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Original Articles

Cell-specific uptake of mantle cell lymphoma-derived exosomes by malignant and non-malignant B-lymphocytes

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ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive and incurable mature B cell neoplasm. The current treatments are based on chemotherapeutics and new class of drugs (e.g. *Ibrutinib*®), which in most cases ends with tumor resistance and relapse. Therefore, further development of novel therapeutic modalities is needed. Exosomes are natural extracellular vesicles, which play an important role in intercellular communication. The specificity of exosome uptake by different target cells remains unknown. In this study, we observed that MCL exosomes are taken up rapidly and preferentially by MCL cells. Only a minor fraction of exosomes was internalized into T-cell leukemia and bone marrow stroma cell lines, when these cells were co-cultured with MCL cells. Moreover, MCL patients' exosomes were taken up by both healthy and patients' B-lymphocytes with no apparent internalization to T lymphocytes and NK cells. Exosome internalization was not inhibited by specific siRNA against caveolin1 and clathrin but was found to be mediated by a cholesterol-dependent pathway. These findings demonstrate natural specificity of exosomes to B-lymphocytes and ultimately might be used for therapeutic intervention in B cells malignancies.

Introduction

Mantle cell lymphoma (MCL) is a well-defined, aggressive, lymphoid neoplasm characterized by proliferation of a distinctive population of mature B-lymphocytes [1]. The neoplastic cells tend to colonize the mantle zone of the lymphoid follicles and spread throughout the body, infiltrating lymphoid tissues, bone marrow, peripheral blood and extranodal sites [2]. Conventional chemotherapy induces remission in the majority of newly treated patients. However, within a few years, most patients experience relapse with severe drug-resistant phenotype that often leads to death with relatively short median survival duration of 5–7 years [3]. Therefore, the discoveries of novel biological pathways involved in MCL progression and resistance may lead to new therapeutic agents with improved treatment outcomes [4].

Exosomes are small extracellular membrane-enclosed vesicles of 30–150 nm in diameter. Exosomes originate from the inward budding of the endosomal membrane, forming multivesicular bodies (MVBs). They are secreted into the extracellular environment or into biological fluids as a result of fusion of intracellular MVBs with the plasma membrane and are considered as messengers in intercellular communication [5,6]. Exosomes are released by different types of healthy cells such as leukocytes (B lymphocytes [7,8], T lymphocytes [9] and natural killer cells (NK) [10]) as well as by cancer cells, lymphocytic cell lines [10,11] and by primary chronic lymphocytic leukemia (CLL) and acute myelogenous leukemia (AML) cells [12,13]. Their biological role depends on the cell of origin and recipient cell. The putative function of exosomes in cancer is based on the recently described findings of transfer of genetic material and signaling proteins, resulting in increased angiogenesis, metastasis, drug resistance and immunosuppressive environment [5,14–16]. Increasing evidence emphasize the role of exosomes secreted by various leukemic cells in reprograming the microenvironment and supports disease progression. Exosomes released by K562 cells, a chronic myeloid leukemia (CML) cell line induce angiogenic activity of human umbilical endothelial cells (HUVEC) [17]. These exosomes when released from K562 cells overexpress microRNA-92a, were able to transfer this microRNA to HUVEC, and enhance their migration and blood vessel formation [18]. Recently, CML-derived exosomes were shown to promote, through an autocrine mechanism, the proliferation and survival of tumor cells, both *in vitro* and *in vivo*, by activating anti-apoptotic pathways [19]. Primary and cell line AML-derived exosomes

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transfer mRNA and alter the secretion of growth factors from bone marrow stromal cells, thereby supporting the survival of the neoplastic cells [13]. Furthermore, leukemia and lymphoma derived exosomes were shown to immunosuppress the cytotoxic activity of NK cells by binding the NK group 2 receptor (NKG2D), thus assisting in the immune evasion of malignant cells [20].

Both endocytosis [21–23] and phagocytosis [24] pathways involved in delivery of functional cargo to recipient cells [25,26]. Proteomic analysis of exosomes reveals elevated levels of adhesion proteins that promote absorption to the cell surface [27,28]. In addition, exosomes have been shown to attach to recipient cells via phosphatidylserine receptors [29]. Previous studies have suggested that tumor derived exosomes are more readily associated with cancer cells as compared to normal cells ex-vivo [30,31]. However, specificity of exosomes to recipient cells and the membrane molecules that are involved in the recognition of exosomes by recipient cells are mostly unknown. In this study, we characterized exosomes of the MCL cell line and primary cells, elucidate their internalization mechanism and decipher their cell specificity.

Materials and methods

Simvastatin, Dynasore, PKH-26 and PKH-67 were purchased from Sigma-Aldrich, Israel. Nystatin was purchased from Biological Industries Ltd, Israel. Gefitinib was pursued from Cayman Chemicals, USA. All other materials were of chemical grade. MEBCYTO® apoptosis kit was purchased from MBLI.

siRNA sequences

siCLTC and siCAV1 were purchased from siGENOME SMARTpool – Thermo Scientific Dharmacon. The following combination of four different siRNA oligos for clathrin heavy chain (CLTC) and caveolin1 (CAV1) were employed:

siCLTC – (GCAAUGAGCUGUUUGAAGA; GAAAGAAUCUGUAGAGAAA; UGACAAAGGUGGAUAAAUU; GGAAAUGGAUCUCUUUGAA).
siCAV1 – (GCAAAUACGUAGACUCGGA; AUUAAGAGCUUCCUGAUUG; GCAGUUGUACCAUGCAUUA; CUAAACACCUCAACGAUGA). The luciferase gene as control sequence (siLUCsense strand: CUUACGCUGAGUACUUCGA) were designed and screened by Alnylam Pharmaceuticals (Cambridge, MA, USA).

Cell culture

Mantle cell lymphoma cell line, Jeko-1 (CRL-3006) was purchased from Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany) and Mino cell line (CRL-3000) was purchased from the American Type Culture Collection (ATCC). Jeko-1 and Mino cell lines were cultured in RPMI-1640 (Gibco, Life Technologies), supplemented with 20% or 15% exosome-depleted, Fetal Bovine Serum (FBS) (Biological Industries Ltd, Israel), respectively supplemented with 2% glutamine (Gibco, Life Technologies) and 1% penicillin/streptomycin (Biological Industries Ltd, Israel). Jurkat (TIB-152), human acute T cell leukemia cell line and HS-5 (CRL-11882) human bone marrow derived stroma cell line were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies), supplemented with 10% exosome-depleted FBS (Biological Industries Ltd, Israel), 2% glutamine (Gibco, Life Technologies) and 1% penicillin/streptomycin (Biological Industries Ltd, Israel).

Healthy and MCL patients' mononuclear cell isolation

Peripheral blood (PB) and serum samples were obtained from healthy donors and from patients with MCL who were treated at the Rabin Medical Center (Petah Tikva, Israel) and the Rambam Medical Center (Haifa, Israel) after obtaining institutional review board-approved informed consent. The clinical characteristics of the patients whose PB samples were studied are presented in Table 1. To isolate low-density cells, PB cells were fractionated using Ficoll-Paque™ PLUS (GE Healthcare, Life Sciences). Fractionated cells were used immediately or frozen for additional studies.

Exosome isolation and labeling

Exosomes were isolated by differential centrifugation [32] and were labeled by PKH-26 red/PKH-67 green fluorescent cell linker cell membrane labeling (Sigma-Aldrich) as described in Supplemental Materials and Methods. Exosomes were isolated from serum by total exosome isolation reagent from serum (Life Technologies).

Exosome analysis by flow cytometry

Anti CD81 coated latex beads were bound to exosomes and analyzed by flow cytometry as described in Supplemental Materials and Methods.

Structural analysis of exosomes by electron microscopy

MCL Exosomes were loaded onto formvar carbon coated grids (Ted Pella Inc, Redding, USA). Next, the exosomes were fixed in 2% paraformaldehyde and washed. The exosomes were immunostained with anti-CD81 antibody (BioLegend) followed by staining with a 12 nm gold-conjugated secondary antibody (Jackson ImmunoResearch). Staining with 12 nm gold-conjugated secondary antibody only was used as a negative control. The exosomes were subsequently fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate and embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%). The preparations were examined with Jeol 1200EX TEM (Jeol, Japan).

Nanoparticle tracking analysis (NTA)

Size distribution analysis of exosomes based on Brownian motion was assayed by NanoSight LM20 (NanoSight, Amesbury, United Kingdom) using NTA2.3 software upon exosomes dilution into PBS pH 7.4.

Exosomes internalization assays

Exosomes were labeled with PKH-26 for confocal microscopy experiments or with PKH-67 for flow cytometry analysis experiments, and uptake was determined by flow cytometry and confocal microscopy as described in Supplemental Materials and Methods.

Western blot analysis

Lysates of cells and exosomes were separated by SDS/PAGE, transferred to nitrocellulose membranes, and incubated with antibodies as described in Supplemental Materials and Methods.

Electroporation

1 nmole of each of the RNA duplexes (siCLTC, siCAV or siLUC) was electroporated into 10×10^6 Jeko-1 cells using the Amaxa 4D-nucleofactor system (CM-119 program, SF cell line solution).

Table 1
MCL patient characteristics.

MCL patient no.	PB/BM	Sex	Age Y	WBCs $10^3/\mu l$	Lymphocytes (%)	Hemoglobin g/dl	Platelets $10^3/\mu l$	Immuno-histochemistry cyclin D1	IgH/CCND1 FISH	Alive = A Death = D	Previous treatment
1	PB	M	65	16.7	10	13.6	78	Positive	Positive	D	
2	PB	M	50	3.37	19	8.3	79	Positive	Positive	A	
3	BM	M	51	6.38	33	13.1	139	Positive	Positive in PB	A	RCHOP
4	PB	F	57	223	81	9.8	112	Positive	Positive	D	
5	PB	M	62	104	94	10.2	96	Positive	Positive	D	
6	PB	M	75	94.35	86	12.3	93	Positive	NA	A	
7	BM	M	65	15.56	10	9.3	33.8	Positive	Positive	A	
8	PB	M	75	94.35	92	12.3	93	Positive	positive	A	

MCL indicates mantle cell lymphoma; PB, peripheral blood; BM, bone marrow; M, male; F, female; WBC, white blood cell; CCND1, cyclin D1; NA, not available; RCOP, rituxan, cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone.

Quantitative real-time PCR

Total RNA was isolated using EZ-RNA kit (Biological Industries, Israel) and cDNA was generated with high capacity cDNA kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturers' protocols. qRT-PCR was performed with Fast SYBR® Green Master Mix and the ABI StepOnePlus™ instrument (Life Technologies).

CLTC (Fwd: TCGCTACCTGGTACGTCGAAA; Rev: GCCTTTACAGTTACTGACACTTCTTCA) or Caveolin1 (Fwd: GTCAACCGCGACCCTAAAC; Rev: TTCCAAATGCCGTCAAAACT) expression was normalized to the housekeeping genes eIF3a (F:TCCAGAGAGC CAGTCCATGC, R:CCTGCCACAATTCA TGCT) and eIF3c (F:ACCAAGAGAGTTGTCCGCAGTG, R:TCATGGCATTACG GATGGTCC). Analysis was done with the StepOne™ software V 2.1 (Life Technologies) using the multiple endogenous controls option. When using multiple endogenous controls, the software treats all endogenous controls as a single population, and calculates the experiment-appropriate mean to establish a single value against which the target of interest is normalized.

Results

Structural and biochemical characterization of exosomes derived from MCL cells

To isolate and characterize exosomes of MCL cells, we utilized a classic exosome isolation protocol that exploits their differential sedimentation properties [33]. Exosomes were isolated from the condition media of Jeko-1 cells, a human MCL cell line, 72 h post cell culturing. Transmission electron microscopy (TEM) analysis of these vesicles showed spherical, membrane encapsulated particles. A typical electron micrograph of Jeko-1 stained vesicles illustrated a mean diameter of 150 nm (Fig. 1A). To visualize further these vesicles, we utilized immunogold labeling technique in combination with an anti-CD81 mAb, a tetraspanin known to be expressed on exosomes. The detection of immune-colloidal gold on the outer layer of Jeko-1 vesicles by TEM analysis, confirmed that these vesicles are exosomes (Fig. 1B). Nanoparticle tracking analysis demonstrated a bell-shaped curve, indicating a homogeneous population of Jeko-1 exosomes with a mean diameter of 121 ± 87 nm (Fig. 1C). Expression of exosomes proteins markers [34] was characterized by flow cytometry and western blot analyses. Western blotting using protein lysates of exosome preparations revealed the presence of the multivesicular bodies' protein TSG101 and enrichment in CD81 compared to whole cell lysate of Jeko-1 cells. Lysates of Jeko-1 exosomes do not express the endoplasmic protein, calnexin (Fig. 1D). Exosomes were bound to aldehyde-sulfate latex beads coated with anti-CD81 mAb. Flow cytometry analysis revealed the presence of exosomal marker proteins CD63 and CD81 as well as the B-cells marker, CD19 in 92% of the beads (Fig. 1E).

To strengthen our results, we have isolated exosomes from peripheral blood mononuclear cells (PBMC) of primary MCL patients (MCL4, MCL5 and MCL6 see Table 1). Analyzing these patients' monocular cells by flow cytometry revealed that 90–95% of the cells are MCL malignant B-cells ($CD5^+CD19^+$), therefore the vast majority of the exosomes released by these patients' PBMCs are originated from MCL cells. Exosomes were isolated from the conditioned media as described in the Materials and methods section. TEM analysis illustrates the size distribution of ~100 nm in the diameter of primary MCL exosomes (Fig. 2A). Nanoparticle tracking analysis showed a homogeneous population of MCL primary exosomes with a mean size diameter of 94 ± 62 nm (Fig. 2B). Next, we wanted to know if MCL primary cells release exosomes and therefore it will be possible to purify these exosomes directly from MCL patients' serum. To this end, exosomes were purified from serum of three MCL patients, two patients with high white blood cells (WBC) count and one with low WBC count (MCL4, MCL8 and MCL7, respectively) (Table 1). MCL exosomes were subjected to flow cytometry analysis and identified by expression of CD81 and CD63 and the B-cell marker, CD20. MCL derived exosomes are present in the serum of all three MCL patients (Fig. 2C), with higher amount in patients with higher WBC count.

MCL derived exosomes are rapidly and preferentially internalized by B-lymphocytes

In order to study the uptake of MCL derived-exosomes by MCL cells, we incubated PKH-26 labeled Jeko-1 exosomes with Jeko-1 cells at different time points and examined their internalization by confocal microscopy analysis. Exosome internalization was observed as early as 10 min post incubation. Longer incubation time resulted in higher accumulation of exosomes inside the cells. One-hour post incubation, ~80% of the cells internalized exosomes (Fig. 3A). To study the kinetics of exosome accumulation, we performed quantitative flow cytometry. PKH-67 labeled exosomes were incubated with Jeko-1 cells and the fluorescence intensity was detected. Increase in the fluorescence signal was detected as early as 10 min post incubation and increased in time, saturated at 120 min (Fig. 3B). This rapid internalization suggested that MCL cells have a high affinity for the Jeko-1-derived exosomes. In order to study the cell specificity of MCL-derived exosomes, we exposed co-culture of Jeko-1, Jurkat (human acute T-cells leukemia) and HS-5 (human stromal cells) cells to Jeko-1-derived exosomes and measured the internalization kinetics to the different cell populations. Only a small amount of exosomes were taken up by Jurkat (4.5 ± 0.9%) or HS-5 (7.86 ± 5.88%) cells, while 78.8 ± 8.3% of Jeko-1 cells internalized exosomes 120 min post incubation (Fig. 4A). The specificity of MCL exosomes to MCL cells was further demonstrated with Mino-derived exosomes, an additional MCL cell line. Co-culture of Mino, Jurkat and HS-5 cells was exposed to PKH-67 labeled Mino-derived exosomes for several times periods. Similar to the results obtained with Jeko-1-derived exosomes, Mino exosomes were uptaken rapidly and preferentially by Mino cells. 120 min post incubation, only 37.52% ± 4 of HS-5 cells and 11.5% ± 1.95 of Jurkat cells, while 84.2% ± 0.95 of Mino cells internalized Mino-derived exosomes (Fig. 4B).

Internalization of Jeko-1 exosomes to different cells was analyzed also by confocal microscopy. Jurkat, Jeko-1 and HS-5 cells were co-cultured with Jeko-1-derived exosomes for 60 min. After incubation, Jurkat and Jeko-1 cells were removed and the cell membrane was stained with either anti AlexaFluor 488 anti CD3 or anti AlexaFluor 488 anti CD19. The HS-5 cells were stained by Wheat germ agglutinin (WGA)-AlexaFluor 488. Confocal microscopy analysis confirmed localization of exosomes inside Jeko-1 cells, with no apparent internalization into Jurkat and HS-5 cells (Fig. 4C). These results affirm that Jeko-1 exosomes have higher affinity for MCL cells.

To further test the hypothesis that MCL exosomes have a unique affinity toward B-lymphocytes in general and MCL cells in particular, we investigated the cell specificity of MCL exosomes upon simulation of their endogenous environment. We purified PBMCs from healthy individuals and MCL patients, and exposed them to primary MCL exosomes for several designated time periods. Primary MCL exosomes were isolated from culture medium of PBMC from MCL patients. Uptake of MCL primary exosomes by monocytes, B-lymphocytes, natural killer (NK) cells and T-lymphocytes was measured by flow cytometry and confocal microscopy analysis. Healthy B-lymphocytes internalized exosomes efficiently as opposed to healthy T-lymphocytes and NK cells that barely internalized MCL exosomes (Fig. 5A and C). As demonstrated by flow cytometry analysis, 180 min post incubation of MCL5 exosomes with healthy PBMC, 50.76 ± 10.89% of B-lymphocytes internalized exosomes in contrast to 5.127 ± 1.224% of T-lymphocytes and 8.145 ± 2.044% of NK cells. Similarly, MCL patients' derived exosomes were preferentially internalized by patients B-lymphocytes. MCL6 B-lymphocytes internalized MCL exosomes in a time dependent manner, however T-lymphocytes as well as NK cells hardly took up MCL exosomes (Fig. 5B and D). Similar results were obtained when PBMCs from healthy individuals and MCL patients were exposed to exosomes of a different patient, MCL4 (Fig. S1). Although B-lymphocytes from

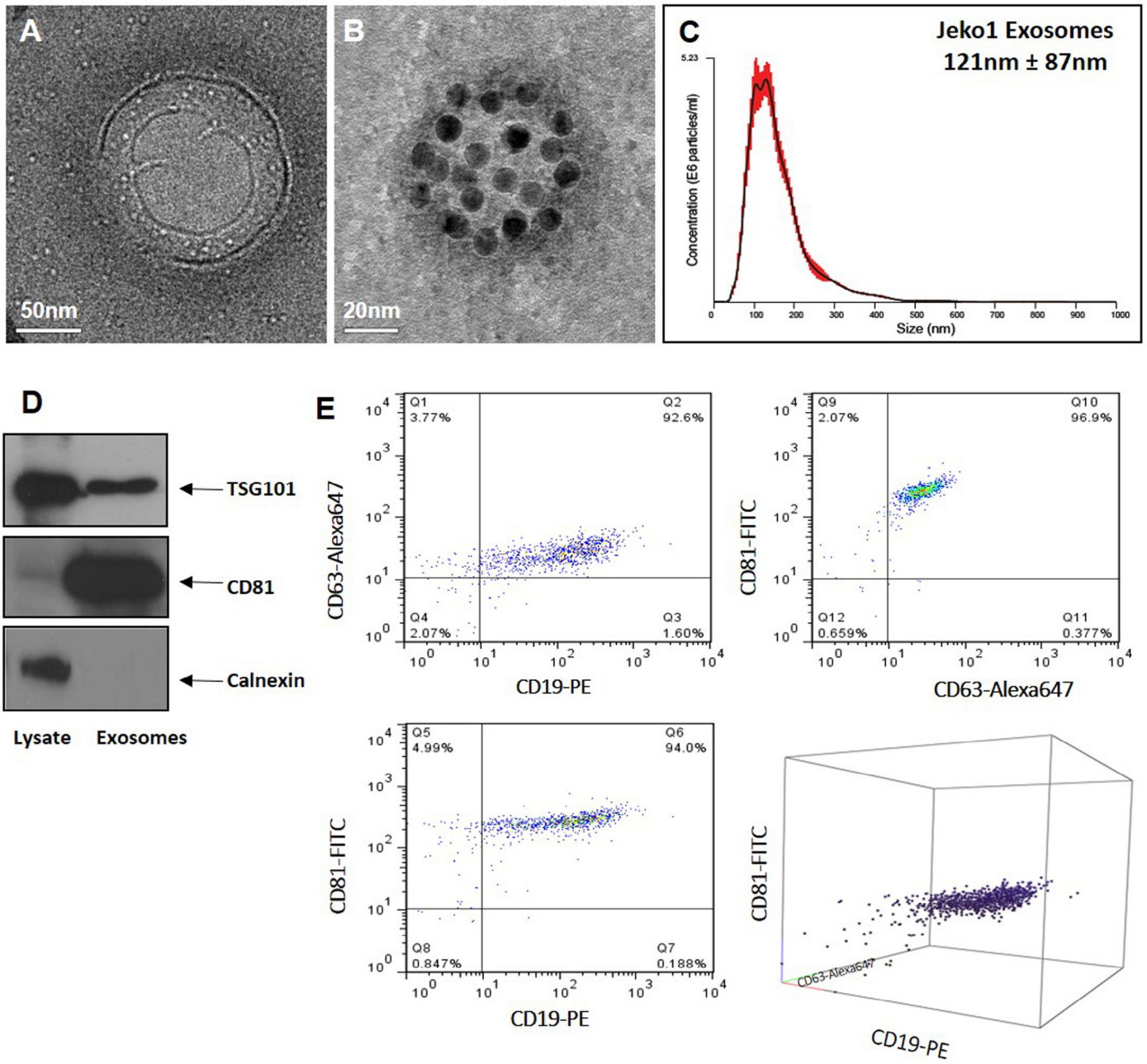

Fig. 1. Structural and biochemical characterization of Jeko-1 exosomes. (A) Exosomes were isolated from the culture media of Jeko-1 cells and analyzed by transmission electron microscopy (TEM). (B) Immuno-electron microscopy of isolated Jeko-1 exosomes incubated with anti-CD81 followed by IgG 12-nm in diameter gold nanoparticle-conjugated mAb. (C) Measurement of Jeko-1 exosome mean diameter by nanoparticle tracking analysis system. (D) Western blot analysis of the exosome markers CD81, TSG1 and the intracellular protein Calnexin. (E) Flow cytometry analysis of the exosomal surface proteins CD81, CD63 and the B-lymphocytes marker CD19 on Jeko-1 exosomes. Representatives of the three independent experiments are shown.

various patients showed divergence kinetics of exosomes uptake, in all the patients exosomes were preferentially internalized into B-lymphocytes with no apparent internalization to patients' T-lymphocytes (Fig. S1). Taken together, these data suggest that MCL derived exosomes are preferentially taken up by B-lymphocytes.

The uptake of exosomes by healthy monocytes preceded that of B lymphocytes. 10 min post incubation, 70.16 ± 20.38% of the healthy monocytes uptake exosomes while only 8.47 ± 1.9% of B-lymphocytes internalized the exosomes (Fig. 5A). Similarly, the monocytes of MCL patient's uptake exosomes are faster than B-lymphocytes in part of MCL patients (Figs. 5B and S1D), however in other patients, the uptake of MCL exosomes by monocytes and B-lymphocytes was at the same rate (Fig. S1B and C).

Internalization of MCL exosomes is mediated by lipid raft/cholesterol endocytosis pathway

Next, we explored the internalization mechanism(s) of MCL exosomes. The process of endocytosis involves multiple mechanisms in mammalian cells. The internalization pathway is dependent on the type of cargo and its intracellular fate. In order to identify if clathrin-dependent endocytosis mediates internalization of MCL

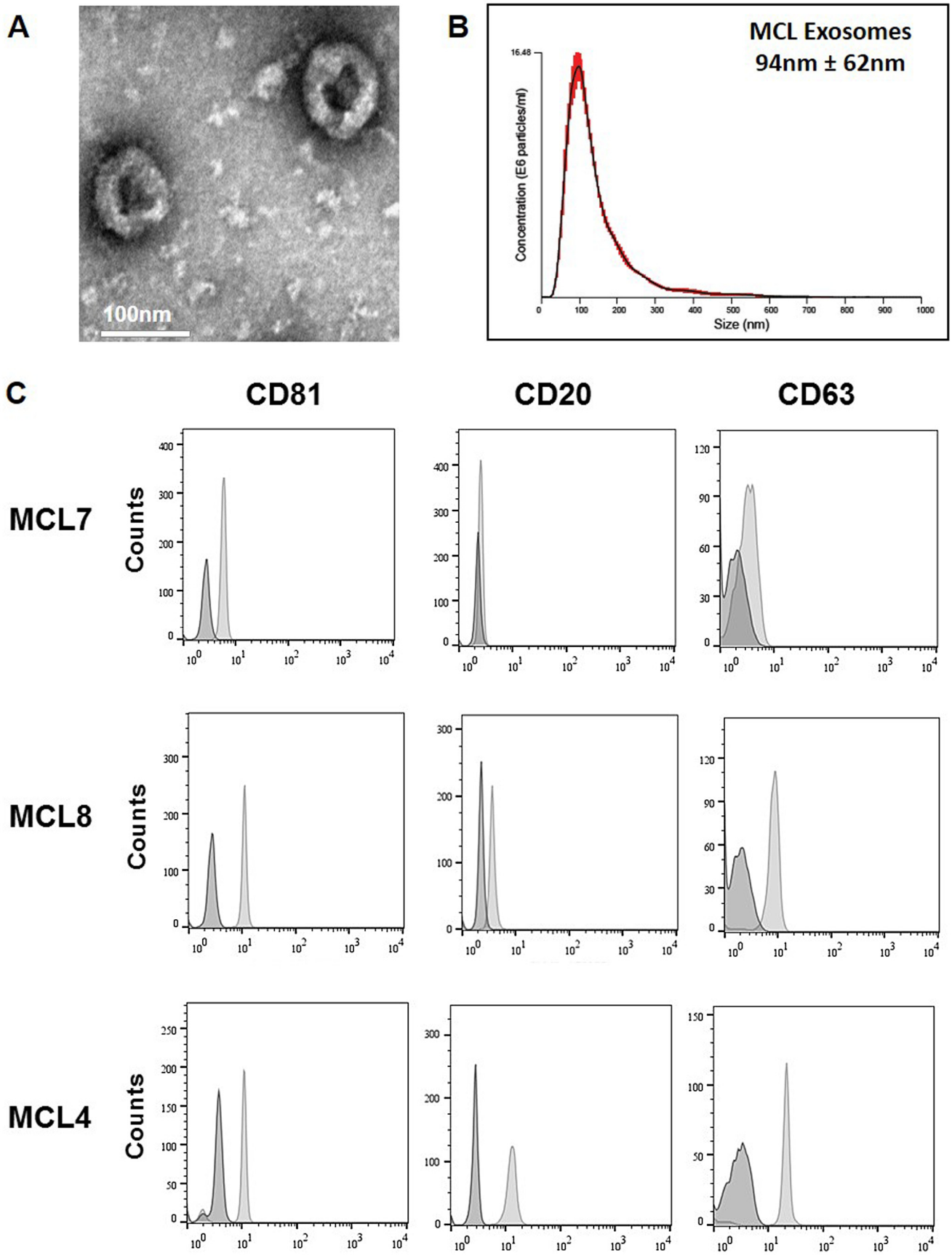

Fig. 2. Characterization of MCL primary exosomes. (A) Exosomes were isolated from the culture media of MCL patient(s) cells and analyzed by transmission electron microscopy (TEM). (B) Measurement of MCL primary exosomes mean diameter by nanoparticle tracking analysis system. (C) Flow cytometry analysis of exosomal surface protein (CD81 or CD63) and the B-lymphocytes marker (CD20) on primary exosomes from serum of patients MCL7, MCL8, and MCL4.

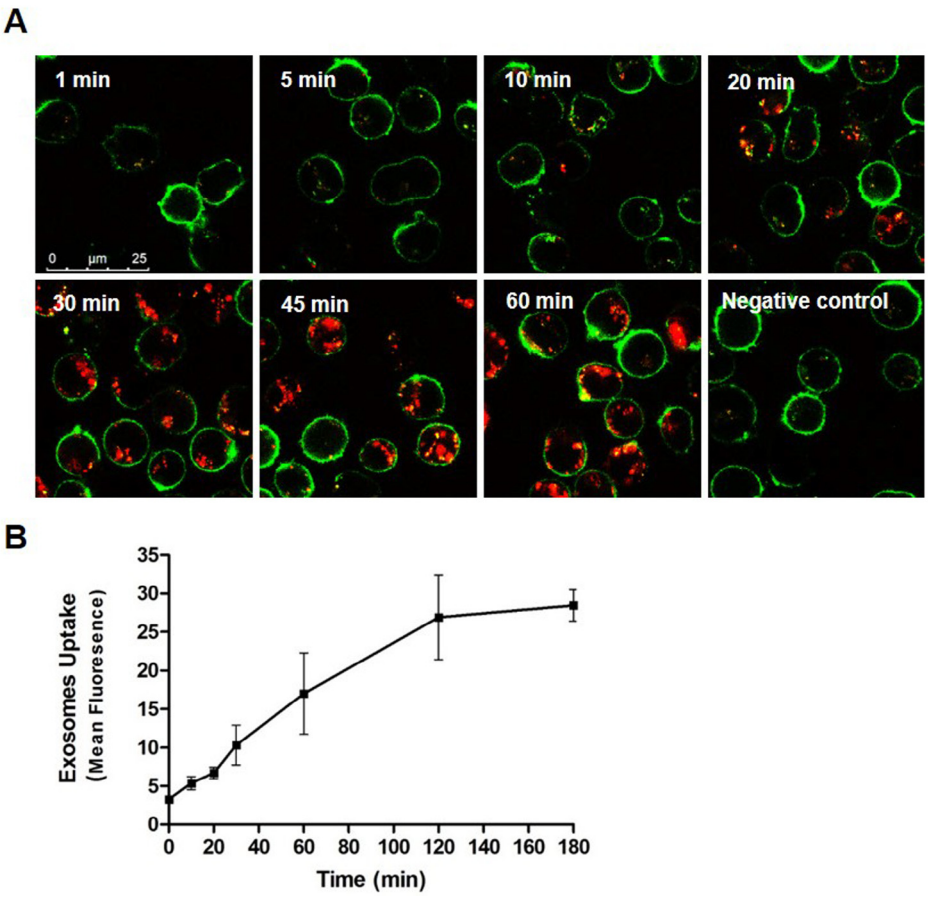

Fig. 3. Rapid uptake of Jeko-1 exosomes by Jeko-1 cells. Kinetics of Jeko-1 exosomes uptake by Jeko-1 cells measured by confocal microscopy and flow cytometry analysis. (A) PKH-26 labeled Jeko-1 exosomes were incubated with Jeko-1 cells, the reaction was stopped at different time points (1, 5, 10, 20, 30, 45 and 60 min) and cells were analyzed by confocal microscopy. The cell membrane of Jeko-1 cells was stained with AlexaFluor 488-anti CD44 mAb. Negative control – Jeko-1 cells with no addition of labeled exosomes. The data are representative of three independent experiments. Scale bar equals 25 µm. (B) Flow cytometry analysis of Jeko-1 cells, mean fluorescence after incubation with PKH-67 labeled Jeko-1 exosomes for the indicated time points (10, 20, 30, 60, 120 and 180 min). The data are presented as mean ± SD of four independent experiments.

exosomes, we have knocked down clathrin heavy chain by specific siRNA. Jeko-1 cells were electroporated with Clathrin-siRNA (siCLTC) or Luciferase siRNA (siLUC) as control. Electroporation of Jeko-1 cells with siCLTC resulted in significant decrease in Clathrin mRNA (62 ± 2.2% of inhibition, relative to siLUC treated cells) and Clathrin protein levels, 72 h post electroporation (Fig. 6A and B). Exosomes were incubated for 60 min with untreated Jeko-1 cells (mock), siLUC or siCLTC-treated Jeko-1 cells. The uptake of exosomes was quantified by flow cytometry and visualized using confocal microscopy. Inhibition of clathrin expression did not affect the uptake of exosomes by Jeko-1 cells (Fig. 6C and D), while uptake of transferrin, a well-established protein that internalizes through clathrin-dependent mechanism, was significantly decreased. Transferrin CF640 bound to the plasma membrane but only slightly internalized in siCLTC-treated cells (Fig. 6D).

These results suggest that clathrin independent pathway mediates the endocytosis of MCL exosomes by MCL cells. Described mechanisms of clathrin independent endocytosis include macropinocytosis, caveolae-mediated uptake and non-classical pathways involving non-clathrin, non-caveolae-mediated endocytosis [35–37].

To identify if caveolae dependent pathway mediates the endocytosis of MCL exosomes, Jeko-1 cells were electroporated with Caveolin1-siRNA (siCAV1) or Luciferase siRNA (siLUC) as control. Electroporation of Jeko-1 cells with siCAV1 resulted in a significant decrease in caveolin1 mRNA (58 ± 9.7% of inhibition, relative to siLUC treated cells) (Fig. 6E). Inhibition of caveolin1 expression did not affect the uptake of exosomes by Jeko-1 cells (Fig. 6F and G), while uptake of cholera toxin, that partially internalizes by caveolae-dependent mechanism, decreased (Fig. 6G). The decreased uptake of transferrin or cholera toxin in Jeko1 treated siCLTC and siCAV1, respectively, could not be quantified by FACS since they still bound to the plasma membrane. We next ask if uptake of MCL exosomes is mediated by dynamin, tyrosine kinases or is associated with cholesterol-enriched membrane microdomains. Jeko-1 cells were incubated for 30 min with Dynasore (80 µM from stock solution of 3.6 mM in DMSO), an inhibitor of dynamin-dependent endocytosis [38], Gefitinib, a tyrosine kinase inhibitor (10 µM from stock solution of 10 mM in DMSO) and Nystatin (40 µg/ml from stock solution of 50 mg/ml in H_2O), an inhibitor of the lipid raft-mediated endocytosis pathway [39,40] or cultured in the presence of Simvastatin (2 µM from stock solution of 4 mM in DMSO), an inhibitor of cholesterol synthesis [41] for 16 h. Control cells were incubated with DMSO at the same concentration. PKH-67 labeled Jeko-1 exosomes were added to the treated cells for 60 min. Exosome internalization was quantified by flow cytometry and analyzed by confocal microscopy. Uptake of MCL exosomes was significantly inhibited by Dynasore (88% ± 8.6 inhibition) and Gefitinib (83.8% ± 6.6 inhibition) and partially inhibited by Nystatin (62% ± 7.1 inhibition) and by Simvastatin (61.2% ± 7.1 inhibition) (Fig. 7A). Confocal microscopy analysis revealed that almost none of the cells internalized exosomes when treated by Dynasore or Nystatin (Fig. 7B).

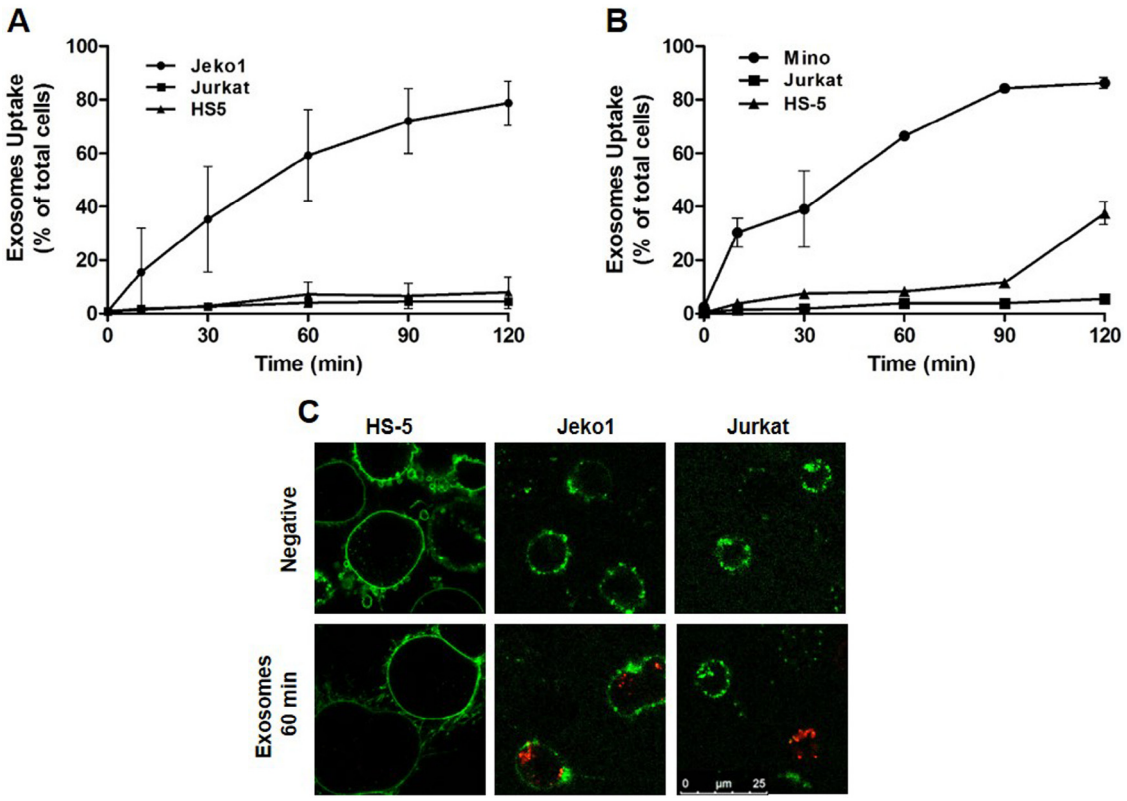

Fig. 4. MCL exosomes specifically internalized into MCL cells. (A) Time course of Jeko-1 derived exosomes internalization to Jeko-1, Jurkat and HS-5 cells. PKH-67 labeled Jeko-1 exosomes were incubated for the indicated time points with co-culture of Jeko-1, Jurkat or HS-5 cells. Internalization was measured by flow cytometry using specific antibodies (PE-anti CD19 for Jeko-1 cells, PerCP-anti CD3 for Jurkat cells and AlexaFluor 647-anti CD105 for HS-5 cells). The data present the percentage of cells that uptake labeled exosomes and are expressed as mean ± SD of three independent experiments. (B) Time course of Mino derived exosomes internalization to Mino, Jurkat and HS-5 cells. PKH-67-labeled Mino exosomes were incubated for the indicated time points with co-culture of Mino, Jurkat or HS-5 cells. Internalization was measured by flow cytometry using specific antibodies (PE-anti CD19 for Mino cells, PerCP-anti CD3 for Jurkat cells and AlexaFluor 647-anti CD105 for HS-5 cells). The data present the percentage of cells that uptake labeled exosomes and are expressed as mean ± SD of three independent experiments. (C) PKH-26 labeled Jeko-1 exosomes were incubated for 60 min with co-culture of Jeko-1, Jurkat or HS-5 cells and uptake of exosomes was analyzed by confocal microscopy. After incubation Jurkat and Jeko-1 cells were removed from the HS5 cells and the cell membrane was labeled with AlexaFluor 488-anti CD3 (Jurkat) or AlexaFluor 488-anti CD19 (Jeko-1) HS-5 cells were labeled by AlexaFluor 488-wheat germ agglutinin. Negative control – Jeko-1, Jurkat and HS-5 cells with no addition of labeled exosomes. The data are representative of three independent experiments. Scale bar is 25 μm.

At the same concentration and time points, these inhibitors did not affect the viability of the cells (94.7% ± 2.1, 92.5% ± 1.386 and 89.3% ± 3.3 live cells in the control, Dynasore- Nystatin- and Simvastatin-treated cells, respectively, Fig. S2). These results suggest that a non-classical pathway involving dynamin, tyrosine kinase and cholesterol mediated endocytosis of MCL exosomes by MCL cells.

Discussion

In this study, we have described the characterization of human MCL exosomes. Using electron microscopy analysis, Jeko-1-derived exosomes and MCL patients' cell-derived exosomes displayed a typical spherical morphology. Size distribution of isolated exosomes from primary MCL cells (94 ± 62 nm) fits the presumed size range of exosomes (30–150 nm), while exosomes derived from Jeko-1 cells were found to be larger (121 ± 87 nm). Using flow cytometry analysis, 92% of these Jeko-1-derived exosomes carry the exosomal markers CD81 and CD63 that are frequently known to be present on exosomes from other cell types [5]. Western blot analysis of exosomes-derived from Jeko-1 cells further demonstrated the expression of exosomal protein, TSG101, and was enrichment in CD81. Taken together, these results demonstrated that the MCL-derived exosomes are a relatively pure population.

Exosomes are released by most types of cells into the extracellular milieu upon fusion of the MVBs with the plasma membrane. The presence of exosomes in urine [42], circulating blood [43], ascites and cerebrospinal fluid [44] *in vivo*, together with recent data indicating that exosomes can transfer proteins, messenger RNAs (mRNAs) and microRNAs to neighboring cells and thus affect their biological activity [6], raises the question whether exosomes have target cell specificity. Previous report suggests that extracellular vesicles can be taken up by every cell type tested [45], however, others have shown cell-specific uptake [46]. Our results provide evidence for the preferential internalization of MCL exosomes by normal and malignant B-cells. This is based on several lines of experimental evidence. We observed extremely rapid internalization of Jeko-1-derived exosomes to Jeko-1 cells. Ten min post administration of exosomes we were able to quantify and visualize them within MCL cells. Internalization was linearly increased up to 60 min and reached a plateau after 120 min. When MCL exosomes (Jeko-1 or Mino) were administrated to a co-culture of MCL cell line, Jurkat and HS-5 cells, almost no detectable internalization was observed in Jurkat and HS-5 cells even after 120 min of incubation. Finally, when MCL exosomes were introduced to mononuclear cells, a mixture of lymphocytic and monocyte populations that include B-lymphocytes, NK cells and various T-lymphocytes from healthy control or MCL patients' PB, a preferential internalization into B-lymphocytes subsets was observed. These results support the hypothesis raised in this study that MCL exosomes have unique specificity to B-lymphocytes.

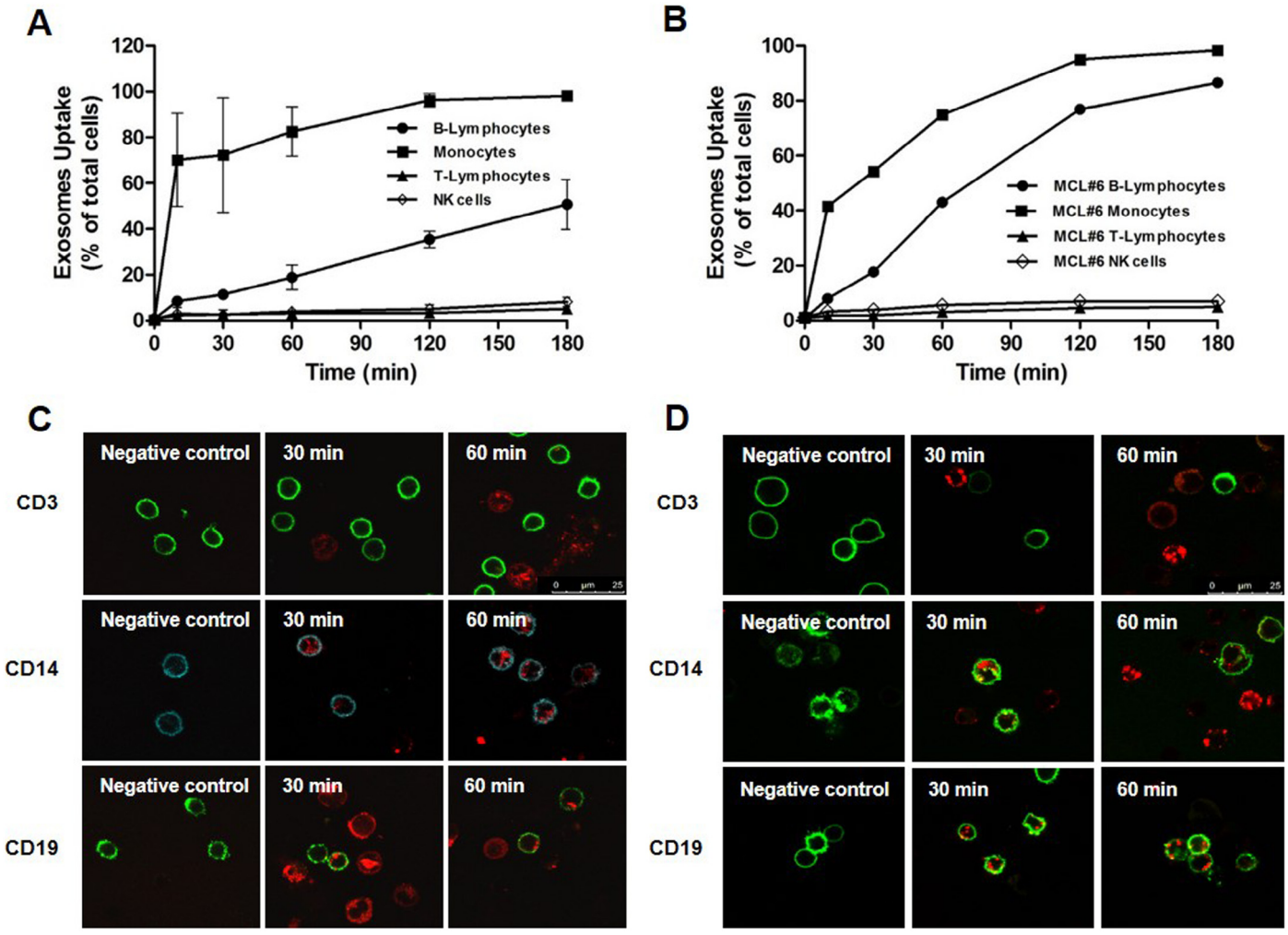

Fig. 5. Normal B-cells and MCL cells preferentially uptake MCL exosomes. Exosomes derived from MCL patient's cells (MCL5 and MCL6) were incubated for the indicated time points (0, 10, 30, 60, 120 and 180 min) with PBMCs of healthy individuals or MCL patients. Internalization of PKH-67 labeled exosomes by healthy (A) PBMCs or (B) MCL patient's PBMCs was assayed by flow cytometry using specific antibodies to T-lymphocytes (PerCP-anti CD3), B-Lymphocytes (PE-anti CD19), NK cells (APC-anti CD56) and monocytes (APC-anti CD14). The data are expressed as the percentage of cells that taken up labeled exosomes. Results of healthy PBMCs are expressed as mean ± SD of three independent controls. PKH-26 labeled MCL exosomes were incubated for 30 or 60 min with (C) healthy individuals PBMCs or (D) MCL patient's mononuclear cells. Internalization was assayed by confocal microscopy. The cell membrane was labeled with AlexaFluor 488-anti CD3 (T-lymphocytes), AlexaFluor 488-anti CD19 (B-lymphocytes) or AlexaFluor 488-anti CD14 (monocytes). Negative control – cells without addition of exosomes. The data are representative of the two independent experiments. Scale bar is 25 μm.

We have shown that monocytes of both healthy subjects and MCL patients are extremely efficient in the uptake of MCL exosomes. The different kinetics of exosome uptake by monocytes and B-lymphocytes can reflect on two different processes of exosome uptake, while monocytes phagocyte exosomes, B-lymphocytes internalized them by endocytosis. The uptake of exosomes by monocytes was previously described and occurs through phagocytosis mechanism [24]. A role for CD169 in the capture of B-cell derived exosomes by macrophages in the marginal zone of the spleen and in the sub-capsular sinus of the lymph node was recently found [47]. Although the uptake of MCL exosomes by monocytes is an effective process, we have shown that in competitive conditions when exosomes were exposed to PBMC, a substantial amount of B-lymphocytes uptake exosomes and in part of MCL patients in a similar rate as monocytes. These results further support the high affinity of B-lymphocytes to MCL exosomes. The exceeded uptake of exosomes by monocytes was previously shown for rat pancreatic adenocarcinoma exosomes, however these exosomes were uptaken by all lymphocyte subsets and no difference was observed between B and T-lymphocytes [45]. The preferential internalization of MCL exosomes by B-lymphocytes is probably based on protein–protein interaction of the B-lymphocytes and MCL exosomes, however this mechanism is unknown and is currently under investigation.

The presence of MCL derived exosomes *in vivo* was verified in the serum of MCL patients. Primary MCL-cells derived exosomes could be detected in the serum of MCL patients with high WBC count (MCL4 and MCL8) but also in the serum of patients with relatively low WBC count (MCL7). This raises the future possibility of purifying MCL derived exosomes from a patient's serum and harnessing them for the delivery of therapeutic payloads while exploiting their natural specifically toward MCL cells. Since exosomes could be taken up by monocytes as well, exosomes might be loaded with specific anti MCL molecules, such as siRNA molecules for cyclin D1, which was previously shown by us to decrease viability of MCL cells and induce cell apoptosis [48].

Finally, we explored the internalization mechanism of MCL exosomes by MCL cells. Previous studies have shown that exosomes released from Epstein–Barr virus-infected B cells are internalized via caveolae-dependent endocytosis mediated by viral gp350 protein [49].

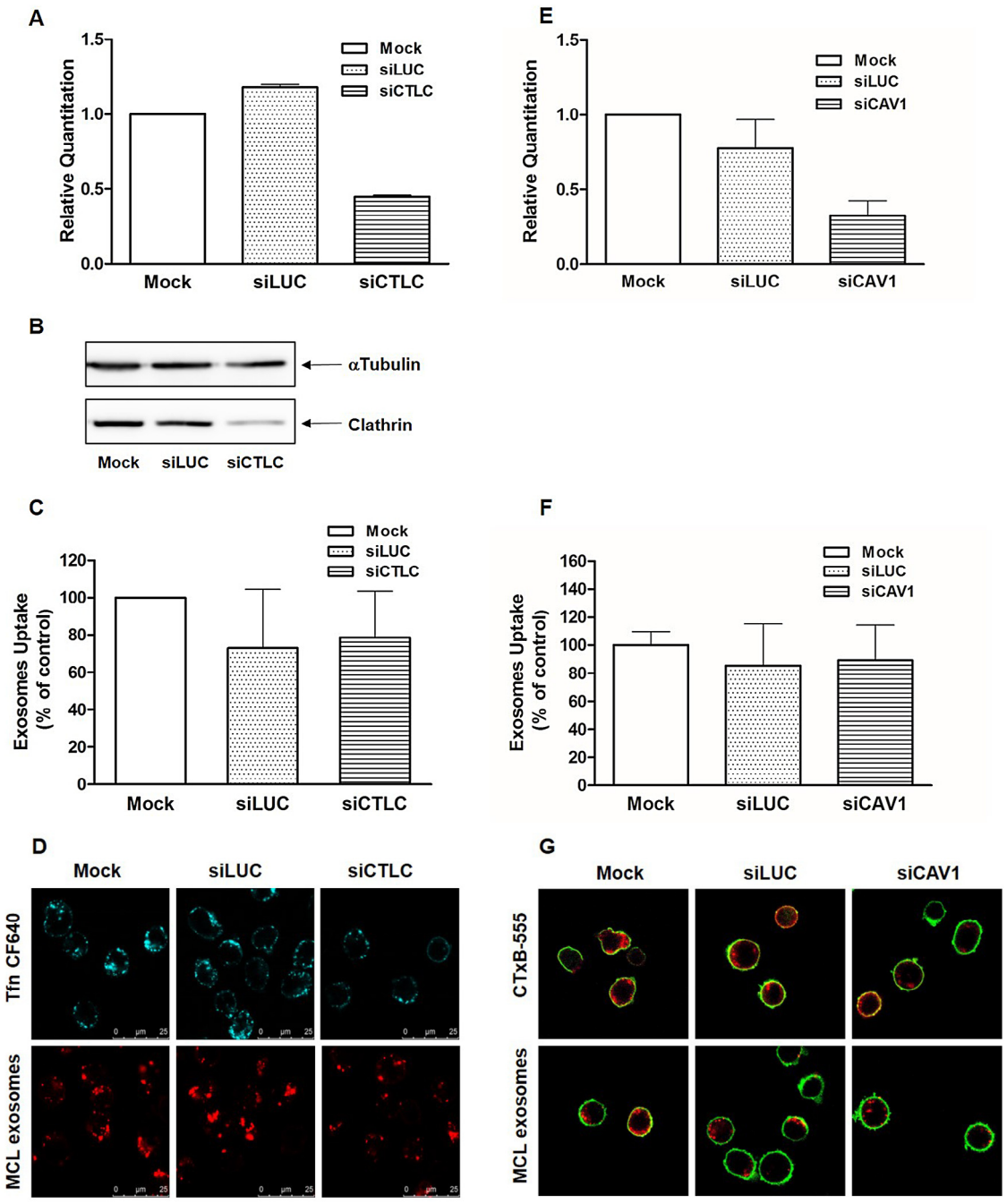

Fig. 6. MCL exosomes internalized by Clathrin and Caveolin1-independent endocytosis pathway. Clathrin heavy chain (CLTC) or Caveoin1 (CAV1) downregulation. qPCR analysis of (A) CLTC and (E) CAV1 mRNA levels 72 h post electroporation of siCLTC or siCAV1 in Jeko-1 cells. Expression was normalized to housekeeping genes eIF3a and eIF3c and depicted as mRNA concentration relative to untreated cells (Mock). The data are presented as mean ± SD of thee independent experiments. (B) Representative Western Blot analysis of Clathrin heavy chain expression 72 h post electroporation. α-Tubulin was used as a loading control. (C, F) PKH-67 labeled Jeko-1 exosomes were incubated with untreated (Mock), siLUC electroporated cells, siCLTC or siCAV1 treated cells, 72 h post electroporation for 60 min. Internalization was measured by flow cytometry analysis. The data are expressed as the percent of cells that uptake labeled exosomes as mean ± SD of three independent experiments. (D) Representative confocal microscopic images of untreated (Mock), siLUC electroporated cells or siCLTC treated cells, 72 h post electroporation, incubated with PKH-26 labeled Jeko-1 exosomes or Transferin CF640 for 60 min or 15 min, respectively. Scale bar is 25 μm. (G) Representative confocal microscopic images of untreated (Mock), siLUC or siCAV1 treated cells, 72 h post electroporation, incubated with PKH-26 labeled Jeko-1 exosomes or CholeraToxin-555 for 60 min. The cell membrane was labeled with AlexaFluor 488-anti CD19. Scale bar is 25 μm.

Internalization of MCL exosomes was not affected by knockdown of caveolin1 or clathrin, therefore a non-classical pathway, clathrin and caveolae independent mechanism mediates this process. Several endocytosis pathways are clathrin and caveolin1 independent; these include dynamin dependent mechanisms and dynamin-independent mechanisms. The inhibition of MCL endocytosis by dynasore excluded the possibility of endocytosis by macropinocytosis since this process is dynamin independent [50]. The lipid raft endocytosis is a dynamin dependent process mediated by cholesterol rich domains [51]. The inhibition of MCL exosome uptake by Nystatin and Simvastatin suggest that cholesterol/lipid raft-dependent but clathrin- and caveolin1-independent endocytosis mediates this process.

The uptake of exosomes was recently shown to be affected by lipid raft associated proteins. Internalization of exosomes derived from

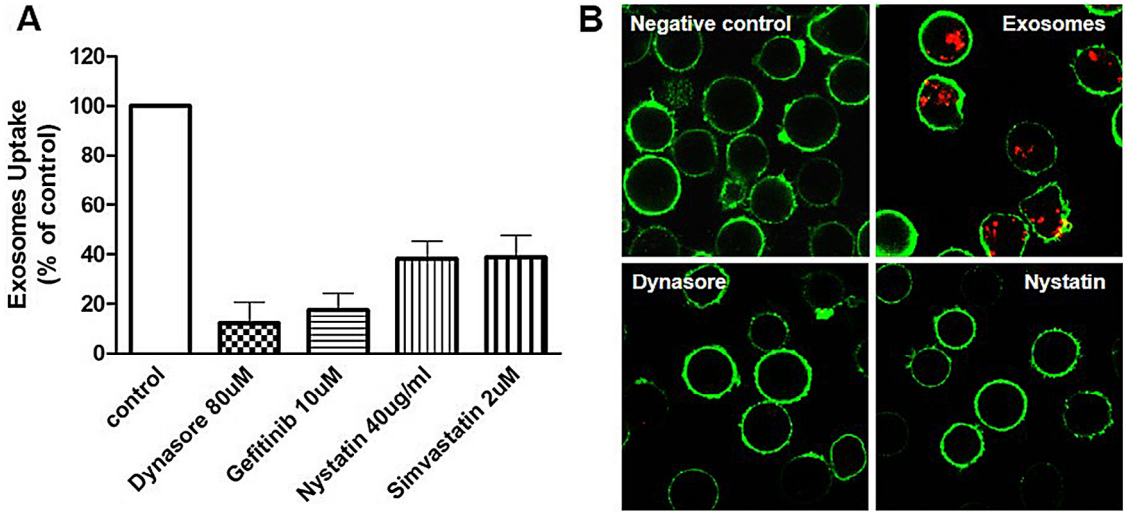

Fig. 7. MCL exosomes internalized by cholesterol dependent endocytosis pathway. (A) Jeko-1 cells were pre-treated with 10 μM Gefitinib, 80 μM Dynasore, or 40 μg/ml Nystatin for 3h 30 min and 60 min, respectively. For cholesterol synthesis Jeko-1 cells were cultured in serum free RPMI media supplemented with 0.1% BSA and 20 mM Hepes in the presence of 2uM Simvastatin for 16 h. PKH-67 labeled Jeko-1 exosomes were incubated with untreated (control) or treated cells for 60 min and internalization was measured by flow cytometry analysis. The data are expressed as the percent of cells that uptake labeled exosomes as mean ± SD of three independent experiments. (B) PKH-26 labeled Jeko-1 exosomes were incubated with untreated (control) or treated cells (Dynasore or Nystatin) and internalization was measured by confocal microscopy. Jeko-1 cell membrane was labeled with AlexaFluor 488-anti CD44. Scale bar is 25μm.

glioblastoma cells is negatively regulated by lipid raft-associated protein caveolin-1 and induces the phosphorylation of several downstream targets known to associate with lipid rafts, such as extracellular signal-regulated kinase-1/2 and heat shock protein 27 [52]. This raised the possibility that selective uptake of MCL-derived exosomes by B-Lymphocytes, as demonstrated in our study, contributes to proteins associated with cholesterol/lipid raft microdomains.

To summarize, in the present study, we characterized MCL exosomes derived from cell line and primary MCL cells and from the serum of MCL patients. We then showed for the first time the cell-specific uptake of MCL exosomes by normal and MCL patients' B-lymphocytes in a lipid raft-dependent manner. These findings can be utilized for therapeutic and imaging of MCL patients. Exosomes will be purified from an MCL patient's cells, molecularly labeled or loaded with therapeutic payloads and will be administered back to the same patient.

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Conflict of interest

D.P. has financial interests in Quiet Therapeutics Ltd.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.04.026.

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