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Effect of the oral thrombin inhibitor dabigatran on allergic lung inflammation induced by repeated house dust mite administration in mice

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de Boer JD, Berkhout LC, de Stoppelaar SF, Yang J, Ottenhoff R, Meijers JC, Roelofs JJ, van't Veer C, van der Poll T. Effect of the oral thrombin inhibitor dabigatran on allergic lung inflammation induced by repeated house dust mite administration in mice. *Am J Physiol Lung Cell Mol Physiol* 309: L768–L775, 2015. First published August 28, 2015; doi:10.1152/ajplung.00102.2015.—Asthma is a chronic disease of the airways; asthma patients are hampered by recurrent symptoms of dyspnoea and wheezing caused by bronchial obstruction. Most asthma patients suffer from chronic allergic lung inflammation triggered by allergens such as house dust mite (HDM). Coagulation activation in the pulmonary compartment is currently recognized as a feature of allergic lung inflammation, and data suggest that coagulation proteases further drive inflammatory mechanisms. Here, we tested whether treatment with the oral thrombin inhibitor dabigatran attenuates allergic lung inflammation in a recently developed HDM-based murine asthma model. Mice were fed dabigatran (10 mg/g) or placebo chow during a 3-wk HDM airway exposure model. Dabigatran treatment caused systemic thrombin inhibitory activity corresponding with dabigatran levels reported in human trials. Surprisingly, dabigatran did not lead to inhibition of HDM-evoked coagulation activation in the lung as measured by levels of thrombin-antithrombin complexes and D-dimer. Repeated HDM administration caused an influx of eosinophils and neutrophils into the lungs, mucus production in the airways, and a T helper 2 response, as reflected by a rise in bronchoalveolar IL-4 and IL-5 levels and a systemic rise in IgE and HDM-IgG1. Dabigatran modestly improved HDM-induced lung pathology ($P < 0.05$) and decreased IL-4 levels ($P < 0.01$), without influencing other HDM-induced responses. Considering the limited effects of dabigatran in spite of adequate plasma levels, these results argue against clinical evaluation of dabigatran in patients with asthma.

asthma; house dust mite; dabigatran; thrombin

ASTHMA IS A CHRONIC AIRWAY disease characterized by symptoms of reversible airway obstruction, wheezing, and dyspnoea (25). The prevalence of asthma is high, with 5–10% of the population affected in most Western countries (10). The majority of

asthma patients has an allergic phenotype (4), characterized by a variable state of allergen-evoked lung inflammation. Common allergens triggering asthma symptoms in sensitized patients are pollen, cockroaches, and house dust mite (HDM) allergens (23). When allergic lung inflammation is severe or chronic, lung function may further decline, and the reversible character of asthma may be lost by structural alterations of the lung tissue by a process called airway remodeling (19). Although corticosteroids are the cornerstone of anti-inflammatory treatment in asthma, an important subgroup of patients is unresponsive to corticosteroids and suffers from frequent asthma exacerbations (1). New anti-inflammatory treatment strategies need to be developed that may improve outcome of allergic lung inflammation, especially for those patients that are difficult to control clinically and are at high risk to proceed to airway remodeling despite corticosteroid therapy. In this study, we explored thrombin inhibition by dabigatran as a potential anti-inflammatory treatment option in a mouse asthma model.

In the last two decades, the coagulation system has been recognized as an inseparable component of inflammatory responses (8, 21). While coagulation activation can be beneficial to promote protective inflammatory reactions directed at invading pathogens (12), coagulation activation may be detrimental for the host in chronic and/or exaggerated inflammatory reactions, such as in the case of allergic lung inflammation in asthma patients (8, 21, 34). Tissue injury exposes and induces tissue factor expression and activates the coagulation cascade. As a result, thrombin is generated, which converts fibrinogen to the end-product of the coagulation system fibrin. Besides mediating the production of fibrin, thrombin has been described to have a variety of cellular effects. These effects may be of importance for allergic lung inflammation as well, and include activation of human bronchial rings (17), fibroblast proliferation (27), and smooth muscle cell activation (18). We recently developed a HDM-based mouse model for allergic lung inflammation (9). We here used this model to investigate whether treatment with the new oral thrombin inhibitor

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dabigatran influences the extent and/or characteristics of HDM-induced allergic lung inflammation in mice.

MATERIALS AND METHODS

Animals. Sex- and age-matched 8- to 9-wk-old wild-type C57Bl/6 mice were purchased from Charles River (Maastricht, the Netherlands). Experiments seeking to document the thrombin-inhibitory capacity of dabigatran were done in 5 mice/group and all other experiments with 10 mice/group. Animals were housed in standardized specific pathogen-free conditions, and experiments were approved by the Animal Care and Use Committee of the University of Amsterdam.

HDM-induced mouse asthma model. HDM allergen whole body extract (Greer Laboratories, Lenoir, NC), derived from the common European HDM species *Dermatophagoides pteronyssinus*, was used to induce allergic lung inflammation as described previously (9). Briefly, mice were inoculated intranasally on days 0, 1, and 2 with 25 μ g HDM and on days 14, 15, 18, and 19 with 6.25 μ g HDM. Controls received isotonic sterile saline intranasally. Inoculum volume was 20 μ l for every HDM, and saline exposure and inoculation procedures were performed during isoflurane inhalation anesthesia. The experiment was ended at day 21 by killing the mice and the subsequent collection and processing of samples: in one experiment bronchoalveolar lavage fluid (BALF) and citrated blood were collected, and in a separate experiment one lung was obtained for pathology and one lung for homogenization using procedures as described before (9).

Dabigatran treatment. Dabigatran (dabigatran etexilate; Boehringer Ingelheim, Ingelheim am Rhein, Germany) was mixed with normal flour for mouse chow (AM-II, code 2141; Arie Blok Diervoeder, Woerden, the Netherlands) in a 10 mg/g chow concentration as used by others (3, 30). This was mixed with water to make a paste and subsequent pellets and dried for 7 days in a flow cabinet. Normal mouse chow served as placebo. In a study to measure the thrombin-inhibitory effect of dabigatran, mice were fed dabigatran or placebo chow ad libitum and killed at time (t) = 48 h or t = 12 days to obtain citrated plasma. Dabigatran levels were measured in citrated plasma with the Hemoclot Thrombin inhibitor test (Hyphen BioMed, Neuville-sur-Oise, France) (33). Dabigatran or placebo chow was provided ad libitum to mice challenged with HDM or sterile saline starting at day 0.

Histology. Histological analysis was performed as described before (9). Shortly, after fixation in 10% formalin, paraffin-embedded 4- μ m-thick sections were stained with hematoxylin-eosin (H&E) for general pathology analysis and with Periodic acid-Schiff (PAS)-D for mucus detection. A pathologist blinded for treatment group quantified H&E and PAS-D stainings. The H&E semiquantitative score was based on the features of peribronchitis, endothelialitis, epithelialitis, edema, and pleuritis; each element was scored 0–4 (in which a score of 4 is the most severe). Additionally, we digitally quantified eosinophils in lung tissue stained by an antibody against major basic protein (MBP, kindly provided by Dr. Nancy Lee and James Lee, Mayo Clinic Arizona, Scottsdale, AZ).

Assays. ELISAs were used to measure the concentrations of lung IL-4, IL-5 (both R&D Systems, Abingdon, UK), and thrombin-antithrombin complexes (TATc; Siemens Healthcare Diagnostics, Marburg, Germany). D-dimer was measured using Western blotting as described previously (20) with slight modifications, using rabbit anti-mouse fibrinogen (MyBioSource, San Diego, CA) and goat anti-rabbit IgG- and horseradish peroxidase-linked antibody as second antibody (Bioké, Leiden, the Netherlands). BALF total protein was determined by using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Total IgE in plasma was measured using rat anti-mouse IgE as a capture antibody and biotin rat-anti-mouse IgE as a detection antibody; purified mouse IgE was used as standard (all from BD Biosciences Pharmingen, Breda, the Netherlands). HDM-specific IgG1 antibodies were measured using HDM (1 μ g *D. pteronyssinus*

1/ml) as capture, biotinylated anti-mouse IgG1 (2 μ g/ml) as detection antibody, and purified mouse IgG1 as standard (both from BD Biosciences Pharmingen, Breda, the Netherlands).

Statistical analysis. Values are expressed as means $\pm$ SE. Comparison between two variables was done by Student's *t*-test or Mann Whitney where appropriate; D'Agostino and Pearson omnibus test was used to assess for normality. GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) was used for all analyses. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Dabigatran does not influence local activation of coagulation during HDM-induced lung inflammation. Mice were fed dabigatran or placebo chow ad libitum and killed at 48 h or 12 days after the start of the experiment. Mice on dabigatran chow reach plasma concentrations of 0.30–0.36 μ g/ml dabigatran as measured with a thrombin inhibition assay (Fig. 1), which is similar to those reported by others (30) and relates well to human therapeutic concentrations ($\sim$ 0.18 μ g/ml) (33). Repeated administration of HDM induced local activation of the coagulation system in the lungs, as reflected by increases in the concentrations of TATc ($P < 0.001$ vs. saline; Fig. 2A) and D-dimer (at least $P < 0.01$ vs. saline; Fig. 2, B–D) in whole lung homogenates. Remarkably, dabigatran did not influence this pulmonary procoagulant response. Total protein in BALF was determined as a marker of extravascular plasma leakage. HDM increased BALF total protein levels ($P < 0.001$ vs. saline; Fig. 2E); this effect was not influenced by dabigatran treatment.

Dabigatran does not impact cell recruitment in the lungs induced by HDM. Repeated HDM exposure via the airways induced an increase in leukocytes in BALF ($P < 0.001$ vs. saline; Fig. 3A). As expected, this increase was mainly due to the influx of eosinophils ($P < 0.001$ vs. saline; Fig. 3B), and to a lesser extent of neutrophils ($P < 0.001$ vs. saline; Fig. 3C). HDM also caused increases in BALF numbers of macrophages ($P < 0.05$ vs. saline; Fig. 3D) and lymphocytes ($P < 0.001$ vs. saline; Fig. 3E). Dabigatran had no impact on leukocyte numbers or leukocyte subsets in BALF. Because eosinophils are the predominant cell type of allergic lung inflammation, we also investigated if dabigatran affected eosinophil accumulation in

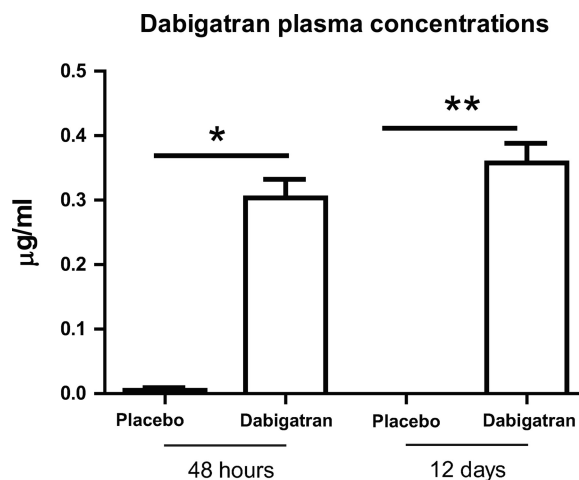


Fig. 1. Dabigatran plasma levels are increased in dabigatran-treated mice after 48 h and 12 days. Data are means $\pm$ SE of 10 mice/group. * $P < 0.05$ and ** $P < 0.01$.

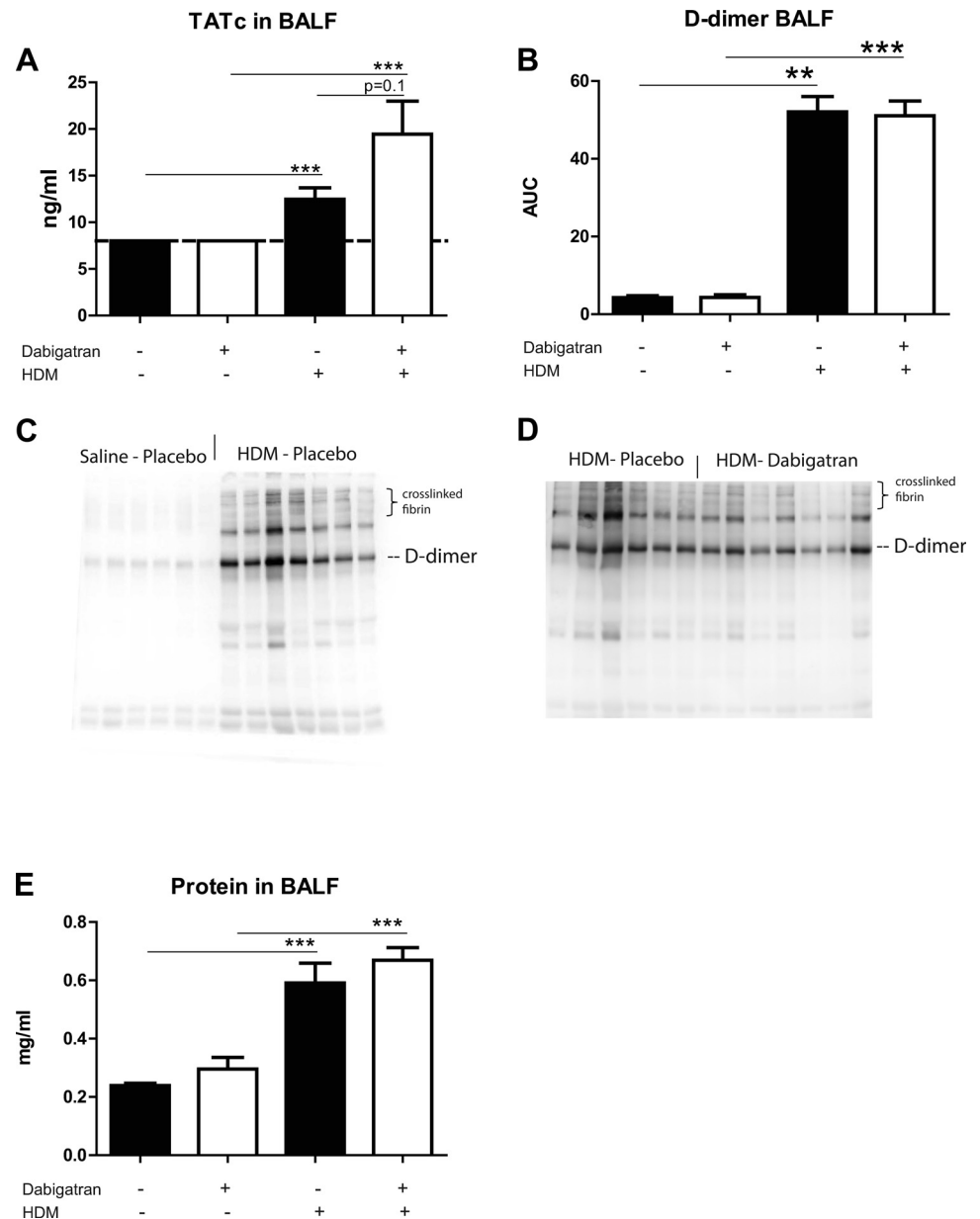


Fig. 2. Dabigatran does not influence local activation of coagulation during house dust mite (HDM)-induced lung inflammation. Concentrations of bronchoalveolar lavage fluid (BALF, A) thrombin-antithrombin complexes and D-dimer (B). C and D: representative Western blots showing D-dimer bands at ~195 kDa. E: saline- and dabigatran-treated mice have similar protein leakage in BALF. Data are means $\pm$ SE of 10 mice. $**P < 0.01$ and $***P < 0.001$. Filled bars, placebo-challenged animals; open bars, dabigatran-challenged animals. In addition, challenges (saline and HDM) are indicated beneath the x-axes.

lung tissue by staining with an eosinophil-specific MBP antibody (Fig. 4). Whereas HDM instillation clearly induced accumulation of eosinophils in the lungs, dabigatran did not modify this response. Together, these results show that dabigatran does not influence HDM-induced leukocyte influx in the lungs.

Dabigatran reduces HDM-evoked lung pathology. To assess the potential impact of dabigatran on HDM-evoked lung pathology, we analyzed H&E-stained lung slides using a semi-quantitative score described in METHODS and before (9). Consecutive airway challenges with HDM resulted in increased lung pathology ($P < 0.001$ vs. saline; Fig. 5). Treatment with dabigatran improved general lung pathology ($P < 0.05$ vs. saline), but only to a small extent. The formation of mucus in smaller and larger bronchioles is an important aspect of allergic lung inflammation (32). HDM exposure increased mucus production in the airways, as visualized by PAS-D staining ($P <$

0.001 vs. saline; Fig. 6). Dabigatran treatment had no effect on the amount of mucus in the airways.

Impact of dabigatran on T helper 2 responses. We further explored markers of a T helper 2 response. HDM exposure to the airways elicited increased pulmonary levels of the T helper 2 cytokines IL-4 ($P < 0.001$ vs. saline; Fig. 7A) and IL-5 ($P < 0.001$ vs. saline; Fig. 7B). Dabigatran strongly reduced pulmonary concentrations of IL-4 ($P < 0.01$ vs. saline) while not affecting IL-5 levels. HDM elicited strong increases in the plasma concentrations of total IgE ($P < 0.001$ vs. saline; Fig. 7C) and HDM-IgG1 ($P < 0.05$ vs. saline; Fig. 7D); dabigatran did not alter these responses.

DISCUSSION

Although steroids and bronchodilating medication often suffice as primary therapy of asthma, a subgroup of patients is

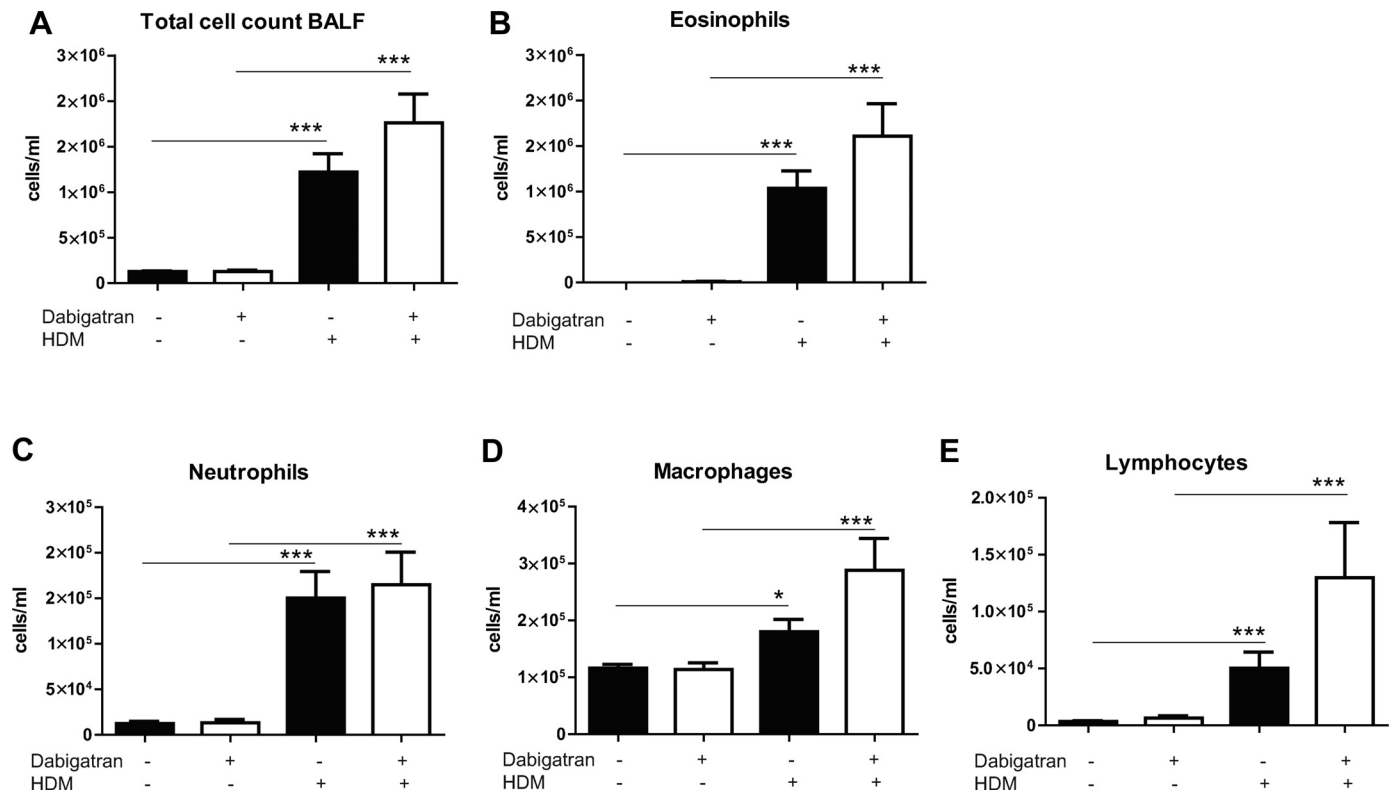


Fig. 3. Dabigatran does not impact cell recruitment in the lungs induced by HDM. Total cell count (A), eosinophils (B), neutrophils (C), macrophages (D), and lymphocytes (E) in BALF. Data are means + SE of 10 mice/group. * $P < 0.05$ and *** $P < 0.001$. Filled bars, placebo-challenged animals; open bars, dabigatran-challenged animals. In addition, challenges (saline and HDM) are indicated beneath the x-axes.

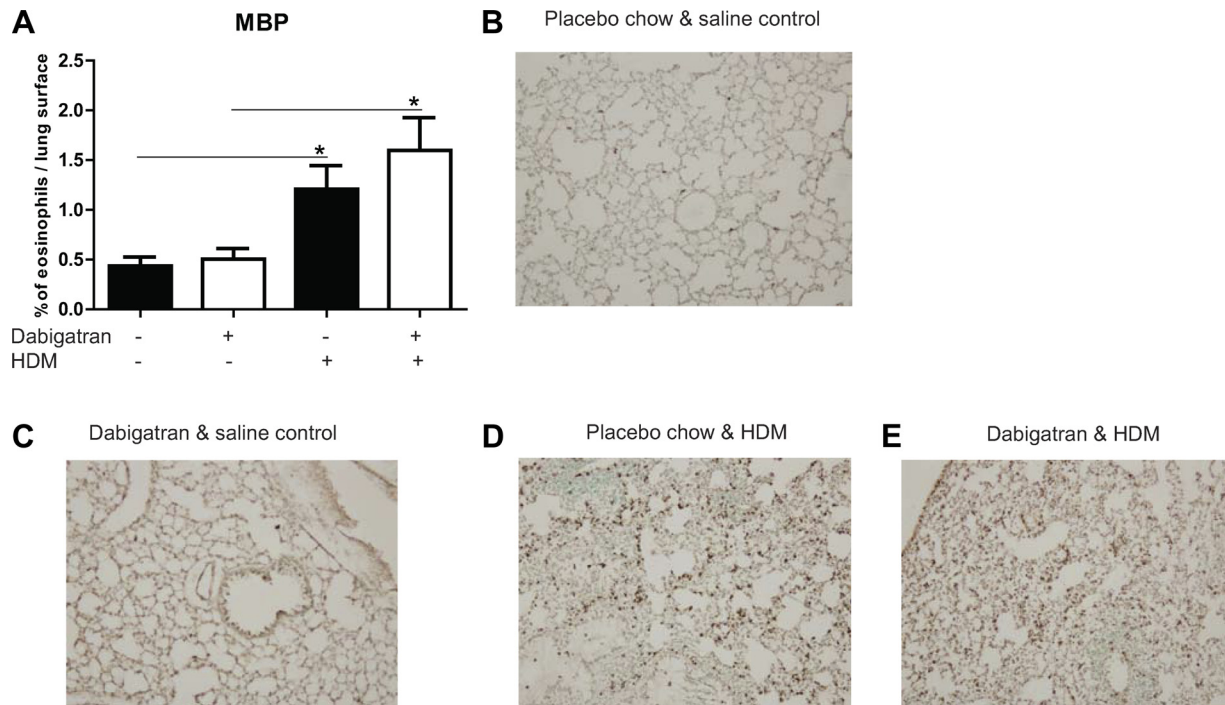


Fig. 4. Dabigatran does not influence HDM-induced lung eosinophil influx. A: lung tissue eosinophils expressed as percentage of surface area as analyzed by major basic protein (MBP) staining to quantify eosinophils in lung tissue. B–E: representative slides of MBP-stained lung tissue ($\times 100$ magnification). Data are means + SE of 10 mice/group. * $P < 0.05$. Filled bars, placebo-challenged animals; open bars, dabigatran-challenged animals. In addition, challenges (saline and HDM) are indicated beneath the x-axes.

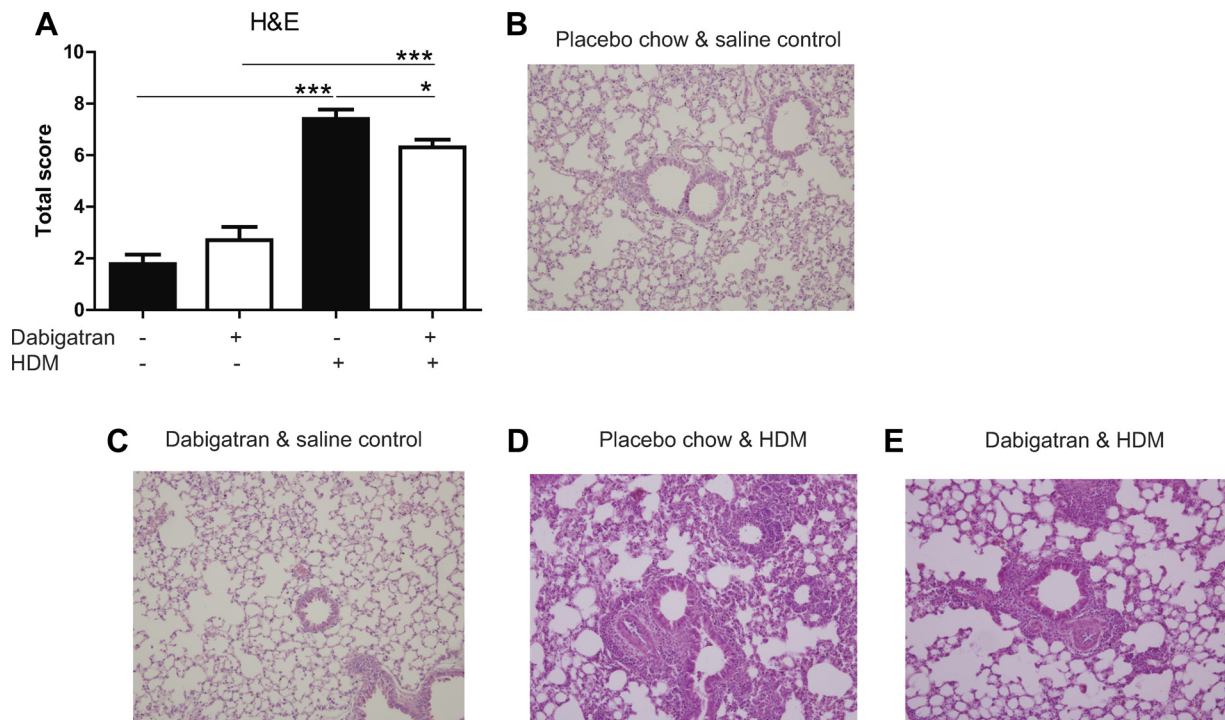


Fig. 5. Dabigatran reduces HDM-evoked lung pathology. *A*: overall score for HDM-induced lung pathology based on the features of peribronchitis, endothelialitis, epithelialitis, edema, and pleuritis. *B–E*: representative hematoxylin-eosin (H&E)-stained slides of lung tissue ($\times 100$ magnification). Data are means $\pm$ SE of 10 mice/group. * $P < 0.05$ and *** $P < 0.001$. Filled bars, placebo-challenged animals; open bars, dabigatran-challenged animals. In addition, challenges (saline and HDM) are indicated beneath the *x*-axes.

refractory to currently available treatments (25). Because there is no definite cure for asthma and because inadequately controlled allergic lung inflammation may lead to irreversible loss of normal lung function (19), new anti-inflammatory treatment options are urgently needed. Here, we explored the effects of dabigatran, an oral direct thrombin inhibitor (13), in allergic lung inflammation induced by the clinically relevant allergen HDM in mice. Repeated HDM exposure via the airways reproduced major features of asthma, including an influx of eosinophils and neutrophils in the lungs, mucus production in the airways, and local and systemic T helper 2 responses. In addition, HDM elicited local activation of the coagulation system in the lungs, as reflected by elevated lung levels of TATc and D-dimer. Although dabigatran clearly induced a strong thrombin inhibitory effect in the circulation, it had only a modest effect on HDM-induced lung inflammation. Considering that dabigatran plasma levels were within the range reported in humans treated with this compound (33), and considering that high doses increase the risk for bleeding complications (11), our results suggest that dabigatran is less likely to be useful as adjunctive therapy in refractory asthma.

Recently, attention has been drawn toward pulmonary coagulopathy as an active contributor to allergic lung inflammation accompanying asthma (5, 8, 16, 21, 24, 26). Thrombin is the key enzyme of the coagulation cascade converting fibrinogen to the coagulation end-product fibrin. Thrombin is a known constituent of sputum of asthma patients (16), and multiple studies have shown increased thrombin activity after antigen provocation in asthma patients (28, 31). Thrombin is a protease with differential effects: besides its procoagulant function, thrombin can exert proinflammatory effects via the G

protein-coupled protease-activated receptor (PAR) 1 (6, 7). Proinflammatory effects of thrombin include chemotactic properties (14), activation of fibroblast proliferation (27), contraction of human bronchial rings (17), and induction of lung fibrosis (6). Dabigatran is a new oral anticoagulant that directly inhibits thrombin with the clinical advantage that its pharmacological profile does not require monitoring of blood coagulation (13). Dabigatran has been shown to prevent activation of PAR1 by thrombin (3), potentially inhibiting both its procoagulant and proinflammatory effects. A previous investigation studied the effect of dabigatran in a bleomycin mouse model for interstitial lung disease; both lung inflammation and lung fibrosis were significantly reduced by dabigatran (3). In the present study we found a modest reduction of lung pathology in dabigatran-treated mice. The much stronger effects of dabigatran in bleomycin-induced lung pathology may in part be explained by differences in the severity and type of lung injury caused by bleomycin and HDM. Although thrombin induced PAR1-dependent chemotaxis of eosinophils in vitro (14), we did not detect an in vivo effect of dabigatran on eosinophil recruitment. Inhibition of coagulation factor Xa resulted in attenuation of airway hyperresponsiveness and reduced collagen and mucus production in mice with allergic lung inflammation induced by ovalbumin; this intervention did not modify cell influx in the lungs (29). Direct comparison with our findings in HDM-induced inflammation is hampered by the considerable differences with the ovalbumin model (15).

The mechanism by which dabigatran attenuated pulmonary IL-4 release remains to be determined. Dabigatran did not have a general inhibitory effect on T helper 2 responses, considering the unaltered local IL-5 and systemic IgE and IgG1 responses.

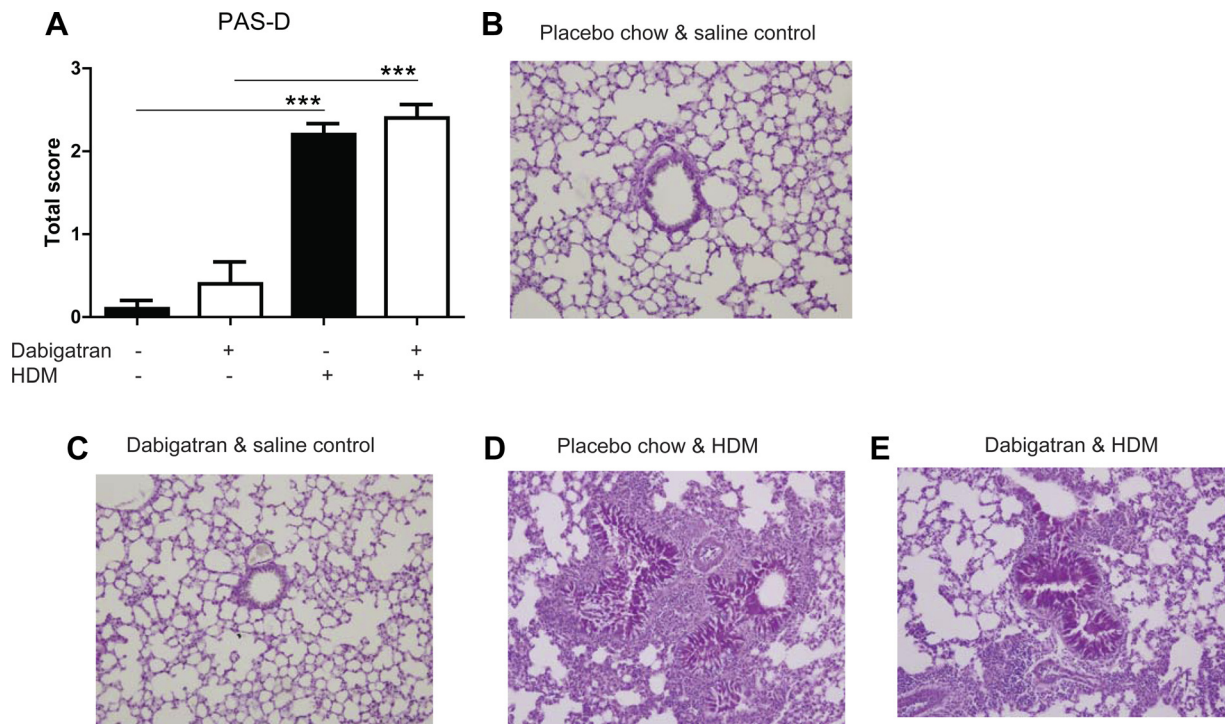


Fig. 6. Unaltered mucus production in dabigatran-treated mice. *A*: semiquantitative score of HDM-induced mucus production. *B–E*: representative Periodic acid-Schiff (PAS)-D-stained lung tissue slides ($\times 100$ magnification). Data are means $\pm$ SE of 10 mice/group. *** $P < 0.001$. Filled bars, placebo-challenged animals; open bars, dabigatran-challenged animals. In addition, challenges (saline and HDM) are indicated beneath the *x*-axes.

Lung tissue stromal cells, such as fibroblasts and macrophages, are reported to be very sensitive for IL-4; IL-4 significantly stimulated the production of procollagen I by bronchial fibroblasts of healthy volunteers and asthma patients (2). Additionally, IL-4 can drive macrophages to undergo functional skew-

ing to the M2 subtype, and aberrant activation of M2 macrophages has been associated with airway remodeling and fibrosis (22). Dabigatran has been demonstrated to reduce the fibrotic effects of thrombin on fibroblasts (3). The HDM model is not characterized by, and too short for the development of,

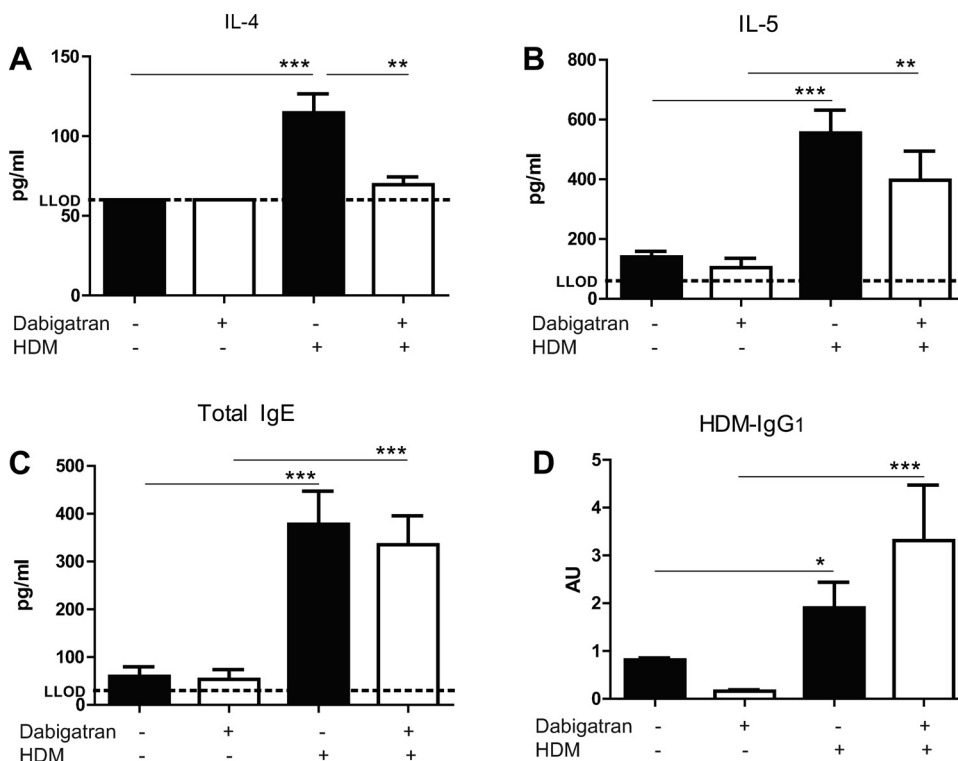


Fig. 7. Dabigatran reduces IL-4 BALF concentrations while not affecting HDM-evoked IL-5 and plasma IgE and HDM-IgG1. BALF concentrations of IL-4 (*A*), IL-5 (*B*), plasma total IgE (*C*), and HDM-IgG1 (*D*). LLOD, lower limit of detection. Data are means $\pm$ SE of 10 mice/group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Filled bars, placebo-challenged animals; open bars, dabigatran-challenged animals. In addition, challenges (saline and HDM) are indicated beneath the *x*-axes.

fibrosis; therefore, the effect of dabigatran on airway remodeling and lung fibrosis cannot be adequately studied herein. HDM administration was associated with profound lung pathology characterized by peribronchitis, endothelialitis, edema, and pleuritis. Treatment with dabigatran caused a small but significant reduction in the overall lung pathology score composed of these separate parameters of inflammation. The relatively small sample and effect size did not allow for detection of the specific element of pathology that was most influenced by dabigatran.

Remarkably, dabigatran did not influence the activation of the coagulation system in the lungs of HDM-challenged mice. In part, this can be explained by functioning of dabigatran: it prolongs the time needed to generate fibrin, but this does not implicate that dabigatran decreases the total amount of fibrin produced. HDM-induced protein content in BALF was similar in both placebo- and dabigatran-treated mice; this indicates that dabigatran did not impact on extravascular plasma leakage. We did not measure lung dabigatran levels. Yet, since high systemic levels were reached, we consider it likely that pulmonary levels of dabigatran were adequate, also in light of the fact that the lung is abundantly perfused (especially during inflammation). Furthermore, the HDM asthma model is characterized by considerable protein leakage in the bronchoalveolar space, which adds to pulmonary dabigatran availability. Nonetheless, it is possible that local administration of dabigatran, for example, by inhalation, can result in higher lung levels without the risk of systemic bleeding complications; the effect of inhaled dabigatran in experimental asthma warrants further research.

In conclusion, we explored the oral thrombin inhibitor dabigatran as a potential new therapy for asthma using a HDM-based mouse model for allergic lung inflammation. Whereas repeated HDM instillation in the airways reproduced many features of asthma, dabigatran had a very limited effect hereon. Our results argue against the clinical evaluation of dabigatran in patients with asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors. The authors claim not to have potential conflict of interest.

AUTHOR CONTRIBUTIONS

Author contributions: J.D.d.B., L.C.B., S.F.d.S., J.Y., R.O., J.C.M., J.J.T.H.R., C.v.t.V., and T.v.d.P. conception and design of research; J.D.d.B., L.C.B., S.F.d.S., and J.Y. performed experiments; J.D.d.B., J.C.M., J.J.T.H.R., and C.v.t.V. analyzed data; J.D.d.B., J.C.M., J.J.T.H.R., C.v.t.V., and T.v.d.P. interpreted results of experiments; J.D.d.B. prepared figures; J.D.d.B. and T.v.d.P. drafted manuscript; J.D.d.B. edited and revised manuscript; J.D.d.B., L.C.B., S.F.d.S., J.Y., R.O., J.C.M., J.J.T.H.R., C.v.t.V., and T.v.d.P. approved final version of manuscript.

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