Vol.65: e22210234, 2022
https://doi.org/10.1590/1678-4324-2022210234
ISSN 1678-4324 Online Edition

Article - Human and Animal Health

Proliferative Effects of Lung Cancer Cells Derived Exosomes on T Cells

Shahid Hussain[1]
https://orcid.org/0000-0002-0117-5262

Shaukat Iqbal Malik[1]*
https://orcid.org/0000-0003-4839-3168

[1]Capital University of Science and Technology, Faculty of Health and Life Sciences, Department of Bioinformatics and Biosciences, Islamabad Pakistan.

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Paulo Vitor Farago

Received: 13-Apr-2021; Accepted: 25-Apr-2022.

*Correspondence: iiuibiotech@gmail.com; Tel.: +92-343-3232031 (S.I.M.).

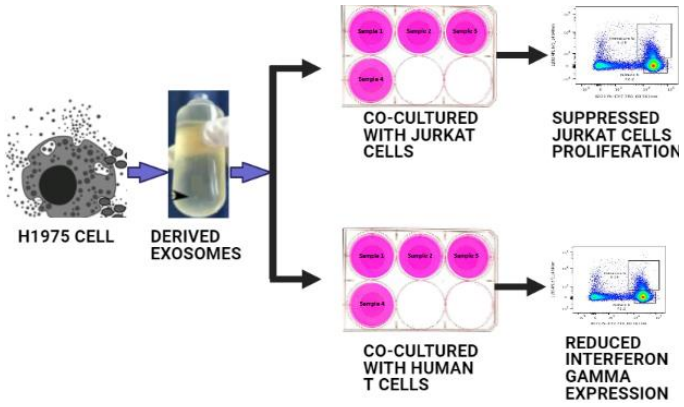
HIGHLIGHTS

- The expression of exosomes is higher in cancer environment.
- Exosomes effects T cells proliferation.
- The expression of CD4-IFNγ and CD8-IFNγ are deregulated by lung cancer exosomes.

Abstract: Lung cancer patients have higher expression of Exosomes (EXs) carrying several bioactive molecules including lipids, protein, carbohydrates, and different types of RNAs. Upon delivery of EXs into the recipient cells different behavioral changes are observed in the biological mechanisms of the cell. In the current study, we aimed to compare the EXs production in lung cancer cells (H1975) in two different conditions including normal conditions (NC) (20% O_2, 5% CO2, 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin added media with pH 7) and our established conditions (EC) (1% O_2, 5% CO_2 and FBS/antibiotic-free media with pH 6). We also studied the proliferative effects of these EXs (NC and EC) on Jurkat cells (immortalized T lymphocytes) and proliferation of some of the human T cells population was also observed with co-culture experiments. From the Nano sight analysis, a high quantity of EXs was observed in EC as compared with the NC. EXs isolated from both conditions showed proliferative effects on Jurkat cells. Among the human T cells population, EC-EXs were shown low expression of CD8-IFNγ and CD4-IFN γ in comparison with NC-EXs. These results support the hypothesis that Lung cancer cells derived EXs can interact with the T cells mechanisms leading to the low immune response in the cancer microenvironment and providing a favorable condition for cancer cells. Such studies need validation in vivo to verify the actual mechanisms which could be effective in potential therapeutic strategies.

Keywords: Lung Cancer; Exosomes; T cells proliferation; Interferon-gamma.

GRAPHICAL ABSTRACT



INTRODUCTION

Lung cancer has two sub-types including non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). overall death ratio of lung cancer is declined in the United States [1]. In the last few decades' important therapeutic advancements have contributed to understanding the tumor progression mechanisms, disease biology, early detection advancement, and multimodal care [2]. Among other therapeutic options, molecular targeted therapies are proven to have improved the survival rate in a limited group of NSCLC patients [3]. For years, lung cancer was considered as non-immunogenic, however, elevated levels of B7-H3, and B7-H4 on CD8+ tumor-infiltrating lymphocytes (TILs), programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has now been reported [4, 5]. Among other immune responses, T cells are the core player in the tumor microenvironment which acquires effecter and functional characteristics to act with either direct inflammatory or anti-inflammatory responses [6]. Besides Tumor-associated macrophages (TAMs), T cells are the second most abundant immune cells in the cancer microenvironment and have been studied extensively in different cancer types [7]. During the tumor initiation, an early stage in the presence of immunogenic antigens naïve T cells are primed in the lymph nodes and are activated and migrated to the tumor microenvironment where they initiate a protective effector immune response and finally eliminate cancer cells. Some histo-pathological studies have also demonstrated that T cells go beyond the invasive edge of the tumor and predominate in its hypoxic core [8]. Many aspects have been studied that how cancer cells escape from the T cells. With recent advancements in research, this is also evident that cancer cells can invade the T cell by secreting certain types of extracellular vesicles (EVs) which are sub-cellular components with a group of apoptotic bodies, micro vesicles, and EXs. EVs are mostly focused on the last decade to study their role in various biological systems. This area has sparked the interest of investigators to study the regulatory action and mechanisms. EXs are secreted by almost all cell types and are found in different biological fluids and are responsible for cell to cell communication and elevation of unnecessary materials from the cells [9]. EXs are 30-150nm in size and carry a variety of bioactive compounds including lipids, protein, and nucleic acids which can be transferred inactive form from the originating cell to the recipient cell without being degraded by enzymes like RNases and thus provide a longer life span to its inner constituents to survive in extracellular environment [10]. EXs are interacting in many biological mechanisms, cancer progression, role in inhibition or enhancement of immune response, etc. [11]. The suppressive state of immune cells leads to a weak anti-tumor response, therefore targeting EXs could be a novel therapeutic approach to enhance the immune response in the cancer microenvironment [12]. The different expression level of EXs has been found among healthy and cancerous conditions with differential expression of certain microRNAs (miRNAs). This deregulation of miRNA is found to be of much importance in cancer progression by contributing in alteration of tumor suppressor genes and oncogenes expression [13]. Tumor cell-derived exosomes miRNA research is a promising and dynamic approach in early detection, personalized therapy, and diagnosis and cancer prevention [14]. EXs are also considered to be the key players in drug delivery system and diagnostic biomarkers identification. In this study we have conducted some experiments to identify the effects of lung cancer cells derived EXs on T-cells proliferation.

MATERIAL AND METHODS

In the current study, we analyzed the level of production of EXs from H1975 cells (adenocarcinoma; non-small cell lung cancer cells) in two different conditions (NC & EC) and these two groups of EXs were then co-cultured with Human blood isolated T cells to study the expression level of CD8-IFNγ and CD4-IFN γ.

H1975 Cell culture

H1975 cells were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium for 48 hours to obtain a higher confluence. Then H1975 cells ($3x10^5$ cells/ml) were added into two separate T-150 flasks (NC & EC labeled). NC containing 40 ml RPMI-1640 media (media pH 7 with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin added) and the conditions 20% O_2, 5% CO_2 were set on incubator. EC flask was added with FBS and antibiotic-free 40 ml RPMI-1640 media with pH 6 and a separate designated incubator was set with 1% O_2, 5% CO_2. Both NC and EC flasks were incubated for 48 hours at 37°C in two different incubators. Experiment flow is shown in Figure 1.

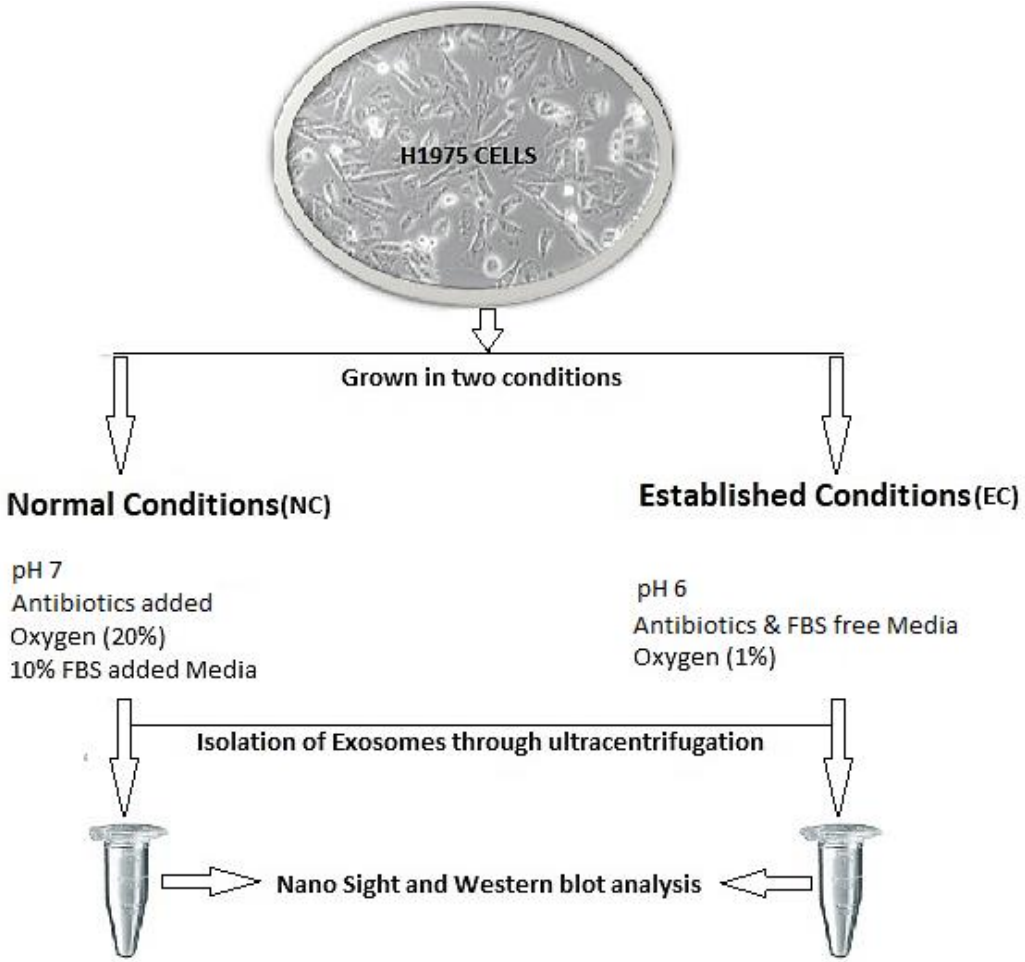


Figure 1. Experimental flow for EXs isolation from H1975 Cells grown in NC and EC.

EXs isolation

EXs were isolated from the media grown with the H1975 cells after 48 hours through a series of centrifugations as shown in Figure 2. This experiment was practiced in high aseptic conditions to avoid EXs contamination. Media was collected from both conditions in two labeled 50 mL centrifuge tubes inside the laminar flow and were centrifuged at 500 g; 10 minutes to remove cell debris and other larger components. Pallets were discarded and the supernatant was transferred to new two labeled 50 mL tubes and was centrifuged at 2500 g; 10 minutes to remove other extracellular vesicles. Supernatants were filtered through ultra-filters (0.22 µm) and media was transferred into two separate ultracentrifugation ultra-crimp 35ml tubes inside the laminar flow and were centrifuged in a pre-cooled (4 °C) ultracentrifuge machine at 100,000 g for 90 minutes. The pellets were washed and re-suspended with 10% PBS and again ultracentrifugation was done at 100,000 g for 90 minutes. The pallets (EXs) were re-suspended in 200µl fresh 10% PBS. A small amount of 10µl has proceeded for quantification while the rest of the EXs were stored at -80 °C for further experiments.

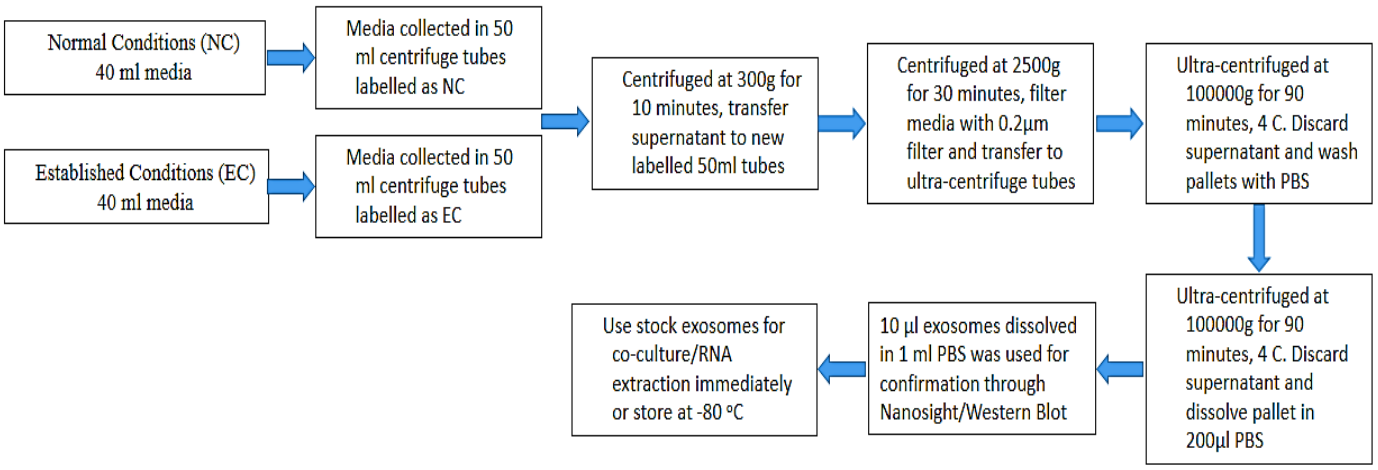


Figure 2. Steps for EXs isolation through centrifugation from H1975 cells grown in two different conditions (NC and EC)

Nano sight, Nanoparticles tracking analysis (NTA)

Nano sight (NTA) is one of the reliable methods used to quantify Nanoparticles. EXs isolated were analyzed through NTA (Nano Sight NS300). First, the Nano sight inner compartment was washed two times by injecting 1 mL ultra-filtered ddH_2O through a syringe, and then each sample was diluted in a labeled Eppendorf tube with the following protocol: 10 µL EXs solution was added into 1 mL ultra-filtered ddH_2O. Each sample was injected through a syringe and the machine was run by following the Nano Sight NS300 manual guide.

Western blotting

Nano sight NTA can detect the particles based on their size. The particles which we assumed as EXs were in the range of the size of EXs (20-150nm) but these results need further validation to confirm the presence of EXs on the molecular level based on their markers (CD63 and CD9). EXs were mixed with RIPA buffer. The samples were incubated on ice for 10-15 minutes and then were centrifuged at 13800 rpm at 4°C for 20 minutes. The supernatant (isolated protein) was separated into new labeled tubes and quantification was done through BCA protein Assay. Protein samples (20 µg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel along with a protein ladder marker (100 KDa) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1 hour at room temperature and probed with primary antibodies CD9 (ab58989) (1:1000) and CD63 (ab231975) (1:1000) at 4°C overnight. The membranes were incubated with an appropriate secondary antibody for 1 hour. Blots were washed five times with Tris-buffered saline with 0.1% Tween (TBST) buffer after each incubation step (5 minutes) at room temperature and visualized through a scanner. Bands were analyzed for the presence of CD9 and CD63.

Blood Collection process and T lymphocytes Isolation

Human T Lymphocyte Isolation was done through the methodology published by Lefort, C. T., & Kim, M. (2010) [15]. In brief, 300 mL fresh blood was obtained from a healthy donor and allowed to cool for 30 minutes before being pipetted 3 mL of Polymorph density gradient media into an 8 mL round-bottom polystyrene tube and followed the same protocol to isolate T lymphocytes. The experiment was covered by ethical approval granted by the Research Ethics Committee.

T-cells proliferation assay

After the confirmation of extracted EXs from both NC and EC, they were co-cultured with the Jurkat Cells (Plate 1) and Human T cells (Plate 2). Plate 1) Four wells were added with 1mL RPMI1640 Media and 1 × 105 numbers of Jurkat cells were added to each labeled well as shown in Figure 3A. The following set of experiments was designed for co-culture with four groups. 1) Jurkat cells 2. Jurkat cells and phytohemagglutinin (PHA) / phorbol 12-myristate 13-acetate (PMA) (T cell stimulators) 3. Jurkat cells, PHA/PMA and NC EXs 4. Jurkat cells, PHA/PMA and EC EXs. Plate 2) Human T Cells were isolated through standard PBMC protocol from human fresh blood provided by the University hospital with written consent and prior approval. A six-well plat was taken and four wells were added with 1ml RPMI1640 Media and 1 × 10^5 numbers of human T cells were added to each labeled well as shown in Figure 3B. Four groups were

categorized. 1. Human T cells 2. Human T cells and PHA/PMA 3. Human T cells, PHA/PMA, and NC EXs 4. Human T cells, PHA/PMA, and EC EXs. Both the plates were incubated for 24 hours at 37ºC.

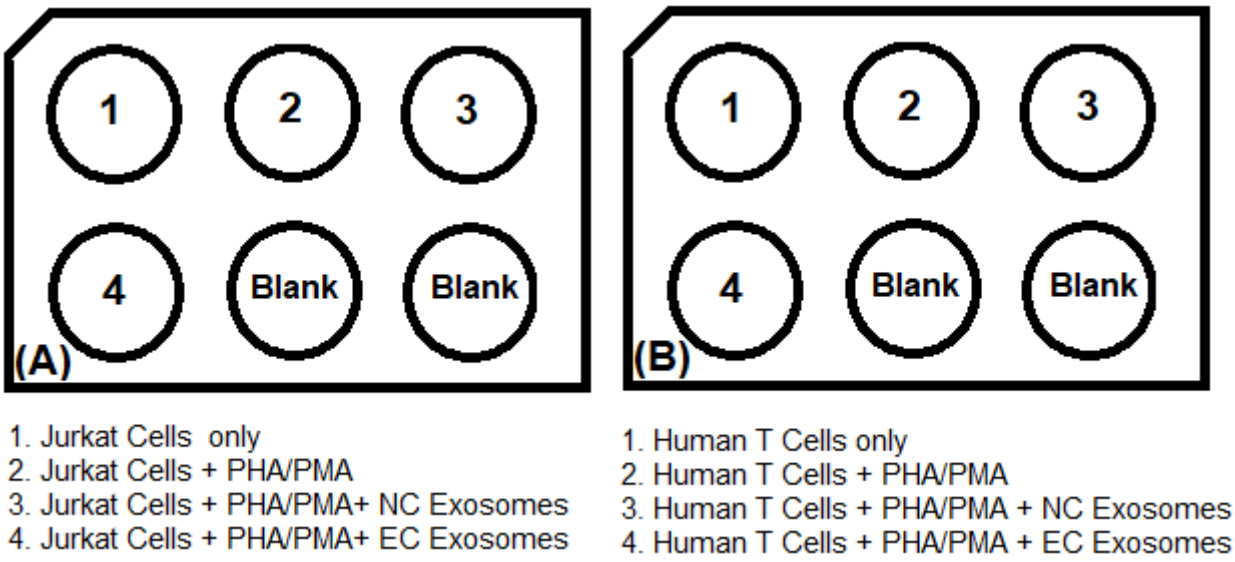


Figure 3. (A) experimental design for samples arrangements plate-1 with Jurkat Cells. (B) Plate-2 with Human T Cells.

Flow cytometer analysis

After 24 hours of incubation eight centrifuge tubes were labeled accordingly for plates 1 and 2 and the media was transferred. All tubes were centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and cells from both plates were collected in the form of pallets. Each cells pellet was washed with 10% PBS and again centrifuged with the same protocol used before. Each washed pallet of plate 1 (Jurkat cells) was re-suspended into 100 µL Jurkat cells markers (CD69 and CD25) solution. The samples were incubated at 4˚C for 30 minutes. All four samples were run through the flow cytometer and data was analyzed. While plate 2 (Human T cells) pallets were treated with two types of antibodies i.e., 1) Cell surface antibodies: Anti-CD45 antibody, (ab40763), CD3 Monoclonal Antibody, eBioscience™ (Catalog # 11-0032-82), Anti-CD4 antibody (ab269349) and CD8 Monoclonal Antibody (Catalog # MHCD0801). 100 µL cell surface markers solution was added to each sample. The samples were incubated at 4ºC for 30 minutes and were centrifuged at 500xg for 10 minutes, the supernatants were removed and 100ul membrane breaking buffer was added and mixed. These tubes were incubated at 4˚C overnight and were centrifuged at 500xg for 10 minutes and pallets were washed with 10% PBS. 2) Intracellular antibodies were added (100 µL IFN γ eBioscience™ Catalog # 53-7319-42) to each previously treated pallet of Human T cells. The tubes were incubated at room temperature for 30 minutes and were run through a flow cytometer and data was analyzed.

RESULTS

EXs quantification

H1975 Cell-derived EXs from both EC and NC were analyzed through Nano sight. All particles size were in the range of 100-130nm as shown in Figure 4A&B. NC isolated EXs concentration was $6.7x10^8$ particles/ml, while EC yielded $8.8x10^8$ particles/ml. We also confirmed the presence of EXs through western blotting by using EXs surface markers (CD9 and CD63). The results confirmed the isolated EXs and the difference in band size showed a difference in the EXs production in both NC and EC as shown in Figure 4C.

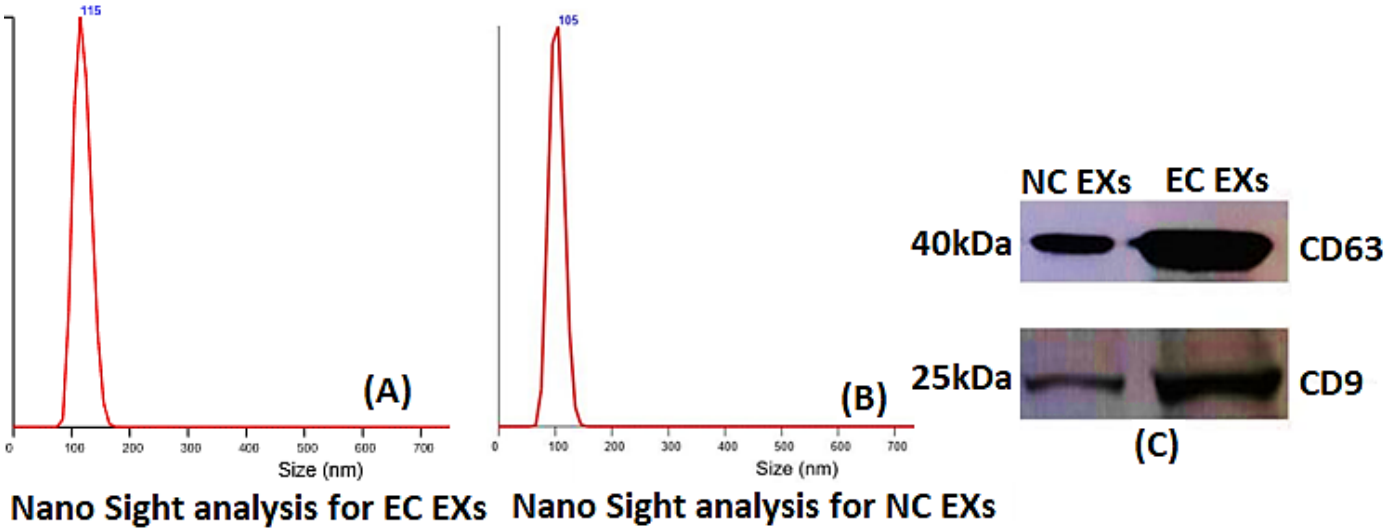


Figure 4. Quantification and confirmation of EXs. (A, B) Nano Sight (NTA) analysis of EC and NC EXs. (C) Western blotting

Co-Culture of Jurkat cells with EXs from H1975 Cells

The EXs isolated from both NC and EC were co-cultured with Jurkat Cells and incubated for 48 hours. The flow cytometer analysis with two markers (CD69 and CD25) revealed that there was a difference in the expression of both markers indicating the suppressive role of cancer-derived EXs in conditions. EC EXs co-cultured had a more decreased number of cells as compared to NC EXs. Here we tested two markers, the CD69 expression was found different in Jurkat cells only (21%), Jurkat cells and phytohemagglutinin (PHA) / phorbol 12-myristate 13-acetate (PMA) (T cell stimulators) (77.6%), Jurkat cells, PHA/PMA and NC EXs (58.2%) and Jurkat cells, PHA/PMA and EC EXs (39.5%) as results given by flow cytometry shown in Figure 5A. Similarly, the CD25 expression was also different in Jurkat cells only (38.8%), Jurkat cells and phytohemagglutinin (PHA) / phorbol 12-myristate 13-acetate (PMA) (T cell stimulators) (94.6%), Jurkat cells, PHA/PMA and NC EXs (80%) and Jurkat cells, PHA/PMA and EC EXs (49.3%) as results given by flow cytometry shown in Figure 5B. Both the markers confirmed the decreased level of Jurkat cells when treated with cancer-derived exosomes. The effects were seen more in EC-EXs in comparison with NC-EXs.

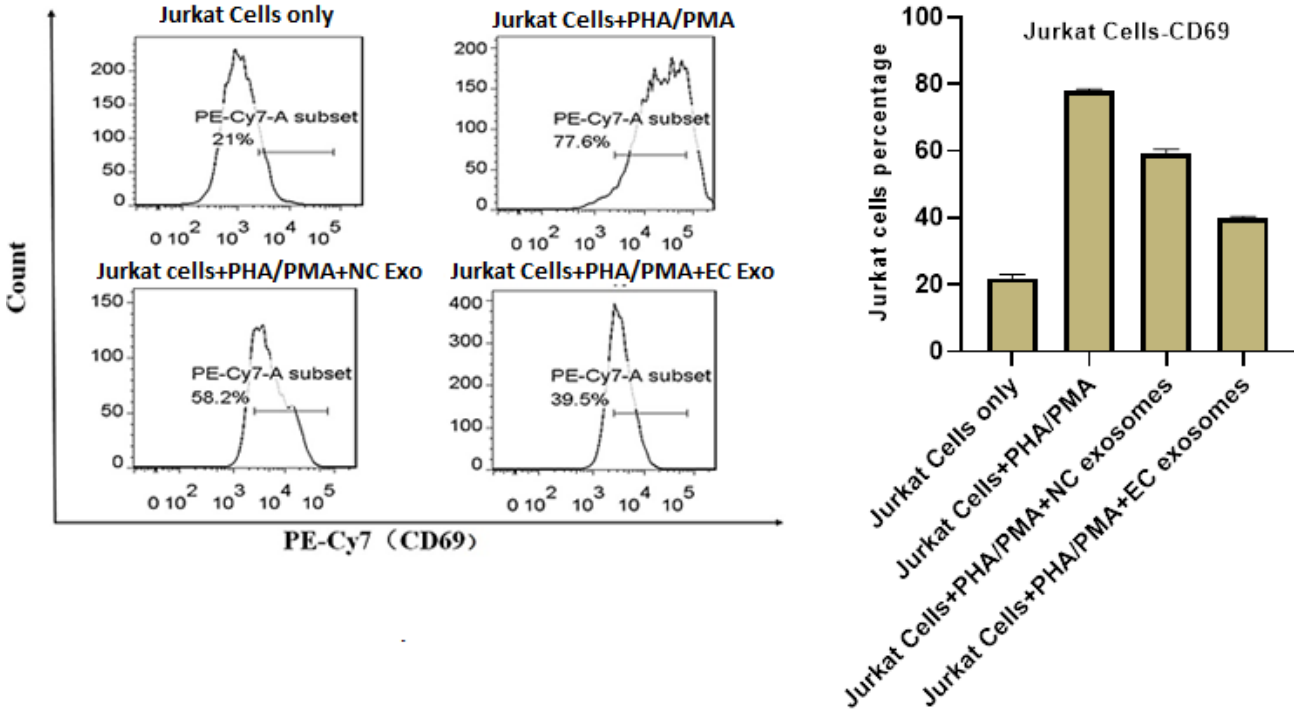


Figure 5A. Flow cytometer analysis of CD69 labeled Jurkat cells.

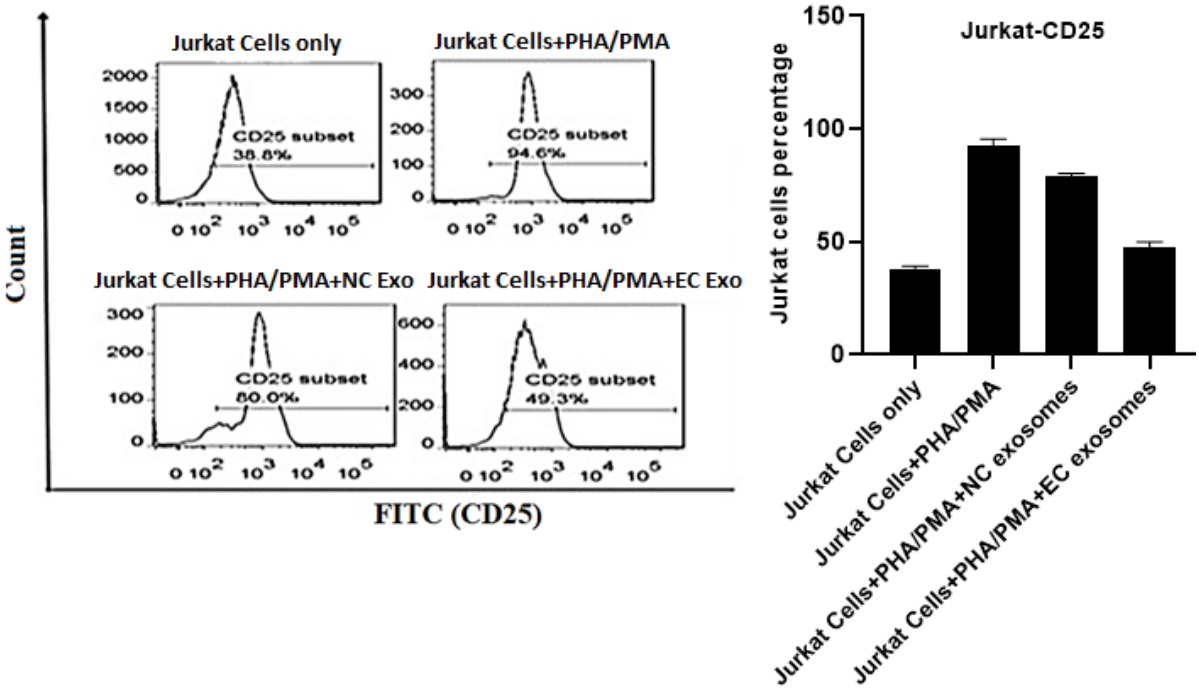


Figure 5B. Flow cytometer analysis of CD25 labeled Jurkat cells.

Co-Culture of Healthy Human T cells with EXs from H1975 Cells

For human T cells, we analyzed the expression of CD4- IFN γ and CD8-IFNγ only. There was a little difference observed in the expression of CD4-IFNγ among T cells only (2.67%), T cells and phytohemagglutinin (PHA) / phorbol 12-myristate 13-acetate (PMA) (T cell stimulators) (3.42%), T cells, PHA/PMA and NC EXs (0.55%) and T cells, PHA/PMA and EC EXs (0.32%) as results given by flow cytometer shown in Figure 6A. Similarly, the CD8-IFNγ expression was also different in T cells only (2.8%), T cells and phytohemagglutinin (PHA) / phorbol 12-myristate 13-acetate (PMA) (T cell stimulators) (9.05%), T cells, PHA/PMA and NC EXs (7.4%) and T cells, PHA/PMA and EC EXs (3.27%) as shown in Figure 6B. Together these results indicated the proliferative effects of lung cancer (H1975 cells) derived EXs on CD4 – IFNγ and CD8-IFNγ expression.

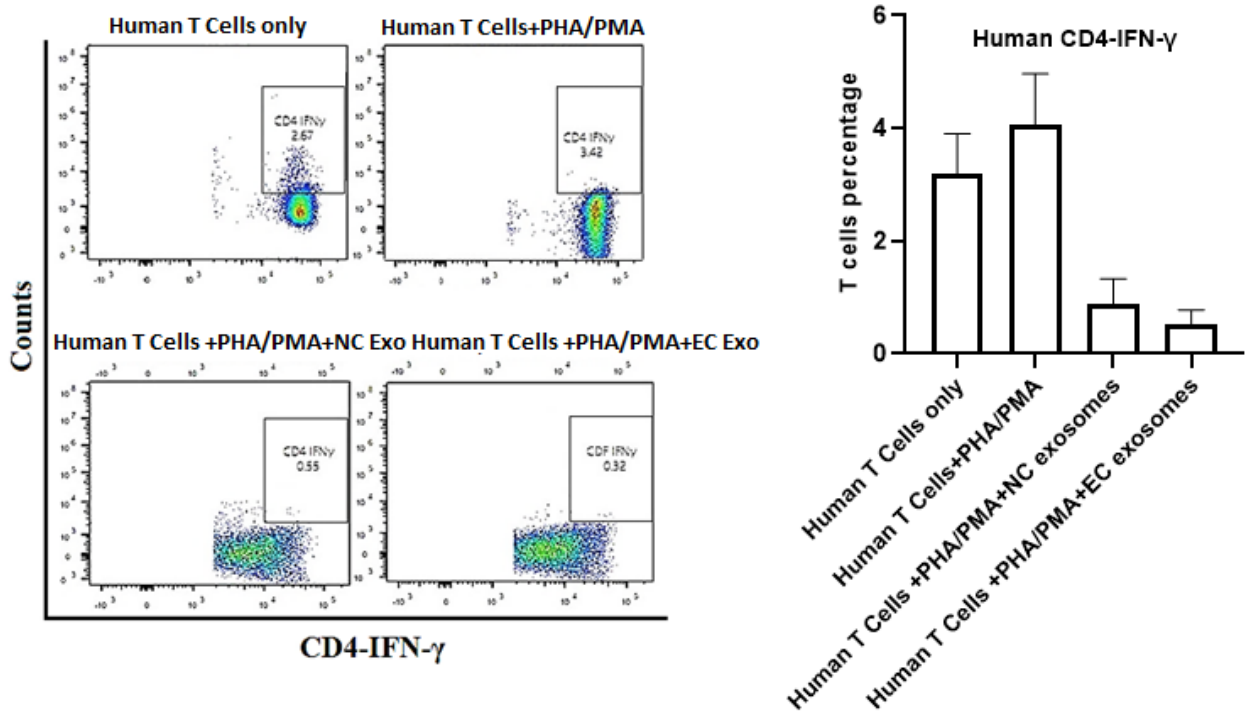


Figure 6A. Flow cytometer analysis of CD4-IFNγ labeled Human T cells.

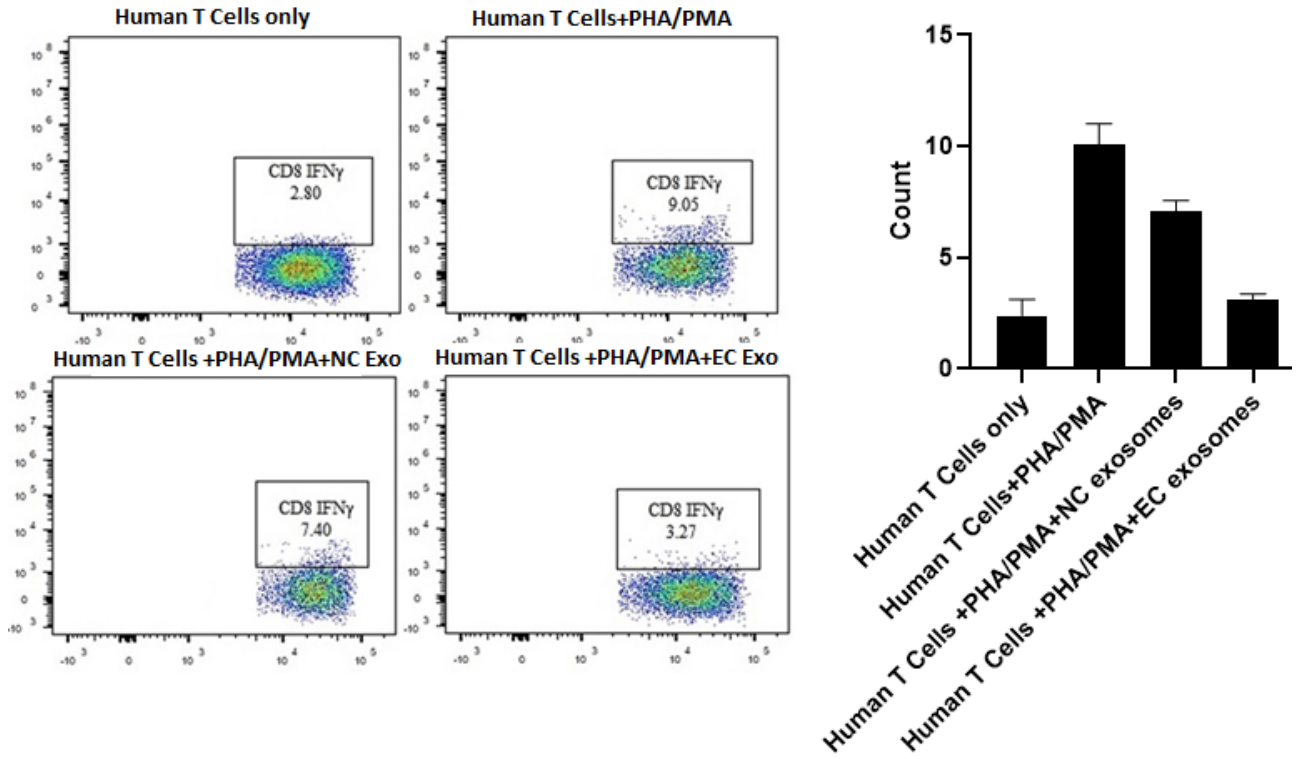


Figure 6B. Flow cytometer analysis of CD8-IFNγ labeled Human T cells.

DISCUSSION

Since the discovery of EXs in 1980 this area of research has a great deal of interest because of the biologically important nature of EXs. These tiny particles are produced in both normal and pathological conditions [16]. Their normal functions include cell to cell communication and eliminating cellular waste in the extracellular region. In the early years, these agents were only considered to be responsible for keeping the cellular environment clean but with the advancement in techniques, several other roles of EXs we unveiled. Many studies have revealed the importance of EXs in disease conditions as they are produced in aberrant form with a difference in their contents of cargo in comparison with health samples [17]. This nature of EXs has sparked the area of diagnostic biomarkers identification [18]. The role of EXs has also been studied in detail regarding cancer but still many questions are left unsolved. During the cancer microenvironment, there are different morphological and physiological changes observed with various identified agents playing role in its establishment. Among them, EXs are also targeted to evaluate them for their functions in such situations. The role of EXs in immune modulation has also been studied in wide. In the current study we analyzed the EXs production in two different conditions and to find its effects on Jurkat cells proliferation. Along with these we also studied the effects of cancer EXs on the expression of CD4-IFN γ and CD8-IFN γ. We have analyzed the proliferative effects of H1975 derived EXs on Jurkat cells and their suppressive role in IFN-γ in both CD4 and CD8 cells. These results are cell type-specific and need detailed validation in vivo and could be also assessed in more cell lines to confirm the role of EXs in immune cells proliferation and cytokines expression. If such results correlate with more detailed studies, then we can conclude that the cancer microenvironment potentially targets the immune suppression leading to the severity of cancer. Because there are diverse biological actions of IFN-γ which may implicated in pregnancy, obesity, allergies, autoimmune diseases, as well as in cancer. In cancer biology, results from early studies established IFN-γ as a prototypical antitumor cytokine. However, findings that endogenous IFN-γ not only controls tumor initiation and progression but also shapes tumor immunogenicity and promotes the outgrowth of tumor cells with immune-invasive properties. In a tumor microenvironment, numerous cells of innate and adaptive immunity produce IFN-γ. Two main producers of IFN-γ are natural killer (NK) cells, providing signals from their activating receptors that prevail over signals stemming from inhibitory receptors, and tumor-specific cytotoxic CD8+ T lymphocytes (CTLs) following antigen stimulation of T cell receptor (TCR) or in TCR-independent way that involves synergistic stimulation with cytokines IL-12 and IL-18 [19]. Th1 polarized CD4+ T helper cells, known for their ability to help in the promotion and maintenance of anti-tumor CTL responses, also secrete IFN-γ that recruits various cells of innate and adaptive immunity to tumor sites and promote their activation. Early studies of IFN-γ effects on various cancer types revealed its extensive anti-tumor potential. Among them, the best known IFN-γ-mediated effect is the augmentation of the cytotoxic function of NK cells and CTLs as potent effectors of anti-

tumor responses [20]. It is also very well known that IFN-γ enhances antigenicity of tumor cells via up-regulation of the major histocompatibility complex (MHC) class Ia membrane expression. As tumor cells express antigens that differ from their non-transformed counterparts, e.g., neo-antigens resulting from gene mutations, overexpressed cellular antigens or viral antigens, IFN-γ stimulates expression of tumor antigen-presenting MHC molecules to increase immunogenicity of tumor cells and makes them more susceptible to immune recognition and destruction.

Funding: This research received no external funding.
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Howlader N, Forjaz G, Mooradian MJ, Meza R, Kong CY, Cronin KA. The Effect of Advances in Lung-Cancer Treatment on Population Mortality. N. Engl. J. Med. 2020;383(7):640-9.
2. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nat. 2018;553(7689):446-54.
3. Massarelli E, Papadimitrakopoulou V, Welsh J, Tang C, Tsao AS. Immunotherapy in lung cancer. Trans.Lung Canc.Res. 2014;3(1):53.
4. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Canc. 2012;12(4):252-64.
5. Somasundaram A, Burns TF. The next generation of immunotherapy: keeping lung cancer in check. J. Hem Onco. 2017;10(1):1-12.
6. Speiser DE, Ho P-C, Verdeil G. Regulatory circuits of T cell function in cancer. Nat. Rev. Immun. 2016;16(10):599-611.
7. Donadon M, Hudspeth K, Cimino M, Di Tommaso L, Preti M, Tentorio P, et al. Increased infiltration of natural killer and T cells in colorectal liver metastases improves patient overall survival. J. Gast.Surg. 2017;21(8):1226-36.
8. Kirilovsky A, Marliot F, El Sissy C, Haicheur N, Galon J, Pagès F. Rational bases for the use of the Immunoscore in routine clinical settings as a prognostic and predictive biomarker in cancer patients. Int. Immun. 2016;28(8):373-82.
9. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. Nat. 2015;523(7559):177-82.
10. Gutiérrez-Vázquez C, Villarroya-Beltri C, Mittelbrunn M, Sánchez-Madrid F. Transfer of extracellular vesicles during immune cell-cell interactions. Immuno. Rev. 2013;251(1):125-42.
11. Xie M, Xiong W, She Z, Wen Z, Abdirahman AS, Wan W. Immunoregulatory effects of stem cell-derived extracellular vesicles on immune cells. Front.in Immun. 2020;11.
12. Maybruck BT, Pfannenstiel LW, Diaz-Montero M, Gastman BR. Tumor-derived exosomes induce CD8+ T cell suppressors. J.for Immun. of Canc. 2017;5(1):1-15.
13. Dakubo GD. Advanced Technologies for Body Fluid Biomarker Analyses. Canc. Biomark. in Body Fluids: Spring; 2016:55-74.
14. Dilsiz N. Role of exosomes and exosomal microRNAs in cancer. Future science OA. 2020;6(4):FSO465.
15. Lefort CT, Kim M. Human T lymphocyte isolation, culture and analysis of migration in vitro. JoVE. 2010(40):e2017.
16. Kalishwaralal K, Kwon WY, Park KS. Exosomes for non-invasive cancer monitoring. Biotech. J. 2019;14(1):1800430.
17. Kok VC, Yu C-C. Cancer-derived exosomes: their role in cancer biology and biomarker development. Int. J. Nanomed. 2020;15:8019.
18. Lin J, Li J, Huang B, Liu J, Chen X, Chen X-M, et al. Exosomes: novel biomarkers for clinical diagnosis. The Sci. World J. 2015;2015.
19. Harlan LM, Djeu JY. Current and Future Role of Natural-Killer Cells in Cancer Immunotherapy. Immuno. Canc: Spring; 2006. p. 45-58.
20. Ijzermans JN, Marquet RL. Interferon-gamma: a review. Immuno. 1989;179(4-5):456-73.