The innate immune protein S100A9 protects from Th2-mediated allergic airway inflammation

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ONLINE DATA SUPPLEMENT

Supplementary Information

METHODS

Histopathology of lungs

Tissue sections (5 µm) were stained with hematoxylin and eosin stain to assess inflammation, or periodic acid-Schiff (PAS) to assess mucus cell metaplasia as a measure of mucin expression. Immunohistochemistry staining was performed using the Leica BOND-MAX autostainer (Wetzlar, Germany). Lung S100A9 production was assessed by immunohistochemistry staining with Cell Signaling Technologies clone D3U8M (Danvers, MA). To assess smooth muscle hypertrophy and hyperplasia, immunohistochemistry staining for □-smooth muscle actin (SMA) with Abcam ab7817 (Cambridge, United Kingdom) was performed. Slides were examined and scored by a pathologist who was blinded to the experimental groups. S100A9 was scored according to the following scale: 0, no staining; 1, normal staining of type II pneumocytes only; 2, staining of type II pneumocytes and all alveolar epithelium with mild intensity; 3, staining of type II pneumocytes and all alveolar epithelium with moderate intensity; 4, staining of type II pneumocytes and all alveolar epithelium with marked intensity.

Inflammation was scored according to the following scale: 0, no inflammatory infiltrates observed in the examined sections; 1, rare perivascular inflammatory infiltration; 2, few perivascular inflammatory infiltrates with rare extenstion into the interstitium; 3, moderate perivascular inflammatory infiltration and with multifocal interstitial infiltration; 4, marked perivascular inflammatory infiltrates with extensive involvement of the interstitium and alveolar spaces. Mucus production was scored according to the following scale: 0, PAS positive cell are not observed in the examined sections; 1, less than 10% of cells in medium and small airways are PAS positive; 2, 10-20% PAS positive cells in medium and small airways; 3, greater than 20%

cells in medium and small airways are PAS positive and hyperplasia of PAS positive cells is observed; 4, greater than 20% PAS positive cells in medium and small airways, hyperplasia of PAS positive cells and mucous plugging of airways. Immunohistochemistry (IHC) anti-SMA slides were digitally scanned on a Leica SCN400 Slide Scanner and quantified for % positive cells and % positive total tissue area using Ariol analysis scripts (Leica).

Flow cytometry staining

Right lungs were digested to single cell suspensions with 1 mg/mL collagenase type I and 0.1 mg/mL DNase. Digestion was stopped by the addition of EDTA to 1 mM. Digested lungs were passed through a 70-μm filter and treated with red blood cell lysis buffer (BioLegend, San Diego, CA). Cells were restimulated with 50 ng/mL PMA, 1 μg/mL ionomycin, and 0.07% golgi stop for 6 hours at 37°C in RPMI + 10% FBS. Meanwhile, live cells were counted by trypan blue exclusion on a hemocytometer. Following restimulation, 2 million lung cells were stained with viability dye from Tonbo (San Diego, CA), blocked with an anti-FcR antibody (clone 2.4G2), and stained with antibodies listed in Table E1. Th2 cells were surface stained with anti-CD90, anti-CD45, anti-CD4, anti-CD3, and anti-FCεR1. ILC2 cells were surface stained with anti-Lineage cocktail, anti-CD3, anti-CD25, anti-CD127, anti-CD45, and anti-ST2. After washing, ILC2 cells were stained with fluorochrome-labeled streptavidin. Cells were then fixed and permeabilized using FoxP3 Fix/Perm Buffer Set by BioLegend and intracellularly stained with anti-IL-5 and anti-IL-13. Th2 cells were pre-gated as $CD90^{+}CD45^{+}CD3^{+}CD4^{+}$ and assessed for IL-5 and IL-13 staining (Fig. S1A). ILC2s were pre-gated as $lineage^{-}CD3^{-}CD45^{+}$ $ST2^{+}CD127^{+}$ and assessed for IL-5 and IL-13 staining (Fig. S1B). T regulatory (Treg) cells were quantified from left lungs on day 10 following BAL. Left lungs were digested to single cell suspensions, and 2 million cells were stained for viability (Tonbo, San Diego, CA), surface stained for CD4 and CD25, fixed and permeabilized as

above, and intracellularly stained with anti-FoxP3. T regulatory cells were pre-gated as $CD4^+FoxP3^+$ and assessed for CD25 and FoxP3 (Fig. S2A). Differences in Treg proportion and CD25 expression were confirmed in littermate controls (one experiment shown in Fig. 6). Flow analysis was conducted on LSRII or Fortessa flow cytometers by BD Biosciences (San Jose, CA), and all data was processed using FlowJo software by FlowJo LLC (Ashland, OR).

Methacholine challenge

Airway hyperresponsiveness (AHR) and airway elastance (AE) was measured on day 10 after Alt Ext challenge. Each mouse was anesthetized by pentobarbital and the trachea was cannulated with a 20-gauge metal stub adapter. The animal was placed on a FlexiVent small-animal ventilator (SCIREQ, Montreal, Canada), with 150 breaths/min and a tidal volume of 10 mL/kg of body weight. Airway responsiveness was assessed by administering incremental concentrations of aerosolized methacholine (0, 12.5, 25, 37.5, and 50 mg/mL in saline) via an Aeroneb in-line ultra-nebulizer (SCIREQ, Montreal, Canada). The SCIREQ software calculates the respiratory system resistance (Rrs in cm H_2O*s/mL) by dividing the change in pressure by the change in flow from single frequency forced oscillation technique; elastance (Ers in cm H_2O/mL) captures the elastic stiffness and is reciprocal of compliance.

In vitro T regulatory cell suppression assay

Wild-type $CD4^+CD25^-$ T effector (Teff) cells and $CD4^+CD25^-$ Treg cells were isolated using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit for mouse from Miltenyi Biotec Inc. (Auburn, CA). Sorting buffer was RPMI + 1% FBS, and complete medium was RPMI + 10% FBS + 1% penicillin/streptomycin + 1% sodium pyruvate + 1% non-essential amino acids. Wild-type Teff cells were labeled with CellTrace Violet from Invitrogen (Carlsbad, CA) in PBS for 20 min at 37°C and the dye reaction was quenched with complete medium. Wild-type Teff cells were

incubated with either wild-type or *S100A9*$^{-/-}$ Treg cells at the indicated ratios for 4 days prior to staining with anti-CD4 PE-Cy7 as for Treg cells above, with and without endotoxin-free recombinant murine calprotectin. Anti-CD3 (1 μg/mL) and anti-CD28 (4 μg/mL) included to stimulate Teff proliferation were Ultra-LEAF purified from Biolegend (San Diego, CA). Teff proliferation was assessed on single cells following the gating scheme outlined in Fig. S2B. Percent suppression was calculated as 100 X ((%divided Teff no Treg)-(%divided Teff with Treg))/(%divided Teff no Treg).

Table E1. Flow cytometry antibodies.

Epitope	Clone	Manufacturer	Fluorophore	Dilution
CD90	353-2.1	BD Horizon	BV786	1:1250
CD45	30-F11	eBioscience	AF700	1:1250
CD4	H129.19	BD Pharmingen	FITC	1:500
CD3	145-2C11		Pacific Blue	1:500
FcεR1	MAR-1	eBioscience	PE-Cy7	1:500
Lineage cocktail	130-092-613	Miltenyi Biotec	biotin	1:100
CD3	17A2	eBioscience	biotin	1:500
	Streptavidin	BD Pharmingen	APC-Cy7	1:1000
CD25	PC61.5	eBioscience	AF488	1:125 (ILC2); 1:1667 (Treg)
CD127	A7R34	eBioscience	PE-Cy7	1:250
CD45	30-F11	eBioscience	AF700	1:250
ST2	eBio927	eBioscience	PerCP-eFluor 710	1:357
IL-5	TRFK5	BD Biosciences	APC	1:67
IL-13	eBio13A	eBioscience	PE	1:67
CD4	RM4-5	BD Biosciences	PE-Cy7	1:1667
FoxP3	FJK-16S	eBioscience	APC	1:100
KLRG1	2F1/KLRG1	Biolegend	PE/Dazzle™ 594	1:200
LAG3	C9B7W	BD Biosciences	BV786	1:100
CTLA4	UC10-4B9	Biolegend	APC	1:50

SUPPLEMENTARY FIGURES AND LEGENDS

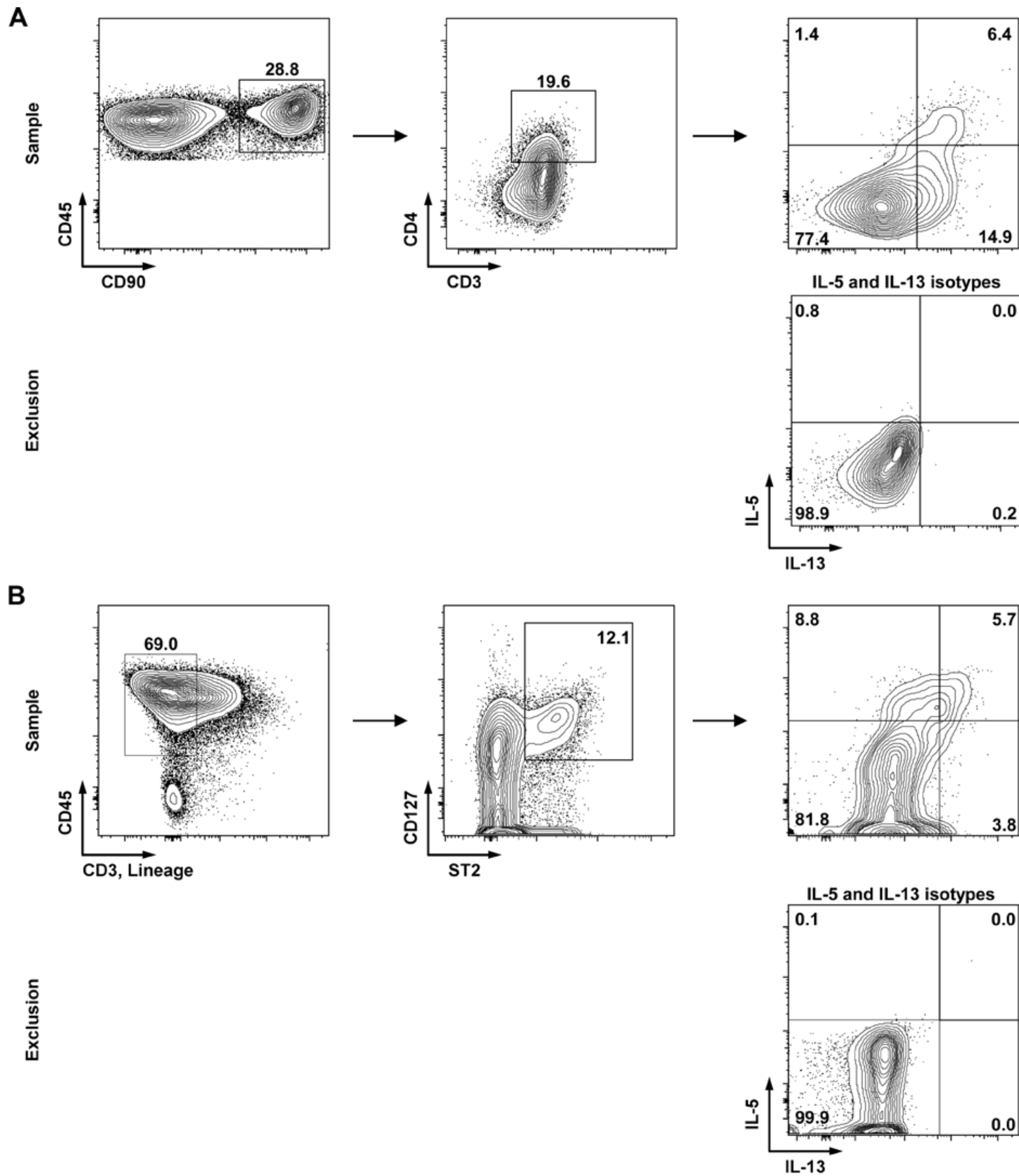

Figure E1: Gating strategies for Th2 cells and ILC2. Data are representative of the gating strategy used to quantify Th2 cells (**A**) and group 2 innate lymphoid cells (**B**).

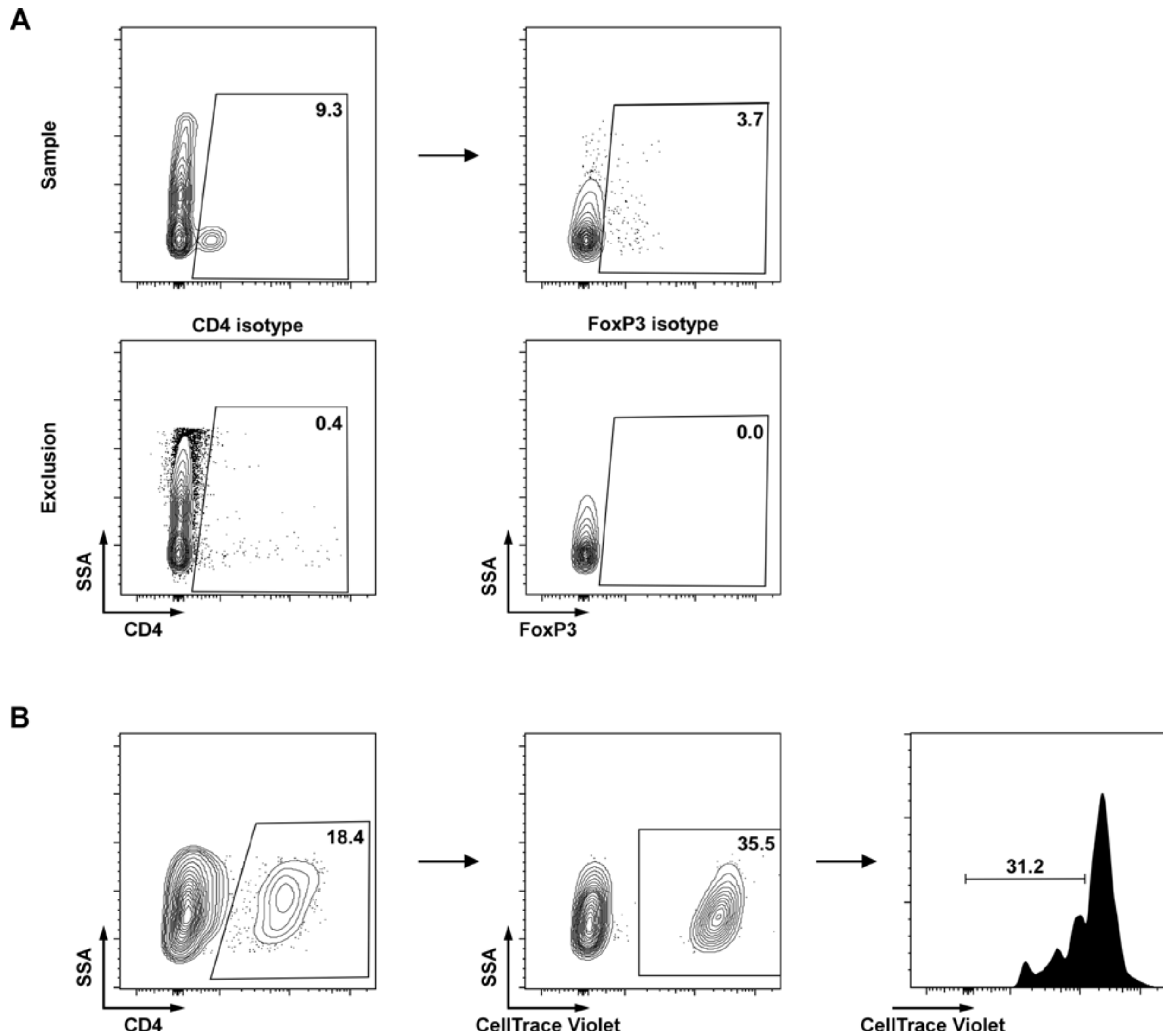

Figure E2: Gating strategies for T regulatory cells. (**A**) Data are representative of the gating strategy used to quantify T regulatory cells in the lungs. (**B**) Data are representative of the gating strategy used to quantify T regulatory cell suppression of T effector cell proliferation in vitro.

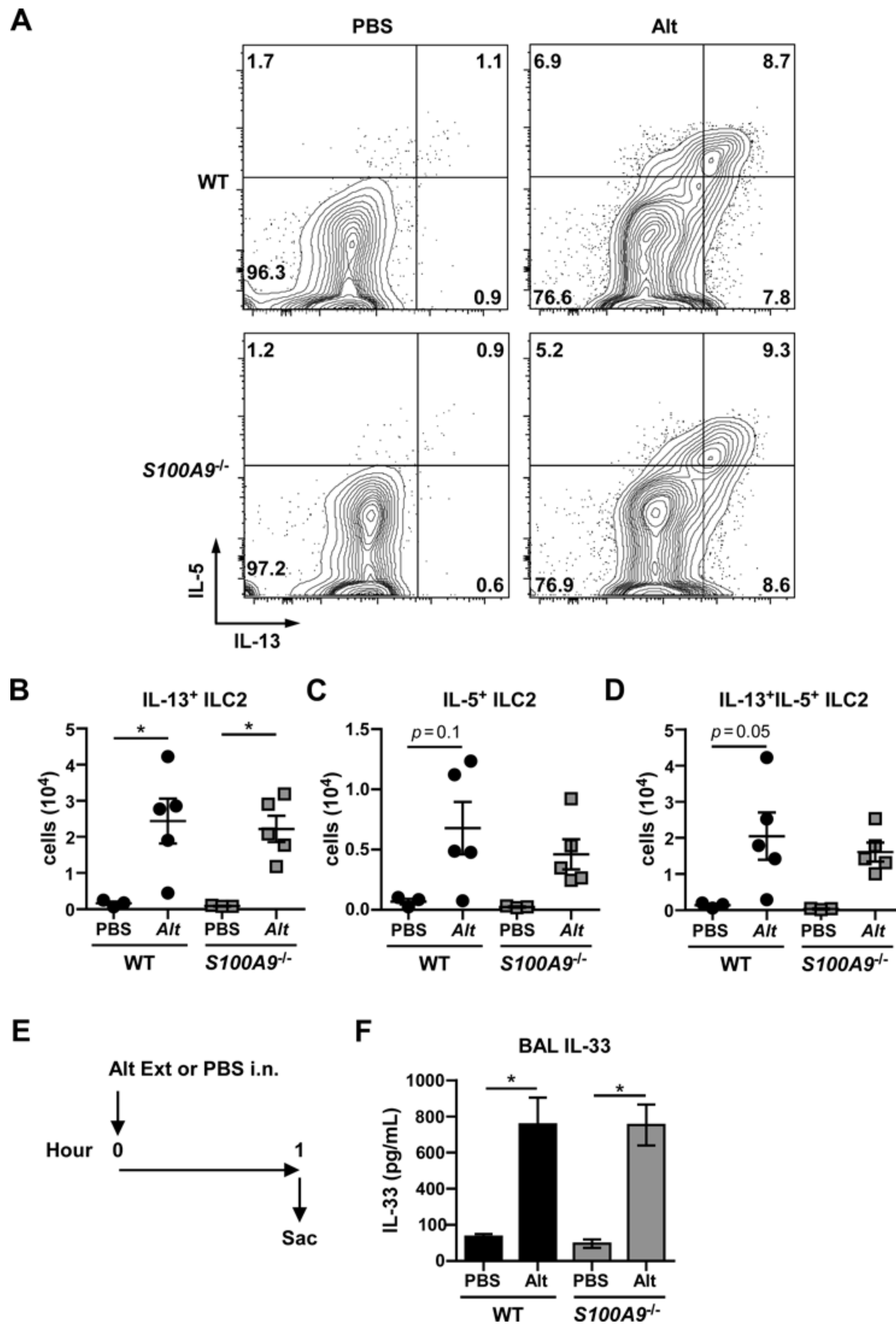

Figure E3: Wild-type and *S100A9*$^{-/-}$ mice have no difference in group 2 innate lymphoid cell (ILC2) numbers or IL-33 release in response to Alt Ext. Representative flow plots with percentages of the parent ST2^{+}CD127^{+} ILC2s shown (**A**), and aggregated data for the total number of (**B**) IL-13^{+}, (**C**) IL-5^{+}, and (**D**) IL-13^{+}IL-5^{+} double positive ILC2s. (**B-D**) Data are representative of three experiments and include 5 Alt Ext challenged and 3 mock challenged mice per genotype. (**E**) Mice were challenged with Alt Ext or mock challenged with PBS at hour 0 and humanely euthanized 1 h later. (**F**) IL-33 was measured in BAL. Data are from one experiment with 9-10 Alt Ext challenged and 4-5 mock challenged mice per genotype.

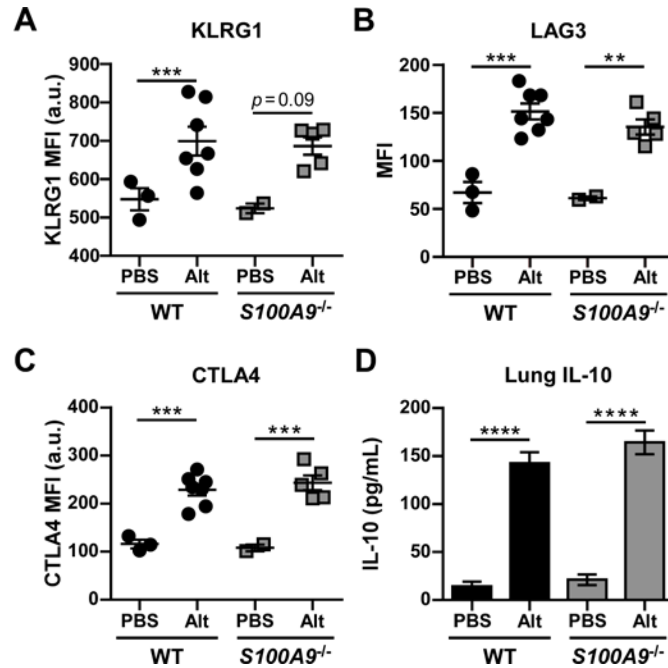

Figure E4: Wild-type and *S100A9*$^{-/-}$ mice have no difference in Treg functional markers or IL-10 production in response to Alt Ext. Wild-type and *S100A9*$^{-/-}$ were challenged with Alt Ext or mock challenged with PBS and humanely euthanized on day 10. Expression of lung Treg functional markers on CD4+Foxp3+ Treg cells were assessed by flow cytometry, including KLRG1 (**A**), LAG3 (**B**), and CTLA4 (**C**) IL-5^{+}. (**D**) IL-10 was measured in lung tissue homogenate by ELISA; data represent 3 mock-challenged mice and 5 Alt Ext challenged mice.