Biometals (2023) 36:617–627
https://doi.org/10.1007/s10534-022-00443-z

A cytotoxic effect of human lactoferrin fusion with Fc domain of IgG

Ewa Zaczyńska · Maja Kocięba · Jolanta Artym · Iwona Kochanowska · Marian L. Kruzel · Michał Zimecki

Received: 14 May 2022 / Accepted: 6 September 2022 / Published online: 22 September 2022

Abstract Lactoferrin (LTF) is a natural iron-binding protein with a potential for clinical utility in many human immune disorders, including cancer. A fusion of LTF with the Fc domain of IgG2 (FcLTF) was designed with inherent properties of an extended the half-life in circulation. Furthermore, the effects of LTF and FcLTF were assessed for influence on the activity of natural killer (NK) cells isolated from human peripheral blood, on the NK-92 cell line, and on human monocytes. The NK cytotoxic activity induced by LTF and FcLTF was determined against the human leukemia K562 cell line, and also for monocytes, by measuring TNFα and granzyme B production, and in an assay for Jurkat cell viability. Selected gene expression in NK-92 cells and monocytes, induced by LTF and FcLTF, was performed by Real Time PCR. No significant difference was observed in NK-92 cytotoxicity stimulated by LTF and FcLTF. The effects on NK cells isolated from the human peripheral blood were varied, possibly due to the immunoregulatory nature of LTF sensing the immune status of donors. Furthermore, only the FcLTF group strongly stimulated production of TNFα and granzyme B in isolated monocytes. In addition, only supernatants from the monocyte cultures treated with FcLTF decreased the viability of Jurkat cells. The ability of FcLTF to induce TNFα in monocytes was strongly inhibited by anti-CD32 and moderately inhibited by anti-CD14 antibody. Lastly, it was demonstrated that FcLTF, strongly induced expression of PI3K, with subsequent activation of AKT/mTOR signaling pathway. Overall, it was demonstrated that this novel fusion molecule may be a perferred choice for clinical utility than the wild type LTF.

Keywords Lactoferrin · Fc-lactoferrin · NK cells · TNFα · Monocytes · Granzyme B

Present Address:
E. Zaczyńska · M. Kocięba · J. Artym · I. Kochanowska · M. Zimecki
Hirszfeld Institute of Immunology and Experimental Therapy, Laboratory of Immunobiology, Polish Academy of Sciences, R. Weigla Str. 12, 53-114 Wrocław, Poland

M. L. Kruzel (✉)
PharmaReview Corporation, 7505 Fannin Str., Third Floor 313, Houston, TX 77054, USA
e-mail: Marian.Kruzel@gmail.com

Introduction

Lactoferrin (LTF) is an evolutionary old, multifunctional protein, playing a key role in maturation and regulation of the immune system function of mammals, protection against pathogens and maintenance of homeostasis (Mayeur et al. 2016; Kruzel 2017). A bewildering spectrum of activities opens perspectives of clinical application of LTF, both as a preventive or therapeutic measure (Hao et al. 2019). Among many experimental therapeutic approaches LTF was considered as an agent capable of enhancing natural killer (NK) cells activity towards tumors or viral infections.

Oral-delivered LTF appeared to have some advantage in patients with tumors, who were not responsive to conventional therapy (Cutone et al. 2020), was very effective in inhibiting tumor growth in mice (Bezault et al. 1994), and directly induced apoptosis in tumor cells (Olszewska et al. 2021). The protective effect of LTF is clearly associated with stimulation of NK cell activity and their expansion (Bezault et al. 1994; Kuhara et al. 2006). LTF was also shown to enhance efficacy of chemotherapy in tumor bearing mice (Sun et al. 2012) and BCG-infected mice (Nguyen et al. 2022). In addition, LTF protected mice against viral infection alone (Shimizu et al. 1996), or by means of a synergistic action with interferon gamma (Lu et al. 1991) via an apparent involvement of NK cells. Initial in vitro studies revealed that adherent fraction of human peripheral blood mononuclear cells (PBMCs) responded to LTF with increased cytotoxicity (Nishiya and Horwitz 1982; Horwitz et al. 1984). McCormick, et al. (1991) demonstrated that a prolonged (18 h) incubation of large granular lymphocytes from a PBMC population, equivalent to NK cells, led to a decay of cytotoxic activity, which was not restorable by LTF. On the other hand, the monocytic fraction retained its cytotoxic activity, which could be further significantly stimulated by LTF. Damiens et al. (1998) convincingly proved a significant effect of LTF on NK cells from PBMC population, which exerted cytotoxic activity against selected epithelial tumor cell lines at low concentration. They also showed binding of LTF to a majority of the heterogenous CD56 dim/bright NK cell population. LTF indeed interacts at least with two cell types in the human PBMC population to elicit cytotoxic activities towards target cells.

A fusion of many biotherapeutics with Fc domain of IgG opens new clinical perspectives (Czajkowsky et al. 2012) based on the concept of facilitating uptake by cells bearing surface Fc-receptors. During the last decade, drugs using the Fc-fusion strategy have been thoroughly assessed, and as of 2012 there were six Fc-fusion-based drugs on the market, with several additional compounds in phase III clinical trials (Strohl and Knight 2009; Strohl 2015). Recently, a fusion of LTF with the Fc domain of human IgG1 (Shiga et al. 2015; 2017) and IgG2 (Zhao et al. 2021) were constructed; both were proven to extend the half-life of FcLTF in rats. The effector functions of IgG is different for each isotype (IgG1, IgG2, IgG3, IgG4) with IgG1, showing the strongest complement dependent cytotoxicity (CDC), and antibody dependent cellular cytotoxicity (ADCC). A therapeutic potential of LTF fusion with Fc domain of IgG2 was recently demonstrated in rats with experimentally induced intracerebral hemorrhage (ICH). Of importance, in this randomized and blinded studies of ICH, it was reported that use of FcLTF considerably reduced edema and improved neurological deficits (Zhao et al. 2021).

The experiments outlined in this study use human peripheral blood natural killer cells and monocytes, as well as a NK-92 cell line, in a well-defined model to assess generation of cytotoxic mediators upon activation. We hypothesized that LTF, being a part of FcLTF complex, would have increased availability to cells and thus induce stronger cell activation. In this work, apart from investigating potential cytotoxic activities induced by LTF and FcLTF in these cell models, regulatory mechanisms of both LTF and FcLTF to induce cytotoxicity were investigated by assessing specific cytotoxic factors and cellular signaling pathways.

Materials and methods

Reagents

The human recombinant lactoferrin (LTF) and fusion of LTF with human IgG2 Fc domain (FcLTF), both expressed in Chinese hamster cell line (CHO), were provided as a lyophilized powder by PharmaReview Corporation (Houston, TX, USA; < 0.5 endotoxin units per mg). FcLTF construct was designed according to the sequence published in US Patent No. 10,442,852 B2 (Kruzel and Aronowski 2019). A short linker (GS) was introduced to the sequence between LFT and hinge region of Fc IgG2 (ERKCCVECPPCP) for the structure highest flexibility. The concentration of FcLTF was normalized for LTF equivalency in each experiment. Fetal bovine serum (FCS) was from HyClone, L-glutamine, penicillin and streptomycin solution, 2-mercaptoethanol, folic acid, actinomycin D, lipopolysaccharide (LPS) O111:B4, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Lymphocyte Separation Medium 1077, PBS, RPMI-1640 and HBSS medium

were obtained from Biowest; IL-2 was from Immunotools, α-MEM and horse serum were from Gibco-BRL (Grand Island, NY, USA). Human IgG2-Fc recombinant protein cat. # 13504-HNAH was from Sino Biological Inc. (Beijing, China). Other materials: Pierce LDH Cytotoxicity Assay Kit (Thermo-Fisher Scientific), TNFα ELISA Kit (Thermo-Fisher Scientific), Human Granzyme B DuoSet ELISpot (R&D Systems), MACSxpress NK Cell Isolation Kit, human (MACS Miltenyi Biotec GmbH), MACSxpress Erythrocyte Depletion Kit (MACS Miltenyi Biotec GmbH), anti-CD14—anti-human CD14 antibody (R&D systems cat. # AB3832), anti-CD16—anti-human CD16 antibody (Biolegend cat. # 302,014), anti-CD32a—anti-human Fcγ RIIA/CD32a antibody (R&D systems cat. # AF1875). Methanol, isopropanol and ethanol (POCh, Poland); RNA Extracol and NG dART RT Kit (EURx, Poland); AmpliQ 5×HOT EvaGreen qPCR Plus (Rox) (Novazym, Poland); pair primers (Genomed, Poland) (Table 1).

Table 1 Primers for human signaling molecules

Gene	Primer	Sequence [5′–3′]
pSyk	F	GTGCTGGACATACGATGTGG
	R	TGACAAGTTGTGGGCATGTT
PI3K	F	TGGCCTCTTCCCTGACTTCT
	R	AGCATCTGCCCCATGAACAA
AKT1	F	TTCTGCAGCTATGCGCAATGTG
	R	TGGCCAGCATACCATAGTGAGGTT
mTOR	F	GCTTGATTTGGTTCCCAGGACAGT
	R	GTGCTGAGTTTGCTGTACCCATGT
GAPDH	F	AGTCAGCCGCATCTTCTTTT
	R	TGAGGTCAATGAAGGGGTCA
ERK1	F	GTGGCCCCAGTTCAATCTC
	R	GGGTTTGAATGAGATGAGGGG
ERK2	F	TATTACGACCCGAGTGACGA
	R	AAGAACACCGATGTCTGAGC
p38α	F	TATGCGTCTGACAGGAACAC
	R	GGGCCGCTGTAATTCTCTTA
p38β	F	ACAGTGGATATCTGGTCCGT
	R	ATATATGTCCGGGCGTGTTC
p38γ	F	CTGAGGTATATCCACGCTGC
	R	TGTAGCGCATCCAATTCAAGA
p38δ	F	GGCACATGGCCTGTGTAATA
	R	TAGGAAATGTCCCCCACCTT
JNK	F	GCTCTCCAACACCCGTACAT
	R	TGTGCTAAAGGAGAGGGCTG

Cell lines and cell culture

NK-92 cell line—human natural killer cells were obtained from American Type Culture Collection (ATCC, TM 2407) and maintained in α-MEM with 12.5% FBS, 12.5% horse serum, supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.2 mM myc-inositol, 2 µM folic acid, 0.05 mM 2-mercaptoethanol, antibiotics (penicillin–streptomycin), and 200 IU/ml human recombinant IL-2, and maintained in humidified 5% CO_2 atmosphere at 37 °C. K562—human leukemia cell line (ATCC CCL 243), Jurkat—human leukemic T cell line (ATCC, TIB 152) and A549—an epithelial lung cancer cell line (ATCC, CCL 185) were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, antibiotics in 5% CO_2 at 37 °C. The K562, A549 and Jurkat cell lines were obtained from the Cell Line Bank of the Institute of Immunology and Experimental Therapy (Wrocław, Poland).

Isolation of NK cells from human peripheral blood

Briefly, EDTA anti-coagulated blood was obtained from healthy volunteers. NK isolation using the MACSxpress NK Cell Isolation Kit was performed according to the manufacturer's instructions. Residual erythrocytes were removed by magnetic depletion using the MACSxpress Erythrocyte Depletion Kit. The supernatant containing NK cells was transferred into a new 15 ml tube, filled up with RPMI 1640 with 1% FCS media and centrifuged at 400×*g* for 8 min to pellet the cells. After 1×wash with RPMI 1640 with 1% FCS, cells were resuspended in RPMI 1640 medium containing 1% FCS and counted using a Bürker chamber, following staining with trypan blue. NK cells (effector cells) were adjusted to a concentration of 5×10^4 cells per 50 µl (RPMI with 1% FCS).

NK cell cytotoxicity test

Two types of effector cells were used for the cytotoxicity tests: NK-92 cell line and human blood derived primary NK cells. NK cells were incubated with or without IL-2, LTF and FcLTF for 0, 21 or 24 h at 37 °C in a cell culture incubator. IL-2 was used in concentration of 20 or 100 U/ml. LTF and FcLTF

was added at equal concentrations of (10–25 µg/ml) taking into account a difference in molecular weight between LTF and FcLTF.

After indicated pre-incubation time, K562 cell line (target cells) was re-suspended at 1×10^5 cells/ml. 50 µl (5×10^3 cells) of K562 cells were added to each well containing NK cells (effector cells) to obtain E:T ratios of 10:1 (in triplicates in "U" bottom 96-well plate). Plain media, maximum lactate dehydrogenase (LDH) release (using 1% Triton X-100) from K562 cells and spontaneous LDH release activities from K562 cells and NK cells controls were included, respectively. The plate was incubated for 3 or 4 h at 37 °C in a cell culture incubator. NK cell cytotoxicity towards K562 cell line was assessed by LDH level, according to manufacturer's protocol. The percentage of cytotoxicity was calculated according to equation: % cytotoxicity = (experimental value – effector cells spontaneous control – target cells spontaneous control)/(target cell maximum control – target cells spontaneous control) × 100.

Isolation and treatment of monocytes

The monocytes were isolated from human peripheral blood as described previously with some modification (Borregaard and Kragballe 1980). The heparinized blood was centrifuged over Lymphoprep. In brief, PBMC population (0.5 ml) at a density of 2×10^6/ml was incubated in RPMI 1640 medium supplemented with 5% of autologous serum and antibiotics in 48-well culture plate for 3 h at 37 °C in a humidified atmosphere containing 5% CO_2. After the incubation the supernatant was removed and the wells were washed 5 × with warm HBSS containing 2.5% of autologous serum to remove non-adherent cells. The collected monocytes were supplemented with RPMI 1640 medium containing 10% of autologous serum and antibiotics. Then cells were incubated for 20 h in the presence of LTF (20 µg/ml) or FcLTF (an equivalent of 20 µg/ml of LTF alone). In the control experiments Fc fragment and mixture of Fc and LTF were included (an equivalent of 20 µg/ml of FcLTF alone). To investigate the effects of blocking CD16, CD32a and CD14 receptors, the monocyte cultures were incubated at 4 °C for 30 min with 5 µg/ml of the anti-CD32a, anti-CD16 and CD14 antibodies, followed by addition of FcLTF (20 µg/ml equivalent of LTF) and incubation at 37 °C for 20 h. After the incubation the supernatants were harvested and stored at -80 °C until use. The levels of TNFα and granzyme B were assessed by the commercial ELISA kits in accordance with manufacturer instructions.

For the combinatorial viability assay Jurkat cells were used at a density of 2×10^6/ml in 25 µl/well in 96-flat bottom microtiter plates, suspended in the culture medium RPMI 1640 supplemented with 10% of autologous serum. The cultures contained actinomycin D (10 ng/ml) and the supernatants, obtained as described above, in a volume of 50 µl/well. Appropriate additional control cultures of Jurkat cells were prepared without actinomycin D (data not shown) and with a control monocyte supernatant. The determination of cell viability was performed by MTT method after 48 h incubation.

Colorimetric MTT assay

Briefly, 25 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) from stock solution (5 mg/ml) was added per well at the end of cell incubation period and the plates were incubated for additional 3 h in a cell culture incubator. Then, 100 µl of the extraction buffer (20% SDS with 50% DMF, pH 4.7) was added. After an overnight incubation the optical density (OD) was measured at 550 nm with the reference wavelength of 630 nm.

RNA isolation

Total RNA was isolated from human cell lysates using RNA Extracol according to the manufacturer's recommendations. Briefly, the cell pellet was suspended in 1 ml of mentioned above reagent, shaken, and incubated 10 min at room temperature (RT). Phase separation was conducted by adding of 0.2 ml of chloroform, vigorous shaking for 15 s, 3 min long incubation at RT, and centrifugation at 12,000 × g for 15 min at 4 °C. RNA precipitated from the water phase with 0.5 ml of isopropanol after 10 min incubation at RT, and centrifugation 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 µl of sterile

diethylpyrocarbonate-treated Mili-Q water. RNA samples were stored at −20 °C.

Reverse transcription

Complementary DNA (cDNA) was synthesized with oligo(dT)20 primers from 5 µg of total RNA using NG dART RT Kit according to the manufacturer's instruction.

Quantification of gene expression by Real Time PCR

Expression of selected genes was measured using AmpliQ 5 × HOT EvaGreen qPCR Mix. The reaction was performed in Bio-Rad CFX thermocycler starting with 5 min of preincubation at 95 °C followed by 40 amplification cycles as follows: 15 s of denaturation at 95 °C, 30 s of annealing at 53 °C and 30 s of elongation at 72 °C, and final elongation for 10 min at 72 °C. The results are presented as absolute values and reflect the fold change in gene expression. They were estimated after normalization of crude results and equilibrating the amount of mRNA in each reaction mixture by the housekeeping gene expression valuation. The gene expression measured in the control cell culture (untreated monocytes) was the control/comparative value for determinations in treated monocytes. Values less than 1 indicate a decrease in gene expression, while values above 1 indicate increased gene expression. GAPDH was used as a housekeeping gene for arbitrary unit. The sequences of primers are listed in Table 1.

Statistics

The results were subjected to statistical analysis using analysis of variance (one-way ANOVA) in STATISTICA 7 for Windows (StatSoft, Poland). Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by post hoc comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal–Wallis analysis of variance. Significance was determined at $p < 0.05$. Two/three independent assays were conducted and the results are presented as mean values from triple determinations for a given experiment ± standard error (SE).

Results

Effects of LTF and FcLTF on cytotoxic activity of NK-92 cell line and NK cells isolated from human blood

Preliminary experiments (not shown), aimed at evaluation of potential cytotoxicity on reference and target cells for LTF and FcLTF, showed that the preparations had no trace of cytotoxic action towards reference A549 cells at a concentration range used in this study. Moreover, NK-92 cells showed unchanged or even higher viability, measured by the MTT assay, if cultured in the presence of both preparations up to 200 µg/ml.

Figure 1A presents effects of LTF and FcLTF on cytotoxic activity of NK-92 cells against target K562 cells depending on incubation time in the mixed cell culture. The mean cytotoxic effects are stronger after 4 h incubation and increased by 4% and 6% for LTF and FcLTF, respectively. In preliminary experiments LTF and FcLTF were tested for their potential toxicity against a reference A549 cell line and non-toxic effect was recorded (data not shown).

Dose-dependent effects of FcLTF on cytotoxic activity in NK-92 cells when the cells were preincubated for 24 h with FcLTF 10–25 µg/ml concentration range are presented in Fig. 1B. In this model the lowest concentration of FcLTF (10 µg/ml) was most effective.

On the other hand, when lactoferrins were added to the mixed cell culture (1:10, E:T cells ratio, lack of preincubation) at the beginning of NK cytotoxic assay, the results show comparable activities of both constructs (Fig. 1C). The higher cytotoxic activity observed in case of preincubation with FcLTF may be explained by the longer contact of cells with the chimera (Fig. 1A) or preferential binding on the surface of K562 cells when FcLTF is added simultaneously to effector and target cells at the beginning of cytotoxic assay (Fig. 1C).

To further assess the potential effects of LTF and FcLTF on cell signaling in NK-92 cell line, the cells were cultured overnight with 10 µg/ml of LTF or

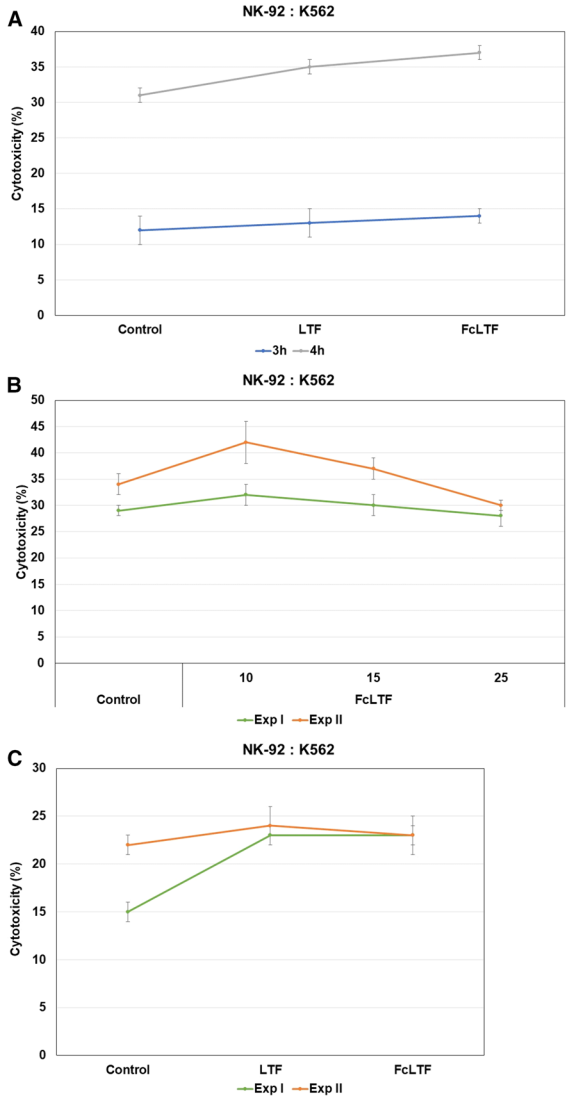

Fig. 1 The effects of LTF and FcLTF on cytotoxic activity of NK-92 cell line. **A** NK-92 cells were pre-incubated for 24 h with LTF (15 µg/ml) and IL-2 (100 U/ml), washed extensively and tested for cytotoxicity against K562 cell line after 3 or 4 h. **B** NK-92 cells were pre-incubated for 24 h with FcLTF at indicated concentrations in µg/ml (10–25 µg/ml), NK-92 cells were incubated for 4 h with K562 cells for measurement of cytotoxicity. **C** NK-92 were not preincubated with the preparations. NK-92 were incubated with K562 cells for 4 h for measurement of cytotoxicity. LTF and FcLTF (10 µg/ml), were added simultaneously to effector and target cells at the beginning of cytotoxic assay. Equivalent concentrations of FcLTF were used in each experiment. The data are presented as mean cytotoxicity values (%) from triplicate wells ± SE

FcLTF (the concentration of FcLTF was normalized for LTF equivalency) and 1 µg/ml of LPS for control (Table 2). The experiment demonstrated a significant increase in expression of ERK-1 gene by LTF. FcLTF, in turn, triggered, in addition, increase expression of p38β, p38δ and JNK genes. In LPS control only p38β gene expression was significantly elevated.

In search for a potential clinical application of FcLTF we directed our next study into human blood derived NK cells and monocytes obtained from human healthy volunteers. The results presented on Fig. 2 showed that cytotoxicity of NK cells was increased by IL-2 from 10 to 25%, depending on a blood donor. The cytotoxicity was further elevated by LTF in 4 out of 5 donors but decreased by FcLTF in 4 donors in relation to IL-2 cultures.

Effects of LTF and FcLTF on production of cytotoxic mediators by monocytes from human blood

Previous studies reported that NK activity was influenced by several cytokines, including TNFα enhancing NK cell cytotoxicity. The experiments with FcLTF and LTF in human PBMC culture (the model representing a natural environment which enables interactions of NK with other cell types), mixed with K562 cells, revealed a significant increase in TNFα production in the FcLTF culture (data not shown). Subsequently, we identified monocytes as sources of TNFα present in PBMC culture. Figure 3A shows the results of TNFα determination in monocytes cultures from 5 blood donors. It appeared that in highly enriched monocyte population, very low levels of TNFα, comparable to control cultures, were present in LTF-treated cultures. In contrast, the cultures treated with FcLTF contained several fold higher concentration of TNFα. In addition the Fc fragment alone and the mixture of Fc fragment with LTF used as control (20 µg/ml FcLTF equivalent) did not induce significant levels of TNFα (60 pg/ml and 110 pg/ml, respectively, data from 3 experiments, not shown).

The supernatants from monocyte cultures from four blood donors, stimulated by LTF and FcLTF were also assayed for presence of granzyme B (Fig. 3B). It appeared that in all cases FcLTF, but not LTF, induced high levels of granzyme B in 20 h monocyte cultures.

Table 2 Effects of LTF and FcLTF on expression of MAP kinase genes in NK-92 cells. NK-92 cells were incubated overnight with 10 µg/ml of LTF, FcLTF (for FcLTF 10 µg/ml LTF equivalent) or 1 µg/ml of LPS

	ERK-1	ERK-2	p38α	p38β	p38γ	p38δ	JNK
LTF	6.3	0.9	0.6	1.8	0.7	2.1	1.5
FcLTF	5.9	0.8	1.5	10.0	1.3	81.1	6.2
LPS	1.8	1.4	2.3	18.5	1.1	1.6	3.7

The values presented in the table reflect relative fold changes in response of the cells to LTF or FcLTF, when the GAPDH gene expression was a control and assumed as equal to one

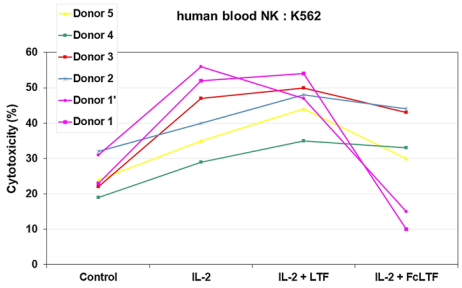

Fig. 2 Induction of cytotoxicity against target K562 cells by human blood derived NK cells by IL-2, LTF and FcLTF. NK cells from individual donors were cultured for 21 h in the presence of 20 units of IL-2 and investigated lactoferrins (10 µg/ml, for FcLTF 10 µg/ml LTF equivalent) and tested for cytotoxicity against K562 cells. The data are presented as mean cytotoxicity values (%) from triplicate wells. For donor #1, two independent experiments were performed

In addition, the induction of cytotoxic mediators produced by monocytes treated with LTF or FcLTF was established in a viability test on Jurkat cells. It appeared that only FcLTF had the ability to lower viability by 20% of Jurkat cells, LTF did not induce a measurable level of cytotoxicity (Fig. 3C).

The morphology of monocytes cultured alone or in the presence of FcLTF was significantly different. The FcLTF-treated monocytes were spread and visibly lost their contours (data not shown). Such an appearance indicates a firm adherence of these cells to plastic surface.

The production of TNFα induced by FcLTF was strongly inhibited upon preincubation of monocytes with anti-CD32 and to a lesser degree by anti-CD14 antibodies (Fig. 4).

Finally, we explored the effects of LTF and FcLTF on gene profiling in human blood monocytes with a specific focus on gene expression related to PI3K/Akt/mTOR signaling pathway. The results presented in Table 3 are absolute values and reflect the change in gene expression. Statistically important changes were noted in three cases after FcLTF treatment, i.e. Akt and SYK gene expression was completely silenced, while PI3K gene expression grew four times. The influence of LTF significantly upped the expression of only one gene, i.e. PI3K. Both substances, FcLTF and LTF, did not impress on mTOR gene expression.

Discussion

This is the first report on direct comparison of human recombinant lactoferrin (LTF) and its fusion with the Fc domain of human IgG2 (FcLTF) to assess their clinical potential based on cytotoxic effects of NK cells. Here we demonstrate that LTF and FcLTF enhanced cytotoxic activity of NK cells towards K562 cells, both for the NK-92 cell line as well as for NK cells isolated from the peripheral human blood of several donors. A major observation of this study was also a difference in degree of TNFα and granzyme B production by the monocytes derived from human blood and treated with LTF or FcLTF. While LTF treatment did not increase the production of these two cytotoxic mediators, the fusion protein was highly stimulatory. In addition, supernatants from monocyte cultures, treated with FcLTF, exhibited a stronger decrease in viability of Jurkat cells, as compared to the LTF. The herein presented results are consistent with previous findings on indirect effects of LTF on NK activity, since those reports suggested modulation of monocytes immune status by LTF (Nishiya and Horwitz 1982; Horwitz et al. 1984; McCornick et al. 1991). This report also supports a previous finding on a direct action of LTF on highly purified NK cells from the PBMC population (Damiens et al. 1998).

Of interest, the morphology of monocytes incubated with FcLTF differed from those incubated with

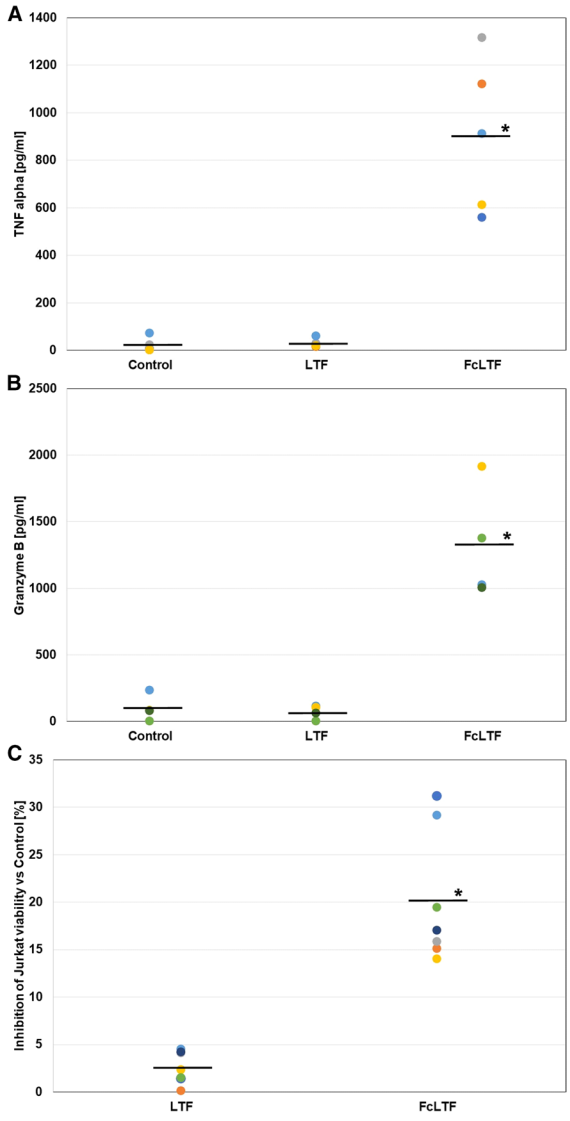

Fig. 3 Induction of TNFα **(A)**, granzyme B **(B)** and monocyte-released cytotoxic mediators **(C)** by LTF and FcLTF in human monocyte cultures. Blood monocytes from individual 4, 5 or 7 donors (respectively) were incubated for 20 h with LTF or FcLTF at concentration of 20 µg/ml (and FcLTF equivalents). The levels of TNFα and granzyme B in monocyte supernatans were measured by ELISA Kits. Induction of cytotoxic mediators produced by monocytes treated with LTF or FcLTF was established in a viability test in Jurkat cells. The data are presented as individual (filled circle) and mean (solid line) TNFα and granzyme B levels and % of inhibition of Jurkat cell line viability. For every donor, individual data are presented as mean value from triplicate wells. *$p < 0.05$ versus control

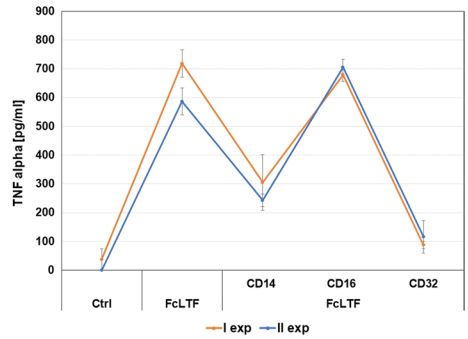

Fig. 4 Inhibition of TNFα production induced in human monocytes by FcLTF by anti-CD32 and anti-CD14 antibodies. Blood monocytes from individual donors were incubated at 4 °C for 30 min with 5 µg/ml of the anti-CD32a, anti-CD16 and CD14 antibodies, followed by addition of FcLTF (20 µg/ml equivalent of LTF) and incubation at 37 °C for 20 h. The levels of TNFα were measured by ELISA Kit. The data are presented as mean TNFα level in monocytes cultures from triplicate wells ± SE

Table 3 Effects of LTF and FcLTF on AKT/mTOR signaling pathway gene expression of in human blood monocytes

	Akt	mTOR	PI3K	SYK
LTF	0.9	0.8	1.8	0.6
FcLTF	0	0.5	4.2	0

The values presented in the table reflect relative fold changes in response of the cells to LTF or FcLTF, when the GAPDH gene expression was a control and assumed as equal to one. Values less than 1 indicate a decrease in gene expression, while values above 1 indicate increased gene expression

LTF or control culture, i.e. monocytes incubated with FcLTF strongly adhered to the plastic (photomicrographs not shown). Such a behavior indicates activation of cells (Kasahara et al. 1991; Stout et al. 1992) in which potent NK activity was only observed when monocytes were in an adherent state (Nishiya and Horwitz 1982).

Nevertheless, in the NK-92 cell line, a significantly elevated expression of MAP kinases, induced by FcLTF but not LTF, did not lead to increased cytotoxicity when compared with LTF. Apparently, another signaling pathway is relevant to trigger cytotoxic action as in NK cells isolated from human blood cells, as PI3K/Akt/mTOR pathway reported here and for NK cells (Nandagopal et al. 2014) and monocytes (Li et al. 2019). Such a signaling pathway may not be elicited in this particular cell line since it lacks Fc

receptors. The increases of expression of members of MAP kinase family (ERK-1, p38β, p38δ, JNK), induced by FcLTF, suggests promotion of such processes as activation and/or differentiation of the cell line (Sutherland et al. 1996; Cheon et al. 2008; Zhong et al. 2011; Kanade and Eckert 2012; Wei et al. 2020; Gagliardi et al. 2020).

In isolated NK cells from individual donors, LTF generally induced higher cytotoxic activity, as compared with the control NK cultures, stimulated only with IL-2. Addition of IL-2 to the cultures was intentional in our experimental model to increase basal activity of NK cells (Abrams and Brahmi 1986; Frey et al. 1987). The stimulatory effects of LTF seem to depend on an initial activation status of the NK cells and are typical for the immunoregulatory action of LTF, as demonstrated in various published models (Zimecki et al. 1998, 2001; Artym et al. 2018). However, the action of FcLTF was suppressive in most cases in comparison with IL-2 control. We have no satisfactory explanation for this effect, and theorize that possible spatial hindrance by the Fc fragment relative to respective sites on LTF molecule are responsible for interaction with specific cell receptors.

We subsequently turned attention to monocytes as their role in generation of cytotoxic activity by LTF surpassed the one displayed by NK cells. Interestingly, neither Fc fragment alone nor the equivalent mixture of Fc and LTF was able to induce high TNFα production, indicating necessity of covalent linkage between these molecules to demonstrate such activity. It is possible that FcLTF, bearing a structural resemblance to immune complexes, could induce release of other cytolytic factors by monocytes, as shown by others (Li et al. 2019). Indeed, our experimental model also identified granzyme B as a second cytotoxic factor, besides TNFα, released by activated monocytes. Granzymes are serine proteases that act in conjunction with perforins to induce in target cells proapoptotic cell signaling (Hay and Slansky 2022). Although mainly attributed to NK cells, these molecules can also be induced in monocytes by the ADCC mechanism (Elavazhhagan et al. 2015). However, it remains unknown if the effector function of IgG2, as part of FcLTF construct, can induce such proapoptotic cell signaling.

The induction of TNFα by FcLTF was strongly inhibited by anti-CD32 and to a lesser degree by anti-CD14, revealing that cell receptors are involved in the interaction and signal transduction upon contact with the FcLTF complex. Blocking of CD16 receptors was not effective. Similar results were obtained by Li et al. (2019) and Hu et al. (2017) using monocytes and anti-LTF immune complexes. The interaction of the complex with corresponding cell receptors is associated with triggering of PI3K/AKT/mTOR signaling pathway (Li et al. 2019). In our experiments, a strong elevation of PI3K expression was also shown. Although other members of the PI3K/AKT/mTOR signaling pathway were not elevated, it should be emphasized that the production of granzyme B is already significantly inhibited by 10 × lower application of PI3K inhibitor than by AKT inhibitors, providing evidence for major involvement of PI3K in mediation of granzyme B production (Li et al. 2019).

Given the many identified beneficial effects of LTF, the fusion of LTF with Fc domain of human IgG2 opens a new therapeutic opportunity for its clinical utility, especially for cytotoxic therapies such as treatment of neoplasms that kill cancer cells or slow their growth. LTF indeed has the ability to reduce the growth and migration of human lung cancer cells with no cytotoxic effect on normal human bronchial cells (NHBC) (Olszewska et al 2021). Of importance, LTF in combination with etoposite (chemotherapeutic), enhances anticancer activity against human lung cancer while reducing etoposite-induced cytotoxicity to normal cells (Olszewska et al. 2021). Based on the cytotoxic effect of FcLTF presented in our studies, it is likely that this fusion molecule would be an improved candidate for an adjunct therapy with current chemotherapeutic protocols in cancer. Although, our in vitro studies need to be confirmed in vivo, this report provides new insight into the immune modulatory of LTF and FcLTF and their potential for treatment of cancer.

In conclusion, although interaction of FcLTF with NK cells was comparable with regard to effect on cytotoxicity, interaction of FcLTF with monocytes could generate a double enhancing effect on cytotoxicity by 1) induction of granzyme B from monocytes, and 2) indirect stimulation of NK cells by TNFα derived from monocytes as reported elsewhere (Ostensen et al. 1987). An additional cytotoxic component, derived from activated NK cells, may also play a role in mounting overall cytotoxicity against target cells. Thus, FcLTF has therapeutic potential in enhancement of antitumor and antiviral immunity.

Acknowledgements The work was supported by statuary grant from the Institute of Immunology and Experimental Therapy in Wrocław # 501-4/2021, and by PharmaReview Corporation Gift UD 07/05/2019

Author contributions EZ: designing experimental protocols and performing experiments. MK: designing experimental protocols, performing experiments, preparation of figures, and statistical analysis. JA: editing the final version of the manuscript. IK: performing molecular studies. MLK: designing and providing testing articles (LTF and FcLTF), evaluation and revision of the final version of the manuscript, partial funding. MZ: general concept, supervision of the studies and writing the draft.

Declarations

Conflict of interest MLK is a founder of PharmaReview Corporation and a donor of LTF and FcLTF for this study. Potential conflicts of interest are managed by documentation registered between PharmaReview Corporation and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland.

References

Abrams SI, Brahmi Z (1986) The functional loss of human natural killer cell activity induced by K562 is reversible via an interleukin-2-dependent mechanism. Cell Immunol 101(2):558–570. https://doi.org/10.1016/0008-8749(86)90166-8

Artym J, Kocięba M, Zaczyńska E, Adamik B, Kübler A, Zimecki M, Kruzel ML (2018) Immunomodulatory properties of human recombinant lactoferrin in mice: Implications for therapeutic use in humans. Adv Clin Exp Med 27(3):391–399. https://doi.org/10.17219/acem/68440

Bezault J, Bhimani R, Wiprovnick J, Furmanski P (1994) Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice. Cancer Res 54(9):2310–2312

Borregaard N, Kragballe K (1980) Role of oxygen in antibody-dependent cytotoxicity mediated by monocytes and neutrophils. J Clin Invest 66(4):6766–6783. https://doi.org/10.1172/JCI1099

Cheon S, Song SB, Jung M, Park Y, Bang J-W, Kim TS, Park H, Kim C-H, Yang Y-H, Bang SI, Cho D (2008) Sphingosine kinase inhibitor suppresses IL-18-induced interferon-gamma production through inhibition of p38 MAPK activation in human NK cells. Biochem Biophys Res Commun 374(1):74–78. https://doi.org/10.1016/j.bbrc.2008.06.091

Cutone A, Rosa L, Ianiro G, Lepanto MS, Bonaccorsi di Patti MC, Valenti P, Musci G (2020) Lactoferrin's anti-cancer properties: safety, selectivity, and wide range of action. Biomolecules 10(3):456. https://doi.org/10.3390/biom10030456

Czajkowsky DM, Hu J, Shao Z, Pleass RJ (2012) Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med 4(10):1015–1028. https://doi.org/10.1002/emmm.201201379

Damiens E, Mazurier J, El Yazidi I, Masson M, Duthille I, Spik G, Boilly-Marer Y (1998) Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells. Biochim Biophys Acta 1402(3):277–287. https://doi.org/10.1016/s0167-4889(98)00013-5

Elavazhagan S, Fatehchand K, Santhanam V, Fang H, Ren L, Gautam S, Reader B, Mo X, Cheney C, Briercheck E, Vasilakos JP, Dietsch GN, Hershberg RM, Caligiuri M, Byrd JC, Butchar JP, Tridandapani S (2015) Granzyme B expression is enhanced in human monocytes by TLR8 agonists and contributes to antibody-dependent cellular cytotoxicity. J Immunol 194(6):2786–2795. https://doi.org/10.4049/jimmunol.1402316

Frey JR, Kamber M, Peck R (1987) Recombinant interferons or interleukin-2 increase cytotoxicity by human monocytes and NK cells. Lymphokine Res 6(3):215–227

Gagliardi M, Pitner MK, Park J, Xie X, Saso H, Larson RA, Sammons RM, Chen H, Wei C, Masuda H, Chauhan G, Kondo K, Tripathy D, Ueno NT, Dalby KN, Debeb BG, Bartholomeusz C (2020) Differential functions of ERK1 and ERK2 in lung metastasis processes in triple-negative breast cancer. Sci Rep 10(1):8537. https://doi.org/10.1038/s41598-020-65250-3

Hao L, Shan Q, Wei J, Ma F, Sun P (2019) Lactoferrin: major physiological functions and applications. Curr Protein Pept Sci 20(2):139–144. https://doi.org/10.2174/1389203719666180514150921

Hay ZLZ, Slansky JE (2020) Granzymes: the molecular executors of immune-mediated cytotoxicity. Int J Mol Sci 23(3):1833. https://doi.org/10.3390/ijms23031833

Horwitz DA, Bakke AC, Abo W, Nishiya K (1984) Monocyte and NK cell cytotoxic activity in human adherent cell preparations: discriminating effects of interferon and lactoferrin. J Immunol 132(5):2370–2374

Hu L, Hu X, Long K, Gao C, Dong H-L, Zhong Q, Gao XM, Gong F-Y (2017) Extraordinarily potent proinflammatory properties of lactoferrin-containing immunocomplexes against human monocytes and macrophages. Sci Rep 7(1):4230. https://doi.org/10.1038/s41598-017-04275-7

Kanade SR, Eckert RL (2012) Protein arginine methyltransferase 5 (PRMT5) signaling suppresses protein kinase Cδ- and p38δ-dependent signaling and keratinocyte differentiation. J Biol Chem 287(10):7313–7323. https://doi.org/10.1074/jbc.M111.331660

Kasahara K, Strieter RM, Chensue SW, Standiford TJ, Kunkel SL (1991) Mononuclear cell adherence induces neutrophil chemotactic factor/interleukin-8 gene expression. J Leukoc Biol 50(3):287–295. https://doi.org/10.1089/jir.2006.26.489

Kruzel ML, Zimecki M, Actor J (2017) Lactoferrin in a context of inflammation-induced pathology. Front Immunol 8:1438. https://doi.org/10.3389/fimmu.2017.01438

Kruzel ML and Aronowski J (2019) Methods and compositions for treatment of symptoms associated with intracranial hemorrhage. US Patent No. 10442852 B2

Kuhara T, Yamauchi K, Tamura Y, Okamura H (2006) Oral administration of lactoferrin increases NK cell activity in mice via increased production of IL-18 and type I IFN in the small intestine. J Interferon Cytokine Res 26(7):489–499. https://doi.org/10.1089/jir.2006.26.489

Li Y, Li J, Gong Z, Pan X-H, Zi-H Ma, Ma SY, Wang H-M, Dong H-L, Gong F-Y, Gao X-M (2019) M860, a

monoclonal antibody against human lactoferrin, enhances tumoricidal activity of low dosage lactoferrin via Granzyme B induction. Molecules 24(20):3640. https://doi.org/10.3390/molecules24203640

Lu L, Shen RN, Zhou SZ, Srivastava C, Harrington M, Miyazawa K, Wu B, Lin ZH, Ruscetti S, Broxmeyer HE (1991) Synergistic effect of human lactoferrin and recombinant murine interferon-gamma on disease progression in mice infected with the polycythemia-inducing strain of the Friend virus complex. Int J Hematol 54(2):117–124

Mayeur S, Spahis S, Pouliot Y, Levy E (2016) Lactoferrin, a pleiotropic protein in health and disease. Antioxid Redox Signal 24(14):813–836. https://doi.org/10.1089/ars.2015.6458

McCormick JA, Markey GM, Morris TC (1991) Lactoferrin-inducible monocyte cytotoxicity for K562 cells and decay of natural killer lymphocyte cytotoxicity. Clin Exp Immunol 83(1):154–156. https://doi.org/10.1111/j.1365-2249.1991.tb05606.x

Nandagopal N, Ali AK, Komal AK, Lee S-H (2014) The Critical Role of IL-15-PI3K-mTOR Pathway in Natural Killer Cell Effector Functions. Front Immunol 5:187. https://doi.org/10.3389/fimmu.2014.00187

Nguyen TKT, Niaz Z, Kruzel ML, ML, Actor JK, (2022) Recombinant Human Lactoferrin Reduces Inflammation and Increases Fluoroquinolone Penetration to Primary Granulomas during Mycobacterial Infection of C57Bl/6 Mice. Arch Immunol Ther Exp 70(1):9. https://doi.org/10.1007/s00005-022-00648-7

Nishiya K, Horwitz DA (1982) Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. J Immunol 129(6):2519–2523

Olszewska P, Pazdrak B, Marian L, Kruzel ML (2021) A novel human recombinant lactoferrin inhibits lung adenocarcinoma cell growth and migration with no cytotoxic effect on normal human epithelial cells. Arch Immunol Ther Exp 69(1):33. https://doi.org/10.1007/s00005-021-00637-2

Ostensen ME, Thiele DL, Lipsky PE (1987) Tumor necrosis factor-alpha enhances cytolytic activity of human natural killer cells. J Immunol 138(12):4185–4191

Shiga Y, Murata D, Sugimoto A, Oshima Y, Tada M, Ishii-Watabe A, Imai K, Tomii K, Takeuchi T, Kagaya S, Sato A (2017) Hinge-deficient IgG1 Fc fusion: application to human lactoferrin. Mol Pharmaceutics 14(9):3025–3035. https://doi.org/10.1021/acs.molpharmaceut.7b00221

Shiga Y, Oshima Y, Kojima Y, Sugimoto A, Tamaki N, Murata D, Takeuchi T, Sato A (2015) Recombinant human lactoferrin-Fc fusion with an improved plasma half-life. Eur J Pharm Sci 67:136–143. https://doi.org/10.1016/j.ejps.2014.11.005

Shimizu K, Matsuzawa H, Okada K, Tazume S, Dosako S, Kawasaki Y, Hashimoto K, Koga Y (1996) Lactoferrin-mediated protection of the host from murine cytomegalovirus infection by a T-cell-dependent augmentation of natural killer cell activity. Arch Virol 141(10):1875–1889. https://doi.org/10.1007/BF01718201

Stout RD, Ferguson KP, Li YN, Lambe DW Jr (1992) Staphylococcal exopolysaccharides inhibit lymphocyte proliferative responses by activation of monocyte prostaglandin production. Infect Immun 60(3):922–927. https://doi.org/10.1128/iai.60.3.922-927.1992

Strohl WR, Knight DM (2009) Discovery and development of biopharmaceuticals: current issues. Curr Opin Biotechnol 20(6):668–672. https://doi.org/10.1016/j.copbio.2009.10.012

Strohl WR (2015) Fusion proteins for half-life extension of biologics as a strategy to make biobetters. BioDrugs 29(4):215–239. https://doi.org/10.1007/s40259-015-0133-6

Sun X, Jiang R, Przepiorski A, Reddy S, Palmano KP, Krissansen GW (2012) "Iron-saturated" bovine lactoferrin improves the chemotherapeutic effects of tamoxifen in the treatment of basal-like breast cancer in mice. BMC Cancer 12:591. https://doi.org/10.1186/1471-2407-12-591

Sutherland CL, Heath AW, Pelech SL, Young PR, Gold MR (1996) Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. J Immunol 157(8):3381–3390

Wei G, Gao N, Chen J, Fan L, Zeng Z, Gao G, Li L, Fang G, Hu K, Pang X, Fan H-Y, Clevers H, Liu M, Zhang X, Li D (2020) Erk and MAPK signaling is essential for intestinal development through Wnt pathway modulation. Development 147(17):dev185678. https://doi.org/10.1242/dev.185678

Zhong J, Lardinois D, Szilard J, Tamm M, Roth M (2011) Rat mesothelioma cell proliferation requires p38δ mitogen activated protein kinase and C/EBP-α. Lung Cancer 73(2):166–170. https://doi.org/10.1016/j.lungcan.2010.12.003

Zhao X, Kruzel M, Ting S-M, Sun G, Savitz SI, Aronowski J (2021) Optimized lactoferrin as a highly promising treatment for intracerebral hemorrhage: Pre-clinical experience. J Cereb Blood Flow Metab 41(1):53–66. https://doi.org/10.1177/0271678X20925667

Zimecki M, Stepniak D, Szynol A, Kruzel ML (2001) Lactoferrin regulates proliferative response of human peripheral blood mononuclear cells to phytohemagglutinin and mixed lymphocyte reaction. Arch Immunol Ther Exp 49(2):147–154

Zimecki M, Właszczyk A, Cheneau P, Brunel AS, Mazurier J, Spik G, Kübler A (1998) Immunoregulatory effects of a nutritional preparation containing bovine lactoferrin taken orally by healthy individuals. Arch Immunol Ther Exp 46(4):231–240