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SHORT COMMUNICATION

# Antiviral Resistance of Splenocytes in Aged Mice

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Abstract

We compared the susceptibility to viral infection of splenocytes, isolated from young versus old CBA mice, and evaluated the antiviral actions of lactoferrin in splenocytes infected with *Encephalomyocarditis virus* (*EMCV*). Recombinant mouse lactoferrin (rmLF) and bovine lactoferrin (bLF) were used. There were no differences in the susceptibility to *EMCV* infection in the studied age categories. Both types of lactoferrins were protective in young and old mice. The study confirmed the undisturbed viral resistance in old mice and the protective actions of lactoferrin in viral infection. The antiviral action of the homologous mouse lactoferrin was demonstrated for the first time.

Key words: immune ageing, lactoferrin, mice, splenocytes, viral resistance

Senescence is associated with gradual impairment of physiological processes, although the functions of the immune cell types may not be uniformly suppressed (Kogut *et al*., 2012; Sansoni *et al*., 2008; Scholz *et al*., 2013). The immune ageing is correlated with preferential diminution of the T-cell compartment, increased ratio of the memory cell phenotype and lower CD28 expression (Simioni *et al*., 2007). Although B cells from aged mice show decreased antigen-induced expansion, the ability of aged B cells to respond appropriately to T-dependent antigens and differentiate into antibody-secreting cells seems to be intact (Dailey *et al*., 2001). In aged humans, the antigen-presenting function of peripheral blood cells in response to staphylococcal enterotoxin is poor in comparison to young individuals (Castle *et al*., 1999). The susceptibility to viral infections in old animals has been a subject of consideration. Twenty two to twenty four month old mice, infected with respiratory syncytial virus, demonstrated diminished virus specific $CD8^+$ cytolytic response and IFN γ production (Zhang *et al*., 2002). In elderly human donors, the majority of the clonally expanded, virus-specific $CD8^+$ cells, was dysfunctional (Ouyang *et al*., 2003). However, the frequency of viral antigen-specific $CD8^+$ T cells was high in the majority of subjects older than 85 years and serologically positive for the viral epitopes (Scholz *et al*., 2013). These data indicate importance of a chronic antigenic stimulation, induced by persistent viral infections during a lifetime that may result in enhanced resistance to viral infections.

Lactoferrin is a multifunctional protein, present in excretory fluids and circulating neutrophils, involved in iron metabolism (Zimecki and Kruzel, 2007). The protein interferes with viral infection by means of several mechanisms, such as inhibition of virus replication and direct interaction with viruses (Välimaa *et al*., 2009; Picard-Jean *et al*., 2014) or blocking cell receptors for viruses (Pietroantoni *et al*., 2015; Zheng *et al*., 2012). The aim of this study was to compare the susceptibility of splenocytes from young versus aged organisms to viral infection and to investigate antiviral actions of a homologous, recombinant mouse and native bovine lactoferrin.

Three and 13 months old CBA mice, provide by the institute of Laboratory Medicine, Łódź, Poland, were used for the study. The local ethics committee approved the study. Low endotoxin (< 1.0 EU/mg) recombinant mouse lactoferrin (rmLF) produced by Chinese hamster ovary cells was obtained from PharmaReview Corporation, Houston, USA, and bovine milk-derived lactoferrin

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(bLF) was generously provided by Morinaga Co., Japan. The culture media were purchased from Cytogen and fetal calf serum (FCS) from Gibco. L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics were from Sigma. *Encephalomyocarditis virus* (*EMCV*) was obtained from The Laboratory of Virology, Institute of Immunology and Experimental Therapy, Wrocław, Poland. L929 cells (ATCC CCL 1) was purchased from ATCC. We followed the procedures of preparation of splenocyte suspension and fractionation of splenocytes on glass wool columns which were described elsewhere (Russo *et al.*, 1979). Such a procedure allowed to remove from the splenocyte suspension strongly adherent cells (macrophages), erythrocytes, debris and dead cells, as well as a part of T cell population, resulting in a B-cell enriched population. The determination of the cell phenotype of this fraction indicated (FACS measurement, not shown) that it was about 65–72% CD19 positive (a pan B cell marker). One of the reasons, we have chosen such a model, was because the activity of B cell compartment is well preserved in aged mice.

For the infection of cells we used *EMCV*, which was propagated and titrated in L929 cells. The titer of the virus was expressed in reference to the value of TCID50 (tissue culture infectious dose), based on the cytopathic effect caused by the virus in 50% of infected cells. In the present study we applied two approaches to evaluate the protective effects of lactoferrins. In the first model, the cells were infected with the virus, followed by the addition of lactoferrins (50 μg/ml). The viral replication was determined after 24 h and 5 days of cell culture. In the second model, the cells ($2 \times 10^6$/ml), re-suspended in the culture medium consisting of RPMI-1640 medium, 10% of FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, were incubated for 1 h at 37°C with the studied lactoferrins (50 μg/ml). Then, the cells were washed twice with Hanks' medium, re-suspended in the culture medium and infected with *EMCV*. The

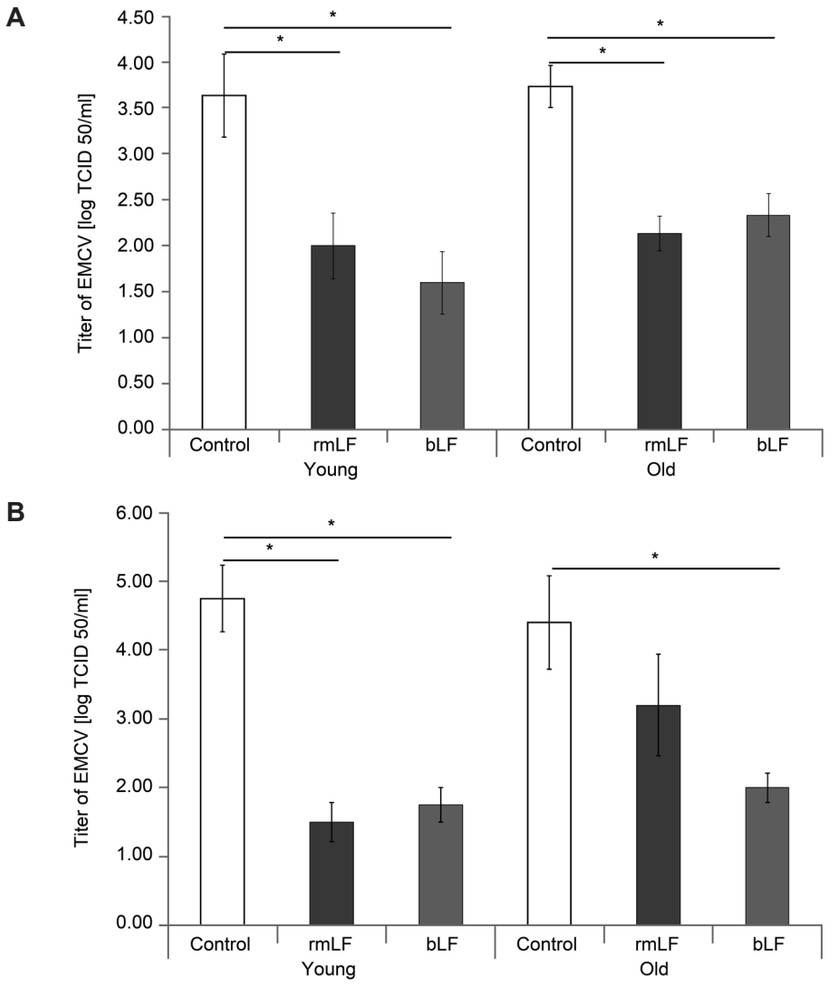


Fig. 1. The effect of lactoferrins on *EMCV* replication in the B-cell enriched splenocyte population from young and old mice.
Lactoferrins (50 μg/ml) were added to the cells after *EMCV* adsorption. Samples of culture medium from LFs-treated and non-treated cells, incubated at 37°C, were collected after 24 h (**A**) or 5 days (**B**) and titrated in L929 cells.

viral replication was determined after 5 days of culture. The cells were infected with a dose of 100 $TCID_{50}$/ml of *EMCV* in a presence or absence of lactoferrins. After 45 min of adsorption at 37°C, the virus was disposed by 5 times wash of the cells in Hanks' medium. The infected cells were then re-suspended in the culture medium and transferred to a 96-well plate. After 24 h or 5 days of incubation at 37°C, samples of the supernatants were collected and frozen at –20°C. The supernatants were then thawed, serially diluted and plated in the presence of target L929 cells to determine the viral titer, indicated as the TCID50. For the statistical evaluation of the data, the analysis of variance (one-way ANOVA) was applied due to a homogeneity of variance between the groups, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of differences between the groups. The significance was determined at $p < 0.05$ and indicated as*. The results are presented as mean values ± standard error (SE). The result of one representative experiment was shown, out of three experiments performed.

The splenocytes from young and old mice were investigated for their susceptibility to viral infection. The effects of lactoferrins on these parameters were also determined. In one model, the cells were infected with the virus, washed and incubated with the lactoferrins for 24 h or 5 days (Fig. 1A-B), followed by determination of the virus titer. Thus, in this model, lactoferrin was designed to act solely as an inhibitor of virus replication. It appeared that after 24 h culture the rate of infection in cells from both young and old mice was the same (Fig. 1A). Both types of lactoferrins significantly reduced the virus titer in cells derived from young and old mice. The inhibitory effects of lactoferrins were stronger in young mice, particularly in the case of bLF. After 5-day exposure to the virus and the lactoferrins, the infection rates of cells from young and old mice were comparable (Fig. 1B). The inhibitory effects of lactoferrins were stronger in young mice, both lactoferrins being equally active. On the other hand, the virus replication in old mice was significantly inhibited only by bLF. In the second model the cells were incubated for 1 h with lactoferrins, washed and exposed to the virus for 5 days (Fig. 2) in order to test an ability of this protein to interfere with virus binding to the cells. The susceptibility of cell populations from both age categories was comparable. While the suppressive effects of bLF on the virus titers were statistically significant in both splenocyte populations, the action of rmLF was weaker and not significant.

Although we demonstrated more potent actions of bovine lactoferrin on resistance of splenocytes to viral infection, as compared to recombinant mouse lactoferrin, the efficacy of the homologous lactoferrin in the antiviral resistance was shown here for the first time. Small differences in the efficacy of bovive and mouse lactoferrin in the antiviral resistance of splenic B cells could be due to different structures of glycan moieties in the two types of lactoferrins. These structures are responsible for blocking CD21 receptors on B cells (Zheng *et al.*, 2012) which are also used for viruses to enter cells. The heparan sulfate receptors for viruses are, in turn, blocked by a highly cationic N-terminal end of lactoferrin (Pietroantoni *et al.*, 2015).

Our results on the suceptibility to viral infection in old versus young individuals are, in part, consistent with a hypothesis (Scholz *et al.*, 2013) that the viral resistance in the elderly may be even enhanced due to a cumulative, frequent contacts with viral antigens

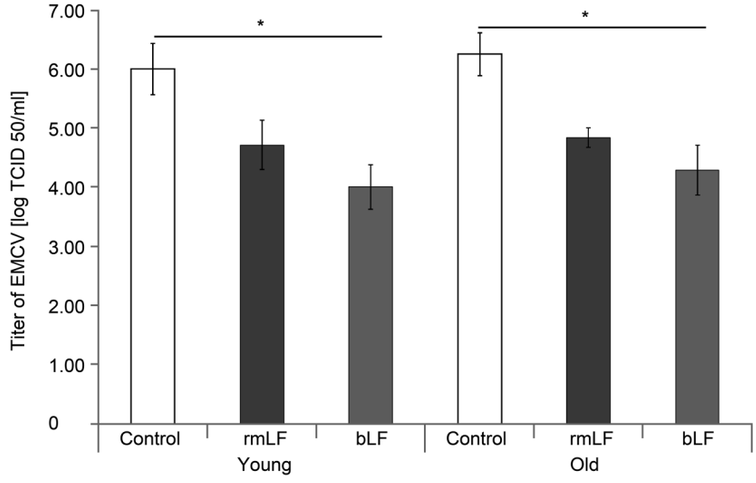


Fig. 2. Effect of preincubation of the B-cell enriched splenocytes from young and old mice, with lactoferrins on viral replication.
The cells were re-suspended in the culture medium and cultured with lactoferrins (50 µg/ml) for 1h at 37°C. After the incubation, the cells were washed twice with Hanks' medium, re-suspended in the culture medium and infected with *EMCV*. Samples of culture medium were collected after 5 days and titrated in L929 cells.

resulting in a presence of a bigger pool of antigen-specific memory cells. Although mouse (Scholz *et al*., 2013; Zhang *et al*., 2002) and human studies (Labeur *et al*., 1991) indicated impaired susceptibility of aged organisms to viral infection, it is also clear (Dailey *et al*., 2001; Castle *et al*., 1999) that the activation status and functional capacity of B cells from old mice are higher or at least not lower than those in young ones.

*EMCV* infects several cell types beside lymphocytes and the mechanisms of interference with virus replication by LF are probably common for most cell types. We also think that interference of other cell types and cytokines on the susceptibility of B cells to viral infection may be negligible. Besides, we obtained similar results with a T-cell-enriched cell fraction (not shown).

In view of the literature on the protective role of lactoferrin in viral infection (Pietroantoni *et al*., 2015; Välimaa *et al*., 2009; Zheng *et al*., 2012) it was not unexpected that also in this study lactoferrins demonstrated antiviral properties in the applied models, *i.e.* potential block of virus receptors on target cells and interference with virus replication. We also suggest still another mechanism of the antiviral activity of lactoferrin, since the protein is an enhancer of COX-1 expression (Kruzel *et al*., 2013), important in maintaining antiviral resistance (Carey *et al*., 2010). In addition, a lack of age-related difference in COX-1 protein levels found by others (Hayek *et al.*, 1997) further supports our findings on the comparable susceptibility to viral infection in both age categories. Our results provided also the evidence that the homologous (mouse lactoferrin) may play a physiological role in the antiviral resistance in mice and that the protective effect of lactoferrin is not species specific. It was also encouraging to find that cells from old individuals were at least equally resistant to viral infection as those derived from young ones. In conclusion, we showed that the resistance to viral infection was preserved in the senescent mice. We also demonstrated, for the first time, the antiviral actions of the homologous mouse lactoferrin. Based on the results, regarding the protective effect of bovine lactoferrin on virally infected splenocytes from old mice, we also suggest a potential benefit of diet supplemented with bovine lactoferrin for the elderly in enhancing antiviral resistance.

**Statement**

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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