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Regulatory Effects of Lactoferrin and Lipopolysaccharide on LFA-1 Expression on Human Peripheral Blood Mononuclear Cells

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Abstract. The aim of this study was to investigate effects of human lactoferrin (hLF) with regard to LFA-1 expression on unstimulated and lipopolysaccharide (LPS)-activated human peripheral blood mononuclear cells (PBMC). The investigations were carried out on 30 healthy volunteers, males and females, 24–58 years old. We found that hLF, at an optimal dose of 5 μg/ml/10^6 cells in 24-hour culture, exerted regulatory effects on LFA-1 expression, depending on distribution of this molecule on cells in control cultures and on the effects of LPS. First, we revealed several patterns of LFA-1 distribution and density of this marker among studied individuals. The effects of LPS and hLF on LFA-1 expression patterns were differential. LFA-1 expression was stimulated by individual actions of LPS or hLF, additive or synergistic effects of both factors, it could be also inhibited by hLF alone or in combination with LPS. In about one third of cases no significant effects of LPS or hLF on LFA-1 expression were seen. Removal of monocytes from the PBMC population diminished LFA-1 expression in control cultures and abolished LPS- or hLF-elicited changes. The regulatory effects of hLF were also blocked by treatment of PBMC cultures with anti-tumor necrosis factor alpha (TNF-α) antibodies. Taken together, the data showed that hLF and LPS had immunoregulatory properties with respect to LFA-1 expression on human PBMC and that these actions were mediated by monocytes and TNF-α.

Key words: lactoferrin; lipopolysaccharide; LFA-1 (CD11a); peripheral blood mononuclear cells; regulation.

Introduction

Adhesion molecules play a key role both in the induction and in the effector phase of the immune response. Leukocyte function associated antigen (LFA-1, CD11a) and its ligand ICAM-1 were among the first integrins described[21, 29]. They participate in cell-to-cell interaction during antigen presentation to T cells as accessory molecules[18] and play a pivotal role in transendothelial cell migration[39]. In the fraction of human peripheral blood mononuclear cells (PBMC) the distribution of LFA-1 antigen is usually bimodal, the marker is associated with $CD4^+$, $CD8^+$ (two peaks) and NK cells[29]. Expression of LFA-1 may be upregulated by several factors including bacterial products and cytokines[10, 13, 21]. The density of LFA-1 antigen may be elevated[2, 25, 32, 43, 45] or suppressed[17, 30] in disease. It has been suggested that lymphocytes expressing high LFA-1 density may belong to memory[16, 27] or recently activated cells[10, 16, 44]. LFA-1 expression is age-related[27] which may be as-

sociated with accumulation of memory cells. It is also of interest that the environmental rather than ethnic factors determine LFA-1 expression[16].

Expression of LFA-1 is regulated by cytokines elicited by the inflammatory process. Lactoferrin belongs to proteins released into circulation during inflammation from secondary granules of neutrophils. Recently this protein has attracted much attention as a multifunctional regulatory factor, integrally associated with the cytokine network[3, 7, 23]. Specific receptors for LF have been described on several cell types, among them on macrophages[5]. LF may transduce signals analogous to those induced by mitogens[11]. This protein can promote differentiation of T[48] and B lymphocytes[49] and their functions[47-50]. LF has been also known for its antibacterial[38, 46], antiviral[34], antiparazite[37], antifungal[41] and antitumor[4] properties. Our *in vitro* studies on surgical and septic patients revealed that bovine lactoferrin exerted regulatory actions on several immunological parameters[1, 42] depending on initial reactivity of patients cells and serum cytokine levels. The regulatory and protective actions of LF were also demonstrated in other systems[7, 9, 24, 51-53].

It was possible that some of these properties of LF could be attributed to effects of this protein on expression of adhesion molecules. LF has been shown to increase cell adhesiveness[28] and its action could be prevented by a treatment with anti-P-selectin antibodies[20]. Therefore, the aim of our study was to investigate the effects of hLF on expression of a major cell adhesion molecule – LFA-1. Since the release of LF is usually preceded by action of endotoxins which can modify expression of adhesion molecules, we decided to study in parallel the effects of both agents on LFA-1 distribution on human PMBC.

Materials and Methods

Preparation of cells for culture. Venous blood from healthy volunteers was taken to heparinized syringes, diluted 2 times with PBS, applied onto Lymphoprep (Pharmacia) and centrifuged for 20 min at 1100×g. The cells from the interphase PMBC were washed 3 times with Hanks' medium and resuspended at a concentration of 10^6/ml of a culture medium consisting of RPMI 1640, 10% fetal calf serum, 2 mM L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics. The PBMC cell fraction consisted of 80% lymphocytes and 20% monocytes as determined by staining with Giemsa and May-Grünwald reagents.

Removal of monocytes. PBMC were resuspended in the culture medium (5×10^5/ml) in 75 cm^2 cell culture flask (Falcon) and incubated for 2 h in a cell culture incubator. After the incubation the nonadherent cells were gently decanted. The nonadherent cell fraction contained less than 1% of monocytes as jugded by ingestion of latex particles.

Pulsing PBMC with LPS. PBMC, at a concentration of 10^6 cells/ml of the culture medium, were incubated in 50 ml plastic tubes (Falcon) with addition of 10 μg/ml of LPS from *E. coli* serotype 055: B5 (Sigma) for 2 h in a cell culture incubator. After the incubation the cells were washed 3 times with a big volume of Hanks' medium.

Culture of PBMC for FACS studies. PBMC, resuspended in the culture medium, were placed in 24-well culture plates at a density of 10^6/ml/well. Human LF was added at a concentration of 5 μg/ml. Antibodies directed against TNF-α were added in the beginning of the culture at a concentration of 2 μg/ml (Genzyme, monoclonal mouse anti-human TNF-α, IgG1).

Staining the cells and FACS studies. After overnight incubation the cells were harvested by a vigorous pipetting, transferred into FACS tubes, washed 2 times with Hanks' and the pellets were treated with anti-LFA-1 mouse anti-human antibody – final dilution 1:50 (Dako, Denmark). After 1 h incubation in ice the cells were washed 3 times with PBS containing 1% BSA and incubated for 1 h with FITC-conjugated anti-IgG mouse antibodies (Sigma Immunochemicals, St. Louis, USA, sample F8264, dilution 1:80).

Analysis of the cells was performed on a FACScan cytofluorimeter (Becton Dickinson, Mountain, View, CA) with a 15 mW argon ion laser at 488 nm excitation. Live gating of the forward and side scatter channels was used to exclude debris and to acquire selectively events for lymphocytes. Data were recorded on a logarithmic scale and 5000 particles of each gated population were analyzed.

Results

Stimulatory effect of LPS on LFA-1 expression on peripheral human mononuclear cells

Investigation of 30 blood samples from healthy donors (males and females, 22–58 years old) revealed that LFA-1 expression on PBMC varied considerably among individuals. The patterns of LFA-1 expression were usually bimodal although one- or three-peak LFA-1 distributions were also observed. We have found that depending on LFA-1 density on PBMC, in untreated, control cultures, the actions of LPS and hLF were differential. Below we describe all registered examples of

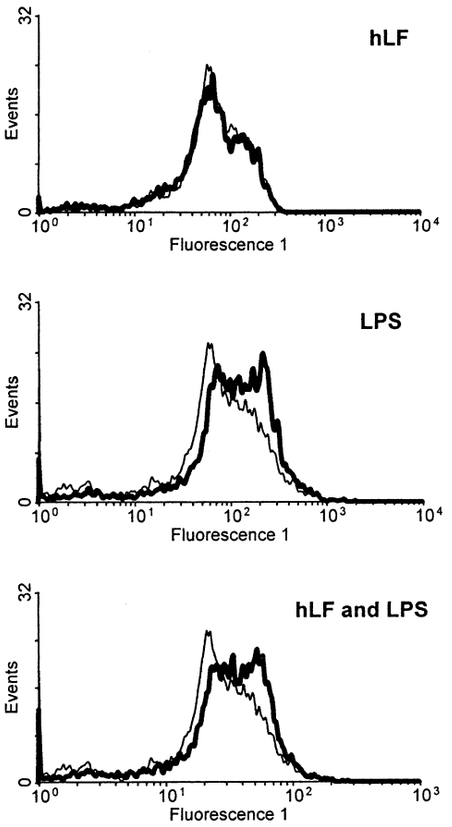

Fig. 1. Stimulatory effect of LPS on LFA-1 expression on PBMC: no regulatory effects of hLF. All procedures and details regarding culture conditions and treatment of cells for Fig. 1–6 are described in the Materials and Methods. Mean log fluorescence: control – 63.26, hLF – 59.49, LPS – 95.13, hLF+LPS – 91.61

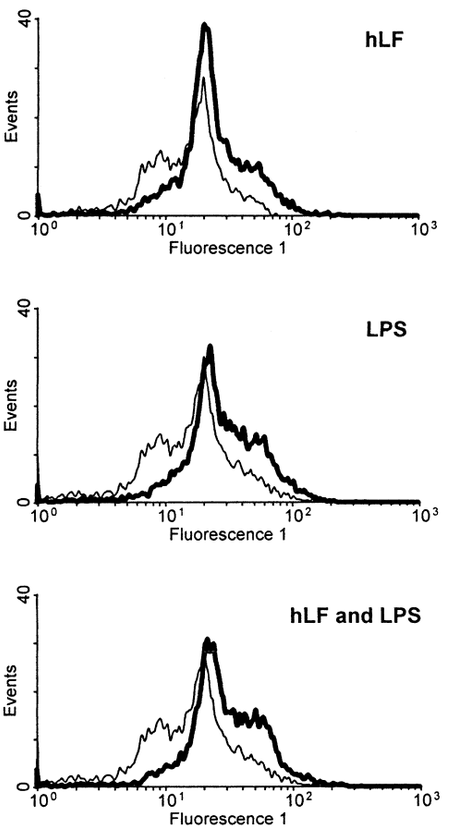

Fig. 2. Stimulatory effects of hLF and LPS on LFA-1 expression on PBMC: an additive action. Mean log fluorescence: control – 34.77, hLF – 59.63, LPS – 67.91, hLF+LPS – 72.63

regulatory effects of LPS and hLF. Figure 1 illustrates a case, where the expression of LFA-1 was stimulated only by addition of LPS. There was no further effect of hLF on the expression of the studied marker. We observed at least 2 cases clearly showing the upregulatory effects of LPS only. These examples represented a moderate LFA-1 expression.

Additive and synergistic stimulatory action of hLF and LPS on expression of LFA-1 on PBMC

Figure 2 depicts a representative case showing independent, stimulatory action of LPS and HLF. These were examples of low-to-moderate LFA-1 expression. A synergistic effect of LPS and hLF is presented in Fig. 3. Such effects were most frequent among the studied individuals (5 cases). They were characterized by a lack or small regulatory effects in the presence of LPS or hLF alone, both agents however, exerted a distinct, stimulatory action resulting in appearance of an additional peak. In the case shown in Fig. 3 LPS was without any effect but hLF was slightly stimulatory. Both agents, however, exhibited a strong stimulatory effect. Figure 4 shows synergistic inhibitory action of LPS and hLF on LFA-1 expression. In this case two distinct peaks of LFA-1 expression (high and low density) were reduced to one peak of low-level LFA-1 expression.

Downregulatory effects of LF and LPS on LFA-1 expression on PBMC

LF alone also exerted some inhibitory action of LFA-1 distribution, both agents, however, demonstrated a very strong downregulatory effect. Downregulatory effect of LF, and to a lesser degree of LPS, is also shown in Fig. 5. It was also of interest, that in about one third of studied cases no or a very minor effect of LPS or hLF on LFA-1 expression were observed.

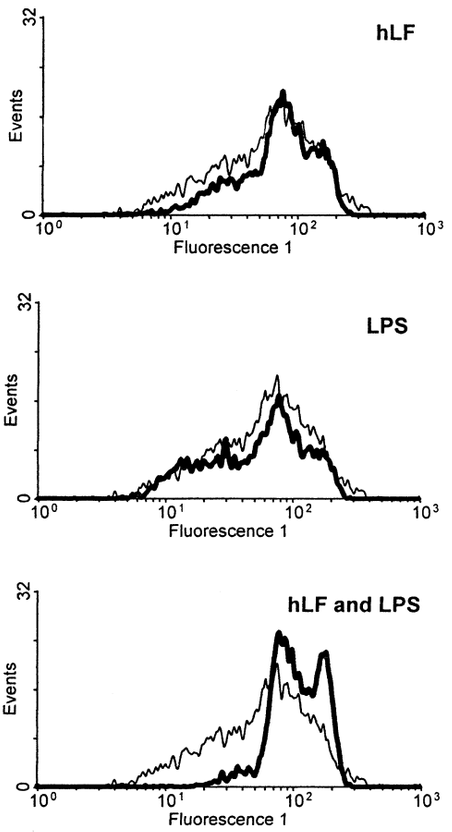

Fig. 3. Synergistic, stimulatory effect of hLF and LPS on LFA-1 expression on PBMC. Mean log fluorescence: control – 170.16, hLF – 275.41, LPS – 193.69, hLF+LPS – 494.37

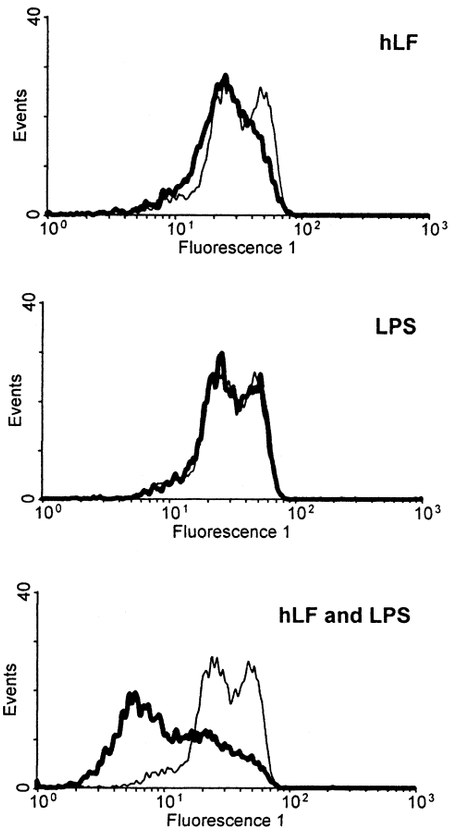

Fig. 4. Synergistic inhibitory action of hLF and LPS on LFA-1 expression on PBMC. Mean log fluorescence: control – 89.52, hLF – 68.85, LPS – 87.76, hLF+LPS – 25.11

Effects of monocyte removal and block of TNF-α activity on the regulation of LFA-1 expression

The regulatory effects of lactoferrin are associated with the cytokine network and activity of immunocompetent cells. Therefore, we decided to find out whether removal of monocytes – major cytokine producers and target cells for LPS action – will abolish LPS- and hLF--induced changes in LFA-1 expression. Monocytes were separated from other mononuclear cells as described in the Materials and Methods. Removal of monocytes (Fig. 6B) resulted in a change of LFA-1 distribution – only a low-density cell fraction was observed. The addition of LPS and hLF to unseparated cell population caused inhibition of LFA-1 expression (disappearance of the second peak) (Fig. 6A). Removal of monocytes caused that the above mentioned LPS and hLF-induced changes disappeared (Fig. 6C).

The inclusion of anti-TNF-α antibodies to PBMC cultures also resulted in abolishment of hLF-elicited changes in LFA-1 distribution. These kinds of experiments were repeated 4 times with similar results and a representative experiment is shown in Fig. 7. In this experiment a synergistic, stimulatory effect of LPS and hLF was blocked by anti-TNF-α antibody.

Discussion

In this report we revealed several interesting phenomena associated with expression of adhesion molecule LFA-1 on human PBMC and demonstrated that expression of that molecule may be regulated independently or in combination by LPS and human lactoferrin in many ways, depending on LFA-1 distribution on PBMC in a given individual or day of experiment. The regulatory actions of LPS and hLF were mediated by monocytes and their product – TNF-α. In a substantial part of cases LFA-1 expression was not affected by LPS or hLF.

Our first observation was that LFA-1 may be expressed on 1, 2 or even 3 cell fractions. In fact expression of LFA-1 is usually biomodal, where the first, low density peak corresponds to $CD4^+$ and one subfraction

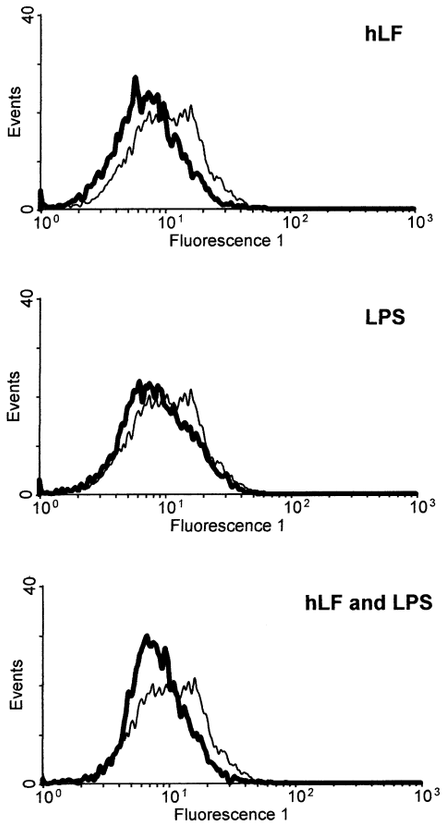

Fig. 5. Downregulatory effects of hLF and LPS on LFA-1 expression on PBMC. Mean log fluorescence: control – 21.40, hLF – 13.66, LPS – 17.31, hLF+LPS – 16.34

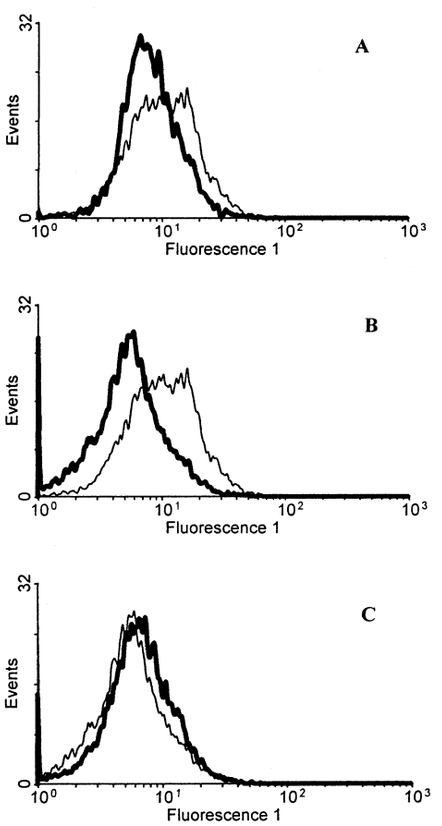

Fig. 6. Effect of monocyte removal on hLF-induced changes in LFA-1 expression on PBMC. A – effect of LPS+hLF on LFA-1 expression in unseparated cell population (mean log fluorescence: control – 21.40, hLF and LPS – 16.34); B – effect of monocyte removal on LFA-1 expression (mean log fluorescence: control – 21.40, monocyte removal – 9.38); C – abolishment of LPS+hLF induced changes in LFA-1 expression after monocyte depletion (mean log fluorescence: monocyte removal – 9.38, LPS+hLF after monocyte depletion – 12.05)

of $CD8^+$ cells, whereas the second one comprises a second subfraction of $CD8^+$ cells and natural killer cells[29]. The differences in LFA-1 density between these two peaks could be as a high as 5-fold[29]. In our studies we have not differentiated cells for respective subpopulations of lymphocytes but were rather interested in studying the overall effect of LPS and/or hLF actions on LFA-1 expression (downregulation/upregulation).

It has been suggested that cell fractions of high LFA-1 density exhibit effector functions such as cytotoxicity, they may also belong to memory cells[16, 27] and show increased adherence to endothelial cells[40]. The feature of increased adherence to endothelial cells and subsequent ability to infiltration into adjacent tissues may be most relevant in our case when we investigate activities of bacterial products and lactoferrin which both are involved in inflammation[3, 12]. Our studies supported the observation that LFA-1 density may be age--dependent and higher LFA-1 expression may derive from accumulation of memory cells resulting from previous immunizations with pathogens[27]. Majority of lymphocyte samples taken from young volunteers (22–26 years) expressed low LFA-levels, usually lacking or expressing small peak of “bright” cels. Lymphocytes from such individuals were, however, very susceptible to the actions of LPS and LF which resulted in appearance of a second-high density marker cell subset. Older individuals expressed higher levels of LFA-1 distributed in one peak and lacked “dull” LFA-1 fraction. They were also usually refractory to the regulatory actions of LF and LPS. However, there were two instances, where one young (22 years) and one older (50 years) individual expressed diametrically different LFA-1 distribution patterns in the time interval of one week without any apparent reason (for example infection). When tested within one weak interval they showed one peak of low density or bimodal (dull and bright peaks) LFA-1 expression.

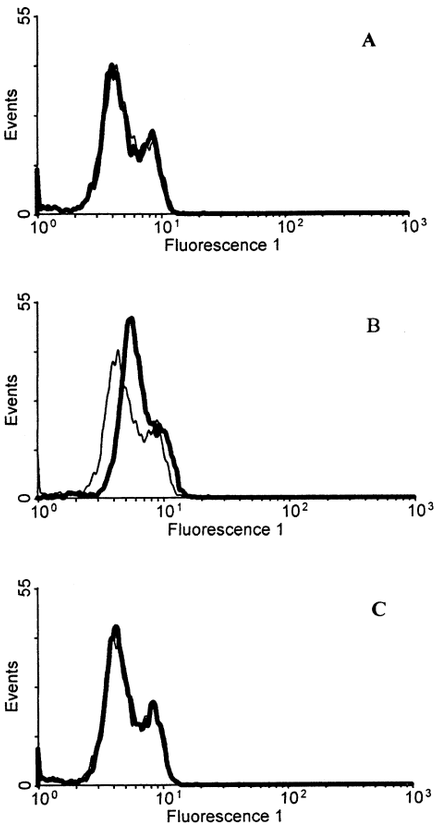

Fig. 7. Effect anti-TNF-α treatment on hLF and LPS-induced changes in LFA-1 expression on PBMC. A – no effect of anti-TNF-α antibodies on LFA-1 expression (mean log fluorescence: control – 8.34); B – LPS+hLF synergistic, stimulatory effect on LFA-1 expression (mean log fluorescence: control – 8.34, LPS+hLF – 11.6); C – as "B" but anti-TNF-α antibodies added (mean log fluorescence: anti-TNF-α antibodies – 8.12, LPS+hLF+anti-TNF-α antibodies – 8.45)

The regulatory effects of LPS and hLF on LFA-1 distribution were differential. The system used in our study allowed for elimination of endogenously released lactoferrin by neutrophils (neutrophil-depleted PBMC). In this way we could observe individual, additive or synergistic effects of both LFA-1 regulating factors. These regulatory actions, mediated by TNF-α, appeared to be logical, leading to optimalization of LFA-1 expression. In general, low LFA-1 expression was stimulated, either separately by LPS or hLF, or in a synergy, optimal (moderate) densities were not significantly affected (usually when represented by one predominant peak) and bimodal but high-density LFA-1 fractions were inhibited either by hLF alone or in combination with LPS. It is possible that in the latter case the fraction exhibiting high adherent properties is affected by LPS and hLF which consequently may lead to diminution of the inflammatory process. hLF effects were probably not associated with competition of LPS binding to monocytes[12, 22] since similar results were obtained with LPS-pulsed monocytes and when LPS was present all the time in cell culture. Our study revealed that the phenomenon of regulation of LFA-1 expression was abrogated following blocking TNF-α or removing monocytes. Although there have been conflicting reports describing effects of TNF-α on LFA-1 levels[14, 26, 39, 40] there is a general agreement that an interdependence exists between activation or blocking LFA-1 molecule and cytokine levels, including TNF-α[8, 33, 35]. Moreover, the process of adhesion is stimulated by TNF-α[6]. Some authors suggest that TNF-α may, in fact, upregulate LFA-1 expression[19]. Here, however, we report for the first time that LFA-1 expression may be regulated by LPS and LF depending on its constitutive density (distribution in a given individual/day) and that this modulation is mediated by TNF-α produced by monocytes. Our finding, revealing that LF may regulate LFA-1 expression *per se*, is probably associated with its ability to induce TNF-α in monocytes[1, 42] and macrophages[36].

The regulatory nature of lactoferrin was described in several reports[9, 24, 38, 49, 50] and reviews[7, 23]. It could depend on iron saturation[15], the types of immune response[50] and magnitude of the immune response[42]. It was also dependent on immune reactivity of surgery patients[42] and category of septic patients[1]. Our recent studies on healthy volunteers indicated that LF was regulatory, when taken per os, in optimalization of some immune parameters[52]. Except of studying the effects of LPS and hLF on LFA-1 expression we were also interested in looking for potential effects of these agents on ICAM-1 expression since ICAM-1 is a ligand for LFA-1[21]. We have not found any substantial influence of LPS and hLF on expression of that marker. However, we revealed a synergistic inhibitory effect of both agents on ICAM-1 expression on an endothelial cell line ECV-1 (data not shown). Such a finding suggests that lactoferrin may also affect ICAM-1 levels on endothelial cells, thus modulating LFA-/ICAM-1 interactions and leukocyte passage through endothelium.

Taken together, the regulatory effects of lactoferrin on expression of adhesion molecule LFA-1 are consistant with immunoregulatory nature of LF in the inflammatory processes. This report also revealed high variability in LFA-1 distribution patterns among healthy individuals.

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