Figure 1: Asthmatic phenotypes are age-dependent in mice when provoked by viruses but not allergens. **(A)** Cartoon depicting the SeV infection model. Time periods denoting acute SeV bronchiolitis, peak lung viral load, and the development of chronic lung pathology are depicted by shaded bars. **(B)** Representative Periodic Acid Schiff (PAS)-stained lung sections obtained 49 days post-SeV infection (DPI, 1.5 x 10^5 pfu/mouse) from juvenile mice (6 weeks old) or mature mice (11 months old). PBS-treated juvenile mice are shown as a negative control. Scale bar=500μm. **(C)** Quantification of mucous cell metaplasia via PAS staining (mean ± SE) in PBS-treated mice (n=3) and SeV-infected mice (n=7-11). **(D, E).** Induction of lung *MUC5AC* expression **(D)** and IL-13 expression **(E)** at 49 DPI as a function of mouse age and SeV dose. Each bar represents the mean ± SE normalized to PBS-treated control mice. Open bar, PBS (n=5-6); grey bar, 1.5 x 10^5 pfu/mouse SeV (n=6-9); black bar, 7.5 x 10^5 pfu/mouse SeV (n=5-6). Data are representative of 2 dose response experiments. **(F)** Induction of *MUC5AC* expression by SeV (1.5 x 10^5 pfu) at 49 DPI in mice of various ages. Bars represent the mean ± SE normalized to PBS-treated controls. Open bars, PBS-treated mice (n=4); black bars, SeV-infected mice (n=7-12). Data are pooled from 3 independent experiments. **(G)** Airway resistance as a function of methacholine exposure at SeV 49 DPI (mean ± SE). Red circles, SeV-infected juveniles (n=10); blue squares, SeV-infected mature mice (n=8); pink circles, PBS-treated juvenile mice (n=6); light blue squares, PBS-treated mature mice (n=3). **(H)** Lung *MUC5AC* expression after Ova sensitization (see Methods). Each bar represents the mean ± SE normalized to PBS treatment. PBS-treated mice (n=3); Ova-treated mice (n=8-9). [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data.

Figure 2: **Chronic lung disease after influenza A virus (IAV) is age-dependent**. Data were pooled from 2 independent experiments. **(A)** Cartoon depicting our protocol for producing IAV-induced chronic lung disease with time periods of acute IAV respiratory infection and peak viral load highlighted. Mice were inoculated with 5 pfu IAV per mouse. Lungs were assessed for chronic pathology at 21 days post-infection (DPI). **(B)** IAV viral gene expression in whole lung homogenates at 5 DPI (n=6 per group). Bars represent mean expression normalized to PBS control lungs ± SE. **(C)** Representative PAS-stained lung sections at 21 DPI after IAV infection (5 pfu). Scale bar=500 μm. **(D)** Airway resistance as a function of methacholine dose (mean ± SE). Red circles, IAV-infected juveniles (n=12); blue squares. IAV-infected mature mice (n=13); pink circles, PBS-treated juveniles (n=3); light blue squares, PBS-treated mature (n=6) mice. **(E)** Quantification of mucous cell metaplasia at IAV 21 DPI as measured by PAS^+ morphometry (mean ± SE, n=4-9). **(F)** Induction of *MUC5AC* expression relative to PBS-treated controls 21 days after IAV inoculation (5 pfu) in juvenile (6 weeks old) and mature (9-12 months old) mice treated with PBS (n=6) or IAV (n=7-19). [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data.

Figure 3: Age-dependent differences in post-viral lung pathology persist in the setting of microbiome suppression. Data are representative of 2 independent experiments. **(A)** Schematic depicting the experimental approach. Juvenile (6 weeks old) and mature mice (12 months old) were provided drinking water containing a broad spectrum antibiotic cocktail (grape Koolaid™ with vancomycin, neomycin, ampicillin, and metronidazole, or VNAM) or control sweetener (grape Koolaid™) for 3 weeks (starting on -21 DPI) and then challenged with SeV (1.5 x 10^5 pfu/mouse) or PBS. Treatment with VNAM or control sweetener continued until 7 DPI, and then mice were provided regular water for the duration of the experiments. Lung pathology was examined at 49 DPI. **(B)** Stool DNA content at various timepoints (mean ± SE, n=6-9). **(C)** Effect of VNAM treatment on the observed α-Diversity of stool samples as determined by 16S sequencing (see Methods). Each datapoint represents a single biological sample. Note, fewer measurements at SeV 0 DPI and 5 DPI were possible due to the paucity of stool DNA content at these time points. **(D)** Interindividual differences in stool DNA samples over the course of VNAM treatment as determined by PCoA analysis. Each datapoint represents a single biological sample. Note that group differences between juvenile and mature mice are most evident prior to VNAM treatment (-21 DPI). **(E)** SeV viral RNA expression at 5 DPI (mean ± SE, n=3 per group). **(F)** Airway response to methacholine at 49 DPI in control (left panel) versus VNAM treated mice (right panel). Datapoints represent means ± SE (n=4-9). **(G)** Lung expression at SeV 49 DPI of *MUC5AC* (left panel), *IL33* (middle panel), and *TREM2* (right panel). Data are expressed as fold induction over age-matched PBS-challenged controls (mean ± SE, n=6-9). [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data.

Figure 4: Age-associated transcriptional signatures in post-viral lung pathology. **(A)** Approach for comparing lung transcriptional signatures based on mouse age at induction and the method for inducing asthmatic phenotypes. Juvenile (6 weeks old) or mature (10 months old) mice were infected with SeV (1.5 x 10^5 pfu, n=3 pools, 3 mice per pool) or were subjected to Ova sensitization (see Methods). Remodeled lungs were harvested at day 49 or 23 post-treatment for SeV and Ova groups, respectively, and analyzed by RNAseq. Lungs from PBS-treated juvenile male (n=3) were included as a control. **(B)** Venn diagram of all genes displaying significant differential expression when compared to PBS-treated juvenile mice (adjusted p<0.05 and greater than two-fold change). A subset of differentially expressed genes (circled) corresponding to genes whose expression is significantly affected by age in SeV-treated mice but not in Ova-treated mice is highlighted for further analysis (panel **(C)**). **(C)** Induction of gene expression in SeV-treated juvenile mice versus SeV-treated mature mice (relative to PBS controls) at 49 DPI. Genes depicted are the subset whose induction relative to PBS-controls was age-sensitive in the SeV model but not the Ova model (n=250, circled area in **(B)**). A line of equivalence (grey dashes) denotes equal induction across age groups. Specific genes of interest are labeled (red circles). **(D)** Venn diagram of genes significantly regulated by mouse age (adjusted p<0.05 and greater than two-fold change) based on direct comparison between juvenile SeV-treated mice and mature SeV-treated mice, or juvenile Ova-treated mice and mature Ova-treated mice. The subset of genes that were determined to be age sensitive in the SeV model via this analysis are further examined in panel **(E)**. **(E)** Induction of gene expression in SeV-treated juvenile mice versus SeV-treated mature mice (relative to PBS controls) at 49 DPI. Genes depicted are the subset whose induction was significantly affected by mouse age in the SeV model based on direct comparison between juvenile and mature SeV-infected mice (n=136, panel **(D)**). A line of equivalence (grey dashes) denotes equal induction across age groups. Specific genes of interest are labeled (red circles). **(F)** Expression of M2-associated markers in SeV infected lungs (1.5 x10^5 pfu/mouse) on 49 DPI as a function of mouse age as validated by qPCR. Each bar represents the mean ± SE relative to age-matched PBS-treated controls (n=6-14 per group, pooled from 2 independent experiments). Pink, PBS-treated juvenile mice (5-6 weeks old); light blue, PBS-treated mature mice (11-12 months old); red, juvenile mice infected with SeV; dark blue, mature mice infected with SeV. (**G, H)** Lung *IL33* (**G**) and *KRT17* (**H**) expression as a function of mouse age at various points after SeV infection (1.5 x10^5 pfu, n=3-8 per time point). (**I**) Representative lung micrographs at various points post-SeV infection stained with the cilia marker acetylated α-tubulin (green), KRT17 (red), and DAPI (blue). Scale bar=10 μm. The presence of $KRT17^+$ cells outside the airway at 49 DPI is denoted by an arrow. [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data.

Figure 5: Acute differences in SeV bronchiolitis between juvenile and mature mice. Data points represent means ± SE throughout. **(A)** Weight loss indexed to starting weight (9-12 mice per group) in juvenile (5 weeks old) and mature (11 months old) mice infected with $1.5x10^5$ or $7.5x10^5$ pfu SeV/mouse. Data are representative of 2 independent dose-response experiments. **(B, C)** Lung SeV RNA expression **(B)** and BAL cell counts (**C**) at various times post-SeV infection ($1.5x10^5$ pfu/mouse) in juvenile mice (5-6 weeks old, n=4-8)and mature mice (10-12 months old, n=4-7 per group). Data was pooled from 2 independent time course experiments. **(D-F)** BAL differential cell count for macrophages (**D**), neutrophils (**E**), and lymphocytes (**F**) at various times post-SeV infection. **(G-I)** Airway resistance as a function of methacholine exposure at 5 days ((**G**), n=5-13 per group), 8 days ((**H**), n=5-13 per group), and 12 days (**I**, n=3-13 per group) post-SeV infection ($1.5x10^5$ pfu/mouse). Data were pooled from 2 independent time-course experiments. **(J)** Representative immunofluorescence staining of juvenile (5 weeks old) and mature (10 months old) mouse lungs at various times post-SeV infection ($1.5x10^5$ pfu/mouse). Green stain, SeV; blue stain, DAPI. Scale bar= 50 μm. **(K)** Quantification of SeV^+ airway cell frequency (n=15 microscopic fields and 3-4 biological replicates per group). [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data.

Figure 6: Juvenile responses to SeV have a type 2 cytokine bias. **(A)** Heat map of BAL cytokine abundance as a function of time after SeV infection ($1.5x10^5$ pfu/mouse) and mouse age at the time of infection (juvenile, 5 weeks old; mature, 11 months old). Dark blue represents lowest median normalized cytokine concentrations, yellow represents highest concentration (n=4-12 per cell). **(B)** Principal Component Analysis (PCA) of cytokine expression in juvenile mice (5 weeks) and mature mice (11 months) at various times after SeV infection. Each point represents the analysis of a single animal. **(C, D)** Differential BAL cytokine expression in juvenile vs. mature mice at SeV 5 DPI (**C**, n=5 per group), and 49 DPI ((**D**), n=8-12 per group). Each bar represents the mean $\log_2$ expression ratio for a given cytokine. Right facing bars represent excess cytokine expression in juvenile mice and left facing bars represent excess cytokine expression in mature mice. Statistically significant differences a denoted by red or blue colored bars (favoring production in juvenile or mature mice, respectively). **E, F**. Biphasic lung expression of IL-4 and IL-5 post-SeV infection in juvenile and mature mice. Data points represent the mean ± SE (n=4-12). **G.** Rank order analysis depicting the positive correlation between airway resistance at SeV 49 DPI and lung expression of IL-4 (black circles) and IL-5 (yellow diamonds) in 18 mice (10 juvenile and 8 mature). Regression lines are plotted as a visual aid. Pearson's r and statistical significance are depicted to the right of the graph. [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data.

Figure 7: Mass cytometry identifies age-specific leukocyte responses to SeV. Data are representative of 2 independent mass cytometry analyses. **(A)** Principal Component Analysis (PCA) of lung leukocyte burden in juvenile (5 weeks old) and mature mice (11 months old) at various times after SeV infection ($1.5x10^5$ pfu/mouse). Each symbol represents data from an individual mouse. **(B)** PCA clustering of leukocyte dynamics after SeV infection. Each data point represents an individual cell type. Co-clustering cell types are circumscribed by ovals. Stacked line graphs depicting the profiles in each of the 3 identified clusters is shown in the center of the panel. Examples of key cell types comprising the clusters are listed to the right. **(C)** Volcano plot depicting relative overall abundance of specific cell types in juvenile versus mature mice, combining all time points pre and post-SeV infection. Cell types with statistically significant differences (p<0.05, Student's 2-Tailed t-Test of log-normalized data) are depicted with colored symbols. **(D-G).** Lung burden of B-cells **(D)**, Polymorphonuclear Leukocytes (PMNs, **(E)**), Eosinophils **(F),** and Alveolar Macrophages **(G)** at various points after SeV infection. Data points represent the mean ± SE (n=4-7). [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature mice, Student's 2-Tailed t-Test performed on log-normalized data. For additional cell types see **Supplementary Fig. 4.** Representative gates are shown in **Supplementary Fig. 6**

Figure 8: Changes to alveolar macrophages with aging. **(A)** viSNE analysis of mass cytometry from juvenile (5 weeks old) and mature mice (11 months old). The top row represents data from naïve mice (0 DPI) and the bottom row SeV 49 DPI ($1.5x10^5$ pfu/mouse). Specific cell types are denoted and AM populations are labeled in red. **(B)** Representative flow cytometric contour plot depicting $CD45^+$, $CD11b^{mid/lo}$, $CD11C^+$ cells from juvenile and mature healthy mice. AM populations are distinguished by high surface expression of Siglec-F and dendritic cells (DCs) by lower surface expression. Data are pooled from two independent experiments (n=5, 300,000 events per age group). See **Supplementary Fig. 7a** for representative gates. **(C)** Frequency of $MHC\text{-}II^{Hi}$ AMs as a function of age (mean ± SE, n=5 per group). Statistical significance by 1-way ANOVA of log-normalized data is depicted. **(D)** Expression of MHC-II protein in various lung myeloid cell types as determined by mass cytometry. Bars represent the median metal intensity (MMI) ± SE in healthy juvenile (n=7) and mature mice (n=7). **(E)** Effect of SeV infection ($1x10^5$ pfu/mouse) on the frequency of $MHC\text{-}II^{Hi}$ AMs (mean ± SE, n=4-7 per time point) expressed as a percentage of total AMs. **(F)** Antigen processing activity in various lung myeloid cell types from 6 week juvenile and 12 month mature mice as measured by the DQ-OVA assay (see Methods). Bars represent mean ± SE (n=10 per group pooled from two independent experiments). See **Supplementary Fig. 7b** for representative gates. **(G)** BAL expression of *H2-AA*, *H2-EB1*, and *CIITA* in healthy mice as a function of age as measured by bulk RNAseq. Data represents mean Lima-Voom transformed Log_2 CPM ± SE (n=3 pools/timepoint each composed of 3 mice). Statistical significance via one-way Kruskal-Wallis test is depicted for each gene. **(H)** Histogram analysis of 1,024 genes demonstrating age-sensitive expression in BALs (p<0.05, one-way Kruskal-Wallis test). The x-axis represents $\log_2$ mean expression ratios for mature (≥7 months old) divided by juvenile gene expression (≤3 months old). The bin containing *H2-AA*, *H2-EB1*, and *CIITA* is highlighted (arrow). **(I)** KEGG pathway functional enrichment analysis of 1,024 genes demonstrating age-sensitive expression in BALs. Statistically enriched pathways (FDR<0.05) are depicted. Blue highlighting reflects that the enrichment of these pathways is dependent in genes overexpressed in mature vs. juvenile mice. [a]p<0.05 SeV-infected vs. PBS-treated; [b]p<0.05 juvenile vs. mature, Student's 2-Tailed t-Test on log-normalized data.

Figure 9. Single cell transcriptomic analysis of AM aging. **(A)** UMAP principal component analysis of scRNAseq data obtained from pooled mouse BALs from juvenile (6 weeks old, n=10, 7,276 cells) and mature mice (12 months old, n=9, 1,616 cells). Major cell types observed are depicted. **(B)** BAL cell differential in healthy juvenile and mature mice. **(C)** UMAP visualization of *H2-AA* expression (orange dots) in juvenile and mature AMs. **(D)** Top 25 differentially expressed genes in AMs. Genes differentially expressed greater than twofold in juvenile and mature mice are depicted with red and blue dots, respectively. The dashed line represents equal expression in juvenile and mature AMs ($\log_e$ CPM). **(E)** Dot plot analysis of genes differentially expressed greater than twofold in juvenile and mature mice (see colored symbols, panel **(D)**). Expression in AMs is highlighted (arrow). **(F)** Dot blot analysis of marker genes specifying embryonically derived tissue resident AMs postnatal (bone marrow-derived) AMs[41].

Figure 10: Alveolar macrophage depletion blunts age differences in post-SeV airway disease. Data are pooled from 3 independent experiments comparing juvenile (6-8 weeks old) to mature (10-12 months old) mice. **(A)** Cartoon depicting our experimental approach for macrophage depletion (also see Methods). Mice received 2 intranasal doses of clodronate or control liposomes prior to infection with SeV ($5x10^5$ pfu/mouse). **(B)** Representative micrographs of Diff-Qwik stained BALs from juvenile mice receiving control liposomes, clodronate liposomes, or no treatment. Scale bar=40 μm. **(C)** Flow cytometric quantification of AMs as a percentage of $CD45^+$ live cells on the day of SeV infection (mean ± SE, n=3-8 pooled from two independent experiments). **(D)** Representative PAS-stained micrographs depicting lung pathology 49 days post-SeV infection. Scale bar=400 μm. Arrows point to corresponding methacholine challenge data (panel **(E)**). (**E)** Airway resistance (mean ± SE) as a function of methacholine exposure at SeV 49 DPI. The left panel depicts SeV-infected juvenile (n=10-11) and mature (n=7-8) mice pre-treated with control liposomes and the right mice treated with clodronate liposomes. **(F)** Morphometric quantification of mucous cell metaplasia via airway PAS staining at SeV 49 DPI (mean ± SE) in SeV-infected juvenile (n=7-11) and mature (n=7-8) mice. **(G, H)** Lung *MUC5AC* (**F**) and *IL-13* (**G**) expression on SeV 49 DPI, expressed as fold induction over age-matched PBS-treated control mice (mean ± SE, n=6-17 pooled from two independent experiments). $^a p<0.05$ SeV-infected vs. PBS-treated; $^b p<0.05$ juvenile vs. mature mice, Student's 1-Tailed t-Test performed on log-normalized data.