Chemotherapy-Associated Extracellular Vesicles Modulate T Cells Activity and Cytokine Release

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Abstract: Colorectal cancer (CRC) remains one of the most widely diagnosed cancers worldwide. Despite the advances in medical research, there is still a lot to be explored between cancer cells and the tumor microenvironment, namely immune cells. Extracellular vesicles (EVs) have been shown to mediate communication between cells and can modulate the activity of immune cells. External stimuli such as stress and chemotherapy can influence the activity of the released EVs. Nevertheless, the relationship between chemotherapy, EVs and immune cells has yet to be fully explored. In this study, we aimed to elucidate the immune-related functional mechanisms of EVs isolated from pre- and post- FOLFOX chemotherapy from CRC patients. The EVs were isolated from the serum of matched patients and characterized via dynamic light scattering. The EVs were then co-incubated with primary CD8 T cells isolated from healthy donors and Jurkat cells. The apoptosis, cell cycle profile, gene expression and cytokines were evaluated. Upon treatment with EVs, the T cells underwent apoptosis however no differences were seen in the cell cycle phases. Gene expression related to cytokine release was also differentially expressed namely IRF4. The level of cytokines that were released also differed between the two groups. Our study has shown that there are some minor differences in the activity of the EVs after induction with chemotherapy.

Keywords: Exosomes, lymphocytes, FOLFOX, colorectal cancer.

INTRODUCTION

Colorectal cancer (CRC) is a global disease that affects millions of people worldwide and is the second most common cancer in the US [1]. In Malaysia, CRC is the second most common cause of cancer-related deaths in both men and women [2]. Among the standard treatment for CRC include surgery and chemotherapy. It has long been known that chemotherapy could affect immune cells especially lymphocytes [3]. Cancer cells can communicate with other cells through various mechanisms including releasing extracellular vesicles (EVs) [4]. EVs are a type of nano-sized vesicles that are released by cells into the extracellular environment. These EVs have been shown to mediate communication between cells and alter the activity of recipient cells [4].

Cancer-derived EVs could affect multiple processes in the tumor microenvironment. A recent study by Yasodha *et al*., showed that EVs isolated from metastatic colon cancer cells could promote proliferation and metastasis of cancer cells [5]. Cancer therapy such as chemotherapy or radiotherapy has been shown to affect the functional activity of EVs. For instance, proton treatment in head and neck squamous cell carcinoma resulted in less amount of EVs being released [6]. The authors also observed that both the treated EVs were able to suppress the release of IFN-γ [6]. The relationship between EVs and T cells has been well-documented in various studies before. For instance, in viral infection, it has been observed that the EVs released modulated activated T cells [7]. Meanwhile, in cancer, cancer-derived EVs could also affect T cells. Our previous study has demonstrated that EVs from CRC patients were able to induce apoptosis in CD8 T cells [8]. Nevertheless, the effects of chemotherapy on the activity of EVs in relation to immunosuppression have not been reported yet. Hence, this study is aimed at doing a preliminary investigation on the effects of FOLFOX-based chemotherapy on the functional activity of EVs.

MATERIALS AND METHOD

Sample Collection

Ethics approval was obtained from the Universiti Kebangsaan Malaysia's Ethics Committee with the approval number JEP-2019-089. Samples were taken at two time points; pre- and post-chemotherapy. The pre-chemotherapy samples were taken before any chemotherapy procedures were initiated. The post-chemotherapy samples were taken at HCTM Daycare Oncology Ward from various cycles of administration. All patients were diagnosed with colorectal cancer by pathological assessment.

EV Isolation

Due to the small volume of samples that we were able to retrieve, we utilized the precipitation method for EV isolation. In brief, a total of 50-100 µl of serum was

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added to an equal volume of the Total Exosomes Isolation Reagent (Thermo Fisher Scientific, USA). The homogenized mixture was incubated at 4 C for 30-45 minutes, followed by centrifugation at 12,000xg for 20 minutes. Afterward, the pellet was resuspended in an equal volume of 1XPBS and stored at -80 C until further usage.

Dynamic Light Scattering

We performed dynamic light scattering analysis using the ZetaSizer Nano ZS (Malvern Instruments, UK) according to the manufacturer's instructions.

T Cells Isolation

We utilized CD8 T cells as the main population to study due to its function as an effective effector cells against cancer. PBMCs were retrieved from healthy donors using Ficoll-Paque (Invitrogen, USA). Then, CD8+ population was further isolated using a magnetic-based isolation kit, EasySep CD8 Isolation Kit (StemCell Technologies, Canada). The purity of the population was analyzed using a flow cytometer (FACSVerse, BD) based on the CD3+CD8+ population.

Annexin V Assay and Cell Cycle Assay

Approximately, $1x10^5$ cells per well were seeded in each well of a 96-well plate. Then, the cells were activated with a CD3/CD28 activator (Immunocult, Stem Cell Technologies) overnight. Afterward, roughly 5 ug of EVs were added into each well and were left to incubate for 24-48 hours. The cells were then collected and subjected to staining using the Annexin V-FITC Apoptosis Kit according to the manufacturer's instructions (BD Pharmingen, United States). The cells were then evaluated using a flow cytometer (FacsVerse, BD). Apoptotic populations were considered in the FITC+PI- and FITC+PI+ populations. For the cell cycle assay, the treated cells were harvested and stained with PI using the Cell Cycle Kit (BD, USA). The stained cells were also acquired using the FacsVerse flow cytometer.

RNA Extraction

Total RNA was extracted using the GeneJET™ Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The quality and quantity of the RNA were evaluated using the NanoDrop spectrophotometer. The RNA was stored at -80 C until further usage. The co-incubation of EVs were done individually, but the CD8 T cells were pooled into 4 groups for the RNA extraction and qPCR analysis, two groups of pre-chemo and two groups of post-chemo.

cDNA Conversion

We performed cDNA synthesis using the Tetro™ cDNA Synthesis Kit (Bioline, UK) according to the manufacturer's instructions. Briefly, 20 ng of RNA was added to the reaction mixture and was subjected to various incubation periods for the cDNA synthesis.

qRT-PCR

After the cDNA synthesis was completed, we performed qRT-PCR analysis using the SensiFAST™ SYBR Green Kit (Bioline, UK). The primers were obtained from the AccuTarget™ Human qPCR Screening Kit (T Cell Anergy and Immune Tolerance) (Bioneer, South Korea). The qRT-PCR reaction was run on the CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA).

Cytokine Analysis

The supernatant from the co-culture experiment was harvested and stored at -80 C. The cytokine profile was analyzed using the Human CD8 / NK Panel LEGENDplex™ Multi-Analyte Flow Assay Kit (BioLegend, USA) according to the user's manual.

RESULTS

EVs were Isolated from the Serum of CRC Patients

The demographic data of the patients that were used in this study is listed in Table **1**. We managed to obtain 8 patients who had pre-chemotherapy serum previously stored. The post-chemotherapy serum was taken at Daycare Oncology, HCTM at various cycles. All the patients underwent FOLFOX-based therapy but were either given adjuvant or palliative therapy. To physically characterize the EVs, the EVs were subjected to dynamic light scattering (DLS) measurement. As shown in Figure **1A**, the isolated EVs were typically sized around 250 nm for the mean, and approximately around 100 nm for the mode diameter.

EVs Pre- and Post Chemotherapy affect the Proliferation of CD8 T Cells and Jurkat Cells

We performed cell cycle analysis on the isolated CD8 T cells and Jurkat cells upon co-incubation with the different EVs from all of the listed patients. After 24 hours, the average percentage of the cells in the G1,

Table 1: Demographic Data of the Selected Patients

Patient Number	Ethnicity	Gender	Number of Cycles	Type of Administration	Regimen
Patient 1	Malay	F	Cycle 9	Palliative	FOLFOX
Patient 2	Malay	F	Cycle 7	Adjuvant	FOLFOX
Patient 3	Chinese	F	Cycle 2	Adjuvant	FOLFOX
Patient 4	Malay	F	Cycle 1	Palliative	FOLFOX
Patient 5	Malay	F	Cycle 2	Palliative	FOLFOX
Patient 6	Malay	F	Cycle 8	Adjuvant	FOLFOX
Patient 7	Malay	F	Cycle 6	Adjuvant	FOLFOX
Patient 8	Chinese	M	On 5 years of surveillance	Adjuvant	FOLFOX

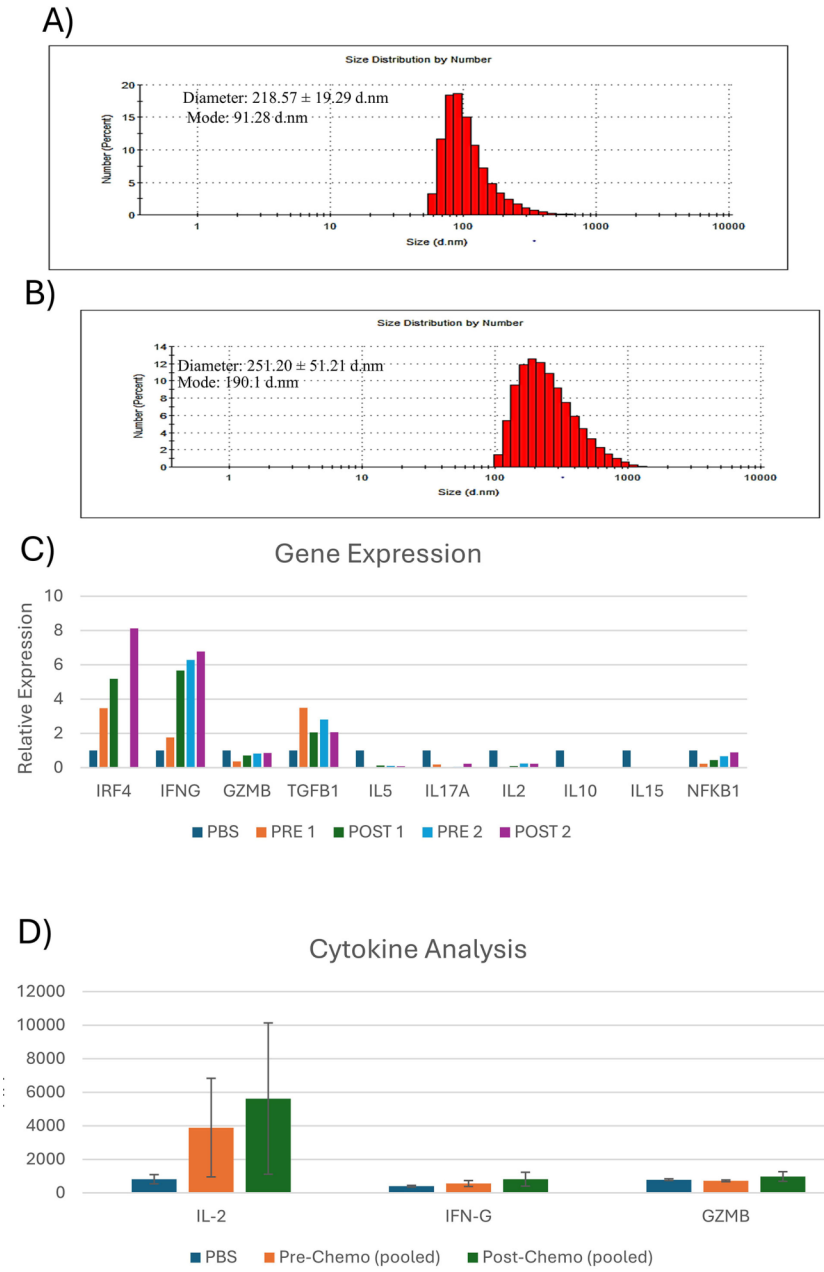

Figure 1: A) DLS analysis of a representative pre-chemo EV **B**) DLS analysis of a representative post-chemo **C**) Gene expression profile of selected genes upon treatment with different EVs. **D**) Fold change of cytokine expression upon treatment with different EVs

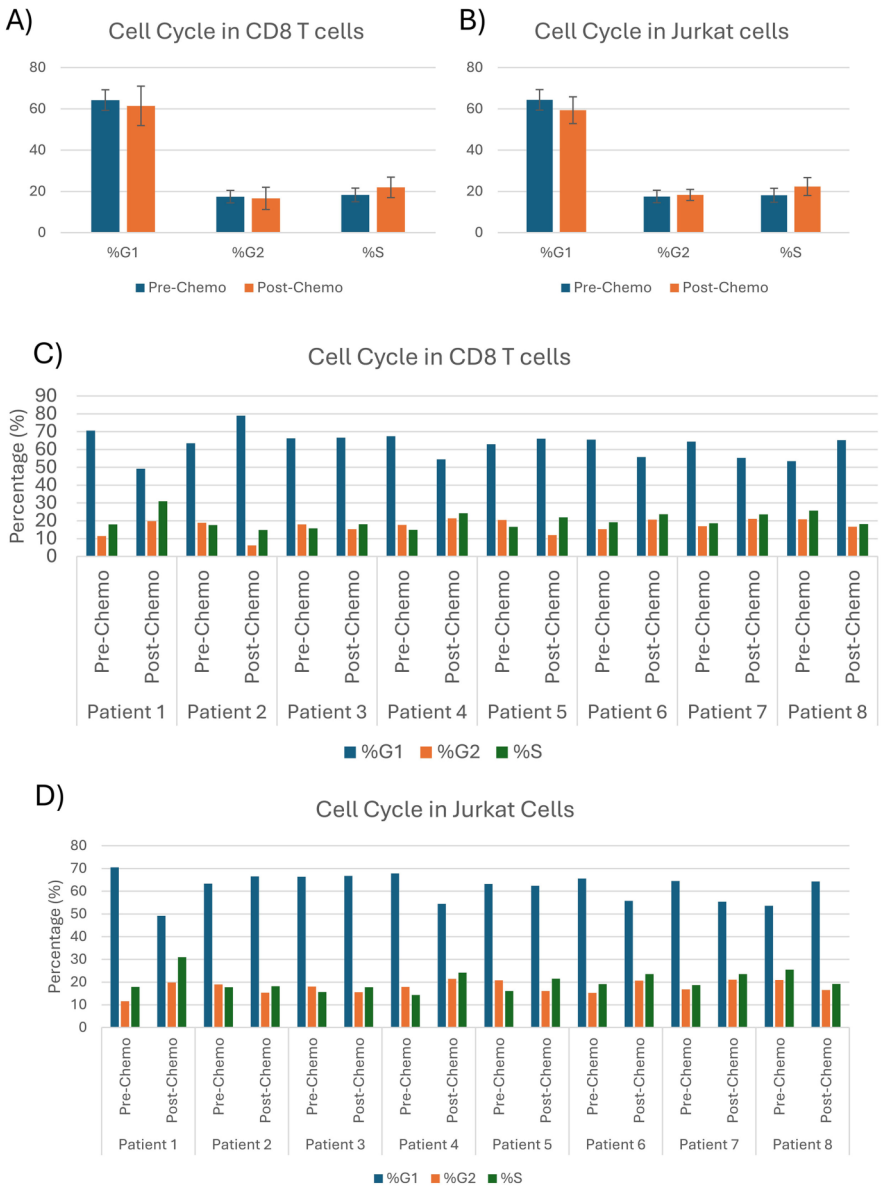

Figure 2: A) Pooled analysis for cell cycle in CD8 T cells. **B**) Pooled analysis for cell cycle in Jurkat cells. **C**) Individual analysis for cell cycle assay in CD8 T cells. **D**) Individual analysis for cell cycle assay in Jurkat cells.

G2 and S phase were measured. Based on the pooled analysis as shown in Figure **2A**, there were no significant differences in the different phases of the cell cycle when compared between pre- and post-chemo EVs in the CD8 T cells. Individually, there were slight variations in the percentage of cells between patients. For instance, in Patient 1 and 4, the percentage of cells in the S phase was higher in the post-chemo treated group as compared to the pre-chemo. Meanwhile, in Patient 2 and 8, the percentage of cells in S phase was lower. In Jurkat cells, a similar pattern was also observed in both the pooled analysis and the individual analysis. This indicates that the individual response to chemotherapy could vary based on different factors such as the number of cycles, thus releasing a functionally different population of EVs.

EVs Modulate the Cell Viability of T Cells

Apart from the cell cycle analysis, we also analyzed the apoptotic state of the cells upon treatment with the different EVs. As shown in Figure **3**, both pre and post-chemo EVs managed to induce cell death in CD8 T cells and Jurkat cells. The overall pattern shows that post-chemo EVs induced a slightly higher percentage of apoptosis as compared to pre-chemo EVs. This observation is similar in both CD8 T cells and Jurkat cells.

Expression of Immune-Related Genes

We also performed qPCR analysis to evaluate the expression of several key genes important in CD8 T

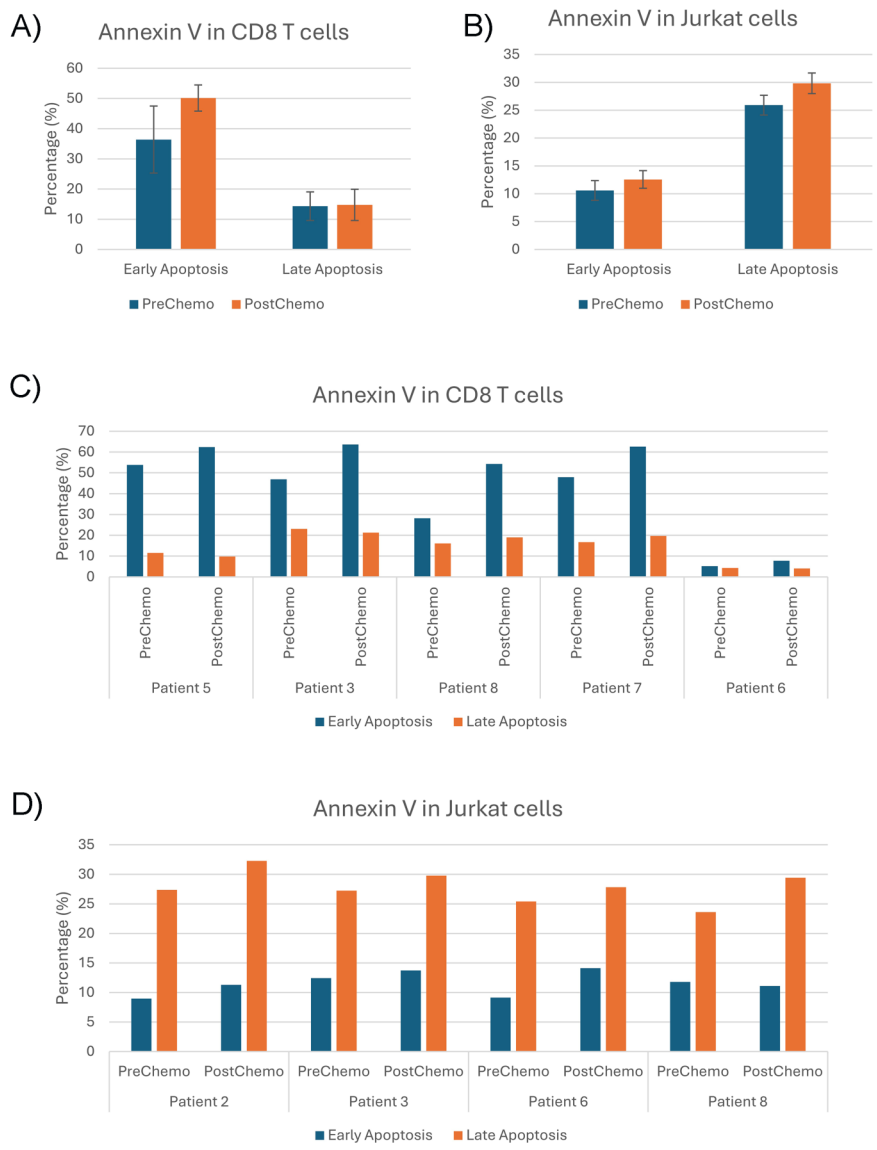

Figure 3: A) Pooled analysis for apoptosis assay in CD8 T cells. **B**) Pooled analysis for apoptosis assay in Jurkat cells. **C**) Individual analysis for apoptosis assay in CD8 T cells. **D**) Individual analysis for apoptosis assay in Jurkat cells.

cells function. Of the 10 selected genes, the *IRF4* gene had the highest differential expression. In both pooled groups, *IRF4* was upregulated in the CD8 T cells treated with the post-chemo group as compared to the pre-chemo group. A similar observation was also observed in the *IFN3* and *NFKB1* expression, as shown in Figure **1**. Interestingly, TGFB1 expression has a higher increase in the pre-chemo pooled group as compared to the post-chemo pooled group. A number of cytokines-related genes such as *IL-2, IL-17A, IL-10* and *IL-15* had a decreased expression in both pre and post-chemo -treated groups.

Cytokine Release

Cytokine detection was performed on the supernatant collected from the co-treated CD8 T cells. Based on our analysis, there was a decrease in the release of granzyme A in both pre-chemo and post-chemo groups. A slight increase was observed in the release of IFN-gamma and Granzyme B as shown in Figure **1**.

DISCUSSION

EVs are known to be mediators of cellular immunity even in the tumor microenvironment. Various external stimuli can affect the release and function of EVs, including stress, infections and also drugs. Our initial findings here show that EVs that are released after chemotherapy treatment, within the same patient possess slightly different functional activities. In this study, EVs were isolated using the precipitation method, which was the most feasible method available

to us at the time of the study. Studies have shown that this method of isolation may also precipitate other entities apart from EVs [9]. Therefore, we cannot conclusively state that the effects seen were purely due to the EVs. Although, the size of the EV population was also within range of what was expected, cautious interpretation of the results is still needed.

Previously, we have shown that EVs isolated from the serum of CRC patients could modulate the activity of T cells as compared to healthy-donor EVs [8]. In this study, a similar observation was also seen whereby EVs isolated from CRC patients also affected the activity of CD8 Tcells and Jurkat cells. Multiple studies have shown that tumor-derived EVs are able to regulate the proliferation and viability of T cells [8]. For instance, some studies have shown that tumor-derived EVs are able to regulate the proliferation and viability of T cells [8]. A study by Ludwig *et al*., showed that exosomes isolated from patients who had undergone oncologic therapies induced a lower rate of apoptosis in CD8 T cells, compared to the exosomes isolated from active disease patients [10]. A different study by Mondal *et al*., observed that EVs isolated from the plasma of breast cancer patients could induce apoptosis in CD8 and CD4 T cells [11]. Similarly, our study also showed that EVs from CRC patients could induce cell death in CD8 T cells and Jurkat cells. However more studies are needed to confirm the differentiation effects of chemotherapy due to the varying cycles of chemotherapy.

IRF4 is a transcription factor that has important roles in the development and function of CD8 T cells. Our analysis showed that EVs did indeed regulate IRF4 expression regardless of whether it was pre- or post-chemo EVs. Interestingly, post-chemo EVs induced a much higher expression of IRF4. Based on the current literature, no studies are reporting on the link between EVs and IRF4. Interestingly,a recent study has shown that IRF4 plays a role in the anti-tumor immunity of CD8 T cells [13]. The authors showed that the overexpression of IRF4 facilitates the anti-tumor efficacy of engineered CD8 T cells including the infiltration ability of the cells [13]. We could therefore postulate that post-chemo EVs may be able to improve the anti-tumor activity of CD8 T cells by increasing the expression of IRF4. A recent study by Hirsch *et al*., showed that IRF4 promoted T-cell proliferation and also affected cell cycle and NFKB-related genes [12]. A study by Vulpis *et al*., has shown that EVs isolated from multiple myeloma can affect natural killer cells through NFKB [14]. Additionally, the regulation of IFN-G in the CD8 T cells was also different between the pre- and post-treated EVs. IFN-G is an important cytokine in maintaining the activity of T cells and thus affecting its regulation is also important in maintaining the cellular dynamics [15].

CONCLUSION

Overall, this study has shown that EVs isolated from pre- and post-chemotherapy patients affected the viability and cell cycle profile of CD8 T cells and Jurkat cells differently. Additionally, the expression of certain genes including IRF4 also differed between these two treatments. Due to our small sample size and varying cycles of chemotherapy, the effects that we observed may not be substantial enough to conclude that chemotherapy indeed affected the EVs. Therefore, using a larger cohort of samples with a more homogenous chemotherapy profile should be included for future research. Moreover, in-depth mechanism-based studies could further shed light on the different pathways that are involved in chemotherapy-induced EVs.

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REFERENCES

[1] Siegel RL, Wagle NS, Cercek A, Smith RA, Jemal A. Colorectal cancer statistics, 2023. CA Cancer J Clin 2023; 73(3): 233-54. https://doi.org/10.3322/caac.21772

[2] Muhamad NA, Ma'amor NH, Rosli IA, Leman FN, Abdul Mutalip MH, Chan HK, *et al*. Colorectal cancer survival among Malaysia population: data from the Malaysian National Cancer Registry. Front Oncol 2023; 13: 1132417. https://doi.org/10.3389/fonc.2023.1132417

[3] Lissoni P, Fumagalli L, Brivio F, Rovelli F, Messina G, Di Fede G, *et al*. Cancer chemotherapy-induced lymphocytosis: a revolutionary discovery in the medical oncology. Journal of Biological Regulators and Homeostatic Agents 2006; 20(1-2): 29-35.

[4] Pitt JM, Kroemer G, Zitvogel L. Extracellular vesicles: masters of intercellular communication and potential clinical interventions. The Journal of Clinical Investigation 2016; 126(4): 1139-43. https://doi.org/10.1172/JCI87316

[5] Yasodha K, Lizha Mary L, Surajit P, Satish R. Exosomes from metastatic colon cancer cells drive the proliferation and migration of primary colon cancer through increased expression of cancer stem cell markers CD133 and DCLK1. Tissue and Cell 2023; 84: 102163. https://doi.org/10.1016/j.tice.2023.102163

[6] Chimote AA, Lehn MA, Bhati J, Mascia AE, Sertorio M, Lamba MA, *et al*. Proton Treatment Suppresses Exosome Production in Head and Neck Squamous Cell Carcinoma. Cancers (Basel) 2024; 16(5). https://doi.org/10.3390/cancers16051008

[7] Rausch L, Flaskamp L, Ashokkumar A, Trefzer A, Ried C, Buchholz VR, *et al*. Phosphatidylserine-positive extracellular vesicles boost effector CD8+ T cell responses during viral infection. Proceedings of the National Academy of Sciences 2023; 120(16): e2210047120. https://doi.org/10.1073/pnas.2210047120

[8] Abu N, Othman N, Ab Razak NS, Bakarurraini N, Nasir SN, Soh JEC, *et al*. Extracellular Vesicles Derived From Colorectal Cancer Affects CD8 T Cells: An Analysis Based on Body Mass Index. Front Cell Dev Biol 2020; 8: 564648. https://doi.org/10.3389/fcell.2020.564648

[9] Welsh JA, Goberdhan DCI, O'Driscoll L, Buzas EI, Blenkiron C, Bussolati B, *et al*. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. Journal of Extracellular Vesicles 2024; 13(2): e12404. https://doi.org/10.1002/jev2.12451

[10] Ludwig S, Floros T, Theodoraki MN, Hong CS, Jackson EK, Lang S, *et al*. Suppression of Lymphocyte Functions by Plasma Exosomes Correlates with Disease Activity in Patients with Head and Neck Cancer. Clinical cancer research: an official journal of the American Association for Cancer Research 2017; 23(16): 4843-54. https://doi.org/10.1158/1078-0432.CCR-16-2819

[11] Mondal SK, Haas D, Han J, Whiteside TL. Small EV in plasma of triple negative breast cancer patients induce intrinsic apoptosis in activated T cells. Communications Biology 2023; 6(1): 815. https://doi.org/10.1038/s42003-023-05169-3

[12] Hirsch T, Neyens D, Duhamel C, Bayard A, Vanhaver C, Luyckx M, *et al*. IRF4 impedes human CD8 T cell function and promotes cell proliferation and PD-1 expression. Cell Reports 2024; 43(7). https://doi.org/10.1016/j.celrep.2024.114401

[13] Yan H, Dai Y, Zhang X, Zhang H, Xiao X, Fu J, *et al*. The transcription factor IRF4 determines the anti-tumor immunity of CD8(+) T cells. iScience 2023; 26(11): 108087. https://doi.org/10.1016/j.isci.2023.108087

[14] Vulpis E, Cecere F, Molfetta R, Soriani A, Fionda C, Peruzzi G, *et al*. Genotoxic stress modulates the release of exosomes from multiple myeloma cells capable of activating NK cell cytokine production: Role of HSP70/TLR2/NF-kB axis. OncoImmunology 2017; 6(3): e1279372. https://doi.org/10.1080/2162402X.2017.1279372

[15] Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. Frontiers in Immunology 2018; 9. https://doi.org/10.3389/fimmu.2018.00847

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