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CARMA1 Controls Th2 Cell-Specific Cytokine Expression through Regulating JunB and GATA3 Transcription Factors

Marzenna Blonska,* Donghyun Joo,*† Patrick A. Zweidler-McKay,‡ Qingyu Zhao,*† and Xin Lin*†

The scaffold protein CARMA1 is required for the TCR-induced lymphocyte activation. In this study, we show that CARMA1 also plays an essential role in T cell differentiation. We have found that the adoptive transfer of bone marrow cells expressing constitutively active CARMA1 results in lung inflammation, eosinophilia, and elevated levels of IL-4, IL-5, and IL-10 in recipient mice. In contrast, CARMA1-deficient T cells are defective in TCR-induced expression of Th2 cytokines, suggesting that CARMA1 preferentially directs Th2 differentiation. The impaired cytokine production is due to reduced expression of JunB and GATA3 transcription factors. CARMA1 deficiency affects JunB stability resulting in its enhanced ubiquitination and degradation. In contrast, TCR-dependent induction of GATA3 is suppressed at the transcriptional level. We also found that supplementation with IL-4 partially restored GATA3 expression in CARMA1-deficient CD4+ splenocytes and subsequently production of GATA3-dependent cytokines IL-5 and IL-13. Therefore, our work provides the mechanism by which CARMA1 regulates Th2 cell differentiation. The Journal of Immunology, 2012, 188: 000–000.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; ChIP, chromatin immunoprecipitation; IKK, IκB kinase; ph, phospho; PKC, protein kinase C; PKCθ, protein kinase C θ; WT, wild-type.
regulates JunB and GATA3 expression, which are crucial for the initiation and propagation of Th2 polarization. CARMA1-deficient CD4+ T cells have severely impaired production of IL-4 because of the reduced JunB level and defective GATA3 induction. We also found that CARMA1-deficient CD4+ splenocytes could induce GATA3 in the presence of exogenous IL-4, and the supplementation with IL-4 partially restored production of GATA3-dependent cytokines IL-5 and IL-13.

Materials and Methods

Reagents and plasmids

Abs specific for phospho-(ph)-c-Jun (S63/73), c-Jun, ph-ERK1/2 (Thr202/Tyr204), ph-JB110 (Ser27/76), ph-INK (Thr185/Tyr186), JNK2, and CARMA1 were purchased from Cell Signaling Technology. Abs specific for JunB, JunD, GATA3, T-bet, c-Maf, NFAT, IkBa, Myc, laminB, Ub (P4D1), and actin were obtained from Santa Cruz Biotechnology. Ab that recognizes ubiquitin linked at Lys64 (catalog number 05-1307) and Lys65 (catalog number 05-1308) was purchased from Millipore. JNK inhibitor (SP600125; catalog number S5567) and IKKβ-specific small-molecule inhibitor (TPCA-1; catalog number T1452) were obtained from Sigma-Aldrich. CARMA1 lentivirus expression vectors were constructed by inserting CARMA1 or its mutant into the BamHI and EcoRI sites in pRlV3 vector that contains bicistronic IRES-GFP cassette downstream of the multiple cloning sites. CARMA1del mutant was generated by overlapping PCR using wild-type (WT) CARMA1 cDNA and verified by sequencing.

Cell lines and transfection

Jurkat T cells deficient in CARMA1 were described earlier (17). Jurkat T cells deficient in NEMO were provided by Dr. S.-C. Sun (University of Texas MD Anderson Cancer Center). Jurkat and primary T cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin, and streptomycin. Human embryo kidney 293 and Phoenix cells were maintained in DMEM supplemented with 10% FBS and the antibiotics. Cells were transfected using the calcium phosphate coprecipitation method. Stable Jurkat transfection of Jurkat and CARMA1-deficient cells with CARMA1wt and CARMA1del was established by lentiviral infection. First, human embryo kidney 293T cells were transfected with expression vector (pRlV3) together with packaging plasmids (pHep and pEnv) using the calcium phosphate precipitation method. Jurkat cells were resuspended in viral supernatant in the presence of polybrene and incubated for 6 h. Next, cells were washed and cultured in the complete medium. The efficiency of viral infection was determined by flow cytometry or Western blot analysis.

Mice

CARMA1 null mice were described previously (16). Animals were maintained under pathogen-free conditions in the institutional animal facility. All experiments were performed in compliance with the institutional guidelines and according to the protocol approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center.

Bone marrow chimeric mice

Four days before bone marrow harvesting, the donor mice were injected with 5-fluorouracil (Acros Organics). Bone marrow cells were collected from femurs and tibias and cultured in conditioned media supplemented with murine stem cell factor, IL-3, and IL-6 for 16 h. Cells were infected by spinoculation for two consequent days and injected to sublethally irradiated recipient mice. The infection efficiency (15–20%) was determined by flow cytometry. Peripheral blood samples were collected from tail veins and analyzed by automatic hematological counter (Advia; Bayer). Leukocyte differential was verified by microscopic evaluation of blood smear (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining). At autopsy, organs were fixed with 10% formalin and embedded in paraffin. Sections were stained with H&E (May–Grunwald–Giemsa staining).

Ova-induced allergic lung inflammation

Mice were immunized with two i.p. Ova/alum injections (100 µg; days 0 and 7) and intranasally challenged with 50 µg Ova in PBS (days 25–27). Two days after the last challenge, bronchoalveolar lavage (BAL) was collected by passing 1 ml PBS (three times), and cells recovered from the BAL were counted using an automatic cell counter (Coulter). Ova-specific IgE was measured in mouse serum using an ELISA kit from DS Pharma Biomedical.

CD4+ T cell isolation and activation

CD4+ T cells were isolated from mouse spleens and peripheral lymph nodes using magnetic beads (StemCell Technologies). The cells were purified by negative selection with a panel of biotinylated Abs directed against CD8, CD11b, CD19, CD45RB/B220, CD49b, TER119 (StemCell Technologies), and CD25 (eBioscience). After 3 h of resting, the cells were activated with plate-bound anti-CD3e (2 µg/ml) and anti-CD28 (1 µg/ml) (both from BD Biosciences). For Th2 polarization, 10 µg/ml anti–IFN-γ (XMGL12) and 10 ng/ml murine IL-4 (PeproTech) were added. Because CARMA1-deficient T cells are defective in IL-2 production, every cell culture was supplemented with rIL-2 (30 U/ml).

ELISA

Cytokines were measured in cell culture supernatants and mouse plasma by sandwich ELISA. IL-4, IL-5, IL-10, IL-13, and IFN-γ Ready-SET-GO kits were purchased from eBioscience, and IL-2 OptEIA kit was obtained from BD Biosciences. Results are presented as arithmetic mean of triplicate cultures ± SD or mean + SEM for each group of mice.

Immunoblotting and immunoprecipitation

The cells were lysed in the buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM NaVO4, 1 mM NaF, 1 mM PMSF, 1 mM DTT, and a protease inhibitor mixture (Roche Diagnostics). The cell lysates were subjected to SDS-PAGE and Western blot or immunoprecipitated with JunB Ab (sc-73; Santa Cruz Biotechnology). The immunoprecipitates were washed with lysis buffer four times and eluted with 2× SDS loading buffer. After boiling (4 min), the samples were fractionated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Immobionobots were incubated with specific primary Abs, followed by HRP-conjugated secondary Abs, and were developed by the ECL method according to the manufacturer’s protocol (Pierce).

Proliferation assay

For [3H]thymidine incorporation assay, the cells (3 × 10^6) well in a 96-well plate) were seeded in triplicate in normal growth medium. At the indicated time points, cells were pulsed with 1 µCi [3H]thymidine for 6 h and harvested onto glass fiber filters. The incorporated radioactivity was measured by scintillation counting. Results are presented as the arithmetic mean of triplicate cultures plus SD.

EMSA

Nuclear proteins were extracted from 3 to 4 × 10^6 cells as described before (24). Nuclear extracts (4 µg) were incubated with a [32P]-labeled, double-stranded, AP-1– or Oct-1–specific probe for 15 min at room temperature, fractionated on a 5% polyacrylamide gel, and visualized by autoradiography. AP-1 and Oct-1 probes were purchased from Promega.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChiP) was performed using a ChiP assay kit (Upstate Biotechnology). In brief, after cross-linking with 1% formaldehyde for 10 min at room temperature, cells were lysed in SDS buffer (50 mM Tris-Cl [pH 8.1], 10 mM EDTA, 1% SDS, and protease inhibitors). Lysates were sonicated using Bioruptor and diluted with a ChiP dilution buffer (20 mM Tris-Cl [pH 8.1], 1 mM EDTA, 150 mM NaCl, and 0.3% Triton X-100). Lysates were then precleared with protein G beads and incubated with JunB (sc-73; Santa Cruz Biotechnology) or control rabbit IgG at 4˚C overnight. Protein G–agarose was added to the lysates and incubated for 2 h at 4˚C. Ab/protein/DNA complexes were eluted and reverse cross-linked with 1% formaldehyde cross-linking, and DNA was recovered by a PCR purification kit (Qiagen). DNA fragments were amplified by PCR with specific primers.

Real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen) and reverse transcribed using SuperScriptIII (Invitrogen). Quantitative PCR was performed in triplicates using Power SYBR Green PCR Master Mix (Applied Biosystems). The amounts of transcript were normalized to GAPDH. Melt curves were run to ensure amplification of a single product.

Luciferase assay

Cells were transfected in triplicate with reporter plasmid encoding 5× NF-κB-luc and pEF-Ten-3. Luciferase expression vectors by electroporation. Twenty hours later, cell lysates were prepared, and luciferase activities were measured with Dual-Luciferase assay kits (Promega).
NF-κB activities were determined by normalization of NF-κB–dependent firefly luciferase to Renilla luciferase activity.

Results
Expression of constitutively active CARMA1 increases the level of Jun proteins and promotes T cell proliferation

Our previous studies demonstrate that stimulation of the TCR induces rapid accumulation of c-Jun and JunB transcription factors, and this effect is completely diminished in CARMA1-deficient Jurkat T cells (Fig. 1A) (24). Moreover, prolonged stimulation resulted in persistent accumulation of Jun proteins (Supplemental Fig. 1A). Because CARMA1 is directly phosphorylated by PKC (20, 21), we also stimulated cells in the presence of PKC inhibitors and found that treatment with PKC inhibitors completely suppressed TCR-induced c-Jun and JunB accumulation in Jurkat cells (Fig. 1B). In contrast, an IKK inhibitor that effectively blocks NF-κB activation had no effect on Jun protein level (Supplemental Fig. 1B), indicating that NF-κB does not control signal-dependent c-Jun and JunB induction. Taken together, our results raised possibility that prolonged CARMA1 activation might enhance T cell proliferation through upregulation of c-Jun and JunB transcription factors.

To test this hypothesis, we generated constitutively active CARMA1 by deletion of the linker region between the coiled-coil and PDZ domains (CARMA1del) (Fig. 1C). This construct is active because of destabilization of an inhibitory conformation of CARMA1 (21). Jurkat cells were stably transfected with CARMA1del, WT (CARMA1wt), or empty vector as a control. We found that cells expressing a high amount of CARMA1wt or expressing CARMA1del had elevated levels of c-Jun and JunB (Fig. 1D, upper panel), and c-Jun was phosphorylated at Ser73, which is known to induce its activity (29). Consistent with these data, AP-1 binding activity was enhanced in the presence of CARMA1del (Fig. 1D, lower panel). To test whether constitutive activity of CARMA1 promotes cell proliferation, we used a [3H]thymidine incorporation assay (Fig. 1E). Indeed, the cells expressing CARMA1del proliferated slightly faster than mock cells. Taken together, these results demonstrate that constitutively active CARMA1 results in elevated levels of c-Jun and JunB and promotes cell proliferation in vitro.

Upregulation of CARMA1 in vivo results in Th2 cell-associated inflammation

Previous studies suggest that deregulation of NF-κB and/or AP-1 signaling pathways leads to inappropriate immune responses and contributes to the development of lymphoma (29–32). Because our studies demonstrate that CARMA1 controls NF-κB, c-Jun, and JunB transcription factors in activated lymphocytes (17, 20, 24), we decided to investigate biological consequences of

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**FIGURE 1.** Constitutively active CARMA1 increases the basal level of c-Jun and JunB and promotes T cell proliferation. (A) Jurkat and CARMA1-deficient Jurkat T cells (6 × 10⁶ cells/sample) were stimulated with CD3 plus CD28 Abs (3 and 2 μg/ml, respectively) for different time points. Whole-cell lysates were subjected to SDS-PAGE and analyzed by Western blot using indicated Abs. (B) TCR-induced accumulation of c-Jun and JunB is PKC dependent. Jurkat T cells were pretreated with PKC inhibitors Ro31-822 and GF109203 (100 nM) for 30 min and then stimulated for indicated time points. The cell lysates were subjected to SDS-PAGE and examined by Western blots using indicated Abs. (C) A schematic diagram of CARMA1 constructs. WT CARMA1 (CARMA1wt) is composed of an N-terminal caspase recruitment domain (CARD), a coiled-coil domain (C-C), a PDZ domain, an Src homology (SH3) domain, and a guanylate kinase-like (GUK) domain in the C terminus. Constitutively active CARMA1 (CARMA1del) was generated by deletion of the linker region between the C-C and PDZ domains (436–660 residues). CARMA1-deficient Jurkat T cells were transfected in triplicate with empty vector, WT CARMA1 (CARMA1wt), or constitutive active mutant, CARMA1del, together with reporter plasmids encoding 5' NF-κB-luc and pEF-Renilla-luc. NF-κB activation was determined by normalization of NF-κB–dependent luciferase to Renilla luciferase activity. Expression levels of transfected proteins were determined in cells lysates using CARMA1 Abs. (D) CARMA1-dependent accumulation of c-Jun and JunB. Jurkat T cells were stably transfected with empty vector WT CARMA1 (CARMA1wt) or constitutive active CARMA1del. The cells were lysed and subjected to SDS-PAGE, followed by Western blot analysis using indicated Abs. Nuclear extracts from tested stable cell lines were analyzed by EMSA using 32P-labeled probes containing AP-1 or Oct-1 binding sites. (E) Proliferation assay. The cells (3 × 10⁴/well in a 96-well plate) were seeded in triplicate in normal growth medium. At the indicated time points, cells were pulsed with 1 μCi [3H]thymidine for 6 h and harvested onto glass fiber filters. The incorporated radioactivity was measured by scintillation counting. Results are presented as mean of triplicate cultures ± SD.
CARMA1 upregulation in vivo. We adoptively transferred bone marrow cells transduced with CARMA1del, CARMA1wt, or mock control into sublethally irradiated mice (Supplemental Fig. 2A). Recipient mice were observed for 20 wk, focusing on their weight (Supplemental Fig. 2B) and hematological parameters. RBC parameters and platelet count were comparable during the whole observation period in all groups (Supplemental Fig. 2C). Although we did not find significant differences in total WBC count (Fig. 2A), we observed increased number of eosinophils in peripheral blood from mice expressing CARMA1del (Fig. 2B, 2C). Consistent with this finding, CARMA1del mice had elevated levels of IL-5 (Fig. 2D), which is known to support growth, survival, and effector function of eosinophils. We also detected significantly higher concentrations of IL-2, IL-4, and IL-10 (Fig. 2D) but not IFN-γ or IL-6 (Supplemental Fig. 2D) in all plasma samples. Furthermore, tissue analysis at week 20 post-transplantation revealed lung inflammation (five of six mice) and enlarged peripheral lymph nodes (three of six mice) in CARMA1del mice (Fig. 2E, 2F). In contrast, control mice did not develop any significant pulmonary inflammation. One mouse from CARMA1del group was sacrificed at week 15 because of severe symptoms, such as short breath, hunched posture, itching, rush, and skin lesions (Supplemental Fig. 2E). These results suggest that constitutive activity of CARMA1 might be sufficient to initiate Th cell differentiation to IL-4− and IL-5− producing effectors and Th2-like inflammation in vivo. Because Th cells require GATA3 transcription factor for optimal Th2 differentiation and GATA3 controls transcription of IL-5 (4), we examined whether CARMA1del could induce GATA3 expression in T cells. Indeed, Jurkat cells transfected with CARMA1del expressed much higher levels of GATA3 than control cells (Supplemental Fig. 3A).

**CARMA1 is required for expression of Th2 cytokines and transcription factors in CD4⁺ T cells**

To examine whether CARMA1 is required for Th2 differentiation, we isolated CD4⁺ T cells from the spleens of WT and

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**FIGURE 2.** Upregulation of CARMA1 in bone marrow chimeric mice results in Th2 cell-mediated inflammation. (A) WBC count (WBC) in peripheral blood of control mice (mock; n = 5) and mice expressing WT CARMA1 (CARwt; n = 6) or CARMA1del mutant (CARdel; n = 6) at week 20 post-transplantation. Blood samples were collected from tail veins and analyzed by an automatic hematological counter (Advia; Bayer). (B) Populations of WBC were analyzed by an automatic hematological counter, and leukocyte differentials were verified by microscopic evaluation of blood smear (May–Grunwald–Giemsa staining). (C) Eosinophilia in CARMA1del mouse. Statistical analysis was performed using Prism 5 software (GraphPad), and p value was determined using Kruskal–Wallis test for three experimental groups. (D) Cytokine level in plasma was measured by ELISA. Results are presented as mean plus SEM for each group of mice, and p value was determined using Kruskal–Wallis test. (E) Comparison of lung inflammation in CARMA1wt and CARMA1del mouse. Lung tissues (week 20 post-transplantation) were fixed with 10% formalin and embedded in paraffin. Tissue sections were stained with H&E and examined by light microscopy (×10 objective). The images were taken with an Olympus IX70 inverted FL microscope and edited with DP Controller software. (F) Enlarged inguinal lymph node in CARMA1del mouse.
CARMA1-deficient (CARMA1KO) mice and stimulated the cells with plate-bound anti-CD3 and CD28. Every culture was supplemented with rIL-2 because CARMA1-deficient cells are defective in production of this growth factor, resulting in reduced proliferation and cell survival. TCR-dependent production of cytokines was measured in supernatants collected at 24-, 48-, and 72-h time points. We found that CARMA1-deficient CD4+ splenocytes secreted significantly reduced levels of IL-4, IL-5, and IL-10, whereas IFN-γ production was slightly higher in comparison with WT cells (Fig. 3A). Taken together, these results indicate that CARMA1 is required for the expression of Th2 cytokines.

It is well established that GATA3 directly controls IL-5 and together with JunB enhances IL-4 production (2, 4, 6, 8). JunB is also implicated in IL-10 expression (33). Therefore, we examined TCR-dependent induction of these transcription factors in the absence of CARMA1. We used CD4+ T cells isolated from peripheral lymph nodes or spleens of WT and CARMA1-deficient mice. The cells were stimulated with plate-bound anti-CD3/CD28 plus IL-2 for 2 and 3 d, and whole-cell lysates were analyzed by immunoblotting (Fig. 3B, 3C). We found that CARMA1-deficient cells did not upregulate both GATA3 and JunB, but Th1-specific factor T-bet was expressed at comparable level in examined cells. Consistent with the results from mouse primary T cells, Jurkat T cells were also defective in GATA3 and JunB induction in the absence of CARMA1 (Fig. 3D). These data indicate that CARMA1 is required for induction of Th2 transcription factors, GATA3 and JunB, and controls IL-4, IL-5, and IL-10 production.

**CARMA1 regulates JunB through a posttranslational mechanism**

IL-4 is a key Th2 cytokine that regulates humoral immunity, and excessive expression of IL4 may cause allergic inflammation. IL4 gene regulation occurs on the transcriptional level and is controlled by multiple transcription factors (2). Because the initial amount of IL-4 secreted from T cells is induced by JunB (6), we assessed the recruitment of JunB to the IL-4 promoter in the absence of CARMA1. Indeed, we found that the binding of JunB to this promoter was not observed in CARMA1-deficient cells during the first 16 h of TCR stimulation (Fig. 4A).

To determine how CARMA1 regulates JunB, we first measured the JunB mRNA expression in WT and CARMA1-deficient cells and found that CARMA1 deficiency did not significantly affect the transcription of JunB upon TCR ligation (Fig. 4B). Because previous studies suggest that JunB protein level is controlled by a ubiquitination-dependent mechanism (34), we examined the level of JunB ubiquitination in the absence of CARMA1. We found that JunB was highly ubiquitinated in CARMA1-deficient cells (Fig. 4C, 4D), which correlated with the low expression of JunB protein (Fig. 4C, 4D). We also confirmed that the majority of detected Ub chains was linked through Lys48 (Fig. 4D, middle panel), but Lys63-linked chains were barely detectable (Fig. 4D, lower panel). These results suggest that CARMA1 regulates JunB level through inhibiting an unknown ubiquitin E3 ligase that induces Lys48-linked JunB polyubiquitination and proteasome-mediated degradation. Of note, we have found that c-Jun ubiquitination also depends on CARMA1 (Supplemental Fig. 4), and it is highly probable that the same E3 ligase is involved in this process.

**CARMA1 regulates GATA3 through a transcriptional mechanism**

To confirm that CARMA1 is required for GATA3 expression, we reconstituted CARMA1-deficient Jurkat T cells with wt CARMA1. Indeed, stable re-expression of CARMA1 efficiently rescued the TCR-dependent induction of GATA3 (Fig. 5A). To further deter-
mine the mechanism by which CARMA1 regulates GATA3 expression, we examined GATA3 mRNA level in CARMA1-deficient T cells following TCR stimulation. We found that the GATA3 transcript was gradually augmented during first 18–36 h of stimulation in Jurkat cells but not in the absence of CARMA1 (Fig. 5B). These results indicate that CARMA1 regulates GATA3 on the transcriptional level.

To determine whether TCR-induced NF-κB activation is required for GATA3 expression, we used a Jurkat T cell line deficient in NEMO (also known as IKKγ), a subunit of the IKK complex, which is completely defective in NF-κB activation. We found that signal-dependent induction of GATA3 was comparable in NEMO-deficient Jurkat T cells and control cells re-expressing wt NEMO (Fig. 5C). This result indicates that GATA3 expression does not require the intact NF-κB activation. Therefore, CARMA1-dependent GATA3 expression is regulated through an NF-κB-independent mechanism.

It has been established that GATA3 expression is induced by both TCR signaling and the IL-4R/STAT6 signaling (35), which raises the possibility that stimulation of CARMA1-deficient cells in the presence of IL-4 may rescue signal-dependent GATA3 expression. Indeed, we found that GATA3 expression could be partially rescued in the presence of exogenous IL-4 in CARMA1-deficient CD4⁺ splenocytes (Fig. 5D) and in CARMA1-deficient Jurkat T cells (Supplemental Fig. 3B). Consistent with above result, the supplementation with IL-4 could also partially restore the production of GATA3-dependent cytokines IL-5 and IL-13 in CARMA1-deficient cells (Fig. 5E). Importantly, we detected the comparable level of IL-4R on the surface of WT and CARMA1KO lymphocytes (Supplemental Fig. 3C), suggesting that attenuation of GATA3 expression in CARMA1-deficient cells is only partially attributable to the lack of CARMA1-dependent IL-4 production.

CARMA1-deficient mice are less susceptible for T cell-mediated airway inflammation

To provide the functional significance of CARMA1-dependent activation of JunB and GATA3 in vivo, we immunized WT or CARMA1-deficient mice with OVA and then rechallenged these mice with OVA peptides intranasally. Consistent with a previous study (36), CARMA1-deficient mice were less susceptible to the allergic airway inflammation (Fig. 6A). We observed less inflammatory cells in BAL fluid and a lower level of IL-5 in BAL (Fig. 6B) and a lack of Ova-specific IgE in serum (Fig. 6C) in CARMA1KO mice. On the basis of the results previously mentioned, we propose a model of CARMA1-mediated regulation of Th2 cytokines (Fig. 6D) in which initial IL-4 production is induced by TCR in a CARMA1- and JunB-dependent manner. In turn, IL-4 enhances expression of GATA3 through the IL-4R/STAT6 signaling leading to optimal secretion of IL-4, IL-5, and IL-13. In contrast, CARMA1-dependent activation of NF-κB is required for IL-2 production and expansion of effector cells.

Discussion

CARMA1 is a scaffold molecule expressed in the hematopoietic cells. Its role in AgR-induced NF-κB activation is well established, but its effect on other transcription factors has not been well studied. In this study, we show that stable expression of the constitutively active mutant of CARMA1 results in accumulation of two Jun family members, c-Jun and JunB, and activation of AP-1 in the absence of exogenous IL-4 (Fig. 5A). Consistent with above result, the supplementation with IL-4 could also partially restore the production of GATA3-dependent cytokines IL-5 and IL-13 in CARMA1-deficient cells (Fig. 5D). Importantly, we detected the comparable level of IL-4R on the surface of WT and CARMA1KO lymphocytes (Supplemental Fig. 3C), suggesting that attenuation of GATA3 expression in CARMA1-deficient cells is only partially attributable to the lack of CARMA1-dependent IL-4 production.

FIGURE 4. CARMA1 regulates JunB through a posttranslational mechanism. (A) ChIP assay. CD4⁺ splenocytes were activated with plate-bound anti-CD3/CD28 for 2 or 16 h. JunB–DNA complexes were precipitated with anti-JunB following with real-time PCR. Results are presented as means plus SD of triplicates after normalization to input. (B) Jurkat and CARMA1-deficient Jurkat T cells were stimulated with CD3/CD28 Abs for indicated time points. Total RNA was reverse transcribed, and quantitative PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems). The amounts of the JunB transcript were normalized to GAPDH. The graphs show mean plus SD of triplicates. (C and D) The cells were stimulated with CD3/28 (3 and 2 μg/ml, respectively) for 24 h (C) or PMA plus ionomycin (P/I; 20 and 100 ng, respectively) for 2 h (D). Lysates from these cells were precipitated with JunB Abs. The immunocomplexes were subjected to Western blot analysis using ubiquitin or JunB Abs.
of JunB transcription factor, because a previous study indicates that JunB is selectively expressed in developing Th2 cells, and JunB transgenic mice have upregulated Th2 cytokines (6). In contrast, we have found that CARMA1 deficiency results in a decreased expression of JunB and Th2 cytokines, which is consistent with the finding that loss of JunB in T cells results in

FIGURE 5. CARMA1 regulates GATA3 through a transcriptional mechanism. (A) Jurkat and CARMA1-deficient Jurkat T cells with or without reconstitution of CARMA1 were stimulated with CD3 plus CD28 Abs (3 and 2 µg/ml, respectively) for 48 h. Whole-cell lysates were subjected to Western blot analysis using indicated Abs. (B) Jurkat and CARMA1-deficient Jurkat T cells were stimulated with CD3/CD28 Abs for indicated time points. Total RNA was reverse transcribed, and quantitative PCR was performed with SYBR Green PCR Master Mix. The amounts of GATA3 transcript were normalized to GAPDH. The graphs show mean plus SD of triplicates. (C) NF-κB activation is not required for TCR-induced GATA3 expression. NEMO-deficient Jurkat T cells were stably transfeceted with empty vector (mock) or NEMO (NEMOwt). These cells were stimulated with PMA or PMA plus ionomycin. Whole-cell lysates were subjected to SDS-PAGE and analyzed by Western blot using indicated Abs. (D) CD4+ splenocytes were stimulated with plate-bound anti-CD3e and anti-CD28 (2 and 1 µg/ml, respectively) in the presence of rIL-2 (30 U/ml), anti–IFN-γ (10 ng/ml), and recombinant murine IL-4 (10 ng/ml) for 48 h. Cells were lysed and subjected to Western blot analysis using indicated Abs. (E) Supernatants from cells treated as in (D) were collected, and indicated cytokines were measured by ELISA. Results are shown as means plus SD of triplicate cultures.

FIGURE 6. Ova-induced lung inflammation. (A) Inflammation around airways and blood vessels. Mice were immunized with two i.p. Ova/ alum injections (100 µg; days 0 and 7) and intranasally challenged with 50 µg Ova in PBS (days 25–27). The lungs were fixed in 10% formalin and processed into paraffin. Tissue sections were stained with H&E and examined by light microscopy (×10 objective). The images were taken with an Olympus IX70 inverted FL microscope and edited with DP Controller software. (B) BAL was collected by passing 1 ml PBS (three times). Cells recovered from the BAL were counted using an automatic cell counter (Coulter). IL-5 concentration was measured by ELISA. (C) Serum levels of OVA-specific IgE were determined by ELISA. (D) A model of CARMA1-mediated cytokine production (description in the main text).
a reduced production of IL-4 (37). Interestingly, JunB-deficient mice also exhibit an impaired allergen-induced airway inflammation (37), similarly to CARMA1KO mice (this study and Refs. 36 and 38).

To date, the molecular mechanism by which CARMA1 regulates JunB expression has not been revealed. Our previous study demonstrates that CARMA1 is required for TCR-induced activation of JNK2 (24), the key regulator of c-Jun and JunB stability (26–28). In this work, we show that CARMA1 deficiency results in accelerated K48-linked JunB polyubiquitination, and the similar effect is observed for c-Jun. Therefore, we speculate that CARMA1 may suppress an E3 ligase that is responsible for inducing Jun protein ubiquitination. Indeed, previous study suggests that JunB and c-Jun can be regulated by Itch, an E3 ligase, following TCR stimulation (34). Because CARMA1 appears to suppress an E3 ligase activity even under unstimulated conditions, CARMA1-dependent regulation of JunB ubiquitination is unlikely mediated through suppression of Itch. Therefore, it remains to be determined which E3 ligase is regulated by CARMA1 in T cells.

The role of GATA3 transcription factor in regulation of IL-4, IL-5, and IL-13 is well established (4, 8), but the mechanism that regulates GATA3 expression is not fully defined. It has been demonstrated that gata3 gene expression can be induced by the TCR, IL-4R/STAT6, and IL-2R/STAT5A signaling pathways during Th cell differentiation (35, 39). Our current work reveals that CARMA1 is required for TCR-dependent induction of GATA3, and Jurkat T cells expressing constitutively active CARMA1 have upregulated GATA3. This finding suggests that CARMA1 may transduce signals from the receptor to the gata3 gene-regulating transcriptional complex. Because CARMA1 is required for activation of the NF-κB transcription factor and a previous study demonstrates that mice deficient in the p50 subunit of NF-κB have reduced GATA3 expression upon TCR stimulation (40), it is conceivable that CARMA1-dependent NF-κB activation contributes to the GATA3 expression. However, we have found that GATA3 induction is intact in NEMO-deficient cells, suggesting that TCR-induced NF-κB activation is not required for GATA3 expression. Instead, our data indicate that CARMA1-dependent IL-4 production may contribute to the induction of GATA3, because exogenous IL-4 can partially rescue the defect of GATA3 expression in CARMA1-deficient cells. Nevertheless, we are unable to rule out the possibility that CARMA1 links TCR to another transcription factor that controls gata3 gene.

A previous study demonstrates that CARMA1KO mice do not develop allergic airway inflammation upon immunization and aerosol challenge with chicken OVA (36). The authors provide in vivo evidence that CARMA1-deficient mice do not generate Ag-specific Th2 effecter cells (36). Similar results have been obtained using PKCδ-deficient mice (41), which is consistent with the role of PKCδ as a direct activator of CARMA1 (20, 21). Although the studies mentioned previously link CARMA1 to Th2 differentiation, the work by Jun et al. (42) demonstrates that mice expressing inactive CARMA1 mutant do not respond to Bordetella pertussis immunization, which is associated with Th1 response. In addition, Morinello and Alegre report in their meeting abstract that CARMA1-deficient mice do not develop Th17-driven experimental autoimmune encephalomyelitis (43). Therefore, CARMA1 could be required for expansion of effector cells, and the observed defects could be attributable to reduced T cell proliferation. In our study, we provide in vivo and in vitro evidence that CARMA1 preferentially directs Th2 differentiation by regulating JunB and GATA3 transcription factors. Moreover, we have shown that persistent CARMA1 activity leads to Th2-like inflammation with elevated eosinophils and Th2 cytokines IL-4 and IL-5. To our knowledge, this is the first study that addresses biological consequences of prolonged CARMA1 activation in vivo in T cells.

In summary, our study provides the molecular link between CARMA1 and Th2 cell differentiation. We demonstrate that CARMA1 is a critical signaling molecule in the regulation of JunB and GATA3 transcription factors and subsequent production of Th2 cytokines. Therefore, the selective role of CARMA1 in directing the immune response makes it a very attractive target for immunosuppressive therapy.

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References


