Administration of plasmacytoid dendritic cell-stimulative lactic acid bacteria is effective against dengue virus infection in mice

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Abstract. Dengue virus (DENV), a mosquito-borne flavivirus, causes an acute febrile illness that is a major public health problem in the tropics and subtropics globally. However, methods to prevent or treat DENV infection have not been well established. It was previously demonstrated that *Lactococcus lactis* strain plasma (LC-plasma) has the ability to stimulate plasmacytoid dendritic cells (pDCs). As pDCs are key immune cells that control viral infection by producing large amounts of type I interferons (IFN), the present study evaluated the effect of LC-plasma on DENV infection using a mouse infectious DENV strain. Mice were divided into two groups and the test group was orally administered LC-plasma for two weeks. Two weeks following administration, the mice were infected with DENV and the relative viral titers and the expression of the inflammatory genes in DENV-infected tissue were measured using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The relative viral titers were notably lower in the DENV-infected tissues compared with the control group when LC-plasma was orally administered prior to DENV infection. Furthermore, the expression of the inflammatory genes associated with DENV infection was also reduced by LC-plasma administration. To investigate how LC-plasma administration controls DENV infection, the present study examined anti-viral gene expression, which is critical for the viral clearance induced by type I IFN. Two weeks subsequent to the administration of LC-plasma, the expression of anti-viral gene was measured using RT-qPCR. Oral intake of LC-plasma enhanced anti-viral gene expression in DENV-infected spleen tissue. To clarify the detailed mechanism, *in vitro* co-culture studies using bone-marrow derived DC (BMDC) were performed. BMDC were stimulated with LC-plasma in combination with anti-IFN-α/β antibody and the expression of anti-viral genes was measured. *In vitro* studies revealed that the effect of LC-plasma on anti-viral genes was dependent on type I IFN. Based on these results, LC-plasma may be effective against DENV infection by stimulating pDCs, which results in the increased production of anti-viral factors.

Introduction

Dengue viruses (DENV) are single-stranded RNA viruses in the genus *Flavivirus* (1). They are one of the most widespread mosquito-borne viruses and are categorized into four serotypes (DENV 1-4) (2). DENV infection results in dengue fever and its more severe forms, dengue hemorrhagic fever and dengue shock syndrome (3,4). Dengue hemorrhagic fever and dengue shock syndrome are characterized by increased vascular permeability, plasma leakage into tissues, thrombocytopenia and hemorrhaging within internal organs, which are thought to be immune-mediated diseases (5). According to the World Health Organization, 50-100 million individuals are estimated to develop dengue fever annually in tropical and subtropical regions of the world such as South-East Asian and South American countries, and ~20,000 cases of mortality from dengue fever are estimated to occur each year (6,7). At present, however, there are few preventative antiviral drugs or vaccines against DENV infection. Therefore, effective therapeutics and/or prophylaxis against DENV infection are urgently required (8).

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Type I interferons (IFNs) are cytokines that enhance immunity to viruses or intracellular pathogens (9,10). By signaling through specific receptors, type I IFNs activate a janus kinase/signal transducer and activator of transcription-dependent signaling cascade that increases the expression of hundreds of IFN-stimulated genes (ISGs) to induce the antiviral state (11). A number of reports have demonstrated that DENV is sensitive to type I IFNs, and that type I and II IFN-receptor-deficient mice are highly susceptible to DENV infection (12-15). In addition, a number of IFN-inducible anti-viral genes have been reported to inhibit DENV infection (16-18).

Plasmacytoid dendritic cells (pDCs) represent a unique immune cell type that is characterized by the production of large quantities of type I IFNs in response to viruses or self-nucleic acids (19,20). pDCs express Toll-like receptor (TLR) 7 and 9 within their endosomes. TLR7 recognizes RNA viruses, endogenous RNA and synthetic oligoribonucleotides, and TLR9 senses non-methylated CpG DNA derived from viruses and bacteria, endogenous DNA and synthetic CpG oligodeoxyribonucleotides (ODN) (21-23). Recognition of these nucleic acids by TLR7 or TLR9 in pDCs results in the production of type I IFNs through the myeloid differentiation primary response gene 88 (MyD88)-IFN regulatory factor 7 pathway (24).

Lactic acid bacteria (LAB) have been reported to have immune-modulatory effects on hosts through the activation of innate immune cells, including macrophages or conventional DCs (cDCs) (25). Although a number of pathogenic bacteria have been demonstrated to stimulate pDCs (26), beneficial bacteria, including LAB, have been less studied in terms of activating pDCs (27). It was previously reported that *Lactococcus lactis* strain plasma (LC-plasma, also known *Lactococcus lactis* subsp. *lactis* JCM 5805) stimulates murine pDCs to produce large quantities of type I IFNs in association with cDCs (28). It was further demonstrated that the effect of LC-plasma on the activation of pDCs is dependent on the TLR9-MyD88 signaling pathway. As pDCs serve a pivotal function in anti-viral immune responses through type I IFN production, the effect of LC-plasma on virus infection was verified using virus infection mouse models. It was demonstrated that murine para-influenza virus-infected mice that were fed LC-plasma demonstrated a markedly improved survival rate compared with controls (29). Furthermore, the oral administration of LC-plasma protected mice against rotavirus infection (30). Therefore, the activation of pDCs by LAB administration appeared to defend the host against viral infection.

In the present study, the effect of a pDC-stimulating LAB, LC-plasma, on DENV infection in mice was examined. To verify the effectiveness of LC-plasma against DENV infection, the virus titer and the expression of inflammatory genes in DENV-infected tissues when LC-plasma was orally administered were examined. In addition, to elucidate the mechanism of protection against DENV infection by LC-plasma administration, the present study focused on anti-viral gene expression, which is assumed to be effective for preventing DENV infection (31). Finally, an *in vitro* study was performed and examined the influence of type I IFNs induced by LC-plasma on the enhancement of anti-viral gene expression. To date, whereas certain types of LAB have been reported to have immune-modulatory effects on host immunity, it has remained unclear whether LAB exerts an effect on DENV infection, which is a major threat to human health. This is the first report, to the best of our knowledge, to investigate whether the administration of LAB is effective against DENV infection *in vivo*.

Materials and methods

Mice. A total of 56 C57BL/6J female mice (6 week-old; weight, 15-25 g) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). All animals were maintained in a specific pathogen-free facility under a 12 h light/dark cycle with *ad libitum* access to water and a basic diet of AIN93G pellets (Oriental Yeast Co., Ltd., Tokyo, Japan). The temperature in the facility was maintained at 22-25˚C and 40-60% humidity. All animals were sacrificed using 5% isoflurane exposure until 2-3 min after the breathing had ceased. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Juntendo University (Tokyo, Japan) and Kirin Company, Ltd. (Kanagawa, Japan). The study was ethically approved by the Committee for Animal Experimentation of Juntendo University and Kirin Company, Ltd. All efforts were made to minimize animal suffering.

LAB strains. LAB strains tested in this study were purchased from collections held at the Japan Collection of Microorganisms (Tsukuba, Japan) and American Type Culture Collection (ATCC; Manassas, VA, USA). *Lactobacillus rhamnosus* strain GG (53103; ATCC) was grown at 37˚C for 48 h in MRS broth (Oxoid; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and LC-Plasma was grown at 30˚C for 48 h in M17 broth (Oxoid; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cultured LAB strains were washed twice with sterile distilled water, heat-killed at 100˚C, lyophilized and suspended in phosphate-buffered saline for the *in vitro* study.

Oral intake of LC-plasma. Six week-old C57BL/6J female mice were acclimatized for 1 week with *ad libitum* access to water and a basic diet of AIN93G pellets. Control groups were fed with 4 g/day AIN93G pellets and test groups were fed with 4 g/day AIN93G pellets containing 1 mg heat-killed LC-plasma during the course of the study.

Virus infection study. A total of 32 mice were divided into two groups (control; n=16, LC-plasma; n=16) and housed with 4 animals/cage following LC-plasma oral administration for 2 weeks as described above. A $5x10^7$ pfu/ml DENV virus solution was prepared, and mice were intraperitoneally injected with 200 μl solution. Mice were monitored every day subsequent to viral infection by monitoring the intake of food and water, the state of breathing and other abnormal behaviors. As the virus titer peaks at 2-3 days following virus infection, mice were sacrificed 2-3 days subsequent to infection, and spleens, liver and blood were harvested. The spleen and liver were homogenized in TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) for 1 min at room temperature followed by RNA extraction. Total RNA was extracted by phenol/chloro-

form/isoamyl alcohol extraction method. Blood samples were collected in EDTA-treated tubes (Terumo Co., Tokyo, Japan) that were subjected to centrifugation at 400 x g for 20 min at room temperature. Subsequent to centrifugation, plasma was obtained from blood samples for NS1 enzyme-linked immunosorbent assay (ELISA).

Non-virus infection study. A total of 20 mice were divided into two groups (control, n=10; LC-plasma, n=10) and housed at 1/cage following LC-plasma oral administration for 2 weeks as described above. After 2 weeks of oral intake of LC-plasma, the mice were sacrificed and the spleens were collected. The spleen was minced in Mg^{2+}- and Ca^{2+}-free Hank's Balanced Salt solution (Gibco; Thermo Fisher Scientific, Inc.) and digested with 1 mg/ml collagenase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 20 min at 37˚C. EDTA (Invitrogen; Thermo Fisher Scientific, Inc.) was adjusted to 30 mM and the sample was incubated for 10 min at room temperature. Tissue lysates were filtered through a 100 μm nylon cell strainer and erythrocytes were removed by exposure to 2 ml of Red Blood Cell Lysis Buffer (Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. Tissue lysates were filtered through a 70 μm nylon cell strainer again and filtered tissue lysates were used for splenocytes. CD11c+ cells were separated from splenocytes using CD11c MicroBeads (Miltenyi Biotec, Inc., Cambridge, MA, USA). The mediated splencytes and CD11c+ cells were suspended in RNAlater Stabilization solution (Thermo Fisher Scientific, Inc.).

Viral strains and growth conditions. DENV strain Eden2 was used in the present study. DENV Eden2 was provided by Dr. Ashley L. St. John of Duke-NUS Medical School (College Road, Singapore) and C6/36 cells and Baby hamster kidney 21 cells were provided by Dr. Yo-ichi Suzuki of Osaka Medical College (Osaka, Japan). DENV Eden2 was grown in *Aedes albopictus* C6/36 cells. C6/36 cells were seeded in plastic flasks and grown in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Moregate Biotech, Bulimba, Australia) and 100 U/ml penicillin-streptomycin (Thermo Fisher Scientific, Inc.). Once the cells were fully confluent, DENV Eden2 was transferred to C6/36 flasks at a multiplicity of infection of 0.01. Viral samples were incubated for >1 h at 28˚C, and subsequently the medium was substituted using RPMI-1640 medium supplemented with 2% inactivated fetal bovine serum. Cells were cultured for 5-7 days, and culture supernatants were collected. Cell debris was removed by centrifugation at 800 x g for 10 min at 4˚C, and supernatants containing the virus were stored at -80˚C. Viral titers were determined by plaque assays using Baby hamster kidney 21 cells. Cells were seeded in 24-well plates in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum and 100 U/ml penicillin-streptomycin. Cells were maintained in a humidified incubator at 5% CO_2 and 37˚C until they reached confluence. Then, the medium was removed and the monolayer was infected with 200 ml of 10-, 10^2-, 10^3-, 10^4-, 10^5- and 10^6-fold diluted virus samples. The plates were incubated for 1 h at 37˚C. After 1 h, cells were washed and then 0.5 ml 1% carboximethylcellulose (Sigma-Aldrich; Merck KGaA) mixed with RPMI-1640 medium supplemented with 2% inactivated fetal bovine serum

and 100 U/ml penicillin-streptomycin was added to each well. The plates were maintained in a humidified incubator at 37˚C and 5% CO_2 for 5-6 days. Subsequent to this period, the cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at room temperature for 1 h and the carboxymethylcellulose plugs were removed by washing with water. Plaques were visualized by staining with a solution of 1% crystal violet (Wako Pure Chemical Industries, Ltd.) in 20% ethanol for 2-3 min at room temperature.

NS1 ELISA. Concentrations of NS1 antigen in plasma samples were measured using a commercially available DENV NS1 ELISA kit purchased from Biocompare (South San Francisco, CA, USA).

In vitro co-culture assays. BM cells were isolated from C57BL/6J mice, and erythrocytes were removed by exposure to 1 ml of Red Blood Cell Lysis Buffer (Sigma-Aldrich; Merck KGaA) for 1 min at room temperature. Cells were cultured at a density of $5x10^5$ cells/ml for 7 days in RPMI-1640 medium supplemented with 1 mM sodium pyruvate (Life Technologies; Thermo Fisher Scientific, Inc.), 2.5 mM HEPES (Life Technologies; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin-streptomycin (Life Technologies; Thermo Fisher Scientific, Inc.), 50 μM 2-mercaptoethanol (Life Technologies; Thermo Fisher Scientific, Inc.), 10% fetal bovine serum and 100 ng/ml FMS-like tyrosine kinase 3 ligand (Flt-3L; R&D Systems, Inc., Minneapolis, MN, USA) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. After 7 days of culturing, cells were washed with RPMI-1640 medium and then incubated at a density of $2x10^5$ cells/ml with 1 μM CpG ODN (InvivoGen, Toulouse, France) or 10 μg/ml LC-plasma or *Lactobacillus rhamnosus* strain GG (ATCC 53103) at 37˚C for 24 h. To block TLR9 signals, a TLR9 antagonist (InvivoGen) was added with LC-plasma. To neutralize IFN-α/β, anti-IFN-α and anti-IFN-β antibody (PBL Assay Science, Piscataway, NJ, USA) was added with LC-plasma. Subsequent to culturing, the supernatant was collected and other BMDC or splenocytes were cultured with the collected supernatant for 24 h. Subsequent to culturing with the collected supernatant, each cell sample was collected for RNA extraction.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from spleen and liver was extracted using TRIzol reagent (Ambion; Thermo Fisher Scientific, Inc.). Subsequent to the extraction of tissue total RNA, a purification step was performed using an RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Total RNA from splenocytes, CD11c+ cells or BMDC was extracted using an RNeasy mini kit. To synthesize complementary DNA, purified total RNA samples were reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol. RT-qPCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara Bio, Inc, Otsu, Japan) and a LightCycler 480 (Roche Diagnostics, Basel, Switzerland). The PCR conditions were as follows: 2-Step Cycling, 95˚C for 10 sec hold, 45 cycles of 95˚C for 5 sec and 60˚C for 20 sec. All values were normalized to the expression of GAPDH

and calculated using the $2^{-\Delta\Delta Cq}$ method (32). Specific forward and reverse primer pairs that were used are listed as follows: GAPDH forward, 5'-TGTGTCCGTCGTGGATCTGA-3' and reverse, 5'-TTGCTGTTGAAGTCGCAGGAG-3'; interleukin (IL)-6 forward, 5'-CCACTTCACAAGTCGGAGGCT TA-3' and reverse, 5'-TGCAAGTGCATCATCGTTGTTC-3'; tumor necrosis factor α (TNFα) forward, 5'-ACTCCAGGC GGTGCCTATGT-3' and reverse, 5'-GTGAGGGTCTGGGCC ATAGAA-3'; IL-1β forward, 5'-TCCAGGATGAGGACATGA GCAC-3' and reverse, 5'-GAACGTCACACACCAGCAGGT TA-3'; monocyte chemoattractant protein 1 (MCP-1) forward, 5'-AGCAGCAGGTGTCCCAAAGA-3' and reverse, 5'-GTG CTGAAGACCTTAGGGCAGA-3'; IFNγ forward, 5'-CGG CACAGTCATTGAAAGCCTA-3' and reverse, 5'-GTTGCT GATGGCCTGATTGTC-3'; interferon-induced GTP-binding protein Mx1 (Mx1) forward, 5'-AATGTGGACATTGCTACC ACAGA-3' and reverse, 5'-GGTTCCGCATCACATCCA AG-3'; Viperin forward, 5'-CCGCAGTAACTCAGCTCA TGGA-3' and reverse, 5'-CTGAGCATTAGACCTCATCTG GACA-3'; 2'-5'-oligoadenylate synthase 1A (Oas1a) forward, 5'-TGCTGCCAGCCTTTGATGTC-3' and reverse, 5'-TCC TCGATGAGGATGGCATAGA-3'; ISG15 forward, 5'-CTG TGAGAGCAAGCAGCCAGA-3' and reverse, 5'-GAGTTA GTCACGGACACCAGGAA-3'.

In vivo virus quantitation. Methods for total RNA extraction and purification were performed as described above. RT-qPCR was performed using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, Inc.) and a LightCycler 480 (Roche Diagnostics). The PCR conditions were as follows: 2-Step Cycling, 50°C for 5 min hold, 45 cycles of 95°C for 20 sec and 60°C for 30 sec. All values were normalized to the expression of GAPDH and calculated using the $2^{-\Delta\Delta Cq}$ method (32). The specific forward and reverse primer pairs and probes used are listed as follows: GAPDH forward, 5'-CTTCACCACCATGGAGAA GGC-3' and reverse, 5'-GGCATGGACTGTGGTCATGAG-3'; GAPDH probe, 5'-(6-FAM)-CCTGGCCAAGGTCATCCA TGACAACTTT-(TAMRA)-3'; DENV Eden2 forward, 5'-CAT ATTGACGCTGGGAAAGA-3' and reverse, 5'-AGAACCTGT TGATTCAAC-3'; DENV Eden2 probe, 5'-(6-FAM)-CTGTCT CCTCAGCATCATTCCAGGCA-(TAMRA)-3'.

Statistical analysis. The data of virus titer and NS1 ELISA were presented as the mean + the value of each individual. All other data were presented as the mean ± standard error of the mean. Data from the *in vivo* study were analyzed using an unpaired Student's t-test. Data from the *in vitro* study were analyzed using one-way analysis of variance followed by Dunnett's test. All statistical analyses were performed using the Excel-Toukei 2012 software program (Social Survey Research Information, Tokyo, Japan). P<0.05 was considered to indicate a statistically significant difference.

Results

Oral administration of LC-plasma inhibits DENV infection. To investigate whether the pDC-stimulative LAB, LC-plasma, is effective for the prevention of DENV infection in mice, the mouse-infectious DENV strain Eden2 was used. As DENV Eden2 has been previously reported to infect the spleen and

liver and the virus infection peaks at 48 or 72 h (33), the relative virus titers were assessed at 48 and 72 h following DENV infection to examine the effect of LC-plasma on DENV infection. The study design is presented in Fig. 1A. The mice were orally administered LC-plasma for two weeks, and subsequently were infected with a titer of 1×10^7 pfu of DENV Eden2 by intraperitoneal injection. Oral intake of LC-plasma was continued for 2-3 days subsequent to DENV infection. A total of 2-3 days following DENV infection, 8 mice from each group were sacrificed, and the spleens, liver and blood were collected. No animals succumbed to DENV infection and any behavioral change was not observed. As presented in Fig. 1B, the relative virus titer in the spleens of the LC-plasma group 48 h subsequent to infection was significantly lower compared with that in the control group (P<0.01). On the other hand, there was no significant difference in the relative virus titers in the spleens at 72 h after infection between the two groups. The relative virus titer in the liver is presented in Fig. 1C. In the LC-plasma group, the relative virus titers at 48 and 72 h following infection were significantly lower compared with that of the control group (P=0.10 and P=0.11, respectively). NS1 antigen, which is a non-structural protein of the DENV (34), was also measured in the blood and the amount of NS1 at 48 h in the LC-plasma group was significantly lower compared with the control group (P<0.05). However, there was no difference in the amount of NS1 between the two groups 72 h after infection (Fig. 1D). These results suggested that oral administration of LC-plasma is effective in the prevention of DENV infection.

LC-plasma alleviates DENV-mediated inflammation. To evaluate the effect of LC-plasma on the suppression of inflammatory responses induced by DENV infection, the expression levels of inflammatory cytokine genes in the spleen and liver at 48 and 72 h subsequent to DENV infection were assessed by RT-qPCR. As presented in Fig. 2A, in the LC-plasma group, the expression levels of IL-6 (P<0.01), MCP-1 (P<0.05) and IFN-γ (P<0.05) in the spleen 72 h after infection were significantly lower compared with that in the control group. In addition, TNF-α expression levels in the spleen at 48 and 72 h after infection were non-significantly lower compared with that of the control group (P=0.08 and P=0.08, respectively). However, only TNF-α expression levels in the liver 48 h after infection were significantly lower compared with the control group (P<0.05), whereas the expression of other cytokine genes in the liver remained unchanged. These results suggest that the oral administration of LC-plasma alleviated inflammatory responses in DENV infected tissue.

Oral administration of LC-plasma enhances anti-viral factors in the spleen and CD11c+ cells in the absence of the virus. It was demonstrated that oral intake of LC-plasma is effective against DENV infection and the associated inflammatory response. To elucidate how orally administered LC-plasma exerts an inhibitory effect on DENV, the expression of anti-viral factor genes in the spleen were evaluated, which is a primary site of DENV infection (33). Subsequent to two weeks of LC-plasma intake, 10 mice from each group were sacrificed, the spleens were harvested and the expression of anti-viral factor genes in the spleen were measured using RT-qPCR.
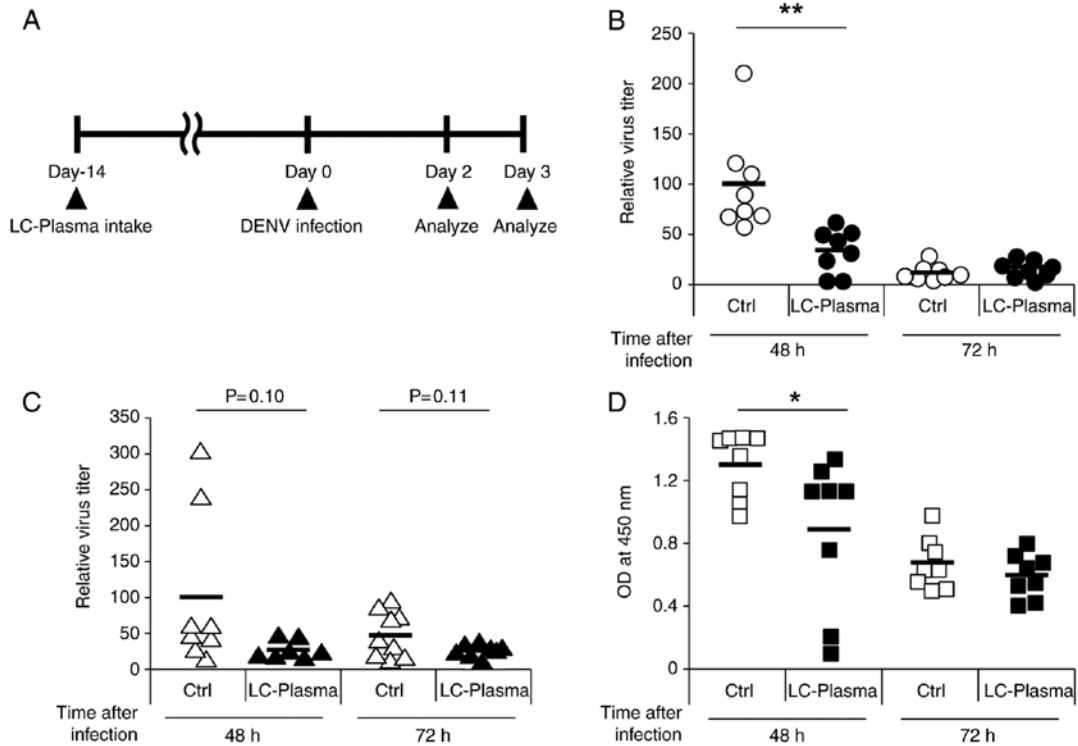
Figure 1. Effect of LC-plasma on DENV infection. (A) Experimental procedure for DENV infection. Mice in the control and LC-plasma groups were fed a diet with or without 1 mg/head/day LC-plasma during the study period (day-14 to 3). Mice were intraperitoneally infected with DENV Eden2 at a titer of $1x10^7$ pfu on day 0. A total of 2-3 days after DENV infection, a total of 8 mice were sacrificed from each group, and spleens, livers and blood were harvested. Total RNA was then extracted from spleen and liver. Relative virus titers in the (B) spleen and (C) liver were measured by reverse transcription-quantitative polymerase chain reaction normalized to GAPDH. (D) Quantity of NS1 peptide in blood measured using an enzyme-linked immunosorbent assay. Each dot corresponds to an individual mouse. The line indicates the mean concentration. $^*P<0.05$ and $^{**}P<0.01$ with comparisons shown by lines. LC-plasma, *Lactococcus lactis* strain plasma; DENV, dengue virus; Ctrl, control; OD, optical density.
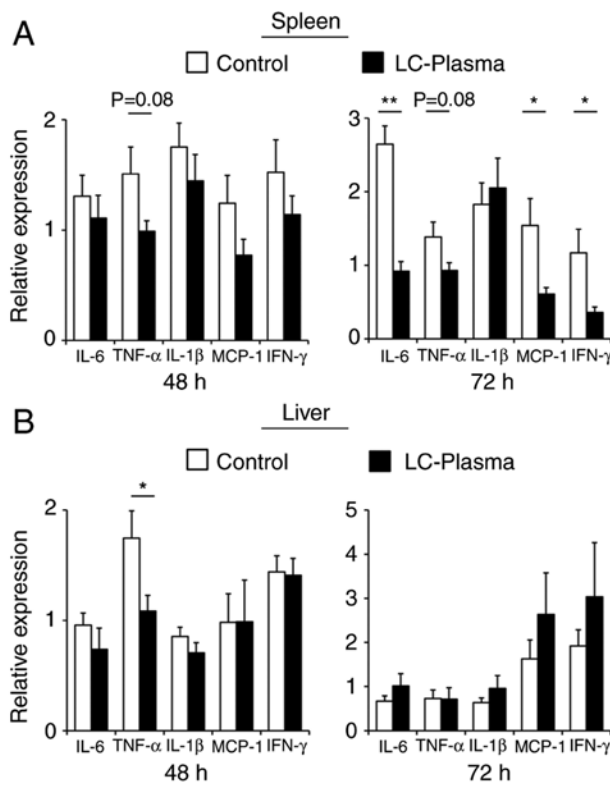

Figure 2. Effect of LC-plasma on DENV-mediated inflammation. Total RNA was extracted from the (A) spleen and (B) liver in each group. Expression levels of IL-6, TNF-α, IL-1β, MCP-1 and IFN-γ genes were evaluated by reverse transcription-quantitative polymerase chain reaction normalized to GAPDH. Data are presented as the mean ± standard error of the mean. $^*P<0.05$ and $^{**}P<0.01$ with comparisons shown by lines. LC-plasma, *Lactococcus lactis* strain plasma; DENV, dengue virus; IL, interleukin; TNF-α, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein 1; IFN-γ, interferon γ.
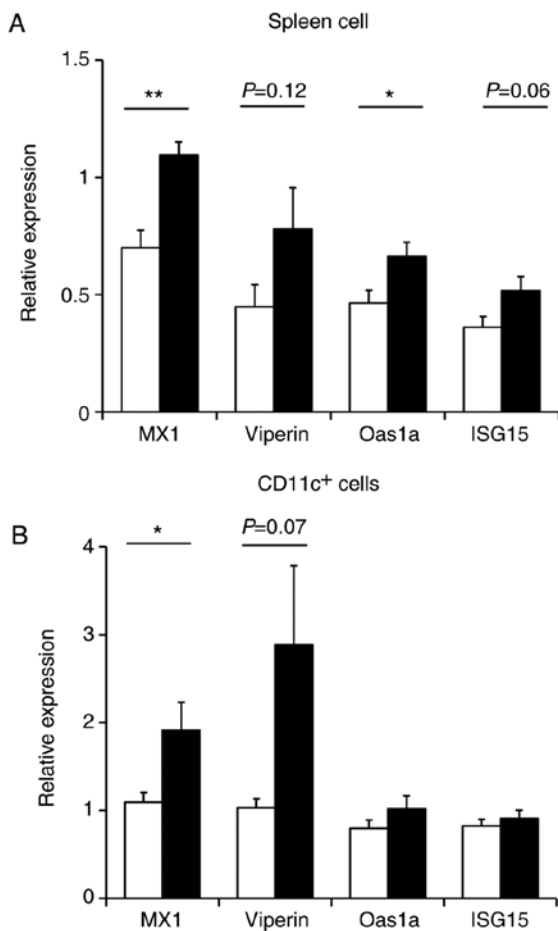
Figure 3. Effect of LC-plasma on anti-viral factor gene expression in spleen and CD11c$^+$ cells derived from splenocytes. Mice in the control and LC-plasma groups (n=10 each) were fed a diet with or without 1 mg/head/day LC-plasma for 2 weeks. A total of 2 weeks later, spleens were harvested from each mouse. Splenocytes were prepared and a portion of the splenocytes were collected for RNA extraction. CD11c$^+$ cells were separated from the remaining splenocytes by magnetic separation and collected for RNA extraction. Total RNA was extracted from the (A) splenocytes and (B) CD11c$^+$ cells to analyze Mx1, Viperin, Oas1a and ISG15 expression levels by reverse transcription-quantitative polymerase chain reaction normalized to GAPDH. Data are presented as the mean ± standard error of the mean. *P<0.05 and **P<0.01 with comparisons shown by lines. LC-plasma, *Lactococcus lactis* strain plasma; Mx1, interferon-induced GTP-binding protein Mx1; Oas1a, 2'-5'-oligoadenylate synthase 1A; ISG15, IFN-stimulated gene 15.
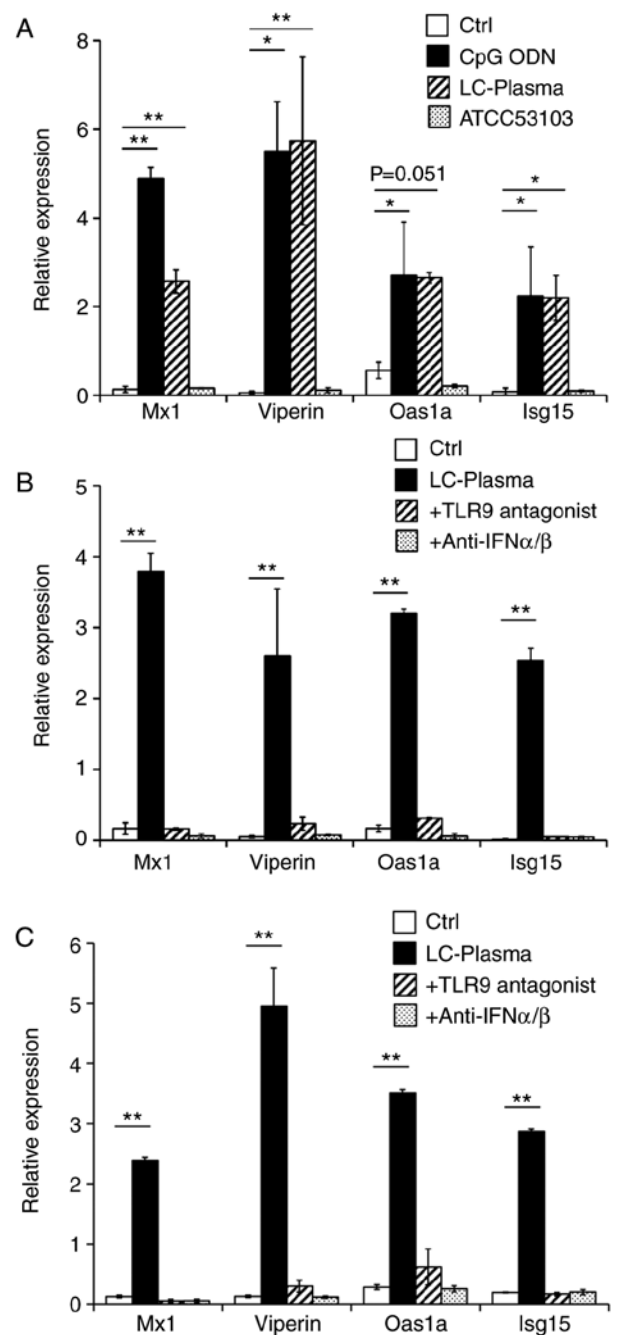

Figure 4. Enhancement of anti-viral factor gene expression mediated by LC-plasma via type I IFNs *in vitro*. (A) Flt-3L induced BMDC were cultured with CpG ODN, LC-plasma or ATCC 53103 for 24 h. Subsequent to culturing, BMDC were collected for RNA extraction. (B) Flt-3L-induced BMDC were cultured with LC-plasma in the presence or absence of a TLR9 antagonist or anti-IFN-α/β antibody for 24 h. After culturing, the supernatant was collected and another sample of Flt-3L-induced BMDC was cultured with the collected supernatant for 24 h. Subsequent to culturing with the supernatant, BMDC were collected for RNA extraction. (C) Flt-3L-induced BMDC were cultured with LC-plasma in the presence or absence of TLR9 antagonist or anti-IFN-α/β antibody for 24 h. Subsequent to culturing, the supernatant was collected and the splenocytes were cultured with the collected supernatant for 24 h. After culturing with the supernatant, splenocytes were collected for RNA extraction. Total RNA was extracted from each cell culture to analyze Mx1, Viperin, Oas1a and ISG15 expression by reverse transcription-quantitative polymerase chain reaction normalized to GAPDH. Data are presented as the mean ± standard error of the mean. *P<0.05 and **P<0.01 with comparisons shown by lines. LC-plasma, *Lactococcus lactis* strain plasma; IFN, interferon; Flt-3L, FMS-like tyrosine kinase 3 ligand; BMDC, bone-marrow derived dendritic cells; ODN, oligodeoxyribonucleotides; TLR9, Toll-like receptor 9; Mx1, interferon-induced GTP-binding protein Mx1; Oas1a, 2'-5'-oligoadenylate synthase 1A; ISG15, IFN-stimulated gene 15.

As presented in Fig. 3A, oral administration of LC-plasma significantly enhanced the expression levels of Mx1 (P<0.01) and Oas1a (P<0.05) compared with the control group. In addition, LC-plasma non-significantly increased the expression levels of Viperin and ISG15 compared with the control group (P=0.12 and P=0.06, respectively). Thus, LC-plasma administration caused the induction of anti-viral factor gene expression in the spleen. Among the various immune cells in the spleen, CD11c$^+$ cells are the major target cells for DENV infection (33). Therefore, anti-viral gene expression in CD11c$^+$ cells was also measured, which were magnetically separated from the spleen tissue. As presented in Fig. 3B, LC-plasma administration significantly induced the expression levels of Mx1 (P<0.05) and also non-significantly enhanced Viperin expression levels (P=0.07) in CD11c$^+$ cells from the spleen compared with the control group. On the other hand, Oas1a and

ISG15 expression levels in CD11c^{+} cells were not influenced by LC-plasma administration. These results demonstrate that LC-plasma may alleviate DENV infection through an increase of anti-viral factor gene expression in infected tissue.

LC-plasma enhances anti-viral factors through type I INF in vitro. *In vivo* studies revealed that the oral treatment of LC-plasma enhanced the anti-viral factors in DENV infected tissue, which may be one mechanism used by LC-plasma to alleviate DENV infection. As LC-plasma strongly induces type I IFNs through pDC activation, it was investigated whether the effect of LC-plasma on the enhancement of anti-viral factors was dependent on type I IFNs through the use of *in vitro* studies. To assess this hypothesis, Flt-3L-induced bone-marrow derived DC (BMDC) were used, which includes pDC and cDC (35). BMDC were stimulated with the TLR9 agonist CpG ODN, or with the LAB strains of LC-plasma or *Lactobacillus rhamnosus* strain GG (ATCC 53103). After 24 h of stimulation, BMDC were collected and the expression levels of anti-viral genes were analyzed. As presented in Fig. 4A, stimulation of BMDC with CpG ODN or LC-plasma significantly increased the expression of the anti-viral factor genes compared with the control group (P<0.05; Fig. 4A). In contrast, ATCC 53103 did not influence the expression of the anti-viral genes. To investigate whether this LC-plasma effect is dependent on soluble factors including interleukins or interferons produced by BMDC, the supernatants were collected and another sample of BMDC was re-stimulated with these supernatants. As presented in Fig. 4B, the supernatants from BMDC stimulated with LC-plasma also significantly enhanced anti-viral gene expression in another BMDC sample (P<0.01). However, this effect was completely abolished by TLR9 antagonist treatment. Furthermore, anti-IFNα/β antibody treatment with BMDC stimulated with LC-plasma also blocked this effect. To investigate whether this LC-plasma effect also occurs in the spleen, which is the major DENV infected tissue, supernatants collected from BMDC stimulated with LC-plasma were added to splenocytes. As presented in Fig. 4C, LC-plasma supernatants also significantly enhanced anti-viral gene expression levels compared with the control group (P<0.01) and this effect was completely abolished by TLR9 antagonist or anti-IFNα/β antibody treatment. Based on these results, it appears that LC-plasma may attenuate DENV infection through type I IFN production by pDCs.

Discussion

To date, despite major efforts, there exists no effective treatment or vaccine for DENV infection. It has been previously reported that the specific LAB, LC-plasma, stimulates pDCs to induce the production of a large quantity of type I IFNs (28). As pDCs and type I IFNs serve a pivotal role in anti-viral immune responses, it was predicted that pDC-stimulative LAB would be effective against DENV. In the present study, the effect of LC-plasma on DENV infection was examined using a mouse model.

A major technical barrier in developing therapeutics or vaccines against DENV infection is the absence of a suitable animal model that mimics dengue disease. The first established animal models used immune-competent mice in which a high dose of DENV was administered intracranially (36). Other animal models included immunocompromised mice lacking type I and/or type II IFNs receptors (37,38). However, neither of these models is suitable to evaluate treatment or vaccines for DENV as the route of infection is different from the usual DENV infection route in one model, and the other uses an immunocompromised mouse lacking the relevant immune receptor(s) (39). Previously, a DENV strain capable infecting mice, DENV Eden2, was reported (33,40). This strain was used to infect wild-type mice by intraperitoneal injection and it was revealed be capable of replicating in the spleen and liver. These organs are also DENV-target organs in humans, so this strain is appropriate for evaluating the effectiveness of therapeutics for DENV. Therefore, the present study used the DENV Eden2 strain to evaluate the effect of LC-plasma on DENV infection *in vivo,* and revealed that LC-plasma is effective against DENV infection by measuring the virus titer in the spleen and the liver and the NS1 expression level in blood.

It was also revealed that inflammatory gene expression in DENV infected tissue was inhibited by oral administration of LC-plasma. DENV infection in children and adults usually results in dengue fever accompanied by a combination of symptoms that includes headache, retro-orbital pain, myalgia and hemorrhagic manifestations (41,42). Certain patients including newborns and elderly people occasionally have an onset of dengue hemorrhagic fever, the severest form of dengue disease. Patients with dengue hemorrhagic fever manifest a cytokine storm, with high levels of circulating pro-inflammatory cytokines and chemokines, which results in endothelial damage and vascular leakage with hemorrhaging and shock (6,42). Therefore, excessive inflammation induced by DENV infection results in severe dengue disease and the inhibition of inflammatory cytokines or chemokines may result in a reduction of DENV-associated inflammatory symptoms (43,44). Thus, LC-plasma administration may contribute to the suppression of DENV pathogenesis.

A number of reports have demonstrated that type I IFNs serve a principal role in inhibiting DENV replication (12-15). One of the essential roles of type I IFNs is the induction of anti-viral factors, which are involved in the suppression of viral replication and release (16-18). Thus, it was predicted that LC-plasma administration may inhibit DENV replication through the enhancement of anti-viral factors in DENV infected tissues. To reveal the mechanism of the LC-plasma effect against DENV infection, it was investigated whether LC-plasma enhances the expression of anti-viral genes in DENV infected tissues and cells. The expression of four important anti-viral genes, Mx1, Viperin, Oas1a and Isg15, were assessed. Mx1 is an interferon-induced GTP-binding protein that prevents transcription of DENV by interacting with viral polymerase (45). Viperin is a multifunctional antiviral factor that inhibits the replication of DNA and RNA viruses including hepatitis C virus, cytomegalovirus, influenza virus and DENV (46). Isg15 is an ubiquitin-like interferon-stimulated protein that conjugates viral proteins, resulting in the suppression of viral release (47). Oas1a is a 2',5'-oligoadenylate synthase that synthesizes higher oligomers of 2'-5'-oligoadenylates from ATP. The oligoadenylates activate RNase L, which degrades viral RNAs (48). Oral administration of LC-plasma significantly enhanced Mx1 and

Oas1a and non-significantly enhanced Viperin and Isg15 in the spleen. Furthermore, LC-plasma induced Mx1 and Viperin in CD11c⁺ cells, which is one of the cell types that DENV infects. The results of the present study indicate that LC-plasma may suppress DENV replication by inducing the expression of these anti-viral genes.

The *in vitro* experiments in the present study clearly demonstrated that LC-plasma increases the expression of anti-viral genes; and this effect was completely abolished by TLR9 antagonists or anti-type I IFN antibody treatment. However, another strain of LAB, ATCC 53103, which does not induce type I IFNs, did not have the ability to enhance anti-viral gene expression. These results demonstrate that pDC-stimulative LAB strongly inhibit viral infection through type I IFN production.

To date, various strains of LAB have been reported to have immune-modulatory effects by affecting immune cells, resulting in anti-pathogenic effects or improving allergy symptoms (49-51). In the present study, it was revealed for the first time that the pDC-stimulating LAB, LC-plasma, is effective against DENV infection. As LC-plasma also has this effect on different types of viruses (29,30), pDC-stimulating LAB may additionally be effective against other viruses.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS and NoY designed the experiments. HS, RT, MS and NoY performed the experiments and analyzed the data. NaY, NoY and OK supervised the experiments. HS wrote the paper and all authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in strict accordance with the guidelines for the care and use of laboratory animals of Juntendo University (Tokyo, Japan) and Kirin Company (Kanagawa, Japan). The studies were approved by the Committee for Animal Experimentation of Juntendo University and Kirin Company.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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