THE ROLE OF THE COMPLEMENT AND CONTACT SYSTEMS IN ASTHMA

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General introduction

GENERAL INTRODUCTION

Asthma is one of the most common chronic lung diseases, affecting over 300 million individuals of all ages worldwide[1]. Asthma is characterized by reversible airflow obstruction, bronchial hyperresponsiveness and airway inflammation. The pathophysiology of asthma is complex and its clinical presentation heterogeneous[2]. New insights in the immunology of asthma has spurt the introduction of classifying asthma subtypes based on distinct functional or pathophysiologic mechanisms, named phenotypes[2-4]. So far, two phenotypes have been identified amongst asthma patients; one with a T-helper cell type 2 (Th2) high- and the other with a Th2 low profile. The nomenclature has gradually shifted to 'type 2' rather than Th2 since it has become evident that beside Th2 cells, other immune cells, like innate lymphoid cells (ILCs) also produce Th2 associated cytokines[3]. Features of the type 2 high asthma phenotype correspond with the traditional dogma of asthma which dictates a Th2 (or ILC2) response accompanied by airway eosinophilia. The type 2 low phenotype shows the absence of a Th2 signature and is subdivided into neutrophilic and paucigranulocytic (normal airway leukocyte count) inflammation[5]. Recognizing the distinct phenotypes advances our understanding of the heterogeneous character of asthma and allows for the development of therapeutics targeting relevant inflammatory pathways.

Type 2 disorder in asthma

This thesis is mainly focused on the type 2 airway inflammation of asthma. Type 2 inflammatory responses are often initiated by environmental stimuli such as viruses, or allergens like house dust mite (HDM), animal dander and fungal spores[6] (Figure 1). The airway epithelium is part of the innate immune system and a pivotal orchestrator of airway inflammation by virtue of its capacity to secrete cytokines such as IL-33, IL-25 and thymic stromal lymphopoietin (TSLP). In non-allergic eosinophilic airway inflammation, group 2 ILCs (ILC2s) produce large amounts of IL-5 and IL-13 following stimulation by IL-33 and IL-25, thereby promoting early type 2 inflammation[6,7]. Atopy, defined as elevated serum IgE concentrations is absent in this type of inflammation. In atopic/allergic eosinophilic airway inflammation, dendritic cells (DCs) primed by TSLP and DC mediated antigen presentation induce the differentiation of Th2 cells, resulting in subsequent secretion of the typical type 2 cytokines IL-4, IL-5 and IL-13. These cytokines are responsible for many of the symptoms and histological changes seen in asthma[8]. IL-5 plays an integral role in the maturation, migration and survival of eosinophils. The biology of IL-13 and IL-4 shows a

high resemblance as both cytokines signal via the IL-4 receptor α chain and activate the transcription factor signal transducer and activator of transcription 6 (STAT6)[9,10]. Nonetheless, IL-13 and IL-4 play distinct roles during the course of allergic asthma. IL-13 has been documented to play an important role in airway hyperresponsiveness, goblet cell hyperplasia and mucus hypersecretion[8], whereas Th2 differentiation and cytokine production depend on IL-4 signaling. Moreover, IL-4 is essential for IgE and IgG1 isotype switching in B-cells[11]. The secretion of IgE is an important event in the early response following allergen exposure. Mast cells and basophils express the high-affinity receptor Fc epsilon RI (FcεRI) and are activated by IgE cross-linking. Upon activation, mast cells and basophils secrete bronchoconstricting contents and proinflammatory mediators that contribute to progression of type 2 immunity[6]. Notably, the classification of allergic and non-allergic responses are gradually fading as coexistence of Th2 and ILC mediated responses is observed in patients, thus underlining the complex heterogeneous character of asthma.

Major advances in the understanding of type 2 immunity has prompted the development of 'biologics', drugs that target key mediators of the type 2 immune response. Omalizumab, a monoclonal antibody neutralizing IgE, was the first clinically approved biologic for treatment of severe allergic asthma[12]. Since then, biologics targeting type 2 cytokines (IL-4, IL-5 and IL-13) have been intensively studied in clinical trials. In patients with a pronounced type 2 airway inflammation, antagonists targeting IL-5 (mepolizumab[13,14] and reslizumab[15,16]), the IL-5 receptor (benralizumab[17]) or IL-4 receptor α (dupilumab[18]) have shown impressive beneficial effects in alleviating asthma morbidity. This has spurt rapid development of drugs targeting upstream cytokines such as IL-33, IL-25 and TSLP[19]. Clinical trials evaluating the effectiveness and safety of these biologics are currently ongoing. Despite the success of these biologicals, about half of all asthma patients, characterized as type 2 low phenotype, do not respond to these drugs. Therefore, an unmet need remains to identify new inflammatory pathways that are involved in the pathogenesis and/or pathophysiology of asthma. One important and so far underexposed inflammatory pathway is the complement system.

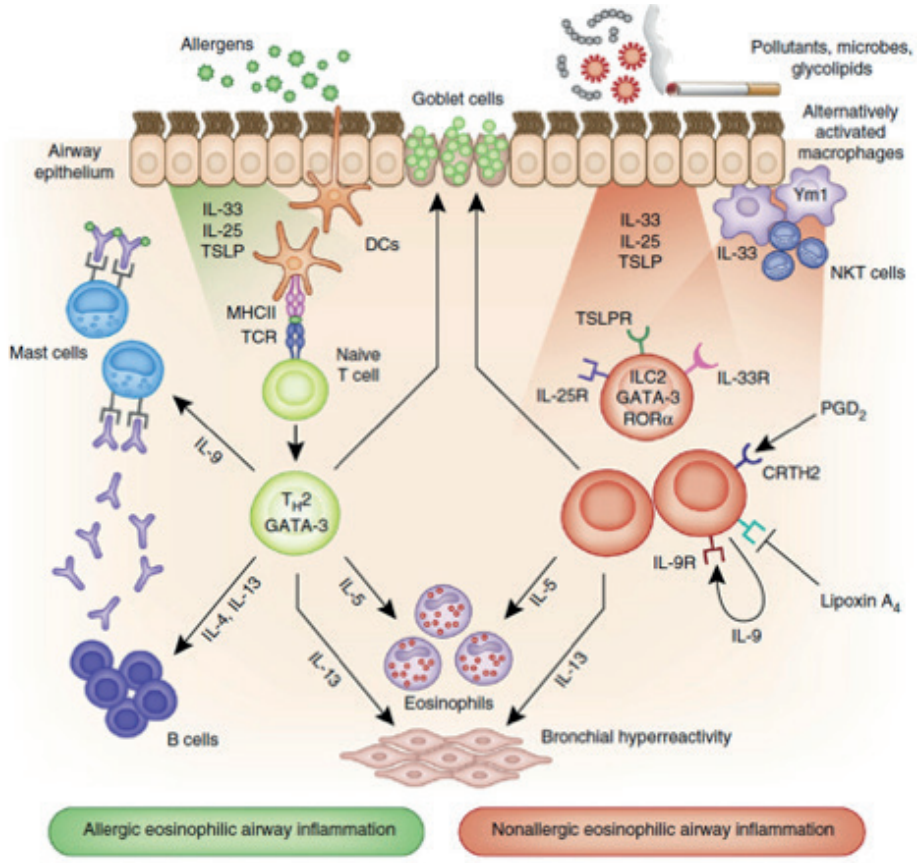

Figure 1. Schematic overview of the type 2 inflammatory response in two types of eosinophilic asthma. In atopic asthma (left), allergen exposure leads to DC activation which stimulates Th2 cells to secrete IL-5, IL-13 and IL-4. This results in eosinophilic airway inflammation, airway hyperresponsiveness (AHR) and IgE release from B-cells. In nonatopic asthma, ILC2s are the key producers of the type 2 cytokines IL-5 and IL-13 to elicit airway eosinophilia and AHR. Due to the absence of a specific allergen and IL-4 production, the involvement of an IgE response is lost in this asthma type. This figure is adapted from a previous review[6].

The complement system and asthma

Complement has a cardinal role in innate immunity to combat pathogens and altered host cells. The complement system consists of more than 40 plasma proteins that operate in plasma, tissues, on cell membranes and even within the cell[20]. Many of the complement proteins exert their function following cleavage activation. Activation of the complement system occurs via intrinsic and extrinsic mechanisms[21,22]. Intrinsic activation commences via one of the three

complement activation pathways: the classical, mannan binding lectin (MBL) and alternative pathway. Under asthmatic conditions, classical pathway activation occurs when allergen and specific antibodies aggregate and form complexes that activate C1q. This elicits conformational changes in C1q resulting in a cascade activation of C1r and C1s. Next, C1s cleaves C4 and C2 into fragments (C4a/b and C2a/b) that together are assembled as C3 convertase (C2aC4b). The MBL pathway is activated when polysaccharide structures on allergens are recognized by Mannan-binding lectin Associated Serine Protease (MASP) which leads to C4 and C2 cleavage and the formation of C3 convertase[23]. The enzyme C3 convertase then cleaves C3 into the functionally active fragments C3a and C3b (Figure 2). Subsequently, this leads to the formation of C5 convertase, that cleaves C5 into fragments C5a and C5b. Extrinsic activation of the complement system does not involve convertase formation, but rather depends on the presence of proteases that directly cleave C3 or C5 into their active fragments.

Until recently, anaphylatoxins (e.g. C3a and C5a) were solely regarded as pro-inflammatory mediators, but in the last decades researchers uncovered novel roles for the complement system, in particular the anaphylatoxins, in regulating the innate and adaptive immune systems[24,25]. Major advances have been made in understanding the role of anaphylatoxins in allergic lung diseases such as asthma[22,23,26]. The interest for the role of complement in asthma ignited following the observation of enhanced anaphylatoxin production in the airways of asthma patients, but not in healthy individuals, after allergen challenge[27]. Several studies demonstrated that C3a or C3a receptor signaling aggravates hallmarks of type 2 high asthma including a type 2 inflammatory profile, mucus hypersecretion and airway hyperresponsiveness[28,29]. C5a and C5a receptor signaling, on the other hand, play a dual role during the course of allergen induced asthma. During allergen sensitization, C5a/C5a receptor signaling protect against the onset of type 2 immune response[30,31], while in an established type 2 environment C5a/C5a receptor signaling contribute to the progression of Th2 inflammation[32,33]. These new insights suggest that the anaphylatoxins C3a and C5a are interesting therapeutic targets in the treatment of the type 2 asthma endotype.

The contact system and asthma

The contact system refers to a proteolytic system which is involved in blood coagulation and inflammation. This system consists of four major components: Factor XI (FXI), Factor XII F(XII), kallikrein and High Molecular Weight Kininogen (HMWK). Activation of the contact system can occur through auto activation of FXII (Figure 2). Activated FXII (FXIIa) converts the zymogen prekallikrein

into kallikrein which reciprocally activates FXII, thus amplifying this activation loop[34]. These events lead to FXIIa mediated FXI activation thereby initiating the subsequent activation of the intrinsic coagulation pathway. Secondly, kallikrein cleaves HMWK to liberate the proinflammatory protein, bradykinin[35,36] (Figure 2).

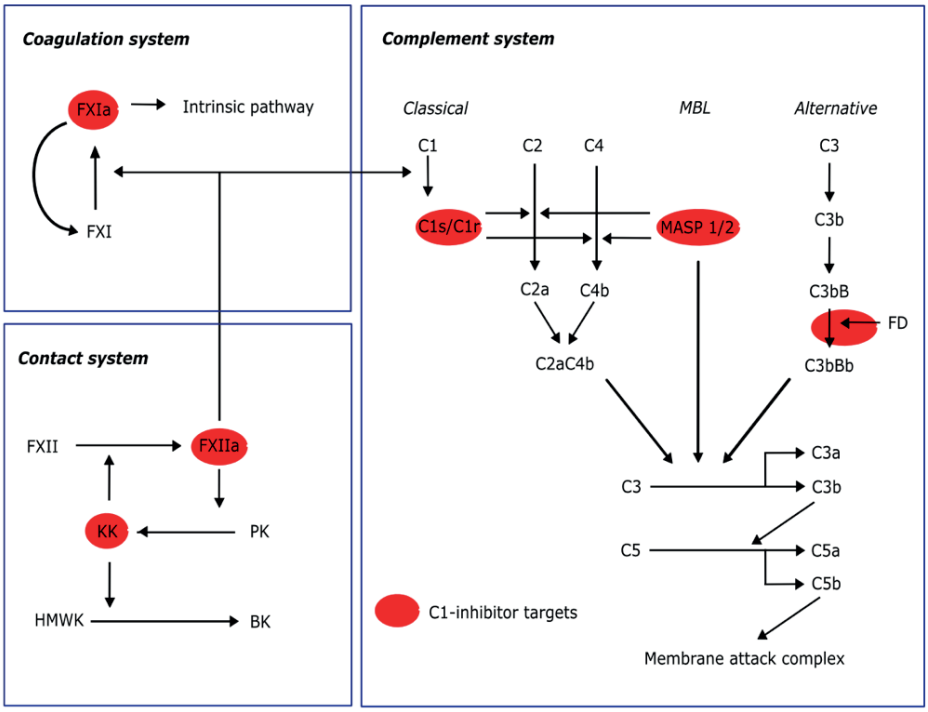

Figure 2. A simplified schematic representation depicting C1-inhibitor's regulatory functions in the plasma cascade systems. F: clotting factor, C: complement component, MBL: mannan-binding lectin, MASP: mannan-binding lectin associated serine protease, FD: Factor D, PK: prekallikrein, KK: kallikrein, HMWK: High Molecular Weight Kininogen, BK: bradykinin. Modified from[44].

Compelling evidence from animal studies demonstrated that intrabronchial administration of bradykinin induces bronchoconstriction, mucus secretion and vascular permeability[37]. During allergic lung inflammation, lung mRNA of the bradykinin B_2 receptor is upregulated with concurrent enhancement of bradykinin mediated bronchoconstriction in allergen sensitized animals[38,39]. Moreover, bradykinin receptor antagonists attenuate allergen induced airway hyperresponsiveness[40,41] and reduce the release of inflammatory mediators such as prostaglandins, thromboxane and leukotrienes[40] in a sheep asthma model. The potential role of the contact system in humans has been suggested

by the finding that inhaled bradykinin elicited bronchoconstriction in asthma patients but not in healthy individuals[42]. In addition, kinin levels were increased in the bronchoalveolar lavage fluid collected from asthma patients after allergen challenge or during symptomatic relapse and correlated with enhance tissue kallikrein activity[43]. Despite previous work, our current understanding regarding the involvement of different key components of the contact system in asthma is limited.

C1-inhibitor as regulator of the complement and contact system

C1-inhibitor is a plasma protein that belongs to the family of serine protease inhibitors (serpins). With its protease inhibitory properties, C1-inhibitor controls the activation of plasmatic cascade systems, in particular the complement and the contact system, thus reducing the generation of proinflammatory mediators (Figure 2). C1-inhibitor's highly glycosylated N-terminal domain may exert additional protease independent anti-inflammatory effects since it can directly bind lipopolysaccharide (LPS) and due to its involvement in neutrophil–endothelial cell interactions[44]. C1-inhibitor is a pivotal regulator of the complement system by virtue of its capacity to inhibit both the classical pathway (by inhibiting C1s/C1r) and the MBL pathway (by inhibiting MASP 1/2)[43]. Moreover, it can inhibit the alternative pathway by reversible binding of C3b in a non-serpin manner[45]. In addition, C1-inhibitor regulates other inflammatory cascades, in particular the contact system by inhibiting the generation of FXIIa and kallikrein and thereby their reciprocal activation.

The importance of C1-inhibitor is illustrated in patients with hereditary angioedema, in whom a functional deficiency of C1-inhibitor leads to uncontrolled activation of the contact and complement system. As a consequence, uncontrolled generation of vasoactive peptides occurs, facilitating the onset of potentially life-threatening subcutaneous edema[44]. The availability of plasma derived C1-inhibitor in the clinic together with C1-inhibitor's diverse regulatory functions has encouraged investigators to conduct explorative studies in other complement-related diseases. Preclinical studies in animals have provided evidence that C1-inhibitor might be an effective therapeutic in sepsis[46,47], ischemia-reperfusion injury[48], transplantation[49] and pneumococcal meningitis[50]. In accordance, C1-inhibitor administration has shown marked inhibition of complement system activation and beneficial anti-inflammatory effects in humans with sepsis[51-53] and ischemia-reperfusion injury[54].

Experimental models used in this thesis

In order to study the effect of C1-inhibitor on airway inflammation in asthma patients, we used a clinically relevant model of segmental provocation with HDM and LPS. This combination is used since HDM is an important domestic allergen with high sensitization rate[55] and a frequent cause of asthma exacerbations[56]. Moreover, LPS is a widespread environmental pollutant and abundantly present in house dust[57] together with HDM, indicating that natural exposure to both HDM and LPS is a likely event. In this model, asthma patients are challenged by bronchoscopy with HDM and LPS in a segment of the lung and with saline in a segment of the contralateral lung as control. A bilateral bronchoalveolar lavage is performed after seven hours, which coincides with the early entry of leukocytes (in particular eosinophils[58]) into the airways. Previously, it has been documented that a simultaneous challenge with HDM/LPS induces a strong influx of eosinophils and neutrophils into the airways of HDM sensitized asthma patients[43,59]. Recent evidence has linked such mixed eosinophilic and neutrophilic airway inflammation with a corticoid unresponsive asthma phenotype[60], which makes the current challenge model relevant for evaluating new therapeutic compounds.

In the second part of this thesis we utilized a HDM induced asthma mouse model to study the role of the complement and contact systems in the pathogenesis of asthma. In this acute asthma model, mice were intranasally challenged with HDM during the sensitization (day 0, 1, 2) and the challenge phase (day 14, 15, 18, 19), thereby attempting to mimic the course of asthma in humans. This HDM induced asthma mouse model allows for the evaluation of the hallmark features also observed in patients with allergic asthma[6], such as a pulmonary type 2 immune response, a pronounced eosinophilic airway inflammation, elevated plasma IgE concentrations, mucus hypersecretion and airway hyperresponsiveness. Utilizing mice with genetic deficiency in or pharmaceutical agents blocking a component belonging to either the complement or contact system, enabled us to investigate the role of these systems in HDM induced airway inflammation.

Scope of this thesis

The aim of this thesis is to better understand inflammatory mechanisms of asthma other than type 2 cytokine-driven responses by investigating the therapeutic potential of C1-inhibitor in asthma patients and to study role of the complement and contact systems (both regulated by C1-inhibitor) in lung inflammation associated with type 2 high asthma. **Chapter 2** entails the primary project of this thesis, the CAST study: a double blind randomised

placebo controlled study investigating the prophylactic effect of C1-inhibitor administration on allergen (HDM/LPS) induced inflammatory markers. In **chapter 3** we assessed genome-wide transcriptome changes in alveolar macrophages harvested from asthma patients after intrabronchial HDM/LPS (or saline control) challenge. In **chapter 4** we studied the effect of C1-inhibitor administration on airway inflammation and hyperresponsiveness in HDM induced allergic lung inflammation in mice. Chapters 5 and 6 are focused on the role of anaphylatoxins (C3a and C5a), activation products of the complement system, on immune cells with a pivotal role in eosinophilic type 2 high asthma. **Chapter 5** describes the chemoattractant effect of C3a on eosinophil migration using an adoptive transfer model of bone marrow derived eosinophils, complemented by an *ex vivo* chemotaxis assay. In **chapter 6** we studied how C5 inhibition in sensitized mice affects the HDM induced type 2 immune response, thereby highlighting alterations in the enumeration and differentiation of Th2 cells and ILC2s. Mast cells have an important proinflammatory effect in the early phase following allergen exposure and the anaphylatoxin C5a is a strong activator of mast cells. In **chapter 7** we investigated the role of mast cells in the HDM-induced allergic pulmonary response using mast cell deficient mice. Chapters 8 and 9 address the role of the contact system in the pathogenesis of allergen induced lung inflammation. In **chapter 8** we report on the role of FXI and FXII in HDM induced asthma mouse model. **Chapter 9** presents findings on how kininogen deficiency or depletion affect asthma hallmarks in the same model. Finally, the findings of our investigation are summarized and discussed in **chapter 10**.

REFERENCES

1. To T, Stanojevic S, Moores G, et al. Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC Public Health.* 2012;12:204.
2. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet.* 2018;391(10122):783-800.
3. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol.* 2015;15(1):57-65.
4. Kaur R, Chupp G. Phenotypes and endotypes of adult asthma: Moving toward precision medicine. *J Allergy Clin Immunol.* 2019;144(1):1-12.
5. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology.* 2006;11(1):54-61.
6. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* 2015;16(1):45-56.
7. Kuruvilla ME, Lee FE, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clin Rev Allergy Immunol.* 2019;56(2):219-233.
8. Gour N, Wills-Karp M. IL-4 and IL-13 signaling in allergic airway disease. *Cytokine.* 2015;75(1):68-78.
9. Munitz A, Brandt EB, Mingler M, Finkelman FD, Rothenberg ME. Distinct roles for IL-13 and IL-4 via IL-13 receptor alpha1 and the type II IL-4 receptor in asthma pathogenesis. *Proc Natl Acad Sci U S A.* 2008;105(20):7240-7245.
10. Kuperman D, Schofield B, Wills-Karp M, Grusby MJ. Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med.* 1998;187(6):939-948.
11. Snapper CM, Finkelman FD, Paul WE. Differential regulation of IgG1 and IgE synthesis by interleukin 4. *J Exp Med.* 1988;167(1):183-196.
12. Israel E, Reddel HK. Severe and Difficult-to-Treat Asthma in Adults. *N Engl J Med.* 2017;377(10):965-976.
13. Ortega HG, Liu MC, Pavord ID, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med.* 2014;371(13):1198-1207.
14. Bel EH, Wenzel SE, Thompson PJ, et al. Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma. *N Engl J Med.* 2014;371(13):1189-1197.
15. Castro M, Zangrilli J, Wechsler ME, et al. Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet Respir Med.* 2015;3(5):355-366.
16. Corren J, Weinstein S, Janka L, Zangrilli J, Garin M. Phase 3 Study of Reslizumab in Patients With Poorly Controlled Asthma: Effects Across a Broad Range of Eosinophil Counts. *Chest.* 2016;150(4):799-810.
17. Nair P, Wenzel S, Rabe KF, et al. Oral Glucocorticoid-Sparing Effect of Benralizumab in Severe Asthma. *N Engl J Med.* 2017;376(25):2448-2458.
18. Castro M, Corren J, Pavord ID, et al. Dupilumab Efficacy and Safety in Moderate-to-Severe Uncontrolled Asthma. *N Engl J Med.* 2018;378(26):2486-2496.
19. Eger KA, Bel EH. The emergence of new biologics for severe asthma. *Curr Opin Pharmacol.* 2019;46:108-115.
20. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. *Front Immunol.* 2015;6:257.
21. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol.* 2015;6:262.
22. Laumonnier Y, Wiese AV, Figge J, Karsten C. Regulation and function of anaphylatoxins and their receptors in allergic asthma. *Mol Immunol.* 2017;84:51-56.
23. Zhang X, Kohl J. A complex role for complement in allergic asthma. *Expert Rev Clin Immunol.* 2010;6(2):269-277.

24. Kolev M, Le Friec G, Kemper C. Complement--tapping into new sites and effector systems. *Nat Rev Immunol.* 2014;14(12):811-820.
25. Liszewski MK, Kolev M, Le Friec G, et al. Intracellular complement activation sustains T cell homeostasis and mediates effector differentiation. *Immunity.* 2013;39(6):1143-1157.
26. Schmudde I, Laumonnier Y, Kohl J. Anaphylatoxins coordinate innate and adaptive immune responses in allergic asthma. *Semin Immunol.* 2013;25(1):2-11.
27. Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med.* 2001;164(10 Pt 1):1841-1843.
28. Drouin SM, Corry DB, Hollman TJ, Kildsgaard J, Wetsel RA. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J Immunol.* 2002;169(10):5926-5933.
29. Drouin SM, Corry DB, Kildsgaard J, Wetsel RA. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J Immunol.* 2001;167(8):4141-4145.
30. Zhang X, Lewkowich IP, Kohl G, Clark JR, Wills-Karp M, Kohl J. A protective role for C5a in the development of allergic asthma associated with altered levels of B7-H1 and B7-DC on plasmacytoid dendritic cells. *J Immunol.* 2009;182(8):5123-5130.
31. Drouin SM, Sinha M, Sfyroera G, Lambris JD, Wetsel RA. A protective role for the fifth complement component (c5) in allergic airway disease. *Am J Respir Crit Care Med.* 2006;173(8):852-857.
32. Kohl J, Baelder R, Lewkowich IP, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest.* 2006;116(3):783-796.
33. Baelder R, Fuchs B, Bautsch W, et al. Pharmacological targeting of anaphylatoxin receptors during the effector phase of allergic asthma suppresses airway hyperresponsiveness and airway inflammation. *J Immunol.* 2005;174(2):783-789.
34. Kaplan AP, Ghebrehiwet B. The plasma bradykinin-forming pathways and its interrelationships with complement. *Mol Immunol.* 2010;47(13):2161-2169.
35. Renne T. The procoagulant and proinflammatory plasma contact system. *Semin Immunopathol.* 2012;34(1):31-41.
36. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost.* 2016;14(1):28-39.
37. Abraham WM, Scuri M, Farmer SG. Peptide and non-peptide bradykinin receptor antagonists: role in allergic airway disease. *Eur J Pharmacol.* 2006;533(1-3):215-221.
38. Hannon JP, Tigani B, Williams I, Mazzoni L, Fozard JR. Mechanism of airway hyperresponsiveness to adenosine induced by allergen challenge in actively sensitized Brown Norway rats. *Br J Pharmacol.* 2001;132(7):1509-1523.
39. Fozard JR, Hannon JP. Species differences in adenosine receptor-mediated bronchoconstrictor responses. *Clin Exp Allergy.* 2000;30(9):1213-1220.
40. Abraham WM, Burch RM, Farmer SG, Sielczak MW, Ahmed A, Cortes A. A bradykinin antagonist modifies allergen-induced mediator release and late bronchial responses in sheep. *The American review of respiratory disease.* 1991;143(4 Pt 1):787-796.
41. Soler M, Sielczak M, Abraham WM. A bradykinin-antagonist blocks antigen-induced airway hyperresponsiveness and inflammation in sheep. *Pulm Pharmacol.* 1990;3(1):9-15.
42. Proud D, Baumgarten CR, Naclerio RM, Ward PE. Kinin metabolism in human nasal secretions during experimentally induced allergic rhinitis. *J Immunol.* 1987;138(2):428-434.
43. de Boer JD, Berger M, Majoor CJ, et al. Activated protein C inhibits neutrophil migration in allergic asthma: a randomised trial. *Eur Respir J.* 2015;46(6):1636-1644.
44. Zeerleder S. C1-inhibitor: more than a serine protease inhibitor. *Semin Thromb Hemost.* 2011;37(4):362-374.

45. Jiang H, Wagner E, Zhang H, Frank MM. Complement 1 inhibitor is a regulator of the alternative complement pathway. *J Exp Med.* 2001;194(11):1609-1616.

46. Jansen PM, Eisele B, de Jong IW, et al. Effect of C1 inhibitor on inflammatory and physiologic response patterns in primates suffering from lethal septic shock. *J Immunol.* 1998;160(1):475-484.

47. Liu D, Lu F, Qin G, Fernandes SM, Li J, Davis AE, 3rd. C1 inhibitor-mediated protection from sepsis. *J Immunol.* 2007;179(6):3966-3972.

48. Horstick G, Heimann A, Gotze O, et al. Intracoronary application of C1 esterase inhibitor improves cardiac function and reduces myocardial necrosis in an experimental model of ischemia and reperfusion. *Circulation.* 1997;95(3):701-708.

49. Salvatierra A, Velasco F, Rodriguez M, et al. C1-esterase inhibitor prevents early pulmonary dysfunction after lung transplantation in the dog. *Am J Respir Crit Care Med.* 1997;155(3):1147-1154.

50. Zwijnenburg PJ, van der Poll T, Florquin S, et al. C1 inhibitor treatment improves host defense in pneumococcal meningitis in rats and mice. *J Infect Dis.* 2007;196(1):115-123.

51. Caliezi C, Zeerleder S, Redondo M, et al. C1-inhibitor in patients with severe sepsis and septic shock: beneficial effect on renal dysfunction. *Crit Care Med.* 2002;30(8):1722-1728.

52. Zeerleder S, Caliezi C, van Mierlo G, et al. Administration of C1 inhibitor reduces neutrophil activation in patients with sepsis. *Clin Diagn Lab Immunol.* 2003;10(4):529-535.

53. Igonin AA, Protsenko DN, Galstyan GM, et al. C1-esterase inhibitor infusion increases survival rates for patients with sepsis*. *Crit Care Med.* 2012;40(3):770-777.

54. de Zwaan C, Kleine AH, Diris JH, et al. Continuous 48-h C1-inhibitor treatment, following reperfusion therapy, in patients with acute myocardial infarction. *Eur Heart J.* 2002;23(21):1670-1677.

55. Peat JK, Tovey E, Toelle BG, et al. House dust mite allergens. A major risk factor for childhood asthma in Australia. *Am J Respir Crit Care Med.* 1996;153(1):141-146.

56. Platts-Mills TA, Tovey ER, Mitchell EB, Moszoro H, Nock P, Wilkins SR. Reduction of bronchial hyperreactivity during prolonged allergen avoidance. *Lancet.* 1982;2(8300):675-678.

57. Carnes MU, Hoppin JA, Metwali N, et al. House Dust Endotoxin Levels Are Associated with Adult Asthma in a U.S. Farming Population. *Ann Am Thorac Soc.* 2017;14(3):324-331.

58. Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin Exp Immunol.* 1998;114(2):137-146.

59. Berger M, de Boer JD, Bresser P, et al. Lipopolysaccharide amplifies eosinophilic inflammation after segmental challenge with house dust mite in asthmatics. *Allergy.* 2015;70(3):257-264.

60. Martinez FD, Vercelli D. Asthma. *Lancet.* 2013;382(9901):1360-1372.

Effect of C1-inhibitor in adults with mild asthma: a randomized controlled trial

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ABSTRACT

Complement system activation occurs in the airways of asthma patients when exposed to allergens. Multiple preclinical studies showed that complement activation products promote allergic inflammatory responses. We aimed to study the effect of C1-inhibitor (C1-INH) on airway inflammation induced by segmental pulmonary instillation of house dust mite (HDM) and lipopolysaccharide (LPS) in allergic asthma patients. Patients were randomised to receive intravenous C1-INH (100 U·kg–1; n=12) or placebo (n=12). Two hours after the start of infusion, patients were challenged in one lung segment with saline (control) and in a contralateral segment with HDM/LPS, thereby mimicking environmental house dust exposure. Measurements were done in bronchoalveolar lavage fluid (BALF) collected from each challenged lung segment by bronchoscopy seven hours after intrabronchial instillation. C1-INH prevented HDM/LPS induced complement activation at the level of C3a and C4a. HDM/LPS induced influx of eosinophils and neutrophils, as well as degranulation of these cells (eosinophil peroxidase, myeloperoxidase, elastase) together with release of 15 cytokines and chemokines; these responses were not influenced by C1-INH. HDM/LPS instillation resulted in increased vascular leak into the bronchoalveolar space (albumin, α2-macroglobulin, IgM), which was abrogated by C1-INH infusion. These results suggest that intravenous C1-INH administration prior to intrabronchial HDM/LPS challenge prevents complement activation and vascular leak without attenuating allergic lung inflammation in patients with HDM allergy and asthma.

INTRODUCTION

A substantial subset of asthma patients suffers from uncontrolled disease under current standard treatments[1]. The majority of these patients display a chronic airway inflammation that is characterized by airway eosinophilia, neutrophilia or a mixed feature[2,3]. This chronic inflammatory response contributes to deteriorating lung function, frequent asthma exacerbations and unresponsiveness to conventional medication such as inhaled corticosteroids, long acting beta-adrenergic receptor agonists and leukotriene receptor antagonists[4]. New therapeutics have been developed that target specific components of a Th2 response[1]. Although these drugs are highly effective to treat patients with a pronounced type 2 inflammation phenotype, they have not been clinically beneficial in a broad asthma population. Beside a Th2 inflammation, most patients with adequately treated and still uncontrolled asthma demonstrate a neutrophilic or a mixed type of inflammation[3]. Therefore, a need remains to develop new anti-inflammatory treatments that provide benefit beyond current conventional therapeutics.

A promising target for new therapeutic agents in asthma is the complement system. In recent years, this system has been implicated in the pathogenesis of Th2 asthma[5,6]. Allergen derived proteases and allergen-antibody complexes can activate the complement system, and elevated levels of anaphylatoxins, activation products of the complement system, have been shown in the airways of asthma patients following local allergen provocation[7,8]. Anaphylatoxins regulate the inflammatory response by interacting with their corresponding receptors expressed on various myeloid cells including macrophages, neutrophils and eosinophils. Under allergic inflammatory conditions, enhanced anaphylatoxin receptor expression was observed in bronchial epithelial and smooth muscles cells, showing the potential of anaphylatoxins to influence a variety of cells considered important in asthma[5,6]. Furthermore, functional roles for the anaphylatoxins C3a[9,10] and C5a[11,12] in asthma have been established in experimental studies in mice, showing that these proinflammatory mediators act synergistically and drive allergic inflammation, including enhanced recruitment of eosinophils, Th2 cytokine release and mucus production, during the effector phase in a sensitized host[5].

The complement system consists of three biochemical cascades: the classical, lectin and alternative pathway[13]. C1-inhibitor (C1-INH) is an endogenous protein with a pivotal regulatory function in the complement system by virtue

of its capacity to inhibit both the classical and lectin pathway. C1-INH is also an important inhibitor of the kallikrein-kinin system through its capacity to bind activated Factor XII (FXIIa) and to inhibit its activity[13]. In addition, C1-INH mitigates kallikrein activity, which leads to a decreased formation of bradykinin. Bradykinin induces vasodilation and promotes asthma associated vascular permeability[14]. Vascular leak often occurs as consequence of allergen induced inflammation in the airway of patients with asthma[15]. C1-INH is an approved therapeutic for the treatment of hereditary angioedema and has been tested in explorative studies involving other complement-related diseases like sepsis[16,17] and ischemia-reperfusion injury[18]. We hypothesized that C1-INH administration inhibits complement activation and attenuates allergen-induced airway eosinophilia in patients with mild asthma. In this proof-of-concept study we studied the effect of C1-INH on airway inflammatory responses evoked by intrabronchial allergen challenge in patients with mild asthma.

METHODS

This single center, randomised, double-blind, placebo-controlled, parallel proof-of-concept study assessed the effect of intravenous C1-INH following segmental allergen challenge in 24 house dust mite (HDM)-allergic asthma patients. The Medical Ethics Committee of the Academic Medical Center, Amsterdam (the Netherlands), approved the study and all patients gave written informed consent. The study is registered at ClinicalTrials.gov (identifier: NCT03051698).

Patients and design

Two hours (t=-2h) before allergen challenge, patients were randomly assigned to start on continuous intravenous infusion with nanofiltered human plasma-derived C1-INH (Cinryze®, Shire ViroPharma Inc., Lexington, MA, USA) 100 U·kg–1·h–1 or placebo (figure 1). Randomization was done by the hospital pharmacy in a block-wise manner (groups of four), allowing balanced assignment. Both patients and investigators were blinded for treatment allocation. Allocation key was given after study completion. Segmental challenge by bronchoscopy was conducted as previously described[8]. Briefly, a flexible fiberoptic videobronchoscope was used to instill one lung segment (middle lobe or lingula) with normal saline (serving as control) followed by instillation of the contralateral segment (middle lobe or lingula) with a combination of HDM extract (50 biological units, *Dermatophagoides pteronyssinus* origin;

Allergopharma, Zeist, the Netherlands) and lipopolysaccharide (LPS; from *Escherichia coli*, Clinical Center Reference Endotoxin, 75ng, kindly provided by Anthony Suffredini, National Institute of Health, Bethesda, MD, USA), thereby seeking to imitate a natural allergen exposure in the airways[19,20]. Seven hours post-challenge, a second bronchoscopy with bronchoalveolar lavage (BAL) of the two instilled segments was performed for analyses[8,21]. The primary outcome was influx of eosinophils and neutrophils, defined as number of cells/ml, into the bronchoalveolar space. As secondary outcomes we determined markers for eosinophil and neutrophil activation, cytokine release and vascular permeability. Further details about measurements and assays are described in the online supplementary material.

Screening procedures

The screening procedures and criteria were similar to a previous study from our group[8]. In brief, the screening consisted of medical history taking, physical examination, routine laboratory tests and spirometry to determine baseline lung function and airway hyperresponsiveness (PC20 defined as concentration methacholine bromide required to achieve 20% fall in Forced Expiratory Volume in 1 second, FEV1). Inclusion criteria were intermittent-to-mild asthma according to criteria of the Global Initiative for Asthma[4], sensitization to HDM confirmed by a positive skin prick test and a positive radioallergosorbent test, FEV1 higher than 70% of the predicted value, PC20 between 1.2 – 9.6 mg/ml (corresponding with increased airway hyperresponsiveness)[22], no clinically significant abnormalities during physical examination, hematological and biochemical screening, signed informed consent, controlledasthma defined as Asthma Control Questionnaire score <1.5, age between 18 and 45 years, no smoking for at least 1 year and less than 10 pack years of smoking history and not pregnant.

Bronchoalveolar lavage handling

We lavaged the saline-challenged subsegment of the lung with eight successive 20 ml aliquots of saline. This procedure was repeated in the HDM+LPS challenged subsegment of the contralateral lung. BAL fluid (BALF) was pooled (per lung subsegment) and centrifuged at 4°C and 400g for 10 minutes. Before centrifugation, 10mM ethylenediaminetetraacetic acid (EDTA), 10mM benzamidine and 0.2mg/ml soybean trypsin inhibitor were added to BALF to inhibit *ex vivo* activation and degradation of complement products. After centrifugation, the cell-free supernatant was snap frozen in liquid nitrogen and stored at -80°C until analysis. Blood was collected in tubes containing citrate, EDTA or heparin, before the start of C1-INH or placebo treatment, and directly

before the first and second bronchoscopy. Blood samples were centrifuged at 4°C, 1000g for 10 minutes. Plasma was snap frozen in liquid nitrogen and stored at -80°C until analysis.

Assays and flow cytometry

See supplemental material.

Statistical analysis

Patient characteristics were compared using a T-test or Mann-Whitney U-test where appropriate. Comparisons of BALF measurements were done between samples obtained from the saline and HDM/LPS challenged site in each treatment group (C1-INH and placebo) using Wilcoxon ranked test. Serial comparison of plasma measurements were analyzed by two-way analysis of variance and Sidak's test to correct for repeated measurements. A p-value of <0.05 was considered statistically significant.

RESULTS

Patients

The study was originally planned to enroll 20 patients per group. With 40 patients, the study would have had a power of 80% to detect a 50% reduction in eosinophil counts with a significance level of 0.05 considering a standard deviation of $10^5/l^8$. Data from an interim analysis of 24 patients (12 per treatment group) showed no differences nor a possible trend in eosinophil or neutrophil influx between the placebo and C1-INH group. Considering the invasive character of the bronchoscopies and the unlikely effect of C1-INH on the primary outcome measure, the study was discontinued. At that moment 25 patients had been randomized. One patient (placebo) did not undergo a second bronchoscopy as a result of intolerance to introduction of the bronchoscope. Analyses are based on the results of the 24 patients from whom BALF was obtained (figure 1). Baseline patient characteristics were similar across treatment groups (table 1).

Clinical responses

C1-INH administration was well tolerated. Segmental bronchial challenge with HDM and LPS did not induce clinical symptoms. One patient from the placebo group experienced dyspnea and had temporary hypoxaemia after completing the BAL procedures.

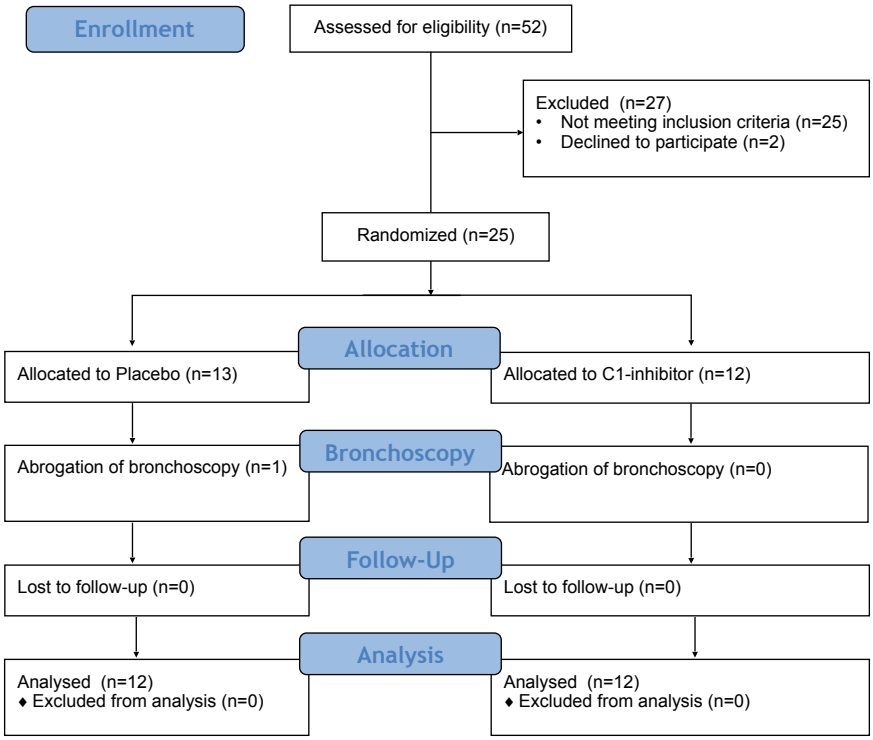

Figure 1. Flow diagram of the study

Table 1. Baseline patient characteristics

	Placebo	C1-inhibitor	Reference range	p-value
General				
Patients with asthma# n	12	12		
Female	9	9	NA	0.999
Age, years	23.7 (0.53)	25.3 (1.08)	NA	0.202
Asthma related symptoms¶ %				
Wheezing	75	83.3	NA	0.633
Cough	75	58.3	NA	0.409
Dyspnea	83.3	91.7	NA	0.557
Chest tightness	50	58.3	NA	0.698
Seasonal variability	75	66.7	NA	0.670
Symptom progression at night	33.3	16.7	NA	0.368
Eczema	58.3	33.3	NA	0.237
Allergic rhinitis	75	66.7	NA	0.670
Family history of atopy	58.3	83.3	NA	0.193
Allergy				
House dust mite %	100	100	NA	
Total IgE, kU/L	349.5 (69.2)	452.72 (88.35)	0-100	0.368
D. pteronyssinus IgE, kU/L	43.9 (10)	69.7 (8.83)	0-0.35	0.068
Lung spirometry				
FEV1 L	3.76 (0.26)	3.82 (0.24)	NA	0.858
FEV1 % of predicted	98.2 (3.32)	99.6 (2.85)	100	0.749
FVC L	4.8 (0.46)	5.02 (0.38)	NA	0.730
FVC % of predicted	96.7 (9.41)	112.2 (3.71)	100	0.140
Methacholine PC20, mg/mL†	2.6 (1.7-4.5)	2.4 (2.1-4.0)	1.2-9.6	

Data are presented as mean ±SEM unless otherwise stated. D. pteronyssinus: Dermatophagoides pteronyssinus; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; PC20: provocative dose causing a 20% fall in FEV1; NA: not applicable. #: Global Initiative for Asthma stage I/II.¶ Symptoms occurred in last 6 months prior to study participation. † Data are expressed as median with interquartile range

C1-INH antigen and activity levels in plasma and BALF

At baseline patients from both groups showed similar plasma C1-INH antigen levels. Two hours after the initiation of C1-INH infusion (i.e., at the time of the bronchial challenge), median plasma C1-INH antigen concentrations were four times higher in C1-INH infused patients when compared to vehicle infused controls (figure 2A); plasma C1-INH concentrations remained elevated during the following seven hours. Segmental HDM/LPS challenge resulted in increased

C1-INH antigen levels in BALF when compared to saline instillation in both treatment groups (figure 2C). C1-INH concentrations were higher in BALF from patients infused with C1-INH than in BALF from those who received placebo. C1-INH activity levels in plasma and BALF were similar to C1-INH antigen concentrations (figure 2B,D), indicating that the vast majority of C1-INH (both endogenous and infused) was biologically active.

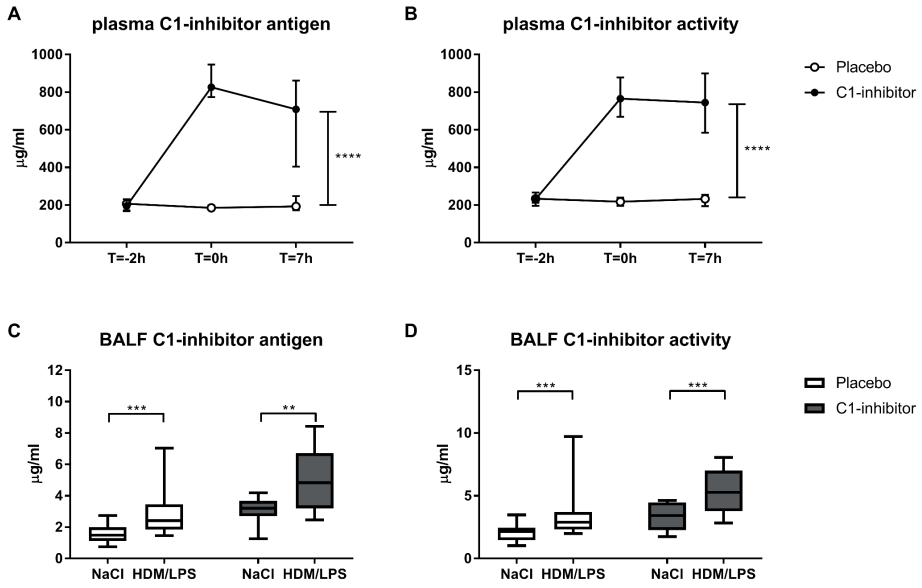

Figure 2. C1-inhibitor administration results in elevated plasma and bronchoalveolar C1-INH antigen and activity. (A) plasma C1-INH antigen, (B) plasma C1-INH activity, (C) BALF C1-INH antigen, (D) Plasma C1-INH activity. Data are expressed as median with interquartile range, the smallest and largest observation. **: *P*<0.01, ***: *P*<0.001, ****: *P*<0.0001 BALF: bronchoalveolar lavage

Effect of C1-INH on complement activation following bronchial HDM/LPS challenge

HDM/LPS challenge induced elevated C4a concentrations as compared to saline challenge in the placebo group (figure 3A). In the C1-INH group BALF C4a levels were not different between the saline and HDM/LPS challenged sites. Consistently, using an assay that detects the C4 activation products C4b, C4bi, and C4c (collectively referred to as C4bc[23]) we detected an increase in C4 activation in the lung subsegment exposed to HDM/LPS in patients infused with placebo but not in those infused with C1-INH (figure 3B).

We next assessed whether C1-INH could influence the common activation pathway. To that end, we measured the anaphylatoxin C3a, which is released following C3 cleaved activation[24]. Similar to C4a, HDM/LPS challenge elicited increased BALF C3a in the placebo group, but not in the C1-INH treatment group (figure 3C). In agreement, using an assay that detects the C3 activation products C3b, C3bi, and C3c (collectively named C3bc[23]) we detected increased C3 activation in the HDM/LPS challenged lung in patients infused with placebo but not in those administered with C1-INH (figure 3D). These data indicate C1-INH infusion prevents C4a and C3a generation in the airways upon a bronchial challenge with HDM/LPS.

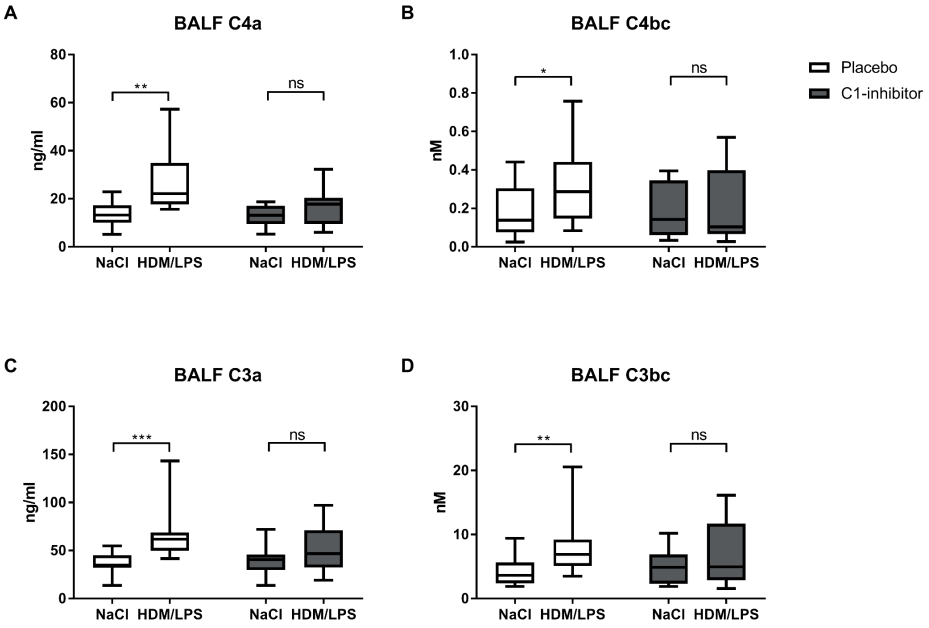

Figure 3. Intravenous C1-inhibitor administration prevents C3a and C4a formation after HDM/LPS challenge in the airways of asthma patients. (A) BALF C4a levels, (B) BALF C4bc levels, (C) BALF C3a levels, (D) BALF C3bc levels. Data represent the median with interquartile range, the smallest and largest observation. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, ns: not significant, BALF: bronchoalveolar lavage

Effect of C1-INH on leukocyte influx induced by HDM/LPS challenge

HDM/LPS instillation augmented total cell counts in BALF compared to saline, which was mainly due to influx of eosinophils and neutrophils (figure 4A-C). C1-INH did not modify this allergen-induced response. HDM/LPS also induced degranulation of eosinophils (reflected by eosinophil peroxidase

levels) and neutrophils (myeloperoxidase, elastase and lactoferrin levels) in the bronchoalveolar space (figure 5A-D). These responses were not affected by C1-INH with the exception of lactoferrin release, which was inhibited by C1-INH (figure 5B). C1-INH did not impact the HDM/LPS-induced upregulation of neutrophil CD11b expression (figure S1 in online supplement). HDM/LPS challenge did not modify alveolar macrophage numbers (figure S2A). HDM/LPS challenge elicited a small increase in BALF CD4 T-cells numbers which did not differ significantly between the placebo and C1-INH group (figure S2B).

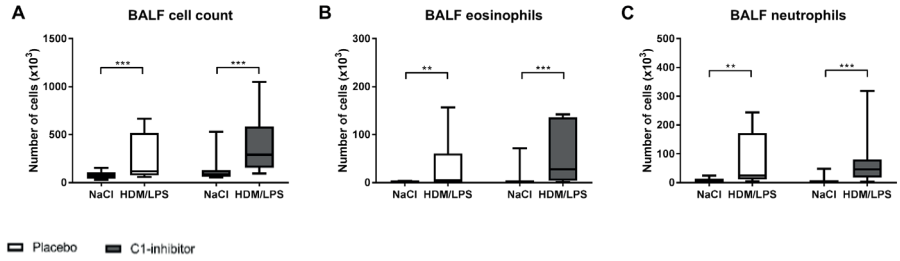

Figure 4. Intravenous C1-inhibitor infusion does not modify leukocyte influx after HDM/LPS challenge in the airways of asthma patients. (A) Total cell number in BALF, (B) eosinophils in BALF, (C) neutrophils in BALF. Data represent the median with interquartile range, the smallest and largest observation. **: *P*<0.01, ***: *P*<0.001, BALF: bronchoalveolar lavage

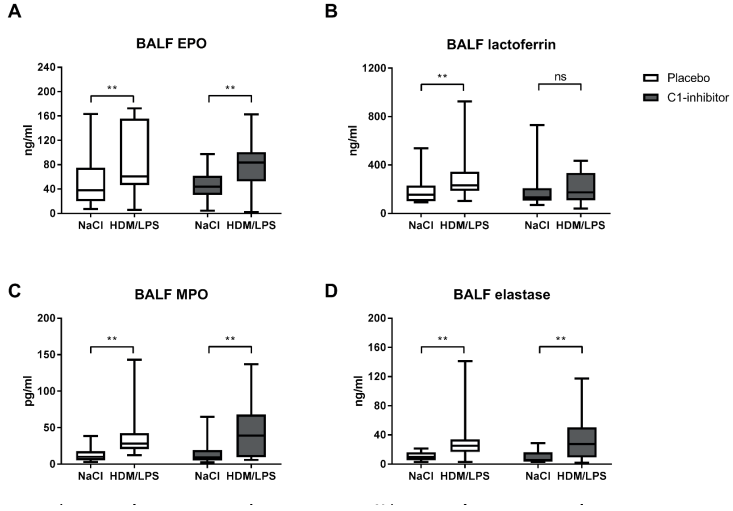

Figure 5. Effect of C1-inhibitor infusion on eosinophil and neutrophil degranulation after HDM/LPS challenge in the airways of asthma patients. (A) BALF eosinophil peroxidase (EPO), (B) BALF lactoferrin, (C) BALF myeloperoxidase (MPO), (D) BALF elastase. Data represent the median with interquartile range, the smallest and largest observation. **: *P*<0.001, ns: not significant, BALF: bronchoalveolar lavage

Effect of C1-INH on cytokine or chemokine release following HDM/LPS challenge

To obtain further insight into the inflammatory response upon HDM/LPS challenge and the effect of C1-INH hereon, we measured a broad spectrum of cytokines and chemokines relevant for allergic inflammation. Of the 35 cytokines and chemokines measured, 15 were detectable in BALF (table 2, table S1). Consistent with our previous study[8], HDM/LPS induced increases in neutrophil chemoattractants such as interleukin (IL)-8, IL-1β, tumor necrosis factor-α, macrophage inflammatory proteins 1α and 1β (table 2). Likewise, HDM/LPS elicited rises in the eosinophil attractants eotaxin-1 and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted). C1-INH did not modify the release of these mediators. HDM/LPS induced the release of growth related oncogene-α, stromal cell-derived factor-1α and IL-18 in the placebo group; while these rises were also detected in the C1-INH group statistical significance was not reached.

Effect of C1-INH on activation of the kallikrein-kinin system

We measured C1-INH/Factor XII and C1-INH/kallikrein complexes in BALF to determine the extent of kallikrein-kinin system activation. However, these complexes were not detectable in the BALF harvested from either saline or allergen challenged lung subsegments.

Effect of C1-INH on vascular leak following HDM/LPS challenge

Vascular leak often occurs as consequence of allergen induced inflammation in the airway of patients with asthma[15]. We here determined the quotients of albumin and α2-macroglobulin levels in BALF and plasma (QAlb and QA2M, respectively) and the relative coefficient of excretion (QA2M/QAlb) as measures of the permeability of the blood–airway barrier[8]. Intrabronchial HDM/LPS challenge was associated with significant increases in QAlb (figure 6A), QA2M (figure 6B) and relative coefficient of excretion (figure 6C) in the placebo group. These effects were abrogated in the C1-INH group (figure 6A-C). In agreement, HDM/LPS induced a rise in BALF IgM concentrations in placebo infused patients, but not in C1-INH treated subjects (figure 6D).

Table 2. C1-INH does not alter cytokine or chemokine release following HDM/LPS challenge

	Placebo		**C1-INH**	
	Saline (N=12)	HDM/LPS (N=12)	Saline (N=12)	HDM/LPS (N=12)
Eotaxin-1	2.5±0.51	10.6±1.14***	2.9±0.57	10.37±2.51**
GRO-α	108.1±14.89	182±24.39***	133.5±13.58	163.4±22.38
IL1-RA	530±275.2	10532±2610***	262±189	9295±2454**
IL-1β	3.1±0.95	12.11±2.55**	2.7±0.54	11.6±3.37*
IL-2	2.2±1.11	10.9±2.29*	1.6±0.90	5.5±1.56*
IL-6	51.8±21.7	1151±265.3***	24.3±6.96	885±254.4***
IL-8	67.1±19.0	415±79.7**	48.0±13.0	456±243.4**
IL-18	8.1±2.12	11.5±2.05***	7.8±2.43	11.2±2.65
IP-10	72±18.14	607.1±141.4***	56.4±7.07	635.4±155.6***
MCP-1	102.9±31.66	348.7±90.04**	77.6±23.94	239±53.45**
MIP-1α	9.9±4.96	139.4±36.72**	6.5±2.41	134.2±42.12**
MIP-1β	199.5±78.6	935.6±226**	121.8±23.12	838.9±262**
RANTES	2.2±0.43	7.7±1.25***	3.3±0.87	6.2±1.15**
SDF-1a	301.7±56.87	590±80.58***	396.5±45.70	506.6±86.48
TNF-α	1.5±0.43	17.98±6.97**	3.03±1.09	8.7±3.36**

Data are presented as mean ±SEM (pg/ml). Bronchoalveolar lavage fluid concentrations of cytokines and chemokines. Growth related oncogene-α (GRO-α), Interleukin (IL)1- Receptor Antagonist (IL1-RA), interferon-inducible protein 10 (IP-10), Monocyte Chemoattractant Protein (MCP), Macrophage Inflammatory Protein (MIP), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), Stromal cell-Derived Factor (SDF)1α, Tumor Necrosis Factor (TNF)-α. * p<0.05 versus saline; ** p<0.01 versus saline; *** p<0.001 versus saline, no significant differences were found between placebo and C1-inhibitor.

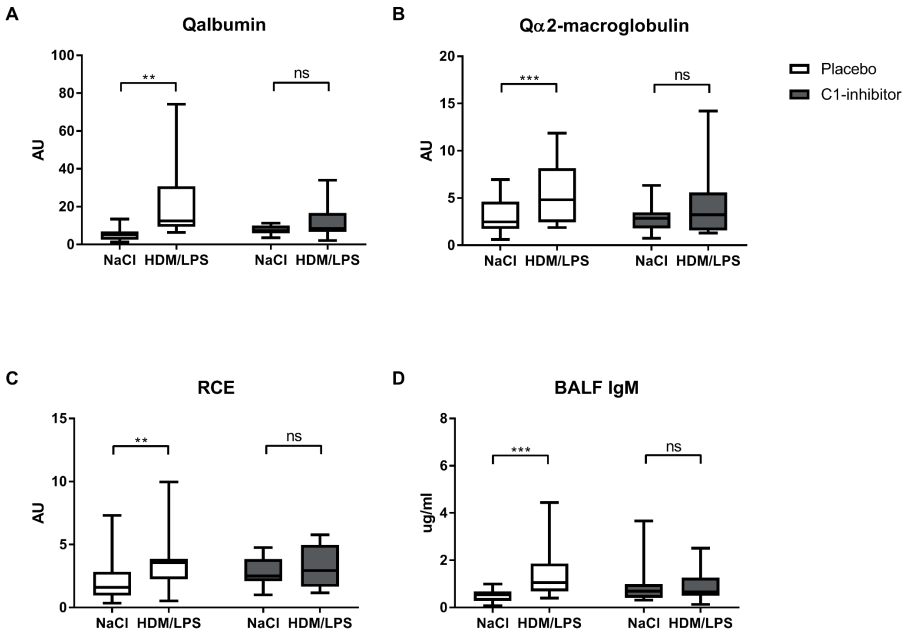

Figure 6. Intravenous C1-inhibitor infusion reduces vascular leak following HDM/LPS challenge in the airways of asthma patients. (A) Qalbumin: BALF albumin / plasma albumin, (B) Qα2-macroglobulin: BALF α2-macroglobulin / plasma α2-macroglobulin, (C) Relative coëfficient of excretion (RCE): Qα2M/Qalbumin, (D) BALF IgM levels. Data represent the median with interquartile range, the smallest and largest observation. **: *P*<0.01, ***: *P*<0.001, ns: not significant, BALF: bronchoalveolar lavage

DISCUSSION

In this proof-of-concept study we investigated the effect of intravenous C1-INH on allergen-induced lung inflammation in patients with asthma using a model of segmental HDM/LPS challenge. Our main findings were that C1-INH prevented HDM/LPS-induced complement activation without influencing leukocyte influx or activation, or local cytokine and chemokine release. C1-INH did prevent protein leak into the bronchoalveolar space upon HDM/LPS challenge, suggestive of a protective effect on epithelial-endothelial barrier integrity.

We used a combination of HDM/LPS to evoke airway inflammation. HDM is an important domestic allergen that triggers asthma exacerbation upon inhalation by sensitized asthma patients[25]. LPS is a component of gram-negative bacterial cell wall and a natural pollutant in house dust[26]. We utilized a LPS dose that

corresponds with dust endotoxin levels detected in homes[19]. House dust is a complex mixture consisting of HDM allergen and microbial components such as LPS and the co-exposure to both HDM and LPS is a likely clinical event. Previously, we have shown that simultaneous challenge with HDM/LPS induced a strong influx of eosinophils and neutrophils into the airways of HDM sensitized asthma patients[8]. Compelling evidence has linked such mixed eosinophilic and neutrophilic airway inflammation with a corticoid unresponsive asthma phenotype[4], which makes the current challenge model relevant for evaluating new therapeutic compounds.

This is one of the few studies that investigated the effect of inhibition of allergen induced complement system activation on airway inflammation in patients with asthma. Two previous clinical trials, only published in abstract form, evaluated an oral inhibitor of the C5a receptor and Eculizumab, a monoclonal antibody directed against C5, reporting negative or inconclusive results regarding lung function tests[27]. Intravenous administration of C1-INH prevented allergen induced complement activation at the level of C3 and C4. The effect of C1-INH on BALF C5a levels could not be evaluated since C5a remains undetectable in BALF of mild asthma patients, even after challenge with HDM/LPS[8]. In mouse asthma models C3a and C5a induced chemotaxis and activation of eosinophils and neutrophils, and in addition stimulated T-helper 2 responses [11,28,29]. In contrast to our original hypothesis, the current study argues against a role for complement in allergen-induced influx of eosinophils and neutrophils. C1-INH also did not modify the release of cytokines or chemokines into the bronchoalveolar space upon HDM/LPS challenge. C1-INH inhibited the release of lactoferrin (a glycoprotein restricted to specific granules of neutrophils) while not influencing the secretion of myeloperoxidase and elastase (both restricted to azurophilic granules of neutrophils)[30]. The mechanism underlying this possible differential C1-INH effect on degranulation of different neutrophil granules remains to be established. The finding that C1-INH did not influence the upregulation of neutrophil CD11b upon HDM/LPS instillation further suggests a limited effect on neutrophil activation. C1-INH has been reported to attenuate neutrophil activation and accumulation in inflammatory conditions other than asthma[16,31,32].

Beside the complement system, C1-INH is an important regulator of the kallikrein-kinin system due to its inhibitory effect on FXIIa and kallikrein activity[13]. The presence of kallikrein-kinin system components has been described in the airways of patients with asthma following allergen challenge[33]. Activation

of the kallikrein-kinin system gives rise to bradykinin release which among various proinflammatory responses, induces vasodilatation and increases vascular permeability leading to enhanced plasma extravasation[34]. Bradykinin is rapidly degraded upon release, which complicates direct measurements. We determined C1-INH/FXII and C1-INH/kallikrein complexes as measure for kallikrein-kinin system activation. In BALF, however, these complexes were below detection limit. Hence, the current challenge model is not suitable to study the contribution of the kallikrein-kinin system in allergen induced inflammation and the effect of C1-INH hereon. Notably, our group recently found no evidence for a role of the kallikrein-kinin system in allergic airway inflammation in mice induced by repeated HDM administration[35,36].

Increased vascular permeability is an important feature of asthma and is associated with deterioration of asthma control[37]. Furthermore, it has been suggested that enhanced plasma extravasation promotes airway remodeling[38,39]. C1-INH administration abrogated protein leak into the bronchoalveolar space in HDM/LPS challenged lung segments, as indicated by measurements of QA2M/QAlb and IgM. In agreement, complement products can increase permeability of the endothelial-epithelial barrier in the lung, and complement inhibition protected against protein leak in preclinical asthma models[40,41]. These data suggest that C1-INH protects the endothelial-epithelial integrity by inhibiting the complement system.

The strength of this study is its novel endeavor to translate the accumulated knowledge about the effect of complement system inhibition in allergic asthma from preclinical studies to patients with asthma. The influx of eosinophils into the airways has been described to occur after four hours[42] and to reach a peak around 42 hours[43] following allergen challenge. Our study is limited by a time span of seven hours, thus allowing analysis of the early phase of the allergen-induced inflammatory response. A possible C1-INH mediated effect on HDM/LPS challenge later on may have been missed. In addition, the Th2 response could not be fully examined due to the fact that relevant cytokines remained undetectable. Likewise, components of the kallikrein-kinin system could not be detected and thus the importance of this system on vascular permeability remains elusive. Moreover, the current trial was done in a limited number of patients in which local inflammation was induced by HDM/LPS instillation and thereby only provides proof-of-concept information about the potential effect of C1-INH in asthma.

In conclusion, we show that intravenous C1-INH administration prior to intrabronchial HDM/LPS challenge prevents complement activation and vascular leak without attenuating allergic lung inflammation in patients with HDM allergy and asthma. Suppressing vascular leakage could help improve symptoms in patients who do not respond adequately to currently available drugs.

REFERENCES

1. Israel E, Reddel HK. Severe and Difficult-to-Treat Asthma in Adults. *N Engl J Med.* 2017;377(10):965-976.
2. Moore WC, Hastie AT, Li X, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol.* 2014;133(6):1557-1563 e1555.
3. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet.* 2018;391(10122):783-800.
4. Martinez FD, Vercelli D. Asthma. *Lancet.* 2013;382(9901):1360-1372.
5. Zhang X, Kohl J. A complex role for complement in allergic asthma. *Expert Rev Clin Immunol.* 2010;6(2):269-277.
6. Laumonnier Y, Schmudde I, Kohl J. The role of complement in the diagnosis and management of allergic rhinitis and allergic asthma. *Curr Allergy Asthma Rep.* 2011;11(2):122-130.
7. Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med.* 2001;164(10 Pt 1):1841-1843.
8. de Boer JD, Berger M, Majoor CJ, et al. Activated protein C inhibits neutrophil migration in allergic asthma: a randomised trial. *Eur Respir J.* 2015;46(6):1636-1644.
9. Drouin SM, Corry DB, Kildsgaard J, Wetsel RA. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J Immunol.* 2001;167(8):4141-4145.
10. Drouin SM, Corry DB, Hollman TJ, Kildsgaard J, Wetsel RA. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J Immunol.* 2002;169(10):5926-5933.
11. Kohl J, Baelder R, Lewkowich IP, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest.* 2006;116(3):783-796.
12. Schmudde I, Strover HA, Vollbrandt T, et al. C5a receptor signalling in dendritic cells controls the development of maladaptive Th2 and Th17 immunity in experimental allergic asthma. *Mucosal Immunol.* 2013;6(4):807-825.
13. Zeerleder S. C1-inhibitor: more than a serine protease inhibitor. *Semin Thromb Hemost.* 2011;37(4):362-374.
14. Zanini A, Chetta A, Imperatori AS, Spanevello A, Olivieri D. The role of the bronchial microvasculature in the airway remodelling in asthma and COPD. *Respir Res.* 2010;11:132.
15. Fick RB, Jr., Metzger WJ, Richerson HB, et al. Increased bronchovascular permeability after allergen exposure in sensitive asthmatics. *J Appl Physiol (1985).* 1987;63(3):1147-1155.
16. Zeerleder S, Caliezi C, van Mierlo G, et al. Administration of C1 inhibitor reduces neutrophil activation in patients with sepsis. *Clin Diagn Lab Immunol.* 2003;10(4):529-535.
17. Igonin AA, Protsenko DN, Galstyan GM, et al. C1-esterase inhibitor infusion increases survival rates for patients with sepsis*. *Crit Care Med.* 2012;40(3):770-777.
18. de Zwaan C, Kleine AH, Diris JH, et al. Continuous 48-h C1-inhibitor treatment, following reperfusion therapy, in patients with acute myocardial infarction. *Eur Heart J.* 2002;23(21):1670-1677.
19. Park JH, Spiegelman DL, Burge HA, Gold DR, Chew GL, Milton DK. Longitudinal study of dust and airborne endotoxin in the home. *Environ Health Perspect.* 2000;108(11):1023-1028.
20. Michel O, Kips J, Duchateau J, et al. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med.* 1996;154(6 Pt 1):1641-1646.
21. Schouten M, MA VDP, Levi M, T VDP, JS VDZ. Early activation of coagulation after allergen challenge in patients with allergic asthma. *J Thromb Haemost.* 2009;7(9):1592-1594.
22. Sterk PJ, Fabbri LM, Quanjer PH, et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl.* 1993;16:53-83.

23. Wolbink GJ, Bollen J, Baars JW, et al. Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA for the quantification of complement activation via the classical pathway. *J Immunol Methods.* 1993;163(1):67-76.
24. Sarma JV, Ward PA. The complement system. *Cell Tissue Res.* 2011;343(1):227-235.
25. Platts-Mills TA, Tovey ER, Mitchell EB, Moszoro H, Nock P, Wilkins SR. Reduction of bronchial hyperreactivity during prolonged allergen avoidance. *Lancet.* 1982;2(8300):675-678.
26. Carnes MU, Hoppin JA, Metwali N, et al. House Dust Endotoxin Levels Are Associated with Adult Asthma in a U.S. Farming Population. *Ann Am Thorac Soc.* 2017;14(3):324-331.
27. Smith SG, Watson B, Clark G, Gauvreau GM. Eculizumab for treatment of asthma. *Expert Opin Biol Ther.* 2012;12(4):529-537.
28. Humbles AA, Lu B, Nilsson CA, et al. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature.* 2000;406(6799):998-1001.
29. Staab EB, Sanderson SD, Wells SM, Poole JA. Treatment with the C5a receptor/CD88 antagonist PMX205 reduces inflammation in a murine model of allergic asthma. *Int Immunopharmacol.* 2014;21(2):293-300.
30. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* 2003;5(14):1317-1327.
31. Caliezi C, Wuillemin WA, Zeerleder S, Redondo M, Eisele B, Hack CE. C1-Esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol Rev.* 2000;52(1):91-112.
32. Davis AE, 3rd, Lu F, Mejia P. C1 inhibitor, a multi-functional serine protease inhibitor. *Thromb Haemost.* 2010;104(5):886-893.
33. Christiansen SC, Proud D, Sarnoff RB, Juergens U, Cochrane CG, Zuraw BL. Elevation of tissue kallikrein and kinin in the airways of asthmatic subjects after endobronchial allergen challenge. *Am Rev Respir Dis.* 1992;145(4 Pt 1):900-905.
34. Kaplan AP, Ghebrehiwet B. The plasma bradykinin-forming pathways and its interrelationships with complement. *Mol Immunol.* 2010;47(13):2161-2169.
35. Stroo I, Yang J, de Boer JD, et al. Factor XI deficiency enhances the pulmonary allergic response to house dust mite in mice independent of factor XII. *Am J Physiol Lung Cell Mol Physiol.* 2017;312(2):L163-L171.
36. Yang J, van 't Veer C, Roelofs J, et al. Kininogen deficiency or depletion reduces enhanced pause independent of pulmonary inflammation in a house dust mite induced murine asthma model. *Am J Physiol Lung Cell Mol Physiol.* 2018.
37. Khor YH, Teoh AK, Lam SM, et al. Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. *Clin Exp Allergy.* 2009;39(11):1659-1667.
38. Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med.* 1997;156(1):229-233.
39. Orsida BE, Ward C, Li X, et al. Effect of a long-acting beta2-agonist over three months on airway wall vascular remodeling in asthma. *Am J Respir Crit Care Med.* 2001;164(1):117-121.
40. Khan MA, Nicolls MR, Surguladze B, Saadoun I. Complement components as potential therapeutic targets for asthma treatment. *Respir Med.* 2014;108(4):543-549.
41. Khan MA, Assiri AM, Broering DC. Complement mediators: key regulators of airway tissue remodeling in asthma. *J Transl Med.* 2015;13:272.
42. Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin Exp Immunol.* 1998;114(2):137-146.
43. Lommatzsch M, Julius P, Kuepper M, et al. The course of allergen-induced leukocyte infiltration in human and experimental asthma. *J Allergy Clin Immunol.* 2006;118(1):91-97.

SUPPLEMENTAL METHOD

Design and segmental allergen challenge

The dose and timing of C1-inhibitor administration were based on previous clinical studies with this compound[1,2]. We randomised HDM and LPS challenge to the left or right lung segments to cater for potential biases due to anatomical differences.

Assays and flow cytometry

The following assays were used in accordance with the manufacturers' instructions or as described: C1-INH antigen and C1-INH activity (Sanquin, Amsterdam, The Netherlands), C3bc[3], C4bc[3], C3a and C4a (both BD Biosciences, San Jose, CA, USA). Cell populations in BALF were determined by flow cytometry. BALF cells were stained with, CD4 Alexa fluor 700 (eBiosciences, San Diego, CA, USA), CD45 phycoerythrin (PE)-CF594, CD71 Brilliant Violet (BV)421, viability dye APC-Cy7 (all BD Biosciences), CD16 BV650, Siglec-8 Allophycocyanin (APC) (Biolegend, San Diego, CA, USA). All singlet cells were selected for CD45+ and viability dye-. Subsequently, eosinophils (CD71-CD4-CD16-Siglec-8+), neutrophils (CD71-CD4-CD16+Siglec-8-), alveolar macrophages (CD71+) and CD4 T-cells (CD71-CD4+) were identified. Data were collected on a BD FACSAria™ III flow cytometer and analyzed using FlowJo software (Treestar, Palo Alto, CA, USA). Lactoferrin, elastase-α1-antitrypsin complexes (Sanquin), myeloperoxidase (MPO)[4] and EPO (LifeSpan BioSciences, Seattle, WA, USA). Luminex bead assays (Thermo Fisher, the Netherlands) were used to measure Eotaxin-1, RANTES, Granulocyte-macrophage colony-stimulating factor (GM-CSF), GRO-α, Interferon (IFN)-α, IFN-γ, interleukin (IL)-1β, IL-1α, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7 IL-8, IL-9 IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, Interferon gamma-induced protein 10 (IP-10), Monocyte chemoattractant Protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, Stromal cell-derived factor (SDF)1α, tumor necrosis factor (TNF)-α. Contact system activation was assessed by C1-INH/Factor XII and C1-INH/kallikrein complex[5]. Vascular permeability was assessed by measuring IgM (Sanquin), albumin and α2-macroglobulin [4]. We calculated the quotients of albumin (QAlb) and α2-macroglobulin (QA2M) in BAL fluid and paired plasma samples. Relative coefficient of excretion (QA2M/QAlb) was determined to account for variable dilution of epithelial lining fluid during lavage[6].

Supplemental table

Table S1. Cytokines and chemokines that remained undetectable in BALF in all groups (with detection limits)

Cytokine/chemokine	Detection limit (pg/mL)
GM-CSF	10
IFN-α	7.2
IFN-γ	12
IL-1α	0.73
IL-4	8.84
IL-5	7.42
IL-7	0.73
IL-9	7.57
IL-10	2.26
IL-12p70	6.86
IL-13	3.27
IL-15	3.22
IL-17A	2.33
IL-21	9.69
IL-22	20
IL-23	17
IL-27	20
IL-31	9.64
TNF-β	6.54

Bronchoalveolar lavage fluid concentrations of cytokines and chemokines. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Interferon (IFN), Interleukine (IL), Tumor Necrosis Factor (TNF).

Supplemental figures

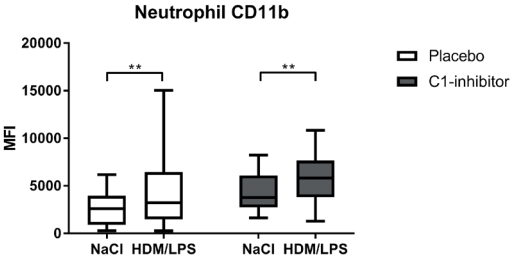

Figure S1. C1-inhibitor infusion does not modify CD11b expression on neutrophils in BALF of asthma patients following HDM/LPS challenge. Data represent the median with interquartile range, the smallest and largest observation. **: *P*<0.01.

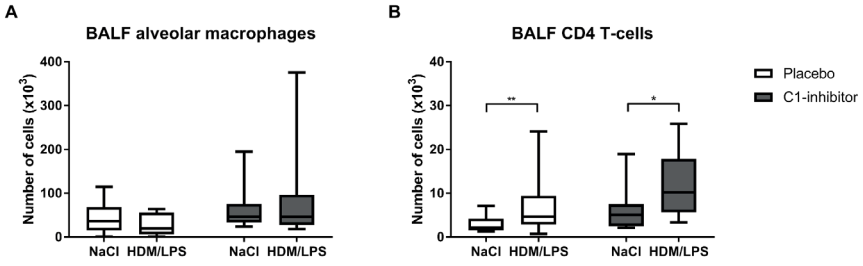

Figure S2. C1-inhibitor infusion does not alter the numbers of alveolar macrophages and CD4 T-cells in BALF of asthma patients following HDM/LPS challenge. (A) number of alveolar macrophages in BALF, (B) number of CD4 T-cells in BALF. Data represent the median with interquartile range, the smallest and largest observation. *: *p*<0.05 **: *P*<0.01.

SUPPLEMENTAL REFERENCES

1. Zeerleder S, Caliezi C, van Mierlo G, et al. Administration of C1 inhibitor reduces neutrophil activation in patients with sepsis. *Clin Diagn Lab Immunol.* 2003;10(4):529-535.
2. Caliezi C, Zeerleder S, Redondo M, et al. C1-inhibitor in patients with severe sepsis and septic shock: beneficial effect on renal dysfunction. *Crit Care Med.* 2002;30(8):1722-1728.
3. Wolbink GJ, Bollen J, Baars JW, et al. Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA for the quantification of complement activation via the classical pathway. *J Immunol Methods.* 1993;163(1):67-76.
4. de Boer JD, Berger M, Majoor CJ, et al. Activated protein C inhibits neutrophil migration in allergic asthma: a randomised trial. *Eur Respir J.* 2015;46(6):1636-1644.
5. Minnema MC, Wittekoek ME, Schoonenboom N, Kastelein JJ, Hack CE, ten Cate H. Activation of the contact system of coagulation does not contribute to the hemostatic imbalance in hypertriglyceridemia. *Arterioscler Thromb Vasc Biol.* 1999;19(10):2548-2553.
6. Nocker RE, Weller FR, Out TA, de Riemer MJ, Jansen HM, van der Zee JS. A double-blind study on the effect of inhaled corticosteroids on plasma protein exudation in asthma. *Am J Respir Crit Care Med.* 1999;159(5 Pt 1):1499-1505.

Transcriptional changes in alveolar macrophages from adults with asthma after intrabronchial allergen challenge

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Submitted

To the Editor,

Under homeostatic conditions, macrophages are the most abundant immune cells in the lung. Pulmonary macrophages are a heterogeneous cell population that can be classified in at least two distinct subpopulations, i.e., interstitial macrophages, located within the lung parenchyma, and alveolar macrophages (AM) which reside in the airway lumen, allowing direct contact with the environment (e.g., allergens, particulate matter, and commensal bacteria)(1). In recent years, AM have been shown to play an important role in environmental allergen-induced airway inflammation in asthma(2). Elimination of resident AM resulted in enhanced type 2 airway inflammation in a mouse asthma model, while depletion of blood monocytes resulted in abrogation of newly formed AM after allergen challenge and a decreased type 2 immune response(3, 4). Knowledge of phenotypic alterations of AM in allergic asthma in humans is limited(1, 2). In this study, we investigated the effect of house dust mite (HDM) and lipopolysaccharide (LPS) challenge on the transcriptome of AM from patients with mild asthma. We have shown previously that intrabronchial HDM/LPS challenge induces a mixed eosinophilic and neutrophil airways inflammation in asthma patients(5). Therefore, we hypothesize that exposure of AM to HDM/LPS would upregulate genes associated with eosinophil and neutrophil signaling.

AM were harvested from asthma patients who participated in a double blind randomized placebo controlled trial investigating the effect of C1-inhibitor on allergen-induced airway inflammation(5). For the current analysis 12 patients (treated with placebo) with intermittent–to–mild asthma, defined according to the criteria of the Global Initiative for Asthma guideline(6), and HDM allergy, confirmed by a positive skin prick and radioallergosorbent test, were challenged via bronchoscopy with saline (as internal control) in one lung segment and HDM extract (50 biological units, *Dermatophagoides pteronyssinus* origin; Allergopharma, Zeist, the Netherlands), combined with LPS (75 ng; from *Escherichia coli*, Clinical Center Reference Endotoxin, kindly provided by Anthony Suffredini, National Institute of Health, Bethesda, MD, USA) in the contralateral lung segment. The addition of LPS to HDM mimics a natural allergen exposure as LPS is a widespread pollutant and coexisting with HDM in house dust(7). After seven hours, a bilateral bronchoalveolar lavage (BAL) was performed. BAL fluid was pooled (per lung subsegment) and centrifuged at 4°C and 400g for 10 minutes. Cells were stained with CD45 phycoerythrin (PE)-CF594 and CD71 Brilliant Violet (BV)421 (both BD Biosciences, San Jose, CA, USA). AM, defined as cells expressing CD45 and CD71, were isolated by flow cytometry sorting

using FACSAria III (BD Biosciences) with high purity (>95%). Sorted AM were stored in RNA later (Qiagen, Venlo, Netherlands) at -80 °C until analysis. Total RNA was isolated using RNAeasy isolation kit (Qiagen) and quantified by Qubit® 2.0 (Life Technologies, Carlsbad, CA). Paired samples (AM from saline and HDM/LPS challenged lung segments of one individual) from eight patients had a RNA integrity number > 6 and were selected for further analysis. RNA-seq libraries were prepared using the KAPA RNA HyperPrep with RiboErase (Roche) according to manufacturer's instructions and sequenced using the Illumina HiSeq4000 (Illumina, San Diego, CA) instrument. Sequences were evaluated for quality (FastQC version 0.11.5, Babraham institute, Babraham, Cambridgeshire, United Kingdom) and trimmed (Trimmomatic version 0.36)(8). The remaining reads were used to align against the Genome Reference Consortium human genome build 38 (GRCh38) using HISAT2 (version 2.1.0)(9) with parameters as default. The HTSeq and DESeq2 methods were used to calculate and compare sequence counts, respectively. Pathway analysis was done using Ingenuity Pathway Analysis (QIAGEN bioinformatics) software. Throughout, Benjamini & Hochberg adjusted p-values < 0.05 demarcated significance. Sequence libraries are publicly available through the National Center for Biotechnology Information under the following accession number: GSE144576. The baseline characteristics of these patients are depicted in Table 1.

Table 1. Baseline patient characteristics

General	
Number	8
Female	7
Age, years	23.7 (0.53)
Asthma related symptoms¶ %	
Wheezing	75
Cough	75
Dyspnea	83.3
Chest tightness	50
Seasonal variability	75
Symptom progression at night	33.3
Eczema	58.3
Allergic rhinitis	75
Family history of atopy	58.3
Allergy	
House dust mite %	100
Total IgE, kU/L	349.5 (69.2)
D. pteronyssinus IgE, kU/L	43.9 (10)
Lung spirometry	
FEV1 L	3.76 (0.26)
FEV1 % of predicted	98.2 (3.32)
FVC L	4.8 (0.46)
FVC % of predicted	96.7 (9.41)
Methacholine PC20, mg/mL†	2.6 (1.7-4.5)

Data are presented as mean ±SEM unless otherwise stated. D. pteronyssinus: Dermatophagoides pteronyssinus; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; PC20: provocative dose causing a 20% fall in FEV1; NA: not applicable. #: Global Initiative for Asthma stage I/II.¶ Symptoms occurred in last 6 months prior to study participation. † Data are expressed as median with interquartile range

Principal component analysis of 24,555 transcripts (sequence counts ≥ 10) revealed clear separation of saline or HDM/LPS challenges (Fig. 1A), showing that HDM/LPS induced a marked transcriptomic response in AM. Comparing HDM/LPS challenge to saline identified 1013 significantly altered transcripts (fold expression ≤ -1.2 or ≥ 1.2), of which 485 were upregulated and 528 downregulated as consequence of HDM/LPS challenge (Fig. 1B). Pathway analysis of upregulated genes revealed predominantly pro-inflammatory responses such as inducible nitric oxide synthase, Toll-like receptor, p38 mitogen-activated protein kinase and interleukin (IL)-1, IL-8, IL-17 signaling (Fig. 1C). IL-1 signaling

has been implicated in neutrophilic asthma and increased IL-1β production has especially been documented in this asthma subtype(2). Likewise, IL-17 has been identified as an important cytokine in the pathogenesis of asthma due to its capacity to promote neutrophilic airway inflammation, which is associated with a severe asthma phenotype(10). In agreement, our data shows upregulation of *CXCL1*, a chemoattractant for neutrophils, suggesting that AM aid in neutrophil recruitment following HDM/LPS challenge (Fig. 1D). Interestingly, genes associated with the anti-inflammatory IL-10 signaling pathway were also upregulated. This could be explained by heterogeneity among AM including both pro- and anti-inflammatory subpopulations and/or concurrent induction of both pro- and anti-inflammatory pathways in the same AM. This observation suggests a regulatory role for AM to maintain homeostasis by counteracting the pro-inflammatory processes inflicted by allergen challenge. Among downregulated transcripts were genes associated with cell cycle and growth (T cell receptor, role of NFAT in cardiac hypertrophy), DNA damage and repair (breast cancer regulation by stathmin 1) and metabolic pathways (e.g., insulin receptor and leptin signaling, Fig. 1C and D).

To the best of our knowledge, this is the first investigation to examine gene expression profiles in AM from asthma patients challenged with HDM and LPS *in vivo*. Using a well-controlled study design allowing paired analysis of saline and HDM/LPS effects in the same patient, we identified different transcriptional profiles in AM as consequence of local exposure to HDM and LPS. These findings may provide a better understanding of AM functions in (exacerbations of) allergic asthma.

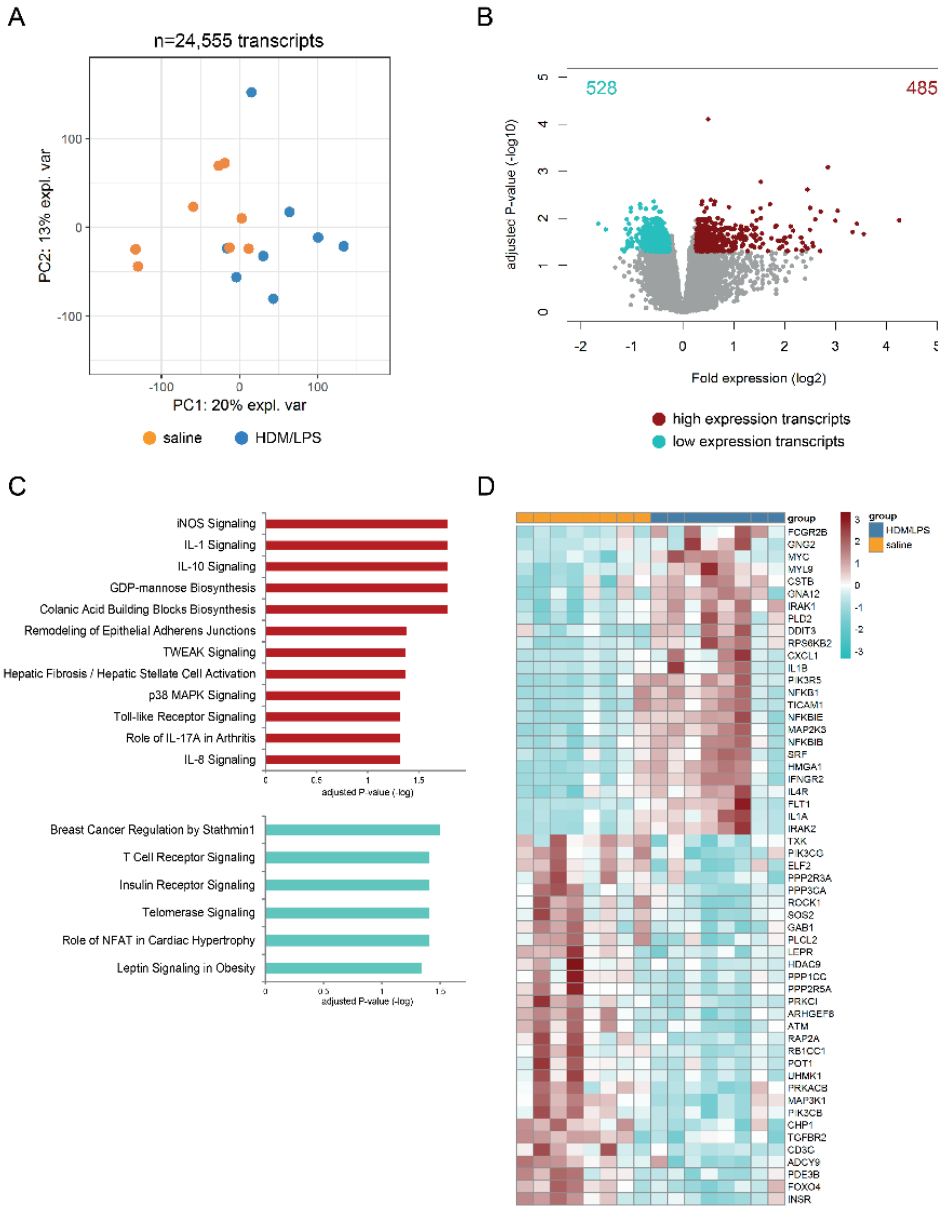

Figure 1. Genome-wide analysis of alveolar macrophage response to HDM/LPS challenge. (A) Principal component analysis (PCA) of the 24.555 transcripts showing explainable variances of PC1 20% and PC2 13% between saline or HDM/LPS samples (B) Volcano plot depicting the differences in gene expression following either saline or HDM/LPS exposure. Horizontal line represents the multiple-test adjusted significance threshold ($P < 0.05$) (C) Ingenuity pathway analysis of the canonical pathway gene expression profiles in HDM/LPS samples relative to saline samples. Red bars, overexpressed pathways; green bars, under expressed pathways (D) Heatmap plot of the top significant genes between saline and HDM/LPS samples.

REFERENCES

1. Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. Pulmonary macrophages: key players in the innate defence of the airways. Thorax. 2015;70(12):1189-96.
2. Fricker M, Gibson PG. Macrophage dysfunction in the pathogenesis and treatment of asthma. Eur Respir J. 2017;50(3).
3. Zaslona Z, Przybranowski S, Wilke C, van Rooijen N, Teitz-Tennenbaum S, Osterholzer JJ, et al. Resident alveolar macrophages suppress, whereas recruited monocytes promote, allergic lung inflammation in murine models of asthma. J Immunol. 2014;193(8):4245-53.
4. Mathie SA, Dixon KL, Walker SA, Tyrrell V, Mondhe M, O'Donnell VB, et al. Alveolar macrophages are sentinels of murine pulmonary homeostasis following inhaled antigen challenge. Allergy. 2015;70(1):80-9.
5. Yang J, van Engelen TSR, Haak BW, Bonta PI, Majoor CJ, van 't Veer C, et al. Effect of C1-inhibitor in adults with mild asthma: A randomized controlled trial. Allergy. 2019.
6. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention (GINA). Available at https://ginasthma.org; 2019.
7. Carnes MU, Hoppin JA, Metwali N, Wyss AB, Hankinson JL, O'Connell EL, et al. House Dust Endotoxin Levels Are Associated with Adult Asthma in a U.S. Farming Population. Ann Am Thorac Soc. 2017;14(3):324-31.
8. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
9. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015;12(4):357-60.
10. Chesne J, Braza F, Mahay G, Brouard S, Aronica M, Magnan A. IL-17 in severe asthma. Where do we stand? Am J Respir Crit Care Med. 2014;190(10):1094-101.

Human plasma-derived C1 esterase inhibitor concentrate has limited effect on house dust mite-induced allergic lung inflammation in mice

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ABSTRACT

C1 esterase inhibitor (C1-INH) can inhibit multiple pathways (complement, contact-kinin, coagulation, and fibrinolysis) that are all implicated in the pathophysiology of asthma. We explored the effect of human plasma-derived C1-INH on allergic lung inflammation in a house dust mite (HDM) induced asthma mouse model by daily administration of C1-INH (15 U) during the challenge phase. NaCl and HDM exposed mice had comparable plasma C1-INH levels, while bronchoalveolar lavage fluid (BALF) levels were increased in HDM exposed mice coinciding with slightly reduced activation of complement (C5a). C1-INH treatment reduced Th_2 response and enhanced HDM-specific IgG_1. Influx of eosinophils in BALF or lung, pulmonary damage, mucus production, procoagulant response or plasma leakage in BALF was similar in both groups. In conclusion, C1-INH dampens Th2 responses during HDM induced allergic lung inflammation.

INTRODUCTION

Asthma is a chronic airway inflammatory disease that results from an excessive immune response to common environmental allergens such as house dust mite (HDM)[1]. In 2013, the WHO estimated that worldwide 235 million people suffer from asthma and the incidence is still rising[2]. Symptoms include recurrent episodes of wheezing, coughing, chest tightness and shortness of breath in response to an allergen. Currently, asthma cannot be cured, however, a combination of inhaled corticosteroids (to suppress inflammation) and a short- or long-acting β-adrenergic agonist (to open the constricting bronchial smooth muscle cells) can control the disease and improve quality of life[3]. While the majority of patients with asthma can be treated effectively with the currently available medications, adequate disease control cannot be achieved in a significant proportion of patients. Because of the high incidence and burden on our health care system, there is an urgent need to explore new treatment options.

The complement system is one of the inflammatory pathways activated during asthma. The complement cascade consists of a number of plasma- and membrane-bound proteins that can be activated via three distinct routes; the classical, lectin or alternative pathway of complement. All three pathways converge at the level of C3 which following activation subsequently activates C5. This activation cascade leads to the formation of the anaphylatoxins C3a and C5a. Anaphylatoxins possess many proinflammatory and immunomodulatory characteristics; for example they are chemotactic factors for eosinophils[4]. In the bronchoalveolar lavage fluid (BALF) of asthmatic patients C3a and C5a levels are increased following allergen challenge and both anaphylatoxins correlate with influx of eosinophils[5]. Moreover, experimental asthma models suggest that C3a and C5a regulate Th_2 response during the sensitization and challenge phase[6].

Activation of both the classical and lectin pathway of complement is tightly regulated by C1 esterase inhibitor (C1-INH). The plasma glycoprotein C1-INH belongs to the family of serpins (serine protease inhibitors). Serpins are characterized by a typical mechanism of action; target proteases attack the " fake" substrate conformation of the serpin, leading to the formation of a covalent complex between protease and serpin (deadly handshake)[7]. Next to complement inhibition, C1-INH is 1) a major regulator of the contact-kinin system by blocking of activated factor XII (FXIIa) and plasma kallikrein, 2) the main inhibitor of activated factor XI (FXIa), the central player in the intrinsic

coagulation, and 3) an inhibitor of fibrinolysis via blocking plasmin and tissue-type plasmin activator[7]. These functions of C1-INH are all exerted via its protease inhibitor domain. However, several experimental studies have shown an inhibitory effect for C1-INH in the production of cytokines and attraction of leukocytes that is independent of its protease inhibitory activity[8-10]. Asthma is associated with activation of the coagulation system[11] and contact-kinin system[12]. The contact-kinin system is an interesting target for the treatment of asthmatic exacerbations. Activation of this system leads to the formation of bradykinin, a small molecule that causes smooth muscle contraction, increases vascular permeability, and enhances mucus secretion[12]. In a sheep model of allergen-induced airway inflammation, the bradykinin B_2-receptor antagonist NPC349 blocked the airway hyperresponsiveness and reduced inflammatory mediators[13,14]. Recently, a bradykinin B_1-receptor antagonist impaired eosinophil influx in a murine ovalbumin asthma model[15]. Although these results are promising, studies exploring the role of the contact-kinin system in asthma are limited.

Taken together, as C1-INH targets multiple biological systems that are activated during asthma (being complement, contact-kinin, and coagulation pathways) it might be a promising therapy to alleviate asthmatic symptoms. Moreover, C1-INH is already used by patients suffering from hereditary angioedema and is proven to be safe and efficacious in humans. In the present study we treated mice that were subjected to our HDM asthma model during the challenge phase daily with human plasma-derived C1-INH and determined the inflammatory response.

METHODS

Mice

Female C57Bl/6J wild-type (WT) mice were purchased from Charles River Inc. (Maastricht, The Netherlands). Mice were housed under specific pathogen-free conditions receiving food and water *ad libitum*. Age-matched mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments (permit numbers: DIX102020AQ and DIX102791). Intranasal inoculation was performed under isoflurane anesthesia and mice were euthanized under general anesthesia, all efforts were made to minimize suffering.

Kinetics C1-INH

Nine-weeks old mice received a single injection of 5 U (1 U equals ~250 µg) or 15 U of human plasma-derived C1-INH (Cetor, Sanquin, Amsterdam, Netherlands) intravenously (i.v.) or intraperitoneally (i.p.). Mice were randomly assigned to 3 groups and blood samples were taken at 1 and 2 hours (group I), 6 and 10 hours (group II), or 48 hours (group III) from the facial vein into EDTA tubes (Microvette®, Sarstedt, Etten-Leur, Netherlands) (n=3 per group). Mice were euthanized and blood was obtained from the vena cava inferior 4 (group I), 24 (group II) or 72 hours (group III) following injection. Blood samples were centrifuged to collect plasma. All samples were stored at -80°C until further analysis.

HDM asthma model

HDM allergen whole body extract (Greer Laboratories, Lenoir, N.C., USA), derived from the common European HDM species Dermatophagoides pteronyssinus, Der p, was used to induce allergic lung inflammation as described previously[16]. In short, 8-weeks old mice (n=10) were inoculated intranasally on day 0, 1 and 2 with 25 µg HDM (sensitization phase) and on day 14, 15, 18 and 19 with 6.25 µg HDM (challenge phase). Controls (n=6) received sterile saline intranasally on each occasion. Inoculum volume was 20 µl for every HDM and saline exposure and inoculation procedures were performed under isoflurane inhalation anesthesia. During the challenge phase (day 14 till 19) mice received daily 15 U human plasma-derived C1-INH i.p. or vehicle (provided by Sanquin) containing all excipients of C1-INH (i.e. sodium chloride, saccharose, sodium citrate, L-Valine, L-Threonine, L-Alanine). Both NaCl and HDM exposed mice were treated at the same days; all mice were included in a single experiment. Mice were euthanized at day 20 and blood was collected from the vena cava inferior into citrate (4:1

v/v), after which 10 mM EDTA, 10 mM benzamidine, and 0.2 mg/ml SBTI (all end concentrations) was added to specifically inhibit coagulation, contact, and complement activation. One-sided BALF was collected by instilling and retrieving 0.8 ml of sterile PBS containing 10 mM EDTA, 10 mM benzamidine and 0.2 mg/ml SBTI in 400 µl aliquots via the trachea. Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and differential cells counts by cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). BALF supernatant was collected for protein analysis. The right lung was fixed 24 hours in 10% formalin. The mediastinal lymph nodes (MLN) were dissected and single cell suspension were prepared by mashing the cells through a 70 µm cell strainer. Cell counts were determined in a hemocytometer for each MLN sample and $2x10^5$ cells/well were seeded in a 96-well round bottom plate and restimulated for 4 days with 50 µg/ml HDM or NaCl. Supernatants were collected for cytokine production.

Histology

Formalin-fixed tissue was embedded in paraffin using standard procedures. Four µm 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, perivascular inflammation, and edema on a scale from 0-4 (0: absent; 1: mild; 2: moderate; 3: severe; 4: very severe)[16]. 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 reagens 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 mucus plug formation; 0-4 for the extent of goblet cell hyperplasia)[16]. For eosinophil staining, sections were digested for 20 min in 0.25% pepsin in 0.1 M 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)[17]. Next, sections were incubated for 30 min with poly HRP-anti-rabbit IgG (Brightvision, Immunologic, Duiven, the Netherlands) and stained using 3,3"-diaminobenzidine 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

Human C1-INH antigen (Ag) and activity (Act) were measured in plasma by ELISA using as capture antibody monoclonal mouse anti-human C1-INH (clone RII, Sanquin) and as detection antibody biotinylated rabbit anti-human C1-INH (Sanquin) or biotinylated C1s (Calbiochem, Merck Millipore, Amsterdam, Netherlands) respectively[18]. Normal human plasma with known C1-INH concentration was used as standard. Complement activation in plasma and BALF was determined by C5a ELISA. Purified rat anti-mouse C5a (clone I53-1486) was used as capture antibody, biotinylated rat anti-mouse C5a (clone I52-278) was used as detection antibody (all from BD Biosciences). A standard curve for C5a was generated by serial dilutions of an in-house standard of maximal activated mouse serum, by incubating normal mouse serum at 37°C for 1 week in the presence of sodium azide. Purified recombinant mouse C5a (BD Biosciences) was used to determine concentration of C5a in maximal activated mouse serum. 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 IgG_1 was determined using HDM as capture, and biotinylated rat-anti-mouse IgG_1 (from BD Biosciences) as detection. HDM specific IgG_1 is expressed as percentage compared to WT HDM group. IL-4, IL-5, IL-13 (MLN supernatants) and E-selectin were measured using DuoSet ELISA kits (R&D Systems, Abingdon, UK) according to the supplied protocol. BALF IL-4, IL-5 and IL-13 were determined by a Mouse Magnetic Luminex Screening Assay according to the manufacturer's protocol (R&D Systems) and analyzed on a Bio-Rad BioPlex® 200 (Bio-Rad Laboratories, Veenendaal, the Netherlands). BALF 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 anti-thrombin complexes (TATc) were measured by ELISA according to manufacturer's instructions (Stago BNL, Leiden, the Netherlands).

Statistical analysis

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 6 (GraphPad Software, San Diego, CA, USA). Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

C1-INH plasma levels after single injection

In order to design a C1-INH treatment schedule for our mouse asthma model, we needed to obtain more information about the behavior of this human plasma derived protein in mice. Therefore mice were injected with a single dose of C1-INH (5 U or 15 U) either i.v. or i.p.. At indicated time points up to 72 hours after injection, blood was collected and C1-INH antigen and activity levels were determined in plasma (Figure 1a-d). Following i.v. injection, C1-INH antigen levels declined gradually with an estimated elimination half-life of 10 hours for both doses. Plasma levels of C1-INH antigen was on average 4.0 ± 0.2 fold higher in 15 U compared with 5 U treated mice. After i.p. administration of C1-INH, plasma antigen levels increased steadily reaching after approximately 6 hours the maximal plasma concentration (55 µg/ml for 5 U, and 600 µg/ml for 15 U). Plasma levels of C1-INH antigen following i.p. administration were on average 12.8 ± 1.7 fold higher in 15 U compared with 5 U dosage. Elimination half-life after i.p. administration was approximately 12 hours for both doses. C1-INH activity was comparable with antigen level for both routes and doses, indicating that C1-INH was still biologically active.

For our experimental HDM asthma mouse model, our goal was to achieve plasma levels of 500-1000 µg/ml (comparable to 2- to 4-times normal human plasma levels, i.e. ~250 µg/ml). Additionally, we were aiming for stable plasma levels throughout the whole experiment, which is more easily reached with i.p. injections. To fulfill these criteria, a treatment schedule of daily i.p. injections with 15 U C1-INH was chosen.

Daily C1-INH treatment results in local C1-INH that slightly inhibits complement activation.

During the challenge phase of the HDM asthma model, mice received daily 15 U C1-INH or vehicle i.p.. One day after the last challenge, and hence C1-INH administration, exogenous C1-INH antigen and activity levels were determined by ELISAs specific for human C1-INH. Similar plasma levels of either C1-INH antigen (Figure 2a) or C1-INH activity (Figure 2b) were observed following NaCl or HDM exposure. Although much lower than in plasma, presence of C1-INH, both antigen and activity, was confirmed in the BALF of NaCl and HDM treated mice (Figure 2c-d). Interestingly, C1-INH levels were significantly higher in BALF from HDM exposed mice as compared with the NaCl group. Of note, C1-INH antigen or activity could not be detected in vehicle treated NaCl or HDM mice indicating that there was no cross reactivity with mouse C1-INH.

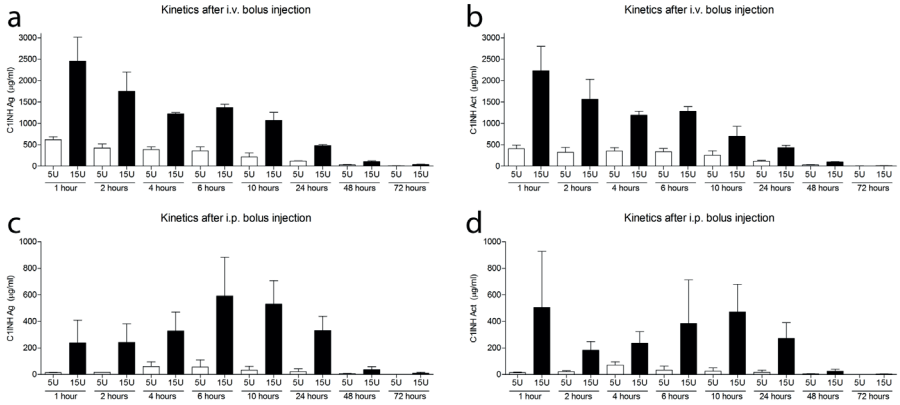

Figure 1. C1-INH plasma levels after i.v. or i.p. bolus injection. Human plasma derived C1-INH antigen (Ag) and activity (Act) in plasma after bolus injection of 5U (white bars) or 15U (black bars) C1-INH either i.v. (a-b) or i.p. (c-d) at indicated time points up to 72 hours following injection. Data are presented as mean ± SEM, n=3 per time point.

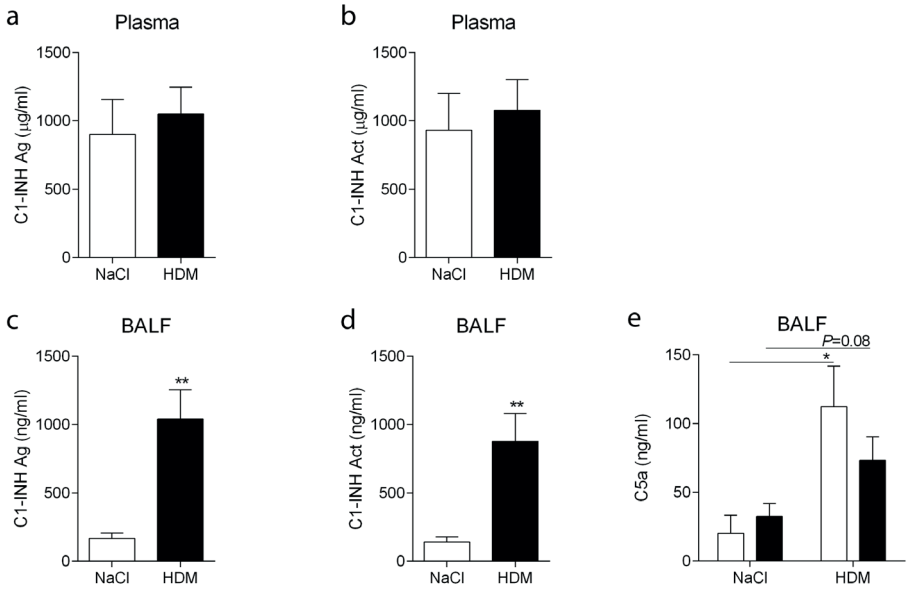

Figure 2. C1-INH and C5a levels in plasma and BALF. Human plasma derived C1-INH antigen (Ag) and activity (Act) in plasma (a-b) and BALF (c-d) of NaCl (white bars) and HDM (black bars) exposed C1-INH treated mice. Complement activation was determined in BALF (e) by C5a ELISA in vehicle (white bars) and C1-INH (black bars) treated NaCl and HDM exposed mice. Data are presented as mean ± SEM, n=6 (NaCl) and n=10 (HDM). *P<0.05, **P<0.01

To determine whether exogenous C1-INH was effective, complement activation was detected by an ELISA specific for a neoepitope on C5a. In BALF we could detect significant activation (4.2 ± 1.5 fold increase in ctr HDM versus ctr NaCl) of the complement system in vehicle treated mice, and a trend (P=0.08; 2.1 ± 0.5 fold increase in C1-INH HDM versus C1-INH NaCl) towards higher BALF C5a in C1-INH treated mice following HDM exposure (Figure 2e). Although BALF C5a was lower in HDM exposed C1-INH treated mice, as compared with vehicle treated mice, this was not significant most likely due to intragroup variation. In plasma we could not detect activation of the complement system following HDM exposure.

C1-INH treatment does not alter eosinophil influx

One of the hallmarks of an asthmatic response is the influx of eosinophils in the lung. At time of euthanize the BALF was analyzed for total cell influx (Figure 3a) and the number of eosinophils, neutrophils, macrophages, and lymphocytes was assessed (Figure 3b). Following HDM exposure there was an approximately 3-fold increase in number of leukocytes, which was almost exclusively caused by eosinophils. C1-INH treatment had no effect on total leukocyte or leukocyte differential numbers in BALF of either NaCl or HDM exposed mice. Additionally, eosinophil influx was determined in lung tissue by immunohistochemical staining for MBP, an eosinophil specific marker. In line with BALF influx data, more eosinophils were present in lungs of HDM exposed mice and no effect of C1-INH treatment was observed (Figure 3c).

C1-INH treatment has no effect on lung pathology

Following HDM exposure histopathological changes, including perivascular inflammation, interstitial inflammation, and edema, occur in the lungs[16]. A pathologist, blinded for the treatment, analyzed these hallmarks in a semi-quantitative fashion and the total pathology score is depicted in Figure 4a. Although HDM exposure increased lung pathology, no effect of C1-INH treatment was observed.

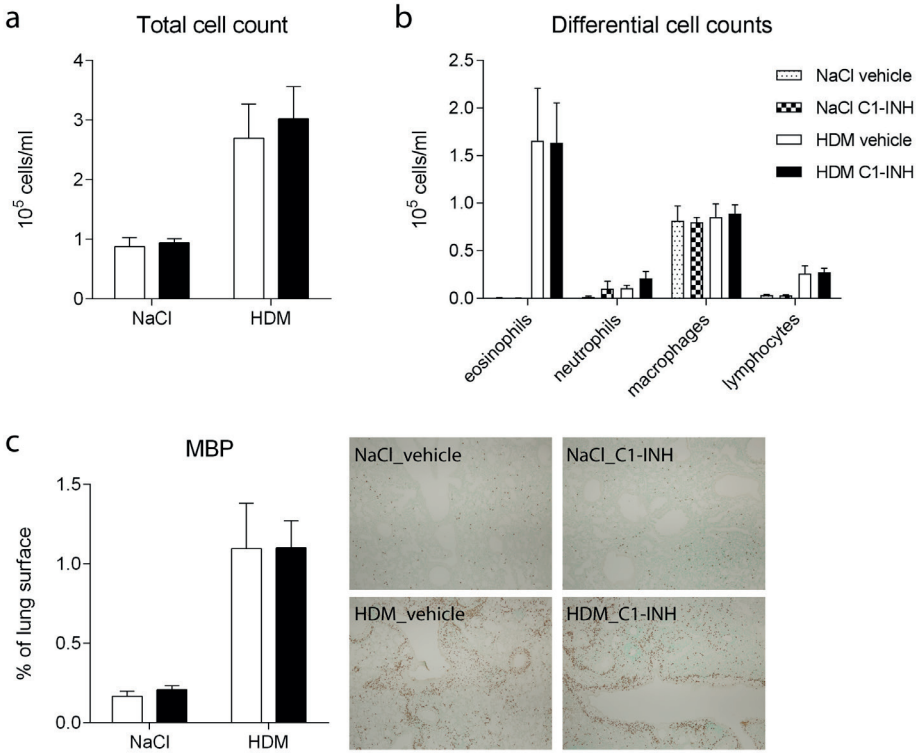

Figure 3. Eosinophil influx in BALF and lung. (a) Total cell count in BALF of NaCl or HDM challenged vehicle (white bars) or C1-INH (black bars) treated mice. (b) Differential cell count in BALF showing eosinophil, neutrophil, macrophage, and lymphocyte numbers in NaCl challenged vehicle (dotted bars) and C1-INH treated (blocked bars) mice and in HDM challenged vehicle (white bars) and C1-INH treated (black bars) mice. (c) Influx of eosinophils in the lung was determined by digital analysis of Major Basic Protein (MBP) stained lung sections in C1-INH (black bars) and vehicle (white bars) treated mice following challenge with NaCl or HDM. Representative pictures of each group are shown, magnification 4x. Data are presented as mean ± SEM, n=6 (NaCl) and n=10 (HDM)

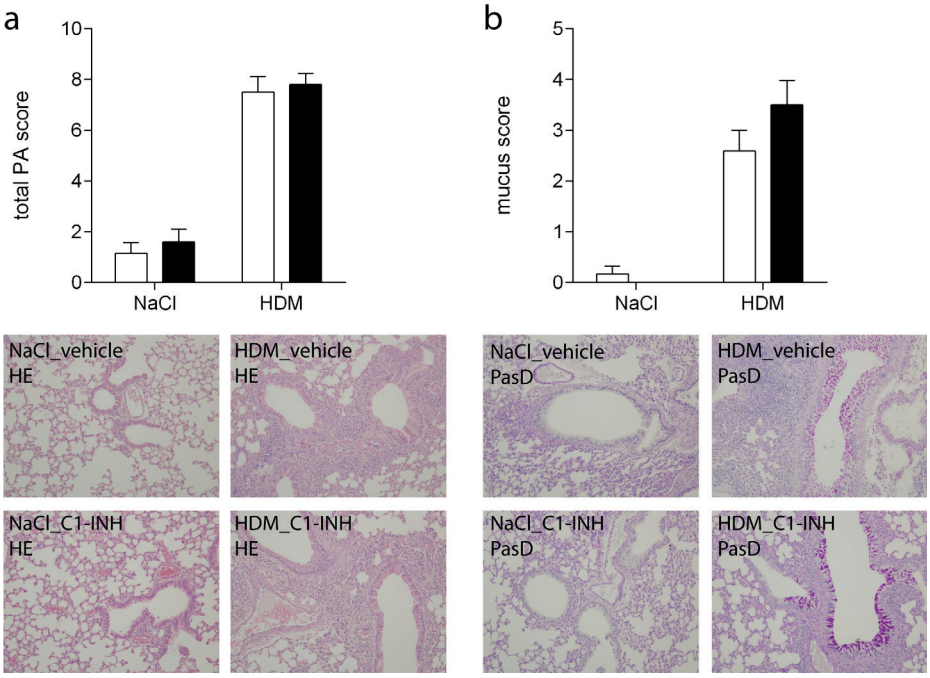

Figure 4. Lung damage and mucus production. Lung sections were stained with HE (a) or PasD (b) to determine damage, depicted as total pathology, or mucus production, respectively in NaCl or HDM exposed mice treated with C1-INH (black bars) or vehicle (white bars). Representative pictures of each staining per group are shown, magnification 10x. Data are presented as mean ± SEM, n=6 (NaCl) and n=10 (HDM).

One important feature of asthma is the increased production of mucus by bronchial epithelial cells[19]. Using a PAS-D staining to visualize mucus, lung sections were analyzed for the presence and localization of mucus resulting in a mucus score (Figure 4b). In line with total pathology, HDM exposure enhanced mucus production while C1-INH treatment had no effect hereon.

C1-INH treatment inhibits Th_2 response in MLN but not in BALF

In MLN, the draining lymph nodes of the lung, priming of T cells occurs[3]. To investigate the Th_2 response following HDM challenge, we dissected MLN. Macroscopically we observed larger MLNs in HDM exposed mice as compared with NaCl groups. Interestingly, MLN in the C1-INH treated HDM group were smaller as compared with vehicle treated HDM mice. Equal numbers of MLN cells were seeded and restimulated with HDM or NaCl. IL-4, IL-5, and IL-13 secretion was measured as a determinant of the Th_2 response. Only MLNs that were taken from mice challenged *in vivo* with HDM were used for this

analysis. Following HDM restimulation MLNs from C1-INH treated mice had significantly lower IL-13 and a trend (P=0.06) towards lower IL-4 and IL-5 production as compared with MLNs from vehicle treated mice (Figure 5a-c). Incubation of MLN with NaCl did not induce a detectable cytokine response in either treatment group.

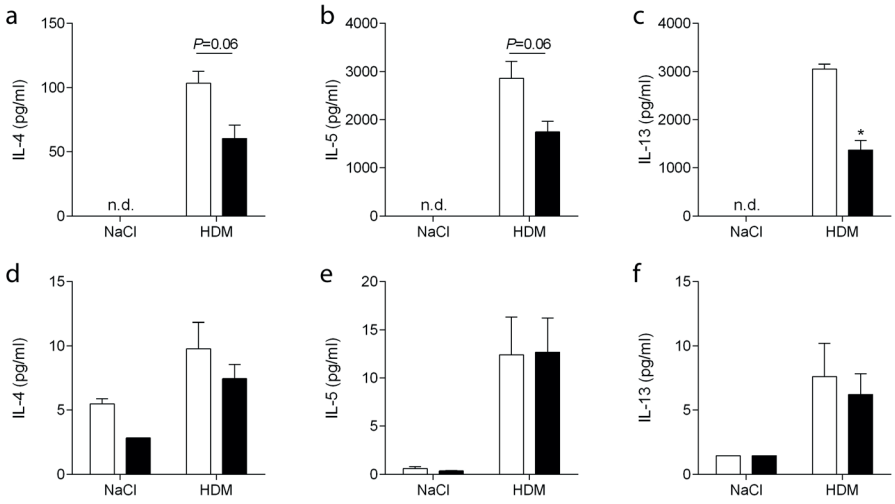

Figure 5. Th_2 response in MLN and BALF. Following NaCl or HDM exposure MLN were dissected from mice treated with C1-INH (black bars) or vehicle (white bars). Equal numbers of MLN cells from C1-INH (black bars) or vehicle (white bars) treated HDM exposed mice were restimulated ex vivo with HDM and afterwards production of IL-4 (a), IL-5 (b), and IL-13 (c) was determined in the medium. In BALF of NaCl or HDM exposed mice treated with C1-INH (black bars) or vehicle (white bars) the cytokines IL-4 (d), IL-5 (e) and IL-13 (f) were determined by a magnetic luminex screening assay. Data are presented as mean ± SEM, n=6 (NaCl) and n=10 (HDM). *$P<0.05$

Next we evaluated Th_2 cytokines IL-4, IL-5, and IL-13 in BALF. All three cytokine levels were low, however, increased following HDM exposure (Figure 5d-f). C1-INH treatment did not significantly alter IL-13, IL-4, or IL-5 levels in the BALF.

C1-INH treatment enhances HDM specific IgG_1

Plasma IgE significantly increased following HDM exposure, however, C1-INH treatment did not affect this response (Figure 6a). To see whether the HDM specific antibody response was altered, we determined HDM specific $IgG_{1;}$ there was a remarkably higher HDM specific plasma IgG_1 response in C1-INH treated mice compared with vehicle treated mice. HDM treatment induces vascular permeability[20] and C1-INH might affect this. We first measured plasma E-selectin as a marker of endothelial activation; C1-INH treatment had

no effect on E-selectin plasma levels (Figure 6c). Additionally, we determined E-selectin, total protein and IgM in the BALF as markers of plasma leakage. Both increased following HDM exposure, however C1-INH treatment did not alter these markers (Figure 6d-f). C1-INH might reduce coagulation via inhibiting the coagulation proteins FXIa or FXIIa[7]. Therefore we analyzed TATc levels in BALF to determine activation of coagulation. HDM exposure induced TATc in the BALF of both vehicle and C1-INH treated mice (Figure 6g). C1-INH treatment had no effect on the extent of coagulation activation.

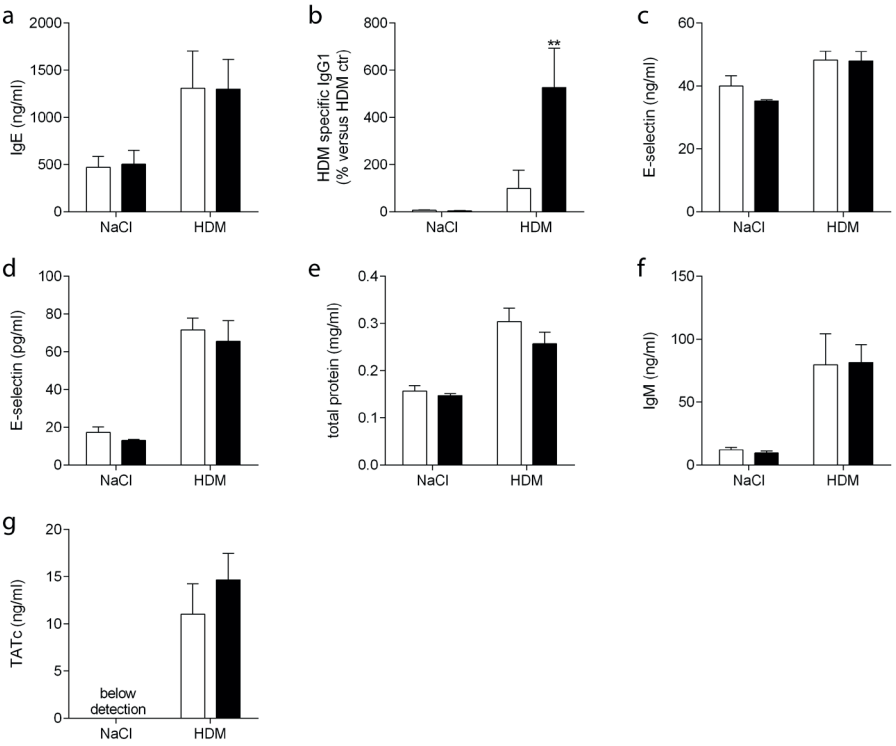

Figure 6. Plasma and BALF protein levels. In plasma of NaCl or HDM exposed mice treated with C1-INH (black bars) or vehicle (white bars) IgE (a), HDM specific IgG_1 (b) and E-selectin (c) were determined. In BALF of C1-INH (black bars) or vehicle (white bars) treated NaCl or HDM exposed mice E-selectin (d), total protein (e), IgM (f) and TATc (g) were determined. Data are presented as mean ± SEM, n=6 (NaCl) and n=10 (HDM). $^{**}P<0.01$

DISCUSSION

In the present study we determined the effect of human plasma-derived C1-INH in a clinically relevant HDM-induced asthma mouse model. C1-INH has already been proven to be beneficial in various inflammatory conditions. During lethal endotoxin shock, C1-INH protects against endothelial cell apoptosis, (micro)vascular permeability and mortality most likely via a direct effect on LPS that is independent of C1-INH protease inhibitor activity[21-23]. C1-INH protects against ischemia/reperfusion injury of the myocardium[8] and brain[24], early vein graft remodeling[25], and development of atherosclerosis[26]. Also in a TRALI (Transfusion Related Acute Lung Injury) model C1-INH has been proven to be effective in inhibiting complement activation and improving lung injury scores[27]. In our experimental setting we used a therapeutically relevant approach by administering C1-INH i.p. daily during the challenge phase, thereby circumventing a possible effect of C1-INH during the sensitization phase. It is for instance known that blocking C5a/C5aR signaling during the sensitization phase induces airway inflammation and hyperresponsiveness, while these parameters were reduced upon blocking of C5a/C5aR signaling during the challenge phase[28]. Although others have shown beneficial effects of C1-INH in inflammatory mouse models[8,21-27], we did not observe a clinical effect of C1-INH treatment in our setting. In addition, recently, de Beer et al. reported no effect of C1-INH treatment in two different rat pneumonia models[29,30].

Although several studies describing the effect of C1-INH in various experimental mouse models have been published, information about the pharmacokinetics of C1-INH in mice is limited. Therefore we analyzed the plasma C1-INH antigen and activity levels up to 72 hours after a single i.v. or i.p. injection. In humans the half-life of C1-INH is 28 hours[31], while the reported half-life in rats is 4½ hours[18]. To our knowledge, we are the first to describe the half-life of C1-INH in mouse, which is roughly 12 hours. Our aim was to reach plasma levels of 500-1000 µg/ml C1-INH in our experimental asthma model by administering once daily 15 U. At time of euthanize mice had C1-INH antigen plasma level of on average 900 µg/ml, 3 to 4-fold the human plasma concentration. C1-INH plasma levels were independent of HDM challenge. Human C1-INH antigen and activity BALF levels were elevated following HDM exposure. This is most likely due to an increase in plasma leakage, as confirmed by elevated BALF total protein and IgM. Human C1-INH antigen and activity levels were comparable in both plasma and BALF, indicating that all human C1-INH retained its protease inhibiting capacity. In line with previous studies in mice[16] and asthmatic

patients[5], local activation of complement was seen in BALF. This was slightly inhibited by C1-INH treatment.

Following repeated exposure to HDM, mice developed a Th_2 response as indicated by elevated levels of the cytokines IL-4, IL-5, and IL-13 in BALF and after restimulation of MLN. Following treatment with C1-INH, the Th2 cytokine response was lower in HDM restimulated MLN. Recently, a role for FXII in autoimmunity, independent of coagulation or contact-kinin system, was described[32]. In an experimental autoimmune encephalomyelitis mouse model, FXII deficiency or pharmacological blocking of FXIIa inhibited neuroinflammation and reduced influx of T cells in the brain[32]. The authors suggested that FXIIa acts on dendritic cells, thereby shaping T-cell differentiation and adaptive immunity[32]. As C1-INH is a potent inhibitor of FXIIa[7] and our results suggest a role for C1-INH in directing adaptive immunity, our data points into the same direction as the study by Göbel et al.[32]. Therefore, it would be of interest to see whether C1-INH would have therapeutic potential in autoimmune diseases such as multiple sclerosis.

Although the Th_2 response was inhibited in MLN of C1-INH treated mice, systemic total IgE was comparable and HDM specific IgG_1 was elevated following C1-INH treatment. From a classical point of view, a reduced Th_2 response should go hand in hand with lowered (allergen-specific) antibody responses. However, allergen specific IgG competes with allergen specific IgE and thereby protects against allergic immune response, a mechanism that is used during allergen-specific immunotherapy[33]. Indeed, high-dose HDM exposure can induce IgG-mediated protection against HDM-specific anaphylaxis[34]. Therefore we hypothesize that in our HDM exposed asthma model elevated HDM specific IgG_1 dampens adaptive immunity. Unfortunately we were not able to detect HDM specific IgE, as has been previously reported by others using the same HDM preparation[35,36].

C1-INH is a major regulator of the contact-kinin system by blocking of activated FXII and plasma kallikrein, thereby suppressing formation of bradykinin. In experimental allergic airway models, several bradykinin receptor antagonists have been proven to be beneficial by, amongst other anti-inflammatory effects, reducing plasma leakage (reviewed in [12]). Moreover, in C1-INH deficient mice, human plasma-derived C1-INH reverses enhanced vascular permeability[37]. In the present study, however, we did not see an effect of C1-INH treatment on plasma leakage as similar IgM and total protein levels were detected in C1-INH and vehicle treated groups.

Several reports show an effect of C1-INH treatment on leukocyte chemotaxis[8-10]. This anti-inflammatory effect of C1-INH is presumably independent of its protease inhibitory activity and therefore cannot be ascribed to an indirect effect of complement pathway inhibition. In the present study, however, we did not observe an effect of C1-INH treatment on influx of eosinophils.

C1-INH is an inhibitor of fibrinolysis and may therefore lead to increased risk of thromboembolic complications. Indeed, several reports suggest an association between C1-INH use and risk of thrombosis[38], especially beyond the approved clinical indications and doses. However, the effect of high dose of C1-INH on thrombotic events is under debate. Recently, Schürmann et al. investigated the prothrombotic potential in rabbits and found no evidence for thrombosis at doses up to 800 IU/kg, but even a slight protective (anti-coagulant) effect of C1-INH in the $FeCl_3$-induced arterial thrombosis model[39]. In line with the study from Schürmann, we did not observe a difference in coagulation activation using approximately the same dose of C1-INH. The observed adverse prothrombotic effects in clinical studies may be attributed to other causes such as the use of catheters or underlying thromboembolic risk factors, and further research is needed to confirm an association between thrombosis and C1-INH use[38,40].

In the mouse C1-INH is highly expressed in the liver, but also relatively high in the heart and lung[41]. Moreover, airway epithelial cells induce expression of C1-INH in dendritic cells[42]. It can be speculated that the mouse lung contains high levels of C1-INH, which might even be increased following allergen exposure, to exert its function in the HDM asthma model. Hence human C1-INH treatment might result in excess C1-INH, and it is therefore no longer possible to delineate the effects of endogenous and exogenous C1-INH. A limitation of this study is the lack of endogenous C1-INH data in plasma and BALF. As far as we know, no data is available on the levels of C1-INH in asthmatic patients or experimental asthma models. It would be of interest to study the effect of C1-INH in mice that lack endogenous C1-INH.

In conclusion, C1-INH dampens the adaptive immune response by inhibiting Th_2-induced cytokine production in MLN and by enhancing HDM-specific IgG_1. These effects are most likely not mediated via blocking the complement, contact-kinin, or coagulation systems. Recent evidence suggests that C1-INH via blocking FXII might directly shape the adaptive immune response.

REFERENCES

1. Martinez FD, Vercelli D. Asthma. *Lancet.* 2013;382(9901):1360-1372.
2. World Health Organization. WHO factsheet 307: Asthma. Available at: http://www.who.int/mediacentre/factsheets/fs307/en/. 2013.
3. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* 2015;16(1):45-56.
4. DiScipio RG, Schraufstatter IU. The role of the complement anaphylatoxins in the recruitment of eosinophils. *Int Immunopharmacol.* 2007;7(14):1909-1923.
5. Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med.* 2001;164(10 Pt 1):1841-1843.
6. Khan MA, Nicolls MR, Surguladze B, Saadoun I. Complement components as potential therapeutic targets for asthma treatment. *Respir Med.* 2014;108(4):543-549.
7. Zeerleder S. C1-inhibitor: more than a serine protease inhibitor. *Semin Thromb Hemost.* 2011;37:362-374.
8. Lu F, Fernandes SM, Davis AE, III. The effect of C1 inhibitor on myocardial ischemia and reperfusion injury. *Cardiovasc Pathol.* 2013;22(1):75-80.
9. Lu FX, Fernandes SM, Davis AE. The role of the complement and contact systems in the dextran sulfate sodium-induced colitis model: the effect of C1 inhibitor in inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol.* 2010;298:G878-G883.
10. Thorgersen EB, Ludviksen JK, Lambris JD, Sfyroera G, Nielsen EW, Mollnes TE. Anti-inflammatory effects of C1-Inhibitor in porcine and human whole blood are independent of its protease inhibition activity. *Innate Immun.* 2010;16:254-264.
11. de Boer JD, Majoor CJ, van t, V, Bel EH, van der Poll T. Asthma and coagulation. *Blood.* 2012;119:3236-3244.
12. Abraham WM, Scuri M, Farmer SG. Peptide and non-peptide bradykinin receptor antagonists: role in allergic airway disease. *Eur J Pharmacol.* 2006;533(1-3):215-221.
13. Abraham WM, Burch RM, Farmer SG, Sielczak MW, Ahmed A, Cortes A. A bradykinin antagonist modifies allergen-induced mediator release and late bronchial responses in sheep. *Am Rev Respir Dis.* 1991;143(4 Pt 1):787-796.
14. Soler M, Sielczak M, Abraham WM. A bradykinin-antagonist blocks antigen-induced airway hyperresponsiveness and inflammation in sheep. *Pulm Pharmacol.* 1990;3(1):9-15.
15. Vasquez-Pinto LM, Nantel F, Sirois P, Jancar S. Bradykinin B(1) receptor antagonist R954 inhibits eosinophil activation/proliferation/migration and increases TGF-beta and VEGF in a murine model of asthma. *Neuropeptides.* 2010;44(2):107-113.
16. Daan de BJ, Roelofs JJ, de Vos AF, et al. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *Am J Respir Cell Mol Biol.* 2013;48:382-389.
17. Lee JJ, McGarry MP, Farmer SC, et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *JExpMed.* 1997;185(12):2143-2156.
18. de Smet BJ, de Boer JP, Agterberg J, Rigter G, Bleeker WK, Hack CE. Clearance of human native, proteinase-complexed, and proteolytically inactivated C1-inhibitor in rats. *Blood.* 1993;81:56-61.
19. Thomson NC, Chaudhuri R, Messow CM, et al. Chronic cough and sputum production are associated with worse clinical outcomes in stable asthma. *Respir Med.* 2013;107(10):1501-1508.
20. Wang JY. The innate immune response in house dust mite-induced allergic inflammation. *Allergy Asthma Immunol Res.* 2013;5(2):68-74.
21. Liu DX, Cai SH, Gu XG, Scafidi J, Wu X, Davis AE. C1 inhibitor prevents endotoxin shock via a direct interaction with lipopolysaccharide. *J Immunol.* 2003;171:2594-2601.

22. Liu DX, Zhang D, Scafidi J, Wu X, Cramer CC, Davis AE. C1 inhibitor prevents Gram-negative bacterial lipopolysaccharide-induced vascular permeability. *Blood*. 2005;105:2350-2355.
23. Mejia P, Davis AE, III. C1 Inhibitor Suppresses the Endotoxic Activity of a Wide Range of Lipopolysaccharides and Interacts With Live Gram-negative Bacteria. *Shock*. 2012;38:220-225.
24. De Simoni MG, Rossi E, Storini C, Pizzimenti S, Echart C, Bergamaschini L. The powerful neuroprotective action of C1-inhibitor on brain ischemia-reperfusion injury does not require C1q. *Am J Pathol*. 2004;164:1857-1863.
25. Krijnen PAJ, Kupreishvili K, de Vries MR, et al. C1-esterase inhibitor protects against early vein graft remodeling under arterial blood pressure. *Atherosclerosis*. 2012;220:86-92.
26. Shagdarsuren E, Bidzhekov K, Djalali-Talab Y, et al. C1-esterase inhibitor protects against neointima formation after arterial injury in atherosclerosis-prone mice. *Circulation*. 2008;117:70-78.
27. Muller MC, Stroo I, Wouters D, et al. The effect of C1-inhibitor in a murine model of transfusion-related acute lung injury. *Vox Sang*. 2014;107(1):71-75.
28. Kohl J, Baelder R, Lewkowich IP, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest*. 2006;116(3):783-796.
29. de Beer FM, Aslami H, Hoeksma J, et al. Plasma-derived human C1-esterase inhibitor does not prevent mechanical ventilation-induced pulmonary complement activation in a rat model of Streptococcus pneumoniae pneumonia. *Cell Biochem Biophys*. 2014;70(2):795-803.
30. de Beer F, Lagrand W, Glas GJ, et al. Nebulized C1-Esterase Inhibitor does not Reduce Pulmonary Complement Activation in Rats with Severe Streptococcus Pneumoniae Pneumonia. *Cell Biochem Biophys*. 2016;74(4):545-552.
31. Caliezi C, Wuillemin WA, Zeerleder S, Redondo M, Eisele B, Hack CE. C1-Esterase inhibitor: an anti-inflammatory agent and its potential use in the treatment of diseases other than hereditary angioedema. *Pharmacol Rev*. 2000;52(1):91-112.
32. Gobel K, Pankratz S, Asaridou CM, et al. Blood coagulation factor XII drives adaptive immunity during neuroinflammation via CD87-mediated modulation of dendritic cells. *Nat Commun*. 2016;7:11626.
33. Jay DC, Nadeau KC. Immune mechanisms of sublingual immunotherapy. *Curr Allergy Asthma Rep*. 2014;14(11):473.
34. Hirai T, Yoshioka Y, Takahashi H, et al. High-dose cutaneous exposure to mite allergen induces IgG-mediated protection against anaphylaxis. *Clin Exp Allergy*. 2016.
35. Post S, Heijink IH, Petersen AH, de Bruin HG, van Oosterhout AJ, Nawijn MC. Protease-activated receptor-2 activation contributes to house dust mite-induced IgE responses in mice. *PLoS One*. 2014;9(3):e91206.
36. Post S, Nawijn MC, Hackett TL, et al. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax*. 2012;67(6):488-495.
37. Han Lee ED, Pappalardo E, Scafidi J, Davis AE, III. Approaches toward reversal of increased vascular permeability in C1 inhibitor deficient mice. *Immunol Lett*. 2003;89(2-3):155-160.
38. Gandhi PK, Gentry WM, Bottorff MB. Thrombotic events associated with C1 esterase inhibitor products in patients with hereditary angioedema: investigation from the United States Food and Drug Administration adverse event reporting system database. *Pharmacotherapy*. 2012;32(10):902-909.
39. Schurmann D, Herzog E, Raquet E, et al. C1-esterase inhibitor treatment: preclinical safety aspects on the potential prothrombotic risk. *Thromb Haemost*. 2014;112(5):960-971.
40. Crowther M, Bauer KA, Kaplan AP. The thrombogenicity of C1 esterase inhibitor (human): review of the evidence. *Allergy Asthma Proc*. 2014;35(6):444-453.
41. Lener M, Vinci G, Duponchel C, Meo T, Tosi M. Molecular cloning, gene structure and expression profile of mouse C1 inhibitor. *Eur J Biochem*. 1998;254:117-122.
42. Rate A, Bosco A, McKenna KL, Holt PG, Upham JW. Airway epithelial cells condition dendritic cells to express multiple immune surveillance genes. *PLoS One*. 2012;7(9):e44941.

Complement factor C5 inhibition reduces type 2 responses without affecting group 2 innate lymphoid cells in a house dust mite induced murine asthma model

5

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ABSTRACT

Background: Complement factor C5 can either aggravate or attenuate the T-helper type 2 (T_H2) immune response and airway hyperresponsiveness (AHR) in murine models of allergic asthma. The effect of C5 during the effector phase of allergen-induced asthma is ill-defined.

Objectives: We aimed to determine the effect of C5 blockade during the effector phase on the pulmonary T_H2 response and AHR in a house dust mite (HDM) driven murine asthma model.

Methods: BALB/c mice were sensitized and challenged repeatedly with HDM via the airways to induce allergic lung inflammation. Sensitized mice received twice weekly injections with a blocking anti-C5 or control antibody 24 hours before the first challenge.

Results: HDM challenge in sensitized mice resulted in elevated C5a levels in bronchoalveolar lavage fluid. Anti-C5 administered to sensitized mice prior to the first HDM challenge prevented this rise in C5a, but did not influence the influx of eosinophils or neutrophils. While anti-C5 did not impact the recruitment of CD4 T cells upon HDM challenge, it reduced the proportion of T_H2 cells recruited to the airways, attenuated IL-4 release by regional lymph nodes restimulated with HDM *ex vivo* and mitigated the plasma IgE response. Anti-C5 did not affect innate lymphoid cell (ILC) proliferation or group 2 ILC (ILC2) differentiation. Anti-C5 attenuated HDM induced AHR in the absence of an effect on lung histopathology, mucus production or vascular leak.

Conclusions: Generation of C5a during the effector phase of HDM induced allergic lung inflammation contributes to T_H2 cell differentiation and AHR without impacting ILC2 cells.

INTRODUCTION

Asthma is a heterogeneous disease characterized by airway hyperresponsiveness (AHR) and usually chronic airway inflammation dominated by a T-helper type 2 (T_H2) response[1,2]. The vast majority of allergic asthma patients are sensitized to house dust mite (HDM) and exposure to this abundantly present allergen causes respiratory symptoms such as coughing, wheezing and reversible airway obstruction[3].

The complement system is an important part of the innate immune system and consists of a network of proteins that when activated releases proteolytic fragments with pro-inflammatory properties. Activation of the complement system can occur through three pathways (i.e., the classical, lectin and alternative pathways), which lead to downstream proteolytic cleavage of C3 and C5, resulting in the release of the anaphylatoxins C3a and C5a[4,5]. Recent investigations revealed a novel role for these anaphylatoxins and their receptors in the pathogenesis of asthma[6,7]. In asthma patients, elevated levels of C3a and C5a were detected in the airways following allergen challenge[8]. While C3a signaling aggravates AHR[9] and drives allergic inflammation in different asthma models[10-12], C5a can exert both protective and detrimental effects during the course of an allergic inflammation. Prior to allergen sensitization, genetic deletion or pharmacological blockade of C5 or the C5a receptor (C5aR) resulted in a strongly enhanced allergic phenotype[13,14]. Mechanistically, C5a/C5aR signaling regulated dendritic cells (DC) function, thereby favoring plasmacytoid DCs to suppress T-cell activation[13]. In addition, C5a/C5aR signaling in myeloid DCs hampered production of the chemokines CCL17 and CCL22, leading to an impaired recruitment of T_H2 cells into the lung[15]. Furthermore, C5a/C5aR signaling can induce IL-12 production in antigen presenting cells and potentiates skewing toward T_H1 responses[16]. In contrast, eliminating C5a/C5aR signaling after the sensitization phase reduced allergic lung inflammation [17] and AHR [18,19]. The underlying mechanisms for this C5a mediated proallergic effect in an established inflammation environment is not well understood.

Group 2 Innate lymphoid cells (ILC2s) have been recognized to play an important role in type 2 immune responses[20,21]. As an innate counterpart of T_H2 cells, ILC2s orchestrate the allergic immune response by producing T_H2 associated cytokines (IL-5 and IL-13) and presenting antigen to naïve T-cells for an effective T_H2 cell development[22]. In the absence of T and B cells, the presence of ILC2s is sufficient to initiate and maintain an allergic lung inflammation and AHR

in distinct mouse asthma models[23], emphasizing the significant contribution of ILC2s to the hallmarks of asthma. In earlier experimental asthma models blocking the C5a/C5aR axis, the type 2 response was primarily attributed to T_H2 cells without assessing the contribution of ILC2s[13,18]. We here studied the effect of C5 inhibition during the effector phase on the type 2 responses in the lung and AHR in a HDM induced asthma model.

METHODS

Mice

Female BALB/c mice (8-12 weeks old) were purchased from Charles River (Maastricht, the Netherlands). 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 in mice, repeated HDM extract intranasal challenges were performed as described previously[24]. Briefly, 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. Prior to intranasal administration of HDM, all mice were anesthetized with isoflurane. BALB/c mice were injected intraperitoneally with a rat anti-mouse C5 monoclonal antibody (clone BB5.1; 1mg/mouse)[25] or an irrelevant control antibody twice weekly (on days 13, 14, 17 and 18) during the challenge phase. 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) fluid was collected by airway lumen lavage with 2x 0.5 ml PBS containing 10mM EDTA, 10mM benzamidine and 0.2mg/ml soy bean trypsin inhibitor as described[24]. Cell counts were measured using a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and cell differentiation was made by flow cytometric analysis. In one experiment the lavaged lungs were minced, followed by enzymatic digestion in RPMI medium with 5% Fetal Bovine Serum, 1% penicillin/streptomycin, liberase™ and DNAse at 37°C for 30 minutes. Next, incubated cells were dissociated by aspiration through a 19-gauge needle to obtain single cells. Erythrocytes were lysed with sterile lysis buffer (Qiagen, Hilden, Germany). Unflushed lung, collected in a separate experiment, was used for pathology examination to avoid structural disruption as a consequence of BAL.

Measurement of enhanced pause (PenH)

PenH was measured at day 19 by whole-body plethysmograph in conscious mice (Buxco Electronics, Troy, NY, USA) as described[24]. Mice were first subjected to aerosolized saline to determine nonspecific responsiveness, followed by increasing concentrations of aerosolized methacholine (3.1, 12.5, 25 and 50 mg/mL in saline for 3 min; Sigma-Aldrich). For each methacholine dose PenH values were measured over five minutes.

Flow cytometry

Cells in BAL fluid were stained with CD3 FITC, CD11c PercP, Siglec F 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, and CD45 PE-eFluor610 (eBiosciences, San Diego, CA, USA) in the presence of Fc blocker (CD16/CD32, eBiosciences). Single cell suspensions from lungs were stained with CD4 FITC, CD45 PerCP-Cy5.5 (eBiosciences), GATA-3 Alexa 647, and viability dye APC-Cy7 (BD Biosciences). The following markers were used for the analysis of ILCs in lung tissue: Lineage (Lin) markers including CD3e, CD19, GR1, B220, Ter119, FcaR1 (all FITC, Biolegend), CD45 Alexa700, CD90 PE, ST2 Brilliant Violet 421 (Biolegend), CD49b PE-Cy7 (eBiosciences) and CD3 Percp-Cy5.5 (BD biosciences). Mediastinal lymph nodes (mLN) cells were stained with CD45 PerCP-Cy5.5, CD4 FITC, GATA-3 Alexa 647 (BD Biosciences) and IL-4 APC (Biolegend). For intracellular/intranuclear staining, cells were permeabilized and fixed using a FOXp3 Staining Buffer set (eBioscience) and subsequently stained with the appropriated markers. All appropriate Fluorescence Minus One (FMO) controls were used. Data were collected on a BD Biosciences Canto II flow cytometer or BD FACSAria™ III and analyzed using FlowJo software (Treestar, Palo Alto, CA, USA).

Assays

C5a was measured in BAL fluid by ELISA. Purified rat anti-mouse C5a (clone I52-1486) was used as capture antibody, purified recombinant mouse C5a as standard and biotinylated rat anti-mouse C5a (clone I52-278) as detection antibody (all from BD Biosciences). Cytokines (IL-4, IL-5, IL-13), myeloperoxidase (MPO) and elastase were measured by ELISA (R&D systems, Minneapolis, MN, USA). 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) as described[24]. Plasma HDM-specific IgG1 was determined using HDM as capture and biotinylated rat-anti-mouse IgG1 as detection (BD Biosciences). BAL fluid IgM was determined as described[24],

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. Total protein in BAL fluid was measured using Bio-Rad protein assay (Bio-Rad Laboratories, Veenendaal, Netherlands).

Ex vivo stimulation of mediastinal lymph nodes (mLN)

Stimulations of mLN ex vivo were performed as described[24]. Briefly, mLN were harvested 24 hours after the last challenge and filtered through 100 µm strainers. Single cells were seeded at a density of 2 x 10^5 cells/well in 96-well round bottom plates (Greiner Bio-One, Alphen a/d Rijn, Netherlands) and incubated with 25 µg/ml HDM or PBS for four days at 37°C with 5% CO. Supernatants were collected and stored at -80°C until analysis. In a separate experiment IL-4 production by CD4 T-cells in mLN were determined. To that end, mLN cells were stimulated with PMA (10ng/ml) and ionomycin (1500ng/ml; both Sigma-Aldrich) for five hours in the presence of Golgiplug (BD Biosciences) for the final three hours.

Histology

Histological analysis was performed as described[24]. Briefly, after fixation in 10% formalin, four µm thick sections were stained with Hematoxylin and Eosin (H&E) to determine allergic inflammation properties such as edema, endothelialitis, peribronchial and perivascular inflammation and interstitial inflammation on a scale from 0 to 4 (0: absent; 1: mild; 2: moderate; 3: severe; 4: very severe). 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. Slides were coded and scored by a pathologist in a blinded fashion.

Statistical analysis

Data were analyzed by Mann-Whitney U-test for comparison between groups. Two-way analysis of variance test followed by Tuckey's multiple comparison test was used for groups of three or more. Experimental groups consisted of 6-8 mice. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.

RESULTS

C5 inhibition does not modify leukocyte influx in the airways upon HDM challenge

To investigate the contribution of C5 activation to allergic lung inflammation HDM sensitized mice were treated with a neutralizing anti-C5 mAb during repeated HDM challenges via the airways. HDM challenge resulted in increased C5a levels in BAL fluid. Treatment with anti-C5 mAb during the challenge phase reduced HDM-induced C5a concentrations to levels detected in unchallenged mice (Fig. 1A). Repeated HDM challenge triggered an influx of leukocytes into BAL fluid (Fig. 1B), which was the result of recruitment of eosinophils (Fig. 1C) and neutrophils (Fig. 1D). The number of alveolar macrophages in BAL fluid was lower in HDM challenged mice when compared with control animals (Fig. 1E).

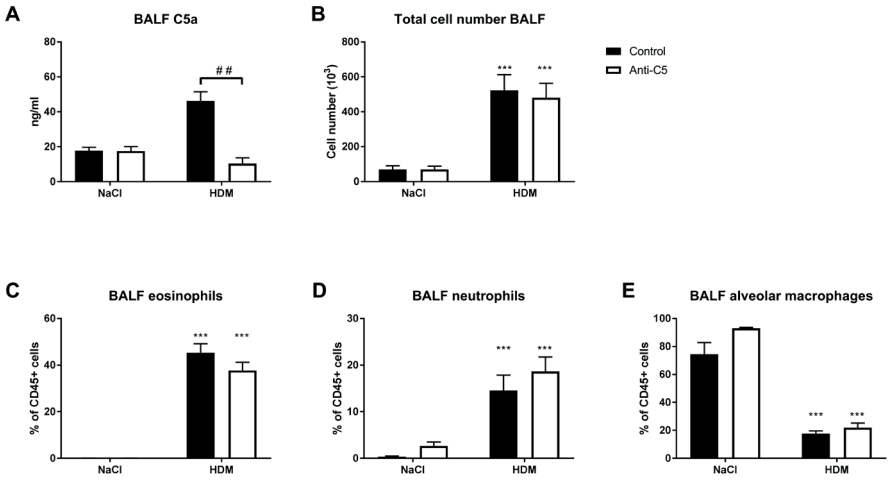

Figure 1. C5 inhibition does not modify leukocyte influx in the airways. Mice were injected intraperitoneally with anti-C5 or control antibody twice weekly during the challenge phase. (A) C5a concentration in bronchoalveolar lavage fluid (BALF) from saline (NaCl) or HDM challenged mice. (B) Total cell count in BALF. (C-E) Percentage of eosinophils, neutrophils and alveolar macrophages in BALF. Data are expressed as means ± SEM (n=6-8 per group). ##P<0.01, ***P<0.001 for comparison between NaCl and HDM within control or anti-C5 antibody injected mice.

Anti-C5 did not modify leukocyte numbers or composition in BAL fluid. HDM challenge also caused neutrophil degranulation, as reflected by elevated concentrations of MPO and elastase in BAL fluid; this response was not altered by anti-C5 (Fig. 2A,B).

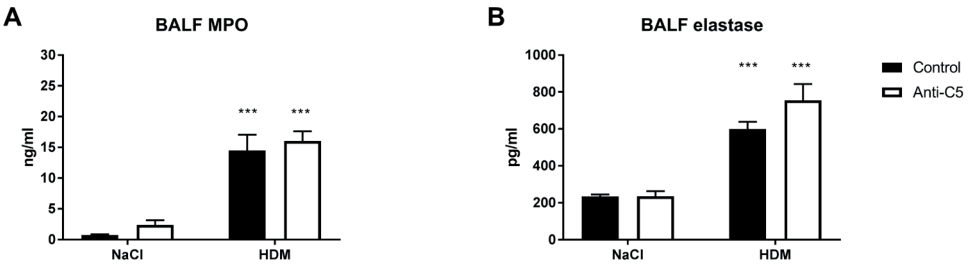

Figure 2. C5 inhibition does not alter neutrophil degranulation. (A) Myeloperoxidase and (B) elastase concentration in bronchoalveolar lavage fluid (BALF). All data are presented as means ± SEM (n=6-8 per group). ***P <0.001 for comparison between NaCl and HDM within control or anti-C5 mice.

C5 inhibition reduces T_H2 but not ILC2 cell numbers following HDM challenge

ILC2s and T_H2 cells are both essential for the initiation and propagation of a type 2 immune response in allergic airway inflammation[22]. Therefore, we determined whether C5 inhibition influences the influx or expansion of these cells upon HDM challenge in sensitized mice. Total ILCs were defined as $CD45^+Lin^-CD3^-CD49b^-CD90^+$ cells, and within the total ILC population, ILC2s were further identified by expression of ST2 (Additional file 1: Figure S1). C5 inhibition during the challenge phase did not affect the proportion of total ILC or ILC2s in the lungs (Fig. 3A). Likewise, the percentage of CD4 T-cells in the lungs was similar between the anti-C5 treated and control group following HDM challenge (Fig. 3B). However, the percentage of T_H2 cells, defined as $CD4^+GATA\text{-}3^+$ cells, within the total CD4 T-cell population (Additional file 2: Figure S2) was significantly lower in anti-C5 treated mice.

C5 inhibition reduces type 2 responses to HDM

To further evaluate type 2 responses, we measured T_H2 cytokines in BAL fluid and supernatants of mLNs re-stimulated with HDM. As expected, HDM challenge elicited enhanced IL-5 and IL-13 release in BAL fluid compared with saline controls (Fig. 4A,B). C5 inhibition did not alter the levels of these T_H2 cytokines in BAL fluid. IL-4 remained below detection limit in BAL fluid, consistent with previous results from our group[24].

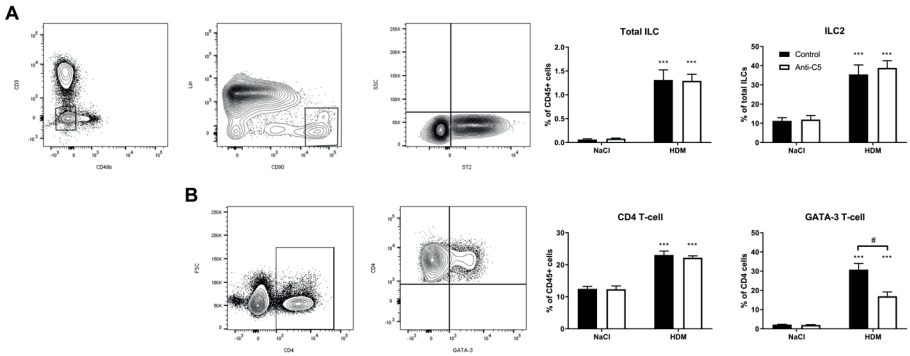

Figure 3. C5 inhibition reduces T_H2 cells but not ILC2 following HDM challenge. Flow cytometry analysis of group 2 innate lymphoid cells (ILC2) and T-helper 2 cells (T_H2) in lung tissue. All cells depicted here are pre-gated as single, viable and $CD45^+$ cells. (A) Total ILCs were defined as $CD45^+Lin^-CD3^-CD49b^-$ $CD90^+$ cells. Within the total ILC population $ST2^+$ cells were regarded as ILC2 cells. (B) Total T-helper cells were defined as $CD45^+CD4^+$ cells. T_H2 cells within total T-helper cell population stain positive for the transcription factor GATA-3. All data are means ± SEM (n=8 per group). *#P* <0.05 ****P* <0.001 for comparison between NaCl and HDM within control or anti-C5 mice.

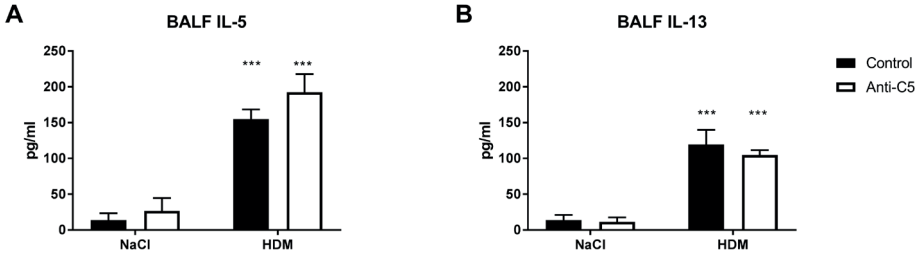

Figure 4. C5 inhibition does not modulate cytokine release in BALF following HDM challenge. (A,B) IL-5, IL-13 in bronchoalveolar lavage fluid (BALF) Data are depicted as means ± SEM (n=6-8 per group). ****P*<0.001 for comparison between NaCl and HDM within control or anti-C5 mice.

Re-stimulation of mLNs obtained from HDM challenged mice with HDM resulted in release of IL-4, IL-5 and IL-13 (Fig. 5A-C). C5 inhibition during the HDM challenge phase was associated with diminished IL-4 release by mLN upon re-exposure to HDM, while IL-5 and IL-13 release were not modified. The attenuated IL-4 release by mLN in C5 inhibited mice was accompanied by reduced intracellular IL-4 production by mLN derived CD4 T-cells (Fig. 5D) and a lower proportion of $CD4^+GATA\text{-}3^+$ T-cells in mLN (Fig. 5E). During allergic inflammation, IL-4 mediates the class switch recombination of IgM to IgE and IgG1 in B-cells [22].

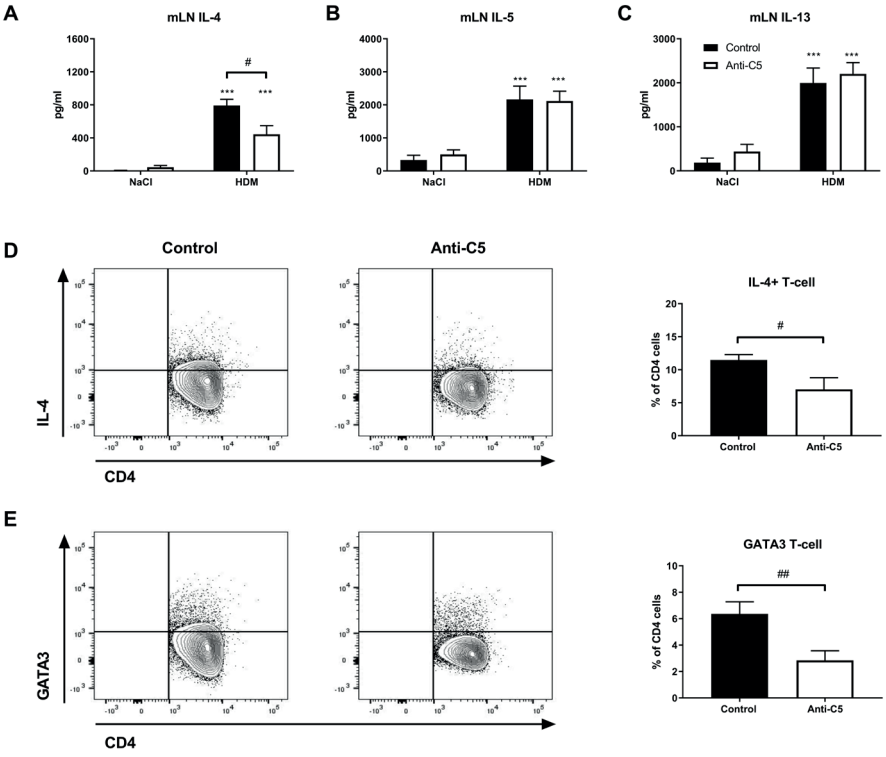

Figure 5. C5 inhibition mitigates IL-4 production and percentage of T_H2 cell in the mLN following HDM challenge. Mediastinal lymph nodes (mLN) were harvested from HDM sensitized and challenged mice in both control and anti-C5 groups. In one experiment mLN cells were stimulated with either saline (NaCl) or HDM for four days. In cell-free supernatants the cytokines IL-4, IL-5 and IL-13 (A-C) were measured. In a separated experiment mLN cells were incubated with PMA and ionomycin in the presence of Golgiplug, followed by intracellular staining of IL-4 (D) and intranuclear staining of GATA-3 (E). Data are depicted as means ± SEM (n=6-8 per group). #P<0.05, ##P<0.01, ***P<0.001 for comparison between NaCl and HDM within control or anti-C5 mice.

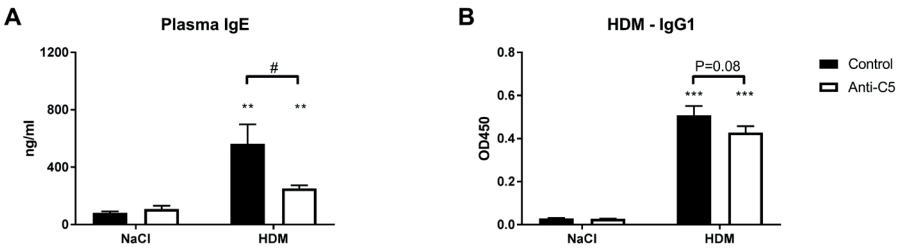

Figure 6. C5 inhibition reduces immunoglobulin release in plasma following HDM challenge. (A) IgE concentration and (B) absorbance (OD450) of HDM-specific IgG1 in plasma. Data are express as means ± SEM (n=6-8 per group). #$P<0.05$, **$P<0.01$ and ***$P<0.001$ for comparison between NaCl and HDM within control or anti-C5 mice.

Repeated HDM challenge led to elevated plasma IgE and HDM-specific IgG1 concentrations (Fig. 6A,B). C5 inhibition reduced total IgE and tended to lower HDM-specific IgG1 ($P=0.08$). Together these data show that C5 inhibition during the HDM challenge phase attenuates type 2 responses without affecting ILC2s.

C5 inhibition does not modify lung pathology 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[24] (Fig. 7A,B). Anti-C5 treated mice displayed HDM-induced lung inflammation to the same extent as control mice, as reflected by the semi-quantitative scoring system described in the Methods section. Likewise, HDM challenge evoked mucus production was unaltered in anti-C5 administered mice. Complement C5 has been implicated to play a role in vascular permeability[26,27]. We measured total protein and IgM in BAL fluid as measures for vascular leak. C5 inhibition in HDM challenged mice did not attenuate allergen induced vascular leak (Fig. 7C,D).

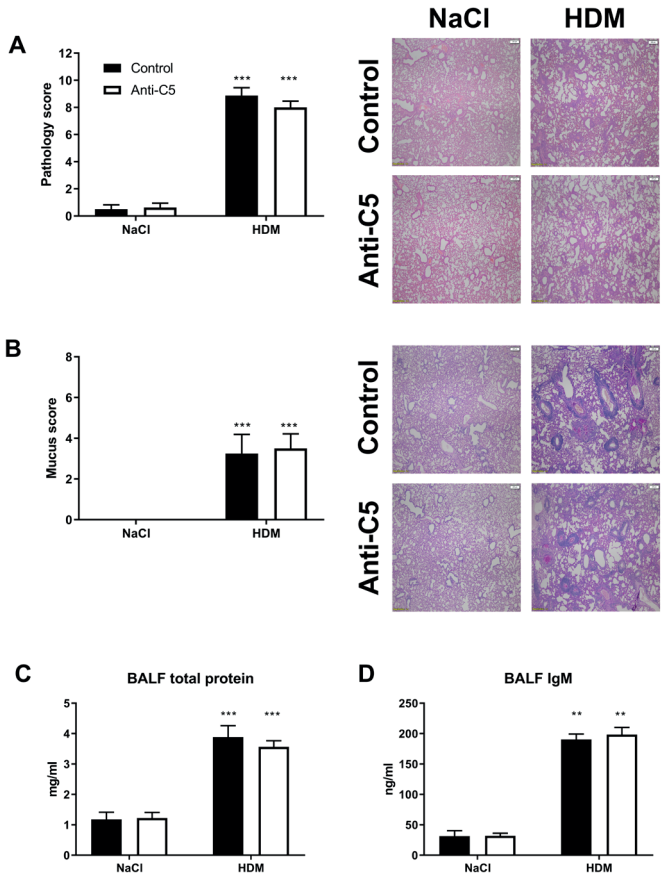

Figure 7. C5 inhibition does not alter lung pathology and vascular permeability following HDM challenge. (A) Total pathology score of hematoxylin and eosin (H&E) stained lung sections (x4 magnification) from control (black bar) and BB5.1 (white bar) treated mice and (B) total mucus score of PAS-D stained lung sections(x4 magnification) (n=6-8 per group). Protein leakage was assessed using (C) total protein and (D) IgM in BALF (n=6-8 per group). $^{**}P<0.01$ and $^{***}P<0.001$ for comparison between NaCl and HDM within control or anti-C5 mice.

C5 inhibition reduces airway hypersensitivity as measured by PenH

To obtain insight into the potential functional consequences of attenuated T_H2 responses in C5 inhibited mice, we measured PenH as a marker for AHR (Fig. 8). Consistent with our previous studies[24] HDM sensitization and challenge elicited enhanced AHR in comparison to saline challenge. C5 inhibition significantly attenuated allergen induced AHR.

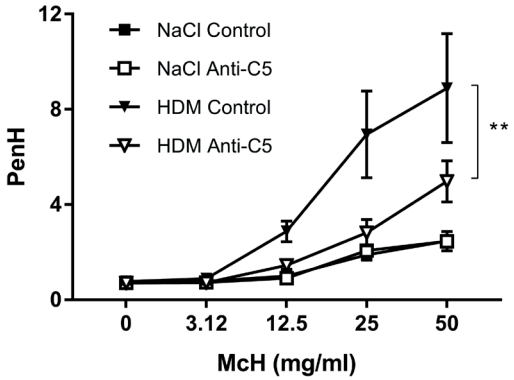

Figure 8. C5 inhibition attenuates AHR following HDM challenge. Airway hypersensitivity (AHR) expressed as enhanced pause (PenH) after dose-response to methacholine (Mch) exposure on day 19. Data are presented as means ± SEM (n=6-8 per group). ***P*<0.01

DISCUSSION

The anaphylatoxin C5a has been unveiled to play an important role in orchestrating the maladaptive T_H2 immune response in murine asthma models[15,16]. The mechanism by which C5a amplifies T_H2 inflammation in an inflamed pulmonary environment is unclear. In the present study we demonstrate that C5 inhibition during the effector phase does not modulate the proportion of ILC2s but decreases T_H2 cells in the lung and mLN, which is accompanied by reduced type 2 responses and an attenuated AHR.

Our HDM driven asthma model reproduces important features of allergic asthma such as pulmonary T_H2 inflammation, AHR and airway mucus production[28]. In accordance with similar asthma models, HDM elicited an expansion of both ILCs and T_H2 cells in the lungs[29,30]. We here demonstrate for the first time that C5 inhibition during the effector phase does not modify the total ILC or ILC2 population in the lung, indicating that C5a signaling is not involved in ILC proliferation or differentiation. This finding is consistent with a study showing the lack of C5aR expression on ILC2s[31], rendering direct C5a signaling unlikely. Furthermore, specific deletion of C5aR on cells expressing the LysM promoter (such as neutrophils, macrophages and dendritic cells) in an OVA-model showed an equally strong increase in allergen evoked ILC2 cells compared to wild-type mice[32]. Our data indicate explicitly that C5 blockade during the effector phase hampers the differentiation of T_H2 cells, resulting in reduced IL-4 production in mLN with unaltered IL-5 and IL-13 BALF levels. This discrepancy in T_H2 cytokines has also been observed in previous studies in which C5aR was blocked during the effector phase in a murine asthma

model[13,18]. While the exact mechanism remains enigmatic, our data suggest that ILC2 derived IL-5 and IL-13 may have compensated a presumable lower IL-5 and IL-13 release from reduced T_H2 cells numbers. Indeed, several studies reported enhanced IL-5 and IL-13 secretion by expanding ILC2s[33,34], while ILC2s are generally considered either not to produce IL-4[20,22] or produce it in negligible amounts[35]. We found that C5 inhibition impeded IL-4 production in mLN which was associated with decreased plasma IgE and HDM-specific IgG1 responses. This finding is corroborated by a study which prevented C5 activation during the effector phase in an OVA-induced asthma model[19]. Conversely, abrogating C5 activation prior to sensitization yielded enhanced IgE production[14], emphasizing the time dependent dual character of C5a signaling during allergic inflammation. Remarkably, previous investigations demonstrated decreased eosinophil numbers without affecting IL-5 release in the airways following C5a signaling blockade[13,18,19]. The mechanism behind this disparity was not addressed. In our study, the absence of an effect on IL-5 and the eosinophil attractant CCL11 (data not shown) by anti-C5 treatment corresponded with a lack of differences in total leukocyte, especially eosinophil, influx in the airways. The unaltered airway leukocyte recruitment is in contrast to studies that likewise investigated C5a signaling in established asthma. Different allergens (i.e. OVA[19] or Aspergillus fumigatus[18]) used in these studies may have contributed. C5aR antagonists appeared to be potent to mitigate allergen induced leukocytes infiltration, including eosinophils, neutrophils and lymphocytes[13,18] while blocking the anaphylatoxin C5a only limits neutrophil influx[19] or fails to affect leukocyte influx as shown by this study. Beside eosinophils, neutrophils express C5aR (CD88) on their cell membrane making direct C5a signaling possible[31]. C5a has been shown to be chemotactic for neutrophils and to promote release of neutrophil intracellular content[36]. Nonetheless, data from present study contradict an effect of C5a on neutrophil recruitment and neutrophil degranulation in HDM induced lung inflammation.

Consistent with earlier studies eliminating C5a/C5aR signaling during the effector phase[13,18,19], we observed an attenuated AHR in sensitized anti-C5 treated mice. Multiple factors may have contributed to this finding. First, a decreased total IgE concentration due to C5 inhibition could impair mast cell degranulation and consequently mast cell derived histamine induced bronchoconstriction. As C5a is an activator of mast cells, inhibited C5a activity could also influence AHR in a mast cell dependent manner[15]. Alternatively, C5a might aggravate AHR directly via activation of the C5aR on bronchial smooth muscle cells[37]. Our data argue against T_H2 cytokine mediated attenuation of AHR, since IL-13 is essential for the regulation of AHR[38] and unaffected by C5 inhibition. IL-4 and IL-13 express

high resemblance and transmit signals via shared functional receptor complexes (IL-4Rα/IL-13Rα1)[38]. Despite these similarities, a number of *in vivo* functional experiments have shown that IL-4 and IL-13 facilitate different features of allergic asthma. Specifically, IL-4 is regarded a regulator of T_H2 cell proliferation and IgE synthesis[39-41] while IL-13 is thought to mediate AHR, mucus production, airway smooth muscle thickening and sub-epithelial fibrosis[42-45]. In accordance with the unaltered IL-13 concentration in BAL fluid, C5 inhibition did not modulate HDM induced mucus production. Although activation of C5 has been shown to enhance vascular permeability [46], C5 inhibition did not modulate vascular permeability in our HDM induced asthma model.

As result of C5 inhibition we observed reduced C5a levels which implies a simultaneously reduced formation of the membrane attack complex (MAC), since the assembly of the MAC occurs after cleavage of C5 into C5a and C5b[4]. While MAC drives numerous proinflammatory events and regulates cell signaling, its role in asthma has not been studied and the contribution of MAC in HDM-induced responses remains to be elucidated.

We used unrestrained whole body plethysmography (PenH) as measure for AHR. The use of this tool to measure airway resistance is under debate. However, the merit of this technique has been shown in various mouse models in which PenH results correlate well with results from invasive measurement techniques[47-49]. A close correlation between invasive measurements and whole body plethysmography has been validated in a comparable HDM induced asthma mouse model[48]. Moreover, AHR, measured by invasive methods, is enhanced following HDM challenge in models resembling ours[50,51]. Nonetheless, interpretation of our Penh data without validation by invasive measurements is a limitation of this study.

CONCLUSION

In conclusion, our data demonstrate that complement C5 activation during the challenge phase drives T_H2 responses via T_H2 cells but not ILC2s and aggravates allergen induced AHR. Further research is warranted to elucidate the underlying mechanisms of the C5a mediated effect on IL-4 production. Despite the promising beneficial effect of C5 inhibition on airway inflammation and AHR in murine asthma models, clinical investigations are needed to evaluate the therapeutic potential of C5 inhibition in asthma patients.

REFERENCES

1. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol.* 2015;15(1):57-65.
2. Reddel HK, Bateman ED, Becker A, et al. A summary of the new GINA strategy: a roadmap to asthma control. *Eur Respir J.* 2015;46(3):622-639.
3. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 2011;32(9):402-411.
4. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement System Part I - Molecular Mechanisms of Activation and Regulation. *Front Immunol.* 2015;6:262.
5. Merle NS, Noe R, Halbwachs-Mecarelli L, Fremeaux-Bacchi V, Roumenina LT. Complement System Part II: Role in Immunity. *Front Immunol.* 2015;6:257.
6. Laumonnier Y, Wiese AV, Figge J, Karsten C. Regulation and function of anaphylatoxins and their receptors in allergic asthma. *Mol Immunol.* 2017;84:51-56.
7. Khan MA, Nicolls MR, Surguladze B, Saadoun I. Complement components as potential therapeutic targets for asthma treatment. *Respir Med.* 2014;108(4):543-549.
8. Krug N, Tschernig T, Erpenbeck VJ, Hohlfeld JM, Kohl J. Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. *Am J Respir Crit Care Med.* 2001;164(10 Pt 1):1841-1843.
9. Bautsch W, Hoymann HG, Zhang Q, et al. Cutting edge: guinea pigs with a natural C3a-receptor defect exhibit decreased bronchoconstriction in allergic airway disease: evidence for an involvement of the C3a anaphylatoxin in the pathogenesis of asthma. *J Immunol.* 2000;165(10):5401-5405.
10. Drouin SM, Corry DB, Kildsgaard J, Wetsel RA. Cutting edge: the absence of C3 demonstrates a role for complement in Th2 effector functions in a murine model of pulmonary allergy. *J Immunol.* 2001;167(8):4141-4145.
11. Drouin SM, Corry DB, Hollman TJ, Kildsgaard J, Wetsel RA. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J Immunol.* 2002;169(10):5926-5933.
12. Zhang X, Lewkowich IP, Kohl G, Clark JR, Wills-Karp M, Kohl J. A protective role for C5a in the development of allergic asthma associated with altered levels of B7-H1 and B7-DC on plasmacytoid dendritic cells. *J Immunol.* 2009;182(8):5123-5130.
13. Kohl J, Baelder R, Lewkowich IP, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest.* 2006;116(3):783-796.
14. Drouin SM, Sinha M, Sfyroera G, Lambris JD, Wetsel RA. A protective role for the fifth complement component (c5) in allergic airway disease. *Am J Respir Crit Care Med.* 2006;173(8):852-857.
15. Zhang X, Kohl J. A complex role for complement in allergic asthma. *Expert Rev Clin Immunol.* 2010;6(2):269-277.
16. Wills-Karp M. Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. *Proc Am Thorac Soc.* 2007;4(3):247-251.
17. Staab EB, Sanderson SD, Wells SM, Poole JA. Treatment with the C5a receptor/CD88 antagonist PMX205 reduces inflammation in a murine model of allergic asthma. *Int Immunopharmacol.* 2014;21(2):293-300.
18. Baelder R, Fuchs B, Bautsch W, et al. Pharmacological targeting of anaphylatoxin receptors during the effector phase of allergic asthma suppresses airway hyperresponsiveness and airway inflammation. *J Immunol.* 2005;174(2):783-789.
19. Peng T, Hao L, Madri JA, et al. Role of C5 in the development of airway inflammation, airway hyperresponsiveness, and ongoing airway response. *J Clin Invest.* 2005;115(6):1590-1600.
20. Martinez-Gonzalez I, Steer CA, Takei F. Lung ILC2s link innate and adaptive responses in allergic inflammation. *Trends Immunol.* 2015;36(3):189-195.

21. Cosmi L, Liotta F, Maggi L, Annunziato F. Role of Type 2 Innate Lymphoid Cells in Allergic Diseases. *Curr Allergy Asthma Rep.* 2017;17(10):66.
22. Kubo M. Innate and adaptive type 2 immunity in lung allergic inflammation. *Immunol Rev.* 2017;278(1):162-172.
23. Morita H, Moro K, Koyasu S. Innate lymphoid cells in allergic and nonallergic inflammation. *J Allergy Clin Immunol.* 2016;138(5):1253-1264.
24. Yang J, van 't Veer C, Roelofs J, et al. Kininogen deficiency or depletion reduces enhanced pause independent of pulmonary inflammation in a house dust mite-induced murine asthma model. *Am J Physiol Lung Cell Mol Physiol.* 2019;316(1):L187-L196.
25. Copland DA, Hussain K, Baalasubramanian S, et al. Systemic and local anti-C5 therapy reduces the disease severity in experimental autoimmune uveoretinitis. *Clin Exp Immunol.* 2010;159(3):303-314.
26. Jose PJ, Forrest MJ, Williams TJ. Human C5a des Arg increases vascular permeability. *J Immunol.* 1981;127(6):2376-2380.
27. Kawatsu R, Sanderson SD, Blanco I, et al. Conformationally biased analogs of human C5a mediate changes in vascular permeability. *J Pharmacol Exp Ther.* 1996;278(1):432-440.
28. Martinez FD, Vercelli D. Asthma. *Lancet.* 2013;382(9901):1360-1372.
29. Li BW, de Bruijn MJ, Tindemans I, et al. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. *Eur J Immunol.* 2016;46(6):1392-1403.
30. Ferrini ME, Hong S, Stierle A, et al. CB2 receptors regulate natural killer cells that limit allergic airway inflammation in a murine model of asthma. *Allergy.* 2017;72(6):937-947.
31. Ender F, Wiese AV, Schmudde I, et al. Differential regulation of C5a receptor 1 in innate immune cells during the allergic asthma effector phase. *PLoS One.* 2017;12(2):e0172446.
32. Wiese AV, Ender F, Quell KM, et al. The C5a/C5aR1 axis controls the development of experimental allergic asthma independent of LysM-expressing pulmonary immune cells. *PLoS One.* 2017;12(9):e0184956.
33. McKenzie ANJ, Spits H, Eberl G. Innate lymphoid cells in inflammation and immunity. *Immunity.* 2014;41(3):366-374.
34. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* 2015;16(1):45-56.
35. Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity.* 2012;36(3):451-463.
36. Sadik CD, Miyabe Y, Sezin T, Luster AD. The critical role of C5a as an initiator of neutrophil-mediated autoimmune inflammation of the joint and skin. *Semin Immunol.* 2018;37:21-29.
37. Drouin SM, Kildsgaard J, Haviland J, et al. Expression of the complement anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J Immunol.* 2001;166(3):2025-2032.
38. Gour N, Wills-Karp M. IL-4 and IL-13 signaling in allergic airway disease. *Cytokine.* 2015;75(1):68-78.
39. Coyle AJ, Le Gros G, Bertrand C, et al. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol.* 1995;13(1):54-59.
40. Brusselle G, Kips J, Joos G, Bluethmann H, Pauwels R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol.* 1995;12(3):254-259.
41. Tepper RI, Levinson DA, Stanger BZ, Campos-Torres J, Abbas AK, Leder P. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell.* 1990;62(3):457-467.
42. Wills-Karp M, Luyimbazi J, Xu X, et al. Interleukin-13: central mediator of allergic asthma. *Science.* 1998;282(5397):2258-2261.
43. Grunig G, Warnock M, Wakil AE, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science.* 1998;282(5397):2261-2263.
44. Zhu Z, Homer RJ, Wang Z, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest.* 1999;103(6):779-788.

45. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol.* 2000;165(1):108-113.

46. Pandey MK. Molecular basis for downregulation of C5a-mediated inflammation by IgG1 immune complexes in allergy and asthma. *Curr Allergy Asthma Rep.* 2013;13(6):596-606.

47. Crosby JR, Guha M, Tung D, et al. Inhaled CD86 antisense oligonucleotide suppresses pulmonary inflammation and airway hyper-responsiveness in allergic mice. *J Pharmacol Exp Ther.* 2007;321(3):938-946.

48. McKnight CG, Jude JA, Zhu Z, Panettieri RA, Jr., Finkelman FD. House Dust Mite-Induced Allergic Airway Disease Is Independent of IgE and FcepsilonRIalpha. *Am J Respir Cell Mol Biol.* 2017;57(6):674-682.

49. Karras JG, Crosby JR, Guha M, et al. Anti-inflammatory activity of inhaled IL-4 receptor-alpha antisense oligonucleotide in mice. *Am J Respir Cell Mol Biol.* 2007;36(3):276-285.

50. Liu C, Yuan L, Zou Y, et al. ITGB4 is essential for containing HDM-induced airway inflammation and airway hyperresponsiveness. *J Leukoc Biol.* 2018;103(5):897-908.

51. Yocum GT, Turner DL, Danielsson J, et al. GABAA receptor alpha4-subunit knockout enhances lung inflammation and airway reactivity in a murine asthma model. *Am J Physiol Lung Cell Mol Physiol.* 2017;313(2):L406-L415.

SUPPLEMENTAL FIGURES

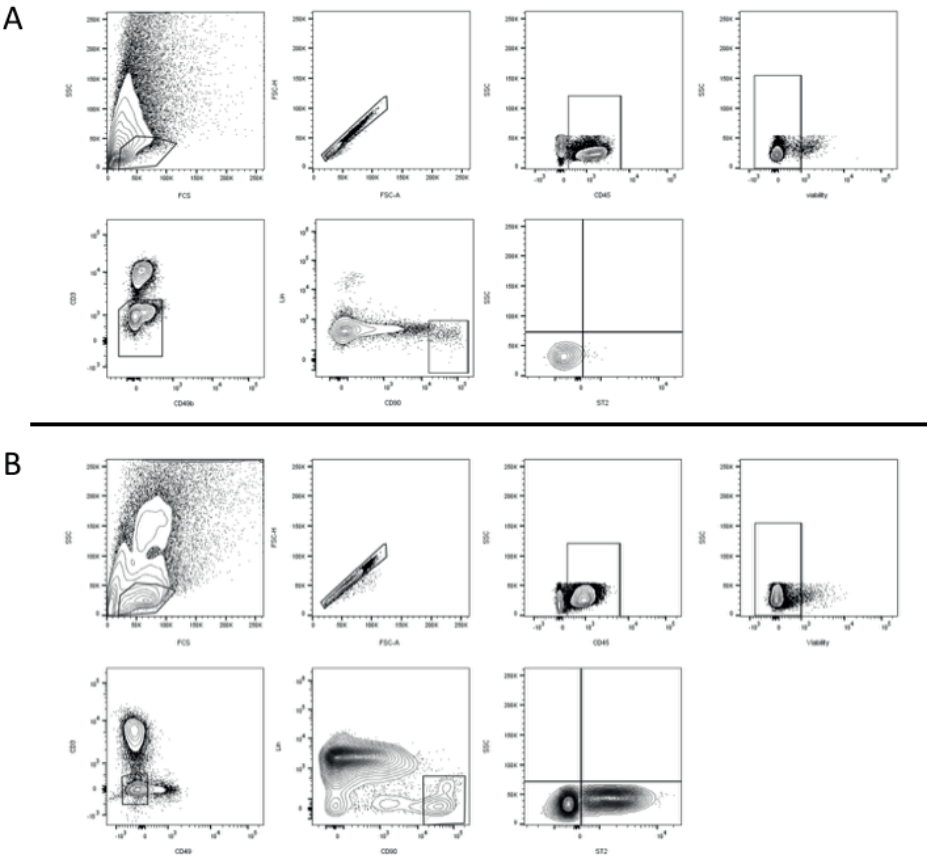

Figure S1. Gating strategy for ILCs and ILC2s. Flow cytometry plots of lung tissue from (A) NaCl or (B) HDM challenged mice. From left to right; within the lymphocyte gate, single CD45 positive and viable cells expressing $CD3^{-}CD49b^{-}Lin^{-}CD90^{+}$ were defined as ILCs. Cells expressing ST2 positivity within the ILC population were defined as ILC2s.

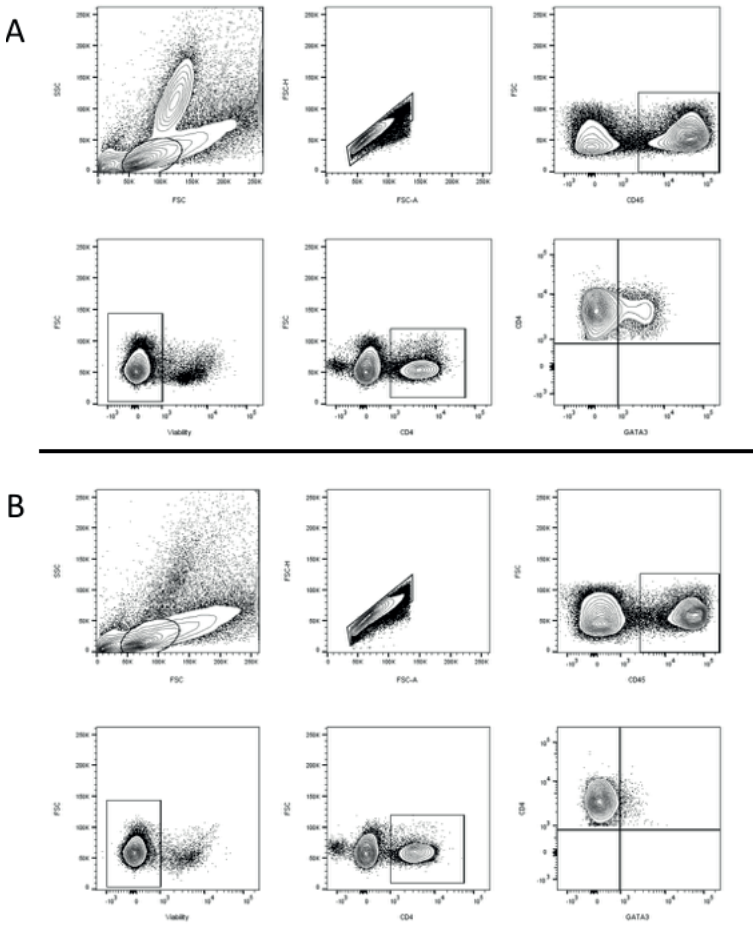

Figure S2. Gating strategy for CD4 T-cells and T_H2 cells. Flow cytometry plots of lung tissue from (A) NaCl or (B) HDM challenged mice. From left to right; within the lymphocyte gate, single CD45 positive and viable cells expressing CD4 positivity were defined as CD4 T-cells. Cells expressing GATA-3 positivity within the CD4 population were defined as T_H2 cells.


6

C3a signaling is not involved in eosinophil migration during experimental allergic lung inflammation in mice

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6

Allergy, 2019; in press

To the Editor,

Eosinophilia of the lungs and airways is a hallmark feature in allergic asthma. Eosinophils can exert pro-inflammatory and epithelial-damaging effects[1]. New therapies in the field of asthma targeting eosinophils have shown benefit in patients with high blood and sputum eosinophilia, including fewer exacerbation and improved lung function[2], thereby pointing to the potential importance of therapeutics that interfere with eosinophil recruitment in the treatment of asthma. In recent years, data from precliniical studies unveiled an important role for anaphylatoxins, components of complement system activation, in the pathogenesis of allergic airway disease[3]. Signaling of the anaphylatoxin C3a through its receptor (C3aR) was documented to promote the onset of Th2 responses in different allergen induced asthma models. Deficiency in or pharmacological blocking of either C3a or C3aR attenuated the allergen-induced Th2 response, which included a marked reduction of the eosinophilia in lung and airways[3]. While many studies focused on the interaction between C3a signaling and cells of the adaptive immune system during allergic inflammation[4], only few investigated a potential direct effect of C3a signaling on eosinophil function. Infusion of C3a induced eosinophil adherence to postcapillary venules of IL-1β stimulated mesenteric blood vessels of rabbits, but did not influence subsequent transmigration of eosinophils, suggesting a selective effect of C3a on eosinophil adhesion5. It remains elusive if C3a signaling in eosinophils affects their migration to lungs during an allergic response. This study aimed to investigate the role of C3a signaling in eosinophils in their recruitment to the airways during allergic lung inflammation.

We used an established mouse model of airway sensitization and challenge with the clinically relevant allergen house dust mite (HDM) (for details see Fig S1 and online supplement). HDM challenge elicited similar increases (P=0.94) in C3a concentrations in bronchoalveolar lavage (BAL) fluid from HDM sensitized WT and eosinophil lineage deficient ΔdblGATA KO mice (Fig 1A).

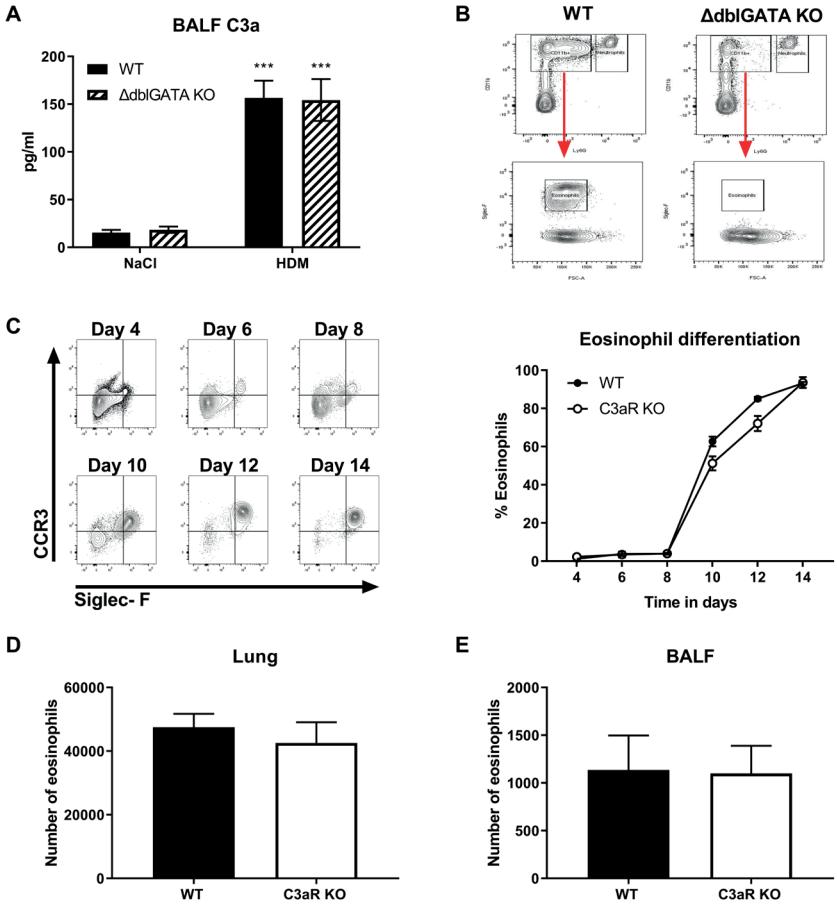

Figure 1. Generation of bone marrow derived eosinophils *ex vivo* and adoptive transfer in HDM challenged eosinophil deficient mice. (A) BALF C3a in WT and ΔdblGATA KO mice 24 hours after the last saline or HDM challenge. (B) Identification of bone marrow derived eosinophils in the lung by flow cytometry in WT and ΔdblGATA KO mice 24 hours following last HDM challenge. (C) Identification of eosinophils by double positive staining for CCR3 and Siglec-F in response to IL-5 on each indicated time point. Percentage of eosinophils following incubation with IL-5 from day 4 till 14. Number of eosinophils in the (D) lung or (E) BALF of recipient (ΔdblGATA KO) mice following adaptive transfer of either WT or C3aR KO bmEos. A parametric t-test was used for the comparison between groups. Data are representatives of at least two independent experiments and expressed as means ± SEM from 6-8 separate cultures or mice per group. ***p<0.001

As expected, HDM challenge induced eosinophilia in the lungs and airways of WT mice whereas ΔdblGATA KO mice showed a complete deficit in eosinophils in both lung tissue and BALF (Fig 1B). Next, we harvested bone marrow cells from wild-type (WT) and C3aR knockout (KO) mice and differentiated these into mature bone marrow derived eosinophils (bmEos) *ex vivo* (Fig S2A) as described in detail in the online supplement. We confirmed that C3aR mRNA was present in WT bmEos but completely abrogated in C3aR KO bmEos after 14 days in culture (Fig S2B). Total cell numbers grew similarly from WT and C3aR KO bone marrow to approximately 30 x 10^6 cells on day 14 (P=0.91)(Fig S2C). Over a time span of 14 days, a high purity of eosinophils, defined as CCR3 and Siglec-F double positive cells, was achieved from WT and C3aR KO mice (> 95% at day 14, P=0.92; Fig 1C). Giemsa staining showed the stereotypical bilobed nucleus and eosinophilic granules in eosinophils from both WT and C3aR KO (Fig S2D) cultures[6]. Moreover, circular nuclear morphologies, previously described in mouse eosinophils from peripheral blood were also observed[6]. Together these data indicate that signaling through C3aR is not essential for the proliferation and differentiation of eosinophils.

Eosinophils are not required for the generation of memory T-cells during the sensitization phase[7]. Thus, we adoptively transferred 5 x 10^6 WT or C3aR KO bmEos (intravenously) 24 hours after the first challenge (on day 15) into ΔdblGATA KO mice. Previously, bmEos were shown to have a half-life of eight days following chemotaxis into the lung[8], enabling the investigation of migrated eosinophils after completing the challenge phase in our HDM model. 24 hours after the last challenge (on day 20), saline challenged mice demonstrated a lack of bmEos accumulation in both lung tissue and BAL fluid (data not shown). HDM challenged mice showed similar numbers of WT and C3aR KO bmEos in their lungs (P=0.53) (Fig 1D) and BAL fluid (P=0.94) (Fig 1E). Together, these data suggest that C3aR deficient eosinophils are not impaired in their migration towards the lung and airways following HDM challenge in spite of the presence of elevated C3a levels in the airways.

We next examined the potential of C3a as an chemoattractant for bmEos *in vitro* using a Transwell system (for protocol see online supplement). The potency to attract bmEos was expressed as chemotactic index (CI) defined as: number of cells migrated in response to chemoattractant / number of cells migrated in response to vehicle control. As expected, WT bmEos migrated toward hCCL24, a chemoattractant for both human and murine eosinophils[9], in a dose dependent fashion with a maximum CI of 11 at 1 μM hCCL24 (Fig 2).

C3a mediated chemotaxis of WT eosinophils exhibited a maximum response of CI=1.3, indicating that C3a at best is a very weak chemoattractant for eosinophils, thereby corroborating the *in vivo* findings. In addition, these *in vitro* results are consistent with the findings from a previous study[5].

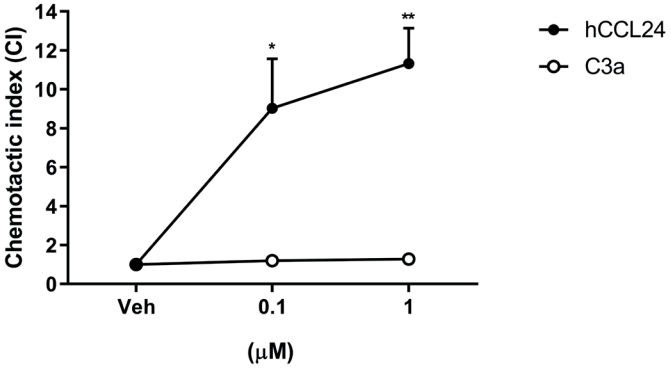

Figure 2. C3a is not an important chemoattractant for bone marrow derived eosinophils *ex vivo*. Chemotaxis of WT bmEos in response to increasing dose of hCCL24 or C3a. Chemotactic index (CI) is used as measure for the extent of *ex vivo* chemotaxis. A parametric t-test was used for the comparison between groups. Data are representatives of at least two independent experiments (n=6-8 per group) and expressed as means ± SEM. *p<0.05, **p<0.01 for comparison between hCCL24 and C3a at the indicated dose.

In conclusion, this study shows that C3a does not exert important chemoattractant activity on bmEos during HDM-induced allergic lung inflammation in mice. As such, this report supports the notion[3] that C3a signaling promotes eosinophilia during allergic inflammation via an altered Th2 response rather than through a direct effect on eosinophils.

REFERENCES

1. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol.* 2015;15(1):57-65.
2. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet.* 2018;391(10122):783-800.
3. Zhang X, Kohl J. A complex role for complement in allergic asthma. *Expert Rev Clin Immunol.* 2010;6(2):269-277.
4. Laumonnier Y, Wiese AV, Figge J, Karsten C. Regulation and function of anaphylatoxins and their receptors in allergic asthma. *Mol Immunol.* 2017;84:51-56.
5. DiScipio RG, Daffern PJ, Jagels MA, Broide DH, Sriramarao P. A comparison of C3a and C5a-mediated stable adhesion of rolling eosinophils in postcapillary venules and transendothelial migration in vitro and in vivo. *J Immunol.* 1999;162(2):1127-1136.
6. Varga SM, Beckman NA, Chu M, Braciale TJ. Sensitive detection and quantitation of mouse eosinophils in tissues using an enzymatic eosinophil peroxidase assay: its use to rapidly measure pulmonary eosinophilia during experimental respiratory syncytial virus infection of mice. *J Immunol Methods.* 2002;262(1-2):111-120.
7. Jacobsen EA, Lee NA, Lee JJ. Re-defining the unique roles for eosinophils in allergic respiratory inflammation. *Clin Exp Allergy.* 2014;44(9):1119-1136.
8. Wen T, Besse JA, Mingler MK, Fulkerson PC, Rothenberg ME. Eosinophil adoptive transfer system to directly evaluate pulmonary eosinophil trafficking in vivo. *Proc Natl Acad Sci U S A.* 2013;110(15):6067-6072.
9. Ochkur SI, Jacobsen EA, Protheroe CA, et al. Coexpression of IL-5 and eotaxin-2 in mice creates an eosinophil-dependent model of respiratory inflammation with characteristics of severe asthma. *J Immunol.* 2007;178(12):7879-7889.

SUPPLEMENTAL METHODS

Mice

ΔdblGATA (C.129S1(B6)-Gata1^{tm6Sho}/J,) and C3aR (C3ar1^{tm1Cge}) knockout (KO) mice on a BALB/c background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Wild-type 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.

Bone marrow isolation and eosinophil cultures

The protocol for isolation and *ex vivo* culture of mouse bone marrow-derived eosinophils (bmEos) was adapted from a previous study [1]. Briefly, bone marrow cells were harvested from murine hind legs and red blood cells were lysed using sterile lysis buffer (Qiagen, Hilden, Germany). Bone marrow cells were cultured in IMDM containing 10% FBS, 1% penicillin and streptomycin, 2 mM glutamine (Thermo Fisher Scientific, Waltham, MA, USA), 25 mM HEPES and 50 μM β-mercapto-ethanol in the presence of 100 ng/ml stem cell factor (SCF) and 100 ng/ml FLT-3 ligand (Peprotech, London, UK) from day 0 to day 4. From day 4 on, SCF and FLT-3 ligand in the medium were substituted by 10 ng/ml recombinant mouse (rm) IL-5 (Peprotech). Half of culture medium containing rmIL-5 was refreshed every other day and cell density was kept at 1×10^6 cells/ml. Eosinophil proliferation was measured concurrently with medium change starting on day 4 using a hemocytometer (Beckman Coulter, Fullerton, CA, USA). bmEos maturity was determined by flow cytometric analysis of Siglec-F and CCR3 (see below). Eosinophil morphology was visualized by Cytospin preparations stained with Giemsa (Diff Quik, Dade Behring AG, Düdingen, Switzerland). For adoptive transfer and ex vivo experiments bmEos cultured for 14 days were used.

House dust mite induced lung inflammation and adoptive transfer of eosinophils

Allergic lung inflammation was established by repeated intranasal challenge with HDM [2]. Briefly, 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. All mice were anesthetized by isoflurane inhalation prior to intranasal administration of HDM. Adoptive transfer of 5x10^6 wildtype (WT) or C3aR KO bmEos was done on day 15 by intravenous injection in the tail vein of ΔdblGATA mice. All mice were euthanized 24 hours after the last challenge. In all experiments bronchoalveolar lavage (BAL) fluid was collected

by airway lumen lavage with 2x 0.5 ml PBS. BAL fluid samples were enumerated by hemocytometer (Beckman Coulter). To assess the amount of pulmonary eosinophils, the lavaged lungs were mechanically minced and digested in RPMI containing 5% FCS, 1% penicillin/streptomycin, liberase™ and DNAse at 37°C for 30 minutes. Next, cells were dissociated by aspiration through a 19-gauge needle to obtain single cells. Erythrocytes were lysed with sterile lysis buffer (Qiagen). In both BAL fluid and digested lung tissue samples, cell differentiation was determined by flow cytometry.

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 (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 fluorescence minus one (FMO) controls were used. Data were collected on a BD Biosciences Canto II flow cytometer and analyzed using FlowJo software (Treestar, Palo Alto, CA, USA).

Eosinophil *ex vivo* chemotaxis assay

Eosinophil migration was determined largely as described [3]. Shortly, mature bmEos (cultured for 14 days) were washed and resuspended at $1x10^7$ cells/ml in RPMI medium supplemented with 1% FCS and 10 mM Hepes. Using a 96-wells HTS Transwell© plate with 5.0 µm pore membrane (Corning Life Sciences, Radnor, PA, USA), 100 µl of cell suspension were transferred into the upper wells, and 100 µl medium containing recombinant hCCL24 (Peprotech), recombinant mouse C3a (R&D Systems, MN, USA) or PBS control was added into the bottom wells. Plates were incubated for three hours at 37°C with 5% CO_2 after which migrated cells were counted by hemocytometer (Beckman Coulter). Chemotaxis towards hCCL24 and C3a was expressed as chemotactic index (CI):

$$CI = \frac{\text{number of cells migrated in response to chemoattractant}}{\text{number of cells migrated in response to vehicle control}}$$

RNA isolation and qRT-PCR

Total RNA was isolated from bmEos 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: *C3aR1* forward: TCAAGTGCAGAAACGCCCTGAAGC; *C3aR1* reverse: CTCCACCCACGTGTCCTTCCCAATG; Hprt forward: ACAGGCCAGACTTTGTTGGAT; Hprt reverse: ACTTGCGCTCATCTTAGGCT; 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. All data have been tested for normal distribution using the Shapiro-Wilk test. Comparison between groups were analyzed using a parametric t-test. Data are representatives of at least two independent experiments. *In vivo* experimental groups consisted of 6-8 mice. $P \leq 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.

SUPPLEMENTAL FIGURES

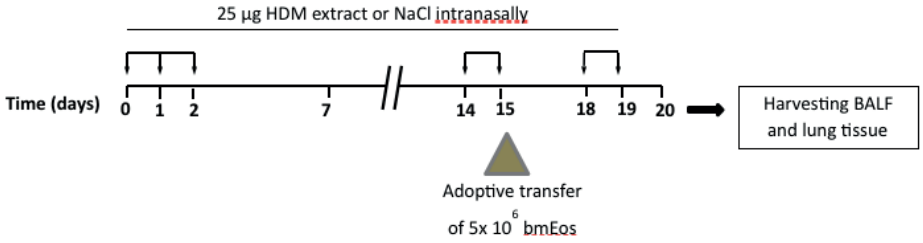

Figure S1. Schematic representation of bone marrow derived eosinophils (bmEos) adaptive transfer during the challenge phase of a HDM induced allergic model.

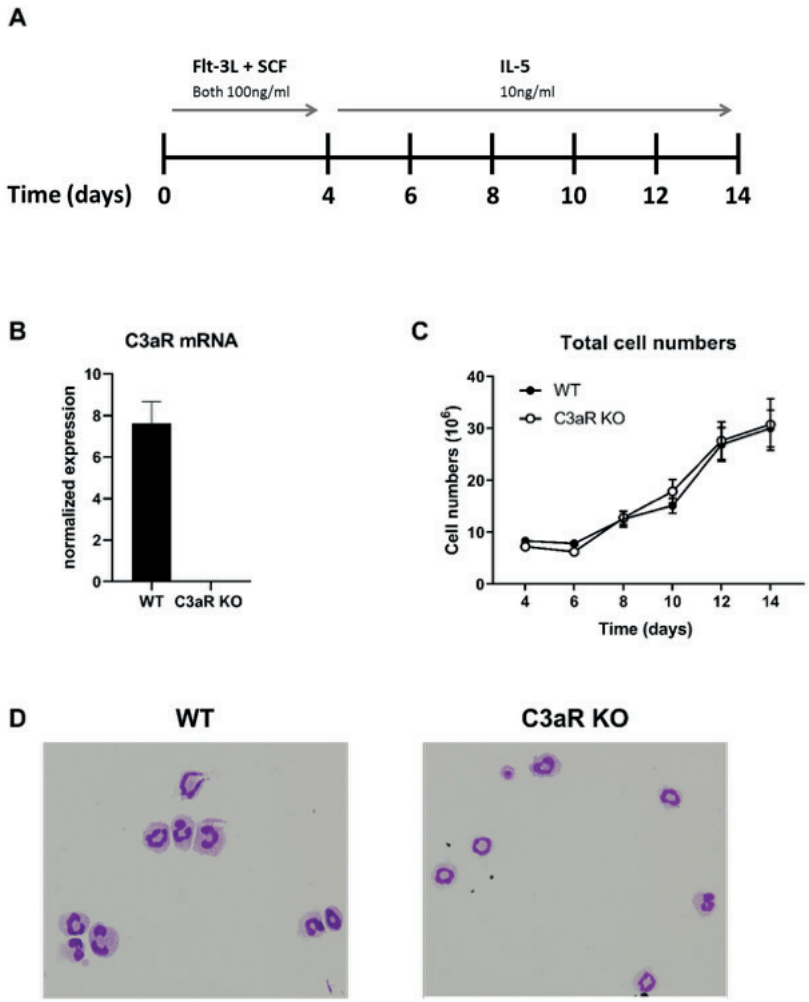

Figure S2. Generation of eosinophils from unselected bone marrow cells. (A) Culture scheme of eosinophil differentiation from unselected bone marrow cells. (B) mRNA expression of C3a receptor (C3aR) in WT and C3aR KO eosinophils after 14 days of culture. (C) Number of WT and C3aR KO bone marrow derived cells in response to IL-5 on each indicated time point. (D) Visualization of Giemsa stained cells from WT and C3aR KO mice after 14 days culture. Data are representatives of at least two independent experiments and expressed as means ± SEM from 4-6 separate cultures.

SUPPLEMENTAL REFERENCES

1. Dyer KD, Moser JM, Czapiga M, Siegel SJ, Percopo CM, Rosenberg HF. Functionally competent eosinophils differentiated ex vivo in high purity from normal mouse bone marrow. *J Immunol*. 2008;181(6):4004-4009.
2. Yang J, van 't Veer C, Roelofs J, et al. Kininogen deficiency or depletion reduces enhanced pause independent of pulmonary inflammation in a house dust mite-induced murine asthma model. *Am J Physiol Lung Cell Mol Physiol*. 2019;316(1):L187-L196.
3. Dyer KD, Garcia-Crespo KE, Percopo CM, Sturm EM, Rosenberg HF. Protocols for identifying, enumerating, and assessing mouse eosinophils. *Methods Mol Biol*. 2013;1032:59-77.

Mast Cell-Deficient Kit W-sh Mice Develop House Dust Mite-Induced Lung Inflammation despite Impaired Eosinophil Recruitment

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ABSTRACT

Background: Mast cells are implicated in allergic and innate immune responses in asthma, although their role in models using an allergen relevant for human disease is incompletely understood. House dust mite (HDM) allergy is common in asthma patients. Our aim was to investigate the role of mast cells in HDM-induced allergic lung inflammation. Methods: Wild-type (Wt) and mast cell-deficient $Kit^{w\text{-}sh}$ mice on a C57BL/6 background were repetitively exposed to HDM via the airways. Results: HDM challenge resulted in a rise in tryptase activity in bronchoalveolar lavage fluid (BALF) of Wt mice, indicative of mast cell activation. $Kit^{w\text{-}sh}$ mice showed a strongly attenuated HDM induced recruitment of eosinophils in BALF and lung tissue, accompanied by reduced pulmonary levels of the eosinophil chemoattractant eotaxin. Remarkably, $Kit^{w\text{-}sh}$ mice demonstrated an unaltered capacity to develop lung pathology and increased mucus production in response to HDM. The increased plasma IgE in response to HDM in Wt mice was absent in $Kit^{w\text{-}sh}$ mice. Conclusion: These data contrast with previous reports on the role of mast cells in models using ovalbumin as allergen in that C57BL/ $Kit^{w\text{-}sh}$ mice display a selective impairment of eosinophil recruitment without differences in other features of allergic inflammation.

INTRODUCTION

Asthma is a disease characterized by episodes of reversible airway obstruction, dyspnea and wheezing. In Western countries asthma prevalence has reached 10% on average and remains to increase towards epidemic proportions[1]. The pathophysiology of asthma frequently involves a varying degree of allergen-induced lung inflammation that can be difficult to manage clinically and can lead to airway remodeling[2]. House dust mite (HDM) allergy - and subsequent HDM-induced allergic lung inflammation - is common in asthma patients: in most populations the majority of asthma patients are allergic for HDM[3-5]. Better understanding of the role of different cellular subsets contributing to HDM-induced allergic lung inflammation could lead to new anti-inflammatory treatment approaches.

Mast cells are resident tissue cells that are described to have important immunoregulatory functions in allergic lung inflammation and asthma[6,7], but their precise involvement in HDM-induced allergic lung inflammation is not fully clarified[8]. Upon activation mast cells are able to release proinflammatory mediators such as histamine, tryptase, serotonin, heparin sulfates, lipid mediators such as PGE2 and LTB_4, and a vast range of interleukins[9,10]. Mast cells can be activated by both IgE dependent and IgE independent pathways[8,11]. A simplistic view of mast cells as 'merely' secretory proinflammatory and secretory cells has changed due to new insights in the involvement of mast cells in wound healing, UV irradiation protection, tumor biology (reviewed in [10]) and pulmonary fibrin and fibrinolysis homeostasis in asthma (reviewed in [12]). Mast cell-deficient mice are a well-known tool to study the role of mast cells in mouse asthma, but have not been investigated in a C57BL/6 strain-based model of HDM-induced allergic lung inflammation.

The hematopoietic system develops progenitor mast cells that further mature into mast cells at the target resident peripheral tissue. Mast cells especially reside around blood vessels, nerves and in vascularized organs such as the skin, gastrointestinal tract and lung[8,10]. The expression of c-Kit tyrosine kinase receptor (CD117) on mast cells is essential for the appropriate development and proliferation of progenitor mast cells from the hematopoietic system[13]. Two genetic c-Kit mutant mouse strains have been investigated most frequently in asthma models: c-$Kit^{W/W\text{-}v}$ and c-$Kit^{W\text{-}sh/W\text{-}sh}$ ($Kit^{w\text{-}sh}$) mice[14]. In contrast to $Kit^{w\text{-}sh}$ mice, which have an inversion mutation on chromosome 5 at the transcriptional site of c-Kit [15,16], c-$Kit^{W/W\text{-}v}$ mice have significant comorbidity (e.g. anemia,

infertility, dermatitis, skin ulceration), which makes the latter mouse strain less suitable for asthma studies examining the role of mast cells.

Here, we investigated the impact of mast cell deficiency using mast cell-deficient $Kit^{w\text{-}sh}$ mice with a C57Bl/6 in a recently developed HDM-evoked mouse asthma model[17]. We show that mast cell-deficiency attenuated the recruitment of eosinophils and was associated with lower pulmonary levels of eotaxin. Remarkably, in this HDM-induced model $Kit^{w\text{-}sh}$ mice were able to develop increased mucus production and allergic lung pathology equivalent to Wt mice.

METHODS

Mice

C57Bl/6 wild type (Wt) mice were purchased from Charles River Inc. (Maastricht, The Netherlands). Mast cell-deficient $Kit^{w\text{-}sh}$ on a C57Bl/6 background were from Jackson Laboratories (Bar Harbor, Maine), housed in standardized specific pathogen free conditions and sex and age-matched. Experiments started when animals were 8-9 weeks old. Each group consisted of 8 mice (except for one of the Wt saline groups, n=5; see Figure legends). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

HDM asthma model

HDM allergen whole body extract (Greer Laboratories, Lenoir, NC), derived from the common European HDM species *Dermatophagoides pteronyssinus*, Der p, was used to induce allergic lung inflammation as described[17]. Briefly, mice were inoculated intranasally on day 0, 1 and 2 with 25 mg HDM (sensitization phase) and on day 14, 15, 18 and 19 with 6.25 mg HDM (challenge phase). Controls received isotonic sterile saline intranasally on each occasion. Inoculum volume was 20 ml for every HDM and saline exposure and inoculation procedures were performed during isoflurane inhalation anesthesia. The experiment was ended at day 21 by euthanizing the mice and the subsequent collection and processing of samples: in one experiment bronchoalveolar lavage fluid (BALF) and citrated blood was collected, in a separate experiment one lung was obtained for pathology and one lung for homogenization.

Bronchoalveolar lavage and tissue handling

BALF was harvested after exposing the trachea through a midline incision and instilling and retrieving 1 mL of sterile saline 0.9% (in 500-µl aliquots)[17]. Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA) and differential cell counts by cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). In independent experiments non-lavaged lungs were homogenized in 5 volumes of sterile 0.9% saline using a tissue homogenizer (Biospec Products, Bartlesville, Okla, USA) or fixed in 10% formalin.

Histology

Lungs were embedded in paraffin after fixation in 10% formalin; 4-µm-thick sections were stained with hematoxylin and eosin (HE). Parameters of allergic lung inflammation were scored by an experienced histopathologist who was blinded for treatment and strain of mice. The following parameters were scored: interstitial inflammation, peribronchial inflammation, edema, endothelialitis and pleuritis, each graded on a scale of 0–4 (0: absent, 1: mild, 2:

moderate, 3: severe, 4: very severe). The total pathology score was expressed as the sum of the score for all parameters. Periodic acid Schiff (PAS)-D staining for carbohydrates in mucus was performed to quantify the amount of mucus. The amount of mucus per lung section was scored by a histopathologist in a semiquantitative fashion on a scale of 0–8 (0–4 for plug formation, 0–4 for mucus extent).

Immunohistochemistry and Digital Imaging Analysis

Eosinophil staining was performed using a monoclonal antibody against granule major basic protein (GMBP; kindly provided by Dr. Nancy Lee and Prof. James Lee, Mayo Clinic Arizona, Scottsdale, Ariz., USA)[17]. Entire sections were digitized with a slide scanner using the 10× objective (Olympus dotSlide, Tokyo, Japan). Images were exported in the TIFF format for quantification. Influx of eosinophils was determined by measuring the GMBP immunopositive

area by digital image analysis (ImageJ 1.46, National Institute of Health, Bethesda, Md., USA), and subsequently expressed as a percentage of the total lung area. The average of ten pictures was used for analysis of eosinophilic pulmonary influx.

Assays

Plasma IgE was measured as described using rat anti-mouse IgE as capture antibody, purified mouse IgE as standard and biotinylated rat-anti-mouse IgE as detection (all reagents from BD Biosciences Pharmingen, Breda, the Netherlands)[17]. Concentrations of lung eotaxin, IL-4, IL-5 and IL-13 were measured using Elisa (R&D Systems, Abingdon, UK). Tryptase activity was determined as described[18] using an amidolytic assay with chromogenic tryptase substrate S-2288 (Chromogenix Instrumentation Laboratory, Milan, Italy) was added to 70 µl 57-mM Tris-HCl with pH 8.3 in a 96-well microtiter plate. After initiating the reaction by adding 40 µl of BALF sample the ΔA405 n M was developed in 1 h with subtraction of the baseline measurement and monitored in a plate reader at 37 ° C (Biotek Instruments, Winooski, Vt., USA). Differences were calculated relative to optical density at the zero time point.

Statistical analysis

Values are expressed as mean ± SEM. Differences between groups were tested by Mann-Whitney U test. 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

HDM airway exposure results in enhanced tryptase activity in BALF of Wt mice

First, we established whether repeated HDM exposure resulted in enhanced mast cell degranulation. For this we measured tryptase activity in BALF at day 21, 2 days after the last challenge (Figure 1)[18,19]. Tryptase activity was very low in saline-treated mice. HDM treatment increased tryptase activity in Wt mice ($P < 0.01$ versus saline controls), but not in $Kit^{w\text{-}sh}$ mice ($P < 0.05$ versus HDM treated Wt mice). These data indicate that our HDM asthma model is associated with mast cell activation in the bronchoalveolar compartment.

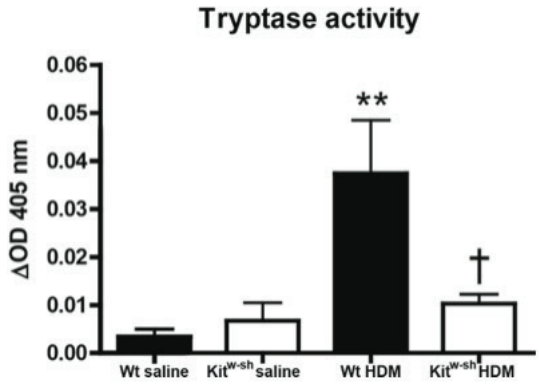


Figure 1. HDM airway exposure induces tryptase acivity in BALF of Wt but not of Kit$^{w\text{-}sh}$ mice. Tryptase activity levels (DOD vs. OD at baseline). Data are means ± SE of 8 mice per group. ** $P < 0.01$ versus saline, † $P < 0.05$ versus HDM exposed Wt mice.

Kit$^{w\text{-}sh}$ mice have reduced influx of cells in BALF after HDM exposure due to decreased recruitment of eosinophils

Upon HDM exposure of the airways both Wt and Kitw-sh mice show increased total cell influx in BALF (Figure 2A, $P < 0.001$ and $P < 0.01$ versus their respective saline controls). Total cell influx in BALF was significantly reduced in Kitw-sh mice after HDM instillation compared to Wt mice (Figure 2A, $P < 0.05$). The reduction in total cell influx was explained by a decrease in HDM-evoked eosinophil recruitment in Kitw-sh mice compared to Wt mice (Figure 2B, $P < 0.05$). Relative to saline controls, Wt and Kitw-sh mice showed similar increases in HDM-induced influx of neutrophils (both $P < 0.01$) and lymphocytes ($P < 0.01$ and $P < 0.05$, respectively). Together, these data indicate that Kitw-sh mice have decreased cell numbers in BALF in the HDM asthma model caused by a decreased migration of eosinophils to the bronchoalveolar compartment.

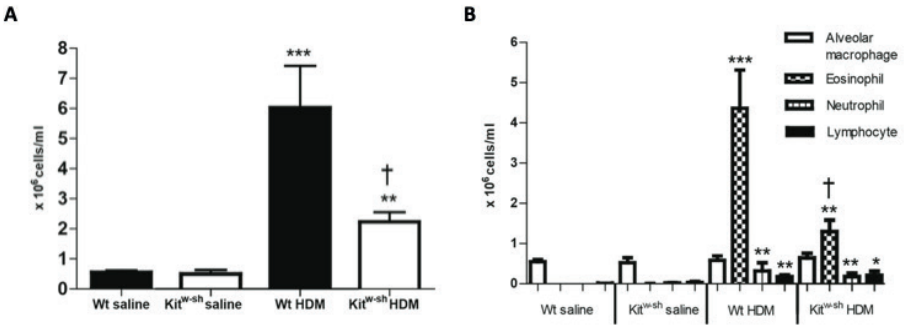
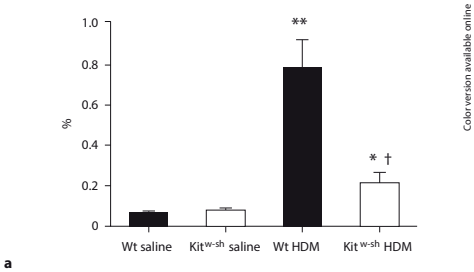
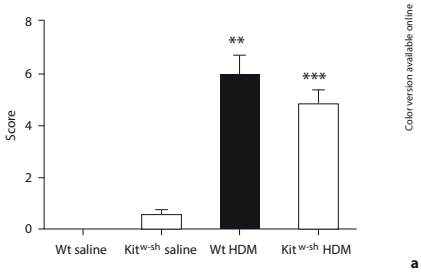


Figure 2. Kit$^{w\text{-}sh}$ mice have decreased total cell counts in BALF after HDM challenge due to lower eosinophil influx. Total cell counts (A) and differential cell counts (alveolar macrophages, eosinophils, neutrophils and lymphocytes) (B). Data are means ± SE (10^5 cells/ml) of 8 mice per group. * $P < 0.05$ and ** $P < 0.01$ versus saline challenged mice of the same genotype; † $P < 0.05$ versus Wt mice challenged with HDM.

Reduced eosinophil accumulation in lung tissue in Kit$^{w\text{-}sh}$ mice upon HDM administration

Lung tissue eosinophils were detected by GMBP staining, analysed by digital imaging and expressed as the percentage of lung surface occupied by eosinophils (Figure 3). HDM instillation caused an increase in pulmonary eosinophils in both Wt and Kit$^{w\text{-}sh}$ mice compared to saline (Figure 3A, $P < 0.01$ and $P < 0.05$, respectively). The number of eosinophils in lung tissue of Kit$^{w\text{-}sh}$ mice was decreased by 74% compared to Wt mice ($P < 0.05$), corroborating the findings in BALF shown in Figure 2 and indicating decreased HDM-induced pulmonary recruitment of eosinophils in Kit$^{w\text{-}sh}$ mice.





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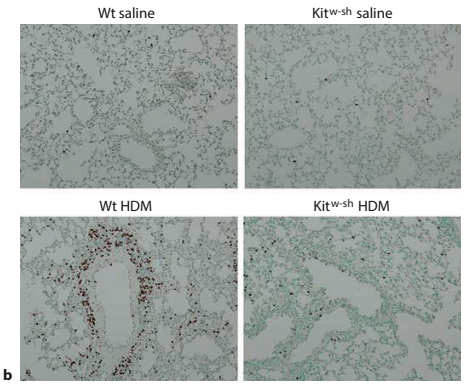

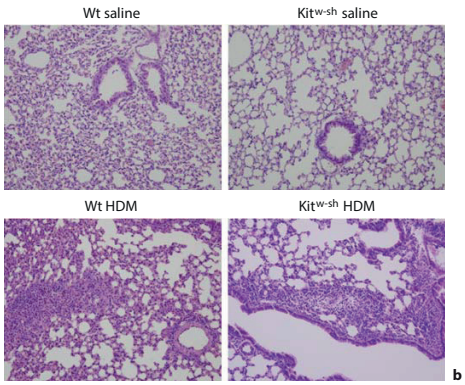

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Figure 3. Kit$^{w\text{-}sh}$ mice demonstrate a reduced influx of eosinophils in lung tissue after HDM challenge. (A) Percentage of lung surface stained positive for eosinophils quantified by digitally imaging of major basic protein (MBP) staining (see Methods section). Data are means ± SE of 8 mice per group except for Wt saline (n=5). * $P < 0.05$ and ** $P < 0.01$ versus saline challenged mice of the same genotype; † $P < 0.05$ versus Wt mice challenged with HDM. Representative MBP staining of lung tissue slides of Wt mice exposed to saline (B, upper left), Kit$^{w\text{-}sh}$ mice exposed to saline (B, upper right), Wt mice exposed to HDM (B, lower left), Kit$^{w\text{-}sh}$ mice exposed to HDM (B, lower right). Original magnification 40x

Figure 4. Kit$^{w\text{-}sh}$ and Wt mice demonstrate similar lung pathology after HDM challenge. (A) Semiquantitative pathology scores (described in the Methods section). Data are means ± SE of 8 mice per group (except Wt saline: n=5). ** $P < 0.01$ and *** $P < 0.001$ versus saline challenged mice of the same genotype. Representative HE stained lung tissue slides of Wt mice exposed to saline (B, upper left), Kit$^{w\text{-}sh}$ mice exposed to saline (B, upper right), Wt mice exposed to HDM (B, lower left), Kit$^{w\text{-}sh}$ mice exposed to HDM (B, lower right). Original magnification 40x

Kit$^{w\text{-}sh}$ mice develop HDM-evoked lung pathology to a similar extent as Wt mice.

HE-stained slides of lung tissue were scored for parameters of allergic lung inflammation in a semiquantitative fashion as described in the Methods section (Figure 4). Repeated HDM exposure resulted in lung pathology in both Wt and Kit$^{w\text{-}sh}$ mice ($P < 0.01$ and $P < 0.001$ versus their respective saline controls). Surprisingly, there was no difference in the extent of lung pathology between Wt HDM and Kit$^{w\text{-}sh}$ HDM challenged groups. Moreover, the scores of distinct pathology features (i.e. perivascular inflammation, interstitial inflammation, endothelialitis, peribronchitis, edema) were not different between HDM exposed groups (data not shown).

HDM-induced pulmonary mucus production is similar in Wt and Kit$^{w\text{-}sh}$ mice

Lung tissue slides were PAS-D stained and subsequently scored for mucus production by procedures described in the Methods section (Figure 5). HDM challenge led to increased mucus scores in both Wt and Kit$^{w\text{-}sh}$ mice compared to saline controls ($P < 0.01$ and $P < 0.001$, respectively). However, HDM-induced mucus production did not differ between Wt and Kit$^{w\text{-}sh}$ mice.

Lung levels of eotaxin are reduced in Kit$^{w\text{-}sh}$ mice after HDM airway challenge

Since upon HDM exposure mast cell deficiency in Kit$^{w\text{-}sh}$ mice impacted specifically on eosinophil recruitment in BALF and lung tissue, but not lung pathology or mucus production, we investigated whether mast cell deficiency was associated with a reduced production of mediators implicated in eosinophil recruitment: IL-4, IL-5, IL-13[20] and eotaxin[21]. Lung IL-4, IL-5 and IL-13 concentrations were low in all groups and not significantly different between saline and HDM challenged mice (data not shown). In contrast, HDM induced a significant increase in lung eotaxin levels in Wt mice, but not in Kit$^{w\text{-}sh}$ mice (Figure 6, $P < 0.05$).

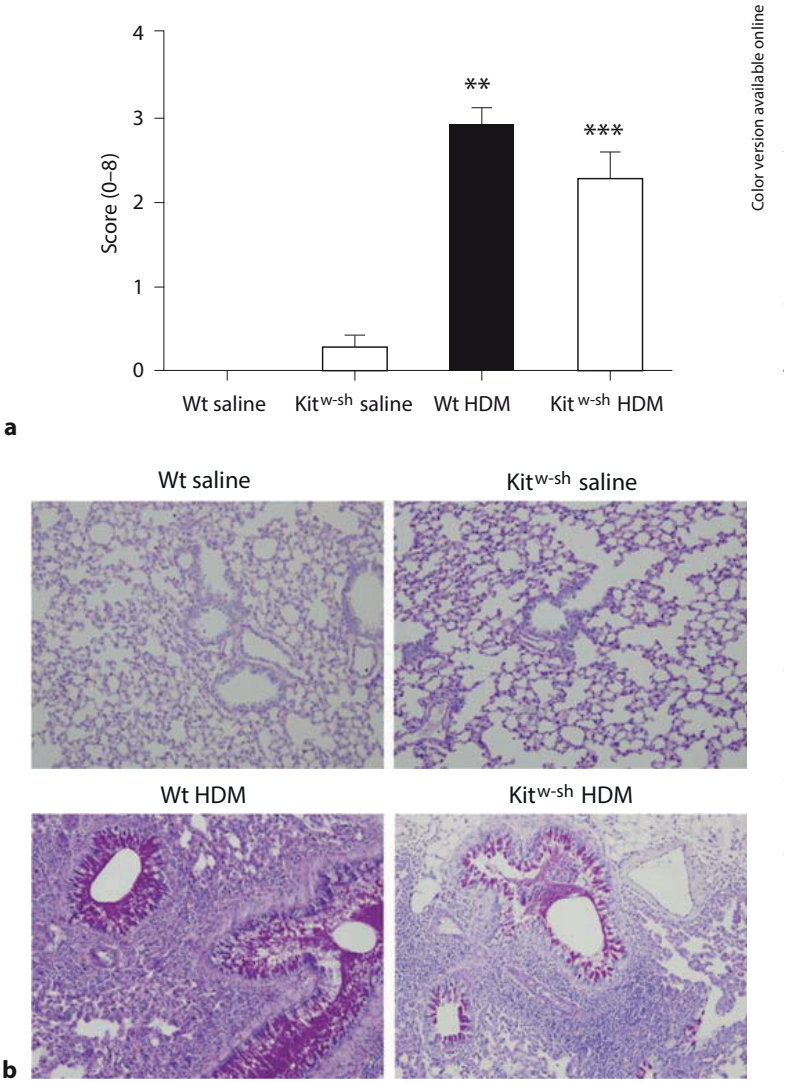

Figure 5. Kit$^{w\text{-}sh}$ mice mice show unaltered mucus production in their airways upon HDM challenge. (A) Semi-quantitative mucus scores (described in the Methods section). Data are means ± SE of 8 mice per group (except Wt saline n=5). ** P < 0.01 and ***P < 0.001 versus saline challenged mice of the same genotype. Representative PAS-D stained lung tissue slides of Wt mice exposed to saline B, upper left), Kit$^{w\text{-}sh}$ mice exposed to saline (B, upper right), Wt mice exposed to HDM (B, lower left), Kit$^{w\text{-}sh}$ mice exposed to HDM (B, lower right). Original magnification 40x

Kit$^{w\text{-}sh}$ mice fail to produce IgE upon HDM exposure

Plasma IgE was below detection limit in saline control mice. HDM airway exposure resulted in a strong increase in plasma IgE in Wt, but not in Kit$^{w\text{-}sh}$ mice (Figure 7, $P < 0.05$).

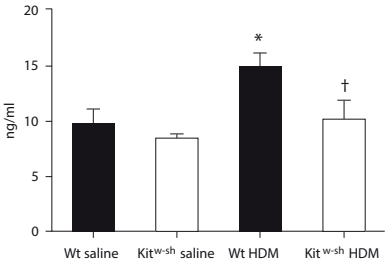

Figure 6. Pulmonary eotaxin levels are decreased in Kit$^{w\text{-}sh}$ mice after HDM exposure. Lung concentrations of eotaxin. Data are means ± SE of 8 mice per group (except Wt saline n=5). * $P < 0.05$ versus saline challenged Wt mice; † $P < 0.05$ versus HDM challenged Wt mice.

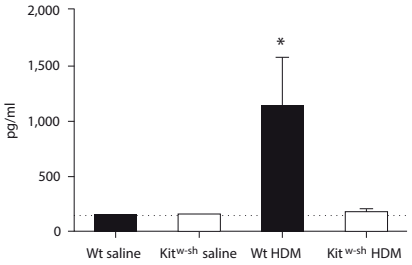

Figure 7. Kit$^{w\text{-}sh}$ mice do not show a plasma IgE response after HDM challenge. Plasma concentrations of IgE. Data are means ± SE of 8 mice per group. *$P < 0.05$ vs. saline and † $P < 0.05$

DISCUSSION

Mast cells have been implicated as important players in the pathophysiology of allergic lung inflammation and asthma. Notably, however, the role of mast cells in allergic responses in the airways has been investigated predominantly in ovalbumin (OVA)-based mouse models[21-23]. Important differences between OVA and HDM exist. In asthma patients allergy for HDM is highly prevalent[4], while OVA is not a relevant human allergen. Furthermore, whereas HDM can influence mast cell activity, OVA does not[24]. Moreover, relative to OVA-induced lung inflammation, the HDM-based model puts more emphasis on barrier protection, mucosal defense and the role of the epithelium, which is likely to be of influence on locally residing mast cells[25,26]. Taken together, mouse models using HDM as allergen relate better to human asthma, yet are sparsely investigated in mast-cell deficient settings. Here we investigated mast cell-deficient Kit$^{w\text{-}sh}$ mice in a recently developed HDM asthma model[17]. We showed that HDM administration via the airways resulted in a local increase in tryptase

activity, indicative of mast cell activation. Kit$^{w\text{-}sh}$ mice demonstrated decreased eosinophil numbers in BALF and lung tissue after HDM exposure, which was associated with lower eotaxin levels in the bronchoalveolar compartment. Remarkably, lung pathology and mucus production after instillation of HDM were similar in Kit$^{w\text{-}sh}$ and Wt mice. Together, these data point to a crucial role of mast cells in HDM-induced recruitment of eosinophils to the lungs, potentially in part via an eotaxin-dependent mechanism. Our results further establish that mast cells do not contribute to HDM-induced lung pathology or mucus production, suggesting that these responses occur via pathways that do not rely on recruited eosinophils.

The phenotype of the Kit$^{w\text{-}sh}$ mice in the current model of HDM-induced lung inflammation was mainly defined by a decreased pulmonary recruitment of eosinophils. This finding is in accordance with previous studies that investigated Kit$^{w\text{-}sh}$ mice in allergic lung inflammation elicited by OVA[19,20,27]. While the levels of IL-4, IL-5 and IL-13 remained low in all mice after HDM challenge, pulmonary concentrations of eotaxin, a key chemoattractant for eosinophils[21], were significantly reduced in Kit$^{w\text{-}sh}$ mice. The role of mast cells and eotaxin in eosinophil attraction was previously studied in allergen-challenged skin, identifying a role for histamine released from mast cells in inducing eotaxin expression by endothelial cells and subsequently the recruitment of eosinophils[17]. It would be interesting for future research to investigate interactions of mast cells and endothelium in HDM-induced lung inflammation.

Mast cell deficiency had no effect on HDM-evoked lung pathology in our study, which contrasts with data from earlier studies using OVA as allergen showing attenuated pulmonary inflammation in Kit$^{w\text{-}sh}$ mice[19,20,27]. This illustrates differences between OVA and HDM effects in the airways: while OVA-induced response are almost completely dependent on mast cells, the heterogeneity of the HDM extract probably induces a broader symphony of allergenic effects that also involve activation of innate immunity (reviewed in [16]), initiating both mast cell dependent and independent effects (reviewed in [3]). It is also important to recognize differences in mouse strains in this respect. While BALB/c mice are skewed for Th-2 dependent inflammatory reactions, C57Bl/6 mice (the genetic background of the Kit w-sh mice used here), are more prone to Th1 inflammation (reviewed in[28]). A potential 'Th2 bias' may occur when using BALB/c mice and/or OVA which may result in underestimation of the effect of Th1-dependent inflammatory reactions. The mouse strain used has been shown to be of importance for asthma models in a series of experiments[29], but

only in an OVA Th2-dependent model. Importantly, the effects of HDM are not exclusively Th2 dependent: effects of HDM extract includes activating toll-like receptor 2- and 4-dependent pathways[30] and proteolytic activity targeted at airway epithelial tight junctions[26,31,32]. Additionally, HDM preparations contain protease-activated receptor agonists, which have diverse proinflammatory effects[33]. This extensive activation of multiple inflammatory pathways involves distinct cellular, epithelial and humoral components besides mast cells and they are hypothetically underestimated in OVA-based protocols. Comparable with the unaffected lung pathology between Wt and $Kit^{w\text{-}sh}$ mice, HDM-evoked mucus production was not dependent on mast cells in our HDM model. Of note, it is known that $Kit^{w\text{-}sh}$ mice have been described to develop lung pathology resembling emphysema beyond the age of 14 weeks[34]. We used mice at a younger age and lung pathology of saline challenged $Kit^{w\text{-}sh}$ mice was unremarkable and not different from Wt mice.

The levels of total IgE were absent in $Kit^{w\text{-}sh}$ mice after HDM challenge compared to Wt mice, indicating as expected that the mast-cell dependent recruitment of eosinophils is partly IgE dependent. However, since mast cells are essential for the initiation of immunoglobulin production, it could well be that the differences in IgE are due to lack of mast cell-induced sensitization capacities of $Kit^{w\text{-}sh}$ mice compared to Wt mice. Strongly attenuated IgE production in response to HDM was previously also shown in mast cell-deficient BALB/c mice; other parameters of allergic lung inflammation were not investigated in this study[35].

In conclusion, we have shown that mast cells play a key role in the recruitment of eosinophils to the lungs after airway exposure to HDM. Unexpectedly, mast cells did not impact on HDM-induced lung pathology or mucus production, contrasting with earlier findings in experimental allergic pulmonary inflammation elicited by OVA and adding important new information on the function of mast cells in the airway response to a clinically relevant human allergen.

REFERENCES

1. Braman SS. The global burden of asthma. *Chest.* 2006;130(1 Suppl):4S-12S.
2. Murphy DM, O'Byrne PM. Recent advances in the pathophysiology of asthma. *Chest.* 2010;137(6):1417-1426.
3. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 2011;32(9):402-411.
4. Nelson RP, Jr., DiNicolo R, Fernandez-Caldas E, Seleznick MJ, Lockey RF, Good RA. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. *The Journal of allergy and clinical immunology.* 1996;98(2):258-263.
5. Lodge CJ, Lowe AJ, Gurrin LC, et al. House dust mite sensitization in toddlers predicts current wheeze at age 12 years. *The Journal of allergy and clinical immunology.* 2011;128(4):782-788 e789.
6. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *The New England journal of medicine.* 2002;346(22):1699-1705.
7. Wardlaw AJ, Brightling CE, Green R, Woltmann G, Bradding P, Pavord ID. New insights into the relationship between airway inflammation and asthma. *Clin Sci (Lond).* 2002;103(2):201-211.
8. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annual review of immunology.* 2005;23:749-786.
9. Lorentz A, Schwengberg S, Sellge G, Manns MP, Bischoff SC. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *J Immunol.* 2000;164(1):43-48.
10. Kalesnikoff J, Galli SJ. New developments in mast cell biology. *Nature immunology.* 2008;9(11):1215-1223.
11. Chodaczek G, Bacsi A, Dharajiya N, Sur S, Hazra TK, Boldogh I. Ragweed pollen-mediated IgE-independent release of biogenic amines from mast cells via induction of mitochondrial dysfunction. *Molecular immunology.* 2009;46(13):2505-2514.
12. de Boer JD, Majoor CJ, van 't Veer C, Bel EH, van der Poll T. Asthma and coagulation. *Blood.* 2012;119(14):3236-3244.
13. Kirshenbaum AS, Goff JP, Kessler SW, Mican JM, Zsebo KM, Metcalfe DD. Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells. *J Immunol.* 1992;148(3):772-777.
14. Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. *The American journal of pathology.* 2005;167(3):835-848.
15. Lyon MF, Glenister PH. A new allele sash (Wsh) at the W-locus and a spontaneous recessive lethal in mice. *Genetical research.* 1982;39(3):315-322.
16. Jacquet A. The role of innate immunity activation in house dust mite allergy. *Trends in molecular medicine.* 2011;17(10):604-611.
17. Daan de Boer J, Roelofs JJ, de Vos AF, et al. Lipopolysaccharide inhibits th2 lung inflammation induced by house dust mite allergens in mice. *American journal of respiratory cell and molecular biology.* 2013;48(3):382-389.
18. Ishizaki M, Tanaka H, Kajiwara D, et al. Nafamostat mesilate, a potent serine protease inhibitor, inhibits airway eosinophilic inflammation and airway epithelial remodeling in a murine model of allergic asthma. *Journal of pharmacological sciences.* 2008;108(3):355-363.
19. Payne V, Kam PC. Mast cell tryptase: a review of its physiology and clinical significance. *Anaesthesia.* 2004;59(7):695-703.
20. Wardlaw AJ. Molecular basis for selective eosinophil trafficking in asthma: A multistep paradigm. *The Journal of allergy and clinical immunology.* 1999;104(5):917-926.

21. Dent G, Hadjicharalambous C, Yoshikawa T, et al. Contribution of eotaxin-1 to eosinophil chemotactic activity of moderate and severe asthmatic sputum. *American journal of respiratory and critical care medicine*. 2004;169(10):1110-1117.

22. Kung TT, Stelts D, Zurcher JA, et al. Mast cells modulate allergic pulmonary eosinophilia in mice. *American journal of respiratory cell and molecular biology*. 1995;12(4):404-409.

23. Reuter S, Dehzad N, Martin H, et al. Mast cells induce migration of dendritic cells in a murine model of acute allergic airway disease. *International archives of allergy and immunology*. 2010;151(3):214-222.

24. Herrerias A, Torres R, Serra M, et al. Subcutaneous prostaglandin E(2) restrains airway mast cell activity in vivo and reduces lung eosinophilia and Th(2) cytokine overproduction in house dust mite-sensitive mice. *International archives of allergy and immunology*. 2009;149(4):323-332.

25. Holgate ST. Pathophysiology of asthma: what has our current understanding taught us about new therapeutic approaches? *The Journal of allergy and clinical immunology*. 2011;128(3):495-505.

26. Post S, Nawijn MC, Hackett TL, et al. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax*. 2011.

27. Yu M, Tsai M, Tam SY, Jones C, Zehnder J, Galli SJ. Mast cells can promote the development of multiple features of chronic asthma in mice. *The Journal of clinical investigation*. 2006;116(6):1633-1641.

28. Spellberg B, Edwards JE, Jr. Type 1/Type 2 immunity in infectious diseases. *Clin Infect Dis*. 2001;32(1):76-102.

29. Becker M, Reuter S, Friedrich P, et al. Genetic variation determines mast cell functions in experimental asthma. *J Immunol*. 2011;186(12):7225-7231.

30. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med*. 2009;15(4):410-416.

31. Wan H, Winton HL, Soeller C, et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *The Journal of clinical investigation*. 1999;104(1):123-133.

32. Wan H, Winton HL, Soeller C, et al. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2001;31(2):279-294.

33. Cho HJ, Lee HJ, Kim SC, et al. Protease-activated receptor 2-dependent fluid secretion from airway submucosal glands by house dust mite extract. *The Journal of allergy and clinical immunology*. 2012;129(2):529-535, 535 e521-525.

34. Lindsey JY, Ganguly K, Brass DM, et al. c-Kit is essential for alveolar maintenance and protection from emphysema-like disease in mice. *American journal of respiratory and critical care medicine*. 2011;183(12):1644-1652.

35. Coyle AJ, Wagner K, Bertrand C, Tsuyuki S, Bews J, Heusser C. Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J Exp Med*. 1996;183(4):1303-1310.

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

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ABSTRACT

Asthma is associated with activation of coagulation in the airways. The coagulation system can be initiated via the extrinsic tissue factor dependent pathway or via the intrinsic pathway, in which the central player factor XI (FXI) can be either activated via active factor XII (FXIIa) or via thrombin. We aimed to determine the role of the intrinsic coagulation system, and its possible route of activation, in allergic lung inflammation induced by the clinically relevant human allergen house dust mite (HDM). Wild-type (WT), FXI knockout (KO) and FXII KO mice were subjected to repeated exposure to HDM via the airways, and inflammatory responses were compared. FXI KO mice showed increased influx of eosinophils into lung tissue, accompanied by elevated local levels of the main eosinophil chemoattractant eotaxin. While gross lung pathology and airway mucus production did not differ between groups, FXI KO mice displayed an impaired endothelial/epithelial barrier function, as reflected by elevated levels of total protein and IgM in bronchoalveolar lavage fluid. FXI KO mice had a stronger systemic IgE response with an almost completely absent HDM-specific IgG_1 response. The phenotype of FXII KO mice was, except for a higher HDM-specific IgG_1 response, similar to that of WT mice. In conclusion, FXI attenuates part of the allergic response to repeated administration of HDM in the airways by a mechanism that is independent of activation via FXII.

INTRODUCTION

Asthma is a chronic inflammatory disease of the airways that affects up to 300 million people worldwide[1,2]. Asthma is associated with bronchial hyper-reactivity, mucus overproduction and airway remodeling, leading to episodes of reversible airway obstruction, dyspnea and wheezing[1,2]. Most asthma patients have an allergic phenotype. House dust mite (HDM) is amongst the most common allergens triggering respiratory symptoms in sensitized patients[3].

In patients with asthma, leakage of clotting factors into the bronchoalveolar space and expression of tissue factor (TF) on various cell types (e.g., alveolar epithelium, macrophages and eosinophils) result in a local procoagulant state[4]. Whereas limited activation of coagulation may enhance epithelial repair, more pronounced activation can, together with the inflammatory response, worsen pathophysiology (reviewed in[4]). The end product of coagulation is fibrin, which is formed after cleavage of fibrinogen by thrombin. Two different pathways are known to lead to thrombin formation, the TF (or extrinsic) pathway and the intrinsic pathway of coagulation. Soluble TF as well as thrombin-antithrombin complexes have been detected in bronchoalveolar lavage fluid (BALF) or sputum of asthmatic patients[5,6], suggesting that the TF pathway of coagulation is activated during asthma. To our knowledge, it is not known whether the intrinsic pathway of coagulation is triggered during asthma.

The central player in the intrinsic coagulation system is factor XI (FXI). Plasma activators of FXI include activated factor XII (FXIIa)[7], thrombin[8-10], and FXIa (autoactivation)[9,11]. Interestingly, it has been reported that a proteinase from HDM can directly activate FXII[12]. Apart from initiating the intrinsic coagulation system via FXI, FXIIa additionally can convert prekallikrein into kallikrein which on its turn cleaves high molecular weight kininogen releasing the potent proinflammatory and vasoactive small molecule bradykinin[13].

When coagulation is initiated by low concentrations of TF the thrombin-FXI amplification loop contributes to coagulation activation[11,14-16]. Recently, a bimodal role of thrombin in allergic airway inflammation induced by ovalbumin was described in mice; herein complete inhibition of thrombin by hirudin or instilling a high dose of thrombin worsened ovalbumin induced asthma, while a low dose of thrombin ameliorated the inflammatory response[17]. Considering the role of coagulation and thrombin in experimentally induced allergic airway inflammation, and considering that thrombin is amplified via the thrombin-

FXI loop, we found it of interest to establish the role of FXI during asthma. Hence, the aim of the present study was to determine the contribution of the intrinsic coagulation system to the allergic airway inflammation accompanying asthma. Additionally, if the intrinsic coagulation is involved in the development of asthma, we wanted to investigate whether this is regulated via FXIIa. For this we used mice deficient for either FXI or FXII and subjected them to our model of allergic lung inflammation induced by repeated administration of HDM via the airways[18].

METHODS

Mice

C57Bl/6 wild-type (WT) mice were purchased from Charles River Inc. (Maastricht, The Netherlands). Factor XI knockout (FXI KO) and Factor XII knockout (FXII KO) mice were generated as described before[19,20], and bred in the animal facility of the Academic Medical Center in Amsterdam. Mutant mice were backcrossed to C57Bl/6 at least 7 generations. Mice were housed under specific pathogen-free conditions receiving food and water ad libitum. Age-matched (9-12 weeks at start of the experiments) female mice were used in all experiments. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

HDM asthma model

HDM allergen whole body extract (Greer Laboratories, Lenoir, N.C., USA), derived from the common European HDM species Dermatophagoides pteronyssinus, Der p, was used to induce allergic lung inflammation as described previously[18]. In short, mice were randomized to receive (by intranasal inoculation) 25 µg HDM (sensitization phase) on day 0, 1 and 2 and 6.25 µg HDM (challenge phase) on day 14, 15, 18 and 19, or sterile saline intranasally on each occasion (controls). Inoculum volume was 20 µl for every HDM and saline exposure and inoculation procedures were performed during isoflurane inhalation anesthesia. The complete mouse model was performed twice (experiment I and II) and on each occasion different materials were obtained which are specified hereafter. Mice were sacrificed at day 21 and blood was collected from the vena cava inferior into citrate (4:1 v/v, experiment I). The right lung was fixed 24 hours in 10% formalin (experiment II). The left lung and part of the liver were homogenized in 4 volumes of saline after which a sample was taken for RNA isolation; the remaining homogenate was lysed in equal volume of lysis buffer containing

150mM NaCl, 15mM Tris, 1mM MgCl, 1mM $CaCl_2$, 1% Triton and 1% protease inhibitor cocktail (Roche Applied Science, Almere, Netherlands) and used for protein analysis (all experiment I). Two-sided BALF was collected by instilling and retrieving 1ml of sterile PBS containing 10mM EDTA, 10mM benzamidine and 0.2mg/ml SBTI in 500µl aliquots via the trachea (experiment II). Cell counts were determined for each BALF sample in a hemocytometer (Beckman Coulter, Fullerton, CA, USA) and differential cells counts by cytospin preparations stained with Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland). BALF supernatant was collected for protein analysis.

Histology

Formalin-fixed tissue was embedded in paraffin using standard procedures. Four-µm 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[18]. 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)[18]. 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)[21], 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 IgG_1 was determined using HDM as capture, and biotinylated rat-anti-mouse IgG_1 (from BD Biosciences) as detection. HDM-specific IgG_1 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[22]. 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[23]. 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[18], 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 $P \leq 0.05$ 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[18]. 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).

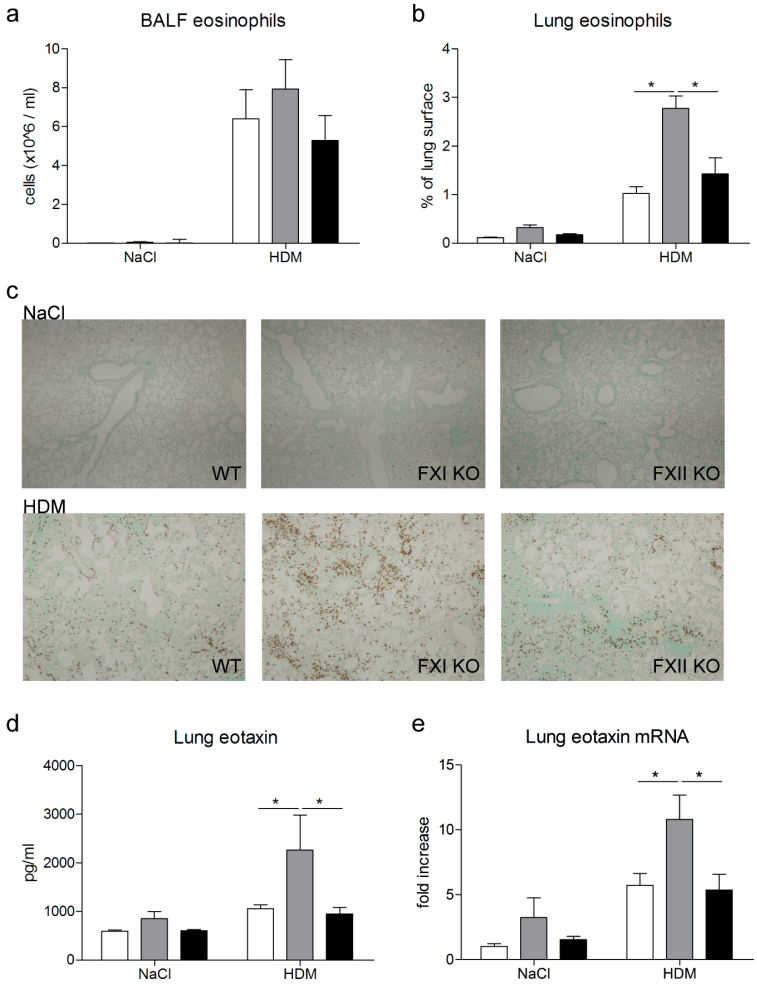

Figure 1. FXI KO, but not FXII KO, mice have increased eosinophil influx and eotaxin levels in lungs upon HDM exposure via the airways. (a) Influx of eosinophils in BALF of WT (white bars), FXI KO (grey bars) or FXII KO (black bars) following NaCl or HDM exposure. Accumulation of eosinophils in the lung 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

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 [24], we next set out to investigate whether FXI or FXII deficiency impacted on this feature by PasD 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.

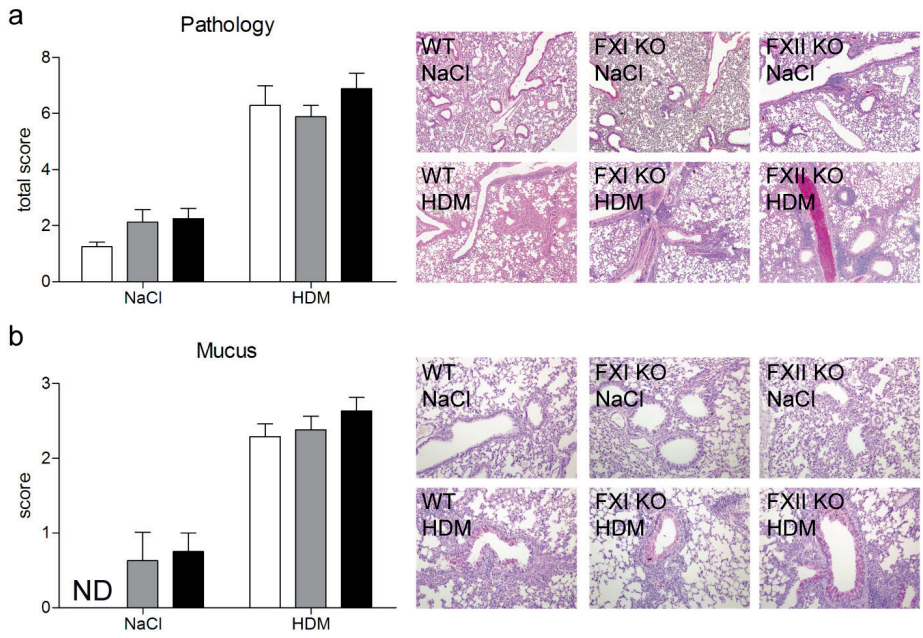

Figure 2. FXI KO and FXII KO mice show similar lung pathology and mucus production after HDM administration via the airways. Total pathology score of HE stained lung sections (a) and mucus score of PasD stained lung sections (b) of WT (white bars), FXI KO (grey bars) and FXII KO (black bars). Data are 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

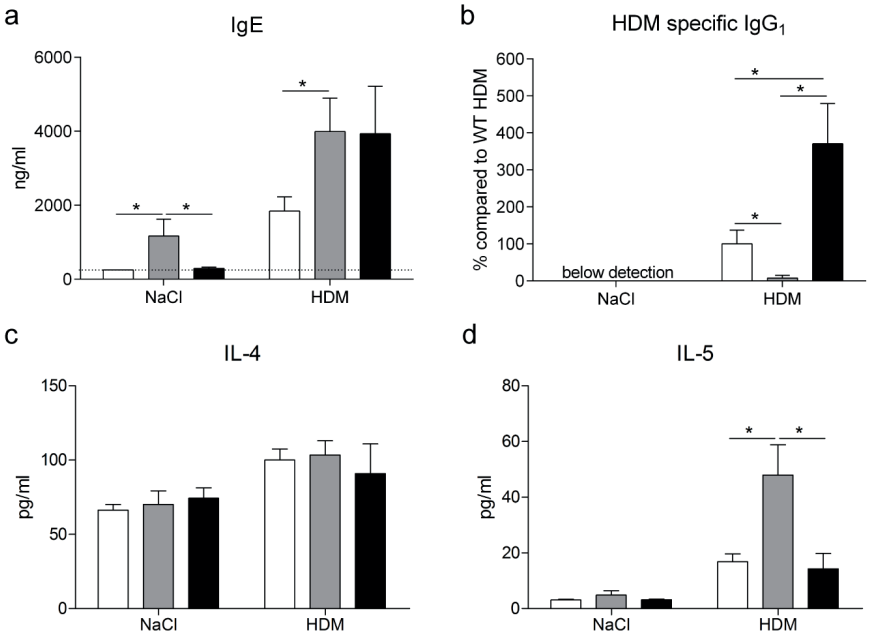

Figure 3. Impact of FXI or FXII deficiency on type 2 immune responses. Plasma IgE (a) and HDM-specific IgG_1 (b) and lung IL-4 (c) and IL-5 (d) levels in WT (white bars), FXI KO (grey bars) and FXII KO (black bars) in NaCl and HDM exposed mice. Dashed line represents lower detection limit. Data are means + SEM (n=8 per group, experiment I), *P<0.05

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 IgG_1 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 IgG_1 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.

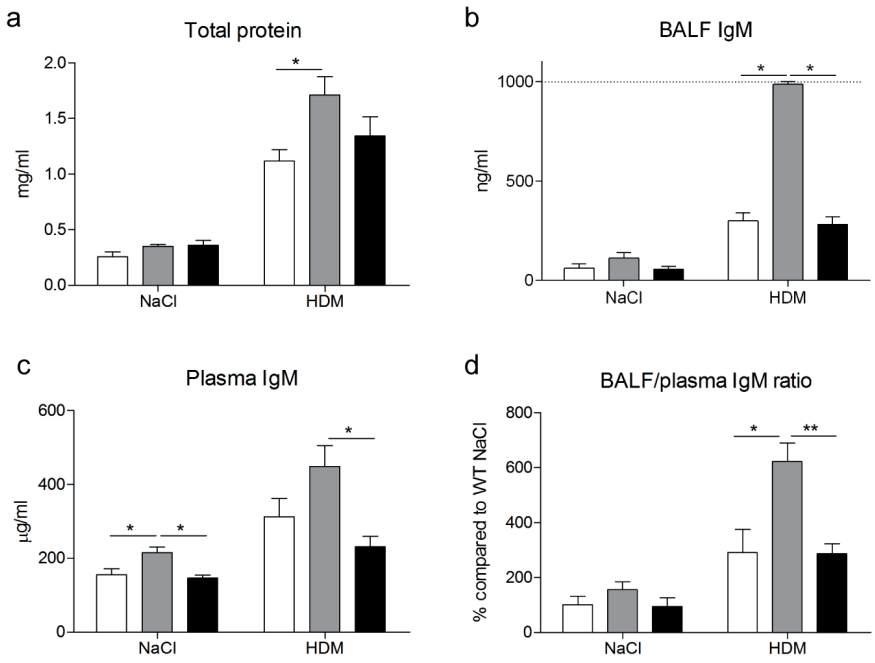

Figure 4. FXI KO, but not FXII KO, mice show increased protein leak in BALF. Protein leakage was determined by BALF total protein (a) and IgM (b) levels in WT (white bars), FXI KO (grey bars) and FXII KO (black bars) mice. 7 out of 8 HDM exposed FXI KO mice had IgM levels above detection (detection limit 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

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.

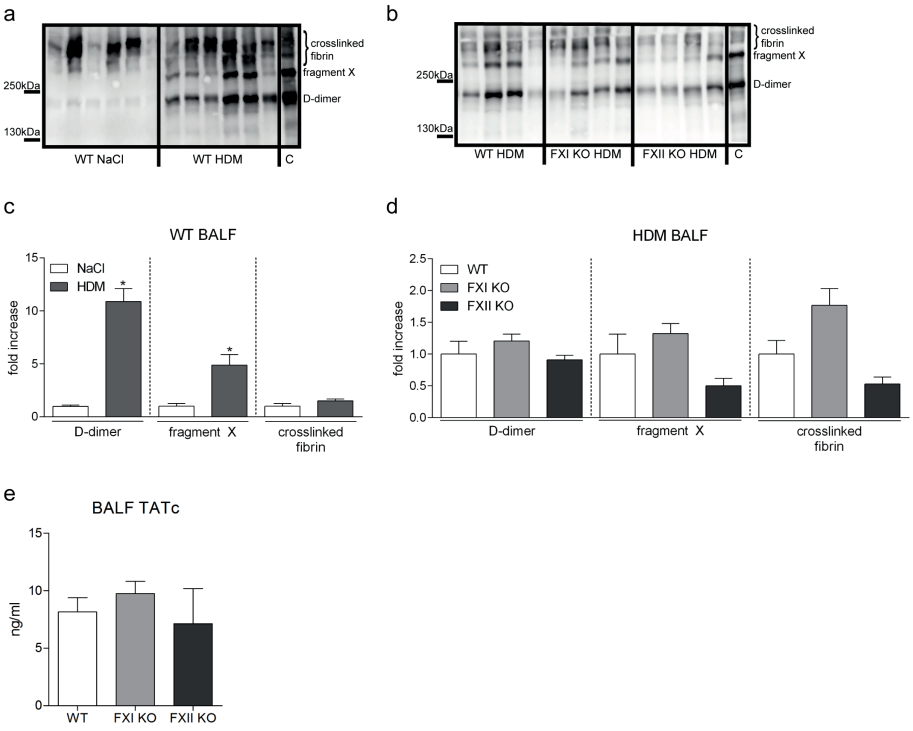

Figure 5. HDM exposure via the airways induces local activation of coagulation and fibrinolysis in all three strains. (a) Fibrin degradation WB of WT NaCl and HDM exposed BALF. (c) Digital analysis of WB is depicted as fold increase in HDM (black bars) compared with NaCl (white bars) exposed WT BALF. (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

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[25] and via the interaction of fibrinogen cleavage products with Toll-like receptor 4[26]. Recently it was shown that low levels of thrombin are beneficial while high levels of thrombin are detrimental in an ovalbumin asthma model[17]. 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[27], 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[17]. 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[28]. 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[29]. In line, activated FXI has been shown to inhibit neutrophil migration in a chemotactic assay[30]. 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 Th_2 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[31]. 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[32]. Several clinical trials have reported reduced eosinophilia following anti-IL-5 therapy, although clinical responses have been quite variable[33]. 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 Th_2 response is enhanced in FXI KO mice.

The Th_2 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[34,35]. HDM-specific IgG_1 was detectable in our mouse model, however almost undetectable in FXI KO mice. Total IgG_1 was not lower in NaCl or HDM exposed FXI KO mice (data not shown); thus the low HDM-specific IgG_1 cannot be explained by an impaired IgG_1 production. The equivalent of murine IgG_1 in humans is IgG_4[36]. In a recent clinical trial, HDM immunotherapy induced a dose-dependent increase in allergen-specific IgG_4 that was accompanied by a reduction in asthma exacerbations[37]. Moreover, desensitization with ovalbumin enhanced ovalbumin-specific IgG_1, and reduced IL-4 and IL-5 in mice[38]. 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 IgG_1 in our HDM exposed FXI KO mice. The mechanism how FXI influences HDM-specific IgG_1 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[39]. 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[40]. 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[20]. 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[41].

FXII KO mice showed, except for HDM-specific IgG_1, similar HDM-induced inflammatory response as WT mice. FXII can be activated either directly via HDM proteinases[12] or indirectly via the release of negatively charged endogenous molecules, for instance via heparin released by activated mast cells[42], 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[43]). Moreover, FXII-independent activation of the kallikrein-kinin system has been reported in allergic asthma[44]. 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[5,6]. 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.

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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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[45-47]. 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.

REFERENCES

1. Braman SS. The global burden of asthma. *Chest.* 2006;130(1 Suppl):4S-12S.
2. Martinez FD, Vercelli D. Asthma. *Lancet.* 2013;382(9901):1360-1372.
3. Lodge CJ, Lowe AJ, Gurrin LC, et al. House dust mite sensitization in toddlers predicts current wheeze at age 12 years. *The Journal of allergy and clinical immunology.* 2011;128(4):782-788 e789.
4. de Boer JD, Majoor CJ, van t, V, Bel EH, van der Poll T. Asthma and coagulation. *Blood.* 2012;119(14):3236-3244.
5. Gabazza EC, Taguchi O, Tamaki S, et al. Thrombin in the airways of asthmatic patients. *Lung.* 1999;177(4):253-262.
6. Schouten M, MA VDP, Levi M, T VDP, JS VDZ. Early activation of coagulation after allergen challenge in patients with allergic asthma. *Journal of thrombosis and haemostasis : JTH.* 2009;7(9):1592-1594.
7. Bouma BN, Griffin JH. Human blood coagulation factor XI. Purification, properties, and mechanism of activation by activated factor XII. *The Journal of biological chemistry.* 1977;252(18):6432-6437.
8. Gailani D, Broze GJ, Jr. Factor XI activation in a revised model of blood coagulation. *Science.* 1991;253(5022):909-912.
9. Naito K, Fujikawa K. Activation of human blood coagulation factor XI independent of factor XII. Factor XI is activated by thrombin and factor XIa in the presence of negatively charged surfaces. *The Journal of biological chemistry.* 1991;266(12):7353-7358.
10. Kravtsov DV, Matafonov A, Tucker EI, et al. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood.* 2009;114(2):452-458.
11. von dem Borne PA, Meijers JC, Bouma BN. Feedback activation of factor XI by thrombin in plasma results in additional formation of thrombin that protects fibrin clots from fibrinolysis. *Blood.* 1995;86(8):3035-3042.
12. Maruo K, Akaike T, Matsumura Y, et al. Triggering of the vascular permeability reaction by activation of the Hageman factor-prekallikrein system by house dust mite proteinase. *Biochimica et biophysica acta.* 1991;1074(1):62-68.
13. Renne T. The procoagulant and proinflammatory plasma contact system. *Seminars in Immunopathology.* 2012;34(1):31-41.
14. Keularts IM, Zivelin A, Seligsohn U, Hemker HC, Beguin S. The role of factor XI in thrombin generation induced by low concentrations of tissue factor. *Thromb Haemost.* 2001;85(6):1060-1065.
15. He R, Xiong S, He X, et al. The role of factor XI in a dilute thromboplastin assay of extrinsic coagulation pathway. *Thromb Haemost.* 2001;85(6):1055-1059.
16. Cawthern KM, van 't Veer C, Lock JB, DiLorenzo ME, Branda RF, Mann KG. Blood coagulation in hemophilia A and hemophilia C. *Blood.* 1998;91(12):4581-4592.
17. Miyake Y, D'Alessandro-Gabazza CN, Takagi T, et al. Dose-dependent differential effects of thrombin in allergic bronchial asthma. *Journal of thrombosis and haemostasis : JTH.* 2013;11(10):1903-1915.
18. Daan de BJ, Roelofs JJ, de Vos AF, et al. Lipopolysaccharide inhibits Th2 lung inflammation induced by house dust mite allergens in mice. *AmJRespirCell MolBiol.* 2013;48(3):382-389.
19. Gailani D, Lasky NM, Broze GJ, Jr. A murine model of factor XI deficiency. *Blood CoagulFibrinolysis.* 1997;8(2):134-144.
20. Iwaki T, Castellino FJ. Plasma levels of bradykinin are suppressed in factor XII-deficient mice. *ThrombHaemost.* 2006;95(6):1003-1010.
21. Lee JJ, McGarry MP, Farmer SC, et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *JExpMed.* 1997;185(12):2143-2156.
22. Ruijter JM, Pfaffl MW, Zhao S, et al. Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. *Methods.* 2013;59(1):32-46.

23. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*. 2012;9(7):671-675.
24. Thomson NC, Chaudhuri R, Messow CM, et al. Chronic cough and sputum production are associated with worse clinical outcomes in stable asthma. *Respiratory medicine*. 2013;107(10):1501-1508.
25. Peters T, Henry PJ. Protease-activated receptors and prostaglandins in inflammatory lung disease. *British journal of pharmacology*. 2009;158(4):1017-1033.
26. Millien VO, Lu W, Shaw J, et al. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science*. 2013;341(6147):792-796.
27. Fulkerson PC, Fischetti CA, McBride ML, Hassman LM, Hogan SP, Rothenberg ME. A central regulatory role for eosinophils and the eotaxin/CCR3 axis in chronic experimental allergic airway inflammation. *Proc Natl Acad Sci U S A*. 2006;103(44):16418-16423.
28. Maas C, Meijers JC, Marquart JA, et al. Activated factor V is a cofactor for the activation of factor XI by thrombin in plasma. *Proc Natl Acad Sci U S A*. 2010;107(20):9083-9087.
29. Cheng Q, Zhao Y, Lawson WE, et al. The effects of intrinsic pathway protease deficiencies on plasminogen-deficient mice. *Blood*. 2005;106(9):3055-3057.
30. Itakura A, Verbout NG, Phillips KG, et al. Activated factor XI inhibits chemotaxis of polymorphonuclear leukocytes. *Journal of Leukocyte Biology*. 2011;90(5):923-927.
31. Schuijs MJ, Willart MA, Hammad H, Lambrecht BN. Cytokine targets in airway inflammation. *Current opinion in pharmacology*. 2013;13(3):351-361.
32. Mould AW, Matthaei KI, Young IG, Foster PS. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *The Journal of clinical investigation*. 1997;99(5):1064-1071.
33. Garcia G, Taille C, Laveneziana P, Bourdin A, Chanez P, Humbert M. Anti-interleukin-5 therapy in severe asthma. *European respiratory review : an official journal of the European Respiratory Society*. 2013;22(129):251-257.
34. Post S, Heijink IH, Petersen AH, de Bruin HG, van Oosterhout AJ, Nawijn MC. Protease-activated receptor-2 activation contributes to house dust mite-induced IgE responses in mice. *PLoS One*. 2014;9(3):e91206.
35. Post S, Nawijn MC, Hackett TL, et al. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax*. 2012;67(6):488-495.
36. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172(5):2731-2738.
37. Virchow JC, Backer V, Kuna P, et al. Efficacy of a House Dust Mite Sublingual Allergen Immunotherapy Tablet in Adults With Allergic Asthma: A Randomized Clinical Trial. *JAMA*. 2016;315(16):1715-1725.
38. Fox EM, Torrero MN, Evans H, Mitre E. Immunologic characterization of 3 murine regimens of allergen-specific immunotherapy. *J Allergy Clin Immunol*. 2015;135(5):1341-1351 e1341-1347.
39. van Iwaarden F, de Groot PG, Bouma BN. The binding of high molecular weight kininogen to cultured human endothelial cells. *The Journal of biological chemistry*. 1988;263(10):4698-4703.
40. Oehmcke S, Herwald H. Contact system activation in severe infectious diseases. *Journal of molecular medicine*. 2010;88(2):121-126.
41. Shaver CM, Grove BS, Putz N, et al. Lung Epithelial Tissue Factor Regulates Alveolar Pro-coagulant Activity and Permeability in Direct Acute Lung Injury. *American journal of respiratory cell and molecular biology*. 2015.
42. Oschatz C, Maas C, Lecher B, et al. Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. *Immunity*. 2011;34(2):258-268.
43. Joseph K, Kaplan AP. Formation of bradykinin: a major contributor to the innate inflammatory response. *Adv Immunol*. 2005;86:159-208.
44. Christiansen SC, Proud D, Cochrane CG. Detection of tissue kallikrein in the bronchoalveolar lavage fluid of asthmatic subjects. *The Journal of clinical investigation*. 1987;79(1):188-197.

45. Kelada SN, Wilson MS, Tavarez U, et al. Strain-dependent genomic factors affect allergen-induced airway hyperresponsiveness in mice. *Am J Respir Cell Mol Biol.* 2011;45(4):817-824.

46. Atochina EN, Beers MF, Tomer Y, et al. Attenuated allergic airway hyperresponsiveness in C57BL/6 mice is associated with enhanced surfactant protein (SP)-D production following allergic sensitization. *Respir Res.* 2003;4:15.

47. Gueders MM, Paulissen G, Crahay C, et al. Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res.* 2009;58(12):845-854.

Kininogen deficiency or depletion attenuates airway hyperresponsiveness 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 enhanced pause (Penh) measurements 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 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 Penh independently 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[1,2]. 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[3]. 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[4].

High molecular weight kininogen (HK) is a component of the kallikrein-kinin system together with factor XII (FXII) and prekallikrein[5,6]. 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[7]. 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[5,6]. 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[8,9]. In the absence of FXII, however, tryptase release following mast cell degranulation likewise elicits bradykinin release from HK[10].

Inhalation of bradykinin causes immediate bronchoconstriction in asthmatic patients but not in healthy subjects[11,12]. 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[13]. Like in humans, various animal models have demonstrated enhanced bradykinin induced bronchoconstriction after allergen exposure[14,15]. Antagonists that interfere with bradykinin receptor signaling are able to attenuate airway hyperresponsiveness[16,17]. 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 enhanced pause (Penh) measurements 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.

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[7]. 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[18]. 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[19]. 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

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 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. 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[18]. 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 10^5 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[20]. 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

Data were analyzed by either two-way ANOVA or Mann-Whitney U-test. Experimental groups consisted of 6-8 mice. $P \leq 0.05$ 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 increase in Penh 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[21]. We determined Th2 cells in the lungs by intracellular staining of the transcription factor GATA3 in CD4+ lymphocytes[22] and in addition assessed the activation status of CD4+ T cells by measuring CD69 expression[23]. *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).

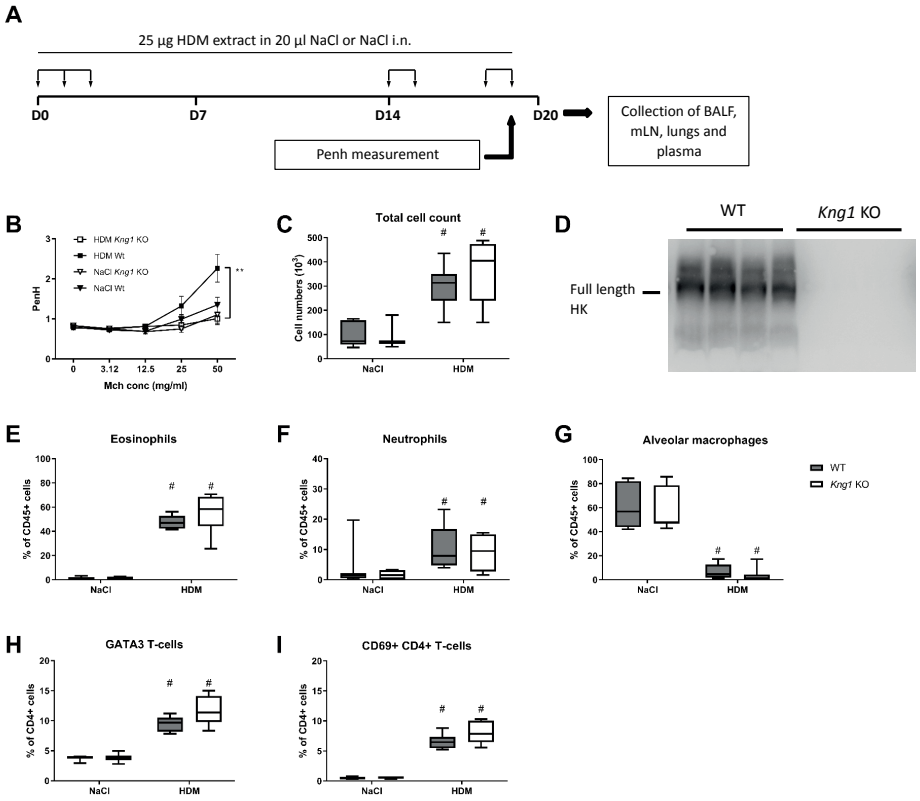

Figure 1. *Kng1* KO mice show an attenuated HDM induced rise in Penh with unaltered airway leukocyte influx. (A) HDM induced asthma model. (B) Penh after dose-response to methacholine (Mch) exposure on day 19. Data are pooled from two independent experiments. (C) Total cell count in bronchoalveolar lavage 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 expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation(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.

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.

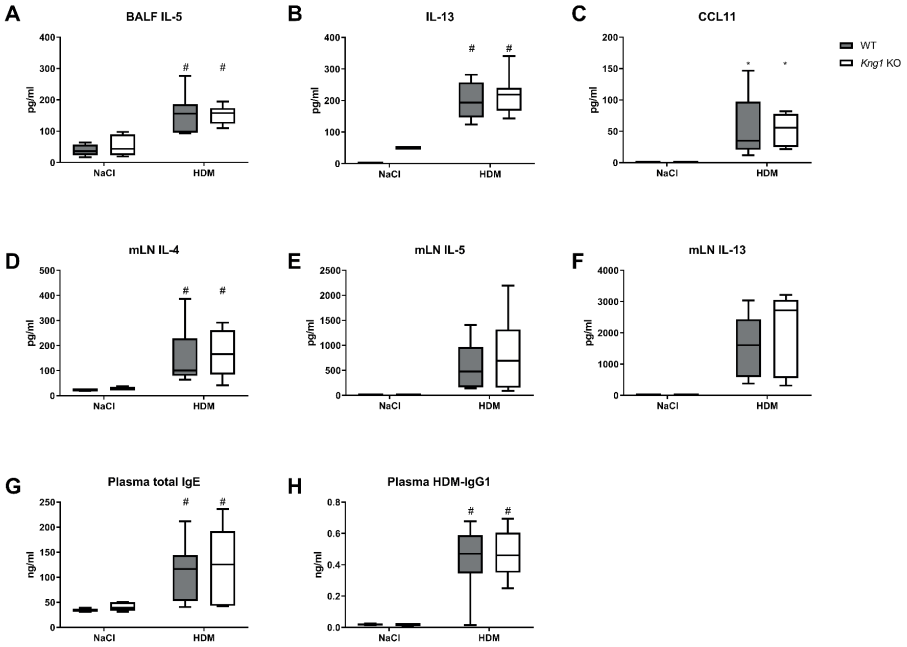

Figure 2. *Kng1* KO mice demonstrate unaltered Th2 responses during HDM induced lung inflammation. IL-5, IL-13 and CCL11 (A,B,C) in BALF and IL-4, IL-5 and IL-13 (D-F) in supernatant of *ex vivo* HDM stimulated mediastinal lymph nodes (mLN) are displayed. (G,H) Plasma total IgE and plasma HDM-IgG1. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. (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.

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[20]. *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[24]. 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[18]. 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).

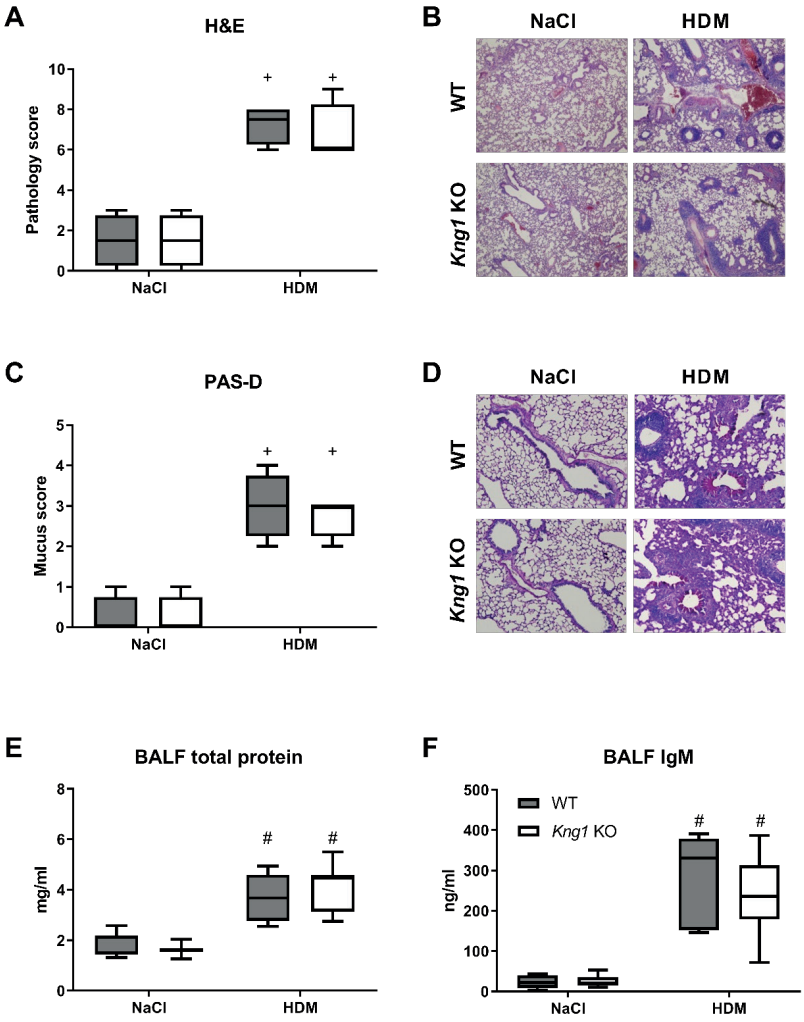

Figure 3. *Kng1* KO mice display similar lung pathology, mucus production and protein leakage to WT mice following HDM challenge. (A,B) Total pathology score of hematoxylin and eosin (H&E) stained lung sections (x4 magnification) from wild-type (WT)(black bars) and *Kng1* KO (white bars) and (C,D) total 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 shown as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. +P<0.05 and #P<0.001 for comparison between NaCl and HDM within *Kng1* KO or wild-type (WT) mice.

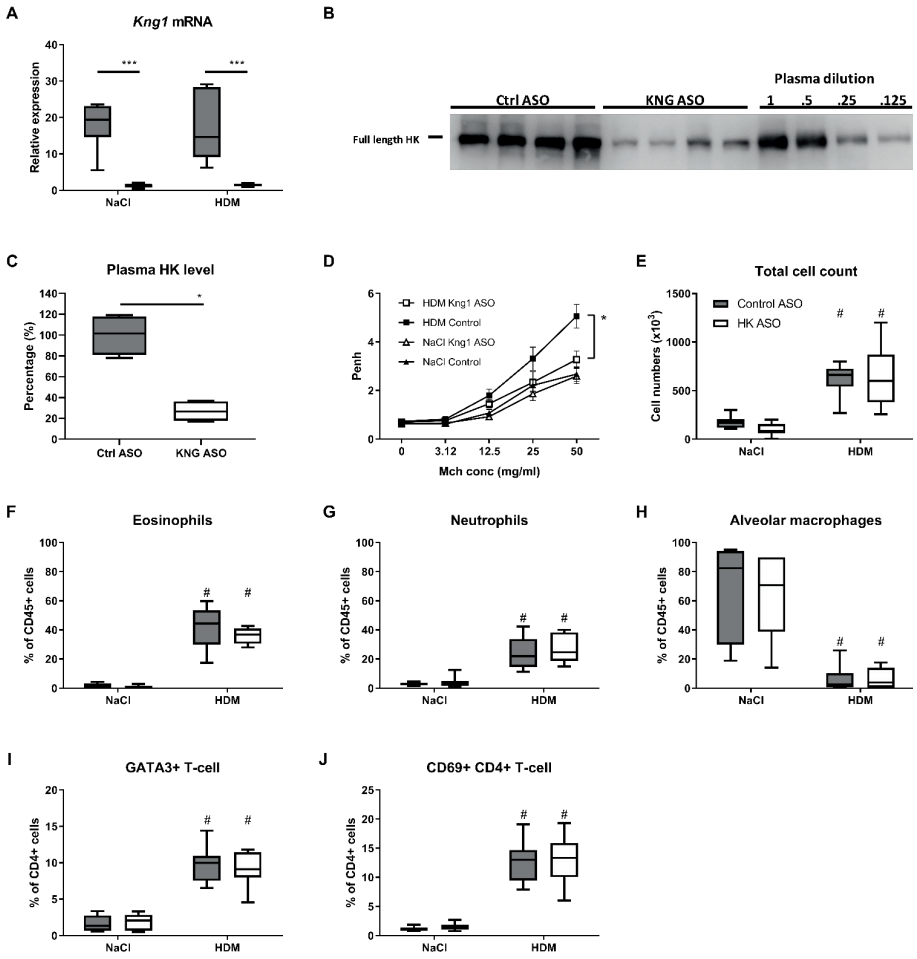

Figure 4. KNG depletion after HDM sensitization attenuates the HDM induced rise in Penh without affecting leukocyte influx. KNG ASO injections (40mg/kg) were given twice weekly after the sensitization phase starting on day 3. (A) *Kng1* mRNA normalized to HPRT in the liver 24 hours after last challenge 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 represented as box-and-whisker plots with 6-8 mice per group depicting the smallest observation, lower quartile, median, upper quartile and largest observation. *$P<0.05$ and #$P<0.001$ for comparison between NaCl and HDM within KNG and Ctrl ASO treated mice.

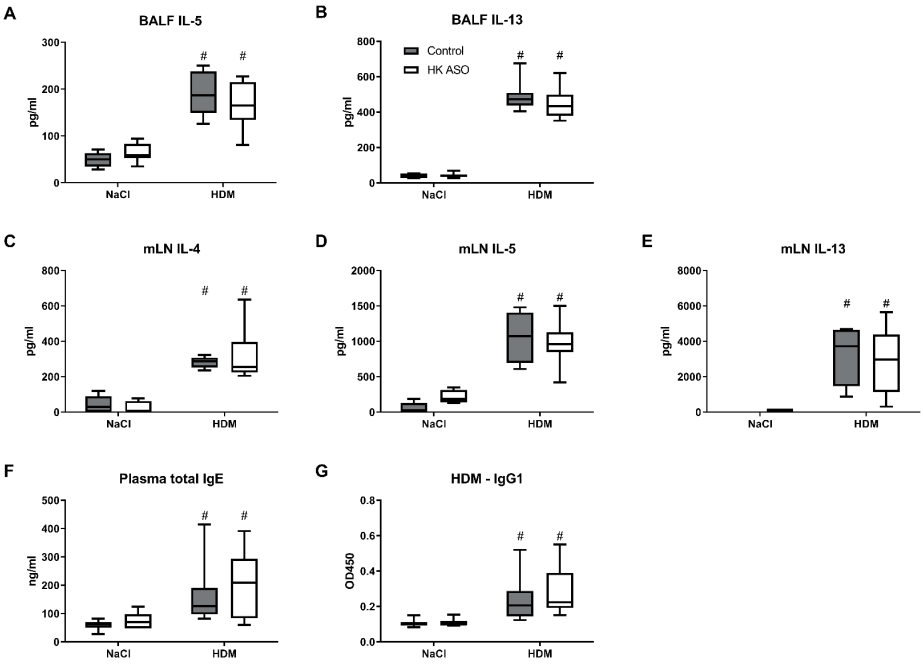

Fig. 5 KNG depletion after HDM sensitization does not influence Th2 responses following HDM challenge. KNG ASO injections (40mg/kg) were given twice weekly after the sensitization phase starting on day 3. (A,B) IL-5 and IL-13 in BALF and (C-E) IL-4, IL-5 and IL-13 in supernatant of *ex vivo* HDM stimulated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

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.

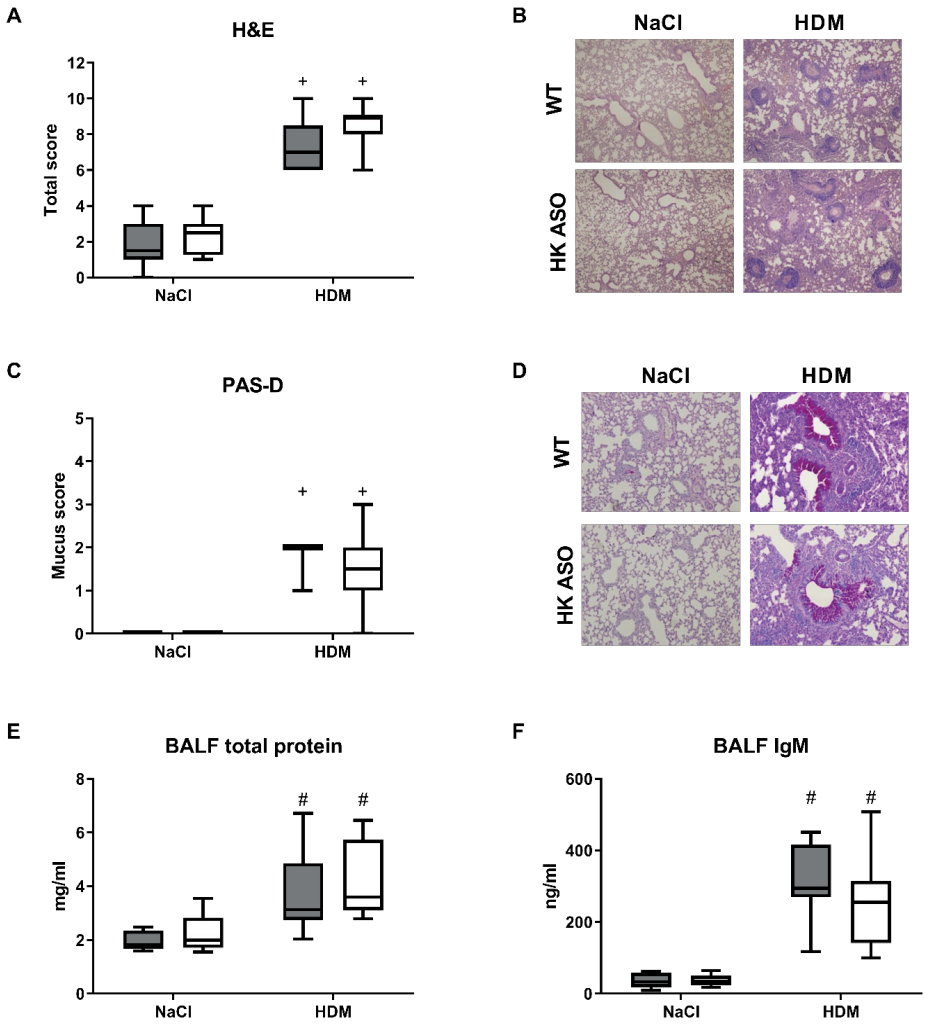

Figure 6. KNG depletion after HDM sensitization does not alter lung pathology, mucus production and protein leakage following HDM challenge. (A,B) Total pathology score of hematoxylin and eosin (H&E) stained lung sections (x4 magnification) from Ctrl ASO (black bar) and KNG ASO (white bar) treated mice 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 represented as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. +P<0.05 and #P<0.001 for comparison between NaCl and HDM within KNG and Ctrl ASO treated mice.

DISCUSSION

Several studies have suggested a role for the kallikrein-kinin system in allergic airway disease such as asthma[11,13]. 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[25] and a direct bradykinin challenge in rats and sheep likewise enhanced airway hyperresponsiveness (AHR)[14,15]. 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[11]. It is still in debate which of the bradykinin receptors is responsible for bronchoconstriction, B1R[26,27] or B2R[16,28]. 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[29,30]. Furthermore, blocking B1R in murine asthma models making use of ovalbumin sensitization and challenge resulted in lower airway eosinophilia[26,31]. 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[32]. 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[32]. It is possible that the small differences in eosinophil influx observed in ovalbumin models[26,31] were overwhelmed in our HDM model. Alternatively, B1R and B2R may have opposite roles in eosinophil influx [27] , 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[19], 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[21]. 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[33,34]. A possible mechanism of kininogen derived bradykinin induced increase in Penh 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[35]. 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[36]. 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[38,39]. In asthmatic patients an endotype

named paucigranulocytic asthma has been identified[40,41]. 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[40]. 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[42]. Moreover, different mouse strains could affect Penh outcomes, causing conflicting conclusions[43]. Notably, the severity of allergen induced lung inflammation modifies Penh results significantly[44], 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[45-47]. 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[44]. In a comparable HDM model, a direct correlation between whole body plethysmography and direct/invasive measurements has been reported[47]. Likewise, AHR is enhanced in HDM challenged groups compared to controls in models that are very similar to ours[48,49]. 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.

REFERENCES

1. Fahy JV. Type 2 inflammation in asthma--present in most, absent in many. *Nat Rev Immunol.* 2015;15(1):57-65.
2. Reddel HK, Bateman ED, Becker A, et al. A summary of the new GINA strategy: a roadmap to asthma control. *Eur Respir J.* 2015;46(3):622-639.
3. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* 2011;32(9):402-411.
4. Israel E, Reddel HK. Severe and Difficult-to-Treat Asthma in Adults. *N Engl J Med.* 2017;377(10):965-976.
5. de Maat S, Tersteeg C, Herczenik E, Maas C. Tracking down contact activation - from coagulation in vitro to inflammation in vivo. *Int J Lab Hematol.* 2014;36(3):374-381.
6. Long AT, Kenne E, Jung R, Fuchs TA, Renne T. Contact system revisited: an interface between inflammation, coagulation, and innate immunity. *J Thromb Haemost.* 2016;14(3):427-437.
7. Merkulov S, Zhang WM, Komar AA, et al. Deletion of murine kininogen gene 1 (mKng1) causes loss of plasma kininogen and delays thrombosis. *Blood.* 2008;111(3):1274-1281.
8. Brunnee T, Reddigari SR, Shibayama Y, Kaplan AP, Silverberg M. Mast cell derived heparin activates the contact system: a link to kinin generation in allergic reactions. *Clin Exp Allergy.* 1997;27(6):653-663.
9. Maruo K, Akaike T, Matsumura Y, et al. Triggering of the vascular permeability reaction by activation of the Hageman factor-prekallikrein system by house dust mite proteinase. *Biochim Biophys Acta.* 1991;1074(1):62-68.
10. Coffman LG, Brown JC, Johnson DA, et al. Cleavage of high-molecular-weight kininogen by elastase and tryptase is inhibited by ferritin. *Am J Physiol Lung Cell Mol Physiol.* 2008;294(3):L505-515.
11. Abraham WM, Scuri M, Farmer SG. Peptide and non-peptide bradykinin receptor antagonists: role in allergic airway disease. *Eur J Pharmacol.* 2006;533(1-3):215-221.
12. Fuller RW, Dixon CM, Cuss FM, Barnes PJ. Bradykinin-induced bronchoconstriction in humans. Mode of action. *The American review of respiratory disease.* 1987;135(1):176-180.
13. Christiansen SC, Proud D, Sarnoff RB, Juergens U, Cochrane CG, Zuraw BL. Elevation of tissue kallikrein and kinin in the airways of asthmatic subjects after endobronchial allergen challenge. *Am Rev Respir Dis.* 1992;145(4 Pt 1):900-905.
14. Abraham WM, Ahmed A, Cortes A, et al. Airway effects of inhaled bradykinin, substance P, and neurokinin A in sheep. *J Allergy Clin Immunol.* 1991;87(2):557-564.
15. Hannon JP, Tigani B, Williams I, Mazzoni L, Fozard JR. Mechanism of airway hyperresponsiveness to adenosine induced by allergen challenge in actively sensitized Brown Norway rats. *Br J Pharmacol.* 2001;132(7):1509-1523.
16. Abraham WM, Burch RM, Farmer SG, Sielczak MW, Ahmed A, Cortes A. A bradykinin antagonist modifies allergen-induced mediator release and late bronchial responses in sheep. *The American review of respiratory disease.* 1991;143(4 Pt 1):787-796.
17. Soler M, Sielczak M, Abraham WM. A bradykinin-antagonist blocks antigen-induced airway hyperresponsiveness and inflammation in sheep. *Pulm Pharmacol.* 1990;3(1):9-15.
18. Ding C, Van't Veer C, Roelofs J, et al. Limited role of kininogen in the host response during gram-negative pneumonia derived sepsis. *Am J Physiol Lung Cell Mol Physiol.* 2017:ajplung 00288 02017.
19. Stroo I, Yang J, de Boer JD, et al. Factor XI deficiency enhances the pulmonary allergic response to house dust mite in mice independent of factor XII. *Am J Physiol Lung Cell Mol Physiol.* 2017;312(2):L163-L171.
20. de Boer JD, Yang J, van den Boogaard FE, et al. Mast Cell-Deficient Kit Mice Develop House Dust Mite-Induced Lung Inflammation despite Impaired Eosinophil Recruitment. *J Innate Immun.* 2014;6(2):219-226.
21. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* 2015;16(1):45-56.

22. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*. 1997;89(4):587-596.
23. Kimura MY, Hayashizaki K, Tokoyoda K, Takamura S, Motohashi S, Nakayama T. Crucial role for CD69 in allergic inflammatory responses: CD69-Myl9 system in the pathogenesis of airway inflammation. *Immunol Rev*. 2017;278(1):87-100.
24. Schlemper V, Medeiros R, Ferreira J, Campos MM, Calixto JB. Mechanisms underlying the relaxation response induced by bradykinin in the epithelium-intact guinea-pig trachea in vitro. *Br J Pharmacol*. 2005;145(6):740-750.
25. Forteza R, Botvinnikova Y, Ahmed A, et al. The interaction of alpha 1-proteinase inhibitor and tissue kallikrein in controlling allergic ovine airway hyperresponsiveness. *Am J Respir Crit Care Med*. 1996;154(1):36-42.
26. Vasquez-Pinto LM, Nantel F, Sirois P, Jancar S. Bradykinin B(1) receptor antagonist R954 inhibits eosinophil activation/proliferation/migration and increases TGF-beta and VEGF in a murine model of asthma. *Neuropeptides*. 2010;44(2):107-113.
27. Gama Landgraf R, Jancar S, Steil AA, Sirois P. Modulation of allergic and immune complex-induced lung inflammation by bradykinin receptor antagonists. *Inflamm Res*. 2004;53(2):78-83.
28. Sylvin H, van der Ploeg I, Alving K. The effect of a bradykinin B2 receptor antagonist, NPC-567, on allergen-induced airway responses in a porcine model. *Inflamm Res*. 2001;50(9):453-459.
29. Baraniuk JN, Lundgren JD, Mizoguchi H, et al. Bradykinin and respiratory mucous membranes. Analysis of bradykinin binding site distribution and secretory responses in vitro and in vivo. *The American review of respiratory disease*. 1990;141(3):706-714.
30. Hirayama Y, Miyayasu K, Yamagami K, Imai T, Ohkubo Y, Mutoh S. Effect of FK3657, a non-peptide bradykinin B2 receptor antagonist, on allergic airway disease models. *Eur J Pharmacol*. 2003;467(1-3):197-203.
31. Eric J, Gabra BH, Sirois P. Implication of the bradykinin receptors in antigen-induced pulmonary inflammation in mice. *Br J Pharmacol*. 2003;138(8):1589-1597.
32. Johnson JR, Wiley RE, Fattouh R, et al. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med*. 2004;169(3):378-385.
33. Utsch L, Logiantara A, van Ree R, van Rijt LS. Experimental food allergy to peanut enhances the immune response to house dust mite in the airways of mice. *Clin Exp Allergy*. 2017;47(1):121-128.
34. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med*. 1998;157(1):4-9.
35. Deshpande DA, Penn RB. Targeting G protein-coupled receptor signaling in asthma. *Cell Signal*. 2006;18(12):2105-2120.
36. Jain D, Keslacy S, Tliba O, et al. Essential role of IFNbeta and CD38 in TNFalpha-induced airway smooth muscle hyper-responsiveness. *Immunobiology*. 2008;213(6):499-509.
37. Polosa R. Contribution of histamine and prostanoids to bronchoconstriction provoked by inhaled bradykinin in atopic asthma. In: GD P, ed. ST H, trans. Vol 45: Allergy; 1990:174-182.
38. Proud D. The kinin system in rhinitis and asthma. *Clin Rev Allergy Immunol*. 1998;16(4):351-364.
39. Heitsch H. Bradykinin B2 receptor as a potential therapeutic target. *Drug News Perspect*. 2000;13(4):213-225.
40. Svenningsen S, Nair P. Asthma Endotypes and an Overview of Targeted Therapy for Asthma. *Front Med (Lausanne)*. 2017;4:158.
41. Ntontsi P, Loukides S, Bakakos P, et al. Clinical, functional and inflammatory characteristics in patients with paucigranulocytic stable asthma: Comparison with different sputum phenotypes. *Allergy*. 2017;72(11):1761-1767.
42. Bates JH, Irvin CG. Measuring lung function in mice: the phenotyping uncertainty principle. *J Appl Physiol (1985)*. 2003;94(4):1297-1306.

43. Adler A, Cieslewicz G, Irvin CG. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *J Appl Physiol (1985)*. 2004;97(1):286-292.

44. Verheijden KA, Henricks PA, Redegeld FA, Garssen J, Folkerts G. Measurement of airway function using invasive and non-invasive methods in mild and severe models for allergic airway inflammation in mice. *Front Pharmacol*. 2014;5:190.

45. Crosby JR, Guha M, Tung D, et al. Inhaled CD86 antisense oligonucleotide suppresses pulmonary inflammation and airway hyper-responsiveness in allergic mice. *J Pharmacol Exp Ther*. 2007;321(3):938-946.

46. Karras JG, Crosby JR, Guha M, et al. Anti-inflammatory activity of inhaled IL-4 receptor-alpha antisense oligonucleotide in mice. *Am J Respir Cell Mol Biol*. 2007;36(3):276-285.

47. McKnight CG, Jude JA, Zhu Z, Panettieri RA, Jr., Finkelman FD. House Dust Mite-Induced Allergic Airway Disease Is Independent of IgE and FcepsilonRIalpha. *Am J Respir Cell Mol Biol*. 2017;57(6):674-682.

48. Yocum GT, Turner DL, Danielsson J, et al. GABAA receptor alpha4-subunit knockout enhances lung inflammation and airway reactivity in a murine asthma model. *Am J Physiol Lung Cell Mol Physiol*. 2017;313(2):L406-L415.

49. Liu C, Yuan L, Zou Y, et al. ITGB4 is essential for containing HDM-induced airway inflammation and airway hyperresponsiveness. *J Leukoc Biol*. 2018;103(5):897-908.

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Summary and Discussion

Summary and Discussion

For a long time asthma has been regarded as a lung disease associated with lung inflammation, airflow obstruction, coughing and mucus hypersecretion. The first asthma guidelines were published almost 30 years ago and recommended the use of inhaled corticosteroids (ICS) and short acting β2-agonists[1-4]. Although this treatment has greatly benefited many patients, it does not achieve disease control in a substantial group with difficult-to-treat or severe asthma[5,6]. Nevertheless, ICS and bronchodilators are still the cornerstone of asthma management nowadays[7], indicating that advancement in this field has somewhat stagnated. In recent years, we have started to appreciate that underneath the similar set of symptom manifestations, the asthma syndrome (rather than disease) encompasses complex and heterogeneous pathophysiological mechanisms[8,9]. This has resulted in the introduction of biologicals targeting specific type 2 cytokines with great clinical improvements in patients with type 2 high asthma[10]. These biologicals exemplified how tailored therapy provides tremendous (additional) benefits to patients. Much effort in ongoing research is spent on identifying undiscovered pathophysiological traits that characterize distinct subsets of asthma patients (phenotyping), which may aid in defining targets for drug development[11-14].

This thesis can be regarded as part of that continuous effort. We here investigated the therapeutic potential of C1-inhibitor in asthma models, considering that C1-inhibitor exerts immunomodulatory effects as a regulator of both the complement and contact system. These systems have been implicated to play a role in the pathogenesis and pathophysiology of asthma, making them interesting for further research to elucidate their potential as candidates for targeted therapy.

The role of C1-inhibitor in human and murine asthma models

In **chapter 2** we report on the results of a randomized placebo-controlled double-blind parallel study, named the CAST-study. In this proof-of-concept study we investigated the effect of C1-inhibitor on house dust mite (HDM) and lipopolysaccharide (LPS) induced airway inflammation in adults with mild asthma. We included 24 eligible patients who were randomized to placebo and C1-inhibitor treatment groups. A mixure of HDM/LPS was instilled in one lung segment to induce airway inflammation while saline was given in the contralateral lung segment as internal control. A bilateral bronchoalveolar lavage was performed seven hours after the HDM/LPS challenge. We opted

for a HDM/LPS provocation since HDM is an important domestic allergen with high sensitization rate[15] while LPS is a widespread environmental pollutant and abundantly present in house dust[16], which makes a co-exposure of both HDM and LPS a likely event. We observed that the intravenously administered C1-inhibitor did reach the airways and mitigated HDM/LPS induced complement activation at the level of C4 and C3. As primary outcome, we observed that prophylactic C1-inhibitor treatment did not modulate eosinophil or neutrophil numbers in the bronchoalveolar lavage fluid of our patients after intrabronchial HDM/LPS challenge. Likewise, C1-inhibitor treatment did not influence the release of a series of cytokines and chemokines in the lung subsegment challenged with HDM/LPS. C1-inhibitor administration was associated with an attenuated HDM/LPS induced protein leakage across the mucosal barrier. Though airway epithelial cells have been recognized as an important regulator of the type 2 immune response[17], it is not clear how exactly disrupted epithelial barrier function translates to the clinical manifestation of asthma. Nonetheless, enhanced vascular permeability is associated with the loss of asthma control[18] and airway remodeling[19]. Our data indicate that allergen induced eosinophil and neutrophil migration into the airways is not significantly dependent on mucosal integrity. In addition, we show that the intracellular content from eosinophils and neutrophils does not significantly contribute to the disruption of the mucosal barrier in the early phase following allergen challenge. This does not preclude a more significant role for these inflammatory cells at a later time point. The peak for eosinophil influx into the airways occurs 24 hours following allergen challenge[20]. This may explain, at least in part, the discrepancy between our finding and mechanistic studies conducted in animal[21,22] or *in vitro*[23,24] models.

Beside eosinophils and neutrophils, alveolar macrophages have been shown to play a role in airway inflammation[25,26]. This cell population is the most abundantly present cell type in the airways under homeostatic conditions[27]. Their numbers remain remarkably consistent between patients[28] and are not affected by allergen challenge[29,30]. This may be a reason for the limited amount of research on alveolar macrophages regarding their potential role in the pathogenesis of asthma. In **chapter 3** we describe transcriptomic changes in alveolar macrophages from asthma patients as consequence of HDM/LPS challenge. We isolated alveolar macrophages from the bronchoalveolar fluid of both saline and HDM/LPS challenged lung segments, harvested from a subset of the placebo patients from the CAST study. Using RNA sequencing we identified 1013 differently expressed genes after HDM/LPS challenge relative to control. Ingenuity pathway analysis demonstrated upregulation of

pro-inflammatory responses such as inducible nitric oxide synthase, Toll-like receptor, p38 mitogen-activated protein kinase and interleukin (IL)-1, IL-8, IL-10, and IL-17 signaling, whereas metabolic pathways (e.g., insulin receptor and leptin signaling) were downregulated. Some of these enriched pathways (IL-1, IL-10 and TLR-signaling) were previously demonstrated to be upregulated in healthy individuals following an intrabronchial challenge with LPS[31], suggesting that activation of these pathways are LPS induced and not exclusively in alveolar macrophages in asthma patients. However, the extent to which these pathways are activated between alveolar macrophages from healthy individuals and asthma patients remains to be determined. Direct comparison between data from the current study and data from our previous study[31] is hampered due to differences in challenge (HDM/LPS versus LPS alone) and transcriptome analysis (RNA sequencing versus micro-array). To our knowledge, this is the first investigation to examine gene expression profiles in alveolar macrophages from asthma patients challenged with HDM and LPS *in vivo*. These findings may provide a better understanding of alveolar macrophage functions in (exacerbations of) allergic asthma.

To gain more understanding of the effect of C1-inhibitor on airway inflammation in asthma, in **chapter 4** we sensitized mice to HDM in the first week and challenged them with HDM in the second and third week during which C1-inhibitor was administered. In line with our finding in asthma patients, C1-inhibitor treatment did not alter HDM induced eosinophil and neutrophil influx in the airways. Moreover, C1-inhibitor did not modify the type 2 response in the airways, but seemed to modestly decrease the release of type 2 cytokines in the mediastinal lymph nodes of HDM challenged mice. In contrast to asthma patients, prophylactic C1-inhibitor treatment in HDM challenged mice was not associated with a restored mucosal barrier integrity in the lung. Similarly, HDM induced pulmonary pathological changes were unaffected by C1-inhibitor administration. Differences between species, model and treatment dose could have contributed to the disparity in the results on mucosal permeability.

The role of anaphylatoxins in asthma

The anaphylatoxin C3a or signaling via its receptor (C3aR) have been shown to promote type 2 inflammation in mouse asthma models[32]. C3aR is expressed in all myeloid derived leukocytes and several lung parenchyma cells such as smooth muscle, endothelial and epithelial cells[33], enabling C3a to exert a broad inflammation modulating effect. Many recent studies have elucidated some of the possible mechanisms by which C3a modulates the adaptive immune system

and indirectly facilitates allergen induced eosinophilia in the airways[32,34,35]. Few studies have addressed the direct C3a effects on eosinophils in the context of allergic inflammation[36,37]. In **chapter 5** we assessed the chemoattractant effect of C3a signaling on eosinophils. To this end, we adoptively transferred normal or C3aR deficient bone marrow derived eosinophils in HDM sensitized and challenged mice deficient of eosinophils. We found similar eosinophil numbers in the lungs and airways of the recipient mice, indicating that HDM induced eosinophil migration is independent of C3a signaling. In line with the *in vivo* experiment, we observed that C3a lacks chemotactic activity towards eosinophils *ex vivo*. However, this latter finding requires further investigation as contradicting results have been reported[37]. While C3a appears to be irrelevant for murine eosinophil chemotaxis, it may have an effect in human eosinophils. Differences in eosinophil function between species are not uncommon[38] and often complicate the translation of findings in animal derived eosinophils to human eosinophils. This is an important limitation in experiments using murine eosinophils to study eosinophil functions and interactions. Yet, some essential characteristics are conserved between species[38,39], which together with their practical availability make murine eosinophils still meaningful to study.

Experimental studies in animals have demonstrated a remarkable dual role for complement factor C5a in the pathogenesis of asthma[40-43]. While it exerts an inhibitory effect prior to allergen sensitization, C5a enhances the propagation of Th2 inflammation in allergen sensitized animals. The underlying mechanisms for the C5a mediated proallergic effect in an established inflammation environment are not well understood. The previous studies investigating the C5a/C5aR elimination in asthma models were mainly focused on the role Th2 cells without assessing the contribution of group 2 innate lymphoid cells (ILC2s). The recently recognized ILC2s are regarded as the innate counterpart of Th2 cells and together they orchestrate type 2 immune responses due to their capacity to produce type 2 cytokines. In **chapter 6** we aimed to investigate the role of C5 inhibition in the HDM induced type 2 response, focusing on both Th2 cells and ILC2s. We found that C5 activation aided in the differentiation of Th2 cells but did not affect the differentiation or proliferation of ILC2s. This is consistent with a recent study showing that ILCs do not express C5a receptors on their cell membrane[44]. We observed reduced intracellular IL-4 synthesis in Th2 cells residing in the mediastinal lymph nodes as result of C5 inhibition during the challenge phase. Though we did not determine intracellular IL-5 and IL-13 synthesis in the same cells, it is very likely that the synthesis of those cytokines was also decreased since the expression of transcription factor GATA-3, which

drives the production of type 2 cytokines[45], in Th2 cells was significantly lower in C5 inhibited mice. Nonetheless, the extracellular release of IL-5 and IL-13 in bronchoalveolar fluid and in the supernatant of restimulated mediastinal lymph nodes was similar, thus indicating that other sources for these cytokines are in play. It is conceivable that ILC2s are responsible as they are well established producers of IL-5 and IL-13[46]. The fact that their numbers (and probably function) were not affected by C5 inhibition further supports that notion. Lung pathology and mucus production in the airways showed no apparent changes, which can be explained by a sustained IL-13 production. Whether and to which extent C5 or C5a signaling blockade influences type 2 inflammation remains under debate. Some groups[40,42] reported significant attenuation whereas others[47] did not observe reduced inflammation. The outcome seems to be variable depending on the model and the designated target (C5 or C5a receptor) of the antagonist. Interestingly, airway hyperresponsiveness was alleviated in all studies that examined the effect of C5 signaling inhibition during the challenge phase. This confirms the notion that airway hyperresponsiveness and airway inflammation are two separate phenomena in asthma[48] and suggests that despite its contribution, a type 2 response is not necessarily the key factor in airway hyperresponsiveness. A growing body of evidence has made clear that airway hyperresponsiveness is a hallmark feature in asthma with complex underlying mechanisms involving smooth muscles, structural airway remodeling, nerve stimulation and a plethora of inflammatory mediators[49,50]. The exact mechanism by which C5 inhibition attenuated airway hyperresponsiveness in our study remains elusive. These results suggest that blocking C5 in established asthma (as is the case in adult asthma patients) could exert beneficial effects. The therapeutic potential of C5a signaling inhibition has been recognized and tested in asthma patients with inconclusive results so far[51]. More investigations addressing both inflammatory and clinical parameters in carefully selected patients are warranted to determine the value of C5 inhibition as treatment for asthma patients.

Mast cells have been implicated in the pathogenesis of asthma due to their ability to secrete pro-inflammatory and bronchoconstrictive mediators[52]. The effector function of mast cells are closely related to the binding of IgE and have been shown to play an important role in the early phase of asthma. We aimed to investigate the role of mast cells in HDM induced airway inflammation in **chapter 7.** We observed that mast cell deficiency in HDM challenged mice reduced airway eosinophilia and eosinophil specific chemokine (eotaxin-1) levels. The underlying mechanism of this finding is not well understood. Mast cell derived

histamine can induce eotaxin release from endothelial cells[53] and may partially explain decreased eotaxin concentrations, resulting in reduced eosinophil chemotaxis in mast cell deficient mice. In the recent years, knowledge about the role of the 'newly' discovered ILCs has vigorously expanded. New evidence unveiled that not only mast cells but also ILCs are diminished in c-kit deficient mice[54,55]. We have not measured ILCs in this study and type 2 cytokines in BAL fluid were below detection, precluding a full assessment of the type 2 response. Insight in the type 2 response could have provided a possible explanation for the reduction in airway eosinophils and plasma IgE concentrations in c-kit deficient mice after HDM challenge. IL-5 is an essential cytokine for the maturation, migration and survival of eosinophils[46] and IL-4 is essential for IgE class switch in B-cells[56]. Our data did not support suppressed IL-13 production in c-kit mice since airway mucus production appeared to be unaltered. In addition, HDM induced lung pathology was not affected in c-kit mice, arguing against a significantly attenuated (type 2) inflammatory response.

The role of the contact system in asthma

In chapter 8 and 9 we addressed the role of the contact system in allergic asthma. In **chapter 8** we investigated the role of factor XI (FXI) and FXII deficiency in allergic asthma using the HDM induced asthma mouse model. FXI appeared to have a beneficial role in allergic asthma by keeping a selective part of type 2 inflammation at bay: FXI deficient mice showed a marked increase in lung eosinophil numbers. This could be explained in part by enhanced maturation, survival (reflected by increased IL-5 levels) and chemotaxis (reflected by enhanced eotaxin-1 levels). It is challenging to pinpoint the underlying mechanism responsible for this interesting and relevant finding. FXI activation initiates the coagulation system via the intrinsic pathway. In our model HDM induced coagulation and fibrinolysis, yet FXI deficiency did not influence these responses, suggesting that FXI mediated suppression of lung eosinophilia did not occur via an effect on coagulation activation. The effect of FXI on eosinophil recruitment seems to occur independently of FXII activation since it was absent in FXII deficient mice. Our data suggest that FXI has a, so far unidentified, modulatory role in the course of allergic inflammation. On the other hand, these data imply that FXI does not simply impair the whole set of type 2 effector cell functions since other type 2 (dependent) responses were not affected in FXI deficient mice.

The kallikrein-kinin system refers to the cleavage of high-molecular-weight-kininogen by kallikrein to liberate bradykinin, which is part of the contact system[57]. **Chapter 9** describes the effect of kininogen deficiency and depletion on hallmarks of asthma in the HDM induced mouse model. We observed that kininogen deficiency throughout the course of HDM sensitization and challenge did not modulate airway inflammation. This result is in accordance with our findings in FXII deficient mice (chapter 8), indicating that contact system activation lacks significant impact on HDM induced inflammatory responses. Kininogen deficiency markedly alleviated HDM induced airway hyperresponsiveness. Additionally, we depleted kininogen in mice only during the challenge phase to mimic a therapeutically relevant approach and, in complete agreement with kininogen deficiency, observed attenuation of airway hyperresponsiveness with unaltered inflammatory markers, thereby excluding a sensitization dependent dual role for kininogen such as demonstrated for C5a in allergen induced asthma. Further research is warranted to elucidate the mechanisms by which kininogen aggravates airway hyperresponsiveness. The discovery of the paucigranulocytic asthma phenotype has fueled interest in inflammation independent airway obstruction[58]. For patients with this type of asthma, conventional (and probably new emerging biological) drugs are ineffective as they all aim to mitigate inflammation driven symptoms. In that regard, kininogen depletion could provide beneficial effects, which should encourage clinical testing. At the time of this writing, a direct interaction between kininogens and immune or parenchymal cells has not been documented. Therefore, we hypothesize that kininogen derived bradykinin is the key driver for the observed airway responses. Bradykinin has been reported to induce bronchoconstriction via signaling through its receptors (brB1 and brB2) on smooth muscles and sensory neurons[59]. Furthermore, bradykinin receptor antagonists have shown their merit in alleviating bradykinin induced bronchoconstriction in both animals[60,61] and humans[62,63]. To date, only one phase II clinical trial, addressing the role of bradykinin receptor antagonists in asthma, has been conducted. This study investigated the efficacy of Icatibant, a bradykinin B2 receptor antagonist, in moderate to severe asthma patients reporting inconclusive results[64]. Meanwhile new generations of bradykinin receptor antagonists have been developed with allegedly higher specificity and efficacy[50]. Convincing data from preclinical studies justify and warrant clinical trials in asthma patients using bradykinin receptor antagonists to determine their therapeutic potential.

Concluding remarks

This thesis has extended current knowledge about the roles of the complement and contact system in the context of allergen induced asthma. We have shown a complex role for the complement system in asthma associated type 2 airway inflammation; though its activation influences selective elements of the inflammatory response, redundant counteracting mechanisms may simultaneously occur. Our findings suggest that inhibiting the contact system could yield beneficial effects on features of asthma that are independent of inflammation. Better understanding of these processes are warranted to be able to determine the full potential of both systems as potential targets for asthma treatment.

REFERENCES

1. Woolcock A, Rubinfeld AR, Seale JP, et al. Thoracic society of Australia and New Zealand. Asthma management plan, 1989. *Med J Aust.* 1989;151(11-12):650-653.
2. Warner JO, Gotz M, Landau LI, et al. Management of asthma: a consensus statement. *Arch Dis Child.* 1989;64(7):1065-1079.
3. Hargreave FE, Dolovich J, Newhouse MT. The assessment and treatment of asthma: a conference report. *J Allergy Clin Immunol.* 1990;85(6):1098-1111.
4. Guidelines for management of asthma in adults: I--Chronic persistent asthma. Statement by the British Thoracic Society, Research Unit of the Royal College of Physicians of London, King's Fund Centre, National Asthma Campaign. *BMJ.* 1990;301(6753):651-653.
5. Israel E, Reddel HK. Severe and Difficult-to-Treat Asthma in Adults. *N Engl J Med.* 2017;377(10):965-976.
6. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention (GINA). In: Available at https://ginasthma.org; 2019.
7. McCracken JL, Veeranki SP, Ameredes BT, Calhoun WJ. Diagnosis and Management of Asthma in Adults: A Review. *JAMA.* 2017;318(3):279-290.
8. Anderson GP. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet.* 2008;372(9643):1107-1119.
9. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. *Lancet.* 2018;391(10122):783-800.
10. Eger KA, Bel EH. The emergence of new biologics for severe asthma. *Curr Opin Pharmacol.* 2019;46:108-115.
11. Hekking PP, Loza MJ, Pavlidis S, et al. Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J Allergy Clin Immunol.* 2018;141(4):1280-1290.
12. Richards LB, Neerincx AH, van Bragt J, Sterk PJ, Bel EHD, Maitland-van der Zee AH. Biomarkers and asthma management: analysis and potential applications. *Curr Opin Allergy Clin Immunol.* 2018;18(2):96-108.
13. Moore WC, Meyers DA, Wenzel SE, et al. Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med.* 2010;181(4):315-323.
14. McDowell PJ, Heaney LG. Different endotypes and phenotypes drive the heterogeneity in severe asthma. *Allergy.* 2019.
15. Peat JK, Tovey E, Toelle BG, et al. House dust mite allergens. A major risk factor for childhood asthma in Australia. *Am J Respir Crit Care Med.* 1996;153(1):141-146.
16. Carnes MU, Hoppin JA, Metwali N, et al. House Dust Endotoxin Levels Are Associated with Adult Asthma in a U.S. Farming Population. *Ann Am Thorac Soc.* 2017;14(3):324-331.
17. Hammad H, Lambrecht BN. Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity.* 2015;43(1):29-40.
18. Khor YH, Teoh AK, Lam SM, et al. Increased vascular permeability precedes cellular inflammation as asthma control deteriorates. *Clin Exp Allergy.* 2009;39(11):1659-1667.
19. Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med.* 1997;156(1):229-233.
20. Brown JR, Kleimberg J, Marini M, Sun G, Bellini A, Mattoli S. Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients after allergen inhalation. *Clin Exp Immunol.* 1998;114(2):137-146.
21. Yoshikawa S, Kayes SG, Parker JC. Eosinophils increase lung microvascular permeability via the peroxidase-hydrogen peroxide-halide system. Bronchoconstriction and vasoconstriction unaffected by eosinophil peroxidase inhibition. *Am Rev Respir Dis.* 1993;147(4):914-920.
22. Fujimoto K, Parker JC, Kayes SG. Activated eosinophils increase vascular permeability and resistance in isolated perfused rat lungs. *Am Rev Respir Dis.* 1990;142(6 Pt 1):1414-1421.

23. Brottman GM, Regelmann WE, Slungaard A, Wangensteen OD. Effect of eosinophil peroxidase on airway epithelial permeability in the guinea pig. *Pediatr Pulmonol.* 1996;21(3):159-166.
24. Minnicozzi M, Gleich GJ, Duran WN, Egan RW. Increased microvascular permeability induced by eosinophil proteins. *Int Arch Allergy Immunol.* 1995;107(1-3):348.
25. Fricker M, Gibson PG. Macrophage dysfunction in the pathogenesis and treatment of asthma. *Eur Respir J.* 2017;50(3).
26. Draijer C, Peters-Golden M. Alveolar Macrophages in Allergic Asthma: the Forgotten Cell Awakes. *Curr Allergy Asthma Rep.* 2017;17(2):12.
27. Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. Pulmonary macrophages: key players in the innate defence of the airways. *Thorax.* 2015;70(12):1189-1196.
28. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology.* 2006;11(1):54-61.
29. de Boer JD, Berger M, Majoor CJ, et al. Activated protein C inhibits neutrophil migration in allergic asthma: a randomised trial. *Eur Respir J.* 2015;46(6):1636-1644.
30. Yang J, van Engelen TSR, Haak BW, et al. Effect of C1-inhibitor in adults with mild asthma: A randomized controlled trial. *Allergy.* 2019.
31. Reynier F, de Vos AF, Hoogerwerf JJ, et al. Gene expression profiles in alveolar macrophages induced by lipopolysaccharide in humans. *Mol Med.* 2012;18:1303-1311.
32. Zhang X, Kohl J. A complex role for complement in allergic asthma. *Expert Rev Clin Immunol.* 2010;6(2):269-277.
33. Laumonnier Y, Wiese AV, Figge J, Karsten C. Regulation and function of anaphylatoxins and their receptors in allergic asthma. *Mol Immunol.* 2017;84:51-56.
34. Kolev M, Le Friec G, Kemper C. Complement--tapping into new sites and effector systems. *Nat Rev Immunol.* 2014;14(12):811-820.
35. Strainic MG, Liu J, Huang D, et al. Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells. *Immunity.* 2008;28(3):425-435.
36. DiScipio RG, Daffern PJ, Jagels MA, Broide DH, Sriramarao P. A comparison of C3a and C5a-mediated stable adhesion of rolling eosinophils in postcapillary venules and transendothelial migration in vitro and in vivo. *J Immunol.* 1999;162(2):1127-1136.
37. Daffern PJ, Pfeifer PH, Ember JA, Hugli TE. C3a is a chemotaxin for human eosinophils but not for neutrophils. I. C3a stimulation of neutrophils is secondary to eosinophil activation. *J Exp Med.* 1995;181(6):2119-2127.
38. Lee JJ, Jacobsen EA, Ochkur SI, et al. Human versus mouse eosinophils: "that which we call an eosinophil, by any other name would stain as red". *J Allergy Clin Immunol.* 2012;130(3):572-584.
39. Dyer KD, Percopo CM, Xie Z, et al. Mouse and human eosinophils degranulate in response to platelet-activating factor (PAF) and lysoPAF via a PAF-receptor-independent mechanism: evidence for a novel receptor. *J Immunol.* 2010;184(11):6327-6334.
40. Kohl J, Baelder R, Lewkowich IP, et al. A regulatory role for the C5a anaphylatoxin in type 2 immunity in asthma. *J Clin Invest.* 2006;116(3):783-796.
41. Drouin SM, Sinha M, Sfyroera G, Lambris JD, Wetsel RA. A protective role for the fifth complement component (c5) in allergic airway disease. *Am J Respir Crit Care Med.* 2006;173(8):852-857.
42. Baelder R, Fuchs B, Bautsch W, et al. Pharmacological targeting of anaphylatoxin receptors during the effector phase of allergic asthma suppresses airway hyperresponsiveness and airway inflammation. *J Immunol.* 2005;174(2):783-789.
43. Staab EB, Sanderson SD, Wells SM, Poole JA. Treatment with the C5a receptor/CD88 antagonist PMX205 reduces inflammation in a murine model of allergic asthma. *Int Immunopharmacol.* 2014;21(2):293-300.
44. Wiese AV, Ender F, Quell KM, et al. The C5a/C5aR1 axis controls the development of experimental allergic asthma independent of LysM-expressing pulmonary immune cells. *PLoS One.* 2017;12(9):e0184956.

45. Zheng W, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell.* 1997;89(4):587-596.

46. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* 2015;16(1):45-56.

47. Peng T, Hao L, Madri JA, et al. Role of C5 in the development of airway inflammation, airway hyperresponsiveness, and ongoing airway response. *J Clin Invest.* 2005;115(6):1590-1600.

48. Brusasco V, Crimi E, Pellegrino R. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax.* 1998;53(11):992-998.

49. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. *Clin Exp Allergy.* 2015;45(4):706-719.

50. Abraham WM, Scuri M, Farmer SG. Peptide and non-peptide bradykinin receptor antagonists: role in allergic airway disease. *Eur J Pharmacol.* 2006;533(1-3):215-221.

51. Smith SG, Watson B, Clark G, Gauvreau GM. Eculizumab for treatment of asthma. *Expert Opin Biol Ther.* 2012;12(4):529-537.

52. Mendez-Enriquez E, Hallgren J. Mast Cells and Their Progenitors in Allergic Asthma. *Front Immunol.* 2019;10:821.

53. Menzies-Gow A, Ying S, Phipps S, Kay AB. Interactions between eotaxin, histamine and mast cells in early microvascular events associated with eosinophil recruitment to the site of allergic skin reactions in humans. *Clin Exp Allergy.* 2004;34(8):1276-1282.

54. Brown MA, Weinberg RB. Mast Cells and Innate Lymphoid Cells: Underappreciated Players in CNS Autoimmune Demyelinating Disease. *Front Immunol.* 2018;9:514.

55. Fonseca W, Rasky AJ, Ptaschinski C, et al. Group 2 innate lymphoid cells (ILC2) are regulated by stem cell factor during chronic asthmatic disease. *Mucosal Immunol.* 2019;12(2):445-456.

56. Gour N, Wills-Karp M. IL-4 and IL-13 signaling in allergic airway disease. *Cytokine.* 2015;75(1):68-78.

57. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost.* 2016;14(1):28-39.

58. Tliba O, Panettieri RA, Jr. Paucigranulocytic asthma: Uncoupling of airway obstruction from inflammation. *J Allergy Clin Immunol.* 2019;143(4):1287-1294.

59. Ricciardolo FLM, Folkerts G, Folino A, Mognetti B. Bradykinin in asthma: Modulation of airway inflammation and remodelling. *Eur J Pharmacol.* 2018;827:181-188.

60. Ellis KM, Cannet C, Mazzoni L, Fozard JR. Airway hyperresponsiveness to bradykinin induced by allergen challenge in actively sensitised Brown Norway rats. *Naunyn Schmiedebergs Arch Pharmacol.* 2004;369(2):166-178.

61. Folkerts G, Vlieger JW, de Vries A, et al. Virus- and bradykinin-induced airway hyperresponsiveness in guinea pigs. *Am J Respir Crit Care Med.* 2000;161(5):1666-1671.

62. Ricciardolo FL, Timmers MC, Geppetti P, et al. Allergen-induced impairment of bronchoprotective nitric oxide synthesis in asthma. *J Allergy Clin Immunol.* 2001;108(2):198-204.

63. Ricciardolo FL, Di Stefano A, Silvestri M, et al. Exhaled nitric oxide is related to bronchial eosinophilia and airway hyperresponsiveness to bradykinin in allergen-induced asthma exacerbation. *Int J Immunopathol Pharmacol.* 2012;25(1):175-182.

64. Akbary AM, Wirth KJ, Scholkens BA. Efficacy and tolerability of Icatibant (Hoe 140) in patients with moderately severe chronic bronchial asthma. *Immunopharmacology.* 1996;33(1-3):238-242.

10

Nederlandse samenvatting

Astma is een ziekte van de luchtwegen waarbij er meestal sprake is van een chronische ontsteking. Deze ziekte wordt verder gekenmerkt door reversibele luchtwegvernauwing, luchtweg hyperreactiviteit en overmatig slijmproductie. Wereldwijd zijn meer dan 300 miljoen mensen van alle leeftijden gediagnosticeerd met astma, waarbij er sprake is van een toenemende incidentie en prevalentie. De behandeling voor deze ongeneesbare ziekte bestond jarenlang louter uit inhalatiecorticosteroïden en luchtwegverwijders. Hoewel deze behandeling zeer effectief is voor een groot deel van de patiënten, is het voor een substantieel groep patiënten onvoldoende om hun ziekte onder controle te krijgen. Recentelijk hebben nieuwe wetenschappelijke onderzoeken aangetoond dat de onderliggende ontstekingsreacties wezenlijk kunnen verschillen in patiënten met vergelijkbare astma gerelateerde symptoommanifestatie. Een gerichte behandeling van een specifieke ontstekingsreactie blijkt in correct geselecteerde patiënten buitengewoon effectief om luchtwegontsteking te remmen en daarmee astmaklachten te verminderen. De introductie van 'biologicals', antilichamen die specifieke cytokinen onschadelijk maken, staat symbool voor deze hoopgevende benadering en spoort toekomstige onderzoeken aan nieuwe mechanismen te identificeren die relevant zijn voor de pathofysiologie van astma.

Dit proefschrift richt zich vooral op de rol van het complement- en het contactsysteem op type 2 inflammatie, één van de ontstekingsreacties die geassocieerd is met astma. Het complement- en contactsysteem zijn beide netwerken van eiwitten in het bloed die, eenmaal geactiveerd, het immuunsysteem beïnvloeden. Eerdere preklinische onderzoeken hebben aangetoond dat beide systemen betrokken zijn bij astma gerelateerde inflammatoire processen. We hebben getracht nieuwe inzichten te verwerven met het verrichten van onderzoeken in astmapatiënten; aangevuld met experimenten in astma muismodellen, die ons in staat stellen om onderliggende mechanismen beter te begrijpen.

Hoofdstukken 2 en 4 beschrijven het effect van C1-inhibitor op allergeen geïnduceerde ontstekingsprocessen in de longen. C1-inhibitor is een eiwit in het bloed dat een remmende werking heeft op het complement- en contactsysteem. Het is tevens beschikbaar als medicijn en wordt gegeven aan mensen die, als gevolg van C1-inhibitor te kort, ernstige zwellingen in de huid (angio-oedeem) ervaren. In **hoofdstuk 2** tonen we aan dat een profylactische behandeling met C1-inhibitor in patiënten met een milde vorm van astma leidt tot de preventie van slijmvliesbarrière beschadiging, een verschijnsel dat optreedt in de luchtwegen van astmapatiënten na allergeencontact. In een klinisch

onderzoek genaamd CAST-studie, hebben we hiervoor 24 volwassen patiënten geselecteerd die in gelijke aantallen zijn gerandomiseerd in een placebo of C1-inhibitor behandeling groep. In de luchtwegen van deze patiënten werden vervolgens een ontstekingsreactie opgewekt middels intrabronchiale provocatie met huisstofmijt (HDM) en lipopolysaccharide (LPS). C1-inhibitor remde de complementsysteemactivatie die optreedt als gevolg van de allergeenprovocatie. Daarnaast tonen we aan dat C1-inhibitor een beschermd effect had op de slijmvliesbarrière maar niet in staat was om de allergische inflammatoire respons te remmen. Alveolaire macrofagen zijn immuuncellen die in grote aantallen aanwezig zijn in de luchtwegen. Ze kunnen de ontstekingsreactie bij astmapatiënten beïnvloeden. In **hoofdstuk 3** hebben we alveolaire macrofagen verkregen uit de luchtwegen van deelnemende astmapatiënten uit de CAST-studie. Vervolgens hebben we gekeken naar veranderingen van genen in macrofagen die wel of niet in contact zijn geweest met HDM en LPS. Het blijkt dat een groot aantal genen die geassocieerd zijn met inflammatoire processen verhoogd tot expressie komen in alveolaire macrofagen die blootgesteld zijn aan allergenen. In **hoofdstuk 4** onderzochten we het effect van C1-inhibitor in een astma muismodel. We vonden een vergelijkbaar resultaat als in het onderzoek met astmapatiënten, namelijk dat C1-inhibitor geen duidelijk remmend effect heeft op de allergische ontstekingsprocessen in de luchtwegen.

Hoofdstukken 5 en 6 beschrijven de rol van anafylatoxinen, activatieproducten van het complement systeem op astma gerelateerde ontstekingscellen en ontstekingsreacties. Eosinofielen zijn belangrijke ontstekingscellen die frequent worden waargenomen in de luchtwegen van astmapatiënten. Recent ontwikkelde geneesmiddelen tegen interleukine (IL)-5 reduceren het aantal eosinofielen sterk en zijn zeer effectief gebleken in de behandeling van astmaklachten. Het is daarom belangrijk om nieuwe aangrijpingspunten te vinden die de functies van eosinofielen kunnen beïnvloeden. In **hoofdstuk 5** hebben we gekeken of anafylatoxine C3a signalering een chemotactisch effect heeft op eosinofielen. We hebben met *in vivo* en *ex vivo* experimenten laten zien dat C3a signalering geen directe rol speelt in de migratie en chemotaxis van eosinofielen. C3a deficiënte muizen tonen verminderde eosinofiel aantallen in hun luchtwegen na allergeenprovocatie, wat suggereert dat C3a dit op een indirecte manier bewerkstelligt. De anafylatoxine C5a speelt een merkwaardige dubbele rol in de pathogenese van allergische astma. Verschillende studies hebben aangetoond dat C5a het immuunsysteem ondersteunt om de allergische inflammatoire respons in toom te houden vóór allergeensensitisatie, terwijl na sensitisatie C5a acteert als een pro-inflammatoir signaal. In **hoofdstuk 6** onderzochten

we nader hoe C5a luchtweginflammatie beïnvloedt. We observeerden dat C5a een stimulerend effect had op Th2 cellen, maar niet op ILC2s. Dit verklaart deels onze bevinding dat de type 2 inflammatie slechts deels is onderdrukt als gevolg van C5 blokkade na sensitisatie. We vonden een evident verminderde luchtweghyperreactiviteit als gevolgd van C5 remming. Dit wijst erop dat C5 remming potentieel een kandidaat is voor verder onderzoek in astmapatiënten.

Mestcellen zijn een van de immuuncellen die betrokken zijn bij het initiëren van een allergische reactie en geactiveerd kunnen worden door C5a. Deze cellen worden in de vroege fase na allergeencontact geactiveerd waarna ze mediatoren afgeven die astma gerelateerde symptomen kunnen uitlokken. In **hoofdstuk 7** bestudeerden we de rol van mestcellen in muizen die gesensitiseerd zijn voor huisstofmijt en vonden dat selectieve immuunresponsen verminderd waren in mestcel deficiënte muizen. Dit leidde echter niet tot veranderingen in de allergeen gedreven longpathologie en slijmproductie in de luchtwegen. Hierdoor is het niet duidelijk vast te stellen welke rol mestcellen precies spelen in het moduleren van de allergische ontstekingsreactie.

In de hoofdstukken 8 en 9 presenteren we onderzoeken met de vraag of het contactsysteem een rol speelt in de pathofysiologie van astma. Hierbij hebben we gekeken naar verschillende modulaties in het contact systeem en welke gevolgen deze modulaties hebben op belangrijke uitkomsten die gerelateerd zijn aan astmapathologie. In **hoofdstuk 8** analyseerden we FXI en FXII deficiënte muizen in het huisstofmijt gedreven astma muismodel. FXI bleek luchtwegeosinofilie in toom te houden door signaaleiwitten (chemokines) te verminderen die de aanwezigheid van eosinofiel kunnen bevorderen. FXII had geen evident effect op allergisch inflammatoire parameters. Een ander component van het contact systeem is high-molecular-weight-kininogen (HMWK). Het enzym (kallikrein) kan via verschillende routes geactiveerd worden en knipt het substraat HMWK waarbij een eiwit (bradykinine) vrijkomt dat ontstekingsreacties uitlokt. In **hoofdstuk 9** onderzochten we wat het effect is van HMWK deficiëntie op de luchtweginflammatie en luchtweghyperreactiviteit in huisstofmijt gesensitiseerde muizen. We vonden net als in hoofdstuk 8 dat het contactsysteem geen invloed heeft op de luchtweginflammatie. Verder toonden we aan dat HMWK bijdraagt aan allergeen geïnduceerde luchtweghyperreactiviteit. Een ander experiment, waarbij HMWK in reeds gesensitiseerde muizen werd geremd, gaf dezelfde resultaten weer.

Dit proefschrift heeft de kennis over het complement- en contactsysteem in de context van allergeen gedreven astma verder uitgebreid. De complexiteit waarmee het complement system luchtweginflammatie beïnvloedt, is uit de reeks van experimenten in dit proefschrift duidelijk gebleken. Hoewel complement systeem activatie bepaalde immuun responsen moduleert, worden deze effecten grotendeels gecompenseerd of tegengewerkt door andere immuunmechanismen. Het contactsysteem blijkt een effect te hebben op de luchtweghyperreactiviteit. Daarnaast wordt het feit dat de luchtweghyperreactiviteit deels onafhankelijk is van de luchtwegontsteking meermaals onderstreept. Resultaten uit deze preklinische onderzoeken rechtvaardigen verdere evaluatie van deze systemen in klinische studies.

ANTARCTICA

Addendum

A

LIST OF PUBLICATIONS

Yang J, Scicluna BP, Van Engelen TSR, Bonta PI, Majoor CJ, Van 't Veer C, De Vos AF, Bel EH, Van der Poll T. Transcriptional changes in alveolar macrophages from adults with asthma after allergen challenge. (submitted)

Yang J, Van Engelen TSR, Haak BW, Bonta PI, Majoor CJ, Van 't Veer C, De Vos AF, Kemper EM, Lutter R, Van Mierlo G, Zeerleder SS, Bel EH, Van der Poll T. Effect of C1-inhibitor in adults with mild asthma: a randomised controlled trial. Allergy, 2019

Yang J, van 't Veer C, ten Brink MS, de Vos AF, van der Poll T. C3a signaling is not involved in eosinophil migration during experimental allergic lung inflammation in mice. Allergy. 2019

Yang J, Ramirez Moral I, Van 't Veer C, de Vos AF, de Beer R, Roelofs JJTH, Morgan BP, van der Poll T. Complement factor C5 inhibition reduces type 2 responses without affecting group 2 innate lymphoid cells in a house dust mite induced murine asthma model. Respiratory Research. 2019

Yang J, van 't Veer C , Roelofs J.J.T.H, van Heijst J.W.J, de Vos A.F, McCrae K.R, Revenko A.S, Crosby J, van der Poll T. Kininogen deficiency or depletion attenuates airway hyperresponsiveness independent of pulmonary inflammation in a house dust mite induced murine asthma model. Am J Physiol Lung Cell Mol Physiol. 2019

Ding C, Scicluna BP, Stroo I, **Yang J,** Roelofs JJ, de Boer OJ, de Vos AF, Nürnberg P, Revenko AS, Crosby J, Van't Veer C, van der Poll T. Prekallikrein inhibits innate immune signaling in the lung and impairs host defense during pneumosepsis in mice. Journal of Pathology 2020

Ding C, **Yang J,** Van't Veer C, van der Poll T. Bradykinin receptor deficiency or antagonism do not impact the host response during gram-negative pneumonia-derived sepsis. Intensive Care Med Exp. 2019

Stroo I, Ding C, Novak A, **Yang J,** Roelofs JJTH, Meijers JCM, Revenko AS, van 't Veer C, Zeerleder S, Crosby JR, van der Poll T. Inhibition of the extrinsic or intrinsic coagulation pathway during pneumonia-derived sepsis. Am J Physiol Lung Cell Mol Physiol. 2018

Stroo I, **Yang J,** Anas AA, de Boer JD, van Mierlo G, Roem D, Wouters D, Engel R, Roelofs JJTH, van 't Veer C, van der Poll T, Zeerleder S. Human plasma-derived C1 esterase inhibitor concentrate has limited effect on house dust mite-induced allergic lung inflammation in mice. Plos One. 2017

Stroo I, **Yang J,** de Boer JD, Roelofs JJ, van 't Veer C, Castellino FJ, Zeerleder S, van der Poll T. Factor XI deficiency enhances the pulmonary allergic response to house dust mite in mice independent of factor XII. Am J Physiol Lung Cell Mol Physiol. 2017

Anas AA, **Yang J,** Daan de Boer J, Roelofs JJ, Hou B, de Vos AF, van der Poll T. General, but not myeloid or type II lung epithelial cell, myeloid differentiation factor 88 deficiency abrogates house dust mite induced allergic lung inflammation. Clin Exp Immunol. 2017

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. 2015.

de Boer JD, **Yang J,** van den Boogaard FE, Hoogendijk AJ, de Beer R, van der Zee JS, Roelofs JJ, van 't Veer C, de Vos AF, van der Poll T. Mast cell-deficient kit mice develop house dust mite-induced lung inflammation despite impaired eosinophil recruitment. J Innate Immun. 2014.

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ABOUT THE AUTHOR

Junjie (Jack) Yang was born in Guangzhou, China. At young age, he moved to the Netherlands where he graduated from Johan van Oldenbarnevelt gymnasium in Amersfoort (2007). Following high school, he moved to the Dutch capital and studied Medicine at the University of Amsterdam. As second and third year student, he was introduced in the field of biomedical science through two internships at the department of Center for Experimental and Molecular Medicine (CEMM) supervised by dr. Daan de Boer and dr. Kees van ‘t Veer. During this time, he contributed to projects which later on were published in peer reviewed journals. Following this scientific intermezzo, he started a two years lasting clinical rotation and as part of these clinical internships, he had the opportunity to work and learn at the pediatrics department in Manipal, India. After graduating from Medical school, he briefly worked as resident at the department of internal medicine in Tergooi ziekenhuis, Hilversum. In 2014, he started his PhD project on the ‘role of the complement and contact systems in asthma’, supervised by prof. dr. Tom van der Poll, prof. dr. Liesbeth Bel and dr. Kees van 't Veer. In October 2019 Jack returned to healthcare as Medical doctor. Determined to become a pulmonologist, he has started working as resident at the pulmonology department in Noordwest Ziekenhuis, Alkmaar.

PHD PORTFOLIO

Courses	Year	ECTS
Basic course legislation and Organization (BROK)	2015	1
Basic laboratory Safety	2015	1
Laboratory Animals	2015	2.5
Advanced Immunology	2015	2.5
Practical biostatistics	2015	1
Advanced qPCR	2016	0.5
Scientific Writing	2018	1
Seminars and Masterclasses		
Journal Club	2014-2019	2
CEMMposium	2014-2018	1
CEMMinars	2014-2018	1
Retreat Infection and Immunity	2014-2018	2
(Inter)national conferences		
New Frontiers in Innate Immunity and Inflammation, Cluj-Napoca	2018	1
European Academy of Allergy and Clinical Immunology congress, Helsinki	2017	1
17th Congress of European Shock Society, Paris	2017	1
EMBO conference Innate Lymphoid cells, Berlin	2016	1
Congress European Society of Thrombosis and Haemostasis, the Hague	2016	1
International Society of Thrombosis and Haemostasis, Toronto	2015	1
Poster presentation		
A randomised controlled trial of C1-esterase inhibitor in adults with mild asthma	2018	0.5
Inhibiting activation of complement C5 attenuates airway hyperresponsiveness and allergic lung inflammation in a house dust mite induced model	2017	0.5

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momenten die we samen beleefd hebben. Intussen ben je helemaal gesettled op je nieuwe baan als klinisch chemicus in spe. Ik hoop dat je nog eens terugkomt naar de bewoonde wereld als je straks uit Amsterdam verhuist.

Lieve collega's van het NWZ,
Wat fijn dat jullie me meteen zo geweldig in jullie groep hebben opgenomen en geduldig ingewerkt! Ik weet dan ook zeker dat we sterker uit de COVID-19 pandemie gaan komen. Ik hoop nog veel van jullie te leren in de toekomst.

Ooit begonnen als een stel kattenkwaad uithalende mafkezen in een studentenflat in Diemen. Intussen hebben we allen ons pad gevonden. Tijd heeft ons gelukkig niet ver uit elkaar gedreven. Die mannenavonden houden stand! Daniël, Hessel, Martijn, bedankt voor jullie vriendschap!

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Mijn SKØLL maten: Jan, Maarten, Eelco, Pascal, Jasper, Duncan, Sjoerd, Eva en Döpp. Na 4 LPD's kan ik nu eindelijk zeggen: het is af! En nee, het heeft niet "tot in de MILJOENEN" jaren geduurd.

Dani, ik ben blij dat je de cursus Nederlands hebt gevolgd en Iris hebt ontmoet, anders heb ik nu geen squashmaatje gehad. Roy, je bent veel meer dan mijn squashmaatje en dat weet je. We zijn de afgelopen jaren nauw naar elkaar gegroeid en het is fijn om zo'n goede vriend als jij te hebben. Helaas moeten we een nieuwe squashlocatie vinden nu jij naar Westzaan verhuist. Kan je er niet één in je tuin bouwen, naast het voetbalveldje voor Alex?! Fijn dat jullie onze vrienden zijn en ik kijk uit naar wat er nog komt.

Liebe Schwiegerfamilie, danke, dass ihr mich so herzlich in eure Familie aufgenommen habt.

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Kleine broer, ik ben er niet altijd, maar weet dat ik er altijd voor je zal zijn.

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Pauline en Iris, jullie hebben een speciale plek in mijn hart en verdienen een speciale plek in dit boekje.

Pauline, mijn kleine schat! Jij bent het zonnetje in mijn leven. De zomer komt eraan, we gaan lekker veel buiten spelen.

Iris, lieverd, het is ons gelukt! Onze PhD babies zijn er! Jij en ik zijn jaren geleden gelijktijdig begonnen aan een promotie avontuur en nu zijn we tegelijk klaar, wat een droomscenario! We hebben elkaar er doorheen geloodst met veel liefde. Ik hou zielsveel van je en kijk uit naar alle avonturen die de toekomst ons gaat brengen!