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Neonatal Exposure to Pneumococcal Phosphorylcholine Modulates the Development of House Dust Mite Allergy during Adult Life

Preyam S. Patel and John F. Kearney

Currently, ~20% of the global population suffers from an allergic disorder. Allergies and asthma occur at higher rates in developed and industrialized countries. It is clear that many human atopic diseases are initiated neonatally and herald more severe IgE-mediated disorders, including allergic asthma, which is driven by the priming of Th2 effector T cells. The hygiene hypothesis attempts to link the increased excessively sanitary conditions early in life to a default Th2 response and increasing allergic phenomena. Despite the substantial involvement of IgE Abs in such conditions, little attention has been paid to the effects of early microbial exposure on the B cell repertoire prior to the initiation of these diseases. In this study, we use Abs-binding assays to demonstrate that Streptococcus pneumoniae and house dust mite (HDM) bear similar phosphorylcholine (PC) epitopes. Neonatal C57BL/6 mice immunized with a PC-bearing pneumococcal vaccine expressed increased frequencies of PC-specific B cells in the lungs following sensitizing exposure to HDM as adults. Anti-PC IgM Abs in the lung decreased the interaction of HDM with the lung, IgE production, development of airway hyperresponsiveness, and Th2 T cell priming. Thus, exposure of neonatal mice to PC-bearing pneumococci significantly reduced the development of HDM-induced allergic disease during adult life. Our findings demonstrate that B cells generated against conserved epitopes expressed by bacteria, encountered early in life, are also protective against the development of allergic disease during adult life. The Journal of Immunology, 2015, 194: 5838–5850.

In the past few decades, there has been a dramatic rise in the incidence of asthma and other atopic diseases among individuals living in developed countries (1, 2). The hygiene hypothesis (1) proposes that this increasing incidence may result from a decreased frequency of childhood infection and perinatal exposure to microbes, leading to a long-lasting imbalance between the Th1 and Th2 T cell subsets initiated at this early stage of life (3). However, empirical data supporting such a mechanism are conflicting (4, 5). We previously demonstrated that, in early life, the B cell repertoire diversity is more amenable to change by bacterial exposure than it is during adult life (6); however, little is known about the long-term effects of such exposure on allergic disease initiation. Increasing evidence suggests that primary sensitization to environmental Ags occurs early in life, but airway disease may not develop until after elements of the respiratory immune system functionally mature (7). Because evidence is mounting that the possibility of reversing the disease declines with time after onset (8), early therapeutic intervention is essential to achieve this goal.

Approximately 40% of individuals with allergic rhinitis, the most common allergic disease among adults (9), and 89% of asthmatics demonstrate sensitivity to indoor allergens derived from the house dust mite (HDM) species Dermatophagoides pteronyssinus (Der p) (10, 11). More than 75% of these individuals express IgE-mediated sensitivity to the protease allergen Der p 1 (12). We and others have observed that HDM contains phosphorylcholine (PC) epitopes (13, 14) similar to those integrated into the cell wall of Streptococcus pneumoniae (pneumococcus) bacteria (15). In mice, natural TEPC15 (T15) idiotype-bearing natural anti-PC Abs generated by the B1a B cell subset (16) are germline encoded and are protective against the development of pneumococcal disease and atherosclerosis (17, 18). These observations, and our previous studies on allergic airway responses to the fungus Aspergillus fumigatus (19), suggested that B cells and Abs with PC specificity might also be protective against HDM-induced allergic disease development.

In the current study, we investigated the effects of neonatal (day 3 of life) bacteria-associated PC exposure on the later induction of HDM-induced allergic disease during adult life. Analysis of these mice demonstrated that there was a broad decrease in cellular and humoral mediators of allergic disease following challenge with HDM. The results we present argue strongly for a central role of B cells, and their Ab products, in the protection against the development of HDM-induced allergic airway disease.

Materials and Methods

Animals

C57BL/6 and μMT mice purchased from The Jackson Laboratory and TEP15 (T15) IgH gene knock-in (KI) mice, initially generated in the laboratory of K. Rajewsky (University of Cologne) (20), were maintained under specific pathogen-free conditions using approved animal protocols from the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.
In vivo intratracheal challenge and the HDm allergen model

For intratracheal (i.t.) challenge, 6- to 8-wk-old (adult) C57BL/6 mice were anesthetized using 3–5% isoflurane before being immobilized on a vertebro board using a suture string looped around their upper incisors. The tongue was extended from the oral cavity using blunt-end forceps so that liquid could be pipetted into the oral cavity. The nares were manually plugged to facilitate inhalation of the liquid suspension. Adult mice were challenged i.t. with 5 μg processed HDM particulate allergen resuspended in 50 μL PBS. Mice were rested for 7 d before being challenged i.t. daily for consecutive 5 d with 5 μg HDM in 50 μL PBS. Following the last challenge with HDM allergen, mice were rested for 2 d before euthanization. When stated, the above procedure was also conducted using Alexa Fluor 647–labeled HDM for all sensitizations and subsequent challenges. For in vivo Ab measurements, mice were given 5 μg Alexa Fluor 647–labeled HDM, Alexa Fluor 647–labeled HDM, and 50 μg anti-PC IgM Ab or Alexa Fluor 647–labeled HDM and 50 μg irrelevant isotype control Ab i.t. and then euthanized 24 h later.

Bronchoalveolar lavage fluid, lung, and mediastinal lymph node collection

Following sacrifice, trachea were cannulated to extract cellular infiltrates from the bronchoalveolar space via a 5-mL lavage with PBS. Mice were perfused by cardiac puncture with PBS plus 1% heparin prior to lung removal. For cell isolation, lungs were minced and treated with 1 mg/ml collagenase (Sigma-Aldrich) in 5 mL HBSS for 40 min at 37°C, followed by 40-μm filtration and lymphocyte separation (Celtigro). To identify CD138+ IgM−secreting B cells and PC−specific B cells, lungs were minced and treated with 5 mg collagenase plus 50 μU DNase (Sigma-Aldrich) for 40 min at 37°C. Mediastinal lymph node (MedLN) cells were collected by mechanical isolation. All cells were manually enumerated using a hemocytometer.

ELISA

ELISAs were performed to measure anti-PC IgM and IgG3 Abs along with total IgE in the bronchoalveolar lavage fluid (BALF) and Der p 1–specific serum IgE. PC-specific ELISAs were conducted using high-binding enzyme immunoassay/RIA plates (Costar) coated with 2 μg/ml PC-BSA (Biosearch Tech) and detected with alkaline phosphatase–conjugated goat anti-mouse IgM, or IgG3 (Southern Biotechnology, Birmingham, AL), respectively. Standard curves were prepared using known quantities of PC-specific IgM (BHS)-coated HDM. For all ELISAs, p-nitrophenyl phosphate substrate (Sigma-Aldrich) was added, and color development was detected with a SPECTROstar Omega Reader (BMG Labtech) at 405 nm.

EliSpot assay

High-binding flat-bottom enzyme immunoassay/RIA plates (Costar) were coated with either 5 μg/ml unlabelled goat anti-mouse IgM (Southern Biotechnology; catalogue 1021), goat anti-mouse IgA (Southern Biotechnology; catalogue 1040-01), or 5 μg/ml PC-BSA (Biosearch Technologies), respectively, to quantitate PC-specific Ab-secreting cells. HDM particulates were pelleted at 10,000 rpm for 5 min. HDM-exposed mice were perfused by cardiac puncture, and their lungs were digested with a mixture of 1 mg/ml collagenase (Sigma-Aldrich) with 50 U DNase in 5 mL HBSS (Life Technologies). Single-cell suspensions were prepared in RPMI 1640 (Life Technologies) supplemented with 2% ultralow IgFBS (Life Technologies), and then 5 × 10^5 cells were added to one well and diluted 2-fold in RPMI 1640 plus 2% low IgG FCS. Plates were then incubated for 18 h at 37°C, and cells were lysed with water plus 0.05% Tween 20. Plates were then washed three times with PBS plus 0.05% Tween-20, and then incubated with 500 μg/ml anti-IgM or anti-IgA alkaline phosphatase (Southern Biotechnology) in a solution of PBS plus 0.05% Tween 20, plus 0.1% gelatin for 37°C. Plates were then incubated overnight at 37°C, washed 3 times with PBS plus 0.05% Tween and developed for 18 h at 4°C in substrate buffer (pH 10.25) containing 1 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich), 0.1% Triton-X405 (Sigma-Aldrich), and 0.01 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Sigma-Aldrich). Plates were then washed in dH2O, and resulting spots were enumerated visually.

Ab reagents, flow cytometry, and cell identification

Hybridomas and plasmacytomas were grown in serum-free RPMI 1640 (hybridoma SfM; Life Technologies). IgM Abs were purified from supernatants on Sepharose-6B columns coupled with R36A, 1 anti-IgH6a mAb, and IgA Abs were purified on Sepharose 4B affinity columns coupled with rat monoclonal anti-IgA. HDM particulates and pneumococcal bacterial vaccine preparations were stained with purified mAbs BHI (anti-PC IgM) and SI07 (anti-PC IgA), followed by secondary goat anti-mouse FITC Abs against respective isotypes (Southern Biotechnology). BALF, MedLN, and lung cells were stained for flow cytometry using fluorochrome-conjugated Abs specific for the following molecules, transcription factor, and cytokines: CD3, CD4, CD5, CD11c, CD19, CD44, CD62L, CD86, CD117, CD127, CD138, B220, IgE, SiglecF, Ly6G, GATA3, IL-2, IFN-γ, IL-4, IL-6, and IL-13 from BD Biosciences, and anti-IgM from eBioscience. Anti–CD11c-Alexa Fluor 488, TC08-Alexa Fluor 647, B220-Alexa Fluor 700, and PC-BSA (Biosearch Technologies)-Alexa Fluor 647 were conjugated in our laboratory, according to manufacturer’s instructions. Leukocytes were identified using flow cytometry to detect specific markers based on the following gating strategy (see Supplemental Fig. 1): alveolar macrophages (SiglecF+FDC1c), T cells (CD3^+CD4^+), B cells (B220^CD19^), eosinophils (SiglecF-FDC1c), macrophages (CD11b^+CD1c^+), dendritic cells (DCs; CD11b^+CD1c^+), immature DCs (CD11c^-CD1c^+), neutrophils (CD11b^+Ly6G^+), IgE-bound basophils (B220^IgE^+^), basophils (B220^IgE^+^), and mast cells (B220^IgE^+^). IgM-expressing lung B were identified by gating on B cells (B220^CD19^) that bound anti-mouse IgM; additionally, lung plasmablasts were detected by gating on viable B220^IgM^- cells that also expressed CD138. PC-specific B cells were identified as B cells (B220^CD19^) that bound both PC-BSA-Alexa Fluor 647 and the anti-idiotypic reagent TC08 Alexa Fluor 647. T cells (CD3^+CD4^) from the MedLN were stained with isotype control Ab or anti-GATA3 Ab in conjunction with the eBioscience Foxp3 staining buffer kit (00-5523-00). Following restimulation, permeabilization, and fixation, MedLN T cells (CD3^+CD4^) were stained with isotype control Ab as well as for production of Th1 and Th2 cytokines listed above. Macrophages (CD11b^CD1c^-), DCs (CD11b^-CD1c^-), immature DCs (CD11b^-CD1c^-), and alveolar macrophages (SiglecF^-CD1c^-) were also analyzed for uptake of Alexa Fluor 647–labeled HDM. All flow cytometry analyses were performed on a FACSAccuri (BD Biosciences) or LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Single-frequency forced oscillation technique for mechanical ventilation

Mice were anesthetized with ketamine xylazine (100 mg/kg) and pancuronium bromide (0.8 mg/kg), and their tracheas were cannulated with an 18-G tube connected to the inspiratory and expiratory ports of a Flexivent ventilator (SCTEREQ), in which mice were ventilated at a rate of 160 breaths...
per minute. After initial resistance measurements (0 mg/ml methacholine), increasing concentrations (10–50 mg/ml) of methacholine (Sigma-Aldrich) were vaporized and total respiratory resistance was recorded every 12 s continuously for up to 3 min. Averages from each methacholine dose were taken from four to six mice per group to determine airway hyperresponsiveness (AHR).

**Histology and fluorescence staining**

For paraffin-embedded sections, mouse lungs were fixed in 4% PFA, dehydrated by sequentially increasing concentrations of ethanol, and, after xylen incubation, embedded in paraffin (Leica EG1150H). Six-micron-sections were cut and subjected to sterile collagenase digestion, followed by lymphocyte enrichment with lymphocyte separation medium (Cellgro; Sigma-Aldrich). BALF cells were seeded at 200 cells/well and lung digests at 100,000 cells/well and lung digests at 100,000 cells/well in 6-well plates and then rested for 12 h. BALF cells from naive unmanipulated mice were primarily SiglecF+CD11c+ alveolar macrophages, whereas leukocyte preparations from lung digests contained multiple pulmonary APCs: CD11b+CD11c+ resident macrophages, CD11c+ DCs, and CD11c+Siglec-F+ alveolar macrophages. The RAW 264.7 cell line (ATCC) was maintained in DMEM (Life Technologies) plus 10% FCS (Hyclone) and 100 μM 2-ME (Sigma-Aldrich) at 37°C. For ex vivo Ab-mediated blocking experiments, 10 μg Alexa Fluor 647–HDM was incubated with either 20 μg IgM isotype control Ab or 10 or 20 μg anti-PC IgM (BH8) for 30 min in serum-free DMEM before being added to cultures of seeded BALF cells or lung digests or unmanipulated BALF cells. In this work, we found that PC-specific IgM and IgA Abs bound small milled and sonicated HDM particles in addition to intact HDM (Fig. 1C–E). Because the bacterially induced anti-PC Abs reacted with a significant human allergen, we asked whether early-life microbial PC exposure could reduce the development of HDM-induced allergic disease (Fig. 1F). Three-day-old individual C57BL/6 littermate mouse pups were immunized with R36A, a PC-bearing unencapsulated pneumococcus strain (Fig. 1A); JY2190, an unencapsulated PC-deficient pneumococcal mutant (Fig. 1B); or treated with PBS alone. In this study, we used littermate mice to avoid the variability in immune responses resulting from differing commensal microbiota among separately caged mice. In addition to these mice, we included groups of age-matched T15 IgH gene KI (T15 KI) mice. T15 KI mice contained a high frequency of PC-specific B cells in the absence of deliberate microbial exposure. At 6–8 wk of age, these groups of mice were sensitized i.t. with HDM on day 0 and then challenged i.t. with HDM particulates daily on days 7–11 to induce allergic disease (Fig. 1F). Mice were euthanized 14 d following initial sensitization (day 0), and all analyses were performed at this time point, unless otherwise stated.

We observed that bronchoalveolar lavage of naive unmanipulated mice contained mostly alveolar macrophages (data not shown); however, HDM sensitization and challenge resulted in the accumulation of T cells, eosinophils, neutrophils, basophils, and mast cells into both the bronchoalveolar space and the pulmonary parenchyma (Figs. 1G–L, 2A, 2B). BALF from mice immunized with PC-bearing R36A as neonates and from the T15 KI mice had half as many T cells, eosinophils, neutrophils, APCs, mast cells, and basophils infiltrating the bronchoalveolar space following HDM exposure as adults compared with mice immunized with JY2190 as neonates or treated with PBS alone (Fig. 1G, 1H). The numbers of alveolar macrophages collected from all groups of mice were statistically similar, as they are normally resident cells of the lung, and are not known to be mobilized in large numbers after HDM exposure (Fig. 1H). Wright stains of BALF cytopsin confirmed that all groups of mice had similar numbers of alveolar macrophages per field (Fig. 11–L). However, fewer neutrophils, eosinophils, and lymphocytes were detected in the BALF of C57BL/6 mice immunized with R36A as neonates and the T15 KI mice compared with mice exposed to JY2190 or PBS as neonates (Fig. 11–L). Thus, mice immunized with PC-bearing R36A as neonates and the T15 KI mice had a significantly decreased infiltration of allergy-associated cells into their bronchoalveolar space following HDM exposure compared with mice exposed to PC-deficient JY2190 or PBS early in life.

Prior to HDM exposure, the lungs of naive unmanipulated mice contained 10 times fewer T cells, neutrophils, macrophages, and DCs compared with those exposed to HDM. Following HDM sensitization and challenge, we enumerated inflammatory and allergy-associated cells in the pulmonary parenchyma. Both C57BL/6 mice immunized with R36A as neonates and the T15 KI mice had significantly decreased numbers of infiltrating T cells, eosinophils, neutrophils, APCs, mast cells, and basophils in their pulmonary parenchyma compared with mice receiving JY2190 or PBS early in life (Fig. 2A, 2B). The infiltration of allergy-associated cells was validated by fluorescence microscopy of cyrosctions of lungs from mice exposed to HDM (Fig. 2C–F). C57BL/6 mice vaccinated

**Results**

**Neonatal exposure to PC-bearing R36A reduces infiltration of cells into the BALF and lungs following adult exposure to HDM**

To better understand the relationship between early microbial exposure and the development of allergic disease, we began by testing HDM for reactivity with mAbs raised against conserved epitopes on a panel of bacteria. In this work, we found that PC-specific IgM and IgA Abs bound small milled and sonicated HDM particles in addition to intact HDM (Fig. 1C–E). Because the bacterially induced anti-PC Abs reacted with a significant human allergen, we asked whether early-life microbial PC exposure could reduce the development of HDM-induced allergic disease (Fig. 1F). Three-day-old individual C57BL/6 littermate mouse pups were immunized with R36A, a PC-bearing unencapsulated pneumococcus strain (Fig. 1A); JY2190, an unencapsulated PC-deficient pneumococcal mutant (Fig. 1B); or treated with PBS alone. In this study, we used littermate mice to avoid the variability in immune responses resulting from differing commensal microbiota among separately caged mice. In addition to these mice, we included groups of age-matched T15 IgH gene KI (T15 KI) mice. T15 KI mice contained a high frequency of PC-specific B cells in the absence of deliberate microbial exposure. At 6–8 wk of age, these groups of mice were sensitized i.t. with HDM on day 0 and then challenged i.t. with HDM particulates daily on days 7–11 to induce allergic disease (Fig. 1F). Mice were euthanized 14 d following initial sensitization (day 0), and all analyses were performed at this time point, unless otherwise stated.

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**Sample size and statistics**

Values represent the mean ± SEM from three to five independent experiments with 5–10 mice per group. Statistical calculations described below were performed with Prism 4.0 software (GraphPad). Comparison of three or more groups was performed by a one-way ANOVA test, followed by Tukey’s post hoc analysis. Data from only two groups were analyzed by a two-tailed unpaired t test to determine statistical significance. In the figures, statistically significant results are represented as values of *p < 0.05, **p < 0.01, and ***p < 0.001.
cytometry, as stated in the BALF were enumerated and identified by flow cytometry. Whole mounts of HDM were stained with HDM allergen. (A–C) Ab binding was detected by flow cytometry. Whole mounts of HDM were stained with (D) isotype control Ab or (E) fluorescently labeled anti-PC IgM Ab (BH8) and viewed with a Leica/Leitz DMRB microscope. (F) Littermate C57BL/6 mice that were either treated i.p. with PBS, or immunized with JY2190 or R36A at day 3 of life, as well as the T15 KI mice were sensitized with HDM on day 0 and challenged with HDM daily on days 7–11 at 6–8 wk of age. Following challenge with HDM, mice were euthanized on day 14, tracheas were cannulated, and a 5-ml wash of the bronchoalveolar space was removed. (G and H) Cells in the BALF were enumerated and identified by flow cytometry, as stated in Materials and Methods, as well as demonstrated in Supplemental Fig. 1. (I–L) Equal volumes of BALF were cytocentrifuged onto glass slides and stained with modified Wright stain to identify alveolar macrophages (A.M.), eosinophils (eo), and neutrophils (neut). Values represent the mean ± SEM from five independent experiments with 5–10 mice per group. Data were analyzed by ANOVA, in which statistically significant results are represented as **p < 0.01 and ***p < 0.001.

with R36A as neonates and the T15 KI mice had decreased eosinophilic infiltration surrounding their bronchioles compared with mice immunized with JY2190 or treated with PBS early in life (Fig. 2C–F). Because initiation of HDM-induced allergic disease depends on the activation of DCs in the lung (23), we assessed APC activation by quantifying alveolar and tissue-resident DC and macrophage expression of CD86, a MHC-II costimulatory molecule. Tissue-resident APCs, but not alveolar macrophages, in the pulmonary parenchyma of C57BL/6 mice immunized with R36A and the T15 KI mice expressed significantly less CD86 compared with C57BL/6 mice immunized with JY2190 or treated with PBS as neonates (Fig. 2G, 2H). Therefore, exposure to PC-bearing R36A as neonates and the presence of anti-PC Abs in T15 KI mice each significantly inhibited lung-resident APC activation and diminished the infiltration of allergy-associated cells into the pulmonary parenchyma following adult exposure to HDM compared with C57BL/6 mice exposed to PC-deficient JY2190 or PBS early in life.

Neonatal exposure to PC-bearing R36A diminishes mediastinal lymph node T cell activation, IgE secretion, AHR, and allergic disease development

Upon natural inhalation of allergen, scavenging APCs migrate from the pulmonary parenchyma and present allergen to naive T cells in draining lymph nodes to prime a Th2-polarized response (24). To assess HDM priming of this Th2 response, we collected the mediastinal lymph node (MedLN) following HDM sensitization and challenge. The MedLN of HDM-exposed C57BL/6 mice were ∼30 times larger (∼2.8 mm) in diameter than those of naive unmanipulated mice (∼0.1 mm) (Fig. 3A and data not shown). C57BL/6 mice immunized as neonates with R36A and the T15 KI mice each possessed MedLNs that were half the size and weight of those collected from C57BL/6 mice immunized with JY2190 or treated with PBS as neonates (Fig. 3A, 3B). As expected, the smaller size of MedLNs corresponded with lower lymph node cellularity, including half as many T cells, B cells, neutrophils, eosinophils, and basophils (Fig. 3C, 3D). In addition to the decreased T cell population in the MedLN of C57BL/6 mice immunized with R36A as neonates and of the T15 KI mice, these T cells expressed significantly decreased levels of CD44, a marker of Ag experience (Fig. 3E–G). Thus, in C57BL/6 mice exposed to PC-bearing R36A as neonates, and in T15 KI mice, T cell activation in the MedLNs is inhibited following HDM exposure compared with C57BL/6 mice exposed to PC-deficient JY2190 or PBS early in life.

T cells primed in the MedLN can induce B cell class switching to IgE, a hallmark of allergic disease (25). We thus quantified the amount of total IgE from the BALF and Der p 1–specific IgE in the serum. Mice immunized with PC-bearing R36A as neonates and the T15 KI mice each had significantly decreased total IgE levels in their lung (Fig. 4A) and Der p 1–specific IgE in the serum (Fig. 4B) compared with C57BL/6 mice exposed to PC-deficient JY2190 or PBS early in life.

Th2-associated cytokines that induce IgE production are also significant contributors to the AHR development, production of mucus in bronchioles, and recruitment of allergy-mediating cells around bronchioles (26). To assess AHR development, following HDM exposure, mice were mechanically ventilated and a forced oscillation technique was used to measure total resistance of the respiratory system after vaporized doses of methacholine (Fig. 4C).
Following exposure to HDM, C57BL/6 mice immunized with PC-bearing R36A as neonates and the T15 KI mice had airway resistance measurements similar to naive mice that had not received HDM (Fig. 4C). These C57BL/6 mice had significantly lower airway resistance prior to methacholine exposure and following the highest dose (50 mg/ml) of methacholine compared with mice treated with PC-deficient JY2190 or PBS as neonates (Fig. 4C).

Periodic acid–Schiff–stained lung paraffin sections demonstrated that HDM-treated, but not naive unmanipulated mice, developed increased mucin-producing goblet cell frequencies in bronchioles throughout the upper and lower airways (Fig. 4D, 4F, 4H, 4J). However, mucin-producing goblet cells in the bronchioles (magenta) of C57BL/6 mice immunized with R36A as neonates (Fig. 4H) and T15 KI mice (Fig. 4I) were dramatically lower in number compared with C57BL/6 mice exposed to JY2190 or PBS as neonates (Fig. 4D, 4F). H&E-stained lung sections of naive mice not treated with HDM were free of inflammatory infiltrates (results not shown). The extensive leukocytic infiltrates associated with the bronchioles of PBS- or JY2190-immunized HDM-exposed C57BL/6 mice (Fig. 4E, 4G) were not observed around the bronchioles of mice first treated with R36A as neonates or of the T15 KI mice (Fig. 4I, 4K). These results demonstrate that neonatal exposure to PC-bearing R36A and endogenous production of anti-PC Ab in the T15 KI mice reduces IgE production, AHR development, overproduction of mucin in the bronchioles, and infiltration of leukocytes around the airways compared with mice treated with PC-deficient JY2190 or PBS as neonates.

IgM-secreting PC-specific B cells dominate the local immune response to HDM among mice immunized with PC-bearing R36A as neonates

Although mice immunized with R36A as neonates and the T15 KI mice have reduced numbers of allergy-associated cells infiltrating their lungs, they had approximately twice as many B cells in their pulmonary parenchyma following HDM exposure compared with mice first treated with JY2190 or PBS as neonates (Fig. 5A). More than 90% of these B cells expressed the IgM isotype (Fig. 2B, 2G). The frequencies of lung IgM+ B220+CD19+ B cells and IgM+ CD138+ B220low plasmablasts in both C57BL/6 mice immunized with R36A as neonates and in T15 KI mice were higher compared with those in C57BL/6 mice immunized with JY2190 as neonates (Fig. 5C, 5D). ELISPOT analysis demonstrated that C57BL/6 mice immunized with R36A as neonates and T15 KI mice have more than twice as many IgM-secreting cells in their pulmonary parenchyma compared with mice immunized with JY2190 (Fig. 5E, 5F).

To determine the location of these B cells, lung cryosections were stained for IgM (green) and laminin (gray) (Fig. 5I–L). C57BL/6 mice immunized with R36A early in life and the T15 KI mice both had higher numbers of IgM+ cells proximal to their bronchioles compared with mice immunized with JY2190 (Fig. 5H–L). Thus, mice immunized with PC-bearing R36A as neonates and the T15 KI mice had an increased frequency of IgM-expressing B cells in their lungs located proximal to their bronchioles compared with mice treated with PC-deficient JY2190 or PBS as neonates.

PC is the immunodominant epitope on unencapsulated pneumococcus such as R36A (27–29). Therefore, we next probed for PC-specific B cells in the lung. ELISPOT assays following repeated HDM exposure revealed that mice immunized with R36A early in life had ~10-fold more PC-specific IgM-secreting cells in their lungs compared with mice immunized with JY2190 as neonates (Fig. 6A, 6B). By contrast, none of the groups of mice analyzed had detectable numbers of PC-specific IgA-secreting cells in their lungs (data not shown). Using flow cytometry, PC-specific B cells were identified as B cells that bound both PC-BSA and TC68, an Ab reagent that detects the PC-specific T15 IgH (VH15) (Fig. 6D, 6E). Similar to our ELISPOT analysis, mice immunized with R36A as
neonates and the T15 KI mice had an increased percentage of PC-specific B cells in their lung compared with mice immunized with JY2190 or PBS as neonates. Following exposure to HDM, (A) MedLNs were removed and (B) weighed. Cells removed from the MedLN were (C and D) enumerated and identified by flow cytometry, as stated in Materials and Methods, as well as demonstrated in Supplemental Fig. 1. CD44 expression on CD4+ T cells from MedLNs are presented as (E) flow plots and (F) histograms and (G) quantified as CD44 mean fluorescence intensity. Values represent the mean ± SEM from five independent experiments with 5–10 mice per group. Data were analyzed by ANOVA, in which statistically significant results are represented as *p < 0.05, **p < 0.01, and ***p < 0.001.

That PC-specific B cells did not express CD5 (data not shown); however, these cells did express CD138 (Fig. 6D). To further determine the phenotype and distribution of these PC-specific B cells following HDM exposure, we stained lung cryosections with anti-mouse IgM (green), anti-CD138 (red), PC-BSA (yellow), and CD5 and CD138 expression to determine their B1a or plasmablast phenotypes, respectively. This analysis demonstrated that PC-specific B cells did not express CD5 (data not shown); however, these cells did express CD138 (Fig. 6F). To further determine the phenotype and distribution of these PC-specific B cells following HDM exposure, we stained lung cryosections with anti-mouse IgM (green), anti-CD138 (red), PC-BSA (yellow), and AB CDF E

FIGURE 4. Mice exposed to PC-bearing R36A as neonates produced decreased IgE, did not develop severe AHR, and exhibited diminished mucin production in their bronchioles and cellular infiltration around these airways. Following exposure to HDM, (A) total IgE Ab in the BALF was quantified from the first milliliter of the BALF and (B) Der p 1–specific IgE from the serum by ELISA. (C) Groups of HDM-exposed mice were mechanically ventilated, and total airway resistance was measured following challenges with vaporized methacholine. Following exposure to HDM, 6-μm paraffin-embedded lung sections were stained with (D, F, H, and J) periodic acid–Schiff stain or (E, G, I, and K) H&E. (D, F, H, and J) Upper images are magnifications of marked areas in lower periodic acid–Schiff–stained bronchioles. Values represent the mean ± SEM from three to five independent experiments with 5–10 mice per group. Data were analyzed by ANOVA, in which statistically significant results are represented as *p < 0.05, **p < 0.01, and ***p < 0.001.
and anti-laminin (gray) (Fig. 6G–J). IgM+ CD138+ cells were detected in lung sections from mice treated with JY2190 or PBS as neonates, but these cells did not react with PC (Fig. 6G, 6H). However, in the lungs of mice treated with R36A as neonates (Fig. 6I) and the T15 KI (Fig. 6J) mice, large CD138+ IgM-expressing PC-specific cells were detected at a higher frequency than those identified in PBS-treated mice (Fig. 6G) or those immunized with JY2190 as neonates (Fig. 6H). Mice immunized with R36A as neonates also had some clusters of non-PC–binding IgM-expressing cells in their lungs (data not shown). Thus, mice immunized with PC-bearing R36A as neonates and the T15 KI mice had an increased frequency of CD138+ IgM-expressing PC-specific cells in their lungs following exposure to HDM compared with mice treated with PC-deficient JY2190 or PBS as neonates.

We next quantified the amount of PC-specific IgM in the BALF of these mice after HDM exposure (Fig. 6C). Prior to HDM exposure, IgM, IgA, and IgG3 anti-PC Abs in the BALF and PC-specific B cells in the pulmonary parenchyma of these mice were undetectable (data not shown). However, following repeated HDM exposure, mice immunized with R36A as neonates secreted higher amounts of PC-specific IgM and IgG3 Ab, but not IgA, into their bronchoalveolar space compared with those immunized with JY2190 (Fig. 6C and data not shown). Thus, in mice exposed to PC-bearing R36A early in life and in T15 KI mice, there was an increased secretion of PC-specific Abs into the bronchoalveolar space compared with mice treated with PC-deficient JY2190 or PBS as neonates.

Anti-PC IgM Abs decrease ex vivo uptake of HDM by resident APCs and the in vivo trafficking of HDM to the MedLN and priming of a Th2 response

In the presence of complement, anti-PC IgM Abs are efficient at promoting the opsonization of PC-bearing microbes and apoptotic cells (30). However, the lung contains 500 times less the concentration of complement components C3, C5, and factor B compared with those present in serum (31). To better understand the function of anti-PC IgM Abs in the lung, we designed an ex vivo system to determine mechanisms involved in the modulation of APC activation by Ab interactions with HDM. Alveolar macrophages and pulmonary APCs isolated from mouse lungs were cultured ex vivo. Next, 10 μg Alexa Fluor 647–labeled HDM was combined with 20 or 10 μg purified monoclonal anti-PC IgM (BH8), or 20 μg isotype control Ab was added to serum-free cultures containing alveolar macrophages and pulmonary APCs (resident DCs and macrophages), and to the mouse macrophage cell line (RAW 264.7 cells) for 3 h. HDM uptake by APCs under these conditions was determined by flow cytometry. Anti-PC IgM Abs decreased the uptake of Alexa Fluor 647–labeled HDM by alveolar macrophages and pulmonary APCs isolated from mouse lungs were cultured ex vivo. Next, 10 μg Alexa Fluor 647–labeled HDM was combined with either 20 or 10 μg purified monoclonal anti-PC IgM (BH8), or 20 μg isotype control Ab was added to serum-free cultures containing alveolar macrophages and pulmonary APCs (resident DCs and macrophages), and to the mouse macrophage cell line (RAW 264.7 cells) for 3 h. HDM uptake by APCs under these conditions was determined by flow cytometry. Anti-PC IgM Abs decreased the uptake of Alexa Fluor 647–labeled HDM by alveolar macrophages, pulmonary APCs, and RAW 264.7 cells in a dose-dependent manner (Fig. 7A). In addition to decreased uptake of HDM particulates, anti-PC IgM Abs also decreased the activation of alveolar macrophages and pulmonary APCs as demonstrated by decreased expression of CD86 (Fig. 7B).

To determine whether these IgM Abs functioned similarly in vivo, purified monoclonal anti-PC IgM Ab (BH8) or isotype-control Ab
were administered i.t. to mice along with Alexa Fluor 647–labeled HDM. Uptake of HDM by APCs in the lung was measured by flow cytometry at 24 h. Purified anti-PC IgM mAb (BH8) decreased the interaction of HDM with alveolar macrophages, resident DCs, and macrophages in the lung by about half compared with mice treated with HDM and isotype control (ISO) or HDM alone (Fig. 7C). Alveolar macrophages collected from the BALF of mice treated with HDM plus anti-PC IgM Ab also expressed decreased amounts of CD86 compared with mice treated with HDM plus isotype control Ab (Fig. 7C). Collectively, these results demonstrate that anti-PC IgM interferes with the uptake of HDM by APCs in vitro, ex vivo, and in vivo.

We next determined the amount of Ab coated on HDM particles in the lung following HDM sensitization and challenge. We used a sandwich ELISA technique to capture HDM particles from the BALF and quantified the amount of IgM bound to these particles. Significantly more IgM was bound to HDM from the BALF of C57BL/6 mice immunized with R36A and from T15 KI mice than was bound to HDM from mice immunized with JY2190 (Fig. 7D). To trace the uptake and trafficking of HDM by APCs in the lung, sensitized and challenged mice with Alexa Fluor 647–labeled HDM. Labeling of HDM with Alexa Fluor 647 did not affect the outcome of HDM-induced allergic disease (data not shown). We observed a decreased percentage of alveolar macrophages, and lower numbers of resident DCs, macrophages, and neutrophils in the lungs of C57BL/6 mice immunized with R36A and the T15 KI mice that had taken up Alexa Fluor 647–labeled HDM (Fig. 7E, 7F). Additionally, these mice also had 10 times fewer in number and

FIGURE 6. Neonatal exposure to PC-bearing R36A resulted in the accumulation of CD138+ IgM-expressing PC-specific cells in the lung following exposure to HDM. Following exposure to HDM, lungs were perfused and enzymatically digested. Cells isolated from perfused lungs were incubated on PC-coated plates for ELISPOT analysis and were (A and B) visually enumerated. (C) The first milliliter of the BALF was collected to quantify (A) anti-PC IgM by ELISA. (D and E) The percentage of B cells that were PC specific was identified by flow cytometry, as stated in Materials and Methods, as well as demonstrated in Supplemental Fig. 1.
4 times lower percentage of HDM-bearing DCs in the MedLN compared with DCs isolated from the MedLN of C57BL/6 mice immunized with JY2190 as neonates (Fig. 7G, 7I). To determine whether early exposure to R36A influences the degree of Th2 priming in adults, we quantified the number of MedLN T cells expressing GATA3, a Th2-commitment factor. C57BL/6 mice immunized with R36A and the T15 KI mice had 4 times less GATA3-expressing T cells in their MedLN compared with mice immunized...
with JY2190 or treated with PBS as neonates (Fig. 7H). These results demonstrate that mice immunized with PC-bearing R36A as well as the T15 KI mice have decreased HDM trafficking by DCs and a significantly inhibited generation of a Th2 response in the MedLN compared with mice treated with PC-deficient JY2190 or PBS as neonates.

**Early microbial exposure does not influence the Th1 or Th2 T cell subset balance**

It has been hypothesized that early microbial exposures generate a robust Th1 T cell response capable of suppressing the overt development of Th2 responses (3, 4). BALF collected from naive unmanipulated mice prior to HDM exposure did not contain detectable levels of cytokines or chemokines (data not shown). Following HDM exposure, the C57BL/6 mice immunized with either pneumococcal strain as neonates had statistically similar levels of Th1-associated cytokines, such as IL-2, IL-12, and IFN-γ, in the airways (Fig. 8A). There was also no statistical difference in the frequency of IL-2−, IL-12−, or IFN-γ−producing MedLN T cells (Fig. 8D). However, the lungs of C57BL/6 mice treated with R36A as neonates and the T15 KI mice contained significantly lower concentrations of Th2-associated cytokines, such as IL-4, IL-5, IL-6, IL-13, and IL-9 compared with mice exposed to JY2190 or PBS as neonates (Fig. 8B). These protected mice also had a significantly decreased frequency of IL-4−, IL-6−, and IL-13−producing T cells in their MedLN (Fig. 8E). Therefore, mice immunized with PC-bearing R36A as neonates secrete significantly decreased Th2-associated cytokines in their airways and had a significantly reduced frequency of Th2 cytokine-producing cells in their MedLN compared with mice immunized with PC-deficient JY2190. A comparison of Th1- and Th2-associated cytokine production in C57BL/6 mice treated with PBS, and those immunized with JY2190 or with R36A, as well as T15 KI mice demonstrated no statistical differences in the amounts of Th1-associated cytokines secreted into the lungs or produced by T cells in the MedLN (Fig. 8B, 8E). Additionally, nonspecific early microbial exposure, in the case of mice that received JY2190 as neonates, is not sufficient to significantly decrease the production of Th2-associated cytokines or Th2 T cell generation.

Because the decreased secretion of Th2-associated cytokines into the lung (Fig. 8B) could result from either a lower number of cells secreting Th2-associated cytokines or impaired production of chemokines that recruit these cells, we quantified the level of chemokines produced in the lung that are potentially capable of recruiting Th2-associated cells into the lung. C57BL/6 mice immunized with R36A as neonates and the T15 KI mice secreted decreased concentrations of CXCL1, CXCL2, RANTES, and CCL4 compared with C57BL/6 mice exposed to JY2190 or PBS as neonates (Fig. 8C). Therefore, neonatal immunization with PC-bearing R36A leads to decreased chemokine secretion into mouse lungs compared with immunization with PC-deficient JY2190. These observations suggest that immunization with PC-containing formulations can decrease the influx of allergy-associated cells into the lung.

**Discussion**

There is a clear association between the rise in incidences of atopic conditions and decreased rates of infection among newborns (1, 32); however, a mechanism to explain this trend has remained elusive. Although a permanent skewing of the Th1 and Th2 T cell subsets has long been proposed (3, 4, 33), empirical evidence to support this claim has been confounding. Less effort has been made to understand how the developing neonatal immune system is affected by early exposure to microbes. We, and others, have demonstrated that neonatal bacterial exposure can select rare B cell clones for expansion, permanently skewing the overall diversity of the developing B cell repertoire (6, 34, 35). As IgE Ab producers, B cells initiate multiple manifestations of allergic pathologies, and are thus clearly considered important instigating components of atopic diseases (25). In this study, we now demonstrate that B cells, and their

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**FIGURE 8.** Early microbial exposure did not influence the Th1 or Th2 T cell subset balance. Following HDM exposure, the first milliliter of the BALF was collected for quantification of (A) Th1-associated and (B) Th2-associated cytokines and (C) chemokines using Milliplex magnetic beads and MILLIPLEX Analyst V5.1 software. (D and E) T cells isolated from the MedLN were restimulated with PMA + ionomycin for 6 h before being fixed and permeabilized to stain for the production of (D) Th1 and (E) Th2 cytokines, as stated in Materials and Methods, as well as demonstrated in Supplemental Fig. 1. Data were analyzed by ANOVA, in which statistically significant results are represented as *p < 0.05, **p < 0.01, and ***p < 0.001. (F) Exposure to PC-bearing microbes during early life generates PC-specific B cells that secrete Abs locally in the lung following incidence with PC-bearing HDM. These B cells and their non-IgE Ab products diminished the development of Th2 responses and allergic pathology.
Ab products, can exert an opposing effect to this accepted patho-
logical function by impeding the development of allergic disease in
response to the PC-bearing HDM allergen.
Mice exposed to PC-bearing pneumococcus as neonates had a 10-fold increased CD138+ IgM-expressing PC-specific B cells in
the pulmonary parenchyma, and higher concentrations of secreted Abs in their lung following exposure to HDM compared with mice exposed to PC-deficient pneumococcus. When passively admin-
istered or endogenously produced, these anti-PC IgM Abs reduced
the interaction of HDM with APCs in the lung. Increased anti-PC
IgM Ab titers were also associated with decreased trafficking of
the allergen to the draining lymph node and significantly reduced a Th2 T cell response. Because Th2-associated cytokines induce the pathology and inflammation associated with allergic disease (36), we hypothesize that the diminished priming of a Th2 response in the MedLN was directly responsible for the observed decrease in
IgE production and the recruitment of allergy-associated inflam-
matory mediators into the lung. This effect was accompanied by a
moderate development of airway resistance, inhibited production of mucin-producing goblet cells, and reduced numbers of allergy-
associated effector cells in the bronchoalveolar space and pulmo-
nary parenchyma. In addition to an overall decreased cellular infil-
tration, APCs and effector T cells in lung tissues expressed lower levels of cell surface markers indicative of inflammation and activation. Thus, a single immunization of neonatal mice with PC-
bearing pneumococcus 3 d after birth had a potent and long-lasting impact on susceptibility to HDM-induced allergic inflammation in adult mice (Fig. 8F).
A tenet of the hygiene hypothesis is that microbial exposure during
development tempers the initiation of allergic diseases later in adult life via a global redirection of Th2 to Th1 responses (1, 4, 37). However, C57BL/6 mice immunized with PC-deficient JY2190 pneumococcus as neonates developed manifestations of allergic disease, upon HDM challenge, similar to those seen in mice treated with PBS. Additionally, the limited development of allergic disease observed in the T15 KI mice that had received no deliberate bacterial exposure, which could alter the Th1/Th2 balance, also strongly supports a protective contribution of bacterial-induced specific Ab in this model of HDM-induced allergic disease. Based on these findings, we infer that neonatal exposure to PC-bearing pneumococcus modulates the development of HDM-induced aller-
genic disease by B cell–dependent mechanisms, and not the stimula-
tion of Th1 cells. To further strengthen our hypothesis that B cells and Abs play a pivotal role in the protection against allergic airway
disease development, we immunized 3-d-old B cell–deficient µMT mice with PC-bearing pneumococcus R36A, or PC-deficient JY2190, or PBS alone (Supplemental Fig. 2). At 8 wk of age, these mice were challenged with HDM. µMT mice challenged with HDM developed similar allergic manifestations whether they had been treated with PBS, or immunized with JY2190 or R36A as neonates. Compared with our observations in similarly treated C57BL/6 mice, these results demonstrate that R36A-triggered pro-
tection against HDM-induced allergic disease does not occur in the
absence of B cells (Supplemental Fig. 2).
Our studies also demonstrate that the decreased development of HDM-induced allergy by early bacterial exposure required the PC epitope to be expressed by both the challenging bacteria and the allergen. The T15 KI mice and C57BL/6 mice treated with pneu-
 mococcal strains JY2190 or R36A were not protected from allergic disease resulting from repeated exposure to the PC-deficient timothy grass pollen allergen (data not shown). We also investigated the sig-
nificance of timing of microbial exposure. Adult mice immunized with either strain of pneumococcus exhibited similar development of HDM-induced allergic disease (Supplemental Fig. 3). These findings
are consistent with our previous results demonstrating that neonatal, but not adult, exposure to group A streptococci significantly dam-
pens development of Aspergillus-induced airway disease (19). We further determined whether anti-PC IgM Abs were sufficient to
dampen the development of HDM-induced allergic disease. Mice
administered anti-PC IgM Abs 1 h prior to sensitization and every challenge with HDM displayed significantly decreased development of HDM-induced allergic disease compared with mice administered
isotype control Ab (Supplemental Fig. 4). Thus, our combined ob-
servations of 1) the protective effects elicited by neonatal exposure to
PC-bearing pneumococcus but not PC-negative bacteria in B cell–
sufficient mice; 2) the optimal protection in T15 KI mice that have not been deliberately immunized with PC-containing bacteria; 3) the lack of protection observed in µMT mice; and 4) the protection afforded by passive administration of highly purified IgM anti-PC mAbs argue strongly for the central role for B cells and IgM Abs in protection against the development of allergic airway disease in this
HDM model of allergic airway disease.
PC is a conserved molecule detectable on HDM particles con-
taining allergenic cargo such as HDM-associated Der p 1 (38). Mammalian leukocytes have multiple innate receptors for PC, including CD36 and platelet-activating factor receptor on leuko-
cytes, epithelial cells, and APCs (39). Because PC associated with
HDM particles can potentially ligate these PC-recognizing innate
receptors, CD36 and platelet-activating factor receptor may pro-
duce entry points for phagocytosis on cells that express these proteins. Subsequent processing could then prepare the Der p 1 allergenic cargo for presentation, resulting in the activation of Th2
T cells. Although numerous pulmonary cells bearing PC-ligating
receptors are potentially involved in the initiation and development of allergic disease, DCs are specifically essential in the development of HDM-induced allergic disease (40). Thus, interference of HDM allergen uptake by the DCs may be one way that anti-PC Abs disrupt a critical step in the induction of allergic disease. Thus, we hypothesize that Abs, by impeding the interaction of PC-decorated
HDM particles with APCs in the lung, play a major role in inhib-
iting the development of HDM-induced allergic disease.
Pneumococcal infections among infants are a major cause of
morbidity and mortality in both underdeveloped and developed
countries (41). Despite the recent introduction of neonatal vacci-
nation with Prevnar 13 and decreased infection rates of the serotype-specific pneumococcal strains covered by this vaccine
(42), serotype replacement is prevalent (43), with the consequence
that pneumococcal infections still occur, even in developed
countries. Neonatal responses to purified polysaccharides are poor,
but, in children colonized or infected with pneumococcus, some mount high Ab responses to PC, even at 2–3 y of age, whereas others do not (44). Whether childhood pneumococcal infections correlate with decreased HDM sensitivity is unknown; however, there is evidence that high IgE titers to pneumococcus are associ-
ated with decreased risk for asthma (45). Additionally, non-
asthmatics have Abs to a larger number of pneumococcal serotypes
compared with asthmatics, and when PBMCs from these non-
asthmatics were cultured with HDM, they released less IL-5 than those from asthmatics (46). Many other organisms such as Neisseria
sp, Haemophilus influenzae, Pseudomonas aeruginosa, and hel-
imnths also contain PC epitopes (39). Helminth infections are in-
versedly correlated with allergic disease incidence (47). This inverse correlation has mostly been attributed to the potent induction of regulatory T cells; however, alternative mechanisms such as an Ab response to PC-associated glycoprotein, ES-62, could also be
modulating allergic disease development (48).
Anti-PC Abs secreted by B1a cells have been shown to modulate the outcome of both pneumococcal disease and atherosclerosis (49).
Although our PC-specific B cells do not express CD5, B1a cells lose their CD5 expression as they become plasma cells (50); therefore, we cannot exclude the possibility that B1a cells induced or expanded by our PC-based vaccine modulate the development of HDM-induced allergic disease. Currently, there is no Food and Drug Administration-approved PC-based vaccine or cure for allergies or asthma, and management of most atopic diseases involves treatment of symptoms. Immunotherapy has successfully induced allergen tolerance; however, this anti-inflammatory response is restricted to the highly variable peptides and extracts administered during immunotherapy. Immunotherapy generates blocking Abs that can potentially inhibit allergen-induced mediator release from mast cells and basophils or Ag presentation to T cells (51). However, the mechanism by which these Abs reduce allergic disease development is limited to the Ag specificity of IgE Abs, and would most likely only function after IgE generation and progression of allergic disease. Because we have shown that the PC-specific B cells generated by neonatal exposure to PC-containing bacteria have the potential to inhibit HDM-induced allergic disease development, we suggest that a regimen of suitable vaccine exposure, informed microbiota manipulation, or probiotic use in at-risk children could be used to prevent the development or further progression of respiratory allergies and atopic diseases later in life.

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