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The sickle cell mouse lung: pro-inflammatory and primed for allergic inflammation

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Abstract

Co-morbid asthma in sickle cell disease (SCD) confers higher rates of vaso-occlusive pain and mortality, yet the physiological link between these two distinct diseases remains puzzling. We utilized 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 (IP) route followed by daily, nose-only OVA-aerosol challenge to induce AAD. The lungs of naïve SCD mice showed signs of inflammatory and immune processes: (1) histologic and cytochemical evidence of airway inflammation as compared to naïve wildtype mice; (2) bronchoalveolar lavage fluid (BAL) contained increased total lymphocytes, %CD8⁺ T cells, G-CSF, IL-5, IL-7, and CXCL1, and (3) lung tissue and hilar lymph node (HLN) had increased CD4⁺, CD8⁺, and regulatory T cells (Tregs). Further, SCD mice at AAD demonstrated significant changes compared to the naïve state: (1) BAL with increased %CD4⁺ T cells and Tregs, lower %CD8⁺ T cells, and decreased IFN γ , CXCL10, CCL2, and IL-17, (2) serum with increased OVA-specific IgE, IL-6, and IL-13, and decreased IL-1 α and CXCL10, (3) no increase in Tregs in the lung tissue or HLN, and (4) hypo-responsiveness to methacholine challenge. In conclusion, SCD mice have an altered immunologic pulmonary milieu and physiologic responsiveness. These findings suggest that the clinical phenotype of AAD in SCD mice differs from that of wildtype mice and suggests that individuals with SCD may also have a unique, divergent phenotype perhaps amenable to a different therapeutic approach.

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Introduction

Pulmonary diseases, such as asthma and acute chest syndrome, are major determinants of mortality in individuals with SCD¹. In fact, asthma is an independent predictor of mortality in patients with SCD²⁻⁵. Airway hyper-responsiveness 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 airway hyper-responsiveness in SCD are unknown.

Interestingly and conversely, in a cohort of 99 children with SCD, 30% were found to be hypo-responsive to maximal doses of methacholine raising the question of whether what appears to be asthma in SCD is really related to classic bronchoconstriction¹⁰. Investigations of experimental asthma in the SCD mouse model to date reveal histopathologic evidence of an enhanced asthma phenotype, increased total plasma IgE, and elevated bronchoalveolar lavage fluid pro-inflammatory cytokines including IL-13 and IL-5¹¹.

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 levels¹²⁻¹⁴. The pathogenesis and immunology of this disease are complex with mast cells, dendritic cells, T- and B-lymphocytes, and eosinophils all playing significant roles¹⁵. The increase in eosinophils and CD4+ T lymphocytes in the bronchial mucosa and bronchoalveolar lavage (BAL) fluid are characteristic features 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 asthma patients 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 pro-inflammatory 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 al* have shown that low-dose LPS challenge in transgenic SCD mice provokes an exaggerated inflammatory response with elevated levels of TNF α , IL-1 β , and soluble VCAM-1 in the serum and BAL fluid²⁴. Our group has previously reported an altered baseline immunophenotype in the serum, gut, and spleen of SCD mice²⁵, 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, Nandekar *et al* demonstrated histopathological evidence of enhanced pulmonary inflammation in SCD mice under an experimental asthma protocol²⁷. 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.

Co-morbid asthma in SCD is a major contributor to mortality^{3,4}, yet mechanistic insights into its pathogenesis have been sparse. Herein, we utilized a mouse model of SCD to study inflammatory factors involved in the genesis of asthma by employing a well-characterized OVA-induced allergic airway disease (AAD) model. We hypothesized that altered immune responses to allergens occur in SCD mice which 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, ME, USA) and were 8–12 weeks of age and weighed 15–25 g at the time of sacrifice. Berkeley sickle cell transgenic mice [Tg(Hu-miniLCR 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 SCD²⁸. 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 22–24°C with a daily light/dark cycle (light from 0600 to 1800h). 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 Figure 1, mice were immunized with three weekly intraperitoneal (i.p.) injections of a suspension containing 25 μ g of OVA (grade V, Sigma Chemical Co., St. Louis, MO) 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 physiologic saline, 1 hour/day, for 3 days²⁹. 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, NJ) into a 7.6-L inhalation exposure chamber to which restraint tubes were attached. Chamber airflow was 6 L/minute 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 (GSDs) were 1.4 and 1.6 μ m, respectively. The estimated daily inhaled OVA dose approximated 30–40 μ g/mouse³⁰.

Harvesting of Samples: Blood, BAL, Lung tissue, and Hilar Lymph Node

Twenty-four hours after the final aerosol exposure, the mice were sacrificed by ketamine/xylazine overdose and samples collected from the various tissues and compartments as previously described^{30–32}. Briefly, whole blood was immediately collected via cardiac puncture into non-heparinized tubes and allowed to clot at room temperature. Serum was collected after centrifugation at 4,000 rpm and frozen for later use at -80°C . A bronchoalveolar lavage (BAL) was performed immediately after sacrifice *in situ* with five 1 mL aliquots of sterile saline. Lung draining (hilar) lymph nodes (HLN) were harvested and mechanically disrupted into a single-cell suspension. In addition, lungs were isolated from un-lavaged mice, minced, and treated with collagenase solution (150 U/mL, Gibco, Grand Island, NY) for 1 hour at 37°C . The minced tissue was mechanically disrupted through a 40- μm nylon filter (BD Falcon, Bedford, MA). 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. 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. BAL protein concentrations were measured in the supernatants by bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard (Pierce Rockford, IL).

Antibodies

The following monoclonal antibodies were utilized 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 utilized.

FACS analysis

Cell pellets containing lymphocytes from tissue preparations were resuspended in PBS solution (containing 0.2% BSA and 0.1% NaN_3) at a concentration of $1 \times 10^5 - 1 \times 10^6$ white blood cells/mL, and 100 μL of the cells were diluted 1:1 with 100 μL of the appropriate monoclonal antibody and incubated at 4°C for 30 min. Foxp3⁺ Treg cells were identified by staining lymphocytes with anti-CD3, anti-CD4, and anti-CD25, followed by permeabilization using fixation/permeabilization buffer, 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% NaN_3) and relative fluorescence intensities were determined using an LSR-II (Becton-Dickinson, San Diego, CA) and analyzed with FlowJo flow cytometry analysis software (Ashland, OR). Fluorescence minus one (FMO) controls were used to assist with gating.

Cytokine/Chemokine Measurements

Frozen serum samples obtained from mice were thawed and utilized for analysis. Samples were processed using a Milliplex Mouse Cytokine/Chemokine 22-plex kit (Cat #MPXMCYT070KPMX22; Millipore; Billerica, MA) following the manufacturer's

instructions and utilizing 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 ELISA using isotype-specific capture monoclonal antibodies³³. IgE was captured from serum (diluted 10- to 1,280-fold) using Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, VA) coated with anti-mouse IgE (R35-72) (BD Pharmingen, San Diego, CA) at 2 µg/ml in 0.1 mol/L carbonate, pH 9.5. Detection was with an OVA-digoxigenin conjugate followed by horseradish peroxidase-conjugated anti-digoxigenin, essentially as described³⁴. Development was with the TMB microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Histology

After sacrifice, unmanipulated (not subject to BAL) and non-inflated 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 five lobes were examined in their entirety in a blinded manner for qualitative analysis.

Airway Hyperreactivity

Airway responses to methacholine were assessed by whole-body barometric plethysmography, as previously described³⁶. Mice were studied 24 hours after the third OVA aerosol. Mice were placed in individual chambers (Buxco Electronics, Troy, NY) 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 (Penh) 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, NC, USA) and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). For comparisons between three or more groups, a Tukey post-hoc test was performed. All data were expressed as means $\pm$ standard error of the mean. Methacholine response curves were compared by computing the area under the curves (AUC) and comparing AUCs using ANOVA 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 naïve to OVA-induced AAD in wild-type C57 mice as previously demonstrated²⁹. Naïve C57 mice show lung histopathologic findings devoid of peri-bronchial inflammation and mucus staining (Figure 2A). Upon induction of AAD, the C57 mice show airway luminal narrowing, increased mucus staining (short arrows) and increased peri-bronchial/peri-vascular cellularity (Figure 2B). Interestingly, naïve SCD mice exhibit a baseline increase in perivascular cellular infiltration (Figure 2E). SCD mice show even greater enhancement of airway mucus production and perivascular cellularity at AAD as compared to the naïve state (Figure 2F). Hemizygous mice were histopathologically similar to C57 mice in both the naïve and AAD states (Figures 2C and 2D).

AAD induces enhanced BAL leukocyte and eosinophil counts in SCD mice as compared to C57 wildtype mice

Although the total number of leukocytes in the BAL in the naïve C57, hemizygous and SCD are similar (0.7 , 1.0 and 0.8×10^5 , respectively, Figure 3A), the SCD mice have a significantly increased total BAL lymphocyte count (0.05×10^5) compared to C57 and hemizygous mice (0.01 and 0.02×10^5 , respectively, $p=0.0006$ Figure 3B). At AAD, all three groups of mice demonstrate significant increases in BAL total leukocyte count compared to the naïve state (Figure 3A, $p=0.004$). However, SCD mice display a significantly more robust BAL leukocytosis and eosinophilia at AAD (Figures 3A and 3C) suggestive of more profound allergic response to inhaled OVA (both $p<0.03$). There were no eosinophils observed in the BAL of any of the mouse groups in the naïve state. Leukocyte differentials are reported in Table 1. There is no difference in BAL protein concentration among SCD mice compared to the C57 and hemizygous control mice in the naïve state, nor at AAD (Figure 3D). However, there is a statistically significant increase in BAL protein concentration within each mouse group when comparing the naïve to the AAD state ($p<0.01$ for each group).

BAL T cell sub-populations 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 naïve state and after induction of AAD (Figure 4). The BAL of naïve SCD and hemizygous mice had a higher percentage of CD8+ T cells (Figure 4B, $p=0.007$) as compared to naïve C57 mice but no difference in %CD4+ T cells (Figure 4A) or %Treg cells (Figure 4C). In AAD SCD and AAD hemizygous mice, there were significantly higher %CD4+ T cells in BAL than C57 mice with a concomitant decrease in %CD8+ T cells (Figures 4A and 4B, $p<0.004$). Although all mouse groups had increases in %Treg cells in the BAL at AAD ($p<0.0003$) compared to the naïve state, consistent with previous results in C57 mice³², SCD mice displayed a significantly higher percentage of Treg cells at AAD compared to 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 of SCD, hemizygous and C57 mice (Figure 5A–D). G-CSF, IL-5, and IL-7 were significantly higher in concentration in SCD mice compared to both C57 and hemizygous mice in the naïve state ($p=0.004$, 0.049 , and 0.008 , respectively for each cytokine). The chemokine CXCL1 was significantly increased in the BAL of naïve SCD mice as compared to C57 and hemizygous mice ($p=0.01$). There was no difference in levels of the cytokines GM-CSF, IFN γ , IL-1b, IL-2, IL-4, IL-9, IL-19, IL-12, IL-13, IL-15, IL-17, or TNF α (data not shown). Additionally, there was no difference in levels of the chemokines CCL2, CCL3, CCL5, or CXCL10 (data not shown).

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 of SCD mice as compared to control mice (Data not shown). However, as demonstrated in Figure 6, two cytokines and two chemokines were significantly decreased in the BAL of SCD mice compared to 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 ($p=0.03$, Figure 7). Although serum OVA-specific IgE levels were higher in hemizygous mice compared to C57s, this was not statistically significant ($p=0.14$). As shown in Figure 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 as compared to 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 sub-populations in lung tissue in the naïve state, but not at AAD

Immunophenotyping of lung tissues demonstrated that in the naïve state the SCD mouse lung has increased total lymphocytes, CD4 $^{+}$ T cells and CD8 $^{+}$ T cells compared to C57 control mice (Figure 9A–C; all $p<0.02$). Further sub-characterization of the CD4 $^{+}$ T cell subset identifies an increase in total Treg cells as well (Figure 9D; $p<0.0001$).

Interestingly, at AAD, there were no observed differences in total lung lymphocyte counts between SCD mice and both C57 and hemizygous mice (Figure 9A). 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 (Figures 9B–D).

SCD mice have altered T cell sub-populations in the naïve state as well as at AAD

Hilar lymph node (HLN) immunophenotyping of T cells was also performed to assess for 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 naïve HLN was no different

between mouse groups. However, at the induction of AAD, there was a greater increase in the HLN lymphocyte count in SCD mice as compared to C57 controls (Figure 10A; $p=0.04$). T cell immunophenotyping of naïve HLN lymphocytes (Figures 10B and 10C) demonstrated an increase in total CD4⁺ and CD8⁺ T cells in the SCD and hemizygous mice compared to C57 mice (both $p=0.01$). Although all three 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 (Figures 10B; $p=0.04$). Naïve SCD and hemizygous mice had significantly greater numbers of Treg cells in their HLN compared to C57 mice (Figure 10D; $p=0.007$). However, while C57 mice demonstrated increased Tregs in the HLN at induction of AAD ($p=0.01$), SCD and hemizygous mice did not (Figure 10D).

Airway Responsiveness

Airway responsiveness to methacholine challenge was assessed by whole-body barometric plethysmography. As we have observed previously³⁵, C57 mice show airway hyper-responsiveness at AAD, physiologically confirming allergic airway disease. Interestingly, the SCD mice had significantly decreased airway hyper-responsiveness (AHR) as compared to C57 mice at AAD (Figure 11; $p < 0.01$ by one-way ANOVA). 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 (Figure 11).

Discussion

Asthma is a major contributor to morbidity and mortality in SCD, yet relatively little is known of the pathophysiologic basis of the asthma phenotype in SCD or the interactions between these two disease states. Here, we report that transgenic SCD mice exhibit baseline pulmonary inflammation and a distinct cytokine, chemokine, and cellular milieu at AAD as compared to C57 control mice. Naïve SCD mice are characterized by increased levels of pro-inflammatory cytokines and chemokines in the BAL, elevated 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 as compared to 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 suggests that naïve SCD mice exist in a pro-inflammatory 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 SCD³⁹. The immune system in SCD may be primed to respond aggressively to inhaled allergen by virtue of this pro-inflammatory 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 Nandekar *et al*, where high mortality was present exclusively in SCD mice undergoing experimentally-induced asthma²⁷. 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 we

observed in SCD mice at AAD. Higher naive SCD BAL 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 cytokine¹⁴. Targeted therapy to reduce the Th2 response, such as the anti-IL-5 monoclonal antibody mepolizumab, should be investigated pre-clinically as a means to reduce the robust allergic response in SCD.

Additionally, the pro-inflammatory 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 pro-inflammatory 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 SCD⁴², and its presence could bias the immune system toward a pro-inflammatory state and influence subsequent development of allergic airway disease.

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 like IL-13 at AAD. Immunophenotyping revealed many of the cells in the lungs and BAL of naive mice to be CD4+ and CD8+ T, and these may be pre-positioned 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 many-fold reduced in the BAL of SCD mice at AAD when compared to 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 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 Tregs in AAD SCD mice would seem to predict heightened capacity for regulation and disease suppression. However, there is some evidence that Tregs 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 Tregs⁴⁶ and regulatory B cells (Bregs)⁴⁸. We and others have shown that Tregs^{32,49,50} and Bregs^{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 suggests 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.

Interestingly, while lung lymphocytes were increased in SCD as compared to C57 controls in the naïve state, total numbers did not increase at AAD, in sharp contrast to C57s, where the increase is over 6-fold. The lack of increase of lung Tregs at AAD in SCD when compared to C57 controls is particularly noteworthy given the role of these cells in modulating allergic diseases³². 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 adherent⁵⁶⁻⁵⁹, 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 node⁶⁰. In SCD, blood levels of S1P are elevated and have been shown to be associated with sickling and general disease progression⁶¹. It may be that elevated 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 co-morbid SCD and asthma is warranted.

It is also possible that the initial sites of inflammation in the lung parenchyma, visible even in the naïve 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 vascular permeability, which could also produce elevated BAL cellularity, would be expected to raise the concentration of protein in the BAL.

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 a third were hypo-responsive to methacholine challenge¹⁰, suggesting a similar disease process might be occurring in the SCD mouse and human SCD patients. Furthermore, others have also described evidence of airway obstruction despite a lack of airway hyper-reactivity (AHR) in SCD⁶², and there is some evidence that bronchodilators provide diminished symptomatic relief in SCD⁶³. 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 reexamination 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 SCD⁶³, is that vascular congestion and corresponding vascular enlargement could be producing airway obstruction in the absence of AHR, consistent with reported poor efficacy of bronchodilators in the SCD population.

The disturbed cytokine/chemokine milieu provides for a possible mechanistic explanation for the airway hypo-reactivity. Both IFN γ and CXCL10 have been shown to be important for the development of airway hyper-reactivity, 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 naïve 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 al* performed a bone marrow transplant into wild type mice from a transgenic SCD mouse¹¹. 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.

Co-morbid 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 pathophysiologic 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 pro-inflammatory Th2 state prior to induction of AAD with elevated CD4+ T cells in the HLN and lung tissue. It may be this predisposition to Th2-mediated inflammation evidenced by elevated naïve BAL 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 as well as increased overall cellularity and eosinophil number in BAL at AAD. Surprisingly, but consistent with previous clinical reports, SCD and hemizygous mice are hypo-responsive to methacholine challenge at AAD. SCD mice have low levels of IFN γ at AAD as compared to C57 controls, and this cytokine may be a crucial player in the lack of AHR. Lack of AHR in the mouse model of allergic airway disease suggests standard clinical therapy with bronchodilators may be ineffective in this population and warrants further investigation. Observed similarities between SCD and hemizygous 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 utilized herein may provide important insights into disease pathophysiology and drive development of new therapeutic modalities for these intertwined conditions.

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Brief Commentary

Background

Co-morbid 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 two biologically distinct processes remains elusive. Herein, we utilize 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. Further, since airway hyper-reactivity is a major component in the diagnosis of asthma, it may be under-diagnosed in this population.

OVA AAD Model:

Figure 1. OVA-Induced AAD (asthma) Model

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

Figure 2. Sickie AAD mice have increased lung inflammation and mucus

Images are 20x magnification. Short arrows denote PAS+ stain indicative of mucus, whereas long arrows represent the peri-vascular/peri-bronchial inflammation. A= naïve C57, B = AAD C57, C= naïve hemizygous, D = AAD hemizygous, E = naïve SCD, F = AAD SCD. aw=airway. bv=blood vessel.

Figure 3. AAD induces shifts in BAL leukocyte counts and differentials in SCD mice but no difference in protein

Bronchoalveolar lavage (BAL) fluid was assessed for total leukocyte count (A), differentials (B, C) and protein concentration (D) from naïve C57 (n=6), hemizygous (n=6) and SCD (n=5) mice and compared to BAL from C57 (n=4), hemizygous (n=10) and SCD (n=7) mice after three weekly i.p. 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 one-way ANOVA with multiple post-hoc pair-wise comparisons between groups using a student's t-test. Differences between the naïve and AAD state of an individual mouse group is denoted by an asterisk (*). Differences between SCD mice and both C57 and hemizygous mice is denoted by two asterisks (**).

Figure 4. BAL T cell sub-populations 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). CD4⁺ T cells are CD3⁺CD4⁺ lymphocytes. CD8⁺ T cells are CD3⁺CD8⁺ lymphocytes. Treg cells are CD3⁺CD4⁺CD25⁺Foxp3⁺ lymphocytes. Bars represent mean levels $\pm$ SEM.

Significant differences were determined by one-way ANOVA with multiple post-hoc, pairwise comparisons between groups using a student's t-test. Differences between the naïve 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 two asterisks (**). Differences between C57 controls and both hemizygous and SCD mice in the naïve state or at AAD are denoted by three asterisks (***).

Figure 5. Naïve SCD mice have increased BAL G-CSF, IL-5, IL-7, and CXCL1

BAL was assessed from naïve C57 (n=6), hemizygous (n=6), and SCD (n=5) mice for cytokine and chemokine concentration via Luminex bead assay. Bars represent mean levels $\pm$ SEM. Significant differences were determined by one-way ANOVA with multiple post-hoc, pair-wise comparisons between groups using a student's t-test. Differences between SCD mice and both C57 and hemizygous mice are denoted by two asterisks (**).

Figure 6. AAD SCD mice have lower BAL concentrations of IFN γ , IL-17, CXCL10, and 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 $\pm$ SEM. Significant differences were determined by one-way ANOVA with multiple post-hoc, pair-wise comparisons between groups using a student's t-test. Differences between C57 mice and both hemizygous and SCD mice are denoted by three asterisks (***).

Figure 7. Serum OVA-specific IgE is 10-fold higher in SCD mice at AAD

Serum OVA-specific IgE was measured using a capture ELISA. C57 (n=9), hemizygous (n=9) and SCD (n=10) mice were analyzed after induction of AAD. Bars represent mean levels $\pm$ SEM. Significant differences were determined by one-way ANOVA with multiple post-hoc, pair-wise comparisons between groups using a student's t-test. Differences between SCD and C57 mice are denoted by two asterisks (**).

Figure 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 $\pm$ SEM. Significant differences were determined by one-way ANOVA with multiple post-hoc, pair-wise comparisons between groups using a student's t-test. Differences between SCD mice and both C57 and hemizygous mice are denoted by two asterisks (**). Differences between C57 mice and both hemizygous and SCD mice are denoted by three asterisks (***).

Figure 9. Lung T cell sub-populations are increased in the naïve state but not at AAD in SCD mice

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. CD4⁺ T cells are CD3⁺CD4⁺ lymphocytes. CD8⁺ T cells are CD3⁺CD8⁺ lymphocytes. Treg cells are CD3⁺CD4⁺CD25⁺Foxp3⁺ lymphocytes. Bars represent mean levels $\pm$ SEM. Significant differences were determined by one-way ANOVA with multiple post-hoc, pair-wise comparisons between groups using a student's t-test. Differences between the naïve and AAD state of an individual mouse group is denoted by an asterisk (*). Differences between SCD mice and C57 control mice at the naïve stage is denoted by two asterisks (**).

Figure 10. HLN lymphocyte sub-populations are altered in the naïve state and at AAD in SCD mice

Lymphocyte flow cytometric analysis of hilar lymph node (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. CD4⁺ T cells are CD3⁺CD4⁺ lymphocytes. CD8⁺ T cells are CD3⁺CD8⁺ lymphocytes. Treg cells are CD3⁺CD4⁺CD25⁺Foxp3⁺ lymphocytes. Bars represent mean levels $\pm$ SEM. Significant differences were determined by one-way ANOVA with multiple post-hoc, pair-wise comparisons between groups using a student's t-test. Differences between the naïve and AAD state of an individual mouse group is denoted by an asterisk (*). Differences between SCD and hemizygous mice compared to C57 mice at the naïve stage is denoted by two asterisks (**). Differences between C57 mice and SCD mice at AAD are denoted by three asterisks (***).

Figure 11. SCD and hemizygous mice are hyporesponsive to methacholine challenge at AAD
 Airway hyperresponsiveness was assessed at AAD following 3 OVA i.p. sensitizations and 3 days of OVA aerosol challenge. Sensitivity to methacholine challenge was compared between SCD (n=7, closed triangles), hemizygous (n=7, open boxes), and C57 control (n=6, closed circles) mice. Increasing concentrations of methacholine are denoted on the x-axis. Area under the curve (AUC) was computed for each mouse and groups compared using one-way ANOVA and Tukey post-hoc test. Data represent mean $\pm$ SEM levels of Penh responses. The overall effect of genotype on methacholine response is significant ($p < 0.01$). $p < 0.01$ for C57 versus SCD. $p = 0.06$ for C57 versus Hemizygous.

Table 1

Comparison of BAL Leukocyte Differentials in Naïve and AAD Mice

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

All three 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 polymorphonuclear (PMN) cells at AAD between mouse groups. Data represent mean ± SEM values.