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# The protective effects of lactoferrin against murine norovirus infection through inhibition of both viral attachment and replication

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ABSTRACT

The purpose of this study was to evaluate the effects of bovine lactoferrin against norovirus infection using mouse norovirus (MNV) and Raw264.7 cell *in vitro*. When Raw264.7 cells were infected with MNV in the presence or absence of lactoferrin, the cytotoxic damage to the infected Raw264.7 cells significantly and dose-dependently decreased and completely inhibited in the presence of 15 or 20 μg/well of lactoferrin as compared with the absence of lactoferrin. Correspondingly, the MNV titers in the culture medium and intracellularly were significantly decreased in infected Raw264.7 cells treated with lactoferrin compared to control infected Raw264.7 cells. The mechanisms responsible for the protective effects of lactoferrin against MNV infection were attributed to both its inhibition of the initial MNV attachment to cells and the subsequent interference with MNV replication. Moreover, it was revealed that lactoferrin could rapidly induce the expression of anti-viral cytokine mRNA, such as IFN-α and IFN-β which involved in inhibition of MNV replication in infected Raw264.7 cells, in the early phase of infection. It was concluded that lactoferrin exerts protective effects against MNV infection through inhibition of both viral attachment and replication. The present results provide evidence that lactoferrin may be useful as a preventive and/or therapeutic anti-norovirus agent.



## 1. Introduction

Norovirus causes the majority of acute infectious nonbacterial gastroenteritis in humans of all ages worldwide, and the highest frequency of norovirus infection is in the winter season. Norovirus infects its hosts through the intake of contaminated water and food. Moreover, norovirus spreads very rapidly through direct contact with waste materials derived from infectious hosts, and it spreads easily from person to person, especially in closed communities such as nursing homes, hospitals and schools. In addition, norovirus is highly infectious in humans, and can cause infection after an intake of only 1000 pfu [1]. The symptoms caused by norovirus infection include nausea, forceful vomiting, watery diarrhea and abdominal pain within 24 h after the infection. The risk of morbidity and mortality are increased in infants, the elderly and immunocompromised hosts. Moreover, no vaccine or effective anti-viral drug for norovirus infection has been developed, and the host's memory immunity to norovirus infection is generally transient and incomplete [2]. Therefore, norovirus causes recurring infections.

Unfortunately, human norovirus remains difficult to study, because there is a lack of cell culture and animal models. Recently, MNV that grows in a murine cell line, which is RAW264.7 cell, has been reported. It can provide the first cell culture system to investigate the pathogenesis and molecular mechanisms of norovirus replication [3–5].

Lactoferrin is present in the saliva, tears and breast milk of several mammalian species, and has many physiological functions involved in host defense system, and one of effects of lactoferrin is to protect the host from parasitic, bacterial and viral infections [6]. Lactoferrin prevents infections with numerous viruses, regardless of whether the virus has an envelope or is non-enveloped. Non-enveloped viruses, such as rotavirus, enterovirus and adenovirus, cause infections of the gastrointestinal tract, and their infections are prevented by lactoferrin [7–9]. The protective effects of lactoferrin against viral infections are considered to be attributable to its preventing viral attachment to cells, internalization of the virus and/or to inhibition of viral replication in cells. However, it remains unclear whether lactoferrin also has any effect against norovirus infection, and the potential mechanisms of protection against norovirus infection have yet to be elucidated.

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Therefore, the purpose of the present study was to determine whether lactoferrin has protective effects against norovirus infection using MNV and Raw264.7 cells *in vitro*. We herein demonstrate not only that lactoferrin has protective effects, but also the potential mechanisms by which lactoferrin exerts these effects against MNV infection.

## 2. Materials and methods

### 2.1. Cells, virus and lactoferrin

Raw264.7 cells were purchased from RIKEN BioResource Center Cell Bank (Tsukuba, Japan). The cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, MO, USA) in 5% $CO_2$ at 37 °C. MNV was originally isolated from a laboratory mouse in Japan, and the genome sequence was determined (Genbank accession No. AB435515) [10]. MNV was used to infect confluent Raw264.7 cells in 150 $cm^2$ tissue culture flasks, and the Raw264.7 cells were subsequently cultured in 5% $CO_2$ at 37 °C for 3 days. Then, supernatants including the MNV were collected and centrifuged at 1750*g* for 15 min. After filtration, the aliquots of virus fluid were frozen at −80 °C until use. The bovine lactoferrin used in this study was kindly provided by Morinaga Milk Industry CO., Ltd.

### 2.2. Virus titer

The virus titers were determined by the $TCID_{50}$ methods. Briefly, $2 \times 10^5$ Raw264.7 cells were cultured in 96 well flat bottom plates overnight. To measure the virus titer, the confluent Raw264.7 cells were infected with titrated MNV, followed by incubation at 37 °C for 48 h. The cells were then fixed and stained with a 20% methanol solution including 0.1% crystal violet. The $TCID_{50}$ of the virus titer was calculated using the Reed and Muench method [11]. In some experiments, the cDNA generated from the MNV RNA, which is determined based on the virus titer obtained by the $TCID_{50}$ methods was used as a standard for the virus titer for quantitative real-time PCR.

### 2.3. Purification of mRNA and synthesis of cDNA

For purification of viral mRNA in the culture supernatant and synthetic cDNA, a MagMax viral RNA isolation kit and a TaqMan gene expression Cells-to-CT kit were used (Ambion, TX, USA). Purification of mRNA in infected cells and synthetic cDNA was performed by a TaqMan gene expression Cells-to-CT kit according to the manufacturer's instructions (Ambion).

### 2.4. Quantitative real-time PCR

The probes and primers for quantitative real-time PCR used in this study are indicated below. For detection of the MNV titer, a forward primer 5′-AATCTATGCGCCTGGTTTGC-3′ and a reverse primer 5′-CTCATCACCCGGGCTGTT-3′ were employed to amplify a specific region of the virus RNA sequence, and a Taqman probe with the sequence 5′-CTGTACACGCCACTCCGC-3′ was generated with 5′-FAM dye and 3′-BHQ dye (Sigma, MO, USA) based on a sequence as modified of previously described by Barron et al. [12]. TagMan gene expression assays for mouse IFN-α (Assay ID; Mm01703465_s1), mouse IFN-β (Assay ID; Mm00439552_s1) and mouse GAPDH (Assay ID; Mm03302249_g1) were purchased from Applied Biosystems (CA, USA). Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system with included software program. In some experiments, each sample was calibrated to the internal standard (GAPDH) level and normalized to the average value of control samples.

### 2.5. Cytotoxicity assay

To assess the cytotoxicity of MNV infection, confluent Raw264.7 cells were infected with MNV at 400 pfu with various concentrations of lactoferrin, and the cells were incubated at 37 °C for 48 h. Then, the cells were fixed and stained with a 20% methanol solution including 0.1% crystal violet to estimate the cell viability. Wells with a decreased number of cells were considered to have experienced a cytotoxic effect.

### 2.6. MNV attachment and reproduction assays

To measure the inhibition of MNV attachment to cells by lactoferrin, confluent Raw264.7 cells were incubated with 20 μg/well of lactoferrin at 37 °C for 1 h. Subsequently, the cells were exposed to 400 pfu MNV at 37 °C for 1 h, followed by gentle washing three times with PBS to remove unbound virus. The RNAs from infected cells were isolated and purified and the virus titer was determined by a quantitative real-time PCR.

To determine whether there was inhibition of MNV replication by lactoferrin, confluent Raw264.7 cells were infected with 400 pfu MNV at 37 °C for 1 h, followed by gentle washing three times with PBS to remove unbound virus. The cells were then incubated with DMEM medium including 20 μg/well of lactoferrin at 37 °C for various lengths of time, then the RNAs were obtained from the cells and the virus titer was measured by quantitative real-time PCR. In some experiments involving neutralization of IFN-α or/and IFN-β, 25 μg/ml of anti-IFN-α antibody (Ab) (clone: RMMA-1) or/and anti-IFN-β Ab (clone: RMMB-1) was added in the wells at same time as the addition of 20 μg/well of lactoferrin after MNV infection. The RNAs were purified from the cells 12 h later.

### 2.7. Statistical analysis

The statistical analyses were performed using the Prism ver. 5.0 software program (GraphPad software Inc., CA, USA). The statistical significance of the findings was calculated using the unpaired *t*-test for all of experiments. In some experiments, the statistical significance of dose-dependent responses in the *in vitro* assay were calculated by a linear regression analysis. *P*-values of $<0.05$ were considered to be statistically significant. All values are presented as the means ± standard deviation.

## 3. Results

### 3.1. Lactoferrin inhibits the cytotoxic damage caused by MNV in association with a decreased MNV titer in the culture medium

To investigate whether lactoferrin is effective against MNV infection, Raw264.7 cells were cultured with various concentrations of lactoferrin and 400 pfu of MNV for 48 h. As shown in Fig. 1A, a protective effect of lactoferrin against the cytotoxicity of MNV was indicated, and occurred in a concentration-dependent manner. Moreover, the cytotoxicity of MNV was completely inhibited in all of the wells treated with 15 and 20 μg/well of lactoferrin. The MNV titer in the culture medium also significantly decreased in the presence of lactoferrin, with a concentration-depend effect observed in wells treated with 2.5–20 μg/well, compared to cells incubated without lactoferrin (Fig. 1B). However, when Raw264.7 cells were infected with MNV at 4000 pfu, the inhibition of cytotoxic damage was not observed even in the presence of 20 μg/well of lactoferrin (data not shown). These results suggest that

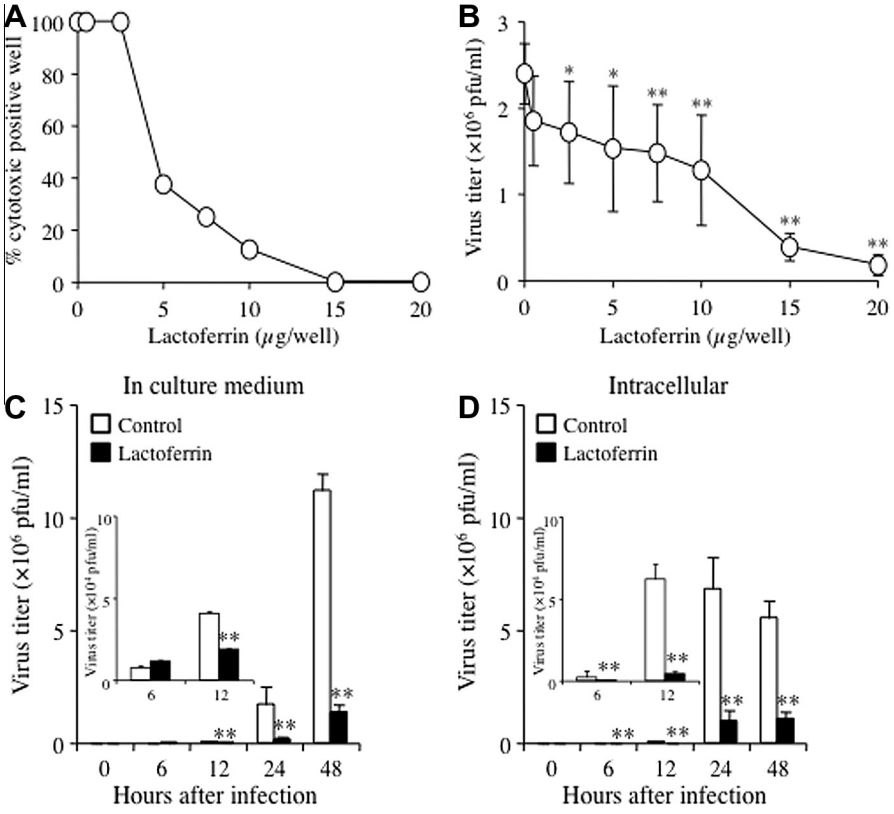


**Fig. 1.** Inhibition of the cytotoxicity and viral reproduction of MNV by lactoferrin. Confluent Raw264.7 cells were cultured with serially diluted lactoferrin and MNV of 400 pfu for 48 h. The cells were then fixed and stained with a 20% methanol solution including 0.1% crystal violet to identify damaged cells (A) and the culture supernatants were collected for a quantitative real-time PCR analysis to measure the virus titer (B). Data from eight wells were used for each group (A and B). Confluent Raw264.7 cells were cultured with 20 μg/well of lactoferrin and 400 pfu MNV for 48 h. The MNV titers were determined by quantitative real-time PCR. Samples from six wells were used for each group (C and D). $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the virus titer of the infected cells without lactoferrin treatment. Similar results were obtained from three independent experiments.

lactoferrin inhibits the cytotoxicity of MNV, but not high dose of MNV infection, in association with a reduction in the number of MNV in culture.

### 3.2. Lactoferrin decreases the number of MNV in infected cells followed by reduced the viral titer in culture medium

To determine whether lactoferrin inhibits MNV replication in cells and/or the viral release from infected cells, Raw264.7 cells were cultured with 400 pfu MNV and 20 μg/well of lactoferrin, which was sufficient to inhibit the cytotoxic damage induced by infection with 400 pfu of MNV, for 6, 12, 24 and 48 h. As shown in Fig. 1C and D, the MNV titer in the culture medium was significantly decreased in lactoferrin-treated wells compared to those of control infected cells at 12, 24 and 48 h after viral infection. The intracellular MNV titer in lactoferrin-treated cells was also significantly reduced at 6, 12, 24 and 48 h compared to infected control cells, thus suggesting that lactoferrin is responsible for decreasing the number of MNV in infected cells followed by reducing the release of MNV from infected cells.

### 3.3. Lactoferrin inhibits MNV attachment to cells

Based on the above results, two putative mechanisms by which lactoferrin inhibits MNV infection were considered. The first was that it inhibits the attachment to cells and the second was that it inhibits MNV replication in infected cells. Therefore, each possible protective mechanism was investigated. First, to determine whether lactoferrin inhibited MNV attachment to cells, Raw264.7 cell were treated with 20 μg/well of lactoferrin followed by infection with MNV for 1 h. After washing the cells, the MNV titer on/in the cells was measured by quantitative real-time PCR. As shown in Fig. 2, the MNV titer on/in cells was significantly reduced in lactoferrin-treated cells compared to that in control cells.

### 3.4. Lactoferrin inhibits MNV replication and increases the expression of anti-viral cytokines

Next, we investigated whether lactoferrin inhibited MNV replication. MNV infected Raw264.7 cells were washed, and then 20

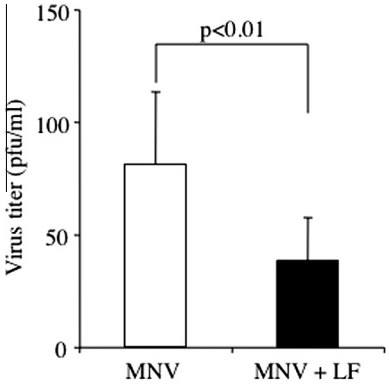


**Fig. 2.** Inhibition of MNV attachment to cells by lactoferrin. Confluent Raw264.7 cells were cultured with 20 μg/well of lactoferrin for 1 h, followed by infection of the cells with 400 pfu MNV. At 1 h after virus infection, the cells were gently washed three times with PBS. Total RNAs were extracted from the cells to measure the MNV titers by quantitative real-time PCR. LF means an abbreviation for lactoferrin. Samples from eight wells were used for each group. These results are representative of two independent experiments.

μg/well of lactoferrin was added in the wells. The MNV titer in infected cells was significantly decreased by lactoferrin treatment compared to that in control cells at 6, 12 and 24 h after virus infection (Fig. 3A).

As it is well known that IFN-α and IFN-β induce potent anti-viral responses in the early phase of viral infection [13], changes in the IFN-α and IFN-β mRNA expression in infected cells induced by lactoferrin were measured by quantitative real-time PCR. As shown in Fig. 3B, the expression of both IFN-α and IFN-β mRNAs in infected cells significantly increased in the lactoferrin-treated cells compared to control-infected cells, which exhibited no increase in IFN-α or IFN-β mRNA expression during the viral infection. These results suggest that lactoferrin induces anti-viral cytokines in the early phase of infection, leading to inhibition of MNV replication.

### *3.5. Neutralization of IFN-α or/and IFN-β abrogates the inhibition of MNV replication induced by lactoferrin*

To evaluate the inhibitory effects of IFN-α and/or IFN-β on MNV replication, neutralizing assay tests of IFN-α and/or IFN-β were performed. MNV infected Raw264.7 cells were washed, and then lactoferrin and the anti-IFN-α Ab and/or anti-IFN-β Ab was added in the wells. As shown in Fig. 4, lactoferrin inhibited the MNV replication, consistent with the above results. When the anti-IFN-α Ab or/and anti-IFN-β Ab was added in the culture wells in the presence of lactoferrin, the inhibition of MNV replication induced by lactoferrin was significantly abrogated, although the effects of the anti-IFN-α Ab on MNV replication were not complete.

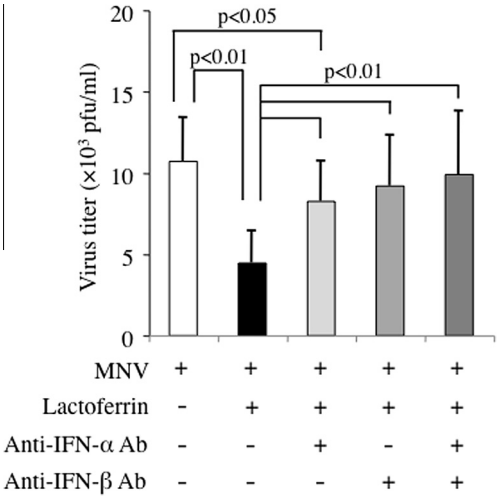


**Fig. 4.** Inhibition of MNV replication by lactoferrin is abrogated by the neutralization of IFN-α or/and IFN-β. Confluent Raw264.7 cells were infected with 400 pfu MNV at 37 °C for 1 h, followed by gentle washing three times with PBS. The cells were then cultured in supplemented DMEM containing 20 μg/well of lactoferrin, and 25 μg/ml of anti-IFN-α Ab and/or anti-IFN-β Ab at 37 °C. Twelve hours later, total RNAs were extracted from the cell lysates, and the virus titers were determined by real-time PCR. Samples from ten wells were used for each group. Similar results were obtained from two independent experiments.

## 4. Discussion

Norovirus infects many people worldwide every year, especially during the winter. There are currently not any vaccines or anti-viral drugs available for norovirus, and the immunity against norovirus is incomplete after infection. While human norovirus cannot be adequately investigated because there is no suitable animal model or susceptible cell culture system available for such experiments, cell culture experiments using MNV have been performed [4,5]. Therefore, we evaluated the effects of lactofferin against norovirus infection using MNV in this study. To the best of our knowledge, this is the first report that lactoferrin inhibits not only MNV attachment to cells, but also the replication of the virus, in association with an enhancement of anti-viral immunity via the induction of IFN-α and IFN-β. As a result of these activities, lactoferrin decreased the viral burden of MNV infection, leading to inhibition of the cytopathical effects.

Lactoferrin is secreted by mucosal tissues, and is present in the saliva, tears and milk of several mammalian species. Lactoferrin has non-immune natural defense functions. The effects of lactoferrin against microbial infections including those by parasites, bacteria and viruses have been reported in a large number of *in vivo* and *in vitro* studies [6]. Among these effects, the anti-viral functions of lactoferrin *in vitro* are considered to be mediated through its inhibition of viral attachment to and entry into cells, which are the first steps of viral infection, or its inhibition of the replication of viruses including cytomegalovirus [14], herpes simplex virus [15], Epstein-Barr virus [16] human immunodeficiency virus [17], human hepatitis C virus [18], rotavirus [7,19], enterovirus [8], and adenovirus [9]. Our present study showed that the pre-treatment of cells with lactoferrin prevents MNV from binding to and/or entering into cells. It has been previously reported that lactoferrin binds to cell surface glycosaminoglycans and low density lipoprotein receptors, which are used as receptors of several viruses, and that lactoferrin

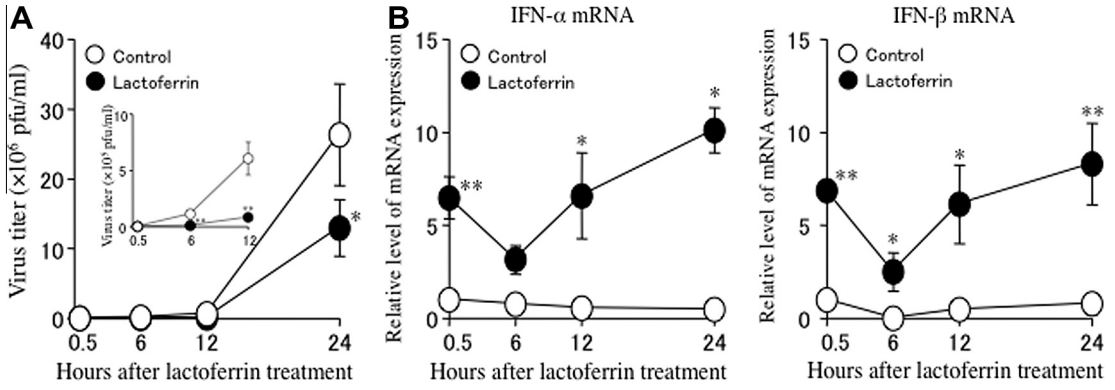


**Fig. 3.** Lactoferrin decreases MNV replication as a result of the induction of anti-viral cytokines. Confluent Raw264.7 cells were infected with 400 pfu MNV at 37 °C for 1 h followed by gentle washing three times with PBS. Then the cells were cultured in supplemented DMEM containing 20 μg/well of lactoferrin at 37 °C for each incubation time point. Total RNAs were extracted from the cell lysates for real-time PCR. The virus titer (A), and IFN-α and IFN-β mRNA expression (B) are shown. Samples from six wells were used for each group. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to control infected cells at the same time point. Similar results were obtained from three independent experiments.

competitively inhibits the binding of viruses to these molecules, leading to the prevention of virus infection [16,20]. Lactoferrin can bind to many viral receptors because it is a positively-charged glycoprotein. Although the histo-blood group antigen (HBGA) in humans was identified as a putative receptor or ligand of protrusion (P) domain of the human norovirus capsid protein [21,22], the receptors on cells for MNV have not yet been confirmed. Therefore, further studies are needed to provide direct evidence of whether lactoferrin can interact with viral receptors, such as the HBGA in mice, and with the P domain of the virus.

Our present results also showed that lactoferrin enhances anti-viral cytokine production including IFN-α and IFN-β. Moreover, neutralization of IFN-α or/and IFN-β by antibodies significantly abolished MNV replication inhibited by lactoferrin. Therefore, it plays an important role in the autocrine effects of IFN-α and IFN-β. Signaling via type I IFN mediated through the IFN-α/β receptor results in the induction of distinct proteins with diverse biological effects, including hundreds of different interferon-stimulated genes, which block viral replication and limit the cell-to-cell spread [13,23,24]. It has been reported that mice lacking the IFN-α/β receptor are more susceptible to MNV infection than wild-type mice [3]. Moreover, the signal transducer and activator of transcription 1 (STAT-1) molecule, which is downstream of IFN-α/β receptors signaling, is also associated with the innate immune response for preventing lethal norovirus infection [3]. Therefore, IFN-α/β signaling is involved in preventing MNV infection. It has recently been clearly shown that melanoma differentiation-associated gene 5 (MDA-5), but not toll-like receptor-3 (TLR-3) or retinoic-acid-inducible gene I (RIG-I), among the various pattern recognition receptors binding non-self RNA ligands, has a dominant role as an initial innate host sensor for MNV, and there are viral clearance delays in MDA-5 deficient cells infected with MNV [25,26]. In addition, activation of MDA-5 in response to a viral infection has been shown to initiate the innate immune response, including type I IFN production [25,27,28]. Type I IFN also enhances MDA-5 expression effectively creating a type I IFN-positive feedback loop [28]. Our results and those of other reports may indicate that both increased type I IFN production and enhanced MDA-5 expression cooperatively regulate the responses to norovirus infection.

In conclusion, we herein provided the first description of the effects of lactoferrin against MNV infection, including inhibition of the spread and pathogenesis of the virus. Our results demonstrated that lactoferrin treatment before the viral infection inhibits MNV attachment to cells, and that lactoferrin administration after the development of a viral infection prevents the viral reproduction because it rapidly leads to enhanced type I IFN production. These results suggest that lactoferrin has broad utility as a preventive and therapeutic agent against norovirus infection. Future research is needed to examine whether lactoferrin inhibits norovirus binding to cells through the specific receptor on the cells or to the viral binding domain, and to confirm the anti-viral signaling pathway by which type I IFN is induced by lactoferrin.

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