

Development of atopy by severe paramyxoviral infection in a mouse model

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Background: Atopic diseases have been increasing in prevalence, yet the initial inciting events that lead to atopy are not understood. Paramyxoviral infections have been suggested to play a role; however, much of these data are correlative.

Objective: To determine whether exposure to a nonviral antigen during a paramyxoviral infection is sufficient to drive IgE production against the bystander antigen and whether clinical disease against this antigen would result.

Methods: Wild-type C57BL/6 mice or mice deficient in *FcεRIα* (*FcεRIα*^{-/-}) or *IgE* (*IgE*^{-/-}) were inoculated with Sendai virus (SeV) or UV-inactivated SeV (UV-SeV) and subsequently exposed to ovalbumin (OVA) intranasally. Mice were further challenged 3 times with intranasal OVA on days 20 to 22 after inoculation with SeV, and airway hyperreactivity and mucous cell metaplasia were determined.

Results: Exposure to OVA during SeV infection led to significant OVA specific IgE production (median, 548 vs 0 ng/mL; *P* = .03; SeV vs UV-SeV). This induction of OVA specific IgE production depended on FcεRI because *FcεRIα*^{-/-} mice produced significantly less IgE (112 ng/mL; *P* = .03; vs wild-type mice). Furthermore, in wild-type mice OVA exposure and challenge significantly enhanced SeV-induced airway hyperreactivity and mucous cell metaplasia, but this failed to occur in either *FcεRIα*^{-/-} or *IgE*^{-/-} mice.

Conclusion: A single exposure to a bystander allergen during a paramyxoviral infection is sufficient to drive allergen specific IgE production in a partial FcεRI-dependent mechanism. These data begin to provide mechanistic insight into how viral infections might drive development of atopic disease.

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INTRODUCTION

Asthma and atopic diseases have been increasing in prevalence in westernized countries.¹ The reasons for this increase are unknown. In addition, the mechanisms that drive the development of atopic disease are unknown, although genetic and other hypotheses have been suggested.^{2,3} One association that has been well documented is that severe respiratory paramyxoviral infections early in life lead to greatly increased risk of asthma and atopic disease.^{4,5} We undertook these studies to see whether exposure to a nonviral antigen during a respiratory viral infection was sufficient to drive the development of atopic disease.

Sendai virus (SeV), the murine parainfluenza virus type 1, is a paramyxovirus and natural rodent pathogen. In a strain-dependent fashion, mice that survive a severe infection with SeV develop long-lasting interleukin 13 (IL-13)–dependent mucous cell metaplasia and airway hyperreactivity.⁶ We have

shown that the acute development of postviral atopic disease (occurring within the first 3 weeks after viral inoculation) depends on expression of the high-affinity receptor for IgE (FcεRI) on lung conventional dendritic cells (cDCs).⁷ Cross-linking of FcεRI by antiviral IgE led to production of CCL28, a chemokine that attracted T_H2 cells in an antigen nonspecific fashion.^{7,8} Although these initial observations were made in mice, we have shown that the cDC FcεRI-CCL28 axis appears operative in humans.⁹ Furthermore, it has been shown that expression of FcεRI on human peripheral blood cDCs is increased by respiratory viral infection.¹⁰ Therefore, although not completely validated in the human, critical portions of the cDC FcεRI-CCL28 pathway appear intact in both mice and humans.

We also have shown that during SeV infection the resident lung cDCs become mature and activated, which suggests that they could present antigen to passing T cells.¹¹ Therefore, we hypothesized that the mature and activated cDCs would be able to present nonviral bystander antigen to T_H2 cells that had been recruited in an antigen nonspecific fashion during the infection. Furthermore, this would lead to an atopic response against the nonviral antigen, characterized by specific IgE production, increased mucous cell metaplasia, and airway hyperreactivity. Therefore, we undertook these studies to test this hypothesis and see whether mice exposed to ovalbumin (OVA) once during SeV infection would make IgE against OVA without the addition of an adjuvant and, if they were able to make IgE, whether a subsequent exposure to the antigen would lead to enhanced

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airway hyperreactivity and mucous cell metaplasia (i.e., clinical disease in the mouse model).

We now report that a single exposure to a nonviral antigen during an active antiviral immune response is sufficient to drive significant and marked IgE production against the nonviral antigen. We also show that generation of IgE is dependent, at least partly, on expression of FcεRI. Moreover, postviral airway hyperreactivity and mucous cell metaplasia were augmented in wild-type mice exposed to and subsequently challenged by the nonviral antigen. Interestingly, although the standard mouse OVA asthma model does not appear to be fully dependent on IgE, the augmented disease seen in our model is dependent on both IgE and FcεRI.^{12,13} These results have important implications for how atopic disease might develop and provide direction for future investigations into the development of postviral atopic disease.

METHODS

Mouse Generation and Handling

C57BL6 mice were obtained from the Jackson Laboratory. Mice deficient in FcεRIα (*FcεRIα*^{-/-}) on a C57BL6 background were a gift of J.P. Kinet (Harvard Medical School, Boston, Massachusetts). IgE-deficient mice (*IgE*^{-/-}) on a C57BL6 background were generated by backcrossing *IgE*^{-/-} Balb/c mice (gift of H. Oettgen, Harvard Medical School) 10 or more generations into C57BL6 mice. Mice 6 to 20 weeks old were used for all experiments. Mice were housed and handled and experiments performed according to protocols approved by the Institutional Animal Care and Use Committee.

Some mice received 2 intraperitoneal injections of 20 μL of 1 mg/mL OVA plus alum (1:1) 1 week apart, followed by an intranasal challenge with OVA on day 14 as described (Figure 1A).¹⁴ Other mice were inoculated intranasally with 2 × 10⁵ PFU of SeV (strain 52; American Type Culture Collection) or UV-inactivated SeV (UV-SeV), followed 8 days later by a single intranasal OVA administration (15 μL of a 2-mg/mL solution; Figure 1B). Mice in all groups were monitored daily for weight and activity. Twenty-one days after SeV inoculation or initial intraperitoneal OVA and alum administration, OVA specific IgE was determined in the serum by standard enzyme-linked immunosorbent assay (ELISA). In some mice, intranasal OVA (15 μL of 2-mg/mL solution) was administered daily on days 20, 21, and 22. On day 23, airway resistance was measured and mice were euthanized for evaluation of mucous cell metaplasia (Figure 1C). We limited our experiments to day 23 so that our findings would not be confused by the chronic alternatively activated macrophage disease that develops by 7 weeks after inoculation in C57BL6 mice.¹⁵

Measurement of IgE

Standard ELISA was performed as previously reported.⁷ Briefly, 96-well MaxiSorp (Nalge Nunc) plates were coated overnight with OVA. After being washed, mouse sera was added to the wells, and OVA specific IgE was determined using a detection anti-IgE antibody (BD Biosciences Phar-

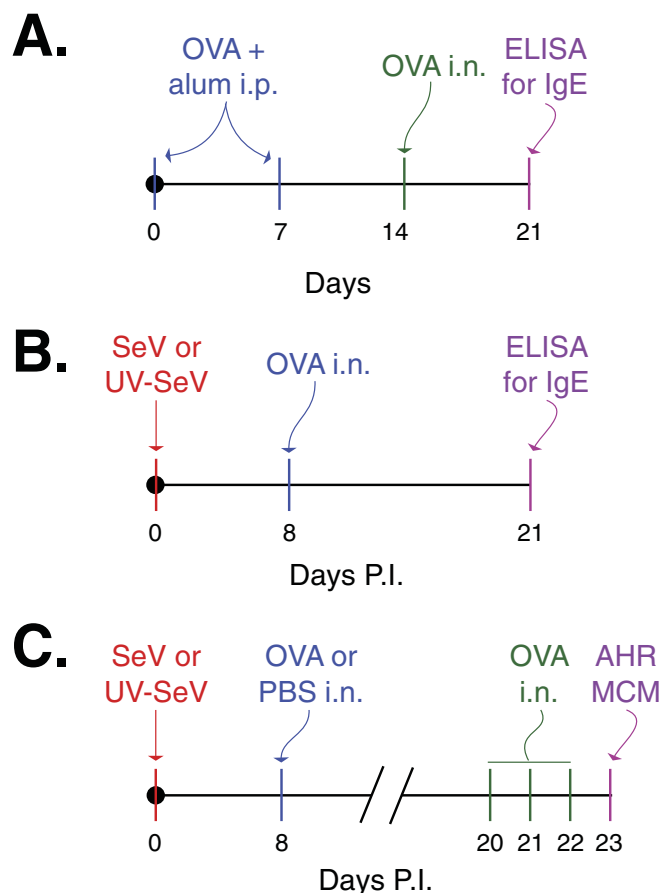


Figure 1. Study design. The generation of ovalbumin (OVA) specific IgE was assessed after 2 different sensitization or challenge models. A, The traditional mouse model that involves 2 intraperitoneal (i.p.) injections of OVA plus alum 1 week apart, followed by an intranasal (i.n.) challenge with OVA on day 14.¹⁴ In this model, OVA specific IgE in the serum is determined 1 week later by enzyme-linked immunosorbent assay (ELISA). B, To determine whether exposure to a nonviral antigen (OVA) during a viral infection leads to IgE production against the nonviral antigen, mice were inoculated with Sendai virus (SeV) or UV-inactivated SeV (UV-SeV). Eight days later, OVA was administered intranasally. On day 21 post inoculation (P.I.), OVA specific IgE was determined in the serum using ELISA. C, To see if this anti-OVA IgE was sufficient to induce allergic airway disease, mice that were treated as in B were challenged with i.n. OVA on P.I. days 20, 21, and 22 and airway hyperreactivity (AHR) and mucous cell metaplasia (MCM) assessed the following day.

mingen, San Diego, California). Purified mouse OVA specific IgE (clone 2C6; AbD Serotec, Raleigh, North Carolina) was used to construct a standard curve to calculate concentration of the anti-OVA IgE.

Histochemistry

Mouse lung (at 25 cm H₂O of pressure) was fixed in 10% zinc formalin and processed by the Medical College of Wisconsin histology core for standard hematoxylin-eosin and periodic acid-Schiff (PAS) staining. Quantification of mucous cells

was performed by a blinded observer counting the number of PAS-positive cells per millimeter of basement membrane using Image-J software (National Institutes of Health, Bethesda, Maryland) as previously reported.⁷

Real-time PCR Assay

Messenger RNA (mRNA) was isolated with Trizol (Sigma-Aldrich, St Louis, Missouri) from which complementary DNA was generated with the QuantiTect reverse transcription kit (Qiagen, Valencia, California) as per the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) assays were performed using TaqMan fast master mix and the StepOnePlus PCR system (Applied Biosystems, Carlsbad, California). All quantitative PCR data are presented as *Muc5ac* copy number normalized to *GAPDH* copy number. *GAPDH* and *Muc5ac* primer probe sets were obtained as previously reported.⁷

Airway Reactivity Measurements

Invasive measurement of airway hyperreactivity (resistance) after exposure to nebulized vehicle (phosphate-buffered saline [PBS]) or doubling concentrations of methacholine (0.625–10 mg/mL) was performed using the flexiVent system per the manufacturer's instructions (Scireq, Montreal, Quebec, Canada).

Statistical Analyses

Unless otherwise stated, all data are presented as mean (SEM). The *t* test was used to assess the statistical significance of differences between 2 means for data that are normally distributed. The Mann-Whitney test was used for comparison of 2 medians for data that are clearly not normally distributed. For comparison of airway hyperreactivity data, repeated-measures analysis of variance with the Tukey multiple comparison test was used. In all cases, significance was set at $P < .05$.

RESULTS

A Single Exposure to a Nonviral Antigen During a Severe Paramyxoviral Respiratory Infection is Sufficient to Drive Specific IgE Production Against the Nonviral Antigen

We first examined whether a single intranasal exposure to a nonviral antigen during an SeV infection was sufficient to drive IgE production against this nonviral antigen (see Figure 1A and B for design). Wild-type mice were inoculated with SeV, and on day 8 after inoculation, when viral-induced upregulation of FcεRI on lung cDCs is well developed, a single dose of OVA was administered intranasally. This single exposure to OVA without an adjuvant was sufficient to produce significant amounts of specific IgE against OVA when compared with mice inoculated with UV-SeV before receiving OVA intranasally on day 8 after viral inoculation (median [interquartile range], 548 [317–1470] vs 0 [0–133] ng/mL; $P = .03$; Figure 2). The level of anti-OVA IgE produced was actually greater than that seen with the traditional 2 intraperitoneal injections of OVA and alum, followed

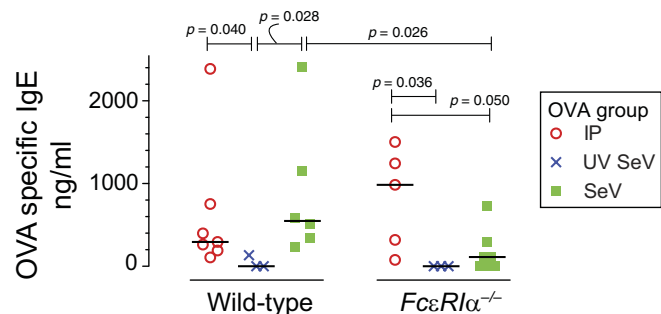


Figure 2. Nonviral antigen exposure during a severe paramyxoviral infection drives specific IgE production and is partially dependent on FcεRI. Wild-type mice or mice deficient in the high-affinity receptor for IgE, *FcεRIα*^{-/-}, were treated as indicated in Figure 1A and B. The concentrations of serum anti-ovalbumin (OVA) IgE were analyzed and individual values are shown. IP (intraperitoneal) indicates the design in Figure 1A; SeV (Sendai virus) and UV SeV (UV-inactivated SeV) refer to the design in Figure 1B. The number of mice per group are as follows: wild-type, IP = 7, UV SeV = 3, and SeV = 6; *FcεRIα*^{-/-}, IP = 5, UV SeV = 3, and SeV = 7. Bar indicates median; significant and trending *P* values are shown (Mann-Whitney test).

by intranasal OVA exposure (see Figure 1A for design and Figure 2 for results).

Development of IgE Against a Nonviral Antigen During a Paramyxoviral Infection is Dependent on FcεRI Expression

When we performed the same experiment using mice genetically deficient in the high-affinity receptor for IgE (*FcεRIα*^{-/-} mice), we saw a significant reduction in the level of OVA specific IgE produced (Figure 2). In fact, only 2 of 5 *FcεRIα*^{-/-} mice made any appreciable anti-OVA IgE, whereas all 6 of the wild-type mice made anti-OVA IgE.

Exposure to a Nonviral Antigen During SeV Infection is Sufficient to Drive IgE-Mediated Augmented Airway Hyperreactivity

We next wanted to determine whether our model could lead to clinically relevant disease in mice exposed to OVA during a SeV infection. The design of these experiments is presented in Figure 1C. Mice were inoculated with SeV or UV-SeV intranasally and then treated with either OVA or PBS intranasally on day 8 after viral inoculation. Subsequently, all mice were challenged with OVA intranasally on days 20, 21, and 22 after viral inoculation, with airway resistance to a methacholine challenge performed on day 23. In wild-type mice, airway resistance was significantly increased in those mice infected with SeV and exposed to OVA at day 8 after inoculation with SeV when compared with mice given PBS at day 8 after viral inoculation (Figure 3A). As expected, mice inoculated with UV-SeV did not develop airway hyperreactivity regardless of whether they received OVA or PBS at day 8 after viral inoculation. *FcεRIα*^{-/-} mice developed a modest increase in airway hyperreactivity when infected with SeV, but the administration of OVA or PBS at day 8 after viral inoculation had little further effect (Figure 3B). These data

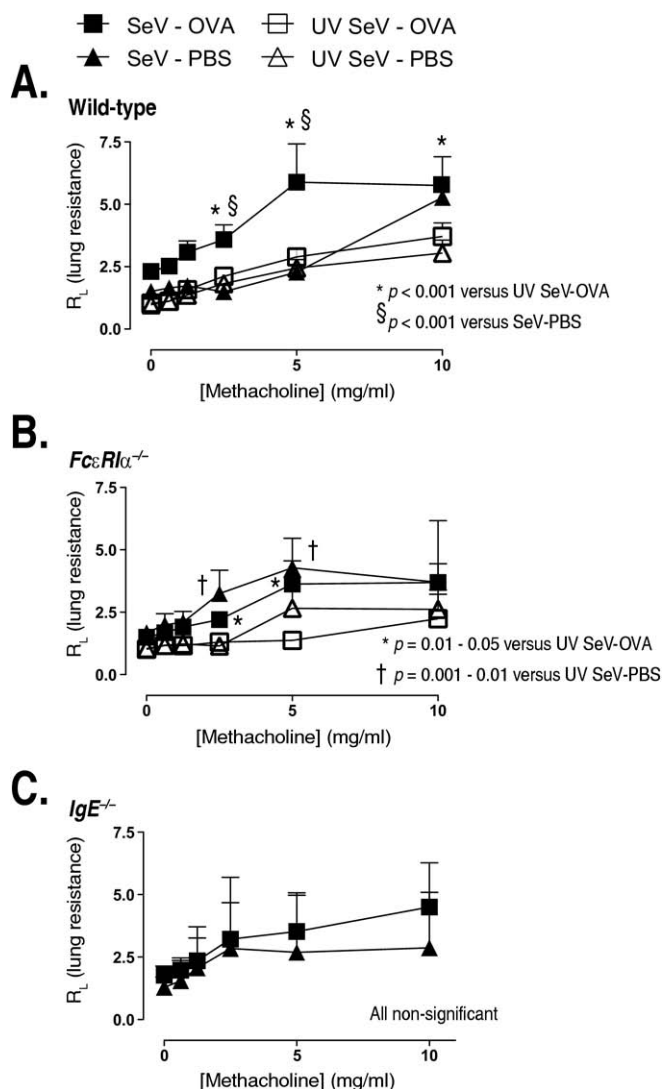


Figure 3. Nonviral antigen exposure during a severe paramyxoviral infection induces airway hyperreactivity through an IgE-dependent mechanism. Mice were treated as outlined in Figure 1C, and airway resistance to a graded methacholine challenge was measured after 3 ovalbumin (OVA) intranasal challenges. Note that all mice received 3 OVA challenges regardless of the sensitization group (phosphate-buffered saline [PBS] or OVA). A, Wild-type mice developed significantly increased airway resistance only when they had been exposed to OVA during an active viral infection (Sendai virus [SeV]-OVA). The number of mice per group were as follows: SeV-OVA = 7, SeV-PBS = 3, UV-inactivated SeV-OVA (UV SeV-OVA) = 8, and UV SeV-PBS = 8. B, This response appeared to depend on the high-affinity receptor for IgE because *FcεRIα*^{-/-} mice did not exhibit this increased airway hyperactivity. The number of mice per group were as follows: SeV-OVA = 6, SeV-PBS = 7, UV SeV-OVA = 6, and UV SeV-PBS = 8. C, This response was IgE dependent because *IgE*^{-/-} mice failed to demonstrate increased airway hyperactivity when given OVA during an active viral infection. The number of mice per group were as follows: SeV-OVA = 4 and SeV-PBS = 3. All data are presented as mean (SEM) of airway resistance for each dose of methacholine. Statistical comparisons are as indicated using repeated-measures analysis of variance with the Tukey multiple comparison test.

suggest that the OVA-mediated augmentation of airway hyperreactivity required expression of FcεRI.

To test whether IgE played a role in this response, we examined the response in IgE-deficient mice (*IgE*^{-/-} mice). Similar to what was seen with the *FcεRIα*^{-/-} mice, no increase in airway hyperreactivity was seen in the *IgE*^{-/-} mice when given OVA (Figure 3C), supporting the idea that IgE and FcεRI are important in this model.

Exposure to a Nonviral Antigen During SeV Infection is Sufficient to Drive IgE-Mediated Augmentation of Mucous Cell Metaplasia

Because mucous cell metaplasia is one of the cardinal features of asthma and clearly has been shown to be dependent on IL-13 in the SeV model, we examined the development of mucous cell metaplasia after exposure and challenge to a nonviral antigen. Using mice treated as outlined herein, we quantified mucous cell metaplasia in lung sections from mice inoculated with SeV or UV-SeV followed by OVA or PBS intranasally on day 8 after viral inoculation and OVA intranasal challenge daily on days 20, 21, and 22 with lungs harvested the next day. As expected, SeV infection in wild-type mice induced PAS-positive mucous cell metaplasia (Figure 4). However, a significant and marked increase in mucous cells and *Muc5ac* mRNA was seen in wild-type mice if they were exposed to OVA on day 8 after viral inoculation (Figure 4). In *FcεRIα*^{-/-} mice, there was a significant but small increase in PAS-positive mucous cells in the OVA-treated group compared with the PBS-treated group (Figure 4B). However, this increase was not seen in the level of *Muc5ac* mRNA (Figure 4C). Further evidence of the IgE-FcεRI axis being important for mucous cell metaplasia came from the fact that the *IgE*^{-/-} mice demonstrated no increased level of mucous cells (either by PAS staining or mRNA) with OVA exposure (Figure 4B and C).

Taken together, these data demonstrate that a single exposure to a nonviral antigen during SeV infection is sufficient to drive IgE production against this antigen. Furthermore, the development of airway hyperreactivity and mucous cell metaplasia after subsequent challenge with OVA depends on both IgE and FcεRI, even though this response has been reported to be IL-13 T-cell dependent in the traditional OVA and alum model.¹²

DISCUSSION

The mechanisms underlying the development of atopy remain unclear, although a strong association between early life severe respiratory viral infection and atopy has been documented.^{4,5,16} Our prior studies have suggested that severe paramyxoviral infection is sufficient to drive an IL-13-dependent atopic response; however, these studies did not explore whether the atopic response could spread to nonviral bystander antigens.⁷ The results of our current study support the hypothesis that a single exposure to nonviral antigen during the antiviral response is sufficient to drive atopic sensitization against the antigen. Although previous studies

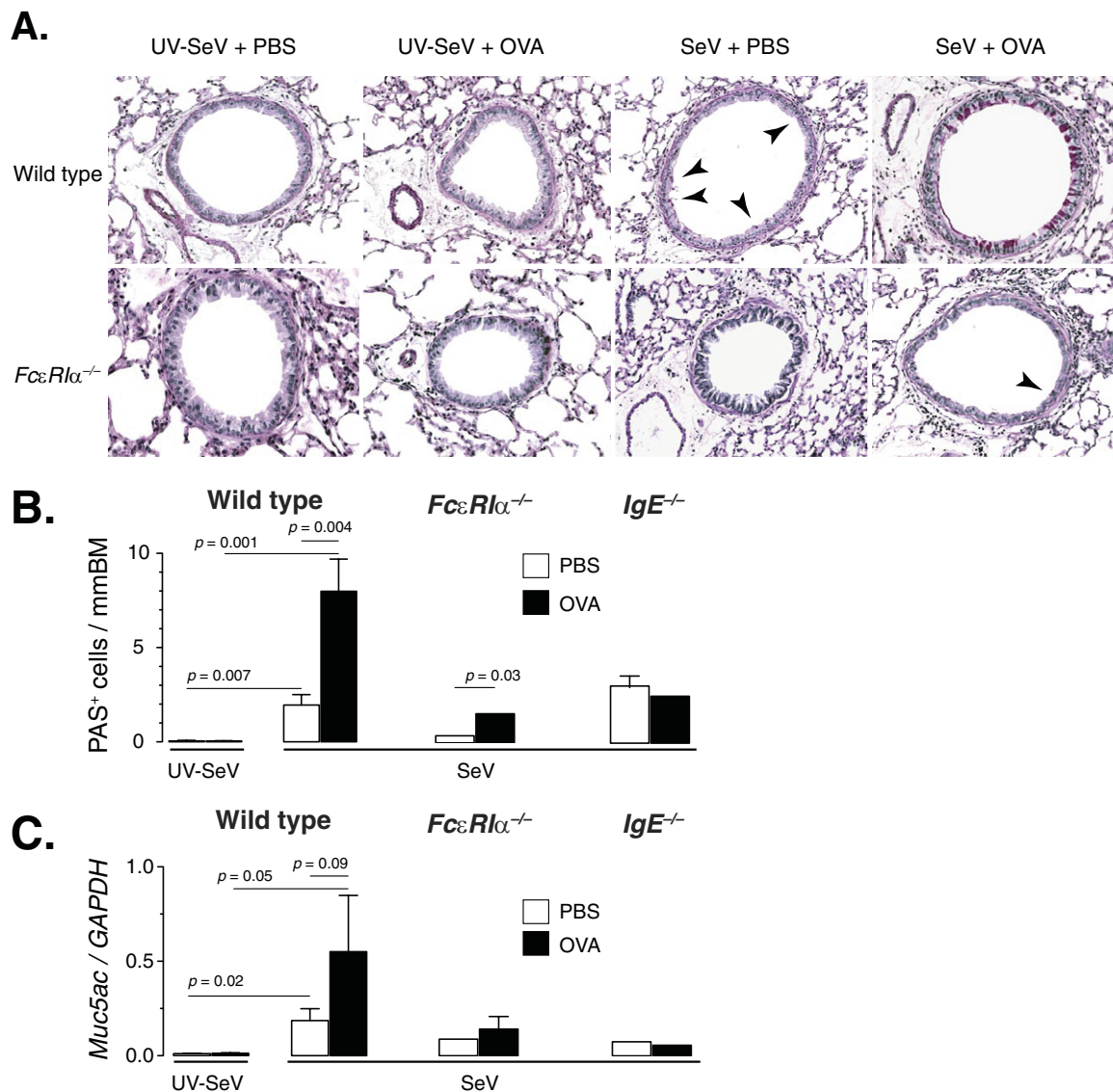


Figure 4. Nonviral antigen exposure during a severe paramyxoviral infection induces mucous cell metaplasia primarily through an *FcεRI*-dependent process. A, Representative periodic acid–Schiff (PAS) staining of lung sections from C57BL/6 or *FcεRIα*^{-/-} mice inoculated with either 2×10^5 PFU of Sendai virus (SeV) or UV-inactivated SeV (UV-SeV) intranasally, exposed to OVA or phosphate-buffered saline (PBS) intranasally on day 8 after viral inoculation, and challenged with OVA intranasally daily as outlined in Figure 1C. SeV infection induced a modest level of PAS-positive cells (red staining cells in bronchial epithelium, indicated by arrowheads) in wild-type mice, but exposure to OVA during the viral infection greatly enhanced this response (the arrowheads were omitted because of the much greater level of staining). Mice deficient in *FcεRIα* had few PAS-positive cells in their airways (see arrowhead), regardless of treatment. B, Quantification of the number of PAS-positive cells per millimeter of basement membrane (mmBM) in the indicated mouse strain inoculated with UV-SeV (wild-type only) or SeV and then given PBS (open bars) or OVA (filled bars) and treated as in A. C, Level of expression of *Muc5ac* gene product in whole lung (normalized to *GAPDH* levels) for each of the groups as discussed in B. Data are presented as mean (SEM), with the number of mice as outlined in the legend to Figure 3. Statistical comparisons are as indicated using the 2-tailed, unpaired *t* test.

have examined the role of exposure to nonviral antigens during a viral infection, these studies have either sensitized the mice by intraperitoneal injection before viral exposure or exposed the mice to daily OVA for 10 days before measurement of outcomes, with less than ideal physiologic correlation.^{17–19} To our knowledge, our study is the first to examine

the effect of a single antigenic exposure without adjuvant; therefore, we think this model is more reflective of what may occur in humans with severe respiratory viral infections.

The presence of IgE does not in itself indicate atopic disease. It is only with the development of clinical disease on subsequent exposure to the antigen that atopic disease can

truly be diagnosed. In this vein, we showed that the IgE generated against the nonviral antigen in our model was sufficient to induce a marked increase in mucous cell metaplasia and airway hyperreactivity on re-exposure to the allergen. On the basis of our prior studies, we believe that the IgE was able to bind and signal through FcεRI because the presence of both IgE and FcεRI was critical for the exacerbation of the phenotype upon subsequent allergen challenge. This is a novel finding because previous reports on the development of airway hyperreactivity and mucous cell metaplasia in the intraperitoneal OVA and alum model have been shown to be primarily dependent on T cells and IL-13.^{12,13} In fact, our prior studies have shown that mice infected with SeV make a robust anti-SeV IgE response. We believe that this anti-SeV IgE may be critical for the recruitment of T_H2 cells in an antigen nonspecific manner. Once recruited to the airways, these T_H2 cells could respond to any nonviral antigen that is encountered (such as OVA in our model). This sensitization phase will then leave the mouse susceptible to a subsequent exposure to the nonviral antigen and the development of atopic disease. Our current model of how exposure to a nonviral antigen during a viral infection drives the development of initial atopic disease is shown in the eFigure. In fact, because cross-linking IgE on lung cDCs drives further recruitment of T_H2 cells, this model predicts that there could be development of an amplification loop (the so-called atopic cycle) that would drive further atopic sensitization.³

One caveat to our current studies is the fact that SeV infection itself drives airway hyperreactivity and mucous cell metaplasia.⁶ In our current study we found that OVA exposure and challenge exacerbated this baseline-increased airway disease. Therefore, we believe this response is due to the OVA exposure on top of the viral infection. The exacerbated atopic disease due to OVA exposure involved both increased airway hyperreactivity and mucous cell metaplasia. This is interesting because we have previously reported that *FcεRIα*^{-/-} mice do not develop mucous cell metaplasia with SeV infection, although in that study we did not examine airway hyperreactivity.⁷ Therefore, it appears that the mechanisms behind IL-13-dependent mucous cell metaplasia (which is FcεRIα dependent) and airway hyperreactivity (not FcεRIα dependent based on our current results) are disparate, at least at 21 days after inoculation with SeV. This finding supports a prior study that examined the genetics of airway hyperreactivity and mucous cell metaplasia and found these 2 traits segregate separately in the SeV model.²⁰

The data from our mouse model provide the first direct evidence that development of atopy may be due to environmental exposures during a severe respiratory viral infection. On the basis of these studies, we would predict that infants hospitalized with a severe paramyxoviral infection would likely develop initial sensitization to indoor allergens. In fact, it has been shown that young children tend to develop IgE against perennial allergens earlier than seasonal allergens.²¹ Whether specific allergen sensitivity in humans can be directly related to environmental allergen exposure awaits fur-

ther study. Our model uses a severe paramyxoviral infection to initiate the allergic sensitization, but it is not known whether other viruses or less severe infections are capable of driving a similar response. In fact, in addition to paramyxoviruses (eg, respiratory syncytial virus, parainfluenza 1 and 3), rhinoviruses have been associated with the development of asthma, and it is tempting to speculate that similar mechanisms are active during rhinovirus infections.³ Given our finding that exposure to antigen during a viral infection can lead to atopic sensitization against the antigen, we cannot help but wonder whether the increase in atopic disease seen in the westernized world could be related to an increase in population density with an associated possible increase in respiratory viral infections. Finally, our study helps elucidate the mechanisms involved in the translation of viral infection into atopic disease and lays the groundwork for future studies exploring therapeutic strategies to reduce or prevent the development of atopic disease.

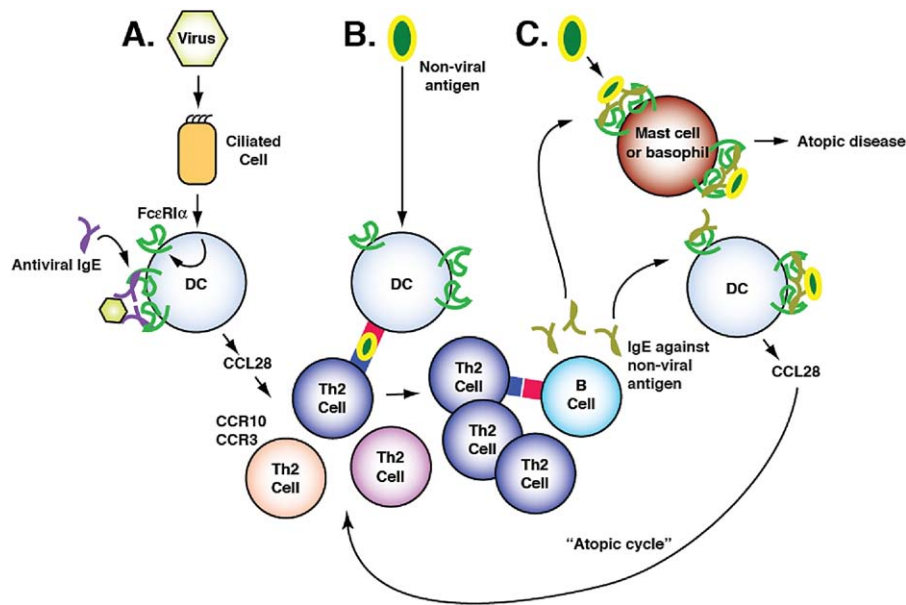
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eFigure. Proposed model of development of atopy. A, Viral respiratory infection drives expression of FcεRI on lung conventional dendritic cells (DCs) and IgE against virus leads to cross-linking of FcεRI on the conventional DCs. This leads to release of chemoattractant (CCL28) for the recruitment of T_H2 cells in an antigen nonspecific fashion. B, Exposure to a nonviral antigen could lead to expansion of T_H2 cells, which would instruct B cells to make nonviral antigen specific IgE. C, Subsequent exposure to the antigen would lead to clinical relevant atopic disease. If IgE against the nonviral antigen is cross-linked on the conventional DCs in this second exposure, then further antigen nonspecific recruitment of T_H2 cells could occur, leading to an "atopic cycle."