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Original Article

Effects of tumor derived exosomes on T cells markers expression

Efeitos de exossomos derivados de tumor na expressão de marcadores de células T

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Abstract

Exosomes are 30-120nm bio particles transferred from donor to recipient cells leading to modification in their regulatory mechanisms depending upon the coded message in the form of loaded biomolecule. Cancer cells derived exosomes the true representatives of the parent cells have been found to modify the tumor surrounding/distinct regions and participate in metastasis, angiogenesis and immune suppression. Tis study was aimed to study the effects of tumor mice derived exosomes on the normal mice spleen isolated T cells by using co-culture experiments and flow cytometer analysis. We mainly focused on some of the T cells population and cytokines including IFN-γ, FOXP3+ regulatory T (Treg) cells and KI67 (proliferation marker). Overall results indicated random changes in different set of experiments, where the cancer derived exosomes reduced the IFN-γ expression in both CD4 and CD8 T cells, similarly the Treg cells were also found decreased in the presence of cancer exosomes. No significant changes were observed on the Ki67 marker expression. Such studies are helpful in understanding the role of cancer exosomes in immune cells suppression in tumor microenvironment. Cancer exosomes will need to be validated in vivo and in vitro on a molecular scale in detail for clinical applications.

Keywords: cancer exosomes, immune suppression, T cells expression.

Resumo

Os exossomos são biopartículas de 30-120 nm transferidas de células doadoras para células receptoras, levando à modificação em seus mecanismos reguladores, dependendo da mensagem codificada na forma de biomolécula carregada. Verificou-se que exossomos derivados de células cancerosas – os verdadeiros representantes das células-mãe – modificam as regiões circundantes / distintas do tumor e participam da metástase, angiogênese e imunossupressão. Este estudo teve como objetivo estudar os efeitos de exossomos derivados de camundongos com tumor nas células T isoladas de baço de camundongos normais, usando experimentos de cocultura e análise de citômetro de fluxo. Concentrou-se, principalmente, em algumas populações de células T e citocinas, incluindo IFN-γ, células T reguladoras FOXP3 + (Treg) e KI67 (marcador de proliferação). Os resultados gerais indicaram mudanças aleatórias em diferentes conjuntos de experimentos, em que os exossomos derivados de câncer reduziram a expressão de IFN-γ em células T CD4 e CD8, da mesma forma que as células Treg também foram encontradas diminuídas na presença de exossomos de câncer. Nenhuma mudança significativa foi observada na expressão do marcador Ki67. Esses dados são úteis para a compreensão do papel dos exossomos do câncer na supressão de células do sistema imunológico no microambiente tumoral. Exossomos de câncer precisarão ser validados in vivo e in vitro em escala molecular com detalhes para aplicações clínicas.

Palavras-chave: exossomos de câncer, imunossupressão, expressão de células T.

1. Introduction

Cancer patients are still poorly investigated (Jeske et al., 2018) and angiogenesis has been one of the major focal points in cancer research (Katona et al., 2015). Most of the recent therapeutic approaches used to treat cancer have adverse side effects (Almalki et al., 2021). T-cell immunotherapy is frequently being used to cure solid tumors, including non-small cell lung cancer (NSCLC). This needs a greater understanding of the T cells found in the lungs of NSCLC patients (Reuben et al., 2020). NSCLC has a large mutational burden (Alexandrov et al., 2013), which has been related to tumor-specific antigens known as neo-antigens, which can activate anti-tumor T cell responses in the host. This has rekindled interest in medicines that target the T cell repertoire, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) checkpoint inhibition (CTLA-4) (Hodi et al., 2010), PD-1,

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and PD-L1 (Brahmer et al., 2012). Immunohistochemistry was initially used to identify immune cells in NSCLC. These investigations discovered that NSCLC tumors include a wide range of immune cells, including T cells, B cells, NK cells, macrophages, and dendritic cells (DCs), and that there are several relationships between immune cell density and patient survival (Schalper et al., 2015). Tumor microenvironment is made up of a number of intricate mechanisms and actions through which tumor cells infiltrate the surroundings, suppressing immune cells. Many of the mechanisms are still unclear, but over the last two decades, extracellular vesicles, particularly exosomes, which are produced by cells for communication and to clean up the cellular environment, have gained popularity. However, multiple studies have revealed that these tiny nano biomolecules have a significant impact on various cancers. Angiogenesis, EMT, invasion, migration, and the formation of a pre-metastatic niche are all stages of the invasion and metastasis cascade where cancer cells produce and secrete abnormal quantities and quality of exosomes in the extracellular environment, which travel through the bloodstream to different distinct and nearby organs and play important roles in various stages of the invasion and metastasis cascade (Becker et al., 2016). Exosomes reflect the donor cell's behavior and regulatory mechanism. (Valadi et al., 2007). According to one of the recent update listings of the exosome database, exosomes include a range of components, including 3408 mRNAs, 9769 proteins, 1116 lipids and 2838 miRNAs (Xie et al., 2019). Exosomes cargo can be used as prognostic markers (Lai and Friedman, 2017) and/or a grading system for cancer progression. In tumor cells, it also controls tumor development, metastasis, and angiogenesis (Sharma, 2014), as well as mediating treatment resistance. (Mimeault and Batra, 2014). Exosomes are nano-sized phospholipid carriers produced by cells that deliver large amounts of bioactive molecules to particular target areas. This exosomes mediated cell to cell communication has an impact on several hallmarks of cancer, including changing the extracellular matrix composition, providing cancer cells with drug resistance traits, and even influencing immunological responses. Exosomes may potentially be used in numerous new immunological techniques to trigger adaptive and innate immune effector cells to make an effective anticancer immune-surveillance due to their immune-modulatory capability and endogenous activities. (Dutta, 2021). For cell survival and response to paracrine and endocrine signals, intercellular interactions between surrounding and distant cells are critical (Francis and Palsson, 1997). Tumor genesis requires cell-to-cell communication as well. Tumors are not separate entities; they are the result of a complex interplay between altered and non-transformed cells that promotes tumor progression, growth, metastasis and angiogenesis (Maia et al., 2018). The exchange of material between cells is necessary for cell communication and survival. Exosomes and other extracellular vesicles (EVs) are emerging as new cell-cell communication mediators in both healthy and pathological circumstances (Tetta et al., 2013). Exosomes are distinguished from extracellular vesicles such as apoptotic bodies and micro vesicles (MVs) by their biogenesis, release mechanisms, size, composition, and function. MVs are created by direct outward budding of the cell membrane, whereas apoptotic bodies are released into the extracellular space during cell death. Exosomes are formed by inward budding of the membrane of early endosomes, which eventually mature into multi-vesicular bodies (MVBs) (Doyle and Wang, 2019). Exosomes generated from tumor cells play a significant role in communication by transferring numerous biomolecules such as proteins, lipids, DNA, and RNA, according to recent studies (Rahbarghazi et al., 2019). Exosomal cargo has a striking resemblance to the parent cell's internal components. The detection of these exosomal constituents in real time could provide crucial information for diagnosis, prognosis, and management of diseases (Javeed and Mukhopadhyay, 2017). Exosomes can be used as diagnostic biomarkers or even as carriers of anticancer medicines in the clinic. Because of their clathrin-coated membrane, exosomes are exceptionally durable and resistant to degradation enzymes like RNases, making them a valuable diagnostic and therapeutic tool (Gurunathan et al., 2019). Exosomes have a wide range of applications due to their unique biological and pathological features. Exosomes' significance in immunological control has been shown by a growing body of research in recent years. This has encouraged additional research into the use of these vesicles in anticancer therapies, such as delivery agents or immunological regulators (Wang et al., 2017).

2. Materials and Methods

In the current study we analyzed the level of production of exosomes in normal and tumor mice models and the isolated exosomes were co-cultured with normal mice spleen isolated T cells to study the expression level of different markers related to T cells subpopulation and secreting cytokines.

2.1. Tumor genesis study and blood collection from mice

In the current study C57BL/6 mice were enrolled from the experimental animal center and were housed in a pathogen-free facility with required conditions of temperature, light, humidity along with the water and food. C57BL/6 mice, 8-9 weeks old (weighing 21.6 ±0.8 g) were intra-peritoneally injected with (1x106 cells) H1975 cells and growth factor reduced matrigel in the right forelimb. After the tumor production started in mice and reached to approximately 70-100 mm3, blood was collected from the venous sinus in red topped tubes. The same procedure was followed to collect blood from normal mice to be used as a control.

2.2. Serum preparation

The collection tubes were kept at room temperature for blood clotting for 15-30 minutes. In the refrigerated centrifuge clots were separated by centrifugation at 1500 x g for 10 minutes. Serum as a supernatant was separated in new polypropylene tube using a Pasteur pipette and stored at 4^{o}C

2.3. Isolation of exosomes

A series of differential centrifugations were used for the isolation of exosomes. Large cell fragments were eliminated in the first centrifugation at 1500 x g for 10 min at 4°C. Further small debris were removed by centrifugation at 2500 x g at 4°C for 10 min. Then the supernatant was transferred to a new tube and ultracentrifugation was done through Sorvall WX80 ultracentrifuge (Thermo Scientific) at 120,000 x g for 120 min at 4°C to pellet down the small vesicles. To eliminate potential contaminants supernatant were discarded and the pellets were resuspended in 5ml PBS and filtered through 0.22um syringe filters followed by centrifugation at 120,000 xg for 120 min, the pellets were resuspended in 200ul PBS and 10ul of the purified exosomes are used for Nano sight quantification, while the rest of the samples were preserved at -80°C for further experiments.

2.4. Quantification of exosomes through nanosight

Nano sight (NS300) a nanoparticle tracking analysis (NTA) system was used to quantify isolated exosomes. The Nano sight inner compartment was washed two times by injecting 1 ml ultra-filtered ddH2O through a syringe, and then each sample was diluted in a labeled Eppendorf tube with the following protocol: 10 µl exosomes solution was added into 1 ml ultra-filtered ddH2O. Each sample was injected through a syringe and the machine was run by following the Nano Sight NS300 manual guide and the data was recorded.

2.5. Western blot analysis

Nano sight NTA can detect the particles based on their size. The particles which we assumed as exosomes were in the range of the size of exosomes (20-150nm) but these results need further validation to confirm the presence of exosomes on the molecular level based on their markers (CD63 and CD9). Exosomes were added with 100ul RIPA buffer. The samples were incubated on ice for 10-15 minutes and then were centrifuged at 13800 rpm at 4°C for 15 minutes. The supernatant (isolated protein) was separated in new labeled tubes and quantification was done through Bradford 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 (TBS) 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.

2.6. Immune cells isolation from mice spleen

Cells were collected from mouse spleen by following the standard protocol. In brief a healthy C57BL/6 mouse spleen was isolated from a normal mouse and washed multiple time with PBS and transferred to RPMI media. The spleen was crush on the sieve and serially washed with 10ml of PBS to the sieve after crushing it nicely and collected in 50ml tube. The filtered solution was centrifuged at 1200xg for 5 minutes and the pellet was washed with 10 ml 1X PBS and resuspended. Another tube containing10ml percoll was taken and gently add the spleen cells to the falcon with percoll using 10ml pipette by slightly tilting the falcon while considering not to mix cells and percoll. The tube was centrifuged for 10 minutes at 1200xg. A buffy layer in the middle of the tube was observed. This was transferred to another 50ml tube carefully without mixing it with the debris. The tube was filled with 1X PBS. Centrifuge it for 10 mins, 1200xg. Discarded the supernatant and added 1ml PBS to the pellet and cells were count and grown on media at 37°C.

2.7. Mice T cells and tumor mice and normal mice isolated exosomes co-culture

Mouse T cells (2x106/ml) were co-cultured in six-well plates with the exosomes (200ul) from both normal and tumor derived mice. The plates were then incubated at 37 °C for 24 hours and the expression of various markers of mice T cells were observed by using a flow cytometer.

3. Results

3.1. Exosomes quantification

Serum collected from a group of five mice in a single tube was processed for exosomes isolation as shown in Figure 1a. This was practiced for both normal and tumor mice. All exosomes tubes to be used for co-culture experiments were kept at -80°C, while one tube from each group was processed for quantification through Nanosight and western blot analysis.

From the nanoparticle tracking analysis through nanosight the Tumor derived exosomes concentration was found $9x10^9$ particles / ml and were heterogeneous in size ranging from 30 to 300nm as shown in Figure 2a, Mean size was 187.7 nm and mode was 142.9 nm. For the normal mice exosomes concentrations was $8x10^7$ particles / ml and were heterogeneous in size ranging from 30 to 200nm as shown in Figure 2b, Mean size was 133.2 nm and mode was 133.8 nm. We also confirmed the presence of exosomes through western blot analysis by using exosomes surface markers (CD9 and CD63). The results confirmed the isolated exosomes and the difference in band intensity showed difference in the concentration in both normal and tumor exosomes as shown in Figure 2c.

3.2. Analysis of T cells co-culture with normal and tumor mice exosomes

This experiment was conducted in a six well plate. The first well was loaded with mice T cells only ($2x10^6$ cells) in 2 ml media. Similarly same number of mice T cells was added with normal exosomes (200µl) in the second well and the third well was added with 200µl tumor mice exosomes and mice T cells. phytohemagglutinin

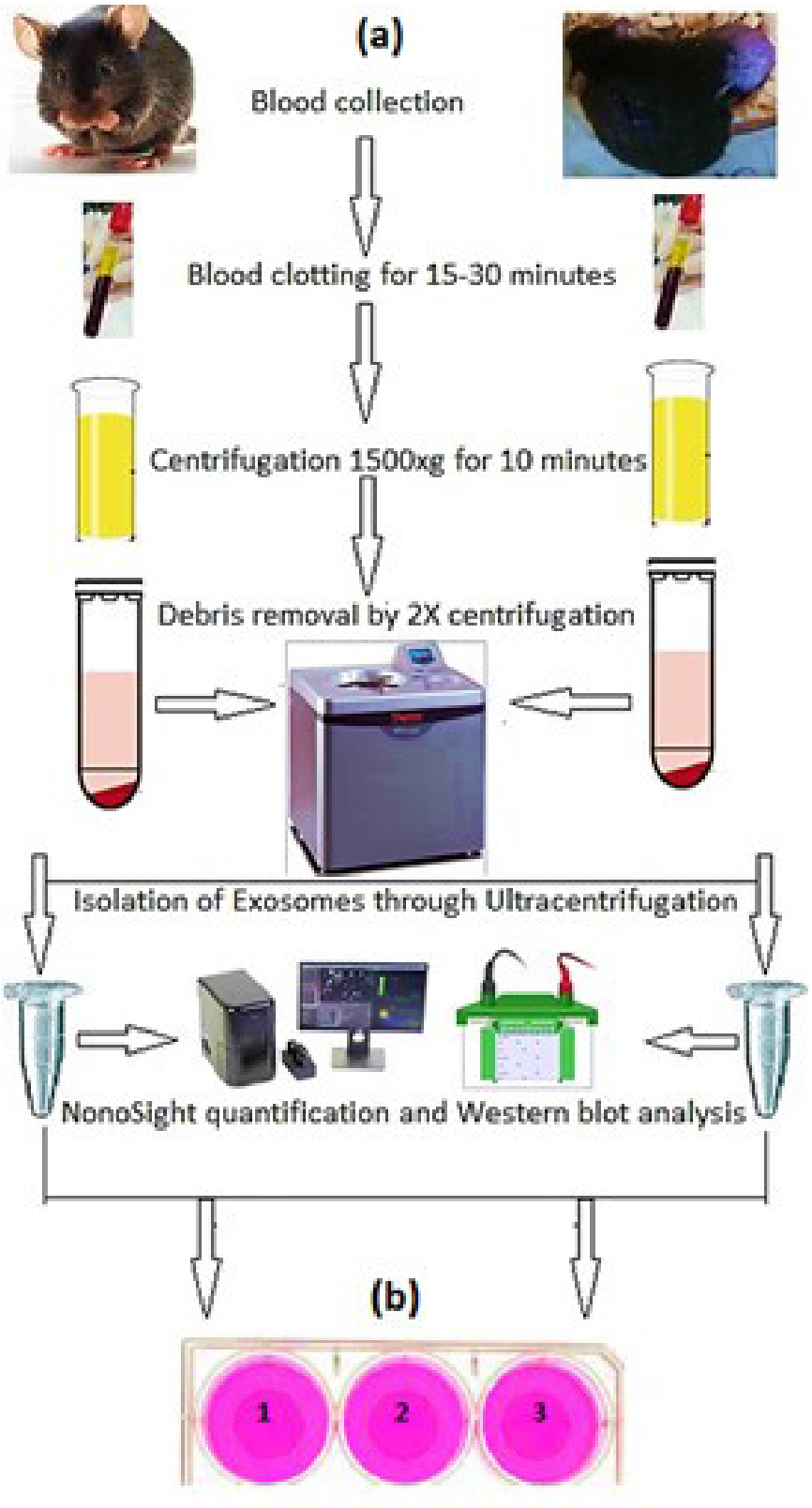

Figure 1. (a) Experimental flow of exosomes isolation from Normal and Tumor Mice. (b) Co-culture experimental design plat with normal mice T cells and Tumor mice derived exosomes.(1) Mice T cells only in media, (2) Mice T cells+ Normal mice exosomes, (3) Mice T cells+ Tumor mice exosomes.

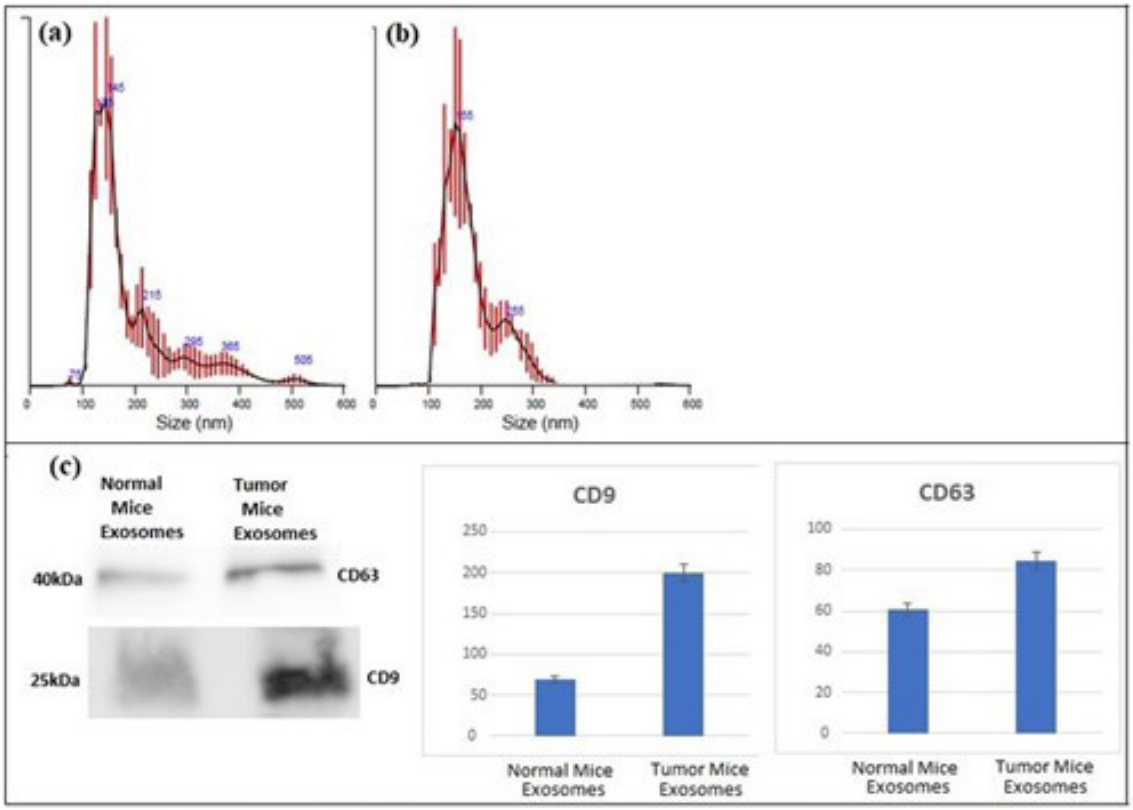

Figure 2. Quantification and confirmation of Exosomes. (A) Nano Sight results of tumor and (B) normal mice exosomes. (C) and their Western blot analysis through exosomes specific CD9 and CD63 antibodies.

(PHA) / phorbol 12-myristate 13-acetate (PMA) (T cell stimulators) was added to all wells for T cells activation. After the 24 hours of incubation the cells were separated from the media through centrifugation and were analyzed through Fluorescence-activated cell sorting (FACS). The results were analyzed through FlowJo software. Each experiment was repeated three times. The data shown here is a mean of all experiments. In the first set of our experiments we analyzed the expression of Interferon gamma in CD4+ T cells. Interferon-gamma is secreted mainly by activated lymphocytes such as CD4 T helper cells and CD8 cytotoxic T cells. Our results showed a significant decrease in CD4-IFNγ expression when treated with the tumor mice derived exosomes. On the other hand a slight rise was seen in the IFNγ expression after the T cells were treated with normal mice exosomes as shown in Figure 3. This result shows a variation in the mode of action of normal and cancer exosomes on the IFNγ expression in CD4 T cells.

In the second round of experiment IFNγ expression was also analyzed in CD8 T cells where both the normal and tumor exosomes were found with a reduced CD8-IFNγ expression. Comparatively high reduction was observed in the tumor exosomes treated cells as shown in Figure 4. Thus tumor exosomes may affect the IFN-γ associated anti-proliferative, pro-apoptotic and antitumor mechanisms in cancer microenvironment.

Further the effect of cancer exosomes were also analyzed on the expression of Ki67 marker in CD8 T cells. Ki67 is a cancer antigen that's found in growing, dividing cells but is absent in the resting phase of cell growth. These results had a variation in every repeated experiment but overall the expression of Ki67 was stable after the T cells were treated with both normal and cancer exosomes. No significant changes were observe as shown in Figure 5. We also identified that cancer exosomes reduced the Treg cells (23%) which were 28.9% in normal exosomes treated T cells while the untreated T cells were shown 35% of the Treg population as shown in Figure 6.

4. Discussion

In the current study we tried to isolate exosomes from normal and tumor mice serum. After the quantification and quality of exosomes was confirmed, we analyzed the effects of these exosomes on the mice spleen isolated T cells. Where we found some hints from our results that cancer exosomes may have a different role on the proliferation of T cells sub population and also the T cells cytokine. Our results demonstrated that CD4 T cells were shown a lower expression of IFNγ (3.8%) in the presence of tumor exosomes in comparison with normal exosomes (13.7%) and none treated T cells (11.4%). Similarly the cancer exosomes

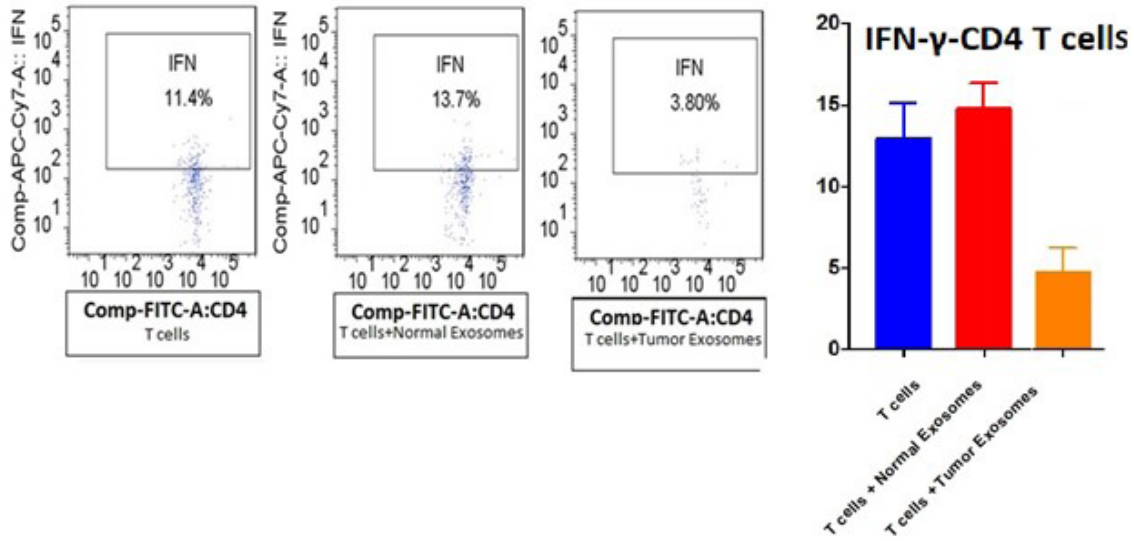

Figure 3. Flow cytometer analysis of CD4+ Interferon gamma (CD4-IFNγ).

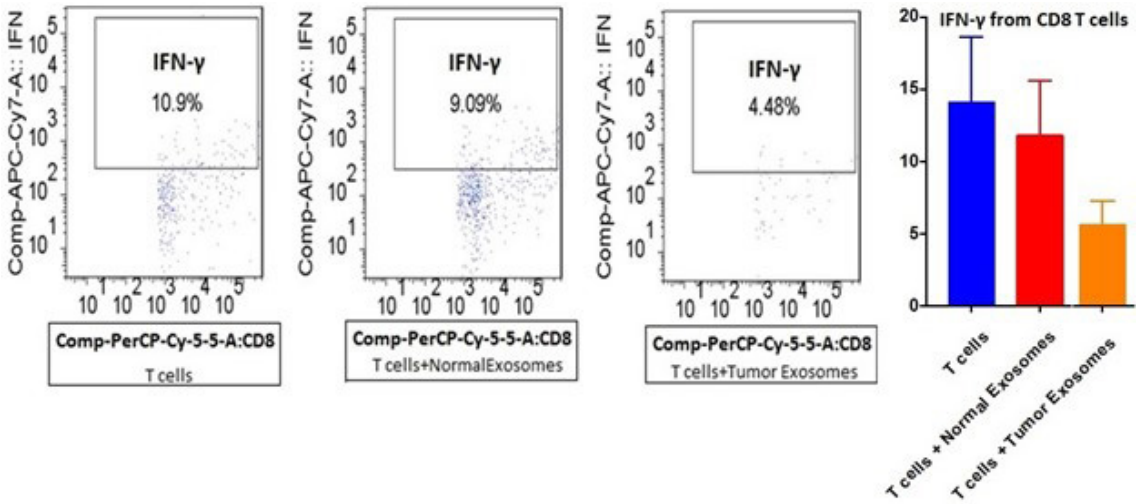

Figure 4. Flow cytometer analysis of CD8+ Interferon gamma (CD8-IFNγ).

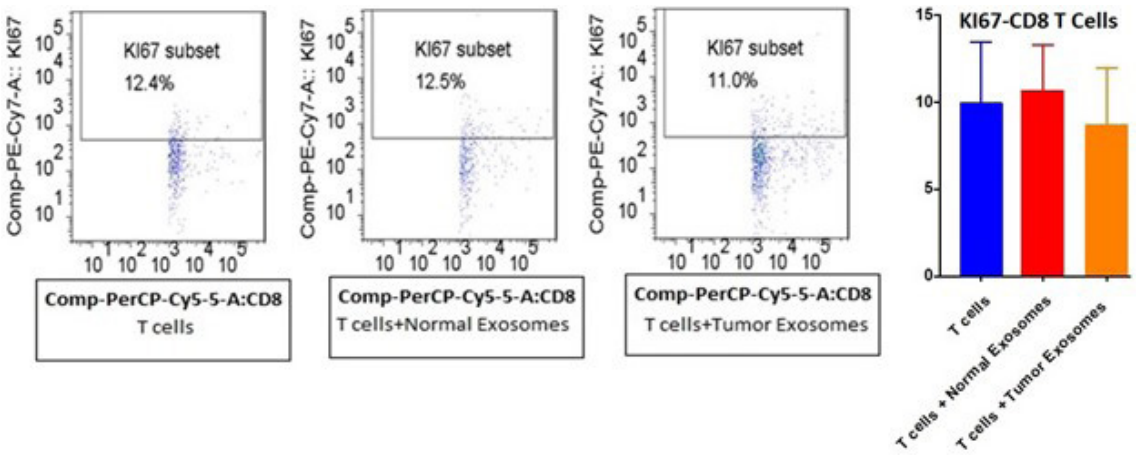

Figure 5. Flow cytometer analysis of CD8⁺ KI67 expression.

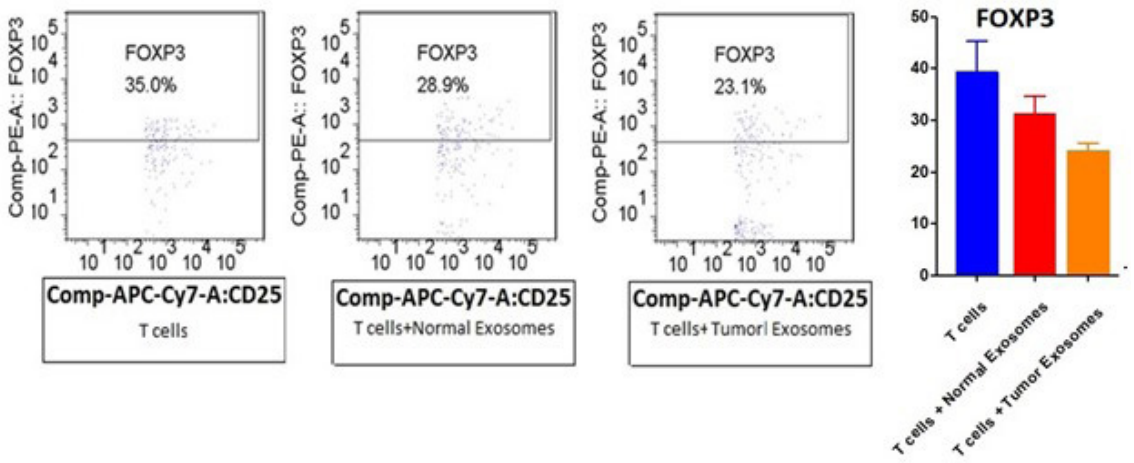

Figure 6. Flow cytometer analysis of Regulatory T cells (Tregs).

not only effected the expression of IFNγ in CD4 T cells but was also reduced in CD8 T cells, where T cells treated with tumor exosomes were 4.48%, normal exosomes treated cells were shown the expression of CD8-IFNγ as 9.9% and the results were more similar between normal exosomes treated T cells (10.9%) and none treated T cells (9.9%). We also analyzed the expression of Ki67 maker where no significant changes were seen between normal exosomes, tumor exosomes and none treated T cells. There was a significant decrease in the Treg population. None treated T cells were 35%, where the normal exosomes treated cells were 28.9% and the tumor exosomes treated T cells had only 23.1% Treg population. On the basis of above results we can suggest to study such effect of cancer exosomes not only T lymphocytes and their cytokines but also on other types of immune cells with more detail both in vitro and in vivo. Cancer cells potentially target the immune cells through exosomes leading to more complications and severity. Because there are diverse biological actions of every member of the immune system and down regulation or deactivation of any of them will lose its contribution in fighting cancer. Tumor exosomes are emerging as novel immune-regulatory factors. It's remarkable and provocative to convey and deliver inhibitory or stimulatory signals to immune effector cells. The cellular composition of the tumor micro environment and the types of cells targeted by exosomes may be the key to tumor exosomes dual functional potential. Tumor exosomes-driven interactions can be direct or indirect, with the presence or absence of immunological recipient cells in the tumor microenvironment determining the outcome of tumor exosomes signaling. Immune cells infiltrating tumor have a strong incentive to produce immunosuppressive tumor exosomes in order to disarm antitumor effector cells. Immunotherapies that block suppressive pathways induced in immune recipient cells by tumor exosomes delivering juxtacrine or paracrine signaling may be effective against these tumors. Tumor exosomes are primarily used as a remarkably effective mechanism that favors tumor escape in the tumor microenvironment, where tumor cells are actively involved in suppressing antitumor immunity mediated by infiltrating activated T cells. In this instance, with tumor exosomes committed to directly support tumor progression, immune therapies are likely to be ineffective, and conventional therapies designed to inhibit tumor progression warrant consideration. At the same time, it's important to note that all cells in the tumor cells generate exosomes, and that immune stimulatory signaling induced by tumor exosomes, whether directly or indirectly, can remodel the environment to support immune activities rather than tumor growth. The tumor's fate, and also the activities of the local immune response, will be determined by the balance of all of these intracellular interactions in the TME. In the future, molecular, genetic, or immunological therapies could be used to control tumor exosomes activity. Today, efforts are directed at the better understanding of molecular mechanisms driving tumor exosomes release, uptake by recipient cells and transcriptional or translational changes they induce. Such research could point to the possibility that cancer exosomes play a role in promoting metastasis by influencing immune system responses. Exosomes have a unique repertoire of mRNAs, miRNAs, and proteins, which has been thoroughly described. Exosomes from tumor cells can be transported to immune cells, affecting gene expression and cell behavior. The tumor-derived exosomal protein galectin-1 generated a suppressor phenotype in T cells, suggesting that it could be a viable therapeutic target for preventing T cell malfunction and enhancing anti-tumor immune responses. Cancer exosomes manipulate the immune system to create an immune-suppressive microenvironment. Because tumor derived exosomes affects gene expression and cytokine secretion patterns, CD8+ T cells are the major immune cell to attack the tumor. This could be linked to the accumulation of the contents in tumor derived exosomes. Exosomes are released at a high rate by cancer cells, and their cargo is critical for cancer growth. Considering the complexity and heterogeneity of cancer, understanding of the specific biological and molecular factors that lead to immune escape of tumor

derived exosomes and its components (protein and RNA) in vitro and in vivo would be essential for developing future therapeutic strategies.

5. Conclusion

In the last two decades exosomes are widely studied in different diseases including cancer. These nano bodies are the key players in immune therapeutics. Previously the role of exosomes in cancer have shown to affect the nearby and distinct regions in the body and exosomes are mainly involved to convey the cancer cells message in the form of certain cancer biomolecules. We also concluded that cancer exosomes may participate in deregulatory mechanisms of the T cells in mice which needs further validation to explore the molecular mechanism of these effects both in vitro and in vivo. Such studies will help to assess the pathological effects of cancer exosomes on immune cells dysfunction and lead to highlight pathways for therapeutic purpose.

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