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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 (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 proinflammatory 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: 683–694.

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 adventitious 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...
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 CARMA3 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, liver, lung, 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 (ATP-S) 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 germine transmission of this altered CARMA3 allele (CARMA3F/F). These mice were crossed with actin-flippase recombinase mice to delete the flippase recognition target–flanked neomycin cassette. The resulting CARMA3F/F mice were born in the predicted Mendelian distribution and were viable and fertile. Transgenic mice that express a TCR specific for chicken OVA323–330 in the context of I-α1 (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 aerosolization with 10 mg/ml OVA in PBS or PBS alone (control mice) for 20 min daily for 3 d. For OVA-PBS (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 days 1 and 7. On days 14 and 15, mice were challenged by aerosolization 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−) 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 × 107 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 inflated 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 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, and house dust mite (HDM) were purchased from Greer Laboratories (Lenoir, NC). The left lung was inflation fixed in 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 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, CD69, CD11c, CD11b, MHC class II (I-A, Gr-1/Ly6g, CD80, CD86, DX40L (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 then centrifuged (1600 rpm [155 × 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-
ory 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 were used 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 nylon mesh 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), SSIβ4 cells, allowing stable ALI (1:75; 95-8134-41, Life Technologies) were stained with EPCAM–PE-Cy7 (1:50; 25-5791-80, eBioscience) or EPCAM-allophycocyanin (1:50; 17-5791, eBioscience). Samples were assayed in duplicate. Primer sequences used were selected using the Massachusetts General Hospital PrimerBank (http://pga.mgh.harvard.edu/primerbank/).

Quantitative PCR

RNA from stimulated cells and lung lobes was isolated using TRIzol (Invitrogen) or EPCAM-allophycocyanin (1:50; 17-5791, eBioscience). 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 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/GM-CSF, and that the expression was dependent on CARMA3 (47). 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

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CARMA3 MEDIATES ALLERGIC AIRWAY INFLAMMATION

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

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 CARMA3F/F mice. These mice were then crossed to SPCCre/CARMA3F/F mice. These mice were then crossed to SPCCre/CARMA3+/+ mice, 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.

Deletion of CARMA3 in AECs attenuates allergic airway inflammation.

We assessed whether deletion of CARMA3 expression in AECs would also attenuate AHR. Using mechanically ventilated mice, airway resistance (Rn) and airway compliance (C) were calculated from data obtained with a forced oscillation technique as described previously (64). OVA challenge of both SPCCre/CARMA3+/+ and SPCCre/CARMA3F/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 does not attenuate AHR

We assessed whether deletion of CARMA3 expression in AECs would also attenuate AHR. Using mechanically ventilated mice, airway resistance (Rn) and airway compliance (C) were calculated from data obtained with a forced oscillation technique as described previously (64). OVA challenge of both SPCCre/CARMA3+/+ and SPCCre/CARMA3F/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 SPCCre/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 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-3a, 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.
FIGURE 3. Generation of SPCcre/CARMA3F/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) SPCcre/CARMA3+/+, (D) SPCcre/CARMA3F/F, and (E) CARMA3F/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 SPCcre/CARMA3+/+ and SPCcre/CARMA3F/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 SPCcre/CARMA3+/+ and SPCcre/CARMA3F/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 (SPCcre/CARMA3+/+ compared with SPCcre/CARMA3F/F). (I) Immunofluorescence of lungs from PBS- and OVA-immunized and challenged SPCcre/CARMA3+/+ 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-

FIGURE 4. Attenuation of airway inflammation in OVA-immunized and -challenged SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup> mice. (A) Histopathologic analysis of lung sections stained with H&E from SPC<sup>Cre</sup>/CARMA3<sup>+/+</sup>-PBS–, SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup>-PBS–, SPC<sup>Cre</sup>/CARMA3<sup>+/+</sup>-OV A–, and SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup>-OV A–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 (OV A treatment compared with same genotype that was treated with PBS or SPC<sup>Cre</sup>/CARMA3<sup>+/+</sup> OV A compared with SPC<sup>Cre</sup>/CARMA3<sup>F/F</sup> OV A). ND, not detected.
mDCs, CD80+ mDCs, CD86+ mDCs, OX40L+

lymph nodes were isolated and the number of

OV A administration the (OV A i.t. on day 7. Twenty-four hours after DQ-
expression of PAR2 by AECs allows the recognition of protease-
activation (72–75) and are linked to common allergens. The ex-
initiation and modulation of allergic lung responses via NF-
stimulation in AECs, suggesting that the airway epithelium is
we found that these specific receptors were elevated without
inhaled into the airways. AECs express a diverse array of GPCRs,
abundant array of particles, Ags, and infectious pathogens that are

FIGURE 5. DCs isolated from SPC Cre/
CARMA3F/F mice show impaired maturation, migra-
tion, and Ag processing. SPC Cre/CARMA3+/+ and
SPC Cre/CARMA3F/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-
OV A 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 experi-
ments. *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

Results of the present study demonstrate an important rela-
tionship 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 migra-
tion, 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 inflam-
mation 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 ex-
pression 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 proin-
flammatory 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 re-
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 air-
ways of allergen-challenged patients and contributes to disease
pathogenesis via signaling at P2Y receptors expressed at the ep-
ithelial 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 eos-
nophils 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 findings support our notion of the central role of airway epithelial CARMA3 signaling in allergic airway inflammation, the precise GPCR–agonist interaction that triggers CARMA3 and NF-κB signaling remains to be identified.

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 SPCCre/CARMA3F/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 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-kB 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 allergic challenge and could also mediate GPRC activation of AECs in vivo (56, 106, 107).

Global targeting of NF-kB is not a viable therapeutic option, as total inhibition of NF-kB activity would interrupt vital physiologic processes important for tissue and immune homeostasis. However, these studies suggest that targeting the NF-kB in a cell- and pathway-specific manner could be beneficial in treating asthma. Consistent with this, selectively inhibiting NF-kB in endothelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J. Exp. Med. 204: 253–258.


References

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Disclosures

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Acknowledgments

References


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

Single cell suspension of lung and TLNs were analyzed by flow. After gating on live cells, we identified CD11c\textsuperscript{+}/CD11b\textsuperscript{+}/MHCII\textsuperscript{+}/Gr-1\textsuperscript{-} 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\textsuperscript{Cre}/CARMA3\textsuperscript{+/+} mice that received no DQ-OVA, (B) SPC\textsuperscript{Cre}/CARMA3\textsuperscript{+/+} mice that received DQ-OVA and (C) SPC\textsuperscript{Cre}/CARMA3\textsuperscript{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.