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RESEARCH ARTICLE

Senescence in the Lung

Inflammatory and antiviral responses to influenza A virus infection are dysregulated in pregnant mice with allergic airway disease

Rebecca L. Vanders,[1,2] Henry M. Gomez,[1,2] Alan C. Hsu,[1,2] Katie Daly,[1,2] Peter A. B. Wark,[1,2] Jay C. Horvat,[1,2] and Philip M. Hansbro[1,2,3]

[1]*Priority Research Centre for Healthy Lungs, The University of Newcastle, Newcastle, New South Wales, Australia;* [2]*Vaccines, Infection, Viruses and Asthma Research Program, Hunter Medical Research Institute, Newcastle, New South Wales, Australia; and* [3]*Faculty of Science, School of Life Sciences, Centre for Inflammation, Centenary Institute and University of Technology Sydney, Sydney, New South Wales, Australia*

Abstract

Influenza A virus (IAV) infections are increased during pregnancy especially with asthma as a comorbidity, leading to asthma exacerbations, secondary bacterial infections, intensive care unit admissions, and mortality. We aimed to define the processes involved in increased susceptibility and severity of IAV infections during pregnancy, especially with asthma. We sensitized mice to house dust mite (HDM), induced pregnancy, and challenged with HDM to induce allergic airway disease (AAD). At midpregnancy, we induced IAV infection. We assessed viral titers, airway inflammation, lung antiviral responses, mucus hypersecretion, and airway hyperresponsiveness (AHR). During early IAV infection, pregnant mice with AAD had increased mRNA expression of the inflammatory markers *Il13* and *IL17* and reduced mRNA expression of the neutrophil chemoattractant marker *Kc*. These mice had increased mucous hyperplasia and increased AHR. miR155, miR574, miR223, and miR1187 were also reduced during early infection, as was mRNA expression of the antiviral β-defensins, *Bd1*, *Bd2*, and *Spd* and IFNs, *Ifnα*, *Ifnβ*, and *Ifnλ*. During late infection, *Il17* was still increased as was eosinophil infiltration in the lungs. mRNA expression of *Kc* was reduced, as was neutrophil infiltration and mRNA expression of the antiviral markers *Ifnβ*, *Ifnλ*, and *Ifnγ* and *Ip10*, *Tlr3*, *Tlr9*, *Pkr*, and *Mx1*. Mucous hyperplasia was still significantly increased as was AHR. Early phase IAV infection in pregnancy with asthma heightens underlying inflammatory asthmatic phenotype and reduces antiviral responses.

NEW & NOTEWORTHY Influenza A virus (IAV) infection during pregnancy with asthma is a major health concern leading to increased morbidity for both mother and baby. Using murine models, we show that IAV infection in pregnancy with allergic airway disease is associated with impaired global antiviral and antimicrobial responses, increased lung inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR). Targeting specific β-defensins or microRNAs (miRNAs) may prove useful in future treatments for IAV infection during pregnancy.

allergic airway disease; asthma; infection; influenza; pregnancy

INTRODUCTION

Asthma is a global significant health problem, and is the most common chronic disease to affect pregnant women (1–4). In a Canadian population-based retrospective cohort study consisting of 134,188 pregnant women, asthma was the most prevalent comorbidity during pregnancy, occurring in 6,978 subjects (5.2%) (4). When compared with the year before pregnancy, they observed that the presence of a comorbidity resulted in higher hospitalization rates during pregnancy compared with pregnant women without any comorbidity {rate ratio 7.9 [95% confidence interval (CI) 5–12.5] vs. 5.1 [3.6–7.3]}.

Respiratory viruses, like influenza A virus (IAV), are the most common cause of exacerbations among pregnant women with asthma (5–8). This is exemplified in a prospective study where 71% of pregnant asthmatic women experienced a questionnaire detected cold compared with 46% of pregnant nonasthmatic women (9). These women were also significantly more likely to experience multiple colds during pregnancy (33% vs. 16%) and had greater symptom severity compared with pregnant nonasthmatic women [median total common cold questionnaire (CCQ) score 8 (5, 10) vs. 6 (5, 8)]. Asthma exacerbations increase in frequency during pregnancy, as evidenced by a study by Murphy et al. (10), where the ratio of hospitalizations to emergency department

Correspondence: P. M. Hansbro (Philip.Hansbro@uts.edu.au); R. L. Vanders (Rebecca.vanders@newcastle.edu.au).
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presentations for severe asthma exacerbations was substantially higher during pregnancy (2.97 events/year/person) compared with before pregnancy (0.403 events/year/person). In another study, these authors also showed that among pregnant asthmatic women with PCR-confirmed respiratory virus infections, 60% of infections was associated with uncontrolled asthma and increased prevalence of preeclampsia (9).

During pregnancy, maternal immunity alters to become tolerant to the presence of a growing fetus, however, respiratory viruses can take advantage of these changes, increasing the susceptibility and severity of viral infection (11). In turn, infection exacerbates and increases the severity of the asthma phenotype (8, 12, 13). Previously, we showed that peripheral blood mononuclear cells (PBMCs) isolated from pregnant women and infected in vitro with IAV (H1N1pdm09) had increased inflammatory responses (14). In addition, PBMCs and primary nasal epithelial cells (pNECs) from pregnant women with and without asthma had attenuated antiviral responses following infection with human rhinovirus and IAV H1N1pdm09 (15–18).

To date, our research has been limited to in vitro models of infection in pregnancy and asthma. However, to determine the processes that underpin increased disease severity of IAV infections in pregnancy with asthma, we have used in vivo murine models of IAV infection in pregnant mice with allergic airway disease (AAD). We found increases in early inflammatory responses and microRNAs (miRNAs) were associated with reduced global antiviral responses during both early and late infection with IAV in pregnancy with asthma. Infection also prolonged exaggerated AHR.

METHODS

Mice and Animal Ethics

Female BALB/c mice 6–8 wk of age were obtained from Central Animal House at the University of Newcastle and acclimatized for 1 wk. All experiments were performed in the Hunter Medical Research Institute (HMRI) animal facility, under specific pathogen-free conditions. All animal works and protocols used in this study were approved by the Animal Ethics Committee of the University of Newcastle, Australia.

Induction of Time-Mated Pregnancy

Mice were time-mated by first inducing the estrus cycle for 3 days by placing two females per box into housing previously occupied by a male mouse (Fig. 1*A*). Females were mated for 2 days with the male mouse that occupied the housing previously. The third day was considered *day 1* of pregnancy.

IAV a/PR/8/34 Propagation

The mouse adapted IAV, A/Puerto Rico/8/34 was propagated in Madine–Darby canine kidney (MDCK) cells using UltraMDCK media (Lonza Bioscience). Viral titers in stocks and tissue samples were determined by plaque assays as we previously described (12, 19, 20).

Mouse Model of IAV Infection in Pregnancy with AAD

To characterize the impact of experimental asthma, mice were lightly anesthetized with isoflurane (1.5 L/min) and then sensitized by intranasal inoculation with house-dust mite (HDM) extract (Greer Laboratories, Lenoir, NC) at 50 μg/50 μL of sterile saline on the 3 consecutive days that matched the induction of the estrus cycle (Fig. 1*A*) (21). Fourteen days later, mice were challenged with HDM (5 μg/50 μL) for a further 3 consecutive days (21–24). IAV infection was induced on the last day of HDM challenge (Fig. 1*A*). Pregnant mice with and without AAD and age-matched control females were intranasally inoculated with a sublethal dose of the mouse-adapted strain of IAV H1N1 A/PR/8/34 [10 plaque-forming units (pfu) in 50 μL of ultraMDCK media) or media only (25–27). This time point coincides with *day 11* of pregnancy and corresponds with the second trimester of pregnancy in humans. The timing of both sensitization and challenge is an important determinant that was carefully planned when designing these experiments (21–24). We have shown that mice resolve symptoms of acute AAD over 20 days (12). To develop effective acute AAD in mice, sensitization needs to be performed 14 days before challenge to allow sufficient time for Th2 cells to develop. Then mice are challenged to induce AAD and at the same time influenza infection during the mid-second week of gestation in mice. This is analogous to infection during the second trimester in pregnant women, which is when women are most likely to have influenza and develop asthma exacerbations during pregnancy, which is modeled here (4, 16, 28). After 3 or 7 days postinfection (dpi), lung function was assessed, mice were euthanized, and lung tissue was collected for subsequent analyses (Fig. 1*A*) (25, 26, 29–33).

Bronchiolar Lavage Fluid Collection

Bronchiolar lavage fluid (BALF) was collected by tying off the right lung lobe and washing the left lobe only twice with 500 μL of DMEM (Sigma-Aldrich, Australia) (25, 26, 34). Erythrocytes were lyzed by addition of red blood cell lysis buffer (200 μL, 5 min, 4°C) and the remaining cell suspension was centrifuged (200 *g*, 5 min, 4°C). Supernatants were aspirated and stored for subsequent protein analyses and the remaining cell pellets were resuspended in DMEM (160 μL, Sigma-Aldrich, Australia). Total leukocytes were quantified using the trypan blue exclusion method using a hemocytometer with the outer four squares counted before calculating total cells/mL [(count of four squares)/$4 \times 2 \times 0.16 \times 10^4$ cells/mL]. The remaining leukocytes were centrifuged onto microscope slides using a cytospin (Shandon, Cheshire, England; 300 *g*, 10 min, room temperature). Differential enumeration of inflammatory cells was determined after May Grunwald–Giemsa staining based on morphology (35).

Lung mRNA and miRNA Expression

To obtain mRNA and miRNA, snap-frozen lung tissue from one lobe of the multilobed right lung was homogenized and extracted using TRIzol reagent, according to the manufacturer's instructions (Life Technologies Pty Ltd, Australia) (36, 37). Total RNA was reverse-transcribed using BioScript (Bioline, Alexendria, Australia) and random hexamer primers (for mRNAs) or reverse primers U6, U44, and U49 (for miRNAs) (Invitrogen). Relative abundance of complementary DNA was determined using custom-designed primers and

SYBR FAST (Sigma-Aldrich, Australia) compared with the reference gene hypoxanthine-guanine phosphoribosyltransferase, analyzed using a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Australia) (12, 38, 39).

Viral Titers by Plaque Assay

Plaque assays were used to measure both the viral stock concentrations and recovered virus from BALF. MDCKs (80% confluent) were infected with fivefold serial dilutions of lung homogenate and subsequently overlaid with Leibovitz-15 media containing 1.8% agarose and trypsin [tosylamido-2-phenylethyl chloromethyl ketone (TPCK)]. Plates were incubated at 35°C for 3 days and plaques were subsequently counted (19, 20, 25, 26).

Histology

Following perfusion with 0.9% saline solution, the large-lobed left lung was fixed in 10% formalin solution (Sigma-Aldrich, Australia), embedded in paraffin, and sectioned at 5 μm thickness. Lung sections were then stained with periodic acid Schiff-Alcian blue (PAS-AB), and the numbers of mucus-secreting cells around the airways were counted using a grid and ×100 magnification (35, 40, 41). Ten counts were averaged and are represented as mucus-secreting cells per 100 μM of airway.

Lung Function

Airway hyperresponsiveness (AHR) was measured in anesthetized, cannulated mice using a Buxco Electronic resistance and compliance system (Sharon, CT) in response to increasing doses of methacholine. This machine induces a deep inflation in the lungs before each dose including at baseline, which normalizes the resistance between the groups at this baseline time point. Data are represented as dose response curves ranging from 0 to 50 mg/mL of methacholine (12, 32, 40, 42).

Statistical Analyses

Data were analyzed using GraphPad Prism 8 (San Diego, CA). Normality of the data was assessed using the D'Agostino–Pearson normality test. Differences between data with three or more groups were assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data), with Tukey's adjustment for multiple comparisons. For lung function data, a two-way ANOVA using a mixed model with repeated measures and Tukey's adjustment for multiple comparison was performed. For viral titers, data were assessed using the unpaired two-tailed Student's *t* test (or rank-sum test for nonparametric data). Data are represented as means ± SE.

RESULTS

For clarity throughout the results, we have focused on comparing infection in pregnancy with AAD with IAV infection in pregnancy alone, since we are examining how asthma affects infection in pregnancy. Other comparisons are included in the Supplement Data and are highlighted in the DISCUSSION (https://doi.org/10.6084/m9.figshare.22193362.v2).

IAV Infection in Pregnancy Alters Viral Titers during Early and Late Infection and Reduces Pregnancy Success

We first explored the impact of IAV infection on an asthmatic phenotype in pregnancy (Fig. 1). Plaque assays showed that at the peak of infection [i.e., 3 days postinoculation (3 dpi)], viral titers in pregnant mice with AAD were increased, though not statistically significant, compared with pregnant mice with IAV infection only (Fig. 1*B*). However, following 7 dpi with IAV, pregnant mice with AAD showed significantly lower viral titers compared with pregnant mice with IAV only (Fig. 1*C*). To examine the effect of IAV infection and AAD on the success of pregnancy following 3 days of mating, we measured pregnancy success rates and found significant reductions in pregnancy success among all three groups compared with controls. The combined effect of IAV infection with AAD produced the greatest reduction in pregnancy success (Fig. 1*D*).

IAV Infection in Pregnancy with AAD Reduces Neutrophil but Increases Eosinophil Influx into the Airways

We next assessed the impact of IAV in pregnancy with AAD at the peak (3 dpi, Fig. 2*A*) and persistence (7 dpi, Fig. 2*B*) on inflammatory cell influx into the airways. At 3 and 7 dpi, IAV infection in pregnant mice with AAD resulted in a significant decrease in the influx of neutrophils compared with pregnant mice with IAV only (Fig. 2, *A* and *B*). By 7 dpi, IAV-infected pregnant mice with AAD also had significantly reduced total leukocytes, monocytes, and lymphocytes compared with pregnant mice with IAV only (Fig. 2*B*). At 3 dpi, pregnant mice with IAV infection and AAD showed no difference in eosinophil response compared with pregnant mice with IAV only, however there was a significantly blunted eosinophil response compared with pregnant mice with AAD only. By 7 dpi, pregnant mice with IAV infection and AAD showed significantly increased eosinophil production compared with pregnant mice with IAV only, and comparable levels when compared with pregnant mice with AAD only.

IAV Infection in Pregnancy with AAD Increases Proinflammatory Cytokine Responses in the Lung

The major cause of morbidity and mortality following IAV infections is excessive acute cytokine responses and a cytokine storm. Thus, we next assessed the impact of IAV infection in pregnancy with AAD on lung mRNA expression of key inflammatory cytokines including interleukin (Il) *Il5*, *Il13*, *Il17*, *Il1β*, *Kc*, *IL6*, and tumor-necrosis factor alpha (*Tnfα*) (Fig. 3). At 3 dpi, IAV infection in pregnant mice with AAD resulted in significantly increased mRNA expression of the proasthmatic and proinflammatory cytokines *Il13* and *Il17a*, compared with pregnant mice with IAV infection only (Fig. 3*A*). There was no difference in the expression of *Il1β*, *Il6*, and *Tnfα* mRNA levels between IAV-infected pregnant mice with AAD and IAV-infected pregnant mice without AAD. By 7 dpi, *Il17* expression was still significantly increased (Fig. 3*B*). At both 3 and 7 dpi, keratinocyte-derived chemokine (*Kc*, mouse equivalent of human IL-8) levels were significantly decreased in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV infection only; corresponding with the significantly reduced neutrophil counts we observed in the BALF in Fig. 2.

IAV Infection in Pregnancy with AAD Leads to Dysregulated miRNA Expression

We next examined the expression of four microRNAs (miR155, miR574, miR223, and miR1187) known to be induced during IAV infections (Fig. 4). At 3 dpi, all four miRs were

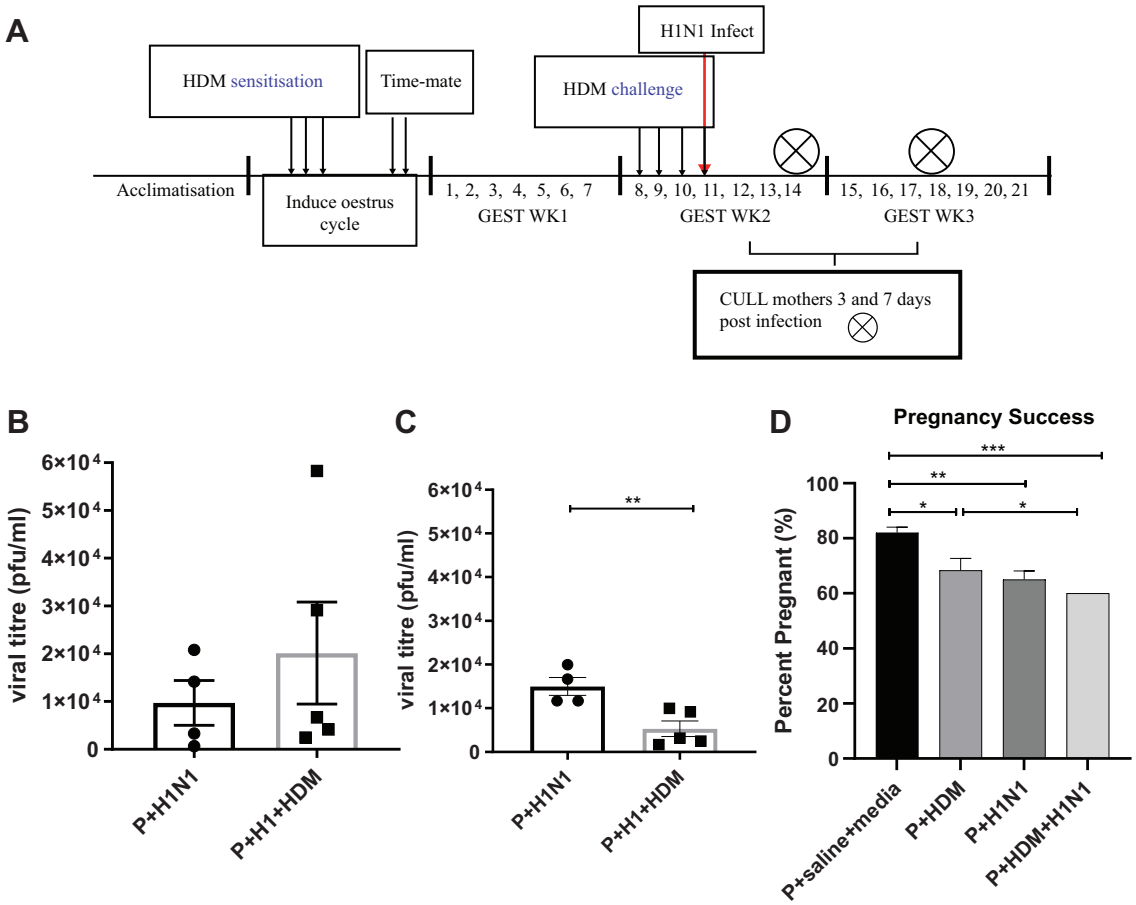


Figure 1. IAV infection in pregnancy with AAD leads to early increase of viral titers. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Control females were administered saline and media only. *A*: mice were induced into estrus, sensitized with three doses of house dust mite (HDM) extract and then time-mated. During the second week of gestation and 14 days after the last sensitization, mice were challenged with three further doses of HDM to induce AAD. Control females were administered saline and media only. One day later mice were inoculated with IAV H1N1 A/PR/8 or media. Endpoints were assessed at 3 and 7 days postinoculation. At 3 (*B*) or 7 (*C*) days postinoculation, lung tissue was homogenized and plaque assays used to determine IAV titers. *D*: pregnancy success was also assessed by counting the total number of mice that were pregnant at the end of treatment (assessed by visual observation for the presence of pups in the uterus at the time of culling) compared with total number of mice impregnated at the start of treatment. $n = 4$–8 females per group. Viral titers were assessed using the unpaired two-tailed Student *t* test (or rank-sum test for nonparametric data), pregnancy success was assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. $^*P < 0.05$; $^{**}P < 0.01$. AAD, allergic airway disease; IAV, influenza A virus; H1N1, APR8; P, pregnant; pfu, plaque-forming unit.

significantly decreased in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV infection only (Fig. 4*A*). By 7 dpi, no significant difference was observed in the expression of these miRNAs in IAV-infected pregnant mice with AAD compared with all control groups (Fig. 4*B*).

IAV Infection in Pregnancy with AAD Impairs Antiviral Responses

IFNs provide a critical antiviral response during IAV infection (26, 43), and at 3 dpi, we found that IAV-infected pregnant mice with AAD had significantly decreased mRNA expression of interferon *Ifnα*, *Ifnβ*, and *Ifnλ* compared with pregnant mice with IAV only (Fig. 5*A*). At this same time point, both *Ifnγ* and IFNγ-inducible protein (*Ip)10* (*Cxcl10*) were significantly increased in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only. Expression of the viral RNA-sensing Toll-like receptors (TLRs), *Tlr3*, *Tlr7*, and *Tlr9*, was also significantly increased in the lungs of IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only (Fig. 5*A*). The downstream early induced IFN-stimulated genes (ISGs), protein kinase (*Pkr*) and myxovirus resistant 1 (*Mx1*), showed no significant difference in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only.

By 7 dpi, mRNA expression of *Ifnb*, *Ifnl*, *Ifng*, and *Ip10* was all significantly decreased in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only (Fig. 5*B*). At this time point, *Tlr3* and *Tlr9* (but not *Tlr7*), as well as the ISGs *Pkr* and *Mx1*, were also significantly reduced in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only (Fig. 5*B*).

IAV Infection in Pregnancy with AAD Impairs Antimicrobial Responses

β-defensins (BDs) and surfactant proteins (SPs) are small peptides also known to play an important protective role in IAV infection as well as in the clearance of secondary bacterial infections (44, 45). Consequently, we measured the mRNA expression of two of the most common BDs, *Bd1* and *Bd2*, and the surfactant protein-D (*SpD*) (Fig. 6). We found that at 3 dpi, *Bd1*, *Bd2*, and *SpD* were all significantly

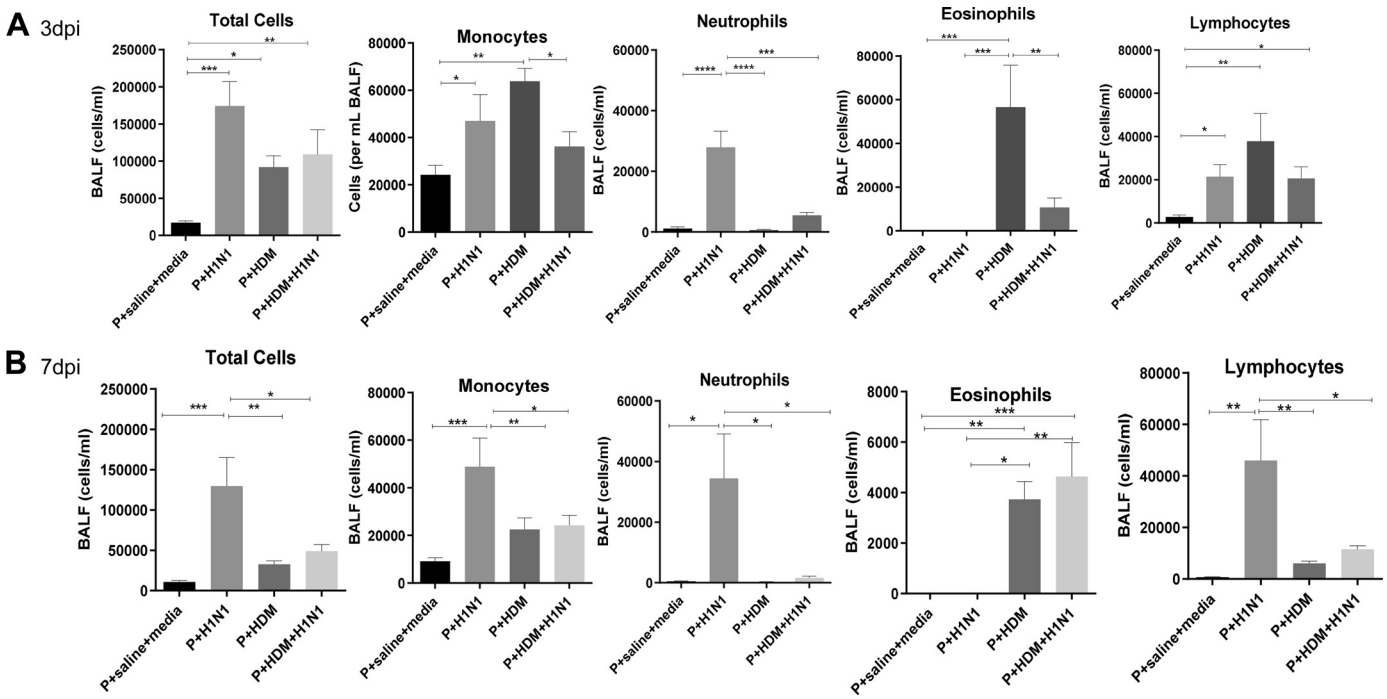


Figure 2. IAV infection in pregnancy with AAD leads to increased inflammatory cells and reduced neutrophils. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Control females were administered saline and media only. At 3 (*A*) or 7 (*B*) days postinoculation, bronchoalveolar lavage fluid was obtained, cytocentrifuged onto microscope slides, stained with hematoxylin and eosin, and total and differential inflammatory cell counts enumerated according to morphology. $n = 6$–8 females per group. Data were assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. AAD, allergic airway disease; BALF, bronchiolar lavage fluid; dpi, days postinoculation; IAV, influenza A virus; H1N1, APR8; HDM, house dust mite; P, pregnant.

decreased in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only (Fig. 6*A*). By 7 dpi, these differences had largely resolved (Fig. 6*B*).

IAV Infection in Pregnancy with AAD Results in High Levels of Mucus Production and Protracted AHR

We next investigated the impact of infection in pregnancy with AAD on some of the major pathological features of asthma, i.e., mucus hypersecretion and airway hyperresponsiveness (AHR) (Fig. 7). Histological analyses of lung tissues showed that at both 3 and 7 dpi, IAV-infected pregnant mice with AAD had significantly increased numbers of mucus-secreting cells around the airways compared with that of pregnant mice with IAV only (Fig. 7, *A* and *B*).

At 3 dpi, pregnant mice with AAD only as well as pregnant mice with AAD and IAV infection had significantly increased dynamic airway resistance in response to 50 mg/mL methacholine compared with uninfected pregnant controls without AAD (Fig. 7*C*). However, by 7 dpi, pregnant mice with IAV only and pregnant mice with AAD and IAV infection showed significantly increased airway resistance compared with uninfected pregnant controls without AAD. This shows that although AAD increases early (i.e., 3 dpi) AHR in this acute model, IAV infection is the major determinant in prolonging exaggerated AHR in pregnancy following IAV infection.

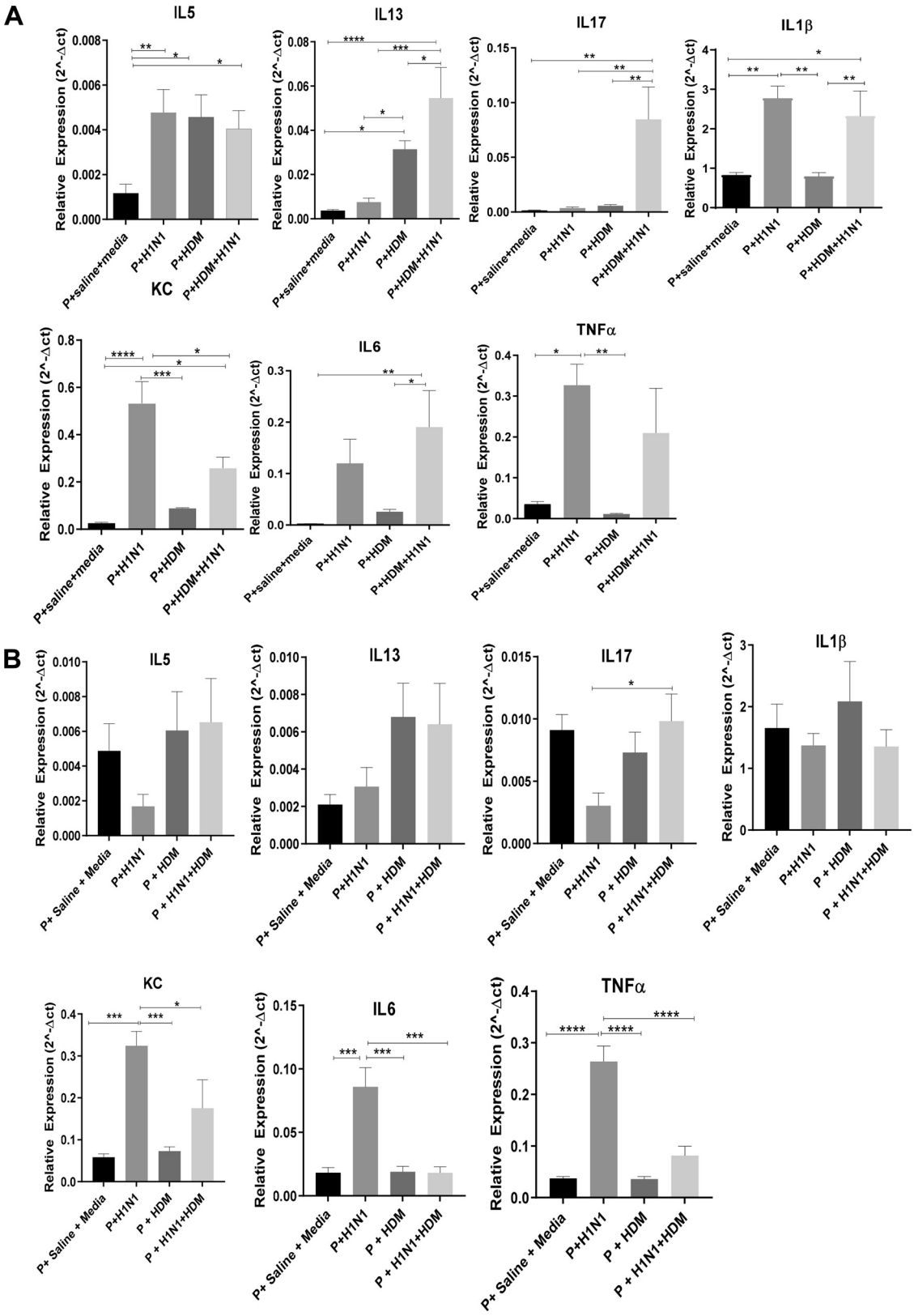
DISCUSSION

Here we make substantial progress in understanding the impact of IAV infection in pregnancy and interactions with an asthmatic phenotype, and the processes involved in increased disease severity and pathogenesis of infection in pregnancy with AAD. IAV infection in pregnant mice with AAD significantly increases inflammation compared with infection alone, evidenced by increased airway eosinophil infiltration and mRNA expression of the key asthmatic inflammatory cytokines, *Il13* and *Il17*. We also showed decreased global antiviral and antimicrobial protective immunity, including reduced neutrophil counts and *Kc* expression, reduced mRNA expression of type I and III IFNs, ISGs, and BDs, and also significant decreases in key miRNAs, known to be upregulated during IAV infections. Finally, we showed that IAV infection during pregnancy with AAD prolongs AHR in the mother and leads to reduced fertility (Fig. 8).

In our study, we showed that IAV infection in pregnant mice with AAD reduced protective neutrophil responses, which was accompanied by a reduction in mRNA expression of *Kc* at both 3 and 7 dpi. We also showed a concomitant increase in inflammatory eosinophil infiltration into the lungs at 7 dpi and also increased proasthmatic *Il13* and *Il17* expression at 3 dpi. The early *Il17* response was still significantly upregulated at 7 dpi. These cytokines are innate and adaptive inducers of Th2 responses and contribute to asthma pathogenesis (46). IL-13 alone can induce all of the hallmark features of asthma(30), and IL-17 has important roles in the cytokine storm that occurs during pathogenic IAV infection (47). Collectively, these findings indicate that in an environment of pregnancy and asthma, where there is already a Th2-skewed phenotype

(48), IAV infection substantially worsens the underlying inflammatory conditions associated with lung damage.

Neutrophils are one of the early immune cells to influx into affected areas in response to infection (49). Their recruitment to the site of infection and inflammation occurs in three phases, which include early neutrophil recruitment, amplification of infiltration, and resolution (50). A host of signals are involved in each of these phases, with the CXCL8



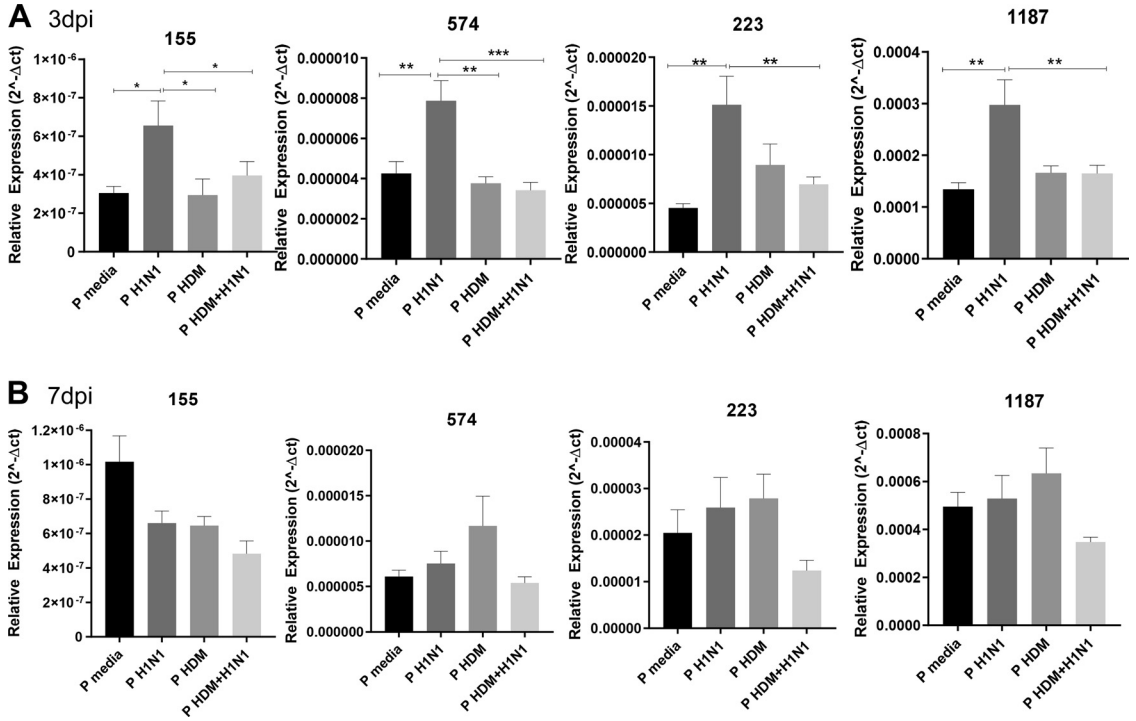


Figure 4. IAV infection in pregnancy with AAD leads to reduced expression of influenza-induced miRNAs. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Control females were administered saline and media only. At 3 (*A*) or 7 (*B*) days postinoculation, miRNA was extracted from homogenized lung tissue and the relative abundance of miR155, miR574, miR223, and miR1187 was assessed. $n = 6–8$ females per group. Data were assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. AAD, allergic airway disease; dpi, days postinoculation; IAV, influenza A virus; H1N1, APR8; HDM, house dust mite; P, pregnant.

family (including KC) being one of the most important signals in all three. Conversely, IL-17, although a key marker of influenza infections (51), increases at the late phase of neutrophil recruitment and resolution and appears to play a more prominent role in persistent neutrophil inflammation that can occur following IAV infections (50). As such, the reduction in KC expression that we saw at both early 3 dpi and later 7 dpi likely accounts for the marked reduction we observed in neutrophil infiltration at both early and late time points.

Neutrophils are professional phagocytes that have essential roles in host defense against respiratory pathogens including IAV (52). Although it is well established that neutrophils can contribute to lung injury during various pathological conditions, a decrease in their numbers during mild IAV infection enhances the development of severe clinical disease (53, 54). This protective effect of neutrophils against IAV infection has also been observed in a background with chronic obstructive pulmonary disease (COPD) (26). This is because these innate immune cells are important in the effective clearance of IAV, suppressing the infection and therefore assisting in controlling inflammation (52, 53, 55). One of the most important ways they do this is by leaving neutrophil trails enriched with CXCL12, which guides CD8 T cells to the site of IAV infection (49).

Despite the decrease in neutrophil influx at both 3 and 7 dpi, there was still a decrease in viral titers observed by 7 dpi in pregnant mice with AAD and IAV infection compared with pregnant mice with IAV infection only. This may be explained by the changes we observed in eosinophil influx during 3 and 7 dpi. Although at 3 dpi, there was a blunted eosinophil response in this group compared with pregnant mice with IAV only, by 7 dpi, we saw a significant increase in airway eosinophils in pregnant mice with AAD and IAV infection.

During the 2009 influenza pandemic, it was observed that although asthmatics were more likely to be hospitalized with IAV infection, they had less severe IAV-induced morbidity compared with nonasthmatics (56). More recently, several studies have demonstrated that acute allergic asthma protects mice from severe influenza. These studies used murine models of ovalbumin (OVA)-sensitized mice with eosinophil transfer from mice infected with IAV. They showed that eosinophils are short-lived granulocytes, capable of phagocytosing virus

Figure 3. IAV infection in pregnancy with AAD increases proasthmatic and proinflammatory cytokine responses in the lung. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Control females were administered saline and media only. At 3 (*A*) or 7 (*B*) days postinoculation, mRNA was extracted from homogenized lung tissue and the relative abundance of proasthmatic interleukin (*Il*)*5* and *Il13* and proinflammatory *Il17*, *Il1b*, keratinocyte-derived chemokine (*Kc*), *Il6*, and tumor necrosis factor-α (*Tnfα*) were assessed compared with the reference gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). $n = 6–8$ females per group. Data were assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. AAD, allergic airway disease; IAV, influenza A virus; H1N1, APR8; HDM, house dust mite; P, pregnant.

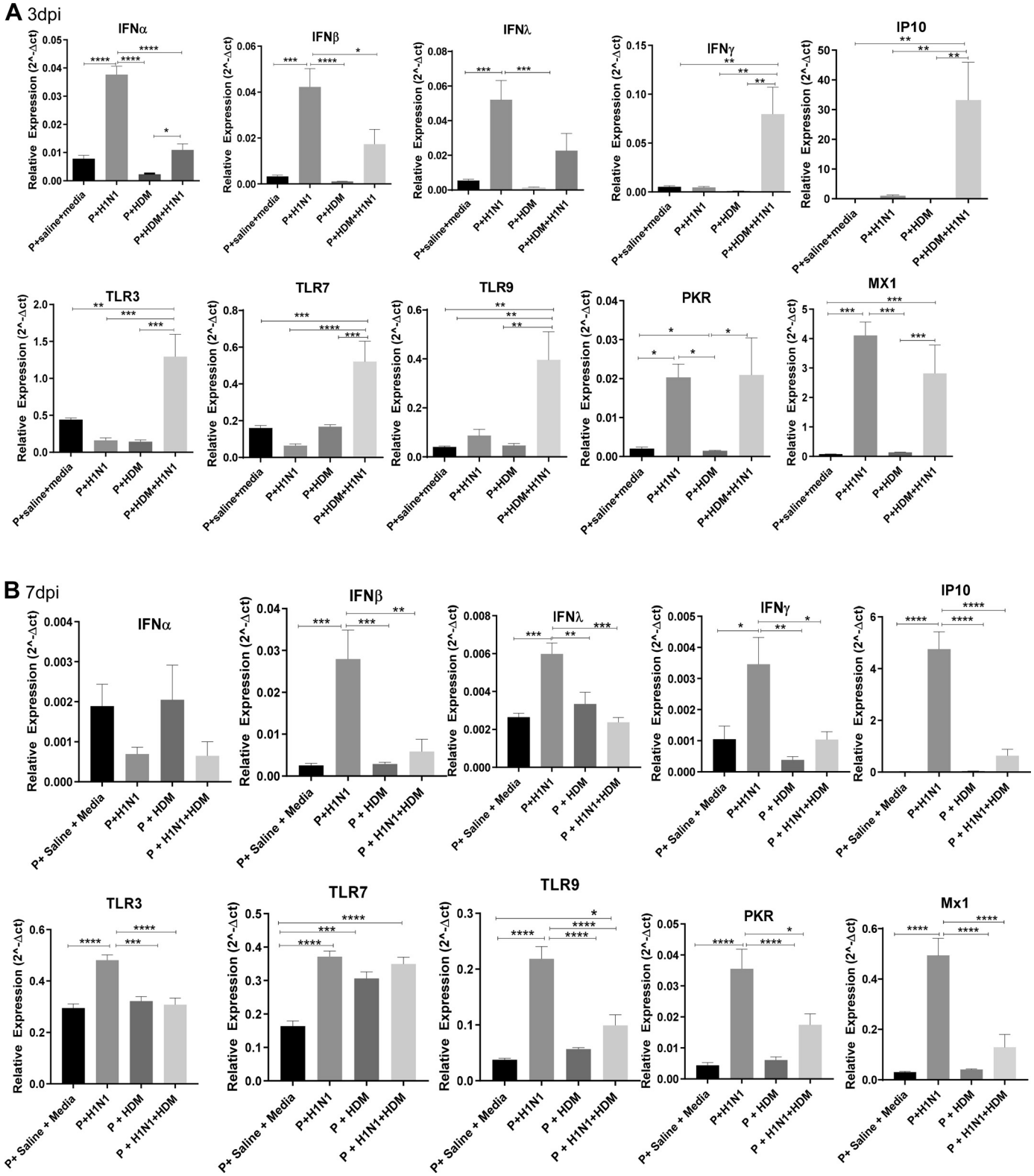


Figure 5. IAV infection in pregnancy with AAD leads to global reduction in the expression of antiviral mRNAs. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Control females were administered saline and media only. At 3 (*A*) or 7 (*B*) days postinoculation, mRNA was extracted from homogenized lung tissue and the relative abundance of interferon-α (*Ifna*), *Ifnb*, *Ifnl*, *Ifng*, IFNγ-inducible protein (*Ip)10*, Toll-like receptor (*Tlr*)3, *Tlr7*, *Tlr9*, protein kinase (*Pkr*), *Mx1*, and *Rigi* was assessed compared with the reference gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). $n =$ 6–8 females per group. Data were assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. AAD, allergic airway disease; dpi, days postinoculation; IAV, influenza A virus; H1N1, APR8; HDM, house dust mite; P, pregnant.

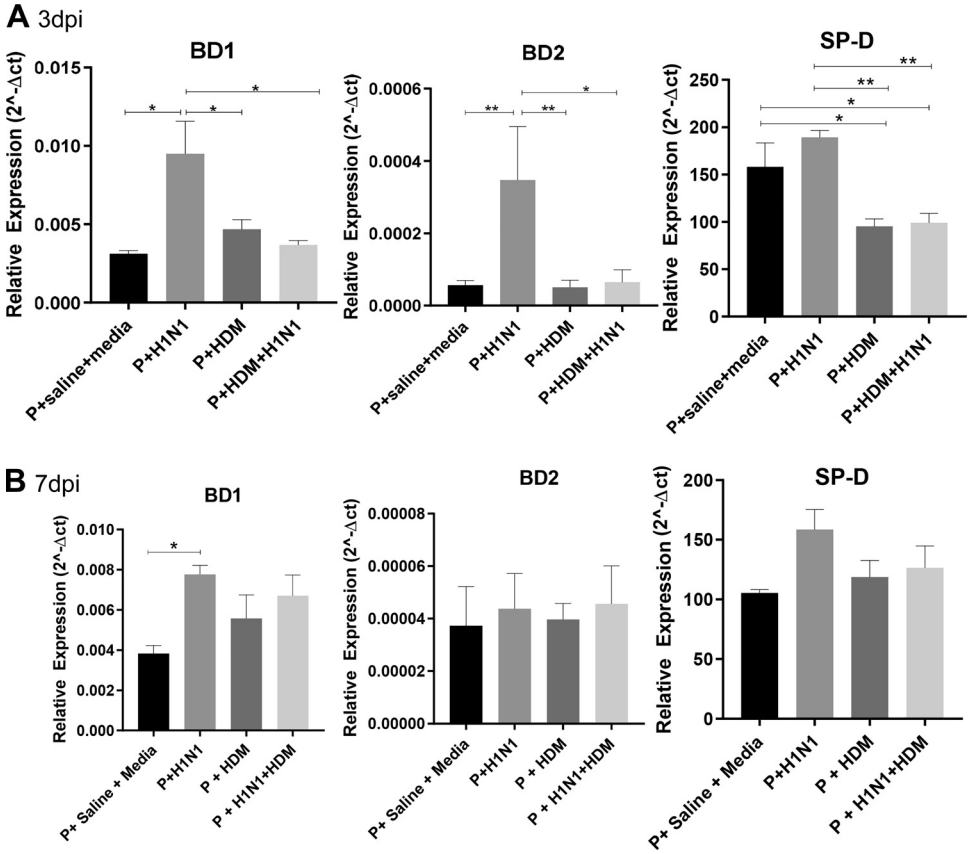


Figure 6. IAV infection in pregnancy with AAD leads to reduced expression of the antimicrobials, β-defensins, and surfactant proteins. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Control females were administered saline and media only. At 3 (*A*) or 7 (*B*) days postinoculation, mRNA was extracted from homogenized lung tissue and the relative abundance of *Bd1*, *Bd2*, and *Sp-d* was assessed compared with the reference gene hypoxanthine-guanine phosphoribosyltransferase (*Hprt*). $n = 6–8$ females per group. Data were assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. $^{*}P < 0.05$; $^{**}P < 0.01$. AAD, allergic airway disease; BD, β-defensins; dpi, days postinoculation; IAV, influenza A virus; H1N1, APR8; HDM, house dust mite; P, pregnant; SP, surfactant protein.

particles, and can themselves be infected with IAV. This results in their ability to present viral particles on their surface, which can in turn activate CD8 T cells, leading to increased viral clearance (57–59). Thus, our findings demonstrate why pregnant mice with AAD and IAV infection have an early blunted eosinophil response that markedly increased by 7 dpi and why despite having reduced neutrophil counts, there was also reduced titers. Collectively, these findings provide important insight as to why pregnant asthmatics have worsened symptom severity with increased risk for hospitalization but reduced viral load during infection.

To date, more than 15,000 miRNAs have been identified, including hundreds of viral miRNAs, each of which has the potential to target numerous genes during viral infections (60, 61). In this study, we examined the expression of four miRNAs (miR-155, miR-223, miR-574, and miR-1187), known to be induced during IAV infection and to have important antiviral activity (61). For example, high airway levels of miR-155 have been linked to reduced severity of infection (60, 62), and mice deficient in miR-155 are unable to establish protection against IAV infection as they have altered humoral and cell-mediated immunity (63). Conversely, it appears to have no or redundant roles in AAD (64). Increasing evidence also suggests a role for miR-223 in limiting inflammation to prevent lung damage during infection (60). In this study, we showed that following IAV, all four miRNAs were significantly decreased in pregnant mice with AAD compared with pregnant mice with IAV infection only. These findings indicate a dysregulation in miRNA expression that occurs because of having an underlying inflammatory lung condition during pregnancy. The fact that this decrease was observed at 3 dpi but not so markedly at 7 dpi highlights that miRNA dysregulation occurs early during viral infection.

During the 2009 swine flu pandemic, pregnant women, especially those with underlying lung disease like asthma, commonly presented with febrile illness that developed into secondary pneumonia, resulting in increased hospitalization rates (28). BDs and surfactant proteins, like SP-D, are small peptides that have essential roles in effective clearance of IAV infection as well as bacterial infections that are secondary to them (41, 44, 65, 66). In a recent study by Pinkerton et al. (32), it was shown that therapeutic administration of hBD2 by intranasal inoculation in mice with AAD resulted in a significant reduction in the influx of inflammatory cells in the BALF. This was associated with a reduction in key inflammatory cytokines like IL13 as well as a reduction in airway hyperresponsiveness.

Like miRNAs, these peptides function as part of the early innate immune response to IAV infection (44). Defensins are produced predominantly by respiratory epithelial cells and by neutrophils. Neutrophils express α-defensins, whereas epithelial cells express β-defensins (67). Thus, in our study, the cellular source of β-defensins can be attributed to the respiratory epithelial cells infected by IAV. In our study, we found that at 3 dpi, there were significant reductions in the mRNA expression of *Bd1*, *Bd2*, and *SpD* in IAV-infected pregnant mice with AAD compared with pregnant mice with IAV only. By 7 dpi, there was no significant difference in expression. These changes in β-defensins at 3 and 7 dpi also correlate with the *Il13* expression we found in the lungs and that observed previously by Pinkerton et al. (32).

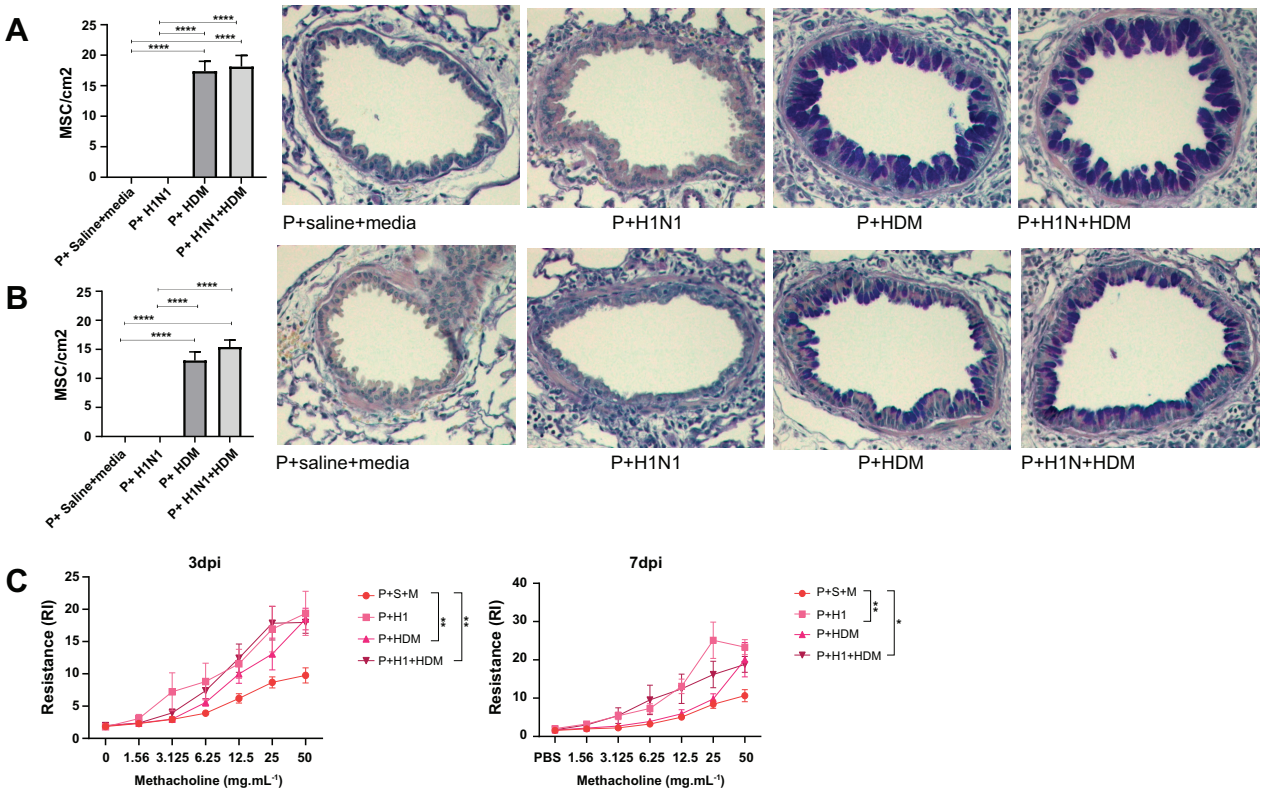


Figure 7. IAV infection in pregnancy with AAD maintains mucus hypersecretion and induces airway hyperresponsiveness. Pregnant mice with AAD were inoculated with IAV H1N1 A/PR/8 or media. Controls were administered saline and media only. At 3 (*A*) or 7 (*B*) days postinoculation, mouse lungs were collected, perfused, and inflated, stained with periodic acid Schiff-Alcian blue and the numbers of mucus-secreting cells enumerated. *C*: At 3 or 7 days postinoculation, mice were anesthetized, cannulated, and airway resistance in response to increasing doses of methacholine (0–50 μg/mL) were assessed. $n = 6$–8 females per group. Mucous hypersecretion was assessed using the one-way ANOVA (or Kruskal–Wallis test for nonparametric data) with adjustment for multiple comparisons. Airway hyperresponsiveness was measured by two-way ANOVA using a mixed model, with repeated measures and adjustment for multiple comparisons. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$. AAD, allergic airway disease; dpi, days postinoculation; IAV, influenza A virus; H1N1, APR8; HDM, house dust mite; MSC, mucus-secreting cell; P, pregnant.

Consequently, our findings highlight the importance of the BDs in both IAV infection and AAD. Reductions in the effectiveness of the BD pathway during IAV infection may be part of the mechanism causing increased lung inflammation and secondary bacterial infection leading to IAV-induced pneumonia. (55, 68, 69). Harnessing the potential of BDs and miRNAs as antiviral agents could prove to be of great value in restoring maternal immune responses before or during IAV infections.

In our study, we found that IFN-γ and IP-10, as well as the downstream ISGs, PKR, and Mx1, and the viral-sensing TLRs (3, 7 and 9) were increased in pregnant mice with AAD following infection at 3 dpi but reduced at 7 dpi. These data match with our viral titers, showing increased viral counts at 3 dpi but a reduction by 7 dpi, highlighting the immune system's attempt to mount a sufficient antiviral response against the infection. However, we also found that infected pregnant mice with AAD had impaired early and late antiviral type I and III IFN responses. We found that at both 3 and 7 dpi, mRNA expression of *Ifnβ* and *Ifnλ* was significantly reduced in the lung tissue of pregnant mice with AAD following IAV infection when compared with pregnant mice with IAV infection only. Previously, we have shown that PBMCs from pregnant women with and without asthma display similar impaired antiviral responses following in vitro infection with respiratory viruses like IAVs (16, 17). IFN-β and IFN-λ are produced early in infection by both epithelial cells and innate cells, like dendritic cells, and lead to the effective clearance of the invading virus, preventing excessive host tissue damage (43, 70–72). As such, our findings indicate that a decrease in these key antiviral agents may lead to increased host tissue damage and altered lung function even when viral titers have reduced later in infection.

We found that HDM-induced AAD induces mucus-secreting cell hyperplasia, which was maintained during IAV infection in pregnant mice with AAD. In addition, we found that pregnant mice with AAD and IAV infection showed increased AHR at 3 dpi, which was maintained at 7 dpi. In contrast, AHR induced by AAD alone was increased at 3 dpi but resolved by 7 dpi. Numerous studies in murine models have shown that IAV infection induces AHR even in the absence of AAD (73, 74). This may be mediated by IAV-induced damage to the epithelial lining (evidenced by edema) as well as increases in Th2-type cytokines like IL13 (73, 74). Indeed, in our study, we observed that pregnant mice with IAV infection, but no AAD, had increased levels of total protein in BALF (see Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.22193362.v2) indicative of lung damage as well as increased levels of IL13 at both 3 and 7 dpi. These findings help to explain why IAV infection even in the

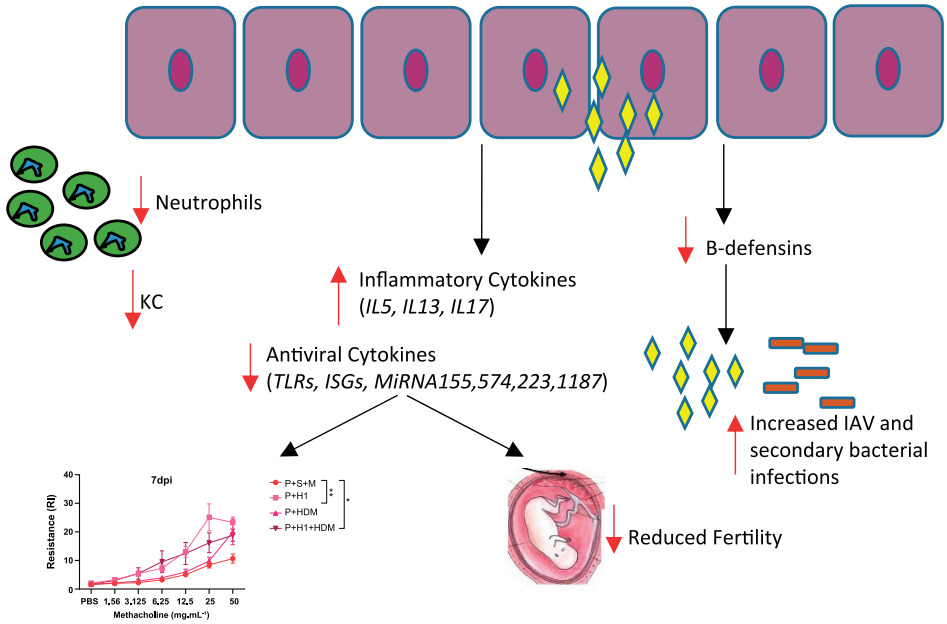


Figure 8. Schematic summary of the results: IAV infection in pregnancy with AAD is associated with reduced neutrophils and neutrophil chemokines, antiviral cytokines, and miRNA responses and the antimicrobials, β-defensins, and surfactants. Collectively, these changes lead to prolonged AHR and potential secondary bacterial infections. These findings provide mechanisms to explain the worsened outcomes observed in pregnant women with asthma following influenza infections as well as the adverse fetal outcomes that subsequently occur in these women.

absence of AAD still elicits AHR and why pregnant mice with AAD have worsened outcomes following IAV infection. Collectively, these findings show that in an environment of asthma and pregnancy, where there is already a Th2-skewed phenotype (48), IAV infection worsens the underlying inflammatory condition, increasing asthma symptoms.

A limitation of our study is that we used an acute model of AAD in mice. This model resolves over 10–20 days (12). In humans, most women have established asthma before falling pregnant. Thus, our model represents an exacerbation of asthma during pregnancy, which occurs most commonly during pregnancy following a respiratory viral infection. This is likely to influence the immune cell types that are involved in the immune responses, which is why we have included data for both 3 dpi and 7 dpi.

Conclusions

Our findings show that the combination of IAV infection in pregnancy with AAD leads to increased lung inflammation, mucus hypersecretion, and AHR. This is associated specifically with impaired global antiviral and antimicrobial responses. This provides a plausible explanation for the increase in IAV symptom severity and its effects in pregnant asthmatic women, which is observed especially during influenza pandemics. It also highlights the potential benefit that may come from targeting specific BDs or miRNAs as a prevention and/or treatment for IAV infection and secondary bacterial infections during pregnancy.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Results and Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.22193362.v2.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.L.V., J.C.H., and P.M.H. conceived and designed research; R.L.V., H.M.G., and K.D. performed experiments; R.L.V. and A.C.H. analyzed data; R.L.V., J.C.H., and P.M.H. interpreted results of experiments; R.L.V. prepared figures; R.L.V. drafted manuscript; R.L.V., J.C.H., and P.M.H. edited and revised manuscript; R.L.V., H.M.G., A.C.H., K.D., P.A.B.W., J.C.H., and P.M.H. approved final version of manuscript.

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