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Allergic airway inflammation is exacerbated during acute influenza infection and correlates with increased allergen presentation and recruitment of allergen-specific T-helper type 2 cells

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Summary

Background Respiratory viral infections are a leading cause of the hospitalization of asthmatics, however, the cellular immunological interactions which underlie these two diseases remain elusive.

Objective We sought to characterize the effect influenza viral infection has on allergic airway inflammation and to identify the cellular pathways involved.

Methods We have used an ovalbumin (OVA) model of allergic airway inflammation, which involves sensitization of animals with OVA adsorbed in alum adjuvant followed by an intranasal challenge with OVA in phosphate-buffered saline. To study T cell recruitment into the lung, we adoptively transferred *in vitro* activated T cell receptor-transgenic T cells, which were subsequently identified by fluorescence-activated cell sorting (FACS) analysis. In addition, to study *in vivo* dendritic cell (DC) migration, we administered fluorescently labelled dextran and identified DCs that had phagocytosed it by FACS analysis.

Results We found that different stages of influenza infection had contrasting effects upon the outcome of OVA-induced allergic airway inflammation. The allergic response against OVA was exacerbated during the acute stage of influenza infection; however, mice were protected against the development of airway eosinophilia at late time-points following infection. We investigated the mechanisms responsible for the virus-induced exacerbation and found that the response was partially independent of IL-4 and that there was increased delivery of inhaled allergens to the draining lymph node during the acute stage of the infection. In addition, virus-induced inflammation in the lung and draining lymph node resulted in the non-specific recruitment of circulating allergen-specific effector/memory cells.

Conclusion In addition to virus-mediated damage to the lung and airways, influenza viral infection can also enhance unrelated local allergic responses.

Keywords asthma, exacerbation, influenza, T cell, virus

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Introduction

Clinical and epidemiological investigations have yielded conflicting results regarding the effect of respiratory viral infections such as influenza virus, respiratory syncytial virus (RSV) and rhinovirus on asthma. Although many investigations have shown that viral infections can both increase the development [1–4] and exacerbation [5–7] of atopic asthma, recent epidemiological studies by Illi et al. [8] have shown that certain types of viral infections are actually protective against the development of atopy. The paradigm of T-helper (Th) cell differentiation predicts that Th1 responses, such as those mounted against viral infections or intracellular bacteria, would suppress the development of subsequent asthma-inducing Th2 responses [9]. Indeed, a characteristic feature of virus-induced inflammation is the absence of Th2 cytokine-producing T cells, IgE antibodies and eosinophils, however, the Th cell paradigm alone is insufficient to explain the interactions between respiratory viral infections and asthma. Instead, the time of viral infection, patient history and viral and allergen load may all contribute to the complex interplay between viral infections and clinical asthma making investigations of the cellular immunological mechanisms that underlie these processes difficult.

Influenza virus causes an acute infection that is generally limited to the respiratory epithelium [10]. Infection results in the accumulation of neutrophils and virus-specific $CD4^+$ and $CD8^+$ T cell populations in the lung parenchyma and airways. $CD8^+$ T cells produce high titres of IFN-γ in response to influenza infection, and represent an integral aspect of the immune response as shown by a dramatically delayed clearance of the virus in mice treated with anti-IFN-γ antibodies

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[11, 12]. By contrast, allergic airway inflammation is characterized by an infiltrate, which only occurs in individuals who have been pre-sensitized to the specific allergens, and is comprised of $CD4^+$ Th2 cells, macrophages, and large numbers of eosinophils [13, 14]. The activity of these inflammatory cells results in swelling of the bronchioles, smooth muscle contraction, reversible airway obstruction and mucus production [15] over a period of 4–6 days. In chronically exposed individuals, the airways become fibrotic and there is smooth muscle hypertrophy with resulting increases in airway hypersensitivity [16].

The experiments detailed in this paper were designed to identify the cellular immunological mechanisms key to respiratory viral infection-induced exacerbation of atopic asthma. We found that allergen-sensitized mice exhibited a greatly exacerbated allergic airway inflammation when concurrently infected with influenza virus. An important feature of this viral infection induced exacerbation was that it only occurred within a 4–14 day window following viral infection. Thereafter, prior viral infection was found to markedly suppress allergic airway inflammation. The exacerbation of allergen-specific responses in mice previously infected with influenza virus was characterized by a greatly increased number of eosinophils recruited into the airways, increased airway hyper-responsiveness (AHR) and an increase in the number of IL-4-producing Th2 cells within the airways. Furthermore, concurrent influenza infection increased migration of allergen-carrying dendritic cells (DCs) to the draining lymph node and the migration of allergen-specific effector/memory Th2 cells into the airways, even in the absence of inhaled allergen. We also noted the surprising finding that the exacerbated allergic response is not solely dependent on IL-4, although this cytokine is still clearly important for the development of Th2 responses in the airways. Taken together, these data provide further clarity to the influence of viral infections on allergic individuals, and highlight some of the key immunological pathways associated with virus-induced exacerbation of allergic airway inflammation.

Materials and methods

Mice

C57BL/6J mice were bred from mice originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice genetically deficient in IL-4 were bred from mice generated by Professor Manfred Kopf (Molecular Biomedicine, ETH, Zürich, Switzerland). OT-II mice were backcrossed to C57BL/6 mice and maintained by breeding males to OT-II females. The transgenic T cell receptor (TCR) was derived from a $CD4^+$ T cell hybridoma, clone 1.1, specific for the ovalbumin (OVA) peptide fragment 323–339 in the context of MHC class II $I\text{-}A^b$ molecule and was kindly provided by Dr F. R. Carbone (Department of Pathology and Immunology, Monash Medical School, Victoria, Australia). All mice were maintained at the Biomedical Research Unit of the Wellington School of Medicine (Wellington, New Zealand). Animals used in the experiments were between 6 and 10 weeks of age. All animal experimental procedures were approved by the Wellington School of Medicine Animal Ethics Committee and conducted in accordance with the guidelines of the University of Otago (Dunedin, New Zealand).

Infection with influenza virus

The A/HKx31 (H3N2) influenza A virus is a laboratory-generated recombinant with external surface components of A/Aichi/2/68 (H3N2) and internal components of A/PR8/8/34 (H1N1). Virus stocks were grown in the allantoic cavities of 10-day-old embryonated chicken eggs and stored as infectious allantoic fluid at $-80\,^{\circ}C$. Virus stock was shown to be endotoxin free by the *Limulus* amebocyte lysate assay (Sigma, St Louis, MO, USA). Virus titre was determined by agglutination of human erythrocytes and is expressed as haemagglutinating units (HAU). On day 0 mice were anaesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine (Phoenix, Auckland, New Zealand), and 12 HAU of virus in a 30-μL volume of phosphate-buffered saline (PBS) was administered by intranasal (i.n.) inoculation.

OVA-induced airway inflammation

Mice were injected i.p. with 2 μg OVA in 200 μL alum adjuvant (SERVA, Electrophoresis GmbH, Heidelberg, Germany) on days 1 and 14. Seven days later, mice were infected with either 12 HAU influenza virus in a volume of 30 μL or mock-infected with PBS. After the time-point indicated, mice were anaesthetized and 100 μg of OVA in a 50 μL volume of PBS was administered i.n. At the indicated time-points, mice were examined for AHR or killed and bronchoalveolar lavage (BAL) fluid and tissues taken for analysis.

Quantitative and qualitative measurement of inflammatory cells in the BAL

At the indicated time-points post-OVA i.n. challenge, mice were killed with a lethal dose of anaesthetic. The trachea was cannulated and BAL performed by flushing the airways three times with 1 mL PBS. BAL cells were counted and spun onto glass slides using a Cytospin 2 (Shandon Southern Products Ltd, Astmoor, UK). Slides were stained with Diff-Quik (Dade International, Düdingen, Switzerland) according to the manufacturer's instructions. Percentages of macrophages, lymphocytes, neutrophils and eosinophils were determined microscopically by counting 200 cells/sample using standard morphological and cytochemical criteria. Total numbers of eosinophils were derived by multiplying the total number of leucocytes counted with a Neubauer haemocytometer (Assistent, Sondheim, Germany) by the percentage of eosinophils identified morphologically.

Measurement of AHR

On day 4 post-i.n. challenge with OVA, mice were placed in individual unrestrained whole-body plethysmograph chambers (Buxco Electronics Inc., Petersfield, UK). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methylcholine-chloride (MetCh) (Aldrich Chemie, Steinheim, Germany). This procedure estimates total pulmonary airflow in the upper and lower respiratory tracts. The chamber pressure was used as a measure of

the difference between thoracic expansion (or contraction) and air volume removed from (or added to) the chamber during inspiration (or expiration). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics Inc.). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh and monitoring the breathing pattern for 5 min after initiation of aerosol dose.

Activation of cells for cytokine analysis

On day 4 post-OVA i.n. challenge, mice were killed and BAL performed. The total number of cells in the BAL fluid was quantified. A total of 5×10^5 BAL cells/mL were cultured with 1×10^5 BMDC/mL (previously pulsed for 2 h with 1 mg/mL OVA protein) in a total volume of 200 μL in 96-well plates (Falcon, Becton Dickinson, Mountain View, CA, USA) for 6 h at 37 °C. Brefeldin A (Sigma) was added for the final 2 h of culture. Cells were fixed in 2% paraformaldyhyde and intracellular cytokine staining performed as described previously [17].

Administration of fluorescein isothiocyanate (FITC)–Dextran and in vivo *DC tracking*

Mice were infected with influenza as described previously. At day 10 post-infection, 50 μL of a 2 mg/mL solution of FITC–Dextran (Molecular Probes, Eugene, OR, USA) diluted in PBS was administered i.n. At the indicated time-points, a single cell suspension was made from the draining mediastinal lymph node (MLN) and the percentages of $CD11c^{high}$, MHC II^{high}, FITC+ cells were determined. Total numbers were derived from the proportion of FITC+ DCs as determined by fluorescence-activated cell sorting (FACS) analysis, multiplied by the total number of cells isolated from the MLN.

OVA-specific $CD4^+$ TCR-transgenic T cell adoptive transfer model

Cell suspensions were prepared from lymph nodes of OVA-specific TCR-transgenic OT-II mice. For generation of Th2 effector cells, lymphocytes were cultured at a concentration of 2×10^6 cells/mL in a total volume of 1 mL per well in 24-well plates (Falcon, Becton Dickinson) in the presence of $OVA_{323–339}$ peptide and 1000 U/mL recombinant mouse IL-4 supernatant, 20 U/mL recombinant human IL-2 (IL-2L6 supernatant) and 10 ng/mL recombinant human IL-6. At the end of the culture the percentage of Vα2/Vβ5.1/5.2, TCR-expressing lymphocytes from the *in vitro* Th2 cultures was determined by FACS analysis. A total of 5×10^6 $V\alpha2^+V\beta5.1/5.2^+$ Th2 cells were injected into the tail vein of sex-matched naïve C57BL/6 mice in a total volume of 500 μL of Iscove's Modified Dulbecco's Medium. Recipient mice were immediately infected with influenza virus or mock-infected with PBS. Mice were killed 10 days later and the indicated tissues removed for FACS analysis.

FACS analysis

Anti-FcγRII (2.4G2) Ab was affinity purified from tissue culture supernatants using protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden). Anti-CD4, anti-Vα2, anti-Vβ5.1/5.2, anti-CD11c, anti-I-A^b and anti-IL-4 were obtained from PharMingen (San Diego, CA, USA). Cells were stained in PBS containing 2% fetal calf serum and 0.01% sodium azide and analysed on a FACSort using Cellquest software (Becton Dickinson).

Statistics

Statistical significance was analysed by Student's *t*-test. Unless otherwise indicated data represents mean ± standard deviation with data giving *P*-values <0.05 being considered statistically significant.

Results

Allergic airway inflammation is exacerbated during the acute stages of an influenza infection, but is suppressed following resolution of infection

In this study, OVA protein was used as a model allergen to induce allergic airway inflammation. Mice were sensitized to OVA by two i.p. injections of OVA adsorbed on alum adjuvant at days 0 and 14. One week later, mice were inoculated i.n. with influenza virus. On days 0, 8, 14, 21, 25 and 32 post-influenza or PBS mock infection, previously OVA-sensitized mice were challenged i.n. with OVA protein. We have previously established that the peak of eosinophil infiltration into the airways occurs 4–6 days following OVA challenge (data not shown); accordingly we enumerated the number of eosinophils in the airways 4 days after each OVA challenge (i.e. days 4, 12, 18, 25, 29 and 36 post-influenza infection). Figure 1 shows that at early time-points following infection, the infiltration of eosinophils into the airways was dramatically exacerbated as compared with similarly OVA-sensitized and challenged, but mock-infected mice. Importantly, these data show that the allergen-induced response is still characteristic of a Th2 immune response as the migration of eosinophils is dependent on the Th2 cytokine IL-5 [18, 19]. In striking contrast, at late time-points following influenza infection the infiltration of eosinophils into the airways was suppressed as compared with mock-infected controls. Variation between experiments in the degree of influenza infection resulted in a window lasting between 10 and 14 days post-infection where the allergic response would be exacerbated. We conclude that the exacerbation is only evident during the acute infection and is not long lasting; accordingly, the experiments in the following sections were performed between 10 and 14 days post-infection. We have shown previously that the cellular mechanism underlying the influenza-induced suppression of allergic airway inflammation involves IFN-γ production by effector/memory $CD8^+$ T cells that remain in the airways following influenza infection [20].

Influenza infection affects the magnitude but not the kinetics of allergic airway inflammation

To further characterize the influenza-induced exacerbation of airway eosinophilia, we investigated whether influenza infection also altered the duration of the eosinophil response. OVA-sensitized mice were infected with influenza virus or

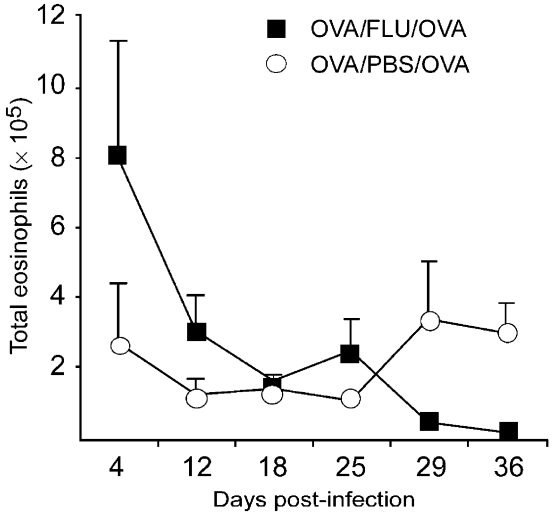


Fig. 1. Allergic airway eosinophilia is exacerbated at early time-points and suppressed at late time-points following influenza (FLU) infection. Mice were sensitized to ovalbumin (OVA) protein by intraperitoneal (i.p.) immunization on days 1 and 14. Seven days later, these OVA-sensitized mice were inoculated i.n. with either influenza virus or phosphate-buffered saline (PBS). On day 0, 8, 14, 21, 25 or 32 post-influenza or PBS inoculation, three to four mice per group were challenged i.n. with OVA protein in PBS. Four days following i.n. OVA challenge bronchoalveolar lavage was performed (i.e. days 4, 12, 18, 25, 29 and 36) and the total number of eosinophils determined by cell counts and morphological differentiation. Data represents the mean total number of eosinophils detected in the airways ± standard deviation. This experiment was repeated twice with comparable results using three to four mice per group, per time-point examined.

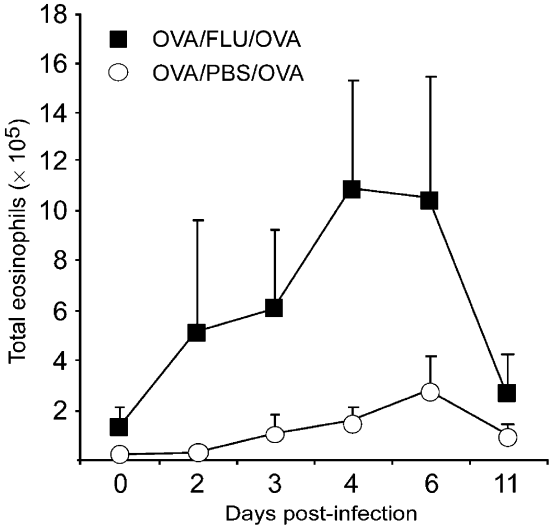


Fig. 2. Influenza (FLU) infection increases the total number of airway eosinophils, but does not affect the kinetics of eosinophil infiltration. Mice were sensitized to ovalbumin (OVA) protein by intraperitoneal (i.p.) immunization on days 1 and 14. Seven days later, these OVA-sensitized mice were inoculated intranasally (i.n.) with either influenza virus or phosphate-buffered saline (PBS). Fourteen days later, all mice received an OVA i.n. challenge. Immediately post-OVA i.n. challenge, or on days 2, 3, 4, 6 or 11 post-OVA challenge, bronchoalveolar lavage was performed and the total number of eosinophils determined by cell counts and morphological differentiation. Data represents the mean total number of eosinophils detected in the airways ± standard deviation. This experiment was repeated twice with comparable results using three to four mice per group, per time-point examined.

mock infected with PBS. Fourteen days later, mice were challenged i.n. with OVA and the number of eosinophils that had migrated into the airways was quantified at the indicated time-points (Fig. 2). The infiltration of eosinophils into the airways peaked between days 4 and 6 post-OVA challenge and returned to baseline levels by day 11, irrespective of prior influenza or mock infection. However, in agreement with Fig. 1, the total number of eosinophils in the airways was greater in influenza-infected mice as compared with mock-infected, but similarly challenged mice. These data show that influenza infection causes a profound exacerbation of OVA-induced allergic airway inflammation, without altering its intrinsic kinetic characteristics.

Influenza infection exacerbates allergen-induced AHR and increases the number of IL-4-producing CD4$^+$ T cells in the airways

We sought to ascertain whether other hallmark features of allergic airway inflammation were increased, similar to the infiltration of eosinophils. Four days following OVA challenge, mice were placed in a whole-body unrestrained plethysmograph and exposed to increasing concentrations of aerosolized MetCh. OVA-sensitized and challenged mice, which had a concurrent influenza infection, exhibited an increased AHR as compared with uninfected but similarly OVA-challenged control mice (Fig. 3a). Mice which were OVA-sensitized and infected with influenza, but did not receive an OVA i.n. challenge, did not exhibit AHR to increasing doses of MetCh indicating that the increased AHR was due to the exacerbated allergic response rather than virus-mediated AHR (Fig. 3a). We next isolated lymphocytes from the airways to quantify the number of Th2 cells present. To specifically identify the CD4$^+$ T cells responding to OVA, rather than influenza, we cultured the isolated cells with OVA-pulsed bone-marrow-derived DCs for 6 h and then determined the number of IL-4-producing CD4$^+$ T cells by intracellular staining and flow cytometry. In line with the data presented above, the total number of OVA-specific CD4$^+$ T cells-producing IL-4 was increased in mice that had a concurrent influenza infection, as compared with uninfected, but similarly OVA-sensitized and challenged mice (Fig. 3b).

Influenza-induced exacerbation of allergic airway inflammation is partially independent of IL-4 production

We examined mice genetically deficient for the cytokine IL-4 to determine whether this prototypic Th2 cytokine was essential for influenza-induced exacerbation of allergic airway inflammation. OVA-sensitized wild-type C57BL/6 mice and IL-4-deficient mice were infected with influenza or mock-infected with PBS. Fourteen days post-infection, mice were challenged i.n. with OVA and 4 days later the total number of eosinophils that had infiltrated into the airways was examined. As expected, influenza infection exacerbated the OVA-induced airway eosinophilia in wild-type mice in comparison with mock-infected wild-type controls (Fig. 4). IL-4 is critical for the development of an OVA-induced airway eosinophilia as highlighted by the absence of eosinophils in OVA-sensitized and challenged, but mock-infected IL-4-deficient mice (Fig. 4). However, significant numbers of eosinophils were detected in the airways of influenza-infected OVA-sensitized and challenged, IL-4-defi-

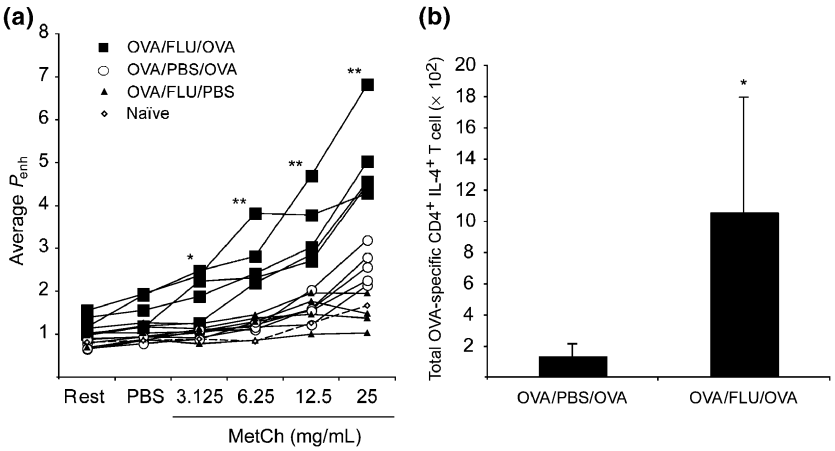


Fig. 3. Influenza (FLU) infection exacerbates allergen-induced airway hyper-responsiveness (AHR) and increases the total number of IL-4-producing allergen-specific $CD4^+$ T cells in the airways. Mice were sensitized to ovalbumin (OVA) protein by intraperitoneal (i.p.) immunization on days 1 and 14. Seven days later, these OVA-sensitized mice were inoculated intranasally (i.n.) with either influenza virus or phosphate-buffered saline (PBS). On day 10 post-infection, mice were challenged i.n. with either OVA protein or PBS alone. Four days following i.n. challenge, (a) AHR to increasing doses of aerosolized methylcholine-chloride (MetCh) was measured using a whole-body unrestrained plethysmograph, (b) bronchoalveolar lavage was performed and isolated lymphocytes were cultured with DCs pulsed with OVA protein for 6 h. The total number of OVA-specific IL-4-producing T cells was determined by flow cytometry and total cell counts. Data represents (a) individual mice or (b) mean total number of IL-4-producing $CD4^+$ T cells in the airways ± standard deviation. Statistical significance determined by Student's *t*-test between OVA/FLU/OVA and OVA/PBS/OVA groups is designated by asterisk(s) (**$P<0.005$; *$P<0.05$). This experiment was repeated with comparable results using four to five mice per group.

cient mice. The number of eosinophils was reduced compared with that seen in influenza-infected wild-type mice, but was comparable with that seen following OVA sensitization and challenge of mock-infected wild-type mice. This indicates that the exacerbation of airway eosinophilia by influenza infection of wild-type mice occurs through both IL-4-dependent and IL-4-independent mechanisms. However, the IL-4-independent mechanism appears only to be possible within the pro-inflammatory environment induced by concurrent influenza infection.

Influenza infection increases the total number of allergen-carrying DCs that migrate to the draining lymph node following allergen inhalation

We next set out to determine the key cellular pathways responsible for the increased allergic airway eosinophilia observed during influenza infection. One possible mechanism was increased presentation of allergen following viral infection. We therefore designed an experiment to examine whether there was increased allergen uptake by DCs resident in the lung and draining lymph node of influenza-infected mice. Mice were either infected with influenza or mock-infected with PBS, and 10 days later given FITC–Dextran i.n. as a model allergen. Draining MLN cells were isolated at the indicated time-points and DCs (identified as $CD11c^{high}$/MHC II^{high} cells) were analysed by flow cytometry for FITC fluorescence. At each time-point examined, a 3–4-fold increased total number of DC containing FITC–Dextran were detected in the MLN of mice that had previously been infected with influenza, in comparison with mock-infected controls (Fig. 5). The infiltration of DC into the MLN appeared to peak around 25 h post-i.n. challenge. These data indicate that during an influenza infection, the presentation of inhaled allergens is increased in the MLN due to a striking increase in DC migration. This viral-induced increase in allergen uptake is likely to result in an increase in allergen presentation and

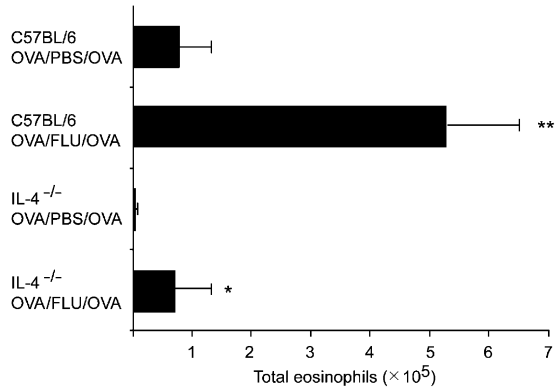


Fig. 4. Influenza (FLU)-induced exacerbation of allergic airway eosinophilia involves both IL-4-dependent and IL-4-independent pathways. Both wild-type and IL-4-deficient mice were sensitized to ovalbumin (OVA) protein by intraperitoneal (i.p.) immunization on days 1 and 14. Seven days later, these OVA-sensitized mice were inoculated intranasally (i.n.) with either influenza virus or phosphate-buffered saline (PBS). Ten days following infection, all mice received an OVA i.n. challenge and 4 days later bronchoalveolar lavage was performed and the total number of eosinophils determined by cell counts and morphological differentiation. Data represents the mean total number of eosinophils detected in the airways ± standard deviation. Statistical significance determined by Student's *t*-test between C57BL/6 OVA/FLU/OVA and C57BL/6 OVA/PBS/OVA or IL-$4^{-/-}$ OVA/FLU/OVA and IL-$4^{-/-}$ OVA/PBS/OVA groups is designated by asterisk(s) (**$P<0.005$; *$P<0.05$). This experiment was repeated twice with comparable results using three to four mice per group.

T cell activation, and thus be at least partially responsible for the observed exacerbation of allergic airway inflammation.

Influenza infection induces the non-specific migration of allergen-specific Th2 cells into the airways and MLN

Pre-sensitized allergic individuals have existing allergen-specific effector/memory cells present in the circulation and these cells are recruited into the lung and airways immediately following inhalation of allergen [21, 22]. To determine

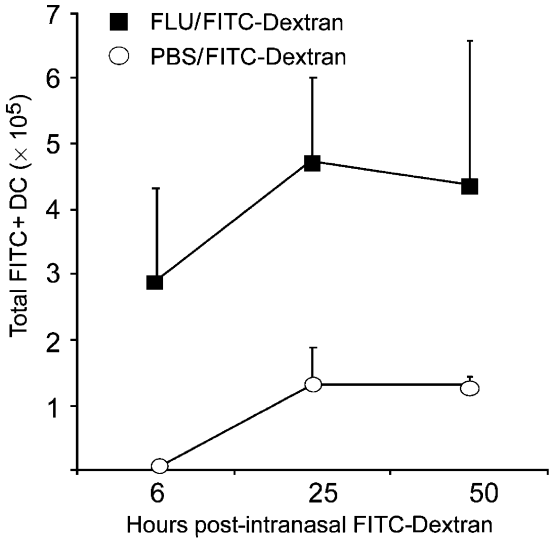


Fig. 5. Concurrent influenza (FLU) infection during allergen challenge increases the number of allergen-carrying DCs that migrate to the draining mediastinal lymph node (MLN). Naïve mice were infected with influenza virus or mock-infected with phosphate-buffered saline (PBS). Ten days after infection, mice were challenged intranasally (i.n.) with fluorescein isothiocyanate (FITC)–Dextran, and either 6, 25 or 50 h later the draining MLN was removed and the number of FITC+ DCs quantified. DCs were identified as cells staining positive for CD11c and high MHC II expression. Data represents mean total number of FITC+ DCs ± standard deviation at each time-point investigated. This experiment was repeated twice with comparable results using three to four mice per group, per time-point examined.

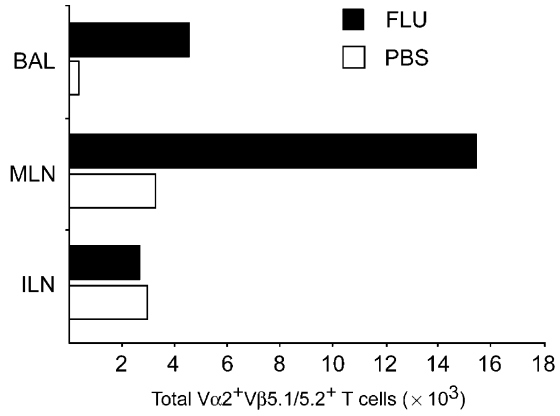


Fig. 6. Influenza (FLU) infection causes the recruitment of allergen-specific effector/memory T cells into the airways and draining lymph node, in the absence of specific allergen. Naïve mice were injected intravenously (i.v.) with 5×10^6 $V\alpha2^+V\beta5.1/5.2^+$ *in vitro* cultured T-helper type 2 (Th2) cells, then immediately inoculated intranasally (i.n.) with influenza virus or phosphate-buffered saline (PBS). Ten days following adoptive transfer and infection, bronchoalveolar lavages (BALs) were performed and draining mediastinal lymph nodes (MLNs) and non-draining inguinal lymph nodes (ILNs) removed. Total numbers of infiltrating $V\alpha2^+V\beta5.1/5.2^+$ T cells were determined by cell counts and flow cytometry. Data is from pooled samples and corrected to represent the total number of $V\alpha2^+V\beta5.1/5.2^+$ T cells detected in individual mice. This experiment has been repeated twice using four to five mice per group.

whether influenza infection also induces recruitment of such allergen-specific cells into the airways, we intravenously (i.v.) injected *in vitro* activated TCR-transgenic Th2 cells from OT-II mice, and immediately infected recipient mice with influenza or mock-infected with PBS. Ten days later, the cellular infiltrate within the airways, draining MLN and non-draining inguinal lymph node (ILN) were examined and the total number of TCR-transgenic Th2 cells was determined by flow cytometry. TCR-transgenic Th2 cells were detected in the airways of recipient mice that had been infected with influenza virus while only minimal numbers of these cells were present in the airways of mock-infected mice (Fig. 6). A similar trend was evident in the draining MLN where an increased number of TCR-transgenic Th2 cells were evident in influenza-infected mice. No difference in TCR-transgenic Th2 cell number was apparent in the ILN indicating the increased recruitment of circulating effector/memory cells was limited to the sites of virus-induced inflammation. These data provide evidence that circulating effector/memory allergen-specific Th2 cells are recruited to the airways during influenza infection, even in the absence of their specific antigen. Thus, allergen inhalation during a respiratory viral infection would be expected to result in a greater allergic airway response due to the increased frequency of allergen-specific T cells present at this site.

Discussion

It has long been established that respiratory viral infections can exacerbate asthma [5–7]. In the current study, we have used a murine model of allergic airway inflammation to elucidate key cellular mechanisms involved in this phenomenon. Specifically, we have shown that the stage of influenza infection determines the intensity and nature of concurrent allergic immune responses against inhaled allergens. In agreement with clinical observations, allergen challenge of sensitized mice during the acute phase of viral infection resulted in a marked exacerbation of allergic airway inflammation and hyper-responsiveness.

Due to the significant clinical impact of viral-induced exacerbation of asthma, we proceeded to determine the key cellular mechanisms involved. We found that while influenza infection increased the intensity of the allergic response, it did not affect the kinetics of the allergen-induced eosinophilia. This data indicates that the exacerbation is antigen driven and that the mechanism is not solely 'leakiness' of an inflamed tissue, but rather, a specific enhancement of the allergic response. Allergen-induced AHR was also exacerbated during the influenza infection. This AHR was due to an enhanced Th2 response as OVA-sensitized mice that were infected with influenza but not challenged with OVA did not exhibit AHR. We also found an increased number of IL-4-producing $CD4^+$ T cells in the airways of OVA-challenged mice concurrently infected with influenza, as compared with mice mock-infected with PBS. However, we did not find any evidence of IL-4 production by influenza-specific $CD8^+$ cells indicating that the Th2 response did not influence the cytokine polarization of cells responding to the virus (data not shown). Similar data have previously been published showing that IL-4-transgenic mice did not develop IL-4- or IL-5-producing $CD8^+$ T cells *in vivo* [23], although such cells can be produced *in vitro* [23, 24].

Since the influenza-induced exacerbation of allergic airway inflammation appeared to be allergen specific, we hypothesized that influenza infection may result in increased presentation of inhaled allergens by DCs in the draining lymph node. To address this question, we administered a

model allergen, FITC–Dextran, i.n. to both influenza-infected and mock-infected control mice. This model provided us with a means of tracking the number of DCs that had taken up the inhaled allergen and migrated to the draining lymph node. At each time-point examined, an increased number of FITC–Dextran+ DCs were found in the draining lymph node of influenza-infected mice in comparison with uninfected controls. Presumably influenza infection results in the production of pro-inflammatory cytokines that increase activation signals for DCs to take up antigen and migrate to the draining lymph node. Clearly, these signals not only increase viral antigen presentation, but also boost responses against any antigen encountered at the site of inflammation, including allergens. A similar mechanism of enhanced antigen presentation has been associated with the development of some autoimmune diseases [25] and the breaking of tolerance to aeroallergens [26].

When a tissue is inflamed, increased adhesion molecules and chemokine gradients allow the migration of effector/memory cells to this site [27, 28]. Since allergic individuals have a preponderance of effector/memory allergen-specific cells, we speculated that another mechanism for the enhanced allergen response during influenza infection might be the increased recruitment of allergen-specific memory/effector cells into the airways by signals induced by viral infection alone. To test this possibility, we adoptively transferred *in vitro* cultured TCR-transgenic Th2 cells of known antigen specificity into influenza- or mock-infected mice and tracked their migration. We found that these cells were in fact specifically recruited to the airways and draining lymph node during influenza infection, but did not migrate to these tissues in the absence of inflammation. Upon activation following allergen challenge, this increased local population of allergen-specific cells is likely to participate in the exacerbation of allergic airway inflammation.

To our surprise, we found that IL-4 is not absolutely required for viral-induced exacerbation of allergic airway inflammation. In agreement with published results, mock-infected IL-4-deficient mice did not develop a robust allergen-induced airway eosinophilia [29]. However, OVA-challenged IL-4-deficient mice that were concurrently infected with influenza did develop an airway eosinophilia, at a level comparable to non-infected wild-type controls. Although the total number of eosinophils was reduced as compared with similarly infected and OVA-challenged wild-type mice, these data show that an IL-4-independent pathway for airway eosinophilia is induced during influenza infection. A possible candidate for mediating this IL-4-independent response is the cytokine IL-13 which is known to share some biological functions with IL-4 [30]. Increased local production of IL-13 following allergen challenge during a concurrent viral infection could potentially compensate for the deficiency of IL-4 [31, 32]. Of note, our data show that concurrent viral infections are capable of inducing an allergic airway response, which would otherwise not have developed, providing indirect support that viral infections may have a role in promotion of atopic development.

Taken together, the data presented here identifies three cellular mechanisms associated with viral-induced exacerbation of allergic airway inflammation: the enhancement of an IL-4-independent pathway of Th2 inflammation; the increased accumulation of allergen-carrying DC in the lung draining lymph node; and the non-specific recruitment of allergen-specific effector/memory cells into the airways during viral infection. In this manner, influenza not only causes inflammation and tissue damage during its infectious cycle, but the immune response against it also has the potential to enhance concurrent local allergic immune responses.

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