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# The effect of dietary bovine colostrum on respiratory syncytial virus infection and immune responses following the infection in the mouse[§]

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**Human respiratory syncytial virus (hRSV) is the most common cause of respiratory tract infection among young children because of immature T cell immunity of them against hRSV. CD8 T cells play a pivotal role in clearing hRSV and preventing subsequent infection. We examined the effects of dietary bovine colostrum on virus infection and CD8 T cell responses following hRSV infection in the mouse model. Mice received bovine colostrum for 14 days prior to hRSV challenge, and lung indexes (severity of symptom) and lung virus titers were analyzed. In addition, the activation of CD8 T cells in the bronchoalveolar lavage fluids (BALFs) of mice receiving bovine colostrum were compared with those in the BALFs of mice receiving phosphate-buffered saline (PBS) or ribavirin, post virus challenge. The severity of infection and lung virus titers were reduced in the mice receiving bovine colostrum, compared to those receiving PBS. Moreover CD8 T cell responses were selectively enhanced in the former. Our results suggest that dietary bovine colostrum exerts the effects to inhibit hRSV and ameliorate the symptom by hRSV infection, and enhances the CD8 T cell response during the hRSV infection.**

***Keywords***: bovine colostrum, CD8 T cell, respiratory syncytial virus

## Introduction

Human respiratory syncytial virus (hRSV) is a negative sense single stranded RNA virus belonging to the family *Paramyxoviridae*, and a leading cause of lower respiratory tract infection (Domachowske and Rosenberg, 1999). The large number of hospitalizations resulting from hRSV infection are a health and economic burden, especially in developing countries (Nair *et al.*, 2010). It is estimated that 200,000 children below five die annually from hRSV infection, mostly in developing countries (Nair *et al.*, 2010). hRSV infection is very frequent in infants and young children: 70% of infants under one year are infected with hRSV, and almost 100% of children under two are infected with it at least once (Glezen *et al.*, 1986; Shay *et al.*, 1999). It has been suggested that the high prevalence of hRSV in infants and young children is due to the lack of a response of T cell immunity against hRSV, or an inadequate response (Rossey *et al.*, 2014). hRSV is also a significant pathogen in adults; it frequently causes upper and lower respiratory illness in adults, although the symptoms are relatively mild and resemble the common cold (Walsh and Falsey, 2012). Unfortunately, there is no commercially available prophylactic vaccine for preventing hRSV infection. Approaches to preventing and treating RSV infection merit priority attention.

Colostrum is the first milk produced in mammals after parturition. The primary role of the colostrum is to protect neonates, which have immature immune system, from infectious agents, but it is also believed to contribute to the development of the brain and gastrointestinal tract of neonates (Wolinski *et al.*, 2012; Pierzynowski *et al.*, 2014). Fundamentally the effect of colostrum is species-specific. However, numerous studies have indicated that it can act across species. Thus for example there is evidence that dietary bovine colostrum enhances immunity to respiratory tract infection in humans. Cesarone *et al.* (2007) reported that it reduced flu episodes, and they suggested that supplementation with colostrum might be more effective than vaccination against influenza virus. Recently it was reported that bovine colostrum reduced the severity of viral upper respiratory tract infections in IgA deficient children (Patiroglu and Kondolot, 2013), and in infants (Uchida *et al.*, 2010). However, the effect of dietary bovine colostrum on hRSV infection has not been investigated.

Current evidence indicates that hRSV inhibits CD8 T cell priming and effector function (Rossey *et al.*, 2014). In other words, it is likely that the development of an adequate CD8 T cell response against hRSV is crucial for preventing and treating the infection. In this study we investigated the effect of dietary bovine colostrum on immune response and virus clearance following hRSV challenge in the mouse model.

## Materials and Methods

### Ethics

Five-week old female Balb/c mice were purchased from Orient Bio and acclimatized for one week. All animal experiments were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and with the Guidelines for Animal Experiments of Chung-

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### Preparation and titration of virus

Hep-2 cells were cultured in MEM-α medium (Gibco) containing 10% fetal bovine serum (v/v; GenDEPOT) and 1% penicillin/streptomycin (v/v; Invitrogen). At 80–90% confluence they were infected with 0.1–0.5 plaque forming unit (PFU) per cell of hRSV virus (A2 strain). Cells were harvested and disrupted with a Dounce homogenizer (WHEATON) 3 days after infection, and the lysates was clarified by centrifugation at 2,500 rpm for 10 min, and the supernatants stored at -80°C. Virus was titrated by plaque assay with modifications (Cherukuri *et al.*, 2012). For titrations, monolayer of Hep-2 cells in 96-well cell culture plates (BD Falcon) were incubated with serial dilutions of RSV for 2 h at 37°C, and cultured in MEM-α medium containing 2% FBS and 0.75% methyl cellulose (Sigma). After 6 days at 37°C, the cells were fixed with 2% glutaraldehyde (Sigma) and stained with 0.1% crystal violet in 20% ethanol and plaques were counted by microscope.

### Sample administration and virus challenge

Mice were divided into three groups. The phosphate-buffered saline (PBS) group and ribavirin group received 200 μl of PBS per day by oral gavage for 14 days prior to virus challenge. The colostrum group received 0.01 mg of bovine colostrum (based on protein determined by the Bradford assay; Ildong Foodis) per day by oral gavage for 14 days prior to virus challenge. The period (14 days) for oral administration of the bovine colostrum is based on previous report (Xu *et al.*, 2013). Protein contents of bovine colostrum used in this study are presented in Supplementary data Table S1. Mice in the ribavirin group were given ribavirin syrup (0.8 mg/day; Daewoo Pharmaceutical) for 3 days post virus challenge. The regimens of the PBS and colostrum groups were continued for 3 days post virus challenge. All mice received intraperitoneally 2 mg of cyclophosphamide (Sigma), an immunosuppressive agent, 5 days prior to virus challenge, and the indicated amount of hRSV by the intranasal route.

### Determination of lung indexes of lung virus titers

The virus challenge and lung harvest were conducted as described previously (Anderson *et al.*, 1990). hRSV A2 strain is known to make peak lung titer in Balb/c mouse at day 4 post virus challenge (Anderson *et al.*, 1990). Mouse lungs were collected at day 4 post virus challenge to determine lung indexes and lung virus titers. Lung indexes were calculated as follows: lung indexes = (lung weight / body weight) × 1,000 (Luo *et al.*, 2012). Mouse lungs were disrupted with a Dounce homogenizer (WHEATON), centrifuged at 2,500 rpm for 10 min at 4°C, and frozen quickly in a cell container containing 70% ethanol at -80°C. Hep-2 cells in 96-well or 24-well tissue culture plates (all TPP) were infected with serial dilutions of lung homogenates and cultured in MEM-α medium containing 2% fetal bovine serum and 0.75% methylcellulose (Sigma) for 6 day. Thereafter plaques were counted as described above.

### Measurement of anti-hRSV IgG titers in serum

Mouse sera were collected on day 9 post hRSV infection, and anti-hRSV immunoglobulin G (IgG), IgG1, and IgG2a titers were determined as described previously, with modifications (Kamphuis *et al.*, 2012). 96-well enzyme-linked immunosorbent assay plates (Greiner Bio One) were coated with hRSV and blocked with 3% skim milk in PBS containing 0.05% Tween 20 for 3 h at 37°C. The plates were incubated with serial dilutions of mice sera for 2 h at 37°C. Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bethyl), anti-mouse IgG1 (Serotec) and anti-mouse IgG2a (Serotec) were used to detect the anti-hRSV IgG, anti-hRSV IgG1, and anti-hRSV IgG2a bound to the immobilized hRSV, respectively. The reactions were developed with *o*-phenylenediamine (Sigma) and the optical densities were measured at 492 nm.

### Flow cytometry analysis

It has been known that T cells of mouse lung are developed and have cytotoxic effects against hRSV between 6 and 12 day after hRSV challenge in Balb/c mouse (Anderson *et al.*, 1990). Therefore, bronchoalveolar lavage fluid (BALF) cells were isolated nine days post virus challenge to analyze the T cell responses. To detect CD3, CD8, CD69 and interferon-γ (INF-γ), peridinin chlorophyll-efluor 710-labeled anti-CD3e, allophycocyanin-labeled anti-CD8, phycoerythrin-labeled anti-CD69 and PE-labeled anti-INF-γ antibodies were used. All antibodies were from eBioscience. Cell sorting was performed on a FACSCalibur flow cytometer (BD Bioscience). To measure % of CD8 T cell and CD8 T cell expressing CD69, CD3-, and CD8-positive cells and CD3-, CD8-, and CD69-postive cells were scored, respectively. Lymphocytes were selected from the forward and side scatters.

To assess the proportion of CD8 T cells producing INF-γ, BALF cells were stimulated with a multiplicity of infection of 3 of hRSV together with phorbol myristate acetate (Sigma) and ionomycin (Sigma) for 6 h at 37°C, and treated with Golgiplug™ (BD bioscience) to inhibit secretion of the INF-γ. CD3-, CD8-, and INF-γ-positive cells were scored. Fifty thousand events were acquired by flow cytometry and analyzed with Flowing Software 2.5 (www.flowingsoftware.com).

### Statistical analysis

*P* values were determined by two-tailed *t*-tests. Statistical analysis was performed with Graph Pad Prism 5 Software. *P* values less than 0.05 or 0.01 were considered statistically significant.

## Results

### Morbidity and lung virus titers in mice receiving bovine colostrum following hRSV infection

The PBS and colostrum groups received orally PBS and bovine colostrums, respectively, for 14 days prior to hRSV challenge, and each regimen was continued for 3 day post virus challenge. The ribavirin group received PBS orally for 14 days prior to hRSV challenge and ribavirin for 3 days

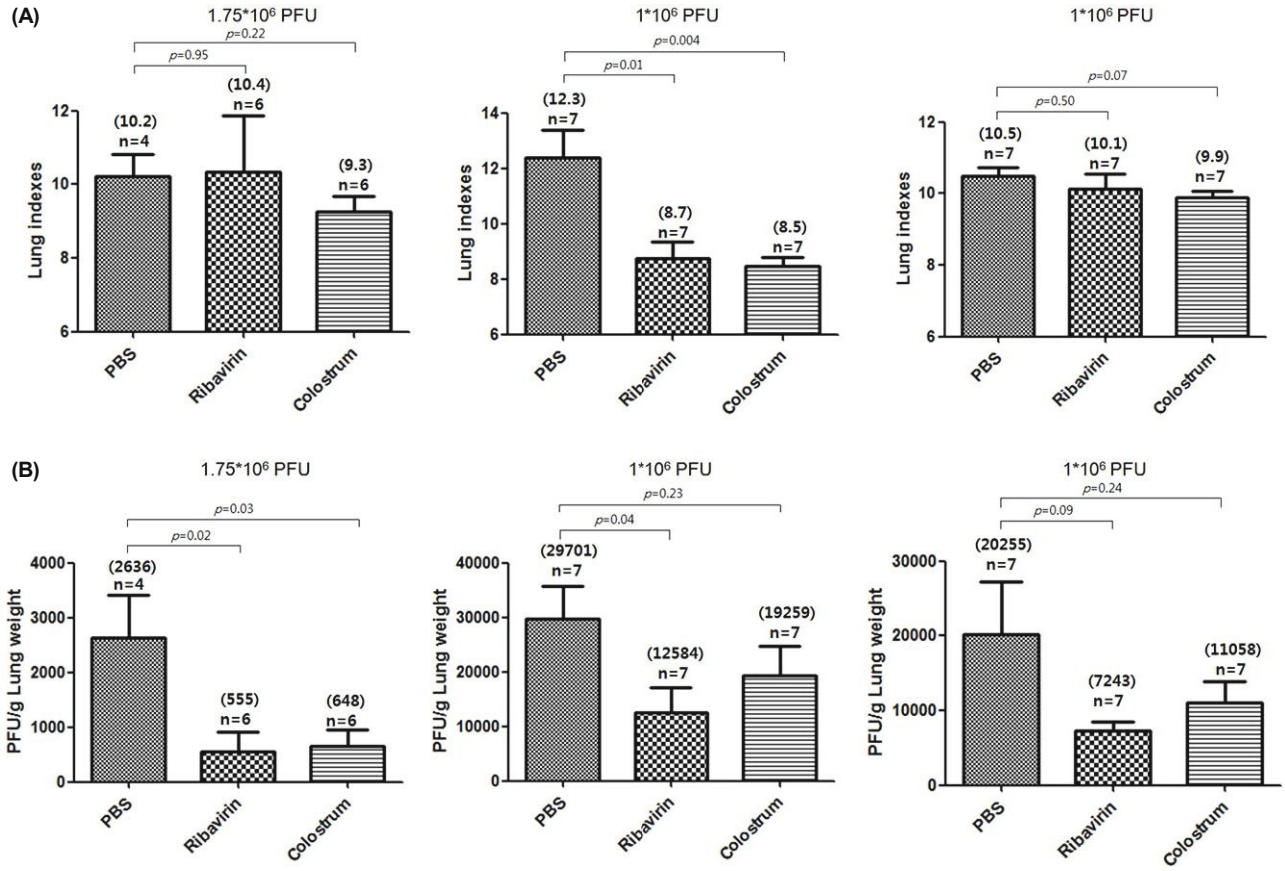


**Fig. 1. Lung indexes and lung virus titers following hRSV challenge.** Mice were challenged with $1.75 \times 10^6$ or $1 \times 10^6$ PFU of hRSV, and lung indexes (A) and lung virus titers (B) were determined as described in 'Materials and Methods' on day 4 post virus challenge. Three independent mouse experiments were conducted. Lung index reflects severity of symptom following hRSV infection and was assessed as follows: lung index = (lung weight / body weight) × 1,000. Data are mean ± standard error of mean (SEM). Numbers in parenthesis are mean values.

post challenge. Each group was challenged with $1 \times 10^6$ or $1.75 \times 10^6$ PFU of hRSV (Fig. 1). Severity of hRSV infection was assessed by lung index [(lung weight / body weight) × 1,000]. Lung indexes and lung virus titers were measured on day 4 post virus challenge, at the peak hRSV (Anderson *et al.*, 1990). As shown in Fig. 1A, the lung indexes of the colostrum group were lower than those of the PBS group. The colostrum group also had lower lung virus titers (Fig. 1B). Lung virus titers were in the decreasing order PBS, colostrum and ribavirin group, while the lung indexes were

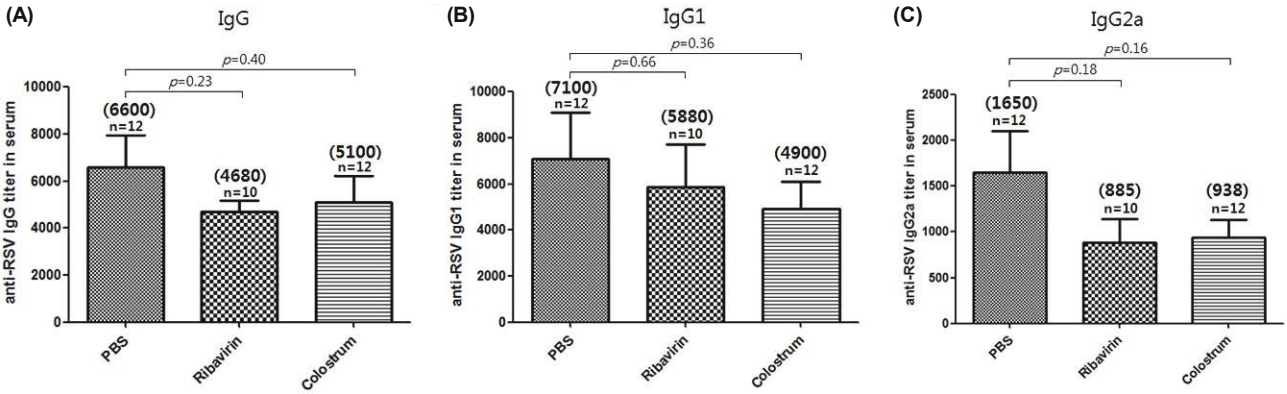


**Fig. 2. Anti-hRSV IgG titers following RSV challenge.** Mice were challenged with $5 \times 10^6$ PFU of RSV, and sera were collected on day 9 post virus challenge. Titers of anti-hRSV IgG (A), anti-hRSV IgG1 (B) and anti-hRSV IgG2a (C) were determined by ELISA described in 'Materials and Methods.' Data are mean ± SEM of individual mice (n = 12 or 10). Numbers in parenthesis are mean values. Statistical significances between groups were not found.

in the order PBS, ribavirin and colostrum. These results demonstrate that orally administered bovine colostrum is effective in inhibiting hRSV and in ameliorating the symptoms of hRSV infection.

### Humoral and cellular immune responses in mice receiving bovine colostrum following hRSV infection

We found that IgG titer was in the decreasing order PBS, colostrum and ribavirin group (Fig. 2A). Therefore, the trend of the antibody titer (Fig. 2A) was similar to that of the lung virus titers (Fig. 1B). Meanwhile, reduced anti-hRSV IgG1 (Fig. 2B) and anti-hRSV IgG2a (Fig. 2C) responses in ribavirin and colostrum group were found after hRSV challenge.

Evidence from the mouse model has indicated that CD8 T cells promote the clearance of RSV, and recovery from infection (Rossey *et al.*, 2014). Also, previous studies have suggested that hRSV suppresses the effector activity of CD8 T cells, which plays an important role in the control of secondary infection (Rossey *et al.*, 2014). CD69 is expressed rapidly and transiently in activated lymphocytes, but not in resting lymphocytes (Gonzalez-Amaro *et al.*, 2013). Expression of CD69 on T cells is thought to be involved in the induction of IL-2 and INF-γ, which eventually induce cell proliferation (Testi *et al.*, 1989). To investigate the expression of CD69 on CD8 T cell following hRSV infection, mice were given different amounts of RSV, and the proportions of activated CD8 T cells in BALF lymphocytes were measured on day 9 post virus challenge. There were no CD8 T cells expressing CD69 in mice not challenged with RSV (Table 1). Overall, the proportion of CD8 T cell expressing CD 69 in the colostrum group appeared to be higher than in the PBS and ribavirin groups (Table 1). The colostrum group had a significantly higher proportions of CD8 T cell expressing CD69 than the PBS or ribavirin group when the mice were challenged with $3.8 \times 10^2$ or $2.4 \times 10^4$ PFU of hRSV (CD8 T cell expressing CD 69% in Table 1). Overall, moreover, the BALF lymphocytes of the colostrum group had a higher proportion of CD8 T cells than those of PBS or ribavirin groups (CD8 T cell % in Table 1).

Expression of CD69 on the CD8 T cells in the BALF did not increase with hRSV dose: the highest expression of CD69 was observed in the mice receiving $2.4 \times 10^4$ PFU of RSV (CD8 T cell expressing CD 69% in Table 1), whereas the proportions of CD8 T cells among BALF lymphocytes were higher in the PBS and colostrum groups when they received $3 \times 10^6$ or $5 \times 10^6$ PFU of RSV (CD8 T cell % in Table 1). CD69 is expressed transiently in an early stage of T cell activation and CD69 expression induces T cell proliferation (Testi *et al.*, 1989; Gonzalez-Amaro *et al.*, 2013). Therefore we believe that the CD8 T cells in the mice receiving $2.4 \times 10^4$ PFU of RSV were in the early activation stage while those receiving $3 \times 10^6$ PUF or $5 \times 10^6$ PFU of hRSV were in the post activation stage (in which CD69s disappears).

In addition we measured the proportion of CD8 T cells producing INF-γ on day 9 post challenge. BALFs were collected and stimulated with hRSV, and the INF-γ-producing CD8 T cells were analyzed by staining intracellular cytokines. The colostrum group appeared to have a higher proportion of INF-γ-producing CD8 T cells than the PBS group (Table 2). This supports the evidence that bovine colostrum enhances the CD8 T cell response during RSV infection.

In summary, our results indicate that dietary bovine colostrum enhances the CD8 T cell response during hRSV infection and the ability to eliminate the hRSV; it also ameliorates the symptoms of hRSV infection.

**Table 1. The proportions of CD8 T cell and CD8 T cell expressing CD69 in mouse BALFs following hRSV challenge**
Mice were challenged with $3.8 \times 10^2$, $2.4 \times 10^4$, $3 \times 10^6$ or $5 \times 10^6$ PFU of hRSV by intranasal instillation. Four independent experiments were conducted. BALF were collected on day 9 post virus challenge and analyzed by flow cytometry. Lymphocytes of BALF were selected from the forward and side scatters. RSV(-) refers to mouse group without hRSV infection. Data are mean ± SEM.

| Infection dose of hRSV | Group | Mouse (n =) | CD8 T cell (%) | CD8 T cell expressing CD 69 (%) |
|---|---|---|---|---|
| $3.8 \times 10^2$ PFU | RSV (-) | 1 | 0 | 0 |
| | PBS | 5 | 4.0 ± 0.9 | 0.3 ± 0.1 |
| | Ribavirin | 5 | 3.8 ± 1.5 | 0.1 ± 0.1 |
| | Colostrum | 5 | 4.8 ± 1.0 | 0.6 ± 0.2*[b] |
| $2.4 \times 10^4$ PFU | RSV (-) | 2 | 3.5 ± 3.5 | 0 |
| | PBS | 6 | 22.6 ± 0.9 | 4.8 ± 0.8 |
| | Ribavirin | 6 | 24.0 ± 1.8 | 5.3 ± 0.9 |
| | Colostrum | 6 | 25.1 ± 0.9 | 7.7 ± 0.9*[a] |
| $3 \times 10^6$ PFU | RSV (-) | 1 | 0 | 0 |
| | PBS | 5 | 19.4 ± 1.4 | 2.4 ± 0.4 |
| | Ribavirin | 5 | 15.6 ± 1.6 | 2.3 ± 1.2 |
| | Colostrum | 5 | 45.9 ± 11.3*[ab] | 3.2 ± 0.8 |
| $5 \times 10^6$ PFU | RSV (-) | 2 | 0.4 ± 0.0 | 0 |
| | PBS | 6 | 39.5 ± 3.2 | 2.6 ± 0.2 |
| | Ribavirin | 6 | 18.7 ± 1.3**[c] | 1.9 ± 0.1*[c] |
| | Colostrum | 6 | 31.2 ± 4.5*[b] | 3.0 ± 0.4 |

[a] The value of colostrum group was compared to that of PBS group
[b] The value of colostrum group was compared to that of ribavirin group
[c] The value of ribavirin group was compare to that of PBS group
* $P < 0.05$
** $P < 0.01$

**Table 2. The proportion of INF-γ producing CD8 T cells following hRSV challenge.** INF-γ-producing CD8 T cells were analyzed by intracellular cytokine staining. BALF cells were isolated on day 9 post virus challenge and stimulated with hRSV together with PMA and ionomycin. CD3-, CD8-, and INF-γ-positive cells were scored by flow cytometry. RSV(-) refers to mouse group without hRSV infection. n=2; PBS, n=6; ribavirin, n=5; colostrum, n=6. Data are mean ± SEM. Statistical differences between three groups were not significant.

| | RSV(-) | PBS | Ribavirin | Colostrum |
|---|---|---|---|---|
| INF-γ-producing CD8 T cell (%) | 0 | 5.3 ± 0.8 | 6.2 ± 0.9 | 6.3 ± 0.4 |

## Discussion

There is evidence that oral supplementation with colostrum can reduce the episodes of respiratory tract infection (Cesarone *et al.*, 2007; Uchida *et al.*, 2010; Patiroglu and Kondolot, 2013). In addition there is a recent report that orally administered bovine colostrum (skimmed and concentrated) ameliorated the symptoms of influenza A (H1N1) virus and enhanced NK cell activity in the Peyer's patch, splenocytes and lung cells of mice (Uchida *et al.*, 2012). We showed previously that dietary intake of the acidic protein fraction of bovine colostrum increased the survival and reduced the weight loss

in mice receiving a lethal infection with influenza A (H1N1) virus (Xu *et al.*, 2013). Also, supplementation with bovine colostrum enhanced NK cell cytotoxicity and stimulated the immune response to primary influenza virus infection in mice (Wong *et al.*, 2014). In the present work, mice receiving bovine colostrum contained a higher proportion of CD69 expressing-CD8 T cell and INF-γ-producing CD8 T cell post virus challenge, than those receiving PBS or ribavirin (Tables 1 and 2). Moreover, the colostrum group had lower lung indexes than the PBS group (Fig. 1A). Thus bovine colostrum again augmented immunity to hRSV infection.

Ribavirin is a broad spectrum inhibitor of RNA viruses (Mondelli, 2014) and was licensed for the treatment of hRSV disease (Hall *et al.*, 1993). Ribavirin has been suggested as an inhibitor of viral RNA dependent RNA polymerase, host inosine monophosphate dehydrogenase or viral capping enzymes (Mondelli, 2014). Therefore, ribavirin has been considered as a direct inhibitor of hRSV although its mechanism of action has remained unclear. Indeed, mice receiving ribavirin showed significantly reduced lung virus titer, compared to those receiving PBS (Fig. 1B). However, the reduction rates of anti-hRSV antibodies in mouse sera (Fig. 2) and those of $CD8^+$ T cell and $CD8^+$ T cell expressing CD69 in BALF (Table 1) of mice receiving ribavirin were not comparable to that of lung virus titer. Moreover, mice of ribavirin group showed rather enhanced proportion of $CD8^+$ T cells producing INF-γ in BALF (Table 2). Therefore, the trends of immune responses of ribavirin group didn't follow that of lung virus titer. Meanwhile, it was confirmed that bovine colostrum orally administered reduced the lung virus titer (Fig. 1B) while that didn't inhibit plaque formation of hRSV *in vitro* (data not shown). Moreover, the bovine colostrum enhanced the responses of $CD8^+$ T cell and $CD8^+$ T cell expressing CD69 (Table 1). Therefore, it is likely that bovine colostrum orally administered controls hRSV infection indirectly through modulating immune system. Taken all together, it is thought that the immune responses following hRSV challenge are relatively independent from lung virus titer and there may be independent pathway for up-regulation or down-regulation of the immune responses in mouse model.

Bovine colostrum contains immunomodulatory components such as immunoglobulins, transforming growth factor-β, cytokines, and lactoferrin (Playford *et al.*, 2000). Two questions arise in relation to the systemic immunomodulatory effect of orally administered bovine colostrum. One is whether bovine colostrum components are active in other mammals, and another is how proteins that are readily digested can have systemic immunomodulatory effects. There is increasing evidence that orally administered bovine colostrum can have systemic immunomodulatory effects in other species (Yoshioka *et al.*, 2005; Stoy *et al.*, 2014; Xu *et al.*, 2014), and that components of colostrum can be absorbed through the gastrointestinal tract. It has been suggested that bovine IgG can bind to human Fcγ receptor and activate not only human T cells but also antigen presenting cells (Kramski *et al.*, 2012). The various types of glycosylated molecules are found in mammal's colostrum (Petzold *et al.*, 2004). Certain glycosylated molecules in human milk are resistant to the harsh condition of the GI tract (Dallas *et al.*, 2012), can have systemic physiological effects (Rudloff and Kunz, 2012), and can be detected in urine (Rudloff *et al.*, 1996), indicating that glycoconjugates can be absorbed through the gastrointestinal tract. Taken together, these evidences indicate that the components of bovine colostrum exert immunomodulatory effects through absorption in GI tract.

The immature immune systems of infants and the compromised immunity of elderly persons can increase susceptibility to hRSV infection (de Bree *et al.*, 2005; Cusi *et al.*, 2010; Rossey *et al.*, 2014). Moreover, an inadequate CD8 T cell response against hRSV is thought to be the main cause of repetitive infection by hRSV (Kruijsen *et al.*, 2010). Our results suggest that orally administered bovine colostrum is effective in enhancing CD8 T cell responses during hRSV infection. It also reduced lung virus titers and symptoms of hRSV infection.

## Competing interests

The authors declared that there are no conflicts of interest.

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