Research Paper

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

CrossMark

Laura A. Doorley[a,b], Kim S. LeMessurier[b,c], Amy R. Iverson[d], Maneesha Palipane[b,c], Amali E. Samarasinghe[a,b,c,*]

[a] College of Graduate Health Sciences, University of Tennessee Health Science Center, Memphis, TN 38103, United States
[b] Children's Foundation Research Institute, Memphis, TN 38103, United States
[c] Department of Pediatrics, University of Tennessee Health Science Center, Memphis, TN 38103, United States
[d] Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN 38105, United States

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 IgG$_1$ 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 to identify the mechanisms involved with B cell activation and function in allergic hosts facing respiratory viral infections.

1. 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 (Bateman et al., 2008; WHO, 2017). Allergic asthma is the most prevalent phenotype of asthma, and can result in severe disease when triggered. Fungal species such as *Aspergillus* and *Alternaria* 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 (Denning et al., 2006). 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 (Samarasinghe et al., 2011a). Respiratory viruses can trigger asthma exacerbations and asthmatics are considered a high-risk group for severe disease from respiratory pathogen infections (Busse et al., 2010).

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 (Jain et al., 2009). Interestingly, hospitalized asthmatics had less severe influenza morbidity compared to non-asthmatics during this pandemic (Van Kerkhove et al., 2011; Gilca et al., 2011; Bramley et al., 2012; Louie et al., 2009). In

order to investigate the association between asthma and influenza further, we generated novel combinatorial models by combining our mouse model of SAFS (Hoselton et al., 2010; Samarasinghe et al., 2010) with a well-established model of influenza (Samarasinghe et al., 2014). 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 (Samarasinghe et al., 2014). Others have shown similar protection with different allergen-based model systems and mouse strains (Furuya et al., 2015). Investigations into mechanisms by which this protection may occur have identified roles for eosinophils (Samarasinghe et al., 2017a) and transforming growth factor-β (Furuya et al., 2015) 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 (Waffarn and Baumgarth, 2011). While B cells are undoubtedly important in both diseases 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.

2. Materials and methods

2.1. 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.

2.2. 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 (Samarasinghe et al., 2011a; Hoselton et al., 2010). 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 min, 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 ($TCID_{50}$) pH1N1 (A/California/04/2009) in 50 μL intranasally under isoflurane anesthesia one week after the second fungal inhalation challenge as previously published (Samarasinghe et al., 2014). 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 h prior to infection with IAV as above. Whole lungs from these recipient mice were harvested one day after infection for viral titer determination.

2.3. 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-quik (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 μm 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.

2.4. Flow cytometric analyses

Approximately four million cells were re-suspended in PBS/5% FBS + 1 μg/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 min 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 Cytofix/Cytoperm™ kit (BD Biosciences). Samples were washed twice and incubated in the dark on ice with intracellular Abs: IgG_1 (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.

2.5. 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)