# Role of endogenous interferon and LPS in the immunomodulatory effects of bovine lactoferrin in murine peritoneal macrophages

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**Abstract: Lactoferrin (Lf) plays an important role in host defense against infection and excessive inflammation. Although the mechanisms underlying its immunomodulatory properties have not been fully elucidated yet, recent evidence suggests that some of these effects may be related to its capacity to form complexes with LPS. We report that the culture of resting mouse peritoneal macrophages (PM) with bovine Lf (bLf), prior to infection with the vesicular stomatitis virus (VSV), resulted in a significant reduction of virus yield with respect to control cultures. The antiviral activity of bLF was related to its capacity of inducing IFN-α/β expression, which in turn inhibited VSV replication. Indeed, the accumulation of IFN-β but not of IFN$\alpha_{1\text{-}2}$ transcripts was up-modulated markedly early after bLf addition. Furthermore, bLf did not exert any antiviral activity in the presence of neutralizing antibodies to IFN-α/β in PM from wild-type mice, as well as in PM from mice genetically defective for the response to IFN. The antiviral activity of bLf relied on its intrinsic capacity to bind LPS, as this protein did not induce IFN expression in PM from LPS-hyporesponsive mice. It is interesting that this LPS-binding property was dispensable for the production of TNF-α, which also occurred in LPS-hyporesponsive mice. Overall, these results indicate that some of the immunomodulatory effects ascribed to Lf may be related to its capacity to favor Type I IFN expression and argue in favor of an important role of the LPS-binding feature and TLR4 in some of the effects ascribed to this molecule. *J. Leukoc. Biol.* 82: 347–353; 2007.**

***Key Words:*** *cytokines · vesicular stomatitis virus*

## INTRODUCTION

Lactoferrin (Lf) is an 80-kD, iron-binding glycoprotein, abundantly found in exocrine secretions of mammals, in particular, in milk and fluids of the digestive tract and released by mucosal epithelia and neutrophils during inflammation [1]. It is now well recognized that Lf plays a direct, antimicrobial role in secretions and at the surface of epithelia, by limiting the proliferation and adhesion of microbes and/or by killing them. These properties are related mainly to its ability to sequester iron in biological fluids or to destabilize the membranes of microrganisms [2]. Iron-independent, microbicidal activities, requiring direct interaction between Lf and microbial surface components, have been demonstrated subsequently [1, 2]. In addition to the antimicrobial properties of Lf, its ability to modulate the overall immune response and to protect against viral infections and septic shock has been described in numerous in vitro and in vivo studies [1, 2]. Although the cellular and molecular mechanisms accounting for the immunomodulatory effects of Lf are far from being fully elucidated, it is now clear that at least some of its biological activities do not depend merely on its capacity to bind iron but may arise from its interaction with a variety of molecules [1]. In particular, Lf contains a strongly basic region close to the N terminus, which binds anionic, biological molecules, including lipid A of bacterial LPS [3]. Lf binds to lipid A with high affinity, and this LPS-binding property of Lf has been suggested recently to play an important role in the immunomodulatory activity of this molecule [4].

Macrophages are considered important elements in natural resistance against infection [5]. During viral infection, macrophages are among the first cells in any organs to be exposed to the intruders and are generally considered to be the major producers of Type I IFN soon after infection [5]. Among several viruses, murine macrophages are infected by the vesicular stomatitis virus (VSV), an arthropod-borne, enveloped virus carrying a single-strand RNA genome with negative polarity, which replicates entirely in the cytoplasm of target cells [6]. This virus gains access into the cell through the binding of its surface glycoprotein to phosphatidylserine, a near-universal component of cell surface membranes, enabling VSV to infect virtually any animal cell [6]. However, several animal viruses, including VSV, do not multiply in macrophages when these cells are first placed in culture, suggesting that macrophages

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may limit viral dissemination in vivo by restricting viral multiplication [7]. These cells are related intimately to the IFN system, and the existence of a so-called "IFN-macrophage alliance" has been postulated [5]. Several studies performed by our group over the years have revealed that low levels of Type I IFN, mainly IFN-β, are expressed spontaneously in resting mouse peritoneal macrophages (PM) and are responsible for the natural, antiviral state of these cells [7, 8]. The IFN-β-mediated, antiviral state of freshly harvested PM is progressively lost when these cells are maintained in vitro for a few days, and it is associated with the decay of IFN-β mRNA expression [9].

Although first characterized on the basis of their potent, antiviral functions, IFN-α/β also mediates a variety of immunomodulatory effects [10]. These immune-modulating functions provide strong evidence for an important role of the host response to viruses, which are affected profoundly by Type I IFN. Early in viral infection, IFN interferes with viral replication and activates NK cells. Later in the adaptive immune response, these cytokines up-regulate MHC Class I expression and promote cross-priming, thus favoring the killing of virally infected cells by cytotoxic lymphocytes [11, 12].

In the present study, we report that bovine Lf (bLf) stimulates Type I IFN expression in resting mouse PM and that the IFN produced is in turn responsible for the restriction of VSV replication. Our results indicate that the capacity of bLf to induce Type I IFN expression is linked intimately to its capacity to bind LPS, as bLf does not induce IFN-α/β expression in PM harvested from LPS-hyporesponsive mice, thus suggesting a dependency of Lf-LPS signals on TLR4 for the induction of IFN-α/β, but not of TNF-α, expression. Overall, these results indicate that some of the immunomodulatory effects ascribed to Lf may be related to its capacity to favor Type I IFN expression and argue in favor of an important role of the LPS-binding feature and TLR4 in some of the effects ascribed to this molecule.

## MATERIALS AND METHODS

### Mice

Male, wild-type, C3H/HeN and 129sv mice were purchased from Harlan (Horst, The Netherlands), whereas LPS-hyporesponsive C3H/HeJ were obtained from Charles River, Italia S.p.A. (Milan, Italy). Breeding pairs of IFN-α/β receptor$^{-/-}$ (IFN-α/βR$^{-/-}$) mice (Strain 129sv) were obtained through the courtesy of Dr. Ion Gresser (Institute de Recherches Scientifiques sur le Cancer, Villejuif, France). A colony was bred and maintained at the Istituto Superiore di Sanità (Rome, Italy). Mice were kept under pathogen-free (C3H/HeN, C3H/HeJ, and 129sv) or germ-free conditions (IFN-α/βR$^{-/-}$). All mice were 5–7 weeks old and were used within 2 weeks from their arrival.

### PM cultures

Total peritoneal cells were harvested by washing the peritoneal cavity of mice with cold PBS (BioWhittaker, Walkersville, MD, USA, 5 ml per mouse). Total peritoneal cells were centrifuged and suspended in RPMI-1640 medium containing 10% heat-inactivated FBS supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml; complete medium). Cells were cultured in 24 ($1\times10^6$ cells/well in 500 μl)- or 48-well, tissue-culture plates ($5\times10^5$ cells/well in 250 μl; Costar, Cambridge, MA, USA) at 37°C in 5% $CO_2$-humidified air. After 1 h, nonadherent cells were removed by three washings with medium without serum, and then complete medium was added. Experiments were undertaken when the cells were firmly adherent to the culture wells after vigorous washing. Over 95% of the cells stained for nonspecific esterase and were stained positively with PE-conjugated antibody against F4/80, as described previously [7, 13].

### Reagents

All culture reagents were purchased from Hyclone (Logan, UT, USA) as endotoxin-free lots and assessed further by the Limulus amebocyte assay (LAL; BioWhittaker). LPS from *Escherichia coli* (Serotype 026:B6) was purchased from Sigma-Aldrich (Italy). In some experiments, polymyxin B (PMB; purchased from Sigma-Aldrich) was used to inhibit LPS activity. In these experiments, PMB was incubated with bLf or LPS at the final concentration of 50 μg/ml for 15 min at 37°C prior to addition to the cell monolayer. Polyclonal antibodies against IFN-α/β (neutralizing titer $6.4\times10^6$ against 4 U mouse IFN-α/β) as well as normal sheep globulins, obtained as described elsewhere [14], were used at the final dilution of 1:1000.

Highly purified bLf was kindly provided by Morinaga Milk Industries Co., Ltd. (Tokyo, Japan). bLf was checked for purity by SDS-PAGE and found to be contaminant-free, with a single band displayed at 80 kDa upon staining with silver nitrate. Furthermore, bLf was found to contain a little amount of LPS, i.e., 0.7 EU/mg protein, corresponding to 100 pg/ml free LPS, by conventional LAL (Pyrochrome kit, PBI International, Italy). However, at this LPS concentration, the VSV replication in PM as well as their capacity to produce TNF-α were not affected (data not shown). The iron saturation of bLf was ~25%, as determined by optical spectroscopy. Before biological assays, bLf preparations were sterilized by filtration using 0.2 μ Millex HV at low protein retention (Millipore Corp., Bedford, MA, USA).

### Assay for induction of antiviral state in macrophages

Adherent macrophages were cultured for 3 days and then treated with bLf, LPS, or left untreated for 24 h. At Day 4, 0.2 ml of a viral dilution of VSV, corresponding to a multiplicity of infection (MOI) of 0.2 pfu/cell, was added to each well. After 1 h of incubation at 37°C in 5% $CO_2$-humidified air, the cell monolayer was washed thoroughly, and 1 ml complete medium was added. Eighteen hours later, the cell-free supernatants were frozen at −80°C and stored until virus titration.

### VSV titration

The origin, methods of preparation, and assay of VSV (Indiana strain) in mouse L929 cells have been described previously [7].

### Real-time RT-PCR

Total RNA isolated with the Qiagen RNeasy mini kit (Hilden, Germany) was treated with DNase I (Qiagen) and retrotranscribed into cDNA by using polydT. Real-time RT-PCR was performed on an ABI-Prism 7700 PCR cycler (Applied Biosystems, Foster City, CA, USA). PCR was performed by using the Quantitect SYBR Green PCR kit according to the manufacturer's instructions (Qiagen), and thermal cycler conditions were as follows: 1 × 15 min at 95°C, 40 cycles of denaturation (15 s at 94°C), and combined annealing/extension (30 s at 55°C for GAPDH and 62°C for IFN-$\alpha_{1\text{-}2}$ and -β). The sequences of GAPDH and IFN primers have been described previously [15, 16]. Relative quantification was performed by using the comparative threshold cycle (Ct) method [17]. Arithmetic formulas were used to calculate relative expression levels and compared with a calibrator (in our conditions, untreated, 3-day-cultured PM). The amount of target, normalized to the endogenous housekeeping gene (GAPDH) and relative to the calibrator (Day 3 PM), is then given by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ (target) $- \Delta Ct$ (calibrator), and $\Delta Ct$ is the Ct of the target gene subtracted from the Ct of the housekeeping gene. The equation thus represents the normalized expression of the target gene in the unknown sample, relative to the normalized expression of the calibrator sample.

### Detection of secreted TNF-α

To determine secreted TNF-α levels in culture supernatants, a mouse TNF-α ELISA kit (R&D Systems, Milan, Italy) was used. Assays were performed according to the manufacturer's instructions.

## Statistical analysis

Statistical comparison between various groups was performed by the Student's *t*-test for independent samples. Differences were considered significant when $P$ values were $<0.05$.

## RESULTS

### bLf inhibits VSV replication in in vitro, aged PM by postentry mechanisms

We have demonstrated previously that PM are naturally resistant to VSV infection soon after their isolation [7]. However, this antiviral state is lost during the in vitro culture, and it is associated with a progressive decline of Type I IFN expression, mainly IFN-β [9]. In vitro treatment of PM with different biological response modifiers, including LPS, IFN-γ, TNF-α, and M-CSF, restores the IFN-β-mediated, antiviral state of PM [18, 19]. To establish whether bLf exhibits the capacity to interfere with VSV replication in PM, we examined the effect of this molecule by measuring the induction of an antiviral state in virus-permissive PM upon 3 days of in vitro culture. **Figure 1** shows the results of a representative experiment in which PM were treated for 24 h, before VSV infection, with different concentrations of bLf. The addition of bLf at the concentration of 1000 μg/ml (corresponding to 12.5 μM) resulted in an ~100-fold reduction of VSV yield ($P<0.001$), whereas at lower concentration, such as 100 μg/ml, a modest but still significant inhibition of virus replication ($P<0.05$) was observed. At concentrations below 100 μg/ml, bLF did not exert any significant effect on VSV replication.

It has been reported previously that Lf exerts a potent, antiviral activity against several viruses through its binding to host cells and/or viral particles, thus impeding viral entry into target cells [2]. Thus, to evaluate whether the antiviral activity of bLf was related to its capacity to interfere with early steps of VSV infection in PM, we carried out experiments in which bLf was added at different time-points prior to VSV infection. As shown in **Figure 2**, the addition of bLf to PM 30 min prior to their infection with VSV completely failed to protect the cells from infection, whereas a 24-h treatment resulted in the expected protection ($P<0.01$), thus suggesting that the bLf-induced, antiviral state could require the expression of an antiviral factor. As a control, some PM cultures were also treated with LPS, a well-known, potent inducer of an IFN-mediated, antiviral state in PM [19]. As shown in Figure 2, similarly to the effect observed after a short treatment with bLf, 30 min of LPS exposure did not inhibit VSV replication significantly in PM, whereas a 24-h treatment resulted in an almost complete inhibition of viral replication in these cells ($P<0.001$).

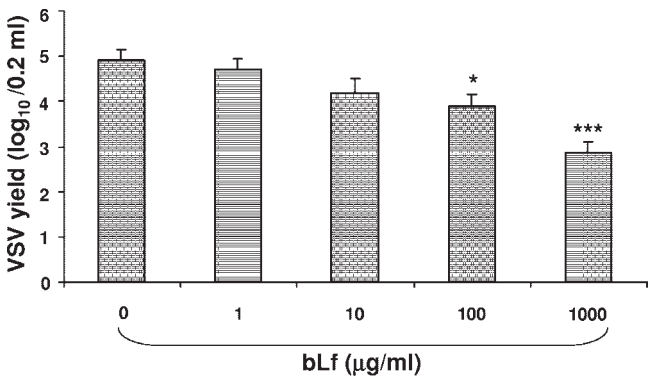


**Fig. 1.** bLf induces an antiviral state to VSV in in vitro-cultured PM. PM were harvested from wild-type C3H/HeN mice and cultured for 3 days to render them permissive to viral replication, and then cells were treated with different concentrations of bLf. Twenty-four hours later, cells were infected with VSV (MOI 0.2 pfu/cell) as described in Materials and Methods. Cell supernatants were collected 18 h after infection, centrifuged, and frozen at −80°C until titration of virus yields on a monolayer of L929 mouse fibroblasts. Results are presented as means ± SE of two experiments performed, run in triplicate. $P$ values were calculated by Student's *t*-test and indicate significant reduction of VSV yield with respect to the untreated cultures (*, $P<0.05$; ***, $P<0.001$).

### The bLf-induced, antiviral state in PM is mediated by Type I IFN

We have reported previously that virus-permissive PM are capable of responding to a variety of stimuli for IFN production, which in turn restores their capacity to restrict viral replication [18, 19]. We thus investigated whether the antiviral activity of bLf in PM was somehow related to Type I IFN expression. As shown in **Figure 3A**, treatment of PM with bLf in the presence of antibodies specific for IFN-α and -β did not result in any significant, bLf-induced, antiviral state ($P>0.05$) with respect to PM cultures treated with bLf alone ($P<0.001$). As expected, antibodies to IFN-α/β also inhibited the LPS-induced, antiviral effect ($P>0.05$) [18, 19]. In contrast, the addition of normal serum globulins did not exert any effect on the antiviral state induced by bLf and LPS. Likewise, the addition of antibodies to IFN-α/β did not exert any effect per se on the extent of VSV replication with respect to control, untreated PM (Fig. 3A). To confirm further that the bLf-induced, antiviral effect was indeed mediated by Type I IFN expression, experiments were carried out in PM harvested from mice genetically defective for IFN-α/β$R^{-/-}$ expression. As shown in Figure 3B, treatment of PM harvested from IFN-α/β$R^{-/-}$ mice with bLf and LPS failed completely to reduce the VSV yield as compared with PM harvested from the corresponding wild-type mice, thus confirming that bLf-mediated induction of the antiviral state was dependent on Type I IFN expression. We have reported previously that the amount of Type I IFN secreted by PM is low and may not be detected in the culture medium by using sensitive cell assay systems, as IFN is removed continuously by binding to cellular receptors [7, 8, 19, 20]. Furthermore, neutralization studies with mAb to mouse IFNs indicated that IFN-β was the major component of peritoneal cell IFN and that IFN-α was a minor component (13–17%) [21]. We therefore evaluated whether bLF exposure of PM resulted in any accumulation of Type I IFN transcripts. The analysis of IFN-$\alpha_{1\text{-}2}$ and -β transcripts by real-time PCR assay is shown in **Figure 4**. As expected, 3 days cultured PM did not express IFN-$\alpha_{1\text{-}2}$ and IFN-β mRNA, as in vitro culture of PM results in a rapid decay of IFN transcript accumulation [9]. However, a marked accumulation of IFN-β mRNA was observed in bLf-treated PM (~20-fold of increase), although to a much lesser extent than in LPS-treated PM (685-fold of

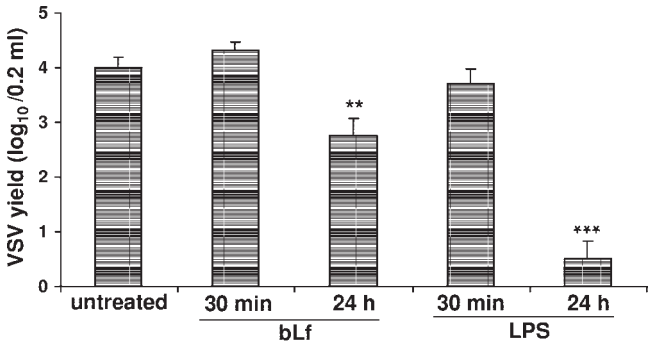


**Fig. 2.** bLf does not interfere with early steps of VSV replication. PM were harvested from C3H/HeN mice and cultured as described in the legend to Figure 1. Three days cultured PM were then treated with bLf (1000 μg/ml) or LPS (100 ng/ml) or were left untreated for 30 min or 24 h prior to VSV infection. At Day 4, cells were infected with VSV (0.2 pfu/cell), and after 18 h, supernatants were collected and processed as described in the legend to Figure 1. Results are shown as means ± SE of two experiments run in triplicate. *P* values indicate significant reduction of VSV yield measured in 24 h-treated cells with respect to the untreated cultures (**, $P<0.01$; ***, $P<0.001$).

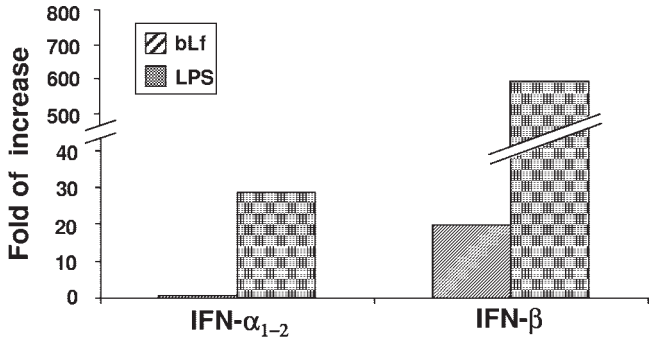


**Fig. 4.** bLf up-modulates IFN-β mRNA expression. Adherent PM from C3H/HeN mice were treated for 4 h with bLf (1000 μg/ml) or LPS (100 ng/ml), and then cells were collected and RNA extracted as described in Materials and Methods. One representative experiment out of three performed is shown. Results, analyzed by the relative quantification method ($2^{-\Delta\Delta Ct}$ method), are presented as fold increase of IFN-$\alpha_{1-2}$ and IFN-β gene expression in LPS- and bLf-treated cells with respect to control, 3-day cultures.

increase), after 4 h of treatment with respect to control, untreated cells. In contrast, IFN-$\alpha_{1-2}$ mRNA accumulation was up-modulated moderately upon LPS treatment (~29-fold of increase), whereas bLf did not exhibit any effect (Fig. 4). In keeping with our previous results, we found secretion of Type I IFN barely detectable in PM cultures treated with bLf (1 U/ml) and only a modest secretion in LPS-treated PM (32 U/ml), despite the marked accumulation of IFN transcripts (Fig. 4).

## The bLf-mediated induction of antiviral state to VSV requires TLR4

Structurally, Lf contains a highly basic region close to the N terminus, which binds to a variety of anionic, biological molecules, including lipid A. Lf binds to lipid A with high affinity [3], and this LPS-binding property of Lf has been suggested recently to be involved in the immunomodulatory functions of this molecule [4]. Furthermore, a recent study showed that bLf activates macrophages via TLR4-dependent and -independent signaling pathways [22]. To address this issue, we therefore

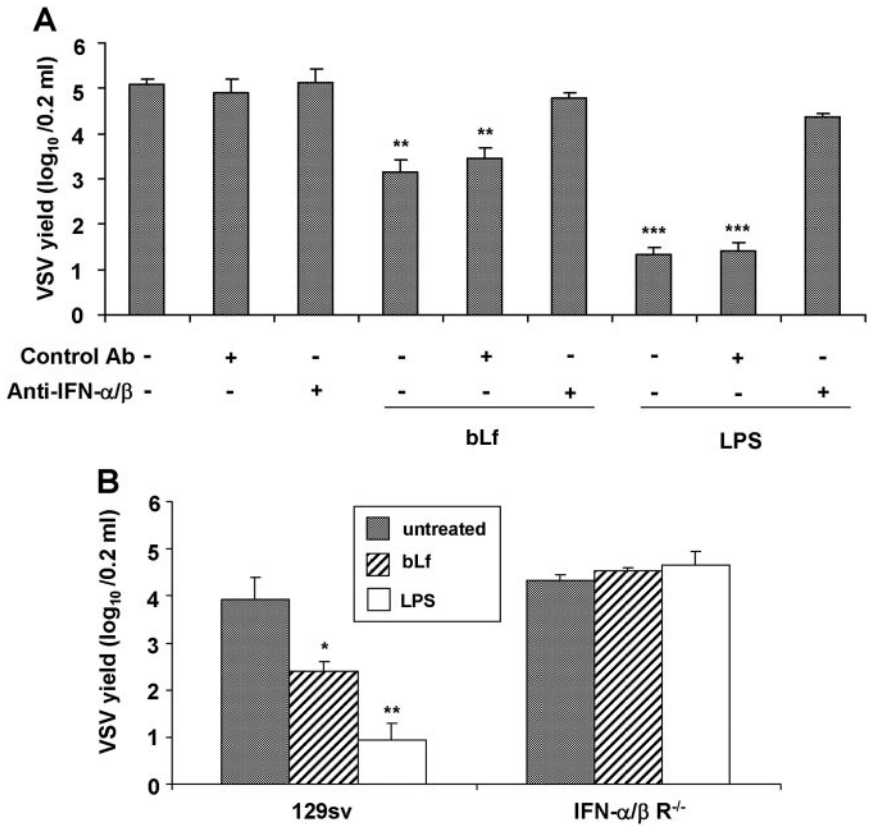


**Fig. 3.** The bLf-induced, antiviral state in PM is mediated by Type I IFN. (A) PM were harvested from C3H/HeN mice and cultured as described in the legend to Figure 1. Three days cultured PM were treated with bLf (1000 μg/ml) or LPS (100 ng/ml) in the presence or in the absence of antibodies (Ab) against IFN-α/β (1:1000 final dilution) or sheep normal serum or were left untreated. At Day 4, cells were infected with VSV, and after 18 h, supernatants were collected and processed as described in the legend to Figure 1. Data are presented as means ± SE of one representative experiment out of two performed, run in triplicate. (B) Three days cultured PM from wild-type 129sv and IFN-α/βR$^{-/-}$ mice were treated with bLf (1000 μg/ml) or LPS (100 ng/ml) for 24 h prior to VSV infection, as described in the legend to Figure 1. Data are presented as means ± SE of two experiments performed, run in triplicate. *P* values indicate significant differences with respect to the untreated control (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

investigated whether bLf was capable to induce an IFN-mediated, antiviral state in PM harvested from C3H/HeJ mice, a mouse strain bearing a mutation in the *Lps* locus, which confers hyporesponsiveness to the immunostimulatory effects of LPS [23]. TLR4 has been characterized as the gene product of *Lps* locus, which is responsible for the lack of response [24]. As shown in **Figure 5**, neither LPS nor bLf induced any antiviral state in PM harvested from C3H/HeJ LPS-hyporesponsive mice as compared with C3H/HeN LPS-responsive, wild-type mice, in which both agents induced a remarkable, antiviral effect ($P<0.001$). To confirm further that the bLf-induced, antiviral state could be a result, at least in part, of its capacity to bind LPS, we performed experiments by using PMB, a well-known, LPS-binding molecule, which binds lipid A moiety and neutralizes LPS activity [25]. As shown in **Figure 6**, the addition of PMB to control PM cultures infected with VSV did not result in any change in the extent of viral replication with respect to control-infected cells. However, when PMB was preincubated with bLf or LPS prior to the addition to PM cultures, the antiviral activity of bLf and LPS was abrogated completely.

## The bLf-mediated induction of TNF-α does not require TLR4 expression

To further investigate the role of TLR4 in the immunomodulatory effects of bLf, we assessed the capacity of this molecule to induce the production of another important immune mediator, such as TNF-α, typically induced by LPS in macrophages [26] and also reported to be induced in these cells by bLf [27]. It is interesting that as shown in **Figure 7A**, bLf stimulation of PM harvested from LPS-hyporesponsive mice resulted in TNF-α secretion ($P<0.001$), although to a reduced extent with respect to PM from wild-type mice ($P<0.001$), whereas LPS was completely ineffective. In addition, bLf preincubation with PMB did not abrogate the capacity of bLf to induce TNF-α secretion ($P<0.05$), whereas it blocked the LPS effect completely on TNF-α secretion (Fig. 7B).

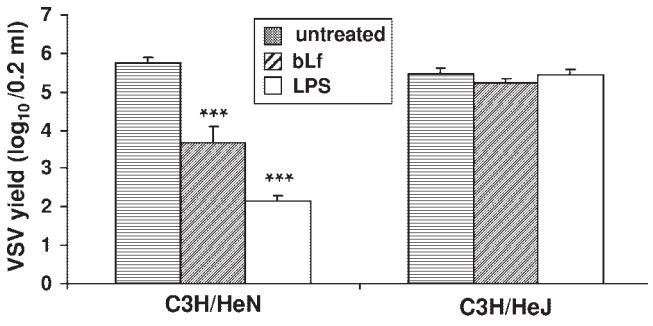


**Fig. 5.** bLf does not induce an antiviral state in PM harvested from LPS-hyporesponsive mice. Three days cultured PM from C3H/HeN (LPS-responsive) and C3H/HeJ (LPS-hyporesponsive) mice were treated with bLf (1000 μg/ml) or LPS (100 ng/ml) for 24 h and then infected with VSV. Virus yields were measured as described in the legend to Figure 1. Results are presented as means ± SE of three experiments run in triplicate. Significance of differences between treated and untreated cells is shown (***, $P<0.001$).

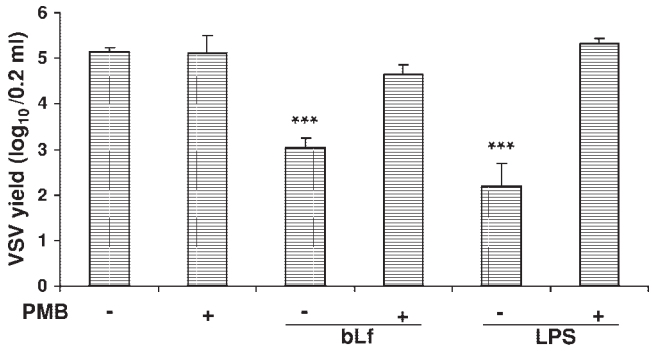


**Fig. 6.** PMB abolish bLf antiviral activity completely. Three days cultured PM from C3H/HeN mice were treated with 1000 μg/ml bLf or with 100 ng/ml LPS. Some cultures were treated with bLf or LPS preincubated with PMB at the final concentration of 50 μg/ml for 15 min at 37°C prior to addition to the cell monolayer. Twenty-four hours later, cells were infected with VSV and virus titers determined on L929 cells. Data are presented as means ± SE of two independent experiments run in triplicate. *P* values were calculated by comparing treated cultures with untreated controls (***, $P<0.001$).

## DISCUSSION

Lf is a key element in the host defense system. This assumption is based mainly on the antimicrobial properties of this molecule, which include iron sequestration, direct lytic activities, and/or impaired binding of microbes to host cells [2]. More recently, however, it has becoming evident that its protective effect also extends to the modulation of the host response to infections. Depending on the immune status of an individual, Lf can have anti-inflammatory properties, which are explained mostly by its capacity to interact with exogenous, proinflammatory molecules, mainly LPS and its CD14 receptor, thus decreasing the immune response and preventing septic shock and damage to tissues. However, Lf can also favor the activation, differentiation, and proliferation of immune cells, and this promoting activity has been related to a direct effect of Lf on immune cells [28]. In this study, we report that bLf induces Type I IFN expression in mouse PM by up-modulating the accumulation of IFN-β but not of IFN-$\alpha_{1\text{-}2}$ transcripts, although to a lesser extent than LPS. Conversely, up-modulation of IFN-$\alpha_{1\text{-}2}$ mRNA is only observed upon LPS treatment. In keeping with our previous results [7, 8, 19, 20], barely detectable levels of Type I IFN are secreted by PM upon bLf treatment. However, the bLf-stimulated, Type I IFN expression is entirely responsible for the reduced VSV replication, as bLf does not exert any antiviral activity in the presence of neutralizing antibodies to IFN-α/β in PM from wild-type mice, as well as in PM from mice genetically defective for the response to IFN. The antiviral activity of Lf has been largely demonstrated for a number of viruses [2]. Several studies indicated that Lf may exert its antiviral effects through inhibition of viral entry rather than stimulation of the immune cells [2]. Our findings clearly indicate that bLf also exerts indirect effects on viral replication by stimulating the production of antiviral factors, such as Type I IFN, which in turn, allows restriction of viral replication. In keeping with our observation, previous studies have suggested that Lf could somehow act on the IFN system by promoting its expression. In this regard, it has been dem-

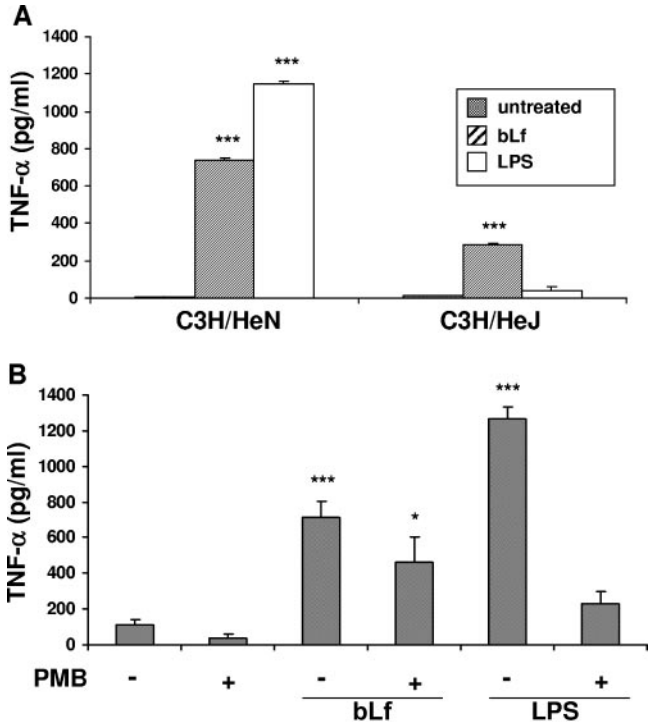


**Fig. 7.** bLf induces TNF-α production in PM from LPS-hyporesponsive mice. (A) PM from wild-type, LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice were treated with 1000 μg/ml bLf or LPS (10 ng/ml). Supernatants were collected 24 h later and TNF-α measured by ELISA. Results are presented as means ± SE of two experiments out of three performed, run in triplicate. (B) PM from C3H/HeN mice were treated with 1000 μg/ml bLf or with 100 ng/ml LPS. Some cultures were treated with bLf or LPS, preincubated with PMB to block LPS activity. Twenty-four hours later, cell supernatants were collected, and the content of TNF-α was measured by ELISA. Results are presented as means ± SE of two experiments run in triplicate. *P* values represent significant differences between treated cultures and controls (*, $P<0.05$; ***, $P<0.001$).

onstrated that oral administration of bLf modulates the expression of immunity-related genes in small intestines of mice. It is interesting that a significant up-modulation of transcripts coding for IFN-β was observed in animals, which received bLf, with respect to control mice [29]. Likewise, oral administration of bLf was reported to increase the expression of IFN-α and IFN-β mRNA in Peyer's patches and mesenteric lymph nodes, as well as the levels of IL-18 protein in the portal circulation [30]. Of note, administration of liposomal Lf also induced a significant up-modulation of IFN-α levels in healthy human volunteers [31]. It is well known that Type I IFN induces NK cell activation by enhancing its cytotoxic activity and IFN-γ production [10]. It is interesting that in studies describing the fact that oral Lf administration stimulates Type I IFN production, an increased NK cell activity was also found [30, 31].

In this study, we also report that the capacity of bLf to induce a Type I IFN-mediated, antiviral state relies on the presence of TLR4. In fact, bLf did not exert any effect in PM harvested from LPS-hyporesponsive mice or following incubation with PMB, a well-known, LPS-binding molecule, which binds lipid A moiety and neutralizes LPS activity [25]. In keeping with our results, it is now well established that the ability of Lf to bind free LPS may account, at least in part, for the anti-inflammatory activities of this protein. In particular, the protective effect of exogenous Lf against endotoxin shock in various animals was reported extensively [32–35], as well as its in vitro capacity to inhibit the LPS-induced IL-8, E-selectin, and ICAM-1 expression in human endothelial cells [36, 37] and reactive oxygen species production in neutrophils [38, 39]. However, Na and co-workers [4] reported recently that when LPS and purified Lf were mixed and formed a complex, production of proinflammatory mediators rather than inhibition of LPS challenge was observed in RAW 264.7 cells and PM harvested from C3H/HeN mice. Comparative studies carried out with LPS-responsive and LPS-hyporesponsive mice demonstrated a strong dependency of the Lf-LPS-triggered signals on TLR4, leading to the conclusion that the immunomodulatory properties of Lf could be a result, at least in part, of LPS binding [4]. Lf is a highly cationic glycoprotein, which binds to the lipid A portion of LPS via a charge-charge interaction. The portion of Lf that binds to anionic molecules, including lipid A, is limited to its N terminus, arginine-rich domain [40]. Thus, it is likely that bound LPS can still expose the unbound part of lipid A, which is recognized by LPS receptors such as TLR4. Such a Lf-LPS recognition would result in macrophage activation [4]. Of note, the lipid A backbone is also the epitope being recognized in the LAL, thus explaining why the Lf-LPS complex is found to be LAL-positive [4, 41]. Collectively, these results suggested that lipid A can be recognized even after the Lf-LPS complex has been formed and that this complex retains the capacity to activate macrophages through TLR4. However, the intimate relationship between Lf and LPS does not completely account for the different biological activities ascribed to this molecule. Our results, showing that the presence of functional TLR4 is not essential for the production of TNF-α, strongly suggest that bLF induces macrophages activation via TLR4-dependent and -independent mechanisms. Accordingly, it has been reported recently that bLf-induced, IL-6 secretion and CD40 expression in PM from BALB/c mice were achieved via TLR4-independent and TLR4-dependent mechanisms, respectively, thus indicating potentially separate pathways for bLf-mediated, macrophage events in innate immunity [22]. Thus, Lf binding to LPS may represent an important aspect, but does not account entirely, for some immunomodulatory effects of this molecule, at least in those cell types such as the macrophage, in which TLR4 function is of critical importance in the regulation of their activity.

Growing evidence is accumulating about the role of Lf as a key host-defense factor. Indeed, its presence in mucosal secretions identifies it as in the front line against attack by a wide variety of pathogens. Our finding that bLf stimulates Type I IFN expression, a cytokine playing an essential function in the antiviral response, as well as in linking innate to adaptive immune responses, extends the role of this molecule far beyond its direct, antimicrobial activity and argues in favor of Lf as an interesting candidate for promising protective and therapeutic strategies against human viral infections.

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