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Human lactoferrin induces phenotypic and functional changes in murine splenic B cells

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SUMMARY

The immunotropic activities of human lactoferrin were studied with respect to phenotypic and functional changes in murine splenic B cells. Phenotypic changes were induced by human lactoferrin in splenic B-cell fractions separated by buoyant density. B cells from 7–8-day-old BALB/c mice isolated from a 50/60% Percoll gradient, gained characteriztic features of more mature B cells manifested by an increase of surface IgD and complement receptor expression. Incubation of the analogous B-cell fraction from adult mice with human lactoferrin resulted in minor changes in relation to IgM and IgD expression. Besides induction of phenotypic changes on immature B cells, human lactoferrin enabled B cells from normal newborn and adult immunodeficient CBA/N mice to present antigen to an antigen-specific T-helper type 2 (Th2) cell line. We conclude that human lactoferrin acts as a maturation factor for B cells with regard to their phenotype and function.

INTRODUCTION

Lactoferrin is an iron-binding protein of the transferrin family present in secretory fluids of mammals[1] and contained in secondary granules of neutrophils.[2] The specific receptor for lactoferrin has been reported on several cell types including, mitogen-stimulated human peripheral blood lymphocytes,[3] brush border intestinal cells,[4] macrophages,[5] platelets,[6] epithelial cells of human mammary gland[7] and bacteria.[8] It is believed that receptor binding is the first step in cell functions related to lactoferrin. For instance, interaction of lactoferrin with cells of the immune system induces a regulation of the release of cytokines[9,10] and can protect mice against a lethal dose of *Escherichia coli* in experimental infection *in vivo*.[11] An increase of proliferation of phytohaemagglutinin-stimulated human peripheral blood lymphocytes,[3] as well as both human B- and T-lymphocytic cell lines,[12] has been observed in the presence of lactoferrin.

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Abbreviations: APC, antigen-presenting cell; C3, third component of complement; FCS, fetal calf serum; HSA, human serum albumin; IL-1, interleukin 1; IL-4 interleukin 4; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PMS, phenazine methosulphate; SRBC, sheep red blood cells; XTT, sodium 3-3′-phenylamino/carbonyl-3,4-tetrazolium-bis sulphonic acid hydrate.

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Recently, we have found that lactoferrin may also directly affect maturation of double-negative $CD4^-$ $CD8^-$ thymocytes by inducing expression of mature cell markers on these cells.[13] These phenotypic changes were accompanied by acquirement of helper function in the antibody response to sheep red blood cells (SRBC). Having established that lactoferrin promotes maturation of T-cell precursors, we turned our attention to effects of lactoferrin in relation to immature B cells.

Maturation of B lymphocytes in ontogeny is characterized by the sequential appearance of cell markers. B cells from spleen of newborn mice bear mainly surface IgM,[14] followed by expression of IgD[15] and later by formation of complement receptors (C3R).[16] We and others have recently established that B cells from neonatal mice are very poor antigen-presenting cells (APC) for T-helper type 2 (Th2) cell lines.[17,18] A similar defect in APC function is also found in B cells from chromosome X-linked immunodeficient mice (Xid mice).[17] Xid mice lack the subset of mature B cell that is characterized by the expression of Lyb-5 alloantigen.[19–21] This developmental defect can be overcome by preincubation of the cells with mast cell growth factor, which promotes further maturation of these cells to stromal cells.[22] We have recently found that the inability of B cells from Xid mice to present antigen could be partially abrogated by preincubation of these cells with interleukin-1 (IL-1) or IL-4, and that this phenomenon was correlated with the induction of Lyb-5 antigen.[23]

The aim of the present work was to demonstrate that lactoferrin promotes maturation of B cells from newborn mice by inducing characteriztic changes in the ratio of IgM- to

IgD-bearing cells and in the appearance of complement C3 receptors. In addition, we show that preincubation of immature B cells from newborn or immunodeficient mice significantly enhances their APC function.

MATERIALS AND METHODS

Animals

Immunodeficient Xid CBA/N and CBA mice, bred at the Animal Facility of Harrington Cancer Center (Emory University, Atlanta, GA), were kindly provided by Dr B. Saha. BALB/c mice were derived from the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). The adult mice were allowed a standard laboratory pellet food and tap water *ad libitum* and were used for experiments at 8–12 weeks of age: the newborn mice were used at 7–8 days of age.

Proteins

Lactoferrin was prepared from human skimmed milk and isolated by ion-exchange chromatography on a SP-Sephadex column, as described previously.[24] Lactoferrin was iron saturated[25] and found to be pure by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Apyrogenic human serum albumin (HSA) was obtained from Behringwerke AG (Marburg, Germany).

Preparation of B cells and B-cell subset from BALB/c spleen

A spleen cell suspension was obtained by pressing the organs through a plastic sieve into precooled Hanks' medium. After two washes in Hanks' medium, the cells were resuspended in RPMI-1640 medium containing 10% heat-treated fetal calf serum (FCS), and then applied onto nylon wool columns. The columns were left in a cell culture incubator for 1 hr and then non-adherent cells, mostly T cells and erythrocytes, were washed away with warm (37°) Hanks' medium, while the adherent cells (B-cell enriched population) were recovered by teasing the nylon wool with forceps in ice-cold Hanks' medium. The cell pellets, after centrifugation, were incubated in an ice bath for 45 min, with monoclonal anti-Thy-1 antibodies diluted 1/100 (Pharmingen, San Diego, CA). A non-toxic complement (baby rabbit complement; Sera Lab Ltd, Sussex, UK) was added and the cells were kept at 37° for another 45 min. Cell debris was separated on a Lymphoprep gradient (Pharmacia, Uppsala, Sweden). Cells from the interphase were harvested and washed twice with Hanks' medium, resuspended in 1·5 ml of RPMI-1640 culture medium supplemented with 10% FCS, glutamine and antibiotics, and applied onto a Percoll (Pharmacia) discontinuous gradient, at 30%, 50%, 60%, 75% and 85% concentrations. Cells were centrifuged for 20 min at 600 *g* and layers from the interphases were aspirated separately and washed three times with Hanks' medium. Before separation on Percoll, B cells from both adult and newborn mice were more than 90% positive when stained with fluorescent rat anti-immunoglobulin mouse antibodies.

Detection of C3 receptors

The presence of receptors for complement C3 was detected as previously described.[26] SRBC delivered by the Laboratory of Media and Biopreparates of the Institute of Immunology and Experimental Therapy (Wroclaw, Poland) were used within 7 days. SRBC were washed three times with phosphate-buffered saline (PBS) and 1 ml of 5% SRBC suspension was then incubated for 45 min at 37° with 1 ml of rabbit anti-SRBC IgM antibody (final dilution 1600; Biomed, Cracow, Poland). After incubation, the SRBC were washed once with PBS and then incubated with 1 ml of mouse complement/fresh mouse serum at a final dilution of 1 : 20 for an additional 45 min at 37°. SRBC–IgM–complement complexes were washed three times with PBS and resuspended as a 1% suspension. For the preparation of rosettes, 0·1 ml/10^6/BALB/c splenocytes, preincubated with either human lactoferrin or control protein HSA, was mixed with 0·1 ml 1% SRBC–IgM–complement, and allowed to stand for 30 min at 37° before pelleting by centrifugation for 5 min at 120 *g*. The cell pellet was then resuspended by tapping the tube, diluted to 0·5 ml with PBS, and 50 μl of 0·1% orange acridine added. The rosettes were counted in a fluorescent microscope. The result was expressed as the percentage of cells forming rosettes. Up to 1000 lymphocytes were counted in a single sample.

Double staining of IgM and IgD markers

B cells from the 50/60% Percoll interphase, prepared as described above, were incubated for 48 hr in 24-well plates at a cell density of 10^6/ml/well in the presence of 20 μg/ml of human lactoferrin or 20 μg/ml of HSA. After the incubation, the cells were washed twice with Hanks' medium and once with PBS containing 1% bovine serum albumin (BSA) and 0·02% sodium azide. After washing, the cells were incubated with biotin-derived anti-mouse IgD (L : ED50-k 251; dilution 1 : 50; Interchim, Montluçon, France) for 1 hr in an ice bath. Then the cells were washed twice with PBS containing BSA and sodium azide and incubated for an additional 1 hr with fluorescein-conjugated goat anti-mouse IgM immunoglobulin, anti-μ specific (L : 012H8855; dilution 1 : 50; Sigma, St Louis, MO), and avidin-conjugated R-phycoerythrin (dilution 1 : 50; Pierce, Rockford, IL). After the incubation, the cells were washed once with PBS and analysed for green and red fluorescence.

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

Antigen presentation by B cells to antigen-specific T-cell lines

A sheep insulin-specific T-cell line, KSins 1, was derived from the lymph nodes of BIO.BR (H-2^k) mice primed with 100 μg of sheep insulin, as described previously.[27] This line responds to H-2^k-bearing APC; it has been maintained in our laboratory (Institute of Immunology and Experimental Therapy) for over 2 years and is not specific for insulins from other species (beef, pork). The cell line can be restimulated with irradiated (3300 rads) syngeneic filler spleen cells and sheep insulin (30 μg/ml), and produces IL-4 but not IL-2 nor interferon-γ (IFN-γ); it can be therefore classified as Th2 type cell line. For the measurement of antigen-induced proliferative responses, T cells were cultured at 2×10^4 cells/well in flat-bottomed microtitre plates (Linbro, Flow Laboratoires, Puteaux, France) in culture medium supplemented with antibiotics. Sheep insulin was used at culture initiation at a concentration

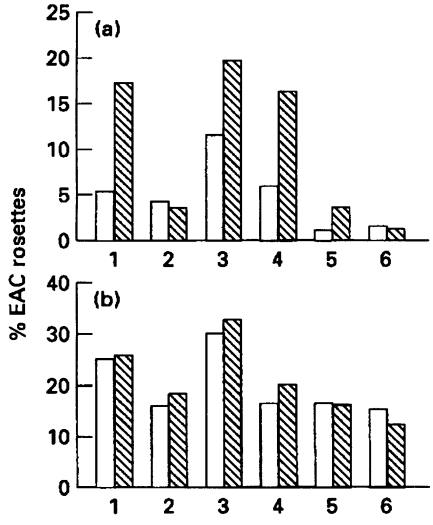

Figure 1. Effects of human lactoferrin on the expression of complement C3 receptors on B cell fractions isolated from newborn and adult BALB/c mice. B cells were prepared from spleens of newborn and adult mice and separated on a discontinuous Percoll gradient as described in the Materials and Methods. The cells were incubated for 48 hr with 20 μg/ml of human lactoferrin (▨) or human serum albumin, (control protein) (□), and tested for formation of EAC rosettes. (a) Newborn mice; (b) adult mice; 1: whole B-cell population; 2–6, Percoll fractions 0–30% (2), 30–50% (3), 50–60% (4), 60–75% (5) and 75–85% (6).

of 30 μg/ml. APC (2×10^4 cells/well) were unfractionated B cells from normal newborn, normal adult or adult immunodeficient mice, preincubated overnight with human lactoferrin (20 μg/ml) or recombinant IL-4 (Dnax, Palo Alto, CA, Lot 96; 1 μg/ml = $12{\cdot}7 \times 10^4$ HT-2 U/ml) at a concentration of 200 U/ml. Control B cells were incubated with apyrogenic HSA at concentration of 20 μg/ml. Proliferation of cells was determined 2 days later by using XTT.[28] Briefly, 50 μl of XTT solution (Diagnostic Chemical Ltd, Charlottetown, Canada), at 1 mg/ml containing 0·01 M PMS (Sigma), was added to the culture of proliferating T cells. After 2–6 hr incubation at 37°, the optical density was read in an automated enzyme-linked immunosorbent assay (ELISA) reader (Dynatech 5000) at 450 nm.

RESULTS

Induction of complement receptors on immature B cells by human lactoferrin

Appearance of receptors for complement C3 (C3R) is a characteristic of mature B cells, and a significant increase of C3R expression is first registered between the second and fourth week of postnatal life.[16] C3R were identified in our studies by Erythrocytes/Antibody/Complement (EAC) rosette test. As depicted in Fig. 1a, b, lactoferrin significantly increased the percentage of B cells bearing C3R in newborn mice, whereas no such effect could be found with adult mice B cells, which already had a high percentage of C3R-bearing B cells. As the population of B cells was heterogeneous, although it is not established that the IgM and IgD status is related to cell density, it was however convenient to separate the cells according to their density, and we decided to study the effects of lactoferrin on more homogeneous cell fractions.

Separation of cells according to cell density was achieved by using centrifugation on a discontinuous Percoll gradient. The whole B-cell population was separated into five fractions: 0–30%, 30–50%, 50–60%, 60–75% and 75–85% of Percoll. The whole B-cell population and cell fractions were incubated with lactoferrin, 20 μg/ml, for 48 hr and EAC rosettes were determined. As shown in Fig. 1a, cells bearing C3R were located predominantly in the less dense fractions (30–50% and 50–60% Percoll) in newborn mice. In adult BALB/c mice (Fig. 1b) these cells were distributed more evenly across all fractions, although with some emphasis in the 30–50% Percoll fraction. The 50–60% Percoll fraction had the highest relative increase of EAC rosettes of all the fractioned cell populations, and was therefore used subsequently for experiments on human lactoferrin-induced phenotypic changes.

Human lactoferrin-induced changes in the expression of IgM and IgD receptors on B cells

To demonstrate further the immunotropic activity of lactoferrin on immature B cells, we analysed the expression of IgM and IgD markers on Percoll B-cell fractions from newborn and adult mice. The experiments were carried out on the 50–60% Percoll cell fraction. In some cases, we also studied the phenotypic alterations on the 60–75% cell fraction (data not shown). Figure 2 demonstrates the distribution of cell populations within the 50–60% cell fraction with regard to IgM and IgD expression. The cells were divided arbitrarily into four quadrants, and three main subpopulations were defined with respect to IgM and IgD status: fraction I, $IgM^{low}IgD^{low}$; fraction II, $IgM^{high}IgD^{low}$; fraction III, $IgM^{high}IgD^{high}$.

In adult mice (Fig. 2a), most B lymphocytes were located in fraction III (71·3%) whereas, fraction II represented a minor subpopulation (13·6%). In contrast, as shown in Fig. 2c, B cells isolated from newborn mice had fewer cells in fraction III (41·6%) and relatively more cells in fraction II (37·7%).

The action of lactoferrin with regard to cell phenotypic changes of B cells from adult mice was not significant (Fig. 2b), since no change (fraction II) and slight alterations were registered in fractions I and III, showing a drop in IgD expression from 71·3% to 61·3%, and a small increase from 15·1% to 24·1% of the $IgM^{low}IgD^{low}$ B-cell pool. We never registered an increase of IgD expression in the 50/60% Percoll fraction from adult mice, and the changes in the level of IgM-bearing cells were also limited. On the other hand, marked changes were observed in the newborn B-cell population following incubation with lactoferrin (Fig 2d). Firstly, there was a significant drop in the content of fraction II from 37·7% to 20·1%. Secondly, the percentage of cells in fraction III was increased from 41·6% to 69·8%, approaching the value of fraction III in untreated cells from adult mice (71·3%). Thirdly, the cell number in fraction I was diminished from 20·7% to 9·8%. These results indicate that the increase of the fraction III cell pool in newborn B cells was caused by a transformation of approximately half of the fractions I and II into cells that acquired IgD receptors.

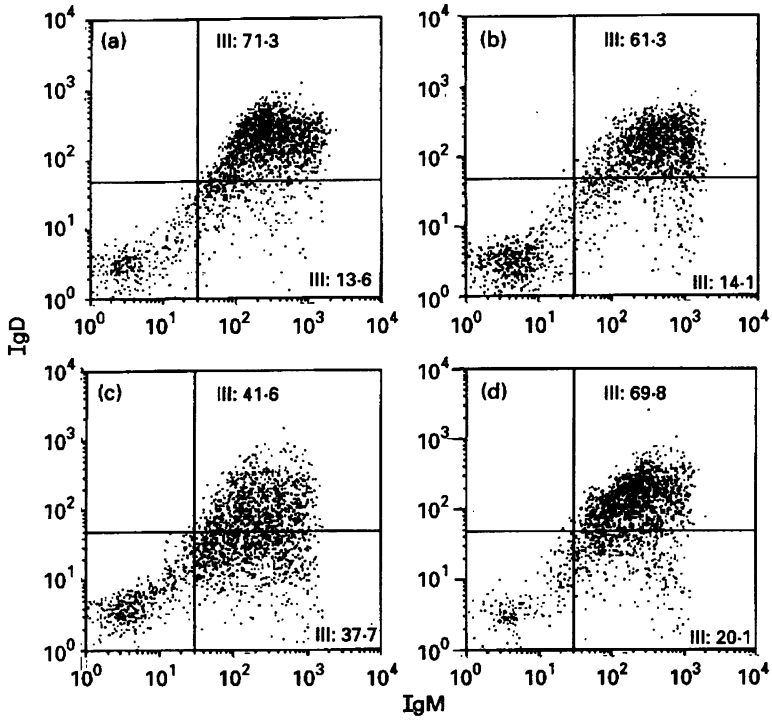

Figure 2. Determination of IgD and IgM expression on B lymphocytes. Two-colour immunofluorescence analyses of the 50–60% Percoll gradient layer were performed on lymphocytes from adult BALB/c (a, b) and newborn (c, d) mice. Cells were treated with either 20 μg/ml of HSA (a, c) or human lactoferrin (b, d) and then stained with fluorescein-conjugated anti-IgM immunoglobulins (green fluorescence) and with phycoerythrin-conjugated IgD immunoglobulins (red fluorescence). Ordinates: logarithm of fluorescence intensity.

Effect of human lactoferrin on antigen presentation by B cells from newborn and immunodeficient CBA/N mice

Our previous studies have shown that B cells from immature newborn mice as well as from Xid CBA/N mice, are unable to present antigen effectively to antigen-specific T-cell lines.[17] We have also demonstrated that cytokines involved in B-cell differentiation/activation, such as IL-1, IL-4 and IL-6, significantly enhance the APC activity of these cells.[23] Therefore, we preincubated B cells from newborn and adult Xid mice with lactoferrin and compared their APC function with that of B cells from normal adult CBA mice. In addition, IL-4 was used as positive control. Figures 3 and 4 show the results of two representative experiments on the action of lactoferrin with regard to the APC function of B cells from newborn CBA and immunodeficient mice, respectively. The data are presented as antigen-specific proliferation of sheep insulin-specific T cells (the KSins 1 cell line). The results suggest that lactoferrin enables immature B cells, from newborn (Fig. 3) or Xid CBA/N (Fig. 4) mice, to present antigen effectively to T cells, albeit to a lesser degree than IL-4. Human lactoferrin had no stimulatory effect on antigen presentation by B cells from normal mice.

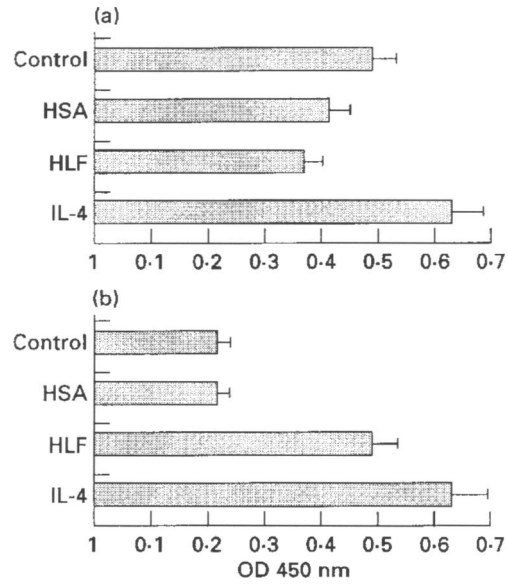

Figure 3. Effects of human lactoferrin and IL-4 on antigen presentation by B cells from newborn CBA mice. B cells from adult CBA (a) and newborn (b) mice were preincubated overnight with HSA (20 μg/ml), human lactoferrin (HLF) (20 μg/ml) or IL-4 (200 U/ml), washed twice, and used as APC for KSins 1 cells. The antigen proliferative response was measured using the XTT reagent, and the optical density (OD) was read at 450 nm. The data are presented as mean values from triplicate determinations ± SE.

DISCUSSION

Our work has revealed an interesting immunotropic activity of human lactoferrin in relation to immature B cells. B cells from newborn mice acquired cells markers characteriztic of a mature B-cell phenotype, and became fully functional as APC.

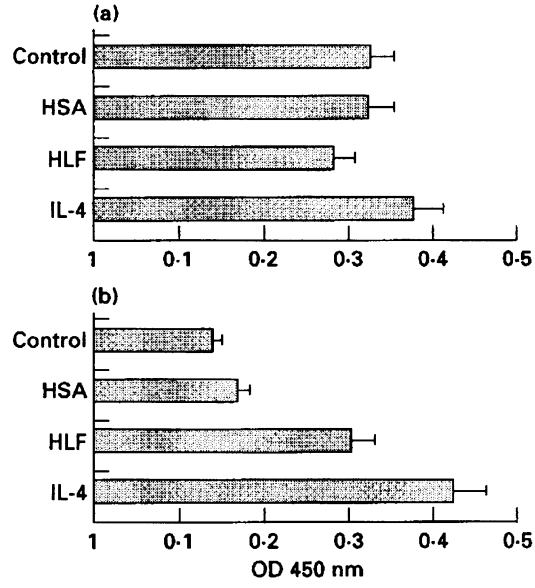

Figure 4. Effects of human lactoferrin and IL-4 on antigen presentation by B cells from immunodeficient CBA/N mice. B cells from adult CBA mice (a) and adult Xid CBA/N mice (b) were treated as described in Fig. 3. The data are presented as mean values from triplicate determinations ± SE.

In preliminary experiments using whole B-cell populations, we did not obtain a significant increase of IgD expression on B cells from newborn mice following incubation with lactoferrin. It was subsequently found that less dense fractions of neonatal B cells, in particular the 50/60% Percoll fraction, were most appropriate for demonstrating a lactoferrin-induced increase of IgD expression. This cell fraction was still heterogeneous and apparently contained precursors of $IgM^{high}IgD^{high}$ cells (Fig. 2). Cells from denser (60/75%) Percoll fractions from newborn mice that already expressed more IgD (data not shown) showed no further increase of surface IgD. The 50/60% cell fraction from adult mice, analogous in density and size to the immature cell subset from newborn mice, did not significantly change its phenotype after incubation with lactoferrin indicating again that the primary role of lactoferrin was to elicit a maturation process. However, in adult mice the changes were unremarkable, except for a small drop in IgD expression. These results suggest that lactoferrin may trigger, depending on the target cell, two opposite processes, the first, and also more pronounced, promoting the maturation of B cells, and the second, and less distinct, converting mature B cells into less mature or activated cells. That B cells from adult, but not from newborn, mice are activated by lactoferrin is also indicated by our recent finding (unpublished data) revealing a very significant increase (20%) of CD23 antigen on adult but not on neonatal B cells following incubation with lactoferrin. CD23 markers appeared later in ontogeny, but their expression was strongly correlated with IgM and was transient.[29] It is also tempting to speculate that the shift from fractions II and III of adult B cells to fraction I (double-negative cells) may represent a class switch into a memory B-cell pool that may be generated in the absence of intentional immunization.[30]

Our data show, in addition, that lactoferrin can induce a significant increase in C3R-bearing B cells from neonatal mice B cells in the whole populations and in both B-cell fractions. As C3R appears later in ontogeny[16] than IgD[15] markers, this finding suggests that lactoferrin can promote B-cell differentiation at a later stage. Coincidently, we have shown recently[17] that B cells gain the capability to present antigen when mice are around 14 days old. At this stage of ontogeny, B cells acquire complement receptors.[16]

Besides affecting the phenotypic changes in the immature B-cell populations, lactoferrin also has an effect on the antigen-presenting function of these cells. Both B cells isolated from neonatal mice and from adult, immunodeficient CBA/N mice gained the ability to present antigen (insulin) to the antigen-specific T-cell line KSins 1. These results suggest that lactoferrin exerts a stimulatory effect on different stages of B-cell maturation.

Our recent[17] and other studies[18] have revealed that the ability to present antigen is a feature of mature B cells that are absent from normal newborn or immunodeficient adult Xid mice. The inability of immature B cells to stimulate T cells is probably due to a lack of membrane structures responsible for transmitting signals during T–B-cell co-operation, and not to a failure in antigen processing.[17,31] Cytokines known to elicit B-cell activation and differentiation enhance the ability to present antigen.[23] In this context, lactoferrin clearly displays activities similar to IL-4[23,31,32] by enhancing the APC function of immature B cells to the level of B cells from adult mice.

The results presented in this work broaden our knowledge on the role of lactoferrin in the maturation of cells of the immune system and, together with the demonstration of the involvement of lactoferrin in the maturation of T cells,[13] provide a satisfactory explanation of why this protein enhances the induction phase of the immune response. In addition, our data suggest that lactoferrin, contained in maternal milk, may play a role in promoting maturation of the intestinal immune system in a newborn.

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