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Adaptive immune stimuli altered the cargo proteins of exosomes released by supraneural myeloid body cells in *Lampetra japonica*

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ABSTRACT

As one of the representatives of jawless vertebrates, lamprey is an important animal model for research on the evolution of adaptive immune system. Although it is widely accepted that the key characteristic of the immune response in jawed vertebrates is the functional cooperation between different immune effector cell lineages, whether immune cells of lamprey can communicate with each other is still unclear. Recently, mounting evidence has revealed the emerging role of exosomes in mediating intercellular communication. In this study, by means of ultrafiltration followed by size exclusion chromatography, exosomes are purified from conditioned growth medium of the primary supraneural myeloid body cells (SMB cells) in LPS-immunized and control *Lampetra japonica* (Japanese lamprey). The proteomic profiling and qualitative comparison are performed between protein components enriched in exosomes released by SMB cells under quiescent and activated conditions. Our results demonstrate that SMB cells can release exosomes with well-defined characteristics comparable to their mammalian counterparts on their size, morphology and protein markers, which supports exosomes are evolutionarily conserved between lamprey and other higher vertebrate species. In terms of comparison with exosomes released by quiescent SMB cells, activated SMB cell-derived exosomes contain more and significantly different protein components. The unique exosomal protein 'fingerprint' might reflect that exosomes from the SMB cells with different states of activation have distinguishing functional roles as well as targeting properties. Further bioinformatic analysis suggests that compared to quiescent exosomes, activated SMB cell-derived exosomes mainly participate in distinctive biological processes including activation of cellular component biogenesis and regulation of cell proliferation. Moreover, activated exosomes may function through the specific signaling pathways associated with the regulation of cell mitosis and immune response. Our results give valuable clues about the immunomodulatory functions of SMB cell-derived exosomes and provide the foundation for further investigation in the regulation mechanism of the adaptive immune response in lamprey.

1. Introduction

Adaptive immune system was considered to exist only in jawed vertebrates until the unusual adaptive immunity was discovered in lamprey several decades ago (Finstad and Good, 1964). Lamprey, the ancient species with a history of more than 350 million years, is one of a few remaining representatives of jawless vertebrates. Recently, several transcriptomic analyses have revealed a lot of homologous genes involved in immune response of lamprey, especially the antibody-like molecules were found to be composed of three types of variable lymphocyte receptors (VLRs), which are membrane-bound proteins responsible for antigen recognition based on their highly diverse sequences of variable leucine-rich repeats (LRR) (Kasamatsu et al., 2010; Pancer et al., 2004a; Rogozin et al., 2007). Among them, VLRAs and VLRCs are expressed by two distinct lymphocyte-like cells lineages, respectively, which resemble the αβ and γδ TCR bearing T-cells in jawed vertebrates. On the other hand, VLRBs are expressed by B-like lymphocytes lineages (Guo et al., 2009; Hirano et al., 2013). Thus, there

Abbreviations: SMB cells, supraneural myeloid body cells; VLRs, variable lymphocyte receptors; LRR, leucine-rich repeats; EVs, extracellular vesicles; MVBs, multivesicular endosomes; LIP, lamprey immune protein; LPS, lipopolysaccharides; SEC, size exclusion chromatography; TRPS, tunable resistive pulse sensing; LC–MS/MS, liquid chromatography–tandem mass spectrometry; PPI, protein-protein interaction; MWCO, molecular weight cutoff; TEM, transmission electron microscopy; ALIX, ALG-2-Interacting Protein X; FLOT1, Flotillin 1; GRP78, Glucose-Regulated Protein 78 kDa; RACE, rapid-amplification of cDNA ends; pAb, polyclonal antibodies; IL-2, interleukin-2

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are similarities in the nature of the design principles and mechanisms of the adaptive immune system between jawed and jawless vertebrates despite totally different antigen recognition receptors adapted by the two species (Boehm et al., 2018). Although the major progress has been made on the characterization of homologous immune molecules and lymphocyte-like cells, there is a lack of enough studies illuminating how different immune effector cells lineages communicate with each other for functional cooperation in lamprey except some indirect evidence. For example, the gene expression patterns of the homologous genes of several cytokines and their receptors such as IL-8/IL-8R or IL-17/IL-17R are indicative of the functional interactions between T-like cells and B-like cells in the adaptive immune systems of lamprey (Guo et al., 2009; Han et al., 2015). The interaction or 'cross-talk' between immune cells is a core feature of the adaptive immune system in jawed vertebrates, which is essential for coordinating the effective immune response. Therefore, further research on whether immune cells in the lamprey have similar characteristics is very important to understand how this unique immune system works. Recently, mounting evidence has revealed the emerging role of exosomes in mediating intercellular communication. Exosomes are defined as the subtype of extracellular vesicles (EVs) with a diameter between 30 nm–100 nm. Exosomes originate from the fusion of the multi-vesicular endosomes (MVBs) with the plasma membrane, and then the secreted exosomes target to the recipient cells, thereby affecting their physiology (Colombo et al., 2014). During biogenesis of exosomes, a series of cargoes including proteins, RNAs, and miRNAs are specifically sorted into the vesicles. Analysis of the exosomal protein components by proteomic profiling is crucial for understanding the function of exosomes (Kreimer et al., 2015). Numerous studies have indicated that exosomes have important modulatory functions in the innate and adaptive immune responses (Robbins and Morelli, 2014; Thery et al., 2009). Previous reports mainly focused on the function of exosomes in the regulation of the classic immune system of jawed vertebrates, especially the mammals. Whether exosomes could be released by immune cells of lamprey and mediate immune stimulation or suppression remains to be clarified.

In this study, exosomes released by the supraneural myeloid body cells of *Lampetra japonica* (Japanese lamprey) were isolated and characterized. In the feeding adult lampreys, the supraneural myeloid body which extends from the third gill pouch to the anterior part of the second dorsal fin replaces the larval typhlosole and nephric fold after metamorphosis as the main, almost exclusive hemopoietic organ. The supraneural myeloid body is comparable to the bone marrow of higher vertebrates since the precursors of blood cells form small clusters among the adipose cells in this connective tissue (Ardavin and Zapata, 1987; George and Beamish, 1974). Cells isolated from the supraneural myeloid body were shown to produce agglutinins that were antigen-specific in response to immunization of human O-type erythrocytes (Hagen et al., 1983), and can also secrete the lamprey immune protein (LIP) as host defense molecule with cytocidal actions against tumor cells (Pang et al., 2017). Thus, the supraneural myeloid body cells (SMB cells) could play crucial roles in the immune response of adult lampreys. Here, we compared the protein compositions of exosomes between quiescent and LPS-activated SMB cells through LC–MS/MS analysis. The proteomic analysis suggested that exosomes from lamprey have evolutionary conservation compared with jawed vertebrates. Further data mining based on bioinformatic methods revealed the immunomodulatory role of activated SMB cell-derived exosomes.

2. Materials and methods

2.1. Animals and cell culture

The handling of lampreys and all experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit Number: SCXK2008 − 0002). Adult Japanese lampreys (*Lampetra japonica*) were obtained from the Tongjiang Valley, a branch of the Songhua River in Heilongjiang Province in China. Healthy individuals with a size of around 25 cm were kept in fiber-reinforced plastic (FRP) tanks with fresh water at 10 °C. For immune stimulation, the intraperitoneal injection was performed with 100 ug LPS (lipopolysaccharides; Sigma-Aldrich) or saline thrice at 7-day intervals and cells were collected 5 days later.

For isolation of SMB cells, briefly, lampreys were anesthetized (100 mg/l MS222; Aladdin) and sterilized with 70% ethanol, then the supraneural myeloid body tissues were stripped from lampreys with a scalpel and forceps and placed in HBSS (Hanks balanced salt solution; Hyclone). After removal of attached muscle and adipose tissues, SMB tissues were cut into tissue blocks with a diameter of about 3 mm, and then transferred to 25 cm^2 cell culture flasks containing 30 ml 0.1% type 1 collagenase (Sigma-Aldrich) at 4 °C overnight. The digested cells suspension was filtered with a cell strainer followed by centrifugation at 400 × g for 10 min. The crude SMB cells preparation was further purified by density gradient centrifugation. In Ficoll-Histopaque density gradient centrifugation, resuspended SMB cells were layered on the Ficoll-Histopaque (Sigma-Aldrich) solution and centrifuged at 400 × g for 30 min. After centrifugation, SMB cells were retained at the interface of the density gradient whereas the contaminants including adipocytes, dead cells together with cell debris were removed. After density gradient centrifugation was repeated once, purified SMB cells were washed by PBS then transferred to Leibovitz L-15 medium supplemented with antibiotics (100 IU/ml of penicillin sulfate and 100 µg/ml of streptomycin) and 2 mM L-glutamine. Cells (2×10^6 cells/ml) were maintained in a low-temperature incubator at 18 °C. Cell cultures were regularly tested by trypan blue staining and confirmed that the proportion of dead cells was less than 5%.

2.2. Exosomes isolation

SMB cells were grown in suspension for 24 h in serum-free Leibovitz L-15 medium. Exosomes were isolated from the conditioned medium as described (Boing et al., 2014) with some modifications. Briefly, conditioned medium from 2×10^8 cells were collected and cleared by centrifugation at 400 × g for 10 min, then the resulting supernatants were centrifuged at 2000 × g for 10 min to remove dead cells and cellular debris. For further elimination of cellular debris and membranes, the supernatants were filtered through a 0.22 µm syringe filter device (Millipore). The cleared conditioned medium was further concentrated into 1.5 ml using ultrafiltration devices (Amicon Ultra Columns, Millipore) with 100 kDa molecular weight cutoff (MWCO) by centrifugation at 3500 × g. Next, the concentrated medium was subjected to size exclusion chromatography (SEC) for isolating exosomes. A 10 ml plastic syringe stacked with 10 ml sepharose CL-2B (GE Healthcare) was used as the SEC column. The concentrate (1.5 ml) was loaded on the column, followed by elution with PBS/0.32% citrate (pH 7.4). The eluate was collected in 26 sequential fractions of 0.5 ml. The fractions 9~12 where the highest concentration of exosomes exists were pooled for further downstream analysis.

2.3. Transmission electron microscopy

EM analysis of whole-mount exosomes was performed using a commercial kit (Exosome-TEM-easy kit; 101bio) according to the manufacturer's instructions. Briefly, Formvar-carbon coated EM grid was floated on the drop of freshly isolated exosomes. After 20 min the exosomes were absorbed into the grid membrane. The grid was transferred to the wash buffer drop to wash for 30 s. The wash step was repeated once. Next, the grid was transferred to the EM solution drop for negative staining and incubated for 10 min. The grid was washed twice followed by air dry at room temperature for overnight. The grid is imaged using the H-600 A electron microscope (Hitachi) operated at 80 kV.

2.4. Tunable resistive pulse sensing (TRPS) analysis

The TRPS measurement of the concentration of exosomes was performed with a qNano platform (Izon Science). Sample diluted 5 × with PBS was dispensed into the top fluid cell. Trans-membrane voltage was applied and adjusted using Izon Control Suite (ICS v 3.3.2) software. The pore size NP150 (150 nm particles) was used in these experiments. A triplicate measurement was preceded per sample.

2.5. Immunoblot analysis

The cultured 10^7 SMB cells were centrifuged for 5 min at 400 × g, 4 °C, the cell pellet was resuspended and washed twice by PBS. Then cell pellet was homogenized in 200 µl RIPA lysis buffer containing 50 mM Tris − HCl (pH 7.5), 1% (v/v) Triton X-100, 0.25% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.15 M NaCl, 1 mM EDTA and protein inhibitor cocktail (Sigma-Aldrich). The cell lysate was centrifugated for 20 min at 20,000 × g. The total protein concentration in the whole cell lysate and secreted exosomes was determined by Bradford's method (Bio-Rad) and fatty acid-free bovine serum albumin as a standard. For detection of various proteins in exosomes and SMB cells, equal total protein amounts (10 µg) of exosomes and cell lysates were subjected to immunoblot analysis by a standard procedure, probed with the indicated antibodies and visualized by Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific). The polyclonal anti-L-Alix, L-Flot1, L-CD63, and L-Grp78 antibodies were generated in New Zealand white rabbits against His-tagged full-length lamprey proteins, respectively. For immunoblot analysis of L-CD63, SDS-PAGE under the non-reducing condition was performed.

2.6. Protein identification by nano-LC–MS/MS analysis

Exosomes samples from quiescent and activated SMB cells were solubilized in 4 × Laemmli sample buffer (Bio-Rad) containing 50 mM DTT, heated to 70 °C for 10 min, then resolved on 4–20% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) followed by visualization using Imperial™ Protein Stain (Thermo Fisher Scientific). Then the individual gel lanes were excised into equal slices (20 × 2 mm). The proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS was performed at Shanghai Applied Protein Technology Co. Ltd, China). In brief, the gel slices were destained by 30% ACN / 100 mM NH_4HCO_3, then treated with 100 mM DTT and 200 mM IAA to reduce and alkylate the proteins. In-gel digestion was performed with 10 ng/µl trypsin for 20 h at 37 °C. The peptides were extracted with 60% ACN / 0.1% TFA. Then peptides were separated with reversed-phase EASY-nLC1000 HPLC system (Thermo Fisher Scientific), in which buffer A (0.1% Formic acid) and buffer B (0.1% Formic acid and 84% ACN solution) were used. Samples were loaded onto Thermo Easy column traps 100 µm × 20 mm in 100% buffer A. Then, samples were separated with Thermo Easy column traps 75 µm × 100 mm in buffer B at a flow rate of 400 nl/min for about 120 min. Separated peptides were subjected to mass spectrometer analysis with Q exactive™, a high-performance quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific). MS data were acquired by choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Ten mass fragmentography were acquired in each full scan. Survey scans were acquired at a resolution of 70,000 at m/z 200 and 17,500 at m/z 200. MS analysis was performed in triplicates. MS data were searched against UniProt Petromyzontidae_15173 _20170320 database (15,173 total entries, downloaded at 2017 − 03-20) using Mascot 2.2 software (Matrix Science). The searching parameters were as follows: enzyme: trypsin; dynamic modification: oxidation (M); fixed modification: carbamidomethyl (C); 2 trypsin missed cleavages were allowed; unique peptide ≥ 2, filter by score ≥ 20.

2.7. Bioinformatic analysis

For annotation of proteins identified by MS with the corresponding human homologs, amino acid sequence alignment was performed using local NCBI BLAST + (NCBI-blast-2.2.28+ win32.exe). For gene ontology analysis, the identified proteins datasets were allocated to biological process categories defined by the GO consortium (Level 3). The GO annotation process by Blast2GO software (https://www.blast2go.com) can be summarized into four steps: sequence alignment (Blast), GO entry extraction (Mapping), GO annotation and annotation augmentation. Briefly, the identified protein sets were aligned with the appropriate protein sequence database using the localized sequence alignment tool NCBI BLAST + and the first 10 alignment sequences meeting E-value ≤ 1e-3 were subsequently analyzed. Secondly, using the Blast2GO Command Line to extract the GO entries associated with the target protein set and the eligible alignment sequences in the previous step (Database version: go_201504.obo, download at www.geneontology.org). During the annotation process, the Blast2GO Command Line annotated the target protein sequences with the GO entries extracted during the mapping process. Next, in order to further improve the annotation efficiency, using InterProScan to search for the conserved motifs in the EBI database that match the target protein, and annotate the target protein sequences with the motif-related functional information. And running ANNEX to further supplement the annotation information and establishing contact between different GO categories to improve the accuracy of the annotation. The comparative GO analysis of biological processes, biological pathway enrichment analysis, as well as protein-protein interaction (PPI) networks analysis, were performed using Funrich software (version: 3.1.3, http://www.funrich.org) according to the developer's instructions.

3. Result

3.1. Purification and characterization of exosomes derived from supraneural myeloid body cells

So far, whether exosomes could be released by SMB cells has not been reported. Therefore, we develop a strategy based on the isolation protocol for mammalian exosomes. Briefly, SMB cells isolated from adult lamprey were grown in suspension for 24 h in serum-free L-15 medium. Then conditioned medium was collected and cleared by differential centrifugation and filtering through a 0.22 µm syringe filter device to remove cells, dead cells, and cellular debris. The conditioned medium was further concentrated into a small volume using ultrafiltration devices with 100 kDa molecular weight cutoff (MWCO), followed by transferring concentrated media to the size exclusion chromatography (SEC) column, the eluate was collected in sequential fractions of 0.5 ml. Finally, the fractions 9˜12 where the highest concentration of exosomes exist as previously reported (Boing et al., 2014) were pooled for further downstream analysis.

Identification of membrane vesicles we isolated as exosomes, as well as evaluation of the purity of exosomes, were determined according to a series of criteria for characterization of mammalian exosomes (Lotvall et al., 2014; Witwer et al., 2013). Firstly, the transmission electron microscopy (TEM) studies revealed that the exosomes we isolated were spherical membrane vesicles in a size range of 50 ~ 100 nm and free of lipid particles (10˜20 nm), apoptotic debris and other contaminants which may confound analysis (Fig. 1A). Next, exosomes were subject to tunable resistive pulse sensing (TRPS) analysis, which can quantify particle-by-particle size and concentration of EV populations with high accuracy and resolution. The histogram in Fig. 1B represents the size distributions of SMB cell-derived exosomes, in which the mean diameter is 134 ± 46.5 nm. The concentration of exosomes isolated from 100 ml of SMB cells conditioned medium is 6.59e + 08 ± 1.45e + 07 particles/ml, and vesicles with 100 nm in diameter show the highest concentration in EV populations.

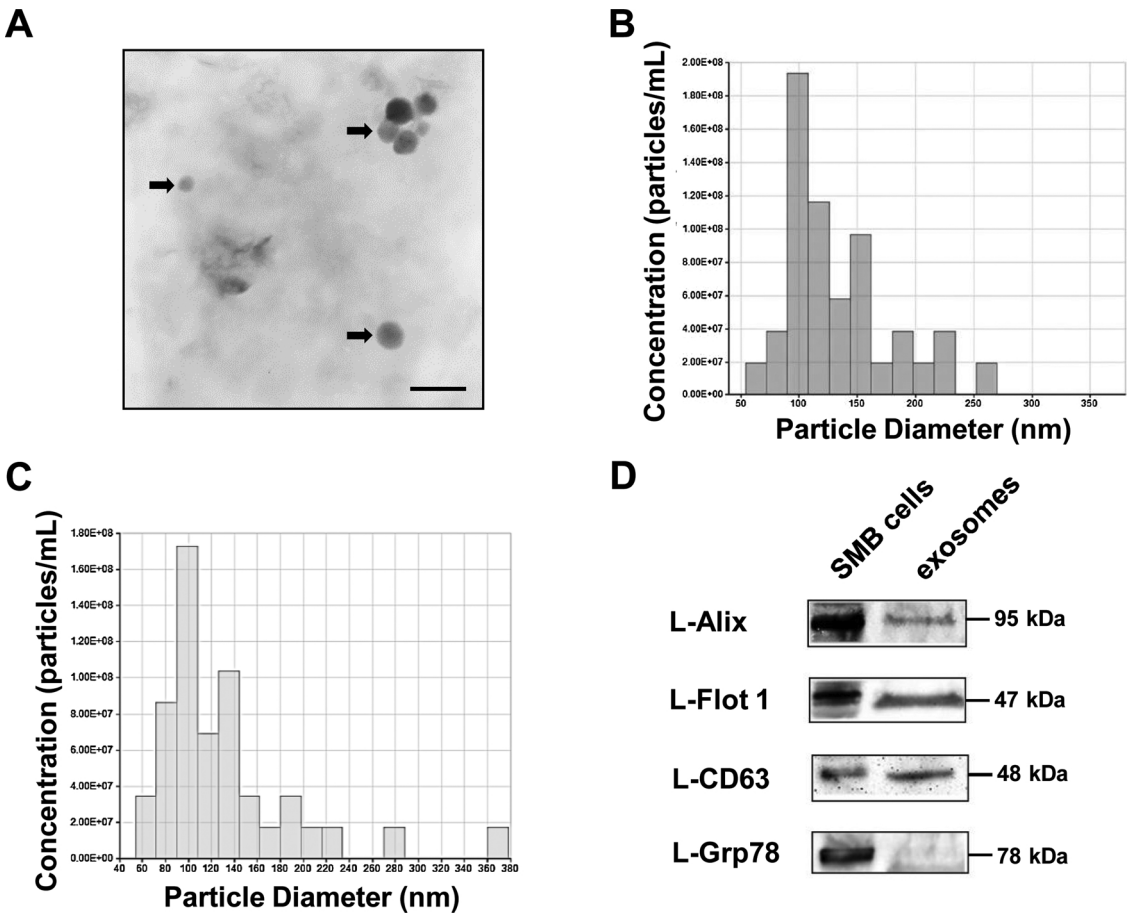

Fig. 1. Characterization of exosomes released by supraneural myeloid body cells (SMB cells) of lamprey. (A) Transmission electron micrograph of exosomes isolated from SMB culture supernatant as indicated by arrows. Scale bar = 200 nm. (B) Tunable resistive pulse sensing (TRPS) analysis for particle size shows the mean diameter of quiescent SMB cell-derived exosomes was 134 ± 46.5 nm (mean ± SD), and the concentration of exosomes isolated from 100 ml of SMB cells conditioned medium was 6.59e + 08 ± 1.45e + 07 particles/ml. (C) Similar to quiescent SMB cell-derived exosomes, the mean diameter of LPS-stimulated SMB cell-derived exosomes was 132 ± 62.8 nm (mean ± SD), and the concentration of exosomes was 6.22e + 08 ± 1.24e + 07 particles/ml. (D) Several homologous protein markers of exosomes are specifically enriched in SMB cell-derived exosomes, in which GRP78, the protein marker of ER, is absent. The MW (kDa) of expected signals is indicated.

To further characterize the purified vesicles as exosomes, we determined whether the common exosomal markers, which were specifically enriched in mammalian exosomes, were also present in SMB cell-derived exosomes preparations by immunoblot analysis. Protein sequence alignment analysis in cDNA library of *Lampetra japonica* constructed by our lab revealed that several mammalian exosomal markers had highly similar homologs in *L. japonica*, including tetraspanins such as CD63, and cytosolic proteins with membrane binding capacity such as ALIX (ALG-2-Interacting Protein X), FLOT1 (Flotillin 1). Furthermore, the endoplasmic reticulum marker GRP78 (Glucose-Regulated Protein, 78 kDa), which was expected to be absent in exosomes and can be used as a negative control for determination of exosomes purity, also have the homolog in *L. japonica*. Among these candidate markers, the cDNA sequences with the complete open reading frames (ORF) of lamprey homologs such as *L-CD63* (GenBank accession: KT225459) and L-*Flot1* (Xu et al., 2017) have been identified by other members of our lab. So we amplified the cDNA sequences of *L-Alix* and *L-Grp78* by 5' and 3'-RACE (rapid amplification of cDNA ends) methods from kidney cDNA library of *L. japonica* (data not shown). The ORF of L-*Alix* is 2628 bp in length and encodes a protein containing 875 amino acid residues with a theoretical molecular weight of 98 kDa. The amino acid sequence of L-Alix shares 68% identity with human ALIX and 75% identity with mouse Alix. In addition, the 1992 bp ORF of *L-Grp* encodes a protein containing 663 amino acid residues with a theoretical molecular weight of 73 kDa, which shares 88% amino acid identity with human and mouse GRP78. Finally, anti-L-Alix, L-Flot1, L-CD63 and L-Grp78 polyclonal antibodies (pAb) were developed and used to detect the presence of the candidate markers in SMB cells and released exosomes by immunoblot analysis (Fig. 1D). As a result, the corresponding protein bands for all of the markers can be detected respectively at their predicted molecular weight in the total lysates prepared from SMB cells. Importantly, the homologs of exosomal markers, including L-Alix, L-Flot1 and L-CD63, were specifically enriched in SMB cell-derived exosomes, whereas L-Grp78, the ER marker GRP78 homolog, was absent in exosomes. Thus, all these morphological and immunoblot analyses support that SMB cells can release exosomes with well-defined characteristics comparable to their mammalian counterparts, and the purity of exosomes preparation meets the requirements for further proteomic profiling.

3.2. Proteomic characterization of exosomes released by quiescent and activated SMB cells

As mentioned above, SMB cells might play an essential role in the adaptive immune system of lamprey. In order to investigate the possible function and significance of SMB cell-derived exosomes in the regulation of immune function, we compared the protein composition between 2 types of exosomes produced by quiescent and activated SMB cells respectively. Firstly, SMB cells were isolated from adult lamprey injected by lipopolysaccharides (LPS) or saline thrice at 7-day intervals.

Then, by means of ultrafiltration followed by SEC methods, exosomes were purified from conditioned growth medium of LPS-immunized and control SMB cells. Notably, similar with quiescent SMB cell-derived exosomes (6.59e + 08 ± 1.45e + 07 particles/ml) (Fig. 1B), the concentration of exosomes isolated from the same volume of conditioned medium collected from LPS-stimulated SMB cells was 6.22e + 08 ± 1.24e + 07 particles/ml (Fig. 1C). Also, there was no difference in the mean diameters of exosomes between quiescent (134 ± 46.5 nm) and activated SMB cells (132 ± 62.8 nm). Therefore, our results show that cellular activation by LPS has no significant impact on the morphology and amount of SMB cell-derived exosomes.

The protein components of the exosomes from quiescent and activated SMB cells were identified by nano-LC–MS/MS analysis of peptides obtained by in-gel tryptic digestion of SDS-PAGE gel slices. LC–MS/MS spectra were searched against the lamprey UniProt database using Mascot (Matrix Science, UK). Our database searching identified a total of 1660 redundant proteins, which were further condensed to 392 unique exosomal proteins in quiescent SMB cells and 770 unique proteins in LPS-activated SMB cells. Next, we compared the proteomic profiles of activated exosomes with quiescent exosomes. As revealed in the Venn diagram (Fig. 2A), 206 proteins are common to the two data sets, which account for 52.4% in quiescent exosomes and 26.8% in activated exosomes, respectively, whereas 186 and 564 proteins are uniquely identified in the quiescent (47.4%) and activated exosomes (73.2%), respectively. Therefore, the comparison between the two datasets suggests that after the activation of SMB cells by LPS there are more and significantly different exosomal protein components.

Furthermore, in order to reveal the presence of lamprey homologs of marker proteins previously identified in exosomes derived from mammalian sources, especially human cells, the corresponding human homologs of each exosomal proteins from lamprey SMB cells were identified based on amino acid sequence alignment using local NCBI BLAST. A total of 363 homologs and 690 homologs were recognized in quiescent and activated SMB cell-derived exosomes proteome, respectively. The Venn diagram (Fig. 2B) shows that 61.4% of quiescent exosomal homologous proteins and 63.2% of activated exosomal homologous proteins are overlapped with exosomes markers which are defined as exosomal proteins with an identification count of more than 10 in the Evpedia database (https:// evpedia.info) (Kim et al., 2015). As shown in Table 1, the top 10 proteins identified simultaneously between quiescent and activated exosomes are listed based on their identification counts in the Evpedia database. Similarly, the top 10 unique proteins identified only in quiescent exosomes or only in activated exosomes are recognized, respectively (Supplementary Table 1–2). In particular, L-Alix and L-Flot1, which were cloned and verified as the homologs of exosomal markers ALIX and FLOT1 by immunoblot analysis (Fig. 1D), were also identified in SMB cell-derived exosomes proteome. The similarity among exosomal protein markers as shown in our analyses reveals that exosomes appear to be evolutionarily conserved between jawless and jawed vertebrates.

3.3. Exosomes released by quiescent and activated SMB cells were involved in different biological processes as revealed by comparative gene ontology analysis

Although the proteomic profiles revealed the existence of common proteins between exosomes derived from SMB cells under different physiological states, there were significantly different exosomal protein components between them. To investigate the functional significance of the similarities and differences between the two proteomic datasets, we performed data mining using bioinformatic tools to annotate and compare the biological processes in which quiescent and activated exosomal proteins are involved. Firstly, by means of Blast2GO software (https://www.blast2go.com), the identified proteins in both datasets were allocated to biological process categories defined by the GO consortium. A total of 276 of the 392 proteins identified in quiescent exosomes and 531 of the 770 proteins in activated exosomes could be assigned to the biological process categories. Then we used the Funrich software (http://www.funrich.org) (Pathan et al., 2015, 2017) to determine the common biological processes in which both quiescent and activated exosomal proteins were participated. The top 10 common biological processes are listed in Fig. 3A according to the percentage of proteins annotated as a given biological process in the total number of proteins from quiescent or activated exosomes, including cellular metabolic process, regulation of cellular process, biosynthetic process, cellular component organization, establishment of localization, cellular response to stimulus, cell communication, signal transduction, cell proliferation and regulation of immune system process. Furthermore, in order to determine the functional significance of different protein profile of activated exosomes compared with quiescent exosomes, GO enrichment analysis was performed using Funrich software. Bar charts in Fig. 3B represent significantly enriched GO terms of biological processes in activated exosomes, which take the corrected p values < 0.05 as a threshold defined by the hypergeometric test. One of the significantly enriched GO terms was positive regulation of cellular component biogenesis (corrected p values = 0.038), the other was cell cycle process (corrected p values = 0.037). Thus, the two identified biological processes suggest that exosomes released by activated SMB cells may play distinctive physiological roles in activating cellular component biogenesis and cell mitosis compared to quiescent exosomes.

3.4. Exosomes released by activated SMB cells were involved in unique signal transduction pathways as revealed by pathway enrichment analysis

As mentioned above, based on comparative GO analysis of biological processes between quiescent and activated exosomal proteins, signal transduction was identified as one of the top 10 common biological processes (Fig. 3A). Next, we further distinguished signal pathways regulated by activated exosomes from quiescent exosomes. In order to perform this analysis, the identified proteins in both types of exosomes were firstly annotated with the corresponding human

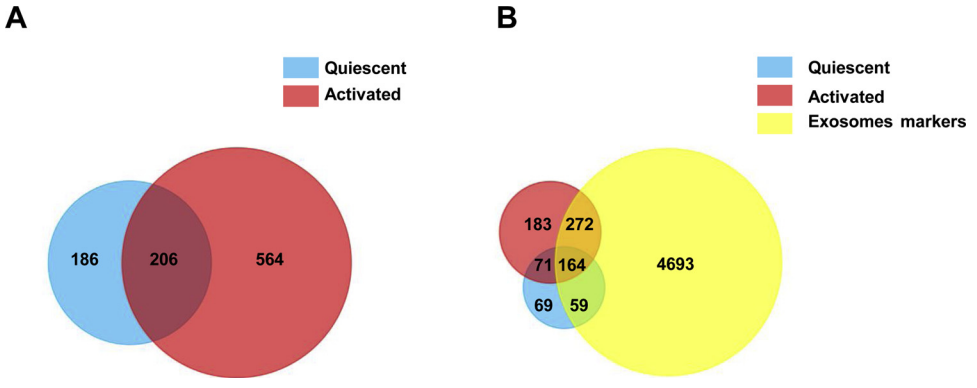

Fig. 2. Comparison between identified exosomal proteins released by quiescent and activated SMB cells. (A) Venn diagram summarizes the number of unique and common proteins in exosomes isolated from quiescent and activated SMB cells. (B) Venn diagram demonstrates that the homologous proteins of SMB cell-derived exosomes have significant overlap with human cell-derived exosomes markers which are defined as proteins identified in more than 10 datasets of the Evpedia database.

Table 1
Top 10 proteins identified in both quiescent and activated SMB cell-derived exosomes.

Identified proteins		Evpedia EV markers		
UniProt accession	Protein name	UniProt accession	Protein name	Identification number
S4RN92	similar to 14-3-3 protein epsilon	P62258	14-3-3 protein epsilon (14-3-3E)	366
S4R4N9	Clathrin heavy chain (Clh)	Q00610	Clathrin heavy chain 1 (CLH-17)	347
A0A1B1SLK1	Heat shock protein 90 (Hsp90)	P07900	Heat shock protein HSP 90-alpha (HSP90A)	343
Q6F3E7	Elongation factor 1 (Eef1a)	P68104	Elongation factor 1-alpha 1 (EEF1A)	331
S4RRW6	similar to Alix	Q8WUM4	Programmed cell death 6-interacting protein (ALIX)	337
D0V2A5	Integrin beta (Itgb1)	P05556	Integrin beta-1 (ITGB1)	314
S4RNL1	similar to Annexin	P50995	Annexin A11 (ANXA11)	271
S4R9V8	similar to Rab1a	P62820	Ras-related protein Rab-1A (RAB1A)	270
A0A0K0XHH6	CD81	P60033	CD81	247
S4RWV1	similar to Lamp2	P13473	Lysosome-associated membrane glycoprotein 2 (LAMP-2)	196

The top 10 proteins in the overlap between quiescent and activated exosomes are listed according to their identification number in the Evpedia database.

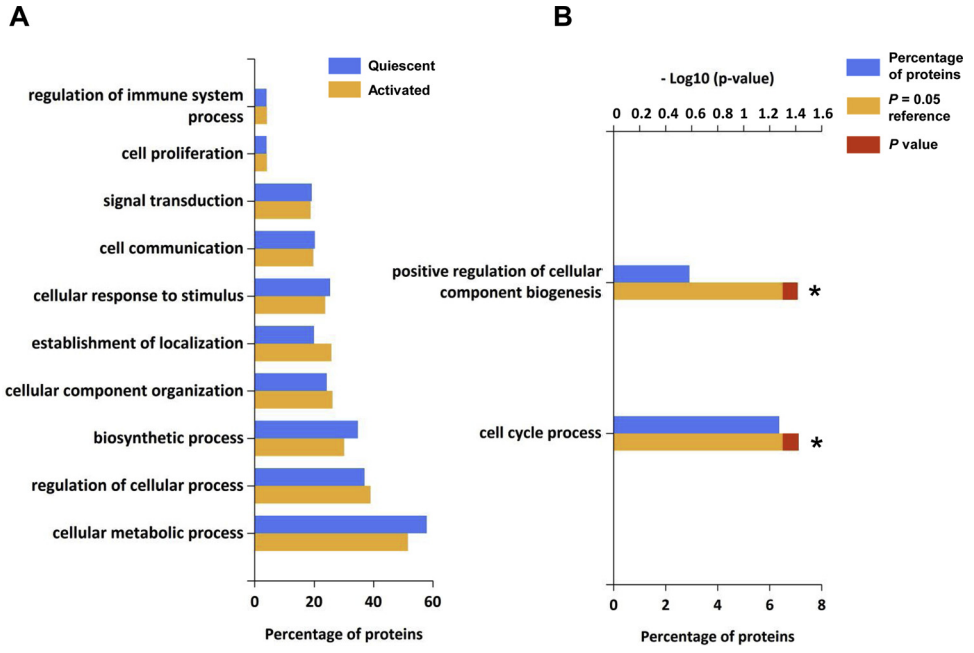

Fig. 3. GO annotation and functional enrichment analysis of exosomal proteins from quiescent and activated SMB cells. (A) The proteins identified in both quiescent and activated SMB cell-derived exosomes are allocated to biological process categories defined by the GO consortium. Bar charts show the top 10 classifications according to GO terms of biological processes. The percentages for proteins in quiescent and activated SMB cell-derived exosomes are indicated by bars respectively. (B) GO enrichment analysis recognizes the main biological processes in which the unique proteins of activated SMB cell-derived exosomes could be involved. Bar charts show the significantly enriched GO terms. The percentages for proteins contained by each GO term in the unique proteins of activated exosomes and corrected p values of enrichment analysis are indicated by bars respectively. Asterisk (*) indicates corrected p values < 0.05.

homologs, then the annotated exosomal proteins were assigned with K numbers in the KEGG Orthology system by way of BlastKOALA (KEGG Orthology And Links Annotation) software (https://www.kegg.jp/blastkoala) (Kanehisa et al., 2016). After these steps, biological pathway enrichment analysis was performed using Funrich software. The results are represented in Fig. 4A, where the top 10 signal pathways significantly enriched in activated exosomes are listed according to the fold number of enrichment and the corrected p values ($p < 0.05$ as a threshold defined by hypergeometric test), including IL2 signaling events mediated by PI3K (fold number = 4.18, $p = 0.04$), Integrin cell surface interactions (fold number = 3.96, $p = 0.02$), cell cycle mitotic (fold number = 2.94, $p = 0.02$), mitotic G1-G1/S phases (fold number = 2.68, $p = 0.007$), signaling by EGFR (fold number = 2.55, $p = 0.004$), adaptive immune system (fold number = 2.35, $p = 0.004$), mitotic M-M/G1 phases (fold number = 2.25, $p = 0.002$), M/G1 transition (fold number = 2.06, $p = 0.001$), antigen processing: ubiquitination & proteasome degradation (fold number = 1.99, $p = 0.00005$), antigen processing-cross presentation (fold number = 1.7, $p = 0.00002$). Furthermore, the top 5 most significant pathways defined by enrichment analysis were represented by protein-protein interaction (PPI) networks. As indicated in Fig. 4B–F, lists of exosomal proteins enriched in particular pathways, which directly interact with each other, are highlighted by nodes within the interactions network, respectively. Thereby, the significantly enriched signal pathways indicate that compared to quiescent exosomes, activated SMB cell-derived exosomes appear to mainly participated in the regulation of the signal pathways associated with cell mitosis and immune response.

4. Discussion

The purity of exosomes preparation is a key factor affecting the quality of the downstream analysis data, especially the proteomic profiling data. Because of the high resolution of Q Exactive™ mass spectrometry used in our study, the co-isolation of contaminating proteins can generate a significant artifact. Although ultracentrifugation and density gradient sedimentation are the most widely used methods in exosomes isolation, several reports have indicated the significant drawbacks of these isolation methods to recover exosomal proteins, including the contaminating cellular debris, low exosomes yield, vesicle aggregation and the highly time-consuming separation process (Cvjetkovic et al., 2014; Mathivanan et al., 2010; Tauro et al., 2012; Taylor et al., 2011). In addition, the damage to the structure and integrity of the exosomal membrane caused by ultracentrifugation can result in a serious loss of exosomal protein components. In our study, we successfully combined ultrafiltration with size exclusion chromatography (SEC) for isolating exosomes from the conditioned growth medium of SMB cells. As an emerging technique for exosomes preparation, size exclusion chromatography has been used and thought to

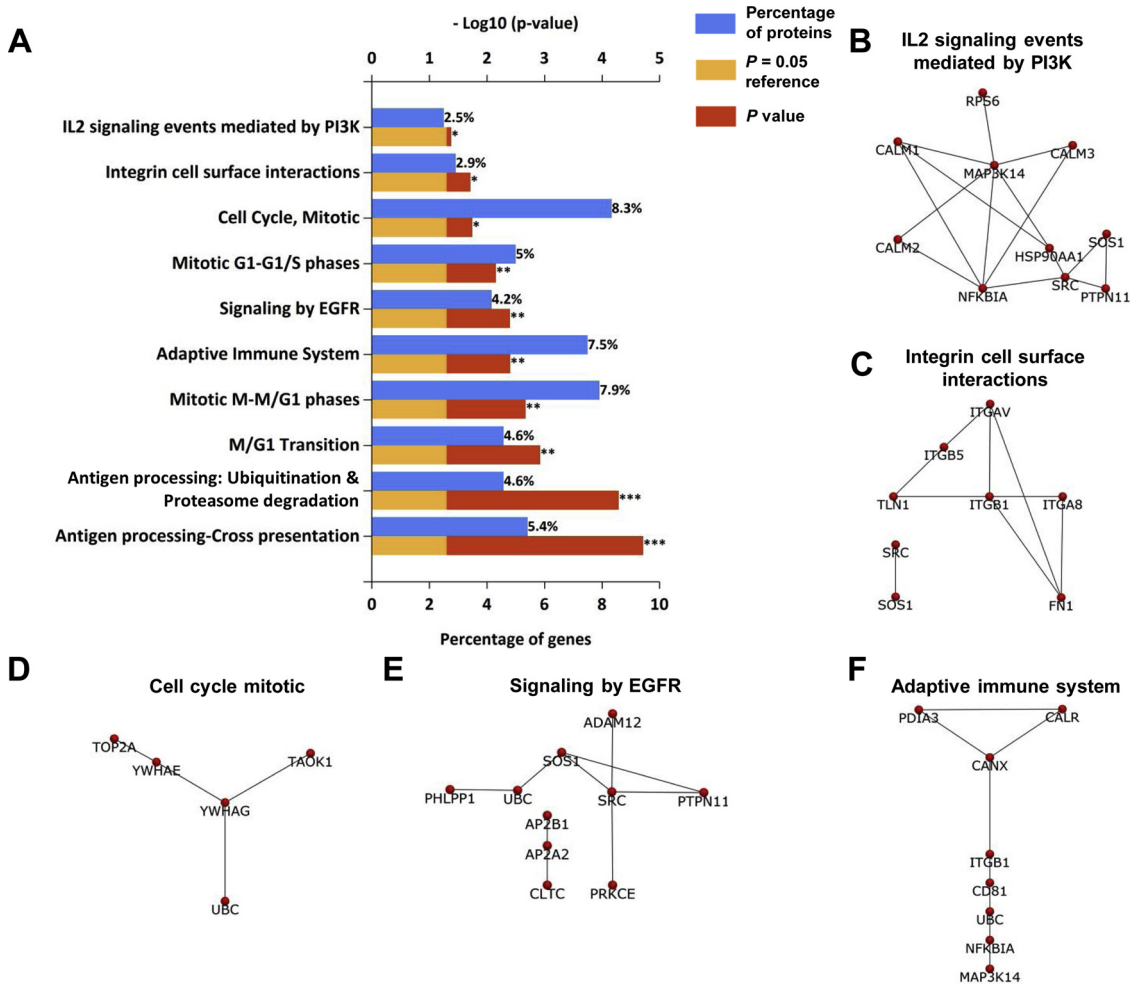

Fig. 4. Pathway enrichment analysis and interaction network analysis of homologous proteins from activated SMB cell-derived exosomes. (A) Pathway enrichment analysis identifies the main signal transduction pathways in which the unique proteins of activated SMB cell-derived exosomes could be involved. Bar charts show the top 10 significantly enriched pathways. The percentages for proteins contained by each pathway in the unique proteins of activated exosomes and corrected *p* values are indicated by bars respectively. *: corrected *p* values < 0.05, **: < 0.01, ***: < 0.001. (B–F) Interaction networks analysis shows protein-protein interactions of the top 5 significantly enriched signal pathways as indicated in (A), including IL2 signaling events mediated by PI3K (B), integrin cell surface interactions (C), cell cycle mitotic (D), signaling by EGFR (E), and adaptive immune system (F). Nodes in the networks represent homologs of unique proteins in activated SMB cell-derived exosomes.

be a better alternative (de Menezes-Neto et al., 2015; Mol et al., 2017; Muller et al., 2014; Nordin et al., 2015). Here, the modified methods of SEC coupled with ultrafiltration are proved to isolate exosomes efficiently and with high purity which meet the criterion for defining the isolated vesicles as exosomes and for proteomic studies.

Although exosomes released from mammalian cells have been intensively studied, exosomes derived from diverse lower organisms also have been reported, such as amoebae, parasitic helminths, C. elegans, and even Gram-negative bacteria (Colombo et al., 2014). To our knowledge, there has been no previous study with respect to detailed characterization of exosomes released by lamprey, a living representative of several extinct early jawless vertebrate lineages. Our proteomic study shows that 70% (146 homologs in total) of the homologous proteins identified simultaneously in both of the quiescent and activated exosomes are overlapped with human exosomes markers. Thus, this finding demonstrates that SMB cells could release exosomes which resemble mammalian cell types-derived exosomes on their size, morphology and protein markers. Our results in lamprey, together with other reports of exosomes-like vesicles in lower organisms, indicate that exosomes appear to be evolutionarily conserved among different species.

It is known that different physiological or activation states of the secreting cells could determine the nature of the immunomodulatory effects of exosomes by inducing changes in exosomes composition (Robbins and Morelli, 2014; Thery et al., 2009). In this study, we used lipopolysaccharides (LPS), a kind of endotoxin of gram-negative bacteria which can activate B cells, monocytes, dendritic cells, macrophages in mammals, to stimulate SMB cells and induce the adaptive immune response of lamprey *in vivo* by an immunization procedure. Next, we compared the characteristics and specific cargo between exosomes derived from quiescent and activated SMB cells. Firstly, the TRPS analysis shows that there are no significant differences in the morphology and quantity of exosomes before and after LPS activation. In contrast with the constitutive secretion of exosomes from SMB cells as shown in our study, it has been reported that the quantity of secreted exosomes depended on the different context of the donor cells, especially the immune cells in mammals. For instance, B or T cells released quite a few exosomes at the quiescent state but these lymphocytes enhanced exosomes secretion significantly following the activation through interactions with T cells (Blanchard et al., 2002; Mittelbrunn et al., 2011) or with the B cell receptor (Arita et al., 2008; Muntasell et al., 2007; Saunderson et al., 2008). Similarly, DCs increased exosomes release by cognate interactions with CD4 + T lymphocytes (Buschow et al., 2009; Nolte-'t Hoen et al., 2009). Secondly, our

proteomic profiling shows that there are significantly increased unique proteins in activated exosomes compared to quiescent exosomes (Fig. 2A). Consistent with our observation, many studies have indicated that inflammatory signals which stimulate the donor cells could induce changes in exosomes compositions in mammals. For instance, mature DCs stimulated by LPS could secret exosomes which have different protein compositions and enhanced ability for priming naive T cells compared with immature DCs (Segura et al., 2005). TNF-α-induced activation also could affect the protein and RNA compositions of exosomes released by endothelial cells (de Jong et al., 2012). Additionally, the IFN-γ stimulation could alter the protein contents of the exosomes derived from mesenchymal stromal cells (Kilpinen et al., 2013). Here, our finding reveals that the protein compositions of exosomes are modulated by the state of activation of the SMB cells.

There has been much research to reveal that exosomes can positively and negatively modulate the immune response. Previous studies showed that SMB cells play an essential role in the immune response of lamprey, but little is known about the function of SMB cell-derived exosomes in regulating the homeostasis of adaptive immune system. In this study, we annotated quiescent and activated exosomal proteins with the biological processes categories defined by the Gene Ontology. Firstly, our result shows that proteins present in both quiescent and activated exosomes might be implicated in several identical processes, which especially contain cell communication, signal transduction, and regulation of immune system process. These significant over-representation biological processes are consistent with the proposed role of SMB cell-derived exosomes in intercellular communication between SMB cells and other recipient cells for immune regulation. Moreover, GO enrichment analysis was performed to reveal the functional implication of the unique protein 'fingerprint' in activated exosomes compared to quiescent exosomes. The significantly enriched GO terms of biological processes suggest that activated exosomes may play distinctive functional roles in activation of cellular component biogenesis and regulation of cell proliferation. It is noteworthy that several reports have shown that exosomes released by activated lymphocytes in human can induce proliferation of recipient lymphocytes. For instance, B cell-derived exosomes which were loaded with peptides from allergen induced a dose-dependent T cell proliferation (Admyre et al., 2007). Additionally, activated CD3 + T cell-derived exosomes coupled with IL-2 (interleukin-2) promoted the proliferation of autologous quiescent CD3 + T cells (Wahlgren et al., 2012). Hence, our results suggest that exosomes from quiescent and activated SMB cells are likely to have distinguishing immunomodulatory roles.

It is known that once exosomes bind to recipient cells or are endocytosed by recipient cells, their components can induce changes in the signal pathways of recipient cells (Colombo et al., 2014). Thus, in order to determine the possible pathways that activated exosomes may affect compared to quiescent exosomes, we performed biological pathway enrichment analysis on the 240 unique proteins in activated exosomes. The significantly enriched pathways include cell cycle mitotic, mitotic G1-G1/S phases, mitotic M-M/G1 phases, and M/G1 transition. This result is consistent with one of the significantly enriched biological processes in the activated exosomes, namely cell cycle process, which further highlights that the activated exosomes may regulate proliferation of the specific recipient cells. In addition, several signal pathways are found uniquely enriched in activated exosomes, including IL2 signaling events mediated by PI3K, Integrin cell surface interactions, and signaling by EGFR. Although these pathways were not fully studied in lamprey, our analysis shows that the homologs of multiple elements in these pathways can be found in lamprey. Importantly, the enrichment analysis provides valuable clues about how activated exosomes induce changes in the physiology of recipient cells through the specific signal pathways. For instance, the IL-2 signaling pathway plays an important role in orchestrating immune responses (Boyman and Sprent, 2012). The IL-2 is a cytokine secreted by antigen-activated T cells and is capable of stimulating the growth and differentiation of various immune cells (e.g. T cells, B cells, natural killer cells). IL-2 could activate PI3K and finally stimulate the transcription of NF-κB target genes involved in promoting cell survival (Lauder et al., 2001). Whether exosomes could regulate the immune response of lamprey through the IL-2/PI3K pathway may be worthy of further study. In addition to the enriched pathways discussed above, the remaining three enriched pathways, including the adaptive immune system, antigen processing: ubiquitination & proteasome degradation, and antigen processing-cross presentation, highlight the possible role of exosomes in the adaptive immune system of lamprey. Numerous studies have revealed that exosomes could play a critical role in direct or indirect antigen presentation (Robbins and Morelli, 2014; Thery et al., 2009). For instance, B cell line-derived exosomes bearing MHC class II and co-stimulatory molecules can directly activate CD4 + T cell clones (Raposo et al., 1996). In addition, exosomes carrying adhesion molecules such as Integrins can bind to the surface of dendritic cells (DCs), indirectly presented peptide-MHC complexes to T cells (Mallegol et al., 2007; Segura et al., 2007; Thery et al., 2002). Although the VLRs-based antigen recognition system and B/T-like lymphocyte lineages in lamprey have been intensely studied, little is known about the mechanism of antigen processing and presentation, even the homologs or functional equivalents of MHC molecules fail to be found in jawless vertebrates (Pancer et al., 2004b; Suzuki et al., 2004). Our finding that the activated exosomes are associated with antigen processing-related pathways may provide insights into the possible role of exosomes in antigen presentation of lamprey.

In summary, based on modified methods for isolation and characterization of exosomes derived from SMB cells in lamprey, our proteomic analysis illustrates that exosomes are evolutionarily conserved between lamprey and other higher vertebrate species on their protein markers. The further bioinformatic analysis compares the specific composition of exosomes derived from the quiescent and activated SMB cells, and provides clues about the possible role of exosomes in immune regulation of lamprey. Our research could shed light on the evolution of exosomes from early jawless vertebrates (such as lampreys) to jawed vertebrates (such as mammals), may also extend our knowledge about the regulation mechanism of the VLRs-based adaptive immune response. Future functional studies are needed to elucidate the specific recipient cells and verify the roles of exosomes released by SMB cells in lamprey, and related works are already in progress.

Competing interests

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.04.004.

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