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ADAM10 mediates the house dust mite-induced release of chemokine ligand CCL20 by airway epithelium

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Abstract

Background: House dust mite (HDM) acts on the airway epithelium to induce airway inflammation in asthma. We previously showed that the ability of HDM to induce allergic sensitization in mice is related to airway epithelial CCL20 secretion.

Objective: As a disintegrin and metalloprotease (ADAM)s have been implicated in chemokine shedding, we sought to determine their involvement in HDM-induced release of chemokines, including CCL20, by airway epithelial cells.

Methods: We studied the effects of pharmacological ADAM inhibitors as well as ADAM10 and ADAM17 siRNA downregulation on chemokine release using (multiplex) ELISA in supernatants from HDM-exposed human bronchial epithelial 16HBE cells and primary normal human bronchial epithelial cells (NHBE) at 4–24 h.

Results: House dust mite markedly increased CCL20 levels in both 16HBE and NHBE cells (16–24 h). In 16HBE cells, the HDM-induced increase was observed as early as 4 h upon exposure and the use of specific inhibitors indicated the involvement of ADAM10/17-mediated shedding. siRNA knockdown of ADAM10, but not of ADAM17, significantly reduced the HDM-induced release of CCL20 in both 16HBE and NHBE cells. A similar effect was observed for HDM-induced CCL2, CCL5, and CXCL8 release in NHBE cells. The HDM-induced increase in CCL20 levels was not affected by protein synthesis inhibitor cycloheximide nor protein transport inhibitor monensin, indicating that HDM induces surface shedding of chemokines.

Conclusion: Our data show for the first time that ADAM10 activity contributes to HDM-induced shedding of chemokines, including CCL20. The ADAM10/CCL20 axis may be a target for novel therapeutic strategies in asthma.

Allergic asthma is characterized by allergen-induced airway inflammation, airway hyper-responsiveness, and remodeling. Inhaled allergens first encounter the airway epithelium, which acts as a barrier to prevent their access to the submucosa. Loss of airway epithelial barrier function in patients with asthma may facilitate transepithelial transport of allergens to immune cells in the submucosa, leading to allergic sensitization. In addition to its barrier function, the airway epithelium is a source of pro-inflammatory cytokines, chemokines, and growth factors, acting on immune

Abbreviations
ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin motifs; Ca2+, calcium; CCR6, CC chemokine receptor 6; DCs, dendritic cells; EGFR, epidermal growth factor receptor; GPCRs, G protein-coupled receptors; HDM, house dust mite; MMP, matrix metalloproteinase; NHBEs, normal human bronchial epithelial cells; PAR-2, protease-activated receptor; PBECs, primary bronchial epithelial cells; RAGE, receptor for advanced glycation endproducts; TIMP, tissue inhibitor of metalloproteinase.
and structural cells in the submucosa to promote airway inflammation and remodeling, especially when damaged (1, 2). House dust mite (HDM), a major aeroallergen in asthma, can disrupt epithelial barrier function and induce pro-inflammatory activity (3–6), the latter by pattern recognition of β-glucan moieties, leading to the release of CCL20 (6). We recently demonstrated that HDM-induced epithelial barrier dysfunction and CCL20 production are related to the allergenicity of HDM in a mouse model and are independent of serine/cysteine protease activity (5). Furthermore, we observed that HDM activation of pattern recognition receptors on epithelial results in a stronger increase in CCL20 in cells from patients with asthma than from healthy controls (7). CCL20 is a chemotractant for CC chemokine receptor 6 (CCR6+ T helper (Th)-17 cells and dendritic cells (DCs), and allergen challenge results in an increase in both CCL20 levels and CCR6+ DC counts in the airways of patients with asthma (8). Moreover, increased CCL20 levels have been observed in severe compared to nonsevere patients with asthma (9).

Cytokines/chemokines, growth factors, and their receptors are typical substrates of the a disintegrin and metalloproteinase (ADAMs) family, membrane-anchored proteases that are able to cleave the extracellular domains of membrane-bound proteins (10, 11). Expression of ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17 and ADAM with thrombospondin motifs (ADAMTS)-1, ADAMTS2, and ADAMTS12 has been demonstrated in lung tissue (12). Specifically, lung epithelial cells have been shown to express ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, and ADAMTS1. Furthermore, aberrant levels of ADAM8, ADAM9, ADAM12, ADAMTS1, and ADAM15 mRNA were reported in induced sputum from asthmatic patients (12), while a genomic study demonstrated that ADAM10, ADAM17, ADAM28 and ADAMTS4, ADAMTS9, and ADAMTS15 are over-expressed in a chronic mouse model of asthma (13). By the so-called ectodomain shedding of pro-inflammatory cytokines (e.g. TNF-α or heparan sulfate-bound chemokines, ADAMs may contribute to airway inflammation in asthma. The use of a specific ADAM10 inhibitor in a mouse model of asthma has indicated that ADAM10 is important for maintaining Th2-mediated inflammation in the lung (14). Of note, ADAM10-dependent ligand shedding is induced by calcium (Ca2+) signaling (10, 15) and we recently showed that the HDM-induced secretion of CCL20 is mediated by intracellular Ca2+ signaling (7). However, the mechanism by which HDM-induced Ca2+ signaling leads to the secretion of CCL20 remained unclear. It is of interest to further dissect this mechanism given the implications for CCL20 in asthmatic airway inflammation. Therefore, we sought to determine whether specific ADAMs may be involved in the HDM-induced release of chemokines by airway epithelial cells. Using pharmacological inhibitors and siRNA downregulation of ADAM10 and ADAM17, we show that specifically ADAM10 is involved in the release of various chemokines including CCL20 from human bronchial epithelial cells.

Material and Methods

Cell culture

The human bronchial epithelial cell line 16HBE14o- (16HBE) was kindly provided by 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 (Hyclone, Logan, UT, USA) in collagen-coated flasks as described before (2). Normal human bronchial epithelial cells from nonsmoking individuals’ cells were obtained from Lonza (Walkersville, MD, USA) and grown in Bronchial Epithelium Growth Medium (BEGM; Lonza) in fibronectin/collagen-coated flasks. Cells were seeded in 24-well plate at 5 × 104 cells/well, grown to 90–95% confluence, serum-deprived (16HBE) or hormonally/growth factor-deprived (NHBE) overnight and treated with 50 μg/ml HDM protein extract (Greer Laboratories, Lenoir, NC, USA) or 0.1 μM ioneomycin (Sigma-Aldrich, St Louis, MO, USA) for 4, 16, or 24 h. Prior to HDM stimulation, cells were incubated with/without 20-μM broad-spectrum matrix metalloproteinase (MMP)-inhibitor TAPI-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1 to 10 μM specific ADAM10 inhibitor GI254023, and 1 to 10 μM dual ADAM17/ADAM10 inhibitor GW280264 (both kindly provided by M. Johnson, GSK, UK), 1 μg/ml cycloheximide (Sigma-Aldrich), 1 μM monensin (Sigma-Aldrich), or 1 μM epidermal growth factor receptor (EGFR) inhibitor AG1478 (Sigma-Aldrich) for 30 min. Vehicle controls for GW280264 and GI254023, that is 0.2% and 0.05% dimethylsulfoxide (DMSO), respectively, did not affect our experimental readout (data not shown).

Small-interfering (si)RNA transfection

Nontargeting siRNA (OR-0030-NEG05) and ADAM10 siRNA (target sequence: GUUGGGAACUCCUUUAAA) were purchased from Eurogentec (Eurogentec Nederland BV, Maastricht, the Netherlands). ADAM17 siRNA (sc-36604) was obtained from Santa Cruz Biotechnology. Cells were grown in 24-well plate to ~50% (16HBE) or ~80% (NHBE) confluence, washed three times with OPTIMEM (Life Technologies Europe BV), and transfected with 20 μM of siRNA oligos using Lipofectamine 2000 transfection reagent (Life Technologies Europe BV). Two days after the transfection, cells were serum-deprived overnight and harvested for Western blot analysis or stimulated with/without 50 μg/ml of HDM for 4 h (16HBE) or 16 h (NHBE).

Cytokine assay in cell supernatants

CCL20 levels were measured in cell-free supernatants using a Duoset ELISA Development Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. CCL2, CCL5, CXCL8, IL-6, GM-CSF, and VEGF were measured by a multiplex ELISA kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instructions.
Western blot analysis

Total cell lysates were obtained as described previously (2). Immunodetection was performed by standard procedures, according to the manufacturer’s guidelines (ECL; Amersham, Buckinghamshire, UK), using anti-ADAM10 (Abcam, Cambridge, UK), anti-ADAM17 and anti-ß-actin (both obtained from Santa Cruz Biotechnology).

Statistical analysis

Experiments in the cell line were analyzed by Student’s t-test for paired observations. Experiments in primary cells were analyzed by the nonparametric Wilcoxon signed rank test for paired observations.

Results

ADAM10 activity contributes to HDM-induced CCL20 release by human bronchial epithelial cells

First, we investigated the effect of HDM on the release of CCL20 in 16HBE cells. We observed that exposure of 16HBE cells to 10 μg/ml HDM did not increase CCL20 levels, whereas both 50 and 100 μg/ml of HDM induced a variable but significant increase in the release of CCL20, without a significant difference between these concentrations (Fig. 1A). The increase in CCL20 secretion was already observed after 4 h and still present after 24 h of exposure to 50 μg/ml HDM (Fig. 1B). The early increase in CCL20 levels suggests that HDM promotes CCL20 release by mechanisms other than de novo synthesis, for instance by surface shedding. To test this, we used several sheddase inhibitors, that is the broad-spectrum MMP inhibitor TAPI-2, ADAM10/ADAM17 inhibitor GW280264, and ADAM10 inhibitor GI254023. It has been demonstrated that GW280264 potently blocks ADAM17 and ADAM10, with IC₅₀ values of 8.0 and 11.5 nM, respectively, while GI254023 displays more than 100-fold stronger affinity for ADAM10 (IC₅₀: 5.3 nM) than for ADAM17 (IC₅₀: 541 nM) (16). Pretreatment of the 16HBE cells with TAPI-2 and GW280264 attenuated the CCL20 release both at baseline and after 24 h HDM stimulation (Fig. 1C). TAPI-2 (20 μM) only modestly inhibited the HDM-induced CCL20 secretion, while a stronger effect was observed for the ADAM10/ADAM17 inhibitor GW280264 (1–10 μM, Fig. 1C). In addition, pre-treatment with the ADAM10 inhibitor GI254023 significantly reduced HDM-induced CCL20 release in a concentration of 3 and

![Figure 1](https://example.com/figure1.png)

Figure 1 Inhibition of ADAM10 attenuates HDM-induced CCL20 release in human bronchial epithelial 16HBE cells. Cells were grown to confluence, serum-starved, and exposed to 10 μg/ml, 50 μg/ml, or 100 μg/ml HDM and HDM (50 μg/ml) for 4 or 24 h. (A and B) CCL20 levels (mean ± SEM, n = 4–5) were measured in cell-free supernatants. (C) CCL20 levels (mean ± SEM, n = 5) upon pretreatment (30 min) of the cells with and without TAPI-2 (20 μM) or GW280264 (GW; 1–10 μM) expressed as percentage of the levels upon treatment with HDM alone (4 h). (D) CCL20 levels (mean ± SEM, n = 5) upon pretreatment (30 min) of the cells with and without GI254023 (GI; 1–10 μM) expressed as percentage of the levels upon treatment with HDM alone (4 h). *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated values.
10 μM, but not 1 μM (Fig. 1D), indicating that the HDM-induced release of CCL20 is, at least partly, mediated through ADAM10. At baseline, GI254023 did not affect CCL20 levels (Fig. 1D), indicating that this effect is specific for HDM stimulation.

To assess whether ADAM10 and/or ADAM17 is involved in the HDM-induced CCL20 secretion at 4 h, we treated 16HBE cells with ADAM10 siRNA and ADAM17 siRNA. We verified the downregulation of both ADAMs by siRNA treatment using Western blotting, showing a strong, ~80% downregulation for both ADAM10 and ADAM17 compared to cells treated with nontargeting siRNA (Fig. 2A,B). Furthermore, we observed that siRNA downregulation of ADAM10 significantly reduced the HDM-induced CCL20 secretion (4 h) to 67 ± 13%, while treatment with siRNA of ADAM17 did not significantly reduce HDM-induced CCL20 secretion (Fig. 2C), although it unexpectedly tended to increase baseline levels of CCL20. Together, our results suggest that ADAM10 rather than ADAM17 activity contributes to HDM-induced CCL20 release in bronchial epithelial cells.

ADAM10 activity contributes to the HDM-induced release of CCL20, CCL2, CCL5, and CXCL8 and the ionomycin-induced release of CCL20 by primary bronchial epithelial cells

To increase the relevance of our findings, we additionally studied the effect of ADAM10 siRNA downregulation in primary NHBE cells. As CCL20 levels were below the detection limit at 4 h (both at baseline and upon HDM exposure), we studied the levels after 16 h. Here, we observed that HDM significantly increases the release of CCL20. In further line with the effects in 16HBE cells, we observed that ADAM10 siRNA treatment resulted in ~70% downregulation of ADAM10 and significantly reduced the HDM-induced CCL20 release (Fig. 3A,B), while it also slightly but significantly reduced baseline levels of CCL20.

Next, we assessed whether HDM increases the release of additional chemokines through a similar mechanism, involving ADAM10-dependent shedding. HDM significantly increased the secretion of CCL2, CCL5, and CXCL8, which was attenuated upon treatment with ADAM10 siRNA (Fig. 3C–E), while the HDM-induced increase in the cytokines IL-6, GM-CSF, and VEGF was unaffected by ADAM10 siRNA treatment (Fig. 3F–H).

We have previously shown that HDM increases CCL20 secretion from primary bronchial epithelial cells in a Ca²⁺ signaling-dependent manner (7). As ADAM10-dependent ligand shedding can be induced by Ca²⁺ signaling (10, 15), we investigated the effect of the Ca²⁺ ionophore ionomycin. Indeed, siRNA downregulation of ADAM10 significantly reduced the ionomycin-induced CCL20 release in NHBE cells (Fig. 3I).

HDM increases CCL20 secretion by surface shedding

We further elucidated the mechanisms of this HDM-induced release of chemokines in 16HBE cells. ADAM10 is a well-known sheddase of EGF (17), and ecto-domain shedding of EGF and subsequent EGFR downstream signaling has been shown to induce CCL20 transcription (18). We used EGFR tyrosine kinase inhibitor AG1478 to study the involvement of EGFR in HDM-induced CCL20 production. While prepared and subjected to Western blotting for the detection of ADAM10 and ADAM17. (A) Representative for relative expression (OD/mm²) of ADAM10 in 16HBE cells normalized to actin. (B) Representative for relative expression (OD/mm²) of ADAM17 in 16HBE cells normalized to actin. (C) CCL20 levels. (Mean ± SEM, n = 3). *P < 0.05 and **P < 0.01 between the indicated values.

Figure 2 siRNA downregulation of ADAM10, but not ADAM17, reduces the HDM-induced CCL20 secretion in 16HBE cells. 16HBE were transfected with nontargeting (control) ADAM10 or ADAM17 siRNA and grown to confluence for 3 days. Subsequently, cells were serum-deprived overnight and treated with or without HDM (50 μg/ml) for 4 h for the measurement of CCL20 in cell-free supernatant or total cell lysates were

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treatment of the 16HBE cells with AG1478 significantly inhibited mRNA expression of CCL20 (see Fig. S1A), we observed that AG1478 did not significantly affect the HDM-induced release of CCL20 (Fig. 4A), indicating that ADAM10-dependent shedding of EGFR ligands is not involved in the observed effect of HDM on CCL20 secretion.

Figure 3 siRNA downregulation of ADAM10 reduces HDM-induced release of CCL20, CCL2, CCL5, and CXCL8, but not IL-6, GM-CSF, and VEGF in normal human bronchial epithelial cells. NHBE cells (n = 6) were transfected with nontargeting (control) and ADAM10 siRNA and grown to confluence. A day after transfection, cells were hormonally/growth factor-deprived overnight and treated with or without HDM (50 μg/ml) for 16 h for collection of cell-free supernatants or preparation of total cell lysates for immunodetection of ADAM10. (A) Representative for relative expression (OD/mm²) of ADAM10 normalized to actin. (B) CCL20, (C) CCL2, (D) CCL5, (E) CXCL8, (F) IL-6, (G) GM-CSF, and (H) VEGF levels in cell-free supernatants. (I) NHBE cells were treated with ionomycin (0.1 μM) for 16 h, and CCL20 levels were analyzed in cell-free supernatants. ND = not detected, *P < 0.05 and **P < 0.01 between the indicated values.
To assess whether the HDM-induced release of CCL20 at 4 h is indeed mediated by its shedding or involves de novo synthesis, we treated 16HBE cells with the protein synthesis inhibitor cycloheximide (CHX) prior to HDM exposure. CHX did not significantly inhibit CCL20 secretion at baseline or upon HDM stimulation, indicating that the observed increase in CCL20 release does not derive from de novo synthesis of CCL20 (Fig. 4B). In contrast, CHX did significantly reduce (HDM-induced) VEGF secretion, confirming its effectiveness at the used concentration (1 μg/ml, Fig. S1B).

Finally, we used monensin to study whether the increase in CCL20 levels could be the consequence of its release from preformed vesicles. Treatment with monensin did not prevent the HDM-induced release of CCL20 (Fig. 4C). Together, these data indicate that the HDM-induced CCL20 release by bronchial epithelial cells involves direct ADAM10-dependent surface shedding of CCL20.

**Discussion**

We previously observed that the HDM-induced epithelial secretion of CCL20 is related to the allergenicity of HDM in a mouse model and involves Ca$^{2+}$ signaling, although the mechanisms by which Ca$^{2+}$ signaling leads to the secretion of CCL20 remained unclear. In the present study, we show that HDM-induced CCL20 release by human bronchial epithelial cells is mediated by ADAM10-dependent surface shedding. The pharmacological ADAM17/ADAM10 inhibitor GW280264 and ADAM10 inhibitor GI254023 both significantly reduced the release of CCL20 by human bronchial epithelial cells, and treatment with specific siRNA revealed that ADAM10 is involved in this inhibitory effect. The inhibitory effect of ADAM10 siRNA was observed in both the human bronchial epithelial 16HBE cell line and primary NHBE cells and not specific for CCL20, as the HDM-induced release of other chemokine ligands, that is CCL2, CCL5, and CXCL8, but not pro-inflammatory cytokines IL-6, GM-CSF, and VEGF, was also reduced by ADAM10 siRNA treatment. The use of AG1478, CHX, and monensin revealed that the HDM-induced chemokine release involves direct surface shedding by ADAM10, rather than de novo synthesis induced by transcriptional activation of CCL20 expression upon shedding of EGFR ligands (19, 20).

As described in the introduction, CCL20 is thought to play an important role in airway inflammation in asthma, acting as chemotactant for DCs and Th17 cells. Furthermore, CCL20 has been implicated in mucus hypersecretion, increasing MUC5AC production in NCI-H292 cells (21). In addition to CCL20, the chemokines CXCL8, CCL2, and CCL5 may play a role in the pathogenesis of asthma, acting as attractants of neutrophils, monocytes/dendritic cells, and eosinophils/T cells, respectively (22, 23). Indeed, increased levels of these chemokines have been found in the airways of (severe) patients with asthma compared to healthy individuals (24, 25). Various chemokines, including CXCL8 (26), CCL2 (27), and CCL5 (28), can bind the heparin sulfate chain on the ectodomain of the transmembrane proteoglycan syndecan 1–4, which can be shed by metalloproteinases (29). Furthermore, colocalization of CCL20 and syndecan-1 has been observed in murine choroid plexus epithelium (30). Future studies will be required to confirm that CCL20 can indeed bind to the heparin sulfate ectodomain of syndecans in bronchial epithelium. Both ADAM10 and ADAM17 have been described to shed syndecan-1 (31), although our data indicate that the HDM-induced secretion of CCL20 is more likely mediated by ADAM10 activation. Accordingly, TAPI-2 has relatively low affinity for ADAM10 (32) and only marginally inhibited HDM-induced CCL20 secretion at a concentration as high as 20 μM.

This novel role of ADAM10 in allergen-induced CCL20 secretion may have implications for the development of allergic airway inflammation in asthma, although further studies are required to show whether ADAM10 is capable of shedding syndecan-bound CCL20. Recently, we observed that

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**Figure 4** HDM-induced CCL20 release is not affected by the inhibition of the EGFR receptor, new protein synthesis, or vesicle release. 16HBE cells were exposed to HDM (4 hrs) in the presence or absence of the EGFR inhibitor AG1478, the protein synthesis inhibitor cycloheximide (CHX), or the protein transport inhibitor monensin. CCL20 levels after (A) AG1478 treatment, (B) CHX treatment, and (C) monensin treatment. Levels were analyzed in cell-free supernatants (Mean ± SEM, n = 4–8), *P < 0.05, **P < 0.01, and ***P < 0.001 between the indicated values.
HDM induced a stronger increase in CCL20 secretion in bronchial epithelial cells from patients with asthma than from healthy individuals (7) and that HDM induces a stronger Ca\(^{2+}\) influx in asthma-derived epithelial cells (7). Here, we show that siRNA downregulation of ADAM10 significantly reduces the ionomycin-induced increase in epithelial CCL20 secretion, indicating that Ca\(^{2+}\)-dependent ADAM10 activation may contribute to the HDM-induced CCL20 secretion. The effect of HDM on CCL20 secretion was not reduced as effectively by siRNA downregulation of ADAM10 as the effect of ionomycin. This suggests that additional pathways than the Ca\(^{2+}\)-induced activation of ADAM10 are involved in the effect of HDM on CCL20. Indeed, HDM is known to act on various pattern recognition receptors, for example, TLR4, purinergic receptors, and protease-activated receptors, activating multiple signaling pathways that may lead to the secretion of CCL20 (33–35).

It is currently unknown whether bronchial epithelium from patients with asthma expresses higher levels of ADAM10 than in healthy individuals, which will be of interest to assess in future studies.

In addition to its role in CCL20 production, we previously demonstrated that Ca\(^{2+}\) signaling contributes to HDM-induced epithelial barrier dysfunction by disruption of E-cadherin (7). Interestingly, ADAM10 has been shown to shed E-cadherin (36), and it is therefore tempting to speculate that ADAM10 may also play a role in the HDM-induced barrier dysfunction.

In conclusion, we show for the first time that ADAM10 contributes to the allergen-induced release of chemokines, including CCL20. Our findings may provide a novel insight into avenues for therapeutic strategies for allergic asthma aimed at the chemotraction of immune cells to the airways.

**Conflict of interest**
None of the authors has a conflict of interest.

**Author contributions**
S. Post analyzed and interpreted the data and drafted the manuscript. D. Rozeveld performed all experiments and analyzed the data. M.R. Jonker performed the cell culture and provided technical support for the experiments. R. Bischoff and A.J.M. van Oosterhout were involved in the design and supervision of the study. I. Heijink coordinated, designed, and supervised the study and was involved in the manuscript writing. All authors critically read and revised the manuscript.

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

**Figure S1.** The mRNA expression of CCL20 in 16HBE cells is reduced after inhibition of the EGFR receptor and the release of VEGF is affected by cycloheximide.

**References**


