

ORIGINAL ARTICLE

Presence of commensal house dust mite allergen in human gastrointestinal tract: a potential contributor to intestinal barrier dysfunction

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ABSTRACT

Background Abnormal gut barrier function is the basis of gut inflammatory disease. It is known that house dust mite (HDM) aero-allergens induce inflammation in respiratory mucosa. We have recently reported allergen from *Dermatophagoides pteronyssinus* (*Der p1*) to be present in rodent gut.

Objective To examine whether *Der p1* is present in human gut and to assess its effect on gut barrier function and inflammation.

Design Colonic biopsies, gut fluid, serum and stool were collected from healthy adults during endoscopy. *Der p1* was measured by ELISA. Effect of HDM was assessed on gut permeability, tight-junction and mucin expression, and cytokine production, in presence or absence of cysteine protease inhibitors or serine protease inhibitors. *In vivo* effect of HDM was examined in mice given oral HDM or protease-neutralised HDM. Role of HDM in low-grade inflammation was studied in patients with IBS.

Results HDM *Der p1* was detected in the human gut. In colonic biopsies from healthy patients, HDM increased epithelial permeability ($p<0.001$), reduced expression of tight-junction proteins and mucus barrier. These effects were associated with increased tumour necrosis factor (TNF)- α and interleukin (IL)-10 production and were abolished by cysteine-protease inhibitor ($p<0.01$). HDM effects did not require Th2 immunity. Results were confirmed *in vivo* in mice. In patients with IBS, HDM further deteriorated gut barrier function, induced TNF- α but failed to induce IL-10 secretion ($p<0.001$).

Conclusions HDM, a ubiquitous environmental factor, is present in the human gut where it directly affects gut function through its proteolytic activity. HDM may be an important trigger of gut dysfunction and warrants further investigation.

INTRODUCTION

The human GI mucosa is the largest mucosal surface in the body continually exposed to dietary and microbial antigens. The intestinal epithelium, located at the interface between the external environment and the internal mucosal immune system, plays a central role in immune regulation.¹ As a physical protection against external environmental insults, the integrity of the epithelial barrier relies on epithelial permeability, mucus production,

Significance of this study

What is already known on this subject?

- Incidence of gut inflammatory diseases is increasing at an alarming rate.
- Breakdown of gut epithelial barrier is implicated in onset of gut inflammation.
- Triggers responsible are largely unknown.

What are the new findings?

- Respiratory house dust mite (HDM) allergen (*Dermatophagoides pteronyssinus* (*Der p1*)) is present in human gut.
- HDM in the gut is functional; it reduces gut epithelial barrier integrity and stimulates immunity.
- These processes are driven by cysteine-proteases contained in the HDM.
- HDM further deteriorates gut epithelial function in patients with IBS.
- Prior allergic sensitisation to *Der p1* is not required for these detrimental effects.

How might it impact on clinical practice in the foreseeable future?

- HDM may be an important environmental trigger for initiation and/or maintenance of gut dysfunction in susceptible individuals. These results have important clinical implications in prevention strategies and/or treatment of patients with IBS and other gut inflammatory disease such as environmental control of respiratory allergen exposure.

robust innate immune response and microbial sensing through pattern-recognition-receptors including Toll-like receptors (TLR), as well as immune regulatory circuits (Tregs).^{1 2} Clinical studies have demonstrated a loss or breakdown of the epithelial barrier during chronic inflammatory diseases, including IBD, IBS, food allergies, type 1 diabetes, celiac disease and multiple sclerosis.³ This leaky-gut barrier enables access of luminal content to underlying tissue, initiating an inflammatory cascade characterised by innate proinflammatory cytokine responses and activation of innate

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lymphoid cells (ILCs) and by adaptive Th1, Th2 and Th17 responses.^{2–4} Although gut exposure to dietary and microbial proteins has been well studied in context of oral tolerance^{5–7} and gut inflammation/homeostasis,² little is known about environmental respiratory antigens and their effect on human GI tract (GIT).

House dust mite (HDM) is an ubiquitous and perennial source of allergen, considered to be the most important cause of allergic respiratory disease such as asthma.⁸ For this reason, most of what we know about HDM comes from respiratory literature. While *Dermatophagoides pteronyssinus* (*Der p*) and *Dermatophagoides farinae* (*Der f*) are the most common mites in temperate climates, mites from *Blomia tropicalis* (*B. tropicalis*) are most prominent in tropical regions. *Der p* contains both cysteine (*Der p1*) and serine (*Der p3* and *Der p6*) proteases as well as components of Gram-negative bacteria such as lipopolysaccharide (LPS).⁹ The strong protease activity of *Der p1* disrupts respiratory epithelial tight-junction (TJ) proteins; namely zonula occludens protein 1 (ZO-1) and occludin-1,¹⁰ which anchor adjoining epithelial cells together, as well as stimulate protease-activated-receptors (PARs).¹¹ This disruption facilitates the passage of allergens across epithelial surface¹⁰ and induction of potent proinflammatory cytokines as well as enhancement of *Der p*-specific IgE,⁹ all which can lead to allergic disease in genetically prone and/or atopic individuals. Interestingly, in non-allergic asymptomatic patients who represent 60%–80% of the population, immune response to *Der p* is also observed but is qualitatively different and consists mainly of regulatory immune response characterised by interferon (IFN)- γ , transforming growth factor- β , and interleukin (IL)-10 secretion.^{12–13}

It has been long known that proteases are present in the human GIT where they function as protein-degrading enzymes in food digestion. More recently, proteases have been shown to play an important role in integrity of the intestinal barrier.^{1–2, 14–18} Moreover, we and others have shown that the disruption of epithelial barrier in IBD and IBS is attributed to destruction of ZO-1 and Occludin-1 TJs.^{1–2, 14–18} The mechanisms proposed include elevated luminal cysteine¹⁹ and serine²⁰ protease activity resulting in direct TJ protein destruction and/or indirect activation of PAR-2.^{14–21, 22} Using experimental models of colitis, PAR-2 antagonist have been shown to be effective in ameliorating IBD-like pathology and symptoms;^{18–21, 22} however their effect remains to be tested in humans.

For over 30 years now, we have known that inhaled antigen can be found in the rodent gut²³ and that young children often ingest >100 mg dust/ day during play.²⁴ In this context, our most recent results showing oral exposure to *Der p* in neonatal mice to prime for allergic sensitisation in adulthood²⁵ warrant exploration of HDM in the gut. Here, we set out to determine if *Der p* can be found in the human gut and if so, what role it plays in gut barrier function. HDM as a potential source of exogenous protease in the gut is a novel concept in understanding the pathophysiology of gut inflammation in humans.

METHODS

Participants

Healthy controls (n=38) and IBS (n=8) patients were recruited from Gastroenterology Department, Hôpital-Archet undergoing endoscopy. Control patients were asymptomatic and on macroscopic examination their colon had normal colon mucosa. IBS was assessed using Rome III criteria and IBS severity score. Patients were enrolled between 2011 and 2015. All patients completed questionnaires detailing past medical history and use

of medications. Patients who were taking corticosteroids and/or who had autoimmune disease were excluded from the study. All patients followed the Helsinki protocol and gave written informed consent. The study was approved by the hospital ethics committee No-07-047 and the General Health Delegation (GHD) No-2007-0456.

Collection of biological specimens

Initially, to examine if *Der p* was present in the human gut, duodenal fluid was collected from non-selected population by aspiration (n=28). Subsequently, distribution of *Der p1* was studied by aspirating standardised volume of gut fluid from each patients' six different GIT regions (duodenum, jejunum, right colon, left colon, transverse colon and rectum) (n=10). Colonic biopsies (2–5 mm in diameter, n=6–8 per patient) were taken during endoscopy and immediately transported to laboratory for histology processing, measurement of gut permeability and cytokine production. Peripheral blood was taken for measuring *Der p*-specific IgE (n=13). Stool samples were taken from healthy volunteers (n=12) and frozen immediately at –80°C. On the day, 1 g stool was diluted 7 $\times$ in phosphate-buffered saline (PBS). After centrifugation (10 min/4500 g), pellets were discarded and faecal supernatants filtered with 0.8 μ m-sized syringe filters (Nalgene, San Diego, USA). *Der p1* in faecal supernatant and GI fluid was measured by an in-house ELISA as previously described.²⁵ Detection level was 5 pg/mL.

Measurement of colonic permeability and tissue resistance

Paracellular permeability was measured as previously described.¹⁵ Four colonic biopsies from the same patient were weighed and mounted in Ussing Chambers (Transcellab, Paris, France) with 0.02 cm² of exposed surface. Apical and serosal sides of the biopsies were bathed in media (Ham's Nutrient Mixture, HAM's F12 Glutamax; Invitrogen) and continually oxygenated (95% O₂/5% CO₂) at 37°C. After 10 min equilibration period, 150 μ L media from apical side of the chamber was taken and replaced with 150 μ L fluorescein-5-(and-6)-sulfonic-acid-trisodium-salt (FITC-sulfonic acid, Invitrogen; 1 mg/mL) containing HDM extract alone (*Der p* species, containing 0.1–100 ng/mL *Der p1*) (Stallergenes, Paris) or HDM incubated with cysteine protease inhibitor (E64, 10 μ M, Sigma) or serine protease inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM, Santa Cruz). Control chambers contained no HDM or inhibitors. Aliquots (150 μ L) from both apical and serosal sides were taken every 30 min for 3 h and fluorescence measured using a spectrophotometer (Tecan Infinite F500, Tecan SA, France). Concentrations of fluorescein (ng/mL) were obtained by converting fluorescence intensity values using a standard curve. Potential difference was measured at the beginning and at the end of the experiment using the electrode bridge (3M KCl containing 4% agarose) (Biomecatronics, Ruitz, France). Transepithelial current (I_{TE}) was set to 10 mA and transepithelial resistance (R_{TE}) was calculated using Ohm's law.

Immunofluorescence

ZO-1 and occludin-1 immunofluorescence staining in human colonic biopsies were performed on 5 μ m thick paraffin sections. Prior to staining, tissue was deparaffinised and antigen sites unmasked by bringing slides to boil in 1 mM EDTA pH 8, followed by 30 min at 95°C. Tissue was permeabilised (Triton 0.1%; 2 min) and blocked (30% PBS/bovine serum albumin for 1 h) prior to incubation with rabbit anti-ZO-1 (1:100, Invitrogen) or rabbit anti-occludin (1:100, Zymed) overnight at

4°C in moisture chamber. Next day, secondary antibody was applied to both (goat anti-rabbit Alexa Fluor 594, 1:200, Invitrogen) for 1 h at room temperature and 4',6-diamidino-2-phenylindole (DAPI) added (1:100, 1 min) for nuclear staining. Slides where primary antibody was omitted were used as controls. All slides were examined with an inverted confocal microscope (Zeiss Axiovert 200M, Gottingen, Germany). Quantitative analysis of immunostained sections were performed using ImageJ processing software.

Cytokine production from colonic biopsies

Biopsies were cultured in 100 µL HAM's media, in presence or absence of HDM and with or without protease inhibitors as described above. In subset of studies, biopsies were incubated with purified *Der p1* 100 ng/mL (from native HDM, Stallergenes) or with LPS (*Escherichia coli*, 10 ng/mL, Sigma) in presence or absence of Polymyxin B (LPS inhibitor, 1 ng/mL, Life Technologies). After 24 h incubation, supernatant was collected and stored at -20°C for analysis. TNF-α and IL-10 were measured using a commercially available high-sensitivity ELISA kits with signal amplification (eBiosciences, Vienna, Austria). Detection limits for TNF-α was 0.3 pg/mL and for IL-10 was 0.4 pg/mL.

Electron microscopy

Following *in vitro* culture, gut biopsies were placed in 1–2 mL of 2% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.4 and left to fix overnight. Next day, tissue was rinsed in 0.1 M sodium cacodylate buffer (no glutaraldehyde) and shipped for processing and analysis. Ultrastructure of the tissues was examined using a JEOL 1011 transmission electron microscope at an acceleration voltage of 80 kV.

Allergen-specific IgE

IgE-specific against *Der p* was measured from serum using commercially available fluoroenzyme-ImmunoCAP technology with an ImmunoCap250 for IgE testing (Thermo Fisher Scientific, Uppsala, Sweden). Values greater than 0.1 kU/L were considered positive.

Animals

BALB/c 8–12 weeks old adult mice were purchased from Janvier Labs (France) and housed under specific-pathogen-free conditions at Centre Méditerranéen de Médecine Moléculaire. Experiments were performed according to CIEPAL animal ethics agreement NCE/2012–81.

Inactivation of proteases in HDM extract

HDM extract was suspended in water to obtain 12 mg/mL protein concentration. 4 mL was incubated with serine protease inhibitor AEBSF (Santa Cruz, 1 mM) and cysteine protease inhibitor E64 (Sigma, 100 µM) for 15 min at 37°C. To eliminate free remaining protease inhibitors, treated extract was desalted by gel filtration on PD10 columns (GE Healthcare). Mock-treated extract was not incubated with protease inhibitors but underwent similar desalting procedure. Protease activity was quantified in mock and treated desalted extracts using Boc-Gln-Ala-Arg-AMC (100 µM) as substrate in a reaction buffer composed of sodium phosphate 100 mM pH 6, EDTA 10 mM, dithiothreitol (DTT) 1 mM. Release of AMC fluorescence was followed over time using a Varioskan Flash apparatus (Thermo Scientific). The protease inhibitor treatment allowed 95% reduction in protease activity.

In vivo exposure of mice to HDM

Mice were given 20 µg of protease-inactivated HDM intragastrically (i.g.) or same concentration of mock-treated HDM. Control animals received only water. Food was removed and 4 h later their colon was harvested for measurement of baseline permeability. Gut content was collected for measurement of *Der p1*.

In vitro T-cell differentiation

Mesenteric lymph nodes (MLNs) were harvested from wild-type (WT) mice given HDM (20 µg i.g.) or water for 7 days, digested (collagenase 400 U/mL and DNase 100 µg/mL for 20 min at 37°C) and stimulated *in vitro* with 10 µg/mL HDM. Four days later, supernatant was collected and T-helper cytokines measured using ELISA. Detection limits for IFN-γ was 30 pg/mL, for IL-5/IL-17 was 20 pg/mL and for IL-13 was 60 pg/mL.

Mucus staining

To examine the effect of HDM on mucus, histological sections from WT mice given HDM (20 µg i.g.) or water for 7 days were stained with periodic acid Schiff (PAS). Mucosal thickness and PAS-positive area (magenta in colour) were assessed using SlidePath Gateway software (Leica, Nanterre, France) as previously described.²⁶

Statistical analyses

Results are represented as individual data or as box-and-whisker plots with 5–95 percentiles line representing median of each group. Significance between groups at 95% confidence level was determined by Mann–Whitney non-parametric unpaired t tests, using Graphpad Prism (V6.0) software package for Mac (Graphpad, San Diego, USA). Statistical significance was set at $p < 0.05$.

RESULTS

Antigens from house dust mites are present in the human gut

The presence of HDM antigens in the human gut remained unknown so far. *Der p1* antigen was detected in the diluted duodenal fluid of 13/28 healthy subjects and in all volunteer stool samples (figure 1A). To analyse *Der p1* distribution along the gut, we standardised aspirate volume. In positive individuals, *Der p1* was found throughout the gut, decreasing from upper to lower GIT, and its content varied between 20 and 420 pg/mL (figure 1B). We failed to detect *Der p2* antigen in the human gut.

HDM reduces barrier function and increases local inflammation in healthy human gut

Having shown that *Der p1* is found in the human colon, we set to study its effect on colonic barrier function and local cytokine production. We assessed paracellular permeability by measuring transfer of FITC-tagged sulfonic-acid-trisodium salt from apical to basolateral side of the gut mucosa and modification of R_{TE}. Addition of graded doses of HDM extract (0.1–100 ng/mL *Der p*) to healthy biopsies induced a dose-dependent increase in transfer of FITC-sulfonic acid (figure 2A and B). This increase was significant at 100 ng/mL ($p < 0.01$) (figure 2A), inducing a greater than fourfold increase in permeability compared with baseline responses ($p < 0.01$) (figure 2B); however, borderline significance can be seen even at 1000-fold lower dose ($p = 0.06$). Epithelial permeability increased progressively over time, reaching significance 60 min after HDM addition (figure 2C).

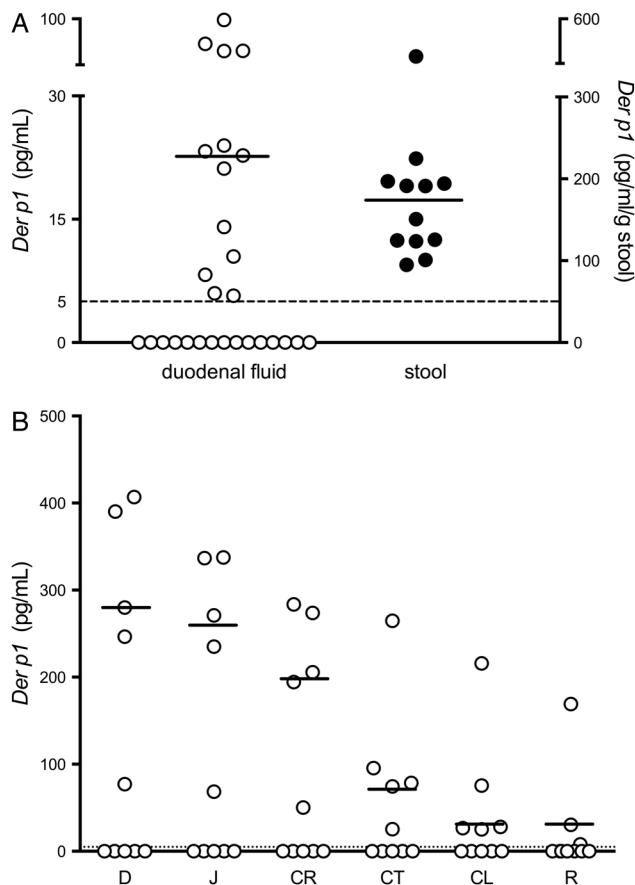


Figure 1 Presence of *Dermatophagoides pteronyssinus* (Der p1) in the human gut. Global Der p1 was measured in diluted duodenal fluid (n=28) and stool samples (n=12) of healthy patients (A). Distribution of Der p1 antigen in non-selected patients (n=10) is represented in (B) where collection of fluid from the duodenum (D), jejunum (J), right colon (CR), transverse colon (CT), left colon (CL) and the rectum (R) was standardised. Data are shown as individual patients with bars representing median values.

Increased permeability was associated with a dose-dependent reduction in R_{TE} (figure 2D) ($p<0.01$).

To examine whether HDM-induced permeability was associated with inflammation, we incubated colonic biopsies with HDM for 24 h and measured *in vitro* cytokine production from cultured supernatants. HDM induced a significant increase in both TNF- α and IL-10 production compared with non-stimulated controls ($p<0.05$) (figure 2E). These effects were already significant at HDM 10 ng/mL. Together, these data demonstrate that HDM can affect both permeability and cytokine secretion from healthy colon and its effect is dose-dependent.

HDM interrupts TJ proteins in healthy human colon

To examine whether HDM-induced changes in permeability and resistance may be attributed to interruptions and/or damage of epithelial TJ proteins, we have examined ZO-1 and occludin-1 expression by immunofluorescence and electron microscopy. Initially, in the human intestinal epithelial cell line Caco-2, we have shown that HDM 100 ng/mL caused significant damage and disruption in ZO-1 staining evidenced by loss of TJ mesh-like network normally seen in control, non-exposed epithelial cells (see online supplementary figure S1). Disruption of ZO-1 and occludin-1 was confirmed in healthy human colonic

biopsies following HDM exposure (figure 3A). Quantitative assessment of slides demonstrates reduced expression and intensity of ZO-1 and occludin-1 staining post HDM incubation (figure 3B). Electron microscopy images of healthy colon show continuous epithelial brush border and extensive zipper-like TJ networks (figure 3C left), which are destroyed and interrupted following HDM exposure (figure 3C right). Together, these images demonstrate that HDM in the human gut directly affects epithelial TJ proteins and induces damage to the gut epithelial layer.

HDM-induced changes in the human colon are dependent on cysteine protease activity

HDM extract contains both proteases and LPS. To test whether HDM-induced effects on gut inflammation are protease-dependent or LPS-dependent, we incubated biopsies with HDM 100 ng/mL with or without cysteine protease inhibitor (E64), serine protease inhibitor (AEBSF) or LPS inhibitor (Polymyxin B) and measured cytokine production. E64 abolished the *in vitro* HDM-induced TNF- α (figure 4A) ($p<0.001$) and IL-10 production (figure 4B) ($p<0.001$) and responses were similar to baseline. AEBSF had no effect on HDM-induced TNF- α production but there was a trend for reduced IL-10 production ($p=0.09$). Addition of Polymyxin B failed to modify HDM-induced cytokine production in the colon, indicating the lack of role for LPS in this response. E64, AEBSF or Polymyxin B alone had no effect and responses were similar to unstimulated controls. LPS-mediated responses were completely abolished by Polymyxin B (figure 4A,B). These data indicate that the effect of HDM is not driven by LPS or by serine proteases but is cysteine protease-dependent. Addition of purified Der p, a cysteine protease, was sufficient to reproduce the HDM effect (figure 4A,B). To assess whether HDM-induced increase in paracellular permeability was also cysteine protease-dependent, colonic biopsies were incubated with AEBSF or E64 in presence of HDM. While AEBSF had no effect, E64 abolished the HDM-induced increase in permeability and responses were similar to baseline (figure 4C,D) ($p<0.05$). Together, these results indicate that cysteine protease activity in the HDM is predominantly responsible for HDM-induced *in vitro* changes in permeability and cytokine production in human colon.

HDM augments colonic permeability *in vivo* in mice in protease-dependent manner

To assess whether HDM can induce gut barrier defect *in vivo* and whether Der p protease activity persists in the colon, we administered HDM orally to naïve adult mice. Mice given HDM had twofold increased paracellular permeability compared with control mice (n=6, $p=0.043$) (figure 4E). These *in vivo* changes were of similar magnitude to that seen following exposure of naïve mice to 3% dextran sodium sulphate for 7 days, which is an agent commonly used to induce colitis in mice (data not shown). To see whether *in vivo* HDM effect was also protease-dependent, we administered HDM in which protease activity was inactivated and 4 h later we measured paracellular permeability. Mice treated with protease-inactivated HDM failed to show augmentation in permeability and responses were similar to control (figure 4E). HDM-induced increase in permeability was associated with 1.6-fold reduced R_{TE} in HDM-treated mice ($p=0.0143$) but not in mice given inactivated HDM (figure 4F). Increased *in vivo* permeability in HDM-exposed mice was observed at Der p1 concentration of 1.2 (0.8–2.5) ng/mL (median (IQR)) while it was undetectable in controls ($p<0.001$, n=7).

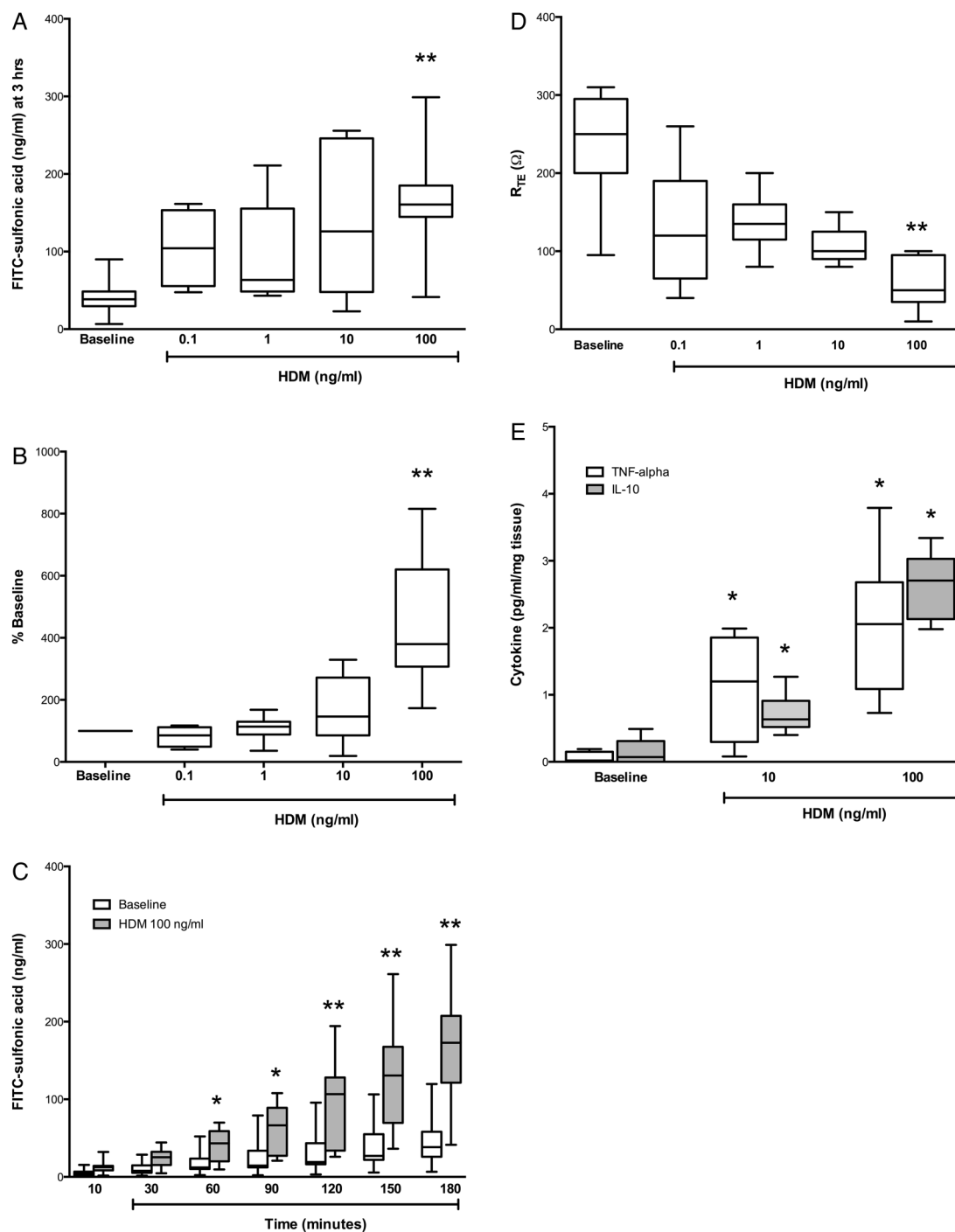


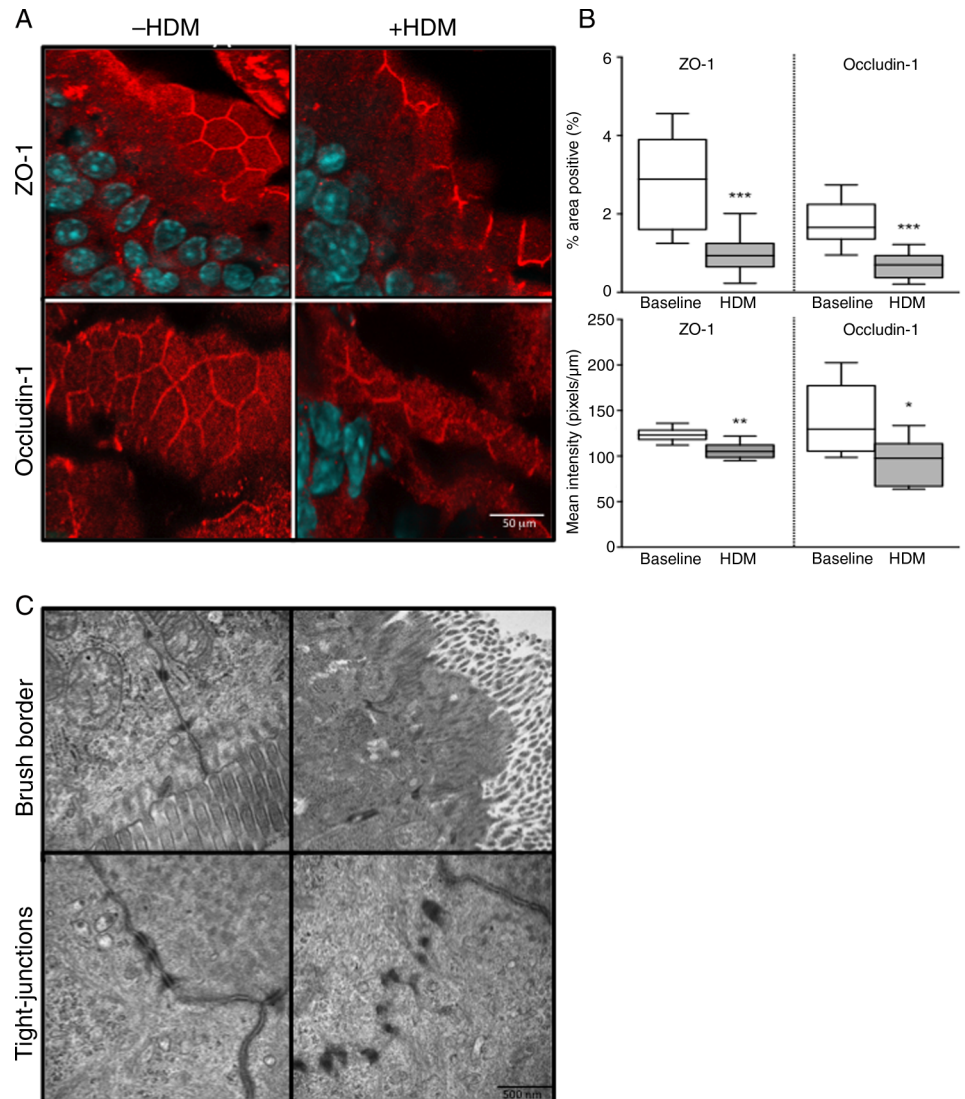
Figure 2 House dust mite (HDM)-induced changes in gut function. Effect of increasing concentration of HDM (0.1–100 ng/mL, $n=4-12$) on *in vitro* gut permeability in Ussing Chambers (A–C), transepithelial resistance R_{TE} (D) and *in vitro* cytokine production (E) before and after incubation with HDM are shown. Permeability is shown as concentration of transferred sulfonic acid (A) or percentage change from baseline (B) 3 h after incubation. Time course of HDM-induced permeability (100 ng/mL, grey bars, $n=10$) compared with baseline (white bars, $n=10$) is shown in (C). Cytokine production is measured 24 h after addition of HDM. Data are shown as box-and-whiskers plots with 5–95 percentile and lines represent median values; * $p<0.05$ and ** $p<0.01$ versus respective baseline. FITC, fluorescein isothiocyanate.

HDM impacts the mucus layer in the colon

We know that HDM has a detrimental effect on mucus production in the lungs.²⁷ Recently, it has been shown that cysteine proteases from bacteria can degrade the protective colonic mucous layer.²⁸ To see if HDM can directly impact intestinal mucus layer, we measured mucosal thickness and mucus-positive cells before and after HDM exposure in mice using PAS staining. Images in [figure 5A](#) illustrate patchy PAS staining along

epithelial surface following HDM exposure (top right panel) and accumulation of mucus in the gut lumen (bottom right panel) compared with non-exposed tissue (left panels). HDM significantly reduced the mucosal thickness (155 μm (131–179) to 124 μm (108–136); median (IQR)) ($n=6$, $p<0.05$) and the PAS-positive area ($n=6$, $p<0.05$) compared with controls ([figure 5B](#)). These results demonstrate that HDM directly impairs the mucus layer in the gut.

Figure 3 House dust mite (HDM)-induced destruction of tight junctions. (A) Immunostaining of tight-junction proteins zonula occludens protein 1 (ZO-1) and occludin-1 in human colonic biopsies before (left) and after HDM 100 ng/mL exposure (right) is shown. DAPI nuclear staining is shown in blue and tight-junction proteins in red. (B) Quantitative assessment of percentage (%) area positive for tight-junction proteins (top graph) and mean intensity of tight-junction staining (bottom graph), before and after HDM exposure in human colon (n=8) are shown. Results are presented as box-and-whisker plots with 5–95 percentile and lines represent median values; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus respective baseline. (C) Electron microscopy images of human colonic biopsies before (left) and after HDM 100 ng/mL exposure (right), showing HDM-induced destruction of epithelial brush border and tight-junctions between gut epithelial cells.



HDM-induced effects on gut barrier are Th2-independent

As HDM is known to promote allergic Th2-mediated immune responses in the lungs,^{29,30} we wondered whether HDM-induced changes in the gut required pre-existing specific allergic immunity to HDM. This was initially tested in HDM-sensitised and HDM-non-sensitised subjects. Baseline colonic permeability was similar between *Der p1*-specific IgE-negative individuals (non-allergic, n=8) and *Der p1*-specific IgE-positive individuals (allergic, n=5) (figure 6A). HDM caused a similar degree of increase in permeability within each group (3.8-fold and 5-fold increase, respectively) (figure 6A). Greater heterogeneity was observed among atopics. These results suggest that gut paracellular permeability does not require *prior* sensitisation to HDM. Second, to examine if HDM-induced increased gut permeability is associated with induction of Th2 differentiation, we measured Th1 and Th2 cytokine production by gut draining lymph node cells from naïve mice given HDM i.g. for 7 days. HDM significantly increase both Th1 (IFN- γ) and Th2 (IL-5, IL-13) cytokine production compared with non-stimulated controls (figure 6B). Together, these data show that HDM-induced changes in the gut do not require prior allergic sensitisation to *Der p* antigen nor are mediated by Th2-specific immunity.

HDM compromises barrier function in patients with IBS

To examine the effect of HDM in subjects with known gut epithelial dysfunction, we measured colonic permeability and cytokine production in patients with IBS with and without HDM exposure. As we previously showed,¹⁵ patients with IBS (with no HDM exposure, n=8) have increased permeability compared with healthy controls (n=18, $p < 0.01$) (figure 7A). IBS mucosa exposed to HDM showed significantly increased permeability compared with IBS non-exposed mucosa (n=8, $p = 0.001$) (figure 7A). This increase was significantly higher than the increase seen in healthy mucosa exposed to HDM (n=13, $p = 0.05$). While the ratio of IL-10 to TNF- α production was in favour of a regulatory balance in colon of healthy subjects exposed to HDM (1.3 (0.9–2.8); median (IQR)) (n=10, $p < 0.001$), it was strongly shifted towards a proinflammatory response in patients with IBS (0.1 (0.03–1.85); median (IQR)) (n=8, $p < 0.05$) (figure 7B).

DISCUSSION

HDM impact on respiratory epithelial cells, innate and specific immune system activation has been well documented in respiratory allergic disease but has never been assessed in the context of inflammatory diseases of the gut. In this paper, we demonstrated that HDM major allergen (*Der p1*) is found in the

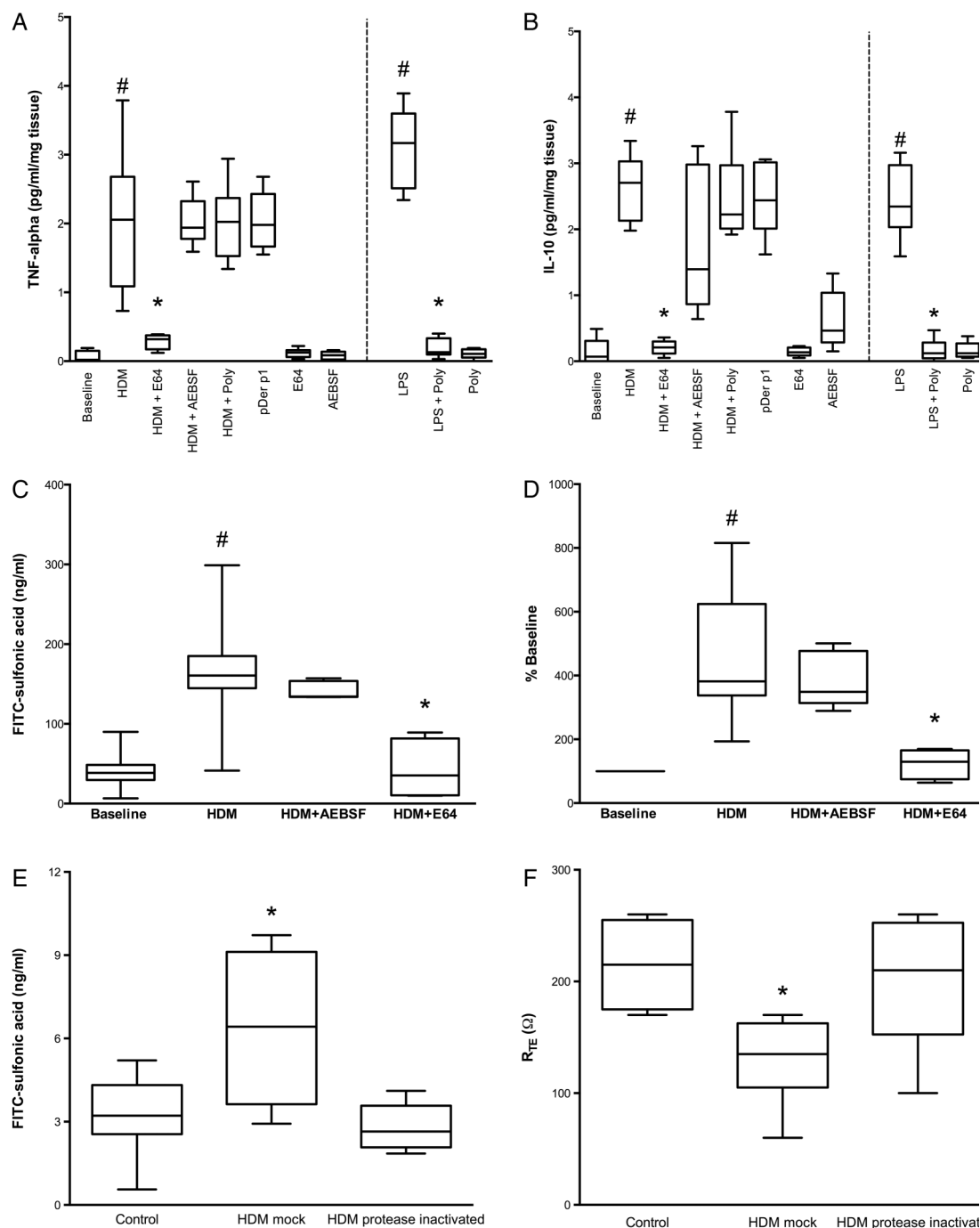


Figure 4 House dust mite (HDM)-induced gut inflammation and permeability are driven by cysteine proteases. Effect of cysteine protease inhibitor (E64 10 μ M, n=6), serine protease inhibitor (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) 1 mM, n=6) or LPS-inhibitor (Polymyxin B 1 ng/mL, n=6) on *in vitro* HDM-induced cytokine production (A and B) and gut permeability (C and D) in human colon is shown. Specificity of HDM response was tested with purified *Dermatophagoides pteronyssinus* (Derp1) (n=5). LPS inhibitor (n=6) and protease inhibitors (n=6) alone were used as controls. Cytokine production is measured at 24 h and permeability at 3 h after *in vitro* addition of HDM. *In vivo* effect of proteases on HDM-induced colonic gut permeability (E) and trans-epithelial resistance (R_{TE}) (F) was tested in mice 4-h after administration of HDM (mock-treated, n=6) or HDM in which all proteases have been inactivated (n=6) (both at 20 μ g intragastrically). Control mice received same volume of water (n=8). Results are represented as box-and-whisker plots with 5–95 percentile (n=6–12) and lines represent median values; #p<0.05 versus baseline and *p<0.05 versus HDM or LPS respective controls. FITC, fluorescein isothiocyanate; R_{TE} , transepithelial resistance; TNF, tumour necrosis factor.

human gut where it has detrimental and profound impact on gut function. We have shown that HDM directly augments colonic permeability, induces proinflammatory and anti-inflammatory cytokine secretion, destroys TJs between gut epithelial cells and degrades the protective mucus barrier on gut

epithelia. These effects are mediated by cysteine proteases contained in the HDM. Role of HDM proteases were confirmed *in vivo* in naïve mice after acute exposure. Importantly, these *in vivo* findings indicate that proteases from the HDM survive gut digestion and remain functional until the distal gut.

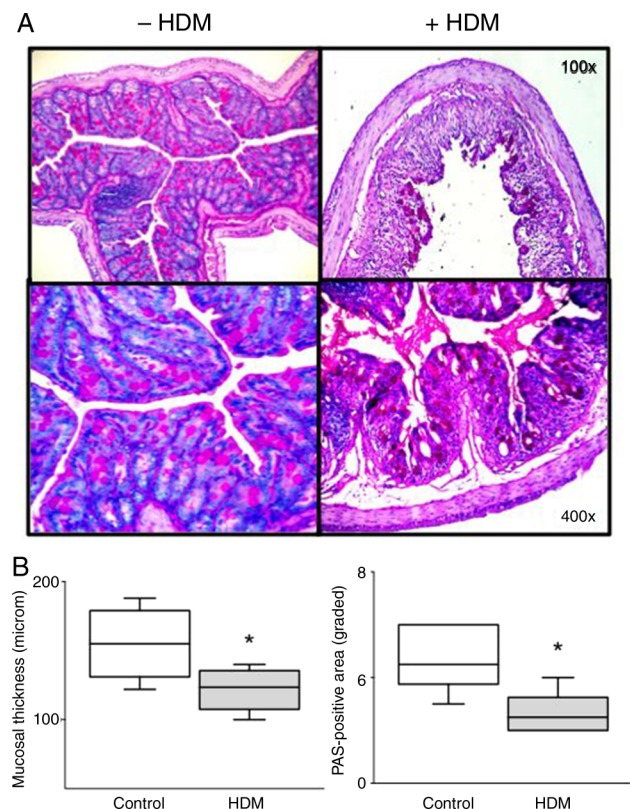


Figure 5 House dust mite (HDM) impacts the mucus layer in the gut. Mucus (and goblet cells) was stained with periodic acid-Schiff (PAS) stain before and after HDM exposure (A). Mucus-positive area and epithelial brush border stain are magenta in colour. Semi-quantitative analysis of mucosal thickness (μm , left) and PAS-positive area (right) are shown in (B) ($n=6$). Data are represented as box-and-whiskers plots with 5–95 percentile and lines represent median values; $*p<0.05$ versus control.

Furthermore, in gut with barrier dysfunction such as in patients with IBS, HDM further compromises the epithelial barrier integrity. Prior immunity to *Der p* is not required for such HDM-driven effects. Together these findings shed light on a novel concept of a ubiquitous environmental factor which may

directly drive induction of inflammatory diseases of the gut and contribute to exacerbation of the disease in susceptible individuals.

Der p1 was present in the gut of $\sim 50\%$ of subjects tested; it was distributed throughout their GIT and its content was higher in proximal regions of the gut, concentrations ranging from 20 to 420 pg/mL. For correct interpretation of these results, one must take into account that *Der p1* levels were measured in patients who were prepared for an endoscopic examination (involved extensive washing of GIT) and 5 mL liquid were further added for aspiration; therefore, the real concentration is likely to be much higher. Our data in mice suggest that ~ 1 ng/mL *Der p1* in the gut is sufficient to produce detrimental functional effects of the allergen on gut permeability *in vivo*. Interestingly, this is also the same dose range required for *Der p* enzymatic activity.^{9 31} Furthermore, we must remember that *in vivo*, the intestinal tissue is subject to chronic environmental HDM exposure and we can also be exposed to other respirable mites such as *Der f* or to *B. tropicalis*; therefore, multiple exposures may increase total concentration of mites in the faeces. Our ELISA was *Der p*-specific and therefore results are applicable to *Der p* mites only. Whether other mites are present in the human gut or if they have physiological relevance remains to be tested.

In the human gut, we have demonstrated that the mechanisms involved in HDM-induced increase in gut permeability and reduction in gut resistance is associated with disruption of TJ proteins, ZO-1 and occludin-1 as well as reduced integrity of the mucus barrier. Although the effect of HDM on TJ proteins has been previously established for respiratory epithelium, here we demonstrate this also to be the case for human gut. These events weaken the gut barrier, making it less resistive and prone to inflammation due to easier access of luminal content to lamina propria. Additional HDM-induced mechanisms involve TNF- α secretion. TNF- α has been shown to be a crucial cytokine in pathophysiology of IBD.^{2–4} Its mechanism of action involves increased gut permeability suggesting an additional, indirect effect of HDM.³² In healthy, non-atopic individuals, such local-driven inflammatory effects are likely to be dampened by endogenous regulatory mechanisms and/or cysteine protease inhibitors, as well as production of immune-regulatory cytokines such as IL-10. However, in those who have defective regulatory mechanisms and/or insufficient endogenous levels of protease

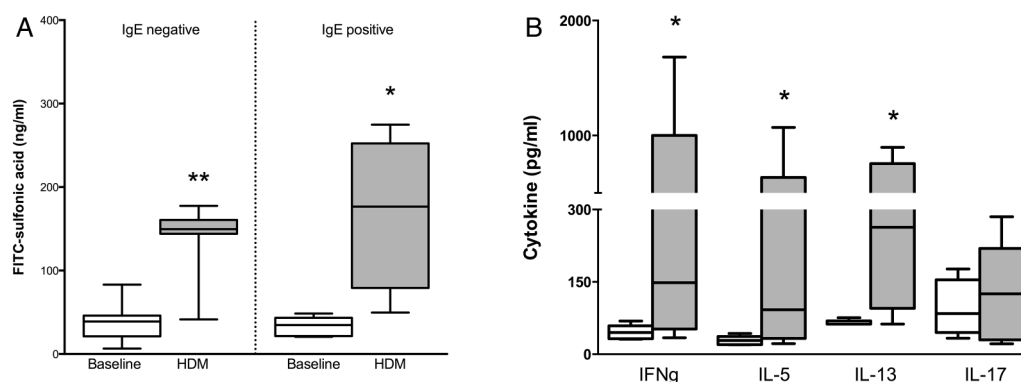


Figure 6 House dust mite (HDM)-induced changes in the gut are Th2-independent. HDM-induced changes in (A) colonic permeability (*Dermatophagoides pteronyssinus* (*Der p*) 100 ng/mL, grey bars) in *Der p*-specific IgE-negative individuals ($n=8$) and *Der p*-specific IgE-positive individuals ($n=5$) and (B) Th1 (interferon (IFN)- γ) and Th2 (interleukin (IL)-5, IL-13 and IL-17) cytokine production in cultured mesenteric lymph nodes from mice exposed to HDM (20 μg i.n for 7 days) prior to *in vitro* stimulation with HDM 10 μg on day 8 (grey bars, $n=5$). Results are represented as box-and-whisker plots with 5–95 percentile and lines represent median values; $*p<0.05$ versus control (white bars, $n=5$). FITC, fluorescein isothiocyanate.

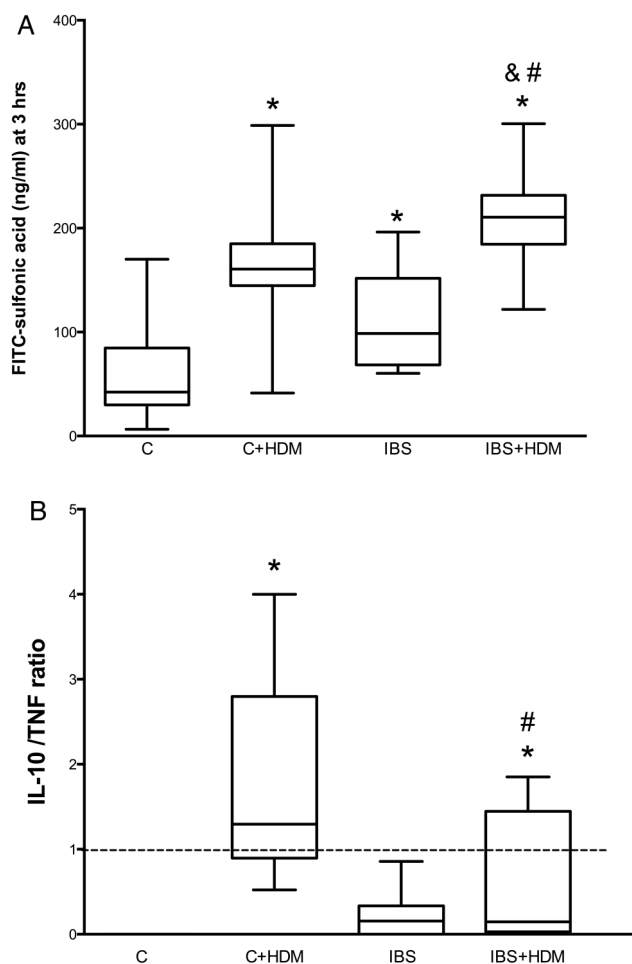


Figure 7 House dust mite (HDM) further exacerbates permeability and inflammation in patients with IBS. Effect of HDM 100 ng/mL on *in vitro* gut permeability (A) and *in vitro* interleukin (IL)-10 and tumour necrosis factor (TNF)- α cytokine production (B) in healthy controls (n=6) or in patients with IBS (n=8) is shown. Permeability is shown as concentration of transferred fluorescein isothiocyanate (FITC)-sulfonic acid and cytokines are expressed as IL-10:TNF- α ratio. Data are shown as box-and-whiskers plots with 5–95 percentile and lines represent median values; * $p < 0.05$ versus control, # $p < 0.05$ versus C+HDM and § $p < 0.05$ versus IBS. C, controls.

inhibitors, these local inflammatory changes may be sufficient to initiate disease or exacerbate ongoing pathology. Here we report large production of IL-10 in healthy biopsy cultures post HDM exposure which may be indicative of a regulatory response. Interestingly, similar increase in IL-10 production to HDM was not seen in patients with IBS and may reflect failed immune-regulatory and protective mechanism(s) which may contribute to ongoing abdominal pain and gut inflammation in IBS; however, this remains to be confirmed.

The role of proteases in IBD and IBS has received much attention (reviewed in Ref. 16); however, their direct contribution has been difficult to elucidate due to their pleiotropic functions *in vivo*. Whether an exogenous source of proteases to the gut may be contributing to its pathology is unknown. Experiments shown here suggest that proteases from the environmental dust may contribute to its detrimental effects. This was demonstrated both *in vitro* in human biopsies using specific cysteine-protease inhibitor E64 and *in vivo* in mice by neutralising proteases from HDM extract, both which abolished the

HDM-induced damage to the gut. Moreover, purified *Der p1* (a cysteine protease) mimicked inflammation seen with HDM extract. *Der p* from HDM is also likely to be involved in cleavage of intestinal mucins as supported by our data showing reduced PAS staining and mucus thickness in HDM-exposed gut. Cysteine proteases from other pathogens^{33 34} and bacteria²⁸ are known to degrade the colonic mucous layer through their mucinase activity. Our results do not support the contribution of LPS or serine protease in HDM-induced changes in colonic function. In this context, we would envisage specific cysteine protease inhibitors to play a beneficial role in patients with inflammatory diseases of the gut, particularly in patients with elevated cysteine protease activity such as those with IBS.¹⁹

For HDM to have effect on gut mucosa, we have shown that specific immunity to *Der p* is not required and *Der p*-induced damage in the colon is not Th2-mediated. This can be concluded from two of our observations. First, in human biopsies, similar level of augmentation in HDM-induced permeability was seen between HDM atopic and HDM non-atopic subjects, suggesting that HDM can contribute to gut dysfunction independent of patient's allergic status. Second, detrimental *in vivo* effects of HDM were seen in naïve mice in which HDM was capable of stimulating MLN production of both Th1 and Th2 cytokines.

In light of recent literature in the field of gut–lung cross-talk,^{35–37} it is likely that HDM exposure in the gut may prime for sensitisation at another site. These novel data, along with increased awareness of potential link between asthma (where HDM is clearly implicated) and IBD,³⁷ it is tempting to speculate a central role of HDM in the link between these two diseases. In addition, we can envisage that HDM effect may be potentiated in inflammatory conditions where lower GIT becomes susceptible to IgE-mediated responses.^{38 39} Indeed, in this paper, we have shown HDM to further increase epithelial permeability in patients with IBS. Of course, the question remains whether patients with IBS have higher GIT *Der p* content than healthy controls and this remains to be fully explored in a larger cohort of subjects.

Currently, there is no epidemiological evidence linking environmental HDM exposure to gut inflammatory disease. This is largely due to HDM being often thought of as a respiratory allergen and not a respirable antigen that is ingested (via food or inhalation). Here we show evidence for a direct role of HDM in human gut which gives us an insight into an environmental trigger, previously overlooked, that may contribute to gut disease. We hope these findings stimulate epidemiological studies to look at the potential link between environmental exposure to HDM and incidence of gut inflammatory disease. Although identifying one's primary sources of HDM exposure and those individuals who would benefit from HDM avoidance will be of challenge,⁴⁰ the ever-increasing presence of HDM in our modern, well-isolated homes^{41 42} warrants exploration of its impact on susceptibility of gut disease.

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REFERENCES

- Pastorelli L, De Salvo C, Mercado JR, *et al.* Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Frontiers Immunol* 2013;4:280.
- Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 2011;474:298–306.
- Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009;124:3–20; quiz 21–22.
- Geremia A, Biancheri P, Allan P, *et al.* Innate and adaptive immunity in inflammatory bowel disease. *Autoimmunity Rev* 2014;13:3–10.
- Verhasselt V. Oral tolerance in neonates: from basics to potential prevention of allergic disease. *Muc Immunol* 2010;3:326–33.
- Faria AM, Weiner HL. Oral tolerance. *Imm Rev* 2005;206:232–59.
- Pabst O, Mowat AM. Oral tolerance to food protein. *Muc Immunol* 2012;5:232–9.
- Sporik R, Chapman MD, Platts-Mills TA. House dust mite exposure as a cause of asthma. *Clin Exp Allergy* 1992;22:897–906.
- Gough L, Schulz O, Sewell HF, *et al.* The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999;190:1897–902.
- Wan H, Winton HL, Soeller C, *et al.* Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* 1999;104:123–33.
- Asokanathan N, Graham PT, Stewart DJ, *et al.* House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J Immunol* 2002;169:4572–8.
- Akdis CA, Akdis M. Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. *J Clin Invest* 2014;124:4678–80.
- Akdis M, Verhagen J, Taylor A, *et al.* Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* 2004;199:1567–75.
- Bueno L, Fioramonti J. Protease-activated receptor 2 and gut permeability: a review. *Neurogastroenterol Motil* 2008;20:580–7.
- Piche T, Barbara G, Aubert P, *et al.* Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut* 2009;58:196–201.
- Turk B. Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Disc* 2006;5:785–99.
- Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. *Ann Rev Physiol* 2006;68:403–29.
- Vergnolle N. Protease-activated receptors as drug targets in inflammation and pain. *Pharmacol Therap* 2009;123:292–309.
- Annahazi A, Ferrier L, Bézirard V, *et al.* Luminal cysteine-proteases degrade colonic tight junction structure and are responsible for abdominal pain in constipation-predominant IBS. *Am J Gastroenterol* 2013;108:1322–31.
- Roka R, Rosztóczy A, Leveque M, *et al.* A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2007;5:550–5.
- Hyun E, Andrade-Gordon P, Steinhoff M, *et al.* Protease-activated receptor-2 activation: a major actor in intestinal inflammation. *Gut* 2008;57:1222–9.
- Lohman RJ, Cotterell AJ, Suen J, *et al.* Antagonism of protease-activated receptor 2 protects against experimental colitis. *J Pharmacol Exp Therap* 2012;340:256–65.
- Willoughby JB, Willoughby WF. In vivo responses to inhaled proteins. I. Quantitative analysis of antigen uptake, fate, and immunogenicity in a rabbit model system. *J Immunol* 1977;119:2137–46.
- Calabrese EJ, Stanek EJ III, Pekow P, *et al.* Soil ingestion estimates for children residing on a superfund site. *Ecotoxicol Environ Safety* 1997;36:258–68.
- Macchiaverni P, Rekima A, Turfkruyer M, *et al.* Respiratory allergen from house dust mite is present in human milk and primes for allergic sensitization in a mouse model of asthma. *Allergy* 2014;69:395–8.
- Sakamoto K, Hirose H, Onizuka A, *et al.* Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats. *J Surg Res* 2000;94:99–106.
- Cho HJ, Lee HJ, Kim SC, *et al.* Protease-activated receptor 2-dependent fluid secretion from airway submucosal glands by house dust mite extract. *J Allergy Clin Immunol* 2012;129:529–35.
- Van Der Post S, Subramani DB, Backstrom M, *et al.* Site-specific O-glycosylation on the MUC2 mucin protein inhibits cleavage by the *Porphomonas gingivalis* secreted cysteine protease (RgpB). *J Biol Chem* 2013;288:14636–46.
- Comoy EE, Pestel J, Duez C, *et al.* The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN-gamma. *J Immunol* 1998;160:2456–62.
- Kalsheker NA, Deam S, Chambers L, *et al.* The house dust mite allergen Der p1 catalytically inactivates alpha 1-antitrypsin by specific reactive centre loop cleavage: a mechanism that promotes airway inflammation and asthma. *Biochem Biophys Res Comm* 1996;221:59–61.
- Schulz O, Sewell HF, Shakib F. A sensitive fluorescent assay for measuring the cysteine protease activity of Der p 1, a major allergen from the dust mite *Dermatophagoides pteronyssinus*. *Mol Pathol* 1998;51:222–4.
- Zeissig S, Bojarski C, Buerger N, *et al.* Downregulation of epithelial apoptosis and barrier repair in active Crohn's disease by tumour necrosis factor alpha antibody treatment. *Gut* 2004;53:1295–302.
- Lehker MW, Sweeney D. Trichomonad invasion of the mucous layer requires adhesins, mucinases, and motility. *Sex Trans Inf* 1999;75:231–8.
- Lidell ME, Moncada DM, Chadee K, *et al.* Entamoeba histolytica cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *PNAS* 2006;103:9298–303.
- Keely S, Hansbro PM. Lung-gut cross talk: a potential mechanism for intestinal dysfunction in patients with COPD. *Chest* 2014;145:199–200.
- Trompette A, Gollwitzer ES, Yadava K, *et al.* Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* 2014;20:159–66.
- Wang H, Liu JS, Peng SH, *et al.* Gut-lung crosstalk in pulmonary involvement with inflammatory bowel diseases. *World J Gastroenterol* 2013;19:6794–804.
- Bannert C, Bidmon-Fliegenschnee B, Stary G, *et al.* Fc-epsilon-RI, the high affinity IgE-receptor, is robustly expressed in the upper gastrointestinal tract and modulated by mucosal inflammation. *PLoS ONE* 2012;7:e42066.
- Vivinus-Nebot M, Dainese R, Anty R, *et al.* Combination of allergic factors can worsen diarrheic irritable bowel syndrome: role of barrier defects and mast cells. *Am J Gastroenterol* 2012;107:75–81.
- Tovey ER, Willenborg CM, Crisafulli DA, *et al.* Most personal exposure to house dust mite aeroallergen occurs during the day. *PLoS ONE* 2013;8:e69900.
- Korsgaard J. House-dust mites and asthma. A review on house-dust mites as a domestic risk factor for mite asthma. *Allergy* 1998;53:77–83.
- Korsgaard J. Epidemiology of house-dust mites. *Allergy* 1998;53:36–40.

SUPPLEMENTARY FIGURE 1

HDM-induced destruction of ZO-1 in gut epithelial cell line

Caco-2 human intestinal epithelial cell line (EATCC, Port Down, UK) was used to examine the effect of HDM on ZO-1 expression. 6×10^5 cells with or without HDM (100 ng/ml) in 200 μ L Dulbecco's-Modified-Eagle's-Medium (DMEM) were incubated on poly-L-lysine-coated slides (0.1%) for 3 hrs. Cells were fixed (4% paraformaldehyde, 20 minutes), permeabilized (Triton® 0.2%, 2 minutes) and saturated (0.05% saponin 1% BSA, 10 minutes) prior incubation with primary antibody (rabbit anti-ZO-1, 1:100, Invitrogen) for 35 mins at RT in moisture chamber. After 3 PBS washes, cells were incubated with secondary antibody (donkey anti- rabbit Alexa Fluor® 546, 1:1000) for 35 mins. DAPI was added (1:100, 1 min) for nuclear staining and slides covered with Mowiol® 4-88 mounting medium before leaving to dry overnight at 37°C. Slides where primary antibody was omitted were used as controls. All slides were examined with an inverted confocal microscope (Zeiss Axiovert 200M, Gottingen, Germany).

