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RESEARCH ARTICLE
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THE PREDOMINANT ROLE OF CD4+T CELL-DERIVED SMALL EXTRACELLULAR VESICLES IN IMMUNE ENHANCEMENT

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ABSTRACT

Background: The human lymphocyte population provides adaptive and humoral immunity; however, the defending function and activities of lymphocytes were found abridged in many infections. Research has also proved the lack of CD4+ T cells in these infections. Thus, this study was conducted to investigate the role of small extracellular vesicles (sEV) derived from CD4+ T cells in immune enhancement.

Methodology: CD4+ T cells were activated, and sEV was Isolated and identified. Using different immunological techniques, including Flow cytometry, ELISA, Western Blotting, and immunofluorescence, the endorsement of activation, proliferation, and antibody production by lymphocytes was observed.

Result and discussion: The sEV derived from CD4+ T cells have expressed CD4, CD25, and ICOS molecules connected the biological properties of sEV to the parent cells. The result indicates that CD86 and MHCII expressions on the B cell surface were enhanced in the derived sEV-treated group, conforming that CD4+ T cell-derived sEV can promote B cell activation. In addition, the B cells treated with CD4+ T cell-derived sEV have greater CFSE and SSC-Height subsets and prove they can promote B cell proliferation. Furthermore, The IgG level in cells (wells) treated with 50µg CD4+ T cell-derived sEV was four times higher, corroborating its ability to promote antibody production of B cells.

Conclusion: sEV derived from CD4+ T cells enhances immune responses by promoting activation, proliferation, and IgG production by B cells.

Keywords: Small Extracellular Vesicles (sEV), Lymphocytes, T Cell, B Cell, Immune Enhancement

INTRODUCTION

The human lymphocyte population mainly includes T-lymphocytes (T cells), B-lymphocytes (B cells), and natural killer cells (NK cells). T and B cells would be activated, proliferate, and differentiate after recognizing foreign antigens mediating adaptive immunity. The cellular immune response is driven by T lymphocytes, while B cells are responsible for the humoral immune response. T-lymphocytes have two subsets, CD4+ T and CD8+ T cells. CD4+ T cells recognize peptides presented on Major Histocompatibility Complex (MHC) Class I I (MHC-II) by Antigen-presenting cells (APCs). CD4+ T cells, when activated, undergo proliferation and differentiate into the effector T cell. Effector CD4+ T cells, through the secretion of cytokines, induce the activation and proliferation of antigen-specific B cells. Then activated B cells differentiate into plasma cells to secrete large quantities of protective antibodies/ immunoglobulins. At the same time, Memory B and T cells develop for long-term protection [2, 3].

Disrupted CD4+ T cell function is also related to diseases. Previous research has established that HIV depletes the amount of CD4+ T cells to a level that is insufficient to produce antibodies[4]. Moreover, several studies described that normal T cell response, especially CD4+ T cell function, becomes impaired in end-stage renal disease[5], diabetes[6], inflammatory bowel disease[7] patients, and older adults [8], which results in immunocompromised individuals. These studies support the notion that CD4+ T cells are crucial in developing an effective immune response.

Various studies have demonstrated small extracellular vesicles (sEV) as a novel player in intercellular communication. sEV is a 30-120 nm membrane-bound vesicle secreted out by most types of cells, including melanocytes[9], trophoblasts[10], intestinal epithelial cells[11], and immune cells like B lymphocytes[12], T lymphocytes [13] and so on. These sEVs can carry specific biomolecules – sugar, protein, lipid, and nucleic acid – toward recipient cells, thereby influencing target cell function [14]. Several pieces of literature have revealed that sEV transports proteins, mRNA, and microRNA for subsequent protein expression in target cells [15, 16]. Due to its significant endogenous and delivery property, sEV has promising application prospects in disease diagnosis and drug treatment[17].

sEV secreted from immune cells contributes to regulating immune responses, including activation and inhibition. For instance, sEV secreted from dendritic cells would express MHC I or II molecules, present antigen on the surface, and play a role in CD4+ T cell indirect activation[18]. Various studies also suggest that sEV secreted from T cells unidirectionally transfers miRNA to APCs, regulating T cell activation and subsequent immune responses [19, 20]. There are applications for using sEV produced by immune cells (dendritic cells and T cells) for vaccination [17].

Collectively, these studies outline the critical role of CD4+ T cells and sEV in immune development. However, from these literatures, it is unclear whether sEV can work on CD4+ T cells to promote immunity or not. Therefore, this research aims to determine whether the sEV derived from CD4+ T cells that carry specific biological substances of CD4+ T cells would affect immune enhancement, investigated by three aspects; B cell activation, B cell proliferation, and antibody production.

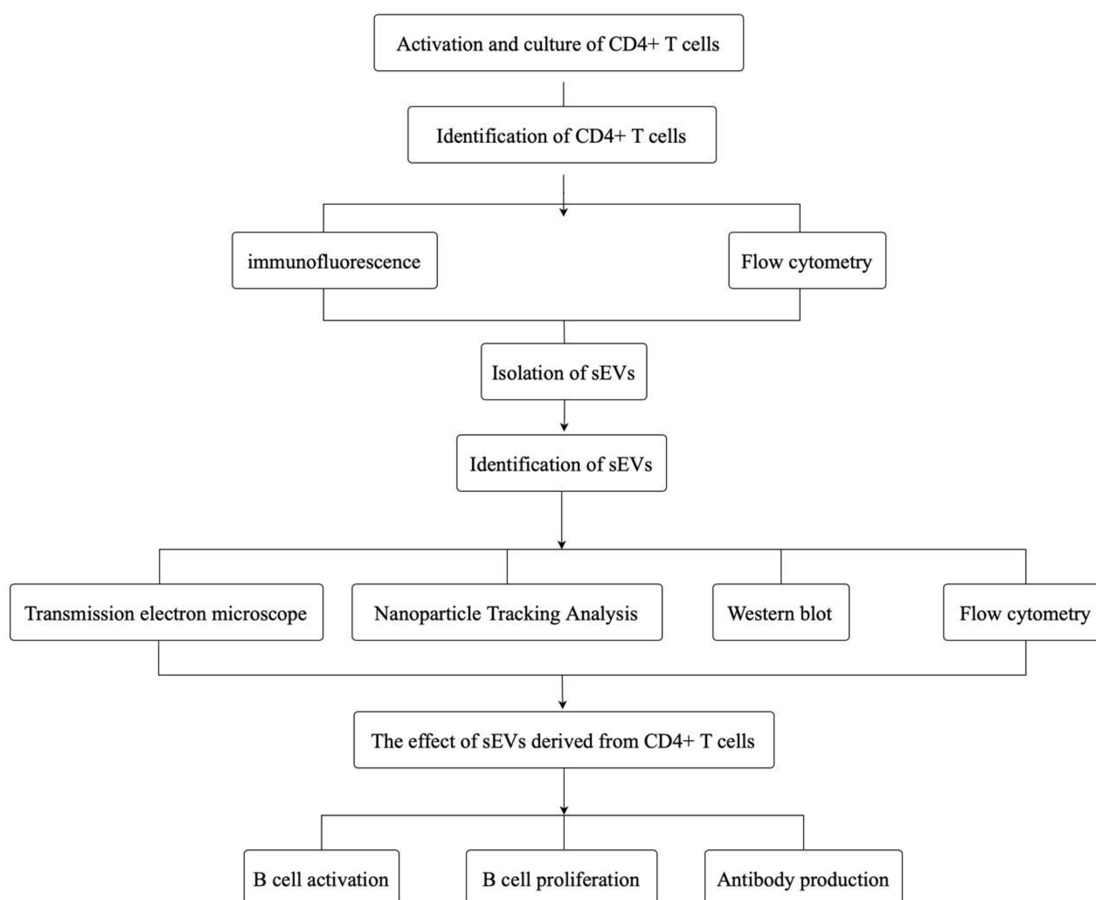
MATERIALS

Reagents and Chemicals	
CD4+ T cells	sEVs
Anti-CD3 antibody	Anti-CD28 antibody
CD63 antibody	HSP-70 antibody
TSG101 antibody	Fluorescein-conjugated monoclonal antibody
ExoQuick-TCTM Exosome Isolation Kit (SBI)	10% Fetal bovine serum (FBS) RPMI 1640 medium
PBS	FITC conjugated Anti-CD4 mouse monoclonal antibody
RIPA Lysis Buffer	SDS-PAGE Sample Loading Buffer
Human sera	Enzyme and zymolyte
TMAH	Blocking Buffer containing 5% BSA

EQUIPMENTS

Equipment/s	
24-well plates (Axygen Scientific)	Immunoplates (Thermo Scientific)
Centrifugal machine (Trikamlal & Sons)	Ultrafiltration centrifuge tube (Millipore)
Transmission electron microscope (Nantong University)	LAS-4000 (Fujifilm Life Sciences)
Flow cytometer (BD Bioscience)	FACS Calibur flow cytometer (Becton Dickinson)
Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany)	Immobilon polyvinylidene fluoride membranes (Bio-Rad, USA)
Enzyme immunometer (BioMérieux)	Enhanced chemiluminescence (ECL)
	(Champion Chemical)

RESEARCH DESIGN FLOWCHARTS



METHODOLOGY

Activation and culture of CD4+ T cells

CD4+ T cells were seeded into 24-well plates (2×10^6/well) coated with anti-CD3 (2μg/mL). 10% fetal bovine serum (FBS) RPMI 1640 medium without sEV was added to the plates. Then CD28 antibody (2μg/mL) was added and CD4+ T cells were incubated for 24h.

sEV Isolation

Culture supernatants were collected and centrifuged many times, removing debris and apoptotic bodies by filtering the supernatant through 0.22μm membranes. Then the filtrate was added to a 100 kDa ultrafiltration centrifuge tube (Millipore) and centrifuged at 1000g for 30 min. ExoQuick-TCTM Exosome Isolation Kit (SBI) was used to isolate sEV, following the manufacturer's instructions.

sEV Identification

The morphology of sEV derived from CD4+ T cells was observed by transmission electron microscope (Nantong University). sEV grain size was assessed by Nanoparticle Tracking Analysis (Vivacell company). sEV protein markers expressions were evaluated by western blot. sEV surface molecule expressions were tested by flow cytometer (BD Bioscience).

Flow cytometry

After washing and resuspension of 10^6 cells, 1□g fluorescein-conjugated monoclonal antibody against tested antigen was added. Cells were then incubated at 4 degrees for 30 min in a dark place. 1mL of PBS was added to stop staining. Cells were centrifuged at 500g for 5 min at 4 degrees and the supernatant was removed. After adding 200□L PBS to resuspend cells, a flow cytometry assay was used to evaluate the processed cells. A FACS Calibur flow cytometer (Becton Dickinson) was used to perform flow cytometry, while FlowJo software was used for statistical analysis.

Immunofluorescence analysis

Cells were incubated with FITC conjugated Anti-CD4 mouse monoclonal antibody. After being washed with PBS, cells were photographed under the Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Western blotting (WB)

Cells were harvested and lysed by RIPA Lysis Buffer. After centrifugation, cell supernatant was collected, evaluated by BCA kit and added by SDS-PAGE Sample Loading Buffer. The mixture was heated to 100°C, cooled down, loaded onto 12% SDS-PAGE gel, and transferred onto immobilon polyvinylidene fluoride membranes (Bio-Rad, USA). After transfer, PVDF membranes were blocked by Blocking Buffer containing 5% BSA for 1-1.5h. Then PVDF membranes were soaked with appropriate primary antibodies overnight at 4°C. After washing, the membranes were incubated by the appropriate secondary antibody for 1-1.5h. The membranes were tested through the enhanced chemiluminescence (ECL) western blotting detection system (Champion Chemical). LAS4000 UVP BioSpectrum Imaging System was used to image and analyze.

ELISA

Human sera were added to wells of immunoplates coated by antigen. After the plates were incubated at 37°C and washed, an enzyme-labeled specific antibody was added to each well. The plates were incubated and washed. Subsequently, zymolyte was added to render colors and TMAH was added to end the reaction. An enzyme immunometer was used to measure the absorbance of each well. The standard curve was drawn from the results to calculate the level of specific antibodies against the coated antigen.

Statistical analysis

Unless stated elsewhere in this article, experimental data represent the mean ± standard error of the mean (SEM). The significance threshold level for all statistical tests was set as 0.05. $P<0.05$ indicates that the difference is statistically significant (**: $P<0.01$, *: $P<0.05$). Comparison between the two groups was evaluated by 2 sample t-test. GraphPad Prism 5.0 software was used for graph processing.

c. Safety, Environmental and Ethical Consideration

All the experiments that would bring into contact with chemical agents, such as gel preparation in western blot, are conducted with gloves and masks. When dealing with cells, if necessary, experiments are conducted on a clean bench, with hands washed with ethanol every time. The alcohol burner and ultra-low temperature freezer are used under regulation and carefully. No animal experiment is included in this study.

RESULTS AND ANALYSIS

Identification of CD4+ T cells

CD3 and CD4 molecules are critical molecules in CD4+ T cell signaling and the surface marker protein for CD4+ T cells. To identify CD4+ T cells in the sample, cell samples were stained by murine CD4 monoclonal antibody and immunofluorescence was performed. The result indicated that cells were in round or oval shapes and expressed CD4 molecules on the surface (Figure 1A). Then, murine CD3 antibody and CD4 monoclonal antibody were used for fluorescent staining of the cells. Flow cytometry was applied to test the cell sample. The result shows that cells expressed CD3 and CD4 molecules simultaneously, proving they are CD4+ T cells and cell purity was greater than 95% (Figure 1B).

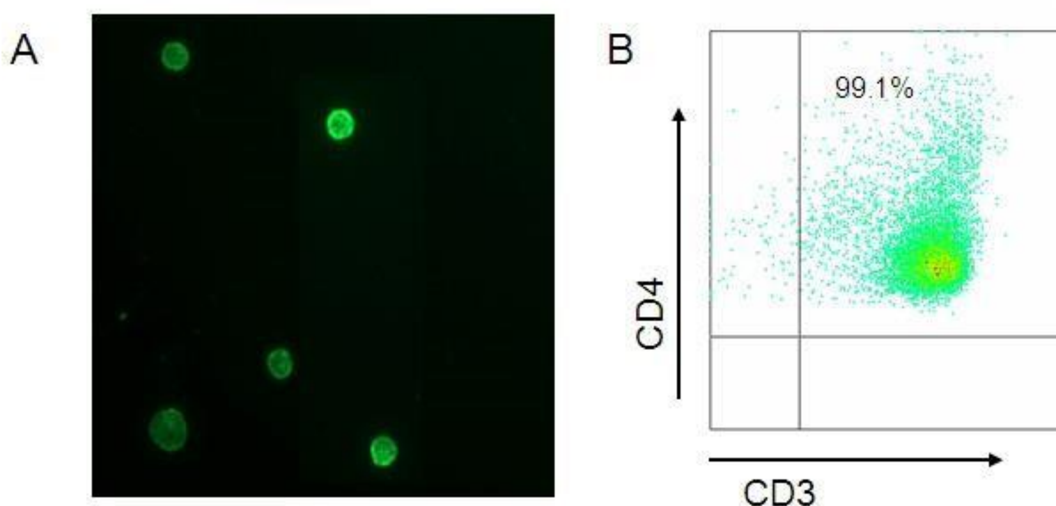


***Figure 1. Identification of CD4+ T lymphocytes**. **A**. Identification of CD4+ T lymphocytes by immunofluorescence**. B**. Identification of CD4+ T lymphocytes by flow cytometry.*

Isolation and identification of sEVs derived from CD4+ T cells

First, to explore the function of sEV derived from CD4+ T cells, anti-CD3 and anti-CD28 antibodies were used to activate CD4+ T cells in vitro. Twenty-four hours later, sEVs were derived from CD4+ T cells and identified according to vesicle morphology, size, and surface marker proteins.

A transmission electron microscope can observe subcellular structure, so it was used to observe the structure of sEV. from this approach, it was discovered that the CD4+ T cell-derived cell samples had a round/oval shape and a bilayer vesicle structure, which is representative of the standard sEV structure. (Figure 2A).

Nanoparticle Tracking Analysis was applied to measure derived sEV grain sizes. The result indicates that sEVs from CD4+ T cells had a similar size, mainly distributed from 50 to 150nm (Figure 2B). The mean grain size was 102nm, which is reasonable identification.

Western blot assay was used to evaluate expression levels of several marker proteins for sEV derived from CD4+ T cells. The result reveals the expression of sEV marker proteins HSP70, CD63, and TSG101, which proved that the derived vesicles express sEV-specific proteins (Figure 2C).

CD4 molecules are crucial for CD4+ T cell signaling: they induce an inducible costimulator (ICOS) to express on activated T cells, regulate the secretion of cytokines after T cell activation, and promote T cell proliferation. After fluorescence staining by a monoclonal antibody, flow cytometry was used to detect a surface molecule of derived sEV from CD4+ T cells. The result demonstrates that sEV derived from CD4+ T cells have expressed CD4, CD25 and ICOS molecules belonging to parent cells (Figure 2D). Therefore, sEV may have some biological properties of the parent cells.

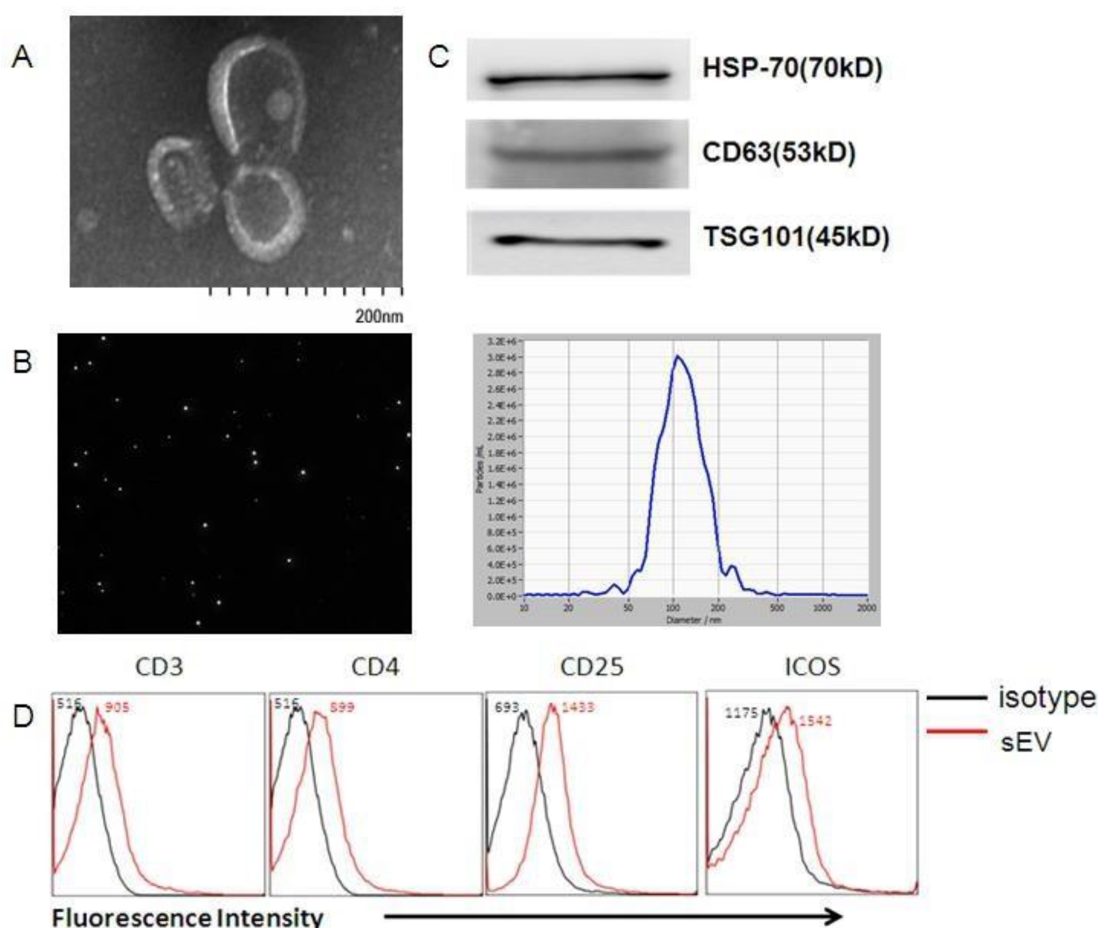


***Figure 2. Identification of sEV derived from CD4+ T lymphocytes**. **A**. Identification of sEV derived from CD4+ T cells by TEM. **B**. Identification of sEV derived from CD4+ T cells by Nanoparticle Tracking Analysis. **C**. Identification of sEV by Western blot. **D**. Identification of sEV derived from CD4+ T cells by FCM.*

CD4+ T cell-derived sEV promotes B cell activation, proliferation and production of IgG

CD4+ T cells play an essential role in B cell activation and antibody production in the immune system. To investigate the biological function of sEV derived from CD4+ T cells, sEV was added to B cell culture, and its role in B cell activation, B cell proliferation and antibody production was evaluated.

CD86 and MHC II molecules have low or no expression in inactivated B cells, but their expression levels rise in activated B cells. To investigate the role of sEV derived from CD4+ T cells in B cell activation, the FCM was used to detect CD86 and MHCII expressions on B cells after incubating with derived sEV for 48h. The result indicates that, compared to the control group, CD86 and MHCII expressions on the B cell surface were enhanced in the derived sEV-treated group (Figure 3A, 3B). Thus, CD4+ T cell-derived sEV can promote B cell activation in vitro.

To investigate the role of sEV derived from CD4+ T cells in B cell proliferation, 10μg of CD4+ T cell-derived sEV was incubated with B cells for 4 days at 37°C. Treated B cells were stained by fluorescent dye CFSE and performed flow cytometry. Compared to the control group, B cells treated by CD4+ T cell-derived sEV have greater CFSE, SSC-Height subset (Figure 3C). Therefore, CD4+ T cell-derived sEV can promote B
cell proliferation in vitro.

The production of antibodies is also an essential factor in determining vaccination effectiveness. To explore whether sEV derived from CD4+ T cells can regulate antibody production by B cells in vitro, sEV were added into B cells and incubated for 96h. The supernatant was collected and the IgG level was evaluated by ELISA. From the result, IgG level in wells treated with 10μg CD4+ T cell-derived sEV is higher than that in the control group. Moreover, the IgG level in wells treated by 50μg CD4+

T cell-derived sEV was even higher, approximately four times that in the control group (Figure 3D). To conclude, sEV derived from CD4+ T cells can promote antibody production of B cells.

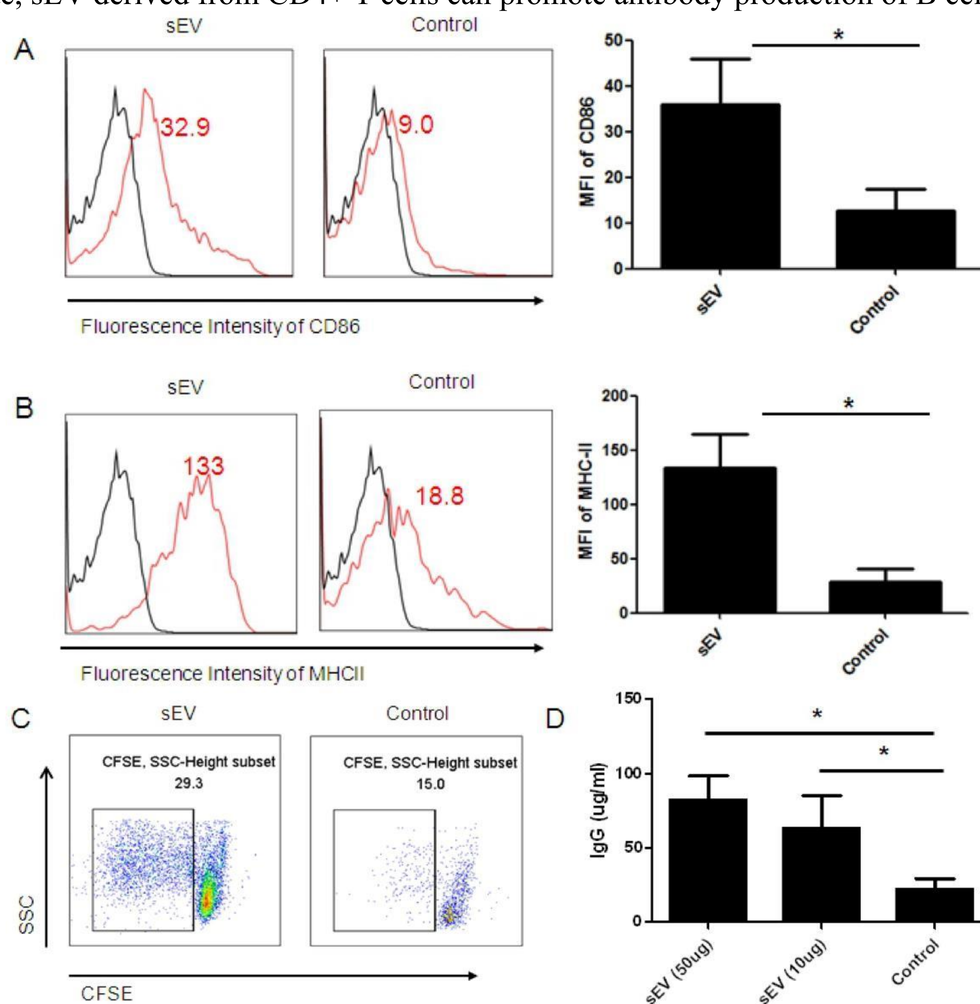


Figure 3. sEV derived from CD4+ T cells promote B cell activation, proliferation and antibody production. A. *CD4+ T cell-derived sEV enhanced expression of B cell surface molecule CD86, tested by FCM.* ***B.*** *CD4+ T cell-derived sEV enhanced expression of B cell surface molecule MHCII, tested by FCM.* ***C.*** *FCM was used to detect CFSE, SSC-Height subset of treated B cells.* ***D.*** *ELISA was used to measure IgG levels in treated B cells. * $p < 0.05$*

DISCUSSION

Small extracellular vesicles (sEVs) are small membrane-bound vesicles secreted from most cells for intercellular communication. They carry out functions of delivering materials between cells or sending signals to target cells. During synthesis, sEVs enwrap specific lipids, proteins, carbohydrates and RNAs to trigger specific regulations at different target cells. Due to its biological and physiological properties, prior studies have noted the importance of sEVs as novel therapeutic agents, such as discovering antigens for vaccination. In a study conducted by Lifang Cheng et al. (2017), it was shown that sEVs derived from M1-polarized macrophages could enhance the activity of a cancer vaccine by promoting specific cytotoxic T-cell responses.

Moreover, the M1-derived sEVs are more effective with the LCP nanoparticle vaccine during the melanoma growth inhibition study [21]. Several reports have shown that sEV is promising to work on vaccinating some infectious diseases by transporting materials to immune-competent cells, including vaccines against Neisseria meningitides, Bordetella pertussis, enteric pathogens, and tuberculosis [22]. Even some ancient eukaryotes would secrete their sEVs to provide a virus transfer pathway and regulate the parasite's life infectious cycle, such as Leishmania. These sEVs improve virus and parasite transmission and infectivity of the host [23, 24]. Therefore, sEVs research may

provide new diagnostic targets, develop preventive strategies and help to discover new therapeutic pathways.

Despite all the studies presented above, there is no relevant study on sEV derived from CD4+ T cells and its role in immunology. Thus this study highlighted the novel properties of sEV and its interaction with immune-competent cells for the first time.

CONCLUSION

This study identified the isolated sEV from CD4+ T cells with three aspects: vesicle morphology, size, and surface markers. Derived sEV was co-cultured with B lymphocytes, and the effects were evaluated by many means. The results indicate the promotion role of CD4+ T cell-derived sEV on B cell activation, proliferation, and antibody production of B cells (Figure 4). Therefore, sEV derived from CD4+ T cells is proven beneficial for immune enhancement.

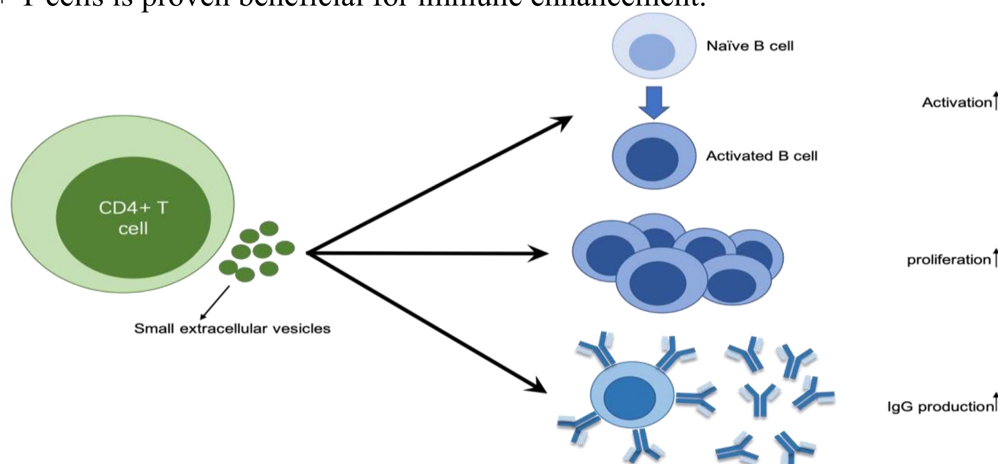


Figure 4. *CD4+ T cell-derived sEV promotes B cell activation, proliferation and antibody production.*

The conclusion drawn from this study provides evidence for the future research and application of sEV on immunity-related diseases and vaccination. sEV is biologically safe, physically stable, and can reproduce in an incredible amount in vitro. These properties make sEV possible and promising in future clinical treatment. The broad prospects of topics about immune cell-derived sEV can be seen from this project.

REFERENCE

1. Pan, L., et al., *A genome-wide association study identifies polymorphisms in the HLA-DR region associated with non-response to hepatitis B vaccination in Chinese Han populations.* Hum Mol Genet, 2014. 23(8): p. 2210-9.
2. Vora, K.A., K. Tumas-Brundage, and T. Manser, *Contrasting the In Situ Behavior of a Memory B Cell Clone During Primary and Secondary Immune Responses.* The Journal of Immunology, 1999. 163(8): p. 4315.
3. Luckheeram, R.V., et al., *CD4⁺T cells: differentiation and functions.* Clin Dev Immunol, 2012. 2012: p. 925135.
4. van den Berg, R., I. van Hoogstraten, and M. van Agtmael, *Non-responsiveness to hepatitis B vaccination in HIV seropositive patients; possible causes and solutions.* AIDS Rev, 2009. 11(3): p. 157-64.
5. Eleftheriadis, T., et al., *Disturbances of acquired immunity in hemodialysis patients.* Semin Dial, 2007. 20(5): p. 440-51.
6. Saco, T.V., A.T. Strauss, and D.K. Ledford, *Hepatitis B vaccine nonresponders: Possible mechanisms and solutions.* Ann Allergy Asthma Immunol, 2018. 121(3): p. 320-327.
7. Sempere, L., et al., *Factors predicting response to hepatitis B vaccination in patients with inflammatory bowel disease.* Vaccine, 2013. 31(30): p. 3065-71.

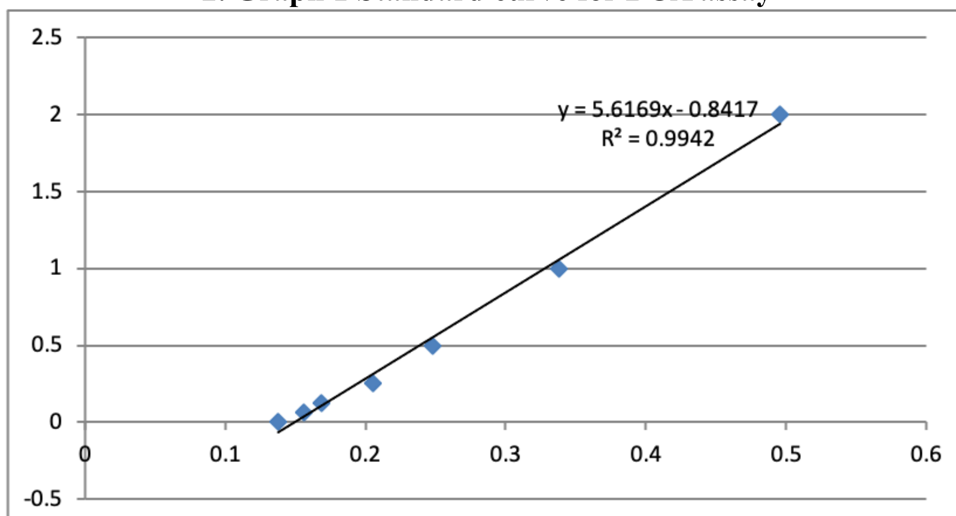
8. Rosenberg, C., et al., *Age is an important determinant in humoral and T cell responses to immunization with hepatitis B surface antigen.* Hum Vaccin Immunother, 2013. 9(7): p. 1466-76.
9. Der, J.E., et al., *A murine monoclonal antibody, MoAb HMSA-5, against a melanosomal component highly expressed in early stages, and common to normal and neoplastic melanocytes.* Br J Cancer, 1993. 67(1): p. 47-57.
10. Abrahams, V.M., et al., *First trimester trophoblast cells secrete Fas ligand which induces immune cell apoptosis.* Mol Hum Reprod, 2004. 10(1): p. 55-63.
11. van Niel, G., et al., *Intestinal epithelial cells secrete exosome-like vesicles.* Gastroenterology, 2001. 121(2): p. 337-49.
12. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. 183(3): p. 1161-72.
13. Blanchard, N., et al., *TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex.* J Immunol, 2002. 168(7): p. 3235-41.
14. Yáñez-Mó, M., et al., *Biological properties of extracellular vesicles and their physiological functions.* J Extracell Vesicles, 2015. 4: p. 27066.
15. Mathivanan, S. and R.J. Simpson, *ExoCarta: A compendium of exosomal proteins and RNA.* Proteomics, 2009. 9(21): p. 4997-5000.
16. Valadi, H., et al., *Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells.* Nat Cell Biol, 2007. 9(6): p. 654-9.
17. Batrakova, E.V. and M.S. Kim, *Using exosomes, naturally-equipped nanocarriers, for drug delivery.* J Control Release, 2015. 219: p. 396-405.
18. Théry, C., et al., *Indirect activation of naïve CD4+ T cells by dendritic cell-derived exosomes.* Nat Immunol, 2002. 3(12): p. 1156-62.
19. Mittelbrunn, M., et al., *Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells.* Nat Commun, 2011. 2: p. 282.
20. Mittelbrunn, M. and F. Sánchez-Madrid, *Intercellular communication: diverse structures for exchange of genetic information.* Nat Rev Mol Cell Biol, 2012. 13(5): p. 328-35.
21. Cheng, L., Y. Wang, and L. Huang, *Exosomes from M1-Polarized Macrophages Potentiate the Cancer Vaccine by Creating a Pro-inflammatory Microenvironment in the Lymph Node.* Mol Ther, 2017. 25(7): p. 1665-1675.
22. Acevedo, R., et al., *Bacterial outer membrane vesicles and vaccine applications.* Front Immunol, 2014. 5: p. 121.
23. Atayde, V.D., et al., *Exploitation of the Leishmania exosomal pathway by Leishmania RNA virus 1.* Nat Microbiol, 2019. 4(4): p. 714-723.
24. Atayde, V.D., et al., *Exosome Secretion by the Parasitic Protozoan Leishmania within the Sand Fly Midgut.* Cell Rep, 2015. 13(5): p. 957-67.

APPENDIX

1. Table 1 Protein sample concentration for western blot is evaluated by BCA kit

Standard OD	Standard Concentration	Sample OD	Diluted sample concentration	Sample concentration	Sample volume
0.496	2	0.295	0.8152855	4.64712735	6.455601007
0.338	1	0.207	0.3209983	1.82969031	16.39621735
0.248	0.5	0.226	0.4277194	2.43800058	12.30516524
0.205	0.25	0.259	0.6130771	3.49453947	8.584822194
0.169	0.125	0.311	0.9051559	5.15938863	5.814642422
0.156	0.0625	0.22	0.394018	2.2459026	13.35765852
0.138	0	0.279	0.7254151	4.13486607	7.255374054

2. Graph 1 Standard curve for BCA assay



3. Table 2 IgG (μg/ml) level tested by ELISA

	sEV(50μg)	sEV(10μg)	control
IgG (μg/ml)	85.0	55.0	29.0
IgG (μg/ml)	97.0	47.0	15.0
IgG (μg/ml)	65.0	88.0	23.0