Transient downregulation of miR-671-5p promotes IL-10 mRNA increase in porcine moDCs stimulated with the probiotic BB12

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Short Report

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Abstract

**Background:** Previous work showed that the microRNA (miRNA) miR-671-5p was upregulated in monocyte-derived dendritic cells (moDCs) stimulated with *Bifidobacterium animalis subsp. lactis* BB12 (BB12) with no increase in IL-10 after six hours of stimulation. In this work, we performed an in silico prediction of genes targeted by miR-671-5p and which are the terms and pathways involved with it. Also, miR-671-5p was transiently downregulated to assess its effect on IL-10 regulation.

**Methods and Results:** First, we performed a Gene Ontology enrichment analysis to predict immune response terms and pathways involved with miR-671-5p. Some of the terms and pathways found were related to the immune response promoted by the probiotic, as the terms “negative regulation of the inflammatory response to an antigenic stimulus” and “cancer” were highlighted. Then, to assess the role of miR-671-5p in IL-10 regulation, moDCs were derived from porcine peripheral blood and later transfected with miR-671-5p antisense oligonucleotide (ASO). Flow cytometry was employed to evaluate the transfection efficiency. Then, the moDCs were stimulated with BB12, and the expression of IL-10 was assessed by RT–qPCR and ELISA. An increase in IL-10 transcript in miR-671-5p-ASO-transfected moDCs stimulated with BB12 was observed compared with moDCs stimulated with BB12 but not transfected. These results suggest the participation of miR-671-5p as a negative regulator of IL-10.

**Conclusion:** These findings suggest that miR-671-5p participates in the downregulation of IL-10, as previously predicted in silico by our work group. miR-671-5p could play an essential role in the immunomodulation promoted by the probiotic BB12.

1. Introduction

Several reports support that the microbiota and probiotic microorganisms influence the host's immune system; nonetheless, the cause-effect of this relationship has not been completely established [1–4]. Although the participation of microRNAs (miRNAs) in gene regulation is still in its early stages, the "probiotic-miRNAs-host/gut microbiota" axis has emerged to incorporate even more complexity into the relationship between microbiota and the host. miRNAs are small noncoding RNAs of ~18–24 bp that hybridize imperfectly with diverse regions of mature mRNAs; consequently, the message is degraded, or its translation is blocked. Thus, they could affect genetic expression by various stimuli. For example, in innate immune cells, antigen processing and presentation cells, miRNAs can be expressed by activating signaling pathways [1–3]. The profile of cytokines in response to microorganisms is strain specific, and a variety of miRNAs are expressed after a stimulus, as demonstrated by different studies. This knowledge is essential to understanding immune response regulation and how microbiota microorganisms and probiotics influence human health and illnesses such as colon cancer or autoimmune diseases. The probiotic BB12 has been one of the most studied probiotics since it was first found in the stools of breastfed healthy babies in 1899 [4]. Previously, our workgroup identified the profile of miRNAs in porcine moDCs in response to the probiotic BB12 [5, 6]. One of these miRNAs was miR-671-5p, which is involved in antigen processing and presentation. In this work, we performed transient downregulation of porcine
moDCs with antisense oligonucleotide of miR-671-5p to investigate its influence on the regulation of IL-10 transcript, a cytokine capable of inhibiting the inflammatory response and guiding the immune response to homeostasis.

2. Materials And Methods

2.1 Target Gene Prediction and GO and KEGG Pathway Analyses

The prediction of target genes of miRNA miR-671-5p was performed by miRWalk (RRID:SCR_016509) [7]. The in silico analysis allowed the interaction of miR-671-5p with the three regions (5'UTR, CDS, and 3'UTR) of its target mRNAs. Genes were selected by their value of minimal free energy (≤-25 kcal/mol) followed by their best target on the same mRNA (Supplementary file 1). Then, a Gene Ontology (GO) analysis was performed to determine whether those genes are involved in immune system processes and the main pathways by KEGG (RRID:SCR_012773) using Cytoscape (RRID:SCR_015784) (http://www.cytoscape.org/) (Supplementary file 2). A hypergeometric test ($p < 0.01$) was used to identify enriched terms of GO and KEGG pathway analysis. The network of pathways was constructed with ClueGO, an application of Cytoscape.

2.2. Monocyte Dendritic Cell (moDC) Derivation

Monocyte isolation was performed as described previously [6]. Briefly, after isolation, monocytes were cultivated for derivation to moDCs for five days in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Co., Carlsbad, California, USA) supplemented with L-glutamine (10 µL/mL), gentamicin (50 µg/mL), penicillin/streptomycin (100 UI/mL), amphotericin B (2.5 µg/mL), β-mercaptoethanol (0.033%), and 10% fetal bovine serum (FBS). Unless otherwise stated, all reagents from Sigma–Aldrich, St. Louis, Missouri, USA (Sigma). The medium was supplemented with recombinant porcine GM-CSF (40 ng/mL) and IL-4 (50 ng/mL) (R&D Systems, Abingdon, UK) on day one and a half of the concentration of both cytokines on day three of derivation to achieve moDC differentiation [8]. Cell cultures were seeded in 6-well plates (Corning Inc., Corning, NY, USA) and incubated at 37°C in 5% CO$_2$.

2.3. Bacteria Culture

*Bifidobacterium animalis* subsp. *lactis* BB12 was cultivated on Man Rogosa Sharpe broth (MRS; BD Difco, Sparks, Maryland, USA) supplemented with 0.05% L-cysteine (Alfa Aesar, Ward Hill, Massachusetts, USA) at 37°C for five days in anaerobiosis (reseeding at 72 h) using effervescent tablets for CO$_2$ production [9]. The bacterial culture was centrifuged, and the precipitate was washed 2X in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, and 4.2 mM KH$_2$PO$_4$) to eliminate residues of MRS broth. The concentration of the bacterial suspension was determined using an optical density (600 nm) and adjusted according to the equation $y = 5E-09x + 0.052$. 
2.4. MoDCs transfection with the antisense oligonucleotide (ASO) miRNA ssc-miR-671-5p and stimulation with BB12 and Pam3CSK4

The miR-671-5p-ASO (5’-mCmCmUmCmAmGmGmCmUmUmCCmU-3’) was designed with Gapmer (shown in bold font in the sequence), containing 2’O-Methyl-RNA bases in the extremes of the oligonucleotide that confer protection to the oligo from nuclease degradation and DNA nucleotides in the center, with the finality to form an RNA/DNA duplex-helix with the microRNA that is inactivated by RNase H cleavage [10, 11]. Then, to ensure the correct function of the transfection system, we used a scrambled oligonucleotide similar to the miRNA but tagged with 5’6-carboxyfluorescein (FAM) fluorochrome (5’FAM-CAGUACUUUUGUGUAGUACAA-3’). The sequences of the mature miRNA ssc-miR-671-5p (5’-AGGAAGCCCUGGAGGGCUGGAGG-3’), the designed miR-671-5p-ASO, and the scramble control (5’FAM-SCR-oligonucleotide) were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa, USA). The moDCs were cultivated in 24-well plates (2 × 10^5 cells/mL in 500 µL DMEM). For the nontransfected groups, on the seventh day of differentiation, the BB12-stimulated moDC group, bacteria were added to the cell culture (1:100 moDCs/bacteria) for 4 h. For the transfected groups, transfection was carried out using Lipofectamine®-RNAiMAX (Thermo Fisher, Waltham, MA, USA) according to the manufacturer’s instructions. Lipofectamine®-RNAiMAX and ASO of ssc-miR-671-5p were employed to transfect moDCs of three groups on the fifth day of differentiation for 48 h. Then, BB12 (1:100 moDCs/bacteria) (Lipo + miR-671-5p-ASO + BB12) or Pam3CSK4 (100 ng) was added as indicated. Nontransfected, transfected cells only with Lipofectamine®-RNAiMAX and miR-671-5p-ASO (Lipofectamine®-RNAiMAX + ASO)-transfected moDCs but not stimulated with BB12 were used as a negative control of transfection in the different experiments. Transfected moDCs with 5’FAM-SCR-oligonucleotide for 48 h were used to estimate the efficiency of the transfection system employing a FACS球星 III™ cytometer (BD Biosciences, San Jose, California, USA) and the FACS Diva program. The antibodies used to characterize the moDCs by flow cytometry are listed in Supplementary file 3.

2.5. RNA Isolation and RT–qPCR Analysis

Total RNA from BB12-stimulated moDCs and Lipo + miR-671-5p-ASO + BB12 moDCs was isolated using the PicoPure™ RNA Isolation Kit (Thermo Fisher Scientific, Baltics UAB, Valencia, Lithuania). The RNA concentration was measured using a NanoDrop One (Thermo Scientific) and then used to perform reverse transcription (RT) to synthesize cDNAs. The RT mix reaction contained 10X PAP buffer, 1 mM ATP, 10 µM RT-primer, 1 mM dNTPs mix, M Mu-LV RT (NEB M0253S, 10000 units), and Poly (A) polymerase (NEBM0276S, 100 units), all from New England BioLabs Inc., Ipswich, Massachusetts, USA. RT was carried out in an MJMini Personal Thermal cycler from BIO-RAD (Irvine, CA, USA) under the following conditions: 1 h at 37°C, 5 min at 95°C, and a final cooling step at 4°C. Then, cDNA was used to perform qPCR with the enzyme 5xHOT FIREpol® EvaGreen® ROX (Solis Biodyne, Tartu, Estonia) and the couple of primers for ssc-miR-671-5p, Fw 5’CAGAGGAAGCCCCTGGAGG3’, Rv 5’CAGTTTTTTTTTTTTTAGCCCAATTG3’. The expression of ssc-U6 was detected using the primers Fw
5′CAGCACATATACTAAAATTGGAACG3′ and Rv 5′ACGAATTTGCGTGTCATCC3′ and used as an endogenous control. qPCR was performed using a QuantStudio 6 Flex thermocycler (Applied Biosystems, Carlsbad, CA, USA) for 20 s at 64°C and 20 s at 72°C for 40 cycles. The expression of miR-671-5p was calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of the IL-10 transcript was carried out using the primers Fw 5′TGAGAACAGCTGCATCCACTT3′ and Rv 5′TCTGGTCCTTCGTTTGAAAGAA3′ from Sigma (Merck, Darmstadt, Germany) and the enzyme Brilliant III Ultra-Fast SYBR® (Life Technologies, Carlsbad, CA, USA) at 55°C for 30 s for 40 cycles.

2.6 ELISA

ELISA was used to quantify the IL-10 cytokine in the moDC groups (control, BB12-stimulated, Lipo + miR-671-5p-ASO, Lipo + miR-671-5p-ASO + BB12, and Lipo + miR-671-5p-ASO + Pam3CSK4). To perform ELISA, 100 µL of the culture supernatant of every group of moDCs, ELISA plates, and reagents from the DuoSet Ancillary Reagent Kit2 (Cat. No. DY008), capture and detection antibodies against porcine IL-10 (Cat. No. DY693B) from R&D Systems, Minneapolis, Minnesota, USA.

2.7. Statistical Analysis

Statistical analysis was performed using NCSS 2022 software. Data are expressed as the mean ± standard error of the mean (SEM). The results were compared using a paired t test. A value of $p < 0.05$ was considered statistically significant.

3. Results And Discussion

3.1 Gene Ontology and KEGG Analysis

miRNAs have been mainly studied in processes such as cancer and cardiovascular events. In contrast, the miRNA profile and its effects in response to probiotics and other microbiota microorganisms are still scarce [12]. Previously, in our workgroup, the miRNA miR-671-5p was upregulated in porcine monocytes stimulated with the probiotic BB12 [(NCBI GEO dataset (GSE132995)] and validated in moDCs [6]. In the present work, we performed functional analysis focused on immune system processes from a list of genes predicted to be targeted by miR-671-5p. The study resulted in 14,841 genes selected with a value of minimal free energy (mfe) ≤ -25 kcal/mol and included only the best target per gene. Then, Gene Ontology (GO) enrichment analysis was performed to analyze the function of immune processes of the target genes, and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated which of the predicted target genes were mainly enriched in pathways. The ClueGo network of terms and pathways [13] is presented in (Fig. 1). The network contains 48 terms in 23 groups of immune system processes and pathways involved with miR-671-5p. Among them, negative regulation of the inflammatory response to an antigenic stimulus, focal adhesion, cell adhesion molecules, defense response to bacteria, immune response-activated cell surface receptor signaling pathway, and MAPK signaling pathway are directly related to the modulation of the immune response associated with symbiosis of gut microbiota and the host [4, 14].
Other functions and pathways are involved in cancer (microRNAs in cancer, pathways in cancer, small cell lung cancer), and there is a recent association between miR-671-5p in human cancers. The decrease in miR-671-5p expression is linked with metastasis and a poor prognosis in breast cancer [15]. Conversely, the overexpression of miR-671-5p is associated with the acceleration of proliferation, migration, and invasion of colon cancer cells [16] and prostate cancer [17]. As such, miR-671-5p has been proposed as a possible colon cancer biomarker. Findings on the expression of miR-671-5p in human cancers are contradictory. They should not be compared since they are tissue dependent, with various factors involving the microenvironment, hormones, cytokines, and activation of immune receptors, among others [18].

3.2 Transient downregulation of miR-671-5p

Previously, according to the in silico prediction, the mRNA of IL-10 could be a target of miR-671-5p (minimal free energy = -41.2 kcal/mol), and a modest decrease in IL-10 was found in moDCs stimulated for six hours with the probiotic BB12 [6]. Then, to investigate whether miR-671-5p downregulates the IL-10 transcript after BB12 stimuli in moDCs, transient silencing of miR-671-5p was carried out using porcine moDCs [20, 21]. Flow cytometry was employed to determine the transfection efficiency. 5’FAM-SCR (a fluorescent scramble sequence) was internalized in the cells using a cationic lipid (Lipofectamine®-RNAiMAX). The flow cytometry strategy used was previously reported by Rodríguez-Gómez et al. [19]; first, moDCs according to the size and complexity parameters (Fig. 2A). Then, myeloid cells (CD172a+) expressed 5’FAM-SCR (Fig. 2B). These cells were later analyzed for the expression of CD172a⁺ and SLA-DR⁺, a marker of mature immune presenting cells (Fig. 2C). The transfection efficiency was 46.3±28.4% (mean ± SD), similar to what has been observed in cell lines using the same transfection system [22, 23]. This decrease was sufficient to downregulate miR-671-5p expression in the Lipo+miR-671-5p-ASO+BB12 moDC group in comparison with the BB12-stimulated moDC group (Fig. 2D). All experiments were performed after five days of differentiation plus 48 h of transfection, followed by four h of stimulation with BB12 if it corresponded (n=3).

3.3. Quantification of IL-10

Subsequently, we expected that miR-671-5p downregulation would increase IL-10 transcripts. Indeed, the results indicate that the downregulation of miR-671-5p increases the mRNA expression of IL-10 (Fig. 3A). Then, the production of IL-10 cytokines was determined in the following groups of moDCs: Control, BB12-stimulated, Lipo+miR-671-5p-ASO, Lipo+miR-671-5p-ASO+BB12. Given that the probiotic BB12 is mainly recognized by Toll-like receptor 2 (TLR2) expressed by antigen-presenting cells, another group of moDCs was stimulated with the synthetic ligand of TLR2 Pam3CSK4 (miR-671-5p-ASO-Pam3CSK4). All groups produced IL-10 cytokines, but no significant differences were found (Fig. 3B). Despite IL-10 cytokine showing a tendency to increase in all the transfected groups of moDCs, it was not remarkable, probably because 4 h of stimuli with the probiotic was not sufficient for that aim. IL-10 is an autocrine cytokine, and an increase in this cytokine was previously found at 24 h of stimulation with BB12 [6, 24], and it was TLR2-dependent in porcine monocytes stimulated with BB12. These findings indicate that miR-671-5p
may participate in the downregulation of IL-10 transcripts during the first four hours of stimulation with probiotic BB12.

Given that pigs are considered a better model to study human microbiota, future *in vivo* studies could help examine the participation of miR-671-5p and others in the regulation of IL-10 with the development of cancers that are in communication with microbiota, such as colon cancer, where the overexpression of miR-671-5p has been linked with poor prognosis [16, 17]. Additionally, in some types of cancers, the immunosuppressive role of IL-10 could facilitate tumor immune escape. However, these findings need further experiments.

**Statements And Declarations**

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**Competing Interests:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Author Contributions:** Conceptualization, M.B.P. and V.M.H.; Data acquisition: M. B.-P, M. R.-S., and V. B.-P. Writing-Original Draft Preparation, M.B.P.; Writing-Review & Editing, J. H. and V. M-H.; Funding Acquisition, V.M.H.

**Ethical Approval:** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**References**


**Figures**

**Figure 1**

ClueGo network of GO enrichment of immune response terms and pathway analysis. Each node represents a pathway. The node size reflects the pathway's enrichment significance; all terms included have a p<0.01 value in the hypergeometric analysis. Node color represents the group to which they belong. Mixed coloring means that the specific node belongs to multiple classes.

**Figure 2**

Transfection efficiency assessed by flow cytometry. **A.** Nontransfected monocyte-derived dendritic cells (moDCs) were selected according to their size and complexity parameters, followed by doublet depletion. **B.** Selection of myeloid and Lipo+miR-671-5p-ASO transfected moDCs population (P4) CD172a+ 5´FAM-SCR+. **C.** P5 shows CD172a+SLA-DR-transfected moDCs, and P6 shows CD172a+SLA-DR+ moDCs. The transfections were performed for 48 h with Lipofectamine®-RNAiMAX and 5’FAM-SCR. The transfection efficiency of the P6 population was 50.2%. **D.** Expression of miR-671-5p by RT–qPCR from moDCs stimulated with BB12 and moDCs transfected with an antisense oligonucleotide (ASO) and stimulated with BB12 is presented in the panel. The t test analysis was used to find significant differences in the expression of miR-671-5p between the groups of cells (*p<0.05), n=3.
Figure 3

Transient downregulation of miR-671-5p increases the expression of IL-10 mRNA but not the cytokine protein level after four h of stimulation with BB12. **A.** Fold change of IL-10 mRNA expression in BB12-stimulated and Lipo+miR-671-5p-ASO+BB12 moDCs groups, n=5. **B.** Cytokine production in all the moDC groups (control, BB12-stimulated, Lipo+miR-671-5p-ASO, Lipo+miR-671-5p-ASO+BB12, and Lipo+miR-671-5p-ASO+Pam3CSK4) n=3 is presented in panel. Statistical differences were assessed using the Wilcoxon rank test and paired t tests (*p<0.05)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1miRWalkmiRNATargets.xlsx
- Supplementaryfile2ClueGOResultTable0.xls
- Supplementaryfile3.AntibodiesFlowcytometry.pdf