

Intranasal Exposure of Mice to House Dust Mite Elicits Allergic Airway Inflammation via a GM-CSF-Mediated Mechanism¹

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It is now well established that passive exposure to inhaled OVA leads to a state of immunological tolerance. Therefore, to elicit allergic sensitization, researchers have been compelled to devise alternative strategies, such as the systemic delivery of OVA in the context of powerful adjuvants, which are alien to the way humans are exposed and sensitized to allergens. The objectives of these studies were to investigate immune-inflammatory responses to intranasal delivery of a purified house dust mite (HDM) extract and to evaluate the role of GM-CSF in this process. HDM was delivered to BALB/c mice daily for 10 days. After the last exposure, mice were killed, bronchoalveolar lavage was performed, and samples were obtained. Expression/production of Th2-associated molecules in the lymph nodes, lung, and spleen were evaluated by real-time quantitative PCR and ELISA, respectively. Using this exposure protocol, exposure to HDM alone generated Th2 sensitization based on the expression/production of Th2 effector molecules and airway eosinophilic inflammation. Flow cytometric analysis demonstrated expansion and activation of APCs in the lung and an influx of activated Th2 effector cells. Moreover, this inflammation was accompanied by airways hyper-responsiveness and a robust memory-driven immune response. Finally, administration of anti-GM-CSF-neutralizing Abs markedly reduced immune-inflammatory responses in both lung and spleen. Thus, intranasal delivery of HDM results in Th2 sensitization and airway eosinophilic inflammation that appear to be mediated, at least in part, by endogenous GM-CSF production. *The Journal of Immunology*, 2004, 173: 6384–6392.

Experimental modeling of allergic airway inflammation, particularly in mice, has contributed dramatically to our understanding of the pathogenesis of asthma. However, because conventionally used models involve i.p. administration of Ag in conjunction with potent chemical adjuvants (generally aluminum hydroxide) to generate unequivocal allergic airway inflammation, they are hampered by design when it comes to elucidating the subtle immunological elements required to generate allergic sensitization. Importantly, the need to use those artifices when eliciting allergic sensitization and airway inflammation is due in large part to the nature of the Ag selected, OVA. Indeed, not only does passive respiratory exposure to OVA lead to inhalation tolerance (1–4), but continuous exposure to OVA of an already sensitized animal results in a diminution and complete abrogation, not an increase or maintenance, of the airway inflammatory response (5). To extend our knowledge of asthmatic inflammation, at least

within the basic experimental domain, it thus seemed imperative to explore immune-inflammatory responses to real-life aeroallergens.

House dust mites (HDM)³ are a significant source of indoor allergens, resulting in a range of atopic symptoms in 10% of individuals (6). Specifically, the species *Dermatophagoides pteronyssinus* is the most frequently implicated source of mite-related allergens in subjects with respiratory allergy (7). To date, the majority of work, in animal models, relating to allergy to HDM Ags has been performed in immunologically deficient animals in the form of a humanized SCID mouse model (8, 9) or in either of the above-mentioned conventional models (10–13). In this regard, Hsiue et al. (14) showed that multiple aerosol inhalations of crude mite extract were insufficient to cause sensitization in guinea pigs and that, in fact, i.p. injections were required to elicit overt Th2 sensitization. In addition, very little work has been performed in systems using whole mite extracts; most studies used single isolated allergens and/or their epitopes. Although whole mite extracts are complex from an immunological perspective, they are ultimately more representative of real-life aeroallergen exposure.

In the present study we have investigated the impact of respiratory mucosal exposure to the HDM *D. pteronyssinus* on the generation of allergic-inflammatory responses. In contrast to Der p 1, which is well known to elicit inhalation tolerance (15; reviewed in Ref. 16), our data demonstrate that daily intranasal exposure to a HDM extract for 10 days without additional adjuvants elicits Th2-type sensitization, robust eosinophilic airway inflammation, and

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³ Abbreviations used in this paper: HDM, house dust mite extract; BAL, bronchoalveolar lavage; DC, dendritic cell; rDerp1, recombinant Der p 1 protein; TSLP, thymic stromal lymphoprotein.

airway hyper-responsiveness in mice. The intranasal administration of a polyclonal GM-CSF-neutralizing Ab concurrent with HDM exposure resulted in a marked attenuation of the airway inflammatory response as well as the production of Th2-associated cytokines. These findings suggest that the presence of GM-CSF in the airway microenvironment critically influences the generation of allergic airway responses to HDM.

Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories (Montreal, Canada). The mice were housed in a specific pathogen-free environment under a 12-h light, 12-h dark cycle. All experiments described in this report were approved by the Animal Research Ethics Board of McMaster University.

Protocol of mucosal sensitization

HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in saline (0.9% NaCl Irrigation Solution, Baxter, Toronto, Canada) at a concentration of 2.5 mg/ml. This suspension was delivered to isoflurane-anesthetized mice intranasally (dropwise, alternating between nostrils) in a 10- μ l volume. The mice were exposed to HDM daily for 10 consecutive days. Initially, experiments were performed in mice that received doses of HDM ranging from 5 to 100 μ g daily for 10 days. HDM elicited inflammation in a dose-response fashion (data not shown), with a peak response at 25 μ g. Responses of a similar nature were noted with 50 and 100 μ g; the 25- μ g dose was used in all subsequent experiments.

In select experiments, groups of mice were exposed to 10 μ l of recombinant Der p 1 protein (rDerp1; Indoor Biotechnologies, Charlottesville, VA) resuspended in PBS at a concentration of 2 mg/ml. Administration of rDerp1 was performed for 10 days in the same manner as described above for HDM. rDerp1 was given in the presence or the absence of GM-CSF, which was delivered by infecting mice with 3×10^7 PFU of an adenoviral construct 1 day before allergen exposure, as previously described (17).

In vivo recall with HDM

After complete resolution of the initial airways inflammation (3 wk to 1 mo after the last HDM exposure), mice were subjected to an in vivo recall challenge with HDM. Mice were re-exposed to 25 μ g of HDM, administered intranasally, daily for 3 consecutive days. Initially, experiments were performed in which mice received 25 μ g of HDM for 2, 3, and 5 days and were killed 2, 3, or 5 days after each rechallenge period (data not shown). The most robust inflammatory response occurred 5 days after 3 HDM re-exposures. Therefore, this protocol was used in all subsequent experiments.

Anti-GM-CSF neutralizing Ab (anti-GM) treatment

In specific instances, mice were treated with anti-GM (R&D Systems, Minneapolis, MN) to block the effects of endogenous GM-CSF production. In these cases, for the first 3 days of the exposure protocol, mice received 50 μ g of anti-GM in a 10- μ l volume immediately preceding their exposure to HDM. The dose of anti-GM was reduced to 25 μ g in a 10- μ l volume for the last 7 days of the sensitization protocol. Polyclonal goat IgG (R&D Systems) was used as a control in these experiments.

Collection and measurement of specimens

Seventy-two hours after the last HDM exposure, mice were killed, bronchoalveolar lavage (BAL) was performed, and differential cell counts were determined according to a previously established protocol (18). In addition, blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C. Finally, lung tissue was fixed in 10% formalin and embedded in paraffin. Three-micron sections were stained with H&E.

Isolation of mRNA from tissues and generation of cDNA

Lungs and thoracic lymph nodes were collected, placed in RNAlater (Ambion, Austin, TX), and stored at -20°C for future RNA extraction. Total RNA was extracted using TriPure isolation reagent (Roche Canada, Laval, Canada). Genomic DNA was eliminated using the DNA-free kit (Ambion). Finally, cDNA was generated using the RETROscript kit (Ambion) using random decamers as primers.

Real-time quantitative PCR (TaqMan)

For TaqMan, primers and FAM-labeled probes were designed for GATA-3 with PrimerExpress version 1.5 software (Applied Biosystems, Foster City, CA) and were used as described by Ritz et al. (19). The IL-4, IL-5, CCR4, and CCR8 primers; FAM-labeled probes; GAPDH primers; and VIC-labeled probe were obtained from Applied Biosystems. PCR was performed using TaqMan Universal PCR Master Mix in the ABI PRISM 6700 Sequence Detection System operated by Sequence Detector version 1.7 software (Applied Biosystems). Gene expression is quantified relative to the expression of GAPDH, with naive samples defined as 1, and all other values plotted relative to them.

Splenocyte culture

Spleens were harvested into tubes containing sterile HBSS (Invitrogen Life Technologies, Burlington, Canada). Splenocytes were isolated as previously described (18). Cells (8×10^5 /well) were cultured in complete RPMI 1640 either alone or supplemented with 3.125 μ g/well HDM in flat-bottom, 96-well plates (optimal dose as determined by dose response; BD Biosciences, Franklin Lakes, NJ). After 5 days of culture, supernatants were harvested for cytokine measurements.

Cytokine and Ig measurements

Measurements of in vitro cytokine production were performed in one of two ways. 1) ELISA kits for mouse IL-4, IL-5, and IL-13 were purchased from R&D Systems. These assays have thresholds of detection <2 , 1.5, and 7 pg/ml, respectively. 2) Cytokine production was detected using the Luminex 100 Total System (Luminex, Austin, TX) using xMAP multiplexing technology. 5-Plex cytokine kits containing microspheres specific for IL-4, IL-5, IL-10, IL-13, and IFN- γ were purchased from Upstate Biotechnology (Charlottesville, VA).

Measurement of HDM-specific IgG1

Ninety-six-well Maxi-Sorp plates (Nunc, VWR Canlab, Mississauga, Canada) were coated overnight at 4°C with 100 μ l of a 4 μ g/ml solution of HDM in carbonate-bicarbonate buffer (pH 9.6; Sigma-Aldrich Canada, Oakville, Canada). Coated wells were blocked with 1% BSA in PBS for 2.5 h at room temperature. The ELISA was performed using four serial dilutions of each serum sample prepared using the reagent diluent (0.3% BSA in PBS) and biotinylated goat anti-mouse IgG1 (0.25 μ g/ml; Southern Biotechnology Associates, Birmingham AL). The dilutions used were 1/20, 1/200, 1/2000, and 1/20000. Alkaline-phosphatase-streptavidin (Sigma-Aldrich Canada) was used as a second-step reagent for detection of the biotin-labeled Abs. Ab titers were calculated using the formula $1/(x \times \text{ODX}) \times 0.05$, where x equals the dilution factor closest to but greater than twice the background OD reading, and ODX is the OD reading of x .

Measurement of total IgE

Ninety-six-well Maxi-Sorp plates (Nunc, VWR Canlab) were coated overnight at 4°C with 100 μ l of a 5 μ g/ml solution of LO-ME-3-purified capture Ab (Southern Biotechnology Associates) in borate buffer (pH 8.4). Coated wells were blocked with 1% BSA in PBS for 2 h at room temperature. The ELISA was performed using purified mouse IgE (Pharmingen, Mississauga, Canada) over the range of 7.8–500 ng/ml as a standard. Total IgE was detected using biotinylated rat anti-mouse IgE (0.5 μ g/ml; Southern Biotechnology Associates) and alkaline phosphatase-streptavidin (Sigma-Aldrich Canada) as a second-step reagent.

Flow cytometric analysis

Flow cytometric analysis was performed on lung cells isolated as previously described (18). The cells were then stained with a panel of fluorescently conjugated Abs (BD Pharmingen, San Diego, CA). The following Abs were used: anti-CD11c-FITC, anti-MHC II-FITC, anti-B220-FITC, anti-CD4-FITC, anti-CD8-FITC, anti-CD11b-PE, anti-B7.1-PE, anti-CD3-PE, anti-CD69-PE, anti-MHC II-biotin, anti-B7.2-biotin, anti-CD4-biotin, and anti-CD3-biotin. Appropriate isotype controls were also purchased from BD Pharmingen. The anti-T1/ST2-FITC Ab was provided by Millennium Pharmaceuticals (Cambridge, MA). To minimize nonspecific binding, $4\text{--}8 \times 10^5$ cells were preincubated with Fc block (CD16/CD32; BD Pharmingen). For each Ab combination, $4\text{--}8 \times 10^5$ cells were incubated with mAbs at $0\text{--}4^{\circ}\text{C}$ for 30 min; the cells were then washed and treated with second-stage reagents. Streptavidin-PerCP (BD Biosciences, San Jose, CA) was used as a second-step reagent for detection of biotin-labeled Abs. Titration was used to determine the optimal concentration for each Ab. Cells were fixed in 1% paraformaldehyde and counted on a FACScan (BD Biosciences, Mountain View, CA). A minimum of 100,000 events were acquired. Analysis was performed using WinMDI software

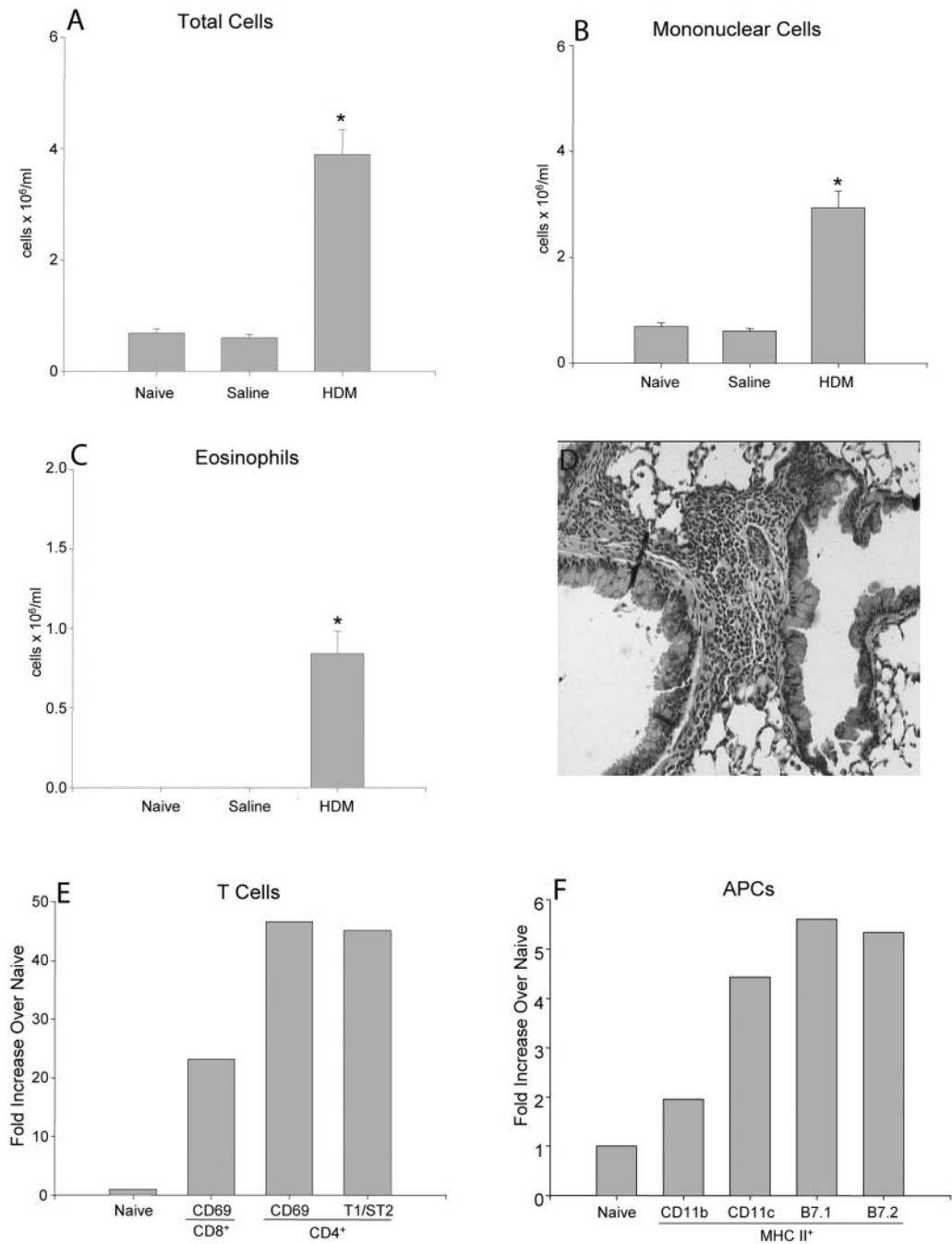


FIGURE 1. Lung/airway inflammation in BALB/c mice exposed to HDM. *A–C*, Airway inflammation in the BAL fluid of BALB/c mice exposed to HDM or saline. Daily, over a period of 10 consecutive days, mice were exposed intranasally to 25 μ g of HDM or saline (both in a 10- μ l volume). Data were obtained 72 h after the last exposure ($n = 8–28$; *, $p < 0.001$, by Fisher’s post-hoc test). *D*, Light photomicrograph of a paraffin-embedded section of lung tissue obtained 72 h after HDM exposure. Section stained with H&E ($\times 100$ magnification). *E*, Fold increase over naive of activated CD8⁺ cells (CD3⁺/CD8⁺/CD69⁺), activated CD4⁺ cells (CD3⁺/CD4⁺/CD69⁺), and Th2 effector cells (CD3⁺/CD4⁺/T1-ST2⁺), as assessed by flow cytometry. Over a period of 10 consecutive days, mice were exposed daily to HDM. Cells were collected 1 h after the last exposure. *F*, Expansion and activation of APCs in the lungs of mice 1 h after the last HDM exposure. The percentages of macrophages (MHC II⁺/CD11b⁺) and DCs (MHC II⁺/CD11c⁺) were assessed by flow cytometry. Activation of the APCs was determined on the basis of expression of the costimulatory molecules B7.1 and B7.2.

(The Scripps Research Institute, La Jolla, CA). The percentages of the various cell types were used to approximate the number of each cell type based on the total number of cells isolated per mouse and are expressed as fold increases over naive, with naive being represented as 1.

Airway hyper-responsiveness measurements

Airway responsiveness was measured using the flow interrupter technique (20–22) on the basis of the response of total respiratory system resistance to saline and increasing doses (10, 33, 100, and 330 μ g) of i.v. methacho-

line. Briefly, mice were anesthetized using 2,2,2-triethanol (Avertin; 240 mg/kg, i.p.; Aldrich Chemical, Milwaukee, WI), and the trachea was exposed and cannulated using a blunted 18-gauge needle. The needle was then attached to a ventilator (RV5; Voltek Enterprises, Toronto, Canada) designed to deliver constant inspiratory flow despite the disturbances to the function of the respiratory system that occur during methacholine challenge. Using the pattern of ventilation described by Inman et al. (20), total respiratory system resistance was measured, and the difference in resistance at each dose of methacholine was assessed.

Data analysis

Data are expressed as the mean $\pm$ SEM. Statistical analysis was performed using SigmaStat version 2.03 (Jandel, San Ramon, CA). Differences were considered significant at $p < 0.05$ (by one-way ANOVA unless otherwise indicated).

Results

Inflammatory responses in the airway

Intranasal delivery of HDM elicited a robust inflammatory response in the lungs/airways of mice that was measurable in the BAL and was clearly evident histopathologically. Exposure to HDM resulted in an increase in total cells to $\sim 4 \times 10^6$ (from 7×10^5 in naive mice; Fig. 1A). The cellular distribution consisted of 76% mononuclear cells and 22% eosinophils (Fig. 1, B and C). Infiltration of neutrophils was negligible (3%; data not shown). Intranasal delivery of saline did not yield any inflammatory response. In agreement with our findings in the BAL, lungs from mice exposed to HDM showed evidence of severe peribronchial and perivascular inflammation as well as goblet cell hyperplasia (Fig. 1D).

To further define the types of immune effector cells present within this inflammatory infiltrate, we isolated lung mononuclear cells on day 9 of the exposure protocol and performed a flow cytometric analysis (Fig. 1, E and F). HDM exposure led to an expansion of both activated CD8⁺ and CD4⁺ cells (based on CD69 expression), 23- and 47-fold over that in naive cells, respectively. Th2 effector cells (CD3⁺/CD4⁺/T1ST2⁺) were also greatly expanded (45-fold over naive cells; Fig. 1E). The APC population was also examined by flow cytometry (Fig. 1F). We observed a 2-fold increase in macrophages (MHC II⁺/CD11b⁺) and a 4.5-fold increase in dendritic cells (DCs; MHC II⁺/CD11c⁺). The degree of APC activation was examined by assessing the number of APCs expressing the costimulatory molecules B7.1 and B7.2. Our data show that for cells expressing MHC II, there was an ~ 5 -fold increase in both B7.1 and B7.2 expression.

In some groups of mice, the inflammation caused by the initial exposure to HDM was allowed to resolve, and the mice were re-challenged with HDM. The resolution of the initial inflammatory response was determined by killing mice 3 wk to 1 month after the exposure protocol and assessing the nature of the inflammatory infiltrate in the BAL. HDM re-exposure resulted in a recapitulation and, in fact, an augmentation of the initial inflammatory response. This response consisted of ~ 6.6 million total cells in the BAL fluid 120 h after recall challenge (approximately twice the number of total cells observed in the initial inflammatory response). Eosinophils constituted $\sim 25\%$ of this population (Fig. 2).

Airway responsiveness measurements

To determine whether the inflammatory response in the lung contributed to airway dysfunction, we evaluated the total respiratory resistance in mice exposed to saline and mice exposed to 10 days of HDM using a flow interrupter technique. HDM exposure resulted in a higher, although not significant, background level of airway resistance compared with that in controls. Mice exposed to HDM were hyper-responsive to increasing doses of methacholine compared with saline-exposed controls. This reached statistical significance at a dose of 33 $\mu\text{g/kg}$ and remained significantly elevated at increased doses of methacholine (Fig. 3).

Evidence of Th2 sensitization and effector activity in HDM-exposed mice

Evidence of Th2 sensitization in thoracic lymph nodes was evaluated using TaqMan to measure the relative expression of the Th2-associated transcription factor GATA-3 and the Th2-affiliated chemokine receptors CCR4 and CCR8. We observed a significant

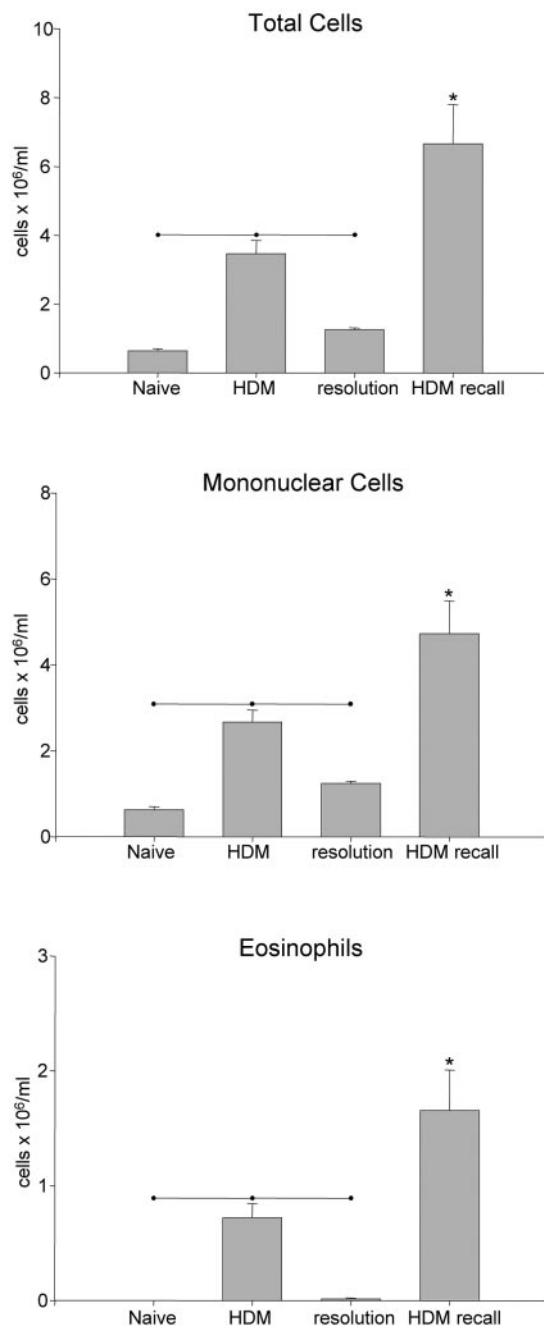


FIGURE 2. Memory-driven immune-inflammatory response to HDM. After the initial 10-day HDM exposure, resolution of the inflammatory response was allowed to occur. After resolution, mice were rechallenged *in vivo* with HDM daily for 3 consecutive days. Data were collected 120 h after the last exposure ($n = 3$ –25; *, significant to all, $p < 0.001$; lines represent significance of $p < 0.03$; by Student-Newman-Keuls post-hoc test).

up-regulation of GATA-3 that peaked 24 h after the first HDM administration at a level ~ 12 -fold over that in naive cells (Fig. 4A). We also noted significant up-regulation of CCR4 and CCR8, both of which reached their highest levels 24 h after three HDM exposures (~ 43 - and 85-fold over naive, respectively; Fig. 4, B and C).

Th2 activity in the effector organ (i.e., the lung) was assessed using TaqMan to examine the expression of Th2-associated cytokines. We detected a significant up-regulation in the relative expression of IL-4 and IL-5 in the lungs of mice exposed to HDM *in vivo*. As shown in Fig. 5, A and B, IL-4 expression showed a

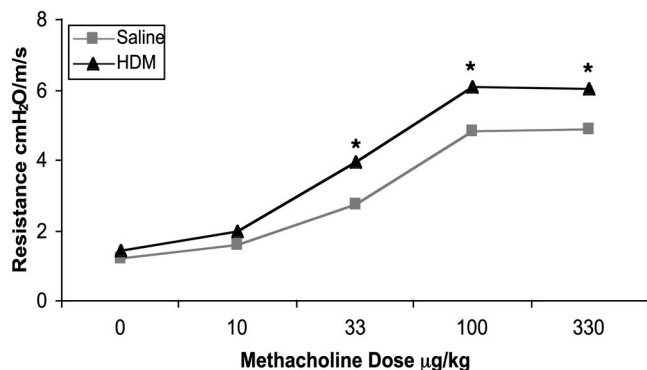


FIGURE 3. Airway responsiveness in BALB/c mice exposed to HDM. Over a period of 10 consecutive days mice were exposed daily to HDM or saline. Total respiratory system resistance was measured in response to increasing doses of i.v. methacholine. Measurements were performed 72 h after the last exposure ($n = 18$; *, $p < 0.001$, by Duncan's post-hoc test).

220-fold increase, and IL-5 expression showed a 14-fold increase over that in naive cells.

Evidence of systemic Th2 sensitization was obtained by detecting significant Th2 cytokine production by splenocytes from HDM-exposed animals upon HDM recall in vitro. Levels of IL-4, IL-5, and IL-13 were significantly elevated to 160, 1,470, and 19,900 pg/ml, respectively (Fig. 5C). In addition, we were able to measure HDM-specific IgG1 and total IgE in the sera of mice exposed to HDM in vivo. Both these Th2-associated Igs were significantly elevated compared with levels in naive mice (Fig. 5D).

GM-CSF-neutralizing Ab treatment diminishes the response

To test the putative role of GM-CSF in this process, we delivered anti-GM to mice immediately before HDM exposure. First, we observed a marked attenuation of the level of inflammation in the BAL fluid. Indeed, total cells were decreased from 4×10^6 in HDM-exposed mice to $\sim 2 \times 10^6$ in anti-GM-treated, HDM-exposed mice (Fig. 6A). Eosinophilia in these mice was also considerably decreased from 8×10^5 to $\sim 1.5 \times 10^5$ (corresponding to a decrease in eosinophils from $\sim 21\%$ of total cells to 7%). Mice that received control Ab and HDM had similar numbers of cells in their BAL as mice that were exposed to HDM alone.

Systemically, splenocytes from anti-GM-treated, HDM-exposed mice showed a significant decrease in their production of IL-5 and IL-13 when restimulated with HDM in vitro compared with mice that were treated with HDM alone or in conjunction with control Ab in vivo. Compared with mice treated with control Ab, anti-GM treatment resulted in a 63% decrease in IL-5 (Fig. 6B; 1,350 vs 500 pg/ml) and a 49% reduction in IL-13 (Fig. 6C; 14,800 vs 7,500 pg/ml).

Immune-inflammatory responses in mice exposed to rDerp1

As expected, intranasal delivery of only rDerp1 to mice resulted in a BAL cellular profile indistinguishable from that in naive mice. However, if rDerp1 was delivered into an airway microenvironment enriched with GM-CSF, a robust inflammatory response was observed (Fig. 7, A–C). Indeed, mice exposed to GM-CSF and rDerp1 exhibited an increase in total BAL cells (Fig. 7A) to 4.4×10^6 (from 9.3×10^5 in naive mice). This population consisted of mononuclear cells (Fig. 7B) and eosinophils (Fig. 7C). Specifically, the number of eosinophils increased to 3.6×10^5 (with no eosinophils present in the lungs of naive mice). Interestingly, the number of eosinophils in mice exposed to rDerp1 in the context of GM-CSF was very similar to the number in mice exposed to HDM

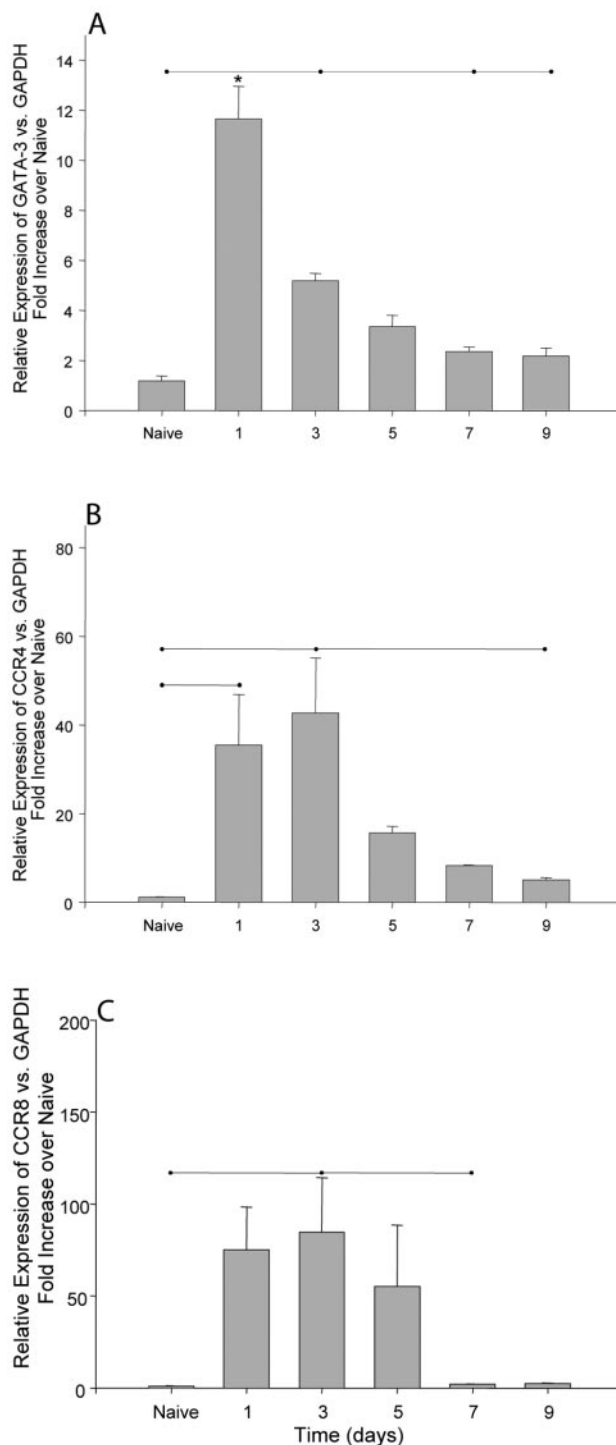


FIGURE 4. Evidence of Th2 sensitization in HDM-exposed mice. Mice were exposed to 10 intranasal instillations of HDM. RNA was isolated from thoracic lymph nodes at various time points during the exposure protocol as shown, and GATA-3 (A), CCR4 (B), and CCR8 (C) relative expression was measured by TaqMan ($n = 2$ –3 mice; statistical analysis performed on triplicate measurements; GATA-3: *, $p < 0.001$, by Tukey's post-hoc test; CCR4 and CCR8: $p < 0.0$, by Kruskal-Wallis one-way ANOVA on ranks and Dunn's post-hoc test).

alone. Virtually no eosinophils were observed in mice exposed to GM-CSF in the absence of allergen (17).

Evidence of systemic Th2 sensitization was acquired through the detection of an increase in Th2-associated cytokine production by splenocytes from mice exposed to rDerp1 in the context of

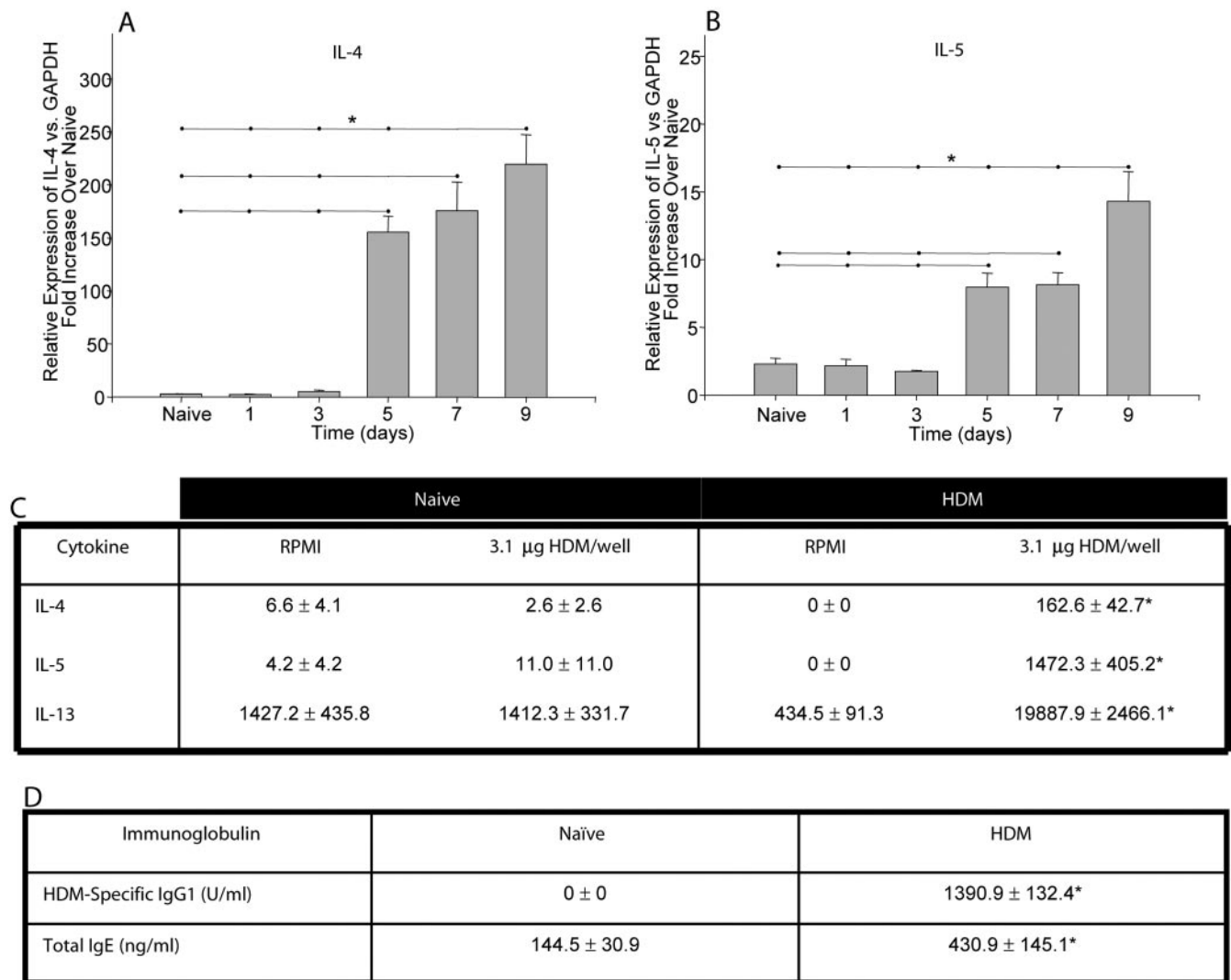


FIGURE 5. Evidence of Th2 effector function and systemic sensitization in HDM-exposed mice. *A* and *B*, Mice were exposed to 10 intranasal instillations of HDM. RNA was isolated from lung tissue at various time points during the exposure protocol as shown, and IL-4 (*A*) and IL-5 (*B*) relative expression was assessed by TaqMan ($n = 3$ –4 mice; statistical analysis performed on triplicate measurements: *, $p < 0.02$, by Fisher's post-hoc test). *C*, In vitro production of HDM-specific IL-4, IL-5, and IL-13 by splenocytes. Cells were obtained 72 h after the last exposure to HDM and were cultured in RPMI 1640 alone or RPMI 1640 plus 3.125 μ g of HDM/well (data are expressed in picograms per milliliter; $n = 3$ –4; *, $p < 0.014$, by Fisher's post-hoc test). *D*, Serum Ig levels in BALB/c mice exposed to HDM. Mice were killed 72 h after 10 intranasal instillations of HDM, and the Ig content in the serum was measured by ELISA ($n = 4$ –7; *, $p < 0.011$, by Student's *t* test).

GM-CSF. When these cells were restimulated with rDerp1 in vitro, levels of IL-4 (Fig. 7D), IL-5 (Fig. 7E), and IL-13 (Fig. 7F) were significantly elevated relative to control values.

Discussion

Dust mites are a significant source of indoor aeroallergens. Despite this significance, there is relatively little information available regarding airway immune-inflammatory responses to HDM in murine systems. A great deal of information concerning allergic airway inflammation has been compiled in murine experimental systems using OVA as a surrogate allergen. However, OVA, when used without adjuvants, elicits tolerance. Our data show that the administration of this whole mite extract to mice in the absence of exogenous adjuvants leads not to tolerance, but to a robust inflammatory response. Although this extract is known to contain LPS, it is highly unlikely that it is mediating the effects seen in this study. Indeed, LPS is known to be the most powerful stimulator of IL-12, which preferentially drives Th1-type responses.

The inflammatory response to HDM is characterized by an infiltrate comprising a considerable proportion of eosinophils and Th2 effector cells (as indicated by the expression of T1/ST2, a putative marker of Th2 effector cells) (23) as well as by genetic up-regulation of Th2-affiliated cytokines. These findings are corroborated histopathologically by evidence of peribronchial/perivascular inflammation and mucous production and are correlated physiologically with airway hyper-responsiveness. Systemically, this process is associated with increased serum levels of Th2-affiliated Igs, total IgE, and HDM-specific IgG1. Conclusive evidence for the generation of a Th2 immune response is provided by early genetic up-regulation in the thoracic lymph nodes of Th2-affiliated molecules (GATA-3, CCR4, and CCR8), splenocyte production of Th2-affiliated cytokines upon HDM recall in vitro, and a very robust inflammatory response upon long term in vivo recall.

We have previously reported an experimental model in which the expression of exogenous GM-CSF, a cytokine clearly relevant

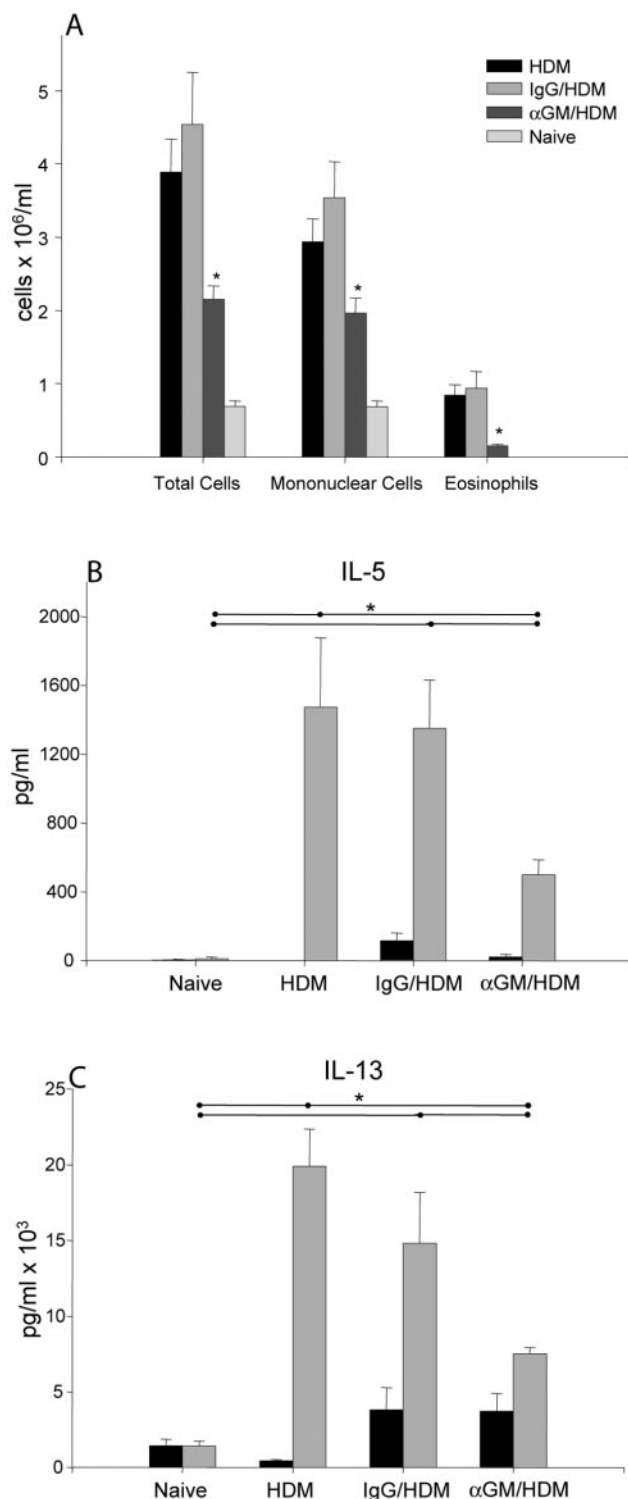


FIGURE 6. Putative role of GM-CSF in the immune-inflammatory process. **A**, Airway inflammation in the BAL fluid of BALB/c mice exposed to HDM in the context of anti-GM. Over a period of 10 consecutive days mice were exposed daily to HDM immediately after 10 μl of either IgG or anti-GM. Data were obtained 72 h after the last exposure ($n = 6-29$; *, significant decreases compared with IgG/HDM: $p < 0.013$, by Fisher's post-hoc test). **B** and **C**, In vitro production of HDM-specific IL-5 and IL-13 by splenocytes. Over a period of 10 consecutive days mice were exposed daily to HDM after receiving 10 μl of either IgG or anti-GM. Cells were obtained 72 h after the last exposure to HDM and were cultured in RPMI 1640 alone (■) or RPMI 1640 plus 3.125 μg HDM/well (▤; $n = 3-6$; *, significant decreases compared with IgG/HDM: $p < 0.004$, by Fisher's post-hoc test).

in asthma (24), in the airway environment facilitated allergic sensitization and airway eosinophilic inflammation to an otherwise innocuous Ag, OVA (17). That intranasal exposure to HDM alone generated such a similar response prompted us to consider the role of endogenously produced GM-CSF in this process. However, detection of GM-CSF in the BAL or lung of HDM-exposed mice proved exceedingly difficult. Given GM-CSF's powerful nature, we did not expect to measure high levels of this cytokine; moreover, increased difficulty in its measurement was probably introduced by the proteolytic nature of the extract delivered. In addition, GM-CSF is known to become embedded in extracellular matrix (25), which may have prevented the passage of sufficient (i.e., detectable) amounts into the BAL fluid. Thus, another approach for testing our hypothesis regarding the involvement of GM-CSF in the immune-inflammatory response to HDM had to be developed. GM-CSF is not only indispensable for DC differentiation and maturation from bone marrow progenitors and peripheral blood monocytes in vitro, but administration of GM-CSF to the mouse lung leads to a massive accumulation and activation of DCs. Hence, we set out to 1) examine changes in the APC compartment upon HDM exposure, and 2) determine whether the HDM-elicited immune-inflammatory response could be affected by neutralizing GM-CSF in vivo.

We first evaluated the APC compartment in the lungs of mice that had been exposed to HDM. We observed an increase in both macrophages and DCs as well as in the activation of APCs based on the expression of B7.1 and B7.2. We then tested the putative role of GM-CSF in this process by delivering anti-GM to mice immediately before HDM exposure. We observed a marked attenuation of the level of inflammation in BAL fluid. Indeed, total cells were decreased by $\sim 50\%$ in anti-GM-treated, HDM-exposed mice. Eosinophilia in these mice was also considerably decreased. Systemically, splenocytes from anti-GM-treated, HDM-exposed mice showed a significant decrease in their production of IL-5 and IL-13 when restimulated with HDM in vitro compared with mice that were treated with HDM alone or in conjunction with control Ab in vivo. Furthermore, we investigated the impact of repeated intranasal exposure to rDerp1. Although rDerp1 alone did not elicit inflammation of the airways, exposure to rDerp1 in the context of a GM-CSF-enriched microenvironment resulted in eosinophilic airway inflammation comparable to that observed in mice exposed to HDM as well as a Th2-polarized cytokine profile upon in vitro stimulation of splenocytes with rDerp1.

It is well known that the main Ags of HDM, Der p 1, 3, 5, and 9, are cysteine and/or serine proteases (26), and a number of in vitro studies have demonstrated that these Ags can stimulate airway epithelial cells to liberate a number of cytokines, including GM-CSF, probably through a protease-activated receptor-2-mediated mechanism (27-29). Our data demonstrate for the first time that GM-CSF plays an important role in the generation of Th2 sensitization and airway eosinophilic inflammation in BALB/c mice exposed to an HDM extract in vivo. However, it is also likely that molecules other than GM-CSF play a role in this process. In this regard, Soumelis et al. (30) have recently demonstrated that epithelial cells, including lung fibroblasts and bronchial epithelial cells, can produce thymic stromal lymphoprotein (TSLP), a molecule capable of inducing marked expansion and activation of DCs. Whether airway epithelial cells produce TSLP upon HDM exposure is not known, and whether GM-CSF and TSLP cooperate in predisposing the APC compartment to generate allergic sensitization awaits further studies. Finally, we suggest that the opportunity to integrate a discrete pathogenic sequence of events, from initiation to conclusion, illustrates one of the advantages of using

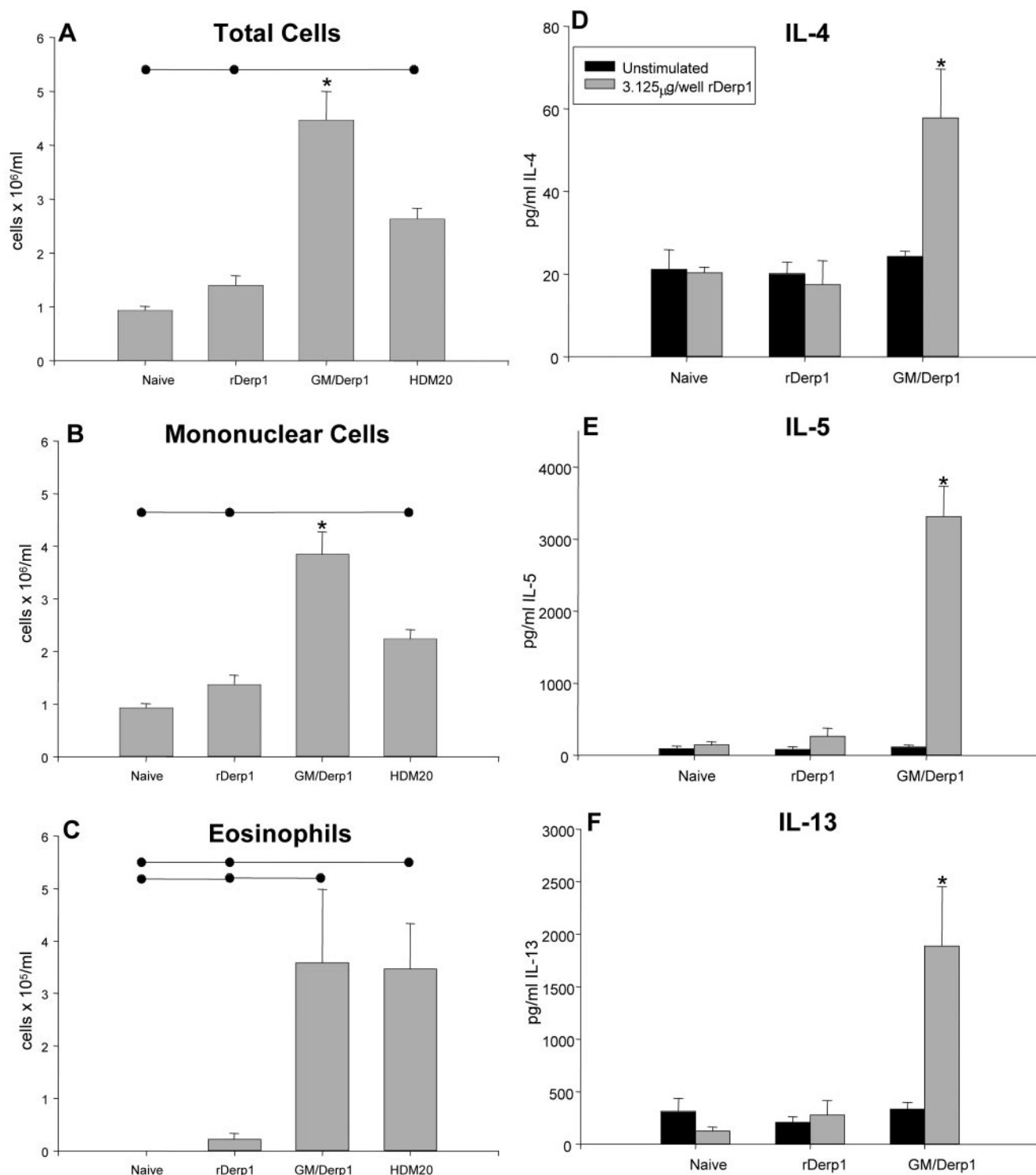


FIGURE 7. Immune-inflammatory responses in BALB/c mice exposed to rDerp1. *A–C*, Lung inflammation in the BAL of BALB/c mice exposed to rDerp1. Daily, over a period of 10 consecutive days, mice were exposed to 20 μ g of rDerp1 or HDM (both in a 10- μ l volume). One day before allergen exposure, one group of mice was infected with an adenovirus expressing GM-CSF (GM/Derp1). Data were collected 72 h after the last allergen exposure ($n = 5–10$; *, significant to all $p < 0.001$; lines represent significance of $p < 0.004$, by Student-Newman-Keuls post-hoc test). *D–F*, In vitro production of rDerp1-specific IL-4, IL-5, and IL-13 by splenocytes. Cells were obtained 72 h after the last exposure to rDerp1 and were cultured in RPMI 1640 alone or RPMI 1640 plus 3.125 μ g/well. After 5 days of culture, supernatants were collected, and cytokine levels were assessed by Luminex ($n = 4–5$; *, significant to rDerp1 stimulated controls: $p < 0.023$, by Student-Newman-Keuls post-hoc test).

real-life aeroallergens. We surmise that the data presented in this report provide a meaningful foundation to explore the biochemical and molecular bases of aeroallergen-induced immunopathology.

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References

- Holt, P. G., J. E. Batty, and K. J. Turner. 1981. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 42:409.
- McMenamin, C., C. Pimm, M. McKersey, and P. G. Holt. 1994. Regulation of IgE responses to inhaled antigen in mice by antigen-specific $\gamma\delta$ T cells. *Science* 265:1869.
- Seymour, B. W., L. J. Gershwin, and R. L. Coffman. 1998. Aerosol-induced immunoglobulin (Ig)-E unresponsiveness to ovalbumin does not require CD8⁺ or T cell receptor (TCR)- $\gamma\delta$ ⁺ T cells or interferon (IFN)- γ in a murine model of allergen sensitization. *J. Exp. Med.* 187:721.
- Swirski, F. K., B. U. Gajewska, D. Alvarez, S. A. Ritz, M. J. Cundall, E. C. Cates, A. J. Coyle, J. C. Gutierrez-Ramos, M. D. Inman, M. Jordana, et al. 2002. Inhalation of a harmless antigen (ovalbumin) elicits immune activation but divergent immunoglobulin and cytokine activities in mice. *Clin. Exp. Allergy* 32:411.
- Swirski, F. K., D. Sajic, C. S. Robbins, B. U. Gajewska, M. Jordana, and M. R. Stämpfli. 2002. Chronic exposure to innocuous antigen in sensitized mice leads to suppressed airway eosinophilia that is reversed by granulocyte macrophage colony-stimulating factor. *J. Immunol.* 169:3499.
- Neeno, T., C. J. Krco, J. Harders, J. Baisch, S. Cheng, and C. S. David. 1996. HLA-DQ8 transgenic mice lacking endogenous class II molecules respond to house dust allergens: identification of antigenic epitopes. *J. Immunol.* 156:3191.
- O'Brien, R. M., W. R. Thomas, and A. M. Wootton. 1992. T cell responses to the purified major allergens from the house dust mite *Dermatophagoides pteronyssinus*. *J. Allergy Clin. Immunol.* 89:1021.
- Duez, C., A. Tscipoulos, A. Janin, I. Tillie-Leblond, G. Thyphronitis, P. Marquillies, Q. Hamid, B. Wallaert, A. B. Tonnel, and J. Pestel. 1996. An in vivo model of allergic inflammation: pulmonary human cell infiltrate in allergen-challenged allergic Hu-SCID mice. *Eur. J. Immunol.* 26:1088.
- Herz, U., N. Schnoy, S. Borelli, L. Weigl, U. Kasbohrer, A. Daser, U. Wahn, E. Kottgen, and H. Renz. 1998. A human-SCID mouse model for allergic immune response bacterial superantigen enhances skin inflammation and suppresses IgE production. *J. Invest. Dermatol.* 110:224.
- O'Brien, R., M. A. Ooi, A. H. Clarke, and W. R. Thomas. 1996. Immunologic responses following respiratory sensitization to house dust mite allergens in mice. *Immunol. Cell. Biol.* 74:174.
- Tournoy, K. G., J. C. Kips, C. Schou, and R. A. Pauwels. 2000. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin. Exp. Allergy* 30:79.
- Lee, Y. L., Y. L. Ye, C. I. Yu, Y. L. Wu, Y. L. Lai, P. H. Ku, R. L. Hong, and B. L. Chiang. 2001. Construction of single-chain interleukin-12 DNA plasmid to treat airway hyperresponsiveness in an animal model of asthma. *Hum. Gene Ther.* 12:2065.
- Tategaki, A., S. Kawamoto, T. Okuda, T. Aki, H. Yasueda, O. Suzuki, K. Ono, and S. Shigeta. 2002. A high-molecular-weight mite antigen (HM1) fraction aggravates airway hyperresponsiveness of allergic mice to house dusts and whole mite cultures. *Int. Arch. Allergy Immunol.* 129:204.
- Hsiue, T. R., H. Y. Lei, A. L. Hsieh, T. Y. Wang, H. Y. Chang, and C. R. Chen. 1997. Mite-induced allergic airway inflammation in guinea pigs. *Int. Arch. Allergy Immunol.* 112:295.
- Hoyne, G. F., R. E. O'Hehir, D. C. Wraith, W. R. Thomas, and J. R. Lamb. 1993. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J. Exp. Med.* 178:1783.
- Hoyne, G. F., K. Tan, M. Corsin-Jimenez, K. Wahl, M. Stewart, S. E. Howie, and J. R. Lamb. Immunological tolerance to inhaled antigen. *Am. J. Respir. Crit. Care Med.* 162:S169, 2000.
- Stämpfli, M. R., R. E. Wiley, G. S. Neigh, B. U. Gajewska, X. F. Lei, D. P. Snider, Z. Xing, and M. Jordana. 1998. GM-CSF transgene expression in the airway allows aerosolized ovalbumin to induce allergic sensitization in mice. *J. Clin. Invest.* 102:1704.
- Gajewska, B. U., D. Alvarez, M. Vidric, S. Goncharova, M. R. Stämpfli, A. J. Coyle, J. C. Gutierrez-Ramos, and M. Jordana. 2001. Generation of experimental allergic airways inflammation in the absence of draining lymph nodes. *J. Clin. Invest.* 108:577.
- Ritz, S. A., M. J. Cundall, B. U. Gajewska, D. Alvarez, J. C. Gutierrez-Ramos, A. J. Coyle, A. N. McKenzie, M. R. Stämpfli, and M. Jordana. 2002. Granulocyte macrophage colony-stimulating factor-driven respiratory mucosal sensitization induces Th2 differentiation and function independently of interleukin-4. *Am. J. Respir. Cell Mol. Biol.* 27:428.
- Inman, M. D., R. Ellis, J. Wattie, J. A. Denburg, and P. M. O'Byrne. 1999. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. *Am. J. Respir. Cell Mol. Biol.* 21:473.
- Leigh, R., R. Ellis, J. Wattie, D. S. Southam, M. De Hoogh, J. Gauldie, P. M. O'Byrne, and M. D. Inman. 2002. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 27:526.
- Volgyesi, G. A., L. N. Tremblay, P. Webster, N. Zamel, and A. S. Slutsky. 2000. A new ventilator for monitoring lung mechanics in small animals. *J. Appl. Physiol.* 89:413.
- Lohning, M., A. Stroehmann, A. J. Coyle, J. L. Grogan, S. Lin, J. C. Gutierrez-Ramos, D. Levinson, A. Radbruch, and T. Kamradt. 1998. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl. Acad. Sci. USA* 95:6930.
- Ritz, S. A., M. R. Stämpfli, D. E. Davies, S. T. Holgate, and M. Jordana. 2002. On the generation of allergic airway diseases: from GM-CSF to Kyoto. *Trends Immunol.* 23:396.
- Gordon, M. Y., G. P. Riley, S. M. Watt, and M. F. Greaves. 1987. Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. *Nature* 326:403.
- Hewitt, C. R., S. Foster, C. Phillips, H. Horton, R. M. Jones, A. P. Brown, B. J. Hart, and D. I. Pritchard. 1998. Mite allergens: significance of enzymatic activity. *Allergy* 53:60.
- King, C., S. Brennan, P. J. Thompson, and G. A. Stewart. 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J. Immunol.* 161:3645.
- Sun, G., M. A. Stacey, M. Schmidt, L. Mori, and S. Mattoli. 2001. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. *J. Immunol.* 167:1014.
- Asokanathan, N., P. T. Graham, D. J. Stewart, A. J. Bakker, K. A. Eidne, P. J. Thompson, and G. A. Stewart. 2002. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J. Immunol.* 169:4572.
- Soumelis, V., P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, et al. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 3:673.