

# German Cockroach Proteases and Protease-Activated Receptor-2 Regulate Chemokine Production and Dendritic Cell Recruitment

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## Key Words

Allergy · Chemokines · Immune response · Dendritic cell · Murine · Pulmonary

## Abstract

We recently showed that serine proteases in German cockroach (GC) feces (frass) decreased experimental asthma through the activation of protease-activated receptor (PAR)-2. Since dendritic cells (DCs) play an important role in the initiation of asthma, we queried the role of GC frass proteases in modulating CCL20 (chemokine C-C motif ligand 20) and granulocyte macrophage colony-stimulating factor (GM-CSF) production, factors that regulate pulmonary DCs. A single exposure to GC frass resulted in a rapid, but transient, increase in GM-CSF and a steady increase in CCL20 in the airways of mice. Instillation of protease-depleted GC frass or instillation of GC frass in PAR-2-deficient mice significantly decreased chemokine release. A specific PAR-2-activating peptide was also sufficient to induce CCL20 production. To directly assess the role of the GC frass protease in chemokine release, we enriched the protease from GC frass and confirmed that the protease was sufficient to induce both GM-CSF and CCL20 production *in vivo*. Primary airway epithelial cells produced both GM-CSF and CCL20 in a protease-

ase- and PAR-2-dependent manner. Finally, we show a decreased percentage of myeloid DCs in the lung following allergen exposure in PAR-2-deficient mice compared to wild-type mice. However, there was no difference in GC frass uptake. Our data indicate that, through the activation of PAR-2, allergen-derived proteases are sufficient to induce CCL20 and GM-CSF production in the airways. This leads to increased recruitment and/or differentiation of myeloid DC populations in the lungs and likely plays an important role in the initiation of allergic airway responses.

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## Introduction

Airborne allergens are known to induce asthma in predisposed individuals. Many of these allergens contain protease activity which modulates the inflammatory response. Endogenous proteases in a variety of allergens, including house dust mite [1], mold [2] and cockroach [3], have been shown to cleave and activate protease-activated receptor (PAR)-2 in cell culture models. PARs are a family of G-coupled receptors through which a number of extracellular proteases can signal directly to cells via the cleavage and activation of the receptors on the cell surface

[4]. PAR-2 is expressed by many cells in the lung, including airway epithelial cells [1], alveolar macrophages [5], neutrophils [6] and mast cells [7]. We recently showed that serine proteases in German cockroach (GC) feces (frass) proteases played a role in modulating airway hyperresponsiveness and mucin production [8] through the activation of PAR-2 [9]. Our data showed that mucosal sensitization to an allergen required the active proteases to mediate a full inflammatory response. What is still unclear, however, is the mechanism by which allergen-derived proteases initiate allergic airway inflammation.

Dendritic cells (DCs) are potent antigen-presenting cells which play a role in both the innate and adaptive immune response. In the lung, DCs are normally found at the epithelial cell layer, where they sample inhaled allergens present in the airways [10]. However, DC turnover is high, and immature DCs are continuously recruited from the circulation along chemokine gradients. Moreover, after allergen inhalation in both humans [11] and mice [12], DC recruitment is further enhanced through the release of CCL20 from the epithelium. CCL20 is a unique ligand for the chemokine CCR6 which is selectively expressed on circulating immature DCs [13]. Recently, house dust mite (HDM) allergen has been shown to increase CCL20 mRNA and protein secretion from human airway epithelium through a  $\beta$ -glucan-dependent pathway [14].

In addition to the production of CCL20, epithelial cells can also enhance immune responses through the release of granulocyte macrophage-colony stimulating factor (GM-CSF), a factor which induces the development of pro-asthmatic myeloid DCs (mDCs) while actively suppressing the development of plasmacytoid DCs [15], a DC subset that has been shown to be tolerogenic in murine asthma models [16]. Overexpression of GM-CSF has been shown to overcome the necessity for adjuvant in the development of eosinophilia and Th2 differentiation in response to purified allergen (recombinant Der p1, ovalbumin) [17, 18]. Neutralization of GM-CSF using a specific antibody was shown to reduce HDM-induced eosinophilia, Th2 cytokine production and airway hyperresponsiveness [17]. However, little is known regarding the induction of CCL20 or GM-CSF by specific allergenic components.

Since allergen-associated proteases and PAR-2 clearly play a role in allergic airway inflammation [8, 9], and due to the importance of GM-CSF and CCL20 in the recruitment and differentiation of mDCs in the lung, we hypothesized that allergen-associated proteases may be responsible for induction of CCL20 and GM-CSF production in vivo. In this report, we demonstrate that serine

proteases in GC frass, through the activation of PAR-2, can regulate CCL20 and GM-CSF production, leading to increased pulmonary mDC levels in the lung. These data highlight the possibility that proteases in allergens are important factor(s) that make GC frass allergenic.

## Materials and Methods

### *Cockroach Frass*

The fecal remnants (frass) from one cage of GCs were transferred to a sterile container and stored at 4°C. GC frass was resuspended in PBS (divalent-cation free; Gibco/Invitrogen, Carlsbad, Calif., USA) made with endotoxin-free double-distilled water (Sigma-Aldrich Corp., St. Louis, Mo., USA) for 2 h at 4°C while rocking. Extracts were centrifuged to remove debris (10,000 g for 5 min at 4°C), supernatants harvested and total protein was measured using the Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, Calif., USA). To inhibit protease activity, frass was pretreated with aprotinin (Sigma-Aldrich; 10  $\mu$ g/ml for 30 min at 37°C) prior to use. The same concentration of aprotinin was added to PBS and used as a control. Protease activity was determined using the Azocoll assay as previously described [19, 20]. GC frass was determined to contain 12.3 U/mg and aprotinin treatment inhibited 85% of the protease activity [21], and will hence be referred to as protease-depleted GC frass. Endotoxin levels were measured using the Limulus Amebocyte Lysate Assay (Lonza, Walkersville, Md., USA). Some GC frass was labeled with AlexaFluor-405 (Invitrogen) according to the manufacturer's specifications.

### *Protease Enhancement of GC Frass*

GC frass was run through a size exclusion column (Sephadex G75 superfine, Amersham Pharmacia, Piscataway, N.J., USA) at 0.5 ml/min using a 50-mM sodium phosphate buffer, pH 7.4. Fractions (1 ml) were assessed for protease activity using the Azocoll assay as previously described [19, 20]. Protease activity was detected in a single peak eluted between roughly 20 and 45 kDa. The fractions in the protease peak were combined, dialyzed against double-distilled H<sub>2</sub>O, and concentrated using a Centrivan (Lab-conco, Kansas City, Mo., USA). A prepacked HiTrap Benzamide FF affinity column (GE Healthcare, Piscataway, N.J., USA) was equilibrated with 5 column volumes of binding buffer (20 mM NaPO<sub>4</sub>, pH 7.5, with 0.5 M NaCl) prior to the addition of the protease sample. The concentrated protease peak was loaded onto the HiTrap column, the column was washed with 10 column volumes of binding buffer and then the protease was eluted with 10 column volumes of binding buffer containing 20 mM *p*-aminobenzamide (Spectrum Chemical Corp, Gardenia, Calif., USA). One-milliliter fractions were collected and protease activity in each fraction was assayed by the Azocoll assay [19, 20]. The fractions containing protease activity were combined, dialyzed against double-distilled H<sub>2</sub>O and measured for protein concentration and protease activity as described above. Table 1 shows the amount of protein, enzymatic activity and endotoxin in the starting material GC frass and in the final column-purified protease sample. The protease-enhanced GC frass was frozen in aliquots and used for the remainder of the studies.

**Table 1.** Characteristics of GC frass and the protease-enriched GC frass

	Protein mg/ml	Activity units	Activity/mg protein	Endotoxin EU/mg protein
GC frass	4.1	50.3	12.3	28,585
Protease	0.35	31.5	90	14.14

The protease activity in GC frass was enriched by removal of other GC frass components via column chromatography. Both the original (GC frass) and the enriched component (protease) were analyzed for protein, protease activity and endotoxin levels.

#### *Animals and Exposure Protocol*

Six-week-old female BALB/c and PAR-2-deficient mice were obtained from Jackson Laboratory (Bar Harbor, Me., USA). The PAR-2 mice were backcrossed onto the BALB/c background for 10 generations. Mice were anesthetized with ketamine (45 mg/kg)/xylazine (8 mg/kg) prior to PBS or GC frass (40 µg/40 µl) exposure by a single instillation as previously described [22]. In some cases, the PAR-2-activating peptide (PAR-2AP: SLIGRL-NH<sub>2</sub>) or PAR-control peptide (PAR-2CP; LSIIGRL-NH<sub>2</sub>; both used at a concentration of 400 µg/40 µl in PBS) obtained from Peptides International (Louisville, Ky., USA) or enriched protease (0.5 units/40 µl) were administered by intratracheal instillation. Mice were given a lethal dose of sodium pentobarbital 1–20 h later. Animal care was provided in accordance with NIH guidelines. These studies were approved by the Cincinnati Children's Hospital Medical Center Institutional Animal Care and Use Committee.

#### *Assessment of Airway CCL20 and GM-CSF Levels*

Lungs were lavaged with 1 ml of Hanks' balanced salt solution without calcium or magnesium. The lavage fluid was centrifuged (300 g for 10 min); the supernatant was removed and immediately stored at –80°C. The bronchoalveolar lavage (BAL) fluid was analyzed for CCL20 and GM-CSF using an ELISA kit purchased from R&D Systems (Minneapolis, Minn., USA). In some cases, whole lungs were removed and snap frozen in liquid nitrogen for PCR analysis.

#### *Mouse Tracheal Epithelial Cells*

Tracheas from 4-week-old wild-type or PAR-2-deficient mice were removed from the thyroid cartilage to the level of bifurcation and incubated in Pronase (1 mg/ml; Roche Applied Science, Indianapolis, Ind., USA) and incubated (18 h × 4°C while rocking). The next day, 10% FBS and 1 mg/ml DNase (Sigma-Aldrich) was added to the tube and inverted multiple times. The trachea was discarded; cells were washed and plated onto a cell culture plate with Primaria surface treatment (BD Biosciences, Bedford, Mass., USA) for 4 h to remove fibroblasts. Nonattached cells were washed, counted and plated in DMEM/F12 (50/50) containing L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 µg/ml) NaHCO<sub>3</sub> (1 mM), FBS (5%), cholera toxin (0.1 µg/ml), mouse EGF (0.5 ng/ml), amphotericin B (0.25 µg/ml), bovine pituitary extract (50 µg/ml), insulin-transferrin-selenium X, and retinoic acid (0.1

ng/ml). Mouse tracheal epithelial cells were grown on collagen-coated 6-well tissue culture plates until confluent. Cells were treated with PBS, aprotinin in PBS, GC frass (1 µg/ml) or GC frass pretreated with aprotinin for 18 h. Supernatants were harvested, clarified, and analyzed for CCL20 and GM-CSF by ELISA.

#### *Flow Cytometry*

Wild-type or PAR-2-deficient mice were given a single intratracheal instillation of PBS or AF405-labeled GC frass (40 µg/40 µg). Twenty hours later, whole lungs were removed and placed in RPMI-1640 containing Liberase CI (0.5 mg/ml; Roche Diagnostics, Indianapolis, Ind., USA) and DNase I (0.5 mg/ml; Sigma, St. Louis Mo., USA) at 37°C for 45 min. The tissue was forced through a 70-µm cell strainer, and red blood cells were lysed with ACK lysis buffer (Invitrogen). Cells were washed with RPMI containing 10% FBS, counted and 1 × 10<sup>6</sup> cells were used for staining. Staining reactions were performed at 4°C following incubation with Fc block (mAb 2.4G2) for 30 min. mDCs (CD11c+, CD11b+, Gr1-, CD317-) were quantified using anti-CD11c-APC (HL3), anti-CD11b-PE-Cy7 (M1/70), and anti GR-1-APC-Cy7 (RB6-8C5), anti-CD317-FITC (eBio129c). Dead cells were excluded using 7-AAD. All antibodies were purchased from eBioscience (San Diego, Calif., USA). Data were acquired with an LSRII flow cytometer (BD Biosciences, San Jose, Calif., USA). Spectral overlap was compensated using the FACSDiVa software (BD Biosciences) and analyzed using FlowJo software (Treestar Inc., Ashland, Oreg., USA).

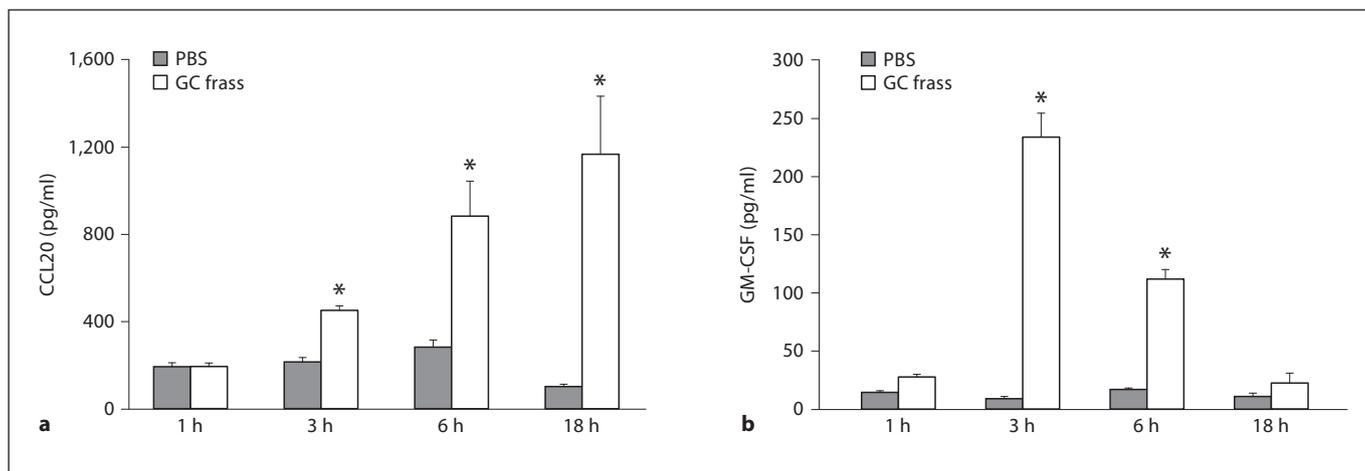
#### *Statistical Analysis*

When applicable, statistical significance was assessed by one-way analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by Student-Newman-Keuls' multiple range test.

## **Results**

### *A Single Intratracheal Instillation of GC Frass Increased CCL20 and GM-CSF Expression in the BAL Fluid*

To measure GC frass-induced production of CCL20 and GM-CSF, naïve wild-type mice were given a single intratracheal instillation of PBS or GC frass (40 µg/40 µl) and BAL fluid was harvested 1, 3, 6, or 18 h later. One hour after instillation, there was no difference in chemokine release into the BAL fluid. However, within 3 h there was a statistically significant increase in the level of both CCL20 (fig. 1a) and GM-CSF (fig. 1b) in the BAL fluid of GC frass-treated mice compared with PBS-treated mice. Interestingly, the levels of CCL20 continued to rise until 18 h after exposure, while the levels of GM-CSF were highest at 3 h after exposure and by 18 h were indistinguishable from those of PBS-treated mice. Together, these data suggest that a single exposure to GC frass is sufficient to induce an early innate immune response in mice;



**Fig. 1.** A single instillation of GC frass induced CCL20 expression in the airways of mice. Balb/c mice were administered a single intratracheal instillation of PBS or GC frass (40  $\mu$ g/40  $\mu$ l) 1, 3, 6, and 18 h later. BAL fluid was harvested, clarified, and production of CCL20 (a) and GM-CSF (b) was analyzed by ELISA. Means  $\pm$  SEM (n = 4–9 mice per group) are reported (\* p < 0.001 compared to PBS control) from 3 separate experiments.

however, the kinetics of CCL20 and GM-CSF release were significantly different following GC frass exposure.

#### *GC Frass Proteases Regulate the Release of CCL20 and GM-CSF into the BAL Fluid*

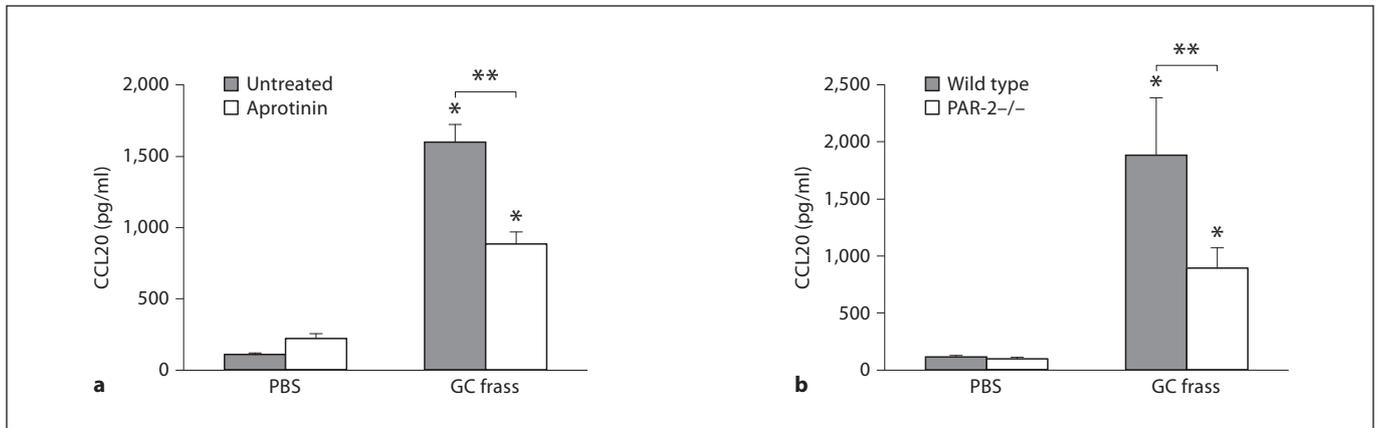
We have previously shown that GC frass contains active serine proteases which can regulate allergic airway inflammation [9]. To test the role of the proteases and PAR-2 activation on CCL20 release into the airways, we compared BAL levels of CCL20 18 h after a single exposure to GC frass or protease-depleted GC frass. We have previously reported that aprotinin treatment inhibits 85% of the protease activity in GC frass [21]. Exposure to GC frass induced a significant increase in airway CCL20 levels, an effect that was significantly reduced when exposure to protease-depleted GC frass occurred (fig. 2a). We then asked whether functional PAR-2 was important in the GC frass-protease mediated CCL20 release. To do this, we compared CCL20 release in wild-type and PAR-2-deficient mice following a single exposure to GC frass. Our data confirmed that a functional PAR-2 was important for optimal CCL20 production (fig. 2b). We also measured CCL20 production 3 h after instillation. At this time point, GC frass induced about a 2-fold increase in CCL20 production, and this production was also partially dependent on the active protease and PAR-2 (data not shown).

We next asked whether the early release of GM-CSF was dependent on PAR-2 activation. Since the kinetics of

GM-CSF are different from those of CCL20, we exposed wild-type and PAR-2-deficient mice to GC frass and isolated the BAL fluid 3 h later. Exposure to protease-depleted GC frass resulted in significantly decreased release of GM-CSF compared to GC frass containing active proteases (fig. 3a). Using PAR-2-deficient mice, we confirmed that GM-CSF release into the airways was partially dependent on PAR-2 (fig. 3b). Analysis of GM-CSF levels in the BAL fluid of mice 18 h after instillation showed insignificant amounts of GM-CSF in the airways at this time point in both PBS- and GC frass-exposed mice (data not shown). Together these data suggest a role for active proteases and PAR-2 in modulating early cytokine production in mice.

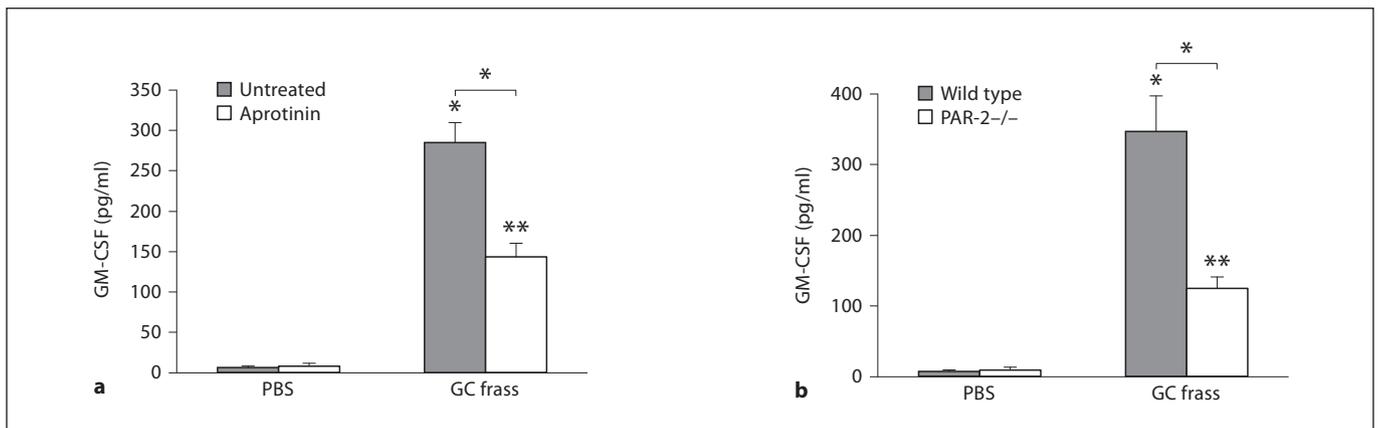
#### *GC Frass Protease Is Sufficient to Induce CCL20 Production*

To further study the role of proteases in CCL20 production, we enriched the protease activity of GC frass using gel filtration. It is interesting to note that while we significantly increased the amount of protease in our preparation, we almost completely removed endotoxin from the GC frass (table 1). We asked whether the enriched protease in GC frass was sufficient to induce the innate immune response as determined by CCL20 production. The protease was administered at a dose comparable to the protease concentration found in GC frass. Our 40- $\mu$ g dose for instillation contains approximately 0.5 activity units; therefore, we administered 0.5 units of



**Fig. 2.** GC frass proteases and PAR-2 regulate CCL20 expression in mice. **a** Balb/c mice were administered a single intratracheal instillation of PBS, aprotinin-treated PBS, GC frass (40  $\mu$ g/40  $\mu$ l) or protease-depleted frass. BAL fluid was harvested 18 h later and clarified, and CCL20 was analyzed by ELISA. Means  $\pm$  SEM (n = 6 mice per group) are reported (\* p = 0.001; \*\* p = 0.01) from

a single experiment. **b** Wild-type or PAR-2-deficient mice were given a single intratracheal instillation of PBS or GC frass and CCL20 levels in BAL fluid were analyzed 18 h later. Means  $\pm$  SEM (n = 8 mice per group) are reported (\* p = 0.001; \*\* p = 0.021) from 2 separate experiments.

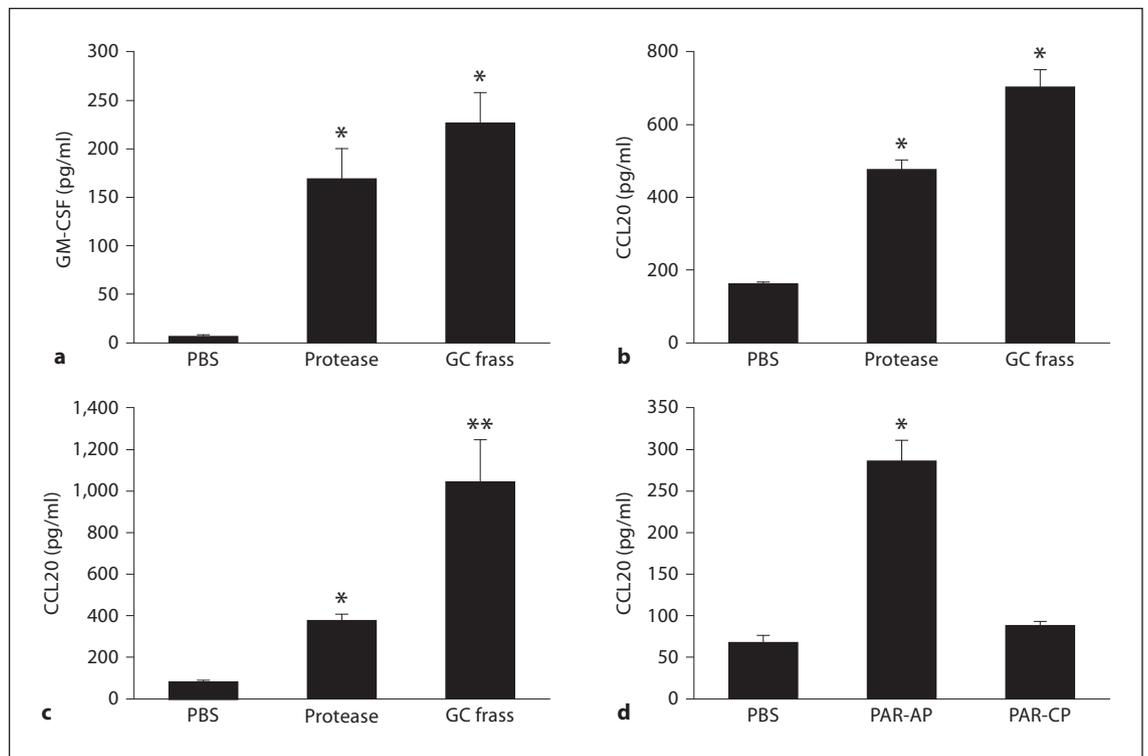


**Fig. 3.** GC frass proteases and PAR-2 regulate GM-CSF expression in mice. **a** Balb/c mice were administered a single intratracheal instillation of PBS, aprotinin-treated PBS, GC frass (40  $\mu$ g/40  $\mu$ l) or protease-depleted frass and harvested 3 h later. BAL fluid was harvested, clarified, and GM-CSF was analyzed by ELISA. Means  $\pm$  SEM (n = 6 mice per group) are reported (\* p < 0.001; \*\* p =

0.01) from 2 separate experiments. **b** Wild-type or PAR-2-deficient mice were given a single intratracheal instillation of PBS or GC frass and GM-CSF levels in BAL fluid were analyzed 18 h later. Means  $\pm$  SEM (n = 8 mice per group) are reported (\* p < 0.001; \*\* p = 0.021) from 2 separate experiments.

enriched protease per mouse and harvested the BAL fluid 3 and 18 h later. We found that the GC frass protease alone was sufficient to induce early GM-CSF (fig. 4a) and CCL20 (fig. 4b) production which was fairly comparable to the levels induced by GC frass. At the 18-hour time point, CCL20 production was also increased compared to PBS-treated mice, but was lower than GC frass (fig. 4c). To determine if selective activation of PAR-2 was also suf-

ficient to mediate CCL20 production in vivo, we performed a single intratracheal instillation of a PAR-2-activating peptide (PAR-2-AP) or a scrambled control peptide (PAR-2-CP). PAR-2-AP activates PAR-2 by bypassing the requirement for proteolytic cleavage. We confirmed that selective activation of PAR-2 was also sufficient to induce CCL20 production, while PAR-2-CP had no effect (fig. 4d). Interestingly, the levels of CCL20 production by



**Fig. 4.** Direct activation of PAR-2 with protease or PAR-2 agonist increased chemokine production. Naïve mice were administered a single instillation of protease-enriched GC frass (0.5 U) or GC frass (40  $\mu$ g) and BAL fluid was harvested 3 or 18 h later. **a** GM-CSF levels at 3 h after instillation (\*  $p < 0.05$ ). **b** CCL20 levels at 3 h after instillation (\*  $p < 0.05$ ). **c** CCL20 levels at 18 h after in-

stillation (\*  $p < 0.05$ ). **d** Naïve mice were administered a single instillation of PBS, PAR-2-activating peptide (PAR-2-AP), or PAR-2 control peptide (PAR-2-CP) and analyzed 18 h after instillation (\*  $p < 0.05$ ). In all cases, means  $\pm$  SEM ( $n = 4-8$  mice per group) are reported from 1 or 2 separate experiments.

the active protease and the PAR-2 agonist were lower than CCL20 production by GC frass at the 18-hour time point. These data suggest an increase in the rate of cleavage or instability of the enriched protease compared to GC frass, or suggests the possibility that other activators may also be required for maximal CCL20 release. GM-CSF levels are below the level of detection by 18 h after instillation.

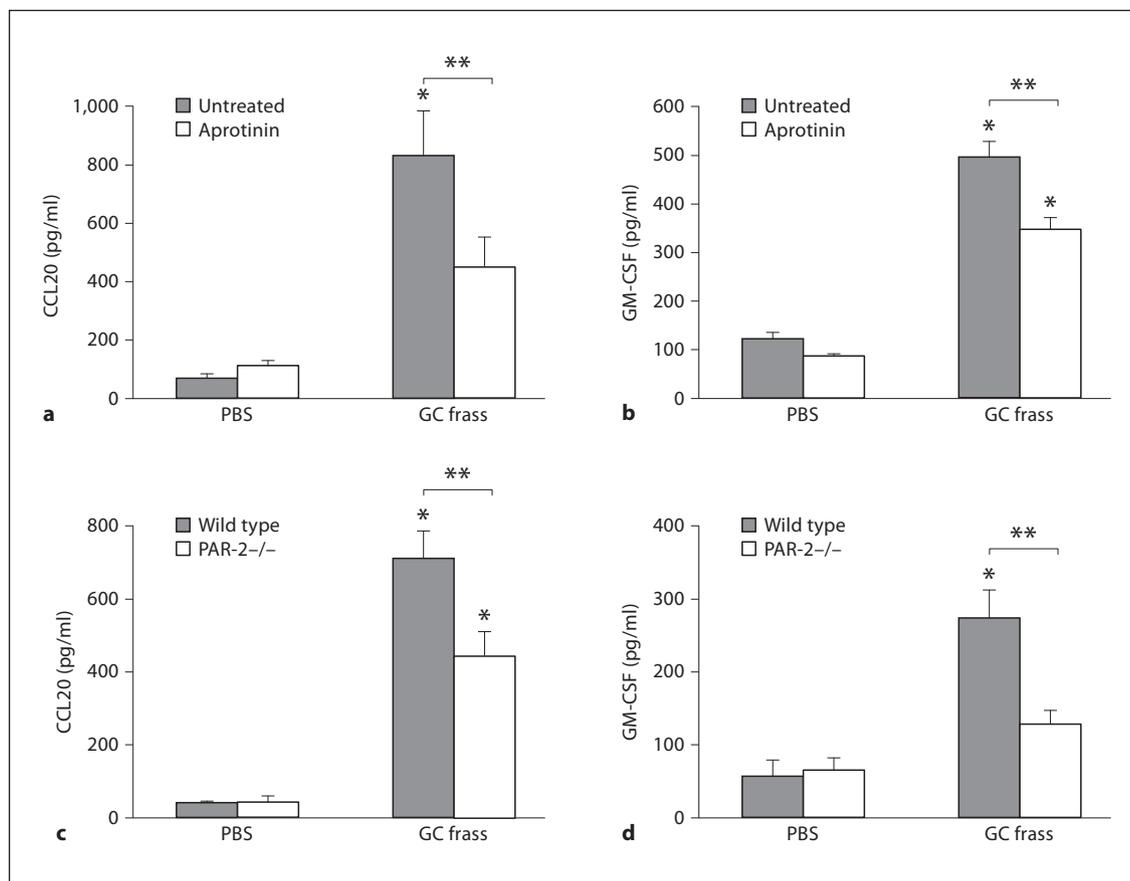
#### *GC Frass Proteases and PAR-2 Regulate CCL20 and GM-CSF Production from Airway Epithelial Cells*

To determine if exposure to GC frass altered airway epithelial cell production of CCL20 and GM-CSF, we cultured primary MTECs from wild-type mice with PBS, aprotinin-treated PBS, GC frass or aprotinin-treated GC frass. Ex vivo treatment of MTECs with protease-depleted GC frass resulted in decreased CCL20 and GM-CSF production when compared to treatment with protease-containing GC frass (fig. 5a, b). To determine if a functional PAR-2 was required for the production of these

chemokines, we cultured primary MTECs from wild-type and PAR-2-deficient mice and treated them with GC frass. We found that PAR-2-deficient MTECs produced less CCL20 and GM-CSF than the MTECs from wild-type mice (fig. 5c, d). These data suggest that the active proteases in GC frass and PAR-2 regulate CCL20 and GM-CSF production from airway epithelial cells.

#### *GC Frass Proteases and PAR-2 Are Sufficient for Increased mDCs in the Lung*

Since GC frass proteases and PAR-2 were sufficient to regulate GM-CSF and CCL20 production, we tested whether protease-PAR-2 activation could alter the recruitment of DCs into the lung. To do this, we performed a single intratracheal instillation of PBS or AF405-GC frass into wild-type or PAR-2-deficient mice. Twenty hours later, lungs were removed, cells isolated and stained for flow cytometry. We classified the mDCs as CD11c+, CD11b+, Gr1- CD317- (data not shown). Exposure to GC



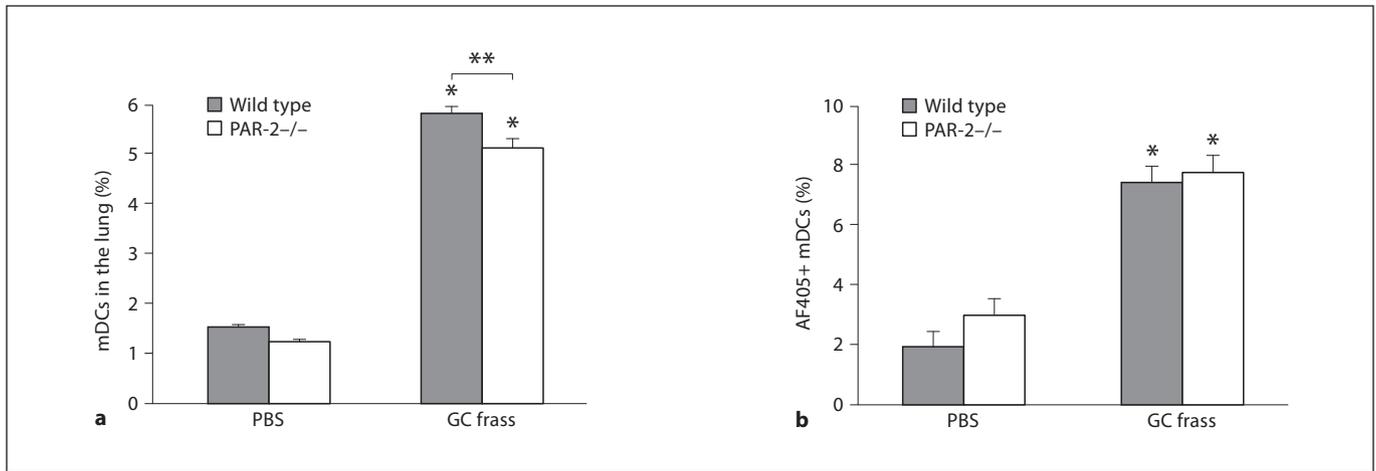
**Fig. 5.** CCL20 and GM-CSF expression from MTECs. Tracheas from mice were isolated and MTECs were cultured in transwell plates until confluent. MTECs were treated with PBS, aprotinin-treated PBS, GC frass (300 ng/ml), or protease-depleted frass GC frass (300 ng/ml) for 18 h. Cell supernatants were harvested, clarified and analyzed for CCL20 (**a**; \*  $p < 0.001$ , \*\*  $p = 0.017$ ) or GM-

CSF (**b**; \*  $p = 0.001$ , \*\*  $p = 0.005$ ) by ELISA. Mean  $\pm$  SEM are reported from 5 separate experiments. MTECs were cultured from wild-type or PAR-2-deficient mice. Cell supernatants were harvested, clarified and analyzed for CCL20 (**c**; \*  $p < 0.001$ , \*\*  $p = 0.019$ ) or GM-CSF (**d**; \*  $p = 0.002$ , \*\*  $p = 0.004$ ) by ELISA. Mean  $\pm$  SEM are reported from 3 or 4 separate experiments.

frass led to an increased percentage of pulmonary mDCs in wild-type mice (fig. 6a). The percentage of mDCs in the airways following GC frass instillation in PAR-2-deficient mice was significantly less than in the wild-type mice. We next asked whether differential uptake of GC frass occurred in PAR-2-deficient mice. We measured the amount of AF405-labeled GC frass in the mDC population in the lung. We found that there was no significant difference in the percentage of mDCs that had taken up AF405-GC frass between the wild-type and PAR-2-deficient mice (fig. 6b). Together, these data suggest that the lower levels of mDCs, but not differential uptake of allergen, may be responsible for the decreased allergic airway inflammation we have previously shown in these mice [9].

## Discussion

Herein we describe a role for allergen-derived proteases and PAR-2 in the release of CCL20 and GM-CSF into the airways of mice following a single exposure to GC frass. GC frass is complex and contains a number of components including active serine proteases [23], endotoxin [23], a TLR2 agonist [24], and a number of unknown components. In this report, our goal was to assess the effect of the protease component(s) in GC frass on their ability to induce chemokine production in vivo. To do this, we used a variety of reagents, including protease-depleted GC frass, protease-enriched GC frass and PAR-2-deficient mice. We found that the kinetics of GM-CSF and CCL20 release are different following in vivo expo-



**Fig. 6.** Percentage of mDCs and allergen uptake in mDCs following AF405-GC frass exposure. Wild-type and PAR-2-deficient mice were exposed to a single intratracheal instillation of PBS or Alexa-Fluor (AF) 405-labeled GC frass (40  $\mu$ g/40  $\mu$ l). Whole lungs were isolated 20 h later and cells were dissociated and stained for flow cytometric analysis. Cells were gated only on

mDCs (CD11c+, CD11b+, Gr1-, CD317-). **a** Percentage of mDCs in the lung (\*  $p < 0.001$ , \*\*  $p = 0.007$ ). **b** Percentage of AF405-positive mDCs in the lung (\*  $p < 0.001$ ). Mean  $\pm$  SEM are reported from 6 mice per group with the samples being run in quintuplicate in a single experiment.

sure to GC frass, in that GM-CSF peaks early while CCL20 has more of a delayed release. While the mechanism responsible for differences in the kinetics of GM-CSF and CCL20 release are unclear, we speculate that the early burst of GM-CSF may be to facilitate DC activation/maturation. As mature DCs will then migrate from the lung to the lung-draining lymph nodes, the subsequent burst of CCL20 release may be responsible for replacing the mature DCs with immature DCs recruited directly from the circulation. In support of this, it has been demonstrated that allergen-bearing, activated DCs can be observed in the lung-draining lymph nodes as rapidly as 6 h after allergen exposure [25].

We confirmed that these chemokines are being synthesized by the airway epithelium, and are likely first responders to allergen exposure. It is not surprising that allergen-derived proteases can act directly on airway epithelium as we have previously shown the role of proteases in regulating signaling pathways following PAR-2 activation [23, 26, 27]. Other reports have also investigated the importance of the allergen-associated protease in regulating CCL20 production in vitro. Pichavant et al. [28] showed that the proteolytically active Der p1 induced CCL20 while the inactive form, pro-Der p1, failed to regulate CCL20 production in BEAS-2B cells. In addition, they showed that chemical inhibition of Der p1 protease activity also inhibited CCL20 production, suggest-

ing that its action is linked to its protease activity. Trypsin has been shown to induce PAR-2-mediated CCL20 production in human gingival epithelial cells [29]. However, Nathan et al. [14] recently showed that HDM-induced CCL20 production in the human airway epithelial cell line 16HBE14o- cells was independent of protease activity. The protease activity in the HDM preparation was not discussed in that study, so it is unclear how much protease activity was initially in the HDM preparation. In the current study, we enriched the protease in GC frass using column chromatography methods. Our data indicate that the enriched protease can induce GM-CSF and CCL20 production at a level similar to that of GC frass at 3 h after instillation. Interestingly though, at 18 h after instillation, the levels of CCL20 production from the enriched protease were substantially lower than those from GC frass. One interpretation of these data is that enrichment of the protease may remove a component in GC frass that aids in the stabilization of the protease or in protection from degradation. It is likely that endogenous pulmonary antiproteases (i.e. serine leukocyte protease inhibitor or  $\alpha_1$  antitrypsin) could inactivate the enriched protease at an increased rate, or with increased potency.

Interestingly, a consequence of the enrichment of the protease was the removal of endotoxin. We have previously attempted to remove the endotoxin using a com-

mercially available endotoxin-removal column; however, we were unsuccessful in removing more than 50% of the endotoxin [K. Page, unpublished observation]. It is important to note that endotoxin was not the only component removed from GC frass during the enrichment procedure and at this point it is unclear what role these unknown components have on chemokine production. Complete removal of endotoxin often includes very harsh conditions (i.e. exposure to acids or alkalis at concentrations equal to or higher than 0.1 M or temperatures of 250°C for 30 min) which would also alter the activity of a serine protease. Since we were unable to selectively remove endotoxin from GC frass while retaining the presence of proteases and other proteins, we cannot conclude in the present study that endotoxin plays a major role in regulating CCL20 and GM-CSF production. In addition, treatment with commercially available 'purified' endotoxin may not be similar to the endotoxin found in GC frass. Thus, while it is possible that endotoxin plays a role in mediating these effects, the overall goal of this study was to examine the ability of the active serine protease in GC frass to regulate chemokine expression *in vivo*.

At this point, we have no evidence to suggest that PAR-2-deficient mice are unable to mount a normal immune response because of an abnormality in the DC population. While Fields et al. [30] showed that DCs do not spontaneously develop from the bone marrow of PAR-2-deficient mice, a subsequent study failed to find a direct role for PAR-2 in the differentiation of bone marrow from wild-type and PAR-2-deficient mice into bone marrow DCs when cultured in the presence of GM-CSF [31]. In addition, there was no difference between DC subset frequencies in the lymph nodes of PAR-2-deficient mice compared to wild-type mice [31]. In our study, there was no difference in the numbers of mDCs in the PBS-stimulated PAR-2-deficient mice compared to wild-type mice. If PAR-2 deficiency was responsible for the development of DCs, we would expect a lack of DCs in the lung even in the unchallenged state. One difference between our work and that of Ramelli et al. is that they found that selective PAR-2 activation increased maturation of bone marrow DCs as evidenced by increased MHC class II and CD86 expression on DCs and that PAR-2-deficient mice demonstrated a reduced frequency of FITC+ DCs following FITC painting [31]. While we did not study the maturation of bone marrow DCs in the presence of GC frass proteases in this study, our results did not show any differences in the uptake of Alexa-Fluor 405-labeled GCs by pulmonary DCs between wild-type and PAR-2-deficient mice. The reasons for this are

unclear, but may reflect differences in mechanisms of allergen uptake between small molecules (FITC) and complex antigens (GC frass) or differences in PAR-2 biology between DCs at epidermal versus mucosal sites. Further study is needed to determine the root cause of these differences.

GM-CSF is clearly a proallergic signal, as overexpression of GM-CSF was shown to induce allergic airway inflammation to ovalbumin exposure compared to ovalbumin exposure alone [18]. Our data demonstrate that the complex allergen GC frass can directly induce GM-CSF expression from epithelial cells in a mechanism at least partially dependent on PAR-2 activation. In support of this, BEAS-2B cells treated with Der p1 or Der p9 were shown to produce GM-CSF [32], and Der p3 and Der p9 were shown to activate PAR-2 and induce the release of GM-CSF [33]. Another study confirmed that recombinant allergens Der f 1 and Der p 1 stimulated the production of GM-CSF in normal human keratinocytes and this could be inhibited by the addition of cystatin A (a cysteine proteinase inhibitor) [34]. Normal and asthmatic bronchial epithelial cells have been shown to release GM-CSF following Der p exposure [35] and, recently, nasal biopsies from patients with chronic rhinosinusitis without nasal polyps had increased PAR-2 expression [36]. Collectively, these studies suggest that PAR-2 activation by protein allergens can induce GM-CSF production by epithelial cells, suggesting a possible mechanism by which allergen exposure could initiate allergic responses.

Overall, the findings in this study show that the protease-PAR-2 plays a role in GM-CSF and CCL20 production in the airways of mice. The subsequent infiltration and/or differentiation of mDCs could be sufficient to initiate allergic airways in the presence of allergen. It is important to consider that the relatively small, but statistically significant change in the percentage of mDCs in the airways of wild-type and PAR-2-deficient mice following GC frass exposure was performed on whole lung. It is possible that if we looked directly at the actual differences in mDC populations in a local microenvironment, these changes might be greater. We anticipate that in the upper airways where the instillation of GC frass is likely to be the most concentrated, there may be even higher levels of mDC recruitment. In our study, it does not appear that PAR-2 plays a role in antigen uptake, but it is possible that an additional role for PAR-2 could be in the regulation of the overall maturation state of the DCs, the ability of DCs to process and present antigen, or in the migration of activated DCs to the draining lymph nodes,

where T cell activation can occur. Elucidation of the mechanism by which proteases associated with allergens can initiate and/or augment the early innate immune response may ultimately lead to a better understanding of how an allergen is able to elicit a shift from tolerance to disease.

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