BASIC SCIENCE ARTICLE

Impact of Holder pasteurization on immunological properties of human breast milk over the first year of lactation

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BACKGROUND: The timing of milk donations to human milk banks ranges from a few days to more than 1 year after delivery, and the Holder method is used for pasteurization. We evaluated the effect of temporal variation and thermal treatment on the immunological properties of milk.
METHODS: We analyzed 73 milk samples, raw and after pasteurization, donated at different lactation stages. We studied antibodies, lysozyme, cytokines, soluble receptors, and factors with impact on barrier function. We also evaluated in vitro the capacity of milk to modulate nuclear factor-κB (NF-κB) signaling in an HT-29 epithelial cell line stimulated with tumor necrosis factor-α (TNF-α).
RESULTS: With few exceptions, immune components exhibited their highest levels in colostrum, and were stable in the various stages of mature milk. Pasteurization altered the immunological composition of milk, and very drastically for some components. Raw milk of the first year reduced NF-κB activation in HT-29 cells treated with TNF-α to approximately the same extent, and Holder pasteurization significantly affected this capacity.
CONCLUSIONS: Overall, the present work reports that mature donated milk is equally valuable over the first year of lactation, but warns about drastic losses of anti-inflammatory properties during Holder pasteurization that could be critical for the health of preterm infants.

INTRODUCTION

Human milk is the best food for both term and preterm newborns (NBs); it provides nutrients and bioactive components adapted to the needs of NBs.[1] The immunological composition of milk has been widely studied, and most research has focused mainly on the more abundant components.[1,2] However, a lot of minor components with relevant functions have been less studied;[3] their concentration depends on each particular component and the timeframe.[2,4,5]

When NBs cannot be fed with their own mother's milk, such as preterm infants in neonatal intensive care units, the human milk donated to human milk banks (HMBs) is an important resource to support their health. According to international guidelines,[6] the milk is pasteurized by the Holder method (62.5 °C for 30 min); however, treatment affects its immunological composition.[4,7,8] Although colostrum contains the maximal concentration of bioactive compounds, mature milk is the most abundant donated milk; thus, it is important to know how the immune components of milk change over time.

Biological evaluation of the benefits of human milk has been poorly studied. Some workers have studied the effect of individual components on in vitro or in vivo models.[9–11] However, few studies have evaluated the effect of whole raw and pasteurized human milk.[12]

In present work, we studied the immunological composition of donated milk. We determined changes associated with time post delivery of donation, and the effect of Holder pasteurization. Finally, we compared the biological effects of both (time of donation and Holder pasteurization), using the HT-29 cell line.

MATERIALS AND METHODS

Milk samples

This is a descriptive study of a convenience sample of donated milk obtained at different days during 1 year postpartum. We recruited donors from the HMB of the Pereira Rossell Hospital (Montevideo, Uruguay). Milk availability was the main inclusion criterion among donors who met the HMB criteria. Exclusion criteria included positive serology for infectious diseases (e.g., HIV, syphilis) and consumption of illicit drugs or medications incompatible with breastfeeding. The HMB in Uruguay processes milk following the guidelines of the Brazilian Network of HMB (2008). Briefly, mothers extract their milk with an electric pump into sterile glass bottles and keep it frozen until collected. Each individual donation is made up of pooled breast milk extracted over one day. The milk that complies with the requirement of off-flavor and physicochemical analysis is pasteurized by the Holder method (62.5 °C for 30 min). Thawed processed milk is used under

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medical prescription. The pasteurization equipment used in our HMB (Dexin-Grupo-Químico, S.R.L., Uruguay) has the ability to process 29 bottles/150 mL each, and has an informatic system to monitor the process. The bottles are partially submerged in water and continuously agitated to guarantee the homogenization of temperature; a milk bottle with the probe of temperature is placed in the middle of the bath. After 30 min at 62.5 °C, milk samples are cooled to room temperature with tap water that circulate continuously in the equipment, and finally to 4 °C with a bath of water/ice. All process takes approximately 70–80 min.

Seventy three women donated one milk sample during March–October 2016, and samples were analyzed before and after Holder pasteurization. This represented about 36% of the woman who made milk donations. Characteristics of mothers and infants are summarized in Table 1. For analysis of changes over time, we arbitrarily divided samples into five groups according to the days/months after delivery: 2–7 days (colostrum, C), 9–30 days (M1), 2–3 months (M2), 4–6 months (M3), and 9–12 months (M4).

The samples were stored at −80 °C until use. Once thawed they were centrifuged in two sequential steps to obtain the aqueous fraction (AF): 1000 rpm, 10 min (4 °C) and 10,000 rpm, 30 min (4 °C) to remove the lipid phase. The AF was aliquoted and stored at −80 °C until use.

The study was approved by the Research Ethics Board of the Pereira Rossell Hospital and written informed consent was obtained from all participating mothers.

Protein content
Protein concentration in the AF was determined by the Bradford method[13] adapted to microplates. Briefly, 25 μL of milk or standard diluted in phosphate-buffered saline (PBS) was added to 96-well plates (Grainer, Frickenhausen, Germany) with 200 μL of Bradford reagent. After 5 min with gentle agitation, optical densities (ODs) were read at 600 nm. Standard curves were constructed using the AF of mature milk with protein concentration evaluated by the Kjeldahl method (11.4 mg/mL).

Analysis of immune components
Antibodies. Immunoglobulin A (IgA), IgM, and IgG were quantified by the capture enzyme-linked immunosorbent assay (ELISA) previously developed.[4] Briefly, microplates (Grainer) were coated with the appropriate capture antibody in PBS overnight (ON; 4 °C). After blocking with 1% gelatin in PBS (1 h, 37 °C), milk samples or standard were added in 0.2% gelatin and 0.05% Tween-20 in PBS and incubated ON (4 °C). Then, horseradish peroxidase (HRP)-conjugated antibodies were added and incubated for 1 h (37 °C). The color reaction was developed with a solution of sodium

acetate (pH 5.5) containing 1.3 mM H_2O_2 and 0.4 mM 3,3′,5,5′-tetramethylbenzidine; the reaction was stopped with acid and OD_{450} registered.

The capture antibodies used were: rabbit anti-human-Fc antibody (Sigma-Aldrich, St. Louis, MO, USA) for IgA and IgM, and goat anti-human-Fcγ antibody (Abcam, Cambridge, MA) for IgG. For detection, appropriate HRP-conjugated antibodies were used: goat anti-human-Fcα-HRP (Sigma-Aldrich) for IgA and goat anti-human-Fcμ-HRP (Sigma-Aldrich) for IgM. Goat anti-human-Fcγ-HRP (Dako, Glostrup, Denmark) absorbed with goat serum was used for IgG. Standard curves were constructed with a commercial IgA (Sigma-Aldrich) and N Protein Standard SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) for IgM and IgG.

Lysozyme. The enzymatic activity was measured in triplicate using the turbidimetric method with *Micrococcus lysodeikticus*,[14] adapted to microplates. Briefly, 50 μL of solutions of AF or commercial lysozyme (Sigma-Aldrich) in 66 mM potassium phosphate buffer (pH 6.2) was added to microplates. Then, 200 μL of 700 μg/mL of *M. lysodeikticus* (Sigma-Aldrich) previously equilibrated to 30 °C were added. ODs at 450 nm were read immediately and after incubating the plates at 30 °C for 3 h. Standard curves were constructed to calculate activity (LU/mL).

Other immune components. Transforming growth factor-β2 (TGF-β2), monocyte chemoattractant protein-1 (MCP-1), soluble Toll-like receptor-2 (sTLR2), soluble cluster of differentiation-14 (sCD14), epithelial growth factor (EGF), Trefoil factor-3 (TFF3), tumor necrosis factor-α (TNF-α), and interleukins IL-1β, IL-6, IL-10, and IL-17A were analyzed. The concentration of these molecules in the AF of milk samples were quantified in duplicate using commercial capture ELISA kits (R&D Systems, Minneapolis, MN, USA; Pepro-Tech Inc., Rocky Hill, NJ, USA), according to the manufacturer's instructions.

In vitro evaluation of modulatory effects of donated human milk
The regulatory effect of AF of milk was evaluated with HT-29-NF-κB-hrGFP F6 reporter cells.[15] Cells were cultured in 96-well plates (5×10^4 cells/well) in RPMI-1640 supplemented with 10% (v/v) of heat-inactivated fetal calf serum (Capricorn Scientific GmbH, Germany) with 5% CO_2 at 37 °C. After 24 h, the medium was renewed and the stimulus was incorporated. For evaluation of direct effect of milk on cell activation, the sterile AF diluted in complete medium at 1, 2, and 5% (v/v) was added. After 24 h, the cells were trypsinized for flow cytometry analysis. For evaluation of the modulatory effect on cell activation by TNF-α, the sterile AF at

Table 1. Maternal and infants characteristics

	Period of lactation				
	2–7 days C	9–30 days M1	2–3 months M2	4–6 months M3	9–12 months M4
Mother					
Number of donors	10	10	20	20	13
Age (years)[a]	24 (5)	29 (5)	32 (6)	32 (4)	33 (4)
BMI (kg/m²)[a,b]	23 (3)	22 (2)	25 (4)	24 (3)	22 (2)
Gestational weight gain (kg)[a]	13.8 (6.8)	13.8 (4.2)	13.2 (2.3)	14.5 (6.3)	13.6 (2.8)
Infant					
Gestational age (weeks)[a]	38 (4)	40 (1)	39 (1)	40 (1)	39 (1)
Weight (kg)[a]	3.1 (1.0)	3.2 (0.3)	3.6 (0.4)	3.6 (0.5)	3.3 (0.3)
Female (%)	60	60	60	40	65

[a]Data are represented as mean with standard deviation
[b]Body mass index (BMI) at the beginning of pregnancy

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1% (v/v) was used. After 6 h, TNF-α was added at the final concentration of 1 ng/mL, and incubated for 18 h. The AF used in in vitro assays were pools of the individual milk samples from each timeframe.

For flow cytometry analysis, 10,000 events gated on forward scatter/side scatter dot-plot (excluding dead cells with propidium iodide, 2 µg/mL) were acquired in a BD FACSCalibur™ (BD Biosciences). Flowing software was used for analysis. The fluorescence threshold was defined for cells incubated with culture medium. Results were expressed as the ratio between the % GFP-positive cells in the sample with respect to the control wells.

Data analysis

The Mann–Whitney test was used to evaluate differences between sample groups, and the Wilcoxon's matched-pair, signed-rank test was used to examine the effect of pasteurization. Box and whiskers graphs were used to represent the results, where the box is defined by the 25 and 75% quartiles and the median of the data group is represented inside; whiskers are the minimum and maximum values.

In vitro assays were done in triplicate in two independent experiments. Results were represented as the mean and SEM of one representative experiment. Differences between groups were evaluated using one-way analysis of variance.

Differences were considered to be statistically significant when P values were ≤0.05, and statistical tendencies were indicated when P values were between 0.05 and 0.1. GraphPad Prism 6.01 (demo) was used for analyses.

RESULTS

Temporal variation of immunological components of donated human milk

Our first aim was to evaluate the variation of immunological composition of donated milk according to lactation stage, during the first year of lactation. To fulfill this objective, we analyzed a group of 73 milk samples derived from different mothers who were donors to the Pereira Rossell's HMB. We studied various peptides/proteins with immunological activity in the AF of milk, which comprises more than 95% of total protein content.[16]

As was previously reported, protein content decreased during the first 6 months postpartum, from 24.8 mg/mL (median) in colostrum to 11.8 mg/mL (median) in milk of 4–6 months (Fig. 1a). In contrast, lysozyme activity was higher at the end of the first year of lactation, with a significant increase of 220% with respect to the colostrum content ($P < 0.001$, Fig. 1a). IgA was the major antibody during the first year of lactation, and decreased by 85% between the colostrum stage and 6 months after delivery; strikingly, a small increase was observed at the end of the year (Fig. 1b). The IgM profile followed a similar pattern, but a more drastic decrease (97%) was observed in the first 6 months postpartum (Fig. 1b). On the other hand, IgG concentration increased from the colostrum stage (4.4 µg/mL, median) to the mature milk of 1 month (17.1 µg/mL, median), after which it remained constant (Fig. 1b).

The results of temporal variation of EGF, TFF3, TGF-β2, MCP-1, sTLR2, and sCD14 are shown in Fig. 2. EGF concentration did not vary significantly during the first year of lactation. TFF3 decreased from 104.0 ng/mL (median) in colostrum to 0.4 ng/mL at 4–6 months, and then it remained constant. TGF-β2 and MCP-1 were detected in almost all samples; the former did not vary significantly during the first year postpartum. MCP-1 seemed to be more concentrated at the beginning of lactation, but no significant differences were observed. The concentration of sTLR2 was higher in colostrum (1.1 ng/mL, median) and decreased until the lactation stage corresponding to 4–6 months after delivery; indeed, in this group, sTLR2 was not detected in 11 out of 20 samples. The concentration of sCD14 was found to be higher in

colostrum (23.1 µg/mL, median) and then remained constant in mature milk (13.8 µg/mL, median).

Since the components being measured were peptides/proteins, we also evaluated the variation of these components as a proportion of total protein content. For antibodies, the patterns observed for content per mg of protein were similar to the concentration per mL (Fig. 1b); this indicates that variations do not just reflect the decrease in protein content. In contrast, we did not observe any clear pattern for the relative amounts of minority peptide components (data not shown); it is in accordance with their production in very lower amounts by different cell types.

We evaluated other cytokines present in milk: TNF-α, and interleukins IL-1β, IL-6,IL-10, and IL-17A. These cytokines were detected in low concentration and/or in few samples (Fig. 3). IL-1β was detected in 63 samples uniformly distributed between groups, and its concentration was very low and did not vary during lactation (Fig. 3). IL-6, IL-10, IL-17A, and TNF-α were principally detected in the early months after delivery (Fig. 3a).

Effect of Holder pasteurization on immunological components of donated human milk

Our second aim was to determine the effect of Holder pasteurization on the immunological components measured. We focused on components that were detected in the most individual samples, and for analysis purposes, we showed the results corresponding to colostrum and mature milk as the main differences were observed between these two milk types; the main results are shown in Fig. 4.

All antibody isotypes were affected by Holder pasteurization, but with different degrees of susceptibility. IgA decreased 34% and IgG 20%, without differences between colostrum and milk. IgM was the most thermosensitive antibody, with losses of 80% in colostrum and 60% in milk. sCD14 is in high levels in colostrum and mature milk; surprisingly, this molecule was almost completely destroyed (99%) by Holder pasteurization in both colostrum and milk samples. The behavior of the other components was variable; lysozyme activity decreased only 18% compared to raw milk independently of the lactation stage, but EGF and TGF-β2 were more severely affected by Holder pasteurization (they decreased by 44 and 59%, respectively). The levels of sTLR2 and TFF3 did not change with Holder treatment.

Modulatory effect of donated human milk

Finally, we aimed to study the impact of the observed composition variations associated with lactation stages and pasteurization processing on the ability of milk to modulate epithelial cell activation. We used the HT-29-NF-κB-hrGFP F6 cell line to analyze the effect of the pooled AF of milk derived from each timeframe of lactation defined above.

We evaluated the capacity of milk to modulate the activation of cells by TNF-α, by pre-incubation of the cells with the AF (1% v/v, for 6 h). We selected this concentration because it was the highest that did not activate the cells (Fig. 5a). Raw colostrum and mature milk significantly decreased the cell activation induced by TNF-α independently of their timeframe. However, Holder pasteurization considerably affected the modulatory effect of milk, by decreasing the milk's capacity to regulate cell activation by TNF-α (Fig. 5b).

DISCUSSION

Immune factors represent up to 10% of total milk proteins,[17] most of them contained in the AF.[16] Most studies of immune proteins in breast milk have been focused on protection of the infant against pathogens; less information is available about some minority components involved in immune regulation. Several components can also directly or indirectly affect the function of the neonatal gut immune system with long-term impact over the individual's lifespan.[18,19] Interestingly, the development of the infant's
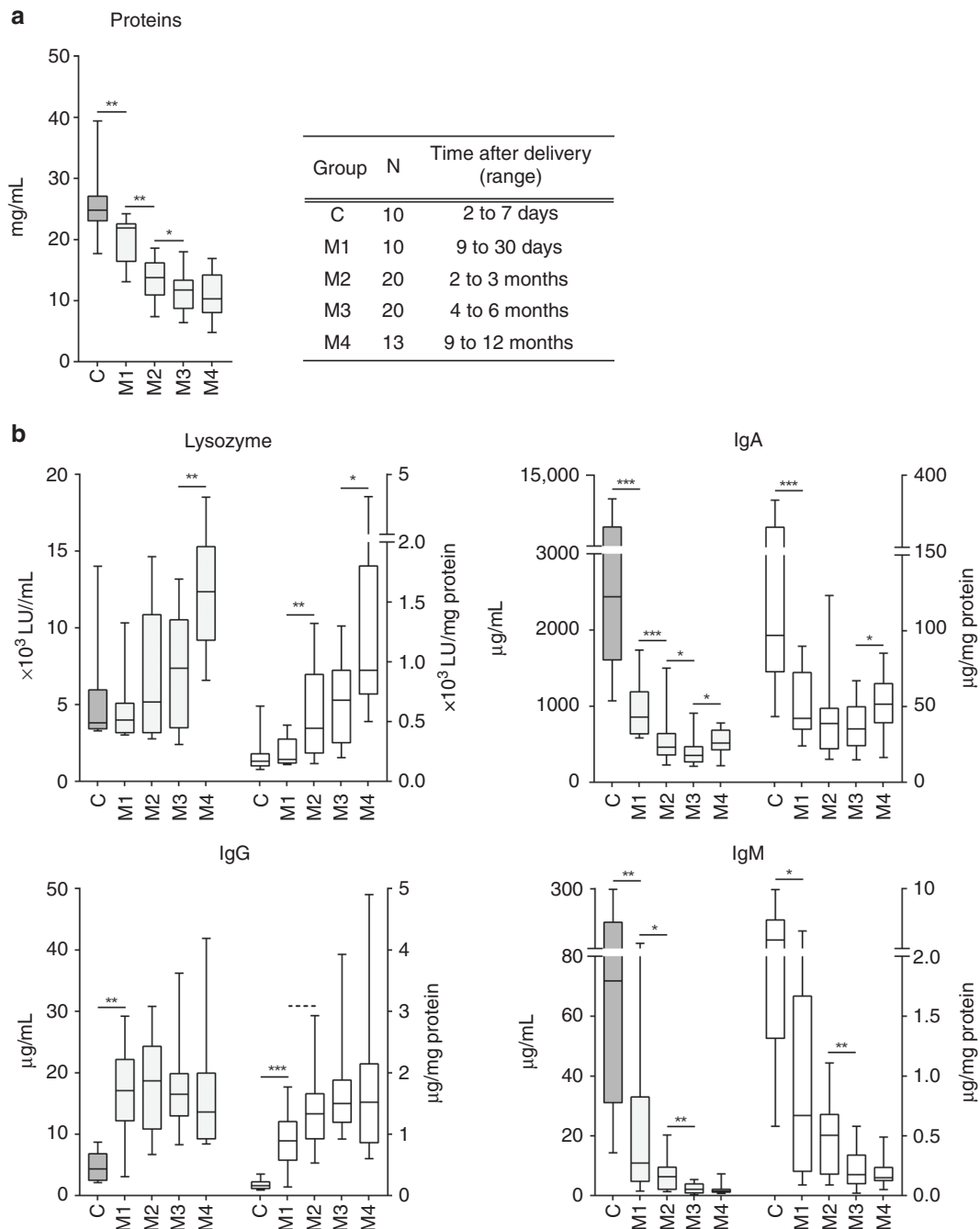
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a Proteins

Group	N	Time after delivery (range)
C	10	2 to 7 days
M1	10	9 to 30 days
M2	20	2 to 3 months
M3	20	4 to 6 months
M4	13	9 to 12 months

b Lysozyme   IgA

IgG   IgM

Fig. 1 Time variations of protein, lysozyme activity, and antibodies in milk. In each box and whisker graph, the box is defined by the 25 and 75% quartiles, and median of data is indicated inside; whiskers correspond to minimum and maximum values. **a** The table describes the number of samples in each study group and the corresponding range of days/months after delivery. Statistical differences between groups are indicated with a line and symbol: *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ (Mann–Whitney test). Dotted lines indicated tendencies

immune system and the immune function of the mammary gland are time-linked processes in normal health; a decline of several immune components can be expected while the child acquires immune self-sufficiency. However, information about the dynamics of several key components is incomplete. Knowledge about the profile of bioactive components is relevant for the health care of hospitalized preterm infants while they cannot be breastfed. Nourishing the preterm infant with donated breast milk is recommended in these situations.[20] Mature milk is the main source of donations, but it is desirable to narrow the gap as much as possible between its composition and that corresponding to the gestational age.

While the time variations of major whey proteins in breast milk (lactoferrin, lysozyme, and antibodies) have been extensively reported,[2,17] scarce data are available regarding the temporal changes of bioactive components with immunoregulatory properties.

We selected for study several soluble factors based on their impact on protection against infections and inflammatory diseases:[18,21,22] (i) antibodies and lysozyme; (ii) factors that contribute to the reinforcement of the infant's gut epithelial barrier (e.g., EGF, TFF3); (iii) chemokines and cytokines that regulate the function of several cell populations (e.g., MCP-1, TGF-β2, IL-10); (iv) soluble receptors that modulate the inflammatory response (sCD14, sTLR2).
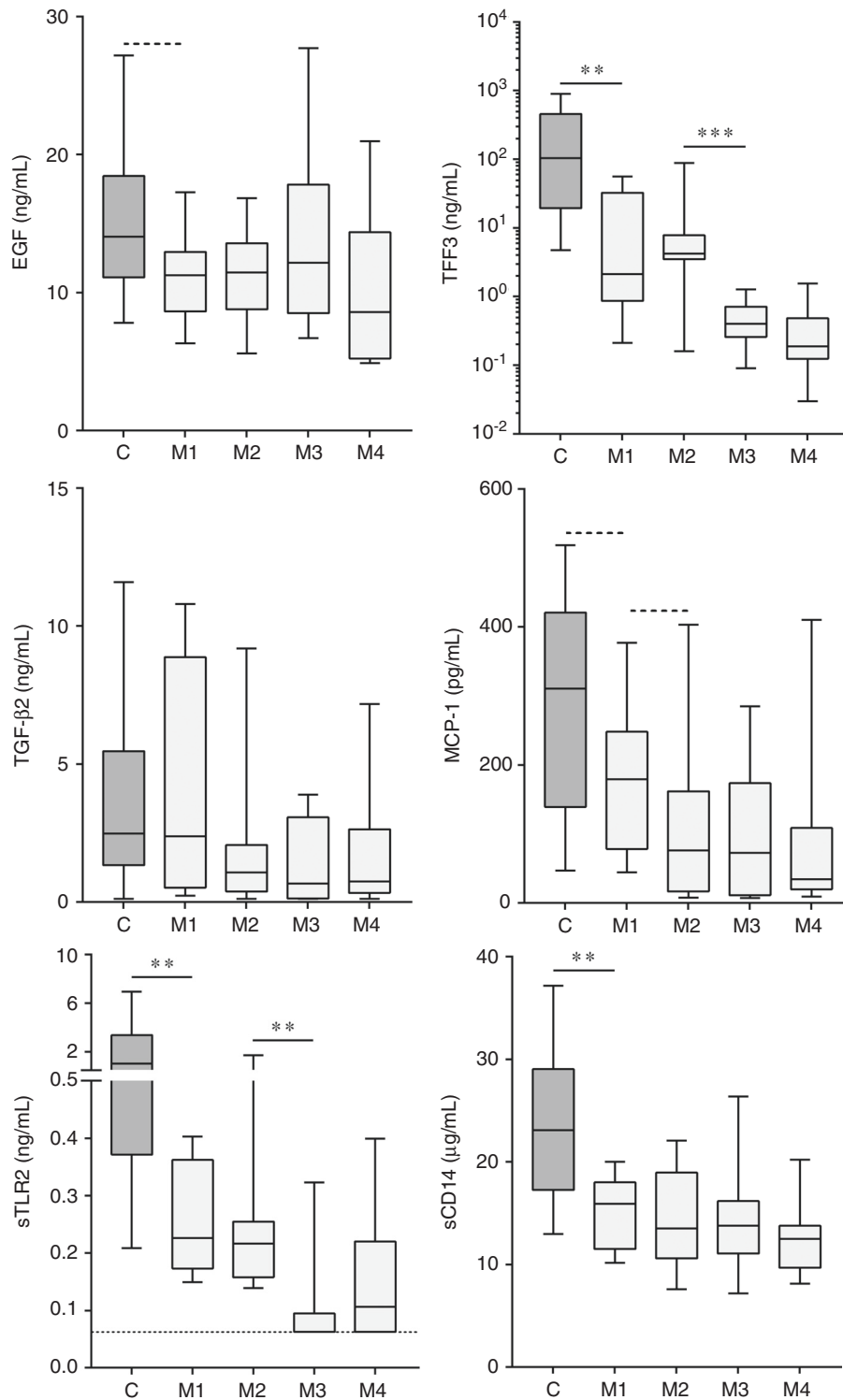
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Fig. 2 Time variations of epithelial growth factor (EGF), Trefoil factor-3 (TFF3), transforming growth factor-β2 (TGF-β2), monocyte chemoattractant protein-1 (MCP-1), soluble Toll-like receptor-2 (sTLR2), and soluble cluster of differentiation-14 (sCD14) in milk. In each box and whisker graph, the box is defined by the 25 and 75% quartiles, and median of data is indicated inside; whiskers correspond to minimum and maximum values. Statistical differences between groups are indicated with a line and symbol: $**P \leq 0.01$, and $***P \leq 0.001$ (Mann–Whitney test); tendencies ($0.05 < P \leq 0.1$) are indicated with a dotted line. The dotted line in sTLR2 graph represents the quantification limit of the ELISA kit

Previous analysis of sIgA was only performed until 90 days postpartum, with a 4.5-fold decrease from colostrum to 3 months.[2] We completed this picture as we analyzed IgA levels until 1 year after parturition, and observed a similar pattern until 3 months, as

a 5.2-fold drop was observed in this timeframe. A striking increase (1.5-fold) after 6 months of lactation was observed. This behavior is in line with recent reports of a significant increase in the concentration of IgA in longitudinal samples of mother's milk
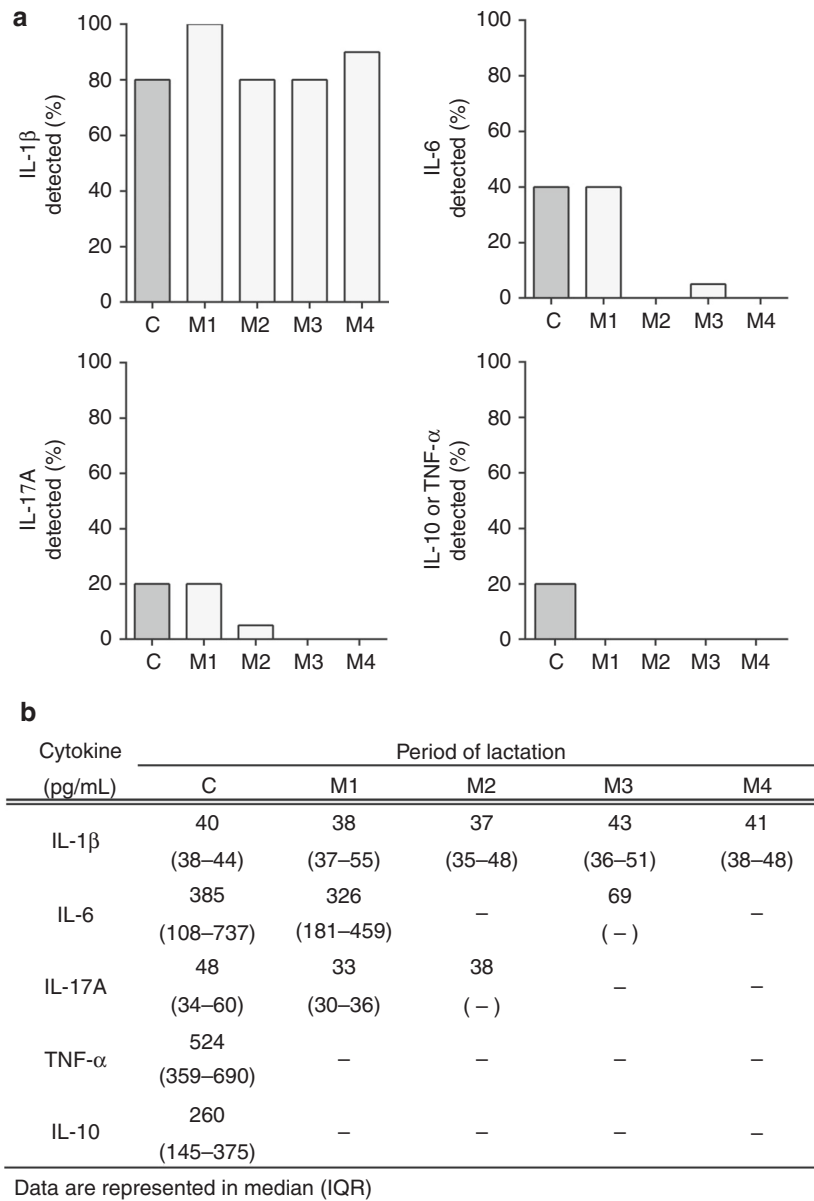
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Fig. 3 Time variations of tumor necrosis factor-α (TNF-α) and the interleukins IL-1β, IL-6, IL-10, and IL-17A in milk. **a** Frequency of samples with detectable cytokine are represented; **b** median concentration and the interquartile range (IQR) are indicated

b

Cytokine	Period of lactation				
(pg/mL)	C	M1	M2	M3	M4
IL-1β	40 (38–44)	38 (37–55)	37 (35–48)	43 (36–51)	41 (38–48)
IL-6	385 (108–737)	326 (181–459)	–	69 (–)	–
IL-17A	48 (34–60)	33 (30–36)	38 (–)	–	–
TNF-α	524 (359–690)	–	–	–	–
IL-10	260 (145–375)	–	–	–	–

Data are represented in median (IQR)

between 11 and 17 months postpartum.[5] The fact that 6 months is the age at which solid foods are introduced into the diet could indicate that this is a key time point linked to functional changes in both infant gut and mammary gland. Minority IgG and IgM antibodies showed different profiles during the first year. IgM is a secretory antibody with a pattern resembling that of IgA, in contrast with IgG, which is derived from serum. We observed stable levels of IgG throughout lactation after an initial increase and this behavior is in line with reports reviewed by Lönnerdal et al.[19]. Longitudinal studies by proteomic approaches also confirmed higher IgG levels in mature milk compared with transitional stage.[23] The late production of IgG by the infant and the catabolism of trans-placental antibodies at about 6 months after parturition could contribute to an IgG deficiency during the first year of life; thus, higher IgG supply through late mature milk would be compensatory at the gut compartment.

Lysozyme is the most conserved protective molecule that participates in innate immunity with antibacterial and anti-oxidant properties.[24] Panneth cells producing lysozyme are absent in neonatal gut, thus lysozyme activity relies completely on breast milk supply.[25] We observed a gradual increase of lysozyme activity throughout lactation, being more evident after 6 months of lactation in line with previous studies.[5,26] The physiological changes of mammary gland function over late lactation are characterized by tissue redistribution. As immune cells play a role in mammary development and involution, the changes of levels of lysozyme and other components could be a consequence of these processes. Nevertheless, the signaling pathways involved in the adaptation of mammary physiology during breastfeeding are poorly understood.

Cytokines are peptides involved in cell chemoattraction and regulation of the immune response. Defective cytokine production is a hallmark of the neonatal immune system, and neonatal gut epithelial cells mount exacerbated responses against inflammatory stimuli. Preterm NBs are highly vulnerable to excessive inflammation, in part because of defective synthesis of
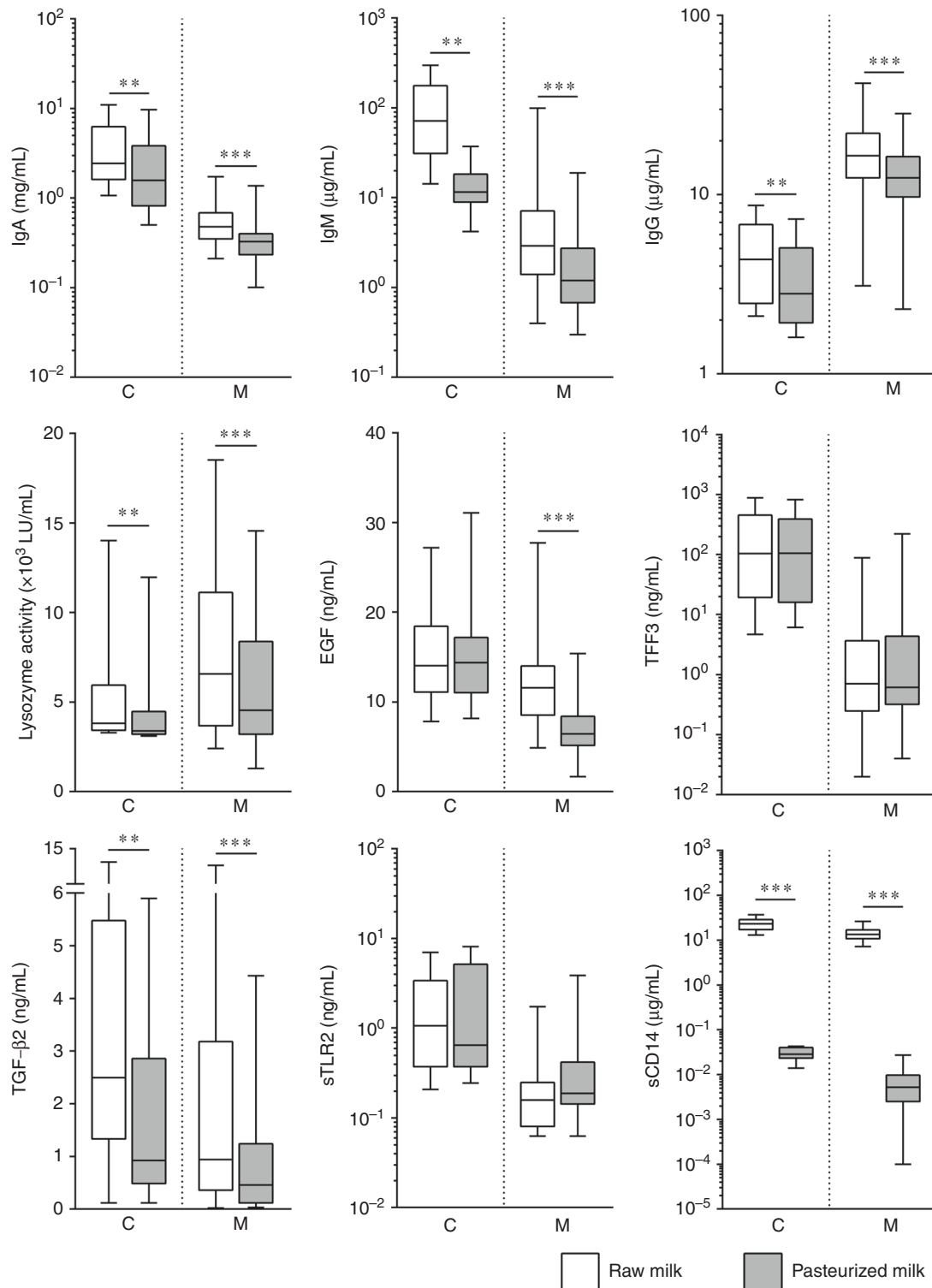
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Fig. 4 Effects of Holder pasteurization on immune components of milk. The graphs compare the level of each immune component measured in raw and pasteurized colostrum ($N = 10$) and all mature-milk samples ($N = 63$). In each box and whisker graph, the box is defined by the 25 and 75% quartiles, and median of data is indicated inside; whiskers correspond to minimum and maximum values. Statistical differences between raw and pasteurized colostrum/mature milk are indicated with a line and symbol: $**P \leq 0.01$, $***P \leq 0.001$ (Wilcoxon's matched-pair, signed-rank test)

immunomodulatory factors.[22] Transforming growth factors and IL-10 are key suppressive cytokines supplied by breast milk that dampen the inflammatory response. While IL-10 detection was limited to 20% of colostrum samples, significant levels of TGF-β2

were measured in most samples over the whole lactation period without significant variations. This pattern is in line with previous work.[3,14] Nevertheless, our results contrast with previous work that found higher rates of detection of proinflammatory cytokines and

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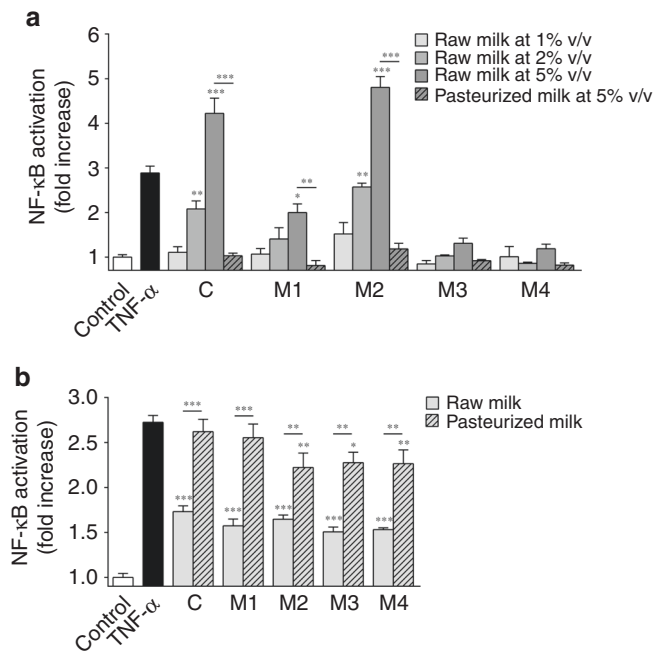C Rodríguez-Camejo et al.

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Fig. 5 Modulatory effect of aqueous fraction (AF) of human milk on epithelial cell activation. **a** HT-29-NFκB-hrGFP cells were incubated for 24 h with tumor necrosis factor-α (TNF-α) (1 ng/mL) or with the AF of milk samples, before and after pasteurization; this was a preliminary experiment in order to establish the concentration of milk that did not activate the cells. **b** The cells were incubated for 6 h with milk samples before the incorporation of TNF-α (final concentration of 1 ng/mL) for additional 18 h. The nuclear factor-κB (NF-κB) activation was analyzed by flow cytometry. For each condition, % GFP+ cells were normalized against the values from cells with culture medium only. One of two individual experiments performed is shown. The bars show mean and SEM of three replicate values. The symbols on the bars represent statistical differences with the control group in **a**, or with the TNF-α condition in **b**, and differences between groups are shown with a line and symbol: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ (one-way analysis of variance (ANOVA))

IL-10.[27] The differences could be attributable to: (i) logistic procedures for milk obtaining and storing until analysis, because freeze-thawing cycles in HMB could account for losses in our work, and (ii) variability of the concentration associated with the location in which the study was made.[28]

Proinflammatory cytokines in milk originate from mammary tissues or from cells transferred to milk (mononuclear and epithelial cells). The dynamics of these cells in milk vary during lactation stages.[29] The profiles of the proinflammatory cytokines TNF-α and IL-6 are in line with the higher immune cell content in colostrum as macrophages represent a main source of these molecules.[30,31] Nevertheless, another cellular source for IL-1β should be taken into account to explain our results. Chemokines are also scarce in neonatal gut, and this can ultimately impact on immunoregulatory mechanisms displayed in lamina propria by dendritic cells and macrophages. We analyzed the profile of monocyte chemoattractant protein-1 (MCP-1/CCL2) because it is a key chemokine that regulates the migration and infiltration of monocytes, which are precursors of these cells. Results indicate that higher levels of MCP-1 are present during early lactation, with a 3-fold decrease in average levels over 2 months.

In addition to cytokines, soluble receptors may regulate the function of epithelial cells by interfering with endogenous or microbial proinflammatory signaling. sCD14 is a very abundant molecule detected in all samples, being more concentrated in

colostrum and stable at lower concentrations throughout lactation stages. In contrast, a striking variable pattern was observed with sTLR2, which was expressed in oscillating levels in mature milk; this may parallel dynamic changes in the immune system of the infant, as the composition of milk responds to variable conditions of the infant.

We also focused on peptide milk components that contribute to the development of the infant gut barrier. EGF promotes epithelial cell growth and proliferation, increases mucus production, and the expression of tight junction proteins.[32] Thus, EGF improves the epithelial barrier function and may be involved in the protection against necrotizing enterocolitis. EGF is also present in amniotic fluid and a continuous supply of EGF is ensured during uterine and postnatal life through breast milk.[33] Interestingly, the EGF levels in breast milk of mothers with severely preterm infants were higher than those from term delivery. All our colostrum samples were from mothers with term delivery, but in line with these considerations we observed a tendency for higher levels at initial lactation stage with no significant variations over lactation, and lower values at final stages in line with gut maturation of the infant. In contrast, a significant decrease of intestinal trefoil factor (TFF3) over the lactation period was observed. TFF3 contributes to the healing of gastrointestinal mucosa and stimulates the production of β-defensin production by gut epithelium, and improves the barrier function of the breastfed infants.[34] The diminishing levels would parallel the maturation of infant gut over the first year.

Taken together, the main differences were observed between colostrum and mature milk, with most components being stable over lactation during the first year. This feature is relevant for HMB policies about the preferable timeframe for donations, taking into account both the protein content and quality.

In relation with our second aim, we analyzed all paired samples before and after treatment and results are shown in colostrum and whole group of mature-milk samples because the temporal profiles of raw and pasteurized samples were similar (data not shown). The results about the effects of Holder pasteurization extend available data about the variable retention of major and minor components in colostrum and mature milk;[8,35] this information is particularly relevant for the HMBs in our country.[4]

Alternative methods based on high temperature and short time (HTST), high pressure processing or ultraviolet-C irradiation have been proposed to improve nutritional and biological properties of milk.[7,36] Particularly, HTST pasteurizers have been recently validated for human milk processing,[37,38] so the information about the impact on immune factors that we studied in present work would be valuable.

Our results point to a drastic decrease of sCD14, a major component involved in the modulation of the inflammatory response induced by lipopolysaccharide in mononuclear and epithelial cells.[39] The impact of pasteurization of both colostrum and mature milk would affect the immunomodulatory effect, in conjunction with significant decrease of TGF-β2.

As a first approach to gain insight about the functional consequences of pasteurization, we compared the modulatory effect of raw and pasteurized milk at lactation stages in response to inflammatory stimulus. In accordance with the deleterious effect of thermal treatment on most components, the modulatory ability of raw milk was higher than pasteurized samples. Unexpectedly, the colostrum and milk of 1 month lacked modulatory effects after thermal treatment, while mature milk obtained after 2 months retained about 40% of activity compared with raw milk (Fig. 5b). The mechanisms involved in the modulation of the inflammatory response induced by TNF-α remain unknown. Several candidate milk soluble factors could be involved. In fact, the inflammatory response elicited by high doses of TNF-α in cultured fetal enterocytes was decreased by

pretreatment with IL-10 and TGF-β.[9] In spite of the differences between immature enterocytes and transformed epithelial cell used in our work, it cannot be ruled out that TGF-β detected in all raw and pasteurized samples contributed to the modulation of low doses of stimulus. In fact, constant levels of TGF-β were observed in accordance with homogeneous inhibition of NF-κB pathway activation among the lactation periods. Globally, the underlying mechanisms involved cannot be concluded from this study. The effects may be attributed to factors that block the stimulus, decrease the expression of the receptors, and/or interfere with signaling pathways.

Globally, present work reports that most immune factors in donated mature milk are equally found over the first year of lactation; this aspect is important to local HMB where most of the milk received is mature. Nevertheless, our work points out there are drastic losses of several immune factors during Holder pasteurization that could be critical for preterm infant's health. This is accordance with the lower modulatory effect obtained by pasteurized milk in the in vitro assay. Identification of the specific components involved in this effect is underway.

Limitations of this study

The selection of donors for this study was based on the availability of milk; thus, there may be a selection bias toward women with high milk production that would affect the compositional description.

As we analyzed the AF of milk for technical reasons, our results cannot strictly be extrapolated to whole milk, although the AF contains 95% of milk proteins. The presence of non-protein bioactive components in the AF, such as human milk oligosaccharides, could contribute to the immunomodulatory effects of raw and pasteurized samples. However, the human milk oligosaccharides are stable to Holder pasteurization,[40] and therefore they are unlikely to be responsible for the decrease of the modulatory effect of colostrum and mature-milk samples by the thermal treatment. The identification of the main components that contribute to the anti-inflammatory effects observed in vitro is complex because several immune factors can interact synergistically. Several experimental approaches are underway to identify key components to optimize milk composition intended for vulnerable NBs.

AUTHOR CONTRIBUTIONS

The author's responsibilities were as follows: A.H., C.R.-C., and A.P. developed the study design; C.R.C. conducted most research activities; E.V., P.A., and C.S. contributed to technical assistance with cell assays, interpretations, and discussion of the data information. A.P., L.F., L.C., and M.C. contributed to samples and data collection at the HMB. A.H. and C.R.-C. wrote the first draft of the manuscript; all authors read and approved the final manuscript.

ADDITIONAL INFORMATION

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