Aryl hydrocarbon receptor-mediated induction of the microRNA-132/212 cluster promotes interleukin-17–producing T-helper cell differentiation

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Aryl hydrocarbon receptor (AHR) plays critical roles in various autoimmune diseases such as multiple sclerosis by controlling interleukin-17 (IL-17)–producing T-helper (T_H17) and regulatory T cells. Although various transcription factors and cytokines have been identified as key participants in T_H17 generation, the role of microRNAs in this process is poorly understood. In this study, we found that expression of the microRNA (miR)-132/212 cluster is upregulated by AHR activation under T_H17-inducing, but not regulatory T-inducing conditions. Deficiency of the miR-132/212 cluster prevented the enhancement of T_H17 differentiation by AHR activation. We also identified B-cell lymphoma 6, a negative regulator of T_H17 differentiation, as a potential target of the miR-212. Finally, we investigated the roles of the miR-132/212 cluster in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis. Mice deficient in the miR-132/212 cluster exhibited significantly higher resistance to the development of experimental autoimmune encephalomyelitis and lower frequencies of both T_H1 and T_H17 cells in draining lymph nodes. Our findings reveal a unique mechanism of AHR-dependent T_H17 differentiation that depends on the miR-132/212 cluster.

dioxin receptor | autoimmunity | immune regulation

Interleukin-17 (IL-17)–producing T-helper (T_H17) cells play important roles in the pathogenesis of autoimmune diseases such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) (1–3). T_H17 differentiation requires retinoid-related orphan receptor (ROR)-γt, a transcription factor that is induced by TGF-β in combination with IL-6, -21, and -23, all of which activate STAT3 phosphorylation (4). Additionally, various host proteins such as aryl hydrocarbon receptor (AHR) (5–7), Runt-related transcription factor 1 (Runx1) (8), Interferon regulatory factor 4 (IRF4) (9), and Basic leucine zipper transcription factor ATF-like (BATF) (10) positively regulate T_H17 differentiation, whereas B-cell lymphoma 6 (Bcl-6) (11, 12), Ets-1 (13), Nuclear receptor subfamily 2 group F member 6 (NR2F6) (14), Peroxisome proliferator-activated receptor gamma (PPAR-γ) (15), Suppressor of cytokine signaling 3 (SOCS3) (16), and Liver X receptor (LXR) (17) inhibit generation of T_H17 cells. However, very few studies have addressed the regulation of T_H17 differentiation by microRNAs, a topic that should be investigated further.

MicroRNAs are noncoding RNA molecules of 20–22 nucleotides in length that regulate many biological systems, including immune systems, by controlling gene expression at the post-transcriptional level (18–20). Typically, the seed sequences of mammalian microRNAs base pair with binding sites in the 3′ untranslated region (3′ UTR) of target mRNAs, leading to translational inhibition and/or degradation of these mRNAs (21). In addition, microRNA can bind to target mRNAs that contain no seed sequence (22) or have G:U wobbles and G bulges within the seed sequence (23–25), indicating that there are multiple mechanisms for microRNA/mRNA interactions.

AHR is a ligand-activated transcription factor that belongs to the basic helix–loop–helix (bHLH)/PER–ARNT–SIM (PAS) family (26–28). Since the original finding that AHR regulates the balance of regulatory T (T_{reg}) and T_H17 cells (5–7), evidence for the role of AHR in immune regulation has continued to accumulate. Furthermore, several groups have demonstrated that AHR plays critical roles in such models of autoimmune diseases as EAE and CIA (5, 6, 29). Although the roles of AHR in immune cells and several inflammatory diseases have been elucidated, the intracellular mechanisms of AHR-mediated immune regulation remain poorly understood. A recent study showed that AHR agonists inhibit the progression of breast cancer cell through induction of microRNA (miR)-335 (30), but it is not known whether specific microRNAs regulate AHR-mediated immune regulation.

In this study, we demonstrate that the miR-132/212 cluster is induced by AHR activation in the context of T_H17 differentiation. These results provide evidence for an important role for the miR-132/212 cluster in T_H17 differentiation and development of EAE.

Results

AHR-Mediated Induction of the miR-132/212 Cluster Under T_H17-Polarizing Conditions. In previous studies, our group and others demonstrated that AHR regulates differentiation of T_{reg} and T_H17 cells (5–7). Therefore, to identify microRNAs that might contribute to AHR-mediated control of T_{reg} and T_H17 cells, we screened microRNA expression under T_{reg}- or T_H17-polarizing conditions in the presence or absence of the AHR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The analysis indicated that the miR-132/212 cluster was highly induced by TCDD under conditions of T_H17, but not T_{reg}, differentiation (Fig. 1 *A* and *B*). In addition, the miR-132/212 cluster was not induced in AHR-deficient naïve T cells under this condition. Another AHR agonist, 6-formylindolo[3,2-b]carbazole (FICZ), also caused significant induction of the miR-132/212 cluster under T_H17-polarizing conditions (Fig. 1*C*). These results indicate that expression of the miR-132/212 cluster depends on AHR under T_H17-polarizing conditions and suggest that this cluster affects T_H17 differentiation.

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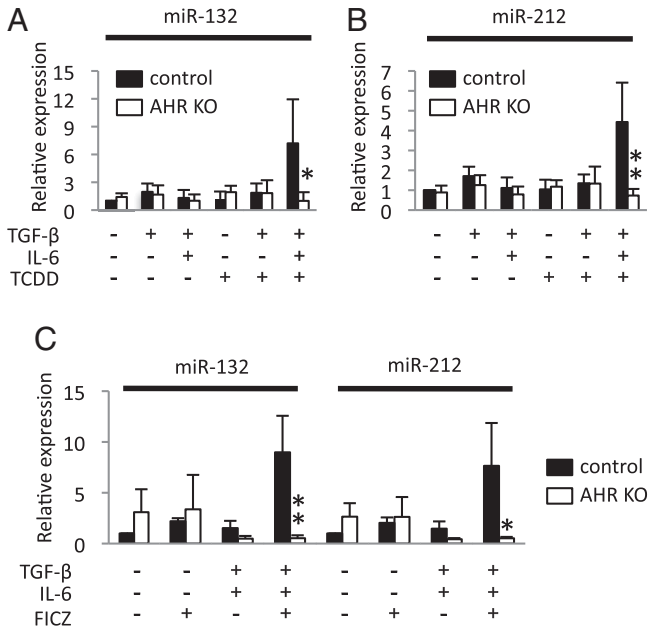

Fig. 1. The miR-132/212 cluster is induced by AHR agonists under T_H17-polarizing conditions. Isolated naïve T cells from control or AHR KO mice were cultured with anti-CD3/CD28 beads and stimulated with the indicated cytokines in the presence or absence of TCDD (*A* and *B*) or FICZ (*C*) for 3 d. Expression of miR-132 and -212 was examined by using quantitative PCR (qPCR). Data show means ± SD of at least three independent experiments. $*P < 0.05$; $**P < 0.01$.

The miR-132/212 Cluster Positively Regulates T_H17 Differentiation. To determine whether the miR-132/212 cluster regulates T_H17 development, we stimulated naïve T cells isolated from B6N (control) mice or miR-132/212 cluster double-knockout (miR-132/212 DKO) mice with IL-6 and TGF-β in the presence or absence of FICZ. Promotion of T_H17 cell differentiation by AHR activation was significantly abrogated by miR-132/212 DKO (Fig. 2 and Fig. S1). The frequency of T_H1 cells did not differ significantly between control and miR-132/212 DKO cells. Consistent with the finding that the miR-132/212 cluster is not induced in T_H17-polarizing conditions in the absence of AHR activation (Fig. 1), T_H17 development induced by IL-6 and TGF-β did not significantly differ between cells from control and miR-132/212 DKO mice. We also found that production of IL-17, but not IL-22, was significantly lower in miR-132/212 DKO than control naïve T cells during T_H17 development in the presence of FICZ (Fig. S2). Because AHR also participates in T_{reg} development (5, 7), we investigated whether the miR-132/212 cluster regulates the differentiation of T_{reg} cells. We found that T_{reg} differentiation from naïve T cells by TGF-β, with or without TCDD or FICZ, did not differ significantly between control and miR-132/212 DKO mice (Fig. S3). Together, these data indicate that the miR-132/212 cluster participates in AHR-mediated development of T_H17, but not T_{reg}, cells.

miR-132/212 Cluster Deficiency Significantly Suppressed EAE Development. To elucidate the pathophysiological roles of the miR-132/212 cluster, we conducted studies of EAE, a T_H17-dependent murine model of the human autoimmune disease multiple sclerosis (MS). Clinical symptoms were assessed in miR-132/212 DKO and control mice after treatment with myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) in complete Freund's adjuvant (CFA). Clinical scores and incidence were significantly lower in MOG-treated miR-132/212 DKO mice than in control mice with EAE (Fig. 3*A*). Data reflecting the effects of miR-132/212 cluster deficiency in EAE-treated mice are summarized in Table 1. To confirm that the miR-132/212 cluster participates in T_H17 generation in EAE, we isolated draining lymph-node cells from MOG-treated control and miR-132/212 DKO mice and examined the population of T_H1, T_H17, and T_{reg} cells (Fig. 3 *B–D*). miR-132/212 DKO mice exhibited lower frequencies of T_H1 and T_H17, but not T_{reg}, cells. Moreover, we observed suppressed expression of IL-6, but not TNF-α, in draining lymph-node cells from miR-132/212 DKO mice (Fig. 3 *E* and *F*) These finding suggested that miR-132/212 cluster deficiency ameliorated EAE development, possibly by the suppression of T_H1 and T_H17 induction.

The miR-132/212 Cluster Promotes T_H17 Cell Differentiation Through a STAT1- and STAT5-Independent Pathway. As shown in Fig. 1, the miR-132/212 cluster is not induced under T_H17-polarizing conditions in the absence of AHR activation. Consistent with this result, miR-132/212 cluster deficiency did not inhibit T_H17 development under the same conditions (Fig. 2 and Fig. S1). Therefore, we hypothesized that transfection of a miR-132 or -212 mimic would promote T_H17 differentiation and thereby substitute for FICZ treatment. To investigate this hypothesis, naïve T cells transfected with a miR-132 or -212 mimic were differentiated into T_H17 cells by stimulation with IL-6 and TGF-β. Both miR-132 and -212 mimics significantly promoted IL-17 secretion into the culture medium (Fig. 4*A*); miR-212 exhibited a stronger effect on T_H17 development than miR-132. In addition, we characterized the expression of T_H17-related genes such as IL-17A, -21, -22, -23R, Rora, and Rorc in naïve T cells transfected with miR-132 or -212 mimic and then stimulated by IL-6 and TGF-β. miR-212 mimic-transfected naïve T cells under T_H17-polarizing conditions significantly increased expression of IL-17A, and increased (not significantly) the expression of IL-23R, relative to the negative-control cells (Fig. 4*B*). Consistent with the result shown in Fig. 4*A*, miR-132 mimic up-regulated IL-17A and -23R to a lesser extent than the miR-212 mimic.

AHR increases T_H17 development by inactivating Stat1 and Stat5 (7, 31). Therefore, we investigated whether the miR-132/212

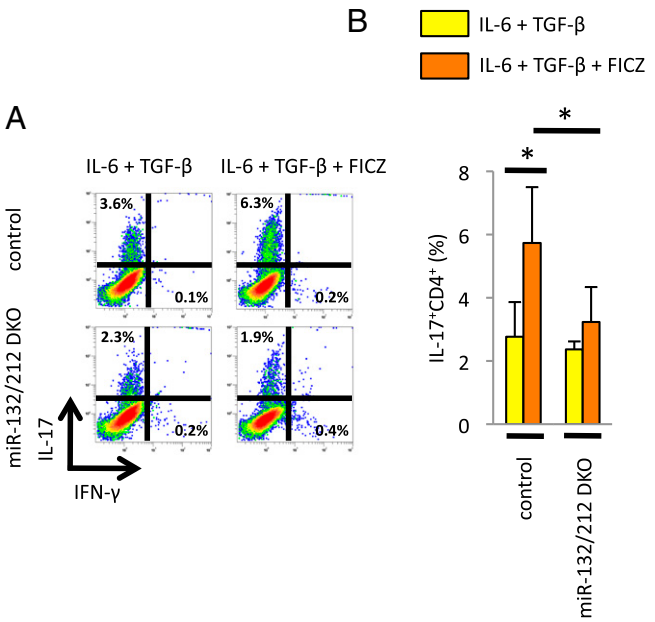

Fig. 2. The miR-132/212 cluster participates in promotion of T_H17 generation by FICZ. (*A*) Purified naïve T cells from control or miR-132/212 DKO mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence of IL-6 and TGF-β, with or without FICZ, for 5 d. Frequencies of IFN-γ– and IL-17–positive cells were determined by flow cytometry. Dot plots and values are representative of at least three independent experiments. (*B*) Bar charts show means ± SD of the frequency of IL-17–positive cells from at least three independent experiments. $*P < 0.05$.

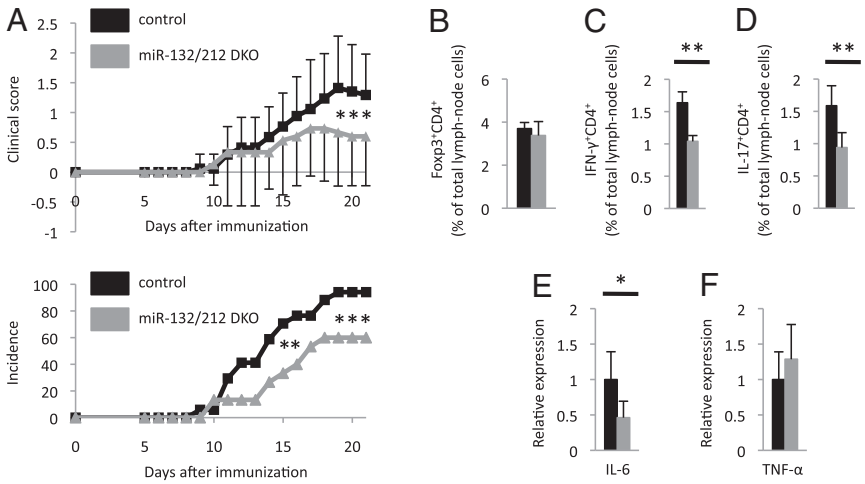

Fig. 3. Deficiency of the miR-132/212 cluster suppresses EAE development. Control and miR-132/212 DKO mice were immunized with MOG emulsified with CFA at the base of the tail on day 0. (*A*) The mean clinical score and incidence were recorded until day 21 (control, $n = 17$; miR-132/212 DKO, $n = 15$). Data are pooled from five independent experiments. (*B–D*) Draining lymph-node cells obtained from mice 21 d after immunization were counted after staining for CD4 in combination with IFN-γ, IL-17, or Foxp3, as indicated (control, $n = 8$; miR-132/212 DKO, $n = 5$). (*E* and *F*) On day 21, RNA was isolated from draining lymph-node cells, and expression levels of proinflammatory cytokines IL-6 (*E*) and TNF-α (*F*) were determined by using qPCR (control, $n = 8$; miR-132/212 DKO, $n = 5$). $^*P < 0.05$; $^{**}P < 0.01$.

cluster participates in the AHR-mediated regulation of Stat1 and Stat5. However, the protein levels of Stat1 and Stat5 were not reduced following transfection of miR-132 or -212 mimic (Fig. 4*C*). These results indicate that the miR-132/212 cluster promotes T_H17 development by a Stat1- and Stat5-independent pathway.

Bcl-6 Is a Potential Target of the miR-212 Cluster Under T_H17-Polarizing Conditions. To identify the miR-132/212 cluster target genes that negatively regulate T_H17 differentiation, we performed gene-expression analysis using DNA microarrays on naïve T cells isolated from control or miR-132/212 DKO mice and stimulated with IL-6, TGF-β, and FICZ. Under these conditions, Bcl-6 was expressed at fourfold higher in miR-132/212 DKO naïve T cells than in control cells (Fig. 5*A*). We also confirmed by Western blotting that the Bcl-6 protein level in miR-132/212 DKO cells is higher than that in controls (Fig. 5*B*). Because Bcl-6 is a negative regulator of T_H17 differentiation, we hypothesized that the miR-132/212 cluster participates in T_H17 differentiation by targeting Bcl-6 (11, 12). To investigate whether the Bcl-6 3′ UTR contains binding sites for miR-132 and/or -212, we used a computational microRNA target-prediction tool, MiRNAMap. This computational analysis demonstrated that miR-212, but not miR-132, potentially binds the Bcl-6 3′ UTR (Fig. 5*C*). Finally, to examine the experimental interaction between miR-212 and the Bcl-6 3′ UTR, we inserted the candidate miR-212 target site from the Bcl-6 3′ UTR into a luciferase reporter and cotransfected the resultant vector along with miR-132 or -212 mimic. The results of luciferase assays revealed that miR-212, but not miR-132, binds to the Bcl-6 3′ UTR (Fig. 5*D*). Together, these data demonstrate that Bcl-6, a negative regulator of T_H17 differentiation, is a possible target of miR-212.

Table 1. Suppression of EAE in miR-132/212 DKO mice

Group	Incidence (%)	Mean day of onset	Mean clinical score*
Control	16 of 17 (94)	13.8 ± 3.0	1.1 ± 0.5
miR-132/212 DKO	9 of 15 (60)	14.6 ± 2.9	0.6 ± 0.8

Values represent means ± SD.
*Calculated from day 14 to termination.

Discussion

In this study, we discovered a unique mechanism for AHR-mediated development of T_H17 cells that acts through induction of the miR-132/212 cluster. Although a number of studies have revealed that AHR plays critical roles in several autoimmune diseases by controlling T_H17 differentiation, the precise intracellular mechanisms still remain unclear (5–7). MicroRNAs have recently emerged as a new class of negative regulators of gene expression at the posttranscriptional level. MicroRNAs are crucial in many biological processes, including differentiation, proliferation, and apoptosis, as well as in disease (e.g., in immunity, autoimmune disease, and cancer) (32, 33). A recent study demonstrated that an AHR agonist inhibits lung metastasis of breast cancer cells through induction of miR-335 (30), suggesting that AHR-mediated induction of several microRNAs might also be important for immune regulation. In this study, we screened for microRNAs involved in AHR function and revealed roles for specific microRNA regulated by AHR during generation of T_H17 cells.

These results provide evidence that expression of the miR-132/212 cluster depends on AHR. Expression of the miR-132/212 cluster is elevated in neurons and is tightly controlled by the transcription factor CREB (camp-response element binding protein) (34, 35). miR-132 can amplify CREB activity, and stimulation of neuronal morphogenesis by CREB is abrogated by inhibition of miR-132 signaling, suggesting that CREB and miR-132 form a positive-feedback loop (36, 37). Likewise, we show here that the miR-132/212 cluster is induced by AHR activation and amplifies AHR-mediated induction of T_H17 cells, suggesting that the miR-132/212 cluster and AHR may also form a positive-feedback loop. cAMP activates AHR and causes the translocation of AHR into the nucleus, similar to the effects of TCDD treatment (38). Together with our data, this observation suggests the possibility of cross-talk between CREB and AHR signaling, which is important for miR-132/212 cluster induction.

Previous studies have revealed the roles of AHR in several immune cells such as T cells, macrophages, and dendritic cells, as well as in several types of autoimmune diseases (5–7, 29, 39, 40). FICZ promotes EAE through T_H17 differentiation, and TCDD ameliorates the disease through induction of T_{reg} cells in a ligand-specific fashion (5). However, the role of AHR endogenous ligands in EAE development is still controversial: Quintana et al.

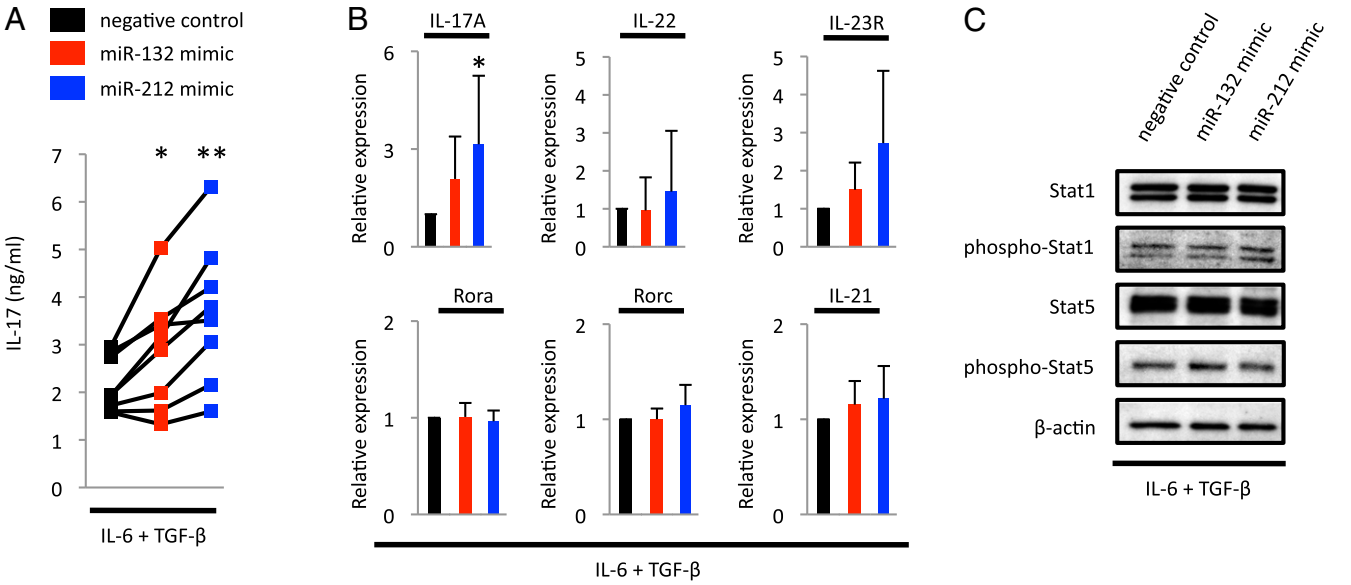

Fig. 4. The miR-132/212 cluster participates in T_H17 differentiation via a Stat1- and Stat5-independent pathway. Purified naïve T cells were transfected with miR-132 mimic, miR-212 mimic, or negative-control mimic and then stimulated with anti-CD3/CD28 beads in the presence of IL-6 and TGF-β for 3 d. The cultures were supplemented with anti–IFN-γ and anti–IL-4 antibodies. (*A*) IL-17 levels in the supernatant were measured by ELISA ($n = 8$ in each condition). (*B*) The expression of T_H17-related genes was determined by qPCR ($n = 7$ in each condition). Data show means ± SD of at least three independent experiments. $*P < 0.05$; $**P < 0.01$. (*C*) Transfected T cells were lysed 24 h after stimulation and subjected to Western blotting. Data are from one representative of at least three experiments.

(5) reported that Ahr^d mice, which carry a mutation in the AHR binding site that reduces the receptor's affinity for its ligands, develop more severe EAE; this finding is contrary to the report by Veldhoen et al. (6) that AHR KO mice exhibit less severe EAE. Therefore, it may be difficult to create a drug that targets AHR.

In this study, we investigated the roles of the miR-132/212 cluster, which is controlled by AHR, in both T_H17 differentiation in vitro and EAE in vivo. We found that the miR-132/212 cluster deficiency inhibits EAE development and lowers the frequencies of T_H1 and T_H17 cells. Thus, as a downstream regulator of AHR signaling, the miR-132/212 cluster represents a target for potential MS therapies based on blocking the generation of T_H17 cells. Although we did not identify the roles of the miR-132/212 cluster in T cells in EAE development by conducting T-cell–specific deletion of the miR-132/212 cluster, these results imply that expression of the miR-132/212 cluster in T cells induced by endogenous AHR agonists contributes to EAE development through promotion of T_H17 generation. Consistent with this idea, multiple reports have shown that the induction of EAE is critically dependent on T_H17 differentiation (1, 41). Conversely, we also observed decreased expression of IL-6 in draining lymph-node cells isolated from miR-132/212 DKO mice with EAE. Given that IL-6 is produced by several types of cells, including antigen-presenting cells such as macrophages and dendritic cells, we cannot exclude the possibility that expression of the miR-132/212 cluster in these cells is important for EAE development. Therefore, it is also necessary to gain an understanding of how the miR-132/212 cluster regulates the immune system in various types of immune cells, such as T cells, macrophages, and dendritic cells, by conducting research similar to our study of AHR in these immune cells (7, 39, 40). For instance, we recently found that AHR deficiency in peritoneal macrophages results in failure to induce the miR-132/212 cluster in response to LPS. Further studies using immune cell type-specific miR-132/212 cluster conditional knockout mice will help to define the functions of the miR-132/212 cluster in immunity and several autoimmune diseases.

We identified Bcl-6 as a possible functional target of miR-212. Although two groups have reported that Bcl-6 negatively regulates T_H17 differentiation, the precise role of Bcl-6 in T_H17 differentiation remains controversial (11, 12). Nurieva et al. reported that Bcl-6 inhibits Rorc transcriptional activity but not Rorc expression (11), whereas Yu et al. reported that Bcl-6 directly binds the promoter region of Rorc and affects Rorc expression (12). However, the targets of miR-212 may not be limited to Bcl-6, but also might include other factors; indeed, our finding that transfection with a miR-212 mimic promotes T_H17 differentiation without Rorc induction is consistent with the findings of Nurieva et al. In a previous study, we showed that Rorc expression in AHR-deficient naïve T cells stimulated by IL-6 and TGF-β did not differ from that of controls (7). In addition, another group demonstrated that AHR activation by FICZ also does not affect the levels of Rorc mRNA under T_H17-polarizing conditions (6). These findings are consistent with our observation that neither miR-132 nor -212 had an effect on Rorc expression, because induction of the miR-132/212 cluster depends on AHR under these conditions. Yu et al. used microarrays to identify several microRNAs down-regulated by Bcl-6, and their data showed that expression of the miR-132/212 cluster is strongly inhibited by Bcl-6 (12). These results suggest that Bcl-6 and miR-212 negatively regulate each other, with Bcl-6 suppressing the induction of miR-132/212 cluster at the transcriptional level and miR-212 causing translational inhibition and degradation of Bcl-6 mRNA. In this study, we did not detect an interaction between miR-132 and the Bcl-6 3′ UTR, indicating that miR-132 and -212 may have different targets. Our finding that miR-212 exerted a stronger effect than miR-132 on T_H17 differentiation may also support this possibility. Furthermore, mRNAs encoding negative regulators of T_H17 differentiation, such as Egr2 (42), Pias3 (43), and Id3 (44), contain potential binding sites for miR-132, but not miR-212, in their 3′ UTRs (MiRNAMap, http://mirnamap.mbc.nctu.edu.tw/, or microRNA.org). In addition to the known negative regulators of T_H17 generation, we should not exclude the possibility that as-yet-unidentified regulators are also regulated by miR-132. Therefore, further investigation is needed to shed light on the network comprising the miR-132/212 cluster and negative regulators of T_H17 differentiation.

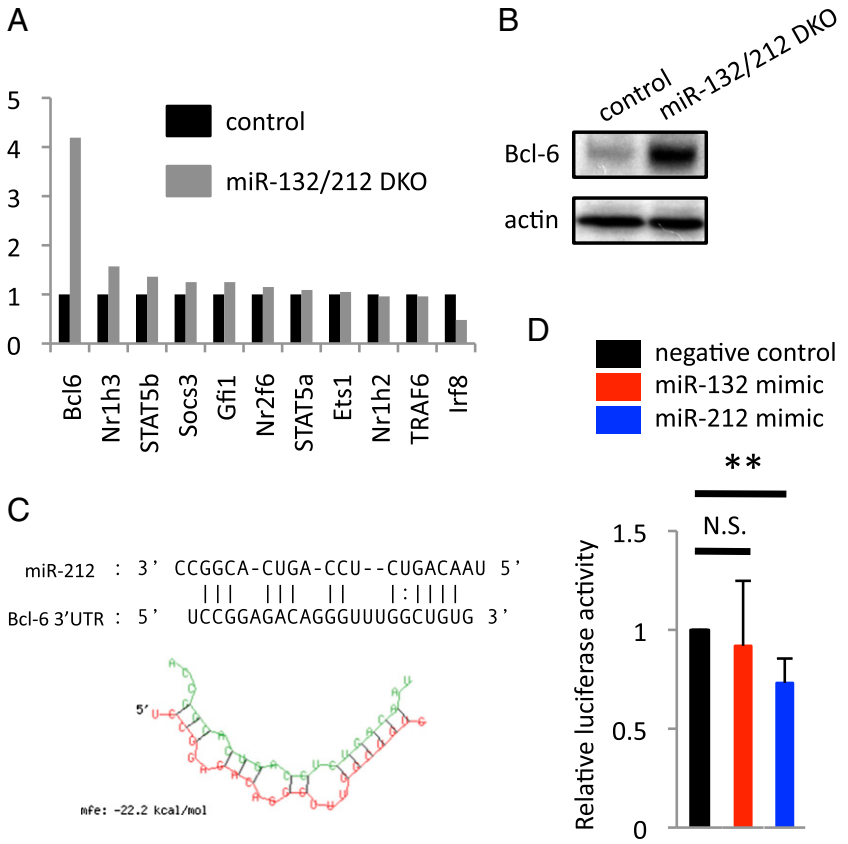

Fig. 5. The miR-132/212 cluster negatively regulates expression of Bcl-6. Isolated naïve T cells from control or miR-132/212 DKO mice were stimulated with anti-CD3/CD28 beads in the presence of IL-6, TGF-β, and FICZ for 3 d (DNA microarray) or 4 d (Western blotting). Neutralizing antibodies against IFN-γ and IL-4 were added in the cultures. (*A*) The expression of genes previously reported as negative regulators of T_H17 differentiation was investigated by DNA microarray. (*B*) Bcl-6 protein levels were determined by Western blotting. Data are from one representative of at least three experiments. (*C*) The free energy of miR-212 binding to its candidate targeting site in the Bcl-6 3′ UTR was calculated, and the predicted structure was determined by using RNAhybrid. Jurkat cells were cotransfected with a luciferase reporter vector containing the Bcl-6 target site along with miR-132 mimic, miR-212 mimic, or negative-control mimic. (*D*) Luciferase activity was measured 48 h after transfection; normalized levels of luciferase activity are shown. Data show means ± SD of at least three independent experiments. $^{**}P < 0.01$; N.S., not significant.

To summarize, we have demonstrated that expression of the miR-132/212 cluster depends on AHR under T_H17-polarizing conditions. We also found that the miR-132/212 cluster participates in AHR-mediated T_H17 generation and that miR-132/212 cluster deficiency suppresses development of EAE. The promotion of T_H17 induction by miR-212, but not miR-132, may be caused by targeting of Bcl-6. Because AHR has various functions in other types of immune cells, such as macrophages and dendritic cells, as well as in autoimmune diseases such as arthritis (45), future studies investigating the roles of the miR-132/212 cluster in these cell types and conditions will be important in advancing our understanding of AHR-mediated immune regulation.

Materials and Methods

Mice. miR-132/212 DKO mice in the C57BL/6N background have been described earlier (46). AHR KO mice in the C57BL/6J background were provided by Y. Fujii-Kuriyama (University of Tsukuba, Tsukuba, Japan). C57BL/6N wild-type and C57BL/6J wild-type mice were purchased from Charles River Japan and CLEA Japan, respectively, and used as controls for the corresponding KO mice. Age-matched (7- to 12-wk-old) and sex-matched mice were used. All animal experiments were performed based on protocols approved by the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience at Osaka University.

Isolation of Naïve T Cells and T-Cell Differentiation. Naïve T cells were purified from spleens of mice by using the $CD4^+$ T Cell Isolation Kit and CD62L MicroBeads (Miltenyi). Purified naïve T cells were stimulated with plate-bound anti-CD3 (R&D Systems) and 1 μg/mL soluble anti-CD28 (BioLegend) or with Dynabeads Mouse CD3/CD28 T Cell Expander (Invitrogen). RPMI or Iscove's modified Dulbecco's medium, as indicated, was supplemented with recombinant cytokines and/or AHR agonists: mouse IL-6 (30 ng/mL; R&D Systems), human TGF-β1 (2 ng/mL; R&D Systems), FICZ (100 nM; Enzo Life Science), or TCDD (160 nM; Cambridge Isotope Laboratories), either alone or in combination. In some experiments, neutralizing antibodies against IFN-γ (10 μg/mL; BioLegend), IL-4 (10 μg/mL; BioLegend), or IL-2 (5μg/mL; BioLegend) were additionally supplemented.

Transfection. Purified naïve T cells were stimulated with anti-CD3/CD28 beads in medium containing anti–IFN-γ and anti–IL-4 neutralizing antibodies 2 d before transfection. Activated T cells in Mouse T Cell Nucleofector solution (Amaxa) were transfected with miR-132 mimic, miR-212 mimic, or negative-control mimic (500 nM; Dharmacon) with program X-001 (Amaxa).

Flow Cytometry. $CD4^+$ T cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Calbiochem) and 800 ng/mL ionomycin (Calbiochem) for 5 h, with GolgiStop (BD PharMingen) added for the final 2 h. Samples were fixed and permeabilized with Cytofix/Cytoperm (BD PharMingen). Cells were stained with phycoerythrin (PE)-conjugated anti–IL-17 (BD PharMingen) and FITC-conjugated anti–IFN-γ (eBioscience) antibodies. For Foxp3 staining, cells were fixed and permeabilized by using Fixation/Permeabilization buffer (eBioscience) for 30 min at 4 °C before intracellular staining with FITC-conjugated anti-Foxp3 antibodies (eBioscience). For ex vivo experiments, isolated draining lymph-node cells from EAE-treated mice were stained with PE–Cyanine 7 (Cy7)-conjugated anti-CD4 antibodies (BioLegend) in combination with each antibody described above. Flow cytometry was performed on a Cytomics FC500 system (Beckman Coulter).

Quantitative PCR. Total RNA was prepared by using the RNeasy kit (Qiagen). cDNA was synthesized by using the ReverTra Ace qPCR RT kit (Toyobo). Quantitative PCR (qPCR) was performed by using TaqMan gene expression assays (IL-17A, Mm00439618_m1; IL-21, Mm00517640_m1; IL-22, Mm01226722_g1; IL-23R, Mm00519943_m1; Rora, Mm01173766_m1; Rorc, Mm01261022_m1; GAPDH, Mm99999915_g1; all from Applied Biosystems) and THUNDERBIRD Probe qPCR mix (Toyobo). For detection of microRNAs, reverse-transcription reactions were performed by using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), and cDNA was amplified by PCR using TaqMan MicroRNA Assays (hsa-miR-132, 000457; mmu-miR-212, 002551; RNU6B, 001093; all from Applied Biosystems) with the TaqMan Universal PCR Master Mix (Applied Biosystems). We used the comparative ΔΔCt method to determine levels of each mRNA (normalized to GAPDH) and microRNA (normalized to RNU6B).

EAE Induction and Evaluation of Disease Severity. CFA (25 mg/mL) was prepared by heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories) in CFA (Sigma). An emulsion was formed by dissolving 1 mg/mL $MOG_{35–55}$ peptide (Peptides International) in PBS and then combined with an equal volume of CFA. Control or miR-132/212 DKO mice were injected with 200 μL of emulsion at the base of the tail on day 0 followed by i.p. injection of pertussis toxin (Enzo Life Science) on days 0 and 2. Clinical signs of EAE were recorded daily from 5 to 21 d after immunization with $MOG_{35–55}$ by using the following scoring system (range, 0–4): 0, normal; 1, limp tail; 2, unsteady gait; 3, hind-limb paralysis; 4, fore-limb paralysis.

DNA Microarrays. Naïve T cells isolated from control and miR-132/212 DKO mice were cultured with anti-CD3/CD28 beads and the indicated cytokines for 3 d. cDNA was synthesized and hybridized to the 3D-Gene Mouse Oligo chip 24k (Toray).

Western Blotting. Stimulated T cells were lysed in lysis buffer [1% Nonidet P-40, 50 mM Tris·HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 10 mM Na_2VO_4, 0.5 mM DTT, and 1:100 protease inhibitor]. Cell lysates were analyzed by Western blotting using antibodies against Stat1, phospho-Stat1, Stat5, phospho-Stat5, and Bcl-6 (Cell Signaling Technology).

Luciferase Assay. A candidate miR-212 binding site from the Bcl-6 3′ UTR was cloned into the pMir-Report vector (Ambion) containing firefly luciferase. The insert sequences were designed to carry the HindIII and SpeI sites for ligation into the vector. The oligonucleotides used in the experiments were 5′-CTAGTGTCGTTATAATTACTCCGGAGACAGGGTTTGGCTGTGTCTAAACTGCATTA-3′ and 5′-AGCTTAATGCAGTTTAGACACAGCCAAACCCTGTCTCCGGAGTAATTATAACGACA-3′. Jurkat cells were transfected with pMir–Bcl-6 and 250 nM miR-132 mimic, miR-212 mimic, or negative-control mimic using DharmaFECT Duo Transfection Reagent (Dharmacon). Cells were lysed, and luciferase activity was determined 48 h after transfection.

Statistical Analysis. Student *t* test was used to identify significant differences in the data. EAE incidence was analyzed by using Fisher's exact test. $P < 0.05$ was regarded as significant.

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