# Effects of Experimental Asthma on Inflammation and Lung Mechanics in Sickle Cell Mice

Kirkwood A. Pritchard, Jr., Ph.D., Thom R. Feroah, Ph.D., Sandhya D. Nandedkar, Ph.D., Sandra L. Holzhauer, William Hutchins, B.S., Marie L. Schulte, Ph.D., Robert C. Strunk, M.D., Michael R. DeBaun, M.D., Cheryl A. Hillery, M.D.

***ONLINE DATA SUPPLEMENT***

## *Supplemental Methods, Discussion and Figure*

### *Animals*

*Engraftment confirmation and Ova sensitization:* Following irradiation and HSCT, mice were treated with prophylactic antibiotics (trimethoprim/sulfamethoxazole in drinking water) for 1 month and routine sterile rodent barrier care for a second month to provide sufficient time for engraftment and development of SCD hematologic, organ and vascular pathologies. Engraftment was assessed by hemoglobin electrophoresis of red blood cells isolated from the chimeric mice (Helena laboratories, Beaumont, Texas). Only mice with 100% sickle or HbA erythrocyte engraftment at 2 months post transplantation were used for these studies.

Engrafted mice were either placed in a non-sensitized (Non-Sen) control group or in an OVA sensitized (OVA-Sen) group as we have previously described.(1) Briefly on day 1, mice were anesthetized (isoflurane; Baxter, Deerfield, IL) and heat-denatured OVA fragment (~80 mg, ~5x2x2 mm) was implanted subcutaneously in the dorsal aspect of the neck. The incision was closed with stainless steel clips and the mouse allowed to recover. Implantation of the heat-denatured OVA fragment initiates sensitization. On day 15, the implanted mice were exposed to aerosolized OVA (10% in PBS, OVA-Sen) or PBS alone (Non-Sen) for 15 min, "low-dose," every other day for 10 days. In the present study OVA-sensitized mice were exposed to low-dose OVA because we observed previously that the OVA-Sen native SCD mice were more susceptible to death when exposed to a full 30 min of aerosolized OVA.(1) Twenty-four hours after the last OVA challenge, the mice were either evaluated for changes in bronchoalveolar lavage fluid (BALF) cytokines and histopathology or were subjected to airway mechanic studies. This yielded 4 groups: 1) chi-HbA Non-Sen (n = 7 [3*]); 2) chi-SCD Non-Sen (n = 9 [4*]); 3) chi-HbA OVA-Sen (n = 5 [3*]); and, 4) chi-SCD OVA-Sen (n = 10 [4*]). The number in square brackets and asterisk (*) equals the number of animals used for histopathology. The chimeric mice were housed in sterile autoclavable microisolation cages and provided food and water ad libitum.

*Collection of blood and tissue samples:* After establishment of deep anesthesia (isoflurane; Baxter, Deerfield, IL) and thoracotomy, ~750μL of blood was drawn into 35 μL of heparin (10,000 units/mL) by cardiac puncture. For BALF samples, the trachae were cannulated with polyethylene tubing. BALF was obtained by flushing the lungs with PBS, first 1 mL followed by 0.5 mL. The rinses were combined and the BALF samples centrifuged at 2000 rpm

for 10 min at 4ºC. The supernatants were removed and stored at -80ºC until analysis. Mice used for collection of BALF were not used for histopathology. The lungs of mice used for histopathology were rapidly isolated and fixed in zinc formalin, embedded in paraffin, cut into 5 μm sections and mounted onto slides for staining.

***Histopathology Studies***

*Eosinophil Infiltration*: Sections were stained with hematoxylin and eosin (H&E) to enhance cytoplasmic and nuclear structures (see Supplemental Figure). Infiltration of inflammatory eosinophils was quantified as follows: Cell counts were performed by a technician who had no prior knowledge of the different treatment groups. Cells were counted in ten - 100 $\mu m^2$ fields randomly selected in the peribronchial and perivascular regions and subsequently averaged for each animal as previously described (1).

*Collagen Deposition*: Sections were stained with McLetchies's Trichrome (Newcomer Supply Co.; Middleton, WI) to visualize collagen deposition and to assess thickening of basement membranes of airway epithelium and pulmonary vessels. Images were captured on a Ziess Imager.Z1 microscope using 40 X objective and Axiocam HRc camera and Axiovision Software (v4.6). Basement membrane and vessel wall thickening were determined by measuring 4 times at 4 different points the perpendicular distance from a tangential line from the outer edge to the inner edge of the basement membrane or vessel wall. The 4 measurements were averaged and the average recorded for each animal as described in our previous report (1).

***Plasma Lactate Dehydrogenase (LDH) and IgE Levels***

To assess effects of SCD and experimental asthma on red cell lysis, we quantified LDH activity in plasma isolated from the Non-Sen and OVA-Sen chimeric mice, using QuantiChromTM Lactate Dehydrogenase Kit (DLDH-100) from BioAssay Systems (Hayward, CA) following the manufacturer's guidelines. Plasma IgE levels were measured as previously described (1).

***BALF Cytokine, Chemokine and VEGF Analysis***

IL-1β, interferon-γ (IFN-γ), IL-4, IL-5, IL-6, IL-10, IL-13 and monocyte chemoattractant protein-1 (MCP-1) from BALF were measured by a multiplex, bead-based assay using Bio-Plex

Cytokine Assay kit following the manufacturer's instructions (Bio-Rad Laboratories). BALF VEGF levels were measured using the Mouse VEGF Quantikine ELISA Kit from R&D Systems (Minneapolis, MN).

***Measurements of Airway and Tissue Mechanics***

To assess airway and lung mechanics, chimeric mice were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (90mg/kg). Anesthetized chimeric mice were placed on a 37° C heating pad to maintain core body temperature. After confirming full anesthesia by the absence of pain reflex (paw pinch), mice were tracheostomized and intubated with a ½ inch long thin-walled, metal tubing (0.05 in OD, 0.042 in ID). Chimeric mice were then connected to a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, PQ, Canada) and ventilated at a tidal volume of 9 mL/kg at a frequency of 150 breaths/min, which is the default rate for mice (2-4). A positive end-expiratory pressure (PEEP) of 3 $cmH_2O$ was applied to the breathing circuit by submerging the outlet of the expiratory line 3 cm below the surface of water in a water trap. A standard 3-lead electrocardiogram (ECG, Grass Instruments) was placed on the mouse to monitor heart rate and pattern of cardiac electrical activity.

After the chimeric mice were instrumented, they were paralyzed with pancuronium bromide (0.04 μg/kg, IP) to eliminate the possibility of involuntary smooth muscle cell contraction during airway studies. The position of the mouse was carefully adjusted and assessed with pulmonary mechanics test (described below) until a minimal airway resistance was verified. The pulmonary mechanics was assessed using the forced oscillation technique (FOT) with Prime-2 perturbations (a 2 sec pause in ventilation) using the flexiVent system. The testing protocol for measuring pulmonary airway and tissue mechanics started with 2 pressure limited total lung capacity maneuvers with an intervening period of 30 sec. Following a 60 sec period of default ventilation, a series of challenges was performed at different levels of PEEP (0, 3, 6, and 9 $cmH_2O$). Each level of PEEP was held for 130 sec with the Prime-2 perturbation repeated twice, once at 60 sec and the other at 120 sec. Testing at different PEEP levels was performed to assess chronic responses to OVA sensitization or lack thereof.

Pulmonary reactivity was then assessed by administering a series of progressively increasing concentrations of methacholine to induce successive increases in bronchoconstriction (5, 6). Methacholine challenge was performed at 5 different concentrations (1.25, 3.125, 6.25,

12.5, 18.0, 25.0 mg/mL in physiological buffered saline), which was aerosolized by an ultrasonic nebulizer (Aeroneb, SCIREQ) and connected in-line to the inspiratory arm of the flexiVent. *These concentrations, which are lower than what are typically used in less fragile mice (7) were used to minimize death of the chi-SCD mice*. The methacholine aerosol was delivered for a total of 20 breaths at a tidal volume of ~0.4 mL at a rate of 30 breaths/min. Immediately after delivery of the methacholine, the nebulizer was quickly removed from the inspiratory arm of the flexiVent and default ventilation re-instated. Then for the next 3 min, a Prime-2 perturbation was repeatedly applied every 20 sec.

***Measurement of Pulmonary Airway and Tissue Mechanics***

The pressure and flow data obtained during application of each Prime-2 perturbation have been described before (2-4). Pressure and flow data obtained during Prime-2 perturbations were used to calculate a complex input impedance of the respiratory system (Zrs). Zrs was then fit to a model consisting of a single airway serving a constant-phase viscoelastic tissue unit (8). The model equation is shown in equation 1.

Equation 1:(9) $Zrs(f) = R_N + i2\pi f Iaw + \frac{G - iH}{(2\pi f)^{\alpha}}$

Here $R_N$ represents Newtonian resistance which is composed mostly of the flow resistance of the conducting pulmonary airways. Iaw reflects the inertness of the gas in the central airways which can be ignored in mice below a breathing frequency of 20 Hz.(5) G reflects viscous dissipation of energy in the respiratory tissues (tissue resistance). H reflects elastic energy storage in the tissues (tissue stiffness), while f stands for frequency, $i = \sqrt{-1}$, and $\alpha$ couples G and H as shown in equation 2 (9).

Equation 2:[9] $\alpha = \frac{2}{\pi}\tan^{-1}\frac{H}{G}$

The above model has been shown to accurately describe respiratory impedance in mice at ventilatory rates below 20 Hz under both control conditions and under the conditions of mild bronchoconstriction(5, 6, 10) as was used in the present study. The advantage of this model is that it allows for distinction between central and peripheral events in the lung. Furthermore, by performing a series of Prime-2 perturbations for 3 min following a specific level of a

methacholine challenge, the time-dependent changes in $R_N$, G, and H can be more clearly ascertained to better determine the maximal response of each parameter.

At each level of PEEP, the values of $R_N$, G, and H were averaged for each mouse. The maximal response of each mouse at each level of methacholine challenge was determined from the multiple Prime-2 maneuvers following aerosolization. Each mouse's maximal response ($R_N$, G, and H) was plotted with respect to the dose of methacholine at a PEEP level of 3 $cmH_2O$.

***Statistical Analyses***

Statistical analysis was by a combination of planned comparisons, 1-way analysis of variance (ANOVA) and 2-way ANOVA with the appropriate post-hoc comparisons when tests for normality were passed. Planned comparisons were performed using the student's t-test to determine the effects of HSCT or the effects of experimental asthma. One-way ANOVA was performed on hematological, histological morphometric, plasma and BALF data. Two-way ANOVA was performed on airway mechanics data to determine the statistical differences in the response curves in Non-Sen and OVA-Sen mice. When tests for normality failed, a Kruskal-Wallis Test with post hoc Dunn's Multiple Comparisons Test was performed. Probability *(P)* values of less than 0.05, which were automatically adjusted for the number of comparisons via the software, were accepted as being statistically significant. Analysis was performed using Prism 5 (Graph Pad, Inc.). Data are expressed as means ± SEM unless otherwise specified.

## *Supplemental Discussion*

The key observations in this study are that SCD exaggerates airway inflammation and hyperresponsiveness both at baseline and in response to experimentally-induced asthma. To investigate mechanisms by which the combination of experimental asthma and SCD increases pulmonary inflammation and impairs airway mechanics, we sensitized chimeric HbA and SCD mice to low dose OVA using established protocols (1). After sensitization, we determined effects on histology, BALF cytokine production, plasma IgE levels, airway hyperresponsiveness, blood counts and plasma LDH, an index of hemolysis and/or tissue injury. HSCT was used to generate chi-HbA and chi-SCD mice on an inbred C57BL/6J background in order to reduce variation in the responses of the lung parenchyma that might have occurred in mice with a mixed Berkeley background. Fully engrafted chimeric SCD mice demonstrated hemolytic anemia and organ pathologies similar to that which are observed in human and murine models of severe SCD.

Pulmonary mechanic studies provide a sensitive means of identifying differences in lung physiology in health and disease. Measurements of $R_N$, G and H can be used to obtain insight into the potential mechanisms mediating airway and tissue disease. For example, a shift toward $T_H2$ cytokine production is known to increase large airway narrowing to increase $R_N$, a measure of central airway resistance. If $R_N$ were to increase at the same time as G increases, it would suggest that the lung has developed an increase in small airway stiffness rather than large airway narrowing. If H were to acutely increase, it could mean that the lung is experiencing an acute increase in de-recruitment (airway closure). However, if $R_N$ increases minimally at the same time as H increases, this suggests that not only are the lung parenchymal tissues more stiff, but also that the increase in stiffness is preventing the lung from contracting during expiration, which is a normal elastic property of a healthy lung. The consequence of such changes is that the energy that would have been stored in healthy elastic lung tissue during inspiration is no longer available for expiration (11, 12).

## *Literature Cited*


E1. Nandedkar SD, Feroah TR, Hutchins W, Weihrauch D, Konduri KS, Wang J, Strunk RC, DeBaun MR, Hillery CA, Pritchard KA. Histopathology of experimentally induced asthma in a murine model of sickle cell disease. *Blood* 2008;112(6):2529-2538.

E2. Hirai T, McKeown KA, Gomes RF, Bates JH. Effects of lung volume on lung and chest wall mechanics in rats. *J Appl Physiol* 1999;86(1):16-21.

E3. Gomes RF, Shen X, Ramchandani R, Tepper RS, Bates JH. Comparative respiratory system mechanics in rodents. *J Appl Physiol* 2000;89(3):908-916.

E4. Schuessler TF, Bates JH. A computer-controlled research ventilator for small animals: Design and evaluation. *IEEE Trans Biomed Eng* 1995;42(9):860-866.

E5. Tomioka S, Bates JH, Irvin CG. Airway and tissue mechanics in a murine model of asthma: Alveolar capsule vs. Forced oscillations. *J Appl Physiol* 2002;93(1):263-270.

E6. Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, Ackerman K, Haley K, Galle PR, Szabo SJ, Drazen JM, De Sanctis GT, Glimcher LH. Development of spontaneous airway changes consistent with human asthma in mice lacking t-bet. *Science* 2002;295(5553):336-338.

E7. Wang W, Xu H, Shi Y, Nandedkar S, Zhang H, Gao H, Feroah T, Weihrauch D, Schulte ML, Jones DW, Jarzembowski J, Sorci-Thomas M, Pritchard KA, Jr. Genetic deletion of apolipoprotein a-i increases airway hyperresponsiveness, inflammation, and collagen deposition in the lung. *J Lipid Res* 2010;51(9):2560-2570.

E8. Gomes RF, Bates JH. Geometric determinants of airway resistance in two isomorphic rodent species. *Respir Physiol Neurobiol* 2002;130(3):317-325.

E9. Hantos Z, Adamicza A, Govaerts E, Daroczy B. Mechanical impedances of lungs and chest wall in the cat. *J Appl Physiol* 1992;73(2):427-433.

E10. Ingenito EP, Mora R, Cullivan M, Marzan Y, Haley K, Mark L, Sonna LA. Decreased surfactant protein-b expression and surfactant dysfunction in a murine model of acute lung injury. *Am J Respir Cell Mol Biol* 2001;25(1):35-44.

E11. Bates JH, Irvin CG. Measuring lung function in mice: The phenotyping uncertainty principle. *J Appl Physiol* 2003;94(4):1297-1306.

E12. Irvin CG, Bates JH. Measuring the lung function in the mouse: The challenge of size. *Respir Res* 2003;4:4.

***Supplemental Figure E1***

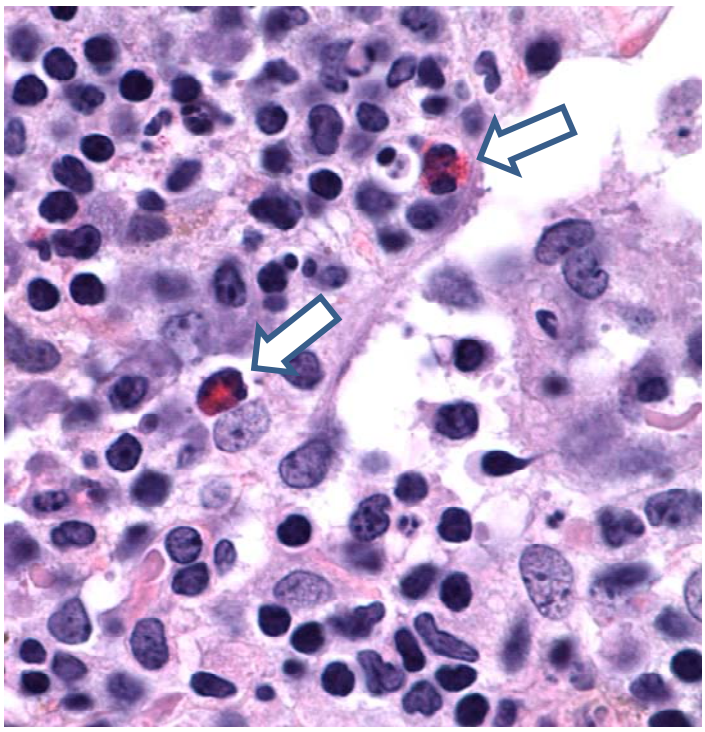

**Figure Legend E1**: **Eosinophil morphology and staining in Ovalbumin-Sensitized chimeric SCD mouse lung section.** A representative example of an inflamed section from the lungs of an ovalbumin-sensitized chimeric SCD mouse stained with H&E is shown at high power (100x) to demonstrate the morphology and staining of eosinophils (arrows) compared to other lung and inflammatory cells.