Journal of Cellular Biochemistry 76:30–36 (1999)

Effect of Lactoferrin on the Production of Tumor Necrosis Factor-α and Nitric Oxide

Yoon-Hyuk Choe and Soo-Won Lee*

Department of Food and Life Science, SungKyunKwan University, College of Life Science and Natural Resources, Suwon, Kyunggi-do 440–746, Korea

Abstract One of the biological functions of lactoferrin is the modulation of the host defense systems, including cytokine production and immune responses. We have tested the effect of lactoferrin on the productions of tumor necrosis factor-α and nitric oxide in some cells. Lactoferrin itself did not induce either tumor necrosis factor-α production or nitric oxide production, but lipopolysaccharide-stimulated tumor necrosis factor-α production of macrophages and monocytes were inhibited by lactoferrin treatment combined with stimulant. The induction of nitric oxide synthesis in stimulated macrophages and vascular smooth muscle cells was not affected by the lactoferrin. J. Cell. Biochem. 76:30–36, 1999.

Key words: lactoferrin; tumor necrosis factor-α; nitric oxide

Lactoferrin is an iron-binding glycoprotein found in milk and other body fluids such as plasma, saliva, tears, and the secondary granules of neutrophils [Baynes and Bezwoda, 1994]. Lactoferrin has a role in iron absorption and is an important antimicrobial component of the host defense system. When released from the granules of neutrophils in response to bacterial infection, lactoferrin binds iron and results in the clearance of iron from the serum [Birgens et al., 1988]. This lactoferrin-associated hyperferremia of acute inflammation and infection is thought to make the environment unfavorable for bacterial growth [Van Snick et al., 1974]. In addition to its ability to chelate iron ions, lactoferrin binds to the bacterial cell membrane; this leads to the destruction of some kind of microorganisms [Ellison et al., 1988], serving as a protective mechanism during infection. There is also evidence that lactoferrin modulates host defense responses, acting through the inhibition of hydroxy-radical formation, the modulation of cytokine production, the inhibition of lipopolysaccharide (LPS)-mediated activation of neutrophils, and stimulation of natural killer cell activation [Nuijens et al., 1996], and has an anti-tumor effect by inhibiting the growth of tumors and the development of the metastases [Bezault et al., 1994].

It has been reported that lactoferrin enters the cell from the serum and is transported into the cell nucleus where it binds to DNA [Garre et al., 1992]. Specific DNA sequences that confer lactoferrin-induced gene transcription have been identified [He and Furmanski, 1995]. These observations have great potential for the role of lactoferrin in the inflammatory response and in the other biological functions. We have been interested in the role of lactoferrin in the production of tumor necrosis factor-α (TNF-α) and nitric oxide (NO), which are important in the immune responses and in some pathogenic conditions. We report the modulatory effect of lactoferrin on TNF-α secretion in stimulated macrophages and monocytes, which possibly suppresses the proinflammatory responses of TNF-α in immune responses.

MATERIALS AND METHODS

Cell Cultures

Raw264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), and antibiotics (100 U/ml of penicillin and 100 mg/ml

Grant sponsor: Ministry of Agriculture and Forest, Korea; Grant number: AG 480-M.

*Correspondence to: S.-W. Lee, Department of Food and Life Science, SungKyunKwan University, College of Life Science and Natural Resources, 300 Chunchun-dong, Changan-gu, Suwon, Kyunggi-do, 440–746, Korea. E-mail: leesw@yurim.skku.ac.kr

Received 5 February 1999; Accepted 11 June 1999

of streptomycin) (Gibco-BRL). Cells were subcultured by scraping to a 1:20 split when 90% confluent. THP-1 cells were maintained in RPMI-1640 containing 10% (v/v) FBS and antibiotics at a cell density of 1×10^5–1×10^6 cells/ml. Vascular smooth muscle cells (VSMCs) were prepared from the aorta of 4- to 6-week-old Sprague-Dawley rat (Charles River, Japan) by the method described in Kim et al. [1994]. Cells were grown in DMEM/F12 containing 10% (v/v) FBS and antibiotics. Cells were passaged at 90% confluence and used between passages 4 and 15.

TNF-α Production and Bioassay

Cells grown in 100-mm plates were harvested and plated into a 96-well plate at the concentration of 2×10^5 cells/well in growth medium. Cells were stimulated with LPS (*E. coli* 0111:B4) (Sigma) or lactoferrin (Sigma), or both, at various times, and the supernatants were collected and used immediately or stored at −20°C up to 1 week before use.

For the TNF-α bioassay, confluent L929 fibroblasts were incubated with the prepared supernatants, diluted in growth medium containing actinomycin D (Sigma) for 18 h. Cell vitality was measured by MTT treatment described by Roehm et al. [1991] and colorimetric changes were measured by spectrophotometer (ELx 800, Bio-Tech Instruments) at 540 nm.

A standard curve for measuring TNF-α production was generated using recombinant mTNF-α (R&D systems) and adapted to calculate the concentration of TNF-α in bioassay.

NO Production and Nitrite Measurement

Cells grown in 100-mm plates were harvested and plated into a 96-well plate at a concentration of 2×10^5 cells/well in growth medium without phenol-red. Cells were stimulated with various combinations of LPS (*E. coli* 0111:B4) (Sigma), IFN-γ (R&D systems), TNF-α (R&D systems), and/or lactoferrin (Sigma) in a volume of 120 µl per well. At 24 and 48 h after stimulation, 100 µl of supernatant was transferred to the fresh wells and 100 µl of Griess reagents (solution I: 0.2% of naphthylethylene diamine, solution II: 2% of sulfamilamide and 10% of phosphoric acid) was added at room temperature. The colorimetric development was measured within 30 min by spectrophotometry at 540 nm; 0–10 µl of 1 mM $NaNO_2$ in 100 µl of phosphate-buffered saline (PBS) was used as the nitrite standard in each experiment.

RESULTS

Effect of Lactoferrin on TNF-α Release

TNF-α can be released from macrophages and monocytes when stimulated by bacterial LPS or PMA. We have tested the effect of lactoferrin on the release of TNF-α. First, we have tested whether lactoferrin itself can stimulate the cells to produce TNF-α. Less than 10% of the death rate has been detected by the treatment of lactoferrin, representing the production of TNF-α by Raw264.7 cells (Fig. 1A) and THP-1 (Fig. 2A) cells. The induction is minor and within error ranges of the data, demonstrating that lactoferrin itself does not have good potential as an inducer of TNF-α.

We have induced TNF-α by LPS stimulation and have tested the effect of lactoferrin on the production of TNF-α in Raw264.7 cells and THP-1 cells. In Raw264.7 cells, lactoferrin inhibited the TNF-α production by LPS-stimulation in a dose-dependent manner (Fig. 1B). Both bovine and human lactoferrin have inhibited TNF-α production. 0.1, 0.5, and 1.0 mg/ml of bovine lactoferrin suppressed TNF-α production to 68%, 50%, and 50% of the control experiment (LPS stimulation only without lactoferrin) (Fig. 1C). Human lactoferrin also suppressed TNF-α production to 74%, 43%, and 35% of the control experiment by the treatment of 0.1, 0.5, and 1.0 mg/ml of lactoferrin (Fig. 1C).

The same experiments were done with human monocytic cells, THP-1 cells, with increased time of LPS-stimulation. As seen in Raw264.7 cells, both bovine and human lactoferrin have shown the inhibitory effect on the TNF-α production in THP-1 cells (Fig. 2B). 0.1, 0.5, and 1.0 mg/ml of bovine lactoferrin suppressed TNF-α production to 56%, 38%, and 50% of the control experiment (LPS-stimulation only without lactoferrin) and 88%, 75%, and 48% of the control value by human lactoferrin (Fig. 2C). Comparing the effect of bovine and human lactoferrin, human lactoferrin has shown less inhibitory effect than bovine lactoferrin.

The specificity of lactoferrin on the modulation of TNF-α release was addressed by comparing the effect of bovine serum albumin (BSA), which did not affect TNF-α production in LPS-stimulated cells. We also tested the activity of lactoferrin on TNF-α bioassay and did not find

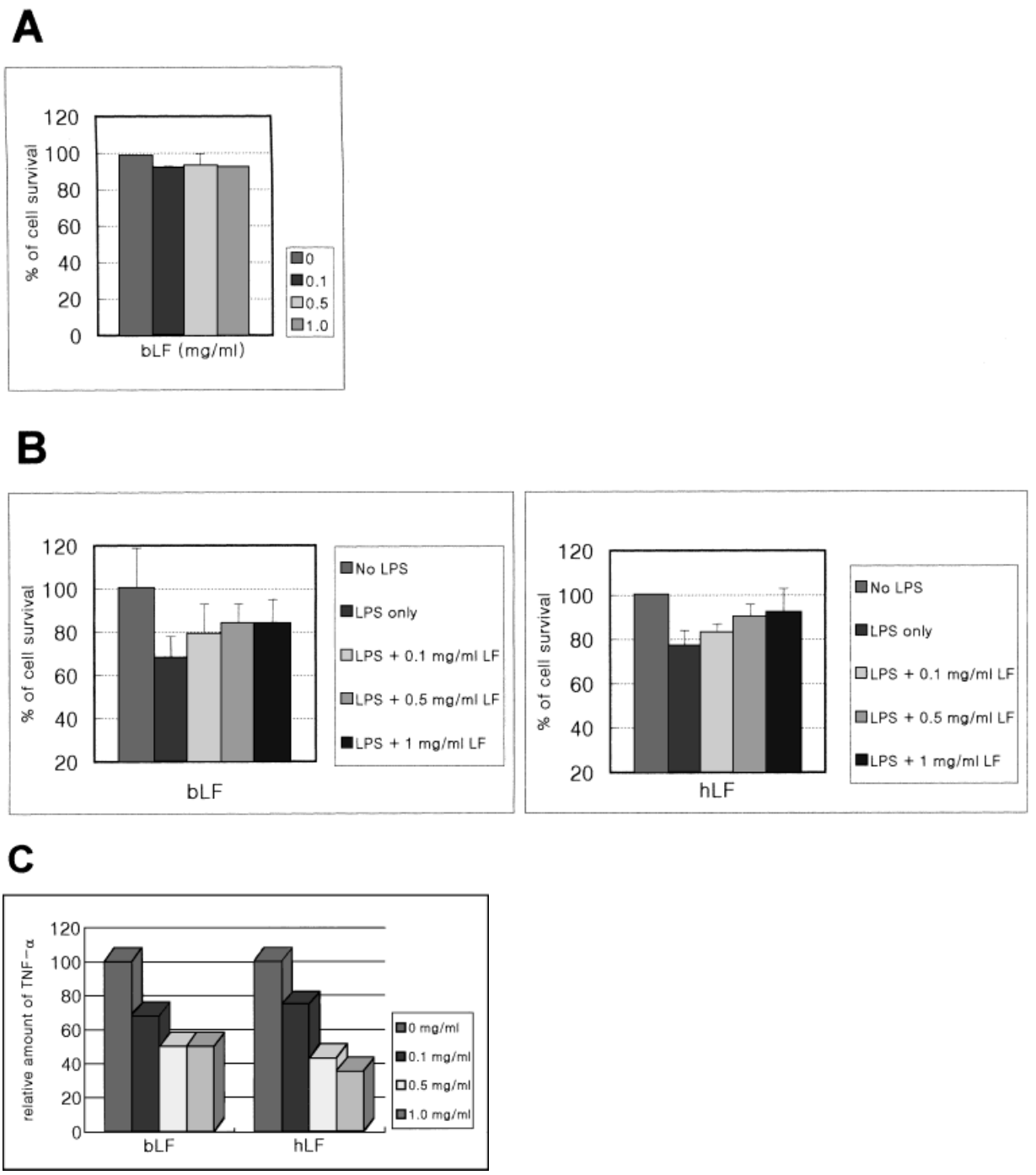

Fig. 1. Effect of lactoferrin on the TNF-α production in Raw264.7 cells. Cells were treated with lactoferrin and LPS (1 μg/ml) for 6 h. After the bioassay for the amount of TNF-α, the percentage of cell survival was adapted as an indicator of the produced TNF-α by Raw264.7 cells. **A:** Treatment of bovine lactoferrin (bLF) without LPS stimulation. **B:** Treatment of bLF and human lactoferrin (hLF) with LPS stimulation. **C:** The relative amount of the TNF-α was calculated by converting the value of cell survivality to the concentration of TNF-α production using recombinant TNF-α as a standard. $P < 0.05$ vs LPS-stimulated cells without lactoferrin treatment: for the treatment of 0.1, 0.5, and 1.0 mg/ml bLF, $P = 0.029$, 0.008, and 0.011, respectively (n = 9). For the treatment of 0.1, 0.5, and 1.0 mg/ml hLF, $P = 0.013$, 0.017, and 0.022, respectively (n = 5).

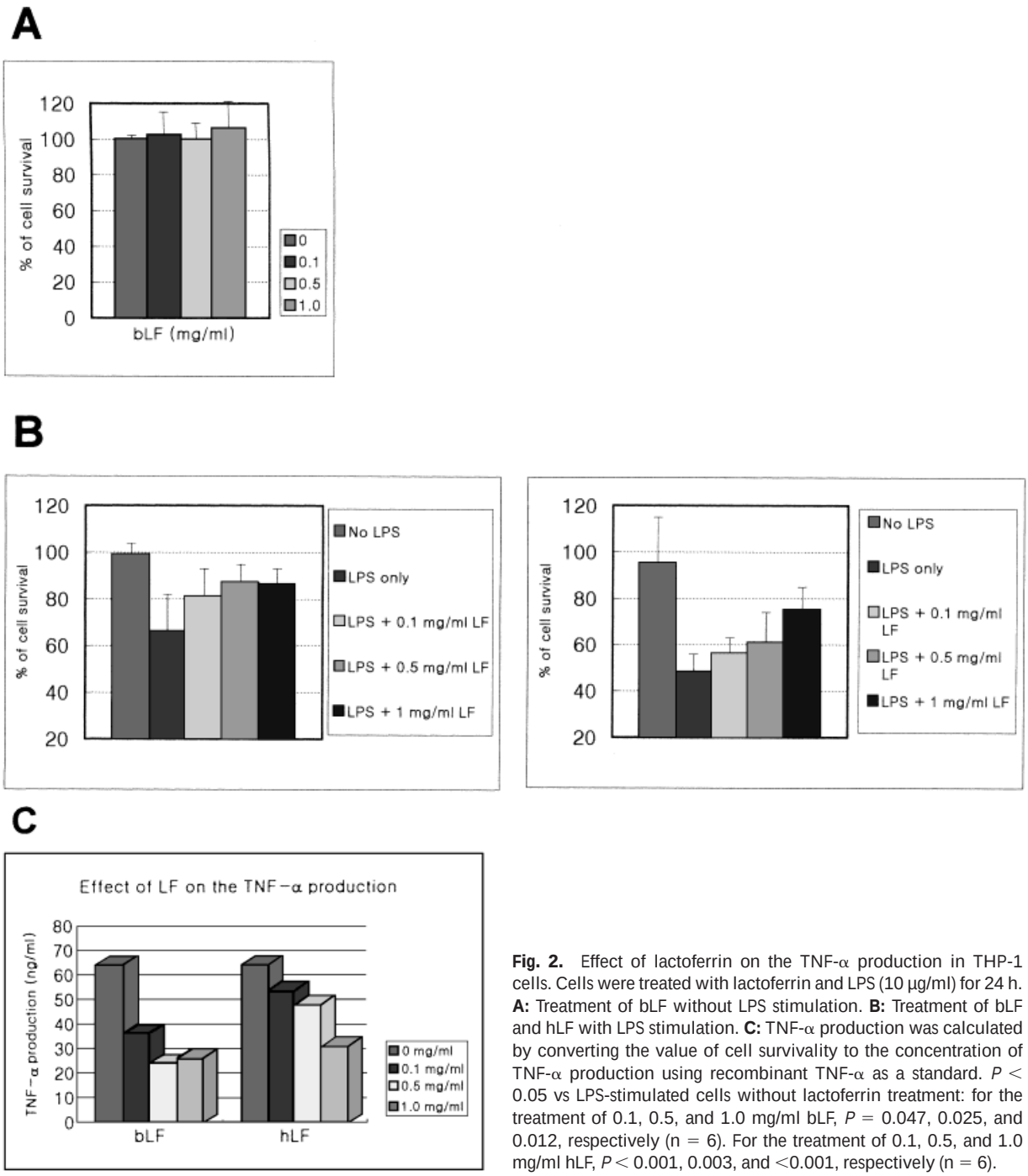

Fig. 2. Effect of lactoferrin on the TNF-α production in THP-1 cells. Cells were treated with lactoferrin and LPS (10 μg/ml) for 24 h. **A:** Treatment of bLF without LPS stimulation. **B:** Treatment of bLF and hLF with LPS stimulation. **C:** TNF-α production was calculated by converting the value of cell survivality to the concentration of TNF-α production using recombinant TNF-α as a standard. $P < 0.05$ vs LPS-stimulated cells without lactoferrin treatment: for the treatment of 0.1, 0.5, and 1.0 mg/ml bLF, $P = 0.047$, 0.025, and 0.012, respectively ($n = 6$). For the treatment of 0.1, 0.5, and 1.0 mg/ml hLF, $P < 0.001$, 0.003, and <0.001, respectively ($n = 6$).

any significant role of lactoferrin in the sensitivity of L929 cells to recombinant TNF-α.

Effect of Lactoferrin on Production of Nitric Oxide

As shown in Figure 3A, treatment of Raw264.7 cells with bovine lactoferrin fails to have a stimulatory effect on production of NO. Cells were stimulated to produce NO by IFN-γ and LPS, and the effect of lactoferrin on the stimulated macrophages was tested (Fig. 3B). Both bovine and human lactoferrin did not significantly change the measured nitrite concentration of the stimulated macrophages.

We have also tested the effect of lactoferrin in aortic VSMCs, which produce NO by IFN-γ and TNF-α stimulations. In VSMCs, lactoferrin itself does not have any stimulatory effect on the NO production as in Raw264.7 cells (Fig. 4A). Stimulated VSMCs can produce NO, but lacto-

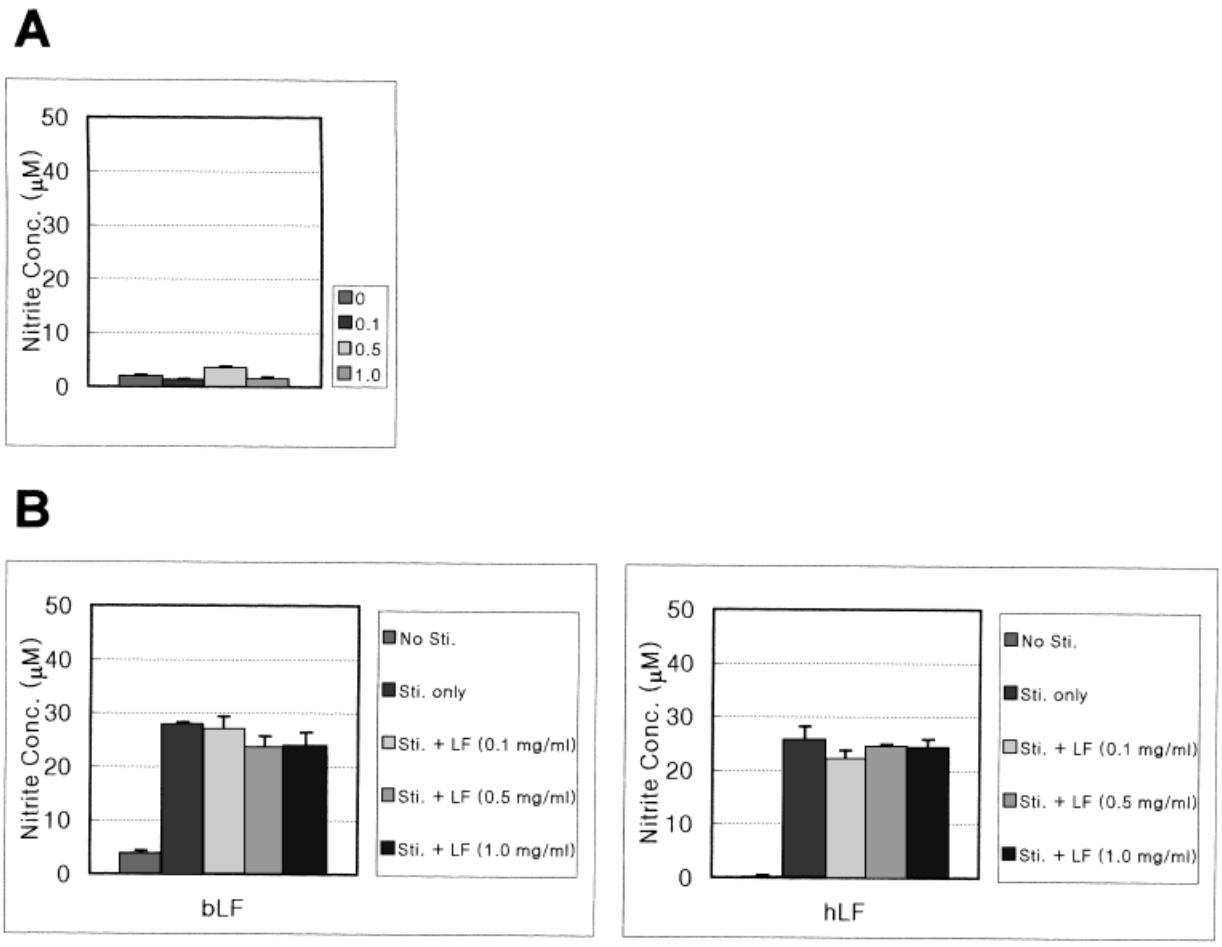

Fig. 3. Effect of lactoferrin on production of NO in Raw264.7 cells. The concentration of nitrite was calculated with the standard values using known concentrations of $NaNO_2$. **A:** Bovine lactoferrin treatment only. **B:** Cells were stimulated with 1 μg/ml of LPS and 50 U/ml of IFN-γ in serum-free DMEM for 24 h. Indicated amount of lactoferrin was added to the medium with stimulants and the supernatant was tested for NO production.

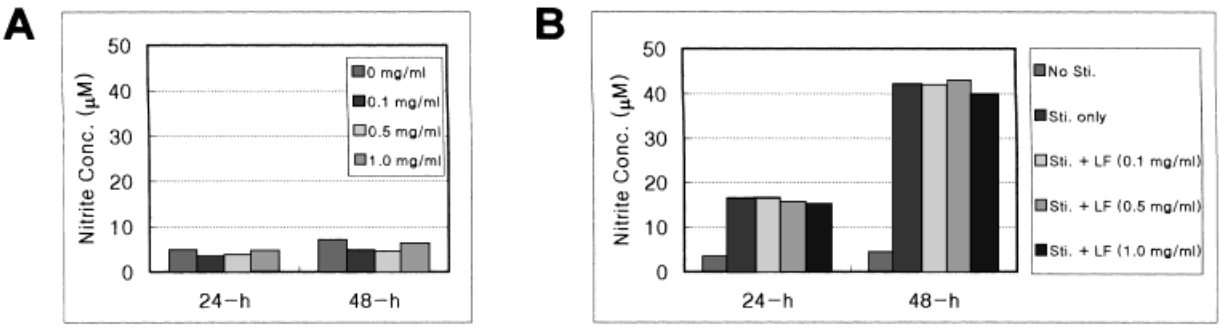

Fig. 4. Effect of lactoferrin on production of NO in VSMCs. Cells were grown confluent and treated with bovine lactoferrin for 24 h and 48 h. **A:** Bovine lactoferrin treatment only. **B:** Cells were stimulated with 10 U/ml of TNF-α and with 50 U/ml of IFN-γ in serum-free DMEM/F12. Bovine lactoferrin was added to the medium with stimulants; the supernatant was tested for production of NO after the indicated time of treatment.

ferrin does not change the production of NO measured by nitrite concentration (Fig. 4B).

DISCUSSION

We investigated the role of lactoferrin in the modulation of the TNF-α production of macrophage and monocytic cells, which is related to the proinflammatory process and the stimulation of T lymphocytes. Because of its role in inflammatory disease, such as the rheumatoid arthritis and allergic inflammation [Firestein, 1994; Manogue et al., 1992], a significant effort

has been focused on the control of TNF-α in several regulatory steps of its production and functional activation [Eigler et al., 1997; Probert and Selmaj, 1997]. The inhibitory role of lactoferrin in TNF-α production by stimulated macrophages is significant both in murine macrophage Raw264.7 cells and in human monocyte THP-1 cells. Although both bovine and human lactoferrins inhibit TNF-α production, human lactoferrin represents less inhibitory effect than bovine lactoferrin in the concentration of 0.1 and 0.5 mg/ml in THP-1 cells. Bovine and human lactoferrins are structurally similar and share 69% homology in their overall amino acid sequences [Metz-Boutigue et al., 1984]. They are highly homologous in functional domains, which are known to be important in their antibacterial functions. It is difficult to determine whether there is a difference in their function in inhibiting TNF-α production between bovine and human lactoferrins without identifying the functional domains of their role for TNF-α production and their working mechanisms.

The mechanism whereby lactoferrin inhibitis the production of TNF-α upon LPS stimulation is unclear. Evidence indicates that lactoferrin modulates the gene expression by sequence-specific binding to DNA [He and Furmanski, 1995; Penco et al., 1995], but the target factors are not well established. It has been demonstrated that the transcription factor NF-κB modulates the transcriptional activity of the TNF-α gene [Udalova et al., 1998] and that lactoferrin may be one of the regulating factors that can affect the expression of TNF-α gene through the direct interference with the transcription of the TNF-α gene or modulate the expression or activation of the NF-κB. If lactoferrin itself acts as a transcriptional factor that binds to the promoter region of the specific gene, it must first be established that lactoferrin can be transported to the nucleus from the outside of the cell. Several researchers have reported on the lactoferrin-specific receptor to which lactoferrin binds, as well as the translocation of the intact lactoferrin into nucleus [Garre et al., 1992; Hu et al., 1990; Roiron et al., 1989; Mazurier et al., 1989]. Once it can be translocated into the nucleus, it binds easily to the DNA [Ravazzollo et al., 1988].

Stimulated macrophages are known to secret NO, as well as cytokines such as TNF-α, interleukin-1β and -6 (IL-1β and IL-6), granulocyte colony-stimulating factor (G-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF) [Stuehr and Marletta, 1985, 1987]. There is no effect with lactoferrin on NO production, showing that the inhibitory role of lactoferrin does not interfere the other functions of stimulated macrophages, at least NO production. This finding leads us to conclude that lactoferrin function does not occur not through the interference of the macrophage activation, but possibly through the TNF-α-specific regulatory mechanism such as transcriptional regulation of TNF-α. Lactoferrin exerts no effect on the NO production in VSMCs, which are stimulated by IFN-γ and TNF-α. We also conclude that lactoferrin does not interfere the function of IFN-γ and TNF-α for induction of NO synthesis.

The production of other cytokines secreted by stimulated macrophages and monocytic cells, IL-1β, IL-2, IL-6, and GM-CSF, is reported to be affected by lactoferrin [Zimecki et al., 1998; Penco et al., 1995; Machnicki et al., 1993; Crouch et al., 1992]. Cytokine production tends to decrease secretion upon treatment with lactoferrin. This finding suggests that lactoferrin can act to prevent the overall recruitment of cytokines at the sites of their actions.

We are currently studying the role of lactoferrin as a transcriptional regulator of several genes, including TNF-α. As there is no evidence that lactoferrin has any cytotoxic effect of the cells, it will be useful to adapt lactoferrin to the therapeutic uses in the TNF-α-related pathogenic diseases, such as rheumatoid arthritis or inflammation.

REFERENCES

Baynes RD, Bezwoda WR. 1994. Lactoferrin and the inflammatory response. Adv Exp Med Biol 357:133–141.

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 54:2310–2312.

Birgens HS, Kristensen LO, Borregaard N, Karle H, Hansen NE. 1988. Lactoferrin mediated transfer of iron to intracellular ferritin in human monocytes. Eur J Haematol 41:52–57.

Crouch SP, Slater KJ, Fletcher J. 1992. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. Blood 80:235–240.

Eigler A, Sinha B, Hartmann G, Endres S. 1997. Taming TNF: strategies to restrain this proinflammatory cytokine. Immunol Today 18:487–492.

Ellison RT III, Giehl TJ, LaForce FM. 1988. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immunol 56:2774–2781.

Firestein GS. 1994. Rheumatoid synovitis and pannus. In: Klippel J, Dieppe P, editors. Rheumatology. St. Louis: CV Mosby. p 3.12.1–3.12.30.

Garre C, Bianchi-Scarra G, Sirito M, Musso M, Ravazzolo R. 1992. Lactoferrin binding sites and nuclear localization in K562(s) cells. J Cell Physiol 153:477–482.

He J, Furmanski P. 1995. Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. Nature 373:721–724.

Hu WL, Mazurier J, Montreuil J, Spik G. 1990. Isolation and partial characterization of a lactoferrin receptor from mouse intestinal brush border. Biochemistry 29:535–541.

Kim DK, Zhang L, Dzau VJ, Pratt RE. 1994. H19, a developmentally regulated gene, is reexpressed in rat vascular smooth muscle cells after injury. J Clin Invest 93:0355–360.

Machnicki M, Zimecki M, Zagulski T. 1993. Lactoferrin regulates the release of tumor necrosis factor alpha and interleukin 6 in vivo. Int J Exp Pathol 74:433–439.

Manogue KR, Van Denventer JH, Cerami A. 1992. A tumor necrosis factor-α or cachectin. In: Thomson A, editor. The cytokine handbook. San Diego: Academic Press. p 241.

Mazurier J, Legrand D, Hu WL, Montreuil J, Spik G. 1989. Expression of human lactoferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes. Eur J Biochem 179:481–487.

Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, Jolles P. 1984. Human lactoferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem 145:659–676.

Nuijens JH, Berkel PHC, Schanbacher FL. 1996. Structure and biological actions of lactoferrin. J Mammary Gland Biol Neoplasia 1:285–295.

Penco S, Pastorino S, Bianchi-Scarra G, Garre C. 1995. Lactoferrin down-modulate the activity of the granulocyte macrophage colony-stimulating factor promoter in interleukin-1β-stimulated cells. J Biol Chem 270:12263–12268.

Probert L, Selmaj K. 1997. TNF and related molecules: trends in neuroscience and clinical applications. J Neuroimmunol 72:113–117.

Ravazzolo R, Garre C, Bianchi-scarra G, Barresi R, Fiorentini P. 1988. Nuclear DNA binding proteins in hemopoietic differentiating cells. In: Bissel M, Deho G, Sironi G, Torriani A, editors. Gene expression and regulation: the legacy of Luigi Gorini. Amsterdam: Excerpta Medica/Elsevier Science. p 291–298.

Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. 1991. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J Immunol Methods 142:257–265.

Roiron D, Amouric M, Marvaldi J, Figarella C. 1989. Lactoferrin-binding sites at the surface of HT29-D4 cells—comparison with transferrin. Eur J Biochem 186:367–373.

Stuehr DJ, Marletta MA. 1985. Mammalian nitrite biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proc Natl Acad Sci USA 82:7738.

Stuehr DJ, Marletta MA. 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. J Immunol 139:518.

Udalova IA, Knight JC, Vidal V, Nedospasov SA, Kwiatkowski D. 1998. Complex NF-κB interactions at the distal tumor necrosis factor promoter region in human monocytes. J Biol Chem 273:21178–21186.

Van Snick JL, Masson PL, Heremans JF. 1974. The involvement of lactoferrin in the hyposideremia of acute inflammation. J Exp Med 1:1068–1084.

Zimecki M, Wlaszczyk A, Zagulski T, Kubler A. 1998. Lactoferrin lowers serum interleukin 6 and tumor necrosis factor alpha levels in mice subjected to surgery. Arch Immunol Ther Exp 46:97–104.