Soluble CD14 in human breast milk and its role in innate immune responses

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Immune factors secreted in milk are important for health in the neonatal gut. We have detected the bacterial pattern recognition receptor, soluble CD14 (sCD14) in human breast milk at different times during lactation. The molecule occurs in a single form in milk, in contrast to human serum, in which there are two isoforms. Produced by mammary epithelial cells, milk sCD14 mediates secretion of innate immune response molecules such as interleukin-8, tumor necrosis factor-α, and epithelial neutrophil activator-78 by CD14-negative intestinal epithelial cells exposed to lipopolysaccharide (LPS) or bacteria. Although present at low concentrations in milk, LPS-binding protein may be implicated in the biological effects observed. Our findings support the premise that milk sCD14 acts as a 'sentinel' molecule and immune modulator in homeostasis and in the defense of the neonatal intestine. In so doing, it may prevent the immune and inflammatory conditions of the gut to which non-breastfed infants are predisposed. □ *Bacteria; breast milk; CD14; intestine; mucosal immunity*

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The gastrointestinal mucosa is a site at which the host encounters a wide variety of microorganisms and through which pathogens can gain entry into the host and induce infection. It is therefore imperative that the host can deploy rapid and effective defense mechanisms in the event of any confrontation. This is achieved through the coordinated action of the epithelial and immunocompetent cells of the intraepithelial and lamina propria compartments, in both adaptive and innate immune responses.

The neonatal immune system is considered to be immature due to its limited exposure to antigen. The early neonatal period is a unique challenge to the inexperienced mucosal immune system and to its precarious homeostasis. After birth the sterile fetal intestine is colonized by vast numbers of bacteria. Excessive predominance of endotoxin-producing gram-negative bacteria may compromise the neonate and induce severe pathological conditions such as necrotizing enterocolitis (NEC) and gut-derived sepsis (1). The lower incidence of such conditions in the breast-fed infant has been attributed to a diversity of protective factors in breast milk (2, 3). However, there may be additional, more specific factors that sense the presence of microorganisms. Signals should then be relayed to the neonate's mucosal immune system to release inflammatory cytokines and chemokines and to stimulate adaptive immune responses.

The monocyte/macrophage membrane glycoprotein CD14 exists in two soluble forms (sCD14) (α and β) in normal human plasma (4, 5). Serum sCD14 recognizes bacteria and their cell wall components and thereafter activates both membrane CD14-positive and -negative cells (6–8). Here, we summarize our studies on the presence of a single isoform of sCD14 in human milk at a concentration considerably higher than its counterpart in normal human serum. In addition, we show that lipopolysaccharide (LPS), non-pathogenic and pathogenic *Eschericia coli* strains induce cytokine secretion from human intestinal epithelial cells through sCD14-dependent mechanisms. It is also shown that LPS-binding protein (LBP) has a regulating role. We propose that, during the early neonatal period, sCD14 in breast milk monitors the bacterial load in the gut and initiates appropriate immune activity in response to commensals and pathogens.

Materials and methods

Reagents

Anti-human CD14 monoclonal antibodies MY4 (Coulter Instrumentation Laboratory, Switzerland) and MEM-18 (Dr. V. Horejsi, Academy of Sciences, Czech Republic), isotype control MOPC 141 (IgG2b) (Sigma-Aldrich, UK), and biotinylated rabbit anti-CD14 (Sanofi-Synethelabo, France) were used. LBP and anti-LBP antibody (Big 412) were purchased from Biometec GmbH (Germany). LPS from non-pathogenic *Escherichia coli* O55:B5 was from Sigma-Aldrich, and the enteropathogenic *E. coli* 0127:H6 (EPEC) was from Prof. Hackler (University of Würzburg, Germany).

Human breast milk and human serum samples

Human breast milk (HM) was from healthy mothers,

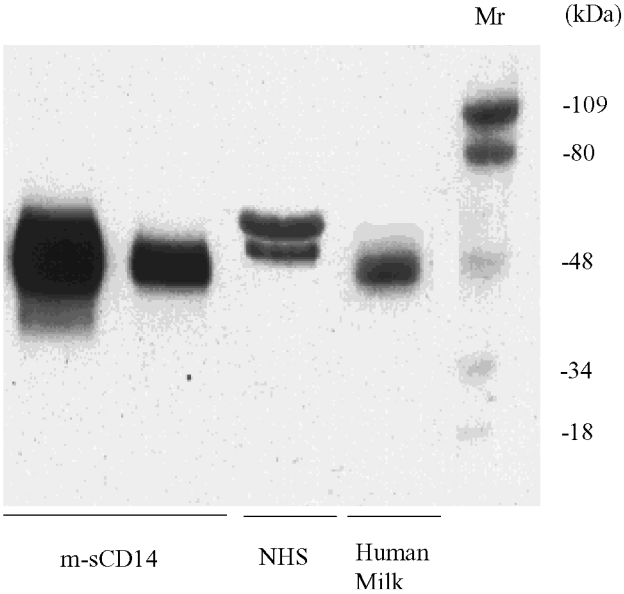

Fig. 1. Characterization of m-sCD14. Affinity purified m-sCD14 (1 μg and 0.2 μg) analyzed by means of Western blot with a rabbit anti-CD14 antibody showed a molecular pattern identical to that of sCD14 detected in human milk (14 days postpartum) but a different pattern from that of sCD14 in normal human serum (NHS).

and pooled human AB^+ serum was from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) kits were used to measure sCD14 (IBL, Germany) and LBP (HyCult Biotechnology, The Netherlands) content. For Western blot (WB) analysis samples were separated by means of 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (PhastSystem®, Pharmacia, Sweden, or Mini Protean II, Biorad, USA) under reducing conditions (5). Soluble CD14 in human skimmed milk (3 months postpartum) was affinity-purified, using MEM-18 mAb coupled to CN-Br Sepharose 4B (9).

Cell lines and cultures

Human colon carcinoma epithelial cells HT29 (American Type Culture Center, USA) and human breast adenocarcinoma cells MCF-7 (European Collection of Cell Culture, UK) were cultured as indicated by the suppliers. After being cultured for 72 h in AIM-V serum-free medium, cell lysates and culture supernatants of MCF-7 were analyzed by means of WB for CD14 content. For stimulation assays, HT-29 cells (90% confluent) were washed twice in serum-free media, incubated for 24 h in serum-free media supplemented with human milk (final concentration of sCD14, 0.3 μg/ml) in the absence or presence of either *E. coli* or *E. coli* LPS, and the supernatants analyzed for interleukin (IL)-8 with a standard ELISA protocol and specific match-paired antibodies (ImmunoKontact, Switzerland).

Results

Isolation and biochemical characterization of human milk sCD14 (m-sCD14) (9)

WB analysis of human breast milk by means of anti-CD14-specific antibodies (Fig. 1) showed a strong, single band corresponding to a polypeptide of approximately 48 kDa. This was identical to the molecular pattern of affinity-purified m-sCD14. Normal human serum (NHS) showed the typical sCD14 α (50 kDa) and sCD14 β (56 kDa) polypeptides (5).

Table 1 shows that human breast milk had higher levels of sCD14 (14.84 ± 6.39 μg/ml, $n = 40$) than those previously reported for NHS (2–3 μg/ml) (4, 5). The highest levels of 20.10 ± 8.74 μg/ml ($n = 10$), detected within the first week postpartum, decreased to 13.09 ± 4.31 μg/ml ($n = 30$) in samples collected later. In contrast to sCD14, LBP is present in breast milk at approximately 1000-fold lower levels than those in serum (Table 1).

Cellular origin of m-sCD14

WB analysis of the human mammary gland epithelial cell line MCF-7 showed strong CD14 polypeptide bands in both total cell lysates and supernatants (Fig. 2). The cell lysate showed two closely migrating CD14 polypeptides of approximately 48 kDa.

Table 1. Concentrations of sCD14 and LBP in human serum and breast milk

	Normal human serum	Human milk (days postpartum)		
		≤6 days	≥8 days	0–71 days
sCD14 (μg/ml)	3.76 ± 0.52 ($n = 20$)	20.10 ± 8.74 ($n = 10$)	13.09 ± 4.31 ($n = 30$)	14.84 ± 6.39 ($n = 40$)
LBP (μg/ml)	9.67 ± 2.11 ($n = 16$)	0.03 ± 0.01 ($n = 8$)	0.01 ± 0.01 ($n = 24$)	0.01 ± 0.01 ($n = 32$)

Data represent the mean ± standard deviation of samples tested with enzyme-linked immunosorbent assay. LBP = lipopolysaccharide-binding protein.

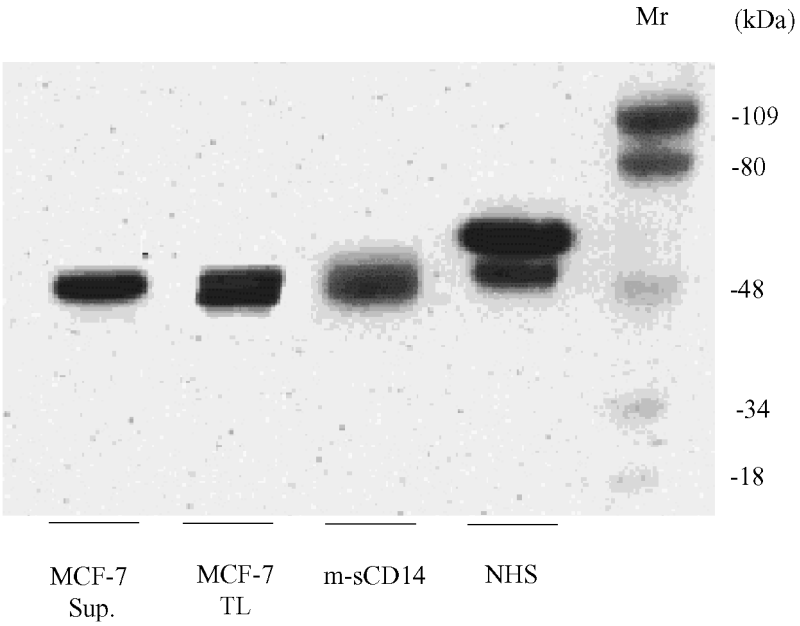

Fig. 2. Mammary epithelial cells produce 48 kDa sCD14. Western blot analysis of sCD14 in 72-h culture supernatants (Sup) and total cell lysates (TL) of MCF-7 cells. Soluble CD14 in milk and normal human serum (NHS) were analyzed in parallel. (Reproduced from The Journal of Experimental Medicine, 2000, Vol. 191, pp. 1807–12, by copyright permission of The Rockefeller University Press.)

Human intestinal epithelial cells (IEC) are activated by endotoxin and by whole bacteria, in an m-sCD14-dependent fashion

During bacterial colonization of the neonatal intestine, human breast milk protects the mucosal surface against bacterial challenge. To test whether m-sCD14 might be involved in the protection against infection with gram-negative microorganisms, the membrane CD14-negative IEC line HT-29 was challenged by bacterial endotoxin in the presence of human milk, and the production of

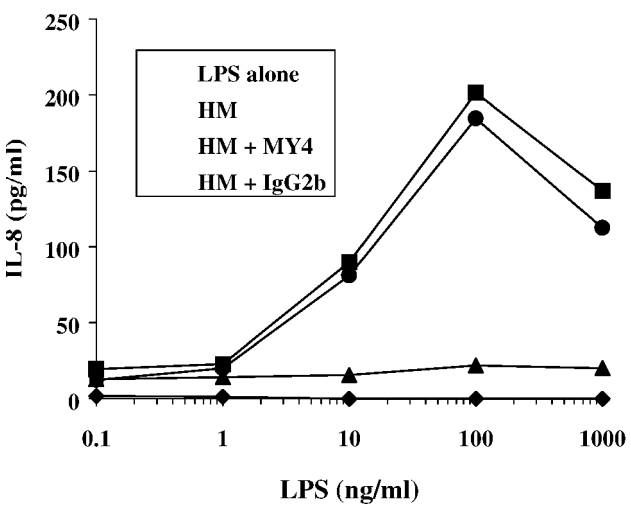

Fig. 3. Intestinal epithelial cells are activated by lipopolysaccharide (LPS) in a dose-dependent manner in the presence of m-sCD14. Production of interleukin (IL)-8 by HT29 cells cultured for 24 h in medium containing human milk (HM) and various doses of LPS. The anti-CD14 monoclonal antibody MY4, but not its isotype-matched control (IgG2b), blocked LPS stimulation.

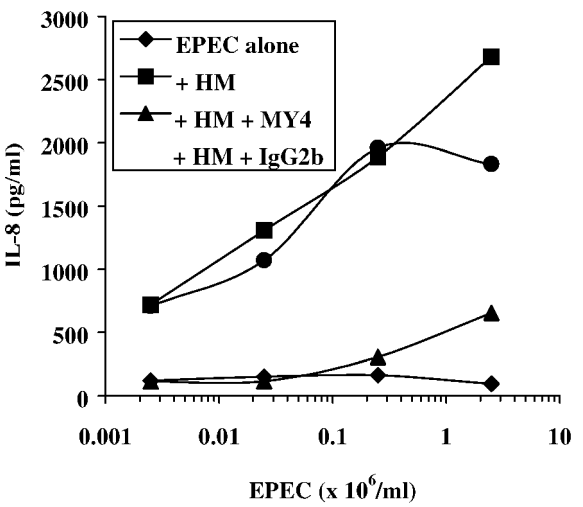

Fig. 4. Intestinal epithelial cells are activated by pathogenic bacteria in an m-sCD14-dependent manner. Production of interleukin (IL)-8 by HT29 cultured for 24 h in medium containing human milk (HM) and various doses of enteropathogenic *Escherichia coli* (EPEC). The anti-CD14 antibody MY4 but not its isotype control blocked the bacterial stimulation.

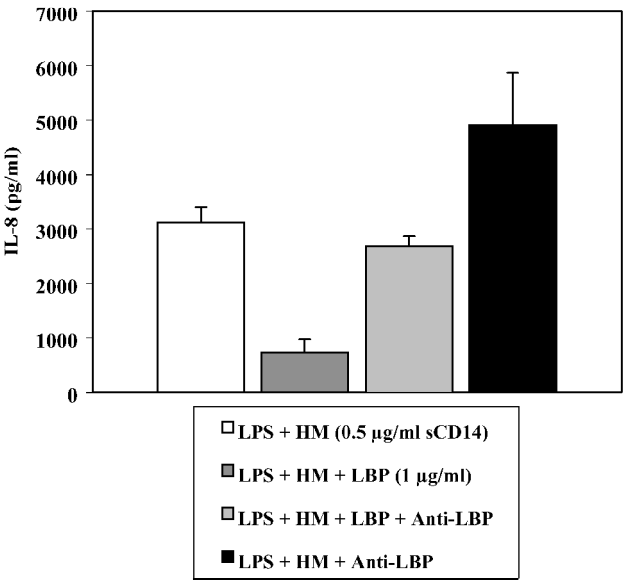

Fig. 5. Intestinal epithelial cell activation by lipopolysaccharide (LPS)/m-sCD14 is inhibited by LPS-binding protein (LBP). Production of interleukin (IL)-8 was measured after 24-h stimulation of HT29 cells cultured in medium containing human milk (HM) and LPS (100 ng/ml) in the presence or absence of LBP (1 µg/ml) and/or anti-LBP (IgG1, ascites, 1:50 dilution). The results shown are from a single representative experiment performed in duplicate.

immune and pro-inflammatory molecules was examined (9). Breast milk mediated IL-8 production by LPS-activated IEC in a dose-dependent manner (Fig. 3). A similar effect was seen for tumor necrosis factor (TNF)-α and epithelial neutrophil activator (ENA)-78 production (not shown). Previously, we have shown that challenge of IEC with whole cells from a non-pathogenic *E. coli* strain also caused production of IL-8, TNF-α, and ENA–78 in a CD14-dependent manner (9). The results in Fig. 4 show that an enteropathogenic *E. coli* (EPEC) strain mediates expression of IL-8 in a similar fashion.

Addition of an antibody to LBP significantly increased the production of IL-8 by IEC exposed to LPS and human milk, whereas addition of recombinant human LBP inhibited its release (Fig. 5). This suggests that, although the levels of LBP in milk are low, LBP may nevertheless be implicated in the response of the IEC to LPS and human milk.

Discussion

During the process of bacterial colonization there is a brief 'physiological' catabolic period, more evident in formula-fed than in breast-fed infants, which lasts for the first few days and is accompanied by weight loss. This situation is more critical in premature and low birth-weight babies and is sometimes related to the development of severe pathological conditions such as NEC, which are almost never seen in breast-fed infants. Our results suggest that recognition of microbial components in the intestinal milieu is aided by milk factors, which may then communicate with immunocompetent cells in the neonatal gut mucosa. We show the presence of a specific isoform of biologically active sCD14 in human milk which is produced by differentiated, mammary epithelial cells. Our in vitro data show that IEC exposed to breast milk and either gram-negative bacteria or endotoxin induces release of molecules such as TNF-α and the chemokines IL-8 and ENA-78, which are involved in cellular recruitment and innate defense at the site of infection. We have previously shown that mediators of epithelial–T cell communication, such as IL-7, IL-15, IL-18, major histocompatibility complex (MHC) I and II molecules, and CD80, are not affected (9).

In contrast to the high concentration of m-sCD14, the levels of LBP are very low in milk. LBP is an acute-phase reactant that catalyzes the interaction between bacterial endotoxin and CD14 and thereby enhances immune recognition of endotoxin and gram-negative bacteria (10). However, high concentrations of LBP decrease endotoxin activity and protect against septic shock (11). We found that LBP, in spite of its low levels in milk, was implicated in the IEC response and appeared to limit excessive production of pro-inflammatory cytokines by the IEC. The recent observation that IEC produce LBP and that this is increased during endotoxemia in vivo (12) suggests that IEC-derived LBP may be contributing to the effects we have observed in our experimental model. The combination of a huge bacterial inoculum in the neonate's intestine and the high concentration of sCD14 in breast milk do not result in any excessive, deleterious immune response. This may be explained by the presence of LBP in the milk. However, direct interaction between m-sCD14 and T cells and B cells, without involvement of LPS, may also be important, since sCD14 participates in the regulation of humoral immune responses (13, 14) and can interact with activated T cells to decrease antigen- and mitogen-induced proliferation (15).

In conclusion, human breast milk contains high concentrations of a single form of sCD14, which is derived from mammary gland epithelial cells. This molecule is involved in innate immune responses and may control mechanisms of homeostasis in the neonatal intestine. A second important molecule for bacterial recognition, LBP, was found at very low levels. However, there may be cooperation between m-sCD14 and IEC-derived LBP in local intestinal responses. Milk sCD14 had biological activity comparable to that of serum-derived sCD14. LPS and *E. coli* induced pro-inflammatory cytokine production by IEC in the presence of m-sCD14. However, this stimulation did not appear to be part of a generalized hyperreactivity. Ingesting milk containing sCD14 may avoid the excessive immune reactivity and tissue damage that is found in neonatal pathologies.

References

1. Peter CS, Feuerhahn M, Bohnhorst B, Schlaud M, Ziesing S, von der Hardt H, et al. Necrotising enterocolitis: is there a relationship to specific pathogens? Eur J Pediatr 1999;158:67–70.
2. Howie PW, Forsyth JS, Ogston SA, Clark A, Florey CD. Protective effect of breast feeding against infection. BMJ 1990; 300:11–6.
3. Lucas A, Cole TJ. Breast milk and neonatal necrotising enterocolitis. Lancet 1990;336:1519–23.
4. Bazil V, Horejsi V, Baudys M, Kristofova H, Strominger JL, Kostka W, et al. Biochemical characterization of a soluble form of the 53-kDa monocyte surface antigen. Eur J Immunol 1986; 16:1583–9.
5. Durieux JJ, Vita N, Popescu O, Guette F, Calzada-Wack J, Munker R, et al. The two soluble forms of the lipopolysaccharide receptor, CD14: characterization and release by normal human monocytes. Eur J Immunol 1994;24:2006–12.
6. Frey EA, Miller DS, Jahr TG, Sundan A, Bazil V, Espevik T, et al. Soluble CD14 participates in the response of cells to lipopolysaccharide. J Exp Med 1992;176:1665–71.
7. Labéta MO, Durieux JJ, Fernandez N, Herrmann R, Ferrara P. Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14. Eur J Immunol 1993;23:2144–51.
8. Jack RS, Grunwald U, Stelter F, Workalemahu G, Schütt C. Both membrane bound and soluble forms of CD14 bind to Gram-negative bacteria. Eur J Immunol 1995;25:1436–41.
9. Labéta MO, Vidal K, Rey-Nores JE, Arias M, Vita N, Morgan BP, et al. Innate recognition of bacteria in human milk is mediated by a milk-derived highly expressed pattern recognition receptor, soluble CD14. J Exp Med 2000;191:1807–12.
10. Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M, et al. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to sCD14. J Exp Med 1994;79: 269–77.
11. Lamping N, Dettmer R, Schröder NWJ, Pfeil D, Hallatschek W, Burger R, et al. LPS-binding protein protects mice from septic shock caused by LPS or Gram-negative bacteria. J Clin Invest 1998;101:2065–71.
12. Vreugdenhil ACE, Snoek AMP, Greve JWM, Buurman WA. Lipopolysaccharide-binding protein is vectorially secreted and transported by cultured intestinal epithelial cells and is present in the intestinal mucus of mice. J Immunol 2000;165:4561–6.
13. Arias MA, Rey Nores JE, Vita N, Stetler F, Borysiewicz LK, Ferrara P, et al. Cutting edge: human B cell function is regulated by interaction with soluble CD14: opposite effects on IgG1 and IgE production. J Immunol 2000;164:3480–5.
14. Filipp D, Alizadeh-Khiavi K, Richardson C, Palma A, Paredes N, Takeuchi O, et al. Soluble CD14 enriched in colostrum and milk induces B cell growth and differentiation. Proc Natl Acad Sci USA 2001;98:603–8.
15. Rey Nores JE, Bensussan A, Vita N, Stelter F, Arias MA, Jones M, et al. Soluble CD14 acts as a negative regulator of human T cell activation and function. Eur J Immunol 1999;29:265–76.