

Uptake of lymphoma-derived exosomes by peripheral blood leukocytes

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Abstract: Exosomes are nanosized lipid vesicles secreted into blood and other body fluids and serve as vehicles for intercellular communication. Despite being an important component of the tumor microenvironment (TME), exosomal targeting and uptake into recipient cells are still not fully understood. Few studies have looked at lymphoma exosomes and their interactions with circulating blood cells. In this study, we examine the exosomal uptake distribution among peripheral blood leukocytes (PBLs) using vesicles derived from a diffuse large B cell lymphoma cell line, WSU-DLCL2. Lymphoma cells survive, proliferate, and are protected from the cytotoxic effects of chemotherapeutic agents by soluble factors or by direct contact with inflammatory and stromal cells within the TME. In an attempt to close the gap in knowledge concerning lymphoma TME immunosuppression, we have treated normal human PBLs with PKH67-labeled lymphoma exosomes and monitored the uptake by measuring fluorescence at different time points using flow cytometry and fluorescent microscopy. Our results show that of the four populations examined, B cells and monocytes demonstrated uptake of PKH67-labeled exosomes, while T cells and NK cells displayed significantly less uptake.

Keywords: exosome, non-Hodgkin's lymphoma, B cell

Introduction

Diffuse large B cell lymphoma (DLCL) is an intermediate grade and the most common form of non-Hodgkin's lymphoma (NHL), affecting 40–50% of adult lymphoma patients in the US.¹ Chemokines, cytokines, and growth factors are critical for the growth and survival of these malignant B cells.² In addition, specific oncogenes such as c-Myc have been shown to have proliferation regulatory ability in malignant B cells both *ex vivo* and *in vivo*.³ The cross talk between the tumor microenvironment (TME) and the DLCL cells is mainly mediated by direct cell-to-cell interactions but has recently been shown to be facilitated through extracellular vesicle-trafficked soluble factors.^{4,5}

Exosomes are small 30–150 nm sized extracellular vesicles important in the intercellular communication between cells.^{6–9} Communication can occur both by transfer of nucleic acids and proteins and by binding cell surface receptors and inducing cell signaling pathways. Both normal and tumor cells release exosomes, although tumor-derived exosomes (TEXs) have been the subject of a wide range of studies. TEXs have been shown to be involved in many aspects of the TME, including immune suppression,^{10,11} antigen presentation,^{12–16} a means of acquiring chemotherapeutic resistance,^{17–21} as biomarker reservoirs,^{22–28} inducers of angiogenesis,^{29–31} and vehicles of niche preparation for metastasis.^{32–36}

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However, the modes and mechanisms of uptake are not completely understood. Cells appear to internalize exosomes through several endocytic pathways, including clathrin- and caveolin-dependent endocytosis, phagocytosis, and lipid raft-mediated internalization. It is likely that cells utilize multiple routes to take up exosomes, depending on the proteins, glycoproteins, and lipids found on the surface of the vesicles and the target cell itself.³⁷ Numerous studies show proficient uptake of TEXs by endothelial cells,^{38–40} epithelial cells,⁴¹ fibroblasts,⁴² myeloid precursors in bone marrow,^{35,40} mesenchymal stem cells,⁴⁰ and other tumor cells.⁴³

There have been few studies investigating uptake of exosomes by peripheral blood cell populations. Zech et al⁴⁴ found that rat pancreatic adenocarcinoma exosomes could be taken up by all leukocyte subpopulations examined, with CD11b+ cells demonstrating higher internalization than T or B cells. At this time, there is only one other publication addressing peripheral blood uptake of lymphoma exosomes – a study by Hazan-Halevy et al⁴⁵ looking at mantle cell lymphoma exosomes and their preferential uptake by B-lymphocytes. DLCL, an aggressive form of lymphoma representing >40% of adult lymphoma patients, has not been investigated. It is therefore important to investigate these interactions between the lymphoma cells and the TME in order to find and exploit new prognostic factors and to design new therapeutic approaches.

Methods

Cell culture

Human lymphoma cell lines WSU-DLCL2 and WSU-FSCCL were developed at Wayne State University and are Epstein–Barr virus-negative.⁴⁶ Cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 10% United States Department of Agriculture-sourced heat-inactivated fetal bovine serum (FBS; Mediatech, Manassas, VA, USA), 4 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 units/mL penicillin and incubated at 37°C and 5% CO₂. Trypan blue staining was used to measure cell density (confluent at 1×10⁶/mL) and viability (>90%).

Peripheral blood from healthy apheresis blood donors were obtained from the Life Stream Blood Bank (San Bernardino, CA, USA) according to our approved Loma Linda University institutional review board (IRB) protocols. The red blood cells were lysed using an ACK lysis buffer containing 8.3 g/L NH₄Cl, 1 g/L KHCO₃, and 1.8 mL 5% EDTA and centrifuged for 5 minutes at 1,500 rpm at 4°C in a Beckman Coulter Allegra X-15R centrifuge, equipped with an SX4750 rotor to obtain a pellet of peripheral blood leukocytes (PBLs). These PBLs were allowed to rest overnight before exosome treatment in 5×10⁶/mL complete RPMI, with or without

100 IU/mL IL-2. This study, in its entirety, was approved by Loma Linda University's IRB.

Exosome isolation

Lymphoma cells were cultured for 24 hours in media depleted of exosomes from FBS (Hyclone Laboratories, Inc., South Logan, Utah, USA) by overnight ultracentrifugation at 100,000× *g*. This conditioned medium was subjected to serial centrifugation, removing cells (300× *g*, 5 minutes) and removing noncellular debris (2,000× *g* for 10 minutes). The supernatant was then centrifuged at 10,000× *g* for 30 minutes. Exosomes were isolated using the commercially available ExoQuick-TC™ (System Biosciences, Mountain View, CA, USA) at a 1:5 ratio of reagent to conditioned medium and incubated overnight at 4°C. A low speed spin at 1,500× *g* for 30 minutes was sufficient to pellet the precipitated vesicles. Exosome pellets were resuspended in 40–70 μL PBS and protein quantified by bicinchoninic acid assay protein assay (#23225; Pierce/Thermo Scientific, Rockford, IL, USA). Size of the vesicles was examined using dynamic light scattering (DLS) with a Nicomp N3000 nanoparticle sizing instrument (Particle Sizing Systems, Port Richey, FL, USA).

Uptake of exosomes

The exosome pellet, obtained from ExoQuick isolation, was labeled with PKH67 Green Fluorescent Cell Linker Kit (Sigma Aldrich; Saint Louis, MO, USA) as per manufacturer's protocol, with modifications. Briefly, exosomes in PBS corresponding to 200 μg were added to 250 μL of diluent C. As a control, the same volume of PBS was also added to 250 μL of diluent C (no exosome control) and processed in parallel. The exosome suspension was added to an equal volume of 2× PKH67 dye mixture and mixed well for 4 minutes. The dye reaction was stopped by addition of 9 mL of media depleted of bovine exosomes by ultracentrifugation and then spun for 90 minutes at 110,000× *g* using an SW41 rotor. The pellet was washed in PBS with a second ultracentrifugation. The PKH67-labeled vesicles were incubated either with PBLs for 1, 4, or 24 hours for dose–curve experiments or with NK cells for 30, 60, or 240 minutes. Cells were washed with PBS, stained with surface marker antibodies, and fixed in 2% paraformaldehyde before proceeding with further analysis by flow cytometry or microscopy.

Flow cytometry

Antibodies directed against the following markers and directly labeled with indicated fluorophore were used to stain PBLs for flow cytometry analysis: CD3-PE, CD1a-PE (Becton, Dickinson [BD] Biosciences, San Diego, CA, USA); CD14-antigen-presenting cells (APC, M5E2; BioLegend;

San Diego, CA, USA); CD56-APC (MY31; Tonbo; San Diego, CA, USA); and CD19-APC (H1B19). Live cell exosome binding was distinguished from dead cells using fixable viability dye eFluor 780 (eBioscience; San Diego, CA, USA). Cells were run on MACSQuant Analyzer (Miltenyi Biotec; Bergisch Gladbach, Germany) and data analyzed using FlowJo software (Tree Star; Ashland, OR, USA). The percentage of cells positive for PKH67+ exosomes was determined for each cell population. The gating strategy used to assess uptake of PKH67-labeled exosomes is shown in Figure S2.

Microscopy

Samples were spun onto poly-L-lysine slides using StatSpin CytoFuge 2 (Beckman Coulter; Brea, CA USA) set at 800 rpm for 4 minutes. One drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) stain to visualize nuclear structures (Vectashield, Vector Laboratories, Burlingame, CA, USA) was placed onto the glass slide before adding glass coverslip and sealing with nail polish. Slides were imaged using a fluorescence light microscope (BIOREVO BZ7000; Keyence; Osaka, Japan) and a Zeiss LSM 710 NLO confocal microscope.

Statistical analysis

All the quantitative data of this study were expressed as mean \pm SD, and statistical analysis was conducted using GraphPad Prism software v.5.01 for Windows (San Diego, CA, USA). To test for statistical significance, nonparametric two-tailed Mann–Whitney analysis was performed. Comparisons between groups were performed using Student's *t*-test with probability $p < 0.05$ considered to indicate a statistically significant difference. Each experiment was repeated at least twice to assess the level of reproducibility.

Results

Cellular uptake characterization of DLCL2 exosomes

Exosome uptake by peripheral blood cells was measured using flow cytometry and fluorescent microscopy, with demonstrated uptake differing between cell lineages and in a dose- and time-dependent manner. Specifically, to study the uptake of lymphoma-derived exosomes by PBLs, vesicles were isolated from the conditioned media from the WSU-DLCL2 cell line. Vesicle size was evaluated by DLS and confirmed to be consistently in the reported range of exosomes (30–150 nm; Figure S1).

The exosomes were labeled with a lipophilic dye, PKH67, after which 25, 50, 100, 200, or 400 μ g of these exosomes were

incubated with PBLs for 1, 4, and 24 hours. Internalization by confocal microscopy was performed and analyzed. Exosomal internalization was observed as early as 1 hour postincubation with longer incubation times and higher concentrations resulting in higher accumulation of exosomes inside the cells (Figure 1).

To study the kinetics of exosome accumulation, we performed quantitative flow cytometry. PKH67-labeled DLCL2 exosomes were incubated with PBLs, and the fluorescence intensity was detected. Uptake was most prominent by B cells and myeloid-derived cells and less so in T cells and NK cells (Figure 2). The uptake of exosomes from healthy B cells was rapid in the higher exosome concentrations, with 28% positive after 1 hour at 400 μ g/mL (Figure 2A and B) and increasing to 69% at 24 hours at 400 μ g/mL (Figure 2B). Cell specificity for uptake of DLCL2-derived exosomes was further studied in a single experiment using B cells (CD19+), T cells (CD3+), NK cells (CD56+), and monocytes (CD14+; Figure 2C and S3). Compared to B cells where 28% to 70% of cells showed exosome uptake in the 400 μ g/mL exosomes over the 24-hour study, NK cells, monocytes, and T cells only maximized 6%, 8%, and 3% uptake, respectively (Figure 2C and S3). After a second independent experiment was concluded, CD19+ B cells and CD14+ monocytes maximized nearly 40% in the 400 μ g/mL incubation while CD3+ T cells and CD 56+ NK cells only proved to be able to uptake nearly 10% (Figure 2D).

The specificity of DLCL2 exosomes was further tested using exosomes derived from FSCCL and HeLa cells. Coculture of B cells and NK cells with 200 mg/mL PKH67-labeled DLCL2, FSCCL, and HeLa-derived exosomes for 1-hour

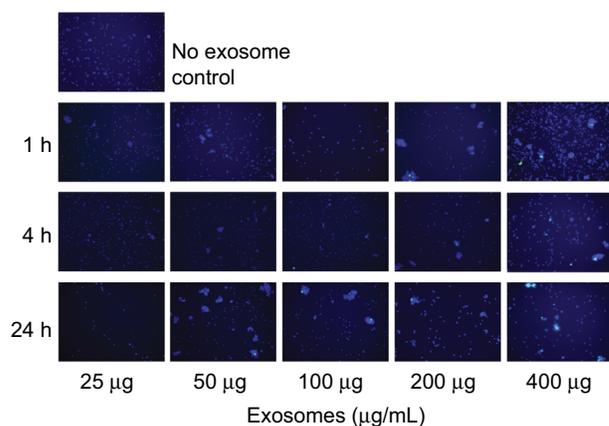


Figure 1 Peripheral blood leukocytes take up DLCL2 exosomes.

Notes: Peripheral blood leukocytes were treated with DLCL2 exosomes (25–400 μ g/mL) and harvested at 1, 4, and 24 hours. Cells were cytospun onto poly-L-lysine-coated slides. Microscopy images acquired with BIOREVO BZ7000 fluorescent microscope (Keyence), 20 \times magnification. Nucleic acids are stained with DAPI (blue), and exosomes bind and internalization is visualized with PKH67 (green). Cells without the addition of exosomes were used as a negative control. Several fields were analyzed for each labeling condition, and representative results are presented. The data are representative of two independent experiments.

Abbreviations: DLCL, diffuse large B cell lymphoma; DAPI, 4',6-diamidino-2-phenylindole; h, hour.

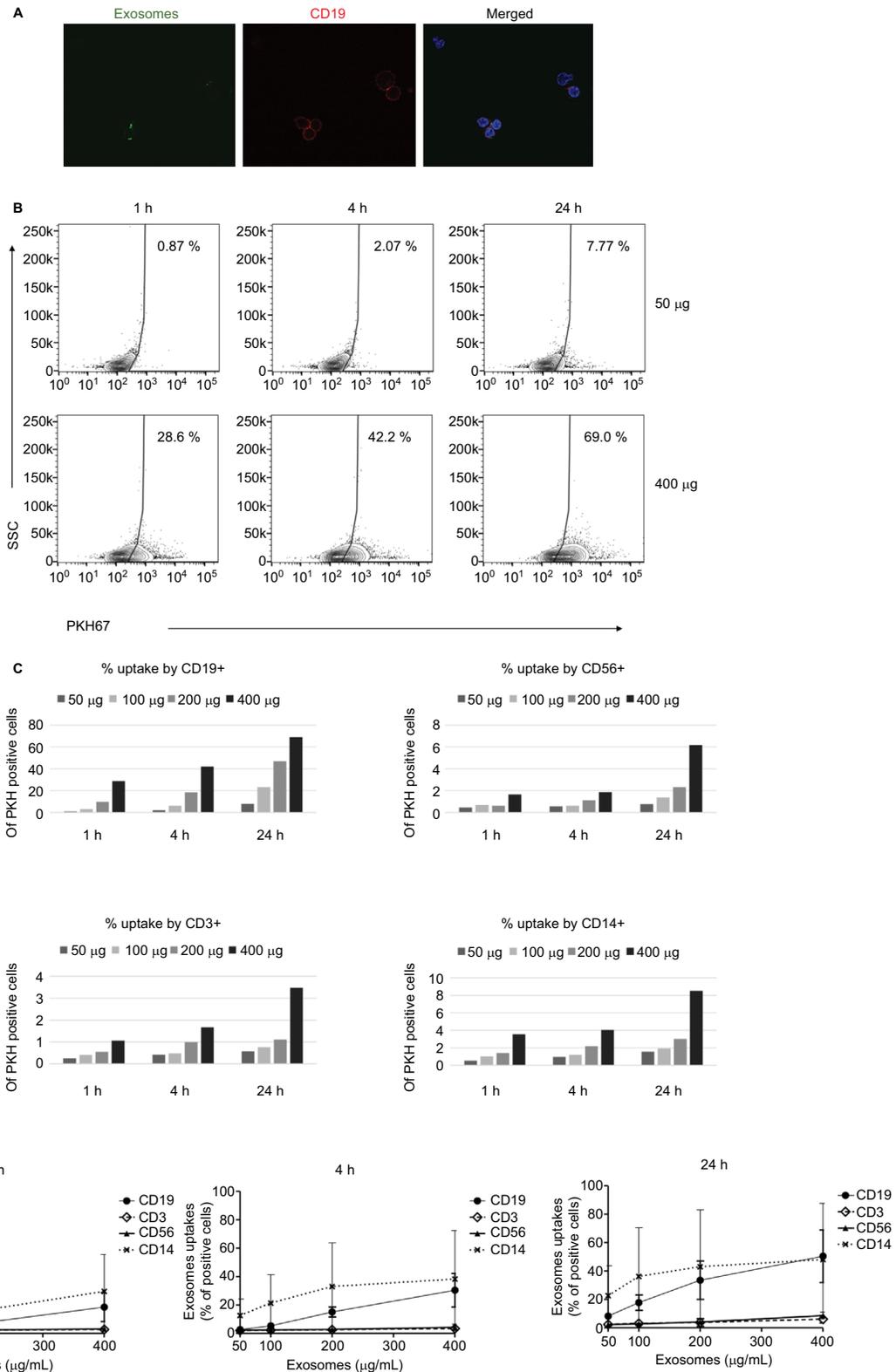


Figure 2 Uptake of PKH67-labeled exosomes in a time- and dose-dependent manner.

Notes: DLCL2 exosomes were labeled with PKH67 and then added to peripheral blood cells for various lengths of time (1, 4, and 24 hours) and treatment amounts (25, 50, 100, 200, and 400 µg/mL). As measured by flow cytometry, the uptake of labeled exosomes proceeded in a time- and dose-dependent manner. **(A)** Uptake of PKH67-labeled exosomes after 4 hours by CD19-APC cells (red). Microscopy images acquired with Zeiss LSM 710 NLO confocal microscope, 60× magnification. **(B)** Representative flow cytometry data of CD19+ cells. **(C)** Graphical representation of the percentage of PKH+ cells in each of the four lineages derived from one donor: B cells (CD19+), monocytes (CD14+), NK cells (CD56+), and T cells (CD3+). **(D)** Combined data from two separate experiments depicting the disparity in uptake between each cell population. Results are expressed as mean ± SD.

Abbreviations: DLCL, diffuse large B cell lymphoma; APC, antigen-presenting cells; h, hours.

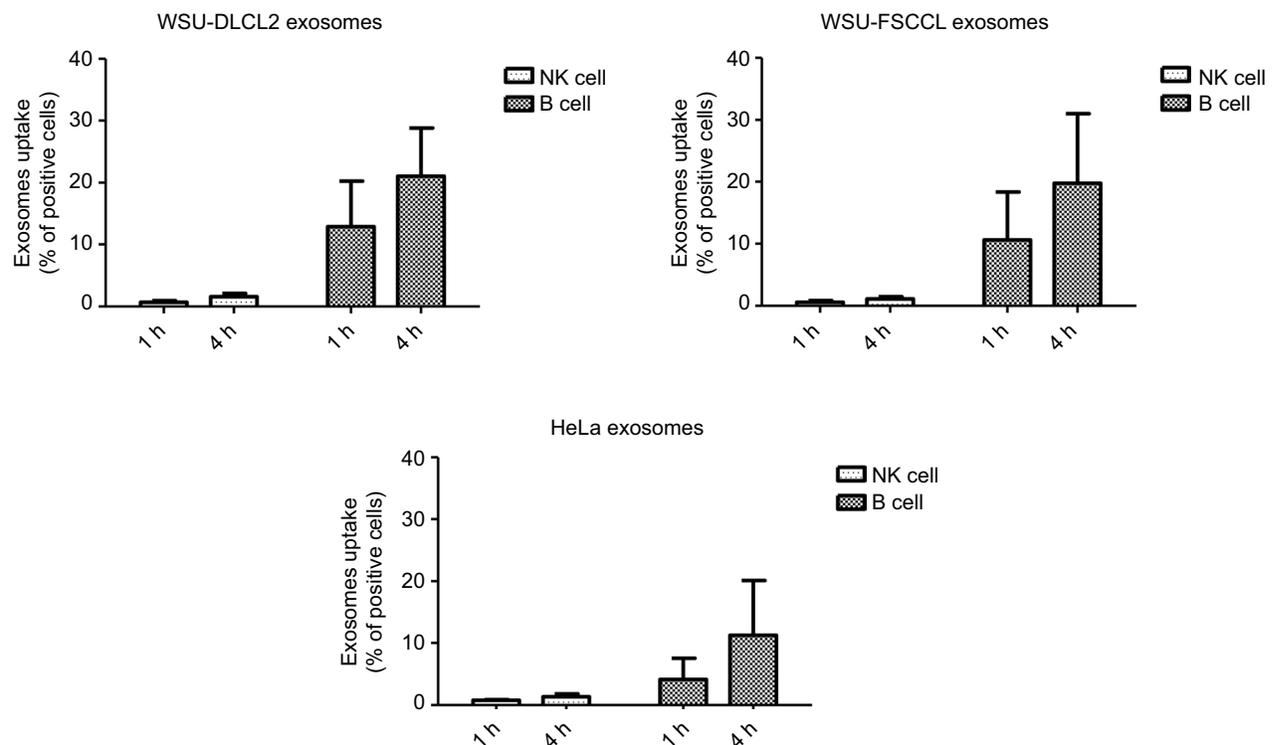


Figure 3 Differences in uptake by NK cells and B cells are not unique to DLCL2 exosomes.

Notes: DLCL2, FSCCL, and HeLa cell-derived exosomes were labeled with PKH67 and then added to peripheral blood for 1 and 4 hours at 200 $\mu\text{g}/\text{mL}$. As measured by flow cytometry, the uptake of labeled exosomes proceeded in a time- and dose-dependent manner as before in lymphocyte lineage B cells but not the NK cells.

Abbreviations: DLCL, diffuse large B cell lymphoma; h, hours.

and 4-hour time points showed very similar results to those previously recorded using DLCL2 exosome (Figure 3). In this study, B cells were able to rapidly and preferentially take up PKH67-stained DLCL2, FSCCL, and HeLa cell-derived exosomes in a time-dependent manner, while the NK cells were significantly less capable of this internalization. Four hours postincubation, 22% \pm 8, 20% \pm 10, and 12% \pm 9 of B cells had taken up DLCL2, FSCCL, and HeLa exosomes, respectively (Figure 3).

Discussion

Although the spleen, liver, and lymph nodes take up the majority of exosomes produced by organs and hematopoietic cells, plasma and other body fluids still contain large quantities of exosomes.⁴⁷ Cancer cells in particular secrete large quantities of TEXs, which can be found in peripherally circulating blood.⁴⁸ Consequently, blood cells are exposed to many exosomes from both normal and malignant cells,⁴⁹ which play key roles in modulating the immune system.⁸

While previous studies have investigated exosomal interactions with leukocytes in lymphoid organs, such as APCs in the spleen⁵⁰ and follicular dendritic cells (DCs) in the

lymph nodes,^{51,52} there is less work done regarding uptake of exosomes by peripheral blood cell populations.

In this study, we tested the hypothesis that some populations of white blood cells will be more receptive to interact with B cell lymphoma exosomes and, therefore, will be more vulnerable to TME modulating effects of these vesicles. In an attempt to close the gap in knowledge concerning lymphoma TME immunosuppression, we have treated normal human PBLs with PKH67-labeled lymphoma exosomes and monitored uptake by measuring fluorescence at different time points using flow cytometry and fluorescent microscopy. We expected to observe a disparity in exosome uptake between blood cells of lymphoid and myeloid lineages that we hypothesized was perhaps due to myeloid-derived cells such as monocytes and macrophages, being better equipped for exosome uptake than lymphocytes such as B cells, T cells, and NK cells. However, this was not what we observed with B cells being the most effective and efficient at TEX uptake.

Our data are not consistent with those of previous studies, which show a disparity in uptake between myeloid-derived cells and lymphocytes.^{44,53} We demonstrated that NK and T cells have lower levels of internalization than monocytes and

B cells. In our study model, B cells showed a high level of uptake, possibly due to the B cell origin of the lymphoma exosomes, supporting the idea of uptake specificity given the exosomes were collected primarily from cells originating from B cell lymphoma disease. These findings are in line with those of Hazan-Halevy et al⁴⁵ and Gutzeit et al⁵⁴ concerning B cells and Riches et al⁵⁵ in their work with breast tissue. In this study, we observed that DLCL2 exosomes were taken up rapidly and preferentially into CD19+ B lymphocytes and CD14+ monocytes. Only a small percentage of T cells and NK cells showed internalization or binding to exosomes, even after 24-hour incubations. We have further investigated to see if the origin of the exosome would influence the preference or rate of uptake on the peripheral cell (Figure 3). Exosomes were taken from two lymphoma cell lines (DLCL2 and FSCCL) and from the cervical cancer cell line HeLa. In our hands there was little difference recorded in exosomal uptake. This may indicate that the uptake is controlled by something specific to the exosome rather than the cell of origin.

The mechanisms of uptake into B lymphocytes remain to be elucidated, whether it be caveolin, clathrin, cholesterol, lipid-raft, or receptor-mediated endocytosis, or something completely novel and not proposed. In this work, we have demonstrated a natural preference of TEXs to B cells, further supporting the concept of targeting therapy to this lymphocyte population. However, to fully appreciate and dissect the mechanism, many more cell line-derived as well as patient-procured exosomes, from varying pathologies, will need to be investigated, a process that has only just begun in our laboratory. Understanding the structure and marker/receptor profiles on the exosome, the cell of origin as well as the recipient cell's membranes and the protein, RNA, and DNA contents from within the exosome will further the ability to regulate the role of TEXs in the pathobiology of hematologic malignancies and to identify novel therapeutic approaches.

In addition to the indirect effects of exosomes through interactions with APCs, and the limited ability of NK cells and T cells for exosome uptake, these cell populations seem to have a wide variety of responses to direct exposure to exosomes. Whether these responses are the result of surface interactions rather than uptake or due to secondary effects from other cells which more readily internalize exosomes is not always apparent. There has been evidence for both possibilities, and it is likely that exosomes interacting with lymphocytes deliver signals by direct surface contact more frequently than internalization.^{42,56} The surface interactions

between exosomes and recipient cells can occur via membrane-bound activating or inhibitory proteins that directly signal through relevant receptors and initiating downstream pathways. TEXs are enriched in proteins specialized for surface interactions, such as integrins, MHC class I and II molecules, co-stimulatory molecules (CD40, CD86), various growth factor receptors, such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER-2), death receptor ligands such as Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and programmed cell death ligand 1 (PDL-1) and inhibitory factors such as prostaglandin E2 (PGE2). Therefore, uptake is not necessary for a cell to be altered by exosomes, and may likely be the case for changes induced by exosomes in T cells and NK cells. In fact, much of the immune suppression mediated by TEXs occurs through surface molecules such as FasL, TGF- β 1, and IL-10.⁵⁷⁻⁵⁹

Perhaps an equally important aspect could be that exosome uptake has downstream direct and indirect effects on PBLs. Through producing cytokines like TNF α , presenting antigen on MHC I and II, and providing costimulatory signals via CD80/CD86 and CD40,^{53,60} many of the exosomal effects observed in T and NK cells may be an indirect result from primary changes induced in macrophages, monocytes, B cells, and DCs, which actually internalize the vesicles. Uptake of exosomes induces monocytes to produce TNF α ,⁵³ macrophages and DCs capture and present antigen to T cells,⁶⁰ and B cells can be activated by antigen-carrying exosomes with subsequent Th1 cell stimulation.⁶¹ Antigen-bearing TEXs seem to require uptake and processing by DCs before they can efficiently stimulate a specific cytotoxic T lymphocyte response.^{12,62} The exosomes from DCs carry MHC I, MHC II, CD80, and CD86 and are therefore equipped to elicit T cell activation through surface interactions.⁶³

To summarize, in the present study, we characterized DLCL2 cell line-derived exosomes on different PBL populations showing that there is preference of targeted uptake. However, it is still not clear whether exosomes are still inducing cellular signaling pathways in the T cell and NK cells through direct cell to cell contact. From these findings, we hope a better understanding of tumor cell/TME communication may result, further leading to increased knowledge of how the tumor cells communicate with and manipulate the TME. By better understanding these signaling pathways, we may better prepare therapeutic modalities to enhance immune cell surveillance and killing of these tumors, which up to now seem immune.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

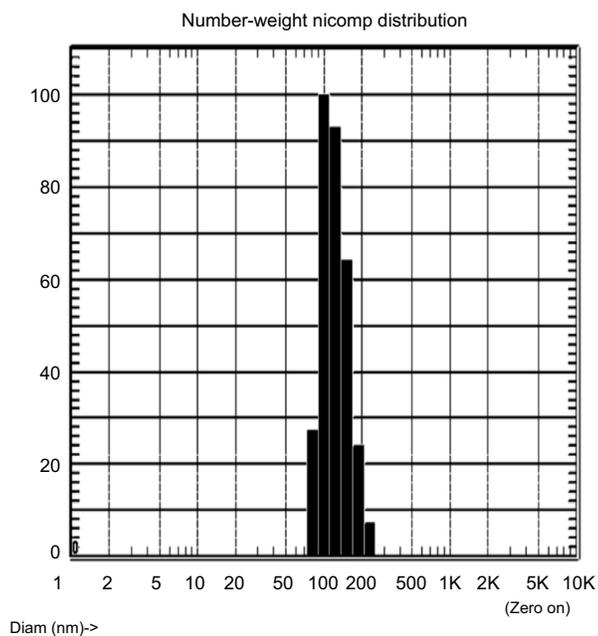


Figure S1 Size determination of WSU-DLCL2 exosomes.

Notes: Number-weight distribution of EV size using dynamic light scattering analysis with Nicomp BZ3000 instrument. Sample was read every minute for 30 minutes, with a calculated average diameter of 117 nm. One representative diameter histogram is shown.

Abbreviations: DLCL, diffuse large B cell lymphoma; Diam, Diameter; EV, extracellular vesicle.

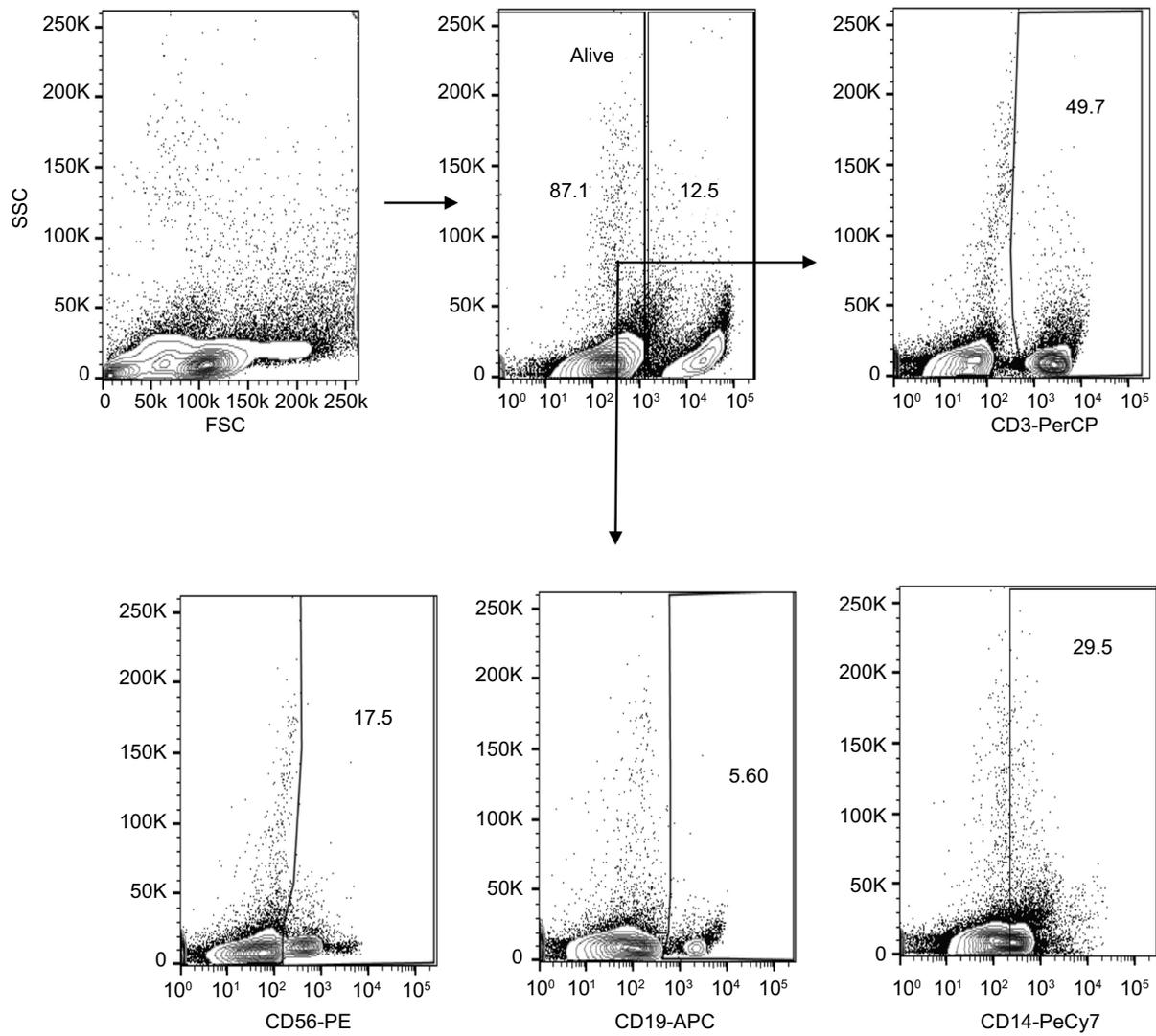


Figure S2 Gating strategy used to assess binding of PKH67-labeled exosomes to human T cells (CD3+), NK cells (CD56+), B cells (CD19+), and monocytes (CD14+) within the peripheral blood leukocytes.

Note: One representative dot plot is shown from two independent experiments.

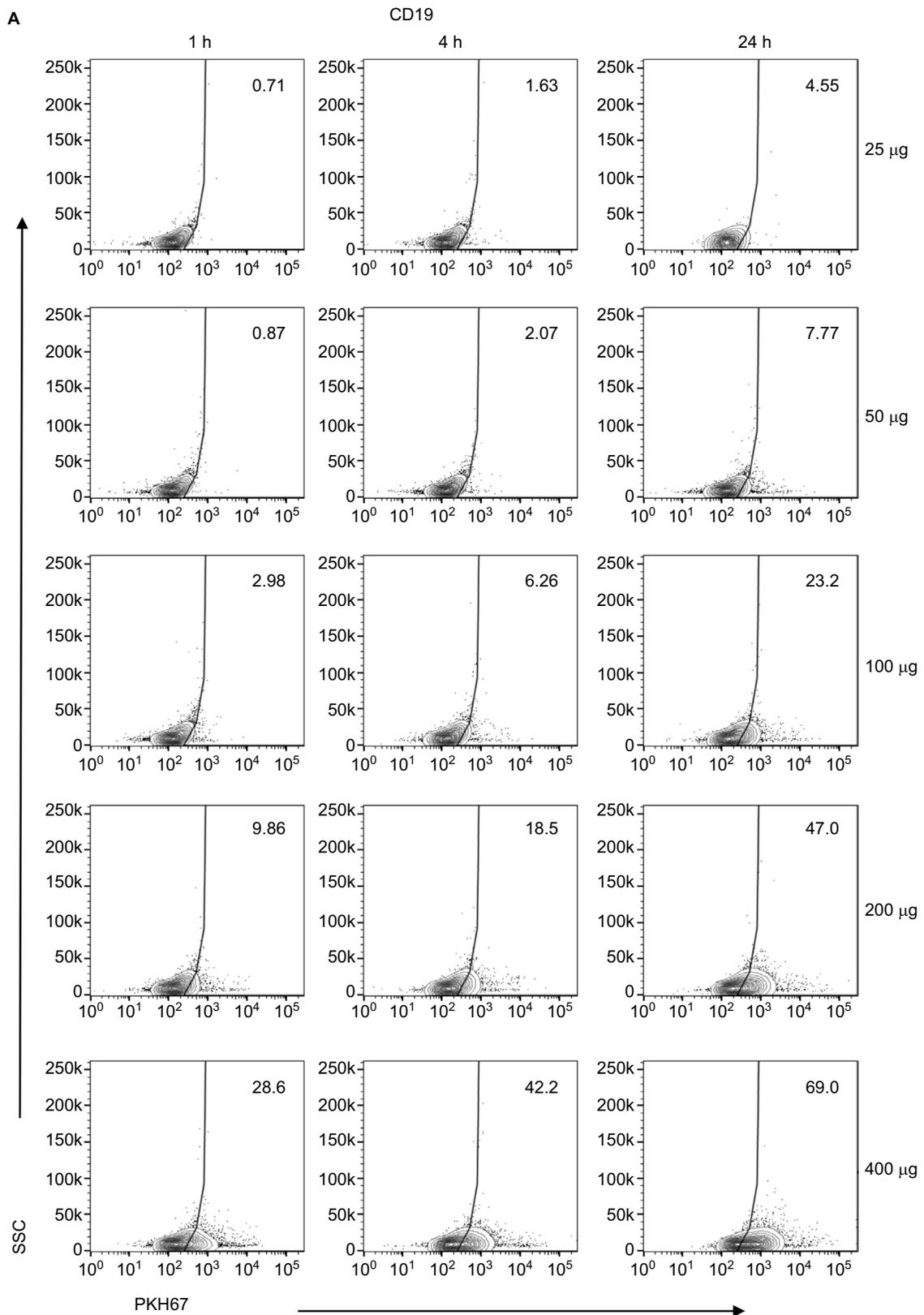


Figure S3 (Continued)

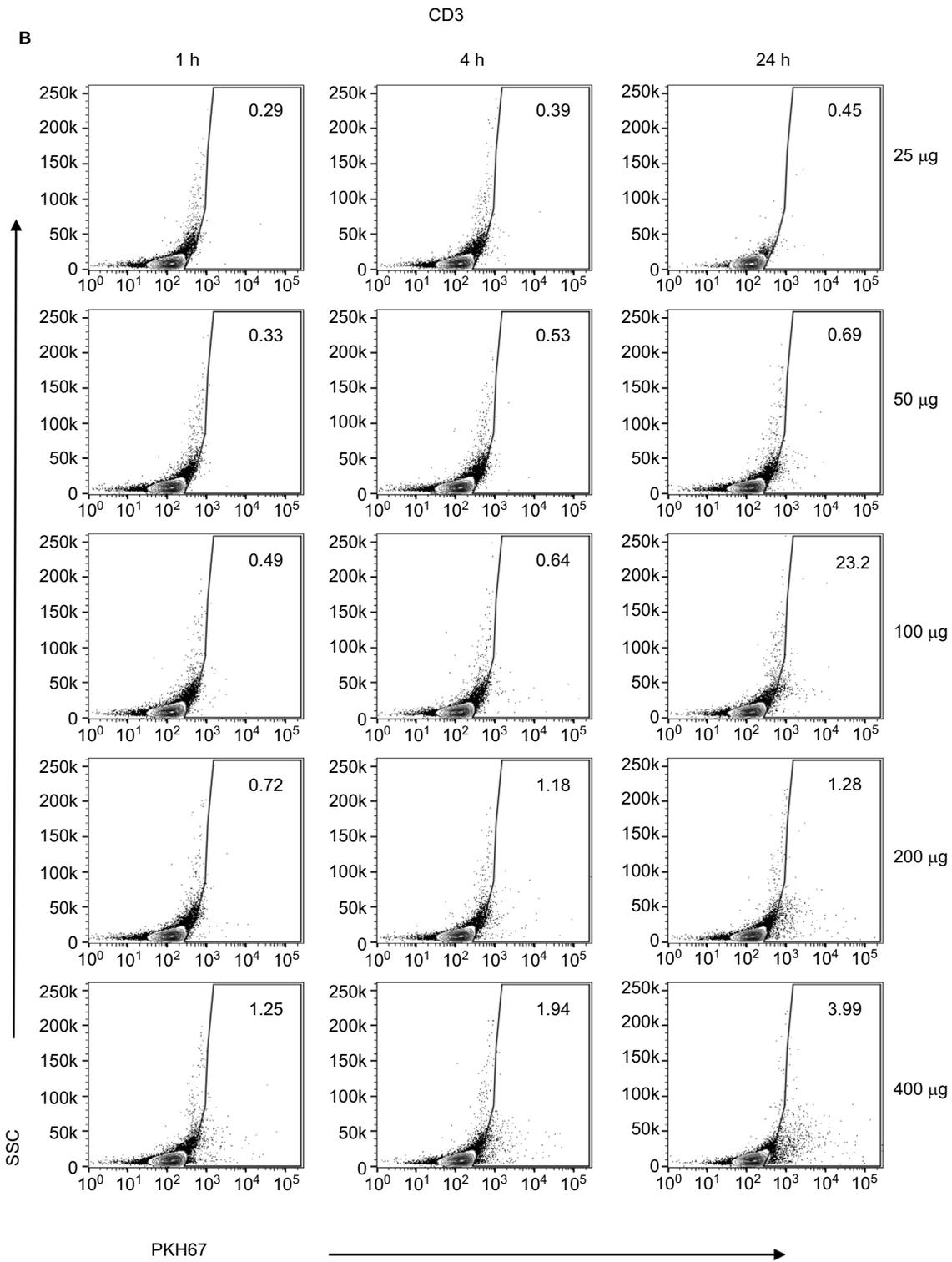


Figure S3 (Continued)

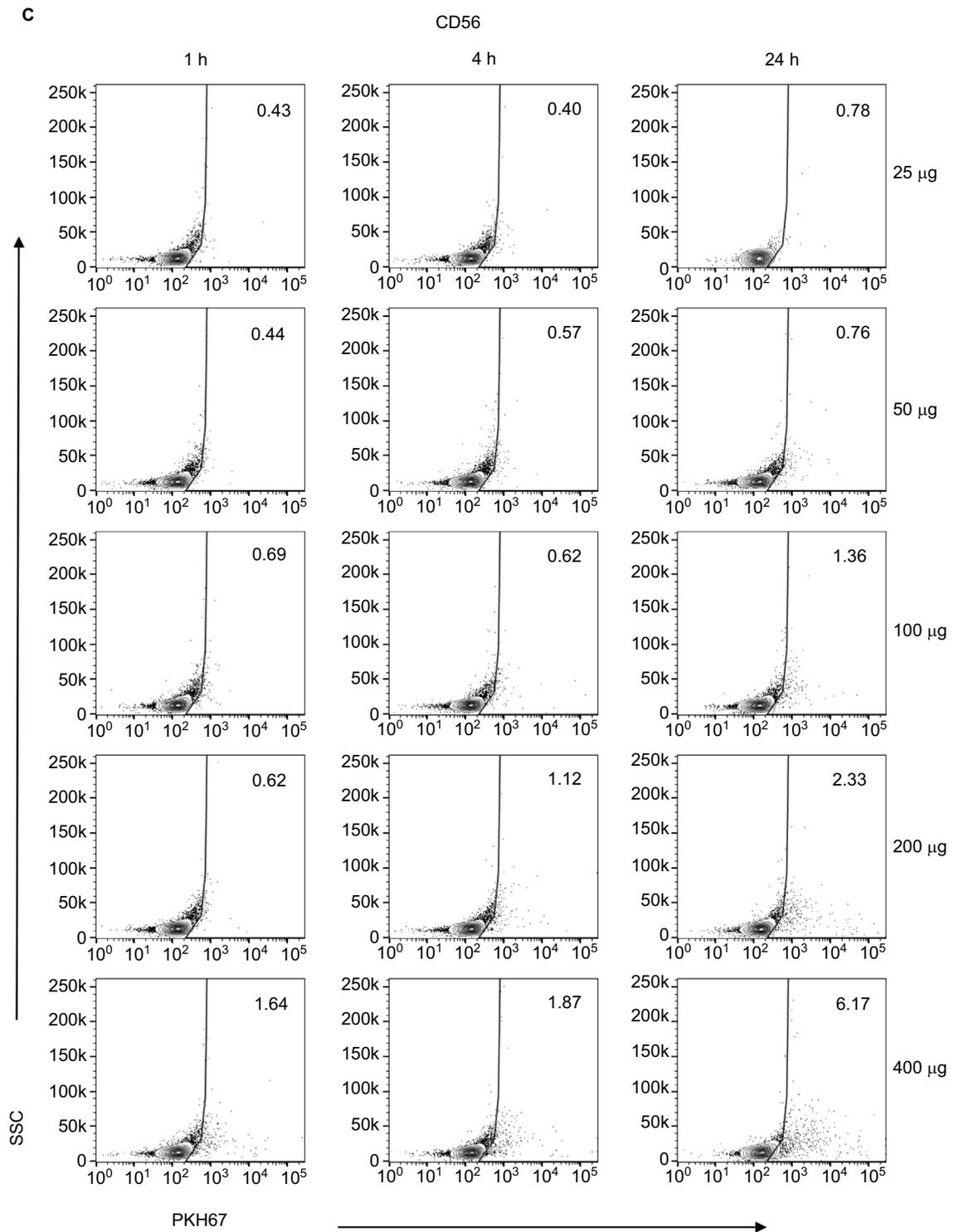


Figure S3 (Continued)

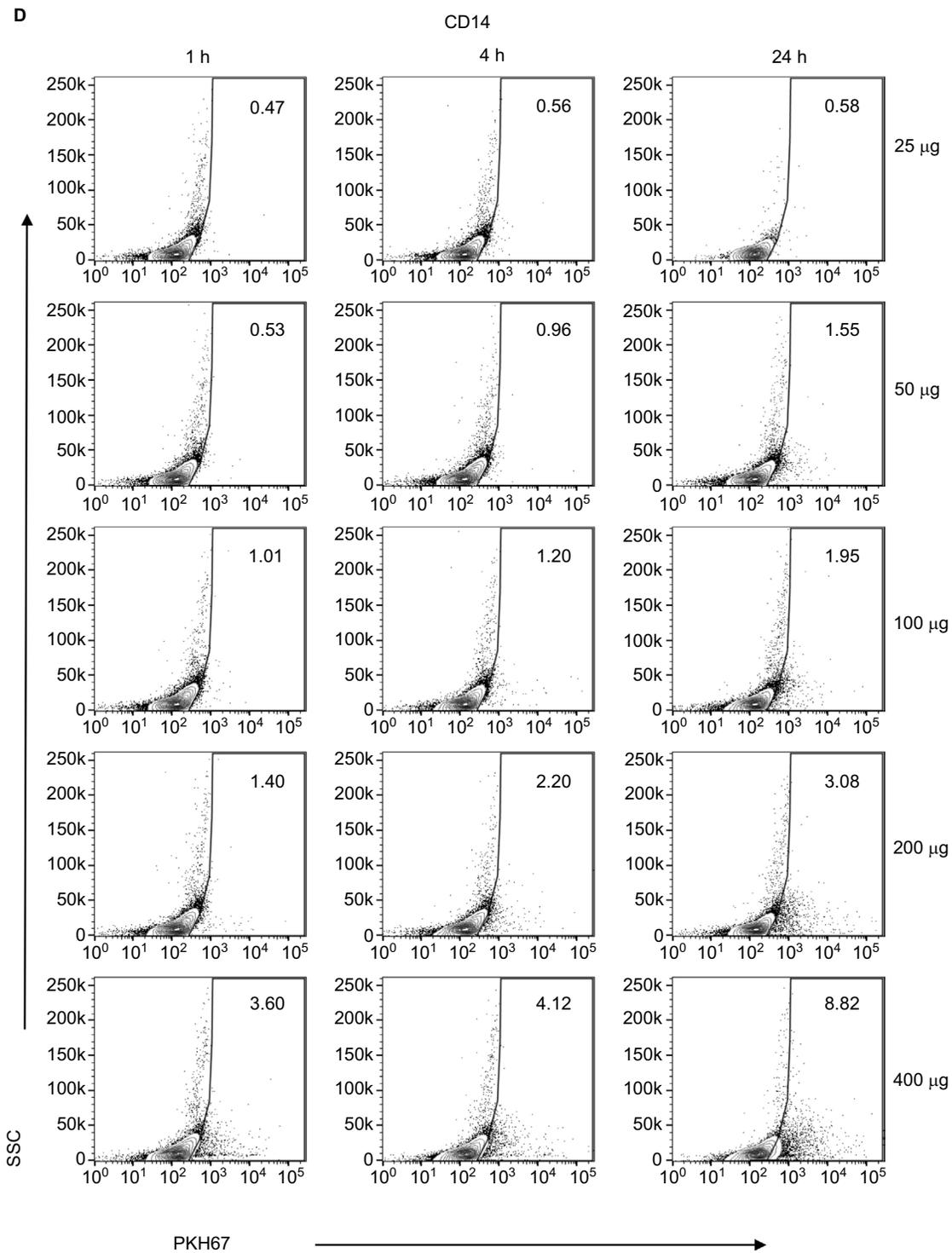


Figure S3 Internalization of PKH67-labeled exosomes by healthy PBLs.

Notes: Leukocytes were assayed by flow cytometry using specific antibodies to (A) B-lymphocytes (APC-anti-CD19), (B) T-lymphocytes (PerCP-anti-CD3), (C) NK cells (PE-anti-CD56), and (D) monocytes (PeCy7-anti-CD14). Representative dot plots are shown for each cell population.

Abbreviation: PBLs, peripheral blood leukocytes; APC, antigen-presenting cell; h, hours.

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