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## Pasteurization of human milk affects the miRNA cargo of EVs decreasing its immunomodulatory activity

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In this report, we evaluated the effect of the pasteurization (P) process of mother's own milk (MOM) on the miRNA content of extracellular vesicles (EVs) and its impact on innate immune responses. Differences in size or particle number were not observed upon pasteurization of MOM (PMOM). However, significant differences were observed in the EV membrane marker CD63 and miRNA profiles. miRNA sequencing identified 33 differentially enriched miRNAs between MOM<sub>EV</sub> and PMOM<sub>EV</sub>. These changes correlated with significant decreases in the ability of PMOM<sub>EV</sub> to modulate IL-8 secretion in intestinal Caco2 cells where only MOM<sub>EV</sub> were able to decrease IL-8 secretion in presence of TNF $\alpha$ . While EVs from MOM<sub>EV</sub> and PMOM<sub>EV</sub> were both able to induce a tolerogenic M2-like phenotype in THP-1 macrophages, a significant decrease in the transcript levels of IL-10 and RNA sensing genes was observed with PMOM<sub>EV</sub>. Together, our data indicates that pasteurization of MOM impacts the integrity and functionality of MOM<sub>EV</sub>, decreasing its EVs-mediated immunomodulatory activity. This data provides biomarkers that may be utilized during the optimization of milk processing to preserve its bioactivity.

Human milk (herein referred to as Mother's own milk, MOM) is a complex source of nutrition and metabolic-endocrine regulators that program the signaling system of infants in a very personalized manner. The bioactive components responsible for these benefits include a wide variety of cytokines, hormones, immunoglobulins, miRNAs, and bacterial cells that are transmitted from mother to child<sup>1,2</sup>. Our group and others have reported that around 200 different bacterial species have been identified in MOM, suggesting that these may not be simply contaminants but integral components that play a beneficial role in development<sup>3,4</sup>. MOM provides important benefits to preterm infants, including immune benefits, nutrition, protection against infections (especially necrotizing enterocolitis), and overall, a decreased risk of mortality<sup>5</sup>.

In the absence of MOM, the American Academy of Pediatrics (AAP) recommends using pasteurized donor human milk (DHM) over formula in very preterm infants<sup>6</sup>. Current practice in the Neonatal Intensive Care Unit (NICU) at Shands Children's Hospital, (University of Florida), is to provide DHM to premature infants of gestational age less than 30 weeks. DHM is obtained from pooled donors and pasteurized, rendering the milk nearly devoid of personalized live commensal bacteria<sup>7</sup>. Furthermore, pasteurization of DHM has also been reported to significantly decrease the levels of sIgA, alkaline phosphatase, bile salt stimulated lipase, lactoferrin and leptin<sup>8,9</sup>. Nonetheless, the role of the pasteurization process on the diversity of microRNA (miRNAs) has not been evaluated until now. miRNAs are small, non-coding RNAs (around 22-nucleotides in length) that have been found in bacteria, animals, and some viruses. More than 3000 recognized or new miRNAs have been identified in human milk<sup>10,11</sup>, most of which have been implicated in immune functions. miRNAs are secreted within milk exosomes or extracellular vesicles (EV). EVs have an endocytic origin and are around 30–150 nm (diameter) in size. They are surrounded by a lipid bilayer membrane and contain a rich cargo composed of proteins, DNA, RNA, peptides, and lipid-derivatives<sup>12,13</sup>. These nanostructures have been isolated from many biological fluids including MOM. Among the rich cargo, miRNAs are considered an outstanding feature due to their role as modulators of a wide variety of processes<sup>14,15</sup>. It has been reported that miRNAs can regulate more than 30% of human genes<sup>16</sup>. Thus, the transfer of exosomes and their miRNA cargo from mother to child contributes to the benefits provided by breastfeeding. miRNAs contained in MOM EVs have high stability at low pH, suggesting that these regulators

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could survive the gastric transit, reaching the intestine where they could modulate the host's immune system<sup>17</sup>. Recently, it was shown that pasteurized or milk subjected to high pressure milk processing introduced shifts in the abundance of several miRNA<sup>18</sup>. This article overcomes a limitation identified in previous reports where the effect of pasteurization on the functionality of miRNAs contained in the exosomes was not evaluated. The most abundant miRNAs found in MOM target several processes important for infant development (reviewed in<sup>19</sup>). Some of those are: miR-182-5p, miR-148a-3p, let-7f-5p and miR-22-3p, targeting genes involved in lipid metabolism; miR26a and miR181b involved in glucose metabolism; miR21 and miR-200 family participating in tight junction stability in the gut; miR-29b and the let-7 family affecting neurogenesis; and the miR29 and miR-148a-3p implicated in epigenetic regulation. Furthermore, miRNAs miR-181a, miR223, miR146b-5p, and miR155 play important roles in the development of both innate and acquired immune systems.

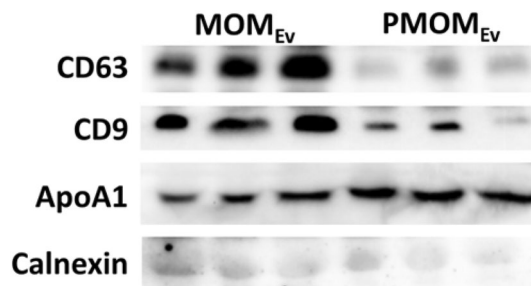
The preservation of these bioactive components in MOM is highly important in preterm infants, where MOM feedings reduce the incidence and severity of infections, especially necrotizing enterocolitis (NEC) and late onset sepsis (LOS)<sup>20,21</sup>. As indicated above MOM feedings have shown superior epidemiological performance when compared to DHM and formula. In this report, we have evaluated the effect of the pasteurization process of MOM on the miRNA content of MOM EVs and their impact on innate immune response in human cell lines. We found that pasteurization of MOM impacts the integrity and the miRNA diversity in the EV cargo. Furthermore, biomarkers of host responses that may be followed during the milk processing to evaluate its bioactivity were identified.

## Results

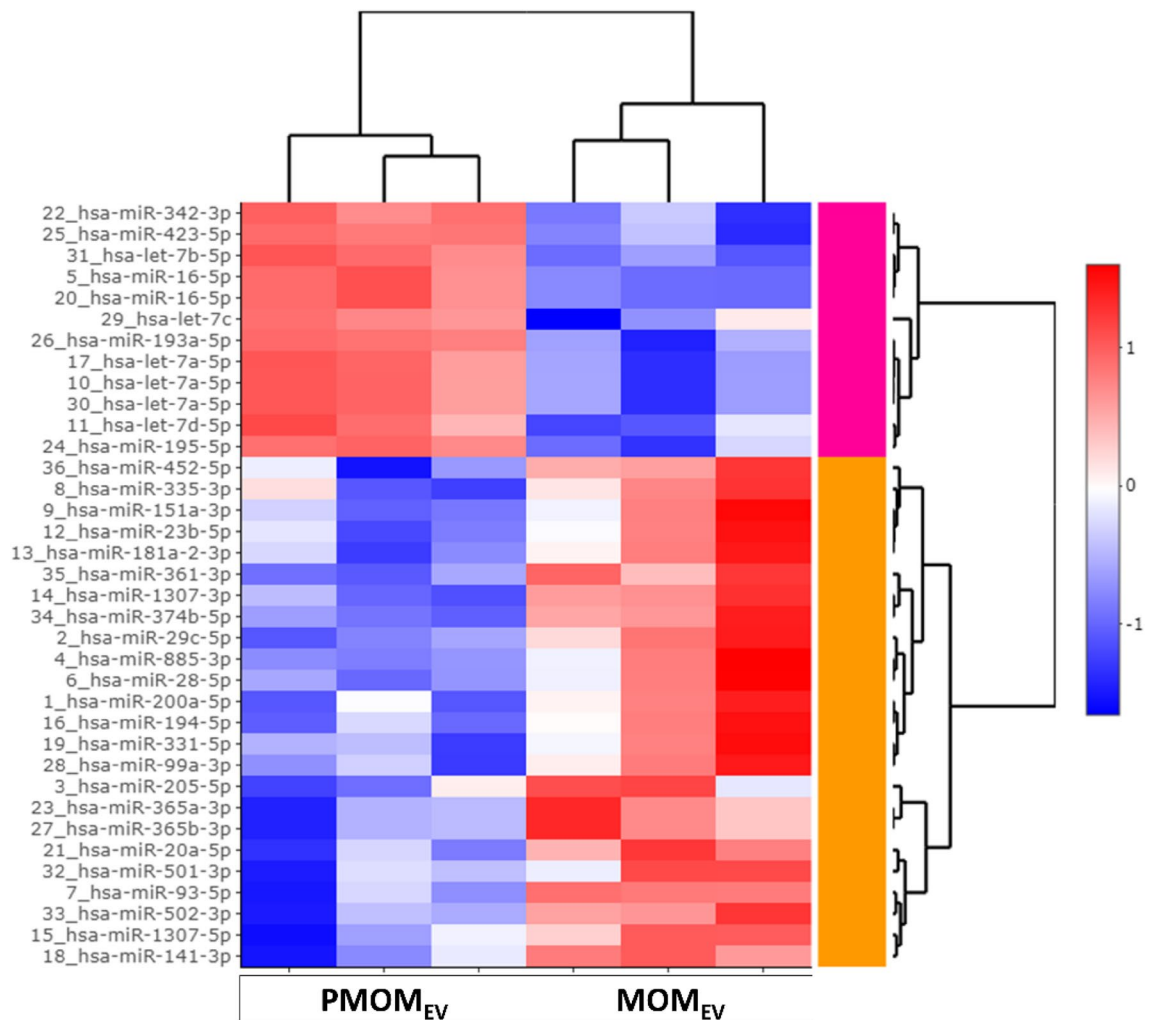
**Pasteurization does not affect the EVs yield of MOM.** To evaluate the role of pasteurization on the stability of miRNAs in human milk, we first developed PMOM (as a proxy for DHM) in which the components are identical to MOM, except that it has been subjected to pasteurization (see “Material and methods” for details). Next, the EVs enriched fractions of MOM and PMOM were assessed for yield and size distribution. A comparable yield of EVs was observed between MOM<sub>EV</sub> and PMOM<sub>EV</sub> with an average concentration of  $1.6 \times 10^{10} \pm 9.6 \times 10^8$  and  $1.7 \times 10^{10} \pm 1 \times 10^9$  EVs per mL of milk, respectively (Suppl. Fig. 1). MOM<sub>EV</sub> and PMOM<sub>EV</sub> also showed a similar size distribution (200 nm in average diameter), and negative zeta potential (MOM<sub>EV</sub> = -13.4 mV, PMOM<sub>EV</sub> = -16.64 mV, respectively). However, immunoblots using the exosome common markers CD63 and CD9 showed that only CD9 was detected in MOM<sub>EV</sub> and PMOM<sub>EV</sub>. On the contrary, CD63 was observed in MOM<sub>EV</sub> samples but was absent in PMOM<sub>EV</sub>, indicating damage or degradation during the pasteurization process. The ER marker Calnexin was not detected in either EVs samples, however the apolipoprotein A1 was detectable in both MOM<sub>EV</sub> and PMOM<sub>EV</sub>, indicating that lipoproteins were co-purified with human milk EVs (Fig. 1).

**MOM<sub>EV</sub> and PMOM<sub>EV</sub> are enriched with different miRNA cargo.** Global miRNA RNAseq was performed on MOM<sub>EV</sub> and PMOM<sub>EV</sub> enriched samples. A total of 166 miRNA were identified with 33 miRNAs of those being differentially enriched. It was found that 9 miRNAs were enriched in PMOM<sub>EV</sub> while 24 were enriched in MOM<sub>EV</sub> (Fig. 2, Suppl. Table 1). To further understand the potential impact of the differences observed between MOM<sub>EV</sub> and PMOM<sub>EV</sub>, we utilized the microRNA target filter tool using the Ingenuity Pathway Analysis software (IPA) (Table 1). Of the differentially enriched mRNAs, 25 were predicted to target 201 messenger RNAs. About fifty percent of those genes (92 mRNAs), are involved in five different pathways: AMPK signaling, NF-κB signaling, STAT3 pathway, T Cell Receptor signaling, and IL-15 production (Fig. 3). The analyses of those five pathways showed that they share 28 genes that may be affected by the miRNAs differentially enriched in MOM<sub>EV</sub> and PMOM<sub>EV</sub>.

Specifically, the miRNAs enriched in MOM<sub>EV</sub> target 41 mRNA while the miRNAs enriched in PMOM<sub>EV</sub> target 51 genes (Suppl. Table 2). To integrate and visualize the IPA results obtained, we used Circos plot to combine in a single plot the potential pathways affected by miRNA from both MOM<sub>EV</sub> and PMOM<sub>EV</sub> (Fig. 4). As shown in the Circos plot, only has-let-7b-5p, has-miR-16-5p, has-miR-193a-5p, and has-miR-423-5p identified in PMOM<sub>EV</sub> are predicted to have an impact on these pathways while 14 miRNAs from MOM<sub>EV</sub> are predicted to impact the mRNA involved in the same pathways (Figs. 3 and 4, Table 1 and Suppl. Table 2). Based on these predictions, miRNAs enriched in PMOM<sub>EV</sub> may have a greater influence in the regulation of the transcription factor NF-κB



**Figure 1.** Pasteurization affects the detection of endocytic EV markers. Western blot analyses were performed on total protein extractions of MOM<sub>EV</sub> and PMOM<sub>EV</sub> enriched fractions using anti-CD63, anti-CD9, as well as anti-Calnexin as a negative control and anti-ApoA1 as a lipoprotein marker.



**Figure 2.** Heatmap summarizing the miRNA sequencing analysis. The distribution of the 33 miRNAs statistically significant enriched in MOM<sub>EV</sub> and PMOM<sub>EV</sub> are shown.

signaling pathway miRNAs enriched in MOM<sub>EV</sub> may have a greater influence in the regulation of IL-15 signaling pathway (Fig. 4). However, both pathways are essential in the development and modulation of multiple aspects of the innate and adaptive immune system<sup>22,23</sup>. These results suggest that miRNA differentially enriched in MOM<sub>EV</sub> and PMOM<sub>EV</sub> may affect the immune system through different but overlapping mechanisms.

**EVs from MOM and PMOM stimulate IL-8 secretion in Caco2 cells.** Based on the previous predictions, the impact of enriched MOM<sub>EV</sub> and PMOM<sub>EV</sub> fractions was evaluated on the nuclear factor kappa B (NF- $\kappa$ B) pathway by following the induction of IL-8 expression<sup>24</sup>. As controls, whole MOM and PMOM as well as exosome free supernatants were tested. A total of 24 fresh MOM samples were pooled into 3 groups and their ability to stimulate IL-8 secretion in Caco2 cells was evaluated. Differential fractionation was performed to evaluate whole milk, EV free supernatant and EV enriched fractions. It was found that while all the treatments induced secretion of IL-8 in Caco2 cells compared to the control, significantly higher secretion levels of IL-8 were observed in Caco2 cells treated with MOM 2%, supernatant and MOM<sub>EV</sub> when compared to their corresponding PMOM fractions (Fig. 5A). The results obtained with the supernatants were expected as the milk contains a variety of cytokines that may nonspecifically stimulate IL8 secretion.

The effect of the human milk EVs was further investigated in a proinflammatory environment by adding TNF $\alpha$  to Caco2 cells in presence or absence of EVs. It was found that MOM<sub>EV</sub> had similar secretion levels of IL-8 as the TNF $\alpha$  control, while the addition of TNF $\alpha$  to Caco2 in presence of MOM<sub>EV</sub> was able to decrease IL-8 secretion. In contrast, PMOM<sub>EV</sub> showed trends (albeit not significant  $p$  value = 0.055) to increase IL-8 in presence of TNF $\alpha$  in Caco2 cells, (Fig. 5B). TNF $\alpha$  is a positive regulator of IL-8 gene expression through NF- $\kappa$ B, which is essential for IL-8 gene transcription<sup>24</sup>. These results are in agreement with predicted regulatory effect of PMOM<sub>EV</sub> miRNAs on the NF- $\kappa$ B signaling pathway (Fig. 4).

Another predicted signaling pathway affected by the miRNAs enriched in both MOM<sub>EV</sub> and PMOM<sub>EV</sub> is STAT3 Pathway (Figs. 3 and 4). The Signal Transducer and Activator of Transcription (STAT) 3 pathway is involved in survival, cell growth, and immune response<sup>25</sup>. This pathway is rigorously regulated by Janus Kinase (JAK) and Epidermal Growth Factor Receptor (EGFR). We identified EGFR as one of the targets regulated by

miRNA	logFC	Adj. <i>P</i> value
hsa-let-7b-5p	4.8	2.85E-06
hsa-let-7a-5p	3.7	4.54E-04
hsa-let-7a-5p	3.7	4.54E-04
hsa-miR-342-3p	3.3	4.54E-04
hsa-miR-16-5p	3.3	6.30E-04
hsa-let-7c	2.9	6.81E-03
hsa-let-7d-5p	2.9	6.57E-03
hsa-miR-193a-5p	2.9	3.84E-03
hsa-miR-195-5p	2.5	9.31E-03
hsa-miR-423-5p	2.1	9.45E-03
hsa-miR-20a-5p	-2.5	9.31E-03
hsa-miR-93-5p	-2.6	9.31E-03
hsa-miR-885-3p	-2.9	9.31E-03
hsa-miR-28-5p	-2.9	9.31E-03
hsa-miR-151a-3p	-3.0	9.31E-03
hsa-miR-331-5p	-3.0	9.45E-03
hsa-miR-141-3p	-3.1	2.63E-03
hsa-miR-361-3p	-3.2	2.63E-03
hsa-miR-194-5p	-3.3	7.54E-03
hsa-miR-23b-5p	-3.3	6.83E-03
hsa-miR-1307-5p	-3.4	5.41E-03
hsa-miR-501-3p	-3.4	8.27E-03
hsa-miR-374b-5p	-3.4	2.41E-03
hsa-miR-200a-5p	-3.5	7.54E-03
hsa-miR-365a-3p	-3.6	4.54E-04
hsa-miR-365b-3p	-3.6	4.54E-04
hsa-miR-99a-3p	-3.7	3.84E-03
hsa-miR-205-5p	-3.8	3.03E-04
hsa-miR-452-5p	-3.8	2.63E-03
hsa-miR-181a-2-3p	-3.8	2.98E-03
hsa-miR-502-3p	-3.9	1.86E-03
hsa-miR-335-3p	-4.4	2.51E-03
hsa-miR-1307-3p	-4.4	4.54E-04
hsa-miR-29c-5p	-4.5	8.79E-04

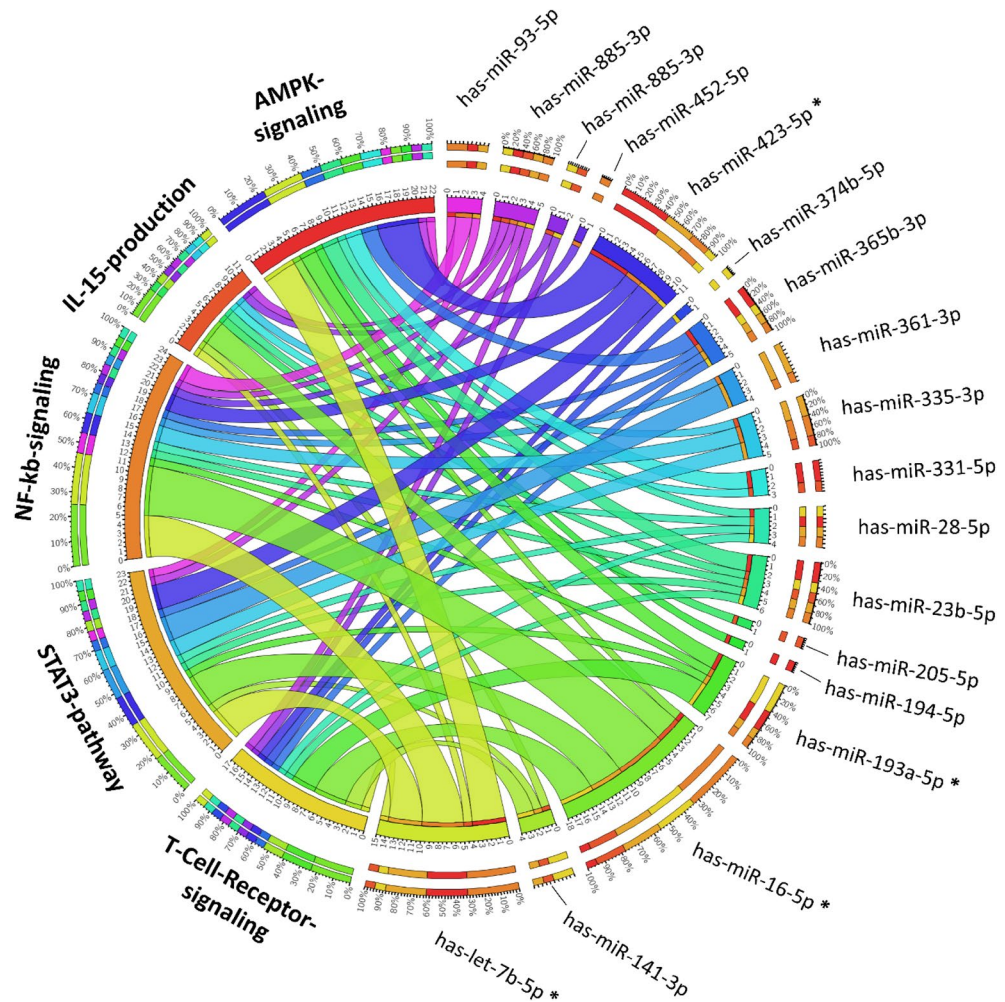
**Table 1.** Statistically significant miRNA enriched differentially between MOM and PMOM EVs. Positive log fold change (logFC) shows enriched miRNA in PMOM<sub>EV</sub>, while negative fold changes show enriched miRNA in MOM<sub>EV</sub>.

hsa-miR-16-5p enriched in PMOM<sub>EV</sub>. In order to elucidate and further understand if the EVs from human milk were able to differentially modulate these pathways, we determined the mRNA expression of some key effectors such as *AKT*, *STAT3*, and *IGF-1* receptor, in Caco2 cells treated with MOM<sub>EV</sub> or PMOM<sub>EV</sub>. A significant decrease in the expression of *IGF-1* receptor was observed with both EVs treatments while no significant differences were observed in the expression of *AKT* compared to the control (Fig. 5C–D). Additionally, a 3.5-fold increase was observed in the expression of *STAT3* after treating Caco2 cells with MOM<sub>EV</sub>. While the expression of *STAT3* was higher in the cells treated with PMOM<sub>EV</sub>, no significant differences were observed when compared to the control (Fig. 5E). However, when *STAT3* was evaluated by Western blot, no significant differences were observed between the treatments and the control (Supplementary Fig. 2). These results suggest that regulation of *STAT3* transcript could be modulated in a hsa-miR-16-5p through EGFR repression in Caco2 cells treated with PMOM<sub>EV</sub>.

### Differential M2 tolerogenic differentiation of THP-1 human macrophages by MOM<sub>EV</sub> and PMOM<sub>EV</sub>.

The potential impact of the enrichment of has-let-7b-5p in PMOM<sub>EV</sub> was evaluated on the mRNA expression levels of IL10<sup>26</sup> using the THP-1 PMA-activated macrophage cell line. Macrophages are broadly located in the human body playing a crucial role in regulating immune responses<sup>27</sup>. Once activated, macrophages can differentiate into two sub-types M1-like macrophages capable of proinflammatory responses and M2-like macrophages capable of tolerogenic responses<sup>28</sup>. To this end, THP-1 monocytes induced with PMA were treated with MOM<sub>EV</sub> and PMOM<sub>EV</sub> at 10<sup>10</sup> particles/well for 6 h. The mRNA levels of IL-10, TNF $\alpha$  and IL-1 $\beta$  were quantified as signature markers of tolerogenic THP-1 (M2) macrophages. It was found that both MOM<sub>EV</sub> and PMOM<sub>EV</sub> induced a M2 phenotype when compared to the vehicle control. Nonetheless, THP-1 macrophages





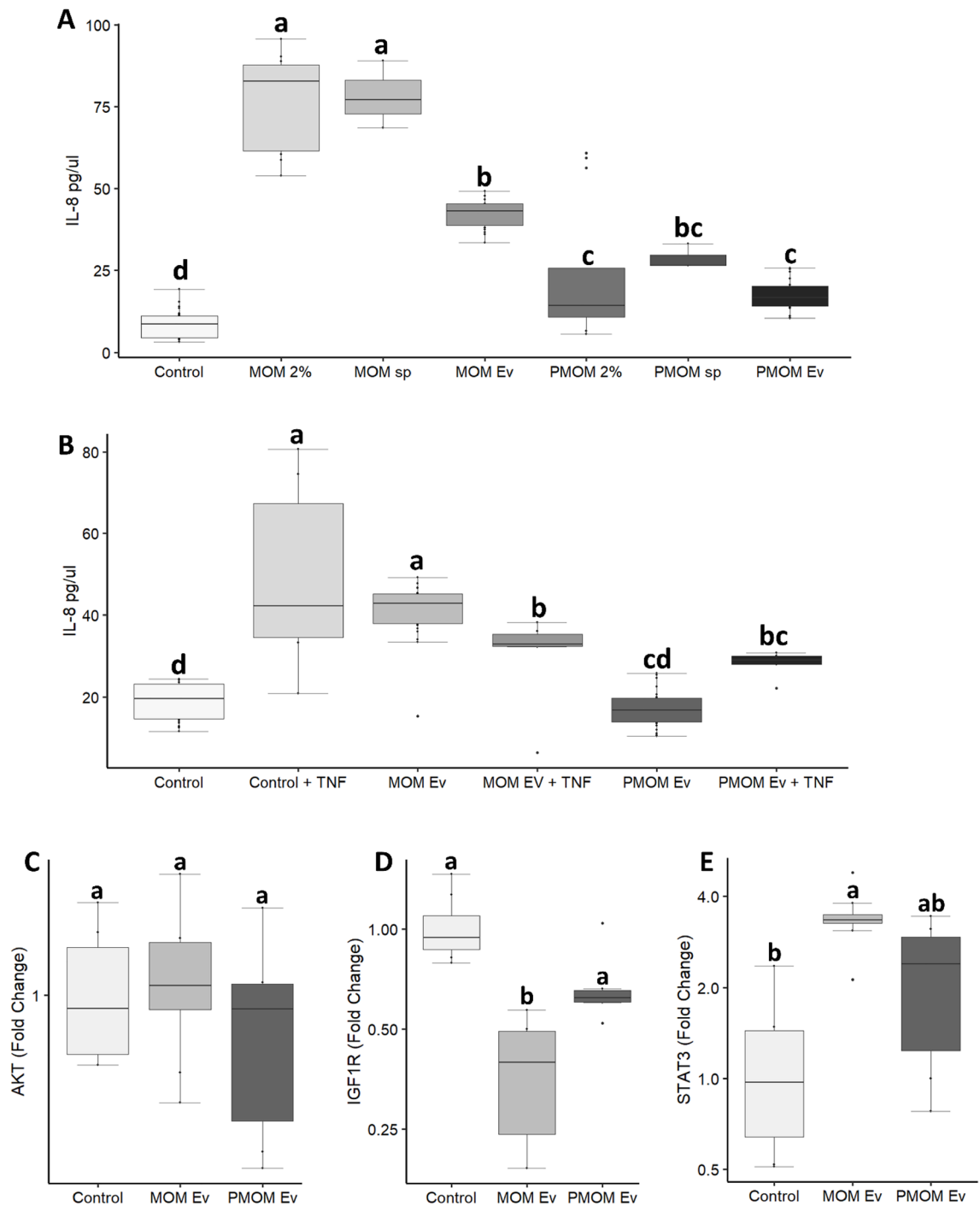
**Figure 4.** Circos Plot representation to visualize miRNA-gene and inference of the biological impact in the different pathways. With “\*” are denoted the miRNA enriched in PMOM<sub>EV</sub>.

levels of IL29, OAS2, RIG-I and INF- $\alpha$  were determined. Although both treatments, MOM<sub>EV</sub> and PMOM<sub>EV</sub>, induced higher expression of these genes when compared to the control, significant higher levels of OAS2, INF- $\alpha$  and IL29 were observed in THP-1 macrophages treated with MOM<sub>EV</sub> compared to PMOM<sub>EV</sub> for  $p < 0.05$  (Fig. 7). Altogether, these results indicate that the miRNA cargo of human milk EVs could be compromised after the pasteurization process decreasing or changing the regulatory effect exerted by human milk miRNA.

**MOM<sub>EV</sub> differentially affects AMP, I $\kappa$ B $\alpha$  and STAT3 signaling pathways.** The impact of MOM<sub>EV</sub> and PMOM<sub>EV</sub> on AMPK signaling, NF- $\kappa$ B signaling (by following the protein basal level and activation of the I $\kappa$ B kinase), and STAT3 pathways, was confirmed by evaluating the protein levels in THP-1 macrophages. It was found that the basal level abundancy of ERK and I $\kappa$ B $\alpha$  were significantly higher in MOM<sub>EV</sub> when compared to the control (Fig. 8). STAT3 showed a similar trend albeit not statistically significant. PMOM<sub>EV</sub> showed significantly lower concentrations of STAT3 and ERK when compared to MOM<sub>EV</sub> while I $\kappa$ B $\alpha$  showed a similar trend albeit not statistically significant. The quantification of the activating phosphorylation's in ERK (P-Thr202/Tyr204 ERK/ERK) showed a significant decrease in PMOM<sub>EV</sub> when compared to MOM<sub>EV</sub>. No significant differences were observed for P-S727 STAT3/STAT3 between MOM<sub>EV</sub>, PMOM<sub>EV</sub> and the vehicle control (Fig. 8). These results are in agreement with the stronger stimulation of innate immune responses observed with MOM<sub>EV</sub>.

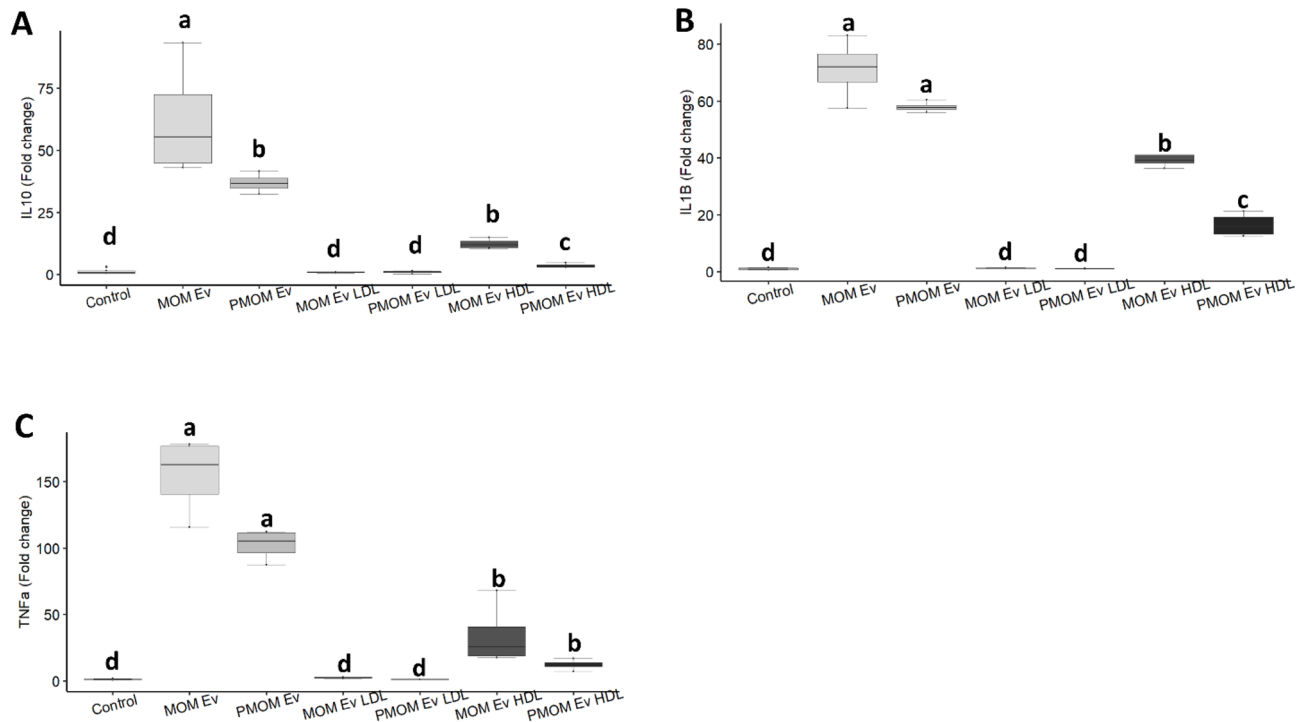
## Discussion

Many mothers that deliver preterm do not produce sufficient volumes of MOM<sup>35</sup>, and as a result, DHM is provided for these infants due to the increased benefits over formula<sup>6</sup>. However, the pasteurization process of DHM significantly reduces the availability of bioactive molecules as well as personalized live commensal bacteria<sup>9</sup>. In this report, we evaluated the impact of pasteurization on human milk on EVs integrity and miRNA cargo. We found that while similar yields and charge of EVs were obtained for MOM and PMOM EVs, a significant decrease in the protein marker CD63 was observed in PMOM<sub>EV</sub>, while CD9 was detectable in both EVs samples. Other



**Figure 5.** Effect of human milk EVs on stimulation of the innate immune response in Caco-2 epithelial cell line. (A–B) ELISA quantification of IL-8 in Caco2 epithelial cells after 6 h of treatment with (A) different milk fractions or (B) with human milk enriched EV fractions in presence and absence of TNF $\alpha$ . Untreated Caco2 cells were used as control. (C–E) Relative expressions of AKT (C), IGF1R (D), and STAT3 (E) are shown as fold change relative to the vehicle control. The experiments were performed with biological and technical triplicates. One way ANOVA were performed, different letters indicate statistical significance at  $p < 0.05$ .

ER markers such as calnexin were not detected on either sample while the Apolipoprotein A1 was present in both MOM<sub>EV</sub> and PMOM<sub>EV</sub>. Lipoproteins and EVs are both in the submicron scale and overlap in size distributions which translates frequently in co-purification of EVs and lipoproteins<sup>36,37</sup>. The presence of lipoproteins could explain the comparable yield and size between MOM<sub>EV</sub> and PMOM<sub>EV</sub> samples. Recent reports in bovine milk-derived EVs evaluated the impact of several industrial processes on EV integrity. Similar to our findings, pasteurization and homogenization significantly affected the levels of CD63 while CD9 was heat stable<sup>38</sup>. Other studies have shown that the stability of exosomes is highly affected by low temperatures as well<sup>39</sup>.



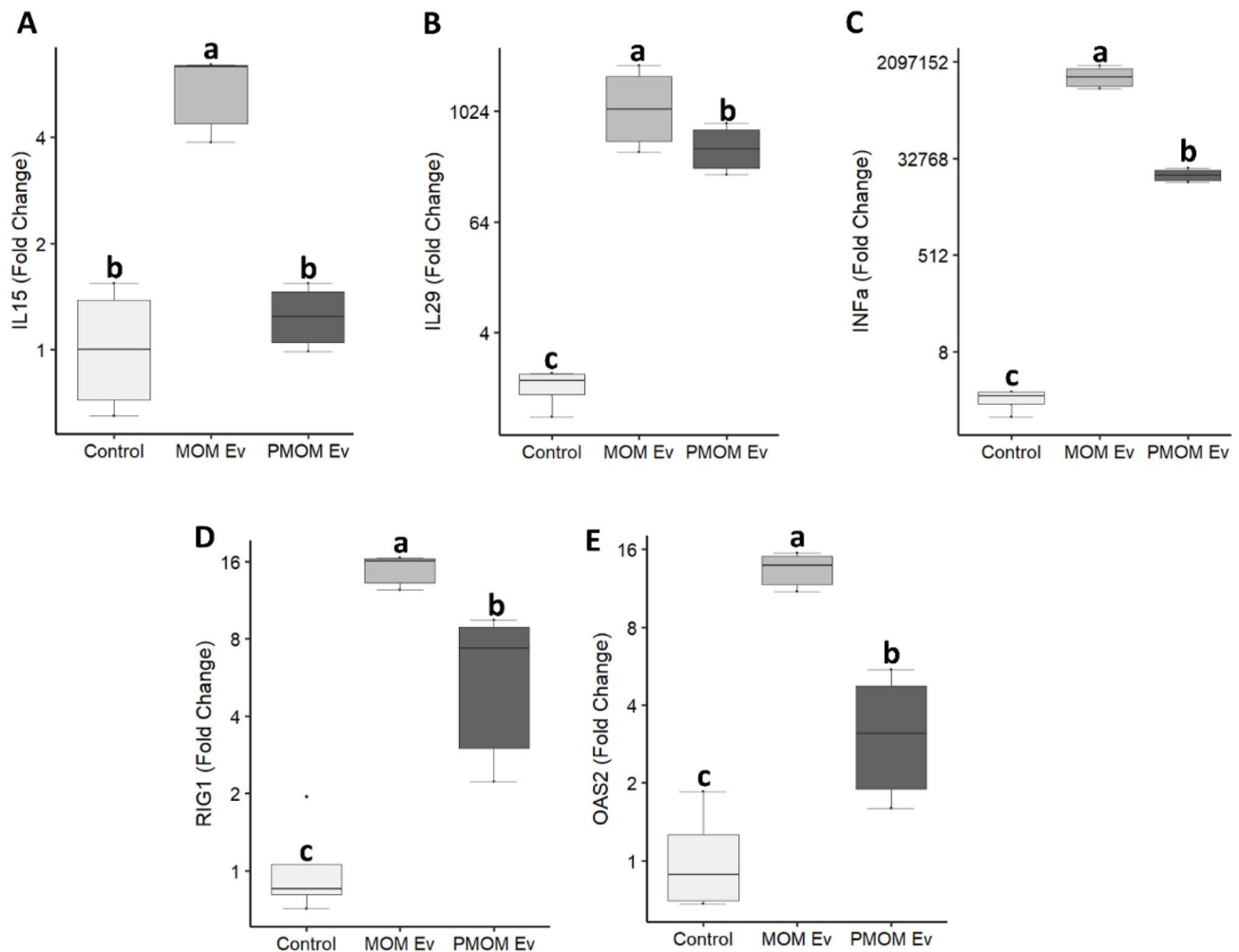
**Figure 6.** Human milk EVs stimulate a M2-tolerogenic phenotype in THP-1 macrophages. The relative expression of *IL-10* (A), *TNFα* (B) and *IL1β* (C) are shown relative to the vehicle control. The experiments were performed with biological and technical triplicates. ANOVA and mean comparisons were performed, different letters indicate statistical significance for  $p < 0.05$ .

Our RNAseq approach also identified significant changes to the miRNA diversity in EVs of MOM upon pasteurization. These results suggest that the decrease in protein stability of the EVs was translated into changes in the miRNA cargo in human milk. The stability and shifts in the miRNA cargo upon heat treatments procedures such as pasteurization and ultra-heat treated (UHT) milk has been studied in human and bovine milk with conflicting results<sup>38</sup>. Smyczynska et al.<sup>18</sup> observed more severe effects of pasteurization of DHM, using Holder Pasteurization (HoP) than with High-Pressure Processing (HPP). A 302-fold decrease in the yield of exosomes was observed with (HoP), with a complete loss of RNA fragments of length typical for miRNA and piRNA. High pressure processing DHM showed less detrimental effect than pasteurization in human milk<sup>18</sup>. However, the impact of these results on host responses were not evaluated. Some studies have reported a significant effect in the abundance and stability of miRNA in bovine milk<sup>38,40</sup>, while others have shown that pasteurization does not have a significant effect<sup>41,42</sup>. These conflicting results may be explained by the differences in methods used for their analyses.

We determined the differential enrichment of 33 miRNA between MOM<sub>EV</sub> and PMOM<sub>EV</sub>. Moreover, the main five pathways predicted to be impacted by the differentially enriched miRNA share multiple genes resulting in overlapping effects on immune stimulation and cell proliferation. To evaluate the impact of the potential loss in integrity or decreased abundance of specific miRNA, we used intestinal epithelial and macrophage cell lines to measure key gene markers. The miRNA enriched in MOM<sub>EV</sub> and PMOM<sub>EV</sub> target 25 genes involved in NF-κB signaling pathway. Of those, only six genes are shared among MOM<sub>EV</sub> and PMOM<sub>EV</sub> (CD40, CXCR5, PIK3R3, TGFBR1, TGFBR2 and TLR4). We hypothesized that the differential enrichment of miRNA after pasteurization would affect signaling through the NF-κB pathway. To this end, we followed the expression of IL-8 in human epithelial cells<sup>24</sup>. Stimulation of IL-8 secretion was observed with all the fractions of human milk tested (human milk, supernatant free of EVs and EVs), however, significant difference in the stimulation of IL-8 secretion was observed when fractions from PMOM were compared with their correspondent MOM fractions. Similar findings have been observed in the stimulation of IL-8 secretion after heat treatment of bovine and goat milk EVs<sup>43,44</sup>. Likewise, a reduction in the expression of IL-6 was observed in a mice model of necrotizing enterocolitis (NEC) when comparing raw and pasteurized human milk EVs<sup>45</sup>.

Another predicted pathway showing overlapping effector genes was the MAPKs and PI3K/AKT. To further investigate the effects of MOM<sub>EV</sub> and PMOM<sub>EV</sub> in Caco2 cells, we evaluated the expression levels of 3 key genes involved in these pathways. Lower expression levels of IGF1R were observed with both MOM<sub>EV</sub> and PMOM<sub>EV</sub> treatment in Caco2 cells. However, only MOM<sub>EV</sub> showed statistically significant differences. IGF1R activation mediates signaling cascades through MAPKs and PI3K/AKT<sup>46,47</sup> impacting many cellular responses including cell proliferation, differentiation, and survival<sup>46,47</sup>. We found that the posttranscriptional regulators of IGF1R, hsa-miR-16-5p and hsa-let-7b-5p, were enriched in PMOM<sub>EV</sub>, consistently with the lower levels in IGF1R mRNA observed. However, the decrease in expression did not reach statistical significance. The indirect compensatory effect of increased expression levels of STAT3 observed could explain those observations.

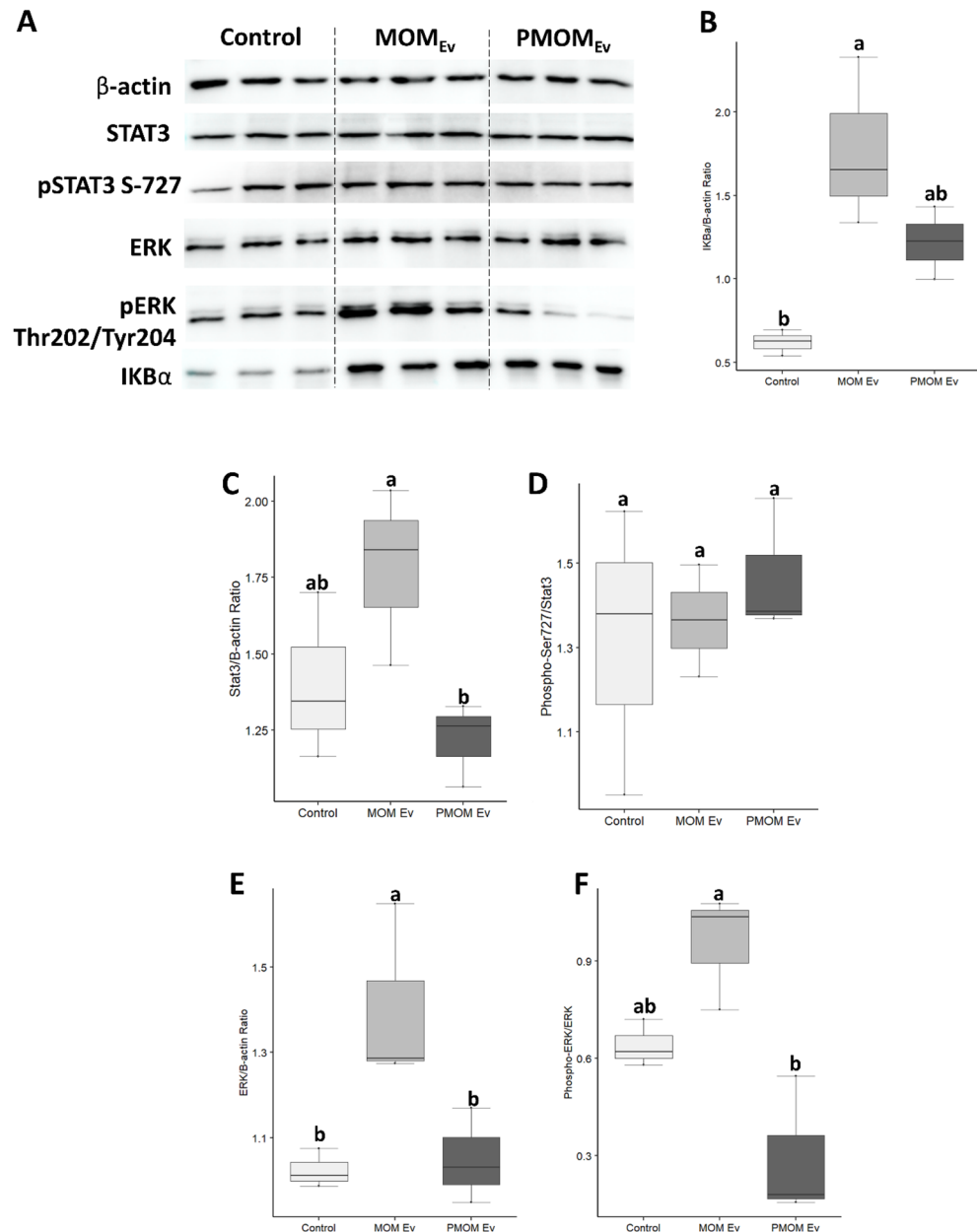




**Figure 7.** Human milk EVs stimulate gene expression of RNA sensing signaling in THP-1 macrophages. The relative expressions of *IL-15* (A), *IL29* (B), *INFα* (C), *RIG1* (D) and *OAS2* (E) are shown as fold change relative to the vehicle control. The experiments were performed with biological and technical triplicates. Single way ANOVA and mean comparisons were performed, different letters indicate statistical significance at  $p < 0.05$ .

The beneficial effects of human milk on immune stimulation have been largely described<sup>1,2</sup>, however little is known about the immune stimulatory properties of human milk EVs. In bovine milk, the lactation-related differential expression of miRNAs suggests that the miRNA produced in the mammary glands may have a specific function<sup>48</sup>. Since many of the enriched miRNA in human milk EVs identified in this work are predicted to impact immune modulatory pathways, we evaluated the impact of MOM<sub>EV</sub> and PMOM<sub>EV</sub> on the stimulation of a M2-like tolerogenic phenotype in macrophages. Interestingly, a stronger stimulation of IL-10 (as gene expression levels) was observed in macrophages treated with MOM<sub>EV</sub> compared to PMOM<sub>EV</sub>. These results concur with the predicted activation of the canonical NF-κB signaling pathway through has-let-7b-5p and has-miR-16-5p enrichment quantified in PMOM<sub>EV</sub>. While the tolerogenic M2-like polarization of macrophages observed in this work has been described earlier for other miRNAs<sup>17,49</sup>, macrophage differentiation and responses could be tissue- and species-dependent. In example, inflammatory M1-like macrophage differentiation, characterized by high levels of IL-6, TNFα, IL-12/23 and decreased IL-10, was observed with bovine milk-derived EVs (BEVs) using a mice model for agricultural dust exposure<sup>50</sup>. In contrast, BEVs administration in two mouse models for arthritis reduced serum levels of MCP-1 and IL-6 correlated with delays in the onset of arthritis and diminished cartilage pathology and bone marrow inflammation<sup>51</sup>.

A remarkable finding was the induction of IL15 in macrophages treated with MOM<sub>EV</sub>. This cytokine has protective roles towards viral and bacterial infections<sup>52,53</sup>. IL15 has been also successfully used as adjuvant in many antiviral vaccines<sup>23</sup>. The stimulation of IL15 positively correlated with higher expression levels of the interferons IL29, INFα, and the RNA sensing genes RIG1 and OAS2 in MOM<sub>EV</sub> when compared to PMOM<sub>EV</sub>. The potential role of milk miRNA in viral interference was explored through in silico methods in the context of the SARS-CoV-2 pandemic. It was found that some of the abundant miRNA in milk (miR29a, miR21 and miR181), can interfere with replication of a wide variety of viruses such as HIV, enterovirus 71 and influenza. However, scarce information is available on the mechanisms that mediate these cross-kingdom interactions<sup>11,19</sup>. Together, these results suggest that human milk EVs may play a significant role in stimulation of RNA sensing pathways to trigger an antiviral response that may be diminished by the pasteurization process.



**Figure 8.** Effect of MOM<sub>EV</sub> and PMOM<sub>EV</sub> on AMP, IκBα and STAT3 signaling pathways. (A) Summary panel of the Western blots performed after incubation of THP-1 macrophages in presence or absence of MOM<sub>EV</sub> and PMOM<sub>EV</sub>. The results of biological triplicates are shown. (B–F) Quantification of the results obtained with (B) total IκBα, (C) total STAT3, (D) STAT3 phosphorylation S-727, (E) total ERK, and (F) ERK phosphorylation Thr202/Tyr204. β-actin was used as a loading control. Quantification of band intensity was performed with ImageJ. One way ANOVA were performed, different letters indicate statistical significance at  $p < 0.05$ .

There is substantial evidence that milk exosomes can be absorbed and delivered to peripheral tissues where they can exert their regulatory effect (For review see Zemleni et al.)<sup>12</sup>. Therefore, the differences observed in the bioactivity of MOM after pasteurization raise concerns regarding the potential impacts of these processes on health outcomes. While the benefits of MOM have been widely reported, the benefits provided by specific bioactive components such as EVs and their cargo in human milk is unknown. It has been shown that physiological concentrations of bovine milk miRNA can affect gene expression in vivo and in cell cultures (Peripheral Blood Mononuclear Cells, HEK-293 Kidney)<sup>54</sup>. These reports suggest that a decrease in MOM EVs concentration or in their integrity affecting their cargo bioactivity can potentially have an impact on the health of infants. The most significant difference observed in this work was a decrease in the stimulation of IL15 as well as RNA sensing genes by PMOM<sub>EV</sub>. The reduced stimulation observed in innate immune responses maybe translated into a decreased response to viral infections. These findings, combined with the reported decreases in numerous

proteins (lactoferrin, lysozyme)<sup>55</sup>, immunoglobulin, and cytokines (like IL-7)<sup>56</sup> reported by others<sup>9,57</sup>, may significantly impact the overall immune bust usually provided by MOM.

The results presented here highlight the need to optimize processes in human milk banks in order to preserve the potential bioactivity of all components in human milk while maximizing its biosafety. There is no direct substitute for the nutritional and immune benefits that MOM provides, but its proven benefits highlight the need for attempting to replicate these factors in alternative feedings as closely as possible. In this work, we identified mechanistic effectors that may be utilized as biomarkers for process optimization. The decreased immunomodulatory activity of pasteurized milk and its derived EVs observed needs to be addressed in future studies, in order to establish better processing strategies and to provide our VLBW infants with the best and personalized nutrition possible.

## Material and methods

**Milk collection and EV enrichment.** For the RNAseq extractions, samples were collected using a sterile Symphony® double breast pump kit at a single expression session with an electric hospital Symphony® breast pump (Medela, McHenry, IL). The protocols used in this study for sample collection were reviewed and approved through the University of Florida Institutional Review Board (IRB RB201400527), a written informed consent was obtained from each donor mother. All methods here were performed in accordance with the relevant guidelines and regulations of the University of Florida Institutional Review Board. Beyond standard hand washing and pumping per NICU protocol, no breast hygiene preparation was performed. The sample was assigned a de-identified subject number, then immediately transported on ice to the microbiology lab for further processing. For the functional analyses using human cell lines, 24 milk samples we obtained from the NICU. Sets of 8 samples (total of 3 sets) were pooled. For both experiments each pool of milk was divided into two fractions, one fraction was immediately processed (MOM) for EV enrichment (MOM<sub>EV</sub>), while the other half was pasteurized to mimic DHM (P-MOM) to obtain PMOM<sub>EV</sub>, following the HMBANA protocol (HMBANA 2020). Briefly, milk was heated at constant temperature 65 °C for 30 min and cooled down immediately after in an ice bath. After pasteurization milk was further processed for EV enrichment as follows. MOM and PMOM fractions were centrifuged at 2246 g for 15 min at 4 °C to remove the fat, following a 45 min centrifugation at 15900 g to eliminate cell debris. Milk supernatants were further filtered sequentially using nitrocellulose filters of 11 µm, 6 µm, 2.5 µm, 0.45 µm and 0.2 µm. After filtration, EVs were enriched by ultracentrifugation at 207,888 g for 2 h. The EVs were washed twice with PBS, quantified, and stored in aliquots at – 80 °C.

**EV physical characterization and quantification.** Nanoparticle tracking analysis (NTA) using a NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK) was utilized to quantify the EVs as well as to determine its size distribution. Videos were recorded for 60 s (five times), with the camera level at 15, and analyzed with NTA software 4.3 (Malvern instruments Ltd, Malvern, UK). An average yield of 10<sup>12</sup> exosomes per µL was obtained. Dynamic light scattering was performed to measure the zeta potential of the EV suspensions using a Zetasizer ultra particle analyzer (Malvern Instruments Ltd, Malvern, UK). The samples were diluted 1:1000 with distilled water. The measurements were conducted in biological and technical triplicates at 25 °C.

**RNA extraction, sequencing, and data analysis.** RNA extractions were performed from three pools of MOM<sub>EV</sub> and PMOM<sub>EV</sub> samples using *mirVana*™ miRNA Isolation Kit (Invitrogen), with small RNA enrichment from total RNA according to manufacturer's instructions. Library construction with fragments around 20–75 bp in length and sequencing using Ion Torrent sequencing platform was performed by PrimBio (PrimBio Research Institute LLC, Exton, PA). FastQC was used to filter high quality reads<sup>58</sup>. For the data analysis Kallisto v0.46.1<sup>59</sup> was used to create an index and map the high-quality raw reads using a reference transcriptome for no coding RNA through EnsDb.Hsapiens.v86.2.99.0 package in RStudio<sup>60,61</sup>. Differential abundance analysis of miRNA was performed using limma 3.52.4, edgeR 3.38.4 and SVA 3.44.0 packages in RStudio<sup>62–64</sup>. All the graphics were generated using ggplot2 3.3.6 R package<sup>65</sup>.

**Protein extractions and Western Blot.** Aliquots normalized to the same particle concentration of MOM<sub>EV</sub> and PMOM<sub>EV</sub> were used for protein extraction and quantification using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Briefly, total proteins were extracted from EVs using Radio Immunoprecipitation Assay Buffer (RIPA) containing 150 mM NaCl, 50 mM Tris (pH 8), 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS), with Halt™ protease inhibitor cocktail (Thermo Fisher, Waltham, MA, USA). The EVs homogenates were centrifuged at 12,000 g for 10 min, at 4 °C and the protein concentration was measured following the manufacturer instruction.

Lipoproteins HDL (High-Density lipoprotein) and LDL (Low-Density lipoprotein) were purified from human milk EVs suspensions using LDL/VLDL and HDL purification kit (STA-608 ultracentrifugation free) following manufacturer protocol (Cell Biolabs, INC). Lipoproteins were solubilized in equal volume of PBS as EVs suspensions.

Human milk EVs were analyzed for exosome surface proteins by Western blot. Anti-CD63 (ab134045) and anti-CD9 (ab263019) were used as positive control. To investigate the presence of endoplasmic reticulum proteins (ER) or lipoproteins co-enriched in the EV preparations, anti-Calnexin (ab133615) and anti-Apolipoprotein A I (ab227455) was used, respectively. Appropriate secondary antibodies were used, and detection conducted using enhanced chemiluminescence reagent (Genesee Scientific, San Diego, CA).

**Cell lines propagation, treatment and western blot.** Human Intestinal Caco-2 and monocyte THP-1 cell lines were obtained from ATCC (Gaithersburg, MD, USA). Caco-2 cells were cultured at 37 °C in Eagle's

minimum essential medium (EMEM), supplemented with 15% heat inactivated fetal bovine serum (FBS) (Invitrogen), 2% of penicillin and streptomycin solution containing 10,000 units of penicillin and 10 mg of streptomycin/ml (Sigma-Aldrich, Saint Louis, MO) in a humidified atmosphere (5% CO<sub>2</sub> and 95% air). For the experiments,  $1 \times 10^6$  Caco-2 cells per well were seeded in 6-well plates. The cells were treated with vehicle control (10  $\mu$ L of EMEM media), 2% of defatted MOM (v/v) in EMEM, 2% defatted PMOM, 2% MOM supernatant (EVs free), 2% PMOM supernatant (exosome free),  $10^{10}$  MOM<sub>EV</sub> in EMEM, or  $10^{10}$  PMOM<sub>EV</sub>. After 24 h of incubation, culture supernatants were collected for IL-8 (BD OptEIA™) by enzyme-linked immunosorbent assay (ELISA) and the cells collected for RNA extractions and/or protein extractions. THP-1 Cells were cultured in RPMI 1640 medium supplemented with 10,000 units of penicillin, 10 mg of streptomycin, and 10% heat inactivated FBS. For the experiments, THP-1 cells were seeded in 6-well plates at  $1 \times 10^6$  cells/well and activated by adding 100 nM phorbol 12-myristate-13-acetate (PMA), for 48 h at 37 °C. The cells were treated with  $10^{10}$ /mL of MOM<sub>EV</sub> or PMOM<sub>EV</sub> for 6 h. Supernatants and cells were processed as indicated above. For protein extractions, RIPA buffer containing 150 mM NaCl, 50 mM Tris (pH 8), 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS), with Halt™ protease inhibitor cocktail (Thermo Fisher, Waltham, MA, USA) was used. The cell homogenates were centrifuged at 12,000 g for 10 min, at 4 °C and the protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). For western blots, primary antibodies against AKT (#9272), pAKT-T308 (#13038), pAKT-S473 (#9271), STAT3 (#4904), pSTAT3-S727 (#94994), p44/42 MAPK (Erk 1/2) (#4695), pp44/42 MAPK (Erk1/2)-Thr202/Tyr204 (#9101), IGF-1R (#9750S) from Cell Signaling Technology, and I $\kappa$ B- $\alpha$  (MAB4299 R&D SYSTEMS) were used. Anti-GAPDH (ab9485 abcam) and anti- $\beta$ -actin (#8457 Cell Signaling Technology) were used as loading controls. All the experiments were performed with biological and technical triplicates. Data was analyzed using ANOVA, and Tukey's 'Honest Significant Difference' method was used to assign statistical significance for a  $p < 0.05$ . All the graphics and statistical analysis were generated using RStudio<sup>61</sup>.

**qRT-PCR and mRNA expression.** RNA was isolated from cell lines using RNeasy® Mini Kit following the manufacturer's protocol (QIAGEN, Germantown, MD). DNA was removed by treatment with DNase (QIAGEN, Germantown, MD) according to the manufacturer's protocol. RNA quality was monitored on 1% agarose gels, and RNA quantification was performed using Thermo Scientific Nanodrop One Microvolume UV-vis spectrophotometer (Thermo Fisher Scientific, Grand Island, NY). qRT-PCR was performed as described<sup>66</sup>. Primer sequences used to determine relative transcript abundance are listed in Suppl. Table 3.

### Data availability

miRNA raw data generated in this study was deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA930463. GEO accession GSE225840 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225840>. Token yxwhykosdbgjxyp.

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## Author contributions

G.L.L., L.A.P. and M.F.T.L. conceptualized the study. M.F.T.L. and C.F.G. contributed to the sample processing, performed the experiments and data analyses. M.F.T.L. wrote the original draft. G.L.L., C.F.G. and L.A.P. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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## Competing interests

The authors declare no competing interests.

## Additional information

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