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Pulmonary α -1,3-Glucan-Specific IgA-Secreting B Cells Suppress the Development of Cockroach Allergy

Preeyam S. Patel, R. Glenn King, and John F. Kearney

There is a higher incidence of allergic conditions among children living in industrialized countries than those in developing regions. One explanation for this is reduced neonatal exposure to microbes and the consequent lack of immune stimulation. Sensitivity to cockroach allergen is highly correlated with the development of severe asthma. In this study, we determined that an Ab to microbial α -1,3-glucan binds an *Enterobacter* species and cockroach allergen. Neonatal, but not adult, mice immunized with this α -1,3-glucan-bearing *Enterobacter* (MK7) are protected against cockroach allergy. Following exposure to cockroach allergen, α -1,3-glucan-specific IgA-secreting cells are present in the lungs of mice immunized with MK7 as neonates but not in the lungs of those immunized as adults. Mice that are unable to generate anti- α -1,3-glucan IgA Abs were immunized with MK7 as neonates and were no longer protected against cockroach allergy. Thus, neonatal, but not adult, exposure to α -1,3-glucan results in suppressed development of cockroach allergy via pulmonary α -1,3-glucan-specific IgA-secreting cells. *The Journal of Immunology*, 2016, 197: 000–000.

The childhood risk for developing an autoimmune or allergic condition is increasing more rapidly in industrialized regions, compared with developing regions, of the world (1–3). A theory termed the “Hygiene Hypothesis” suggests that this increase is the result of decreased exposure to microbes among children living in these developed environments (4–6). A favored paradigm associated with the hygiene hypothesis is that Th1 T cells stimulated by early microbial exposure suppress the development of Th2-associated allergic disease (7, 8). However, little empirical evidence supports the long-term maintenance of Th1 cells or the ability of this T cell subset to suppress allergic disease development throughout the lifetime of the individual. In fact, early microbial exposure may have a multitude of effects on the developing immune system. We described examples of significant alterations to the developing B cell repertoire following neonatal Ag exposure in mice (9).

During neonatal B cell lymphopoiesis, we observe interclonal competition between distinct emerging B cell clones that express different BCRs but have similar Ag specificity. Depending on the time after birth, exposure to particular bacterial Ags causes these emerging B cell clones to wax and wane according to their BCR specificity, such that some remain dominant in the adult repertoire, whereas others become minor players (9–11). In addition, neonatal Ag exposure may be able to stimulate innate-like B1 cells that are

enriched within the neonatal spleen (12) and have unique functions, such as the ability to reside within the pleural and peritoneal cavity, contribute to serum IgM production, self-renew (13), and readily switch to IgA (14). Exposure to particular bacterial Ags during early life can alter the numbers of these developing clones and permanently select them into the adult repertoire (9). We demonstrated previously that altered Ag-specific B cell responses, following neonatal immunization with bacterial vaccines containing polysaccharide or phospholipid moieties shared by *Aspergillus fumigatus* fungi or house dust mite (HDM), suppress allergic disease development in adult animals (15, 16).

Cockroaches are a prominent source of allergens, and cockroach sensitivity affects ~26% of the United States population (17). About 40–60% of patients with asthma are sensitized to the German cockroach (*Blattella germanica*) (18, 19). The major allergenic proteins from the German cockroach, Bla g 1 and Bla g 2, are found in the insect feces, saliva, and exoskeletons (20, 21). Cockroach sensitization among children is often associated with urban environments (18, 22) and low socioeconomic status (17, 23) and is more frequent within the African American community (23). Children that are skin-prick test positive for cockroach allergens are more likely to have difficult-to-control asthma (18, 19) and are three to four times more likely to visit healthcare providers or the emergency room as the result of an asthmatic event (24) compared with those suffering from other allergies. The allergenic potential of cockroach-associated proteins, such as Bla g 1 and Bla g 2, is well described (21, 25), and these proteins are used in immunotherapy treatments (26, 27). However, the association of conserved carbohydrates (28) and lipids (29) on allergen-bearing particles is often overlooked, and the involvement of these epitopes in modulating the allergic immune response is not well appreciated.

In this article, we show that that cockroach particles contain anti- α -1,3-glucan-reactive epitopes. α -1,3-glucans, also referred to as α -1,3-dextran (30), are also expressed by fungi (31, 32) and some types of bacteria (33–35). The largely T-independent mouse Ab response to α -1,3-glucan is well characterized and primarily consists of IgM, IgG3, and IgA isotypes (10, 30, 36–38). Additionally, B cells with specificity for α -1,3-glucan are enriched within the marginal zone and B1b cell populations (37). In this study, we examined the effects of neonatal exposure to α -1,3-glucan

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Abbreviations used in this article: AC, alimentary canal; AHR, airway hyperresponsiveness; ASC, Ab-secreting cell; BALF, bronchoalveolar lavage fluid; CTB, cholera toxin B; HDM, house dust mite; ILC, innate lymphoid cell; i.t., intratracheal(ly); MedLN, mediastinal lymph node; PFA, paraformaldehyde; Rrs, respiratory system resistance; UAB, University of Alabama at Birmingham.

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on the subsequent development of an allergic response to cockroach allergen. Immunization of neonates with α -1,3-glucan, either as a bacteria-derived vaccine or a purified polysaccharide, results in the localization of α -1,3-glucan-specific IgA-secreting cells to the lung and a dramatic reduction in allergic responses to cockroach allergen in mice challenged with cockroach allergen as adults.

Materials and Methods

Animals

C57BL/6 mice and triple-congenic mice (*Gpi1^a Thy1^a Igh^e*) were originally obtained from The Jackson Laboratory. Such congenic mice are referred to in this study as B6.Igh-C^a. IgA-deficient mice (39) that expressed the Igh-C^a haplotype on a C57BL/6 background were obtained from Charles Elson III (University of Alabama at Birmingham [UAB]). C57BL/6 mice (Igh-C^b Igh-2^{+/+}) were crossed to IgA-deficient mice (Igh-C^a Igh-2^{-/-}) to generate F1 mice that do not make anti- α -1,3-glucan IgA (Igh-2^{a/-}). All mice were maintained under specific pathogen-free conditions using approved animal protocols from the Institutional Animal Care and Use Committee at UAB.

Cockroach allergen particulate preparation

Powdered, dried, acetone-defatted German cockroach (RMB46P; Greer Laboratories) was sonicated at 12 W at 4°C in 30-min increments for 15 h before passage through a 10- μ m filter. Particulate size was determined to be smaller than 0.5 μ m by flow cytometric comparison with 0.1–1- μ m latex beads as reference particles (LB1-LB30; Sigma-Aldrich). Cockroach particulates were pelleted at 10,000 rpm for 30 min prior to lyophilization. The lyophilized particulates were resuspended in sterile PBS at 10 mg/ml and stored at –80°C or were diluted to 100 μ g/ml and stored at 4°C until administration to mice.

B. germanica husbandry and dissection

Male and female *B. germanica* (German cockroach) were purchased from Carolina Biological Supply and reared at UAB. Roaches are maintained at 70°C and 80% humidity and are supplied with water, commercial dog food, and potato slices ad libitum. For Ab-binding studies, female roaches carrying an egg case (ootheca) were dissected into head, thorax, wings, abdomen, legs, ootheca, nymphs, and alimentary canal (AC), and these parts were homogenized separately. Fecal pellets were also collected from the colony, and bacteria from AC were enriched in Lysogeny broth and THY broth. Cockroach components or bacteria were stained with a mAb against α -1,3-glucan (A16), as described below ("Ab reagents and flow cytometry").

Enterobacter vaccines and immunizations

Enterobacter cloacae strain cBAN (α -1,3-glucan deficient) (40) or MK7 (α -1,3-glucan bearing) (33) was grown to mid-log phase in Lysogeny broth at 37°C in 5% CO₂. Bacteria were washed, fixed with 1% paraformaldehyde (PFA) for 12 h, resuspended in sterile PBS, and stored at –80°C until use. Neonatal (7-d-old pups) or adult (8–10-wk old) mice from the same litter (littermate) were immunized i.p. with 5 $\times$ 10⁷ of PFA-fixed cBAN or MK7 *Enterobacter* strains or were treated with PBS. Alternatively, neonatal (7–8-d-old) littermate pups were immunized i.p. with 25 μ g β -1,3-glucan (Laminarin; Sigma-Aldrich) or α -1,3-glucan (gift from A. Jeanes, U.S. Department of Agriculture) or were treated with PBS.

In vivo intratracheal challenge and the cockroach allergy model

For intratracheal (i.t.) challenge, 8–10-wk-old (adult) mice were anesthetized with 3–5% isoflurane and immobilized on a vertical board with suture string looped around the upper incisors. The tongue was extended using blunt-end forceps, liquid was pipetted into the oral cavity, and the nares were manually plugged to ease inhalation of the liquid suspension. Adult mice were challenged i.t. with 5 μ g of processed cockroach particulate allergen resuspended in 50 μ l PBS. Mice were rested for 7 d before being challenged i.t. daily for five consecutive days with 5 μ g of cockroach allergen in 50 μ l PBS. Following the last challenge with cockroach allergen, mice were rested for 2 d before euthanization.

Tissue and leukocyte collection and preparation

Following euthanization, tracheae were cannulated to extract cellular infiltrates from the bronchoalveolar space via a 5-ml lavage with PBS, the first milliliter of which was reserved for measuring Abs. Mice were perfused by cardiac puncture with 1% heparin in PBS prior to lung or spleen removal. Lungs were minced and treated with 1 mg/ml collagenase + 50 U DNase

(both from Sigma-Aldrich) in 5 ml of HBSS for 40 min at 37°C, followed by lymphocyte separation (Cellgro). Mediastinal lymph node (MedLN) and spleen cells were collected by mechanical isolation. Bone marrow was flushed from two femurs and two tibia/mouse using a 25-gauge needle. Spleen and bone marrow cell suspensions were subjected to ammonium chloride potassium RBC lysis (Life Technologies). All recovered cell populations were manually enumerated using a hemocytometer.

ELISPOT assay

High-binding flat-bottom EIA/RIA plates (Corning Costar) were coated with 2 μ g/ml of purified α -1,3-glucan to quantify α -1,3-glucan-specific IgM-, IgA-, and IgG3-secreting cells or with 1 μ g/ml anti-mouse IgM or anti-mouse IgA (both from Southern Biotech, Birmingham, AL) to identify total IgM- or IgA-secreting cells, respectively. Plates were coated overnight at 4°C and then blocked for 6 h at 37°C with a 1% gelatin in PBS solution. Single-cell suspensions were prepared in RPMI 1640 supplemented with 2% FBS (both from Life Technologies), and 5 $\times$ 10⁵ bone marrow cells or 2 $\times$ 10⁶ cells from the lung or spleen were added to one well of the plate and serially diluted with the same media. Plates were incubated for 18 h at 37°C. Cells were lysed with 0.05% Tween-20 in water, and plates were washed three times with 0.05% Tween-20 in PBS and incubated with 500 μ g/ml of anti-mouse IgM-AP (Southern Biotech) or anti-mouse IgA-AP (Life Technologies) in a solution of 0.05% Tween-20 in PBS and 1% gelatin for 2 h at 37°C. Plates were washed three times with 0.05% Tween-20 in PBS and developed for 18 h at 4°C in substrate buffer (pH 10.25) containing 1 M 2-amino-2-methyl-1-propanol, 0.1% Triton X-405, and 0.01 g/ml 5-bromo-4-chloro-3-indolyl-phosphate (all from Sigma-Aldrich). Plates were washed in dH₂O, and resulting spots produced by Ab-secreting cells (ASCs) were enumerated visually.

ELISA

ELISAs were performed to measure serum and bronchoalveolar lavage fluid (BALF) anti- α -1,3-glucan-IgM and anti- α -1,3-glucan-IgA Abs, along with total IgE and anti-Bla g 2 IgE in the serum and total IgE in the BALF. Anti- α -1,3-glucan Ab ELISAs were conducted using high-binding EIA/RIA plates (Corning Costar) coated with 2 μ g/ml purified α -1,3-glucan. After incubation with serum or BALF, Ab binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotech) or alkaline phosphatase-conjugated goat anti-mouse IgA (Life Technologies). Standard curves were prepared using known quantities of α -1,3-glucan-specific IgM (A16) or IgA (J558) Abs, purified as described in *Ab reagents and flow cytometry*. Anti-Bla g 2-IgE was detected by coating plates with 2 μ g/ml natural Bla g 2 (Indoor Biotechnologies), and Ab was detected using alkaline phosphatase-conjugated goat anti-mouse IgE (Southern Biotech). Total IgE levels were determined by coating plates with 2 μ g/ml rat anti-mouse IgE (Southern Biotech), and standard curves were prepared with known concentrations of IgE Abs (Southern Biotech). For all ELISA assays, p-Nitrophenyl Phosphate Substrate (Sigma-Aldrich) was added, and color development was detected with a SPECTROstar Omega Reader (BMG LABTECH) at 405 nm.

Flow cytometric or ELISPOT oligosaccharide-inhibition assays

Cockroach particulates were incubated with 500 ng of A16 Ab with 5–100 μ g of nigerose (a disaccharide with α -1,3-glucan linkages), sucrose (a disaccharide of glucose and fructose) (both from Sigma-Aldrich), or PBS alone. A16 Ab binding to cockroach particles was detected by secondary goat anti-mouse FITC Abs by flow cytometry. Additionally, during the ELISPOT protocol, 5–100 μ g of nigerose or sucrose (both from Sigma-Aldrich) in RPMI 1640 + 2% FBS or media alone was incubated with 4 $\times$ 10⁵ cells from the spleen of an adult mouse that was immunized with MK7 as a neonate. Following development, as described above, resulting ASCs were enumerated visually.

Ab reagents and flow cytometry

Hybridoma (A16) and plasmacytoma (J558) were grown in serum-free RPMI 1640 (Hybridoma SFM; Life Technologies). IgM Abs were purified from supernatants on Sepharose-6B columns coupled with RS3.1 anti-mouse IgH6a mAb, and IgA Abs were purified from Sepharose 4B affinity columns coupled with rat monoclonal anti-mouse IgA (clone 11-44). Cockroach particulates, *Enterobacter* vaccine preparations, and timothy grass pollen were stained with purified monoclonal anti- α -1,3-glucan Ab A16 (IgM), anti- β -1,3-glucan Ab 744 (IgM), or anti-hen egg lysozyme isotype-control Ab MD4 (IgM). Cockroach fecal pellets were stained with Abs against Bla g 2 (IgG1) or isotype-control Der p 1 (IgG1) from Indoor Biotechnologies. Primary Ab incubation was followed by secondary goat

anti-mouse FITC Abs against respective isotypes (Southern Biotech, Life Technologies). Cells from the BALF, MedLN, and lungs were stained for flow cytometry with fluorochrome-conjugated anti-mouse Abs specific for the following cell surface markers, transcription factors, and cytokines: CD3 (145-2C11), CD4 (GK1.5), CD5 (53-7.3), CD8 (53-6.7), CD11c (HL3), CD11b (M1/70), CD19 (1D3), CD25 (3C7 & PC61), CD44 (IM7), CD62L (MEL-14), CD86 (GL1), CD90.2 (53-2.1), CD117 (2B8), CD127 (SB/199), B220 (RA3-6B2), IgE (R35-72), SiglecF (E50-2440), Ly6G (IA8), Gr-1 (RB6-8C5), KLRG1 (2F1), ST2 (RMST2-2) TER-119, DX5, GATA3 (L50-823), Foxp3 (R16-715), T-bet (O4-46), IL-2 (JES6-5H4), IFN- γ (XMG1.2), TNF (MP6-XT22), IL-4 (11B11), IL-6 (MP5-20F3), and IL-13 (ebio13A) (BD Biosciences and eBioscience).

Cell identification and T cell stimulation

Leukocytes were identified using flow cytometry by detecting specific cell surface markers based on the following gating strategy (Supplemental Fig. 1): T cells (CD3⁺CD4⁺), alveolar macrophages (Siglec-F⁺CD11c⁺), eosinophils (Siglec-F⁺CD11c⁺), neutrophils (CD11b⁺Ly6G⁺), dendritic cells (DCs; CD11b⁺CD11c⁺), immature DCs (CD11b⁺CD11c⁺), macrophages (CD11b⁺CD11c⁺Ly6G⁺), B cells (B220⁺), IgE-bound non-B cells (B220⁺IgE⁺), basophils (B220⁺IgE⁺Ckit⁺), and mast cells (CD44^{high} (Ag experienced) or CD25⁺ (activated)). Type 2 innate lymphoid cells (ILCs) were identified by excluding cells expressing CD3, CD4, CD8, CD19, B220, CD11b, CD11c, F4/80, GR1, TER119, and DX5 and gating on those that expressed CD90.2, ST2, KLRG1, CD127, CD25, and CD117. To detect cytokine production by T cells, cell suspensions were restimulated with 0.08 μ M PMA, 1.34 μ M ionomycin (eBioscience Cell Stimulation Cocktail) in the presence of 3.0 μ g/ml Brefeldin A (eBioscience) for 6 h. These cells were fixed and permeabilized with a BD Biosciences Cellular Fixation/Permeabilization Kit (554714), according to the manufacturer's instructions, before staining with Abs targeting IL-2, IFN- γ , TNF, IL-4, IL-6, and IL-13 (BD Biosciences). To determine T cell commitment, CD3⁺CD4⁺ T cells were stained with isotype-control Ab or Abs directed against GATA3, Foxp3, or T-bet together with reagents from the eBioscience Foxp3 staining buffer kit (00-5523-00). All flow cytometry procedures were performed on a FACSCalibur (BD Biosciences) or LSR II (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Histology and fluorescence staining

For paraffin-embedded sections, mouse lungs or *B. germanica* roaches (Carolina Biological Supply) were fixed in 4% PFA, dehydrated by sequentially increasing concentrations of ethanol, and, after xylene incubation, embedded in paraffin (Leica EG1150H). Six-micron lung sections were cut (Leica RM2235), rehydrated, and stained with H&E, periodic acid-Schiff, or Masson's trichrome (all from Sigma-Aldrich), according to the manufacturer's instructions, before being dehydrated and mounted in a xylene-based mounting media (Poly-Mount Xylene). Cockroach sections were deparaffinized in Citrisolv (Fisher) and subjected to heat-induced Ag retrieval in 10 mM citrate buffer (pH 6). Slides were quenched with 3% H₂O₂, blocked, and incubated with A16 (IgM) or isotype-control Ab (MD4). Ab binding was detected using HRP-conjugated anti-mouse IgM Ab (Southern Biotech), and slides were developed using diaminobenzidine peroxidase (Vector Laboratories). Cockroach sections were also counterstained with hematoxylin. The sagittal section of the German cockroach (Fig. 1H) is a composite of four sequential images created with Adobe Photoshop. Intact cockroach fecal pellets were stained with monoclonal anti-Bla g 2 (IgG1; Indoor Biotechnologies) or A16 (IgM), followed by anti-mouse IgG-FITC (Southern Biotech) or anti-mouse IgM 647 (Life Technologies) and then mounted with Fluoromount-G (Southern Biotech). For BALF stains, identical volumes of mouse BALF cell suspensions were cytocentrifuged (Shandon Elliott cytocentrifuge) onto glass slides at 1000 rpm for 5 min before staining with modified Wright stain (Sigma-Aldrich). Sections were imaged with a Leica DMRB microscope equipped with Hamamatsu C4742-95 and 3CCD color cameras and appropriate filter cubes. Images involving fluorescence were acquired using Openlab 3.1 software (Agilent Technologies), and bright-field images were acquired using Adobe Photoshop 7.0.

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 the tracheas were cannulated with an 18-G tube connected to the respiratory and expiratory ports of a flexiVent ventilator (SCIREQ); each mouse was ventilated at a rate of 160 breaths per min. After acquiring baseline resistance measurements without challenge, increasing concentrations (10–50 mg/ml) of methacholine (Sigma-Aldrich)

were vaporized, and total respiratory system resistance (Rrs) was recorded every 12 s for up to 3 min. Averages from each methacholine dose were calculated from four to six mice per group to determine airway hyperresponsiveness (AHR).

In vitro T cell-priming assays

A total of 300,000 MedLN cells from each mouse that contained similar frequencies of T cells, B cells, and DCs as the other groups of mice was incubated with 5–50 μ g of cockroach particles (used for allergic challenge) in DMEM (Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin and streptomycin, 2.5 μ g/ml amphotericin, and 100 μ M 2-ME for 5 d. The percentage of CD3⁺CD4⁺ T cells expressing CD25 was measured by flow cytometry.

Sample size and statistics

Data are the mean $\pm$ SEM from three independent experiments with 5–10 mice/group. The statistical calculations described below were performed with Prism 4.0 software (GraphPad). Comparison of three or more groups was performed by one-way ANOVA, followed by the Tukey post hoc analysis, whereas data from only two groups were analyzed by a two-tailed unpaired *t* test to determine statistical significance. In Figs. 2–4, 6, and 7, and Supplemental Figs. 2 and 3, only statistically significant differences between mice immunized with PBS or cBAN and differences between mice immunized with cBAN or MK7 are displayed. In Fig. 5, differences between mice immunized with PBS and β -1,3-glucan and differences between mice immunized with β -1,3-glucan and α -1,3-glucan are displayed.

Results

Cockroach allergen expresses α -1,3-glucan epitopes

Having demonstrated that environmental allergens, such as *A. fumigatus* and HDM, display Ab-reactive epitopes associated with certain bacteria (15, 16), we expanded our studies to analyze German cockroach (*B. germanica*) allergen. Cockroach particles were bound by purified monoclonal anti- α -1,3-glucan Ab, A16 (IgM, λ 1) (Fig. 1A). We (10, 33, 41) demonstrated previously that some strains of *Enterobacter*, such as MK7, express α -1,3-glucan (Fig. 1C), whereas other strains, such as cBAN, do not (Fig. 1B). Binding of mAb A16 to cockroach allergen is inhibited by nigerose, a disaccharide with α -1,3-glucan glycosidic linkages, but not by sucrose (Fig. 1D). Staining of dissected (Fig. 1E) and paraffin-embedded histologic sections of German cockroach (Fig. 1H) revealed that A16 Ab, but not an isotype-control Ab, binds many external and internal structural components of the insect, including cockroach allergen Bla g 2-expressing fecal pellets (Fig. 1E–H). A16 Ab does not bind components of the AC, its contents, bacteria isolated from the AC (Fig. 1E), or the crop of the insect (Fig. 1H). Thus, the α -1,3-glucan epitope is widely expressed on German cockroaches, including Bla g 2-expressing fecal pellets.

Immunization of neonatal B6.Igh-C^a mice with α -1,3-glucan-bearing *Enterobacter* (MK7) results in the accumulation of α -1,3-glucan-specific IgM- and IgA-secreting cells in the lung following sensitization with cockroach allergen

To focus our studies on the protective effects of α -1,3-glucan-specific B cells and Abs on the development of cockroach allergy, we used congenic C57BL/6 mice expressing the Igh-C^a haplotype (B6.Igh-C^a), which, unlike the background C57BL/6 strain, express a J558 Ig variable H chain gene segment that enables them to respond α -1,3-glucan and produce anti- α -1,3-glucan Abs. As a model for early exposure to microbial α -1,3-glucan, neonatal (7–8-d old) B6.Igh-C^a mice were immunized i.p. with *Enterobacter* vaccine MK7. Control littermate mice were immunized with *Enterobacter* vaccine cBAN or treated with PBS, and all groups were rested until they were 8–10 wk of age (Fig. 2A). At this time, the mice were sensitized i.t. with cockroach allergen, rested for 7 d, challenged for five consecutive days with cockroach allergen, and euthanized 2 d later for analysis (Fig. 2A).

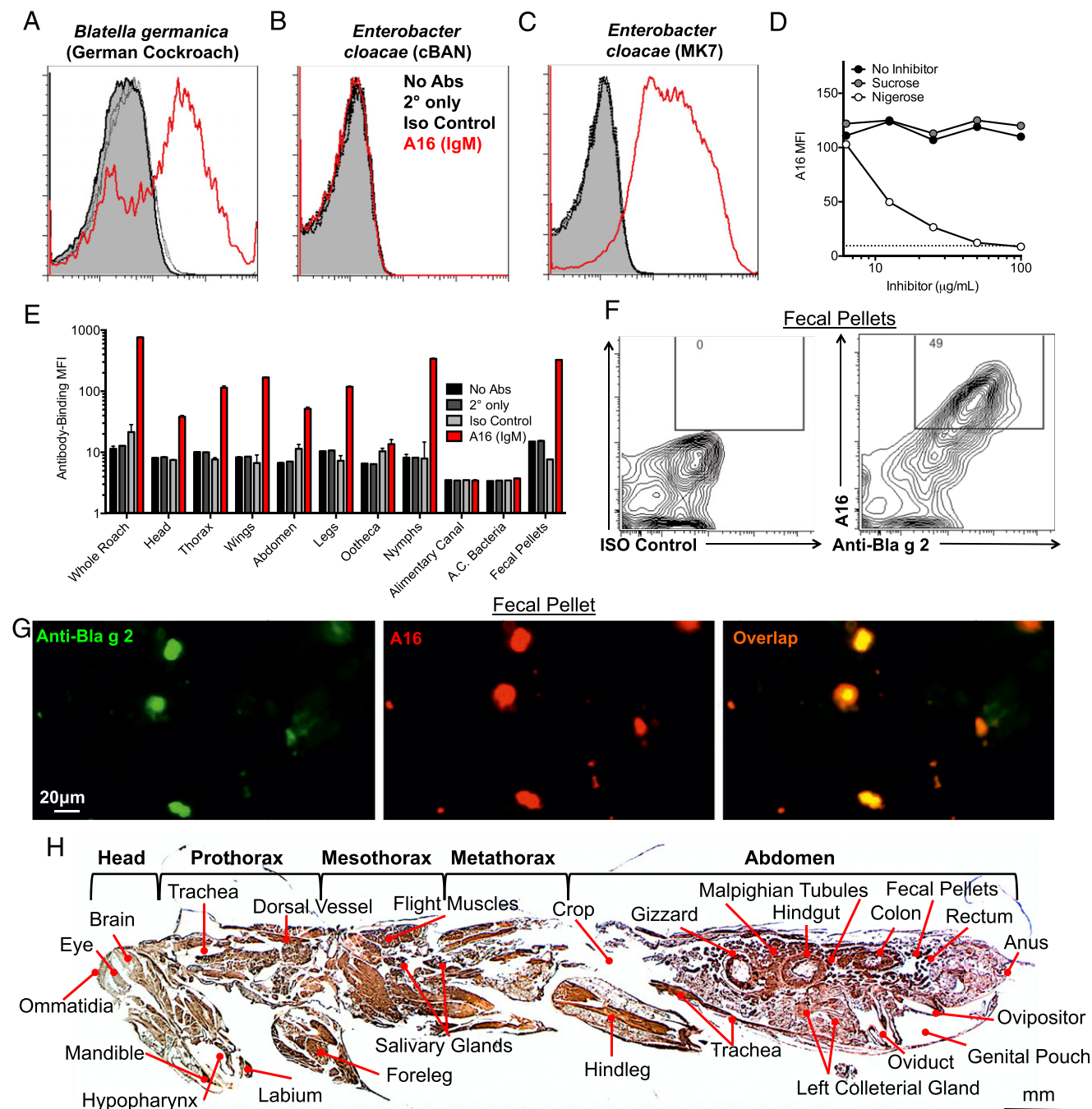


FIGURE 1. Purified monoclonal anti- α -1,3-glucan Ab A16 reacts with epitopes distributed throughout German cockroach (*B. germanica*). German cockroach particles (Greer) (A) or *E. cloacae* vaccine cBAN (B) or MK7 (C) was stained with monoclonal anti- α -1,3-glucan IgM Ab A16 or isotype-control Ab, and Ab binding was determined by flow cytometry. (D) Cockroach particles (dotted line) were incubated with A16 Ab or PBS alone (no inhibitor) in the presence of increasing concentrations of nigerose or control disaccharide sucrose, and the mean fluorescence intensity of A16 binding to cockroach particles was determined. (E) Homogenized cockroach parts (Carolina Biological Supply) or fecal pellets, or gut bacteria, were incubated with A16 Ab or isotype-control Ab, and the mean fluorescence intensity of A16 Ab binding was determined. German cockroach fecal pellets were incubated with anti-Bla g 2 and A16 or isotype-control Abs, and Ab binding was detected by flow cytometry (F) and fluorescence microscopy (G). (H) Whole German cockroaches (Carolina Biological Supply) were embedded in paraffin, and sagittal sections were subjected to Ag retrieval prior to staining with A16 Ab (amber) and counterstaining with hematoxylin.

Compared with controls, mice immunized with MK7 have significantly increased numbers of α -1,3-glucan-specific IgM- and IgA-secreting cells in the spleen (Fig. 2B, 2C) and bone marrow, as well as higher levels of serum anti- α -1,3-glucan Abs (P.S. Patel and J.F. Kearney, unpublished observations). ELISPOTs from splenic α -1,3-glucan-specific IgM-secreting cells were inhibited by nigerose, a disaccharide with α -1,3-glucan linkages, but not by sucrose (P.S. Patel, unpublished observations). α -1,3-Glucan-specific ASCs were not detectable in the lungs of control mice

or mice immunized as neonates prior to sensitization with cockroach allergen (P.S. Patel and J.F. Kearney, unpublished observations). However, following sensitization and challenge with cockroach allergen, only mice immunized with MK7 have detectable frequencies of α -1,3-glucan-specific IgM- and IgA-secreting cells in the lung tissue (Fig. 2B, 2C) and increased levels of corresponding Abs in the BALF (Fig. 2D, 2E) compared with control mice. The increased frequency of pulmonary α -1,3-glucan-specific ASCs is not associated with an overall increase in

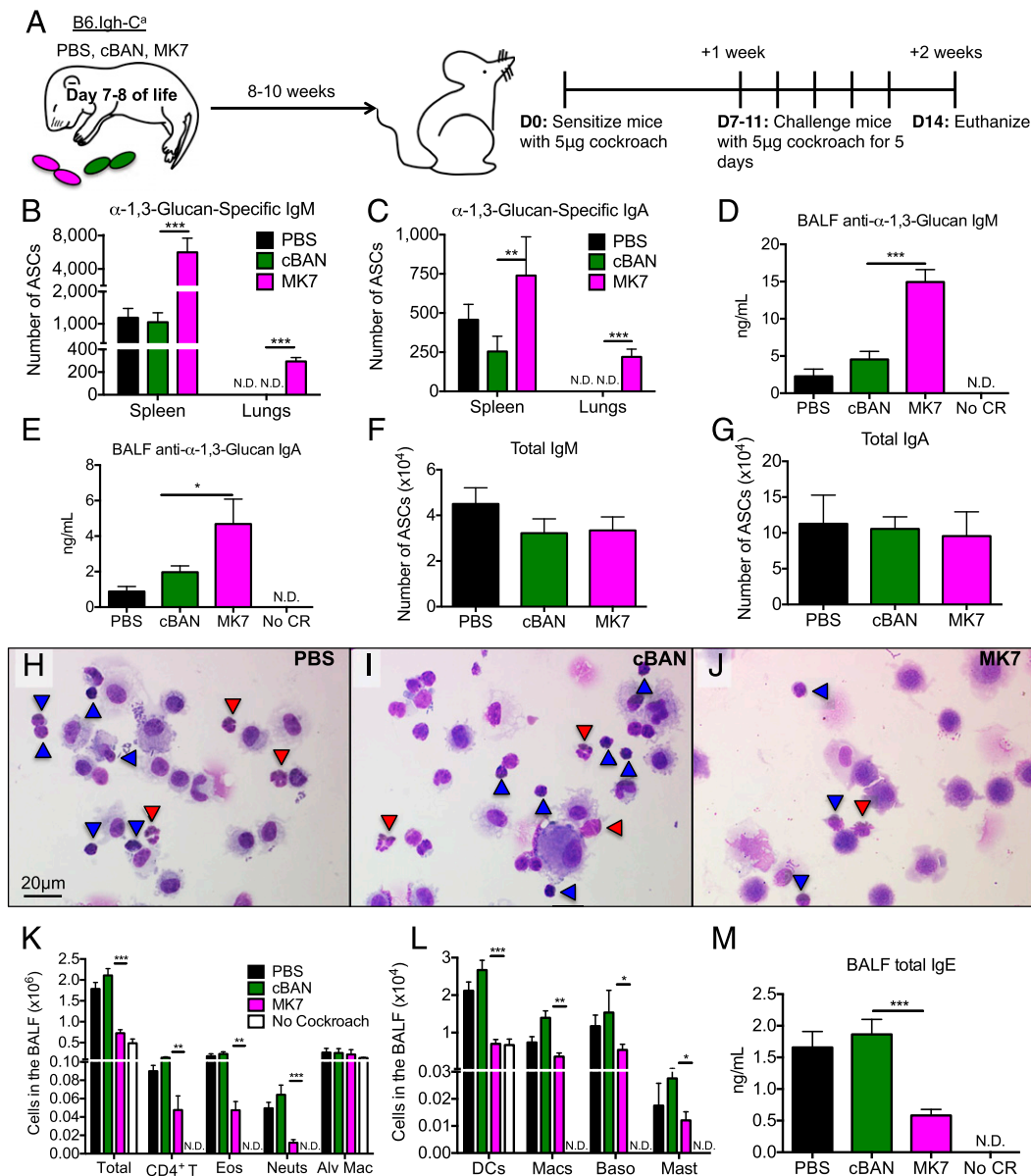


FIGURE 2. Following challenge with cockroach allergen, α -1,3-glucan-specific IgM- and IgA-secreting cells are detectable in the lungs of B6.Igh-C α mice immunized with MK7 as neonates. (A) Neonatal B6.Igh-C α mice were immunized i.p. with 5×10^7 cBAN or MK7 or were treated with PBS. At 8–10 wk of age, these mice were sensitized with cockroach allergen, rested for 7 d, challenged for five consecutive days, and euthanized 2 d later for analysis. The number of α -1,3-glucan-specific IgM and IgA ASCs (B and C) and total IgM- and IgA-secreting cells (F and G) in the spleen and lungs were determined by ELISPOT. (D and E) Levels of anti- α -1,3-glucan IgM and IgA Abs in BALF were determined by ELISA. (H–J) Identical volumes of BALF were cytocentrifuged, and cells were stained with modified Wright stain. Lymphocytes (blue arrowheads) and neutrophils (red arrowheads) are indicated. (K and L) Cells in the BALF were identified by flow cytometry, as documented in Supplemental Fig. 1, and quantified. (M) Total IgE in BALF of indicated mice was measured by ELISA. Data are mean $\pm$ SEM from three independent experiments with 5–10 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001, ANOVA. N.D., not detectable.

total numbers of pulmonary IgM- or IgA-secreting cells (Fig. 2F, 2G). Thus, immunization of neonatal mice with α -1,3-glucan-bearing MK7 and subsequent challenge of these mice with cockroach allergen as adults results in an increased frequency of α -1,3-glucan-specific IgM- and IgA-secreting cells in the lung tissue and Ag-specific Ab in the BALF compared with control mice.

Neonatal immunization with α -1,3-glucan-bearing *Enterobacter* (MK7) suppresses cockroach-induced allergic responses in the lung

Given the reactivity of anti- α -1,3-glucan Abs with cockroach allergens, we asked whether increasing the number of α -1,3-glucan-specific B cells in the adult by neonatal immunization would affect the response to cockroach allergen challenge during

adult life. Staining of cytocentrifuged cells from the BALF with modified Wright stain revealed fewer neutrophils (red arrowheads) and lymphocytes (blue arrowheads) in mice treated with MK7 as neonates compared with control mice (Fig. 2H–J). Additionally, mice immunized with MK7 as neonates had fewer CD4⁺ T cells, eosinophils, neutrophils, APCs, basophils, and mast cells in the BALF (Fig. 2K, 2L) and pulmonary parenchyma, as well as ILCs in their lungs (Fig. 3D, 3E), compared with control mice. Pulmonary DCs, immature DCs, parenchymal macrophages, and alveolar macrophages from mice immunized with MK7 as neonates express decreased amounts of the MHC class II costimulatory molecule CD86 (Fig. 3F) compared with control mice. Neonatal immunization with MK7 is associated with reduced total IgE in the BALF (Fig. 2M) and serum (Fig. 3J), as well as decreased

levels of serum anti-Bla g 2-IgE (Fig. 3K), compared with control mice.

The lungs of unchallenged mice have minimal cellular infiltration around the bronchioles and vessels (Supplemental Fig. 1Q), an absence of mucin-producing cells in the bronchus (Supplemental Fig. 1R), and no evidence of excessive collagen deposition around the vessels and bronchi (Supplemental Fig. 1S). Following sensitization and challenge with cockroach allergen as adults, mice treated with PBS or cBAN as neonates exhibit extensive cellular infiltration around the bronchioles, pulmonary vessels, and the smaller alveolar airways (Fig. 3A, 3B). Epithelial cells within the bronchioles of these animals are hypertrophic, and numerous bronchioles are obstructed by mucin plugs (Fig. 3G, 3H; magenta). Collagen (blue) accumulates around the airways and in the alveolar spaces (Fig. 3M, 3N), and these mice exhibit impaired lung function, as indicated by the increase in Rrs following challenge with vaporized methacholine (Fig. 3L). In contrast, mice immunized with MK7 as neonates have markedly reduced cellular infiltrates around bronchioles and vessels (Fig. 3C), exhibit a lack of mucin-producing cells and mucin plugs in the bronchioles (Fig. 3I), have decreased collagen deposition surrounding the bronchioles and alveolar spaces (Fig. 3O), and have Rrs comparable to normal mice after challenge with vaporized methacholine (Fig. 3L). Collectively, these results demonstrate that neonatal immunization with α -1,3-glucan-bearing MK7 decreases the infiltration of allergy-associated cells into the bronchoalveolar space and lungs along with the production of IgE and dramatically affects airway pathology and pulmonary function resulting from sensitization and challenge with cockroach allergen.

MedLNs of mice immunized with α -1,3-glucan-bearing Enterobacter (MK7) as neonates contain decreased numbers of Th2-committed and cytokine-producing CD4⁺ T cells following cockroach allergy induction

Priming and activation of allergen-specific memory Th2 cells in the lung-draining lymph node (42) are responsible for driving and maintaining the pathogenicity associated with allergic disease (43). MedLNs isolated from mice immunized with MK7 as neonates and challenged with cockroach allergen as adults weigh less (Fig. 4A), are smaller (Fig. 4B), and display reduced cellularity (Fig. 4C) compared with control mice. Mice immunized with MK7 as neonates have decreased numbers of CD4⁺ T cells in their MedLN that are CD44^{high}, CD25⁺, or GATA-3⁺, but there is no difference in the number of CD4⁺ T-bet-expressing cells compared with control mice (Fig. 4D–F). MedLN cells from each mouse that contained similar numbers of CD4⁺ T cells, DCs, and B cells as mice from the other groups were incubated with cockroach allergen in vitro for 5 d to evaluate the ability of T cells to become activated. Mice immunized with MK7 as neonates have a lower percentage of CD4⁺ T cells that expressed CD25 in response to Ag stimulation compared with control mice (Fig. 4G). The number of CD4⁺ T cells from the MedLN that produce Th1-associated cytokines (TNF or IFN- γ /IL-2) or Th2-associated cytokines (IL-4, IL-6, and IL-13) following stimulation is also decreased in mice immunized with MK7 compared with the control group (Fig. 4H–J). Overall, compared with control mice, MedLNs from mice immunized with MK7 as neonates contain fewer CD4⁺ T cells that are CD44^{high}, Th2 committed, cytokine producing, and responsive to cockroach stimulation.

Neonatal immunization with purified α -1,3-glucan, but not β -1,3-glucan, suppresses cockroach allergy

To determine whether the decreased allergic response to cockroach particulates following neonatal immunization with MK7, compared

with cBAN, is due to expression of the α -1,3-glucan epitope by MK7, we treated neonatal littermate B6.Igh-C^a mice with PBS or immunized them with purified α -1,3-glucan or purified β -1,3-glucan as a control (Fig. 5A). The lack of reactivity of anti- β -1,3-glucan mAb 744 (IgM, κ) with cockroach particulates (P.S. Patel and J.F. Kearney, unpublished observations) indicates an absence of β -1,3-glucan epitopes on cockroach allergen. At 8–10 wk of age, immunized mice were sensitized i.t. with cockroach allergen and challenged as described in Fig. 5A. Similar to mice immunized with MK7 as neonates, there were increased numbers of α -1,3-glucan-specific IgM- and IgA-secreting cells in the spleens of mice treated with α -1,3-glucan as neonates (Fig. 5B, 5C), and there were no detectable α -1,3-glucan-specific ASCs in the lungs of adult mice treated with either glucan as neonates prior to sensitization (data not shown). However, following sensitization and challenge with cockroach allergen, α -1,3-glucan-specific IgM- and IgA-secreting cells are detectable in the lungs (Fig. 5B, 5C) along with increased titers of anti- α -1,3-glucan IgM and IgA in the BALF (Fig. 5D, 5E). Mice immunized with purified α -1,3-glucan as neonates exhibit suppressed development of cockroach allergy, as evidenced by decreased infiltration of CD4⁺ T cells, eosinophils, and neutrophils in the BALF (Fig. 5F) and lungs (Fig. 5G), decreased cellularity of the MedLN (Fig. 5H–J), reduced titers of serum anti-Bla g 2 IgE (Fig. 5K), and lower total IgE in the BALF (Fig. 5L) compared with PBS-treated or β -1,3-glucan-immunized mice. Thus, similar to immunization with MK7 bacteria during neonatal life, early life immunization with purified α -1,3-glucan, but not β -1,3-glucan, results in suppression of cockroach allergy following sensitization and challenge to cockroach allergen. As further evidence for the specificity of the α -1,3-glucan Ab response's contribution to the protection against allergic disease, MK7 immunization of C57BL/6 mice (Igh-C^b), which make poor Ab responses to α -1,3-glucan, does not result in protection against the development of cockroach allergy (Supplemental Fig. 2).

Immunization of adult B6.Igh-C^a mice with α -1,3-glucan-expressing Enterobacter (MK7) does not effect the development of cockroach allergy

Because adult mice mount a robust Ab response to α -1,3-glucan (41), we next determined whether immunization of adult mice with MK7 would protect against the development of allergic disease in response to cockroach allergen. Five-week-old adult littermate B6.Igh-C^a mice were treated with PBS or immunized i.p. with cBAN or MK7, and similar to the mice immunized as neonates, these mice were rested for 8 wk before being sensitized and challenged with the allergen, as described in Fig. 6A. Adult B6.Igh-C^a mice immunized with MK7 have increased numbers of α -1,3-glucan-specific IgM- and IgA-secreting cells in the spleen (Fig. 6B, 6C) and bone marrow and increased titers of circulating anti- α -1,3-glucan Abs in sera (P.S. Patel and J.F. Kearney, unpublished observations) compared with control mice. Following sensitization and challenge with cockroach allergen, α -1,3-glucan-specific IgM-secreting cells (Fig. 6B) and anti- α -1,3-glucan IgM Ab (Fig. 6D), but no IgA-secreting cells or respective Abs (Fig. 6C), were detected in the lungs of mice treated with MK7 as adults. Compared with adult mice treated with PBS or cBAN, adult mice immunized with MK7 demonstrate no differences in the number of allergy-associated cells in the BALF (Fig. 6E) or lungs (Fig. 6G), cellular composition of the MedLN (Fig. 6H–J), activation of CD4⁺ T cells following stimulation with cockroach allergen (Fig. 6K), total IgE in the BALF (Fig. 6F) or serum (Fig. 6L), or anti-Bla g 2-IgE in the serum (Fig. 6M). Therefore, unlike neonatal immunization with MK7, adult immunization with MK7 does not

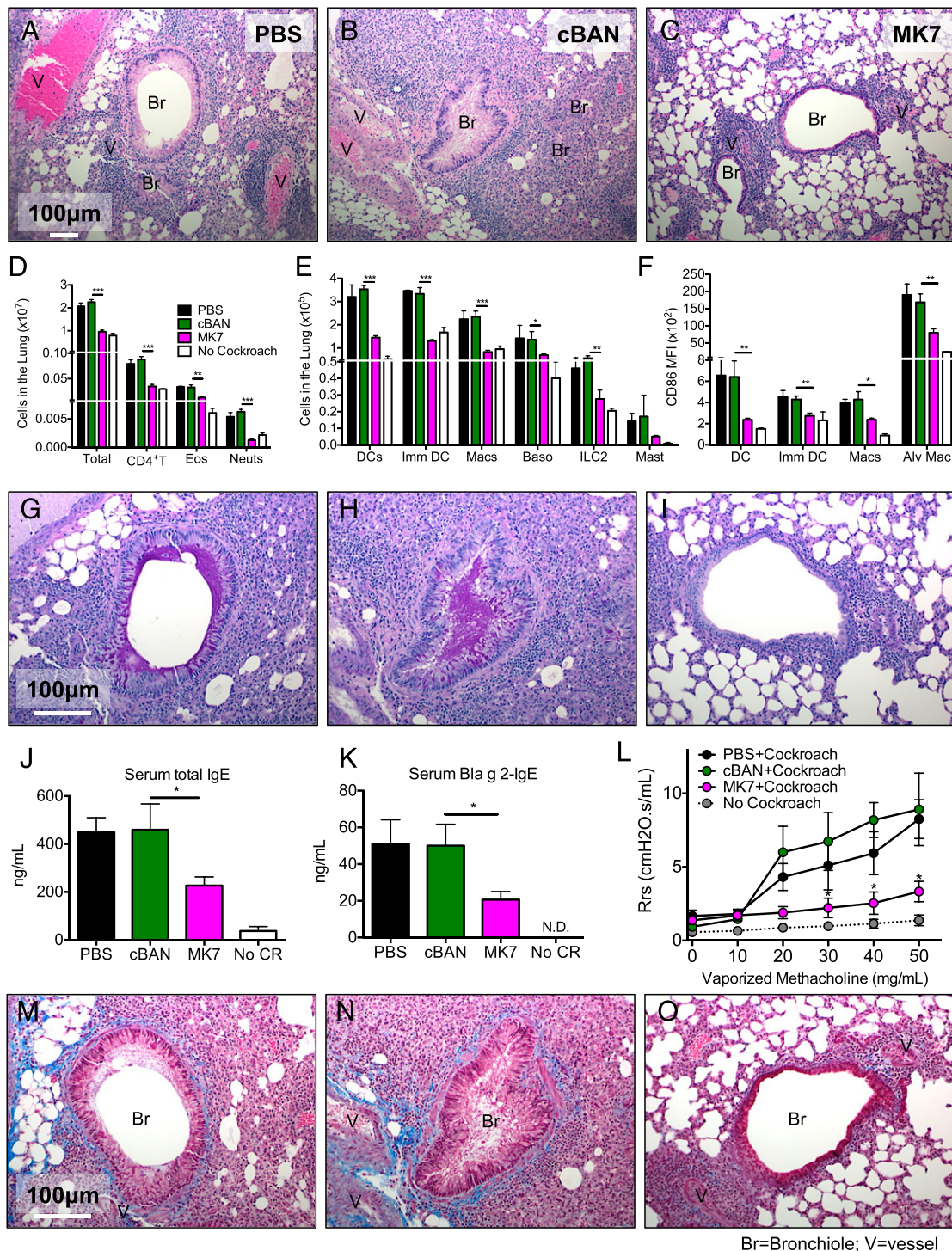


FIGURE 3. B6.Igh-C^a mice immunized with MK7 as neonates exhibit suppressed development of pulmonary airway disease. (A–C) Neonatal B6.Igh-C^a mice were immunized with cBAN or MK7 or treated with PBS. At 8–10 wk of age, these animals were sensitized and challenged with cockroach allergen. Paraffin-embedded lungs were stained with H&E (A–C), periodic acid–Schiff (G–I), or Masson's trichrome (M–O). (D and E) Cells from enzymatically digested lungs of the indicated mice were identified by flow cytometry, as documented in Supplemental Fig. 1, and quantified. (F) CD86 mean fluorescence intensity from the lung digest was determined for the indicated APC populations. Levels of total IgE (J) and anti-Bla g 2-IgE (K) in the sera of the indicated groups of mice, as measured by ELISA. (L) Rrs was calculated from mice that were mechanically ventilated and challenged with the indicated concentrations of vaporized methacholine. Data are mean $\pm$ SEM from three independent experiments with 5–10 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001, ANOVA. N.D., not detectable.

result in allergic suppression. The protective effect of neonatal vaccination is not due to an overall increase in the frequency of regulatory T cells or Th1 cells, because the numbers of these cells are similar in mice immunized with MK7 as neonates or adults (P.S. Patel and J.F. Kearney, unpublished observations). Instead,

neonatal immunization with MK7 results in a unique population of α -1,3-glucan-specific IgA-secreting cells in the lungs following sensitization and challenge with cockroach allergen (Fig. 2C) that is not detectable in the lungs of mice immunized with MK7 as adults (Fig. 6C).

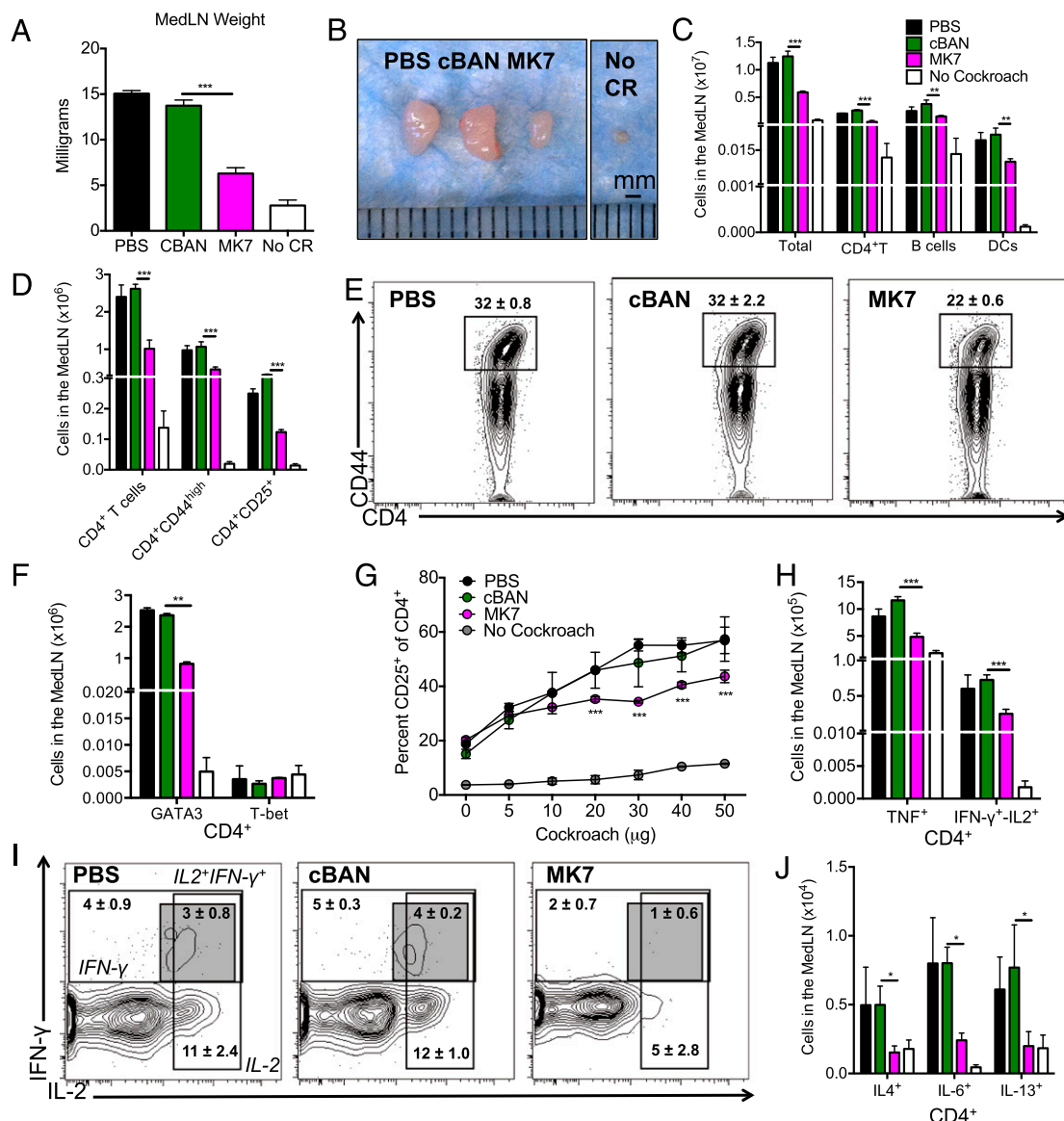


FIGURE 4. MedLNs of mice immunized with MK7 as neonates contain decreased numbers of Th2-committed and cytokine-producing CD4⁺ T cells following cockroach allergy induction. Total weight (A), representative images (B), and cellular composition (C) of MedLNs harvested from B6.Igh-C^a mice following sensitization and challenge with cockroach allergen. The number of CD44^{high} (Ag experienced) or CD25⁺ (activated) CD4⁺ T cells was enumerated from the MedLN (D), and CD44^{high} CD4⁺ T cells are shown (E). (F) Frequency of CD4⁺ and GATA-3⁺ or T-bet⁺ cells from the MedLN. (G) A total of 300,000 MedLN cells from each mouse was incubated with 5–50 μ g of cockroach allergen, and the percentage of CD4⁺ T cells expressing CD25 was determined after 5 d. MedLN cells were stimulated with PMA and ionomycin in the presence of Brefeldin A, and CD4⁺ T cells were assayed for production of Th1-associated cytokines (TNF or IFN- γ /IL-2) (H and I) or Th2-associated cytokines (IL-4, IL-6, and IL-13) (J) by intracellular staining and flow cytometry, as documented in Supplemental Fig. 1. CD4⁺ T cells producing IFN- γ , IL-2, or IFN- γ and IL-2 are shown (mean $\pm$ SEM). Data in (I) are mean $\pm$ SEM from three independent experiments with 5–10 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001, ANOVA.

Immunization of neonatal mice that cannot make IgA Abs to α -1,3-glucan with MK7 does not result in suppressed cockroach allergy

To determine whether anti- α -1,3-glucan IgA is necessary to protect against cockroach-induced allergic disease, we crossed C57BL/6 mice (Igh-C^b) with IgA-deficient mice (Igh-2^{-/-}) that expressed the Igh-C^a haplotype. The F1 generation of these mice simultaneously expresses paternally derived Igh-C^b haplotype-expressing B cells that can make IgA (Igh-2^{+/+}) but cannot make Abs to α -1,3-glucan and maternally derived Igh-C^a haplotype-expressing B cells that can make anti- α -1,3-glucan IgM but not anti- α -1,3-glucan IgA. Therefore, these F1 mice (Igh-2^{a/-}) are specifically deficient in their ability to generate an IgA Ab response to α -1,3-glucan. Neonatal Igh-2^{a/-} littermate pups were

immunized with cBAN or MK7 or treated with PBS. At 8–10 wk of age, these animals were sensitized and challenged with cockroach allergen, as described above (Fig. 7A). As expected, IgA-secreting cells and IgA Abs, but not α -1,3-glucan-specific IgA-secreting cells or specific Abs, were detectable in the serum and multiple tissues analyzed from these mice (Fig. 7B–E). Igh-2^{a/-} mice immunized with MK7 as neonates have increased numbers of α -1,3-glucan-specific IgM-secreting cells in the spleen (Fig. 7F) and bone marrow and anti- α -1,3-glucan IgM Abs in the serum, and there were no detectable α -1,3-glucan-specific ASCs in the lungs of adult mice prior to allergen sensitization (data not shown). Sensitization and challenge with cockroach allergen of Igh-2^{a/-} mice immunized with *Enterobacter* MK7 as neonates result in the localization of α -1,3-specific IgM-secreting cells in

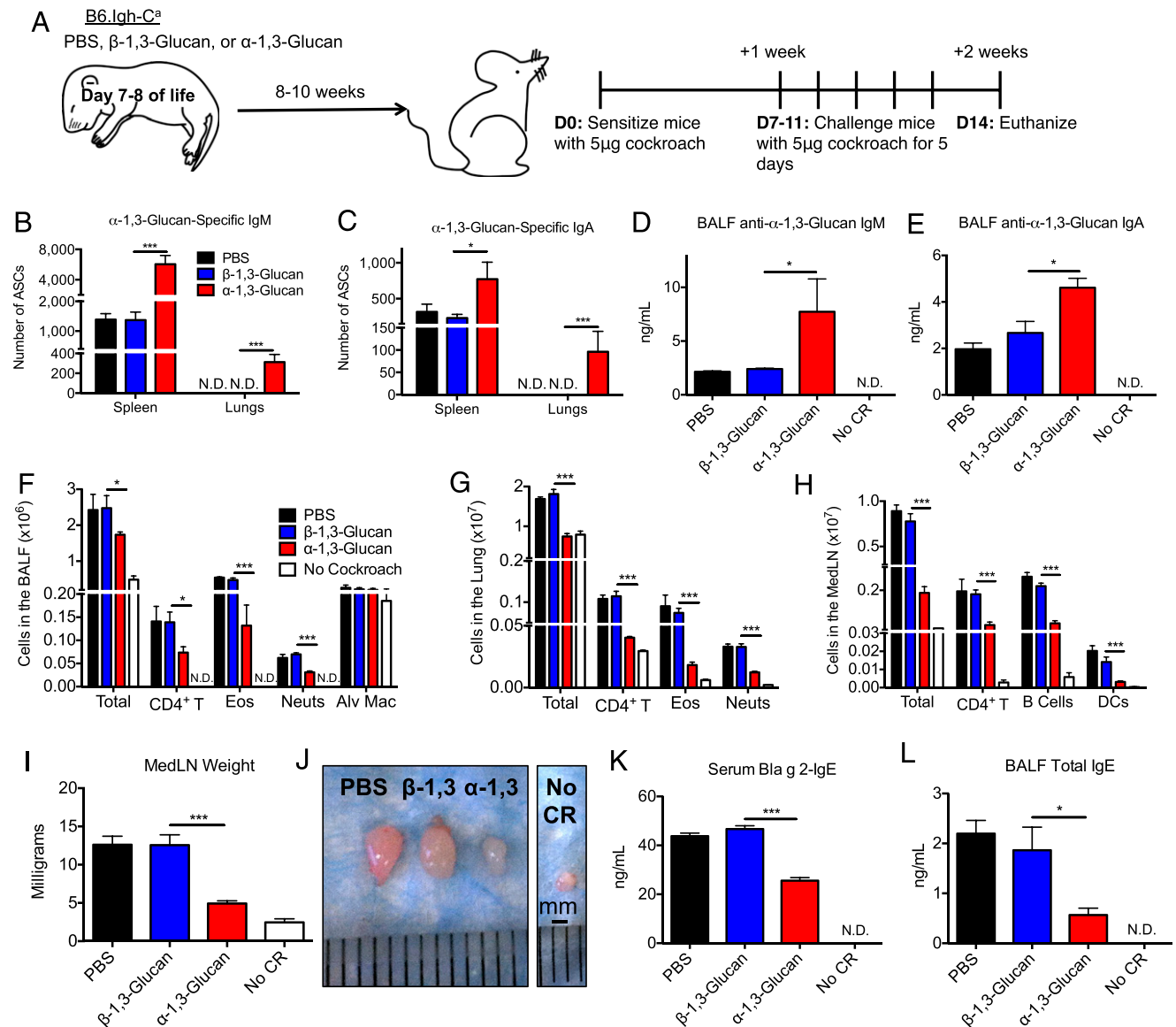


FIGURE 5. Neonatal immunization with purified α-1,3-glucan results in the localization of α-1,3-glucan-specific IgM- and IgA-secreting cells in the lung and protection against cockroach allergy. (A) Neonatal B6.Igh-C^a mice were immunized i.p. with 25 μg β-1,3-glucan or α-1,3-glucan or were treated with PBS. At 8–10 wk of age, these mice were sensitized and challenged i.t. with cockroach allergen. The number of α-1,3-glucan-specific IgM- and IgA-secreting cells from the lungs and spleen was enumerated (B and C), and anti-α-1,3-glucan IgM and IgA from BALF were quantitated by ELISA (D and E). (F and G) Cells were identified from the BALF and enzymatically digested pulmonary parenchyma, as documented in Supplemental Fig. 1, and enumerated. MedLNs were weighed (J) and photographed (H), and CD4⁺ T cells, B cells, and DCs were quantified (I). ELISA was used to determine the amount of serum anti-Bla g 2-IgE (K) and BALF total IgE (L). Values represent the mean ± SEM from two independent experiments with 5–10 mice per group. **p* < 0.05, ****p* < 0.001, ANOVA. N.D., not detectable.

the lungs (Fig. 7F) and increased levels of anti-α-1,3-glucan IgM Ab in the BALF (Fig. 7G) compared with control mice. The cellularity of the BALF (Fig. 7H), lungs (Fig. 7I), and MedLN (Fig. 7J–L), as well as AHR (Fig. 7M) and Bla g 2-IgE levels in the serum (Fig. 7N), is similar within these groups, regardless of neonatal immunizations. These results demonstrate that the protective effects of neonatal immunization with MK7 against the development of cockroach allergy are dependent on the IgA Ab response to α-1,3-glucan.

Discussion

In this study, we demonstrate that neonatal immunization with purified α-1,3-glucan or an α-1,3-glucan-expressing bacterial vaccine derived from *Enterobacter* MK7 provides significant protection against the development of cockroach allergy. This

protection is the result of a specific response to the α-1,3-glucan moiety on the *Enterobacter*, because immunization of B6.Igh-C^a mice with β-1,3-glucan or the α-1,3-glucan-deficient *Enterobacter* strain cBAN or immunization of poorly responding C57BL/6 mice with MK7 does not have an effect on the allergic response to cockroach particles. Additionally, expression of the α-1,3-glucan epitope on the allergen is critical for this immune protection, because sensitization and challenge with α-1,3-glucan-free timothy grass pollen did not result in the localization of α-1,3-glucan-specific IgA-secreting B cells in the lung, and mice immunized with MK7 as neonates did not exhibit suppressed development of timothy grass pollen-induced allergic disease (Supplemental Fig. 3). Despite a robust Ab response, B6.Igh-C^a mice immunized with MK7 as adults are not protected against allergic disease. These results are similar to our findings in the *A. fumigatus* fungal and

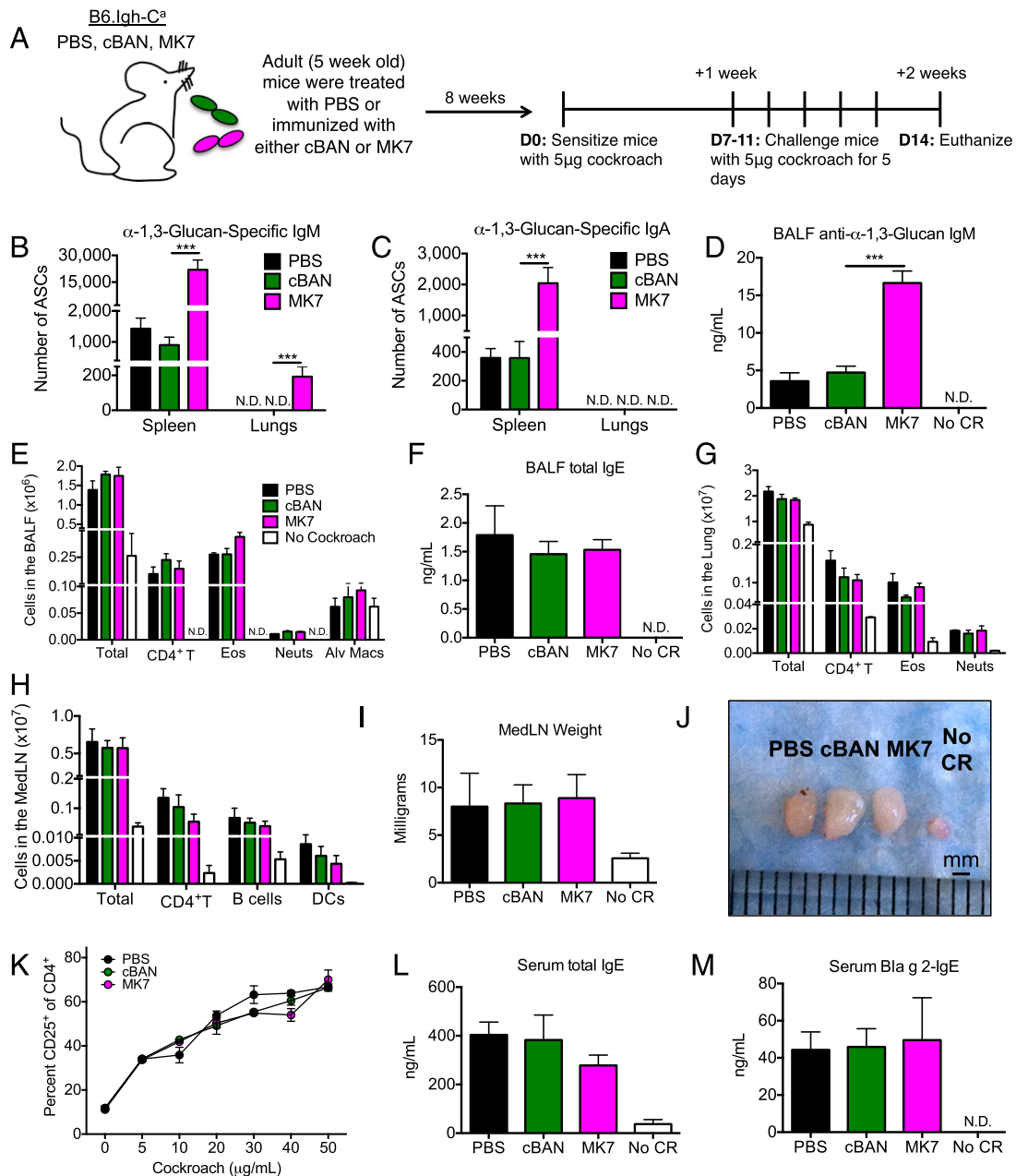


FIGURE 6. Immunization of adult B6.Igh-Ca mice with MK7 has no effect on the development of cockroach allergy. **(A)** Five-week-old B6.Igh-Ca mice were immunized i.p. with 5×10^7 cBAN or MK7 or were administered PBS. These mice were rested for 8 wk, sensitized with cockroach allergen, rested for 7 d, challenged for five consecutive days, and euthanized 2 d later for analysis. The number of α -1,3-glucan-specific IgM- and IgA-secreting cells isolated from the spleen and lungs was quantified by ELISPOT **(B and C)**, and titers of anti- α -1,3-glucan IgM and IgA Abs from BALF were determined by ELISA **(D)**. **(E and G)** Cells from the bronchoalveolar space and pulmonary parenchyma were identified by flow cytometry, as documented in Supplemental Fig. 1, and quantified. **(F)** BALF total IgE was measured by ELISA. MedLNs were photographed **(J)** and weighed **(I)**, and the numbers of CD4⁺ T cells, B cells, and DCs in the LN were quantified **(H)**. **(K)** A total of 300,000 MedLN cells was incubated with 5–50 μ g of cockroach allergen for 5 d, and the percentage of CD4⁺ T cells expressing CD25 was determined. The amount of serum total IgE **(L)** and anti-Bla g 2-IgE **(M)** was measured by ELISA. Data are mean $\pm$ SEM from three independent experiments with 5–10 mice per group. *** $p < 0.001$, ANOVA. N.D., not detectable.

HDM allergy models (15, 16), in which there was a striking age-related dependence for vaccination to induce effective protection against allergic reactions. A similar age-related effect was also shown to be critical for the induction of inducible BALT (44). Suppressed development of cockroach-associated allergic responses following neonatal immunization with MK7 is a result of α -1,3-glucan-specific IgA-secreting cells, which localize to the lung following allergen sensitization and challenge with the allergens. Mice deficient in their ability to make anti- α -1,3-glucan IgA that are immunized with MK7 as neonates and B6.Igh-Ca mice immunized

with MK7 as adults do not exhibit protection from cockroach-induced allergic disease, despite a robust α -1,3-glucan-specific IgM response.

In previous studies, we detected α -1,3-glucan-specific splenic B cell clonal precursors during neonatal life (5–11 d of life) that have the potential to give rise to progeny that predominantly secrete α -1,3-glucan-specific IgM and IgA in culture supernatants. The overall number of B cells secreting anti- α -1,3-glucan IgM remains constant with age (between days 12 and 30 of life); however, the number of cultures containing both IgM and IgA or

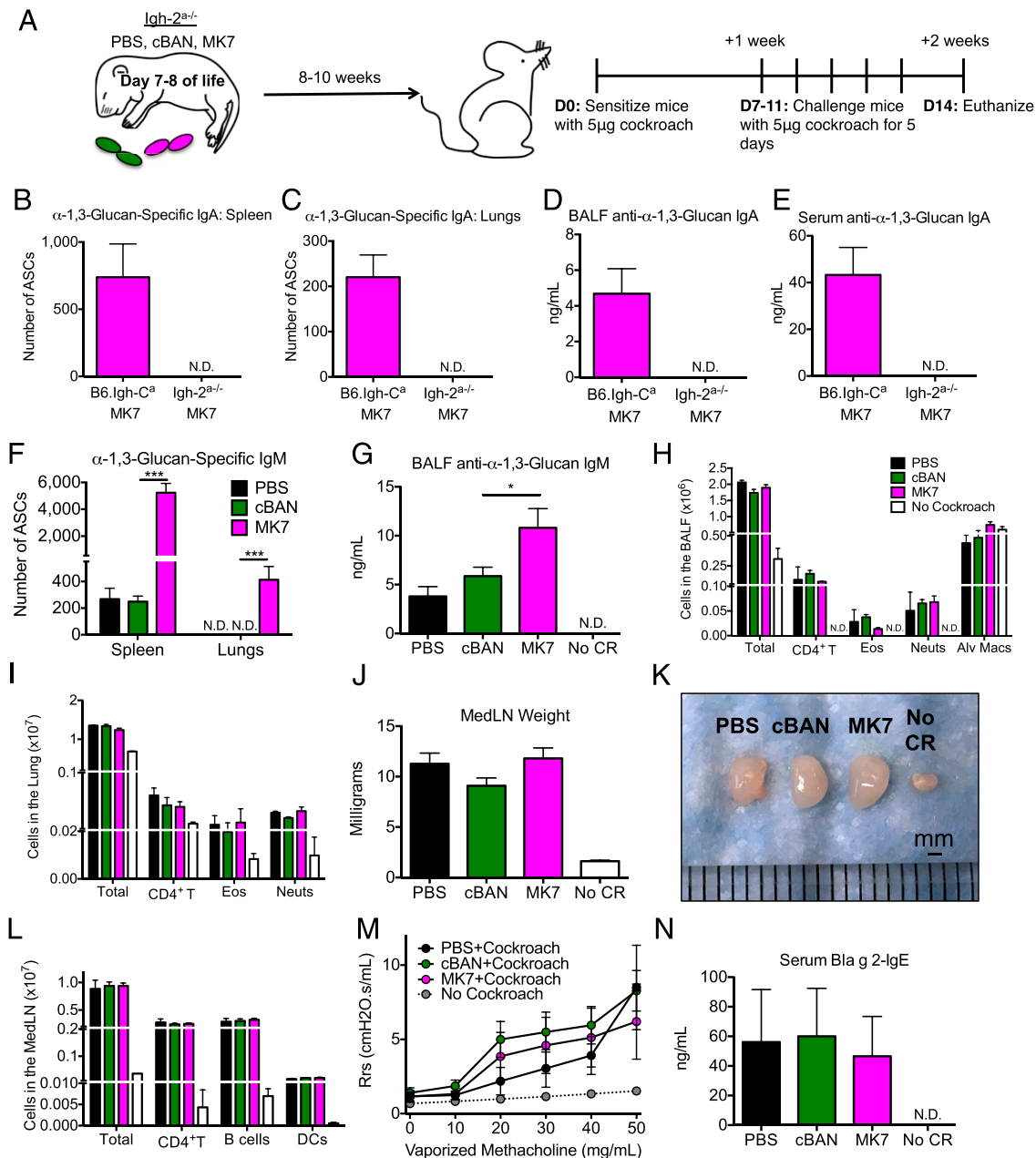


FIGURE 7. Neonatal immunization of mice that do not make IgA Abs to α-1,3-glucan with MK7 does not attenuate cockroach-induced allergic disease. **(A)** C57BL/6 (*Igh-C^b* *Igh-2^{+/+}*) mice were crossed to IgA-deficient mice (*Igh-C^a* *Igh-2^{-/-}*) to generate mice that do not make IgA Abs to α-1,3-glucan (*Igh-2^{a/-}*). Neonatal *Igh-2^{a/-}* mice were immunized i.p. with 5×10^7 of cBAN or MK7 or were treated with PBS. At 8–10 wk of age, these mice were sensitized and challenged i.t. with cockroach allergen. The number of α-1,3-glucan-specific IgA- and IgM-secreting cells from spleen (**B** and **F**) and lungs (**C** and **F**) was determined by ELISPOT, and levels of anti-α-1,3-glucan-specific IgA and IgM from BALF (**D** and **G**) and serum (**E**) was determined by ELISA. CD4⁺ T cells, eosinophils, and neutrophils in BALF (**H**) and enzymatically digested pulmonary parenchyma (**I**) were identified, as documented in Supplemental Fig. 1, and quantified. MedLNs were photographed (**K**) and weighed (**J**), and the number of CD4⁺ T cells, B cells, and DCs of MedLN isolated from these mice was determined (**L**). **(M)** Rrs was calculated from mice that were mechanically ventilated and challenged with the indicated concentrations of vaporized methacholine. **(N)** The amount of anti-Bla g 2-IgE in the serum of the indicated mice was determined by ELISA. Data are mean ± SEM from three independent experiments with 5–10 mice per group. **p* < 0.05, ****p* < 0.001, ANOVA. N.D., not detectable.

only IgA decreases to a much lower frequency (10). A possible reason for this shift is that there is a greater abundance of B1 cells in the spleen of developing neonates compared with adult mice (12), and B1 cells switch more readily to IgA than do other B cell subsets (14, 45). Therefore, neonatal exposure to α-1,3-glucan-bearing *Enterobacter* may select a subset of early CD5⁺ B cells with a higher intrinsic potential for anti-α-1,3-glucan IgA secretion into the adult repertoire, or into mucosal sites, which would account for the protective effects of neonatal immunization with MK7.

Our previous studies demonstrated that IgM Abs stimulated by microbes (*Streptococcus pyogenes* and *Streptococcus pneumoniae*) during early life are protective against the development of *A. fumigatus* and HDM allergy during adult life (15, 16). However, the studies presented in this study clearly demonstrate that anti-α-1,3-glucan IgA, but not IgM, is protective against the development of cockroach allergy. Protection mediated by IgA, but not IgM, may be the net result of several factors. One distinct difference between this model and the two published studies is that immunization of

neonates with phosphorylcholine-expressing *S. pneumoniae* does not stimulate anti-phosphorylcholine IgA Abs (16); however, immunization of neonatal and adult mice with α -1,3-glucan-expressing *Enterobacter* stimulates a robust anti- α -1,3-glucan IgA Ab response (37). Because TLR stimulation is a signal that can promote T-independent class switching to IgA (46), it is possible that IgA Abs are uniquely induced in this model by Gram-negative *Enterobacter* but are not induced by Gram-positive streptococci. α -1,3-Glucan-specific IgM-secreting cells selected into the adult repertoire as a result of neonatal immunization with MK7 express Abs with the M104E idiotype (10), which has 100-fold lower affinity for α -1,3-glucan compared with α -1,3-glucan-specific B cells that express the J558 idiotype (9). Additionally, TdT is necessary for the formation of high-affinity J558 idiotype-expressing Abs to α -1,3-glucan, and expression of TdT is low during neonatal life (47). Therefore α -1,3-glucan-specific IgM Abs stimulated by MK7 during early life express relatively low-affinity BCRs and Abs. Because α -1,3-glucan can be expressed by enteric commensal organisms (33), it is possible that the α -1,3-glucan-specific IgA-secreting cells that localize to the lung following cockroach sensitization express Abs that are more somatically hypermutated and, thus, may have higher affinity for α -1,3-glucan compared with the anti- α -1,3-glucan IgM Abs. Last, although we know that α -1,3-glucan epitopes are expressed in multiple sites in the German cockroach, we do not know how the glycan is arrayed on the allergenic particles. The α -1,3-glucan epitope may be linear or branched, and it could exist as an oligomer or a polymer (48, 49). M104E Abs preferentially bind oligomers that are no larger than trisaccharides, whereas the higher-affinity J558 Ab binds glycans arrayed as penta- and hexasaccharide (49). Depending on the way in which α -1,3-glucan epitopes are arrayed on cockroach particles, it is possible that the allergen could be better recognized by IgA Abs but not IgM Abs. Therefore, Ab affinity and glycan presentation on the allergenic particles may be contributing factors to IgA-mediated, but not IgM-mediated, protection against cockroach allergy.

IgA-mediated neutralization of allergens, bacterial pathogens, and viral particles associated with the mucosa is critical for immune protection (50). Selective IgA-deficient patients have higher rates of atopic disorders than do those without the deficiency (51, 52). In our model, anti- α -1,3-glucan IgA may be especially important in preventing the interaction of cockroach-derived Bla g 2/ α -1,3-glucan complexes with innate receptors on DCs and epithelial cells in the lung, thus decreasing their activation of type 2 ILCs and priming of Th2 cells. IgA Ab binding to pathogens or allergenic particulates may also promote more efficient mucosal clearance via the mucociliary escalator. In line with our studies, it was reported that intranasal administration of an anti-human IgA Ab to a ragweed pollen Ag (Amb a 1) within a few hours of allergen challenge significantly decreased the development of AHR, production of allergen-specific IgE, and secretion of Th2-associated cytokines in mice. This study also demonstrated that Ab-mediated protection was Ag dependent, because the anti-human Amb a 1 Ab did not protect in a model of OVA challenge (53). Another study showed that DCs primed in the presence of cholera toxin B (CTB) and OVA stimulated the production of mucosal IgA from MedLN B cells, which suppressed the development of allergic disease. The investigators suggested that CTB exposure suppressed allergic inflammation via secretory IgA, because the above phenotype was lost in pIgR^{-/-} mice (54). However, IgM is also transported to the mucosa via the polymeric IgR, and it is possible that the immunosuppressive effects of CTB were due to pulmonary mucosa-associated IgM and/or IgA.

Because α -1,3-glucan epitopes are expressed on gut microflora, fungi, and various oral biofilm-forming organisms, it is likely that children can respond to these epitopes via mucosal (gut, pulmonary, or oral) immune responses during early life. Sampling of a cohort of 150 white and African American pediatric patients ranging from ages 1 to 15 y demonstrated that all children have detectable, but variable, levels of serum anti- α -1,3-glucan IgM Abs, irrespective of their asthma status (P.S. Patel and J.F. Kearney, unpublished observations). There is no cure for asthma, and therapeutics for atopic diseases focus on treatment of symptoms. Individuals typified by long-term steroid or antihistamine use or those administered mAbs against Th2-associated cytokines or cytokine receptors can develop resistance to these treatments over time (55, 56). Based on our understanding of the neonatal mouse B cell response to α -1,3-glucan, we suggest that therapeutic prophylaxis in the form of a vaccine or probiotic containing α -1,3-glucans during early life could be used to modulate neonatal B cells, with the potential for long-lived suppression of asthma development to cockroach sensitization.

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Disclosures

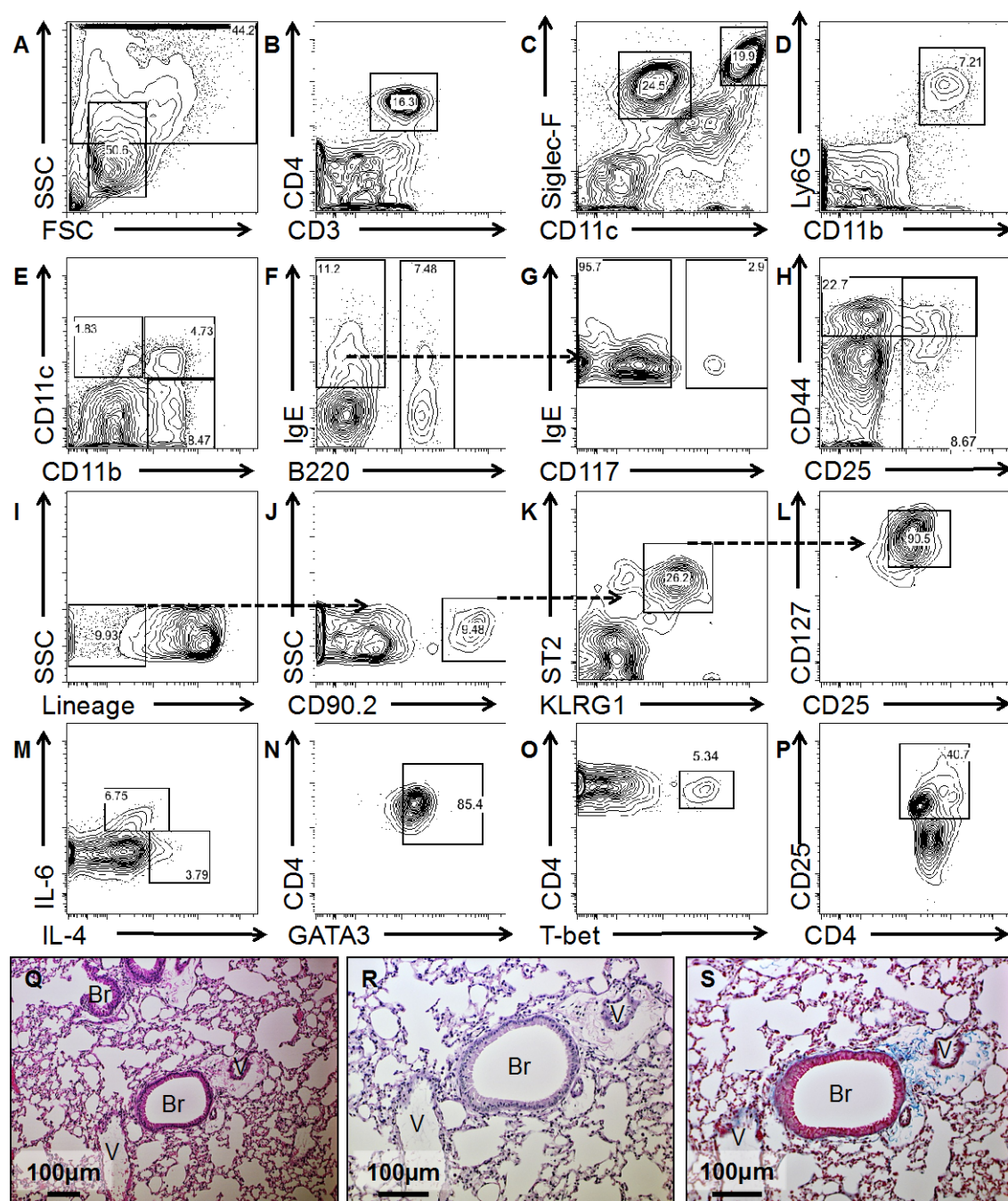
The authors have no financial conflicts of interest.

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Supplemental Figure 1

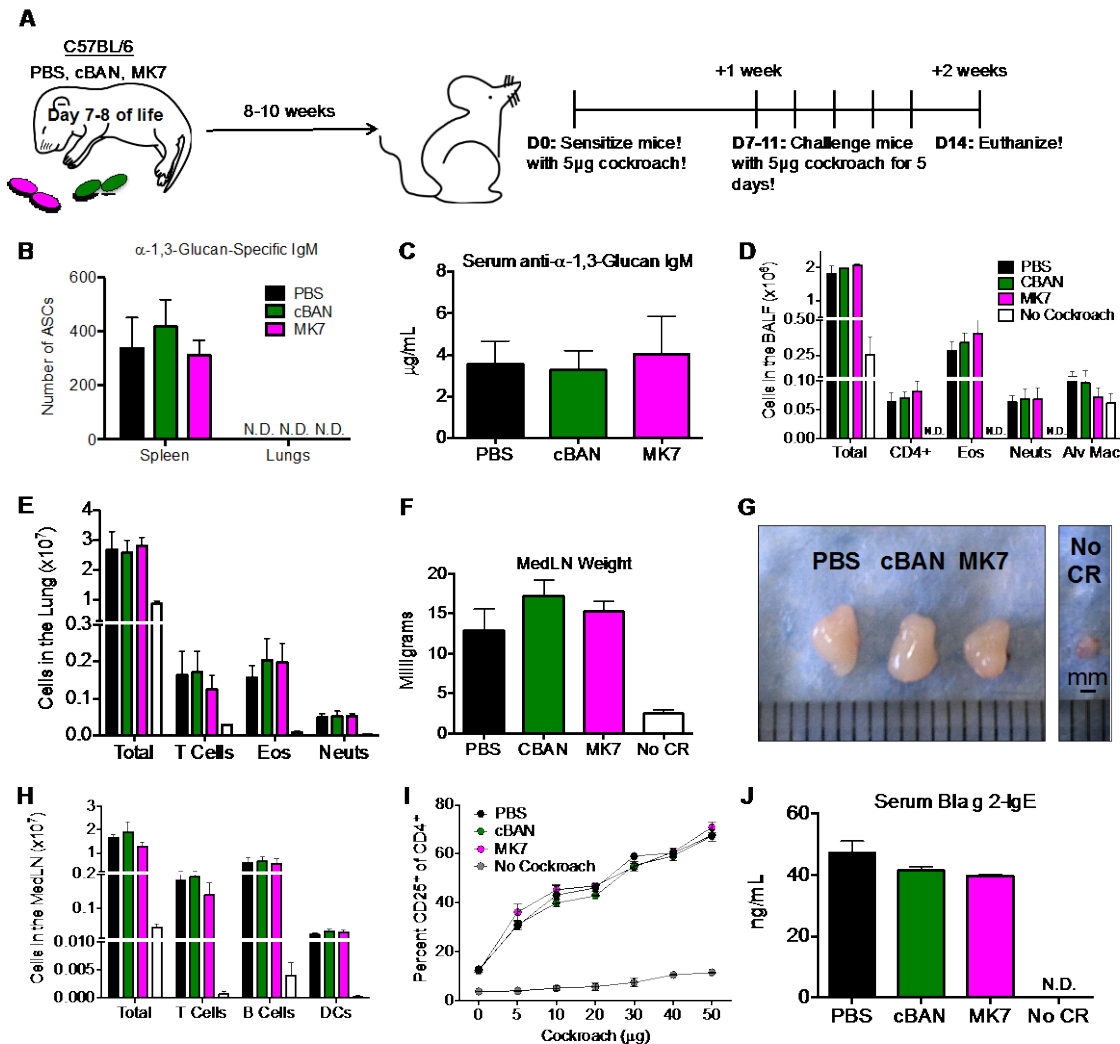


Br=Bronchiole; V=vessel

Supplemental Figure 1: Representative gating scheme for cells detected by flow cytometry and histological stains of paraffin-embedded lungs from mice that have not received any allergens. Cells from suspensions of BALF, lungs, and MedLN were

5 analyzed by flow cytometry to detect specific markers as described below: (A)
 6 representative forward scatter (FSC) and side scatter (SSC) for cells from the lungs (B) T
 7 cells ($CD3^+CD4^+$), (C) alveolar macrophages (Siglec-F $^+CD11c^+$) and eosinophils (Siglec-
 8 F $^+CD11c^-$), (D) neutrophils ($CD11b^+Ly6G^+$), (E) dendritic cells ($CD11b^-CD11c^+$),
 9 immature dendritic cells ($CD11b^+CD11c^+$), and macrophages ($CD11b^+CD11c^-Ly6G^-$) (F)
 10 B cells ($B220^+$) and IgE-bound non B cells ($B220^-IgE^+$), (G) basophils ($B220^-IgE^+Ckit^-$),
 11 and mast cells ($B220^-IgE^+Ckit^+$). (H) T cells ($CD3^+CD4^+$) were further phenotyped as
 12 $CD44^{high}$ or $CD25^+$. (I) ILC2s were identified by excluding lineage cells expressing CD3,
 13 CD4, CD8, CD19, B220, CD11b, CD11c, F4/80, GR1, TER119, and DX5. From the
 14 remaining cells, ILC2s were identified as those that expressed (J) CD90.2, (K) ST2 and
 15 KLRG1, (L) CD127 and CD25, and CD117 (not shown). $CD4^+$ T cells were further
 16 characterized as (M) IL-4 $^+$ or IL-6 $^+$, (N) GATA3 $^+$ (O) T-bet $^+$, or (P) $CD25^+$. Paraffin-
 17 embedded lungs from mice receiving no allergens were stained with (Q) Hematoxylin and
 18 Eosin, (R) Periodic Acid-Schiff, (S) or Masson's Trichrome stains.

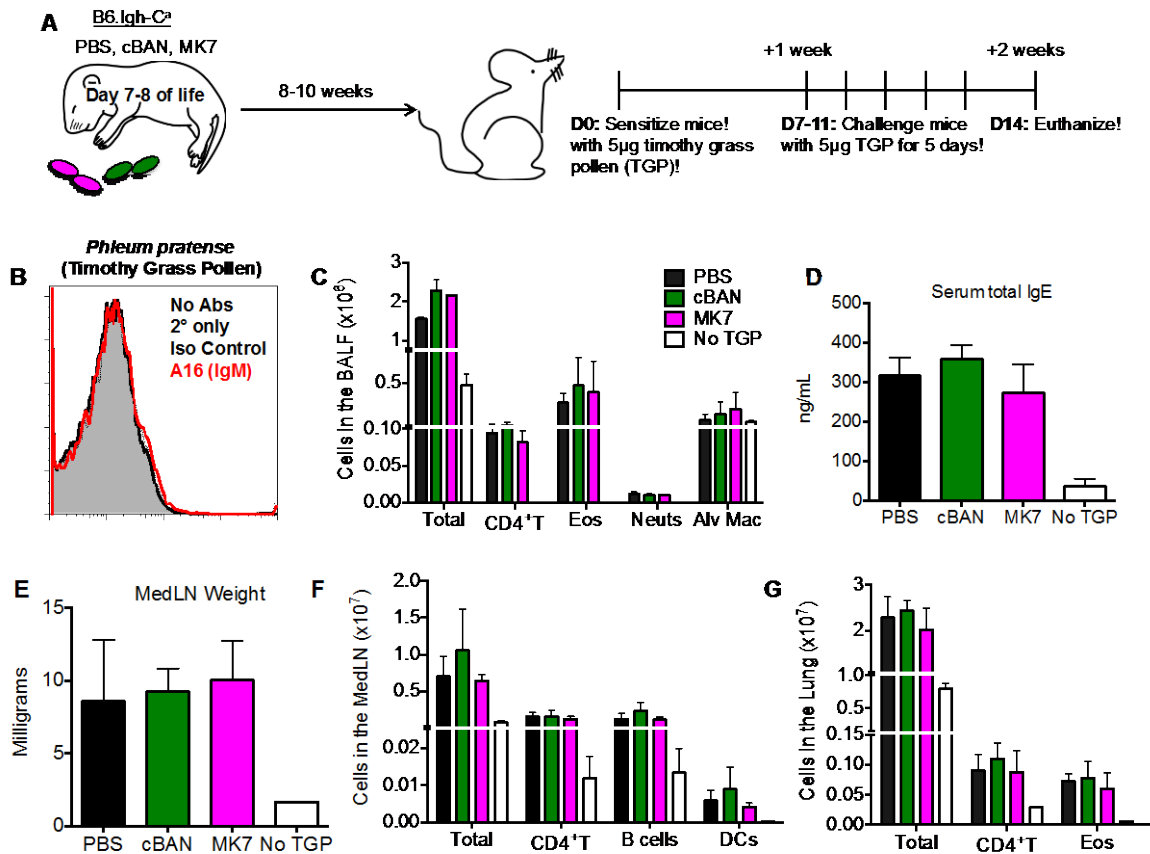
Supplemental Figure 2



19

20 **Supplemental Figure 2: Immunization of neonatal C57BL/6 mice (Igh-C^b) with MK7**
 21 **does not result in suppressed development of cockroach allergy** (A) Neonatal C57BL/6
 22 mice (B6.Igh-C^b) were immunized i.p. with 5 $\times 10^7$ of cBAN or MK7, or treated with PBS.
 23 At 8 to 10 weeks of age, these mice were sensitized and challenged i.t. with cockroach
 24 allergen. (B) The number of α -1,3-glucan-specific IgM- and IgA- secreting cells from the
 25 lungs and spleen were enumerated and (C) anti- α -1,3-glucan-IgM from the serum was
 26 quantified by ELISA. (D, E) Cells were identified from the BALF and lungs as
 27 documented in Supplemental Figure 1 and enumerated. (F-H) MedLNs were
 28 photographed, weighed, and the CD4⁺ T cells, B cells, and DCs were quantified. (I)
 29 300,000 MedLN cells were incubated with indicated amounts of cockroach allergen for
 30 five days and the percent of CD4⁺ T cells expressing CD25 was determined. (J) ELISA
 31 was used to measure levels of serum anti-Bla g 2-IgE. Values represent the mean $\pm$ SEM
 32 from 3 independent experiments with 5-10 mice per group. Values that were not
 33 detectable are noted as "N.D." Data were analyzed by ANOVA and statistically significant
 34 differences are indicated as * p <0.05, ** p <0.01, and *** p <0.001

Supplemental Figure 3



Supplemental Figure 3: B6.Igh-C^a mice immunized with MK7 as neonates are not protected against the development of α -1,3-glucan-free timothy grass pollen (TGP) allergy. (A) Neonatal B6.Igh-C^a mice were immunized i.p. with 5×10^7 cBAN or MK7 or treated with PBS. At 8 to 10 weeks of age, these mice were sensitized with TGP, rested for seven days, challenged for 5 consecutive days, and euthanized two days later for analysis. (B) TGP was stained with monoclonal anti- α -1,3-glucan IgM antibody, A16, or isotype control antibody, MD4, and antibody binding was determined by flow cytometry. (C) Cells were enumerated from the BALF and identified by flow cytometry as documented in Supplemental Figure 1. (D) ELISA was used to determine levels of IgE in the serum. (E) MedLNs were weighed and (F) cells were enumerated from the MedLN and (G) pulmonary parenchyma. Values represent the mean $\pm$ SEM from 2 independent experiments with 5-10 mice per group. Data were analyzed by ANOVA and statistically significant differences are indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.