what mother is telling you

The messages encoded in milk-derived extracellular vesicles

Implications for the immune system and epithelial barrier function

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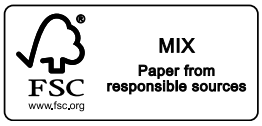
Cover design: Bas van der Leegte, 25twaalf
Layout: Nicole Nijhuis, Gildeprint – The Netherlands
Printed by: Gildeprint – The Netherlands

ISBN: 978-94-6233-655-1



Printing of this thesis was financially supported by Nutricia Research and the Infection & immunity Center, Utrecht

What mother is telling you:

The messages encoded in milk-derived extracellular vesicles

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Wat moeder vertelt:
Extracellulaire membraanblaasjes in moedermelk als boodschappers van moeder naar kind
Implicaties voor het afweersysteem en het functioneren van de epitheliale barrière

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 juni 2017 des middags te 2.30 uur

door

Marijke Irene Zonneveld

geboren op 25 oktober 1986
te *'s-Gravenhage*

Promotoren:	Prof. dr. M.H.M. Wauben
	Prof. dr. J. Garssen
Copromotoren:	Dr. E.N.M. Nolte – 't Hoen
	Dr. F.A.M. Redegeld

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek middels het Partnershipprogramma STW-Danone met projectnummer 11676.

Voor mama,
omdat jij mij laat zien dat alles kan

Voor papa,
omdat jij mij het gevoel geeft dat ik alles kan

Imagination is more important than knowledge.
Knowledge is limited. Imagination encircles the world.

- Albert Einstein

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Chapter 1

General Introduction

Mammalian milk has a high nutritional value in the form of protein, fat, and oligosaccharides and is optimized for supporting the infants' growth during the first months of life[1–3]. Besides providing nutrition, milk assists the further development of the intestinal epithelial mucosa by stimulating the intestinal barrier, the immune system and the bacterial microenvironment[4–8]. Moreover, the beneficial effects of human milk on prevention of diseases, such as necrotizing enterocolitis (NEC) and infectious disease, are well documented[9–11]. Milk can therefore be considered to be natures' first functional food. However, in the past few decades there has been a sharp increase in the development of allergic disease in children[12,13]. Currently, it is uncertain whether there is an association with human milk or changes in its composition[14–16]. Human milk is composed of many bioactive macromolecular structures. The lipid content of milk is mainly present in the form of milk fat globules, which are lipid droplets secreted by the epithelial cells of the mammary gland[17,18]. The most abundant proteins in milk consist of whey and caseins[19], while other components in human milk are oligosaccharides[20], (immune) cells[21–23], growth factors[24,25], hormones[26], cytokines[27], immunoglobulins[28,29], and bacteria[30]. One understudied component of human milk are the extracellular vesicles (EV). As specialized intercellular communication units, EV in milk could be important for the development of the epithelial mucosa, including adaptive immunity. Below, key elements of the epithelial mucosa will be briefly introduced, as well as EV and what is known about their function in milk.

The epithelial barrier

The gastro-intestinal (GI) tract forms the physical and chemical barrier between the environment and the internal organs. This barrier is maintained by a single layer of polarized epithelial cells[31]. This polarization allows them to respond differently to the interior and external environment through the differential distribution of proteins and receptors[32]. This also allows for selective uptake and transcytosis of macromolecular structures[33]. The single layer of cells is interconnected via the adherens junctions and tight junctions, controlling passage of solutes and ions[34]. This selective epithelial barrier needs to be maintained during the rapid growth which occurs during the first few months of life. The expansion of the intestinal surface area occurs in two ways, one is through the duplication of crypts, known as crypt fission, and the other by deepening of the crypts and extending the villi, which is called crypt hyperplasia[35,36]. Postnatally, the primary mode of intestinal growth is crypt fission, and is increasingly substituted by crypt hyperplasia during weaning, becoming the main form of intestinal epithelial growth in adults[35,37,38]. Crypt fission has been shown to be dependent on breast milk, and bottle fed children have increased crypt depth, indicative of crypt hyperplasia[35].

Innate immune sensors

The epithelial cells lining the GI tract do not only constitute an important barrier, but are also specialized sensors, critical for the maintenance of immune homeostasis[32]. One of their tools

for sampling the environment are a class of pattern recognition receptors known as the Toll-like receptors (TLR). TLR are an integral part of the innate immune system, as they are able to quickly recognize conserved bacterial and viral elements and initiate cell signaling cascades which lead to the recruitment of immune cells necessary for resolving infection[39]. However, the intestine is permanently colonized by the bacterial microbiota and intestinal TLR responses therefore need to by attenuated. This attenuation of epithelial TLR signaling occurs postnatally and throughout the entire length of the GI tract. Both buccal and intestinal epithelial cells stimulated directly after birth are highly sensitive to agonist stimulation, but quickly loose this ability and become hyposensitive[40–42]. This hyposensitivity to the intestinal microbiota needs to be maintained throughout life to prevent excessive inflammation which is detrimental to the epithelial barrier. A break in hyposensitivity is thought to lead to diseases, such as inflammatory bowel disease[43]. Besides hyposensitivity, the differential distribution of TLR on the polarized epithelium plays a role in maintaining immune homeostasis. Stimulating a certain TLR basolaterally can result in inflammation, while stimulating the same TLR apically results in hyporesponsiveness[44]. This might be a mechanism through which epithelial cells detect breaks in barrier integrity[45]. There is also mounting evidence that epithelial TLR are capable of discriminating between commensal and pathogenic bacteria, and that the balance between inflammation and tolerance is maintained by tight regulation of the expression levels, as well as the site of expression within the GI tract[44,46–48]. In addition, it has been shown that TLR stimulation in the absence of downstream NF-κB signaling is necessary to allow correct microbial colonization[49].

Mucosal-associated lymphoid tissue

Mucosal surfaces of the body are continuously in close contact with the environment. In order to maintain immune homeostasis at these sites, the mucosa is lined with mucosal-associated lymphoid tissue (MALT)[50]. In the buccal cavity, these consist of the tonsils, while the intestine is lined with mesenteric lymph nodes and Peyer's patches[50,51]. Peyer's patches are tethered to specialized epithelial cells. These cells differ from the surrounding epithelial lining and produce less mucus, have reduced digestive enzymatic activity, and the basal lamina is more permeable than in surrounding cells[51]. In addition, specialized epithelial cells, known as microfold (M) cells, actively transport antigen from the environment to the adaptive immune cells present in the Peyer's patches[32,52]. There, the antigen is processed by antigen presenting cells (APC), such as dendritic cells, and presented to B cells and T cells. It is at these sites that oral tolerance is induced. Oral tolerance is an active immune response which is characterized by the induction of antigen-specific regulatory T cells that inhibit immune activation in response to orally administered antigens[53,54]. The development of allergic responses to these antigens is thought to be the result of incorrect induction of oral tolerance[55], although it is still under investigation which factors determine tolerance versus immunogenicity.

Extracellular vesicles

Extracellular vesicles (EV) are lipid bilayer enclosed particles secreted by cells for the purpose of intercellular communication[56]. EV are typically described as a mix of exosomes and microvesicles, which differ in their biogenesis[57,58]. On the one hand, exosomes are formed by inward budding of the membrane of the late endosome resulting in a multivesicular body containing intraluminal vesicles. Fusion of the multivesicular body with the plasma membrane results in the release of the vesicles, now called exosomes, into the extracellular milieu[58]. On the other hand, microvesicles originate by pinching off from the plasma membrane[58]. Importantly, there are no known markers that can reliably distinguish these different vesicles subsets once they have been secreted.

EV are usually not more than 200 nm in size and contain protein, lipids, and RNA[59]. This cargo differs depending on the type of parental cell, cellular stress and signals from the environment[60]. This dynamic incorporation of cargo makes them excellent vehicles for multicomponent signaling[61]. However, due to their heterogenous origin and dynamic cargo, it is difficult to determine which message(s) an EV is communicating to another cell, and to which cell or cells this is directed. In body fluids, this is even further complicated by the myriad of cell types which can secrete EV. In addition, commonly used protocols for the isolation of EV do not purify EV from soluble proteins, non-EV associated extracellular RNA or lipoprotein particles[62]. All these factors make the study of EV challenging and care should be taken in optimizing isolation techniques and including proper controls for the determination of EV-specific effects[63].

Extracellular vesicles as intercellular communicators in human milk

It is generally agreed upon that human milk is beneficial for the development of the epithelial mucosa. However, the mode of action and the precise macromolecular structures involved are not fully known[64]. In part, this can be attributed to the sheer complexity of milk, as it consists of many molecular structures with bioactive properties, making the isolation and study of individual components difficult. In 2007 the existence of extracellular vesicles (EV) in human milk was first described[65]. Since then, they have also been identified in bovine, porcine, buffalo, and dromedary milk[66–70]. One of the main functions of EV is the communication of complex messages between cells[61]. This can be between proximal or more distant cells in the same individual, or, in the case of milk, between two individuals. There is evidence to support inter-species[68,71,72] and even cross-kingdom communication by EV[73–76], adding to the relevance of milk EV as they can be consumed either from the mother or from other species through dairy products. Several studies have subsequently focused on elucidating the composition of milk EV in order to gain insight into the messages which are transferred from mother to child, with most studies focusing on the small RNA content[66,69,71,77–82]. Small RNA's, such as miRNA's, are potent modulators of cell signaling and can be highly effective even in femtomolar amounts[83]. EV provide a means for cells to transfer RNA to distant cells without degradation in the extracellular milieu[84]. On the whole, these studies find small RNA species associated with EV that are known to possess immune modulatory functions.

Conserved among different species, the most frequently identified small RNA's in milk EV are miR-148a[69,71,78,81,82], let-7a[69,71,77,81,82], and members of the miR-30 family [69,71,77,78,81,82]. These have been described to have modulatory roles in NF-κB signaling[85,86], translation of pro-inflammatory cytokines [87], T helper 17 induction[88], and inflammatory cell-cell adhesion molecules[89]. Most of these studies have focused on porcine and bovine RNA's[66,69,71,77,81,90], while only three studies have performed sequencing on RNA in human milk-derived EV, with only two analyzing small RNAs[78,79,82].

Besides miRNA, some reports have also attempted to elucidate the milk EV proteome[65,91,92]. These studies identified milk proteins, such casein and adipophilin, as well as known EV enriched proteins, such as CD81, CD63, and Annexins[65,92]. Immune-modulatory proteins were also found to be abundantly present. Among these, were HSP70, MHC class I/II, MUC-1, TGF-β, and immunoglobulins[65,93]. In addition, many proteins were linked to the GO term 'metabolic process' indicating that EV might also have a function in digestion and metabolism[92]. Two reports compared the impact of the maternal health status on EV protein composition. Reinhardt, et al., reported that EV significantly upregulated their expression of proteins associated with the GO term 'defense response' in cattle infected with *S. aureus*[94]. In humans, Torregrosa Paredes, et al. reported that allergic-sensitized mothers contained lower levels of MUC-1 on a subset of EV[95]. These studies suggest that the cells which secrete EV in milk are influenced by these inflammatory cues and possibly alter the composition of EV they secrete.

Based on these descriptive studies, EV from milk are hypothesized to play an important role in oral tolerance induction and regulatory T cell formation[96–98]. However, separating milk EV from other biologically relevant components appears to be challenging, because milk consists mostly of milk fat globules, protein and oligosaccharides. Although it is known that each body fluid requires optimization of the EV isolation protocol[63] and that isolation method affects outcome[99–102], the impact of isolation techniques on milk EV composition and function have rarely been studied[102].

The functional and omics studies which have been performed until now, often make use of generally accepted protocols for the isolation of EV from culture medium, such as high speed ultracentrifugation. However, these methods are also used in milk research to obtain casein micelles and other proteins[92,103]. Thus, using only ultracentrifugation, without additional purification steps, is unsuitable as a manner to define milk EV-specific effects. Besides this, ultracentrifugation has sometimes been reported to give rise to an insoluble pellet, which could trap EV and prevent analysis of the entire population[92,94,102]. Due to the often poor characterization of the structures which were isolated, it is difficult to determine which results could be attributed to EV and which to other milk components. The interpretation of the published results is often also hampered by the lack of appropriate milk matrix and procedural controls[63].

Nevertheless, some studies have undertaken efforts to elucidate functional effects of milk EV[65,66,68,69,71,93,104–109]. However, only a hand full of studies have used the same species as the source and target of EV [65,69,72,106], with most studies focusing on cross-species dietary milk EV. It

can therefore be argued that the physiological relevance and natural cellular targets of breast milk EV and their function in milk for the newborn have rarely been studied.

Despite these limitations, in general, studies indicate that milk-derived EV are capable of attenuating inflammation, promoting epithelial proliferation, and preventing viral infection[65,72,104,106]. In addition, there are reports which show that various cell types, including epithelial, endothelial and immune cells, are able to endocytose and process milk EV[66,68,71,104,105,108,109]. Importantly, milk EV have been described to resist digestion[66,69,90,108].

Taking all of these factors into account, the composition of purified human milk EV, as well as the natural cellular targets and effects of milk EV is under investigated and poorly controlled. In addition, little is known about the cells which secrete EV and which factors determine the composition of EV in milk.

Scope of this thesis

In this thesis, we aim to elucidate the molecular composition of purified EV from human milk, as well as their effects on the development of the epithelial mucosa. In addition, we study whether maternal sensitization impacts milk EV composition and function. For this purpose, we designed an isolation method specifically adapted for the isolation of EV from human milk (Chapter 2). Furthermore, we defined an adapted storage method for milk, which minimizes contamination of the naturally present EV population with vesicles released due to cell death or fragmentation of milk fat globules (chapter 2). Using these optimized protocols, we subsequently described the human milk EV proteome. This revealed a novel functional proteome, previously undescribed in milk (Chapter 3). The results from chapter 3 prompted a detailed analysis into the functional role of EV in the development of the gastro-intestinal epithelial barrier, and the role of EV on the innate and adaptive immune systems. Here, we found that human milk EV promoted epithelial barrier formation, as well as immune homeostasis (Chapter 4). Finally, we have set up a clinical study to assess whether EV from milk of allergic and non-allergic mothers differed in content and function, with the ultimate purpose of defining candidate biomarkers for prediction of allergic disease in infants (Chapter 5). The first results of this study revealed that cells of the adaptive immune system were more responsive to activation stimuli in the presence of EV from allergic mothers (Chapter 5).

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Chapter 2

Recovery of extracellular vesicles from human breast milk is influenced by sample collection and vesicle isolation procedures

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Journal of Extracellular Vesicles. 3, doi: 10.3402/jev.v3.24215 (2014)

Abstract

Extracellular vesicles (EV) in breast milk carry immune relevant proteins and could play an important role in the instruction of the neonatal immune system. To further analyze these EV and to elucidate their function it is important that native populations of EV can be recovered from (stored) breast milk samples in a reproducible fashion. However, the impact of isolation and storage procedures on recovery of breast milk EV has remained underexposed. Here, we aimed to define parameters important for EV recovery from fresh and stored breast milk. To compare various protocols across different donors, breast milk was spiked with a well-defined murine EV population. We found that centrifugation of EV down into density gradients largely improved density-based separation and isolation of EV, compared to floatation up into gradients after high-force pelleting of EV. Using cryo electron microscopy, we identified different subpopulations of human breast milk EV and a not previously described population of lipid tubules. Additionally, the impact of cold storage on breast milk EV was investigated. We determined that storing unprocessed breast milk at -80°C or 4°C caused death of breast milk cells, leading to contamination of the breast milk EV population with storage-induced EV. Here, an alternative storage method is proposed to store breast milk samples for EV analysis at later time points. The proposed adaptations to the breast milk storage and EV isolation procedures can be applied for EV-based biomarker profiling of breast milk and functional analysis of the role of breast milk EV in the development of the neonatal immune system.

Introduction

Breast milk not only functions as a source of nutrition, but also delivers immune modulatory factors to neonates. Breast milk components relevant to immunity include cytokines, antibodies, multiple types of immune cells, antimicrobial peptides, microbes, and unique lipids (reviewed in 1–4). Components such as IgA and IgG have been shown to modulate immune reactivity of the child to antigens present in the environment[5,6]. Breast milk is therefore thought to play an important role in the development of the immune system of the child. Direct evidence that the composition of breast milk can influence immune responses in neonates was provided in mouse models of allergic asthma and food allergy[7,8]. In these studies, offspring was breastfed by mothers environmentally exposed to inhaled antigens or food antigens. It was shown that this offspring exhibited profoundly reduced immune reactions to these antigens. The mechanism behind this type of immune modulation via breast milk is not understood, but preliminary data indicated a key role for induction of tolerance and regulatory T cells.

Recently, extracellular vesicles (EV) have been identified in human breast milk[9–11] and in bovine milk[12,13]. EV are released by many different cell types and are recognized as potent vehicles for intercellular communication, capable of transferring messages encoded in proteins, lipids and RNA. The majority of EV is < 200 nm in size and typically consists of lipid bilayer enclosed vesicles. The cargo of EV is regulated by the EV producing cells and depends on activation- and stress-stimuli imposed on these cells[14,15]. EV derived from different cell types can influence various biological processes, with EV from leukocytes playing an active role in the regulation of innate and adaptive immune responses[15]. It is currently unknown whether the EV in breast milk originate from cells present in milk, from epithelial cells lining the mammary gland or from cells elsewhere in the body. A potential role for milk-derived EV in immune modulation was suggested based on their protein and miRNA contents[9,11–13]. Moreover, human breast milk-derived EV were found to facilitate regulatory T cell induction[9]. This has led to the hypothesis that EV in breast milk could be involved in the instruction of the neonatal immune system.

General protocols to isolate EV from cell culture supernatants or body fluids involve steps of differential (ultra)centrifugation and further purification on a density gradient. However, since different body fluids have a highly variable composition, each of the isolation protocols to recover EV from these body fluids may require specific optimization steps[16]. Factors that could complicate isolation of EV from milk are the high fat and protein content of this body fluid. Most of the fat is stored in milk fat globules (MFG), which are lipid droplets that are formed and secreted by the epithelial cells lining the mammary gland[17]. Major milk proteins are casein and whey[18]. The tertiary structures of these proteins add to the complexity of the milk matrix, as casein is organized into micelles[19], while whey consists of globular proteins known for their emulsifying capacities[20]. Besides the complex milk matrix, storage protocols could also affect recovery of EV from breast milk. Previous studies used refrigerated or frozen milk as a source for EV[9–12,21–24]. Cold storage

of milk is common practice and effects on nutritional components and soluble immunological factors have been described[25–27]. However, storage effects on cells and EV populations in breast milk have mostly been ignored[28]. Since disintegration of dead cells leads to the formation of membrane-enclosed vesicles[29], cell death can lead to contamination of the pool of naturally present EV with vesicles induced upon sample storage.

Here, we assessed and optimized the efficiency and reproducibility with which EV can be recovered from breast milk samples. To this end, milk samples were spiked with a well-characterized mouse EV population as an internal standard. The commonly used differential centrifugation and density gradient ultracentrifugation protocol was modified, in order to achieve efficient and quantitative recovery of EV from breast milk. Additionally, we show that cells in refrigerated or frozen breast milk samples die rapidly, leading to contamination of the native milk EV population with vesicles derived from dying or dead cells. Thus, we provide an alternative method for the storage of breast milk samples for efficient and reliable recovery of EV at later time points.

Materials and Methods

Breast milk collection

Fresh, mature milk samples were collected by 17 healthy mothers at < 8 months (15 donors) or 11-20 months (2 donors) after delivery, who were not actively terminating breast feeding. Donors were instructed to collect fore and hind milk of one breast using an electric breast pump, and to gently mix and aliquot 10-50 ml of milk in sterile containers. Milk was transported to the lab and used for experiments within 20 minutes of collection. Milk was prevented from cooling down by transportation in a Styrofoam box, insulated with tissues and containing a heat-generating package. All donors gave their signed informed consent to participate and the study was approved by the local ethics committee.

Preparation of Bone Marrow derived Dendritic Cells (BMDC) for spiking of milk samples

Murine BMDC used for spiking of breast milk samples were generated from C57BL/6 mice as described previously[30,31]. Cells were cultured in EV depleted culture medium, prepared by centrifuging solutions of 30% FCS in IMDM and conditioned medium from GM-CSF producing R1 cells for 15 hrs at 100,000g. On day 14, cells were harvested, counted, washed twice in PBS containing 1% vesicle-depleted FCS, and finally resuspended in PBS/1% FCS at a concentration of $15*10^6$ cells/ml. Vesicle-depleted FCS was obtained by 65 min centrifugation at 100,000g (Beckman Coulter Optima L-90K using a SW28 rotor).

Preparation of murine reference EV for spiking of milk samples

Murine EV were obtained by cognate co-culture of the immature DC-line D1 with p53-specific $CD4^+$ T cells derived from a C57BL/6 $p53^{-/-}$ mouse, as described previously[32]. Briefly, supernatant was harvested after 20 hours of co-culture and centrifuged 2 x 200g and 2 x 500g for 10 minutes, 10,000g for 30 minutes (Beckman Coulter Avanti J-26 XP using a JA-12 rotor), and 100,000g for 65 min (Beckman Coulter Optima L-90K ultracentrifuge using a SW28 rotor). All centrifugation steps were performed at 4°C. The 100,000g pellet was resuspended in PBS supplemented with 0.2% BSA which had been ultracentrifuged O/N at 100,000g. This EV suspension was used immediately for spiking of breast milk samples. For spiking of 1 ml of breast milk, reference EV derived from co-cultures of $1.75*10^6$ DC with $1.75*10^6$ T cells were used. Recovery of these reference EV was calculated as a percentage of the total number of reference EV in control conditions in each individual experiment.

Breast milk EV isolation

Fresh breast milk samples were spiked with murine reference EV and gently inverted to mix or were left unspiked. Milk was centrifuged at RT for 10 minutes at 3000g (Beckman Coulter Allegra X-12R) to remove cells and cream layer (Supplementary figure 1). Supernatant was then transferred to new tubes and centrifuged again at 3000g at RT to remove remaining cells and cream. The supernatant was then either immediately processed or frozen at -80°C. If frozen milk samples were used, they were thawed in a 37°C water bath until a small clump of ice remained and used immediately. The fresh or thawed milk supernatants were subsequently transferred to polyallomer SW40 tubes (Beckman Coulter) and centrifuged at 5000g for 30 minutes at 4°C to remove larger cell debris and residual cream, and subsequently at 10,000g for 30 minutes at 4°C to remove smaller cell debris. For top-down ultracentrifugation (Supplementary figure 1, left side), 6.5 ml of the cleared 10,000g milk supernatant was loaded on top of a sucrose density gradient in an SW40 tube. This density gradient was prepared by layering successive sucrose solutions (15 fractions of 350 µl) of decreasing density (2.0 M-0.4 M) on top of 700 µl 2.5 M sucrose. For bottom-up density gradient ultracentrifugation (Supplementary figure 1, right side), 9.0 ml of the cleared 10,000g milk supernatant was layered on top of a 2-step cushion of 2.0 M and 0.74 M sucrose in an SW40 tube and centrifuged at 100,000g for 125 min. The interphase between the sucrose layers was harvested using a 21G syringe, diluted with PBS/0.1% O/N ultracentrifuged BSA, and pelleted at 100,000g for 65 min. The pellet was resuspended in PBS/0.2% ultracentrifuged BSA, mixed with 1.5 ml 2.5 M sucrose, and overlaid with sucrose solutions (15 fractions of 750 µl) of decreasing density (2.0 M-0.4 M). Both top-down and bottom-up density gradients were ultracentrifuged for 14-21 hours at 192,000g (Beckman Coulter Optima L-90K with a SW40 rotor). Density fractions of 0.5 ml (top-down) or 1 ml (bottom-up) were collected from the bottom of the tube in Eppendorf tubes. Densities were determined by refractometry.

Cryo-electron microscopy

For cryo-electron microscopy (EM), fractions of indicated densities were pooled and diluted with Annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), supplemented with 0.1% ultracentrifuged BSA. Material was pelleted at 100,000g for 65 min using an SW40 rotor and pellets were resuspended in 50 µl Annexin V-binding buffer/0.1% ultracentrifuged BSA and kept at 4°C prior to Annexin V labeling and processing for cryo EM. For Annexin V labeling, a 7 µL aliquot was mixed with 1 µl Annexin V-conjugated gold nanoparticles at $2*10^{16}$ particles/l[33] and 1 µl 2 mM $CaCl_2$ and incubated for 15 min at RT. Then, a 4 µL aliquot was deposited onto an EM grid coated with a perforated carbon film (Ted Pella, Redding, CA, USA), the excess liquid was blotted off with filter paper, and the grid was quickly plunged into liquid ethane using a Leica EM-CPC cryo-chamber. EM grids were stored in cryo-boxes under liquid nitrogen until use, then mounted in a Gatan 626 cryo-holder and transferred in a Tecnai F20 microscope operated at 200 kV. Images were recorded with an USC1000-SSCCD Gatan camera.

SDS-PAGE, Coomassie staining, and western blotting

Density fractions were either pooled or processed individually, as indicated. Fractions were diluted with PBS/0.1% ultracentrifuged BSA and pelleted at 100,000g for 65 min in a Beckman Coulter Optima L-90K, using a SW60 rotor for individual fractions and a SW40 rotor for pools. Pellets were resuspended in non-reducing SDS-PAGE sample buffer, heated at 100°C for 3 minutes and run on a 4 – 20% TGX-Criterion gel (Bio-Rad, Hercules, CA, USA). Gels were stained with Coomassie brilliant blue solution or transferred to PVDF membranes and blocked in PBS containing 0.2% fish skin gelatin and 0.1% Tween-20. Proteins were detected by immunoblotting using rat anti-mouse CD9 (KMC8, eBioscience, Vienna, Austria), rat anti-mouse CD63 (R5G2, MBL, Woburn, MA, USA), rabbit anti-mouse MHC class II (polyclonal, Genscript, Piscataway, NJ, USA), mouse anti-human CD9 (HI9a, BioLegend, Fell, Germany), mouse anti-flotillin-1 (clone 18, BD Biosciences, San Jose, CA, USA), mouse anti-HLA-DP/DQ/DR (CR3/43, Dako, Heverlee, Belgium), mouse anti-human CD63 (TS63), rabbit anti-human β-casein (polyclonal), rabbit anti-human lactoferrin (polyclonal), and rabbit anti-human α-lactalbumin (EPR12460, all from Abcam, Cambridge, UK). Secondary antibodies goat anti-mouse-HRP (Jackson Immuno Research, Suffolk, UK), rabbit anti-rat-HRP, and goat anti-rabbit-HRP (both from Dako) were used. Labeled antibodies were detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Landsmeer, Netherlands). Protein levels were quantified by densitometry using a ChemiDoc XRS and Quantity One Basic software V4.6.9 (Bio-Rad).

Cellular viability assessment

Fresh breast milk was spiked with murine bone marrow derived dendritic cells (BMDC) at a final concentration of $0.75 * 10^6$ cells/ml or was left unspiked. Milk was divided into equal volumes and was either processed immediately or stored at -80°C, 4°C, RT or 37°C for the indicated time periods. Breast milk was centrifuged at 3000g for 10 minutes. Cell pellets were resuspended and

washed twice in IMDM containing 5% FCS and centrifuged at 500g for 10 min. Cell pellets were resuspended in PBS/1% FCS. Cellular viability was determined by trypan blue exclusion.
Alternatively, cells were incubated with APC-conjugated anti-mouse MHC class II (M5/114, eBioscience) or the corresponding isotype control, or Alexa488-conjugated Annexin V (Life Technologies, Bleiswijk, Netherlands) and propidium iodide (PI; eBioscience) as indicated by manufacturer. Cells were analyzed using a FACS Canto II and FACS Diva software (BD Biosciences), and FCS Express software (De Novo software, Los Angeles, CA, USA). Murine cell viability was determined by gating on MHC class II positive cells, and determining the percentage of Annexin V and/or PI positive cells.

Storage of milk supernatant cleared from cells and cream

Fresh breast milk was spiked with murine reference EV, as indicated above, and gently inverted to mix. Milk was centrifuged at 3000g for 10 minutes at RT. Subsequently, the cream layer was removed and the supernatant was transferred to a new tube. Supernatant was then centrifuged again at 3000g for 10 minutes at RT, after which milk was divided into equal volumes and stored for 2-8 weeks at -80°C.

Statistics

Normal distribution of data was determined using the Shapiro-Wilk normality test. Normally distributed data were analyzed by one sample t-test, relative to control values, which were set to 100%, otherwise a Wilcoxon signed-rank test was used. Normally distributed paired data were analyzed using a paired t-test. If multiple parameters were compared, one-way ANOVA was applied, followed by Tukey's posthoc test. Significance was defined as $*p < 0.05$ and $** p < 0.01$, and calculated with GraphPad Prism software V6.02.

Results

Differential centrifugation followed by top-down density gradient ultracentrifugation allows efficient density separation of EV from breast milk

Differential (ultra)centrifugation followed by density gradient ultracentrifugation is a common protocol used for EV isolation from cell culture supernatant[34,35]. Although this procedure has been used in previous studies on breast milk EV, the efficiency with which EV can be isolated from milk using this procedure has not been assessed. Determining such efficiency, however, is hampered by the lack of knowledge on markers characteristic for milk EV, and by the large differences in milk composition observed between individuals and even between feeds of the same individual[36–38]. In order to optimize EV isolation from breast milk, fresh human milk was spiked with a known population of murine EV (hereafter referred to as ref-EV) as an internal standard, prior to the centrifugation procedure. These ref-EV consisted of EV released during *in*

vitro co-culture of murine DC with T cells, which were previously found to be enriched in CD9 and CD63 ([32,39] and data not shown). Based on earlier observations that characteristics such as size (~50-150 nm) and density (1.12 – 1.18 g/ml) appear common to EV throughout species and body fluids, we assumed that the ref-EV and human breast milk EV behaved similarly with regard to sedimentation and density separation[40].

First, the spiked breast milk was subjected to the conventional protocol of differential centrifugation, in which unwanted components are removed in low/intermediate speed centrifugation steps prior to pelleting EV at 100,000g[34,35]. An initial centrifugation step of 3000g was applied to clear the milk from cells and cream layer. In comparison to the more conventional initial centrifugation at lower speed (500g), this higher g-force led to more efficient separation of the cream layer from the milk supernatant, whereas the efficiency of cell pelleting and the viability of these cells were similar (data not shown). The milk supernatant was additionally centrifuged at 5000g and 10,000g to further remove fat and cellular debris. Following 100,000g centrifugation, we observed that the EV-containing pellet was too solid to resuspend in PBS or SDS sample buffer, which prevented further processing and reliable analysis of these EV. The inability to resuspend the 100,000g pellet could be due to highly abundant milk proteins, such as whey and casein, which can complex and sediment at 100,000g, forming a compact protein matrix[19]. In order to remove such large protein complexes prior to 100,000g pelleting, the 10,000g milk supernatant was centrifuged into a 0.74 M/2.0 M sucrose cushion (Supplementary figure 1). The EV-containing interphase was harvested, and, after centrifugation at 100,000g, the EV containing pellet could be resuspended. This material was overlaid with a sucrose gradient, after which EV were separated based on buoyant density in a 'bottom-up' approach (Supplementary figure 1). Next, the distribution and quantity of recovered ref-EV was assessed by western blotting for mouse CD9 (mCD9) and CD63 (mCD63). Based on previous studies in our lab, the murine ref-EV were expected to equilibrate at densities of 1.12 – 1.18 g/ml[32,39,41]. The antibodies used to detect mCD9 and mCD63 did not cross-react with proteins in human milk (data not shown). Although mCD9 was observed in the expected density fractions (1.12 – 1.18 g/ml), a substantial amount of the murine EV-associated protein stayed behind in the high density fractions (1.25 – 1.28 and 1.20 – 1.23 g/ml; Figure 1A). This indicates that the bottom-up approach for density-based purification of EV from milk is not efficient.

Next, we tested whether the ref-EV could be efficiently purified and recovered from milk using top-down density gradient ultracentrifugation (Supplementary figure 1). In this approach, the EV-containing 10,000g supernatant was applied directly on top of a density gradient, thereby circumventing the 100,000g EV pelleting step. Following top-down density gradient ultracentrifugation, most of the mCD9 was present in density fractions 1.13-1.18 g/ml to which the ref-EV were expected to float (Figure 1A). In addition to the 23 and 27 kD monomeric mCD9, high molecular weight complexes (~150 kD) of mCD9 were detected after top-down density gradient ultracentrifugation. These complexes could correspond to CD9 coupled to the β1-integrin subunit[42].

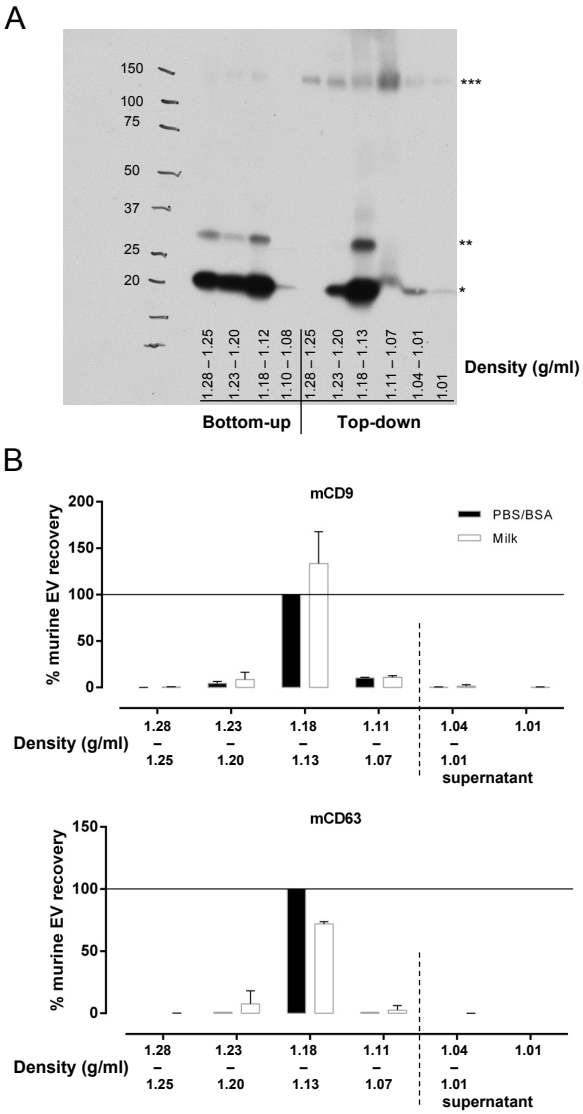

Figure 1: Efficient isolation of reference EV from breast milk by top down density gradient ultracentrifugation. (A) Fresh human breast milk was spiked with murine ref-EV, after which EV were recovered via bottom up or top down density gradient ultracentrifugation. The distribution of recovered ref-EV over the different density fractions was analyzed by western blotting for murine CD9 (mCD9). Indicated are the sizes for monomeric mCD9 (*, ~23 kD), N-glycosylated monomeric mCD9 (**, ~27 kD), and high molecular weight complex mCD9 (***, ~150 kD). This result is representative of 4 independent experiments in 3 different donors. (B) Fresh human breast milk (□) or PBS/BSA control solution (■) were spiked with murine ref-EV, after which EV were recovered via top down density gradient ultracentrifugation. Fractions of the density gradient and the overlying milk supernatant were analyzed for the presence of mCD9 and mCD63 by western blotting. Indicated are the quantified western blot signals (mean values of 2 independent experiments in 2 different donors) normalized to the values detected in the 1.13 – 1.18 g/ml fractions of the PBS/BSA control (set to 100%).

Next, we investigated to what extent the complex composition of breast milk affected the efficiency with which murine ref-EV could be recovered. The ref-EV were spiked in either milk or PBS/0.1% BSA, after which differential centrifugation and top-down density gradient ultracentrifugation were used to isolate EV. The quantity of ref-EV recovered from the two different fluids was determined by western blotting for mCD9 and mCD63. A similar distribution of mCD9 and mCD63 was observed over the different density fractions for EV recovered from breast milk and PBS/BSA, with the majority of signal detected in the 1.13 – 1.18 g/ml density fraction (Figure 1B). The quantity of mCD9 recovered from both solutions was similar, whereas slightly less CD63 was recovered from milk compared to PBS/BSA (Figure 1B). This could be explained by the disparate presence of CD9 and CD63 on different subsets of the ref-EV sample. Based on these data, we conclude that top-down gradient ultracentrifugation is an efficient method for isolation of EV from breast milk.

Native breast milk EV are heterogeneous in size and composition

To confirm that the top-down procedure described above allowed isolation of EV naturally present in human breast milk, unspiked milk samples were subjected to top-down gradient ultracentrifugation and analyzed for the presence of human EV-associated proteins. Western blot analysis of human CD63 (hCD63), human CD9 (hCD9), flotillin-1, and HLA class II showed that these proteins were present in the same density fractions as the mouse reference EV (Figure 2A). Next, we investigated in more detail whether our top-down procedure separated milk EV from milk-specific proteins known to be abundantly present in breast milk. For these experiments, individual gradient fractions rather than pools of fractions were tested. Coomassie blue staining of proteins in the different gradient fractions showed that differently sized proteins distributed unequally over the gradient fractions, with some proteins being predominantly present in the high density bottom fractions and some in the EV containing fractions at lower densities (Figure 2B). Western blot analysis was performed to investigate the presence of the three major milk proteins β-casein, lactoferrin, and α-lactalbumin in the different gradient fractions (Figure 2C). To visualize the location of EV within the gradient, the fractions were also stained for the EV-associated markers HLA class II, hCD63, and hCD9. We observed that β-casein was predominantly present in the high-density fractions of the gradient. In contrast, α-lactalbumin did not migrate into the density gradient, but was abundantly present in the supernatant on top of the gradient (data not shown). The majority of lactoferrin was also present in this supernatant. Interestingly, a small amount of lactoferrin was detected in the EV-containing density fractions (Figure 2C). These data indicate that milk proteins that easily form aggregates or micelles, such as β-casein, are high in density and sediment towards the bottom of the gradient. Smaller soluble proteins, such as α-lactalbumin and lactoferrin, do not migrate into the density gradient, although some lactoferrin could be detected in EV.

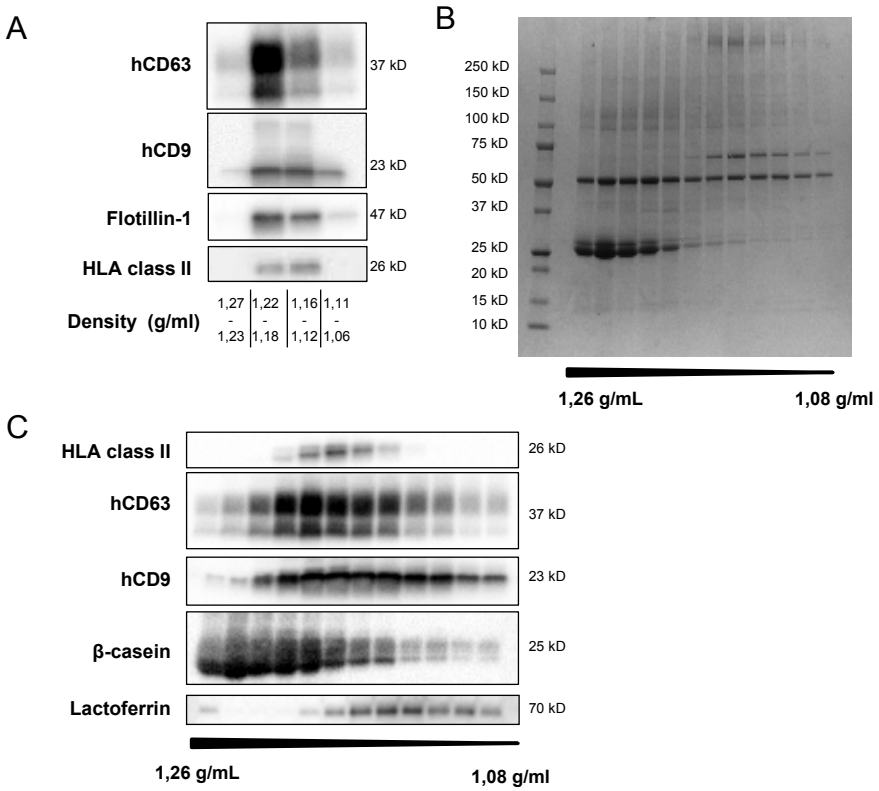

Figure 2: Unequal distribution of native breast milk EV and major milk proteins over density gradient fractions.
Fresh, unspiked human breast milk was subjected to top down density gradient ultracentrifugation, after which the material in the different density fractions was harvested and analyzed. (A) Density fractions were pooled as indicated and analyzed for the recovery of native human breast milk EV by western blotting for human CD63 (hCD63), CD9 (hCD9), flotillin-1, and HLA class II. Results for hCD9 and HLA class II are representative of > 10 independent experiments in > 8 different donors; results for hCD63 and flotillin-1 represent 2 independent experiments in 2 different donors (B, C) The protein content of individual density fractions was assessed by (B) Coomassie blue staining (representative of 2 different donors) and by (C) western blotting for HLA class II, hCD63, hCD9, β-casein, lactoferrin, and α-lactalbumin (representative of 3 different donors).

To visualize and further characterize the size and morphology of breast milk EV in the low-density fractions, EV were labeled with Annexin V-conjugated gold nanoparticles and analyzed by cryo electron microscopy (EM). Both the 1.12-1.18 and 1.18-1.21 g/ml density fractions contained EV, which appeared as spherical structures within the porous carbon film (Figure 3A – D). The ratios between Annexin V-positive and -negative EV populations were similar in both densities (Figure 3A – D). Furthermore, the ratio between small (< 200 nm) and large (> 200 nm) EV was ~ 4:1 in the 1.12-1.18 g/ml fraction, and ~2:1 in the 1.18-1.21 g/ml fraction. In addition to EV, the 1.12-1.18 g/ml fractions contained tubular structures (Figure 3E – G). These tubules did not stain with Annexin V, were enclosed by a lipid bilayer, and had a smooth appearance. The tubular structures

did not contain striations typical for the presence of actin filaments and were approximately 5 times more prominent in the 1.12-1.18 g/ml as compared to the 1.18-1.21 g/ml fraction. However, it should be noted that quantitative analysis of EM samples is difficult due to potential bias for larger objects, which are more easily retained within the perforated carbon film. The data presented here are based on assessing 50-100 EV per density fraction. Results were comparable in two different donors. Taken together, these data suggest that a heterogeneous population of native breast milk EV can be isolated using top-down gradient ultracentrifugation.

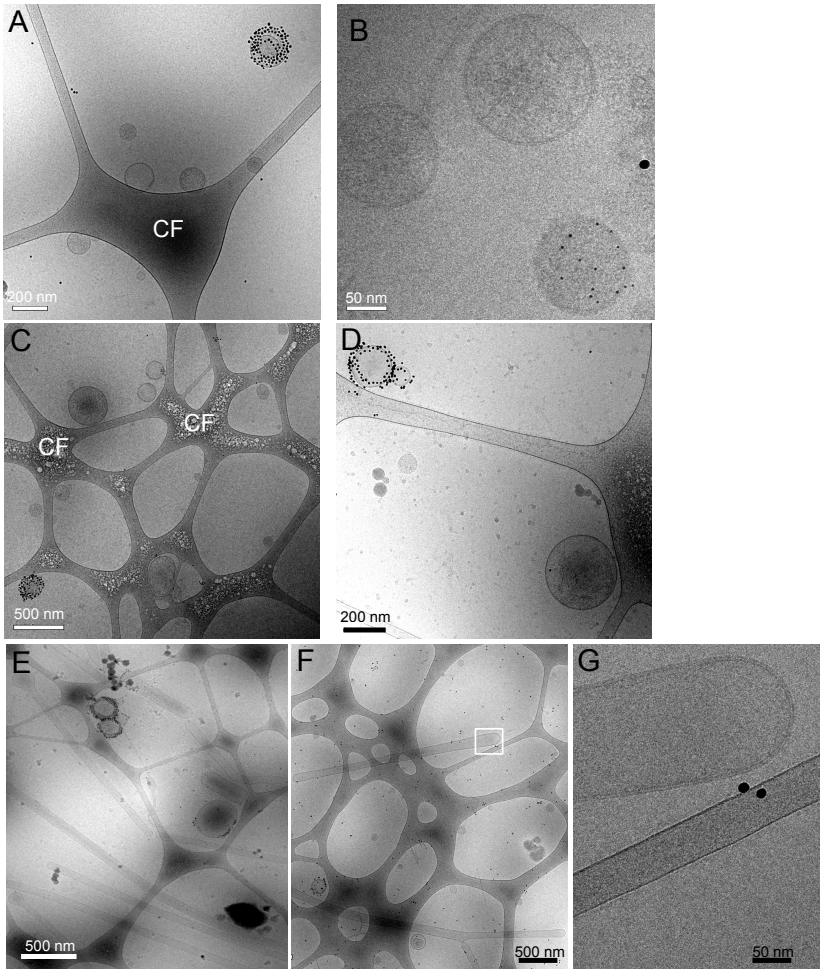

Figure 3: Native breast milk EV are heterogeneous in size and composition.
Fresh, unspiked human breast milk was subjected to top down density gradient ultracentrifugation, after which the material from density fractions 1.12 – 1.18 and 1.18 – 1.21 g/ml was labeled with Annexin V-conjugated gold nanoparticles and analyzed by cryo EM. (A, B, C, D) Representative and close up images of Annexin V positive and negative EV in the (A, B) 1.12 – 1.18 g/ml and (C, D) 1.18 – 1.21 g/ml density fraction. (E, F) Representative images of smooth tubular structures predominantly present in the 1.12 – 1.18 g/ml fraction, with (G) close up image of indicated field in (F). T = tubular structure; CF = carbon film. Data are representative of 3 independent experiments and 2 different milk donors.

Storage of breast milk samples induces milk cell death and contamination of the naturally present EV population

Breast milk is often stored in the refrigerator (4°C) or freezer (-20 or -80°C) for the purpose of feeding the infant, as well as for research. Here, the effects of breast milk storage on milk-derived cells and EV were investigated.

Fresh breast milk was either processed immediately (within 20 min after collection) or stored for two hours at -80°C or 4°C (Figure 4A,B) prior to processing. First, the number of viable human breast milk cells in fresh versus stored milk was assessed. The number of viable cells present in breast milk immediately after collection was largely donor-dependent with a mean cell count of 2.2 ± 2.1* 10^5 cells/ml (mean ± SD). As expected, the number of viable cells was strongly decreased in milk stored at -80°C (Figure 4A). Remarkably, however, the number of viable cells also declined significantly after storage for two hours at 4°C (Figure 4B). Although the strength of the effect varied between milk samples of different donors, the reduction in milk cell viability upon cold storage was observed in all milk samples tested. The decrease in cell viability during storage at above zero temperatures was not specific for 4°C, since storage for two hours at room temperature or 37°C induced similar levels of cell death (Supplementary Figure 2).

Cell death and consequent formation of cell debris can affect the composition of the total pool of milk EV. However, unique markers to distinguish (apoptotic) vesicles released by dying cells from EV actively released by viable cells are lacking. To determine whether cell death induced by cold storage led to contamination of the milk EV population, fresh breast milk samples were spiked with *in vitro* cultured dendritic cells of murine origin (hereafter referred to as ref-cells). The milk was then either processed immediately or stored at -80°C. First, the percentage of ref-cell death in breast milk in response to freezing was determined by analyzing the number of propidium iodide and/or Annexin V positive cells by flow cytometry. Although the murine ref-cells appeared more robust than the human milk cells, 28 - 50% of the spiked cells died or underwent apoptosis due to freezing (Figure 4C). Next, we investigated whether this storage method led to contamination of the pool of actively released milk EV with vesicles induced upon cold storage of the murine cells in milk. EV were isolated from immediately processed or frozen spiked milk samples and tested for the presence of mCD9 and mMHC class II. Although immediately processed control samples were typically not negative for the murine EV markers, a significant increase in mCD9 (22-225%) and mMHC class II (186-2222%) signals was observed in the milk EV population after storage at -80°C compared to immediately processed milk samples (Figure 4D,E). These data indicate that a newly formed pool of vesicles, released by cells in milk during cold storage, contaminated the pool of EV naturally present in breast milk.

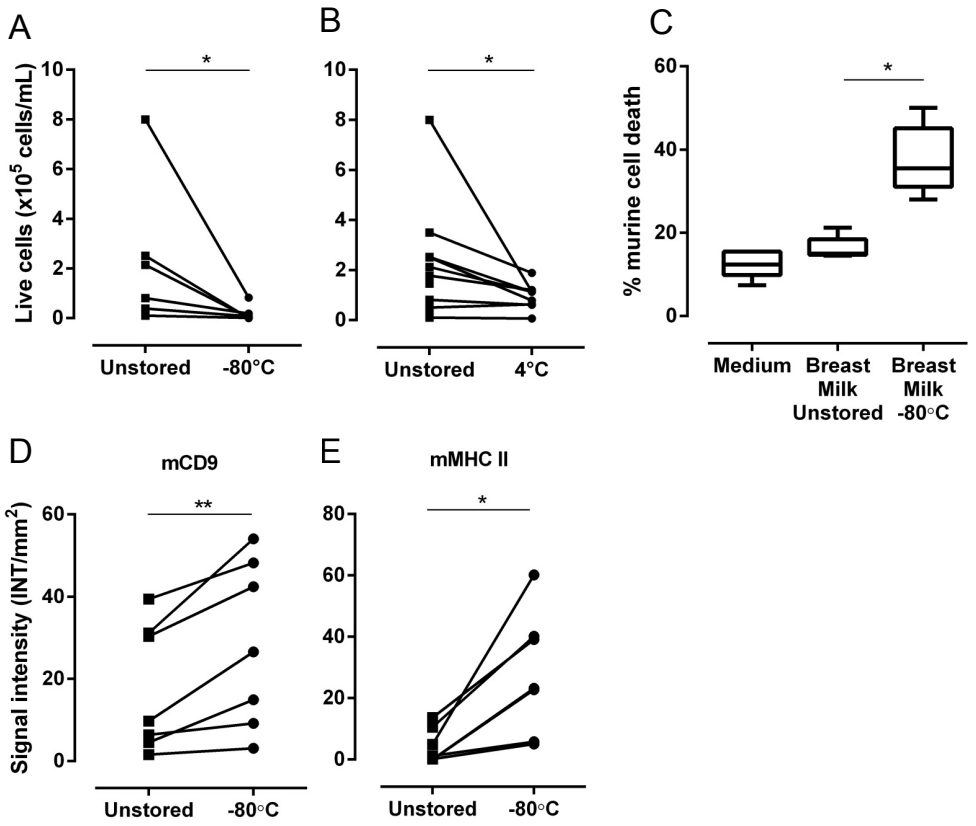

Figure 4: Cell death in stored breast milk samples leads to contamination of the milk EV population. (A, B) Cells were isolated from fresh or stored breast milk and their viability was assessed by trypan blue exclusion. Number of viable cells determined in fresh milk samples and after storage (A) for 2 hours at -80°C (n = 6 different donors) or (B) for 2 hours at 4°C (n = 9 different donors). (C, D, E) Fresh breast milk was spiked with murine cells, after which cells or EV were recovered immediately or after storage at -80°C for ≥ 2 weeks. (C) Flow cytometric analysis of the percentage of dead murine cells in culture medium, or in fresh or stored breast milk samples. Box plots show mean ± SD of 3 independent experiments in 5 donors. (D, E) EV were recovered from breast milk samples by top down density gradient ultracentrifugation. Contamination of the breast milk EV population with vesicles induced upon sample storage was assessed by western blot detection of murine EV markers. Indicated are the quantified western blot signals for (D) mCD9 and (E) mMHC class II (mCD9 n = 7; mMHC class II n = 6). * $p < 0.05$; ** $p < 0.01$.

Freezing milk supernatant cleared of cream and cells is an alternative method for efficient and reliable EV recovery

The release of storage-induced vesicles may be circumvented by clearing milk from cells as soon as possible after milk collection and prior to cold storage. Fresh breast milk spiked with murine ref-EV was cleared from cells and cream layer by two centrifugation steps at 3000g, after which EV were isolated from the milk supernatant either immediately or after storage for at least two weeks at -80°C. The quantity of recovered ref-EV was assessed by western blot analysis of mCD9 and mCD63. No significant differences were found in the amount of mCD9 and mCD63

recovered from freshly processed and stored milk supernatant (Figure 5A,B). These data indicate that freezing milk supernatant devoid of cells and cream layer can be used to store breast milk samples for efficient recovery and reliable analysis of EV at later time points.

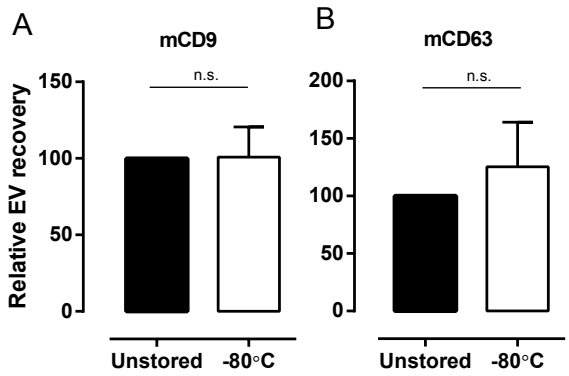

Figure 5: **Milk supernatant cleared from cells and cream can be stored for efficient and reliable recovery of EV.** Fresh breast milk was spiked with murine reference EV and centrifuged twice to remove cells and cream. EV were recovered from milk supernatant before (■) and after (□)storage at -80°C for 2 – 8 weeks and analyzed by western blotting for the presence of (A) mCD9 and (B) mCD63. Indicated are the quantified western blot signals (mean ± SD) normalized to the values detected in unstored milk supernatant (set to 100%). mCD9 n = 5; mCD63 n = 4. N.s. = not significant.

Discussion

We here gained insight into parameters affecting the recovery of EV from (stored) human breast milk samples. Differential centrifugation followed by top-down density gradient ultracentrifugation resulted in efficient and reliable isolation of EV from breast milk. In addition, we showed that freezing of unprocessed milk samples, comprising a variety of milk cell types and cream-forming MFG, led to contamination of the natural milk EV population with EV induced by the storage process. An alternative storage protocol was proposed in which milk supernatant cleared of cells and most of the MFG was frozen for efficient and reproducible recovery of EV in breast milk at later time points.

EV Isolation

Marker proteins for the discrimination of EV, MFG, and cellular debris in human milk are currently lacking. An additional complicating factor in the field of milk research is the high inter- and intra-donor variation with regard to the protein, fat, and cellular content of milk. Hence, the yield of breast milk EV using different EV isolation protocols could not be compared based on detection of human EV-associated markers. To overcome this problem, we spiked breast milk with a well-characterized murine EV population. By profiling defined murine EV-associated proteins, different

protocols could be compared for the recovery of EV from human breast milk samples derived from a range of different donors.

A major difference between the two EV isolation protocols tested here was the presence or absence of a high-speed centrifugation step (100,000g) to sediment EV. In our hands, the 100,000g sedimented material formed a gelatinous pellet that could not be resuspended, as was also observed in several other milk EV studies[13,43]. This gelatinous pellet is most likely caused by condensing of casein micelles and the globular nature of the abundantly present whey proteins, which can form gel-like matrices under high mechanical pressure[44]. Others have applied a filtration step of the milk supernatant prior to the EV pelleting step, and a subsequent overnight incubation step to desolidify the pellet[9]. However, using filtration to remove large protein complexes from the milk supernatant entails a high risk for selective loss of EV subpopulations. As an alternative approach, we have cleared the 10,000g breast milk supernatant of abundant protein complexes using a sucrose cushion. This step was followed by a 100,000g EV pelleting step and bottom-up floatation of EV into a density gradient. Using this protocol, however, a substantial amount of EV still did not efficiently reach their expected buoyant density of 1.12-1.18 g/ml. This could be explained by trapping of EV within residual protein complexes that still contaminated the EV pellet. This bottom-up protocol, including the sucrose cushion, was compared to top-down density gradient ultracentrifugation. In this case, the 10,000g milk supernatant was applied directly on top of a density gradient, thereby circumventing the 100,000g pelleting step. Using this procedure, the majority of spiked murine EV reached their expected buoyant density. Moreover, recovery of the spiked EV from breast milk samples was as effective as from PBS, indicating that the complexity of milk did not hamper quantitative recovery of EV from milk.

The use of spiked reference murine EV allowed close monitoring of the efficiency of different protocols for isolation of EV from milk. However, we cannot fully exclude that spiked murine EV and naturally occurring human breast milk EV behaved differently in these procedures. Nevertheless, we confirmed the presence of native human breast milk EV in the same density fractions as the spiked reference murine EV using western blot and cryo EM (Figures 2 and 3). Interestingly, the breast milk EV floating to the different densities contained relatively different amounts of HLA class II, hCD63 and hCD9. The different distributions of these EV-associated proteins over the gradient, which has also been observed for EV derived from tumor cells[45], indicate that the protein composition of breast milk EV is heterogeneous. The majority of major milk proteins separated from EV, either by sedimentation towards the bottom of the gradient or because their nature prohibited movement into the gradient. A minor proportion of beta-casein and lactoferrin co-localized with EV markers. Whether this denotes small-scale contamination of the EV fractions with these proteins or whether a small amount of these proteins is physiologically associated with EV is currently unknown.

By using cryo EM analysis, we demonstrated that the human breast milk EV population was very heterogeneous with regard to size, electron density, and Annexin V labeling. With regard

to Annexin V labeling, which detects exposed phosphatidylserine (PS), EV were either highly positive or fully negative. This difference in apparent loss of phospholipid asymmetry in the lipid bilayer of these two subtypes of EV might reflect a difference in their biogenesis. Besides EV, lipid bilayer enclosed tubules were identified, which were markedly enriched in the 1.11-1.18 g/ml density fraction (Figure 3E-G), while being almost absent in the higher density (1.18-1.21 g/ml) EV-containing fractions and the lowest density fractions (1.10-1.04 g/ml, data not shown). These tubules were homogenous in diameter (~100 nm) and negative for Annexin V staining. Their smooth appearance suggests a lipid core, as striations typical for actin-like filaments were lacking. The tubes observed in human breast milk are distinct from tubular structures observed in platelet-free plasma[46] and are currently under further investigation.

Storage of milk samples

Until now, most studies on human and bovine milk EV have utilized frozen or refrigerated unprocessed milk[9–12,21–24]. Although the effects of sample storage on recovery of EV from blood plasma has been well documented (reviewed in 16), storage effects on EV in milk have not been studied in detail. We here showed that storage of unprocessed milk, especially at subzero temperature, led to massive death of milk cells. Spiking human breast milk with cells of murine origin allowed us to monitor the induction of storage-induced EV. Upon storage of spiked milk samples we found a large increase in the recovery of murine EV corresponding to an increase in murine cell death. These data indicated that storage-induced stress or death of cells in milk induced EV formation, thereby contaminating the pool of native milk EV. Despite thorough washing of the murine cell preparations used to spike milk samples, low levels of murine EV could also be recovered from fresh spiked milk samples. This could be explained by rapid stimulation-induced release of EV[47] caused by mechanical stress imposed on cells during harvesting from the *in vitro* cultures. Alternatively, dying or dead cells present in the murine cell population at the time of spiking could have led to detectable contamination in the EV population.

In contrast to the predictable death of cells caused by freezing of milk samples, the large-scale cell death detected in milk stored at RT and 4°C was unexpected (Figure 4A-B, Supplementary Figure 2). This cell death may be caused by the action of multiple proteases and lipases, with bile-salt-stimulated lipase (BSSL) being one of the most prominent lipases in milk. It has been described that BSSL loses its dependency on bile salt at low temperature, rendering it constitutively active[48,49]. This could cause cells in milk to lose their membrane integrity and die. Besides cells being a source of storage-induced EV, contamination of the EV population might also occur by storage-induced MFG fragmentation. Although the withdrawal, processing and storage of body fluids will always affect the fluid composition to a certain extent, these effects should be kept to a minimum to allow for accurate analysis of EV naturally present in body fluids. Previous studies demonstrated that it is difficult to discriminate EV from apoptotic bodies and cell debris based on protein profile. Although some proteins (e.g. histones) are more abundant in apoptotic bodies, a large

number of proteins abundantly present in EV from healthy cells, such as MHC class II, also occur in apoptotic bodies and cell debris[29]. Together with our results, this emphasizes the necessity to remove potential sources of storage-induced EV, such as cells and MFG present in milk, as soon as possible after collection of the body fluid. The impact of our findings on the conclusions drawn in previous studies on stored breast milk samples is unknown. Some of the observed effects in these studies could have been due to cell debris, while other EV-specific effects could have been missed. In conclusion, the data presented here provide new insights into storage and isolation methods for breast milk EV. This knowledge can be used to further elucidate the role of breast milk EV in development of the infant immune system and for EV-based biomarker studies in breast milk.

Acknowledgements

We thank H. de Boer-Brouwer for technical assistance, Dr. P. Vos, Prof. Dr. W. Stoorvogel, and Dr. R. Wubbolts for valuable discussions.
This research was performed as part of project 11676 within the framework of a partnership program jointly funded by Nutricia Research and the Dutch Technology Foundation STW, which is part of the Netherlands Organization for Scientific Research (NWO), and is partly funded by the Ministry of Economic Affairs.

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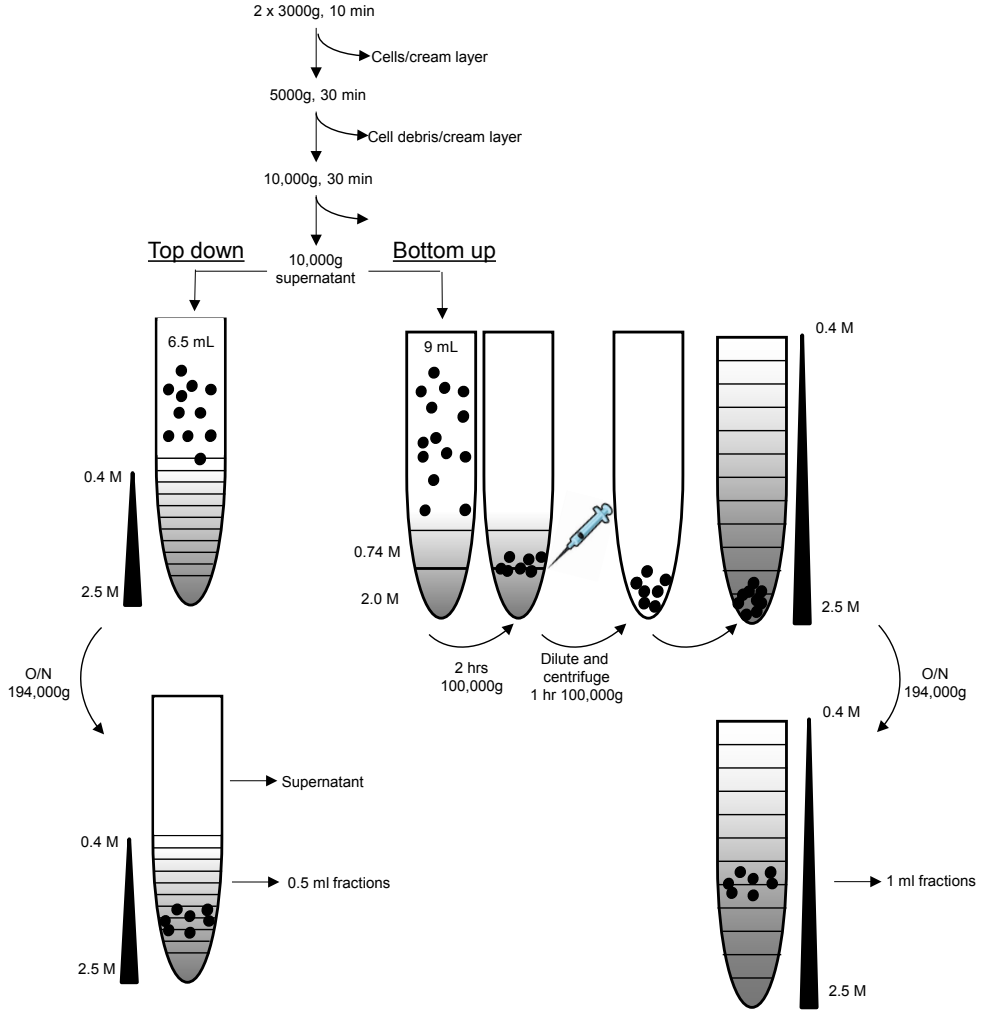
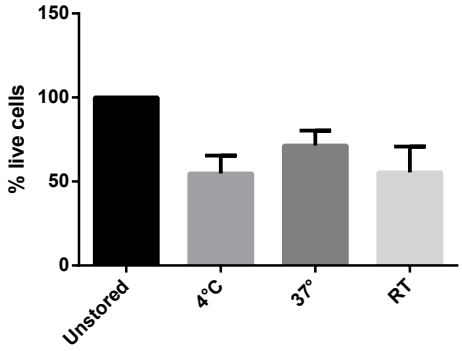


Supplementary Figure 1: Workflow for isolation of EV from breast milk using top down or bottom up density gradient ultracentrifugation. Fresh breast milk was subjected to consecutive differential centrifugation steps in order to remove cells, cell debris and the cream layer. For top down density gradient ultracentrifugation (left), the 10,000g supernatant was loaded on top of a sucrose gradient and centrifuged overnight, after which the different density fractions were harvested. For bottom up density gradient ultracentrifugation (right), the 10,000g supernatant was first centrifuged through a sucrose cushion. The material in the interphase was harvested, pelleted, and overlaid with a sucrose gradient. After O/N centrifugation, density fractions were harvested.



Supplementary Figure 2: Storage of breast milk at above zero temperatures causes milk cell death. Fresh breast milk samples were divided in equal volumes and processed immediately or stored for 2 hours at the indicated temperatures. Cell viability was assessed by trypan blue exclusion and normalized to unstored controls (100%). Bars represent mean ± SD of 3 different donors.

Chapter 3

Comprehensive proteomic analysis of human milk-derived extracellular vesicles unveils a novel functional proteome distinct from other milk components

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Molecular & Cellular Proteomics. 15 (11), 3412-3423 (2016)

Abstract

Breast milk contains several macromolecular components with distinctive functions, whereby milk fat globules and casein micelles mainly provide nutrition to the newborn, while whey contains molecules that can stimulate the newborn's developing immune system and gastrointestinal tract. Although extracellular vesicles (EV) have been identified in breast milk, their physiological function and composition has not been addressed in detail. EV are submicron sized vehicles released by cells for intercellular communication via selectively incorporated lipids, nucleic acids and proteins. Due to the difficulty in separating EV from other milk components, an in-depth analysis of the proteome of human milk-derived EV is lacking. In this study, an extensive LC-MS/MS proteomic analysis was performed of EV that had been purified from breast milk of 7 individual donors using a recently established, optimized density-gradient-based EV isolation protocol. A total of 1963 proteins were identified in milk-derived EV, including EV-associated proteins like CD9, Annexin A5 and Flotillin-1, with a remarkable overlap between the different donors. Interestingly, 198 of the identified proteins are not present in the human EV database Vesiclepedia, indicating that milk-derived EV harbor proteins not yet identified in EV of different origin. Similarly, the proteome of milk-derived EV was compared to that of other milk components. For this, data from 38 published milk proteomic studies were combined in order to construct the total milk proteome, which consists of 2698 unique proteins. Remarkably, 633 proteins identified in milk-derived EV have not yet been identified in human milk to date. Interestingly, these novel proteins include proteins involved in regulation of cell growth and controlling inflammatory signaling pathways, suggesting that milk-derived EVs could support the newborn's developing gastrointestinal tract and immune system. Overall, this study provides an expansion of the whole milk proteome and illustrates that milk-derived EV are macromolecular components with a unique functional proteome.

Introduction

Breast milk is a complex body fluid containing a variety of macromolecular components with distinct functions. Breast milk does not only have a nutritional value for the newborn, it also has various bioactive properties, e.g. nurturing of commensal bacteria, killing of pathogens, promoting intestinal barrier function development, and supporting development and balancing of the newborn's immune system[1–3]. The beneficial effect of breastfeeding is reflected by the observation that breast milk intake results in a reduced risk for developing allergic disorders[2,4]. Although these functional aspects of breast milk have been observed, it is not yet clear which macromolecular components in milk perform these functions and via which molecular pathways they exert these effects.

The composition of milk is generally described as a mixture of macromolecular components which include cells, milk fat globules (MFG), casein micelles and whey[5–8]. Although not mutually exclusive, these major components are often categorized into nutritional components and functional components[9]. The primary nutritional components are MFG, which are the main source of lipids, and caseins, which provide most of the protein. Functionally bioactive properties are mainly allocated to the whey fraction, which contains carbohydrates, antimicrobial proteins and immunomodulatory factors[6–8].

Apart from these well-described macromolecular structures, breast milk contains extracellular vesicles (EV) as well[10–13]. EV are lipid bilayer enclosed vesicles that are released by cells as mediators of intercellular communication[14]. EV released by viable cells are very heterogeneous in composition and size (ranging from approximately 50 nm to >1 μm), with the vast majority <200 nm. EV can originate from either the endosomal route being released from multivesicular bodies as exosomes, or they are released as microvesicles by direct budding from the plasma membrane[15]. Although the biogenesis of exosomes and microvesicles is different, once released into the extracellular milieu (as is the case with breast milk) there are no markers to distinguish these subsets of vesicles[16,17]. The molecular composition of EV (functional proteins, nucleic acids and lipids) is regulated by the producing cell and depends on the type and condition of the cell[18]. Although for human milk-derived EV some microRNAs and proteins have been identified[12,19], a detailed characterization of milk-derived EV is lacking. This makes milk-derived EV an understudied macromolecular component of milk.

Proteomic analysis has allowed for the exploration of the protein composition of distinct macromolecular structures. However, in a complex body fluid such as breast milk, which contains a variety of macromolecular components in the presence of a few highly abundant proteins, detection of low abundant proteins is easily obscured. Hence, to unravel the full milk proteome it is essential to define appropriate isolation methods for the individual components followed by high-resolution mass spectrometry. Recently, we developed a protocol to reliably isolate pure EV from other macromolecular structures in milk, allowing in-depth characterization of milk-derived EV[10].

In this study, in-depth proteomic analysis on purified human milk-derived EV from 7 individual donors was performed and the first comprehensive proteome for milk-derived EV was established. We identified 198 novel EV-associated proteins not present in the EV database Vesiclepedia. By comparing the milk-derived EV proteome to the total milk proteome, which was manually constructed using previously published proteomics data of various isolated milk components, 633 proteins were identified in the milk-derived EV proteome that have not been identified in milk previously. Based on GO analysis, these 633 newly identified milk proteins have distinct functions from other milk proteins, indicating that milk-derived EV are a separate bioactive component of breast milk.

Materials & Methods

Breast milk collection

Breast milk was collected as previously described[10]. Briefly, fresh, mature milk samples were collected by 7 healthy mothers (between 3 and 9 months after delivery) who were not actively terminating breast feeding. Additional donor information can be found in Table 1. Milk was prevented from cooling down and transported to the lab in order to start EV isolation within 20 minutes after collection. Informed consent was signed by all donors and this study was approved by the local ethics committee.

Table 1. Information regarding age, stage of lactation (months after delivery) and number of pregnancies to a viable gestational age (parity) of the individual donors. Additionally, volumes of milk collected and amount of 10,000g milk supernatant that was used for further isolation of milk-derived EV and non-floating complexes is given, as described in the experimental procedures.

Donor	Age donor	Months after delivery	Parity	Amount of milk (ml)	Amount of 10,000g sup (ml)
1	35	6	3	10	6,5
2	35	4	1	35	32,5
3	34	4	1	30	32,5
4	32	5	1	33	32,5
5	32	5	1	30	32,5
6	39	9	3	30	32,5
7	34	3	2	30	32,5

Isolation of milk-derived EV and high-density complexes

Isolation of milk-derived EV was done as previously described[10]. Whole milk was centrifuged at RT for 10 minutes at 3,000 g (Beckman Coulter Allegra X-12R, Fullerton, CA, USA) (Fig. 1 A) in which a white pellet and cream layer were formed. The cream layer, containing MFG, was removed and milk supernatant was harvested without disturbing the cell pellet. Supernatant was

transferred to new tubes and centrifuged at 3,000 g at RT. The supernatant was stored at 80°C until further processing. Later, frozen supernatant was thawed and transferred immediately to polyallomer SW40 tubes or SW28 tubes (Beckman Coulter) and centrifuged at 5,000 g for 30 minutes at 4°C and subsequently at 10,000 g. Next, 6.5 ml aliquots of the 10,000 g supernatant were loaded on top of individual gradients in a SW40 tube. For donor 1, a total of 6.5 ml was used, and for donors 2-7 the total volume was 32.5 ml (Table 1). The gradient consisted of 15 successive layered sucrose fractions of 350 µl (ranging from 2.0 M to 0.4 M sucrose, with an decrement of 0,114 M per fraction) on top of 700 µl 2.5 M sucrose. Ultracentrifugation was done at 192,000 g (in a Beckman Coulter Optima L-90K with a SW40 rotor) for 15-18h. After ultracentrifugation, fractions of 500 µl were harvested from the density gradient starting from the bottom of the SW40 tube and collected in Eppendorf tubes. The density of the collected fraction was measured by refractometry in order to identify the fractions containing milk-derived EV (densities of 1.12-1.18 g/ml sucrose) or high-density complexes (bottom 3 fractions with densities 1.23-1.28 g/ml). After collection of all fractions, the residual pellet was resuspended in 500 µl PBS. Fractions containing EV and fractions containing high-density complexes were pooled (the resuspended pellet was added to the high density complex sample). Pooled fractions were transferred to SW28 tubes and diluted with PBS and samples were pelleted at 100,000 g for 65 minutes. After centrifugation, supernatant was removed and pellets were stored at -80°C.

Protein extraction

For protein isolation, pellets were suspended in lysis buffer containing 8 M urea in 50 mM ammonium bicarbonate, 1 tablet of protease inhibitors Complete mini (Roche ref. 11836170001, Sigma, St. Louis, MO, USA) and 1 tablet of PhosSTOP phosphatase inhibitor mixture (Roche ref. 04906845001). Sample sonication was carried out using a UP100H sonicator (Hielscher Ultrasound Technology, Teltow, Germany) for 6 cycles of 1 sec pulse with a 30 sec gap in between at 60% amplitude. Clear supernatant was collected after centrifugation of the sample at 20,000 g for 15 min at 4°C for isolation of soluble proteins.

SDS-PAGE, in gel digestion and western blotting

For high-resolution LC-MS/MS analysis samples were further processed after sonication. For this, 20 µl or 40 µl of each sample was added to 10 µl 200 mM DTT (Sigma ref. 43815) and 10 µl sample buffer (Biorad ref. 161-0791, Hercules, CA, USA) and incubated for 5 min at 95°C. After a short centrifugation step, the samples were separated on a 12% Tris-HCl gel next to a protein ladder (Thermo ref. 10747012, Waltham, MA, USA). The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ (Millipore, Billerica, MA, USA) (Fig. 1B). Each lane was excised in 6 or 7 bands for in gel digestion using trypsin. After extraction with 100% acetonitrile the samples were dried and reconstituted in 40 µl of 10% formic acid/5% DMSO (Sigma St. Louis, MO).

For western blotting (Fig. 4, Fig. 5B and Fig. 6B), 1 ml of the pooled fractions containing EV and pooled fractions containing high-density complexes from donors 4 to 7 were taken and transferred to SW60 tubes (Beckman Coulter) and diluted with PBS. Next, samples were pelleted at 100,000 g for 65 minutes (in a Beckman Coulter Optima L-90K with a SW60 rotor) and pellets were taken up in reducing buffer (containing 350 mM β-mercaptoethanol) for EHD3 detection, or non-reducing sample buffer for the detection of flottilin-1, CD9, OLAH, PTHLH and MPZL1. Samples were loaded on a 4-20% TGX-Criterion gel (Bio-Rad, Hercules, CA, USA) and separated proteins were transferred to PVDF membranes followed by blocking in PBS containing 0.2% fish skin gelatin and 0.1% Tween-20. Proteins of interest were detected by immunoblotting using mouse anti-human flotillin-1 (clone 18, BD Biosciences, San Jose, CA, USA), mouse anti-human CD9 (HI9a, BioLegend, Fell, Germany), rabbit anti-human OLAH (HPA037948, Atlas Antibodies, Stockholm, Sweden), rabbit anti-human PTHLH (PA5-40799, Thermo Fisher, Rockford, USA), mouse anti-human MPZL1 (SAB1406763, Sigma), mouse anti-human EHD3 (ab194512, Abcam, Cambridge, UK). Secondary antibodies used were goat anti-mouse-HRP (Jackson Immuno Research, Suffolk, UK) and goat anti-rabbit-HRP (Dako, Heverlee, Belgium). Labeled antibodies were detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Landsmeer, Netherlands) and blots were imaged using a ChemiDoc XRS (Bio-Rad).

High-resolution LC-MS/MS analysis

Samples were individually acquired in two separate experiments (milk-derived EV or non-floating complexes from donor 1 and 2 in the first experiment. Milk-derived EV or non-floating complexes from donor 3 to 7 in the second experiment). Experiment 1 was analyzed by nanoflow LC-MS/MS using a Proxeon EASY-nLC 1000 (Thermo Scientific, Odense, Denmark) with an analytical column heater (40°C) coupled to an LTQ-Orbitrap Elite. In gel digestion fractions were dried, reconstituted in 10% FA and delivered to a trap column (ReproSil C18, (Dr Maisch GmbH, Ammerbuch, Germany); 20 mm x 100 µm inner diameter, packed in-house (at the Netherlands Proteomics Centre) at 5 µl/min in 100% solvent A (0.1 M acetic acid in water). Next, peptides eluted from the trap column were loaded onto an analytical column (Poroshell 120 EC-C18 (Zorbax, Agilent Technologies); 40 cm length, 50 µm inner diameter, packed in-house) at approximately 100 nl/min in a 60 min gradient from 0 to 40% solvent B (0.1 M acetic acid in 8:2 (v/v) ACN/water). The eluent was sprayed via distal coated emitter tips butt-connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from m/z 350 to 1500) were acquired in the Orbitrap with a resolution of 60,000 Full width at half maximum (FHMW) at 400 m/z using an AGC setting of 1e6 ions. After the survey scans, the 10 most intense precursors were selected for subsequent decision tree-based ion trap CID or ETD fragmentation. Experiment 2 was analyzed using a Q-Exactive instrument (Thermo Scientific, Bremen) connected to an Agilent 1290 Infinity LC system with an analytical column heater (40°C), a trap column of 20 mm x 100 µm ID Reprosil C18 (Dr Maisch)

and a 400 mm x 50 µm ID Poroshell C18 analytical column (Zorbax, Agilent Technologies), all packed in-house. Solvent A consisted of 0.1M acetic acid (Merck) in Milli-Q (Millipore), while solvent B consisted of 0.1M acetic acid in 80% acetonitrile (Biosolve). Trapping was performed at a flow rate of 5 µl/min for 10 min and peptides were eluted using a passively split flow of 100 nl/min for 60 min. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS. For the MS analysis the ten most intense ions in the survey scan (350 to 1500 m/z, resolution 35,000, AGC target 3e6) were subjected to HCD fragmentation (resolution 17,500, AGC target 5e4), with the normalized collision energy set to 25% for HCD. The signal threshold for triggering an MS/MS event was set to 500 counts. The low mass cut-off for HCD was set to 180 m/z. Charge state screening was enabled, and precursors with unknown charge state or a charge state of 1 were excluded. Dynamic exclusion was enabled (exclusion size list 500, exclusion duration 15 s).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[20] partner repository with the dataset identifier PXD004423.

Data processing

MS raw data were processed with Proteome Discoverer (version 1.3, Thermo Scientific). Generated peak lists were searched against the Uniprot database; Homo sapiens (July 2014, 546,121 entries), supplemented with frequently observed contaminants using Mascot software version 2.3 (Matrix Science, UK). Trypsin was chosen as enzyme and two missed cleavages were allowed. Carbamidomethylation (C) was set as a fixed modification and oxidation (M) was set as variable modification. The searches were performed using a peptide mass tolerance of 50 ppm and a product ion tolerance of 0.05 Da (HCD), followed by data filtering using percolator, resulting in 1% false discovery rate (FDR). Only ranked 1 PSMs with Mascot scores >20 were accepted. Keratins were not considered contamination and therefore were not removed from the dataset, since mammary epithelial cells (as possible EV producers) express keratins. The data from this study can be found in Supplemental File 1 and has been submitted to the Vesiclepedia database, http://www.microvesicles.org/ (accession number: Vesiclepedia_574).

Experimental Design and Statistical Rationale

Mass spectrometry was performed on 7 biological replicates with milk-derived EV and high-density complexes, while western blot analysis was performed on 4 biological replicates. Functional enrichment analysis for Gene Ontology (GO) terms and comparison of datasets (Fig. 2, Fig. 3, Fig. 5 and Fig. 6) was done using FunRich (with the human FunRich database as background; as recent as November 2015)[21]. Comparison between this study and EV database Vesiclepedia was done by importing the online protein data from the Vesiclepedia database (as recent as November 2015) into FunRich. This dataset included all proteins listed in Vesiclepedia (from all tissues and cell types; all cell lines; all isolation methods; all detection methods; all vesicle types). GO analysis

of annotated proteins was performed for cellular component, molecular function, biological processes and site of expression. Enriched terms were ranked by p value (Hypergeometric test) by FunRich. GraphPad Prism software (La Jolla, CA, USA) was used to plot the graphs when comparing datasets. In order to show the highest diversity of enriched GO terms, duplicate or highly similar terms were removed, selecting those with the highest statistical power or with the highest number of genes in the background dataset. For cellular component in Fig. 2B and Fig. 6C, GO terms for 11 major cellular components were selected in order to limit results (nucleus, ribosome, vesicle [named exosomes in FunRich], endoplasmic reticulum [ER], Golgi apparatus, cytoskeleton, mitochondrion, cytoplasm, lysosome, centrosome and plasma membrane) and supplemented with extracellular to include secreted proteins. For site of expression analysis, primary cells reported to reside in the mammary gland or cells known to be present in the circulation were selected. Cell lines, other tissues than breast tissue, or fluids were excluded from the analysis. In comparisons for molecular function and biological process between milk proteins or milk-derived EV proteins in Fig. 6C, enriched GO terms that were significant (GO terms with a p value <0.05 corrected for multiple comparisons by hyper geometric test) in at least one dataset were selected and compared to the other dataset. The number of genes in the background dataset was at least 15 in order to remove underrepresented GO terms.

For statistical comparison of datasets within a single GO-term (Fig. 2B and Fig. 6C), the number of proteins linking to the GO term were tested for significant difference in distribution with chi-square test using GraphPad prism software. Datasets with a p value <0.05 were regarded as significant.

Comparison of identified proteins between all 7 individual donors (Table 3) was done using the Venn diagrams web tool from http://bioinformatics.psb.ugent.be/webtools/Venn/.

Construction of the human milk proteome

Previously published raw data was obtained from 8 studies, with 6 primary studies and 2 review studies that contained a total of 38 individual studies. Supplemental File 2 contains the raw data from these 8 studies and Supplemental Table S1 contains an overview of the individual 38 studies. Datasets from Beck et al. and D'Alessandro et al., which summarized previous published work, were used instead of each individual study reviewed[22,23]. Datasets were converted to the common gene name format in order to construct one dataset that could be compared to our dataset. Genes listed under multiple (alternative) names were condensed to the common gene name as listed in the UniProt database. Proteins without a common gene name ("N/A"), or entries no longer existing in the UniProt database, or listed under cDNA entries were excluded from the milk proteome dataset. The common gene names from all studies were imported into FunRich as a dataset and FunRich was used to compare this dataset in a Venn diagram.

Results

Isolation of milk-derived EV and workflow of the proteomics approach

Isolation of milk-derived EV from breast milk of 7 donors was performed using the protocol by Zonneveld et al. (Fig. 1A)[10]. First, fat (containing MFG), cells and cellular debris were removed by differential centrifugation resulting in 10,000g milk supernatant. The milk supernatant was loaded on top of a sucrose gradient and ultracentrifuged to allow for the separation and concentration of EV. This top-down density gradient ultracentrifugation was used since pelleting EV from milk supernatant via 100,000 g centrifugation results in an insoluble pellet[10]. After ultracentrifugation, individual fractions were collected. To prepare the milk-derived EV sample, EV enriched fractions with densities 1.12 to 1.18 g/ml were pooled. Additionally, high-density fractions (density 1.23 to 1.28 g/ml), enriched in non-membrane-associated protein complexes, were pooled and used as a control.

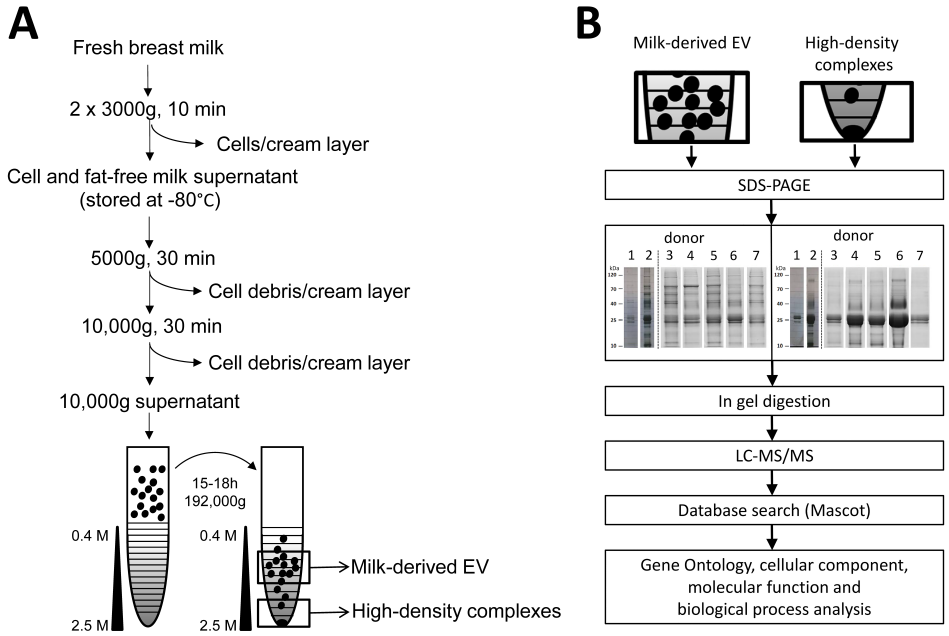


Figure 1. Workflow for the isolation and proteomic analysis of milk-derived EV
A. Workflow for the isolation of milk-derived EV and high-density complexes. Breast milk from 7 donors was processed individually and subjected to differential centrifugation in order to remove live cells, cell debris and cream (containing MFG). Top down ultracentrifugation was performed whereby 10,000 g milk supernatant was loaded onto a sucrose gradient followed by overnight centrifugation. Individual fractions were collected and fractions 6-9 (with a density of 1.12-1.18 g/ml) were pooled to obtain milk-derived EV, while the three bottom fractions plus the pellet were pooled and contained high-density complexes.
B. Workflow for the processing of milk-derived EV or high-density complexes for in-depth proteomic analysis. Individual samples were subjected to SDS page followed by excision of gel bands for trypsin digestion. Dashed lines indicate that donors 1 and 2 were analyzed in a separate mass spectrometry experiment from donors 3 to 7. Peptides were introduced to the mass-spectrometer and identified proteins were subjected to GO analysis.

Next, the isolated milk-derived EV and high-density complexes from the individual donors were further processed (Fig. 1B). Although differences in total protein amount between donors were observed, SDS-PAGE analysis showed large similarities between different donors in the protein patterns obtained for both milk-derived EV and high-density complexes. Milk-derived EV had a more complex banding pattern than high-density complexes, which exhibited a few high abundant proteins (Fig. 1B). For each individual donor, protein bands were excised for in gel digestion and these samples were subjected to LC-MS/MS analysis.

Identification of the milk-derived EV proteome

In-depth proteomic analysis was performed to determine the proteome of milk-derived EV and high-density complexes. The total number of proteins identified was higher for milk-derived EV than high-density complexes. For milk-derived EV, proteins numbered between 650 and 1615, while for high-density complexes this ranged from 194 to 495 individual proteins (Table 2). Remarkably, in the milk-derived EV from donor 2 there were more proteins identified, while this donor was similar to the other donors (Table 1 and high-density complexes in Table 2). In total, 1963 unique proteins were identified in milk-derived EV in all samples, while 739 unique proteins were determined in the high-density complexes (Fig. 2A). To get more insight into the subcellular origin of the identified proteins, functional enrichment analysis for the cellular component was done. These analyses showed that in both milk-derived EV and high-density complexes a high percentage of proteins linked to GO terms like ‘exosomes’ (which are a subclass of extracellular vesicles), ‘lysosome’ and ‘cytoplasm’ (Fig. 2B). We next determined the influence of the overlapping proteins for the enrichment analysis (Fig 2A: 606 proteins). Within the samples, we compared non-overlapping proteins (1357 in milk-derived EV and 133 for high-density complexes) to the proteins identified in the entire sample (1963 for milk-derived EV and 739 for high-density complexes). When the overlapping proteins were excluded for the GO analysis, then especially ‘exosomes’ were significantly lower for milk-derived EV (Fig. 2B, left figure). For high-density complexes especially GO-terms ‘exosomes’, ‘lysosomes’, ‘cytoplasm’ and ‘plasma membrane’ were significantly less enriched when the overlapping proteins were excluded for analysis. This suggests that the overlapping proteins most likely are derived from some high-density EV present in the high-density complexes fractions, while the 133 proteins unique in the high-density complexes are most likely not EV-associated proteins. Therefore, we selected all proteins identified in the milk-derived EV sample (1963) for further analysis.

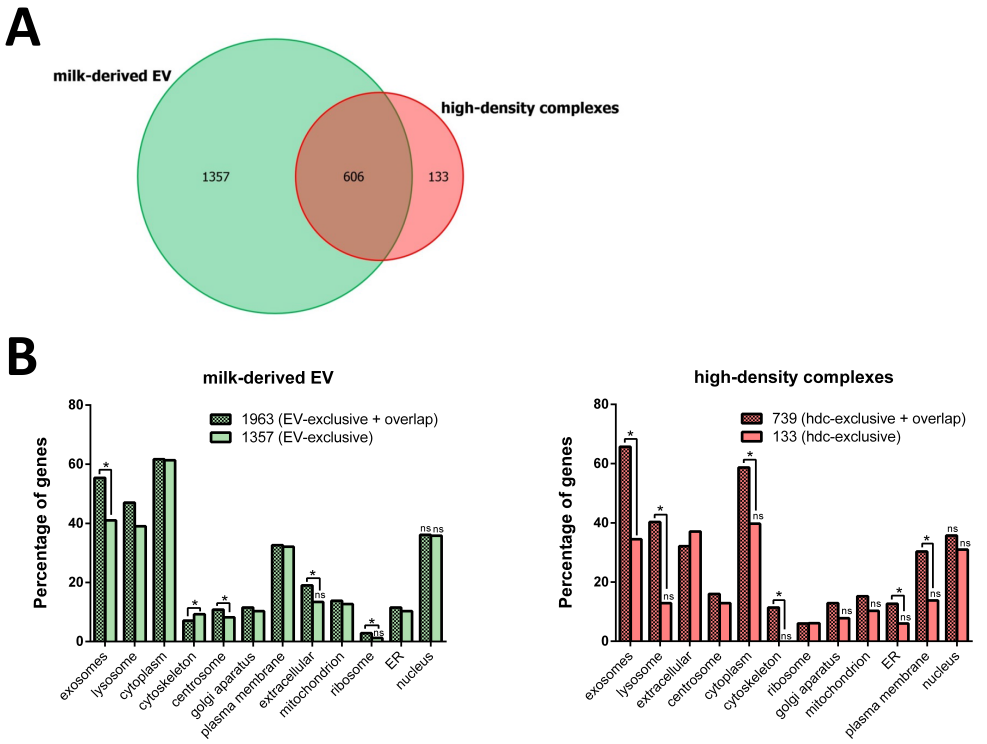


Figure 2. Comparison of the milk-derived EV and high-density complexes proteomes
A. Venn diagram comparing uniquely identified and shared proteins between milk-derived EV and high-density complexes (pooled data from all 7 milk donors).
B. Functional annotations for cellular component of the identified proteins comparing all 1963 proteins identified in milk-derived EV versus 1357 proteins exclusively identified in EV (left figure). Additionally, all 739 proteins identified in the high-density complexes were compared to the 133 proteins exclusively identified in this sample. Enriched terms were ranked by p value (Hypergeometric test) for either dataset. Those GO terms that were not significantly enriched are indicated with "ns". Within individual GO-terms, annotated proteins were compared for distribution via chi-square test. Those datasets that were significantly different are indicated with "*". For both the Hypergeometric test and the chi-square test, a p value <0.05 is regarded as significant.

Table 2. The number of identified proteins in the milk-derived EV samples and the high-density complexes samples per individual donor.

	Number of identified proteins milk-derived EV	Number of identified proteins high-density complexes
Donor 1	857	495
Donor 2	1615	358
Donor 3	817	194
Donor 4	650	157
Donor 5	783	260
Donor 6	814	236
Donor 7	964	304

Identification of the origin of milk-derived EV

In the context of intercellular communication via milk-derived EV between mother and child, it is interesting to consider which maternal cells were the potential producers of milk-derived EV. Apart from cells making up the breast tissue, like mammary epithelial cells and adipocytes, a variety of immune cells have been isolated from breast tissue and milk[5,24] (5, 24) and all these cells could be the source of milk-derived EV. Via GO analysis we determined the site of expression of the identified EV-proteins (Fig. 3). Interestingly, although GO terms for 'breast', 'mammary gland' and 'mammary epithelium' were significantly enriched in the milk-derived EV proteome, the GO terms related to immune cells like 'dendritic cell', 'CD4 T cell', 'platelet', 'monocyte', and 'B cell' were most prominently enriched. These findings indicate that milk-derived EV do not only originate from breast tissue but that immune cells likely contribute to the production of these macromolecular components in breast milk.

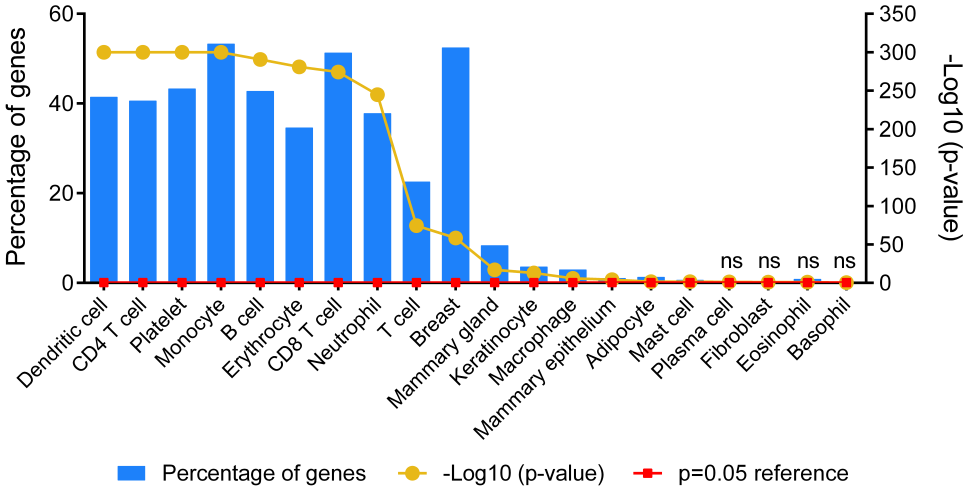


Figure 3. Site of expression of milk-derived EV proteins
The 1963 proteins identified in all 7 milk-derived EV samples were analyzed for the site of expression. The percentage of genes linking to individual GO terms were ranked by p value and are shown, together with the p-value from the Hypergeometric test (-Log10 depicted in yellow) and the reference p=0.05 value (depicted in red). Those GO terms that were not significantly enriched are indicated with "ns".

Identification of common milk-derived EV proteins

Since milk composition is known to vary among individuals we determined the variation of identified proteins in milk-derived EV between individual donors. This revealed that a total of 367 proteins were shared among all 7 donors, which is 19% of the total of 1963 unique proteins in the milk-derived EV proteome (Table 3). Although variation exists whereby some proteins are uniquely identified in specific donors, a substantial number of proteins is shared between the different milk-derived EV samples, indicating that there is a common milk-derived EV proteome.

Importantly, from the proteins shared among all donors, several are considered as EV-associated proteins and are used as markers for EV (as reviewed by Lötvall et al.)[17]. These EV-associated proteins can be categorized according to their subcellular location (Table 4). For instance, transmembrane or lipid-bound extracellular proteins (e.g. tetraspanins CD9, CD63 and CD81), and cytosolic proteins (e.g. annexins and Ras-related proteins) were identified in all milk-derived EV samples, while some intracellular proteins (e.g. histone) that are not expected to be enriched in EV were indeed lacking. In contrast to the milk-derived EV proteome, EV associated proteins are underrepresented or lacking in the proteome of the high-density complexes (Table 4). These data were confirmed by western blotting (Fig. 4), which showed the presence of Flotillin-1 in milk-derived EV while being absent in high-density complexes. CD9 was more abundantly present in milk-derived EV than in high-density complexes.

Table 3. The number of identified proteins shared among donors. The milk-derived EV proteomes from all 7 individual donors were compared in an overlay. From these data the number of shared proteins between donors was calculated. Percentage of total relates to the total of 1963 unique proteins identified in all 7 milk-derived EV samples.

	Number of shared proteins	Percentage of total
Identified in all 7 donors	367	19
Identified in at least 6 donors	527	27
Identified in at least 5 donors	653	33
Identified in at least 4 donors	789	40
Identified in at least 3 donors	954	49
Identified in at least 2 donors	1245	63

Milk-derived EV contain proteins not listed in the Vesiclepedia database

Apart from common EV proteins (Table 4), EV contain subset-specific proteins due to different cells of origin and biogenesis pathways. To identify any known EV proteins in milk-derived EV, we compared the 1963 proteins identified in the milk-derived EV with previously published EV studies included in the (human) Vesiclepedia database[25]. Vesiclepedia contains 8450 unique proteins identified in EV from an extensive number of studies (currently 188 individual human studies) that included the characterization of EV from a wide variety of cellular sources (e.g. bodily fluids, primary cells and cell lines) and vesicle types.

When comparing the overlap we found that 1765 proteins were already reported in Vesiclepedia (which is 90% of the 1963 proteins in the milk-derived EV proteome), however 198 proteins have been identified in this study that have not been listed in Vesiclepedia (Fig. 5A). With the results from this analysis, we can append the current "total" EV proteome to 8648 unique proteins.

Among the proteins that have not previously been reported in other EV were Oleoyl-ACP Hydrolase (OLAH) and Parathyroid Hormone-related Protein (PTHLH). In our proteomic analysis OLAH was detected with a coverage of 27,9% with 5 unique peptides, while PTHLH was detected with a

coverage of 40,7% with 9 unique peptides (Supplemental File 1*). Using western blot analysis we verified the presence of both proteins on all donors tested (Fig. 5B). Interestingly, various isoforms of PTHLH have been identified in human milk[26], which were detected with varying intensities between donors.

Table 4. EV-associated proteins are preferentially identified in milk-derived EV samples. Different categories of proteins are present in EV, according to Lötvall et al. (17). Proteins that were identified in at least 5 out of 7 donors in either sample (milk-derived EV, or high-density complexes) are shown, except for intracellular proteins not expected to be enriched in EV, these are given for all samples.

Proteins	Full name	Milk-derived EV	High-density complexes
transmembrane or lipid-bound extracellular proteins (enriched in EV)			
CD9	CD9 antigen	present (7/7)	present (2/7)
CD63	CD63 antigen	present (7/7)	present (2/7)
CD81	CD81 antigen	present (7/7)	present (7/7)
ICAM1	Intercellular adhesion molecule 1	present (7/7)	present (1/7)
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	Present (7/7)	-
FLOT1	Flotillin 1	present (7/7)	-
GNA11	Guanine nucleotide-binding protein subunit alpha-11	present (7/7)	present (1/7)
GNA13	Guanine nucleotide-binding protein subunit alpha-13	present (7/7)	-
GNAS	Guanine nucleotide-binding protein G(s) subunit alpha	present (7/7)	-
GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha	present (7/7)	-
MFGE8	Lactadherin	present (7/7)	present (7/7)
cytosolic proteins (enriched in EV)			
ANXA2	Annexin A2	present (7/7)	present (6/7)
ANXA4	Annexin A4	present (7/7)	present (2/7)
ANXA5	Annexin A5	present (7/7)	-
ANXA6	Annexin A6	present (7/7)	present (1/7)
ANXA7	Annexin A7	present (7/7)	-
ANXA11	Annexin A11	present (7/7)	-
RAB5A	Ras-related protein Rab-5A	present (7/7)	present (1/7)
RAB7A	Ras-related protein Rab-7A	present (7/7)	present (3/7)
RAB11B	Ras-related protein Rab-11B	present (7/7)	-
RAB25	Ras-related protein Rab-25	present (7/7)	-
RAB35	Ras-related protein Rab-35	present (7/7)	present (2/7)
SDCBP	Syntenin-1	present (7/7)	present (2/7)
intracellular proteins (not expected to be enriched in EV)			
Hsp90B1	Endoplasmin	present (5/7)	present (3/7)
CANX	Calnexin	present (5/7)	present (1/7)
GM130	130 kDa cis-Golgi matrix protein	-	-
CYC1	Cytochrome C	-	-
HIST	Histone	-	-
AGO	Argonaute	-	-

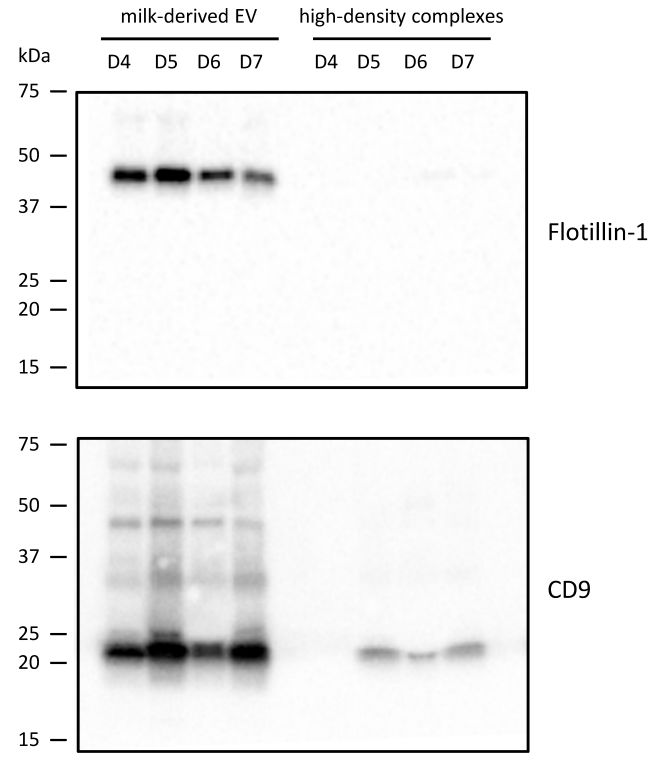


Figure 4. Western blot analysis on EV-associated markers
The presence of flotillin-1 (~48 kDa) and CD9 (~23 kDa) was determined in isolated milk-derived EV or high-density complexes by western blotting for donors 4, 5, 6 and 7.

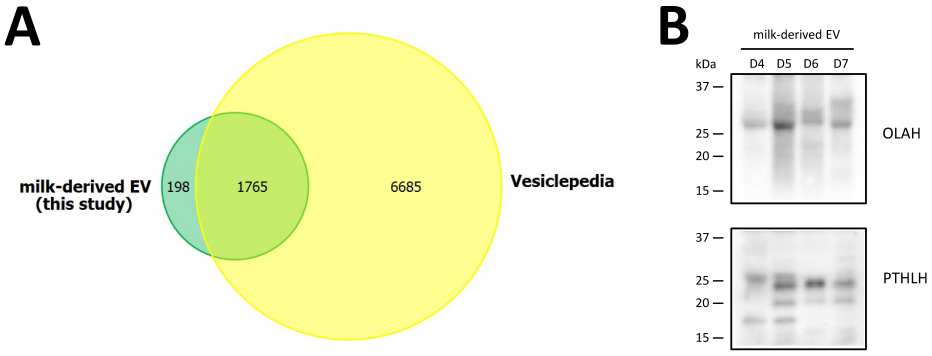


Figure 5. Comparison of the milk-derived EV proteome from this study to the EV database Vesiclepedia
A. All protein entries from Vesiclepedia were downloaded and imported into FunRich for overlay analysis with the milk-derived EV proteome from this study, which is shown in the Venn diagram.
B. Western blot analysis was performed for OLAH (~30 kDa) and PTHLH (~20 kDa and ~24 kDa) on milk-derived EV from donors 4, 5, 6 and 7.

Milk-derived EV contain proteins previously not identified in milk proteomic studies

Breast milk is a complex body fluid that contains a variety of macromolecular structures that harbor proteins. The most frequently studied milk components abundant in proteins are MFG, caseins and whey[6,7,27]. Apart from whole milk and skim milk, the proteomes of milk fat globule membrane (MFGM), caseins and whey have been extensively characterized. Based on these data, we constructed the human milk proteome by combining the data from 8 previously published studies[22,23,28–33], which represent a total of 38 individual studies on whole milk, skim milk, caseins, whey and MFGM (Supplemental Table S1). This "total" milk proteome has a sum of 2698 unique proteins (Supplemental File 2*).

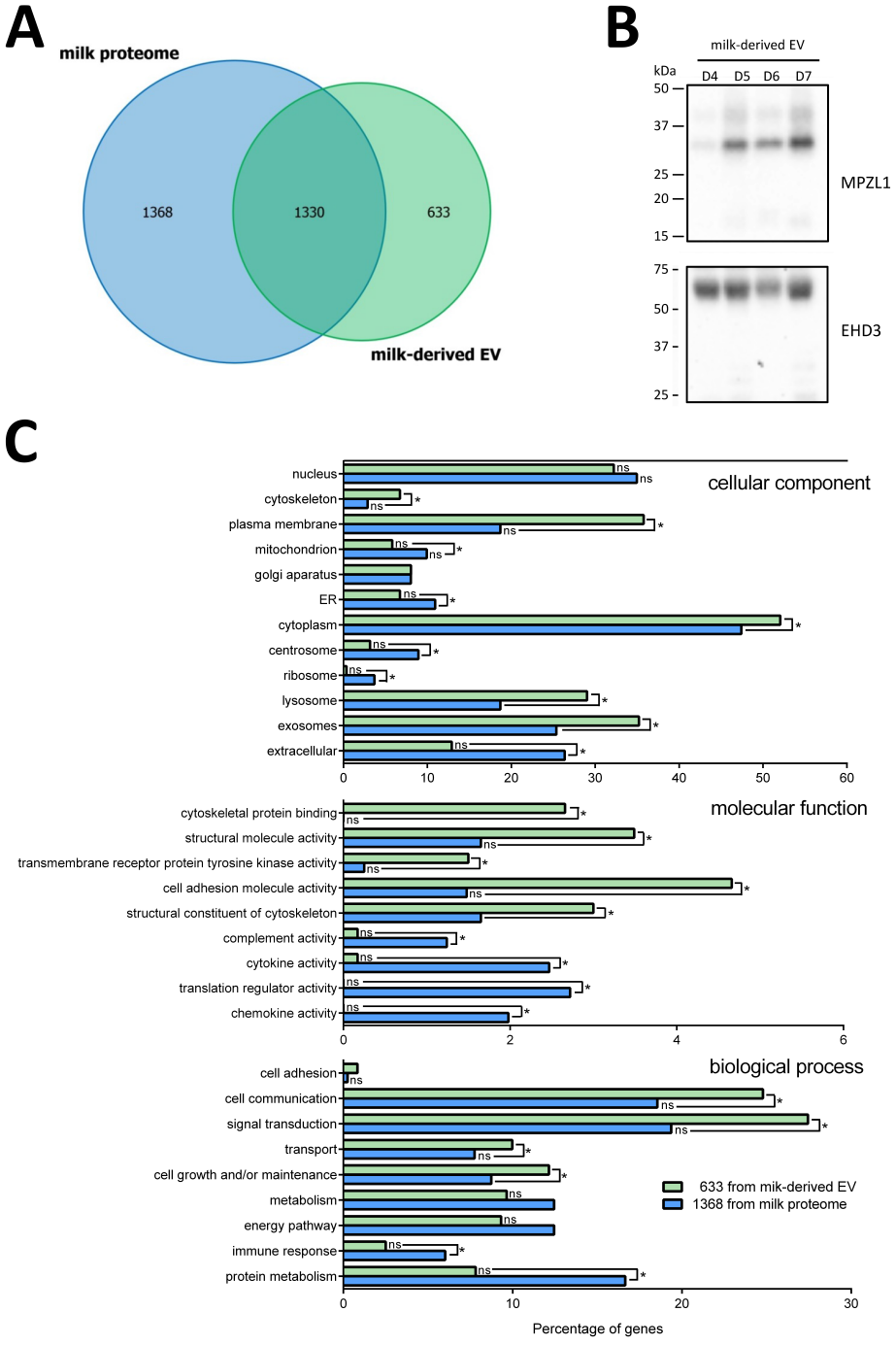
Next, we investigated whether the proteins identified in milk-derived EV have been identified in previous milk studies (since whole milk and skim milk will contain EV), or whether the differential centrifugation of milk followed by density gradient-based isolation of EV improved the detection of proteins that would otherwise be masked by highly abundant milk proteins. When comparing the milk-derived EV proteome from this study to the total milk proteome, 1330 proteins were shared (which is 49% of the total milk proteome of 2698 proteins) between milk-derived EV and the composed milk proteome (Fig. 6A). Interestingly, we identified 633 proteins that have never been described in human milk before. This indicates that isolation of EV as described in this study allows for a better separation of major milk components, which increases the dynamic range in which EV proteins can be detected. Our data reveal that human milk contains more unique proteins than previously known, with now 3331 unique proteins identified. Milk-derived EV contribute substantially to the total milk proteome, because 59% of the proteins (1963 of 3331 total proteins) have been identified in milk-derived EV. We confirmed the presence of two newly identified milk proteins using western blotting. Milk-derived EV contained Myelin protein zero-like protein 1 (MPZL1) and EH domain-containing protein 3 (EHD3) (Fig. 6B).

Figure 6. Comparison of the milk-derived EV proteome to the milk proteome

A. Comparison of the proteins identified in milk-derived EV from all 7 donors in the current study with the milk proteome constructed from previously published milk studies. 38 published (or reviewed) proteomes described in Beck (22), D'Alessandro (23), Dallas (28), Guerrero (29), Hettinga (30), Spertino (31), Yang (32) and Lu (33) were combined such that each protein is represented by its common gene name. The milk proteome was compared to the milk-derived EV proteome from this study and is depicted in the Venn diagram.

B. MPZL1 (~29 kDa) and EHD3 (~61 kDa) were detected on milk-derived EV from donors 4, 5, 6 and 7 using western blot analysis.

C. Functional annotations of proteins uniquely identified in milk-derived EV compared to non-EV milk proteins. The 633 proteins exclusively identified in milk-derived EV (shown as milk-derived EV in green) were compared to the 1368 proteins from the milk proteome that were not shared with milk-derived EV (shown as milk proteome in blue). Enriched terms for cellular compartment, molecular function and biological process term were ranked by p value (Hypergeometric test) for either dataset. Those GO terms that were not significantly enriched are indicated with "ns". Within individual GO-terms, annotated proteins were compared for distribution via chi-square test. Those datasets that were significantly differently distributed are indicated with "*". For both the Hypergeometric test and the chi-square test, a p value <0.05 is regarded as significant.➔


A
milk proteome
1368
1330
633
milk-derived EV
B
milk-derived EV
kDa
D4 D5 D6 D7
50
37
25
20
15
MPZL1
75
50
37
25
EHD3
C
cellular component
nucleus
cytoskeleton
plasma membrane
mitochondrion
golgi aparatus
ER
cytoplasm
centrosome
ribosome
lysosome
exosomes
extracellular
molecular function
cytoskeletal protein binding
structural molecule activity
transmembrane receptor protein tyrosine kinase activity
cell adhesion molecule activity
structural constituent of cytoskeleton
complement activity
cytokine activity
translation regulator activity
chemokine activity
biological process
cell adhesion
cell communication
signal transduction
transport
cell growth and/or maintenance
metabolism
energy pathway
immune response
protein metabolism
633 from mik-derived EV
1368 from milk proteome
Percentage of genes

Additionally, enrichment analysis was performed on the subcellular origin and the possible functions of the newly identified milk-derived EV proteins. We compared the 633 proteins exclusively identified in milk-derived EV to the 1368 proteins from the total milk proteome that are not shared with milk-derived EV (which we will refer to as non-EV milk proteins). We evaluated the cellular distribution of proteins by selecting for major cellular compartments. The 633 proteins exclusively identified in milk-derived EV proteins were significantly enriched for GO terms like 'cytoskeleton', 'plasma membrane', cytoplasm', 'lysosome' and 'exosomes' (Fig. 6C). The 1368 non-EV milk proteins were significantly enriched for cellular component terms like 'ER', 'centrosome', 'ribosome' and 'extracellular'. Subsequently, we compared the molecular function of proteins and found that complement, cytokine and chemokine activity, and 'translation regulator activity' was significantly enriched in the non-EV milk proteome. Interestingly, proteins exclusive to milk-derived EV are significantly enriched in other molecular functions e.g. cytoskeletal/structural activity, 'transmembrane receptor protein tyrosine kinase activity' and 'cell adhesion'. Finally, the annotated biological processes of proteins was assessed. While significantly enriched non-EV milk proteins were involved in 'immune response' and 'protein metabolism', the milk-derived EV proteins were enriched for biological processes related to, 'signal transduction' and 'cell growth and/or maintenance'. Interestingly, the protein EHD3, which was verified with western blotting (Fig. 6 B), was annotated under 'signal transduction' and 'cytoskeletal protein binding'. Together, the GO analysis indicates that proteins from milk-derived EV come from different cellular compartments and have other molecular functions and are involved in distinct biological processes compared to non-EV milk proteins.

Discussion

To unveil the proteome of milk-derived EV, we performed a comprehensive proteomic analysis of purified EV isolated from breast milk from 7 different donors. For EV isolation we used our recently established protocol optimized to purify EV from human milk[10]. Proper sample collection and storage are critical for accurate analysis of EV that are naturally present in body fluids[34]. For breast milk, removal of cells and MFG by differential centrifugation at low speed before long term storage is crucial[10]. Next, isolation of EV from milk supernatant should involve density gradient based ultracentrifugation for further EV enrichment. An alternative approach often used for EV isolation is the direct pelleting of EV via ultracentrifugation (with at least 100,000g), however this approach yields less pure EV[35] and leads to an insoluble pellet when working with breast milk[10]. Therefore, top-down ultracentrifugation of milk supernatant is necessary and allows for an enhanced separation of EV from non-floating complexes with high densities. Using this protocol we isolated milk-derived EV and high density complexes from 7 individual donors and performed proteomics analysis with high-resolution mass spectrometry. We identified a total of 1963 proteins

in the milk-derived EV, while 739 proteins were identified in high-density complexes. In the milk-derived EV samples, common EV associated proteins were frequently present thereby confirming and supplementing earlier work in which CD9, CD63, MHC-class II and Flotillin-1 were detected in milk-derived EV via western blot[10].

Milk composition of an individual changes during lactation[7,36–38]. This is influenced by a variety of factors including diet[39] and age of the mother[40]. We did observe that the milk-derived EV proteome from donor 2 was considerably larger than from the other donors (1615 proteins, while the average of the other donors was 814 proteins), while the high-density complex proteome was similar. We therefore performed additional GO analyses excluding donor 2 and found that this gave similar results and conclusions for all data presented in this report (data not shown). Using the current approach it remains to be determined whether during lactation the total number of EV changes, or that the composition of milk-derived EV alters. However, it is remarkable that the proteomes of the milk-EV from the individual donors, ranging in age from 32 to 39 and between 3 to 9 months after delivery, share 367 proteins (which is 19% of the total milk-derived EV proteome). This indicates that a part of the molecular composition of milk-derived EV is less influenced by these factors. A likely explanation for this finding is the presence of common EV proteins among these shared proteins. Furthermore, since we found that not only breast tissue contributes to the milk-derived EV pool, but that also immune cells are likely producers of milk-derived EV, changes in the EV proteome during lactation might be dependent on other factors than the one regulating soluble and nutritional milk components. It remains to be determined whether the total number of EV changes, or that the composition of milk-derived EV is altered during lactation.

Using the EV database Vesiclepedia[25], which included 8450 unique proteins identified in EV from a broad range of different origins, we were able to determine a large overlap of 1765 proteins between this milk-EV study and previously documented EV-proteins in Vesiclepedia, illustrating a common protein profile of EV regardless of the cell type that produces them, or bodily fluid from which EV are isolated. Additionally, this study reveals 198 proteins identified in milk-derived EV that have not been identified in other EV studies before. The discovery of these EV proteins could be because milk-derived EV are underrepresented in this database, since only few studies have characterized human milk-derived EV[12,13,41], contributing only 81 proteins to Vesiclepedia. Indeed some of the proteins we identified in milk-derived EV that are not listed in Vesiclepedia, OLAH and PTHLH, have been described in milk proteomics studies on whole milk or skim milk[22,42–44]. These proteins are thus specific to EV derived from milk (and have not been identified in other EV preparations). Moreover, OLAH could potentially be used to differentiate between milk-derived EV and MFG, as some studies performed on MFG have reported the presence of PTHLH, while OLAH has never been detected in this milk component[31–33,37]. Both OLAH and PTHLH have been detected in whey[36,45], which contains EV but not MFG, further supporting the idea that milk-derived EV express both OLAH and PTHLH. Most likely, OLAH and PTHLH will be derived from

the EV subset produced by cells in the mammary gland, since these proteins were exclusively mapped to 'breast' and 'mammary gland' respectively in the GO analysis for site of expression in this study. However, besides EV produced by cells in the mammary gland we found by GO analysis that also immune cells might be the origin of milk-derived EV. Taken together the cellular origin of milk-derived EV is diverse and it remains to be determined what the contribution of individual cell types is to the number and composition of milk-derived EV.

Although the analysis of breast milk composition has received considerable attention, milk-derived EV have not yet been recognized as a major component of milk. In order to compare and assess the relative contribution of the milk-derived EV proteome to proteins previously identified in other milk components, we combined the data from previous milk studies (including analysis of whole milk, skim milk, casein, whey and MFG) to construct the proteome of total human milk. Overlap analysis revealed that 1330 proteins that were identified in our study have also been identified in other milk proteome studies and reflect 49% of the total milk proteome. Since the total milk proteome contains proteins identified in whole milk and skim milk, which both contain milk-derived EV, indeed a considerable overlap was expected. Why in previous milk proteomics studies the milk-derived EV proteome was not completely unrevealed could be caused by the relative low number of EV in the whole milk analyzed. This is in contrast to our study, where EV were purified and concentrated. Additionally, milk-derived EV contain proteins also present in other milk components. For instance, CD9 and MUC-1 have also been identified in MFG[37]. Since the isolation procedure of MFG is considerably different from milk-derived EV the most likely explanation for this finding is that the EV-proteins are not derived from contaminating EV in the MFG fraction but that these proteins are indeed present in both macromolecular components. Finally, in the 49% overlap between previous milk studies and this EV study, also highly abundant proteins in milk, like caseins or lactoferrin are present. These proteins are often identified in proteomic studies of milk regardless of the isolation procedure aimed to purify a specific milk component.

Remarkably, 633 milk-derived EV proteins from this study have not been reported in any of the 38 previous milk proteomics studies. The fractionation of the milk with our established protocol to purify EV, using differential centrifugation followed by density gradient ultracentrifugation, allows for an increased dynamic range in detection, since abundantly present milk proteins that would otherwise mask the detection of low abundant proteins are separated from the milk-derived EV. Importantly, this study demonstrates that milk contains much more different proteins than previously known and that a substantial number of these proteins is EV-associated. Remarkably, whereas the proteins of the total milk proteome that are not shared with milk-derived EV are primarily linked to nutritional pathways (reflecting the nutritional characteristics of milk) and immune function based on complement activity and cytokine/chemokine function, the unique milk-derived EV proteins are linked to other bioactive biological pathways like cell signaling and cell growth and/or maintenance.

In conclusion, by using an optimized EV isolation procedure together with in-depth proteomic analysis we unraveled the milk-derived EV proteome in detail and identified novel bioactive proteins associated with milk-derived EV. This indicates that besides commonly described milk components, milk-derived EV need to be recognized as yet another important bioactive macromolecular component in breast milk, which might be involved in transmitting signals from the mother to the newborn.

Acknowledgments

We thank M. Mank and W. Stoorvogel for valuable discussions and C.A.H. van de Lest for discussions on statistical analysis.
This research was performed as part of project 11676 within the framework of a partnership program jointly funded by Nutricia Research and the Dutch Technology Foundation STW, which is part of the Netherlands Organization for Scientific Research (NWO), and is partly funded by the Ministry of Economic Affairs. A.F.M.A. was supported by the Netherlands Organization for Scientific Research (NWO) with a VIDI grant (723.012.102). This work is part of the project Proteins At Work, financed by the Netherlands Organisation for Scientific Research (NWO) as part of the National Roadmap Large-scale Research Facilities of the Netherlands (project number 184.032.201). This work is part of the Utrecht University focus area Future Food Utrecht.

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***Supplemental File 1 and File 2 available online:** http://www.mcponline.org/content/15/11/3412/suppl/DC1

Supplemental Table S1. List of publications of proteomic analysis from human milk. The data from these studies was used to construct the human milk proteome.

Authors	Title	Journal	Milk fraction
Beck KL, Weber D, Phinney BS, et al.	Comparative Proteomics of Human and Macaque Milk Reveals Species-Specific Nutrition During Post-Natal Development	J Proteome Res. 2015 May 1; 14 (5): 2143-57.	whole
Reviewed the following papers:			
Gao X, McMahon RJ, Woo JG, et al.	Temporal Changes in Milk Proteomes Reveal Developing Milk Functions	J Proteome Res. 2012; 11 (7): 3897-907.	whey
Hettinga K, van Valenberg H, de Vries S, et al.	The Host Defense Proteome of Human and Bovine Milk	PLoS One. 2011; 6 (4): e19433.	MFGM/ milk serum
Liao Y, Alvarado R, Phinney B, et al.	Proteomic Characterization of Human Milk Whey Proteins during a Twelve-Month Lactation Period	J Proteome Res. 2011; 10 (4): 1746-54.	whey
Liao Y, Alvarado R, Phinney B, et al.	Proteomic Characterization of Specific Minor Proteins in the Human Milk Casein Fraction	J Proteome Res. 2011; 10 (12): 5409-15.	casein
Liao Y, Alvarado R, Phinney B, et al.	Proteomic Characterization of Human Milk Fat Globule Membrane Proteins during a 12 Month Lactation Period	J Proteome Res. 2011; 10 (8): 3530-41.	MFGM
Mangé A, Tuaillon E, Viljoen J, et al.	Elevated Concentrations of Milk β2-Microglobulin Are Associated with Increased Risk of Breastfeeding Transmission of HIV-1 (Vertical Transmission Study)	J Proteome Res. 2013; 12 (12): 5616-25.	skim
Molinari CE, Casadio YS, Hartmann BT, et al.	Proteome Mapping of Human Skim Milk Proteins in Term and Preterm Milk	J Proteome Res. 2012; 11 (3): 1696-714.	skim
Palmer DJ, Kelly VC, Smit AM, et al.	Human colostrum: Identification of minor proteins in the aqueous phase by proteomics	Proteomics. 2006; 6 (7): 2208-16.	skim
Picariello G, Ferranti P, Mamone G, et al.	Gel-free shotgun proteomic analysis of human milk	J Chromatogr A. 2012; 1227: 219-33.	MFGM/ whey
Zhang Q, Cundiff JK, Maria SD, et al.	Quantitative Analysis of the Human Milk Whey Proteome Reveals Developing Milk and Mammary-Gland Functions across the First Year of Lactation	Proteomes 2013; 1 (2): 128-158.	whey
D'Alessandro A, Scaloni A, Zolla L.	Human milk proteins: an interactomics and updated functional overview	J Proteome Res. 2010; 9 (7): 3339-73.	Multiple (see below)
Reviewed the following papers:			
Armogida SA, Yannaras NM, Melton AL, et al.	Identification and quantification of innate immune system mediators in human breast milk	Allergy Asthma Proc. 2004; 25 (5): 297-304.	whey
Barlet JP, Davicco MJ	Parathyroid hormone-related peptide (PTHrP)	Reprod Nutr Dev. 1990; 30 (6): 639-51.	not specified
Buts JP	Milk-borne bioactive factors	Arch Pediatr. 1998 Mar;5(3):298-306.	not specified

Authors	Title	Journal	Milk fraction
Cavaletto M, Giuffrida MG, Fortunato D, et al.	A proteomic approach to evaluate the butyrophilin gene family expression in human milk fat globule membrane	Proteomics. 2002; 2(7): 850-6.	MFGM
Fortunato D, Giuffrida MG, Cavaletto M, et al.	Structural proteome of human colostral fat globule membrane proteins	Proteomics. 2003;3 (6): 897-905.	MFGM
Furukawa M, Narahara H, Yasuda K, et al.	Presence of platelet-activating factor-acetylhydrolase in milk	J Lipid Res. 1993 Sep;34(9):1603-9.	whole
Hamosh M	Protective function of proteins and lipids in human milk	Biol Neonate. 1998; 74 (2): 163-76.	not specified
Kirshner J, Hardy J, Wilczynski S, et al.	Cell-cell adhesion molecule CEACAM1 is expressed in normal breast and milk and associates with beta1 integrin in a 3D model of morphogenesis	J Mol Histol. 2004; 35 (3): 287-99.	MFGM
Kling PJ	Roles of erythropoietin in human milk	Acta Paediatr Suppl. 2002; 91 (438): 31-5.	not specified
Kulski JK, Hartmann PE	Milk insulin, GH and TSH: relationship to changes in milk lactose, glucose and protein during lactogenesis in women	Endocrinol Exp. 1983; 17 (3-4): 317-26.	whey
Kverka M, Burianova J, Lodinova-Zadnikova R, et al.	Cytokine Profiling in Human Colostrum and Milk by Protein Array	Clin Chem. 2007;53 (5): 955-62.	whey
Nair RM, Somasundaran M, Katikaneni LD, et al.	Studies on LHRH and physiological fluid amino acids in human colostrum and milk	Endocrinol Exp. 1987 Mar;21(1):23-30.	not specified
Ogundele M	Role and significance of the complement system in mucosal immunity: particular reference to the human breast milk complement	Immunol Cell Biol. 2001; 79 (1): 1-10.	not specified
Palmer DJ, Kelly VC, Smit AM, et al.	Human colostrum: Identification of minor proteins in the aqueous phase by proteomics	Proteomics. 2006; 6 (7): 2208-16.	skim
Panchaud A, Kussmann M, Affolter M	Rapid enrichment of bioactive milk proteins and iterative, consolidated protein identification by multidimensional protein identification technology	Proteomics. 2005; 5 (15): 3836-46.	whey
Picariello G, Ferranti P, Mamone G, et al.	Identification of N-linked glycoproteins in human milk by hydrophilic interaction liquid chromatography and mass spectrometry	Proteomics. 2008; 8 (18): 3833-47.	skim
Rajaram S, Baylink DJ, Mohan S	Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions	Endocr Rev. 1997; 18 (6): 801-31.	not specified
Struck J, de Almeida P, Bergmann A, et al.	High concentrations of procalcitonin but not mature calcitonin in normal human milk	Horm Metab Res. 2002; 34 (8): 460-5.	not specified
Takeda S, Kuwabara Y, Mizuno M	Concentrations and origin of oxytocin in breast milk	Endocrinol Jpn. 1986; 33 (6): 821-6.	not specified
Werner H, Katz P, Fridkin M, et al.	Growth hormone releasing factor and somatostatin concentrations in the milk of lactating women	Eur J Pediatr. 1988; 147 (3): 252-6.	whey
Xyni K, Rizos D, Giannaki G, et al.	Soluble form of ICAM-1, VCAM-1, E- and L-selectin in human milk	Mediators Inflamm. 2000; 9 (3-4): 133-40.	skim

Authors	Title	Journal	Milk fraction
Dallas DC, Guerrero A, Khaldi N, et al.	Extensive in vivo Human Milk Peptidomics Reveals Specific Proteolysis Yielding Protective Antimicrobial Peptides	J Proteome Res. 2013; 12 (5): 2295-304.	skim
Guerrero A, Dallas DC, Contreras S, et al.	Mechanistic Peptidomics: Factors That Dictate Specificity in the Formation of Endogenous Peptides in Human Milk	Mol Cell Proteomics. 2014; 13 (12): 3343-51.	skim
Hettinga KA, Reina FM, Boeren S, et al.	Difference in the Breast Milk Proteome between Allergic and Non-Allergic Mothers	PLoS One. 2015; 10 (3): e0122234.	milk serum
Spertino S, Cipriani V, De Angelis C, et al.	Proteome profile and biological activity of caprine, bovine and human milk fat globules	Mol Biosyst. 2012; 8 (4): 967-74.	MFGM
Yang Y, Zheng N, Zhao X, et al.	Proteomic characterization and comparison of mammalian milk fat globule proteomes by iTRAQ analysis	J Proteomics. 2015; 116: 34-43.	MFGM
Liu, Wang, Zhang, et al.	Comparative proteomics of milk fat globule membrane in different species reveals variations in lactation and nutrition	Food Chem. 2016; 196: 665-672	MFGM

Chapter 4

Extracellular vesicles in human milk support the epithelial barrier by promoting reepithelialization and modulating innate and adaptive immune responses

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To be submitted

Abstract

Mammalian milk has co-evolved to provide support for the postnatal development of the gastrointestinal (GI) tract and the immune system. However, which milk constituents contribute to these beneficial effects is not fully understood. Previous research has strongly focussed on the identification of individual milk components, while their functional effects remain understudied. We recently isolated and analysed extracellular vesicles (EV) from human milk and showed that these milk constituents contain a functional proteome unique from other milk components. Extracellular vesicles (EV) are submicron lipid bilayer enclosed entities secreted by cells for intercellular communication. Here, we show that purified EV from human milk, used at physiological concentrations, modulate multiple functions of the epithelial mucosa. Human milk EV increase epithelial cell migration, while inhibiting agonist-induced TLR3 and TLR9 activation. Furthermore, milk EV strongly inhibit activation of $CD4^+$ T cells without inducing tolerance or suppressive regulatory T cells. Our results demonstrate that EV are overlooked functional entities that might play a role in the healthy development of the infant GI tract and maintenance of immune homeostasis.

Introduction

The epithelial mucosa is an important barrier between the external environment and the internal organs. Two of the main functions of the mucosa is the maintenance of an intact physical barrier and preservation of immune homeostasis[1]. In the neonate, the epithelial barrier needs to grow and mature, while the adaptive immune system is still developing[2,3]. Human milk plays an integral part in these processes. It enhances the physical barrier by increasing epithelial tight junctions, proliferation, and migration, and it supports expansion of the total gut epithelial surface area, important for nutrient uptake[4–6]. In addition, human milk helps to maintain immune homeostasis and to prevent the disintegration of the epithelial barrier, e.g. due to (excessive) inflammation[7]. Human milk also influences the development of the neonatal adaptive immune system, and is thought to support oral tolerance induction[8,9]. These beneficial effects of human milk on the epithelial mucosa are underscored by findings that breastfed infants are at reduced risk for contracting and succumbing to infectious diseases or developing necrotizing enterocolitis (NEC)[10–12]. In addition, human milk is protective for the development of non-communicable diseases, such as obesity and diabetes[13].

Despite these beneficial effects of human milk, the milk components which are at the root of these effects are still not identified. In part, our limited understanding is due to the complexity of this body fluid, as it is composed of several macromolecular components with bioactive properties, while most studies have focused on the functional analysis of single molecules[14–16]. One of the understudied macromolecular components in milk are the extracellular vesicles (EV). EV is a collective term for all lipid bilayer enclosed vesicles released by cells[17]. Typically EV secreted by viable cells are < 200 nm in size and contain selected proteins, lipids and nucleic acids[18]. Nowadays ample evidence is available that EV play an important role in intercellular communication in and between organisms [19,20]. Despite being described in human milk in 2007[21], their impact on the development of the infant's epithelial mucosa and immune homeostasis has scarcely been studied. To date, three studies have attempted to study the effect of EV on intestinal epithelial cells and indicated a possible role in the induction of proliferation[22–24]. With regard to immune development, milk EV are suggested to inhibit viral infection[25] and stimulate regulatory T cell (T_{reg}) development[21]. However, whether these effects were due to EV or other factors known to co-isolate with the methods used in these papers[26,27], remains to be determined. The technical complications in separating EV from other bioactive macromolecular components in human milk has severely hampered the progression in studying the characteristics and physiological functions of milk EV. Previously, we have established an isolation and biobanking procedure specifically designed for the reliable isolation of EV from human milk[28]. Using this procedure, we were able to perform in-depth analysis of the human milk EV proteome, thereby unveiling a novel functional proteome, hitherto undescribed in milk[29]. EV from milk were significantly enriched for proteins involved in biological processes of 'cell growth & maintenance', 'cell communication', and 'signal

transduction'[29]. Based on the protein profile, we determined that immune cells were an abundant source of EV in human milk and that milk EV contain immune modulatory proteins, such as MHC molecules, galectins, and co-stimulatory molecules[29]. These results led us to hypothesize that EV from human milk could be important contributors to both the development of the epithelial barrier and maintenance of immune homeostasis.

In the present study, we provide evidence that EV promote the maintenance of the physical epithelial barrier by increasing the reepithelialization rate through p38 MAPK signaling. In addition, innate immune signaling was attenuated by human milk EV, as EV were capable of inhibiting agonist stimulation of TLR3 and TLR9, but not TLR2 and TLR4 by influencing NF-κB and MAPK signaling. Finally, we discovered that human milk-derived EV strongly inhibited T cell activation, proliferation, and cytokine secretion in an antigen-independent manner without inducing tolerance or regulatory T cells.

Materials & Methods

Human milk collection

Human milk was collected as previously described[28]. Briefly, fresh, mature milk samples were collected from 11 healthy mothers, with a mean age of 34 ± 2.3 years, who were not actively terminating breast feeding. Donors were asked to provide an aliquot of a full breast expression and donations were made between 3 and 15 months after delivery, with an average of 8 ± 3 months. The milk was prevented from cooling down and transported to the lab in order to start EV isolation within 20 minutes after collection. Informed consent was signed by all donors and this study was approved by the local ethics committee.

Human milk EV isolation & EV-depleted control

Isolation of milk EV was performed as previously described with some modifications for functional analysis[28]. The workflow is described in Supplementary Figure 1. Whole milk was centrifuged, within 20 minutes after collection, at RT for 10 minutes at 3,000 g (Beckman Coulter Allegra X-12R, Fullerton, CA, USA). The cream layer was removed and milk supernatant was harvested without disturbing the cell pellet. Supernatant was transferred to new tubes and centrifuged at 3,000 g at RT. The supernatant was stored at -80°C until further processing. Later, frozen supernatant was thawed and transferred immediately to polyallomer SW40 tubes (Beckman Coulter) and centrifuged at 5,000 g for 30 minutes at 4°C and subsequently at 10,000 g (Beckman Coulter Optima L-90K with a SW40Ti rotor). Next, 6.5 ml aliquots of the 10,000 g supernatant were loaded on top of a 60% – 10% iodixanol gradient (Optiprep™, Progen Biotechnik GmbH, Heidelberg, Germany) in a SW40 tube. Gradients were ultracentrifuged at 192,000 g (Beckman Coulter Optima L-90K with a SW40Ti rotor) for 15-18 hrs. Following this centrifugation, the

resulting EV-depleted supernatant (6.5 ml) left on top of the gradient was harvested and loaded onto a new iodixanol gradient and ultracentrifuged once more for 15-18 hrs and further processed identically to milk EV samples, described below, to obtain a donor-matched procedural. The gradients of milk EV and EV-depleted samples were harvested in fractions of 500 µl. The density of the fractions was measured by refractometry and densities 1.06-1.19 g/ml, previously shown to contain milk EV[28,29], were pooled. Iodixanol was removed from samples by size exclusion gel filtration using a 20 ml column (Bio-Rad Laboratories, Hercules, CA, USA) packed with SephaDex g100 (Sigma-Aldrich, St. Louis, MO, USA) and elutriating 24 fractions of 1 ml with phenol red free medium (Gibco™, Invitrogen, Carlsbad, CA, USA). Eluates 3 – 9 contained EV, while iodixanol and protein contaminants were found in eluates 15 – 22. Eluate fractions 3 – 9 of were pooled and supplemented with 10% heat-inactivated fetal calf serum (FCS) Ultraglutamine and pen/strep to obtain EV-enriched or EV-depleted samples in complete medium. For reepithelialization assay, FCS was substituted with 0.1% BSA. Samples were frozen at -80°C until use. Concentration of EV used in functional assays were estimated to be within the physiological range, as the volume of the 10,000 g milk supernatant (6.5 ml) from which the milk EV were isolated was similar to that of the pooled eluates (7 ml).

Reepithelialization assay

The Ca9-22 (JCRB0625) cell line, derived from a gingival squamous epithelial carcinoma, was used as a model for intestinal epithelial responses[30]. Cells were maintained in DMEM + high glucose + GlutaMAX (Invitrogen) + 10% FCS + pen/strep. Cells were seeded at 3.5 x 10^4 cells/well in 96-well flat bottom tissue culture treated plates (BD Biosciences, San Jose, CA, USA) and left to reattach and form a confluent monolayer overnight. Cells were starved in FCS-free DMEM for 2 hours prior to experiments and labeled during the last 20 minutes of starvation with 2 µM CellTracker™ Red CMTPX and 4 µg/ml Hoechst 33342 (both from Molecular Probes, Eugene, OR, USA). A gap was made in the cell monolayers of every well using an HTS Scratcher (Peira, Antwerpen, Belgium). Cells were washed twice with PBS after which 100 µl of milk EV, EV-depleted control or medium control (DMEM + high glucose + GlutaMAX + 0.1% BSA) were added to the wells. To indicated wells, 10 µM of p38 inhibitor (SB203580) or MEK 1/2 inhibitor (U0126) was added (both from Cell Signaling Technology, Danvers, MA, USA). 4 ng/ml TGF-α (R&D Systems, Minneapolis, MN, USA) was used as a positive control, while 10 µM SB203580 + U0126 served as negative controls. Cells were monitored by live cell imaging using a BD Pathway 855 Bioimaging System (BD Biosciences). Images of each well were acquired every 20 minutes for 5 hours or until the gaps in the positive controls were closed. Image segmentation and analysis were carried out using CellProfiler 2.1.1 (https://www.cellprofiler.org/) and FCS Express 4 Plus (De Novo Software, Glendale, CA, USA). A nonlinear least squares regression of the modified Gompertz funtion[31] of each condition was calculated from technical triplicates to determine the reepithelialization rate at the linear growth phase in cells/minute.

TLR reporter assay

HEK-Blue™ -hTLR2, HEK-Blue™ -hTLR3, HEK-Blue™ -hTLR4, and HEK-Blue™ -hTLR9 reporter cell lines (all from Invivogen, San Diego, CA, USA) were cultured in DMEM supplemented with 8.5% heat-inactivated FCS, 50 units/mL penicillin, 50 µg/mL streptomycin and 100 µg/mL Normocin. For experiments, cells were harvested and seeded in 96-well flat bottom tissue culture treated plates and incubated with medium, milk EV, or EV-depleted controls, in the presence or absence of the TLR appropriate agonist. Specifically, 100 ng/mL Pam3CSK was added to HEK-Blue™ -hTLR2, 5 µg/ml Poly I:C to HEK-Blue™ -hTLR3, 10 ng/mL LPS-EK to HEK-Blue™ -hTLR4, and 1 µg/ml CpG ODN2006 to HEK-Blue™-hTLR9 (all from Invivogen). Cells were incubated for 16 hours with indicated conditions after which supernatant was harvested and SEAP reporter protein secretion was determined using QUANTI- Blue™ detection medium (Invivogen) and measuring absorption at 650 nm on a 550 Microplate reader (Bio-Rad) as per manufacturer's instructions.

TLR stimulation Ca9-22 cells

Ca9-22 cells were incubated with medium, milk EV, or EV-depleted controls in the presence or absence of 5 µg/ml Poly I:C. After 5 hours, cells were washed with PBS, harvested and stored at -80°C in RBL buffer with β-mercaptoethanol from the RNeasy Mini prep Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions until cDNA synthesis.

RT-qPCR and RT² Profiler PCR array

Total RNA was extracted from HEK-Blue™-hTLR9 reporter cells or Ca9-22 cells after 4-5 hours of culture using the RNeasy Mini prep Kit and cDNA was prepared using the RT² First Strand Kit (Qiagen) or the qScript™ cDNA Synthesis Kit (Quanta Biosciences, MA, USA) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was performed on a Rotor-Gene Q2plex real-time cycler (Qiagen). For relative gene expressions of *IL6* and *CXCL8,* delta Ct-values were log-transformed with *GAPDH* and *ACTB* as internal controls. For gene expression profiling, cDNA was added to the Human Toll-like receptor signaling pathway RT² Profiler PCR array (Qiagen) and run on an iCycler MyiQ (Bio-Rad). The relative expression levels of each gene was normalized to the expression level of 4 reference genes (*B2M, GAPDH, HPRT1, RPLP0*) included in the array. Delta Ct-values were log-transformed and analyzed by the web-based software GeneGlobe Data Analysis Center (Qiagen).

CD4⁺ T cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from Buffy coats by Lymphoprep™ density gradient centrifugation (Axis-shield, Dundee, United Kingdom and Nycomed, Zurich, Switzerland). PBMC were resuspended in complete medium for experiments or MACS buffer (PBS/0.5% BSA/2mM EDTA) for CD4⁺ T cell isolation. CD4⁺ T cells were isolated using CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's

instructions and were used directly in experiments or fractionated into CD45RA$^+$ and CD45RO$^+$ CD4$^+$ subsets using anti-CD45RA-PE (UCHL-1, DAKO, Agilent Technologies, Santa Clara, CA, USA) and anti-PE magnetic beads (Miltenyi Biotec), as described previously[32].

T cell stimulation

Purified CD4$^+$ T cells were seeded at 0.5x10^6 cells/ml in plates coated with 0.5 – 1.5 µg/ml αCD3 (CLB-T3/4.E, 1XE) and cultured with 70 ng/ml – 1 µg/ml soluble αCD28 (CLB-CD28/1, 15E8; both antibodies from Sanquin, Amsterdam, The Netherlands), medium, 750 µl milk EV or EV-depleted controls in a total volume of 1 ml and cultured for the indicated amount of time. For Figure 5A, total PBMC were seeded at 2x10^6 cells/ml and stimulated with 10 ng/ml soluble α-CD3.

Mixed lymphocyte response

Monocyte-derive dendritic cells (DC) were generated as previously described[33]. CD4$^+$CD45RA$^+$CD45RO$^-$ T cells were activated for 3 days with 1.5 µg/ml plate bound αCD3 and 1 µg/ml soluble αCD28 in medium, or in the presence of EV or EV-depleted controls. T cells were subsequently harvested, washed, counted and replated at a 4:1 ratio with allogeneic DC. Cells were incubated for 3 days total. ^{3}H-thymidine was added during the last 16 hours of culture.

Suppressor assay

Suppressor assays were performed as described previously[32]. In brief, CD4$^+$CD45RA$^+$CD45RO$^-$ T cells were activated for 5-6 days with 1.5 µg/ml plate bound αCD3 and 1 µg/ml soluble αCD28 in medium, or in the presence of EV or EV-depleted controls. T cells were subsequently harvested, irradiated, washed and counted. Cells were replated at a 2:1 ratio with CFSE (Sigma-Aldrich) labeled CD4$^+$CD45RO$^+$CD45RA$^-$ responder cells in the presence of allogeneic DC. Cells were incubated for 5-6 days and CFSE dilution was determined on a BD Canto II (BD Bioscience).

Multiplex cytokine analysis

Supernatants of purified CD4$^+$ T cells were harvested on day 2 and day 6 of culture and analyzed using the LEGENDplex™ human Thelper 1/2/9/17 multiplex kit (BioLegend, San Diego, CA, USA). Beads were acquired on a BD Canto II (BD Bioscience) and analyzed with LEGENDplex™ V7.0. Depending on the optimal secretion dynamics of the αCD3/αCD28 stimulated controls, the results of day 2 or day 6 are shown.

Flow cytometry

Proliferation of purified CD4$^+$ T cells was assessed by labeling cells with 2 µM CellTrace Violet (Invitrogen) or 0.5 µM CFSE (Sigma-Aldrich) prior to culture. Following culture, cells were harvested and stained with fluorescent conjugated antibodies. Antibodies used in this study: CD25-Alexa488, FoxP3-APC (both from eBioscience, San Diego, CA, USA), CD45RA-PE, CD127-PE,

CD3-PE-Cy7, CD4-PerCP-Cy5.5, and CD45RO-APC-Cy7 (all from BioLegend). FoxP3 staining was performed with FoxP3 staining buffer set according to manufacturer's instructions (eBioscience). Cells were acquired on a BD Canto II (BD Bioscience), and analyzed by FlowJo (v10.1).

Statistical analysis

Data were analyzed by paired t-test or one-way ANOVA with Tukey's multiple comparison test in GraphPad Prism Software V6.07. Significance was defined as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

Human milk-derived EV enhance reepithelialization through the p38 MAPK pathway

In order to determine whether EV play a role in maintenance of the physical epithelial barrier, a gap was made in a monolayer of Ca9-22 epithelial cells, after which the cells were incubated with a physiological concentration of milk EV or the EV-depleted milk control, or TGF-α as a positive control. The EV-depleted milk control was prepared by subjecting EV-depleted milk supernatant to the same density gradient-based EV isolation protocol as the EV-enriched samples (Supplementary Figure 1). The EV-depleted milk control serves as a control not only for the EV isolation procedure, but also for donor-specific background effects of non-EV associated milk components. The reepithelialization rate of the gap was monitored by live cell imaging. EV of all milk donors significantly increased the reepithelialization rate of cells compared to both medium and EV-depleted controls, while EV-depleted samples did not affect reepithelialization (Figures 1A and 1B). In order for reepithelialization to occur, a combination of proliferation and migration of cells needs to take place. These processes are downstream effects of two major cell signaling cascades, with migration acting primarily through p38 MAPK signaling, and proliferation mainly through ERK1/2, although crosstalk is known to occur[34]. To establish which pathways were involved in the increased reepithelialization, cells were incubated with milk EV in the absence or presence of pharmacological inhibitors of p38 MAPK (SB203580) and MEK1/2 (U0126), which is directly upstream of ERK1/2. Inhibition of p38 MAPK completely abolished the increased reepithelialization rate induced by milk EV (Figure 1C). In contrast, inhibiting MEK1/2 showed only a minor and non-significant reduction of EV-mediated reepithelialization (Figure 1D). These data indicate that human milk-derived EV enhance epithelial barrier maintenance mainly through p38 MAPK signaling.

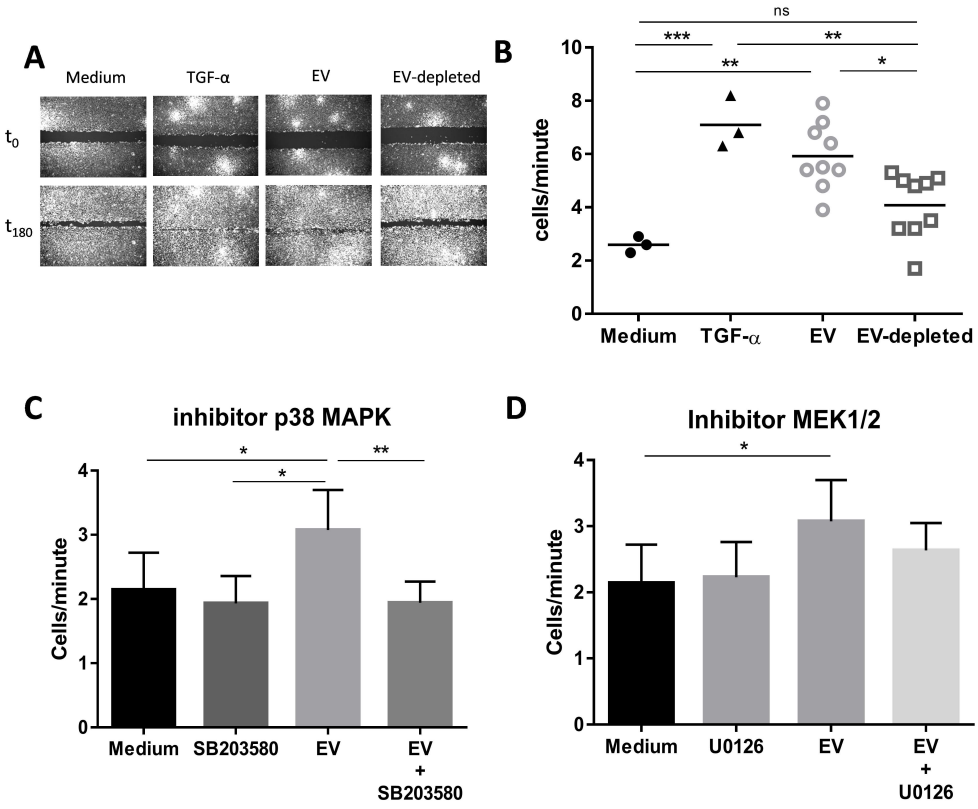

Figure 1: Human milk EV enhance the formation of the epithelial barrier through p38 MAPK
A gap was made in a confluent monolayer of Hoechst stained Ca9-22 cells and subsequently incubated with the indicated components. Cell migration was tracked in triplicate conditions by live cell imaging. Images were acquired every 20 minutes for 5 hours or until gap in TGF-α was closed.
A) Representative images of Hoechst staining at the start of experiment (t_0) and after 180 minutes (t_{180}) incubation with medium, 4 ng/ml TGF-α, EV or the donor-matched EV-depleted control. Images are representative of 2 experiments with 3 milk donors per experiment.
B) Quantification of Ca9-22 cells migrating into the gap in cell per minutes during the linear growth phase. Linear growth phase was calculated for each well using nonlinear least squares regression of the modified Gompertz function and plotted as a single point. Data are representative of 2 experiments with 3 milk donors in each experiment.
C) + D) Quantification of Ca9-22 cells migrating into the gap in cell per minutes during the linear growth phase. 10 µM of inhibitors SB203580 or U0126 were added to wells containing medium or EV (n=2 different donors). Data is representative of 2 independent experiments. Bars represent mean ± SD.
Significance was calculated by one-way ANOVA with * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; ns: not significant.

Human milk-derived EV actively interfere in agonist-induced TLR3 and TLR9 signaling, but not TLR2 and TLR4 signaling

Besides supporting migration and growth of the epithelial barrier, damage of this barrier by excessive inflammation needs to be prevented by attenuating neonatal immune responses[35]. This attenuation is also necessary to allow for appropriate colonization of the GI tract by commensals[36,37]. Epithelial cells lining the digestive tract, including the oral cavity, express Toll-like receptors (TLR), which are important for initiating innate immune responses against pathogens, but which should not respond vigorously to commensals[30,38,39]. TLR expression by epithelial cells varies throughout the GI tract[36]. To determine whether EV in human milk interfered in signaling of specific TLR, we made use of TLR reporter cell lines for human TLR2, TLR3, TLR4, and TLR9. In these cells, the secreted embryonic alkaline phosphatase (SEAP) reporter gene is fused to a promotor region containing NF-κB and AP-1 responsive elements, and activation of TLR signaling will lead to SEAP production and secretion[40]. First, we determined whether purified human milk EV could act as TLR agonists. Each reporter cell line was incubated with medium, the TLR specific agonist (TLR2: Pam3CSK; TLR3: Poly I:C; TLR4: LPS; TLR9: CpG ODN2006), or human milk-derived EV or their EV-depleted milk controls. Both TLR2 and TLR4 reporter lines showed some activation in response to EV, although SEAP levels were variable (Figures 2A and 2B, left of dashed line). This variability was not only observed between different donors, but also between different EV preparations of the same milk sample (data not shown). In addition, no significant differences were found in TLR2 or TLR4 activation between EV and EV-depleted controls, indicating that the activation of TLR2 and TLR4 is not dependent on EV-specific effects, but rather is caused by a soluble mediator present in milk or by a contaminant introduced during the experimental procedure. In contrast, TLR3 and TLR9 reporter lines were only triggered by their respective agonist, while neither EV nor EV-depleted controls showed stimulatory effects (Figures 2C and 2D, left of dashed line).

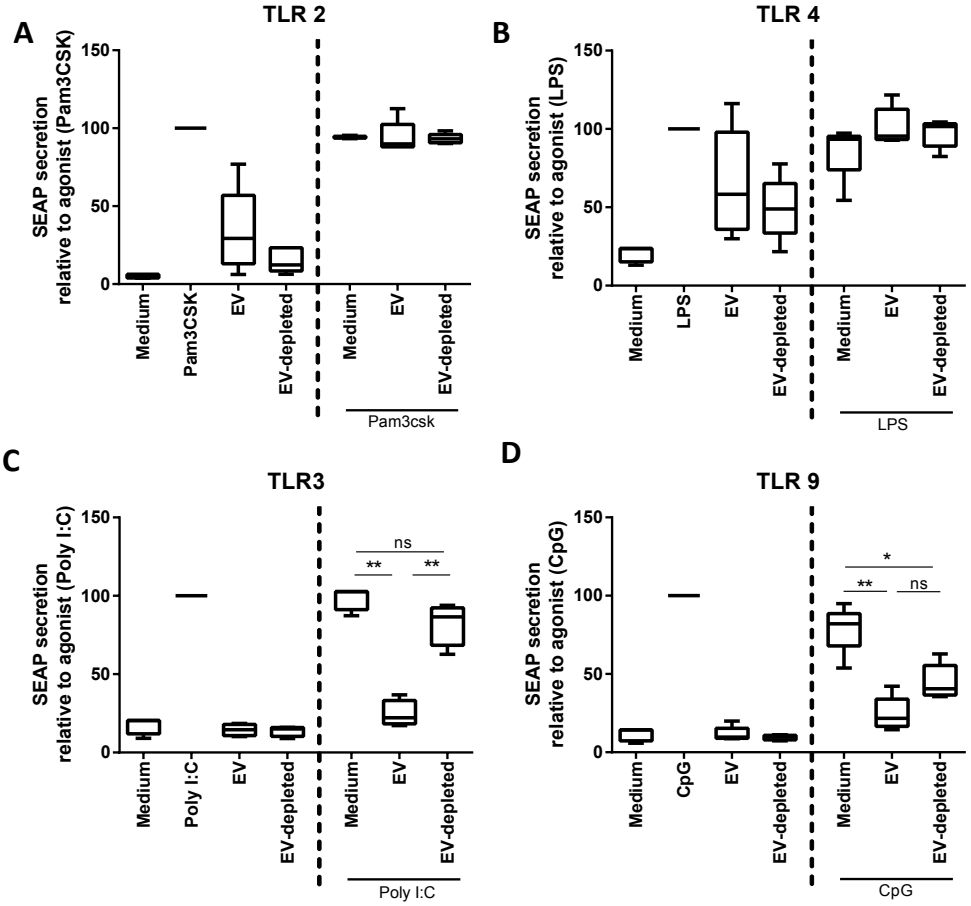

Figure 2: Human milk EV inhibit agonist-induced activation of TLR3 and TLR9, but not of TLR2 and TLR4

HEK-Blue TLR reporter cell lines for TLR2 (A), TLR4 (B), TLR3 (C), TLR9 (D) were incubated overnight with the indicated conditions in triplicate. Left of the dashed lines, cells were incubated with medium, the TLR-specific agonist, EV, or EV-depleted controls. Right of dashed lines, cells were incubated with medium, EV, or EV-depleted controls plus TLR-specific agonist. SEAP reporter protein was detected in the supernatant by Quanti-Blue detection reagent and measuring absorption at 650 nm. SEAP secretion in response to agonist was set to 100%. A) HEK-Blue TLR 2 reporter cells were stimulated with 100 ng/ml Pam3csk as agonist. B) HEK-Blue TLR 4 reporter cells were stimulated with 10 ng/ml LPS-EK as agonist. C) HEK-Blue TLR 3 reporter cells were stimulated with 5 µg/ml Poly I:C as agonist. D) HEK-Blue TLR 9 reporter cells were stimulated with 1 µg/ml CpG ODN2006 used as agonist. Box and whisker plots contain data of 3 independent experiments with a total of 4 different milk donors. Significance was calculated by one-way ANOVA with * $p < 0,05$; ** $p < 0,01$; ns: not significant.

In order to determine whether milk-derived EV could alter the ability of the reporter cell lines to respond to TLR stimulation, the cells were exposed to their specific agonist in the presence of milk-derived EV or EV-depleted milk controls. Both TLR2 and TLR4 showed equal stimulation by the agonist in the presence of milk EV or EV-depleted milk controls, which was comparable to

agonist stimulation only (Figures 2A and 2B, right of dashed line). This indicates that EV neither enhanced, nor inhibited TLR2 and TLR4 signaling in response to agonistic stimulation. However, human milk-derived EV significantly inhibited the responses of TLR3 reporter lines to Poly I:C and of TLR9 reporter lines to CpG ODN2006, while EV-depleted milk controls had no or minor effects on agonist induced stimulation (Figures 2C and 2D, right of dashed line). Thus, EV from human milk selectively inhibited agonist-stimulation of the endosomal TLR3 and TLR9, but not of TLR2 and TLR4, which are expressed on the cell surface[41]. To investigate how milk EV affect endosomal TLR signaling at the transcriptional level, the TLR9 reporter cell line was incubated with medium, agonist only, or agonist in the presence of milk EV or the EV-depleted milk control and the expression of n=84 genes involved in TLR signaling pathways were analyzed[41]. A heatmap was generated in order to visualize global chances in gene expression. Expression levels of pro-inflammatory mediators *CXCL8, TNF,* and NF-κB subunit *NFKB1* were found to be downregulated specifically in agonist in the presence of milk EV compared to agonist in the presence of EV-depleted milk controls or agonist only (Figure 3A). Conversely, *TICAM1, MAPK8,* and *MAPK8IP3*, were among those genes which were specifically upregulated in the presence of milk EV (Figure 3A), possibly indicating activation of the JNK pathway[42]. The analysis also identified genes that were consistent in all agonist containing conditions, showing that all cells responded to CpG stimulation and that milk EV did not prevent CpG binding to TLR9. Strikingly, many of the genes analyzed showed similar expression levels for agonist in the presence of milk EV or EV-depleted milk controls, which differed from the agonist only or medium conditions, e.g. *TIRAP, ECSIT,* or *IL2* (Figure 3A). This indicates that a vast amount of gene alterations were induced by non-EV milk components or procedural steps, highlighting the necessity to take along a milk and procedural control to discern EV-specific effects[17]. Our results show that milk EV are actively interfering with agonist-mediated TLR9 signaling, ultimately leading to reduced SEAP secretion in the TLR9 reporter cell line. In the reporter cell line, SEAP is under the control of transcription factors NF-κB and AP-1. In order to determine whether downstream targets of NF-κB and AP-1 were also impacted in epithelial cells, Ca9-22 cells were stimulated with the TLR3 agonist Poly I:C and TLR9 agonist CpG in the presence or absence of milk EV of two different donors. No effect of CpG stimulation was seen on Ca9-22 cells, indicating that TLR9 is not expressed by these cells (data not shown). Stimulation with Poly I:C strongly increased gene transcription of *IL6* and *CXCL8*, which are known physiological target genes of NF-κB and AP-1 (Figure 3B)[43]. The addition of human milk-derived EV strongly reduced transcription of these inflammatory mediators in response to Poly I:C stimulation (Figure 3B), confirming the results obtained in the TLR3 reporter cell line (Figure 2C). Taken together, these data indicate that human milk EV actively interfere in the transcription of NF-κB and AP-1 regulated genes in TLR3 and TLR9 stimulated cells.

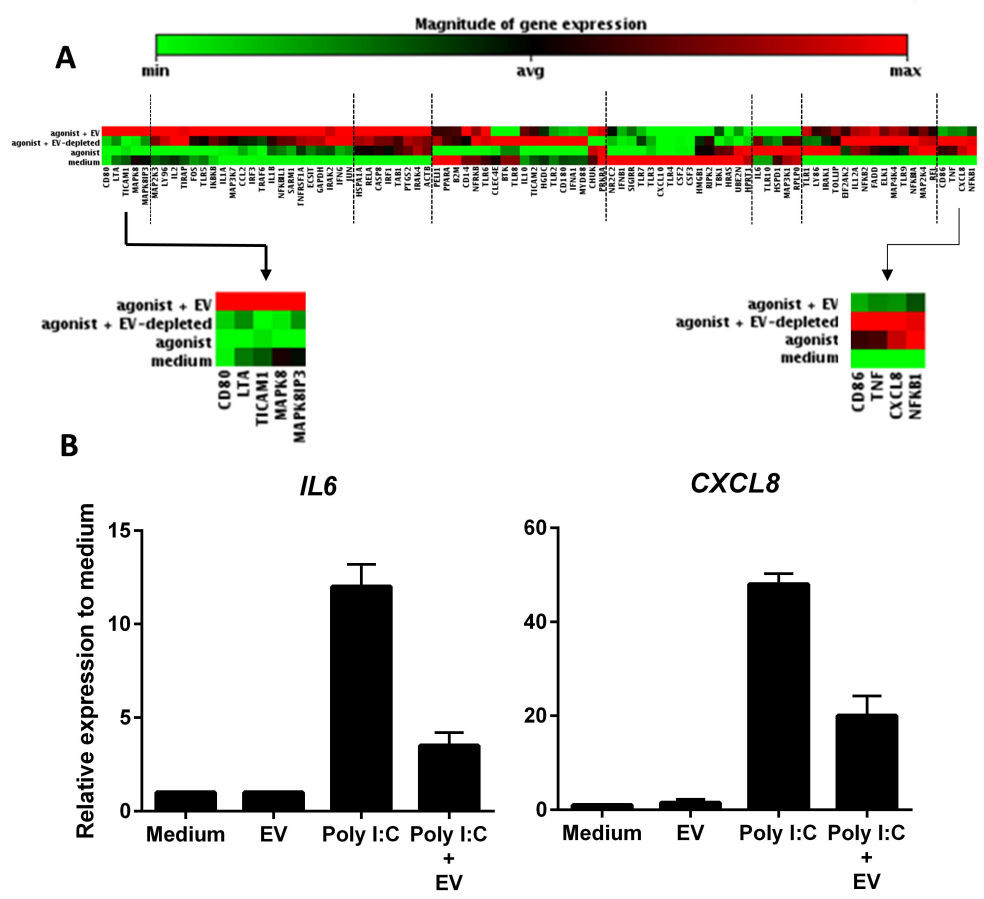

Figure 3: Human milk EV actively interfere in agonist-induced TLR3 and TLR9 signaling
A) Transcriptomic analysis was performed of HEK-Blue TLR9 reporter cells incubated for 4 hours with medium, agonist (1 µg/ml CpG ODN2006), agonist + EV or agonist + EV-depleted control. Cellular gene expression profiles were made using Human Toll-like receptor signaling pathway RT^2 Profiler PCR array. For each gene, the average gene expression across the 4 different conditions was determined and the results for the individual conditions represented in a gradient running from minimal gene expression (green) to maximal expression (red). Data is derived from 1 experiment.
B) Ca9-22 epithelial cells were incubated for 5 hours with medium, EV, 5 µg/ml Poly I:C or 5 µg/ml Poly I:C + EV and gene expression of *IL6* and *CXCL8* was assessed by RT-qPCR. Delta Ct-values to *ACTB* were calculated and expressed relative to medium controls. EV of 2 different milk donors were used and PCR reaction was performed twice. Results are summarized in bar graphs as mean ± SD.

Human milk EV inhibit CD4$^+$ T cell activation

Human milk is known to support postnatal development of the adaptive immune system[2,3,9,15]. As EV are able to traverse traditional barriers, such as the epithelial mucosa and the blood-brain barrier[44,45], it is plausible that milk-derived EV can reach mucosal lymphoid tissue and can interact with adaptive immune cells in the gastrointestinal tract-associated lymphatic tissue. Since CD4$^+$ T cells are critical components of the adaptive immune system, we studied the effects of milk EV on

$CD4^+$ T cell activation and differentiation. Addition of milk EV to purified $CD4^+$ T cells did not result in T cell activation (Data not shown). However when purified $CD4^+$ T cells were stimulated with αCD3/αCD28 in the presence of milk EV, milk EV induced a significant reduction of proliferation, while EV-depleted milk controls did not affect proliferation (Figure 4A + 4B). In order to determine whether EV generally impaired T cell activation or only interfered in T cell receptor signaling, the ratio of CD45RA to CD45RO T cells was analyzed. In peripheral blood, resting, naïve $CD4^+$ T cells typically express the RA isoform of the tyrosine phosphatase CD45, while activated and antigen experienced cells express CD45RO[46,47]. Therefore, monitoring this transition within the different culture conditions provides insight into the activation status of T cells. As expected, culturing T cells with only αCD3/αCD28 or αCD3/αCD28 in the presence of EV-depleted milk controls skewed the $CD4^+$ T cell population towards CD45RO (37% to 83%) at the expense of CD45RA (47% to 12%) (Figure 4C and 4D). However, T cells incubated with αCD3/αCD28 in the presence of milk EV maintained levels of CD45RA and CD45RO similar to medium controls (Figures 4C and 4D). To determine whether milk EV not only selectively impacted activation and expansion of antigen experienced CD45RO T cells but also prevented the activation of naïve CD45RA T cells highly purified naïve CD45RA T cells were isolated[32] and stimulated using αCD3/αCD28 and cultured in the presence of EV or EV-depleted controls. As shown in Figure 4E, the proliferation of naïve T cells was also significantly reduced in EV exposed conditions, indicating that human milk EV negatively affect activation of naïve T cells and their transition to a memory phenotype.

Human milk EV do not induce suppressive regulatory T cells

Regulatory T cells (T_{reg}) suppress $CD4^+$ T cell activation[48] and are involved in the induction of antigen-specific oral tolerance[49]. Therefore, we investigated whether purified human milk EV induced functional T_{reg}. Stimulating PBMC with αCD3/αCD28 in the presence of milk EV or the EV-depleted milk controls revealed no difference in the percentage of $CD4^+CD25^+CD127^-FoxP3^+$ T_{reg} (Figure 5A). Moreover, $CD4^+$ T cells activated in the presence of EV showed a significantly reduced secretion of the anti-inflammatory cytokine IL-10 compared to cells treated with EV-depleted milk controls (Figure 5B). In order to assess the suppressive capacity of EV-exposed T cells, $CD4^+CD45RA^+CD45RO^-$ T cells were incubated with αCD3/αCD28 in the absence or presence of milk-EV or EV-depleted milk controls for 5 days washed, irradiated and their inhibitory capacity in was analyzed in an allogeneic mixed lymphocyte reaction (MLR) using autologous memory responder $CD4^+CD45RO^+CD45RA^-$ T cells and allogeneic DCs. T cells primed in the presence of human milk-derived EV showed no difference in suppressive capacity compared to controls (Figure 5C). Taken together, we found no indication for the induction of suppressive T_{reg} by human milk EV.

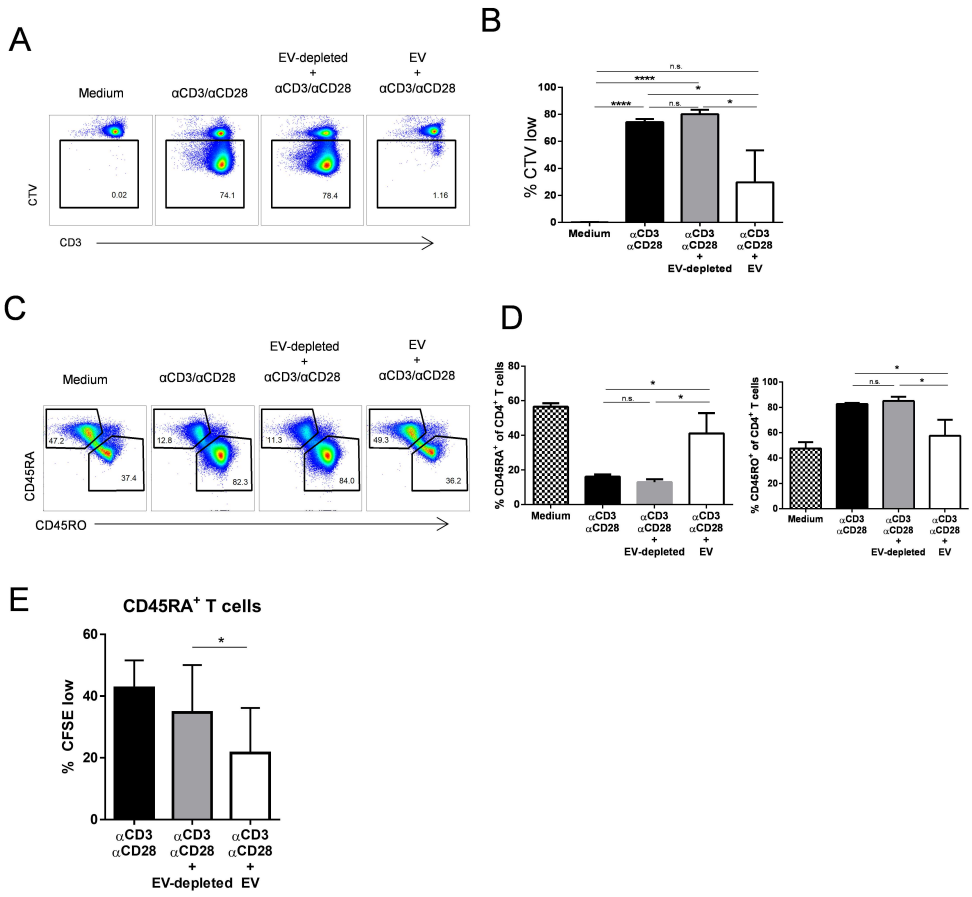

Figure 4: Human milk EV inhibit αCD3/αCD28 CD4⁺ T cell proliferation and cells retain a more naïve phenotype

A – D) Purified CD4⁺ T cells were labeled with CellTrace Violet (CTV) and incubated with medium, αCD3 (0.5 µg/ml) and αCD28 (70 ng/ml), or αCD3/αCD28 with EV or donor-matched EV-depleted controls. After 6 days cells were harvested and analyzed by flow cytometry.

A) Representative dot plots of CTV dilution in response to the indicated conditions. Percentage CTV^{low} cells (gate) is expressed as a fraction of total CD4⁺ T cells.

B) Quantification of the percentage CTV^{low} CD4⁺ T cells following incubation with the indicated conditions. Bars summarize mean ± SD of 2 independent experiments using different T cell donors (n=2) and different milk donors (n = 2 and 3).

C) Representative dot plots of CD4⁺ T cells stained for CD45RA (x-axis) and CD45RO (y-axis) after incubation with the indicated conditions. Percentage of $CD45RA^+/CD45RO^-$ and $CD45RO^+/CD45RA^-$ cells in gates are expressed as a fraction of total CD4⁺ T cells.

D) Quantification of the percentage CD45RA⁺ and CD45RO⁺ cells of total CD4⁺ T cells following incubation with the indicated conditions. Bars summarize mean ± SD of 2 independent experiments using different T cell donors (n=2) and different milk donors (n = 2 and 3).

E) Highly purified $CD45RA^+CD45RO^-$ CD4⁺ T cells of 3 different donors were labeled with CFSE or CTV and stimulated with 1.5 µg/ml αCD3 and 1 µg/ml αCD28 in the presence or absence of EV and EV-depleted controls for 6 days. Cells were subsequently harvested and CFSE/CTV dilution was determined by flow cytometry. Bars represent mean ± SD of 2 independent experiments using different T cell donors (n=1 and n=2) and 3 different milk donors (n=3).

Significance was calculated with one-way ANOVA (A and D) or paired t-test (E) and significance defined as * $p < 0,05$; **** $p < 0,0001$; n.s.: not significant.

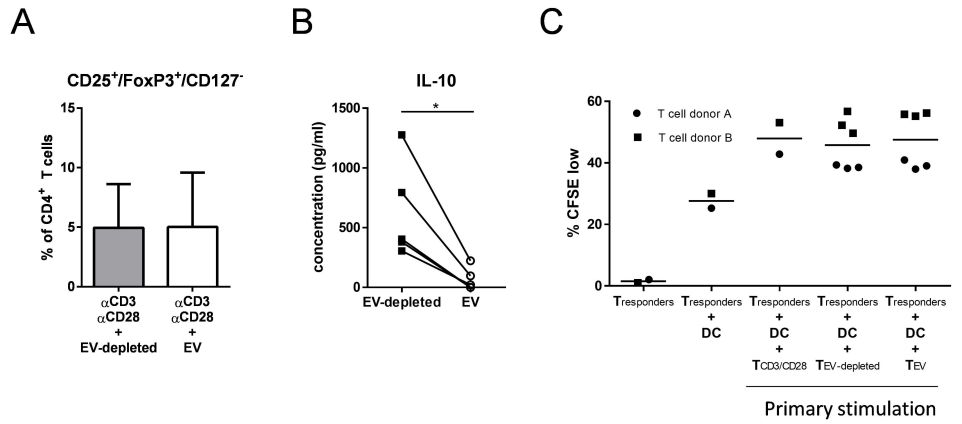

Figure 5: Human milk EV do not induce suppressive regulatory CD4⁺ T cells
A) PBMC were stimulated with 10 ng/ml αCD3 in the presence of EV or donor-matched EV-depleted controls for 6 days and stained for CD4, CD25, CD127, and FoxP3 and analyzed by flow cytometry. Regulatory T cells were defined as $CD4^+CD25^{high}FoxP3^+CD127^-$. Results are expressed as a percentage of total CD4⁺ T cells. Bars represent mean ± SD of 4 independent experiments performed with 4 different PBMC donors and in total 6 different milk donors.
B) Purified CD4⁺ T cells of two different donors were cultured with 0.5 µg/ml αCD3 and 70 ng/ml αCD28 in the presence of human milk EV (clear circles) or paired EV-depleted controls (black squares). Supernatants were collected on day 2 and day 6 and analyzed in a multiplex assay. Results are shown for day 6, as more IL-10 secretion was detected in αCD3/αCD28 control conditions on this day (330,2 ± 243,7 pg/ml).
C) Highly purified CD45RA⁺ CD4⁺ T cells of 2 different donors (symbols) received primary stimulation with αCD3 (1.5 µg/ml) and αCD28 (1 µg/ml; $T_{CD3/CD28}$) or with αCD3/αCD28 in the presence of EV (T_{EV}) or the EV-depleted controls ($T_{EV\text{-}depleted}$) of three different milk donors for 6 days. Cells were subsequently harvested, irradiated and added to autologous CFSE labeled $CD45RO^+$ $CD4^+$ T responder ($T_{responder}$) cells at a 2:1 ratio and allogeneic monocyte-derived DC. After 5 days, cells were harvested and CFSE dilution of $T_{responder}$ cells was determined by flow cytometry. Plotted are single points for the average of triplicates, shown as a percentage of total $T_{responder}$ cells. Data is representative of 2 independent experiments.
*P < 0,05; **P < 0,01; ****P < 0,0001; n.s.=not significant; p-values were determined by paired t-test (A and B) or one-way ANOVA (C).

Human milk EV cause a general inhibition of cytokine secretion by CD4⁺ T cells

Next, we assessed whether milk EV impacted T helper subsets other than T_{reg}, by determining the cytokine profile. In the presence of milk EV, most Th1, Th2, Th17, and Th9 associated cytokines were reduced, with significantly reduced secretion of IFN-γ, IL-5, IL-9, IL-13, IL-17A, IL-17F, and IL-22 (Figure 6). IL-21 was not detected in any of the culture conditions. Cytokine secretion in the presence of EV-depleted controls was similar to secretion by T cells stimulated with αCD3/αCD28 only (see legend of Figure 6 for values), with the exception of IL-17A and IL-17F, which were enhanced in EV-depleted controls. This suggests that either milk components or procedure-related contaminations in the EV-depleted controls affect Th17 responses, underscoring the necessity to include this control[17]. These data show that milk EV inhibit the release of a wide range of cytokines by activated CD4⁺ T helper cells, suggesting a general suppressive effect of milk EV.

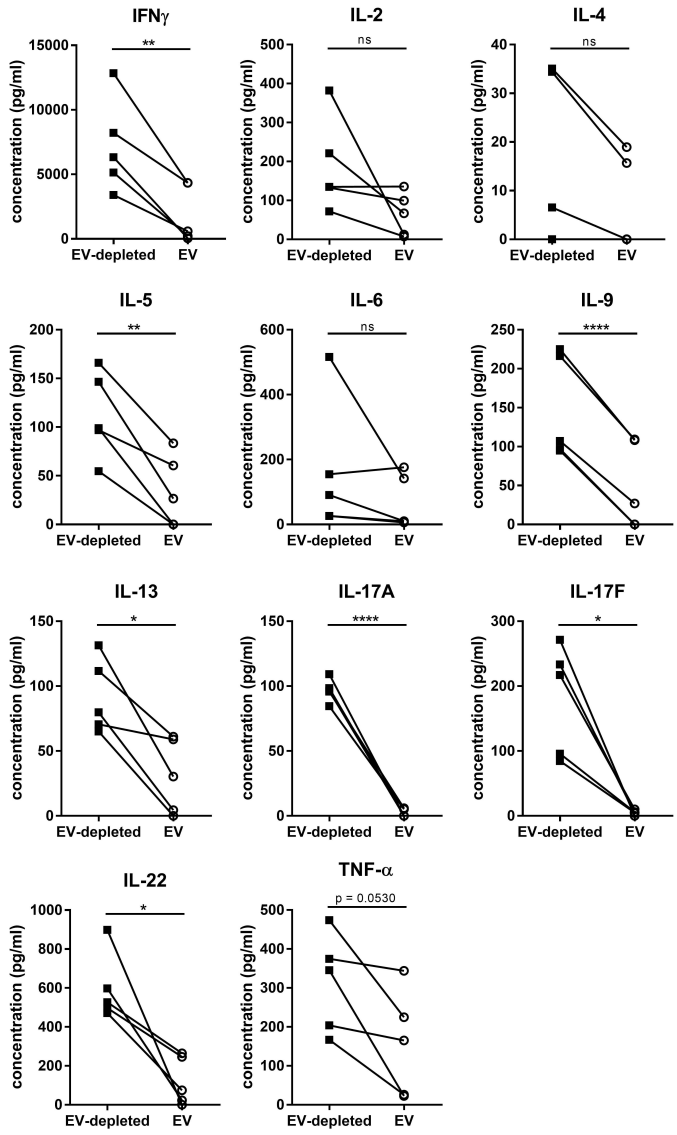

Figure 6: Human milk EV inhibit αCD3/αCD28 induced cytokine secretion by CD4⁺ T cells
Purified CD4⁺ T cells were cultured with αCD3 (0.5 µg/ml) and αCD28 (70 ng/ml) in the presence of human milk EV (clear circles) or paired EV-depleted control (black squares). Supernatants were collected on day 2 and day 6 and analyzed in a multiplex assay. For comparison, the following values were obtained for αCD3/αCD28 stimulated controls (mean ±SD):
IFN-γ: 4514 ± 1757 pg/ml, IL-2: 162,6 ± 44,41 pg/ml, IL-4: 14,23 ± 17,73 pg/ml, IL-5: 89,34 ± 30,34 pg/ml, IL-6: 13,72 ± 3,948 pg/ml, IL-9: 123,2 ± 37,70 pg/ml, IL-13: 99,10 ± 22,57 pg/mL, IL-17A: 13,15 ± 7,616 pg/ml, IL-17F: 68,43 ± 47,42 pg/ml, IL-22: 346,9 ± 59,61 pg/ml, TNF-α: 308,4 ± 176,0 pg/ml. IL-21 was not detected above threshold. Data are shown for day 6 as highest secretion was seen for αCD3/αCD28 controls on this day, except for IL-2, IL-4, and TNF-α, which had a higher secretion on day 2 (data not shown). Graphs summarize results of 2 independent experiments, each using 1 T cell donor with 2 or 3 different milk donors (n=5 different milk donors in total). *P < 0,05; **P < 0,01; ****P < 0,0001; n.s.=not significant; p-values were determined by paired t-test.

Inhibition of CD4+ T cell activation by human milk EV is transient

Next, we tested whether the inhibition of T cell activation by human milk EV was transient and dependent on the immediate presence of milk EV, or whether T cell responses remained suppressed after primary EV exposure. Naïve T cells were primed with αCD3/αCD28 or with αCD3/αCD28 in the presence of milk EV or EV-depleted controls, after which the cells were washed and restimulated with allogeneic dendritic cells. Interestingly, T cells rendered non-responsive during incubation with human milk EV responded equally well to restimulation in the absence of milk EV as control T cells that had been pre-incubated with EV-depleted milk controls (Figure 7). These results demonstrate that the immediate presence of human milk EV causes a transient block in T cell proliferation and cytokine release, and that milk EV do not cause permanent changes in T cell responsiveness.

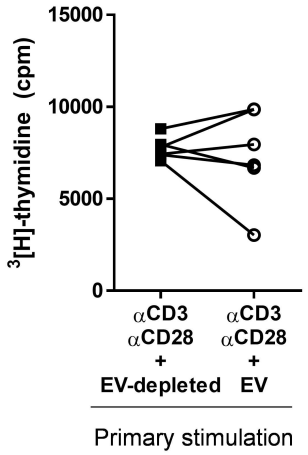

Figure 7: Inhibition of CD4+ T cell activation by human milk EV is transient
Highly purified CD45RA+ CD4+ T cells of 2 different donors received primary stimulation with αCD3 (1.5 µg/ml) and αCD28 (1 µg/ml) in the presence of EV or the donor-matched EV-depleted controls from 3 different milk donors for 6 days. Cells were subsequently harvested, washed and restimulated with allogeneic monocyte-derived DC at a 4:1 ratio for 72 hours in the absence of EV and EV-depleted controls. ^{3}H-thymidine was added during the last 16 hours of culture. Restimulation was performed in triplicate and the average values plotted as a single point. Data are expressed as counts per minute (cpm). Significance was tested by paired t-test.

Discussion

Human milk is known to aid in the postnatal development and maturation of the GI tract, and in the maintenance of immune homeostasis[6–8]. However, the macromolecular structures which induce these effects are still elusive. An interesting, newly discovered player in the field of milk research are the EV[21]. Due to technical complications in purifying EV from other components in milk, the investigation of the specific function of EV in milk has been greatly hampered. Following

up on our initial study, in which we designed an optimized procedure to isolate EV from human milk[28], we recently showed that milk EV are composed of a functional proteome, unique from other bioactive milk components[29]. EV were significantly enriched for proteins involved in biological processes of 'cell growth & maintenance', 'cell communication', and 'signal transduction'[29]. Here, we explored the functional effects of purified milk EV on the epithelial mucosa by assessing their effects in *in vitro* models for epithelial barrier formation, innate immune signaling, and adaptive immune responses.

We demonstrate for the first time that purified EV from human milk enhance reepithelialization of the epithelial barrier, with EV significantly boosting reepithelialization compared to donor-matched EV-depleted milk controls and medium. We further show that human milk EV-mediated reepithelialization is, at least in part, induced by p38 MAPK, a kinase downstream of many replicative and migratory stimuli[50]. Recent reports using high-speed centrifugation or ExoQuick have suggested a role for EV from milk of rat, porcine, and bovine origin in increasing proliferation of rodent intestinal epithelial cells[22–24]. Although these methods will isolate other bioactive components besides EV[27,29,51], it might indicate that epithelial barrier formation is a central and evolutionary conserved function of maternal EV delivered through milk, or of EV in general. Recently, EV from sources other than milk have been implicated in stimulating cell growth, wound repair, and migration[52–56]. Recently, it was suggested that epithelial cell-derived EV enhance wound healing of epithelial cells via Annexin A1 in the outer leaflet of the EV membrane[53]. Although Annexin A1 was also present in the human milk EV proteome, as well as in the milk EV preparations used in the present study ([29] and data not shown), we could not confirm that the enhanced reepithelialization rate witnessed in Ca9-22 cells was due to this single molecule (data not shown).

Epithelial cells of the digestive tract do not only form a physical barrier between the environment and the internal organs, but are also specialized sensors, critical for the maintenance of immune homeostasis[1]. Toll-like receptors expressed on epithelial cells play an important role in sampling the environment. Human milk has been described to modulate TLR signaling through multiple components, such as non-digestible oligosaccharides, soluble CD14, and bacteria present in human milk[57–60]. In addition, breastfed infants are known to be at reduced risk for the development of NEC, a disease associated with excessive TLR4 expression and stimulation in the intestine[61,62]. In the present study, we describe that human milk EV specifically inhibited TLR3 and TLR9 signaling, but not TLR2 and TLR4 signaling, by modulating NF-κB and MAPK pathways. This selective inhibition of TLRs could be essential for the colonization of the intestine, as it has been shown that stimulation of TLR9 without downstream NF-κB activation is essential for immune homeostasis and tolerance to commensal bacteria[63]. Alternatively, this can indicate that different milk components induce hyporesponsiveness of different TLR to maintain immune homeostasis. For example, Menckeberg, et al., showed that NF-κB inhibitor SLPI, which is present in soluble form in milk, inhibits downstream activation of TLR2 and TLR4[30].

Intestinal epithelial cells (IEC) are known to selectively transport macromolecular structures from the apical to basolateral compartments of the intestine via a process known as transcytosis[64]. Moreover, EV are known to cross traditional barriers, such as the blood-brain barrier[44], and bovine milk EV have been shown to be taken up by IEC[45]. By passing the epithelial barrier, EV from human milk could come into contact with adaptive immune cells in the lymphoid tissue of the GI tract and might be able to influence adaptive immune responses[65,66]. $CD4^+$ T cells are imperative for mounting and regulating antigen-specific immune responses. In this study, we show for the first time that EV purified from human milk inhibit activation of $CD4^+$ T cells by αCD3/αCD28. Our data show that EV create a general immunosuppressed state, rather than inducing suppressive T_{reg}, important for antigen-specific tolerance[49]. This was indicated by reduced $CD4^+$ T cell proliferation, cytokine secretion and persistence of a naïve phenotype in the absence of phenotypic and functional T_{reg} induction. Importantly, in the absence of milk EV T cells were still capable of responding to stimulation after being exposed to milk EV. Previously, the 100,000g pellet from human milk has been reported to induce $FoxP3^+$ expression in $CD4^+$ T cells, implying T_{reg} induction[21]. Importantly, the 100,000g pellet contains immunomodulatory components besides EV[26,27,29], making it difficult to determine the specific contribution of EV. In addition, recently activated $CD4^+$ T cells are also known to upregulate $FoxP3^+$ expression[67]. Here, we found no evidence for the induction of suppressive T_{reg} by purified human milk EV. However, an increased activation threshold of T cells could indirectly contribute to the development of antigen-specific tolerance. By increasing the threshold for $CD4^+$ T cell activation, high antigen doses from human milk and the environment would result in suboptimal stimulation of the immune system. As previous studies have shown that suboptimal immune responses favor the development of antigen-specific T_{reg}[49,68,69], this could be a previously undescribed mechanism through which human milk contributes to peripheral tolerance induction. Based on our current findings, we propose that milk EV create a temporary increased threshold for T cell activation. Raising the activation threshold could be a useful mechanism to help the infant cope with the high antigenic load to which it is continuously exposed and create low inflammatory conditions more optimal for organ development[2,6,14]. In addition, it could facilitate microbial colonization of the intestine.

In summary, we propose that milk EV play a role in different functions of epithelial mucosa and provide evidence that milk EV (1) support epithelial barrier formation, (2) attenuate innate immune signaling, and (3) temporarily prevent activation of adaptive immune cells (Figure 8).

Our results demonstrate that the isolation and analysis of intact macromolecular components present in breast milk is a prerequisite to understand the mode of action of breast milk and that EV are overlooked functional entities that might play a role in the healthy development of the infant GI tract and immune homeostasis.

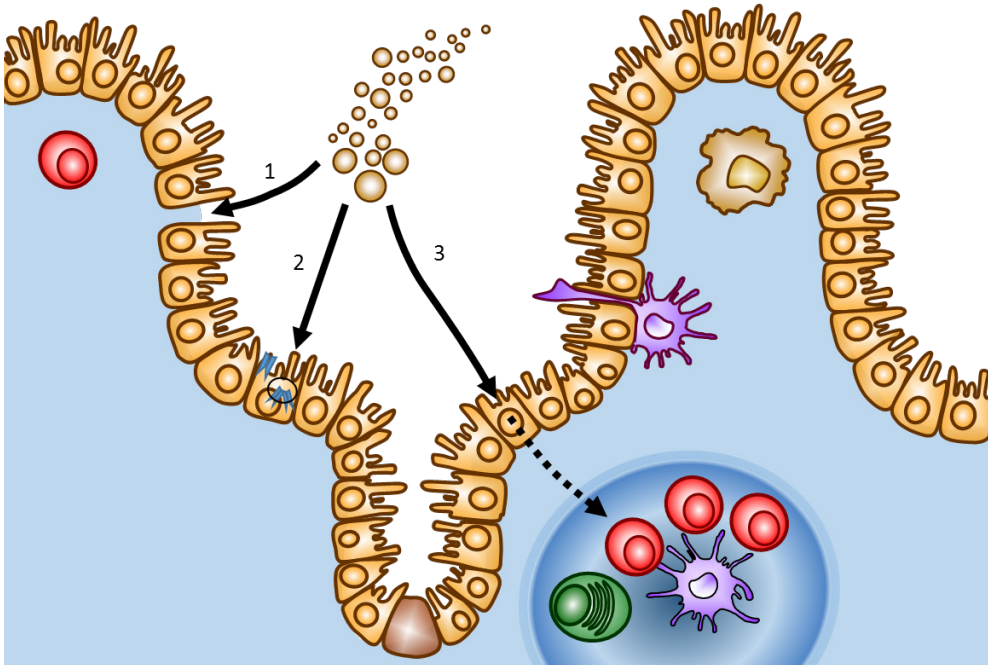

Figure 8: Proposed model for regulated development of the GI-tract and the immune system by maternal EV delivered via milk.
1) Human milk EV stimulate the development of the physical epithelial barrier by enhancing epithelial cell migration.
2) Human milk EV maintain innate immune homeostasis by attenuating TLR signaling.
3) Human milk EV maintain adaptive immune homeostasis by temporarily inhibiting $CD4^+$ T cell proliferation and activation.

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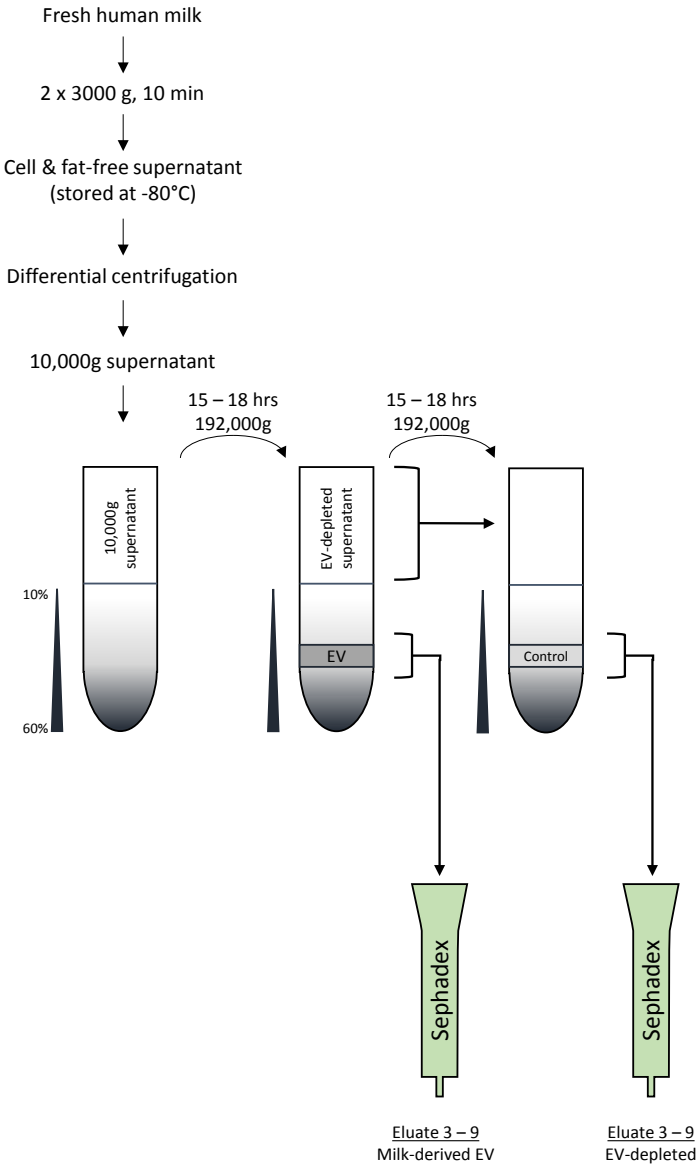

Supplementary Figure 1: schematic overview of preparation of EV and EV-depleted control conditions.
Fresh human milk is centrifuged within 20 minutes of donation and the cell & fat-free supernatant stored at -80°C until use. Thawed supernatant is further centrifuged until 10,000g supernatant is obtained. The supernatant is layered on top of an 10 – 60% iodixanol gradient in an SW40 tube and ultracentrifuged 15 – 18 hours at 192,000 g. EV containing fractions, as well as the EV-depleted supernatant are harvested. EV fractions are eluated over a column packed with Sephadex g100 to obtain EV in culture medium, free of iodixanol. Meanwhile, the EV-depleted supernatant is loaded onto a new iodixanol gradient and ultracentrifuged for 15 - 18 hours at 192,000g. The gradient is further processed identically to the EV-containing gradient in order to obtain a donor and procedure matched EV-depleted control.

Chapter 5

Extracellular vesicles from milk of allergic versus non-allergic mothers differentially affect T cell activation

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In preparation

Abstract

Allergic disease is a mounting health hazard and contributes substantially to child morbidity. Although human milk is known to support the development of neonatal adaptive immunity, it is currently unclear whether breastfeeding impacts the development of allergy. Moreover, it is unknown how the allergic status of the mother influences immune development of breastfed children. We recently identified human milk extracellular vesicles (EV) as potent inhibitors of $CD4^+$ T cell activation. Here, we used *in vitro* studies to assess functional differences between human milk EV from allergic and non-allergic mothers. In addition, the molecular composition of EV from allergic and non-allergic milk donors was analyzed using LC-MS/MS proteomics and small RNA deep sequencing. We found that EV from allergic mothers were significantly less capable of inhibiting $CD4^+$ T cell activation, indicated by increased CD25 expression, TNF-α and IL-6 production, and gene transcription of several activation associated genes, such as *CDK4*, *NFKB1*, and *NFATC1*. Omics-based comparison of the composition of EV from allergic and non-allergic mothers identified the proteins EHD4, CYFIP1, TF, PICALM, CBR3, PDZK1IP1, and RPLP2, and miRNAs miR-223-3p, miR-142-3p, miR-582-3p/5p, miR-10a-3p/5p, and miR-378e/h as promising new leads for future studies into infant allergy development. We conclude that T cells exposed to EV from allergic mothers show partial responsiveness to activation stimuli, whereas EV from non-allergic mothers cause a general block of $CD4^+$ T cell activation.

Introduction

Allergic disease is an escalating health hazard and contributes substantially to child morbidity[1–3]. Despite extensive experimental and epidemiological research, the impact of breastfeeding on allergy development remains unclear[4–12]. In order to gain full understanding of the immune modulatory mechanisms of human milk, it is important to understand the effects of its individual components. Extracellular vesicles (EV), which are nano-sized lipid bilayer enclosed intercellular communication units[13–16], were discovered in human milk in 2007[17]. However, isolation of EV from milk is technically difficult[18] and the impact of milk EV on immune development has been understudied[17,19]. Using our optimized milk EV isolation protocol, we recently uncovered a novel functional proteome for milk EV, which is distinct from other milk components[18,20]. In addition, we showed that EV from human milk can affect multiple cells the gastrointestinal (GI) tract. Milk EV were found to increase epithelial barrier function and to transiently inhibit $CD4^+$ T cell responses, keeping T cells temporally in a naïve state (Zonneveld, et al., in prep). These EV-induced effects may be beneficial for microbial colonization of the intestine, development of antigen-specific tolerance, or maintenance of the $CD4^+$ T cell repertoire[21]. It has been shown that the sensitization status of the mother influences milk composition[22–26], although functional consequences for the infant are unclear. As allergy is a $CD4^+$ T cell-driven disease, we investigated whether EV from allergic and non-allergic mothers differentially affect $CD4^+$ T cell responses. We found that EV from allergic mothers were less capable of inhibiting $CD4^+$ T cell responses than EV from non-allergic mothers. We subsequently assessed the protein and miRNA content of EV from milk of allergic and non-allergic mothers using omics-based approaches. Using stringent corrections for multiple comparisons in the proteomics and transcriptomics data, the statistical power in our experiments was too low to find significant differences in the protein and miRNA content of EV from milk of non-allergic vs. allergic mothers. However, several interesting protein and miRNA candidates were identified that will be further explored in future studies on how the sensitization status of the mother impacts the composition of EV in human milk and their effects on immune function. This study shows for the first time that EV from allergic and non-allergic mothers differ in their ability to dampen $CD4^+$ T cell activation, and provides an indication of which EV-associated molecules may be involved.

Materials & Methods

Donor selection and classification of allergic and non-allergic subjects

This study was performed as part of the Comparison of Human Milk Extracellular Vesicles in Allergic and Non-allergic Mothers (ACCESS) study (NL 47426.099.14; RTPO 914), which focusses on biomarker identification and functional analyses of EV in human milk of allergic and non-

allergic mothers. Donors were lactating women of good general health, aged 18 years or above, who had given birth to a full-term newborn via vaginal delivery. Exclusion criteria were: use of immune suppressive or immune modulatory medication, immune-related diseases (other than allergy), severe atopic dermatitis, smoking, drug abuse, pre-eclampsia, and alcohol consumption during lactation. Milk and serum samples were provided during routine hospital check-ups between 4 – 13 weeks after delivery. Donors were classified as 'Allergic' if total serum IgE ≥ 50 kU/ml and/or specific IgE was detected for grass pollen, tree pollen, house dust mite, cat dander, or dog dander by positive Phadiatop assay (Ratio > 1.0; Thermo Scientific, Uppsala, Sweden). Non-allergic donors had IgE < 50 kU/ml and Phadiatop assay was negative (Ratio ≤ 1.0). Individual donor information is provided in Table 1. All donors signed an informed consent form and the study was approved by the local medical ethics committee.

Table 1: Donor information
Data of donors used in the present study. Table lists donor ID according the ACCESS study register, age, parity, weeks after birth at time of donation, total IgE titers (kU/ml), ImmunoCAP Phadiatop ratios, allergen specific IgE in maternal serum, and whether donors were classified as non-allergic or allergic in this study. The last column lists whether donors were included in functional T cell assays (T), proteomic analysis (P), or RNA deep sequencing (R).

	Donor	Age (yrs)	Parity	Weeks after delivery	Total IgE (kU/ml)	ImmunoCAP Phadiatop Ratio	Specific IgE against allergen	Analysis performed*
Non-allergic	D1	28	2	6	6,2	0,1	all below detection limit	T, P, R
	D4	29	1	10	11,8	0,4	all below detection limit	T, P, R
	D36	31	1	5	30,4	0,1	all below detection limit	T, P, R
	D43	32	1	6	23,5	0,3	all below detection limit	P, R
	D49	27	1	10	17,6	0,3	all below detection limit	P, R
	D30	36	1	8	38,9	0,2	all below detection limit	R
	D18	35	2	13	15,8	0,2	all below detection limit	R
	D7	37	4	5	42,1	0,2	all below detection limit	R
	D10	34	4	5	24,7	0,2	all below detection limit	R
	D16	29	1	5	8,1	0,1	all below detection limit	R
	D17	31	2	5	27,8	0,2	all below detection limit	R
	D5	30	1	8	4	0,1	all below detection limit	R
	D32	27	2	7	7,3	0,3	all below detection limit	R
	D6	32	3	6	4,1	0,2	all below detection limit	R
	D15	34	3	4	9,8	0,2	all below detection limit	R
	D39	26	2	4	2,5	0,8	all below detection limit	R
	D23	26	1	10	42,4	0,2	all below detection limit	R
	D34	37	1	5	14,3	0,1	all below detection limit	R
	D11	37	3	5	42,8	0,6	all below detection limit	R
	D40	33	3	6	9,6	0,2	all below detection limit	R

Allergic	D2	29	1	5	70,3	27,2	grass pollen 0.39; tree pollen 1.9; house dust mite 9.6; cat dander <0.35; dog dander <0.35	T, P, R
	D27	30	3	8	259	13,6	grass pollen 0.53; tree pollen 0.40; house dust mite 5.2; cat dander <0.35; dog dander <0.35	T, P, R
	D33	26	1	10	65	22,8	grass pollen <0.35; tree pollen <0.35; house dust mite 12; cat dander <0.35; dog dander <0.35	T, P, R
	D20	29	2	6	28,5	21,4	grass pollen <0.35; tree pollen <0.35; house dust mite 9.9; cat dander <0.35; dog dander <0.35	P, R
	D21	29	2	6	388	91,8	grass pollen 11; tree pollen <0.35; house dust mite 75; cat dander 1.8; dog dander <0.35	P, R
	D14	34	2	6	243	53,7	grass pollen 0.97; tree pollen 40; house dust mite 4.2; cat dander <0.35; dog dander <0.35	R
	D24	35	2	4	82,8	3	grass pollen <0.35; tree pollen <0.35; house dust mite 0.70; cat dander <0.35; dog dander <0.35	R
	D8	30	2	9	73,6	13,8	grass pollen <0.35; tree pollen 7.6; house dust mite 1.0; cat dander <0.35; dog dander <0.35	R
	D31	25	1	7	81,9	27,4	grass pollen 1.3; tree pollen 7.7; house dust mite 4.4; cat dander 0.49; dog dander 1.1	R
	D19	37	4	5	87,5	45,2	grass pollen 32; tree pollen 5.0; house dust mite 6.4; cat dander <0.35; dog dander <0.35	R
	D41	34	1	5	139	2,5	all below detection limit	R
	D28	35	3	11	257	0,9	all below detection limit	R
	D37	26	1	8	11,1	1,3	grass pollen <0.35; tree pollen <0.35; house dust mite 0.53; cat dander <0.35; dog dander <0.35	R
	D44	33	2	9	37	6	grass pollen <0.35; tree pollen 0.45; house dust mite <0.35; cat dander <0.35; dog dander <0.35	R
	D35	33	1	6	49,2	4,5	grass pollen <0.35; tree pollen 0.78; house dust mite 1.2; cat dander <0.35; dog dander 0.64	R
	D29	29	1	8	226	58,6	grass pollen 0.36; tree pollen 0.35; house dust mite 41; cat dander 1.1; dog dander 0.64	R
	D22	37	2	4	11	7,9	grass pollen <0.35; tree pollen 4.3; house dust mite <0.35; cat dander <0.35; dog dander <0.35	R
	D25	32	2	6	141	6,6	grass pollen <0.35; tree pollen <0.35; house dust mite 3.8; cat dander <0.35; dog dander <0.35	R
	D42	28	1	5	20,8	8,8	grass pollen <0.35; tree pollen 9.5; house dust mite <0.35; cat dander <0.35; dog dander <0.35	R
	D45	30	1	6	75,3	0,4	all below detection limit	R

Human milk collection

Human milk was collected as previously described[18]. In brief, milk was prevented from cooling down and centrifuged twice at 3,000 g for 10 minutes at RT, within 20 minutes of collection. The cell and fat-free supernatant was then stored at -80°C and transported to the lab on dry ice for EV isolation.

Human milk EV isolation & EV-depleted control

The workflow is schematically represented in supplementary Figure 1. The milk supernatant was thawed and transferred immediately to polyallomer SW40 tubes (Beckman Coulter, Krefeld, Germany) and centrifuged at 5,000 g for 30 minutes at 4°C and subsequently at 10,000 g (Beckman Coulter Optima L-90K with a SW40Ti rotor). For proteomic analysis 6.5 ml, and for deep sequencing 3.5 – 4.0 ml aliquots of the 10,000 g supernatant were loaded on top of a 2.5 M – 0.4 M sucrose gradient in an SW40 tube. Alternatively, 6.5 ml of 10,000 g supernatant was loaded on top of a 60% – 10% iodixanol gradient (Progen Biotechnik GmbH, Heidelberg, Germany) for functional experiments. Gradients were ultracentrifuged at 192,000 g with a SW40Ti rotor (Beckman Coulter) for 15 - 18h. For functional experiments, donor-matched EV-depleted controls were prepared by harvesting 6.5 ml of the supernatant that was left on top of the gradient after the 192,000 g ultracentrifugation step described above. This EV-depleted supernatant was loaded onto a new iodixanol gradient and ultracentrifuged 15-18h and processed identically to EV-enriched samples (Supplementary Figure 1). The gradients of EV-enriched and EV-depleted samples were harvested in fractions of 500 µl. The density of the fractions was measured by refractometry and densities, previously shown to contain milk EV (iodixanol: 1.06-1.19 g/ml (data not shown); sucrose: 1.12-1.22 g/ml[18,20]), were pooled and processed further for proteomic analysis, RNA sequencing, or functional experiments.

EV preparation for T cell assays

For functional experiments, EV and EV-depleted preparations in iodixanol were processed as previously described (Zonneveld et al., in prep). In brief, iodixanol was removed from EV-enriched and EV-depleted samples by size-exclusion chromatography using a 20 ml column (Bio-Rad Laboratories, Hercules, CA, USA) packed with SephaDex g100 (Sigma-Aldrich, St. Louis, MO, USA). Samples were elutriated in 24 fractions of 1 ml with phenol red free RPMI 1640 (Gibco™, Invitrogen, Carlsbad, CA, USA). Eluates 3-9 contained EV, while iodixanol and protein contaminants were found in eluates 15 – 22. Fractions 3-9 were pooled and supplemented with 10% FCS, Ultraglutamine and pen/strep (Supplementary Figure 1). The concentration of EV used in functional assays are estimated to be within the physiological range, as volume of the 10,000 g supernatant (6.5 ml) was similar to that of the pooled eluates (7 ml).

EV isolation for LC-MS/MS mass spectrometry and RNA sequencing

EV-enriched fractions from sucrose gradient isolation were diluted with PBS (Invitrogen) and pelleted at 192,000 g in a SW40Ti rotor. Pellets were either frozen directly at -80°C until protein extraction for LC-MS/MS or resuspended in 700 µl Qiazol and frozen at -80°C until RNA isolation for sequencing.

EV protein extraction

EV pellets of 5 non-allergic and 5 allergic donors (Table 1) were resuspended in lysis buffer containing 8 M urea in 50 mM ammonium bicarbonate, 1 tablet of protease inhibitors Complete mini (Sigma-Aldrich, St. Louis, MO, USA) and 1 tablet of PhosSTOP phosphatase inhibitor mixture (Sigma-Aldrich). Samples were sonicated using a UP100H sonicator (Hielscher Ultrasound Technology, Teltow, Germany) for 6 cycles of 1 sec pulses with 30 sec pauses at 60% amplitude. Clear supernatant was collected after centrifugation of the sample at 20,000 g for 15 min at 4°C for protein isolation.

FASP protein digestion

Protein digestion was performed with filter aided sample preparation (FASP)27, with a buffer containing 8 M urea in 1 mM Tris-HCl at pH 8.0 and pH 8.5; using filters with cutoff of 10 kD (Merck Millipore, Billerica, MA, USA). Proteins were reduced with 10 mM DTT alkylated with 200 mM iodo acetamide (both from Sigma-Aldrich) and digested for 4 hrs with 2 mg/ml LysC (Wako Chemicals GmbH, Neuss, Germany) in a ratio of 1:50 and O/N with 0.1 µg/µl trypsin (Promega, Madison, WI, USA) in a ratio of 1:50. The digested samples were desalted using Oasis HLB 96-well µelution plate (Waters Chromatography B.V., Etten-leur, The Netherlands). Finally, samples were dried and reconstituted in 40 µl of 10% formic acid/5% DMSO (Sigma-Aldrich).

High resolution LC-MS/MS method

The samples were analyzed with a QExactive Plus instrument (Thermo Scientific, Waltham, MA, USA) connected to an Agilent 1260 Infinity LC system. The UHPLC system was equipped with a 20 mm x 100 µm ID Reprosil C18 (Dr Maisch HPLC GmbH, Ammerbuch, Germany) trap column and a 450 mm x 75 µm ID Poroshell C18 analytical column (Agilent Technologies, Santa Clara, CA, USA), all packed in-house. Solvent A consisted of 0.1 M acetic acid (Merck Millipore) in Milli-Q, while solvent B consisted of 0.1 M acetic acid in 80% acetonitrile (Biosolve B.V., Valkenswaard, The Netherlands). Trapping was performed at a flow rate of 5 µl/min for 10 min and peptides were eluted using a passively split flow of 180 nl/min (120 min LC method). The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS. For the QExactive analysis the 20 most intense ions in the survey scan (375 to 1600 m/z, resolution 35, 000, AGC target 3e6) were subjected to HCD fragmentation (resolution 17,500, AGC target 5e4), with the normalized collision energy set to 25% for HCD. The signal threshold for triggering an MS/MS event was set to 500 counts. The low mass cut-off for HCD was set to 180 m/z. Charge state screening was enabled, and precursors with unknown charge state or a charge state of 1 were excluded. Dynamic exclusion was enabled (exclusion size list 500, exclusion duration 18 s).

LC-MS/MS data processing

MS raw data were processed with Proteome Discoverer (version 1.4.4.14, Thermo Scientific). Generated peak lists were searched against Uniprot Homo *sapiens* database, (April 2015, 548,329 entries) supplemented with frequently observed contaminants using Mascot software version 2.4.01 (Matrix Science, London, UK). Trypsin was chosen with two missed cleavages allowed. Carbamidomethylation (C) was set as a fixed modification and oxidation (M) was set as variable modification. The searches were performed using a peptide mass tolerance of 50 ppm and a product ion tolerance of 0.05 Da (HCD), followed by data filtering using percolator, resulting in 1% false discovery rate (FDR). Only ranked 1 peptide spectral match (PSM) with Mascot scores >20 were accepted. Comparison of identified proteins between individual donors per group was done using the Venn diagrams web tool from http://bioinformatics.psb.ugent.be/webtools/Venn/. Comparison of pooled individual donors with Venn diagrams was done using FunRich[28]. P-values were calculated with an unpaired two-tailed t-test with Holm-Sidak corrections of multiple comparisons using GraphPad Prism Software (V6.07). Significance was defined as $*p < 0.05$. The annotated functions and the linked references of selected proteins were searched for in the UniProt database.

Isolation of small RNA from EV for RNA sequencing

Small RNA (< 200 nt) was isolated using the miRNeasy micro kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quality and quantity of the RNA samples was assessed using Agilent 2100 Bioanalyzer pico-RNA chips (Agilent Technologies Netherlands B.V., Amstelveen, The Netherlands).

Small RNA sequencing library preparation

Input quantity of small RNA was between 10 and 158 ng per sample. The Illumina® Truseq small RNA Sample Prep Kit was used to process the samples according to the kit-specific guidelines. Size fractions containing inserts of 20-200 nt in length were excised. The quality and yield after sample preparation was measured with the Fragment Analyzer. Small RNA sequencing was done on the Illumina NextSeq 500 platform with 75 bp single-end sequencing. A minimum of 7 million sequencing reads per sample was obtained. The sample preparations were done in two separate batches.

Small RNA sequence data analysis

Small RNA reads were processed using an in-house pipeline (https://git.lumc.nl/biopet/biopet/blob/develop/docs/pipelines/tinycap.md) which incorporates several open-source NGS tools. First, remaining adapter sequence (TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC) was checked and clipped from all sequence reads at the 3' end using cutadapt (version 1.9.1, with settings of "-a TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC –error-rate 0.1 –times 1 -m 15"). Then, the

low quality bases were trimmed from all sequence reads using sickle (version 1.33, with settings of "-t sanger -l 15"). After this QC, all sequence reads shorter than 15 bases were discarded. The processed reads were aligned to human reference genome GRCh38 using bowtie (version 1.1.1, with settings of "--best –strata –seedlen 28 –seedmms 3 -k 3"), allowing for alignments to a maximum of three loci with equal alignment scores, and a maximum of 3 mismatches.
HTSeq-count (version 0.6.1p1 with default settings) was used to count the number of aligned reads for each annotated small RNA. The small RNA annotation track was obtained from miRBase version 21.

Statistical analysis of differential miRNA expression

MiRNA-expression levels were tested for significant differential expression between the libraries of allergic vs. non-allergic milk donors using the edgeR package in R (version 3.2.4)[29]. Data were normalized using the TMM method (weighted trimmed mean of M-values). A generalized linear model was fit after estimating the common, trended (over expression values) and tagwise dispersion, with allergy status and batch as fixed effects in the model. The loglikelihood ratio test was used to evaluate differential expression between allergic and non-allergic donors. P-values were adjusted for multiple testing using Benjami and Hochberg's false discovery rate (FDR). Differences in expression with FDR ≤ 0.05 were considered to be significant. Linear associations with Phadiatop or IgE levels were evaluated using the same procedures but containing the log-transformed Phadiatop or IgE levels and batch in the generalized linear model.

$CD4^+$ T cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from Buffy coat by Lymphoprep™ density gradient centrifugation (Axis-shield, Dundee, United Kingdom). PBMC were washed and resuspended in MACSbuffer (PBS/0.5% BSA/2mM EDTA) for $CD4^+$ T cell isolation using $CD4^+$ T cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions.

T cell stimulation

Purified $CD4^+$ T cells were seeded at 0.5*10^6 cells/ml in plates coated with 0.5 µg/ml αCD3 (CLB-T3/4.E, 1XE) and 70 ng/ml soluble αCD28 (CLB-CD28/1, 15E8; both from Sanquin, Amsterdam, The Netherlands). Cells were cultured in a total volume of 1 ml consisting of medium only or of 750 µl EV or EV-depleted controls + 250 µl medium for the indicated amount of time.

Multiplex cytokine analysis

Supernatants from stimulated T cells were harvested on day 2 and day 6 of culture and analyzed using the LEGENDplex™ human Thelper 1/2/9/17 multiplex kit (BioLegend, San Diego, CA, USA). Beads were acquired on a BD Canto II (BD Bioscience) and analyzed with LEGENDplex™

V7.0. Depending on when the biggest difference between allergic and non-allergic donors was observed, the results of day 2 or day 6 are shown.

Flow cytometry

Proliferation of stimulated CD4+ T cells was assessed by labeling cells with 2 μM CellTrace Violet (Invitrogen) prior to culture. Following culture, cells were harvested and stained with fluorescent conjugated antibodies. Antibodies used in this study: CD25-Alexa488 (eBioscience, San Diego, CA, USA), CD45RA-PE, CD3-PE-Cy7, CD4-PerCP-Cy5.5, and CD45RO-APC-Cy7 (all from BioLegend). Cells were acquired on a BD Canto II (BD Bioscience), and analyzed by FlowJo (V10).

RT² Profiler PCR array

Total RNA was extracted from CD4+ T cells after 16 hours of culture using the RNeasy Mini prep Kit and cDNA was synthesized using the RT² First Strand Kit according to the manufacturer's instructions and added to the Human T cell Tolerance & Anergy RT² Profiler PCR array (all from Qiagen, Hilden, Germany). The array was run on an iCycler MyiQ (Bio-Rad). The relative expression levels of each gene were normalized to the expression level of 4 reference genes (B2M, GAPDH, HPRT1, RPLP0) included in the array. Delta Ct-values were log-transformed and analyzed using Graphpad Prism software V6.07.

Statistical analysis of T cell assays

Differences between EV and EV-depleted samples and differences between allergic and non-allergic donors were analyzed simultaneously by repeated measures two-way ANOVA and Sidak's multiple comparisons test. Calculations were made in GraphPad Prism Software V6.07. Significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Results

Human milk EV from allergic versus non-allergic mothers differentially affect CD4+ T cell activation

CD4+ T cells are instrumental in the development of allergic disease. We previously showed that EV from human milk are potent inhibitors of CD4+ T cell activation (Zonneveld, et al. manuscript in prep). In order to determine whether the allergic status of the mother impacted this function of EV, CD4+ T cells were stimulated with α-CD3/CD28 in the presence of EV or EV-depleted controls from milk of non-allergic or allergic mothers. Compared to donor-matched EV-depleted controls, EV significantly inhibited CD4+ T cell proliferation and CD25 upregulation, regardless of allergic status of the mother (Figure 1A and 1B). Additionally, transition from naïve CD45RA+ T cells towards memory or effector CD45RO+ T cells was suppressed by EV (Supplementary Figure

2). Interestingly, CD4+ T cells exposed to EV of allergic donors showed a significantly increased expression of CD25 compared to those exposed to EV from non-allergic donors (Figure 1A), indicating reduced inhibition of CD4+ T cell responses. In addition, CD4+ T cells exposed to EV of allergic donors showed a trend towards increased proliferation, as demonstrated by the dilution of the cytoplasmic dye CellTrace Violet (CTV; Figure 1B). The expression of CD45RA and CD45RO was not significantly altered between T cells exposed to EV from allergic or non-allergic donors (Supplementary Figure 2). Analysis of cytokine profiles showed that secretion of the pro-inflammatory cytokines IL-6 and TNF-α was significantly increased in T cells exposed to EV from allergic mothers (Figure 2). For the other cytokines tested, no differences were observed between T cells exposed to allergic or non-allergic EV, with EV from both groups inhibiting overall cytokine secretion compared to EV-depleted controls (Supplementary Figure 3). Importantly, no differences were observed in activation markers or cytokine secretion between CD4+ T cells incubated with EV-depleted controls of allergic or non-allergic mothers (Figure 1 and 2, Supplementary Figure 2 and 3). Thus, differential effects on CD4+ T cell activation and cytokine secretion are specific to EV derived from the milk samples.

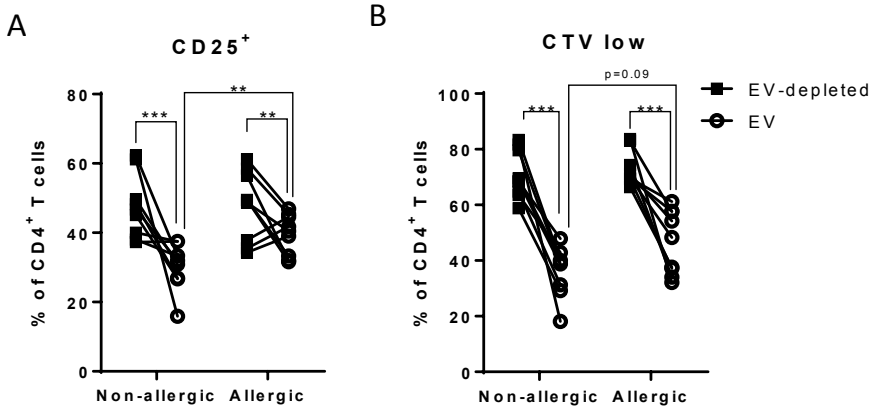

Figure 1: CD4+ T cells exposed to EV from allergic mothers vs. non-allergic mothers display a higher activation status.
CD4+ T cells of 3 different donors were labeled with CellTrace Violet (CTV) and stimulated with CD3/CD28 in the presence of EV (o) or its EV-depleted control sample (▪) from allergic (n=3) or non-allergic (n=3) mothers for 6 days. Cells were harvested and stained with anti-CD25-A488 and analyzed by flow cytometry. Graphs show the paired results obtained for each EV donor and its EV-depleted control.
A) Quantification of the percentage of CD4+ T cells which showed a positive staining for CD25.
B) Quantification of the percentage of CD4+ T cells which had undergone one or more proliferation cycles, and were thus lower in CTV signal intensity than non-proliferating T cells.
Significance was calculated using repeated measures two-way ANOVA with Sidak's multiple comparisons test. P-values are defined as **p <0.01 and ***p < 0.001.

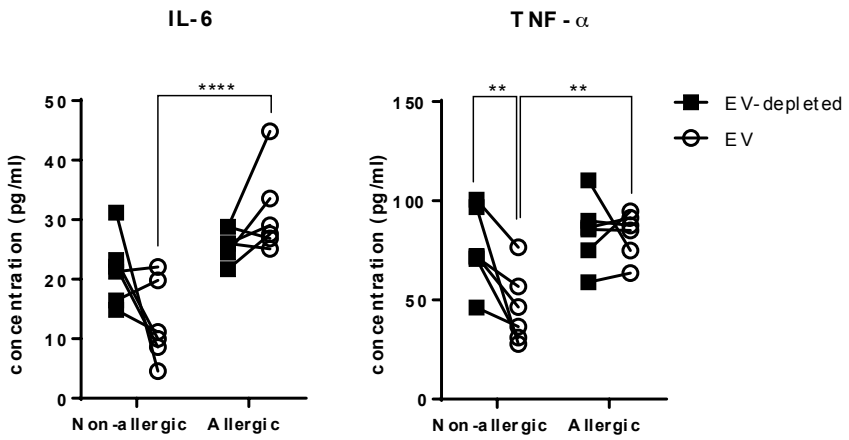

Figure 2: CD4⁺ T cells exposed to EV from allergic mothers secrete more pro-inflammatory cytokines. CD4⁺ T cells of 2 different donors were stimulated with CD3/CD28 in the presence EV (o) or its EV-depleted control sample (▪) from allergic (n=3) or non-allergic (n=3) mothers for 48 hours after which supernatants were harvested and analyzed for cytokines. Graphs show the paired results obtained for each EV donor and its EV-depleted control in pg/ml. Significance was calculated using repeated measures two-way ANOVA with Sidak's multiple comparisons test. P-values are defined as **p < 0.01 and ****p < 0.0001.

In order to determine whether EV from allergic or non-allergic donors induced a different transcriptional program in CD4⁺ T cells, we analyzed the expression of genes involved in T cell activation, anergy, and tolerance. Transcription of several genes associated with T cell activation were inhibited specifically by EV from non-allergic mothers, but not by EV from allergic mothers (Figure 3). These genes included transcription factors (*JUN, NFKB1, NFATC1*), co-stimulatory and adhesion molecules (*CD40, CD40LG, ICAM1, ICOS, TNFRSF4, TNFRSF18, TNFSF14*), cyclin-dependent kinases (*CDK4*), growth factors (*CSF2, TGFB1*), cytokines (*IL5, IL6, IL13, LTA*), and cytokine receptors (*IL2RA*; Figure 3). Despite the observed increase in transcription of *IL5, IL6,* and *IL13,* which is associated with a Th2 phenotype[30], IL-5 and IL-13 protein secretion was not significantly increased under the influence of allergic milk-EV (Supplementary Figure 3). In contrast, increased transcription levels of *IL6* and *IL2RA* (mRNA encoding CD25) were confirmed at the protein level (Figures 1A and 2A). Gene expression of CD4⁺ T cells exposed to EV-depleted samples did not significantly differ between groups, with the exception of *IL7R*, which was increased under the influence of EV-depleted samples of allergic mothers (Supplementary Figure 4). This indicates that certain components in the EV-depleted control are capable of inducing immune responses and underscores the necessity of taking along this control for the accurate interpretation of EV-mediated effects[31]. Together, these data indicate that EV from allergic donors are less capable of inhibiting CD4⁺ T cell activation and cytokine release than EV from non-allergic mothers. No clear indications were found that EV from allergic mothers skewed towards a Th1 or Th2 phenotype. As milk EV from allergic and non-allergic mothers differentially affected CD4⁺ T cell activation, we next investigated whether EV of these two groups differed in their molecular composition.

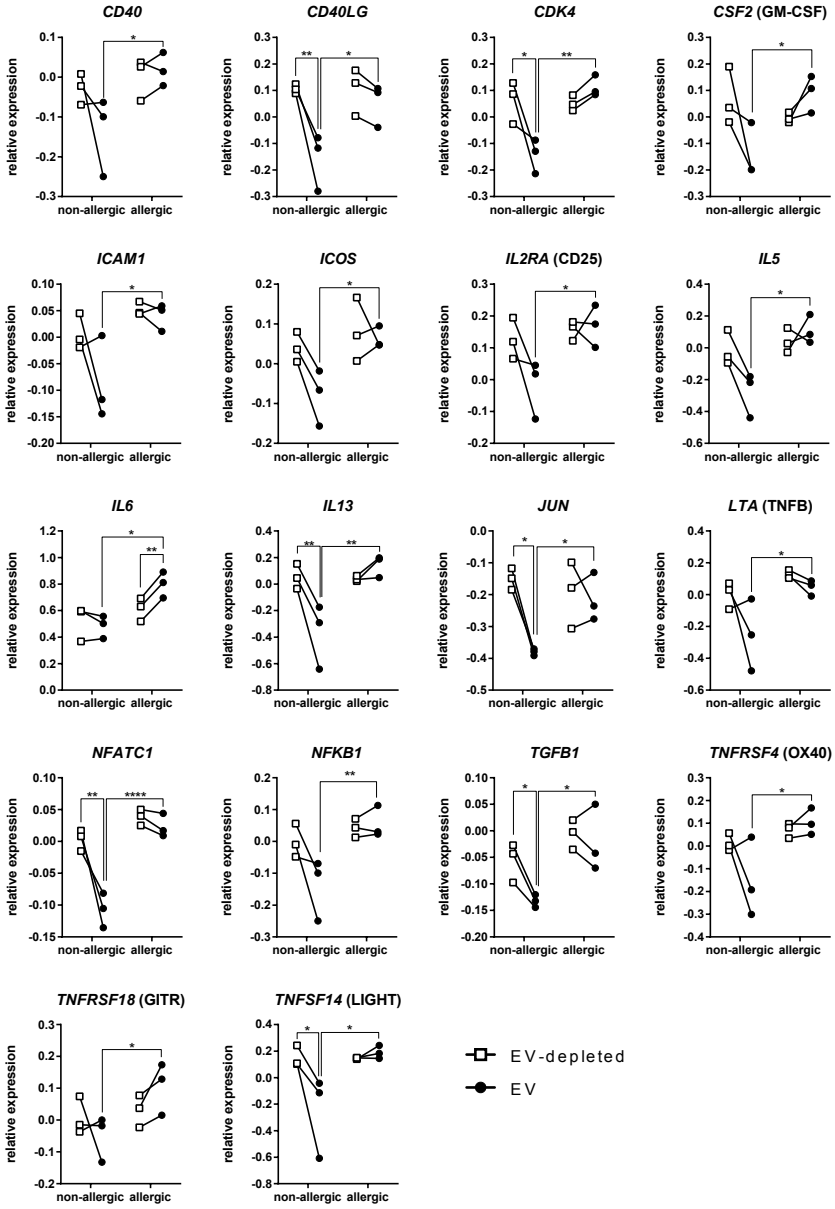

Figure 3: CD4⁺ T cells exposed to EV from allergic mothers vs. non-allergic mothers increase the transcription of activation associated genes.

CD4⁺ T cells were stimulated with CD3/CD28 in the presence of EV (●) or their EV-depleted controls (□) from allergic (n=3) or non-allergic (n=3) mothers for 16 hours after which T cell RNA was extracted and gene expression analysis was performed. Gene expression was normalized to 4 housekeeping genes and delta Ct-values to CD3/CD28 stimulated T cell controls were calculated and log-transformed (CD3/CD28 control values are 0). Shown are the paired results of each EV donor with its EV-depleted control. Significance was calculated using repeated measures two-way ANOVA with Sidak's multiple comparisons test and p-values defined as *p<0.05, **p<0.01, and **** p < 0.0001.

Milk EV from non-allergic and allergic mothers contain potent immune-modulatory miRNA species

EV are known to contain small RNAs, such as miRNAs, which are potent regulators of cell signaling[32]. First, we determined which portion of extracellular small RNAs in milk was EV-associated. To this end, the 10,000 g supernatant was loaded on top of a sucrose density gradient and subjected to high-speed density gradient ultracentrifugation. In this gradient, EV migrate to low density fractions, while protein aggregates and nucleoprotein complexes containing RNA reside in high-density fractions at the bottom of the tube. Next, we isolated small RNA from the protein pellet, as well as from the EV-containing fractions. We consistently found the majority of extracellular small RNA, ranging from 56 – 81% of the total yield of small RNAs to be associated with EV (Supplementary Figure 5). Subsequently, we performed next-generation RNA deep sequencing on EV-associated small RNAs from milk of non-allergic or allergic mothers (n = 20 donors per group). In total, 578 different miRNA species were detected, with no unique species detected in either group. Ranking the top 20 most abundant miRNA species in EV of non-allergic and allergic donors showed remarkable similarities between the two groups. Many of these miRNAs are known to play immune modulatory roles in T cell activation and differentiation, and Toll-like receptor responses and NF-κB signaling[33–48] (Figure 4A). This may suggest that one of the main functions of EV-associated miRNA in milk is the maintenance of immune homeostasis. Next, we searched for miRNAs which were differentially present in EV of allergic vs. non-allergic mothers. A high degree of similarity was observed between the relative abundance (average counts per million (cpm)) of miRNAs present in milk EV from allergic versus non-allergic mothers (Figure 4B). However, 9 miRNAs were found to differ between the two groups with a fold change >2 and with non-adjusted p-values of < 0.05 (Figure 4C). A total of 6 miRNAs were enriched in EV of non-allergic mothers, i.e. miR-223-3p, miR-142-3p, miR-378e, miR-3614-5p, and miR-582-3p/5p. The 3 miRNAs enriched in EV of allergic mothers were miR-378h and miR-10a-3p/5p. However, none of these miRNAs reached statistical significance after multiple testing correction due to lack of power in the current study (Table 2). Follow-up studies are therefore needed to validate whether the miRNA candidates described above truly correlate with the allergic stage of the milk donors.

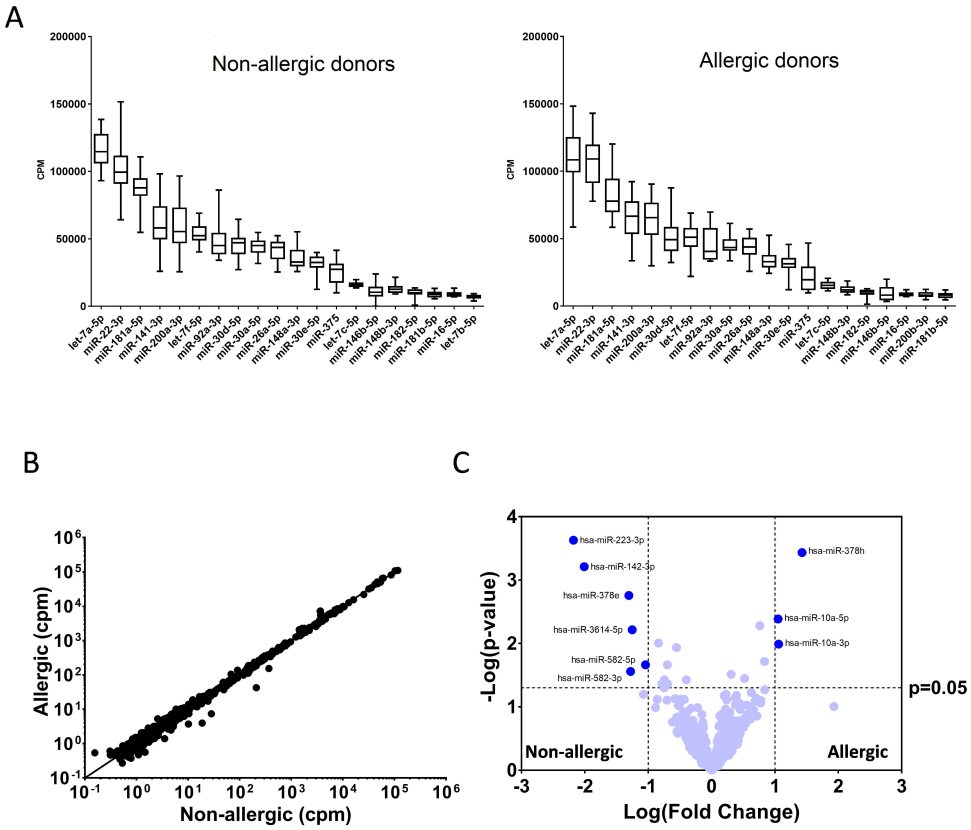

Figure 4: miRNA content of EV from allergic and non-allergic mothers is similar.
RNA deep sequencing was performed on milk EV of allergic (n=20) and non-allergic (n=20) mothers and the abundance of detected miRNA species expressed as counts per million (cpm) total reads annotated to miRNAs.
A) Ranking of the top 20 most abundant miRNAs detected in EV of non-allergic (left panel) and allergic donors (right panel) based on cpm values. Shown are box and whiskers plots showing the mean, minimum and maximum cpm values detected in 20 donors.
B) Scatterplot showing the average cpm of non-allergic (x-axis) versus allergic (y-axis) samples of every miRNA species detected. A line was plotted with y = x to facilitate determination of quantitative differences between allergic and non-allergic groups.
C) Volcano-plot indicating the log fold change (allergic vs non-allergic) of the averaged cpm values for each miRNA (x-axis) plotted against the -log of the non-adjusted p-value (y-axis). The horizontal dashed line indicates a non-adjusted p-value of 0.05. Indicated in dark blue are miRNA species which were found to differ > 2-fold between allergic and non-allergic EV samples and which had a non-adjusted p-value < 0.05. Significance was calculated using the loglikelihood ratio test without correcting for multiple testing.

Table 2: List of miRNAs found to be most different between allergic and non-allergic mothers
RNA deep sequencing was performed on milk EV of allergic (n=20) and non-allergic (n=20) mothers. The abundance of detected miRNAs was expressed as cpm and the mean per miRNA was compared between groups. Significant differences were calculated using the loglikelihood ratio test (p-values), or the loglikelihood ratio test corrected for multiple testing with Benjami and Hochberg's false discovery rate (Adj. p-values).

miRNA	P-value	Adj. p-value
hsa-miR-223-3p	0.000235194	0.106714785
hsa-miR-378h	0.000369255	0.106714785
hsa-miR-142-3p	0.000616806	0.118837867
hsa-miR-378e	0.001750164	0.252898649
hsa-miR-10b-5p	0.004117633	0.397643486
hsa-miR-10a-5p	0.004127787	0.397643486
hsa-miR-940	0.005240948	0.432752523
hsa-miR-3614-5p	0.006096279	0.440456145
hsa-miR-556-5p	0.009864298	0.592335493
hsa-miR-10a-3p	0.010248019	0.592335493
hsa-miR-548ah-5p	0.011621889	0.610677432
hsa-miR-4449	0.019227043	0.896221347
hsa-miR-582-5p	0.021704862	0.896221347
hsa-miR-3929	0.021707783	0.896221347
hsa-miR-582-3p	0.027700441	0.952214948
hsa-miR-423-5p	0.030618406	0.952214948
hsa-miR-1285-3p	0.035737516	0.952214948
hsa-miR-6726-3p	0.037451579	0.952214948
hsa-miR-32-3p	0.037486718	0.952214948
hsa-miR-3177-3p	0.043451464	0.952214948
hsa-miR-6853-3p	0.043844369	0.952214948
hsa-miR-504-5p	0.049953101	0.952214948

Human milk-derived EV from allergic and non-allergic mothers contain proteins that can affect proliferation

In addition to exploring the EV-RNA content, we assessed whether differences in protein composition of EV in milk from allergic and non-allergic mothers could clarify differences in $CD4^+$ T cell activation. Proteomic analysis revealed a total of 1148 proteins identified in non-allergic EV, and 1071 in allergic EV (Figure 5A and 5B). A similar degree of variation was observed between individual donors regardless of allergy status (Figure 5A). Upon pooling all proteins identified in each group, overlay analysis showed a considerable overlap of 951 proteins (Figure 5B). Additionally, 197 proteins were exclusively identified in EV isolated from non-allergic mothers and 120 proteins in EV from allergic mothers, suggesting distinct protein profiles between these groups. However, very few of these unique proteins were identified robustly in all donors of each group (data not shown), which suggests that donor variation is a significant contributor to these profiles. In addition, we compared the relative abundance of individual proteins between groups

using the peptide spectral matches (PSM). EDH4, CYFIP1, TF, PICALM, and CBR3 were enriched in EV from allergic donors (Figure 5C, left of dashed line), while PDZK1IP1 and RPLP2 were enriched in the non-allergic group (Figure 5C, right of dashed line). Remarkably, 4 of these 7 proteins have been reported to be involved in cellular proliferation (Table 3), which could partially explain their differential impact on CD4⁺ T cells. Although these proteins are interesting candidates for further investigation, the differences between allergic and non-allergic groups did not reach significance following multiple testing correction.

Taken together, our data show that the maternal allergy status impacts the ability of milk-derived EV to inhibit CD4⁺ T cell activation, but that no significant miRNA or protein candidates have been uncovered yet that can explain the functional differences between EV from allergic and non-allergic mothers.

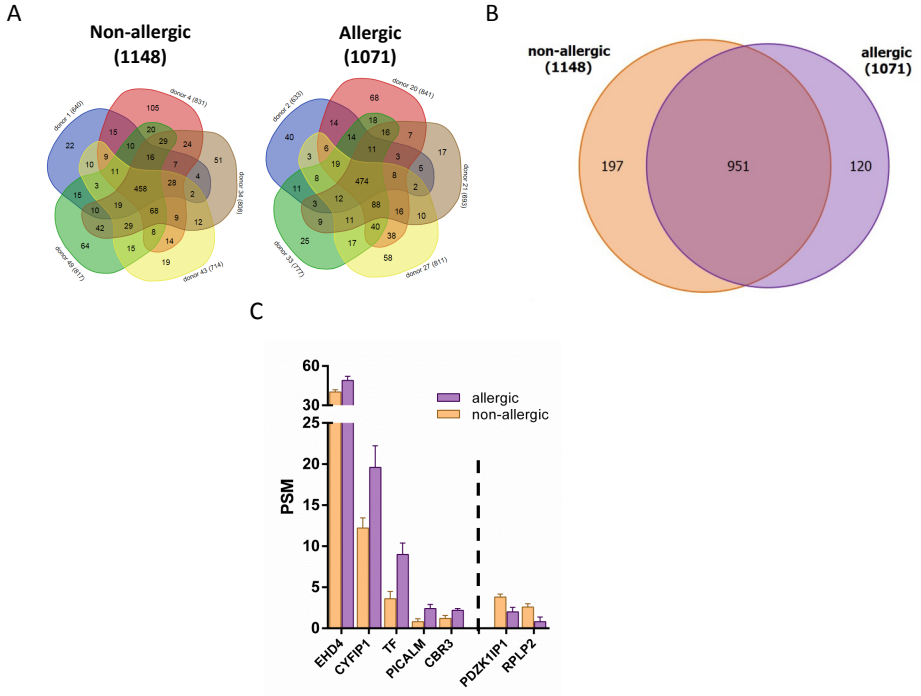

Figure 5: The variation in protein distribution is similar between allergic and non-allergic donors
A) Venn diagrams comparing uniquely identified and shared proteins between EV of non-allergic (n=5) and allergic (n=5) mothers. The number of proteins identified per donor is given, as well as the total number of proteins identified per group.
B) Comparison of the total milk-derived EV proteome from non-allergic mothers to allergic mothers reveals a considerable overlap between groups, with 197 uniquely identified proteins in non-allergic donors and 120 proteins in allergic mothers.
C) The average peptide spectral match (PSM) of individual proteins was used to compare relative protein abundance per group. Shown are the proteins which were significantly different in PSM between groups using an unpaired two-tailed t-test without correcting for multiple testing. Significance was classified as $p < 0.05$. Protein description and functions are depicted in Table 3.

Table 3: Protein description and UniProt annotation of candidate protein biomarkers
Description and function of proteins that were significantly different in PSM between milk-derived EV from breast milk from non-allergic (n=5) and allergic (n=5) mothers. For each protein the description and references used to annotate their function as described in UniProt are provided. Additionally, the number of donors in which the protein was identified and the p-value calculated in Figure 5C are shown.

GeneID	Protein	Description	Functions	Reference	identified in		Non. Adj. P-value
					non-allergic	allergic	
30844	EHD4	EH-domain containing 4	regulation of early endosomal transport	75	5/5	5/5	0.040
23191	CYFIP1	cytoplasmic FMR1 interacting protein 1	involved in cell proliferation	71	5/5	5/5	0.034
			involved in neuronal development	76			
7018	TF	Serotransferrin	involved in iron binding transport	77	5/5	5/5	0.011
			stimulates cell proliferation	72			
8301	PICALM	phosphatidylinositol binding clathrin assembly protein	coated-pit formation	78	3/5	5/5	0.035
			affects TF receptor and proliferation	73			
874	CBR3	carbonyl reductase 3	oxidoreductase activity	79	4/5	5/5	0.046
10158	PDZK1IP1	PDZK1 interacting protein 1	decreases cell proliferation	74	5/5	5/5	0.027
6181	RPLP2	ribosomal protein, large, P2	catalyzes protein synthesis	80	5/5	2/5	0.034

Discussion

Maternal sensitization is known to alter the composition of milk[22,49,50], although the functional consequences for the infant are not always clear. Recently, we have identified extracellular vesicles present in human milk as potent inhibitors of CD4+ T cell activation (Zonneveld, et al. in prep). As allergy is a CD4+ T cell-driven disease, we here studied the impact of EV from non-allergic and allergic mothers on CD4+ T cell activation under non-skewing conditions. We found that, although EV from milk of both allergic and non-allergic mothers inhibited T cell activation compared to EV-depleted controls, EV of allergic mothers were significantly less effective in suppressing T cell responses. Data supporting this conclusion include increased levels of the IL-2 receptor CD25, as well as increased secretion of the potent pro-inflammatory cytokines TNF-α and IL-6 in the presence of allergic EV. In addition, CD4+ T cells stimulated in the presence of EV from allergic mothers significantly increased transcription of several activation-associated genes and showed a trend towards increased T cell proliferation. Overall, our data suggests that EV from allergic mothers are less potent suppressors of CD4+ T cell activation.

In this study, no clear skewing of CD4+ T cells towards a Th1 or Th2 phenotype was seen under the influence of EV from allergic donors. While increased transcription of *IL5, IL6*, and *IL13* under the influence of EV from allergic mothers could indicate a Th2 phenotype, only IL-6 protein secretion was confirmed to be augmented (Figure 2 and Supplementary Figure 3). This suggests that EV from allergic mothers do not have the intrinsic capacity to skew T cell responses towards Th1 or Th2 immunity. Moreover, no significant differences in EV composition were identified that pointed towards a preference for induction of a Th1 or Th2 phenotype. However, the results presented here do provide new insights into previously published data. Most reports have described that their study outcome, be it aggravating or protective of allergy, is more pronounced in children breastfed by sensitized mothers[4,5,51,52]. Baïz, *et al*. have suggested a role for the antigen itself to explain discrepancies from their own group between tolerance induction and sensitization[11,51–53]. Based on our results, we propose that CD4+ T cells receiving stimulatory cues are attenuated by human milk EV, and as EV from allergic mothers are less potent inhibitors of CD4+ T cell responses, T cell responses will be stronger in individuals breastfed by allergic mothers. Whether this ultimately results in a skewing of T cell responses in e.g. Th1 or Th2 responses needs to investigated.

In an attempt to pin-point the cause of the difference in inhibitory capacity of EV on CD4+ T cell responses, we performed in-depth characterization of the miRNA and protein content of EV from allergic and non-allergic mothers. The top 20 most abundant miRNA species in both groups principally consisted of immune modulatory miRNAs[33–48], which could partially account for the immunosuppressive effect of milk EV on CD4+ T cells. In this top 20 ranking, we noticed a high degree of overlap with miRNA analyses presented in other studies, in which RNA from less pure EV preparations or even whole milk was analyzed[54–56]. This could indicate that these 20 most

abundant EV-associated miRNAs represent a high percentage of the total pool of extracellular small RNAs in milk. Alternatively, it is possible that the same miRNA species are allocated to multiple macromolecular structures[54]. This redundancy, along with the low degree of variation between donors and between studies, would imply an important and highly conserved function for these miRNAs in the infant.

The donors used in this report were enrolled in the ACCESS study, which aims to define milk EV-based biomarkers for allergy development in the infant. Due to a lack of power, no significant differences were detected in protein or miRNA content of EV of allergic and non-allergic mothers using omics-based approaches with n = 20 and n = 5 donors per group for RNA sequencing and proteomics, respectively. While no statistical significance was reached, 9 miRNAs and 7 proteins were found to be differentially present in EV from allergic or non-allergic donors, with non-adjusted p-values < 0.05 (Figures 4C & 5C). Below, we list considerations to include or exclude these molecules in future studies on milk EV-based biomarkers for allergy. Both miR-223-3p and miR-142-3p were found to be more abundant in non-allergic donors (Supplementary Figure 6A) and have been shown to downregulate IL-6, TNF-α, as well as CXCL-8[57–62], which fits our present data that non-allergic EV, in contrast to allergic EV, inhibit IL-6 and TNF-α secretion (Figure 2). Moreover, we recently showed that human milk EV inhibited both IL-6 and CXCL-8 in TLR3 stimulated epithelial cells, indicating that milk EV might use these same mechanisms in different cell systems (Zonneveld et al, in prep). Although the possible functions of these miRNAs make them interesting candidates to validate as biomarkers, further inspection of the cpm values revealed that the mean fold increase of these miRNAs seem to depend disproportionately on 2 or 3 donors (Supplementary Figures 6A and 6B). In addition, no correlation to serum IgE titers or Phadiatop ratio could be discerned (Supplementary Figure 6A and 6B). Nevertheless, these miRNAs might play an important role in the overall immune modulatory effect of human milk EV. Other miRNAs with higher abundance in EV from non-allergic mothers include miR-582-3p and miR-582-5p, which have been linked to reduced cellular proliferation[63], along with miR-142-3p[64]. In addition, a target of miR-582 is FoxO1[65], the downregulation of which has been shown to ameliorate allergic asthmatic inflammation[61]. However, overall cpm of these miRNA species were low (Supplementary Figure 6C and 6D), which negatively impacts their reliability as biomarker.

In EV from allergic mothers, increased levels of miR-10a-3p and 5p were observed (Figure 4C). These miRNAs have been reported to be expressed in high amounts in natural regulatory T cells (T_{reg}), while they are present in low amounts in inducible T_{reg} and differentiated T cells[66–68]. It is possible that EV introduce miR-10a to T cells in order to retain the naïve T cell repertoire in allergic mothers, despite increased T cell activation. Additionally, miRNA-10a shows a reasonable correlation to IgE and Phadiatop (Supplementary Figure 6E and 6F) making it an interesting candidate for further study.

Different family members of miR-378 were found to be upregulated in either allergic or non-allergic groups (Figure 4C). Although little is known about the miR-378h and miR-378e forms,

based on homology with miR-378a, vimentin is a possible target[69]. Vimentin has been shown to interact with p38 MAPK to mediate CCL2 production by mast cells[70]. We have previously demonstrated that human milk EV enhanced epithelial migration through p38 MAPK (Zonneveld et al in prep). Thus, it is possible that miR-378h/e act on vimentin to attenuate p38 MAPK signaling. Although overall cpm of miR-378 was low, miR-378h showed a reasonable correlation to IgE titers and Phadiatop ratio (Supplementary Figure 6G and 6H).

Finally, several proteins which were found more abundant in allergic EV have been described to be involved in cellular proliferation, such as CYFIP1, TF and PICALM[71–73] (Figure 5C & Table 3). In contrast, PDZK1IP1 has been shown to decrease cell proliferation and is found more abundant in non-allergic EV[74] (Figure 5C and Table 3). Although milk EV from allergic donors did not increase $CD4^+$ T cell proliferation significantly, replication often correlates with T cell activation. As overall activation of $CD4^+$ T cells was increased by human milk EV from allergic donors, it would be interesting to determine whether these proteins play a significant role in these processes.

Overall, the miRNAs and proteins discussed above provide a starting point for further study aimed at validating their differential presence and function in milk samples from allergic and non-allergic mothers. Ultimately, we will attempt to correlate the RNA and protein data to the allergic outcome of the infants within the ACCESS study, which could lead to a predictive marker in human milk for infant allergy development.

The results presented here provide the first evidence that EV from allergic and non-allergic mothers vary in their capacity to functionally inhibit T cell activation. Whether this is detrimental or beneficial for infant immune development is not yet known and most likely depends on additional factors in milk, the environment, and/or genetic predisposition. The results obtained in this study increase our understanding of the development of $CD4^+$ T cell immunity, and helps focus research on defining T helper skewing factors in the environment, diet, and breast milk, which act in concert with human milk EV in the development of neonatal immunity.

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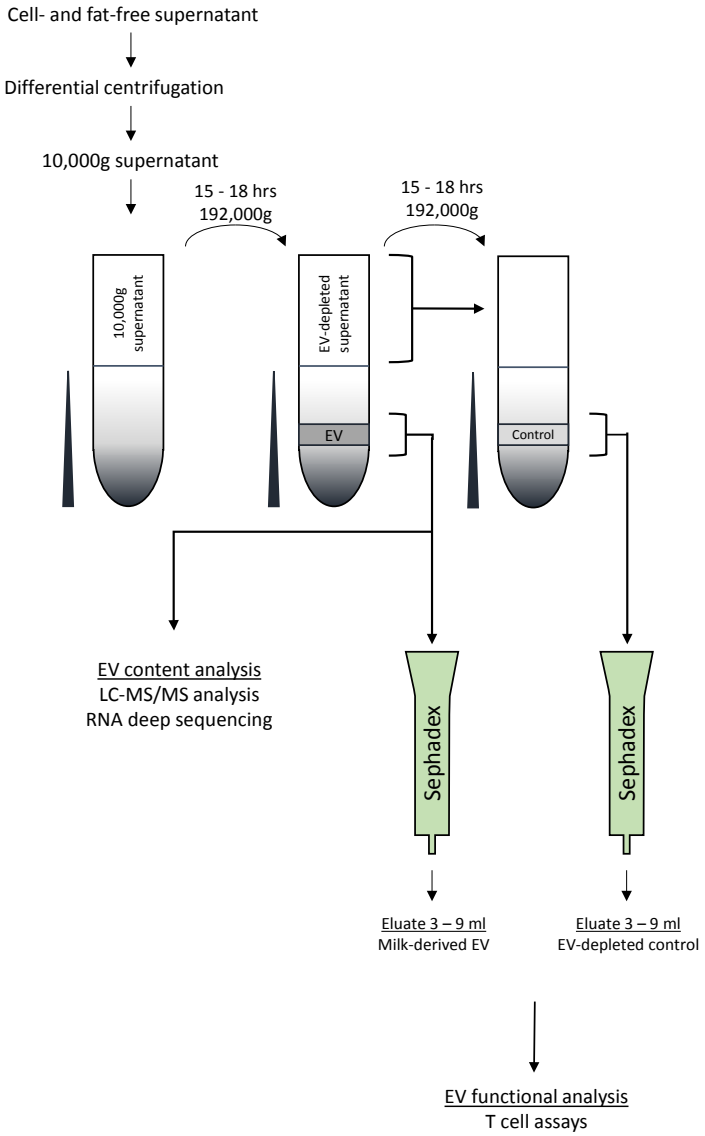
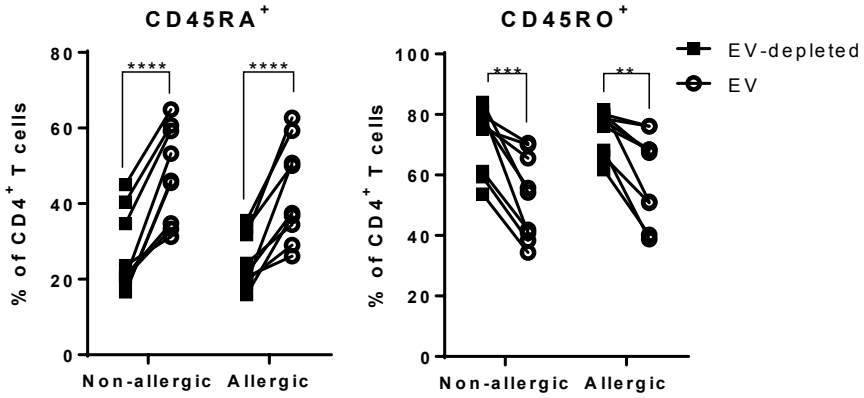
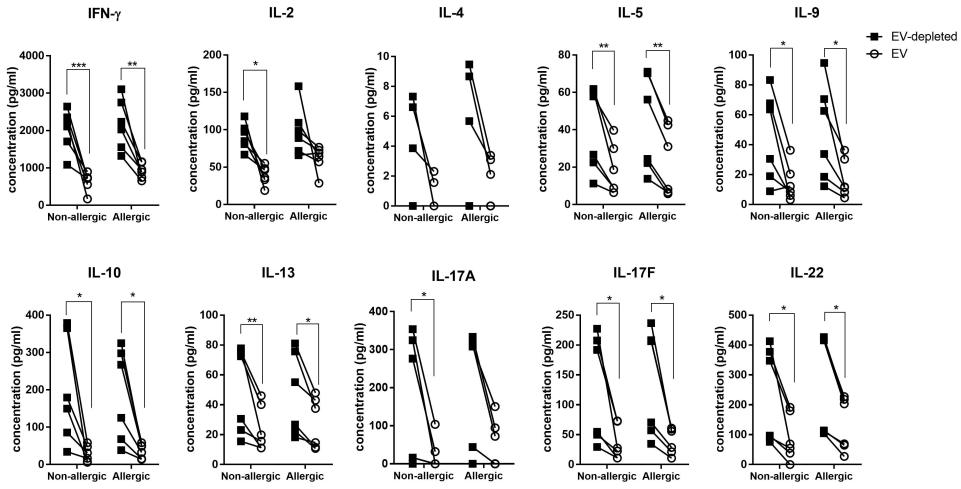


Supplementary Figure 1: schematic overview of the preparation of EV and EV-depleted control conditions.
Fresh human milk was centrifuged within 20 minutes of donation and the cell & fat-free supernatant stored at -80°C until use. Thawed cell- and fat-free supernatant was further centrifuged until 10,000g supernatant was obtained. The supernatant was layered on top of an iodixanol gradient (T cell assays) or a sucrose gradient (LC-MS/MS and RNA deep sequencing) in an SW40 tube and ultracentrifuged 15 – 18 hours at 192,000 g. EV fractions were harvested and protein and RNA content analyzed or used in functional experiments. For functional experiments, a donor-matched EV-depleted control was prepared by harvesting EV-depleted supernatant and loading it onto a new iodixanol gradient and ultracentrifuging for 15 – 18 hours at 192,000g. Fractions from this 'EV-depleted' density gradient corresponding to EV-enriched samples were harvested. To remove Iodixanol, EV-enriched and EV-depleted samples were both eluted over a column packed with Sephadex g100.



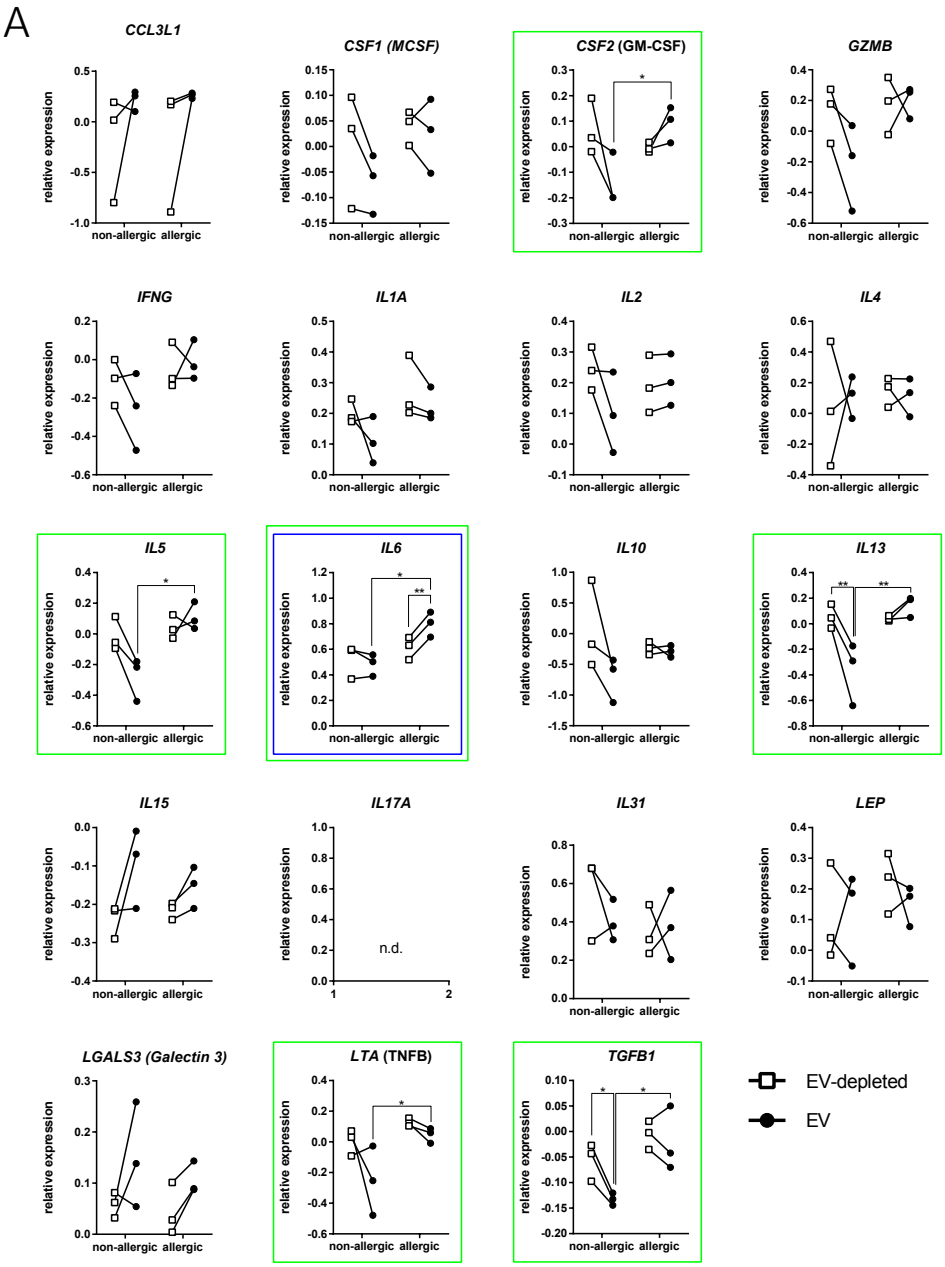
Supplementary Figure 2: Human milk EV from non-allergic and allergic mothers both prevent CD45RA to CD45RO transition of CD4⁺ T cells

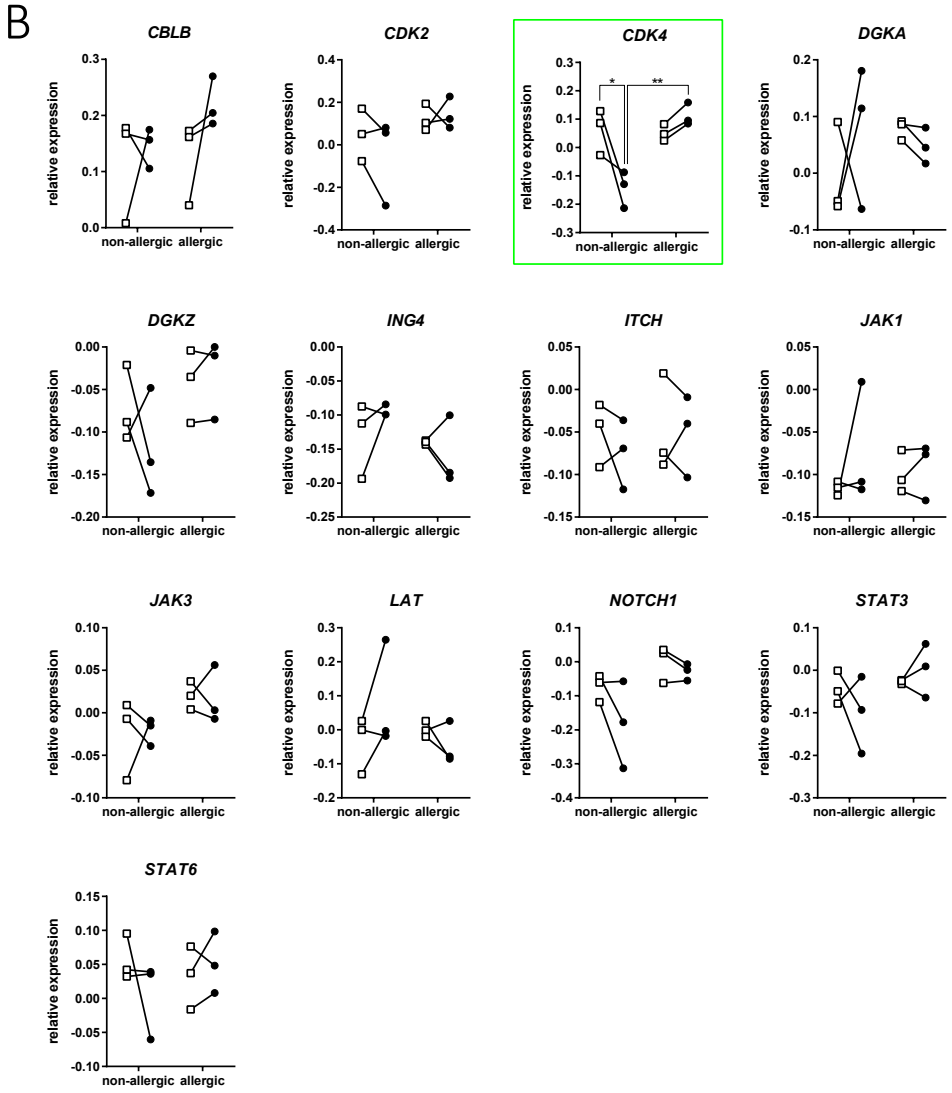
CD4⁺ T cells of 3 different donors were stimulated with CD3/CD28 in the presence of EV (o) or its EV-depleted control sample (▪) from allergic (n=3) or non-allergic (n=3) mothers for 6 days. Cells were harvested and stained with anti-CD45RA-PE or anti-CD45RO-APC-Cy7 and analyzed by flow cytometry. Plotted are the percentage of CD4⁺ T cells which showed a positive staining for CD45RA (left panel) and CD45RO (right panel). Graphs show the paired results obtained for each EV donor and its EV-depleted control. Significance was calculated using repeated measures two-way ANOVA with Sidak's multiple comparisons test. P-values are defined as **p <0.01, ***p < 0.001, and ****p < 0.0001.



Supplementary Figure 3: Human milk EV from non-allergic and allergic mothers inhibit secretion of various Th1/2/9/17 related cytokines by CD4⁺ T cells

CD4⁺ T cells of 2 different donors were stimulated with CD3/CD28 in the presence EV (o) or its EV-depleted control sample (▪) from allergic (n=3) or non-allergic (n=3) mothers for 6 days. Supernatants were harvested and analyzed for cytokines after 2 and 6 days of culture. Except for IL-2 and IL-4, data of day 6 is shown. Choice for day 2 or day 6 was made depending on when the optimal resolution between EV of allergic or non-allergic donors was obtained. Graphs show the paired results obtained for each EV donor and its EV-depleted control in pg/ml. Significance was calculated using repeated measures two-way ANOVA with Sidak's multiple comparisons test. P-values are defined as * < 0.05, **p < 0.01 and ***p < 0.001.


A
CCL3L1
CSF1 (MCSF)
CSF2 (GM-CSF)
GZMB
IFNG
IL1A
IL2
IL4
IL5
IL6
IL10
IL13
IL15
IL17A
IL31
LEP
LGALS3 (Galectin 3)
LTA (TNFB)
TGFB1
relative expression
non-allergic allergic
n.d.
EV-depleted
EV


B
CBLB
CDK2
CDK4
DGKA
DGKZ
ING4
ITCH
JAK1
JAK3
LAT
NOTCH1
STAT3
STAT6
relative expression
non-allergic
allergic
*
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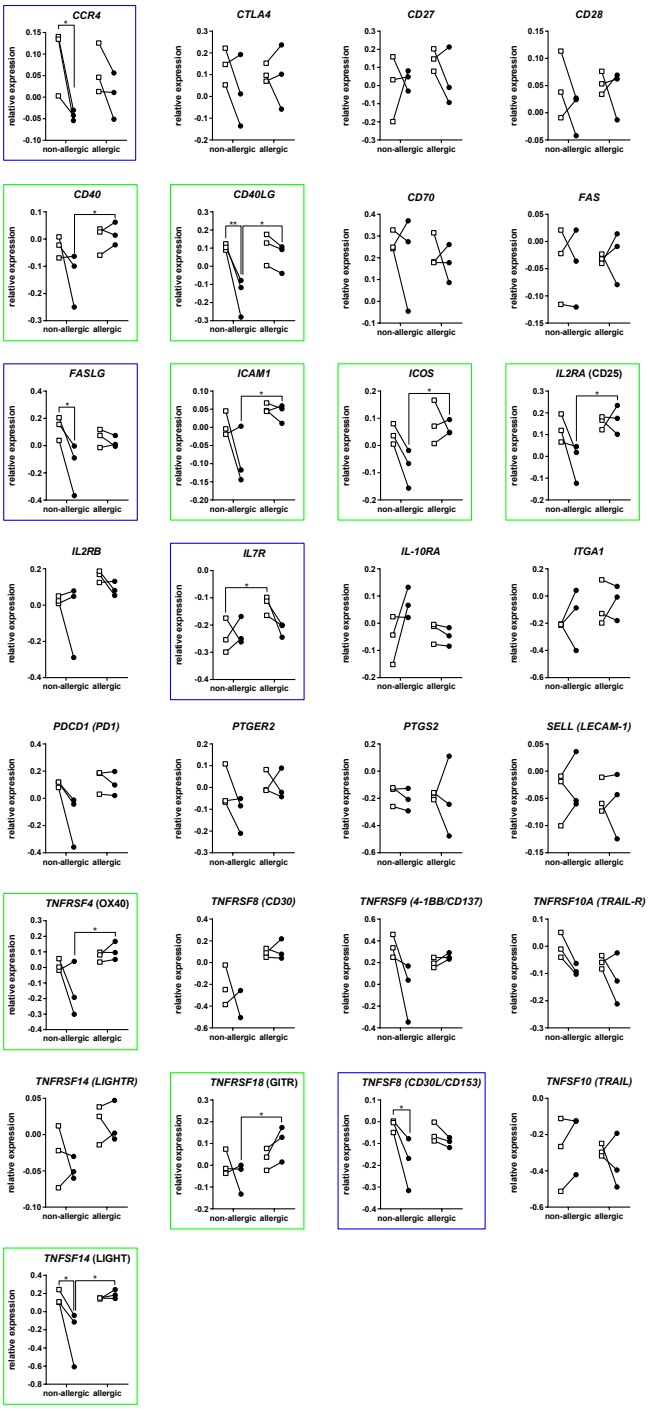
C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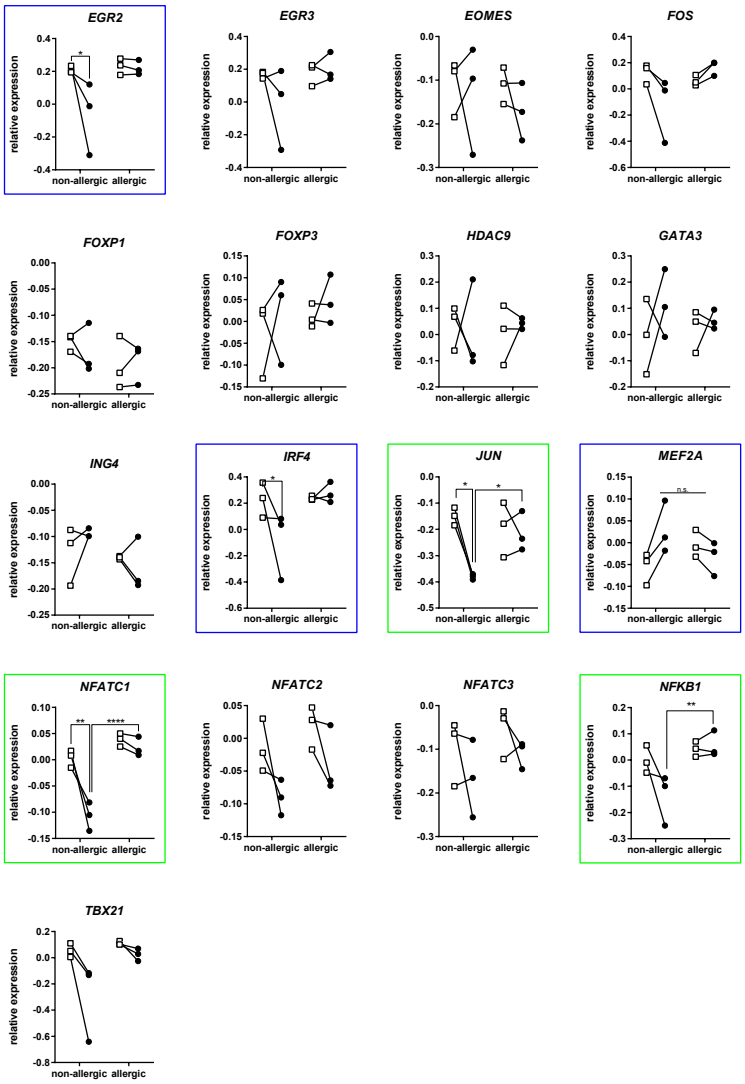
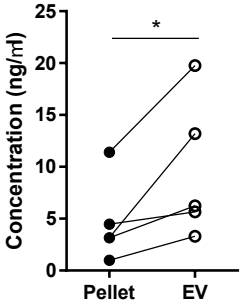
CTLA4
CD27
CD28
CD40
CD40LG
CD70
FAS
FASLG
ICAM1
ICOS
IL2RA (CD25)
IL2RB
IL7R
IL-10RA
ITGA1
PDCD1 (PD1)
PTGER2
PTGS2
SELL (LECAM-1)
TNFRSF4 (OX40)
TNFRSF8 (CD30)
TNFRSF9 (4-1BB/CD137)
TNFRSF10A (TRAIL-R)
TNFRSF14 (LIGHTR)
TNFRSF18 (GITR)
TNFSF8 (CD30L/CD153)
TNFSF10 (TRAIL)
TNFSF14 (LIGHT)
relative expression
non-allergic allergic

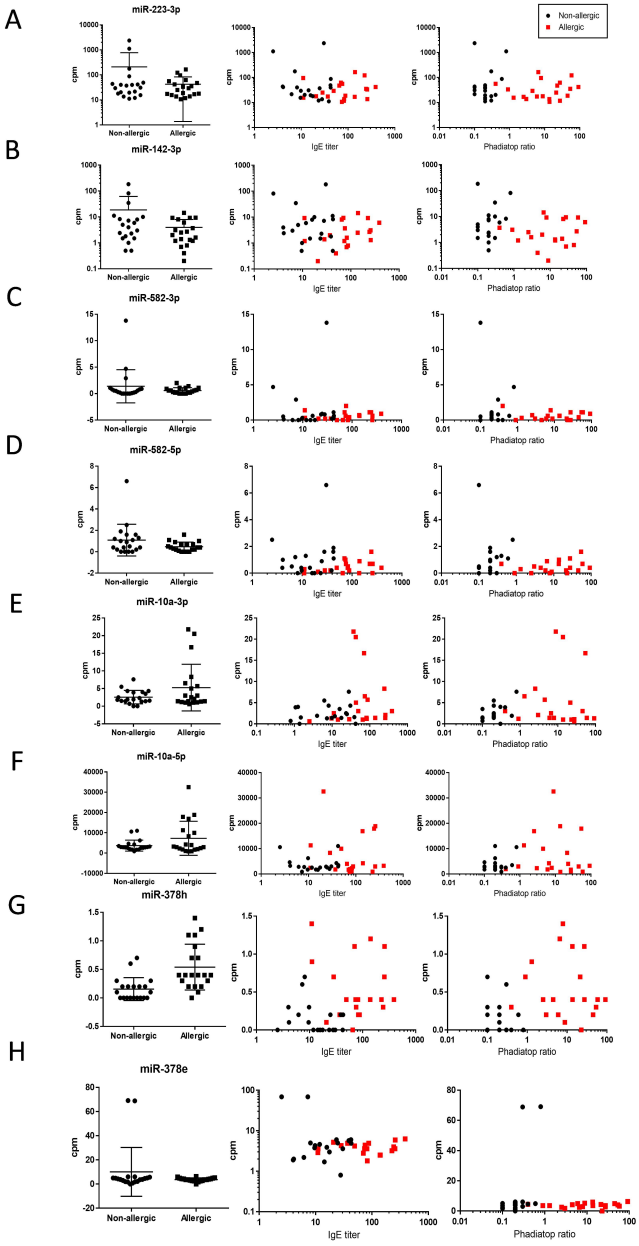
D



Supplementary Figure 4: Gene expression analysis of CD4⁺ T cells stimulated with CD3/CD28 in the presence of EV or EV-depleted controls from allergic or non-allergic mothers.
CD4⁺ T cells were stimulated with CD3/CD28 in the presence of EV (●) or their EV-depleted controls (□) from allergic (n=3) or non-allergic (n=3) mothers for 16 hours after which T cell RNA was extracted and gene expression analysis was performed. Gene expression was normalized to 4 housekeeping genes and delta Ct-values to CD3/CD28 stimulated T cell controls were calculated and log-transformed (CD3/CD28 control values are 0). Shown are the paired results of each EV donor with its EV-depleted control. Shown is the gene expression analysis of **(A)** secretory mediators, **(B)** Intracellular signaling molecules, **(C)** extracellular receptors and ligands, and **(D)** transcription factors. Green boxes indicate genes which were found to significantly differ between EV from allergic and non-allergic mothers and are used in Figure 3. Blue boxes indicate that significant differences were detected in conditions other than allergic and non-allergic EV. Significance was calculated using repeated measures two-way ANOVA with Sidak's multiple comparisons test and p-values defined as *p<0.05, **p<0.01, and **** p < 0.0001.



Supplementary Figure 5: RNA yield from pellets and EV fractions of density gradients.
Small RNA was extracted from the bottom (pellet; 1.26 – 1.23 g/ml) of the density gradient used to separate EV. Likewise, RNA was extracted from EV-enriched (EV; 1.12-1.22 g/ml) density fractions. RNA concentrations from identical milk volumes of 5 different donors were determined using Agilent 2100 Bioanalyzer pico-RNA chips. Shown is the concentration in ng/ul. Differences in RNA concentration between pellet and EV samples were calculated by paired t-test and significance was defined as $*p < 0.05$. For this experiment, milk was donated by healthy volunteers, which were not enrolled in the ACCESS study.



Supplementary Figure 6: Identification of miRNA candidates for biomarker validation studies
RNA deep sequencing was performed on milk-EV of allergic (n=20) and non-allergic (n=20) mothers and the abundance of detected miRNA species expressed as counts per million (cpm). **A-H)** Graphs show cpm of allergic and non-allergic donors (left panel), cpm versus serum IgE titers (middle panel), and cpm versus Phadiatop ratios (right panel). Each dot represents 1 donor. Red and black dots indicate whether donors were classified as allergic (red) or non-allergic (black) during analysis. Shown are miR-223-3p **(A)**, miR-142-3p **(B)**, miR-582-3p **(C)**, miR-582-5p **(D)**, miR-10a-3p **(E)**, miR-10a-5p **(F)**, miR-378h **(G)**, and miR-378e **(H)**.

Chapter 6

Summarizing Discussion

Human milk supports the development of the infant gastro-intestinal (GI) tract and immune system[1–4]. However, the mechanisms and macromolecular structures responsible are not fully known. Human, bovine, porcine, dromedary, and most recently, buffalo milk have all been described to contain extracellular vesicles[5–8]. As EV are mediators of intercellular communication[9], their function in milk is hypothesized to be to transfer messages from maternal cells to cells of the infant[10]. Despite their discovery in human milk in 2007[5], (pure) EV from milk have not extensively been studied. Furthermore in most published studies, claimed EV-mediated effects can be disputed due to difficulties in separating EV from other milk structures and lack of milk matrix controls. EV are often isolated by differential centrifugation culminating in a 100,000 g pellet[11]. Although this might suffice for more simple fluids, such as culture medium, body fluids are more complex and require further purification steps[12]. In the case of milk, abundantly present proteins, such as whey and caseins, can pellet and form gelatinous, non-resuspendable aggregates during high-speed centrifugation[13–15]. This makes pelleting of EV by differential (ultra) centrifugation without additional purification measures, unsuitable for this body fluid[16]. In the present work, we have addressed this issue and developed an isolation method for the reliable and quantitative recovery of EV from human milk (Chapter 2). By avoiding high-speed pelleting at 100,000 g, but instead directly applying EV-containing milk supernatant on top of a density gradient, the entrapment of EV in the gelatinous pellet is avoided. In addition, this protocol circumvents possible aggregation and fusion of EV by ultracentrifugation[17], which could influence characterization, as well as functional outcome. Others have used different measures to cope with gelatinous pellet formation, such as the addition of sodium citrate[18] or dissolving the pellet by overnight agitation[5: personal communication, 18]. Munagala, et al., have discarded the entire 100,000 g pellet, continuing experiments with a 135,000 g pellet[19]. It should be noted that the goal of this particular study was not to describe milk EV content or function, but rather to assess their potential as drug-carriers. Reinhardt, et al. has stated that the firm pellet they encounter following 100,000 g centrifugation consists of casein, while the more loose pellet consisted of 'crude' exosomes[20]. However, it is unclear whether Reinhardt's casein-pellet has been characterized and is known to be devoid of EV. A filtration step prior to high-speed pelleting has often been applied to remove aggregates, and a non-resuspendable pellet has not been mentioned in studies using filtration[21–27]. Nevertheless, it should be considered that filtration might also remove EV subsets or cause fragmentation of milk fat globules (MFG), of which the latter could contaminate the naturally present pool of EV.

Density gradient separation allows EV to be separated from soluble proteins and non-EV associated extracellular RNA. The most often used density gradient material is sucrose, followed by iodixanol. However, for functional analysis of EV it is prudent to isolate EV from the density gradient medium, as both sucrose and iodixanol can alter cellular responses (personal communication (unpublished data)). To this end, we have added a size exclusion column (SEC) filtration step after density gradient separation. SEC not only clears iodixanol from EV samples (unpublished data),

but additionally purifies EV from lipoprotein particles, which cannot be reliably achieved with density gradient separation alone ([28] and Vergauwen, et al. in prep).

Besides developing an isolation protocol for EV from human milk, we have proposed an adapted storage protocol for milk biobanking from which EV are intended to be isolated. In this thesis, we have described that storage of full milk directly after expression impacts cell viability and possibly MFG integrity, thereby contaminating the pool of naturally present EV (Chapter 2)[29,30]. Based on our results, we recommend that milk should be maintained at body or room temperature during transport and is centrifuged as quickly as possible (< 30 minutes after expression) to separate cells and MFG. Following this depletion, it is possible to freeze the milk supernatant until analysis, without loss of EV recovery (Chapter 2).

In Chapter 3, we used our optimized EV isolation protocol to perform in-depth analysis of the human milk EV content. Although whole milk and milk fractions have already been extensively characterized using proteomics, by using our milk EV isolation protocol we identified proteins which have never before been identified in milk, indicating that fractionation of milk enhances sensitivity of detection. These newly detected milk EV-associated proteins comprised a novel functional proteome, enriched for proteins annotated to the biological processes "cell growth & maintenance", "cell communication", "signal transduction", and "transport". This is in contrast to the molecular functions which were associated with the already known milk proteome, which showed enrichment for soluble mediators of immunity, such as cytokine, chemokine, and complement activity. These results suggest that different milk components fulfill different functions.

Besides protein content, we also determined the miRNA content of milk EV (Chapter 5). This revealed that the majority of EV-associated miRNAs have an immune modulatory capacity. Remarkably, the most abundantly present miRNA's in human milk EV were similar to those identified in other studies, even to those that had not specifically isolated EV[31–33]. It is possible that these abundant miRNA's fulfill a highly conserved and important function, and that less abundant miRNAs might vary relatively more with changing conditions. Also other RNA species, such as mRNA and non-coding RNA's, which are also present in milk EV[34], could show a higher degree of variation. Some studies have suggested that most RNA in milk resides within EV[33]. However, we and others have observed that a substantial amount of RNA is also present in other milk fractions ([31] and chapter 5, supplementary Figure 5). Nevertheless, it is possible that the most abundant miRNAs are EV-associated and comprise a relatively high percentage of the total miRNA content. Alternatively, these miRNAs could be associated to various macromolecular milk structures, such as MFG[31]. The high degree of similarity in RNA from whole milk and from isolated milk EV could provide a welcome outcome for RNA-based biomarker studies in milk, as this might make the laborious and time consuming isolation of EV unnecessary for these purposes. On the other hand, less abundant EV-specific RNA's might show a higher dependency on maternal health or diet and could therefore have a greater biomarker potential.

The molecular composition of milk EV led us to hypothesize that EV play a role throughout the entire epithelial mucosa of the GI tract. The GI tract continues to develop and mature postnatally, indicated by a rapid increase of cell surface area to enhance nutrient uptake, and a decrease in intestinal permeability to enhance the epithelial barrier function[1]. Besides requiring rapid growth and maturation of epithelial cells, immune homeostasis needs to be maintained in response to the high antigenic load encountered by the infant after birth[35]. Not only does the neonate need to cope with environmental and dietary antigens, but it also needs to maintain immune homeostasis during the process of microbial colonization[36]. Failure to control inflammatory responses leads to disintegration of the intestinal barrier, which is detrimental to infant health. In order to exert an effect in the infant GI tract, EV should be relatively resistant to digestion. Preliminary data from our lab suggests that EV are able to survive the initial steps of digestion, as they can still be recovered from the stomach compartment of the TNO *in vitro* gastrointestinal model (TIM-1). This is in line with Benmoussa, et al., who were able to isolate EV from cow's milk following digestion by various compartments of TIM-1[18]. In addition, multiple other reports show that EV from milk are resistant to relatively harsh conditions, such as low pH and enzymatic digestion[6,7,20,32,37]. Moreover, milk is a pH buffer, effectively neutralizing gastric pH[38], which is already higher in infants than in adults[39]. Therefore, we find it plausible that EV are able to pass through the digestive tract intact and reach the cells of the epithelial mucosa.

We proceeded to test the functional effects on selected key elements of the epithelial mucosa *in vitro* (Chapter 4). First, we found that purified milk EV contribute to intestinal barrier function by enhancing epithelial cell migration. Second, our results show that milk EV actively attenuated innate immune signaling by inhibiting TLR3 and TLR9, but TLR2 and TLR4 pro-inflammatory signaling. Finally, we show for the first time that EV from human milk inhibit activation of $CD4^+$ T cells. EV are known to consist of heterogeneous subpopulations. It is likely that various EV subpopulations exert their function throughout the GI tract and interact with different cells. It is even possible that some EV subsets need the influence of digestive enzymes in order to perform their function. Our tests on buccal epithelial cells show that human milk EV stimulate migration and suppress inflammation. Previously, Menckeberg, et al. showed that buccal epithelial cells are a good model for intestinal epithelial responses[40]. Once cells reach the duodenum, EV can be transported through the epithelial layer via M cells, transcytosis, or be taken up by antigen presenting cells in order to reach Peyer's patches or the mesenteric lymph nodes in which the cells of the adaptive immune system reside[41]. Rather than inducing suppressive T_{reg}, important in antigen-specific tolerance, our data show that EV induce a general immunosuppressed state, indicated by reduced $CD4^+$ T cell proliferation, cytokine secretion and expression of activation markers. Importantly, T cells were still capable of mounting immune responses in the absence of milk EV after being exposed to milk EV. Therefore, we propose that milk EV create a temporary increased threshold for T cell activation. Strikingly, this threshold depended on the sensitization status of the mother, as T cell activation was less inhibited during exposure to EV from milk of

allergic mothers compared to those exposed to EV from non-allergic mothers (Chapter 5). In order to understand these differences in function, we aimed to pin-point the origin of human milk EV, as well as differences in protein and miRNA content. By analyzing the site of expression of milk EV enriched proteins, we were able to determine that EV were mainly enriched for proteins typically expressed in immune cells, although cells of the breast tissue, such as mammary epithelial cells and adipose tissue were also significant contributors (Chapter 3). As milk contains immune cells, such as macrophages, DC, and T cells[31,42–45], these are likely the parental cells of milk EV, although it is possible that immune cells elsewhere in the body can secrete EV that end up in breast milk. In a recent study, Larsson, et al. likewise used proteomics to determine the origin of milk EV and corroborated our finding that immune cells are a likely source[23]. If immune cells are indeed the source of milk EV, it stands to reason that their composition will vary with maternal health status. In our omics-based miRNA and protein analyses of EV content no significant differences were detected, due to lack in statistical power. However, interesting candidates for future studies were identified and are currently awaiting validation (Chapter 5).

Currently, controversy exists on whether maternal allergy status impacts the development of the infant immune system. Maternal sensitization has been associated with higher breast milk sIgA titers, lower TGF-β concentrations, and milk EV have been reported to contain less MUC1[46–48]. MUC1 from human milk has been shown to block the pattern recognition receptor DC-sign[49] and a reduction in MUC1 might therefore cause an increased inflammatory response to microbes. Conversely, sIgA has been shown to suppress $CD4^+$ T cell responses suggesting increased immune suppressive capacity in sensitized mothers[50]. In this thesis, we describe that maternal allergy status impacts the ability of EV to inhibit $CD4^+$ T cell responses. However, it remains to be determined what the functional impact is on the newborn, and whether this is protective or detrimental to allergy development.

Taken together, we here provided direct evidence that maternal EV delivered via milk are multicomponent signaling devices which can interact with and modulate cells of the epithelial mucosa, and we speculate that these milk EV create a window for regulated development of the GI tract and the immune system.

Future perspectives

In this thesis, we have attempted to build on our understanding of the unique role of milk EV and how they influence infant development. In light of the findings presented in chapter 4 on the development of the epithelial mucosa, it would be interesting to determine therapeutic effects of milk EV on the risk of contracting diseases, such as necrotizing enterocolitis (NEC). NEC is a syndrome causing uncontrollable, destructive inflammation of the intestine and is mostly seen in pre-mature infants[51]. There are many reports showing that human milk significantly reduces

the risk of contracting NEC[52–54]. In pre-mature infants, the gastro-intestinal system is less mature and milk assists in enhancing barrier function and modifying the intestinal microbiota[2]. As we have determined that milk EV play a significant role in these processes, it would be interesting to determine whether these effects are due to EV and to investigate whether human milk EV could be used to prevent or treat NEC. Besides NEC in pre-mature infants, it is interesting to investigate whether human milk EV might be able to ameliorate symptoms of inflammatory bowel disease (IBD) in adults. IBD is thought to arise from a break in tolerance to the intestinal microbiome resulting in epithelial barrier breakdown and excessive inflammation[55,56]. Therefore, human milk EV might aid in rebuilding the physical barrier, downregulating inflammation, and reestablishing a healthy gut microbiome. Likewise, human milk EV could prove to be a novel treatment strategy for other diseases associated with intestinal barrier breakdown or inappropriate activation of T cells. These could encompass diseases as diverse as obesity, allergy, diabetes, arthritis, and other auto-immune disorders.

In this light, it would be of added value to determine whether these effects of human milk EV are evolutionary conserved, as cow's milk would provide a readily accessible source of EV as an alternative to human milk. Cross-species experiments have already indicated that preparations containing cow's milk EV can be taken up and processed by human intestinal cells[27], and that these preparations can ameliorate immune diseases, such as experimental arthritis in mice[21]. In addition, it would be worthwhile to determine the effects of homogenization, heating or freeze drying of commercial cow's milk on the EV content. Epidemiological evidence suggests that consumption of raw cow's milk decreases the risk of allergic disease development[57–60]. Based on our findings, we now hypothesize that these benefits are partially mediated through cow's milk EV and might be altered or lost during processing for commercial consumption. Further understanding of the role of milk EV in health and disease can contribute to harnessing these qualities to improve artificial milk formulas, which would greatly benefit infants of mothers which are incapable of breastfeeding.

Understanding the role of milk EV and what influences their composition can also increase their clinical value as a minimally invasive biomarker. The mothers analyzed in Chapter 5 were enrolled in the ACCESS study, of which the ultimate aim is to identify EV-based biomarkers in milk to predict allergy development in the child. In time, the results we have obtained thus far will be complemented by data on allergy development at 1 year of age. These data can provide additional clues as to the determinants of allergic outcome in the child. This knowledge can eventually help to identify infants which would benefit most from breastfeeding, given environmental circumstances and genetic makeup.

In conclusion, the results presented in this thesis provide a first comprehensive insight into the technical challenges and diverse functions of human milk EV. However, these results are only the tip of the iceberg and should be considered a stepping stone towards a more comprehensive understanding of the role of EV in milk. This understanding will contribute to the utilization of this knowledge for the promotion of health, not only of humans, but also of other mammals.

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Appendices

Nederlandse Samenvatting

Ons maagdarmstelsel is een belangrijk orgaan met meerdere functies. De meest bekende zijn de verwerking en opname van voedingsstoffen uit ons eten. Daarnaast kent het maagdarmstelsel een belangrijke functie als barrière tussen de buitenwereld en de rest van ons lichaam. Aangezien dit het grootste contactoppervlakte is met de omgeving, is een goede werking van de darmbarrière van essentieel belang voor onze gezondheid. De darmbarrière bestaat uit een fysieke barrière en uit componenten van het immuunsysteem. In het pasgeboren kind zijn zowel de fysieke, als de immunologische barrière nog volop in ontwikkeling. De cellen die de darmwand bekleden, de epitheelcellen, moeten snel delen om de fysieke barrière van de snelgroeiende darm te waarborgen. Tevens moet het immuunsysteem geïnstrueerd worden om onderscheid te maken tussen pathogenen en ongevaarlijke substanties, zoals voedselcomponenten en de bacteriële darmflora die na de geboorte de darm koloniseert. Over het algemeen zijn te sterke afweerreacties in het kind onwenselijk, aangezien deze een normale en gezonde ontwikkeling van organen, en van het kind in het algemeen, zouden kunnen belemmeren. Het is bekend dat moedermelk de delicate balans tussen immuunactivatie en -tolerantie bevordert en bijdraagt aan een juiste instructie van het afweersysteem van het kind. Tegelijkertijd bevordert moedermelk de ontwikkeling van de fysieke barrièrefunctie van het maagdarmstelsel. Echter, de bijdragen van individuele melkcomponenten aan deze functies zijn nog onduidelijk.

Recentelijk zijn er extracellulaire membraanblaasjes in moedermelk ontdekt. Extracellulaire membraanblaasjes, in het Engels *extracellular vesicles* (EV) genaamd, zijn structuren van ongeveer 50 – 500 nm groot, die uitgescheiden worden door cellen om met elkaar te communiceren. EV bestaan uit lipiden, eiwitten en genetisch materiaal, zoals DNA en RNA. De precieze samenstelling van deze verschillende componenten in EV varieert per celtype en de directe omgeving waarin een cel zich bevindt. Deze factoren beïnvloeden niet alleen de boodschap die EV overbrengen, maar ook naar welke cel deze boodschap overgebracht wordt. Op deze manier kan de communicatie tussen cellen nauw gereguleerd worden. Derhalve zijn EV in staat om complexe boodschappen over te brengen tussen verschillende cellen van een individu of, in het geval van moedermelk, van moeder naar kind.

Moedermelk bevat naast EV nog vele andere biologisch relevante structuren. De vetten bestaan voornamelijk uit triglyceriden en zijn voornamelijk aanwezig in de *milk fat globules* (MFG) die ontstaan doordat de epitheelcellen die de melkklier bekleden hun vetdruppels uitscheiden. De voornaamste eiwitten in melk zijn wei en caseïne. Andere componenten zijn de oligosacharides, (immuun) cellen, groeifactoren, hormonen, cytokinen, antistoffen en bacteriën. Om specifiek de samenstelling en effecten van EV uit moedermelk te kunnen bestuderen, hebben wij in **hoofdstuk 2** een isolatiemethode beschreven waarin we EV kunnen scheiden van andere melkcomponenten. Naast een isolatiemethode, hebben wij in dit hoofdstuk aangetoond dat een snelle verwerking van melkmonsters (binnen 30 minuten na afkolven) wenselijk is, om te voorkomen dat materiaal

afkomstig van afstervende cellen in melk de oorspronkelijke EV populatie contamineren. Wanneer het toch noodzakelijk is om moedermelk op te slaan voordat EV geïsoleerd kunnen worden, hebben wij aangetoond dat het invriezen van moedermelk nadat de MFG en cellen verwijderd zijn middels centrifugatie nauwelijks verlies van EV oplevert of contaminaties veroorzaakt.
Middels het in hoofdstuk 2 beschreven protocol, waren wij in staat de eiwitcompositie van EV uit moedermelk in kaart te brengen. Deze bevindingen staan beschreven in **hoofdstuk 3**. In dit hoofdstuk tonen wij allereerst aan dat eiwitten die veelvuldig aanwezig zijn in melk, zoals caseïne en wei, de gevoeligheid van detectie verminderen. Door EV te isoleren waren wij in staat om 633 eiwitten te detecteren die nog nooit eerder in moedermelk gevonden waren. De analyse van deze eiwitten toonden aan dat dit voornamelijk eiwitten betrof die afkomstig waren van epitheel- en immuun cellen. Op basis hiervan concludeerden wij dat EV uit melk voornamelijk geproduceerd worden door (borst)epitheel- en immuuncellen. Verdere analyses lieten zien dat EV verrijkt zijn in eiwitten die belangrijk zijn in 'cellulaire communicatie', 'signaal transductie', 'transport', en 'celgroei en -onderhoud'. Deze resultaten gaven aan dat EV mogelijk een belangrijke component zijn in de vorming en onderhoud van de barrièrefunctie van het maagdarmstelsel. Deze hypothese hebben wij vervolgens getest in *in vitro* celsystemen. In **hoofdstuk 4** laten wij zien dat EV uit moedermelk ervoor zorgen dat de epitheliale barrière zich sneller vormt en dat dit effect gemedieerd wordt door de activiteit van p38 MAPK in de epitheelcel. Tevens testten wij de invloed van moedermelk EV op het aspecifieke afweersysteem. Het aspecifieke afweersysteem is actief vanaf de geboorte en herkent evolutionaire geconserveerde patronen op bacteriën en virussen (PAMPs; *pathogen-associated molecular patterns*) via gespecialiseerde receptoren die zich op cellen bevinden. Door het herkennen van PAMPs kan al binnen een paar uur een globale afweerreactie plaatsvinden. Het aspecifieke afweersysteem is dan ook sneller dan het specifieke systeem, welke pas na enkele dagen op gang komt. Een klasse van PAMP receptoren zijn de Toll-like receptoren (TLR), die zich onder andere op epitheelcellen bevinden. De epitheelcellen kunnen derhalve niet alleen als fysieke barrière, maar ook als component van de immunologische barrière gezien worden. De samenstelling van de TLR op epitheelcellen verschilt tussen de kant die in contact staat met de buitenwereld (apicale zijde) en de kant die in contact staat met het lichaam (basolaterale zijde). Door dit verschil in verdeling van de TLR, kunnen epitheelcellen snel verstoringen in de fysieke barrière signaleren en het herstelproces in gang zetten. De TLR die wij getest hebben in **hoofdstuk 4** herkennen bepaalde bacteriële lipiden (TLR2 en TLR4) of genetisch materiaal van virussen of bacteriën (TLR3 en TLR9). Om te kijken of melk EV invloed hebben op de immuunrespons van deze verschillende TLR, hebben wij ze proberen te activeren in de aan- en afwezigheid van melk EV. In het geval van TLR2 en TLR4 had de aanwezigheid van EV geen invloed op de mate van activatie. Echter, in het geval van TLR3 en TLR9 dempte melk EV significant de immuunrespons. Deze resultaten geven aan dat moedermelk EV een actieve rol spelen in de homeostase van het aspecifieke afweersysteem en dat er mogelijk een affiniteit bestaat van EV voor TLR die genetisch materiaal herkennen.

Van EV is bekend dat zij epitheliale barrières kunnen passeren. Derhalve hebben wij ook onderzocht of melk EV een effect hadden op een belangrijke component van de specifieke afweer, namelijk de T cellen. T cellen bezitten een T cel receptor (TCR), die geactiveerd kan raken door kleine stukjes eiwit of lipide (epitoop) wanneer dit in een complex zit met een MHC eiwit. Bij mensen heten de MHC eiwitten *human leukocyte antigens*, oftewel HLA. HLA bevinden zich op alle lichaamseigen cellen, behalve rode bloedcellen. Op deze manier kan een T cel epitopen op alle cellen van het lichaam herkennen. De TCR varieert van T cel tot T cel, waardoor een breed repertoire van epitopen kan worden herkend. Wanneer een T cel zijn epitoop herkent en geactiveerd raakt, zal deze cel gaan delen, zodat vele T cellen met dezelfde TCR ontstaan. Hierdoor kan, in het geval van een pathogeen, deze uiteindelijk opgeruimd worden. Echter, omdat er maar een aantal T cellen het pathogeen zullen herkennen en er vervolgens veel cellen uit die enkele T cellen moeten ontstaan, duurt deze afweerreactie langer dan bij de aspecifieke afweer. Na de opruiming van de pathogeen, blijven er een aantal T cellen met deze receptor achter in het lichaam, waardoor immunologisch geheugen ontstaat. Dit immunologisch geheugen vormt de basis van vaccinaties. Epitopen zijn echter niet alleen afkomstig van pathogenen, maar ook van lichaamseigen cellen, voedsel, of andere omgevingsfactoren. Derhalve zijn T cellen zijn niet alleen belangrijk voor het opzetten van een afweerreactie, maar zijn zij ook essentieel voor het behoud van immuuntolerantie. In het proces van immuuntolerantie worden ook epitopen herkend door T cellen, maar worden immuunresponsen actief gedempt. Deze immuundempende T cellen zijn de regulatoire T cellen (T_{reg}). De educatie van het immuunsysteem bepaalt of een T cel een epitoop herkent als immuunactiverend of tolerogeen. Fouten in de inductie van immuuntolerantie leiden tot immuunziekten zoals allergie en auto-immuunziekten. In de darm staan T cellen in nauw contact met de buitenwereld en dit is dan ook de plek waar er een delicate balans gehanteerd moet worden tussen immuunactivatie en -tolerantie.

In **hoofdstuk 4** laten we zien dat in de aanwezigheid van EV uit moedermelk T cellen die het CD4 molecuul bevatten *in vitro* minder goed geactiveerd kunnen worden via hun TCR. In tegenstelling tot wat eerder beschreven was voor moedermelk EV, werden er geen T_{reg} geïnduceerd, maar was er sprake van een algehele vermindering van de T cel respons. Dit bleek afhankelijk van de directe aanwezigheid van melk EV, aangezien CD4 T cellen in de afwezigheid van EV alsnog gestimuleerd konden worden. Een algehele vermindering van de immuunrespons door melk EV zou kunnen bijdragen aan het creëren voor optimale voorwaarden voor het induceren voor immuuntolerantie. Tegelijkertijd worden (te) sterke afweerreacties die schadelijk kunnen zijn voor de ontwikkeling van het kind, voorkomen.

De laatste jaren is er een scherpe toename in allergie bij kinderen. Op dit moment is het onduidelijk of er een associatie is met een afname in (duur van) borstvoeding als primaire voedselbron. Tevens is onbekend of bepaalde componenten in moedermelk beschermend of juist bevorderlijk zijn voor het ontwikkelen van allergie. Aangezien maternale allergie een risicofactor is voor ontwikkeling van allergie in kinderen, hebben wij in **hoofdstuk 5** gekeken naar verschillen in EV

van allergische of niet allergische moeders. EV waren daarbij afkomstig uit gelijke hoeveelheden moedermelk. Wij toonden aan dat EV van beide groepen moeders in staat waren T cel activatie te dempen. Echter, EV van allergische moeders waren hierin minder efficiënt dan EV van niet-allergische moeders. Vervolgens hebben wij de microRNA (miRNA) en de eiwitsamenstelling tussen deze twee groepen vergeleken. Voor zowel de miRNA, als eiwitinhoud van EV geldt dat deze grotendeels gelijk zijn tussen de twee groepen. We hebben echter enkele miRNA en eiwitkandidaten geïdentificeerd waarvoor het interessant zou zijn om in vervolgonderzoek nader te bepalen of hun aanwezigheid in melk EV van allergische en niet-allergische moeders significant verschilt. Tevens kan in toekomstige studies onderzocht worden of deze miRNA en eiwitten ten grondslag liggen aan het verschil in vermogen om T cel activatie te dempen en zullen wij proberen uit te zoeken wat dit betekent voor de gezonde ontwikkeling van het kind.

Conclusies

EV uit moedermelk worden zeer waarschijnlijk geproduceerd door epitheel- en immuuncellen en bevatten een unieke eiwitsamenstelling in vergelijking tot andere componenten uit melk. Deze EV zijn als gespecialiseerde cel communicatiemiddelen in staat om de fysieke darmbarrière te verstevigen, en (te) sterke reacties van het aspecifieke en specifieke immuunsysteem te kunnen dempen. In het geval van allergische moeders lijken er een aantal verschillen op te treden in de samenstelling van moedermelk EV, en is de activatie van CD4 T cellen minder gedempt in de aanwezigheid van EV van deze allergische moeders. In de toekomst moet blijken welke implicaties dit heeft voor het kind.

List of Publications

This thesis

Marijke I. Zonneveld, Alain R. Brisson, Martijn J. van Herwijnen, Sisareuth Tan, Chris H.A. van de Lest, Frank A. Redegeld, Johan Garssen, Marca H.M. Wauben, and Esther N.M. Nolte-'t Hoen. *Recovery of extracellular vesicles from human breast milk is influenced by sample collection and vesicle isolation procedures.* Journal of Extracellular Vesicles, 2014; 3. Doi: 10.3402/jev.v3.24215

Martijn J.C. van Herwijnen, Marijke I. Zonneveld, Soenita Goerdayal, Esther N.M. Nolte-'t Hoen, Johan Garssen, Bernd Stahl, A.F. Maarten Altelaar, Frank A. Redegeld, Marca H.M. Wauben. *Comprehensive proteomic analysis of human milk-derived extracellular vesicles unveils a novel functional proteome distinct from other milk components.* Molecular & Cellular Proteomics, 2016; 15 (11), 3412-3423

Marijke I. Zonneveld, Marcela M. Fernandez-Gutierrez*, A. Marit de Groot*, Martijn J.C. van Herwijnen, Marije Kleinjan, Toni M.M. van Capel, Alice J.A.M. Sijts, Leonie S. Taams, Johan Garssen, Esther C. de Jong, Michiel Klerebezem, Esther N.M. Nolte-'t Hoen#, Frank A. Redegeld#, Marca H.M. Wauben. *Extracellular vesicles in human milk support the epithelial barrier by promoting reepithelialization and modulating innate and adaptive immune responses.* 2017 (To be submitted)

Marijke I. Zonneveld*, Martijn J.C. van Herwijnen*, Marije Kleinjan, Soenita Goerdayal, Arianne van Bruggen - de Haan, Tom A.P. Driedonks, Hailiang Mei, Wai-Yi Leung, Henk P.J. Buermans, Ruurd M. van Elburg, Gerbrich N. van der Meulen, Johan Garssen, A.F. Maarten Altelaar, Peter A.C. 't Hoen, Frank A. Redegeld, Esther N.M. Nolte – 't Hoen, Marca H.M. Wauben. *Extracellular vesicles from milk of allergic versus non-allergic mothers differentially affect T cell activation.* 2017 (In preparation)

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Others

Jan Van Deun, …, Marijke I. Zonneveld, …, and An Hendrix. *EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research.* Nature Methods, 2017; 14 (3), 228–232

Anita N. Kremer, Marijke I. Zonneveld, Andreas E. Kremer, Edith D. van der Meijden, J.H. Frederik Falkenburg, Esther N.M. Nolte-'t Hoen, Marca H.M. Wauben and Marieke Griffioen. *Natural T cell ligands can be transferred between cells by extracellular vesicles.* 2017 (To be submitted)

Tammy Oth, Thomas H.P.M. Habets, Wilfred T.V. Germeraad, Marijke I. Zonneveld, Gerard M.J. Bos, Joris Vanderlocht. *Pathogen recognition by NK cells amplifies the pro-inflammatory cytokine production of monocyte-derived DC via IFN-γ*. 2017 (Submitted)

Published abstracts

Marijke I. Zonneveld, Esther N.M. Nolte-'t Hoen, Frank A. Redegeld, Johan Garssen and Marca H.M. Wauben. *Comparison of protocols for efficient isolation and characterization of extracellular vesicles in human breast milk*. Second International Meeting of ISEV 2013: Boston, USA, April 17th-20th, 2013. Published in Journal of Extracellular Vesicles, 2013; 2 (1), 20826
Selected for poster presentation

Marijke I. Zonneveld, Martijn J.C. van Herwijnen, Alain Brisson, A.F. Maarten Altelaar, Frank A. Redegeld, Johan Garssen, Marca H.M. Wauben and Esther N.M. Nolte - 't Hoen. *Human breast milk contains various subpopulations of extracellular vesicles carrying immune modulatory proteins*. Third International Meeting of ISEV 2014: Rotterdam, The Netherlands, April 30th – May 3rd, 2014. Published in Journal of Extracellular Vesicles, 2014; 3 (1), 24214
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Marijke I. Zonneveld, Martijn J.C. van Herwijnen, Jos Brouwers, Johan Garssen, Frank A. Redegeld, Esther N.M. Nolte - 't Hoen and Marca H.M. Wauben. *Defined breast milk EV subsets boost the immune response and skew the T-cell balance towards a regulatory phenotype*. Fourth International Meeting of ISEV, ISEV2015, Washington, D.C., USA, 23-26 April 2015. Published in Journal of Extracellular Vesicles, 2015; 4 (1), 27783
Selected for oral presentation

Marijke I. Zonneveld, Martijn J.C. van Herwijnen, Marije Kleinjan, A. Marit de Groot, Marcela M. Fernandez-Gutierrez, Alice Sijts, Michiel Kleerebezem, Leonie S. Taams, Johan Garssen, Esther N.M. 't-Hoen, Frank A. Redegeld and Marca H.M. Wauben. *Breast milk-derived extracellular vesicles influence the development of the epithelial and immunological gastrointestinal barrier*. The Fifth International Meeting of ISEV, ISEV2016, Rotterdam, The Netherlands, 4 - 7 May, 2016. Published in Journal of Extracellular Vesicles, 2016; 5 (1), 31552
Selected for oral presentation

Curriculum Vitae

Marijke Irene Zonneveld was born on October 25th 1986 in 's-Gravenhage, The Netherlands as daughter to Paulien Zonneveld-Lind and Kees Zonneveld. At the age of 5, she and her family moved to Switzerland in the greater Zurich-area where she attended the International Community School Zurich. Eventually, they moved back to The Netherlands and Marijke attended the Dr. Nassau College, Quintus in Assen from 1999-2005, attaining her gymnasium diploma. After a year at University College Maastricht, she decided to pursue her interest in biology and enrolled in the Bachelor Molecular Life Sciences at Maastricht University, which she completed in 2009. In 2011 she attained her Masters in Oncology & Developmental Biology from the same university. During her Masters she performed her 16 week internship at Université Pierre et Marie Curie – Paris 6 in the Laboratoire de Biologie du Développement under the supervision of dr. Muriel Umbhauer. Her subject was the role of PAX8 in the development of the kidney in a *Xenopus laevis* model. Marijke performed her 32 week internship at Maastricht University's department of Internal Medicine under the supervision of dr. Joris Vanderlocht and Tammy Oth. Here, she studied the crosstalk between dendritic cells and natural killer cells in the context of dendritic cell-based vaccines against cancer. In 2011 Marijke started her PhD at Utrecht University under the supervision of prof. dr. Marca Wauben, prof. dr. Johan Garssen, and under the daily supervision of dr. Esther Nolte-'t Hoen and dr. Frank Redegeld. Her project on human milk-derived extracellular vesicles was a close collaboration between the department of Biochemistry and Cell Biology at the Faculty of Veterinary Medicine, the department of Pharmaceutical Sciences at the Faculty of Science, and Nutricia Research. During her studies she received a ME-HaD COST action STSM for young researchers to perform a three week traineeship in the lab of prof. dr. Leonie Taams at King's College London. The results of Marijke's research on milk-derived extracellular vesicles are described in this thesis. Currently, Marijke continues to work on extracellular vesicles as a postdoctoral researcher in the lab of dr. Kasper Rouschop at the department of Radiotherapy at Maastricht University.

Dankwoord

Zo. Dit was het dan. Mijn promotietraject is zo goed als afgerond en er rest mij alleen nog de mensen te bedanken die dit gedurende de afgelopen 6 jaar mogelijk hebben gemaakt. De weg was lang, de weg was zwaar. Maar wat heb ik ervan genoten! En dat komt door jullie. En mocht je jezelf niet terugzien in dit dankwoord, weet dan dat als jij dit boekje vast hebt, je er op een of andere manier, direct of indirect, aan hebt bijgedragen en dat ik je daarvoor dankbaar ben.

Allereerst gaat mijn dank uit naar mijn promotor, prof. dr. Wauben, beste Marca. Bedankt dat ik in jouw groep heb mogen promoveren. Ik herinner mij als de dag van gister dat ik bij jou, Esther en Frank op sollicitatiegesprek kwam en dat ik me verbaasde over hoezeer ik mij op mijn gemak voelde. Ik ben nog altijd dankbaar dat jullie mij uitgekozen hebben om als AIO te starten. Ik kan me niet voorstellen dat ik me onder een andere promotor zozeer had kunnen ontwikkelen. Jouw begeleiding kenmerkt zich door je enorme betrokkenheid, aanstekelijk enthousiasme en op de persoon toegespitste aanpak. De vele beren die ik op mijn weg zag heb je altijd vakkundig weggenomen. Hierdoor lag de weg voor mij vrij om me verder te ontwikkelen, als onderzoeker, maar ook als persoon. Marca, je hebt altijd het uiterste van mij gevraagd, maar gaf mij ook het vertrouwen dat ik aan die verwachtingen kon voldoen. Bedankt dat jij altijd het beste uit mij en dit project hebt willen halen, zonder concessies.

Dan is daar natuurlijk mijn copromotor, dr. Nolte-'t Hoen, lieve Esther. Jij bent een van de mensen in mijn leven die ik het meest bewonder. Ik ben continu van jou aan het leren. Experimentele opzet, time management, data analyse, presenteren en schrijven. Jij kunt het allemaal. En altijd maak jij tijd voor een praatje of *ad hoc* overleg. Hoewel dat *ad hoc* waarschijnlijk meer aan mij ligt. Meer dan eens ben ik bij jou op kantoor binnengevallen met een vraag waar ik acuut het antwoord op moest weten, omdat ik het experiment al aan het inzetten was. Jij hebt alles vanaf het begin van dichtbij meegemaakt en weet als geen ander wat er allemaal bij is komen kijken voor mij om dit punt te bereiken. De laatste paar jaar kenmerkte jouw begeleiding zich vooral in het altijd klaarstaan met een luisterend oor en praktisch toepasbare adviezen. Met name als ik weer mee was gesleurd in Marca's ideeën-wervelwind, kon jij ervoor zorgen dat alles weer behapbaar werd. Jij en Marca vormen een heus Yin en Yang op het gebied van persoonlijke begeleiding en zorgden ervoor dat ik het beste uit mezelf kon halen. Ik ben dankbaar dat ik van jullie begeleiding heb mogen profiteren. Dankzij jouw geduld, inzet en steun en (soms) die broodnodige schop onder de kont, sta ik nu hier. Dank je wel.

Ik heb het voorrecht gehad niet 1, maar 2 mensen mijn copromotor te mogen noemen. Dr. Redegeld, beste Frank. Voor mijn gevoel bestonden de eerste paar maanden van mijn PhD uit veel en lekker eten. Het was heel normaal om de ene week een uitgebreid 5-gangen diner met

een professor van Harvard te nuttigen en de volgende week naar Zeist te rijden om frietjes te eten uit een foodtruck die op jouw oprit geparkeerd stond. Jammer genoeg ben je al na 6 maanden vertrokken naar de VS en is deze gewoonte nooit in ere hersteld. Frank, bedankt voor je steun en je vermogen om het vaste rustpunt te zijn in de werkbesprekingen en al mijn projecten te faciliteren waar je kon.

Mijn begeleidingscommissie was een waar Full House, want ik had ook nog een 2e promotor. Prof. dr. Garssen, beste Johan. Ik vond het altijd een feestje om mijn resultaten aan jou te presenteren. Altijd was jij enthousiast en leerde ik weer nieuwe superlatieven voor 'mooie resultaten' kennen. Mijn favoriete werkbespreking was toch wel toen de functionele resultaten begonnen te lopen en ik veel van de data die nu in hoofdstuk 4 beschreven staan in één keer aan jou kon laten zien. Na alle vormen van 'fantastisch' en 'geweldig' wel te hebben gehad, kon jij op een gegeven moment alleen nog in stilte met je handen op je hoofd van verbazing, toekijken. Bedankt voor je steun en interesse in mijn welzijn en mijn werk en voor je periodieke spontane telefoontjes en appjes om te checken hoe het me verging. Ik zal Wilfred de groeten doen!

En dan zijn daar natuurlijk mijn collegaatjes van *The Wauben group. Do you remember my talk?!* Martijn, ik ben jou voor het eerst tegengekomen op het NVvI, waarna je voor mij bekend stond als 'die man in pak', en nog steeds vind ik het heel vreemd om je te zien in zoiets normaals als een spijkerbroek. Bedankt voor alle gezelligheid bij de koffietafel, je vermakelijke verhalen (je krijgt er zóveel voor terug), Vinex-Vesicle BBQs, Star Wars impressies (Alle mannen in koor nu: Maaahriiiijjjjkuuuuh) en lessen Venny. Je bent de koning van het relativeringsvermogen (zelfs een aap kan promoveren!) wat een mooie tegenhanger was met mij als drukte-makertje. Wat een goede zet is het geweest om jou als postdoc aan te nemen op dit project! Jij nam veel zaken die ik niet leuk vond moeiteloos van mij over en zorgde er daardoor enkelhandig voor dat ik promoveren als een feestje ging zien. Ik ben vereerd dat jij mijn paranimf bent. Als ik tijdens de plechtigheid maar niet ga bedenken hoeveel köttbullar er per jaar doorheen gaan…

Susanne, wat ben ik blij dat jij als PhD bij B&C bent begonnen! Eindelijk was ik niet langer de enige AIO van Celbiologie! Daarnaast vormde je een prima bliksem(be)geleider, waardoor ik lekker mijn gang kon gaan. Je hebt mijn laatste jaar (alle 3 ervan!) een stuk lichter gemaakt. Er gaat niks boven gedeelde smart! Samen hebben wij een wereld van stappen gezet, doorgepakt tot onze handen bloedden, en zoveel op het vizier gekregen dat we van voren niet meer wisten wat er van achteren leefde. Bedankt dat je deze laatste stap van mijn PhD samen met mij wil maken als mijn paranimf. Ik ga onze favoriete gezamenlijke hobby erg missen. Hoewel ik wel minder vaak misselijk ben, nu ik niet meer zo vaak rondjes draai op mijn stoel! Inmiddels zit ook jij in je laatste jaar en heb je de geuzennaam 'Senior-AIO' van mij overgenomen (werd misschien ook wel tijd dat ik die afstond na 4 jaar…). Succes met de afronding van je eigen PhD. Echt waar (ik mag het nu zeggen, hè), het komt goed! Heus! Beloofd!

Man, man, man, Sten. Man, man, man, alles moet je ze ook uitleggen! Samen vormden wij toch een beetje het ouwe getrouwde stel van B&C. Niets fijner dan lekker op elkaar vitten en zonder enige moeite het bloed onder elkaars nagels vandaan halen…of… nou ja… ik in ieder geval onder die van jou. Maar één ding is zeker, nu ik het land van Maas en Vlaai woon, zal ik dà kupke wel houwe, war! Wist je trouwens dat Vlaai maar op 3 plekken in Limburg wordt gemaakt?! Sten, ik zal je missen. Niet doorvertellen hè! Ik onthoud het wel!

Lieve Tom, TGK! jij bent door mijn hele promotie heen een immer aanwezige (klein intermezzo bij Dr Phil daargelaten), fijne, stabiele factor geweest. Samen waren wij de newbies in EV-land die even het eerste ISEV congres meepakte. Ik heb jou zien groeien als postdoc en heb altijd veel van je geleerd. Zoals dat de primaire kweek een prima ruimte is om goeie gesprekken te voeren, bijvoorbeeld! Wat vreemd om te bedenken dat jij midden in dit promotie-circus zat op het moment dat wij elkaar leerden kennen. Vroegah, toen ik nog op vakantie ging en daardoor zomaar een promotie kon mislopen! Ik ben blij dat je hiervan geleerd hebt en dat je nog even met me overlegd hebt om jouw laatste B&C werkdag goed in te plannen. Fijn dat je dat zo mooi gesynchroniseerd hebt met die van mij, het wordt gewaardeerd! Succes in A'dam en A'nem met Eva en de rest van je gezin.

Marije! Het was ons bijna gelukt! Ons masterplan om heel Team Frank naar B&C over te hevelen! Misschien probeer ik hetzelfde trucje wel weer in Maastricht. Interesse?! Ik ga onze gesprekken aan de koffietafel heel erg missen. Ze begonnen altijd met data, maar eindigde altijd heel ergens anders. Altijd sta je voor iedereen klaar, wat jou niet alleen heel goed maakt in je werk, maar ook een mooi mens. Bedankt dat ik altijd bij je langs kon komen voor alle kleine (en grote) wissewasjes. Zoals jij is er maar één!

Tommie D(J)! Ook jij kwam met je gezellige aanwezigheid Team PhD versterken! Met je droge humor, opvallende overhemden en oog voor detail vrolijkte jij menig werkbespreking op [insert headbangende banaan hier]. Bedankt dat jij op geheel eigen wijze aan mijn promotie bij wil dragen door goeie (of was het nu toch foute?) muziek te gaan draaien! En we moeten volgend jaar maar even testen hoe het Mestreechse Carnaval zich verhoudt tot het Eindhovense! Succes met je eigen PhD de komende jaren!

Janneke, jij weet als geen ander: het is niet niks, nie! Allebei hebben we de rollercoaster van die laatste paar maanden (of was het toch langer?) beleefd. Bedankt voor het delen van alle laatste-jaars-promotie-perikelen. Om maar te zwijgen van de bijna-werkeloos-zijn-en-ik-heb-geen-idee-wat-ik-wil-stress. En natuurlijk ook bedankt dat je me van dat laatste af geholpen hebt! Succes met de allerlaatste loodjes!

Ger, Ellen, Marthe en Henrike, bedankt voor jullie gezellige aanwezigheid. Ger, laten we hiermee nu definitief het 'FIETS!' tijdperk achter ons? Bedankt voor al die lessen Influx. Ik ben blij dat het nu toch iets makkelijker is geworden en wat minder afhankelijk van kookwekkers! Ellen, birthday-buddy! Elke verjaardag vanaf nu zal ik toch even aan je moeten denken! Veel geluk met je kersverse gezinnetje! Marthe, bedankt dat je me altijd een mini-donor-dienst wilde bewijzen! Heel veel sterkte met je eigen PhD project, je kunt het! Henrike, ik ben altijd jaloers geweest op jouw gigantische mok. Zo zonde om daar thee in te doen! En ik sta nog steeds versteld van jouw mooie Sponge Bob taart!

Willem, bedankt voor je input tijdens de werkbesprekingen en de (soms verrassende) gesprekken die ontstonden tijdens de verjaardagsvlaai. Richard, jij bent voor mij een soort levende Willie Wortel. Het is altijd weer afwachten wat je in elkaar gaat knutselen. Je notitieblok niet meer laten slingeren, hè! Esther V., als Richard Willie Wortel is, ben jij zijn Lampje dat hem behoed voor zichzelf! Petra, je weet dat je altijd welkom bent in het Zonnige Zuiden. Je bent te lief voor deze wereld! Ruud, de pilaar waar heel B&C op steunt. Hopelijk lukt het je ooit om orde te scheppen uit chaos! En dan zijn daar natuurlijk ook nog mijn vele andere B&C collega's, Chris (bedankt voor de last minute statistiek!), Martijn M, Aike, Jos, Jeroen, Bart, Bas, Louise, Bernd, Coos, Rachid, Theo, Carla, Caspaar, Martin, Dora, Jaël, Nick, Jie, Nafiseh. Bedankt allemaal voor een onvergetelijke tijd!

Els, Marian, Toine en Mieke. Mijn Celbio crew van het eerste uur! Els & Marian, Nimfies4Life! Hoewel jullie al een tijdje niet meer op het Nieuw Gildestein rondlopen, hebben jullie mijn promotie op een afstandje kunnen volgen. Bedankt voor al jullie adviezen, steun en bovenal 'lachen is het beste medicijn' mentaliteit! Ik hoop jullie nog lang mijn vriendinnen te mogen noemen! Toine, jouw geest is altijd een beetje rond blijven dwalen op B&C in de vorm van een grote collectie CD's. De posters zijn echter wel de prullenbak in verdwenen… Mieke, jij was mijn eerste kamergenootje en ik jouw laatste. Je stond altijd klaar met een luisterend oor en een daverende lach! Je timer heeft het ook nog lang gedaan nadat je met pensioen was! Ik ben blij dat ik je eindelijk dit boekje toe kan sturen, zodat je kan zien wat er van al dat gestuntel met melk gekomen is!

Bij Farmacologie wil ik alle lieve huidige en oud PhD's, postdocs, analisten en staf bedanken die mij als 'verdwaalde' AIO altijd weer adopteerden (of tolereerden?) in hun midden. Bedankt Atanaska, Kim, de Saskia's O. en B, Caroline, Lieke, Gemma, Mara, Suzan, Marlotte, Suzanne, Arash, Peyman, Mojtaba, Betty, Simone, Astrid, Kirsten, Soheil, Laura, Gerard.
Bart, bedankt dat ik altijd al jouw spullen mocht jatten! Hopelijk mogen we in de toekomst ooit echt een keer samen werken!
Paul, het Uithof Open was ons helaas niet gegund! Ger, focus houden op de presentaties, hè!

Op congressen was het ook altijd een dolle boel! Op de immunologie congressen waren daar Anouk, Susan en Marit! Met jullie was het altijd lachen. Anouk en Marit, bedankt voor de gezelligheid, biertjes, en mooie gespreksonderwerpen in Wenen (van voor naar achter? Of van achter naar voor?). Marit, ik heb altijd met veel plezier met je samengewerkt! Heel veel succes met jouw laatste loodjes!
En dan was daar natuurlijk ook de ISEV-bende! Hoewel de meesten gewoon uit Utrecht kwamen, zagen we elkaar vooral op dit congres. En helaas dus iets te vaak in Rotterdam. Susan, Sander, Olivier, Niek, Vera en Bas; van dansen op ABBA in Göteborg, tot een underground club in Boston, en van een Hotel Lobby in Washington.. of.. nee.. North Bethesda, tot een kerk in Rotterdam. Overal maken wij er een feestje van! Ik hoop dat ik jullie op nog vele ISEV's tegen mag komen!

Marcela, thank you for the great week I had in Wageningen. The Palm intocht definitely contributed to this! Thank you for taking me along for the ride! Good luck with finishing your own PhD.

Joris, Tammy, Birgit, Wilfred. Als ik niet bij jullie op de afdeling stage had gelopen, was ik waarschijnlijk nooit in Utrecht terecht gekomen, en misschien zelfs wel helemaal niet gaan promoveren. Bedankt voor de leuke en leerzame tijd! Ik kijk ernaar uit jullie weer wat meer tegen te gaan komen. Birgit, er is altijd tijd voor koffie!

Mijn inmiddels-niet-zo-nieuwe-collega's bij Maastro. Bedankt dat jullie mij allemaal zo welkom hebben laten voelen op de afdeling. Kasper, jij in het bijzonder bedankt voor je luisterend oor, begrip en geduld de afgelopen maanden. En nu gaan we vlammen! Trouwens, wanneer is de BBQ? Tom, mijn eigen Lab-Yoda! The Pipet is strong with this one. Met jou is het altijd een feestje. Ik ben wel blij dat je af en toe niet weet waar de tapejes nou blijven. Kim, bench-mate! Ik kijk ernaar uit samen nog veel te lachen en de receptor te vervloeken! Hanneke, ik ben blij dat jij weet over wie ik het heb! Als jij erachter komt hoe het in elkaar steekt, laat je het me weten toch? Marike, kersverse roomie! Als dat maar goed gaat… Lekker koffie drinken (lekker slaat zeer zeker niet op de koffie), totdat we er maagzweren van krijgen. Auke, Nicolle en Marike: wat fijn om met jullie al die promotie stress te hebben kunnen delen!

En dan zijn we aanbeland bij de categorie mensen die vooral aan mijn mentale gezondheid hebben bijgedragen gedurende de afgelopen paar jaar! Wat is het toch heerlijk om het af en toe niet over werk te hebben! Hoewel dat de afgelopen periode ook niet helemaal, of zeg maar gerust, helemaal niet, meer lukte!
Bedankt Kim, Marjon, Ilse, Irene, Krista, Laurie, Stephanie, Rolo, Stefan, Wouter, Dennis, Daan, Odin, Tommie, Arthur, Manon, Maurice, Harry, Opa & Oma M., Pieter, en natuurlijk Gerben. Tennis, wintersport, festivals, Oud & Nieuw, Miami, BBQs, competities, verjaardagen. Er is altijd

wel een reden voor een biertje of twee, al is die tegenwoordig soms alcoholvrij. Helaas was de Jäger dat tijdens de afgelopen wintersport zeker niet. Hopelijk vergeeft onze Pomp-meister het me ooit!
Lieve Kim, Marjon, Ilse, Irene, Krista en Laurie, bedankt voor jullie vriendschap en alle afleiding in de vorm van tennis, kaasfondue, stappen, ladies nights, Ibiza, Miami, Lake Dance en nog vele andere kleine en grote dingen!
Kimmie, ik ben blij dat wij juist in de afgelopen jaren Buuf&Buuf waren en er voor elkaar konden zijn. Ik mis het nu al om op mijn sloffen bij je op de koffie (met 43…soms ook zonder koffie) te komen. En doodmoe zijn, maar toch in de stad eindigen (nee, we gaan echt niks drinken!). Wanneer gaan we weer samen relaxen?
Rolo, bedankt voor alle mooie gesprekken, (te) vele biertjes, en avondjes in de Irish/Wildeman/Lempke/etc/etc. Wat zijn wij toch fantastisch!

Mijn vrienden van Helix! Evelien, Marjolein, Judith, Guido, Maarten, Dennis & Nadja, en Jo. Ook jullie kon ik te pas en vooral te onpas lastigvallen met alles van promotie-stress tot feestjes. Evelien, ik ben blij dat ik weer wat dichterbij je woon. Ik heb je gemist! Marjolein, Judith en Guido, succes met het afronden van jullie eigen promotie! Maarten, ik ben vereerd dat ik jouw paranimf mocht zijn. Ik kom je snel opzoeken in Stockholm! Jo, Dennis en Nadja, ik kom snel een keer bij jullie mijn Duits oefenen! Waar is Ralph eigenlijk?!

Als laatste wil graag mijn familie bedanken. Promoveren is niet alleen afzien voor degene die promoveert, maar ook voor degene die je lief zijn! Gelukkig heb ik altijd de onvoorwaardelijke steun en liefde gehad van mijn broertje, zusje, papa en mama.
Saskia, lief zusje, vakanties inplannen of even lekker een shop weekend. Vaak lukte het allemaal niet of alleen last minute. Zullen we daar de komende tijd verandering in brengen? Bedankt voor al die keren dat je met congressen en de sporadische vakantie op Karel & Frits hebt gepast. Het was altijd een mooi excuus om toch even bij te kletsen. Veel plezier in jullie nieuwe huis. Zorg je er wel voor dat voor dat Karel & Frits niet verwilderen?
Peter, van op-en-top student, naar hardwerkende Yup! Amsterdam doet je duidelijk goed. Bedankt voor al je verhalen, gezelligheid en betrokkenheid. Hoewel het wel beter is dat we niet gaan samenwerken als ik rood ben en jij geel!
Ik ben er trots op dat ik jullie zus ben.
Papa en mama, zonder jullie was dit allemaal niet mogelijk geweest, dus deze is voor jullie. Van jongs af aan hebben jullie ons alle drie vrij gelaten om ons eigen geluk te vinden. Zo vanzelfsprekend als jullie dat vinden, is bijzonder. Bij jullie ben ik altijd thuis. Ik hou van jullie.