Gut commensals suppress interleukin-2 production through microRNA-200/BCL11B and microRNA-200/ETS-1 axes in lamina propria leukocytes of murine large intestine

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Abstract

The role of microRNAs (miRNAs) in how microbiota influence the host intestinal immune system is not fully understood. We compared the expression profiles of miRNAs and mRNAs in lamina propria leukocytes (LPL) in the large intestines of germ-free (GF) and specific pathogen-free (SPF) mice. Microarray analysis revealed different expression profiles of miRNAs and mRNAs between GF and SPF mice. Quantitative real time-PCR (qRT-PCR) showed that the level of miR-200 family members was significantly higher in SPF mice than in GF mice. *In silico* prediction followed by qRT-PCR suggested that *Bcl11b, Ets1*, *Gbp7*, *Stat5b,* and *Zeb1* genes were downregulated by the miR-200 family. Western blotting revealed that the expression of BCL11B and ETS-1, but not ZEB1, in large intestinal LPL was significantly lower in SPF mice than in GF mice. Interleukin (IL)-2 production in cultured LPL upon stimulation with phorbol 12-myristate 13-acetate and ionomycin for 24 h was significantly lower in SPF mice than in GF mice. Conventionalization of GF mice substantially recapitulated SPF mice in terms of the expression of miR-200 family members and their target genes and IL-2 production in large intestinal LPL. Considering that BCL11B and ETS-1 reportedly function as transcription factors to activate the *Il2* gene, we propose that the presence of gut commensals suppresses IL-2 production in large intestinal LPL, at least in part through post-transcriptional downregulation of *Bcl11b* and *Ets1* genes by miR-200 family members.

Keywords: Gut microbiota; MicroRNA; BCL11B; ETS-1; Interleukin-2; Large intestine

1. Introduction

Small non-coding RNAs including microRNAs (miRNAs) function in post-transcriptional regulation of gene expression. miRNAs bind to partially complementary sequences in target mRNAs, and the mRNA molecules are then degraded or inhibited their translation into proteins [1, 2]. In the intestines, miRNAs contribute to gene expression regulatory networks to control mucosal immune development and responses [3, 4]. However, the entire picture of miRNA regulation of the intestinal immune system remains to be elucidated.

Gut microbiota modulate the development and function of the host intestinal immune system [5-7]. Although miRNAs have attracted attention for their roles in the interaction between gut microbiota and the host intestinal immune system [3, 8], limited information are available. Our present study therefore compared the global expression of miRNAs and mRNAs in the lamina propria leukocytes (LPL) in the large intestines of GF and SPF mice and identified differentially expressed miRNAs and their target genes.

2. Materials and methods

2.1. Animals

This study was approved by the Hokkaido University Animal Use Committee (approval no. 19-0017), and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University. Seven-week-old male SPF and GF BALB/c mice (n=6 in each group), which were purchased from Japan SLC (Hamamatsu, Japan), were euthanized as described below immediately upon being received from the distributor. In a separate experiment, six-week-old male GF mice (n=6) were conventionalized by intragastric administration of a fecal suspension obtained from six-week-old male SPF mice and co-housing with those SPF mice (n=6). Three to five pellets of fresh feces, which were obtained from SPF mice, were suspended in 600 μL of PBS followed by centrifugation for 10 min at 500 × *g*. The supernatant (200 μL in each mouse) were then administered to GF mice daily for three consecutive days. These GF and SPF mice were co-housed in a standard plastic cage in a

temperature-controlled (23 ± 2˚C) room under a 12-h light:dark cycle. They were allowed free access to tap water and standard laboratory rodent feed (Oriental Yeast, Tokyo, Japan). After 7 days of initial fecal administration, the conventionalized GF (Conv-GF) and SPF mice were euthanized as described below.

2.2. Isolation of large intestinal LPL

Large intestinal LPL were isolated according to Weigmann *et al.* [9] with some modifications. In brief, mice were euthanized by cervical dislocation under sevoflurane anesthesia. A laparotomy was made, and the entire length of the large intestine, i.e., cecum and colon, was excised. After removing the cecal patch, the intestine was opened longitudinally, thoroughly washed with ice-cold PBS, and then incubated in 10 mM dithiothreitol in 5% (v/v) fetal bovine serum (FBS, FB-1061/25, Biosera, Nuaille, France)/Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS (-)) for 10 min at RT with mixing. The tissue was further incubated in 10 mM EDTA in 5% (v/v) FBS/HBSS (-) for 20 min at 37°C with mixing followed by straining through gauze. The tissue retained by the gauze was cut into approximately 2-mm pieces and then incubated in 1.5 mg/mL collagenase D (Roche Diagnostics, Mannheim, Germany), 0.1 mg/mL DNase I (Roche Diagnostics), and 3 mg/mL dispase II (Roche Diagnostics) in 5% (v/v) FBS/complete HBSS for 60-90 min at 37°C under slow rotation. Thereafter, the solution was passed through a 40-μm cell strainer followed by centrifugation for 10 min at 450 × *g*. The precipitate was resuspended in 6 mL of 45% (v/v) Percoll solution (GE Health Care, Milwaukee, WI), and the cell suspension was then overlaid with 3 mL of 75% (v/v) Percoll solution. Following centrifugation for 20 min at 800 × *g* without braking, the LPL at the interphase of the two different Percoll solutions were collected and subjected to RNA isolation, western blot analysis, and cytokine production assays as described below.

2.3. Isolation of RNA and microarray analyses

Total RNA including small RNA was isolated from LPL using an miRNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Pooled samples in GF and SPF

mice were subjected to expression profiling of miRNA and mRNA. Microarray analyses including labeling, hybridization, scanning, and data processing were performed by Toray Industries (Tokyo, Japan) using 3D-Gene mouse miRNA and mRNA oligo chips that contain 1,900 and 23,474 antisense probe spots, respectively (Toray Industries). Using the background-subtracted signal intensity of all miRNAs and mRNAs in each microarray, the expression level of each miRNA and mRNA was globally normalized such that the median of all miRNAs and mRNAs for each sample was 25. Microarray data are deposited as a MIAME compliant study in NCBI's Gene Expression Omnibus [10] and are accessible through GEO Series accession number GSE157029 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE157029). For the prediction of miRNA targets, the web-based tool TargetScan (http://www.targetscan.org/) was used. For mRNAs, enrichment of gene ontology (GO) was calculated by the gene-ontology tool Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 online tool (https://david.ncifcrf.gov).

2.4 Quantitative real-time PCR (qRT-PCR) analysis

qRT-PCR analysis for miRNA and mRNA was performed as previously described [11]. The sequences of primers used for miRNA and mRNA are described in **Supplementary Tables 1** and **2**, respectively. For miRNA, synthetic *Caenorhabditis elegans* miRNA (Syn-cel-miR-39-3p, Qiagen) was spiked in. Relative expression levels of mRNA and miRNA were normalized to that of *Actb* and cel-miR-39-3p, respectively.

2.5. Western blot analysis

LPL were lysed with a buffer composed of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% (w/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, and cOmplete protease inhibitor cocktail (Roche Diagnostics). SDS-PAGE, western blotting, immune detection, and quantification of band intensities were performed as previously described [12]. Primary and secondary antibodies are listed in **Supplementary Tables 3** and **4**, respectively.

2.6. IL-2 production by LPL ex vivo

LPL were adjusted to 2×10^6 cells/mL, and 100 μL were added to each well of a 96-well round-bottom culture plate. The cells were cultured in RPMI1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μM β-mercaptoethanol, 25 ng/mL phorbol 12-myristate 13-acetate (PMA, Fujifilm Wako Pure Chemical, Osaka, Japan), and 1 μg/mL ionomycin (Fujifilm Wako Pure Chemical) at 37ºC in a humidified atmosphere of 5% CO_2-95% air. After 24 h, cultures were centrifuged for 10 min at 450 × *g*, and cell-free culture supernatants were harvested and then subjected to measurement of IL-2 by using an IL-2 Mouse ELISA Kit (M2000, R&D Systems) according to the manufacturer's instructions. The cell precipitates were subjected to RNA isolation and qRT-PCR analysis as described above.

2.7. DNA isolation and qRT-PCR for bacterial enumeration

DNA isolation and qRT-PCR for bacterial enumeration in the cecal contents were performed as previously described [12]. Eub338F (ACTCCTACGGGAGGCAGCAG) and Eub518R (TTACCGCGGCTGCTGG) were used as primers for qRT-PCR. For qRT-PCR standard, subcloned 16S rRNA gene fragment was prepared from *Blautia coccoides* (JCM 1395T) as previously described [12].

2.8. Statistical analysis

Results are presented as standard box plots with medians and interquartile ranges with minima and maxima. To compare the mean values of two groups, an unpaired *t* test was used. Data were analyzed using GraphPad Prism software for Macintosh (version 8, GraphPad Software, San Diego, CA). *P* values of <0.05 were considered to be statistically significant.

3. Results

3.1. miRNA expression profiles in large intestinal LPL differ between GF and SPF mice

To determine the effects of the presence of gut commensals on the expression of miRNAs in large intestinal LPL, we compared GF and SPF mice. As shown in the MA plot (**Fig. 1A**), microarray analysis for pooled samples in each group revealed that 23 miRNAs were higher (≥ 2-fold) in SPF mice than in GF mice. Among them, eight miRNAs were detected by qRT-PCR in individual mice, and the levels were significantly higher in SPF mice than in GF mice (**Fig. 1B**). Of note, five miRNAs (miR-141-3p, -200a-3p, -200b-3p, -200c-3p and -429-3p) are members of the miR-200 family. In contrast, 35 miRNAs were lower (≤ 0.5-fold) in SPF mice than in GF mice as shown by microarray analysis (**Fig. 1A**), and qRT-PCR detected seven miRNAs and showed that the levels of two miRNAs (miR-150-5p and -3971) were significantly lower in SPF mice than in GF mice (**Fig. 1C**).

3.2. mRNA expression profiles in large intestinal LPL differ between GF and SPF mice

We compared mRNA expression profiles in large intestinal LPL between GF and SPF mice. Microarray analysis for pooled samples in each group showed that 1,943 mRNAs were higher (≥ 2-fold) and 775 mRNAs were lower (≤ 0.5-fold) in SPF mice than in GF mice (**Fig. 2A**). Focusing on the lower mRNAs in SPF mice than in GF mice, GO analysis revealed a significant enrichment of genes associated with infection defense and the immune system (**Fig. 2B**). We predicted the target genes of eight miRNAs in which the level was significantly higher in SPF mice than in GF mice (**Fig. 1B**) and further narrowed down the target genes such that their annotated GO-biological process terms are included in those in **Fig. 2B**. The resultant predicted genes of individual miRNAs are shown in **Supplementary Table 5**. qRT-PCR analysis revealed that the mRNA levels of these genes were significantly lower in SPF mice than in GF mice (**Fig. 2C**). These genes are shown as red-colored symbols in **Fig. 2A**.

3.3. Protein expressions of BCL11B and ETS-1 in large intestinal LPL are lower in SPF mice than in GF mice

Western blot analysis was employed to estimate the protein expression levels of BCL11B, ETS-1, and ZEB1, which are encoded by predicted target genes *Bcl11b*, *Ets1*, and *Zeb1*,

respectively, in large intestinal LPL. Antibodies against BCL11B, ETS-1, and ZEB1 detected bands of ~120, ~55, and ~210 kDa, respectively, being consistent with the manufactures' technical information (**Fig. 3A**). The relative expression levels of BCL11B and ETS-1 were significantly lower in SPF mice than in GF mice, whereas no difference was observed in the levels of ZEB1 between the two groups (**Fig. 3B**).

3.4. IL-2 production in large intestinal LPL is lower in SPF mice than in GF mice

IL-2 concentrations in the culture supernatants of large intestinal LPL upon 24-h stimulation with PMA and ionomycin were significantly lower in SPF mice than in GF mice (**Fig. 3C**). Similarly, *Il2* mRNA levels tended to be lower in SPF mice than in GF mice (**Fig. 3D**).

3.5. Conventionalization of GF mice recapitulates SPF mice

The number of total bacteria in the cecal contents estimated by qRT-PCR of the bacterial 16S rRNA gene fragment was the same between Conv-GF and SPF mice (**Fig. 4A**). In large intestinal LPL, there was no significant difference in the level of miR-200a-3p, -200b-3p, -200c-3p, and -429-3p between Conv-GF and SPF mice (**Fig. 4B**). In addition, no significant difference in the mRNA level of *Bcl11b*, *Ets1*, and *Zeb1* genes was observed between Conv-GF and SPF mice (**Fig. 4C**). Likewise, the relative expression level of BCL11B, ETS-1, and ZEB1 did not differ between Conv-GF and SPF mice (**Fig. 4D**). IL-2 concentrations in the culture supernatants of large intestinal LPL upon 24-h stimulation with PMA and ionomycin were the same between Conv-GF and SPF mice (**Fig. 4E**), whereas *Il2* mRNA levels were significantly higher in SPF mice than in Conv-GF mice (**Fig. 4F**).

4. Discussion

The present study clearly showed that miRNA expression profiles in large intestinal LPL are different between GF and SPF mice. LPL are composed mainly of lymphocytes, dendritic cells, and macrophages. Our data therefore suggest that the presence of gut commensals influences the expression of specific miRNAs in the immune cells existing in the lamina propria of large

intestinal mucosa. Notably, all members of miR-200 family, i.e., miR-141-3p, -200a-3p, -200b-3p, -200c-3p, and -429-3p, were upregulated in SPF mice as compared to GF mice. A previous study described that miR-200 family members inhibit the terminal differentiation of effector $CD8^+$ T cells and promote formation of memory $CD8^+$ T cells, which is partly due to suppressing target gene *Zeb2* expression [13]. However, the present microarray analysis showed no difference in the mRNA level of *Zeb2* gene in large intestinal LPL between GF and SPF mice.

We examined the mRNA expression profiles in large intestinal LPL to explore target genes for miRNAs upregulated by gut commensals. Clearly, GO analysis of genes in which the presence of gut commensals decreased the mRNA levels showed a significant enrichment of genes associated with infection defense and the immune system. Among them, we predicted five genes of miR-200 family targets, i.e., *Bcl11b*, *Ets1*, *Gbp7*, *Stat5b,* and *Zeb1*, and qRT-PCR analysis confirmed that the mRNA levels of these genes were lower in SPF mice than in GF mice. Repression of *Ets1* by the miR-200 family is reportedly implicated in the erythropoietin inhibition of epithelial-mesenchymal transition (EMT) [14], a biological program during which epithelial cells lose their cell identity and acquire a mesenchymal phenotype, and in the angiogenic response of endothelial cells [15]. In addition, repression of *Stat5b* by the miR-200 family has been shown to promote progesterone metabolism in the pregnant uterus [16]. Furthermore, regulation of EMT *via* the miR-200/*Zeb1* axis has been extensively studied in terms of cancer progression, including of colorectal cancer [17]. Thus, it has been already demonstrated that the miR-200 family regulates the expression of *Ets1*, *Stat5b,* and *Zeb1* genes.

The present study showed that IL-2 production in large intestinal LPL was lower in SPF mice than in GF mice. It could be that the decreased production of IL-2 in large intestinal LPL isolated from SPF mice was due to a smaller number of IL-2-producing cells, i.e., mainly $CD4^+$ T cells. However, the number of large intestinal lamina propria $CD4^+$ T cells is reportedly higher in SPF mice than in GF mice [18]. We thus focused on three target genes, *Bcl11b*, *Ets1,*

and *Zeb1*, which are reportedly involved in the regulation of IL-2 production in T cells [19-21]. BCL11B has been shown to activate the *Il2* gene as a transcription factor in $CD4^+$ T cells [19]. Similarly, ETS-1 functions as a transcription factor to promote *Il2* gene expression through the nuclear factor of activated T-cells [20]. In contrast, ZEB1 reportedly suppresses *Il2* gene activation by cooperating with C-terminal-binding protein 2 and histone deacetylase 1 [21]. In the present study, western blot analysis showed that BCL11B and ETS-1 expressions in large intestinal LPL were decreased by the presence of gut commensals, being consistent with changes in their mRNA levels. These results suggest that gut commensals suppress BCL11B and ETS-1 expression *via* degradation of their mRNAs, which would be due to post-transcriptional regulation by the miR-200 family. In contrast, the ZEB1 protein level was not altered by gut commensals despite a significant decrease in its mRNA level. This is probably due to post-translational regulation of ZEB1 expression, including ubiquitination and proteasomal degradation [22]. Furthermore, conventionalization of GF mice substantially recapitulated the SPF mice in terms of the expression of miR-200 family members and their target genes and IL-2 production. Collectively, it is conceivable that miR-200/BCL11B and miR-200/ETS-1 axes contribute to the decrease of IL-2 production in large intestinal LPL, probably in $CD4^+$ T cells, by the presence of gut commensals.

It remains unclear how gut commensals influence miRNA expression in large intestinal LPL. Our initial prediction was that gut microbial metabolites and/or cellular constituents might activate directly the expression of miR-200 family members in lamina propria T cells in the large intestine. However, our preliminary experiments showed that supplementation with short-chain fatty acids (SCFAs), gut microbial fermentation products of indigestible carbohydrates, failed to alter the levels of miR-200 family members in cultured LPL isolated from the large intestines of SPF mice (**Supplementary Fig. 1**). In addition, LPL isolated from the large intestines of GF mice were cultured with fecal extracts prepared according to Brimnes *et al.* [23] from cecal contents of GF and SPF mice. The levels of miR-200 family members were unaffected not only by SCFAs but also by fecal extracts (**Supplementary Fig. 2**). We thus

suspect that indirect action of gut commensals, e.g., through epithelial cells, may alter the expression of miR-200 family members in large intestinal LPL. Further studies are needed to elucidate the cellular and molecular mechanisms by which gut commensals influence miRNA expression in large intestinal LPL.

It is worth discussing the physiological implications of commensals suppression of IL-2 production in large intestinal LPL. IL-2 is produced mainly by activated $CD4^+$ T cells and plays a crucial role not only in protective immunity by inducing effector Th1 and Th2, but also in peripheral immune tolerance by maintaining Tregs [24]. It is thus possible that commensal suppression of IL-2 production in large intestinal LPL, probably in $CD4^+$ T cells, results in the reduction of both protective immunity and immune tolerance. Nevertheless, from an evolutionary perspective, suppression of IL-2 production may contribute to the mutualism of gut commensals and the host through reduced protective immunity, which results in both permitting colonization and inhibiting inflammation.

In conclusion, we propose that the presence of gut commensals suppresses IL-2 production in large intestinal LPL, at least in part through post-transcriptional downregulation of *Bcl11b* and *Ets1* genes by miR-200 family members.

Acknowledgments

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Figure legends

Figure 1 Comparison of miRNA expression profiles in large intestinal LPL between GF and SPF mice. Chart *A* shows the MA plot of average of miRNA expression (log2, x-axis) and fold change (log2, y-axis) calculated from microarray data of pooled samples in each group. Red- and blue-colored symbols with miRNA names represent higher (≥ 2-fold) and lower (≤ 0.5-fold) miRNAs in SPF mice, respectively, which were validated by qRT-PCR in individual mice as shown in charts *B* and *C*. In charts *B* and *C*, results are presented as standard box plots composed of medians and interquartile ranges with minima and maxima, n=6 in each group. White and gray boxes represent GF and SPF mice, respectively. Data for SPF mice are shown relative to their levels in GF mice, which were set to 1. Mean values between groups were compared using an unpaired *t* test. * $P<0.05$ *vs.* GF mice.

Figure 2 Comparison of mRNA expression profiles in large intestinal LPL between GF and SPF mice. Chart *A* shows the MA plot of average of mRNA expression (log2, x-axis) and fold change (log2, y-axis) calculated from microarray data of pooled samples in each group. Red-colored symbols with gene symbols represent lower (≤ 0.5-fold) mRNAs in SPF mice, which were validated by qRT-PCR in individual mice as shown in chart *C*. Charts *B* shows the GO-biological process terms of the genes in which the mRNA level was lower (≤ 0.5-fold) in SPF mice as analyzed by microarray. In chart *C*, results are presented as standard box plots composed of medians and interquartile ranges with minima and maxima, n=6 in each group. White and gray boxes represent GF and SPF mice, respectively. Data for SPF mice are shown relative to their levels in GF mice, which were set to 1. Mean values between groups were compared using an unpaired *t* test. * $P<0.05$ *vs.* GF mice.

Figure 3 Protein expressions of BCL11B, ETS-1, and ZEB1 and IL-2 production in large intestinal LPL of GF and SPF mice. Chart *A* shows western blots of BCL11B, ETS-1, ZEB1, and β-actin. Chart *B* shows the relative levels of BCL11B, ETS-1, and ZEB1, which were estimated from band intensities of western blots. Charts *C* and *D* show IL-2 concentration in the

culture supernatant and *Il2* mRNA level in the cells, respectively. The cells were cultured in the presence of 25 ng/mL PMA and 1 μg/mL ionomycin for 24 h. In charts *B* and *D*, the levels of BCL11B, ETS-1, ZEB1, and *Il2* mRNA are shown relative to their levels in GF mice, which were set to 1. The levels of BCL11B, ETS-1, and ZEB1 were normalized to the levels for β-actin. Results are presented as standard box plots composed of medians and interquartile ranges with minima and maxima. White and gray boxes represent GF (n=5) and SPF (n=6) mice, respectively. Mean values between groups were compared using an unpaired *t* test. * $P<0.05$ *vs.* GF mice.

Figure 4 Expression of miR-200 family members and their target genes and IL-2 production in large intestinal LPL of conventionalized GF (Conv-GF) and SPF mice. Chart *A* shows the number of total bacteria estimated by qRT-PCR of the bacterial 16S rRNA gene fragment in the cecal contents. Charts *B* and *C* show the relative level of miR-200 family members and their target genes, respectively. Chart *D* shows the relative levels of BCL11B, ETS-1, and ZEB1, which were estimated from band intensities of western blots. The levels of BCL11B, ETS-1, and ZEB1 were normalized to the levels for β-actin. Charts *E* and *F* show IL-2 concentration in the culture supernatant and *Il2* mRNA level in the cells, respectively. The cells were cultured in the presence of 25 ng/mL PMA and 1 μg/mL ionomycin for 24 h. Results are presented as standard box plots composed of medians and interquartile ranges with minima and maxima. White and gray boxes represent Conv-GF (n=6 in chart *A*; n=4 in charts *B*-*F*) and SPF (n=6) mice, respectively. In charts *B, C*, and *F*, data for Conv-GF mice are shown relative to their levels in SPF mice, which were set to 1. Mean values between groups were compared using an unpaired *t* test. * $P<0.05$ *vs.* SPF mice.