The Journal of Immunology

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J Immunol 2007; 179:1969-1978; ;
doi: 10.4049/jimmunol.179.3.1969
http://www.jimmunol.org/content/179/3/1969

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Exosomes with Immune Modulatory Features Are Present in Human Breast Milk[1]

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Breast milk is a complex liquid with immune-competent cells and soluble proteins that provide immunity to the infant and affect the maturation of the infant's immune system. Exosomes are nanovesicles (30–100 nm) with an endosome-derived limiting membrane secreted by a diverse range of cell types. Because exosomes carry immunorelevant structures, they are suggested to participate in directing the immune response. We hypothesized that human breast milk contain exosomes, which may be important for the development of the infant's immune system. We isolated vesicles from the human colostrum and mature breast milk by ultracentrifugations and/or immuno-isolation on paramagnetic beads. We found that the vesicles displayed a typical exosome-like size and morphology as analyzed by electron microscopy. Furthermore, they floated at a density between 1.10 and 1.18 g/ml in a sucrose gradient, corresponding to the known density of exosomes. In addition, MHC classes I and II, CD63, CD81, and CD86 were detected on the vesicles by flow cytometry. Western blot and mass spectrometry further confirmed the presence of several exosome-associated molecules. Functional analysis revealed that the vesicle preparation inhibited anti-CD3-induced IL-2 and IFN-γ production from allogeneic and autologous PBMC. In addition, an increased number of Foxp3$^+$CD4$^+$CD25$^+$ T regulatory cells were observed in PBMC incubated with milk vesicle preparations. We conclude that human breast milk contains exosomes with the capacity to influence immune responses. *The Journal of Immunology,* **2007, 179: 1969–1978.**

Breast milk contains a potent mixture of diverse components such as milk fat globules (MFG),[3] immune competent cells, and soluble proteins like IgA, cytokines, and antimicrobial peptides (1) and is considered to provide protection against early infections in the infant (2). It has further been suggested that breast milk might have a role in tolerance induction (3) and may protect infants from allergy development (4); however, this is controversial (5).

Exosomes are MHC class I- and class II-bearing nanovesicles, ~30–100 nm in size, produced from different cell types including dendritic cells (6), macrophages, and lymphocytes (7, 8) as well as epithelial (9) and tumor cells (10). They have been shown to be present in physiological fluids such as bronchoalveolar lavage (11), human plasma (12), malignant effusions (13), and urine (14) and on the surface of follicular dendritic cells (15). Although exosomes have been implicated in cell-to-cell signaling, their physiological role in vivo is largely unknown. They have been found to be involved in both immune stimulation and tolerization, depending on the cell origin (7, 16), and several studies have suggested the potential use of exosomes in immunotherapy (17, 18). Two phase one clinical trials using exosomes in cancer therapy have recently been published showing promising results (19, 20).

In the present study we investigated whether human breast milk contains exosomes that might contribute to immune regulatory functions in the infant. We isolated vesicles from colostrum and mature milk by differential ultracentrifugation and sucrose gradient fractionation and characterized them both morphologically by electron microscopy (EM) and phenotypically by flow cytometry, Western blot and mass spectrometry (MS). We found that these vesicles display similar shapes, sizes, and densities as those of previously described exosomes. They also expressed molecules that are characteristic of exosomes such as MHC, tetraspanins and heat shock proteins. Furthermore, functional analyses showed that the vesicle preparations inhibited anti-CD3-induced IL-2 and IFN-γ production in vitro and increased the number of Foxp3$^+$CD4$^+$CD25$^+$ T regulatory cells. Taken together, our results indicate that exosomes with immunomodulatory features are present in human breast milk. This novel finding might shed light on how breast milk can modulate the development of the infant's immune system.

Materials and Methods

Subjects and sampling of milk and peripheral blood

Colostrum (within 4 days after delivery) and mature milk (within 1–6 mo after delivery) were collected by healthy mothers (Table I) using a manual breast pump in sterile tubes. Smoking or medication during pregnancy, known autoimmune disorder, diabetes, or allergic asthma excluded women from participation in this study. All mothers had vaginal deliveries at full term of healthy, normal birth weight infants. The colostrum samples were

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Received for publication July 31, 2006. Accepted for publication May 25, 2007.

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[1] This study was supported by Swedish Research Council Grants 15243-01, 7924-18A, and 71P-14158 and by the Centre for Allergy Research at the Karolinska Institute, the Hesselman Foundation, the Jeansson Foundation, the European Science Foundation, the Åke Wiberg Foundation, the Magnus Bergvall Foundation, the Swedish Cancer and Allergy Foundation, and the Swedish Asthma and Allergy Association's Research Foundation.

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[3] Abbreviations used in this paper: MFG, milk fat globule; ACN, acetonitrile; CBA, cytometric bead array; EM, electron microscopy; Hsc70, heat shock cognate protein 70; LC, liquid chromatography; MDDC, monocyte-derived dendritic cell; MS, mass spectrometry; MS/MS, tandem MS; MUC-1, mucin-1; SFC, spot-forming cell.

Table I. *Demographic data of milk donors*

Milk Donor	Age (Years)	No of Previous Deliveries	Colostrum[a] (ml)	Mature Milk (ml)	Mature Milk Collection (Months after Delivery)
1	30	1	40	195	1.5
2	34	1		80	3
3	35	3	15	100	2.5
4	31	1		143	3
5	35	1		91	2.5
6	39	2		113	3
7	37	1		108	3.5
8	33	2	73	66	2.5
9	35	1		109	6
10	37	2	55	76	3.5
11	31	1	30	90	1
12	31	1		138	3
13	31	1	45	138	3
14	34	1	20	69	3
15	34	2	15	53	3
16	34	1		42	3
17	34	2		18	3
Median (range)	34 (30–39)	1 (1–3)	35 (15–73)	91 (18–195)	3 (1–6)

[a] Milk collected within 4 days after delivery.

frozen at −80°C directly at the maternity unit. The mature milk was collected at home and kept at 4°C until delivered to the laboratory, where the samples were centrifuged at 300 × *g* to remove cells and stored at −80°C within 24 h of sampling. No difference in phenotype of milk vesicles was detected when using fresh milk or milk that had been directly frozen in −80°C (data not shown). Cells from mature milk were counted by trypan blue exclusion and the cell number ranged between 2.3×10^4 and 2.4×10^5 (median 7.6×10^4, $n = 16$) cells per ml of milk. The cell viability was between 46 and 99%, median 84%. Forty milliliters of peripheral blood was taken from the mothers in heparinized glass tubes and PBMC were isolated by density centrifugation on Ficoll Paque according to the manufacturer's instructions (Amersham Biosciences). The cells were frozen in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone) and 10% DMSO (Merck) and stored at −80°C. The study was approved by the local ethics committee and informed consent was obtained from each donor.

Preparation of exosome-like vesicles by differential ultracentrifugation

Vesicles were isolated by serial ultracentrifugation as described previously for exosomes (7) with some modifications. After thawing, the whole colostrum or mature milk supernatants were subjected to 300 × *g* followed by 3000 × *g* centrifugation and filtered sequentially through 1.2-, 0.8-, 0.4-, and finally 0.2-μm filters (Advantec MFS) to remove cell debris. The supernatants were centrifuged at 10,000 × *g* for 30 min at 4°C to further remove cell debris and then at 100,000 × *g* for 70–90 min at 4°C to pellet the vesicles. The pellet was left over night in PBS at 4°C to dissolve. Vesicles were then washed twice and resuspended in PBS. Exosomes were also prepared from day 7 culture supernatants of monocyte-derived dendritic cells (MDDCs) generated from buffy coats derived from 14 different healthy blood donors as previously described (11, 21). Cell lysate from human EBV-transformed B cells (a gift from Dr. B. Bohle, Medical University of Vienna, Vienna, Austria) was prepared by exposing cells to ice-cold buffer containing 2% Nonidet P-40, 10 mM EDTA, 1 mM PMSF (Sigma-Aldrich), and a phosphatase and protease inhibitor mixture (Roche Diagnostics) for 1 h followed by centrifugation at 16,110 × *g* for 30 min at 4°C. The protein contents of some of the vesicle preparations and the cell lysate were measured by a DC protein assay (Bio-Rad Laboratories) and a BCA protein assay (Pierce), respectively. The protein concentration ranged between 41.0 and 153 μg (median 90.9 μg) of protein per milliliter of original volume of colostrum ($n = 6$), and between 19.8 and 319 μg (median 54.0 μg) of protein per milliliter of original volume of mature milk vesicles ($n = 16$), and there were no significant differences between colostrum and mature milk ($p = 0.22$) based on statistical analysis using the Mann-Whitney *U* test.

Preparation of milk fat globules

MFG from mature milk were prepared as previously described (22) with the modification that the cream layer was separated by centrifugation at 3,000 × *g* for 30 min.

Transmission electron microscopy

Vesicles captured on anti MHC-class II beads were gold-immunolabeled for CD63 and HLA-DR as described previously (11) and then fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose and 3 mM $CaCl_2$ (pH 7.4) at 4°C over night and centrifuged to a pellet. The pellet was rinsed in 0.15 M sodium cacodylate buffer containing 3 mM $CaCl_2$ (pH 7.4) followed by postfixation in 2% osmium tetroxide in 0.07 M sodium cacodylate buffer containing 1.5 mM $CaCl_2$ (pH 7.4) at 4°C for 2 h. The pellet was further dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd Research Industries). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope (FEI) at 80 kV. Digital images were captured by a Mega View III digital camera (Soft Imaging System).

SDS-PAGE and Western blot analysis

The pellet from 100,000 × *g* centrifugations or from each sucrose fraction was denatured in Laemmli sample buffer (Bio-Rad Laboratories), separated on 12% polyacrylamide gels (Bio-Rad Laboratories) and used for immunoblotting. After electrophoretic transfer to Immobilon-P transfer membrane (Millipore) and blocking overnight with ECL advance blocking agent (Amersham Biosciences), primary Abs i.e., anti-HLA-DR, anti-CD81, anti-calnexin (Santa Cruz Biotechnology), or anti-heat shock cognate protein 70 (Hsc70) (StressGen Biotechnologies) were added to the membrane and incubated for 1 h with gentle shaking, followed by washing and the application of HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). Blots were developed using an ECL chemiluminescence developing kit (Amersham Biosciences).

Flow cytometry

Pelleted vesicles or 100 μl of sucrose fractions were adsorbed onto 4.5-μm Ø Dynabeads precoated with anti-MHC class II clone HKB1 (Dynal) in PBS containing 0.1% BSA and 0.01% sodium azide overnight at room temperature. The beads were coated with an amount of milk exosomes corresponding to 1.5 ml of the original volume of the milk to 1-μl beads. The exosomes were stained with anti-HLA-DR FITC, anti-MHC class I FITC, anti-CD40 FITC, anti-CD54 PE, anti-CD63 PE, anti-CD80 FITC, anti-CD81 PE, anti-CD86 FITC (BD Biosciences), anti-mucin-1 (MUC-1) FITC (Abcam), and anti-A33 (a gift from Assoc. Prof. E. Telemo, Göteborg University, Göteborg, Sweden, with the permission of Dr. J. K. Heath, Ludwig Institute for Cancer Research, Victoria, Australia), biotinylated anti-mouse IgG2a (DakoCytomation), and streptavidin PE (BD Biosciences) and compared with isotype-matched controls (BD Biosciences). Samples were analyzed by a FACSCalibur flow cytometer (BD Biosciences) and the data were analyzed using CellQuest Pro software (BD Biosciences). A minimum of 5×10^3 beads per sample were examined.

Sucrose gradient

Pelleted vesicles from colostrum and mature milk were layered on top of a linear sucrose gradient (0.25–2 mM sucrose (Sigma-Aldrich and 20 mM

HEPES/NaOH (pH 7.4)). The gradients were centrifuged for 21 h at 79,000 × *g* at 4°C. Eighteen fractions (1 ml each) were collected from the bottom of the tube and the density of each fraction was determined by refraction index measurements. Each fraction was then either washed in 3 ml of 20 mM HEPES and solid material was pelleted by centrifugation at 200,000 × *g* for 35 min at 4°C for Western blot analysis or directly loaded onto anti-MHC class II Dynabeads (Dynal) for flow cytometry analysis.

Preparation and trypsin digestion of exosome proteins for mass spectrometry analysis

Milk vesicles from sucrose fractions with high HLA-DR expression by flow cytometry (densities between 1.08 and 1.15 in the two tested individuals) were pooled and concentrated using Microcon centrifugal filter units with 3-kDa cutoff (Millipore). Total protein concentrations were measured by a DC assay (Bio-Rad Laboratories). Fifty micrograms of proteins from each sample were concentrated further by a vacuum centrifuge and then dissolved in a denaturing buffer (6 M urea, 0.05% SDS, 5 mM EDTA, and 50 mM Tris-HCl (pH 8.5)) and reduced for 45 min with 1 mM Tris 2-carboxyethyl phosphine hydrochloride at 37°C followed by the addition of 3 mM iodoacetamide and incubation for 90 min at 37°C. The alkylation reaction was stopped by adding 15 mM DTT for 5 min at room temperature. The urea concentration of the samples was diluted with Milli-Q H_2O to 1 M and then the proteins were digested with 2 μg of trypsin (sequencing grade modified trypsin; Promega) overnight at 37°C. The resulting peptides were purified by using cation exchange columns according to the manufacturer's instructions (Applied Biosystems). Eluted peptides were then dried and resuspended in 0.1% HCOOH. Peptides were purified with pipette tips filled with a piece of C18 Empore membrane (3M), washed with 0.1% HCOOH, and finally eluted with 60% acetonitrile (ACN) in 0.1% HCOOH. Eluted peptides were dried in a vacuum centrifuge and suspended to 0.1% HCOOH before tandem MS (MS/MS) analysis.

MS/MS and data analysis

Peptides digested from milk vesicle proteins were separated and analyzed by a liquid chromatography (LC) MS/MS instrument, which consisted of a nano-HPLC (Famos, Switchos II, and Ultimate; LC Packings) coupled to QSTAR Pulsar mass spectrometer (Applied Biosystems/MDS Sciex). A linear 120-min gradient from 2% buffer A (2% ACN in 0.1% HCOOH) to 35% buffer B (95% ACN in 0.1% HCOOH) was used for peptide separation. The mass spectrometer was set to automatically collect the first 1 s of MS data and then MS/MS data for 3 s from the two most intense peaks of the MS spectrum fulfilling the criteria specified in the Information Dependent Acquisition (Applied Biosystems/MDS Sciex) method. Mascot (Matrix Science) was used to process the MS and MS/MS data. Mass tolerance of 0.3 Da was used for both MS and MS/MS spectra. In data processing, the modifications of carbamidomethylated cysteine (fixed) and oxidized methionine (variable) as well as one missed cleavage were allowed when the data were searched against the human proteins of the Swiss-Prot database (http://www.expasy.org/sprot/). Protein identifications with a significant Mascot score of >25 in samples from both of the two tested individuals were filtrated from the Mascot results. The confidence of these protein identifications were further evaluated by processing the data also with ProID software (Applied Biosystems). Proteins identified by ProID with a confidence score of >50 were matched with Mascot-identified proteins to further confirm the significance of the identifications. InforSense 2.0 software was used to match the results.

Preparation of anti-CD81-coated latex beads

Four-micrometer aldehyde/sulfate latex beads (Interfacial Dynamics) were incubated in 50 μl of PBS with anti-CD81 (Santa Cruz Biotechnology) at room temperature. After 15 min, the volume was made up to 400 μl with PBS and rotated overnight at room temperature. The reaction was stopped by incubation with 100 mM glycine for 30 min, after which the beads were extensively washed in PBS. Exosomes were coated to the beads by incubation in <50 μl of medium for 15 min at room temperature. The volume was then made up to 400 μl with complete medium (see below) and rotated overnight.

IL-2 ELISPOT

PBMC from six mothers (donor nos. 1–6 in Table I) were incubated in duplicate at a concentration of 1 × 10^6 cells/ml with or without various concentrations of autologous or allogeneic mature milk vesicles (50–500 μg/ml) that were prepared by differential ultracentrifugation as described above. In additional experiments the milk vesicle preparation was coated to anti-MHC class II Dynabeads and/or anti-CD81 latex beads, and the fractions not bound to the beads were added to the allogeneic PBMC from healthy blood donors. In some experiments a pool of MDDC-derived exosomes obtained from 14 different healthy blood donors were added to allogeneic PBMC from healthy blood donors. The cells and the milk-derived or MDDC-derived vesicles were incubated for 4 days in complete medium consisting of RPMI 1640 (Invitrogen Life Technologies) supplemented with 25 μg/ml gentamicin (Invitrogen Life Technologies), 10% heat-inactivated FCS (HyClone), 2 mM L-glutamine, 100 IU/ml penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Invitrogen Life Technologies), and 50 μM 2-ME (KEBO-lab) in a 37ºC humidified incubator with 6% CO_2. After 4 days the viability of the PBMC was ~80% for all culture conditions as analyzed by using propidium iodide in a FACS (BD Biosciences). The PBMC were then added to 96-well ELISPOT plates (ELIIP; Millipore) that had been precoated with 15 μg/ml anti-IL-2 (Mabtech) and 1/100 dilution of anti-CD3 (OKT3 supernatant) overnight at 4°C. After 48 h of incubation at 37°C supernatants were collected for further cytokine analysis, the cells were washed away, and the plates were developed as described previously (21). Spots were counted with an ELISPOT reader using AID ELISPOT software (Autoimmun Diagnostika). Results are expressed as the mean of duplicates of spot-forming cells (SFC) per 2 × 10^5 PBMC.

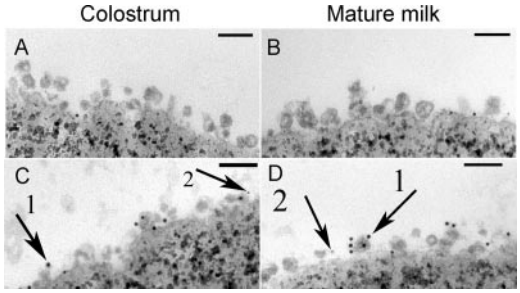

FIGURE 1. Exosome-like vesicles in breast milk detected by EM. The 100,000 × *g* pellets from human colostrum and mature breast milk were coated to anti-MHC class II beads. Isotype control Abs (*A* and *B*) or Abs against CD63 (arrow 1) and HLA-DR (arrow 2) (*C* and *D*) were detected by gold-conjugated secondary Abs, 10 and 5 nm, respectively. Samples were fixed and analyzed by electron microscopy. Scale bar, 100 nm.

Cytometric bead array

Supernatants from IL-2 ELISPOT analyses were centrifuged to remove cells and then maintained frozen at −20°C until analysis. The cytometric bead array (CBA) human Th1/Th2 kit (BD Biosciences) was used to assay IFN-γ, TNF-α, IL-4, IL-5, and IL-10 in accordance with the manufacturer's instructions. The lower limits of detection were 5 pg/ml.

Incubation of PBMC with milk exosomes for regulatory T cell analysis

Human PBMC from seven different healthy blood donors were prepared as described above. When abundant, RBC were lysed by incubating the cells for 5 min in ammonium/chloride potassium (ACK) lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA). PBMC, 1 × 10^6/ml, were coincubated with 500, 50, or 5 μg/ml mature milk-derived exosomes from allogeneic healthy mothers or, as a control, with the same amount of PBS in complete exosome-depleted medium in a 37°C humidified incubator (5% CO_2). On day 4, the cells were harvested and total cell counts and viability were established by trypan blue exclusion. Viability was also assessed by annexin-V FITC (BD Biosciences) and propidium iodide (Sigma-Aldrich) staining was analyzed by flow cytometry. There were no significant differences in total cell counts or viability for the different conditions (data not shown). For regulatory T cell analysis, cells were stained, fixed, and permeabilized using a regulatory T cell staining kit (eBioscience) with anti-CD4, anti-CD25, and anti-Foxp3 according to the manufacturer's instructions. Additionally, anti-CD8-PerCP (BD Biosciences) and anti-CD3 Pacific Blue (BD Biosciences) were included as well as their appropriate isotype controls (BD Biosciences). This five-color panel was analyzed on a FACSAria cytometer (BD Biosciences). Regulatory T cells are expressed as the percentage of $Foxp3^+$ cells of $CD4^+$ lymphocytes.

Statistics

Wilcoxon's matched pairs test and Mann-Whitney *U* test were performed using Statistica 7.1 software package (StatSoft Scandinavia). Values of $p <$ 0.05 were considered statistically significant.

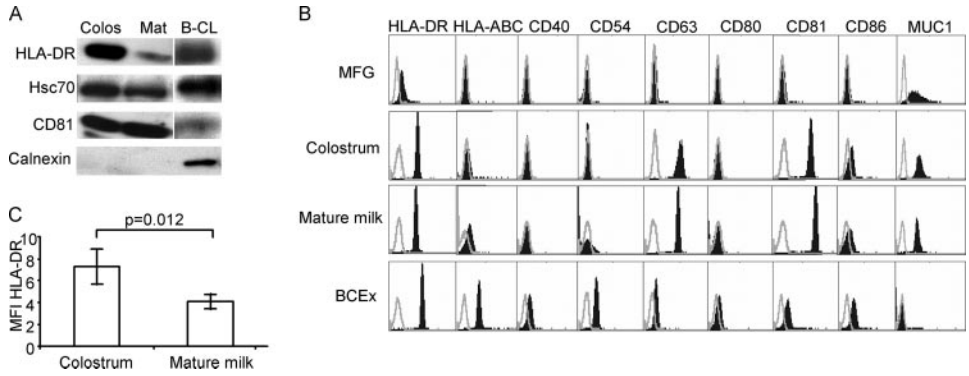

FIGURE 2. Exosome-like vesicles in colostrum and mature milk contain exosomal proteins but not the endoplasmic reticulum-related protein calnexin. *A*, The protein profile of exosomes was analyzed by Western blotting using Abs against HLA-DR, Hsc70, CD81, and calnexin. Five micrograms of colostrum (Colos)-derived and mature milk (Mat)-derived, exosome-like vesicles or 2 μg of B cell lysate (B-CL) were separated on 12% acrylamide gel, transferred to PVDF membrane, and incubated with specific Abs followed by incubation with HRP-conjugated secondary Abs and detected by a chemiluminescence kit. Results are shown as one representative experiment of four using milk from different individuals. *B*, Flow cytometry analysis of the surface expression of exosome-associated proteins. Pelleted vesicles from colostrums and mature milk, MFG, and B cell line-derived exosomes (BCEx) were captured on anti-MHC class II-coated magnetic beads and stained with a panel of specific mAbs for flow cytometry. All Abs used were compared with isotype-matched controls. Results are shown as one representative experiment of eight using milk from different individuals. *C*, Mean fluorescence intensity (MFI) values for colostrum and mature milk vesicles were compared for HLA-DR. Results show mean values and SD, $n = 8$. Statistical analysis was performed using Wilcoxon's matched pairs test.

Results

Morphology of isolated vesicles

Vesicles were obtained from both colostrum and mature milk by differential ultracentrifugations. To analyze the morphology of the obtained vesicles, they were coated to anti-MHC class II beads and analyzed by EM. The vesicles were strikingly homogenous in size (~50 nm in diameter) in both colostrum (Fig. 1, *A* and *C*) and mature milk (Fig. 1, *B* and *D*), and this is within the size range previously reported for exosomes (23). Immunolabeling revealed the presence of both CD63 and HLA-DR (Fig. 1, *C* and *D*).

Phenotypic analysis of the vesicles

Western blot analysis of the 100,000 $\times$ *g* fractions revealed that both colostrum-derived and mature milk-derived vesicles were positive for the exosome-associated proteins HLA-DR, Hsc70, and CD81 but negative for calnexin, which was detected only in the cell lysate (Fig. 2*A*), indicating that the vesicles are not of endoplasmatic reticulum origin.

We further examined the expression of surface proteins on the vesicles from colostrum and mature milk by flow cytometry. Colostrum and mature milk from eight individuals were analyzed and compared with MFG and exosomes prepared from B cells. The vesicles of both colostrum and mature milk from all individuals showed strong signals for HLA-DR and the tetraspanin molecules CD63 and CD81 (Fig. 2*B*). However, HLA-DR expression was significantly higher for colostrum-derived vesicles than for mature milk-derived vesicles (Fig. 2*C*). Vesicles from mature milk were positive for HLA-ABC for all individuals, whereas vesicles of colostrum from five of eight individuals were positive for this molecule. CD86 was also identified on the surface of vesicles derived from both the colostrum and mature milk of four of eight individuals at moderate levels. MUC-1, a glycoprotein that is present in

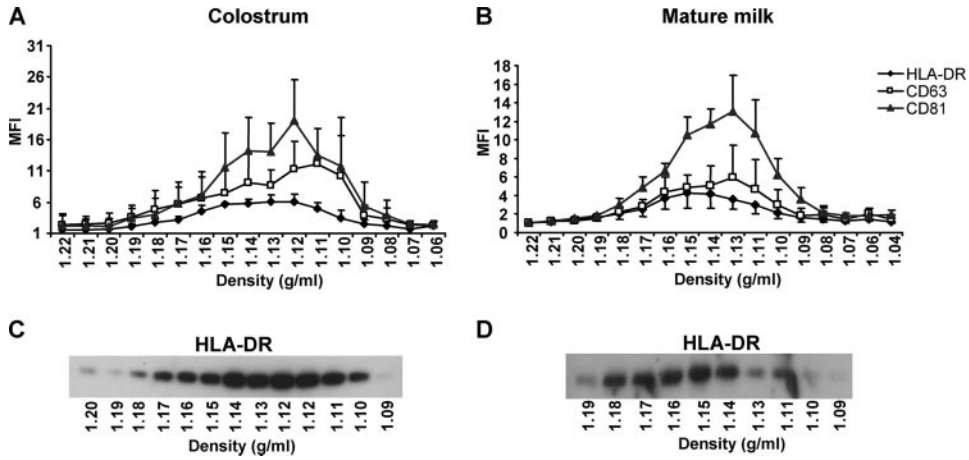

FIGURE 3. Vesicles from both colostrum and mature milk have similar densities as exosomes. Pelleted (100,000 $\times$ *g*) exosome-like vesicles from colostrum (*A* and *C*) and mature milk (*B* and *D*) were loaded on continuous sucrose density gradients and ultracentrifuged. Fractions were collected and either directly analyzed by flow cytometry for mean fluorescence intensity (MFI) after immunomagnetic isolation with MHC class II coated beads (*A* and *B*) and staining with Abs against HLA-DR, CD81, and CD63 or fractions were further spun down and the pellets were analyzed by Western blotting using HLA-DR-specific Abs (*C* and *D*). A minimum of 5×10^3 beads per sample were analyzed. The results are shown as mean value and SD (*A*, $n = 4$; *B*, $n = 5$) or as one representative experiment of three using colostrum and mature milk from different individuals (*C* and *D*).

Table II. *Proteins identified by MS in colostrum and mature milk exosomes*

Swiss-Prot Accession No.[a]	Protein Identification[a]	Colostrum[b]				Mature Milk[b]				Function[c]	Cell Types and References[d]
		S_1	P_1	S_2	P_2	S_1	P_1	S_2	P_2		
(P60709)[e]	Actin (β-actin)	43	2	154	8	176	5	452	15	Cytoskeleton	Mast/B/Mov/MC/IEC
(Q99541)[e]	Adipophilin (ADRP)	67	5	173	8	106	2	422	23	Milk lipid globules	
(P84077)[e]	ADP-ribosylation factor 1	28	1	110	6	43	1	269	8	Vesicle budding	Mov
(P14550)[e]	Alcohol dehydrogenase					68	1	97	3	Enzyme	
(P06733)[e]	α-Enolase	168	4	197	8	197	6	329	12	Enzyme	B/MC/IEC
(P47710)[e]	α-S1-casein precursor	79	2	26	3	118	2	103	4	Calcium transport	
(Q9BY76)[e]	Angiopoietin-related protein 4 precursor					33	1	39	2	Homeostasis regulation	
(P07355)	Annexin A2					56	2	32	1	Calcium regulation	Mov/MC,/DC
(P08133)[e]	Annexin A6					82	2	63	5	Calcium regulation	Mov/MC/DC
(P02649)[e]	Apolipoprotein E precursor (Apo-E)					77	1	102	3	Lipoprotein particle transport	
(Q9UNQ0)[e]	ATP-binding cassette sub-family G member 2	48	2	119	8	146	3	327	14	ABC transporter	
(P15291)[e]	β-1,4-Galactosyltransferase 1					72	1	61	1	Lactose production	
(P61769)[e]	β-2-Microglobulin precursor	33	1	45	1					Antigen presentation, MHC I	
(P05814)[e]	β-Casein precursor	36	3	41	4					Micelle/mammary gland specific	
(P19835)[e]	Bile-salt-activated lipase precursor	95	4	149	7	298	8	408	17	Enzyme	
(Q13410)[e]	Butyrophilin subfamily 1 member A1 precursor (BT)	60	2	204	8	161	6	476	20	Milk fat droplet secretion	
(P16671)[e]	CD36 Platelet glycoprotein IV (GPIV)	274	7	294	12	209	6	353	11	Cell adhesion/fatty acid transport	
(P60033)[e]	CD81 antigen (TAPA-1)	44	1	86	2	36	1	86	1	Cell growth/signal transduction	IEC
(P60953)	Cell division control protein 42 homolog					41	1	73	2	Cell signaling/cell division control	Mast
(Q6WN34)	Chordin-like protein 2 precursor					28	1	38	1	Matrix	
(P10909)[e]	Clusterin precursor					36	1	51	2	Not known/apoptosis	
(P23528)[e]	Cofilin-1	39	1	104	3	81	2	140	4	Cytoskeleton	Mov/DC
(Q96KP4)	Cytosolic nonspecific dipeptidase	32	1	58	1	73	2	67	4	Not known	
(Q9H4M9)	EH domain-containing protein 1 (testilin)	26	1	48	2	58	1	43	1	Endocytic membrane fusion	IEC
(Q9H223)[e]	EH domain-containing protein 4	50	2	59	2					Not known	
(P68104)[e]	Elongation factor 1-α1	75	3	141	4	93	2	165	5	Protein biosynthesis	Mast/DC/B/Mov
(P13639)[e]	Elongation factor 2 (EF-2)					33	1	137	3	Protein biosynthesis	
(P27105)[e]	Erythrocyte band 7 integral membrane protein (stomatin)	112	3	230	7	99	2	263	11	Regulate cation conductance/lipid rafts	DC
(P15311)[e]	Ezrin (p81) (cytovillin) (Villin-2)	107	3	52	6	114	3	258	12	Cytoskeleton membrane interactions	DC/MC
(P49327)[e]	Fatty acid synthase	428	10	1055	49	760	21	1263	48	Enzyme	DC
(P05413)[e]	Fatty acid-binding protein	37	1	199	6	43	1	290	11	Fatty acid transport	
(P15328)[e]	Folate receptor α precursor (FR-α)					34	1	149	5	Binds folate/cell membrane	
(P04075)[e]	Fructose-bisphosphate aldolase A	70	1	40	1	45	1	80	3	Enzyme	B
(Q9NZH0)	G protein coupled receptor family C group 5 member B precursor	53	2	42	1					Not known	
(P04899)[e]	Guanine nucleotide-binding protein G(I), α-2 subunit	37	1	43	3					Transmembrane signaling	
(P62879)[e]	Guanine nucleotide-binding protein G(I)/G(S)/G(T) β subunit 2	38	1	194	10					Transmembrane signaling	
(P50148)[e]	Guanine nucleotide-binding protein G(q), α subunit	54	1	57	2	49	1	99	4	Transmembrane signaling	DC
(P08107)[e]	Heat shock 70 kDa protein 1 (HSP70.1)	29	1	33	4					Chaperone	IEC
(P11142)[e]	Heat shock cognate 71 kDa protein	55	2	68	7					Chaperone	MC/Mov/B/DC
(P01903)	HLA class II histocompatibility antigen, DR α-chain precursor	71	2	40	2					Antigen presentation	B/Mov/IEC
(Q96S86)[e]	Hyaluronan and proteoglycan link protein 3 precursor					48	2	67	4	Matrix	
(P01876)[e]	Ig α-1-chain C region					93	3	116	5	Immunoglobulin (IgA)	

(*Table continues*)

Table II. (*Continued*)

Swiss-Prot Accession No.[a]	Protein Identification[a]	Colostrum[b]				Mature Milk[b]				Function[c]	Cell Types and References[d]
		S_1	P_1	S_2	P_2	S_1	P_1	S_2	P_2		
(P01834)[e]	Ig κ-chain C region	35	1	42	2	56	2	65	2	Immunoglobulin	
(P01842)[e]	Ig λ-chain C regions	28	1	69	3					Immunoglobulin	
(P01871)	Ig μ-chain C region	130	6	110	6					Immunoglobulin	
(P04220)	Igμ heavy chain disease protein (BOT)					58	3	35	4	Immunoglobulin	
(P23229)[e]	Integrin α-6 precursor (VLA-6, CD49f)	40	1	37	1					Hemidesmosome structure	IEC/Mov/DC
(P07498)[e]	κ-Casein precursor					66	4	88	5	Micelle formation	
(Q08431)[e]	Lactadherin precursor (MFG-E8)	672	29	917	32	477	14	795	30	Cell binding	DC/Mov/IEC
(P02788)[e]	Lactotransferrin precursor	148	3	1305	53	194	4	349	18	Antimicrobial, iron binding	
(P06858)[e]	Lipoprotein lipase precursor (LPL)	77	1	72	1	96	2	105	2	Enzyme	
(P07195)[e]	L-Lactate dehydrogenase B chain (LDH-B)	87	2	32	2	41	1	89	3	Enzyme	
(O95573)[e]	Long-chain fatty acid CoA ligase 3	101	3	56	4					Lipid synthesis	
(P14174)[e]	Macrophage migration inhibitory factor (MIF)					56	1	88	2	Cell binding	
(Q14764)	Major vault protein (MVP)					108	3	45	2	Not known	DC/IEC
(Q8N9U0)[e]	Membrane targeting tandem C2 domain containing protein 1	74	2	113	3					Not known	
(P26038)[e]	Moesin (membrane-organizing extension spike protein)	100	3	58	6	135	4	135	10	Cytoskeleton membrane interactions	IEC/MC/Mov/B/DC
(P15941)[e]	Mucin-1 precursor (MUC-1)	57	1	98	4					Cell binding	
(O00159)[e]	Myosin Ic (Myosin I beta)	82	2	49	4					Intracellular motility	MC/DC
(P62937)[e]	Peptidyl-prolyl *cis-trans* isomerase A (rotamase) (cyclophilin A)	94	5	61	5	128	4	168	6	Protein biosynthesis	DC/Mov
(Q06830)	Peroxiredoxin 1 (thioredoxin peroxidase 2)	34	1	34	2	25	1	28	1	Redox regulating	
(P01833)[e]	Polymeric-immunoglobulin receptor precursor (PIGR)	125	3	144	5	172	3	151	5	IgA and IgM binding	
(P07737)[e]	Profilin-1 (profilin I)	46	1	53	2	34	1	62	5	Cytoskeleton	Mov
(P50395)[e]	Rab GDP dissociation inhibitor β	122	4	81	2					Signaling	
(P35241)[e]	Radixin	85	3	73	6	51	2	157	11	Cytoskeleton	DC
(P46940)[e]	Ras GTPase-activating-like protein IQGAP1	50	2	30	1	42	1	29	2	Cell signaling/cell division control	
(Q13228)[e]	Selenium-binding protein 1	140	4	132	7	39	1	142	6	Not known	
(O95436)[e]	Sodium-dependent phosphate transport protein 2B					37	1	101	1	Phosphate transport	
(O00560)[e]	Syntenin-1	124	3	146	6	59	2	226	5	Cytoskeletal/signaling	DC
(O60603)[e]	Toll-like receptor 2 precursor (CD282)					33	1	106	6	LPS-response	
(P09758)[e]	Tumor-associated calcium signal transducer 2 precursor	41	1	33	1					Putative growth factor receptor	
(P62988)[e]	Ubiquitin	62	1	60	2					Protein degradation	DC
(P47989)[e]	Xanthine dehydrogenase/oxidase	754	26	1079	40	969	32	1597	65	Enzyme	

[a] The Mascot algorithm was used to identify the protein names and the Swiss-Prot accession nos. (www.expasy.org/sprot/).

[b] Colostrum and mature milk were analyzed from two individuals indicated by the numbers 1 and 2 in subscript. Only proteins identified in both individuals are shown. Mascot score (S) cut-off was 25 and numbers of matching peptides (P) are indicated. Mass tolerance of 0.3 Da was used for both MS and MS/MS spectra, and the modifications of carbamidomethylated cysteine and oxidized methionine as well as one missed cleavage were allowed.

[c] Suggested functions are derived from the Swiss-Prot database.

[d] Cell types and references (in parentheses): Mast, Mast cell (43); B, B cell (44); Mov, immortalized neuroglial cell (45); MC, mesothelioma cell (46); IEC, intestinal epithelial cell (9, 47); and DC, dendritic cell (48).

[e] Indicates additional confirmation using the ProID algorithm at confidence levels typically >90 but with cutoff at confidence of >50.

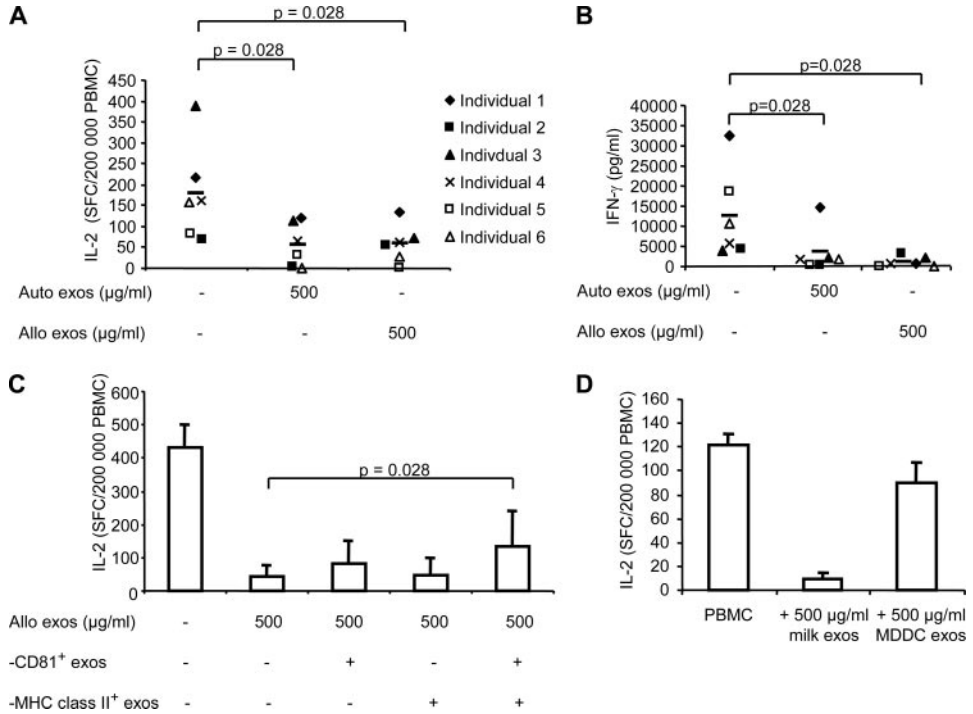

FIGURE 4. Breast milk exosome preparations can inhibit IL-2 production from PBMC. *A–C*, Exosome preparations (500 μg/ml) from mature milk were incubated for 4 days with autologous (Auto) or allogeneic (Allo) PBMC. In the allogeneic cultures the mother's milk exosomes (exos) and PBMC were tested against each other as follows: nos. 1 and 2, nos. 3 and 4, and nos. 5 and 6 (Table I). The cells were thereafter stimulated in duplicate with immobilized anti-CD3 Ab, IL-2 production was measured after 48 h with ELISPOT (*A*), and the supernatants were collected for IFN-γ analysis using CBA (*B*). The results are expressed as the mean of duplicates of SFC per 2×10^5 PBMC (*A*) or as pg/ml supernatants (*B*) for each individual. Each individual is depicted by a separate symbol. Bars show the mean value. Mature milk exosome preparations were in some experiments coated to anti-MHC class II Dynabeads and/or anti-CD81 latex beads and the remaining material not bound to the beads was added to allogeneic PBMC for 4 days, which were thereafter stimulated with anti-CD3. IL-2 production was measured with ELISPOT after 48 h (*C*). Results show mean and SD values of triplicates of SFC per 2×10^5 PBMC from six individuals. Statistical analysis was performed using Wilcoxon's matched pairs test and significant differences are indicated by *p* values (*A*). *D*, Allogeneic PBMC were incubated for 4 days with milk-derived exosomes or with a pool of exosomes derived from the culture supernatants of day 7 MDDC, after which they were stimulated with anti-CD3 and the IL-2 response was detected with ELISPOT after 48 h. The results show mean and SD values of triplicates of SFC per 2×10^5 PBMC derived from one representative experiment of three using PBMC from different donors.

large amounts in MFG (22), was detected in these vesicle fractions (Fig. 2*B*). MFG only showed the presence of MUC-1 and HLA-DR (Fig. 2*B*). B cell exosomes stained positive as expected for CD40, CD54, and CD80 (24) in addition to the other molecules that were found on milk-derived vesicles but were negative for MUC-1 (Fig. 2*B*).

Density analysis of the exosome like vesicles

To further verify the exosomal nature of the extracted vesicles, the 100,000 $\times$ *g* fractions from both colostrum and mature milk were layered on a continuous sucrose gradient (0.25–2.0 M sucrose). The distribution of HLA-DR, CD81, and CD63 over the gradient fractions was analyzed by flow cytometry and Western blotting. We compared the Western blotting to flow cytometry and observed that sucrose fractions could be analyzed by direct coating onto beads and visualized by flow cytometry (Fig. 3), thereby reducing the need for material as well as the labor intensity of the analyses. As shown by both Western blotting and flow cytometry, the HLA-DR molecules were distributed mainly in fractions with densities between 1.10–1.18 g/ml (Fig. 3), which corresponds to the density previously shown for exosomes (7). Similarly, CD81 and CD63 were also enriched in the fractions corresponding to the same densities (Fig. 3, *A* and *B*). Taken together, the results strongly support the hypothesis that exosomes are present in breast milk, and the vesicles will hereafter be referred to as exosomes.

Proteomic analysis of milk exosomes by MS/MS

Sucrose-fractionated exosomes from colostrum and mature milk of two healthy donors were analyzed by LC-MS/MS. Table II shows a list of the total protein identifications that were confirmed in both individuals. Our results verified the presence of several previously described exosome-associated proteins from different exosomal sources. Molecules detected in flow cytometry and Western blotting such as MHC class II, CD81, MUC-1, and heat shock proteins were confirmed by MS/MS (Table II). Lactadherin (MFG membrane protein E8 (MFG-E8)), which has been suggested to be involved in the clearance of apoptotic cells (25), was highly abundant in the exosome milk preparations as has been previously described for dendritic cell-derived exosomes (26, 30). In addition, proteins involved in cell binding, several enzymes, and cytosolic components were found. Notable was the presence of proteins involved in vesicle budding (ADP-ribosylation factor 1) (28) and in endocytic membrane fusion (EH domain-containing protein 1 (Testilin)) (29) which supports the endocytic origin hypothesized for exosomes. Molecules found in our exosome preparations that have not previously been detected on other exosomes include, among others, CD36, butyrophilin, and polymeric-Ig receptor precursor.

Inhibition of cytokine production from PBMC

Next, we investigated whether milk exosomes could regulate T cell responses. Because the infant has half of the genes from the mother

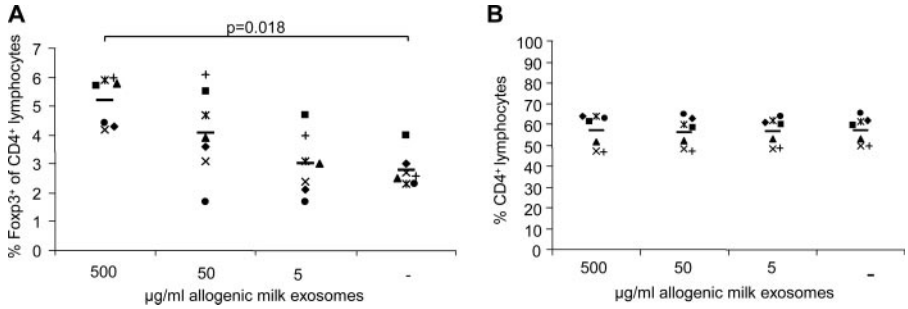

FIGURE 5. Incubation of PBMC with allogeneic milk exosomes induces T regulatory cells. Healthy blood donor PBMC (1×10^6/ml) were incubated with or without 5, 50, or 500 μg/ml allogeneic mature milk exosomes for 4 days. Cells were washed and stained for FACS analysis. The results are presented as the percentage Foxp3$^+$ cells of CD4$^+$ lymphocytes (*A*) and the percentage CD4$^+$ cells of total lymphocytes (*B*). Statistical analysis was performed using Wilcoxon's matched pairs test and significant differences are indicated by *p* values. Each individual is depicted by a specific symbol, $n = 7$. Bars, mean values.

and the other half from the father, exosomes from the mother would be semi-autologous for the child. We therefore incubated the pelleted exosome preparations from mature milk with the mother's autologous PBMC and with allogeneic PBMC from another mother for 4 days. To investigate whether the PBMC were affected by the exosomes, they were activated with anti-CD3 and IL-2 production was measured by ELISPOT. Strikingly, an inhibition of IL-2 production was detected when autologous PBMC were incubated with 500 μg/ml milk exosome preparation ($p = 0.028$; $n = 6$) (Fig. 4*A*). With the same concentration of exosome preparation, inhibition was also seen in the allogeneic situation ($p = 0.028$; $n = 6$) (Fig. 4*A*). When analyzing the supernatants from the IL-2 ELISPOT assays with CBA we could detect a significant reduction of the IFN-γ response from the PBMC when incubated with either autologous or allogeneic milk exosomes at 500 μg/ml ($p = 0.028$; $n = 6$) (Fig. 4*B*). No significant difference was seen for 50 μg/ml exosomes (data not shown). A significant reduction ($p = 0.046$, $n = 6$) was also detected for TNF-α when PBMC were incubated with 500 μg/ml autologous milk exosomes, and a significant increase in IL-5 production ($p = 0.046$, $n = 6$) was seen with 50 μg/ml allogeneic milk exosomes (data not shown). No significant differences were detected for IL-4 and IL-10 (data not shown). To investigate whether this inhibition was due to exosomes or other coisolated factors, milk vesicle preparations were coated to anti-MHC class II Dynabeads and/or anti-CD81 latex beads, the fractions not bound to the beads were added to allogeneic PBMC, and the response was detected with IL-2 ELISPOT as described above. A significant reduction of the inhibition of IL-2 production was seen when the exosomes had been incubated with both anti-CD81 and anti-MHC class II beads (Fig. 4*C*). To investigate whether this inhibitory effect of milk exosomes can also be seen when using exosomes from other sources and to control for inhibition caused by the exosomes sterically hindering the binding of the anti-CD3 Ab to CD3 on the T cells, we repeated the IL-2 ELISPOT assay by incubating allogeneic PBMC with either a pool of MDDC-derived exosomes obtained from different healthy blood donors or milk-derived exosomes. Interestingly, MDDC-derived exosomes could not induce the strong inhibitory effect seen for milk-derived exosomes (Fig. 4*D*). Taken together, these results demonstrate that milk-derived exosomes can inhibit CD3-induced production of IL-2, IFN-γ, and TNF-α while they increase IL-5 production.

Milk exosomes induce regulatory T cells

We hypothesized that the observed milk exosome modulation of IL-2 release could be reflecting an effect on the regulatory T cell fraction of the lymphocyte population. The breast feeding situation is semi-autologous, and because we had seen comparable IL-2 inhibition using either autologous or allogeneic PBMC, we used allogeneic PBMC for regulatory T cell analysis. PBMC were co-incubated with titrated amounts of mature milk exosomes for 4 days, thus reaching the same state as those consecutively stimulated with anti-CD3 Abs in our ELISPOT experiments. Cells were stained for regulatory T cell analysis using intracellular mAb against Foxp3 combined with surface stainings for CD3, CD4, CD8, and CD25. Interestingly, milk exosomes induced a dose-dependent increase of the proportion of Foxp3$^+$ cells (Fig. 5*A*). Significant differences compared with control PBMC were seen for 500 μg/ml milk exosomes. Importantly, neither of the fractions of CD4$^+$ nor CD4$^+$CD25$^+$ lymphocytes changed significantly in comparison to the controls (Fig. 5*B* and data not shown), showing that the number of Foxp3$^+$ cells was increased in PBMC incubated with milk-derived exosomes.

Discussion

We show here the presence of vesicles in milk that display all the hallmarks of exosomes (7, 24): 1) shape and size ~50 nm; 2) density between 1.10 and 1.18 g/ml; and 3) expression of different exosome-associated molecules on the surface such as the high expression of MHC and tetraspanins and the lack of the endoplasmatic reticulum-specific marker calnexin. The milk exosomes show differences in surface staining compared with dendritic cell-derived exosomes (24) such as very low or no staining for MHC class I and CD54 and no detectable levels of CD40 and CD80 but the high presence of MUC-1. They also showed a low level of CD86. This suggests that the milk exosomes may originate from other cells such as the macrophages and lymphocytes present in breast milk (30) or from breast epithelial cells. Alternatively, they could be homing to the milk from other compartments of the body, because serum has been shown to contain exosome-like vesicles (12).

Colostrum-derived exosomes showed significantly higher levels of HLA-DR than mature milk-derived exosomes. The same amount of protein was analyzed in Western blots from colostrum and mature milk derived exosomes and we did not see a significant difference in the protein levels between colostrum and mature milk exosomes, suggesting that the difference is not due to a higher exosome content in colostrum but to a higher expression of HLA-DR on colostrum exosomes. One explanation for this could be that the exosomes in milk are derived from a different composition of cells or cells in a different maturation stage during the first days of lactation.

The fact that we have coated exosomes to anti-MHC class II beads for morphological analysis may lead to the selection of exosomes expressing high MHC class II, and it is possible that MHC class II-negative exosomes are also present in the milk. Preliminary flow cytometry analysis of exosomes bound to anti-CD81 coated latex beads show that exosomes bound to these beads express CD63, CD81, and MUC-1 (data not shown). A positive signal for HLA-DR was detected but was much lower than that for exosomes bound to anti-MHC class II beads, indicating that there are probably also MHC class $II^{low/-}$ exosomes present in the milk.

MS analysis further revealed the presence of proteins not previously identified in exosomes such as butyrophilin, CD36, polymeric-Ig receptor precursor, and immunoglobulins. CD36 on cells has been reported to have diverse functions such as mediation of phagocytosis, cell adhesion, and binding of oxidized low-density lipoproteins (31). The functions of CD36 on milk exosomes need to be further established. It should be noted that even though the exosomes for MS analysis were sucrose fractionated, the coisolation of soluble milk proteins cannot be excluded.

Human breast milk is known to be an important immunologic support system for the infant during the first months of life. The milk exosomes could have dual functions in the infant, because exosomes have been found to be involved in both immune stimulation (7) and tolerization (16). Their expression of MHC class II together with CD86 could suggest a CD4 T cell stimulatory capacity. However, initial experiments adding allogeneic milk exosome pellets to PBMC for 48 or 96 h did not show an increased T cell stimulation compared with autologous exosomes (data not shown). Inversely, both autologous and allogeneic milk exosomes preparations were shown to inhibit anti-CD3 induced IL-2 production. The inhibition was seen with 500 μg/ml exosomes, which corresponds to exosome preparations from ~2 ml of milk (with $\sim 1.5 \times 10^5$ milk cells) per 2×10^5 PBMC. Significant reductions were also seen for IFN-γ and TNF-α at 500 μg/ml exosomes, showing that the inhibition was not only specific for IL-2. This is similar to the results by Taylor et al. (32), who found an inhibitory effect of pregnancy-associated exosomes on PHA-induced IL-2 production in Jurkat T cells in the same concentration range. We further showed that the inhibitory effect seen is not a common feature for all types of exosomes, because MDDC-derived exosomes were unable to induce the strong inhibition seen for milk-derived exosomes. This result also demonstrates that the inhibition seen is not merely due to the exosomes binding to the cells and thereby blocking the anti-CD3 Ab from binding to CD3. The inhibition of IL-2 production could be reduced by removing milk-derived exosomes with anti-CD81 and anti-MHC class II beads, showing that exosomes are truly contributing to the observed inhibition. No significant difference was detected when removing exosomes with only anti-CD81 or anti-MHC class II beads, suggesting that there is a synergistic effect between exosomes that highly express MHC class II and exosomes with lower MHC class II for the inhibition. The blocking of the inhibition was only partial, which could be due to the possibility that not all exosomes were removed with the beads or that there are other factors involved in the milk. Another factor(s) in milk responsible for the inhibition of anti-CD3-induced cytokine production by PBMC besides exosomes could be molecules like IL-10 and TGF-β that are possibly coisolated with the exosomes. It has previously been shown that breast milk is anti-inflammatory, an effect partly mediated by TGF-β (33). However, neither TGF-β nor IL-10 were present in our proteomics data. Another possibility is the presence of MUC-1, which has been shown to be able to inhibit T cell proliferation (34). It has previously also been demonstrated that a high concentration of colostral milk protein can have an inhibitory effect on mitogen-induced T cell proliferation, whereas a low concentration of colostral milk protein can enhance T cell proliferation. The inhibitory effect was not seen for late milk proteins and was suggested to be associated with glycoprotein (35). Sucrose purification of the pelleted exosomes would probably not have given a different outcome, because MS analysis of the flanking sucrose fractions showed the presence of very few proteins (data not shown). The level of inhibition varied between individuals, and this could be due to the time point of sampling, life style factors, or the phenotype and genotype of the mother. Due to a lack of material, functional tests were only performed with mature milk and it is possible that exosomes from colostrum might have different effects.

The mechanism for the reduction in cytokine production could be the suppression of effector T cells due to a higher number of T regulatory cells. In support of this, we here show that PBMC incubated with mature milk exosomes contain a significantly higher number of $CD4^+CD25^+Foxp3^+$ T regulatory cells compared with PBMC not incubated with milk exosomes. It has been shown that $CD4^+CD25^+$ regulatory T cells suppress T cell responses by inhibiting IL-2 production (36). This fits well with our observations where the IL-2 production is reduced when the number of T regulatory cells is increased. How exosomes could influence the regulatory T cells remains to be investigated along with the mechanism behind the exosome-induced increase in IL-5 production. It is possible that exosomes in milk come from different cellular sources and that only a subpopulation of exosomes is inducing regulatory T cells. Exosomes from intestinal epithelial cells, which are positive for the intestinal epithelial-specific marker A33 (37, 38), have been found to induce tolerance (16). Our preliminary data indicate that our milk exosomes are negative for A33 (data not shown), suggesting that the exosomes in milk have not been homing to the milk from the intestinal epithelium.

The role of exosomes in breast milk in vivo remains to be elucidated. Our hypothesis is that either tolerizing or immune-stimulating exosomes can be released into breast milk depending on the route of Ag administration and the immune status of the mother. The age and immune status of the child could also be important for the outcome. The gastric pH is >5 in the neonate and decreases with time to reach adult levels (pH 1–3) at 2 years of age (39). MHC class II expression on exosomes is stable down to at least pH 4 (our unpublished data), which suggests that the exosomes would tolerate the gastric environment of the infant and could be taken up in the intestine and therefore be able to influence the immune system in the infant. However, peptides in the MHC cleft could possibly be eluted due to the low pH in the gastric environment, and it is not known how the exosomes would tolerate the digestive enzymes in the gastrointestinal tract. The effects of breast feeding on the development of inflammatory diseases like allergies are controversial (4, 5). A recent study using a mouse model showed that breast milk from OVA-sensitized mice contains factors that are sufficient to increase the susceptibility of the offspring to the development of allergic airway disease (40). The mediator or mediators in breast milk responsible for this were not identified (40), and whether the exosome could be one of those mediators needs to be further explored.

In conclusion, we here demonstrate for the first time the presence of exosomes in colostrum and mature human breast milk and show that they express MHC class II, CD86, and the tetraspanin proteins CD63 and CD81. We further demonstrate that these milk exosome preparations can inhibit anti-CD3-induced cytokine production from PBMC and increase the number of $Foxp3^+CD4^+CD25^+$ T regulatory cells. This suggests that exosomes in human breast milk have the potential to influence the immune system of the infant.

Acknowledgments

We are grateful to the Proteomics and Mass Spectrometry Unit of the Turku Centre for Biotechnology, Turku, Finland for access to the mass spectrometer. We also thank Dr. Kjell Hultenby with staff, of the Electron Microscopy Unit at the Karolinska University Hospital, Huddinge, Sweden for excellent assistance with the electron microscopy.

Disclosures

The authors have no financial conflict of interest.

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