

ORIGINAL ARTICLE

The sickle cell mouse lung: proinflammatory and primed for allergic inflammation

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Comorbid asthma in sickle cell disease (SCD) confers higher rates of vaso-occlusive pain and mortality, yet the physiological link between these 2 distinct diseases remains puzzling. We used a mouse model of SCD to study pulmonary immunology and physiology before and after the induction of allergic airway disease (AAD). SCD mice were sensitized with ovalbumin (OVA) and aluminum hydroxide by the intraperitoneal route followed by daily, nose-only OVA-aerosol challenge to induce AAD. The lungs of naive SCD mice showed signs of inflammatory and immune processes: (1) histologic and cytochemical evidence of airway inflammation compared with naive wild-type mice; (2) bronchoalveolar lavage (BAL) fluid contained increased total lymphocytes, %CD8+ T cells, G-CSF, interleukin 5 (IL-5), IL-7, and CXCL1; and (3) lung tissue and hilar lymph node (HLN) had increased CD4+, CD8+, and regulatory T (Treg) cells. Furthermore, SCD mice at AAD demonstrated significant changes compared with the naive state: (1) BAL fluid with increased % CD4+ T cells and Treg cells, lower %CD8+ T cells, and decreased interferon gamma, CXCL10, CCL2, and IL-17; (2) serum with increased OVA-specific immunoglobulin E, IL-6, and IL-13, and decreased IL-1 α and CXCL10; (3) no increase in Treg cells in the lung tissue or HLN; and (4) hyporesponsiveness to methacholine challenge. In conclusion, SCD mice have an altered immunologic pulmonary milieu and physiological responsiveness. These findings suggest that the clinical phenotype of AAD in SCD mice differs from that of wild-type mice and that individuals with SCD may also have a unique, divergent phenotype perhaps amenable to a different therapeutic approach. (Translational Research 2015; ■:1–15)

Abbreviations: ■ ■ ■ = ■ ■ ■ ■

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AT A GLANCE COMMENTARY

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Background

Comorbid asthma is an independent predictor of morbidity and mortality in sickle cell disease (SCD). Although both diseases are hallmarked by altered inflammatory states, the pathophysiological link between these 2 biologically distinct processes remains elusive. Herein, we use a mouse model of SCD to study inflammatory and lung mechanics changes in experimentally induced asthma.

Translational Significance

Our work has the potential to drive development of new therapeutic modalities for humans living with both SCD and asthma. Lack of airway hyper-reactivity in the mouse model suggests standard clinical therapy with bronchodilators may be ineffective in this population and warrants further investigation. Furthermore, because airway hyper-reactivity is a major component in the diagnosis of asthma, it may be underdiagnosed in this population.

INTRODUCTION

Pulmonary diseases, such as asthma and acute chest syndrome, are major determinants of mortality in individuals with sickle cell disease (SCD).¹ In fact, asthma is an independent predictor of mortality in patients with SCD.²⁻⁵ Airway hyper-responsiveness (AHR) is detectable in up to 83% of adults and children with SCD, significantly more frequently than in healthy controls.^{3,6-9} Exact mechanisms leading to AHR in SCD are unknown.

Interestingly and conversely, in a cohort of 99 children with SCD, 30% were found to be hyporesponsive to maximal doses of methacholine raising the question of whether what appears to be asthma in SCD is really related to classic bronchoconstriction.¹⁰ Investigations of experimental asthma in the SCD mouse model to date reveal histopathologic evidence of an enhanced asthma phenotype, increased total plasma immunoglobulin E (IgE), and increased bronchoalveolar lavage (BAL) fluid proinflammatory cytokines including interleukin 13 (IL-13) and IL-5.¹¹

Allergic asthma is a chronic allergic disorder characterized by airway smooth muscle hyper-responsiveness, bronchial inflammation with increased mucus secretion,

airway remodeling, and increased serum IgE levels.¹²⁻¹⁴ The pathogenesis and immunology of this disease are complex with mast cells, dendritic cells, T lymphocytes and B lymphocytes, and eosinophils all playing significant roles.¹⁵ The increase in eosinophils and CD4+ T lymphocytes in the bronchial mucosa and BAL fluid is a characteristic feature of the inflammatory response in patients with asthma and in animal models of allergic airway disease (AAD).¹⁶⁻¹⁹ Increased numbers of CD4+ T cells isolated in bronchial mucosa and peripheral blood in patients with asthma appear to correlate with the severity of the disease and have been shown to secrete IL-2 and Th2 cytokines such as IL-4, IL-5, and IL-13, which attract and activate eosinophils and regulate the synthesis of IgE from B cells.^{15,17-21}

Individuals living with SCD have increased levels of circulating endothelial cells and proinflammatory cytokines at baseline and even higher at times of vaso-occlusion, providing evidence that endothelial inflammation and injury play important roles in mechanisms involved in vascular dysfunction.^{22,23} Holtzclaw et al²⁴ have shown that low-dose lipopolysaccharide challenge in transgenic SCD mice provokes an exaggerated inflammatory response with increased levels of tumor necrosis factor α , IL-1 β , and soluble VCAM-1 in the serum and BAL fluid. Our group has previously reported an altered baseline immunophenotype in the serum, gut, and spleen of SCD mice,²⁵ raising speculation that disruption in SCD splenic lymphofollicular morphology results in impaired systemic immunity. Furthermore, we have shown sensitization with systemically administered ovalbumin (OVA)/alum leads to increased mortality, antigen-specific serum IgE, and BAL fluid IL-6 and IL-1 β .²⁶ Additionally, Nandedkar et al²⁷ demonstrated histopathologic evidence of enhanced pulmonary inflammation in SCD mice under an experimental asthma protocol. Altogether, these findings support the contention that both humans and mice with SCD live in states of heightened baseline inflammation, which might translate into robust immune responses to antigenic challenge. This, in turn, may predispose them to exaggerated allergic airway responses to inhaled allergens and, at least in part, explain the pulmonary complications observed in SCD.

Comorbid asthma in SCD is a major contributor to mortality,^{3,4} yet mechanistic insights into its pathogenesis have been sparse. Herein, we used a mouse model of SCD to study inflammatory factors involved in the genesis of asthma by using a well-characterized OVA-induced AAD model. We hypothesized that altered immune responses to allergens occur in SCD mice that give rise to a heightened asthma phenotype. The purpose of our study was to specifically

investigate the immune response to AAD in SCD mice, which may give mechanistic insights into novel therapies for SCD-related asthma.

MATERIALS AND METHODS

Animals. Three groups of female mice were used: C57BL/6 (C57 control), transgenic sickle cell mice (SCD mice), and hemizygous sickle cell littermates (hemizygous). All mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and were aged 8–12 weeks and weighed 15–25 g at the time of sacrifice. Berkeley sickle cell transgenic mice (Tg[Hu-mini-LCR α 1G γ A γ δ β S] Hba $^{-/-}$ Hbb $^{-/-}$) expressing human HBA and HBB^S and no longer expressing mouse Hba and Hbb were used as a murine model of SCD.²⁸ The stock background of this strain is a mixture of FVB/N, 129, DBA/2, Black Swiss, and >50% C57BL/6 genomes. It was backcrossed to C57BL/6 one generation after importation to the Jackson Laboratory. Littermate controls of the Berkeley transgenic SCD mouse (generated on the same mixed background of strains) that express no mouse Hba, but do express one copy of mouse Hbb, human HBA and HBB^S giving rise to a hemizygous, sickle cell trait-like genotype were used as a second control arm.

All mice were housed conventionally in plastic cages with corncob bedding. The mouse room was maintained at 22°C–24°C with a daily light/dark cycle (light from 6 AM to 6 PM). Chow and water were supplied ad libitum. The protocols for mice used were approved by the Animal Care Committee at the University of Connecticut Health Center and conform to ethical guidelines for animal research.

OVA exposure protocol. As depicted in Fig 1, mice were immunized with 3 weekly intraperitoneal injections of a suspension containing 25 μ g of OVA (grade V; Sigma Chemical Co, St. Louis, Missouri) and 2 mg of aluminum hydroxide (alum) in 0.5 mL of saline. One week after the last injection, the mice were exposed to 1% aerosolized OVA in physiological saline, 1 hour/d, for 3 days.²⁹ The mice were placed in plastic restraint tubes (Research and Consulting Co, Basel, Switzerland) for nose-only aerosol exposure. The aerosols were generated by a BANG nebulizer (CH Technologies, Westwood, New Jersey) into a 7.6-L inhalation exposure chamber to which restraint tubes were attached. Chamber airflow was 6 L/min and aerosol particle size of OVA was monitored by gravimetric analysis with a Mercer cascade impactor (In-Tox Products). The mass median aerodynamic diameter and geometric standard deviations were 1.4 and 1.6 μ m, respectively. The estimated daily inhaled OVA dose approximated 30–40 μ g per mouse.³⁰

OVA AAD Model:

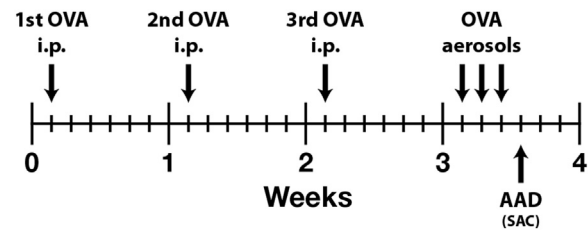


Fig 1. OVA-induced AAD (asthma) model. To investigate the mechanisms and therapeutic targets of allergic asthma, we use a well-characterized OVA-induced AAD model. After a 3-week period of OVA sensitization (1 i.p. per week), mice are exposed to nose-only OVA-aerosol challenge for 1 hour per day for 3 days. AAD, allergic airway disease; i.p., intraperitoneal; OVA, ovalbumin; SAC, sacrifice.

Harvesting of samples: blood, BAL fluid, lung tissue, and hilar lymph node. Twenty-four hours after the final aerosol exposure, the mice were sacrificed by ketamine/xylazine overdose and samples were collected from the various tissues and compartments as previously described.^{30–32} Briefly, whole blood was immediately collected via cardiac puncture into nonheparinized tubes and allowed to clot at room temperature. Serum was collected after centrifugation at 4000 rpm and frozen for later use at -80°C . A BAL was performed immediately after sacrifice in situ with 5 1 mL aliquots of sterile saline. Lung draining hilar lymph nodes (HLNs) were harvested and mechanically disrupted into a single-cell suspension. In addition, lungs were isolated from unlagged mice, minced, and treated with collagenase solution (150 U/mL; Gibco, Grand Island, New York) for 1 hour at 37°C . The minced tissue was mechanically disrupted through a 40- μ m nylon filter (BD Falcon, Bedford, Massachusetts). Red blood cells were lysed with Tris ammonium chloride. For all tissue samples, total nucleated cell counts were obtained using a hemocytometer with nigrosin dye exclusion as a measure of viability. Aliquots containing 10^6 cells were resuspended in FACS buffer.^{Q11} Leukocyte differentials were determined in BAL fluid using cytocentrifuged preparations stained with May-Grünwald/Giemsa. The remaining cells were analyzed phenotypically for subpopulations using specific antibodies and fluorescence flow cytometry.^{Q12} BAL protein concentrations were measured in the supernatants by bicinchoninic acid protein assay using bovine serum albumin (BSA) as a standard (Pierce Rockford, Illinois).

Antibodies. The following monoclonal antibodies were used in this study for flow cytometry: CD3-Cy5.5 (145-2C11; BD Biosciences [BD]), CD4-APC-Cy7 (RM4-5; BioLegend), CD8-FITC (53-6.72; BD), CD25-PE (PC61.5; eBioscience), Foxp3-APC

(FJR-16s; eBioscience). Corresponding isotype controls were also purchased and used.

FACS analysis. Cell pellets containing lymphocytes from tissue preparations were resuspended in phosphate-buffered saline (PBS) solution (containing 0.2% BSA and 0.1% NaN₃) at a concentration of 1×10^5 – 1×10^6 white blood cells/mL, and 100 μ L of the cells were diluted 1:1 with 100 μ L of the appropriate monoclonal antibody and incubated at 4°C for 30 minutes. Foxp3⁺ regulatory T (Treg) cells were identified by staining lymphocytes with anti-CD3, anti-CD4, and anti-CD25, followed by permeabilization using fixation/permeabilization buffer as per the manufacturer's instructions. Cells were then stained using an anti-Foxp3 antibody. After staining, cells were washed twice with PBS solution (0.2% BSA and 0.1% NaN₃) and relative fluorescence intensities were determined using an LSR-II (Becton-Dickinson, San Diego, California) and analyzed with FlowJo flow cytometry analysis software (Ashland, Oregon). Fluorescence minus one controls were used to assist with gating.

Cytokine/chemokine measurements. Frozen serum samples obtained from mice were thawed and used for analysis. Samples were processed using a Milliplex Mouse Cytokine/Chemokine 22-plex kit (Cat #MPXMCYTO70KPMX22; Millipore; Billerica, Massachusetts) following the manufacturer's instructions and using a 1:2 dilution. BAL fluid cytokines were processed and analyzed using the same technique as serum, but sterile PBS was used as a diluent for the standard curve. Samples were analyzed using a Bio-Rad Bioplex-200 system, with recombinant murine cytokines as standards, and Luminex bead array software was used to analyze the data.

OVA-specific serum IgE levels. OVA-specific IgE levels in serum samples were measured by an enzyme-linked immunosorbent assay using isotype-specific capture monoclonal antibodies.³³ IgE was captured from serum (diluted 10- to 1280-fold) using Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, Virginia) coated with anti-mouse IgE (R35-72) (BD Pharmingen, San Diego, California) at 2 μ g/mL in 0.1 mol/L carbonate, pH 9.5. Detection was with an OVA-digoxigenin conjugate followed by horseradish peroxidase-conjugated antidigoxigenin, essentially as described.³⁴ Development was with the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland).

Histology. After sacrifice, unmanipulated (not subject to BAL) and noninflated lungs from separate animals (n = 6–8) were removed, fixed with 10% buffered

formalin, and processed in a standard manner. Tissue sections were stained with hematoxylin and eosin, Mallory Trichrome (collagen), and Periodic acid-Schiff (mucus).³⁵ Sections from all 5 lobes were examined in their entirety in a blinded manner for qualitative analysis.

Airway hyper-reactivity. Airway responses to methacholine were assessed by whole-body barometric plethysmography, as previously described.³⁶ Mice were studied 24 hours after the third OVA aerosol. Mice were placed in individual chambers (Buxco Electronics, Troy, New York) and exposed for 2 minutes to aerosolized saline or increasing concentrations of methacholine from 3 to 300 mg/mL. Respiratory system variables including tidal volume, respiratory frequency, inspiratory and expiratory times, and changes in box pressure were recorded before and during aerosolization and for 4 minutes after each exposure. The maximal enhanced pause value response to methacholine was recorded at each dose.

Statistical analyses. Statistical comparisons between groups were made with analysis of variance and unpaired *t* tests using JMP Software (SAS Institute Inc, Cary, North Carolina) and GraphPad Prism 6 (GraphPad Software, Inc, La Jolla, California). For comparisons between 3 or more groups, a Tukey post hoc test was performed. All data were expressed as the means $\pm$ standard error of the mean. Methacholine response curves were compared by computing the area under the curves and comparing area under the curves using analysis of variance and a Tukey post hoc test. Differences were considered significant if *P* < 0.05.

RESULTS

Lung histopathology. Histopathology specimens demonstrate classical evolution of lung pathology from naive to OVA-induced AAD in wild-type C57 mice as previously demonstrated.²⁹ Naive C57 mice show lung histopathologic findings devoid of peribronchial inflammation and mucus staining (Fig 2, A). On induction of AAD, the C57 mice show airway luminal narrowing, increased mucus staining (short arrows), and increased peribronchial/perivascular cellularity (Fig 2, B). Interestingly, naive SCD mice exhibit a baseline increase in perivascular cellular infiltration (Fig 2, E). SCD mice show even greater enhancement of airway mucus production and perivascular cellularity at AAD compared with the naive state (Fig 2, F). Hemizygous mice were histopathologically similar to C57 mice in both the naive and AAD states (Fig 2, C and D).

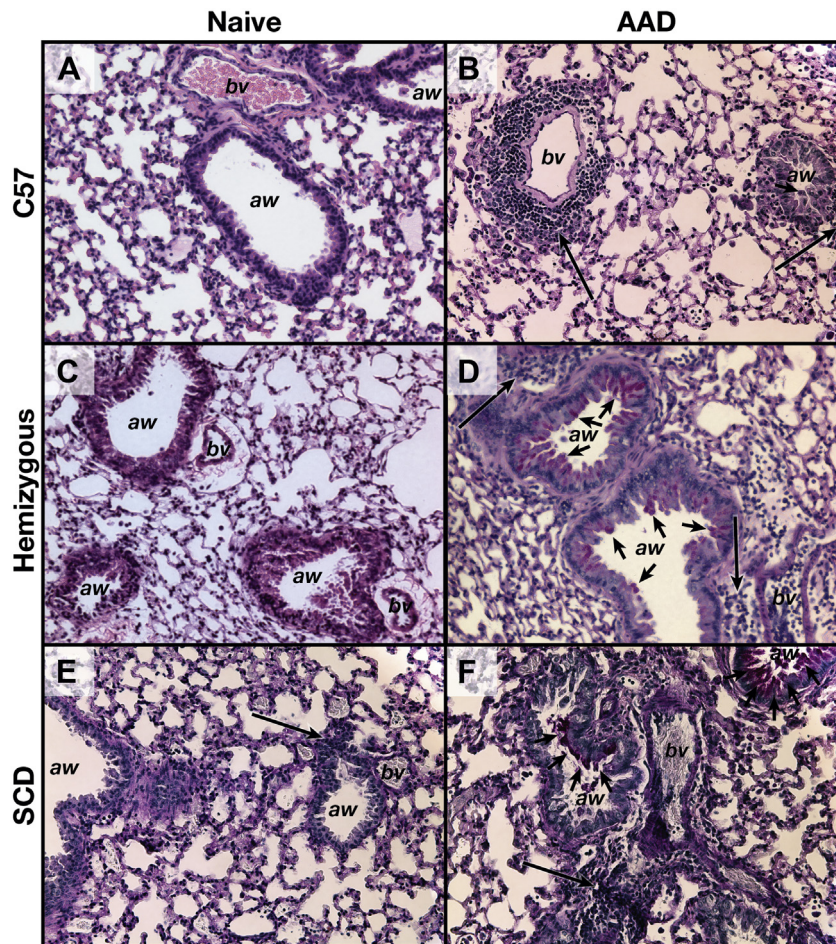


Fig 2. Sickie AAD mice have increased lung inflammation and mucus. Images are $\times 20$ magnification. Short arrows denote PAS + stain indicative of mucus, whereas long arrows represent the perivascular/peribronchial inflammation. (A) Naive C57, (B) AAD C57, (C) naive hemizygous, (D) AAD hemizygous, (E) naive SCD, (F) AAD SCD. AAD, allergic airway disease; aw, airway; bv, blood vessel; SCD, sickle cell disease.

AAD induces enhanced BAL leukocyte and eosinophil counts in SCD mice compared with C57 wild-type mice. Although the total number of leukocytes in the BAL fluid in the naive C57, hemizygous, and SCD are similar (0.7 , 1.0 , and 0.8×10^5 , respectively; Fig 3, A), the SCD mice have a significantly increased total BAL lymphocyte count (0.05×10^5) compared with C57 and hemizygous mice (0.01 and 0.02×10^5 , respectively, $P = 0.0006$; Fig 3, B). At AAD, all 3 groups of mice demonstrate significant increases in BAL total leukocyte count compared with the naive state (Fig 3, A; $P = 0.004$). However, SCD mice display a significantly more robust BAL leukocytosis and eosinophilia at AAD (Fig 3, A and C) suggestive of more profound allergic response to inhaled OVA (both $P < 0.03$). There were no eosinophils observed in the BAL fluid of any of the mouse groups in the naive state. Leukocyte differentials are reported in

Table I. There is no difference in BAL protein concentration among SCD mice compared with the C57 and hemizygous control mice in the naive state, nor at AAD (Fig 3, D). However, there is a statistically significant increase in BAL protein concentration within each mouse group when comparing the naive to the AAD state ($P < 0.01$ for each group).

BAL T cell subpopulations are altered at baseline and at AAD in SCD mice. Given the central role of T cells in the genesis of asthma,^{14,18,19} immunophenotyping of BAL T cells was undertaken at the naive state and after induction of AAD (Fig 4). The BAL fluid of naive SCD and hemizygous mice had a higher percentage of CD8+ T cells (Fig 4, B; $P = 0.007$) compared with naive C57 mice but no difference in %CD4+ T cells (Fig 4, A) or %Treg cells (Fig 4, C). In AAD SCD and AAD hemizygous mice, there were significantly higher %CD4+ T cells in BAL fluid than C57 mice

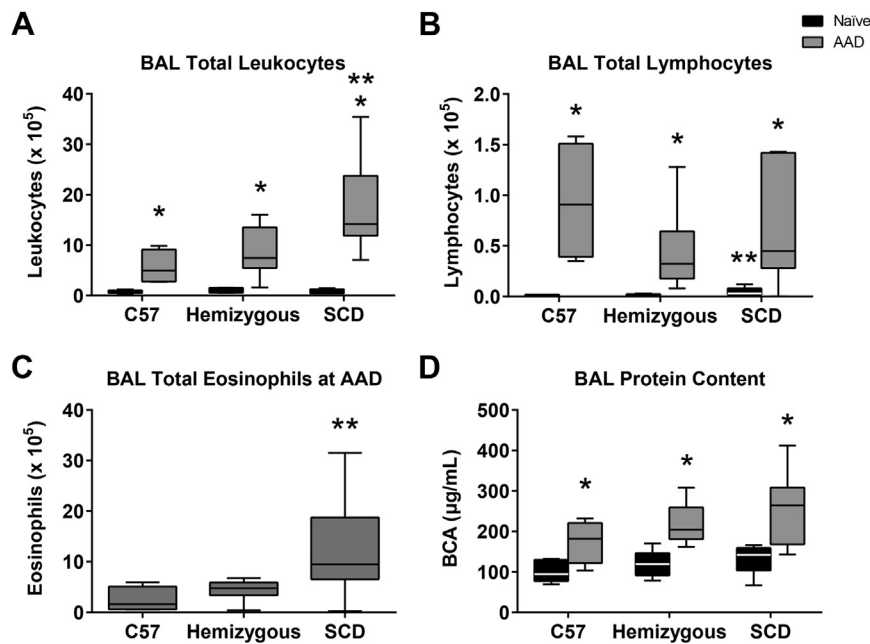


Fig 3. AAD induces shifts in BAL leukocyte counts and differentials in SCD mice but no difference in protein. BAL fluid was assessed for total leukocyte count (A), differentials (B and C), and protein concentration (D) from naive C57 (n = 6), hemizygous (n = 6), and SCD (n = 5) mice and compared with BAL fluid from C57 (n = 4), hemizygous (n = 10), and SCD (n = 7) mice after 3 weekly intraperitoneal OVA-alum sensitizations followed by 3 days of OVA aerosolization. Boxes represent 25–75th percentiles. Whiskers represent minimum and maximum values. Medians are represented by a black or white horizontal line within boxes. Significant differences were determined by 1-way analysis of variance with multiple post hoc pairwise comparisons between groups using a Student's *t* test. Differences between the naive and AAD state of an individual mouse group are denoted by an asterisk (*). Differences between SCD mice and both C57 and hemizygous mice are denoted by 2 asterisks (**). AAD, allergic airway disease; BAL, bronchoalveolar lavage; BCA, bicinchoninic acid; OVA, ovalbumin; SCD, sickle cell disease.

Table I. Comparison of BAL leukocyte differentials in naive and AAD mice

	% Lymphocytes	% PMNs	% Macrophages	% Eosinophils
Naive				
C57	2 (0)	0 (0)	98 (1)	0 (0)
Hemizygous	2 (0)	0 (0)	98 (1)	0 (0)
SCD	5 (1)	0 (0)	95 (2)	0 (0)
AAD				
C57	16 (1)	8 (3)	41 (13)	35 (9)
Hemizygous	6 (1)	4 (1)	40 (4)	51 (5)
SCD	3 (1)	3 (3)	32 (8)	61 (11)

Abbreviations: AAD, allergic airway disease; BAL, bronchoalveolar lavage; PMN, polymorphonuclear; SCD, sickle cell disease. All 3 groups of AAD mice were sensitized and challenged with ovalbumin. Relative to naive unexposed animals, AAD mice demonstrated increases in airway eosinophils and decreases in airway macrophages. There was no significant change in airway PMN cells at AAD between mouse groups. Data are represented as the mean $\pm$ standard error of the mean values.

with a concomitant decrease in %CD8⁺ T cells (Fig 4, A and B; $P < 0.004$). Although all mouse groups had increases in %Treg cells in the BAL fluid at AAD

($P < 0.0003$) compared with the naive state, which is consistent with previous results in C57 mice,³² SCD mice displayed a significantly higher percentage of Treg cells at AAD compared with C57s ($P = 0.04$). There was no significant difference at AAD between SCD and hemizygous mice.

Naive SCD mice have increased BAL G-CSF, IL-5, IL-7, and CXCL1. We measured 17 cytokines and 5 chemokines in the BAL fluid of SCD, hemizygous, and C57 mice (Fig 5, A–D). G-CSF, IL-5, and IL-7 were significantly higher in concentration in SCD mice compared with both C57 and hemizygous mice in the naive state ($P = 0.004$, 0.049 , and 0.008 , respectively for each cytokine). The chemokine CXCL1 was significantly increased in the BAL fluid of naive SCD mice compared with C57 and hemizygous mice ($P = 0.01$). There was no difference in levels of the cytokines GM-CSF, interferon gamma (IFN- γ), IL-1b, IL-2, IL-4, IL-9, IL-19, IL-12, IL-13, IL-15, IL-17, or tumor necrosis factor α (data not shown). Additionally, there was no difference in levels of the chemokines CCL2, CCL3, CCL5, or CXCL10 (data not shown).

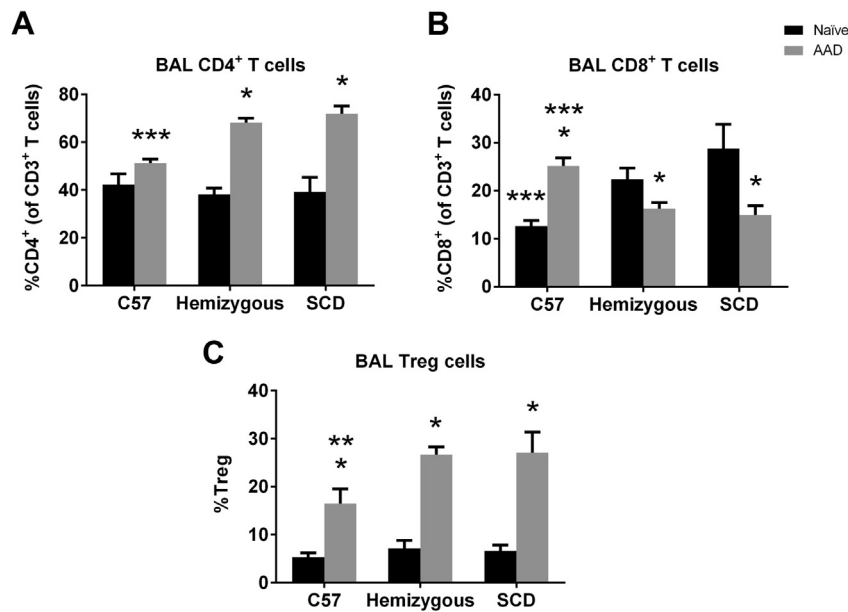


Fig 4. BAL T cell subpopulations are altered at baseline and at AAD in SCD mice. Lymphocyte flow cytometric analysis of BAL cells from C57, hemizygous, and SCD at baseline ($n = 6, 6,$ and 5 , respectively) and at AAD ($n = 4, 10,$ and 7 , respectively). (A) CD4⁺ T cells are CD3+CD4⁺ lymphocytes. (B) CD8⁺ T cells are CD3+CD8⁺ lymphocytes. (C) Treg cells are CD3+CD4+CD25+Foxp3⁺ lymphocytes. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's t test. Differences between the naive and AAD state of an individual mouse group are denoted by an asterisk (*). Differences between C57 controls and SCD mice at AAD are denoted by 2 asterisks (**). Differences between C57 controls and both hemizygous and SCD mice in the naive state or at AAD are denoted by 3 asterisks (***). AAD, allergic airway disease; BAL, bronchoalveolar lavage; SCD, sickle cell disease; Treg, T regulated.

SCD mice have lower BAL IFN- γ , IL-17, CXCL10, and CCL2 at AAD. At AAD, none of the 22 measured cytokines/chemokines were increased in the BAL fluid of SCD mice compared with control mice (data not shown). However, as demonstrated in Fig 6, 2 cytokines and 2 chemokines were significantly decreased in the BAL fluid of SCD mice compared with C57 and hemizygous mice: IFN- γ , IL-17, CXCL10, and CCL2 ($P = 0.003, 0.001, 0.0002,$ and 0.04 , respectively).

At AAD, SCD mice demonstrate enhanced serum OVA-specific IgE, IL-6, and IL-13, but decreased IL-1 α and CXCL10. Serum OVA-specific IgE levels in the SCD mice were significantly greater than in C57 mice at AAD ($P = 0.03$; Fig 7). Although serum OVA-specific IgE levels were higher in hemizygous mice compared with C57s, this was not statistically significant ($P = 0.14$). As shown in Fig 8, SCD mice at AAD have significantly increased serum IL-6 and IL-13 as well as decreased serum IL-1 α and CXCL10 levels compared with C57 controls (all $P < 0.04$). Except for significantly decreased serum levels of IL-6 at AAD, the hemizygous mice were similar to SCD mice.

SCD mice have increased T cell subpopulations in lung tissue in the naive state, but not at AAD. Immunophenotyping of lung tissues demonstrated that in the naive state the SCD mouse lung has increased total lymphocytes, CD4⁺ T cells, and CD8⁺ T cells compared with C57 control mice (Fig 9, A–C; all $P < 0.02$). Furthermore, subcharacterization of the CD4⁺ T cell subset identifies an increase in total Treg cells as well (Fig 9, D; $P < 0.0001$).

Interestingly, at AAD, there were no observed differences in total lung lymphocyte counts between SCD mice and both C57 and hemizygous mice (Fig 9, A). Although C57 mice demonstrated a robust increase in total CD4⁺, CD8⁺, and Treg cells (all $P < 0.02$), there was no increase in the T cell subsets in SCD mice after AAD induction (Fig 9, B–D).

SCD mice have altered T cell subpopulations in the naive state and at AAD. HLN immunophenotyping of T cells was also performed to assess potential changes in lymphocyte trafficking from the lung to its draining lymph node. In contrast to the lung tissue, the total lymphocyte count in the naive HLN was no different between mouse groups. However, at the induction of AAD, there was a greater increase in the HLN

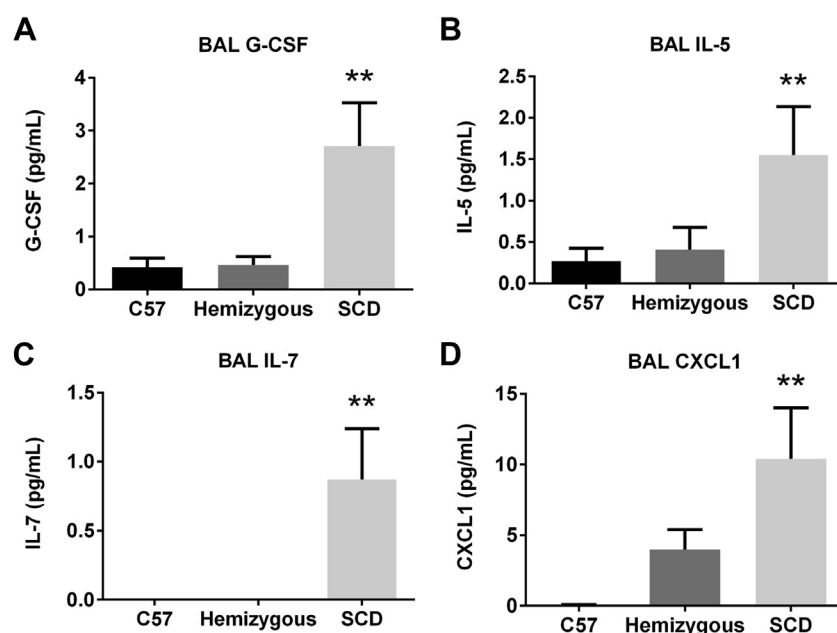


Fig 5. Naive SCD mice have increased (A) BAL G-CSF, (B) IL-5, (C) IL-7, and (D) CXCL1. BAL was assessed from naive C57 (n = 6), hemizygous (n = 6), and SCD (n = 5) mice for cytokine and chemokine concentration via Luminex bead assay. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's *t* test. Differences between SCD mice and both C57 and hemizygous mice are denoted by 2 asterisks (**). BAL, bronchoalveolar lavage; IL, interleukin; SCD, sickle cell disease.

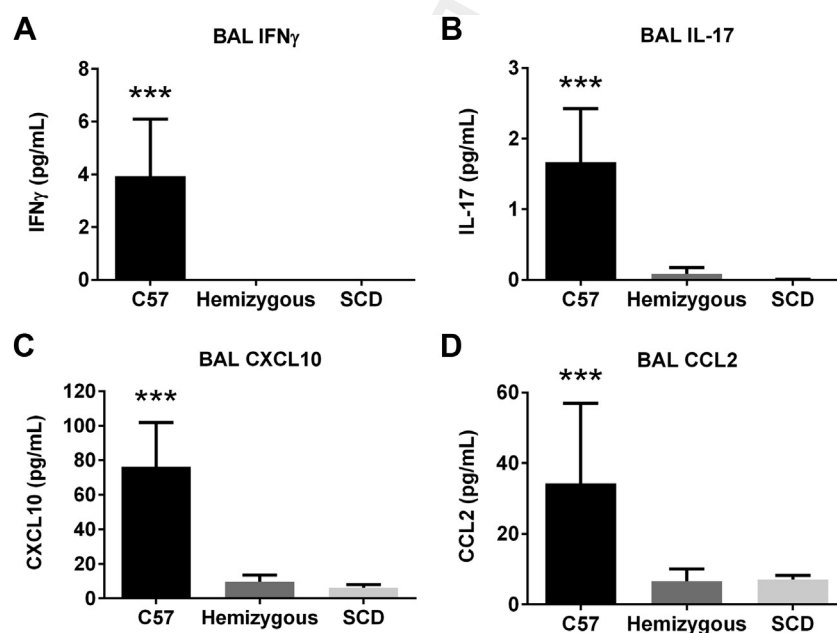


Fig 6. AAD SCD mice have lower BAL fluid concentrations of (A) IFN- γ , (B) IL-17, (C) CXCL10, and (D) CCL2. BAL was assessed after induction of AAD from C57 (n = 4), hemizygous (n = 10), and SCD (n = 7) mice for cytokine and chemokine concentration via Luminex bead assay. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's *t* test. Differences between C57 mice and both hemizygous and SCD mice are denoted by 3 asterisks (***). AAD, allergic airway disease; BAL, bronchoalveolar lavage; IFN- γ , interferon gamma; IL, interleukin; SCD, sickle cell disease.

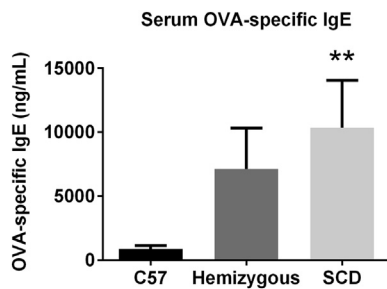


Fig 7. Serum OVA-specific IgE is 10-fold higher in SCD mice at AAD. Serum OVA-specific IgE was measured using a capture enzyme-linked immunosorbent assay. C57 ($n = 9$), hemizygous ($n = 9$), and SCD ($n = 10$) mice were analyzed after induction of AAD. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's t test. Differences between SCD and C57 mice are denoted by 2 asterisks (**). AAD, allergic airway disease; IgE, immunoglobulin E; OVA, ovalbumin; SCD, sickle cell disease.

lymphocyte count in SCD mice compared with C57 controls (Fig 10, A; $P = 0.04$). T cell immunophenotyping of naive HLN lymphocytes (Fig 10, B and C) demonstrated an increase in total CD4+ and CD8+ T cells in the SCD and hemizygous mice compared with C57 mice (both $P = 0.01$). Although all 3 mouse groups demonstrate increases in total CD4+ and CD8+ T cells at induction of AAD (all $P < 0.04$), SCD mice had significantly higher increases in total CD4+ T cells than C57 mice (Fig 10, B; $P = 0.04$). Naive SCD and hemizygous mice had significantly greater numbers of Treg cells in their HLN compared with C57 mice (Fig 10, D; $P = 0.007$). However, although C57 mice demonstrated increased Treg cells in the HLN at induction of AAD ($P = 0.01$), SCD and hemizygous mice did not (Fig 10, D).

Airway responsiveness. Airway responsiveness to methacholine challenge was assessed by whole-body barometric plethysmography. As we have observed previously,³⁵ C57 mice show AHR at AAD, physiologically confirming AAD. Interestingly, the SCD mice had significantly decreased AHR compared with C57 mice at AAD (Fig 11; $P < 0.01$ by 1-way analysis of variance). This is in contrast to the observed enhanced cellular inflammatory response on corresponding histopathology and cytochemical analysis of lung lavage. Hemizygous mice demonstrated an intermediate state between SCD and C57 mice (Fig 11).

DISCUSSION

Asthma is a major contributor to morbidity and mortality in SCD, yet relatively little is known of the

pathophysiological basis of the asthma phenotype in SCD or the interactions between these 2 disease states. Here, we report that transgenic SCD mice exhibit baseline pulmonary inflammation and a distinct cytokine, chemokine, and cellular milieu at AAD compared with C57 control mice. Naive SCD mice are characterized by increased levels of proinflammatory cytokines and chemokines in the BAL fluid, increased levels of lung CD4+ and CD8+ T cells, and evidence of perivascular cellular infiltration in the lungs. At AAD, SCD mice have increased BAL eosinophils, 10-fold greater serum OVA-specific IgE levels, and an altered cytokine profile compared with C57 controls but do not differ in total cell counts in the lung. Intriguingly, SCD mice are hyporesponsive to methacholine challenge at AAD.

Histopathology and immunophenotyping suggest that naive SCD mice exist in a proinflammatory immune state, in agreement with previous reports from our laboratory^{25,26} and that of other groups in both mouse models^{37,38} and in humans with SCD.³⁹ The immune system in SCD may be primed to respond aggressively to inhaled allergen by virtue of this proinflammatory state, providing one explanation for the high morbidity and mortality seen in SCD patients with asthma. Evidence for such priming can be seen in our previous report of OVA-alum vaccination in SCD mice and in work by Nandedkar et al,²⁷ where high mortality was present exclusively in SCD mice undergoing experimentally induced asthma. This priming is likely the driving force behind the 10-fold higher levels of serum OVA-specific IgE and several-fold higher BAL leukocyte and eosinophil counts were observed in SCD mice at AAD. Higher naive SCD BAL fluid levels of G-CSF, IL-5, and IL-7 may collectively be major drivers of eosinophil recruitment and lung remodeling, particularly IL-5, an important Th2-skewing cytokine.¹⁴ Targeted therapy to reduce the Th2 response, such as the anti-IL-5 monoclonal antibody mepolizumab, should be investigated preclinically as a means to reduce the robust allergic response in SCD.

Additionally, the proinflammatory immune state we describe may arise from an erythrocytic source: free heme. SCD is characterized by a state of chronic hemolysis, resulting in release of free heme into the circulating plasma. Free heme is a potent proinflammatory mediator through its induction of cytokine release, leukocyte activation, and TLR4-mediated activation of the innate immune system.^{40,41} Indeed, heme-induced TLR4 activation of endothelial cell adhesion molecules has been found to play a role in vaso-occlusion in mouse models of SCD,⁴² and its presence could bias the immune system toward a proinflammatory state and influence the subsequent development of AAD.

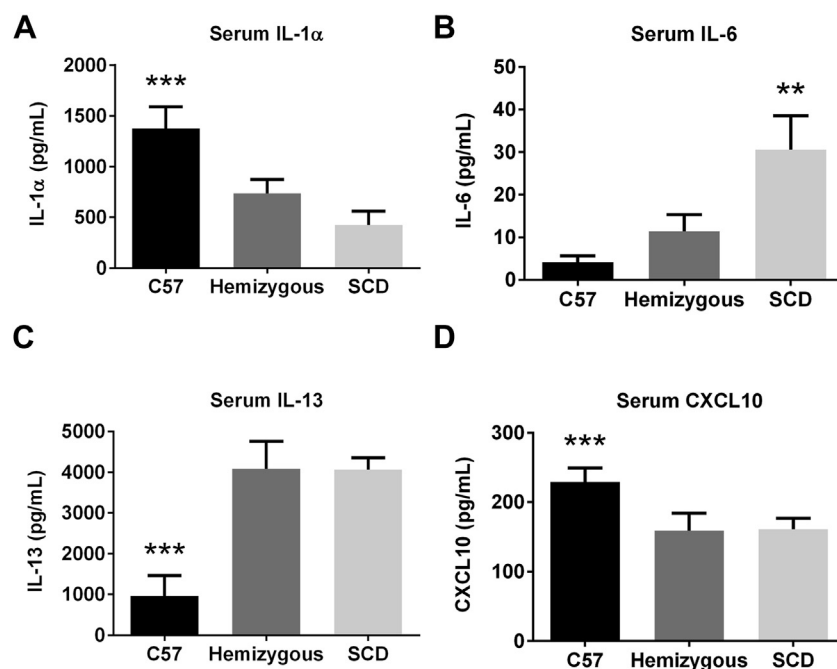


Fig 8. AAD induces an altered serum cytokine profile in SCD mice. Serum was assessed for AAD C57 (n = 8), hemizygous (n = 8), and SCD (n = 8) mice for cytokine and chemokine concentration via Luminex bead assay. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's *t* test. Differences between SCD mice and both C57 and hemizygous mice are denoted by 2 asterisks (**). Differences between C57 mice and both hemizygous and SCD mice are denoted by 3 asterisks (***). AAD, allergic airway disease; IL, interleukin; SCD, sickle cell disease.

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The cellular disturbances we note in SCD mice may provide additional mechanistic explanations for the severity of asthma in human SCD. The perivascular clusters of inflammatory cells in the lungs of naive mice may at least partially account for the increase in IgE and related cytokines such as IL-13 at AAD. Immunophenotyping revealed many of the cells in the lungs and BAL fluid of naive mice to be CD4⁺ and CD8⁺ T, and these may be prepositioned to receive stimulus from antigen-presenting cells that process OVA and initiate a robust inflammatory response. The presence of increased CD8⁺ T cell numbers is particularly noteworthy, as multiple reports have suggested that CD8⁺ T cells can modulate allergic responses by influencing CD4⁺ T cells.⁴³⁻⁴⁵ However, these same reports also pointed to CD8⁺ T cell production of IFN- γ as a key mediator of disease suppression, and IFN- γ is manifold reduced in the BAL fluid of SCD mice at AAD when compared with C57 controls. This suggests that SCD may be associated with a defect in IFN- γ production by CD8⁺ T cells, and it is perhaps for this reason that the increase in percentage of CD4⁺ T cells in the BAL fluid from naive to AAD is greater in SCD than in C57 controls.

Interestingly, such a defect may not be limited to CD8⁺ T cells. The presence of increased Treg numbers in the lung and HLN in naive SCD mice as well as the sharp increase in BAL Treg cells in AAD SCD mice would seem to predict heightened capacity for regulation and disease suppression. However, there is some evidence that Treg cells in SCD display an altered phenotype and may have reduced immunosuppressive capacity, although the latter remains debated.^{46,47} Of particular note, alloimmunization associated with frequent blood transfusion, a mainstay of treatment of SCD, may produce dysfunction in both Treg cells⁴⁶ and regulatory B cells.⁴⁸ We and others have shown that Treg cells^{32,49,50} and regulatory B cells^{30,31,51} play important roles in the regulation of allergy and asthma. Taken together, these findings suggest that the presence of regulatory immune cells may not be sufficient to alleviate the burden of asthma in SCD and suggest dysfunctional immune regulation and suppression by these cells. Future studies of the regulatory function of these cell populations are warranted to determine the specific contribution of each to asthma pathophysiology in SCD.

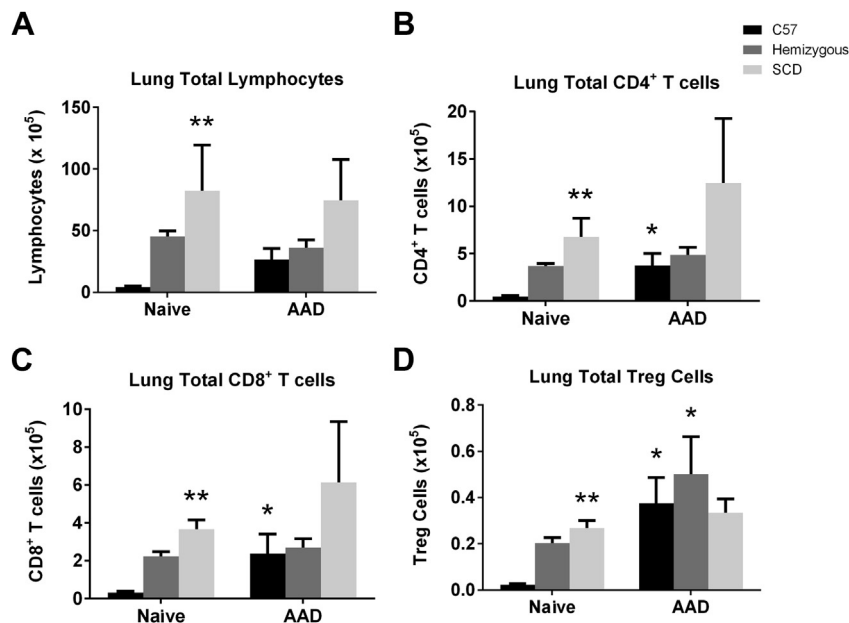


Fig 9. Lung T cell subpopulations are increased in the naive state but not at AAD in SCD mice. (A) Lymphocyte flow cytometric analysis of lung mononuclear cells from C57, hemizygous, and SCD mice at baseline ($n = 6, 6$, and 4 , respectively) and at AAD ($n = 4, 10$, and 7 , respectively) was performed. (B) CD4⁺ T cells are CD3⁺CD4⁺ lymphocytes. (C) CD8⁺ T cells are CD3⁺CD8⁺ lymphocytes. (D) Treg cells are CD3⁺CD4⁺CD25⁺ Foxp3⁺ lymphocytes. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's t test. Differences between the naive and AAD state of an individual mouse group are denoted by an asterisk (*). Differences between SCD mice and C57 control mice at the naive stage are denoted by 2 asterisks (**). AAD, allergic airway disease; SCD, sickle cell disease; Treg, T regulated.

Interestingly, although lung lymphocytes were increased in SCD compared with C57 controls in the naive state, total numbers did not increase at AAD, in sharp contrast to C57s, where the increase is more than 6-fold. The lack of increase of lung Treg cells at AAD in SCD when compared with C57 controls is particularly noteworthy given the role of these cells in modulating allergic diseases.³² This disruption in lymphocyte kinetics may have multiple etiologies. It is increasingly recognized that SCD is associated with profound disturbances in endothelial cells, including increased numbers of circulating, activated endothelial cells²²; disruption of nitric oxide and regulation of vascular caliber^{52,53}; and poor response of endothelial cells to shear stress.^{54,55} Endothelial cells are important mediators of transmigration of lymphocytes from the circulation into tissues, and disturbance of these cells could be expected to impair migration. It is also possible that the dysfunction involves endothelial cells but is not directly related to their lack of function. Multiple reports have noted that erythrocytes in SCD are more adherent,⁵⁶⁻⁵⁹ particularly to activated endothelium. In the presence of a multitude of adherent erythrocytes, competition for adhesion sites on endothelial cells may be too great for the

relatively smaller number of lymphocytes to successfully adhere and pass through the vascular wall. It will be important for future investigations to examine lymphocyte migration, or lack thereof, in SCD.

A further possibility concerns the sphingolipid sphingosine-1-phosphate (S1P), an important mediator of lymphocyte trafficking. Under normal circumstances, activated lymphocytes leave the lymph node by following a gradient of S1P into the blood, where it is higher than in the node.⁶⁰ In SCD, blood levels of S1P are increased and have been shown to be associated with sickling and general disease progression.⁶¹ It may be that increased S1P is another impediment to lymphocytes migrating from the blood into tissues, as they are subjected to a constant tug back into the blood by the unusually high S1P concentration. Further evaluation of the specific role of S1P in comorbid SCD and asthma is warranted.

It is also possible that the initial sites of inflammation in the lung parenchyma, visible even in the naive state, provide a source of cells that can proliferate and migrate into the BAL fluid as disease develops despite poor migration from the blood. The lack of increased protein leak in SCD relative to C57 controls is consistent with a local generation of these additional cells, as increased

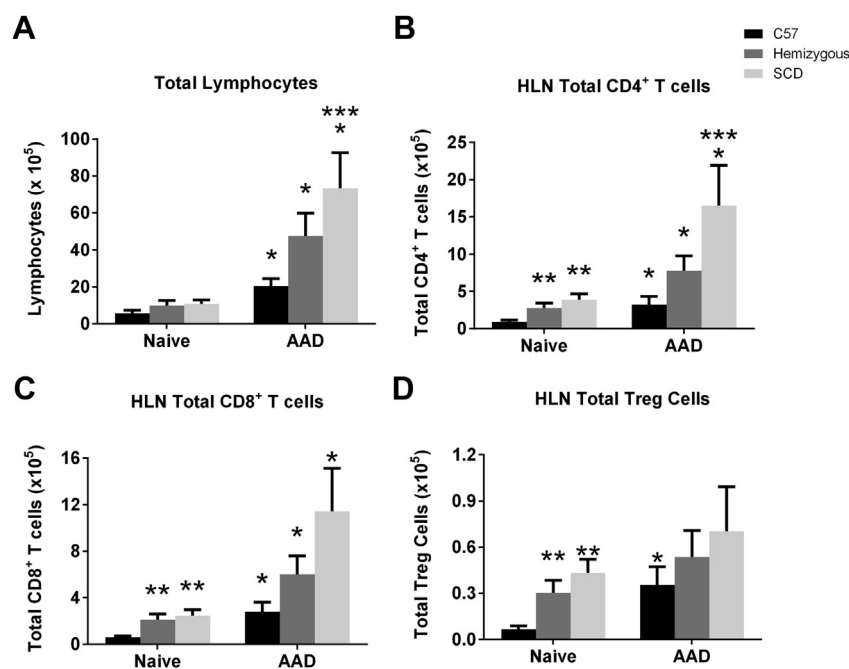


Fig 10. HLN lymphocyte subpopulations are altered in the naive state and at AAD in SCD mice. (A) Lymphocyte flow cytometric analysis of HLN mononuclear cells from C57, hemizygous, and SCD mice at baseline ($n = 6, 6$, and 4 , respectively) and at AAD ($n = 9, 9$, and 10 , respectively) was performed. (B) CD4⁺ T cells are CD3⁺CD4⁺ lymphocytes. (C) CD8⁺ T cells are CD3⁺CD8⁺ lymphocytes. (D) Treg cells are CD3⁺CD4⁺CD25⁺ + Foxp3⁺ lymphocytes. Bars represent mean levels $\pm$ standard error of the mean. Significant differences were determined by 1-way analysis of variance with multiple post hoc, pairwise comparisons between groups using a Student's *t* test. Differences between the naive and AAD state of an individual mouse group are denoted by an asterisk (*). Differences between SCD and hemizygous mice compared with C57 mice at the naive stage are denoted by 2 asterisks (**). Differences between C57 mice and SCD mice at AAD are denoted by 3 asterisks (***). AAD, allergic airway disease; HLN, hilar lymph node; SCD, sickle cell disease; Treg, T regulated.

vascular permeability, which could also produce increased BAL cellularity, would be expected to raise the concentration of protein in the BAL fluid.

Despite increased baseline pulmonary inflammation and a robust serum IgE response to OVA administration, SCD mice proved resistant to methacholine-induced airway constriction after induction of AAD. A recent report of a cohort of children with SCD noted that nearly one third were hyporesponsive to methacholine challenge,¹⁰ suggesting a similar disease process might be occurring in the SCD mouse and human patients with SCD. Furthermore, others have also described evidence of airway obstruction despite a lack of AHR in SCD,⁶² and there is some evidence that bronchodilators provide diminished symptomatic relief in SCD.⁶³ Taken together, this could indicate that the obstruction present may not arise from airway smooth muscle constriction but rather from some other obstructive force not responsive to methacholine challenge. Such findings would necessitate a re-examination of current clinical practices for the treatment of asthma in SCD, perhaps supplementing or replacing conventional asthma treatment with alternative therapies in this unique disease

state. One possibility, recently raised by studying children with SCD,⁶³ is that vascular congestion and corresponding vascular enlargement could be producing airway obstruction in the absence of AHR, which is consistent with reported poor efficacy of bronchodilators in the SCD population.

The disturbed cytokine/chemokine milieu provides a possible mechanistic explanation for the airway hyporeactivity. Both IFN- γ and CXCL10 have been shown to be important for the development of airway hyperreactivity, and we found these to be deficient in SCD mice at AAD. Both are also deficient in hemizygous mice, potentially explaining the attenuated response to methacholine challenge also seen in these animals.

Intriguingly, our findings contrast with those of Pritchard et al,¹¹ who reported significantly increased AHR in SCD mice at AAD and in the naive state. One possible explanation for this difference concerns the unique aspects of our respective SCD models. The SCD model examined in our study is a transgenic mouse expressing human globins, including sickle beta globin, from birth. Contrastingly, Pritchard et al¹¹ performed a bone marrow transplant into wild-type mice from a

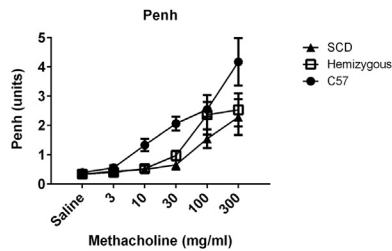


Fig 11. SCD and hemizygous mice are hyporesponsive to methacholine challenge at AAD. Airway hyper-responsiveness was assessed at AAD after 3 OVA intraperitoneal sensitizations and 3 days of OVA-aerosol challenge. Sensitivity to methacholine challenge was compared between SCD ($n = 7$, filled triangles), hemizygous ($n = 7$, open boxes), and C57 control ($n = 6$, filled circles) mice. Increasing concentrations of methacholine are denoted on the x-axis. Area under the curve was computed for each mouse and groups compared using 1-way analysis of variance and Tukey post hoc test. Data are represented as the mean $\pm$ standard error of the mean levels of Penh responses. The overall effect of genotype on methacholine response is significant ($P < 0.01$). $P < 0.01$ for C57 vs SCD. $P = 0.06$ for C57 vs hemizygous. AAD, allergic airway disease; Penh, enhanced pause; OVA, ovalbumin; SCD, sickle cell disease.

transgenic SCD mouse. Those mice were allowed to recover for 8 weeks and demonstrated many of the hallmark pathologies of SCD, but they did not develop from birth in the presence of sickling erythrocytes and consequently their organ function and pathology may vary from an animal exposed to sickling cells for the entire postnatal period.

Comorbid asthma in SCD is a predictor of future morbidity, mortality, and consequently quality of life. Much effort has been directed at the clinical interaction of asthma and SCD, but the underlying pathophysiological process remains unclear. Herein, we describe the application of a well-established OVA-induced model of murine AAD to SCD. Naïve SCD mice exist in a proinflammatory Th2 state before induction of AAD with increased CD4⁺ T cells in the HLN and lung tissue. It may be this predisposition to Th2-mediated inflammation evidenced by increased naïve BAL fluid levels of the Th2 cytokine IL-5 that produces the enhanced allergic response seen at AAD. SCD mice have 10-fold greater levels of OVA-specific IgE in the blood than C57 controls and increased overall cellularity and eosinophil number in BAL fluid at AAD. Surprisingly, but consistent with previous clinical reports, SCD and hemizygous mice are hyporesponsive to methacholine challenge at AAD. SCD mice have low levels of IFN- γ at AAD compared with C57 controls, and this cytokine may be a crucial player in the lack of AHR. Lack of AHR in the mouse model of AAD suggests standard clinical therapy with bronchodilators may be ineffective in this population and warrants further investigation. Observed similarities between SCD and hemi-

zygous mice suggest evaluation of sickle cell trait as a potential risk factor for asthma susceptibility should be investigated clinically.

In summary, further study of models of asthma in SCD such as that used herein may provide important insights into disease pathophysiology and drive development of new therapeutic modalities for these intertwined conditions.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

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