodies diluted in PBS. Following a 1 hour incubation at 4°C, cells were washed and resuspended in the streptavidinBV421 (diluted 1:200). Following a 30 minute incubation at 4°C, cells were washed and fixed and permeabilized using the eBioscience FoxP3 staining kit per manufacturer's instructions. Intracellular antibodies, anti-CD45.2, and anti-CD90.2 antibodies were added and incubated at 4°C for 1 hour. Cells were then washed, resuspended, and analyzed on a BD LSR II Flow Cytometer with compensation via OneComp eBeads (eBioscience). Data analysis was conducted using FlowJo software (Treestar).

Antibodies used in these panels were diluted 1:200 in PBS unless otherwise specified and include: GATA3-FITC, diluted 1:5 (BD Pharmingen, Clone: L50-823), TCRβ-PerCP-Cy5.5 (eBioscience, Clone: H57-597), TCRγδ-PE (eBioscience, Clone: GL3), CD90.2-PE-Cy7, diluted 1:1500 (eBioscience, Clone: 53-2.1), RORγT-APC (BD Pharmingen, Clone: Q31-378), CD45.2-APC-e780 (eBioscience, Clone: 104), Streptavidin-BV421 (BioLegend, Cat: 405226), CD11b-Biotin (eBioscience, Clone: M1/70), F4/80-biotin (eBioscience, Clone: BM8), NK1.1-biotin (BD Pharmingen, Clone: PK136), CD11c-biotin (BD Pharmingen, Clone: HL3), CD19-biotin (BD Pharmingen, Clone: 1D3), TER119-biotin (BD Pharmingen, Cat: 553672). APC panel: GR-1-e450 (BD Biosciences, Clone: RB6-8C5), CD11b-APC (Biolegend, Clone: M1/70), F4/80-APC-e780 (eBioscience, Clone: BM8), SiglecF-PE (BD Biosciences, E50-2440), CD11c-FITC (eBioscience, Clone: N418).

Lung Mechanics and Airway Hyperresponsiveness Measurements

Pulmonary function was assessed during mechanical ventilation of anesthetized (90 mg/kg pentobarbital i.p.) and tracheotomized mice using a computer-controlled small-animal mechanical ventilator (FlexiVent; SCIREQ, Montreal, Quebec, Canada). Briefly, mice were mechanically ventilated at 200 breaths/min with a tidal volume of 0.25mL and a positive end expiratory pressure of 3 cmH_2O (mimicking spontaneous ventilation). The quasi-static mechanical properties of the lung (compliance and hysteresis) were calculated using pressure-volume curves derived from a step-wise inflation of the lung to a maximum pressure of 30cmH_2O. Newtonian resistance (Rn), tissue damping (G), and tissue elastance (H) were assessed prior to and following airway challenge with increasing doses of aerosolized methacholine (0, 0.75, 3.125, 12.5, and 50 mg/mL) using the forced oscillation technique and constant phase model as previously described. Multiple linear regression was used to fit measured pressure and volume in each individual mouse to the model of linear motion of the lung. Model fits that resulted in a coefficient of determination less than 0.8 were excluded.

Online Supplement Figure Legends

Supplementary Figure 1. Pathway analysis on RNA sequencing shows upregulation of pathways associated with type II immunity. A.) KEGG pathways significantly upregulated in wild type mice compared to $CD4^+$ T-cell depleted mice. B.) Individual gene expression associated with each KEGG pathway expressed as log(Fold change). C.) Disease pathways significantly upregulated in wild type mice compared to $CD4^+$ T-cell depleted mice. D.) Individual gene expression associated with each disease pathway log(Fold change).

Supplementary Figure 2. $CD4^+$ T-cells are sufficient to induce type II mediated pathology. A.) *Rag1* -/- mice received PBS (no transfer, *n*=4) or 5 x 10^5 purified $CD4^+$ T-cells (*n=4)* via intravenous injection and were infected with *Pneumocystis murina*. Six weeks post-infection, *Rag1* -/- receiving $CD4^+$ T-cells had increased gene expression of type II related genes in lung homogenate (*p<0.05, **p<0.01, student's t-test).

Supplementary Figure 3. **PCAg treated mice have increased Th2 cells and eosinophil recruitment in the lung.** A.) Following PCAg treatment, lung cells were isolated from lungs of PCAg treated or naïve mice and stimulated with PCAg or CD3/CD28 beads for 72 hours and analyzed for expression of *Gata3* and *Rorc* (** p<0.01). B.) Flow cytometry on $CD4^+$ T-cells demonstrates an increase in intracellular GATA3, but not RORγT, following PCAg treatment. C.) Quantification of Th2 $GATA3^+$ cells and Th17 $ROR\gamma T^+$ cells in naïve and PCAg treated mice (p<0.05, student's t-test). D.) qRT-PCR on *ex vivo* stimulated lung cells shows increases in *Il4* and *Il13* in

response to PCAg and a log increase in expression of *Il4*, *Il5*, and *Il13* following CD3/CD28 stimulation (*p<0.05). E.) Flow cytometry on whole lung cells shows an increase in eosinophils (SiglecF^{+}CD11b^{+}) following PCAg treatment. F.) Flow cytometry on whole lung cells shows minimal recruitment of neutrophils (CD11b^{+}Gr1^{+}) following PCAg treatment. G.) Quantification of eosinophils and neutrophils by flow cytometry shows significant increase in eosinophils following PCAg treatment (p<0.01, student's t-test).

Supplementary Figure 4. **PCAg and HDM induce similar type II responses in BALB/c mice**. A.) BALB/c mice were treated with either PCAg or HDM and type II response in whole lung was analyzed by qRT-PCR (*n*=6 per group). HDM and PCAg induce comparable type II responses in BALB/c mice (* p<0.05, ** p<0.01, one-way ANOVA with Tukey's multiple comparisons).

Supplementary Figure 5. **ST2-antibody treatment partially abrogates the pathologic Th2 response to PCAg**. C57BL/6 mice were untreated (naïve), treated with PCAg, or treated with PCAg and anti-ST2 blocking antibody (*n*=6-7). A.) Flow cytometry gating strategy for isolating ILC2 cells from whole lung. Cells were gated on lineage negative, γδ-TCR negative, CD90 high, TCRβ negative, GATA3 high (*n*=4). B.) Quantification of ILC2 cells (TCRβ^{-}GATA3^{+}) and Th2 cells (TCRβ^{+}GATA3^{+}). C.) Anti-*Pneumocystis* antigen IgG and IgE responses are unchanged with anti-ST2 treatment. D.) qRT-PCR for type II immune response genes showing decrease in type II cytokine production in anti-ST2 treated mice, but no change in mucus associated genes. E.) PAS

staining demonstrating mucus production in PCAg and PCAg mice treated with anti-ST2. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, one-way ANOVA with Tukey's multiple comparisons).

Supplementary Figure 6. Individual antibody titer curves for healthy control and severe asthma patients against *Pneumocystis* and *S. pneumoniae*. A.) Individual curves for HC and SA patients showing a shift to the right in a subset of patients with SA against PCAg. B.) Individual curves for HC and SA patients showing similar antibody responses against *S. pneumoniae* (SP) antigen preparation.

Supplementary Figure 7. **Serum from a healthy control and severe asthma patient demonstrate limited reactivity to mouse lung protein**. A.) Serum samples from a healthy control and a patient with severe asthma were probed for reactivity against lung homogenate from naïve mice (N) or mice treated previously treated with PCAg (D14). Unlike the PCAg western, the same human serum samples displayed a limited reactivity to mouse protein.

Supplementary Figure 8. **Allergic sensitization in healthy control and severe asthma patients**. A.) Serum IgE shows elevation in SA patients compared to healthy controls, but no difference based on anti-PC IgG status. B.) Allergen skin testing analysis for HC, PC IgG low, and PC IgG high patients.

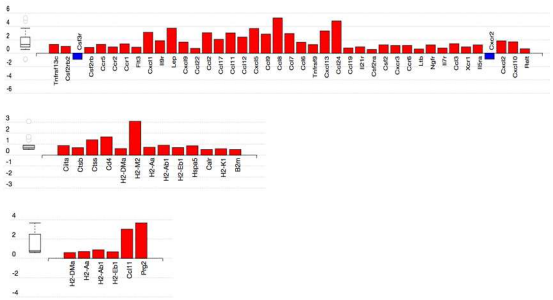
Supplementary Figure 9. **Peripheral blood and bronchoalveolar lavage cell analysis in healthy control and severe asthma patients**. A.) Number of neutrophils, lymphocytes, and eosinophils in peripheral blood in HC, PC IgG low, and PC IgG high patients (* $p<0.05$, ** $p<0.01$, one-way ANOVA with multiple comparisons). B.) Percentage of neutrophils, lymphocytes, eosinophils, and macrophages in the bronchoalveolar lavage of HC, PC IgG low, and PC IgG high patients.

Supplemental Figure 1

A

KEGG Pathway	p-value
Cytokine-cytokine receptor interaction	6.67×10^{-7}
Antigen processing and presentation	8.95×10^{-4}
Asthma	6.00×10^{-3}

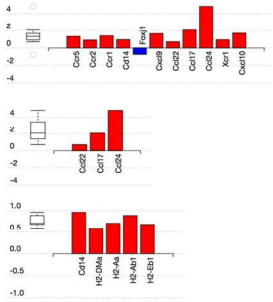
B



C

Disease Pathway	p-value
Allergic Rhinits	2.97×10^{-7}
Eosinophilic esophagitis	0.002
Asthma	0.029

D



Pathway analysis on RNA sequencing shows upregulation of pathways associated with type II immunity.
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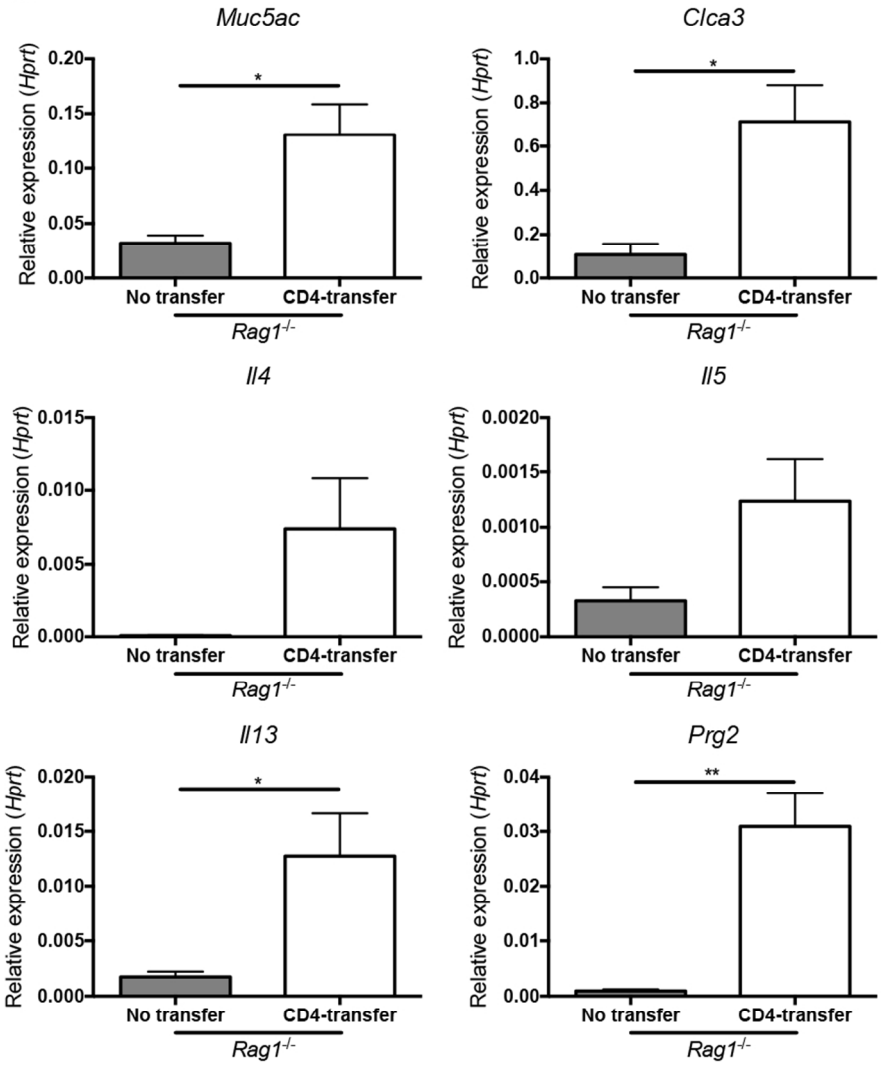
Supplemental Figure 2

A



CD4+ T-cells are sufficient to induce type II mediated pathology.
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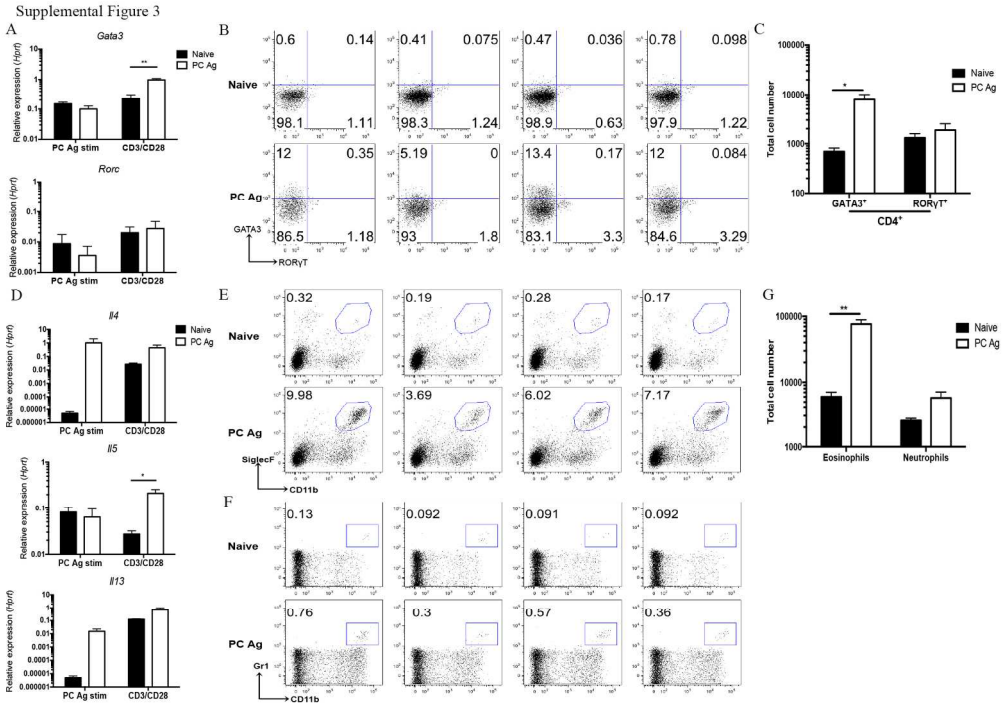

PCAg treated mice have increased Th2 cells and eosinophil recruitment in the lung.
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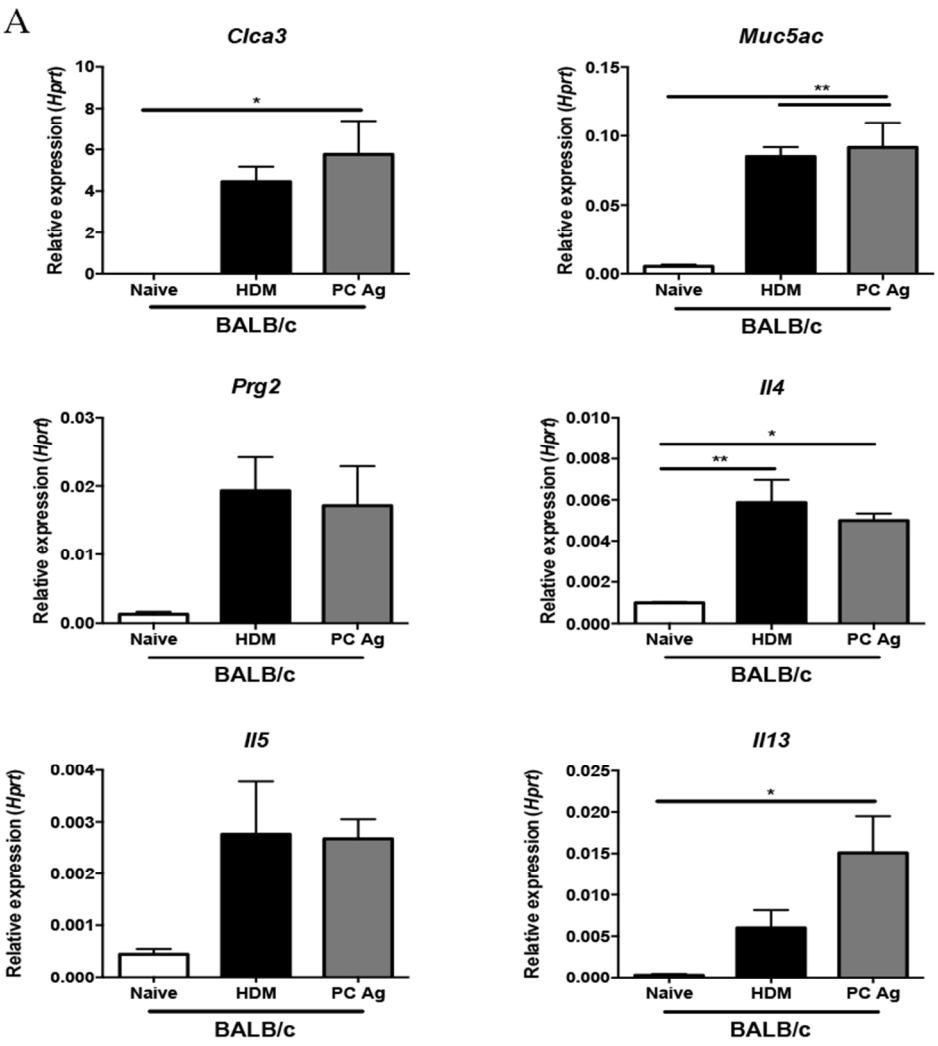
Supplemental Figure 4



PCAg and HDM induce similar type II responses in BALB/c mice.
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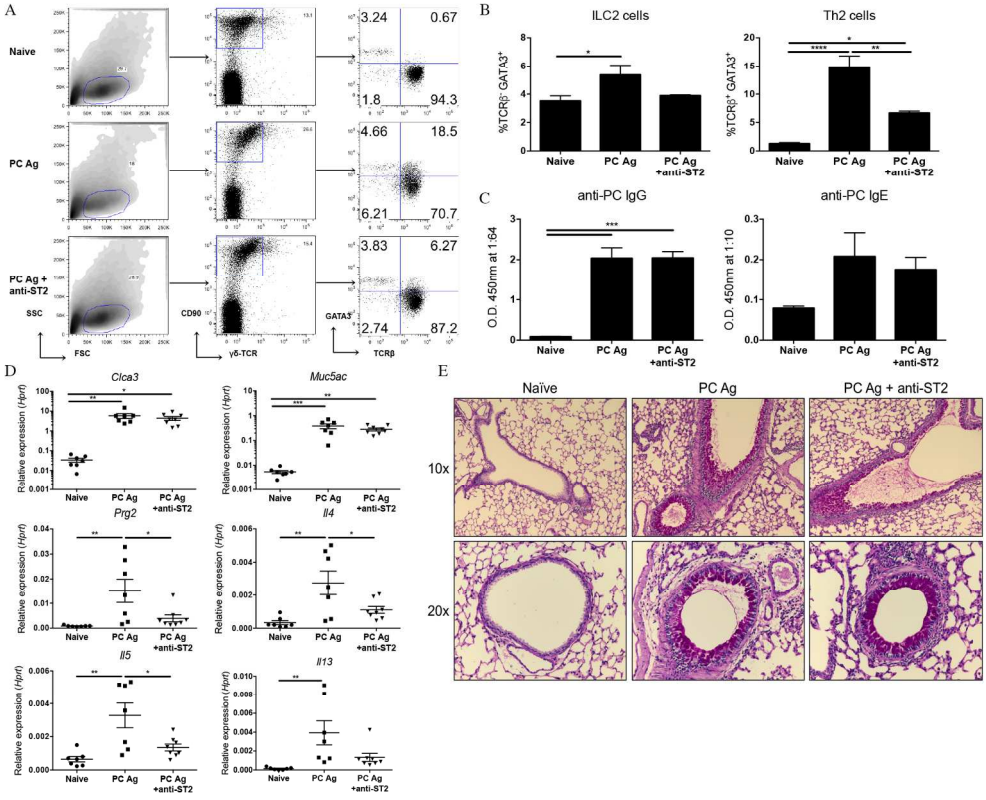
Supplemental Figure 5



ST2-antibody treatment partially abrogates the pathologic Th2 response to PCAg
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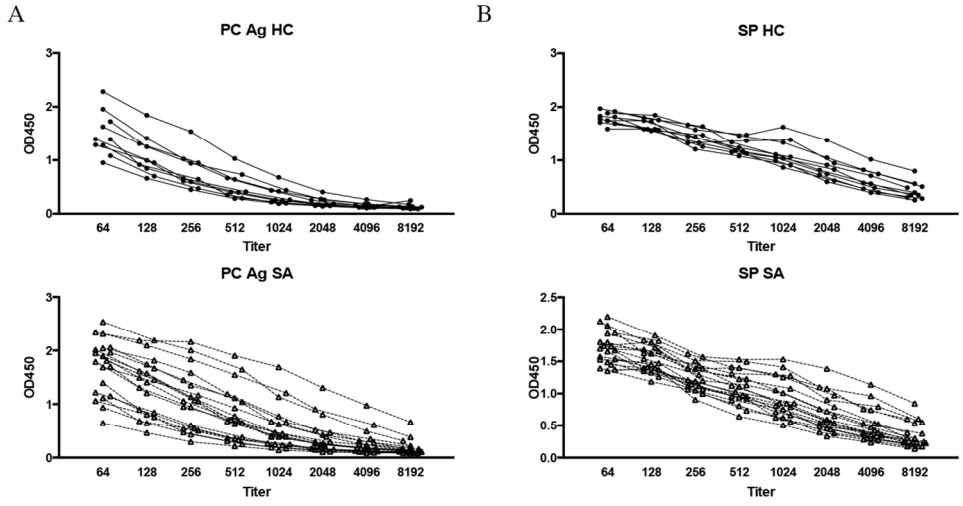
Supplemental Figure 6



Individual antibody titer curves for healthy control and severe asthma patients against Pneumocystis and S. pneumoniae.
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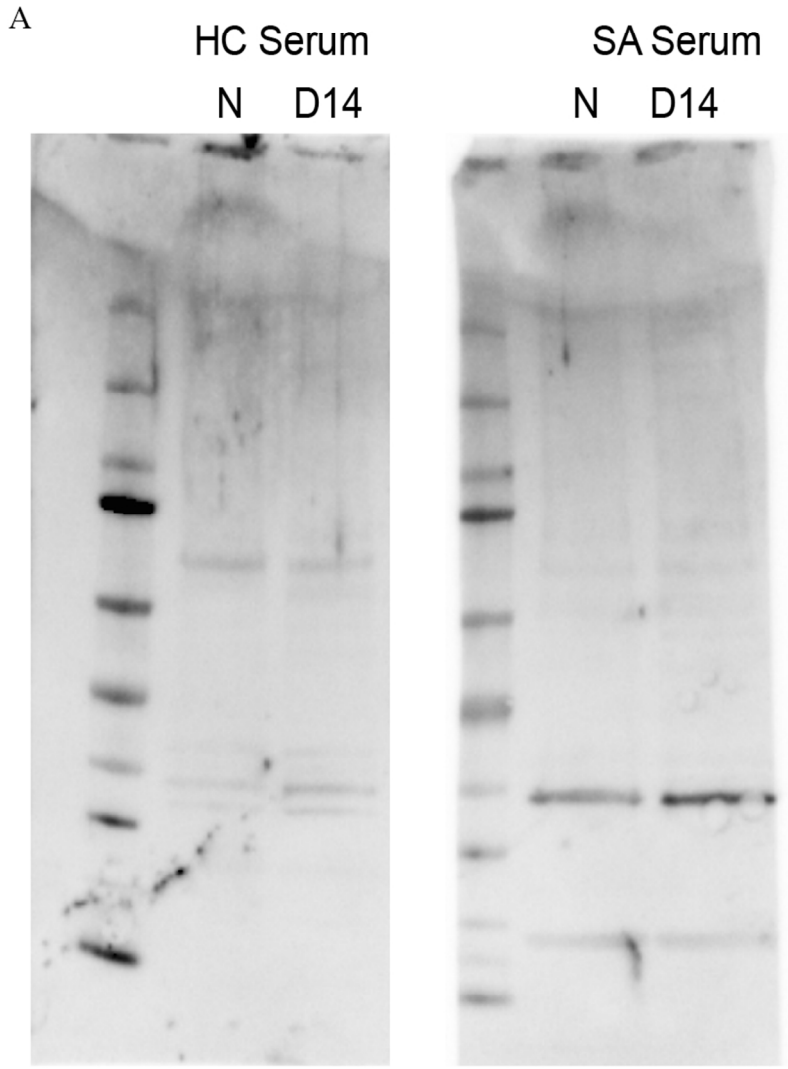
Supplemental Figure 7



Serum from a healthy control and severe asthma patient demonstrate limited reactivity to mouse lung protein.
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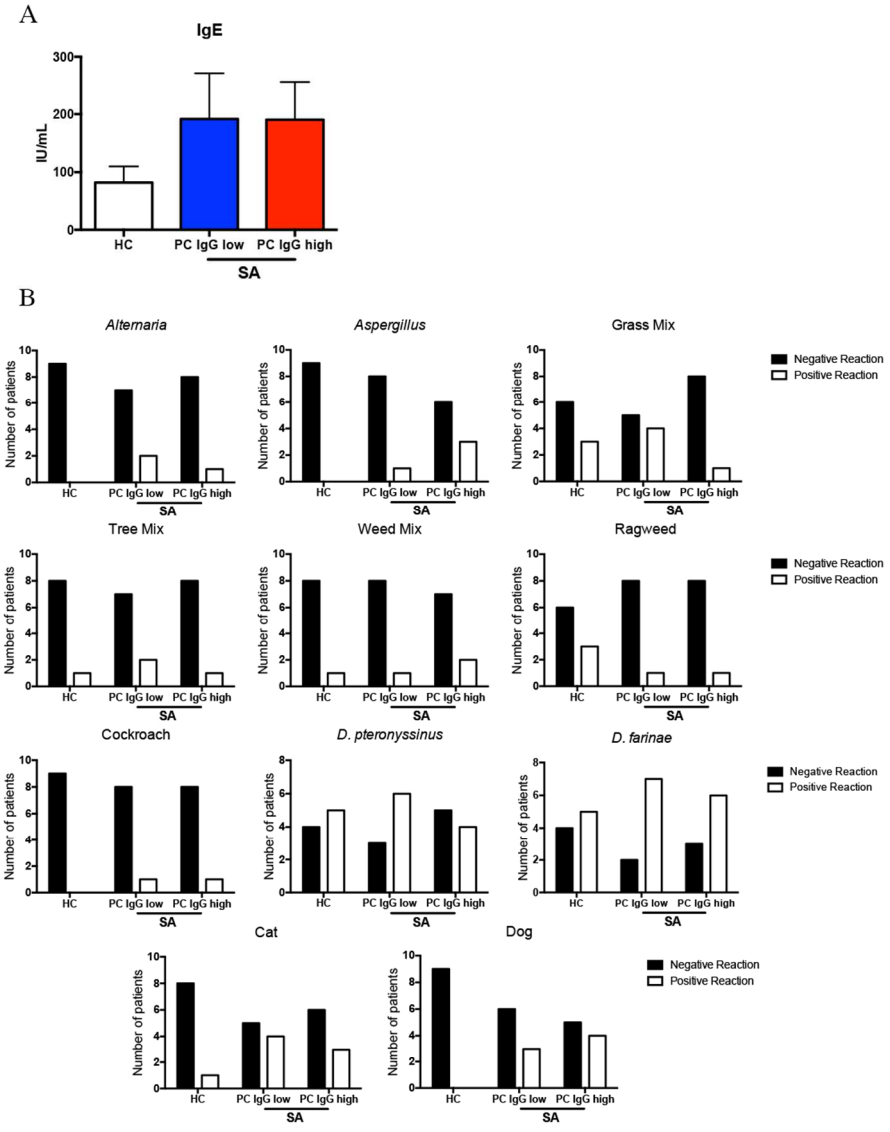
Supplemental Figure 8



Allergic sensitization in healthy control and severe asthma patients.
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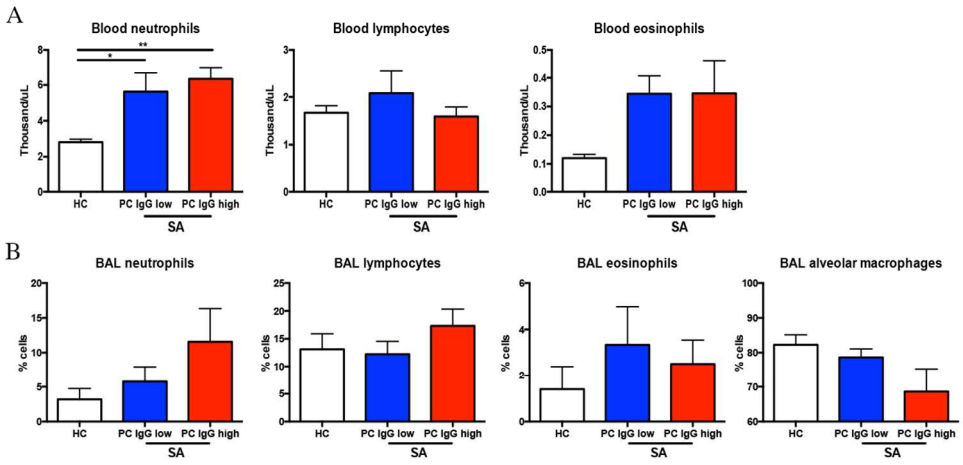
Supplemental Figure 9



Peripheral blood and bronchoalveolar lavage cell analysis in healthy control and severe asthma patients.
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