

Acute, but Not Resolved, Influenza A Infection Enhances Susceptibility to House Dust Mite-Induced Allergic Disease¹

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The impact of respiratory viral infections on the emergence of the asthmatic phenotype is a subject of intense investigation. Most experimental studies addressing this issue have used the inert Ag OVA with controversial results. We examined the consequences of exposure to a low dose of the common aeroallergen house dust mite (HDM) during the course of an influenza A infection. First, we delineated the kinetics of the immune-inflammatory response in the lung of mice following intranasal infection with influenza A/PR8/34. Our data demonstrate a peak response during the first 10 days, with considerable albeit not complete resolution at day 39 postinfection (p.i.). At day 7 p.i., mice were exposed, intranasally, to HDM for 10 consecutive days. We observed significantly enhanced eosinophilic inflammation, an expansion in Th2 cells, enhanced HDM-specific IgE and IgG1 responses and increased mucous production. Furthermore, lung mononuclear cells produced enhanced IFN- γ and IL-5, unchanged IL-13, and reduced IL-4. These immunologic and structural changes lead to marked lung dysfunction. This allergic phenotype occurs at a time when there is a preferential increase in plasmacytoid dendritic cells over myeloid dendritic cells, activated CD8⁺ T cells, and increased IFN- γ production, all of which have been proposed to inhibit allergic responses. In contrast, the inflammatory response elicited by HDM was reduced when exposure occurred during the resolution phase (day 40 p.i.). Interestingly, this was not associated with a reduction in sensitization. Thus, the proinflammatory environment established during an acute influenza A infection enhances Th2-polarized immunity to a low dose of HDM and precipitates marked lung dysfunction. *The Journal of Immunology*, 2009, 182: 3095–3104.

Allergic asthma is a chronic inflammatory disease mediated by a Th2-polarized immune response involving eosinophilic inflammation, mucous overproduction, bronchial hyperactivity, and, eventually, airway remodeling. Allergic asthma occurs following sensitization to naturally occurring aeroallergens such as house dust mites (HDM),³ roaches, pollens, or animal dander. We have previously shown that HDM administered through the mucosal route and without the use of additional adjuvant leads to the generation of Th2-mediated inflammation with all of the cardinal features of asthma (1, 2). Furthermore, we have recently furnished a comprehensive computational view of the impact of dose and length of allergen exposure on allergic sensitization and inflammation (3). Although many facets of

asthma have been uncovered, the origins of asthma pathogenesis remain unclear. Importantly, allergen exposure does not ever occur in isolation and, thus, exposure to other entities such as biologics and chemicals may impact the immune status of the lung such as to alter the levels and thresholds of allergen responsiveness.

A growing number of epidemiologic studies have shown that certain respiratory viral infections in infancy are associated with increased atopy to common allergens and an overall increased risk of asthma in school-aged children (4, 5). Such an outcome seems intuitive in the case of infections with respiratory syncytial virus (RSV) which induces Th2-polarized immunity (6, 7) and can, thereby, establish a lung environment that facilitates subsequent sensitization to allergens. In contrast to RSV infections, infections with influenza A, also a significant cause of lower respiratory illness in young children (8, 9), involve the generation of archetypical Th1 immunity (10) that has been historically regarded as able to inhibit or down-regulate Th2-mediated processes.

In this study, we have investigated the immunologic, structural, and functional impact of exposure to a threshold concentration of HDM in mice during the course of an influenza A infection. Our data show that exposure to HDM during the acute phase of a flu infection, but not during the resolution phase, reduces the threshold responsiveness to allergen exposure, resulting in a robust allergic inflammatory response that is associated with enhanced mucous production and a marked alteration in lung mechanics. Importantly, this response emerges in a lung environment that contains a preferential increase in plasmacytoid dendritic cells (pDCs) over myeloid dendritic cells (mDCs), the presence of activated CD8⁺ T cells, and high levels of IFN- γ . Thus, these findings intimate that allergen exposure in a flu-induced innate proinflammatory lung environment might precipitate overt allergic disease, hence increasing the susceptibility to develop asthma.

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³ Abbreviations used in this paper: HDM, house dust mite; BAL, bronchoalveolar lavage; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; RSV, respiratory syncytial virus; p.i., postinfection; PAS, periodic acid-Schiff; MCh, methacholine; Rn, airway resistance; G, tissue resistance; H_{TE}, tissue elastance; MHCII, MHC class II; KLH, keyhole limpet hemocyanin; NES, *Nippostrongylus brasiliensis*.

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Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories. The mice were housed under specific pathogen-free conditions and maintained on a 12-h light-dark cycle with food and water ad libitum. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, Ontario, Canada).

Influenza A virus and infection protocol

Influenza type A virus strain A/PR/8/34 (H1N1) was prepared as described previously (11) and provided by MedImmune, Inc.. The viral stock suspension (10^9 PFU/ml) was diluted and 10^6 PFU were administered intranasally to isoflurane-anesthetized BALB/c mice in 35 μ l of sterile PBS solution. Animals were monitored for signs of illness twice daily for a period of 10 days following infection.

Sensitization protocols

Allergen administration. HDM extract (Greer Laboratories) was resuspended in sterile PBS at a concentration of 0.5 mg (protein)/ml and 10 μ l (5- μ g dose) was administered to isoflurane-anesthetized mice intranasally.

Allergen exposure during acute phase infection. Animals that were infected with 10^6 PFU of PR8 flu virus were exposed intranasally daily to 5 μ g of HDM for 10 consecutive days, starting at day 7 postinfection (p.i.) (F plus H group). Age-matched flu-infected animals, received 10 μ l of sterile saline (F group). Uninfected animals received either 5 μ g of HDM (H group) or 10 μ l of sterile saline (SAL group). Three days after the last exposure, animals were sacrificed and the inflammatory response was assessed.

Long-term rechallenge protocol. Animals were infected with A/PR8 virus on day 0 and then groups of animals were exposed to 10 days of allergen (or saline) as described above. After the last exposure, mice were rested for a period of 30 days, at which point they were rechallenged with 5 μ g of HDM daily for 3 days. Seventy-two hours after the last challenge, animals were sacrificed and the inflammatory response was assessed.

Allergen exposure during resolution phase. Groups of animals that were infected with 10^6 PFU of A/PR8 were exposed daily to 5 μ g of HDM for 10 consecutive days, starting at day 40 p.i. (F plus H group). Age-matched flu-infected animals received 10 μ l of PBS for 10 consecutive days (F group). Uninfected animals received either 5 μ g of HDM (H group) or 10 μ l of PBS (SAL group) for 10 consecutive days. Animals were sacrificed 3 days after the last exposure and the inflammatory response was assessed.

Collection and measurement of specimens

Bronchoalveolar (BAL) fluid, lungs, and blood were collected at the time of sacrifice. BAL was performed as previously described (12). Briefly, lungs were dissected and the tracheae were cannulated with a polyethylene tube (outer/inner diameter = 0.965/0.58 mm; BD Biosciences). Lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml) and ~0.25–0.3 ml of the instilled fluid was consistently retrieved. Total cell counts were then determined using a hemocytometer. Each BAL sample was then centrifuged and the supernatants were collected and stored at -20°C . Cell pellets were subsequently resuspended in PBS and smears were prepared by centrifugation (Shandon) at 300 rpm for 2 min. A protocol Hema 3 stain set (Fisher Scientific) was used to stain all smears. Differential cell counts of BAL were determined from at least 300 leukocytes using standard hemocytologic criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Peripheral blood was collected by retro-orbital bleeding and serum was obtained and stored at -20°C . Where applicable, after BAL collection, lungs were inflated with 10% formalin at a constant pressure of 20 cm of H_2O and then fixed in 10% formalin for 48–72 h until further processing.

Histology and morphometric analysis

Lung tissues were collected, the left lung was dissected and embedded in paraffin, and 3- μ m-thick sections were cut and stained with H&E and periodic acid-Schiff (PAS). Multiple images (9–12 photographs) of the primary airway were taken. Images for morphometric analysis were captured with OpenLab software (version 3.0.3; Improvision) via Leica camera and microscope (Leica Microsystems). Image analysis was performed using a custom-computerized analysis system (Northern Eclipse software version 5; Empix Imaging). Analysis of PAS-stained sections was performed as previously described (13). Briefly, a line is drawn immediately below the airway epithelium. The software creates a parallel line 30 μ m away, creating a “band” which encompasses the airway epithelium/goblet cells/mucous. Within this region of interest, a color range is selected that allows for

the selection of mucous and excludes nonmucous elements. A weighted average of the region of interest positive for PAS staining is calculated for each mouse.

Lung cell isolation and flow cytometric analysis of lung cells

Total lung cells were isolated as previously described (14). Briefly, total lung cells were isolated by collagenase digestion (collagenase type I; Life Technologies) and washed twice in PBS and stained with a panel of Abs. To minimize nonspecific binding, cells were first preincubated with FcBlock (anti-CD16/CD32; BD Pharmingen). For each Ab combination, 2×10^6 cells were incubated with mAbs at 4°C for 30 min. Cells were then washed in FACS buffer (PBS/0.5% BSA) and data were collected using an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star and Stanford University, Palo Alto, CA). The following Abs were used for the identification of mDCs, pDCs, macrophages, and B cells (15–17): CD45-allophycocyanin-Cy7, MHC class II (MHCII)-allophycocyanin, CD11b-PE, CD11c-FITC, B220-allophycocyanin, GR1-PE-Cy7, and CD19-PE-Cy5.5. T cells were identified using CD3-PerCP-Cy5.5 or PE-Cy7, CD4-FITC or allophycocyanin, CD8-allophycocyanin-Alexa Fluor 750 (eBioscience) or PerCP, CD69-PE-Cy7 or PE, CD25-allophycocyanin (all BD Biosciences), and T1/ST2-FITC (MD Bioscience). Where applicable, total lung eosinophils were quantified on the basis of the following analysis: briefly, total lung cells were stained with anti-CD45-allophycocyanin-Cy7 (to select leukocytes) and anti-GR1-PE-Cy7 (to exclude neutrophils-Gr-1^{bright} population). Eosinophils were then identified on the basis of forward scatter and side scatter as shown in supplemental Fig. 4S.⁴ All appropriate isotype controls were used (BD Biosciences). Abs were titrated to determine optimal concentration. See online supplemental material for additional details on the methods used to make these measurements.

Mononuclear cell isolation and in vitro cytokine production

Total lung cells were isolated by collagenase digestion and mononuclear cells were purified over a Percoll gradient as described previously (14). After washing in RPMI 1640, cells were resuspended in complete RPMI 1640 (RPMI 1640, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% 2-ME) and 5×10^5 cells/well (in 100 μ l of complete RPMI 1640) plated on 96-well plates. Cells were stimulated with 3 μ g of HDM extract (2.5 μ g/ μ l) and incubated at $37^{\circ}\text{C}/5\% \text{CO}_2$ for 5 days. Thereafter, supernatants were collected and stored at -70°C for cytokine analysis.

Cytokine analysis and Ig measurements

Levels of proinflammatory cytokines in BAL were measured using a Luminex 100 Total System based on xMAPmultiplexing technology. 5-Plex cytokine kits containing microbeads with capture Ab and biotinylated reporter specific for mouse IL-6, TNF- α , IFN- γ , and IL-10 were purchased from Upstate Biotechnology while type I IFN- α and IFN- β were measured by ELISA kits purchased from PBL Biomedical Laboratories. Levels of IL-5, IL-4, IL-13, and IFN- γ in supernatants from mononuclear cell cultures were measured by DuoSet ELISA kits purchased from R&D Systems and used according to the manufacturer's instruction. Levels of HDM-specific IgE, IgG1, and IgG2 were measured by sandwich ELISA as previously described (2, 12).

Preparation of influenza A/PR8 flu lysate and measurement of influenza-specific IgG1 and IgG2a

Madin-Darby canine kidney cells were seeded at 10^7 cells/15-cm dish and grown to confluency. Cells were washed twice with PBS and infected with A/PR8/34 at an multiplicity of infection of 2. Cells were incubated for 24 h in serum-free medium (α -MEM, 1% L-glutamine, and 1% penicillin/streptomycin) at 37°C in 5% CO_2 or until 50% lysis was achieved. Cells were harvested and spun at 1200 rpm/ 4°C for 10 min. The cell pellet was resuspended in 1 ml of PBS and then lysed by three sequential freeze-thaw cycles in liquid N_2 followed by a 37°C water bath. Cell lysates were spun down and supernatants were collected and stored at -70°C . Protein concentration was determined using a Bradford assay (Bio-Rad) as per the manufacturer's instructions.

For the detection of influenza-specific IgG1 and IgG2a, 96-well Maxi-Sorp plates (Nunc and VWR) were coated overnight at 4°C with 50 μ l of 10 μ g/ml solution of flu-PR8 cell lysate in PBS. Coated wells were blocked with 0.05% BSA in TBS with 0.05% Tween 20 for 2 h at room temperature. After washing, serum samples (diluted 1/20, 1/200, 1/2,000, and 1/20,000 for IgG1 and 1/50, 1/500, 1/5,000, and 1/50,000 for IgG2a, 50 μ l/well) were added to the wells and incubated overnight at 4°C , washed,

⁴ The online version of this article contains supplemental material.

and then incubated with 0.25 $\mu\text{g}/\text{ml}$ biotin-labeled IgG1 or IgG2a (Southern Biotechnology Associates) overnight at 4°C. Plates were then incubated with alkaline phosphatase-streptavidin (Zymed Laboratories) in 50 $\mu\text{l}/\text{well}$ at a concentration of 1/1000. The color reaction was developed with *p*-nitrophenyl phosphatase tablets (Sigma-Aldrich) in 50 $\mu\text{l}/\text{well}$ and stopped with 25 $\mu\text{l}/\text{well}$ 2 N NaOH. ODs were read at 405 nm. Blank OD values were based on the average of 20 control wells that were loaded with diluent instead of sample. Flu-specific IgG1 and IgG2a units corresponded to the maximal dilution that resulted in an OD that exceeded the average OD value of 20 zero standard replicates plus 2 SDs. The formula used to calculate is as follows: relative units = (OD reading – OD blank) $\times$ dilution of OD reading.

Airway responsiveness measurements

Airway responsiveness was assessed 2 days after the last exposure to HDM in response to increasing doses of nebulized methacholine (MCh; Sigma-Aldrich) using a previously described protocol (3). Briefly, mice were anesthetized with inhaled isoflurane (3% with 1 liter/min of O₂), paralyzed with pancuronium bromide (1 mg i.p.), tracheotomized with a blunted 18-gauge needle, and mechanically ventilated with a small animal computer-controlled piston ventilator (flexiVent; SCIREQ) (18). Mice received 200 breaths/min and a tidal volume of 0.25 ml; the respiratory rate was slowed during nebulization (10 s) to provide five large breaths of aerosol at a tidal volume of 0.8 ml. The response to nebulized saline and increasing doses (3.125, 12.5, and 50 mg/ml) of MCh were measured and the data fit with the constant phase model. Model parameters of airway resistance (R_n), tissue resistance (G), and tissue elastance (H_{TE}) were calculated as described previously (19). Model fits that resulted in a coefficient of determination <0.8 were excluded.

Data analysis

Data were analyzed using SigmaStat version 3.1 (SPSS). Data are expressed as mean $\pm$ SEM. Results were interpreted using ANOVA Fisher's least significance difference post hoc test, unless otherwise indicated. Differences were considered statistically significant when *p* values were <0.05.

Results

Experimental schematics

To comprehensively investigate the impact of aeroallergen exposure during the course of an influenza infection, we designed the four protocols outlined in Fig. 1. First, we determined the kinetics of the inflammatory response to a flu infection (Fig. 1A). Then, we chose the time point at which acute inflammation is at its peak and exposed mice to a low dose of 5 μg of HDM daily. This dose of HDM was chosen because it induces a mild eosinophilic inflammation and no airway dysfunction. The inflammatory and functional responses were evaluated 3 days after the last challenge (Fig. 1B). To investigate whether changes in the inflammatory response are transient in nature or, in fact, induced long-lasting immune changes, we recapitulated the protocol in Fig. 1B, but allowed animals to rest for a period of at least 30 days, after which they were re-exposed to 5 μg of HDM for 3 consecutive days. The inflammatory responses were then evaluated 3 days after the last exposure (Fig. 1C). Lastly, we examined whether the response to HDM was affected by the phase of the influenza infection. To that end, we repeated the infection protocol but then allowed the inflammatory response to resolve. At day 40 p.i., animals were then exposed to the same low dose of HDM and the inflammatory response was evaluated 3 days after the last exposure (Fig. 1D).

Kinetics of influenza A/PR8/34 infection in BALB/c mice

As described in Fig. 1A, mice were inoculated intranasally with 10 PFU of PR8/34 virus at day 0. This concentration of virus was chosen after an initial dose-response study revealed that this was a sublethal dose that induced a robust inflammatory response from which all animals fully recovered. Groups of mice were sacrificed at days 1, 3, 5, 7, 10, and 28, up to and including day 39 p.i. After infection, we observed that the acute inflammatory response in the BAL peaks at days 3–7 p.i. and was considerably, albeit not com-

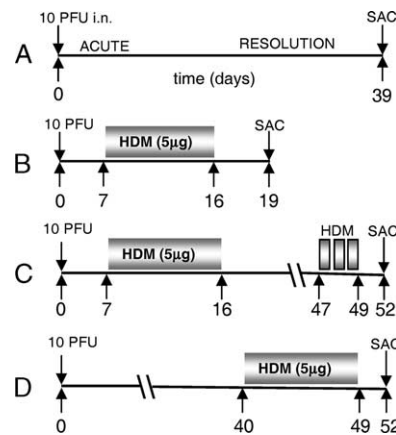


FIGURE 1. Experimental schematics. *A*, Mice were infected with flu strain A/PR8/34 intranasally at a dose of 10 PFU in 35 μl of sterile PBS. Separate groups of mice were sacrificed at various time points and the inflammatory response was delineated through the acute and resolution phase. *B*, Mice were infected as described in *A* and then separate groups of mice were exposed to 5 μg of HDM or SAL daily for a period of 10 days, starting at day 7 p. i. Seventy-two hours after the last exposure, animals were sacrificed and the inflammatory response was examined. *C*, Separate groups of animals were treated as described in *B*, but then allowed to rest for a period of 30 days after the last exposure to HDM. Animals were then re-exposed to 5 μg of HDM on 3 consecutive days and the inflammatory response was assessed for long-term immune changes. *D*, Separate mice were infected with flu-PR8 as described in *A* and allowed to rest for a period of 40 days. Groups of mice were then exposed to 5 μg of HDM, SAL, or no treatment daily for 10 consecutive days. Animals were sacrificed 3 days after the last exposure to HDM and the inflammatory response was assessed.

pletely, resolved at day 10 p.i. (Fig. 2A and supplemental Fig. 1S). At day 3, the inflammatory response consisted mainly of infiltrating neutrophils and macrophages while at day 7 this response was replaced by infiltrating mononuclear cells. Inflammatory cells remained elevated over PBS-treated groups even at days 28–39 p.i.

Flu A infection on cytokine production

In addition to cellular infiltrate, we evaluated the cytokine response. An initial antiviral response, involving the generation of type I IFNs, can be detected as indicated by increased levels of IFN- α and IFN- β in the BAL at day 3 (Fig. 2B). This increase was transient and significantly diminished by day 5 p.i. At day 10 p.i., type I IFNs were no longer detectable. The levels of the proinflammatory cytokines IL-6, IFN- γ , and TNF- α were significantly elevated in the BAL at days 3 through 7. TNF- α and IL-6 peaked early in the response, at days 3–7 p.i., while peak levels of IFN- γ and IL-10 were detected at day 7 p.i. and coincided with the influx of mononuclear cells. Again, these increases were transient and all proinflammatory cytokines were undetectable at day 10 p.i.

Lung immune status at days 7 and 10 following flu A infection

Next, we sought to evaluate the immune status of the lung by identifying the different types of APCs and T cells during the acute phase of the inflammatory response, i.e., at days 7–10. As can be seen in Fig. 3A (and supplemental Fig. 2S), we identified different populations of APCs by flow cytometry. The total number of MHCII⁺ cells was dramatically increased in flu-infected animals vs PBS control mice (supplemental Fig. 2S). In addition, in flu-infected animals, there was a remarkable 26-fold increase in the absolute number of pDCs present in the lung as compared with

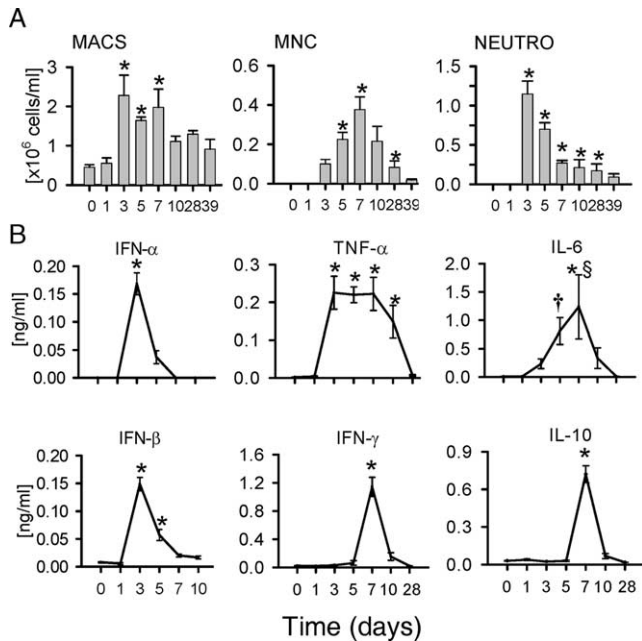


FIGURE 2. Kinetics of influenza A/PR8-induced BAL inflammation and cytokine responses. **A**, Animals were infected intranasally with 10 PFU of PR8 virus and the inflammatory response in BAL was evaluated at days 0, 1, 3, 5, 7, 10, 28, and 39 p.i. Cellular differentials showing absolute number of macrophages, neutrophils, and mononuclear cells. $n = 5$ mice/group. One of two representative experiments is shown. Data represent mean $\pm$ SEM. *, $p < 0.05$ compared with day 0 (Dunnett's post hoc test). **B**, Kinetics of type I IFNs and proinflammatory cytokine levels in BAL fluid as evaluated by ELISA at various time points. $n = 5$ mice/group. One of two representative experiments is shown. Data represented as mean $\pm$ SEM. *, $p < 0.001$ compared with day 0 and †, $p < 0.05$ and §, $p < 0.05$ compared with days 3 and 6, respectively. MACS, Macrophages; MNC, mononuclear cells; NEUTRO, neutrophils.

PBS controls, whereas mDCs experienced an ~ 5 -fold increase. Similarly, there was a 24-fold increase in B220^{low}CD11c^{int}CD11b^{high} cells, a population representing alveolar macrophages and an ~ 2.5 -fold increase in B cells. Although the absolute number of mDCs increased with infection, it was notable that the relative percent contribution of mDCs did not change after flu infection (3% before and after flu A). In contrast, the pDC percent contribution increased from 1 to 5% at day 7 after flu infection (data not shown).

In addition to APCs, we also assessed T cell populations (Fig. 3B). At days 7 and 10 after flu infection, both CD4⁺ and CD8⁺ T cells increased in absolute numbers, with CD8⁺ T cells showing a 4-fold increase while CD4⁺ T cells increased by ~ 2.7 -fold. We observed an increased number of activated cells as indicated by CD25 and CD69 expression at days 7 and 10 in both CD4 and CD8 T cells. The number of CD25⁺CD8⁺ T cells was 10-fold higher than in CD4⁺ T cells, with a notable 44-fold increase as compared with a more modest increase of ~ 4.8 -fold in CD25⁺ activated CD4⁺ T cells. Furthermore, the relative contribution of CD25⁺ activated T cells increased to 20% after flu A, corresponding to a 1.7- and 10.5-fold increase for CD4⁺ and CD8⁺ T cells, respectively (data not shown). Similarly, CD69⁺CD8⁺ T cells experienced a 55-fold expansion, whereas CD69⁺CD4⁺ T cells increased 31-fold over PBS-treated animals; this corresponded to a 15-fold increase in the activation state for CD8⁺CD69⁺ T cells (2.5% before vs 32% after flu A), whereas the relative contribution of CD4⁺CD69⁺ changed from 1.2% before vs 12.8% after infection (data not shown). Overall, flu infection changed the CD4:CD8

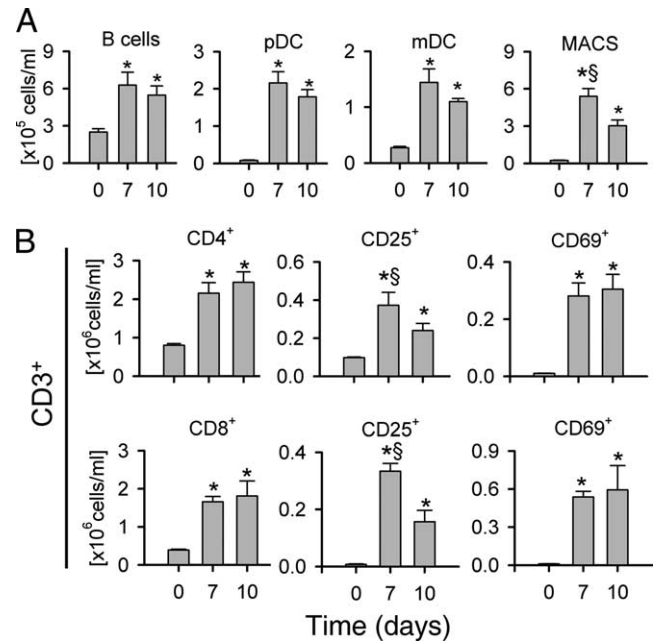


FIGURE 3. Immune status of lung at days 7 and 10 after PR8 infection. Total lung cells were isolated at days 7 and 10 p.i. and stained with MHCII, CD11b, CD11c, B220, and GR1 cell surface markers. More than 300,000 events were collected for each group. **A**, Number of B cells, pDCs, mDCs, and macrophages at days 7 and 10 p.i. as compared with control mice at day 0. $n = 5$ mice/group. One of two representative experiments is shown. Data represent mean $\pm$ SEM and $p < 0.05$ compared with *, day 0 and §, day 10, respectively (Tukey's post hoc test). **B**, Absolute number of CD4 and CD8 T cells (CD3⁺) and their activation states (CD25⁺ and CD69⁺) at days 7 and 10 after PR8 infection. $n = 5$ mice/group. One of two experiments with similar results is shown. Data represent mean $\pm$ SEM. $p < 0.05$ compared with *, day 0 and §, day 10, respectively. MACS, Macrophages.

ratio from 2:1 in PBS-treated animals to 1:1 after flu infection, thus indicating a preferential increase in activated CD8⁺ over activated CD4⁺ T cells.

Taken together, these results indicate that a proinflammatory environment is rapidly established during the acute phase of flu infection and is associated with an expansion in the APC as well as the T cell compartment, with a preferential increase in both pDCs and activated CD8⁺ T cells.

Responses to HDM exposure in the context of flu-induced acute inflammation

Next, we investigated the impact of a viral-induced proinflammatory environment on the response to allergen exposure. To this end, we exposed groups of animals to the protocol outlined in Fig. 1B. We found enhanced BAL inflammation in mice previously infected with flu virus (F plus H) as compared with HDM (H) alone, those infected only with flu (F), or saline (SAL)-treated animals (Fig. 4A). The increase in total inflammation in HDM-exposed animals previously infected with flu could be mainly attributed to an increase in both mononuclear cells and eosinophils. Indeed, prior flu infection resulted in a doubling of the proportion of eosinophils in the BAL from 7% in HDM-treated animals to 15% in the flu plus HDM group (data not shown).

To further evaluate the nature of T cell subsets in the lung, total lung cells were analyzed by flow cytometry. The total number of CD4⁺ or CD8⁺ T cells in mice exposed to HDM alone, flu alone, and HDM after flu infection was not significantly different compared with saline at this time point (supplemental Fig. 3S and data not shown). However, we observed

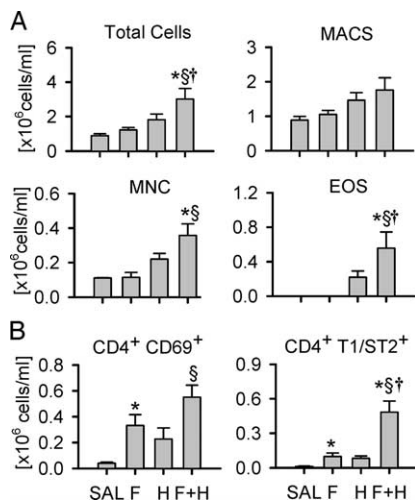


FIGURE 4. Impact of influenza A infection on subsequent HDM exposure. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure and the inflammatory response was evaluated in BAL fluid and lung. *A*, Cellular profile in BAL fluid showing the number of total cells, macrophages, mononuclear cells, and eosinophils. *n* = 5 mice/group. One of three representative experiments is shown. Data represent mean $\pm$ SEM. *p* < 0.05 compared with *, SAL; †, F; and §, HDM, respectively. *B*, Flow cytometric analysis of lung immune cells showing the absolute number of CD4⁺ T cells expressing the activation marker CD69 and Th2 marker T1/ST2. *n* = 5 mice/group. Data represent mean $\pm$ SEM. *p* < 0.05 compared with *, SAL; †, F; and §, HDM, respectively. MACS, Macrophages; MNC, mononuclear cells; EOS, eosinophils.

a significant increase in the number of activated cells, as evaluated by CD69⁺ expression (Fig. 4*B*). In animals exposed to flu only, we found an 8.2-fold increase in CD69-activated CD4⁺ cells and a 2.1-fold increase in CD8⁺ T cells (supplemental Fig. 3*S*). HDM exposure further increased this by 1.6- and 1.5-fold for CD4⁺ and CD8⁺ T cells, respectively. Indeed, the level of CD4⁺ T cell activation in flu-infected animals increased from 26 to 39% after HDM exposure (data not shown). In contrast, exposure to 5 μ g of HDM alone led to only a modest increase in CD69 expression as compared with control animals.

Finally, to examine whether the pronounced eosinophilic response was associated with an expansion in Th2 cells, we evaluated the expression level of T1/ST2⁺, a cell surface marker expressed on effector Th2 cells (20, 21). We observed a modest increase in CD4⁺T1/ST2⁺ cells in animals exposed to 5 μ g of HDM over saline-treated animals; this level was significantly increased in the flu plus HDM-treated group (5.9-fold) as compared with HDM and corresponds to a doubling in the percent contribution of this cell type in the flu-infected and HDM-exposed group vs HDM alone, ~8% vs ~4%, respectively (data not shown).

Cytokine recall responses of lung mononuclear cells

Next, we explored whether the enhanced inflammatory and eosinophilic response was associated with changes in immune responsiveness. We examined the production of Th1/Th2 cytokines by lung mononuclear cells cultured with 25 μ g of HDM. We found that in animals exposed to HDM alone, the average level of IL-5 production was 621 pg/ml, whereas in animals infected with flu virus and subsequently exposed to HDM, this level increased to an average of 1613 pg/ml (Fig. 5). Similarly to IL-5, IFN- γ production was also increased significantly in

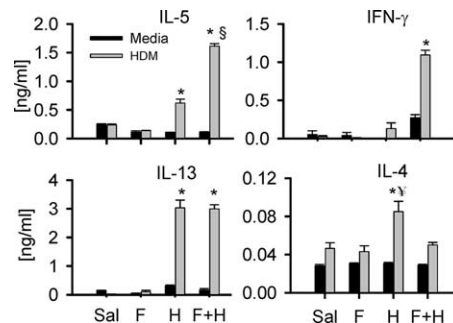


FIGURE 5. Cytokine production by lung mononuclear cells. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure and the inflammatory response was evaluated. Evaluation of IL-4, IL-5, IL-13, and IFN- γ expression by lung mononuclear cells cultured for 5 days in the presence of HDM (■) or medium alone (□). Samples were pooled within groups and plated in triplicate (*n* = 5). Data represent mean $\pm$ SEM. *p* < 0.001 compared with §, HDM; ¥, F + H; and *, F or SAL, respectively.

animals previously infected with flu and subsequently exposed to HDM, from 130 pg/ml in the HDM group to 1094 pg/ml in the flu plus HDM-treated animals.

In contrast to increases in IL-5 and IFN- γ , we observed a significant decrease in IL-4 production in animals previously infected with flu and exposed to HDM, 85 pg/ml vs 50 pg/ml, in HDM vs flu plus HDM, respectively. Finally, we observed no difference in IL-13 production between HDM and flu plus HDM-treated animals, but found it to be significantly increased over flu alone or SAL control.

To determine the impact on Ig production, we evaluated flu and HDM-specific IgGs. Whereas, flu-specific IgG responses were not affected by subsequent HDM exposure, there was a significant increase in both HDM-specific IgG1 and IgG2a responses in F plus H compared with HDM alone (Fig. 6). HDM-specific IgE responses at this time point could not be evaluated due to the short duration of the experimental protocol used.

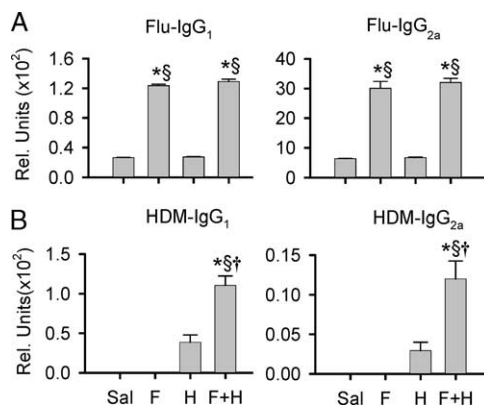


FIGURE 6. Impact of influenza A infection on HDM-specific Igs. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure. Serum Ig levels measured by ELISA showing flu-specific IgG1 and IgG2a (*A*) and HDM-specific IgG1 and IgG2a (*B*). *n* = 5 mice/group. One of two representative experiments is shown. Data represent mean $\pm$ SEM. *p* < 0.05 compared with *, SAL; §, HDM; and †, flu, respectively.

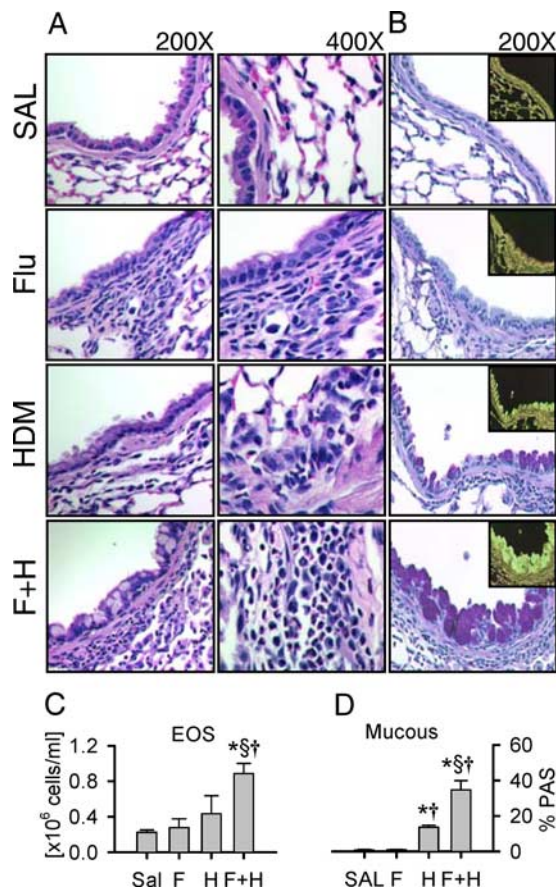


FIGURE 7. Lung histopathology after HDM exposure in the context of a prior flu infection. *A* and *B*, Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure. Lung histopathology was evaluated by H&E to show degree of lung eosinophilia (*A*) and PAS staining (*B*) indicating mucous production by epithelial goblet cells (magenta; *insets* show color-inverted image used for morphometric analysis). *C*, Quantitative analysis of tissue eosinophils by flow cytometry. *D*, Morphometric measurement of PAS-stained tissues. $n = 5$ mice/group. One of two representative experiments is shown. Micrographs were taken at $\times 200$ and $\times 400$ original magnification as noted. Data represent mean $\pm$ SEM. $p < 0.05$ compared with *, SAL; †, F; and §, HDM.

The changes in inflammation observed in the BAL were also associated with changes in tissue inflammation, goblet cell metaplasia, and enhanced mucous production. Animals exposed to 10 days of HDM exhibited a very modest degree of tissue inflammation characterized by peribronchiolar mononuclear cell and eosinophil accumulation, which was enhanced in animals previously exposed to flu virus (Fig. 7*A*). In contrast, tissue inflammation in animals exposed to flu alone was limited to mononuclear cell infiltration with no accumulation of eosinophils. The degree of tissue eosinophilia was evaluated both qualitatively by H&E staining and quantitatively by flow cytometric analysis (Fig. 7*C* and supplemental Fig. 4*S*), which confirmed the elevated eosinophilic inflammation observed in the BAL of flu plus HDM-treated animals.

Based on the increased inflammatory responses, we examined whether flu infection also impacted structural remodeling, in particular goblet cell metaplasia and mucous production (Fig. 7*B*). After 10 days of HDM exposure, goblet cell metaplasia and mucous production was evident in HDM-treated animals. This effect was almost doubled in the HDM-treated group previ-

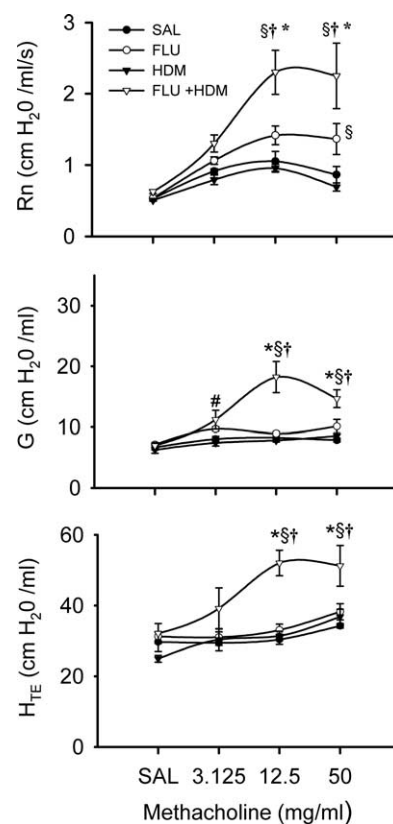


FIGURE 8. Impact of HDM exposure on airway responsiveness in the context of acute flu infection. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure. Airway responsiveness to increasing doses of MCH was assessed and shown as maximum Rn, G, and H_{TE}. $n = 6$ mice/group. One of two representative experiments is shown. Data represent mean $\pm$ SEM. $p < 0.01$ compared with *, SAL; §, HDM; and †, flu, respectively. #, $p < 0.05$ compared with HDM or SAL. Two-way ANOVA (Tukey's post hoc test).

ously infected with flu virus (Fig. 7*D*). In contrast, control animals treated with flu or saline alone did not exhibit any mucous production.

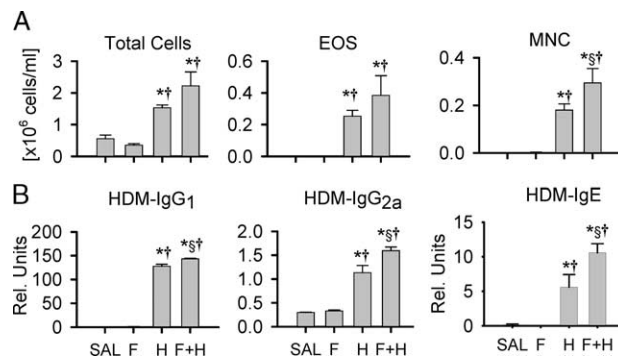


FIGURE 9. Impact of acute flu infection on long-term allergen-specific immunity. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. After a 30-day rest period, mice were re-exposed to three doses of 5 μ g of HDM or SAL. The inflammatory response was evaluated 3 days after the last exposure. *A*, Cellular profile in BAL fluid showing the number of total cells, eosinophils, and mononuclear cells. *B*, HDM-specific IgG1, IgG2a, and IgE levels in the serum. One of two representative experiments is shown. Data represent mean $\pm$ SEM. $p < 0.05$ as compared with *, SAL; †, flu; and §, HDM, respectively. EOS, Eosinophils; MNC, mononuclear cells.

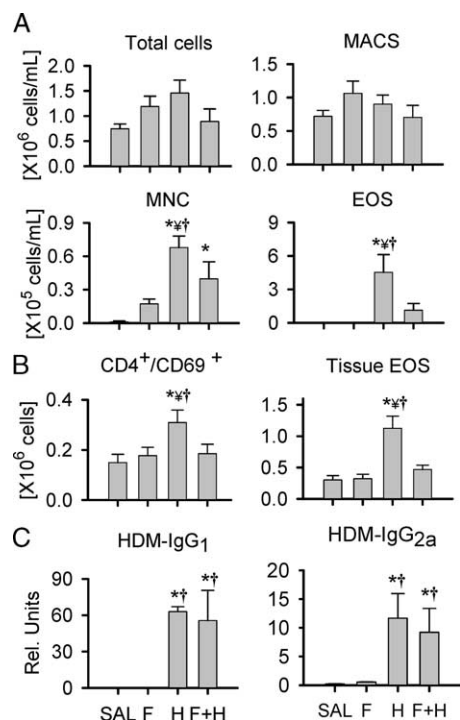


FIGURE 10. Allergen exposure during the resolution phase of flu infection. Mice were infected with 10 PFU of PR8 virus or mock infected (PBS) and then allowed to rest for 40 days. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 40 p.i. Mice were sacrificed 3 days after the last exposure and the inflammatory response was evaluated. *A*, BAL inflammation showing the number of total cells, macrophages, mononuclear cells, and eosinophils. *n* = 5 mice/group. One of three representative experiments is shown. Data represent mean $\pm$ SEM. *p* < 0.05 compared with *, SAL; †, F; and ¥, F + H, respectively. *B*, Flow cytometric analysis of lung cells showing the absolute number of activated CD4⁺ T cells expressing CD69 and tissue eosinophils. *C*, Serum Ig levels of HDM-specific IgG1 and IgG2a. *n* = 6 animals/group. One of two representative experiments is shown. Data represent mean $\pm$ SEM. *p* < 0.05 compared with *, SAL; †, F; and ¥, F + H, respectively. MACS, Macrophages; MNC, mononuclear cells; EOS, eosinophils.

Lung function after HDM exposure in the context of flu-induced acute inflammation

We investigated whether the enhanced allergic response to HDM in flu-infected animals resulted in altered lung function by evaluating respiratory mechanics; this was accomplished by evaluating Rn, G, and H_{TE}. A low dose of HDM exposure (5 μ g for 10 days), which caused a mild inflammatory response, did not elicit any measurable changes in lung function (Fig. 8). In contrast, animals previously infected with flu and subsequently exposed to HDM exhibited significantly enhanced dysfunction as measured by Rn, G, and H, and this was statistically significant at both 12.5 and 50 mg/ml of MCh exposure. Interestingly, animals infected with flu only also experienced a small but significant increase in Rn over HDM and saline-treated animals at the highest dose of MCh used (Fig. 8).

Long-term immune-inflammatory changes

To investigate whether the flu-mediated changes in allergen responsiveness were transient in nature, we examined long-term immunologic events. Thus, flu-infected, HDM-exposed animals were rechallenged at day 31 after the last HDM exposure for 3 consecutive days (Fig. 1C).

Similar to what we observed at day 19, we found an increase in total BAL inflammation in animals with a prior flu infection as compared with the HDM-only group and the respective control groups (Fig. 9A). Furthermore, this increase in inflammation was characterized by a significant increase in mononuclear cells as well as eosinophils; although the latter did not reach statistical significance. In addition, we found that the serum HDM-specific IgG1 and IgG2a responses remained statistically elevated at day 52 p.i. in flu plus HDM-treated animals, as compared with HDM only (Fig. 9B). Importantly, HDM-specific IgE levels were significantly increased at this time in animals previously infected with flu virus and exposed to HDM over the HDM-alone group or the respective control animals.

HDM exposure during the resolution phase

The responses to HDM exposure observed during the acute phase of influenza infection raised the question whether exposure to HDM long after resolution of the acute phase would lead to a similar outcome. To that end, animals were exposed to the experimental protocol outlined in Fig. 1D. We found that animals infected with flu and subsequently exposed to HDM had lower total inflammation and that this was associated with a 4-fold reduction in eosinophils in BAL and lung tissue (Fig. 10, A and B). Furthermore, we observed a significant decrease in BAL mononuclear cells, which upon further examination by flow cytometry revealed a significant decrease in the number of activated CD4⁺CD69⁺ cells (Fig. 10B). At variance with these findings, we found similar serum levels of HDM-specific IgG1 and IgG2a in animals exposed to HDM and previously infected with flu as compared with the HDM-only group (Fig. 10C) suggesting that allergic sensitization was not affected.

Discussion

There has been a great deal of interest to uncover the impact of respiratory viral infections on asthma. Understandably, much of this research has focused on the potential of such infections to exacerbate asthma; much less evidence is available investigating their ability to facilitate the emergence of the asthmatic phenotype. This is due, at least in part, to the fact that it is nearly impossible to directly investigate the development of asthma in humans. With respect to experimental research, the vast majority of studies investigating the impact of flu infection on allergic asthma have been conducted using the innocuous Ag OVA as a surrogate allergen (22–27). Indeed, this model system has become a prolific tool to elucidate specific molecular mechanisms of Th2-mediated inflammation. It should be noted that, in the absence of exogenous adjuvants, mucosal exposure to OVA leads to the induction of tolerance and, in this regard, Tsitoura et al. (26) clearly demonstrated that a concurrent flu infection is able to prevent the induction of tolerance. In addition, others have shown that a flu infection can either enhance or suppress allergic responses, interestingly both effects being mediated by IFN- γ (28, 29). The controversial and, in some instances, even contradictory nature of these and other data in OVA-based systems intimate that models of allergic asthma that require the introduction of Ag into the peritoneal cavity along with chemical adjuvants to elicit productive immunity are limited. Indeed, not only do they introduce confounding variables, but, importantly, preclude the investigation of allergic sensitization via the mucosal route. Thus, the precise impact mucosal allergen exposure has following influenza A infection has not been examined.

In this study, we have investigated the impact of a preexisting lung viral infection on cardinal immune, structural, and functional features of the asthmatic phenotype. The experimental system that we used has three central characteristics. First, we used the most

pervasive and common aeroallergen worldwide, HDM, a biochemically complex material with a wide array of protein and nonprotein components with numerous immunogenic properties (30–32). These constituents enable these extracts to initiate Th2-polarized immune-inflammatory responses when delivered intranasally without additional adjuvants (1, 2), thus permitting the study of incipient mucosal responses. Second, we used a low concentration of HDM, 5 μ g per day for 10 days, that elicits only very mild airway inflammation and, most importantly, no lung dysfunction. Third, a preexisting immune-inflammatory environment was established with influenza A, a virus that elicits an archetypic Th1-type response. This is in contrast to many studies that have investigated the impact of RSV infection which by itself tends to promote a Th2 environment.

To precisely identify the time points at which aeroallergen exposure was to be conducted, we first delineated the inflammatory response to a sublethal dose of influenza A/PR8 virus. Our data show that the pulmonary environment after flu infection exhibits an initial antiviral as well as a proinflammatory state characterized by a robust, albeit transient, infiltration of inflammatory cells largely consisting of neutrophils and mononuclear cells. In addition to cellular immunity, this acute phase is characterized by increased amounts of an array of proinflammatory cytokines in the BAL including TNF- α , IFN- γ , IL-10, and IL-6.

To better define the immune status of the lung, we evaluated the profile of immune cells at the peak of the PR8-induced mononuclear cell infiltration, i.e., at days 7 and 10 p.i. We first evaluated the APCs compartment because it is clear that APCs play an important role in enhancing allergen responses after flu infection (24, 25, 27). Our findings demonstrate an overall increase in all APC populations, namely, macrophages, B cells, mDCs, and pDCs. Notably, our data also show a particularly dramatic increase in pDCs and macrophages as compared with mDCs and B cells. These findings are consistent with the idea that pDCs are the major cell type responding after respiratory viral infections and the major producers of type I IFNs (15). With respect to T cell subsets, our data show that although the number of both CD4⁺ and CD8⁺ T cells increased, there was a preferential increase in the activation of CD8⁺ T cells over CD4⁺ T cells. This preferential increase is expected after a viral infection and particularly interesting in terms of its potential impact on the outcome of subsequent allergen exposure.

This detailed kinetic study allowed us to clearly separate the response to PR8 into acute and resolution phases. Exposure to a low dose of HDM during the acute phase of a flu infection led to a significantly increased number of mononuclear cells as well as eosinophils in the lung. A closer examination by flow cytometry revealed that the increase in mononuclear cells was associated with a significant increase in the number of activated CD4⁺ T cells and, particularly, activated CD4⁺ T cells expressing T1/ST2, a cell surface marker expressed by effector Th2 cells (20, 21) over and above that induced by this concentration of HDM alone. In terms of humoral immunity, our data show that HDM exposure during the acute phase of a flu infection results in significantly increased levels of HDM-specific IgG1 and IgG2a, although this time point is too premature to detect a HDM-specific IgE response. In contrast, the levels of flu-specific Igs remained unaltered. Collectively, these data indicate that the environment established during the acute phase of an infection with flu-PR8 virus leads to enhanced sensitization and attendant allergic inflammation to a low dose of aeroallergen administered through the mucosal route. Interestingly, this occurs at a time when there is an accumulation of activated CD8⁺ T cells in the lung and increased IFN- γ production, both of which have been proposed to inhibit allergic airway in-

flammation (33). Thus, our findings demonstrate that a vigorous flu-induced Th1 response does not deviate HDM-induced Th2 immunity.

We next asked whether the enhancement in allergic sensitization and inflammation was transient in nature. Thus, animals that had been exposed to allergen during the acute response of a flu infection were allowed to rest for a period of 30 days so that the HDM-mediated inflammatory response would fully resolve. Allergen rechallenge, at this time point, led to a substantial increase in BAL inflammation, eosinophilia, and mononuclear cells. Similar to the observations during the acute phase, HDM exposure at this time resulted in enhanced humoral immunity and was now associated with increases in HDM-specific IgE. These data show that HDM exposure during the acute phase of a flu infection resulted in long-lasting immune changes.

Our findings may appear to contrast with those of Wohlleben et al. (29) showing that, in a conventional OVA model, a prior flu infection inhibited airway eosinophilia and Th2 cell recruitment and that these effects were dependent on IFN- γ . However, the timing of viral and allergen exposures were fundamentally different from those used in our study. Indeed, Wohlleben et al. (29) examined already sensitized mice that were subsequently infected with influenza A 1–9 wk before OVA challenge. Hence, this study examined the impact of a flu infection on OVA-specific recall responses rather than on mucosal allergic sensitization.

Our data demonstrate that the presence of a large number of pDCs and macrophages in the lung at the time of allergen exposure did not result in inhibition of allergic sensitization and inflammation. Rather, our study shows that these responses were enhanced. Interestingly, in a model of experimental asthma to the inert Ag OVA, it has been shown that pDCs inhibit allergen sensitization and, in fact, promote tolerance (17), while mDCs are thought to mediate allergic sensitization (16). These observations led to the suggestion that immature pDCs possess tolerogenic properties and have poor Ag-presenting capacity, whereas activated pDCs can convert to an immunogenic phenotype able to prime T cells and drive potent Th1 polarization. However, there is a considerable amount of conflicting data regarding this simple dichotomy. Indeed, pDCs have been shown capable of mediating both Th1 and Th2 polarization (15, 34). Furthermore, studies in humans show that both mDCs as well as pDCs are present in the allergic immune response to allergen (35, 36) and, in patients with allergic asthma, allergen challenge increased both mDC and pDC numbers with an overall greater percent increase in pDCs (37). Clearly, the precise role of different dendritic cells subsets in allergic diseases is far from resolved. With respect to experimental studies, it seems increasingly apparent that the Ag used, an inert protein such as OVA or a complex allergen with inherent immunogenic properties, may have a decisive impact on the outcome.

The immune interactions that occur between respiratory viral infection and aeroallergen exposure defy a simple classification into Th1- or Th2-type responses. For example, our data show that lung mononuclear cells from HDM-treated animals previously infected with flu virus express, upon *in vitro* stimulation with HDM, an atypical profile characterized by increased amounts of IL-5 as well as IFN- γ while IL-4 is significantly down-regulated and IL-13 production is unchanged. Whether enhanced allergic responses to HDM in the context of an ongoing flu infection involve an IL-4-independent mechanism is not known. However, a recent study by Kurowska-Stolarska et al. (38) has shown, in an OVA system, that IL-33, the ligand for the T1/ST2 receptor expressed on CD4⁺ T cells, can induce IL-5 and IL-13 production and promote allergic airway inflammation in the absence of IL-4. It is, thus, conceivable

to postulate that the increased IFN- γ level that we detected down-regulates IL-4 production, whereas the enhanced IL-5 production is driven by IL-33. However, whether a flu infection induces the expression of IL-33 is not known. We should also note that other innate molecules may contribute to the enhanced allergic responses that we have documented in flu- infected, HDM-exposed animals. These may include, for example, GM-CSF and IL-6, both of which are elevated in flu- infected animals. Studies to uncover the mechanisms underlying the enhanced allergic responses that we have documented are receiving intense attention in our laboratory.

Within a larger perspective, studies have demonstrated that several hundred genes are up-regulated shortly after a flu infection (39, 40). There are no similar data available to date for HDM-elicited immune-inflammatory responses, although a similarly rich array of gene up-regulation would be expected given the immunogenic potential of HDM extracts (30–32). Furthermore, the interplay that surely occurs between flu-induced and HDM-induced gene products would elevate the degree of complexity to a higher level. In regard to the mechanisms underlying the enhancement in allergic sensitization and inflammation that we have documented when HDM exposure occurs during the acute phase of a flu infection, we can say that such an enhancement is connected to the presence of an innate, proinflammatory environment and that it seems unlikely that a single molecule is responsible for this effect.

To elucidate the health impact of those immunologic events, we investigated their structural and functional consequences. Airway remodeling is, indeed, a typical hallmark of asthma and a growing number of studies have found that remodeling, or at least certain aspects of it does contribute to airway dysfunction and, ultimately, clinical symptoms (41, 42). Typical features of remodeling occurring below the epithelial lining, such as collagen deposition, take several weeks to develop in mice. Thus, within the interval of the short protocol of HDM exposure that we used (10 days), only goblet cell metaplasia and mucous production can be evaluated. We observed that exposure to even a low dose of HDM induced mild but significant mucous production and that influenza A infection alone did not lead to any mucous production. However, animals that were exposed to HDM in the context of an acute flu infection had significantly greater goblet cell metaplasia and mucous production as compared with those treated with a low dose of HDM only. Thus, this provides evidence that a prior flu infection can enhance aspects of airway remodeling that could have an impact on function.

Previous studies, using conventional OVA models, have found either no effect (28) or shown increased airway hyperreactivity (24) after flu infection. Not only are the outcomes controversial but the actual methodology used to assess lung function performed in these studies (enhanced pause (Penh)) has serious drawbacks such that its physiologic significance has been questioned (43, 44). We evaluated lung physiology using a protocol that furnishes data on lung mechanics including Rn, G, and H_{TE}, an indicator of the degree of airway closure in response to MCh exposure. This approach has been validated in other models and is considered a superior predictor of changes in lung function (45). Exposure of animals to a low dose of HDM did not result in any changes in lung mechanics as compared with saline-treated animals. Intriguingly, flu infection alone induced significant changes in airway resistance. Moreover, a low dose of HDM exposure during an acute flu infection increased the response to MCh challenge, leading to enhanced values for Rn, G, and H_{TE}. Thus, the immunologic and structural changes that occurred as a result of flu infection lowered the threshold of allergen needed to generate significant lung dysfunction. In other words, a low dose of HDM that in an

otherwise healthy lung environment would not lead to significant lung dysfunction results in severe functional abnormalities if exposure had occurred during the acute phase of a flu infection.

Lastly, to address whether timing of allergen exposure played a role in the response to HDM, we exposed animals to HDM starting at 40 days after flu infection. In contrast to the enhancement observed during the acute phase of infection, the inflammatory response, including eosinophils, elicited by HDM exposure was significantly reduced compared with uninfected mice. Importantly, these changes in the BAL were substantiated by a reduction in CD4⁺CD69⁺ T cells and eosinophils in the tissue. The decrease in lung inflammation cannot be attributed to decreased allergic sensitization because the levels of HDM-specific IgG1 and IgG2a were similar between animals that had been infected with the flu virus 40 days earlier and uninfected animals. Moreover, we did not detect any alteration in lung function (data not shown). There is no direct evidence of reference for these findings. However, two studies have examined the consequences of exposure to a heterologous Ag during the resolution phase of a flu infection with divergent results (28, 33). Our findings are in sharp contrast to those of Dahl et al. (28) in that these authors reported an increase in lung eosinophilia as well as primary, but not secondary, Ag-specific IgG1 and IgE responses upon exposure to the surrogate allergen keyhole limpet hemocyanin (KLH) 30 days after flu infection, an effect that was shown to be mediated by activated MHCII/CD11c⁺ cells (28). Not only the Ag but also the experimental protocol was different from that which we used in that, at day 30, mice had to be exposed first to KLH i.p. twice along with aluminum hydroxide to achieve sensitization and later exposed to KLH intranasally. At variance with these findings, Marsland et al. (33) reported a reduction of allergic airway inflammation in mice exposed to the parasite *Nippostrongylus brasiliensis* (NES) 14 days after a flu infection, an effect mediated by CD8⁺ T cells and IFN- γ (33). Although it may be argued whether 14 days represents true resolution, this time point is indeed past the acute proinflammatory phase of the flu infection. In this study as well, mice were exposed first to a NES extract i.p. along with aluminum hydroxide and exposed to NES intranasally 7 days later. Thus, neither of these studies was able to investigate the effect of a resolving, or resolved, flu infection on mucosal sensitization to allergen. With this background, our study shows that mucosal exposure to the common aeroallergen HDM during the resolution phase of a flu infection does not alter allergic sensitization and is associated with a decrease of allergic airway inflammation.

In summary, our findings demonstrate that mucosal exposure to the common aeroallergen HDM during the acute proinflammatory phase of a flu infection results in increased allergic sensitization and enhanced allergic inflammation, goblet cell metaplasia, and mucous production. In addition, allergen exposure under these conditions lowers the threshold for the generation of a definitely abnormal functional response (lung mechanics). The direct, and particularly important, implication of these findings is that the environment established during the acute phase of a flu infection increases the susceptibility to elicit allergic disease. However, this increased susceptibility is transient since allergic inflammation is, in fact, reduced when allergen exposure occurs during the resolution phase. It should be noted that the enhancement of allergic responses observed when allergen is introduced during the acute phase of the flu infection occurs at a time when there is a preferential increase in the lung of pDCs over mDCs as well as the presence of increased numbers of activated CD8⁺ T cells and high levels of IFN- γ , all of which have been proposed to inhibit allergic responses. Hence, perhaps a broader implication of our findings is that of caution against attachment to oversimplified paradigms.

The biologic responses induced by HDM and influenza A are each complex; it seems that decoding the regulation of this complexity may require ontologies more sophisticated than traditional reductionist dichotomies.

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Disclosures

A.J.C. is an employee of MedImmune and J.R. is a former employee of MedImmune, Inc.

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Figure Legends

Figure 1S: *Kinetics of influenza A/PR8 induced BAL inflammation:*

Mice were infected intranasally with 10 PFU PR8 virus and the inflammatory response evaluated at days 0, 1, 3, 5, 7, 10, 28, and 39 post infection. Figure showing total cells in BALF. One of two representative experiments shown. Data represent mean $\pm$ SEM.

* $P < 0.05$ compared with day 0. Dunnett's post hoc test.

Figure 2S: *APC gating strategy.*

Groups of mice were infected with 10 PFU PR8 virus or PBS. Total lung cells were isolated at day 7 and 10 p.i. and stained with MHCII, CD11b, CD11c, B220 and GR1 cell surface markers. In all plots single cells were identified on the basis of FSC-W and FSC-A. (A) Shows the number of MHCII⁺ cells in the lung at various time points post flu infection. (B) Shows the gating strategy used to identify four different APC populations, B cells, plasmacytoid DCs (pDC), myeloid DCs (DC) and macrophages (MACS). To identify these populations, single cells were first gated on MHCII⁺ and then differentiated on the basis of B220, CD11b, CD11c, expression as shown. More than 300,000 events were collected for each group Shaded histogram shows isotype control for CD11b. Data represent mean $\pm$ SEM and * $P < 0.05$ compared to day 0.

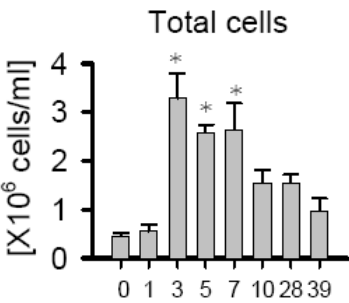
Figure 3S: *CD69 expression on CD8 T cells in flu infected animals exposed to HDM.*

Groups of mice were infected with 10 PFU PR8 virus or PBS. Animals were exposed to 5 μ g of HDM for 10 days starting at day 7 p.i. and sacrificed three days after last exposure at day 19. Total lung cells were isolated and stained with CD45, CD3, CD4, CD8 and the activation marker CD69 and analyzed by flow cytometry. Cells were gated on CD45/CD3 expression and the absolute number of cells expressing CD8 and CD69 quantified. $n = 5$ mice/group. Data represent mean $\pm$ SEM. $P < 0.005$ compared to †HDM and *SAL.

Figure 4S: *Eosinophil quantification using flow cytometric analysis.*

Whole leukocytes were first selected by gating on CD45⁺ cells in order to exclude nonleukocytes, then single cells were selected on the basis of FSC-W and FSC-A. Three distinct populations were subsequently selected, on the basis of FSC and SSC, and sorted. Cytospins were prepared for each sorted population and differential cell counts were performed using standard hemocytological criteria to classify the cells as neutrophils (NEUT), eosinophils (EOS), monocytes/macrophages (MONO/MACS) or lymphocytes (Lymph).

Figure 1S



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Figure 2S:

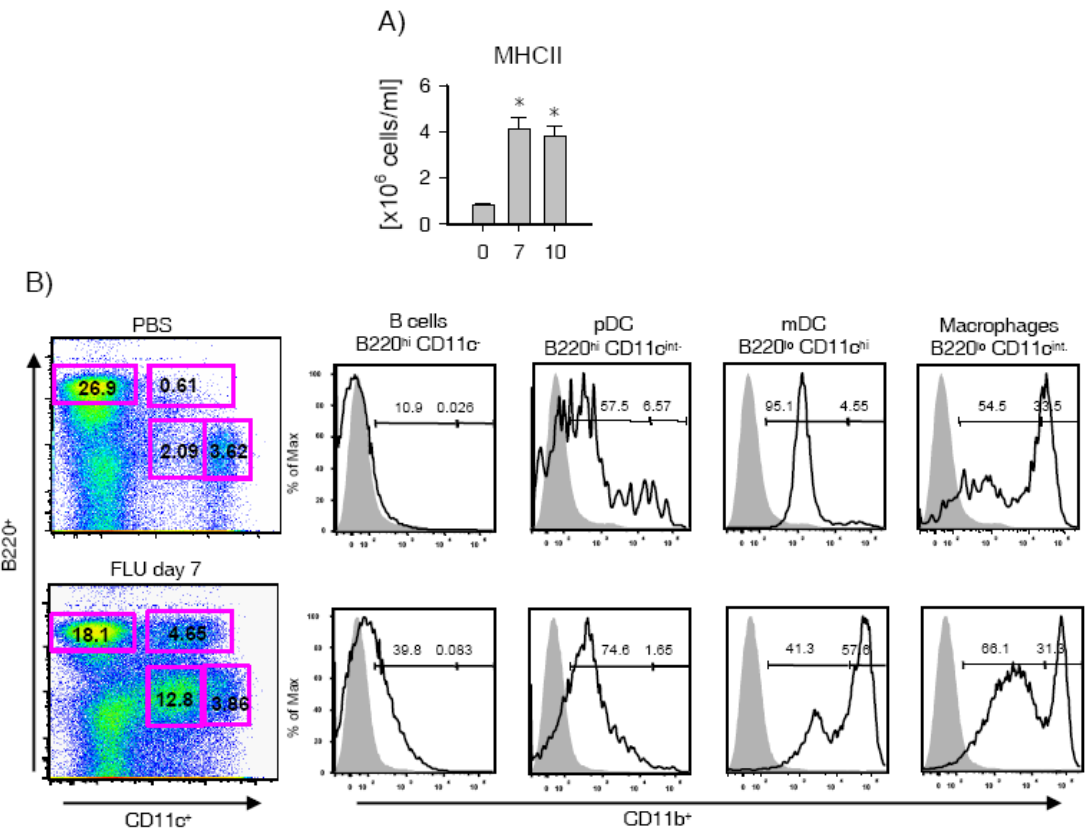
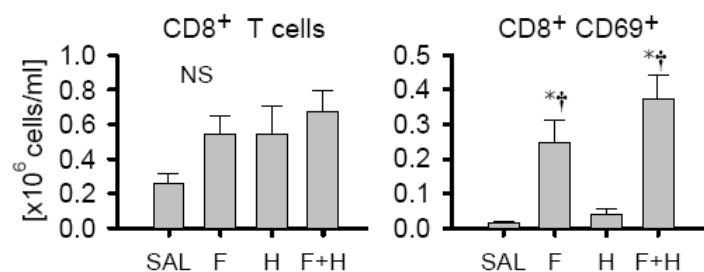


Figure 3S



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Figure 4S

