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Humoral immune responses during asthma and influenza co-morbidity in mice

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Abstract

Humoral immunity serve dual functions of direct pathogen neutralization and enhancement of leukocyte function. Antibody classes are determined by antigen triggers, and the resulting antibodies can contribute to disease pathogenesis and host defense. Although asthma and influenza are immunologically distinct diseases, since we have found that allergic asthma exacerbation promotes antiviral host responses to influenza A virus, we hypothesized that humoral immunity may contribute to allergic host protection during influenza. C57BL/6J mice sensitized and challenged with *Aspergillus fumigatus* (or not) were infected with pandemic influenza A/CA/04/2009 virus. Negative control groups included naïve mice, and mice with only 'asthma' or influenza. Concentrations of antibodies were quantified by ELISA, and *in situ* localization of IgA- and IgE-positive cells in the lungs was determined by immunohistochemistry. The number and phenotype of B cells in spleens and mediastinal lymph nodes were determined by flow cytometry at predetermined timepoints after virus infection until viral clearance. Mucosal and systemic antibodies remained elevated in mice with asthma and influenza with prominent production of IgE and IgA compared to influenza-only controls. B cell expansion was prominent in the mediastinal lymph nodes of allergic mice during influenza where most cells produced IgG₁ and IgA. Although allergy-skewed B cell responses dominated in mice with allergic airways inflammation during influenza virus infection, virus-specific antibodies were also induced. Future studies are required

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contributions

LAD performed immunohistochemistry, tissue harvest, cell staining, and FlowJo analysis; KSL performed tissue harvest, and cell staining; ARI performed the microneutralization assay and analysis; MP helped with tissue harvest; AES designed the experiments and performed tissue harvest, cell staining, ELISA, analyzed data, and wrote the manuscript. All authors edited the manuscript and approved the final version.

to identify the mechanisms involved with B cell activation and function in allergic hosts facing respiratory viral infections.

Keywords

Fungal allergy; Pandemic influenza; Antibody; Lung; Host-pathogen interactions

Introduction

Diseases of the respiratory system like asthma and influenza affect millions worldwide and available therapeutics are not always effective. Asthma is a syndrome of the airways that affects approximately 300 million people globally resulting in a large economic burden due to hospitalization and controller medication costs (1, 2). Allergic asthma is the most prevalent phenotype of asthma, and can result in severe disease when triggered. Fungal agents such as *Aspergillus* and *Alternaria* species are prominent allergens that are difficult to avoid, and severe asthma with fungal sensitization (SAFS) is estimated to occur in 30–70% of patients with uncontrolled asthma (3). Characteristics of allergic asthma broadly include increased serum immunoglobulin E (IgE), eosinophilic inflammation, mucus cell hyperplasia and increased mucus production, airways hyperresponsiveness, and airway wall remodeling events. These characteristics are effectively recapitulated in our *Aspergillus fumigatus* based mouse model of SAFS (4). Respiratory viruses can trigger asthma exacerbations and asthmatics are considered a high-risk group for severe disease from respiratory pathogen infections (5).

While immunologically distinct, asthma and influenza can co-occur in the same patient frequently due to high incidence of each in the population. In fact, asthma was identified as a risk factor for hospitalization during the influenza pandemic of 2009 (6). Interestingly, hospitalized asthmatics had less severe influenza morbidity compared to non-asthmatics during this pandemic (7–10). In order to investigate the association between asthma and influenza further, we generated novel combinatorial models by combining our mouse model of SAFS (11, 12) with a well-established model of influenza (13). Using these models, we showed that the pathogenesis of influenza in the allergic host was time dependent; mice exhibiting the characteristics of an acute asthma exacerbation were protected from influenza compared to those that had a more chronic form of asthma with limited allergic inflammation but heightened airway wall remodeling (13). Others have shown similar protection with different allergen-based model systems and mouse strains (14). Investigations into mechanisms by which this protection may occur have identified roles for eosinophils (15) and transforming growth factor- β (14) as mediators of anti-influenza responses in the allergic host. Both asthma and influenza are immunologically complicated diseases, and the mechanisms that underlie the interactions between influenza A virus (IAV) and the allergic host are likely multifaceted, thereby necessitating further investigation.

Humoral immune responses are dominated by IgE in allergy and IgG in influenza, while IgA may play a role in both diseases due to the involvement of the pulmonary mucosa. Antibody (Ab) producing cells residing in the lung draining lymphoid organs contribute to the

majority of Abs available during the course of influenza (16). While B cells are undoubtedly important in each disease and have well-established functions in each, their role in an allergic host with influenza is less clear. As a first step in delineating B cell function in asthma and influenza co-morbidity, we investigated B cell kinetics in the lymphoid organs and lungs of mice with acute inflammatory asthma during the course of a primary infection with the 2009 pandemic IAV strain until infectious virus clearance occurred in the lungs. We found that in spite of dynamic regulation of B cell populations, the Ab profile remained largely T_H2-biased during influenza in the allergic host.

Materials and Methods

Ethics Statement

All studies were performed in strict accordance with protocols approved by the Institutional Animal Care and Use Committees at St. Jude Children's Research Hospital and the University of Tennessee Health Science Center.

Mouse models

Six week-old female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and acclimatized in microisolator cages with α -dri bedding and *ad libitum* autoclaved chow and water for one week. Mice were then subjected to our *A. fumigatus* model of SAFS as previously described in detail (4, 11). Briefly, mice were sensitized *via* intraperitoneal and subcutaneous administration routes to *A. fumigatus* extract (Greer Labs, Lenoir, NC, USA) in the presence of Alum (Pierce, Rockford, IL, USA) and rested for two weeks. Mice were then administered fungal extract intranasally once weekly for three weeks. Finally, anesthetized animals were exposed to airborne conidia from mature *A. fumigatus* cultures *via* inhalation route for 10 minutes, rested for two weeks, and repeated. Mice that were in the Acute Asthma and Influenza (AA+Flu) groups were infected with 1000 tissue culture infectious dose 50 (TCID₅₀) pH1N1 (A/California/04/2009) in 50 μ L intranasally under isoflurane anesthesia one week after the second fungal inhalation challenge as previously published (13). All infected mice were monitored on a daily basis for signs of severe morbidity. Naïve (N) control mice were administered PBS instead of *A. fumigatus* and virus, while those in the Asthma (A) group were mock-infected with PBS, and mice in the Flu-control groups were not taken through the SAFS model.

In Ab neutralization studies, pooled sera from naïve, AA+Flu or Flu-control mice at day 9 were diluted 1:1 in PBS and injected intraperitoneally into recipient mice 48 hours prior to infection with IAV as above. Whole lungs from these recipient mice were harvested one day after infection for viral titer determination.

Sample Harvest and Processing

Mice were euthanized at predetermined time points to collect tissue. Bronchoalveolar lavage (BAL) was performed with two 1 mL aliquots of PBS through a cannula used to intubate. Left lung lobes were fixed *ex vivo* with 10% normal buffered formalin for histologic analyses. In studies where viral titers were to be determined, whole lungs were collected and

snap frozen in liquid nitrogen and stored at -80°C until use. In addition, blood, spleen, and mediastinal lymph nodes (MLNs) were collected.

BAL contents were spun down to separate cells and fluid. The cells were cytospun and stained with Diff-quick (StatLab, McKinney, TX), and the BAL fluid was stored at -80°C until use. Blood was centrifuged to collect serum which was stored at -80°C until use. Fixed lungs were embedded in paraffin and $4\text{ }\mu\text{m}$ sections across the coronal plane were affixed onto glass slides. Spleens were homogenized in PBS with 0.6 mM EDTA using the gentleMACS tissue dissociator (Miltenyi Biotec, San Diego, CA). Following red blood cell lysis with cold water, single cell suspensions were enumerated with a Countess (Invitrogen, Grand Island, NY) and stained for flow cytometry. Cells in the MLNs were liberated by grinding the tissue gently with a plunger from a 1 mL syringe in PBS/EDTA, washed, enumerated, and stained for flow cytometry.

Flow Cytometric Analysis

Approximately four million cells were re-suspended in PBS/5% FBS + $1\text{ }\mu\text{g/mL}$ GolgiPlug (BD Biosciences, San Jose, CA). Samples were incubated with human gamma-globulin to inhibit non-specific binding and DAPI (Biolegend, San Diego, CA) for 30 minutes on ice in the dark. Washed cells were then incubated with fluorescent Abs for surface markers on various B-cell populations: CD19 (clone 6D5)-BV605, B220 (clone RA3-6B2)-A700, CXCR4 (L276F12)-APC (all from BioLegend) and Ly6K (clone B33fc8k)-PE (eBioscience, San Diego, CA). Samples were washed and permeabilized using Cytotfix/Cytoperm™ kit (BD Biosciences). Samples were washed twice and incubated in the dark on ice with intracellular Abs: IgG₁ (clone RMG1-1)-PE-Cy7, IgG_{2a} (clone RMG2a-62)-PerCP-Cy5.5, IgA (clone RMA-1)-Biotin (all from Biolegend) and IgE (clone R35-72)-BV650 (BD Biosciences). A third incubation was performed to conjugate IgA-Biotin to Streptavidin-PE-CF594 (Biolegend). Controls for flow cytometry included unstained cells, single color, and both all-inclusive isotype and intracellular isotype cocktails. Data were acquired with an LSRFortessa™ (BD Biosciences) and analyzed with FlowJo v10 (Ashland, OR, USA) software.

Isotype controls were used to eliminate background fluorescence from non-specific binding. Cells were gated to eliminate debris and singlets were selected prior to all other gating. A lymphocyte gate was used prior to the gating on the B220⁺ then CD19⁺ population. Cells that expressed both B220 and CD19 were then gated on CXCR4 and Ly6K expression, and cells in each quadrant were then examined for the presence of intracellular Abs.

Immunohistochemistry

Lung sections were de-paraffinized prior to antigen retrieval performed with the IHC-Tek™ epitope retrieval steamer and buffer following manufacturer's guidelines (IHCWorld, Woodstock, MD). The Anti-Goat HRP-AEC Cell Staining kit (R&D Systems, Minneapolis, MN) was used per manufacturer's recommended protocol. Following serum blocking to minimize non-specific binding, sections were incubated in either goat anti-mouse IgE (Bethyl Laboratories, Montgomery, TX, USA) or goat anti-mouse IgA (abcam, Cambridge, MA, USA) at 1:250 and 1:500 dilutions respectively. Sections were washed and incubated in

secondary Ab, color developed, and coded for analyses. Slides were scored on a scale of 0–3 (where 0 was no staining and denoted as - and 3 was maximum staining intensity and denoted as +++) by an investigator blinded to the study conditions. Mononuclear cells that stained homogeneously for either IgE or IgA were enumerated in 20 fields at 600× on each slide based on their proximity to similar-sized airways and similar-sized blood vessels.

ELISA

The amount of Abs in the BAL fluid and serum was determined by ELISA following manufacturer's guidelines. Kits for IgG₁ and IgA were purchased from Bethyl Labs (Montgomery, TX) and IgE and IgG_{2a} kits were purchased from BD Biosciences.

Microneutralization Assay

The presence of virus-specific Abs were determined by microneutralization assay as previously described against IAV nucleoprotein (17). Briefly, 2000 TCID₅₀/mL pH1N1 was incubated in known dilutions of serum for 2 hours and used to inoculate monolayers of Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) MDCK cells for 18 hours. Cells were incubated for a further 18 hours in media supplemented with 2 µg/mL TPCK- trypsin (Worthington Biochemical, Lakewood, NJ) after removing inocula. Cells were then fixed in 80% acetone and viral nucleoprotein (NP) was detected as a marker of virus with anti-influenza A NP Ab (Millipore, Darmstadt, Germany) at a 1:2000 dilution. Microneutralization titers are reported as the final dilution at which virus neutralization occurred.

Viral titer determination

Whole lung homogenates were serially diluted 1:10 and used to inoculate monolayers of MDCK in quadruplicates for 1 hour at 37°C–5% CO₂. Unbound virus was washed and cells were incubated in the presence of 1 µg/mL TPCK-trypsin for 72 hours at 37°C and 5% CO₂. The presence of infectious virus particles was determined by incubating supernatants 1:1 in 0.5% chicken red blood cells and observing the presence/absence of a pellet. The viral titer was calculated with the Reed-Muench formula.

Hemagglutination (HA) assay

Two-fold serial dilutions were performed in each sample prior to addition of 0.5% chicken red blood cells to each sample at a 1:1 dilution. The presence/absence of pellets were observed and noted after a 30 minute incubation. The HA titer was determined as the last dilution in which no pellet was observed.

Statistical Analyses

The reproducibility of the animal models used was validated by repeating at least three times by different investigators. Mice that were naïve to any treatment were used as baseline controls while mice that were rendered allergic (*A. fumigatus*-sensitized and -challenged) were used to determine mediator levels in asthma. Time points were pre-selected to capture changes throughout the viral growth curve with and without asthma and terminated at day 9 after virus infection at which infectious virus was completely cleared from the lungs (13).

Data are represented as the mean and standard deviation of 4–5 mice per treatment group per time point. Data in each treatment group were compared to naïve controls by Mann-Whitney test and denoted by hash (#) symbol when $P < 0.05$ and considered statistically significant. Data were compared between Flu-controls and AA+Flu at each timepoint by two-way ANOVA with Sidak's multiple comparisons test and denoted by asterisk (*) symbol when $P < 0.05$ and considered statistically significant. Statistical software in GraphPad Prism v6.01 was used for analyses.

Results

Antibodies are an important component of immune system activation due to their ability to enhance innate immunity. Their role in the immune response is invaluable and manipulated as vaccines to protect against common pathogens. We noted that B cells in the airways (marked by CD19 expression) were present but not dynamically regulated during pandemic IAV infection of mice with allergic asthma (data not shown). While T_H cells were not affected in this model of asthma and influenza co-morbidity, cells of the innate immune system were regulated (13). We have previously shown that acute asthma exacerbation in mice protects against influenza morbidity marked by an absence of weight loss in spite of an equivalent viral burden at early timepoints (13, 14). However, since the role of humoral immunity in asthma and influenza co-morbidity has not been investigated, herein, we assessed the role of B cell populations and their products during the course of a primary IAV infection in mice with preexisting SAFS.

Antibody secretion was elevated in mice with allergic asthma and influenza

Secreted Abs play a role in pathogen neutralization, complement activation, and enhancing the functions of innate immune cells. Systemic levels of Abs can serve as a marker of B cell responses in the primary lymphoid organs, while Abs in the airways may represent both serum- and locally-derived Abs. Allergen sensitization and challenge led to the production of IgE, and IgG₁ which were available in the BAL fluid and serum (Fig 1A–D). While IgE level was not affected by IAV infection alone in allergic mice (Fig 1A&B), IgG₁ increased in the BAL fluid and serum of allergic mice (Fig 1C&D). IgG_{2a} is an important activator of innate immunity through complement activation and high affinity Fc γ receptor binding. Mice that recover from influenza have increased IgG_{2a} in the serum and its presence is associated with vaccine efficacy (18). While there were subtle changes in IgG_{2a} in the BAL fluid in treatment groups compared to baseline (Fig 1E), this Ab remained equivalent to baseline levels in the serum of treatment groups (Fig 1F). Although IgA was increased in the BAL fluid and serum of allergen-exposed mice, IAV infection did not seem to have a further impact in IgA availability in these mice, nor was IgA induced by IAV infection alone (Fig 1G&H). The dominant Ab in the BAL fluid was IgA, while IgG₁ was higher in the serum (Fig 1).

IgE and IgA antibody production may occur locally in the lungs in mice during disease

The presence of IgE and in the BAL fluid is a hallmark of allergic asthma. Cells localizing both IgE and IgA have been previously shown to occur in the lungs in response to *A. fumigatus* exposure (19, 20). Since IgE and IgA were found to be most abundant in the BAL

fluid, we performed immunohistochemistry to determine if these Abs localized to mononuclear cells in the lungs and enumerated cells that had uniform (not punctate) staining in the cytoplasm in an attempt to avoid counting cells that may have bound IgE/IgA through Fcε/Fcα receptors respectively.

The overall intensity after anti-IgE staining was absent or minimal in naïve and Flu-control mouse lungs, while it was present but low in the lungs of allergen-exposed mice with/without IAV infection (Table 1, Fig 2). IgE⁺ cells present in the AA+Flu lungs were frequently located near major airways and blood vessels and spread throughout the lung parenchyma (Fig 2A). The peribronchial and perivascular cell counts suggest a more pronounced localization of the IgE⁺ cells in co-morbid mice (Fig 2B). The overall staining intensity for IgA was higher in the lungs of mice with underlying allergic asthma (Table 2, Fig 3). Similarly, IgA⁺ cells were localized to the airways and blood vessels, and distributed across the lung parenchyma of mice in the AA+Flu group (Fig 3A). There were more IgA⁺ cells in the peribronchovascular areas of these mice compared to mice with influenza alone (Fig 3B). It is also noteworthy that more lung resident cells were positive for IgA than IgE. Staining in the bronchial epithelium that occurred may be due to Abs bound to these cells as part of the mucosal response (Fig 2&3).

Allergic asthma and influenza result in B cell proliferation in the lung draining lymph nodes

Single cells harvested from the secondary lymphoid organs were analyzed by flow cytometry for those expressing B220 and CD19 and were considered to be B cells (Fig 4A). The number of splenocytes did not change as a result of disease states (Fig 4B), while the cell number within the MLNs increased during disease (Fig 4C). The B220⁺CD19⁺ B cell population was increased in secondary lymphoid organs compared to naïve controls (Fig 4D&E). This population expansion in the virus-only controls was comparable to mice in the asthma and influenza group by day 3 after virus infection in both lymphoid organs assayed (Fig 4D&E).

Plasma cell populations are dynamically regulated in asthma and influenza co-morbidity

Ly6K is a marker of plasma cells in mice (21), and CXCR4 expression is important in germinal center positioning (22) as well as bone marrow homing (23). Since we did not observe significant differences in the number of CD19⁺ B cells between treatment groups, we quantified Ly6K and CXCR4 expression on B220⁺CD19⁺ B cells as markers of plasma cells and mature plasma cells able to migrate to the bone marrow during disease (Fig 5A). Total plasma cells in the spleen and MLNs were unaltered until late timepoints after IAV infection (Fig 5B&C). However, those expressing CXCR4 were reduced in spleens of allergic mice after IAV infection and maintained at levels equivalent to naïve controls (Fig 5D). These cells increased significantly in the MLNs of AA+Flu group at day 9 (Fig 5E) at which time IAV is cleared from the lungs (13).

Niche specific differences in antibody production occurred in plasma cell populations

Since plasma cells were regulated in numbers and maturity during IAV infection, and draining lymph nodes are re-constituted during pulmonary infections (24), we investigated

the classes of antibodies produced by these cells in the spleens and MLNs during disease in comparison to naïve and asthma-only conditions (Figs 6&7). Antibody classes were identified through intracellular staining on B220⁺CD19⁺Ly6K⁺ cells that did/did not express CXCR4 (Figs 6&7, A&B). A lower frequency of CXCR4⁺ plasma cells produced Abs in the spleens compared to MLNs (Fig 6 B&D). The most abundantly produced Ab by splenic CXCR4⁺ plasma cells was IgA, and IgA⁺CXCR4⁺ cells were maintained at levels equivalent to steady state in the asthma-only controls. However, IgA producing CXCR4⁺ plasma cells were reduced during disease states while plasma cells producing other classes of Abs were increased in the spleen (Fig 6C). As expected, MLN plasma cells were more abundant in disease conditions than at steady state (Fig 6D). Overall IgA⁺CXCR4⁺ plasma cells were more abundant in the MLNs, and there were significantly greater IgG-producing plasma cells in the AA+Flu mouse MLNs at the initial phase of adaptive immunity (Fig 6D). The reduction in CXCR4⁺ plasma cells in the MLN may be due to their conversion to CXCR4⁺ cells or attrition as the viral antigen burden decreases at the site of infection.

Plasma cells that upregulate CXCR4 gain the ability to migrate to tissues with CXCL12 including the bone marrow, and we noted that CXCR4⁺ plasma cells were less abundant in the lymphoid organs than CXCR4⁺ (Fig 5) possibly due to migration out of these niches. Unlike the CXCR4⁺ population of plasma cells, the abundance of CXCR4⁺ plasma cells in the spleens and MLNs varied by class of Ab being produced (Fig 7 C&D). While IgE- and IgA-producing CXCR4⁺ plasma cells in the spleen were maintained at levels equivalent to naïve controls in general, plasma cells producing these Abs were more regulated by disease states in the MLNs (Fig 7 C&D). IgG₁-producing plasma cells increased in response to IAV-infection in both lymphoid organs while IgG_{2a}-producing cells increased significantly in the spleen, especially at late time points (Fig 7 C&D). In general, however, the Ab-producing plasma cell trends in both lymphoid organs tested were similar irrespective of CXCR4 expression, although the cell frequencies were higher for the CXCR4⁺ plasma cell population (Figs 6&7).

Influenza-specific antibodies were generated after IAV infection in both groups

We have previously shown that the lung viral burden was equivalent between allergic and non-allergic mice infected with IAV at early timepoints although viral clearance was enhanced in the AA+Flu group (13). Since the availability of neutralizing Abs favor viral clearance and indicative of effective B cell memory, we measured the availability of viral NP-specific Abs in the serum of mice through microneutralization assays. Quantification of virus NP-specific Abs showed an increase in Ab titers at day 9 after IAV infection, wherein the titer in Flu-control ranged from >10 to >1280 while in AA+Flu mice the range was from >80 to >2560. However, this increase in NP-specific Ab titer noted in the AA+Flu group was not statistically significant ($p < 0.11$). Nonetheless, since there was a greater IAV-specific Ab titer in the AA+Flu group, we performed a serum transfer to determine if these sera were able to induce passive immunity. The lung viral burden was reduced equally in mice that received pooled 1:1 diluted serum from Flu-control donors ($\log_{10} 4.06 \pm 0.79$ TCID₅₀/mL) and from AA+Flu donors ($\log_{10} 4.11 \pm 0.38$ TCID₅₀/mL) compared to recipients of serum from naïve donors ($\log_{10} 4.66 \pm 0.20$ TCID₅₀/mL) 24 hours after infection. This reduction in viral burden in recipients of serum from infected mice was also not statistically significant.

However, stock virus incubated in serum from naïve mice had an HA titer of 1:64, virus incubated in serum from Flu-control mice and AA+Flu mice at day 9 had an HA titer of 1:32 suggesting that the Abs available at the resolution of IAV infection were able to neutralize viral hemagglutinin equally between the two groups.

Discussion

Immune responses triggered during the pathogenesis of diseases like asthma (25) and influenza (26) are complicated and involve the activation and function of numerous cell types and their products. Abs play a vital role in human health by alleviating threats posed by invading infectious agents and their byproducts and Ab classes are dictated by antigen, T cell involvement, and cytokine signal. In general, allergens are thought to promote class switching to IgE and IgG₁ (27), while IgG₁ and IgG_{2a} are expected to dominate during IAV infection (18, 28), and IgA production will occur as part of the mucosal response during both diseases (29, 30). The manner in which B cells are regulated during two immunologically distinct diseases that affect the same organ system such as asthma and influenza has not been fully elucidated. Therefore, we investigated the changes in Abs and Ab-producing cells in allergic mice during a primary infection with IAV as an initial step toward understanding the regulation of humoral immunity during respiratory co-morbidity. We found that IgE and IgA dominated the humoral response in allergic mice infected with IAV, while IgA and IgG₁ prevailed in Flu-control mice. However, there were only minor variations in the Ab-producing plasma cells within the lymphoid organs between disease conditions. Given that this study was aimed at demarcating the topography of humoral immunity during the course of the viral replication cycle, and the greatest virus-specific Ab titer was detected at day 9 after infection, the likelihood that our study design precluded the identification of changes in long-lived humoral immune responses between allergic and nonallergic mice after IAV infection is high.

Mucosal IgE in allergic hosts has been shown to perpetuate inflammation (31), while mucosal IgA is important in antigen neutralization in asthma (32). These mechanisms could be in effect as IgE and IgA governed the mucosa of allergic hosts throughout the course of influenza. Lung viral load in allergen-sensitized and -challenged mice was equivalent to non-allergic controls at early timepoints while viral clearance was accelerated in allergic mice in this model (13). Since IAV-specific Ab titers were highest during viral clearance, it is unlikely that immediately available Abs in the BAL fluid play a role in IAV neutralization in the allergic hosts. Interestingly, IAV infection results in the increase of both IgE (33) and IgA (34) in humans. Despite observing an increase in IgE⁺ and IgA⁺ cells in the spleen, MLNs, and lungs of Flu-control mice, neither Ab increased over baseline in the BAL fluid or serum. Although local and systemic IgE and IgA were maintained during the virus growth curve in the allergic hosts, plasma cells producing these Abs decreased in the MLN. Therefore, it is likely that local production of these Abs in the allergic hosts was maintained by mucosal B cells, while splenic plasma cells contributed to the maintenance of systemic Ab levels. Since primary IgE response to inhaled antigens occurs in the draining lymph nodes followed by local production in the lungs (35), we may have missed the initiating steps in our fungal asthma model due to the time points selected for analyses. Although passive immunization with convalescent serum showed trends at reducing the HA titer of

IAV and the viral burden in recipient mice, perhaps BAL fluid may have shown more benefit as mucosal Abs are thought to be more important than systemic Abs during host defense to pulmonary antigenic stimuli (29), and IgE and IgA stained cells were prominent in the lungs.

IgG subclasses play a dominant role within the lungs after infection with IAV (29). Previous findings that C57BL/6 mice have an IgG₁ dominant response to IAV infection (28), were reiterated in our studies. While the importance of IgG_{2a} in anti-influenza immunity had been demonstrated in the past (18), there were no marked differences in secreted IgG_{2a} in our models in spite of IgG_{2a}⁺ plasma cell population availability in the lymphoid organs. The role of IgG_{2a} between our data and that of Huber *et al.* may differ due IAV (H1N1 vs H3N2) and mouse (C57BL/6 vs BALB/c) strains utilized in the studies (18). Increased availability of serum IgG₁ in allergic mice coordinated with an increase in IgG₁-producing plasma cells in the MLNs compared to Flu-control mice. The recovery of non-allergic mice infected with this IAV dose in spite of the lack abundance in IgG_{2a} could suggest that functions of IgG_{2a} are shared by other IgG subtypes such as IgG₁ during influenza. We have previously demonstrated that asthma and influenza co-morbidity results in significantly less IFN α in the BAL fluid than in IAV infection alone (11). Interactions between type I IFNs and their receptors on B cells reduce early B cell proliferation and non-specific Ab production in response to IAV infection in the regional lymph nodes (31, 32). Therefore, the early expansion of B220⁺CD19⁺ cells and increased production of IgG₁ in the MLNs of AA+Flu mice could potentially be due to non-specific clonal expansion of B cells occurring in the absence of type I IFN receptor-mediated regulation. While this possibility needs further investigation in our model, it presents intriguing questions for alternative signaling involved in the activation, proliferation, and migration of polyclonal B cells during IAV infection in the allergic host.

Germinal center formation in the lymphoid tissues including the rapid growth in MLN size occurs as part of the inflammatory cascade in response to immune triggers (24, 36). We used the recently identified plasma cell marker Ly6K (21) in combination with CXCR4 to determine populations of plasma cells in secondary lymphoid organs that may be able to migrate to areas of high CXCL12 such as the bone marrow (23, 37) and lungs (38–40) during asthma and influenza. Although B cell expansion was more evident in the MLNs compared to the spleen during disease, the percentage of CXCR4⁺ plasma cells were equivalent between the two organs. The reduction in splenic Ly6K⁺CXCR4⁺ B cells may suggest that this receptor was downregulated in the spleen to allow cells to gain entry to the light zone or that cells expressing CXCR4 in the spleen migrated out of the spleen to other tissues with high CXCL12 availability such as the lungs or bone marrow. Lymphoid tissue resident B cells may be T cell in/dependent for activation. Since we did not measure the entire spectrum of Abs for IAV specificity, nor did we measure the viral specificity for each class of Ab secreted during the course of disease, additional information regarding the variations noted in this model are unavailable. Differences in CD8⁺ T cell responses in co-morbidity (41) suggesting alternative pathways of adaptive immune system activation exist in hosts with underlying asthma, it is conceivable that different antigenic epitopes are targeted by B cell receptors during disease. The recent innovative advances made in identification of epitope-specific T cell receptor repertoires during viral (42) and bacterial

(43) infections offer confidence that similar assays will be used in the future to determine B cell receptor specificities providing information necessary to create effective humoral responses to infectious agents.

Although specialized at Ab production, like most leukocytes, B cells also perform numerous other key roles to enhance immunity (44–46). Characteristics of asthma are reduced in B cell deficient mice (47–49), underscoring their role as regulators of allergic inflammation. Herein, we determined trends in B kinetics during the course of influenza in hosts with underlying allergic asthma. We propose that B cell populations in the MLNs contribute to systemic IgG₁ levels while those in the spleen contribute to IgA and IgE in the serum in allergic mice with influenza (Fig 8). In addition, our data suggest that IgA and IgE may be produced locally during influenza in the allergic host, and this local availability may relegate the necessity for Ab-producing cell expansion in the lymphoid organs. We have previously shown a negative correlation between eosinophils and IFN α in the airways of allergic mice during IAV infection (13). Resident eosinophils in the gut regulate local plasma cell differentiation and mucosal antibody secretion (50) while type I IFNs downregulate nonspecific B cell expansion following viral infection (51). While pathways mediating this protection are likely multifaceted, since eosinophils have been shown to regulate B cell responses (52, 53), and the trend in local B cells and Ab availability coincided with that of eosinophils in our model (13), future studies are aimed at delineating whether B cells are regulated by eosinophils during influenza in allergic hosts. Herein, we provide evidence that, while B cells are regulated during pulmonary co-morbidity, allergy-associated B cell responses dominate in allergic mice throughout the course of IAV infection although we found no evidence that these humoral responses were important in antiviral immunity in the allergic host.

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Abbreviations

AA+Flu	Acute asthma and influenza
BAL	bronchoalveolar lavage
IAV	influenza A virus
MLNs	mediastinal lymph nodes
NP	nucleoprotein
TCID₅₀	tissue culture infectious dose 50

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Figure 1. Airway and systemic antibody profiles differed in allergic mice after influenza A virus infection

Elevated IgE in the bronchoalveolar lavage (BAL) fluid and serum of allergic mice was unaffected by virus infection (**A** and **B**). While IgG₁ was elevated in the BAL fluid in virus infected mice with and without allergy, there was no difference between the groups (**C**). However, IgG₁ elevation in the serum of allergic mice with influenza was more prominent than mice with influenza alone (**D**). Amount of IgG_{2a} was only elevated at some timepoints in the BAL fluid (**E**), but largely unaffected in the serum of both groups (**F**). Mucosal IgA

was increased in allergic mice while IgA remained equivalent to basal levels in the BAL fluid of mice with influenza (**G**), and allergic mice also had significantly elevated levels of IgA in the serum compared to basal expression in mice with influenza alone (**H**). Data are represented as the mean and standard deviation in n=4–5 mice/group, of one representative study of three. N=naïve; A=asthma. # represents $P<0.05$ when compared to naïve levels by Mann-Whitney test. * represents $P<0.05$ when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak's multiple comparisons test.

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Table 1. IgE staining intensity

Group	Days after virus infection					
	0	1	3	5	7	9
Naïve	-					
Asthma	+					
Flu-only		-	-	-	-/+	-
Asthma+Flu		+	+	+	+	++

(-) indicates a score of 0, while (+) indicates a score range from 1-3. Flu=influenza

Figure 2. Peribronchovascular availability of IgE was not altered in allergic mice during virus infection

There were no IgE staining (table) or IgE⁺ cells visible in naïve mice and those with influenza during early time points, although some IgE⁺ mononuclear cells were visible at late timepoints after virus infection. IgE staining was prominent in the extracellular matrices surrounding the large airways of allergen-sensitized and -challenged mice in addition to IgE⁺ mononuclear cells spread throughout the parenchymal areas. Peribronchovascular IgE⁺ cells were enumerated and found to be significantly elevated in mice with allergic asthma which remained unaltered throughout the course of the IAV infection (**B**). Scale bar = 100 μm. Data are represented as the mean and standard error of the mean in n=20 fields/lung and representative of one study of three. # represents P<0.05 when compared to naïve levels by Mann-Whitney test. * represents P<0.05 when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak’s multiple comparisons test. N=Naïve; A=Asthma.

Table 2. IgA staining intensity

Group	Days after virus infection					
	0	1	3	5	7	9
Naïve	-					
Asthma	++					
Flu-only		+	+	+	+	+
Asthma+Flu		++	+++	+++	+++	++

(-) indicates a score of 0, while (+) indicates a score range from 1-3. Flu=influenza

Figure 3. Peribronchovascular availability of IgA was increased in allergic mice during virus infection
Lungs of naïve mice had minimal IgA staining while mice infected with IAV had slightly more IgA⁺ cells mostly around the large airways. Allergen-sensitized and -challenged mice had IgA staining in the bronchial epithelium as well as infiltrates of IgA⁺ cells in the peribronchial areas. While there was a slight reduction in the overall IgA staining intensity in lungs of allergic mice during early timepoints of IAV infection, staining pattern and IgA⁺ cells were elevated at late time points (table). Peribronchovascular IgA⁺ cells were significantly elevated in mice with allergic asthma which remained unaltered throughout the course of the IAV infection. Scale bar = 100 µm. Data are represented as the mean and standard error of the mean in n=20 fields/lung with n=5 mice/group and representative of one study of three. # represents P<0.05 when compared to naïve levels by Mann-Whitney test. * represents P<0.05 when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak’s multiple comparisons test. N=Naïve; A=Asthma.

Figure 4. Total cell numbers in secondary lymphoid organs differed by disease burden leading to an increase in B cells

Lymphocytes were gated on scatter properties and single cells were selected for further analyses based on expression of markers used to determine B cell populations (**A**, shown Day 3 Spleen). The number of splenocytes were unaffected by disease until the last time point after virus infection (**B**) while the number of cells in the mediastinal lymph nodes (MLNs) increased over time in response to all disease conditions (**C**). The percentage of B220⁺CD19⁺ cells did not change in either lymphoid compartment (**D**&**E**). Data are

represented as the mean and standard deviation in n=4–5 mice/group. # represents $P<0.05$ when compared to naïve levels by Mann-Whitney test. * represents $P<0.05$ when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak's multiple comparisons test. N=naïve; A=asthma.

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Figure 5. Plasma cells in the lymphoid organs were dynamically regulated during disease
 Plasma cells were identified based on expression of Ly6K and CXCR4 wherein double positive populations were considered to be mature plasma cells (A, shown Day 7 spleen). Plasma cells were increased in the spleen at day 7 after virus infection with significantly more plasma cells in allergic mice (B). MLN plasma cells increased at day 9 after virus infection with no difference between allergic and non-allergic mice (C). Mature plasma cells in the spleen were mostly decreased in the Flu-control mice and transiently increased at day 5 in the AA+Flu mice (D). Mature plasma cells in the MLN were significantly decreased in

the AA+Flu group at day 5 and increased by day 9 (E). Data are represented as the mean and standard deviation in n=4–5 mice/group. # represents $P<0.05$ when compared to naïve levels by Mann-Whitney test. * represents $P<0.05$ when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak's multiple comparisons test. N=naïve; A=asthma.

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Figure 6. Antibody production in B220⁺CX19⁺Ly6K⁺CXCR4⁻ plasma cells was similar between allergic and non-allergic mice after influenza

Ab isotypes produced by plasma cells was determined by intracellular staining and flow cytometry following gating on lymphocyte singlets that were positive for B220, CD19, and Ly6K and negative for CXCR4 (A&B, shown Day 7 after influenza A virus). Overall regulation of antibodies were lower in the spleens (C) compared to the MLNs. Except for minor changes at specific timepoints, similar frequencies of cells producing each class of antibody were noted in both niches. Data are represented as the mean and standard deviation in n=4–5 mice/group. # represents P<0.05 when compared to naïve levels by Mann-Whitney test. * represents P<0.05 when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak's multiple comparisons test. N=naïve; A=asthma.

Figure 7. Antibody production in B220⁺CX19⁺Ly6K⁺CXCR4⁺ plasma cells was similar between allergic and non-allergic mice after influenza

Ab isotypes produced by plasma cells were determined by intracellular staining and flow cytometry following gating on lymphocyte singlets that were positive for B220, CD19, Ly6K and CXCR4 (A&B, shown Day 7 after virus infection). Plasma cells (Ly6K⁺CXCR4⁺) producing IgE were higher in allergic mice at early timepoints while IgG₁ producing cells were maintained throughout the virus infection in these mice (B). IgA producing plasma cells increased after virus infection and returned to baseline by day 9 in both groups (B). Flu-only control mice had no increase in IgE producing plasma cells while IgG producing cells gradually increased over time (B). Mature plasma cells (Ly6K⁺CXCR4⁺) in the MLN followed similar patterns in Ab production except more mature plasma cells produced IgG₁ and IgG_{2a} (C) to CXCR4⁺ plasma cells (B). Data are represented as the mean and standard deviation in n=4–5 mice/group. # represents P<0.05 when compared to naïve levels by Mann-Whitney test. * represents P<0.05 when comparing influenza (Flu)-control to acute asthma and influenza (AA+Flu) by two way ANOVA with Sidak's multiple comparisons test. N=naïve; A=asthma.

Figure 8. Schematic representation of B cell dynamics during IAV infections in allergic hosts
 B cell populations in the mediastinal lymph nodes (MLNs) in allergic mice may contribute to IgG1 levels in the serum while plasma cells in the spleens may secrete IgA and IgE. Local populations of B cells in the lungs may contribute to mucosal IgA and IgE in the allergic hosts during influenza virus infections. These local B cells and their products may be regulated *in situ* by eosinophils and cytokines in the allergic hosts.