

Factor XI deficiency enhances the pulmonary allergic response to house dust mite in mice independent of factor XII

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24 **Abstract**

25 Asthma is associated with activation of coagulation in the airways. The coagulation system can be
26 initiated via the extrinsic tissue factor dependent pathway or via the intrinsic pathway, in which the
27 central player factor XI (FXI) can be either activated via active factor XII (FXIIa) or via thrombin. We
28 aimed to determine the role of the intrinsic coagulation system, and its possible route of activation, in
29 allergic lung inflammation induced by the clinically relevant human allergen house dust mite (HDM).
30 Wild-type (WT), FXI knockout (KO) and FXII KO mice were subjected to repeated exposure to HDM
31 via the airways, and inflammatory responses were compared. FXI KO mice showed increased influx of
32 eosinophils into lung tissue, accompanied by elevated local levels of the main eosinophil
33 chemoattractant eotaxin. While gross lung pathology and airway mucus production did not differ
34 between groups, FXI KO mice displayed an impaired endothelial/epithelial barrier function, as reflected
35 by elevated levels of total protein and IgM in bronchoalveolar lavage fluid. FXI KO mice had a stronger
36 systemic IgE response with an almost completely absent HDM-specific IgG₁ response. The phenotype
37 of FXII KO mice was, except for a higher HDM-specific IgG₁ response, similar to that of WT mice. In
38 conclusion, FXI attenuates part of the allergic response to repeated administration of HDM in the
39 airways by a mechanism that is independent of activation via FXII.

40

41 **Keywords:** allergy, coagulation, factor XI, factor XII, house dust mite

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43 Introduction

44 Asthma is a chronic inflammatory disease of the airways that affects up to 300 million people
45 worldwide(3, 26) . Asthma is associated with bronchial hyper-reactivity, mucus overproduction and
46 airway remodeling, leading to episodes of reversible airway obstruction, dyspnea and wheezing(3, 26).
47 Most asthma patients have an allergic phenotype. House dust mite (HDM) is amongst the most
48 common allergens triggering respiratory symptoms in sensitized patients(24).

49 In patients with asthma, leakage of clotting factors into the bronchoalveolar space and
50 expression of tissue factor (TF) on various cell types (e.g., alveolar epithelium, macrophages and
51 eosinophils) result in a local procoagulant state(8). Whereas limited activation of coagulation may
52 enhance epithelial repair, more pronounced activation can, together with the inflammatory response,
53 worsen pathophysiology (reviewed in(8)). The end product of coagulation is fibrin, which is formed
54 after cleavage of fibrinogen by thrombin. Two different pathways are known to lead to thrombin
55 formation, the TF (or extrinsic) pathway and the intrinsic pathway of coagulation. Soluble TF as well as
56 thrombin-antithrombin complexes have been detected in bronchoalveolar lavage fluid (BALF) or
57 sputum of asthmatic patients(11, 41), suggesting that the TF pathway of coagulation is activated
58 during asthma. To our knowledge, it is not known whether the intrinsic pathway of coagulation is
59 triggered during asthma.

60 The central player in the intrinsic coagulation system is factor XI (FXI). Plasma activators of
61 FXI include activated factor XII (FXIIa)(2), thrombin(12, 22, 32), and FXIa (autoactivation)(32, 47).
62 Interestingly, it has been reported that a proteinase from HDM can directly activate FXII(27). Apart
63 from initiating the intrinsic coagulation system via FXI, FXIIa additionally can convert prekallikrein into
64 kallikrein which on its turn cleaves high molecular weight kininogen releasing the potent
65 proinflammatory and vasoactive small molecule bradykinin(38).

66 When coagulation is initiated by low concentrations of TF the thrombin-FXI amplification loop
67 contributes to coagulation activation (4, 16, 21, 47). Recently, a bimodal role of thrombin in allergic
68 airway inflammation induced by ovalbumin was described in mice; herein complete inhibition of
69 thrombin by hirudin or instilling a high dose of thrombin worsened ovalbumin induced asthma, while a
70 low dose of thrombin ameliorated the inflammatory response(30). Considering the role of coagulation
71 and thrombin in experimentally induced allergic airway inflammation, and considering that thrombin is
72 amplified via the thrombin-FXI loop, we found it of interest to establish the role of FXI during asthma.

73 Hence, the aim of the present study was to determine the contribution of the intrinsic coagulation
74 system to the allergic airway inflammation accompanying asthma. Additionally, if the intrinsic
75 coagulation is involved in the development of asthma, we wanted to investigate whether this is
76 regulated via FXIIa. For this we used mice deficient for either FXI or FXII and subjected them to our
77 model of allergic lung inflammation induced by repeated administration of HDM via the airways(7).
78

79 **Material and methods**

80 **Mice**

81 C57Bl/6 wild-type (WT) mice were purchased from Charles River Inc. (Maastricht, The Netherlands).
82 Factor XI knockout (FXI KO) and Factor XII knockout (FXII KO) mice were generated as described
83 before(13, 18), and bred in the animal facility of the Academic Medical Center in Amsterdam. Mutant
84 mice were backcrossed to C57Bl/6 at least 7 generations. Mice were housed under specific pathogen-
85 free conditions receiving food and water *ad libitum*. Age-matched (9-12 weeks at start of the
86 experiments) female mice were used in all experiments. The Animal Care and Use Committee of the
87 University of Amsterdam approved all experiments.

88

89 **HDM asthma model**

90 HDM allergen whole body extract (Greer Laboratories, Lenoir, N.C., USA), derived from the common
91 European HDM species *Dermatophagoides pteronyssinus*, Der p, was used to induce allergic lung
92 inflammation as described previously(7). In short, mice were randomized to receive (by intranasal
93 inoculation) 25 µg HDM (sensitization phase) on day 0, 1 and 2 and 6.25 µg HDM (challenge phase)
94 on day 14, 15, 18 and 19, or sterile saline intranasally on each occasion (controls). Inoculum volume
95 was 20 µl for every HDM and saline exposure and inoculation procedures were performed during
96 isoflurane inhalation anesthesia. The complete mouse model was performed twice (experiment I and
97 II) and on each occasion different materials were obtained which are specified hereafter. Mice were
98 sacrificed at day 21 and blood was collected from the vena cava inferior into citrate (4:1 v/v,
99 experiment I). The right lung was fixed 24 hours in 10% formalin (experiment II). The left lung and part
100 of the liver were homogenized in 4 volumes of saline after which a sample was taken for RNA
101 isolation; the remaining homogenate was lysed in equal volume of lysis buffer containing 150mM
102 NaCl, 15mM Tris, 1mM MgCl, 1mM CaCl₂, 1% Triton and 1% protease inhibitor cocktail (Roche
103 Applied Science, Almere, Netherlands) and used for protein analysis (all experiment I). Two-sided
104 BALF was collected by instilling and retrieving 1ml of sterile PBS containing 10mM EDTA, 10mM
105 benzamidine and 0.2mg/ml SBTI in 500µl aliquots via the trachea (experiment II). Cell counts were
106 determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and

differential cells counts by cytopsin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düringen, Switzerland). BALF supernatant was collected for protein analysis.

Histology

Formalin-fixed tissue was embedded in paraffin using standard procedures. Four- μ m thick sections were cut and used for all (immuno)histochemical stainings. For examining allergic lung inflammation, sections were stained with Hematoxylin and Eosin (HE) and analyzed by a pathologist in a blinded fashion. HE-stained sections were scored for interstitial inflammation, peribronchial inflammation, edema, endothelialitis, and pleuritis on a scale from 0-4 (0: absent; 1: mild; 2: moderate; 3: severe; 4: very severe), each parameter was scored separately(7). Total pathology score was expressed as the sum of the scores for the different parameters. For examining mucus production, sections were stained with periodic acid-Schiff reagents after diastase digestion (PasD). The amount of mucus per lung section was assessed, by a pathologist in a blinded fashion, semi-quantitatively on a scale from 0-8 (0-4 for plug formation; 0-4 for mucus extent)(7). For eosinophil staining, sections were digested for 20 min in 0.25% pepsin in 0.1M HCl, incubated overnight at 4°C with rabbit-anti-mouse MBP (Major Basic Protein; kindly provided by Dr. Nancy Lee and Prof. James Lee, Mayo Clinic Arizona, Scottsdale, Ariz., USA)(23), incubated for 30 min with poly HRP-anti-rabbit IgG (Brightvision, Immunologic, Duiven, the Netherlands) and stained using 3,3'-diabminobenzidine dihydrochloride (DAB). Entire sections were digitized with a slide scanner using the 10x objective (Olympus dotSlide, Tokyo, Japan). Influx of eosinophils was determined by measuring the MBP positive area by digital image analysis (ImageJ 1.43, National Institute of Health, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>) and expressed as a percentage of the total lung area.

Assays

Plasma total IgE was determined using rat-anti-mouse IgE as capture antibody, purified mouse IgE as standard and biotinylated rat-anti-mouse IgE as detection (all from BD Biosciences, Pharmingen, Breda, the Netherlands). Plasma HDM-specific IgG₁ was determined using HDM as capture, and biotinylated rat-anti-mouse IgG₁ (from BD Biosciences) as detection. HDM-specific IgG₁ is expressed

as percentage compared to WT HDM group. Eotaxin DuoSet ELISA kit (R&D Systems, Abingdon, UK) was performed according to the supplied protocol. Lung IL-4 and IL-5 were determined by a Mouse Magnetic Luminex Screening Assay according to the manufacturers protocol (R&D Systems) and analyzed on a Bio-Rad BioPlex® 200 (Bio-Rad Laboratories, Veenendaal, the Netherlands). BALF and plasma IgM was determined using rat-anti-mouse IgM as capture antibody, purified mouse IgM as standard and biotinylated goat-anti-mouse IgM as detection (all from BD Biosciences). BALF total protein was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories). Thrombin-antithrombin complexes (TATc) were determined in BALF by ELISA according to manufacturer's protocol (Kordia, Leiden, the Netherlands).

RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from the lung and liver using Nucleospin® RNA II isolation kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using oligo dT primer and M-MLV RT (Invitrogen, Breda, the Netherlands). Primer sequences were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD, USA): Eotaxin forward: CACGGTCACTTCCTTCACCTC; eotaxin reverse: CAGCACAGATCTCTTTGCCCA; Hprt forward: ACAGGCCAGACTTTGTTGGAT; Hprt reverse: ACTTGCGCTCATCTTAGGCT. Quantitative real-time RT-PCR was performed on a LightCycler® 480 System using LightCycler® 480 SYBRGreen I Master Mix (Roche Diagnostics). Data were analyzed using the LinRegPCR program(39). The expression of eotaxin was normalized towards the reference gene Hprt and expressed as fold increase compared to NaCl WT group.

Western blot for fibrin degradation products

Samples for western blotting (WB) were boiled for 5 min in Laemlli buffer and loaded onto 6% SDS-PAGE gels. After electrophoresis the content of the gel was transferred onto PVDF membrane (Roche Diagnostics, Almere, the Netherlands). The membranes were blocked in 5% BSA (Roche Diagnostics) in TBS-T at room temperature for 60 min. Rabbit anti-mouse fibrinogen IgG fraction (MyBiosource.com, San Diego, CA, USA) was diluted 1:10000 in 5% BSA in TBS-T and incubated overnight at 4°C. Next, the membranes were incubated for 1 hour with anti-rabbit-HRP conjugated

secondary antibody (Cell Signaling Technology, Leiden, the Netherlands) in 1% BSA in TBS-T and blots were imaged using LumiLight Plus ECL (Roche Diagnostics) on a LAS4000 chemiluminescence imager (GE Healthcare Biosciences, Pittsburgh PA, USA). Quantification was performed using ImageJ Software(40). This fibrinogen antibody detects fibrinogen and fibrin and its degradation products, among which D-dimer and fragment X. To generate a D-dimer positive control, mouse citrated plasma was recalcified and incubated for 1 hour at 37°C in the presence of the plasminogen activator Activase® (Genentech USA Inc., South San Francisco, CA, USA).

Statistical Analysis

All quantitative assays were done in an unblinded fashion. A sample size of 8 per group was based on variation in eosinophil numbers in lung tissue in previous studies(7), providing a power of 80% to detect a difference of 50% between the genotypes with a *P* of 0.05. Results are expressed as mean ± standard error of the mean (SEM). Comparison between two groups was done by Mann-Whitney U test. For experiments with more than two groups, the Kruskal-Wallis test was used as a pretest, followed by Mann-Whitney U test where appropriate. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Values of *P* ≤ 0.05 were considered statistically significant.

Results

FXI KO, but not FXII KO, mice show enhanced eosinophil influx into lung upon repeated HDM exposure

To obtain insight into the role of FXI and FXII in allergic lung inflammation, WT mice and mice deficient for either FXI or FXII were subjected to repeated administration of HDM via the airways according to a previously established protocol(7). Eosinophil influx, which is a hallmark of allergic asthma, was observed in all three strains in both BALF and lung tissue following HDM exposure. While eosinophil numbers in BALF were similar in the three groups (Fig. 1a), FXI KO mice showed higher eosinophil numbers in lung tissue, as visualized by quantitative MBP staining, in comparison with either WT or FXII KO mice (Fig. 1b,c). Eosinophils were mainly localized in the interstitium. Eotaxin, a main chemoattractant for eosinophils, was significantly higher in HDM challenged lungs of FXI KO mice compared with WT and FXII KO mice (Fig. 1d). Eotaxin was below detection in BALF (data not shown). Analysis of eotaxin mRNA in lung, to determine local production, revealed a similar expression profile as for eotaxin protein (Fig. 1e).

FXI or FXII deficiency does not influence lung pathology upon HDM challenge

Lung pathology was assessed by semi quantitative scoring of HE stained lung sections for several characteristics of pulmonary damage including perivascular inflammation, interstitial inflammation, endothelialitis, peribronchitis and edema. HDM induced significant rises in the lung pathology scores, which was not affected by either FXI or FXII deficiency (Fig. 2a). Since human asthma is characterized by increased airway mucus production (44), we next set out to investigate whether FXI or FXII deficiency impacted on this feature by PAS staining of lung tissue (Fig. 2b). Following exposure to HDM, there was an increased presence of mucus in the bronchial epithelial cells of all three strains. The mucus producing epithelial cells were mainly located in the bronchi and to a lesser extent in the bronchioles. No differences were observed between FXI KO, FXII KO and WT mice.

Impact of FXI or FXII deficiency on type 2 responses

Plasma IgE levels increased in all three strains following HDM exposure. IgE plasma levels were significantly higher in HDM and NaCl FXI KO mice compared with the respective WT groups (Fig. 3a).

Although not significant, most likely due to high intergroup variation, after HDM exposure IgE levels were higher in FXII KO mice as compared with WT mice (WT: 1841 ± 385 ng/ml; FXII KO: 3934 ± 1285 ng/ml). IgE plasma levels were comparable between FXI KO and FXII KO mice following HDM exposure. Remarkably, HDM-specific IgG₁ was almost undetectable in plasma of HDM exposed FXI KO mice while it was present in HDM exposed WT mice (Fig. 3b). FXII KO showed significantly elevated HDM-specific IgG₁ levels. The cytokines IL-4 (Fig. 3c) and IL-5 (Fig. 3d) were measured in the lung as additional markers of a type 2 response. Both IL-4 and IL-5 were significantly increased in HDM compared with NaCl exposed WT mice. While IL-4 levels were similar in all strains following HDM exposure, IL-5 concentrations were 3-fold higher in FXI KO HDM mice compared with WT HDM mice. Pulmonary IL-4 and IL-5 levels in FXII KO mice were comparable with WT mice.

FXI KO, but not FXII KO, mice show increased protein leak in BALF

HDM induced increases in the BALF concentrations of total protein and IgM, indicative of an impaired integrity of the endothelial-epithelial barrier (Fig. 4a,b). FXI KO mice had higher protein and IgM levels in their BALF upon HDM exposure than WT and FXII KO mice. Plasma IgM levels were higher in FXI KO mice both following NaCl and HDM exposure (Fig. 4c), however, this could not explain high BALF IgM in FXI KO mice after HDM challenge as the ratio between BALF and plasma IgM was still significantly elevated in this group (Fig. 4d).

FXI or FXII deficiency do not impact local activation of coagulation or fibrinolysis after HDM exposure

HDM instillation induced a rise in the fibrin degradation products D-dimer and fragment X in BALF of WT mice (Fig. 5a,c). Relative to WT mice, FXI KO and FXII KO mice showed similar rises in BALF D-dimer and fragment X levels as well as crosslinked fibrin (Fig. 5b,d). TATc was undetectable in BALF of NaCl exposed mice, however following HDM exposure this coagulation marker was present in BALF of all three strains with no difference between the strains (Fig. 5e). Together these results indicate that a deficiency for FXI or FXII does not impair the ability to activate coagulation or fibrinolysis.

Discussion

Several studies have indicated that activation of coagulation contributes to the pathophysiology of asthma. In addition to the formation of fibrin clots, activation of the coagulation system induces an inflammatory response via the interaction of thrombin with protease activated receptors(35) and via the interaction of fibrinogen cleavage products with Toll-like receptor 4(29). Recently it was shown that low levels of thrombin are beneficial while high levels of thrombin are detrimental in an ovalbumin asthma model(30). Here we studied the role of the intrinsic coagulation pathway and its route of activation in a mouse model of allergic asthma induced by repeated airway exposure to HDM. We show that the central player of the intrinsic coagulation system, FXI, dampens part of the allergic response following HDM exposure independent of activation via FXII.

FXI deficiency was associated with increased influx of eosinophils into lung tissue upon HDM administration via the airways. The apparent effect of FXI on eosinophil migration likely at least in part was mediated by an effect on locally produced eotaxin, a main chemoattractant for eosinophils(10), which was higher in FXI KO mice. Increased eosinophil influx was also observed in BALF of ovalbumin exposed mice treated with inhaled hirudin compared with saline treated mice(30). Hirudin is a specific thrombin inhibitor that tightly binds to the active site of thrombin and thereby blocks thrombin activity including the activation of FXI(25). We did not observe an altered eosinophil influx in FXII KO mice. Although these results suggest that the enhanced eosinophil influx observed in FXI KO mice is independent of activation via FXII, additional experiments using FXI/FXII double KO mice are necessary to support this hypothesis. Effects of FXI on inflammatory processes not related to coagulation have been reported previously. Plasminogen and FXI double KO mice show an enhanced influx of inflammatory cells into the lung while no difference in fibrin deposition was observed compared with WT or plasminogen KO mice(5). In line, activated FXI has been shown to inhibit neutrophil migration in a chemotactic assay(17). Taken together, FXI might affect eosinophil influx either via its interaction with thrombin or directly. More research is needed to elucidate the exact mechanism at play.

We evaluated the type 2 responses by measuring pulmonary IL-4 and IL-5, two main cytokines produced by Th₂ lymphocytes. Both cytokines play different roles in the allergic inflammatory response. IL-5, that is additionally produced by mast cells and eosinophils, is crucial to eosinophil growth, maturation, activation, and suppression of apoptosis(42). Elevated lung IL-5 levels in FXI KO

mice coincided with higher eosinophil numbers, suggesting that eosinophils may be a source for IL-5 in these animals. Conversely, interactions between IL-5 and eotaxin promote eosinophil release from the bone marrow and their homing to tissues(31). Several clinical trials have reported reduced eosinophilia following anti-IL-5 therapy, although clinical responses have been quite variable(14). In this respect it is interesting to further investigate the mechanism behind increased IL-5 and eotaxin levels in FXI KO mice. As IL-4 levels were not affected by FXI deficiency, we cannot conclude that the overall Th₂ response is enhanced in FXI KO mice.

The Th₂ response in asthma induces isotype switching of B cells to IgE synthesis. IgE is bound to mast cells via its high-affinity receptor FcεR1. IgE-dependent activation of mast cells occurs when the bound IgE molecules are crosslinked by multivalent antigen triggering the release of inflammatory mediators and cytokines which cause the symptoms of immediate hypersensitivity such as plasma extravasation, smooth muscle contraction and itching. Indeed, in our murine asthma model we observed elevated plasma levels of IgE following exposure to HDM. IgE levels were higher in plasma of both saline and HDM exposed FXI KO mice as compared with WT mice. To determine the HDM-specific humoral response, we tried to measure HDM-specific IgE. Unfortunately we were not able to detect HDM-specific IgE, as has been previously reported by others using the same HDM preparation(36, 37). HDM-specific IgG₁ was detectable in our mouse model, however almost undetectable in FXI KO mice. Total IgG₁ was not lower in NaCl or HDM exposed FXI KO mice (data not shown); thus the low HDM-specific IgG₁ cannot be explained by an impaired IgG₁ production. The equivalent of murine IgG₁ in humans is IgG₄(28). In a recent clinical trial, HDM immunotherapy induced a dose-dependent increase in allergen-specific IgG₄ that was accompanied by a reduction in asthma exacerbations(46). Moreover, desensitization with ovalbumin enhanced ovalbumin-specific IgG₁, and reduced IL-4 and IL-5 in mice(9). Together this would argue for a immunomodulatory role wherein HDM-specific IgG₁ dampens adaptive immunity and blocks (HDM-specific) IgE in our mouse model. This would explain enhanced levels of IL-5 and IgE and low/absent HDM-specific IgG₁ in our HDM exposed FXI KO mice. The mechanism how FXI influences HDM-specific IgG₁ remains, however, unclear.

The airway allergic inflammatory response induced by HDM alters endothelial/epithelial permeability. FXI deficiency enhanced this permeability as reflected by higher total protein and IgM levels in BALF. In the circulation FXI is found in complex with high molecular weight kininogen. Apart

from binding FXI, high molecular weight kininogen forms a complex with prekallikrein and thereby facilitates on its surface the activation of the intrinsic coagulation and contact system via activating FXII or FXI directly. High molecular weight kininogen binds to endothelial cells and enhances the activation of its binding partners on these cells(45). One could argue that if FXI is absent, high molecular weight kininogen is more accessible for the contact system factors resulting in enhanced liberation of the vasoactive molecule bradykinin, possibly leading to reduced barrier function. Detection of bradykinin is difficult because of its extremely short (<10sec) half-life(33). Additionally, the precursor of bradykinin, high molecular weight kininogen, interferes with the currently available assays and has to be eliminated from the samples by extraction or filtration methods(18). Therefore, our samples are not suitable for reliable detection of bradykinin. On the other hand, enhanced permeability might be explained by an impaired coagulation on the endothelial-epithelial barrier. This has recently been reported in epithelial-specific TF KO mice(43).

FXII KO mice showed, except for HDM-specific IgG₁, similar HDM-induced inflammatory response as WT mice. FXII can be activated either directly via HDM proteinases(27) or indirectly via the release of negatively charged endogenous molecules, for instance via heparin released by activated mast cells(34), during an asthmatic exacerbation. Our data, however, suggests that there is no significant role for FXII, either via activating FXI or the kallikrein-kinin system, in HDM provoked asthma. Studies that have shown release of bradykinin in the absence of FXII support our findings (reviewed in (19)). Moreover, FXII-independent activation of the kallikrein-kinin system has been reported in allergic asthma(6). These authors reported bradykinin, mainly originating from tissue kallikrein, which in contrast to plasma kallikrein is not activated via FXIIa, in the BALF of asthmatic subjects.

Coagulation factors, including FXI and FXII, are mainly produced in the liver and secreted into our bloodstream. Together with increased vascular permeability, resulting in leakage of plasma into the BALF, activation of the TF pathway of coagulation has been reported in asthma(11, 41). In the present study we found no impairment in coagulation activation in either FXI KO or FXII KO mice. These results suggest that 1) the intrinsic pathway does not make a significant contribution in local activation of coagulation, or 2) increased plasma leakage in FXI KO mice results in enhanced activation of the TF pathway with as a net result similar levels of D-dimer formation as compared with WT mice. As FXI is activated by thrombin, one could argue that lack of FXI enhances availability of

thrombin for other purposes such as activation of fibrinogen, however we did not observe enhanced coagulation as assessed by TATc and fibrin degradation products.

A limitation of our study is the lack of airway responsiveness testing. Our FXI KO and FXII KO mice are on a C57Bl/6 background. Several studies have shown that allergic airway hyperresponsiveness is not coupled to inflammation in C57Bl/6 mice, while it is in BALB/c mice(1, 15, 20). In our opinion, FXI KO and FXII KO mice should be backcrossed on a BALB/c background to assess airway hyperreactivity following HDM exposure.

In conclusion, FXI dampens the allergic response following HDM exposure independent of activation via FXII. To the best of our knowledge, to date no study has been published on an association between allergic asthma and FXI deficiency in humans. The exact mechanism of this FXI phenotype remains to be elucidated. As there is no evidence for altered coagulation, the role of FXI in asthma may be independent of coagulation and FXI may directly influence inflammatory processes.

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Legends to figures

Figure 1: FXI KO, but not FXII KO, mice have increased eosinophil influx and eotaxin levels in lungs upon HDM exposure via the airways. a) Influx of eosinophils in BALF of WT (white bars), FXI KO (grey bars) or FXII KO (black bars) following NaCl or HDM exposure. Accumulation of eosinophils in the lung was determined by immunohistochemical staining for Major Basic Protein (MBP). b) MBP positive staining as percentage of total lung surface is depicted for WT (white bars), FXI KO (grey bars) or FXII KO (black bars) following NaCl or HDM exposure. c) Representative pictures of MBP stained NaCl and HDM exposed lung sections. Lung eotaxin protein (d) and mRNA (e) after NaCl or HDM exposure in WT (white bars), FXI KO (grey bars) or FXII KO (black bars). Data from two separate experiments (n=8 per group); experiment I: b-e; and experiment II: a. Data are means + SEM, * $P < 0.05$

Figure 2: FXI KO and FXII KO mice show similar lung pathology and mucus production after HDM administration via the airways. Total pathology score of HE stained lung sections (a) and mucus score of PasD stained lung sections (b) of WT (white bars), FXI KO (grey bars) and FXII KO (black bars). Data are means + SEM (n=8 per group, experiment I). Right panels show representative pictures of HE (4x magnification) and PasD (10x magnification) stained lung sections. ND = non-detected

Figure 3: Impact of FXI or FXII deficiency on type 2 immune responses. Plasma IgE (a) and HDM-specific IgG₁ (b) and lung IL-4 (c) and IL-5 (d) levels in WT (white bars), FXI KO (grey bars) and FXII KO (black bars) in NaCl and HDM exposed mice. Dashed line represents lower detection limit. Data are means + SEM (n=8 per group, experiment I), * $P < 0.05$

Figure 4: FXI KO, but not FXII KO, mice show increased protein leak in BALF. Protein leakage was determined by BALF total protein (a) and IgM (b) levels in WT (white bars), FXI KO (grey bars) and FXII KO (black bars) mice. 7 out of 8 HDM exposed FXI KO mice had IgM levels above detection (detection limit is 1000ng/ml, represented by dashed line). Plasma IgM (c) and ratio between IgM levels in BALF and plasma (d) in WT (white bars), FXI KO (grey bars) and FXII KO (black bars) mice.

Ratio is given as a percentage compared to WT NaCl exposed mice. Data are means + SEM (n=6-8 per group, experiment II), * P <0.05

Figure 5: HDM exposure via the airways induces local activation of coagulation and fibrinolysis in all three strains. a) Fibrin degradation WB of WT NaCl and HDM exposed BALF. c) Digital analysis of WB is depicted as fold increase in HDM (black bars) compared with NaCl (white bars) exposed WT BALF. b) Fibrin degradation WB of BALF from WT, FXI KO and FXII KO mice following HDM exposure. d) Digital analysis of 2 independent WBs, analyzing all individual mouse samples from the three strains, is depicted as fold increase in FXI KO (grey bars) or FXII KO (black bars) compared with WT (white bars) HDM exposed BALF. e) TATc levels in BALF of WT (white bar), FXI KO (grey bar) and FXII KO (black bar) following HDM exposure. In a+c) and b+d) similar WT HDM samples are used. c=control D-dimer from mouse plasma, data are means + SEM (n=6 per group [NaCl vs HDM] or n=8 per group [WT vs FXI KO vs FXII KO], experiment II), * P <0.05

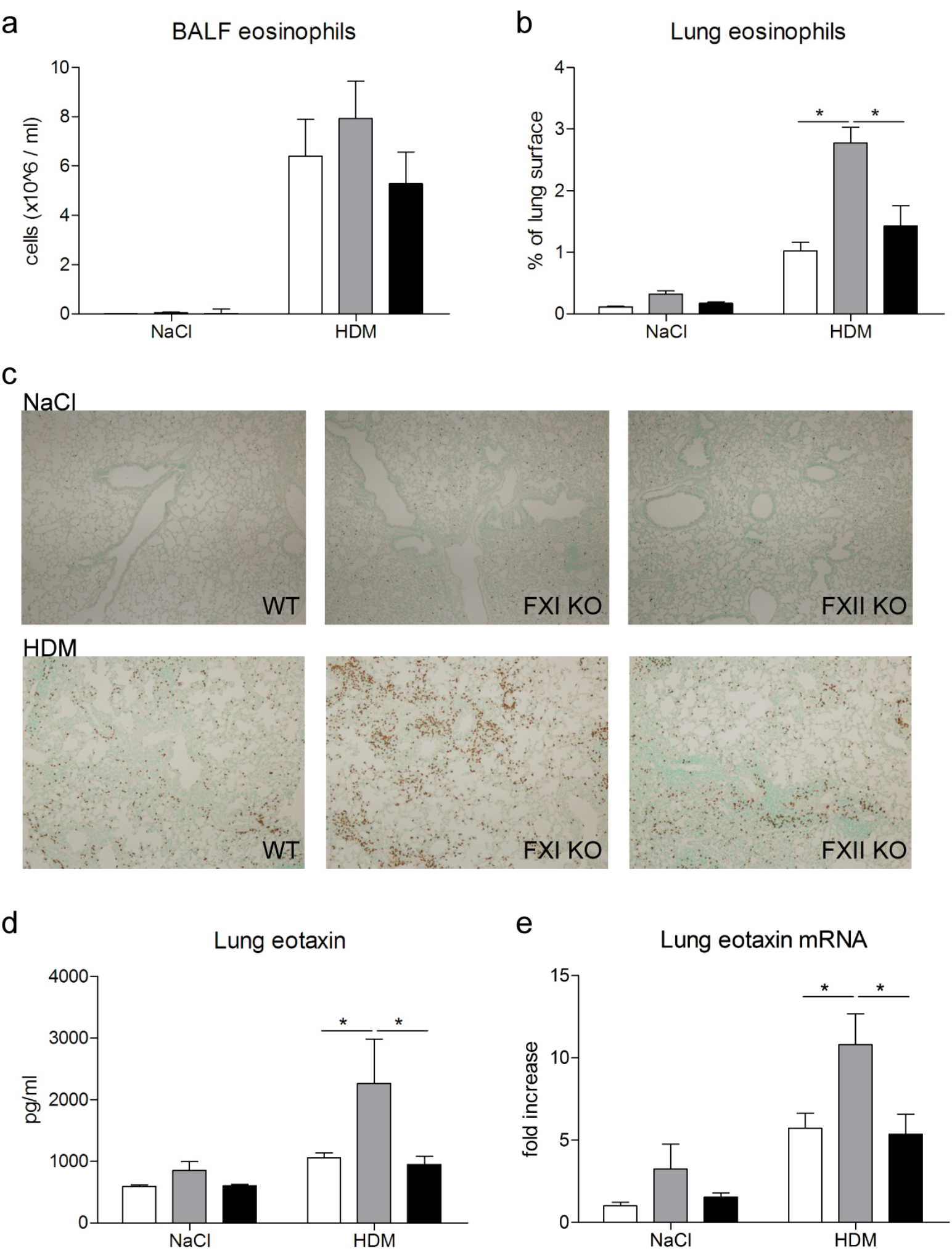


Figure 1

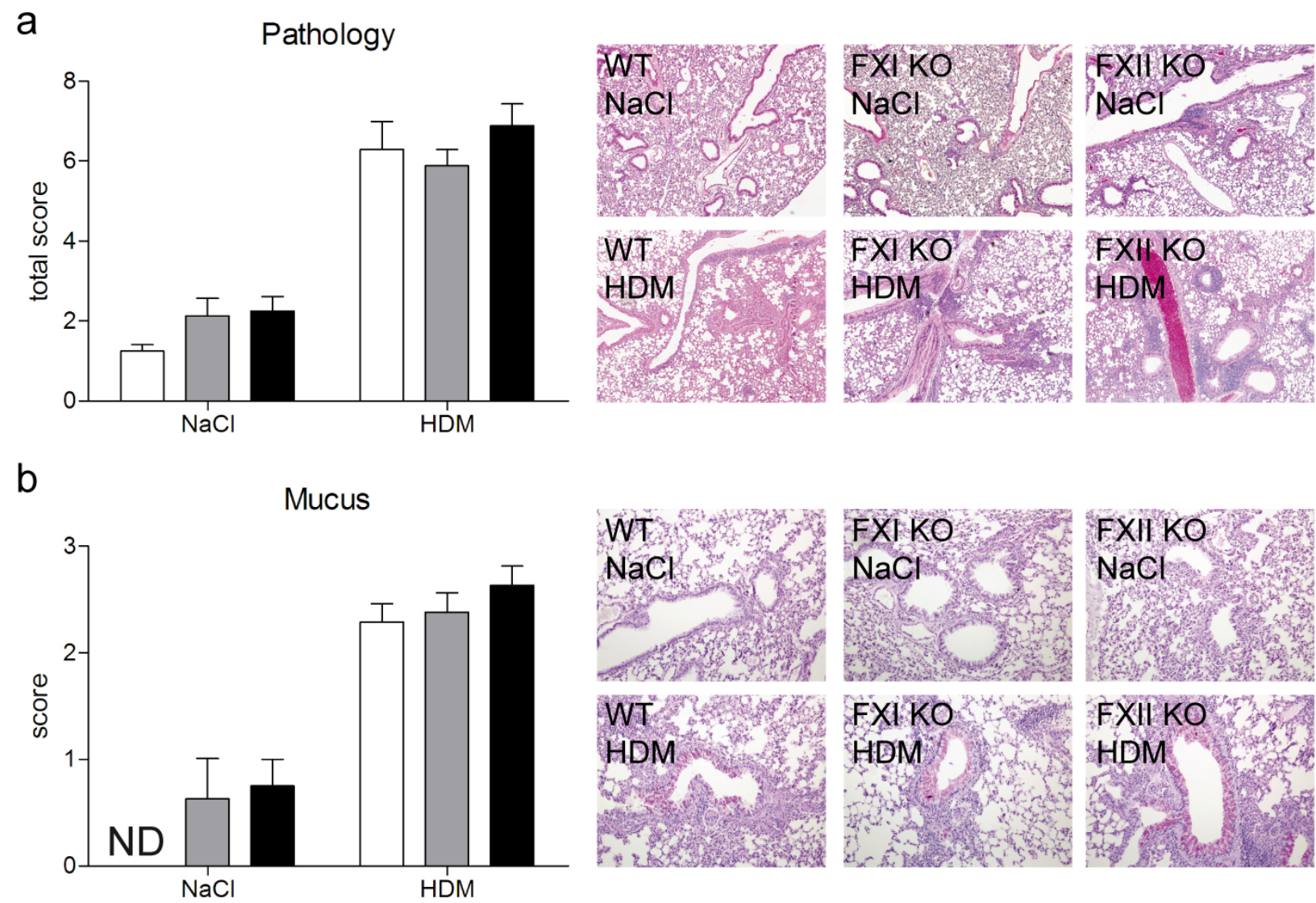


Figure 2

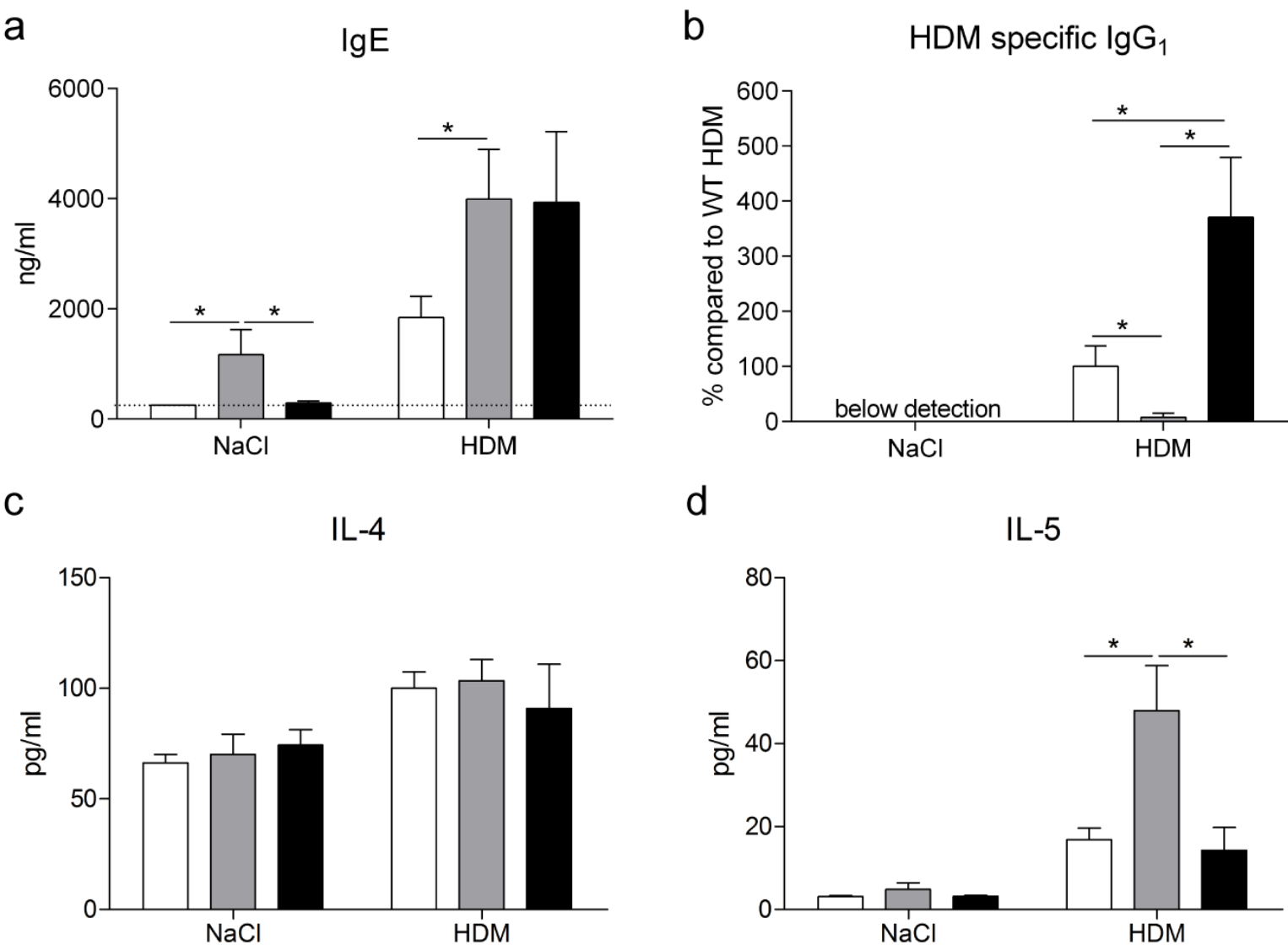


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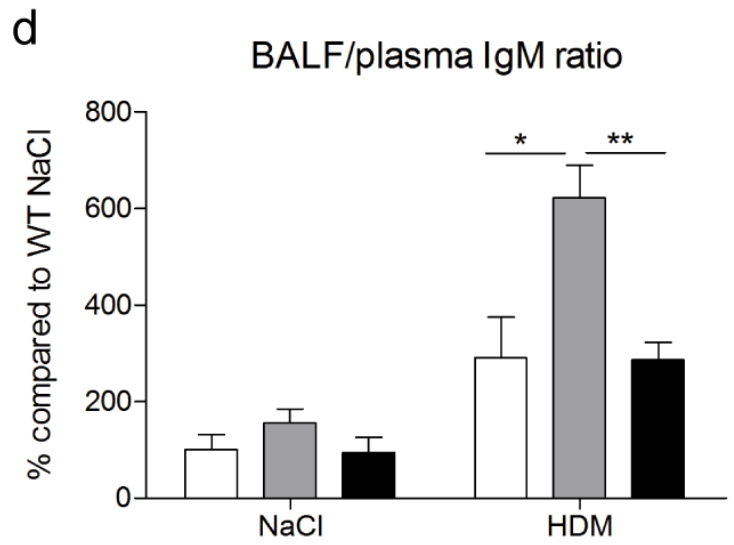
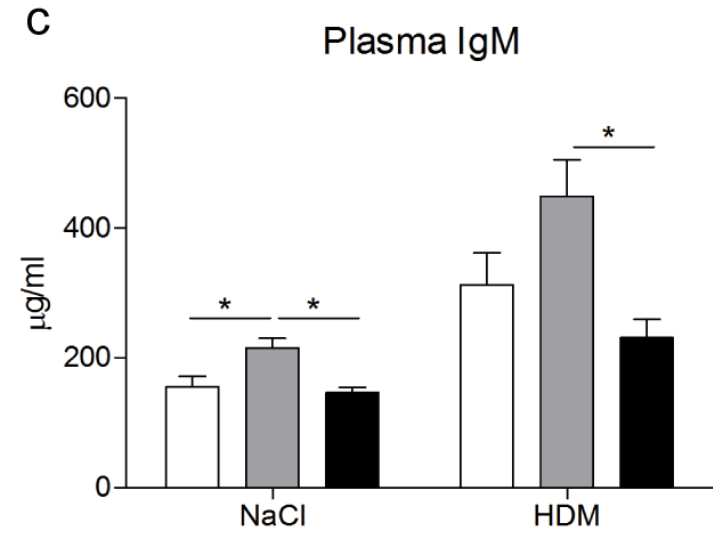
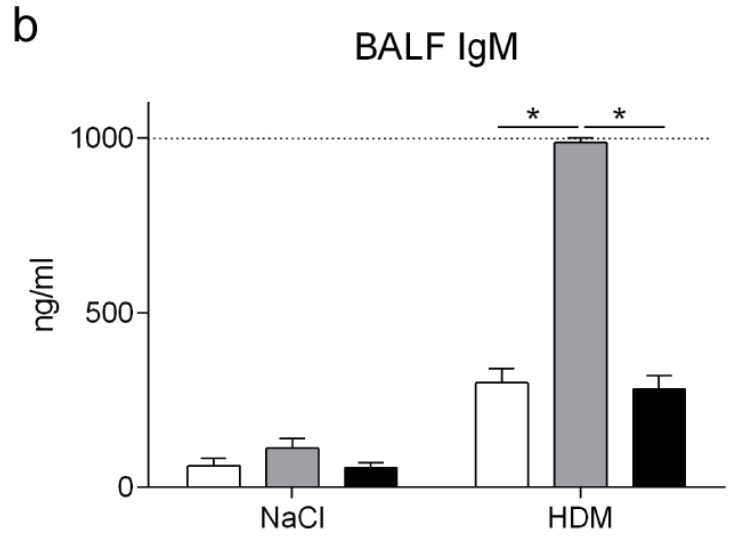
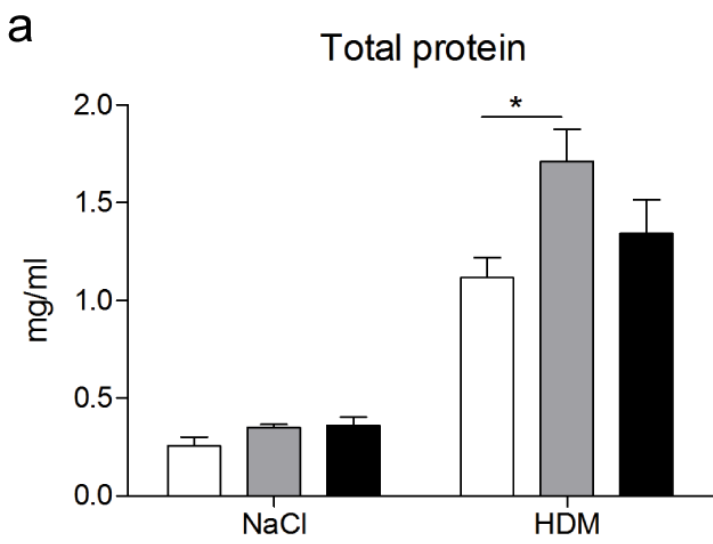


Figure 4

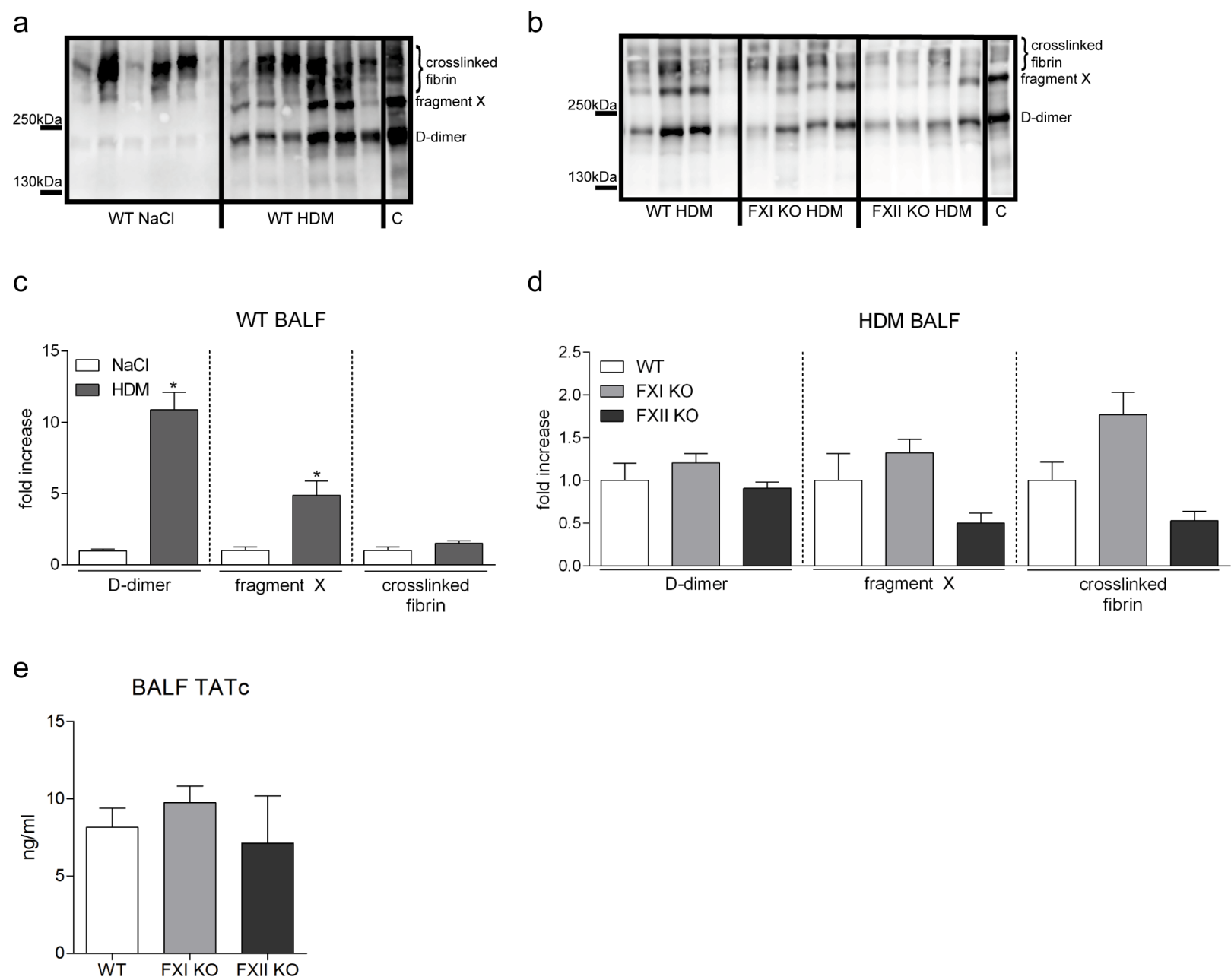


Figure 5