**Kininogen deficiency or depletion reduces enhanced pause independent of pulmonary inflammation in a house dust mite induced murine asthma model.**

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**Abstract**

Background: High molecular weight kininogen is an important substrate of the kallikrein-kinin system. Activation of this system has been associated with aggravation of hallmark features in asthma.

Objectives: We aimed to determine the role of kininogen in airway function and lung inflammation in a house dust mite (HDM) induced murine asthma model.

Methods: Normal wild-type mice and mice with a genetic deficiency of kininogen were subjected to repeated HDM exposure (sensitization on days 0,1 and 2; challenge on days 14, 15, 18 and 19) via the airways to induce allergic lung inflammation. Alternatively, kininogen was depleted after HDM sensitization by twice weekly injections of a specific anti-sense oligonucleotide (KNG ASO) starting at day 3.

Results: In kininogen deficient mice HDM induced in enhanced pause (Penh) was completely prevented. Remarkably, kininogen deficiency did not modify HDM induced eosinophil/neutrophil influx, Th2 responses, mucus production or lung pathology. KNG ASO treatment started after HDM sensitization reduced plasma kininogen levels by 75% and reproduced the phenotype of kininogen deficiency: KNG ASO administration prevented the HDM induced increase in Penh without influencing leukocyte influx, Th2 responses, mucus production or lung pathology.

Conclusions and clinical relevance: This study suggests that kininogen could contribute to HDM induced rise in Penhindependently of allergic lung inflammation. Further research is warranted to confirm these data using invasive measurements of airway responsiveness.

**Introduction**

Asthma is a heterogeneous disease characterized by airway hyperresponsiveness (AHR) and usually chronic airway inflammation(17, 39). Allergic asthma is the most common phenotype. The vast majority of asthmatic patients are sensitized to house dust mite (HDM) and repetitive contact with this allergen elicits respiratory symptoms such as coughing, wheezing and reversible airway obstruction(21). Long acting beta-adrenergic receptor agonists and corticosteroids are still the cornerstone in the treatment of asthma. Between 3% to 10% of all adults with asthma, however, are irresponsive to these therapeutics and experience a serious disease burden(25).

High molecular weight kininogen (HK) is a component of the kallikrein-kinin system together with factor XII (FXII) and prekallikrein(13, 32). Humans have one gene that encodes HK, which also produces low molecular weight kininogen by alternative splicing. In mice, two genes (*Kng1* and *Kng2*) encode HK and low molecular weight kininogen, of which *Kng1* exclusively contributes to plasma kininogens(35). In vitro activation of the kallikrein-kinin system occurs when FXII binds negatively charged surfaces resulting in auto-activation. Activated FXII cleaves the zymogen prekallikrein to generate kallikrein which subsequently digests HK in order to facilitate the release of bradykinin(13, 32). In an allergic inflammation setting, activation of the kallikrein-kinin system is possible in both a FXII-dependent and FXII-independent manner. Proteinase from HDM and mast cell derived heparin proteoglycan are capable of FXII activation which ultimately leads to HK cleavage(7, 33). In the absence of FXII, however, tryptase release following mast cell degranulation likewise elicits bradykinin release from HK(9).

Inhalation of bradykinin causes immediate bronchoconstriction in asthmatic patients but not in healthy subjects(3, 19). Asthmatic patients have elevated levels of HK and its activation products in their airways following allergen challenge, which further supports the role of the kallikrein-kinin system in asthma(8). Like in humans, various animal models have demonstrated enhanced bradykinin induced bronchoconstriction after allergen exposure(1, 22). Antagonists that interfere with bradykinin receptor signaling are able to attenuate airway hyperresponsiveness(2, 41). These studies have been mainly focused on the potential of bradykinin to cause bronchoconstriction. However, how the kallikrein-kinin system affects airway hyperresponsiveness in relation to airway inflammation is not well understood. In this study we aimed to investigate whether inhibition of the kallikrein-kinin system by depleting HK affects airway function and lung inflammation in a HDM induced asthma model. To this end we subjected *Kng1* knockout (KO) mice to repetitive HDM challenge in the airways to induce allergic lung inflammation. Additionally, we mimicked a therapeutically relevant approach by depleting HK exclusively in the challenge phase.

**Material and Methods**

**Mice**

BALB/c mice (8-12 weeks old) were purchased from Charles River (Maastricht, the Netherlands). *Kng1* KO mice on C57BL/6J background were generated as described before(35). Wild-type (WT) littermates were used as controls. Mice were housed under specific pathogen-free conditions receiving food and water ad libitum. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center.

**HDM asthma model**

To induce allergic lung inflammation, mice were sensitized on day 0, 1, 2 and challenged on day 14, 15, 18, 19 with 25µg HDM extract (Greer Laboratories, Lenoir, N.C., USA) or sterile saline intranasally. Prior to intranasal administration of HDM, all mice were anesthetized with isoflurane. BALB/c mice were treated with kininogen anti-sense oligonucleotide (ASO) (KNG ASO; sequence GGCTATGAACTCAATAACAT) or control anti-sense oligonucleotide (Ctrl ASO; sequence CCTTCCCTGAAGGTTCCTCC) by subcutaneous injection twice weekly (40 mg/kg per injection) starting immediately after the sensitization phase(15). Mice were euthanized 24 hours after the last challenge. In all experiments citrate blood was collected from the vena cava inferior (4:1 v/v) and bronchoalveolar lavage (BAL) was collected by airway lumen lavage with 2x 0.5 ml PBS containing 10mM EDTA, 10mM benzamidine and 0.2mg/ml SBTI as described(42). Cell counts were determined for each BAL sample in a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and cell differentiation was made by flow cytometric analysis. To obtain single cells, the flushed lungs were mechanically minced followed by digestion in RPMI with 5% FCS, 1% penicillin/streptomycin, liberase TM and DNAse at 37°C for 30 minutes. After 30 minutes incubation cells were dissociated by aspiration through a 19 gauge needle. Erythrocytes were lysed with sterile lysis buffer (Qiagen, Hilden, Germany). In a separate experiment the unflushed lung was collected for pathology examination. To determine *kng1* mRNA expression, the liver was homogenized and a sample was taken for RNA isolation.

**Measurement of enhanced pause**

Enhanced pause (Penh) was measured at day 19 by whole-body plethysmograph in conscious mice (Buxco Electronics, Troy, NY, USA). Nonspeciﬁc responsiveness was measured by exposing mice to aerosolized saline, followed by increasing concentrations of aerosolized methacholine (3.1, 12.5, 25 and 50 mg/mL in saline for 3 min; Sigma-Aldrich). Penh (enhanced pause) values were measured during five minutes after each methacholine dose.

**Flow cytometry**

Cells in BAL ﬂuid were stained with CD3-FITC, CD11c-PercP, SiglecF-Alexa 647, CD11b PE-Cy7, viability dye APC-Cy7(all BD Biosciences, San Jose, CA, USA), Ly6G-Alexa700 (Biolegend, San Diego, CA, USA), MHCII-PE, CD45-PE-eFluor610 (eBiosciences, San Diego, CA, USA) in the presence of Fc blocker (CD16/CD32, eBiosciences). Single cell suspension from lungs were stained with CD4-FITC, CD45-PerCP-Cy5.5, CD69-PE (eBiosciences), GATA3-Alexa 647, viability dye APC-Cy7 (BD Biosciences). For nuclear staining, cells were stained using a FOXp3 Staining Buffer set (eBioscience). All appropriate isotype controls were used. Data were collected on a BD Biosciences Canto II ﬂow cytometer and analyzed using FlowJo software (Treestar, Palo Alto, CA, USA).

**Assays**

Plasma total IgE was determined using rat-anti-mouse IgE as a capture antibody, purified mouse IgE as a standard, and biotinylated rat-anti-mouse IgE as detection (all from BD Biosciences). Plasma HDM-specific IgG1 was determined using HDM as capture and biotinylated rat-anti-mouse IgG1 as detection (BD Biosciences). BALF IgM was determined using rat-anti-mouse IgM as capture antibody, purified mouse IgM as standard and biotinylated goat-anti-mouse IgM(all from BD Biosciences) as detection. Cytokines (IL-4, IL-5, IL-13), CCL11 (eotaxin 1) and Prostaglandin E2 (PGE2), were measured by ELISA’s (Duoset, R&D systems, Minneapolis, MN, USA). For western blotting plasma proteins were separated by 10% SDS-PAGE gels. Plasma HK levels were measured by immunoblotting using a rabbit anti-human HK domain 5, obtained as described(15). Relative intensity between bands was determined using ImageJ. Total protein in BALF was measured by using Bio-Rad protein assay (Bio-Rad Laboratories, Veenendaal, Netherlands).

***Ex vivo* stimulation of mediastinal lymph nodes**

Mediastinal lymph nodes (mLN) were harvested and single cells were obtained by filtering through 100 μm strainers. Cells were seeded at a density of 2 x 105 cells/well in 96-well round bottom plates (Greiner Bio-One, Alphen a/d Rijn, Netherlands) and stimulated with 25 μg/ml HDM or PBS for 4 days at 37°C with 5% CO.

**Histology**

Histological analysis was performed as described before(12). Briefly, following fixation in 10% formalin, 4µm-thick paraffin-embedded sections were stained with hematoxylin- eosin (H&E). These sections were scored for interstitial inflammation, peribronchial and perivascular inflammation, edema, endothelialitis on a scale from 0 to 4. To examine mucus production, sections were stained with Periodic acid-Schiff (Pas-D) and scored for extent of goblet cells and mucous plugs on a scale from 0 to 3. Both total pathology score and mucus production were determined by a pathologist in a blinded fashion.

**RNA isolation and PCR**

Total RNA was isolated from lung or liver homogenates using RNA isolation kit (Nucleospin RNA, Macherey-Nagel, Düren, Germany) and reverse transcribed using oligo(dT) primer and M-MLV RT (Promega Benelux, Leiden, Netherlands). Primer sequences for gene expression were: *Kng1* forward: ATCACAGCCACCTCTTTACTCTC; *Kng1* reverse: TCCTCTACATTCACCATCATCAC; Hprt forward: ACAGGCCAGACTTTGTTGGAT; Hprt reverse: ACTTGCGCTCATCTTAGGCT; COX-2 forward: TTCAACACACTCTATCACTGGC; COX-2 reverse: AGAAGCGTTTGCGGTACTCAT Quantitative real-time quantitative PCR was performed on a LightCycler 480 System using Sensifast SybrGreen mix (Bioline, London, UK). Data were analyzed using the LinRegPCR software (v.2014.4).

**Statistical Analysis**

Results are expressed as mean ± SEM . Data were analyzed by either two-way ANOVA or Mann-Whitney U-test. Experimental groups consisted of 6-8 mice. P ≤ 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.

**Results**

***Kng1* KO mice show attenuated AHR following HDM challenge with unaltered leukocyte influx into the airways**

To study whether kininogen deficiency influences hallmark features of asthma, allergic airway inflammation was induced by repetitive intranasal administration of HDM in *Kng1* KO and WT mice (Fig. 1A). As expected, plasma HK was completely absent in *Kng1* KO mice (Fig. 1D). HDM challenge did not change plasma HK levels in WT mice, whereas HK could not be detected in BAL fluid (data not shown). Baseline PenH values were similar in *Kng1* KO and WT mice. After HDM challenge, methacholine induced AHR was reduced in *Kng1* KO mice relative to WT mice (Fig. 1B). Strikingly, Penh values did not differ between HDM and saline challenged *Kng1* KO mice, indicating that the HDM induced airway response was completely abrogated in kininogen deficient animals. Next, we examined the extent and character of allergic lung inflammation. HDM challenge resulted in an equally strong influx of leukocytes into BAL fluid from *Kng1* KO and WT mice (Fig. 1C). As expected, leukocytes recruited to the bronchoalveolar space predominantly consisted of eosinophils and to a lesser extent neutrophils; the percentage of eosinophils and neutrophils in BAL fluid did not differ between *Kng1* KO and WT mice (Fig. 1E,F). In accordance with the lack of an effect on eosinophil influx, kininogen deficiency did not affect the concentrations of CCL11, an important chemoattractant for eosinophils, in BAL fluid (Fig. 2C). These changes were accompanied by a similar decrease in the proportion of alveolar macrophages in BAL fluid from *Kng1* KO and WT mice (Fig. 1G). In HDM induced asthma models T helper (Th) 2 cells play a pivotal role in orchestrating pulmonary allergic inflammation(30). We determined Th2 cells in the lungs by intracellular staining of the transcription factor GATA3 in CD4+ lymphocytes(49) and in addition assessed the activation status of CD4+ T cells by measuring CD69 expression(29). *Kng1* deficiency had no effect on the number of GATA3+ (Fig. 1H) or CD69+ CD4 T cells (Fig. 1I) in lungs following HDM challenge. These results suggest that kininogen deficiency prevents the HDM induced increase in Penh without influencing the recruitment of eosinophils, neutrophils or Th2 lymphocytes to the lung compartment. The cyclooxygenase pathway has been implicated in the effects of bradykinin on airway responsiveness. We therefore measured COX2 mRNA in the lung and PGE2 in BALF, but found no difference between *Kng1* KO and WT after allergen challenge (data not shown).

***Kng1* KO mice display unaltered Th2 responses following HDM challenge**

To obtain further insight into the influence of kininogen deficiency on Th2 responses we measured Th2 cytokines in BAL fluid and supernatants of mLN re-stimulated with HDM. BAL fluid IL-5 and IL-13 levels similarly increased in *Kng1* KO and WT mice upon HDM challenge (Fig. 2A,B). IL-4 remained below the limit of detection in BAL fluid of all groups. Re-stimulation of mLN with HDM yielded high levels of IL-4, IL-5 and IL-13, but without differences between *Kng1* KO and WT mice (Fig. 2D-F). Likewise, repeated HDM administration resulted in similar increases in plasma IgE and HDM-specific IgG1 in *Kng1* KO and WT mice (Fig. 2G,H). Together, these results suggest that kininogen deficiency does not modify Th2 responses during HDM induced allergic lung inflammation.

***Kng1* KO mice demonstrate similar lung pathology to WT mice following HDM challenge**

This model of HDM induced allergic inflammation reproduces important features of asthma such as perivascular and interstitial inflammation, peribronchitis, endothelialitis and oedema, as well as mucus production(12). *Kng1* KO mice developed HDM-induced lung inflammation to a similar extent as WT mice, as reflected by the semi-quantitative scoring system described in the Methods section (Fig. 3A,B). HDM challenge also induced similar mucus production in *Kng1* KO and WT mice (Fig. 3C,D). Bradykinin is capable to interact with epithelial cells via signaling through bradykinin receptors on the surface of most epithelium(40). To investigate if kininogen deficiency affects epithelium integrity we measured total protein concentration and IgM levels in BAL fluid; no differences were found between *Kng1* KO and WT mice (Fig. 3E,F).

***Kng1* depletion after sensitization is sufficient to attenuate the HDM induced rise in Penh**

Having established that genetic deficiency of kininogen abolishes the HDM induced increase in Penh, we next wished to investigate whether depleting kininogen during the challenge phase mediates a similar effect. To that end mice were dosed subcutaneously with KNG ASO or Ctrl ASO after sensitization (starting at day 3). Treatment with KNG ASO resulted in more than 90% reduction of *Kng1* mRNA expression in the liver (Fig. 4A), the main source of plasma kininogen production, confirming previous findings(15). Consistently, KNG ASO treatment reduced plasma HK levels to ̴26% of normal HK levels (Fig. 4B,C). Similar to genetic kininogen deficiency, Kng ASO treatment alleviated the increase in Penh after HDM challenge (Fig. 4D). In addition, consistent with the data in *Kng1* KO mice, the influx of leukocytes, eosinophils, neutrophils, Th2 lymphocytes and activated CD4+ lymphocytes was not affected by KNG ASO administration (Fig. 4E-J). Similarly, KNG ASO treatment did not modify Th2 responses, as reflected by unchanged IL-5 and IL-13 levels in BAL fluid (Fig. 5A,B), unaffected IL-4, IL-5 and IL-13 concentrations in supernatants of mLN re-challenged with HDM (Fig. 5C-E) and unchanged rises in plasma IgE and HDM-specific IgG1 (Fig. 5F,G). Finally, KNG ASO administration did not influence the extent of lung pathology (Fig. 6A,B), mucus production (Fig. 6C,D) or BAL fluid concentrations of total protein and IgM (Fig, 6E,F). These results demonstrate that depletion of kininogen after HDM sensitization reproduces the findings in *Kng1* KO mice in this model of HDM induced allergic lung inflammation.

**Discussion**

Several studies have suggested a role for the kallikrein-kinin system in allergic airway disease such as asthma(3, 8). To the best of our knowledge, this is the first study on kininogen elimination or depletion in an allergic asthma model. We here show that kininogen deficiency and depletion inhibit the increase in Penh without influencing the main characteristics of allergic lung inflammation.

In the present study a HDM induced model was employed to study the main features of allergic asthma, including AHR to methacholine, Th2 driven inflammation, increased mucus production and plasma leakage. In this setting, we found that kininogen is essential for the methacholine induced increase in Penh in sensitized mice. In accordance, an earlier study reported that inhaled HK elicited bronchoconstriction in allergic sheep, which was predominantly driven by bradykinin(18) and a direct bradykinin challenge in rats and sheep likewise enhanced airway hyperresponsiveness (AHR)(1, 22). Bradykinin exerts its effect after binding to either bradykinin receptor 1 (B1R) or bradykinin receptor 2 (B2R). Blocking these bradykinin receptors has been shown to attenuate AHR in various asthma models(3). It is still in debate which of the bradykinin receptors is responsible for bronchoconstriction, B1R(20, 46) or B2R(2, 44). Rather than by blocking bradykinin receptors, we here interfered with bradykinin signaling by complete or partial ( ̴75%) elimination of kininogen, the substrate for bradykinin release. The fact that kininogen deficiency (tested in mice of a BALB/c genetic background) and ASO mediated inhibition of kininogen production (tested in C57Bl/6J mice) show very similar results, indicates that partial removal of kininogen in already sensitized mice is sufficient to prevent the rise in Penh to methacholine and that the role of kininogen does not depend on the mouse strain used. Together our data suggest that kininogen contributes to the methacholine induced increase in Penh in HDM sensitized mice.

Kininogen derived bradykinin has been implicated in inflammatory responses in asthma, including mucus production and vascular permeability(5, 24). Furthermore, blocking B1R in murine asthma models making use of ovalbumin sensitization and challenge resulted in lower airway eosinophilia(16, 46). In our HDM induced asthma model, neither genetic kininogen deficiency nor kininogen depletion post sensitization were associated with altered airway eosinophilia, mucus production or IgM levels in BALF (reflecting plasma leakage). These discrepancies might in part be explained by different allergens used between experimental protocols. Mucus production is highly influenced by the Th2 cytokines IL-4 and IL-13, which are more abundant in an HDM model compared to those in an ovalbumin model(27). Our data indicate that kininogen did not affect Th2 responses, and therefore a possible small effect of kininogen derived bradykinin on mucus production might not be detected in the present study. Against our expectations, HK elimination did not protect against plasma leakage into the bronchoalveolar space, which may be explained by an absent role for kininogen/bradykinin herein and/or by HDM derived proteases disrupting barrier function in a manner that outweighed bradykinin mediated vascular permeability. The Th2 response including airway eosinophilia is much stronger after HDM than after ovalbumin challenge(27). It is possible that the small differences in eosinophil influx observed in ovalbumin models(16, 46) were overwhelmed in our HDM model. Alternatively, B1R and B2R may have opposite roles in eosinophil influx (20) , which may partially explain the similar numbers of airway eosinophils regardless of the presence of kininogen. Our laboratory recently reported unaltered allergic inflammation in FXII KO mice after HDM challenge(42), corroborating the data from the present study that kallikrein-kinin system activation does not influence allergic lung inflammation.

Compelling studies indicated that AHR and pulmonary inflammatory responses are closely interconnected in asthma as many Th2 inflammatory mediators are involved in the induction of AHR(30). Our present study provided an interesting mechanism of Penh attenuation in a Th2 inflammation independent fashion. Supporting this finding, observations in experimental models and asthmatic patients demonstrated that Th2 responses are not necessarily correlated to AHR(10, 45). A possible mechanism of kininogen derived bradykinin induced bronchoconstriction could be mediated by direct or indirect effects on bronchial smooth muscles. Airway smooth muscles express bradykinin receptors that after binding to bradykinin evoke smooth muscle contraction(14). This bradykinin mediated contraction seems to be a complex process in which the TNF-α induced IFN-β-CD38 pathway is involved in priming smooth muscle cells rendering them responsive to bradykinin(26). Bradykinin induced bronchoconstriction can be prevented by pretreatment with the non-selective cyclooxygenase inhibitor indomethacin, indicating the involvement of arachidonic acid metabolites(37). In our study, lung COX-2 mRNA expression and BALF PGE2 levels did not differ between WT and *Kng1* KO mice, suggesting that alterations in the cyclooxygenase pathway are not involved in the changes in Penh (data not shown). A third possible mechanism is bradykinin stimulation of cholinergic and sensory nerves resulting in increased synthesis and release of neuropeptides that lead to bronchial muscle contraction(23, 38). In asthmatic patients an endotype named paucigranulocytic asthma has been identified(36, 43). Patients with this type of asthma do not have eosinophilia or neutrophilia in their lung, but nonetheless suffer from intermittent flow limitations. It is suggested that their symptoms are smooth muscle related and therefore therapeutics specifically targeting these cells could be beneficial(43). Taken together, these data support our finding that allergen induced AHR can be independent of allergic lung inflammation.

Unstrained whole body plethysmography (Penh) is considered to be a controversial tool to measure AHR in mice. In various experimental settings, changes in breathing patterns influence Penh(6). Moreover, different mouse strains could affect Penh outcomes, causing conflicting conclusions(4). Notably, the severity of allergen induced lung inflammation modifies Penh results significantly(47), further questioning the reproducibility and validity of this technique. On the other hand, numerous publications showed that in certain models Penh measurements correlate closely to invasive methods(11, 28, 34). In this study, we strived to cater for confounding factors like strain differences, by exploiting two different mouse strains (BALB/c and C57BL/6). In addition HDM as a clinically relevant allergen in our model induces severe lung inflammation, which in contrast to mild lung inflammation, correlates more closely to Penh results(47). In a comparable HDM model, a direct correlation between whole body plethysmography and direct/invasive measurements has been reported(34). Likewise, AHR is enhanced in HDM challenged groups compared to controls in models that are very similar to ours(31, 48). These studies demonstrated a marked increase of AHR in sensitized animals following methacholine provocation using either invasive measurements or whole body plethysmography. This suggests that Penh can be a useful tool to access AHR in HDM models. Nonetheless, the lack of validation of our Penh data by invasive measurements is an important limitation in our study.

In conclusion, our data show that kininogen aggravates allergen induced airway function (Penh) without modifying allergic lung inflammation. Further research is warranted to confirm these data using invasive measurements of airway function and determine whether this finding is solely dependent on bradykinin signaling or that other underlying mechanisms are at play. Additionally, kininogen depletion in asthmatic patients could be of interest, since this isolated effect on airway function could potentially benefit asthmatic patients without elevated airway inflammation.

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**Figure legends**

**Fig. 1** ***Kng1* KO mice show an attenuated HDM induced rise in Penh with unaltered airway leukocyte influx.** (A) HDM induced asthma model. (B) Penh after dose-response to methacholine (Mch) exposure on day 19. Data are pooled from two independent experiments. (C) Total cell count in bronchoalveolar lavage fluid (BALF) of saline (NaCl) or HDM challenged mice. (D) HK plasma levels of *Kng1* KO mice. Plasma proteins were diluted 1:10 in 3 x SDS buffer containing β-mercaptoethanol. From each sample 10 μl was loaded on and separated by 10% SDS-PAGE gels. Plasma HK levels were determined by immunoblotting using a rabbit anti-human HK domain 5. Four representative samples from both wild-type (WT) or *Kng1* KO are displayed. (E-G) % Eosinophils, neutrophils and alveolar macrophages in BALF. (H) Th2 cells expressed as CD4+GATA3+ and (I) activated T-cells expressed as CD69+CD4+ cells in the lung. All data are means ± SEM of 8 mice per group. *\*\*P* <0.01 for comparison between *Kng1* KO and wild-type (WT) mice; *#P*<0.001 for comparison between NaCl and HDM within *Kng1* KO and WT, respectively.

**Fig. 2 *Kng1* KO mice demonstrate unaltered Th2 responses during HDM induced lung inflammation.** IL-5, IL-13 and CCL11(A,B,C) in BALF and IL-4, IL-5 and IL-13(D-F) in supernatant of ex vivo HDM stimulated mediastinal lymph nodes (mLN) are displayed. (G,H) Plasma total IgE and plasma HDM-IgG1. Data are means ± SEM (n=7-8 per group) *#P*<0.001 for comparison between NaCl and HDM within *Kng1* KO or wild-type (WT) mice.For BALF CCL11 levels \*p<0.05 using a Wilcoxon Signed Rank test for the comparison between NaCl and HDM within WT and *kng1* KO treated mice.

**Fig. 3 *Kng1* KO mice display similar lung pathology, mucus production and protein leakage to WT mice following HDM challenge.** (A,B) Total pathology score of hematoxylin and eosin (H&E) stained lung sections (x4 magnification) from wild-type (WT)(black bars) and *Kng1* KO (white bars) and (C,D) total mucus score of PAS-D stained lung sections(x10 magnification) (n=4 per group). Protein leakage was determined by (E) total protein and (F) IgM in BALF (n=7-8 per group). Data are means ± SEM. *+P*<0.05 and *#P*<0.001 for comparison between NaCl and HDM within *Kng1* KO or wild-type (WT) mice.

**Fig. 4 KNG depletion after HDM sensitization attenuates the HDM induced rise in Penh without affecting leukocyte influx.** KNG ASO injections (40mg/kg) were given twice weekly after the sensitization phase starting on day 3. (A) *Kng1* mRNA normalized to HPRT in the liver 24 hours after last challenge on day 20. (B) Plasma HK levels of NaCl treated mice treated with Ctrl ASO or KNG ASO (n=4) were determined by western blot and (C) quantified by densitometry of HK bands at 120 kD and compared to a serial dilution of plasma as shown in B. (D) PenH values after increasing doses of methacholine. (E) Total cell count in BALF. (F-H) % Eosinophils, neutrophils and alveolar macrophages in BALF. (I,J) Th2 cells expressed as CD4+GATA3+ and (H) activated T-cells expressed as CD69+CD4+ cells in the lung. Data are means ± SEM of 6-8 mice per group. *\*P*<0.05 and *#P*<0.001 for comparison between NaCl and HDM within KNG and Ctrl ASO treated mice.

**Fig. 5** **KNG depletion after HDM sensitization does not influence Th2 responses following HDM challenge.** KNG ASO injections (40mg/kg) were given twice weekly after the sensitization phase starting on day 3. (A,B) IL-5 and IL-13 in BALF and (C-E) IL-4, IL-5 and IL-13 in supernatant of *ex vivo* HDM stimulated mediastinal lymph nodes (mLN). (F,G) Plasma total IgE and plasma HDM-IgG1. Data are means ± SEM (n=7-8 per group) *#P*<0.001 for comparison between NaCl and HDM within KNG and Ctrl ASO treated mice.

**Fig. 6 KNG depletion after HDM sensitization does not alter lung pathology, mucus production and protein leakage following HDM challenge** (A,B) Total pathology score of hematoxylin and eosin (H&E) stained lung sections (x4 magnification) from Ctrl ASO (black bar) and KNG ASO (white bar) treated mice and (C,D) total mucus score of PAS-D stained lung sections(x10 magnification) (n=4 per group). Protein leakage was determine by (E) total protein and (F) IgM in BALF (n=7-8 per group). Data are means ± SEM. *+P*<0.05 and *#P*<0.001 for comparison between NaCl and HDM within KNG and Ctrl ASO treated mice.