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The aryl hydrocarbon receptor/microRNA-212/132 axis in T cells regulates IL-10 production to maintain intestinal homeostasis

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Abstract

Aryl hydrocarbon receptor (Ahr), a transcription factor, plays a critical role in autoimmune inflammation of the intestine. In addition, microRNAs (miRNAs), small non-coding oligonucleotides, mediate pathogenesis of inflammatory bowel diseases (IBD). However, the precise mechanism and interactions of these molecules in IBD pathogenesis have not yet been investigated. We analyzed the role of Ahr and Ahr-regulated miRNAs in colonic inflammation. Our results show that deficiency of Ahr in intestinal epithelial cells in mice exacerbated inflammation in dextran sodium sulfate-induced colitis. Deletion of Ahr in T cells attenuated colitis, which was manifested by suppressed T_h17 cell infiltration into the lamina propria. Candidate miRNA analysis showed that induction of colitis elevated expression of the miR-212/132 cluster in the colon of wild-type mice, whereas in *Ahr*$^{-/-}$ mice, expression was clearly lower. Furthermore, miR-212/132$^{-/-}$ mice were highly resistant to colitis and had reduced levels of T_h17 cells and elevated levels of IL-10-producing CD4$^+$ cells. *In vitro* analyses revealed that induction of type 1 regulatory T (Tr1) cells was significantly elevated in miR-212/132$^{-/-}$ T cells with increased c-Maf expression. Our findings emphasize the vital role of Ahr in intestinal homeostasis and suggest that inhibition of miR-212/132 represents a viable therapeutic strategy for treating colitis.

Keywords: aryl hydrocarbon receptor (Ahr), dextran sodium sulfate (DSS)-induced colitis, inflammatory bowel diseases (IBD), microRNA-212/132 (miR-212/132), type 1 regulatory T (Tr1) cells

Introduction

The two types of inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis, can be distinguished by their clinical, histopathological and endoscopic features (1, 2). Differentiated helper T cells such as T_h1, T_h2, T_h17 and

regulatory T (T_{reg}) cells exhibit effector and regulatory functions in the pathogenesis of IBD (3, 4).

Aryl hydrocarbon receptor (Ahr) is a receptor for aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 6-formylindolo[3,2-b] carbazole (FICZ) (5). Activation of Ahr inhibits GATA-3 expression and modulates the T_h1/T_h2 balance in favor of T_h1 dominance *in vivo* (6). Other studies have demonstrated that Ahr regulates differentiation of T_h17 and T_{reg} cells, which modulate the pathogenesis of experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (7–9). We previously demonstrated that Ahr participates in T_h17 cell differentiation through the regulation of Stat1 activation (10), and that Ahr in macrophages forms a complex with Stat1 and negatively regulates the NF-κB-mediated pro-inflammatory responses (11). We have also reported that the absence of Ahr in dendritic cells leads to enhanced T_h17 cell differentiation and reduced T_{reg} cell induction (12). Moreover, Ahr promotes type 1 regulatory T (Tr1) cells, which produce IL-10 in a c-Maf-dependent manner (13). Recent work has shown that RORγt$^+$ innate lymphoid cells require Ahr expression in order to expand and produce IL-22 (14, 15). Thus, Ahr plays multiple roles in distinct immune cell populations.

Expression of *AHR* is suppressed in the intestines of IBD patients (16, 17), and activation of Ahr by ligands such as TCDD or 3,3′-diindolylmethane (DIM) suppresses T_h17 cell differentiation and/or induces T_{reg} cells in murine colitis models (18, 19). In addition, *Ahr*$^{-/-}$ mice exhibit severe symptoms of dextran sodium sulfate (DSS)-induced colitis and produce high levels of pro-inflammatory cytokines (20). Thus, many studies have suggested that the function of Ahr in the intestine is protective. However, the precise mechanism by which Ahr regulates intestinal inflammation remains unclear; Ahr has been implicated in a variety of responses depending on cell type.

We previously reported that miR-132 and miR-212 are induced by Ahr ligand stimulation and promote T_h17 cell differentiation by inhibition of Bcl-6 (21). These microRNAs (miRNAs) are located in a cluster on chromosome 11, and they possess similar seed sequences (22). Their expression is also induced by cAMP response element-binding protein (CREB) (23) and repressor element 1 silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF), as a transcriptional repressor (24). A recent study suggested that the level of miR-132 is higher in the intestine of patients with active IBD (25). However, the detailed roles of these miRNAs in IBD have not been fully elucidated.

In this study, we analyzed the role of Ahr and the Ahr-inducible miR-212/132 cluster in the pathogenesis of DSS-induced colitis. Our findings demonstrated that Ahr exhibits different functions in various immune cell populations under intestinal inflammatory conditions, and further that the Ahr-inducible miR-212/132 cluster promotes inflammatory responses by inducing T_h17 cells and suppressing the development of IL-10-producing T cells.

Methods

Animals

C57BL/6J and C57BL/6N mice were purchased from CLEA Japan Inc. *Ahr*$^{-/-}$ mice in the C57BL/6J background were provided by Y. Fujii-Kuriyama (University of Tsukuba, Japan). Mice carrying a *loxP*-flanked *Ahr* allele (*Ahr*^{flox}), *Vil*^{cre} mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice, *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice and *Vil*^{cre}: *Ahr*$^{flox/flox}$ mice were generated by breeding *Ahr*$^{flox/flox}$ mice and C57BL/6J/129 background *LysM*^{cre} or ICR and C57BL/6J background *Lck*^{cre} knock-in mice, respectively. miR-212/132$^{-/-}$ mice in the C57BL/6N background were provided by K. Chowdhury (26).

Induction of colitis

For all *in vivo* experiments, 6- to 8-week-old sex-matched mice were used. For induction of colitis, mice were given 1 or 2% DSS (molecular weight: 36000–50000+; MP Biomedicals, Santa Ana, CA, USA) dissolved in drinking water provided *ad libitum*. Littermate mice were used as controls for *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice, *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice and *Vil*^{cre}: *Ahr*$^{flox/flox}$ mice. For analysis, mice were treated with 1% DSS for 4 days, followed by provision of ordinary water for 1 day. On the sixth day, mice were anesthetized and sacrificed. All *in vivo* animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the Graduate School of Frontier Bioscience, Osaka University.

Assessment of severity of DSS-induced colitis

The colitis Disease Activity Index (DAI) was calculated daily for each mouse on the basis of weight loss, diarrhea and hematochezia. Body weight score was assessed and modified scoring of diarrhea and hematochezia were performed as previously described (27; Supplementary Methods and Supplementary Figure S1, available at *International Immunology* Online). Details of the scoring of each parameter are as follows. Body weight loss score: 0, no weight loss; 1, 1–3% weight loss; 2, 3–6% weight loss; 3, 6–9%; 4, >9% weight loss. Changes in body weight were calculated as follows: body weight change (%) = [(weight on a given day/weight on day 0) × 100]. Diarrhea score: 0, normal stool; 1, loose stool; 2, diarrhea stool; 3, watery stool. Hematochezia score: 0, normal stool; 1: bloody stool; 2, bloody stool and dried blood on the anus; 3, gross bleeding with a maximum DAI score of 10.

Cytokine ELISA

Colons were cultured at 37°C for 24 h in 500 μl RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) containing penicillin and streptomycin (Nacalai Tesque, Kyoto, Japan). Cytokine levels in tissue culture supernatants were measured by using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

Isolation of intestinal epithelial cells and lamina propria lymphocytes from colons

Intestinal epithelial cells (IECs) and lamina propria lymphocytes (LPLs) were isolated from the colon of mice. In brief, the entire colon was treated with buffer containing 1 mM dithiothreitol (DTT) and 1.5 mM EDTA (Nacalai Tesque) to remove mucus. After mucus removal, colon segments were treated with 10 mM EDTA, and supernatant containing crude IECs was collected. IECs were negatively selected using anti-CD45 antibody and a magnetic cell sorting (MACS) system

(Miltenyi Biotec, Bergisch Gladbach, Germany). The remaining colon segments were washed and digested with 400 U/ml Collagenase D and 10 μg/ml DNase I (Roche, Mannheim, Germany). Crude cells were separated into LPLs and monocytes using Percoll (GE Healthcare, Little Chalfont, UK) as previously described (28). The lymphocyte-enriched population was recovered from the interface between 40 and 75% Percoll, and the monocyte-enriched population was collected from the population on the bottom of the tube.

Flow cytometry

Isolated colonic LPLs were stimulated with 50 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich) and 750 ng/ml ionomycin (Calbiochem, CA, USA) for 3 h, and then GolgiStop (BD PharMingen, San Jose, CA, USA) was added to inhibit protein trafficking. After stimulation, cells were stained with PerCP-Cy7-conjugated anti-CD4, PE-conjugated anti-IL-17 (BD PharMingen) and FITC-conjugated anti-IFN-γ (BioLegend, CA, USA). Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm™ Kit (BD PharMingen). Staining for Foxp3 was performed using the Anti Mouse/Rat Foxp3 staining set FITC (eBioscience, San Diego, CA, USA). Induced Tr1 cells were stained with PerCP-Cy7-conjugated anti-CD4 and FITC-conjugated anti-IL-10 (eBioscience) after stimulation with PMA plus ionomycin. Stained cells were analyzed on a CYTOMICS™ FC 500 (Beckman Coulter, Brea, CA, USA).

Immunohistochemistry

Paraffin-embedded mouse colonic tissues were sliced into 6-μm sections, and then incubated with blocking solution (Nacalai Tesque), primary antibody at 4°C overnight and secondary antibody at room temperature for 1 h. After completion of the reaction, the slides were counterstained with hematoxylin. Specimens were then covered with FluorSave Reagent (Calbiochem). Harvested colons were fixed by 4% PFA after sucrose replacement, and then embedded in Tissue-Tek optimal cutting temperature embedding medium for frozen tissue specimens (Sakura Finetek, Tokyo, Japan).

Western blotting

Cell lysates were prepared with lysis buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.1 mM sodium orthovanadate, 10 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 10 mM protease inhibitor cocktail (Nacalai Tesque)] and protein concentration was quantitated using the BCA Protein Assay kit (Thermo Scientific, Hercules, CA, USA). Following SDS–PAGE electrophoresis and transfer, the membrane was blocked with 5% skimmed milk in Tris-Buffered Saline and Tween (TBST). Rabbit anti-p-MEK1/2, anti-Erk1/2, anti-p-Erk1/2 anti-p-MKK4, anti-JNK, anti-p-MKK3/6 and anti-β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-p-JNK, p38, p-p38 and anti-c-Maf antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies were diluted with Can Get Signal (TOYOBO, Osaka, Japan). Bands were detected by using Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA, USA) and exposed to Amersham Hyperfilm-MP (GE Healthcare, Little Chalfont, UK). Membranes were stripped with 2 M glycine buffer (pH 2.8) and re-blotted with β-actin-specific mAb (Cell Signaling Technology) to confirm equal loading.

Real-time quantitative PCR

Total RNA was prepared using RNeasy (Qiagen, Hilden, Germany); reverse transcription reaction of mRNA was performed with ReverTra Ace (TOYOBO); miRNA reverse transcription was performed with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Gene expression was detected using TaqMan Gene Expression Assays (Applied Biosystems) in stems; mRNA and miRNA expression were normalized against the levels of *Gapdh* mRNA and *U6* RNA, respectively. Quantitative PCR (qPCR) was carried out on a PRISM 7900 HT system (Applied Biosystems).

Isolation of naive T cells and Tr1 differentiation

Naive T cells were purified from spleens using the $CD4^+$ T cell Isolation Kit and CD62L MicroBeads (Miltenyi Biotec). Purified naive T cells were stimulated with the pre-coated mouse anti-CD3 antibody and CD28 T-cell Expander (Invitrogen) for 3 days. As indicated, cultures were supplemented with recombinant cytokines: 30 ng/ml mouse IL-27 (R&D Systems) and human 2 ng/ml transforming growth factor (TGF)-β1 (R&D Systems) combined. Cytokines in cell culture medium were measured by ELISA.

Luciferase assay

A candidate miR-132 and miR-212 binding site from the c-Maf 3′-untranslated region (UTR) was cloned into the pMir-Report vector (Ambion) encoding firefly luciferase. The insert sequences were designed to carry *Hind*III and *Spe*I sites for ligation into the vector. The oligonucleotides used in this construction were 5′-CTAGTAAAGACC GATGCACTA AATTGTTTACTGTTGTGATGTTAAGGGGGGGTAG AGTTTGCAAGGGGACTGTTTAAAAAGTAGA-3′ and 5′-ATT TCTGGCTACGTGATTTAACAAATG ACAACACTACAATTCCC CCCATCTCAAACGTTCCC CTGACAAA TTTTTCATCTTCG A-3′. The luciferase reporter vector containing the c-Maf 3′-UTR sequence, along with 250 nM miR-132 mimic, miR-212 mimic or negative-control mimic, was transfected to Jurkat cells using the DharmaFECT Duo Transfection Reagent (Dharmacon, CO, USA). Cells were lysed and luciferase activity was determined 48 h after transfection.

Statistical analyses

Statistical analyses were performed in Microsoft Excel using a two-way analysis of variance test, *F*-test or Student's *t*-test as appropriate. Data from a minimum of three experiments are presented as means ± SEs. P values <0.05 were considered significant.

Results

Lack of Ahr *in epithelia, but not in macrophages, exacerbates DSS-induced colitis*

Previous studies revealed important roles of Ahr in the pathogenesis of IBD (16, 19, 29); however, due to the complex cell type-specific functions of Ahr, the precise mechanism is still

unclear (7, 11, 12). Therefore, to elucidate the cell-specific functions of Ahr in intestinal inflammation, we implemented a conditional knockout strategy. Mice with intestinal epithelia-specific Ahr deficiency (*Vil*^{cre}: *Ahr*$^{flox/flox}$), but not those with macrophage-specific Ahr deficiency (*LysM*^{cre}: *Ahr*$^{flox/flox}$), were more sensitive than control mice to DSS-induced intestinal inflammation (Fig. 1A and B). The background of *LysM*^{cre}: *Ahr*$^{flox/flox}$ (C57BL/6J/129) and *Vil*^{cre}: *Ahr*$^{flox/flox}$ (C57BL/6J) is different. This may explain why controls of Ahr KO mice and *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice showed moderate disease induction and progression, compared to that in *Vil*^{cre}: *Ahr*$^{flox/flox}$ mice. In histological analysis, the number of caspase-3 or TUNEL-positive cells was elevated in Ahr-deficient colonic epithelia (Fig. 1C). In addition, the JNK and p38 MAPK pathways were highly activated in Ahr-deficient colonic epithelial cells (Fig. 1D). These findings suggest the possibility that Ahr deficiency in epithelia might mediate high susceptibility to DSS-induced intestinal inflammation, accompanied by enhanced inflammatory responses and apoptosis of the epithelia.

Ahr deficiency in T cells reduces the severity of DSS-induced colitis and T_h17 cell infiltration in the intestinal lamina propria

Because several studies have demonstrated various roles for Ahr in differentiation of T cells, including T_h17, T_{reg} and Tr1 cells (7, 8, 10, 13), we examined DSS-induced colitis in T cell-specific Ahr-deficient mice (*Lck*^{cre}: *Ahr*$^{flox/flox}$ mice). Unexpectedly, *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice exhibited much less severe DSS-induced colitis (Fig. 2A). Consistent with previous findings indicating that Ahr participates in T_h17 cell differentiation (7, 8, 10), infiltration of T_h17 cells in intestinal lamina propria was substantially reduced in DSS-treated *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice. However, the frequencies of T_h1 and T_{reg} cells in the lamina propria of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice were similar to those in controls (Fig. 2B). Likewise, production of cytokines, such as IL-1β, IL-6, IL-10 and IL-22, in colonic tissues isolated from DSS-administrated *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice was similar to those in control mice (Fig. 2C). Thus, the introduction of Ahr deficiency in T cells ameliorated DSS-induced colitis and reduced the number of T_h17 cells in the lamina propria.

The miR-212/132 cluster is abundantly expressed in intestinal LPLs of DSS-treated mice

We previously demonstrated that Ahr induces expression of the miR-212/132 cluster, which promotes T_h17 cell development (21). Therefore, we investigated whether the miR-212/132 cluster is involved in the reduction in the number of T_h17 cells in the colonic lamina propria of DSS-administrated *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice. To this end, we first analyzed expression of the miR-212/132 cluster in colons isolated from DSS-treated *Ahr*$^{-/-}$ mice. The expression of miR-132 and miR-212 was severely reduced in *Ahr*$^{-/-}$ mice (Fig. 3A). In addition, colonic expression of both miR-132 and miR-212 was increased after DSS administration in wild-type mice (Fig. 3B and Supplementary Figure S2, available at *International Immunology* Online). To identify which cell populations express the miR-212/132 cluster, we isolated LPLs, monocytes and epithelial cells from DSS-treated mice, and then measured the levels of miR-132 and miR-212 by real-time qPCR. Both miR-132 and miR-212 were highly expressed in LPLs, relative to monocytes or epithelial cells (Fig. 3C). Taken together, these findings indicate that the miR-212/132 cluster expression is induced in colonic LPLs by DSS treatment in the presence of Ahr.

miR-212/132$^{-/-}$ *mice exhibit higher resistance to DSS-induced colitis*

Next, we analyzed the sensitivity of miR-212/132$^{-/-}$ mice to DSS-induced intestinal inflammation. miR-212/132$^{-/-}$ mice exhibited less severe DSS-induced colitis than control mice (Fig. 4A and B). Ahr expression in epithelial cells, monocytes and LPLs isolated from miR-212/132$^{-/-}$ mice was comparable to that in wild-type mice (Supplementary Figure S3, available at *International Immunology* Online). Previous studies reported that miR-132 and miR-212 suppress expression of IL-1β and IL-6 mRNA and negatively regulate IL-6 production (30, 31). Consistent with these findings, IL-1β and IL-6 production in the colonic tissues of DSS-treated miR-212/132$^{-/-}$ mice was elevated, despite the presence of only modest inflammation (Fig. 4C). On the other hand, DSS-treated miR-212/132$^{-/-}$ mice produced elevated level of IL-10 in the colon (Fig. 4C). To determine the frequency of infiltrated effector and regulatory T cells in DSS-administered mice, we stained CD4$^+$ T cells in the intestinal lamina propria with antibodies against IFN-γ, IL-17 and Foxp3 (Fig. 4D). Consistent with our findings in DSS-treated *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice (Fig. 2B), miR-212/132$^{-/-}$ mice exhibited a reduced frequency of IL-17-producing cells. However, in contrast to *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice, miR-212/132$^{-/-}$ mice had elevated levels of IFN-γ-producing T_h1 cells and lower levels of Foxp3$^+$ T cells. Furthermore, the frequency of IL-10-producing T cells was substantially elevated in the lamina propria of DSS-treated miR-212/132$^{-/-}$ mice, despite the reduced number of Foxp3$^+$ T cells (Fig. 4E). Thus, miR-212/132$^{-/-}$ mice exhibited higher resistance to DSS-induced colitis together with reduced levels of IL-17-producing T cells and elevated levels of IFN-γ- and IL-10-producing T cells in LPL.

miR-212 negatively regulates Tr1 cell differentiation

IL-10-producing Foxp3$^-$ CD4$^+$ T cells, which are induced by IL-27 and TGF-β *in vitro*, have been defined as Tr1 cells (13). Tr1 cells produce IFN-γ together with IL-10 (32). Ahr promotes Tr1 cell development (13). However, *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice did not exhibit reduced IL-10 production after DSS administration (Fig. 2C). To induce IL-10-producing T cells, we cultured naive CD4$^+$ T cells in the presence of IL-27 and TGF-β. In miR-212/132$^{-/-}$ T cells, the proportion of IL-10-producing cells was elevated by this treatment (Fig. 5A). Secretion of IL-10 and IFN-γ from the *in vitro* differentiated miR-212/132$^{-/-}$ T cells was also substantially elevated (Fig. 5B). To identify possible targets of the miR-212/132 cluster in T cells, we used two databases for target prediction of miRNA, microRNA.org (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/). The computational analysis revealed that *c-Maf*, a positive regulator of Tr1 differentiation, has a potential binding site in its 3′-UTR (Fig. 5C). To determine whether miR-132 and miR-212 interact with the 3′-UTR of *c-Maf*, we transfected cells with a vector encoding luciferase

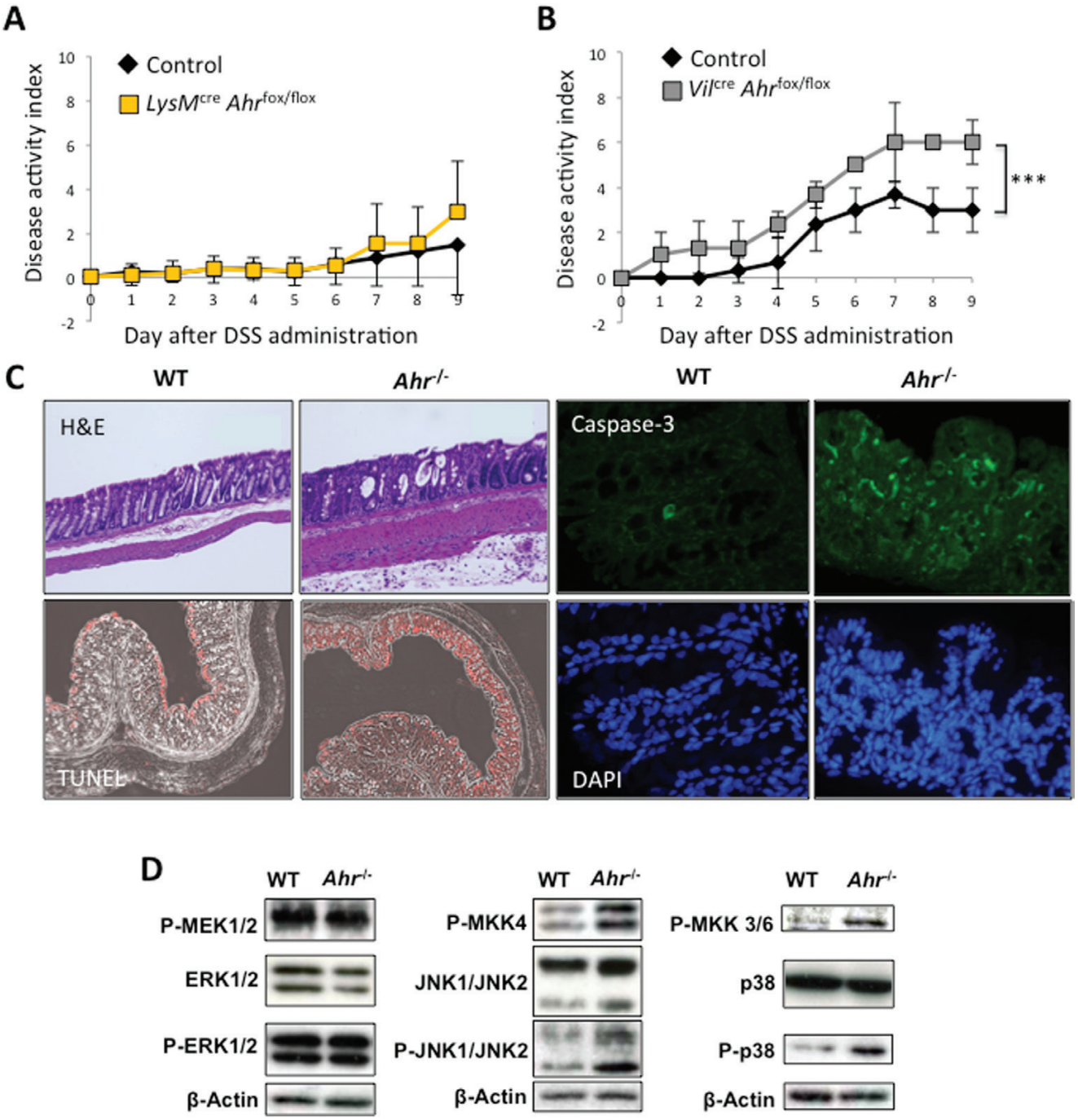

Fig. 1. Ahr deficiency in intestinal epithelial cells promotes DSS-induced colitis and reduced resistance to DSS. (A) DAI in *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice (n = 7 in each group) and (B)*Vil*^{cre}: *Ahr*$^{flox/flox}$ (n = 5 in each group) were given 1% DSS under specific pathogen free conditions. Six- to eight-week-old female mice were used. Body weight, diarrhea and hematochezia scores were measured daily. More than three independent experiments were performed and data were pooled from two representative experiments. ***$P < 0.0001$ (two-way analysis of variance test). (C) Histological analysis of colons of *Ahr*$^{-/-}$ and wild-type mice. After induction of DSS-induced colitis, colon segments were prepared and subjected to hematoxylin and eosin staining, TUNEL staining, active caspase-3 staining and 4′,6-diamidino-2-phenylindole staining. Data are from one representative of at least three experiments. (D) Activation of the ERK, JNK and p38 MAPK pathways in intestinal epithelial cells isolated from colons of *Ahr*$^{-/-}$ and wild-type mice was monitored by immunoblotting assay. Data are from one representative of at least three experiments.

fused to the portion of the *c-Maf* 3′-UTR containing the putative miR-132 or miR-212 binding sites, along with miR-132 or miR-212 mimics. Co-transfection of miR-212, but not miR-132 mimic, attenuated luciferase activity (Fig. 5D). In addition, c-Maf expression was elevated at the protein level in miR-212/132-deficient T cells (Fig. 5E). Taken together, these data indicate miR-212, rather than miR-132, negatively regulates Tr1 generation by targeting *c-Maf*.

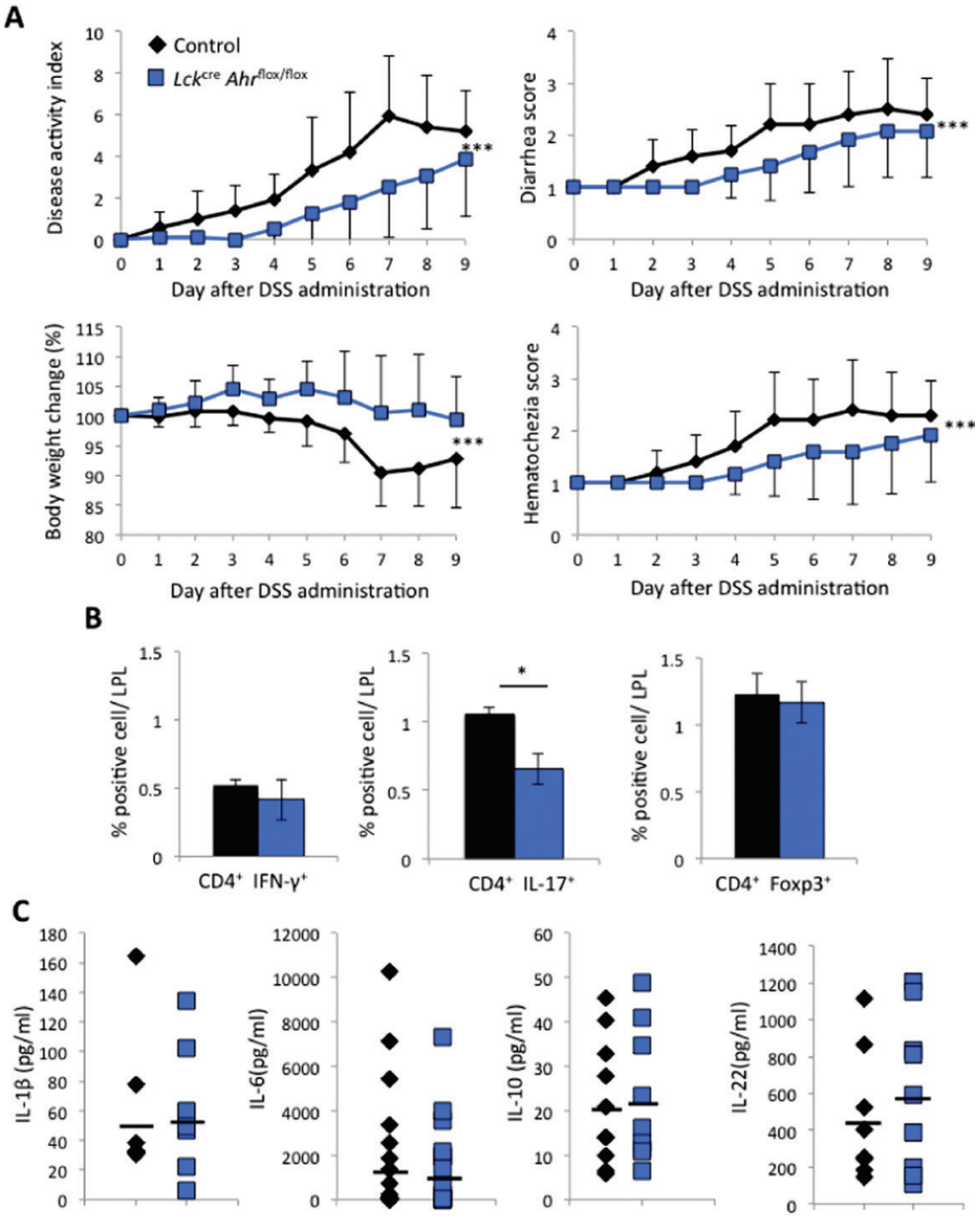

Fig. 2. T-cell-specific Ahr deficiency prevents DSS-induced colitis. (A) Daily symptom scores of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice and control mice were determined. DAI was calculated from daily clinical score and body weight after administration of 1% DSS (n = 7 in each group). More than three independent experiments were performed, and data were pooled from two representative experiments. *$P < 0.05$ (two-way analysis of variance test). (B) LPLs were isolated from colon of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice and control mice, and then infiltrated T_h cell subsets were analyzed by FACS after 1% DSS administration. Data show means ± SD of at least three independent experiments. *$P < 0.05$ (Student's t-test). (C) Levels of cytokines in colon organ culture supernatant were detected by ELISA. After administration of 1% DSS, distal colons were cultured for 24 h at 37°C in RPMI-1640 ($n > 5$ in each condition).

Discussion

In this study, we showed that Ahr in IECs inhibits the development of colitis symptoms by protecting cells against DSS-induced epithelial apoptosis. T cell-specific Ahr deficiency increased resistance to DSS-induced intestinal inflammation, in association with reduced T_h17 cell infiltration in the lamina propria *in vivo*. The Ahr-inducible miR-212/132 cluster mediates T_h17 cell development (21). Accordingly, miR-212/132$^{-/-}$ mice were resistant to DSS-induced colitis and had fewer T_h17 cells in the intestine. In addition, the level of IL-10-producing CD4$^+$ T cells was elevated in DSS-treated miR-212/132$^{-/-}$ mice.

Many studies have reported that activation of Ahr by its ligands, such as TCDD, FICZ and DIM, improves the outcome of colitis in various ways. The underlying mechanisms include elevated production of anti-inflammatory molecules such as prostaglandin E_2 and IL-22, reduced production of pro-inflammatory cytokines such as IL-6 and IL-12 and modulation of T_h cell development (18, 19, 33, 34). Because we found

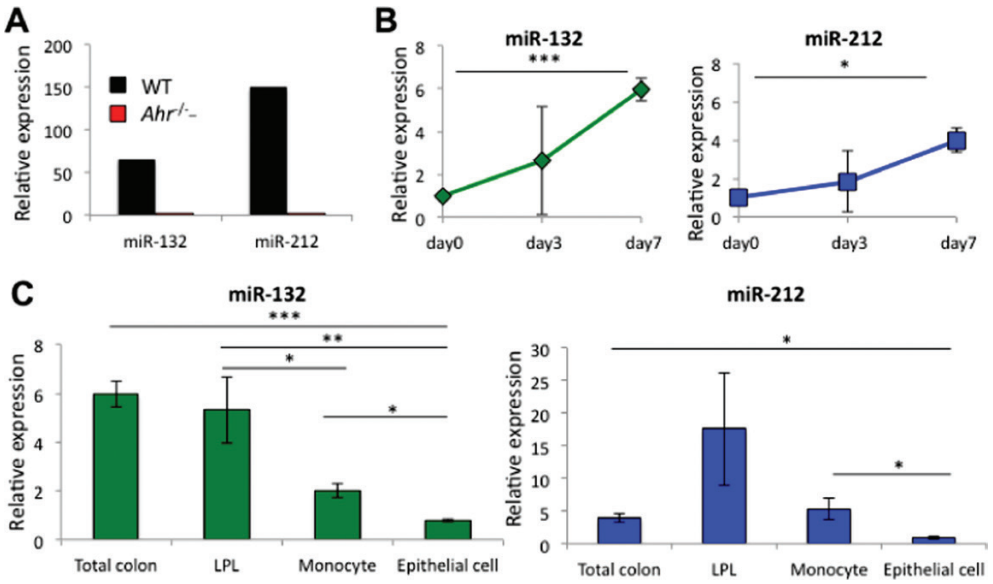

Fig. 3. miR-132 and miR-212 are induced in colons of wild-type but not *Ahr*$^{-/-}$ mice after induction of DSS-induced colitis. (A) qPCR analysis of the expression of miR-132 and miR-212 in whole colon samples obtained from *Ahr*$^{-/-}$ and wild-type mice after induction of colitis with 1% DSS. (B) Time-dependent change in miR-132 and miR-212 expression levels in whole colon samples of wild-type mice during 1% DSS administration. Data show means ± SD of at least three independent experiments. **P* < 0.05 (Student's *t*-test). (C) Expression of miR-132 and miR-212 in whole colon, LPLs, monocytes and epithelial cells of wild-type mice. Colons were harvested after 1% DSS treatment. After epithelial cells were isolated from the colon with EDTA, LPLs and monocytes infiltrated into the lamina propria were isolated by the Percoll method. Data show means ± SD of at least three independent experiments. **P* < 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001 (Student's *t*-test).

previously that Ahr deficiency in macrophages increases the production of pro-inflammatory cytokines (11), we expected Ahr-deficient macrophages to promote DSS-induced colitis by increasing the production of pro-inflammatory cytokines and T_h cell activation. Contrary to our expectations, the symptoms of DSS-induced colitis and production of pro-inflammatory cytokines in colon of *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice were comparable to those in control mice (data not shown). Our previous report using the same *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice strain showed the expression of Ahr in macrophages (9). We also demonstrated that Ahr induction in thioglycollate-elicited peritoneal macrophages isolated from *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice stimulated by LPS was significantly lower compared to controls. It is also reported that intestinal Ahr expression is induced under DSS-induced colitis (20). Therefore, in the current experiment, we considered that Ahr expression in macrophages isolated from *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice was defective. However, progression of colitis was milder and there was no difference in disease activity between *LysM*^{cre}: *Ahr*$^{flox/flox}$ mice and their controls. Previous studies demonstrated that Ahr binds to E2F1 and inhibits apoptosis (35), and that Ahr deficiency induces apoptosis by decreasing AKT activity (36). Furthermore, apoptosis and inflammation are elevated in bladder epithelial cells of *Ahr*$^{-/-}$ mice (37). On the basis of these findings and our current results, we expect that Ahr decreases DSS susceptibility and prevents apoptosis in the intestinal epithelium. These findings indicate that Ahr deficiency in epithelia is one of possibilities that mediate high susceptibility to DSS-induced intestinal inflammation, accompanied by enhanced inflammatory responses and apoptosis of the epithelia. However, further studies in *Vil*^{cre}: *Ahr*$^{flox/flox}$ would be required to substantiate these findings.

Many studies have reported that activation of Ahr by its ligands, such as TCDD and FICZ, regulates differentiation of T_h cell subsets such as T_h17 and T_{reg} (7, 8, 10). We previously reported that the miR-212/132 cluster coordinately enhances T_h17 cell development by suppression of Bcl-6, which is a suppressor of T_h17 (21). Our findings showed that *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice and miR-212/132$^{-/-}$ mice are resistant to DSS-induced intestinal inflammation and exhibit reduced numbers of T_h17 cells. On the basis of these findings, we hypothesize that due to the lack of Ahr, miR-212/132 is not induced through the Ahr pathway in naive T cells of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice. Furthermore, T_h17 cells are significantly induced by IL-6, TGF-β and FICZ in wild-type naive T cells, but not in that of miR-212/132$^{-/-}$ mice, and the T_h17 population is reduced in draining lymph nodes of an *in vivo* EAE model (21). Therefore, Ahr activation and the resultant induction of miR212/132 are important factors in the induction of T_h17 cells. Furthermore, in a colitis model, Ahr is induced by DSS administration in colon tissue (20). Thus, it is referable that decreased T_h17 infiltration in lamina propria is due to the lack of Ahr and the lack of consequent miR-212/132 induction in naive T cells of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice and miR-212/132$^{-/-}$ mice.

Ahr binds to c-Maf and synergistically induces Tr1 cells (13). In this study, IL-10 production and IL-10$^+$ CD4$^+$ cell infiltration were elevated in the colons of miR-212/132$^{-/-}$ mice after DSS administration. Intracellular c-Maf expression was also augmented following *in vitro* Tr1 induction in miR-212/132-deficient naive T cells. After DSS administration, it is conceivable that increased IL-10 production and IL-10$^+$ CD4$^+$ cell infiltration were induced in miR-212/132$^{-/-}$ mice because of the elevation of Ahr and c-Maf expression. On the other hand, we observed resistance to DSS-induced colitis in the colon of

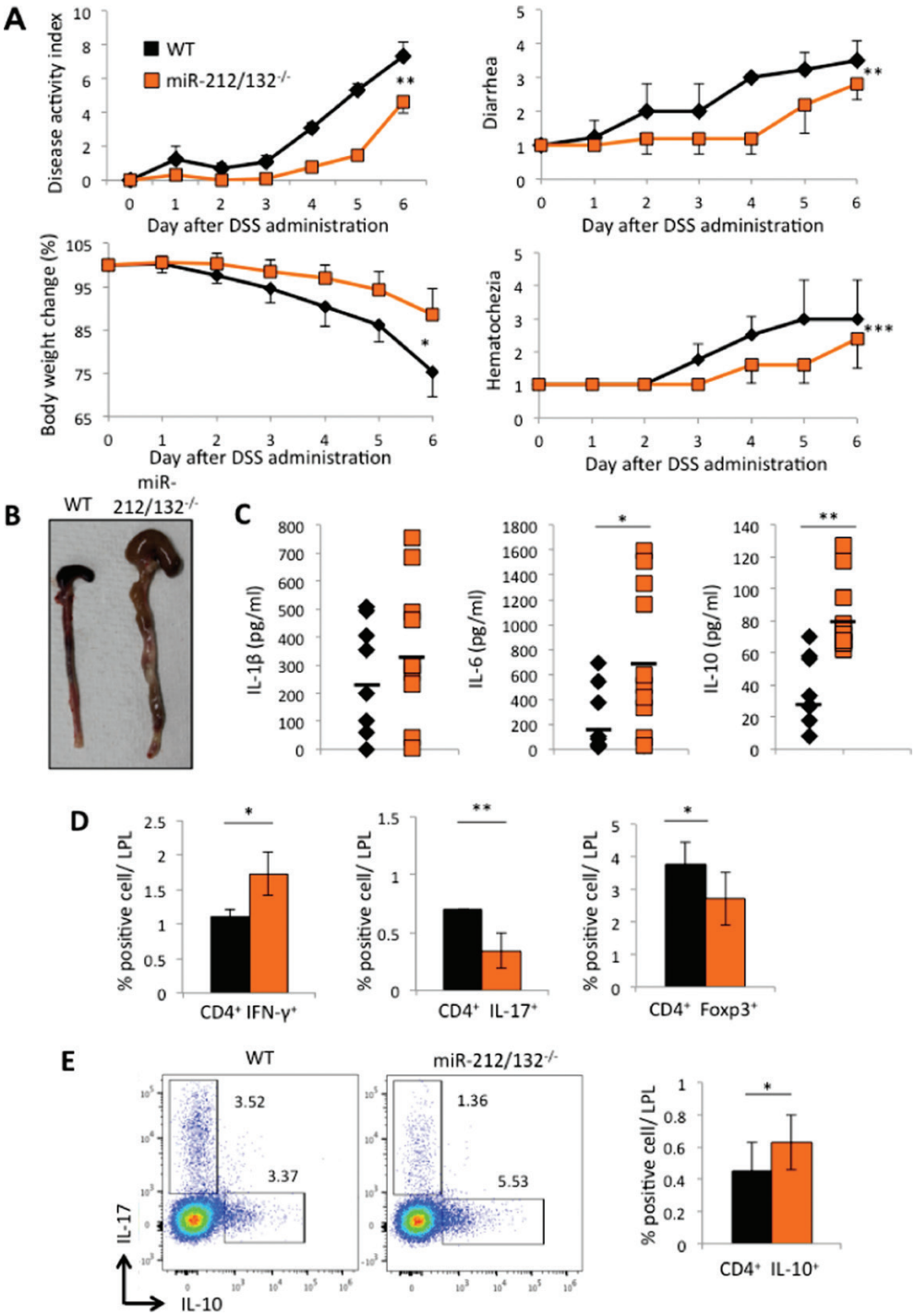

Fig. 4. miR-212/132$^{-/-}$ mice are less affected by DSS and have elevated levels of IL-10 and CD4$^+$ IL-10$^+$ cells. (A) DAI and daily scores of body weight, diarrhea and hematochezia while mice received 2% DSS in drinking water (n = 9). More than three independent experiments were performed, and data were pooled from two representative experiments. *P < 0.05, **P < 0.005, ***P < 0.0001 (two-way analysis of variance test). (B) Colons of miR-212/132$^{-/-}$ and wild-type mice on day 7 after administration of 2% DSS. Data are from one representative of at least three experiments. (C) Cytokine levels of colon organ culture supernatant were measured by ELISA. After administration of 2% DSS, distal colons were removed and cultured in serum-free RPMI-1640 for 24 h at 37°C (n = 8 in each condition). Data show means ± SD of at least three independent experiments. (D) Frequency of IFN-γ$^+$, IL-17$^+$ or Foxp3$^+$ CD4$^+$ T cell subsets infiltrated into the lamina propria of miR-212/132$^{-/-}$ and wild-type mice. (E) Frequency of the IL-10$^+$ CD4$^+$ T cell subset infiltrated into the lamina propria of miR-212/132$^{-/-}$ and wild-type mice. LPLs were isolated from colons after administration of 2% DSS, and cells were analyzed by flow cytometry. The flow cytometry data are from one representative of eight samples. Data in the column graphs show means ± SD of at least three independent experiments. *P < 0.05, **P < 0.005 (Student's t-test).

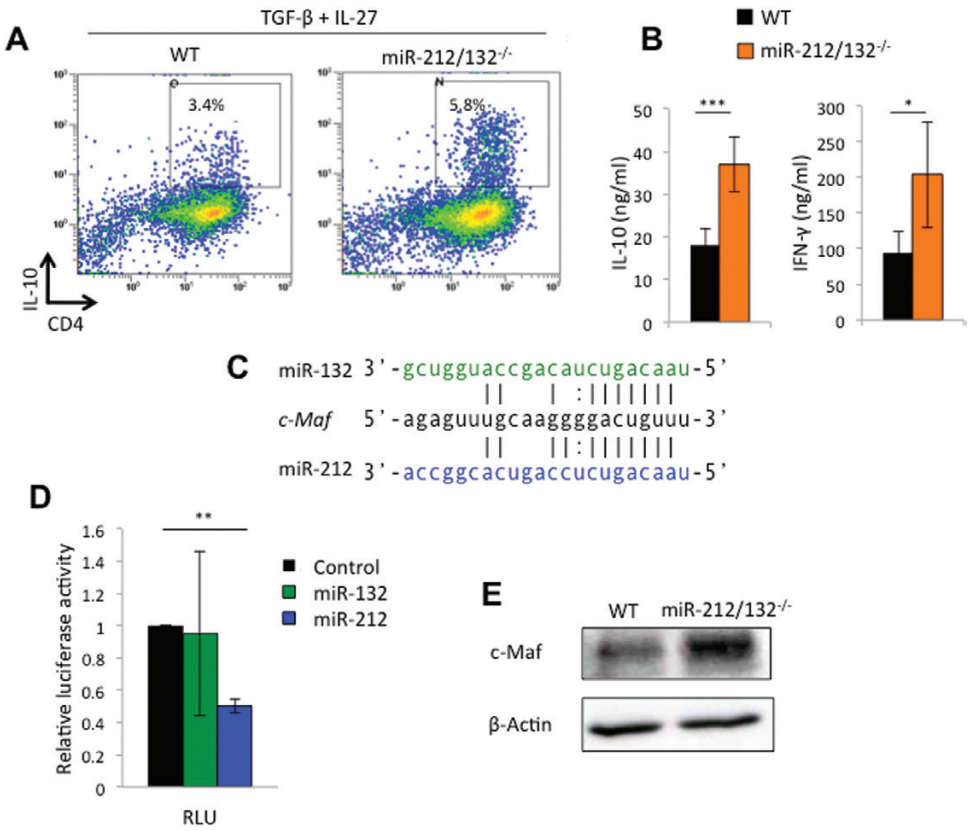

Fig. 5. miR-212/132$^{-/-}$ naive T cells exhibit enhanced differentiation of Tr1 and elevated IL-10 production. (A) IL-10 expression in CD4^{+} cells obtained from miR-212/132$^{-/-}$ and wild-type mice were analyzed by flow cytometry. (B) IL-10 and IFN-γ levels in culture medium were measured by ELISA. Naive CD4^{+} T cells were isolated from total splenocytes, and then cultured for 3 days under IL-27 and TGF-β stimulation (n = 5). Data are from one representative of at least three samples. *P < 0.05, ***P < 0.0001 (Student's t-test). (C) Predicted miR-132 and miR-212 binding site in the 3′-UTR of *c-Maf*. Data used for the computational analysis data were obtained from microRNA.org (http://www.microrna.org/microrna/home.do), a web database containing target site predictions scored by miRanda and mirSVR. (D) Luciferase activity of naive T cells co-transfected with a luciferase reporter construct containing the *c-Maf* promoter and mimics of miR-132 or miR-212 was measured 48 h after transfection; data shown were normalized against luciferase activity in cells co-transfected with empty expression vector (n = 5). **P < 0.005 (Student's t-test). (E) Immunoblotting of c-Maf protein expressed in naive T cells from miR-212/132$^{-/-}$ and wild-type mice. Data are from one representative of three samples.

Lck^{cre}: *Ahr*$^{flox/flox}$ mice; however, IL-10 production was not elevated. Furthermore, Ahr was not expressed in naive T cells of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice. Notably, even though stimulation of Ahr by its ligands can also induce the expression of miR-212/132, the intrinsic expression of miR-212/132 is comparable in Ahr-deficient and wild-type naïve CD4^{+} T cells (21). In this regard, it has been suggested that the expression of miR-212/132 might be regulated by other transcription factors, such as CREB and REST, which are positive and negative regulators of the miR-212/132 cluster, respectively (23, 24). Certainly, both Ahr and c-Maf are important molecules for Tr1 induction; however, even in the absence of one of these factors, the other might be able to activate the *Il10* promoter on its own, albeit less efficiently (13, 38). In naive T cells of *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice, although the Ahr-dependent enhancement of miR-212/132 expression was not active, the miR-212/132 cluster was still expressed. Thus, synergistic Tr1 induction by Ahr and c-Maf was observed in miR-212/132$^{-/-}$ mice, but not in *Lck*^{cre}: *Ahr*$^{flox/flox}$ mice.

Co-transfection of vector encoding luciferase fused to the portion of the *c-Maf* 3′-UTR and miR-212 mimic significantly suppressed luciferase activity, whereas expression of miR-132 did not. miR-132 and miR-212 may form a cluster under steady-state conditions, and it is conceivable that this configuration could affect the suppression of c-Maf. Alternatively, Lagos and colleagues reported that miR-212/132 or a miR-132 mimic decreased the protein level of p300, a co-activator of CREB (31). Additionally, we previously reported that miR-212 but not miR-132 negatively regulates Bcl-6 (21). Although these miRs share the same primary transcript and have similar mature sequences (22), miR-132 has one fewer base than miR-212 in its binding site to the *c-Maf* 3′-UTR. Although further confirmation is required, we tentatively conclude that the number of binding sites and small differences in the sequences of miR-132 and miR-212 may cause differences in their binding magnitude and functions.

In the results described here, IL-6 and IL-1β levels were elevated in colon of DSS-treated miR-212/132$^{-/-}$ mice, in apparent contradiction with the amelioration of colitis symptoms. Thus, the effect of anti-inflammatory responses, such as elevated populations of IL-10-producing and Tr1 cells and

reductions in the population of T_h17 cells in the lamina propria, predominated over the effect of increased pro-inflammatory cytokines. A previous study showed that reduced miR-132 expression negatively correlates with, and has the capability to down-regulate, IL-6 production in adipose tissue (30). Furthermore, lymphatic endothelial cells, transduced with miR-212/132, expressed lower levels of IL-1β and IL-6 mRNA (31). Additionally, IL-6 induces c-Maf via STAT3 (39) and is involved in IL-10 production in $CD4^+$ T cells and induction of Tr1 cells (40). Therefore, it is likely that secreted IL-6 also contributes to induction of Tr1 cells.

In conclusion, it is possible that Ahr and miR-212/132 regulate each other by negative feedback in Tr1 cell differentiation in order to avoid exorbitant immune tolerance due to Tr1 and IL-10 induction. The gut's unique immune system has evolved in response to the huge variety of food-derived antigens, microbiota and chemicals to which this tissue is exposed. Ahr in intestinal epithelia may help to maintain a healthy intestinal environment and thereby prevent pathogenic changes such as colitis, whereas the miR-212/132 cluster may modulate immune tolerance by suppressing Tr1 differentiation. Because Ahr serves a wide range of functions in distinct cell types, further studies will be required in order to fully elucidate the precise molecular mechanisms of pathogenesis, and to apply this knowledge to effective treatment of various diseases.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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