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Review

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Lactoferrin in Health and Disease

Rola laktoferryny w procesach fizjologicznych oraz stanach chorobowych

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Summary

Lactoferrin, an iron-binding glycoprotein, can be regarded as a cell-secreted mediator that bridges innate and adaptive immune function by regulating target cell response. It is a major pleiotropic mediator that directly assists in the development of T-helper cell polarization. The aim of this minireview is to provide a summary of the most recent work presented at the Lactoferrin Minisymposium at the University of Texas, Health Science Center at Houston, Texas, USA, regarding role of lactoferrin in maintaining immune homeostasis. The data presented here lay emphasis on the significance of lactoferrin in the resolution or progression of the immune responses, thus giving lactoferrin bookend properties in controlling the initial reactions to infectious assault, trauma, and injury. These findings may be critically important in the development of therapeutically relevant protocols.

Key words: **Lactoferrin • immunomodulation • innate immunity • adaptive immunity**

Streszczenie

Laktoferryna (LF), glikoproteina wiążąca żelazo, może być uznana za mediator łączący wrodzone i nabyte reakcje obronne ustroju przez regulację reaktywności komórek docelowych. Jest to wielokierunkowy mediator, który jest m.in. bezpośrednio zaangażowany w proces polaryzacji pomocniczych komórek T. Celem artykułu jest zwięzłe przedstawienie wyników zaprezentowanych na minisympozjum, które odbyło się w the University of Texas, Health Science Center at Houston, Texas, USA, dotyczących roli LF w utrzymaniu immunologicznej homeostazy. Prezentowane dane kładą nacisk na znaczenie LF w wygaszaniu lub promowaniu progresji odpowiedzi immunologicznej, uświadamiając w ten sposób przypisywane laktoferrynie zdolności do kontrolowania reakcji zapoczątkowanych przez: atak patogenów, traumatyczną ingerencję w homeostazę ustroju lub odniesione rany. Wyniki te mogą być niezwykle ważne w zaprojektowaniu stosownych protokołów terapeutycznych.

Słowa kluczowe: **laktoferryna • immunomodulacja • odporność wrodzona • odporność nabyta**

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Introduction

The clinical implication of immune dissonance is often overlooked when the life-threatening symptoms of acute infection, inflammation, or trauma require immediate therapeutic or surgical intervention. Imbalances in immune homeostasis become a secondary issue until the critical conditions of patient become manageable, in large part because of inherent difficulties in the diagnostic evaluation of immune dissonance or dysregulation related with specific ensuing pathologies. The immune system represents a complex network of specialized cells responsive to insult and injury that release soluble products responsible for the initiation and maintenance of both the innate and adaptive responses. The innate immune response is essentially the first line of defense responsive to environmental insults (e.g. an invading pathogen or a trauma) and it is mediated by pattern recognition receptors, especially Toll-like receptors, present on specialized cells, such as macrophages and dendritic cells (DCs). These cells, especially DCs, play a central role in the interface between innate and adaptive immunity, directly due to the induction of innate inflammatory responses characterized by the secretion of specific cytokines and chemokines. Depending on the initial stimulus (e.g. antigen or chronic conditions), cellular maturation occurs to allow the activation of naive precursor T cells (Thp) (Figure 1). The DC is critical in this primary process; conditions that promote transcription of IL-12 favor Th1 differentiation, while the production of a high level of IL-4 correlates with the Th2 phenotype [21]. Under certain conditions, some DCs may also secrete IL-10 (DC-Tr1) promoting differentiation into regulatory T-cell phenotypes. This novel concept of innate immunity driving adaptive immunity has been applied to mechanisms underlying disease pathogenesis and the development of vaccines [13]. Recent evidence suggests that lactoferrin may be an early modulator of antigenic response by T cells, perhaps by directly influencing innate regulatory function [17].

Lactoferrin, an iron-binding glycoprotein, can be regarded as a cell-secreted mediator that bridges innate and adaptive immune function by regulating target cell response. Lactoferrin is synthesized by epithelial cells and granulocytes and is considered a first-line defense protein involved in protection against a multitude of microbial infections [28,35] and prevention of systemic inflammation [7,8,25]. Indeed, lactoferrin is well documented as having direct antimicrobial activity, including an iron-dependent bacteriostatic property and non-iron-dependent bacteriocidal action on LPS-bearing Gram-negative bacteria [10,39,44]. While suppressing microbial growth, lactoferrin also directly exerts its first-line defense activity with its significant impact on the development of adaptive immune responses. Sequestration of iron by lactoferrin reduces insult-induced oxidative stress, thus altering the magnitude and specific production of cytokines [24]. Lactoferrin indeed has a profound modulatory action on the adaptive immune system [46,49,50] by promoting the maturation of T-cell precursors into competent helper cells and by the differentiation of immature B cells into efficient antigen-presenting cells [48]. In addition, lactoferrin augments the delayed type hypersensitivity (DTH) response to antigens, leading to a strong induction of cell-mediated immunity (CMI) in mice [1,45]. The ability of lactoferrin to bind large quantities of iron may also provide protection against pathogens and their metabolites by enhancing phagocytosis and cell adherence and controlling the release of pro-inflammatory cytokines [2,3].

Lactoferrin-specific cell surface receptors have been identified on macrophages [30,40], platelets [27], and intestinal cells [14]. The low-density lipoprotein receptor-related protein-1 and -2 (LRP1 and LRP2), which are multi-ligand receptors, are considered primary lactoferrin receptors. Although members of the LRP family are generally considered endocytic receptors, it has been recently demonstrated that LRP1 also functions as a signaling receptor. Lactoferrin can also elicit mitogenic activity, as evidenced in primary osteoblasts via phosphorylation of p42/44 MAP kinases [32]. Finally, administered lactoferrin can potentiate the restoration of humoral immune response in immunocompromised hosts [4,5], suggesting a mechanism for either direct or indirect restimulation of adaptive cell reconstitution through proliferative pathways.

Lactoferrin and Immunologic Dissonance

Consideration of the physiological circumstances in which lactoferrin exerts its action may provide a guide through the complex network of reactions. During the initial development of a response to a Gram-negative bacterial infection, vascular inflammation occurs within minutes which coincides with a burst of pro-inflammatory cytokines, such as tumor necrosis factor (TNFα), interleukin-1β (IL-1 β), and colony-stimulating factor (CSF), all derived from activated monocytes/macrophages. Subsequently, the number of circulating neutrophils acutely increases. Within an hour after the initial response to infection, the bone marrow may speed up the production and the release of fresh neutrophils fivefold. The feedback control of this inflammatory process begins with the degranulation of neutrophils and a massive release of lactoferrin [26]. This, in turn, may affect the monocyte/macrophage system in two ways: 1) by attenuating the production of cytokines when lactoferrin binds to the specific receptor [51] or 2) by reducing the

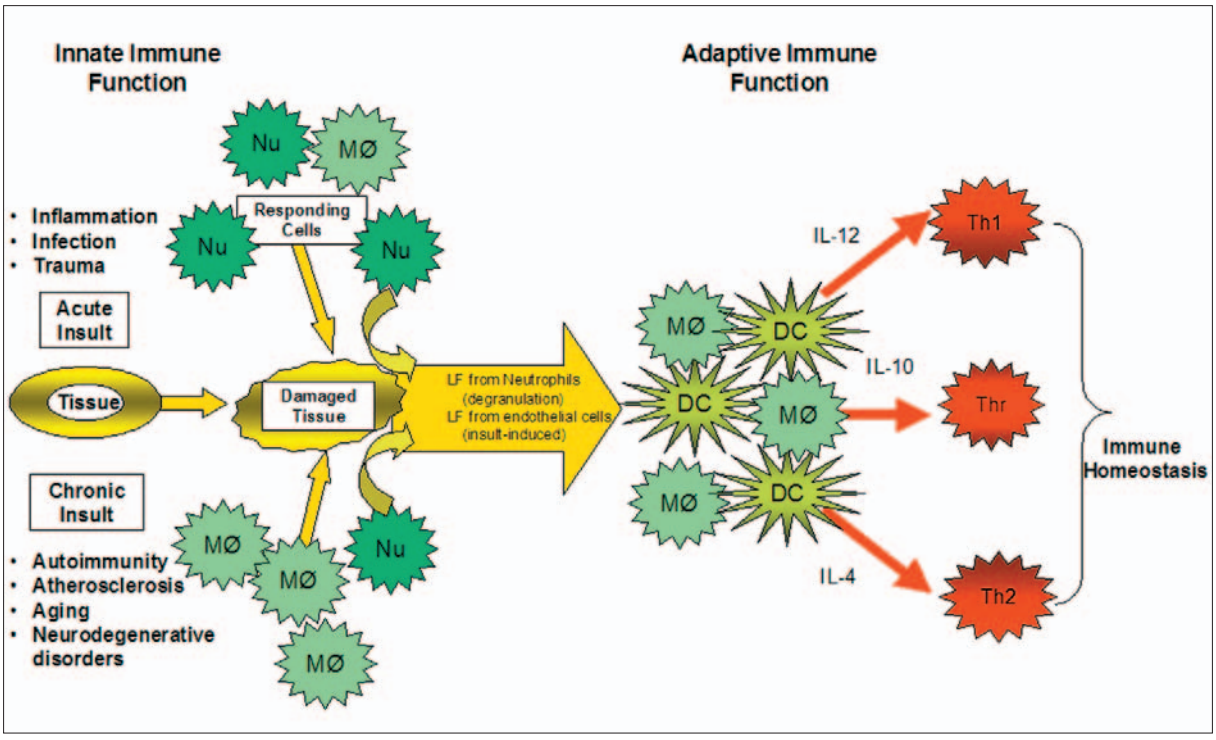

Figure 1. Bridging the innate and adaptive responses. Insult, defined as infection, trauma, or chronic illness, leads to activation of the monocyte/macrophage system (MØ), including neutrophils (Nu), and dendritic cells (DCs). Activated neutrophils (Nu) degranulate at the site of injury and release massive amounts of lactoferrin. Lactoferrin, in turn, may reduce insult-induced oxidative stress and/or binding to LRP1 for signaling. Depending on the magnitude and/or duration of insult, DCs may mature to various forms expressing substantially different cytokines: IL-12 (DC IL-12), IL-10 (DC IL-10), IL-4 (DC IL-4) and promoting Th polarization into Th1, Threg, and Th2 respectively.

number of primed neutrophils for superoxide formation when lactoferrin binds to the CD14 receptor as a complex with LPS. Either way, the initial direct effect of lactoferrin (binding to LPS and/or an identified LPS receptor) is translated into a cascade of immune responses with a variety of activities that often are not attributed to lactoferrin. Although the signals by which lactoferrin is released from neutrophils are different depending on the type and severity of insult as well as the insult's duration and location, lactoferrin responds to these signals in a quantitative matter. It is therefore apparent that lactoferrin is able to bridge the innate and adaptive immune responses during insult-induced metabolic imbalance.

Lactoferrin is generally considered an important nonspecific host defense component for protection against various pathogens. Its concentration in blood is normally low (0.2–0.6 μg/ml), with only a transient increase upon insult-induced activation of neutrophils [15]. In fact, a high level of lactoferrin in plasma has been suggested to be a predictive indicator of sepsis-related morbidity and mortality [8]. In addition, progression in chronic inflammatory disorders, such as Alzheimer's disease (AD), or autoimmune disorders, such as multiple sclerosis (MS), does not correlate with lactoferrin elevation in physiological fluids [18,34]. It is therefore evident that the endogenous production of lactoferrin is either insufficient or does not trigger the pathway(s) of molecular events to aid against chronic insult. Again, it is important to consider that chronic disorders develop over a long time period, during which lactoferrin actually protects against oxidative stress by scavenging free iron. When immune dissonance progresses, regardless of the reason (e.g. natural aging, environmental conditions, genetic make-up, or the intensity of insult), lactoferrin is not able to compensate the growing injury directly, but rather is available to reduce the toxic effects associated with the chronic conditions. This problem is perhaps the best illustration that lactoferrin is only one factor in the regulation of homeostasis. Therefore, it would be difficult to postulate lactoferrin as a single-agent therapy for any chronic inflammatory disorder.

It has become evident that lactoferrin is a pleiotropic agent which may play a role *in vivo* as an "immune sensor" directing specific immune responses to obtain immune homeostasis. In studies on human volunteers orally administered a low dose of lactoferrin (40 mg/day), a differential effect of immune responsiveness was demonstrated. In particular, the effects of lactoferrin on the proliferative response of peripheral blood mononuclear cells (PBMCs) to mitogens and the ability of PBMC cultures to produce IL-6 and TNFα both spontaneously and upon LPS activation were evaluated in healthy individuals [51,53]. Two categories of individuals were selected based on the initial immune responsiveness of PBMCs relative to IL-6: high and low responders (Figure 2). An additional, moderate group was selected relative to TNFα (Figure 2). The *in vivo* effects of lactoferrin appeared to be regulatory and depended on the immune status of a given individual prior to the treatment. The down-regulatory action of lactoferrin could be seen one day after treatment in the high-responding group, while the low-responding individuals showed an up-regu-

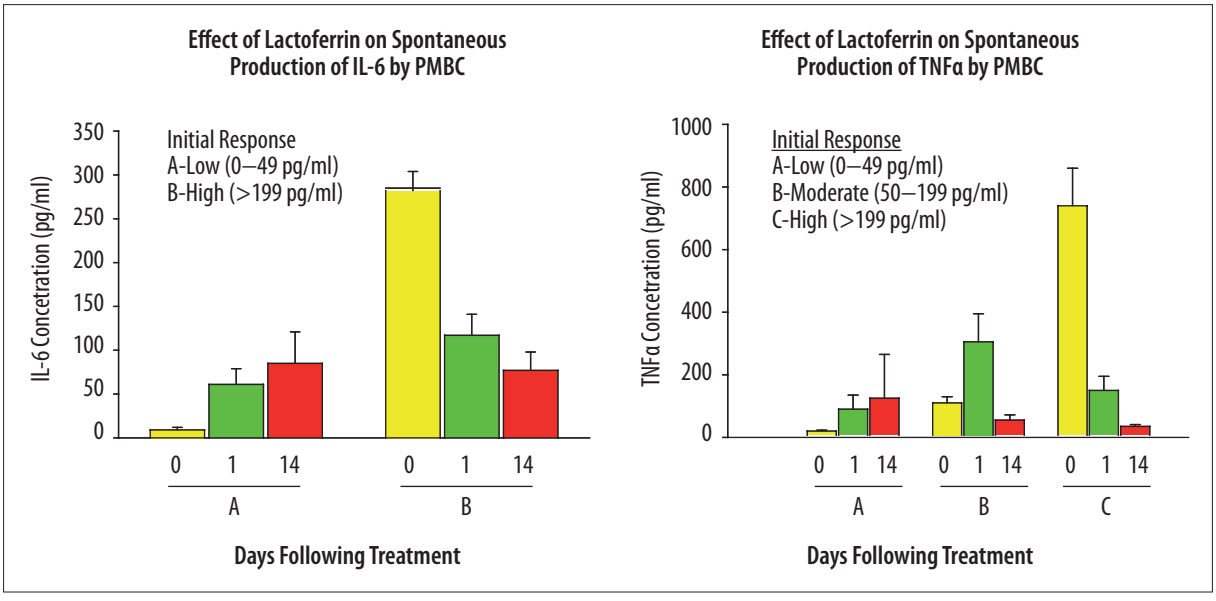

Figure 2. The pleiotropic effect of lactoferrin on the spontaneous production of IL-6 and TNFα in human PMBCs. Data presented in part from [23].

latory effect of lactoferrin. Lactoferrin affected to a much greater degree the ability of PBMCs to produce TNF α and IL-6 spontaneously. When the individuals were classified as low, moderate, and high responders (Figure 2), the effects of lactoferrin become clearly regulatory, e.g. initially low cytokine production was stimulated and high production was inhibited. Spontaneous cytokine production is dependent on cell-to-cell interaction via receptors [52], including accessory adhesion molecules such as LFA-1. Indeed, models demonstrate that lactoferrin's regulatory effects on cytokine production were associated with the modulation of LFA-1 receptor expression [50]. A third parameter reflecting the effects of oral treatment of volunteers with lactoferrin was a twofold increase in the content of neutrophil precursors in the circulating blood (not shown). This phenomenon resembles events seen in the circulation elicited by infection or elective surgery due to the release of endogenous lactoferrin from neutrophils.

Lactoferrin as a Bridge from Innate to Adaptive Response

The importance of lactoferrin as a bridge between innate and adaptive immune function can clearly be seen during the elicitation of response during vaccination. Lactoferrin is capable of enhancing the T cell-mediated delayed type hypersensitivity (DTH) response, as measured by footpad swelling, to a variety of antigens, including sheep red blood cells (SRBCs), ovalbumin (OVA), and *Mycobacterium bovis* Calmette-Guerin (BCG) [22,46,47]. Recently, Lactoferrin was demonstrated to augment the BCG vaccine to protect against subsequent challenge with virulent MTB [20] with increased IFN-γ recall response to BCG antigens. The molecular mechanisms underlying this observation have not been defined; however, preliminary studies address the effects on professional antigen-presenting cells to produce IL-12, a mediator that promotes naïve T-cell development towards the T_H1 phenotype [36,37]. The production of IL-12 is, in part, regulated by IL-10; a high IL-10 level compared with IL-12 will lead to an environment that decreases the promotion of T_H1 generation [16,31]. A number of *in vivo* studies demonstrated increased IL-12 with a concurrent decrease in IL-10 levels following administration of lactoferrin [1,38,41,42]. The relative production of IL-12p40 and IL-10 from macrophages is directly affected by lactoferrin, specifically during subotpimal stimulation of cultures. For example, lactoferrin was able to alter the ratio of IL-12 to IL-10 when adherent F4/80+ splenocytes cells were stimulated with LPS (Table 1). Similar studies have confirmed these affects in murine primary cell lines as well as in human cells lines and PBMCs (data not shown). The ability of lactoferrin to increase *in vivo* the production of IL-12 indicates the potential to direct immune responses towards T_H1, a concept that has been accepted by others [17].

Table 1. Lactoferrin raises the IL-12: IL-10 ratio in LPS stimulated adherent splenocytes.

Lactoferrin (μg/mL)	–	1	10	100
LPS	+	+	+	+
Ratio IL-12:IL10	**1.28**	**1.14**	**0.93**	**2.03**
SD	0.11	0.07	0.01	0.15

Adherent splenocytes (F4/80+ enriched) were stimulated with LPS (400ng/mL) with or without increasing concentrations of lactoferrin (1, 10, 100 μg/mL). Supernatants were collected at 48 hrs and analyzed by ELISA. Concentrations of IL-12p40 were divided by IL-10. SD, standard deviation; *$P<0.05$ relative to non-lactoferrin treated controls.

Lactoferrin and Oxidative Stress

Oxidative stress occurs when there is an imbalance between the amount of antioxidant properties and reactive oxygen species (ROS). ROS are continuously generated during physiological metabolic conditions. Excess ROS is implicated in a variety of pathological and chronic de-

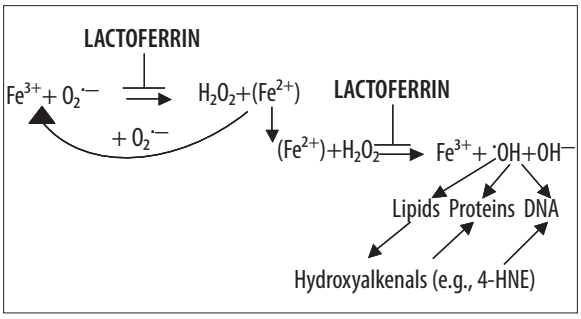

Figure 3. Lactoferrin as an antioxidant in iron-dependent oxidative stress. Under normal physiologic conditions, superoxide will spontaneously or enzymatically dismutate and produce hydrogen peroxide. However, in the presence of free iron, $O_2^{\cdot -}$ dismutation is much faster. The generated Fe^{2+} reduces H_2O_2 to highly reactive hydroxyl radical. These reactions are regulated not only by metal chelators, such as lactoferrin, but also by oxidative stress controlling enzyme activities such as superoxide dismutase.

generative processes, including atherosclerosis, inflammation, aging, and neurodegenerative disorders [29,42]. Superoxide anion ($O_2^{\cdot -}$) is a primary radical generated by the enzymatic reduction of molecular oxygen. For example, the activation of neutrophils, monocytes, macrophages, and eosinophils by cellular and non-cellular agents leads to the activation of NADPH oxidases, which produce superoxide anion, although in response to various stimuli, xanthine oxidoreductases, lipoxygenases, monooxygenases, p450 cytochrome oxidases, and others generate $O_2^{\cdot -}$. Though these oxidases are essential in immune defenses, drug metabolism, etc, they are responsible for only 10% of $O_2^{\cdot -}$ generation. It has been shown that 90% of $O_2^{\cdot -}$ originates from mitochondria; therefore they are the main sources of cellular oxidative overload [12]. In response to an environmental stimulus or trauma, mitochondria increase the rate of $O_2^{\cdot -}$ generation by several fold. Many studies have shown that regardless of where $O_2^{\cdot -}$ is generated, they are poorly reactive in aqueous solution and therefore cannot account for most of the damage observed in the cell in which they are generated. It is therefore likely that cellular physiological changes and/or damage induced are due to a more reactive species, such as H_2O_2 and the hydroxyl radical (·OH). At physiological pH, spontaneous dismutation of $O_2^{\cdot -}$ leads to the production of H_2O_2, which is a reactive oxygen species rather than a free radical, as it has an even number of electrons [19]. The rate of dismutation increases enzymatically by superoxide dismutases.

Iron has two main valency states, the divalent ferrous (Fe^{2+}) and the trivalent ferric (Fe^{3+}) forms. As shown in Figure 3, the two oxidation states allow iron to participate in redox processes, making it an essential biological redox catalyst [19,43]. Therefore, a major determinant of the cell toxicity of $O_2^{\cdot -}$ is dependent on the availability and location of iron, which may be directly affected by the presence of lactoferrin.

Investigations have discovered that some of lactoferrin's physiological effect is associated with its iron binding. In this regard, lactoferrin is considered an antioxidant because its iron binding ability inhibits the iron-catalyzed formation

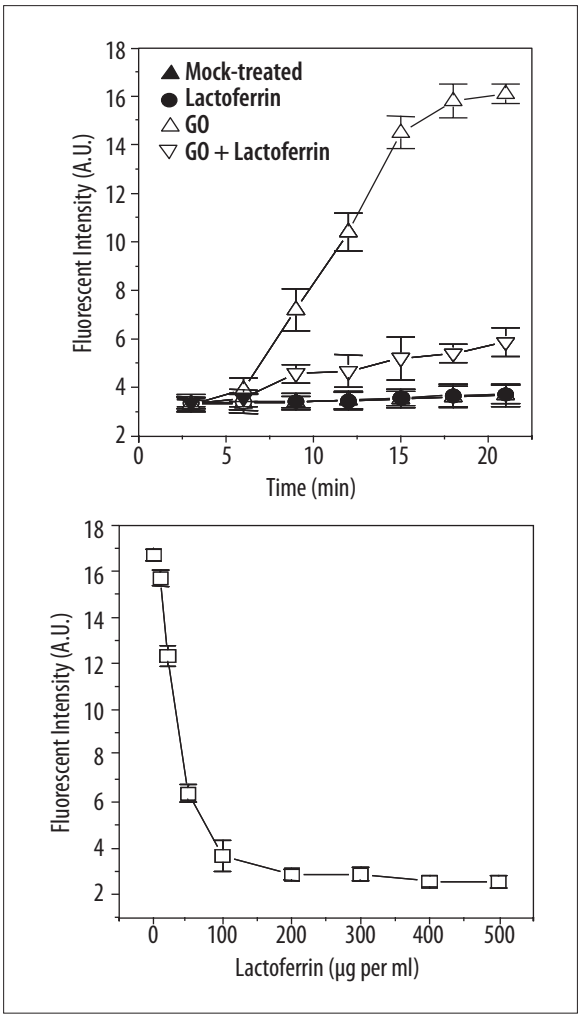

Figure 4. Lactoferrin decreases intracellular ROS level. Cells were lactoferrin-treated, loaded with H2DCF-DA (5 µM final concentration for 15 min, at 37°C), and stressed oxidatively by glucose oxidase (GO, 100 ng/ml). Changes in ROS level were determined by flow cytometric analysis (Becton Dickinson FACScan) at excitation/emission wavelengths of 485/528 nm. Each data point represents the mean fluorescence for 12,000 cells from three independent experiments. Data are expressed ±*SEM*.

of H_2O_2 and ·OH [6]. The Fe^{3+} produced in this process is coordinated by lactoferrin and is safely transported to the macrophages of the reticulo-endothelial system, where it can be stored in ferritin.

Reactive oxygen species, in particular the hydroxyl radical, can react with all biological macromolecules (lipids, proteins, nucleic acids, and carbohydrates). Among the more susceptible targets are polyunsaturated fatty acids [9,11,33]. The anti-oxidant properties of lactoferrin have been tested in human embryonic diploid fibroblasts (MRC5) and on the monocyte origin cell line U937 using 2',7'-dichlorodihydro-fluorescein diacetate (H2DCF-DA; Molecular Probes Eugene, OR). Cells in iron-containing growth medium were treated with increasing concentrations (0, 10, 20, 50, 100, 200, 300, 400, and 500 µg/ml) of lactoferrin. Lactoferrin-pretreated cells were loaded with H2DCF-DA and stres-

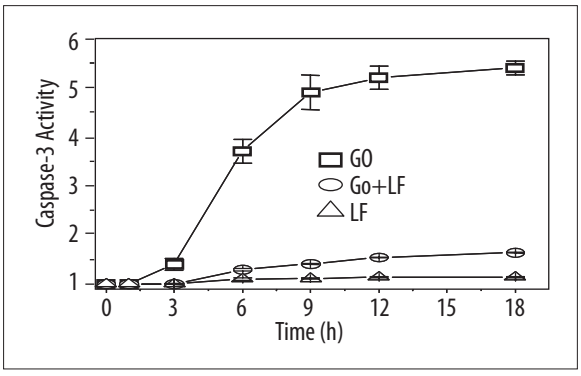

Figure 5. Lactoferrin inhibits oxidative stress-induced apoptosis. Cells were pre-treated with lactoferrin and GO (300 ng/ml) was added. Caspase-3 activities were determined in clarified cell lysates using colorimetric assays (R&D Systems, Inc.). In this assay, changes in O.D. at 405 nm are proportional to the activity of caspase-3. Each data point represents the mean of three independent experiments.

sed oxidatively with glucose oxidase (GO). Changes in the DCF fluorescence was determined by flow cytometry. Lactoferrin significantly inhibited/decreased intracellular ROS levels in a dose-dependent manner (Figure 4). Our data analysis showed that lactoferrin at a concentration of 32 μg/ml mediated a 50% decrease in intracellular ROS levels. Higher concentrations of lactoferrin decreased cellular ROS nearly to background levels. Interestingly, lactoferrin also decreased cellular ROS levels when the cells were grown in iron-free medium (data not shown). These results suggest that lactoferrin may exert its effect on cellular redox metabolism in an iron-independent manner. Similar results were obtained using U937 cells. Addition of lactoferrin alone did change intracellular ROS levels.

We also investigated whether or not lactoferrin decreases ROS-induced apoptosis. U937 cells were pre-treated with 100 μg/ml of lactoferrin and 300 ng/ml of GO was added. GO at this concentration induces apoptosis in 90% of U937 cells and increased caspase-3 activity. Lactoferrin-pretreated, lactoferrin plus GO-exposed, and control cells were harvested after addition of GO and the subsequent caspase-3 activities were determined. The data shown in Figure 5 suggest that lactoferrin was capable of preventing oxidative stress-induced apoptosis. Inhibition of ROS-induced apoptosis by lactoferrin was confirmed in the Annexin V assay using U937 cells using flow cytometry (not shown). When the cells were grown in iron-free medium, GO-induced cell death decreased from 95% to <40%. Pre-treatment of cells with lactoferrin further decreased cell death and the percentage of apoptotic cells were then under 12%. Taken together, these data indicate that iron chelation by lactoferrin is the only mechanisms by which lactoferrin protects cells from oxidative injury.

Conclusion

Immune homeostasis is the maintenance of equilibrium in a biological system by means of positive and negative feedback mechanisms. At the cellular level, immune homeostasis is controlled by the network of immune cells and their products, which provide a successful transition of innate first-line defenses to adaptive immune function. It is becoming increasingly clear that lactoferrin plays a central role in the development and full expression of the adaptive host response. Lactoferrin is indeed a pleiotropic immune modulator that directly assists in the orchestration of the development of T-helper cell polarization. The mechanism of lactoferrin's action is largely dependent on its ability to influence early responses, including the modulation of intracellular ROS production. By virtue of controlling oxidative stress, lactoferrin modulates the innate immune responsiveness accountable for the transition to the active and directed adaptive immune function. Our data also support the significance of lactoferrin in the resolution or progression of the immune responses, thus giving lactoferrin bookend properties in controlling the initial reactions to infectious assault, trauma, and injury. These findings may be critically important in the development of therapeutically relevant protocols to limit pathological damage by disease.

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