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Review

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The Effects of Lactoferrin on Myelopoiesis: Can we resolve the Controversy?

Wpływ laktoferryny na mielopoezę: czy można rozstrzygnąć istniejące kontrowersje?

Jolanta Artym, Michał Zimecki

Department of Experimental Therapy, The Institute of Immunology and Experimental Therapy, Wrocław

Summary

Lactoferrin (LF) is an iron-binding protein contained in the secretory fluids of mammals and secondary granules of neutrophils. LF is a key element in innate immunity and exhibits a wide spectrum of activity against pathogens. Although the effects of LF on the maturation and effector function of immune system cells are well recognized, its regulatory function on myelopoiesis has been a matter of controversy for a long time. The majority of early studies demonstrated the role of LF as a negative feedback regulator of myelopoiesis. There were also reports that revealed no significant involvement of LF in that process. However, a number of experimental data, together with clinical observations, indicate a stimulatory action of LF in myelopoiesis. The aim of this article is to review the available literature data regarding the effects of LF on myelopoiesis in mice and humans. Possible reasons for opposing views on this subject are presented.

Key words: **lactoferrin • myelopoiesis • negative regulation • positive regulation • disorders of myelopoiesis • mice • humans**

Streszczenie

Laktoferryna (LF) jest białkiem wiążącym jony żelaza, występującym w płynach wydzielniczych ssaków i w drugorzędowych ziarnistościach neutrofilów. Białko to stanowi kluczowy element odporności wrodzonej i prezentuje szerokie spektrum aktywności skierowanych przeciw patogenom. LF promuje także dojrzewanie oraz reguluje aktywność efektorową komórek układu immunologicznego. Wpływ LF na regulację mielopoezy budzi kontrowersje. Większość wczesnych prac z lat 70-tych i 80-tych wskazywało na rolę LF jako negatywnego regulatora mielopoezy. Niektóre badania nie wykazały jakiegokolwiek działania LF na mielopoezę. Jednakże, szereg danych doświadczalnych oraz obserwacji klinicznych wskazuje na stymulującą rolę LF w regulacji mielopoezy. Celem tego artykułu jest przegląd dostępnych danych literaturowych dotyczących wpływu LF na proces mielopoezy u myszy i ludzi. Przedstawiono również przypuszczalne powody powstania przeciwstawnych opinii na temat aktywności LF w tym procesie.

Słowa kluczowe: **laktoferryna • mielopoeza • negatywna regulacja mielopoezy • pozytywna regulacja mielopoezy • zaburzenia mielopoezy • myszy • ludzie**

Abbreviations: **LF** – lactoferrin; **BLF** – bovine lactoferrin; **HLF** – human lactoferrin; **holo-LF** – iron-saturated lactoferrin; **apo-LF** – iron-free lactoferrin; ***i.v.*** – intravenously; ***i.p.*** – intraperitoneally; ***s.c.*** – subcutaneously; **BFU-E** – early erythropoietic cell-forming erythroid colonies (Burst-Forming Unit-Erythrocyte); **CFU-GEMM** – pluripotential bone marrow stem cell-forming granulocyte, erythrocyte, macrophage, and megakaryocyte colonies (Colony-Forming Unit-Granulocyte, Erythrocyte, Macrophage, Megakaryocyte); **CFU-GM** – granulomonopoietic progenitor cell (Colony-Forming Unit-Granulocyte, Macrophage; formerly CFU-C); **CSA** – Colony-Stimulating Activity; **GM-CSF** – Granulocyte, Macrophage Colony-Stimulating Factor; **G-CSF** – Granulocyte Colony-Stimulating Factor; **CML** – Chronic Myelogenous Leukemia; **AML** – Acute Myelogenous Leukemia.

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Author's address : Prof. dr hab. Michał Zimecki, Zakład Terapii Doświadczalnej Instytutu Immunologii i Terapii Doświadczalnej PAN, ul. Weigla 12, 53-114 Wrocław, e-mail: zimecki@iitd.pan.wroc.pl

Lactoferrin (LF) represents a key element of innate immunity in mammals. This protein, involved in iron metabolism, was discovered in the early 1960s, initially in bovine and human milk and later in other body fluids (saliva, tears, excretions of nasal mucus, trachea, vagina, and uterus, seminal plasma, and others) [60,73,86] and lastly in the secondary granules of neutrophils [87]. Secretory LF is generated by secretory epithelial cells of various organs and is released to the respective excretions [13,85]. LF contained in neutrophils may be released from these cells into blood plasma, where its concentration in health individuals does not exceed 400–500 ng/ml [18,94]. In some conditions (inflammation, infection) both local and plasma concentrations of LF significantly increase, accompanied by infiltration and degranulation of neutrophils [15,62,137]. Since the time of LF's discovery, numerous studies, aimed at determining its biological properties have been undertaken. Early studies revealed antimicrobial properties of LF, comprising activities directed against bacteria, viruses, fungi, and parasites [96,97]. Subsequent investigations demonstrated a variety of other activities of LF, such as involvement in iron metabolism, ability to neutralize endotoxin, promotion of lymphocyte maturation, regulation of myelopoiesis, anti-inflammatory and immuno-regulatory properties, anti-tumor and anti-analgesic actions, regulation of bone metabolism, participation in embryo development, procreative function, and others [138]. Evidence also exists indicating involvement of LF in the normal development of newborns [2].

Studies to date have shown that the actions mediated by endogenous LF may also be exhibited by exogenous LF, introduced by various routes. More importantly, oral administration of LF seems to be very effective and, due to lack of any toxicity, is also preferred in future clinical protocols. Many studies indicated relative resistance of LF to proteases in the digestive tract; the ingested protein hardly passes the gut/blood barrier (with the exception of newborns) [64,69,77,131]. Nevertheless, LF given orally affects the organism systemically, which may be related to the local activation of gut-associated lymphoid tissue and systemic propagation of immune system responses [46,70,77,116,133]. Interestingly, not only native LF, but also its proteolytic enzyme fragments may express biologic activities. In fact, one such fragment, lactoferricin, has much stronger anti-microbial action than native LF itself [136]. Based on the hitherto accumulated data it cannot be excluded that the protein or/and its active fragments may, at least to a limited degree, be absorbed from the gastrointestinal tract, reach the target tissues from the circulation, and interact locally with immunocompetent cells. This would also regard the actions of LF on myelopoiesis. Studies on the effects of LF on myelopoiesis have been conducted for over half a century but have not yet lead to any definite conclusions. The aim of this article is to review both *in vitro* experimental data as well as *in vivo* results, including clinical observations, regarding the effects of LF on the processes of myelopoiesis.

Myelopoiesis

Myelopoiesis is a dynamic process dependent on a variety of factors which may stimulate or inhibit the proliferation and maturation of granulocyte and macrophage progenitors. The mutual interactions of many cell types and factors secreted by these cells maintain the numbers of granulocytes and monocytes/macrophages at constant levels inherent to the physiological state. These cells constitute the first line of defense against pathogens; therefore the regulation of their recruitment and release into the circulation is of major importance. Particularly important is the regulation of granulopoiesis, since the complete development of granulocytes lasts relatively long (10–14 days), although these cells live very shortly (4–6 h) after being released from bone marrow [107]. The cells must thus be continuously generated and released into the circulation. The demand for neutrophils greatly increases during infection. Neutrophils and macrophages are recruited from pluripotent cells of the bone marrow, common to the whole process of blood cell formation. Pluripotent cells are stem cells with the ability of self-renewal, differentiation, and generation of progenitor cells of two lineages: the myelopoie-

tic and the lymphopoietic systems. Stem cells for the myelopoietic system give rise to precursor cells for erythro-, granulo-, mono-, and megakaryopoiesis. Myelopoiesis is promoted by a number of cytokines described as regulators of hematopoiesis, hematopoietic growth factors, or hematopoietins. Myelopoietic growth factors include, among others, IL-1, -3, -6, -8, TNF-alpha, GM-CSF, G-CSF, and M-CSF. These cytokines are produced by various cell types (monocytes/macrophages, fibroblasts, endothelial cells, epithelial cells, lymphocytes) from many tissues and organs, including cells of the bone marrow microenvironment [72,83].

There have been major disparities regarding the regulatory function of LF on myelopoiesis. Most of the available data are from studies conducted in the 1970s and '80s (both *in vitro* and *in vivo* studies). Some authors found that LF functioned as a negative regulator of myelopoiesis (Broxmeyer, Bagby and Fletcher). Sawatzki and Rich, on the other hand, showed that LF up-regulated the process of myelopoiesis. Here we present and discuss the results of both groups along with our own observation regarding the role of LF in myelopoiesis. Clinical observations are also presented which confirm laboratory and preclinical data on LF in myelopoiesis.

Lactoferrin as a negative regulator of myelopoiesis

The earliest report suggesting a participation of neutrophil-derived factors in the feedback regulation of myelopoiesis was written fifty years ago [98]. The author postulated that mature cells of the granulocytic lineage contain factors limiting the proliferative activity of their precursors and that this regulatory mechanism may be disturbed in leukemic patients. In 1960, Bullough and Lawrence provided experimental proof for the control of epithelial cell proliferation by products of mature cells in cell cultures [40]. The authors proposed a theory of so-called "chalons", tissue-specific products of mature cells acting as proliferation inhibitors of the early cells of the same lineage. Since that time, many experiments have been performed indicating the existence of chalons for various cell lineages, including cells of the granulocyte-macrophage lineage [66,99]. The proposed feedback mechanism of myelopoiesis regulation is probably mediated by suppressive factors secreted by neutrophils, and LF appeared to be one such factor. Among the research teams which initiated studies on the potential myelopoietic activity of neutrophil-derived factors was the laboratory of Broxmeyer. The early studies by his group utilized neutrophil suspensions and extracts from human and mouse blood or bone marrow neutrophils obtained by multiple rapid freezing-thawing procedures. Neutrophil-conditioned culture media were also tested [10,23,34].

The initial studies were based on *in vitro* tests. Neutrophil extracts and neutrophil-conditioned media lowered the synthesis and secretion of CSA (colony-stimulating activity) by monocytes and macrophages, as determined by the numbers of granulocyte-macrophage colonies formed by early cells directed to granulo- and macrophagopoiesis (Colony Forming Unit in Culture, CFU-C, now termed CFU-GM). The formation of colonies by both human and mouse bone marrow cells was inhibited. On the other hand, the neutrophil extracts did not affect colony formation in the presence of exogenous CSA. The inhibitory activity derived from neutrophils was named Colony Inhibiting Activity, or CIA [23,34]. The sensitivity of the target cells to inhibition was lower during the constant presence of neutrophils in the studied cell cultures and the removal of neutrophils significantly increased the sensitivity of the target cells to CIA. Addition of LPS to the experimental system totally reversed or masked the inhibitory action of the neutrophil extracts.

The interesting results of the *in vitro* tests encouraged the Broxmeyer laboratory to initiate *in vivo* studies [23]. It appeared that neutrophil extracts administered *i.v.* do not influence bone marrow and spleen cellularity and the total numbers of endo- and exogenously stimulated CFU-C in the bone marrow and spleens of naive mice. Therefore they decided to eliminate endogenous neutrophils by application of cyclophosphamide (CP). That enabled, in addition, registering a distinct renewal of granulopoiesis. On days 3 and 4 after CP administration, profound losses in metamyelocytes, bands, and mature granulocytes in the bone marrow and circulation were noted. At the same time, a significant renewal of myelopoiesis was observed, as evidenced by increases in blast, promyelocyte, and myelocyte numbers in the bone marrow. A considerable increase in bone marrow CFU-C was also found, these numbers reaching at that time normal values. The level of splenic CFU-C was highest on days 5–11 after administration of CP. The colonies consisted mainly of granulocytes. The *in vitro* studies of the granulocyte-macrophage lineage isolated from these mice during intensive myelopoiesis unanimously showed that the ability to inhibit CFU-C formation was a property of extracts from metamyelocytes, bands, and mature granulocytes only. Thus, earlier forms of cells, dominating on days 3 and 4 after administration of CP, could not inhibit the renewal of myelopoiesis. Repeated intravenous administration of neutrophil extracts lowered CFU-C numbers in the bone marrow and spleen even by 50–60%. The neutrophil extracts did not affect the numbers of splenic B-lymphocyte colonies. Extracts from neutrophils isolated from CML patients were devoid of the inhibitory activity. Administration of LPS reversed or masked the inhibitory activity of the extracts.

In subsequent studies, Broxmeyer's research team identified lactoferrin as the active factor in the neutrophil extracts [38]. In cultures of human and mouse bone marrow in semi-liquid agar, a decrease in CFU-C numbers after addition of neutrophil extracts was observed. Biochemical and isoelectric studies with application of anti-LF antibodies and comparative studies using a pure LF allowed identifying beyond any doubt the inhibitory factor as LF. The activity of LF distinctly depended on the degree of iron saturation; holo-LF, already at very low concentrations (10^{-17}–10^{-15} M), inhibited the production of GM-CSF by human bone marrow cells and blood monocytes [27,30,38] and mouse peritoneal macrophages [57]. In the *in vitro* tests conducted by Bagby and Bennet, holo-LF from human milk inhibited the formation of CFU-C by human bone marrow cells when added to the culture at concentrations of 10^{-17}–10^{-15} M [6]. In similar *in vitro* tests, apo-LF was completely inactive at concentrations of 10^{-8}–10^{-7} M [27,30,38,57]. The activity of LF was "reconstituted" after saturation with iron,

regardless of a method used for iron saturation [27]. This kind of effect could be associated with a higher degree of binding of a native form of LF (8% saturation) and holo-LF to monocytes [30]. Of interest is that LF saturated with other metals (Cu or Zn) did not inhibit CSA production, lowering, at the same time, the ability of the protein to bind to monocytes [30]. Cu and Zn ions also lowered the activity of iron-saturated LF. Warming holo-LF to 100°C totally inactivated the protein [57]. LF did not inactivate exogenous CSA added to cell culture nor did it not diminish the susceptibility of CFU-C to CSA; it inhibited, however, the production and/or release of CSA from adherent mononuclear cells present in the culture [38]. Neutrophil extracts selectively depleted of LF by means of specific antibodies lost their inhibitory activity [6]. The inhibitory action of LF was reversed by addition of LPS to the cell culture: completely when LPS was added together with LF and partially when added 24 h later. The authors suggested that the phenomenon could be associated with an ability of endotoxin to bind iron [38]. Similar results were obtained in *in vivo* studies: a single *i.v.* LPS injection totally abolished the inhibitory action of neutrophil extracts administered to mice [23]. Later studies confirmed the described action of LPS on the LF-mediated inhibition of myelopoiesis [88]. According to the authors, this phenomenon may be explained by the formation of LF-LPS complexes, which prevents the interaction of LF with its cell receptors and the transduction of signals regulating myelopoiesis. Such a situation occurs during bacterial infection when neutrophils are in demand to combat infection. In such a case, abolishment of the suppressive action of LF is fully justified and beneficial. *In vitro* tests demonstrated that cells stimulated with endotoxin or antigen not only increased the production of CSA, but become resistant to LF's action [38]. It appeared that the inhibitory action of LPS can be abolished by glucocorticosteroids added to cell cultures [30]. This may be relevant in the *in vivo* situation when the levels of these hormones are elevated. The inhibitory action of LF may also be inactivated by other factors besides LPS present in the organism or by exogenous factors such as testosterone, estradiol (but not progesterone), and lithium [30].

Subsequent *in vitro* studies showed that neutrophil- and milk-derived LF had similar activities in inhibiting myelopoiesis; both kinds of iron-saturated human LF inhibited the production of GM-CSF to the same degree by human blood mononuclear cells. These activities were blocked by anti-LF antibodies [27]. Comparative experiments with bovine and human LF showed, in addition, that both proteins inhibited the release of GM-CSF from human mononuclear blood cells and mouse resident peritoneal macrophages [32]. Human LF and iron-saturated proteins exhibited higher activities than bovine LF and iron-free proteins. For example, holo-BLF (in the concentration range 10^{-13}–10^{-6} M) inhibited the release of GM-CSF from the human cells, whereas apo-BLF was without effect. Holo-HLF was active in the concentration range of 10^{-16}–10^{-6} M. Both apo- and holo-BLF and -HLF inhibited the release of growth factors from mouse macrophages, in this case the highest activity also having holo-HLF [32].

In subsequent studies, the inhibitory effect of a purified human LF on mouse myelopoiesis was confirmed; the protein lowered myelopoiesis in healthy mice and suppressed the renewal of myelopoiesis in CP-treated animals [39,57]. Intravenous and intraperitoneal administration of LF lowered the numbers of granulocytes and monocytes and inhibited the formation of CFU-C in the bone marrow and spleen. It also slowed the cell cycle progression of CFU-C-forming cells, i.e. lowered the number of cells in S phase. This inhibition was significant, within the range of 30–80% in the case of CFU-C in the bone marrow. Whereas 70% of the CFU-C-forming cells were in S phase in control mice, in mice receiving LF it was only 35–45%. These actions of LF were found both in healthy mice and in mice previously treated with CP, although in the latter group of mice these actions were more distinct. Also, better inhibitory effects were expressed by LF administered *i.v.* than *i.p.* LF subjected to thermal inactivation was not active. Interestingly, apo-LF, not active *in vitro*, appeared to be active *in vivo*, suggesting that LF could acquire iron in the organism (in *in vitro* tests the possibility of binding iron by LF is smaller). Binding of iron probably changes the conformation of the protein, rendering it active (possibly enabling the binding of the protein with cellular receptors). LF was active at a wide range of concentrations; it lowered the number of mononuclear cells and CFU-C at 0.1 ng to 100 μg/mouse, whereas the inhibition of cell progression required a more than 10 μg dose. The action of LF was reversible; 15 days following the last LF dose, no inhibitory effect was further observed. The authors suggested that the action of LF results from the inhibition of some factors regulating the cell cycle of progenitor cells of the granulocyte-macrophage lineage. That factor may be identical to GM-CSF, the growth factor essential for the proliferation and differentiation of the granulocyte-macrophage lineage. The myelosuppressive action of LF, both in healthy and in CP-treated mice, was also confirmed later in *in vivo* studies [39]. In the first case, intravenous injection of LF decreased the percentage of bone marrow cells and splenocytes in cell cycle and lowered the number of granulocyte-macrophage progenitors (CFU-GM), early erythroid progenitors (BFU-E), and myelopoietic stem cells (CFU-GEMM) in the bone marrow and spleen. A similar action was observed when LF was given to healthy mice.

The kind of feedback inhibition mechanism ensured by LF may be of great significance in maintaining normal myelopoiesis. It has been assessed that the amounts of LF contained in every mature granulocyte (3–6 pg) are sufficient to inhibit the production of CSA by over 200,000 monocytes by 60%. Milligram quantities of LF, released daily from granulocytes, have, therefore a great significance in the regulation of myelopoiesis [38]. The abnormalities in this delicate regulatory system found in patients suffering from acute and chronic leukemias and myelodysplastic syndromes are proof of the regulatory role of LF [23,26,33,34,103]. Studies on patients with chronic myelogenous leukemia showed a triple defect in neutrophils: a quantitative deficit in the inhibitory activity (LF), lack of LF activity in inhibiting CSA release, and reduced susceptibility of CSA-producing cells to the inhibitory effect of LF derived from normal neutrophils. This phenomenon could be associated with a smaller number of LF receptors on these cells [31,33]. The studies by Philip et al. showed, in turn, that although granulocytes isolated from CML patients contained granulopoiesis inhibitor, a defect in the re-

lease of that inhibitor was found in 11 out of 12 individuals [103]. In some blood neoplasias (Hodgkin's lymphoma and leukemias), high serum copper levels are found. As mentioned above, Cu interferes with the inhibitory activity of LF [30]. This is possibly another cause of the abnormalities found in these diseases. A defect in the feedback regulation of myelopoiesis may lead to an accumulation of cells of the granulocytic lineage at various maturation stage, a characteristic feature of these leukemias.

Broxmeyer et al. showed that only some neutrophil subpopulations contain active forms of LF which are able to bind to monocytes and inhibit GM-CSA (GM-CSF) production [37]. Active LF was contained only in neutrophils forming rosettes with sheep erythrocytes coated with rabbit IgG, i.e. with neutrophils bearing receptors for the Fc fragment. LF inhibited the formation of GM-CSA already at concentrations of 10^{-16}–10^{-15} M. The other neutrophil subpopulation produced and released LF which was unable to bind to monocytes and was characterized by a smaller ability to inhibit the production of growth factors (the inhibition occurred only at a high concentration of LF: 10^{-5} M and higher). The authors suggest that this may be caused by inactivation of LF by proteolytic enzymes derived from neutrophils devoid of Fc receptors. LF released from Fc receptor-positive neutrophils lost its activity in the presence of neutrophil extracts devoid of such receptors. It should be underlined that the majority of neutrophils expresses Fc receptors, although their detection depends on the sensitivity of the test. In the tests applied in the cited article, the presence of Fc receptors was detected on 52–66% of neutrophils. The authors suggest that these neutrophils may represent more mature cells which have lost the ability to produce enzymes inactivating LF. The studies indicate heterogeneity of the neutrophil population and may explain the discrepancy in the results the regarding effects of LF on myelopoiesis.

The results of the Broxmeyer's research group allowed the assumption that granulopoietic inhibitory activity may depend on the ability of LF to interact with some cell types. Such cells are susceptible to the inhibitory action of LF. For example, binding of LF to human monocytes was found [30]. The majority of LF-positive cells was noted at 30 min. following initiation of incubation and the amount of bound protein did not depend on the incubation temperature (similar results were obtained at 4° and 37°C). Apo-LF was bound to only 41% of cells, but native LF (8% iron-saturated) and holo-LF to 91 and 78% cells, respectively. In the presence of other metal ions (Zn and Cu), significantly fewer cells bound LF. In the same studies, binding of neither partially nor totally iron-saturated LF to human blood lymphocytes was shown [30]. The presence of LF receptors was also found on the surface of resident peritoneal mouse macrophages [27]. The mean number of receptors amounted to 114,500/cell and the binding of labeled LF was almost entirely inhibited by an excess of unlabelled LF, which suggests specificity of the reaction. The cited studies showed that only monocytes/macrophages expressing LF receptor were sensitive to the inhibitory action of the protein. LF inhibited the release of GM-CSA by human monocytes and mouse peritoneal macrophages, as demonstrated by the number of CFU-GM colonies formed in the cultures of human or mouse bone marrow. Cells susceptible to the inhibitory action of LF were characterized as large or medium-sized, adherent, phagocytic, esterase-positive, and bearing Fc receptor [27]. Of interest is that among this cell population, cells were observed which did not respond to the inhibitory effect of LF despite the presence of LF receptors [27]. On the other hand, LF inhibited neither spontaneous nor PHA- or ConA-induced release of GM-CSA by human lymphocytes, which possibly correlated with the lack of LF receptors on these cells [30].

The authors subsequently showed that the inhibitory activity of LF did not exclusively depend on the presence of LF receptors on target cells (monocytes and macrophages) [30,37], but also on other antigen determinants, such as Ia antigens (the mouse equivalent of human MHC class II – HLA-DR) [36]. LF inhibited the production of GM-GSF only from Ia-positive mouse macrophages in the absence of T lymphocytes, endothelial cells, and fibroblasts. A similar action LF was exerted with regard to human monocytes possessing HLA-DR antigens [24]. The inhibitory effect of LF was abolished by monoclonal antibodies directed against the I-A and I-E subregions of Ia antigens and did not occur when Ia-positive cells were removed from the culture [36]. The population of cells responding to LF had the characteristics of mature macrophages; the cells were adherent, phagocytosing, and releasing large amounts of lysozyme, contained a high level of intracellular hydrolases, and bore Fc receptors and Ia antigens [36]. The significance of Ia antigens in the inhibitory action of LF was also demonstrated in a culture of the human U937 monoblast cell line [31]. Formation of colonies by these cells was inhibited (by about 45%) by LF, transferrin, and acidic ferritins. Separation of the cells into two populations, Ia+ and Ia–, demonstrated that only the Ia+ cells responded to the suppressive effect of LF and acidic izoferritins [32]. It is known that LF may regulate the expressions of some genes by binding to cell receptors or translocating to the nucleus [49,58,65]. It can therefore be assumed that the regulation of myelopoiesis by LF may share a similar mechanism. In electron microscopy it was possible to discover LF deposits internalized in the nuclei of human blood monocytes [121]. The deposits were localized in the euchromatin, whereas heterochromatin was free of these deposits. This may suggest that transcriptionally active chromatin is the site of LF activity: the inhibition of transcription of growth factors. The receptors for LF or Ia antigens can bind LF and enable the protein to express its activity [36]. The role of Ia antigens in the activity of LF was indirectly proven by the fact that these antigens disappeared from target cells for LF within one week after Friend virus infection; this was associated with a loss of response of the cells to the inhibitory action of LF [36]. The latest studies of Broxmeyer and co-workers confirmed that the inhibitory effect of LF on myelopoiesis is associated with the presence of MHC class II antigens on cells [28]. Addition of LF to the culture significantly lowered the ability of colony formation by myelopoietic progenitor cells, i.e. erythroid (BFU-E), CFU-GM, and CFU-GEMM of the mouse bone marrow, expressing MHC class II antigens. On the other hand, the cells isolated from mice with impaired expression of these antigens were refractory to the action of LF; however, reconstitution of Ia expression restored the cell activity. The authors conclude that the myelosuppressive action of LF required the presence of Ia antigens on the-

se cells. The expression of Ia antigens is probably regulated, in turn, by CIITA (Class II Transactivator), a transcriptional factor [28].

In the above-described studies [36], the inhibitory action of LF was affected by such factors as cell density in culture and the presence of endogenous prostaglandins (PGE) and acidic isoferritins. Both compounds inhibit colony formation by CFU-GM populations by lowering the sensitivity threshold to CSA, among other mechanisms [32,78,101]. Release of these compounds by macrophages depends on cell density (it increases with cell density). LF may partially inhibit the production of PGE by monocytes and macrophages and block the production of acidic isoferritins [32,101]. Inhibition of colony formation by LF was distinct at cell densities 5×10^4 or 1×10^5/ml, but disappeared at higher densities [36]. Visualization of the inhibitory activity of LF under such conditions required the addition of indomethacin (a PGE inhibitor) to the culture medium and antibodies against acidic isoferritins. The authors concluded that high levels of PGE and isoferritins have, by themselves, strong inhibitory action on CFU-GM formation. The inclusion of LF to the culture partially abrogated that effect, giving an apparent effect of an increase in GM-CSF release. This does not indicate, however, a stimulatory action of LF, but merely abrogation of the inhibitory action mediated by PGE and acidic isoferrins. The inhibitory activity of LF was evident even at high cell density when indomethacin and antibodies against acidic ferritin were added [36].

The results of the Broxmeyer's laboratory suggest an interesting mechanism of regulation of myelopoiesis by LF and PGE [101]. The ability of LF to reduce PGE synthesis (probably by lowering CSF concentration) indicates that both these factors are important for the proliferation of neutrophil and macrophage progenitors. In a healthy state, the proliferation and differentiation of neutrophils and macrophages remain in equilibrium maintained by stimulators (CSF) and inhibitors (neutrophil LF and macrophage PGE). LF may additionally act as a "safety-valve", protecting against excessive extinction of monocytopoiesis by PGE. Bacterial infection leads to increased proliferation of myeloid cell precursors resulting from elevated CSF concentrations which avoid control by physiologic concentrations of LF and PGE. In the presence of high CSF concentrations, the production/release of PGE by monocytes/macrophages increases, which restricts monocytopoiesis, having no effect on granulopoiesis. Accumulating neutrophils release large amounts of LF, which exerts a small inhibitory effect in the presence of bacterial endotoxins. However, when the source of infection is eliminated, high LF inhibits the production/release of CSF. At the same time, the PGE level is dropping. Thanks to these regulatory mechanisms, myelopoiesis returns to the equilibrium state.

The action of LF on myelopoiesis was shown to be selective; the protein inhibited only the production of CSF by monocytes/macrophages, but not by lymphocytes, which did not bind LF [30]. LF did not cause the production/release of CSA for B cells and erythropoietin [30,38]. In addition, LF, affecting the production/release of CSF from macrophages, did not change the release of other macrophage-derived factors, i.e. lysozyme, beta-glucuronidase, and plazminogen activator [30].

The studies by Broxmeyer's group presented above suggest that LF acts directly on macrophages and monocytes by diminishing the amounts of released CSF, since the inhibitory action of LF was demonstrated in macrophage and monocyte cell cultures where other cell types (T lymphocytes, endothelial cells, and fibroblasts) were removed [30,32,36]. However, investigations by other groups and later studies by Broxmeyer suggested indirect actions of LF in that process: inhibition of monokine release (IL-1, IL-6, TNF-alpha), which may induce the release of CSF by stromal cells (fibroblasts, endothelial vascular cells) or T cells [8,9,56,61,145]. It is known that monokines, released by monocytes/macrophages present, for example, in the bone marrow stroma, regulate the release of growth factors by other cells (fibroblasts and endothelial cells) as well as by other cell types, including lymphocytes. Studies *in vivo* by Bagby and co-workers revealed interactions of various factors: lactoferrin, monocytes, and T lymphocytes [9] and lactoferrin, monocytes, and fibroblasts [8] in the regulation of myelopoiesis. It appeared that the production of CSA increased by two-fold after addition of even a small number of unstimulated T lymphocytes to the monocyte culture. LF inhibited the production/release of CSA by monocytes only to a small degree and did not inhibit the production/release of CSA by T lymphocytes; however, when these two types of cells were combined in the culture, the inhibition ranged from 22 to 70% of the initial value. The authors concluded that LF almost entirely inhibited the production or release of some factors (monokines) which could stimulate T lymphocytes to CSA production [9]. T lymphocytes, interacting with monocytes and LF, expressed HLA-DR and T3 antigens, but not T4 and T8, and were glucocorticoid resistant. Likewise, a monocyte-conditioned medium contained a factor which increased the CSA production by fibroblasts by 17- to 50-fold [8]. Addition of LF to the monocyte culture inhibited the activity of the monokine by 75–100%, which corresponded to the inhibition of CSA production by fibroblasts. LF did not directly inhibit the production or release of CSA by these cells. Later studies by Bagby allowed the identification of the above-mentioned monokine as IL-1 [7]. IL-1 induces the release of colony-stimulating factors (GM-CSF, G-CSF, and M-CSF) and IL-6 from fibroblasts, endothelial cells, and mononuclear phagocytes in bone marrow and circulating blood. Studies by Broxmeyer and co-workers also indicated a role for IL-6 in mediating LF activity [56,61]. LF lowered the survival of CFU-GM and BFU-E in a human, whole bone-marrow cell culture, but lost its activity when adherent cells (monocytes) and T lymphocytes were removed from the culture. The myelosuppressive effect of LF was abrogated by the addition of IL-1 and IL-6 to the culture medium [56,61]. Further studies suggested that another factor, released by monocytes, may exist (TNF-alpha) whose expression is induced by hematopoietic growth factors. TNF-alpha may also induce the expression of growth factors and some interleukins participating in hematopoiesis.

The indirect myelosuppressive action of LF was also confirmed in another, two-step *in vitro* test [145]. Holo-HLF (10^{-8}–10^{-7} M) was added to monocyte cultures and supernatants from these cultures were used for fibroblast stimulation. In turn, supernatants from fibroblasts cultures were used in clonogenic tests. An inhibition of colony formation was noted: CFU-GM by 30–70% and BFU-E and

CFU-GEMM by 90–100% in relation to control cultures where the cells were incubated without LF. Antibodies against LF completely inhibited LF activity, whereas anti-IL-1 antibodies abrogated the ability of the supernatants from the monocyte cultures to stimulate CSA release by fibroblasts. Determination of IL-1 activity in the monocyte supernatants showed that LF lowered the production of this cytokine by 60–77%.

Therefore, the myelosuppressive action of LF could result from a direct inhibition of CSF production by monocytes and macrophages and may also be a consequence of an indirect inhibition of the secretion of monokines (IL-1, IL-6, TNF-alpha) required for CSF synthesis by T lymphocytes, fibroblasts, and vascular endothelial cells.

Studies by Bagby and co-workers turned attention to still another factor affecting the activity of LF in the regulation of myelopoiesis. Significantly diluted neutrophil extracts added to a bone marrow cell culture inhibited the production/release of CSA; however, the same but undiluted extracts, similarly as at higher concentrations of purified LF (10^{-9}–10^{-6} M) lost such activity (the inhibitory activity was noted at an LF concentration range of 10^{-17}–10^{-9} M) [6]. The reason for this phenomenon was, according to the authors, a calcium-dependent polymerization of the protein already occurring at 10^{-10}–10^{-9} M; the monomeric form of LF exhibited the inhibitory activity, whereas the polymeric form was inactive. Addition of EDTA inhibited protein polymerization by removing Ca^{++} from the environment. The presented concentrations corresponded to 77 and 7.7 ng of LF/ml and are lower than those found in physiological conditions in the blood plasma (the average concentration of LF is 300–500 ng/ml). The quoted results indicate that in physiological conditions, LF may undergo reversible polymerization, which causes loss of its inhibitory action on myelopoiesis. Some studies indicate that polymerization of LF may be favored by binding Fe ions [84]. Polymerization of LF was also described *in vivo*: the polymeric forms of the protein prevailed in physiological fluids during the inflammatory states when high levels of the protein are found [12,16,63,126]. The studies by Bennet and Kokocinski showed that neutrophilic LF occurs in the polymeric or complexed form and plasma LF may occur in both these forms [14]. This phenomenon may have physiological significance: during inflammation, the number of granulocytes and monocytes increases and inhibition of myelopoiesis would be not beneficial. The *in vitro* studies indicated that in such a situation, cells stimulated with bacterial antigens not only produce/release much higher amounts of CSA, but become resistant to the inhibitory action of LF [30]. Similar mechanisms may occur in the organism. It may be expected that in patients suffering from CML, where serum LF is found in elevated concentrations (even 12 μg/ml) [15], a large portion of plasma LF occurs in the inactive polymeric form, which does not inhibit CSA production. To definitively answer the question whether polymerization of LF may really be responsible for the abnormalities seen in CML, further studies are needed. Some studies did not confirm the phenomena of aggregation or polymerization of LF [67,114].

The relevance of LF polymerization for its activity is further complicated by recently discovered facts. Studies by Semenov and co-workers and Kanyshkova and co-workers demonstrated that polymerization of LF may depend not only on the presence of various metal ions, but also on ATP and NAD molecules, commonly present in the organism [74,115]. It appeared that binding of ATP by LF led to dissociation of the olygomeric forms of the protein and changes in the interaction of LF with polysaccharides, DNA, and proteins. For example, in the presence of NAD and ATP and bivalent metal ions, a significantly increased APT-ase activity of LF was noted [74] which may be associated with transition of the protein into the active monomeric form.

In the cited studies by Broxmeyer's group, the majority of observations related to the inhibitory effect of LF on colony formation by granulocyte-macrophage progenitors (CFU-GM). In addition, in some of these studies the effect of LF on colony formation by early erythroid progenitor cells (BFU-E) and myelopoietic stem cells (CFU-GEMM) was described. One of the early studies showed that LF did not affect the formation of BFU-E in human and mouse bone marrow [38], whereas later studies indicated that LF inhibited the activity of erythroid progenitors [56,61]. The inhibition of both BFU-E and CFU-GEMM was also described in other studies [28,29,39].

Broxmeyer and co-workers continue studies aimed at elucidation of the myelosuppressive activity of LF. Very recently they confirmed that the inhibitory activity of LF did not depend on the expression of SDF-1/CXCL12 (Stromal Cell-Derived Factor-1), promoting the survival of hematopoietic stem cells and progenitor cells [29], and that this activity required the expression of MHC class II antigens [28].

The inhibitory effects of LF on myelopoiesis, beside those mentioned above, were also confirmed in other studies. Already in the early 1980s, Fletcher's group reported the release of a factor from phagocyting granulocytes which inhibited the production of CSA by peripheral blood leukocytes used as a feeder layer in the test of granulocyte-macrophage colony formation in semi-liquid agar [103,104]. Later studies by this group demonstrated that the inhibitory activity was identical to that of LF [52]. In the *in vitro* studies, a medium conditioned by phagocytosing human neutrophils and human milk-derived LF was used. It appeared that already small amounts of undiluted medium added to the bone marrow cell culture inhibited the formation of granulocyte-macrophage colonies by about 25–50%. The observed inhibitory action was similar to that obtained after addition of purified milk LF. In both cases the inhibition was abrogated by LF-specific antibodies. Interestingly, also in both cases the inhibitory action of LF disappeared at a concentration of 10^{-12}–10^{-11} M, and saturation of the protein with iron (to 50% total saturation) retained the inhibitory properties at much lower concentrations (10^{-16} M). These results are not entirely consistent with Broxmeyer's and Bagby's findings. The results of Broxmeyer showed that the inhibitory activity of LF occurred at concentrations of 10^{-15}–10^{-6} M and after total saturation with iron even at 10^{-18} M [30]. Bagby, on the other hand, demonstrated that LF lost its activity already at 10^{-10}–10^{-9} M, and interpreted that phenomenon by protein polymerization taking place at these or higher concentrations. Therefore the inhibito-

ry concentration of LF ranges from 10^{-18}–10^{-9} M for holo-LF (85% saturation with iron) [6].

The cited studies by Fletcher provided still another interesting observation: relatively more CSA was produced by mononuclear blood cells cultured at a higher density, i.e. in round-bottom tubes, in comparison with flat surfaces. Importantly, only in the first case did the inhibitory action of LF take place [52]. That finding suggested that cell-to-cell interactions are crucial for the production of such a fraction of CSA which would be susceptible to inhibition by LF. The results confirmed earlier observations by Broxmeyer's group suggesting that the inhibitory effect of LF could depend on culture conditions, particularly on the density of the target cells producing CSA [36].

Lutynski and co-workers in the early 1980s found that human neutrophils isolated from healthy donors contained a factor(s) which inhibited the production or release of CSA by autologous lymphocytes [81]. Lymphocytes were initially cultured in the presence of neutrophils, which were then removed, and the lymphocytes alone or in combination with monocytes were used for conditioning a medium which stimulated the growth of granulocyte-macrophage colonies in agar. The inhibition depended on the number of neutrophils and was the most profound after a short (2-h) incubation. The authors concluded that lymphocytes, in the presence of neutrophils, lose the ability to produce CSA and also diminish the ability of monocytes to synthesize that factor.

Later studies by Wang and co-workers showed, in turn, that LF inhibited the production of a macrophage factor which increased the activity of GM-CSF [132]. The factor was described as "granulomonopoiesis enhancing activity" and was not identical to IL-1. The inhibition was observed only in the physiological LF concentration range (10^{-13}–10^{-8} M) and was proportionally dependent on dose and time of incubation with the factor.

In the majority of the cited studies, CSA or CSF was determined as an "activity" promoting formation of granulocyte-macrophage colonies. However, no attempts were undertaken to identify specific factors which could be responsible for such activity, although in the mid 1980s various purified or recombinant growth factors (GM-CSF, G-CSF, and M-CSF) or interleukins (IL-1, IL-2, IL-3, IL-6) were available. Nevertheless, it is known from that research period that for the formation of granulocyte-macrophage colonies, growth factors such as GM-CSF and G-CSF present in the culture media were responsible [109]; therefore, we may suppose that the described "activity" was mediated by these factors. Further studies confirmed that LF could inhibit the secretion of GM-CSF and that this is accomplished by transcriptional regulation of the GM-CSF gene. Already in 1987, Thorens and co-workers found that LF at a concentration of 10^{-8} M lowered the level of GM-CSF mRNA in mouse macrophages stimulated with fetal calf serum [127]. In the mid 1990s, Penco and co-workers showed that LF inhibited the activity of GM-CSF promoter and the synthesis of the cytokine by a IL-1-beta-stimulated bladder tumor cell line and human fibroblasts [102]. That inhibitory effect was apparent when the cells produced endogenous LF following gene transfection, but was minor when the cells were only incubated with LF.

Lactoferrin as a positive regulator of myelopoiesis

Evidence for the positive regulation of myelopoieis by LF was first delivered by studies by Sawatzki and Rich [114], which were originally undertaken to confirm the negative regulatory hypothesis of Broxmeyer [38]. However, that hypothesis was not confirmed; quite the contrary, the authors showed that LF functions rather as a stimulator and not as an inhibitor of the renewal of the granulocyte and macrophage pool.

Sawatzki and Rich, discussing the results of their studies, referred to earlier investigations, suggesting that the regulation of myelopoiesis is rather accomplished by a mechanism of "supply and demand", where a "demand" signal induces the formation of a new granulocyte-macrophage cell line [89,106,113]. This theory contradicts the negative feedback inhibition theory put forward by Broxmeyer. Quesenberry and co-workers found time-dependent correlations between the production of CSF and the number of circulating neutrophils after injection of endotoxin to mice: a decrease in the neutrophil number preceded by an increase in CSF level was noted [106]. Morley and co-workers, in turn, demonstrated an inverse correlation between the level of plasma CSF and the number of circulating neutrophils in irradiated animals [89]. In mouse serum a factor appeared which stimulated the formation of granulocyte colonies and neutropenia led to an increased production of that factor. The authors noted that increased production of CSF was accompanied by a lower granulocyte number in blood, which suggests that a direct stimulation of CSA release by macrophages and other cells does not occur; it seems rather that the phenomenon is mediated by a factor(s) released by granulocytes in circulating blood. The release of that mediator(s) depends on the turnover of circulatory granulocytes. The available information on LF and the regulation of myelopoiesis led the researchers to suppose that LF could be that factor.

In their studies, the authors evaluated the effect of *i.v.* injection of LPS and *S. typhimurium* on serum LF level [114]. Six hours after injection, a significant dose-dependent increase in LF blood level was noted. For example, administration of 500 µg LPS caused elevation of the mean serum LF concentration from 255 ng/ml to 1154 ng/ml (a fourfold increase). To prove that the increase in plasma LF concentrations was associated with neutrophil turnover, subsequent studies involved neutropenic mice. In the blood of these mice, much lower LF concentrations were found (mean: 15 ng/ml) compared with normal mice (mean: 194 ng/ml). However, after administration of LPS, an increase in the plasma LF concentrations in both groups was registered, up to 98 and 890 ng/ml, respectively (a 5- to 6-fold increase). It is worth noting that the LF level in neutropenic mice, even after administration of a high LPS dose, did not attain the normal value found in healthy mice. The results clearly indicate that the LF blood level is correlated with the neutrophil number. What is more, there exists a certain fraction of cells, not dependent on the absolute granulocyte number, which undergoes degranulation and releases LF after LPS induction.

Is there, however, any correlation between plasma concentration of LF and CSF involved in myelopoiesis? It could

be anticipated that in the case of inhibition of colony stimulating factors by LF, their concentrations should be inversely correlated with the LF level. The studies by Sawatzky and Rich, however, did not reveal such a correlation [114]. Intravenous injection of species-specific LF (2 mg/mouse, three times, every 12 h) resulted only in a minor decrease in bone marrow cellularity, although, at the same time, a significant increase in the formation of granulocyte-macrophage colonies by the bone marrow cells was observed. The number of CFU-GM obtained 12 and 24 h after LF injection was indeed lower, but still exceeded the values seen in control animals. An increase in spleen cellularity, but not in the number of spleen CFU-GM, was observed, which indicated a lack of stimulation of granulopoiesis in this organ. A maximal increase in plasma CSF concentrations was noted 12 h after administration of LF, followed by a decrease, not differing from the initial values after 48 h. It seems therefore possible that the increase in bone marrow CFU-GM registered after 48 h following LF administration resulted from the increase in plasma growth factor concentration observed earlier. In summary, the presented results of the *in vivo* studies did not demonstrate any inhibition of myelopoietic growth factor production by LF; on the contrary, evidence was provided for the stimulation of their production, which preceded the increase in myelopoiesis [114].

In vitro studies confirmed the above-described results [114]. In these tests, mouse or human LF (10^{-7}–10^{-6} M) were added to cultures of mouse bone marrow cells and adherent peritoneal macrophages. The 48-h supernatants from these cultures were harvested for conditioning of bone marrow cell cultures in a semi-liquid medium with addition of methylcellulose. After seven days of culture, the granulocyte-macrophage colonies were counted. It appeared that the supernatants derived from cell cultures stimulated with LF increased CSF production, which was dependent on the cell density in the culture. The addition of indomethacin (a PGE inhibitor) further increased the production of growth factors by the cultures incubated with mouse or human LF. The incubation of bone marrow cells or peritoneal macrophages with mouse or human LF, irrespective of indomethacin addition, resulted in stimulation of CSF activity by as much as 200–300%. It must be underlined that in both the *in vitro* and the *in vivo* tests teh LF preparations were endotoxin free (application of polymyxin B affinity chromatography). LF was isolated from mouse or human milk.

Sawatzki and Rich applied relatively high LF concentrations: 2 mg/mouse *in vivo* and 10^{-7}–10^{-6} M *in vitro*. Broxmeyer and co-workers used the protein at considerably lower concentrations: 10^{-17}–10^{-6} M *in vitro* and 100 or 300 µg/mouse [30,57]. Sawatzki and Rich were motivated to extend the applied doses by a necessity to reach high LF plasma levels, similar to those occurring upon infection. One million neutrophils contain about 5 µg of LF; the daily production of these cells amounts to 10^{12}, which corresponds to 5 g of LF per day. In a healthy state, about 10% of this amount is released from cells. During infection, however, significantly higher turnover of neutrophils and LF levels are observed: the neutrophil turnover increases as much as 50-fold, which is accompanied by an increased destruction of these cells which releases 30% of LF. The authors estimated that under such conditions as much as 30 g of LF per day may be produced, of which 10 g may be released into the circulation. Therefore, taking into consideration the concentration of LF required for the saturation of cell-binding sites and the short half-life of the protein, the amount of protein should significantly exceed normal plasma levels in order to demonstrate a given action of the protein [114].

Based on their results, Sawatzki and Rich classified LF as a myelopoiesis-stimulating factor. According to the authors, recruitment of neutrophils is not under a mechanism of negative regulation, but is rather regulated by a “supply and demand” mechanism or by “demand” signals liberated by neutrophils. Each dying neutrophil releases such a signal, which subsequently stimulates macrophages to the production of growth factors for early cells of the granulocyte-macrophage lineage. Lactoferrin seems to represent such a signal. In the case of an increased demand for neutrophils, LF may also stimulate macrophages (present, among other sites, in the reticulo-endothelial system) to cytokine production (e.g. IL-1, TNF-alpha) which may, in turn, induce the release of growth factors by endothelial cells and fibroblasts. T lymphocytes may also have a certain role in stimulating myelopoiesis; these cells, by interaction with Ia antigens on macrophages, stimulate the release of growth factors by these cells [110,114]. The model of positive regulation of myelopoiesis proposed by Sawatzki and Rich resembles another one presented earlier by Robinson and Mangalik [113], except that LF was defined at that time as the factor responsible for the stimulation of granulocyte colonies. The model of positive regulation of myelopoiesis, taking into account the role of LF, is presented in Figure 1.

In response to the published studies by Sawatzki and Rich, a critical commentary by Bagby appeared [5]. Sawatzki and Rich responded to the criticism of their studies [111]. The polemics represents an additional contribution to elucidating many doubts associated with the studies on the myelopoietic activity of LF regarding, among others, technical aspects of the performed tests, the origin and purity of the LF preparations, the doses of LF applied, and the choice of experimental models. In Table 1, some of the critical remarks by Bagby and the responses by Sawatzki and Rich are presented.

The mechanism of Lactoferrin action on myelopoiesis

Assuming the participation of LF in the positive regulation of myelopoiesis, the following mechanism(s) of LF’s action in this process in plausible. Elucidation of the precise mechanisms of LF action in the process of myelopoiesis needs further research, but even now we propose that LF indirectly regulates the activity of various hematopoietic cytokines. It has been shown that LF affects the production and/or release of some of them: IL-1, IL-6, IL-8, TNF-alpha, as well as GM-CSF and G-CSF growth factors. As mentioned before, the stimulation of GM-CSF and G-CSF production by LF was shown by Sawatzki and Rich [114]. The stimulation of IL-1 secretion by LF was found in our studies [139] as well as by others [75,118]. The stimulation of IL-6 production was shown in many studies, including our reports [75,76,82,141–143]. The stimulation of IL-8 secretion under the influence of LF or its peptides was confirmed in other studies [76,117,119], similarly as the stimulation of TNF-alpha secretion [51,75,76,119].

Figure 1. Participation of lactoferrin in a model of positive regulation of myelopoiesis. During turnover of neutrophils, both in a healthy state and during infection, LF is liberated, constituting a "demand signal" for macrophages which triggers these cells to produce growth factors for cells of the granulocyte-macrophage lineage. When the demand for neutrophils increases, LF may also stimulate macrophages outside bone marrow (among others in RES) to producte cytokines, such as IL-1 and TNF-alpha, which may, in turn, stimulate the release of growth factors by endothelial cells and fibroblasts. In the presented model, a role for T lymphocytes in the stimulation of myelopoiesis was not considered; these cells, by interaction with macrophages, may also be stimulated to secrete growth factors. CSF – factors stimulating myelopoiesis (according to 110; 114)

The described cytokines belong to the most important regulators of hematopoiesis, including myelopoiesis. For example, IL-1 stimulates the proliferation of precursors cells of the granulocyte-macrophage, erythroid, and megakaryocyte lineages. It acts on the erythropoietic system mainly indirectly, by inducing the production of GM-CSF, G-CSF, IL-2, IL-6, and TNF-alpha. IL-6, besides its effects on the proliferation and differentiation of pluripotent stem cells and progenitor cells of many lineages, including erythrocytes, granulocytes, macrophages, and megakariocytes [100], represents an important factor regulating the function of stromal cells [47,68]. The cytokine, by activating erythropoiesis, acts synergistically with IL-3. It also augments the action of other erythropoietic factors. IL-8 acts on neutrophils, activating, among others, respiratory processes, chemotaxis, and degranulation and increasing cytotoxicity. It belongs to the factors releasing granulocytes from the bone marrow [71]. TNF-alpha is an important function mediator of the granulocyte-macrophage mature cell lineage, increasing their phagocytic, cytotoxic, and bacteriocidic properties. It also accelerates the release of granulocytes from the bone marrow. The role of TNF-alpha in the regulation of myelopoiesis has been not entirely elucidated. The majority of studies indicate that TNF-alpha inhibits the proliferation of progenitor cells of the granulocyte-macrophage lineage; on the other hand it stimula-

Table 1. Responses of Sawatzky and Rich to the critisism of Bagby regarding the authors' studies on the effect of LF on myelopoiesis

Critical remarks by Bagby [5]	Response of Rich and Sawatzki [111]
Application of the whole population of bone marrow cells as the cellular source of the conditioning medium, i.e. cells releasing growth factors for the granulocyte-macrophage cell lineage. Such a medium may contain other cytokines (IL-1 and TNF-alpha) besides growth factors (CSF), which may directly stimulate growth of CFU-GM. The presence of such cytokines was not measured. From the bone marrow cell population no cells were removed which could, potentially, release high amounts of growth factors after induction by these cytokines.	The factors other than growth factors and cells which could be induced for release of CSF by these factors were not removed on purpose. The experimental model was designed to be as close as possible to the *in vivo* conditions, although the *in vivo* conditions cannot be restored *in vitro* for various reasons. Even initial removal of such substances and cells from the culture system does not guarantee that the culture will be still devoid of them because of a possible "influx" of the cells from the compartment of more early progenitor cells present in the bone marrow population.
At the lowest bone marrow cell number, used for conditioning of medium, i.e. 2×10^4 cells/ml, with the addition of indomethacin in the presence of mouse LF, an inhibition of CFU-GM formation was noted. That fact was ignored by the authors; it may result from an improper application of control cultures.	Indeed, in the case of that cell number, used for conditioning of the medium, the inhibition of CSF production was observed. However, no such inhibition was registered in cases of 10^5 and 5×10^5 cells/ml as well as with peritoneal macrophages used for the same purpose. It seems that the observed inhibition may be regarded as an exception and not as the rule.
Lack of demonstration, by means of specific antibodies, that LF was responsible for the described effects, i.e. no specificity of the described effects was shown.	In the experiments the use of specific anti-LF antibodies was not foreseen because such antibodies were not available.
Lack of confidence that the observed results are not caused by activities mediated by contaminations present in LF preparations and not by LF itself. Electrophoretic studies do not exclude a presence of contaminations, for example growth factors, endotoxins, interferons or cytokines (IL-1), able to affect myelopoiesis. Likewise, application of LAL test does not exclude contamination with LPS.	The preparations of human and mouse LF were purified according to generally accepted multi-step biochemical procedures. The procedures included: ammonium sulfate precipitation, ion exchange chromatography, affinity chromatography in the presence of heparin and gel filtration. Such procedures should eliminate the mentioned contaminations (growth factors, IFN and IL-1). During gel filtration and electrophoresis, as well as after the purification procedures, such contaminations were not found in the LF preparations. In addition, all preparations were also purified on polimyxin B-sepharose to eliminate potential LPS contamination.
The application, besides bone marrow cells, of peritoneal macrophages as the source of growth factors in the conditioning medium. Peritoneal macrophages are, in this case, anatomically not adequate source of growth factors since hemopoiesis does not occur in the peritoneal cavity of healthy animals. Moreover, among peritoneal macrophages a large fraction of mesothelial cells can be found, which are difficult to discriminate microscopically from macrophages.	The biggest significance in studying myelopoiesis have bone marrow cells, including macrophages. Additional tests on peritoneal macrophages were undertaken to confirm that this cell type, irrespectively on its place of residence, has an ability to produce growth factors in response to certain signals. Mesothelial cells were nor discriminated from macrophages; these two cell types are not only morphologically similar but also with respect to release some growth factors.
The administration of too high LF doses to mice (according to Bagby's calculations a 10^{-5} M LF solution was given). It could be expected that LF underwent polymerization already in the syringe and became inactive. The induction of CSA in the mouse serum reflected rather activation of macrophages through the polymerized protein (biologically not active) capable, however, to stimulate the inflammatory response accompanied by IL-1 release, which, in turn, induced expression of growth factors genes.	During gel filtration and electrophoresis no polymerization of LF was noted. Before administration to mice LF preparations were diluted with physiological saline which additionally restricted a possibility of polymerization. The applied LF doses, both *in vitro* (10^{-7} M) and *in vivo* (2.6×10^{-8} M), are, in fact, several times higher than those found in serum of healthy mice (about 10^{-8} M), but one has to remember that the LF concentrations significantly increase during bacterial infections or after injection of endotoxin.
The studies were based on functional tests, i.e. on determination of the number of colonies, formed by progenitors of macrophages and granulocytes. The proliferative activity of these cells was not, however, evaluated, i.e. it was not determined how many of these are in the cell cycle.	
The binding of LF to target cells (macrophages) was not determined, (the activity of LF may depend on the interactions with these cells).	In these studies we referred to earlier studies showing an ability of LF to bind to mouse [128] and human [17] macrophages.

tes the final differentiation and maturation of granulocytes [50]. TNF-alpha may, in addition, strongly augment the activities of other cytokines which stimulate myelopoiesis, for example IL-3 and GM-CSF [41].

GM-CSF has a broad spectrum of biological activity. It stimulates the proliferation of multipotent myelopoietic stem cells (CFU-GEMM), precursor cells of the erythroid (BFU-E), granulocyte-macrophage (CFU-GM), and me-

gakariocyte (CFU-Meg) lineages. G-CSF has a more narrow spectrum of activity in comparison with GM-CSF. It stimulates the proliferation and differentiation of essentially committed cells of the granulocyte lineage (CFU-G). In addition, it stimulates the formation of granulocyte colonies by CFU-G, although it may also initiate the growth of granulocyte-macrophage colonies formed by CFU-GM [112]. Although alone it does not affect colony formation by CFU-GEMM, it acts synergistically on these cells with IL-3 and modulates their biological activity in response to IL-1 and IL-6 [107,112]. Both growth factors similarly inhibit apoptosis of mature granulocytes [44], extending the life spans of these cells, and stimulate the activity of mature cells of the granulocyte-macrophage lineage.

It must be underlined that the above-mentioned hematopoietic factors affect each other mutually; among others, the production of IL-1 and TNF-alpha results in increased production of growth factors (GM-CSF and G-CSF), IL-6, and IL-1 in an autocrine way. The growth factors increase, in turn, the production of IL-1, IL-6, IL-8, and TNF-alpha [112,130,144]. Some of the cytokines act synergistically, mutually enhancing their activities.

The results of our preliminary, unpublished studies suggest, in addition, that LF may affect myelopoiesis by direct or indirect (via IL-1 and IL-6) stimulation of the HPA axis, which leads to the release of corticosteroids. These hormones affect the granulocyte pool in several ways. Corticosteroids have the ability to stimulate myelopoiesis, as demonstrated both *in vivo* [53,79] and in cultures of the bone marrow cells [59]. The hormones increase, in addition, the recruitment of immature and mature granulocytes from the bone marrow reservoir [45] and prolong the life-spans of mature granulocytes by lowering their susceptibility to apoptosis [80]. Investigations are underway to confirm our preliminary findings regarding the effect of LF on endogenous steroid release.

Corticosteroids may, in turn, inhibit the release of LF from neutrophils. In *in vitro* tests. corticosteroids (including dexamethasone) inhibited the degranulation of neutrophils and, consequently, the release of LF [43]. Similar results were obtained in *ex vivo* tests. Granulocytes isolated from SLE patients, treated with various doses of methylprednisone, showed a lowered ability for adhesion, degranulation and LF release, and the uptake and killing of bacteria [19]. As shown by Broxmeyer, hydrocortisone and dexamethasone did not affect the process of GM-CSF production by LF [30]. On the other hand, they block the inhibitory action of LPS on LF activity. As a result, the inhibitory action of LF on myelopoiesis is restored. Thus corticosteroids on the one hand hamper the release of LF from cells, but on the other they restore the inhibitory action of the protein on myelopoiesis, which may result in a diminished supply of granulocytes and macrophages in the presence of bacterial endotoxins (a feedback inhibition mechanism). In summary, the following scenario regarding the regulation of myelopoiesis during infection can be proposed. Under the influence of bacteria-derived factors (LPS) and pro-inflammatory cytokines, LF concentrations increase due to granulocyte degranulation. LF, in the presence of LPS, loses its inhibitory activity on myelopoiesis and, by stimulating corticosterone release, additionally enhances the process of myelopoiesis. Elevated concentrations of the hormones eventually inhibit LF release from granulocytes and restore the ability of LF to inhibit myelopoiesis, abrogated by LPS.

Lactoferrin does not participate in the regulation of myelopoiesis

There are reports on myelopoiesis that do not confirm any regulatory function of LF. The data are derived from both *in vitro* and *in vivo* experiments. Winton and co-workers conducted *in vitro* tests aimed at demonstrating the effects of LF on myelopoiesis [135]. A homologous model was applied (a mouse milk-derived LF and mouse cells). Iron-saturated LF in the concentration range of 10^{-13}–10^{-7} M was used in various models serving to evaluate myelopoieis *in vitro*. The media, conditioned by various cells (bone marrow, lung, or peritoneal adherent cells), were inspected for CSA content in tests of colony formation in semi-liquid agar or in tests measuring the incorporation of labeled thymidine to DNA. LF was added to the bottom layer of feeder cells in agar, which produced growth factors for the bone marrow cells placed in the upper layer. The authors could not demonstrate any effect of LF on the production of growth factors and the formation of granulocyte-macrophage colonies in the performed tests, despite the fact that the applied concentrations of LF were within the range or even exceeded the physiological concentrations of LF in serum (10^{-7} M equals about 8 μg/ml). They suggested several possible reasons why the effect of LF on myelopoiesis could not be demonstrated, among others the application of too low LF concentrations and to the small numbers of cells which were the source of growth factors for granulocyte precursors [135]. A next interesting presumption is associated with the ability of LF to bind negatively charged molecules. In such a case, the inhibitory action of LF on myelopoiesis could be due to inhibitory properties of other, acidic compounds bound to the LF molecule. The purity/homogeneity of the LF preparations used by various laboratories could also contribute to the different results [135].

Studies carried out by Stryckmans and co-workers [122] also could not reveal any effect of LF on myelopoiesis. In the studies, human milk-derived LF was used. Iron-saturated protein was added to endogenously stimulated bone marrow cells or to cultures of mononuclear cells used as the feeder cell layer for bone marrow cells. In both tests, the number of granulocyte-macrophage colonies, reflecting the production of CSA by macrophages and monocytes, was counted. Despite the application of a wide range of LF concentrations (10^{-18}–10^{-8} M), no inhibition of CFU-GM formation could be demonstrated. The authors suggested several possible reasons for the lack of LF activity [122]. A presence of PGE, masking the action of LF, could be one of the reasons. However, addition of indomethacin to the culture did not change the results, which excluded the involvement of PGE in the studied system. The presence of acidic izoferritins also had no effect on the obtained results because of their negligible concentrations in the culture. A further possible cause for the lack of LF activity would be masking of its activity by bovine LF present in fetal calf serum (100 ng/ml), even after long-term heating at 56°C. However, when LF was remo-

ved by means of specific antibodies, the results were similar. Also, the binding of LF to plastic surfaces could not be the cause of lack of LF activity, since only 3% of the protein was bound to plastic even after six hours of incubation. The activity of LF was not modified by the applied procedures and the composition of the culture media (no effect of various concentrations of Ca^{++} and Fe^{++} ions). Of interest is that the authors could not demonstrate binding of LF to human monocytes and lymphocytes, similarly as endocytosis of LF by these cells. On the other hand, such interactions were demonstrated for LF and rat hepatocytes. Lack of interaction of LF with the target cells may explain the lack of activity of the protein since, as mentioned above, the action of LF is conditioned by its binding (or endocytosis) to cells.

A lack of LF effect on myelopoiesis was also demonstrated by Poppas and co-workers [105]. A mouse model of myelopoiesis renewal after administration of a sublethal CP dose was applied. Mice were injected *i.v.* or *i.p.* with human LF. Determination of the number of femoral bone marrow cells and blood leukocytes and the incorporation of labeled thymidine by bone marrow cells did not show statistically significant differences between the control and LF-treated groups. Nor a way of LF administration and the age and sex of mice change the parameters of myelopoietic renewal.

Studies by Galbraith delivered inconsistent results on the effects of LF on myelopoiesis [54]. Several types of *in vitro* experiments were performed with the use of holo-LF at a concentration range of 10^{-12}–10^{-10} M. LF was added to cultures of human nonadherent bone marrow cells producing endogenous growth factors or stimulated with growth factors present in conditioned media by mononuclear human blood cells. After an established time interval, the number of CFU-GM colonies were counted and their size was also determined. LF did not significantly stimulate the studied parameters both in an early (day 4) and a later (day 7) phase of colony growth. The action of LF on the stimulated cultures was rather inhibitory, although an increase in colony size in the early phase of growth was noted, suggesting an enhancement by LF of the activity of the preformed growth factors present in the culture. The author suggested that the inhibition observed in the late phase of colony growth represents rather a result of a two-phase kinetics of the colony growth than actual suppression: after the initial, LF-induced, quicker proliferation of cells, a more early cell death occurs. LF did not have any effect on *de novo* production/release of growth factors after their addition to cultures of human mononuclear leukocytes. Nevertheless, when added to the medium after the cells' removal, it affected the preformed CSF, either by increasing or by decreasing their activity. In addition, LF influenced the cell cycle of bone marrow cells by changing the cell fraction in the G2 and M phases of the cycle. That effect is probably mediated by the regulation of DNA synthesis and may be both positive as well as negative. That probably depends on the origin of the studied cells and the cell composition. For example, when the bone marrow was depleted of monocytes, LF increased DNA synthesis. Therefore, because of discrepancies in the results (different results were observed many times in repeated experiments, indicating stimulation, inhibition, or no LF activity), the author suggested complexity of LF action and imperfection of the tests used for evaluating LF activity.

The available data regarding the effect of LF on myelopoiesis are presented in Table 2.

Possible reasons for discrepancies in Lactoferrin action on myelopoiesis

The results of the LF effect on myelopoiesis are exceptionally divergent. Below a list of possible causes is presented which may explain the existing discrepancies.

Regarding the inhibitory activity of LF, the cell composition may be of importance; some studies showed that the action of LF may be indirect and require the presence of cells other than monocytes/macrophages (T lymphocytes, fibroblasts, endothelial cells) [8,145].

Monocytes and macrophages have the ability to synthesize E prostaglandins (PGE) and acidic isoferritins, which lower the sensitivity of cells forming CFU-C on colony-stimulatory activity [78]. Neutrophil extracts and LF preparations should not contain PGE and acidic isoferritins, which can obscure the picture of the inhibitory action of the extracts or LF preparations.

The regulation of the production/release of GM-CSF depends on many factors, and even *in vitro*, more complex interactions may be expected *in vivo*. The following factors may, among others, influence the LF activity: the molecule structure, saturation with iron, presence of other metals and modifying molecules (sex hormones and glucosteroids), the presence of sensitive target cells able to bind and respond to the protein, and the existence of mutual interactions between these cells.

Contamination of LF with endotoxin may mask the inhibitory action of the protein on myelopoiesis [23]. Similar significance may have contamination of neutrophil extracts and LF preparations with CSA and/or other factors able to stimulate CSA production.

Some studies [6,38] indicate that threshold concentrations of LF, still able to inhibit myelopoiesis, are unusually low (10^{-17} M). Fetal calf serum added to the culture media provides higher LF concentrations (100 ng/ml) [122]. Thus, even in control media an inhibition of colony formation may be expected which, however, will be regarded as the "background" for other experimental cultures containing the studied LF preparations. Target cells incubated in the constant presence of LF may also be refractory to LF added to a culture. The addition of LF may not cause further inhibition, all the more because higher protein concentrations favor its polymerization and the formation of inactive forms of LF. Some researchers use serum devoid of LF by means of specific antibodies, whereas others claim that the protein undergoes inactivation upon standard heating of serum (30 min, 56°C) [32]. Currently, chemically defined, serum-free media are commercially available for the purpose of hematopoietic cell cultures. Application of such media allows elimination of the effects of some serum components (hormones, growth and inhibition factors), enabling a strict control of culture conditions and facilitating the interpretation of results.

Table 2. The available data regarding the effect of LF on myelopoiesis

Effects of LF on myelopoiesis	Description of the experimental models and results	References
Inhibition	Studies with application of human blood leukocytes as a feeder layer, stimulating the formation of colonies by human bone marrow cells	[10]
Inhibition	Studies of the activity inhibiting formation of granulocyte-macrophage colonies (CIA), contained in neutrophil extracts and media conditioned by mouse and human neutrophils. As target cells for CIA monocytes and macrophages were used.	[34]
Inhibition	*In vivo* and *ex vivo* studies on healthy mice and mice treated with a sublethal dose of cyclophosphamide (renewal of myelopoiesis). Extracts of human neutrophils and mouse bone marrow cells were studied. CSA levels in the blood serum and in media conditioned by marrow, heart and lung cells from mice receiving *i.v.* neutrophil extracts. Functional tests were applied where the amount of secreted CSA was evaluated by counting granulocyte-macrophage colonies. The effect of LPS injections on reversal of neutrophil extracts action was studied.	[23]
Inhibition	*In vitro* studies on bone marrow and blood cells from healthy humans and WEHI-3 cell line, and *in vivo* and *ex vivo* in mice treated with sublethal dose of CP with application of neutrophil extracts and human milk-derived LF. Functional tests were applied where the amount of secreted CSA was evaluated by counting granulocyte-macrophage colonies. The inhibitory activity on colony formation was identified as LF. The effect of iron saturation of the LF molecule on its activity and addition of LPS to the cell culture on the reversal of the inhibitory LF action, were studied.	[38]
Inhibition	*In vitro* studies with human monocytes with use of holo-LF (no origin given) at a concentration of 10^{-7}–10^{-6} M. The amount of secreted CSA was evaluated by the number of granulocyte--macrophage colonies. A population of human monocytes was identified, bearing Ia antigens as cells responding to the inhibitory action of LF.	[24]
Inhibition	*In vitro* studies with human monocytes with application of holo-LF or lysates of iron-saturated extracts of human neutrophils. The secreted CSA was determined by the number of granulocyte-macrophage colonies. It was found that active LF was derived from neutrophils forming rosettes with sheep erythrocytes coated with IgG, i.e. with cells bearing receptors for Fc fragment of IgG.	[37]
Inhibition	*In vitro* studies on bone marrow and blood cells from healthy individuals and on mouse macrophages were conducted with application of human milk-derived LF. CSA was evaluated by the number of granulocyte-macrophage colonies. The roles of binding different metal ions, corticosteroids, sex hormones, lithium and LPS in the activity of LF were studied. The specificity of LF action was confirmed: the protein bound to human monocytes and mouse peritoneal macrophages but not to lymphocytes, and inhibited CSA secretion from these cells and not other substances.	[30]
Inhibition	*In vitro* studies were performed on bone marrow and blood cells from healthy individuals using human milk-derived LF, both native and iron-saturated. CSA was determined by counting granulocyte-macrophage colonies. To the medium, conditioned by the cells, in the presence of LF, fetal calf serum depleted of endogenous LF was added without indomethacin. Interactions of LF with monocytes and T lymphocytes in regulating myelopoiesis was demonstrated: LF inhibited the production of monokines by monocytes which stimulated T lymphocytes to CSA secretion.	[9]
Inhibition	*In vitro* studies were performed with human mononuclear leukocytes using extracts of neutrophils from human peripheral blood. CSA was evaluated by counting granulocyte-macrophage colonies. Extract devoid of LF by means of specific antibodies lost the ability to inhibit CSA secretion. A process of Ca^{++}-dependent LF polymerization was discovered which abrogated the LF activity.	[6]
Inhibition	*In vitro* studies were conducted on human fibroblasts and monocytes using milk-derived human LF. CSA was determined by the number of granulocyte-macrophage colonies. Fetal calf serum devoid of endogenous LF and indomethacin was added to the culture medium. Interactions of LF with monocytes and fibroblasts in regulating myelopoiesis was demonstrated: LF inhibited the production of monokines by monocytes, which stimulated fibroblasts toi CSA release.	[8]
Inhibition	*In vitro* studies on mouse peritoneal macrophages and *in vivo* on healthy mice and mice treated with a sublethal dose of CP were conducted. Human LF was administered *i.v.* or *i.p.* CSA was determined by enumeration of granulocyte-macrophage colonies. The effects of heating and iron saturation of LF on its activity were investigated. The effect of LF on the cell cycle was also checked.	[57]
Inhibition	Comparative studies between bovine and human LF as well as the apo- and holo-forms of both proteins in their ability to inhibit the release of CSA by human mononuclear blood cells and mouse peritoneal macrophages were performed. Indomethacin and complete fetal calf serum were added to the cultures and the cells were incubated at low O_2 concentration. CSA was determined by the number of granulocyte-macrophage colonies. In addition, experiments on human monoblastic U937 cell line, positive for Ia antigens, were done. Experiments with neutrophils isolated from a child suffering from neutrophilia of unknown etiology confirmed the role of LF in the negative regulation of myelopoiesis.	[32]

Table 2 continiued. The available data regarding the effect of LF on myelopoiesis

Effects of LF on myelopoiesis	Description of the experimental models and results	References
Inhibition	*In vitro* studies on mouse cells using human, milk-derived LF were performed. Complete fetal calf serum and indomethacin were added to the culture. The amount of CSA was evaluated by counting granulocyte-macrophage colonies. A population of mouse peritoneal macrophages, bearing Ia antigens, as the target cells for the inhibitory LF action, was identified.	[36]
Inhibition	In the *in vitro* tests the ability to inhibit CSF secretion by human, mononuclear blond cells, was investigated. Holo-LF activity, isolated from neutrophil extracts and from milk, was studied in the functional tests. Indomethacin and a complete fetal calf serum was added to the culture. LF receptors on mouse peritoneal macrophages were identified.	[27]
Inhibition	In *in vitro* tests the ability to inhibit CSF secretion by human mononuclear blond cells was investigated. Activity of human LF derived from phagocyting neutrophils and from milk, was studied by applying functional tests. Complete calf serum (but no indomethacin) was added to the cell cultures. The effect of the density of the cells producing GM-CSF in culture as well as the effect of iron-saturation of LF preparations on LF activity were evaluated.	[52]
Inhibition	*In vitro* studies with application of human holo-LF were carried out. CSA was determined by counting: granulocyte-macrophage colonies, CFU-GEMM, and BFU-E. The interactions of LF with monocytes and fibroblasts in the regulation of myelopoiesis were demonstrated: LF inhibited the production of IL-1 by monocytes, the cytokine stimulating fibroblasts to CSA secretion.	[145]
Inhibition	*In vitro* studies with application of human holo-LF were carried out. CSA was determined by counting: granulocyte-macrophage colonies, CFU-GEMM, and BFU-E. The interactions of LF with monocytes and fibroblasts in the regulation of myelopoiesis were demonstrated: LF inhibited the production of IL-1 by monocytes, the cytokine stimulating fibroblasts to CSA secretion.	[61]
Inhibition	*In vitro* studies on human bone marrow cells, using holo-LF, were conducted. CSA was evaluated by counting granulocyte-macrophage colonies and BFU-E. A role for IL-6 in the myelosuppressive action of LF, mediated by monocytes, was demonstrated.	[56]
Inhibition	*In vitro* studies with mouse bone marrow cells were performed. The amount of secreted CSA was evaluated by counting granulocyte-macrophage colonies, CFU-GEMM and BFU-E. It was found that the myelosuppressive action of LF was not affected by SDF1/CXCL12 (Stromal Cell-Derived Factor 1), a factor promoting survival of hematopoietic stem cells and progenitor cells.	[29]
Inhibition	*In vitro* tests on mouse bone marrow cells, using human LF. CSA was assessed by enumerating granulocyte-macrophage colonies, CFU-GEMM and BFU-E. It was confirmed that the myelosuppressive action of LF requires expression of MHC class II antigens on bone marrow cells. A significance of the transcriptional factor CIITA, regulating MHC class II expression, was suggested.	[28]
Stimulation	*In vitro* studies with mouse bone marrow cells and peritoneal macrophages and *in vivo* and *ex vivo* on mice using mouse and human LF isolated from milk and iron-saturated. *In vitro* LF was used at 10^{-7}–10^{-6} M, *in vivo* LF was given *i.v.* at a dose of 2 mg/mouse in 1–3 doses. The cellularity of bone marrow and spleen and CSF serum content was evaluated. CSA was determined by counting granulocyte-macrophage colonies. LPS was removed from LF preparations and the endotoxin content was measured in the preparations. Complete fetal calf serum and indomethacin were added to the cell culture. No polymerization or aggregation of LF was confirmed.	[114]
No effect	*In vitro* studies with human bone marrow and peripheral blood monocytes using human holo-LF (10^{-18}–10^{-8} M) isolated from milk. CSA was measured by counting granulocyte-macrophage colonies. Indomethacin and a fetal calf serum, devoid of endogenous LF, were added to the cell cultures. Various methods of cell separation were used for the tests as well as different culture media. The possibility of significant adherence of LF molecules to plastic surfaces in culture having any effect on the results was excluded.	[122]
No effect	*In vitro* studies in the homologous system: mouse LF-mouse cells. Holo-LF was applied at 10^{-13}–10^{-7} M. As the source of CSA peritoneal macrophages, alveolar cells and bone marrow from femur were used. CSA was determined by counting granulocyte-macrophage colonies. Complete fetal calf serum (and no indomethacin) was added to the cultures.	[135]
No effect	*In vivo* studies with application of human LF administered to mice *i.v.* or *i.p.* The total cellullarity of the bone marrow, the number of leukocytes in the peripheral blood and the incorporation of labeled thymidine to bone marrow cells, were evaluated.	[105]
No effect	*In vitro* studies with application of holo-LF (of unknown origin) at 10^{-12}–10^{-10} M, were performed. The effect of LF on the clonogenic activity of granulocyte precursors in human bone marrow and production of CSF by mononuclear leukocytes were evaluated. The effect of LF on the cell cycle of bone marrow cells was also analyzed.	[54]

As indicated in some studies, polymerization of the protein may occur already by low LF concentrations, as manifested by the presence of various oligomeric forms of the protein (monomers, dimers, trimers, and tetramers). Mutual transformations of these forms are very likely, are favored by the high "fluidity" of the LF molecule [1], and may depend not only on the protein concentrations, but also on the presence of other factors, such as iron and other metal ions, ATP, NAD, low-molecular-weight ligands, and solution ionic strength [74,84,115].

The activity of LF may be significantly affected, among others, by iron ions, easily scavenged by the protein. Many studies proved that apo-LF has a smaller effect of myelopoiesis regulation than native LF; this, in turn, being less active than holo-LF. For example, apo-LF inhibited myelopoiesis at 10^{-7} M and iron-saturated LF at such a low concentration as 10^{-17} M.

The LF preparations themselves may also affect the results. Not only the purity of LF preparations may have significance (traces of cytokines, growth factors, or endotoxins), but also whether the LF was used in a homo- or heterologous model. In the *in vitro* tests, mouse and human cells were used and human, bovine, or mouse LF. Therefore, in some of the experiments, homologous models were applied. In the majority of the *in vivo* studies on mice, the heterologous human or bovine protein was used because mouse LF was not available. The studies by Sawatzki and Rich, Winton and co-workers were an exception: the researchers used *in vitro* both mouse and human LF (no differences in the activities were demonstrated) and *in vivo* mouse LF [114]. It appeared that even LF of the same origin, but isolated in different laboratories, may significantly differ in some properties, for example in the ability to polymerize. As Bagby comments [5], milk-derived LF isolated in his laboratory polymerized already at 10^{-10} M. The same batches of LF also polymerized in the studies of Broxmeyer, but LF isolated by Broxmeyer did not polymerize in the experiments performed by Bagby [5]. Sawatzki and Rich showed in their studies [114] a lack of polymerization of both milk-derived human and mouse LF, even at high protein concentrations (10^{-8} M). Some significance for the results on the myelopopietic activity of LF may have the cellular origin of the protein, since Spik and co-workers demonstrated that human neutrophil-derived LF is devoid of fucose in contrast to milk-derived LF [48,120], and high-affinity cellular LF receptors recognize a sugar moiety on the LF molecule [125]. However, the comparative studies applying both neutrophil- and milk-derived LF showed that both preparations had the same inhibitory activity on GM-CSF release from mononuclear blood cells [27,52]. The activity of LF may also depend on the homogeneity of the preparations, since it is known that the protein binds various negatively charged molecules, which could affect the interaction of LF with target cells.

The results may also differ depending on the applied experimental model. In the case of *in vitro* studies, the differences may result at least from the origin and composition of the cells serving as the source of growth factors for granulocyte-macrophage colonies, acting both directly and indirectly, including such cytokines as IL-1 or TNF-alpha. Regarding *in vivo* experiments, the initial immune status of the animals is crucial. It may be expected that exogenous LF will act differently in healthy animals, where myelopoiesis is in the equilibrium state ("steady-state"), than in immunocompromised mice subjected to total body irradiation or treatment with cytostatics which destroy the pool of granulocytes ("induced granulopoietic hyperplasia"). In the latter case, exogenous LF acts in the situation of a large deficit of mature granulocytes and their precursors, accompanied by idiopathic self-renewal of the granulocyte lineage. In Broxmeyer's studies, neutrophil extracts did not show any effect on myelopoiesis in healthy mice, whereas in neutropenic mice, after CP administration, the effect was inhibitory [23]. Quite different results were found in our studies in mice given bovine LF orally in drinking water. Mice given a sublethal dose of CP or subjected to a "conditioning" regimen by administration of busulfan and CP followed by bone marrow cell transfer showed a significantly accelerated process of myelopoiesis after LF treatment [3,4]. In control, naive mice, the LF effect on myelopoiesis was negligible [3]. The discrepancy between Broxmeyer's results and ours may be due to the different models used.

For the demonstration of LF activity, the culture conditions may be also important, for example the cell density in the culture. Some results indicate that a higher CSA is produced by cells crowded on a small surface of a culture dish than those dispersed over a larger area. Only in the first case was inhibition of CSA production by LF noted. It cannot be excluded that only fractions of growth factors released cells in physical contact are sensitive to inhibition by LF. The oxygen pressure may also be critical for the release of growth factors during the incubation of cell cultures. It appears that a low oxygen pressure for example 5%, on a cell culture, increases the ability of progenitor cells to form colonies by increasing the susceptibility of the cells to growth factors. Some studies indicate that such conditions are necessary to detect the inhibitory action of LF [25,39]. The results may also be affected by some ingredients of the culture media, at least agar, commonly used in tests, which may be toxic to the progenitor cells [54].

Clinical observations associated with the participation of lactoferrin in the regulation of myelopoiesis

Besides the data from numerous laboratory studies and experiments with animals presented above, many clinical observations may be cited where changes in the level of plasma LF affected the numbers of circulating neutrophils.

The synthesis of LF may be lowered during chronic, devastating diseases, such as neoplastic diseases, during long-term starvation, and myeloablative and immunosuppressive treatment [55]. In some other diseases (some types of leukemias and neutrophilia) an elevated concentration of LF in the circulation is found. These observations prompt the following questions. Are the abnormal concentrations of LF found in patients associated with disturbances in myelopoiesis? Or do the abnormal concentrations of LF accompanying the primary disturbances of myelopoiesis perhaps rather reflects the outcome of the diseases and not their cause? Is there any causality between defined concentrations of LF and an intensification of myelopoietic processes, and if so, what kind? Have available clinical

observations indicating the participation of LF in the regulation of myelopoiesis been confirmed in many laboratory and preclinical studies?

Plasma concentrations of LF may significantly decline in states of neutrophil deficit, this being the result of disturbances in myelopoiesis. Marked periodic decreases in plasma LF levels were registered in patients with cyclic neutropenia. These were correlated with the number of circulating neutrophils [22,94]. Of importance, an increase an LF levels always preceded, by several days, an increase in circulating neutrophils, which may indicate that not only circulating cells may be the source of plasma LF, but also their immature forms present in bone marrow [94]. In a patient with persistent heavy neutropenia, constant, very low plasma LF concentrations were found which correlated with a very low number of circulating neutrophils [94]. In a female patient with transient drug-induced neutropenia, a significant transient decrease in plasma LF was noted which correlated with lower cellularity of the bone marrow and a lack of neutrophils in the periphery [94]. Beginning six days from administration of the drug, an increase in the number of blood neutrophils was noted, accompanied by a rapid increase in the plasma LF concentration, preceding the appearance of new granulocytes in the blood. In the majority of studied patients with chronic idiopathic neutropenia, no changes in plasma LF levels were found [22]. Based on the above clinical observations, it cannot be claimed that LF acts as a feedback inhibitor of granulopoiesis, since the increased protein concentrations were not accompanied by decreases in neutrophil numbers; quite the contrary, the elevation of LF concentrations correlated well with the increase in neutrophil numbers, sometimes preceding that increase, which rather indicates participation of LF in positive regulation of myelopoiesis. The case of a patient described by Broxmeyer and co-workers who had a normal granulocyte number despite a significant LF deficit in neutrophils does not confirm the participation of LF in the regulation of myelopoiesis [26]. The LF released by the neutrophils of that patient was active as a suppressor of CSA release, both by the patient's cells as well as by those of a healthy donor. The patient's cells correctly responded to inhibition by milk-derived LF.

However, clinical data are also available regarding neutrophilia of unknown etiology in a child which indicate that LF participated in the negative regulation of granulocyte formation [32]. In that child, a significantly elevated granulocyte number was observed from birth. Neutrophils from the patient contained significantly less LF than cells of healthy persons: in bone marrow neutrophils only 10% and in the blood neutrophils 1% of the normal LF amount was found. In addition, the LF present in extracts of peripheral and marrow neutrophils and medium conditioned by these cells was inactive as a suppressor of GM-CSF production by mononuclear cells of a healthy donor. What is more, mononuclear blood cells isolated from that patient were refractory to inhibition by normal iron-saturated LF. The progenitor cells forming CFU-GM of that patient were, in addition, refractory to inhibition by acidic isoferritins. As the authors suggest, the evident lack of response of the patient's cells to the actions of inhibitors may have resulted from the lack of detectable Ia antigens on progenitor cells [32].

Changes in LF concentration are also observed in tumor patients. Lowered concentrations of plasma LF are found in patients with disturbed protein synthesis during abnormal myelopoiesis resulting from the disease itself as well as from the myeloablative treatment. In 12 out of 25 children suffering from AML, lowered concentrations of plasma LF were noted which did not correlate with the number of circulating neutrophils, which indicates a disturbance in protein synthesis in the bone marrow [95]. However, in other studies on AML patients, positive correlation between plasma LF concentrations and the number of circulating neutrophils was found [18]. Similar correlations were found in patients subjected to chemotherapy. In some patients who achieved total remission, plasma LF concentrations increased about six days prior to an evident increase in the circulating neutrophil numbers, suggesting that the elevation of plasma LF in these patients may serve as an early sign of bone marrow regeneration [18]. Studies by Oberg and co-workers involving 92 AML patients revealed that plasma LF concentrations differed depending on the type of the disease, these being, for example, were much lower in patients with AML of M1 type (myeloblastic leukemia with no features of maturation) than in patients with AML type M2 (myeloblastic leukemia with maturation features) [90]. LF concentrations had not only diagnostic, but also prognostic value: in patients with low (below 0.1 μg/ml) protein concentration, remission was less frequent (44% of patients) than in patients with higher (0.1–0.4 μg/ml) LF concentrations (77% of patients). The increase in plasma LF concentration preceded the appearance of granulocytes: LF concentrations started to elevate on day 12 after application of chemotherapy, 4 days ahead of the appearance of granulocytes [92]. This sequence of events suggests that plasma LF concentration may represent an indicator of the myelopoietic activity of the bone marrow. On the other hand, the results of Suzuki and co-workers in AML patients subjected to BMT showed that concentrations of plasma LF strictly correlated with the number of circulating neutrophils, but they did not precede a renewal of that pool of cells [124]. Studies on bone marrow reveal that it still shows a hypoplastic structure during elevation of LF concentration and the number of circulating granulocytes. It may therefore be supposed that at an early stage of myelopoiesis renewal, supplementation of the circulating granulocyte pool without storage of the cells in the bone marrow comes first. Later, the bone marrow reservoir of granulocytes is completed. Therefore, the concentrations of plasma LF indicate early processes of myelopoiesis reconstitution and renewal of the circulating, but not the bone-marrow, granulocyte pool [124]. Significantly decreased amounts of cellular and plasma LF were also noted in myelodysplastic patients (a preleukemic state) [42]. The protein levels reflected granulopoiesis in the bone marrow.

The above observations are confirmed by results regarding LF synthesis in bone marrow cells isolated from patients with various types of acute marrow leukemia, where the maturation process was halted at various stages [108]. In the bone marrow of AML type M2 patients according to the FAB classification (myeloblastic leukemia with maturation features), myeloblasts predominated and no LF synthesis was noted in these cell samples. A lack of LF synthesis was also found in bone marrow cell samples from AML type M3 patients (promyelocytic leukemia), where pro-

myelocytes were the prevailing cell type. No LF expression was also noted in the HL-60 promyelocytic human cell line. In the aspirates of bone marrow from the same patients, but taken during remission, a normal synthesis of LF was observed [108].

On the other hand, a significant (2- to 8-fold) increase in plasma LF was registered in CML patients [94]. In addition, correlation between granulocyte number and LF concentration in serum and granulocytes was found. A decrease in LF content was observed in as many as 71% of circulating granulocytes, which indicates a cellular source of serum LF. The low cellular content of LF during the exacerbation phase of CML indicates not only abnormal activation of these cells, resulting from the extended time of their presence in circulation, but also defective maturation of these cells in the bone marrow [93]. The studies by Rado and co-workers showed that in bone marrow aspirates from CML patients, where myelocytes and metamyelocytes were the dominating cell types, LF synthesis occurred with an efficiency similar to that in healthy persons [108].

As mentioned before, other studies conducted in CML patients revealed a deficit of the inhibitory activity or of LF in neutrophils, disturbances in its release, lowered activity of LF in inhibiting CSA release, and a diminished sensitivity of target cells producing CSA [23,26,33,103]. Consequently, some researchers proposed application of exogenous LF in the therapy of chronic leukemias [31], particularly in association with other factors, such as, for example, IFN-gamma, which increases the susceptibility of abnormally reacting cells to the inhibitory action of LF, among others by induction of Ia antigens [35]. Other clinical studies also exist which demonstrated that granulocytes from CML patients exhibited colony formation inhibiting activity (CIA), similar to that of granulocytes isolated from healthy donors [123]. The studied granulocytes were isolated from patients whose bone marrow cells expressed in 100% the presence of Ph chromosome (the CML marker); these patients, were, however, in remission at the time of analysis.

Studies on leukemic and aplastic anemia patients subjected to myeloablative treatment and bone marrow cell transplantation and in patients with leukopenia after treatment of various malignant tumors showed strict correlation of plasma LF concentration with the number of circulating granulocytes [11]. The increase in LF concentrations did not, however, precede an increase in neutrophil numbers in the blood. Oberg and co-workers came to different conclusions in their studies [91]. In leukemia patients subjected to BMT after myeloablative treatment, LF was detected in the blood about four days before the appearance of granulocytes, reflecting the regeneration of myelopoiesis in the bone marrow. Similar results were reported by Brown and co-workers [21]. Significant, transient increases in granulocyte number and LF concentration in the blood were observed after administration of rhG-CSF, a granulopoiesis-stimulating factor, to a patient [124].

Beside the presented alternations in the process of granulopoiesis in the course of CML and myelopoietic renewal after chemotherapy and bone marrow transplantation, still other situations exist in which elevated concentrations of plasma LF accompany an accelerated turnover and recruitment of granulocytes. Acute bacterial infections are a good example of such a situation [62] and support the theory of the positive involvement of LF in the regulation of myelopoiesis.

There are, however, clinical observations which clearly do not confirm a participation of LF in myelopoiesis. For example, patients suffering from innate deficiencies in specific granules and LF in granulocytes did not show hyperplasia of granulocyte-macrophage cell lineage in the bone marrow and blood or abnormality in the granulocyte number [20]. In children suffering from recurrent infections, significantly lower concentrations of plasma LF were noted in comparison with healthy children. Low levels of LF were not, however, accompanied by a lower number of circulating granulocytes [129].

The authors' studies on the effect of Lactoferrin on myelopoiesis

Our studies conducted at the Laboratory of Immunobiology, Institute of Immunology and Experimental Therapy, Wroclaw, Poland, have demonstrated that bovine LF induces hematopoiesis in healthy individuals. It also has the ability to renew hematopoiesis, mainly granulopoiesis and to a lesser degree erythro- and lymphopoiesis in mice after administration of cytostatics. After intravenous injection of high (10 mg/mouse) doses of LF to healthy mice, an increase in the percentage of the granulocytic cell lineage in the bone marrow (myelocytes, band forms, and mature neutrophils) was noted. It has to be mentioned that because of difficulties in differentiating less mature cells of this lineage in bone marrow smears, no further analysis was performed. Also, in the peripheral blood, increases in both the percentages as well as the total numbers of mature and band forms of neutrophils were observed. These changes occurred relatively early after LF administration (after 24–48 h) [139]. Similar results were obtained in trials on volunteers and in a clinical trial [140–142]. In these trials, LF was given orally in the form of capsules or chewable tablets (2, 10, 20, or 50 mg of LF per day). In a patient with *otitis media* resistant to antibiotic treatment and partially to bacteriophage therapy, oral treatment with tablets containing LF led to complete healing, accompanied by fourfold increase in plasma LF levels and a significant increase in immature (band form) neutrophils in the circulating blood [134].

In mice, LF given in drinking water as a 0.5% solution significantly accelerated the renewal of myelopoiesis after administration of a sublethal dose of CP. This effect consisted of a quicker repopulation of the bone marrow by the granulocyte cell lineage and an increase in both the percentage and the total number of immature and mature neutrophils in the peripheral blood. The beneficial action of the protein was most pronounced 15–21 days after CP administration [3]. In another experimental model, mice received myeloablative doses of busulfan and CP and a suboptimal dose of syngeneic bone marrow cells (10^5/mouse) to visualize the engraftment process in the deserted areas of the bone marrow. LF, administered in the drinking water from the day of bone marrow cell transfer, distinctly accelerated the repopulation of the bone marrow. In histological pictures of the bone marrow and spleen, a signi-

ficant acceleration of the repopulation of these organs by the cells of the myelo-, erythro-, and lymphoid cell lineages was noted. For comparison, in mice given Filgrastim® (rhG-CSF), a strong selective stimulation of myelopoiesis was observed [4]. The results suggest that application of LF may lead to even better results compared with the use of growth factors because of its multidirectional renewal of hematopoiesis. The beneficial effect of LF on hematopoiesis was also confirmed *in vitro*. Both human and bovine LF added to a long-term culture of bone marrow cells (Dexter's type) stimulated the secretion of growth factors by cells present in the cultures of bone marrow stromal cells as the medium, which was conditioned by these cells, stimulated the formation of granulocyte-macrophage colonies in the clonogenic assay (unpublished data). Subsequent experiments allowed the identification of the factors responsible for this activity: the presence of GM-CSF, G-CSF, and IL-6 was shown in the conditioned media. Also, the administration of LF to mice resulted in the elevation of plasma GM-CSF, G-CSF, and IL-6 concentrations. Such sera, used in the clonogenic assays, stimulated the formation of granulocyte-macrophage colonies by bone marrow cells of healthy mice. Besides the stimulation of progenitor cells in myelopoiesis confirmed in the clonogenic assays, LF may enhance the renewal of the earliest hematopoietic stem cells, confirmed by the higher number of spleen colonies formed by CFU-S in mice subjected to the myeloablative procedure and bone marrow transplant.

Conclusions

The variety of applied methods represents an obstacle to establishing the exact role of LF in the regulation of myelopoiesis. First of all, the *in vitro* models are of little relevance to *in vivo* situations. For example, to demonstrate a negative effect of LF on myelopoiesis, very special culture requirements must be fulfilled, such as appropriate cell density, the oxygen 0concentration, or the presence of PGE inhibitors. Lactoferrins of different origin (heterologous proteins) are used, and almost exclusively milk-derived preparations. Lactoferrins from milk and granulocytes may substantially differ in their activities in immunological tests (our unpublished data), probably due to different glycan composition. The most appropriate model would be a homologous one, i.e. the administration of mouse granulocyte-derived LF to mice or human granulocyte-derived LF to humans. Moreover, when using a mouse model, particularly *in vivo*, one must consider that rodents have a different distribution of neutrophils in the bone marrow and in the circulating blood from that of humans, i.e. they have a large reservoir of bone marrow neutrophils ready for immediate export into the periphery; this may have an association with the opposite ratio of neutrophils to lymphocytes in the blood compared with humans. It is also obvious that *in vitro* tests may reflect only one step of the regulatory activity of LF on myelopoiesis. The anti-inflammatory response to infectious agents is a multi-step process and LF, as the immunoregulatory protein, should presumably act first by enhancing and then by inhibiting that process, as proposed by some researchers.

Although elucidation of the role of LF in the regulation of myelopoiesis is of interest, from practical reasons the application of exogenous LF in therapy or prophylaxis would be of utmost importance. Clinical studies have proved the safety and beneficial actions of the protein (bovine or recombinant human LF) administered orally in the treatment of diseases and as a prophylactic agent. It is conceivable that LF, as the regulator of myelopoiesis, could be used in the future both to enhance and to suppress the granulocyte pool, depending on the necessity and/or immune status of the patient.

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