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Lactoferrin prevents LPS-induced decrease of the iron exporter ferroportin in human monocytes/macrophages

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Abstract Iron balance is tightly linked to inflammation and it has been demonstrated that many proteins involved in cellular iron management are up- or down-regulated by inflammatory stimuli, ultimately leading to iron retention in the reticuloendothelial system. Ferroportin is a key player in maintenance of correct iron homeostasis, because it is the only known mammalian cellular iron exporter. In this work we show that incubation of THP-1 monocytes/macrophages with lactoferrin prevents the LPS-induced decrease of ferroportin by reducing secretion of IL-6.

Keywords Ferroportin · Lactoferrin · Iron · Inflammation · Interleukin-6

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Introduction

The importance of iron for all eukaryotes, and particularly for humans, is widely recognized. Iron is essential for transport, storage and activation of oxygen, for electron transfer and many other metabolic processes. It is not surprising that defects affecting iron metabolism genes cause severe pathologies in man. Iron homeostasis is also tightly linked to inflammation and to response to pathogens; macrophages are specialized cells that play a key role in both these processes (Cairo et al. 2011). Iron retention in the reticulo-endothelial system is viewed as the main response of body iron homeostasis to inflammation and it is regarded as a host's attempt to withhold iron from the invading pathogens. On the other hand, if not properly controlled, this situation can lead to the anemia of inflammation. As a matter of fact, it has been shown that the expression of many proteins involved in cellular iron management by macrophages varies in response to inflammatory stimuli or in the presence of pathogens.

Ferroportin (SLC40A1, initially also named MTP1 or IREG1) is the only iron exporter identified in mammalian cells, with a key role in maintenance of correct iron balance in the body. Ferroportin was initially localized on the basolateral membrane in cells of the duodenal epithelium and in the uterus of pregnant women, but it is now established that it is generally present in all those tissues that deliver iron in the plasma, including macrophages where it plays a prominent role in iron egress (Ward and Kaplan 2012

and refs therein). Ferroxidases such as the blue multicopper oxidase ceruloplasmin collaborate with ferroportin for efficient export of iron from cells (Musci et al. 2014), facilitating incorporation of ferrous iron transported through ferroportin as ferric iron in an extracellular acceptor. Two isoforms of ceruloplasmin are found in mammals: secreted ceruloplasmin is mainly synthesized by hepatocytes and released into the plasma, while a GPI-anchored form produced by alternative splicing has been identified in numerous tissues, including macrophages and immune cells (Musci et al. 2014 and refs therein). Ceruloplasmin has been recognized to be an acute phase protein many years ago and it is induced in response to pro-inflammatory stimuli, such as IL-1β (Barber and Cousins 1988; Persichini et al. 2010) and IFN-γ (Mazumder et al. 1997).

Ferroportin expression is regulated at multiple levels and by multiple factors. In particular, ferroportin is down-regulated at the transcriptional level by pro-inflammatory cytokines in reticuloendothelial cells, as demonstrated by the finding that treatment with IFN-γ and LPS reduced ferroportin mRNA and iron release from monocytes (Ludwiczek et al. 2003; Yang et al. 2002). Ferroportin mRNA and protein was also found to decrease significantly in astrocytes treated with LPS but not with IL-6 or TNF-α (Urrutia et al. 2013). Interestingly, we have found that in rat C6 glioma cells ceruloplasmin and ferroportin are both up-regulated by IL-1β, suggesting that the response of ferroportin to cytokines might be tissue-specific (Bonaccorsi di Patti et al. 2004; Persichini et al. 2010).

The amount of ferroportin at the plasma membrane is post-translationally regulated by binding of the peptide hepcidin, which induces internalization and lysosomal degradation of ferroportin, leading to decreased export of iron from cells (Nemeth et al. 2004). In turn, hepcidin synthesis is up-regulated in response to iron loading, LPS and inflammation and it is suppressed by anemia and hypoxia (Ganz 2013). Thus, increased hepcidin levels in response to inflammation result in decreased iron export and macrophage iron retention. Hepcidin is synthesized mainly in the liver; however, it has been demonstrated that in human monocytes and in THP-1 cells LPS and IL-6 induce hepcidin mRNA which in turn causes degradation of ferroportin in an autocrine fashion, leading to intracellular iron retention and contributing to the anemia of inflammation (Theurl et al. 2008).

Lack of ferroxidase activity in specific cell types (including macrophages) also leads to degradation of ferroportin (De Domenico et al. 2007), demonstrating that multiple mechanisms act to control iron balance mediated by the transport activity of ferroportin.

Lactoferrin, a glycoprotein able to reversibly chelate two Fe^{3+} per molecule with high affinity, ensures that the proper 10^{-18} M free iron concentration in human fluids is maintained, thus avoiding iron precipitation, reactive oxygen species formation and microbial colonization (Legrand et al. 2008). Lactoferrin is a multi-functional protein whose activities are both dependent and independent of its iron-binding ability. Lactoferrin is synthesized by exocrine glands and neutrophils in infection and inflammation sites and it is endowed with a potent immuno-modulating and anti-inflammatory activity, which contributes to protect mucosa from infections and inflammation (Legrand et al. 2005; Puddu et al. 2009). Although the mechanisms underlying these properties have not been fully elucidated, evidences indicate that lactoferrin acts both at the cellular level by modulating migration and cell activation, and, at the molecular level, by affecting expression of cytokines, chemokines and other effector molecules, thus contributing to the regulation of inflammation (Puddu et al. 2012).

In this work we have investigated the role of lactoferrin on the expression of ferroportin in THP-1 cells challenged with LPS, a condition that mimics exposure to pathogens or inflammation. Our results indicate that bovine lactoferrin can counteract the decrease of ferroportin protein induced by LPS. We show that this effect is related to reduction of IL-6 secretion by THP-1 cells.

Materials and methods

Materials

Highly purified bovine milk derivative lactoferrin (bLf) was kindly provided by Morinaga Milk Industries Co., Ltd. (Tokyo, Japan). LPS contamination of bLf, estimated by Limulus Amebocyte assay (LAL Pyrochrome kit, PBI International), was equal to 0.5 ± 0.06 ng/mg of bLf. The bLf iron saturation was about 22 %. Before biological assays bLf was sterilized by filtration (Millipore).

Cell culture and treatments

THP-1 human acute monocytic leukemia cells (ECACC, European Collection of Cell Cultures) were maintained in RPMI 1640 medium (Euroclone) supplemented with 10 % fetal calf serum, 100 μM penicillin–streptomycin and 2 mM glutamine in an atmosphere of 95 % air and 5 % CO_2. Undifferentiated cells which grow spontaneously in loose suspension under these conditions were subcultured every 3 day by gentle shaking followed by pelleting and reseeding at a density of approximately 10^6 cells per ml. THP-1 cells were differentiated by incubation with 0.16 μM phorbol myristate acetate (PMA, Sigma) for 48 h at 37 °C (Scorneaux et al. 1996). Monocytes and differentiated THP-1 cells were stimulated with 1 μg/ml LPS from *E. coli* (InvivoGen) for 24 and 48 h; 100 μg/ml bLf was added to monocytes or adherent THP-1 cells without removing the culture medium after 3 and 24 h of stimulation with LPS and/or 2 μg/ml hepcidin-25 (Bachem). Afterwards, the cell were recovered and the supernatants were harvested and stored at −80 °C for quantification of cytokines; the pellets were suspended in PBS containing PMSF 1 mM, centrifuged at 2,500×*g* for 5 min and stored at −80 °C for evaluation of ferroportin expression by western blot.

IL-6 analysis

IL-6 concentration in culture media was measured by ELISA using Human IL-6 ELISA Max Deluxe Set (BioLegend).

Western blot analysis

THP-1 cells (5×10^6–1×10^7 cells) were lysed in 300 μl MOPS 25 mM pH 7.4/NaCl 150 mM/Triton 1 % containing PMSF 1 mM, leupeptin and pepstatin 2 μM in ice for 1 h. Total protein content of samples was measured by the Bradford assay. For SDS-PAGE 20 μg of total protein were loaded per lane, for ferroportin analysis SDS sample buffer containing DTT was added and samples were loaded without heat treatment. For western blot analysis, primary antibodies used were: monoclonal anti-ferroportin 31A5 (Amgen, described in Ross et al. 2012) (1:10,000) and monoclonal anti-actin (BD) (1:10,000). After incubation with the appropriate secondary HRP-conjugated antibody, blots were developed with ECL Prime (GE Healthcare). Ferroportin levels were normalized on actin by densitometry analysis, performed with ImageJ.

Ferritin analysis

Intracellular ferritin content was measured by ELISA (Human Ferritin Kit, Abnova), according to the manufacturer's instructions.

Results

To assess the effect of lactoferrin on LPS-induced inflammation in human monocytes we stimulated undifferentiated THP-1 cells with three different concentrations of *E. coli* LPS (0.01, 0.1 and 1 μg/ml) in the absence and presence of bLf at 100 μg/ml for 24 h. As shown in Fig. 1, bLf was able to reduce IL-6 production at higher LPS concentrations, the effect being evident already on cells treated with 0.1 μg/ml LPS, with a reduction of about 15 %, and more notable at 1 μg/ml LPS with a ca. 30 % reduction of IL-6 production. Therefore, the latter concentration of LPS was chosen for all further experiments.

Effect of lactoferrin on IL-6 and ferroportin expression in undifferentiated THP-1 cells

To investigate the effect of lactoferrin on LPS-induced inflammation and iron homeostasis, undifferentiated THP-1 cells were stimulated with 1 μg/ml LPS in the

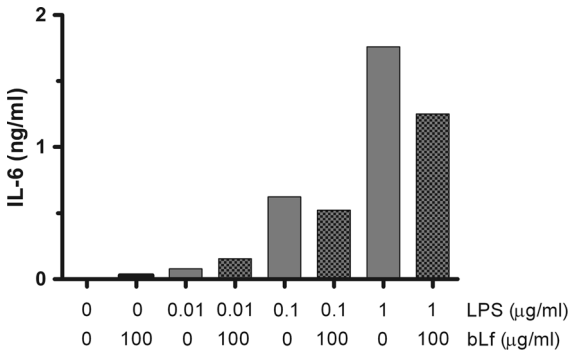

Fig. 1 IL-6 production by monocytic THP-1 cells. Cells were stimulated for 24 h with three different concentration of *E. coli* LPS (0.01, 0.1 and 1 μg/ml), in the presence or absence of 100 μg/ml bLf (added after 3 h of LPS treatment). IL-6 levels were measured by ELISA on culture media

presence and absence of 100 μg/ml bLf. Production of IL-6 and expression of the iron exporter ferroportin was assessed. Lactoferrin was added after 3 h of incubation with LPS, a second addition of bLf was carried out after 24 h to ensure that adequate levels of the iron protein were maintained in the culture medium. We performed the experiment in the presence or absence of hepcidin to further evaluate the role of this peptide in the modulation of ferroportin levels.

The results were significantly reproducible for IL-6, showing the expected large increase of the interleukin levels upon stimulation with LPS and a partial, yet significant decrease in the presence of lactoferrin, as reported in Fig. 1. On the other hand, results were highly variable when ferroportin was considered. Figure 2 reports IL-6 and ferroportin levels measured in two independent experiments. As can be seen, the reproducible increase of IL-6 levels, and the counteracting effect of bLf, could not be related to reproducible variations of ferroportin levels, which were usually not affected (or even increased) by LPS (Fig. 2a), although in some isolated experiments (like the one in Fig. 2b) the protein levels of the iron exporter were severely impaired upon LPS stimulation. In this latter case, however, lactoferrin exerted a rescuing effect, with ferroportin levels going back to normal in its presence.

Hepcidin was found to not affect the LPS-induced IL-6 increase. Treatment with hepcidin, on the other hand, induced a marked reduction in ferroportin protein of about 40 % in all conditions, indicating that, as expected, the immunoreactive band for ferroportin is hepcidin-sensitive (data not shown).

We also measured intracellular ferritin expression by ELISA. In line with the results obtained on ferroportin, ferritin was generally not influenced by LPS and bLf, nor by the presence of hepcidin, as representatively shown in Fig. 2c. Regarding this latter point, it is likely that, in our experimental conditions, the hepcidin-induced decrease of ferroportin was not sufficient to increase intracellular iron levels enough to stimulate ferritin synthesis.

Effect of lactoferrin on IL-6 and ferroportin expression in differentiated THP-1 cells

To investigate the role of differentiation in the response of THP-1 cells to inflammation, cells were

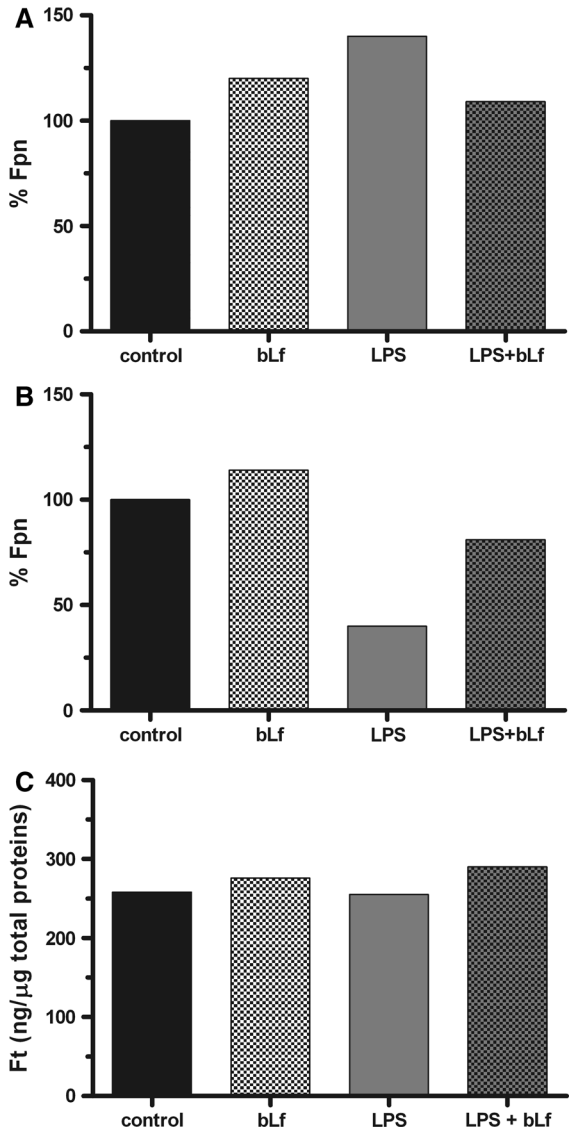

Fig. 2 Expression of ferroportin and ferritin by monocytic THP-1 cells. THP-1 cells were treated for 48 h with 1 μg/ml *E. coli* LPS in the presence or absence of 100 μg/ml bLf (added after 3 and 24 h of LPS treatment). **a, b** Western blot densitometry analysis of ferroportin levels of two different experiments. **c** intracellular ferritin levels measured by ELISA

treated with PMA and differentiated in adherent mature macrophages. First, we analysed whether ferroportin levels were affected by differentiation. To this end, cells were differentiated with PMA for 48 h and then further incubated for 24 or 48 h in the absence or presence of lactoferrin. As shown in Fig. 3a, ferroportin increased ca. twofold after PMA-induced differentiation. Lactoferrin further affected

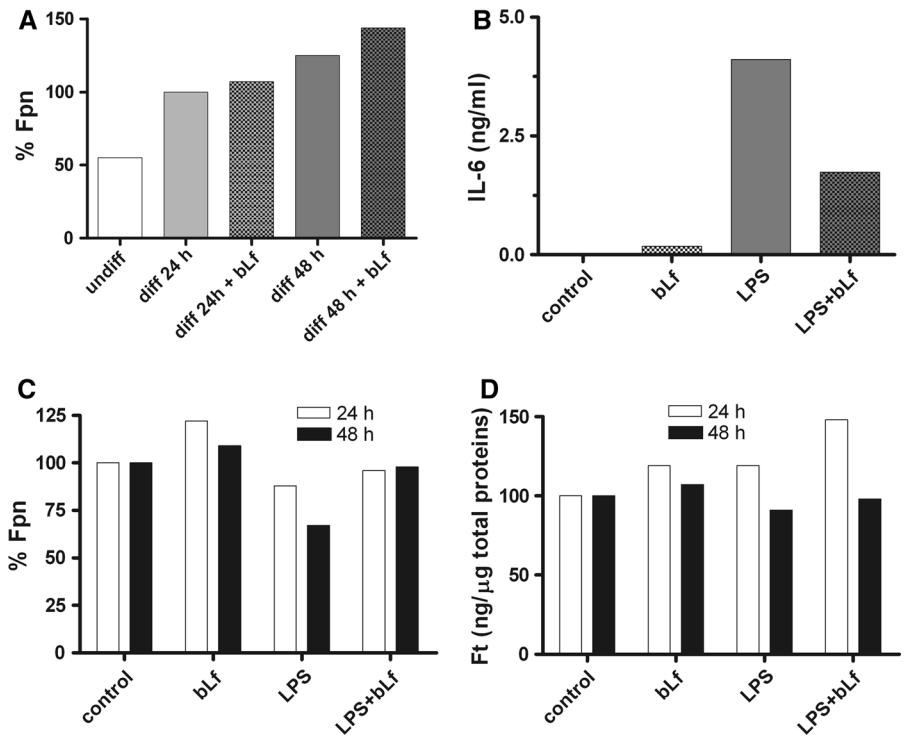

Fig. 3 Expression of IL-6, ferroportin and ferritin by adherent macrophagic THP-1 cells. THP-1 cells were stimulated for 48 h with 0.16 μM PMA until differentiation and adhesion. Adherent cells were treated for 24 and 48 h with 1 μg/ml *E. coli* LPS in the presence or absence of 100 μg/ml bLf (added after 3 and 24 h of LPS treatment). **a** Western blot densitometry analysis of ferroportin on adherent cells compared to undifferentiated THP-1 cells. Cells were differentiated with PMA for 48 h and then further incubated for 24 or 48 h in the absence or presence of lactoferrin. **b** IL-6 production by adherent THP-1 cells after 48 h. **c** Western blot densitometry analysis of ferroportin on adherent THP-1 cells. **d** intracellular ferritin levels measured by ELISA

ferroportin levels, the effect being already evident at 24 h and more evident at 48 h of incubation with a ca. 20 % increase (Fig. 3a). This finding may be related to the iron carrier properties of lactoferrin, as iron released from the protein might lead to induction of ferroportin synthesis to cope with the increased metal load within the cell.

We then tested the response of THP-1 mature cells to LPS in the absence and presence of lactoferrin in terms of IL-6 production and ferroportin expression. At variance with the parallel experiments carried out on undifferentiated cells (see Fig. 2), results were quite reproducible on differentiated macrophages. Again, we show here representative experiments. As reported in Fig. 3b, the ELISA run on secreted IL-6 indicated that differentiated macrophages respond to the LPS treatment with a ca. twofold higher production of the cytokine compared to undifferentiated monocytes. Lactoferrin treatment significantly reduced LPS-triggered IL-6 secretion by about 60 %, indicating a much higher efficiency in comparison to that exerted on monocytes.

Quite interestingly, densitometric analysis of the Western blot on ferroportin showed that the protein levels were significantly reduced by LPS treatment and that bLf significantly reversed this effect, bringing back ferroportin expression close to that of untreated cells (Fig. 3c). The effect was already evident after 24 h of treatment and clearly observable after 48 h, when the protein expression levels in LPS-treated cells were about 30 % lower than those of control cells, and lactoferrin fully restored ferroportin levels.

Ferritin ELISA analysis again did not show significant differences between samples (Fig. 3d). However, as observed for ferroportin, ferritin expression levels were significantly different from those of undifferentiated cells. In particular, ferritin was about twofold lower in mature macrophages that in undifferentiated

THP-1 cells (cf. Figs. 2c, 3d). This result is in line with the higher iron export ability of these cells, i.e. with their higher ferroportin expression.

Discussion

Macrophages play a key role in iron homeostasis, through their ability to scavenge senescent and damaged erythrocytes, recycling iron recovered from hemoglobin. Iron and immunity are tightly linked and many studies have addressed the relationships between iron management by macrophages, the ability of these cells to cope with invading pathogens and inflammation, and the effect of different molecules that can modulate the expression of proteins involved in iron homeostasis in macrophages. Leukemic cell lines such as THP-1 promonocytes are widely used as a model for human monocytes. The transition of normal state circulating monocytes to adherent cytokine-producing inflammatory cells is a step of critical importance in the development of the response to pathogens or inflammation. Biomolecules that can modulate this transition can be viewed as potential therapeutic agents for control of these processes. Lactoferrin has been shown to possess potent antibacterial and anti-inflammatory properties. Previous studies showed that lactoferrin added before treatment with LPS blunted the IL-6 response of THP-1 cells primed with IFN-γ, possibly by interfering with NF-κB signaling (Haversen et al. 2002). Here we show that a similar effect of lactoferrin is obtained in THP-1 cells primed with PMA before stimulation with LPS. Exposure of the cells to a priming agent potentiates the response to subsequent stimuli, and in line with this observation we see a much more effective action of lactoferrin on cells treated with PMA than on undifferentiated cells.

It is known that ferroportin expression is regulated at multiple levels and by multiple factors. In particular, previous studies have shown that ferroportin is down-regulated at the transcriptional level by pro-inflammatory cytokines in reticuloendothelial cells, as demonstrated by the finding that treatment with IFN-γ and LPS reduced ferroportin mRNA and iron release from monocytes (Ludwiczek et al. 2003; Yang et al. 2002). Ferroportin mRNA and protein was also found to decrease significantly in astrocytes treated with LPS but not with IL-6 or TNF-α (Urrutia et al. 2013). Our data are consistent with these findings, moreover we could demonstrate that downregulation of ferroportin can be reverted by the iron protein lactoferrrin playing an anti-inflammatory role. Interestingly, we have also found that in rat C6 glioma cells ceruloplasmin and ferroportin are both up-regulated by IL-1β (Bonaccorsi di Patti et al. 2004; Persichini et al. 2010), suggesting that the response of ferroportin to cytokines might be tissue-specific.

Stimulation with LPS and IL-6 production have been shown to result in increased expression of hepcidin mRNA in human monocytes and THP-1 cells. In turn, this leads to increased hepcidin-dependent degradation of ferroportin and consequently to iron retention in these cells (Theurl et al. 2008). We therefore propose that lactoferrin prevents the decrease of ferroportin levels in THP-1 cells stimulated with LPS because it reduces the LPS- and/or IL-6-dependent induction of hepcidin.

Of note, the expression of ferroportin appeared to be higher in adherent PMA-treated THP-1 cells compared to undifferentiated monocytic cells. Most of the proteins involved in cellular iron homeostasis appear to be differentially expressed in polarized macrophages. Different groups have reported that in vitro polarized murine and human M1 inflammatory macrophages present a ‘storage’ phenotype while M2 tolerogenic macrophages have effective iron-recycling capacity (Corna et al. 2010; Recalcati et al. 2010). In fact, M1 (IFN-γ/LPS) macrophages were found to exhibit high levels of ferritin H and low levels of ferroportin, CD163, TfR1 and HO-1; M2 (IL-4) macrophages instead presented high levels of CD163, TfR1, ferroportin, HO-1, and low levels of ferritin H. Ceruloplasmin and hepcidin were found to be expressed at higher levels in M1 macrophages. Possible explanations for the different iron management by M1 and M2 macrophages are linked to their different physiological roles, whereby inflammatory M1 macrophages must cope with invading intracellular pathogens and therefore must sequester iron. Tolerogenic M2 macrophages, on the other hand, are involved in tissue repair and it has been suggested that their lower iron retaining capacity might impact cytokine production and/or it may affect other cells in the microenvironment (Cairo et al. 2011; Recalcati et al. 2010).

In conclusion, our results demonstrate that lactoferrin can counteract the LPS-induced decrease of

ferroportin protein levels in adherent macrophage THP-1 cells by lowering IL-6 production. Restoring ferroportin at the plasma membrane will affect the iron export ability of these cells with possible beneficial effects, contrasting the anemia of inflammation. Our finding may also have implications in the response of inflammatory macrophages to challenges and confirms once again the tight connection between iron homeostasis and inflammation.

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