ORIGINAL ARTICLE Asthma

Pandemic influenza virus, pH1N1, induces asthmatic symptoms via activation of innate lymphoid cells

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Abstract

Background: The pandemic strain of the influenza A virus (pH1N1) in 2009 caused many complications in patients. In this study, we introduce asthmatic symptoms as a complication of pH1N1 infection in children, not having a relationship with asthma history. The aim of this study was to quantify asthmatic symptoms in pH1N1-infected children and elucidate the underlying mechanisms of airway hyper-responsiveness (AHR) induced in a murine model of pH1N1 infection.

Methods: As a retrospective study, pH1N1-infected children who were hospitalized with moderate to severe acute asthmatic symptoms were enrolled and administered a methacholine challenge test (MCT) at 3 months post-discharge. Additionally, the induction of AHR by pH1N1 infection was measured by MCT in wild-type and $Rag1^{-/-}$ mice. The effect of the innate immune response on the development of AHR following pH1N1 infection was investigated.

Results: More than 70% of the pH1N1-infected children without a pre-infection diagnosis of asthma had a negative response on the MCT. None of these children had recurrent wheezing or asthma during the 3 years following pH1N1 infection. The development of AHR in pH1N1-infected mice was associated with an elevation in IL-33 and innate lymphoid cells 2 (ILC2).

Conclusions: This study demonstrates that pH1N1 infection directly induces transient asthmatic symptoms in patients regardless of their medical history. pH1N1 infection was shown to stimulate the rapid development of AHR and Th2-type cytokine secretion in mice via the activation of ILC2; it may be activated independently of adaptive immunity.

Abbreviations
ILC2, innate lymphoid cells 2; AHR, airway hyper-responsiveness; MCT, methacholine challenge test; FEV1, forced expiratory volume in 1 s; PC_{20}, 20% fall in FEV1; $Rag1^{-/-}$, recombination-activation gene 1 deficient; PFU, plaque-forming unit; BALF, bronchoalveolar fluid; R_L, lung resistance.

Asthma, which is a serious chronic lung disease that afflicts approximately 150 million people worldwide (1), is a heterogeneous and genetically complex disease. Asthma is usually induced by an allergen. Allergen-induced Th2 cell activation results in the activation of eosinophils and mast cells, the activation of which leads to mucus hyperplasia. Respiratory

viruses such as rhinovirus, RSV, and the influenza virus are the most frequent triggers for the allergic exacerbation of asthma (2). Unlike other viruses, the influenza virus induces a Th1 immune response and robust IFN-γ production and consequently activates DC via IFN-γ-bolstered Th2-type immunity against recent or sequentially infected allergens that result in boosting of allergen response. The influenza virus also induces pulmonary lung inflammation (3).

Symptoms after the influenza virus infection is determined by the combination of two surface glycoprotein hemagglutinin and neuraminidase which is the main antigen determinant of influenza virus (4). It also determines tissue and host tropism of the virus and influenza pathogenesis (5). So the virus subtype is important to understand the flu symptoms and pathogenesis. The segmented characteristic of the influenza RNA is what allows the influenza virus to undergo continuous evolutionary antigenic changes during the replication process (6). The emergence of a new strain of the influenza virus can lead to an increase in complications and unexpected symptoms. Several studies have reported that the 2009 pH1N1 virus has been associated with asthma symptoms in a few cases (7, 8).

There are controversies about the role of innate lymphoid cells 2 (ILC2) against influenza infection. Monticelli et al. (9) published that these cells did not affect viral load, but it is important to maintain the integrity of airway epithelial cell barrier and restore tissue homeostasis after viral infection. A recent study on the relationships between H3N1 infection and AHR in mice (10) revealed that H3N1 infection activates resident ILCs, especially ILC2, via IL-33 secretion, which induces type 2 cytokine secretions and leads to airway hyperplasia. ILC2s are the only ILC cell types discovered to date that secrete type 2 cytokines, including IL-5, IL-9, and IL-13 as well as IL-4 in small amounts. ILC2s express the surface markers CD45, IL-7Ra, T/ST2, sca-1, and c-kit, but do not express the lineage cell marker (10–14). ILC2s also express the transcription factor GATA3, which is expressed mainly in Th2 cells.

Existing studies of the effects of influenza infection have focused on asthma exacerbation in patients with a prior asthma history. This study has identified an unprecedented symptom among children diagnosed with the H1N1 virus during the 2009 pandemic. These children experienced acute asthmatic symptoms regardless of their prior asthma history. Animal experiments were also performed to determine the underlying mechanisms related to these influenza-induced asthmatic symptoms in the innate immune system, with a particular focus on ILC2 involvement.

Materials and methods

Subjects

This study included children under 15 years of age who were admitted to the Severance Children's Hospital between October 2009 and February 2010 with respiratory complications resulting from pH1N1 infection confirmed by real-time PCR (15). pH1N1-infected children with acute asthmatic symptoms, confirmed by expert pediatric allergists, were enrolled who showed progressively worsening shortness of breath, coughing, wheezing, and chest tightness (16). The personal and family allergy and asthma history of each patient were collected via a standardized questionnaire (17, 18). Anti-inflammatory drugs and bronchodilators were prohibited 1 week before the MCT. Each patient inhaled increasing concentrations of methacholine (0.075, 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10, 25, and 50 mg/ml) nebulized by a dosimeter (Dosimeter MB3 Mefar, Brescia, Italy) until the forced expiratory volume in 1 s (FEV1) was reduced by 20% from a post-nebulized saline value (19). AHR was defined as the concentration below 16 mg/ml at

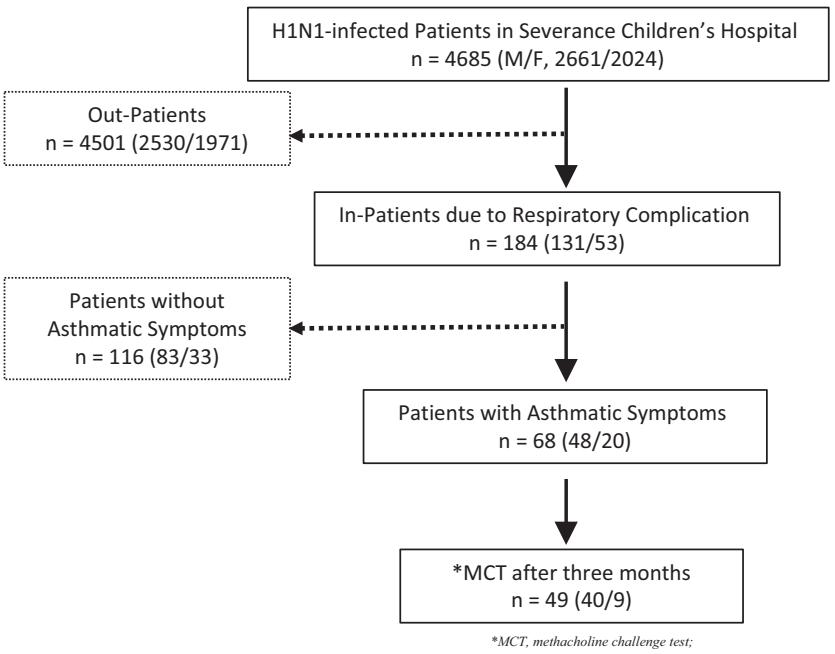

Figure 1 Subject demographics. Among the 4,685 patients confirmed with pH1N1 infection at Severance Children's Hospital, 184 patients (3.9%) were hospitalized due to respiratory complications, 68 of whom experienced moderate to severe acute asthmatic symptoms. Forty-nine children performed the methacholine challenge test (MCT) 3 months post-discharge.

which a 20% fall in FEV1 (PC_{20}) would occur. Patients were reassessed by a telephone interview 3 years post-discharge. This study was approved by the Institutional Review Board of Severance Hospital (4-2014-0127).

Mice and influenza infection

C57BL/6 and C57BL/6 background recombination-activation gene 1-deficient ($Rag1^{-/-}$) mice (8–10 weeks old) were purchased from Orient-Bio (Seoul, Korea). Influenza A virus (A/Seoul/YS-01/2009, pH1N1) was inoculated intranasally with a 1×10^4 plaque-forming unit (pfu) suspended in 20 μl of PBS under anesthetic condition. Experiments were approved by the Institutional Animal Care and Use Committee of the Yonsei University College of Medicine.

Measurement of AHR in the influenza-infected animal

At 5 days post-infection (dpi), mice were prepped for AHR measurement using an animal ventilator (Flexivent; SCIREQ, Montreal, QC, Canada). Anesthetized and tracheotomized mice were treated with aerosol saline and serially diluted (0, 3.125, 6.25, 12.5, 25, and 50 mg/ml) acetyl-b-methylcholine chloride methacholine (Sigma-Aldrich; St. Louis, MO, USA). Lung resistance (R_L) was determined as the average of three measurements at each dose per mouse over three independent experiments.

Total lymphoid cell isolation from the lung

Whole lungs were removed following perfusion and rinsed with PBS. Lungs were digested using a collagenase IV (CSL4, Worthington Biochemicals, Lakewood Township, NJ, USA; Fraction IX; Sigma-Aldrich) and DNase I (50 U/ml; Sigma-Aldrich) in RPMI1640 medium containing 10% FBS. Samples were incubated in a shaking incubator for 60 min at 37°C. Total lymphocytes were then washed and enriched by a discontinuous density gradient of Percoll (Amersham Biosciences, Pittsburgh, PA).

Flow cytometry

Total isolated cells were first incubated with Fc block antibody (2.4G2; BD Pharmingen, San Diego, CA) and stained with fluorescein isothiocyanate (FITC)-conjugated T1/ST2 (DJ8; MD Bioproducts, St Paul, MN, USA), phycoerythrin (PE)-conjugated anti-CD3 (17A2l BD Pharmingen), anti-CD19 (1D3; BD Pharmingen), anti-CD11c (HL3; BD Pharmingen), anti-CD11b (M1/70, BD Pharmingen), anti-CD49b (DX5; Biolegend, San Diego, CA, USA), anti-F4/80 (BM8; eBioscience, San Diego, CA), anti-B220 (RA3-6B2, BD Pharmingen), anti-FcεR1 (MAR-1; eBioscience), PerCP-Cy5.5 rat-anti-mouse CD25 (PC61, BD Pharmingen), allophycocyanin (APC)-conjugated anti-c-Kit (ACK2; Biolegend), AlexaFluor 700-conjugated anti-mouse Ly6A/E (Sca-1, D7; eBioscience), and AlexaFluor 780 conjugated anti-CD45 (30-F11, BD Pharmingen). Cells were analyzed on an LSRII (BD Bioscience, San Jose, CA, USA) with Flowjo 7.6.5 software.

Statistical analysis

All data were expressed as the mean ± s.d. For the human clinical data, numerical parameters with a non-normal distribution were log-transformed before analysis. The SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used. For the *in vivo* animal experiments, statistical comparisons between experimental groups were performed using the Student's *t*-test in GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA).

Results

Epidemiology of pH1N1-infected children

A total of 4,685 children were confirmed with H1N1 infection. A total of 184 (3.9%) of these children were hospitalized due to respiratory complications, including acute asthmatic symptoms, pneumonia, croup, and acute tonsillopharyngitis. Among these 184 inpatients, 68 (37%) exhibited pH1N1-induced acute asthmatic symptoms. Forty-nine children with acute asthmatic symptoms performed the MCT 3 months post-discharge (Fig. 1).

Response to methacholine in patients with pH1N1-induced acute asthmatic symptoms

Forty-nine children were subdivided according to their medical history. Nine children (18.4%) reported a previous history of

Table 1 Comparison between H1N1-induced acute asthmatic subjects with and without previous asthma history

	Previous asthma history	
	Positive	Negative
Sex (M/F)	6/3	34/6
Age (years)	6.7 (5–8.4)	8.1 (4.4–13.7)
BMI	17.93 ± 1.6	17.23 ± 2.6
Blood eosinophils (log/μl)	5.31 ± 1.05	5.34 ± 1.09
Serum total IgE (log kU/l)	6.46 ± 0.82	6.25 ± 1.26
FEV_1 (% pred)	95.26 ± 13.36	102.03 ± 20.62
FVC (% pred)	93.29 ± 15.67	99.72 ± 18.05
FEV_1/FVC (%)	87.73 ± 6.16	86.55 ± 7.59

BMI, body mass index; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

Table 2 Association between AHR and previous asthma history in patients with H1N1-induced acute asthmatic symptoms

Number of subjects (N = 49)	Methacholine challenge test		
	$PC_{20} \geq 16$ mg/ml	$PC_{20} < 16$ mg/ml	Total
Previous asthma diagnosis (%)			
Negative	29 (59.2)	11 (22.4)	40 (81.6)
Positive	3 (6.1)	6 (12.3)	9 (18.4)

asthma, and 40 children (81.6%) did not. No differences in blood eosinophil count, serum total IgE, or other pulmonary function parameters were observed between children with and without a previous asthma history (Table 1). Among the 49 children who performed the MCT, 17 children (34.7%) were positive and 32 (65.3%) were negative. The response to methacholine 3 months following pH1N1-induced acute asthmatic symptoms was negative in 29 children without a previous

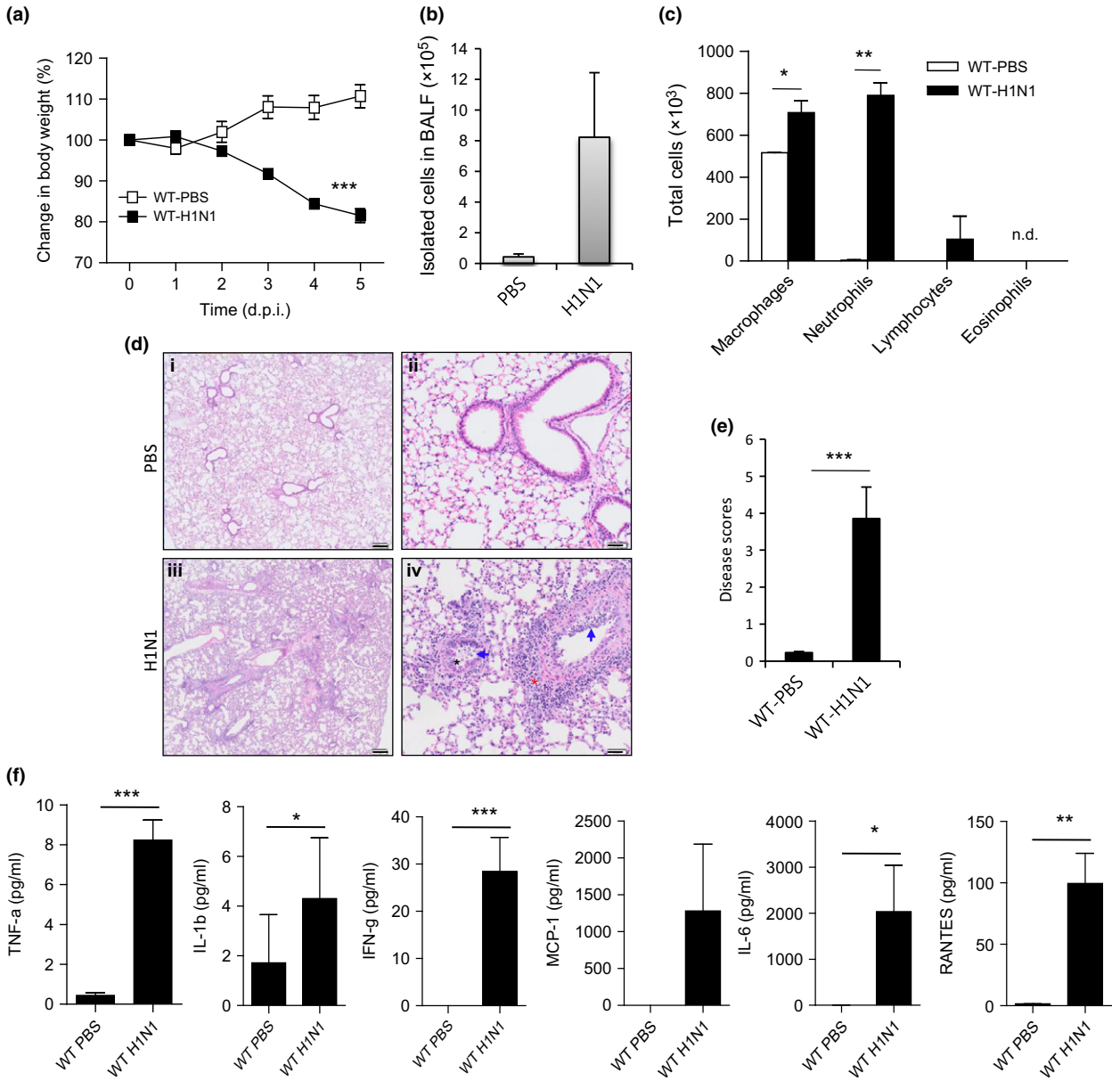

Figure 2 pH1N1 virus isolated from influenza-infected humans induces severe lung inflammation in mouse. (a) Changes in body weight in mock- and virus-infected mice. (b) Number of total infiltrated cells in the BALF. (c) The number of infiltrated inflammatory cells in the BALF following a cytospin assay. (d) Histological features of the lung. Lung samples taken 5 days post virus (iii–iv) or mock (i–ii) infection were stained with H&E. The black asterisk indicates epithelial cell hyperplasia, the blue arrow indicates cell flooding into the cavities of the alveoli, and the red asterisk indicates perivascular cuffing with infiltrated cells. (e) Disease scores based on the levels of destruction of the mucosa, epithelial cells, and alveoli, cell infiltrations into the submucosal region, epithelial cell hyperplasia, cell flooding into the interstitium, and perivascular cuffing with PMN infiltration as well as other lymphoid cells using a blinded test. (f) TNF-α, IL-1β, and IFN-γ in BALF were measured by ELISA, and IL-6, RANTES, and MCP-1 in BALF were measured by CBA. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± s.d. from at least three independent experiments. The bar indicates 200 μm (i, iii) or 50 μm (ii, iv).

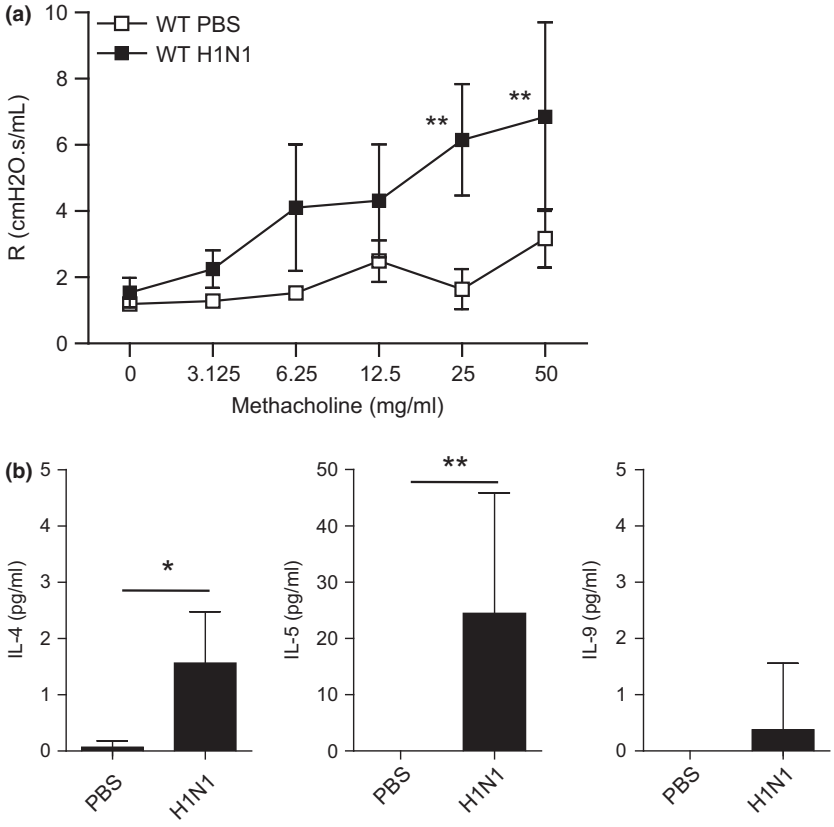

Figure 3 Influenza infection induces AHR in mice. (a) Change in lung resistance (R_L) in 8-week-old C57BL/6 mice (n = 5 per group) was measured by Flexivent following a challenge with serially diluted methacholine nebulized into the trachea under anesthetic conditions on day 5 post-infection with pH1N1 or PBS. (b) Secreted levels of cytokines for IL-4, IL-5, and IL-9 in the BALF. Samples were separated into cells and fluid by centrifugation. Cytokines in the fluid were measured by LSRII following CBA and analyzed by the FCAP CBA analysis program. * $p < 0.05$; ** $p < 0.01$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± s.d. from at least three independent experiments.

history of asthma (Table 2). None of the children with a negative response to methacholine reported any recurrent wheezing or physician-diagnosed asthma during the following 3 years.

Influenza induces severe inflammation in the lung

To confirm the pathogenicity, pH1N1-infected mice monitored for a loss in body weight, and alveolar macrophages and neutrophils were accumulated in the BALF at 5 dpi (Fig. 2a, and b). Infiltrated lymphocytes were detected (Fig. 2c), but eosinophils (Fig. 2c) and mast cells (data not shown) were not observed. Histological features showed severe inflammation in the lung, including perivascular cuffing with infiltrated polymorphonuclear (PMN) cells (Fig. 2d). Damage to the epithelial cell walls was detected, and the multishapes of epithelial cells were increased and expanded in pH1N1-infected mice. The interstitium was thicker due to infiltration compared with the PBS group. Disease scores were significantly increased in the pH1N1-infected group (Fig. 2e).

To confirm this, inflammatory cytokine levels in the BALF were measured. IFN-γ, TNF-α, IL-6, and IL-1β as well as chemokines MCP-1 and RANTES were dramatically increased in the pH1N1-infected mice (Fig. 2f). These data indicate that the intranasal administration of the pH1N1 virus can efficiently infect mice and cause lung inflammation.

AHR in influenza-infected mice

To confirm that the pH1N1 infection directly induced asthmatic symptoms, the change in R_L in pH1N1-infected mice was measured at 5 dpi (Fig. 3a). Increased levels of IL-4 and IL-5 were detected in the BALF in the pH1N1-infected group but not in the mock-infected group (Fig. 3b). The level of IL-9 was not significantly increased in either group.

Influenza infection induces the activation of ILC2

An earlier study showed that the induction of AHR following H3N1 infection may involve the activation of ILC2 and cytokines produced from these cells in the lung (10). To clarify the relationships between influenza-induced AHR and the activation of ILC2, $CD45^+$ lymphocytes were sorted and analyzed for ILC2 markers. An approximately 10-fold difference in Lin^-STII^+ cells was observed between the two groups (0.199% in mock-infected mice and 1.31% in virus-infected mice), and the level of sca-1^+c-kit^+ cells was increased from 1.45% in the mock-infected group to 17.7% in the virus-infected group. Moreover, sca-1^+CD25^+ cells also were increased from 0.513% in the mock-infected group to 2.38% in the virus-infected group (Fig. 4b–d). To further confirm the activation of ILC2, the mRNA levels of IL-4, IL-5, and IL-13 were analyzed. IL-4, IL-5, and IL-13 expression levels were significantly increased in Lin^- cells.

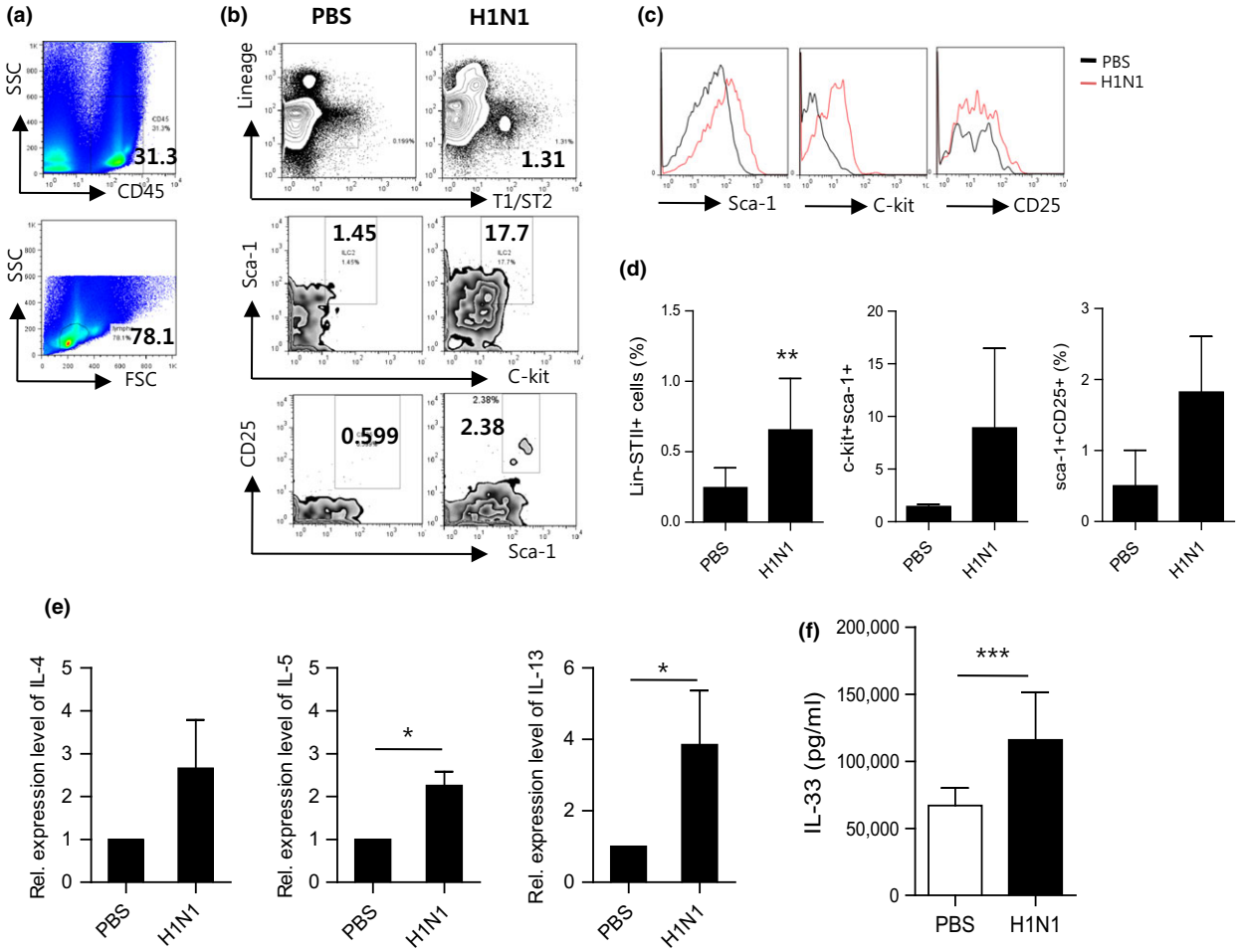

Figure 4 pH1N1 infection results in an increase in ILC2 in the lung. (a) ILC2 in the lung. The gating strategy for ILC2 isolated from total CD45$^+$ lung lymphoid cells. The numbers in the box indicate the percentage of cells in each gate. SSC, side scatter; FSC, forward scatter. (b) The top panels show increased levels of CD45$^+$Lin$^-$ST2$^+$ cells in the lung. The lower panels indicate the expressed levels of sca-1, c-kit, and CD25 from gating on the Lin$^-$ST2$^+$ subset in the top panels. The numbers indicate the percentage of cells in the quadrangle in each panel. (c) B expressed as a histogram. (d) The percentage of Lin$^-$ST2$^+$ cells, Lin$^-$ST2$^+$c-kit$^+$sca-1$^+$ cells, and Lin$^-$ST2$^+$sca-1$^+$CD25$^+$ cells. Each population was statistically analyzed using data from at least three independent experiments. (e) The mRNA expression levels of IL-4, IL-5, and IL-13 in Lin$^-$ cells. The levels of mRNA expression are displayed as the expression levels of each target gene relative to the ct value of expressed GAPDH. (f) ELISA of IL-33 in the total lung lysates. The results are expressed as the mean ± s.d. from three different experiments. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ when compared with the mock-infected group from the same strain.

These results indicate that activated Lin$^-$ cells produce type 2 cytokines, which supports the role of ILC2 in inflammatory responses following pH1N1 infection. Significantly increased levels of IL-33 were observed in the virus-infected group (Fig. 4f). Taken together, these results suggest that influenza induces increased activation of ILC2 and Th2 cytokine levels in mRNA.

Influenza infection induces AHR independent of adaptive immunity

To exclude the possibility of an involvement of the adaptive immune response, including the Th2 cell-secreting cytokines, Rag1$^{-/-}$ mice that lack conventional T and B cells were infected with the pH1N1 virus or PBS. Infected Rag1$^{-/-}$ mice exhibited similar levels of inflammation in response to pH1N1 infection as wild-type mice (Fig. 5). Increased levels of inflammatory cytokines and type 2 cytokines such as IL-4, and IL-5 in the BALF and IL-33 in the lung were detected in Rag1$^{-/-}$ mice (Fig. 6a and b). AHR was observed in the pH1N1-infected group only (Fig. 6c). These results indicate that Rag1$^{-/-}$ mice experience the same symptoms associated with pH1N1-infected wild-type mice. These results suggest that pH1N1-induced acute AHR is not related to an adaptive immune response, especially Th2 immunity.

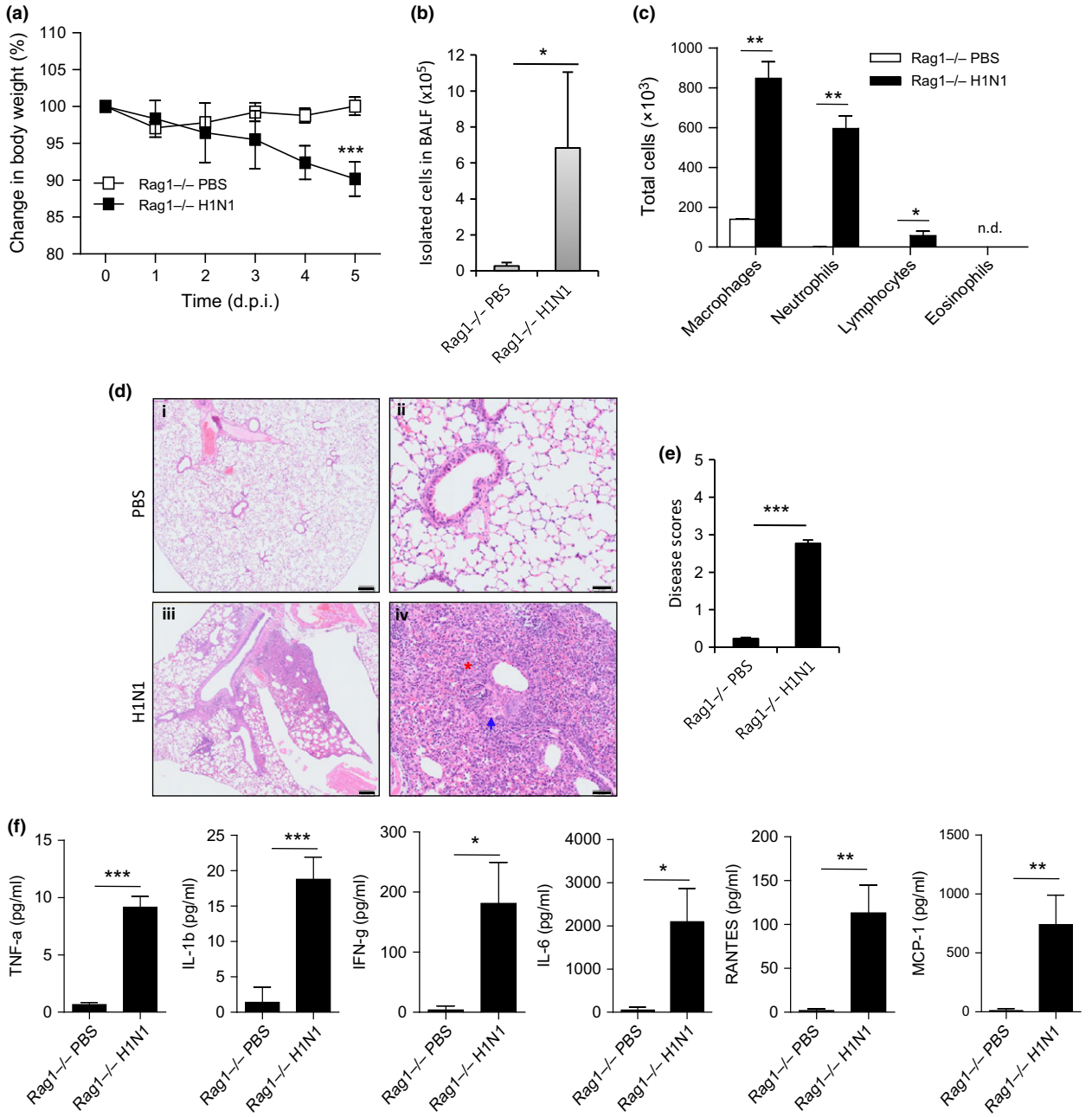

Figure 5 pH1N1 induces severe lung inflammation in mice independent from the adaptive immune response. (a) Changes in body weight (b) Total infiltrated cells in the BALF. (c) Types of infiltrated cells in the BALF. (d) Histological changes in the lung. (e) Disease scores. (f) Levels of inflammatory cytokines in the BALF. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± s.d. from at least three independent experiments. The bar indicates 200 μm (i, iii) or 50 μm (ii, iv).

Discussion

pH1N1 was recorded as the first influenza pandemic of the 21st century. This single institution study revealed the high prevalence of H1N1 infection in young people during the worldwide pandemic period (20, 21). In this study, 184 children were hospitalized due to respiratory complications, 64 of which had acute asthmatic symptoms. Forty-nine of these patients were tested 3 months post-discharge, and more than 70% of the children who did not have a previous history of asthma were negative on the MCT. Laitinen et al. has previously reported that influenza infection causes transient bronchial hyper-responsiveness in healthy adults (22).

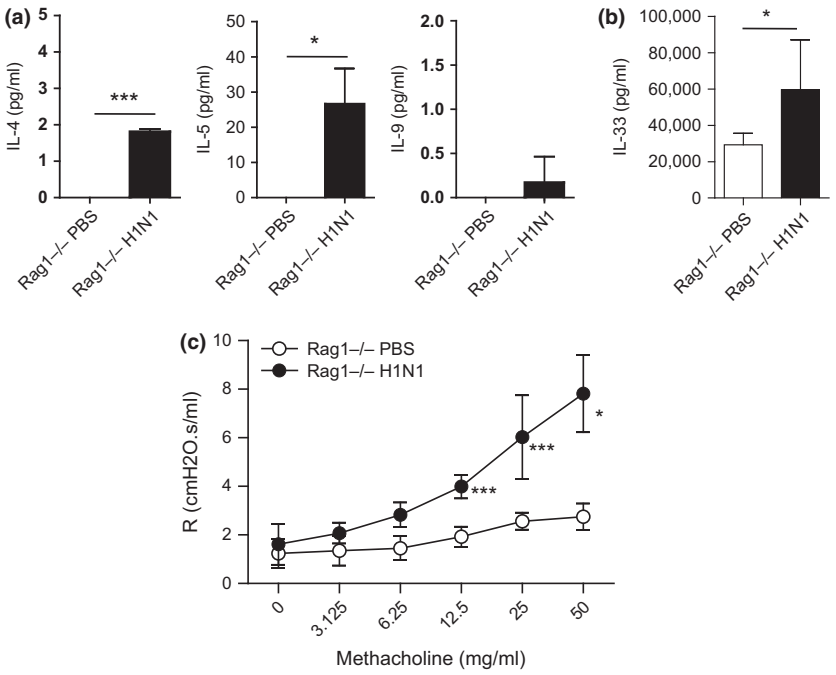

Figure 6 pH1N1 infection induces AHR in Rag1$^{-/-}$ mice. (a) Cytokine levels of IL-4, IL-5, and IL-9 in the BALF. (b) Cytokine levels of IL-33 in the total lung lysates. (c) Change in lung resistance (R_L) in Rag1$^{-/-}$ mice following H1N1 infection. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ when compared with the mock-infected group from the same strain. Data are represented as the mean ± s.d. from at least three independent experiments.

Hasegawa et al. (7) has documented that the pandemic influenza virus infection greatly increased the risk of asthma exacerbation, pneumonia, and atelectasis compared with the seasonal influenza virus. Dahl et al. (3) reported that infection with the influenza A virus affects the subsequent development of allergen-induced lung inflammation in a mouse model. Chang et al. (10) reported the appearance of AHR following H3N1 virus infection and suggested that the main cause of AHR following H3N1 infection is an innate immune cell that does not originate from the common lymphoid precursor in the lung. The present study confirmed that pH1N1 successfully induces lung inflammation in mice (Figs 2 and 6). This infection also induces several inflammatory cytokines, including MCP-1, IFN-γ, RANTES, IL-6, TNF-α, and IL-1β (Figs. 2 f and 5 f) (23, 24). The present study also confirmed that pH1N1 infection induced AHR in mice independent of an adaptive immune response (Figs 3 and 6).

Influenza-induced AHR differs from asthma, which requires repeated allergen sensitization and activates Th2 cells, leading to eosinophil influx and mast cell activation (25). The induction of AHR by the pH1N1 infection was the result of an acute response against the virus in the lungs of mice. No eosinophils (Fig. 2c) or other cells, such as basophils or mast cells were found (data not shown). Additionally, the detection of increased levels of IL-5 and IL-4 but not IL-9 with inflammatory cytokines secretion supports the hypothesis of ILC2 involvement in influenza-induced AHR (Figs 4 and 6a). As expected, ILC2 activation markers such as sca-1 and c-kit and CD25 expression levels on these cells as well as the total number of Lin$^-$ST2$^+$ cells all were increased. This result differs from previous reports in which only the level of ILC2 activation without a change in total population was observed (10). The levels of c-kit$^+$sca-1$^+$ cells were increased in the pH1N1-infected group in a range of approximately 2–20% because the expression level of c-kit varied significantly across each experiment. We therefore speculated that the c-kit molecules were not a sufficient activation marker for ILC2. To further confirm increased ILC2 as a potent effector of Th2-type cytokine secretion, the relative expression levels of IL-4, IL-5, and IL-13 mRNAs were detected by real-time PCR. As a second group of ILCs, the characteristics of these cells resemble Th2 cells, which mediate allergic responses that secrete type 2 cytokines and express transcription factor GATA3 (13). As shown in Fig. 5e, increased levels of IL-4, IL-5, and IL-13 mRNA expression were observed in Lin$^-$ cells following infection with the influenza virus. It is assumed that the type 2 cytokines expressed in Lin$^-$ cells following infection play a role as potent effectors of influenza-induced AHR in this study.

According to several studies, IL-33 has an important role as one of the earliest released signaling molecules following epithelial damage to orchestrate the recruitment and activation of responder cells (26, 27). Haenuki et al. (28) reported that experimental allergic rhinitis is not induced in IL-33$^{-/-}$ mice following a ragweed pollen challenge compared with control wild-type mice. Moreover, Préfontaine et al. (29) has observed an increased level of IL-33 gene and protein expression in endobronchial biopsies from subjects with asthma. Furthermore, anti-IL-33 antibody treatment inhibits airway inflammation in mice (30). In this study, increased levels of IL-33 were detected in the lung homogenate of influenza-infected mice (Figs 4 f and 6 b). Increased IL-33 therefore may directly or indirectly influence AHR and influenza-induced inflammation in mice.

The limitation of this study is that human data, such as BALF inflammatory cells or cytokine responses in the lungs from H1N1-infected patients did not support the murine findings. However, the invasive procedures such as bronchoscopy could not be used for the research purpose in the pediatric patients having asthmatic symptoms. In summary, the present study demonstrated that the pH1N1 infection directly induced asthmatic symptoms in patients regardless of their prior asthma history. Experiments in mice revealed that pH1N1 infection stimulated the rapid development of AHR and Th2-type cytokine secretion via the activation of ILC2. This response may be activated independently of adaptive immunity.

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