

Activation of Mast Cells Is Essential for Development of House Dust Mite *Dermatophagoides farinae*-Induced Allergic Airway Inflammation in Mice¹

Chun-Keung Yu² and Chih-Long Chen

In this study, we demonstrate that *Dermatophagoides farinae* (Der f), a major source of airborne allergens, but not OVA, could rapidly activate mast cells in mice. This was indicated by an elevation of serum mouse mast cell protease 1, a mast cell-specific proteinase, as early as 30 min after intratracheal challenge. Administration of sodium cromoglycate (40 mg/kg, i.p., 1 h before Der f instillation), a mast cell stabilizer, not only suppressed acute mouse mast cell protease 1 production but also attenuated the allergic airway inflammation provoked by repetitive Der f challenge in mice (five times at 1-wk interval). Der f induced the expression of mRNA for TNF- α , IL-1 β , IL-4, IL-6, IL-9, and IL-13 in mastocytoma P815 cells and stimulated both P815 cells and bone marrow-derived mast cells to produce IL-4, IL-6, and TNF- α in a dose- and time-dependent manner. Cycloheximide as well as sodium cromoglycate blocked the Der f-induced IL-4 production, indicating a de novo protein synthesis process. Supernatants of Der f-stimulated mast cells chemoattracted monocytes and T lymphocytes; they up-regulated the expression of costimulatory B7 molecules, eotaxin, RANTES, monocyte chemoattractant protein 1, and IFN-inducible protein 10 mRNA of alveolar macrophages; they supported PHA-induced T cell proliferation; and they promoted Th2 cell development. Our data indicate that mast cells may be an important cell type during the initiation of Der f sensitization in the airway by modulating the function of alveolar macrophages and T cells. *The Journal of Immunology*, 2003, 171: 3808–3815.

Bronchial asthma is a chronic inflammatory disease of the bronchial airways orchestrated by type 2 helper (Th2) T cells and their secreted cytokines (1, 2). Although the pathogenesis of bronchial asthma is not fully understood, studies have shown that innate immunity is involved in the activation and recruitment of cells that are important for the development of allergic sensitization and inflammation (3–6).

Mast cells accumulate at the site of Th2 cell activation and are the primary initiating cells of immediate hypersensitivity reactions, acting by the release of chemical mediators after cross-linking of IgE receptors by Ag triggers (7–9). Mast cells also have been identified as key effectors and regulators in parasite and bacteria defense mechanisms (10, 11). In addition to their role in inflammatory reaction, mast cells can contribute to the regulation of specific immune responses. Mast cells influence the differentiation of naive T cells toward Th2 cells (12, 13) and B cell activation and development (14). The role of mast cells in murine models of asthma has been demonstrated primarily based on studies in mast cell-deficient mice (15–17).

House dust mites (HDMs)³ are the most prominent sources of allergens causing allergic rhinitis and asthma. In vitro studies have

shown that HDM allergens affect a variety of cell types, including bronchial epithelial cells (18), mast cells (19), T cells (20), and B cells (21) and can activate both the kallikrein (22) and complement systems (23). We showed recently that alveolar macrophages (AMs) are sensitive targets for *Dermatophagoides farinae* (Der f), one of the major species of HDMs implicated in allergic asthma (24). These in vitro properties of HDMs are thought to be associated with their allergenicity. However, little is known about the biological sequelae of inhalation of HDM allergens that results in allergic sensitization. Because the airway mucosa is composed of multiple cell types that are known to be affected by HDMs, we surmised that the cross-talk of these cells must represent critical elements in the control of the initiation of allergic sensitization. Our data demonstrate that Der f rapidly activates mast cells in vivo in a non-IgE-mediated process after intra-airway instillation, which is essential for the development of allergic responses. In addition, mast cells are capable of producing multiple cytokines, including those with proinflammatory, chemotactic, and lymphocyte-modulating activities in response to Der f stimulation, which not only promote chemokine expression of AMs but also enhance the proliferation and Th2 polarization of naive T cells.

Materials and Methods

Reagents

Der f (1 g lyophilized whole body extract in ether; Allergon, Engelholm, Sweden) (25), OVA, or LPS (*Escherichia coli* 055:B5; both from Sigma-Aldrich, St. Louis, MO) were dissolved in pyrogenic-free isotonic saline, filtered through a 0.22- μ m filter, and stored at -70°C before use. LPS concentrations of the Der f and OVA preparations were <0.96 and <1.92 endotoxin units/mg, respectively (*Limulus* amoebocyte lysate test, E-Toxate; Sigma-Aldrich).

Mast cell line and generation of bone marrow-derived mast cells (BMMCs)

P815 cells, a mastocytoma cell line from DBA/2 mice, was obtained from American Type Culture Collection (Manassas, VA). BMMCs were obtained from 6- to 8-wk-old BALB/c or DBA/2 mice (Laboratory Animal Center, National Cheng Kung University, Tainan, Taiwan, Republic of

Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, Republic of China

Received for publication April 25, 2003. Accepted for publication July 29, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by Grant NSC90-2320-B-006-067 from the National Science Council, Republic of China.

² Address correspondence and reprint requests to Dr. Chun-Keung Yu, Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, 70101, Republic of China. E-mail address: dckyu@mail.ncku.edu.tw

³ Abbreviations used in this paper: HDM, house dust mite; Der f, *Dermatophagoides farinae*; AM, alveolar macrophage; BMMC, bone marrow-derived mast cell; mMCP-1, mouse mast cell protease 1; i.t., intratracheal; BAL, bronchoalveolar lavage; SCG, sodium cromoglycate; mMCP-1, mast cell protease 1; MCP, monocyte chemoattractant protein; IP-10, IFN-inducible protein 10; PAR, protease-activated receptor.

China). Briefly, the mice were killed with an overdose of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Intact femurs and tibias were removed, and bone marrow cells were harvested by flushing with HBSS. The cell culture was established at a density of 3×10^6 cells/ml in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 1 mM pyruvate, 2 mM L-glutamine, 5×10^{-5} M 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 U/ml IL-4, and 20 U/ml IL-3. Nonadherent cells were transferred to fresh medium every 2–3 days for a total of 21 days to remove adherent macrophages and fibroblasts. The culture consisted of 98% pure mast cells, as assessed by toluidine blue staining. For the last 48 h before harvesting, BMMCs were cultured in the presence of IL-3 and IL-4 in serum-free medium (AIM V; Invitrogen, Carlsbad, CA). All experimental animal care and treatment followed the guidelines set up by the Institutional Animal Care and Use Committee.

Mast cell activation in vivo—mouse mast cell protease 1 (mMCP-1) ELISA

mMCP-1 is a protease specific for mouse mast cells and appears in the blood of mice after mast cell activation (26). To monitor mast cell activation in vivo, groups of six BALB/c mice were intratracheally (i.t.) inoculated with Der f or OVA (50 μ l, 0.5 mg/ml), with or without an i.p. injection of sodium cromoglycate (SCG, 40 mg/kg; Sigma-Aldrich), according to the method previously described (25). Blood samples were collected 30, 60, 120, and 180 min after challenge. After centrifugation, sera were stored at -70°C until assay for mMCP-1 (Moredun Scientific, Midlothian, U.K.) according to the manufacturer's instructions.

Allergen sensitization and assessment of blood eosinophilia and airway inflammation

Groups of six BALB/c mice were i.t. inoculated with five doses of either Der f or OVA (50 μ l, 0.5 mg/ml) at 1-wk intervals. Three days after the last challenge, the number of blood eosinophils was determined using a diagnostic reagent system (Unopette test 5877; BD Biosciences, Rutherford, NJ) with blood samples collected via the orbital sinus under light anesthesia. Mice were then killed and serum samples were collected and stored at -70°C until assay. Bronchoalveolar lavage (BAL) was performed (two washes of 1 ml of ice-cold endotoxin-free saline each) according to the previously described procedure (25). The BAL fluids were separated (2000 rpm, 10 min) and stored at -70°C . After total leukocyte counting, differential counts were performed on cytospin preparations (2×10^4 cells/100 μ l of BAL fluid) stained with Liu stain (Tonyar Diagnostics, Taipei Hsien, Taiwan) in a blind manner. For inhibition studies, mice were i.p. injected with SCG (40 mg/kg) 1 h before each Der f inoculation.

Immunohistochemistry for detection of dendritic cells in lungs

Lungs were removed, embedded in OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Cryosections were placed onto slides coated with poly-L-lysine (Sigma-Aldrich), fixed in ice-cold acetone, and incubated with Abs against CD11c (PE-conjugated, HL3) and CD86 (FITC-conjugated, GL1) (BD PharMingen, San Diego, CA). The number of double-positive staining cells was counted in 20 fields around bronchioles in each of two duplicate slides under a fluorescent microscope ($\times 400$, Leica DM IRE2 and TRB; Leica, Wetzlar, Germany).

Total IgE and Der f-specific IgG1 and IgG2a/2b

An IgE-specific ELISA was used to measure the total IgE Ab levels in serum samples using matching mAb pairs (R35-72 and R35-92; BD PharMingen) according to the manufacturer's instructions. A_{450} readings of the samples were converted to nanograms per milliliter using a standard curve generated with double dilutions of mouse IgE κ isotype standard (BD PharMingen). For Der f-specific Abs, serum samples (1/5 dilution) were added in duplicate onto ELISA plates coated with Der f (20 μ g/ml in 0.1 M NaHCO_3 , pH 8.3). After incubation overnight at 4°C , the plates were washed and incubated with biotinylated rat anti-mouse IgG1 (A85-1) or IgG2a/2b (R2-40) mAb (2 μ g/ml; BD PharMingen) for 1 h, followed by washings and the addition of streptavidin-HRP conjugate (1/1000 BD PharMingen). The plates were washed and developed with a tetramethylbenzidine microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Absorbance of the samples was determined at 450 nm.

Der f stimulation of mast cells

Mast cells (2×10^5), either P815 cells or BMMCs, were treated with medium alone or with 10 μ l of predetermined noncytotoxic doses of Der

f, OVA, or LPS for 2, 12, 24, or 48 h. Supernatants were collected, centrifuged, and stored at -70°C until assayed for IL-4, IL-6, or TNF- α . After extensive washing, cells were subjected to RT-PCR as described below. For preparation of mast cell-conditioned medium, stimulation was performed by immobilized Der f, OVA, or LPS (100 μ l, 0.1 mg/ml) onto plastic wells at 4°C overnight. Wells were washed three times with PBS and mast cells were incubated in serum-free medium at 37°C for 24 h. Culture supernatants were collected, centrifuged, and stored at -70°C until used for stimulation of AMs and T cells. Der f concentration in the supernatants was below 0.01 μ g, as estimated by semiquantitative dot blotting with a serial dilution of Der f (0–10 μ g) to produce a positive control. For inhibition studies, mast cells were incubated with Der f in the presence of cycloheximide (Sigma-Aldrich) or SCG.

Stimulation of alveolar macrophages

BAL cells were collected from BALB/c or DBA/2 mice, pooled, and incubated at 37°C for 30 min in culture plates to remove nonadherent cells. The adhered BAL macrophages ($>95\%$ viability) were recovered and resuspended in serum-free medium. To a volume of 0.5 or 0.75 ml/well, AMs (5×10^5) were seeded in 24-well plates with the presence of 0.5 ml (50%) and 0.25 ml (25%) of mast cell supernatants, respectively. After a 24-h culture, AMs were collected for flow cytometry or RT-PCR. For inhibition experiments, AMs were stimulated with mast cell supernatants in the presence of the following reagents: mAbs to IL-4 (11B11), IL-6 (MP5-20F3), or TNF- α (G281-2626) (5 μ g each; BD PharMingen).

Stimulation of T cells

Autologous plastic-nonadherent splenic cells were collected, and splenic T cells were purified by nylon wool column separation. Single-cell suspensions of splenic T cells ($2 \times 10^5/100 \mu$ l) were stimulated with PHA (1 mg/ml; Murex Biotech Limited, Dartford, U.K.) in combination with 100 μ l of mast cell supernatants plus neutralizing mAb to IL-4, IL-6, or TNF- α (5 μ g each). After a 24-h incubation, 0.5 μ Ci of [^3H]thymidine (Amersham Life Science, Buckinghamshire, U.K.) was added and further incubated for 24 h. Cells were then harvested, and radioactivity as cpm was determined by liquid scintillation counting. For T cell phenotyping, splenic T cells (1×10^6) in 0.5 ml were added to 24-well plates coated with anti-CD3 mAb (10 μ g/ml) in combination with 0.5 ml of mast cell supernatants plus neutralizing mAb to IL-4, IL-6, or TNF- α (5 μ g each). After 96 h, cells were transferred to uncoated 24-well plates with 1 ml fresh culture medium and cultured for an additional 48 h. The cells were collected, washed, and restimulated (1×10^6) with immobilized anti-CD3 mAb (10 μ g/ml) for 24 h. Then supernatants were collected and levels of IL-4 and IFN- γ were determined by immunoassay as described below. After washing, the T cells were subjected to RT-PCR for the expression of T-bet and GATA-3.

Chemotaxis

Splenic T cells and peritoneal macrophages were obtained by nylon wool column separation and peritoneal lavage, respectively. After washing, cells were resuspended in RPMI 1640 containing 0.5% FBS. A total of 1×10^6 cells per 0.3 ml was placed in the upper wells of a 24-well microchemotaxis chamber (BD Labware, Franklin Lakes, NJ). Lower wells contained 0.5 ml of mast cell supernatants. After incubation at 37°C for 4 h, the cells present in the lower wells were counted using a hemocytometer.

Flow cytometric analysis

After 24 h of AM culture in the presence of the supernatants of Der f-stimulated mast cells, cells were collected, resuspended (1×10^6 in 1 ml of HBSS), and incubated with anti-CD16/CD32 mAb (Fc blocker, 2.4G2) followed by FITC-conjugated anti-B7.1 (16-10A1) or anti-B7.2 (GL1) and PE-conjugated anti-I-A ^{d} (AMS-32.1) mAbs (BD PharMingen) for 30 min on ice. After washing, stained cells were quantified by using FACSscan (BD Immunocytometry Systems, San Jose, CA). Isotype-matched mAb-stained cells were used as a background control in all experiments.

RT-PCR

RT-PCR was performed as previously described (24). Total cellular RNA was extracted from lung tissues, pooled AMs, or T cell samples (RNeasy Total RNA kit; Qiagen, Hilden, Germany) and converted to cDNA with StrataScrip H-reverse transcriptase (Stratagene, La Jolla, CA). PCR amplification (GeneAmp PCR System 2400; PerkinElmer, Branchburg, NJ) was performed on a 1- μ l cDNA sample. Amplification cycle numbers and annealing temperatures were optimized for each primer pair and PCR products were obtained within the linear range of amplification. To control for sample-to-sample variation, the amount of cDNA input to all samples was

first adjusted to comparable levels of β -actin or GAPDH transcripts before it was subjected to PCR amplification. Gene-specific primer pairs (sense and antisense, respectively) were used as follows. TNF- α , 5'-AGCCC ACGTCGTAGCAAAACCAACAA-3' and 5'-ACACCCATTCCCTTCACA GAGCAAT-3'; IL-1 β , 5'-TCATGGGATGATGATAACCTGCT-3' and 5'-CCCATACTTTAGGAAGACACGGATT-3'; IL-6, 5'-CTGGTGACA ACCACGGCCTTCCTA-3' and 5'-ATGCTTAGGCATAACGCACTAG GTA-3'; IL-9, 5'-CATCCTTGCTCTGTTTTCG-3' and 5'-CGTCCCCA GGAGACTCTT-3'; IL-13, 5'-CTGCAGTCCTGGCTCTCG-3' and 5'-CTTTCCGCTATGGCCACTG-3'; eotaxin, 5'-CCATCTGTCTCCCTC CACCATG-3' and 5'-ATCCACATCTCTTTTCATGCC-3'; RANTES, 5'-GTACATCACCATGGCGTATG-3' and 5'-TCTTCTCTGGGTTGGC ACACA-3'; monocyte chemoattractant protein (MCP-1), 5'-ACCAGC CAACTCTCACTGAAGC-3' and 5'-CAGAATTGCTTGAGGTGGTTGTG-3'; MCP-2, 5'-AGTGCTTCTTTCCTGCTGCTCATAG-3' and 5'-ATGA GAAACACGCAGCCAGGCACC-3'; MCP-3, 5'-ACGCTTCTGTGCTC GCTGCTCATAG-3' and 5'-GTAAAAATGGGAAAGGGGAGAAT-3'; macrophage-inflammatory protein 2, 5'-AGTGAAGTGCCTGTCAATG-3' and 5'-CTTTGGTCTTCCGTTGAGG-3'; IFN-inducible protein 10 (IP-10): 5'-AGCGATCGGAGAGTTCAAGAGTG-3' and 5'-TTCACCCCAAGAGC CCAGTTTCA-3'; GATA-3, 5'-GAAGGCATCCAGACCCGAAAC-3' and 5'-ACCATGGCGGTGACCATGC-3'; T-bet, 5'-TGCCTGCAGTGCT TCTAACA-3' and 5'-TGCCCCGCTTCTCTCCAACCA-3'; β -actin, 5'-TGGAACTCTGTGGCATCCATGAAAC-3' and 5'-TAAAGCGAGCT CAGTAACAGTCCG-3'; and GAPDH, 5'-GTCTTCACCAACCATGGAG-3' and 5'-CCAAAGTTGTCATGGATGACC-3'. PCR products were electrophoresed on 2% agarose and stained with ethidium bromide. Bands were quantified by using densitometry analysis (Bio-1D; Vilber Lourmat, Marne La Vallée, France).

ELISA of cytokine levels

Concentrations of cytokines were measured by a sandwich ELISA technique using commercial matching mAb pairs, one of which was biotinylated (IL-6: MP5-20F3 and MP5-32C11; TNF- α : G281-2626 and MP6-XT3; IL-4: 11B11 and BVD6-24G2; IFN- γ : R4-6A2 and XMG1.2; BD PharMingen). The reaction was developed as described above. Standards were run in parallel with recombinant cytokines. The detection limits were 7.5 pg/ml for IL-4, IL-6, and TNF- α and 15 pg/ml for IFN- γ .

Statistical analysis

Cytokine contents in culture supernatants and T cell proliferation values were analyzed by the Kruskal-Wallis test. The serum mMCP-1 levels, migratory cell numbers, cellular accumulation, and cytokine contents in BAL fluid and serum Ab titer were analyzed using the Mann-Whitney *U* test. Results are expressed as means $\pm$ SEM. A *p* < 0.05 was considered to be significant.

Results

Der f induces early phase mast cell activation

To examine whether Der f could activate mast cells *in vivo*, we monitored serum mMCP-1 after a single i.t. Der f challenge. A 6-fold increase in serum mMCP-1 was found in mice as early as 30 min after Der f challenge in comparison to naive mice (5.63 ± 0.24 ng/ml serum vs 0.92 ± 0.63 ng/ml serum, *p* < 0.05, *n* = 6 mice per group, Fig. 1). Elevated mMCP-1 levels last for at least 180 min after challenge. Instillation of an equal amount of OVA showed no effect. An i.p. injection of SCG (40 mg/kg) 1 h before Der f challenge significantly blocked the Der f-induced enhanced in serum mMCP-1 by 50–70%. Naive mice did not develop immediate hypersensitivity reaction upon Der f challenge (data not shown), thus the observed Der f effect apparently was not attributed to cross-reactive Ab to other mite species known to infest mice.

Mast cell activation is essential for the development of Der f-induced allergic responses in mice

In agreement with our previous reports (24, 25), repetitive i.t. instillation of Der f elicited blood eosinophilia, pulmonary eosinophilic inflammation, and elevated total serum IgE and Der f-specific IgG Ab productions in mice (Table I). RT-PCR also showed that there was an up-regulation of IL-4, IL-5, IL-13, RANTES, eotaxin, and GATA-3, a Th2-specific transcription factor, in lung

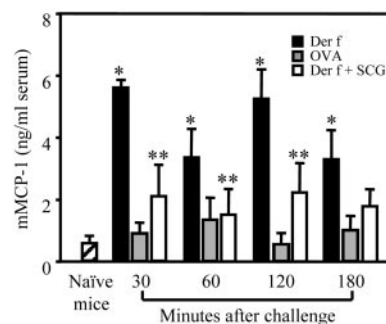


FIGURE 1. Der f activates mast cells *in vivo*. BALB/c mice were i.t. inoculated with Der f or OVA (50 μ l, 0.5 mg/ml), with or without an i.p. injection of SCG (40 mg/kg, 1 h before inoculation), and killed at 30, 60, 120, and 180 min after inoculation. Mast cell activation was evaluated by the determination of the concentration of serum mMCP-1 (see *Materials and Methods*). Data are the means $\pm$ SEM of six mice per group. *, *p* < 0.05 as compared with naive mice; **, *p* < 0.05 as compared with SCG nontreated Der f-challenged mice.

tissues (7.4-, 12-, 2.4-, 28.2-, 5-, and 2.5-fold to basal levels, respectively; Fig. 2A). On the other hand, IL-10 was down-regulated (0.5-fold). OVA-challenged mice had a much weaker BAL cellular responses than Der f-challenged mice. Although OVA challenge up-regulated IL-5 to a level similar to that of Der f challenge, the expression of IL-4, IL-13, RANTES, eotaxin, and GATA-3 in lung tissues was much attenuated (6.5-, 2.4-, 18.9-, 2.2-, and 1.4-fold to basal levels, respectively). Furthermore, in addition to GATA-3, OVA mice also had enhanced expression of T-bet (3.2-fold), a Th1-specific transcription factor (Fig. 2A). To access the contribution of mast cell activation in the subsequent Der f-induced allergic responses, we compared the Der f challenge effects in mice with or without SCG treatment. SCG significantly blocked the blood and pulmonary eosinophilia and elevated IL-4 and IL-5 levels in serum and BAL fluids in Der f mice (Table I). SCG also suppressed the expression of IL-4, IL-5, and IL-13, but enhanced those of IL-10 and T-bet (1.4- and 5.6-fold, respectively Fig. 2A) and significantly reduced the number of infiltrating CD86⁺CD11c⁺ dendritic cells in lungs (24.9 ± 1.5 cells/field vs 18.5 ± 1.7 cells/field, *p* < 0.05; Fig. 2, B and C). Nevertheless, SCG did not alter Ab responses. There were no differences in the levels of total serum IgE and Der f-specific IgG Abs in mice with or without SCG treatment (Table I).

Mast cells produce proallergic cytokines after Der f stimulation

We then further investigated how mast cells responded to Der f stimulation using the P815 mast cell line and BMMCs. RT-PCR showed that Der f up-regulated IL-1 β , IL-4, and IL-6 mRNA in P815 cells as early as 1 h after stimulation (3.8-, 2.6-, and 3.7-fold, respectively). A more intense expression of IL-1 β (5-fold) and IL-6 (5.7-fold) along with an up-regulation of TNF- α (2.7-fold), IL-4 (2.6-fold), IL-9 (10.9-fold), and IL-13 (5.6-fold) was observed at 24 h (Fig. 3A). LPS as well as OVA stimulation also increased the expressions of these cytokines except IL-9. To determine whether Der f stimulated cytokine protein secretion, we measured IL-4, IL-6, and TNF- α concentrations in the culture supernatants of mast cells incubated with Der f for 24 h in comparing with LPS and OVA. In response to Der f, both P815 cells and BMMCs produced IL-4 and IL-6, as well as low but significant concentrations of TNF- α (Fig. 3B). LPS but not OVA induced IL-6 production of P815 cells. Additional experiments showed that both soluble and immobilized (plated-coated) Der f induced IL-4 secretion by P815 cells and BMMCs in a time- and dose-dependent manner (Fig. 4A). Der f induced IL-4 levels as high as those

Table I. Mast cell activation is essential for Der f-induced allergic responses in mice^a

Allergic Responses	Naive Mice	Der f	OVA	Der f + SCG
Total BAL cells ($\times 10^4$ /ml)	25.0 $\pm$ 1.7	63.8 $\pm$ 5.8*	42.0 $\pm$ 4.0*	16.8 $\pm$ 2.3**
Macrophage ($\times 10^4$ /ml)	23.3 $\pm$ 1.6	44.0 $\pm$ 2.2*	37.1 $\pm$ 2.9*	15.7 $\pm$ 2.3**
Lymphocyte ($\times 10^4$ /ml)	0.6 $\pm$ 0.1	7.2 $\pm$ 1.9*	2.4 $\pm$ 0.6*	0.6 $\pm$ 0.3**
Neutrophil ($\times 10^4$ /ml)	0.5 $\pm$ 0.1	8.0 $\pm$ 2.6*	2.1 $\pm$ 0.4*	0.3 $\pm$ 0.1**
Eosinophil ($\times 10^4$ /ml)	0	4.8 $\pm$ 1.0*	0.4 $\pm$ 0.1*	0.1 $\pm$ 0.03**
Blood eosinophils (no. mm ⁻³)	44.0 $\pm$ 0.1	185.0 $\pm$ 10.1*	111.7 $\pm$ 10.5*	84.7 $\pm$ 15.4**
Total serum IgE (ng/ml)	189 $\pm$ 8	227 $\pm$ 4*	233 $\pm$ 5*	223 $\pm$ 2*
Der f-specific IgG ₁ (A ₄₅₀)	0.158 $\pm$ 0.023	1.702 $\pm$ 0.062*	0.160 $\pm$ 0.041	1.812 $\pm$ 0.241*
Der f-specific IgG ₂ (A ₄₅₀)	0.073 $\pm$ 0.018	0.434 $\pm$ 0.154*	0.054 $\pm$ 0.012	0.639 $\pm$ 0.228*
Cytokine (pg/ml)				
BAL IL-4	0	0	0	0
BAL IL-5	40 $\pm$ 10	511 $\pm$ 13*	519 $\pm$ 15*	58 $\pm$ 12**
Serum IL-4	0	561 $\pm$ 26*	0	0**
Serum IL-5	25 $\pm$ 10	566 $\pm$ 50*	453 $\pm$ 6*	10 $\pm$ 5**

^a BALB/c mice were i.t. inoculated with five doses of Der f or OVA (50 μ l, 0.5 mg/ml) at 1-wk intervals. Mice were i.p. injected with SCG (40 mg/kg) 1 h before each Der f inoculation. Mice were killed at day 3 after the last challenge and assayed as described in *Materials and Methods*. Data are the means $\pm$ SEM of six mice per group.

*, $p < 0.05$ as compared to naive mice; **, $p < 0.05$ as compared with SCG-nontreated Der f-challenged mice.

obtained by stimulation with Con A, which has been reported to promote histamine, IL-4, and IL-13 release of basophils and mast cells (27). Cycloheximide significantly inhibited the IL-4 production of Der f-stimulated P815 cells, suggesting that de novo protein synthesis was involved in the process (Fig. 4B). Furthermore, treatment of SCG not only blocked the Der f-induced cytokine expres-

sion (Fig. 3A) but also IL-4 protein production (Fig. 4B). Although mast cells express a number of Toll-like receptors, including Toll-like receptors 2, 4, 6, and 8, and respond to LPS by producing

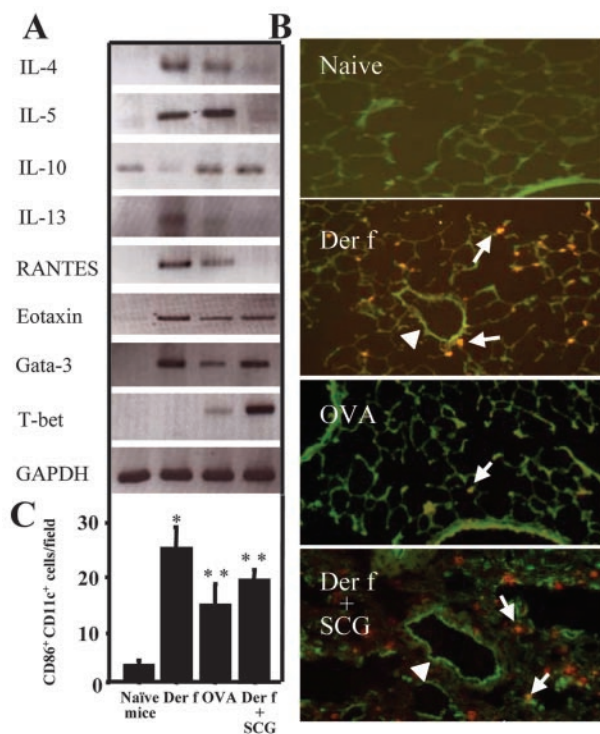


FIGURE 2. SCG diminishes Th2-related gene expression and the number of dendritic cells in lungs of Der f-challenged mice. Mice were treated as described in Table I. After perfusion, total RNA was extracted from whole lung of each mouse and evaluated by RT-PCR (A) or frozen sections of lung were stained with mAbs against CD11c (PE conjugated) and CD86 (FITC conjugated) (original magnification, $\times 400$; arrows, CD86⁺CD11c⁺ cells; arrowheads, bronchioles, B) followed by enumeration of double-positive staining cells in 20 fields ($\times 400$) around bronchioles in each of two duplicate slides. Data are the means $\pm$ SEM of six mice per group. *, $p < 0.05$ as compared with naive mice; **, $p < 0.05$ as compared with SCG-nontreated Der f-challenged mice (C).

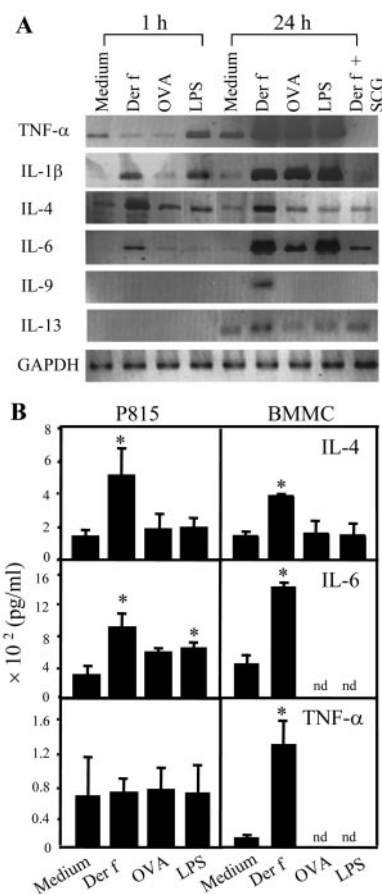


FIGURE 3. Der f induces cytokine productions of mast cells. P815 cells or BMMCs were stimulated with Der f, OVA, or LPS (10 μ l, 1 mg/ml) for 1 or 24 h in the absence or presence of SCG (20 μ l, 10 mg/ml). Total RNA extracted from stimulated P815 cells was evaluated by RT-PCR. One representative of three experiments is shown (A). The accumulation of IL-4, IL-6, and TNF- α in supernatants was evaluated by ELISA (see *Materials and Methods*). Data are the means $\pm$ SEM of three to six separate experiments performed in triplicates. *, $p < 0.05$ as compared with medium control (B). n.d.; not determined.

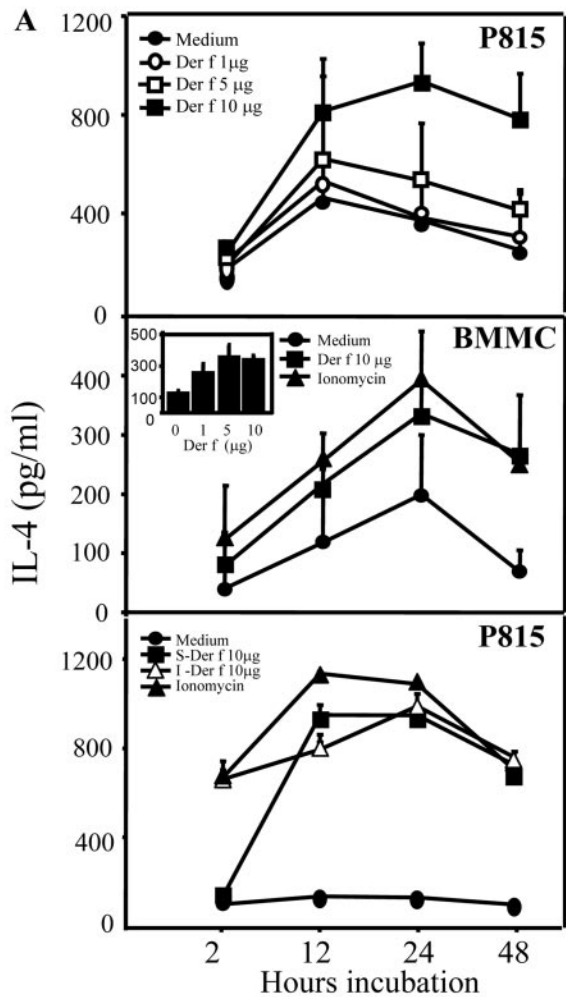


FIGURE 4. Der f dose and time dependently induces IL-4 production of mast cells. P815 cells or BMMCs were stimulated with soluble Der f (10 µl, 0.1–1 mg/ml; S-Der f) or plate-coated Der f (100 µl, 0.1 mg/ml; I-Der f). The accumulation of IL-4 in supernatants was evaluated at various time intervals (see *Materials and Methods*). Ionomycin (1 µM) was used as a stimulator for mast cell activation (A). To assess the requirement for protein synthesis, P815 cells were incubated with Der f (10 µl, 1 mg/ml) in the presence of 100 µl of cycloheximide (0.1–1 mg/ml) or SCG (0.5–2 mg/ml) for 24 h (B). Data are the means ± SEM of six (A) or three (B) separate experiments performed in triplicates. *, $p < 0.05$ as compared with nontreated control.

IL-1 β , TNF- α , IL-6, and IL-13 (28, 29), it seems that our observed Der f effect was not related to LPS contamination. First, the Der f preparations contained very low levels of LPS, <0.96 endotoxin

units/mg Der f. Second, Der f-stimulated and LPS-stimulated mast cells exhibited distinctly different mediator profiles.

Supernatants from Der f-stimulated mast cells induce costimulatory molecule and chemokine expressions of alveolar macrophages and chemoattract T lymphocytes and monocytes

AMs and mast cells represent two prominent innate immune cell types in the lung, so we examined whether the Der f-induced mast cell cytokines would affect the function of AMs. Since maximal release of cytokines was found at concentrations around 10 µg of Der f after an incubation of 24 h, we therefore chose an incubation time of 24 h for assessing the capacity of the mast cell supernatants on expression of costimulatory molecules and chemokines of AMs. Freshly isolated AMs were incubated alone or with supernatants from Der f-stimulated mast cells (referred hereafter as Der f supernatant). Fig. 5A shows that Der f supernatants of both BMMCs and P815 cells (50% concentration) up-regulated surface B7.1 on AMs derived from BALB/c and DBA/2 mice, respectively, by ~2-fold when compared with control supernatants, which could be blocked by neutralizing mAb for TNF- α . However, AMs did not express B7.2 before or after the incubation with Der f supernatants (data not shown). Furthermore, Der f supernatants (both 25 and 50% concentrations) enhanced the expression of

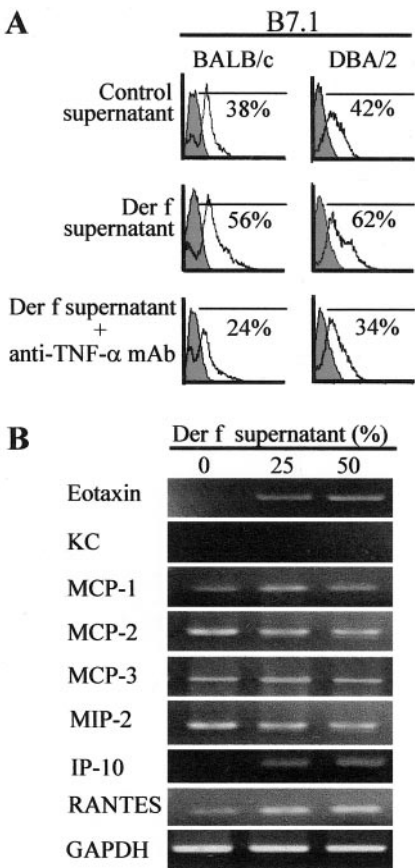


FIGURE 5. Supernatants from Der f-stimulated mast cells up-regulate B7.1 molecules and induce the expression of chemokines in AMs. AMs collected from BALB/c and DBA/2 mice were incubated with supernatants of Der f-stimulated BMMCs and P815 cells, respectively (50% concentration), in the absence or presence of neutralizing mAb to TNF- α (5 µg/ml), and were evaluated by flow cytometry after staining with anti-B7.1 mAb. The population analyzed is I-A^d positive (A). Total RNA extracted from AMs stimulated with 25 or 50% mast cell supernatants was evaluated by RT-PCR. One representative of four experiments for BALB/c mice is shown (B).

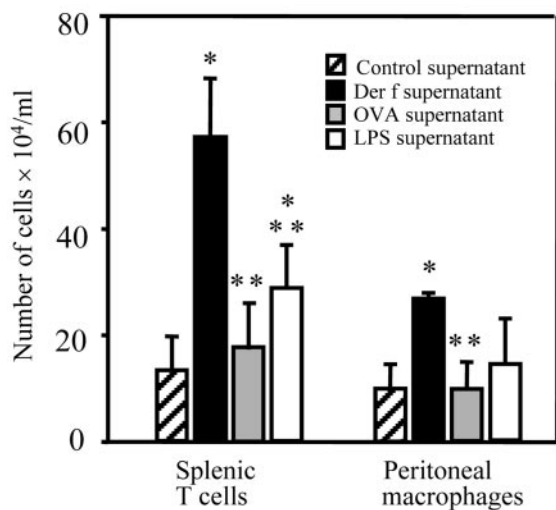


FIGURE 6. Chemotaxis of T lymphocytes and monocytes in response to supernatants from Der f-stimulated mast cells. The migratory response of splenic T cells and peritoneal macrophages to supernatants of Der f-, OVA-, or LPS-stimulated BMMCs was tested (see *Materials and Methods*). The numbers of cells migrating to the lower wells were counted. Data are the means $\pm$ SEM of three separate experiments performed in triplicates. *, $p < 0.05$ as compared with control supernatant; **, $p < 0.05$ as compared with Der f supernatant.

eotaxin, MCP-1, IP-10, and RANTES by 2- to 3-fold, but did not affect basal expression levels of MCP-2, MCP-3, and macrophage-inflammatory protein 2 in AMs (Fig. 5B). Der f supernatants also exerted chemotactic activity for both T cells and peritoneal macrophages, indicating Der f might induce chemokine production of mast cells (Fig. 6). In comparison, LPS but not OVA supernatants contained much less but significant chemotactic activity for splenic T cells.

Supernatants from Der f-stimulated mast cells induce T cell expansion and Th2 cell development

Since the Der f supernatants contained T cell-activating cytokines, we next examined whether Der f supernatants affected T cell activity. Compared with control supernatants, Der f supernatants (50%) elicited a stronger PHA-induced T cell proliferation after 3 days of culture (Fig. 7A). Neutralizing mAbs to IL-4, IL-6, and TNF- α all effectively suppressed the proliferation. Next, we asked whether the Der f supernatants would direct the development of naive CD4⁺ T cells. T cells stimulated initially with anti-CD3 mAb produced IFN- γ but low levels of IL-4 after restimulation (Fig. 7B). However, activation of naive CD4⁺ T cells in the presence of Der f supernatants led to a considerable increase of IL-4 and a decrease of IFN- γ production, indicating a Th2 polarization. Furthermore, these T cells as well as those activated in the presence of ionomycin-stimulated mast cell supernatants expressed GATA-3 (Fig. 7C) but not T-bet (data not shown). This induced development of naive CD4⁺ T cells toward Th2 cells was completely abolished by neutralizing anti-IL-4 mAb.

Discussion

Although HDMs are a major source of environmental inhaled allergens for allergic rhinitis and asthma, factors that contribute to the allergenicity of HDMs are poorly defined. In addition, increasing evidence suggests that airway inflammatory mechanisms contribute significantly to the pathogenesis of bronchial asthma (2, 30, 31). This has prompted us to explore the innate immunity to Der

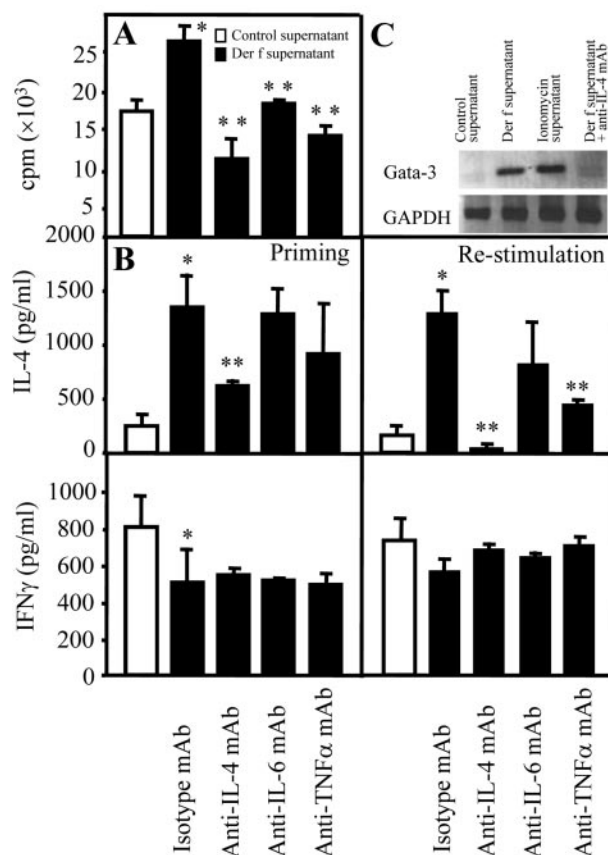


FIGURE 7. Supernatants from Der f-stimulated mast cells augment T cell proliferation and promote Th2 cell development. Splenic T cells from DBA/2 mice were incubated with supernatants from Der f-stimulated P815 cells in the presence of PHA plus neutralizing mAbs to IL-4, IL-6, or TNF- α (5 μ g/ml each). Controls included cultures with isotype-matched mAb. The cells were pulsed with [³H]thymidine and cpm were determined after a 24-h incubation (A). Splenic T cells were stimulated with plate-coated anti-CD3 mAb in the presence of supernatants from Der f-stimulated P815 cells plus neutralizing mAbs to IL-4, IL-6, or TNF- α (5 μ g/ml each). Controls included cultures with isotype-matched mAb. The concentrations of IL-4 and IFN- γ in supernatants were determined by ELISA after a 24-h incubation (priming) or after restimulation (see *Materials and Methods*) (B). Total RNA extracted from restimulated T cells was evaluated by RT-PCR for the expression of GATA-3. Supernatants from ionomycin-stimulated mast cells were used as a control. A representative RT-PCR profile from six independent experiments is shown (C). Data are the means $\pm$ SEM of four separate experiments performed in triplicates. *, $p < 0.05$ as compared with control supernatant; **, $p < 0.05$ as compared with isotype control (A and B).

f, a major species of HDMs. In our previous study (24), we demonstrated an array of immunomodulation and proinflammatory activities of Der f on AMs. In this article, we further demonstrate that mast cells were also a target for Der f activity. Der f could activate mast cells in vivo and trigger P815 cells and BMMCs to express and secrete multiple proallergic cytokines in vitro in an IgE-independent mechanism, which in turn could mediate the cross-talk with AMs and T cells. Der f-induced mast cell activation was found to be necessary for the development of a full-blown allergic airway inflammatory response in sensitized mice. We assume that activation of mast cells by HDM allergens might play an essential role in initiating and maintaining allergic responses.

A previous study showed that Der f could induce mast cell degranulation in vitro (19), and we also have observed mast cell

degranulation after Der f stimulation (C.-L.C. and C.-K.Y., unpublished observation). To a great extent, we demonstrate that Der f could trigger the release of mMCP-1 in mice as early as 30 min after an i.t. inoculation, indicating an early activation of airway mucosal mast cells after an encounter with inhaled HDM allergens. Significantly, OVA, a prototype allergen, did not activate mast cells when inoculated in a similar manner. With regard to their proinflammatory activity to AMs and allergenicity in mice, Der f was found to be superior to OVA (24, 25). In this study, we again observed that Der f challenge resulted in a more severe pulmonary eosinophilic inflammation and blood eosinophilia concomitant with a more pronounced serum IgE and Der f-specific IgG1 Ab response and higher IL-4, IL-5, and IL-13 levels than OVA challenge. More importantly, Der f mice exclusively expressed the Th2-specific transcription factor GATA-3, whereas OVA mice expressed both GATA-3 and the Th1-specific transcription factor T-bet, indicating Der f induced an exclusive Th2 whereas OVA a mixed Th1/Th2 response in mice. Interestingly, Der f challenge suppressed the expression of IL-10. The protective role of IL-10 has been reported in a model of allergic airway inflammation (32). Thus, the decreased expression of IL-10 in Der f-challenged mice may represent a mechanism for promoting allergic response. Collectively, these data support our hypothesis that Der f contains certain properties that are prone to induce Th2 responses, which is probably related to its ability to activate AMs (24) and mast cells. Although Der f challenge provokes TNF- α in BAL fluid of mice (25), considering the multiplicity of cell types that are able to produce cytokines and chemokines, it is difficult to determine whether Der f also triggered mast cells to release these mediators in vivo.

Since Der f could rapidly activate mast cells in vivo, we further investigated the role of this early activation on allergic sensitization and inflammation in the Der f mice by blocking mast cell activity using SCG, a mast cell stabilizer (33). In Der f mice the allergic features were significantly lower or absent after the treatment of SCG, indicating that mast cell activation is essential for the expression of allergic phenotype and that mast cells might participate in early recognition of Der f in the lung before the development of specific immunity. Our data are consistent with that of a nonadjuvant asthma model in which mast cells have been shown to have a critical role in the development of allergic responses in mice (17). It has been suggested that SCG can affect other cells besides mast cells (34); therefore, the observed in vivo events may not be attributed merely to mast cells.

In vivo activation of mast cells appears to be a critical step in the development of the Der f-induced allergic inflammation. It is known that mast cells store and release cytokines such as IL-3, IL-4, IL-5, IL-9, and TNF- α (35, 36). Therefore, we further investigated the cytokine-inducing activity of Der f on mast cells. We demonstrate that Der f was able to up-regulate the expression and secretion of a variety of proallergic cytokines including IL-4, IL-6, IL-9, and IL-13 of mast cells in an IgE-independent manner. This pattern was distinct from those provoked by LPS or OVA. The arrays of cytokines produced by mast cells in response to Der f are known to be able to activate and recruit cells that are important for the development of allergic sensitization and inflammation. Ample studies have demonstrated that IL-4 promotes the development of Th2 cells and IgE Ab production (37). IL-6 directs the differentiation of IL-4-producing CD4⁺ T cells (38). IL-9 is a key mediator that determines the susceptibility of asthma (36). IL-13 can activate eosinophils and B cells, promote IgE production, and promote the induction of airway hyperresponsiveness and mucus hypersecretion (39). Indeed, we observed that the supernatants of Der f-stimulated mast cells were able to chemoattract monocytes and T cells, as well as up-regulate the expression of B7.1, at least

in part mediated by TNF- α , eotaxin (CCL11), MCP-1 (CCL2), IP-10 (CXCL10), and RANTES (CCL5) of AMs. Both eotaxin and RANTES are known to be important for the migration of eosinophils and activated T cells, and MCP-1 is essential for the Th2 response (40). Thus, the up-regulation of chemokines of AMs by mast cell products may be an amplification mechanism for the recruitment of cells that are important for the allergic responses.

Th2 cells require a pulse of IL-4 to initiate their proliferation and production of IL-4 and IL-5. Cells of the innate immunity, including NK1.1 T cells, $\gamma\delta$ T cells, mast cells, and basophils have been suggested to be the cell type responsible for the production of early IL-4 during a primary immune response (37). We show here that Der f could trigger a considerable production of IL-4 by mast cells, which in turn was sufficient to promote T cell proliferation and induce the development of Th2 cells. Our data correlate well with the work of Heuls et al. (12) who found that coactivation of naive CD4⁺ T cells with BMMCs resulted in an IL-4-dependent Th2-like cell development. Collectively, the Der f-induced cytokine production of mast cells may represent an early cellular event in the sensitization process, which can then regulate the development of allergic sensitization and inflammation.

The mechanism by which Der f triggers mast cells to release cytokines can only be speculated upon. Upon IgE receptor-mediated or calcium-ionophore-induced activation, primary mouse BMMCs and permanent mast cell lines produce a panel of different cytokines (8, 35). Since there was no Der f-specific IgE Ab in either our in vitro system or in naive mice, Fc ϵ RI ligation seems not to be involved in providing triggers for the Der f-induced mast cell activation. Der f contains serine protease and elastase, which have been shown to induce a non-IgE-mediated mast cell degranulation (19). In addition Der f III, a trypsin-like protease, could activate the complement system to produce anaphylatoxins C3a and C5a from human C3 and C5 in vitro, respectively (23). Thus, Der f might activate mast cells directly or indirectly via the activation of the complement cascade. We have previously shown that Der f induced production of proinflammatory mediators of AMs via lectin and integrin receptors (24), thus alternatively, Der f might cross-link surface molecules such as Fc ϵ RI or C5a receptors on mast cells. Another possible pathway of the Der f activity is that allergenic peptidases from HDMs may activate the G protein-coupled family of cell surface receptors protease-activated receptors (PARs) (41). PARs are expressed by a variety of cell types including mast cells (42), eosinophils (43), and respiratory epithelial cells (44) and can be activated by thrombin as well as mast cell tryptase. An important role for PARs in modulation of inflammation has been demonstrated in a murine model of airway inflammation (45).

In conclusion, our findings suggest that mast cells might be a source of IL-4 as well as other proallergic cytokines at the onset of allergic sensitization against HDM allergens. The findings also support the concept that the biological activity of HDM allergens on cells of the innate immunity can play a key role in orchestrating allergic responses.

References

1. Kaliner, M. A. 1996. Pathogenesis of asthma. In *Clinical Immunology Principles and Practice*, Vol. 1. R. R. Rich, ed. Mosby-Year Book, St. Louis, pp. 909–23.
2. Holt, P. G., C. Macaubas, P. A. Stumbles, and P. D. Sly. 1999. The role of allergy in the development of asthma. *Nature* 402(Suppl.):B12.
3. Jansen, H. M. 1996. The role of alveolar macrophages and dendritic cells in allergic airway sensitization. *Allergy* 51:279.
4. Zuany-Amorim, C., C. Ruffie, S. Hailé, B. B. Vargaftig, P. Pereira, and M. Pretolani. 1998. Requirement for $\gamma\delta$ T cells in allergic airway inflammation. *Science* 280:1265.
5. Korsgren, M., C. G. Persson, F. Sundler, T. Bjerke, T. Hansson, B. J. Chambers, S. Hong, L. Van Kaer, H. G. Ljunggren, and O. Korsgren. 1999. Natural killer

- cells determine development of allergen-induced eosinophilic airway inflammation in mice. *J. Exp. Med.* 189:553.
6. Humbles, A. A., B. Lu, C. A. Nilsson, C. Lilly, E. Israel, Y. Fujiwara, N. P. Gerard, and C. Gerard. 2000. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature* 406:998.
 7. Metcalfe, D. D., M. Kaliner, and M. A. Donlon. 1981. The mast cell. *Crit. Rev. Immunol.* 3:23.
 8. Ishizaka, T., and K. Ishizaka. 1984. Activation of mast cells for mediator release through IgE receptors. *Prog. Allergy* 34:188.
 9. Schwartz, L. B., and K. F. Austen. 1984. Structure and function of the chemical mediators of mast cells. *Prog. Allergy* 34:271.
 10. McLachlan, J. B., and S. N. Abraham. 2001. Studies of the multifaceted mast cell response to bacteria. *Curr. Opin. Microbiol.* 4:260.
 11. Urban, J. F., Jr., K. B. Madden, A. Svetic, A. Cheever, P. P. Trotta, W. C. Gause, I. M. Katona, and F. D. Finkelman. 1992. The importance of Th2 cytokines in protective immunity to nematodes. *Immunol. Rev.* 127:205.
 12. Huels, C., T. Germann, S. Goedert, P. Hoehn, S. Koelsch, L. Hultner, N. Palm, E. Rude, and E. Schmitt. 1995. Co-activation of naive CD4⁺ T cells and bone marrow-derived mast cells results in the development of Th2 cells. *Int. Immunol.* 7:525.
 13. Hofstra, C. L., I. Van Ark, F. P. Nijkamp, and A. J. Van Oosterhout. 1999. Antigen-stimulated lung CD4⁺ cells produce IL-5, while lymph node CD4⁺ cells produce Th2 cytokines concomitant with airway eosinophilia and hyperresponsiveness. *Inflamm. Res.* 48:602.
 14. Skokos, D., S. Le Panse, I. Villa, J. C. Rousselle, R. Peronet, B. David, A. Namane, and S. Mecheri. 2001. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J. Immunol.* 166:868.
 15. Kung, T. T., D. Stelts, J. A. Zurcher, H. Jones, S. P. Umland, W. Kreutner, R. W. Egan, and R. W. Chapman. 1995. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am. J. Respir. Cell Mol. Biol.* 12:404.
 16. Kobayashi, T., T. Miura, T. Haba, M. Sato, I. Serizawa, H. Nagai, and K. Ishizaka. 2000. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J. Immunol.* 164:3855.
 17. Williams, C. M., and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* 192:455.
 18. Herbert, C. A., C. M. King, P. C. Ring, S. T. Holgate, G. A. Stewart, P. J. Thompson, and C. Robinson. 1995. Augmentation of permeability in the bronchial epithelium by the house dust mite allergen Der p1. *Am. J. Respir. Cell Mol. Biol.* 12:369.
 19. Stewart, G. A., S. M. Boyd, C. H. Bird, K. D. Krksa, M. R. Kollinger, and P. J. Thompson. 1994. Immunobiology of the serine protease allergens from house dust mites. *Am. J. Ind. Med.* 25:105.
 20. Schulz, O., H. F. Sewell, and F. Shakib. 1998. Proteolytic cleavage of CD25, the α 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.
 21. Shakib, F., O. Schulz, and H. Sewell. 1998. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol. Today* 19:313.
 22. Takahashi, K., T. Aoki, S. Kohmoto, H. Nishimura, Y. Kodera, A. Matsushima, and Y. Inada. 1990. Activation of kallikrein system in human plasma with purified serine protease from *Dermatophagoides farinae*. *Int. Arch. Allergy Appl. Immunol.* 91:80.
 23. Maruo, K., T. Akaike, T. Ono, T. Okamoto, and H. Maeda. 1997. Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease. *J. Allergy Clin. Immunol.* 100:253.
 24. Chen, C. L., C. T. Lee, Y. C. Liu, J. Y. Wang, H. Y. Lei, and C. K. Yu. 2003. House dust mite *Dermatophagoides farinae* augments proinflammatory mediator productions and accessory function of alveolar macrophages: implications for allergic sensitization and inflammation. *J. Immunol.* 170:528.
 25. Yu, C. K., C. M. Shieh, and H. Y. Lei. 1999. Repeated intratracheal inoculation of house dust mite *Dermatophagoides farinae* induces pulmonary eosinophilic inflammation and IgE antibody in mice. *J. Allergy Clin. Immunol.* 104:228.
 26. Huntley, J. F., C. Gooden, G. F. Newlands, A. Mackellar, D. A. Lamm, D. Wakelin, M. Tuohy, R. G. Woodbury, and H. R. Miller. 1990. Distribution of intestinal mast cell proteinase in blood and tissues of normal and *Trichinella*-infected mice. *Parasite Immunol.* 12:85.
 27. Haas, H., F. H. Falcone, G. Schramm, K. Haisch, B. F. Gibbs, J. Klauke, M. Poppelman, W. M. Becker, H. J. Gabius, and M. Schlaak. 1999. Dietary lectins can induce in vitro release of IL-4 and IL-13 from human basophils. *Eur. J. Immunol.* 29:918.
 28. Masuda, A., Y. Yoshikai, K. Aiba, and T. Matsuguchi. 2002. Th2 cytokine production from mast cells is directly induced by lipopolysaccharide and distinctly regulated by c-Jun N-terminal kinase and p38 pathways. *J. Immunol.* 169:3801.
 29. Supajatura, V., H. Ushio, A. Nakao, S. Akira, K. Okumura, C. Ra, and H. Ogawa. 2002. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.* 109:1351.
 30. Tsitoura, D. C., S. Kim, K. Dabbagh, G. Berry, D. B. Lewis, and D. T. Umetsu. 2000. Respiratory infection with influenza A virus interferes with the induction of tolerance to aeroallergens. *J. Immunol.* 165:3484.
 31. Whitekus, M. J., N. Li, M. Zhang, M. Y. Wang, M. A. Horwitz, S. K. Nelson, L. D. Horwitz, N. Brechun, D. Diaz-Sanchez, and A. E. Nel. 2002. Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model of ovalbumin sensitization. *J. Immunol.* 168:2560.
 32. Zuany-Amorim, C., S. Hailé, D. Leduc, C. Dumarey, M. Huerre, B. Vargaftig, and M. Pretolani. 1995. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. *J. Clin. Invest.* 95:2644.
 33. Foreman, J. C., and L. G. Garland. 1976. Cromoglycate and other antiallergic drugs: a possible mechanism of action. *Br. Med. J.* 1:820.
 34. Soter, N. A., K. F. Austen, and S. I. Wasserman. 1979. Oral disodium cromoglycate in the treatment of systemic mastocytosis. *N. Engl. J. Med.* 301:465.
 35. Lorentz, A., S. Schwengberg, G. Sellge, M. P. Manns, and S. C. Bischoff. 2000. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *J. Immunol.* 164:43.
 36. Stassen, M., M. Arnold, L. Hultner, C. Muller, C. Neudorff, T. Reineke, and E. Schmitt. 2000. Murine bone marrow-derived mast cells as potent producers of IL-9: costimulatory function of IL-10 and kit ligand in the presence of IL-1. *J. Immunol.* 164:5549.
 37. Haas, H., F. H. Falcone, M. J. Holland, G. Schramm, K. Haisch, B. F. Gibbs, A. Bufer, and M. Schlaak. 1999. Early interleukin-4: its role in the switch towards a Th2 response and IgE-mediated allergy. *Int. Arch. Allergy Immunol.* 119:86.
 38. Rincón, M., J. Anguita, T. Nakamura, E. Fikrig, and R. A. Flavell. 1997. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4⁺ T cells. *J. Exp. Med.* 185:461.
 39. Brubaker, J. O., and L. J. Montaner. 2001. Role of interleukin-13 in innate and adaptive immunity. *Cell. Mol. Biol.* 47:637.
 40. Gu, L., S. Tseng, R. M. Homer, C. Tam, M. Loda, and B. J. Rollins. 2000. Control of T_H2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 404:407.
 41. Coughlin, S. R., and E. Camerer. 2003. PARTICipation in inflammation. *J. Clin. Invest.* 111:25.
 42. Stenton, G. R., O. Nohara, R. E. Déry, H. Vliagoftis, M. Gilchrist, A. Johri, J. L. Wallace, M. D. Hollenberg, R. Moqbel, and A. D. Béfus. 2002. Proteinase-activated receptor (PAR)-1 and -2 agonists induce mediator release from mast cells by pathways distinct from PAR-1 and PAR-2. *J. Pharmacol. Exp. Ther.* 302:466.
 43. Miike, S., A. S. McWilliam, and H. Kita. 2001. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. *J. Immunol.* 167:6615.
 44. Asokanathan, N., P. T. Graham, J. Fink, D. A. Knight, A. J. Bakker, A. S. McWilliam, P. J. Thompson, and G. A. Stewart. 2002. Activation of protease-activated receptor (PAR)-1, PAR-2, and PAR-4 stimulates IL-6, IL-8, and prostaglandin E₂ release from human respiratory epithelial cells. *J. Immunol.* 168:3577.
 45. Moffatt, J. D., K. L. Jeffrey, and T. M. Cocks. 2002. Protease-activated receptor-2 activating peptide SLIGRL inhibits bacterial lipopolysaccharide-induced recruitment of polymorphonuclear leukocytes into the airways of mice. *Am. J. Respir. Cell Mol. Biol.* 26:680.