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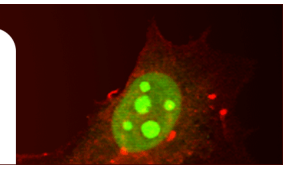

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CARMA3 Is Critical for the Initiation of Allergic Airway Inflammation

Benjamin Causton,*,†,1 Ravisankar A. Ramadas,‡,1 Josalyn L. Cho,*,† Khristianna Jones,*,† Ana Pardo-Saganta,*,§ Jayaraj Rajagopal,*,§ Ramnik J. Xavier,¶,‖ and Benjamin D. Medoff*,†

Innate immune responses to allergens by airway epithelial cells (AECs) help initiate and propagate the adaptive immune response associated with allergic airway inflammation in asthma. Activation of the transcription factor NF-κB in AECs by allergens or secondary mediators via G protein–coupled receptors (GPCRs) is an important component of this multifaceted inflammatory cascade. Members of the caspase recruitment domain family of proteins display tissue-specific expression and help mediate NF-κB activity in response to numerous stimuli. We have previously shown that caspase recruitment domain–containing membrane-associated guanylate kinase protein (CARMA)3 is specifically expressed in AECs and mediates NF-κB activation in these cells in response to stimulation with the GPCR agonist lysophosphatidic acid. In this study, we demonstrate that reduced levels of CARMA3 in normal human bronchial epithelial cells decreases the production of proasthmatic mediators in response to a panel of asthma-relevant GPCR ligands such as lysophosphatidic acid, adenosine triphosphate, and allergens that activate GPCRs such as *Alternaria alternata* and house dust mite. We then show that genetically modified mice with CARMA3-deficient AECs have reduced airway eosinophilia and proinflammatory cytokine production in a murine model of allergic airway inflammation. Additionally, we demonstrate that these mice have impaired dendritic cell maturation in the lung and that dendritic cells from mice with CARMA3-deficient AECs have impaired Ag processing. In conclusion, we show that AEC CARMA3 helps mediate allergic airway inflammation, and that CARMA3 is a critical signaling molecule bridging the innate and adaptive immune responses in the lung. ***The Journal of Immunology*****, 2015, 195: 000–000.**

Asthma is a syndrome broadly defined by inflammation of the airways associated with airway hyperresponsiveness (AHR) and mucus hypersecretion (1). In most cases, the airway inflammation characteristic of asthma results from an allergic-type reaction to an inhaled substance from the environment. In response to allergen exposure, the airways develop a predominantly eosinophilic inflammation with prominent edema and mucus production. One of the earliest steps in the establishment of allergic sensitization is the generation of an Ag-specific T cell response, which results from engagement of T cells by Ag-presenting dendritic cells (DCs) (2). A network of DCs resides beneath the epithelium in the airway mucosa where they can survey the airway for invading pathogens and inhaled Ags (3, 4). When appropriately stimulated, these DCs will mature and present Ag with other secondary activating signals to T cells (5).

It is thought that adjuvant signals from airway epithelial cells (AECs), generated in response to inhaled stimuli, influence the migration and maturation state of DCs and T cells and help determine whether a particular allergen will trigger a Th2-type inflammatory response (3, 6–9). In particular, the production of thymic stromal lymphopoietin (TSLP), GM-CSF, and the chemokine CCL20/MIP-3α by epithelial cells is critical for maturation of airway DCs and for the homing of DCs and T cells to the airways (10–19). Consistent with this, both TSLP and GM-CSF are upregulated in the airways of asthmatics and in response to numerous stimuli known to induce allergic airway inflammation (12, 20–24). Additionally, the production of chemokines and other inflammatory mediators by AECs in response to these stimuli likely augment both the innate and adaptive immune responses (25–27). These data suggest that AEC production of TSLP, GM-CSF, and CCL20/MIP-3α is likely a critical mechanism for the establishment of allergic airway inflammation, and that understanding the mechanisms that regulate their production in AECs may provide novel insight into the nature of the interaction between innate and adaptive immunity in asthma.

The transcription factor NF-κB regulates TSLP, GM-CSF, and CCL20/MIP-3α expression (23, 28–31) and, therefore, is an ideal therapeutic target for inhibiting the production of these important

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Abbreviations used in this article: AEC, airway epithelial cell; AHR, airway hyperresponsiveness; ALI, air–liquid interface; alum, aluminum hydroxide; BAL, bronchoalveolar lavage; CARD, caspase recruitment domain; CARMA, caspase recruitment domain–containing membrane-associated guanylate kinase protein; DC, dendritic cell; ES, embryonic stem; GPCR, G protein–coupled receptor; HDM, house dust mite; i.t., intratracheal(ly); LPA, lysophosphatidic acid; LPAR, LPA receptor; mDC, myeloid dendritic cell; MTEC, murine tracheal epithelial cell; NHBE, normal human bronchial epithelial cell; PAR, protease-activated receptor; P2Y, purinergic receptor; qPCR, quantitative PCR; shRNA, short hairpin RNA; SPC, surfactant protein C; SPC^{Cre}, Cre recombinase–driven by surfactant protein C promoter; TLN, thoracic lymph node; TSLP, thymic stromal lymphopoietin.

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cytokines. Previous research has also demonstrated that NF-κB is involved in multiple other aspects of asthma pathogenesis, including cytokine and mucin production from epithelial cells (32–37), epithelial cell barrier function (38), and airway remodeling (39). Furthermore, NF-κB is activated in airway epithelium in response to numerous asthma-relevant stimuli (27, 28, 33–36, 40–44). These data suggest a critical role for the NF-κB pathway in AECs during the development of allergic inflammation.

Many of the molecular scaffolds that organize and facilitate NF-κB activation downstream of plasma membrane receptor signaling contain caspase recruitment domain (CARD) sequences that facilitate protein–protein interactions (45, 46). To investigate the role of CARD proteins in NF-κB signaling in AECs, we performed a functional screen and identified a specific role for CARD-containing membrane-associated guanylate kinase protein (CARMA)3 (47). The CARMA proteins are a group of three proteins that contain a CARD, a coiled-coil domain, a linker, a PDZ domain, a SH3 domain, and a C-terminal membrane-associated guanylate kinase domain (48). These proteins, known as CARMA1, CARMA2, and CARMA3 (also as CARD11, CARD14, and CARD10, respectively) function as molecular scaffolds for the assembly of multiprotein complexes involved in the activation of NF-κB. CARMA3 is expressed in a wide range of nonhematopoietic cells, including cells in the heart, lung, liver, and kidney (49, 50), and has been linked to NF-κB activation through its interactions with Bcl10, MALT1, and NEMO/IKKγ (51, 52). Previous work has demonstrated that CARMA3 mediates proinflammatory NF-κB activation in response to G protein–coupled receptor (GPCR) activation in parenchymal cells (47, 53–55). Furthermore, our laboratory has demonstrated that CARMA3 is robustly expressed in AECs and is necessary for production of TSLP and CCL20/MIP-3α in response to lysophosphatidic acid (LPA), a GPCR ligand elevated in the lungs of asthmatics (47, 56). However, the specific role of AEC CARMA3 signaling in inflammatory diseases such as asthma has not been investigated.

Materials and Methods

Reagents

The Ab to CARMA3 was purchased from Abcam (Cambridge, MA). A nonhydrolyzable form of ATP (ATPγS) was purchased from Sigma-Aldrich (St. Louis, MO). LPA was purchased from Avanti Polar Lipids (Alabaster, AL) and prepared according to the manufacturer's instructions. *Alternaria alternata* and house dust mite (HDM) were purchased from Greer Laboratories (Lenoir, NC).

Mice

We generated a CARMA3-targeting construct that contained exons 1–3 flanked by *loxp* sites and a flippase recognition target–flanked neomycin cassette. The construct was transfected into C57BL/6N × 129SvEv hybrid embryonic stem (ES) cells by inGenious Targeting Laboratory (Stony Brook, New York). The ES cells were then used to generate knock-in mice with germline transmission of this altered CARMA3 allele ($CARMA3^{FN/+}$). These mice were crossed with actin-flippase recombinase mice to delete the flippase recognition target–flanked neomycin cassette to generate $CARMA3^{F/+}$ mice. We then backcrossed the mice to C57BL/6 mice for two generations and crossed these mice to mice that express Cre recombinase driven by the surfactant protein C promoter (SPC^{Cre} mice, obtained from Dr. Brigid Hogan, Duke University) to generate $SPC^{Cre}/CARMA3^{F/+}$ mice (57, 58). SPC^{Cre} mice have been shown to result in deletion of floxed genes throughout the tracheal epithelium, the bronchiolar epithelium, and in a subset of distal alveolar cells (58, 59). We then crossed these mice to generate $SPC^{Cre}/CARMA3^{F/F}$, $SPC^{Cre}/CARMA3^{+/+}$, and $CARMA3^{F/F}$ mice for experiments. $SPC^{Cre}/CARMA3^{F/F}$ mice were born in the predicted Mendelian distribution and were viable and fertile. Transgenic mice that express a TCR specific for chicken $OVA_{323–339}$ in the context of $I\text{-}A^b$ (OT-II mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were used at 6–8 wk of age and were sex matched for all experiments.

Asthma models

Acute allergic airway inflammation using OVA (Sigma-Aldrich) was induced in mice as previously described (60). Briefly, mice were immunized with two i.p. injections of 10 μg OVA bound to 1 mg aluminum hydroxide (alum; Sigma-Aldrich) in 0.5 ml PBS on days 1 and 7. Starting on day 14, mice were challenged by aerosol nebulization with 10 mg/ml OVA in PBS or PBS alone (control mice) for 20 min daily for 3 d. For DQ-OVA (Molecular Probes/Invitrogen, Carlsbad, CA) experiments, mice were immunized with either no or one i.p. injection of 10 μg chicken OVA bound to 1 mg alum in 0.5 ml PBS on day 1. On day 7, 40 μg DQ-OVA was administered to the mice via intratracheal (i.t.) injection. To assess OT-II cell proliferation, $SPC^{Cre}/CARMA3^{+/+}$ and $SPC^{Cre}/CARMA3^{F/F}$ mice were immunized with two i.p. injections of 10 μg OVA bound to 1 mg alum in 0.5 ml PBS on days 1 and 7. On days 14 and 15, mice were challenged by aerosol nebulization with 10 mg/ml OVA in PBS for 20 min daily. On day 16, single-cell suspensions of thoracic lymph nodes (TLNs) were incubated for 72 h at 37°C with CFSE-labeled Thy1.1 OT-II CD4 cells. The percentage of divided OT-II cells ($Thy1.1^+/CFSE^{low}$) was measured by flow cytometry. Allergic airway inflammation was induced with HDM as previously described (61). Briefly, 25 μg HDM in 25 μl PBS was administered intranasally three times a week for 5 wk. For Alexa Fluor 488–labeled HDM experiments, mice received 25 μg HDM three times a week for 1 wk and a fourth dose of 25 μg Alexa Fluor 488–labeled HDM. HDM was labeled with an Alexa Fluor 488 protein labeling kit (Invitrogen, Carlsbad, CA). For all in vivo experiments, mice were harvested for analysis 24 h after the last inhalation.

Mouse harvest and analysis

Bronchoalveolar lavage (BAL) and harvest of the lungs and TLNs were performed as previously described (60). Differential cell counts were obtained from BAL fluid after spinning 1.5×10^5 cells onto slides and staining with Hema-3 (Fisher Scientific, Pittsburgh, PA). Differential counts were performed on at least 200 cells per slide. Cells were also analyzed by flow cytometry as described below. Single-cell suspensions of TLNs were prepared. The lungs were flushed free of blood by slowly injecting 10 ml PBS into the right ventricle before excision. The superior right upper lobe of the lung was collected for RNA analysis with total RNA isolated using TRIzol (Invitrogen). The left lung was inflation fixed with 10% buffered formalin for histological analysis and stained with H&E. The remaining lung lobes were removed, minced with scissors, and then digested for 45 min in RPMI 1640 with 0.28 Wunsh U/ml Liberase (Roche Applied Science, Indianapolis, IN) and DNase (30 U/ml; Sigma-Aldrich) at 37°C to extract leukocytes from lung tissue. The digested tissues were then strained through a 70-μm filter before RBC lysis. Samples were blocked with purified CD16/CD32 mAb (BD Biosciences, San Diego, CA) and then stained with fluorescently labeled Abs to CD4, CD8, CD69, CD11c, CD11b, MHC class II (I-A), Gr-1/Ly6G, CD80, CD86, OX40L (CD252), and CCR7 (CD197) (R&D Systems, Minneapolis, MN). Flow cytometry was performed on an Accuri C6 or a BD LSR II analytical flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Lung tissue homogenization

Snap-frozen lung tissue samples were homogenized at 50 mg tissue/ml in HBSS (Invitrogen, Paisley, U.K.) containing a protease inhibitor mixture (Roche Diagnostics, Lewes, U.K.). Samples were them centrifuged (1600 rpm [$135 \times g$], 20 min) and supernatant was collected and stored at −80°C.

Immunohistochemistry

Multiple paraffin-embedded 5-μm sections of the entire mouse lung were prepared. Lung sections were dewaxed in xylene, hydrated, and incubated in 5% normal horse serum to preabsorb nonspecific Ig binding sites. The section was flooded with a rabbit polyclonal primary Ab to CARMA3 (1:300; Abcam) and incubated in a humid chamber overnight, followed by a biotinylated goat anti-rabbit secondary Ab (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h and then 1:1000 streptavidin/HRP (Molecular Probes/Invitrogen) for 1 h.

Immunofluorescence and microscopy

Paraffin sections were used and processed as indicated above. Once the paraffin was removed and the tissues were hydrated, Ag retrieval was performed on a pressure cooker for 2 h using citrate buffer. Tissues were blocked using 1% BSA in PBS–0.1% Triton X-100 for 1 h at room temperature and incubated with primary Abs diluted in blocking solution overnight at 4°C. After washing, the sections were incubated with sec-

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ondary Abs diluted in 1% BSA in PBS–0.1% Triton X-100 for 1 h at room temperature and then washed and counterstained with DAPI. The primary Abs used were rabbit anti-CARMA3 (1:100; ab36839, Abcam) and rat anti–E-cadherin (ECCD-2) (1:100; 13-1900, Life Technologies). All secondary Abs were Alexa Fluor conjugates (488 or 594) diluted 1:500 (Invitrogen/Life Technologies). Images were obtained using an Olympus IX81 Inverted microscope (Olympus, Center Valley, PA). Representative images are shown.

Epithelial cell isolation

Airway epithelial cells from the lung were dissociated using papain solution and incubated at 37°C for 2 h. After incubation, dissociated tissues were passed through a cell strainer and centrifuged and pelleted at 500 × *g* for 5 min. Cell pellets were dispersed and incubated with Ovo-mucoid protease inhibitor (Worthington Biochemical) to inactivate residual papain activity by incubating on a rocker at 4°C for 20 min. Cells were then pelleted and stained with EPCAM–PE-Cy7 (1:50; 25-5791-80, eBioscience) or EPCAM-allophycocyanin (1:50; 17-5791, eBioscience), GSIβ4 (*Griffonia Simplicifolia* isolectin β 4)–biotin (L2120, Sigma-Aldrich), SSEA-1 eFluor 650NC (1:75; 95-8813-41, eBioscience), and PE anti-mouse CD24 (1:100; 553262, BD Pharmingen) for 30 min in 2.5% FBS in PBS on ice. After washing, cells were sorted on a BD FACSAria (BD Biosciences) using FACSDiva software.

Epithelial cell culture

Mouse tracheal epithelial cells (MTECs) were cultured using a published protocol (62). Briefly, tracheas were removed and digested overnight with pronase (Roche Applied Science). The released cells were collected and then further selected by removing cells that adhered to a culture dish. The cells were then plated onto collagen-coated Transwells (Fisher Scientific, Pittsburgh, PA) and allowed to grow in media supplemented with epidermal growth factor and retinoic acid as described previously (62). After 5–7 d, an air–liquid interface (ALI) was created and the cells were allowed to grow for an additional 7–10 d. Purity of the culture was determined by the ability to maintain an ALI, the presence of beating cilia, and expression of the AEC transcription factor TTF-1.

Epithelial cell stimulation and CARMA3 knockdown

We have used a technique for knockdown of CARMA3 via lentiviral short hairpin RNA (shRNA) infection of normal human bronchial epithelial (NHBE) cells, allowing stable knockdown of CARMA3 in cells cultured on an ALI (63). For these experiments, NHBE cells (Lonza, Basel, Switzerland) were cultured in T-75 flasks to 75% confluence in B-ALI growth medium (Lonza) and then infected by adding 10 ml packaged CARMA3 shRNA lentivirus or a scrambled shRNA lentivirus with protamine sulfate (5 μg/ml) at a multiplicity of infection of 0.25. DNA lentiviral constructs containing shRNA against human CARMA3 (TRCN107248) or the scrambled shRNA were obtained from the RNAi Consortium (http://www.broadinstitute.org/rnai/public/) supply at the Broad Institute in Cambridge, MA (47). The shRNAs are cloned into the pLKO.1 vector with a puromycin resistance gene. Two days after infection, the cells were cultured for 1 d in puromycin (1, 2, or 5 μg/ml) to select out uninfected cells. The cells were then replated onto collagen-coated 24-well ALI inserts (Costar) with a seeding density of 5×10^4 in B-ALI growth medium (Lonza) according to the manufacturer's instructions. After the cells were confluent on the insert (~7–10 d), the media above the insert were removed to form an ALI using B-ALI differentiation media (Lonza) according to the manufacturer's instructions. After 7 more days the cells were used for experiments. For stimulation, normal culture media were replaced with low-serum (1%) media for 24 h. The cells were then stimulated with LPA (10 μM), *Alternaria* (100 μg/ml), ATP (100 μM), or HDM (100 μg/ml) for 6 h for RNA extraction.

Quantitative PCR

RNA from stimulated cells and lung lobes was isolated using TRIzol (Invitrogen) and a commercial kit (RNeasy, Qiagen, Valencia, CA). cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). GPCR expression was determined via the TaqMan array mouse GPCR panel from Life Technologies (Grand Island, NY). Gene expression was quantified on an iQ5 RT-PCR detection system (Bio-Rad) using SYBR Green (iQ SYBR Green Supermix; Bio-Rad) according to the manufacturer's suggested protocol. All values are shown normalized to GAPDH values. Samples were assayed in duplicate. Primer sequences used were selected using the Massachusetts General Hospital PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

Protein quantification

Supernatants from BAL and lung homogenates were collected and then used undiluted in commercial ELISA kits for KC/mCXCL1, CCL20/MIP-3α, TSLP, GM-CSF, CCL11/eotaxin-1, IL-4, IL-5, and IL-13 (R&D Systems) according to the manufacturer's protocol.

Measurement of lung function in Mice

Lung function measurements were performed using the Flexivent system (Scireq, Montreal, QC, Canada), as described before (64). Briefly, mice were anesthetized with an i.p. injection of xylazine (12 mg/kg) and pentobarbital (70 mg/kg). The trachea of anesthetized mice was cannulated and the mice were ventilated with 6 ml/kg tidal volume at 150 breaths per minute. To suppress spontaneous breathing during measurement of lung function, mice were i.p. injected with pancuronium bromide (2 mg/kg). Incremental doses of nebulized methacholine were used to determine total lung resistance and compliance according to the Snapshot-150 perturbation provided by the Flexivent equipment. Thirteen data points were collected for each methacholine dose, and only data with a coefficient of determination >0.95 were included in analyses. The survival of mice during the procedure was simultaneously monitored using electrocardiograms.

Statistical analysis

Data are expressed as mean ± SEM. Differences between means were tested for statistical significance using unpaired *t* tests as appropriate to the experiment. For multiple comparisons, a two-way ANOVA test was used for lung function analysis. From such comparisons, differences yielding $p < 0.05$ were judged to be significant.

Study approval

All protocols were approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

Results

AECs express asthma-relevant GPCRs

Previous work has demonstrated that CARMA3 is highly expressed in AECs and mediates activation of NF-κB in response to GPCR engagement (47). To characterize the profile of GPCRs expressed in AECs, we used a quantitative PCR (qPCR) array to measure the baseline RNA expression of 380 GPCRs in MTECs cultured on an ALI. Multiple GPCRs were identified that are expressed at moderate (0.002–0.01 copies/copy GAPDH) or high (>0.01 copies/copy GAPDH) levels at baseline (Supplemental Fig. 1). In this list, there were several groups of potentially asthma-relevant proinflammatory receptors that can activate NF-κB, including the protease-activated receptors (PAR1 and PAR2), LPA receptors (LPAR1 and LPAR3), and purinergic receptors ($P2Y_1$, $P2Y_2$, and $P2Y_6$) (Fig. 1).

Knockdown of CARMA3 in AECs decreases cytokine production in response to GPCR ligands

In previous work, it was demonstrated that LPA stimulation of NHBE cells in culture induced expression of TSLP and CCL20/MIP-3α and that the expression was dependent on CARMA3 (47). For those studies, NHBE cells were transfected with plasmids expressing shRNA against CARMA3 to knockdown its expression, but the technique we used only resulted in transient knockdown and was not effective for epithelial cells cultured on an ALI. To induce stable knockdown of CARMA3 expression in AECs cultured on an ALI, we used lentiviral shRNA infection of NHBE cells for knockdown of CARMA3. Analysis of CARMA3 levels demonstrated effective knockdown of CARMA3 protein (Fig. 2A) and RNA (Fig. 2B). NHBEs were then stimulated with either LPA (10 μM), *Alternaria* (100 μg/ml), ATP (100 μM), or HDM (100 μg/ml). LPA- and *Alternaria*-induced expression of IL-8, CCL20/MIP-3α, GM-CSF, and TSLP was attenuated in NHBE cells with CARMA3 knockdown compared with cells infected with a lentivirus containing a nontargeting scrambled shRNA sequence (Fig. 2C–F). Additionally, knockdown of CARMA3 signaling

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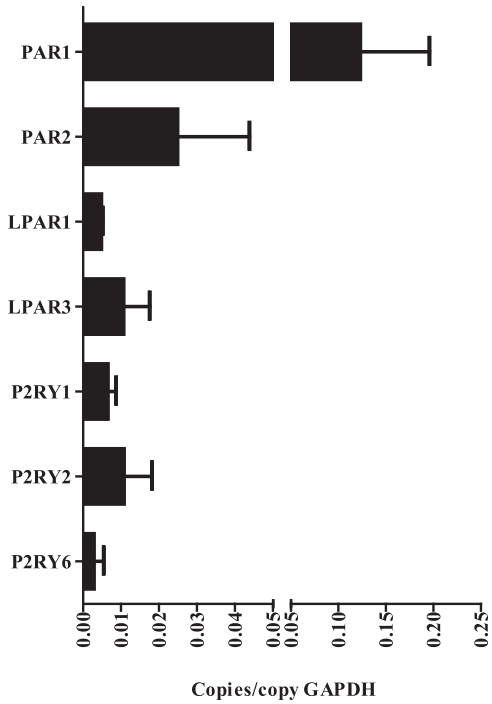

FIGURE 1. Asthma-relevant GPCR profile of mouse tracheal epithelial cells. RNA was isolated from naive, unstimulated mouse tracheal epithelial cells, and the expression profile of a panel of 380 GPCRs was measured using a real-time qPCR mini-array. Shown is an asthma-relevant subset from 67 medium- and high-abundance GPCRs detected. GAPDH was used to normalize the values of GPCR genes tested.

abolished both ATP- and HDM-induced changes in gene expression in NHBE cells (Fig. 2C–F). Taken together, these results suggest that CARMA3 mediates proinflammatory cytokine and chemokine production downstream of multiple different GPCRs.

Generation of mice deficient in AEC-CARMA3 signaling

To study the role of CARMA3 in AECs in vivo, we generated mice capable of cell-specific deletion of CARMA3. A targeting construct with loxp sites flanking exons 1–3 was generated (Fig. 3A, 3B) and used to make CARMA3$^{F/F}$ mice. These mice were then crossed to mice that express Cre recombinase under control of the surfactant protein C promoter (SPC^{Cre}) (59) to generate SPC^{Cre}/CARMA3$^{F/F}$ mice. Although SPC is predominantly expressed by alveolar type II epithelial cells in adult mice, it is expressed in early endoderm during embryogenesis, leading to Cre-mediated deletion of floxed genes in all respiratory epithelium (58). SPC^{Cre}/CARMA3$^{+/+}$ and CARMA3$^{F/F}$ mice had prominent staining for CARMA3 in the airway epithelium (Fig. 3C, 3E). However, lungs from SPC^{Cre}/CARMA3$^{F/F}$ mice had minimal staining in AECs (Fig. 3D). These findings were verified by immunofluorescence with costaining for E-cadherin for airway epithelium. In these experiments, lung sections demonstrated intense CARMA3 staining in the airway epithelium of SPC^{Cre}/CARMA3$^{+/+}$ mice compared with SPC^{Cre}/CARMA3$^{F/F}$ mice (Fig. 3F). To isolate individual AECs, we have developed a protocol to dissociate, isolate, and sort epithelial cell subsets from mouse lungs (65). We isolated pure populations of EpCAM^{+}/GSIβ4^{+} basal cells, EpCAM^{+}/GSIβ4^{-}/SSEA1^{+} secretory cells, and EpCAM^{+}/GSIβ4^{-}/CD24^{+} ciliated cells from dissociated lung. Basal cells displayed the cell markers p63 and CK5 when stained on a glass slide, secretory cells are CC10 (Scgb1a1)$^{+}$ and Scgb3a2^{+}, and ciliated cells were verified by expression of acetylated tubulin and FoxJ1 (data not shown). Using exclusion strategies, we sorted each AEC subtype to 98% purity. In AECs that were sorted from the lungs of naive SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice, qPCR showed that in SPC^{Cre}/CARMA3$^{+/+}$ mice, CARMA3 is expressed in all subtypes but expression is higher in the basal and ciliated cells compared with secretory cells (Fig. 3G). As expected, there was a decrease in CARMA3 RNA expression in basal and ciliated cells in the floxed mice (Fig. 3G) consistent with deletion of the CARMA3 gene. We also measured CARMA3 RNA levels in epithelial cell subtypes isolated from SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice after OVA/alum i.p. injection and a single aerosol administration of OVA. Following OVA inhalation, the CARMA3 levels were comparable in basal and ciliated cells; however, there was upregulation of CARMA3 levels in the secretory cells from SPC^{Cre}/CARMA3$^{+/+}$ mice but not SPC^{Cre}/CARMA3$^{F/F}$ mice (Fig. 3H). Consistent with this, immunofluorescent staining of lung slices from SPC^{Cre}/CARMA3$^{+/+}$ mice following OVA immunization and challenge demonstrated increased staining for CARMA3 in the airway epithelium (Fig. 3I).

Deletion of CARMA3 in AECs attenuates allergic airway inflammation in a murine model of asthma

SPC^{Cre}/CARMA3$^{F/F}$, SPC^{Cre}/CARMA3$^{+/+}$, and CARMA3$^{F/F}$ littermate control mice were immunized and challenged with OVA and then analyzed for airway inflammation. H&E-stained lung sections showed less airway inflammation in the SPC^{Cre}/CARMA3$^{F/F}$ mice compared with the CARMA3-sufficient mice (Fig. 4A). SPC^{Cre}/CARMA3$^{+/+}$ and CARMA3$^{F/F}$ mice showed identical immune responses to OVA and are therefore grouped together as SPC^{Cre}/CARMA3$^{+/+}$ for simplicity hereafter. BAL total cell counts and eosinophil counts were reduced in SPC^{Cre}/CARMA3$^{F/F}$ mice compared with control mice (Fig. 4B). Additionally, protein levels of IL-4, IL-5, and IL-13 were all found to be reduced in the lung tissue of SPC^{Cre}/CARMA3$^{F/F}$ mice compared with control mice (Fig. 4B). Consistent with the data from NHBE cells, the protein levels of GM-CSF, CCL20/MIP-3α, and TSLP in BAL were reduced in SPC^{Cre}/CARMA3$^{F/F}$ mice (Fig. 4D). However, there was no change in the levels of CXCL1/KC or CCL11/eotaxin-1 between the OVA-immunized and -challenged mice (Fig. 4D and data not shown). There was also decreased RNA expression of TSLP and GM-CSF, as well as CCL20/MIP-3α, in the lungs of OVA-immunized and -challenged SPC^{Cre}/CARMA3$^{F/F}$ mice compared with control mice (Fig. 4E). Lung RNA levels of a panel of chemokines did not differ (data not shown). These data demonstrate that CARMA3 expression in AECs has an important role in the development of allergic airway inflammation in vivo.

Deletion of CARMA3 does not attenuate AHR

We assessed whether deletion of CARMA3 expression in AECs would also attenuate AHR. Using mechanically ventilated mice, airway resistance (R_n) and airway compliance (C) were calculated from data obtained with a forced oscillation technique as described previously (64). OVA challenge of both SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice resulted in significant AHR to inhaled methacholine when compared with PBS-challenged mice, however, there was no difference in AHR between the genotypes (Supplemental Fig. 2).

Deletion of CARMA3 in AECs leads to impaired lung DC recruitment, maturation, and Ag processing

The reduced expression of TSLP and GM-CSF by SPC^{Cre}/CARMA3$^{F/F}$ mice suggests that these mice may have impaired lung DC maturation in response to allergens. Additionally, reduced CCL20/MIP-3α levels may affect DC recruitment to the lung. To

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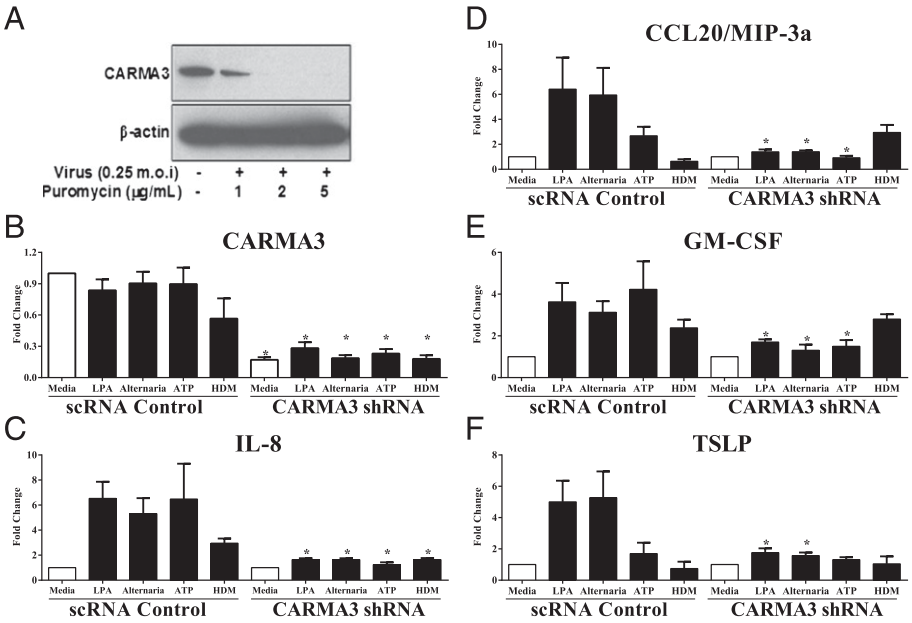

FIGURE 2. CARMA3 mediates proinflammatory cytokine release from normal human bronchial epithelial cells in response to GPCR stimulation. (**A**) A lentivirus containing a shRNA against CARMA3 and a puromycin resistance cassette was used to infect NHBE cells (multiplicity of infection of 0.25). The infected cells were selected for puromycin resistance for 5 d and then Western immunoblotting was used to detect CARMA3. (**B**–**F**) NHBEs were grown on an ALI postinfection with lentivirus containing a nontargeting shRNA (scRNA) or a CARMA3-targeting shRNA. These cells were then stimulated with either 10 μM LPA, 100 μg/ml *Alternaria*, 100 μM ATP, or 100 μg/ml HDM for 6 h. RNA was then isolated from the cells and the levels of (B) CARMA3, (C) CXCL8/IL-8, (D) GM-CSF, (E) CCL20/MIP-3α, and (F) TSLP were determined. GAPDH levels were used to normalize the values of genes tested. Data are expressed as fold change from media. Values are the mean of three to six samples ± SEM. This experiment was repeated twice. $*p < 0.05$ versus scRNA cells by unpaired *t* test. ND, not detected.

assess this, SPC^{Cre}/CARMA3$^{F/F}$ mice and SPC^{Cre}/CARMA3$^{+/+}$ littermate control mice were sensitized to OVA and then challenged i.t. with a fluorescently conjugated form of OVA (DQ-OVA) that emits green fluorescence when the protein is cleaved after cellular uptake. We then assessed DC migration and Ag processing in the lung and lung-draining TLNs. Following OVA sensitization and DQ-OVA challenge, SPC^{Cre}/CARMA3$^{F/F}$ mice had lower numbers of myeloid DCs (mDCs) isolated from the lung and TLNs (Fig. 5). mDCs were identified as CD11c^{+}CD11b^{+} MHC class II^{+}Gr-1^{-} (representative flow plots are shown in Supplemental Fig. 3). Additionally, lung and TLN from SPC^{Cre}/CARMA3$^{F/F}$ mice had fewer mDCs expressing the maturation markers CD80, CD86, OX40L, the chemokine receptor CCR7, and fluorescent DQ-OVA compared with control mice (Fig. 5A), suggesting that DC maturation, migration, and Ag processing ability of DCs is hampered in mice lacking CARMA3 in AECs. However, there was no difference in baseline mDC numbers or maturation marker expression in naive mice (Supplemental Fig. 4).

Deletion of CARMA3 in AECs impairs Ag-specific T cell proliferation

The impairment in DC maturation and Ag processing seen in SPC^{Cre}/CARMA3$^{F/F}$ mice should lead to impairment in Ag-specific T cell activation. To test this, we used a standard model of Ag-specific T cell activation (66). We sensitized SPC^{Cre}/CARMA3$^{F/F}$ mice and SPC^{Cre}/CARMA3$^{+/+}$ littermate control mice to OVA and then challenged them with OVA on 2 consecutive days. One day after the second OVA challenge, single-cell suspensions of the TLN from these mice were incubated with CFSE-labeled naive OVA-specific CD4^{+} T cells isolated from Thy1.1^{+} OT-II mice. Prior work has demonstrated that T cell proliferation in this assay is primarily induced by migratory mDCs from the lung (66). After 72 h, OT-II T cells stimulated with TLN cells from SPC^{Cre}/CARMA3$^{F/F}$ mice proliferated less than did OT-II T cells stimulated with TLN cells from SPC^{Cre}/CARMA3$^{+/+}$ mice (Fig. 6A). These data demonstrate that cells from the TLN of SPC^{Cre}/CARMA3$^{F/F}$ mice have reduced ability to stimulate Ag-specific T cell proliferation.

Deletion of CARMA3 in AECs attenuates allergic airway inflammation in response to HDM

We also tested the role of AEC CARMA3 in the development of allergic airway inflammation in the HDM model of asthma. SPC^{Cre}/CARMA3$^{F/F}$ and SPC^{Cre}/CARMA3$^{+/+}$ mice were given HDM intranasally as previously reported. As in the OVA model of asthma, there was a decrease in eosinophilic airway inflammation in SPC^{Cre}/CARMA3$^{F/F}$ compared with control animals (Fig. 7A). Additionally, when mice were given FITC-labeled HDM there were reduced numbers of FITC-labeled mDCs in the draining lymph nodes of SPC^{Cre}/CARMA3$^{F/F}$ compared with control animals (Fig. 7B). These data confirm the results found with the OVA model of allergic airway inflammation.

Discussion

CARMA3 functions as a molecular scaffold for the assembly of multiprotein complexes involved in the activation of NF-κB, a transcription factor involved in regulation of inflammation and immunity. Its role in the pathogenesis of asthma has been suggested on the basis of evidence of its activation in the bronchiolar epithelium from asthmatics (67) and from studies in mouse models of allergic airways disease (36). Indeed, a crucial role for lung epithelial NF-κB in both OVA (40) and HDM models of allergic airways disease has been described (68). Previous work has demonstrated that CARMA3 mediates proinflammatory NF-κB activation in response to GPCR activation in parenchymal cells (47, 53–55), and our laboratory has demonstrated that CARMA3

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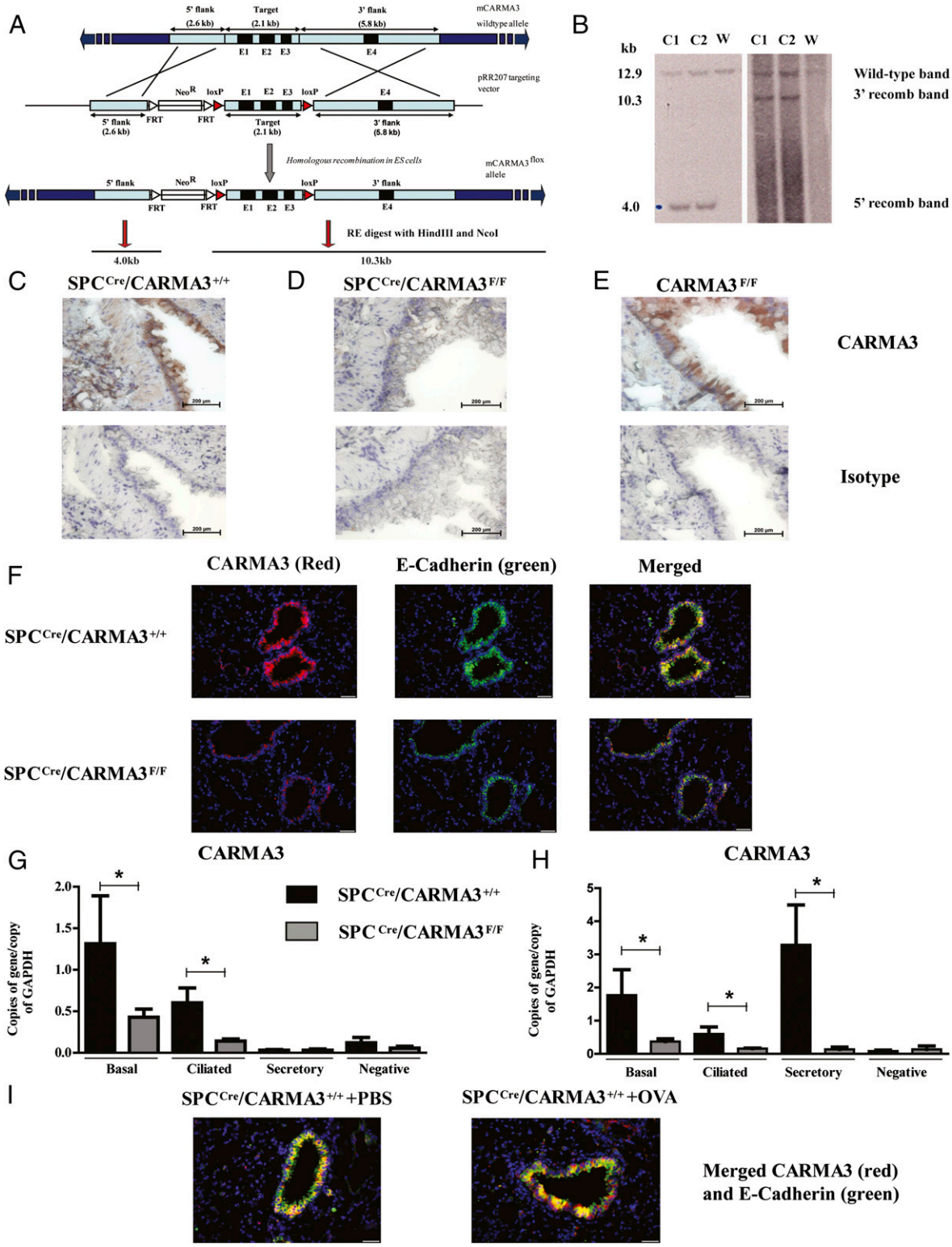

FIGURE 3. Generation of SPC^{Cre}/CARMA3$^{F/F}$ mice. (**A**) Generation of CARMA3 targeting construct. (**B**) Southern blot analysis to identify correctly targeted ES cell clones obtained from ES cells electroporated with the targeting construct. Two ES cell clones (C1 and C2) were identified in which there was the expected 5′ and 3′ recombinations. W, wild-type mouse. Immunohistochemistry of lungs from naive (**C**) SPC^{Cre}/CARMA3$^{+/+}$, (**D**) SPC^{Cre}/CARMA3$^{F/F}$, and (**E**) CARMA3$^{F/F}$ mice stained with an Ab against CARMA3 (*top panels*) or an isotype control Ab (*bottom panels*). Scale bars, 200 μm. (**F**) Immunofluorescence of lungs from naive SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice stained with an Abs against CARMA3 and E-cadherin. All images were taken using the same exposure. Scale bars, 20 μm. Basal, ciliated, and secretory cells sorted from the lungs of SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice that were either (**G**) naive or (**H**) received one OVA/alum immunization and one OVA challenge. RNA levels of CARMA3 were determined by qPCR. Data are means ± SEMs of six mice per group. $*p < 0.05$ (SPC^{Cre}/CARMA3$^{+/+}$ compared with SPC^{Cre}/CARMA3$^{F/F}$). (**I**) Immunofluorescence of lungs from PBS- and OVA-immunized and challenged SPC^{Cre}/CARMA3$^{+/+}$ mice stained for CARMA3. Scale bars, 20 μm.

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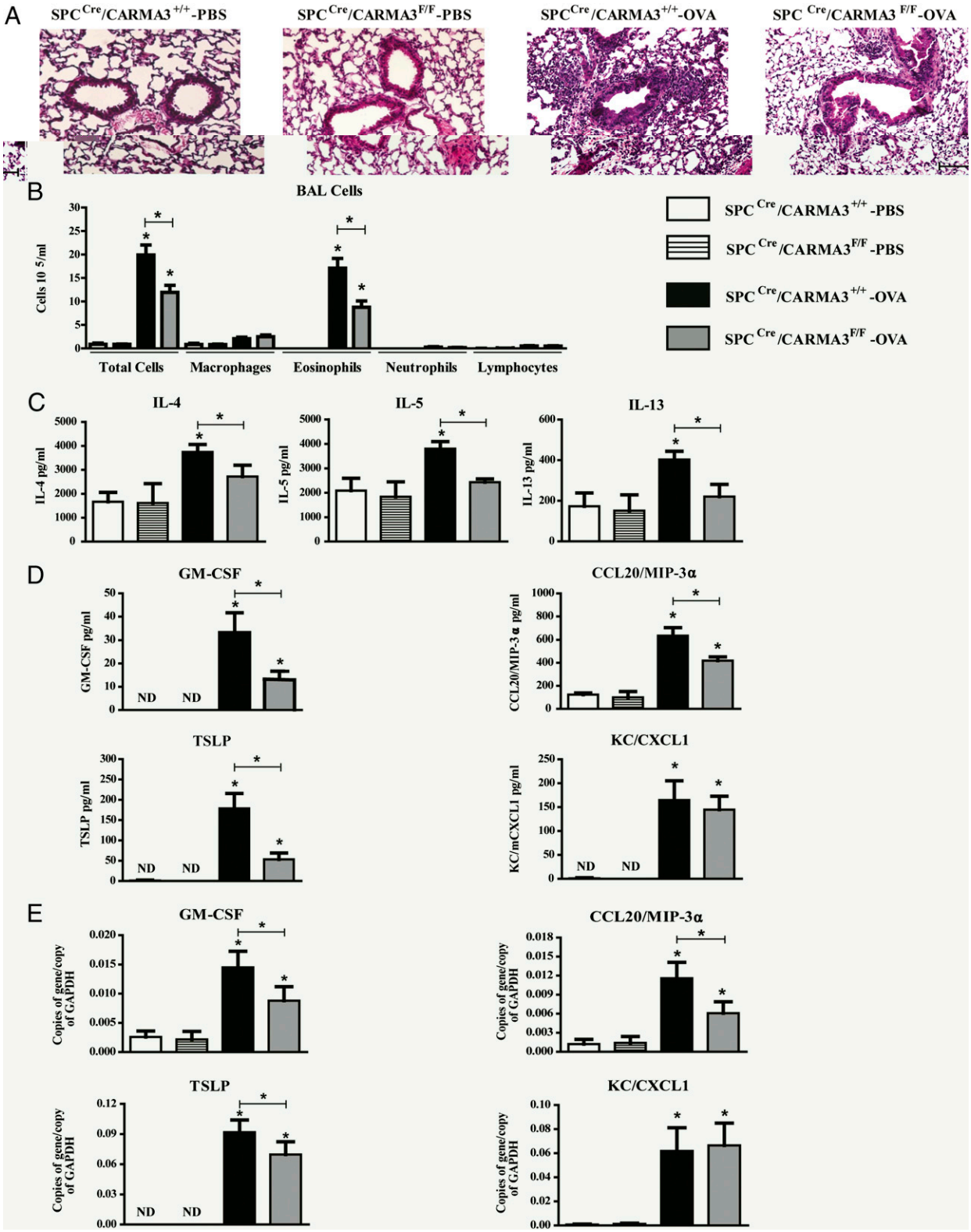

FIGURE 4. Attenuation of airway inflammation in OVA-immunized and -challenged SPC^{Cre}/CARMA3$^{F/F}$ mice. (**A**) Histopathologic analysis of lung sections stained with H&E from SPC^{Cre}/CARMA3$^{+/+}$-PBS–, SPC^{Cre}/CARMA3$^{F/F}$-PBS–, SPC^{Cre}/CARMA3$^{+/+}$-OVA–, and SPC^{Cre}/CARMA3$^{F/F}$-OVA–treated mice. Scale bar, 50 μm. (**B**) Total cells, macrophages, eosinophils, neutrophils, and lymphocytes were enumerated in BAL fluid. (**C**) Protein levels of IL-4, IL-5, and IL-13 in the lung quantified by ELISA. (**D**) Protein levels of GM-CSF, CCL20/MIP-3α, TSLP, and CXCL1/KC in the BAL quantified by ELISA. (**E**) RNA levels of GM-CSF, CCL20/MIP-3α, TSLP, and CXCL1/KC in the lung quantified by qPCR. Data are means ± SEM of eight mice per group from two experiments. *$p < 0.05$ (OVA treatment compared with same genotype that was treated with PBS or SPC^{Cre}/CARMA3$^{+/+}$ OVA compared with SPC^{Cre}/CARMA3$^{F/F}$ OVA). ND, not detected.

is robustly expressed in AECs and is necessary for production of TSLP and CCL20/MIP-3α in response to LPA, a GPCR ligand elevated in the lungs of asthmatics (47, 56). Despite these prior observations, the importance of airway epithelial CARMA3 sig-

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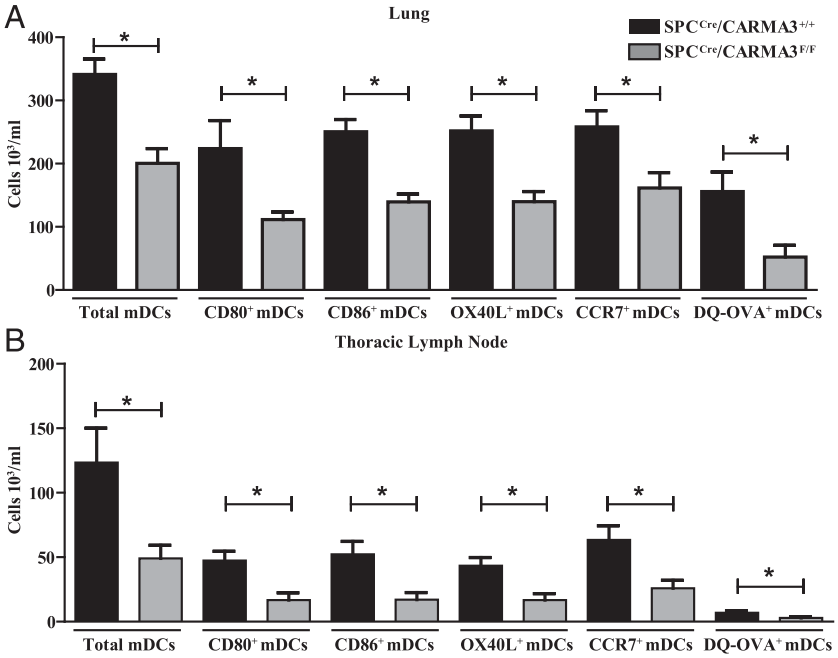

FIGURE 5. DCs isolated from SPC^{Cre}/CARMA3$^{F/F}$ mice show impaired maturation, migration, and Ag processing. SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice received one OVA/alum immunization on day 0, followed by 40 μg DQ-OVA i.t. on day 7. Twenty-four hours after DQ-OVA administration the (**A**) lungs and (**B**) thoracic lymph nodes were isolated and the number of mDCs, CD80$^+$ mDCs, CD86$^+$ mDCs, OX40L$^+$ mDCs, CCR7$^+$ mDCs, and DQ-OVA$^+$ mDCs were determined by flow cytometry. Data are means ± SEM of eight mice per group from two experiments. $*p < 0.05$.

naling and its role in asthma have yet to be determined. Results presented in the present study describe a critical role for AEC CARMA3 in linking the innate and adaptive immune response and promoting airway inflammation in a murine model of allergic asthma.

Results of the present study demonstrate an important relationship between GPCR activation, CARMA3 signaling, and the development of airway inflammation. We show that asthma-relevant GPCRs (PAR1, PAR2, LPAR1, LPAR3, $P2Y_1$, $P2Y_2$, and $P2Y_6$) are elevated in AECs and that shRNA-mediated knockdown of CARMA3 in NHBE cells greatly diminishes IL-8, CCL20/MIP-3α, TSLP, and GM-CSF production in response to the respective asthma-relevant GPCR ligands (HDM, *Alternaria*, LPA, and ATP). Additionally, we demonstrate that OVA- and HDM-driven eosinophilic airway inflammation is reduced in mice lacking CARMA3 specifically in AECs in vivo. Furthermore, proinflammatory cytokine production, DC maturation and migration, Ag processing, and resultant T cell proliferation are impaired in mice deficient in CARMA3; however, the development of AHR is not altered. Taken together, these results suggest a vital role for CARMA3 in the initiation and development of airway inflammation associated with allergic asthma.

The bronchial epithelium is the first line of defense against the abundant array of particles, Ags, and infectious pathogens that are inhaled into the airways. AECs express a diverse array of GPCRs, including PARS, P2Y receptors, and LPA receptors (69–71), and we found that these specific receptors were elevated without stimulation in AECs, suggesting that the airway epithelium is primed to detect inflammatory mediators and inhaled pathogens. Many of the GPCRs expressed on AECs participate in the initiation and modulation of allergic lung responses via NF-κB activation (72–75) and are linked to common allergens. The expression of PAR2 by AECs allows the recognition of protease-active allergens such as HDM and *Alternaria*, and the release of proinflammatory mediators from the airway epithelium has been shown to require PAR2 (73, 76, 77). Upon exposure to protease-active HDM allergens, AECs release a vast array of proinflammatory mediators, including IL-8, GM-CSF (73, 76, 77), and TSLP (72), that attract neutrophils and DCs to the airways and induce DC maturation. The development of *Alternaria*-induced lung inflammation has also been shown to rely on PAR2, with IL-8, GM-CSF, and TSLP being released from AECs upon *Alternaria* exposure (72, 78–80). Thus, proteases can activate PAR2 in the airways to generate leukocyte infiltration and to amplify the response to allergens (81–83). The P2Y receptors and the primary ligand ATP have also been linked to the innate and subsequent adaptive response in asthma (84–86). ATP is released in the airways of allergen-challenged patients and contributes to disease pathogenesis via signaling at P2Y receptors expressed at the epithelial surface (84). Additionally, the bioactive phospholipid LPA is upregulated in the airways of asthmatics and can stimulate AECs to produce additional proinflammatory mediators (57, 87, 88). In the present study, we show that HDM, ATP, LPA, and *Alternaria* stimulation of NHBEs leads to proinflammatory cytokine production. Importantly, we show that knockdown of CARMA3 in vitro attenuates these responses, suggesting that the CARMA3/NF-κB axis acts downstream of multiple GPCR pathways and that CARMA3 contributes to cytokine and chemokine production from AECs.

Thus far, in vivo models studying CARMA3 have been severely limited because permanent genetic deletion of CARMA3 in mice results in neural tube defects leading to high mortality (53). Thus, we used conditional deletion of CARMA3 in AECs to study its cell-specific role in asthma pathogenesis. Using this resource, we demonstrate in this study that deletion of CARMA3 from AECs in mice is sufficient to blunt the eosinophilic inflammatory response observed in the OVA- and HDM-induced models of allergic airways disease. Concomitant with the reduction in airway eosinophils were reduced levels of the Th2 cytokines IL-4, IL-5, and IL-13 in the lung in response to OVA. Additionally, the reduced inflammatory response to OVA in mice with CARMA3-deficient AECs was accompanied by reduced expression of the chemokine CCL20/MIP-3α, as well as TSLP and GM-CSF, which have all been shown to be released by AECs (89). CCL20/MIP-3α is central to early DC recruitment acting via CCR6 (90, 91), and TSLP and GM-CSF can activate DCs and induce maturation, thereby promoting T cell activation and Th2 inflammation (10, 11, 92–94). Consistent with these data, there were lower numbers of CD80$^+$, CD86$^+$, OX40L$^+$, and DQ-OVA$^+$ DCs recovered from both the lungs and TLN of CARMA3-deficient mice. The reduced

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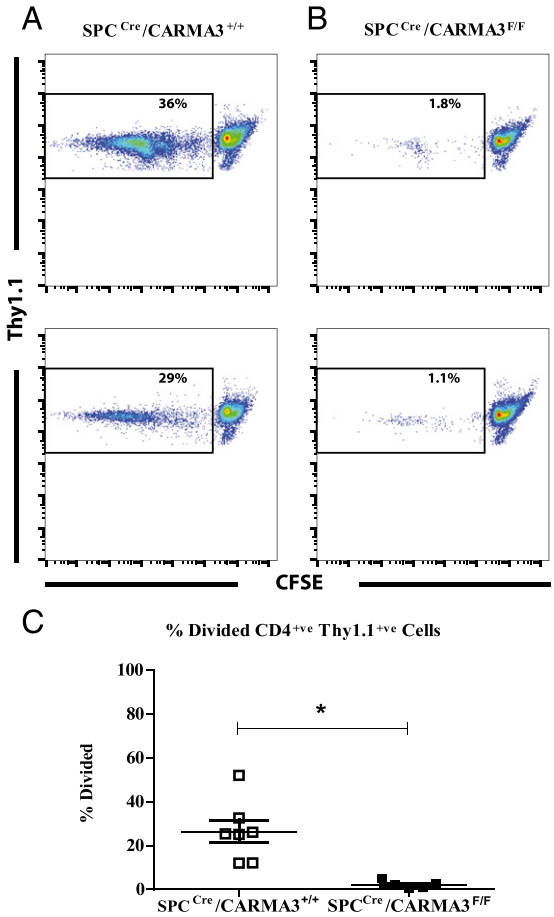

FIGURE 6. Impaired T cell proliferation in SPC^{Cre}/CARMA3$^{F/F}$ mice. SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice received two OVA/alum immunizations on days 0 and 7 followed by two aerosolized OVA challenges on days 14 and 15. On day 16 the thoracic lymph nodes were isolated and single-cell suspensions were incubated with CFSE-labeled Thy1.1^{+} OT-II CD4^{+} T cells for 3 d. Representative flow cytometry plots from (**A**) SPC^{Cre}/CARMA3$^{+/+}$ and (**B**) SPC^{Cre}/CARMA3$^{F/F}$ cultures are shown. (**C**) The percentage of divided OT-II cells (Thy1.1^{+}/CFSE^{low}) was measured. Data are means ± SEM of five to seven mice per group from two experiments. *$p < 0.05$.

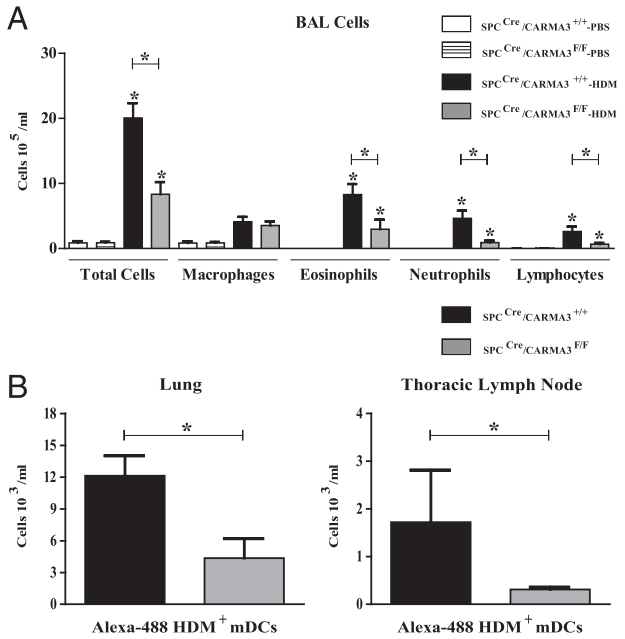

FIGURE 7. Attenuation of airway inflammation in HDM-treated SPC^{Cre}/CARMA3$^{F/F}$ mice. (**A**) Total cells, macrophages, eosinophils, neutrophils, and lymphocytes were enumerated in BAL fluid. Data are means ± SEM of six to eight mice per group from two experiments. *$p < 0.05$ (HDM treatment compared with same genotype that was treated with PBS or SPC^{Cre}/CARMA3$^{+/+}$ HDM compared with SPC^{Cre}/CARMA3$^{F/F}$ HDM). (**B**) SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice received three doses of HDM followed by 25 μg Alexa Fluor 488–labeled HDM intranasally. Twenty-four hours after Alexa Fluor 488–labeled HDM administration, the lungs and TLNs were isolated and the number of Alexa Fluor 488–labeled HDM^{+} mDCs were determined by flow cytometry. Data are means ± SEM of six to seven mice per group from one experiment. *$p < 0.05$.

DC numbers in the TLN as well as the lower numbers of DCs expressing costimulatory proteins and containing processed Ag likely explains the reduced Ag-specific T cell activation (as measured by proliferation) induced by lymph node cells from CARMA3-deficient mice compared with cells from control mice. Overall, these data suggest that the reduced production of TSLP and GM-CSF in CARMA3-deficient mice likely impairs DC maturation and Ag processing, which leads to a defect in T cell activation and Th2 cell development.

The presentation of processed Ags on MHC class II complexes by DCs is a crucial step in T cell activation in asthma (95), and thus our results support the notion that CARMA3 provides an essential link between the innate and adaptive immune response in airway inflammation. These observations, therefore, suggest a mechanism where epithelial GPCR activation and subsequent CARMA3 signaling and NF-κB activation are crucial steps between contact with an allergen and downstream manifestations of airway inflammation. Although the findings support our notion of the central role of airway epithelial CARMA3 signaling in allergic airway inflammation, there are likely additional signaling pathways, additional cell types, and complex interactions between them that contribute to the pathophysiology of allergic airway disease. Consistent with this, despite the significant attenuation of parameters thought to be clinically relevant to the pathophysiology of asthma, the airflow alterations that characterize AHR were not affected in SPC^{Cre}/CARMA3$^{F/F}$ mice. We suspect that alternative signaling pathways activated in airways or the residual inflammatory cells present in the CARMA3-deficient mouse lungs may be responsible for the observed AHR (96). Although the inflammatory state is thought to be integral to the development of AHR, it is also possible that residual inflammation and accompanying secretion of mediators observed in the SPC^{Cre}/CARMA3$^{F/F}$ mice are sufficient to fully drive the AHR. These results are also in agreement with others, where both OVA- and HDM-induced AHR is unaffected with AEC disruption of NF-κB signaling (40, 68). Indeed, our results are also consistent with other data demonstrating that airway inflammation in mice is at least in part uncoupled from AHR, which has been reported in murine models and human subjects (97), and that changes in allergen-induced airway physiology can occur in the absence of airway inflammation (98, 99).

Although our results indicate that CARMA3 contributes to allergic inflammation, the precise GPCR–agonist interaction that triggers CARMA3 and NF-κB signaling remains to be identified. One candidate is ATP acting via the P2Y family of receptors. Extracellular ATP serves as a danger signal to alert the immune system of tissue damage and allergen challenge causes accumulation of ATP in the airways of asthmatic subjects and mice with OVA-induced asthma. Indeed, all the cardinal features of asthma, including eosinophilic airway inflammation, Th2 cytokine pro-

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duction, and AHR, were abrogated when lung ATP levels were neutralized (84). Thus, ATP may act in an autocrine manner at the airway epithelium upon being released. In addition to ATP, PAR2 ligation could also be responsible for the CARMA3/NF-κB cascade. PAR2 has been shown to mediate OVA-induced inflammation and AHR (100), and this again may result from the release of as yet unidentified serine protease in response to OVA acting in an autocrine manner. The specific PAR2 agonist could be mast cell tryptase, which is elevated in the lungs of asthmatics (101–103). Proteases other than tryptase may also activate PAR2 in the airway, such as trypsin-like enzymes, which have been detected in AECs (81, 104) and in airway secretions (81, 105). Finally, LPA and uric acid are released into the airway during allergen challenge and could also mediate GPCR activation of AECs in vivo (56, 106, 107).

Global targeting of NF-κB is not a viable therapeutic option, as total inhibition of NF-κB activity would interrupt vital physiological processes important for tissue and immune homeostasis. However, these studies suggest that targeting the NF-κB in a cell- and pathway-specific manner could be beneficial in treating asthma. Consistent with this, selectively inhibiting NF-κB in endothelial cells in a murine models of peritonitis and rheumatoid arthritis has been shown to ameliorate both disease courses (108). These data, together with the data in the present study, suggest that the NF-κB pathway can be selectively inhibited in a cell type– and activation stage–dependent manner (108).

The model used for these experiments tests the importance of CARMA3 in AECs for allergen sensitization and challenge. In fact, our findings suggest that sensitization to allergens is profoundly impaired with CARMA3 deletion in AECs. Thus, it is not clear whether CARMA3 inhibition in AECs following sensitization would be as effective in reducing allergic airway inflammation. However, TSLP, GM-CSF, and CCL20/MIP-3α are likely important for exacerbations of allergic inflammation after sensitization given their roles in DC maturation, Ag presentation, and recruitment, so we think it is also likely that CARMA3 will have a role in asthma pathogenesis after initial sensitization. Recent data demonstrating that Ab neutralization of TSLP has a therapeutic effect in human asthma support this premise (109). In future experiments, we can use inducible CARMA3 deletion after the initial sensitization step to better determine its role in exacerbations.

In summary, this study demonstrates that CARMA3 signaling in AECs drives allergic airway inflammation and could be a significant contributor to the generation of the adaptive immune responses associated with asthma. These data suggest that CARMA3 could therefore be a promising therapeutic target to reduce airway inflammation in asthma.

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Disclosures

The authors have no financial conflicts of interest.

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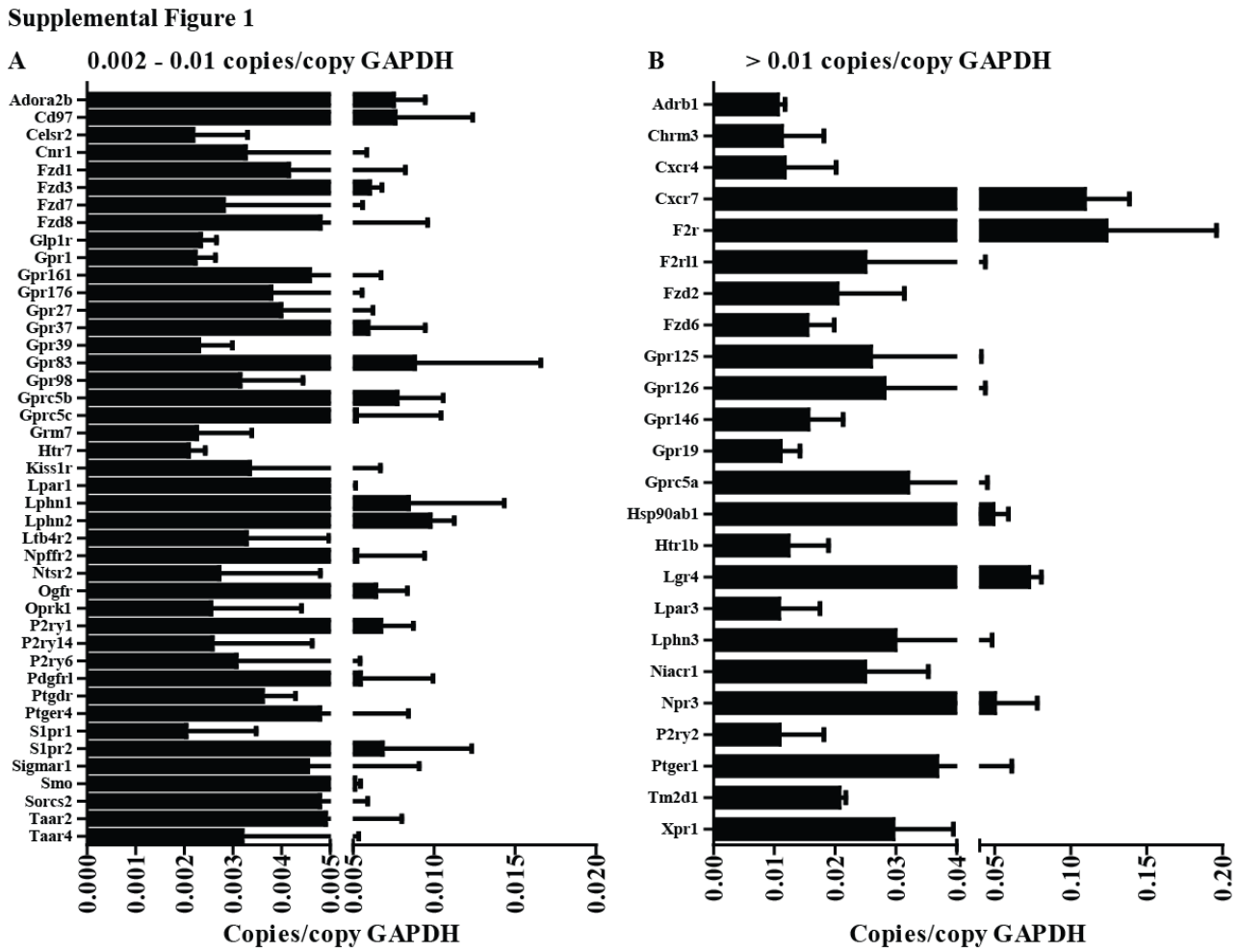
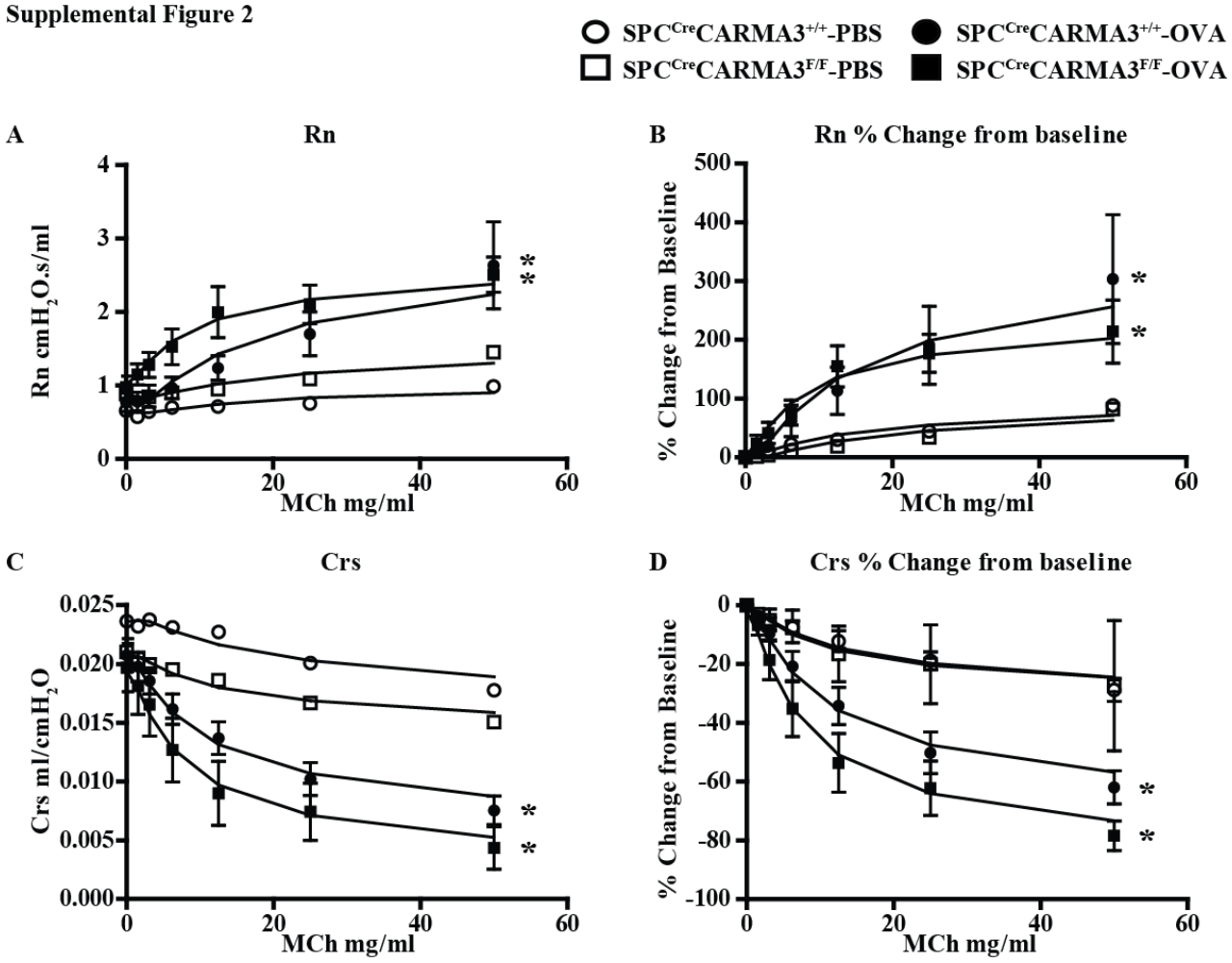
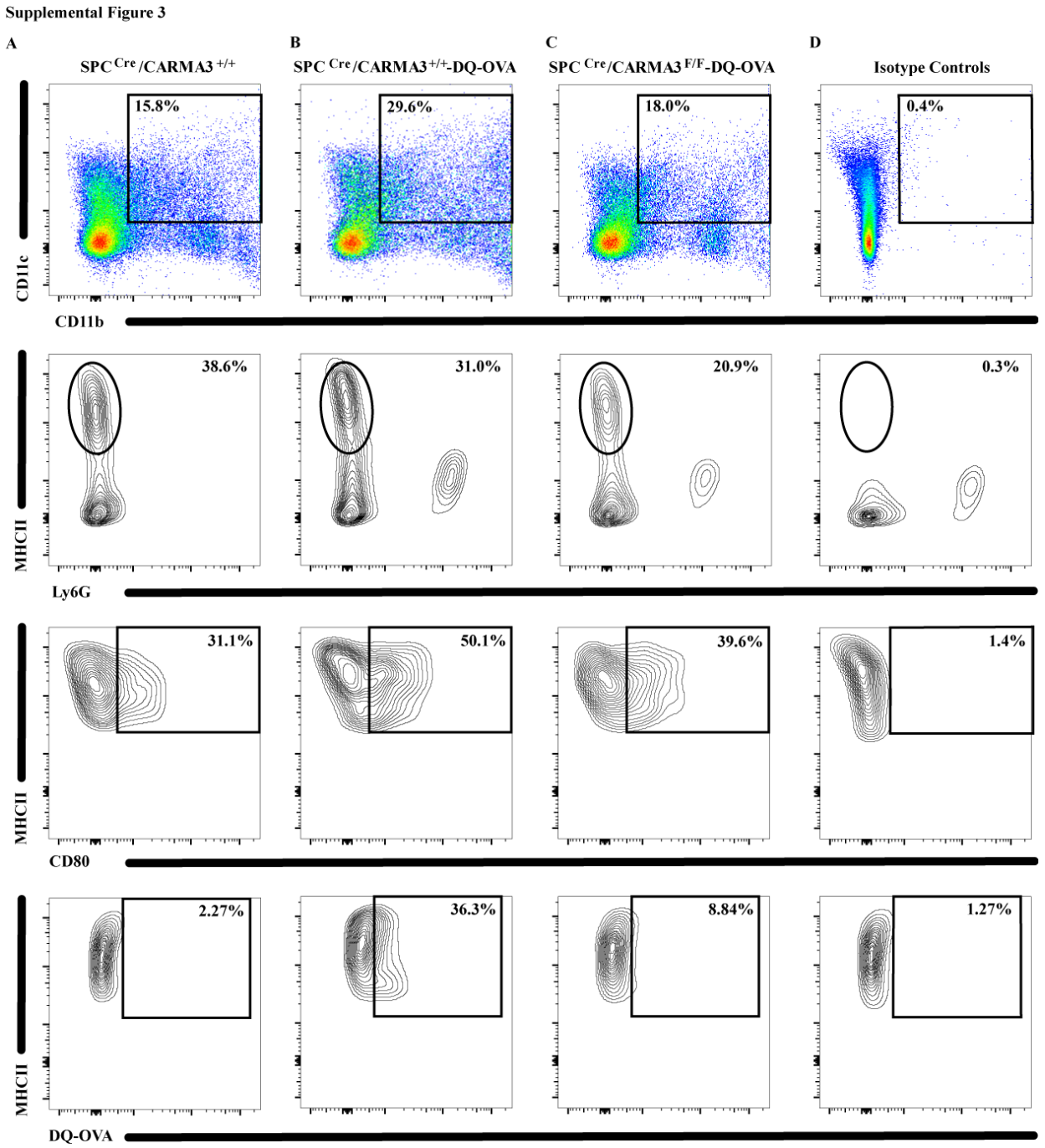


Supplementary Figure 1. GPCR profile of mouse tracheal epithelial cells.
RNA was isolated from naïve, unstimulated mouse tracheal epithelial cells and the expression profile of a panel of 380 GPCRs was measured using a real-time qPCR mini-array. Shown are (**A**) medium and (**B**) high abundance GPCRs. GAPDH was used to normalize the values of GPCR genes tested.

Supplemental Figure 2

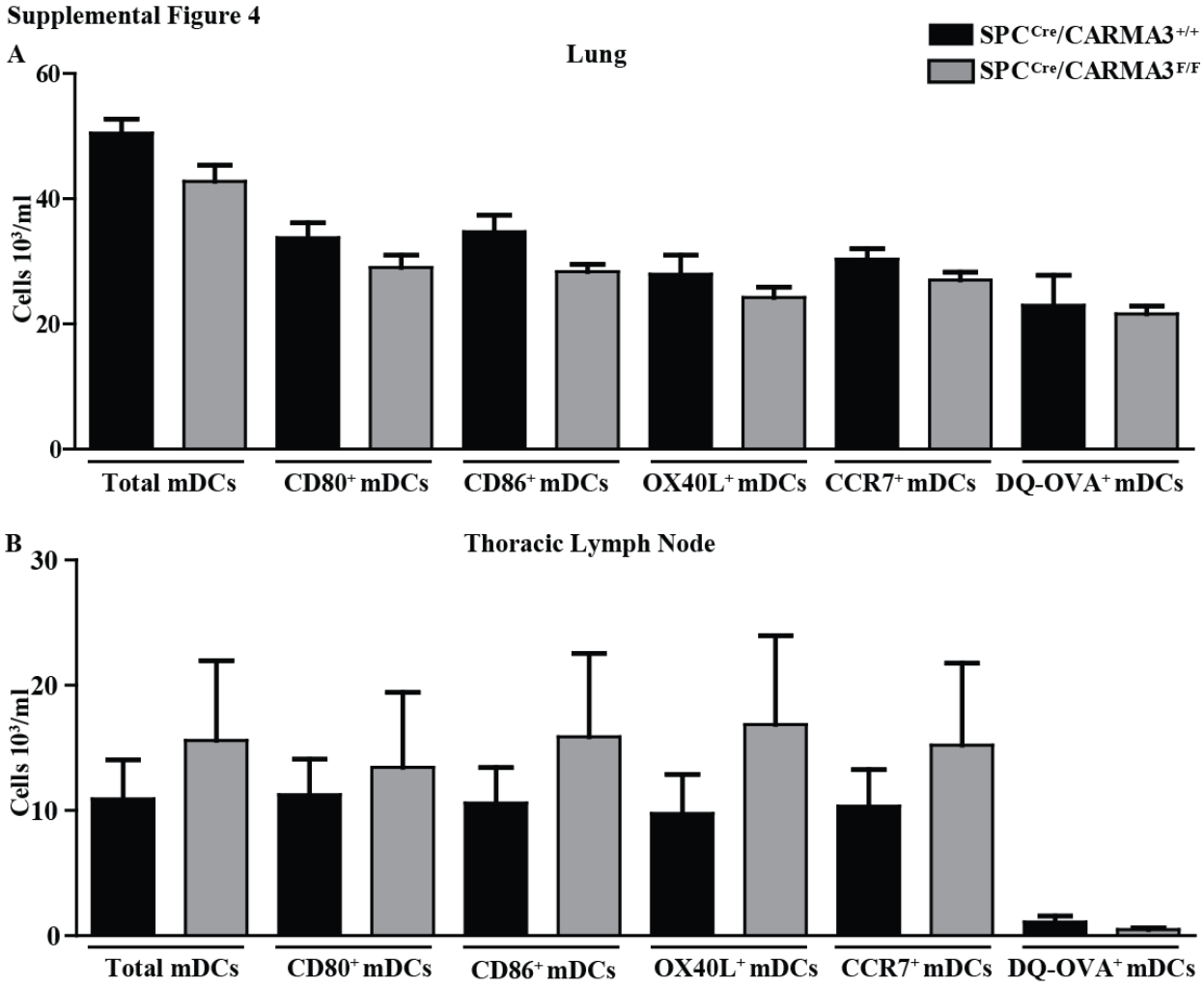


Supplementary Figure 2. Deletion of CARMA3 in airway epithelial cells does not influence the development of airways hyper-responsiveness.
(**A**) Airway resistance of OVA/Alum immunized and either OVA or PBS challenged $SPC^{Cre}/CARMA3^{+/+}$ and $SPC^{Cre}/CARMA3^{F/F}$ mice. (**B**) Airway resistance as expressed as the percentage change from baseline. (**C**) Airway compliance of OVA/Alum immunized and either OVA or PBS challenged $SPC^{Cre}/CARMA3^{+/+}$ and $SPC^{Cre}/CARMA3^{F/F}$ mice. (**D**) Airway compliance as expressed as the percentage change from baseline. Data are means ± SEMs of 8 mice per group from 2 experiments. Multiple comparisons between treatment and control conditions were performed using two-way ANOVA.



Supplementary Figure 3. Representative flow cytometry of mature myeloid DCs isolated from the lungs of SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice.

Single cell suspension of lung and TLNs were analyzed by flow. After gating on live cells, we identified CD11c^{+}/CD11b^{+}/MHCII^{+}/Gr-1^{-} myeloid DCs and assessed expression of CD80, CD86, OX40L, CCR7 and DQ-OVA. Plots shown are CD80 and DQ-OVA expression on myeloid DCs from OVA/alum sensitized (**A**) SPC^{Cre}/CARMA3$^{+/+}$ mice that received no DQ-OVA, (**B**) SPC^{Cre}/CARMA3$^{+/+}$ mice that received DQ-OVA and (**C**) SPC^{Cre}/CARMA3$^{F/F}$ mice that received DQ-OVA. (**D**) Isotype controls for CD11b, MHCII, CD80 and DQ-OVA.



Supplementary Figure 4. There is no difference in naïve DCs isolated from the lung and thoracic lymph nodes of SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice.
The (**A**) lungs and (**B**) thoracic lymph nodes were isolated from naïve SPC^{Cre}/CARMA3$^{+/+}$ and SPC^{Cre}/CARMA3$^{F/F}$ mice and the number of myeloid DCs, CD80$^+$ myeloid DCs, CD86$^+$ myeloid DCs, OX40L$^+$ myeloid DCs, CCR7$^+$ myeloid DCs and DQ-OVA$^+$ myeloid DCs were determined by flow cytometry. Data are means ± SEMs of 8 mice per group from 2 experiments.