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The intracellular source, composition and regulatory functions of nanosized vesicles from bovine milk-serum

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Abstract

A hypothesis that the source of milk-serum derived vesicles (MSDV) is the Golgi apparatus (GA) was examined. By using dynamic light scattering and electron microscopy it was shown that MSDV are composed of globular structures with hydrodynamic size of 70±15 nm. More than 60% of the total protein content of MSDV was associated with MSDV lumen and 30% with MSDV membrane. Casein was the major protein found in MSDV lumen. Conclusive markers of the GA: lactose synthase components (α-lactalbumin and galactosyltransferase) and activity (synthesize of lactose from glucose and UTP-galactose), the presence of casein in micellar form in the MSDV lumen and high luminal content of citric acid were demonstrated in the lumen of MSDV. Though MSDV composed only 0.7% of the milk mass, it account for high proportion of total milk content of reactive (15% Cu and 18% Fe) and toxic minerals (60% Cd and 65% Pb), which strongly suggest that MSDV serve as an avenue to protect mammary epithelial cells from their toxic effect by storing them intraluminaly and secreting them into milk. The presence of micellar casein in the MSDV lumen along with the presence of metal transporters in their membrane was responsible for this impressive storing capacity of reactive and toxic minerals. Exposing a single mammary gland to lipopolysaccharide challenge induced changes in regulatory proteins stored in the lumen of MSDV (tissue plasminogen activator, plasminogen and plasmin) and in the activity of xanthine oxidase and alkaline phosphatase attached to the outer membrane of MSDV. Thus, we have demonstrated that MSDV are under nucleus regulation and response to extracellular signals.

Introduction

The most unique attribute that defines the vertebrate class *mammalia* is the production of milk. Milk is the primary nutrient for the neonate, and has many additional roles, such as support of the newborn immune system[1]. Mammary gland (MG) epithelial cells (MEC) are probably one of the most active tissues in the animal kingdom. This trait reach a climax in mammals selected for high milk production; most notably in the modern dairy cows. Nowadays, dairy cows selected for high milk yield can produce 30-60 liter of milk/day, which typically contains ~10% dry matter composed of 30 to 40 g/L of fat and protein, ~50 g/L lactose and 15 to 20 g/L minerals. Among the components secreted in milk, membranous lipoproteins represent the most complex compounds; both, chemically and structurally[1,2].

Two types of milk components are secreted while being surrounded by lipoprotein membranes[1,2]. Milk fat is secreted by a mechanism unique to the MG: membrane-enclosed cytoplasmic lipid droplets composed mainly of triglycerides are synthesized at the endoplasmic reticulum (ER) and are secreted into the MG lumen as 1 to 5 μm-sized particles by enveloping with the MEC apical membrane[3-5]. The typical number of those milk fat globule membranes (MFGM) in bovine milk is ~10^{10}/mL[6].

In addition, the milk of various mammals contains nanosized vesicles in the range of 50 to 70 nm that resides within the milk serum (whey)[1,2]. In cows, the number of these milk serum derived vesicles (MSDV) was estimated to be ~10^{15}/mL, which account for 40-60% of the total content of ~1.5 g/kg milk lipoproteins[6,7]. Thus, the rate of MSDV secretion is ~5 logarithmic orders greater than the secretion of MFGM.

MSDV of bovine origin were first isolated at the early 1950th and described as MEC-derived xanthine oxidase-rich microsomes[8,9]. MSDV of bovine origin were further characterized in the 1970th and were described as either MEC-derived plasma membrane particles[10-12], or vesicles secondly derived from the MFGM following fat secretion into milk[13]. However, experimental evidence allows concluding that the concept that MSDV derived from MFGM is highly dubious[2]. Meanwhile, in parallel and unrelated studies carried out during the early 1970th, human milk samples were shown to contain nanosized particles that exhibit many of the features characteristic of retroviruses[13]. In particular, these human lipoprotein membrane-surrounded retroviruses-like particles with density of 1.16-1.19 g/mL were found to contain a single-stranded 60 and 70S RNA, which was physically associated with a reverse transcriptase[14]. However, cDNA that was prepared from these particles hybridized exclusively with human genomic DNA, indicating a human origin for the particles and disproof the assumption that retroviruses cause breast cancer[15,16]. The interest in MSDV remained dormant for about 3 decades. Lately, finding of MSDV, which were isolated from human milk and were described as exosomes with immune activity[17] renewed the interest in MSDV[18-22]. In addition to possessing immune activity, it was found in human[23], porcine[24] and bovine[25] milk that MSDV contain microRNA

(miRNA), which could be transferred to other cells and be functional in their new location.

Nowadays, it is well established that exsosomes are virtually derived from all kinds of cells and thus are presented in all kinds of biological fluids, including blood, urine, seminal fluid and milk, and that they possess important biological roles, particularly immunological and regulatory, due to their ability to form intercellular communication[26]. Secretion of exsosomes from cells is related to fusion of multi vesicular bodies (MVB) surrounded by plasma membrane, which release their content (endosomes) to the extracellular space. The source of the endosomes entrapped within MVB is vesicles recycling from the plasma membranes. Thus, secretion of exsosomes is a way to secrete proteins that are located within their double-stranded lipoprotein membrane[26]. The lumen was not considered before 2007 to be a significant contributor to exsosomes function as it entraps only a tiny fraction of cell cytosol[27]. However, in 2007, it was demonstrated that the lumen of exsosomes serves as a cargo of mRNA and miRNA stored in their lumen[28] and since then it became clearer that intraluminal genetic material plays an essential role in exsosomes function in intercellular communication[26].

Nevertheless, in contrast to the current prevailing view that MSDV are exsosomes[20-23], based on the presence of biochemical markers in their lipoproteins, it was suggested that the origin of MSDV is the Golgi apparatus (GA)[2]. Moreover, to the best of our knowledge, no explanation or evidence that could clarify the following question was provided so far: How genomic material is incorporated into vesicles circulating between plasma membrane and MVB. An assumption that vesicles, which are derive from the GA are the source of genomic material that is found in extracellular fluids provides a more plausible explanation to this question because of the direct link between GA, ER - ribosome and cell nucleus. Furthermore, vesicles that are derived from the GA may be endowed with additional functional capacities, such as those related to unique characteristics of proteins stored in their lumen. To the best of our knowledge, hitherto, no particular importance was assigned to proteins entrapped within the lumen of secreted vesicles.

The aim of the present study was to provide direct evidences proving that the source of MSDV is the GA of MEC and to highlight their main feature and physiological role. In order to prove these points we show that MSDV resembles non-swollen- secretory vesicles (SV), which are known to be derived from the GA. The ability to store casein in micellar form[29] and showing that MSDV contain the components of lactose synthase and its ability to synthesize in their lumen lactose from glucose and UDP-galactose[30] were taken as major evidences, which prove this hypothesis. The effect of lipopolysaccharide challenge of a single mammary gland was used as an experimental mean to show that the intra-luminal and membrane composition of MSDV is under nuclear regulation of cells, which secrete these vesicles.

Experimental methods

General Procedures

All the reagents, except otherwise mentioned were obtained from Sigma Chemical Co. (Rehovot, Israel). Protein concentration in different types of samples was determined by the Bradford method.

For the analytical procedures described in this paper, high quality raw milk with superior hygienic quality (somatic cell count $\leq$ 470000 cell/mL and with no bacterial isolate) obtained during the noon milking (out of three daily milkings) from Israeli-Holstein cows was used[6]. Milk samples (~500 ml) were taken over a period of 5 years from ~50 cows that were between 36 and 271 days in lactation and belonged to the experimental herd of the Agricultural Research Organization, as previously described[31]. Soon after sampling, the milk was defatted under cold conditions[6] and the skim milk was stored at -20 °C for further analyses. The content of fat, lactose, total protein and its distribution among casein and whey and somatic cell counts were determined in individual milk samples as described before[31].

Isolation of MSDV

Milk samples were centrifuged first at 5000 g for 30 min at 4 °C to remove milk fat globules (MFGs) as well as somatic cells[6]. The skim milk (in 50 mL tubes) was then subjected to ultracentrifugation (Beckman Coulter Instruments, Fullerton, CA, USA) at 4 °C for 1 h at 100000 *g*. Residual MFGs was removed from the top of the vials, the serum, deprived of vesicles, was collected and stored at –20 °C. The fluffy material that contained the MSDV, which was layered above the firm and white casein sediment was separated and diluted in 50 mL of 10 mM–Tris buffer, pH=7.4, containing 300 mM mannitol and 1 mM KCl and centrifuge at 4 °C for 1 h at 100000 *g*. The final sediment was diluted ×4 in the above solution, snapped frozen in liquid nitrogen and stored at -80 °C for further analyses. Casein sediment was collected and stored at –20 °C.

Hydrodynamic size of MSDV

The distribution and the average hydrodynamic diameter of the MSDV nanoparticles in the aqueous solution were measured by dynamic light scattering on a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Measurements were carried out at a scattering angle of 173° at 633 nm. The average hydrodynamic diameter (±5 nm) was calculated based on the intensity distribution using Stokes-Einstein relation, assuming that particles have a spherical shape using a built-in algorithm. The averaged sizes were obtained from three measurements (10 runs per measurement) for each sample using built-in algorithm for number-weighted statistics.

Electron microscopy

Vesicles of MSDV isolated as described above were negatively stained. A 10 μL drop was added to a 400 mesh form var./carbon grid and incubated for 10 min at room

temperature. Ten µL of 1% phosphotungstic acid in double distilled H_2O was added and rapidly blotted from the grid and examined using a Philips CM10 (The Netherlands) transmission electron microscope (TEM), operated at 80 kV.

SDS-PAGE

MSDV isolated as described above and MFGM prepared as described before[6] were used for SDS-PAGE chromatography. The SDS-PAGE procedures were carried out on MSDV samples pretreated as follow: on MSDV which their membrane was disrupted by radioimmuno-precipitation assay buffer (RIPA) buffer as described before[6]. The disrupted vesicles were ultracentrifugated in 5 ml vials at 4 ^{0}C for 1 h at 100000 *g*. The sediment was washed twice with distilled water and then dissolve in 50 mM Tris buffer, pH 8 for SDS-PAGE.

The molecular weight of separated proteins was estimated by comparing protein mobility against a set of standards, viz: myosin, 200000; β-galactosidase, 116000; phosphorylase b, 92500; bovine serum albumin, 66500; ovalbumin, 45000; carbonic anhydrase, 31000; soyabean trypsin inhibitor, 21500 and lysozyme, 14400.

Immunoelectron microscopy

MSDV samples isolated as described above were processed for detection of the presence and distribution of α_{s1}-casein and milk fat globule-epithelial growth factor 8 protein (MFGE8) between membrane and intraluminal space by immunoelectron microscopy procedures adopted from previously published procedure[32]. The samples were fixed in 1% glutaraldehyde, post-fixed in 0.5% of the latter and then washed in 0.1 M phosphate buffer solution (PBS) pH 7.4 and kept each time for 2 h at 4 °C. The samples were rinsed with shaking in PBS pH 7.4 for 30 min and dehydrated in a series of graded ethanol solution (25, 50 and 75%; 30 min per step), and finally placed in 100% ethanol for 1 h. The samples were infiltrated with LR White resin (CAS Number 94188-59-7), first for 30 min in resin:ethanol (1:2, v/v), then for 30 min in resin:ethanol (2:1, v/v) and finally overnight at 48 °C in resin alone, with two changes. The samples were embedded in fresh LR White resin in BEEM capsules and polymerized at 60 °C for 24 h (electron microscopy procedures manual; sharedresources.fhcrc.org/sites/default/EMProceduresManual.pdf). Grids were placed on droplets of 0.05 M Tris-buffered saline (TBS), pH 7.6, containing 1/30 normal goat antiserum for 10 min to block non-specific binding sites. Samples were incubated overnight at 4 °C with α_{s1}-casein (Santa Cruz, monoclonal mouse IgG1, Cat # sc-373711, CA, USA), and MFGE8 (Santa Cruz, rabbit polyclonal IgG, Cat # sc-33546, CA, USA) antibodies with concentrations in the range 1–50 mg/mL, and in the presence of normal goat antiserum. Grids were then washed in TBS and treated with secondary anti-rabbit antibody coated with colloidal 5 nm gold particles (EY Lab. Inc., San Mateo, CA, USA), diluted 1:20, 1:50, 1:100 in TBS. Samples were placed in 2% glutaraldehyde for 5 min, then 1% OsO_4 for 15 min, and were stained with 3%

uranyl acetate for 1 h at 40 °C and rinsed in water. The samples were then observed with a Philips CM10 TEM operated at 80 kV.

Western blot

Immunoblottings were performed on isolated MSDV with Bio-Rad systems (ChemiDoc™ Touch Gel and Western Blot Imaging System, Berkeley, CA, USA) according to the manufacturer's instructions. After the transfer of proteins from the 12% polyacrylamid gel to the PVDF membrane, the membrane was blocked for 1 h with 3% (w/v) skimmed milk in TBST (a mixture of Tris-Buffered Saline and Tween 20). The antibodies used in this study were: toll-like receptor 2 (TLR2) (Santa Cruz, rabbit IgG Cat # sc-10739), toll-like receptor 4 (TLR4) (Santa Cruz, rabbit IgG Cat # sc-10741, CA, USA) and MFGE8 (Santa Cruz, rabbit polyclonal IgG, Cat # sc-33546, CA, USA).

De novo synthesis of lactose in the MSDV lumen

ß1,4 Galactosyltransferase (GalT) activity was determined in MSDV isolated as described above (under isolation of MSDV) and disrupted with RIPA buffer[6] and in milk serum by previously described procedure[33].

Lactose synthesis was measured in MSDV isolated as described under Isolation of MSDV. Basal concentration of glucose and lactose in MSDV disrupted with RIPA buffer was used as blank (i.e., glucose and lactose values after incubation with experimental solutions were subtracted from glucose and lactose concentration at the beginning of the experiment). The experimental solution was composed of 250 µl of vesicle solution that contained ~10 mg/mL protein, 20 µL of 10 mM glucose dissolved in the buffer salt mixture, 20 µL of 10 mM UDP-galactose dissolved in the buffer salt mixture and 210 µL of buffer salt solution composed of 150 mM mannitol in Tris HCl 10 mM, pH 7.4 that contained 4 mM of KCl, 10 mM of NaCl and 3 mM of $MnCl_2$. The final concentrations in the incubation solution were: glucose and UDP-galactose - 0.4 mM, KCl - 27 mM, NaCl - 67 mM and $MnCl_2$ -15 mM. In addition, controls were made by adding phlorrhizin to the salt solution to reach final concentration of 3 mM in the final incubation solution.

The vesicles (6 replications for each incubation period) were incubated under the above conditions for 30, 60, 90 and 120 min. Reactions were stopped by immersing the vesicles in an ice bath. The vesicles were then separated by centrifugation at 15000 RPM in Eppendorf centrifuge (5424R, Hamburg, Germany), washed ×3 with 10 mM–Tris HCl, pH=7.4 and completed to 0.5 ml volume with RIPA buffer and analyzed for glucose and lactose. Citric acid concentration was measured in non-treated vesicles. The concentration of glucose, lactose and citric acid concentration were related to protein composition on MSDV membranes; i.e., on non-disrupted samples. The concentrations of the following metabolites were determined by previously described procedures: glucose[34], lactose[35], and citric acid[36].

RNA content in the lumen of MSDV

Total RNA was isolated from 300 µL of MSDV (equivalent to ~ 30 mL milk) after being disrupted with RIPA buffer, using Trizol (Invitrogen, Carlsbad, CA, USA). The RNA content was quantified by using e-spect UV–vis spectrometer (Malcom, Tokyo, Japan) and confirmed by using Bioanalyzer (Agilent echnologies, Santa Clara, CA, USA).

Analysis of elements in MSDV and milk serum and determining their mass distribution in whole milk

Elements concentration was carried out on skim milk[6], whey (skim milk deprived from MSDV and casein by ultracentrifugation), on MSDV after their membranes were disrupted with RIPA buffer and on the supernatant of disrupted MSDV following ultracentrifugation (as described above for SDS-PAGE analysis). The concentrations of the elements were measured on these preparations by inductively coupled plasma-atomic emission spectrometry (Spectro, Kleve, Germany) as previously described[37]. The distribution of the elements between MSDV and MSDV following ultracentrifugation and whole milk (ignoring the content of minerals in the fat globules) was calculated on the basis of the mass of MSDV in milk as described before[6].

Effect of intramammary challenge with lipopolysaccharide (LPS) on the activities of tissue-plasminogen activator, plasminogen and plasmin in protein precipitated from MSDV and on alkaline phosphatase (Alp) and acid phosphatase (Acp) activity in MSDV and milk serum

The protocols of this study were approved by the Institutional Animal Care Committee of the Agricultural Research Organization, which is the legitimate body for such authorizations in Israel.

The layout details of the study as well as information on milk yield, milk composition and changes in milk pertaining to immune response and imposition of nitrosative stress in response to LPS challenge were previously described[38]. Briefly, 12 Israeli Holstein heifers with low somatic cell count (47000±5000 cells/mL) and no bacterial detection that produced 33.2 ± 3.1 kg milk/d were divided into two groups of six cows. In the control group, two mammary quarters, one front and one rear were infused with 10 mL of a sterile non-pyrogenic saline solution (Teva Pharmaceutical Industries Ltd, Tel Aviv, Israel). The other two counter quarters served as controls. In the experimental cows, two mammary quarters, one front and one rear were infused with LPS; 10 µg of LPS (*Escherichia coli*, O55B5; Sigma Chemical Co.) dissolved in 10 mL of sterile non-pyrogenic saline, while the two counter quarters served as controls. Intramammary infusion was injected using a special applicator following careful sterile cleaning of the teat surface. During the 4 days of the study (−24, 0, +24, +48 and +72 h, where 0 h refers to the day of infusion), every quarter of each cow was separately milked into individual container and milk samples were taken after recording milk yield. Milk was discarded 7 days following the infusion. In this

publication, we present the effect of the above-described LPS challenge on the activity of tissue-plasminogen activator (t-PA, plasminogen (Plg) and plasmin (PL) in casein micelle isolated by ultracentrifugation from skim milk and protein precipitated from MSDV (as described in Isolation of MSDV and SDS-PAGE subsections). Casein micelle pellets isolated from milk by ultracentrifugation and the proteinaceous pellet derived from disrupted MSDV were dissolved and diluted in 0·05 modified Tris buffer composed of 0·05 M-Tris, 0·1 m-NaCl, 0·01% Tween 80, pH 7.6 to reach a final concentration of ~1 mg/mL. The activities of PL, Plg and t-PA were determined as described before[38] and related to protein concentration. In addition the effects of LPS challenge on the activities of alkaline phosphatase (Alp) and acid phosphatase (Acp) in milk serum in milk serum deprived of MSDV and MSDV were carried out as previously described[38].

Statistical analysis

The results of chemical analysis were presented as mean ± SD. The results in the graphs of the glucose uptake-lactose synthesize experiment and in the LPS challenge experiment were presented as mean ± SEM.

The results of the LPS challenge experiment were analyzed by the statistical model described before[38]. Briefly, the model used was:

$$Yijkm = \mu + Ci + Tj + TjDk + Qm(TjCI) + eijkm$$

where, *Yijkm* is the variable within cow, treatment, quarter and day, *Ci* is the cow class effect, *Tj* is the treatment class effect, *Dk* is the day class effect, *TjDk* is the treatment–day interaction effect, *Qm (TjCI)* is the quarter within cow treatment error term for treatment effect and eijkm is the residual error. LPS treatment did not affect the responses in mammary glands of the running control cows (cows that none of their gland was treated with LPS) and therefore these results are not reported.

Results

Size, appearance and composition

The hydrodynamic size of MSDV as determined by dynamic light scattering was 70±15 nm, consistent with TEM micrographs showing that the MSDV are composed of nearly round vesicles in the range of 50 to 100 nm (Fig. 1A). In general, the size of MSDV is in agreement with previous results of vesicles isolated from the serum of bovine milk [20,25] and human milk[17]. The major proteins, composing the MFGM are well characterized[39]. Comparison of the proteins composition in MFGM and MSDV by a coomassie stained SDS-PAGE gel electrophoresis (Fig. 2A) shows that the major proteins in both membrane sources contain the same proteins. However, even at this low resolution, it becomes obvious that the concentrations of xanthine oxidase (~147 kDa) and butyrophilin (~59 kDa) are higher in MFGM than in MSDV, consistent with previous results[20,40]. On the other hand, casein (CN) subtypes, κ-CN, α_{s1}-CN, α_{s2}-CN and β-CN (19-24 kDa) and the major whey proteins, α-lactalbumin (~14 kDa) and β-

lactoglobulin (~18 kDa), are clearly seen in the disrupted vesicles of MSDV gel, while being absent or barley seen on the MFGM chromatogram (Fig. 2A).

Ultracentrifugation of disrupted MSDV resulted in extensive disappearance of the caseins from the specimens (Fig. 2A). The disappearance of caseins was associated with fading of additional proteins (Fig. 2A). White sediment, which resembles the appearance of micellar casein, was observed at the bottom of the ultracentrifuge tubes with disrupted MSDV. No loss of caseins and formation of precipitates is seen when intact MSDV were ultracentrifuge. SDS-PAGE chromatograms of the proteins in the sediment show the presence of the 4 caseins subtypes and of the proteins that disappeared from the disrupted samples (Fig. 2A). In agreement, TEM micrographs of gold immunestaining with antibodies toward α_{s1}-CN show that the lumen of MSDV is densely filled with α_{s1}-CN (Figs. 1B,C). It is relevant to note, that the presence of casein sediment and the presence of soluble α-lactalbumin and β-lactoglobulin was demonstrated in SV isolated from rat MEC[29,41]. Such findings serve as the basis for the commonly accepted concept that milk proteins are secreted into milk via SV [42]. On the other hand, TEM micrographs of pre-embedded colloidal gold immunostained MSDV with antibodies towards MFGE8 (Fig. 1D) and TLR4 (Fig. 1E) obviously demonstrate that these proteins are located within the MSDV membrane, consistent with previous findings[20,43]. The presence of MFGE8 and TLR2 in MSDV was demonstrated by western blotting (Fig. 1F), consistent to their identifying in MSDV by mass spectroscopy[20].

SDS-PAGE chromatograms of the proteins in non-disrupted MSDV in comparison to MSDV in which their membranes were disrupted with RIPA buffer demonstrated that the markings of caseins in the disrupted vesicles were stained more intensively than in intact MSDV (Fig. 2B). This result is consistent with measurements of protein distribution among MSDV membrane and MSDV lumen (Fig. 3). On the other hand, the staining of proteins that are firmly associated with membranes (XO and butyrophilin) was similar in disrupted and non-disrupted vesicles (Fig. 2B).

Previously, it has been shown[6] that the average yield of MSDV was 6.3 mL/L (SD±1.7) or 7.3 g/kg (±1.8). The specific gravity of MSDV in that study was 1.12 (±0.03), which is fairly consistent with the value of 1.10–1.15 obtained by ultracentrifugation in a sucrose gradient[10] or of vesicles derived from human milk and classified as exsosomes[17]. The protein content of isolated MSDV and MFGM membranous substance was previously found to be ~25 mg/g, which represents ~0.65% of the total proteins in milk[6]. In the present experiment, the typical total protein content of MSDV in the buffer solution after disrupting the membranes was ~10 mg/mL (Fig. 3). The protein content in MSDV, in which the membranes remained intact accounted for ~40% of the total content, whereas the content of the protein precipitated by ultracentrifugation of membrane-disrupted MSDV accounted for additional ~30% (Fig. 3). This means that ~30% of the total protein content in MSDV was not accounted for by the above-described procedure.

Xanthine oxidoreductase (XOR) is a major protein in MSDV[6] (Fig. 1A), which is present in the membrane in two forms, xanthine oxidase and xanthine dehydrogenase.

Xanthine dehydrogenase was found to be firmly associated with the membrane of MSDV on its luminal side. In the next section, we present evidence for the presence of lactose synthase, which is composed of α-lactalbumin and GalT in MSDV. The lactose synthase complex is associated with the lumen of the GA[30,44] and GalT is known to be firmly associated with membranes[45]. Thus, the content of protein in MSDV, which was not explained by our fractionation scheme, may account for proteins that are firmly associated with MSDV membrane from the luminal side and to proteins, such as β-lactoglobulin, which are present in the lumen of MSDV in a 'true' solution and therefore, does not precipitate by ultracentrifugation and to some other proteins, which are lost during the procedure. Previously, it has been shown by us and others that the membranes of MSDV are a rich source of enzymes, including xanthine dehydrogenase, xanthine oxidase, acid phosphatase, alkaline phosphatase, nucleotide pyrophosphatase, γ-glutamyltranspeptidase and sulphydryl oxidase and Mg^{2+}-ATPase[2,40]. Below, we show that the components of lactose synthase are located within the vesicles and that the activity of xanthine oxidase and alkaline phosphatase on the membrane of MSDV is under regulation. All and all, our study show that most proteins (> 60%) in MSDV are not associated with the plasma membrane, as it supposed to be the case with exosomes[26,27].

De novo lactose synthesis

A single previous report demonstrated the presence of GalT in milk[46]. In the present experiment, GalT activity in whey was found to be 28.2 (±5.1) nmole/h/L milk (Fig. 4), whereas GalT activity in the lumen of MSDV was 156.1 (±12.2) nmole/h/L milk (Fig. 4). As the content of MSDV in liter milk is ~6.3 mL, it appears that GalT activity in the lumen of MSDV is ~87 fold more concentrated than in whey. This result is consistent with the tendency of GalT to be firmly bound to membranes[45] and with the overall resembles between the internal composition of MSDV and SV; in particular, both sources of membrane share the content of lactose synthase[29,47].

α-lactalbumin and GalT compose the lactose synthase enzyme, which converts glucose and UDP-galactose into lactose with liberation of free UDP. Incubation of MSDV with glucose and UDP-galactose was associated with time-depended increase in glucose uptake into the vesicles (Fig. 5A) and with parallel increase in lactose content in the vesicles (Fig. 5C), which serve as the basis for calculating the glucose uptake (Fig. 5B) and lactose synthesize (Fig. 5D). The efficiency of conversion of glucose and galactose into lactose was ~40% and was calculated as glucose uptake ×2 (based on their 1:1 ratio in lactose synthesis) divided by lactose synthesis. Glucose uptake rate and lactose synthesis rate show saturation kinetics, consistent with previous experiments with GA apparatus derived vesicles[30] rat homogenized mammary gland acini[48] and bovine and mice SV[29]. Incubation of the MSDV with 10 mM phlorrhizin, an inhibitor of glucose uptake, completely prevented the accumulation of glucose and lactose concentration in the vesicles, hence lactose synthesis, which is in agreement with the results of Kuhan and White[30]. However, the efficiency of lactose synthesis from its precursors in the present experiment was 3-

fold higher than typical conversion efficiency of glucose to lactose at rate of 7-10% in rat homogenized mammary gland acini[30].

Metabolites content in MSDV

In addition to lactose (Fig. 4; 260±15 μmole/g protein), the lumen of MSDV contains high concentration of citric acid (Fig. 4; 515±21 μmole/g protein). When the content of these metabolites is converted into intraluminal concentration (lactose at the range of 6 mM and citric acid at the range of 12 mM), taking into account that typical that protein composition in MSDV is 24 mg/mL[6], it becomes clear that the concentration of these metabolites is much higher than what can be expected to be found in MEC cytosol. The concentration of citrate in MSDV lumen was about equal to that of milk[6,49], but that of lactose is much lower than typical concentration of ~145 mM. Consistently, the concentration of lactose in MSDV is considerably lower than in bovine SV at 1188 μmole/g protein[47]. The RNA content in MSDV isolated from 50 mL milk was 7.2±1.2 μg (Fig. 4), which agrees with previous findings of RNA in nanosized vesicles isolated from bovine milk[25].

Elements content in MSDV

In general, the concentrations of macro- and micro-elements in whole milk, whey and milk micellar casein fraction in the present experiment (Table 1) is consistent with the typical composition of those elements in bovine milk[50]. The concentration of heavy toxic metals in milk can vary over a large range and those found in the present study for cadmium and lead are consistent with relatively low levels of those metals in milk and were smaller than the newly established Codex standard, 20 μg/kg for Pb and 500 μg/kg for Cd[51]. Ca concentration in MSDV mirrored that of whole milk and was mostly associated with MSDV proteins (Table 1). The ratio between Ca content within MSDV and total Ca content in milk (0.72%) was similar to the proportion of MSDV in milk[6]. Like in the case of Ca, the intravesicular concentration of Mg, P, and Zn was similar to that of milk and the proportions of Mg, P, and Zn out of total milk content and their association with intravesicular proteins were similar to that of Ca. Intravesicular concentrations of Na and K were similar to their concentration in milk and thus Na and K contribution to total milk content was similar to the ratio between MSDV and milk mass. However as in whole milk, intravesicular Na and K were not precipitated with proteins by ultracentrifugation, indicating that they are truly soluble in MSDV.

All the minerals mentioned below were mostly associated with intravesicular proteins as indicated by their disappearance upon protein precipitation by ultracentrifugation. Intravesicular concentrations of trace element, Cu, Fe, Mn, Se and Si were much higher than in milk, accounting for 2% (Mn), 3%, (Se), 4% (Si), 15% (Cu) to 18% (Fe) of the respective total milk mineral content (Table 1). The concentrations of toxic minerals were much higher in the intravesicular compartment than in milk, accounting for 8 % (Ba), 60% (Cd) and 65% (Pb) of the total milk content. In the case of Cd and Pb, their concentration in whey fell to below the detectable level and therefore calculating their concentration in casein micelles was

not possible. It is well established that Ca, Cu, Fe, Na, and Zn enter the cells and the intracellular membrane compartments through dedicated transporters[52] and are important in the secretion of trace elements into milk[53]. When the intravesicular concentrations of Ca, Cu, Fe, Na, and Zn was used as independent variables and those of the other intravesicular minerals was used as depended variables the following significant correlations (n=12; and at least $P < 0.05$) were found: Ca was significantly interrelated with Ba, Mg, Zn; Cu was significantly interrelated with Fe, Mn, Pb, Fe, K, Na, Pb; Na was significantly interrelated with Ca, Fe, Mg, Pb; and Zn was significantly interrelated with K, Mg, Na, Sc, Si, Cd.

Effect of intramammary challenge with lipopolysaccharide on the plasmin system, xanthine oxidase, alkaline phosphatase and acid phosphatase activities

The plasmin system which is composed of plasminogen activator (PA), the non activated zymogene, plasminogen and the serine protease plasmin is a ubiquitously enzymatic system expressed in mammalians milk[1]. Degradation of casein and liberation of active components from casein by the plasmin system has been shown to play a regulatory role in day-to-day regulation of milk secretion[54-56] and induction of involution upon initiation of milk stasis[1,31,57]. Recently, it has been shown that t-PA is the PA that degraded casein and that t-PA, Plg and PL are embedded in casein micelles in milk and most likely are secreted from MEC attached to the casein micelles[31].

In a previous experiment[6], it has been shown that 25% of xanthine oxidase (XO) activity is presented in the outer surface of MSDV, whereas 46.7% in solution and the remaining on the outer surface of MFGM. Similarly, the activity of Alp and Acp in milk is distributed between the outer surface of MSDV and MFGM and it is present in a true soluble form in whey. The activity of Alp and Acp on MSDV is about twice their activity in whey[6].

In the present experiment, we looked at the effect of LPS challenge to single mammary glands of cows on the of activity of the plasmin system in the lumen of MSDV and casein micelles in milk and on XO, Alp and Acp in whey, MSDV membrane and whey deprived of MSDV (Figs. 6, 7). LPS treatment of single glands did not induce response of the plasmin system in MSDV or milk micelles and on XO, Alp and Acp activities in the control glands, indicating that responses in enzyme activities were specific to the effect of LPS on the treated glands. LPS challenge of experimental glands induced a transient ~2-fold increase in t-PA activity at 24 h post-treatment in proteins stored in the MSDV lumen and milk casein and a corresponding ~1.7-fold decrease in PG activity, an increase of ~11-fold in PL activity (Figs. 5A-C). These changes in activities of Plg and PL were reflected by a decrease in the PG/PL ratio from ~27 to ~1.5 at 24 h post-treatment in MSDV and casein micelles in milk. The changes of t-PA, Plg and PL activities in milk casein were similar to those in whole milk under similar conditions[38]. Thus, the results of this experiment are consistent with the suggestion that the components of the plasmin system are secreted into milk while being embedded with the casein micelles[31].

LPS treatment did not increase the activity of Acp (a lysosomal enzyme) in whey (Fig. 7A), MSDV and whey deprived of MSDV (data not shown), indicating that the treatment did not induce changes in the secretion of this enzyme in comparison to pre-treatment and control glands. LPS treatment induced a significant transient increase in the activities of XO and Alp in whey, MSDV and whey deprived of MSDV (Figs. 7B,C), which reached a pick at 24 h post-treatment, indicating that LPS induced a dramatic increase in the secretion of those enzymes into the milk. However, when comparing the activity of XO and Alp in MSDV and in whey deprived of MSDV, it becomes clear that most of the increase in the activities of these enzymes in whey is related to the increase in their secretion into milk via the MSDV. In comparison to the response of the plasmin system components and XO activity to LPS challenge, the responses of Alp activity remained high at 48 h post-treatment and at 72 h post-treatment they were still significantly higher than the pre-treatment values, whereas the values of the plasmin system components and XO activity returned at 72 h post-treatment to the pre-treatment levels (Figs. 7B,C).

Discussion

Electron microscopy micrographs of fusions between MVB and the plasma membrane and the consequent release of vesicles have so far been the strongest evidence for the endosomal origin of nanosized extracellular vesicles, commonly defined as exosomes[27]. Alternatively, also by using electron microscopy micrographs technique, it was shown that intraluminal vesicles of MVB can be delivered for degradation into lysosoms[58] (see Fig. 8 for illustration). It would require the use of streaming picturing in electron microscopy resolution to provide direct evidence of the cellular source of endosomes incorporated into MVB. Though, to the best of our knowledge, although there is no direct proof, it is commonly believed that the source of exosomes is the plasma membrane of cells[26,27]. This assumption was based on a similar membrane orientation as the plasma membrane and similar protein composition, which is composed mostly of proteins of the plasma membrane[26,27]. However, because GA releases numerous amounts of SV, which fuse with the plasma membrane and thus determine plasma membrane composition[2-4, 10-12], the same argument may apply to vesicles released by the GA. Indeed, at least on a theoretical ground, it was illustrated in schematic figures in several reviews that vesicles derived from the GA may serve as source of intraluminal endosomes in MVB[27, 59-61]. The present experiment was carried out to prove that nanosized vesicles, which are secreted by MEC and are isolated from milk serum of bovine milk, are derived from the GA. We provide 3 lines of evidence, each of which provides conclusive evidence for that assumption. Casein is the main protein in bovine milk, constituting 2/3 of the total protein content of milk (~ 20 g/L)[62]. After synthesis of the 4 casein sub-types in the ER, a casein macromolecule is assembled in the GA into micelles, which are stored in vesicles[62]. The casein micelles are composed of ~ 40 monomers of casein sub-types with molecular weight of ~ 1500000 Dalton[63]. We show here that the MVB lumen is densely filled with casein in its micellar form, indicating that the source is the GA. On

the other hand, the possibility that their source is the plasma membrane is nil because it lacks storage capacity. Synthesize of lactose is a unique feature of GA in mammals[44]. Thus, the demonstration of lactose synthase components in the lumen of MSDV and demonstration of actual synthesize of lactose when MSDV were incubated with lactose precursors is an additional definite evidence for the GA apparatus origin of MSDV. The third line of evidence is based on demonstrating that extracellular cue (LPS) dramatically affected intraluminal and membrane protein composition of MSDV. This response can be explained as an efferent response: cell nucleus-ER-GA, to the well-defined afferent effect of LPS signaling that affects the cell nucleus. Below, we discuss that evidence in detail as well as data that secretion of MSDV play indispensible physiological role in MEC function.

The evidences that MSDV is derived from the GA

It is well established that lactose is synthesized in the GA of mammals[44]. Lactose is synthesized in the GA by incorporating Glu with UDP-Gal by lactose synthase to yield lactose and UDP[30]. Here we show that lactose synthase components, α-lactalbumin and galactosyltransferase[64], are present in the lumen of MSDV. In previous studies, it was shown that under appropriate incubation conditions, lactose can be synthesized from Glu and UDP-Gal in whole mammary cells[48] (mammary acini), from GA-derived fractions[30] and from SV-rich fractions[29]. In the present experiment, we demonstrated that lactose is synthesized in the lumen of MSDV following their incubation in medium that contains Glu and UDP-Gal. Consistent with the above-mentioned *in vitro* experiments, we show that the uptake of Glu can be inhibited by phlorrhizin, an inhibitor of glucose transport and that inhibition of Glu uptake inhibited lactose synthesis.

SV from several epithelial tissues, including pancreas[65], liver[66] and parotid gland[67], have been shown to be tightly packed with proteins and other organic compounds. Because the intravesicular constituents of those vesicles are composed of high-molecular weight compounds which are osmotically inactive, they are relatively small and un-swollen, and thus stable during homogenization and centrifugal isolation. Here we show that MSDV are small (in the nano-range), packed with proteins and can be stably isolated by ultracentrifugation from milk. In contrast to the features of the above-mentioned types of SV and MSDV, secretory vesicles in lactating mammary epithelial cells are swollen and distended, thus reaching the micron range[29], which makes them exceptionally fragile and unstable under ultracentrifugation[68]. What makes MG SV different from SV in other tissues is that MG SVs are packaged with high concentration of lactose, which is similar to the concentration of lactose in milk[29]. The size of SV immediately after being released from the GA is similar to that of MSDV (Ref. 29 vs. present results; i.e., in the sub-micron range). On their way to the apical membrane, SV of MEC become swollen and distended with diameter in the range of 0.2 to 1.2 μm[29]. It has been hypothesized that osmotic swelling of MG SV on their way from the GA to the apical plasma membrane accounts for secretion of the liquid phase (milk minus the lipid globules) of

milk[42,69]. The presence of high concentration of lactose, an osmotically active molecule, which is impermeable through membranes, packed in the MG SV accounts for their swelling[70]. Lactose concentration in MSDV is much higher than can be expected to be found in the cytoplasm. However, the lactose concentration in MDSV is considerably lower than lactose concentration in milk or in SV, which explains why these vesicles do not swell and their size remained on the nanosized scale. Nevertheless, like in MG SV[29,47], MSDV contain lactose synthase, casein in micellar form, citrate and minerals. The minerals inside MSDV are distributed according to the expected effect of micellar casein on mineral distribution between the micelles and the bathing solution[50]. Thus, the present results unambiguously indicate that the cellular source of MSDV is the GA and it strongly suggests that they are non-swollen subtype of SV that are released intact into the milk (Fig. 8). The size of typical casein micelles in milk is ~150-250 nm is greater than the size of MSDV. However, casein micelle has a sponge-like structure[71], which may explain its ability to be squeezed inside the MSDV lumen and it's swelling inside SV along with an increase of its hydration.

MSDV has important physiological role

Whereas the content of casein and whey proteins inside the MSDV lumen does not significantly contribute to the content of casein in milk, our results strongly suggests that it has an essential role in the regulation of elements secretion into milk. Casein micelles are composed of casein subtypes, α_{s1}-CN, α_{s2}-CN, β-CN and κ-CN, which contain phosphoseryl and carboxyl residues that serve as binding sites for different minerals[72]. In addition to the presence of casein in micellar form, we located proteins such as α-lactalbumin in the MSDV lumen, which can bind minerals[73]. Consistent with the mineral binding capacity of those proteins, we show that except monovalent ions, most of the minerals inside the MSDV were associated with proteins that precipitated following ultracentrifugation.

Modern dairy cows secrete daily between 0.7 to 1.4 kg of minerals in their milk, which originate from the blood. This means that the MEC accumulates many minerals that cannot be metabolized or stored, and some of them can become toxic to MEC. Fe and Cu are minerals that are essential in small amounts, but can easily rich toxic levels due to their tendency to react with peroxides and produce free radicals. The ability to accumulate elements such as Fe and Cu in the lumen of MSDV allows MEC to avoid their accumulation to toxic levels in the cells, in order to prevent formation of free radicals in milk and thus, to prevent deterioration of milk quality during storage in the gland, while preserving the availability of essential micro-elements such as Fe, Cu, Zn, Se to the offspring.

In the case of highly toxic elements such as Ba, Cd and Pb, this function becomes critically important for MEC function, which may explain their very high proportion in MSDV. The dilution of toxic elements in milk may make their presence in milk less critical for the offspring. In any case, from the evolutional point of view, preserving the life of the mother having the capacity to give more birth is more important than the life of her offspring, which carries only half of her gens.

GA is a rich source of metal ions transporters, including several types of Ca^{2+}/Mn^{2+} pumps[74], type IIa Na^{+}/Pi-cotransporter[75], Cu[76], Fe[77] and Zn[78]. Consistently, Ca^{2+}-dependent ATP hydrolysis was found in Golgi vesicles of lactating rat mammary glands[74] and MSDV of bovine milk[40], and type IIa Na^{+}/Pi-cotransporter was localized on MSDV isolated from goat's milk[79]. The Zn transporter, ZIP13, can also transport Fe[77], and NRAMP2 (natural resistance-associated macrophage protein 2)/DMT1 (divalent metal transporter 1), a metal transporter conserved from prokaryotes to higher eukaryotes, which exhibits an unusual broad substrate range, including Fe, Zn, Mn, Cu, Cd, Co, Ni, and Pb[80]. Such overlap in metal transport may explain the high correlation between the minerals stored within MSDV.

In conclusion, the capacity of MSDV to store minerals in proportion that exceed significantly their mass proportion in milk is of such importance that can justify the considerable allocation of resources devoted to its secretion. The high proportion of mineral binding inside the vesicles lumen may be explained by two major factors: i) The presence of various types of metal transporters on MSDV membranes, which allow concentrating them in their lumen, and ii) The presence of intraluminal mineral-binding proteins, mainly in the form of casein micelles, which chelate the transported minerals and prevent their leakage from the vesicles.

The interaction between TLR4 and LPS activates a well defined signal transduction pathway to the cell nucleus, the nuclear factor-kappa B (NF-κB) pathway[81], which in turn rouse the secretion of components of the innate immune system, such as critical proinflammatory cytokines[82] and XO[83] and the plasmin system[57], which is part of the milk-born negative feedback system that regulates milk secretion[57,83]. Synthesis of proteins destined to compartments of the secretory pathway takes place in ribosomes according to information transported with t-RNA from nucleus[84]. After completion of its synthesis and folding, the proteins proceed further to its final destination through the Golgi complex. In polarized cells, such as MEC, the main compartment of protein sorting is the GA, where apical and basolateral cargoes are segregated and targeted to their specific destination[85]. Hence, the increase in the activity of the plasmin system components in the lumen of vesicles in which the mammary gland was challenged with LPS provides direct evidences that it was an efferent response to the afferent effect of LPS on cell nucleus. The changes in the intraluminal activity of the plasmin system reflect the role of this system in ruling casein degradation[31], and are consistent with additional findings on the similarity between the internal composition of MSDV and SV. On the other hand, the changes in XO and Alp in MSDV membranes reflect most likely the contribution of MSDV to the innate immune response, as these enzymes are free to exert their effects while being attached to MSDV[2,57,83].

Previous results ascribed an immunological role to MSDV-like structures (exsosomes), which were isolated from milk[17,18,21]. In the present experiment, we found that MSDV contains on its membrane immunoglobulin G, butyrophilin, MFGE8 and receptors for endotoxins (TLR2 and TLR4). All these components have immunological functions[21], which sustain this possibility and suggest that MSDV may

have a particular function as a platform of scavenger receptors and as a modulator of immune functions.

Defining MSDV as golgisomes and what makes them different from exsosomes?

In milk, MSDV are not exposed to relevant concentrations of glucose and UDP-galactose, like those used to induce lactose synthesis in the vesicles in this study. Thus, the lactose synthase activity in MSDV is most likely a redundant phenomenon that reflects their GA origin. However, the secretion of such large numbers of complicated structures is most likely associated with essential physiological functions, as indeed is supported by the evidences discussed above. To the best of our knowledge, this is the first study that provides evidences that the composition of vesicles secreted from cells to the extracellular environments is under genomic regulation and can be modified in response to environmental signals such as LPS. Consequently, we suggest terming MSDV as golgisomes (Gsomes), to reflect their similarity in size to exsosomes and their GA origin, hence, similarly to SV (Fig. 8). The presence of RNA material in Gsomes is consistent with their GA origin and supports the notion that their internal composition is subjected to regulation by the cell nucleus. Research on exsosomes from various cell types revealed a striking interrelationship between the biological qualities of the vesicles and their cell origin; for instance, exsosomes derived from cancer cells, increase tumorogenicity and vesicles derived from leukocytes posse's immunological functions[26]. This interrelationship between cell type and biological function of vesicles would have made less sense if the source of these vesicles was the plasma membrane, but can be a direct outcome if the source is the GA in the form of Gsomes. Thus, many of the vesicles currently defined as exsosomes might in fact be Gsomes, as it is shown here to be the case for vesicles secreted by MEC.

The association of Gsomes with the GA along with their abundance and simplicity of isolation from milk highlights their potential to serve as a model for exploring the presence and function of GA-derived vesicles, GA-associated transporters, such as metal ion transporters. The presence of toxic elements in milk and milk products is of major public concern because it may pose health problems, especially for infants[51]. The present results indicate that analyzing the mineral content stored in Gsomes may serve as a sensitive indicator for the exposure of dairy cows to toxic elements, hence, as a sensitive gauge for milk safety.

Conclusions

Compelling evidences that milk serum derived vesicles (MSDV) originate from the GA and are secreted intact into the milk were presented. We show that the intraluminal protein composition of those nanosized vesicles is characteristically different from what is expected to be found in exsosomes and more closely resembles those of secretory vesicles of epithelial cells. We also show that proteins associated with the outer surface of the MSDV membrane comprise important physiological functions in milk as components of the innate immune system. Moreover, casein

stored in the lumen of MSDV endowed them to accumulate reactive (Fe, Cu) and toxic metals (Cd, Pb) and thus, prevent their deleterious effect on mammary epithelial cells. We show that the membranous and intraluminal composition of MSDV are under nucleus regulation and response to challenge induced by glandular exposure to LPS. Based on the novel and unique properties of MSDV, we suggest terming them golgisomes. As discussed herein, accepting that many of the vesicles currently termed exsosomes are in fact golgisomes, may explain many currently unexplained characteristics of exsosomes, such as storing RNA and miRNA in their lumen.

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FIGURE LEGENDS

Figure 1. Visualization of MSDV by TEM (1A), presence of α_{s1}-casein in MSDV lumen by Immunoelectron microscopy (1B,1C), presence of MFGE8 (1D) and TLR4 (1E) in MSDV membrane, and presence of MFGE8, TLR2 and TLR4 in MSDV membrane by western blot (1F).

Notes: Fig. 1A shows TEM micrograph of globular bodies of MSDV, which are consistent with the hydrodynamic size of MSDV as determined by dynamic light scattering. In Figs. 1B-1E, high magnifications of immunoelectron microscopy micrographs focusing on single vesicles are presented. The gold nanoparticles used in this procedure perforate the membrane, resulting in staining α_{s1}-casein within and outside the vesicles because of leakage of casein to the surrounding around the vesicles (Figs. 1B,1C). On the other hand, the firm association of MFGE8 (Fig. 1D) and TLR4 (Fig. 1E) with the lipoprotein membrane surrounding MSDV resulted in clear marking of the membrane without evidence of leakage or intra-vesicles markings. Consistently, western blot of proteins from the membranes of intact MSDV show the presence of membrane proteins: MFGE8, TLR2 and TLR4 (Fig. 1F).

Figure 2. Characterization of proteins located on MSDV membranes and MSDV lumen.

Figure 2A. SDS-PAGE chromatography of MFGM and MSDV. Lane M – markers. In lanes 1 to 4 the protein load was 20 μg/10 μL, and in lane 5 to 8 the protein load was 10 μg/10 μL. Lanes 1, 5 – MFGM; Lane 2, 6 – MSDV disrupted with RIPA buffer; Lanes 3, 7 - MSDV disrupted with RIPA buffer after ultracentrifugation; Lanes 5, 8 – chromatography of the proteins precipitated by ultracentrifugation and after re-dissolution in Tris buffer 50 mM, pH 8.

Figure 2B. SDS-PAGE chromatography of MSDV in which the membranes were disrupted with RIPA buffer in comparison to MSDV with intact membranes. Lane M - markers, Lane 1 - non-disrupted MSDV, lane 2 - disrupted MSDV. The protein load was 5 μg/10 μL.

Note: The markings of caseins are clearly shown in the disrupted vesicles while casein is less intensely stained in intact MSDV. On the other hand, membrane-associated proteins (XO and butyrophilin) are stained equally in disrupted and non-disrupted MSDV.

Figure 3. The distribution of protein in MSDV among membrane (protein content in non-disrupted vesicles; green) and intravesicular (protein in disrupted MSDV minus non-disrupted protein; orange) and protein not accounted by this procedure (blue).

Figure 4. Activity of galactosyltransferase (GalT) in milk and MSDV (nmole/h/L milk) and the concentrations of lactose (μmole/g non-disrupted membrane protein)

and citric acid (μmole/g non-disrupted membrane protein) and the content of RNA (μg/L milk) inside MSDV.

Figure 5. Kinetics of glucose uptake and lactose synthesis in MSDV following their incubation with glucose and UTP-galactose with and without 10 mM phlorrhizin. A. Kinetics of glucose concentration in the MSDV lumen. B. Kinetics of glucose uptake into the MSDV lumen. C. Kinetics of lactose concentration in the MSDV lumen. D. Kinetics of lactose synthesis in the MSDV lumen.

Figure 6. Effect of lipopolysaccharide treatment of a single mammary gland on the activity of the plasmin system components in casein micelles and vesicles derived from the milk of treated and contralateral control glands. A. Changes in tissue – plasminogen activator. B. Changes in plasminogen activity. C. Changes in plasmin activity.

Figure 7. Effect of lipopolysaccharide treatment of a single mammary gland on the activity of acid phosphatase, alkaline phosphatase and xanthine oxidase in milk serum, vesicles derived from the milk and milk serum deprived of vesicles from the treated and contralateral control glands. A. Changes in acid phosphatase activity (only in milk serum). B. Changes in alkaline phosphatase activity. C. Changes in xanthine oxidase activity.

Figure 8. An illustration of the voyage made by Golgi apparatus (GA)-derived vesicles to the apical plasma membrane in MEC. On the right side of the scheme, an illustration of the movement and osmotic swelling of secretory vesicles (SV) from the GA to the apical plasma membrane and release of casein micelles following fusion of SV with the membrane. On the left side of the scheme, an illustration of the conventional view of how exsosomes are derived from internalization of plasma membrane vesicles, passing of these vesicles into early and late multi vesicular bodies (MVB) and finally release of exsosomes from late vesicular bodies into the extracellular space following fusion of MVB with plasma membrane. The central part of the scheme describes the formation and secretion of golgisomes. Unique to golgisomes is that they are under direct regulation of the nucleus and that they respond to external signals, as illustrated by the line between the plasma membrane receptor of lipopolysaccharide (LPS), TLR4 and the nucleus. The interrelationship between the nucleus-ER-ribosome and GA is also illustrated.

Table 1. Distribution of Na, K, P, Ca, Mg, Zn, Mn, Se, Si, Ba, Cu, Fe, Cd and Pb among whole milk, milk serum (whey), milk casein, milk-serum derived vesicles (MSDV) and proteins precipitated from disrupted MSDV that underwent ultracentrifugation.

	Na mg/kg	K mg/kg	P mg/kg	Ca mg/kg	Mg mg/kg	Zn µg/kg	Mn µg/kg	Se µg/kg	Si µg/kg	Ba µg/kg	Cu µg/kg	Fe µg/kg	Cd µg/kg	Pb µg/kg
Whole milk	406.0 ±19	1515.0 ±227	1916 ±235	1160.0 ±213	125.0 ±22	3760.0 ±330	255 ±17	275 ±19	609 ±15	365 ±21	164 ±21	210 ±23	11.0 ±5	25.0 ±6
Whey [1]	385.0 ±18	1424.0 ±232	1054 ±198	336.0 ±12	44.0 ±10	1128.0 ±250	82 ±8	77 ±7	213 ±8	135 ±9	36 ±7	54 ±8	b.d.l	b.d.l
% in whey	95.0 ±5	94.0 ±6	55 ±7	29.0 ±4	35.0 ±6	30.0 ±5	32 ±6	28 ±6	35 ±7	37 ±6	22 ±5	25 ±6	-	-
Casein [2]	18.0 ±4	80.0 ±5	844 ±95	814.0 ±192	80.0 ±9	2582.0 ±321	165 ±15	189 ±14	369 ±13	200 ±15	103 ±11	118 ±14	4.0 ±1	10.0 ±2
% in casein	4.0 ±1	5.0 ±1	44 ±6	70.0 ±6	64.0 ±6	69.0 ±7	66 ±7	69±7	61 ±6	55 ±5	63 ±6	57 ±6	40.0 ±6	35.0 ±6
MSDV [1]	3.0 ±0.6	11.0 ±2	18 ±4	10.0 ±2	1.0 ±0.4	50.0 ±5	8 ±1	9 ±1	27 ±3	30 ±3	25 ±5	38 ±5	6.7 ±3.2	16.4 ±5
% in MSDV	0.7 ±0.1	0.7 ±0.2	1 ±0.4	0.8 ±0.3	0.7 ±0.2	1.4 ±0.4	2 ±0.5	3 ±0.4	4.4 ±0.6	8 ±0.7	15 ±2	18 ±2.5	60.0 ±10	65.0 ±6
MSDV PC [3]	0.1 ±0.1	0.6 ±0.22	11 ±2.2	7.0 ±2	0.6 ±0.0.2	36.0 ±5	4 ±2	7 ±2	18 ±3	18 ±3	22 ±4	30 ±4	6.0 ±3.1	14.0 ±6
% of MSDV [3]	5.0 ±1	5.0 ±2	61 ±5	70.0 ±5	60.0 ±7	72.0 ±6	50 ±6	77 ±5	67 ±6	60 ±7	88 ±6	79 ±7	90.0 ±8	85.0 ±7

[1] measured

[2] calculated: Casein = Whole milk – (Whey + MSDV)

[3] % MSDV PC (Precipitable protein) = MSDV Precipitable protein × 100 / MSDV

b.d.l. – below detection level

Fig. 1

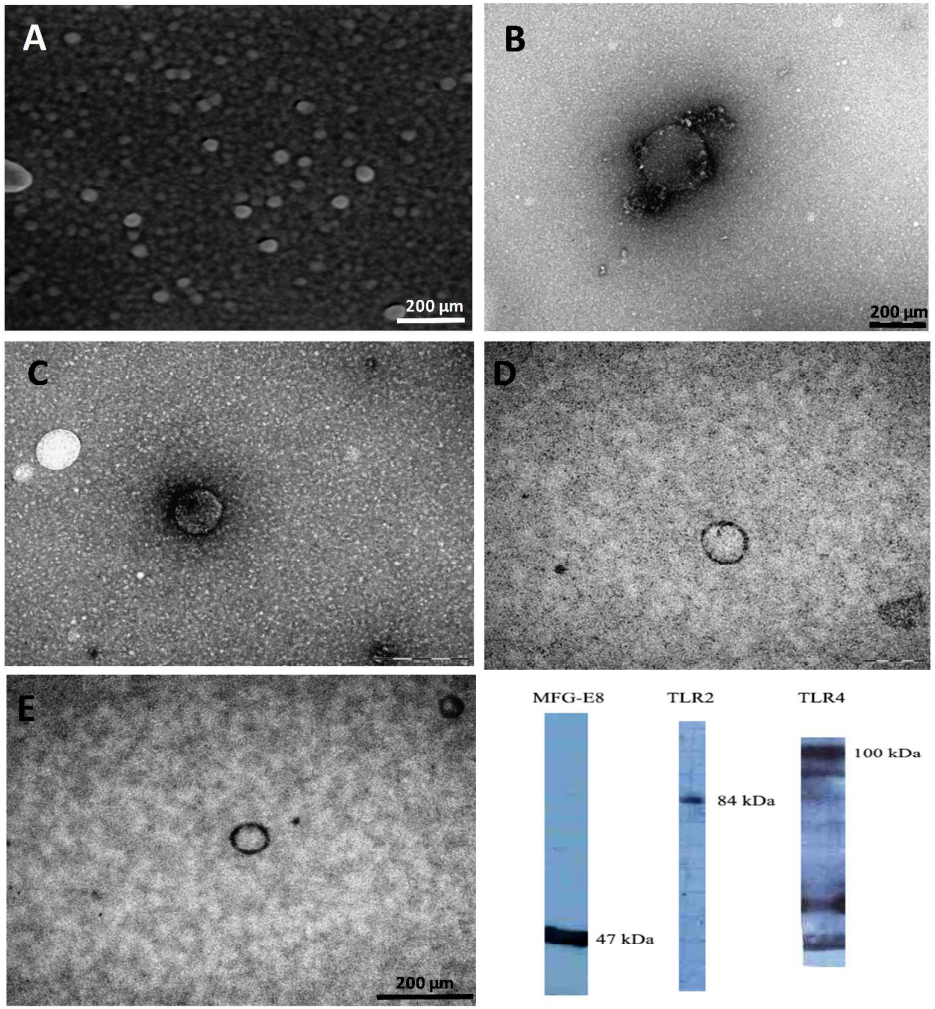


Fig 2

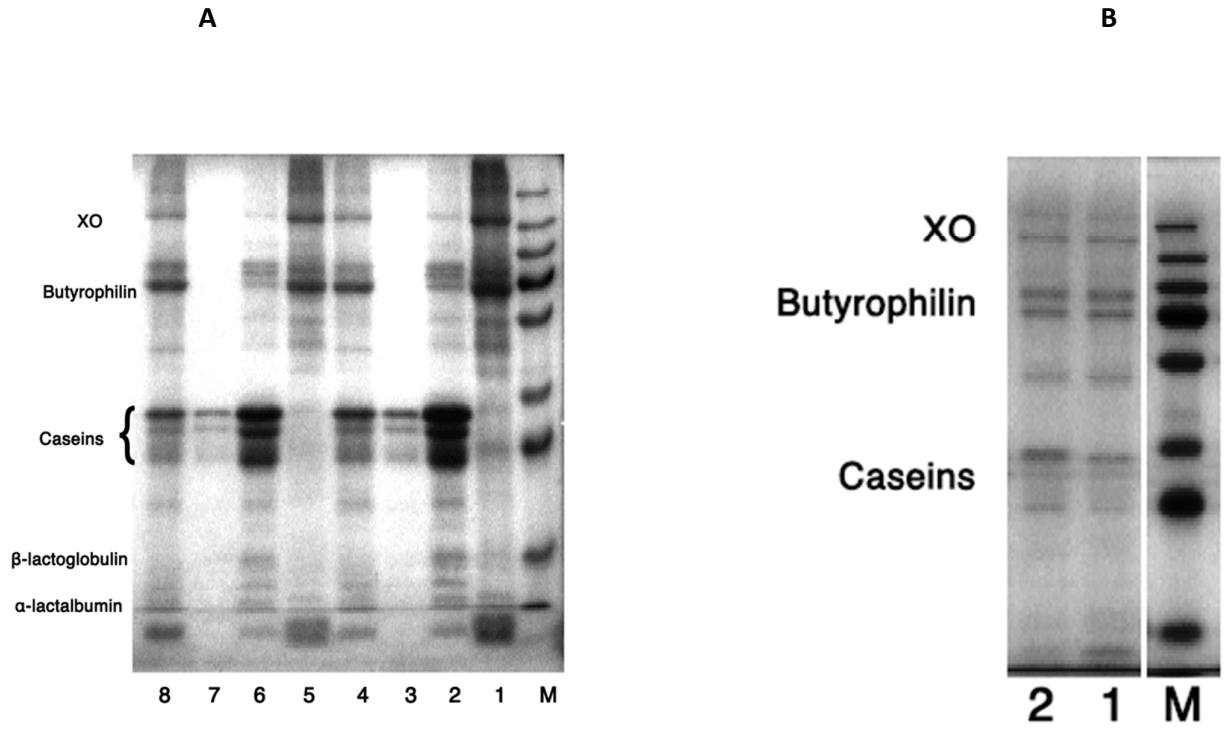


Fig 3

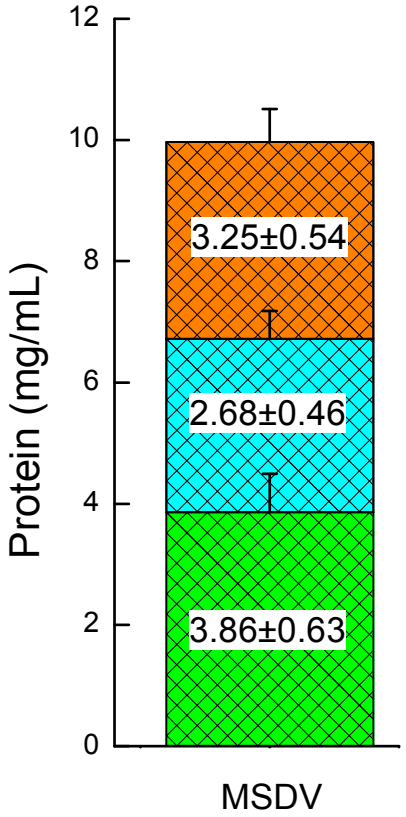


Fig 4

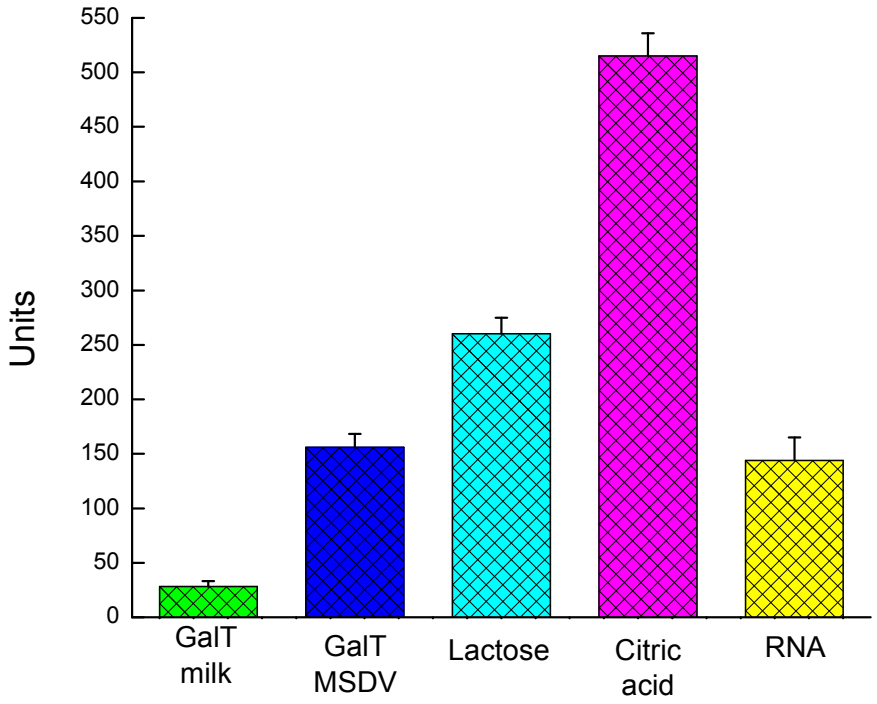


Fig 5

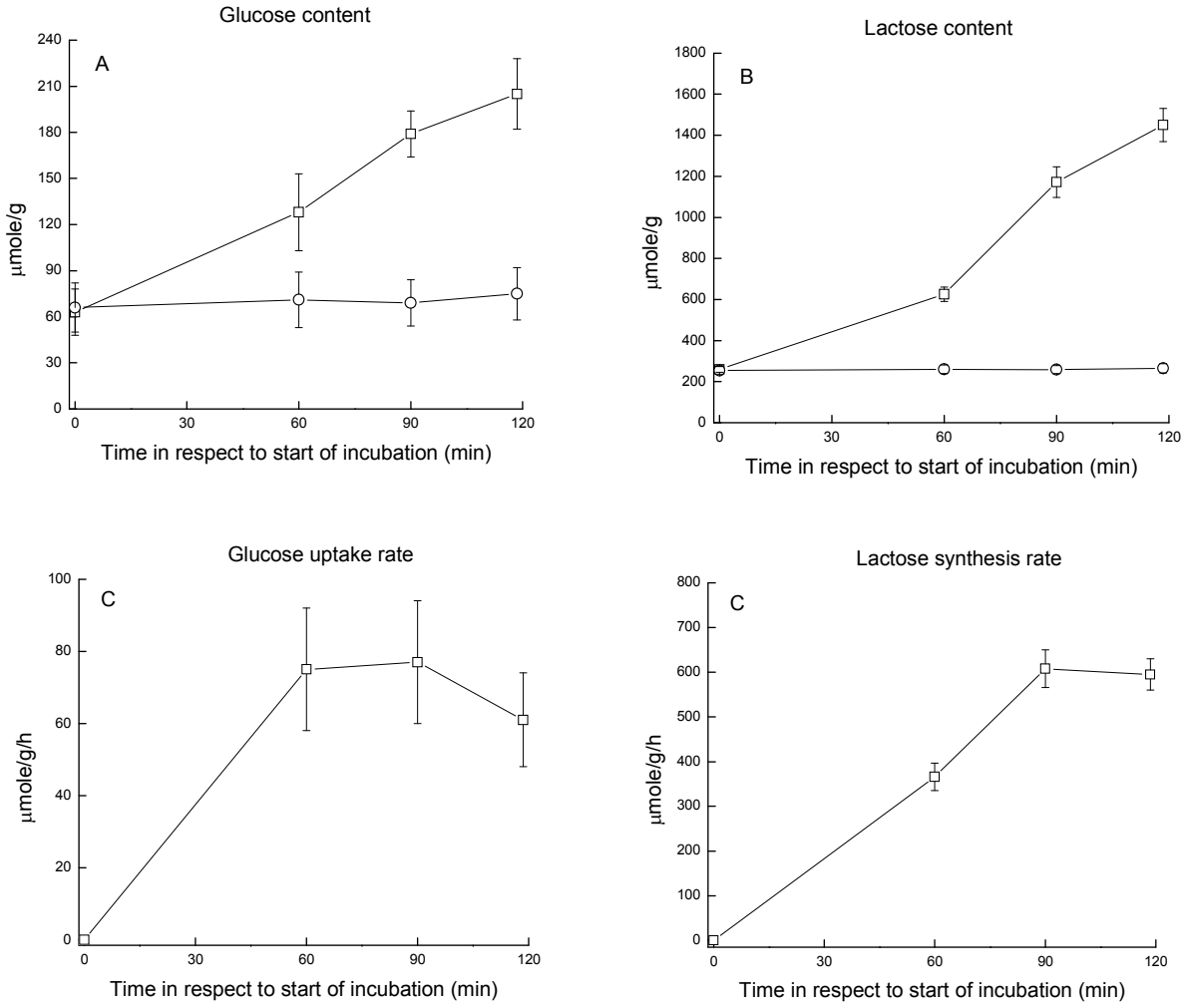


Fig 6

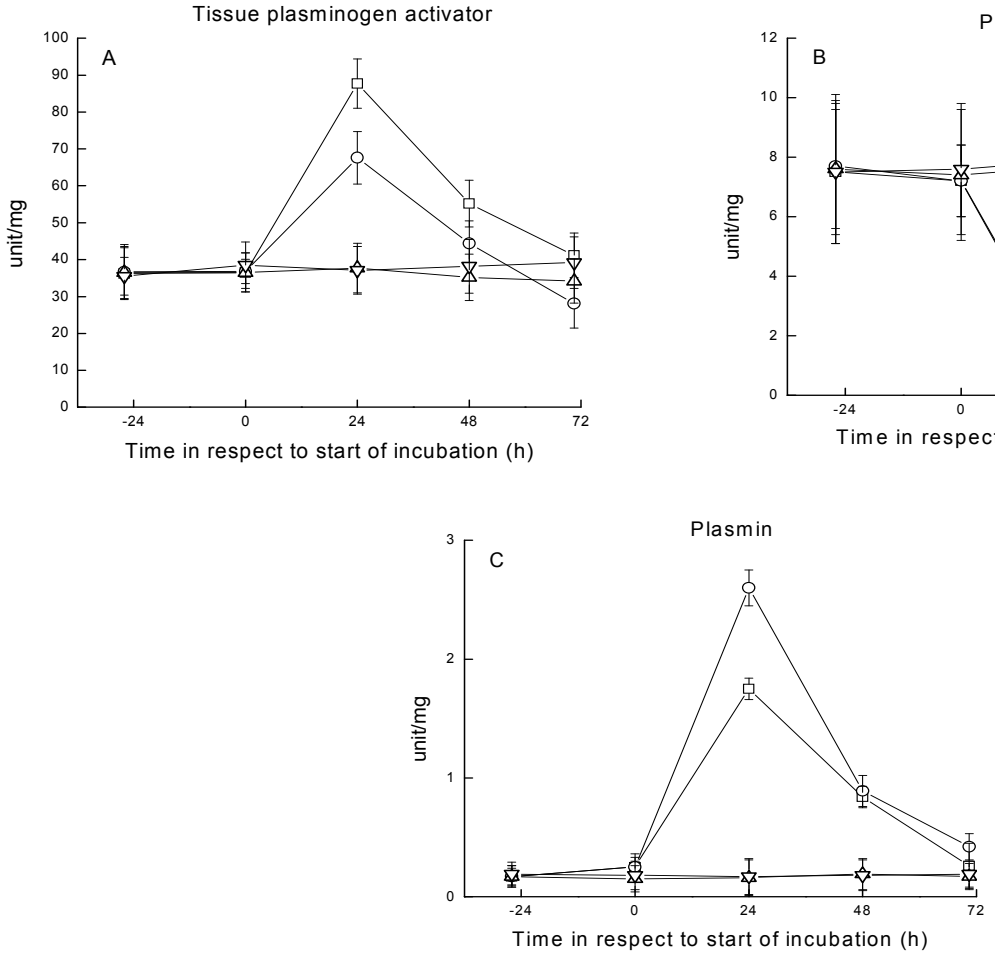


Fig 7

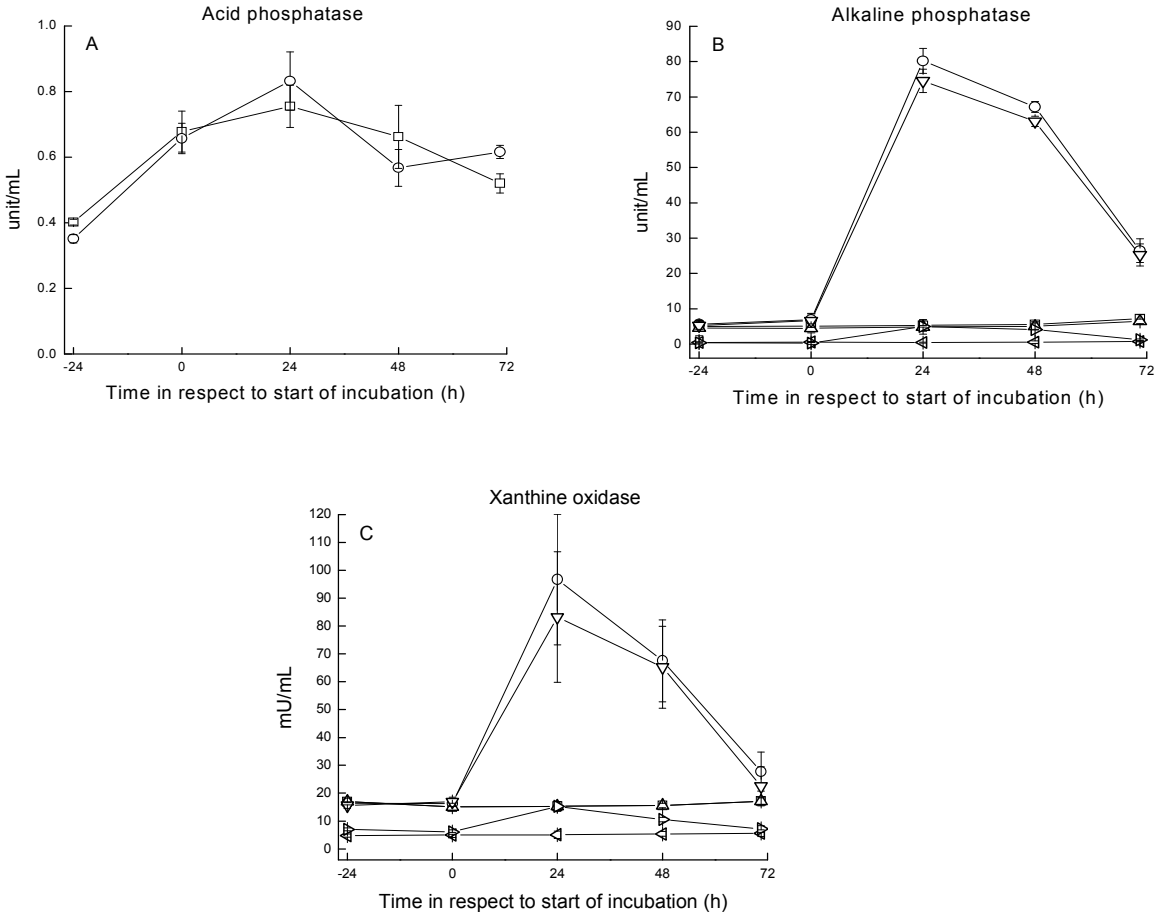
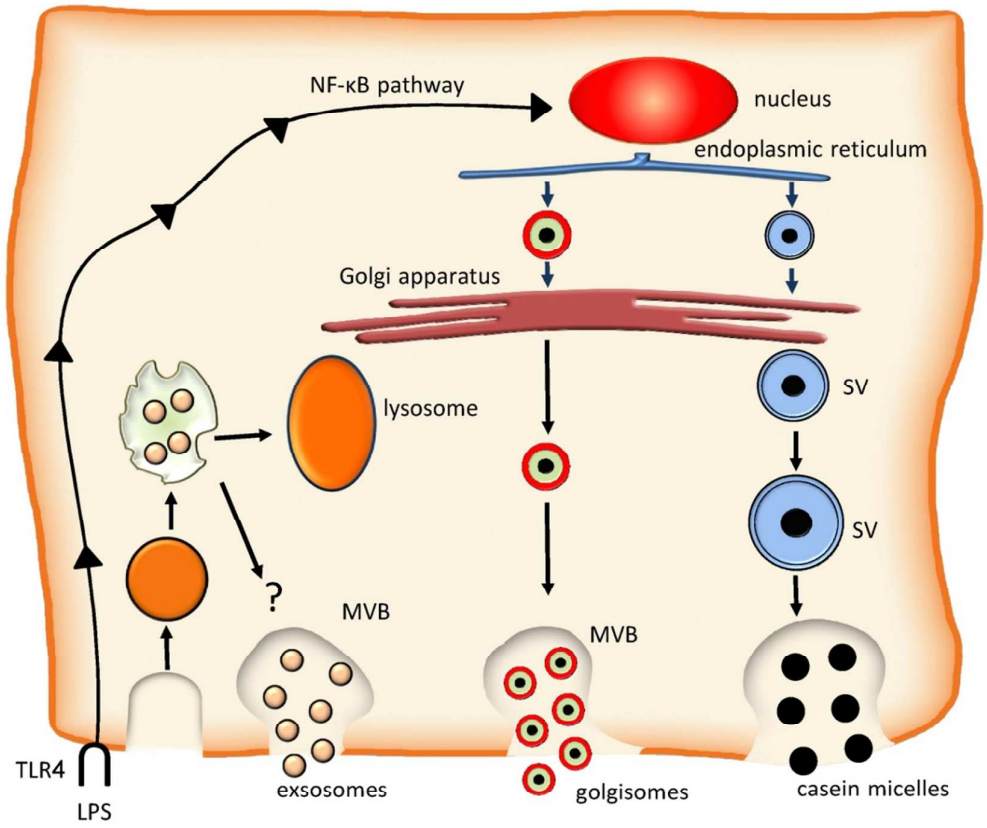


Fig 8



Graphical abstract