Original Article

Lactoferrin Prevents Susceptibility of WEHI 231 Cells to Anti-Ig-Induced Cell Death Promoting Cell Differentiation

(WEHI 231 cells / lactoferrin / apoptosis / signalling molecules / anti-mouse Ig antibodies)

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Abstract. Immature B cells are susceptible to apoptosis due to ligation of surface immunoglobulin receptors. The WEHI 231 cell line represents a useful model to study the mode of action of factors preventing apoptosis. In this work we investigated the protective effects of multi-species lactoferrins in anti-mouse Ig-induced WEHI 231 cell death. Bovine milk-derived lactoferrin (bLF), recombinant human lactoferrin expressed in Chinese hamster ovary cells – rhLF(CHO) or in human endothelial kidney cells – rhLF(HEK), and recombinant mouse lactoferrin expressed in Chinese hamster ovary cells – rmLF(CHO), were used. Goat-anti-mouse Ig antibodies were used to induce cell apoptosis. Survival of WEHI 231 cells in culture was measured using the colorimetric MTT method. Expression of signalling molecules and subunits of interleukin 2 receptor was determined by the RT PCR method. The results showed that anti-mouse Ig antibodies inhibited cell growth in a dose-dependent manner. The lactoferrins alone had no effect on the cell survival. The cells exposed to LFs, prior to anti-Ig treatment, were rescued to a significant degree from cell death. Determination of the signalling molecule expression revealed almost complete suppression of caspase-3 and NF-κB1 by bLF in untreated cells, as well as deep suppression of caspase-3, block of Fas, and 4-fold increase of NF-κB1 in cells incubated with bLF prior to anti-Ig treatment. In addition, differential changes in the expression of interleukin 2 subunits upon bLF treatment were found, indicating a process of cell differentiation. In conclusion, we showed that LF-induced cell differentiation in immature B-cell line WEHI 231 was correlated with partial protection of the cells from anti-Ig-induced cell death.

Introduction

Lactoferrin (LF) is a multifunctional protein, contained in excretory fluids and neutrophils of mammals, exhibiting a wide array of biological activities (Legrand, 2012). Apart from other roles of LF in the host protection and maintenance of homeostasis, and control of inflammation-associated pathology (Kruzel et al., 2007, 2017), the protein was shown to affect development of immunocompetent cells by promoting maturation of T cells (Zimecki et al., 1991), B cells (Zimecki et al., 1995), and antigen-presenting cells (de la Rosa et al., 2008) and to elicit myelopoiesis (Zimecki et al., 2013). The phenomenon of LF effects on restoration of T- and B-cell compartments was also described in a model of immunocompromised mice (Artym et al., 2003a,b). Moreover, the stimulatory effects of LF on immune functions dependent on T and B cells were also found in weanling piglets (Shan et al., 2007). Therefore, the role of LF in maturation of the immune cell system has been firmly established. The protein also has the property to promote growth and differentiation of intestinal epithelial cells, in association with prevention of apoptosis (Blais et al., 2014). Lactoferrin inhibits apoptosis also in other cell types, such as chondrocytes (Tu et al., 2013; Xue et al., 2015), osteoblasts (Hou et al., 2014) and neutrophils (Francis et al., 2011), as well as in virus-infected cells (Pietrantoni et al., 2010). On the other hand, LF

Received October 13, 2017. Accepted April 6, 2018.

This work was supported by the statutory grant from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, No 501-4/2017 and by the Association for Research and Treatment of Sepsis – "Overcome Sepsis".

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Abbreviations: anti-mouse Ig – goat-anti-mouse Ig; bLF – bovine milk lactoferrin; DMEM – Dulbecco's Modified Eagle's Medium; LF – lactoferrin; NF-κB1 – nuclear factor of κ light polypeptide gene enhancer in B cells 1; rhLF(CHO) – recombinant human lactoferrin expressed in Chinese hamster ovary cells; rhLF(HEK) – recombinant human lactoferrin expressed in human endothelial kidney cells; rmLF(CHO) – recombinant mouse lactoferrin expressed in Chinese hamster ovary cells; SE – standard error, WEHI 231 – B lymphoma cells.

induces apoptosis in cancer cells (Zhang et al., 2014; Gibbons et al., 2015) or acts as an agent preventing carcinogenesis in colon mucosa (Fujita et al., 2004).

Immature B cells are highly sensitive to anergy following the encounter with antigen. Such a process is crucial for establishing clonal tolerance to self and orally acquired antigens during neonatal life (Nossal, 1987; Raff et al., 1975; Szewczuk and Siskind, 1977). WEHI 231 cells are typical immature B cells susceptible to apoptosis upon ligation of surface IgM receptors (Bovd and Schrader, 1981; Benhamou et al., 1990). Thus, the WEHI 231 cell line is suitable for investigation of the mechanisms leading to differentiation, activation or apoptosis of immature B cells by various factors including bacterial and viral products or proteins (Jakway et al., 1986; Rott and Cash, 1994).

Nevertheless, the potential ability of lactoferrin to prevent anti-Ig induced apoptosis in immature B cells, for example in the WEHI 231 cell line, has not been explored yet. Such a property of LF, if existing, would provide an additional evidence for its direct effect on elicitation of the maturation process in B cells. Thus, the aim of this work was to investigate whether LF will prevent apoptosis in WEHI 231 cells and to determine how such an effect will correlate with the cell phenotype and expression of selected signalling molecules involved in cell growth and apoptosis.

Material and Methods

Reagents for molecular biology techniques

TRIzol Reagent (Thermo Fisher Scuentific, Waltham, MA), chloroform (POCh, Gliwice, Poland), 2-propanol (Merck, Darmstadt, Germany), ethanol (POCh), agarose (Novazym Poznan, Poland), SuperVerte M-MLV kit (Novazym), KAPA SYBR FAST qPCR Kit (Applied Biosystems, Foster City, CA), primer pairs:
TGTCATTCTCGCTCGGTACG and
AAATGACCCCTTCATCACCA for Casp-3;
ACAATGCCCAGATTTCTCCCTAC and
CAGACAGTATCCCCGAGGTTTG for Casp-8;
AGCCAGATGCTGTCCCATAC and
CAGGAGACAAAACCTGGGAA for Casp-9;
GCGATGAAGAGCATGGTTTAG and
GGCTCAAGGGTTCCATGTT for Fas;
CTCGTCGCTACCGTCGTGACTTCG and
CAGATGCCGGTTCAGGTACTCAGTC for Bcl-2;
GAAATTCCTGATCCAGACAAAAAC and
ATCACTTCAATGGCCTCTGTGTAG for NF-κB1;
ACCACAGTCCATGCCATCAC and
TCCACCACCCTGTTGCTGTA for GAPDH.

Cell line, culture media and reagents

The WEHI 231cell line (ATCC, CRL-1702) was obtained from the Institute of Immunology and Experimental Therapy (Wrocław, Poland). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from the American Type Culture Collection – ATCC (Manassas, VA), Hanks' medium was purchased from CytoGen GmbH (Wetzlar, Germany). Foetal bovine serum was from HyClone (Pittsburgh, PA), L-glutamine, penicillin and streptomycin solution, 2-mercaptoethanol, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich (St. Louis, Mo). Antibody Cat. No. 1010-01, directed against IgM, IgG and IgA classes, was obtained from SouthernBiotech (Birmingham, AL).

Lactoferrins

All lactoferrins showed low endotoxin level (< 1.0 EU/mg) and were obtained from the following sources: recombinant mouse lactoferrin – rmLF(CHO) and recombinant human lactoferrin rhLF(CHO), both expressed in Chinese hamster ovary cells, and recombinant human lactoferrin expressed in human endothelial kidney cells – rhLF(HEK) were from PharmaReview Corporation, Houston, TX and bovine milk lactoferrin (bLF) from Morinaga Milk Industry Co. (Tokyo, Japan).

Proliferation of WEHI 231 cells upon treatment with anti-mouse Ig antibodies

WEHI 231 cells were washed 2 × and and centrifuged at 200 × *g* for 10 min at 4 °C in ion-free Hanks' medium and re-suspended in a culture medium consisting of DMEM supplemented with 10% foetal bovine serum, L-glutamine, 2-mercaptoethanol and antibiotics, at a density of 1×10^6 cells/ml. The cell suspension (100 µl) was then distributed to 96-flat bottom cell culture plates. LFs (10 µg/ml) were added to the cultures for 2 h incubation. Then, anti-mouse Ig antibodies were added and the cells were incubated in a cell culture incubator for 72 h. Cell viability was determined using the MTT colorimetric method (Hansen et al., 1989).

Culture of WEHI 231 cells for molecular studies, total RNA isolation and cDNA synthesis

WEHI 231 cells (10^5/ml) were cultured for 4 h with or without bLF (10 µg/ml) in the culture medium. Then anti-mouse Ig antibodies were added to the cultures at a 1.2 µg/ml concentration, and on the next day, the cells were harvested and centrifuged at 200 × *g* for 10 min at 4 °C. The cell pellets were frozen at –20 °C and stored until RNA isolation. Total RNA isolation was carried out with TRIzol Reagent (Ambion) according to the manufacturer's recommendations. Cell pellet (2×10^6 cells) was suspended in 1 ml of TRIzol reagent, shaken, incubated for 10 min at room temperature (RT), supplemented with 0.2 ml of chloroform, shaken vigorously for 15 s, incubated for 3 min at RT and centrifuged at 12,000 × *g* for 15 min at 4 °C. The water phase was collected, transferred to a new tube, supplemented with 0.5 ml of isopropanol, incubated at RT for 10 min and centrifuged at 12,000 × *g* for 10 min at 4 °C. The RNA pellet was washed with 1 ml of 75% ethanol, dried in air and dissolved in 20–30 ml of sterile diethylpyrocarbon-

ate-treated Mili-Q water. RNA samples were stored at –20 °C. Complementary DNA was synthesized from 5 µg of RNA using a SuperVerte M-MLV kit (Novazym).

Quantification of gene expression by real-time PCR

The expression of genes for: GAPDH, caspases 3, 8, and 9, Fas, Bcl-2, and NF-κB1 was measured using a KAPA SYBR FAST qPCR Kit from Applied Biosystems. The sequences of primers are listed above. The reaction was performed in an Applied Biosystems ViiA7 thermocycler starting with 5 min of pre-incubation at 95 °C followed by 35 amplification cycles as follows: 95 °C for 30 s and simultaneous annealing-extension-data acquisition for 45 s and 60 °C. *GAPDH* was used as a housekeeping gene for arbitrary unit calculation for every tested gene.

Statistics

The results are presented as mean values ± standard error (SE). Analysis of variance (one-way ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the differences between groups. Significance was determined at $P < 0.05$. Statistical analysis was performed using STATISTICA 7 for Windows.

Results

Decreased viability of WEHI 231 cells upon treatment with anti-mouse Ig antibodies

In the first step, the anti-mouse Ig antibodies were titrated to establish an optimal concentration capable to inhibit growth of WEHI 231 cells. As shown in Fig.1, the antibodies at the studied concentration range 5–1.2 µg/ml strongly, and to a similar degree, inhibited cell viability. This effect was gradually lost by using lower concentrations of the antibodies (0.6–0.1 µg/ml).

Effects of lactoferrins and anti-Ig antibodies on the viability of WEHI 231 cells

Since we wished to investigate the potential protective effects of LFs in the model of apoptosis caused by ligation of surface Ig on WEHI 231 cells, we performed a control experiment aimed at determination of the LF effect on growth of these cells. The 10 µg/ml concentration was used based on our year-long experience with the biological activity of lactoferrins. Four types of LFs were used in this and the forthcoming experiments: bovine milk-derived LF, recombinant human LF expressed in CHO cells or in HEK cells, and mouse recombinant LF produced in CHO cells. The cells were incubated with the LFs for 24 h and the cell viability was determined by the colorimetric method (Hansen et al.,1989). No statistically significant differences in the cell viability were found (Fig. 2) upon treatment with 10 µg/ml of the studied LFs. Of importance, the homologous recombinant mouse LF was also without noticeable effect.

Next, we performed experiments aimed at evaluation of the potential ability of LFs to prevent anti-Ig induced apoptosis in WEHI 231 cells. The cells were exposed to LFs at a concentration of 10 µg/ml for 2 h followed by 24 h incubation with anti-mouse Ig antibodies. The viability of cells was determined using the MTT colorimetric method. The results (Fig. 3A) showed that all LFs demonstrated protective effects, best pronounced at anti-Ig concentration of 1.2 µg/ml. However, the LFs were not capable to overcome the pro-apoptotic effect of anti-Ig antibodies at 2.5 µg/ml. The results were confirmed in another experiment (Fig. 3B) at 1.2 µg/ml concentration of anti-Ig antibodies.

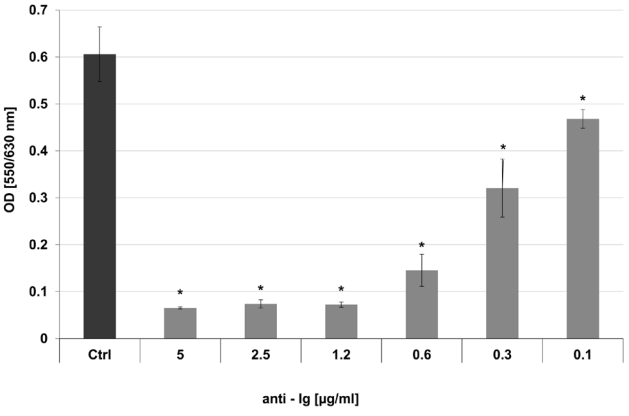

Fig. 1. The effect of anti-mouse Ig antibodies on viability of WEHI 231 cells
WEHI 231 (1×10^6 cells/ml) were cultured in the presence or absence of various concentrations of anti-mouse Ig. Proliferation was assessed at 72 h. Each value is a mean ± SEM of triplicate measurements as compared with the control: *versus Ctrl; $P < 0.05$

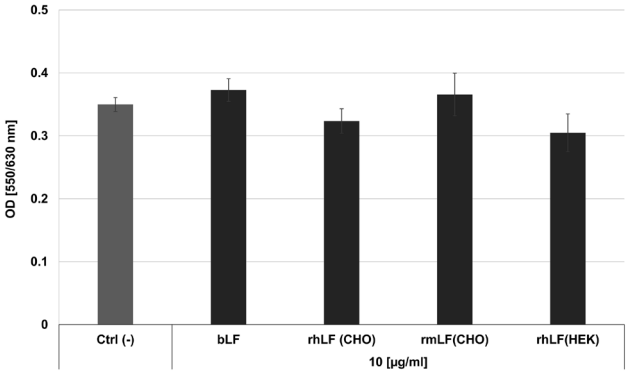

Fig. 2. Effects of lactoferrins on viability of WEHI 231 cells
WEHI 231 were incubated in the presence or absence of 10 µg/ml bLF, rhLF(CHO) or rmLF(CHO), and rhLF(HEK) for 24 h at 37 °C. After the incubation period, the level of cell proliferation was determined using a colorimetric method (MTT). The results are expressed as the mean ± SEM.

Changes in expression of signalling proteins in WEHI 231 cells treated with LFs and anti-Ig antibodies

WEHI 231 cells were incubated for 4 h with 10 μg/ml of bLF. Control cell cultures were without any addition. After the incubation, the cells were exposed to 1.2 μg/ml of anti-Ig antibodies for an overnight incubation. The following combinations of cell cultures were used: 1/ no addition, 2/ addition of anti-Ig antibodies only, 3/ addition of bLF to untreated cells, and 4/ addition of anti-Ig antibodies to bLF-pretreated cells. The cells were centrifuged and subjected to the procedures described in the Methods section.

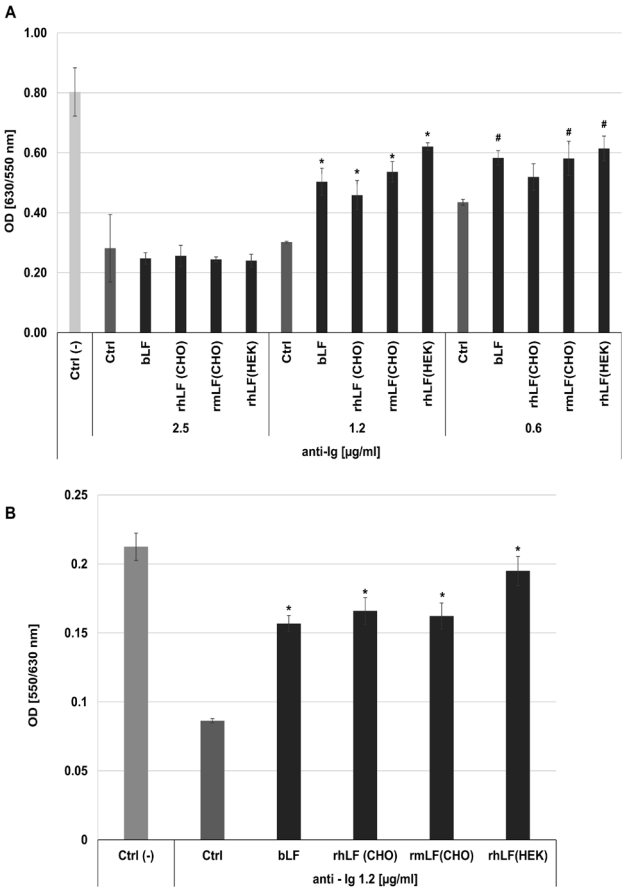

Fig. 3. LFs partially recover the suppressed viability of WEHI 231 cells treated with anti-mouse Ig antibodies
WEHI 231 cells were pre-treated with or without LFs (10 μg/ml) for 2 h before the addition of anti-mouse Ig at concentration: 2.5 μg/ml, 1.2 μg/ml or 0.6 μg/ml for 24 h at 37 °C – Fig. 3A, or only anti-mouse Ig at concentration 1.2 μg/ml for 24 h at 37 °C – Fig. 3B. The degree of cell proliferation was measured using the MTT colorimetric method. The results are presented as mean OD values from three wells ± SE. A. The effect of anti-mouse Ig at concentration 1.2 μg/ml and 0.6 μg/ml were statistically significant (* $P < 0.05$; # $P < 0.05$) compared to the control. B. The effect of anti-mouse Ig at concentration 1.2 μg/ml was statistically significant (* $P < 0.05$) compared to the control.

Figure 4 depicts the values (relative normalized expression in relation to the housekeeping gene) indicating changes in the expression of selected signalling proteins in cell cultures treated with bLF and/or anti-Ig antibodies, as compared to untreated cultures of WEHI 231 cells. The following changes in the expression of selected signalling molecules were registered. The changes in gene expression of cells treated with bLF only (inhibition of caspase-3, almost total inhibition of caspase-8, and 2.3-fold increase of caspase-9) had no impact on the cell viability. Likewise, no association with cell viability was found with Bcl-2, whose expression was inhibited 4-fold. The most striking was almost total inhibition of NF-κB1. The treatment of cells with anti-Ig had no or little effect on the expression of caspases, Fas and NF-κB1, but some inhibitory effect on Bcl-2 expression. On the other hand, the treatment of cells with bLF prior to addition of anti-Ig antibodies resulted in strong inhibition of caspases 3 and 8 accompanied with up-regulation of caspase-9 expression. Further, Fas expression was completely inhibited and that of NF-κB1 strongly up-regulated.

Since the expression of IL-2R is strongly correlated with the maturation stage of B cells (Chen et al., 1994), we investigated expression of three subunits of IL-2R upon incubation with bLF. As presented in Fig. 5, the expression of the α subunit was strongly down-regulated upon bLF addition. Even stronger inhibition was registered with use of anti-Ig antibodies, although that effect was partially reversed by bLF. On the other hand, the expression of the β chain was enhanced 2-fold. The anti-Ig treatment caused only weak up-regulation of this pa-

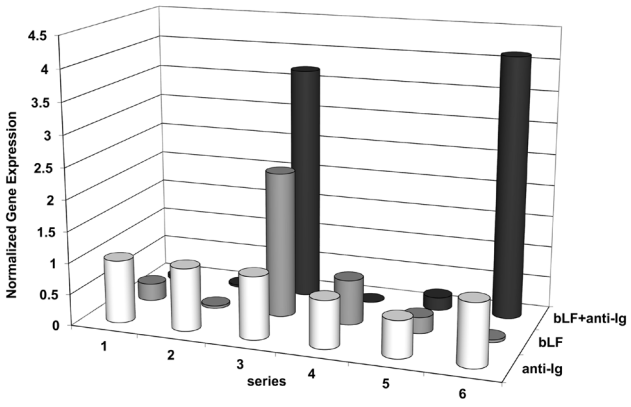

Fig. 4. The relative fold changes in the expression of selected signalling proteins in WEHI 231 cells exposed to anti-Ig antibodies and bLF
WEHI 231 cells (10^5/ml) were cultured for 4 h with or without bLF (10 μg/ml) in the culture medium. Then anti-mouse Ig antibodies were added to the cultures at 1.2 μg/ml concentration and on the next day, the cells were harvested and centrifuged. Total RNA isolation, cDNA synthesis and quantification of gene expression were described in the Methods.
1 – caspase-3; **2** – caspase-8; **3** – caspase-9; **4** – Fas; **5** – Bcl-2; **6** – NF-κB1

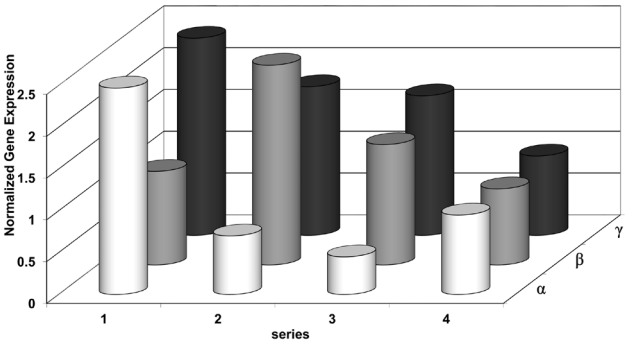

Fig. 5. The relative fold changes in the expression of mouse IL-2R subunits by WEHI cells exposed to anti-Ig antibodies and bLF

WEHI 231 cells (10^5/ml) were cultured for 24 h with: no addition (**1**), 10 μg/ bLF (**2**), 1.2 μg/ml anti-Ig (**3**) and 10 μg/ml bLF + 1.2μg/ml anti-Ig (**4**). Total RNA isolation, cDNA synthesis and quantification of gene expression were described in the Methods.

rameter, and bLF counteracted that tendency. In turn, the changes of expression of the γ chain displayed a similar pattern as in the case of the α subunit, although these alterations were not so marked.

Discussion

The effects of LF on cell differentiation and apoptosis depend on the cell type, stage of maturation and applied experimental model, as described in the Introduction. In this way, lactoferrin fulfils the roles that could be anticipated from such a multifunctional and regulatory protein. In this study we showed that in short-term cultures of WEHI 231 cells, the native and recombinant lactoferrins protected, to a high extent, the viability of cells exposed to anti-mouse Ig antibodies. The lactoferrins alone had no significant effect on the viability of cells, and both the origin and species specificity of LFs had no influence on the activity of LF. This investigation represents a supplement to our original finding showing that LF promoted acquirement of a mature cell phenotype and antigen-presenting function of splenic B cells from neonatal mice. On the other hand, LF had no effects on mature B cells in that study (Zimecki et al., 1995). Therefore, we assumed that the effects of LF on immature B cells should be reflected by some changes in the expression levels of regulatory proteins in the WEHI 231 cell model, corresponding to immature conventional B cells, as well as changes in the cell phenotype. Since LF was also shown to transform neonatal B cells into mature, functional cells (Zimecki et al., 1995), known to be resistant to B-cell receptor ligation (Raff et al., 1975), we expected additional changes in the expression of signalling proteins following incubation of cells with LF prior to anti-Ig treatment.

Our results showed that the expression of caspases 3, 8 and 9 was not changed upon anti-Ig treatment. In fact, caspase-3 was not activated in this model (Brás et al., 1999). In addition, apoptosis induced in WEHI 231 cells by TGF-β was independent of caspase-3 (Brown et al., 1999). The effects of LF in our model were in accord with a report where LF prevented up-regulation of caspase-3 and Fas in dexamethasone-induced apoptosis of chondrocytes (Tu et al., 2013). In our study, a possible protective action of bLF was associated with strong inhibition of caspase-3 and Fas. However, our results do not support the finding that activation of caspase-9 is necessary for anti-Ig-induced apoptosis in WEHI 231 cells (Herold et al., 2002). On the contrary, the protective effect of bLF alone was correlated with some increase (2.3-fold) of caspase-9, the effect further augmented following treatment with anti-Ig antibodies (Fig. 4).

The lack of changes in Fas expression in anti-Ig-treated cells found in our study was consistent with another report (Yoshida et al., 2000). Nevertheless, the combined treatment of cells with bLF and anti-Ig antibodies completely inhibited this apoptosis-associated molecule, suggesting that the cells became resistant to anti-Ig-induced death.

In our model we observed only a modest inhibition of Bcl-2 expression upon Ig ligation, which was stronger following bLF and combined bLF and anti-Ig treatment. In another study (Gottschalk et al., 1994a), no correlation was found between susceptibility of WEHI 231 cells to apoptosis induced by anti-IgM treatment and BcL-2 expression. Further, transfection of WEHI 231 cells with Bcl-2 did not affect immunosuppressant-induced apoptosis (Gottschalk et al., 1994b). It seems, therefore, that the changes in Bcl-2 expression are rather associated with growth arrest leading to cell differentiation (Baixeras et al., 1998). Altogether, Bcl-2 could not be a representative signalling molecule to assess the rate of cell apoptosis in this model.

Quite remarkable changes were noted in the case of NF-κB1 expression. Although no change in the expression of NF-κB1 was seen upon anti-Ig treatment, bLF alone caused strong suppression (25-fold) of this molecule expression, a phenomenon most likely associated with growth arrest and promotion of the maturation process of these cells. On the other hand, the expression of NF-κB1 was increased 4.1-fold (Fig.4) when anti-Ig-treated cells were pre-treated with bLF. This observation is in agreement with other studies showing that activation of NF-κB1 promoted rescue in apoptosis of WEHI 231 cells (Mineva et al., 2007; Graham et al., 2013). The maturation effect of LF on WEHI 231 cells in this study was further demonstrated by characteristic changes in the expression of IL-2R subunits. In general, the expression of IL-2R is correlated with expression of Ig μ heavy chain and is down-regulated during maturation of B cells (Chen et al., 1994).

Although internalization of IL-2 may be mediated via several combinations of IL-2R subunits (Steinberger et al., 1997), a large excess of α chain is required to construct high-affinity IL-2R (Benjamin et al., 1990). In fact, our results showed a significant drop in the expression of α chain following bLF treatment of WEHI 231 cells, suggesting a partial loss of IL-2R function. The maturation

process of these cells was also supported by the increase of β chain expression after LF treatment (Fig. 5), since the expression of β and γ chains without detectable α chain was also found to be associated with B-cell maturation (Tanaka et al., 1988). The changes in the expression of respective IL-2R subunits upon addition of anti-Ig antibodies could reflect the process of cell apoptosis. Interestingly, the changes were partially reversed by bLF. Finally, our results are in accord with other data regarding LF effects on other cell types such as the Jurkat T-cell line (Dhennin-Duthille et al., 2000) and MC3T3-E1 osteoblasts (Liu et al., 2018), where a signalling pathway leading to cell maturation was preferentially elicited.

While discussing the observed alterations in the expression of signalling molecules, one has to consider some limitations of this model. First, the cells are not synchronized in the cell cycle, the protective LF effect is not complete and strongly depends on the concentration of anti-Ig antibodies. Further, the changes in signalling molecule expression vary among WEHI 231 sublines (Rott and Cash, 1994).

It has to be noted that the protective effects of LF do not vary among LFs used, i.e., both native and recombinant LFs, with amino acid homology to different species (bovine, human, mouse), exhibited similar anti-apoptotic actions. It has to be still established what kind of B-cell receptor is responsible for the interaction of LFs with WEHI 231 cells. We found that the CD22 antigen was a prime candidate for the interaction of LF with B cells from mouse splenocytes (Zimecki et al., 2014). In turn, the CD21 antigen belonging to the same family of sialic acid-binding immunoglobulin family lectins interacts with LF on B cells from human peripheral blood (Zheng et al., 2012). Assuming that the LF-CD22 interaction counteracted the pro-apoptotic action of methotrexate (Zimecki et al., 2014), we speculate that in the WEHI 231 model, the LF-CD22 interaction may negatively regulate the pro-apoptotic signals generated by ligation of the B-cell receptor (Sato et al., 1998).

In conclusion, we showed that LF induces cell differentiation/maturation in the immature B-cell line WEHI 231 and partially protects the cells from anti-Ig-induced cell death. These effects were accompanied by characteristic changes in the expression of several signalling molecules involved in the processes of cell activation and apoptosis as well as in phenotypic changes. Thus, the molecular studies provided additional insight to the role of LF in B-cell differentiation.

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