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House Dust Mite-Induced Allergic Lung Inflammation Is Not Exacerbated in Sick Cell Disease Mice

Chenyang Jiang Tyler D. Gavitt Steven M. Szczepanek

Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, USA

Keywords

Sickle cell · Asthma · Allergic airway disease · Eosinophils · Immunoglobulin E · House dust mite

Abstract

Aim: Asthma appears to be a common comorbid condition in children with sickle cell disease (SCD), and such individuals may be at a higher risk for increased morbidity and mortality. However, several reports have indicated that asthma severity is not particularly high in those with SCD, and airway hyperreactivity and wheeze may be independently associated with SCD. In SCD mice, exacerbated allergic airway disease (AAD) has been observed in response to the model antigen ovalbumin (OVA). We sought to determine if allergic lung inflammation is also exacerbated in SCD mice when they are exposed to the human allergen, house dust mite (HDM). **Methods and Results:** Eosinophil counts in bronchoalveolar lavage fluid were determined by cytocentrifugation and increased in both wild-type (WT) and SCD mice after acute exposure to a high dose (25 µg) of HDM, which then decreased in chronically exposed mice. WT mice exposed to a low dose of HDM (1 µg) followed the same pattern of eosinophil flux, but SCD mice did not induce much eosinophilia after acute

exposure to HDM. As was observed in previous studies, lung lesions similarly increased in severity in both WT and SCD mice after acute exposure to HDM, which remained elevated after chronic exposure. Furthermore, serum HDM-specific IgE titers similarly increased and selected serum cytokines were similar in both WT and SCD mice. **Conclusion:** These results contrast with previous reports of exacerbated AAD in SCD mice exposed to OVA and support the alternative hypothesis that asthmatic responses are normal in those with SCD.

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Introduction

Approximately 15–20% of African American children have a doctor's diagnosis of asthma, but children with SCD (mostly African Americans in the USA) have a staggering incidence of 30–70% of comorbid asthma in some reports [1–3]. Furthermore, it is common for children with SCD and comorbid asthma to present with acute

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chest syndrome (ACS) [4–6], a life-threatening and poorly understood lung disease that affects people with SCD, specifically. Indeed, upwards of two-thirds of children with SCD and asthma also have a history of ACS [4], and this trend appears to be upheld in children with the typically milder “SC” variant genotype of SCD [7].

In addition to the high prevalence of asthma in patients with SCD, there appears to be greater severity of disease for those with such comorbid conditions. A well-conducted study of both children and adults (albeit mostly children) with SCD and asthma indicated that this comorbidity results in >2 times the likelihood of early death than having SCD only [8]. This finding was exemplified by a case report of 2 adolescents with SCD that died due to complications from asthma exacerbations [3]. These patients presented with a sudden onset of symptoms and had marked bronchial eosinophilia, airway smooth-muscle hyperplasia, and thickened basement membranes. Similar severe histopathologic findings have been found in the mouse model of SCD, in which mice have allergic airway disease (AAD; essentially murine asthma) induced using the model allergen ovalbumin (OVA) [9, 10]. These mice also exhibit greater airway hyperreactivity (increased airway resistance after methacholine challenge) than WT mice with AAD, as measured by plethysmography [11]. Other consistent findings between humans and mice with SCD and asthma include high levels of allergen-induced immunoglobulin E (IgE) and Th2 cytokines in both the blood and respiratory mucosa [10–15].

Contrary to the above reports, not all in the field are convinced that asthma is more common or more severe in SCD patients than in other cohorts [16–18]. Several groups have shown that wheezing, independent/in the absence of other symptoms of asthma, is common in people with SCD [19–22]. Furthermore, Cohen et al. [23] showed that there was no difference in the mortality rate of asthmatic and non-asthmatic adults with SCD, but those with SCD and wheeze (independent of asthma diagnosis) were at an increased risk of death when compared to those that did not wheeze. Similarly, lung function appears to be decreased in those with SCD, even in the absence of other indicators of asthma [24, 25]. Abnormal lung function values appear to be correlated with increased pulmonary blood volume in those with SCD [26, 27], but do not appear to correlate with increased future morbidity (e.g., vaso-occlusive crisis or ACS) [28, 29].

Given the strong supporting evidence of a correlation of exacerbated OVA-induced AAD in SCD mice, we hypothesized that AAD is more severe in SCD mice than in wild-type (WT) mice when intranasally injected with the common human respiratory allergen, house dust mite (HDM).

Materials and Methods

Animals

Seven-week-old mice harboring knock-ins of the human α -globin transgene, along with either a normal human β -globin or sickle β -globin transgene (“Townes strain”; B6;129-*Hba*^{tm1(HBA)Tow}*Hbb*^{tm2(HBG1,HBB*)Tow}/*Hbb*^{tm3(HBG1,HBB)Tow}/J; Jackson Labs stock No. 013071) were used [30]. Mice homozygous for the normal human β -globin transgene are henceforth referred to as WT mice and those homozygous for the sickle β -globin transgene as SCD mice. Both male and female mice were used and data for both sexes were combined as no differences were observed in disease phenotype between sexes. Housing was maintained at 18–23 °C, with a daily, 12-h light/dark cycle. Chow and water were supplied ad libitum.

HDM-Induced AAD Mouse Model

Mice were lightly anesthetized with 2.5% vaporous isoflurane for 2 min and intranasally instilled with 25- μ L droplets containing 1 μ g (a low dose) or 25 μ g (a high dose) of lyophilized HDM extract (an equal mixture of *Dermatophagoides pteronyssinus* and *D. farinae*, Greer Laboratories, Lenoir, NC, USA), or sterile PBS. Following the model described by Bracken et al. [31], HDM was instilled for 5 consecutive days, followed by 2 days of rest per week, for 5 (acute period) or 11 (chronic period) weeks. Control mice received PBS for 11 weeks. Mice were sacrificed 72 h after the final antigen challenge.

Bronchoalveolar Lavage Fluid Leukocyte Differential Counting

After sacrifice, the lungs of mice were lavaged with 1 mL of sterile PBS to obtain the bronchoalveolar lavage fluid (BALF). BALF was centrifuged to aggregate white blood cells at 300 rpm and 4 °C for 5 min. The cell pellet was resuspended in 100 μ L of sterile PBS for leukocyte differential counting. Cytospin preparations of BALF cells were stained with the Rapid-Chrome Kwik-Diff kit (Thermo, USA). Leukocyte differential counting was performed on 500 cells per slide, using a CX 31 binocular microscope (Olympus, USA). Macrophages, neutrophils, eosinophils, and lymphocytes were identified in the count.

Lung Inflammation Scoring

Lungs were harvested from mice at sacrifice and fixed in 10% buffered formalin. Samples were then processed in a standard manner, and tissue sections were embedded and stained with hematoxylin and eosin (H&E) at the Connecticut Veterinary Medical Diagnostic Laboratory at the University of Connecticut, Storrs, CT, USA. Each slide was examined in its entirety via light microscope by 2 independent scientists blinded to the groups and mouse numbers, and images were taken from representative regions of inflammation. Pathologic scoring for inflammation was presented on a severity scale of 0–3, as previously described [31]. A score of “0” represented no inflammation; “1” was assigned for mild, “2” for moderate, and “3” for severe peribronchiolar/perivascular inflammation, respectively. If lesions appeared to fall in between 2 of these severity scores, a unit of 0.5 was assigned that produced a score of 0.5, 1.5, or 2.5.

Determination of HDM-Specific Serum IgE Levels

Before sacrifice, mice were lightly anesthetized and bled via the submandibular vein with a 5-mm lancet to collect serum. Serum was stored at –80 °C until it was used. 96-well polysorb flat bottom plates were coated with 10 μ g/mL of HDM extract in so-

dium bicarbonate buffer (pH 9.5) overnight at 4 °C. Following washing, plates were blocked with PBST–5% BSA and incubated for 4 h at room temperature. After another washing, serum samples were added following 2-fold serial dilutions in PBST, and then incubated for 1 h at room temperature. Plates were washed 5 times and a 1:1,000 dilution of a secondary goat anti-mouse IgE-HRP antibody (SouthernBiotech, Birmingham, AL, USA) was added and incubated at room temperature for 1 h. After another wash, TMB substrate solution (SouthernBiotech) was applied and incubated in the dark at room temperature for 10 min. TMB stop solution (SouthernBiotech) was used to stop the reactions and dual absorbance was measured at 450/570 nm with a microplate reader.

Serum Cytokine Analysis

Serum samples obtained from HDM-inoculated SCD and WT mice were analyzed using a Bio-Plex Pro mouse cytokine kit (#12002798, Bio-Rad Laboratories, Richmond, CA, USA). Samples were analyzed by the Clinical Research Center at UConn Health, Farmington, CT, USA as per manufacturer instructions. This kit assayed interleukin (IL)-4 (#171G5005M), IL-5 (#171G5006M), IL-10 (#171G5009M, IL-17A (#171G5013M), IL-1 β (#171G5002M), and IFN- γ (#171G5017M) with the following minimum detectable concentrations: IL-4 (0.028 pg/mL), IL-5 (0.056 pg/mL), IL-10 (1.9 pg/mL), IL-17A (0.056 pg/mL), IL-1 β (0.12 pg/mL), and IFN- γ (0.061 pg/mL). All samples were compared against the Bio-Plex Pro mouse cytokine standard (#171I5001M). All cytokines and standards were purchased from Bio-Rad.

Statistical Analyses

Multiple comparisons between groups were made via two-way analysis of variance (ANOVA) with Holm-Sidak multiple comparison tests, since 2 groups of variables (WT/SCD genotypes and PBS/HDM treatments) were included in the experiments. The HDM-specific IgE curves were compared via area under the curve (AUC) analysis. Cytokine data were analyzed by a two-tailed *t* test. For all data, *p* < 0.05 was used as the significance threshold. All statistical analyses were performed using GraphPad Prism v7 (La Jolla, CA, USA).

Results

BALF Leukocyte Differential Counts Were Similar in SCD and WT Mice Exposed to a High or Low Dose of HDM

Leukocytes (500 on each slide) were analyzed, including macrophages, neutrophils, lymphocytes, and eosinophils. In each group, proportions of macrophages and eosinophils were similar in SCD and WT mice. Comparing acute exposure of a high dose of HDM in WT and SCD mice to PBS-inoculated WT and SCD mice (11 weeks of exposure) (Fig. 1a), we found that the eosinophil count in the BALF increased (WT: 2.3–81.8% and SCD: 4.6–78.8%), thereby comprising the majority of

cells, and the macrophages concomitantly decreased (WT: 97.3–15.6%; SCD: 95.4–19.4%). The same pattern was observed in WT mice exposed to a low dose of HDM for an acute period of time compared to their PBS-inoculated counterparts, but no difference was observed in SCD mice (eosinophils in WT: 2.3–27.4% vs. SCD: 4.6–5.2%; macrophages in WT: 97.3–72.5% vs. SCD 95.4–94.4%) (Fig. 1b). The low percentage of eosinophils in the SCD mice after acute exposure to a low dose of HDM appears to have been driven by a lack of response to the antigen in 3/5 mice. The 2 SCD HDM responders had eosinophil counts similar to those observed in WT mice after the same exposure. After chronic exposure to a high dose of HDM extract (Fig. 1a), eosinophils in both genotypes decreased (WT: 81.8–36.7% and SCD: 78.8–41.6%) and macrophages increased (WT: 15.6–59.5% and SCD: 19.4–48.2%), when compared to the acute exposure groups. It should be noted that eosinophils and macrophages did not recover to the same level as in the PBS groups (as observed by Bracken et al. [31]), but differential counts trended in that direction. Low-dose chronic exposure to HDM (Fig. 1b) in WT mice also resulted in a reduction in BALF eosinophils when compared to acute low-dose exposure (27.4–13.0%), with a concomitant increase in macrophages (72.5–86.0%). SCD mice that were chronically exposed to a low dose of HDM actually experienced an increase in BALF eosinophils (5.2–11.9%), which again was likely the result of several SCD mice not responding to low-dose HDM exposure for the acute period. However, it should be noted that the eosinophil counts in SCD mice chronically exposed to a low dose of HDM were similar to those observed in the 2 responding SCD mice that were acutely exposed to a low dose of HDM.

BALF lymphocyte counts in WT and SCD mice acutely exposed to either a low or high dose of HDM were notably higher than in the PBS groups (Fig. 1a, b), but the results were not statistically significant. The same was true for WT mice chronically exposed to either a low or high dose of HDM. Interestingly, lymphocyte counts in SCD mice chronically exposed to either a low or high dose of HDM were significantly higher than in those acutely exposed to HDM or inoculated with PBS. Lymphocytes were also significantly higher in SCD mice after chronic exposure to HDM compared to their WT counterparts. Neutrophil counts showed a similar pattern to lymphocyte counts in each treatment group with a high dose of HDM (although to a lesser degree), including the significant difference of SCD and WT mice after chronic HDM exposure.

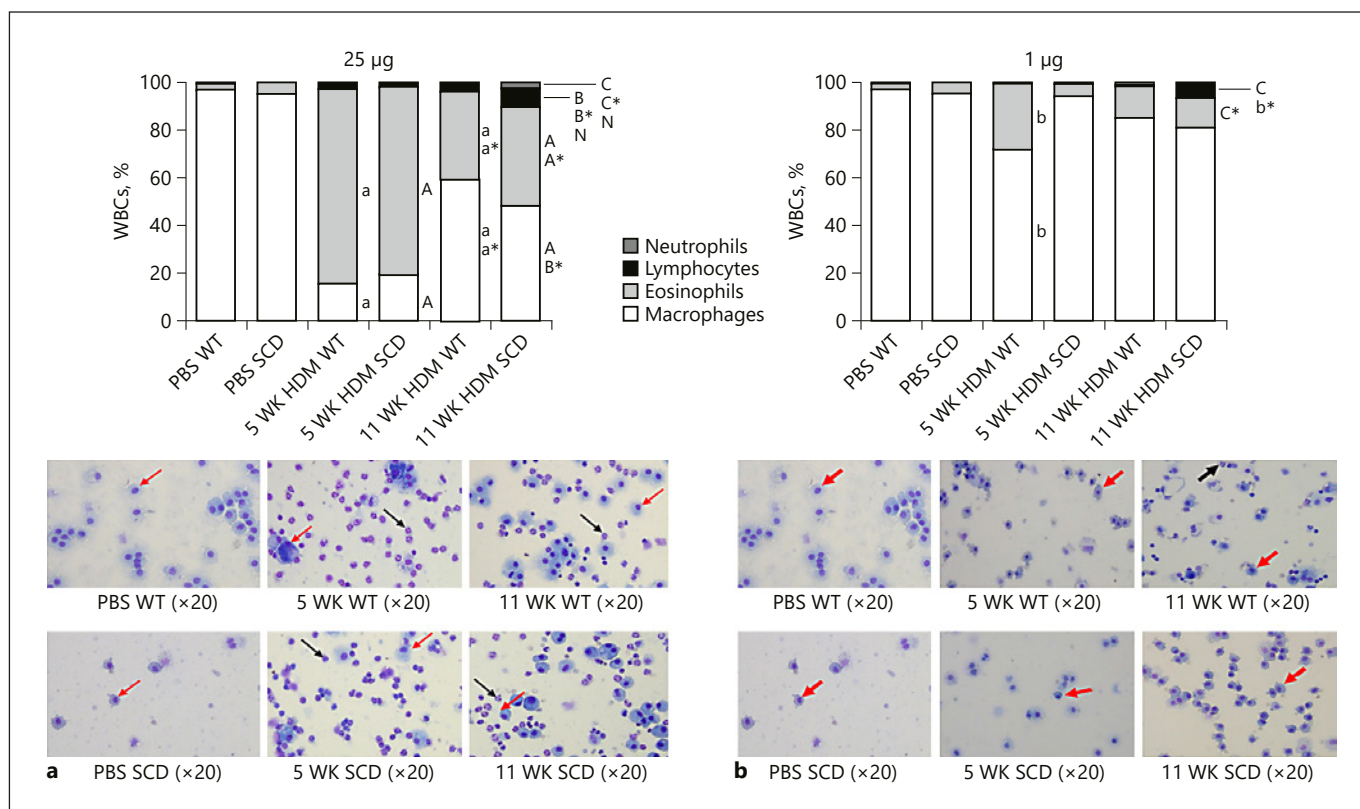


Fig. 1. Bronchoalveolar fluid leukocyte differential counting after HDM or PBS instillation. Differential counts of mice exposed to a high (a) or low (b) dose of HDM or PBS for an acute or chronic period (i.e., 5 or 11 weeks [5 WK or 11 WK]). Representative images of cytopins from each group are provided. Black arrows, eosinophils; red arrows, macrophages. Data were analyzed by two-

way ANOVA, with a Holm-Sidak post hoc test for multiple comparison. ^a $p < 0.0001$, ^b $p < 0.05$ versus PBS WT; ^{a*} $p < 0.0001$, ^{b*} $p < 0.05$ versus 5 WK HDM WT; ^A $p < 0.0001$, ^B $p < 0.001$, ^C $p < 0.05$ versus PBS SCD; ^{A*} $p < 0.0001$, ^{B*} $p < 0.001$, ^{C*} $p < 0.05$ versus 5 WK HDM SCD; ^N $p < 0.05$ versus 11 WK HDM WT. WBCs, white blood cells.

Lung Inflammation Scores Were Similar in WT and SCD Mice Exposed to a High or Low Dose of HDM

Histopathologic evaluation of lungs from WT and SCD mice exposed to HDM or PBS was performed. In both WT and SCD mice, acute exposure to a high dose of HDM triggered severe lesions consisting of peribroncholar/perivascular inflammation (Fig. 2a, black arrows). Additionally, vascular wall thickening was observed in some severely inflamed lungs (Fig. 2a, red arrows). Inflammation scores were lower in WT and SCD mice after acute exposure to a low dose of HDM (Fig. 2b), but there was no significant difference between the groups at this time point. As with the differential counts, 3/5 SCD mice exposed to a low dose of HDM showed no inflammation, as was the case in 2/7 WT mice. This lack of response was not observed in any mice exposed to a high dose of HDM. Lung lesions did not change in WT or SCD mice after

chronic exposure to a low or high dose of HDM, as observed by Bracken et al. [31]. Additionally, no vascular wall thickening was detected in either group chronically exposed to a low dose of HDM. Thus, it appears that the allergic phenotype of AAD (i.e., eosinophil influx) begins to resolve in both genotypes of mice after chronic exposure to HDM, but inflammation persists.

HDM-Specific Serum IgE Levels Were Similar in WT and SCD Mice after Acute or Chronic Exposure to HDM

Serum HDM-specific IgE levels were determined by ELISA, using 2-fold serial dilutions from 1:20 to 1:2,560. There was no significant difference in HDM-specific IgE titers between WT and SCD mice in the PBS, 5-week HDM, and 11-week HDM groups given a high or low dose of HDM (Fig. 3). HDM-specific IgE levels were sig-

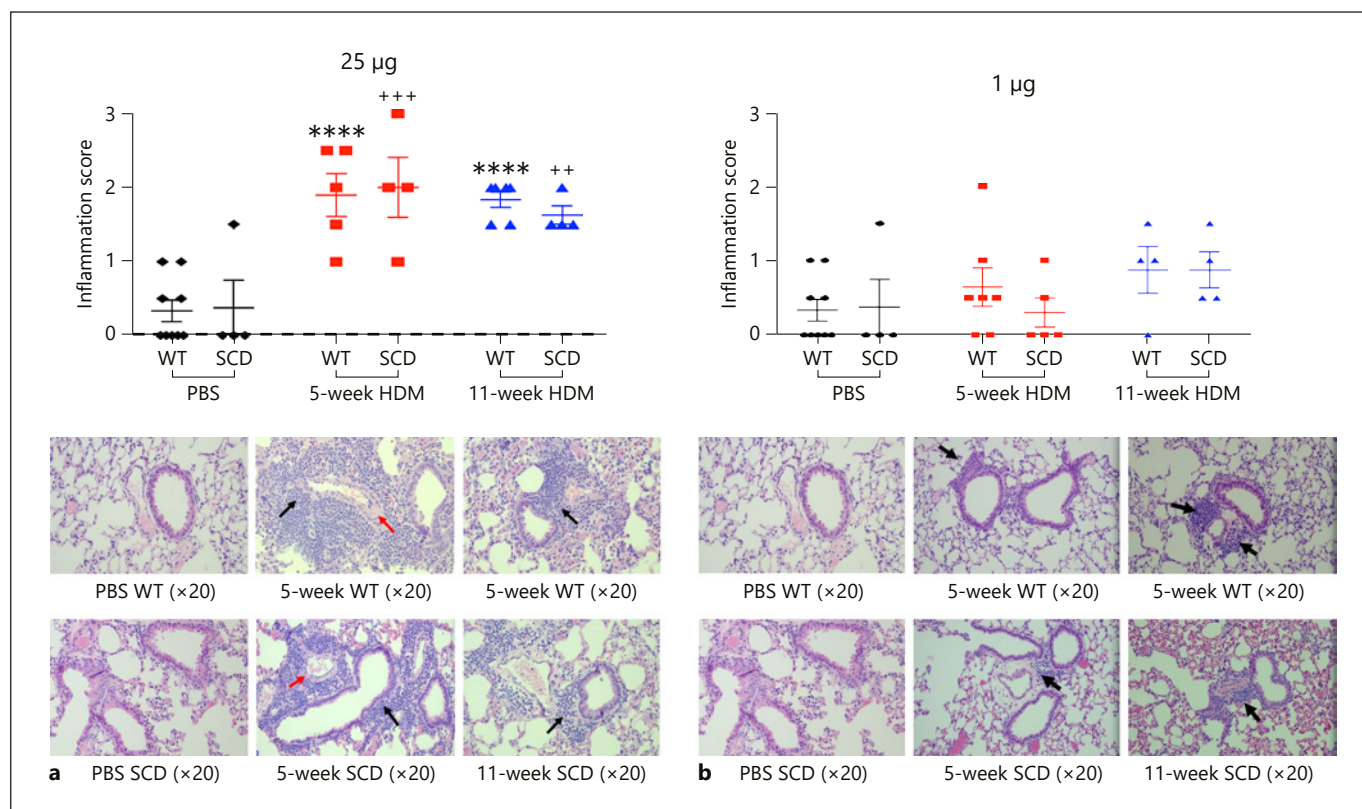


Fig. 2. Lung inflammation scores after HDM or PBS instillation. Severity of histologic lung lesions (0 [no inflammation] to 3 [severe inflammation]) was assessed in mice exposed to a high (a) or low (b) dose of HDM or PBS for an acute or chronic period. Representative histopathology images from each group are provided. Black

arrows, perivascular/peribronchiolar inflammation; red arrows, smooth-muscle hypertrophy. Data were analyzed by two-way ANOVA, with a Holm-Sidak post hoc test for multiple comparison. **** $p < 0.0001$ versus PBS WT; +++ $p < 0.001$ versus PBS SCD; ++ $p < 0.01$ versus PBS SCD.

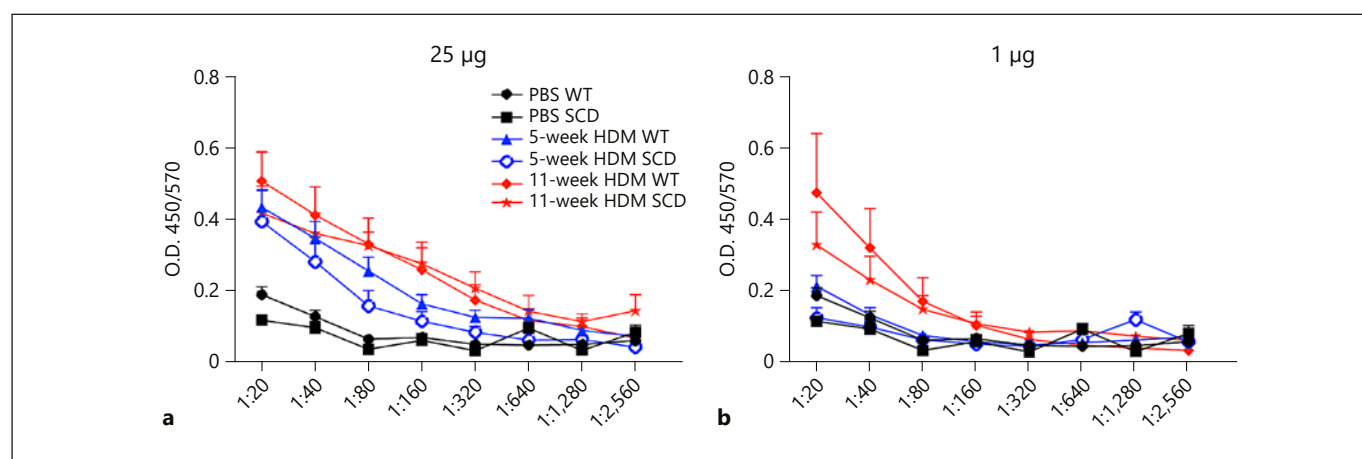


Fig. 3. Serum HDM-specific IgE levels following HDM or PBS instillation. HDM-specific IgE levels in serum collected from mice exposed to a high (a) or low (b) dose of HDM or PBS were determined by ELISA. Dual absorbance at 450 and 570 nm was calculated over the 2-fold serial dilutions, from 1: 20 to 1: 2,560. Data represent mean ± SEM values. Statistical comparisons were con-

ducted using AUC calculations. In the high-dose HDM experiment: $p < 0.05$ AUC of 5-week HDM WT versus PBS WT; $p < 0.001$ AUC of 11-week HDM WT versus PBS WT; $p < 0.01$ AUC of 11-week HDM SCD versus PBS SCD. No significant difference was observed between the curves in mice exposed to a low dose of HDM.

nificantly elevated ($p < 0.05$) in WT mice following acute ($p < 0.05$) or chronic ($p < 0.001$) exposure to high-dose HDM extract, when compared to the WT PBS group. HDM-specific IgE in SCD mice chronically exposed to a high dose of HDM was significantly elevated ($p < 0.01$) when compared to the SCD PBS group. While not significant, SCD mice exposed to a high dose of HDM for an acute period trended toward an elevated HDM-specific IgE titer, but this was also lower than the titer observed in WT mice acutely exposed to a high dose of HDM. The same pattern was observed in SCD mice that were exposed to a low dose of HDM for a chronic period. In both WT and SCD mice, the HDM-specific IgE levels did not change significantly following chronic HDM exposure, compared to after acute high-dose HDM exposure. Of note, HDM-specific IgE titers were essentially the same in both WT and SCD mice exposed to a low dose of HDM for an acute period, and titers were not noticeably different from those observed in PBS mice until 11 weeks of exposure to HDM.

Serum and BALF Cytokines Were Similar in WT and SCD Mice after Acute High-Dose HDM

The cytokines IL-4, IFN- γ , IL-17A, IL-10, IL-5, and IL-1 β were measured by Luminex assay in the serum and BALF of WT and SCD mice exposed to a high dose of HDM for the acute period. All cytokines in both groups were ≤ 1 pg/mL in BALF (data not shown). No significant difference was observed between the groups for any cytokine, although IFN- γ trended towards being significantly higher in the WT group ($p = 0.08$) (Fig. 4).

Discussion

Consistent with the findings of Bracken et al. [31] in WT mice, BALF leukocyte differentials shifted from predominantly macrophages to eosinophils, and then back towards macrophages after chronic exposure to a high dose of HDM in both WT and SCD mice. Also consistent with that study, lung inflammation scores remained essentially the same after both acute and chronic exposure to HDM in both genotypes of mice. While we did not achieve the near-baseline levels of differential counts observed by Bracken et al. [31] after chronic exposure to 25 μ g of HDM, our results trended in that direction. This difference between the studies is likely the result of lot-to-lot differences in the concentrations of components of the HDM extract that may be variable when protein concentration is held constant (e.g., LPS concentration). This

also likely explains why we had much higher levels of eosinophil counts after 5 weeks of HDM exposure than Bracken et al. observed, as the same dose of protein antigen was used. We were concerned that our high BALF eosinophil counts after acute exposure to HDM could be impeding the observation of differences between WT and SCD mice, as the system may have essentially been “maxed out.” Heterozygous mice were used to determine their responsiveness to 10, 5, and 2 μ g of HDM, which resulted in many mice in each group with eosinophil counts similar to mice that received 25 μ g (preventing the observation of statistical differences between the doses). We therefore used 1 μ g of HDM, as this same problem did not arise. Unfortunately, it appears that this dose of HDM was so low that a number of SCD mice did not react at all to the instillations. SCD mice that did respond to a low dose of HDM for the acute period appeared to exhibit similar phenotypes to WT mice. These outcomes reinforce our lack of a finding of a difference in HDM-induced AAD between WT and SCD mice, and, if anything, it appears that the SCD mice may be a bit less responsive to the antigen than their WT counterparts.

Our finding of a lack of a difference between WT and SCD mice in BALF eosinophil/macrophage counts, lung histopathology, serum cytokines, and serum HDM-specific IgE levels stands in stark contrast to previous reports of more severe AAD in OVA/alum-sensitized SCD mice than WT mice. Indeed, several reports [9, 10, 11, 15] by different groups have shown increased mortality, lung histopathology, and eosinophilia in SCD mice when compared to WT mice in the OVA model. Neither a high nor a low dose of HDM resulted in a difference between the genotypes in terms of these inflammatory outcomes. Furthermore, these groups also found that SCD mice have much higher antigen-specific or total IgE titers than WT mice that are run through the OVA model. This does not appear to be the case when SCD and WT mice are exposed to HDM. In fact, in this study, we found slightly lower HDM-specific titers in SCD mice than in WT mice. Additionally, we found no difference between WT and SCD mice acutely exposed to a high dose of HDM in terms of serum cytokine concentrations. BALF cytokines in SCD mice with OVA-induced AAD have higher IL-5 [11], which is responsible for eosinophil chemotaxis and function. The lack of difference of IL-5 secretion in our study is supported by data from the study of Eiymo Mwa Mpollo et al. [32], which also showed a lack of difference in IL-5 levels between (chimeric) SCD and WT mice when exposed to HDM. Indeed, it has been shown that elevated IgE titers induce increased IL-5 production by

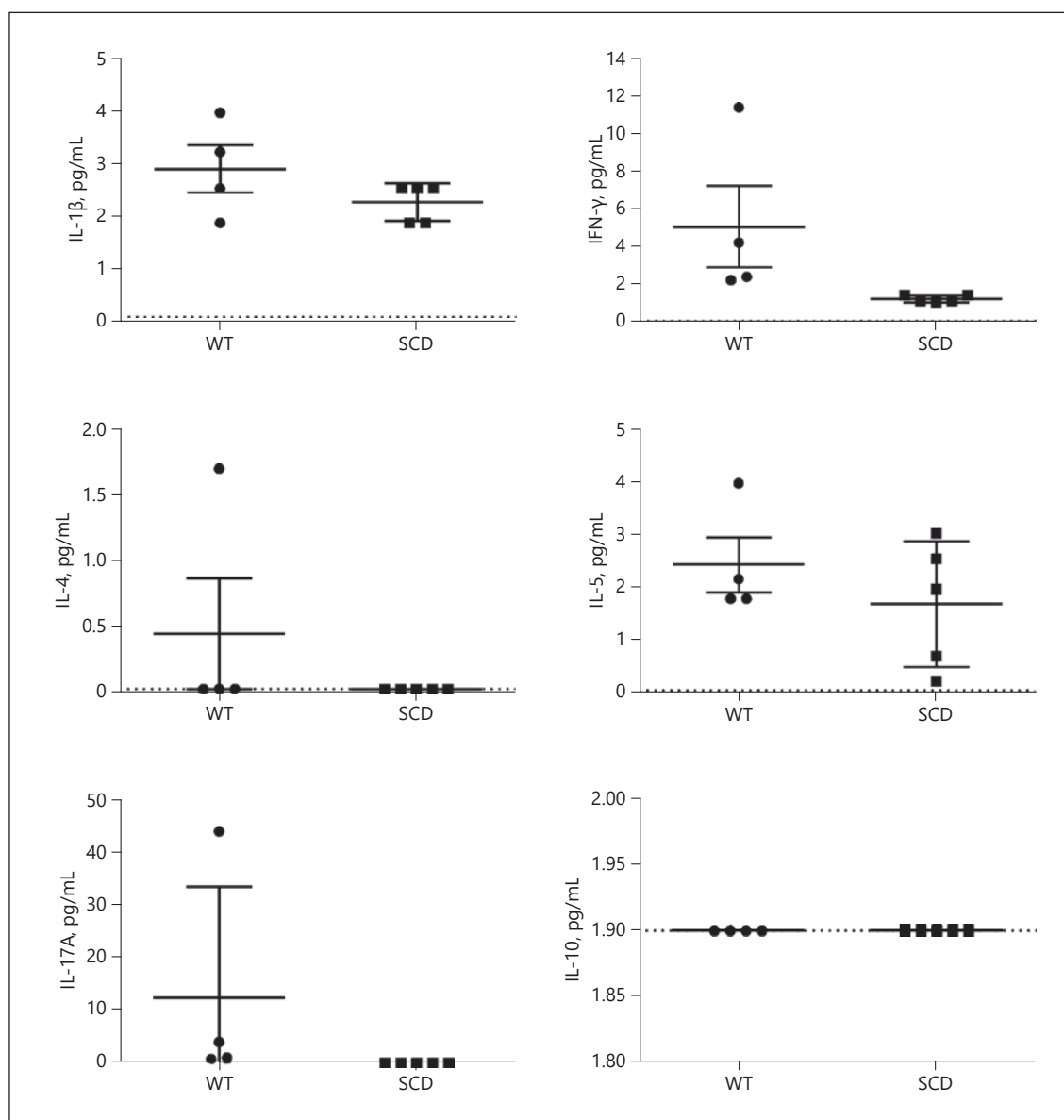


Fig. 4. Serum cytokine levels following high-dose HDM instillation. Concentrations of IL-1 β , IFN- γ , IL-4, IL-5, IL-10, and IL-17A were determined by Luminex assay. Sample concentrations were interpolated from a standard curve and multiplied by the applied

dilution factor, and limits of detection were determined (dotted line). A *t* test was used to compare cytokine concentrations between the groups, but there were no significant differences.

helper T cells [33], and increased IL-5 levels can drive airway eosinophilia [34], possibly giving insight into a key difference between the models that may explain why OVA models induce more robust allergic inflammation in SCD mice, but HDM does not.

The seemingly different conclusions that are reached when SCD mice are compared to WT mice in the OVA and HDM models of AAD mirrors those that are found when

different human studies are compared. Indeed, the mouse SCD/OVA model studies support the idea that comorbid asthma and SCD results in more severe disease than asthma alone, as was found in people with SCD [3, 8]. However, the SCD/HDM model supports contrasting studies in which asthma was not found to be more severe in people with SCD [23, 28, 29]. Additionally, De et al. [35] recently reviewed the literature in terms of the differences and sim-

ilarities between human and murine studies of comorbid asthma in SCD. Based on their interpretation of a human study by An et al. [13] (which showed that there was an increased prevalence of high total IgE titers than diagnosis of asthma in children with SCD), De et al. [35] postulate that the very high titers of IgE that are induced in SCD mice after OVA sensitization are the result of enhanced inflammatory reactions to stimuli, and not necessarily other drivers of AAD. Our lack of high IgE titers and the normal levels of lung inflammation in SCD mice after exposure to HDM support this hypothesis. Additionally, Eiymo Mwa Mpollo et al. [32] found that chimeric SCD mice have greater airway resistance after exposure to HDM than WT mice do. While this may at first seem to contrast with our findings, if taken together, it fits into the clinical hypothesis that children with SCD have impaired lung function but not more severe asthma phenotypes [24, 25]. Indeed, Eiymo Mwa Mpollo et al. [32] observed that chimeric SCD mice have elevated airway resistance at baseline when compared to WT mice, thus supporting this hypothesis. This group was able to link increased levels of placenta growth factor in SCD mice with increased airway resistance. This may be a key molecule that allows SCD mice to develop more severe airway hyperreactivity, in the absence of more severe allergic inflammation (as we have shown), in re-

sponse to HDM. It thus appears that the pathophysiology of SCD is very complicated and is context-dependent. Further studies are needed to help resolve some of the conflicts that are observed in both clinical and murine studies to better tease apart the molecular underpinnings of these differences.

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Statement of Ethics

The protocol for animal use was approved by the Institutional Animal Care and Use Committee at the University of Connecticut (protocol No. A15-039).

Disclosure Statement

The authors declare no financial conflicts of interest with this work.

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