

Allergic airway sensitization impairs antibacterial IgG antibody responses during bacterial respiratory tract infections



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Background: *Mycoplasma pneumoniae*, an atypical human pathogen, has been associated with asthma initiation and exacerbation. Asthmatic patients have been reported to have higher carriage rates of *M pneumoniae* compared with nonasthmatic subjects and are at greater risk for invasive respiratory infections. **Objective:** We sought to study whether prior allergen sensitization affects the host response to chronic bacterial infection.

Methods: BALB/cJ and IL-4 receptor $\alpha^{-/-}$ mice were sensitized with ovalbumin (OVA) and then infected with *M pneumoniae* or *Streptococcus pneumoniae*. Immune parameters were analyzed at 30 days postinfection and included cellular profiles in bronchoalveolar lavage fluid (BALF) and serum IgG and IgE antibody levels to whole bacterial lysate, recombinant P1 adhesin, and OVA. Total lung RNA was examined for transcript levels, and BALF was examined for cytokine protein profiles.

Results: Anti-*M pneumoniae* antibody responses were decreased in allergen-sensitized, *M pneumoniae*-infected animals compared with control animals, but OVA-specific IgG responses were unaffected. Similar decreases in anti-*S pneumoniae* antibody levels were found in OVA-sensitized animals. However, *M pneumoniae*, but not *S pneumoniae*, infection augmented anti-OVA IgE antibody responses. Loss of IL-4 receptor signaling partially restored anti-*M pneumoniae* antibody responses in IgG_{2a} and IgG_{2b} subclasses. Inflammatory cytokine levels in BALF from OVA-sensitized, *M pneumoniae*-infected or *S pneumoniae*-infected animals were reduced compared with those in uninfected OVA-sensitized control animals. Unexpectedly, airway hyperreactivity to methacholine was essentially ablated in *M pneumoniae*-infected, OVA-sensitized animals.

Conclusions: An established type 2-biased host immune response impairs the host immune response to respiratory

bacterial infection in a largely pathogen-independent manner. Some pathogens, such as *M pneumoniae*, can augment ongoing allergic responses and inhibit pulmonary type 2 cytokine responses and allergic airway hyperreactivity. (J Allergy Clin Immunol 2019;143:1183-97.)

Key Words: *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, infection, antibody, allergy, cytokines, animal model

Early microbial exposures in childhood, along with genetic predisposition, have been implicated in the onset of allergic disease.¹ There have been surprisingly few studies comparing immune responses to vaccines or naturally occurring infections among patients with respiratory allergies/asthma and nonallergic control subjects.²⁻⁶ A few recent studies have suggested that short-term infections in conjunction with allergic airway disease can impair antipathogen responses, but few have focused on chronic effects of allergic airway disease on antigen-specific humoral responses.⁷⁻¹⁰ Our work presented here was designed to investigate the hypothesis that the type 2 (T2)-biased pulmonary immune response in asthmatic patients can impair the immune response to infection with select pathogens.

Mycoplasma pneumoniae is one of the smallest self-replicating prokaryotic organisms. It is the leading etiologic agent of bacterial community-acquired pneumonia (CAP) in children, as well as an important agent in adults.¹¹⁻¹⁴ An estimated 30% of all cases of CAP have been attributed to *M pneumoniae*,¹⁵ but diagnosis is difficult because of the need for acute and convalescent sera, specialized culture conditions, or nucleic acid amplification test expertise.¹⁶ Reported carriage rates of the organism in the asthmatic population vary widely based on the detection scheme, but in some cases *M pneumoniae* has been detected in up to 50% of patients with stable asthma.¹⁷

Streptococcus pneumoniae is the leading cause of adult bacterial CAP, as well as an important cause of invasive bacterial disease.¹⁴ Well-established as a causative agent of otitis media, sinusitis, pneumonia, meningitis, and sepsis, *S pneumoniae* is a ubiquitous and occasionally invasive bacterial pathogen, particularly in young children and the elderly, caused by asymptomatic carriage in the nasopharynx. Capsule-specific antibodies are essential mediators of clearance and control during pneumococcal infections alongside other innate immune activators (ie, ficolin-2).¹⁸⁻²⁰

The increased microbial lung burden and susceptibility of asthmatic patients to infections suggest that further study of this population's immune response is warranted. *M pneumoniae* and *S pneumoniae* are convenient model organisms with which to explore the airway allergic immune response in mice.²¹ Although

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Supported by internal funding from the UAB Departments of Pediatrics and Medicine. Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication December 1, 2017; revised June 2, 2018; accepted for publication July 24, 2018.

Available online August 6, 2018.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2018.07.021>

Abbreviations used

ARP:	Animal Resources Program
BALF:	Bronchoalveolar lavage fluid
CAP:	Community-acquired pneumonia
CFU:	Colony-forming unit
CSR:	Class-switch recombination
dpi:	Days postinfection
FITC:	Fluorescein isothiocyanate
HPS:	Histopathologic scoring
HRP:	Horseradish peroxidase
IL-4R:	IL-4 receptor
KO:	Knockout
Muc5AC:	Mucin 5AC
OVA:	Ovalbumin
PAS:	Periodic acid–Schiff
PE:	Phycoerythrin
qPCR:	Quantitative PCR
rP1:	Recombinant P1
T1:	Type 1
T2:	Type 2
THY:	Todd Hewitt broth
UAB:	University of Alabama at Birmingham
WT:	Wild-type

innate immune responses are important for acute-phase control of *M pneumoniae*, chronic models of infection suggest a role for humoral immunity in controlling dissemination of the organism.²² Hypogammaglobulinemic patients, who have impaired IgG antibody responses, also have increased risk of invasive disease caused by *M pneumoniae*, underscoring the importance of humoral responses in sequestering *M pneumoniae* in the lung.^{23,24} Likewise, the highly effective control of invasive pneumococcal disease with polysaccharide and protein conjugate vaccines using select serotype-specific capsular polysaccharides underscores the importance of antibody in controlling *S pneumoniae* infection.²⁵

Mouse models of allergic airway disease and models of infection with *M pneumoniae* and *S pneumoniae* are well-established.^{21,26–31} Hypothesizing that immune responses might be impaired because of allergic/asthmatic comorbidity, we decided to combine sensitization and chronic infection models to examine the effect of prior sensitization on the immune response to infection.

METHODS**Animals and genotyping**

Wild-type (WT) BALB/cJ and BALB/cJ–IL-4 receptor (IL-4R) α^{tm1Sz} (IL-4R $\alpha^{-/-}$, IL-4R α knockout [KO]) animals were purchased from Jackson Laboratory (Bar Harbor, Me). IL-4R $\alpha^{-/-}$ animals were bred in-house for the extent of these studies (find genotyping and phenotyping information in the Methods section in this article's Online Repository at www.jacionline.org). Animal experiments were carried out in accordance with Institutional Animal Care and Use Committee and Animal Resources Program (ARP) guidelines at the University of Alabama at Birmingham (UAB), as well as the National Institutes of Health "Guide for the care and use of laboratory animals" under University of Alabama at Birmingham animal protocol number ARP-10112.

Mouse tissue (tail snips) was used for genotyping to ensure mouse genotypes were correct during breeding. Tissue was homogenized with tissue grinders for disruption (Thermo Fisher Scientific, Waltham, Mass) and

then processed for genomic DNA isolation with the Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's specifications.

For genotyping reactions to examine IL-4R α -sufficient and deficient animals, end-point PCR was used, according to a previously published protocol.³² After determining genomic DNA concentrations (NanoDrop 1000, Thermo Fisher Scientific), agarose gel electrophoresis (1% agarose gels) was carried out on end-point PCR samples. Genotyping gels were imaged under UV conditions with SYBR Safe DNA Gel stain (Thermo Fisher Scientific).

Antibodies, reagents, and buffers

Antibodies used in ELISAs for IgG, IgG subclasses, and IgE were purchased as follows: goat anti-mouse IgG-horseradish peroxidase (HRP) was purchased from Sigma-Aldrich (St Louis, Mo), and the following antibodies were all purchased from Abcam (Cambridge, United Kingdom): goat anti-mouse IgE-HRP, goat anti-mouse IgG₁-HRP, goat anti-mouse IgG_{2a}-HRP, goat anti-mouse IgG_{2b}-HRP, and goat anti-mouse IgG₃. Antibodies for flow cytometric analyses were purchased from BioLegend (San Diego, Calif), as follows: anti-mouse CD45 in fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin, and peridinin-chlorophyll-protein complex/Cy5.5 and anti-mouse CD19-FITC, anti-mouse CD124-PE, and mouse isotype controls in FITC, PE, allophycocyanin, and peridinin-chlorophyll-protein complex/Cy5.5. Antibody against serotype 19 pneumococcal strains (Rb anti-*S pneumoniae* serotype 19) was provided by Dr Moon Nahm and Dr Brady Spencer (University of Alabama at Birmingham, Birmingham, Ala) for this work. Total IgE levels were examined by using a specific kit for Mouse IgE (BioLegend), according to the manufacturer's instructions. *M pneumoniae* P1-adhesin carboxy terminus (recombinant P1 [rP1]) peptide was purchased from ProSpec Biosciences (Ness-Ziona, Israel). Grade V ovalbumin (OVA) was purchased from Sigma-Aldrich for immunization, airway challenge, and enzyme immunoassay.

Bacterial culture

A clinical isolate of *M pneumoniae* (UAB PO1) was obtained from the UAB Diagnostic Mycoplasma Laboratory for this work. Stocks were grown for 5 to 7 days at 37°C until color change in cell-culture flasks with 100 mL of SP4 media. Adherent bacteria were then harvested with cell scrapers (Corning Life Sciences, Corning, NY) and passaged repeatedly through a 27-gauge needle to disrupt aggregates. Bacteria were concentrated in fresh SP4 media, and colony-forming units (CFUs) were determined for cultures in aliquots. Stocks were stored at –80°C, and vials were thawed before each experiment.

S pneumoniae strain EF3030 (ATCC 49619) was grown statically in Todd Hewitt broth (THY) supplemented with 0.5% yeast extract at 37°C (Sigma-Aldrich). Broth cultures were tracked by measuring spectrophotometric absorbance at 600 nm (OD₆₀₀). When OD₆₀₀ reached 0.4, cultures were cooled on ice, and 80% sterile glycerol was added to a final concentration of 10%. Stocks were stored at –80°C, and vials were thawed before each experiment.

M pneumoniae CFUs were determined by using serial dilution and plating on SP4 Agar. Bacteria were diluted on SP4 Agar for 5 to 7 days at 37°C in a 5% CO₂ atmosphere. Plates were visualized with a dissection microscope, and colonies were counted after 5 to 7 days of growth. For *S pneumoniae* CFUs, stocks were serially diluted in THY and plated on blood agar solid media. Plates were incubated for 18 to 24 hours at 37°C in a 5% CO₂ atmosphere. Colonies were visually counted, and CFUs per milliliter were calculated for relative range of frozen stocks.

Preparation of bacterial lysate

M pneumoniae and *S pneumoniae* strains (UAB PO1 and EF3030, respectively) were grown, as outlined above. When cultures had grown for the aforementioned amount of time, cultures were harvested and spun at 10,000g for 20 minutes to pellet bacterial cells. Pellets were resuspended in 5 mL of PBS plus 0.05% thimerosal (Sigma-Aldrich) and incubated at 4°C for 2 to 3 days. After incubation, material was passed through increasingly

smaller needles (18-gauge down to 27-gauge) repeatedly to break apart aggregates. The resulting slurry was then dialyzed in Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific) in PBS, according to the dialysis cassette manufacturer's specifications. Slurry was then vortexed vigorously and placed in aliquots at -20°C until use for ELISA analysis. Antigen preparations were used for no more than 2 freeze-thaw cycles to prevent substrate degradation.

Allergic airway sensitization and infection

Age-matched (4- to 10-week-old) BALB/cJ and IL-4R $\alpha^{-/-}$ mice (Jackson Laboratory) were maintained in filter-top cages in specific pathogen-free environments during the course of experimentation. At the time of sensitization and 7 days later, mice were injected intraperitoneally with 200 μL of OVA/alum (Alumjet; Fisher Scientific) containing 50 μg of OVA per dose. At 14 days after the first immunization and at weekly intervals thereafter, mice received intranasal instillation of OVA (20 μL /naris of 1% OVA-PBS) after achievement of light anesthesia with isoflurane.

Five days after sensitization and initial intranasal challenge, mice were infected with *M pneumoniae* through the intranasal route. Sterile SP4 medium was instilled intranasally in control mice. *M pneumoniae* in SP4 medium (UAB PO1) was thawed to room temperature, sonicated with two 1-second pulses, and then intranasally administered to anesthetized mice to mimic airway infection, as previously described.³³ Bacterial doses for initial infections were 10^7 to 10^8 CFUs for each mouse. *S pneumoniae* stocks were thawed to room temperature directly before infection. Animals were infected through the intranasal route as per earlier *M pneumoniae* infections, with either EF3030 in THY medium or sterile THY alone. Animal groups were killed at 30 days postinfection (dpi) for all experiments.

Mouse euthanasia and sample collection

Mice were anesthetized with vaporized isoflurane (Henry Schein, Melville, NY) before euthanasia for sample processing. Euthanasia was carried out according to specific ARP guidelines.

For histology, lungs were inflated with cold 10% buffered formalin at 24 cm of pressure, and then mainstem bronchi were ligated and clipped. After extraction, lungs were stored in 10% formalin for 24 to 48 hours at 4°C for fixation and then processed. For real-time quantitative PCR (qPCR), lungs were stored in RNeasy Lysis Buffer (Thermo Fisher Scientific) to prevent nucleic acid degradation. Samples were stored at -20°C until processing for total RNA.

BALF analysis

Mouse tracheas were isolated away from the cervical dissection field, pierced with a 21-gauge intravenous catheter (BD Biosciences, San Jose, Calif), and secured in place with 5.0 nylon suture. For collection of bronchoalveolar lavage fluid (BALF), 0.8 to 1 mL of ice-cold sterile PBS with 1% heat-inactivated FCS (Thermo Fisher Scientific) was lavaged into the airway, aspirated, and then maintained at 4°C . Samples were then processed for cytospin analysis and cell differential determination by using a Cytospin cytocentrifuge (Shandon Cytospin III Cytocentrifuge; Thermo Fisher Scientific).

Cytospin slides were air-dried overnight before staining with Modified Wright Stain (Sigma-Aldrich), according to the manufacturer's instructions. Cell types were counted and enumerated based on morphologic staining characteristics. Percentages and absolute cell numbers (cells per milliliter of BALF) were determined based on hemocytometer counts before cytospin. Between 200 and 400 cells were counted per sample in duplicate for analysis.

For protein analysis, BALF was decellularized by means of centrifugation and stored at -80°C with protease inhibitors (Sigma-Aldrich) to prevent degradation. For cytokine protein measurements, 25 μL of neat BALF was analyzed by using the LEGENDplex Mouse Th Cytokine Panel (BioLegend), according to the manufacturer's specifications, on a C6 Accuri Benchtop Flow Cytometer (Becton Dickinson). Data were analyzed with LEGENDplex Software (version 7.0) and represented as medians or normalized mean fluorescence intensity by using the manufacturer's software (BioLegend).

Pulmonary function testing

A 24-gauge Angiocath was inserted into the trachea and fixed with a ligature of 3–0 silk. The flexiVent apparatus (SCIREQ, Montreal, Quebec, Canada) was used to perform measurement maneuvers, including perturbations (predefined pressure of volume waveforms), such as forced oscillations, by using room air in the closed-chest animal. Calibration of the flexiVent is done with a tracheal cannula. Mice are then challenged with the aerosolized bronchoconstrictor methacholine (Sigma-Aldrich) at increasing concentrations (0, 10, 25, 50, and 100 mg/mL). At each dose, lung resistance, compliance, and elastance are calculated by using the single-compartment model.

Histopathologic scoring

After exposure to allergen and infection, histopathologic changes in the lungs were determined by means of histology on formalin-fixed and paraffin-embedded sections by using hematoxylin and eosin staining and airway mucus with periodic acid–Schiff (PAS) staining. For histology, the lungs were inflated with cold 10% buffered formalin at a pressure of 24 cm, mainstem bronchi were ligated, and lungs were immersed in 10% buffered formalin for 24 to 48 hours at 4°C . Fixed tissues were processed routinely into paraffin and sectioned at 5 μm . Duplicate sections from each block were stained with hematoxylin and eosin and PAS-hematoxylin. Slides were examined by an experienced comparative pathologist without knowledge of experimental classifications.

Bronchial lesion scores were based on epithelial hypertrophy and goblet cell metaplasia, mucosal and intramural inflammatory cells, adventitial inflammatory cells, and adventitial edema. Each was scored for severity (0, 1, 2, or 3 for absent, mild, moderate, or severe, respectively) and distribution (1 for $\leq 25\%$, 2 for 25% to 50%, 3 for 50% to 75%, or 4 for 75% to 100% of the section affected). Bronchial lesion scores were calculated as the sum of the products of individual severity and distribution scores.

Alveolar lesion scores were based on perivascular inflammatory cells, septal edema, septal inflammatory cells, type II cell hyperplasia, luminal inflammatory cells, luminal epithelial cells and cellular debris, and luminal edema and fibrin. Each was scored for severity and distribution, and alveolar lesion scores were calculated as above.

Total lesion scores were calculated as the sum of bronchial and alveolar lesion scores.³⁴

RNA isolation and qPCR analysis

After isolation and storage in RNeasy Lysis Buffer, mouse lungs were homogenized with a tissue grinder system (Thermo Fisher Scientific). Total RNA was isolated from tissue by using TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions.

Purified RNA was then used for cDNA synthesis by using the First-Strand cDNA Synthesis kit (Thermo Fisher Scientific), according to the manufacturer's instructions. cDNA was diluted and then used as an input target for qPCR analysis. For immunologic targets, a SYBR-Green Kit (Roche, Mannheim, Germany) was used with specific primers (Table 1)^{35–37} to determine cycle threshold (Ct) expression levels on a LightCycler 480 instrument (Roche). Primers were either obtained from previously described target amplicons or created *de novo* by using the National Center for Biotechnology Information's Primer Blast.^{32,35,38} All qPCR primers were validated for specificity to the gene of interest by showing an amplicon with a single melting peak and a single band in agarose gel. qPCR reactions for specific genes and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; endogenous control) were analyzed by using the $\Delta\Delta\text{Ct}$ method for normalization and comparison of relative expression levels. Data were graphed as $\log_{10}$ mean fold change $\pm$ SD relative to control values.

ELISA for plasma antibodies

For plasma antibody levels, whole blood was obtained from mice by means of cardiac puncture during euthanasia and diluted 1:5 in 1% heparin-PBS (Thermo Fisher Scientific). Plasma was separated by means of centrifugation

TABLE I. Primer pairs used for qPCR

Target gene	Forward primer (5' > 3')	Reverse primer (5' > 3')	Reference
<i>GAPDH</i>	TCCATGACAACCTTGGCATTG	CAGTCTTCTGGGTGGCAGTGA	37
<i>IL4</i>	GGTCTCAACCCCACTAGT	GCCGATGATCTCTCAAGTGAT	37
<i>IL13</i>	CCTGGCTCTTGCTTGCCCT	GGTCTGTGTGATGTTGCTCA	NCBI Primer Blast
<i>IFNG</i>	ACAGCAAGGCGAAAAAGGATG	TGGTGGACCACTCGGATGA	37
<i>IL10</i>	CCTCAGGATGCGGCTGAG	GCTCCACTGCCTTGCTCTTATT	NCBI Primer Blast
<i>CCL11</i>	CCCAACTCCCTGCTGCTTTA	AGATCTCTTTGCCCAACCTG	NCBI Primer Blast
<i>MUC5AC</i>	AAAGACACCAGTAGTCACTCAGCAA	CTGGGAAGTCAGTGTCAAACCA	NCBI Primer Blast
<i>IL5</i>	GCAATGAGACGATGAGGCTTC	GCCCTGAAAGATTCTCCAATG	37
<i>IL17A</i>	ACCGCAATGAAGACCCTGAT	TCCCTCCGATTGACACA	36
<i>IL4RA WT</i>	TGTGGGCTCAGAGTGACCAT	CAGGGAACAGCCAGAAAAG	35
<i>IL4RA Mut</i>	CCAGACTGCCTTGGGAAAAG	CAGGGAACAGCCAGAAAAG	35

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

and then stored at -80°C until ELISA analysis. Ninety-six-well ELISA plates (Costar, Corning Life Sciences) were coated with antigen (20 $\mu\text{g}/\text{mL}$ for bacterial lysate or OVA or 2–5 $\mu\text{g}/\text{mL}$ for rP1 C-adhesin C-terminal peptide) overnight in antigen coating buffer (100 mmol/L bicarbonate/carbonate solution, pH 9.6) at 4°C . In brief, antigen solution was decanted, and plates were blocked with 1% BSA-PBS (Sigma-Aldrich) solution for 1 hour at room temperature. Plasma was diluted 1:50 in 1% BSA-PBS and incubated for 2 hours at 37°C . Plates were washed 3 times with 0.05% Tween-20-PBS (Sigma-Aldrich) before secondary antibody addition. Secondary antibody solution was incubated at room temperature for 1 hour. Plates were washed an additional 3 times before TMB solution addition (Thermo Fisher Scientific), according to the manufacturer's specifications. HRP substrate reactions were halted with 2N sulfuric acid (Sigma-Aldrich), and absorbance was measured at 405 nm by using a Victor X3 Plate Reader (PerkinElmer, Waltham, Mass).

Statistical analysis

Unless otherwise noted, sample means $\pm$ SDs were used for comparison of statistical significance. When data were normally distributed, comparison of more than 2 experimental groups was performed by using 1-way ANOVA with Bonferroni pairwise posttests or the Tukey *ad hoc* posttest. Log₁₀ transformation was used to increase the likelihood of Gaussian distribution on specific data sets. Nonparametric data were analyzed by using the Mann-Whitney *U* test, with a Dunn *post hoc* test for pairwise comparisons. With data sets containing 2 experimental groups, unpaired *t* tests were used for analysis. Pulmonary function data were analyzed by using 2-way ANOVA with the Dunnett multiple comparison posttest. One-tailed tests were used to increase statistical significance where prior data had indicated directionality under 2-tailed conditions. Outliers were determined by using the ROUT method with statistical package software. Data were graphed with GraphPad Prism software (version 7; GraphPad Software, La Jolla, Calif). Differences were considered statistically significant at a *P* value of less than .05.

RESULTS

OVA allergic sensitization induces eosinophilic inflammation and mucus production

Mice were initially sensitized and then dosed intranasally at weekly intervals with OVA as an allergen to induce and sustain T2 inflammatory allergic airway disease (Fig 1, A). The cellular differential of BALF, as determined by using a cytopsin, showed an increased percentage and number of eosinophils in OVA-sensitized groups at 30 dpi (Fig 1, B, and see Fig E1, A and F, in this article's Online Repository at www.jacionline.org). Despite the increased presence of eosinophils in the airways of OVA-sensitized mice, increases in neutrophil (PMN) counts were also seen, suggesting that the sensitization procedure was driving a mixed type 1 (T1)/T2 inflammatory profile (see

Fig E1, A, E, and F). Lymphocytic percentages and numbers were greatly increased in OVA-sensitized animals but not *M pneumoniae*-infected control animals (see Fig E1, D). Conversely, the absolute number of monocyte/macrophages between experimental groups showed no significant difference (see Fig E1, C).

Lung histology using hematoxylin and eosin staining of OVA-sensitized uninfected mice at 1 month after infection showed robust peribronchiolar and perivascular cellular infiltration, goblet cell hyperplasia, and a marked increase in airway mucus by using PAS staining compared with unsensitized mice (see Fig E2 in this article's Online Repository at www.jacionline.org). OVA-sensitized and challenged groups showed significantly increased histopathologic scoring (HPS) compared with allergically naive control groups (Fig 1, C). HPS of OVA-sensitized, *M pneumoniae*-infected mice showed no difference in total HPS from that of OVA-sensitized, uninfected animals (Fig 1, C). Interestingly, although there were no differences in bronchiolitis scores in OVA-sensitized animals (Fig 1, E), alveolitis scores were decreased in *M pneumoniae*-infected, OVA-sensitized animals compared with those in allergen-sensitized control animals (Fig 1, D).

Antibacterial IgG responses are decreased in OVA-sensitized animals with infections

Because previous studies have suggested that asthmatic patients did not seroconvert to IgG with *M pneumoniae* infection,^{4,39} we predicted that IgG antibody titers would be decreased in a mouse model of allergic airway inflammation and *M pneumoniae* infection. Analysis of plasma antibody levels at 30 dpi confirmed these observations and showed that IgG responses against both *M pneumoniae* whole-cell lysate and rP1 are significantly reduced in OVA-sensitized animals with infections compared with *M pneumoniae*-infected animals (unsensitized but infected; Fig 2, A and B). Analysis of P1-specific IgG subclass responses showed that this decrease with OVA sensitization was present in all IgG subclasses (Fig 2, C–F). *M pneumoniae*-specific IgE was undetectable in infected animals in multiple experiments by using either whole bacterial lysate or rP1 (data not shown).

To determine whether total antibody responses were affected by *M pneumoniae* infection, OVA-specific antibody titers were examined. Allergen-specific IgG antibody responses resulted in increased antibody titers in OVA-sensitized groups compared with those in naive control animals (Fig 3, A). No differences

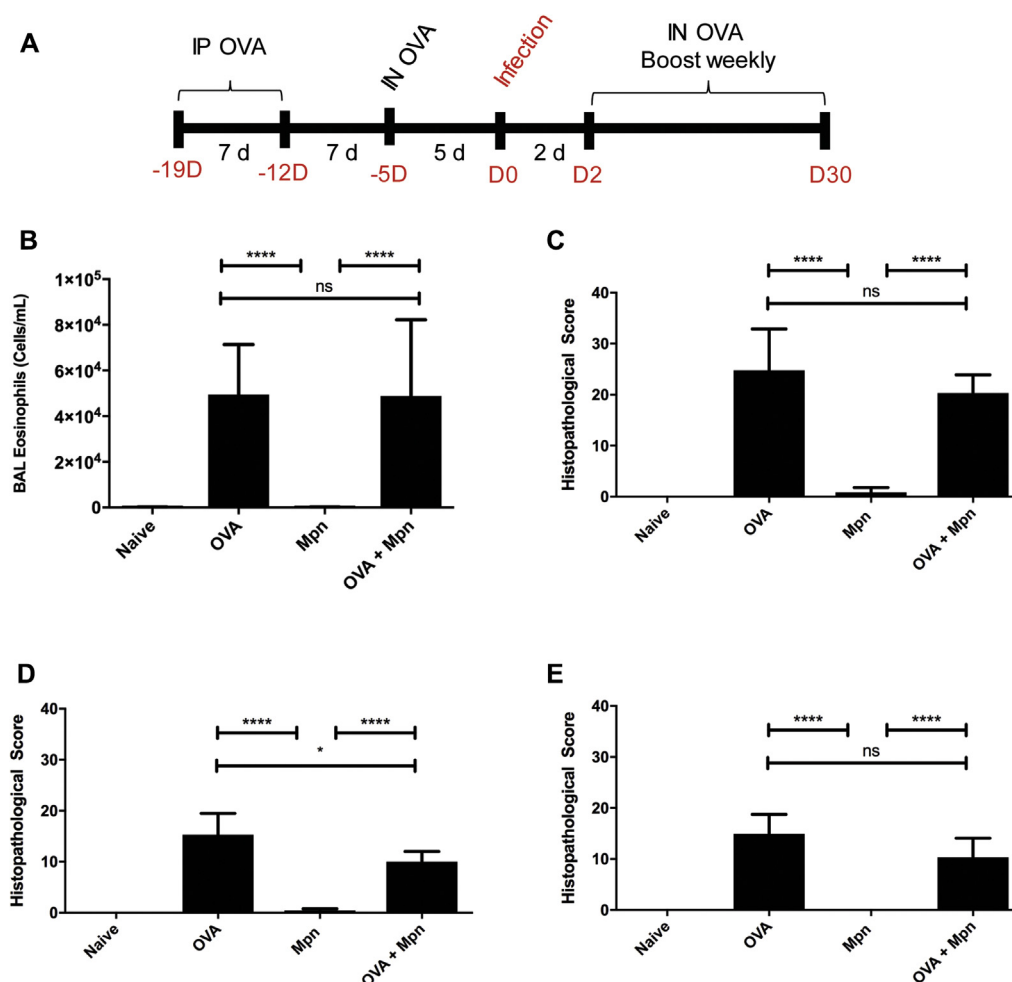


FIG 1. An OVA-based allergic airway sensitization model induces pulmonary eosinophilia and inflammation. **A**, Experimental sensitization protocol for allergic airway inflammation and bacterial infection for the 30-day end point. *IN*, Intranasal; *IP*, intraperitoneal. **B**, BALF cytology and enumeration of eosinophils during allergic airway disease. **C–E**, Histopathologic scores for experimental groups with or without allergic airway sensitization and/or bacterial infection: Fig 1, **C**, total scores; Fig 1, **D**, alveolitis; and Fig 1, **E**, bronchiolitis. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. *Mpn*, *M pneumoniae*; *ns*, no significant difference on statistical analysis. * $P < .05$, **** $P < .001$, and ***** $P < .0001$.

were found in OVA-specific total IgG antibody levels in sensitized mice with or without *M pneumoniae* infection. No differences in anti-OVA IgG subclass responses were seen between the sensitized and sensitized groups with *M pneumoniae* infection (Fig 3, B–E). These observations suggested that defects in bacteria-specific IgG antibody production were not due to a host inability to mount humoral responses against antigen. Interestingly, *M pneumoniae* infection augmented allergen-specific IgE responses significantly compared with OVA control values (Fig 3, F). No differences in total IgE levels were found between the OVA-sensitized and OVA-sensitized and infected groups, suggesting that the selective enhancement of allergen-specific antibody levels was due to *M pneumoniae* infection (Fig 3, G).

Having shown that prior allergic airway sensitization decreased anti-*M pneumoniae* IgG antibody titers, we hypothesized that the reduction of antibacterial titers might be applicable to nonmycoplasmal respiratory tract infections. *S pneumoniae* (pneumococcus) is the second most prevalent bacterial agent of

pediatric bacterial CAP and the leading cause of adult bacterial CAP.¹¹ Because of the lethality of *S pneumoniae* isolates in mice, a serovar 19F strain, EF3030, was chosen for its ability to cause local but not disseminated disease.^{31,36,37} Mice were infected intranasally with *M pneumoniae* or *S pneumoniae* in the absence or presence of preexisting allergic airway sensitization and then examined for antibacterial IgG titers at 30 dpi.

As previously demonstrated in this experiment, allergically naive, *M pneumoniae*-infected animals mounted robust IgG antibody responses against the microbe, which were significantly reduced in the presence of allergic airway disease (Fig 4, A). Similarly, animals infected with *S pneumoniae* mounted high antibacterial IgG titers, whereas allergically sensitized animals showed a similar but less significant decrease in anti-*S pneumoniae* IgG antibody levels (Fig 4, B). As in previous experiments, anti-OVA IgG responses were not significantly different between the OVA-sensitized and OVA-sensitized and infected groups (Fig 4, C).

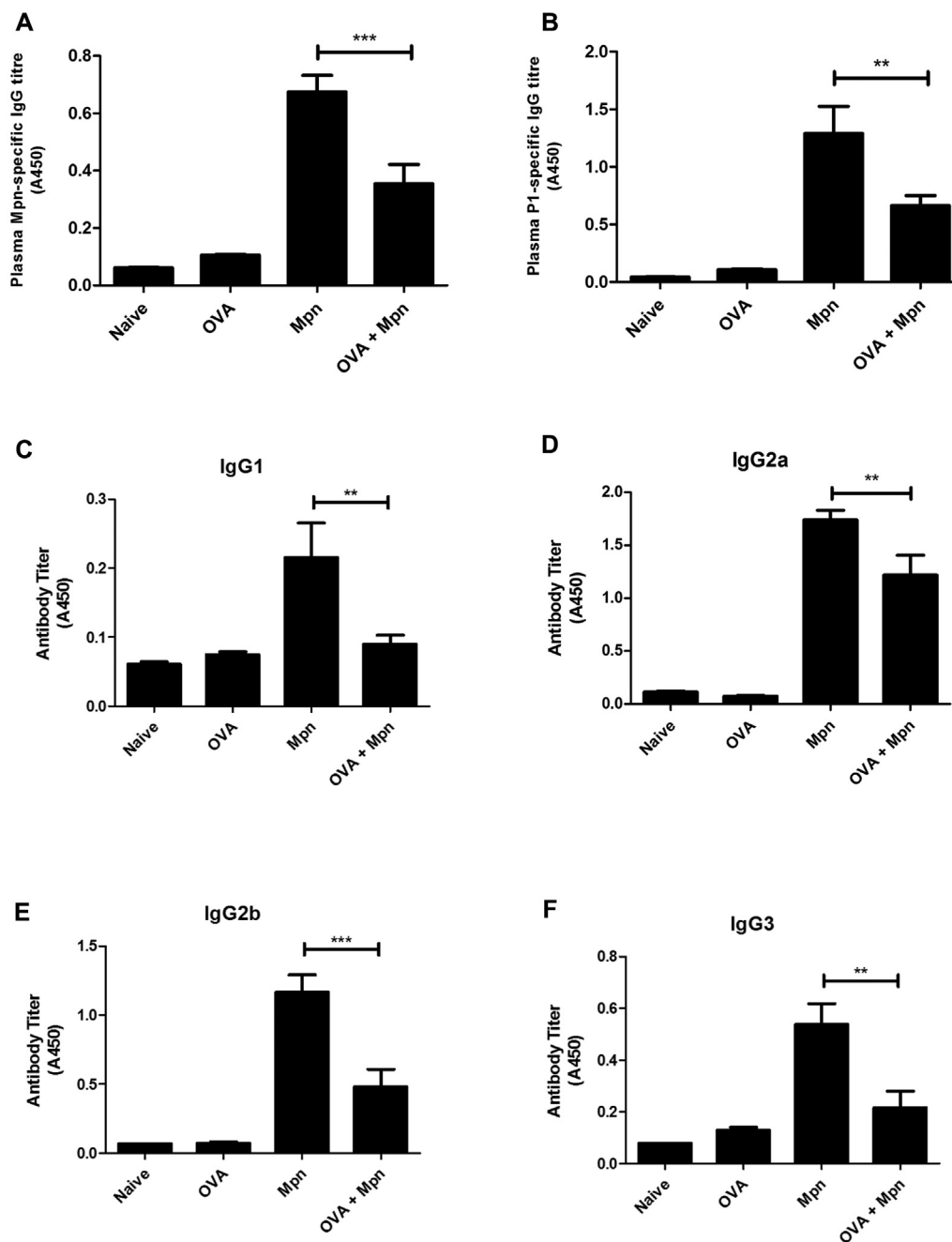


FIG 2. Allergic airway sensitization impairs *M pneumoniae*-specific IgG titers at 30 dpi. **A**, IgG antibody to whole-cell *M pneumoniae* (Mpn) lysate. **B-F**, Antibody to rP1 for IgG (Fig 2, B), IgG₁ (Fig 2, C), IgG_{2a} (Fig 2, D), IgG_{2b} (Fig 2, E), and IgG₃ (Fig 2, F). Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. ** $P < .01$ and *** $P < .001$.

Antibody responses in IL-4R α -deficient mice

We hypothesized that blockade of a T2 inflammatory phenotype would result in restoration of antibacterial antibody responses. To address this hypothesis, IL-4R α -deficient (IL-4R $\alpha^{-/-}$, IL-4R α KO) and sufficient (WT) BALB/cJ animals were examined for the effect of prior allergen sensitization on the antibody response to *M pneumoniae* infection.

First, we confirmed the genetic background and decreased IL-4R α mRNA and protein expression in the knockout strain (see Fig E3 in this article's Online Repository at www.jacionline.org). OVA-induced inflammation was significantly decreased by using HPS in IL-4R α -deficient animals compared with WT animals (see Fig E1, A and E). Lung histology of OVA-sensitized KO animals also showed diminished mucus and airway hyperplasia

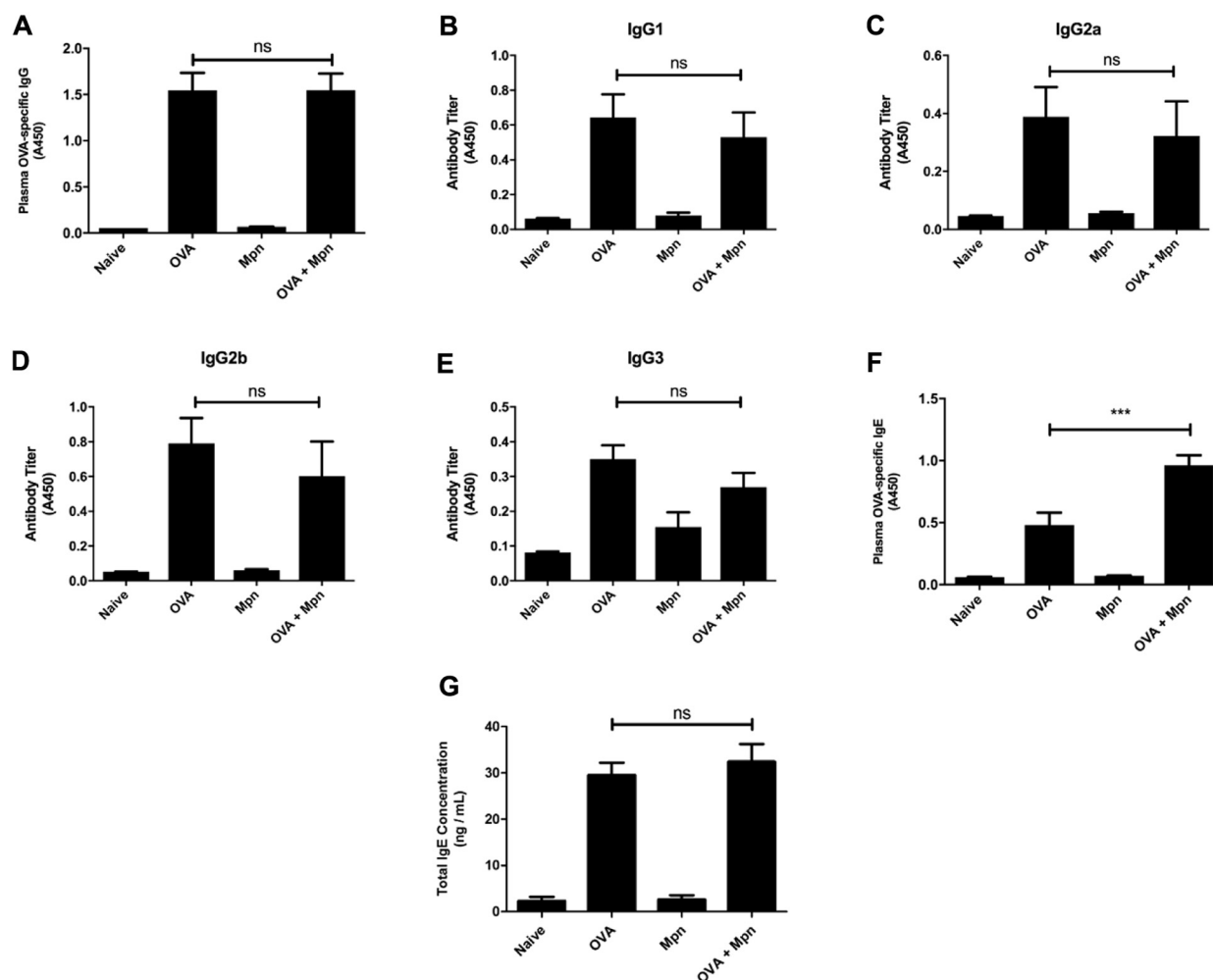


FIG 3. Anti-OVA IgG, IgG subclass, and IgE antibody responses and total IgE in animals with and without OVA sensitization and/or *M pneumoniae* (Mpn) infection at 30 dpi: **A**, total IgG antibody; **B**, IgG₁-specific; **C**, IgG_{2a}-specific; **D**, IgG_{2b}-specific; and **E**, IgG₃-specific responses. **F**, IgE antibody. **G**, Total IgE. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. ns, No significant difference on statistical analysis. *** $P < .001$.

and decreased infiltration of inflammatory cells, suggesting that, as expected, the loss of IL-4 and IL-13 receptor signaling greatly diminished T2 airway disease in our model (see Fig E1, A-C and E; Fig E2, and Fig E4 in this article's Online Repository at www.jacionline.org).

A similar degree of inhibition of *M pneumoniae*-specific IgG antibody production was measured in the OVA-sensitized, *M pneumoniae*-infected IL-4R $\alpha^{-/-}$ mice as seen in the WT control mice by using either a whole-cell lysate (data not shown) or rP1 C-adhesin C-terminal peptide (Fig 5, A). Interestingly, on examination of subclass-specific responses, inhibition of antibody production was virtually abrogated in P1-specific IgG_{2a} and IgG_{2b} subclass titers (Fig 5, B-E). This decrease in inhibition was not seen in either P1-specific IgG₁ or IgG₃, suggesting that other immunomodulatory factors can play important roles in determining subclass-selective isotype switch in this model.

Anti-OVA IgG antibody titers were increased in OVA-sensitized and challenged animals and showed no difference between treatment groups within each strain, although there were

differences between the 2 strains in anti-OVA antibody production (Fig 6, A, and see Fig E4). As previously reported, class-switch recombination (CSR) of B cells to IgE relies on IL-4 signaling.^{40,41} Thus, without functional T1 or T2 receptor complexes, we predicted IgE antibody production would be virtually abolished in the knockout strain. As seen previously, *M pneumoniae* infection in OVA-sensitized WT animals augmented OVA-specific IgE titers, but this increase was not seen in IL-4R $\alpha^{-/-}$ animals (Fig 6, B). Again, no differences in total IgE levels were seen in WT animals, and as hypothesized, IL-4R $\alpha^{-/-}$ animals did not produce IgE at detectable levels (Fig 6, C). Curiously, despite the reported importance of IL-4 in CSR to IgG₁,^{42,43} we found both P1- and OVA-specific IgG₁ titers to be increased in animals deficient in IL-4 signal transduction (Fig 5, B, and see Fig E5 in this article's Online Repository at www.jacionline.org).

Immunologic gene expression

OVA-sensitized and IL-4R α -sufficient groups exhibited increased IL-4, IL-5, IL-10, and IL-13 mRNA levels in whole

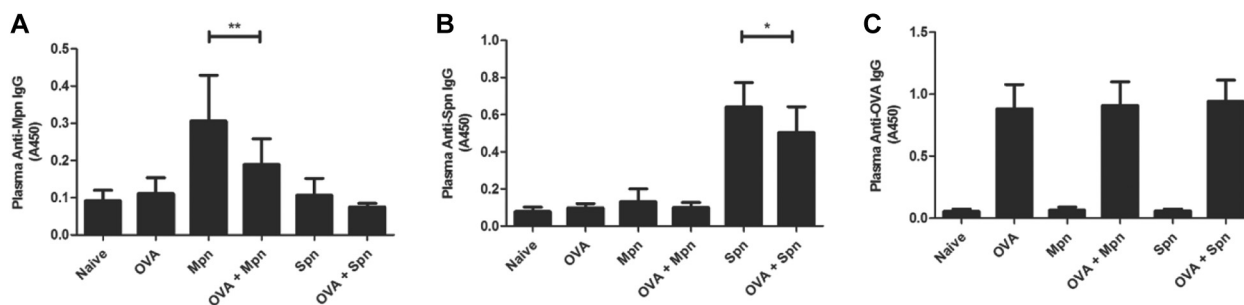


FIG 4. IgG antibody responses during allergic airway disease model with *M pneumoniae* (Mpn) and *S pneumoniae* (Spn) bacterial infections. **A**, Anti-*M pneumoniae* IgG (crude bacterial lysate). **B**, Anti-*S pneumoniae* IgG (crude bacterial lysate). **C**, Anti-OVA IgG. Data are presented as group means $\pm$ SDs for 4 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. * $P < .05$ and ** $P < .01$.

lung by using qPCR compared with levels in allergically naive control animals (Fig 7, A-D). CCL11 (eotaxin-1) qPCR results also showed enhanced levels in OVA-sensitized groups (both *M pneumoniae*-infected and uninfected) compared with levels in naive control animals at 30 dpi (Fig 7, E). Interestingly, mucin 5AC (Muc5AC) and CCL11 mRNA levels were enhanced in *M pneumoniae*-infected and sensitized WT animals compared with those in OVA-sensitized uninfected animals (Fig 7, E and F). As expected, in the knockout animals T2-associated transcripts (IL-4, IL-5, IL-13, CCL11, and Muc5AC) showed significant decreases compared with those in WT IL-4R α -sufficient control animals (Fig 7, A-C, E, and F). Significant increases in *Ifng* and *Il17a* gene expression were present in all OVA-sensitized KO animals compared with WT control animals (Fig 7, G and H).

Cytokine protein levels in BALF and airway resistance during pulmonary infection

Because of the differences between bacterial pathogens in our model, we predicted that the organisms would have different effects on lung cytokine profiles. To determine whether this was the case, BALF was analyzed for cytokine protein levels at 30 dpi and compared for all experimental groups using WT mice (Fig 8, A). As transcript data in previous experiments had shown, T2 cytokine protein expression (IL-4, IL-5, and IL-13) was increased in OVA-sensitized and challenged animals compared with that in their respective naive control animals. No striking differences were apparent between naive control animals and either the *M pneumoniae*- or *S pneumoniae*-infected and allergically naive experimental groups. Interestingly, *M pneumoniae* infection in allergen-sensitized animals resulted in significantly decreased inflammatory cytokine levels (IL-5, IL-6, IL-13, and TNF- α) compared with those in matched OVA-sensitized and uninfected control animals (Fig 8, C-E and G). However, allergen-sensitized, *S pneumoniae*-infected animals had significantly greater IL-6 and TNF- α expression than OVA-sensitized, *M pneumoniae*-infected animals, which were more similar to that in the OVA-sensitized animals, at 30 dpi (Fig 8, E and G). These differences might further clarify why OVA-sensitized, *S pneumoniae*-infected animals also had lower total eosinophil numbers and increased PMN numbers in BALF cellular profiles at 30 dpi when compared with either the OVA or OVA-sensitized, *M pneumoniae*-infected groups (see Fig E6, B and D, in this article's Online Repository at www.jacionline.org).

Having shown decreases in cytokine responses during allergic airway disease and *M pneumoniae* infection, we were curious as to the potential of *M pneumoniae* infection in altering the pulmonary resistance rates. To determine whether *M pneumoniae* augmented airway hyperresponsiveness and pulmonary resistance, pulmonary function tests with methacholine challenge tests were carried out during allergen sensitization and *M pneumoniae* infection by using flexiVent instrumentation. As predicted, OVA-sensitized animals showed increased overall pulmonary resistance rates, elastance, and increases in airway hyperresponsiveness manifested by an increase in pulmonary resistance over baseline during methacholine airway challenge compared with naive animals (Fig 9). Although allergen-sensitized and *M pneumoniae*-infected animals exhibited a greater baseline pulmonary resistance rate (Fig 9, C), contrary to earlier hypothesized differences, they showed diminished airway hyperresponsiveness and no change in pulmonary elastance during exposure to increased doses of methacholine when compared with naive mice (Fig 9, A-C). Interestingly, when the change in resistance with increases in methacholine levels was expressed as a percentage of baseline resistance, the OVA-sensitized, *M pneumoniae*-infected animals exhibited a distinctly blunted response compared with all 3 other groups, including the control animals (Fig 9, D).

DISCUSSION

In the current study we report that allergically sensitized animals with ongoing airway inflammation have an impaired IgG antibody response to either *M pneumoniae* or *S pneumoniae* infection compared with unsensitized and infected control mice. The decreased antibodies against these pathogens suggest that the humoral immune response against pulmonary bacterial infection is impaired in the presence of an ongoing, T2-biased immune response. These results complement and extend the results reported in previous studies by other groups showing T2-mediated impairment of the pulmonary inflammatory response to infection and increased bacterial survival.^{26,44,45} However, our work provides a longer-term look at the humoral and pulmonary immune/inflammatory responses and airway hyperactivity. *M pneumoniae* infection also enhances allergen-specific IgE, emphasizing previous findings that implicate *M pneumoniae* in both allergic sensitization and acute airway exacerbation.^{16,46,47} Further work is needed to understand the effect of acute versus chronic disease models and the effect on host-pathogen interactions.

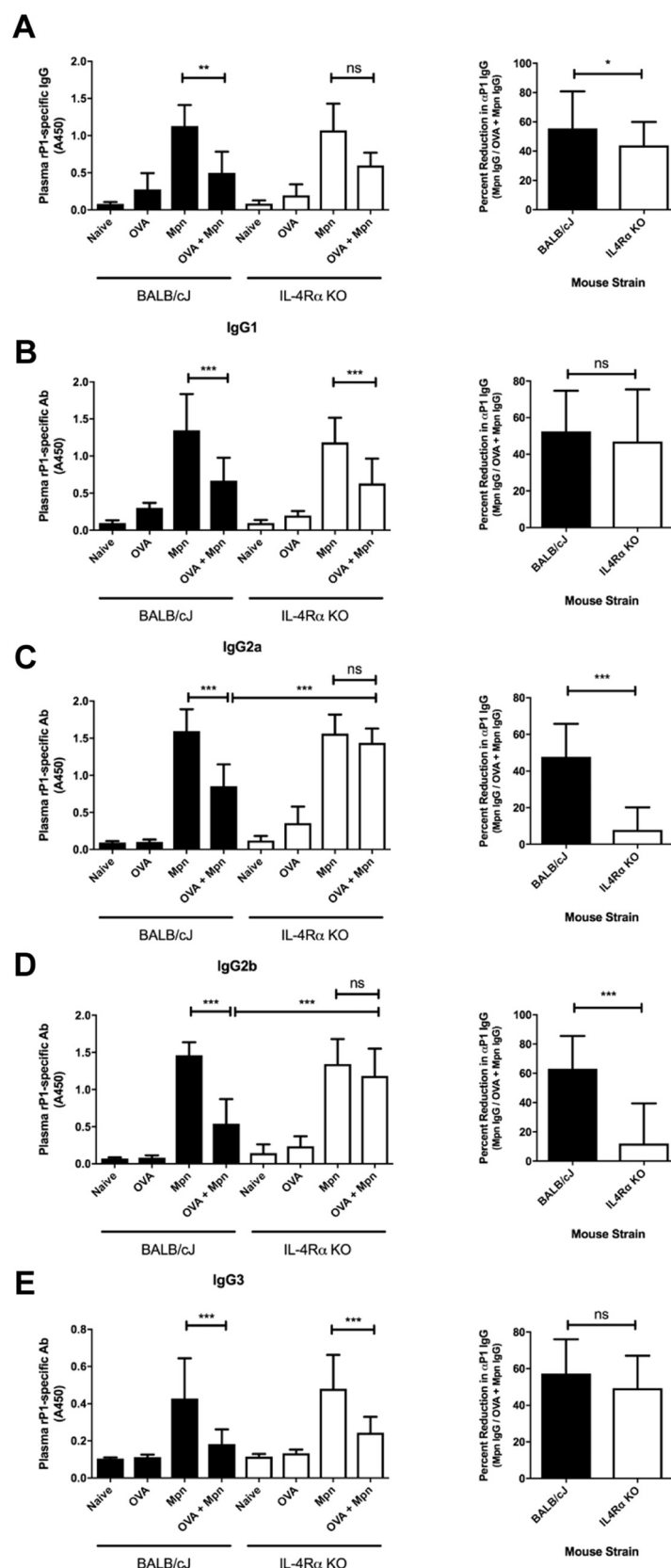


FIG 5. ELISA analysis of anti-*M pneumoniae* (Mpn) IgG and IgG subclass responses to rP1 in IL-4R α -sufficient and deficient animals with or without OVA sensitization and/or *M pneumoniae* infection. *Left panels*, IgG or IgG subclass antibody to rP1, as indicated. *Right panels*, Percentage reduction in antibody comparing respective *M pneumoniae* and OVA plus *M pneumoniae* groups: **A**, total IgG; **B**, IgG₁; **C**, IgG_{2a}; **D**, IgG_{2b}; and **E**, IgG₃. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. *ns*, No statistical difference on analysis. * $P < .05$, ** $P < .01$, and *** $P < .001$.

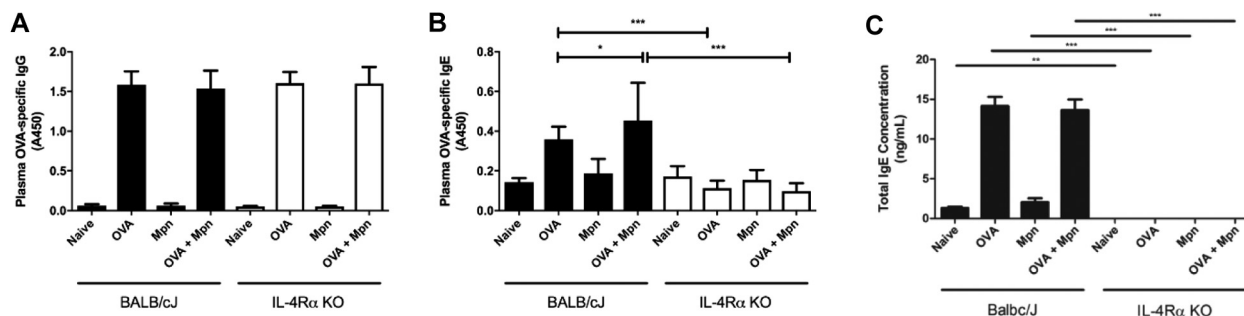


FIG 6. OVA-specific antibody responses and total IgE in WT and IL-4R α -deficient animals with or without OVA sensitization and/or *M pneumoniae* (*Mpn*) infection: **A**, IgG antibody; **B**, IgE antibody; and **C**, total IgE. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. * $P < .05$, ** $P < .01$, and *** $P < .001$.

Our observations demonstrate that antipathogen IgG responses are reduced in the context of allergic airway comorbidity and that antibody responses can be partially restored with loss of functional IL-4R α signaling. IFN- γ and TGF- β have been implicated in IgG_{2a} and IgG_{2b} CSR, respectively, which might in part explain the enhancement of anti-P1 IgG titers in IgG_{2a} and IgG_{2b} subclasses in IL-4R-deficient compared with WT animals (Fig 5, C and D).^{48,49} Our data further suggest that loss of IL-4R α signaling might not hinder CSR to IgG₁ against either P1 or OVA, although some studies have suggested otherwise in other antigen-specific models.^{50,51} Independent of our work, Fish et al⁵⁰ found that IL-4R α -deficient animals were able to mount IgG₁ responses against OVA but had overall lower immunoglobulin levels in sera. IgG₁ seroconversion has also been reported with IL-5 and CD38 activation independent of IL-4 signaling, although these data might not be applicable to our model system.⁵²⁻⁵⁴ These data might prove useful in furthering our understanding of necessary factors for CSR *in vivo*.

In our model we confirm that *M pneumoniae* infection enhances Muc5AC mRNA levels, which was initially reported by Kraft et al⁵⁵ in an *ex vivo* cell-culture system comparing airway cells derived from asthmatic patients and nonasthmatic subjects. Interestingly, for the first time, our observations show that CCL11 mRNA (eotaxin-1) is enhanced with long-term *M pneumoniae* infection. Previous work by Wu et al⁵⁶ showed that in an *M pneumoniae* infection model (FH strain) in the setting of allergic airway disease with OVA sensitization, CCL24 (eotaxin-2) levels were increased, although this was directly correlated with numbers of bacteria used to infect. In addition to bacterial pathogens, rhinoviruses have also been reported to upregulate CCL11 during mouse infections, suggesting that these pro-T2 immunologic signals might represent a form of pathogen-mediated immune modulation to prevent effective pathogen clearance.⁹

Direct comparisons between the host immune response to *M pneumoniae* and *S pneumoniae* infections *in vivo* have been sparse, with one study suggesting differential activation of the immune system. A study by Koh et al⁵⁷ suggested that *M pneumoniae* pneumonia in patients drove increased IL-4 expression (and IL-4/IFN- γ ratio) in BALF samples compared with either control subjects or patients with confirmed *S pneumoniae* pneumonia.⁵⁷ An additional study comparing *S pneumoniae* and atypical bacterial CAP showed that CAP caused by *S pneumoniae* was more acute in symptomatology

and increased PMN numbers compared with the prolonged course of symptomatology with atypical (*M pneumoniae* and *Chlamydia pneumoniae*) infections.⁵⁸ Our data comparing BALF cytokine protein profiles at 30 dpi suggest an interesting difference between acute and long-term experimental infection protocols, as well as between pathogens. *M pneumoniae* can actively influence gene expression to impair host antibacterial immune responses, as *Ureaplasma* species (as well as other *Mycoplasma* species) have been reported to do previously.^{59,60}

In contrast to *M pneumoniae*-modulated host responses, *S pneumoniae* induced significant increases in IL-6 and TNF- α levels during allergic airway disease compared with *M pneumoniae* infection (Fig 8). Disease outcomes between the 2 pathogens might explain some of these differences: *S pneumoniae* infection typically results in high rates of mortality and invasive disease, whereas *M pneumoniae* infection is typically an outpatient condition, and disseminated infections are relatively rare.^{16,61,62} Infection strategy and immune avoidance can play large roles in determining host-pathogen responses, with *S pneumoniae*, a “frontal assault pathogen,” resulting in more T1 immunity compared with the immune response to *M pneumoniae*, which has been referred to as a “stealth pathogen.”⁶³ With the distinct species-specific differences and virulence determinants, it might not be too surprising that *S pneumoniae* induces a somewhat more increased T1 inflammatory cytokine profile than that seen in patients with chronic *M pneumoniae* infection.

Unexpectedly, during examination of pulmonary function testing, downregulation of pulmonary airway responses to methacholine was noted in mice with allergic airway disease and *M pneumoniae* infection. These data intriguingly fit well with our data, suggesting a potent downregulation of inflammatory cytokine responses in the OVA-challenged and *M pneumoniae*-infected animals. These 2 observations directly contradict previous work, which has demonstrated that *M pneumoniae* induces increased inflammatory responses during acute infection.^{22,29,47,64-67} It is likely that the difference lies in the fact that our studies were carried out at a longer time point when most of the inflammation from the acute infection had died down.

Analysis of the host immune response to chronic *M pneumoniae* infection necessarily will involve examining the effects of pulmonary infection at longer timepoints. Chu et al⁴⁵ studied responses in *M pneumoniae*-infected animals with or without prior

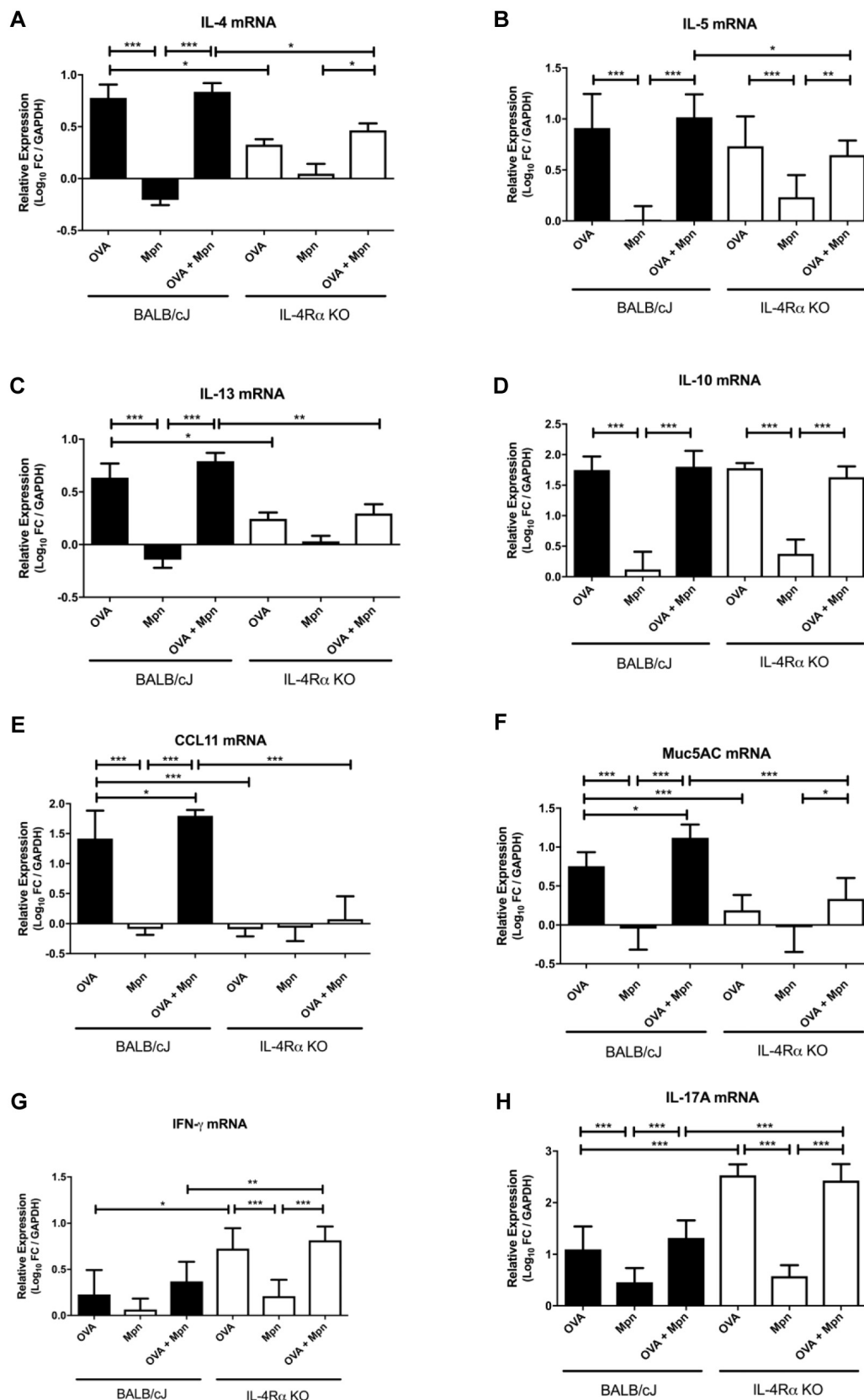


FIG 7. Fold change over control in pulmonary mRNA levels in WT and IL-4R α -deficient BALB/cJ animals at 30 dpi with or without OVA sensitization and/or *M pneumoniae* (*Mpn*) infection. Total lung analysis for pulmonary mRNA expression in WT and KO animals for IL-4 (**A**), IL-5 (**B**), IL-13 (**C**), IL-10 (**D**), CCL11 (eotaxin-1; **E**), Muc5AC (**F**), IFN- γ (**G**), and IL-17A (**H**) is shown. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Data are also presented as log₁₀ fold change of $\Delta\Delta$ cycle threshold analysis for each transcript. Experimental observations were repeated in duplicate at minimum for repeat observations. * P < .05, ** P < .01, and *** P < .001.

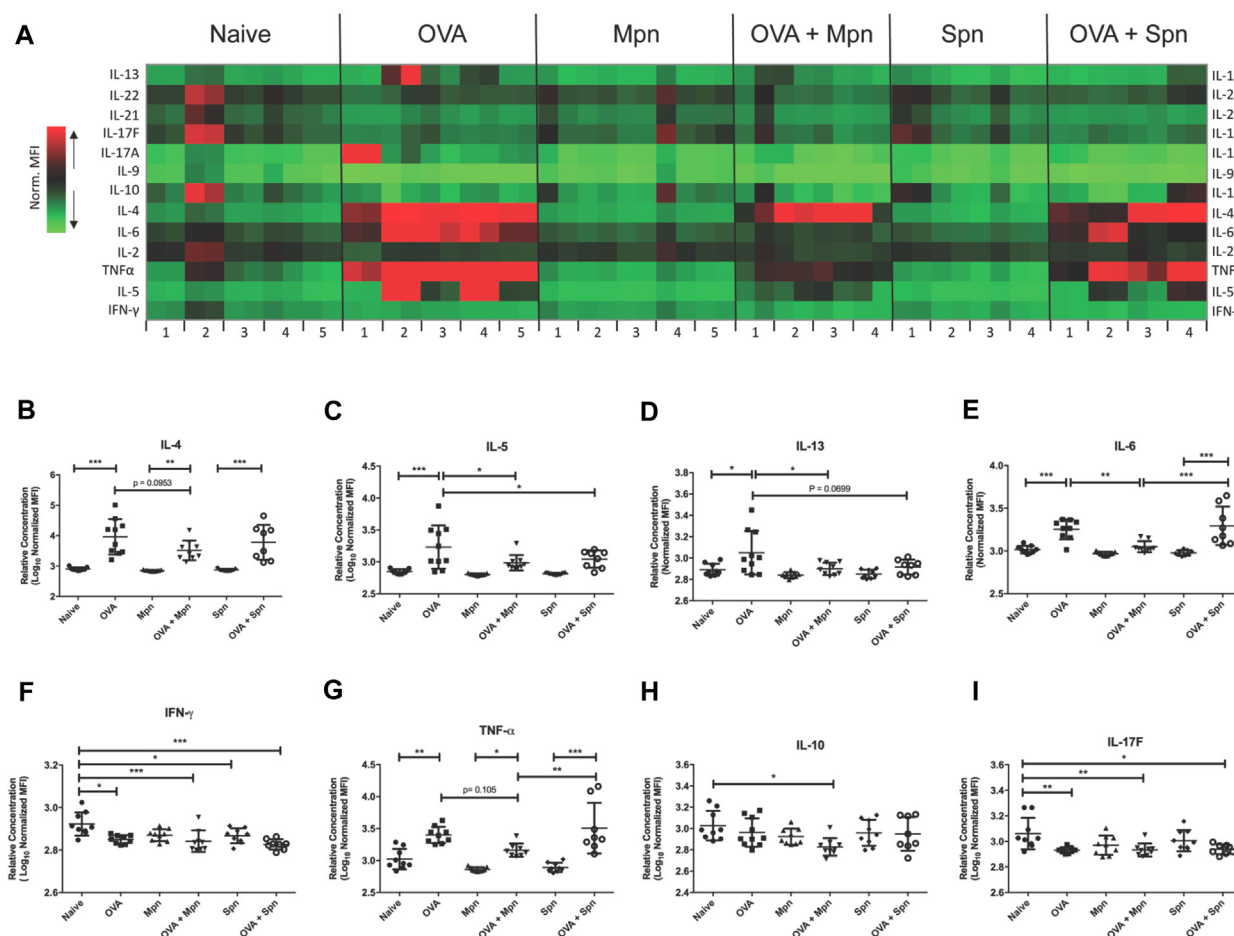


FIG 8. BALF cytokine responses in WT BALB/cJ mice with or without OVA sensitization and/or *M pneumoniae* (*Mpn*)/*S pneumoniae* (*Spn*) infection. **A**, Qualitative analysis of BALF cytokine protein concentrations. Normalized mean fluorescence intensity (MFI) data are represented in heat maps from low (green) to high (red) cytokine concentrations. **A–I**, Analysis of log₁₀ normalized MFI data for the BALF cytokines IL-4 (Fig 8, B), IL-5 (Fig 8, C), IL-13 (Fig 8, D), IL-6 (Fig 8, E), IFN-γ (Fig 8, F), TNF-α (Fig 8, G), IL-10 (Fig 8, H), and IL-17F (Fig 8, I). Data are presented as group means ± SDs for 4 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. **P* < .05, ***P* < .01, and ****P* < .001.

OVA sensitization out to 23 days, and Hardy et al⁶⁸ measured responses in *M pneumoniae*-infected and control animals out to 42 days but did not include allergen-sensitized groups. By day 23, Chu et al⁴⁵ found the airway hyperreactivity to methacholine, which had peaked in infected animals by day 9, was no different than in control animals, whereas Hardy et al,⁶⁸ who was using whole-body plethysmography to measure baseline enhanced pause as a surrogate for direct airway resistance measurements, found an early peak in enhanced pause during the first 3 days after nasal inoculation, which then decreased to near that of the control animals but almost always significantly greater than that of control animals out to day 28. Nevertheless, our finding of strikingly blunted methacholine responses in allergen-sensitized, *M pneumoniae*-infected animals at 30 days has not been reported previously.

Our work has limitations, which are important considerations in interpretation of our model. This model of OVA-induced sensitization uses intraperitoneal induction of OVA with alum before airway challenge. This does not mimic natural allergen sensitization in human subjects, unlike pulmonary challenge

models with house dust mite, pollen, or other bioactive proteases (eg, those from *Aspergillus* or *Alternaria* species).^{69–72} Prolonged allergen challenge can also induce pulmonary desensitization and IL-10 production, limiting the scope of T2-inflammatory responses. Other protocols for allergic airway induction might produce different results.

Additionally, the limitation of our studies to 2 bacterial strains (*M pneumoniae* UAB PO1 and *S pneumoniae* EF3030) might not be true for all *M pneumoniae* or *S pneumoniae* strains or bacterial infection models. Use of some *S pneumoniae* strains (ie, TIGR4) results in mortality, limiting the ability of long-term carriage or infection modeling.^{73,74} Having shown that primary antibacterial humoral immunity is affected during allergic airway disease, our work will need to further clarify whether this defect is present during antigen recall responses. Immunologic memory plays an important role in susceptibility of a host to reinfection, and therefore these observations are vital for further interpretation and work with our model. There is the often-made criticism that animal models of asthma are not as accurate mimics of human asthma as one would like for a variety

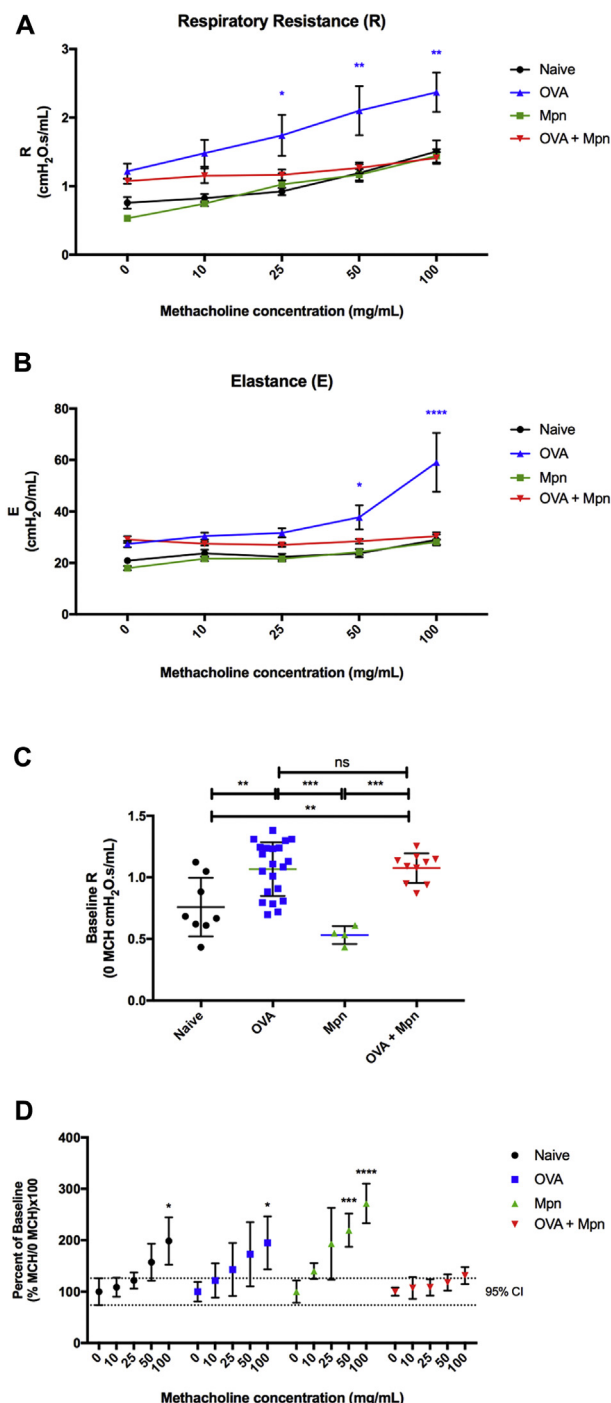


FIG 9. Pulmonary function testing of WT BALB/cJ mice with or without allergic airway sensitization and/or *M pneumoniae* (Mpn) infection during graded methacholine challenge. Airway resistance (A), elastance (B), comparison of baseline airway resistance of all groups (C), and percentage of baseline increases in airway resistance (D) during increasing doses of methacholine challenge compared with allergen-naïve, uninfected animals at baseline are shown. Data are presented as means $\pm$ SEMs for 4 to 10 animals during respiratory resistance analysis. Percentage increases in airway resistance over baseline are represented as means $\pm$ 95% CIs and compared with baseline airway resistance of naïve control subjects. Experimental observations were repeated in duplicate at minimum for repeat observations.

of reasons, including differences in immune function and lung structure/physiology.

Finally, we have only attempted to explore the effects of infection in a model of human T2-high asthma, which is certainly a common subtype, but our results would be of even more uncertain applicability to patients with nonatopic asthma.

In summary, we report antibody responses to respiratory tract infection with *M pneumoniae* or *S pneumoniae* in allergen-sensitized mice while boosting IgE antibodies to an established allergen. The fact that the impairment in antibacterial IgG antibody levels is also seen in a somewhat blunted form during pneumococcal infection suggests that allergic airway inflammation can impair the immune response to a variety of respiratory pathogens. Additionally, during ongoing allergic airway inflammation, *M pneumoniae* infection induced significant decreases in both pulmonary airway resistance and cytokine production through an unknown mechanism. These observations raise questions worthy of further exploration.

We thank Mrs Melanie Fecanin, Dr Warren Simmons, Dr Hong Wang, Dr Preeyam Patel, Dr James Daubenspeck, and Dr Kevin Dybvig for their discussions of this work as a whole. We also thank Dr Brady Spencer and Dr Moon Nahm for the generous donation of antisera against *S pneumoniae* serotype 19 for this work.

Key messages

- Allergic airway sensitization impairs antibody responses to bacterial respiratory tract infection.
- Pulmonary cytokine responses and airway hyperreactivity are diminished with chronic *M pneumoniae* infection in a mouse model of allergic airway inflammation.

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METHODS

For more information on both genotypic and transcriptional WT or KO levels of IL-4R α , see [Fig E4, A and B](#). IL-4R α forms heterodimeric complexes with the common γ -chain and IL-13 receptor α 1, which constitute the T1 and T2 IL-4Rs. Flow cytometric analysis was additionally carried out on WT and IL-4R $\alpha^{-/-}$ splenocytes, showing that protein levels at baseline were lower in KO animals (see [Fig E4, C](#)). Because of insertion of the neomycin resistance cassette in this mouse, specificity for α CD124 showed some binding at the

remaining residues of exon 6-7^{E1} but was overall lower in geometric mean, as well as median mean fluorescence intensity, readings.

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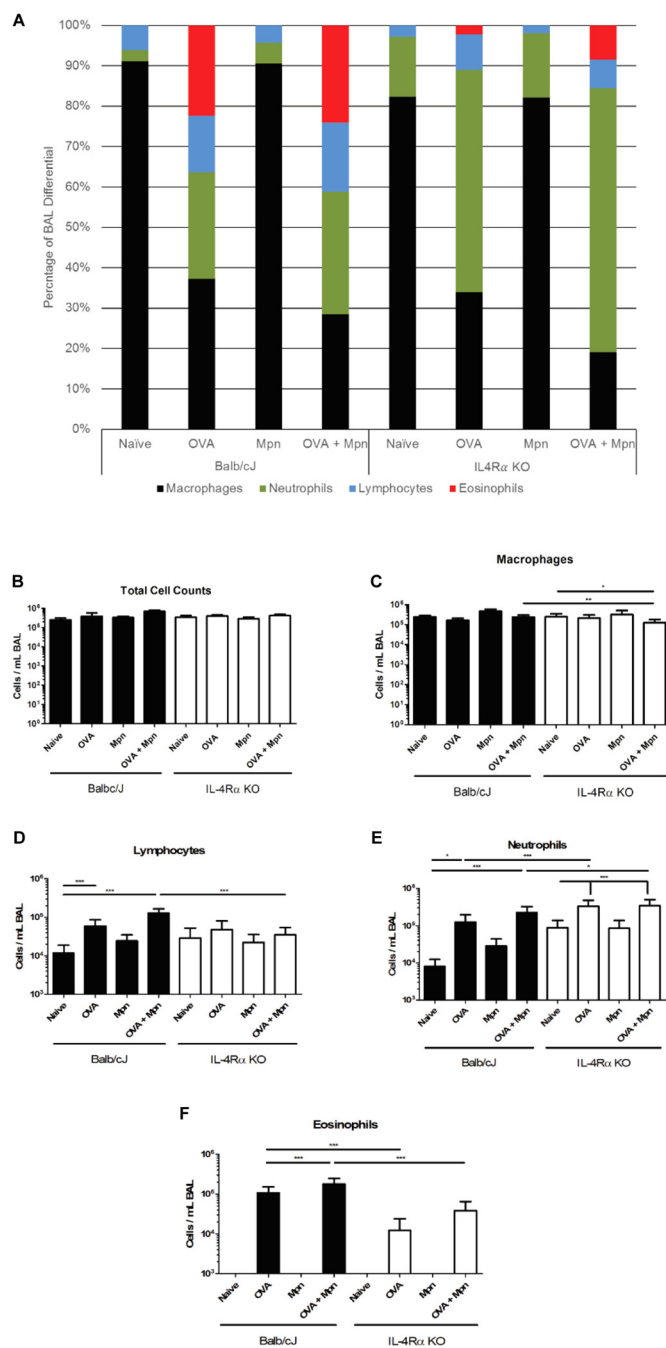


FIG E1. Cytologic analysis of BALF cellular profiles in WT and IL-4R α -deficient BALB/cJ animals with or without OVA sensitization and/or *M pneumoniae* (*Mpn*) infection. **A**, BALF differential in WT and IL-4R α -deficient animals. **B-F**, Absolute cells per milliliter for total cells (Fig E1, **B**), macrophages/monocytes (Fig E1, **C**), lymphocytes (Fig E1, **D**), neutrophils (Fig E1, **E**), and eosinophils (Fig E1, **F**). Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. BAL cell counts are denoted as cells per milliliter of BALF. Experimental observations were repeated in duplicate at minimum for repeat observations. * $P < .05$, ** $P < .01$, and *** $P < .001$.

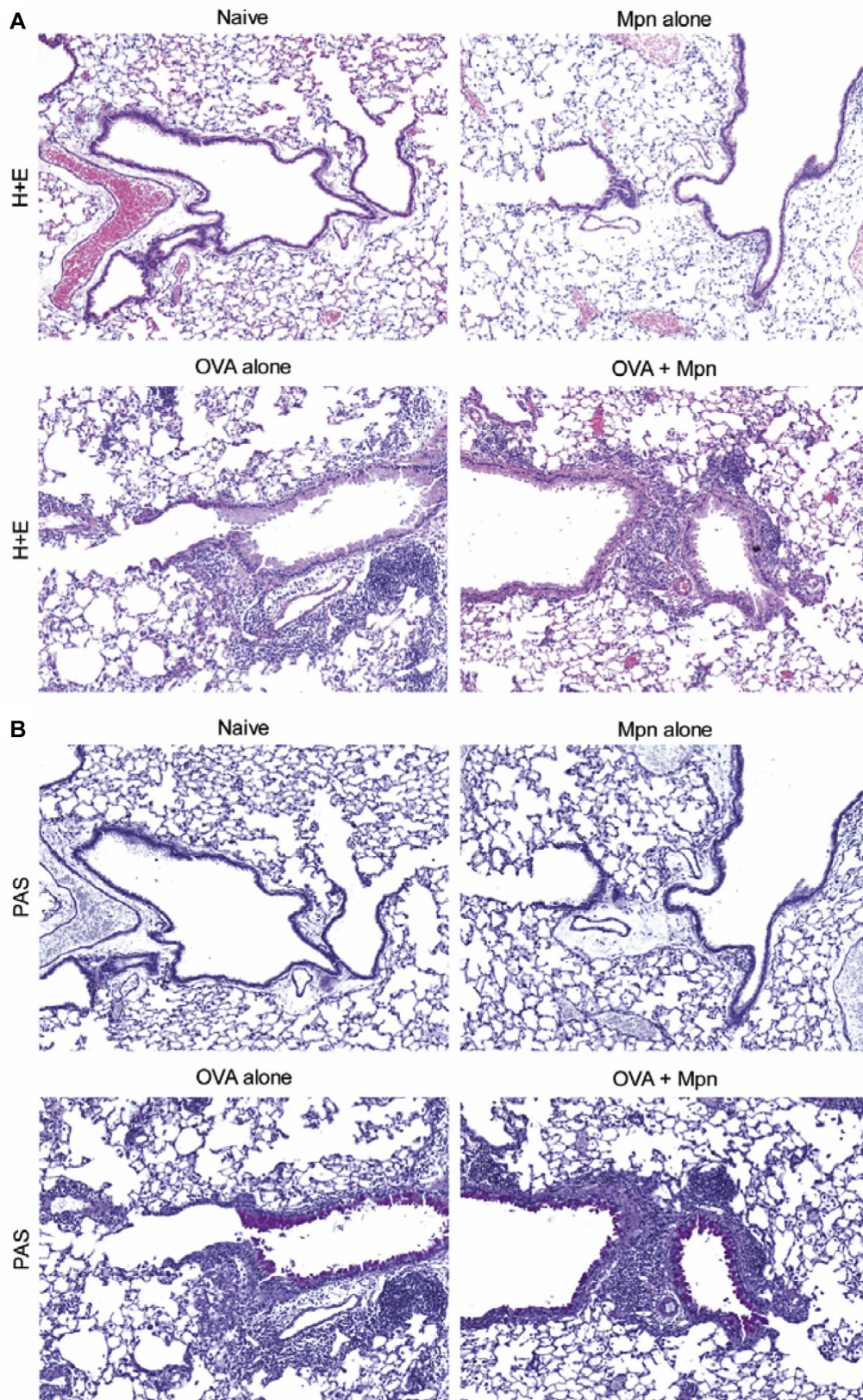


FIG E2. Histopathology of WT BALB/cJ mice with or without OVA sensitization and/or *M pneumoniae* (Mpn) infection. **A**, Representative histologic sections ($\times 10$ magnification) of hematoxylin and eosin (H+E)-stained tissue in WT BALB/cJ animals. **B**, Representative histologic sections ($\times 10$ magnification) of PAS-stained tissue in BALB/cJ animals. Representative sections are chosen from the sample closest to the group median. Experimental observations were repeated in duplicate at minimum for repeat observations.

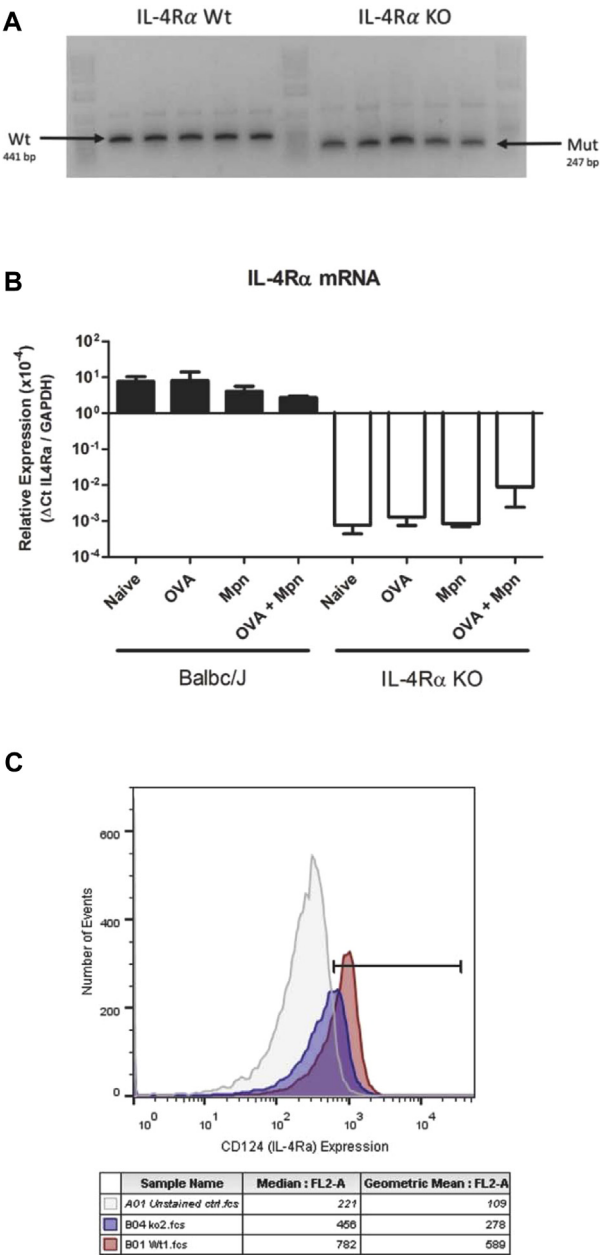


FIG E3. Determination of IL-4Rα expression levels in WT and KO animals. **A**, Representative genotyping gel of IL-4Rα-sufficient WT and IL-4Rα-deficient BALB/cJ animals from cross-breeding. **B**, qPCR analysis of mouse lung tissue for relative expression levels of IL-4Rα. **C**, Representative flow cytometric analysis of CD45 plus splenocytes from WT (red) and IL-4Rα-deficient (blue) animals for CD124 expression levels at baseline. Flow cytometric values are shown as a representative histogram of CD124 expression alongside the calculated geometric mean and median mean fluorescence intensity (*MFI*). Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Results of qPCR analysis are represented as relative expression levels from Δ cycle threshold values. Experimental observations were repeated in duplicate at minimum for repeat observations. *Mpn*, *M pneumoniae*.

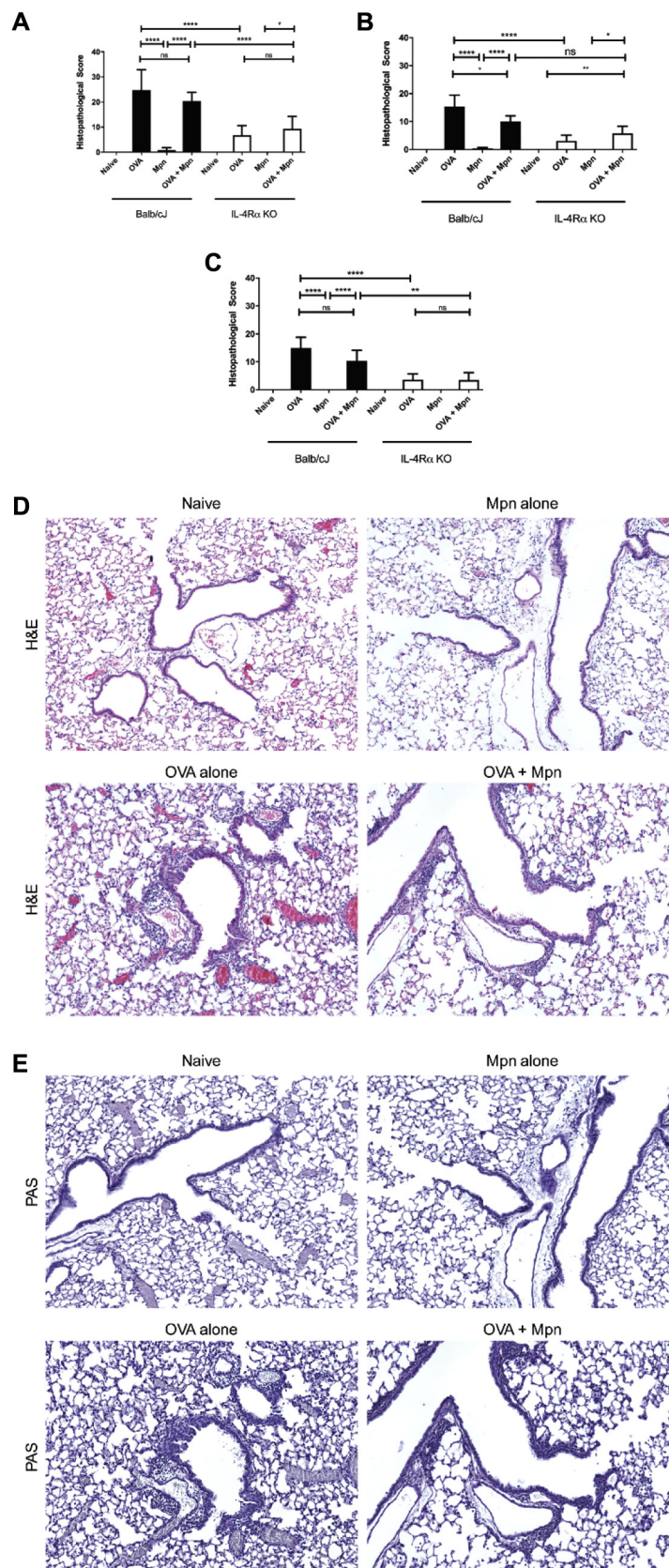


FIG E4. Loss of IL-4R α reduces pulmonary inflammation compared with IL-4R α -sufficient animals with or without allergic airway sensitization and/or bacterial infection. **A–D**, Histopathologic scoring of total scores (Fig E4, A), alveolitis (Fig E4, B), and bronchiolitis (Fig E4, C). **D**, Representative histologic sections ($\times 10$ magnification) of hematoxylin and eosin (H&E)-stained tissue in IL-4R α KO animals. **E**, Representative histologic sections ($\times 10$ magnification) of PAS-stained tissue in IL-4R α KO animals. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Representative sections are chosen from the sample closest to the group median. Experimental observations were repeated in duplicate at minimum for repeat observations. *Mpn*, *M pneumoniae*; *ns*, no statistical difference on analysis. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$.

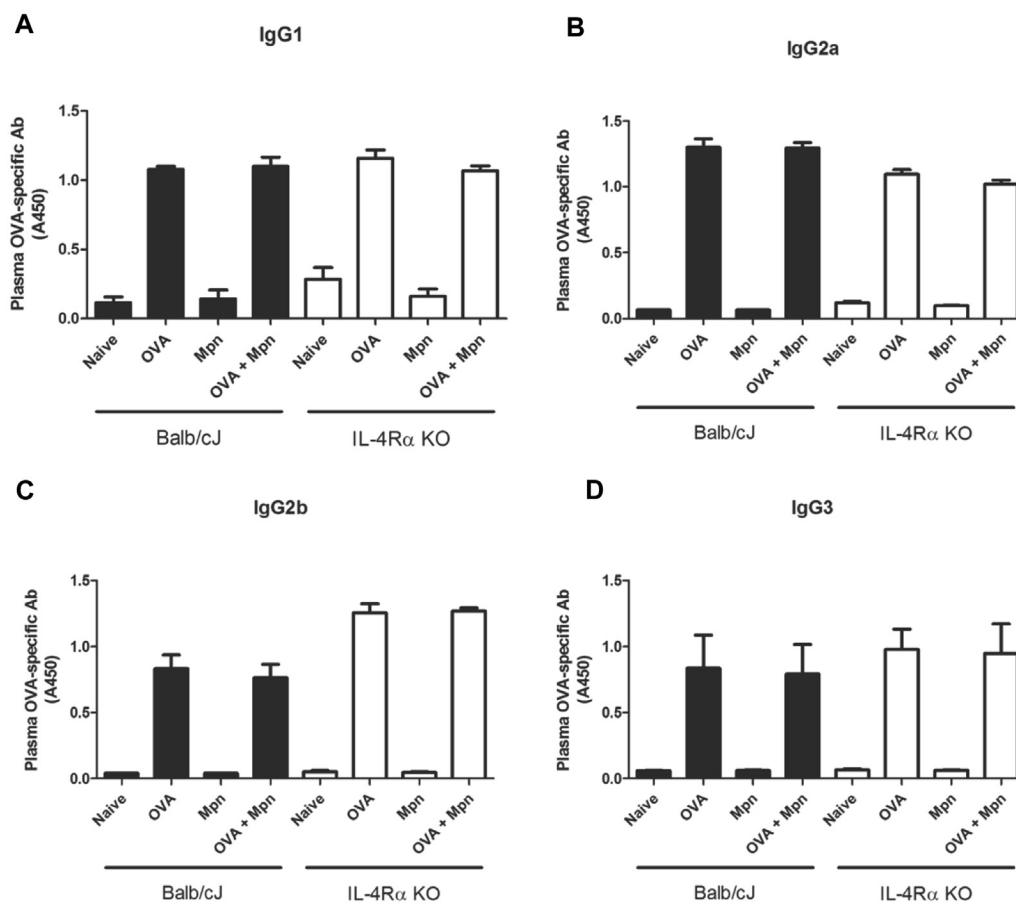


FIG E5. OVA-specific IgG subclass responses in WT and IL-4R α -deficient animals with or without OVA sensitization and/or *M pneumoniae* (Mpn)/*S pneumoniae* infection. **A-D**, ELISA of mouse plasma for OVA-specific responses in IgG₁ (Fig E5, A), IgG_{2a} (Fig E5, B), IgG_{2b} (Fig E5, C), and IgG₃ (Fig E5, D) subclasses. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations.

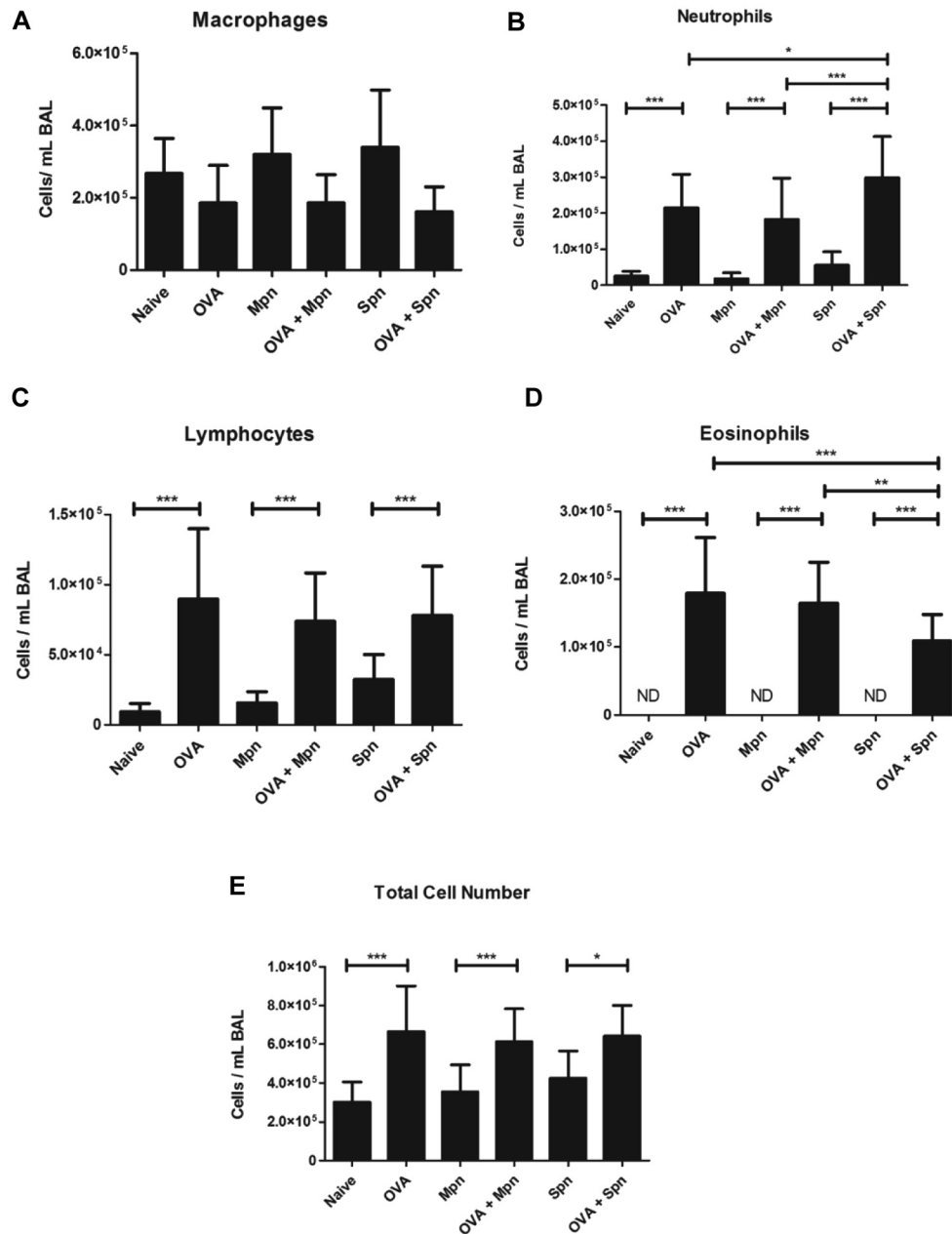


FIG E6. BALF cellular analysis in WT animals with or without OVA sensitization and/or *M pneumoniae* (*Mpn*)/*S pneumoniae* (*Spn*) infection. Absolute numbers (cells per milliliter) of macrophages/monocytes (**A**), neutrophils (**B**), lymphocytes (**C**), eosinophils (**D**), and total cells (**E**) are shown. Data are presented as group means $\pm$ SDs for 5 to 10 animals per group. Experimental observations were repeated in duplicate at minimum for repeat observations. * $P < .05$, ** $P < .01$, and *** $P < .001$.