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*J Immunol* published online 3 June 2015
http://www.jimmunol.org/content/early/2015/06/02/jimmunol.1402983

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/06/02/jimmunol.1402983
3.DCSupplemental

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CARMA3 Is Critical for the Initiation of Allergic Airway Inflammation

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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 (CARMA3) 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.
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 (CARMA3) (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/IκKγ (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<sup>flx<sup>\</sup>/flx</sup>). These mice were crossed with actin-flippase recombinase mice to delete the flippase recognition target–flanked neomycin cassette to generate CARMA3<sup>flx/flx</sup> 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<sup>+</sup> mice, obtained from Dr. Bridig Hogan, Duke University) to generate SPC-Cre<sup>+</sup>/CARMA3<sup>flx/flx</sup> mice (57, 58). SPC-Cre<sup>+</sup> 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<sup>+</sup>/CARMA3<sup>flx/flx</sup>, SPC-Cre<sup>+</sup>/CARMA3<sup>flx/+</sup>, and CARMA3<sup>flx/+</sup> mice for experiments. SPC-Cre/ CARMA3<sup>flx/+</sup> mice were born in the predicted Mendelian distribution and were viable and fertile. Transgenic mice that express a TCR specific for chicken OVA<sub>323-339</sub> in the context of I<sup>A</sup>-<sup>+</sup> (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 inhalation 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<sup>+</sup>/CARMA3<sup>flx/flx</sup> and SPC-Cre<sup>+</sup>/CARMA3<sup>flx/+</sup> 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 Thyl.1 OT-II CD4 cells. The percentage of divided OT-II cells (Thyl.1<sup>+</sup>CFSE<sup>−</sup>−<sup>+</sup>) 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<sup>6</sup> 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 lobe of the lung was collected for RNA analysis with total RNA isolated using TRIzol (Invitrogen). The left lung was inflated with 5% 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 Wunsch 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, Gr-1/Ly6G, CD69, CD11c, CD11b, MHC class II (I-A), and OX40L (CD252), and CCR7 (CD197) (R&D Systems, Minneapolis, MN). Flow cytometry was performed on 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 then centrifuged (1600 rpm at 10°C) and the 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 an Accu-Ct6 or a BDA LSR II analytical flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

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-
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 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 inactive 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-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 on 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 to test the role 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 into the media for 3 h (63). After 48 h, the cells were removed from the ALI by trypsinizing and then cultured in the presence of 0.5% FBS in PBS for 2 d. The purity of the culture was determined by the presence of ciliated cells, as described previously (63).

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.

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 (LPA1 and LPA3), and purinergic receptors (P2Y1, P2Y2, and P2Y6) (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 (Fig. 2C–F). Additionally, knockdown of CARMA3 signaling

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 were 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/).
CARMA3 MEDIATES ALLERGIC AIRWAY INFLAMMATION

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. GADPH 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 lox sites flankung exons 1–3 was generated (Fig. 3A, 3B) and used to make CARMA3F/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/CARMA3F/F mice. These mice were then crossed to mice after OVA/alum i.p. injection and 

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

SPC-Cre/CARMA3F/F, SPC-Cre/CARMA3+/+, and CARMA3F/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/CARMA3F/F mice compared with the CARMA3-sufficient mice (Fig. 4A). SPC-Cre/CARMA3+/+ and CARMA3F/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/CARMA3F/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/CARMA3F/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/CARMA3F/F mice compared with control 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 -challenged SPC-Cre/CARMA3F/F mice compared with control mice (Fig. 4E).

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/CARMA3F/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.
assess this, SPCCre/CARMA3F/F mice and SPCCre/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, SPCCre/CARMA3F/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 SPCCre/CARMA3F/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 SPCCre/CARMA3F/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 SPCCre/CARMA3F/F mice and SPCCre/CARMA3+/+ littermate control mice to OVA and then challenged i.t. 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 SPCCre/CARMA3F/F mice proliferated less than did OT-II T cells stimulated with TLN cells from SPCCre/CARMA3+/+ mice (Fig. 6A). These data demonstrate that cells from the TLN of SPCCre/CARMA3F/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. SPCCre/CARMA3F/F and SPCCre/CARMA3+/+ mice were given HDM intranasally as previously reported. As in the OVA model of allergic airways disease, there was a decrease in eosinophilic airway inflammation in SPCCre/CARMA3F/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 SPCCre/CARMA3F/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...
FIGURE 3. Generation of SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup> 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<sup>Cre</sup>/CARMA3<sup>+/+</sup>, (D) SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup>, and (E) CARMA3<sup>F/F</sup> 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<sup>Cre</sup>/CARMA3<sup>+/+</sup> and SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup> mice stained with 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<sup>Cre</sup>/CARMA3<sup>+/+</sup> and SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup> 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<sup>Cre</sup>/CARMA3<sup>+/+</sup> compared with SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup>). (I) Immunofluorescence of lungs from PBS- and OVA-immunized and challenged SPC<sup>Cre</sup>/CARMA3<sup>+/+</sup> mice stained for CARMA3. Scale bars, 20 μm.
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-
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, P2Y1, P2Y2, and P2Y6) 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 studies modeling 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 airway 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
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 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 SPCCre/CARMA3F/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-
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 proteases 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 response to microbes, trauma, or inflammation and potently activates mast cells. J. Exp. Med. 204: 253–258.


CARMA3 MEDIATES ALLERGIC AIRWAY INFLAMMATION


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.
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\textsuperscript{Cre}/CARMA3\textsuperscript{+/+} and SPC\textsuperscript{Cre}/CARMA3\textsuperscript{F/F} mice.

The (A) lungs and (B) thoracic lymph nodes were isolated from naïve SPC\textsuperscript{Cre}/CARMA3\textsuperscript{+/+} and SPC\textsuperscript{Cre}/CARMA3\textsuperscript{F/F} mice and the number of myeloid DCs, CD80\textsuperscript{+} myeloid DCs, CD86\textsuperscript{+} myeloid DCs, OX40L\textsuperscript{+} myeloid DCs, CCR7\textsuperscript{+} myeloid DCs and DQ-OVA\textsuperscript{+} myeloid DCs were determined by flow cytometry. Data are means ± SEMs of 8 mice per group from 2 experiments.