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Microbiota Downregulates Dendritic Cell Expression of miR-10a, Which Targets IL-12/IL-23p40

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Commensal flora plays important roles in the regulation of the gene expression involved in many intestinal functions and the maintenance of immune homeostasis, as well as in the pathogenesis of inflammatory bowel diseases. The microRNAs (miRNAs), a class of small, noncoding RNAs, act as key regulators in many biological processes. The miRNAs are highly conserved among species and appear to play important roles in both innate and adaptive immunity, as they can control the differentiation of various immune cells, as well as their functions. However, it is still largely unknown how microbiota regulates miRNA expression, thereby contributing to intestinal homeostasis and pathogenesis of inflammatory bowel disease. In our current study, we found that microbiota negatively regulated intestinal miR-10a expression, because the intestines, as well as intestinal epithelial cells and dendritic cells of specific pathogen-free mice, expressed much lower levels of miR-10a compared with those in germ-free mice. Commensal bacteria downregulated dendritic cell miR-10a expression via TLR–TLR ligand interactions through a MyD88-dependent pathway. We identified IL-12/IL-23p40, a key molecule for innate immune responses to commensal bacteria, as a target of miR-10a. The ectopic expression of the miR-10a precursor inhibited, whereas the miR-10a inhibitor promoted, the expression of IL-12/IL-23p40 in dendritic cells. Mice with colitis expressing higher levels of IL-12/IL-23p40 exhibited lower levels of intestinal miR-10a compared with control mice. Collectively, our data demonstrated that microbiota negatively regulates host miR-10a expression, which may contribute to the maintenance of intestinal homeostasis by targeting IL-12/IL-23p40 expression. ***The Journal of Immunology*****, 2011, 187: 5879–5886.**

The intestinal mucosa is home to a massive and diverse microbiota shortly after birth (1). In the large intestine and colon, commensal bacteria can reach a density of 10^{12} organisms/g and comprise >1000 species, including both anaerobes and aerobes (2–4). In addition to providing benefits to their host, including the breakdown of indigestible food, the supply of energy for colonic epithelial cells, and a barrier against invasive pathogenic bacteria, the gut microbiota instructs the postnatal maturation of gut immune defenses, which play a crucial role in maintenance of intestinal homeostasis (5–7). Commensal bacteria modulate the expression of genes involved in many intestinal functions. It was shown that colonization by the commensal bacterium *Bacteroides thetaiotaomicron* is able to modulate the expression of host genes that participate in fundamental physiological functions (8). Colonization with segmented filamentous bacteria induces gut Th17 cell development (9), whereas *Bacteroides fragilis* induce gut IL-10–producing $Foxp3^+$ regulatory T cells (10, 11). Accumulating evidence from reports in multiple experimental inflammatory bowel disease (IBD) models indicates that normal intestinal microbiota is critical to the pathogenesis of IBDs as well, because intestinal inflammation only develops in mice housed in a conventional environment, but not under germ-free (GF) conditions (3, 12). However, it is still largely unknown how the normal host–commensal interaction is regulated.

The recent discovery of microRNAs (miRNAs) has greatly expanded our understanding of the mechanisms that regulate gene expression (13, 14). miRNAs are small, nonprotein-coding RNAs of 19–25 nucleotides that regulate gene expression by targeting mRNA in a sequence-specific manner, either by repressing translation or directly leading to cleavage of mRNA sequences (15). The miRNAs are highly conserved among species and appear to play important roles in both innate and adaptive immunity, because they can control the differentiation of various immune cells, as well as their functions (16, 17). It was shown that specific miRNAs are upregulated during the activation of innate immunity, which is able to influence innate responses to microbial and viral infections (18–20). It is now apparent that abnormal miRNA expression is a common feature of various human diseases, such as cancer, developmental abnormalities, muscular and cardiovascular disorders, and, most recently, inflammatory diseases, including IBDs (21–23). However, it is still largely unknown how microbiota regulates miRNA expression and, thus, contribute to the maintenance of intestinal homeostasis and to IBD pathogenesis. In this report, we demonstrate that microbiota negatively regulates

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The microarray data presented in this article have been submitted to the ArrayExpress database (http://www.ebi.ac.uk/array/express) under accession number E-MEXP-3406.

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Abbreviations used in this article: B6, C57BL/6; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; GF, germ-free; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; KO, knockout; LP, lamina propria; LPDC, lamina propria dendritic cell; miR, microRNA; SPDC, splenic dendritic cell; SPF, specific pathogen-free; UTR, untranslated region; WT, wild-type.

miR-10a expression, in that the intestines, intestinal epithelial cells (IECs), and dendritic cells (DCs) of mice housed under specific pathogen-free (SPF) conditions expressed much lower levels of miR-10a compared with those in mice housed under GF conditions. Stimulation with TLR ligands downregulated the DC expression of miR-10a via the MyD88-dependent pathway. We further identified IL-12/IL-23p40 as a target gene of miR-10a. Furthermore, in colitic IL-10–deficient mice that express high levels of IL-12/IL-23p40, gut miR-10a expression was much lower than that of control wild-type (WT) mice, indicating that miR-10a could negatively regulate intestinal IL-12/23p40 expression in the mice with colitis.

Materials and Methods

Mice

C57BL/6 (B6) mice, B6.IL-10$^{-/-}$ mice, B6.MyD88$^{-/-}$ mice, and B6.RAG$^{-/-}$ mice were obtained from The Jackson Laboratory and maintained in the animal facilities of the University of Alabama at Birmingham and the University of Texas Medical Branch. GF B6 mice were derived by hysterectomy and maintained in Trexler-type isolators, according to standard gnotobiotic techniques in which GF Swiss Webster mice (Taconic) are used as foster mothers (24). Isolators were monitored for contamination monthly by examination of Gram-stained films of fresh fecal samples, aerobic and anaerobic bacterial and fungal cultures of fresh fecal samples, and swabs of water bottle sipper tubes and isolator interiors. We used 8–10-wk-old female mice in these experiments. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Alabama at Birmingham and the University of Texas Medical Branch.

Reagents

RPMI 1640, DMEM, HEPES, penicillin-streptomycin, FBS, 2-ME, L-glutamine, and sodium pyruvate were purchased from Life Technologies (Carlsbad, CA). GM-CSF, anti-CD11c, anti-CD11b, anti-CD80, and anti-CD86 were purchased from BD Biosciences (San Diego, CA). Restriction endonucleases, T4 DNA ligase, psiCHECK-2 vector, and dual-luciferase reporter system were from Promega (Madison, WI). Lipofectamine 2000 and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Collagenase type IV was obtained from Sigma-Aldrich (St. Louis, MO). TaqMan microRNA reverse transcription kits and TaqMan gene expression assays were from Applied Biosystems (Carlsbad, CA). miR-10a precursors and inhibitors were purchased from Ambion (Carlsbad, CA). Nucleotides were synthesized by Fisher Scientific (Pittsburgh, PA).

Generation of bone marrow-derived DCs

Bone marrow cells were isolated, as described previously. Briefly, bone marrow cells were suspended at 2.5×10^5/ml in complete RPMI 1640 media containing 10% heat-inactivated FCS (Atlanta Biologicals, Lawrenceville, GA), 25 mM HEPES buffer, 2 mM sodium pyruvate, 50 mM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Cellgro Mediatech, Manassas, VA). The cells were cultured in the presence of 20 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) in six-well plates at 37°C in 5% CO_2 in humid air. On day 8, bone marrow-derived DCs (BMDCs) were harvested and used as described in the text.

Preparation of IECs and lamina propria cells

To isolate IECs, the intestines were washed and cut into small pieces. Then the latter were incubated with calcium- and magnesium-free HBSS supplemented with 2% FBS and 5 mM EDTA (Sigma-Aldrich) on a magnetic stirrer at 37°C for 30 min. The liberated cells were collected by passage through a stainless steel sieve. The isolated cells were pooled together, and epithelial cells were separated on a 20/75% discontinuous Percoll gradient (Pharmacia). To isolate lamina propria (LP) DCs (LPDCs) and T and B cells, after removal of epithelial cells and intraepithelial lymphocytes, the intestinal tissues were incubated with RPMI 1640 containing 5% FBS and 0.5 mg/ml collagenase type IV for 30 min at 37°C with stirring. The liberated cells were collected by passage through a stainless steel sieve. Then isolated cells were pooled together and separated on a 40/75% discontinuous Percoll gradient (Pharmacia). The cell yield was typically $\sim 2 \times 10^6$ lymphocytes/mouse, with >90% cell viability. DCs, T cells, and B cells were further isolated by MACS for which we used CD11c beads, CD3 beads, and B220 beads. The cell purity was generally ~95% for each cell population.

Microarray analysis for miRNA-expression profile

Total RNA isolation was performed using the TRIzol method (Invitrogen), according to the manufacturer's instructions. Total RNA labeling and hybridization on miRNA microarrays were performed, as previously described. Briefly, we biotin labeled 5 μg total RNA of each testing sample by reverse transcription with a 5′ biotin end-labeled random octomeroligo primer. Biotin-labeled cDNA was hybridized on an miRNA microarray chip (The Ohio State University, Ver. 4.0), containing 1700 miRNA gene-specific oligo probes derived from 474 human miRNA and 373 mouse microRNA genes (http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl; accessed Nov 2006) and printed on arrays in duplicate. Hybridization signals were detected by biotin binding of a streptavidin-Alexa Fluor 647 conjugate on an Axon Scanner 4000B (Axon Instruments, Union City, CA). The images were quantified by GENEPIX 6.0 software (Axon Instruments). The microarray data were deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-3406.

Real-time PCR

Total RNA was extracted with TRIzol reagent and followed by cDNA synthesis with Superscript reverse transcriptase (Invitrogen). Quantitative PCR reactions were performed by using TaqMan Gene Expression Assays for *miR-10a* and *IL-12/IL-23p40* (Applied Biosystems) on a Bio-Rad iCycler (Bio-Rad, Hercules, CA), and all data were normalized to *Gapdh* mRNA expression.

Vector construction and luciferase reporter assays

The dual-luciferase psiCHECK-miR-10a and psiCHECK-IL-12/IL-23p40 vectors were constructed by synthesizing the candidate seed sequences in these genes located in the 3′-untranslated region (UTR) and inserting the annealing products into the psiCHECK-2 vector (Promega) by using NotI (5′-GCGGCCGC-3′) and XhoI (5′-CTCGAG-3′) restriction endonucleases. For mutant constructs psiCHECK-mmiR-10a and psiCHECK-mIL-12/IL-23p40, 3-bp mutations were introduced into the seed sequences. The nucleotide sequences of constructed plasmids were confirmed by enzyme digestion and DNA sequencing.

Murine macrophage RAW264.7 cells were cultured in complete DMEM supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 mM 2-ME at 37°C in 5% CO_2/95% air under humidified conditions. For reporter assays, cells were seeded in 24-well plates (1.5×10^6/well) and transfected with one of the following: 0.8 mg recombinant dual-luciferase vectors alone, vectors plus 30-nM hairpin precursors, or 30-nM inhibitors, with Lipofectamine 2000 reagent (Invitrogen). Luciferase assays were performed 24 h later using the Dual-Luciferase Reporter Assay System (Promega). The *Renilla* and firefly luciferase signals were measured on the Veritas Microplate Luminometer (Promega).

Statistical analysis

Levels of significance were determined by the Student *t* test; p values < 0.05 were considered statistically significant.

Results

miR-10a was predominantly expressed in intestines and was downregulated by microbiota

Although miRNAs have been implicated in the regulation of both innate and adaptive immune responses, there is limited information on microbiota regulation of miRNA expression of the intestinal mucosal system. To determine whether there is a unique miRNA expression profile in the intestines and whether microbiota regulates such miRNA expression, RNA was isolated from the intestines and spleen of B6 mice housed under SPF or GF conditions. miRNA microarray profiling of ~373 mouse microRNA genes was performed. Several miRNAs, including miR-10a, were differentially expressed between the spleen and intestines. As shown in Fig. 1*A*, miR-10a was highly expressed in the intestines but at very low levels in the spleen of GF mice, indicating that the miR-10a was predominantly expressed on the intestines. The intestinal tract is the home of extremely diverse and dense commensal bacteria that are normally nonpathogenic in an immunocompetent host, whereas the spleen remains in a sterile condition. Microbiota were shown to exert a great effect on hosts and, most

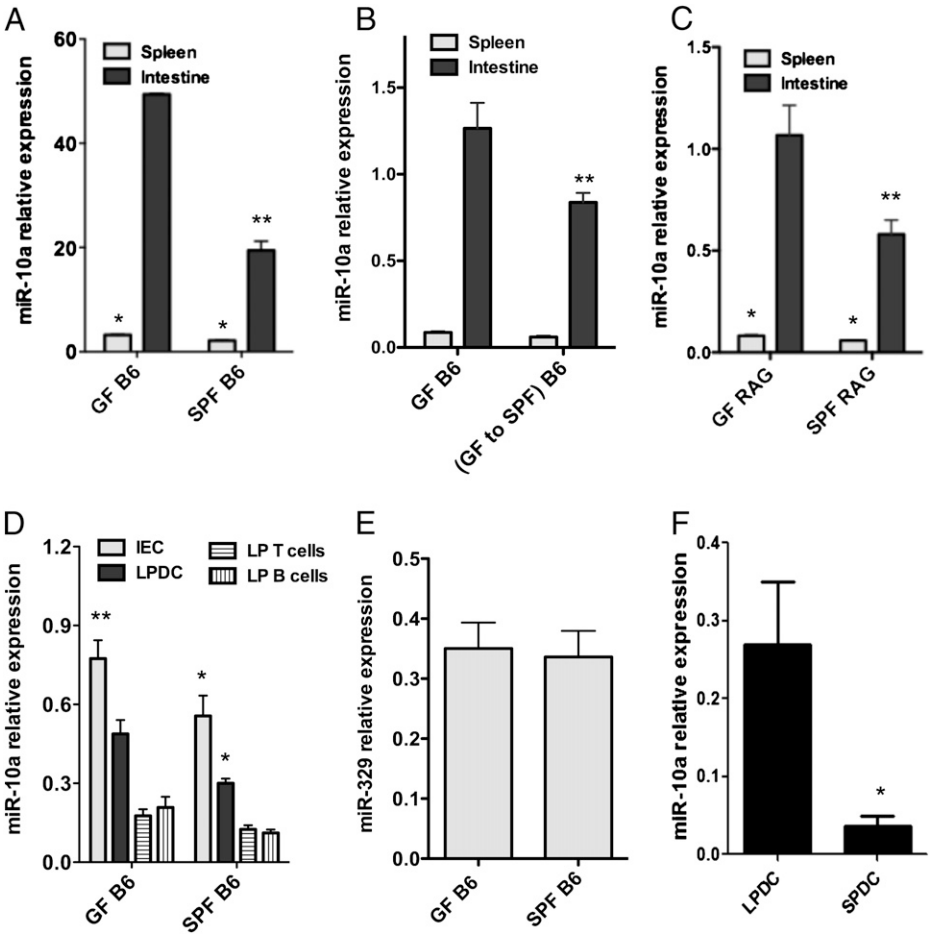

FIGURE 1. miR-10a is predominantly expressed in the intestines by innate cells. *A*, RNA was isolated from the spleen and intestines of B6 mice housed under SPF or GF conditions, and miRNA expression profiles were determined by miRNA microarray. miR-10a expression was normalized to negative control of random sequences of a similar size. $*p < 0.01$ compared with the intestines, $**p < 0.05$ compared with GF B6 intestines. One representative of two experiments is shown. *B*, GF B6 mice were recolonized with normal flora by housing under SPF conditions for 1 mo. RNA was isolated from the spleen and intestines, and miR-10a expression was determined by real-time PCR. Expression of GAPDH was used as a reference gene control. One representative of two experiments is shown. $**p < 0.01$ compared with the intestines of GF mice. *C*, RNA was isolated from spleen and intestines of B6.RAG$^{-/-}$ mice housed under SPF or GF conditions, and miR-10a expression was determined by real-time PCR. Expression of GAPDH was used as a reference gene control. One representative of two experiments is shown.$*p < 0.01$ compared with the intestines, $**p < 0.05$ compared with the intestines of B6.RAG$^{-/-}$ mice. *D*, miR-10a expression of IECs, LPDCs, LP T cells, and LP B cells from SPF and GF B6 mice was determined by real-time PCR. Expression of GAPDH was used as a reference gene control. $*p < 0.05$ compared with GF cells, $**p < 0.05$ compared with LPDCs. *E*, miR-329 expression of LPDCs isolated from GF and SPF B6 mice was determined by real-time PCR and analyzed using the expression of GAPDH as a reference gene. *F*, LPDCs and SPDCs were isolated from SPF B6 mice with CD11c beads, and miR-10a expression was analyzed by real-time PCR. Expression of GAPDH was used as a reference gene control. Data are representative of three experiments. $*p < 0.01$ compared with LPDCs.

particularly, on the intestines. In our study, intestinal miR-10a expression was decreased in mice under SPF conditions compared with that in the GF mice (Fig. 1*A*). Recolonization of the GF mice with normal flora led to a decreased intestinal miR-10a expression (Fig. 1*B*). These data collectively indicated that commensal bacteria could downregulate intestinal miR-10a expression. Notably, miR-10a was expressed predominantly in the intestines but not in the spleen, even in SPF mice.

Positioned at the interface between the intestinal lumen and the host mucosal immune system, epithelial cells and DCs form the first line that senses microbiota, and commensal bacteria modify the mucosal immune response mainly by regulating the mucosal innate response. A recent report demonstrated that miR-10a affects the proinflammatory phenotype in athero-susceptible endothelium by regulating NF-κB activation. When we compared miR-10a expression by the spleen and the intestines of RAG$^{-/-}$ mice housed under SPF and GF conditions, similar to those of WT mice, miR-10a was highly expressed in the intestines, but it was expressed at very low levels in the spleens of GF RAG$^{-/-}$ mice. Furthermore, intestinal miR-10a expression was decreased in SPF RAG$^{-/-}$ mice compared with that in GF RAG$^{-/-}$ mice (Fig. 1*C*). We then investigated whether miR-10a expression varied between WT B6 mice housed under SPF conditions and GF conditions by examining different populations of intestinal cells by real-time PCR. In GF mice, IECs expressed the highest levels of miR-10a, whereas LPDC miR-10a expression was lower than that of IECs but significantly higher than in LP T cells and LP B cells. Furthermore, miR-10a expression by IECs and DCs was decreased under SPF conditions (Fig. 1*D*). In contrast, the expression of control miR-329 by IECs and DCs of GF mice was at a level comparable to that of SPF mice (Fig. 1*E*). Notably, miR-10a expression of LPDCs was much higher than that of splenic DCs (SPDCs) (Fig. 1*F*). Collectively, these data indicated that intestinal IECs and DCs preferably express miR-10a.

Bacterial stimulation downregulated DC miR-10a expression through TLR–TLR ligand interaction

To determine whether commensal bacterial stimulation inhibited DC miR-10a expression, we generated BMDCs from B6 mice by culturing bone marrow cells with GM-CSF for 8 d. BMDCs expressed miR-10a at a level slightly higher than SPDCs but lower than intestinal DCs (Supplemental Fig. 1). BMDCs were stimulated with lysates of *Escherichia coli* isolated from the intestinal lumen of B6 mice, as well as flagellated A4 commensal bacteria that produce immunodominant commensal Ag CBir1 flagellin (25). miR-10a expression was determined by real-time PCR 24 h later. As shown in Fig. 2*A*, BMDC miR-10a expression was downregulated by treatment with *E. coli* and A4 bacteria. Among many bacterial products, TLR ligands stimulate DCs via interaction with TLRs. To determine the roles of TLR ligands in *E. coli* and A4 bacteria downregulation of DC miR-10a expression, we treated BMDCs with various ligands for TLR1/2 bacterial lipopeptides *Pam3CSK* (N-palmitoyl-S-[2,3-bis(palmitoloxy)-(2RS)-propyl]-Cys-Ser-Lys4), TLR4 (LPS), TLR5 (*E. coli* Flagellin FliC), TLR9 (CpG oligodeoxynucleotide), and NOD2 (muramyl dipeptide). miR-10 expression was determined by real-time PCR. As shown in Fig. 2*B*, DC miR-10a expression was downregulated by ligands for TLR1/2, TLR4, TLR5, TLR9, and NOD2. Collectively, these data demonstrated that microbiota-derived TLR ligands inhibited DC miR-10a expression.

It was shown that the TLR–TLR ligand interacts through MyD88 (26–29). To determine the role of the MyD88 pathway in microflora-driven downregulation of miR-10a expression, we determined miR-10a expression by LPDCs obtained from WT and MyD88$^{-/-}$ B6 mice. As shown in Fig. 2*C*, MyD88 deficiency led to an increase in miR-10a expression by LPDCs compared with that in WT mice under SPF conditions. Consistently, treatment with lysates of *E. coli* and A4 bacteria, as well as a variety of TLR ligands, inhibited WT BMDC miR-10a expression but did not downregulate MyD88$^{-/-}$ BMDC miR-10a expression, demonstrating that MyD88 is involved in commensal bacterial downregulation of DC miR-10a expression (Fig. 2*B*).

Activation of NF-κB has been implicated in many functional aspects of TLR–TLR ligand interaction (26, 27). To determine whether the NF-κB pathway is involved in commensal bacteria and TLR ligand downregulation of DC miR-10a expression, we treated BMDCs with LPS in the presence or absence of the NF-κB inhibitor, Bay 11-7082, for 24 h. As shown in Fig. 2*D*, addition of NF-κB inhibitor alone increased the baseline expression of BMDC miR-10a. Furthermore, a blockade of NF-κB activation reversed the inhibition of DC miR-10a expression by LPS. These data indicated that NF-κB activation negatively regulates miR-10a expression and that commensal bacteria and their TLR ligand downregulation of DC miR-10a expression requires NF-κB activation.

IL-12/IL-23p40 is the target gene of miR-10a

miRNAs mainly function as negative regulators by binding with their target genes and blocking mRNA transcription or protein expression. A computer-based miRNA target-detection program was used to predict the potential target genes of miR-10a. IL-12/IL-23p40 was predicted as one of the candidate genes of miR-10a by various programs of miRNA target gene prediction. To determine whether IL-12/IL-23p40 gene is indeed the target of miR-10a, we constructed dual-luciferase reporter vectors containing the predicted seed sequence in the 3′-UTR of IL-12/IL-23p40, as well as the accordingly mutant vectors in which three random nucleotide

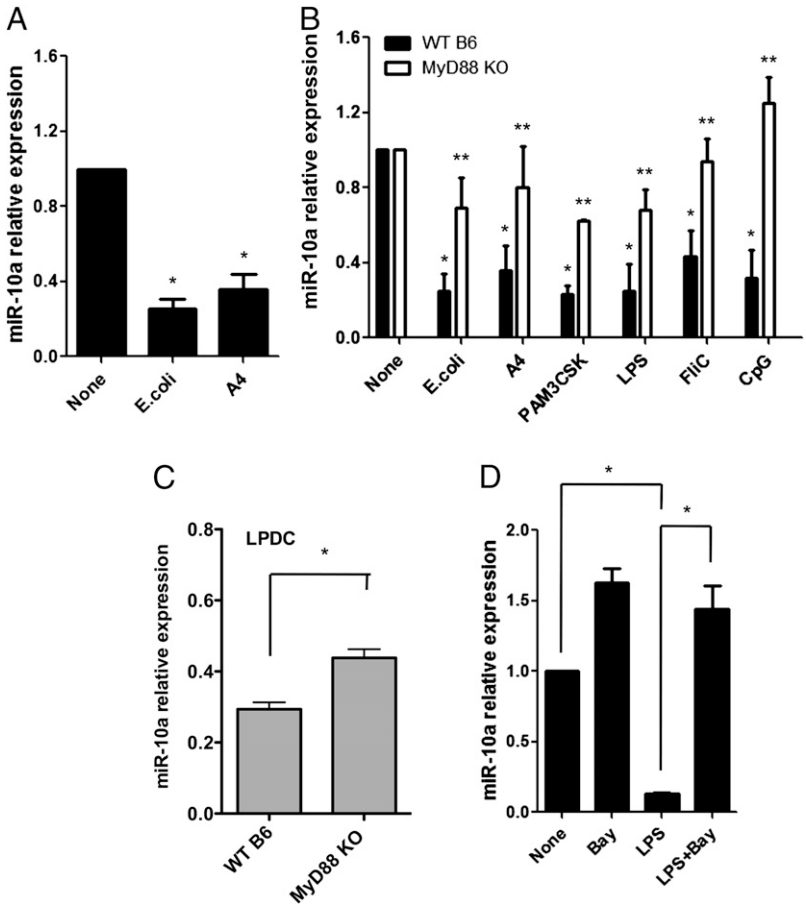

FIGURE 2. Microbiota downregulates miR-10a in BMDCs through TLR–TLR ligand interaction. *A*, BMDCs were generated from WT B6 mice and stimulated with commensal *E. coli* or flagellated A4 bacteria for 24 h. miR-10a expression was determined by real-time PCR. Expression of GAPDH was used as a reference gene control. The baseline expression of BMDC was arbitrarily set to 1.0, and the relative changes were analyzed compared with the baseline expression. $*p < 0.05$ compared with BMDCs treated with media alone. *B*, BMDCs generated from WT B6 and B6.MyD88 knockout (KO) mice were treated with various TLR ligands. miR-10a expression was analyzed by real-time PCR. Data are representative of three experiments. The baseline expression of BMDCs was arbitrarily set to 1.0, and the relative changes were analyzed compared with the baseline expression. $*p < 0.01$ compared with control BMDCs, $**p < 0.05$ compared with WT B6 BMDCs. *C*, miR-10a expression of LPDCs isolated from WT and MyD88$^{-/-}$ mice under SPF conditions was determined by real-time PCR and analyzed using expression of GAPDH as a reference gene. $*p < 0.05$ compared with WT LPDCs. *D*, BMDCs generated from B6 mice were treated with 1 μg/ml LPS in the presence or absence of NF-κB inhibitor (Bay 11-7082) for 24 h. miR-10a expression was analyzed by quantitative PCR. The baseline expression of BMDCs was arbitrarily set to 1.0, and the relative changes were analyzed compared with the baseline expression. $*p < 0.05$ compared with indicated groups. Data are representative of three experiments.

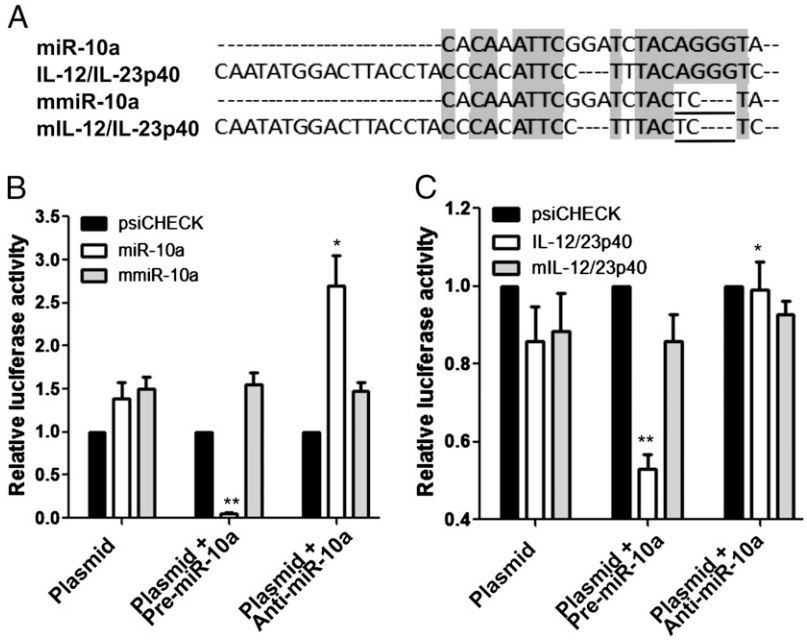

FIGURE 3. IL-12/23p40 is target gene of miR-10a. *A*, Sequence alignment of miR-10a with IL-12/23p40, mutant miR-10a (mmiR-10a), and mutant IL-12/23p40 (mIL-12/23p40); mutant nucleotides are underlined. Dual-luciferase reporter assays of vector construction with miR-10a/mmiR-10a (*B*) and IL-12/23p40–3′-UTR or mIL-12/23p40–3′-UTR (*C*) alone or in the presence of miR-10a precursor or inhibitor. Vector construction with miR-10a and mmiR-10a was used as a positive control. Decrease in *Renilla* luciferase was measured and normalized to firefly luciferase activity, and the constructed vector was normalized to empty psiCHECK-2 vector. $*p < 0.05$, $**p < 0.01$ compared with plasmid-alone group. Data are representative of three experiments.

mutations were introduced into the seed sequences (Fig. 3*A*). The empty vector psiCHECK-2 and the vector containing the theoretical miR-10a seed sequence were used as negative and positive controls, respectively. All of these vectors were used to transfect a murine RAW264.7 macrophage cell line alone or to cotransfect the synthesized precursor (mimics) or inhibitor of miR-10a, and the *Renilla* luciferase was normalized to firefly luciferase. As shown in Fig. 3*B*, when compared with the vector-alone–transfected group, the cotransfection of the miR-10a precursor remarkably repressed the activity of *Renilla* luciferase containing the seed sequence in the 3′-UTR of IL-12/IL-23p40 ($p < 0.001$). Moreover, a >33% reduction in luciferase activity was observed, but the cotransfection of miR-10a inhibitor upregulated the *Renilla* luciferase ~43% in IL-12/IL-23p40 ($p < 0.05$). These

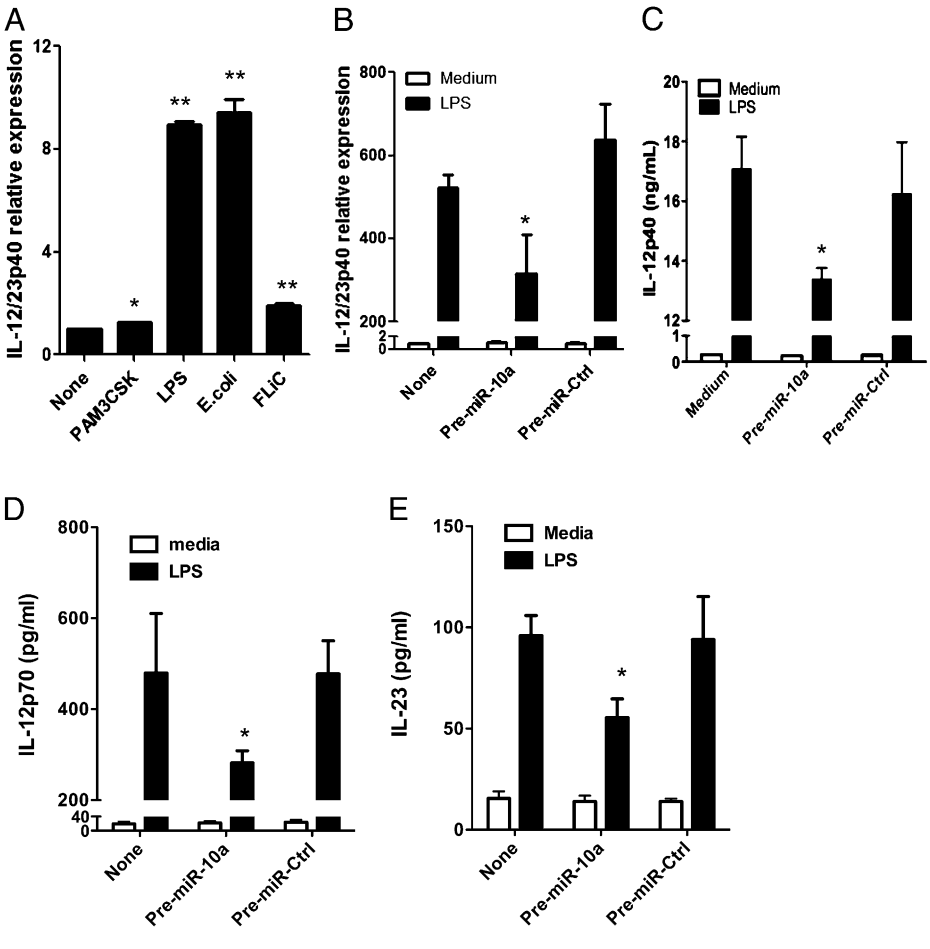

FIGURE 4. miR-10a inhibits BMDC IL-12/23p40 production. *A*, B6 BMDCs were treated with various TLR ligands for 4 h, and the IL-12/23p40 transcription level was analyzed by real-time PCR. Expression of GAPDH was used as a reference gene control. *B* and *C*, BMDCs were transfected with miR-10a precursor (Pre-miR-10a) and stimulated with LPS. IL-12/23p40 expression was analyzed by real-time PCR (*B*) or ELISA (*C*). Production of IL-12p70 (*D*) and IL-23 (*E*) was analyzed by ELISA. Scrambled microRNA precursor (Pre-miR-Ctrl) was used as a negative control. Data are representative of three experiments. $*p < 0.05$, $**p < 0.01$ compared with control BMDCs.

data indicated that miR-10a is constitutively expressed at a very low level in the RAW264.7 cell line, and its function can be strengthened by precursors but weakened by inhibitors. To verify the specific effect of miR-10a on IL-12/IL-23p40, a three-nucleotide mutation was introduced into the theoretical miR-10a binding site of the vector; no significant difference in *Renilla* luciferase activity was found when RAW264.7 cells were transfected with the mutated vector alone or cotransfected with miR-10a precursor or inhibitor (Fig. 3*C*). We observed similar results when these vectors were used to transfect HEK293A cells (data not shown). Collectively, these data indicated that miR-10 specifically downregulates IL-12/IL-23p40 expression.

miR-10a inhibited DC IL-12/IL-23p40 production stimulated by TLR ligands

To determine the role of miR-10a in DC production of IL-12/IL-23p40, we first investigated BMDC expression of IL-12/IL-23p40 stimulated by commensal bacteria and their TLR ligands. As previously reported, commensal *E. coli*, bacterial lipopeptides *Pam3CSK* (N-palmitoyl-S-[2,3-bis(palmitoloxy)-(2RS)-propyl]-Cys-Ser-Lys4), LPS, and *E. coli* Flagellin FliC stimulated BMDC IL-12/IL-23p40 expression at various levels (Fig. 4*A*). We then transfected BMDCs with miR-10a mimics to obtain DC ectopic expression of miR-10a. BMDCs transfected with control miRNA served as a negative control. Twenty-four hours later, BMDCs were stimulated with 100 ng/ml LPS, and the expression of IL-12/IL-23p40 was analyzed by real-time PCR. The overexpression of miR-10a mimics significantly reduced LPS-induced DC expression of IL-12/IL-23p40 mRNA (Fig. 4*B*). To determine whether miR-10a inhibited DC production of IL-12/IL-23p40, as well as that of IL-12p70 and IL-23 protein, we measured IL-12/IL-23p40, IL-12p70, and IL-23 in culture supernatants. The ectopic expression of miR-10a inhibited DC production of IL-12/IL-23p40 (Fig. 4*C*), as well as that of IL-12p70 and IL-23 (Fig. 4*D*, 4*E*). These data collectively indicated that miR-10a inhibits DC production of IL-12/IL-23p40, thus also inhibiting IL-12p70 and IL-23. Considering the requirement of IL-12/IL-23 in the induction of Th1 and Th17 cell differentiation, these results demonstrated that miR-10a acts as a negative regulator of both innate and adaptive immune responses to microbiota and could play a role in the regulation of intestinal immune homeostasis and the pathogenesis of IBD.

Inflamed intestinal tissues in mice with colitis expressed low levels of miR-10a and high levels of IL-12/IL-23p40

It was shown that there is more commensal bacteria translocation through the intestinal epithelium in colitic rather than normal mice, and, as a result, the colitic animals are more susceptible to an enhanced stimulation by the commensal bacteria. To investigate whether miR-10a was differentially expressed in experimental colitis, we assessed the expression of IL-12/IL-23p40 and miR-10a in IL-10–deficient mice that had developed severe colitis. As shown previously, inflamed intestinal tissues of colitic mice produced more IL-12/IL-23p40 compared with those of normal mice (Fig. 5*A*). The increased intestinal IL-12/IL-23p40 was mainly produced by intestinal LPDCs, as intestinal LPDCs from colitic mice produced more IL-12/IL-23p40 compared with normal mice when stimulated with A4 bacteria (Fig. 5*B*). Consistently, intestinal LPDCs from colitic mice produced more IL-17p70 and IL-23 (Fig. 5*C*). Interestingly, although miR-10a expression levels were very low in the spleens of both colitic mice and normal mice, intestinal miR-10a expression was further decreased in colitic IL-$10^{-/-}$ mice compared with that in normal mice (Fig. 5*D*). miR-10a expression by IECs and LPDCs from colitic IL-$10^{-/-}$ mice was also lower compared with that in WT mice (Fig. 5*E*). This effect was not due to a lack of direct effect of IL-10, as it had no effect on miR-10a expression on DCs (Fig. 5*F*).

Discussion

Multiple levels of regulatory mechanisms control host intestinal immune homeostasis to microbiota (3, 30, 31). A coordinated interplay between commensal microbiota and mucosal immune

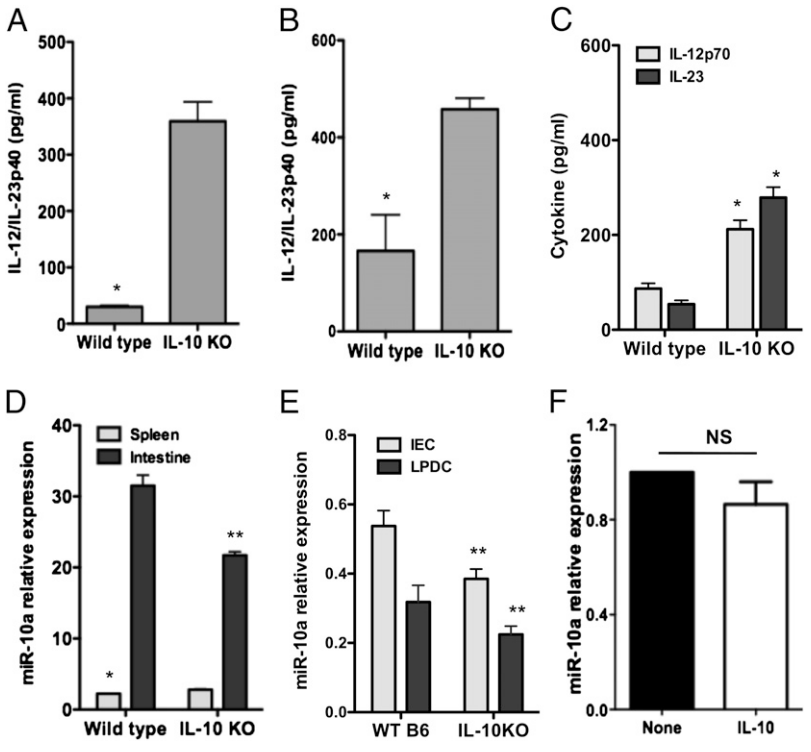

FIGURE 5. Inflamed intestinal tissues of colitic mice express high levels of IL-12/23p40 and low levels of miR-10a. *A*, Colonic tissues of B6.IL-10 knockout (KO) mice with severe colitis were cultured with media for 24 h, and IL-12/23p40 production in the supernatants was measured by ELISA. $*p < 0.01$ compared with colitic IL-10 KO mice. One representative of two independent experiments is shown. *B* and *C*, LPDCs were isolated from control WT B6 mice or colitic B6.IL-10 KO mice and stimulated with A4 bacteria for 24 h. Production of IL-12/23p40 (*B*) and IL-12p70 and IL-23 (*C*) in the supernatants was measured by ELISA. $*p < 0.05$ compared with colitic IL-10 KO mice. One representative of two independent experiments is shown. *D*, RNA was isolated from the intestines of control WT B6 mice or colitic B6.IL-10 KO mice, and miRNA expression profiles were determined by miRNA microarray. miR-10a expression was normalized to negative control of random sequences with the similar size. $*p < 0.01$ compared with intestines, $**p < 0.05$ compared with WT B6 intestines. *E*, miR-10a expression of IECs and LPDCs from WT and IL-10 KO mice was determined by real-time PCR. Expression of GAPDH was used as reference gene control. $**p < 0.05$ compared with WT B6 intestines. *F*, B6 BMDCs were stimulated with IL-10, and miR-10a expression was analyzed by real-time PCR. Expression of GAPDH was used as a reference gene control. Data are representative of three experiments.

responses is reciprocally regulated by each other to maintain the host intestinal immune homeostasis. Microbiota is not only important in shaping the development and function of mucosal immune systems (32–34), it also regulates inflammation as it is crucial for the induction of IBDs, as well as experimental arthritis and experimental autoimmune encephalomyelitis (35–38). However, the mechanisms involved in the regulation of mucosal immunity by microbiota are not well understood. In the current study, we found that microbiota downregulated mucosal DC miR-10a expression through TLR–TLR ligand interaction in a MyD88-dependent manner. miR-10a inhibited DC production of IL-12/IL-23p40 and, thus, could control the host innate response to microbiota, thereby contributing to the maintenance of intestinal homeostasis.

Several miRNAs have been implicated in the regulation of the innate immune response to bacterial stimulation. In response to TLR ligands and proinflammatory cytokines, macrophages and DCs express high levels of miR-147, miR-21, and miR-9 (18, 20). miR-155 expression is induced in bone marrow-derived macrophages in response to various TLR ligands (39). Certain TLR ligands and the cytoplasmic sensor retinoic acid-inducible gene I induce miR-146 expression in an NF-κB–dependent manner (40). Interestingly, those miRNAs can negatively regulate the activation of inflammatory pathways in innate cells. miR-146 was reported to directly inhibit several signaling molecules downstream of the TLRs, including *IRAK1*, *IRAK2*, and TNFR-associated factor 6, all of which are key mediators in inflammation (18, 40). Thus miR-146a suppresses the inflammatory pathway mediated by TLRs by repressing the translation of IRAK1 (18, 40). In contrast, a decrease in the expression of certain miRNAs on innate cells following TLR ligand stimulation has also been reported. Macrophage expression of miR-125b and let-7i is decreased in response to LPS stimulation (41, 42). However, it is still unclear how the microbiota regulates miRNAs and, thereby, contributes to the maintenance of intestinal homeostasis. In this report, we demonstrated that the IEC and LPDC expression of miR-10a was much lower in mice housed under conventional SPF conditions compared with those maintained in GF conditions, and these findings may imply the downregulation of miR-10a expression by microbiota. Indeed, treatment of BMDCs with the commensal bacteria *E. coli* and A4 bacteria, as well as with various TLR ligands, greatly inhibited miR-10a expression in a MyD88-dependent manner, in that MyD88 deficiency abrogated inhibition of miR-10a by microflora and their TLR ligands. Although these data indicated that TLR/MyD88 signaling is sufficient, we still do not know whether this is the only or predominant pathway negatively regulating miR-10a in vivo; other pathways may be involved as well. It is also intriguing that inhibition of the NF-κB pathway reversed TLR ligand inhibition of miR-10a (Fig. 2). However, it is still not clear whether such regulation is mediated directly by NF-κB binding of miR-10a or indirectly through interactions with other pathways; more work needs to be done to pin down the NF-κB pathway assessment of miR-10a expression.

miR-10a has been implicated in the regulation of development and pathogenesis of various tumors. In a recent report, miR-10a expression was shown to promote metastatic behavior of pancreatic tumor cells; repression of miR-10a was sufficient to inhibit invasion and metastasis formation. Thus, miR-10a was demonstrated to serve as a key mediator of metastatic behavior in pancreatic cancer. Our data indicated that miR-10a was predominantly expressed in the intestines and, thus, could play a key role in the regulation of a host response to a huge microbiota challenge. Interestingly, one of the target genes of miR-10a was IL-12/IL-23p40, in that coexpressing miR-10a precursor inhibited IL-12/IL-23p40 expression, whereas coexpressing miR-10a inhibitor promoted IL-12/IL-23p40 expression. IL-12 (p35/p40) and IL-23 (p19/p40) share the same p40 subunit and have emerged as key molecules in the regulation of both innate and adaptive immune responses (43, 44). Many studies have addressed the roles of IL-12 and IL-23 in the regulation of intestinal homeostasis, as well as in the pathogenesis of IBD (45–50). Thus, microflora downregulation of miR-10a allowing for expression of IL-12 and/or IL-23 could set up a low inflammatory environment in the intestines, which is important in promoting intestinal immune homeostasis through increasing epithelial barrier function and protective immunity under steady-state conditions. In contrast, IL-12 and/or IL-23 production stimulated by microflora through inhibition of miR-10a could also contribute to the progression of intestinal inflammation under inflammatory conditions. Indeed, in colitic IL-$10^{-/-}$ mice, miR-10a expression was decreased, whereas IL-12 and IL-23 production was increased in the intestines, as well as in IECs and LPDCs, compared with levels in normal mice, demonstrating a possible relationship between miR-10a regulation of IL-12 and IL-23 and development of colitis. These data indicate the inhibition of IL-12/IL-23p40 by miR-10 in response to a high level of commensal bacterial stimulation in colitic mice; however, more work is needed to understand its contribution to the pathogenesis of IBD.

In summary, our data demonstrated that microbiota regulates DC expression of IL-12/IL-23p40 through the inhibition of miR-10a. Through regulation of innate cell IL-12/IL-23p40 expression in response to microbiota stimulation, miR-10a mediates the host response to microbiota and, thus, could be an important mediator in the maintenance of host immune homeostasis, as well as in the pathogenesis of IBD. Manipulation of miR-10a expression could provide a new avenue of therapeutics for IBD.

Disclosures

The authors have no financial conflicts of interest.

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