Proteomic characterization of thymocyte-derived microvesicles and apoptotic bodies in BALB/c mice

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ABSTRACT

Several studies have characterized exosomes derived from different cell sources. In this work we set the goal of proteomic characterization of two less studied populations of membrane vesicles, microvesicles (100–800 nm) and apoptotic bodies (>800 nm) released by thymus cells of BALB/c mice. The vesicles were isolated by the combination of differential centrifugation and gravity driven multistep filtration of the supernatant of thymus cell cultures. The size distribution of vesicle preparations was determined by transmission electron microscopy. Proteins were released from the vesicles, digested in solution, and analyzed using nano-HPLC/MS/MS. Ingenuity pathway analysis was used to identify functions related to membrane vesicle proteins. In apoptotic bodies and microvesicles we have identified 142 and 195 proteins, respectively. A striking overlap was detected between the proteomic compositions of the two subcellular structures as 108 proteins were detected in both preparations. Identified proteins included autoantigens implicated in human autoimmune diseases, key regulators of T-cell activation, molecules involved in known immune functions or in leukocyte rolling and transendothelial transmigration. The presence and abundance of proteins with high immunological relevance within thymocyte-derived apoptotic bodies and microvesicles raise the possibility that these subcellular structures may substantially modulate T-cell maturation processes within the thymus.

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1. Introduction

Thymus is a central immune organ that provides an environment for positive and negative selections of T lymphocyte maturation. It is the site of central tolerance induction and generation and shaping of the self-tolerant T-cell repertoire. Thus, it substantially contributes to protective cellular immunity and prevents pathological autoimmunity.

Thymus is distinguished from other tissues by two striking features. First, it is characterized by an extremely high apoptosis rate of thymocytes: it is estimated that approximately 95% of T-cell precursors die by apoptosis during thymic development [1]. The other unique feature is the ectopic gene expression of a wide range of tissue-specific antigens. Stromal medullary thymic epithelial cells (MTECs) express thousands of genes whose expression was earlier considered to be
restricted to parenchymal organs [2]. Since the estimated number of MTECs is very low, the possible existence of intercellular antigenic transfer mechanisms (such as membrane vesicular transfer), has been raised [3]. Such a mechanism could efficiently disseminate tissue specific antigens to a high number of thymic antigen presenting cells, and would add another important function to the unexpectedly long list of immune processes in which the role of membrane vesicles has been documented [4].

Extracellular membrane vesicle is the collective term of nm size, subcellular structures originating from different cell types including both prokaryotic and eukaryotic cells (as we reviewed recently [5]). Their formation has been shown to be strongly enhanced upon activating or apoptotic stimuli. They are surrounded by a phospholipid bilayer, and enclose different molecular components (e.g. proteins, mRNA, microRNA) of the donor cell [6]. The best characterized membrane vesicle populations include microvesicles (in clinical studies often referred to as microparticles) with a diameter of 100–800 nm and exosomes that have a diameter <100 nm. Microvesicles (MVs) are formed by outward budding of the plasma membrane, while exosomes are released when the plasma membrane fuses with internal compartments of cells that contain intraluminal vesicles [7]. Surprisingly, apoptotic bodies (ABs) (with a diameter >800 nm), generated by similar mechanism as MVs, are often out of focus of studies of membrane vesicles, in spite of the fact that the principles of the isolation and detection of all subcellular membrane vesicles are similar. Recently, a rapidly increasing number of studies have characterized the exosomal protein composition by using mass spectrometry (for recent review see [8]). Data regarding the exosomal proteome are summarized in the Exocarta database ([http://exocarta.ludwig.edu.au/], [9]). Similarly, numerous works have characterized proteomic profiles of MVs, in particular those in human blood plasma [10–14].

Strikingly, until recently comparative mass spectrometry analysis of MVs and ABs has received little attention, and proteomic data for thymic vesicles are essentially lacking. Given the central role of thymus in immune cell development and immune tolerance induction, we focused on the proteomic characterization of thymocyte-derived ABs and MVs in order to gain an insight into the potential biological significance and functions of these subcellular structures.

2. Materials and methods

2.1. Cell culture

Thymuses of two weeks old BALB/c mice were removed, dissected into small pieces, and digested with 2 mg/mL collagenase D (Roche Applied Science, Indianapolis, IN, USA) in PBS for 30 min at 37 °C. Single cell suspension was made by repeated pipetting of the digested tissue, and was submitted to filtration through a 5 μm filter (Millipore) by gravity in order to remove cell aggregates. Cells were centrifuged at 300 g for 10 min, and the red blood cells were lysed in the pellet by addition of 10 mL of red blood cell lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA in distilled H₂O). After 60 s the cell suspension in lysis buffer was supplemented to a final volume of 50 mL. The non-lysed cells were pelleted, and 1.4×10⁶ cells were transferred to a CELLine bioreactor (Integra Biosciences, Chur, Switzerland). Thymocytes were cultured in the lower compartment of the bioreactor in 15 mL DMEM supplemented with 10% membrane vesicle-free FCS (Gibco, NY, USA) and 2× concentration of glutamine (Sigma-Aldrich Co, St. Louis, MO, USA) and 4.5 g/L glucose. The upper compartment of the bioreactor contained 350 mL DMEM without FCS. After culturing for 24 h, the cells and their supernatant were collected from the lower compartment of the bioreactor. Cells were tested for viability using trypan blue exclusion test and for apoptosis by Annexin V–FITC (BD) binding using flow cytometry. In the population of thymocytes 30.92±4.08% were found positive for Annexin V binding after 24 h culture in CELLine bioreactor.

2.2. Isolation of microvesicles and apoptotic bodies

Fifteen mL supernatant from thymocyte cultures was pelleted at 300 g for 10 min. This was followed by filtration through a 5 μm pore size filter (Millipore Co. Cork, Ireland) by gravity (in order to avoid dispersion upon applying pressure by a syringe plunger).

ABs were pelleted at 2000 g for 20 min, followed by filtration through a 0.8 μm pore size filter (Millipore) by gravity. Next, the filtrate was centrifuged at 12200 g for 40 min to sediment MVs. The pellet was further washed two more times in PBS (by centrifugation at 12200 g for 10 min each time), and then re-suspended in 50 μL deionized water and stored at –80 °C. The presence of contaminating non-vesicular protein aggregates was excluded by 0.05% Triton X lysis of the membrane vesicle preparations as described previously [15].

2.3. Electron microscopy of membrane vesicles

Cellular and vesicular (AB and MV) pellets gained by combined differential centrifugation and filtration steps, were submitted for transmission electron microscopy. Briefly, after short centrifugation the supernatant was carefully removed, and the pellets were fixed at room temperature for 60 min. The fixative contained 2% paraformaldehyde and 2% glutaraldehyde in 0.01 M phosphate buffer, pH 7.4. After washing out the fixative with phosphate buffer, preparations were post-fixed in 1% OsO₄ (Taab; Aldermaston, Berks, UK) for 30 min. Following rinsing with distilled water, the pellets were dehydrated in graded ethanol, including block-staining with 2% uranyl acetate in 70% ethanol for 30 min, and embedded in Taab 812 (Taab). After overnight polymerization at 60 °C, ultrathin sections were analyzed with a HITACHI 7100 electron microscope. Electron micrographs were made by Megaview II (lower resolution, Soft Imaging System, Munster, Germany) digital camera. Brightness and contrast were adjusted when necessary by using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

2.4. Western blot analysis

Whole cell, MV and AB lysates were prepared using ProteoJet cell lysis buffer (Fermentas, Burlington, ON, Canada) supplemented...
with 1 μM leupeptin and aprotinin, 200 μM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate protease inhibitors (all from Sigma-Aldrich). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on pre-cast 12% Bis-Tris gels (Lonza, Basel, Switzerland) according to the manufacturer’s recommendations on a Mini-Cell electrophoresis system (BioRad Laboratories, Hercules, CA, USA) using 5–10 μg of protein/lane. For immunoblotting, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (BioRad Laboratories, Hercules, CA, USA). Anti-mouse galectin-1 antibody (R&D Systems, Minneapolis, MN, USA) or monoclonal anti-mouse α tubulin antibody (AbD Serotec, Kidlington, UK) were used at 1:250 and 1:1000, respectively. Anti-mouse aquaporin 1 antibody (Sigma-Aldrich) was used in 1:500. Mouse anti-rat-HRP (Sigma-Aldrich) and anti-rabbit-HRP (Sigma-Aldrich) were used as secondary antibodies at 1:10000 and 1:250 and 1:1000, respectively. Anti-mouse α-tubulin antibody (AbD Serotec, Kidlington, UK) were used at 1:250 and 1:1000, respectively. Anti-mouse galectin-1 antibody (Sigma-Aldrich) was used in 1:500. Mouse anti-rat-HRP (Sigma-Aldrich) and anti-rabbit-HRP (Sigma-Aldrich) were used as secondary antibodies at 1:10000 and 1:1000, respectively. The proteins were visualized using the ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the protein content of lysates. Identical amounts of proteins were loaded on the gel.

2.5. Flow cytometry

Samples were analyzed by using a FACSCalibur flow cytometer (BD Biosciences). The instrument settings and gating were adopted from a previous work [15]. Microbeads of various sizes (4 μm from Life Technologies, Carlsbad CA, USA and 1 μm from Sigma-Aldrich) were used to select optimal instrument settings and gate. The gates were established using 1 and 4 μm calibration beads as upper right corners of the MV and AB gates, respectively. The lower border was determined after assessing signal/noise ratios and positive event numbers. The gate was set in a way to exclude the highest possible noise, but to detect the highest number of signal events. Samples were tested in 300 μL 0.01 μm pore size membrane filtered PBS (Millipore). Event numbers of equal sample volumes were counted for 30 s. MVs and ABS were isolated from thymuses of mice (as described earlier). Vesicles were suspended in 300 μL filtered PBS, and 1 μL of annexin V antibody (Sigma-Aldrich) was added. Vesicles were incubated with the antibody for 10 min, followed by centrifugation at 20 500 g for 20 min. The supernatant was discarded and vesicles were resuspended in filtered PBS, spun again at 20 500 g for 20 min. Finally, the pellets were suspended in 300 μL filtered PBS, and 1 μL anti rabbit-FITC antibody (Reagent Dickinson, Franklin Lakes, NJ, USA) was added to the tubes, and vesicles were submitted to flow cytometry. Isolated thymocytes were assessed similarly using standard flow cytometry gating for cells.

2.6. Protein extraction and tryptic digestion

The isolated vesicles (0.42–6.96 and 6.56–14.4 ug protein/experiment in case of ABS and MVs, respectively) were submitted to repeated freeze–thaw cycles as described earlier [16]. First, 2 pmol beta-lactoglobulin (Sigma-Aldrich) internal standard (in 2 μL volume) was added to 8 μL thawed up vesicles, as described at Section 2.2. The freeze–thaw cycles included freezing in liquid nitrogen (30 sec) 5 times and –20 °C freezer (1 hour) two times.

Each time thawing was carried out by sonication in a water bath (10 min). Next, the protein content of the vesicles was digested as reported previously [17]. To 10 μL sample (containing 8 μL vesicle + 2 pmol beta-lactoglobulin in water) 1.5 μL 0.2% RapiGest SF (Waters, Milford, MA, USA) and 0.5 μL 200 mM DTT (Sigma-Aldrich) were added and incubated at 60 °C for 30 min to achieve protein unfolding and reduction. Following reduction, 5 μL 200 mM NH4HCO3 and 0.5 μL 200 mM iodoacetamide (Sigma-Aldrich) were added to alkylate the sample which was stored for 30 min in the dark at room temperature. The alkylated samples were digested at 37 °C for 90 min with 4 pmol trypsin (Proteomics grade, Sigma Aldrich). The digestion was quenched by adding 2 μL formic acid (30 min at 37 °C), centrifuged at 17 000 g for 10 min, and then transferred into the micro-vial for analysis.

2.7. Nano-LC–ESI–MS/(MS) analysis of proteins and database search

The LC-MS/(MS) analysis of the tryptic peptide mixtures was performed using a nanoflow UPLC system (nanoAcquity UPLC, Waters) coupled to a Q-TOF Premier mass spectrometer (Waters) equipped with nanoElectrospray source. The electro-spray emitter was purchased from New Objective, Woburn, USA. The tryptic peptides were desalted online on a Symmetry C18 trap column (180 μm i.d. × 20 mm, Waters), and then separated on a reverse phase analytical column (C18, 75 μm i.d. × 150 mm, 1.7 μm BEH particles, Waters). The elution of peptides from the analytical column to the emitter tip was achieved using a flow rate of 400 nL/min and a 150 min long gradient with an increasing concentration ranging from 10% to 40% solvent B (solvent A was water containing 0.1% formic acid and solvent B was acetonitrile also containing 0.1% formic acid). Data were acquired in the data dependent acquisition mode (DDA) using 4 s cycles, consisting of a full scan spectrum (m/z: 400–1999) and MS/MS spectra of the three most abundant ions. MS/MS experiments used Ar collision gas, the collision energies were varied according to the mass and charge state of the precursor ion. Data from the DDA experiment were processed using ProteinLynx Global Server v.2.3 (Waters). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot 2.2) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). X! Tandem was searched against SwissProt_51.6 database assuming trypsin. Mascot was searched against SwissProt_51.6 database with house mouse (Mus Musculus) as taxonomy (12420 entries) assuming digestion enzyme trypsin. One missed cleavage was allowed. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.15 Da and a parent ion tolerance of 50 ppm. Iodoacetamide derivative of cysteine was specified in Mascot and X! Tandem as a fixed modification. Oxidation of methionine and phosphorylation of serine, threonine and tyrosine were specified in Mascot and X! Tandem as variable modifications. Scaffold (version Scaffold_3_00_07, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [18]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides.
Protein probabilities were assigned by the Protein Prophet algorithm [19]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Search against the decoy database gave a false-discovery rate of 0.51% (MV) and 0.40% (AB) when using p<0.01 significance level. Presented results show summarized data from three independent biological replicates, respectively. In case of one sample three technical replicate experiments were also carried out and the results were also taken into consideration.

2.8. **Label-free quantification**

The amount of histones present in ABs and MVs were compared using the label-free quantification technique [20]. This is often used to determine differential expression level of proteins present in biological samples. Briefly, the average of the 3 most intensive peptide signals of detected protein were used as a measure of protein amount (in molar units); and were also compared to the average of the 3 most intensive peptide signals of the BLG internal standard in a single stage MS run. As the concentration of the internal standard is known, quantitative estimation is possible. The amount of histones in ABs and in MVs is shown in Table 1.

2.9. **Pathway analysis**

Proteins identified in thymus derived membrane vesicles were selected by a simple Python script [http://www.python.org] for further proteomic analyses. The proteins were studied for their functions in biological processes and diseases. Basic information about the proteins were extracted automatically from UniProtKB/SwissProt database (release 2010_06) [http://www.expasy.org/sprot] using the ExPASy.get_sprot_raw() and SwissProt.read() functions of BioPython [http://www.biopython.org] to retrieve and parse data. Among the selected data, we were focusing on protein function (members of SwissProt.read(ID).comments starting with -!- FUNCTION), and known interactions with other proteins (starting with -!- INTERACTION).

Possible roles of these proteins were also analyzed using ingenuity pathway analysis (IPA version: 8.6, Release Date: 2010-05-28). In the case of proteins found in membrane vesicles of thymocytes, IDs were translated to GeneIDs using UniProt’s ID Mapping [http://www.uniprot.org/] and the list of these IDs was imported to IPA. Using the IPA Core module, an analysis was performed for biological functions and signal transduction pathways associated with the selected molecules.

3. **Results**

3.1. **Isolation of thymus cell derived apoptotic bodies and microvesicles**

By multistep differential centrifugation and gravity driven filtrations we have isolated thymus derived ABs and MVs. As shown in Fig. 1A, electron microscopy of the ABs showed chromatin condensation and marginalization. ABs in the preparation were in the size range of 800 nm–5 μm. On the contrary, the preparation of MVs, shown in Fig. 1B, contained membrane bound, irregular or spherical structures within the diameter range of 200–800 nm. The electron microphotographs thus, confirmed that the ABs and MVs contained intact structures within the expected diameter ranges.

3.2. **Protein identification and analysis**

Tryptic peptides were analyzed by nano LC–MS/(MS). Protein identification is usually performed based on Mascot scores and number of peptide fragments identified. There are various quality criteria used in the literature to identify a given protein. We have used stringent quality requirement [21]. This requires a minimum Mascot Score corresponding to a reliable hit (p<0.01); and identification of at least two peptide fragments. Of note, the minimum required Mascot score depends on the database used (taxonomy filter).

![Fig. 1 – Transmission electron microphotographs showing apoptotic bodies (A) and microvesicles (B) isolated by differential centrifugation and multistep gravity driven filtration from the tissue culture supernatant of mouse thymocyte cultures. Apoptotic bodies are characterized by the presence of condensed chromatin. Microvesicles are irregular or spherical, structures with 200–800 nm diameter.](image-url)

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**Table 1 – Relative amount of histones in thymus derived ABs and MVs.** The approximate amounts were determined on the basis of label free quantification, using peak intensities. Relative amount of histone families in the samples were also estimated.

<table>
<thead>
<tr>
<th>Description</th>
<th>Apoptotic body %</th>
<th>Microvesicle %</th>
</tr>
</thead>
<tbody>
<tr>
<td>All histones</td>
<td>20.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Histone H1 family</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Histone H2A family</td>
<td>5.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Histone H2B family</td>
<td>5.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Histone H3 family</td>
<td>3.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Histone H4 family</td>
<td>4.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>
In this way 142 proteins were identified in ABs and 195 proteins in MVs released by murine thymus cells. Population-specific and shared protein components of thymus derived ABs and MVs are shown in Fig. 2. The detailed list of proteins is summarized in Supplementary Table 1. Annexin V, a protein identified by MS both in MVs and ABs, was also demonstrated by flow cytometry (Fig. 3). MVs were shown to carry low amounts of surface annexin V as compared to ABs and thymocytes. We also validated some of the MS data by Western blot (Supplementary Fig. 1).

We found a strikingly high number of proteins shared by ABs and MVs (47%), while the number of microvesicle-specific proteins (38%) substantially exceeded that of ABs (15%) (Fig. 2). The ratio of the detected cytoplasmic and membrane proteins was 3.82 in APO and 2.47 in MVs in line with the relative higher membrane surface associated with the smaller size MVs. The identified proteins included enzymes (including several kinases and peptidases), transporters, cytokines, ion channel proteins, transcriptional and translation regulators, transmembrane receptors (including G protein coupled receptors), etc. Their distributions in ABs and MVs were summarized in Fig. 4A and C, while Fig. 4B and D shows the subcellular localization of the identified proteins within the two different vesicle populations. Supplementary Table 2 summarizes the subcellular localization and function of proteins identified by mass spectrometry (MS).

Similarity between AB and MV samples includes the common presence of cytoskeletal (actin and tubulin), cytoskeletal binding-proteins (ezrin, moesin, cofilin 1), metabolic enzymes (GAPDH, alpha-enolase, malate dehydrogenase 1, lactate dehydrogenase A and B) and chaperones (T-complex protein subunits, hsp90). These proteins have also been detected previously in exosomes of various origins [22–24] indicating the presence of common constituents in different types of vesicles.

Using ingenuity pathway analysis we identified top biological functions associated with the identified proteins. Cellular mechanisms and signal pathways are listed in Table 2. As shown by Supplementary Figs. 2 and 3, numerous proteins, identified by our MS analysis of thymus derived MVs, fall into the category of leukocyte extravasation and Rho-regulated, actin-based mobility pathways.

Strikingly, both MVs and ABs contained substantial amounts of different types of histones. Several quantitative estimates were made to compare the relative amount of histones in the samples. Based on the number of peptide fragments, the relative amount of histones in ABs was 20.3% of the total amount of proteins, while it was 6.1% in the case of MVs.

Fig. 2 – Proteins identified by mass spectrometry in thymocyte-derived apoptotic bodies, microvesicles or both. Abbreviations and detailed list of proteins are provided in Supplementary Table 1.
Fig. 3 – Flow cytometry of thymocytes, MVs and ABs. a: MV (rectangular) and AB (elliptic) gates were set using 1 and 4 μm beads (arrows); b: fluorescence intensity was assessed using anti-annexin V and anti rabbit-FITC antibodies in PBS (in the absence of either vesicles or cells); c, e, g: unstained MVs, ABs and thymocytes, respectively; d, f, h: MVs, ABs and thymocytes immunolabeled to demonstrate the presence of surface annexin V, respectively. Thymocytes were assessed within conventional cellular gates (shown in g and h).

Fig. 4 – Distribution of proteins according to their functions and subcellular localization in thymocyte derived apoptotic bodies and microvesicles. Detailed list is provided in Supplementary Table 2.

![Flow Cytometry Diagram](image)
MV preparations (Table 1). Identified histones were grouped into histone families and the relative amount of these families is also included in Table 1.

On an attempt to identify proteins already described by previous studies, we compared our results with Exocarta Database v 2.2 entries. As a first step, we converted GeneID identifiers to SwissProt/Uniprot IDs. The conversion yielded 1507 unique protein IDs, while 171 database identifiers (10.2% of all unique entries) could not be converted. After comparison with our results, we found 25 murine proteins present in ABs, 45 in MVs and 32 present in both populations, but not yet described in Exocarta database.

4. Discussion

We have shown recently that MVs share common biophysical parameters with protein complexes [15]. As a consequence, not only MV detection is comprised by protein aggregates, but also MV preparations may be contaminated by protein complexes [15]. Thus, any proteomic data related to isolated MVs, should be considered cautiously. The present study investigated serum-free tissue culture supernatants of thymocytes. The possibility of contaminating non-vesicle protein aggregates was excluded by 0.05% Triton lysis after which all structures disappeared in our vesicle preparations by flow cytometry.

In the current work we focused on thymus, and investigated the proteomic composition of two populations of thymus cell-derived, membrane bound vesicles (ABs and MVs), surprisingly underrepresented in the literature. Thymic presentation of self epitopes plays a key role in T-cell selection processes. In this study it was a question of particular interest whether thymus derived ABs or MVs could disseminate tissue antigens in order to support selection processes. Our MS work did not identify any ectopically expressed tissue proteins either in thymus derived ABs or MVs. This does not rule out their presence in vesicles in small amounts that did not reach the detection limit of MS. Even though we did not find evidence for tissue antigens in thymocyte-derived membrane vesicles, this study has identified several proteins that suggest previously unidentified roles of these subcellular structures in regulating intrathymic processes.

We have detected several proteins that have been implicated as autoantigens in human autoimmune diseases. Both thymic ABs and MVs contained alpha enolase, a glycolytic enzyme that has been recently suggested to play a role in rheumatoid arthritis and other autoimmune diseases [25–27]. Furthermore, we detected the presence of heat shock proteins implicated as autoantigens in atherosclerosis [28,29].

Our work has also revealed the presence of histones (H1-H4) in both ABs and MVs. Histones are key components of nucleosomes, desoxyribonucleoprotein complexes currently considered as major autoantigenic targets in SLE [30]. The presence of known autoantigens within thymic ABs and MVs is interesting, as impaired induction of central tolerance to autoantigens has been proposed to lead to the development of pathological autoimmunity.

The presence and abundance of histone proteins in ABs was not surprising given the mechanism by which apoptotic blebs are generated. However, unexpectedly we have identified strikingly high number of histone proteins also in MVs. This suggests that a major proportion of thymus derived MVs is possibly generated during apoptosis (and thus, may be referred to as apoptotic MVs).

Another exciting finding of this study was the identification of key regulatory and signaling molecules in membrane vesicles, such as E2F. E2F represents a family of transcription factors involved in cell cycle regulation and synthesis of DNA in mammalian cells, tumorigenesis, apoptosis and differentiation [31]. LCK, a further regulatory molecule that we detected in thymic vesicles, is a tyrosine kinase that plays an essential role in T-cell receptor (TCR)-linked signal transduction pathway. Therefore it is a further molecule of outstanding importance for the selection and maturation of developing T-cell in the thymus and in mature T-cell function. Galectin-1 has been implicated in the induction of apoptosis and in regulation of T-cell death [32,33]. Here we showed the presence of galectin-1 in thymic MVs. This was not only validation of our MS/MS data, but also a strong support for the regulatory roles of membrane vesicles in the thymus.

We detected thymocyte-derived MV-associated presence of calmodulin, a calcium-binding protein that has been documented to regulate a number of different cellular functions including the activation and maturation of lymphocytes [34].

Taken together, these data show the presence of key regulatory molecules in thymic MVs and ABs. Given the extremely high apoptosis rate characteristic for the thymus [1], the magnitude of apoptosis related membrane vesicle generation in the thymus is expected to be very high. Thus, these subcellular structures may mediate significant modulating functions in the thymic microenvironment.
This study has also identified numerous further molecules known to play crucial roles in the immune system such as MHC I and MHC II molecules, CD5 and CD97 (in MVs), CD45 in both types of vesicles. These data suggest a reciprocal membrane vesicular crosstalk between maturing T-cells and antigen presenting cells in the thymus.

Finally, by using pathway analysis, we have identified several proteins of thymic MVs that are implicated in regulation of actin based motility by Rho. RhoA (found both in thymic ABs and MVs), is a small GTPase. The Rac1/Cdc42/Rac1/Rho pathway has been shown earlier to control actin reorganization necessary for based motility by Rho. RhoA (found both in thymic ABs and MVs), is a small GTPase. The Rac1/Cdc42/Pak pathway has been identified in thymic membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci, doi:10.1007/s00018-011-0689-3.

Interestingly, our pathway analysis revealed that several proteins that we have identified in thymic membrane vesicles, are known to be involved in leukocyte rolling on endothelial surfaces as well as in transendothelial leukocyte transmigration (Supplementary Figs. 2 and 3). RhoA was shown to be protective for endothelial barrier integrity under basal conditions, but to become involved in barrier dysfunction after activation of endothelia cells by thrombin [36]. Intrathymic endothelial integrity is required for the maintenance of the blood-thymus barrier and may regulate transendothelial thymocyte export. Our data may shed light on the possible novel role of membrane vesicles in maintaining thymus homeostasis.

5. Conclusion

Unexpectedly, in this study we found high similarity in the protein composition of ABs and MVs. Many of these proteins suggest that both vesicle types are generated predominantly during apoptosis, and have significant immunological and/or regulatory function. Taken together, these data suggest that not only a MVs, but also ABs are likely to have complex functions in regulation of thymus, and that makes them interesting targets for future research.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.05.023.

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REFERENCES


