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CARMA1 Is Critical for the Development of Allergic Airway Inflammation in a Murine Model of Asthma

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CARMA1 has been shown to be important for Ag-stimulated activation of NF-κB in lymphocytes in vitro and thus could be a novel therapeutic target in inflammatory diseases such as asthma. In the present study, we demonstrate that mice with deletion in the CARMA1 gene (CARMA1−/−) do not develop inflammation in a murine model of asthma. Compared with wild-type controls, CARMA1−/− mice did not develop airway eosinophilia, had no significant T cell recruitment into the airways, and had no evidence for T cell activation in the lung or draining lymph nodes. In addition, the CARMA1−/− mice had significantly decreased levels of IL-4, IL-5, and IL-13, did not produce IgE, and did not develop airway hyperresponsiveness or mucus cell hypertrophy. However, adoptive transfer of wild-type Th2 cells into CARMA1−/− mice restored eosinophilic airway inflammation, cytokine production, airway hyperresponsiveness, and mucus production. This is the first demonstration of an in vivo role for CARMA1 in a disease process. Furthermore, the data clearly show that CARMA1 is essential for the development of allergic airway inflammation through its role in T lymphocytes, and may provide a novel means to inhibit NF-κB for therapy in asthma. The Journal of Immunology, 2006, 176:7272–7277.

A member of the caspase recruitment domain-membrane associated guanylate kinase family of proteins, CARMA1 has shown to be essential for Ag-stimulated activation of NF-κB in lymphocytes (1–5). The NF-κB family of transcription factors is ubiquitously expressed in immune cells and is critical for both the innate and adaptive immune response (6–8). NF-κB is activated when inhibitory proteins (IκB complex) are phosphorylated by the IκB kinase (IKK) complex of proteins. This leads to the degradation of IκB, translocation of NF-κB to the nucleus, and then translocation of specific target proteins involved in initiating inflammation. TCR-mediated activation of T cells leads to phosphorylation of CARMA1, a scaffolding protein abundantly expressed in lymphocytes (9–11). CARMA1 then mediates IKK translocation to the immune synapse, which initiates a cascade that leads to NF-κB activation (7, 12–14). Studies in mice with deletion of CARMA1 (CARMA1−/−) have shown that it is necessary for Ag receptor-mediated activation of B cells and T cells (3, 9, 14, 15). These data indicate an important role for CARMA1 in the regulation of the adaptive immune response through its actions in lymphocytes, and suggests that targeted inhibition of CARMA1 would provide a novel means of inhibiting NF-κB activation in lymphocytes. In addition, because CARMA1 activation is regulated by protein kinases, it may be quite amenable to designing pharmacological inhibitors and thus may be a novel therapeutic target in inflammatory diseases. However, the in vivo role of CARMA1 in T cell activation in inflammatory diseases has not been established. In addition, it is unclear whether CARMA1 plays a role in processes other than lymphocyte activation.

Previous research has demonstrated that members of the NF-κB family are important in the pathogenesis of allergic airway inflammation in murine models of asthma (16, 17). Specifically, mice that lack the p50 subunit of NF-κB are unable to develop allergic airway inflammation due to a defect in Th2 cell polarization from a failure to express the transcription factor GATA-3 (18). Other studies using cell-specific blockade of NF-κB in airway lining cells or in alveolar macrophages have demonstrated only partial inhibition of specific aspects of the asthma phenotype (19–21). These data suggest a complex and differential role for the NF-κB pathway in asthma with, however, great potential as a therapeutic target. We hypothesized that CARMA1 would play an essential role in NF-κB activation in T lymphocytes and that deletion of CARMA1 would allow specific inhibition of T cell activation, thus preventing the development of allergic airway inflammation. To address this, we examined the development of allergic airway inflammation in a murine model of asthma using CARMA1−/− mice. To investigate other potential roles for CARMA1 independent of T cell activation, we also examined the development of allergic airway inflammation in a Th2 cell adoptive transfer model of asthma using CARMA1−/− mice.

Materials and Methods

Mice
Mice with a deletion in the CARMA1 gene were generated using modified bacterial artificial chromosome (BAC) technology as previously described (22). In brief, we obtained BAC clones spanning the CARMA1 locus from Research Genetics (Invitrogen Life Technologies). Two short sequences...
flanking exons 3 and 5 were cloned into the 5’ and 3’ insertion sites of the selection cassette of the pSKY replacement vector. BAC host cells were transformed with the pBADΔαβα plasmid, which helped produce electroporation-competent cells. The linear fragment released from the pSKY backbone was then electroporated into the BAC host, and the transformants were selected for simultaneous resistance to chloramphenicol (from the BAC backbone) and zeocin (from the insert). The resulting mutant BAC was shown in Fig. 1A. As shown in Fig. 1B, c3/c4/2p and primers identify a predicted 536-bp PCR product in the mutant BAC and 326-bp PCR product in the wild-type BAC. Fluorescence in situ hybridization was performed as described previously and confirmed targeting in clones 7 and 38 (Fig. 1C). CARMA1−/− mice from clone 38 were born in the expected Mendelian frequency and were healthy. Mice were in a Sv129-C57BL/6 hybrid background (one generation). For the experiments, age- and sex-matched CARMA1−/− and wild-type littermate controls were used at 6–8 wk of age. Mice with a transgenic TCR specific for a peptide of chicken egg albumin (OVA323–339) bound to H-2b (OT-II) in the C57BL/6-Thy1.1 background were provided by Dr. P. Shrikant (Roswell Park Cancer Institute, Buffalo, NY). All protocols were approved by the animal studies committee at Massachusetts General Hospital.

**Mouse models**

Allergic airway inflammation was induced in mice as previously described (23, 24). Briefly, CARMA1−/− mice and wild-type littermate control mice were injected i.p. with 10 μg of chicken egg albumin (OVA) (Sigma-Aldrich) bound to 1 mg of aluminum hydroxide (Type V; Sigma-Aldrich) suspended in 0.5 cc of PBS (Mediatech) on days 0 and 7. This preparation of OVA has been shown to have ~1 U of endotoxin per milligram of OVA (25). Mice underwent aerosol challenge with nebulized OVA (10 mg/ml in PBS) (23). RNA was purified from the lung and analyzed by quantitative PCR as previously described (23, 24). BAL cytokine levels were assessed by a commercial bead array kit (BD Pharmingen) according to the manufacturer’s instructions. Serum OVA-specific IgE concentrations were measured by ELISA as described previously (27).

**Data analysis**

Data are expressed as mean ± SEM. Differences in results were considered to be statistically significant when p < 0.05 using Student’s t test or ANOVA.

**Results**

**CARMA1−/− mice do not develop allergic airway inflammation**

Immunization and challenge of mice with OVA have been shown to lead to prominent allergic airway inflammation associated with recruitment of activated T cells and eosinophils into the peribronchial space and the airways as well as mucus cell hypertrophy (23, 24). We compared the amount of airway inflammation induced by OVA immunization and challenge in CARMA1−/− mice with the inflammation induced in age- and sex-matched wild-type littermate control mice. Wild-type mice developed characteristic eosinophils, no neutrophils, no eosinophils, and very few lymphocytes (Fig. 2Ai) around airways (black arrow) and numerous periodic acid-Schiff (PAS)-positive cells in the airway lining (Fig. 2Aii, black arrow), consistent with mucus cell hypertrophy. In contrast, CARMA1−/− mice did not develop any significant pulmonary inflammation (Fig. 2Aii) or PAS-positive staining in the airways (Fig. 2Aiii). Analysis of cellular recruitment into the airspaces disclosed significantly fewer cells recruited into the BAL in the CARMA1−/− mice compared with wild-type mice (3.31 ± 0.79 × 105 vs 18.79 ± 4.5 × 105; p = 0.0002). The cellular profile in the CARMA1−/− mice was similar to unchallenged mice with >95% macrophages, no eosinophils, no neutrophils, and very few lymphocytes (Fig. 2B and data not shown). However, wild-type mice had significant increases in eosinophils, neutrophils, and lymphocytes in the BAL. We also analyzed T lymphocyte recruitment into the airways. Wild-type mice had prominent recruitment of CD4+ cells.
CARMA1−/− mice have attenuated cytokine production and IgE production

In this murine model of asthma, T cells are polarized to Th2 cells, which produce the cytokines IL-4, IL-5, and IL-13, which stimulate IgE class switching, mucus cell hypertrophy, and eosinophil recruitment. We measured RNA levels of IL-4, IL-5, IL-13, and IFN-γ in the lungs as well as the protein levels of TNF-α, IFN-γ, IL-4, and IL-5 in the BAL of these mice (Fig. 4A). CARMA1−/− mice had significantly lower RNA levels of IL-4, IL-5, and IL-13 and lower protein levels of IL-4 and IL-5 consistent with decreased Th2 lymphocyte polarization and recruitment. We also measured the serum levels of OVA-specific IgE. Serum levels of OVA-specific IgE were significantly greater in the wild-type mice compared with the CARMA1−/− mice (276.7 ± 57.5 vs. 5.8 ± 4.5 ng/ml; p = 0.0001).

CARMA1−/− mice have attenuated AHR

Allergic airway inflammation is associated with greater reactivity of the airway smooth muscle cells to bronchoconstrictors. This hyperresponsiveness can be assessed by measuring changes in airway resistance and dynamic lung compliance (as a reflection of changes in small airway caliber) in response to increasing doses of ACh.
methacholine (29). When we measured changes in airway resistance and dynamic compliance, the OVA-immunized and -challenged wild-type mice had significantly greater reactivity than the OVA-immunized and -challenged CARMA1−/− mice (Fig. 4B). In fact, the CARMA1−/− mice had similar changes in resistance and compliance to unimmunized wild-type and CARMA1−/− mice consistent with the absence of airway inflammation.

Adoptive transfer of Th2 cells restores the asthma phenotype in CARMA1−/− mice

Adoptive transfer of in vitro-generated OVA-specific Th2 cells followed by OVA challenge has been shown to initiate allergic airway inflammation in mice without the need for immunization (26). To determine whether the CARMA1−/− mice do not develop airway inflammation purely due to a defect in lymphocyte activation, we transferred in vitro-generated OVA-specific Th2 cells from OT-II mice into naive CARMA1−/− mice and wild-type control mice. The OT-II mice have been genetically modified such that all CD4+ T cells express a transgenic TCR specific for OVA 323-339 bound to H-2b. The cells were positive for the Thy1.2 allele, allowing us to track the cells in the recipient mice, which were positive for Thy1.2. Adoptive transfer of these OVA-specific cells, after in vitro polarization into Th2 cells, allows the full development of allergic airway inflammation and its consequences independent of the recipient mouse CD4+ T cells and thus should allow us to identify other CARMA1-dependent processes downstream of lymphocyte activation in the CARMA1−/− mice. Following OVA challenge, the wild-type mice and the CARMA1−/− mice developed prominent airway inflammation (Fig. 5A, i and ii) and mucus cell hypertrophy (Fig. 5A, iii and iv). Analysis of T lymphocyte recruitment into the BAL (Fig. 5B) disclosed no differences in the percentage or number of CD4+, CD8+, CD4+CD25+, or CD8+CD25+ T cells recruited into the airspaces. In addition, the number of transferred Th2 cells recruited into the BAL was similar between the two strains of mice. The cell differential in the BAL was also similar between the wild-type mice and the CARMA1−/− mice with prominent recruitment of eosinophils, neutrophils, and lymphocytes into the airways of both strains of mice (Fig. 5C). We examined the profile of T cells in the thoracic lymph nodes of these mice. There was a significantly lower percentage of CD4+CD25+ T cells in the lymph nodes of the CARMA1−/− mice compared with wild-type mice (Fig. 5D). This difference was due to a decrease in the number of native
CD4\(^+\)CD25\(^+\) T cells as demonstrated by an absence of Thy1.1 staining. This likely reflects a failure in T cell activation of the native CARMA1\(^{-/-}\) T lymphocytes. However, when we assessed cytokine RNA and protein levels, we did not see any differences (data not shown), suggesting that the transfer of wild-type Th2 cells was sufficient to restore Th2-type cytokine production. In addition, we saw a similar increase in airway resistance and decrease in lung compliance in the CARMA1\(^{-/-}\) mice after Th2 cell transfer compared with wild-type mice following Th2 cell transfer (Fig. 5E). These data demonstrate that CARMA1 is not necessary for the development of AHR if T cells can be activated. We were unable to assess IgE production in this model because there is no OVA-specific IgE formed in the wild-type mice with Th2 cell transfer.

Discussion

CARMA1 is a protein expressed in lymphocytes that serves as an essential scaffold for the formation of the IKK signaling complex that leads to NF-κB activation (30), and has been shown to be crucial for TCR-mediated activation of T cells (3, 7, 9). Because the adaptive immune response is initiated by Ag engagement of the TCR, these prior studies suggest that CARMA1-mediated T cell activation should have an important role in the development of inflammatory diseases; however, this has not been demonstrated in vivo. In our study, we show that CARMA1 is critical for the development of allergic airway inflammation in a murine model of asthma. However, adoptive transfer of Th2 cells restores the asthma phenotype, suggesting that the critical role of CARMA1 in the development of inflammation is limited to lymphocyte activation.

The murine model of asthma used in these studies is a classic model of a Th2-type adaptive immune response that is dependent on Ag-specific T cell activation (27, 31). In this model, immunization with OVA leads to the activation and polarization of OVA-specific T cells in the mouse. Some of these cells become memory T cells that provide surveillance for further exposure to Ag in organs and lymphoid tissue (32). When the animal is challenged with OVA in the airways, the protein is taken up by APCs and presented to these OVA-specific T cells. The T cells migrate into the airway, proliferate, and then orchestrate the allergic inflammatory response (33). Central to the model is the activation and polarization of T cells into Th2-type lymphocytes, processes that are dependent on the expression of specific NF-κB subunits (18). Our data suggest that deletion of CARMA1 impairs the initial priming of OVA-specific T cells and prevents any reaction to OVA in subsequent airway challenges. However, it is not clear what role, if any, CARMA1 has in T lymphocytes following the initial priming and polarization of Ag-specific T cells. Because memory cells are stimulated through the TCR, one would predict that memory T cell responses would also be impaired. This would have relevance for treatment of patients with established asthma whose T cells would already be primed to relevant allergens.

Although critical for the development of Ag-specific Th2 cells (18), CARMA1 does not seem to be necessary for other aspects in the pathogenesis of asthma, because adoptive transfer of OVA-specific Th2 cells allows full development of allergic inflammation. These data are consistent with other studies that have demonstrated that deletion of the NF-κB subunits c-Rel or p50 impairs the development of allergic airway inflammation (16–18), and as in our experiments, adoptive transfer of Th2 cells into the p50\(^{-/-}\) mice, restored the asthma phenotype. Although other studies have indicated that NF-κB activation in nonlymphocytes in the lung contributes to specific aspects in the development of allergic airway inflammation (19–21), clearly the activation of NF-κB in those cells is independent of CARMA1. We were unable to test whether IgE production was restored in CARMA1\(^{-/-}\) mice following wild-type Th2 cell adoptive transfer. However, because prior studies have indicated that B cell activation is dependent on CARMA1 (34), we suspect that IgE production could remain impaired in these mice.

Following adoptive transfer of Th2 cells into the CARMA1\(^{-/-}\) mice, there was no defect in eosinophil or lymphocyte recruitment into the airways. The lymphocytes recruited into the airways included both transferred Ag-specific Th2 cells, as well as native CARMA1\(^{-/-}\) T cells. Interestingly, some of the native T cells that entered the airway in the CARMA1\(^{-/-}\) mice were positive for the activation marker CD25. These data suggest that T cells can migrate into the airways independently of CARMA1 expression. It is possible that the native CD25\(^+\) T cells recruited into the airway in the CARMA1\(^{-/-}\) mice are “bystander” cells activated through a TCR/CARMA1-independent pathway and brought into the airway by the inflammatory response. However, in the thoracic lymph nodes, there was a clear reduction in the number of CARMA1\(^{-/-}\) CD4\(^+\)CD25\(^+\) T cells, suggesting that deletion of CARMA1 did impair T cell activation in the lymphoid compartment.

Our experiments provide in vivo evidence supporting a critical role for CARMA1-mediated T cell activation in the development of allergic airway inflammation. However, the development of mucous cell hypertrophy, eosinophil and Th2 lymphocyte recruitment, and AHR are independent of CARMA1 expression as demonstrated by the fact that adoptive transfer of wild-type Th2 cells is able to fully restore these components of the asthma phenotype. This suggests that CARMA1 has a specific role in lymphocytes during the development of inflammation. In addition, CARMA1 is essential for Ag-specific activation of T cells, suggesting that targeted inhibition of CARMA1 would provide a novel means of inhibiting NF-κB activation in lymphocytes and could prevent the development of asthma in susceptible patients.

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Disclosures

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References


