

# House dust mite-induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production

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## Keywords

allergens; asthma; bronchial epithelium.

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## Abstract

**Background:** House dust mite (HDM) affects the immunological and physical barrier function of airway epithelium, leading to allergic sensitization, airway remodeling, and eosinophilic inflammation in mouse models, although the mechanisms are still largely unknown.

**Objective:** Given the implications for adenosine triphosphate (ATP)-dependent  $\text{Ca}^{2+}$  signaling in allergic sensitization in mice, we sought to determine the role of intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in HDM-induced barrier dysfunction and pro-inflammatory activity of bronchial epithelium.

**Methods:** We investigated the effect of HDM on accumulation of  $[\text{Ca}^{2+}]_i$  levels, barrier function, and CCL20 release in human bronchial epithelial 16HBE cells and primary bronchial epithelial cells (PBECs) from healthy subjects and asthma patients. Involvement of ATP-dependent activation of purinergic receptors and downstream  $\text{Ca}^{2+}$  influx was studied, using the ATP hydrolyzing agent apyrase, the purinergic receptor agonist PPADS, the calcium chelator BAPTA-AM, and calpain inhibitors.

**Results:** Asthma PBECs were more susceptible to HDM-induced barrier dysfunction, CCL20 secretion, and  $\text{Ca}^{2+}$  influx than healthy PBECs. Furthermore, we show that the HDM-induced increase in CCL20 in PBECs and 16HBE cells and the HDM-induced barrier dysfunction in 16HBE cells are dependent on  $[\text{Ca}^{2+}]_i$  accumulation. Additionally, we demonstrate that  $[\text{Ca}^{2+}]_i$  accumulation is initiated partly through the activation of purinergic receptors, which contributes to HDM-induced epithelial barrier dysfunction by disruption of cell–cell contacts, but not CCL20 secretion.

**Conclusion:** Our data show for the first time that  $\text{Ca}^{2+}$  signaling plays a crucial role in barrier dysfunction and the pro-inflammatory response of bronchial epithelium upon HDM exposure and may thus have important implications for the development of allergic asthma.

## Abbreviations

AJ, adherens junction; AUC, area under the curve; ATP, adenosine triphosphate; ER, endoplasmic reticulum; HDM, house dust mite; NHBE, normal human bronchial epithelium; PAR, protease-activated receptor; PBECs, primary bronchial epithelial cells; SERCA, sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase; TJ, tight junction.

Allergic asthma is characterized by the presence of allergen-specific IgE, Th2 cell-mediated eosinophilic airway inflammation, airway hyper-reactivity (AHR), and airway remodeling. Inhaled allergens first encounter the airway epithelium, which acts as a barrier to prevent their access to the submucosa. Epithelial barrier function is maintained by the formation of cell–cell contacts that consist of tight junction (TJ) proteins, for example, occludin, zona occludens (ZO)-1, and claudins,

which restrict permeability, and adherens junction proteins, for example, E-cadherin, which is a crucial building block for all epithelial junctions (1). Loss of epithelial integrity with reduced expression of ZO-1 and E-cadherin has been reported in the airway epithelium of asthma patients (2, 3).

House dust mite (HDM), the most common allergen in atopic asthma, has a complex composition, containing many biochemical molecules that act on the airway epithelium to cause disruption of cell–cell contacts (4). We and others have shown that HDM not only disrupts the airway epithelial barrier (4, 5), but also promotes airway epithelial cells to produce pro-inflammatory cytokines that attract and activate immune cells (6–9). The proteolytic activity of HDM was initially considered to be a major contributor to epithelial responses and allergic sensitization in asthma (5, 10). However, we recently demonstrated that HDM-induced epithelial barrier dysfunction and CCL20 production, effects that are related to the allergenicity of HDM in a mouse model, are independent on serine- and/or cysteine protease activities (4).

Of interest, the fungal aeroallergen *Alternaria alternata* has been shown to induce protease-activated receptor 2 (PAR)-2 activation by proteases other than serine protease (11). Additionally, exposure of bronchial epithelial cells to *A. alternata* resulted in the extracellular release of adenosine triphosphate (ATP). ATP acts on the P2 purinergic receptors (P2XR and P2YR) to induce an intracellular Ca<sup>2+</sup> influx (12) and has been shown to be critically involved in sensitization to HDM in a mouse model of asthma (13). Similarly, specific components of HDM, that is, proteases and  $\beta$ -glucan, may activate pattern-recognition receptors (PRRs) on airway epithelial cells, including PAR-2 (14) and dectin-1, respectively (15). Activation of these receptors leads to increased intracellular Ca<sup>2+</sup> concentration [Ca<sup>2+</sup>]<sub>i</sub> levels, resulting in the activation of pathways involved in the production of pro-inflammatory cytokines and chemokines, including CCL20, a critical chemokine in allergen-induced airway inflammation in asthma (14, 16). In addition, it is known that Ca<sup>2+</sup> can activate calpain, an intracellular cysteine protease that has been implicated in the cleavage of epithelial junction proteins (17).

We therefore hypothesized that HDM may induce Ca<sup>2+</sup> signaling in bronchial epithelial cells and that this may be crucial for its effects on barrier function as well as pro-inflammatory cytokine production.

## Materials and methods

### Cell culture

The human bronchial epithelial cell line 16HBE14o- (16HBE) was kindly provided by Dr D.C. Gruenert (University of California, San Francisco, CA, USA) and cultured in Eagle's minimum essential media (EMEM; Life Technologies Europe BV, Bleiswijk, the Netherlands) supplemented with 10% FCS in collagen-coated flasks as described previously (4, 18). Primary bronchial epithelial cells (PBECs) were obtained by brushings in healthy and asthmatic individuals as described

previously (18, 19). All subjects were nonsmokers ( $\leq 10$  pack-years, no smoking in the last year), between 18 and 65 years old and free of other lung diseases (see Table 1 for patient characteristics). Asthma patients were included if they had a doctor's diagnosis of asthma and documented bronchial hyper-responsiveness, that is, PC<sub>20</sub> AMP  $< 80$  mg/ml, PC<sub>20</sub> methacholine  $< 8$  mg/ml, or PC<sub>20</sub> histamine  $< 8$  mg/ml. Subjects did not use ICS, long-acting  $\beta$ -agonists, and long-acting anticholinergics for at least 4 weeks preceding the study. The Medical Ethics Committee of the University Hospital of Groningen approved the study, and all participants gave their written informed consent. PBECs and normal human bronchial epithelial cells (NHBE; Lonza, Walkersville, MD, USA) were cultured as previously described (18, 19) in hormonally supplemented bronchial epithelium growth medium (BEGM; Lonza) on collagen/fibronectin-coated flasks.

### Cell stimulation

Cells were grown to 95–98% confluence and serum or hormone/growth factor deprived overnight before stimulation. Cells were stimulated with 50  $\mu$ g/ml HDM (Greer Laboratories, Lenoir, NC, USA), 400  $\mu$ M ATP (Sigma-Aldrich, St Louis, MO, USA), 0.1  $\mu$ M ionomycin (Sigma-Aldrich), 250  $\mu$ g/ml specific protease-activated receptor-2 (PAR-2) agonist peptide (SLIGRL-NH<sub>2</sub>), 250  $\mu$ g/ml control peptide (LRGILS-NH<sub>2</sub>; both from GenScript USA Inc., Piscataway, NJ, USA), or 2  $\mu$ M thapsigargin (Sigma-Aldrich). Prior to HDM stimulation, cells were incubated with or without 15 U/ml apyrase (Sigma-Aldrich), 50  $\mu$ M 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetrakis (acetoxymethyl ester, BAPTA-AM; Tocris Bioscience, Bristol, UK), 30  $\mu$ M pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS; Tocris Bioscience), 50  $\mu$ M calpain I inhibitor (LLNL; Merck, Darmstadt, Germany), or 50  $\mu$ M calpain inhibitor II (ALLM; Merck) for 30 min.

See Data S1 for additional details.

### Statistical analysis

Electric cell-substrate impedance sensing (ECIS) data and Ca<sup>2+</sup> influx data were analyzed by two-way ANOVA. The differences in area under the curve (AUC) between PBECs from asthmatic patients and healthy controls were analyzed with the Mann–Whitney *U*-test. All other experiments were analyzed by the Student's *t*-test for paired observations.

**Table 1** Patient characteristics

Subject	Control ( <i>n</i> = 6)	Asthma ( <i>n</i> = 5)
Age (years)	28 (21–44)	44 (33–50)
Gender (M, %)	2 (40)	3 (60)
Pack-years	0 (0–0)	0 (0–4)
FEV <sub>1</sub> pred (%)	103 (103–112)	84 (76–114)
FEV <sub>1</sub> /FVC (%)	82 (75–94)	73 (58–78)

Median levels ( $\pm$ range) are shown.

## Results

### PBECs from asthmatic patients show stronger barrier dysfunction, E-cadherin delocalization, CCL20 induction, and Ca<sup>2+</sup> influx in response to HDM than healthy PBECs

We have previously demonstrated that HDM-induced epithelial barrier dysfunction and CCL20 production are related to the allergenicity of HDM in a mouse model (4). To enhance the relevance of these findings, we studied the effect of HDM extract on epithelial barrier function and CCL20 production in PBECs from healthy and asthmatic individuals. We analyzed barrier function by electrical resistance measurements using ECIS (18). In PBECs from asthma patients, we observed that HDM exposure caused a reduction in electrical resistance over time, starting within a couple of hours and remaining for at least 24 h (Fig. 1A), with a significant decrease in the AUC over 24 h (Fig. 1B). Importantly, this was not observed in healthy PBECs, where HDM did not induce loss of electrical resistance (Fig. 1A,B). In line with these findings, immunofluorescent staining revealed that HDM exposure (60 min) caused disruption of E-cadherin membrane expression, which was more pronounced in PBECs from asthmatic than from healthy individuals (Fig. 1C). Furthermore, we studied the CCL20 secretion in PBECs from healthy and asthmatic persons after HDM exposure and observed that HDM caused a significantly stronger increase in CCL20 secretion in PBECs from asthma patients than in healthy control PBECs (Fig. 1D and absolute values in Table 2).

To gain more insight in the mechanisms of HDM-induced barrier dysfunction and CCL20 release, we investigated whether HDM is able to induce accumulation of [Ca<sup>2+</sup>]<sub>i</sub> levels. We observed that HDM induces a significant increase in Ca<sup>2+</sup> accumulation at 180 min in PBECs from both asthma patients and healthy controls (Fig. 1E). Of note, [Ca<sup>2+</sup>]<sub>i</sub> levels increased more rapidly upon HDM exposure in PBECs from asthmatic patients, where levels were already significantly increased after 90 min, with a significant difference between the groups at 60 min (Fig. 1E). Furthermore, the HDM-induced CCL20 release in PBECs from asthma patients was significantly reduced after pretreatment with BAPTA-AM, indicating a Ca<sup>2+</sup>-dependent effect (Fig. 1F).

**Table 2** CCL20 levels measured by ELISA in supernatants from PBECs from healthy and asthma patients

PBECs	Absolute values (pg/ml)
Healthy baseline	251.29 ± 45.12
Healthy HDM	511.25 ± 89.34
Asthma baseline	145.99 ± 32.44
Asthma HDM	581.04 ± 125.93

HDM, house dust mite; PBECs, primary bronchial epithelial cells. Mean levels (±SEM) are shown.

### HDM-induced [Ca<sup>2+</sup>]<sub>i</sub> accumulation contributes to epithelial barrier dysfunction and secretion of CCL20

We aimed to further investigate the mechanisms of HDM-induced barrier dysfunction and CCL20 release in 16HBE cells, without the disadvantage of limited cell numbers. We observed that similar to PBECs, HDM induces a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> levels within 30–180 min in 16HBE cells (Fig. 2A). Next, we assessed whether the Ca<sup>2+</sup> influx contributes to HDM-induced barrier dysfunction by measuring electrical resistance in a monolayer of 16HBE cells using ECIS (20). Exposure to HDM induced a transient reduction in epithelial resistance, with a maximum effect at ~10–20 min and recovery to baseline values within 60 min, followed by a second, less steep, and longer-lasting decrease in resistance, similar to the decrease observed in PBECs from asthma patients (18) (Fig. 2B). Because the first rapid effect of HDM in 16HBE was stronger than the second, we focused on the first effect. Of interest, ionomycin induced a similar transient fall in epithelial resistance, from which the cells also recovered within 60 min (Fig. 2B). In line with this involvement of Ca<sup>2+</sup> in epithelial barrier dysfunction, pretreatment of the cells with calcium chelator BAPTA-AM significantly inhibited the HDM-induced reduction in resistance (Fig. 2C).

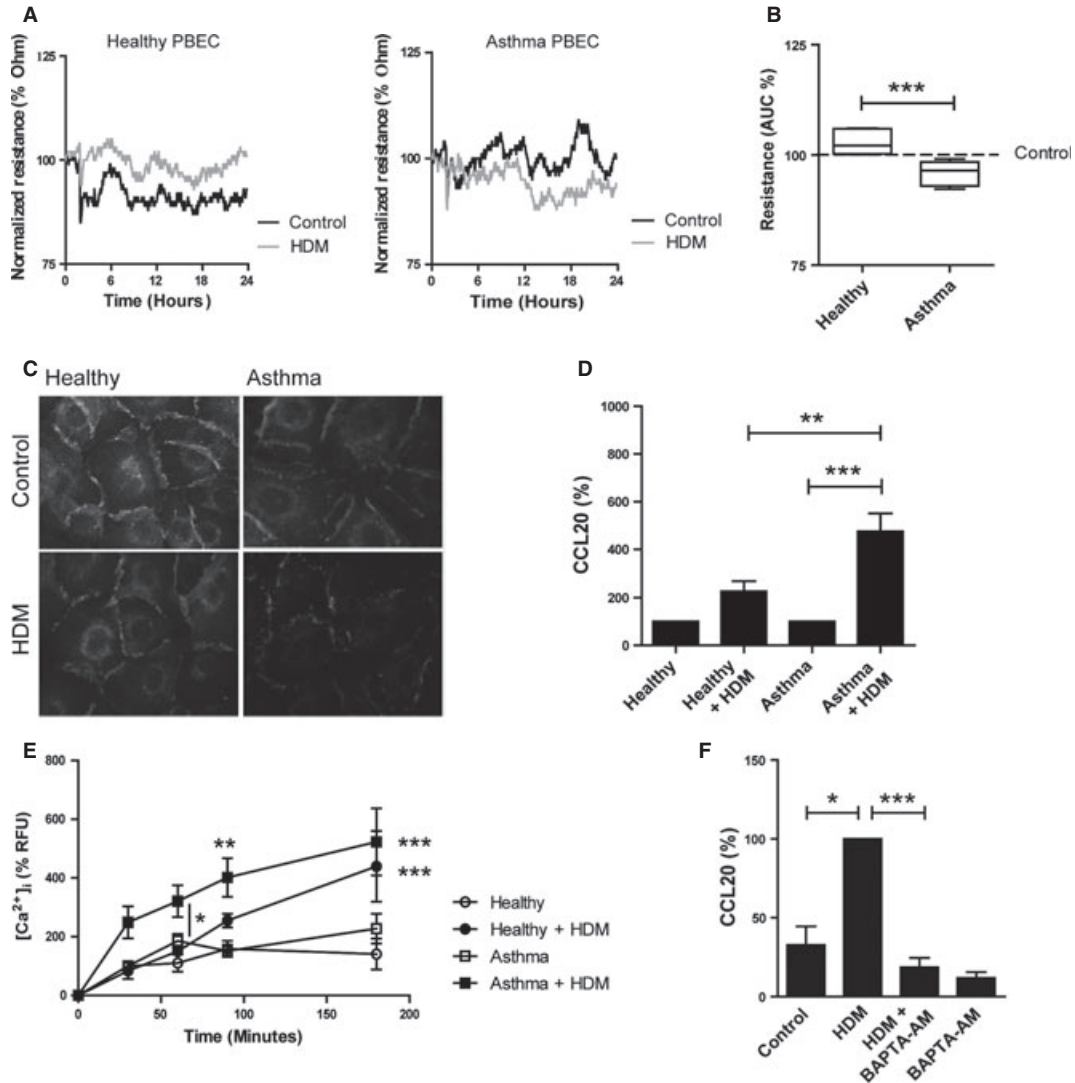
In addition, we were interested to see whether the HDM-induced production of CCL20 also depends on Ca<sup>2+</sup> signaling. We exposed 16HBE cells to HDM and ionomycin in the absence or presence of BAPTA-AM. HDM as well as ionomycin induced a significant increase in CCL20 levels in 16HBE cells (Fig. 2D). Pretreatment of cells with BAPTA-AM was able to inhibit the HDM-induced CCL20 secretion (Fig. 2D). Moreover, we observed that intracellular Ca<sup>2+</sup> accumulation is also involved in CCL20 secretion in PBECs, because BAPTA-AM inhibited the HDM-induced CCL20 secretion in NHBE cells as well (Fig. 2E).

Taken together, these results indicate that increased [Ca<sup>2+</sup>]<sub>i</sub> levels are involved in HDM-induced barrier dysfunction and CCL20 production in bronchial epithelium.

### HDM-induced Ca<sup>2+</sup> signaling responsible for barrier dysfunction is partly mediated through an ATP/purinergic receptor-dependent mechanism

Next, we studied whether HDM may increase Ca<sup>2+</sup> signaling through ATP/purinergic receptor-dependent mechanisms. We treated the cells with purinergic receptor inhibitor PPADS and ATP hydrolyzing enzyme apyrase prior to HDM exposure and measured [Ca<sup>2+</sup>]<sub>i</sub> levels. Treatment with both inhibitors partially, but significantly, inhibited the HDM-induced accumulation of [Ca<sup>2+</sup>]<sub>i</sub> at 180 min (Fig. 3A,B).

To further underscore the role of ATP/purinergic receptor signaling in HDM-induced barrier dysfunction, we assessed the effect of ATP, PPADS, and apyrase on epithelial barrier dysfunction as measured by ECIS. ATP was able to induce a similar transient fall in epithelial resistance as HDM (Fig. 3C), which could also be reduced by treatment with BAPTA-AM (see Fig. S1A). Furthermore, pretreatment with apyrase significantly inhibited the HDM-induced fall in



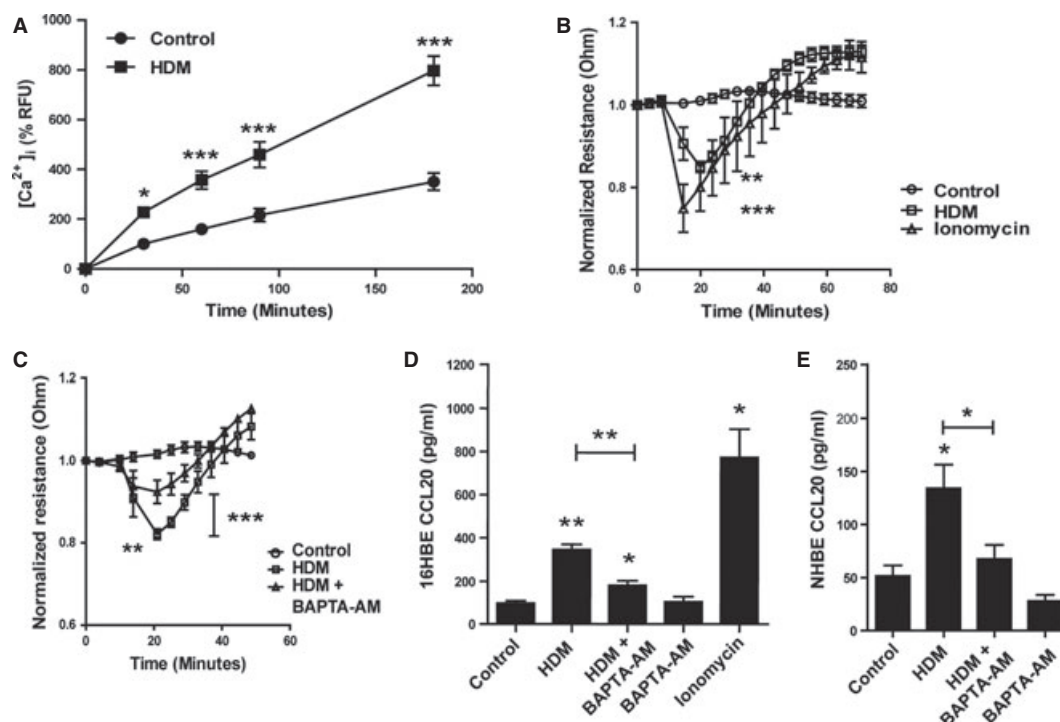
**Figure 1** Primary bronchial epithelial cells (PBECs) from asthma patients respond stronger to house dust mite (HDM) than PBECs from healthy individuals with regard to barrier function, CCL20 secretion, and Ca<sup>2+</sup> influx. Resistance was measured by ECIS, and levels were normalized to the levels prior to HDM (50  $\mu$ g/ml) or medium (control) exposure. (A) A representative of PBECs from a healthy and an asthmatic individual is shown. (B) Median (range) area-under-the-curve (AUC) resistance values of PBECs from healthy ( $n = 6$ ) and asthma patients ( $n = 5$ ; %) calculated over 24 h. (C) Immunofluorescent E-cadherin staining after medium or HDM exposure (60 min). Representative images from four asthma and five healthy PBEC cultures are shown. (D) CCL20 levels

measured by ELISA in supernatants from PBECs from healthy ( $n = 4$ ) and asthma patients ( $n = 5$ ) exposed to HDM (24 h). Baseline levels were set to 100%, and mean levels ( $\pm$ SEM) are shown. (E) Normalized [Ca<sup>2+</sup>]<sub>i</sub> (relative fluorescent unit [%RFU]) levels measured in PBECs from healthy individuals ( $n = 4$ ) and asthmatic patients ( $n = 4$ ) after exposure to HDM (50  $\mu$ g/ml). (F) CCL20 levels measured by ELISA in supernatants from PBECs from asthma patients ( $n = 4$ ) exposed to HDM in the presence of absence of BAPTA-AM (24 h). HDM levels were set to 100%, and mean levels ( $\pm$ SEM) are shown. \* $P < 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P < 0.001$  between control and HDM-treated cells or as indicated.

resistance (Fig. 3D). In addition, using immunofluorescent staining, we observed that E-cadherin was mainly localized at the membrane in untreated cells (Fig. 4A) and that exposure to HDM induced delocalization of E-cadherin in 60 min (Fig. 4A). Similar to the effects observed on electrical resistance, pretreatment with apyrase reduced the HDM-induced delocalization of E-cadherin (Fig. 4A). Western blot analysis

of occludin showed that both HDM and ATP induce cleavage of occludin, as demonstrated by the appearance of smaller fragments of ~31, ~30, and 22 kD, which was prevented by the use of BAPTA-AM, PPADS as well as calpain inhibitors I (LLNL) and II (ALLM; Fig. 4B).

These data indicate that HDM induces ATP/purinergic receptor signaling, leading to [Ca<sup>2+</sup>]<sub>i</sub> accumulation and



**Figure 2** Ca<sup>2+</sup> signaling is involved in house dust mite (HDM)-induced barrier dysfunction and CCL20 production in 16HBE and normal human bronchial epithelium (NHBE) cells. (A) Normalized [Ca<sup>2+</sup>]<sub>i</sub> (relative fluorescent unit [%RFU]) levels after exposure to HDM (50 μg/ml). Electrical resistance levels were measured by ECIS and normalized to values just prior to exposure to (B) HDM or ionomycin or (C) in the presence or absence of BAPTA-AM.

CCL20 levels measured by ELISA in supernatant (8 h) from 16HBE cells (*n* = 4) exposed to (D) HDM or ionomycin in the presence or absence of BAPTA-AM and (E) supernatant (8 h) from NHBE cells (*n* = 5) exposed to HDM and/or BAPTA-AM. Mean levels (±SEM) are shown \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 between control and stimulated cells or as indicated.

downstream calpain activation, and that this effect is involved in the HDM-induced barrier dysfunction.

Additionally, we tested whether ATP/purinergic receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub> accumulation is involved in the increase in CCL20 secretion upon HDM exposure. However, pretreatment of cells with either PPADS or apyrase did not inhibit the HDM-induced CCL20 secretion in 16HBE cells (data not shown), indicating that HDM induces CCL20 release, in contrast to epithelial barrier function, through an ATP/purinergic receptor-independent mechanism.

#### A different pool of [Ca<sup>2+</sup>]<sub>i</sub> contributes to the HDM-induced secretion of CCL20

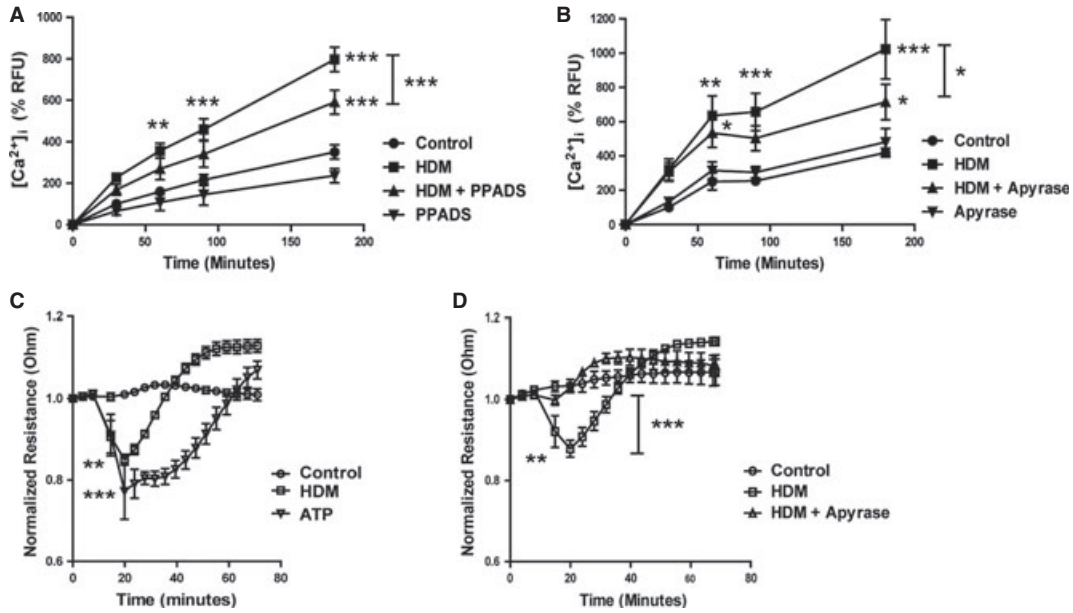
Thus, HDM-induced CCL20 release involves the activation of Ca<sup>2+</sup> signaling through another mechanism. Because HDM contains proteases that can activate PAR-2 and subsequently induce intracellular Ca<sup>2+</sup> signaling in bronchial epithelial cells (6, 21, 22), we examined whether activation of PAR-2 was also able to increase CCL20 secretion. The use of a specific PAR-2 agonist peptide (PAR-2 AP), but not the control peptide, induced a significant increase in CCL20 secretion, which was reduced by BAPTA-AM (Fig. 5A), indicating involvement of Ca<sup>2+</sup>. In contrast, PAR-2 AP did not affect epithelial barrier function (Fig. S1B).

In addition to purinergic receptor and PAR-2 downstream signaling, [Ca<sup>2+</sup>]<sub>i</sub> levels are regulated by Ca<sup>2+</sup> pumps on the endoplasmic reticulum (ER), including sarco-endoplasmic reticulum (ER) Ca<sup>2+</sup> ATPase (SERCA) (23, 24). Inhibition of SERCA leads to increased cytosolic Ca<sup>2+</sup> levels and ER stress (25). To test whether SERCA regulates HDM-induced effects on CCL20, we used the SERCA inhibitor thapsigargin. We exposed the cells to a concentration series of thapsigargin (0.1–5 μM) and observed that thapsigargin in a concentration of 0.1 μM induced a significant increase in CCL20 release, while this upregulatory effect was smaller and not significant at higher concentrations (Fig. 5B). Importantly, in combination with HDM, 2 μM thapsigargin significantly and synergistically enhanced CCL20 secretion, leading to approximately fivefold increase over baseline levels (Fig. 5C).

Overall, these results demonstrate that elevated [Ca<sup>2+</sup>]<sub>i</sub> upon HDM exposure or by PAR-2 activation or by inhibition of SERCA leads to increased CCL20 secretion that presumably contributes to CCL20 secretion in the asthmatic response after HDM exposure.

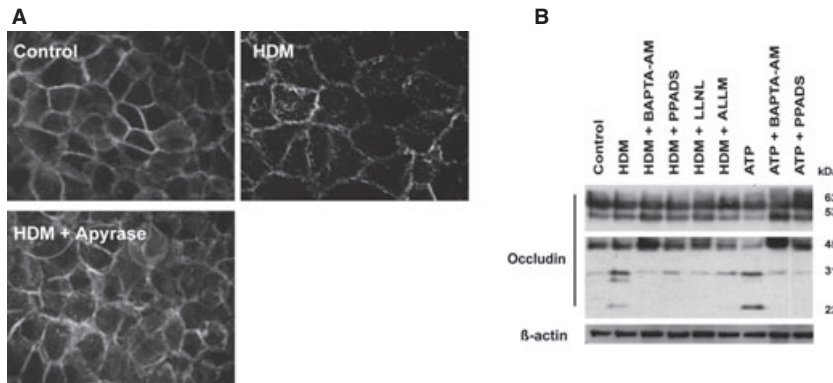
#### Discussion

We have previously shown that HDM-induced epithelial barrier dysfunction and CCL20 secretion are related to



**Figure 3** Adenosine triphosphate (ATP)/purinergic receptor signaling is involved in house dust mite (HDM)-induced [Ca<sup>2+</sup>]<sub>i</sub> accumulation and barrier dysfunction in 16HBE cells. Normalized [Ca<sup>2+</sup>]<sub>i</sub> (relative fluorescent unit [%RFU]) levels after exposure to HDM (50 μg/ml) in the presence or absence of (A) PPADS or (B) apyrase.

Electrical resistance levels measured by ECIS normalized to values just prior to exposure to (C) HDM or ATP (D) in the presence and absence of apyrase. Mean levels (±SEM) are shown (n = 4). \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 between control and HDM-treated cells or as indicated.

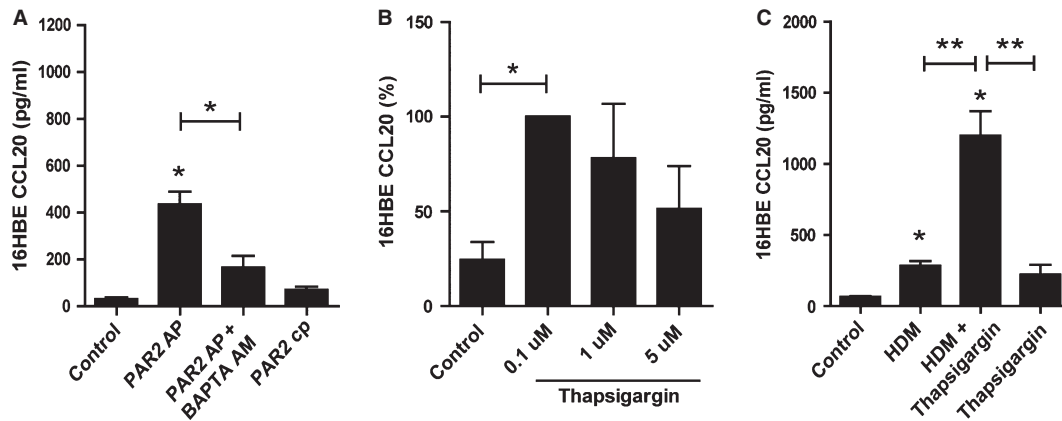


**Figure 4** Adenosine triphosphate (ATP)/purinergic receptor signaling is involved in E-cadherin delocalization and occludin degradation in 16HBE cells. Immunofluorescent staining for E-cadherin upon 15-min exposure to (A) medium (control), house dust mite (HDM),

and HDM in the presence of apyrase in 16HBE cells. (B) Western blot detection of occludin upon 15-min exposure to HDM or ATP with/without BAPTA-AM, PPADS, LLNL, and ALLM in 16HBE cells. Representatives of three independent experiments are shown.

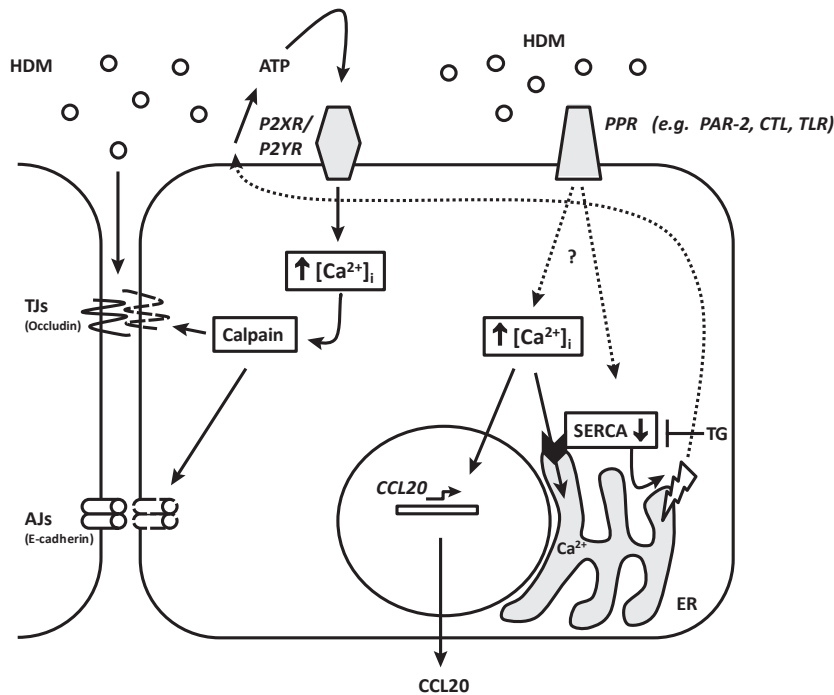
HDM-induced allergic sensitization in mice. In the present study, we demonstrate that PBECs from asthma patients respond stronger to HDM than PBECs from healthy persons with respect to barrier dysfunction, CCL20 secretion as well as Ca<sup>2+</sup> influx. Furthermore, we now provide evidence that HDM induces [Ca<sup>2+</sup>]<sub>i</sub> accumulation involved in the HDM-induced epithelial barrier dysfunction and CCL20 secretion. Our findings are in line with recent data showing that the allergen *A. alternata* induces serine-independent PAR-2 activation (11) as well as the release of ATP in bronchial

epithelial cells, without inducing cell death (26). Our data clearly demonstrate that increased Ca<sup>2+</sup> signaling leads to epithelial barrier dysfunction, the delocalization of E-cadherin, and the degradation of occludin (see Fig. 6 for the proposed model). Based on our data, we suggest that the epithelial barrier dysfunction, measured in a confluent monolayer of cells in the ECIS system, is, at least partly, mediated by ATP/purinergic receptor activation, as evidenced by the use of apyrase and PPADS. Our results further suggest that the activation of Ca<sup>2+</sup> downstream protein calpain (24) may be involved in the



**Figure 5** Protease-activated receptor 2 (PAR)-2 activation and thapsigargin enhance CCL20 secretion in 16HBE cells. CCL20 levels measured by ELISA in supernatants from 16HBE cells (*n* = 3–5) exposed to (A) PAR-2 AP and/or BAPTA-AM, (B) thapsi-

gargin in different concentrations (0.1–5 μM), and (C) house dust mite (HDM) and/or 2 μM thapsigargin (8 h). Mean levels (±SEM) are shown. \**P* < 0.05 and \*\**P* < 0.01 between control and HDM-treated cells or as indicated.



**Figure 6** Proposed model of house dust mite (HDM)-induced Ca<sup>2+</sup> influx and its effect on barrier dysfunction and CCL20 production. Arrows indicate known pathways and/or pathways indicated by our study, and dashed arrows are suggested pathways. AJs, adherens junctions; CTL, C-type lectin receptors; ER, endoplasmic reticulum;

HDM, house dust mite; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; protease-activated receptor (PAR)-2, protease-activated receptor 2; PRRs, pattern-recognition receptors; SERCA, sarco-endoplasmic reticulum Ca<sup>2+</sup> ATPase; TG, thapsigargin; TJs, tight junctions; TLR, Toll-like receptor.

latter effect, in agreement with a previous study showing that calpain is able to cleave occludin (17) (see also Fig. 6). With respect to the potential mechanism regarding E-cadherin delocalization, it has been reported that ATP/purinergic receptor-mediated Ca<sup>2+</sup> signaling can induce A Disintegrin and Metalloproteases-17 (ADAM-17)-dependent epidermal growth factor receptor (EGFR) activation (27), the latter of

which we have shown to be responsible for HDM-induced delocalization of E-cadherin (18). In addition, we observed that the HDM-induced CCL20 secretion in 16HBE cells, NHBE cells, as well as PBECs from asthmatic patients was dependent on Ca<sup>2+</sup> signaling. Accordingly, downstream of Ca<sup>2+</sup>, the calcium–calcineurin–nuclear factor of activated T-cell (NFAT) pathway has been implicated

in the transcriptional activation of CCL20 in airway epithelium (28, 29), although it requires further investigation to confirm the involvement of this pathway. In contrast to the HDM-induced barrier dysfunction, our data exclude a role for ATP/purinergic receptor signaling in HDM-induced CCL20 secretion. This indicates that CCL20 production is mediated through a different intracellular pool of Ca<sup>2+</sup>, with no relation to ATP/purinergic receptor signaling. Of note, PAR-2 activation has been reported to increase [Ca<sup>2+</sup>]<sub>i</sub>, as well as CCL20 secretion in epithelial cells (14, 21). In line with this, we observed that PAR-2 activation induces CCL20 secretion in a Ca<sup>2+</sup>-dependent manner, without affecting epithelial barrier function. We previously reported that HDM-induced CCL20 secretion is independent of serine proteases (4). Indeed, other proteases present in HDM may lead to PAR-2 activation, for instance aspartate protease (11). Future studies will be directed to investigate the involvement of PAR-2 in the HDM-induced effects on CCL20 secretion. In addition to PAR-2 activation, other mechanisms could be involved in the HDM-induced Ca<sup>2+</sup> influx, and the HDM components Der p2, Der p5, and β-glucan structures have been reported to initiate intracellular Ca<sup>2+</sup> accumulation (7, 16). Der p2 can interact with the Toll-like receptor-4 (TLR4) (30) and TLR2 (31), which have both been linked to Ca<sup>2+</sup> signaling (17, 32). Furthermore, HDM can contain endotoxin, which also acts on TLR4 to induce Ca<sup>2+</sup> signaling (33). We have previously demonstrated that the ability of HDM extract to induce epithelial CCL20 secretion is not related to its endotoxin content, but may relate to Der p2 levels (4). Thus, activation of various PRRs may lead to increased [Ca<sup>2+</sup>]<sub>i</sub> levels and subsequent production of CCL20 (see also Fig. 6). In addition, the ER is a well-known regulator for intracellular Ca<sup>2+</sup> levels by the expression of SERCA (23). Inactivation of SERCA leads to increased cytoplasmic levels of Ca<sup>2+</sup> and ER stress (34). We found that the SERCA inhibitor thapsigargin synergistically increased the HDM-induced CCL20 secretion. Thus, reduced levels of SERCA and/or increased ER stress may increase the epithelial release of CCL20 in response to HDM by a mechanism involving increased Ca<sup>2+</sup> accumulation (see Fig. 6). In this respect, it is noteworthy that reduced expression of SERCA2 has been observed in smooth muscle cells from asthmatics (35). Furthermore, *ORMDL3*, which regulates the SERCA pump, has been identified as an important asthma-associated gene (23).

The precise mechanisms of HDM-induced Ca<sup>2+</sup> accumulation in epithelium need further investigation, although we show that the pool of Ca<sup>2+</sup> responsible for barrier dysfunction

is, at least in part, dependent on purinergic receptor signaling. It remains unclear whether and how ATP is released, but it is of interest to note that ER stress has been implicated in the release of ATP (36) (Fig. 6). Future studies will be of interest to reveal whether epithelium from asthma patients displays decreased SERCA levels compared with healthy controls and whether HDM can induce ER stress.

In conclusion, we show for the first time that HDM-induced intracellular Ca<sup>2+</sup> accumulation is involved in barrier dysfunction as well as the pro-inflammatory response of bronchial epithelium to HDM. Our findings may provide novel insight in therapeutic strategies for asthma aimed toward the airway epithelial barrier.

### Author contributions

S. Post performed the 16HBE and NHBE cell experiments and PBEC IF staining, analyzed and interpreted the data, and drafted the manuscript. M. R. Jonker cultured PBECs and performed the calcium influx and BAPTA experiments in these cells. N. Kliphuis performed the culture and additional experiments in PBECs. M. Nawijn designed and supervised experiments and reviewed the manuscript. M. vd Berge recruited the patients and provided the bronchial brushings. A. v Oosterhout supervised the study and reviewed the manuscript. I. Heijink coordinated, designed, and supervised the study and was involved in the manuscript writing.

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### Conflict of interest

Maarten van den Berge has received research support from AstraZeneca. All other authors have no conflict of interest.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** The effect of ATP and PAR-2 AP on epithelial barrier function.

**Data S1.** Materials and methods.

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