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CARMA1 Is Necessary for Optimal T Cell Responses in a Murine Model of Allergic Asthma

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CARMA1 is a lymphocyte-specific scaffold protein necessary for T cell activation. Deletion of CARMA1 prevents the development of allergic airway inflammation in a mouse model of asthma due to a defect in naive T cell activation. However, it is unknown if CARMA1 is important for effector and memory T cell responses after the initial establishment of inflammation, findings that would be more relevant to asthma therapies targeted to CARMA1. In the current study, we sought to elucidate the role of CARMA1 in T cells that have been previously activated. Using mice in which floxed CARMA1 exons can be selectively deleted in T cells byOX40-driven Cre recombinase (OX40<sup>Cre<sup>/CARMA1<sup>F/F</sup>), we report that CD4<sup>+</sup> T cells from these mice have impaired T cell reactivation responses and NF-κB signaling in vitro. Furthermore, in an in vivo recall model of allergic airway inflammation that is dependent on memory T cell function, OX40<sup>Cre<sup>/CARMA1<sup>F/F</sup> mice have attenuated eosinophilic airway inflammation, T cell activation, and Th2 cytokine production. Using MHC class II tetramers, we demonstrate that the development and maintenance of Ag-specific memory T cells is not affected in OX40<sup>Cre<sup>/CARMA1<sup>F/F</sup> mice. In addition, adoptive transfer of Th2-polarized OX40<sup>Cre<sup>/CARMA1<sup>F/F</sup> Ag-specific CD4<sup>+</sup> T cells into wild-type mice induces markedly less airway inflammation in response to Ag challenge than transfer of wild-type Th2 cells. These data demonstrate a novel role for CARMA1 in effector and memory T cell responses and suggest that therapeutic strategies targeting CARMA1 could help treat chronic inflammatory disorders such as asthma. The Journal of Immunology, 2011, 187: 000–000.

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are protected from developing experimental allergic asthma (25, 26). However, there have been no studies addressing the role of CARMA1 in recall responses to allergens in experimental models of asthma. These data would be relevant to therapeutic approaches in asthma that target CARMA1 and NF-κB, as allergen-specific T cells have already been established in asthmatics, and exacerbations of inflammation will largely depend on effector and memory T cell responses.

We hypothesized that deletion of CARMA1 after T cell activation would impair effector and memory T cell responses and attenuate allergic inflammation after re-exposure to allergen. CARMA1-deficient mice do not form effector or memory T cells due to a global defect in T cell activation. Thus, to address our hypothesis and circumvent this problem, we investigated the development of allergenic airway inflammation using conditional CARMA1 mutant mice (OX40CreCARMA1F/F) in which CARMA1 expression is disrupted after T cell activation by an OX40 promoter-driven Cre recombinase that mediates deletion of exons 3 and 4 of CARMA1 (4, 27). Using a combination of in vitro and in vivo studies, we demonstrate that CARMA1 is necessary for optimal T cell responses to TCR engagement and the development of allergic airway inflammation after the initial activation of T cells. These data suggest that decreasing the levels or activity of CARMA1 even after the establishment of chronic asthma may have therapeutic potential.

Materials and Methods

Mice

Mice with floxed CARMA1 (CARMA1F/F) were provided by Dr. Dan Littman (New York University) (4). Mice that express Cre recombinase under control of the OX40 promoter (OX40Cre) were provided by Dr. Nigel McMichael (University of California, San Francisco) (27). Mice with a constitutively expressed modified form of Cre recombinase that translocates to the nucleus in the presence of tamoxifen (Rosa26CreERT2) were provided by Dr. Thomas Ludwig (Columbia University) (28–30). Mice with floxed CARMA1 (CARMA1F/F, isolated CD4+ cells were cultured with irradiated splenic APCs pulsed with 100 ng/ml OVA323–339,1 and 1 μg/ml anti-CD28 on day 0. On day 3, live CD4+ T cells were separated using Lymphocyte (Cedarlane Laboratories, Burlington, NC) and treated with 1 μM 4-hydroxymafloxin (Sigma, St. Louis, MO) or an equal volume of vehicle (ethanol) for 5 d. On day 8, RNA was collected from an aliquot of cells, and the remaining cells were left unstimulated or restimulated with anti-CD3+ and anti-CD28-coated beads. On day 9, activation of CD4+ T cells was evaluated by flow cytometry.

Asthma models

Acute allergic airway inflammation was induced in mice as previously described (17). Briefly, male mice from OX40CreCARMA1F/F, OX40CreCARMA1F/F, and OX40CreCARMA1F/F strains were sensitized with an i.p. injection of 10 μg alum-conjugated OVA (Sigma-Aldrich) on days 0 and 7. Mice were then challenged with 1% OVA aerosol for 20 min on days 14, 15, and 16. For asthma recall experiments, mice were sensitized with an i.p. injection of 10 μg alum-conjugated OVA on days 0 and 7, challenged with 1% OVA aerosol for 20 min on days 14, 15, 16, and rechallenged again on days 58, 59, and 60. In acute and recall asthma experiments, data were collected 24 h after the last OVA challenge. In some recall experiments, lungs and thoracic lymph nodes (TLNs) were collected on day 58 prior to OVA rechallenge. Tissues collected from euthanized mice include bronchoalveolar lavage (BAL) fluid, right upper lobe of the lungs for RNA isolation, and formalin-fixed lung for histological evaluation. Tissues were processed as previously described (17).

The adoptive transfer model of allergic airway inflammation was performed as described previously (17). Briefly, CD4+ T cells were isolated from pooled lymph nodes and spleens of TCR transgenic (OT-II) mice. CD4+ T cells were incubated with irradiated splenic APCs (1:4 CD4+/APC ratio) pulsed with 100 ng/ml OVA323–339,1 and 1 μg/ml anti-CD28 Ab, 1 μg/ml anti-IFN-γ Ab, and 100 ng/ml recombinant mouse IL-4. From day 2, CD4+ T cells were maintained in media supplemented with IL-2. On day 6, an aliquot of cells was separated and stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 6 h. GolgiStop (eBioscience, San Diego, CA) was added during the last 4 h of incubation, and the stimulated cells were analyzed for production of intracellular cytokine IL-4, IFN-γ, and IL-17 of 100 ng/ml OVA323–339,1 from OX40CreCARMA1F/F, OX40CreCARMA1F/F, and OX40CreCARMA1F/F mice. Isolated CD4+ T cells were incubated with irradiated splenic APCs (1:4 CD4+/APC ratio) pulsed with 100 ng/ml OVA323–339,1 and 1 μg/ml anti-CD28 Ab, 1 μg/ml anti-IFN-γ Ab, and 100 ng/ml recombinant mouse IL-4. From day 2, 6- to 8-week-old mice were used in all experiments. All protocols were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

Flow cytometry

CD4+ T cells were isolated from single-cell suspensions of pooled lymph nodes (inguinal, brachial, axillary, and cervical) and spleen from mice using cell separator (EasySep; Stemcell Technologies, Vancouver, Canada). Purity of the CD4+ cell preparations were routinely evaluated and were >90–95% pure. For some experiments, naive CD4+ cells were isolated using a mouse CD4+ CD62L+ isolation kit (Miltenyi Biotec, Auburn, CA). On day 0, isolated CD4+ T cells were activated with anti-CD3 and anti-CD28 Ab-coated beads (Dynabeads; Invitrogen, Carlsbad, CA) in a ratio of 1:1 in media supplemented with IL-2. On day 3, beads were removed from the cells, and total RNA was isolated from an aliquot of cells. The remaining cells were allowed to rest at a concentration of 1 × 10^6 cells/ml in fresh media supplemented with IL-2. Cells were periodically fed fresh media supplemented with IL-2. On day 8, cells were left untreated or were restimulated with anti-CD3 and anti-CD28 Ab-coated beads in the presence of IL-2 and analyzed 24 h later using flow cytometry. In a separate set of experiments, cyttoplasmic and nuclear protein was isolated from cells using the Cytoskeleton Protein Extraction (Cytoskeleton Kit; Pierce, Rockford, IL) left untreated or restimulated for 2 h with anti-CD3+ and anti-CD28-coated beads. For experiments with CreERT2/ CARMA1F/F, isolated CD4+ cells were cultured with irradiated splenic APCs pulsed with 100 ng/ml OVA323–339,1 and 1 μg/ml anti-CD28 on day 0. On day 3, live CD4+ T cells were separated using Lymphocyte (Cedarlane Laboratories, Burlington, NC) and treated with 1 μM 4-hydroxymafloxin (Sigma, St. Louis, MO) or an equal volume of vehicle (ethanol) for 5 d. On day 8, RNA was collected from an aliquot of cells, and the remaining cells were left unstimulated or restimulated with anti-CD3+ and anti-CD28-coated beads. On day 9, activation of CD4+ T cells was evaluated by flow cytometry.

RNA was purified from cells or lung and analyzed by quantitative real-time PCR (QPCR) as previously described (17). In brief, samples were analyzed...
using SYBR Green (Applied Biosystems, Foster City, CA) based QPCR in a Mastercycler EP Realplex thermal cycler (Eppendorf, Hamburg, Germany). Transcript copy numbers of each gene deduced from the cycle threshold (C\textsubscript{T}) values were normalized against copy numbers of an endogenous control gene (GAPDH and/or β-actin) in the corresponding samples. Primer sequences used were obtained from the Massachusetts General Hospital Primerbank (pga.mgh.harvard.edu/primerbank/) or from the National Cancer Institute Primerdepot (http://mouseprimerdepot.nci.nih.gov/).

Detection of OVA-specific CD4\textsuperscript{+} T cells by class II tetramers

Lungs and TLNs were collected from mice. Single-cell suspensions were prepared from pooled TLNs within each group in each experiment and individual lung samples in each experiment. OVA-specific T cells were enriched with a previously described tetramer-based enrichment procedure (32, 33) involving the combined use of two tetramers, OVA-2C and OVA-3C, specific for two immunodominant epitopes of OVA, QA\textsubscript{2}HAAHAINEA and VHA\textsubscript{2}HAAHAEINEA, respectively (34, 35). After enrichement, tetramer bound and unbound cells were analyzed by flow cytometry. Cells were gated for lymphocytes, and OVA-specific T cells were identified and enumerated by a Tetramer\textsuperscript{CD4\textsuperscript{+}CD8\textsuperscript{-}CD11c\textsuperscript{-}CD19\textsuperscript{+}} phenotype.

Statistical analysis

Results are shown as mean ± SEM values. GraphPad Prism software was used to analyze the results. Two groups were compared using a Student t test. Multiple between-group comparisons of means were performed by one-way ANOVA with Newman–Keuls post hoc method. A p value < 0.05 was regarded as a significant difference.

Results

Deletion of CARMA1 from OX40-expressing activated T cells

Deficiency of CARMA1, Bcl11o, or MALT1 reduces NF-κB activation after TCR engagement and thus inhibits the activation of naive T lymphocytes. This makes it difficult to study the role of CARMA1 in T cell functions after activation in CARMA1-deficient cells. To address this issue, we used genetically modified mice withloxP sites inserted flanking exons 3 and 4 of CARMA1 (4). In the original description of this mouse, an intact Neomycin expression cassette was inserted into intron 4 of the CARMA1 gene leading to disruption in the expression of CARMA1 (CARMA1FN/FN mice). We used a modified strain derived from these mice in which the Neomycin cassette was removed, leaving behind a loxP site in intron 4 along with a predesigned, preexisting loxP site in intron 2 (CARMA1F/F mice). CARMA1F/F mice are fully competent in producing full-length CARMA1 protein, and Cre recombinase expression in these mice leads to the deletion of exons 3 and 4 of CARMA1. Exons 3 and 4 encode the C-terminal portion of the CARD and the N-terminus of the coiled-coil domain, and their deletion leads to disruption in functional CARMA1 expression. These mice were bred to mice that express a Cre allele under control of the OX40 promoter (27) to generate OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} mice. Previous work has established that replacement of one of the OX40 alleles with Cre does not affect the functional responses of T cells (27, 36). OX40 is not expressed in naive CD4\textsuperscript{+} or CD8\textsuperscript{+} cells but is upregulated within 24 h in the majority of T cells after activation via TCR engagement (37). Thus, these mice will delete CARMA1 in a large portion of T cells only after activation. To ensure that CARMA1 is efficiently deleted in the OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} mice after T cell activation, naive CD4\textsuperscript{+} T cells were isolated from OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} and OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} mice and stimulated with beads coated with anti-CD3 and anti-CD28 Abs (Fig. 1A). The T cells from both mouse strains activated normally (data not shown). Analysis of RNA expression by QPCR demonstrated that the expression levels of CARMA1 were decreased 1 d after activation in CD4\textsuperscript{+} T cells from both OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} mice and OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} control mice; however, 3 d after activation, the CARMA1 RNA levels in T cells from OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} mice decreased more than in T cells from OX40\textsuperscript{+/+}\textsuperscript{CARMA1F/F} mice (Fig. 1B). This was consistent with the OX40 pattern of expression, which demonstrated a similar peak of RNA expression in T cells from both strains of mice 1 d after activation with a subsequent decline in levels over the next 2 d (Fig. 1C). The T cells from both strains of mice were comparable between OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} and OX40\textsuperscript{+/+}\textsuperscript{CARMA1F/F} mice. Expression levels of Bcl11o and MALT1 also did not differ in the T cells from both strains of mice (data not shown). Western blot analyses confirmed that in unstimulated CD4\textsuperscript{+} cells, CARMA1 protein levels were comparable between OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} and OX40\textsuperscript{+/+}\textsuperscript{CARMA1F/F} mice. Expression levels of Bcl11o and MALT1 also did not differ in the T cells from both strains of mice (data not shown). Western blot analyses confirmed that in unstimulated CD4\textsuperscript{+} cells, CARMA1 protein levels were comparable between OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} and OX40\textsuperscript{+/+}\textsuperscript{CARMA1F/F} mice (Fig. 1D). However, 3 d after activation, CARMA1 protein was markedly reduced in CD4\textsuperscript{+} T cells from OX40\textsuperscript{Cre}\textsuperscript{CARMA1F/F} mice compared with CD4\textsuperscript{+} T cells from OX40\textsuperscript{+/+}\textsuperscript{CARMA1F/F} mice (Fig. 1D). We suspect that there is residual CARMA1 RNA and protein expression in these cells because
OX40 is not upregulated in all T cells after activation. These data demonstrate that CARMA1 can be selectively and efficiently deleted from OX40-expressing activated CD4+ T cells after activation.

**Deletion of CARMA1 after T cell activation reduces T cell reactivation in vitro**

The effects of CARMA1 deletion after T cell activation remains unknown. We hypothesized that deletion of CARMA1 after T cell activation would impair the response of these cells to restimulation via the TCR by inhibiting NF-κB signaling. To address this question, CD4+ T cells were activated with anti-CD3– and anti-CD28–coated beads for 3 d, rested for 5 d in media, and then restimulated with anti-CD3 and anti-CD28 (Fig. 2A). Eight days after activation, CARMA1 RNA levels remained reduced in the CD4+ T cells from OX40+/CreCARMA1F/F mice compared with CD4+ T cells from OX40+/+CARMA1F/F mice after stimulation (Fig. 2B). The expression of OX40 RNA was similar in T cells from both strains of mice 8 d after activation but did not increase as much in CD4+ T cells from OX40+/+CARMA1F/F mice after reactivation compared with T cells from OX40+/+CARMA1F/F mice (Fig. 2C). After the resting period, very few cells expressed CD69, whereas a majority of T cells from both strains of mice expressed CD44, indicative of prior activation (Fig. 2D, 2E).

However, after restimulation, a lower percentage of CD4+ T cells isolated from OX40+/CreCARMA1F/F mice expressed CD69 and CD44 compared with the expression of CD69 and CD44 on CD4+ T cells from OX40+/+CARMA1F/F mice (Fig. 2D, 2E). These data suggest that deletion of CARMA1 after activation impairs subsequent responses of these cells to restimulation via the TCR. To address whether the decrease in reactivation is associated with a concomitant decrease in NF-κB activity, we measured the levels of NF-κB p65 in the nuclear protein extracts isolated from CD4+ T cells 2 h after reactivation with anti-CD3 and anti-CD28 Abs. Decreased nuclear translocation of the p65 subunit of NF-κB was observed in the CD4+ T cells from OX40+/CreCARMA1F/F mice compared with that in CD4+ T cells from OX40+/+CARMA1F/F mice (Fig. 2F).

Given the incomplete deletion mediated by the OX40-controlled Cre, we also tested the effects of CARMA1 deletion postactivation using an inducible Cre system. Mice that constitutively express (via the ROSA26 promoter) a tamoxifen-inducible Cre-ERT2 fusion protein (29, 30) were crossed to the CARMA1F/F line and then to the OT-II line to generate Rosa26CreERT2/+ /CARMA1F/F

![FIGURE 2. Deletion of CARMA1 after T cell activation reduces T cell reactivation in vitro.](image-url)
Deletion of CARMA1 by OX40-driven Cre does not reduce acute allergic airway inflammation

Previous studies from our laboratory have demonstrated that deletion of CARMA1 from naive T cells abrogates allergic airway inflammation by preventing T cell activation (17). We first wanted to determine when OX40–Cre activity would be induced in an OVA-induced model of acute allergic airway inflammation. For these experiments, we used a fluorescent-reporter (mT/mG) mouse that expresses an enhanced GFP in tissues after Cre-mediated excision of the mT cassette (31). OX40+/CremGT/mG mice were immunized with i.p. injections of alum-conjugated OVA and challenged with OVA (Supplemental Fig. 2A). OX40-driven Cre recombinase activity, indicated by the expression of GFP, was evaluated in CD4+ T cells from the lungs, spleen, and BAL from these mice after OVA immunization or after OVA immunization and challenge. Naive mice had very few GFP+ CD4+ T cells in the lung and spleen (data not shown). Immunization alone resulted in generation of small numbers of GFP+ CD4+ T cells in the lungs and spleens of mice and almost no GFP+ cells in the BAL (Supplemental Fig. 2B). However, OV A immunization and challenge resulted in substantial generation of GFP+ CD4+ T cells in the lungs and BAL, but not the spleen (Supplemental Fig. 2C, 2D and data not shown). These studies demonstrated that immunization and challenge with OVA led to Cre activity in activated CD4+ T cells from OX40+/Crem mice. Hence, we would expect OX40+/CremCARMA1F/F mice to effectively delete CARMA1 from activated T cells in an OVA-induced model of allergic airway inflammation.

To determine if deletion of CARMA1 after T cell activation reduces allergic airway responses, OX40+/CremCARMA1F/F, OX40+/CremCARMA1F/F, and OX40+/CremCARMA1F/F mice were sensitized and challenged with OVA in an acute model of allergic asthma (Fig. 3A). We used OX40+/CremCARMA1F/F mice to control for any effects of the deletion of one OX40 allele and for the expression of Cre recombinase in activated T cells. Development of allergic airway inflammation was comparable across all three strains of mice tested. Histology of lung sections did not demonstrate a marked difference in cellular infiltration between OX40+/CremCARMA1F/F, OX40+/CremCARMA1F/F, and OX40+/CremCARMA1F/F mice (Fig. 3B). Furthermore, all three strains demonstrated similar cell counts and cell differential in the BAL (Fig. 3C–E). In addition, similar numbers of CD4+CD69+ T cells were observed in the BAL recovered from OX40+/CremCARMA1F/F and OX40+/CremCARMA1F/F mice (data not shown). These data suggest that OX40–Cre–driven deletion of CARMA1 does not impair acute allergic airway responses.

Deletion of CARMA1 by OX40–Cre attenuates allergic airway inflammation after Ag rechallenge

To determine whether deletion of CARMA1 after T cell activation reduced T cell memory responses in allergic asthma, we used OX40+/CremCARMA1F/F, OX40+/CremCARMA1F/F, and OX40+/CremCARMA1F/F mice in a modified protocol where mice were sensitized and challenged with OVA as in the acute model of allergic asthma, rested for 6 wk, and then rechallenged with OVA (Fig.
Histology of lung sections demonstrated a marked decrease in cellular infiltration in OX40+/CreCARMA1F/F mice compared with that in OX40+/+CARMA1F/F and OX40+/CreCARMA1+/+ mice (Fig. 4B). Evaluation of BAL revealed decreased number of total cells in OX40+/CreCARMA1F/F mice compared with that in controls (Fig. 4C). Differential cell counts of BAL fluid also demonstrated a decrease in the percentage and total number of eosinophils in OX40+/CreCARMA1F/F mice compared with those in controls (Fig. 4D, 4E). In addition, the percentage and total number of macrophages were increased in OX40+/CreCARMA1F/F mice (Fig. 4D, 4E). Finally, QPCR performed on lung RNA revealed that transcript expression of the proallergic cytokines IL-5 and IL-13 were reduced in the lungs of OX40+/CreCARMA1F/F mice compared with that in OX40+/+carma1F/F mice and OX40+/CreCARMA1+/+ mice (Fig. 4F, 4G). We also assessed the levels of IL-4, but the expression was extremely low in all strains of mice (data not shown).

Deletion of CARMA1 by OX40-driven Cre attenuates memory T cell reactivation in allergic airway inflammation after allergen rechallenge

To determine if the reduced eosinophil influx and cytokine production in the lungs of OX40+/CreCARMA1F/F mice is due to reduced memory T cell reactivation, BAL cells from mice subjected to OVA rechallenge were analyzed by flow cytometry for T cell activation and memory markers. The percentage (Fig. 5A) and total number (Fig. 5D) of CD4+ T cells were comparable across the three strains tested. There was a decreased percentage (Fig. 5B) of CD4+ CD62L+ cells in the BAL from OX40+/CreCARMA1F/F mice compared with that in both OX40+/+CARMA1F/F and OX40+/CreCARMA1+/+ mice. There was also a decreased total number (Fig. 5E) of CD4+ CD62L+ cells in the BAL from OX40+/CreCARMA1F/F mice compared with that in the OX40+/+CARMA1F/F mice. However, there was no difference in the percentage (Fig. 5C) or number (Fig. 5F) of central memory (CD4+ CD62L-CD44+) or...
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Deletion of CARMA1 by OX40-driven Cre does not impair the development of memory T cells in the lungs and lymph node

It has been previously demonstrated that activated T cells from mice lacking the CARD domain of CARMA1 undergo increased levels of apoptosis compared with those of T cells from wild-type mice, a defect rescued by the addition of IL-2 in cell cultures (6, 39, 40). These data suggest that deletion of CARMA1 could lead to enhanced apoptosis of activated T cells and impair the formation of memory CD4+ T cells. To test whether there was impaired development of memory T cells in the OX40+/CreCARMA1+/F mice in vivo, we used MHC class II tetramers to measure the levels of OVA-specific CD4+ T cells in the lungs and draining lymph nodes of OX40+/CreCARMA1+/F, OX40+/F, and OX40+/CreCARMA1+/+ mice in the naive state and after OVA sensitization and challenge (Fig. 6A). Consistent with data from others (32), naive mice had nearly undetectable numbers of OVA-specific T cells in the lungs (Fig. 6B). Six weeks after OVA sensitization and challenge, there was a marked increase in the percentage and number of OVA-specific CD4+ T cells in the lungs (Fig. 6B) and lymph nodes (Supplemental Fig. 3) of all three strains of mice without differences between the genotypes (Fig. 6C, 6D). Comparable levels of OVA-specific CD4+ T cells in the three strains of mice suggest that the reduced inflammation observed in OX40+/CreCARMA1+/F mice is not due to a reduced size of Ag-specific memory T cell pool.

Deletion of CARMA1 by OX40-driven Cre attenuates allergic airway inflammation after adoptive transfer of in vitro Th2-polarized CD4+ T cells from OX40+/CreCARMA1+/F mice

To isolate the defect to CD4+ effector T cells, we used an adoptive transfer model of allergic airway inflammation that is dependent on transferred OVA-specific Th2 cells to generate inflammation. For these experiments, Thy1.2+ CD4+ T cells were isolated from OX40+/CreCARMA1+/F, OX40+/F, and OX40+/CreCARMA1+/+ OT-II mice and polarized into Th2 cells in vitro according to a standard 6-d protocol (Fig. 7A) as previously described (17). As expected, Western blot analysis revealed reduced CARMA1 protein in Th2-polarized CD4+ T cells from OX40+/CreCARMA1+/F OT-II mice compared to recipient mice that received OX40+/CreCARMA1+/F OT-II cells (Fig. 7B). Th2-polarized CD4+ T cells from OX40+/CreCARMA1+/F, OX40+/CreCARMA1+/F, and OX40+/CreCARMA1+/+ mice were then adoptively transferred into Thy1.2+ wild-type mice that were subsequently challenged with OVA (Fig. 7A). A reduction in the total cell numbers (Fig. 7C), percentage of eosinophils (Fig. 7D), and total eosinophil and lymphocyte cell numbers (Fig. 7E) was observed in BAL fluid recovered from recipient mice that received OX40+/CreCARMA1+/F OT-II cells compared to recipient mice that received OX40+/F OT-II cells or OX40+/CreCARMA1+/+ OT-II cells. Furthermore, there was a reduced percentage and number of Thy1.2+CD69+ (Fig. 7F, 7H) and Thy1.2+CD44+ (Fig. 7G, 7J) CD4+ T cells in the BAL fluid recovered from recipient mice that received OX40+/CreCARMA1+/F OT-II cells compared to recipient mice that received OX40+/CreCARMA1+/F OT-II or OX40+/CreCARMA1+/+ OT-II cells.

Discussion

CARMA1 is an important component of the TCR-mediated signaling cascade that leads to NF-κB activation (1, 11–14) and naive T cell activation (2, 4, 5). In the study presented in this article, we demonstrate that deletion of CARMA1 from T cells after activation reduces their responses to TCR restimulation, including NF-κB activation and expression of activation markers. We then demonstrate that deletion of CARMA1 after T cell activation in vivo leads to attenuated recall responses in a model of allergic airway inflammation due to a failure to reactivate memory T cells. These data characterize a novel role for CARMA1 in mediating CD4+ effector/memory T cell reactivation and functions in response to TCR stimulation.

Memory CD4+ T cells that develop with initial exposure to pathogens and reside in the lung are the first responders to a secondary infection with the same or similar pathogen (41). Although these memory CD4+ T cells serve to counter the detrimental effects of secondary infections, aberrant activation of T cells specific for non-pathogenic Ags can lead to inflammatory diseases such as allergic asthma (42, 43). Allergen-specific MHC class II-restricted CD4+ T cells are thought to be the primary mediators of allergic airway inflammation (23, 24, 44–46). Specifically, CD4+ Th2-type lymphocytes seem to be central to the pathogenesis of allergic asthma, as the levels of these cells and the cytokines they secrete (IL-4, IL-5, and IL-13) are elevated in the airways of human allergic asthma patients (45, 47–50). Thus, understanding the mechanisms that control TCR signaling in effector and...
memory T cells may reveal potential therapeutic targets that can inhibit T cell-driven chronic inflammatory disorders such as asthma.

In our prior work, we demonstrated that CARMA1-deficient mice do not develop allergic airway inflammation in response to OVA sensitization and challenge due to a defect in naive T cell activation (17), suggesting that CARMA1 could be an effective therapeutic target in asthma. However, this does not fully mimic established allergic asthma in humans where the allergen-specific T cells that drive allergic inflammation have already been activated and reside in the lung as memory or effector T cells. Reactivation of effector and memory T cells requires engagement of the TCR by MHC–peptide-Ag complexes, but the activation of these cells requires less stimuli and secondary signals than that for naive T cells (18, 19). Because CARMA1 likely acts as an amplifier of TCR stimulation (15, 16), it may not be necessary for reactivation of effector and memory T cell subsets. Consistent with this, a prior study of Bcl10, a binding partner of CARMA1, demonstrated that it was not necessary for activation of memory-phenotype cells (20). These data suggest that TCR signaling in memory and effector T cells may not require components of the pathway that are necessary for activation in naive T cells. Thus, prior to the study presented in this article, it was unclear if inhibition of CARMA1 in these T cell subsets would affect their activity.

In the murine model used in our experiments, immunization with OVA leads to the priming and expansion of OVA-specific T cells (51). These cells are then poised to react if re-exposed to OVA in organs and lymphoid tissue (52). When mice are challenged with OVA in the airways, the protein is taken up by APCs and presented to these OVA-specific T cells in the lung and draining lymph nodes. The T cells are activated, migrate to the airways, and then help initiate and orchestrate the allergic inflammatory response (53). After the initial acute response, memory T cells are formed and remain in the lung and draining lymph nodes to provide surveillance for further re-exposure to Ag. This mimics the situation in humans with established allergic asthma where re-exposure in the lung to allergens rapidly leads to an exacerbation of allergic airway inflammation. Central to the model is the activation and reactivation of T cells via TCR stimulation.

To study the role of CARMA1 in effector and memory T cells, we used Cre recombinase-based systems to delete CARMA1 in T cells after the initial activation of these cells. For these experiments, we primarily used mice that express Cre recombinase driven by the OX40 promoter. OX40 (CD134, TNFRSF4) is a member of the TNFR superfamily and functions as a secondary costimulatory molecule on T cells. It is expressed 24 to 72 h after T cell activation and is not expressed on naive T cells or most resting memory T cells (37). It is also expressed on a majority of thymic-derived regulatory T cells and a small proportion of memory-phenotype cells in the periphery of naive mice (27, 54). In our study, anti-CD3– and anti-CD28–driven activation of isolated CD4+ T cells from these mice over a 3-d period was effective at decreasing CARMA1 RNA and protein expression, although the deletion was not complete. Upon restimulation, the OX40+/Cre CARMA1F/F T cells had reduced but not absent expression of activation and memory markers along with defective NF-κB signaling compared with that of control cells. This may also explain the attenuated but not complete inhibition of TCR signaling in these cells. Given the heterogeneity of OX40 expression, we confirmed our findings using Rosa26CreERT2/+/CAR-
MA1^{f/f} OT-II cells that contain a Cre recombinase that is active only in the presence of tamoxifen. When these cells were activated with OVA and then restimulated after tamoxifen exposure, there was more complete deletion of CARMA1 and nearly no upregulation of CD69. These results suggest that the partial but incomplete reactivation responses observed in vitro are likely caused by the incomplete deletion of CARMA1 in the OX40–Cre–based system.

We next sought to corroborate our findings in vivo. Using a mouse model of allergic airway inflammation, we found that acute allergic airway responses were not attenuated in OX40^{d/c} CARMA1^{f/f} mice that were immunized and challenged over 3 d with OVA. Given that OX40 is upregulated over 24 to 72 h, it is likely that CARMA1 expression was not significantly decreased in the majority of effector T cells until the end of the challenge phase, and thus it is not surprising that we did not see differences in acute response to OVA challenge. In contrast to the effects seen in the acute model, when OX40^{d/c} CARMA1^{f/f} mice were immunized, challenged, rested to allow memory T cell formation, and then rechallenged with OVA, there was marked attenuation of allergic airway responses compared with the responses in OX40^{+/+} CARMA1^{f/f} mice and OX40^{d/c} CARMA1^{+/+} mice. The attenuated response was manifested by reduced numbers of airway eosinophils and activated CD4^{+} T cells as well as decreased RNA expression of the Th2 cytokines IL-5 and IL-13. There are two potential mechanisms that explain our findings: 1) the CARMA1-
deficient memory cells have impaired reactivation in response to OVA and thus do not induce as intense inflammation as wild-type T cells; or 2) because CARMA1 may influence T cell survival (39, 40), CARMA1-deficient T cells may undergo apoptosis and thus are not available to react to the recall challenge. Using MHC class II tetramer specific for TCRs that recognize OVA peptides, we were able to demonstrate similar numbers of OVA-specific T cells just prior to OVA rechallenge in the lung and draining lymph nodes of OX40<sup>Cre</sup>/CARMA1<sup>F/F</sup> mice as in control mice, thus suggesting that the impaired inflammatory response is due to impaired memory T cell function rather than apoptosis. It is interesting that the deletion of CARMA1 after T cell activation did not affect the size of the memory T cell population, suggesting that optimal TCR signaling (via CARMA1 amplification) is not necessary for memory T cell formation and maintenance. Despite the attenuated response with recall challenge, the allergic inflammation was not completely eliminated. This may be due to the existence of residual memory T cells with intact CARMA1 expression in the OX40–Cre–driven system as seen in vitro.

The recall experiments also did not give us specific information about the effects of CARMA1 deletion on effector T cell function. The experiment using adoptive transfer of Th2-polarized OX40<sup>Cre</sup>/CARMA1<sup>F/F</sup> OT-II cells limited the CARMA1 deletion to effector T cells and clearly demonstrated that these cells were less effective than control cells in inducing allergic inflammation. In addition, the reduction in inflammation was more dramatic than seen in the recall model suggesting that either CARMA1 is more important for effector T cell function than for memory T cell function or that the OX40-driven Cre-mediated deletion of CARMA1 was more efficient in vitro than in vivo.

An important consideration is that in the OX40<sup>Cre</sup>/CARMA1<sup>F/F</sup> mice, CARMA1 is likely deleted from a majority of regulatory T cells given the expression of OX40 on most thymic-derived regulatory T cells (27, 55). This does not result in a decrease in the number of regulatory T cells in these mice at baseline (data not shown). It is unclear whether this will affect regulatory T cell function; however, in vitro experiments with T cells from a mouse line with a mutant form of CARMA1 that results in very low level expression suggested that CARMA1 was not necessary for suppression by regulatory T cells (16). Furthermore, a regulatory T cell functional defect would not explain our findings as this would be expected to lead to more inflammation rather than less. However, it is possible that a defect in regulatory T cell function will partially counter the effects of CARMA1 deficiency in memory and effector T cells by enhancing inflammation. The result from the adoptive transfer of Th2-polarized CARMA1-deficient OT-II cells into naive mice addresses this issue by limiting CARMA1 deficiency to effector T cells.

Finally, it is important to note that CARMA1 likely participates in non-TCR signaling pathways. For example, a recent report demonstrated that the CARMA1/Bcl10/MALT1 complex also binds to the OX40 signalosome and drives TCR-independent NF-kB activation in T cells after Ag clearance (33). It remains to be determined if the participation of CARMA1 in other T cell signaling pathways contributes to effector and memory T cell function.

In conclusion, our results demonstrate that CARMA1 is necessary for optimal effector and memory T cell function in response to Ag challenge. The effect seems to be mediated in part by impaired TCR signaling in these cells. These data have implications for the treatment of chronic T cell-mediated disorders such as allergic asthma where T cells have already been sensitized and are poised to react to Ag re-exposure. Inhibition of CARMA1 or other components of the TCR signaling pathway could limit the intensity of the recall response initiated by memory T cells. In addition, the inhibition of CARMA1 in effector T cells could limit the amount of inflammation generated by these cells in response to Ag.

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Disclosures

The authors have no financial conflicts of interest.

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