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Potential immunoregulatory role of heme oxygenase-1 in human milk: a combined biochemical and molecular modeling approach

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Abstract

Human milk contains biological factors that are involved in a newborn's growth and immune system regulation. By integrating standard biochemical experimental protocols with computational methods, the present study investigates the presence of heme oxygenase-1 (HO-1), a cytoprotective enzyme, in human milk at different levels of maturation and in milk formulae. Furthermore, we evaluated cytokine and glutathione *S*-transferase (GSH) levels. Samples were collected from colostrum (on Day 1 after birth), from transition milk (on Postdelivery Days 7 and 14) and from mature milk (on Day 30 after delivery) in 14 healthy women. HO-1 protein, GSH and cytokines levels were measured using enzyme-linked immunosorbent assay and flow cytometry. HO-1 protein levels were significantly higher in colostrum (1.33 ng/ml; 5th centile 0.92; 95th centile 2.38) and in transition milk at 14 days (0.97 ng/ml; 5th centile 0.87; 95th centile 1.45) than in mature milk (0.9 ng/ml; 5th centile 0.8; 95th centile 1.38). Levels of HO-1 in milk formulae were similar to those in colostrum. No significant differences in GSH content were observed in mature milk, transition milk and colostrum, whereas significantly higher GSH levels were observed in milk formulae. No significant levels of cytokines, with the exception of interleukin-8, were found. Computational studies on the possible interactions between HO-1 and CD91 were carried out by a battery of softwares, namely, GRAMM (version 1.03), DALI, CLUSTALW (version 2.0), PatchDock and FireDock, mutually counterchecking and validating each other. The computational results, the strong convergence (to the same "solution") of which finally leads to an "experimental-like" character, showed that HO-1 may bind to CD91, thus suggesting its major role as a new chaperokine in immune response regulation. These findings, which connect and integrate biochemical data and computational data interpretation, represent a synergistic and powerful means of conducting biological research.

Keywords: Heme oxygenase; Human milk; Molecular modeling; Glutathione; Newborns; Heat shock proteins

1. Introduction

There is a consolidated body of evidence on the unique role of human milk in infant feeding as regards a reduced risk of cardiovascular disease, diabetes, obesity and cancer [1]. However, breastfeeding is of utmost importance also in the first months of life, when it assures protection against infectious diseases, stimulating the development of the immune system [2–6]. This phenomenon is supported by secretory immunoglobulin A [7,8] and other immunologically active components such as antimicrobial factors, cytokines, chemokines and growth factors [9,10]. Several functions of extreme interest, such as antioxidant property, have been attributed to human milk [11,12], although the mechanisms are still unclear. The explanation may reside in the presence of antioxidant enzymes, as well as free radical scavengers, among which glutathione *S*-transferase (GSH) plays a key role. GSH performs several important physiological functions such as (a) inactivation of oxygen-derived highly reactive species [13,14]; (b) detoxification of various types of xenobiotics [13] and carcinogens [15]; (c) maintenance of the oxidative status of other antioxidants such as ascorbic acid and α-

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tocopherol [16]; and (d) improvement of cellular immune response by activation of lymphocytes [17].

As far as antioxidant enzymes in human milk are concerned, the presence of several proteins (i.e., GSH peroxidase and superoxide dismutase) has been demonstrated [18,19]. Another important group of antioxidant proteins is constituted by heat shock proteins (HSPs), whose presence in biological fluids has been related to the capacity of binding to specific cell surface receptors such as CD91 [20]. Among HSPs, heme oxygenase-1 (HO-1 or HSP32) is the rate-limiting enzyme in heme catabolism that is associated with strong protective effects [21]. The presence of HO-1 has been recently detected in biological fluids (blood and cerebrospinal fluid), reasonably related to its putative protective action [22,23].

The present study investigated (a) the pattern of HO-1 protein, GSH levels and cytokine profile expression over a time course from colostrum to mature milk; (b) the presence of HO-1 and GSH in milk formulae in comparison with that in human colostrum, transition milk and mature milk; and (c) the possible significance of HO-1 in human milk, using computational approaches. This is also the first study to show that HO-1 is detectable in human milk and that its action may be related to CD91 binding, thus suggesting a putative role in immune response regulation.

2. Methods

2.1. Milk samples

Samples were collected from colostrum (24 h after birth; defined according to Playford et al. [24]), from transition milk on Days 7 and 14, and from mature milk on Day 30 after birth from 14 healthy women whose ages ranged from 23 to 28 years (mean age, 25±3.5 years), with consecutive singleton physiological pregnancies (parities 1 and 2) and whose deliveries were made between 37 and 42 weeks' gestation (mean, 39±3.5 weeks). None of the participants smoked tobacco, and all delivered vaginally. Exclusion criteria were as follows: multiple pregnancies, gestational hypertension, diabetes and infections, fever, chromosomal abnormalities, metabolic diseases, diseases of the breast, malnutrition and maternal allergy. The breast milk fatty layer and cellular elements were removed by two centrifugations: at 680×*g* for 10 min at 4°C, after which the supernatants were removed, and at 10,000×*g* for 30 min at 4°C. The resulting translucent whey was immediately stored at −70°C until every measure and assay had been performed. The Ethics Committee of the Giannina Gaslini Children's Hospital, Genoa University, approved the study protocol, and the donors of milk gave informed consent. Twelve different commercially available milk formulae were also assayed.

2.2. HO-1 measurements

A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Stressgen, Victoria, Canada) was used to measure HO-1 protein concentration in milk. The assay was performed in accordance with the protocol provided by the manufacturer. Briefly, each milk sample was incubated with anti-HO-1, anti-rabbit immunoglobulin G and horseradish peroxidase conjugates, in successive order. Absorbance at 450 nM was measured, and HO-1 concentration was calculated from a standard curve generated with purified HO-1. The limits of detection provided by the manufacturer were 0.78–25 ng/ml. Each measurement was performed in triplicate, and averages were reported.

2.3. Interleukin-6 and tumor necrosis factor-α measurements

Commercially available ELISA kits (Biolegend, USA) were used to measure interleukin (IL)-6 and tumor necrosis factor (TNF) α concentrations in human milk samples. The assay was performed in accordance with the protocol provided by the manufacturer. Briefly, each milk sample was incubated with anti-human IL-6 or TNF-α, anti-rabbit immunoglobulin G and horseradish peroxidase conjugates, in successive order. Absorbance at 450 nM was measured, and IL-6 and TNF-α concentrations were calculated from a standard curve generated with purified IL-6 or TNF-α. The limits of detection provided by the manufacturer were 0.78–25 ng/ml. Each measurement was performed in triplicate, and averages were reported.

2.4. Cytokine release assays

Cytokines were detected by Flow Cytometer (Epics XL-MCL; Beckman Coulter, Miami, FL, USA) using the FlowMultiplex kit (Bender MedSystems, Vienna, Austria), which was designed to identify the following cytokines in biological fluids: interferon-γ, IL-1, IL-2, IL-4, IL-5, IL-8, IL-10 and TNF-β (the detection limits were 12, 6.8, 43.8, 9.6, 6.1, 7.8, 9.6, 12.5, 13.2 and 4.8 pg/ml, respectively). Briefly, two sets of microspheres with different sizes were used. Each of the two sets consisted of five bead populations internally dyed with varying intensities of a fluorescent dye. The combination of two different bead sizes and five varying dye intensities allowed 10 bead sets in one fluorescence channel to be distinguished using the same principle as ELISA.

2.5. GSH measurement

Levels of nonproteic thiol groups were measured in 200 μl of milk, in accordance with the method of Hu [25], with partial modifications. This spectrophotometric assay is based on the reaction of thiol groups with 2,2-dithio-bis-nitrobenzoic acid (DTNB) in absolute ethanol to give a colored compound absorbing at λ=412 nm. Since the DTNB method is strongly affected by pH, the possibility of avoiding acids (trichloroacetic or sulfosalicylic acid) to precipitate proteins represents a remarkable advantage to ensure the accuracy of the assay. We then carried out the removal of proteins with an excess of absolute ethanol, followed by centrifugation at 3000×*g* for 10 min at room temperature. Each value represents the mean±S.D. of three experimental determinations for each sample. Results were expressed as micromoles per milliliter of milk.

2.6. Validation of immunoassay for the study of human milk

Because human milk is known to contain soluble receptors and other proteins that can bind to other moieties in human milk, milk specimens from human colostrum, transition milk, mature milk and milk formulae were divided in two equal parts. The first aliquot was not treated, while a known quantity of human HO-1 (4 μg) was added to the second aliquot. The quantities of the protein in both aliquots were then determined using the immunoassay. The percentage of recovery of the added protein was calculated by subtracting the amount in the first aliquot from the quantity in the second aliquot and dividing the difference by the amount of the protein that was added to the second aliquot. The percentages of recovery from the different milks examined were greater than 90% for all (colostrum, 96.5%; transition milk, 97.8%; mature milk, 94.7%; milk formula, 94.3%).

2.7. Computational analysis

Molecular modeling studies were carried out with an INTEL PENTIUM D 3.2-GHz processor running LINUX Fedora Core 8.0 (Kernel 2.6.24.4-64), using the softwares GRAMM (version 1.03) [26–28], CLUSTALW (version 2.0) [29] and DALI [30]. GRAMM [26–28] is a program for molecular docking that predicts the structure of a complex between two proteins using only the atomic coordinates of the two molecules. DALI (http://www.ebi.ac.uk/Tools/dalilite/index.html), the protein–protein docking program PatchDock [31,32] and the molecular structure refinement program FireDock [33,34] (http://bioinfo3d.cs.tau.ac.il/PatchDock/), which are available as Web services, were also used.

GRAMM has been used particularly for the analysis of globular proteins and their ligands [26–28]. CLUSTALW [29] was used to analyze the sequence alignment between two proteins, and DALI [30] was used to obtain the structural alignment between two proteins. Prior to docking simulations, the coordinates of each Protein Data Bank (PDB) file were carefully checked, and the cocrystallized ligand was extracted. Atom-potential-type bond orders were carefully checked to evaluate their correctness with respect to the intended structure. Built structures were minimized to obtain proper bond lengths and angles. Human HO-1 (PDB code 1N3U) interactions in different proteins that bind to chaperonins were analyzed by GRAMM using an exhaustive six-dimensional search (rotation of two three-dimensional molecules in space) routine [26–28]. In particular, a low-resolution generic docking was performed. The low-resolution docking algorithm is conceived for the prediction of the gross features of a complex by identification of the local structural elements of participating molecules and large-scale motifs that facilitate complex formation. It was used to select a list of candidate interactors whose ligands (CD4, PDB code 1WBR; LOX1, PDB code 1YXK; γ-δ receptor, PDB code 1YPZ; CD91, represented by its highly similar protein PDB 1N7D) were compared to HO-1 in terms of sequence and structure. The generic docking [28] mode algorithm was used to explore all ligands' positions and orientations with a grid step size of 6.8 Å, which is enough for the identification of regions that are important for protein conformation. The electrostatic potential used for complex evaluation was calculated on each grid step. Protein sequence alignments are important tools in the identification of conserved sequence regions that usually correspond to key elements in delivery molecular functions such as binding. The sequence and structural alignments to identify structural similarities between proteins were performed by CLUSTALW [29] and DALI [30], respectively. Default settings were used. The pairwise version of DaliLite can be accessed as a Web service, and it provides a global pairwise optimized superimposition of two input structures comparing the contact maps of α carbons. Protein shape comparison tools are also implemented with DALI to identify conserved gene-function-related proteins. The conserved residues of two alignments were compared to heme oxygenase residues that, according to the docking results, may be involved in protein–protein interactions. DS Visualizer was used for visualization. This tool provides functionality for visualizing, analyzing and sharing biological and chemical data which can be viewed from multiple perspectives by providing options through three-dimensional structures, sequences and data tables (Accelrys DS

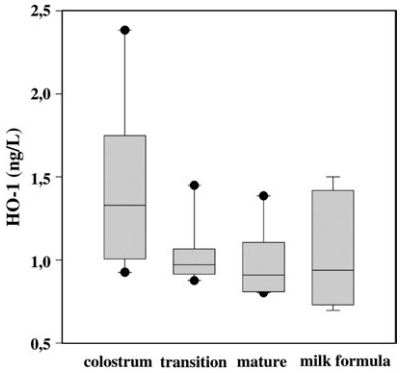

Fig. 1. HO-1 levels in breast milk and milk formulae. The lower and upper bars represent the 5th and 95th centiles, respectively; interquartile range is indicated by the box; and median value is represented by the horizontal line in the box. HO-1 protein milk concentrations in colostrum and transition milk were significantly higher ($P<.001$ for all) than the HO-1 protein milk concentration in mature milk. No significant differences ($P>.05$) in protein concentrations were observed between colostrum and milk formulae.

Visualizer, version 2.0, 2007; Accelrys Software Inc., San Diego; for more details, visit www.accelrys.com).

PatchDock uses a molecular docking algorithm based on shape complementarity principles. The algorithm divides the Connolly dot surface representation of the molecule into concave, convex and flat patches [31,32]. Candidate transformations are generated from complementary patches and evaluated by considering both geometric fit and atomic desolvation energy. Poses are finally refined using the FireDock software [33,34]. FireDock uses an efficient method for the refinement and rescoring of rigid-body docking solutions. The refinement is performed by rearrangement of interface side chains and adjustment of the relative orientation of the molecules.

2.8. Statistical analysis

Maternal parameters are expressed as mean±S.D. Milk concentrations of HO-1, GSH and cytokines are expressed as medians and 5th to 95th centiles. Statistical analysis was performed by between-groups comparison using the Kruskal–Wallis one-way analysis of variance. The correlation between the concentrations in human HO-1 and GSH samples and gestational age at which the samples were taken was assessed by linear regression analysis. Statistical significance was set at $P<.05$.

3. Results

3.1. HO-1 measurement

HO-1 protein was present in all samples of human milk. Its concentrations were highest in colostrum (1.33 ng/ml; 5th centile 0.92; 95th centile 2.38) and transition milk (0.97 ng/ml; 5th centile 0.87; 95th centile 1.45), and significantly ($P<.001$) decreased at 1 month from birth (0.9 ng/ml; 5th centile 0.8; 95th centile 1.38) (Fig. 1). HO-1 was also measurable in all milk formulae (0.98 ng/ml; 5th centile 0.69; 95th centile 1.97). In particular, milk formulae showed no significant differences in HO-1 content when compared to colostrum (Fig. 1).

Chemical complementarity was observed between HO-1 and human CD91. The putative interacting surface of CD91 is represented by a bulge region with specific patterns of charge distribution. The HO-1 model possesses a concave surface with charge distribution. It is likely to allow molecular contacts with CD91 (Fig. 2). The charged and polar residues observed in this region on HO-1 are Glu63, Tyr78, Glu81, Glu82, His84, Lys86, Glu90, Gln91 and Gln102. The charged and polar residues observed on CD91 in the region are Arg571, Thr576, Thr536, Arg553, Trp556 and Ser565. Hydrogen bond interactions were observed between Glu90 and Gly571, between Glu63 and Thr576, and between Gln102 and Thr536. Furthermore, hydrophobic–hydrophobic interactions were observed between Tyr55, Val59 (HO-1) and Val535 (CD91). Salt bridges were also observed between Lys177 (HO-1) and Glu332 (CD91) outside of this region. Since human HSP70 is known to bind to CD91, a pairwise sequence alignment between HO-1 and HSP70 was performed by CLUSTALW to identify conserved residues. Indeed, it is well known that amino acid residues, which are responsible for molecular recognition and protein–protein binding, are usually not allowed to diverge during molecular evolution. HO-1 amino acids in

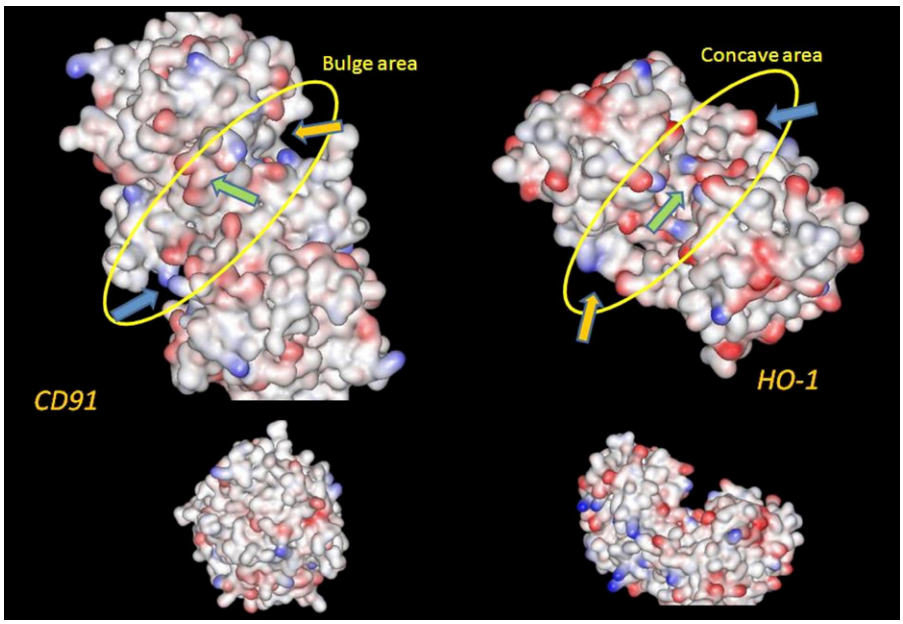

Fig. 2. Details of CD91 and HO1 molecular surfaces. As gathered from docking results, the main possible complex interface could be represented by a bulge region in CD91 and a concave region in HO-1. Electrostatic complementarity is shown on the upper part of the picture. The blue and yellow arrows point to two negatively charged areas in CD91 that correspond to two positive areas in HO-1. The green arrow points to a positive region in the middle of the hydrophobic bulge of CD91 that corresponds to a mainly negative cavity in the center of the HO-1 concave area. The lower part of the picture highlights the shape complementarity of putative binding regions (visualization obtained by Accelrys DS Visualizer).

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hemeox          MERPQPDSMPQDLSEALKEATKEVHTQAENAEFMRNFQKGQVTRDGFKLVMAS-LYHIYV 59
hsp70           ------------------GAMGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKA 41
                                  .:  :.  .:. . :  *: *  * *   *.: . ::.: .

hemeox          ALEEEIERNKESPVFAPVYFPEELHRKAALEQDLAFWYGPRWQEVIPYTPAMQRYVKRLH 119
hsp70           TAGDTHLGGEDFDNRLVNHFVEEFKRKHKKDIS------QNKRAVRRLRTACERAKRTLS 95
                :  :    .::       :* **::**   : .       . : *    .* :*  : *

hemeox          EVGRTEPELLVAHAYTRYLGDLSGGQVLKKIAQKALDLPSSGEGLAFFTFPNIASATKFK 179
hsp70           SSTQASLEIDSLFEGIDFYTSITR----ARFEELCSDLFRS-------TLEPVEKALRDA 144
                .  ::. *:   .    :  .::      :: : . **  *       *:  : .* :

hemeox          QLYRSRMNSLEMTPAVRQRVIEEAKTAFLLNIQLFEELQELLTHDTKDQSPSRA 233
hsp70           KLDKAQIHDLVLVGGSTR------IPKVQKLLQDFFNGRDLNKSINPDEAVAY- 191
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Fig. 3. Sequence alignment of HO-1 with HSP70, which is known to bind to CD91, by CLUSTALW. HO-1 residues that contact CD91 in the docking are shown in negative. An extended binding region from residue 62 to residue 76 is not conserved between HO-1 and HSP70, suggesting a specific binding mode. In the central part of the HO-1 protein sequences, six contacting residues belong to a highly conserved region that contains three amino acid identities (residue E in position 81, residue R in position 85 and residue K in position 86) and one strong chemical similarity (residue Y in position 77).

the CD91 interaction interface, obtained by docking simulation, were selected and mapped on HSP70 versus HO-1 alignment (Fig. 3). Most of the interface residues occupy a conserved position in the alignment, suggesting a putative functional role for HO-1. Both docking softwares GRAMM (version 1.03) and PatchDock showed similar docking poses (Fig. 4). In particular, in a highly homologous central region of nine residues (starting from position 78), glutamic acid 81, arginine 85 and lysine 86, which are conserved as identities in both HO-1 and HSP70, are involved in protein–protein contacts in the predicted complex. Moreover, in accordance with the docking results, tyrosine 78 is also involved in binding. It is replaced by the chemically *cognate* histidine in HSP70. A structural alignment by DALI also showed that some minor structural conservation occurred when comparing HO-1 structure to HSP70 structure (data not shown), but the overall shape similarity was quite small.

3.2. GSH levels

GSH was present in all samples of human milk. Its concentrations in the colostrum taken at 24 h (0.05 μmol/ml; 5th centile 0.01; 95th centile 0.10) and 14 days (0.06 μmol/ml; 5th centile 0.02; 95th centile 0.14), and in mature milk (0.07 μmol/ml; 5th centile 0.02; 95th centile 0.27) were not significantly different (Fig. 5). GSH was also measurable in all milk formulae (0.39 μmol/ml; 5th centile 0.18; 95th centile 0.42), but at significantly higher levels than in human colostrum, transition milk and mature milk ($P<.001$ for all groups) (Fig. 3).

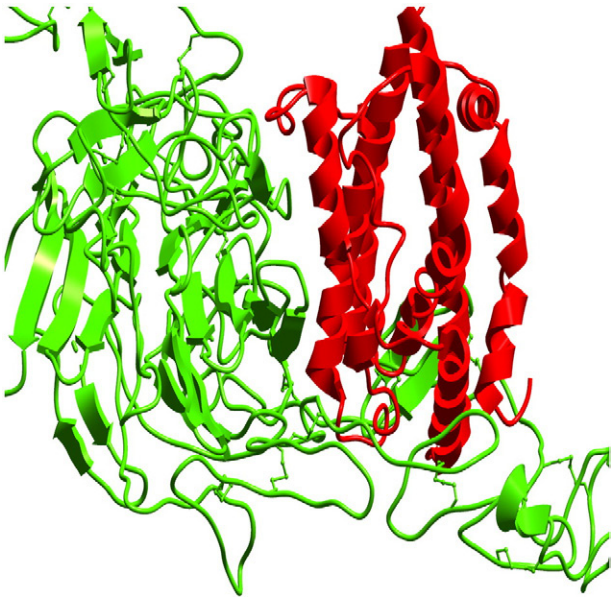

Fig. 4. Close-up view of docked poses at the interface between HO-1 (red) and CD91 (green) proteins.

3.3. Cytokine determination

Cytokines were not detectable by either ELISA or cytofluorimetric analysis, with the exception of IL-8, which was detectable only in six samples of colostrum, in one sample of transition milk and in two samples of mature milk (Fig. 6, Table 1).

4. Discussion

The present data were the first to show that HO-1 is present in human milk and milk formulae at different concentrations, and that its levels in human milk decreased as the milk matured. The findings are in agreement with the notion that the concentrations of colostrum and milk constituents change with suckling time [35]. The presence of a typical intracellular protein such as HO-1 in milk is consistent with previous observations demonstrating the presence of other intracellular proteins (e.g., α-lactalbumin, calmodulin and osteocalcin) in extracellular space and/or biological fluids [36–38]. The presence of such proteins in milk is to be related to an active secretory mechanism and/or to the anatomical and physiological characteristics of the mammary gland (i.e., apocrine mechanism of secretion).

Extracellular stress proteins, including HSP, are emerging as important mediators of intercellular signaling [20,39,40] and transport. Release of such proteins from cells is triggered by physical trauma and behavioral stress, as well as by exposure to

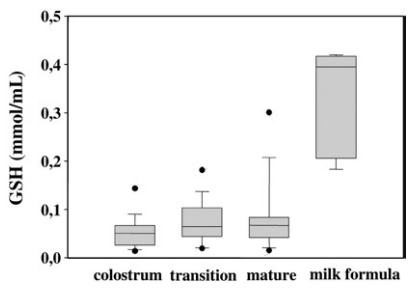

Fig. 5. GSH levels in breast milk and milk formulae. The lower and upper bars represent the 5th and 95th centiles, respectively; interquartile range is indicated by the box; and the median value is represented by the horizontal line in the box. GSH milk concentrations in colostrum were significantly lower ($P<.001$ for all) than those in transition milk and mature milk. GSH levels in milk formulae were significantly higher ($P<.01$) than those in colostrum, transition milk and mature milk.

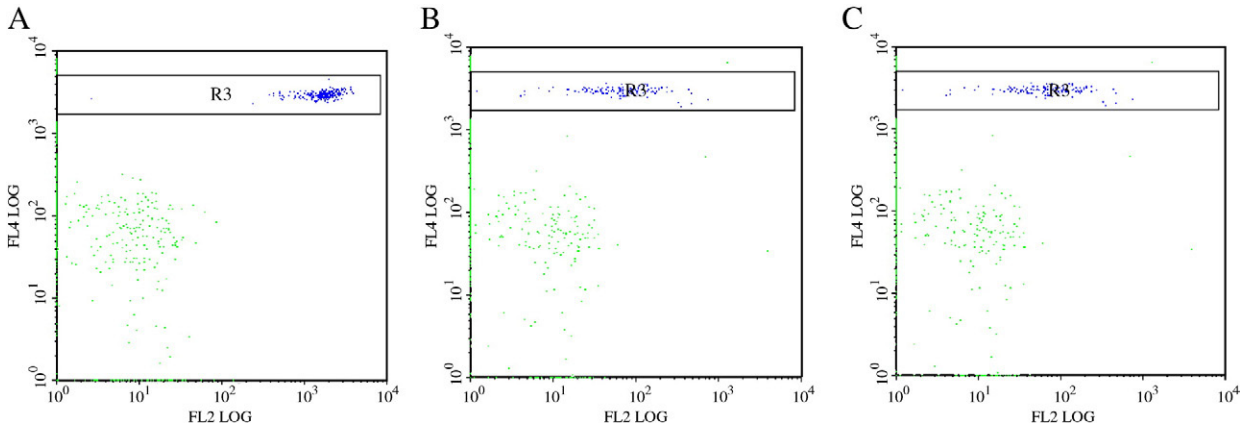

Fig. 6. Flow cytometry analysis of cytokine levels in breast milk. IL-8 was present in six samples of colostrum (A), in one sample of transition milk (B) and in two samples of mature milk (C). FL2 LOG represents the quantity of IL-8, whereas FL4 LOG represents bead size.

immunological "danger signals" [41]. After release into extracellular fluid, HSP may then bind to the surfaces of adjacent cells and initiate signal transduction cascades, as well as transport of cargo molecules such as antigenic peptides [41]. In addition, HSP60 and HSP70 are able to enter the bloodstream and may act at distant sites in the body [20]. However, a similar role for HO-1 has never been suggested before, since only recently was it measured in the extracellular compartment [22,23]. Nevertheless, integrating experimental data with *in silico* data (*vide infra*) also highlighted the putative role for HO-1 as an immunomodulator.

Protein–protein interactions regulate a wide variety of important cellular pathways and, therefore, represent a highly populated and very challenging class of targets for drug discovery [42,43]. In order to elucidate the possible significance of HO-1 in milk, we performed computational studies to screen possible ligands of HO-1. In this respect, it is interesting to note that other HSPs (HSP70 and HSP60) are putatively regarded as chaperokines with an immunomodulatory effect, so that it is tempting to correlate the presence of HO-1 in breast milk with this role. Multiple computational results by different softwares suggest that a remarkable complementarity exists between HO-1 and an important immunoregulatory receptor such as CD91. Moreover, structural and amino acid sequence similarities between HO-1 and HSP70, which is a known interactor of CD91, were found in binding areas. CD91 has been previously identified as a receptor for the serum protein α_2-macroglobulin, a 'natural' protease inhibitor that, similar to HSPs, is found across many species [44]. CD91 was first identified by Binder et al. [45] as a receptor for gp96, and then by Basu et al. [46] as a common receptor for other HSPs such as HSP70, HSP90 and calreticulin. Srivastava [47] and Stebbing et al. [48] interestingly observed that CD91 appears to be a common receptor for all other identified HSPs and that blocking of CD91 with antibodies completely inhibits the phenomenon of re-presentation of peptides that are carried or chaperoned by HSP. The immunological data are already convincing, although it remains to be elucidated whether these correlate with sustained clinical responses. An increasing body of evidence suggests that CD91, also referred to as the common HSP receptor, represents an important route for stimulating a $CD8^+$ T-cell response by major-histocompatibility-complex-class-I-restricted presentation [49]. This may also be a fairly general mechanism by which the innate immune system may stimulate the adaptive arm in viral infections and tumors. Taken together, these findings could be consistent with the hypothesis that HO-1 has an immunoregulatory role that is not dependent on its enzymatic activity but is related to its ability to bind specific receptors, thus regulating the immune response. However, we cannot exclude the possibility that milk HO-1, similar to other milk antioxidant enzymes, may exert protection *via* its antioxidant activity, which is related to the conversion of free heme into three end products: (a) biliverdin, which is rapidly reduced by biliverdin reductase into bilirubin (which possesses antioxidant activity) [50]; (b) carbon monoxide, a potential gaseous neurotransmitter and vasodilator with anti-inflammatory and antiapoptotic activities [21]; and (c) iron (Fe^{2+}), which is sequestered by iron-binding proteins [51,52].

It is noteworthy that HO-1 concentration was higher in human milk than in milk formulae, thus providing additional support to the evidence that the immune system of both term and preterm infants fed on breast milk matures faster than the immune system of infants fed on milk formulae [53] because of their different compositions.

The different concentrations of HO-1 in human colostrum and mature milk (in which the lowest levels of this protein were detected) and in milk formulae could be, at least in part, related to the procedures routinely employed in the commercial preparation of milk and its by-products. In this respect, the drying procedure inevitably causes a reaction in milk that affects protein fraction, leading to the formation of compounds characterized by nonbioavailability of amino acids. Therefore, one explanation for the lower levels of HO-1 protein levels in milk formulae may reside in the possibility that epitopes of the protein could have been modified during these production processes, limiting accuracy in the quantitative measurement of protein. In addition, the possibility that production processes may also affect HO-1 protein, reducing or destroying its biological activity, has to be taken into due account. Further studies aimed at investigating HO-1 modifications during the commercial preparation of milk formula are needed in order to offer conclusive information on the fate of the protein during these processes. If our hypothesis is confirmed, then our data could be useful in limiting this phenomenon and in improving the nutritional properties of industrially prepared milk formulae, as recently demonstrated for other proteins [54].

Table 1
IL-8 in different milk samples

ID sample	Colostrum (pg/ml)	Transition milk (pg/ml)	Mature milk (pg/ml)
ID41	614.89	ND	ND
ID54	1111.1	ND	ND
ID56	3656.2	768.6	697.9
ID57	963.3	ND	ND
ID85	2179.6	ND	ND
ID88	1667.1	ND	811.8

ND, not detectable.

As regards GSH, another important endogenous antioxidant, we found no significant difference in GSH content in colostrum, transition milk and mature milk. The presence of GSH in breast milk is of great nutritional interest, since it may provide antioxidant protection at a period when the newborn may lack the capacity to synthesize GSH or do so efficiently [55]. Feeding on breast milk thus becomes the principal source of GSH for the human infant in early childhood. This practice would be expected to render such infants less susceptible to the effects of oxidants and other toxins, in addition to the provision of colostrum for anti-infective action. In addition, our data demonstrated that milk formulae contain significantly higher levels of GSH when compared to breast milk, thus providing a good source of this protective antioxidant for newborns. To the best of our knowledge, only one study has reported on GSH levels in human milk from Ghanaian women; this study showed decreasing values of GSH at higher milk maturation stages, in apparent contrast with our results [56]. However, racial, social, environmental and nutritional status and methodological differences should be taken into due account.

In order to further corroborate the HO-1 and GSH levels in human milk, we assessed the levels of well-established pro-inflammatory cytokines in order to exclude any inflammatory status of the mother. Our study provides evidence for the presence of IL-8 only among several screened cytokines. High levels of IL-8 in colostrum may play a significant role in the movement of maternal neutrophils, monocytes and lymphocytes to the milk and perhaps subsequently across the neonatal bowel wall [57]. Such trafficking contributes to mucosal defense and the development of the newborn's immune system. We observed statistically significant changes in IL-8 levels in our study. Colostrum showed the highest levels of IL-8, but those decreased quickly in transition milk and mature milk. Similarly, previous studies showed that IL-8 levels in colostrum were significantly higher than the levels in both transitional milk and mature milk [58]. IL-8 is one chemoattractant cytokine that acts as a pro-inflammatory mediator [59]. Furthermore, IL-8 was significantly elevated in mature milk of allergic mothers [60]. However, no history of allergy was recorded for the mothers included in our study. The presence of IL-8 in breast milk might be responsible for the traffic of leukocytes from maternal circulation to breast milk. In addition, the absence of other pro-inflammatory cytokines in human milk further supports the notion that HO-1, which is known to be induced by such inflammatory mediators, may be the result of an active mechanism of secretion under physiological conditions.

HO-1 physiological function, which possibly includes an immunoregulatory role, was supported by a computational analysis of protein–protein interactions analyzing the structural characteristics of protein-binding sites. Protein–protein interactions are at the center of almost every cellular process, from cell motility to DNA replication. Rationalizing protein–protein interactions helps to elucidate the function of a known or novel protein and the role it plays in a known pathway. Proteins that bind to known members of a known pathway are potential new members of the pathway. The reported results might be successfully exploited to derive principles for the discovery of HO-1 mimics that might antagonize or synergize HO-1 interactions, finally acting as immunomodulators.

Although detailed information on the fate of the HO-1 molecule in the gastrointestinal tract is needed to corroborate the hypothesis that HO-1 participates in the nutritional and immunoregulatory effects of milk, the present findings, arising from *reverse pharmacology* [61], open a new clue on the possible role of HO-1 as a new chaperokine through a multidisciplinary "wet-and-dry" approach. Further analytical studies of the HO-1–CD91 complex under native conditions (by direct ESI-Q time-of-flight mass spectrometry analysis) and characterization of the peptides generated by limited proteolysis of the complex (by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) are now warranted in order to fully elucidate the molecular details of such interaction.

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