

Secretoglobin Superfamily Protein SCGB3A2 Alleviates House Dust Mite-Induced Allergic Airway Inflammation in Mice

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Key Words

SCGB3A2 · Lung-enriched cytokine-like protein · Anti-inflammation · Antifibrosis · Growth factor · House dust mite · Allergic airway inflammation · Mouse model

Abstract

Background: Secretoglobin (SCGB) 3A2, a novel, lung-enriched, cytokine-like, secreted protein of small molecular weight, was demonstrated to exhibit various biological functions including anti-inflammatory, antifibrotic and growth-factor activities. Anti-inflammatory activity was uncovered using the ovalbumin-induced allergic airway inflammation model. However, further validation of this activity using knockout mice in a different allergic inflammation model is necessary in order to establish the antiallergic inflammatory role for this protein. **Methods:** *Scgb3a2*-null (*Scgb3a2*^{-/-}) mice were subjected to nasal inhalation of *Dermatophagoides pteronyssinus* extract for 5 days/week for 5 consecutive weeks; control mice received nasal inhalation of saline as a comparator. Airway inflammation was assessed by histological analysis, the number of inflammatory cells and various Th2-type cytokine levels in the lungs and bronchoalveolar lavage fluids by qRT-PCR and ELISA, respectively. **Results:** Exacerbated inflammation was found in the airway of

Scgb3a2^{-/-} mice subjected to house dust mite (HDM)-induced allergic airway inflammation compared with saline-treated control groups. All the inflammation end points were increased in the *Scgb3a2*^{-/-} mice. The *Ccr4* and *Ccl17* mRNA levels were higher in HDM-treated lungs of *Scgb3a2*^{-/-} mice than wild-type mice or saline-treated *Scgb3a2*^{-/-} mice, whereas no changes were observed for *Ccr3* and *Ccl11* mRNA levels. **Conclusions:** These results demonstrate that SCGB3A2 has an anti-inflammatory activity in the HDM-induced allergic airway inflammation model, in which SCGB3A2 may modulate the CCR4-CCL17 pathway. SCGB3A2 may provide a useful tool to treat allergic airway inflammation, and further studies on the levels and function of SCGB3A2 in asthmatic patients are warranted.

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Introduction

Secretoglobin (SCGB) 3A2 is a member of the SCGB gene superfamily of cytokine-like secretory proteins of small molecular weight [1]. SCGB proteins are found only in mammalian lineages, and at high concentrations in se-

M.Y. and L.X. contributed equally to this study.

cretions such as those of the lung, lacrimal gland, salivary gland, prostate and uterus [2]. However, their biological functions are not well understood. SCGB proteins are thought to play a role in the modulation of inflammation, tissue repair and tumorigenesis [2, 3]. SCGB3A2 is predominantly expressed in the epithelial cells of the trachea, bronchus and lung bronchioles [4]. SCGB3A2 plays a role in embryonic lung development and lung inflammation and fibrosis [5–11]. It has also been suggested that it is a candidate marker for lung adenocarcinoma [12, 13]. However, the functional mechanisms for these SCGB3A2 activities are largely unknown.

The human SCGB3A2 gene is located on chromosome 5q31–q34, which harbors a number of genes associated with bronchial asthma, for example, those coding for interleukin (IL)-4, IL-13 and β_2 adrenoreceptor [14]. Moreover, a functional promoter, polymorphism 112G/A, is associated with bronchial asthma in Japanese [15] and Asian [16] populations. In addition, the plasma SCGB3A2 level is associated with the severity of bronchial asthma [17], while the concentration of SCGB3A2 in the sputum of asthma patients is elevated [18]. These findings suggest a role for SCGB3A2 in lung inflammation. This has been demonstrated experimentally using the ovalbumin (OVA)-induced allergic airway inflammation mouse model, in conjunction with recombinant adenovirus-expressing SCGB3A2, where SCGB3A2 suppressed airway allergic inflammation [8]. Furthermore, *Scgb3a2*-null (*Scgb3a2*^{-/-}) mice, when subjected to the OVA-induced airway inflammation model, exhibited increased airway inflammation compared with their respective wild-type littermates [10].

This study, using *Scgb3a2*^{-/-} mice, was carried out to determine whether SCGB3A2 exhibits anti-inflammatory activity in an airway inflammation model of the house dust mite (HDM), one of the most prevalent allergens causing asthma and rhinitis [19, 20]. The results confirm that SCGB3A2 serves as an anti-inflammatory agent in allergic airway inflammation in mice.

Materials and Methods

Mouse Model of HDM-Induced Airway Inflammation

Scgb3a2^{-/-} mice, as previously described [10], were backcrossed 10 times onto a C57BL/6NCr background. Female *Scgb3a2*^{-/-} mice (8–9 weeks old) and their respective wild-type littermates (15–25 mice/group, up to 5 mice per cage) were placed in the same cage and maintained under standard specific-pathogen-free conditions. Airway inflammation was induced by nasal inhalation of *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, N.C., USA, 25 μ g of protein in 10 μ l of saline) under iso-

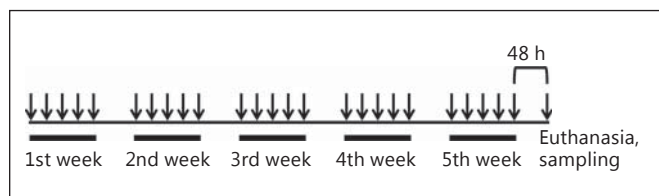


Fig. 1. Experimental protocol. Female *Scgb3a2*^{-/-} mice (8–9 weeks old) and their respective wild-type littermates received nasal inhalation of *D. pteronyssinus* extract (25 μ g of protein in 10 μ l of saline) for 5 days/week for 5 consecutive weeks. Control C57BL/6NCr mice received nasal inhalation of 10 μ l of saline as a comparator. Mice were subjected to necropsy 48 h after the last HDM administration.

flurane anesthesia for 5 days/week for 5 consecutive weeks (fig. 1) [21]. Control C57BL/6NCr mice received nasal inhalation of 10 μ l of saline as a comparator. Mice were subjected to necropsy for end-point analysis 48 h after the last HDM administration. Bronchoalveolar lavage fluid (BALF) was obtained by intratracheal instillation of 1 ml of PBS in the lung. The lung tissues were pooled in RNAlater (Life Technologies, Rockville, Md., USA) and stored at -80°C until RNA purification. Cytospin preparations of BALF were centrifuged onto glass slides through Shandon Cytofunnels (Thermo Fisher Scientific, Rockford, Ill., USA) at 700 rpm for 10 min. Cell differentiation was demonstrated on >200 cells stained with Giemsa (Sigma-Aldrich, St. Louis, Mo., USA) and analyzed by microscopy. All mouse experiments were performed following the guidelines for animal use issued by the National Institutes of Health and approved by the National Cancer Institute Animal Care and Use Committee.

Antibodies

Anti-SCGB3A2 antibody used for immunoblotting and immunohistochemistry was produced by immunizing *Escherichia coli*-produced hexahistidine-tagged protein containing full-length mouse SCGB3A2, as previously described [4]. The anti-GAPDH antibody 6C5 (EMD Millipore, Billerica, Mass., USA) was used to detect GAPDH as a loading control on immunoblots.

Histological Analysis

Lungs were inflated with 10% buffered formalin under a pressure of 25 cm H₂O, fixed in 10% buffered formalin, embedded in paraffin and sectioned at 5 μ m. Lung sections were deparaffinized and stained with hematoxylin and eosin (H&E). Lung lesions and perivascular and peribronchiolar cuffing with inflammatory cells were graded using H&E sections in blind fashion as follows: 0 for no lesions, 1 for 0–25%, 2 for 26–50%, 3 for 51–75% and 4 for 76–100% of the lung section involved. The histological grades depended on the extent of the lesion in the lung and the severity of the lesion itself.

Quantitative RT-PCR Analysis

Total RNA from the lung of each mouse was individually isolated using Total RNA purification kit (Norgen Biotek Corp., Ontario, Canada) according to the manufacturer's protocol with

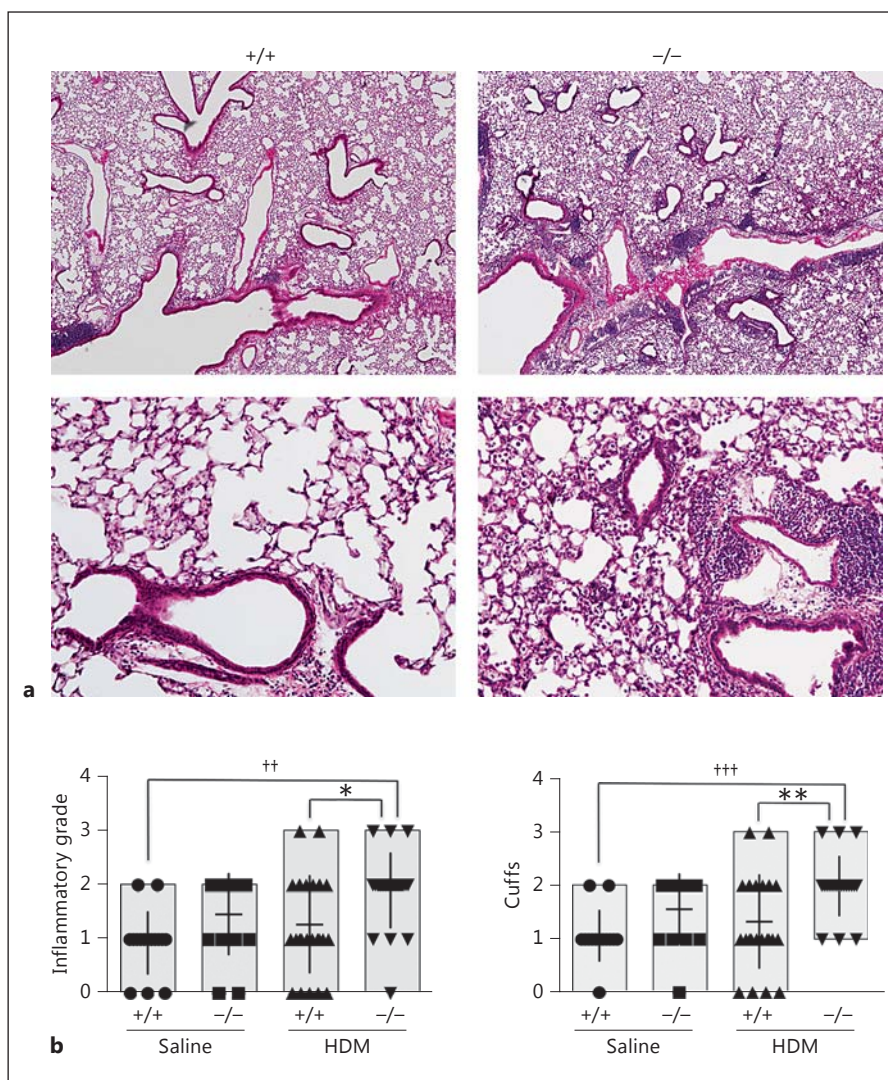


Fig. 2. Histological analysis of HDM-induced allergic airway inflammation. **a** Representative lung H&E sections of wild-type (+/+) and *Scgb3a2*^{-/-} mice (-/-) in HDM-induced airway inflammation. ×40 (upper panel), ×200 (lower panel). **b** Inflammatory grade (left) and perivascular and peribronchiolar cuffs (right) of wild-type and *Scgb3a2*^{-/-} mouse lungs of control (Saline) and challenged (HDM) groups. * $p < 0.05$, ** $p < 0.01$ versus wild-type mice and †† $p < 0.01$, ††† $p < 0.001$ versus saline-treated mice, by two-way ANOVA.

small modifications: 10 mM dithiothreitol was added to a lysis buffer, and 0.5 μ l of RNaseOUT recombinant ribonuclease inhibitor (Life Technologies) was added to each elution tube before eluting RNA. Purified RNA was kept at -80°C until use. Oligo(dT)-primed cDNAs were reverse-transcribed from 1.0 μ g total RNAs by using SuperScript III reverse transcriptase (Life Technologies) according to the supplier's protocol. Real-time RT-PCR analysis was performed in triplicate using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif., USA) with PerfeCta SYBR Green FastMix, ROX (Quanta Biosciences, Gaithersburg, Md., USA). Primer sequences used for qRT-PCR are shown in online supplementary table S1 (www.karger.com/doi/10.1159/000450788). The $\Delta\Delta\text{CT}$ method was used to calculate relative expression levels using that of *Ppia* [peptidylprolyl isomerase A (cyclophilin A)] mRNA as a control.

ELISA Assays

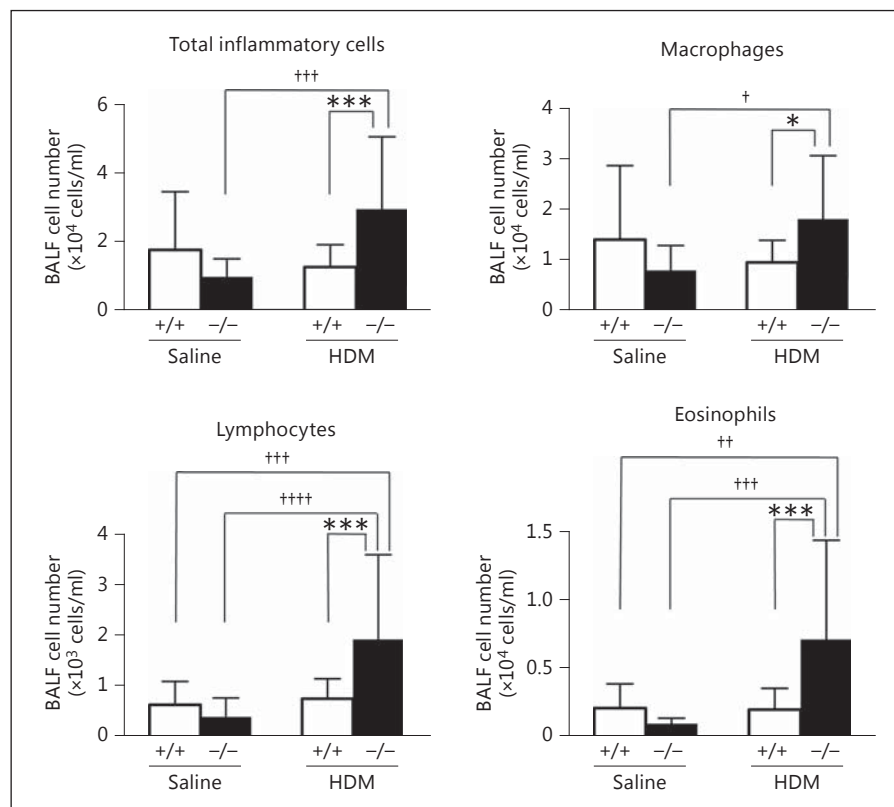
Mouse IL-4, IL-5, IL-9, IL-13 and CCL17 protein levels were quantified by using ELISA kits from R&D Systems (Minneapolis,

Minn., USA), and the CCR4 protein levels were measured with an ELISA kit from LifeSpan BioSciences (Seattle, Wash., USA) according to the manufacturer's protocols. The assay ranges of each ELISA kit were 7.8–500 (IL-4), 15.6–1,000 (IL-5), 46.9–3,000 (IL-9), 7.8–500 (IL-13), 31.2–2,000 (CCL17) and 78–5,000 (CCR4) pg/ml.

HDM Restimulation of Mediastinal Lymph Node Cultures and Measurement of Th2 Cytokines

Single-cell suspensions of mediastinal lymph nodes (MLNs) from either saline- or HDM-challenged mice were prepared by filtering through a 100- μ m cell strainer, as previously described [22]. After red blood cells were lysed with red blood cell lysing buffer (Sigma-Aldrich), MLN cells were resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 250 ng/ml amphotericin B and 3.5 μ l/l 2-mercaptoethanol. Cells were seeded at 4×10^6 cells per well in 96-well plates, and restimulated with HDM (100 μ g/ml) or saline for 96 h before collection. Mouse IL-4,

Fig. 3. Number of total inflammatory cells, lymphocytes, macrophages and eosinophils in the BALF from wild-type (+/+) and *Scgb3a2*^{-/-} (-/-) mice are shown in control (Saline) and challenged (HDM) groups. Significant difference from: * *p* < 0.05; *** *p* < 0.001 versus wild-type mice and † *p* < 0.05, †† *p* < 0.01, ††† *p* < 0.001, †††† *p* < 0.0001 versus saline-treated mice, by two-way ANOVA.



IL-5 and IL-13 production were measured by ELISA kits (R&D Systems) as described above.

Data Analysis

Data are shown as means ± SD. Levels of significance for comparison between samples were determined by two-way ANOVA. *p* values of <0.05 were considered statistically significant. Graph-Pad Prism v7 was used for analysis.

Results

Scgb3a2 Deficiency Exacerbates HDM-Induced Airway Inflammation

To understand the role of SCGB3A2 in HDM-induced allergic airway inflammation, HDM or saline as control was nasally administered to *Scgb3a2*^{-/-} and wild-type mice 5 days/week for 5 consecutive weeks. Histological analysis, using H&E-stained sections of mouse lungs from HDM-challenged *Scgb3a2*^{-/-} and wild-type mice, demonstrated that the inflammation in *Scgb3a2*^{-/-} mouse lungs occurred in much wider areas and was more severe than those of wild-type mice (fig. 2a). In fact, the inflammatory grade and perivascular and peribronchiolar cuff-

ing of these lung lesions were higher, with statistical differences, in HDM-challenged *Scgb3a2*^{-/-} mice than in their wild-type littermates (fig. 2b).

Increased BALF Cells in *Scgb3a2* Knockout Mice

The total number of inflammatory cells and numbers of lymphocytes, macrophages and eosinophils in the BALF were significantly increased in HDM-challenged *Scgb3a2*^{-/-} mice compared with wild-type mice (fig. 3).

Increased Cytokines in *Scgb3a2* Knockout Mice

The expression levels of mRNAs encoding various cytokines were determined from various groups of mouse lungs. Messenger RNAs for IL-4, IL-5, IL-9, IL-13, CCR4 and CCL17 were increased in HDM-challenged *Scgb3a2*^{-/-} mouse lungs compared with saline-challenged *Scgb3a2*^{-/-} mouse lungs (fig. 4). Furthermore, most of these mRNAs were significantly increased in HDM-challenged *Scgb3a2*^{-/-} mouse lungs compared with HDM-challenged wild-type mouse lungs. There was no difference in mRNA levels of these cytokines in HDM-challenged and saline-challenged wild-type mouse lungs. In contrast, the expression levels of mRNAs for Kit ligand

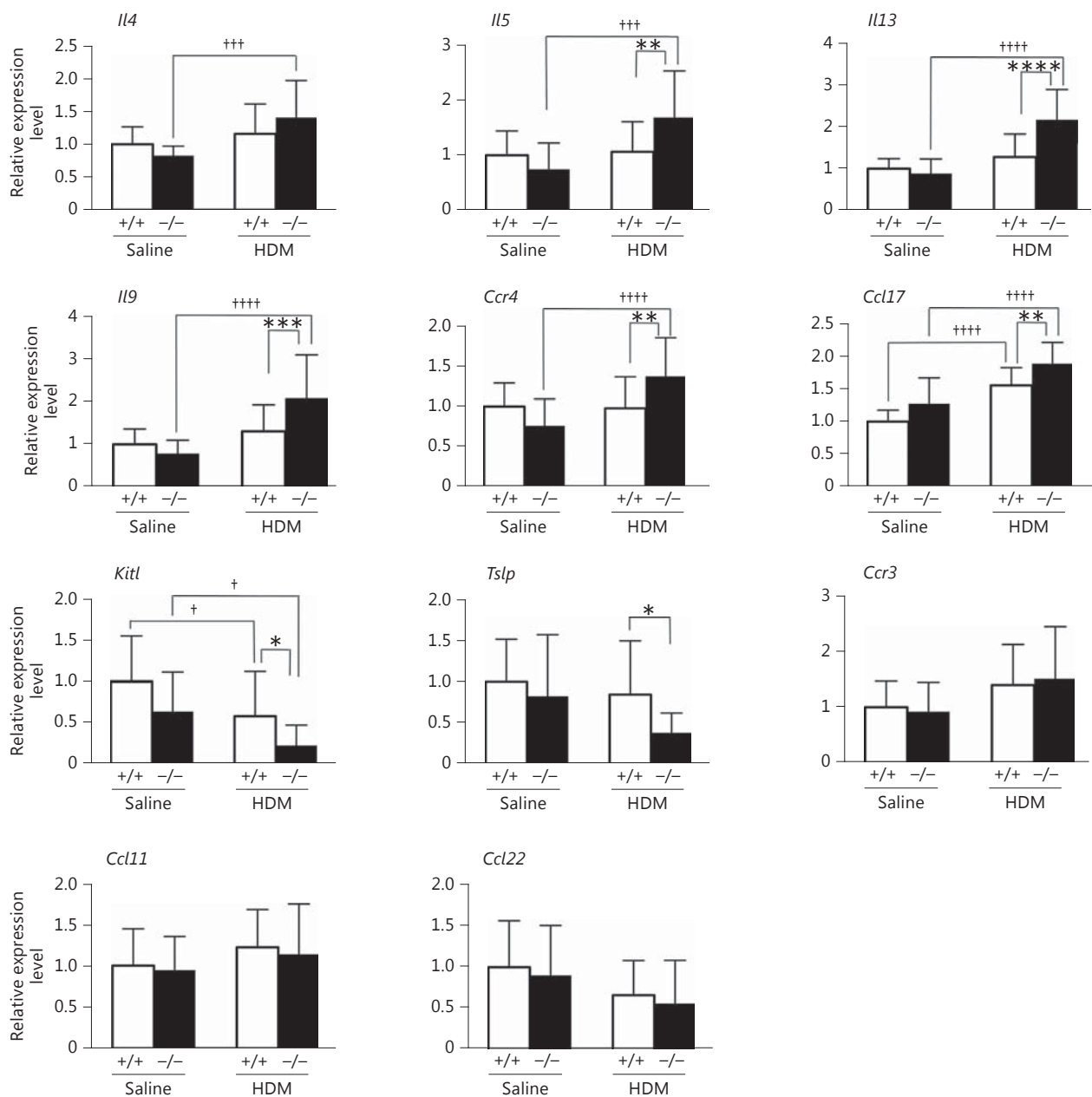


Fig. 4. Expression levels of Th2 cell cytokine mRNAs. The expression levels in the lungs of wild-type (+/+) and *Scgb3a2*^{-/-} (-/-) mice are shown in control (Saline) and challenged (HDM) groups. Relative expression levels of each gene in the lung were measured by qRT-PCR and normalized for that of *Ppia*. The value of saline-

challenged wild-type lungs is set as 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus wild-type mice and † $p < 0.05$, ††† $p < 0.001$, †††† $p < 0.0001$ versus saline-treated mice, by two-way ANOVA.

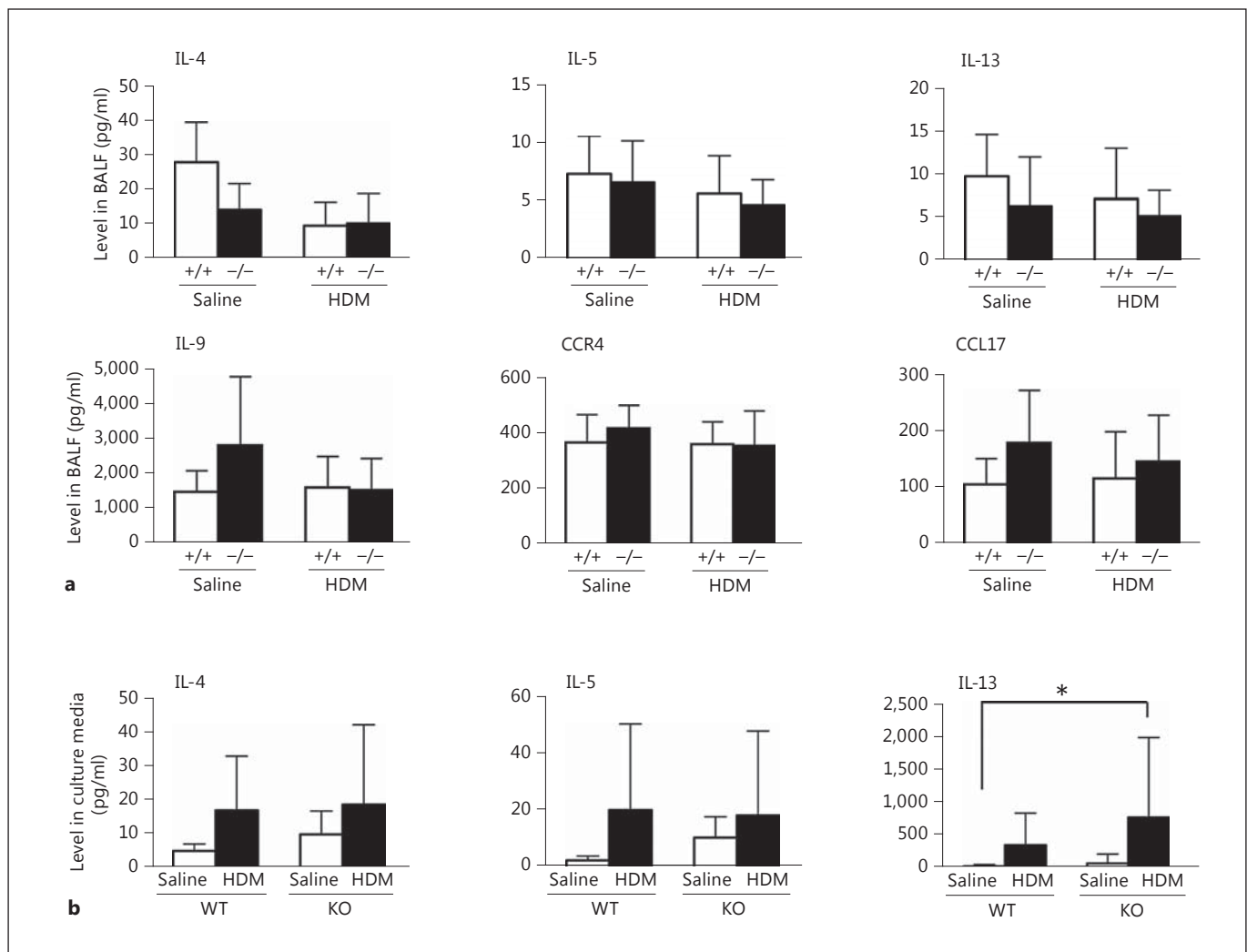


Fig. 5. Expression levels of Th2 cell cytokine proteins. **a** Th2 cell cytokine concentrations in the BALF from wild-type (+/+) and *Scgb3a2*^{-/-} (-/-) mice, which were challenged with control (Saline) and HDM. **b** Th2 cell cytokine productions with restimulation of saline or HDM in the culture media of primary mediastinal lymphocytes from HDM-challenged wild-type (WT) and *Scgb3a2*^{-/-} (KO) mice. Expression levels of each protein in BALF and culture media were measured by ELISA. * $p < 0.05$ by two-way ANOVA.

(*Kitl*, also called stem cell factor, *Scf*) were decreased in HDM-challenged *Scgb3a2*^{-/-} and wild-type mouse lungs compared with the saline-challenged groups of mice, and in HDM-challenged *Scgb3a2*^{-/-} mouse lungs compared with HDM-challenged wild-type mouse lungs. Thymic stromal lymphopoietin (*Tslp*) was also decreased in HDM-challenged *Scgb3a2*^{-/-} mouse lungs compared with HDM-challenged wild-type mouse lungs. No differences were observed in the expression levels of *Ccr3*, *Ccl11* and *Ccl22* mRNAs. ELISA analysis was used to measure levels of IL-4, IL-5, IL-13, IL-9, CCR4 and CCL17 proteins in

BALF from saline- or HDM-challenged *Scgb3a2*^{-/-} and wild-type mouse lungs (fig. 5a). There were no statistically significant differences between the 2 lines of mice within the same treatment or between saline- and HDM-challenged groups within the same genotypes. When IL-4, IL-5 and IL-13 levels were examined using concentrates of ex vivo cultures of MLN cells, significant differences were observed for IL-13 between HDM-treated knockout and saline-treated wild-type mice (fig. 5b).

Discussion

The SCGB superfamily proteins are believed to play a role in inflammation [2, 3]. This study shows that HDM-induced allergic airway inflammation is increased in *Scgb3a2*^{-/-} mouse lungs compared with those of wild-type mice. This is in good agreement with previous results demonstrating that SCGB3A2 has an anti-inflammatory activity using the OVA-induced allergic airway inflammation mouse model in conjunction with intranasal administration of recombinant adenovirus expressing SCGB3A2 [8]. Further, when *Scgb3a2*^{-/-} mice were subjected to the OVA-induced allergic airway inflammation model, they exhibited exacerbated lung inflammation [10]. HDM is one of the most prevalent allergens causing asthma and rhinitis [19, 20] and our results, using the HDM-induced allergic inflammation model, demonstrate a higher relevance of SCGB3A2 in asthma.

The exacerbated lymphocytic and eosinophilic airway inflammation was related to increases in Th2 responses, as revealed by *Il5*, *Il13* and *Il9* expression in the lung. Similarly, the expression of *Ccr4* and *Ccl17* was increased in the lung whereas that of *Ccr3* and *Ccl11* (*eotaxin-1*) was not changed. CCL17, mainly secreted from dendritic cells, recruits T cells via CCR4, but CCL11 recruits eosinophils via CCR3 [23]. CCR3 modulates the early stages of allergen-induced Th2-mediated airway inflammation, while CCR4 is primarily responsible for the long-term recruitment of Th2 cells to the lung in response to chronic allergen stimulation including HDM [24, 25]. Since increased *Ccr4* and *Ccl17* were obtained whereas no increase was observed for *Ccr3* and *Ccl11* in the lungs of HDM-treated *Scgb3a2*^{-/-} mice compared with the wild-type controls, SCGB3A2 may contribute to the modulation of the CCR4-CCL17 pathway.

On the other hand, the expression of *Kitl* (*Scf*) and *Tslp* were decreased in HDM-treated-*Scgb3a2*^{-/-} mice compared with wild-type mice. While the reason for this decrease is not known, it suggests that SCGB3A2 may play a role in modulating the expression of *Kitl* and *Tslp* in the HDM-induced mouse airway inflammation model. In asthmatic patients, the expression of KITL and TSLP in the airways is increased [26–29], while KITL and TSLP are known to promote Th2 cytokine-mediated airway inflammation in asthma [26, 28]. These facts may suggest a reason why HDM-induced inflammation was not severe but mild in this study using *Scgb3a2*^{-/-} mice.

While we demonstrated that *Scgb3a2*^{-/-} mice are more susceptible to HDM-induced allergic airway inflammation, the allergic response was, in general, low. Levels of

IL-9, CCR4 and CCL17 protein in the ex vivo cultures of MLN cells could not be determined, either due to concentrations that were not high enough above the detection limit or the insufficient number of samples available. It is known that C57BL/6 mice are not very sensitive to allergens including HDM [30]. Indeed, a similar low response of *Scgb3a2*^{-/-} mice on the C57BL/6NCr background was observed in the OVA-induced allergic airway inflammation model [10].

In relation to this, even though mRNA levels were found to be significantly different, the levels of IL-4, IL-5, IL-13, IL-9, CCR4 and CCL17 proteins in BALF as well as the ex vivo culture concentrates of MLN cells (for the first 3 cytokines), did not show any significant differences between HDM-treated *Scgb3a2*^{-/-} and wild-type mouse lungs, and/or between saline- and HDM-treated *Scgb3a2*^{-/-} or wild-type mice. We believe that for some cytokines, notably, IL-4, IL-5 and IL-13, the discrepancy found between mRNA and protein levels was partly due to differences in the sensitivity of the detection methods for mRNAs and proteins of generally low expression levels, typical of these cytokines. While changes in expression levels of low-abundance mRNAs could be relatively easily detected by qRT-PCR, the protein levels were close to the ELISA detection limit and so the differences were not easily detected. Furthermore, we used 5-week-old HDM-challenged female mice on the C57BL/6NCr background. Another study reported an induction of immunological tolerance in female C57BL/6-background mice challenged with HDM for 5 consecutive weeks using a protocol similar to ours [31]. In that study, IL-4 and IL-5 protein concentrations in the BALF had not changed in the HDM-challenged mice and PBS-challenged control mice despite the increase in inflammatory cell numbers and in histological inflammatory scores in the HDM-challenged mice. However, lung mRNA levels for these cytokines were not determined. In the lungs of HDM-challenged *Scgb3a2*^{-/-} mice, immunological tolerance might be one of the reasons for the discrepancy found in the relative mRNA and protein levels. It is worthwhile to note that the mRNA levels were determined using lung tissues whereas protein levels were determined using BALF, i.e. not exactly the same source. Immunological tolerance might have different influences on the transcription and secretion of these cytokines. Another possible reason for the discrepancy among gene expression, inflammation and protein levels in our study is that HDM contains numerous compounds which may induce continued innate immune responses that result in persistent inflammation despite the apparent develop-

ment of airway tolerance to HDM. In order to address these questions, levels of Th2 cytokines in the acute and chronic phases, in addition to the current subacute phase, need to be investigated. Nevertheless, our results clearly show that *Scgb3a2*^{-/-} mice experience exacerbated HDM-induced allergic airway inflammation.

Dust mites and their waste products are one of the most common causes of allergy and asthma [20]. Our study suggests that SCGB3A2 expressed in the lungs may be protective to these disorders, and exogenous SCGB3A2 could be a potential therapeutic reagent for the treatment of bronchial asthma. Levels of SCGB3A2 expression in the lungs may play a role in the development and/or pathogenesis of asthma, a possibility that warrants further investigation in asthmatics.

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Disclosure Statement

The authors declare no conflict of interests.

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