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Research Article

Development of a Cellular-Based Assay for Measuring the Anti-Inflammatory Potency of Donor Banked Milk

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Abstract

Background: Meeting extremely preterm infant nutritional needs are one of the main goals of the human milk feeding strategy in the treatment of necrotizing enterocolitis (NEC). Bioactive components of human milk also play an important role in the prevention of NEC. Thus, the objective of our study was to develop an assay to assess anti-inflammatory potential of specific milk samples (DHMs) through the monitoring of NFκB activation, a key factor that regulates inflammation. **Methodology:** Small intestine epithelial cells (FHs74) were seeded in 24-well plate and left to adhere for at least 18 hours after which the medium was removed and cells were incubated with DHMs or controls for 2h. Then, cells were washed with saline prior to the addition of peripheral blood mononuclear cell supernatant used as NFκB activator. After the stimulation, cells were recovered and the activation of NFκB was measured by flow cytometry or by ELISA. **Results:** Using this model, we were able to identify DHMs with strong, moderate and no anti-inflammatory ability. Variation among the triplicate in the same assay was less than 5% and variation between different assay performed at separate times was ≤10% supporting the reproducibility of this assay. **Conclusion:** Coupled to nutritional information, this potency assay could help breast milk banks or neonatal intensive care unit personnel improve their strategy during the selection and preparation of breast milk batches and favor a product with high clinical efficacy for the preterm infant.

Keywords: Breast milk; Potency assay; Clinical efficiency; Inflammation

Introduction

Cell-based assays and analysis are vital experimental tools in life science research and biomanufacturing. They are based on cell culture methods, where live cells are grown in vitro and used as model systems to assess the biochemistry and physiology of both healthy and diseased cells. Cell culture assays provide a means of quantitatively analyzing the presence, amount, or functional activity of a cell or tissue of interest [1].

Necrotizing enterocolitis (NEC) is a multifactorial disease affecting the gastrointestinal tract in 5 to 10% of preterm infants who weigh less than 1500 g at birth. The average mortality caused by NEC is between 20 and 30% and it can reach nearly 50% in infants requiring surgical management [2]. Despite a high volume of research in clinical as well as laboratory settings, we still possess only a limited understanding of the pathophysiological mechanisms of this devastating illness. Multiple etiologic factors including immaturity of the preterm newborn intestinal tract,

formula feeding, infections, and ischemia have been associated with NEC. A combination of these risk factors, perhaps based on genetic predisposition, possibly lead to the mucosal and epithelial injury that is the initiating event of NEC [3, 4].

The intestinal epithelial lining made up of a single layer of multiple cell types that perform different functions including nutrient absorption, preventing entry of pathogenic organisms and unprocessed antigens, innate inflammatory signalling, secretion of molecules that contribute to the mucosal barrier, antigen presentation to underlying immune cells, and production of endocrine signalling molecules [3]. Many studies have shown how cytokines such as IFN-γ, TNF-α and IL-8, induced during intestinal inflammation can further breakdown the intestinal epithelial barrier by causing necrosis as well as inhibition of mucus production by intestinal epithelial cells [5-7].

Among the treatment options that may be considered to prevent NEC, one key strategy has been providing human milk (HM) feedings, both mother's own milk (MOM) and donor human milk (DHM), to preterm infants [2,8]. Indeed, both MOM and DHM contain several bioactive molecules susceptible to promote intestinal barrier function and thus contribute to their ability to prevent NEC, such as antibodies, anti-inflammatory cytokines and growth factors [9]. However, there is currently no *in-vitro* test that can predict the anti-inflammatory potential of MOM and/or DHM.

The transcription factor nuclear factor-κB (NFκB) that consists of five subunits (p50, p65, p52, cRel and RelB) is a major regulator of inflammation by inducing the production of many cytokines, including IL-1, IL-6, chemokines and TNF-α and PAF all known to play an important role in inflammatory response during NEC [10, 11]. NFκB need to homo- or heterodimerize to form active NFκB [12, 13]. Among these, p50-p50 homodimers and p50-p65 heterodimers are the NFκB dimers mostly found in intestinal tissues [9, 10]. In addition, it has been reported that the expression of IκB, the natural repressor of NFκB, is decreased in immature enterocytes and that human breast milk could stimulate IκB expression and thus inhibit NFκB activation. This is a mechanism by which human breast milk could protect neonates from inflammatory bowel diseases such as NEC [16].

Herein, we report on the development of a potency assay to assess the ability of MOM and/or DHM to inhibit inflammation. This method based on the use of FHs74 intestinal cells and the monitoring of NFκB p65 phosphorylation. This method allows for standard evaluation of anti-inflammatory potential of MOM or DHM.

Materials and Methods

Human Donor Milk Collection

This study has been approved by Héma-Quebec's research ethics committee and all participants in this study have signed an informed consent. Non-pasteurized human donor milk samples were provided by the Héma-Québec breast milk bank as well as by the regulatory analysis department of Héma-Québec. DHM was subjected to Holder pasteurization (62, 5˚C for 30 minutes in a water bath).

FHs74 Int cell expansion

The FHs74 Int cells were obtained from the American Type Culture Collection (ATCC; CCL-241; Manassas, VA, USA). These cells were extracted from the small intestine and thus are composed of a mixed population of epithelial cells including enterocytes, paneth cells and potentially some stem cells [17]. FHs74 Int cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific, Waltham, MA, USA), at 37°C, 5% CO_2 supplemented with 20% qualified fetal bovine serum (FBS) (Thermo Fisher Scientific), GlutaMAX 1X (Thermo Fischer Scientific), 10 µg/mL insulin (from bovine pancreas; Sigma Life Science) and 30 ng/mL of recombinant human epidermal growth factor (Thermo Fisher Scientific) to generate a master cell bank. Briefly, cells were seeded at the density of 4000 cells/cm^2 in T175 flasks for cell culture. Medium was changed after four days. At day seven of culture, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Thermo Fisher) and harvested using TrypLE™ Express (Thermo Fisher) for 10 minutes (min) at room temperature (RT). The cell suspension was diluted by adding an equal volume of Plasma-Lyte A (Baxter Canada, Mississauga, ON, Canada) containing 10% Human Serum Albumin (HSA) (Alburex® 25, CSL Behring, Ottawa, ON, Canada). Cells were centrifuged (600 x *g*, 10 min), suspended in Plasma-Lyte A containing 5% HSA and the cell suspension was analyzed (count and viability) on Nucleocounter® NC-250™ (ChemoMetec, Lincoln, NE, USA). FHs74 cells were then reseeded as described above for one or two additional passages and kept frozen in liquid nitrogen vapor phase until use.

Preparation of Peripheral Blood Mononuclear Cell Supernatants

Human Peripheral blood mononuclear cells (PBMCs) were isolated using density centrifugation over Ficoll-Paque (GE Healthcare BioScience) from whole blood collected from healthy volunteers who signed an informed consent. To prepare the supernatants, PBMCs ($5x10^5$ cells/well in a 24-well plate) were activated using 20 ng/mL of anti-human CD3 and CD28 antibodies (respectively clone OKT3 and CD28.2, both from Thermo Fisher). PBMCs were maintained with the antibody mixture at 37 °C, 5% CO_2 four days after which, supernatants were collected and frozen for further use. In addition, cells were also collected to ensure the activation state of the T-cells. Therefore, the latter were labelled with 10 µL of FcR block (Miltenyi Biotech, Auburn, USA), 4 µL of anti-CD3 (SK7 clone; Thermo Fisher) and 12, 3 µL of 7AAD (Thermo Fisher) before being analyzed using flow cytometry.

PBMCs were considered activated with an expansion factor ≥ 3.

Development of the *in Vitro* Inflammation Model

All assays were performed using FHs74 cells at passage four. Thus, upon thaw using Thawstar (BIOLIFE Solutions; Bothell, WA, USA) and centrifugation (130 x *g*, 7 minutes) cells were counted and suspended in IMDM supplemented with 1% FBS. 10^5 cells were seeded onto 24-well plate and allow adhering for at least 18 hours at 37 °C, 5% CO_2.

The next day, culture media was removed and a new culture medium, DHM or milk formulation diluted 1/50 in IMDM (Enfamil A+ Gentlease, Mead Johnson Nutrition, Kanata, ON, CA) was added and incubated for 2 hours with the FHs74 cells according to the following experimental conditions: FH74s alone (baseline); FHs74 stimulated (positive control); FHs74 incubated with milk formulation (negative control); FHs74 incubated with the donor milk to be tested. After this first step, FHs74 cells were washed with DPBS before the addition of different pro-inflammatory factors such as PBMCs supernatants (200 µL; previously filtered with 0.22 µm filter), lipopolysaccharides (LPS) from *Klebsiella pneumoniae* (1 µg/mL); from *Escherichia coli* O55:B5 (1 µg/mL); and from *Pseudomonas aeruginosa* (1 µg/mL; all LPS from Sigma), as well as IFN-γ (1 µg/mL) and TNF-α (1 µg/mL) both from Peprotech, NJ, USA.

FHs74 cells were left in the presence of these different mediators for 30 min and 24 hours (h). Following this stimulation, cells were washed with 1 mL of DPBS (Thermo Fisher) and detached by the addition of 250 µL of TrypleExpress (Thermo Fisher) into each well. 250 µL of IMDM+1% FBS was added to dilute the cell suspensions which were then collected and transferred into tubes for the analysis of the expression of NFκB and Annexin V by flow cytometry. These two parameters were respectively used as an index of inflammation and an index of apoptosis.

Cell Labelling and Flow Cytometry

Cell suspensions were separated in two groups of tubes. The first group was dedicated to measure NFκB phosphorylation whereas, the second group of tubes was used to assess the presence of Annexin V on the cell surface. Thus, to measure NFκB phosphorylation, cells were washed with DPBS+2% FBS and then fixed with formaldehyde (1.5%) for 20 min at room temperature (RT). After the fixation step, cells were washed again and then incubated during 20 min in methanol 90% (v/v DPBS), on ice, to permeabilize the cells. An additional wash was performed and cells were labelled, for 20 min, with 5 µL of Allophycocyanin (APC)-conjugated anti-human phospho-NFkB p65 (Clone B33B4WP; Thermo Fisher). After a final wash, cells were suspended in 200 µL of DPBS+2% FBS before being analyzed by flow cytometry (BD Accuri C6, BD Bioscience). The percent of NFκB signal was determined with the following formula:

$$\text{\% of NF}\kappa\text{B signal} = \frac{\text{Mean Fluorescence Intensity (condition of interest)} * 100}{\text{Mean Fluorescence Intensity of stimulated cells without milk}}$$

The second group of tubes containing the cell suspensions was aimed at measuring the level of apoptosis using APC Annexin V Apoptosis Detection Kit with Propidium Iodide Solution (BioLegend; San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were washed with cold DPBS and suspended in 100 µL of Annexin V Binding Buffer. Then, 5 µL of Annexin V-APC and 10 µL of Propidium Iodide solution were added and allowed to incubate for 15 min at room temperature (RT). Then, cells were re-suspended in 200 µL of Annexin V Binding Buffer and the percent of Annexin V positive cells was assessed.

ELISA

FHs74 cell lysates were generated to measure phosphorylated NFkB p65 and total NFkB using InstantOne ELISA™ Kit (Thermo Fisher) according to the manufacturer's instructions. Briefly, following initial adhesion, incubation of the DHM to be tested and stimulation (as described in section 2.4), cells were lysed using the lysis buffer included in the kit and stored at -80°C until use.

Statistical Analysis

All statistical analyses were performed using the Graph-Pad InStat3 software (GraphPad Software Inc., La Jolla, CA, USA). One-way ANOVA and Tukey-Kramer multiple comparison tests were performed. Values of $p < 0.05$ were considered statistically significant.

Results

PBMC Supernatant Significantly Induces Activation of NFκB

Inflammation and apoptosis are two key features of NEC [18]. We used NFκB and Annexin V respectively as index of inflammation and apoptosis. Thus, to determine the best inflammatory and/or apoptotic agent, FHs74 cells were treated for 30 min and 24 h with PBMC supernatant, *Klebsiella pneumoniae* LPS (1 µg/mL), *Escherichia coli* O55:B5 LPS (1 µg/mL), *Pseudomonas aeruginosa* LPS (1 µg/mL), IFN-γ (1 µg/mL) and TNF-α (1 µg/mL). Our results revealed that PBMC supernatant was the most potent (up to 2-fold increase over the baseline) inducer of NFκB activation among the various activator we used (Figure 1, panels A and B). Indeed, a significant induction of NFκB signal was observed 30 min and 24 h post stimulation with PBMC supernatant. With the exception of TNF-α (which showed an increased NFκB signal at 30 min), no other activator showed a significant induction of the NFκB signal at both time points.

Regarding the apoptosis, after 30 min of stimulation, no apoptosis was observed for the entire activator agent used (Figure 1, Panel C). However, after 24 hours of stimulation, the PBMC supernatant and TNF-α induced a significant 2-fold increase versus the baseline (Figure 1, Panel D). Based on these results, PBMC supernatant was selected to test the effect of human milk on inflammation. Since the activation of NFκB is detectable as early as 30 minutes compared to 24 hours for apoptosis, monitoring of NFκB is the most relevant for the development of a rapid method to determine the anti-inflammatory potential of DHM.

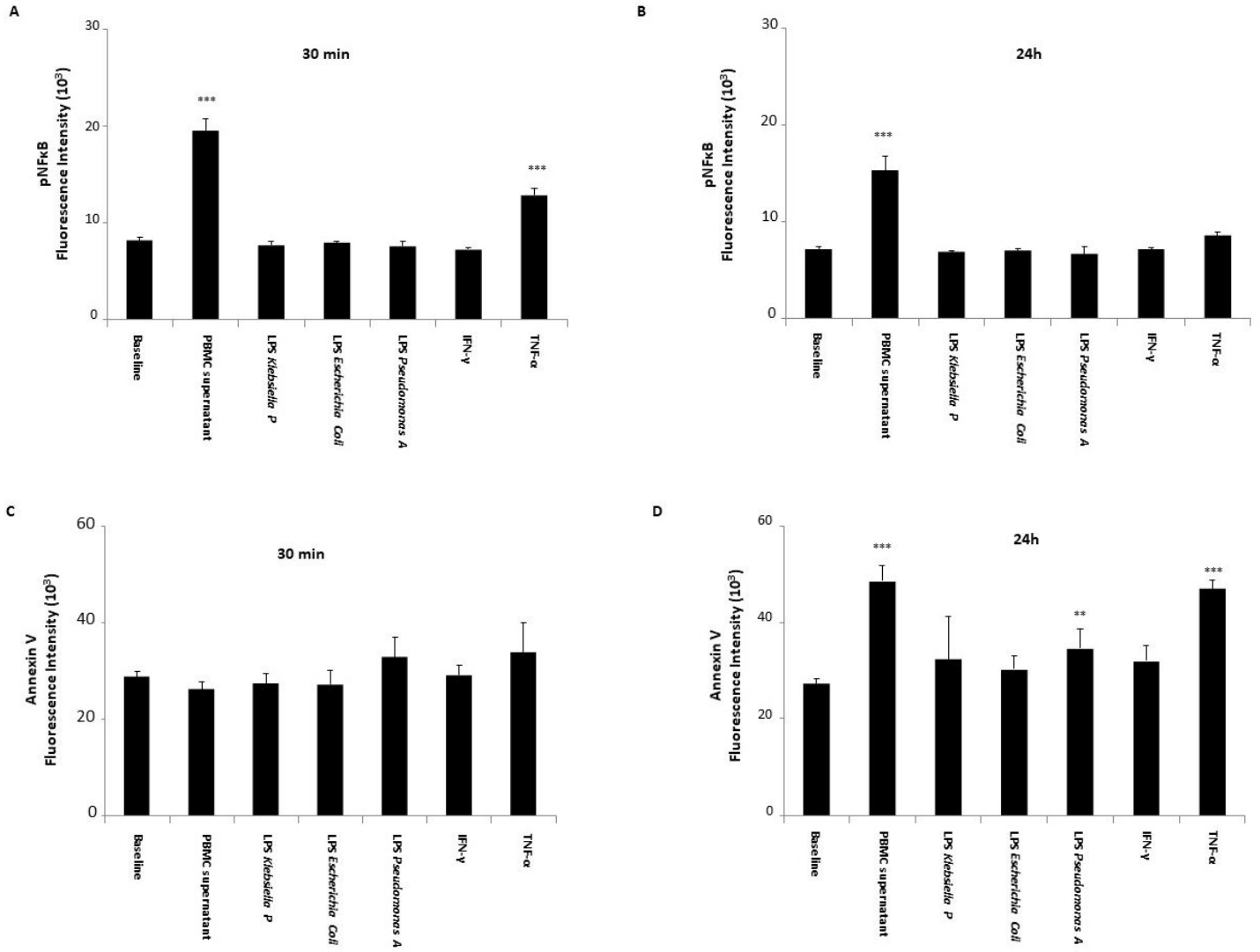

Figure 1: Pro-inflammatory mediators' induction of inflammation and apoptosis. Induction of phosphorylated NFκB signal, after 30 min (A) or 24 h of stimulation (B), with individual pro-inflammatory mediators: LPS from *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* and IFN-γ and TNF-α (all at 1 µg/mL). Induction of apoptosis, after 30 min (C) or 24 h of stimulation (D), with all aforementioned individual pro-inflammatory mediators (all at 1 µg/mL). *$P < 0.05$ vs negative control, **$P < 0.01$ vs negative control, ***$P < 0.001$ vs negative control. Data are presented as mean ± standard deviation (S.D.).

Modulation of NFκB Signal by Donor Human Milk

FHs74 cells were pre-incubated 2 h and 24 h with various DHMs (diluted 1/50) prior to the 30 min stimulation with the PBMC supernatant in order to determine if human milk could prevent the stimulation of the NFκB signal. The use of pure milk or dilution less than 1/50 has resulted in cell detachment (data not shown) which prompted us to use DHM at a dilution of 1/50. Our results showed that

2 h preincubation of FHs74 cells with DHMs resulted in a modulation of the NFκB signal which was variable among the DHMs tested (Figure 2); whereas, formula milk showed a weak modulation of the NFκB signal. However, 24 h preincubation of FHs74 cells with DHMs resulted in cell detachment and consequently to cell loss.

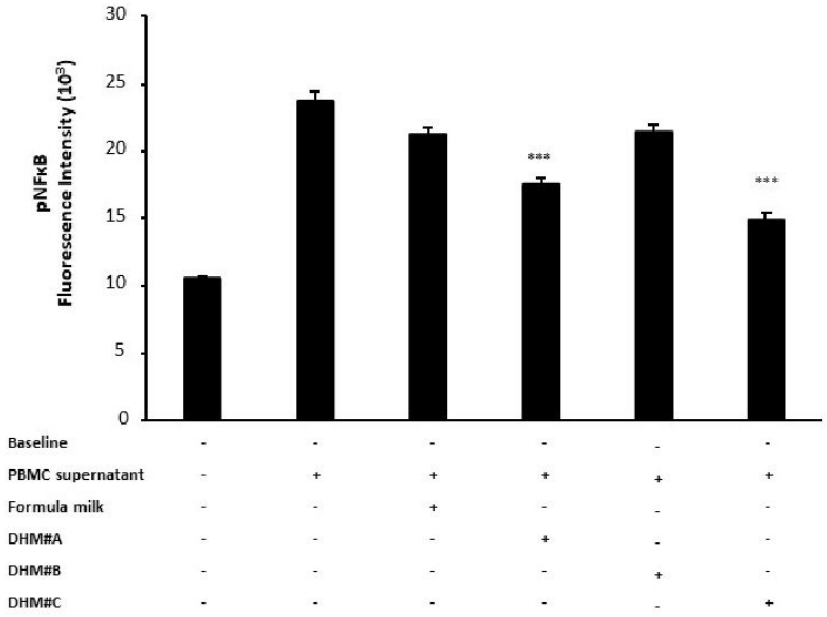

Figure 2: Prevention of NFκB activation by DHM.

Three different DHM samples and formula milk preparations were used to assess their ability to affect NFκB phosphorylation. Briefly, DHM was added to the FHs74 cells for 2 h before stimulation of NFκB phosphorylation with PBMC supernatant. ***$P < 0.001$ vs FHs74 stimulated with PBMC supernatant. Data are presented as mean ± S.D.

Reproducibility of the Potency Model

To assess the reproducibility of the model, intra- and inter-assay variability was evaluated. For intra-assay variability, different conditions were made in triplicate and the variation between wells for the same condition was calculated. For inter-assay, the same experience was repeated three different times to evaluate the variability. These analyses revealed that the variability between three wells for the same condition was approximately 5% and the variability between the same test performed at three different times was approximately 10%, suggesting that the model is reproducible (Figure 3, Panel A and B).

After ensuring that the model was reproducible, we tested different DHM samples with the final parameters of the assay including the conversion of NFκB fluorescence intensity into percentages, described in the Materials and Methods section, as a means to express the anti-inflammatory capacity of different donations. This conversion was made to simplify comparisons between different experiments. As expected, the results showed variable ability of different lots of DHMs to affect NFκB signal with some of them being particularly potent to prevent inflammation while others had no effect at all (Figure 3, Panel C). Our flow cytometry labelling procedure makes it difficult to use this approach to test large numbers of samples. So, as an alternative to flow cytometry, we have tested the use of NFκB p65 ELISA Kit. This kit allows for quantification of both phosphorylated p65 NFκB and total NFκB, which can serve as an internal control to normalize the amount of protein for each condition. Our results revealed, as observed by flow cytometry, that DHM could affect the expression level of the phosphorylated NFκB p65 (Figure 4, Panel A). However, total NFκB whose expression is constitutive in cells also showed variability among the samples. This, prompted us to use a ratio of phosphorylated NFκB over Total NFκB to correctly assess the modulation of NFκB that occurs in our model. This analysis revealed variable anti-inflammatory capacity from the tested DHMs comparable to the results obtained by flow cytometry (Figure 4, Panel C).

Discussion

Human milk is the preferred diet for preterm infants as it protects against a multitude of neonatal intensive care unit challenges, specifically NEC. Meeting extremely preterm infant nutritional needs are one of the main goals of the human milk feeding strategy [19]. However, bioactive components of human milk also play an important role in the prevention of NEC [8, 9, 20]. Herein, we report on the development of a potency assay that could allow for efficient assessment of anti-inflammatory potential of a specific donor human milk and guide for donor pooling strategy.

Inflammation in the gut and apoptosis of intestinal epithelial cells are two of the key manifestation of NEC [18]. Indeed, many factors including bacterial components could result in the activation of the NFκB transcription factor, a major regulator of inflammation [21]. This leads to the production of many inflammatory mediators, including cytokines and chemokines, which lead to immune cell recruitment, especially neutrophils and to intestinal inflammation and ultimately to the disruption of the epithelial barrier.

Thus, to develop our inflammatory model we used LPS from *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa* that have been reported to be involved in the pathogenesis of NEC [22]. However, none of them induced a significant activation of NFκB signal nor was able to induce a quite significant FHs74 cell apoptosis. In addition to bacterial components, we also look at the ability of some pro-inflammatory cytokines such as IFN-γ and TNF-α along to the supernatant of activated PBMCs to activate NFκB. Among these agents, only TNF-α and PBMC supernatant induced a significant increase of both phosphorylated NFκB and Annexin V signals (Figure 1 A and D) suggesting they could be used as activator agents for FHs74 cells and that we can induce inflammation and apoptosis in our model. In order to develop the simplest and fastest model possible, using the PBMC supernatant for 30 minutes with monitoring of NFκB activation seems to be a good option in order to mimic the inflammatory disease. Indeed, compared to the other stimulants used, the supernatant of activated PBMCs is a cocktail of several cytokines, which can explain its higher inflammatory effect compared to the use of different pro-inflammatory cytokines or bacterial LPS individually [23].

The next step in the development of this *in vitro* potency model was to evaluate the time at which breast milk could prevent the induction of inflammation. To do so, FHs74 cells were incubated for 2 h and 24 h with different DHM samples (diluted 1/50) and then NFκB and Annexin V signals were assessed. It is worth mentioning that we used breast milk diluted 1/50, because pure breast milk or breast milk diluted less than (1/50) resulted in cell detachment which prevents us from stimulating the cells and thus to measure NFκB induction or apoptosis. After 2 h, we observed differences in the ability of different DHM samples to inhibit NFκB signal with some samples being potent at inhibiting the NFκB signal when other samples were clearly ineffective (Figure 2). However, pre-incubation of FHs74 cells with DHM for 24 h is not appropriate as all cells lifted from the bottom of the wells and thus it was impossible to stimulate them with the supernatant suggesting that the incubation time with DHM was too long and was altering the cells in these *in vitro* conditions. Thus, we concluded that a 2 h preincubation of the cells with DHM was sufficient to highlight a modulation of the NFκB signal and to achieve our goal, which was to establish whether DHM samples could prevent inflammation induced with PBMC supernatant. This 2-hour pre-treatment period combined with the stimulation of 30 minutes is interesting since it opens the door to performing the test in a single working day.

In order to evaluate the reproducibility of this assay, intra- and inter variability were both evaluated to make sure the model was reproducible and could give reliable results. Our analysis revealed that the variability of the NFκB signal between triplicate for the same condition was approximately 5% and the variability of the NFκB signal between the same assay performed at three different times was approximately 10%, which was considered as acceptable and support the reproducibility of the proposed model (Figure 3, Panel A and B). Thereafter, different acceptance criteria (Table 1) were established so that the user could be able to interpret the test results. Thus, the first acceptance criterion was the level of NFκB activation induced following stimulation that was set to be at least 1.5-fold increase over the baseline (Table 1). Then, we set out a significance threshold of minimally 20% to allow detection of what could be considered as a significant difference between two or more experimental conditions. Thereby, if the difference of two tested conditions is below or equal to 20% then the 2 conditions are considered to have the same inhibitory capacity. Another acceptance criterion for the assay was the result of the formula milk (used as negative control) on the NFκB signal. Indeed, this latter result should be beyond or equal to 80% of NFκB signal obtained with the condition FHs74 cells + PBMC supernatant (considered as the maximum value of the NFκB signal) as milk formula should not or weakly affect NFκB signal. This means that, if a test result showed a negative control value of NFκB signal that is below 80%, the test will not be considered as valid and should be retaken. Moreover, to be considered as significantly inhibitory, a DHM should present a lower inhibitory capacity of at least 20% compared to that obtained with formula milk (represented by the red line in Figure 4B). The implementation of these acceptance criteria led us to test additional donations and to confirm the ability of our test to discriminate anti-inflammatory capacity of different DHM donations (Figure 3C).

Test acceptance criteria		
	Flow cytometry	**ELISA**
NFκB signal fold induction over the baseline.	≥1.5-fold	≥2-fold
Percent of NFκB signal with Formula milk.	≥80%	-
Intra-assay variation.	≤5%	≤10%
Threshold to consider significantly different the variation between conditions.	≥20%	≥20%

Table 1: different acceptance criteria.

Our flow cytometry labelling procedure makes it difficult to use this approach to test large numbers of samples. Thus, to allow the evaluation of the anti-inflammatory capacity of several donations at the same time, we tested the NFκB Instant One ELISA kit, the results of which were comparable to those obtained by flow cytometry (Figure 4). This ELISA kit has some advantages over our cytometry protocol. The most important of them is the ability to measure total NFκB whose expression is constitutive and expected to remain constant among different experimental conditions. Unexpectedly, our results revealed a lot of variability in the expression of total NFκB among our experimental conditions. This prompted us to use a relative approach (Absorbance of phospho-NFκB p65/absorbance of Total NFκB) in order to normalize the expression of phosphorylated NFκB according to the amount of total NFκB expression in each sample. In addition, the kit contains various controls including a positive and negative control for the NFκB signal aimed at assuring that the kit still functional. Coupled with our initial controls (baseline, PBMC stimulated cells, formula milk + PBMC stimulated cells), this increases the level of confidence as to the veracity of the test results. However, it should be noted that our model has one significant limitation. Indeed, it does not fully reflect the conditions of the human gut, as it is only made of epithelial cells but this gives an interesting proxy of the behaviour of immature intestinal cells following their exposure to breast milk.

In conclusion, we have developed a potency assay (summarized in figure 5) that measures the anti-inflammatory capacity of breast milk donors, which could help milk banks to improve their strategy of preparation of breast milk batches. Indeed, recent data seems to suggest that the risk reduction of NEC is statistically significant only for studies in which premature infants are given both their own and donated breast milk [24]. However, given the difference between each donation that our model highlighted, it is appropriate to wonder if all the donations used so far were optimal in terms of bioactive molecules. This aspect is not really considered by the milk banks and in previous studies. It is known that the number of bioactive factors decrease over time, depending on whether the donor has given birth to a premature baby or not [25]. Thus, we believe that our test makes it possible to put the association between nutritional and inflammatory factors back at the heart of the strategy of preparation/selection of breast milk donations with the hope of a more effective product for the preterm newborn.

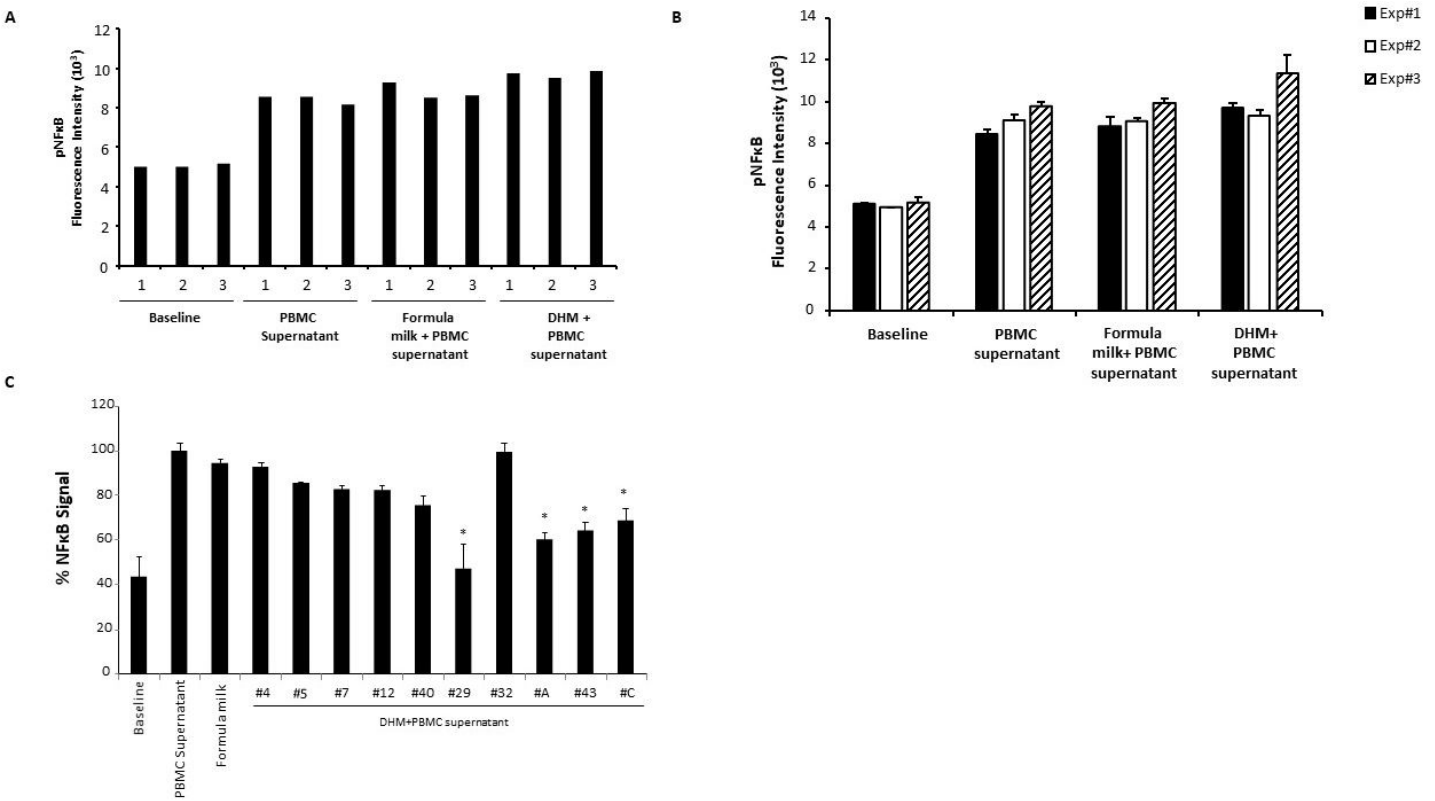

Figure 3: Reproducibility of the model. DHM or formula milk was added to the FHs74 cells for 2h before stimulation of NFκB phosphorylation with PBMC supernatant. Baseline represents unstimulated cells and PBMC supernatant is a condition where the cells were only stimulated with PBMC supernatant. (*A*) Intra-variability, variability between triplicates in the same experiment. (*B*) Inter-variability, variability between the same experiences repeated at three different times in triplicate. Data are presented as mean ± SD. (*C*) Evaluation of the capacity of ten different DHM samples to affect NFκB phosphorylation. Data are expressed as percent of NFκB phosphorylation signal with PBMC stimulated cells considered as 100% of the signal.

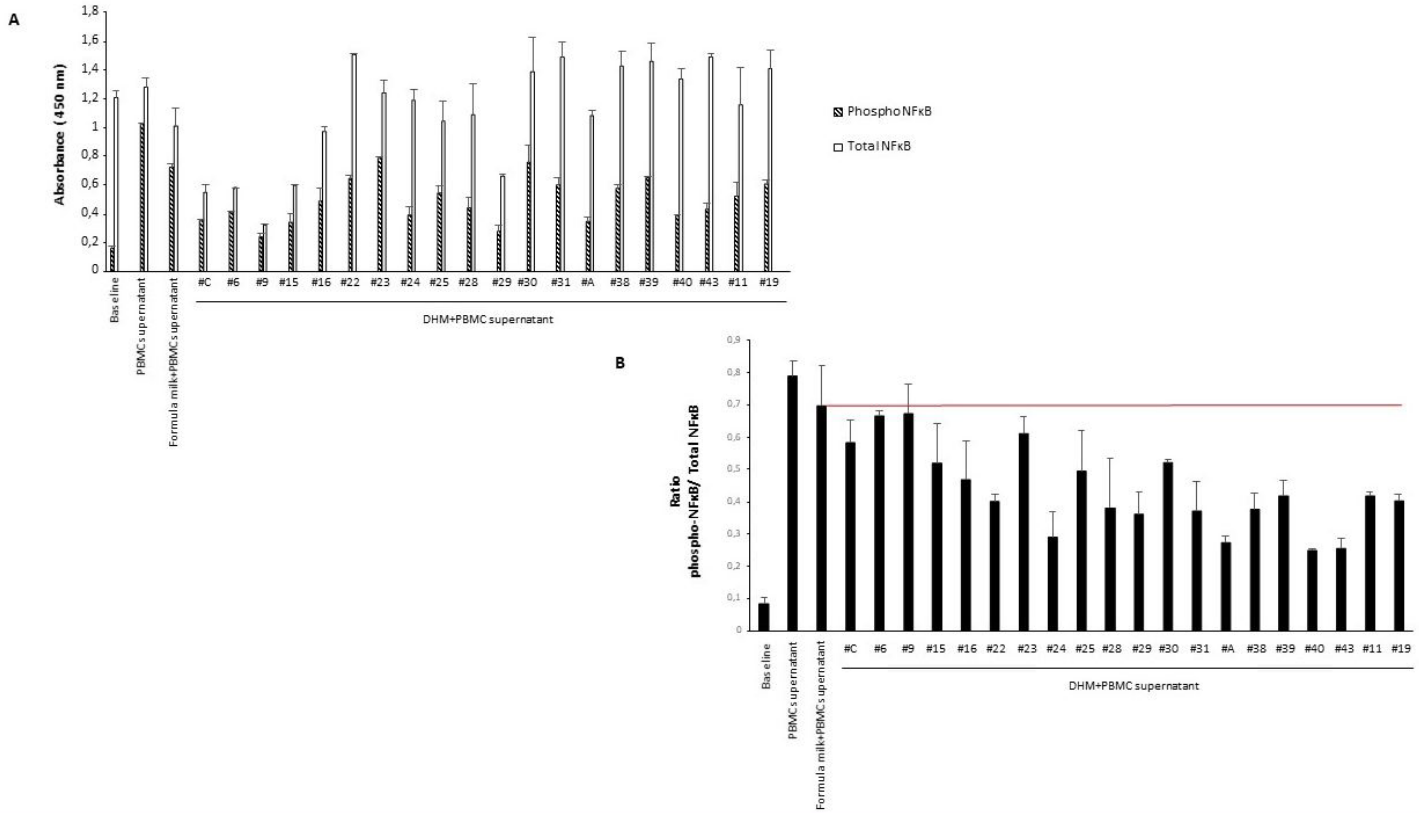

Figure 4: Donor Human Milk Testing by ELISA. Following adhesion and preincubation with DHM to be tested, FHs74 cells were stimulated during 30 min with PBMC supernatant. After the stimulation, supernatant was removed and cells were lysed and the expression of phosphorylated NFκB p65 and total NFκB were assessed using NFκB Instant One™ ELISA kit. (A) Separated absorbance of both phosphorylated NFκB p65 and total NFκB protein expression. (B) Expresion of phosphorylated NFκB p65 relative to total NFκB content in each sample. Data are presented as mean ± S.D.

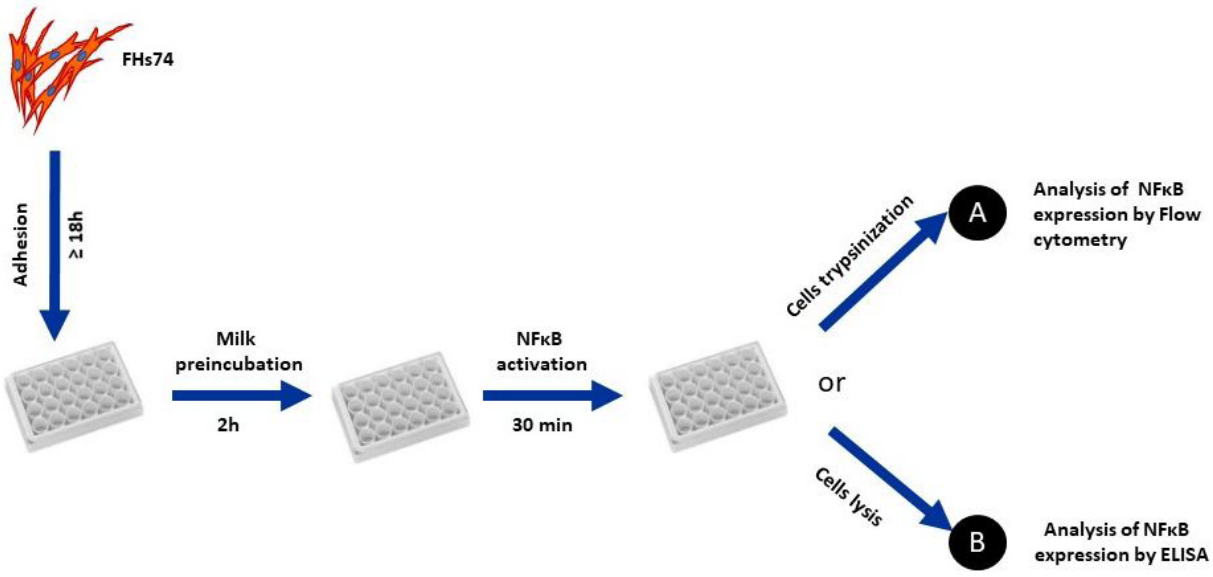

Figure 5: Summary of the potency assay.

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Conflict of Interest Statement

The authors have no conflict of interests to disclose.

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