Allergenic proteases cleave the chemokine CX3CL1 directly from the surface of airway epithelium and augment the effect of rhinovirus

M Loxham[1,2,5], DE Smart[1,5], NJ Bedke[1,5], NP Smithers[1], I Filippi[1,3], C Blume[1], EJ Swindle[1,2], K Tariq[1,4], PH Howarth[1,4], ST Holgate[1] and DE Davies[1,2,4]

CX3CL1 has been implicated in allergen-induced airway CD4$^+$ T-lymphocyte recruitment in asthma. As epidemiological evidence supports a viral infection–allergen synergy in asthma exacerbations, we postulated that rhinovirus (RV) infection in the presence of allergen augments epithelial CX3CL1 release. Fully differentiated primary bronchial epithelial cultures were pretreated apically with house dust mite (HDM) extract and infected with rhinovirus-16 (RV16). CX3CL1 was measured by enzyme-linked immunosorbent assay and western blotting, and shedding mechanisms assessed using inhibitors, protease-activated receptor-2 (PAR-2) agonist, and recombinant CX3CL1-expressing HEK293T cells. Basolateral CX3CL1 release was unaffected by HDM but stimulated by RV16; inhibition by fluticasone or GM6001 implicated nuclear factor-κB and ADAM (A Disintegrin and Metalloproteinase) sheddases. Conversely, apical CX3CL1 shedding was stimulated by HDM and augmented by RV16. Although fluticasone or GM6001 reduced RV16 + HDM-induced apical CX3CL1 release, heat inactivation or cysteine protease inhibition completely blocked CX3CL1 shedding. The HDM effect was via enzymatic cleavage of CX3CL1, not PAR-2 activation, yielding a product mitogenic for smooth muscle cells. Extracts of *Alternaria* fungus caused similar CX3CL1 shedding. We have identified a novel mechanism whereby allergenic proteases cleave CX3CL1 from the apical epithelial surface to yield a biologically active product. RV16 infection augmented HDM-induced CX3CL1 shedding—this may contribute to synergy between allergen exposure and RV infection in triggering asthma exacerbations and airway remodeling.

INTRODUCTION

Asthma is an inflammatory disease of the lower airways exhibiting reversible airflow obstruction and airway remodeling.[1] Asthma exacerbations are a major cause of hospitalization, with the single factor most responsible for exacerbations widely recognized as being viral infection, particularly involving rhinovirus (RV).[2,3] Furthermore, early life wheezing with RV is strongly predictive of subsequent development of asthma, suggesting a link between symptomatic RV infection and disease pathogenesis.[4] There is also a synergistic interaction between viral infection and allergen inhalation in the worsening of asthma symptoms.[5,6] RV16, a major group of RV, can increase airway hyperreactivity, which persists up to 4 weeks postinfection, suggesting that RV16 may prime the airways to overreact to allergen.[7] Indeed, patients receiving hospital treatment have a greater sensitization to, and exposure to, allergen.[6] However, the precise mechanisms of virus–allergen interactions in the development and exacerbation of asthma are yet to be fully elucidated.

CX3CL1 (fractalkine) is a membrane-bound 373 amino-acid chemokine of ~42 kDa, with extensive glycosylation increasing its mass to ~95 kDa.[8,9] The N-terminal chemokine domain (CKD) contains a highly unusual CX3C motif, supported on an extensively glycosylated mucin stalk, with an 18 amino-acid

[1]Academic Unit of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, University Hospital Southampton, Southampton, UK. [2]Institute for Life Sciences, Highfield Campus, University of Southampton, Southampton, UK. [3]Cellular and Molecular Physiology Unit, Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy and [4]NIHR Southampton Respiratory Biomedical Research Unit, University Hospital Southampton, Southampton, UK. Correspondence: M Loxham (m.loxham@soton.ac.uk)
[5]These authors contributed equally to this work.

Received 14 September 2016; accepted 29 May 2017; advance online publication 5 July 2017. doi:10.1038/mi.2017.63

transmembrane domain and an intracellular C-terminal tail with an apparent role in peptide recycling.[10] Almost uniquely for a chemokine, CX3CL1 exhibits different functions depending on its state. Membrane-bound CX3CL1 acts as an adhesion molecule, mediated by firm binding of the CKD to its receptor,[11] CX3CR1, a 7-transmembrane G-protein-coupled receptor.[12] CX3CR1 is found on a variety of cells, including monocytes, macrophages, mucosal dendritic cells, natural killer cells, $CD8^+$ T lymphocytes, and a subset of $CD4^+$ T lymphocytes (see reviews in refs [13,14]). CX3CL1 can also be cleaved constitutively by ADAM10 (A Disintegrin and Metalloproteinase 10), or inducibly by ADAM17 (tumor necrosis factor-α-converting enzyme; TACE).[15,16] Binding of CX3CL1 to CX3CR1 results in $G_{\alpha i}$-mediated intracellular signaling, independent of the adhesive effect of cell surface CX3CL1.[17] Effects of CX3CR1 activation by cleaved soluble CX3CL1 include migration of dendritic cells,[18] macrophages,[19] mast cells,[20] and natural killer cells,[21] promotion of cell survival,[22,23] and proliferation of smooth muscle cells (SMCs)[24] and fibroblasts.[25] CX3CL1 is predominantly expressed by epithelial cells,[26] but its expression can be further induced in airway epithelial, smooth muscle, and endothelial cells in inflammatory conditions and by inflammatory cytokines such as interferon-γ and tumor necrosis factor-α.[27–29] Interestingly, levels of CX3CL1 have been noted to be elevated in the plasma of patients with asthma or allergic rhinitis and in bronchoalveolar lavage fluid after segmental allergen challenge.[30] Although many studies of CX3CL1 release and function *in vitro* have studied endogenous mechanisms of cleavage, there is a paucity of work considering the possibility that CX3CL1 might be cleaved by exogenous proteases. Many aeroallergens linked to asthma exacerbations, including both house dust mite and *Alternaria alternata* fungus, possess proteolytic activity.[31,32] We therefore hypothesized that the proteolytic activity of inhaled allergens might represent a novel mechanism for the cleavage of CX3CL1, which could potentiate the effect of RV16 infection of primary bronchial epithelial cells (PBECs).

RESULTS

The effect of RV16 and house dust mite extract on vectorial CX3CL1 release from PBEC ALI cultures

Differentiated PBEC air–liquid interface (ALI) cultures were grown in Transwells so that challenges could be applied to the apical surface to mimic *in vivo* exposure, and vectorial release of CX3CL1 from the apical and basolateral surfaces measured. ALI cultures were exposed apically to 200 μg ml^{-1} house dust mite (HDM) extract or medium alone for 24 h, followed by RV16 or ultraviolet-treated virus controls (UVRV16) (each at a multiplicity of infection = 5) for 6 h, and then further HDM extract or medium added after washing away unbound virus particles. UVRV16 was used as a control for viral replication as ultraviolet-irradiated RV16 is unable to replicate; in pilot experiments, no difference was observed between the effects of HDM alone and HDM + UVRV16. Apical release of CX3CL1 was not increased by RV16 (**Figure 1**; $P = 0.126$ vs. UVRV16),

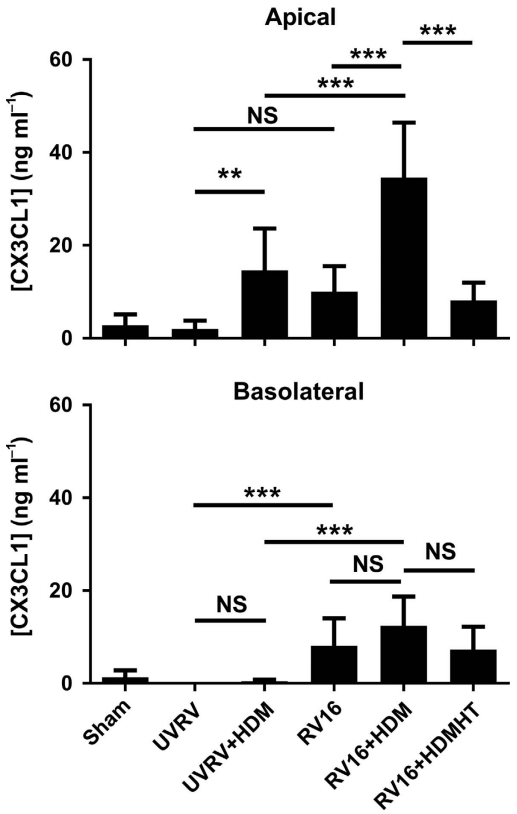

Figure 1 Effect of rhinovirus-16 (RV16) and house dust mite (HDM) extract on CX3CL1 release from primary bronchial epithelial cells (PBECs). Air–liquid interface (ALI) cultures of PBECs were pretreated overnight with HDM extract (200 μg protein per ml) or culture medium, then infected with RV16 or UVRV-16 (multiplicity of infection = 5) for 6 h, and then re-exposed to HDM or medium. HDMHT indicates that the HDM was heat treated (65 °C for 30 min) before use. Supernatants were harvested after 72 h, and CX3CL1 release into the apical and basolateral supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA). Bars represent mean ± s.d., $n = 11$ (sham, RV16, and RV16 + HDM), 7 (UVRV), 5 (UVRV + HDM), or 3 (RV16 + HDMHT) independent experiments with different donors. Data were analyzed by one-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, $^{**}P < 0.01$, $^{***}P < 0.001$.

but was increased up to 72 h by HDM in UVRV16- and RV16-exposed cultures ($P = 0.008$ and $P < 0.001$, respectively). Despite the lack of significant CX3CL1 release in response to RV16 replication alone, apical CX3CL1 was significantly higher in cultures supporting RV16 replication when also treated with HDM ($P < 0.001$ vs. HDM + UVRV16). This augmentation of CX3CL1 release by HDM in RV16-infected cultures was abrogated when heat-treated HDM was used ($P < 0.001$). Conversely, basolateral CX3CL1 concentration was increased by RV16 compared with UVRV16 in either the absence or presence of HDM ($P < 0.001$ for both), whereas heat treatment of HDM did not significantly reduce CX3CL1 release induced by RV16 + HDM ($P = 0.335$). These results suggest that HDM is the key driver of apical CX3CL1 release in this model, although this can be augmented by exposure to RV16. Basolateral release of CX3CL1 is driven primarily by RV16, albeit to a more modest extent than HDM-driven apical release.

Mechanism of increased CX3CL1 release

We next hypothesized that RV16 and HDM might increase mRNA expression of either CX3CL1 or the inducible sheddase ADAM17. At 24 and 72 h neither CX3CL1 nor ADAM17 expression were significantly changed by any of the treatments used (**Figure 2**). However, there was a trend for RV16 to induce CX3CL1 expression at 24 h, and in separate experiments we observed a significant increase in CX3CL1 expression induced by RV16 compared with UVRV and sham after 6 h ($P = 0.030$ and 0.019, respectively), but without any effect on ADAM17 expression (**Figure 3**). HDM tended to suppress the effect of RV16 on CX3CL1 transcription, although this effect did not reach significance ($P = 0.08$). Therefore, further experiments were performed in the presence of the glucocorticoid fluticasone propionate to test the involvement of nuclear factor-κB-mediated transcription of CX3CL1, or the broad-spectrum metalloprotease inhibitor GM6001 to test the involvement of endogenous ADAM sheddases in the release of CX3CL1 (**Figure 4a**). Fluticasone propionate treatment modestly reduced RV16 + HDM-induced apical release of CX3CL1 by 26 ± 17% ($P = 0.040$), whereas GM6001 caused a 41 ± 32% ($P = 0.001$) reduction. In contrast, heat treatment of HDM reduced apical CX3CL1 release by 82 ± 10% ($P < 0.001$), suggesting a role for a heat-labile factor in the HDM. Conversely, fluticasone propionate and GM6001 reduced basolateral release of CX3CL1 by 64 ± 22% ($P < 0.001$) and 47 ± 38% ($P = 0.005$), respectively, while heat treatment of the HDM had no significant reducing effect (32 ± 13%, $P = 0.068$).

The relative potency of heat inactivation of HDM on apical CX3CL1 release suggested a direct effect of a heat-labile component in HDM such as an allergenic protease. However, this did not appear to be mediated by a protease-activated receptor (PAR), as replacement of HDM by a PAR-2 agonist was unable to replicate the CX3CL1-releasing effect of HDM in the presence of RV16 (data not shown). Therefore, we considered that CX3CL1 release into the apical compartment might be partly mediated by direct proteolysis of membrane-bound CX3CL1 by HDM proteases. To test this, we pretreated

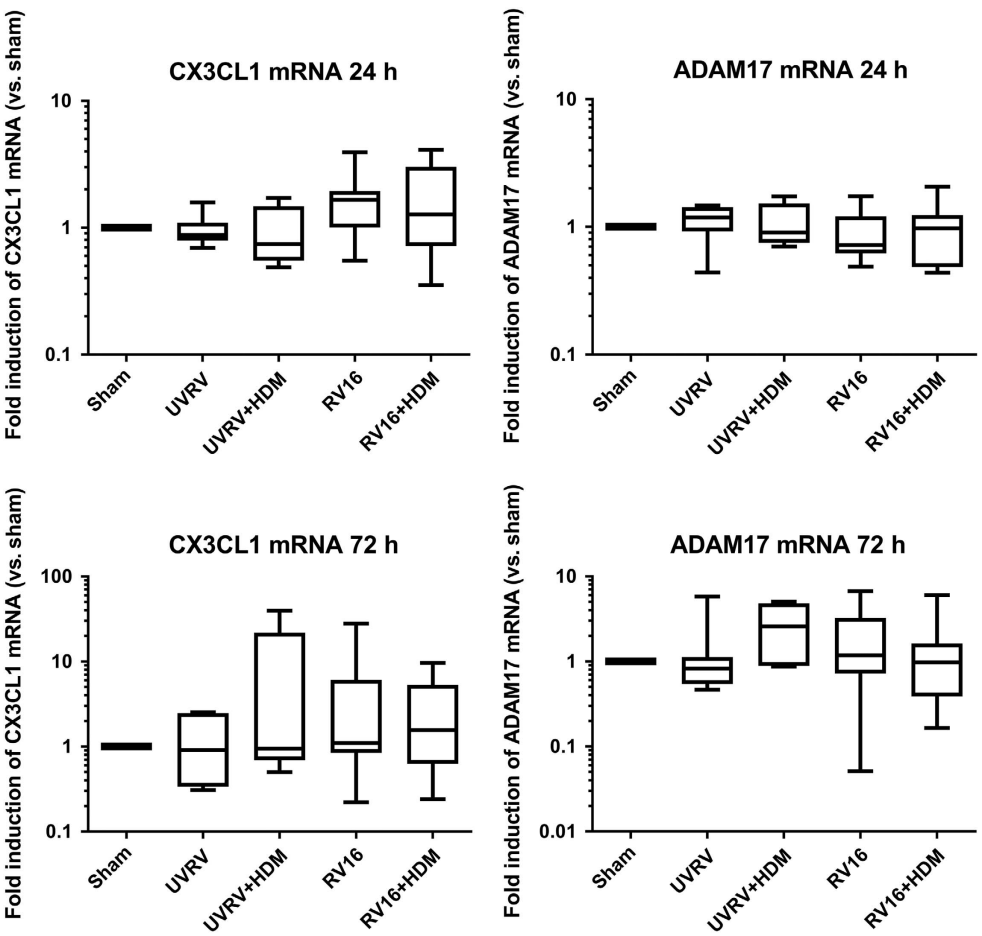

Figure 2 Effect of RV-16 and house dust mite (HDM) extract on CX3CL1 and ADAM17 gene expression. Primary bronchial epithelial cell (PBEC) air–liquid interface (ALI) cultures were treated as described in **Figure 1**. Cells were lysed after 24 or 72 h. RNA was extracted from cell lysates, reverse transcribed to cDNA, and expression of CX3CL1 and ADAM17 genes was determined by quantitative real-time PCR (RT-qPCR). Relative changes in gene expression were calculated using the ΔΔCt method. Boxes represent median with 25th and 75th percentiles, whiskers represent 10th and 90th percentiles, $n = 9$ (sham, RV, RV + HDM), 6 (UV), or 4 (UV + HDM) independent experiments with different donors. Data were analyzed by Friedman's test with Tukey's correction for pairwise comparisons.

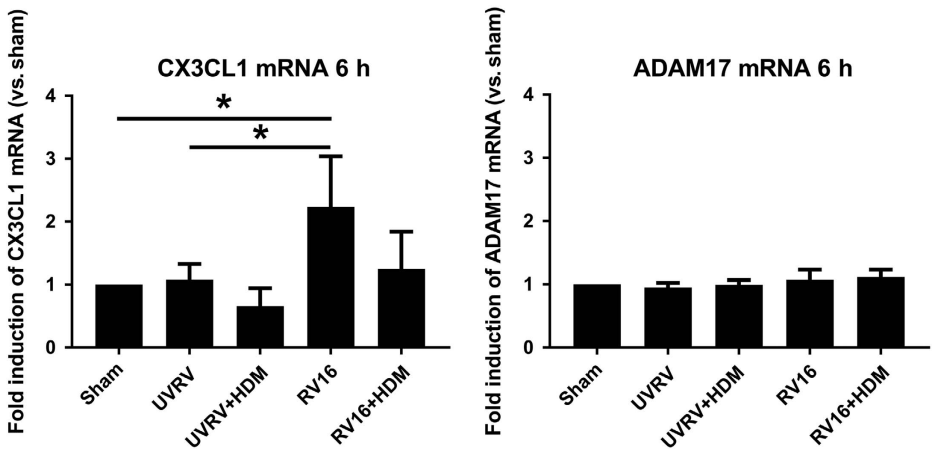

Figure 3 Effect of RV-16 and house dust mite (HDM) extract on CX3CL1 and ADAM17 gene expression at 6 h postinfection. Primary bronchial epithelial cell (PBEC) air–liquid interface (ALI) cultures were treated as described in **Figure 1**. Cells were lysed after 6 h. RNA was extracted from cell lysates, reverse transcribed to cDNA, and expression of CX3CL1 and ADAM17 genes was determined by quantitative real-time PCR (RT-qPCR). Relative changes in gene expression were calculated using the $\Delta\Delta$Ct method. Bars represent mean ± s.d., $n = 4$ independent experiments using cells from one donor. Data were analyzed by one-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, $^{*}P < 0.05$.

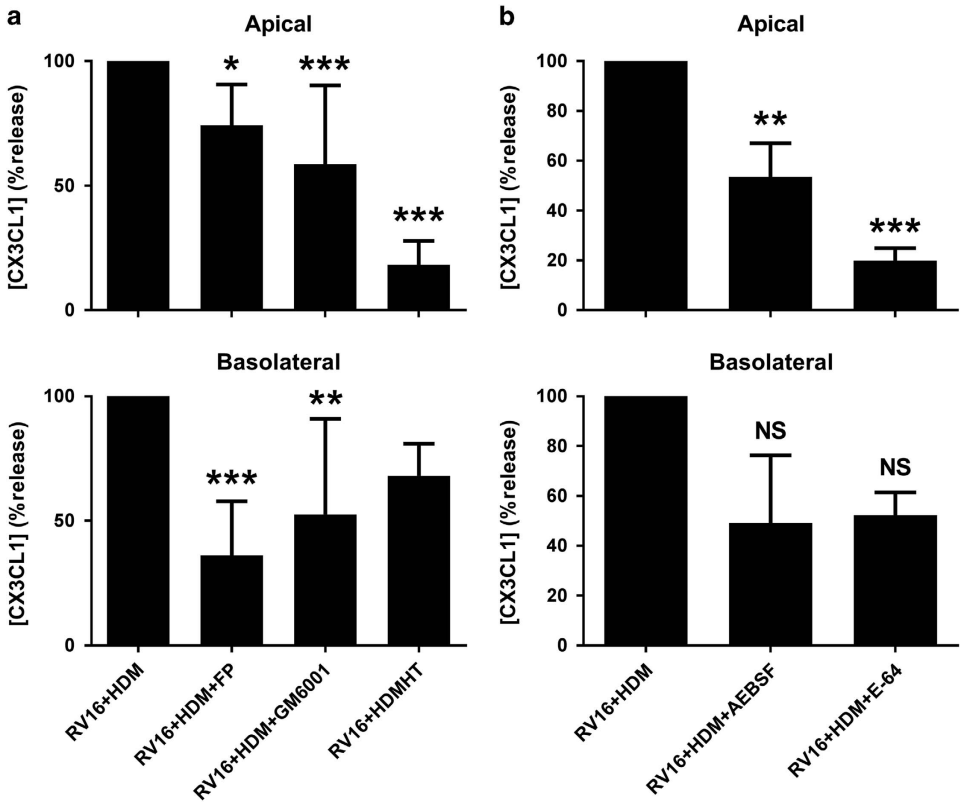

Figure 4 Use of inhibitors to study the mechanism of house dust mite (HDM)-induced CX3CL1 release. (**a**) Primary bronchial epithelial cell (PBEC) air–liquid interface (ALI) cultures were treated as described in **Figure 1**, in the absence or presence of fluticasone propionate (5 nM) or GM6001 (10 μM) to inhibit potential endogenous mechanisms for increased CX3CL1 release. Data were normalized by expressing as a percentage of CX3CL1 release with rhinovirus-16 (RV16) + HDM after correction for CX3CL1 released in the absence of HDM. Bars represent mean ± s.d., $n = 7$ (except RV16 + HDMHT, where $n = 3$) independent experiments with different donors. CX3CL1 release with RV16 + HDM was 40 ± 19 ng ml^{-1} (apical) and 17 ± 11 ng ml^{-1} (basolateral). (**b**) Cells were identically treated, in the absence or presence of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride AEBSF (500 μM) or *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64) (100 μM), to study the protease species responsible for HDM effect. Bars represent mean ± s.d., $n = 3$ independent experiments with different donors. CX3CL1 release with RV16 + HDM was 59 ± 11 ng ml^{-1} (apical) and 28 ± 1 ng ml^{-1} (basolateral). Data were analyzed by one-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RV16 + HDM.

HDM with inhibitors of serine proteases (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; AEBSF) and cysteine proteases (*trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane; E-64) before applying to the cell cultures (**Figure 4b**). RV16 + HDM-induced release of CX3CL1 into the apical compartment was significantly reduced by 46 ± 14% ($P = 0.003$) with AEBSF and by 80 ± 5% ($P < 0.001$) with E-64. Conversely, neither AEBSF (49 ± 27%, $P = 0.056$) nor E-64 (53 ± 9%, $P = 0.069$) were able to significantly inhibit the basolateral release of CX3CL1 elicited by RV16 + HDM. Therefore, we postulated that a substantial amount of the apical release of CX3CL1 by HDM is mediated by a direct action of HDM proteases on membrane-bound CX3CL1.

Transfection of HEK293T to express CX3CL1

To delineate the mechanism of HDM-induced CX3CL1 release more precisely, HEK293T cells, which in their wild type do not express CX3CL1, were transiently transfected to express CX3CL1 (HEK293T/CX3CL1$^+$) (**Figure 5**, inset). Monolayer cultures of the CX3CL1-expressing cells were incubated with HDM for 2 h in the absence or presence of protease inhibitors (**Figure 5**). As before, HDM exposure caused an increase in the release of CX3CL1 ($P < 0.001$). Crucially, this could be significantly inhibited only by E-64 ($P < 0.001$) or prior heat treatment of the HDM ($P = 0.010$). This observation coupled with the lack of effect of PAR-1/2 agonist peptides on CX3CL1 release (data not shown) further supports the notion that the ability of HDM to cause the release of CX3CL1 in this system is via direct action of HDM on CX3CL1, and implicates cysteine proteases as the critical mediators of this effect.

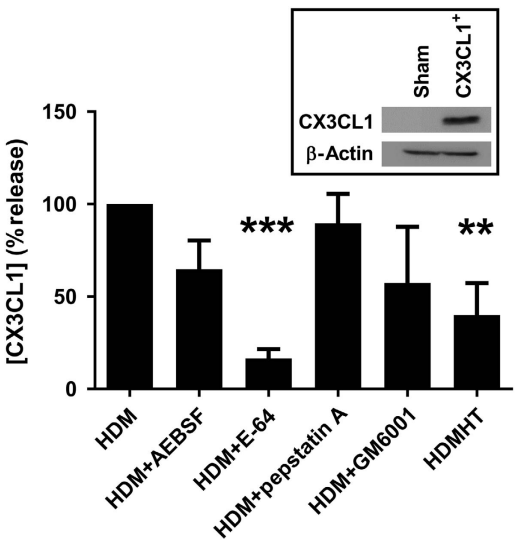

Figure 5 House dust mite (HDM)-induced release of CX3CL1 from HEK293T/CX3CL1$^+$ cells. HEK293T cells were transiently transfected to overexpress CX3CL1, with the specificity of this transfection being confirmed by western blot of lysates of sham-transfected and CX3CL1$^+$ HEK293T cells (inset). β-Actin immunoreactivity was used as a protein loading control. Cells were then treated with HDM (200 μg protein per ml) alone or in the presence or absence of GM6001 (100 nM), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (100 μM), *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64) (10 μM), or pepstatin A (10 μM). Supernatants were harvested after 2 h, and CX3CL1 concentration was analysed by enzyme-linked immunosorbent assay (ELISA). Data were normalized by expressing as a percentage of CX3CL1 release with HDM alone and were corrected for CX3CL1 releases in the presence of medium or inhibitors alone. Bars represent mean ± s.d., $n = 3$ independent experiments. Corrected concentration with HDM = 123 ± 96 ng ml^{-1}. Data were analysed by one-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. HDM.

CX3CL1 cleavage products

CX3CL1 possesses a number of potential cysteine protease cleavage sites (**Figure 6**). To determine whether HDM proteases can directly cleave CX3CL1, we used a cell-free system in which the recombinant extracellular domain of human CX3CL1 was treated with HDM. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting of the untreated CX3CL1 protein yielded two bands, one at 90 kDa and another at ~50 kDa most likely representing glycosylated and nonglycosylated (or partially glycosylated) forms of CX3CL1, respectively. HDM treatment resulted in a decrease in the 90 kDa full-length protein and increases in immunoreactive bands at ~50 and 40 kDa; these bands were reduced when HDM was treated with E-64 or heat inactivated and the 90 kDa band was retained (**Figure 7**), whereas GM6001, AEBSF and pepstatin A had no effect. These data demonstrate direct cleavage of CX3CL1 by a cysteine protease contained in the HDM extract.

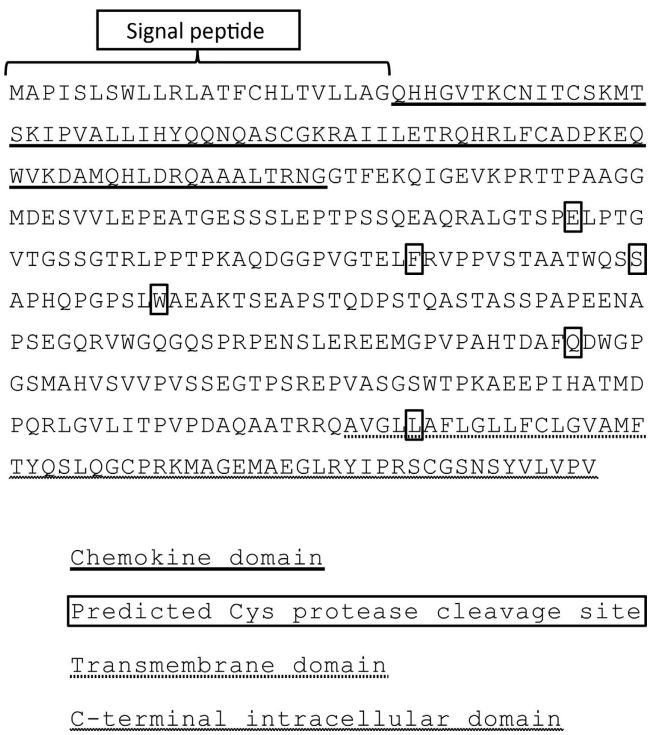

Figure 6 The amino-acid sequence of full-length CX3CL1 and predicted cysteine protease cleavage sites. Note that such cleavage sites are computationally determined on the basis of amino-acid sequence, and do not take into account steric considerations arising as a result of protein tertiary structure.

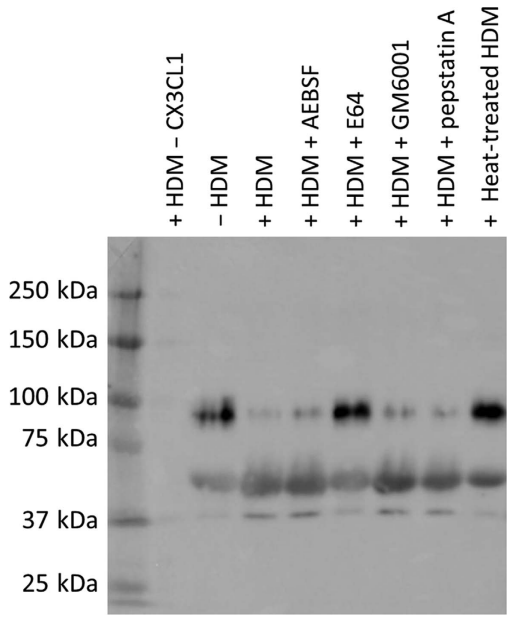

Figure 7 Products of cleavage of recombinant human CX3CL1 precursor by house dust mite (HDM). Recombinant human CX3CL1 (10 ng) was incubated with HDM extract (200 μg protein per ml) in phosphate-buffered saline (PBS) at 37 °C for 2 h alone or in the presence of GM6001 (1 μM), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (100 μM), *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64) (10 μM), or pepstatin A (10 μM). Blot representative of three independent experiments.

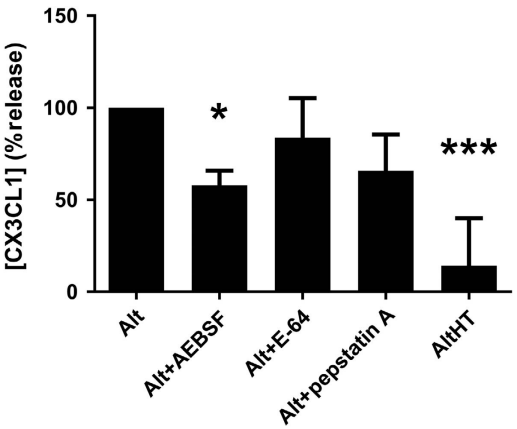

Figure 8 *Alternaria*-induced release of CX3CL1 from HEK293T/CX3CL1$^+$ cells. HEK293T cells were transiently transfected to overexpress CX3CL1, before treatment with *Alternaria* extract (100 μg ml^{-1}) (*Alt*) or heat-treated (65 °C, 30 min) *Alternaria* extract (*AltHT*) alone or in the presence or absence of 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (100 μM), *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64) (10 μM), or pepstatin A (10 μM). Supernatants were harvested after 30 min, and CX3CL1 concentration was analysed by enzyme-linked immunosorbent assay (ELISA). Data were normalized by expressing as a percentage of CX3CL1 release with *Alternaria* alone after correction for the concentration of CX3CL1 in the presence of medium or inhibitors alone. Bars represent mean ± s.d., $n = 3$ independent experiments. Corrected concentration with *Alternaria* was 104 ± 5 ng ml^{-1}. Data were analysed by one-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, *$P < 0.05$, ***$P < 0.001$ vs. *Alt*.

Assessment of the ability of other aeroallergens to elicit CX3CL1 release

To determine whether the ability to mediate CX3CL1 shedding directly is unique to HDM, the transfected HEK293T/CX3CL1$^+$ cells were incubated with extract of *Alternaria alternata* (**Figure 8**). *Alternaria* induced a significant increase in CX3CL1 release (20 ± 18 ng ml^{-1} control vs. 125 ± 21 ng ml^{-1} with *Alternaria*, $P < 0.001$), diminished by AEBSF ($P = 0.015$) or heat treatment of the *Alternaria* extract ($P < 0.001$). Timothy grass pollen extract (equivalent 10 mg pollen per ml), which lacks significant proteolytic activity,[33] did not induce CX3CL1 release.

Bioactivity of cleaved CX3CL1 and localization of CX3CL1 *in vivo*

Although our results demonstrate that HDM proteases can cleave CX3CL1, they do not give any information regarding the bioactivity of the resulting fragment(s), although such fragments do contain the CKD given that the detection antibodies used in the enzyme-linked immunosorbent assay (ELISA) and western blotting bind an epitope in the CKD. To confirm the bioactivity of the cleaved CX3CL1, its ability to stimulate proliferation of SMCs was assessed. The results indicated that supernatant from HDM treatment of HEK293T/CX3CL1$^+$ cells caused a 2.5 ± 1.5-fold increase in SMC proliferation compared with supernatant from HDM-exposed HEK293T cells not expressing CX3CL1, which was significantly greater than the increase seen with conditioned medium from HDM-free HEK293T cells ($P = 0.036$). Conversely, a significant increase in SMC proliferation was not seen with conditioned medium from HEK293T cells exposed to heat-treated HDM (**Figure 9**). Finally, to confirm the relevance of our findings to the *in vivo* setting, we performed IHC on glycol methacrylate-embedded sections of bronchial biopsy tissue and confirmed apical epithelial expression of CX3CL1, as evidenced by immunostaining of the epithelial brush border (**Figure 10**).

DISCUSSION

This study examined the effect of coexposure to RV16 and HDM on the vectorial release of CX3CL1 from fully differentiated mucociliary cultures of PBECs. We observed increased basolateral release of CX3CL1 in response to RV16 in agreement with previous findings,[34] with no significant effect on basolateral CX3CL1 of HDM either alone or in the presence of RV16. In these experiments, we used cells from a number of different donors, giving us confidence that our data are representative. Although it was not always possible to test every condition with every donor, we addressed this through the use of the appropriate repeated-measures statistical tests. We identified an effect of HDM on apical CX3CL1 release, which was only partially ascribable to the ADAM metalloproteases that represent the major endogenous sheddases involved in CX3CL1 release. We used either differentiated epithelial cells or HEK293T/CX3CL1$^+$ cells to show that shedding involved direct contact of a cysteine protease in HDM extract with the membrane-bound CX3CL1, rather than acting through PARs,

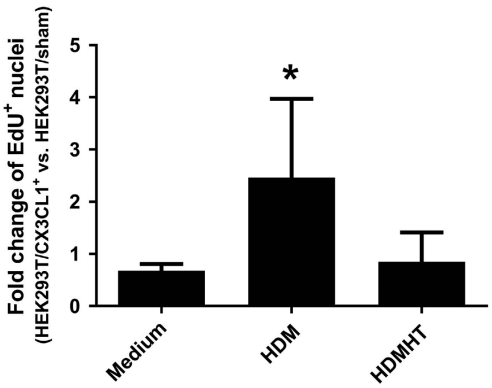

Figure 9 Effect of HEK293T/CX3CL1$^+$ conditioned medium on smooth muscle cell (SMC) proliferation. Primary human bronchial SMCs were exposed to conditioned media samples from HEK293T/CX3CL1$^+$ experiments (see **Figure 5**). After 36 h culture, SMC proliferation was calculated as the percentage of Hoechst-stained nuclei also stained with ethynyl-2-deoxyuridine (EdU), as a marker of proliferation. Data were calculated as the fold increase in percentage of EdU$^+$ nuclei in SMCs exposed to HEK293T/CX3CL1$^+$ conditioned medium over those exposed to HEK293T plasmid control conditioned medium. Bars represent mean ± s.d., $n = 4$ independent experiments, three fields of view per condition. Data were analyzed by one-way repeated-measures analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, *$P < 0.05$ vs. medium control.

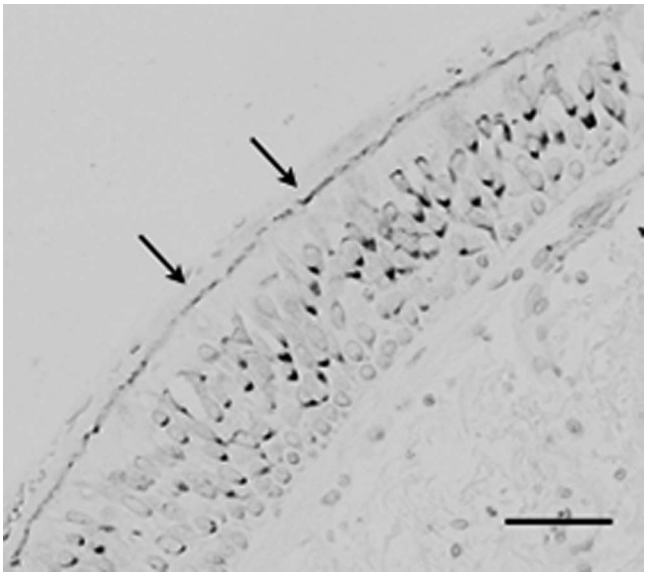

Figure 10 Expression of CX3CL1 in human bronchial biopsy tissue. Bronchial biopsy tissue was fixed and embedded in glycol methacrylate (GMA), and stained using rabbit anti-human CX3CL1 polyclonal antibody and peroxidase-conjugated swine anti-rabbit secondary, visualized with 3,3′-diaminobenzidine. Arrows indicate CX3CL1 immunostaining along the brush border. Bar = 50 μm.

suggesting an enzyme–substrate interaction. We confirmed the direct nature of the cleavage mechanism in a cell-free system. We further demonstrated that *Alternaria* extract also caused shedding of CX3CL1, in a manner dependent on previously demonstrated serine proteases,[32] suggesting that the ability of allergenic proteases to cause CX3CL1 shedding is not restricted to HDM.

Allergens belong to protein families with diverse biological functions.[35] The cleavage of PARs by allergenic proteases or activation of Toll-like receptors (TLR2 and TLR4) by endotoxin contained in crude allergen extracts are potential mechanisms underlying allergen-mediated activation of epithelial cells leading to cytokine release. For example, PAR-2-mediated signaling is involved in HDM-induced IL-25 expression and release,[36] whereas HDM-induced epithelial TLR4 signaling activates nuclear factor-κB to induce granulocyte–macrophage colony-stimulating factor release.[37] Furthermore, it has recently been shown that ADAM10 activity contributes to HDM-induced shedding of chemokines, including CCL20, CCL2, CCL5, and CXCL8 from bronchial epithelial cells.[38,39] However, CCL20 secretion is not protease or TLR2/4 dependent but relies on β-glucan moieties within the HDM extract, as evidenced by the ability of other β-glucans to competitively inhibit its secretion.[38] Thus, these protease-independent effects on CCL20 contrast with the cysteine protease-dependent effects of HDM extract on CX3CL1 shedding. In this study, PAR-activating peptides had no effect on CX3CL1 release, and E-64-inhibitable cleavage of recombinant CX3CL1 precursor in a cell-free system provides clear evidence of direct cysteine-protease-mediated proteolysis of the chemokine.

Although the majority of apical CX3CL1 release by epithelial cells was inhibited by heat treatment of the HDM extract or E-64 treatment, there was some residual shedding. Since HDM extracts used in this study contain a wide range of known and putative allergens, there may be other mechanisms that might influence the residual shedding of CX3CL1 through intracellular pathways. The residual effect might be due to a noncysteine protease component of HDM such as β-glucans, resulting in effects similar to those seen for CCL20 and mediated by ADAM10.[39] This would be consistent with the ability of GM6001 to partially reduce apical CX3CL1 release, as well as RV-induced CX3CL1 release into the basolateral compartment.

CX3CL1 is not unique in being a cytokine that can be cleaved from the cell surface by endogenous proteases. For example, tumor necrosis factor-α is also cleaved to yield a soluble product in a process mediated by ADAM17/TACE,[40] as for CX3CL1. However, we know of no work demonstrating direct cleavage of a cell surface-bound cytokine by exogenous allergenic proteases. Such an effect may not be totally unexpected, given that a range of cell surface molecules including CD23, CD25, CD40, DC-SIGN, and DC-SIGNR have already been identified as major Der p 1 targets.[41–43] The ability of allergenic proteases to modify components of immune signaling pathways suggests multiple mechanisms by which allergenic proteases can modify normal immune responses. The importance of HDM as a factor responsible for the release of CX3CL1 in asthma is underscored by the observation that CX3CL1 concentrations are increased in bronchoalveolar lavage fluid following segmental HDM allergen challenge, correlated with rapid recruitment of circulating CD4$^+$ T-lymphocytes.[30] Although the peak concentration of CX3CL1 in the present study is two orders of magnitude greater than that seen after allergen challenge *in vivo*,[30] this difference may be explained by cytokine dilution

during BAL, and differences in local concentrations of HDM. Studies in mice have shown that CX3CR1 signaling can promote either T-helper type 2 or T-helper type 1 cell survival in inflamed lungs, but not under homeostatic conditions.[22] Thus, the CX3CL1/CX3CR1 axis appears to have an important role in inflammatory pathologies of the lung. Indeed, it would be of considerable interest to examine the effects on CX3CL1 shedding of proteolytic allergens from other sources, such as cockroach[44] and cat,[45] and also of airway mast cell proteases.

Our experiments demonstrated that HDM has the ability to liberate membrane-bound CX3CL1 to yield an immunoreactive protein. However, owing to the presence of multiple potential cleavage sites, cleavage might simply reduce CX3CL1 adhesion without the chemotactic effects of soluble CX3CL1, either by cleavage of the CKD itself or by non-CKD cleavage inducing conformational change of the CKD. Using rhCX3CL1 precursor in a cell-free system, western blotting of the intact protein yielded a band at $\sim$90 kDa; the intensity of this band was greatly reduced by treatment with HDM, with an associated increase in bands at $\sim$50 and 40 kDa, suggesting specific cleavage of CX3CL1 at defined sites. Furthermore, treatment of HDM with E-64 or heat yielded a band pattern similar to untreated CX3CL1. The 90 kDa band is most likely fully glycosylated CX3CL1, similar to the 85–90 kDa full-length CX3CL1 observed by others.[46–48] We postulate that the 40 kDa product might result from cleavage of CX3CL1 by the HDM cysteine protease at F162, resulting in a CKD-bearing peptide chain of 17.3 kDa with 11 O-glycosylation sites. Taking into account that there are 26 such sites in full-length CX3CL1,[8] and that complete de-glycosylation results in a loss of $\sim$50–55 kDa,[8,46] it could be calculated that each glycosylation adds $\sim$2 kDa to the mass of the protein, and so if fully glycosylated, this fragment would have a mass of 39.3 kDa, corresponding to that observed on the blot.

In addition to its role as a chemotactic molecule, CX3CL1 has also been seen to induce proliferation of SMCs.[24] Our data show that SMC proliferation could be stimulated by supernatant from HDM-treated HEK293T/CX3CL1^{+} cells, but not when the HDM had been inhibited with E-64 or heat treated. This suggests that the CX3CL1 cleavage product induces a proliferative response. If HDM is able to cleave surface-bound CX3CL1, and if this effect is increased in the presence of RV16 infection, there is the potential for allergen exposure during infection to induce proliferation of SMCs and smooth muscle, hallmarks of asthmatic airway remodeling. In addition, the activity of CX3CL1 in promoting survival of Th2 CD4^{+} cells, which are crucial to the development of allergic asthma,[22] suggests that there may be multiple pathways through which the HDM-RV16 synergy might affect asthma. Furthermore, as soluble CX3CL1 can promote the migration of dendritic cells,[18] macrophages,[19] mast cells,[20] and natural killer cells,[21] its shedding has the potential to substantially affect immune cell recruitment, which may be important during asthma exacerbations. Indeed, it would be of interest to study the chemoattractant effect on these cells of the cleaved CX3CL1 fragment in our studies, to add even further weight to our findings.

Previous work has shown that properties of the airway epithelium in asthma may contribute to heightened responses during an asthma exacerbation. For example, increased susceptibility to RV infection of the epithelial cells from subjects with moderate–severe asthma has been linked to a defective interferon response,[49] while basal cells, which are sometimes exposed in areas of epithelial damage in asthmatic airways, are more susceptible to RV infection.[50,51] These effects may both heighten and prolong the infection of the airway epithelium, resulting in greater release of CX3CL1 when HDM or other proteolytic allergens are present. Moreover, the integrity of the airway epithelium in asthma is known to be impaired,[52] with epithelial cultures from severe asthmatics being more susceptible to the barrier-weakening effects of *Alternaria*.[32] While the direct effect of HDM on CX3CL1 release was in the apical compartment, there is the potential for passage of shed CX3CL1 or HDM allergen to the underside of damaged epithelium, where further shedding may occur. In this regard, it would be of interest to extend the present study by examining the responses of PBECs from mild, moderate, and severe asthmatics, to determine how CX3CL1 responses vary by donor asthma status. It would also be of interest to evaluate whether this HDM–virus synergy also applies for other viruses. For example, H1N1 influenza virus is known to induce expression of CX3CL1 mRNA.[53] If this is translated to increased cell surface CX3CL1 protein expression, infection might feasibly lead to increased substrate for HDM protease activity and thus increased CX3CL1 release.

In summary, we have shown that cysteine protease activity in HDM is the key driver of apical CX3CL1 shedding and that this is augmented following RV16 infection of PBECs. This release is due, in large part, to direct cleavage of membrane-bound CX3CL1 to liberate a biologically active product which increases SMC proliferation. These effects may contribute to a synergistic interaction between allergen exposure and RV infection in triggering asthma exacerbations and driving airway remodeling.

METHODS

PBEC culture. PBECs were grown from bronchial brushings obtained from nonasthmatic volunteers (mean age 32.9 years, range 19–62 years, M:F 11:3) by fiber optic bronchoscopy.[54] PBECs were expanded and differentiated at ALI as described previously.[32] Briefly, PBECs were expanded in culture in bronchial epithelial growth medium (Lonza, Slough, UK) and, at passage 2, seeded onto Transwell culture inserts (Corning, Tewkesbury, MA), precoated with collagen I (PureCol; Nutacon BV, Leimuiden, Netherlands) and taken to ALI after 24 h, and used at day 21 where transepithelial electrical resistance was $>330\,\Omega\,cm^2$.

Generation and titration of human RV16. RV16 (a gift from Professor Sebastian Johnson, Imperial College, London) was amplified as described previously.[55] RV16 was prepared for use as described previously.[56] Infectivity of RV16 stocks was determined using a HeLa titration assay and 50% tissue culture infective dose assay ($TCID_{50}$) per ml. Ultraviolet-treated virus controls (UVRV16) were prepared by irradiation of virus stocks at 1,200 mJ cm^{-2} on ice for 50 min. Subsequently, virus stocks were diluted in infection medium for use in cell exposure experiments. Infection medium comprised minimum

essential medium supplemented with (final concentrations) fetal bovine serum (4%; Life Technologies, Paisley, UK), nonessential amino-acid solution (1 ×; Life Technologies), penicillin–streptomycin solution (50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin; Life Technologies), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (16 mM; Sigma-Aldrich, Gillingham, UK), $NaHCO_3$ (0.12%; Sigma-Aldrich), tryptose (0.118% (w v^{-1}); Sigma-Aldrich), and $MgCl_2$ (0.3 mM; Sigma-Aldrich).

Challenge of cells with HDM/RV16. Cells were made quiescent by replacing basolateral medium with bronchial epithelial basal medium supplemented with 1% insulin/transferrin/selenium solution (Sigma-Aldrich) and 1.5 μg ml^{-1} bovine serum albumin (Sigma-Aldrich) for 24 h. Cells were treated apically with *Dermatophagoides pteronyssinus* (HDM extract; 200 μg protein per ml; Greer Laboratories, Lenoir, NC) before and after 6 h infection with RV16 at multiplicity of infection equal to 5 or an equivalent UV-irradiated control (UVRV) at 33 °C. Treatments were performed in the absence or presence of GM6001 (10 μM), AEBSF (500 μM), or E-64 (100 μM). Cells were washed with Hanks' balanced salt solution (Life Technologies) between HDM and virus steps, and basolateral culture medium was replenished after initial HDM exposure. Heat-treated HDM was prepared at 65 °C for 30 min. In some experiments, the PAR-2 agonist peptide SLIGKV (100 μM) was used instead of HDM. At a set time from the start of virus exposure, apical and basolateral supernatants were harvested, centrifuged (300 *g*, 5 min), and stored (−80 °C). Cells lysed with Trizol lysis reagent were stored similarly.

HEK293T/CX3CL1^{+} cell culture and challenge with HDM. HEK293T cells were cultured to 50% confluence in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with fetal bovine serum (10%), penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}), NEAA (1 ×), sodium pyruvate (1 mM), and L-glutamine (2 mM) (complete DMEM). A CX3CL1 overexpression plasmid was produced in-house using pcDNA3.1 (Invitrogen, Paisley, UK). HEK293T cells were transiently transfected using TransIT 2020 transfection reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions. At 24 h after transfection, cells were resuspended, seeded into 24-well plates, and cultured for a further 24 h.

HEK293T/CX3CL1^{+} cells were washed with Hanks' balanced salt solution. HDM extract (200 μg protein per ml) was prepared in DMEM/0.5% bovine serum albumin. Some wells were pretreated for 15 min with GM6001 (1 μM), AEBSF (100 μM), E-64 (10 μM), or pepstatin A (10 μM). Further wells were treated with either the PAR-1 agonist TFLLR (1 μM) or the PAR-2 agonist SLIGKV (1 μM). After 2 h, supernatants were collected and processed as above. Further experiments replaced HDM with either *Alternaria alternata* extract (100 μg extract per ml; Greer Laboratories), or *Phleum pratense* (Timothy Grass) pollen extract[33] (equivalent 10 mg pollen per ml; Greer Laboratories) with cultures exposed for 30 min.

RNA extraction and quantitative real-time PCR. RNA was extracted from lysates using a standard phenol–chloroform extraction protocol, and reverse transcribed to cDNA using a Precision Reverse Transcription Kit (PrimerDesign, Southampton, UK) according to the manufacturer's instructions. Expression of ADAM10/ADAM17/CX3CL1 was determined using SYBR green-based qPCR assays (PrimerDesign). Primer sequences (sense and antisense, respectively) were 5′-TTGACCATTCTCCACCTTCCA-3′ and 5′-TAACCAGCCAGCAGCAGAG-3′ (CX3CL1), 5′-AAGGCTATGGAATACAGATAGAGC-3′ and 5′ATCTTCACATCCCAAGCATCC-3′ (ADAM17), and 5′-GGACACATGAGACGCTAACTG-3′ and 5′-TTGGAGATGATGACTTAATAGGTTTC-3′ (ADAM10). Cycling conditions were as per the manufacturer's instructions (95 °C for 10 min, then 50 cycles of 95 °C for 15 s, 60 °C for 1 min). Expression of the housekeeping genes ubiquitin C and glyceraldehyde 3-phosphate dehydrogenase were determined using a probe-based duplex primer mix (PrimerDesign). Data were normalized to the geometric mean of the housekeeping genes and fold change in gene expression relative to controls was determined using the ΔΔCt method.

Quantification of CX3CL1 concentration in culture supernatants. CX3CL1 was quantified using a human CX3CL1 DuoSet ELISA Kit (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Western blotting. Recombinant human CX3CL1 (10 ng) comprising the full extracellular domain Gln25-Arg339 (R&D Systems) was incubated with HDM extract (200 μg protein per ml) in phosphate-buffered saline at 37 °C for 2 h alone or with GM6001 (1 μM), AEBSF (100 μM), E-64 (10 μM), or pepstatin A (10 μM). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed under reducing conditions before western blotting, and immune localization with monoclonal mouse anti-human CX3CL1 (No. 81506; R&D Systems) and goat anti-mouse peroxidase-conjugated secondary antibody (Sigma, Poole, UK).

SMC proliferation assay. Primary human bronchial SMCs were cultured in complete DMEM[57] in 48-well plates at 10,000 cells per well until confluent, before washing, and replacement with DMEM (Lonza, Slough, UK) supplemented with 0.5% fetal calf serum, penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}), and L-glutamine (2 mM) for 24 h. Subsequently, cells were exposed for 36 h to culture supernatants from HEK293T/CX3CL1^{+} cells exposed to HDM and inhibitors (as above), or empty vector control cells similarly exposed. Conditioned media were diluted 1:3 in DMEM/0.5% fetal calf serum. Proliferation was assessed using a Click-iT ethynyl-2-deoxyuridine assay (Molecular Probes, Paisley, UK) as per the manufacturer's instructions.

Staining of bronchial tissue for CX3CL1. Bronchial biopsy tissue taken from a nonasthmatic donor was fixed and embedded in glycol methacrylate,[58] and stained using rabbit anti-human CX3CL1 polyclonal antibody (Atlas, Stockholm, Sweden) and peroxidase-conjugated swine anti-rabbit secondary antibody (Vector Laboratories, Peterborough, UK), and then visualized with 3,3′-diaminobenzidine.

Statistical analysis. Statistical analysis, using GraphPad Prism 6 (GraphPad Software, San Diego, CA) and SigmaPlot 13 (SyStat Software, San Jose, CA), was by one-way repeated-measures analysis of variance/Bonferroni correction (parametric) or Friedman's test/Tukey's correction (nonparametric). Significance threshold was $P<0.05$.

ACKNOWLEDGMENTS

We thank Graham Berreen and Dr Robert Ridley for assistance with cell culture, and Jon Ward (Histochemistry Research Unit, University of Southampton) for assistance with histochemistry. This work was funded by the Medical Research Council (UK) (grant code G0900453) and the Southampton AAIR (Asthma, Allergy, and Inflammation Research) Charity.

AUTHOR CONTRIBUTIONS

M.L., D.E.S., N.J.B., C.B., E.J.S., S.T.H., and D.E.D. designed the study. K.T. and P.H.H. performed bronchoscopy to provide primary cells for the study. M.L., D.E.S., N.J.B., N.P.S., and I.F. performed experiments. M.L., D.E.S., N.J.B., and D.E.D. analyzed the data. M.L. and D.E.D. wrote the manuscript.

DISCLOSURE

The authors declared no conflict of interest.

© 2017 Society for Mucosal Immunology

REFERENCES

1. Holgate, S.T. Innate and adaptive immune responses in asthma. *Nat. Med.* **18**, 673–683 (2012).
2. Nicholson, K.G., Kent, J. & Ireland, D.C. Respiratory viruses and exacerbations of asthma in adults. *BMJ* **307**, 982–986 (1993).

3. Busse, W.W., Lemanske, R.F. & Gern, J.E. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet* **376**, 826–834 (2010).
4. Jackson, D.J. *et al.* Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am. J. Respir. Crit. Care Med.* **178**, 667–672 (2008).
5. Green, R.M., Custovic, A., Sanderson, G., Hunter, J., Johnston, S.L. & Woodcock, A. Synergism between allergens and viruses and risk of hospital admission with asthma: case–control study. *BMJ* **324**, 763–766A (2002).
6. Murray, C.S. *et al.* Study of modifiable risk factors for asthma exacerbations: virus infection and allergen exposure increase the risk of asthma hospital admissions in children. *Thorax* **61**, 376–382 (2006).
7. Lemanske, R.F., Dick, E.C., Swenson, C.A., Vrtis, R.F. & Busse, W.W. Rhinovirus Upper respiratory-infection increases airway hyperreactivity and late asthmatic reactions. *J. Clin. Invest.* **83**, 1–10 (1989).
8. Bazan, J.F. *et al.* A new class of membrane-bound chemokine with a CX(3)C motif. *Nature* **385**, 640–644 (1997).
9. Pan, Y. *et al.* Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* **387**, 611–617 (1997).
10. Andrzejewski, M.G., Koelsch, A., Kogel, T., Dreymueller, D., Schwarz, N. & Ludwig, A. Distinct role of the intracellular C-terminus for subcellular expression, shedding and function of the murine transmembrane chemokine CX3CL1. *Biochem. Biophys. Res. Commun.* **395**, 178–184 (2010).
11. Fong, A.M. *et al.* Fractalkine and CX(3)CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J. Exp. Med.* **188**, 1413–1419 (1998).
12. Imai, T. *et al.* Identification and molecular characterization of fractalkine receptor CX(3)CR1, which mediates both leukocyte migration and adhesion. *Cell* **91**, 521–530 (1997).
13. Jones, B.A., Beamer, M. & Ahmed, S. Fractalkine/CX3CL1: a potential new target for inflammatory diseases. *Mol. Interv.* **10**, 263–270 (2010).
14. White, G.E. & Greaves, D.R. Fractalkine: a survivor's guide chemokines as antiapoptotic mediators. *Arterioscl r. Throm. Vasc.* **32**, 589–594 (2012).
15. Hundhausen, C. *et al.* The disintegrin-like metalloproteinase ADAM 10 is involved in coinstitutive cleavage of CX3CL1 (fractalkine) and regulates CX3CL1-mediated cell-cell adhesion. *Blood* **102**, 1186–1195 (2003).
16. Garton, K.J. *et al.* Tumor necrosis factor-alpha-converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J. Biol. Chem.* **276**, 37993–38001 (2001).
17. Haskell, C.A., Cleary, M.D. & Charo, I.F. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction—rapid flow arrest of CX(3)CR1-expressing cells is independent of G-protein activation. *J. Biol. Chem.* **274**, 10053–10058 (1999).
18. Johnson, L.A. & Jackson, D.G. The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics. *J. Cell Sci.* **126**, 5259–5270 (2013).
19. Truman, L.A. *et al.* CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood* **112**, 5026–5036 (2008).
20. El-Shazly, A. *et al.* Fraktalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J. Immunol.* **176**, 1860–1868 (2006).
21. El-Shazly, A.E., Doloriert, H.C., Bisig, B., Lefebvre, P.P., Delvenne, P. & Jacobs, N. Novel cooperation between CX3CL1 and CCL26 inducing NK cell chemotaxis via CX3CR1: a possible mechanism for NK cell infiltration of the allergic nasal tissue. *Clin. Exp. Allergy* **43**, 322–331 (2013).
22. Mionnet, C. *et al.* CX3CR1 is required for airway inflammation by promoting T helper cell survival and maintenance in inflamed lung. *Nat. Med.* **16**, 1305–U1160 (2010).
23. White, G.E., McNeill, E., Channon, K.M. & Greaves, D.R. Fractalkine promotes human monocyte survival via a reduction in oxidative stress. *Arterioscl r. Thromb. Vasc.* **34**, 2554–2562 (2014).
24. Perros, F. *et al.* Fractalkine-induced smooth muscle cell proliferation in pulmonary hypertension. *Eur. Respir. J.* **29**, 937–943 (2007).
25. Sawai, H., Park, Y.W., He, X.W., Goronzy, J.J. & Weyand, C.M. Fractalkine mediates T cell-dependent proliferation of synovial fibroblasts in rheumatoid arthritis. *Arthritis Rheum.* **56**, 3215–3225 (2007).
26. Lucas, A.D. *et al.* The transmembrane form of the CX3CL1 chemokine fractalkine is expressed predominantly by epithelial cells *in vivo*. *Am. J. Pathol.* **158**, 855–866 (2001).
27. Fujimoto, K., Imaizumi, T., Yoshida, H., Takanashi, S., Okumura, K. & Satoh, K. Interferon-gamma stimulates fractalkine expression in human bronchial epithelial cells and regulates mononuclear cell adherence. *Am. J. Respir. Cell. Mol. Biol.* **25**, 233–238 (2001).
28. Sukkar, M.B., Issa, R., Xie, S.P., Oltmanns, U., Newton, R. & Chung, K.F. Fractalkine/CX(3)CL1 production by human airway smooth muscle cells: induction by IFN-gamma and TNF-alpha and regulation by TGF-beta and corticosteroids. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L1230–L1240 (2004).
29. Muehlhoefer, A. *et al.* Fractalkine is an epithelial and endothelial cell-derived chemoattractant for intraepithelial lymphocytes in the small intestinal mucosa. *J. Immunol.* **164**, 3368–3376 (2000).
30. Rimaniol, A.C. *et al.* The CX3C chemokine fractalkine in allergic asthma and rhinitis. *J. Allergy Clin. Immunol.* **112**, 1139–1146 (2003).
31. Calderon, M.A. *et al.* Respiratory allergy caused by house dust mites: What do we really know?. *J. Allergy Clin. Immunol.* **136**, 38–48 (2015).
32. Leino, M.S. *et al.* Barrier disrupting effects of alternaria alternata extract on bronchial epithelium from asthmatic donors. *PLoS ONE* **8**, e71278 (2013).
33. Blume, C. *et al.* Barrier responses of human bronchial epithelial cells to grass pollen exposure. *Eur. Respir. J.* **42**, 87–97 (2013).
34. Bai, J.W. *et al.* phenotypic responses of differentiated asthmatic human airway epithelial cultures to rhinovirus. *PLoS ONE* **10**, 2 (2015).
35. Gregory, L.G. & Lloyd, C.M. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol.* **32**, 402–411 (2011).
36. Kouzaki, H., Tojima, I., Kita, H. & Shimizu, T. Transcription of interleukin-25 and extracellular release of the protein is regulated by allergen proteases in airway epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* **49**, 741–750 (2013).
37. Hammad, H., Chieppa, M., Perros, F., Willart, M.A., Germain, R.N. & Lambrecht, B.N. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* **15**, 410–416 (2009).
38. Nathan, A.T., Peterson, E.A., Chakir, J. & Wills-Karp, M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *J. Allergy Clin. Immunol.* **123**, 612–618 (2009).
39. Post, S., Rozeveld, D., Jonker, M.R., Bischoff, R., van Oosterhout, A.J. & Heijink, I.H. ADAM10 mediates the house dust mite-induced release of chemokine ligand CCL20 by airway epithelium. *Allergy* **70**, 1545–1552 (2015).
40. Black, R.A. *et al.* A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385**, 729–733 (1997).
41. Schulz, O. *et al.* Cleavage of the low-affinity receptor for human IgE (CD23) by a mite cysteine protease: nature of the cleaved fragment in relation to the structure and function of CD23. *Eur. J. Immunol.* **27**, 584–588 (1997).
42. Schulz, O., Sewell, H.F. & Shakib, F. Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J. Exp. Med.* **187**, 271–275 (1998).
43. Furmonaviciene, R. *et al.* The protease allergen Der p 1 cleaves cell surface DC-SIGN and DC-SIGNR: experimental analysis of *in silico* substrate identification and implications in allergic responses. *Clin. Exp. Allergy* **37**, 231–242 (2007).
44. Bhat, R.K., Page, K., Tan, A. & Hershenson, M.B. German cockroach extract increases bronchial epithelial cell interleukin-8 expression. *Clin. Exp. Allergy* **33**, 35–42 (2003).
45. Ring, P.C., Wan, H., Schou, C., Kristensen, A.K., Roepstorff, P. & Robinson, C. The 18-kDa form of cat allergen *Felis domesticus* 1 (Fel d 1) is associated with gelatin- and fibronectin-degrading activity. *Clin. Exp. Allergy* **30**, 1085–1096 (2000).
46. Schulte, A. *et al.* Sequential processing of the transmembrane chemokines CX3CL1 and CXCL16 by alpha- and gamma-secretases. *Biochem. Biophys. Res. Commun.* **358**, 233–240 (2007).
47. Hundhausen, C. *et al.* Regulated shedding of transmembrane chemokines by the disintegrin and metalloproteinase 10 facilitates detachment of adherent leukocytes. *J. Immunol.* **178**, 8064–8072 (2007).

48. Ostuni, M.A. *et al.* CX3CL1, a chemokine finely tuned to adhesion: critical roles of the stalk glycosylation and the membrane domain. *Biol. Open* **3**, 1173–1182 (2014).
49. Wark, P.A.B. *et al.* Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J. Exp. Med.* **201**, 937–947 (2005).
50. Lopez-Souza, N. *et al.* Resistance of differentiated human airway epithelium to infection by rhinovirus. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **286**, L373–L381 (2004).
51. Jakiela, B., Brockman-Schneider, R., Amineva, S., Lee, W.M. & Gern, J.E. Basal cells of differentiated bronchial epithelium are more susceptible to rhinovirus infection. *Am. J. Respir. Cell. Mol. Biol.* **38**, 517–523 (2008).
52. Loxham, M., Davies, D.E. & Blume, C. Epithelial function and dysfunction in asthma. *Clin. Exp. Allergy* **44**, 1299–1313 (2014).
53. You, H.L., Chen, C.J., Eng, H.L., Liao, P.L. & Huang, S.T. The effectiveness and mechanism of *Toona sinensis* extract inhibit attachment of pandemic influenza A (H1N1) virus. *Evid. Based Complement. Alternat. Med.* **2013**, 479718 (2013).
54. Bucchieri, F. *et al.* Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am. J. Respir. Cell. Mol. Biol.* **27**, 179–185 (2002).
55. Papi, A. & Johnston, S.L. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappa B-mediated transcription. *J. Biol. Chem.* **274**, 9707–9720 (1999).
56. Cakebread, J.A., Haitchi, H.M., Xu, Y.H., Holgate, S.T., Roberts, G. & Davies, D.E. Rhinovirus-16 induced release of IP-10 and IL-8 is augmented by Th2 cytokines in a pediatric bronchial epithelial cell model. *PLoS ONE* **9**, e94010 (2014).
57. Richter, A. *et al.* The contribution of interleukin (IL)-4 and IL-13 to the epithelial–mesenchymal trophic unit in asthma. *Am. J. Respir. Cell. Mol. Biol.* **25**, 385–391 (2001).
58. Britten, K.M., Howarth, P.H. & Roche, W.R. Immunohistochemistry on resin sections—a comparison of resin embedding techniques for small mucosal biopsies. *Biotech. Histochem.* **68**, 271–280 (1993).