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MicroRNA-148/152 Impair Innate Response and Antigen Presentation of TLR-Triggered Dendritic Cells by Targeting CaMKIIα

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MicroRNAs (miRNAs) are involved in the regulation of immunity, including the lymphocyte development and differentiation, and inflammatory cytokine production. Dendritic cells (DCs) play important roles in linking innate and adaptive immune responses. However, few miRNAs have been found to regulate the innate response and APC function of DCs to date. Calcium/calmodulin-dependent protein kinase II (CaMKII), a major downstream effector of calcium (Ca^{2+}), has been shown to be an important regulator of the maturation and function of DCs. Our previous study showed that CaMKIIα could promote TLR-triggered production of proinflammatory cytokines and type I IFN. Inspired by the observations that *dicer* mutant *Drosophila* display defect in endogenous miRNA generation and higher CaMKII expression, we wondered whether miRNAs can regulate the innate response and APC function of DCs by targeting CaMKIIα. By predicting with software and confirming with functional experiments, we demonstrate that three members of the miRNA (miR)-148 family, miR-148a, miR-148b, and miR-152, are negative regulators of the innate response and Ag-presenting capacity of DCs. miR-148/152 expression was upregulated, whereas CaMKIIα expression was downregulated in DCs on maturation and activation induced by TLR3, TLR4, and TLR9 agonists. We showed that miR-148/152 in turn inhibited the production of cytokines including IL-12, IL-6, TNF-α, and IFN-β upregulation of MHC class II expression and DC-initiated Ag-specific T cell proliferation by targeting CaMKIIα. Therefore, miRNA-148/152 can act as fine-tuner in regulating the innate response and Ag-presenting capacity of DCs, which may contribute to the immune homeostasis and immune regulation. ***The Journal of Immunology*****, 2010, 185: 7244–7251.**

Dendritic cells (DCs) are the most potent APCs of the immune system, with a unique capacity to capture, process, transport, and present Ag to T cells, initiating primary T cell responses and linking innate and adaptive immune responses (1, 2). Immature DCs in the periphery uptake Ags efficiently but express low levels of MHC class II (MHC II) and costimulatory molecules. On inflammatory stimulation or uptake of pathogenic Ags, immature DCs migrate to secondary lymph organs undergoing a maturation process, which involves more secretion of cytokines, the upregulation of surface MHC II and costimulatory molecules (CD80, CD86, and CD40), and increase of the ability to stimulate T cells (1, 2). DCs can recognize microbial components via TLRs. Different TLRs expressed on DCs can discriminate distinct pathogen-associated molecular patterns and initiate signaling pathways to induce DC maturation and activation (3). For example, LPS uses TLR4 or CpG oligodeoxynucleotide (ODN) uses TLR9 to induce DC maturation and activate DCs to secrete cytokines including IL-12, which is critical for the differentiation of T cells into Th1 type effector cells (4). In addition to the initiation of immune response, DCs have been found to be able to downregulate immune response or induce immune tolerance, which depends on the immature or mature status of DCs, or different DC subsets (5). Therefore, the regulatory factors of DC maturation attract much attention. For example, TNFR-associated factor 6 plays a critical role in development, maturation, and activation of DCs (6). The suppressor of cytokine signaling 1 negatively regulates Ag presentation of DCs (7).

As we know, calcium (Ca^{2+}) signaling is involved in many biological processes (8, 9). Calcium/calmodulin-dependent protein kinase II (CaMKII), a major downstream effector of calcium (Ca^{2+}), has been shown to be an important regulator of the maturation and function of DC. Inhibition of CaMKII activation in myeloid DCs resulted in reductions of Ag-induced surface expression of MHC II, secretion of IL-12 and IFN-γ, and MHC II-restricted T cell proliferation (10, 11). Recently, CaMKIIα was demonstrated by us to promote TLR-triggered proinflammatory cytokine and type I IFN production by directly binding and activating TGF-β–activated kinase 1 and IFN regulatory factor 3 in macrophages (12). Because DCs also express CaMKII, TLR signaling in DCs should also be positively regulated by CaMKII. However, the molecular mechanisms underlying the regulation of CaMKII-induced DC maturation and activation remain to be fully understood.

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Abbreviations used in this paper: CaMKIIα, calcium/calmodulin-dependent protein kinase II α; CaMKIIα-Luc Ctrl, control mimics; Ctrli, control inhibitor; DC, dendritic cell; DNMT, DNA methyltransferase; MHC II, MHC class II; miR, miRNA; miR-148a-mt, mutant miR-148a; miR-148ai, miR-148a inhibitor; miR-148bi, miR-148b inhibitor; miR-152i, miR-152 inhibitor; miRNA, microRNA; ODN, oligodeoxynucleotide; poly(I:C), polyinosinic-polycytidylic acid; qPCR, quantitative PCR; QRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; 3′-UTR, 3′-untranslated region.

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Recent studies show that a range of microRNAs (miRNAs) are involved in the regulation of immunity, including the development and differentiation of B and T cells (miRNA [miR]-181a, miR-155) (13, 14), proliferation of monocytes and neutrophils (miR-17-5p, miR-20a, miR-106a, miR-223) (15, 16), and release of inflammatory cytokines and type I IFN (miR-146a, miR-155, miR-21) (17–21). However, up to now, only a few studies have demonstrated that miR-155 could regulate the function of conventional DCs (14, 22, 23). Are there any other miRNAs involved in the regulation of DC maturation and function? Therefore, the primary aim of this study was to identify the candidates of miRNAs that may regulate maturation and function of DCs.

A previous study showed that, in *dicer* mutant *Drosophila,* which displayed defect in endogenous miRNA generation, CaMKII expression is significantly higher (24). Considering that CaMKII is a highly conserved molecule in many species (25), it is possible that CaMKII is regulated by miRNA. Because DCs express CaMKII and CaMKII plays important roles in DC maturation and functions, we wondered whether miRNAs could regulate function in DCs via targeting CaMKII. By analyzing with TargetScan 5.1 (http://www.targetscan.org; Whitehead Institute for Biomedical Research, Cambridge, MA) and http://www.microRNA.org (Computational Biology Center at Memorial Sloan-Kettering Cancer Center, New York, NY), we found that CaMKIIα 3′-untranslated region (3′-UTR) contains some miRNA-binding sites, among which the conserver sites for miRNA families broadly conserved in vertebrates are as follows: miR-148/152, miR-217, and miR-129-5p. In this study, we investigated which one(s) of these miRNAs may be involved in DC maturation and function. We demonstrate that miR-148/152 can inhibit TLR-triggered MHC II expression and functional maturation of DCs by targeting CaMKIIα. Therefore, miRNA-148/152 may act as fine-tuner in regulating the innate response and Ag-presenting capacity of DCs.

Materials and Methods

Mice and reagents

C57BL/6 mice (6–8 wk) were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). OVA323–339–specific TCR-transgenic mice (DO11.10) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in specific pathogen-free conditions. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China). Recombinant mouse GM-CSF and IL-4 were purchased from PeproTech (London, U.K.). LPS (0111:B4) was from Sigma-Aldrich (St. Louis, MO). Polyinosinic-polycytidylic acid [poly(I:C)] was from Calbiochem (La Jolla, CA). CpG ODN was synthesized and repurified as described previously. Endotoxin level in CpG ODN was <0.015 endotoxin units/mg CpG ODN (12). Anti–mIa^b-FITC, anti–mCD80-FITC, anti–mCD86-FITC, anti–mCD4-FITC, and isotype Abs were purchased from eBioscience (San Diego, CA). Abs specific to CaMKIIα were from Santa Cruz (Santa Cruz, CA). Ab specific to β-actin was from Sigma-Aldrich.

Culture and transfection of mouse immature DCs and mature DCs

Bone marrow-derived DCs from C57BL/6 mice were generated as described previously (26). In brief, bone marrow progenitors were cultured in 10 ng/ml GM-CSF and 1 ng/ml IL-4. Nonadherent cells were gently washed out on day 4 of culture; the remaining loosely adherent clusters were used on day 6 as immature DCs. Immature DCs were stimulated with 100 ng/ml LPS for 24 h to generate mature DCs. DCs were positively selected using CD11c magnetic microbeads (Miltenyi Biotec, Auburn, CA). For phenotype analysis and cytokine detection, immature DCs were transfected with miRNA mimics or inhibitor using INTERFERin (Polyplus-transfection, Illkirch, France) according to the standard protocol.

Real-time quantitative PCR detection

Total RNA, containing miRNA, was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentrations were determined with a NanoDrop instrument (NanoDrop Technologies, Wilmington, DE). For quantitative real-time PCR (QRT-PCR) analyses of miRNA using SYBR RT- PCR kit (Takara, Dalian, China), RT primers with stem-loop structure were as follows: miR-148a/b: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAAAG-3′; miR-152: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCAAG-3′; miR-155: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCT-3′; miR-217: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCAGT-3′. The forward quantitative PCR (qPCR) primers used were as follows: miR-148a, 5′-ATGCTCAGTGCACTACAGAA-3′; miR-148b, 5′-TGCTCAGTGCATCACAGAA-3′; miR-152, 5′-CTAATGTCAGTGCATGACAGAA-3′; miR-155: 5′-GGCTTAATGCTAATTGTGAT-3′; miR-217: 5′-ATCGCTACTGCATCAGGAACTG-3′. The reverse

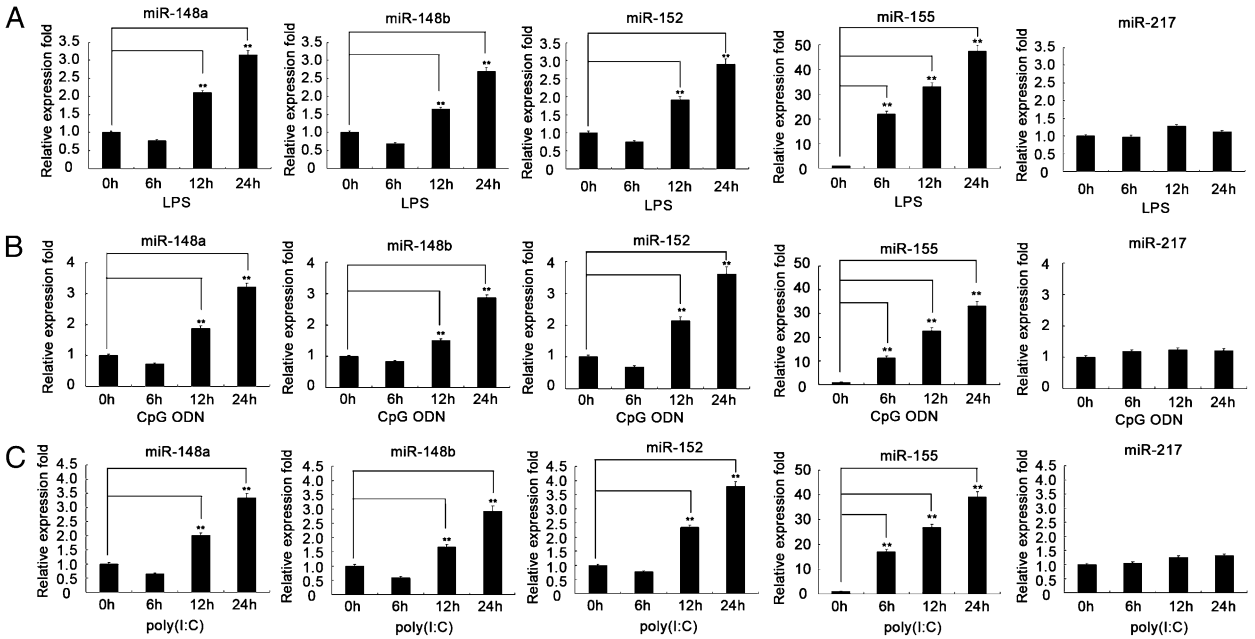

FIGURE 1. Upregulation of miR-148/152 expression in DCs by TLR agonists. *A,* Immature DCs were stimulated with 100 ng/ml LPS (*A*), 0.5 μM CpG ODN (*B*), or 10 μg/ml poly(I:C) (*C*), respectively, for the indicated times. The expressions of miR-148a, miR-148b, miR-152, miR-155, and miR-217 were detected by qPCR and normalized to the expression of U6 in each sample. Data are shown as mean ± SD of three independent experiments. $^{**}p < 0.01$.

qPCR primer 5′-GTGCAGGGTCCGAGGT-3′ was used to the above five miRNA. Similarly, U6 small nuclear RNA was quantified using its reverse primer for RT reaction and its forward and reverse primers for qPCR, which was 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse). QRT-PCR analyses for *miRNAs* were also confirmed using TaqMan miRNA assays from Applied Biosystems (Carlsbad, CA). QRT-PCR analyses were performed using Roche LightCycler (Indianapolis, IN). The relative expression level of miRNAs was normalized to that of internal control U6 by using $2^{-\Delta\Delta Ct}$ cycle threshold method. For mouse β-actin mRNA analysis, the primers were described previously (12). For mouse CaMKIIα, the primers were 5′-ACCTGCACCCGATTCACAG-3′ (forward) and 5′-TGGCAGCATACTCCTGACCA-3′ (reverse). Data were normalized by the level of β-actin expression in each sample as described earlier.

miRNA mimics and inhibitors

miR-148a, miR-148b, and miR152 mimics and control mimics (Ctrl; dsRNA oligonucleotide with chemical modifications) are from GenePharma (Shanghai, China). Ctrl is designed to serve as a negative control for experiments involving miRNA mimics. Its sequence (sense: 5′-UCACAACCUCCUAGAAAGAGUA-3′) is based on *Caenorhabditis elegans* miRNA, which has been confirmed to have minimal sequence identity with miRNAs in human, mouse, and rat. A mutant miR-148a (miR-148a-mt; GenePharma) served as another Ctrl, containing five point substitutions in the "seed region" of miR-148a, which does not target any gene by predicting with TargetScan 5.1. The miRNA inhibitors, as described in the manufacturer's instructions, are hairpin RNA oligonucleotides with secondary structure and chemical modifications designed to specifically bind to and inhibit the function of endogenous miR-148/152, which were from Dharmacon (Lafayette, CO). The control inhibitor served as negative control (Dharmacon).

Detection of IL-6, TNF-α, IL-12, and IFN-β

IL-6, TNF-α, IL-12, and IFN-β levels in the supernatants were measured with ELISA Kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

Flow cytometry

For cell surface marker analysis, cells were incubated for 15 min at 4°C with fluorescein-conjugated Abs in labeling solution. Fluorescein-conjugated, isotype-matched, irrelevant Abs were set to establish background fluorescein. Flow cytometry was conducted on FACS LSRII, and the data were analyzed with FACSDiva (BD Biosciences, San Diego, CA).

Assay for Ag-specific CD4$^+$ T cell proliferation

Splenic CD4$^+$ T cells from DO11.10 OVA323–339–specific TCR transgenic × C57BL/6 F1 hybrid mice were positively selected by MACS (Miltenyi Biotec) for use as Ag-specific responders, then cocultured with DCs treated as indicated in the presence of OVA323–339 peptides (lymphocytic choriomeningitis virus–NP309–328 was used as an Ia^b-restricted peptide control) at a ratio of 1:10 (DC/T cells) in round-bottom 96-well plates (1×10^5 T cells/200 μl/well) for 5 d. Cells were double-stained with anti–CD4-FITC and 7-aminoactinomycin D, resuspended in exactly 300 μL PBS, and acquired for 56 s using a flow cytometer. The number of CD4$^+$ 7-aminoactinomycin D$^-$ live cells was calculated to represent the altitude of Ag-specific CD4$^+$ T cell proliferation (27).

3′-UTR luciferase reporter assays

The wild type and mutant CaMKIIα 3′-UTR luciferase reporter vectors were constructed by amplifying the mouse CaMKIIα mRNA 3′-UTR and cloned into XbaI site of pGL3-promoter vector (Promega, Madison, WI). HEK293 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured according to the standard protocol. HEK293 cells were cotransfected with 80 ng luciferase reporter plasmid, 40 ng thymidine kinase promoter-*Renilla* luciferase reporter plasmid, and indicated miRNA mimics or inhibitor (final concentration, 20 nM) using JetSI-ENDO transfection reagents (Polyplus-transfection), according to the manufacturer's instructions. After 24 h, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Data were normalized for transfection efficiency by dividing Firefly luciferase activity with that of *Renilla* luciferase, as described previously (28).

Immunoblot

Cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with protease inhibitor mixture (Calbiochem, San Diego, CA). Protein concentrations of the extracts were measured with bicinchoninic acid assay (Pierce Chemical, Rockford, IL) and equalized with the extraction reagent. Equal amount of the extracts were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (12).

RNA interference

The sequences of small interfering RNA (siRNA) targeting CaMKIIα were 5′-CACCACCATTGAGGACGAA-3′. The control small RNA sequence was 5′-AATCAGTCACGTTAATGGTCG-3′. siRNA duplexes were transfected into mouse DCs using INTERFERin (Polyplus-transfection) according to the standard protocol.

Statistical analysis

Statistical significance was determined by Student *t* test, with *p* value <0.05 considered to be statistically significant.

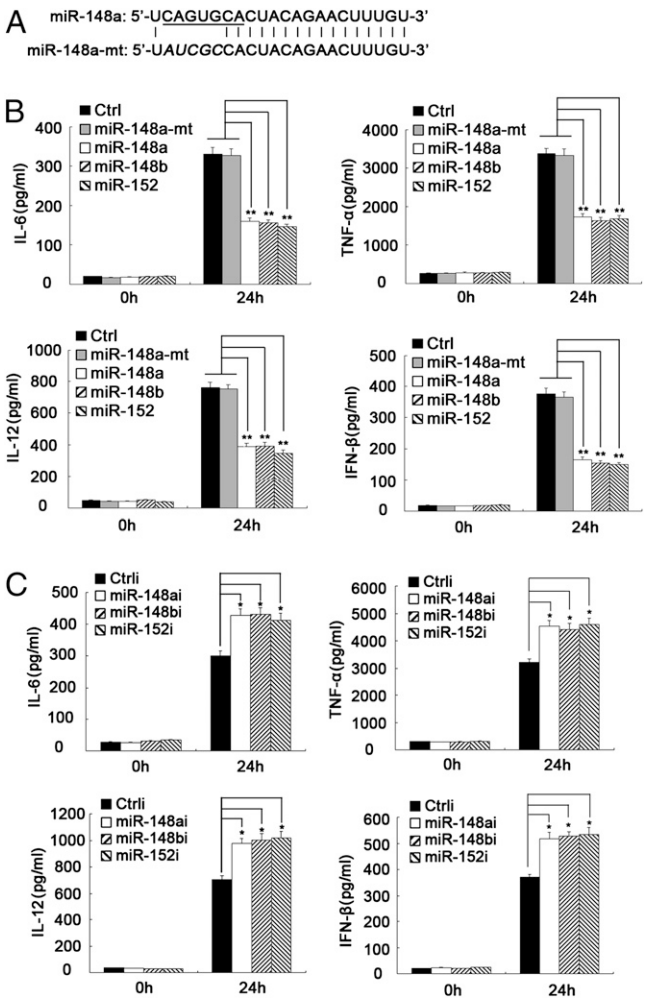

FIGURE 2. miR-148/152 negatively regulate LPS-induced cytokine production in DCs. *A*, Shown is the alignment of miR-148a and miR-148a-mt. Five point substitutions (italics) were introduced into "seed region" (underscore) of miR-148a. *B*, Mouse immature DCs (8×10^5) were transfected with Ctrl, miR-148a-mt mimics, and mimics of miR-148a, miR-148b, or miR-152 at a final concentration of 15 nM. After 48 h, DCs were stimulated with 100 ng/ml LPS for 24 h. IL-6, TNF-α, IL-12, and IFN-β in supernatants were measured by ELISA. *C,* Mouse immature DCs (8×10^5) were transfected with control inhibitor (Ctrli), inhibitor of miR-148a (miR-148ai), miR-148b (miR-148bi), or miR-152 (miR-152i) at a final concentration of 15 nM, and then treated as described in *B*. Cytokines in supernatants were measured by ELISA. Data are shown as mean ± SD of three independent experiments. $*p < 0.05$; $**p < 0.01$.

Results

Upregulation of miR-148/152 expression in DCs stimulated by TLR agonists

First, we analyzed the miRNA expression kinetics in DCs stimulated with TLR agonists using qPCR analysis. As shown in the expression of miR-148a, miR-148b and miR-152, which belong to miR-148 family, significantly increased in DCs stimulated with LPS for 6–24 h, although the expression of miR-217 and miR-129-5p remained almost unchanged (Fig. 1*A* and data not shown). We also detected the expression change of miR-146a and miR-155, and found that their expressions were increased to greater levels than that of miR-148/152 (Fig. 1*A* and data not shown), which is consist with previous studies in LPS-stimulated macrophages (22, 23). CpG ODN and poly(I:C) also markedly induced the upregulation of miR-148/152 expression (Fig. 1*B*, 1*C*). These data demonstrate that miR-148/152 are constitutively expressed in mouse DCs, and their expression is upregulated together with DC maturation and activation induced by TLR agonists, suggesting that miR-148/152, but not miR-217 and miR-129-5p, may be involved in the regulation of DC maturation and function.

miR-148/152 negatively regulate TLR-triggered cytokine production in DCs

To investigate the role of miR-148/152 in regulation of innate immune response of DCs, we examined the effect of overexpression or inhibition of miR-148/152 on the TLR-triggered production of cytokines by DCs. To exclude the off-target effects of miRNA mimics, we generated an miR-148a-mt as another control, containing five point substitutions in the "seed region" of miR-148a (Fig. 2*A*). miR-148a-mt did not target any gene by predicting with TargetScan 5.1 (Fig. 2*A*). As shown in Fig. 2*B*, overexpression of miR-148a, miR-148b, or miR-152 mimics significantly decreased the production of IL-6, TNF-α, IL-12, and IFN-β in LPS-stimulated DCs, as compared with that in DCs transfected with irrelevant sequence Ctrl and miR-148a-mt mimics. In addition, miR-

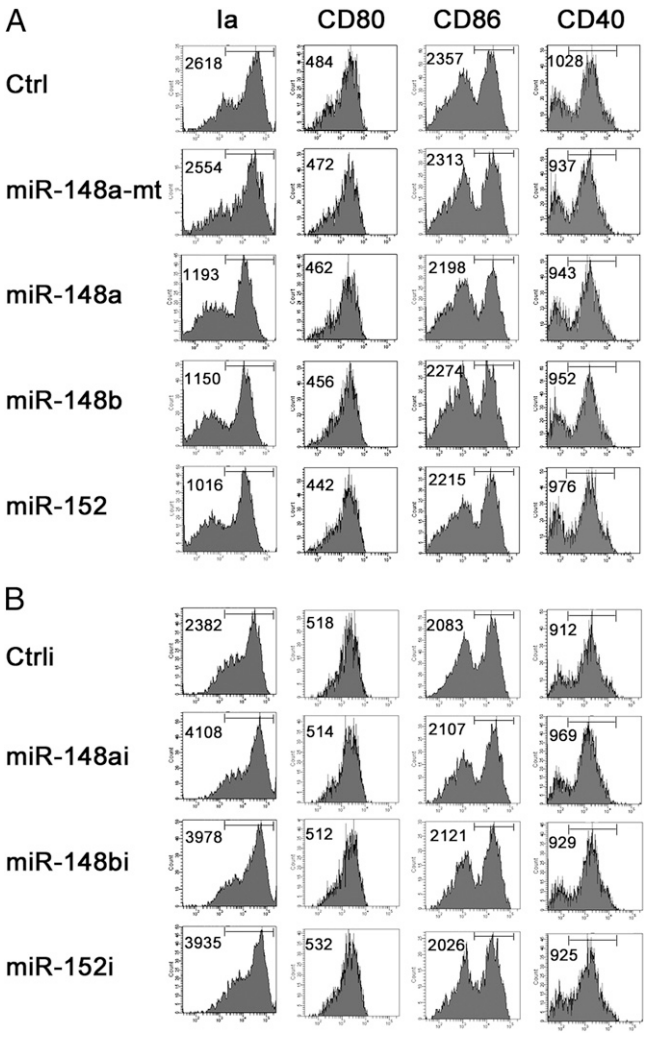

FIGURE 3. Suppression of LPS-upregulated MHC II expression on DCs by miR-148/152. *A,* Mouse immature DCs were transfected with different miRNA mimics: Ctrl and miR-148a-mt mimics (*A*) or different miRNA inhibitor and control inhibitor (*B*) at a final concentration of 15 nM, respectively. After 48 h, DCs were stimulated with 100 ng/ml LPS for 24 h, then stained with specific Ab against MHC II (Ia), CD80, CD86, and CD40, and analyzed by flow cytometry. Numbers in histograms indicate the geometric mean fluorescence of DC in each group. Similar results were obtained in three independent experiments. Ctrli, control inhibitor; miR-148ai, miR-148a inhibitor; miR-148bi, miR-148b inhibitor; miR-152i, miR-152 inhibitor.

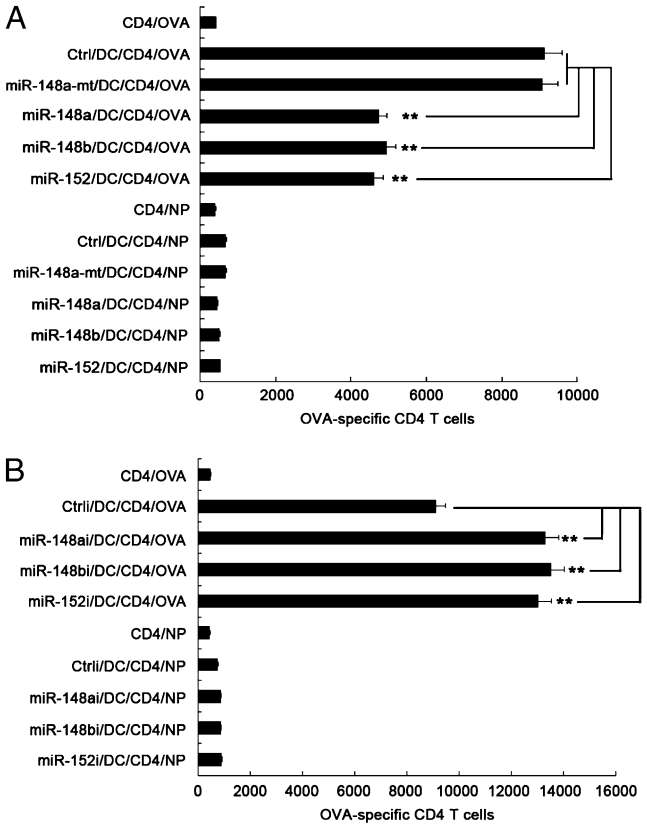

FIGURE 4. miR-148/152 inhibit DC-initiated Ag-specific $CD4^+$ T cell proliferation. Mouse immature DCs were transfected with different miRNA mimics: Ctrl and miR-148a-mt mimics (*A*) or different miRNA inhibitor and Ctrli (*B*) at a final concentration of 15 nM as indicated. After 48 h, DCs were stimulated with 100 ng/ml LPS for 24 h. Purified DO11.10 $CD4^+$ T cells were cocultured with DCs treated in the presence of OVA323–339 at a ratio of 1:10 (DC/T cells); lymphocytic choriomeningitis virus-NP309–328 (NP) was used as an Ia^b-restricted peptide control. After 5 d, cells were collected and double-stained with anti–CD4-FITC and 7-aminoactinomycin D and counted by FACS. Data are shown as mean ± SEM of three independent experiments. $^{**}p < 0.01$. CTrli, control inhibitor; miR-148ai, miR-148a inhibitor; miR-148bi, miR-148b inhibitor; miR-152i, miR-152 inhibitor.

148a-mt overexpression failed to decrease the production of IL-6, TNF-α, IL-12, and IFN-β in LPS-stimulated DCs (Fig. 2*B*). Because miR-148a, miR-148b, and miR-152 have the same "seed region," these data indicate that the effects of miR-148/152 on the cytokine production in DC are specific. Accordingly, inhibition of miR-148a, miR-148b, or miR-152 with miRNA inhibitor increased LPS-induced IL-6, TNF-α, IL-12, and IFN-β production (Fig. 2*C*). Overexpression or inhibition of miR-148a, miR-148b, or miR-152 also inhibited or increased the production of the above cytokines in CpG ODN or poly(I:C)-stimulated DCs, respectively (data not shown). In addition, the transfection of miR-148/152 mimics or inhibitors did not induce significant cell death of DCs and did not affect cell cycle as compared with the transfection of Ctrl, mutated miR-148a mimics, or control inhibitors (data not shown). Together, these data indicate that miR-148/152 may act as negative regulators of TLR-triggered innate response of DCs by suppressing production of proinflammatory cytokines such as IL-6, TNF-α, IL-12, and IFN-β.

miR-148/152 inhibit LPS-induced upregulation of MHC II expression on DCs

To explore the role of miR-148/152 in DC maturation, we examined the effect of overexpression or inhibition of miR-148/152 on DC phenotype. Immature DCs were transfected with miRNA mimics or inhibitor. After 48 h, DCs were stimulated with 100 ng/ml LPS for 24 h; then the phenotype was analyzed by flow cytometry. As shown in Fig. 3, overexpression of miR-148a, miR-148b, or miR-152 mimics significantly reduced LPS-induced surface expression of MHC II on DCs, whereas they had no significant effects on the expression of costimulatory molecules CD80, CD86, and CD40. Consistently, inhibition of miR-148a, miR-148b, or miR-152 increased LPS-induced surface expression of MHC II on DCs (Fig. 3). These data suggest that miR-148/152 inhibit LPS-induced upregulation of MHC II expression on DCs. Considering that production of cytokines, especially including Th1 cytokine IL-12, are integral parts of the maturation of DCs, these results demonstrate that miR-148/152 act as negative

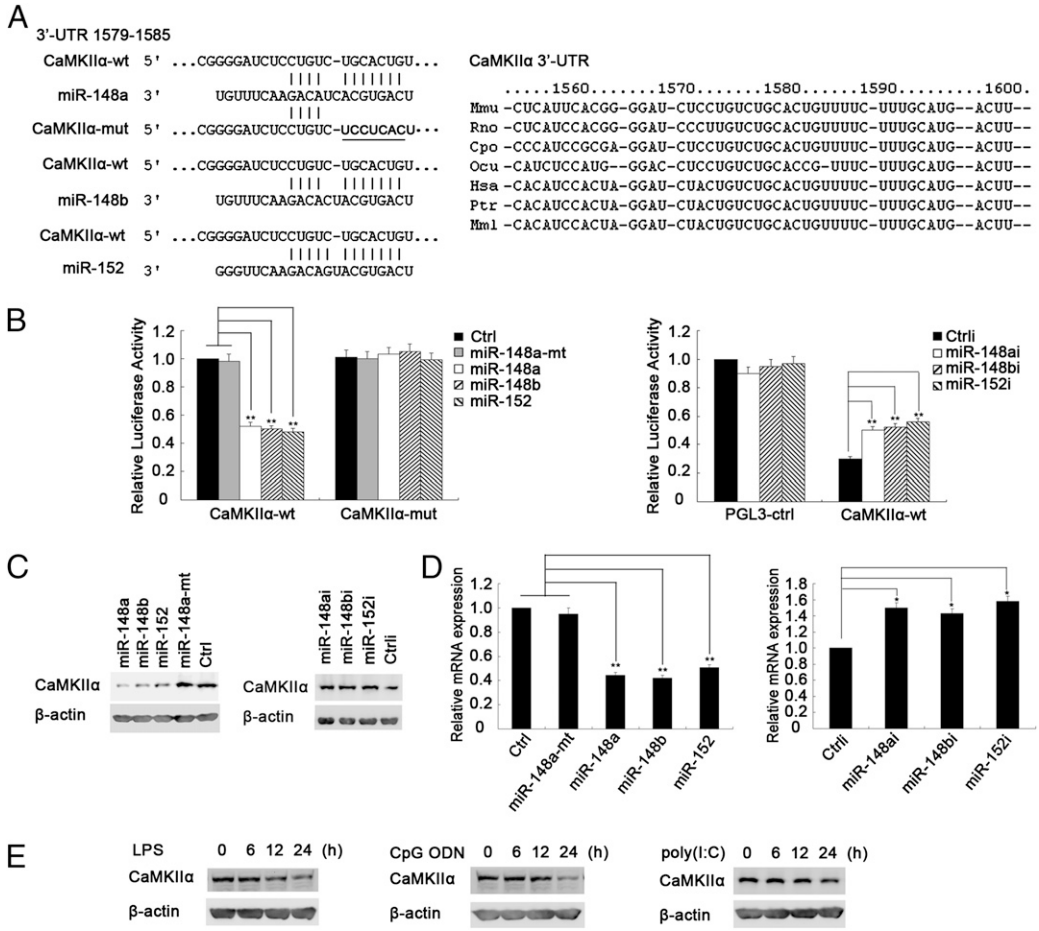

FIGURE 5. miR-148/152 target mouse CaMKIIα. *A*, Mouse CaMKIIα may be a molecular target of miR-148/152. Shown is the alignment of miR-148/152 and its target sites in 3′-UTR of CaMKIIα (*left*) and the sequence alignment of partial CaMKIIα 3′-UTR form different species (*right*). Mutations were generated on "seed region" of the potential binding site (underscore). *B*, HEK293 cells (1×10^4) were cotransfected with 80 ng wild type or mutant pGL3-CaMKIIα 3′-UTR firefly luciferase reporter plasmids, 40 ng thymidine kinase promoter *Renilla* luciferase reporter (pTK-RL) plasmids, together with Ctrl, miR-148a-mt mimics or miR-148/152 mimics (*left*), or Ctrli or miR-148/152 inhibitor (*right*) (final concentration, 20 nM) as indicated. After 24 h, firefly luciferase activity was measured and normalized by *Renilla* luciferase activity. *C* and *D*, Mouse immature DCs (1.6×10^6) were transfected with different miRNA mimics, Ctrl and miR-148a-mt mimics or different miRNA inhibitor and Ctrli at a final concentration of 15 nM as indicated. After 72 h, CaMKIIα protein was detected by immunoblot. β-Actin served as a loading control (*C*). CaMKIIα mRNA was measured by qPCR (*D*). *E*, Immature DCs were stimulated with 100 ng/ml LPS, 0.5 μM CpG ODN, or 10 μg/ml poly(I:C), respectively, for the indicated times. CaMKIIα protein was detected by immunoblot. β-Actin served as a loading control. Data are shown as mean ± SEM of three independent experiments (*B*), data are shown as mean ± SD of three independent experiments (*D*), or similar results were obtained in three independent experiments (*C*, *E*).*$p < 0.05$; **$p < 0.01$. CTrli, control inhibitor; Cpo, guinea pig; Has, human; Mml, rhesus; Mmu, mose; Ocu, rabbit; Ptr, chimpanzee; Rno, rat.

regulators of TLR-triggered functional maturation of DCs, which are attributed to the reduced surface expression of MHC II and the decreased production of cytokines.

miR-148/152 inhibit DC-initiated Ag-specific CD4$^+$ T cell proliferation

The central physiologic function of DC is to present Ags to T cells. Because miR-148/152 impair DC maturation, we further investigated whether miR-148/152 could regulate Ag presentation by DCs. The effect of miR-148/152 on DC-initiated Ag-specific CD4$^+$ T cell proliferation was observed. OVA323–339–specific TCR transgenic CD4$^+$ T cells purified from DO11.10 × C57BL/6 F1 mice were used as responders to miR-148/152–overexpressed DCs, which were loaded with OVA323–339 or lymphocytic choriomeningitis virus–NP309–328 (Ia^b-restricted peptide Ag control) peptides. After 5 d, viable CD4$^+$ T cells present in T cell/DC cultures were counted by flow cytometry. As shown in Fig. 4*A*, overexpression of miR-148a, miR-148b, or miR-152 mimics significantly inhibited mature DC-induced Ag-specific CD4$^+$ T cell proliferation. Accordingly, inhibition of miR-148a, miR-148b, or miR-152 promoted mature DC-induced Ag-specific CD4$^+$ T cell proliferation (Fig. 4*B*). These data provide further evidence that miR-148/152 function as negative regulators of DC maturation and APC function.

miR-148/152 directly target mouse CaMKIIα

Next, we further investigated whether CaMKIIα was a direct target of miR-148/152. CaMKIIα appeared to harbor a common standard target sequence for miR-148a, miR-148b, and miR152 at nucleotides 1579–1585 of its 3′-UTRs (Fig. 5*A*). Sequence analysis indicated that this target sequence was conserved across different species including human, chimpanzee, rhesus, mouse, rat, and rabbit genomes (Fig. 5*A*), suggesting conservational functions of this sequence. To obtain direct evidence that CaMKIIα 3′-UTR is a target of miR-148/152, we integrated a fragment of the CaMKIIα 3′-UTR containing the target sequence, or the fragment whose target sites were mutated, into a luciferase reporter vector. By cotransfection of the reporter plasmids and miR-148/152 mimics or inhibitors in HEK293 cells, we found that miR-148/152 mimics markedly decreased the luciferase activity of wild type CaMKIIα luciferase (CaMKIIα-Luc) but had no effect on mutant CaMKIIα-Luc (Fig. 5*B*). Consistently, miR-148/152 inhibitors increased the luciferase activity of wild type CaMKIIα-Luc (Fig. 5*B*). Furthermore, transfection of miR-148/152 mimics decreased CaMKIIα expression in DCs at both the protein and mRNA levels, whereas miR-148/152 inhibitors increased CaMKIIα expression (Fig. 5*C*, 5*D*), suggesting that CaMKIIα expression could be inhibited by miR-148/152 via both translational inhibition and mRNA degradation. We further observed the protein expression kinetics of CaMKIIα during TLR ligand-induced DC maturation. As shown in Fig. 5*E*, the expression of CaMKIIα significantly decreased in DCs stimulated with LPS, CpG ODN, or poly(I:C) for 6–24 h, suggesting that the negative relation exists between miR148/152 and CaMKIIα expression during TLR ligand-induced DC maturation. Together, these results demonstrate that miR-148/152 selectively target CaMKIIα and downregulate CaMKIIα expression.

CaMKIIα knockdown inhibits LPS-induced MHC II expression and cytokine production in DCs

To further corroborate the results that miR-148/152 target CaMKIIα to negatively regulate DC maturation and function, we observed the effects of knockdown of CaMKIIα on the LPS-induced MHC II expression and cytokine production in DCs. siRNA specific to mouse CaMKIIα significantly downregulated the expression of CaMKIIα (~80%; Fig. 6*A*). CaMKIIα knockdown inhibited LPS-induced surface expression of MHC II (Fig. 6*B*). CaMKIIα knockdown also significantly decreased LPS-induced IL-6 and IL-12 production in DCs (Fig. 6*C*). These data further confirm that the negative regulation of DC maturation and function is mediated by downregulation of CaMKIIα by miR-148/152.

Discussion

miRNAs are short (18–24 nt), endogenous, noncoding RNA and are generated by an RNase III-type enzyme from an endogenous transcript that contains a local hairpin structure. miRNAs bind with imperfect complementarity to 3′-UTRs of target mRNAs, causing translational repression of the target gene or degradation of the target mRNA (29). miRNAs are involved in a range of processes that includes development, proliferation, differentiation, apoptosis, and tumorigenesis (30, 31). Up to now, more than 700 miRNAs have been identified in mammals, whereas biological functions of the majority remain unknown. Emerging evidence suggests that miRNAs play important roles in the regulation of immunological functions including innate immune responses of macrophages; development, differentiation, and function of T and B cells; as well as autoimmune diseases (32–34). Given the central and essential role of DCs in the immune system, the investigation of regulators involved in regulation of DCs is valuable. However, up to now, few miRNAs were reported to regulate DC function. It was reported that the expression level of MHC II and costimulatory molecules of bone marrow-derived DCs in miR-155–deficient mice was normal, whereas the stimulatory ability of such DCs for T cells proliferation was impaired, which suggest that miR-155 may be required for the acquired immune function but not maturation of DCs (14), but the underlying mechanism and the target remain unclear. Another study identified miR-155 as a feedback negative regulator of TLR4/IL-1 signaling by targeting TAB2 in

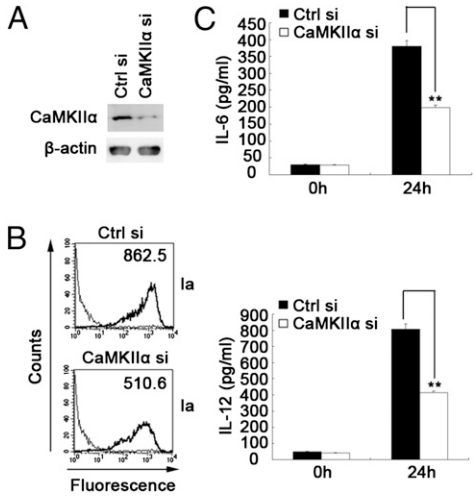

FIGURE 6. Knockdown of CaMKIIα inhibits LPS-induced MHC II upregulation and cytokine production in DCs. *A*, Mouse immature DCs (8×10^5) were transfected with control siRNA (Ctrl si) or CaMKIIα siRNA (CaMKIIα si) at a final concentration of 15 nM. After 72 h, CaMKIIα protein was detected by immunoblot. β-Actin served as a loading control. *B*, Mouse immature DCs were transfected as described in *A*. After 48 h, DCs were stimulated with 100 ng/ml LPS for 24 h, then stained with specific Abs against MHC II (Ia) and analyzed by flow cytometry. *C*, Mouse immature DCs were transfected as described in *A*. After 48 h, DCs were stimulated with 100 ng/ml LPS for 24 h. IL-6 and IL-12 in supernatants were measured by ELISA. Similar results were obtained in three independent experiments (*A, B*), or data are shown as mean ± SD of three independent experiments. $^{**}p < 0.01$.

human monocyte-derived DCs (22). miR-155 silence with anti–miR-155 locked nucleic acid increased the production of inflammatory cytokines including IL-1β, IL-6, TNF-α, and IL-23 in LPS-stimulated DCs, but had no effect on IL-12 production and DC maturation (22). miRNA-155 was also found to modulate the pathogen binding ability of DCs by downregulation of DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) (23). The above three reports indicate that miR-155 may differently regulate the acquired and innate function of DCs, but not DC maturation, by targeting different molecules. miR-146a was found to be constitutively expressed by Langerhans cells and interstitial DCs, and could desensitize cells to TLR2-dependent activation, without affecting phenotypic DC maturation (35). In this study, we proved that miR-148/152 could inhibit LPS-induced upregulation of MHC II expression on DCs. LPS, CpG ODN, or poly(I:C)-induced DC production of cytokines, especially including IL-12, which could promote T cell proliferation, was inhibited by miR-148/152. More importantly, miR-148/152 impaired DC-initiated Ag-specific T cell proliferation. Together with miR-148/152 being upregulated in TLR-triggered DCs, our data provide evidence that miR-148/152 act as negative regulators for the innate response and APC function of DCs, providing another manner to fine-tune the immune response.

DCs are powerful sensors of foreign pathogens and provide the first line of defense against infection. Interaction of DCs with TLR ligands leads to a cascade of proinflammatory cytokines (3). Although the inflammatory response is critical for the control of pathogenic infections, excessive production of proinflammatory cytokines is harmful to the host (36). In addition, it is believed that DCs, which present tissue- or organ-specific peptides derived from necrotic cells under inflammatory conditions, can prime autoreactive T cells, resulting in autoimmune diseases (37, 38). Therefore, mammals have evolved complex genetic programmers that regulate the development and function of immune cells, and enable the immune system to mount specific responses against invading foreign pathogens whereas maintaining tolerance, among which miRNA is a breakthrough. Previous studies showed that miR-146a, miR-155, miR-125b, and miR-21 inhibited TLR-triggered production of inflammatory cytokines (17–21). In this study, miR-148/152 have been confirmed as negative regulators to inhibit the production of inflammatory cytokines and type I IFN in TLR-activated DCs, indicating that miR-148/152, together with miR-146a, miR-155, miR-125b, and miR-21, negatively regulate the activation of immune cells and prevent the overactivation of immune response. The abnormal expression profile of miRNAs in systemic lupus erythematosus and rheumatoid arthritis imply that miRNAs play important regulatory roles in autoimmune diseases (39, 40). miR-148/152 are confirmed to negatively regulate the maturation, activation, and function of DCs, which also suggest that miR-148/152 may be involved in the pathogenesis of autoimmune disorders, and the possibility needs to be investigated in the future.

miRNAs have been thought to target multiple mRNAs, named "targetome," to regulate gene expression. A single miRNA might tune protein expression from thousands of genes by direct or indirect effects (41). A previous study showed that miR-148 could target human DNA methyltransferase (DNMT) 3b protein coding region to repress its expression (42). Other studies also showed that miR-148a and miR-152 play multiple roles as tumor suppressors by targeting mitogen- and stress-activated protein kinase 1 (43) and DNMT-1 (44). Although the roles of DNMT-3b, mitogen- and stress-activated protein kinase 1, and DNMT-1 in immune response remain unclear, whether miR-148/152 regulate DC function through targeting these three genes needs further investigation. *CaMKIIα* is a conserved gene that possesses the common binding site of miR-148/152 in its 3′-UTR. We confirm that miR-148/152 directly target CaMKIIα, leading to an overall diminution of immune responses. Because miR-148/152 have many other potential targets predicted by software, whether miR-148/152 broadly regulate DC function through minor effects on other multiple targets requires further study.

In conclusion, our results demonstrate that miR-148/152 expression is upregulated in DCs on maturation and activation induced by TLR agonists, which, in turn, inhibit the upregulation of MHC II expression, cytokine production, and Ag presentation of DCs by targeting CaMKIIα. miR-148/152 are negative regulators for the innate response and APC function of DCs.

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Disclosures

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