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

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Conflict of interest

The authors have no conflict of interest in relation to this work

Key words

Asthma, allergic airway inflammation, complement system, C1-inhibitor, randomised controlled trial

To the Editor:

Several newly approved monoclonal antibodies targeting type 2 inflammation have shown remarkable beneficial effects in patients with severe asthma. Biologics directed against IL-5 (mepolizumab, reslizumab), IL-5 receptor (benralizumab) or IL-4 receptor α (dupilumab) have shown to reduce asthma exacerbation rate by about 50%^{1,2}. Though promising, these drugs are unable to completely alleviate inflammation induced asthma symptoms. Moreover, a substantial subset of patients without a pronounced type 2 airway inflammation do not benefit from the currently available biologics. Therefore, novel anti-inflammatory treatments targeting other relevant asthma associated pathways are still warranted. In recent years, the complement system has been implicated in the pathogenesis of type 2 asthma³. Elevated levels of anaphylatoxins, activation products of the complement system, have been found in the airways of asthma patients following local allergen provocation⁴. Functional roles for the anaphylatoxins in asthma have been established in experimental studies in mice, showing that these proinflammatory mediators act synergistically and drive allergic inflammation³. C1-inhibitor (C1-INH) is an endogenous protein with a pivotal regulatory function in the complement system by inhibiting both the classical and lectin pathway. We hypothesized that C1-INH administration inhibits complement activation and attenuates allergen-induced airway eosinophilia in patients with mild asthma.

In this randomised, double-blind, placebo-controlled, parallel study 24 adults with asthma and house dust mite (HDM) allergy received a continuous intravenous infusion with human plasma derived C1-INH 100 U·kg⁻¹·h⁻¹ or placebo followed after two hours by segmental challenge with HDM and Lipopolysaccharide (LPS) in one lung and saline in the contralateral lung as control. Bronchoalveolar lavage fluid was obtained seven hours after HDM/LPS or saline challenge. The primary outcome was influx of eosinophils and neutrophils, defined as number of cells/ml, into the bronchoalveolar space. Further details of the study design, subject selection criteria, bronchoalveolar lavage handling, assays and statistical analysis are described in the supplemental section. Baseline patient characteristics were similar across treatment groups (Table S1).

Two hours after the initiation of C1-INH infusion, median plasma C1-INH antigen concentrations were four times higher in C1-INH infused patients compared to vehicle infused controls (Fig S1A). Segmental HDM/LPS challenge resulted in increased C1-INH antigen levels in BALF compared to saline instillation in both treatment

groups (Fig S1C). C1-INH concentrations were higher in BALF from C1-INH infused patients compared to the placebo group. C1-INH activity levels in plasma and BALF were similar to C1-INH antigen concentrations (Fig S1B,D), indicating that C1-INH was biologically active.

HDM/LPS challenge induced elevated C4a concentrations compared to saline challenge in the placebo group (Fig S2A). In the C1-INH group BALF C4a levels were similar 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), C4 activation in the lung subsegment exposed to HDM/LPS was increased in patients infused with placebo but not in those infused with C1-INH (Fig S2B). We next measured the anaphylatoxin C3a, which is released following C3 cleaved activation⁵. Similar to C4a, HDM/LPS challenge increased BALF C3a in the placebo group, but not in the C1-INH treatment group (Fig S2C). In agreement, C3 activation products were elevated in the HDM/LPS challenged lung in patients infused with placebo but not in those administered with C1-INH (Fig S2D). These data indicate that C1-INH infusion prevents C4a and C3a generation in the airways upon a bronchial challenge with HDM/LPS.

HDM/LPS instillation augmented total cell counts in BALF compared to saline, partly as consequence of eosinophils and neutrophils influx (Fig 1A-C). Likewise, HDM/LPS challenge elevated CD4 T-cells, but did not alter the number of alveolar macrophages in BALF (Fig S3A,B). C1-INH did not modify this allergen-induced response. HDM/LPS also induced degranulation of eosinophils and neutrophils in the bronchoalveolar space (Fig S4A-D). These responses were not affected by C1-INH with the exception of lactoferrin release, which was inhibited by C1-INH (Fig S4B).

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 S2, Table S3). HDM/LPS induced increases in neutrophil chemoattractants such as interleukin (IL)-8, IL-1 β , tumor necrosis factor- α , macrophage inflammatory proteins 1 α and 1 β (Table S2). Likewise, eosinophil attractants eotaxin-1 and RANTES were increased upon HDM/LPS challenge. These responses were not influenced by C1-INH. HDM/LPS also induced the release of growth related oncogene- α , stromal cell-derived factor-1 α and IL-18 in the placebo group. Although statistically insignificant, these rises were also detected in the C1-INH group.

Beside the complement system, C1-INH is an important regulator of the kallikrein-kinin system due to its inhibitory effect on FXIIa and kallikrein activity⁶. Elevated levels of kallikrein-kinin system components have been documented in the airways of asthma patients⁷. 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.

Vascular leak often occurs as consequence of allergen induced inflammation in the airway of asthma patients⁸ and has been associated with the loss of asthma control⁹. We determined the quotients of albumin and α 2-macroglobulin levels in BALF and plasma (QA1b and QA2M, respectively) and the relative coefficient of excretion (QA2M/QA1b) as measures of the permeability of the blood–airway barrier⁴. Intrabronchial HDM/LPS challenge was associated with significant increase in QA1b (Fig 2A), QA2M (Fig 2B) and relative coefficient of excretion (Fig 2C) in the placebo group. These effects were abrogated in the C1-INH group (Fig 2A-C). Likewise, HDM/LPS induced elevated BALF IgM concentrations in placebo infused patients but not in C1-INH treated subjects (Fig 2D).

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.

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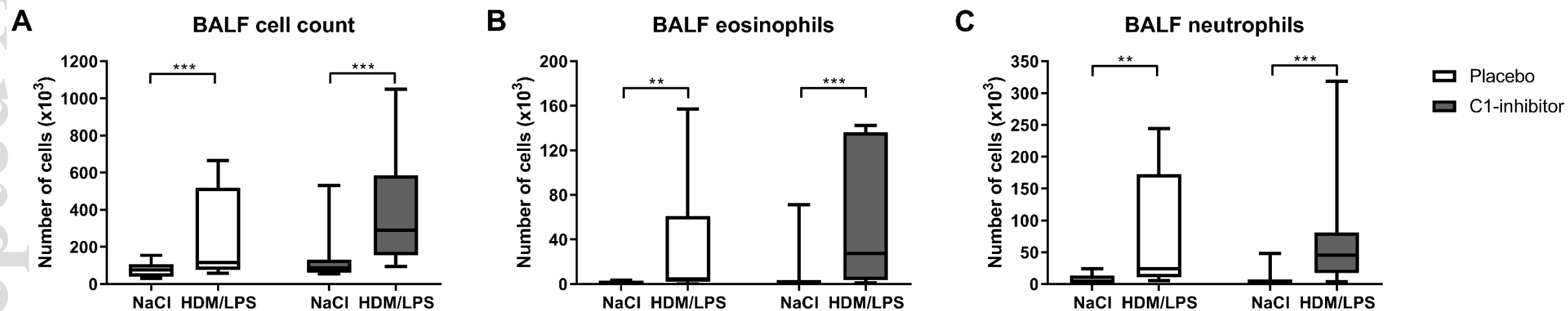
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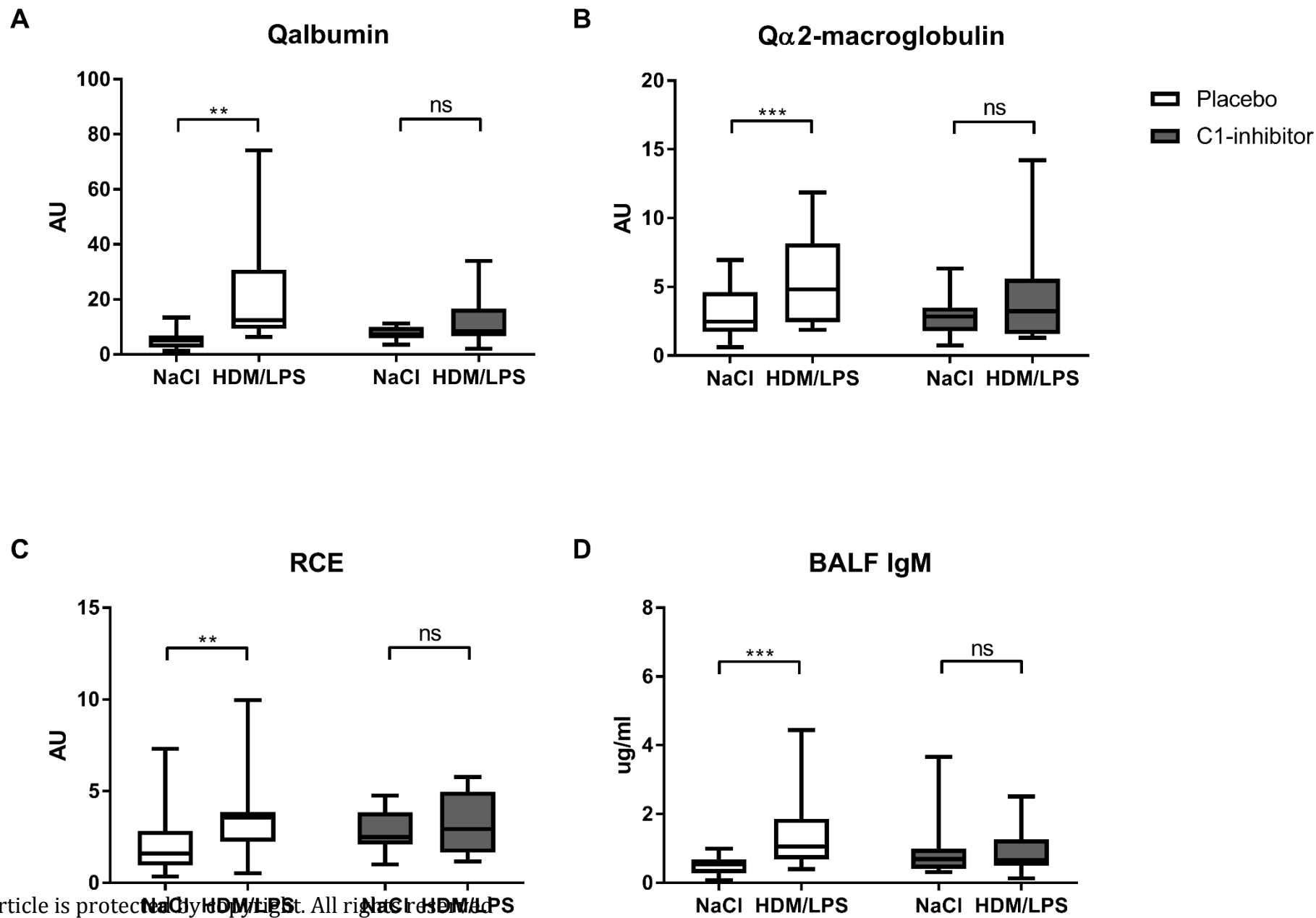
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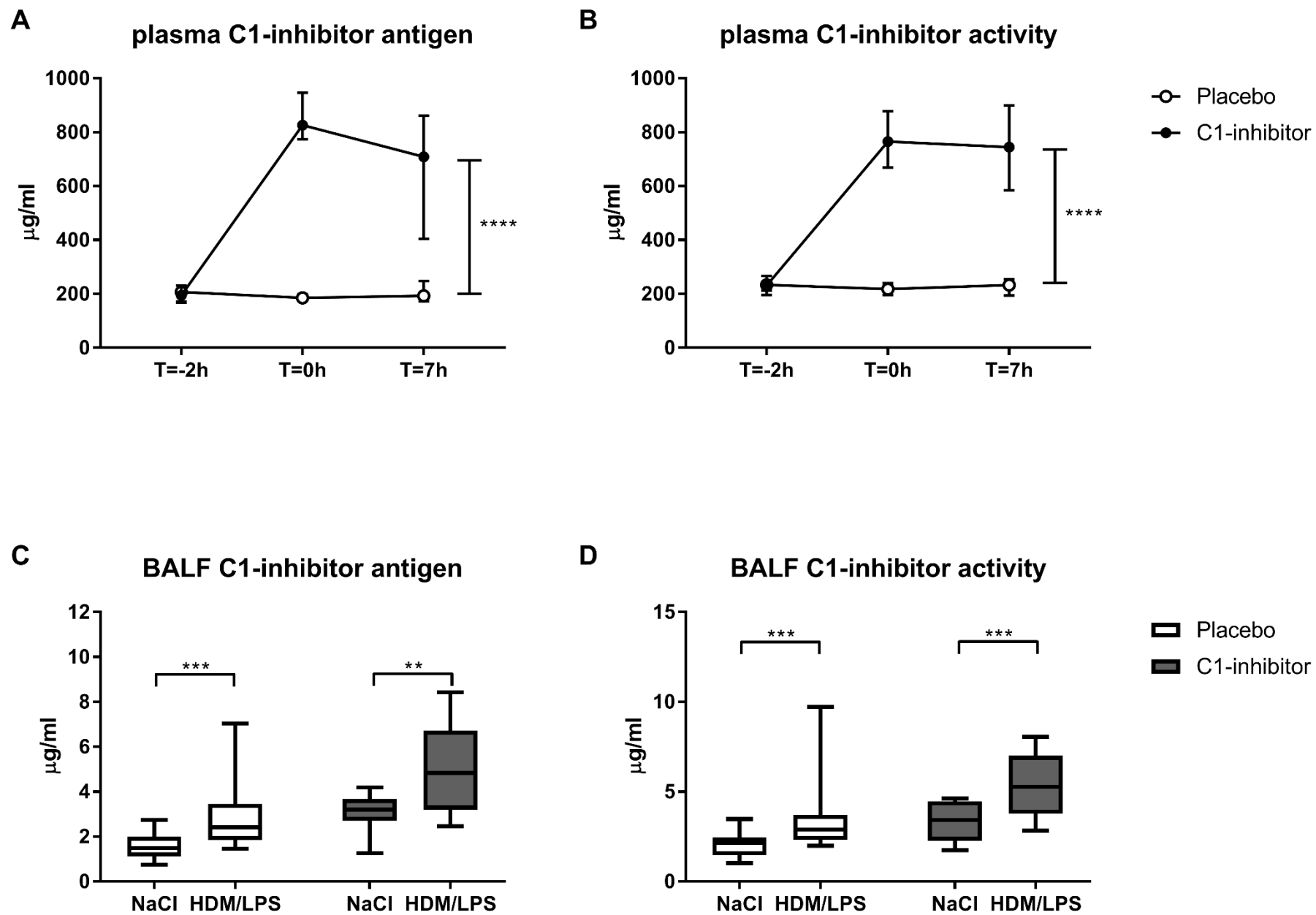
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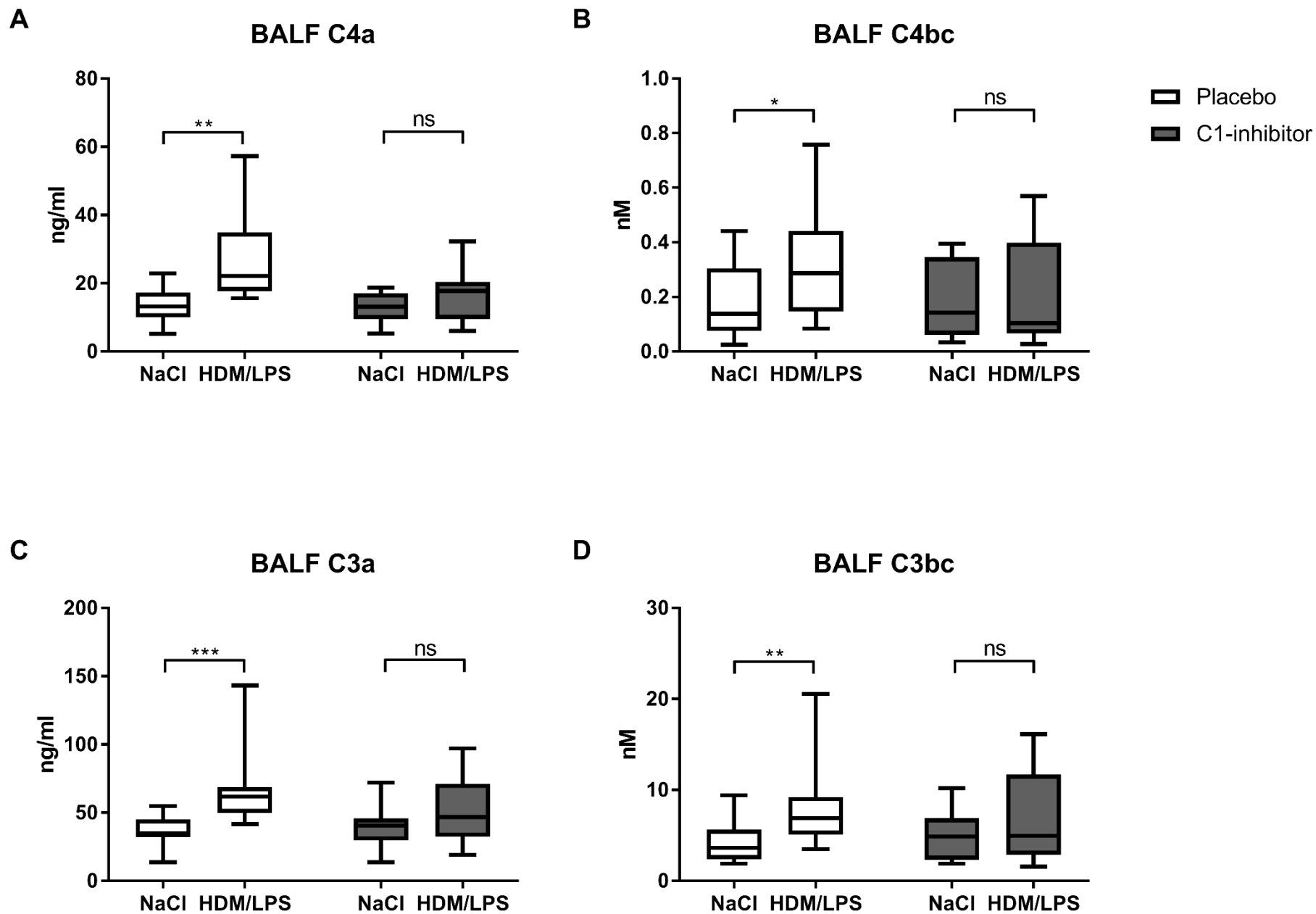
FIGURE 1: Intravenous C1-inhibitor infusion does not modify leukocyte influx after HDM/LPS challenge in the airways of asthma patients. (A) Total cell number in BALF, (B) eosinophils in BALF, (C) neutrophils in BALF. Data represent the median with interquartile range, the smallest and largest observation. **: $P < 0.01$, ***: $P < 0.001$, BALF: bronchoalveolar lavage

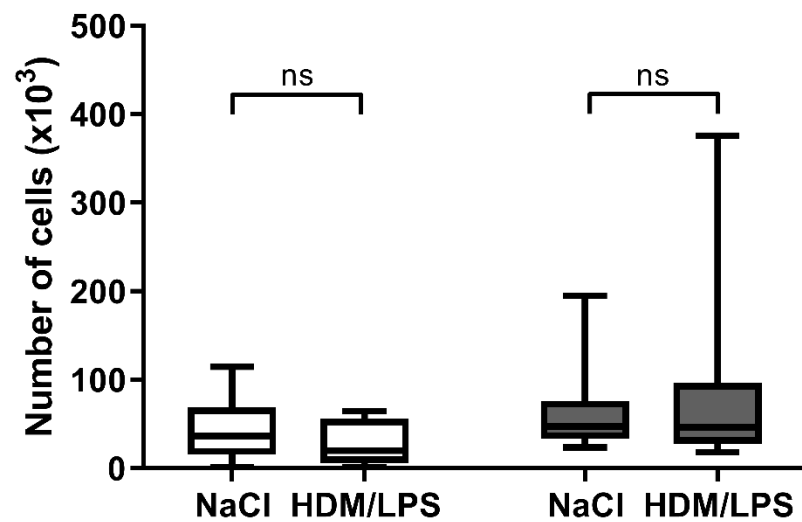
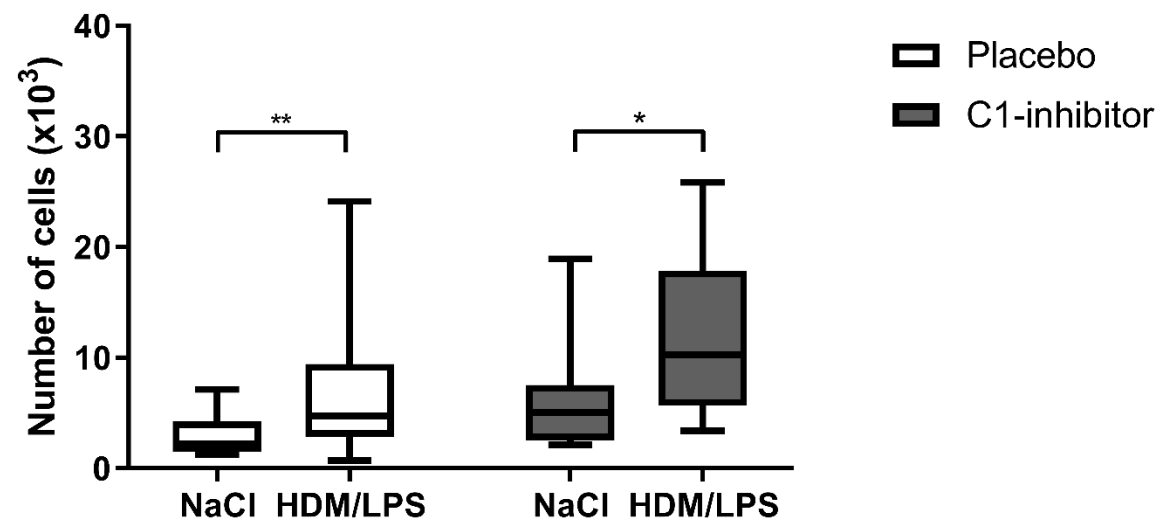
FIGURE 2: Intravenous C1-inhibitor infusion reduces vascular leak following HDM/LPS challenge in the airways of asthma patients. (A) Qalbumin: BALF albumin / plasma albumin, (B) Q α 2-macroglobulin: BALF α 2-macroglobulin / plasma α 2-macroglobulin, (C) Relative coefficient of excretion (RCE): Q α 2M/Qalbumin, (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

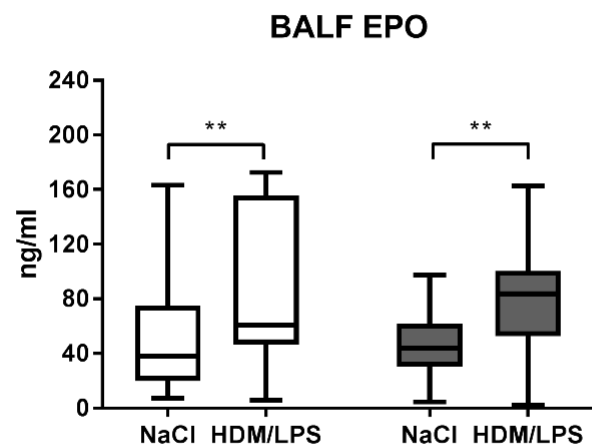
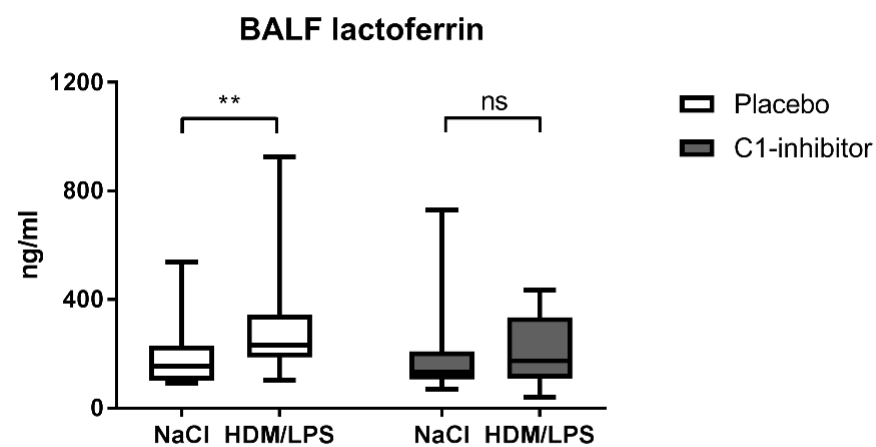
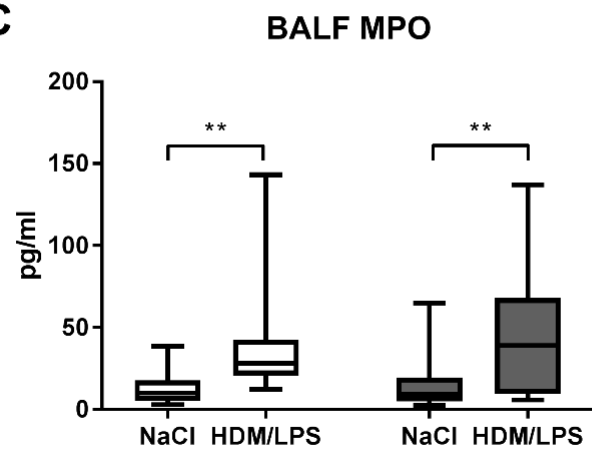
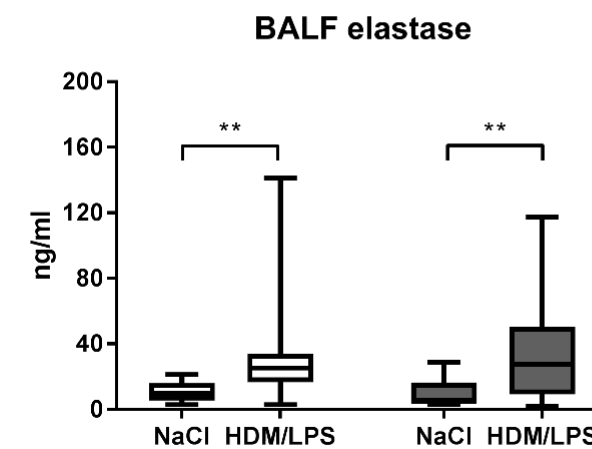








A**BALF alveolar macrophages****B****BALF CD4 T-cells**

A**B****C****D**

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Online Supplemental data

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 IU·kg⁻¹·h⁻¹ or placebo. The dose and timing of C1-INH administration were based on previous clinical studies with this compound¹⁻³. Infusion of 100 IU/kg C1-INH increased C1-INH activity and reduced complement activation in patients without a prior C1-INH deficiency. Moreover, this dose of C1-INH had a good safety profile in those explorative clinical studies. 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 described⁴. 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). 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 patients⁵. LPS is a component of the gram-negative bacterial cell wall and a natural pollutant in house dust⁶. We utilized a LPS dose

that corresponds with dust LPS levels detected in homes⁷. House dust is a complex mixture consisting of HDM allergen and pollutants such as LPS. Co-exposure to both HDM and LPS is not only a likely clinical event, but may be imminent since HDM allergen Der-p2 shares high structural homology with the endogenous LPS-binding protein MD-2, therefore easily binds surrounding LPS⁸. We randomised HDM and LPS challenge to the left or right lung segments to cater for potential biases due to anatomical differences. Seven hours post-challenge, a second bronchoscopy with bronchoalveolar lavage (BAL) of the two instilled segments was performed for analyses^{4,9}. Previous studies have shown that this trial design, involving allergen challenge by bronchoscope and performing a second bronchoscopy for harvesting BAL fluid is safe and well tolerated in asthma patients^{4,10}. In addition, a survey in six asthma patients, one of whom participated in an earlier allergen challenge study, showed that the burden was considered acceptable by patients. 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.

Screening procedures

The screening procedures and criteria were similar to a previous study from our group⁴. 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 Asthma¹¹, 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)¹², no clinically significant abnormalities during physical examination, hematological and biochemical screening, signed informed consent, controlled asthma 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

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), C3bc¹³, C4bc¹³, 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 FACS Aria™ III flow cytometer and analyzed using FlowJo software (Treestar, Palo Alto, CA, USA). Lactoferrin, elastase- α 1-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)-1 β , IL-1 α , 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)-1 α , MIP-1 β , Stromal cell-derived factor (SDF)1 α , tumor necrosis factor (TNF)- α . Contact system activation was assessed by C1-INH/Factor XII and C1-INH/kallikrein complex¹⁴. Vascular permeability was assessed by measuring IgM (Sanquin), albumin and α 2-macroglobulin⁴. We calculated the quotients of albumin (QA1b) and α 2-macroglobulin (QA2M) in BAL fluid and paired plasma samples. Relative coefficient of excretion (QA2M/QA1b) was determined to account for variable dilution of epithelial lining fluid during lavage¹⁵.

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.

Limitations

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-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. We observed a statistically non-significant increase in HDM-specific IgE levels in the C1-INH group. To what extent this could influence the results is not clear. Nonetheless, IgE is not a key driver for airway eosinophilia (primary outcome of this study) as only half of the patients with eosinophilic asthma show signs of atopy¹⁶ and treatment with omalizumab, monoclonal antibody against IgE, does not reduce peripheral blood eosinophils¹⁷. The current trial was conducted in a limited number of patients in whom 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.

Table S1 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
Dyspnoea	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
<i>D. pteronyssinus</i> 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	0.856

Data are presented as mean $\pm$ 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

Table S2 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-1α	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.

Table S3: 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-1 α	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).	

Figure legends

FIGURE S1: 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

FIGURE S2: 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

FIGURE S3: C1-inhibitor administration does not alter alveolar macrophages and CD4 T-cells after HDM/LPS challenge in the airways of asthma patients. (A) BALF alveolar macrophages, (B) BALF CD4 T-cells. Data represent the median with interquartile range, the smallest and largest observation. *: $P<0.05$, **: $P<0.01$, ns: not significant, BALF: bronchoalveolar lavage

FIGURE S4: 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

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