Modifying immune cells using mouse bone marrow-derived cell exosomes during administration of heat-killed lactic acid bacterium *Enterococcus faecalis*

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Abstract. Previously, the mechanism underlying the immunostimulatory effect of orally administered heat-killed *Enterococcus faecalis* (HkEf) mediated by exosomes secreted after intestinal macrophage phagocytosis was clarified. In the present study, mouse bone marrow-derived dendritic cells (DCs) were used as intestinal DCs. *In vitro* studies evaluating the response of mouse spleen cells to exosomes purified and isolated from intestinal DCs stimulated with HkEf showed a significant increase in standard DCs and plasmacytoid DCs (pDCs), indicating a positive effect on DCs, mainly pDCs, and possibly contributing to their immunostimulatory effects. The microRNAs (miRNAs) of exosome fractions derived from intestinal DCs and macrophages were also examined using bone marrow progenitor cells. HkEf stimulation released miRNA-rich exosomes, which acted as an immune response signal mediator, and increased various miRNAs other than miR-146 that are essential for refilling innate immune cells. In the present study, the effect mediated by miRNA-rich exosomes on the distant immune system from intestinal immunity (macrophages, DCs) was observed via orally administered HkEf, a *Lactobacillus* preparation. The findings of the present study revealed that this mechanism can potentially prevent systemic infection.

Introduction

Lactic acid bacteria are probiotics that adhere to the living body and interact with the host. The bacterial cell components also have an immunoregulatory mechanism via the intestinal mucosa to prevent infection. Previously, it has been shown that oral administration of live lactic acid bacteria and their metabolites, as well as dead bacteria, affects the immune system. Oral administration of heat-killed *Enterococcus faecalis* (HkEf) increases immunoglobulin A concentration (1) and exhibits antiviral activity (2).

The authors have previously reported on increased survival rates after oral administration of HkEf in systemic bacterial infection animal models using antimicrobial-resistant bacteria (3,4). Moreover, an increase has been also demonstrated in spleen immune cells in mice that were orally administered with HkEf (5). However, this immunostimulatory effect, which occurred via systemic infection, was difficult to explain only in terms of gut immunity; hence, it was hypothesized that gut cells may have interacted with the remote immune system. The immunostimulatory effects of exosome-mediated γ-aminobutyric acid and carnosine were investigated, and it was clarified that mice orally administered with HkEf released exosomes that functioned as mediators in transmitting immune response signals after phagocytosis via macrophages (6-8). This eventually affected remote immune cells (5).

The present study aimed to elucidate the effect of HkEf phagocytosed by intestinal dendritic cells (DCs) to secrete exosomes, on immune cells and their underlying mechanisms using mouse bone marrow-derived DCs [BMDCs; mainly plasmacytoid (pDCs) or conventional (cDCs)] as intestinal DCs, to examine the effects of exosomes purified and isolated from HkEf-stimulated BMDCs on a subset of immune cells derived from mouse spleen, and analyzing microRNAs (miRNAs) in exosomes derived from BMDCs and macrophages (BMDMs).

Materials and methods

Experiment 1. Effects of mouse BMDC-derived HkEf-stimulated exosomes on mouse spleen cells. HkEf (cat. no. EF-2001; Nihon Berumu Co. Ltd.) was supplied as a heat-killed, dried powder. EF-2001 was also used in Experiments 2 and 3. HkEf (300 mg) was suspended in 3 ml of Penicillin-Streptomycin-Amphotericin B Suspension (x100) (cat. no. 161-23181; FUJIFILM Wako Pure Chemical Corporation) for aseptic processing, and a 100-mg/ml solution was prepared from this suspension.

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Abbreviations: BMDMs, bone marrow-derived dendritic macrophages; HkEf, heat-killed Enterococcus faecalis; NK, natural killer; DCs, dendritic cells; BMDCs, bone marrow-derived DCs; pDCs, plasmacytoid DCs; miRNAs, microRNAs

Key words: exosomes, HkEf, macrophages, DCs, miRNAs, Lactobacillales

Preparation of cells used in the experiment. Dendritic progenitor cells (mouse bone marrow-derived) were obtained from BALB/c mouse bone marrow derivatives [Bone Marrow-Derived Dendritic Precursor (BALB/c); Mouse; cat. no. BMDC01C; Cosmo Bio Co., Ltd.].

Spleen cells were aseptically extracted under anesthesia from five female BALB/c mice aged 4-5 weeks and weighing 14-19 g at the start of the study. The mice purchased from Japan SLC, Inc. were used immediately in the experiments upon arrival. Mice were euthanized via 5% isoflurane and cervical dislocation was used as a secondary euthanasia measure, followed by rapid harvesting of the spleen.

These cells were added into 10 ml of 0.02% EDTA-2Na/PBS (cat. no. 14367-74; Nacalai Tesque, Inc., for dispersion. After centrifugation (190 x g for 7 min at room temperature (~22-25˚C), the supernatant liquid was discarded, and the cell cluster was added to RPMI-1640 culture medium (cat. no. 189-02025; FUJIFILM Wako Pure Chemical Corporation). After pipetting, it was centrifuged (190 x g for 7 min at room temperature; ~22-25˚C), and the supernatant liquid was discarded. This cell cluster was dismantled and stirred for 10 min with RBC Lysis Buffer (cat. no. 420302; BioLegend Inc.) for hemolysis treatment. Following this, the mixture was centrifuged (190 x g for 7 min at room temperature; ~22-25˚C), the supernatant was discarded, cells were resuspended in RPMI-1640 culture medium, to which 5 ml of 2% exosome-depleted fetal bovine serum (FBS; cat. no. EXO-FBSHI-50A-1; System Biosciences) was added, following which the cells were counted.

Induction of BMDCs (pDC/cDC). DCs were induced to differentiate from bone marrow cells according to the standard method (9). Dendritic progenitor cells (mouse bone marrow-derived) were inducted for differentiation using the Flt3 ligand (Flt3-L; cat. no. 060-04803; FUJIFILM Wako Pure Chemical Corporation) to obtain pDCs and cDCs. For their differentiation, Flt3, a cytokine, was essential (10,11). The pDCs and normal DC fraction of cDCs can reportedly be inducted via culturing bone marrow cells in Flt3-L-containing culture medium (11).

Commercially available dendritic progenitor cells were dispersed using 10% exosome-depleted FBS and Dulbecco's modified Eagle's medium (DMEM, High Glucose) with L-Glutamine, phenol red, and sodium pyruvate (cat. no. 043-30085; FUJIFILM Wako Pure Chemical Corporation). Aliquots of the differentiating media for pDCs and cDCs (1 ml/well) were dispensed in six wells of a 12-well plate and cultured for three days under 5% carbon dioxide (CO_2) at 37˚C and 100% humidity. After washing in DMEM at 7˚, the differentiating medium was added and cultured in a CO_2 incubator. Thereafter, the culture medium was changed every other day. Cells cultured on day 11 were used for the study.

Purification and quantification of BMDC (pDC/cDC)-derived exosomes. After the differentiation culture medium was removed, DMEM (producing medium) including 2% exosome-depleted FBS was added. For HkEf (+), 30 μl of aseptic HkEf was added; for HkEf (-), only the Penicillin-Streptomycin-Amphotericin B Suspension was added and cultured for three days, and then centrifuged at 1,000 x g for 15 min.

The supernatant was processed to isolate exosomes using the EXO-Prep kit (cat. no. HBM-EXP-C25; HansaBioMed Life Sciences; https://hansabiomed.eu/). Exosome quantity was determined by the ExoELISA-ULTRA Complete Kit (CD81 Detection) (cat. no. EXEL-ULTRA-CD81-1; System Biosciences; https://www.systembio.com/), following the manufacturer's instructions.

Mouse spleen cell stimulation test with BMDC (pDC/cDC)-derived exosomes. The spleen cell suspension prepared as aforementioned was seeded on a 6-well plate such that the survival cell count was $2x10^6$ cells. After measuring the exosome quantity using ELISA, the following cultures were performed: 1. HkEf (-) group: 40 μl of non-stimulated exosome purified fraction was added (n=5); 2. HkEf (+) group: 40 μl of stimulated pDC/cDC exosome purified fraction was added (n=5). These were cultured for five days in a CO_2 incubator at 37˚C. After culture, the cells were collected, and flow cytometry was performed.

These experiments were approved (approval no. 003) of the Institutional Animal Care and Use Committee of the OBM Research Center (Osaka, Japan).

Analyzing the spleen cell subset using flow cytometry. The spleen cell subset was measured to identify each immune cell via double staining. As shown in Table I, immunocompetent cell-specific markers were red pulp macrophages ($CD11b^{low}F4/80^+$) in macrophages, and cDCs ($CD11c^+MHCII^+$) and pDCs ($CD317^+$ $SiglecH^+$) in DCs. The assay was performed using a Guava® easyCyte HT System flow cytometer (Merck Millipore) and analyzed using guavaSoft™ software (version 4.0; Merck Millipore). Flow cytometry settings were as follows: Count, 50000; Threshold, 9262 (FSC); Gain FSC, 19.9; SSC, 9.93; GTN-B, 9.93; YEL-B, 9.93; RED-B, 1.04.

Experiment 2. MiRNA assay of mouse BMDC-derived HkEf-stimulated exosome fractions. HkEf (-) and HkEf (+) exosome fractions obtained from BMDCs in Experiment 1 were assayed. The following four samples were used as controls: i) Negative control: DNase/RNase-free water (without dissolution step, AC); ii) Negative control: DNase/RNase-free water (with dissolution step, DC); iii) Positive control: RNA control; and iv) Positive control: healthy human pooled serum.

Quantification of miRNA. miRNA quantification was performed using the FirePlex® miRNA Assay-Core Reagent Kit V3 (Abcam). Briefly, purified exosomes were hybridized with FirePlex® Particles and labeled with target miRNAs by PCR (FirePlex® miRNA Panel-Immunology V2; cat. no. ab218369; Abcam). Subsequently, the labeled targets were analyzed using a flow cytometer (Guava® easyCyte™ 5HT, Cytek Biosciences). The sequences of primers have not been provided by the manufacturer. As the FirePlex® miRNA Panel uses homologous human and mouse regions for detection, allowing for the detection of both human and mouse miRNAs.

Experiment 3. MiRNA assay of mouse BMDM-derived HkEf-stimulated exosome fractions. Mouse bone marrow cells were stimulated using GM-CSF (077-04674; FUJIFILM Wako Pure Chemical Corporation). HkEf (-) and (+) exosome fractions were obtained from BMDMs as previously described by Matsuo *et al* (5).

miRNA assay using flow cytometry. According to the Fireplex miRNA Assay V3-Assay Protocol, particles equivalent to each miRNA were assayed via flow cytometry using the FirePlex® miRNA Panel.

Table I. Immunocompetent cell-specific markers.

Immunocompetent cell	Specific marker	
Macrophages		
Red pulp macrophages ($CD11b^{low}F4/80^{+}$)	CD11b	F4/80
Dendritic cells		
Conventional dendritic cells ($CD11c^{+}MHCII^{+}$)	CD11c	MHCII
Plasmacytoid dendritic cells ($CD317^{+}$ $SiglecH^{+}$)	CD317	SiglecH
NK cells ($NK1.1^{+}$)	NK1.1	-
Activated NK cells ($NK1.1^{+}Sca\text{-}1^{+}$)	NK1.1	Sca-1

Statistical analysis. For comparison between HkEf (-) and (+) groups related to the effect on immune subsets of mouse spleen cells in BMDC-derived exosomes, an unpaired t-test was performed using a commercially available statistical program (Numbers; Apple, Inc.), with the significance level set to $P<0.05$. For miRNA, the adjusted P-value was calculated using the Benjamini-Hochberg multiple comparison test, which allows adjustment of family-wise error rates in multiple significance testing. Statistical analysis was performed using a commercially available statistical program (Analysis Workbench FirePlex®; Abcam PLC) with the significance level set to $P<0.05$.

Results

HkEf-stimulated BMDC-derived exosome purification. BMDC-derived exosome production in Experiment 1 was compared with or without HkEf stimulation. The results revealed that HkEf (-) exosomes and HkEf (+) exosomes had almost the same concentration of exosomes, ~$1.21x10^{11}$ particles per 50 μl. Based on this result, 40 μl (~$9.6x10^{10}$ particles) of exosomes were added to mouse spleen cells.

Effect of mouse BMDC-derived HkEf-stimulated exosomes on mouse spleen cells. The number of cDCs ($CD11c^{+}$, MHC II^{+}) in the HkEf (+) group (0.994 ± 0.275) increased more significantly than that in the HkEf (-) group (0.612±0.228) ($P=0.044$; Fig. 1). The number of pDC ($CD317^{+}$, Siglec H^{+}) in the HkEf (+) group (1.024±0.194) increased more significantly than that in the HkEf (-) group (0.692±0.130) ($P=0.013$; Fig. 1).

However, no significant differences were observed in red pulp macrophages ($CD11b^{low}F4/80^{+}$), natural killer (NK) cells ($NK1.1^{+}$), and activated NK cells ($NK1.1^{+}$, $Sca\text{-}1^{+}$) between both groups ($P=0.84$, 0.64, and 0.94, respectively; Fig. 1).

miRNA assay of mouse BMDC-derived HkEf-stimulated exosome fractions. The heatmap of FirePlex®miRNA multiplex assay of BMDC-derived exosome fractions in Experiment 2 is shown in Fig. 2.

In the HkEf (+) group, the exosome fractions significantly increased compared with that in the HkEf (-) group at a fluorescence intensity of ≥2,000 ($P<0.05$, multiple comparison test, Benjamini-Hochberg procedure). The miRNAs detected were miR-146a-5p (13.54-fold), miR-17-5p (12.17-fold), miR-20a-5p (16.32-fold), miR-21-5p (7.12-fold), miR-22-3p (19.37-fold), miR-221-3p (8.25-fold), miR-223-3p (11.01-fold), miR-29b-3p (11.01-fold), miR-29c-3p (11.15-fold), miR-342-3p (10.66-fold) and miR-34a-5p (23.62-fold) (Table II).

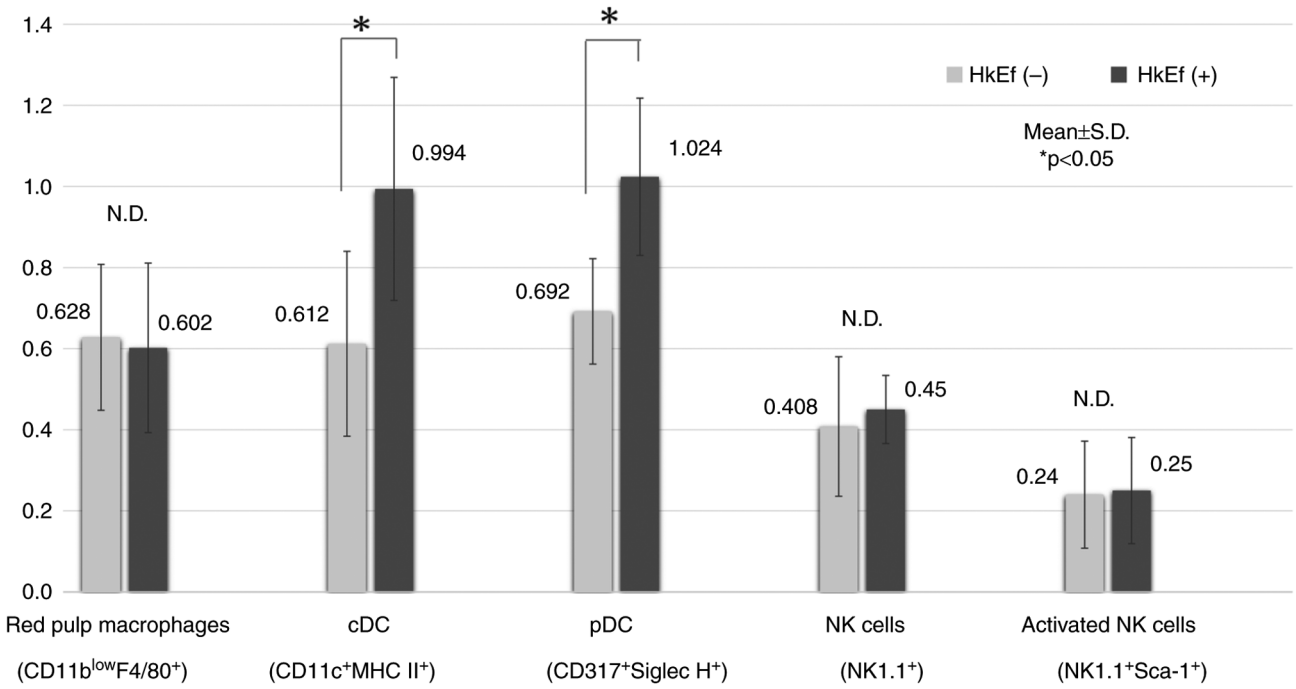
miRNA assay of mouse BMDM-derived HkEf-stimulated exosome fractions. The heatmap of the FirePlex®miRNA multiplex assay of BMDM-derived exosome fractions in Experiment 3 is revealed in Fig. 3.

In the HkEf (+) group, the exosome fractions significantly increased compared with those in the HkEf (-) group at a fluorescence intensity ≥2,000 ($P<0.05$, multiple comparison test, Benjamini-Hochberg procedure). The miRNAs detected were: miR-146a-5p (1.79-fold), miR-17-5p (1.25-fold), miR-20a-5p (1.39-fold), miR-22-3p (2.17-fold), miR-221-3p (2.57-fold) and miR-34a-5p (1.48-fold). A significant decrease in miR-223-3p (0.54-fold) was confirmed (Table III).

Discussion

In a previous study by the authors (5), BMDMs were used as test cells. Orally administered HkEf was suggested to have been phagocyted by gut macrophages after which they released exosomes as signal transmitters for immune regulation. In Experiment 1 of the present study, a stimulation test on mouse spleen cells was also conducted using BMDCs (pDC/cDC) obtained via differentiation with Flt3-L. Quantitative changes were predicted in exosomes upon HkEf stimulation; however, no marked quantitative differences were observed between the HkEf (-) and (+) groups in terms of exosome release. While cDC and pDC significantly increased in the HkEf (+) group compared with that in the HkEf (-) group based on the effect on the immune spleen cell subset, no such influence was observed in NK cells, activated NK cells, and red pulp macrophages. This suggests that the qualitative change of exosomes is accompanied by quantitative changes in cDC and pDC. Furthermore, exosomes related to immune response signals were released from gut immune cells due to HkEf intake and were transported to remote tissues; this finding is supported by a previous study (5) on systemic immunostimulatory regulation.

In Experiment 2, the miRNAs of BMDC-derived exosome fractions with or without HkEf stimulation were further examined. In human monocyte-derived cells, miR-146, which was significantly increased due to HkEf stimulation, is reportedly increased upon inflammatory stimulation,



Immunocompetent	Stimulation	Cell (%)		T-test
		Mean±SD	%CV	
Macrophages				
Red pulp macrophages	HkEf (–)	0.628±0.180	28.6	ND
($CD11b^{low}F4/80^+$)	HkEf (+)	0.602±0.209	34.8	
Dendritic cells				
cDC	HkEf (–)	0.612±0.228	37.2	p<0.05
($CD11c^+$MHC II^+)	HkEf (+)	0.994±0.275	27.7	
pDC	HkEf (–)	0.692±0.130	18.7	p<0.05
($CD317^+$Siglec H^+)	HkEf (+)	1.024±0.194	18.9	
NK cells	HkEf (–)	0.408±0.172	42.1	ND
($NK1.1^+$)	HkEf (+)	0.450±0.084	18.7	
Activated NK cells	HkEf (–)	0.24±0.132	55.2	ND
($NK1.1^+Sca\text{-}1^+$)	HkEf (+)	0.25±0.131	53.2	

Figure 1. Effect of mouse bone marrow-derived DC-derived HkEf-stimulated exosomes on spleen cells. HkEf (-) vs. HkEf (+), as determined by t-test. *P<0.05, mean ± SD. HkEf, heat-killed *Enterococcus faecalis;* NK, natural killer; ND, no significant difference; DCs, dendritic cells; pDC, plasmacytoid DCs; cDCs, conventional DCs.

such as by inflammatory cytokines (tumor necrosis factor-α, interleukin-1β), lipopolysaccharide and peptidoglycan (12). Natural immunity is significantly affected by miR-146. When miR-146 forcibly expressed in mouse hematopoietic stem and progenitor cells and is transplanted in mouse bone marrow after eliminating the cells via radiation, their differentiation to myelocytes in the blood is promoted, whereas their differentiation to lymphocytes is impaired (13). This is because of a filling mechanism; when natural immunity cells combat infectious pathogens, the number of cells is often reduced (14,15). miR-146 is suggested to play a part in this mechanism and preferentially induces the myeloid differentiation pathway rather than the lymphocyte differentiation pathway.

As other miRNAs increased significantly upon HkEf stimulation, miRNAs are related to DC differentiation and maturity. miR-29 has been suggested to play an important role in early hematopoietic stem cell self-renewal and is involved in the onset of acute myeloid leukemia (16). When the expression of miR-146, miR-29b and miR-29c is increased, the survival rate of pDC is lowered (17,18).

Conversely, miR-21 expression has been shown to inhibit apoptosis (19). In the present study, the miRNAs that acted against survival upon HkEf stimulation (specifically, miR-146, miR-29b, miR-29c and miR-21) were greatly increased. An increase in pDC was confirmed in exosome-added mouse spleen cells; hence, it was hypothesized that apoptosis

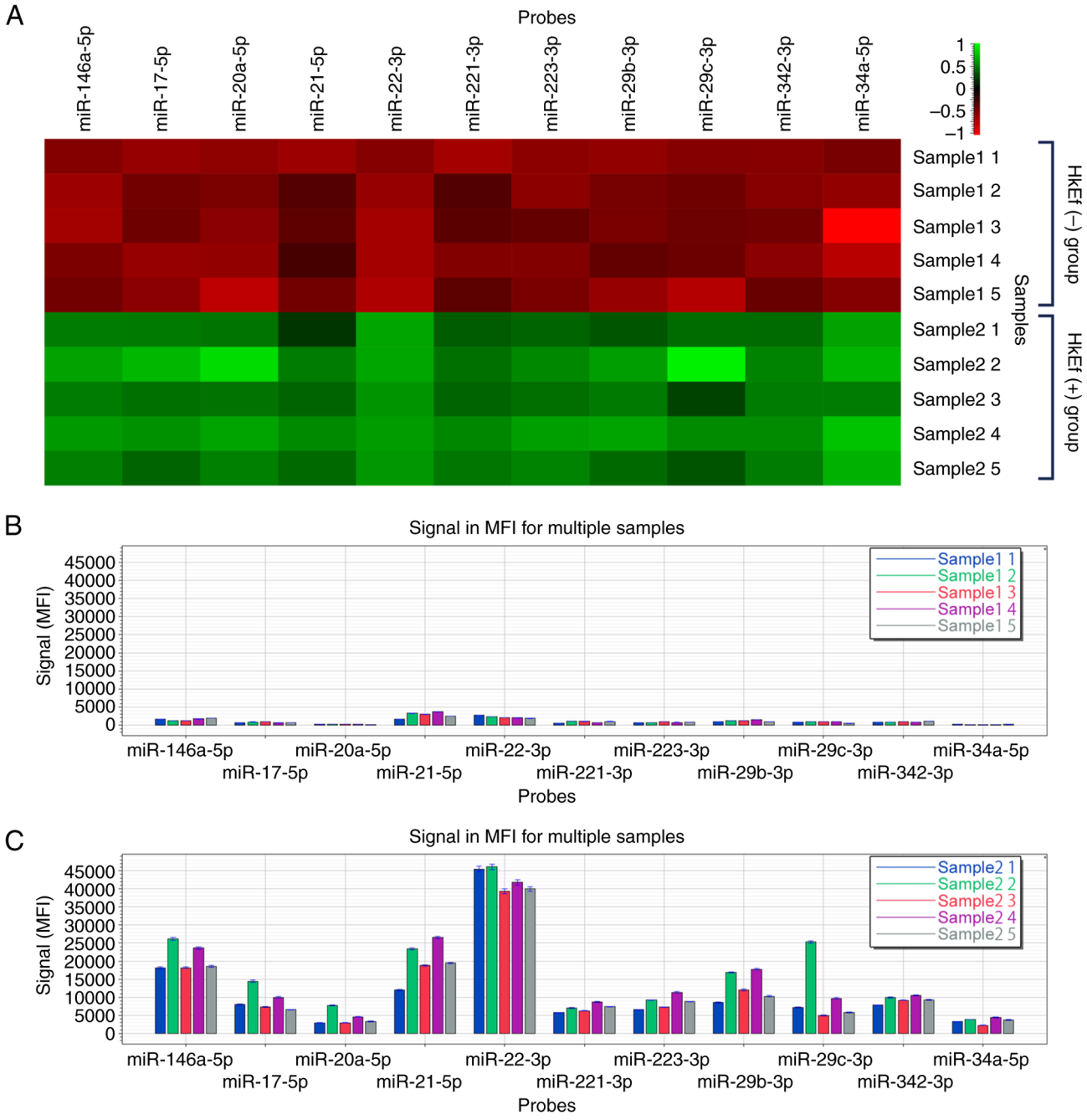


Figure 2. Immuno-miRs of mouse BMDC-derived HkEf-stimulated exosome fractions. (A) A heat map was used to visualize the assayed miRs. Ratio to mean value is shown by log10 conversion. (B and C) MFI of miR shown in (B) HkEf (-) group and (C) HkEf (+) group. In the HkEf (-) group, exosome fractions were released from HkEf-non treated BMDCs (pDC/cDC). In the HkEf (+) group, exosome fractions were released from HkEf-treated BMDCs (pDC/cDC). miRs, microRNAs; BMDCs, bone marrow-derived dendritic cells; HkEf, heat-killed *Enterococcus faecalis*; MFI, mean fluorescence intensity; pDC, plasmacytoid DCs; cDCs, conventional DCs.

can, overall, be inhibited. In miR-34a-overexpressing bone marrow chimera and transgenic mice, increased DCs were confirmed (20). miR-21 and miR-34a promote monocyte to DC differentiation by inhibiting the expression of JAG1 and WNT1, proteins that are necessary for hematopoietic differentiation and generation (21). miR-22 acts as a negative control factor of IRF8, which is a transcription factor that determines the cell fate of neutrophils, monocytes and DCs. Inducing miR-22 expression in DC generation promotes cDC generation with compensation of pDC; miR-22 inhibition shows the opposite effect (22). It influences the differentiation of the DC subset. Upon miR-221 expression, pDC generation is inhibited (23). During DC maturation, miR-221 expression is inversely related to the expression of the cell-cycle regulator $p27^{kip1}$. Therefore, the apoptosis of DCs due to the accumulation of $p27^{kip1}$ appears to be inhibited (24). In the differentiation from monocytes to immature and mature DCs, the expression of miR-29c, miR-21 and miR-342 is increased (24). miR-223 is highly expressed in hematopoietic cells and is essential for natural immune reactions to control bone marrow differentiation and granulocyte function (25,26). In hematopoietic cells, miR-223 overexpression promotes

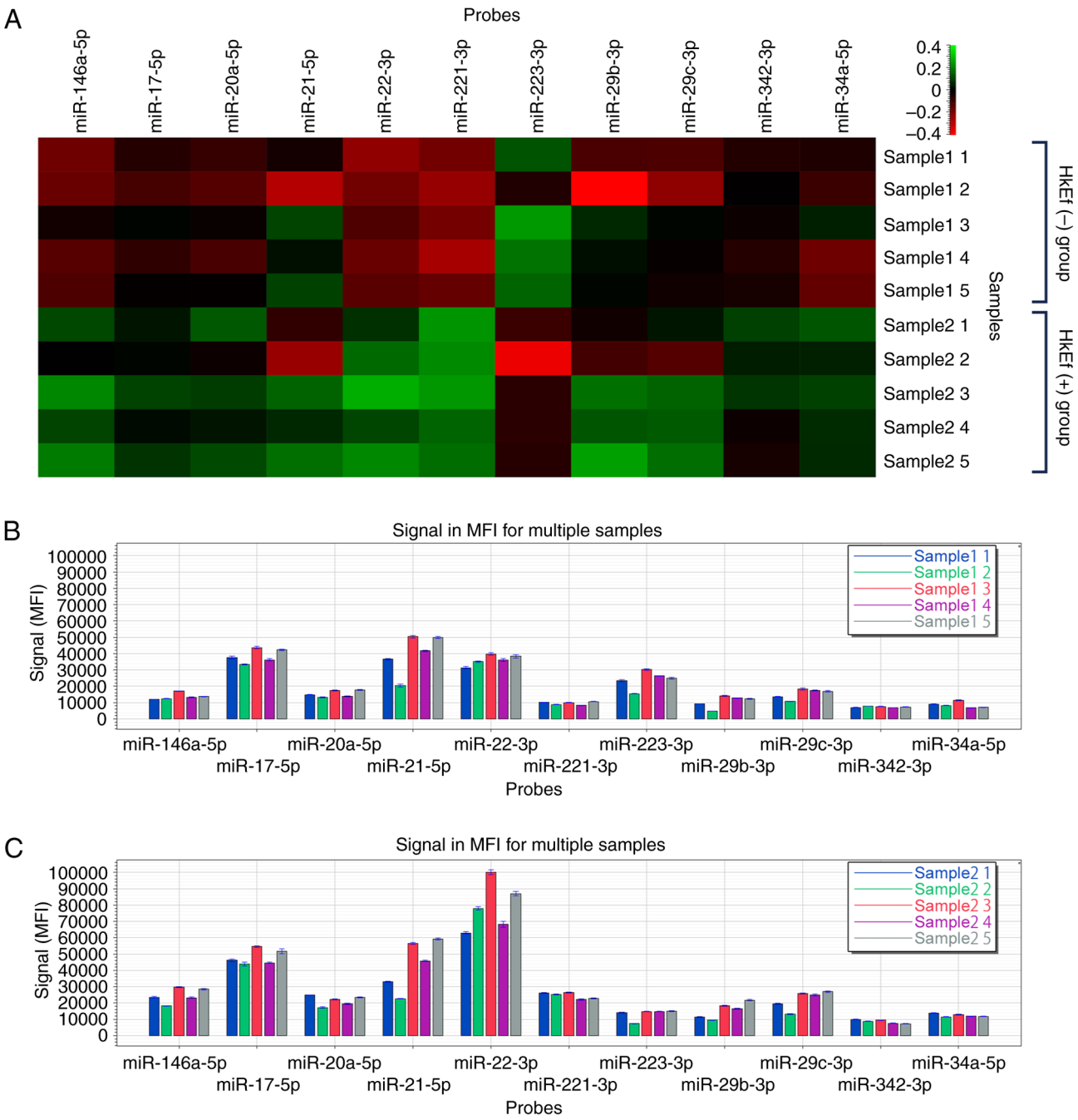


Figure 3. Immuno-miRs of mouse BMDM-derived HkEf-stimulated exosome fractions. (A) A heat map was used to visualize the assayed miRs. Ratio to mean value is shown by log10 conversion. (B and C) MFI of microRNA shown in (B) HkEf (-) group and (C) HkEf (+) group. In the HkEf (-) group, exosome fractions were released from HkEf-non treated BMDMs. In the HkEf (+) group, exosome fractions were released from HkEf-treated BMDMs. miRs, microRNAs; BMDMs, bone marrow-derived macrophages; HkEf, heat-killed *Enterococcus faecalis*; MFI, mean fluorescence intensity.

granulocyte differentiation, whereas the differentiation of erythrocytes and macrophages is inhibited (27). Although miR-223 expression increased in all processes upon the induction of differentiation of human embryonic stem cells to DCs, the expression of the differentiation marker is decreased by the miR-223 inhibitor (28).

In Experiment 3, miRNAs of exosome fractions derived from BMDMs were examined.

The significantly increased miRNAs were almost common among the BMDC- and BMDM-derived exosome fractions, despite small differences in the quantity. Unlike the measured value of BMDC-derived exosome fractions, the rate of increase in miR-17 and miR-20a, which inhibited differentiation to macrophages targeting RUNX1 (29), were smaller in BMDM-derived exosome fractions (Experiment 3: miR-17, 1.25-fold, miR-20a, 1.39-fold; Experiment 2: miR-17, 12.17-fold, miR-20a, 9.59-fold). In a previous study of BMDM-derived mouse spleen cells (5), the number of red pulp macrophages increased. This may be because the rate of increase in BMDM-derived miR-17 and miR-20a was smaller than that of BMDCs, because of which differentiation to macrophages may be induced. Although in Experiment 3 miR-223 decreased more in the HkEf (+) than in the HkEf (-) groups, in Experiment 2, miR-223 conversely increased significantly

Table II. Multiple comparison of immune microRNA of mouse BMDC-derived HkEf-stimulated exosome fractions.

Probe	HkEf (-) group		HkEf (+) group		HkEf (+) group/ HkEf (-) group	Multiple comparison[a]
	Mean (MFI)	CV%	Mean (MFI)	CV%		
miR-146a-5p	1527.05	20.12	20671.12	17.33	13.54	0.00000011
miR-17-5p	731.01	18.59	8899.87	31.83	12.17	0.0000013
miR-20a-5p	244.62	23.60	3993.22	43.21	16.32	0.0000038
miR-21-5p	2721.55	32.14	19376.33	30.91	7.12	0.000020
miR-22-3p	2190.91	15.36	42439.20	7.29	19.37	0.0000000043
miR-221-3p	845.66	33.73	6976.36	15.92	8.25	0.0000039
miR-223-3p	771.19	15.53	8487.27	21.71	11.01	0.0000002
miR-29b-3p	1143.64	20.70	12596.98	32.18	11.01	0.0000023
miR-29c-3p	784.83	29.34	8746.94	72.00	11.15	0.00013
miR-342-3p	873.58	14.15	9310.07	11.26	10.66	0.000000021
miR-34a-5p	144.50	54.89	3413.23	25.77	23.62	0.0000053

[a]miRNA of fluorescence intensity ≥2,000. Multiple comparison between HkEf (+) and (-) groups was performed using Benjamini and Hochberg procedure. Significance level: P<0.05. BMDCs, bone marrow-derived dendritic cells; HkEf, heat-killed *Enterococcus faecalis*; miR, microRNA; MFI, mean fluorescence intensity.

Table III. Multiple comparison of immune microRNA of mouse BMDM-derived HkEf-stimulated exosome fractions.

Probe	HkEf (-) group		HkEf (+) group		HkEf (+) group/ HkEf (-) group	Multiple comparison[a]
	Mean (MFI)	CV%	Mean (MFI)	CV%		
miR-146a-5p	13569.78	13.60	24267.01	19.54	1.79	0.0042
miR-17-5p	38445.69	11.11	48028.35	9.60	1.25	0.034
miR-20a-5p	15252.93	13.46	21277.14	14.98	1.39	0.020
miR-22-3p	35997.20	9.34	78147.10	18.71	2.17	0.00052
miR-221-3p	9514.26	10.11	24486.83	8.28	2.57	0.0000069
miR-223-3p	23559.91	25.82	12793.83	31.86	0.54	0.030
miR-34a-5p	8358.58	21.03	12338.22	7.77	1.48	0.015

[a]miRNA of fluorescence intensity ≥2,000. Multiple comparison between HkEf (+) and (-) groups was performed using Benjamini and Hochberg procedure. Significance level: P<0.05. BMDMs, bone marrow-derived dendritic macrophages; HkEf, heat-killed *Enterococcus faecalis*; miR, microRNA; MFI, mean fluorescence intensity.

(Experiment 3: 0.54-fold; Experiment 2: 11.01-fold). As previously mentioned, the inhibition of miR-223 induces an increase in erythrocytes and macrophages, while differentiation to DCs is inhibited.

Thus, HkEf induces the release of miRNA-rich exosomes, which act as immune response signaling mediators from intestinal DCs and macrophages. These exosomes preferentially induce the myeloid differentiation pathway through the effect of miRNAs, primarily miR-146, in spleen immune cells. Moreover, induction of differentiation to DCs and macrophages appears to be promoted by altered expression of miRNAs, such as miR-223.

The aforementioned discussion is a hypothesis from existing literature. On the other hand, phenotypic analysis revealed that there were no differences in the frequency and cell number of DC and macrophage populations in the spleen of miR-223-deficient mice (25). This may not be due to the effect on spleen immune cells of miRNA-rich exosomes from intestinal DCs and macrophages, but on bone marrow-derived stem cells in the bone marrow.

This result could be explained as an effect on extramedullary hematopoiesis (30) in the spleen, but this was not clear from the experimental design of the present study. The final targets of these changed miRNAs should be clarified, and the biological significance of miRNAs should be confirmed in further studies.

The present study has certain limitations. First, these investigations were animal studies, and a similar effect on humans cannot be guaranteed. The effect of exosomes on remote cells using mouse spleen cells was studied *in vitro*.

The surface of exosomes contains proteins and lipids. Inside the exosomes, mRNA, DNA and various proteins exist; hence, these may play a role in signal transmission as well, requiring further study for more definitive conclusions.

In conclusion, BMDCs induced from mouse bone marrow progenitor cells were stimulated with HkEf, and the released exosomes were purified and isolated. As a result of examining the effect of this exosome on immune cell subsets derived from mouse spleen cells, it can be suggested that this exosome has an immunostimulatory effect mediated by DCs, mainly that of pDC (*in vitro*).

Moreover, as a result of measuring miRNAs in HkEf-stimulated exosome fractions derived from BMDCs (pDC/cDCs) and BMDMs (macrophages), effects mediated by miRNA-rich exosomes such as miR-146, which play a part in the complement mechanism of cells of the innate immune system are hypothesized. Orally administered HkEf releases exosomes involved in immune response signals by intestinal immune cells (macrophages and DCs), and regulates immune activation as an effect on distant immune tissues via blood circulation. Thus, it can be hypothesized that this is one of the mechanisms to prevent systemic infection.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KN conceptualized and designed the study, conducted formal analysis, interpreted the data, and wrote the original draft. TM and IS conceptualized and investigated the study, reviewed and edited the manuscript. KH conceptualized the study, interpreted the data, reviewed the manuscript and supervised the study. KN and KH confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal experiments were conducted under the approval (approval no. 003) of the Institutional Animal Care and Use Committee of the OBM Research Center (Osaka, Japan).

Patient consent for publication

Not applicable.

Competing interests

All authors are employees of NUTRI Co., Ltd.

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