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Effects of Lung Cancer Cells Derived Exosomes on T Cells Proliferation

by

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Effects of Lung Cancer Cells Derived Exosomes on T Cells Proliferation

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To My Parents, Family and Teachers

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It is certified that following publication(s) have been made out of the research work that has been carried out for this dissertation:-

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Abstract

Lung cancer's contribution to 24% of all cancer-related deaths establishes it as a prominent factor in the overall mortality from cancer. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases and is a significant contributor to cancer mortality worldwide. The tumor microenvironment contains high levels of tumor-secreted microvesicles, particularly exosomes, which play a critical role in the severity of the disease. Exosomes are small membrane vesicles (30-150 nm) that contain various biologically active compounds, including nucleic acids, proteins, lipids, and carbohydrates. They are produced within the endosomal compartment of cells and are essential for intercellular communication, both in normal and pathological conditions. Exosomes have diverse biological activities, including the promotion of cancer progression and the suppression of anti-tumor immune responses. This study focuses on investigating the effects of tumor-derived exosomes on T cells in order to gain a better understanding of their role and explore new opportunities for cell-based immunotherapy. The current project was aimed to analyze the effects of NSCLC derived exosomes on T cells proliferation, and also their markers expression. In the designed project a cell culture model mimicking tumor microenvironment was established, by growing NSCLC cells (H1975) in modified growth conditions (MGC). The conditions changed includes RPMI-1640 media (pH-6) without FBS and antibiotics, provided 1% Oxygen for 24 hours. The second condition was normal growth conditions (NGC) which is generally used for the growth of cell lines including RPMI-1640 media (pH-7) with FBS and antibiotics added, provided 20% Oxygen for 24 hours. Exosomes were isolated from the growth media using ultracentrifugation, and their quantification and confirmation was conducted through Nano sight and western blotting (CD63 and CD9 exosomes markers), respectively. Through western blotting, the presence and differential expression of selected NSCLC associated protein (CEACAM1, HVEM and PDL-1), previously found with inhibitory effects on T cells were also analyzed from both groups isolated exosomes. Jurkat cells were treated with the isolated exosomes from both groups to analyze the expression of CD69 and CD25 markers. Human blood isolated T cells were also treated with

these exosomes for the expression analysis of interferon-gamma (IFN-γ). Animal studies involving C57BL/6 mice bearing tumors were also conducted. Exosomes were isolated from the sera of these mice and treated with T cells isolated from the spleens of normal mice. This treatment aimed to identify the effects on natural killer T (NKT) cells quantity, and on the expression of ki-67, IFN-γ, and tumor necrosis factors alpha (TNFα) through flow cytometer. Exosomes from the serum of normal mice were utilized as a control for comparative purposes. NGC and MGC-derived Exosomes of H1975 cells were also studied for the NSCLC associated selected miRNAs through qRT-PCR and the targeted genes and their biological pathways were analyzed through bioinformatics tools. The results of Nanosight and western blot showed a higher production/secretion of the exosomes from the H1975 cells in the MGC in comparison with the NGC. From the Nanosight results, it was also confirmed that most of the particles (exosomes) were in the range of 30-150 nm. Protein markers (CD9 and CD63) from the western blots also confirmed the presence of the exosomes in MGC and NGC groups. CEACAM1, HVEM and PDL1 protein were also found with a differential expression in both groups. From the flow cytometer analysis, it was found that the MGC derived exosomes deregulated Jurkat cells markers (CD25 and CD69) expression. Human T cells were also found with a decreased expression of IFN-γ in both CD8+ and CD4 + T cells after treatment with isolated exosomes. From the animal model investigations, normal mouse spleen cells treated with tumor induced mice derived exosomes showed decreased NKT cells population. Similarly, the expression of TNFα, ki67 and IFN-γ was also found downregulated. The results of miRNAs expression from NGC and MGC groups demonstrated that among twelve selected miRNAs (miRNA- 122-5p, miRNA-20b-5p, miRNA-132-3p, miRNA-451a, miRNA-200b, miRNA-665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p, miRNA-30d-5p, miRNA-338-3p and miRNA-139-5p), The expression of nine miRNAs (miRNA-122-5p, miRNA-20b-5p, miRNA-132-3p, miRNA-451a, miRNA-200b, miRNA-665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p), was found in H1975 derived Exosomes in both conditions, while the three miRNAs (miRNA-30d-5p, miRNA-338-3P and miRNA-139-5p) were not shown any result/expression in both group. Among the nine expressed miRNAs, miRNA-122-5p, miRNA-132-3P, miRNA-29

and miRNA-30b-5p were found with a difference in expression in both groups. The bioinformatics analysis revealed that the expressed miRNAs targeted a range of genes, including those crucial for cell survival and division. In conclusion, this study emphasizes the impact of NSCLC-derived exosomes on T cells, providing insight into their role in immune responses. The analysis of exosomes from NSCLC cells grown in tumor-mimicking microenvironments revealed unique protein and miRNA profiles, highlighting their regulatory influence. The observed changes in T cell markers and functions, demonstrates the potential of exosomes as important contributors to immune modulation in the tumor microenvironment. These findings provide valuable insights for further exploration of cell-based immunotherapy for lung cancer.

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Abbreviations

ACS	American Cancer Society
APCs	Antigen-presenting cells
ATCC	American Type Culture Collection initially
Aβ	Amyloidβ
BCA	Bicinchoninic acid assay
BCL2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BTLA	B- and T-lymphocyte attenuator
CD	Cluster of differentiation
cDNA	Complementary DNA
circRNAs	Circular RNAs
c-MET	Mesenchymal-epithelial transition factor
CT value	Cycle threshold value
CTCs	Circulating tumor cells
ctDNA	Circulating tumor DNA
DCs	Dendritic cells
DHA	Docosahexaenoic Acid
DMSO	Dimethyl sulfoxide
ECM1	Extracellular matrix protein 1
EGFR	Epidermal growth factor receptor
EGFR	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERBB2	Erythroblast oncogene B
EVs	Extracellular vesicles

FABPs	Fatty acid-binding proteins
FACS	Fluorescence activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene ontology
GRB2	Growth factor receptor-bound protein 2
GWAS	Genome-wide association study
HAVCR2	Hepatitis A virus cellular receptor 2
HCC	Hepatocellular carcinoma
HSP70	Heat shock protein 70
HSV gB	Herpes simplex virus glycoprotein B
IFN-γ	Interferon gamma
IL	Interleukin
ILV	Internal luminal vesicles
IMPAD1	Inositol monophosphatase domain-containing protein 1
ISEV	International Society for Extracellular Vesicles
ITIM	Immuno-receptor tyrosine-based inhibitory motif
ITSM	Immuno-receptor tyrosine-based switch motif
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRAS	Kirsten rat sarcoma virus
LLC	Lewis lung carcinoma
lncRNAs	Long non-coding RNAs
LRG1	leucine-rich-alpha2-glycoprotein 1
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells
MEVs	Multi-epitopic vaccines
MGC	Modified growth conditions
MHC	Major histocompatibility molecules
MiRNA	Micro Ribonucliec acid

MITF	Melanocyte Inducing Transcription Factor
mM	Millimolar
MUC 1	Mucin 1
MVs	Microvesicles
MW	Molecular weight
NETs	Neutrophil extracellular traps
NGC	Normal growth conditions
NKT	Natural Killer T cells
NSCLC	None small cell lung cancer
NTA	Nanoparticles tracking analysis
NTA	Nanoparticle Tracking Analysis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PDI	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PHA	Phytohemagglutinin
pHe	Extracellular pH
pHi	Intracellular pH
PKG	Protein kinase Ge
PMA	Phorbol 12-myristate 13-acetate
PRAME	Preferentially expressed Antigen in Melanoma
PS	Phosphatidylserine
RBC	Red blood cells
RIPA	Radioimmunoprecipitation assay
RNU6-1	Endogenous controls U6
RPMI-1640	Roswell Park Memorial Institute Medium
SCLC	Small cell lung cancer
SEVs	Small extracellular vesicles
SILAC	Stable isotope labeling by amino acids in cell culture
SM	Sphingomyelin
SNX	Sorting-nexin
STAT3	Signal transducer and activator of transcription 3

STR	Single Tandem Repeat
TAM	Tumor-associated macrophages
TGF-β	Transforming growth factor-β
TGF-β	Transforming growth factorβ
TGF	Transforming Growth Factort
TGFA	Transforming Growth Factor Alpha
TILS	Tumor-infiltrating lymphocytes
TIM-3	T cell immunoglobulin mucin-3
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF-α	Necrosis factor -α
TNF	Tumor Necrosis Factors
TRAIL	TNF-alpha-related-apoptosis-inducing-ligand
Treg	Regulatory T cells
TRPS	Tunable Resistive Pulse Sensing
TSG	Tumor susceptibility gene
TSG101	Tumor susceptibility gene 101
TTA	Tumor-associated antigens
TTF-1	Thyroid transcription factor
UV light	Ultra violet light
VEGF	Vascular endothelial growth factor
μg	Microgram
μl	Microliter
μm	Micrometer

Chapter 1

Introduction

1.1 Lung Cancer

Cancer represents a group of cells characterized by uncontrolled and unrestrained proliferative potential. These abnormal cancer cells can divide without any external stimuli, invading surrounding tissue. Tissue type and location define the types of cancer, which includes lungs cancer, skin cancer, breast cancer, colon cancer, lymphoma, and prostate cancer. Cancer is the second most common disease and one of the leading causes of death worldwide [1]. By 2040, it is projected that there will be approximately 28.4 million new cancer cases occurring globally. In 2020, the global tally for new cancer diagnoses reached an estimated 19.3 million cases (excluding non-melanoma skin cancer), resulting in around 10.0 million cancer-related deaths. Specifically, breast cancer cases which are increased by 2.26 million, lung cancer by 2.21 million, stomach cancer by 1.089 million, liver cancer by 0.96 million, and colon cancer by 1.93 million. Pakistan is witnessing a rising trend in cancer incidence, with 19 million new cancer cases reported in 2020 [2]. Lung cancer is one of the most prevalent cancer in both men and women that is extremely aggressive and develop rapidly [3]. As per the report published by American Cancer Society, lung cancer makes up roughly 13% of all newly diagnosed cancers in the United States. In the year 2022, it was estimated that there were 236,740 new instances of lung cancer reported in the US, with 117,910 cases in males and 118,830 in females [4]. The estimated number of deaths attributable

to lung cancer in 2022 stood at 130,180, with 68,820 in males and 61,360 in females [5]. As per the American Cancer Society, the small cell lung cancer (SCLC) and the non-small lung cancer (NSLC) are the most prevalent types of cancers, making up around 80-85% of reported cancer cases, while the SCLC contributes to 15 of the total cases.

1.1.1 Non-Small Cell Lung Cancer (NSCLC)

The NSCLC includes not a single type of cancer but a range of distinct types of heterogeneous lungs cancers. This covers different types of adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Adenocarcinoma is most common out of all other lung cancers, with half of the lung's cancer are adenocarcinomas, originating from the distal airways epithelial, alveolar cells type II, which produce secretions like mucus and other substances [6].

Adenocarcinoma usually has glandular histology and has markers expressions, which are similar to the distal portion of lungs, including thyroid transcription factor 1 and keratin 7. The second one is squamous cell carcinoma, which originates from the pseudostratified columnar epithelium of tracheobronchial tree origin, however, most cases are from peripheral regions [7]. Squamous cell carcinoma is strongly linked with cigarette smoking patterns [6]. Adenocarcinoma and squamous cell carcinoma are usually differentiated from each other using immune staining techniques in clinical settings, based on markers like cytokeratin 5 and cytokeratin 6 or the use of transcription factors like p63 or SOX-2 [7]. The third type is large cell carcinomas, a group of NSCLC that includes all other lungs cancer not included in the other two types. The basic reason for the large cell carcinomas in a different category is the poor differentiation and absence of classification markers through immunohistochemistry. They originate from the lungs' central parts, occasionally lymph nodes, or spread to the chest wall or distinct organs [6].

1.2 Role of the Immune System in NSCLC

Tumor microenvironments are crucial to the growth of malignancies, which consists of cancer cells, dendritic cells, vascular endothelial cells, lymphocytes, fibroblasts, macrophages, and extracellular matrix. Cells secrete various types of substances to act as immune modulators, like chemokines, cytokines, enzymes, and growth factors [6]. Epithelial cells in the pulmonary tissue play a vital role in maintaining immune homeostasis within the lungs, they can produce inflammatory intermediaries that entice lymphoid cells and enhance antigen-presenting cells (APCs). In normal physiological situations, the immune cell's quantity and expression are fixed and the fraction of these immune cells linger in a typical range. Furthermore, the immune system plays an important role as it observes, identifies, and destroys cancerous cells. The T lymphocytes attack the cancer cells and cause tumor death. However, the impaired immune system mechanism in the cancer microenvironment disguises the tumor from an immune response by lowering the expression of antigen-presenting cells and co-stimulator molecules, escaping cancer cells from immune system surveillance as it is an important hallmark of cancer [8, 9]. The immune system variation in lungs cancer can act as biomarkers and differentiate between different types of lungs cancer, or even differentiate between subtypes. Furthermore, they can also aid in the prediction of immune therapy at clinical practices [10]. As in the case of non-small cell lungs carcinomas, thyroid transcription factor-1 (TTF-1), acts as a predictor of adenocarcinoma of the lungs. Other immune markers like cytokeratin 5 and cytokeratin 6 help to differentiate between non-small cell lung carcinoma subtypes [7]. Similar techniques are used for tumor markers, as they are linked to the anti-tumor response within the tumor microenvironment. Additionally, tumor-associated antigens (TAAs), typically found in normal tissue, exhibit increased expression in cancerous cells. Notable TAAs include CD19, p53, PRAME, ERBB2, MAGE, and L2A5, and they play a role in treatment responsiveness and tolerance [11, 12]. Moreover, there are also tumor-specific antigens, which are specific to a certain type of tumor due to higher or lower expression of these antigens [13, 14]. In non-small cell lungs

carcinoma, some of the immune cells are associated with good survival like tumor-infiltrating lymphocytes (TILS) which include cytotoxic CD8 T cells and CD4 helper T cells; however, regulatory T cells are highly associated with poor survival when their expression is higher. Furthermore, tumor-associated neutrophils and tumor-associated macrophages have a different effect on a different subtype of NSCLC subtype. Though, in spite of having different types of tumor immune markers and driver mutation in NSCLC, their response to immune-modulating therapy is similar. The subtype of NSCLC represents different immune fractions, as in the case of adenocarcinoma, there were a higher percentage of CD4 T cells, mast cells, and memory B cells as compared to squamous cell carcinoma. However, lower expression of neutrophils and macrophages were measured in correlation with poor survival outcomes in adenocarcinomas. Furthermore, a lower level of CD4 T cells and regulatory T cells in squamous cell carcinomas were associated with poor survival [14].

1.3 Types of Immune Cells and their Function in Cancer

The pulmonary immune system is a complex and organized system, where there is a complex interplay between a large numbers of immune cells, cytokine network and structural elements having variable functions for instance endothelial, epithelial, and mesenchymal cells. In normal circumstances combination of these essentials structural elements are stable, and the ratio of immune cells lies in a standard range. Various types of immune cells make the lungs immune environment to protect lung cells from infectious disease or any type of antigen, which includes T cells lymphocytes, macrophages, B cells lymphocytes, natural killer cells, and dendritic cells.

Macrophages are part of innate immune responses, can detect and respond to tissue injury and infection, initiating restoration mechanism [15]. Macrophages can be divided into two types, one is a pro-tumor and the other one is an antitumor [16].

Pro-tumor macrophages are also known as tumor-associated macrophages (TAM), involved in various secretions like cytokines, epidermal growth factor, and TGF-β. TGF-β is tangled in the generation of immune responses like suppression of the immune system, the impaired dendritic cells and T Lymphocytes. In the case of high-grade cancer, TAM is reported in poor survival and prognosis of patients [17].

Neutrophils are the first line of protection as they are recruited first in case of tissue damages inflammation within cells. They are highly associated to eradicate pathogens and regulate inflammation through processes like phagocytosis, protease-containing granules exocytosis, antibacterial protein secretions, and accumulation of neutrophil extracellular traps (NETs). However, in the case of cancer, they are strongly deregulated leading to a poor prognosis [18]. Further, it has been reported in lung cancer xenografts that the expression of tumor-associated neutrophils is associated with c-MET, expression, which has an anti-cancer and metastatic function. c-MET is expressed in response to TNF- α [19].

T cells are the second common immune cell, part of the adaptive immune system. Due to their abundance and immune response, they are unduly studied in the case of cancer [20]. Their pivotal role in the tumor is majorly associated with the involvement of the drainage of the lymph nodes, leading to transferring to tumor micro-environment, eradicating immunogenic tumor cells. Furthermore, a high level of T cells is reported to be involved with a good prognosis in different cancers like lungs, breast, colorectal, ovarian, gastric cancer [16]. However, cancer cells mask the effect of T cells by suppressing their immunogenic responses. Additionally, the aberrant response of the T cells can be produced in response to the presence of inhibitory signals like programmed cell death protein 1 (PD-1) which is a receptor on the surface of T cells, and programmed death-ligand 1 (PD-L1) is a molecule expressed on the surface of cancer cells (and other cells). When PD-L1 on cancer cells binds to PD-1 on T cells, it sends an inhibitory signal that suppresses the T cell's activity, preventing it from attacking the cancer cell [21].

B-cells are also an important part of the immune system, where they express antibodies in response to several antigens including viruses, bacteria, cancer cells etc [22]. B-cells' role in cancer is not well understood as these have variable responses

in different cancers [17–23]. However, the recent finding suggests the positive role of B-cells in the production of anti-tumor response [24]. Furthermore, other immune cells are also involved in cancer like natural killer cells, which generate an anti-tumor response, but in some cases, it also generates a negative response [25].

In the case of lung adenocarcinoma cancer, higher CD45+ leukocyte expression was reported as compared to the distal lung [26]. Moreover, CD4+ cells and CD8+ cells secrete IFN-γ, which also play a predominant role in cancer cell immunogenicity. CD4+ secreted cells are actively involved in the activation of macrophages, as required for the initiation of immune surveillance in the non-solid tumor [27]. IFN-γ is also associated with non-small cell lungs carcinoma as they respond to the immune checkpoints blockades [28]. However, Jorgovanovic et al (2020), described that the concentration of IFN-γ in non-small cell lungs carcinoma microenvironment defines the type of signaling pathway activation by binding to IFN-γ cell receptor, which confers the different role of IFN-γ in the tumor microenvironment. Significantly low level of IFN-γ in lung cancer was reported and Stankovic et al (2018) stated that 13 types of different immune cells in non-small cell lung carcinoma, which included more than 95% of CD45+ leukocytes, NK cells, CD8+ T cells, B cells, DN T cells, neutrophils, CD1c+ mDCs, eosinophils, macrophages, basophils, mast cells, pDCs, and CD141+ mDCs [26]. These immune cells are reported to be involved in the non-small cells lung carcinoma, which confers the survival rate concerning immune cells density in the tumor region. Furthermore, immune checkpoints are also present to regulate the immune system in a particular tissue, however, in the case of cancer cells these checkpoints are either mutated or have aberrant expression leading to the abnormal immune system.

1.4 Cancer Cells Secretions

Cancer cells secretions are crucial players in the maintenance of the microenvironment and are known facilitators in the mediation of cancerous cells' progression to the secondary site [29]. These cancer cell secretions are protein-based, using a single peptide secreted through different pathways like conventional ER-Golgi

complex pathways or utilization of nonconventional pathways like Exosomes or microvesicles [30]. Extracellular vesicles play a key part in the organization and preservation of cancer hallmarks for tumor initiation and progression, by transferring molecules required for the tumor to distant cells or recipient cells or in the microenvironment [31]. Extracellular vesicles of lungs cancers cells are capable of transmitting signaling molecules, to the receiving cells, to stimulate the epithelial-mesenchymal transition to promote cancer cells migration and invasion [32]. Additionally, deregulation of several pathways results in increased secretions within cancer cells. For example, IMPAD1 and KDELR2 are known to increase Golgi apparatus secretion, which promotes invasion and metastasis in non-small cells lung carcinoma [33]. Moreover, in the case of lungs cancer, secretion of PKM2, which binds immediately with integrin b1 and consequently triggers the FAK/SRC/ERK pathway to stimulate tumor progression and metastasis [34].

1.5 Extracellular Vesicles (EVs)

Cell secrete lipid bound vesicles outside to the extracellular spaces also known as EVs (extracellular vesicles) [35]. Microvesicles (MVs), Exosomes, and apoptotic bodies are the three fundamental subtypes of EVs, and they differ from one another in terms of their biogenesis, modes of release, size, composition, and functions [36]. The cargo of extracellular vesicles (EVs) is made up of lipids, nucleic acids, and proteins—more particularly, proteins related to the plasma membrane, cytosol, and those involved in lipid metabolism [37]. However, a large overlap in protein profiles is frequently discovered [38] as a result of the absence of standardized EV isolation and analysis procedures. Additionally, the isolation process affects the proteomic profiles of EVs from the same source [36]. The use of EVs in the therapeutic setting as biomarker bearers for diagnostics has helped us understand how cells communicate with one another and how cancer metastasizes. [39]. However, before they can be employed in the clinical context, consistent methodologies for EV separation and analysis must be devised.

1.5.1 What are Exosomes?

Almost all the cell types release Exosomes, with mostly reported size range of 30-150 nm [40], but few studies have also ranged their size as 40-150 nm [41]. Small EVs having a multivesicular endosomal origin are referred to as Exosomes [42]. The experimental difficulties connected with studying Exosomes question this classification, as enriched Exosomes preparations obtained using existing techniques may not stringently differentiate from microvesicles released from plasma membrane budding, independent of intracellular multivesicular endosomes [43, 44]. With continuous endeavors to improved identification of their endocytic origin and separate them from other microvesicles, the scientific literature's definition of Exosomes is likely to modify over time. Exosomes are very diverse and the cell phenotypic state that produces them is likely reflected in them. Exosomes, like cells, have a lipid bilayer and can cover all identified molecular elements of a cell at any given time, including proteins, RNA, and DNA [45–47]. Because of repeated enfolding of the lipid bilayer membrane during their formation, Exosomes' lipid bilayer membrane resembles that of the cells from which they originate. [47].

1.5.2 Exosomal Cargo

Exosomes have been thought of as micro representations of the parental cell due to their complex architecture of precisely sorted proteins, lipids, nucleic acids, and related content that is heavily reliant on the status quo of the cell type of origin. Exosomes from various cell types include a wide range of constitutive elements, including roughly 1639 mRNAs, 194 lipids, 764 miRNAs and 4400 proteins demonstrating their intricacy and probable functional multiplicity [48, 49].

Characteristically, Exosomes are made up of proteins like tetraspanins (CD9, CD63, CD81, CD82) that perform multiple functions, some of which include cell penetration, invasion and fusion. Heat shock proteins (HSP70, HSP90)) also makes up the Exosomes that perform antigen binding functions and appear during stress response. Other proteins include the MVB formation proteins (Alix, TSG101), that are involved in Exosomes removal; and proteins associated

with transport through membrane and fusion (annexins and Rab) [50]. Certain members of this group contribute to Exosomes biosynthesis (Alix, flotillin, and TSG101), distinguishing Exosomes from the ectosomes generated when the plasma membrane is shed, though others that are exclusively augmented in Exosomes are commonly utilized for example Exosomal marker proteins (e.g, HSP70, CD63, CD81 and TSG101) [51].

Exosomes consist of various kinds of RNA sequences, which can be fused into receiver cells along with specified proteins. MicroRNAs (miRNAs) were shown to be the most common Exosomal RNA species in human blood plasma, making up approximately 42.32% of all raw readings and 76.20% of the total traceable reads [51]. Ribosomal RNA (9.16% of the total traceable counts), long non-coding RNA (3.36%), piwi-interacting RNA (1.31%), transfer RNA (1.24%), small nuclear RNA (0.18%), and small nucleolar RNA (0.18%) are some of the other RNA species (0.01%). As soon as the miRNAs are packed into Exomes, they can be transferred in a unidirectional manner among cells, forming an intercellular trafficking network that causes recipient cells to suffer transitory or persistent phenotypic alterations [52]. The miRNAs involve in angiogenesis, hematopoiesis, exocytosis, and cancer, including miRNA-214, miRNA-29a, miRNA-1, miRNA-126, and miRNA-320, have been implicated in Exosomes-based cell-to-cell communication [53]. Long RNA species, predominantly long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) have recently been discovered in Exosomes and have been shown to influence several biological processes, including cancer development [53, 54]. They may work together to transmit cells messages to change or sustain local cellular microenvironments. Kogure and his colleagues discovered numerous drastically changed lncRNAs in human hepatocellular carcinoma (HCC) cell-derived Exosomes in early 2013 [54]. The new lncRNA TUC339, which was functionally implicated in controlling tumor cell proliferation and adherence, was the most highly substantially expressed among them. As a result, they hypothesised Exosomes-mediated intercellular communication in HCC as a route of functionally active lncRNA transfer between cells. Conigliaro et al., (2015) also found that lncRNA H19 could be packaged within CD90+Huh7 cell-derived Exosomes and dispersed to endothelial cells (ECs), influencing ECs in a pro-metastatic

way through Exosomes-mediated VEGF increase [55]. These findings imply that Exosomes-mediated lncRNA transport is a key mechanism in tumor growth that it alters important cellular pathways to regulate the tumor microenvironment. lncRNA CRNDE-h in colorectal cancer [56], lncARSR in renal cancer sunitinib resistance [57], lncRNA Hotair in rheumatoid arthritis [58], and lincRNA-p21 and ncRNA-CCND1 in bleomycin-induced DNA damage are some of the other lncRNAs conveyed via Exosomes [59]. CircRNAs were found to exhibit a high level of stability and resistance to exonuclease cleavage, implying that they could be employed as a tumor diagnostic marker. Li et al. reported the presence of circRNAs in Exosomes during RNA sequencing examinations of hepatic MHCC-LM3 cancer cells and cell-derived Exosomes in 2015 [60]. When healthy individuals and colorectal cancer patients were assessed, 67 circRNAs were missing, while cancer patients had 257 new circRNA species found. The reduced expression of circRNA Cdr1as in Exosomes was significantly reduced when miRNA7 was overexpressed in HEK293T and MCF-7 cells, showing that intracellular miRNAs may govern the mechanism of circRNAs entering Exosomes. Exosomal circRNA (100284) was discovered to increase the malignant progress of human hepatic cells via control of EZH2 by miRNA-217 in arsenite-transformed cells [61]. Exosomes are bioactive not only because of the proteins but also due to the presence of lipids e.g. cholesterol, phosphatidic acid, arachidonic acid, sphingomyelin and other fatty acids like leukotrienes and prostaglandins are all abundant in Exosomes, contributing to their structural rigidity and stability. Exosomes also possess a number of lipolytic enzymes producing units of various bioactive lipids according to their own. Exosomal bioactive lipids that are engulfed by the targeted cells tend to congregate lipids mediators within the endosomes. Exosomes have been reported with the presence of prostaglandins and fatty acids, resulting in micromolar prostaglandin concentrations and millimolar fatty acid concentrations, both of which are sufficient to elicit prostaglandin-dependent biological processes [62]. Exosomal lipids may form the cytosolic complex of Arachidonic acid/ Fatty acid-binding protein/ Peroxisome Proliferator Activated Receptor gamma (AA/FABP/PPAR-γ) by interacting with target cytosolic lipid transfer proteins such FABPs or receptors like PPAR-γ for the cyclopentenone prostaglandin (15dPGJ2). It is delivered to the

nucleus. As a result, Exosomes-produced 15dPGJ2 and Prostaglandin-E2 (PGE2) may serve as a natural supply of intracellular PGE2. Exosomes can transport endosomal AA to the endoplasmic reticulum's PGE synthase and cyclooxygenases (COXs), allowing for more PGE2 synthesis. The microsomal antioestrogen binding domain of the host endoplasmic reticulum may also act as a site for the Exosomal binding. This results in a decreased function of the cholesterol epoxide hydrolase which is responsible for converting cholesterol 5,6 epoxide into cholestane triol [63]. Since Exosomes can be created and engaged by the target cells to alter the lipid metabolism of T cells, the design of cholesterol-related storage disorders like atherosclerosis must be taken into consideration during exosomal-mediated intercellular lipid interchange. As the monocytes engulf the Exosomes through phosphatidylserine (PS) receptor, the cholesterol accumulation into lipid droplets is observed to be increase effectively in T cells, showing that Exosomes play a significant role in atherosclerosis formation [64]. Because Exosomes have been linked to lipid-related disorders, their lipid composition, in addition to proteins and RNA biomarkers, might be employed as a genetic hallmark for disease diagnosis and prognosis [64].

1.5.3 Functions of Exosomes

Exosomes and their exact function are unclear. Early theories suggest that Exosomes serve as cellular garbage bags, removing superfluous and/or nonfunctional cellular components. Exosomes are endocytic EVs, that are expected to recycle cell surface proteins and, as a result, influence outside-in signaling [65]. Although this suggests that Exosomes carry explicit ingredients that are existing in surplus in a particular cell, which has yet to be verified. It's unclear if certain cellular elements are traveled into Exosomes through an ordered method or deemed Exosomes packing as a random process. Exosomes content and statistics are expected to vary depending on whether cells are uncovered to various stressors or stimuli, and Exosomes formed by the identical cells can include diverse contents. The contents of Exosomes are believed to replicate the ongoing processes within the cell. However, it's noteworthy that certain proteins are consistently associated with Exosomes, indicating a selective process in the trafficking of proteins into

these vesicles While it remains unclear which proteins are abundant in Exosomes, emerging data suggests that a subset of these proteins plays a role in exosome synthesis. It is possible that plasma membrane anchoring plays a pivotal role in enriching proteins associated with Exosomes [44]. Exosomes components can be transported to a target cells through fusion to impact phenotypic changes, which backs the idea that Exosomes are robust intercellular communication intermediaries [66]. Exosomes are thought to be present in about 2,000 trillion. Exosomes are produced in greater quantities by diseased tissues and aberrant cells inside [42]. The exact source of this rise in Exosomes production is uncertain; however, it is thought to be related to changes in cellular physiology. While determining the rate of Exosomes production and heterogeneous nature of Exosomes extracted during normal physiological conditions through *in vivo* can be challenging, many *in vitro* research suggests that practically all cell types can produce Exosomes, that Exosomes are diverse and complex, and that heterogeneity may be increased in pathological conditions such as cancer [67].

1.5.4 Role of Exosomes in Diseases

Exosomes functions are reported in various diseases. They are associated with the generation of immune response as well as infection, where pathogens use host Exosomes for the infection. Furthermore, they are also involved in various other diseases, which include cancer, neurological diseases, heart issues, liver and lung diseases.

In the case of neurodegenerative disease, they play an active role in neuroinflammation initiation. They act as a carrier in the transmission of neurogenerative disease to the surrounding cells [56]. They are also known to be involved in Alzheimer's disease, where Exosomes spread amyloid β (Aβ) molecules to the surrounding neuronal cells, which are involved in plaque formation within the brain [68]. Furthermore, in the case of Parkinson's, they provide α-synuclein aggregation catalytic surrounding, specifically initiated by Exosomes lipids [69]. This transmission and aggregation of α-synuclein induces apoptosis within neuronal cells [70]. Various studies suggested Exosomes roles in Prion disease, as PrPSc, which

are detectable prion proteins are highly expressed in Exosomes [71]. Other neurogenerative diseases include traumatic encephalopathy, Huntington's disease, and amyotrophic lateral sclerosis, where Exosomes are recognized to play their part in the transmission of disease [72]. Liver-related Exosomes are also indicated to play their part in various liver-related diseases, for instance, HCV, HBV, liver cirrhosis, or even hepatocellular carcinomas. In the case of viral disease, the virus uses cellular Exosomes to transmit proteins and signals to uninfected cells using miRNA and mRNA. Furthermore, they also help in evasion from immune cells for viral and cancerous cells. Additionally, Exosomes are known to enhance HCV RNA stability in hepatocytes [73]. Heart cells release Exosomes for cellular communication as well as in response to a stress condition, for instance, hypoxia [74]. They are known to be involved in Pulmonary arterial hypertension, where they maintain cellular communication using miRNA to increase expression and specifically transport miRNA-143-3p, which can result in heart failure. Mesenchymal cells-derived Exosomes cause hypertension by activation of the STAT3 pathway [75]. Tumor-derived Exosomes are also known to transport regulatory RNAs, which in some cancer causes resistance to chemotherapies. Furthermore, they are also involved in the transportation of oncoprotein, which enhances cancer cells' motility, immunosuppression [76]. Cancer stem cells production is also increasing by the Exosomes signaling through epithelial-mesenchymal transition [77]. In the case of lungs cancer, they are identified to increase the epithelial-mesenchymal transition, high expression of Vimentin, and migration [78]. Moreover, Exosomes have also been found to be associated with the establishment of the hallmarks of cancer [9]. In the breast, cancer plasticity is maintained by the miRNA-200 of the Exosomes, which further enhances the epithelial-mesenchymal transition. Moreover, Exosomes' role in other cancer like melanoma, hepatocellular carcinomas, and pancreatic cancer has also been identified, where Exosomes recruit macrophages and bone marrow progenitor cells for cancer metastasis [79]. In colon cancer, Exosomes maintain the tumor genesis of cancer cells by releasing mutant KRAS in the surrounding cells. Moreover, it has also been observed the presence of the same antigens on tumor-derived Exosomes and immune cells, for instance, CD4+ and CD8+ antigens, conferring immunity to tumor cells. Tumor derived Exosomes

also inhibit cytotoxic T cells as well as CD8+ T cells, which ultimately suppress immune responses within cancer [80]. Cancer cells Exosomes also cause immune suppression by masking antitumor capabilities of the immune system [69].

1.5.5 Role and Function of Exosomes in Cancer

Exosomes have been linked to a number of pathways which further associates with the progression of various cancer types. Micro vesicles produced from tumor cells carried the oncogenic receptor EGFRvIII and delivered miRNAs that prompted non-tumorigenic mammary cells to change and develop cancers [66]. Exosomes produced from gliomas can deliver active mRNAs and miRNAs to preexisting tumors, enhancing tumor progression [66, 81]. Furthermore, autocrine signaling via Exosomes produced from cancer cells can deliver prevailing progression indications. Exosomes obtained from gastric tumor, for example, stimulated tumor spread in vitro in a way that was reliant on MAP kinase and Akt/PI3K signaling [82]. Exosomes from pancreatic cancer patients also help cancer patients live longer by changing signaling through the Notch-1 pathway [83]. Exosomes can be used by cancer cells in a process called homotypic transfer to accelerate the development of the disease overall. A system of various cell types called the local TME, which is involved in all stages of cancer genesis and development, aids in the growth, dissemination, and expansion of tumors. The tumor microenvironment influences cancer hallmarks for instance, continuing propagation, dodging growth suppression, evading immune identification, triggering incursion and metastasis cascades, battling cell death, starting angiogenesis, and aberrent cellular metabolism [84, 85]. Many cell to cell interactions are modulated by the TME via a number of different signaling networks, including juxtracrine and paracrine relationships. Exosomes are essential and growing mode of communication between cells among the paracrine signaling connections [86].

1.5.6 Exosomes in Pre-metastatic and Metastatic Niches

Exosomes are important in establishing pre-metastatic niches, which are good sites for future dispersion and metastatic seeding [87–89]. MET is transferred to

bone marrow progenitor cells via Exosomes, which further regulates pre-metastatic niche development in melanoma, favoring lung metastases [90]. A pre-metastatic environment in the liver is fostered due to the secretion of TGF from the hepatic stellate cells that attract definite macrophage populaces, as mediated by Exosomes from pancreatic cancer cells [91]. Moreover, metastatic colonization is caused when the astrocytes (like fibroblasts) in the brain's metastatic microenvironments secrete miRNAs containing Exosomes that silence PTEN [92]. Exosomes from cancer cells with well-defined metastatic organotropism also contain particular integrins that regulate organotropism [93]. Exosomes content and function to the pre-metastatic niche can also be altered by treatments. Extracellular vesicles from breast cancer cells triggered by chemotherapy have recently been shown to enhance transmitting annexin 6 to activate Ccl2, a monocyte activator, to create a pre-metastatic niche in the lung. Exosomes generated from cancer cells can have brief and far-off impacts on additional cancer cells or host cells, assisting in entire stages of cancer development. With a improved thoughtful of such impacts, crucial Exosomes-related medicines that overcome therapy resistance can be developed [94].

1.5.7 Role of Exosomes in Therapies and Diagnosis

Exosomes are existing in nearly all types of body fluids, which include saliva, breast milk, cerebrospinal fluids, blood, amniotic fluid, urine, ascites, and semen [95–97]. Exosomes derived from cancer cells can be found in biological fluid and can be used as a diagnostic marker or to anticipate therapeutic efficacy. Exosomes's proteins, miRNA, mRNA, and intermediate molecules can act as biomarkers and therapeutic targets. For instance, lower expression of Exosomes miRNA-34c-3p can be an indicator of non-small cell lungs carcinomas [98]. Furthermore, a research by Sandfeld-Paulsen et al presented the prognostic significance of Exosomes, where non-small cell lungs carcinoma showed variable expression and number of exosomal surface proteins, like PLAP, EGFR, and NY-ESO-1 [99]. Another study reported high expression of Exosomes proteins alpha-2-HS-glycoprotein (AHSG) and extracellular matrix protein 1 (ECM1) in non-small cells lungs cancer, which

also specified the predictive rate of Exosomes in NSCLC [100]. Exosomes can be utilized in cancer therapies in four different ways, firstly, which includes targeting cancer-derived Exosomes by inhibiting them, secondly, Exosomes can be manipulated in a way to carry cancer-targeting drugs, thirdly, exploiting them as gene carriers, and lastly by suppressing cancer cells using immune cells derived natural Exosomes [101, 102]. Various studies indicated that transferring miRNA through Exosomes as a vector can confer stability to miRNA. Ohno et al., reported breast cancer growth inhibition after injection of embryonic kidney-derived Exosomes with let-7 expression [103]. Furthermore, plant-based Exosomes are also derived for cancer therapies. Macrophage-derived Exosomes are reported to have the drug-carrying ability and target lung carcinomas [104].

1.6 NSCLC Derived Exosomes

Non-small cell lungs carcinoma is not a single factorial, various factors regulate the initiation and progression through complex processes. Exosomes in non-small cell lungs carcinoma are involved in tumor initiation, angiogenesis, progression, evasion from the immune system, and formation of pre-metastatic microenvironment [105]. Similar to other malignant cells, non-small cell lung carcinoma Exosomes play a significant role in intracellular communication [106]. Rab27 expression is highly involved in the development of cancer, which is a crucial controller of Exosomes secretion. They maintain the tumor niche in the microenvironment for metastasis and also sustain tumor-associated antigen, which impedes immune responses [107]. Moreover, they are involved in the resistance again drugs during treatments as well as tumor growth maintenance [108]. Exosomes of cancer-associated fibroblast were identified to secrete proteins, lipids, and intermediates essential for the cancer cells' survival for energy generation in case of deprivation of nutrients [109]. Furthermore, they are also reported to have a role in the non-small cells lungs carcinomas angiogenesis by regulating receptor tyrosine kinases A3. Moreover, Exosomes also interact with surrounding cells, which also affect normal cells physiology, making it favorable for cancer cells metastasis [110]. cancer-derived Exosomes control the microenvironment by activating oncogenes and inhibiting

tumor suppressors. Likewise, Exosomes miRNA-21 increases VEGF required for epithelial-mesenchymal transformation by activating the STAT3 pathway. VEGF further activates various signaling pathways like Akt and MAPK pathways required for cancer cells communication and signaling [111].

1.7 Role of Exosomes in Immune cells Regulation

Chemokines, growth factors, cytokines, and proteolytic enzymes are secreted by the immune cells in the TME that accelerate tumor development, change immune dodging, or actively kill cancer cells [112]. Furthermore, dynamic signaling governs immune cell acquisition and emigration into the tumor microenvironment [113], and Exosomes play a crucial role in these communications [114]. Exosomes from cancer cells have been shown to stimulate macrophages , natural killer (NK) cells, T cells, and B cells directly [115]. According to recent findings, the immune-activating Exosomes can be generated due to the stimulation of canonical oncogenic signals and these Exosomes can further help to clear tumors [116]. Furthermore, it can work opposite by inhibiting immune system [115]. Exosomes can prevent myeloid-derived suppressor cells (MDSCs) and DCs from differentiating [117, 118]. RNA may hold the crucial to study the interplay between immune system and Exosomes. Myeloid cell populations can be activated by exosomal RNA [119]. The miRNAs have been shown to act as toll-like receptor (TLR) ligands in a variety of malignancies, promoting tumor development [120, 121]. Exosomes control the immune system through a variety of methods that are still being discovered, nevertheless it is apparent that Exosomes have an impact and that their RNA and protein can act as powerful molecules to establish signaling. Exosomes' functions are not limited to secretion, but also play a vital part in the regulation of immune response in normal circumstances. They generate immune reports in response to various pathogens. In the case of pathogen entry, T cells released Exosomes, which interact with dendritic cells, which in turn release antiviral responses like interleukin alpha and beta. The downstream signaling pathways are activated by interleukin which provides defense against viruses [122]. Exosomes also generate

an immune response when irradiated tumor cells transport their double-stranded DNA to dendritic cells for the activation and release of IFN-I, which are intermediates for immune response generation or even act in cancer progression [123]. Exosomes RNAs and DNAs both are responsible for the generation of immune responses [124]. Moreover, inhibition of the immune response in an infected cell can initiate an alternative immune defense system by using Exosomes secretion. Exosomes also carry antigenic peptides from Antigen-presenting cells to T cells for the activation of T cells [80]. Their role in cancer cells is extensively studied, where tumor-associated immune cells also showed the presence of different receptors as compared to the normal one. Like in leukemia T cells Exosomes showed the expression of the membrane-associated ATPase VCP, which were absent in the normal T cells of a healthy individual [125].

1.8 Exosomal miRNAs in NSCLC

In the case of hypoxia in lungs cancer miRNA-23a of the Exosomes, affects prolyl hydroxylase and zonula occludens-1, which are necessary for increased angiogenesis and vascular penetrability [110]. A low level of exosomal miRNA-34c-3p is reported to increase integrin $\alpha 2\beta 1$expression, which helps in NSCLC progression [98]. miRNA-494 and miRNA-542-3p were reported in lungs cancer, where they increase the expression of matrix metalloproteinase by reducing the expression of Cadherin-17 [126]. miRNA-326 lower expression is associated with up-regulation of CCND1 oncogene in non-small cells lungs carcinoma [127]. Circulating Exosomes miRNA-10b is reported to be involved in metastasis of lymph nodes in non-small cells lungs carcinomas [128]. Moreover, Exosomes CD91 is also involved in cancer growth and progression [129]. Wan et al (2018) reported the significant role of miRNA-142-5p in NSCLC, as its upregulation reduced the expression of CD4+ T cells [130]. NSCLC related miRNAs are further summarized by Wang et al, in their study, which includes high levels, miRNA-320b, miRNA-361-5p, and miRNA-181-5p of plasma Exosomes [131].

1.8.1 Regulatory Effects of Exosomal miRNAs in the Immune System

More than 1600 microRNAs are detected in the cell, with a variable role, which includes regulation of expression of proteins, cellular differentiation, metabolism, embryonic development, proliferation and apoptosis, and immune response modulation [132]. Various studies suggest the role of miRNAs regulation of immune cells development, activation, lineage commitment, and function [133, 134]. Testa et al (2017) reported the regulatory role of two miRNA miRNA-146 and miRNA-155 in the variation of immune responses, however, their aberrancy can lead to tumor development [135]. Various miRNAs are reported to control the signaling pathway of nuclear factor- κB (NF-κB), for instance, mi188, 146/miRNA-155, miRN-223, miRNA-17–92 cluster, and miR-23, 27, 24 clusters, thereby regulating inflammation [136]. Furthermore, miRNA-155 directly target SOCS-1 and regulate the CD4+ and CD8+ IFN-γ production [137]. Many miRNAs are associated with various immune-related diseases like infectious diseases and inflammatory diseases due to the variation in expression. MicroRNAs regulate the expression of more than 30%-50% of genes, thereby implying the important role in the proper functioning of the cell [132]. They negatively regulate important immune development genes, in normal, and stress conditions [133]. As miRNA targeting SOCS-1 effectively reduces the expression of IFN-γ [137]. Furthermore, DAP12 expression is also regulated by miRNA, which controls the expression of natural killer cells. MiRNA-146a targets STAT1 and regulates the expression of IFN-γ. FOXPe expression is negatively controlled by miRNA-142-3p which is majorly involved in the T cell regulation [111]. Various miRNAs are associated with the differentiation process of T cell and B-cells like the role of miRNA-125b, miRNA-30, and miRNA-9 in B cell differentiation. miR-221 and miRNA-222 target receptor c-kit to regulate hematopoietic stem cells [138]. miRNA-181a and miRNA-150 are associated with the differentiation and maturation of B-cell and the T cells were reported the important role of miRNA-124 in the immune cell's modulation [139], as it is involved in dendritic cell development, macrophages, and CD4+ T cells

differentiation (also effect by up-regulating IL-2, IFN-γ, and TNF-α. STAT3), conferring miRNAs major role in the immune system regulation [139].

1.8.2 Proteins as Contents of Exosomes

One of the recent studies suggested that, there are approximately 4563 proteins, 194 lipids, 1639 messenger RNAs and 764 miRNAs in Exosomes [140]. Exosomal proteins are of much importance in both normal and disease conditions. These have found to be involved in immune regulation, by processing antigenic peptides due to the presence of enriched molecules such as major histocompatibility molecules (MHC) class I and II. Tetraspanins are unique markers present in Exosomes [141]. Tetraspanins contains adhesion molecules such as CD54, CD11b, CD9, CD63, CD81, and CD82. Exosomes also have activities in cellular response to environmental stress due to presence of enriched heat shock proteins that act as chaperones. Heat shock proteins assist in trafficking and folding of proteins in which several proteins are involved containing Hsp90, Hsp60 and Hsp70 and heat shock protein cognate 70. Exosomes carrying cytoplasmic proteins including Rabs and annexins in addition to the tetraspanins and hsps [142]. The role of these proteins is to assist removal of Exosomes and infusion of multi-vesicular bodies (MVB). The biogenesis of MVBs involve constituents of Exosomes comprise proteins clathrin, Alix, ubiquitin and Tumor susceptibility gene 101 (TSG). Exosomes consist of enzyme that make up its composition such as syntenin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ATPase, protein kinase G (PKG), Signal transduction proteins, CDC42, EGFR, G proteins, and mucin 1 (MUC 1), 14-3-3 [41]. The composition of Exosomes also includes viral proteins such as Herpes simplex virus glycoprotein B (HSV gB), group-specific antigen (Gag), Epstein–Barr virus (EBV) gp350, Human immunodeficiency virus negative regulatory factor (HIV Nef). The Exosomes are involved in alternate pathway of cellular communication only if they are composed of RNA [143]. Therefore, if the Exosomes consist of mRNAs they can be transported to target cells and translated into proteins [144].

1.9 Problem Statement of the Current Project

According to centers for disease control and prevention, if diagnosis and staging of lung cancer are evaluated, certain therapeutic strategies could be used to treat NSCLC including radiation therapy, targeted therapy, chemotherapy, surgery, and immunotherapy or combination of these treatments. Along with some of the satisfactory results from available ways of treatments, there are certain factors including dosage prescribed, patient's health history, or length of the treatment which may not be comfortable for some patients, and several side effects are appeared leading to other severities or complications. In contrast with these treatments from outside, humans immune system is also a potential natural mechanism to destroy cancer cells or to inhibit tumor formation by using innate or adaptive arms [30]. Innate immune responses are mediated by effector cells including polymorphic nuclear leukocytes, mast cells, and natural killer cells as well as antigen-presenting cells including dendritic cells and macrophages. This results in the production of perforin and IFN-γ as well as inflammatory cytokines inducing apoptosis in tumor cells [45]. On the other hand, adaptive immune responses develop slowly, are antigen-specific, and have immune memory. This has both cellular immunity mediated by T cells and humoral immunity mediated by B cells. Therefore, adaptive immunity has a high anticancer immune response and has long-lasting potential. Immature Dendritic cells initiate an anticancer immune response and target antigens released from cancer cells. After getting matured DCs presents antigens to MHC molecule to T cells composed of CD4+ helper T cells and CD8+ cytotoxic T cells. Activated T cells kill tumor cells directly or release cytokines which induce inflammation and recruit other immune cells to eliminate cancer cells. This is a normal mechanism and therefore the immune system is working all the time to clear any cell becoming cancerous in the human body. Once the cancer microenvironment is developed, the cancer cells starts the secretion of different agents including Exosomes which carry normal as well as abnormal biological agents from these sites to the nearby location and upon receiving these Nano agents by receptor cells including immune cells lose their function due to certain mechanisms caused by the materials carried by Exosomes from cancer cells

including miRNA which affects the regulation of important genes required for the maintenance or proliferation of these cells. To analyze these possibilities, it was aimed to optimize and use some of the approaches to enhance the production of Exosomes in NSCLC cells by providing some of the modified growth conditions which mimics the tumor microenvironment and then to identify the effects of Exosomes on Jurkat cells, Human T cells, and Mice T cells. Exosomes obtained from cancer cells and tumor mice were used in our experiments. To study the impacts of cancer resulting Exosomes against these immune cells will not be only helpful in analyzing the impact of tumor Exosomes on T cells proliferation but also on the expression of certain functionally important markers, the proteomic and miRNAs expression analysis my also present a hint for the future studies for further research on therapeutic side. Due to their biocompatibility and size, exosomes which might be utilized as biomarkers in the diagnosis of numerous diseases might also catch the attention of researchers in future investigations.

1.10 Possible Solution of the Problem

Technological advancement takes years of research, and undoubtedly, cancer pathologies are among the most intricate and variable mechanisms, resulting in unique outcomes for each individual. However, there are certain aspects that remain relatively consistent and provide a potential target for various therapeutic outcomes, offering hope for the future of cancer treatment. In this context, we have undertaken a simple initiative with a far reaching goal. In simpler terms, cancer cells infiltrate immune cells, weakening or even killing them, in order to create a conducive environment devoid of resistance for their own growth and proliferation. While much research has focused on direct interactions between cancer cells and immune cells, recent advances have brought attention to indirect mechanisms or pathways adopted by cancer cells to dampen or hinder the activities of the immune cells.

To gain a deeper understanding of these indirect mechanisms, some fundamental validations are required. In our current study, we have identified that cancer Exosomes are overexpressed and exhibit a more detrimental pathological function. We

have confirmed the impact of cancer Exosomes on T cell proliferation and observed alterations in various marker expressions that may contribute to a decrease in the effectiveness of aggressive T cells against cancer cells.

From the findings of our current project, we can speculate that if we can protect immune cells from the secretions (Exosomes) of cancer cells, control the excessive production of Exosomes, or mitigate the pathogenicity of cancer Exosomes, we may be able to reduce the burden of cancer pathogenesis and metastasis. To delve deeper into this realm, our scientific community should start from the basics, taking one step at a time, with the ultimate goal of making the aforementioned approaches viable in the quest to cure or control cancer by bolstering and refining our own immune system.

1.11 Significances of the Study

The immune system takes part in a similar mechanism in cancer prevention just like it recognizes the foreign microorganisms as non-self and destroys these agents. In cancer cells usually, tumor antigens are produced as abnormal protein as the products of mutated DNA, which leads to mark body's own cells as non-self. Thus immune system target and eliminate abnormal cancerous cells. However, there are certain known and unknown mechanisms that allow cancer cells to escape from immune response and develop malignant tumors. The functional loss of immune system surveillance may result in tumorigenesis. Some of the commonly proposed mechanisms are the down regulation of MHC-I, lack of stimulatory signals which helps in presentation of antigens, loss of inflammatory warning signals by the tumors and the most importantly one is the immune-suppressive agents secreted by the cancer cells including Exosomes which carry a variety of biological molecules including DNA, RNA, lipid, proteins. In normal physiological conditions, Exosomes possess normal cargo of the above-mentioned biomolecules while in cancerous cells some abnormal protein or RNA including miRNA or mRNA are transferred from site of origin to other sites of the body and thus leading to tumor formation or affecting the functions of other normal cells plus immune cells. In the current study,

it was focused to identify the effects of these cancer cells secreted Exosomes on T cells proliferation and their markers expression. If the T cells are not proliferated or activated in the required quantity, it will result in the provision of a free environment for cancer cells to grow and divide easily and form tumors in other sites of the body. Our results will provide a clue for the therapeutic research, where the cancer cells secreted Exosomes could be targeted from transferring the aberrant biological agents to enter the normal immune cells and effects it division through the effects of cargo miRNA which may target the replication or other important genes participating in division or to keep immune cells healthy.

1.12 Objectives of the Study

The major objectives of the current study were:

1. To modify the existing protocol for the production and confirmation of high quantity and quality Exosomes from lung cancer cell line and mouse models.

2. To find out the effects of lung cancer cells (H1975 Cells) derived Exosomes on Jurkat cells proliferation.

3. To identify the impact of lung cancer cells derived Exosomes on the proliferation of T cells isolated from human blood.

4. To analyze the effects of tumor C57BL/6 mouse models isolated Exosomes on the proliferation of healthy mice T cells.

5. To analyze selected miRNAs of the Exosomes and their expression level in different physiological conditions, by using qRT PCR.

6. In the last part of our study, bioinformatics techniques were used to analyze the genes targeted by the highly expressed miRNAs found in extracted exosomes.

1.13 Summary

Experimental approaches were used to increase cancer Exosomes production in cancer cells and then analyzed their effects on T cells expression markers and their proliferation. The whole thesis has been structured in a systematic way into 6 chapters including chapter 1, describing the background of thesis title. Chapter 2 highlights the literature reviewed relevant to the field. Methodologies have been described in Chapter 3. Results and discussion have been explained in chapters 4 and 5 respectively. Chapter 6 describes the conclusion and recommendation trailed by references.

Chapter 2

Literature Review

2.1 Exosomes in Cancer Research

From the literature survey, this is understood that, Exosomes are the key player in cellular communications and to keep cellular environment clean and some other functions. These tiny particles have been studied in both normal and pathological conditions to identify their role, contribution and outcomes in both normal and disease conditions. Especially their involvement in cancer have been focused mostly to study angiogenesis, metastasis, immune regulatory mechanism, drug resistance, applications in therapeutics including drug delivery system, and as biomarkers in diagnostics. This is also analyzed that these effects are due to the active biomolecules which Exosomes carry from the cells of origin to the recipient cells. The biomolecules which are abundant constituents of the Exosomes include nucleic acids, protein, lipids and some of the carbohydrates. But the area of nucleic acids mostly the none coding RNAs have been identified with the most prominent functions in modification of recipient cells behaviors. This is also being studied that Exosomes secreted by immune cells can significantly contribute to enhance the immune response against antigens, including foreign substances and cancer cells. On the other hand, a reverse process is also taken under consideration that in tumor and other disease conditions Exosomes may also interfere in the immune cells leading to immune suppression thus hijacking the body immune

system and help the tumor in easy and quick growth, metastasis and angiogenesis as shown in figure 2.1. Many studies have demonstrated that there is a variance in the extent and quality of Exosomes production in normal and pathological conditions, usually the cancer cells that have been found with the increased number of Exosomes and with difference in its constituent molecules, which make them more aggressive and enhance their activities as a negative regulatory mechanism. The role of Exosomes previously discussed in the study are summarized below.

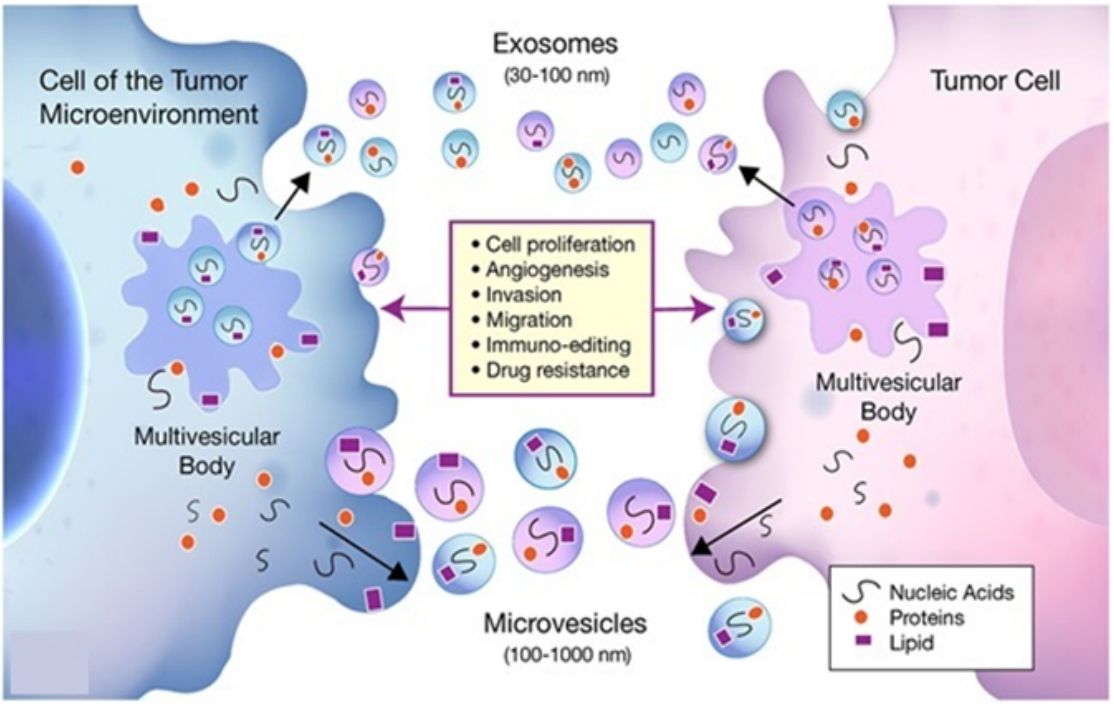

FIGURE 2.1: Tumor cells and cells in the tumor microenvironment communicate in both directions via exosomes and micro vesicles (MVs) [145].

2.2 Exosomes Biogenesis

The Exosomes are produced in cells when the early endosomes are matured into late endosomes during an endocytic pathway involving multiple steps (figure 2.2). Early endosomal membranes fold inward, sequestering proteins, lipids, and other macromolecules into small vesicles known as internal luminal vesicles (ILV). Endosomes harboring ILV have a distinct morphology and are referred to as multivesicular bodies (MVBs). For the removal of harmful compounds from MVBs, they are

sent to the lysosomes where enzymes like hydrolase perform this function appropriately. Due to the presence of particular surface proteins such as tetraspanins, CD63, and the lysosomal-associated membrane proteins LAMP1 and LAMP2, few MVBs escape destruction. The presence of these proteins allows the MVB to escape and are attached to the plasma membrane, releasing vesicles known as Exosomes into the extracellular milieu [142, 146]. During Exosomes biogenesis,

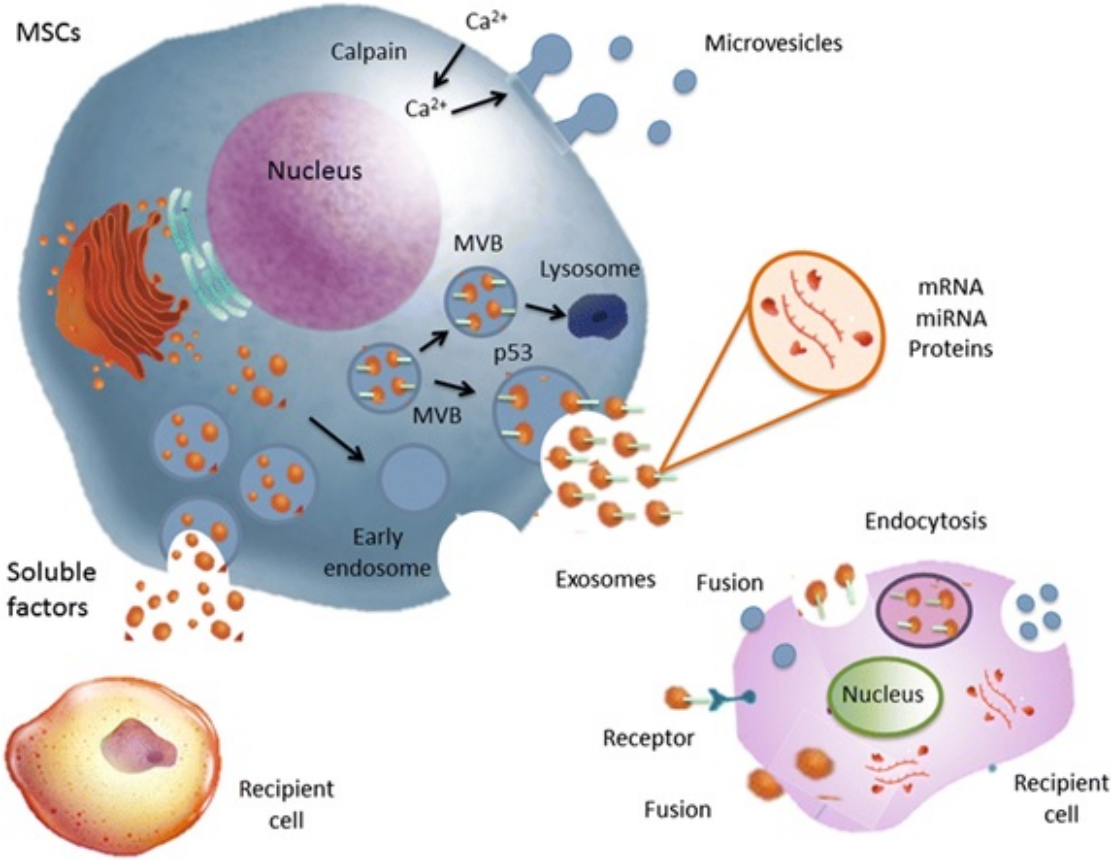

FIGURE 2.2: Illustrative depiction of extracellular vesicle (EV) formation. Vesicles can form directly by budding from the plasma membrane. In contrast, Exosomes originate from intraluminal vesicles (ILVs) produced through the inward budding of the membrane in a subset of late endosomes known as multivesicular bodies (MVBs). These MVBs can be directed toward the cell periphery and, upon fusion with the plasma membrane, release their contents [147]

the entire cellular machinery, consisting of about thirty different proteins, takes part in the selective packing of cellular cargo into Exosomes. The proteins are part of an endosomal sorting complex that is necessary for transport (ESCRT). Colombo et al. (2013) [148] and Stoorvogel (2013) [149] have extensively reviewed the function and significance of ESCRT in Exosomes formation. Four ESCRT

protein members are involved in the packaging of biomolecules into Exosomes lumens and the release of Exosomes from the cell. Exosomes biosynthesis is thought to involve other related or helper proteins like Alix and VSP4 [150]. Exosomes packing and release from cells are also aided by some ESCRT-independent mechanisms. Ceramides, for example, have been demonstrated to have an impact on Exosomes biosynthesis. An inhibitor of sphingomyelinase, which is an enzyme that involved in the ceramide synthesis pathway, was seen to decrease the production of Exosomes as the inhibitor inhibited the formation of ceramides [151, 152].

2.3 Recent Advancements in Exosomal Research Pertaining to Lung Cancer

For histologic and molecular analysis, tumor biopsy is considered to be the most effective and reliable diagnostic method. However, due to poor access to the tumors and the invasiveness of the technique, performing recurrent biopsies as the disease progresses is not always possible. Furthermore, tumor heterogeneity may not be reflected in a biopsy. As a result, finding biomarkers with sufficient sensitivity and specificity for early diagnosis and close monitoring of the condition, as well as assisting in the selection of the optimal medication for customized medicine, is critical. Performing the analysis in liquid biopsy, where repeated sampling is simple, provides an alternative to tissue biopsy [153]. The liquid biopsy in cancer is the process of examining the three forms of tumor-derived precursors in bodily fluids, which include the circulating tumor cells (CTCs), tumor-derived extracellular vesicles (EV), primarily Exosomes, and circulating tumor DNA (ctDNA) [154]. ctDNA has received the most attention, and its analysis is already included in various guidelines for the therapy of NSCLC [155]. Furthermore, some of the previously described medications have already been approved for individuals with actionable mutations found only in ctDNA [156]. Exosomes produced from tumors contribute to the creation of a favorable environment for tumor growth [157]. So, tumor-derived Exosomes play a critical role in the growth of a tumor. Therefore, their examination and understanding can further enhance the understanding of

tumor biology, and they may pose as prime targets for medication therapy or delivery [158]. Exosomes are also appealing because of their suspected functions as cancer biomarkers, that could result in better management of all cancer patients generally and lung cancer patients specifically.

2.3.1 Exosomes Function in Lung Cancer

Secreted Exosomes can be acquired by other cells [159] through receptors for the exosomal proteins via fusion with the plasma membrane, endocytosis, micro pinocytosis, phagocytosis, or receptor-mediated selective binding [160]. Exosomal cargo including miRNAs and lncRNAs as shown in figure 2.4, can perform the function of gene transcription and mRNA translation modulation in target cells [161]. A cellular response is triggered when the transferred materials interact with the target molecules present in the recipient cells. These bioactive chemicals can promote cancer development and metastasis by inducing tumor growth and disturbing the cancer microenvironment [162]. Exosomes have been linked to critical stages of cancer development, including tumor proliferation, epithelial-mesenchymal transition (EMT), tumor migration and metastases, angiogenesis stimulation, and immunosuppression as shown in figure 2.3 [163].

Unchecked cell proliferation, including the onset or disturbance of cell cycle genes and proteins, is the foundation of cancer progression. Exosomes from tumors may include substances that send out signals that encourage the growth of the tumor or even modify cells [164]. Various stages of metastatic development have likely been studied more thoroughly in relation to Exosomal molecules known as miRNAs. For instance, Wu et al. [165] found that the H1299 human lung adenocarcinoma cell line secretes miRNA-96-containing Exosomes that boost Cells proliferation by suppressing the expression of LMO7, a tumor suppressor gene in lung cancer. Another investigation discovered that the A549 lung cancer adenocarcinoma cell line secretes Exosomes that engulf miRNA-21 and miRNA-29a, which bind immune cells' Toll-like receptors TLR8 and activate NFB and release inflammatory cytokines, promoting tumor growth and metastasis [66]. Tyrosine kinase inhibitors

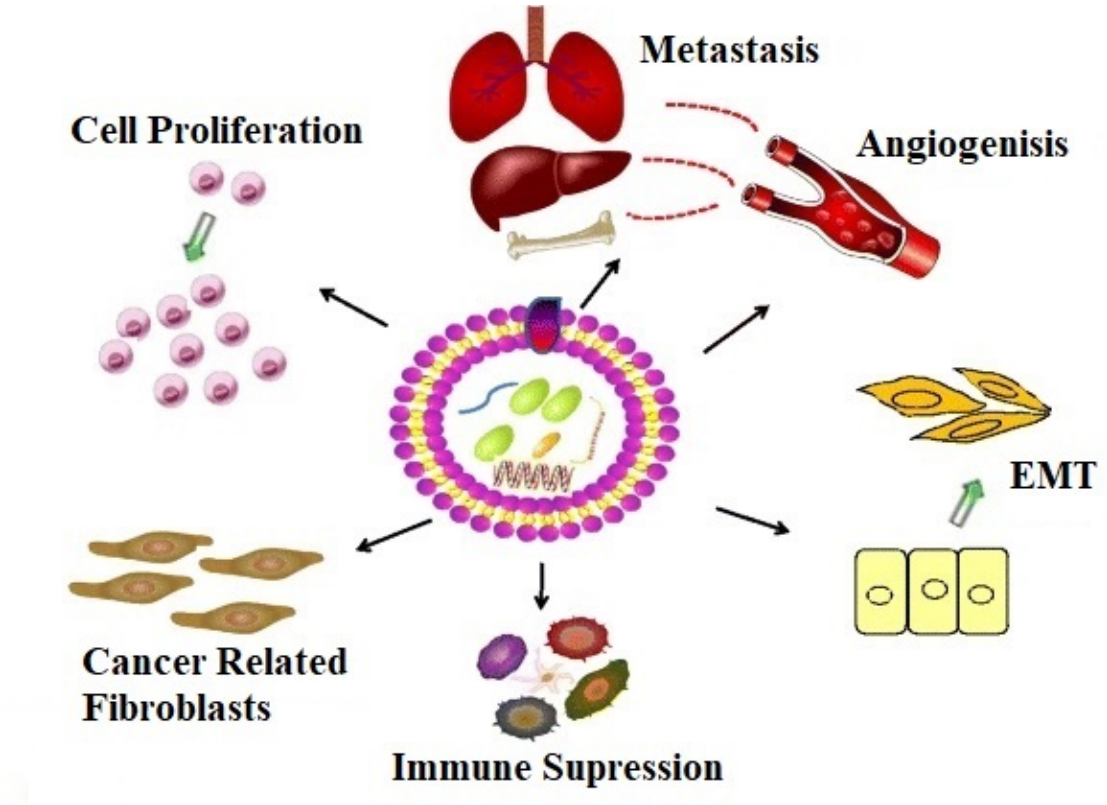

FIGURE 2.3: Exosomes produced by lung tumor cells may play a role in angiogenesis, metastasis, epithelial- mesenchymal transition (EMT), and cell proliferation [163].

(TKIs) have emerged as a first-line treatment for NSCLC, which is characterised by EGFR mutations and gene amplifications; nonetheless, most patients experience relapses as medication resistance increases over time [166]. According to numerous research, Exosomes have been linked to treatment resistance by transferring miRNA or lncRNA from drug-resistant cancer cells to susceptible cells. Gefitinib-resistant PC9 cells and Exosomes showed large amounts of miRNA-214 expression, according to Zhang et al. [167]. These Exosomes delivered miRNA-214 to PC9 cells, causing them to become resistant. By stimulating the PI3K/AKT and MAPK signalling pathways, exosomal transfer of wild type EGFR has been demonstrated to promote resistance to the TKI osimertinib [168]. Exosomes may therefore be exploited as a therapeutic target to prevent the emergence of drug resistance.

In the crucial stage of tumor metastasis, tumor cells lose their epithelial identity by accelerating the expression or production of mesenchymal markers like vimentin,

N-cadherin, and β-catenin. Instead, they develop a mesenchymal phenotype with the ability to migrate and invade [169]. According to numerous studies, Exosomes are implicated EMT observed during the lung cancer, transferring mesenchymal-induced signals and converting tumor cells to a more violent phenotype. For instance, human bronchial epithelial (HBE) cell line vimentin expression and EMT were stimulated by Exosomes produced by highly metastatic lung cancer cells [78]. Exosomes from the lung cancer cell line SPC-A-1-BM with a high miRNA-499a-5p concentration were found to promote proliferation, migration, and EMT when this miRNA was added [170]. Lung cancer cells absorb Exosomes expressing miRNA-210 from cancer-associated fibroblasts, resulting in cell migration, proliferation, invasion, and EMT [171]. After TGF-1-mediated EMT, A549 cells discharge Exosomes with altered cargo, including protein and miRNA content, which result in additional phenotypic changes via autocrine signaling [172].

An initial step among other steps in metastasis is the production of a distant pre-metastatic niche within an appropriate environment to aid the colonization of the tumor cells. Exosomes are essential to this process because they deliver chemically active substances into the circulation, which can change target cells, as seen in figure 2.4 [173].

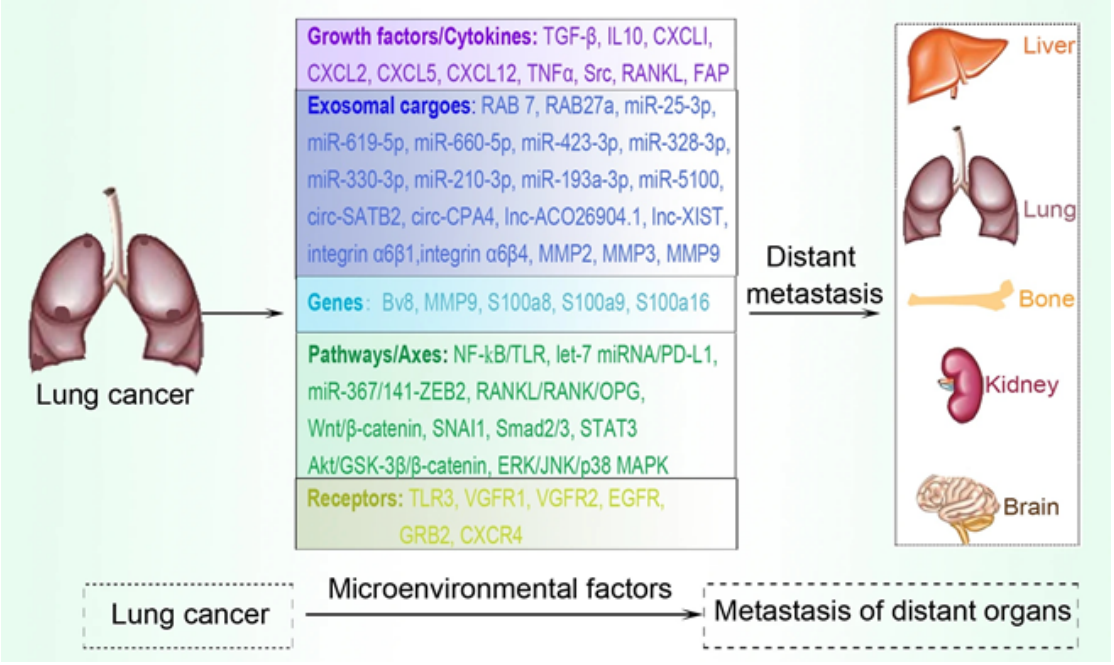

FIGURE 2.4: Lung cancer Exosomes carry different biomolecules in near and distant organs to cause metastasis.

Moreover, a pre-metastatic environment can be produced by transporting molecules which majorly include the integrins and tumor Exosomes, targeting the specific organ or tissues [92]. The inflammatory cytokines are released when the Lewis lung cancer cell line secretes Exosomes consisting of miRNA-3473b and they are ingested by lung fibroblasts and lead to the NF-kB signalling activation [170]. The brain and bones are frequently affected by lung cancer. In accordance with Gang et al. [174], brain microvascular endothelial cells are the target of lung cancer Exosomes, which result in the production of Dkk-1 and a change from M1 to M2 phenotypic microglia, which is more tumorigenic. As a result, metastatic lung cancer cells release less Dkk-1, which lessens the inhibition of microglia that take on a supportive phenotype. Amphiregulin, which binds to EGFR in pre-osteoclasts and activates the pathway that results in the synthesis of proteolytic enzymes, which starts the osteoclastic differentiation process, was found to be present in NSCLC Exosomes by Taverna et al. [175].

2.3.2 Lung Cancer Angiogenesis is Promoted by Exosomes

The creation of new blood vessels from the surrounding tissue is necessary for tumor growth since it depends on nutrients and oxygen-rich blood (Figure 2.5) [176]. Numerous molecules, including miRNAs, have been identified to be transported by tumor Exosomes. These miRNAs, when internalised by endothelial cells, can lead to neo-angiogenesis. As previously mentioned, Exosomes are released as a result of hypoxia, which is particularly prevalent in cancer and encourages angiogenesis. For instance, Hsu et al. [110] discovered that lung cancer cells release Exosomes containing miRNA-23a in hypoxic conditions. The internalization of endothelial cells and miRNA-23a has to chief outcomes in the vasculature. Firstly, it accelerates angiogenesis by sacking prolyl hydroxylase 1 and 2, which causes the buildup of hypoxia-inducible factor-1 (HIF-1). Secondly, it raises vascular permeability (zonula occludens 1 protein). Another study [177] claimed that Exosomes derived from human bronchial epithelial cells that had been altered by cigarette smoke extract that had detectable levels of miRNA-21. Normal human bronchial epithelial cells are the recipients of Exosomes-delivered miRNA-21, that increase

VEGF levels and promote angiogenesis in the endothelial cells of the human umbilical vein. Leucine-rich-alpha2-glycoprotein 1 (LRG1), a protein that controls the onset of angiogenesis, is overexpressed in NSCLC cells, according to Li et al. [178]. They further claimed that the release of Exosomes carrying LRG1, which induced VEGF-A and angiopoietin-1 proangiogenic markers in endothelial cells via a TGF-β-dependent pathway, hence enhancing angiogenesis.

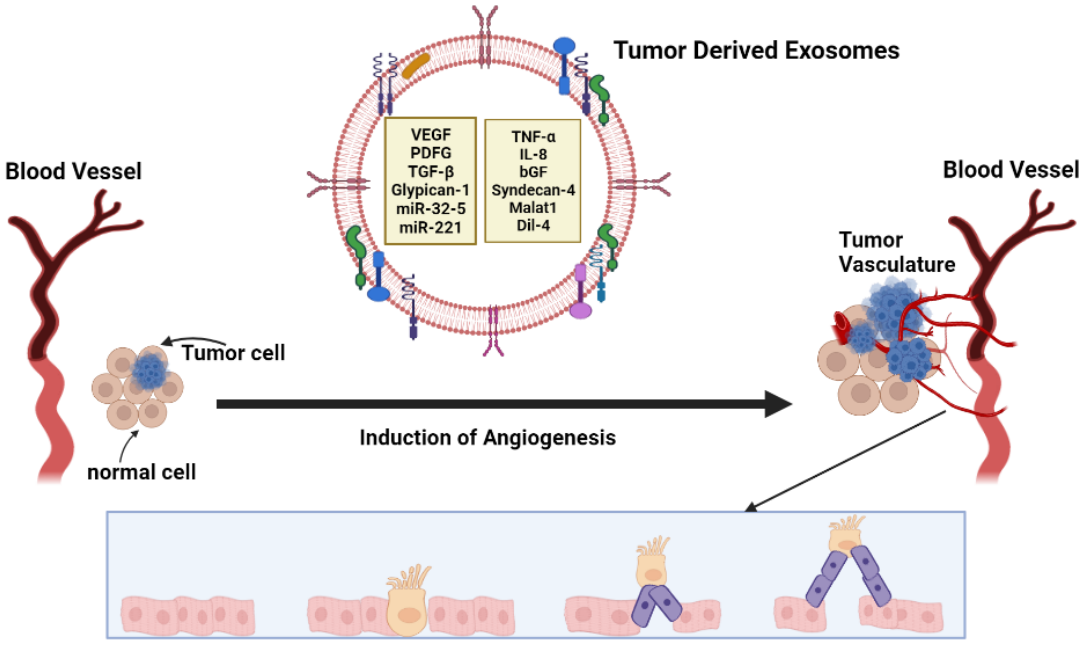

FIGURE 2.5: Exosomes produced by tumors encourage angiogenesis. Vascular development is encouraged by Exosomes from tumors. Basic fibroblast growth factor (bFGF), VEGF, tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), Glypican-1, interleukin-8 (IL-8), transforming growth factor (TGF), Dil4, include syndecan-4, Malat1, and some microRNAs are a few of the angiogenic stimulatory factors that can possibly be transported by the tumor Exosomes [176].

2.3.3 Exosomes Promote Lung Cancer Immune Tolerance

Immune evasion through the establishment of a tolerogenic milieu that avoids cellular death, hence aiding tumor proliferation, is one of the major concerns for tumor development. The payload of tumor Exosomes can decrease immune cell activity in the target cells via two mechanisms: either indirect reprogramming of cells to sack the immunological functions in other cells, or direct blockage

of immune functions. The tumor-derived Exosomes having membrane-associated HSP72 can attach with the TLR2 ligands on the myeloid-derived suppressor cells, causing a STAT3-dependent immunosuppressive activity [179]. Huang et al. [180] demonstrated that the Exosomes involved in lung cancer promote tolerogenic phenotype in dendritic cells. Another study found that under hypoxia, lung tumor cells produce micro vesicles containing TGF-β and miRNA-23a, which decrease NK cell function by decreasing the expansion of the cell surface of the activating receptor NKG2D cells and the cytotoxic marker CD107a/LAMP1 cells [181]. Up regulation of immune checkpoint molecules, like the programmed death-ligand 1 (PD-L1), which bind with their corresponding receptor in T cells and dampen the response, is a frequent strategy of immune evasion. Cheng et al. [182] discovered that metastatic melanoma and breast cancer cells release extracellular vesicles, predominantly Exosomes, with surface PD-L1 that increases in response to IFN.

2.4 Immune Escape Mechanism of Tumor Cells

From the recent studies it is found that Exosomes are extremely useful for the dendritic cells to interact with the rest of the antigen- presenting cells. The Exosomes that were released by the dendritic cells in cluded the MHC I, MHC II, co-stimulatory molecules, and other adhesion molecules [183]. Additionally, Exosomes from dendritic cells contain antigens that the cells have already consumed [183]. Cancer researchers have injected tumor antigens into dendritic cells, collected the Exosomes released by the tumor antigen-pulsed den- dritic cell, and used the Exosomes for immunotherapy as a result of their ability to function as mini-antigen presenting cells. When such Exosomes were given to multiple mice models, they successfully showed the capability of tumor removal [184, 185]. The queries on whether the Exosomes had the tolerance-inducing or immuno-suppressive capabilities arose from the ability of dendritic Exosomes to successfully stimulate the immune system. The question of whether Exosomes could trigger an energy-producing abortive T cells activation pathway arose given that Exosomes had a

high concentration of tumor antigens [185]. The T cells apoptosis-inducing protein FasL has been discovered to be present in many tumor cells and the Exosomes that are generated from them [186]. Various target cells experience apoptosis as a result of FasL's action on CD95/APO1, which is its equivalent receptor. FasL is involved in the creation of immune-privileged status in the testis and eye, as well as a mechanism by which cancers avoid immune-mediated death, in addition to maintaining the homeostasis of activated lymphocytes. FasL is expressed on Exosomes generated by tumor cells in light of the fact that FasL is expressed on them [187].

Exosomes have been proposed to selectively induce antigen-specific T cells apoptosis at the beginning of the neoplastic process by activating the T cells receptor, which up regulates Fas expression on the T cells , and the FasL molecule on the Exosomes induces apoptosis. This hypothesis was made due to Exosomes' capacity to mediate a variety of immunological signals. This process may take place directly between tumor Exosomes and the T cells.

On the contrary, or it may also occur indirectly by tumor Exosomes binding dendritic cells. So, when T cells get attached to the dendritic cells in lymphatic areas, the Exosomes are actually fixed with the dendritic cell and use dendritic cell adhesion/co-stimulatory molecules to form a stable interaction with the T cells, successfully inducing apoptosis. In patients who are suffering with advanced stages of cancer, where Exosomes reach higher systemic concentrations, T cells death is stimulated in a FasL, MHC I-dependent manner that is antigen-nonspecific [186]. There has been a frenzy of research into the immunosuppressive effects of these micro vesicles since it was discovered that the Exosomes generated by tumors are extremely similar to the tumor secreted microvesicles discovered in the 1980s [188].

Pregnant women [189, 190], cancer patients [189], transplant tolerance [190], and oral tolerance induction have all been associated with immunosuppressive microvesicles. Therefore, removing these immunosuppressive microvesicles from circulation with an extracorporeal filter would be a fantastic way to stimulate a cancer patient's immune system [191, 192].

2.5 Biomarkers Potential of the Exosomes in Lung Cancer

Exosomal molecules could be potential diagnostic and prognostic biomarkers, in particular proteins and nucleic acids [193]. Although proteins were once the focus of many studies, interest in miRNAs has grown recently. Utility is frequently dependent on a collection of molecules rather than one in particular. Big-data analysis and bioinformatics are the primary fields doing research and providing solutions in this area since they enable the management and interpretation of enormous volumes of data for the purpose of simply identifying the best markers [194].

2.5.1 Exosomal Proteins as Biomarkers

Proteins support many stages of tumor progression, including metastasis, angiogenesis, and immunomodulation, as well as Exosomes production, membrane trafficking, and fusion. According to the Exocarta database (http://www.exocarta.org), Exosomes include 9,769 proteins. Some Exosomal proteins have the ability to serve as reflectors for producer cells and different phases of diseases [195]. Additionally, these proteins are also involved in the onset and advancement of cancer. Exosomal protein are highly favorable diagnostic and prognostic biomarker in lung cancer because of the said properties [196].Lung cancer Exosomes contain the epidermal growth factor receptor (EGFR), KRAS, extracellular matrix metalloproteinase inducer (EMMPRIN), claudins, and RAB-family proteins. Around 80% of the Exosomes that were recovered from NSCLC tissues contained EGFR, according to Huang et al. [180]. Recently, Exosomal proteins were examined using a triple SILAC quantitative proteomic technique to see whether they were differentially expressed in NSCLC cells and normal bronchial epithelial cells. The researchers found that NSCLC Exosomes include large amounts of proteins involved in extracellular matrix remodeling, cell adhesion, and cell signalling. 721 Exosomal

proteins from three different cell lines were discovered and quantified. The number of signal transduction proteins present in NSCLC Exosomes is enhanced, and these proteins include EGFR and SRC as well as downstream effectors such growth factor receptor-bound protein 2 (GRB2) and RALA. Additionally, it has been depicted that NSCLC Exosomes have higher levels of the MET receptor, RAC1, and KRAS proteins [197]. In addition, Sandfeld-Paulsen showed that 49 Exosomal membrane-bound proteins were examined in a cohort of 276 NSCLC patients of all stages and discovered that nine proteins may be employed as a prognostic marker in NSCLC. They assert that advancing levels of the prognostic markers NYESO-1, EGFR, and PLAP are indicative of a bad prognosis [99]. A proteomic analysis revealed the presence of MHC class I and II proteins, heat shock and cytoskeletal proteins, and proteins involved in signal transduction in Exosomes taken from the human malignant pleural effusions. As parts of Exosomes from various origins, these proteins have already been recognized.

The Sorting-nexin (SNX) family of hydrophilic proteins, which is a congregation of proteins responsible for the intracellular protein trafficking to diverse organelles, is one of the peculiar proteins found in Exosomes from malignant pleural effusions. The elongation step of protein synthesis is greatly aided by the interactions between the elongation factors EF-1 and EF-2 and the acidic ribosomal phosphoproteins from the ribosome's 60S subunit. The abundance of translation-related proteins in the cytoplasm of cancer cells may help to explain why Exosomes include them [198]. A panel of numerous protein markers has exceeded the single Exosomes-protein-focused methodology in the field of biomarker discovery [199]. Exosomes were directly extracted from the plasma of NSCLC patients by Jakobsen and colleagues using an extracellular vesicle array with 37 antibodies, and the results revealed that the combined 30-marker model had a high sensitivity, specificity, and diagnostic accuracy [200]. A recent study using 49 antibodies used the extracellular vesicle array. The most effective Exosomal proteins for separating lung cancer of all histological subtypes from the control are CD151, CD171, and tetraspanin 8. It may be helpful for diagnosing lung cancer in addition to assessing the protein composition of Exosomes from different lung cancer stages and histologies [201].

2.5.2 Exosomal miRNAs as Biomarkers

It has been previously mentioned that Exosomal miRNAs are excessively associated with lung cancer progression through various pathways like angiogenesis, vascular permeability, and metastasis (reviewed in [202]). For the purpose of prognosis and diagnosis, a few of these miRNAs have been studied as biomarkers as well [203]. In most situations, clinical utility is based on a panel of many miRNAs rather than a single miRNA. Using a combination of miRNA-151a-5p, miRNA-30a-3p, miRNA-200b-5p, miRNA-629, miRNA-100, and miRNA-154-3p, researchers distinguished lung cancer from granuloma patients with 96 percent sensitivity and 60 percent specificity [99]. Wang et al. developed a panel of four miRNAs (miRNA-9-3p, miRNA-205-5p, miRNA-210-5p, and miRNA-1269a) with an AUC of 0.91 (77 percent sensitivity and 89 percent specificity) that could distinguish NSCLC patients from healthy controls [204].miRNA-1269a has the best discriminatory ability of all of them. miRNA-20b-5p and miRNA-3187-5p are two further potentials for being diagnostic biomarkers in early-stage NSCLC [205].

Exosomal miRNAs have been studied for their prospective role in diagnosis and prognosis in a number of studies. For example, Dejima et al. [206] looked at miRNA-21 and miRNA-4257 and discovered increased levels of these miRNAs in NSCLC patients. They were also able to link them with clinical parameters such as the tumor size and the TNM stage were associated with miRNA-21 and the histological type, lymphatic invasion and TNM stage were associated with miR-4257.

Lung cancer can be screened for using exosomal profiles in pleural effusions. Out of 254 miRNAs detected in pleural effusion Exosomes, Lin et al. [207] discovered that miRNA-205-5p and miRNA-200b could differentiate between individuals with malignant effusions and those who had pneumonia and tuberculosis. These two miRNAs were also discovered in an exosomal miRNA mixture from peripheral blood (miRNA-429, miRNA-205, miRNA-200b, miRNA-203, miRNA-125b, and miRNA-34b), which had an 84 percent sensitivity and 74 percent specificity for

identifying early-stage patients [208]. Tamiya et al. [209] recently identified another pair of miRNAs, miRNA-182 and miRNA-210, which were capable of differentiating between benign and malignant pleural effusions with AUCs of 0.87 and 0.81, respectively.

Thanks to the creation of novel and user-friendly technical tools, Exosomes research is already being applied in clinical settings. For instance, a recent advancement established the development of a point-of-care device that analyses the salivary and urinary miRNA-205, one of the miRNAs identified as having diagnostic value in some of the earlier studies.

Twenty to forty percent of lung cancer patients have bone metastases, which negatively affects overall survival [210]. Three exosomal miRNAs were shown to express differentially in NSCLC patients depending on whether or not they had bone metastases by Yang et al. [211]. While miRNA-328-3p and miRNA-423-3p were discovered to be upregulated, miRNA-574-5p was found to be down regulated. They're all involved in the Wnt/-catenin signaling pathway, which means they can influence metastatic development.

The exosomal miRNAs have also been explored for the treatment selection and follow-up process. In terms of treatment resistance, Yuwen et al. [212] found that NSCLC patients with low miRNA-146a-5p expression had a short survival period which was free from cancer progression. Furthermore, an advanced expression of miRNA-146a-5p inhibits autophagy, which could reverse chemo resistance to cisplatin in A549 lung cancer cells. In terms of radiation, Zheng et al. [213] demonstrated that miRNA-96 can accurately identify lung cancer patients (AUC = 0.97) but also has the ability to identify radio resistant individuals (AUC = 0.75). Immunotherapy, with PD-1/PD-L1 as one of the targeted checkpoints, is another treatment option that has become a prominent tactic against lung cancer. Exosomes can also provide insight into the probable responsiveness to anti-PD-1 therapies. A profile of three miRNAs from the miRNA-320 family was found to predict the success of this sort of therapy in a study by Peng et al. [214], whereas downregulation of miRNA-125b-5p during treatment identified individuals in partial response.

2.6 Characteristics of Tumor Microenvironment

2.6.1 TME and Hypoxia

Hypoxia is one of the most extensively studied disruptions encountered in the tumor microenvironment (TME). The clinical implications of tumor hypoxia were delineated more than six decades ago, with the discovery that hypoxic tumor cells developed resistance to radiation therapy [215]. Over the subsequent years, hypoxia became a central aspect of tumor growth's pathophysiology, particularly concerning its role in evading cytotoxic treatments [216]. While the original "Hallmarks of Cancer" primarily emphasized genetic alterations driving uncontrolled proliferation, it did recognize hypoxia as a crucial factor in promoting VEGF-mediated angiogenesis [85]. In the era of groundbreaking immunotherapy, hypoxia has been identified as a pivotal link connecting emerging hallmarks such as "tumor-promoting inflammation" and "deregulated cellular energetics" [217]. With resistance to immune checkpoint inhibitors (ICI) and strategies to overcome it taking center stage in oncology, hypoxia and its relationship with various components of the TME have become essential targets for the next wave of immune-based therapies. To embark on these innovative therapeutic journeys, it is paramount to grasp the pathophysiology of hypoxia in the TME. In this section, we will delve into hypoxia's associations with (a) molecular markers, (b) immune cell dysfunction, (c) interactions between tumor and stromal cells, and (d) angiogenesis within the context of a tumor-induced immunosuppressive environment.

While the implications of tumor hypoxia are well-documented, the precise origin of low oxygen levels in the TME may be somewhat less understood. The uncontrolled and rapid expansion of cancer leads to a disordered amalgamation of tumor and stromal cells, resulting in a dysfunctional vascular supply and limited nutrient accessibility [218]. Additionally, as detailed below, the metabolism and energy production of aggressive tumors can transition toward a highly oxidative state, thereby perpetuating hypoxic conditions throughout the TME [219].

2.6.2 Glycolysis and PH

According to Warburg's landmark work from the 1920s [220], glucose was being converted into lactate by cancer cells, with the process of glycolysis, even while there was abundant oxygen. However, recent studies suggests that cancer can be glycolytic in vivo due the hypoxia response mechanisms [221]. Till date, there hasn't been any clear intervention that claims cancer cells to be fundamentally glycolytic. All interventions describe the hypoxia makes up the balance of cellular energy generation to promote glycolysis as a result of the synthesis and subsequent accumulation of lactate [222]. In fact, numerous research [223] have found that the uterine cervix has high median lactate levels, reaching up to 14 mM and around 7 mM, respectively, in head and neck cancers. Contrary to popular belief, lactate can be taken up by normoxic cancer cells via the monocarboxylate transporter-1 and used as a substrate for oxidative phosphorylation in place of glucose [224]. The release and synthesis of the cellular lactate synthesis leads to tumor acidosis. It is apparent that tumor cells contain effective systems for releasing protons into the extracellular space, just like normal cells do [225]. As a result, there is an intracellular pH gradient (pHi) that is greater than the extracellular pH across the tumor cell membrane (pHe). Normal tissues experience the opposite of this gradient, with pHi being lower than pHe [226].

2.6.3 FBS and Antibiotics Use in Growth Media

The culture mediums also typically contain antibiotics. In this line, the drug ciprofloxacin, commonly used to avoid mycoplasma contamination, has been shown to increase the amount of DNA connected with Exosomes and transferred on vesicular surface. A DNA-dependent mechanism is employed by the Exosomes produced by ciprofloxacin-treated cells for biding to fibronectin [227]. Because they are rich in proteins and contain EVs, medium components like fetal bovine serum (FBS) are essential. It has been shown that Exosomes contained in FBS are biologically active, and co-isolation of these Exosomes may interfere with subsequent in vitro or in vivo investigations [228].

Exosomes can be removed from FBS using any method, and several research teams have developed their own methods for removing bovine Exosomes from sera. During Exosomes release, one tactic is to culture cells in serum-free medium; however, the lack of FBS may seriously starve the cells, which can change cellular behavior and Exosomes composition [229]. Although the amount of separated EVs was considerably different, EVs grown in serum-free media did not have significantly different biophysical or size variations from EVs grown in serum-containing media. Additionally, it was discovered that the concentrations of particular vesicular proteins (such as small GTPases, G-protein complexes, mRNA processing proteins, and splicing factors) differed in EVs formed under distinct growth circumstances [230]. Exosomes can be removed from FBS using multiple methods., but the most popular ones are extended ultracentrifugation or filtering [231]. The ISEV recommendations advise using fresh medium that hasn't been cultured with cells as a control in downstream Exosomes tests [232] since complete EV depletion might not be accomplished.

2.7 Various Approaches for Isolation and Quantification of Exosomes

Exosomes must be isolated in a good number and quality from any biological fluid depending on the experiments to examine structure, function, mechanism, bio molecular composition, and other phenomena. A variety of strategies have been optimized and employed up to this point. Some of the most widely used methods are summarized in table 2.1 and figure 2.6 [233].

2.7.1 Centrifugation

Exosomes are extracted in research studies via repeated centrifugation. The cellular debris and unwanted cells are removed through low-speed centrifugation, done at least twice or thrice. The ultra-filtering of isolates is carried with a 0.2 micrometer filter which is ultra-centrifuged at 100,000g for 60-120 minutes. It is

further rinsed with 1X phosphate buffer saline (PBS) and again centrifuged at 100,000g for 90 minutes [232]. However, a demerit of this approach is that it fails to produce completely pure Exosomes at the end. This is majorly because Exosomes can be separated from vesicles of similar size during the centrifugation process. As the small vesicles isolated through a 100,000g pallet can be further distributed into multiple groups if floatation in iodixanol gradients is employed along with immune-isolation (using beads coated with immunoglobulins targeting CD9, CD63, or CD81), they are referred to as small extracellular vesicles (SEVs) rather than Exosomes. In addition, the Exosomes in the serum attached to the lipid droplets and lipo-particles can be extracted with other Exosomes during ultracentrifugation [234]. Ultracentrifugation's throughput is likewise limited by the rotor's capacity. The most used method for isolation Exosomes is the differential centrifugation method as it leads to the production of Exosomes-enriched isolates. Density gradient centrifugation is capable of isolating the purest Exosomes population. Time expenditure and low yields are two disadvantages. [45].

2.7.2 Immuno-Isolation

Purified subpopulations of EVs (extracellular vesicles) can be obtained using immuno-isolation, which uses specific membrane proteins. On the other hand, the technique demands careful protein membrane selection and protocol optimization. It was discovered that immunological affinity Exosomes collection (from colon cancer cell lines) was more effective than density gradient centrifugation or ultracentrifugation [45].

TABLE 2.1: Different techniques used for isolation and purification of Exosomes

Method	**Mechansim**	**Advantages**	**Disadvantages**
Ultra-filtration	molecular weight and Size	Fast and Simple operation, High yield, suitable for RNA extraction	Specificity Issues; scaling issues
Ultra-centrifugation	Density	The gold standard, High purity	Laborious; Time consuming
FFFF or AF4	Field flow (fractionation)	Wide variety of eluents, Wide separation range	Requires fractionation equipment; time taking

Method	**Mechansim**	**Advantages**	**Disadvantages**
SEC	Molecular weight and Size	Fast and Simple operation, high yield; suitable for RNA extraction	Lack of specificity; Difficulty in scaling
Hydrostatic filtration dialysis	Size	Suitable for large EVs, Fast and Simple operation	Centrifugation needed, Molecular weight cut off dependent
Microfluidics based techniques	Size	High purity and low processing time	Advanced instruments required
Exosomes	through kit	Fast operation, High purity; high recovery	Suitable for Low sample volume
Polymer precipitation	Surface (charge)	User-friendly and Easy processing	Scaling and specificity problems
Immuno affinity based techniques	Affinity purification	High purity and specificity	Expensive, low yield

2.7.3 Quantification of Exosomes

The Nanosight instrument, which utilizes a light diffraction mechanism to determine the quantity and size of nanoparticles, is employed for exosome quantification. qNano Gold (Izon Science) is an alternative approach that operates on the premise of Tunable Re- sistive Pulse Sensing (TRPS). Nevertheless, both of these techniques share the drawback of not definitively confirming the presence of Exosomes, as they tend to measure various types of nanoparticles in the solution, irrespective of whether they are Exosomes or not. Some of the most widely used methods are summarized in table 2.2. For example, plasma isolated Exosomes may contain contaminants such as albumin or IgG (stay range of Exosomes), skewing results [235].

TABLE 2.2: Different techniques used for quantification of Exosomes.

Method	**Mechanism**	**Advantage**	**Disadvantage**
Mphology Analysis	EV array	Simple processing	enclosed antigens Detection incapability
	SEM and TEM	Easy process	Scaling and specificity issues
	NTA	Gold standard	Apparatus is Expensive
Proteins based analysis	Western blot	Specific and quantitative method	No number of EV are detected, Size based particles are not ranged
	Flow cytometer	Reproducible analysis, well adapted technique, Easy	Exosomes are not detected through forward scattered light

2.8 Current Research Work and Research Gap

Since Exosomes were first identified thirty years ago, it has become obvious that Exosomes have a role in a variety of physiological and pathological process. As discussed in detail in our studied literature, the MVs including Exosomes have been studied widely with different aspects like their physiology, morphology, cargo, interactions, biogenesis, signaling, pathology, diagnostic biomarkers, Nano carriers, targeted therapy etc. Even most of the studies have also focused their unique role in cancer but as per our knowledge very few studies were found to study the role of Exosomes in immune system during cancer environment. Some researchers have started studying its important roles in pathological conditions. But the effects of cancer Exosomes on immune cells especially T- cells is not clearly identified and studied. To address this gap of research an attempt has been made to conduct a project to compare the production of Exosomes in normal and cancer environments. Then their effects on T cells proliferation and, also to study the expression of different important markers of T cells upon exposure to cancer Exosomes. This project gave rise to different research questions for scientific community to focus on and study these diverse areas to step ahead for immune therapy of NSCLC.

This study is not limited to NSCLC but has also opened an avenue to study the same area for different cancers.

Chapter 3

Materials and Methods

3.1 Methodology Overview

The hypothesis of the current study was to evaluate the effect of cancer derived Exosomes on T cells proliferation and their markers responsible for T cells activities. The experiment was designed to optimize the parameters and conditions to increase the production of Exosomes. The comparison was done between two growth conditions i.e. Normal growth conditions (NGC) and modified growth conditions (MGC) and its validation on Jurkat cells and human T cells. For this purpose, cancer derived Exosomes were treated with Jurkat Cell lines. The results were then validated by using the animal models experiments where the tumor induced mouse isolated Exosomes were treated with the normal mice isolated T cells. In all experiments the T cells proliferation and markers expressions were analyzed through FACS analysis. The H1975 isolated Exosomes were also studied on molecular level to identify their miRNAs and proteins contents.

The identified miRNAs were then analyzed by bioinformatics tool to determine their targeted genes and their role in multiple pathways. Exosomes proteins were also analyzed. Details of the experiments performed is as given below in the following section and summarized in figure 3.1.

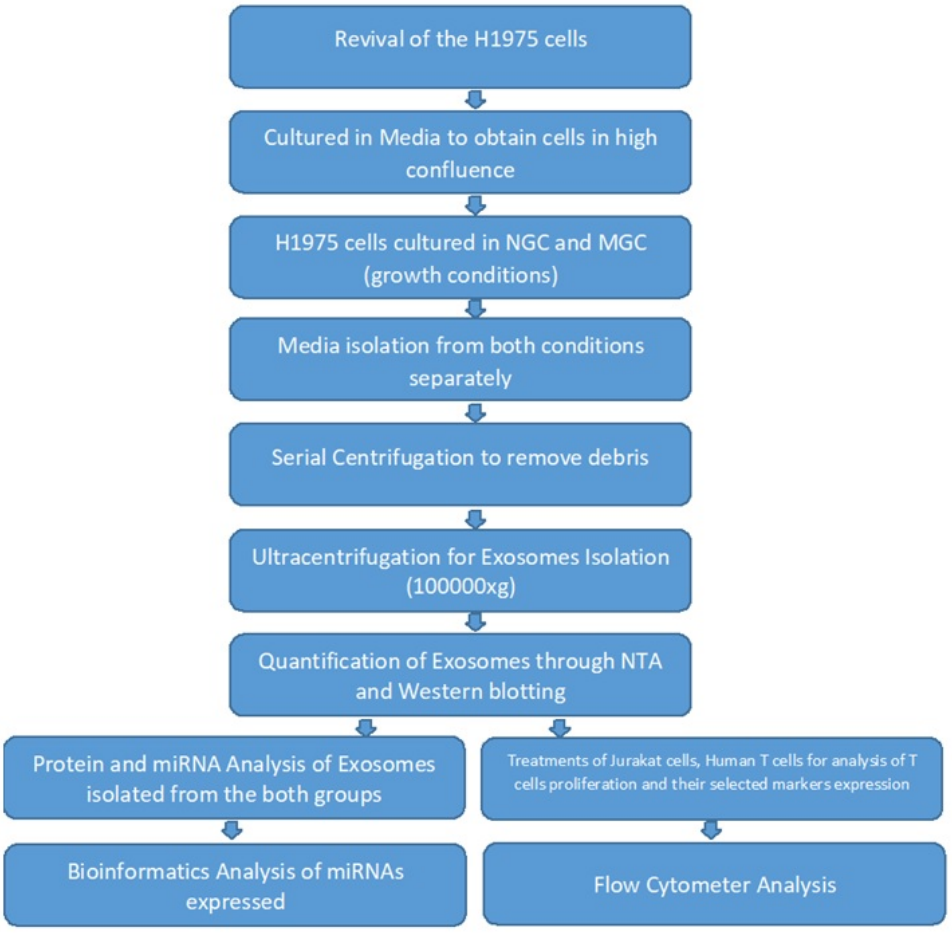

FIGURE 3.1: General overview of the experiments. Revival of the cells, Cells grown in media, Filter centrifugation, Serial centrifugation to remove larger particles, Ultracentrifugation to isolate Exosomes, Diluted Exosomes pellets, Exosomes used for treatment of the T cells, flow cytometry after the treatment, Quantication of the Exosomes through Nano sight and western blotting, Diluted Exosomes for molecular analysis of miRNAs and proteins.

3.2 Cell Culture (H1975) to Obtain a Desired Confluence

The cell line H1975 (NSCLC cells) was used in this study for the Exosomes production. The cell line H1975 is a human lung cancer (NSCLC) epithelial cells and were donated by a none smoker female in 1988. These are usually grown on RPMI-1640 media at 37°C. H1975 are adherent cells (Figure 3.2). The cell line was purchased earlier from the American Type Culture Collection (ATCC). Before

the experiments, absence of *Mycobacterium* contamination was confirmed through PCR. To get higher confluent cells, these cells were grown and maintained by following the standard aseptic techniques and protocol to avoid any contamination during the process.

H1975 cells were taken out from the liquid nitrogen. The cell was further treated with Dimethyl sulfoxide (DMSO) to obtain purified cells. All vials were centrifuged at 1500 rpm for 3 minutes. The pellet was dissolved in 1ml media and af ter cell counting; 1 million cells were grown in pre-warmed RPMI (Roswell Park Memorial Institute)-1640 medium to achieve a desired confluence. A stock of 500 ml RPMI-1640 media (pH=7) was prepared with the addition of 10% FBS, 1% penicillin/streptomycin [236]. 15 ml complete RPMI 1640 media with 1 million cells was added to T-75 flasks (8-15 ml) and were grown in incubator having 20% Oxygen, 5% CO_2, 37°C in for 48 hours. To avoid the DMSO from harming live cells, the media was changed once the cells had attached. After 48-72 hours, when the cells were found confluent, they were grown in two different conditions for the Exosomes production and isolation.

3.2.1 Preparation of Exosomes Free Fetal Bovine Serum (FBS)

Commercially available FBS was processed and purified to remove the naturally occurring Exosomes and to obtain Exosomes free FBS for the production of Exosomes from the H1975 cells to be grown in NGC. FBS was transferred in tubes inside the Biosafety cabinet and the sealed tubes were centrifuged at 1500xg for 10 min. This centrifugation resulted into the debris being pelleted down. Then, the supernatant was shifted into new labeled tubes and again centrifuged at 2500xg to eliminate large components. This supernatant was transferred into 35ml ultracrimp centrifuge tubes and were centrifuged at 100,000xg overnight (12-13 hours) at 4°C. The supernatant FBS was separated in new tubes inside the biosafety cabinets and were placed at -20°C until further use. The conditions were optimized for H1975 cells to enhance the Exosomes production by providing some of

the favorable conditions which mimic the tumor environment and may increase the secretion and production of Exosomes as shown in figure 3.2. In MGC, use of FBS free media was also one of the parameter. The growth of the cells in FBS free media was not effected as the incubation period was only 24 hours to collect Exosomes [237]. Cancer cells can grow and divide without FBSfor 24 hours as also observed by Doyle et al., [238].

3.3 H1975 Cells Culture for Exosomes Production

When the cells from the last experiments were found in a required confluence, they were isolated from the media, washed with PBS and were divided in two groups of T-75 flasks containing media prepared with different formulation for both the groups (NGC & MGC).

3.3.1 Normal Growth Conditions (NGC) for Exosomes Production

One group of the cells were cultured in NGC, with the parameters like RPMI-1640 media having PH=7 and was supplemented with Exosomes free FBS 10%, and 1% penicillin and streptomycin, conditions provided were 20% Oxygen, 5% CO_2, 37°C for 24 hours. Four T-75 flasks were used, each flask containing 12-15ml media and 1 million H1975 cells. These are the normal parameters used to culture cancer cells in Lab [239].

3.3.2 Modified Growth Conditions (MGC) for Exosomes Production

The second set of conditions that mimic the cancer microenvironment [240] was utilized as: RPMI-1640 media (pH-6) without FBS and antibiotics and were grown

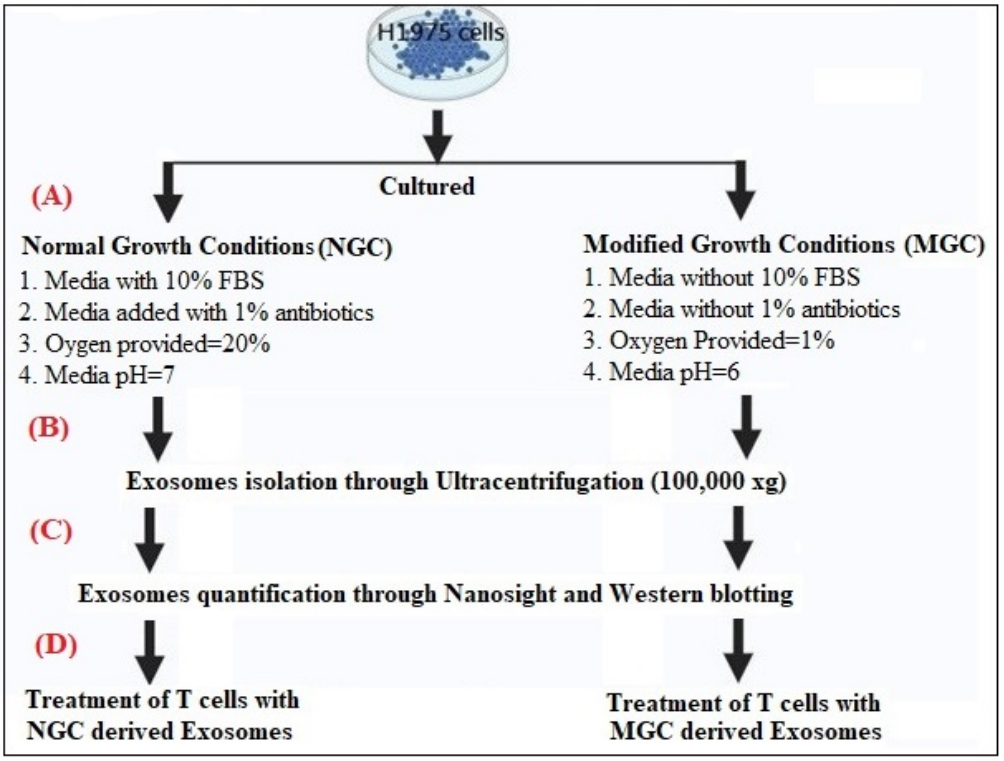

FIGURE 3.2: Experimental flow of Exosomes production from H1975 cells in both NGC and MGC (growth conditions). (A) Both conditions are demonstrated. After the the cells were grown in given conditions, (B) the Exosomes were isolated through ultracentrifugation and (C) their characterization was done through Nanosight and Western bloting. (D) Treatment of T cells was done with isolated Exosomes from both NGC and MGC.

in incubator having 1% Oxygen, 5% CO_2, and 37°C for 24 hours. Same amount of media, cells and flasks were used as in NGC.

3.4 Exosomes Isolation

After 24 hours of incubation the media was harvested from both groups in two different labeled tubes and Exosomes were isolated by using a combination of ultrafiltration and ultracentrifugation. This experiment was practiced in aseptic conditions to avoid Exosomes contamination. Media was collected from both conditions inside the Biosafety cabinet and modified protocol described by The'ry et al [232] was used with a small modifications to isolate Exosomes through centrifugation. Media was centrifuged for 10 min at 300 x g for 10 minutes to pellet down the cells. The supernatant was centrifuged at 2000 x g for 10 minutes to

remove dead cells. Then the supernatant was centrifuged at 10000 x g for 30 minutes to remove cell debris and other larger components. The media supernatants were transferred to new two labeled tubes (NGC & MGC) and were centrifuged at 2500xg for 10 minutes. The filtered media was transferred to new ultracentrifuge tubes (Ultra-crimp 35ml) inside the biosafety cabinet. By following Thermo Scientific^{TM} Sorvall^{TM} WX+ Ultracentrifuge Series standard protocols, the tubes were centrifuged in a pre-cooled (4°C) ultracentrifuge machine at 100,000xg for 120 minutes. The pellets were washed and re-suspended with Phosphate- buffered saline (PBS) and again ultracentrifugation was done at 100,000xg for 120 minutes. The pellets were washed and re-suspended with Phosphate buffered saline (PBS) and again ultracentrifugation was done at 100,000xg for 120 minutes. Exosomes separation through ultracentrifugation may damage some of the particles, but in comparison with other separation methods this technique is preferred, because it is well established and widely used, with high Yield, Purity, Versatility, and Cost-Efficiency [241]. The Pellets (Exosomes) were re-suspended in 200μl PBS (fresh sterile). The Exosomes were kept at -80°C or were preceded for confirmation, quantification and experiments.

3.4.1 Exosomes Quantification and Confirmation through Nano Sight

Nanoparticles tracking analysis (NTA) is performed through Nano Sight, which is the most widely used method for the analysis of Exosomes in scatter and fluorescence mode and is not dependent on any molecular marker(s) [242, 243]. The Nano Sight NS300 (Malvern, UK) NTA instrument used in this project, has the ability to determine size and concentration, which are critical for Exosomes characterization. Exosomes pellet in each tube (NGC and MGC) was dissolved in 200μl 1XPBS buffer [244]. The solution was mixed through pipetting and was loaded in 1 ml syringe to be injected in Nano Sight. The machine was switched on and the software was run and was monitored on the screen to get a better frame of Exosomes. Then the machine was commended for analysis and the data was saved.

3.4.2 Confirmation and Comparison of Selected Protein Expression from NGC and MGC Exosomes through SDS PAGE and Western Blot Analysis

The isolated Exosomes were in the size range of 30-150 nm. To further confirm and analyze total protein of the isolated Exosomes from NGC and MGC, Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting was done. The SDS PAGE was performed to get a complete picture of banding pattern from both the conditions.

Proteins were isolated by following the standard protocol described for RIPA lysis buffer protein isolation method [245]. The protein present in the Exosomes pellet were quantified through western blotting. The bicinchoninic acid (BCA) protein assay [245] was also done before blotting to make sure that similar quantity of protein i.e. 40ug was added in each sample.

3.4.2.1 SDS PAGE of Exosomes Protein

Two gels (7.5%) were prepared (one for a complete image and one for Western Blot analysis). A 5μl protein marker (Precision Plus ProteinT M Unstained Protein Standards, Ct#1610363) was also run in the first well. Then the second well was loaded with NGC Exosomes isolated protein, third well was loaded with MGC Exosomes isolated protein.

For each sample 40μl protein was used. Electrophoresis was performed at 60V for 30 minutes until the samples passed the stacking gel. Then resolving gel was run at 120V for 2 hours or till the dye front reaches the bottom of gel. The gels were separated from plates and one gel out of two was first stained (Commasie blue R-260) and then de-stained until the gel gets transparent and bands are shown in a sharp resolution. Gel Doc was used to process and save the image [246].

3.4.2.2 Western Blot Analysis of Exosomes Protein

The second gel was processed for western blot analysis of the Exosomes specific markers (CD9 (Anti-CD9 antibody, [ab223052], Abcam) & CD63 (Anti-CD63 antibody [EPR21151], abcam) and the expression other selected protein including CEACAM1 (Anti-CEACAM1 antibody [EPR4049], ab108397, Abcam), HVEM (TNFRSF14 Antibody (PA5-29780) in WB, Thermofisher), PDLI (Western blot - Anti-PD-L1 antibody, ab228415), among the NGC and MGC derived Exosomes. These are cancer proteins which are found to affect T cells activities and their proliferation in previous studies. Calnexin (Anti-Calnexin antibody [EPR3633, abcam] - ER Membrane Marker) was used as negative control for Exosomes [247, 248]. For loading control α-Tubulin (Anti-alpha Tubulin antibody [DM1A]- Loading Control, ab7291, abcam) was used. For all western blots, Bio-Rad (Mini-Protean TGX Precast Gels 4-15%), 30μl/well, and 10-well combs were put to use. The samples, 4X buffer, and 10X reducing agent were mixed together to make a solution having 10 μl volume. Western blot protocol described by Mehmood and Yang [249] was followed. The solution was heat-denatured at 70°C for 10 minutes, followed by loading onto precast gels in clamp assemblies. The gels were run at 100V for 1 hour in a chamber filled with 10X Running Buffer. Subsequently, the gels were transferred to nitrocellulose membranes for protein transfer. The loading order on the gel was: pad, filter-paper, equilibrated-gel, nitrocellulose-membrane, filter- paper, fiber-pad. Prior to assembly, gel components, excluding the gel itself, were soaked in 1x Transfer Buffer, and the buffer tank was equipped with a cooling block running at 100V for 30 minutes. To prevent non-specific protein binding, the nitrocellulose membrane was pre-soaked in a 5% milk solution, followed by washing in TBS-Tween buffer. Primary antibodies (CD9 = ab58989, CD63 = ab231975) diluted at 1:1000 were applied to the membrane, followed by overnight incubation. After washing, a 1:300 solution of secondary antibody and Blocking Buffer was added to the membrane and incubated. The membrane was washed again and subjected to substrate treatment, followed by imaging using the Gel Doc system (Bio-Rad). For all western blotting experiments the graphical results were taken as means of three repeated experiments. ImageJ software was used to

get numerical data from the protein bands and Graph Pad Prism 10.0.3 software was used for graphs designing.In both experiments Exosomes isolated from NGC and MGC were analyzed to confirm and compare the presence of selected markers and protein in both groups derived Exosomes and their expression level due to changes in growth conditions.

3.5 Effects of H1975 cells Isolated Exosomes on Jurkat Cells Proliferation

After the confirmation of pure extracted Exosomes from both the NGC and MGC, Isolated Exosomes were treated with the Jurkat cells line (immortalized T lymphocyte cell line) [250] and their microscopic view in different densities are shown in figure 3.3. This experiment was done to analyze the effects of NGC and MGC derived Exosomes on the proliferation of Jurkat cells. Jurkat cells were taken from ATCC; the details are given in table 3.1.

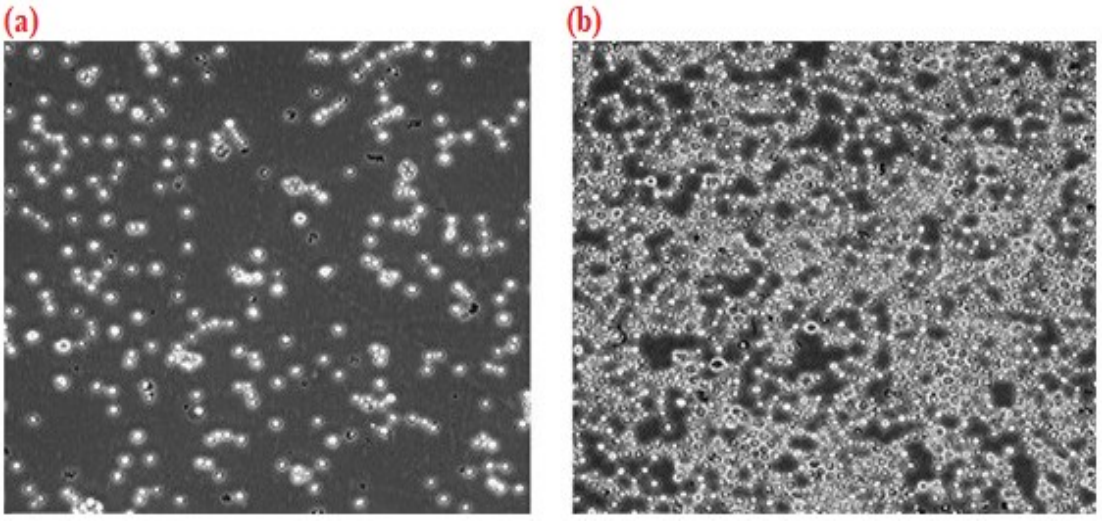

FIGURE 3.3: Microscopic view of Jurkat cells with (a) low and (b) high densities.

A six-well plate was taken and four wells were added with 1ml RPMI-1640 Media and 1x105 numbers of Jurkat cells and were labeled as shown in the Table 3.2. The following sets of experiments were designed for the treatment with four groups.

Table 3.1: Jurkat cell line information provided by ATCC

Organism	Human
Tissue	Blood
Cell Type	T Lymphocyte
Product Format	Frozen
Morphology	Lymphoid
Culture Properties	Suspension
Biosafety Level	2
Disease	T cells leukemia (Acute)
Age	14 years
Gender	Male
Storage Conditions	Liquid-nitrogen
Tumorigenic	Yes
Growth Medium	RPMI-1640
Culture Conditions	37°C
Name of Depositor	Korsmeyer Stanley

Table 3.2: Plate designed for the treatment experiments

Well number	Experiment
1	Jurkat cells only
2	T Jurkat cells and (T cells stimulators) Phyto-Hemagglutinin (PHA) / phorbol 12-myristate 13-acetate (PMA)
3	Jurkat cells and Exosomes isolated from NGC
4	Jurkat cells and Exosomes isolated from MGC

In the first well, only Jurkat cells were added to media, while in the second well Jurkat cells were added 1μl PHA/PMA (T cells stimulators). Similarly, in the third well, Jurkat cells were added with 200μl Exosomes isolated from H1975 cells from NGC. The fourth well Jurkat cells and 200μl of Exosomes isolated from H1975 cells under MGC were added. The Exosomes added wells were also supplemented with PHA/PMA as done for the well # 2. The plate was incubated for 24 hours at 37°C.

3.5.1 Flow Cytometer Analysis of Jurkat Cells

After 24 hours of incubation four centrifuge tubes were labeled accordingly and the media containing Jurkat cells were transferred into these tubes. Modified protocol described by Holmes et al [251] was used to prepare sample for flow cytometer analysis. All tubes were centrifuged for 5 minutes at 1500 xg. The supernatant was discarded and Jurkat cells collected in the form of pellets. The pellets were washed with 1XPBS and again centrifuged with the same protocol used before [251]. Each washed pellet of Jurkat cells was re-suspended into 100 μl Jurkat cells markers Anti-CD69 antibody (Catalog # ab25190) and CD25 Monoclonal Antibody (CD25) eBioscience^{TM} (Catalog # 11-0257-42) solutions. Marker solutions were prepared in PBS with the recommended dilution of the suppliers. The samples were incubated at 4°C for 30 minutes. All four samples were run through the Flow cytometer with the standard parameters used to quantify the cells. Flow cytometry analysis was performed using a FACS Arial III flow cytometer (BD, USA) by gating lymphocytes, then CD4 or CD8 cells and finaly IFN-γ. Data were analysed using FlowJo software (V.10.4, FlowJo, USA). After the evaluation of the effect of cancer Exosomes on Jurkat cells proliferation, it was further evaluated on human blood isolated T- cells where IFN-γ expression was analyzed in CD4 and CD8 T cells.

3.6 Human Blood Collection Process and T Lymphocytes Isolation

Fresh blood required for Isolation of Human T lymphocytes was collected during this study which was covered by ethical approval granted by the Research Ethics Committee (Macau University of Science and Technology, Macau). The protocol published by Efthymiou et al [252] was used with some modifications. A fresh blood sample was obtained from blood bank with all required information and following the proper SOPs. The healthy donor human blood was stored at RT (room temperature). 3 ml blood was slightly pipeted in a round bottom tube

(8ml) with tube containing polymorph density gradient medium forming two layers. The PBMC was segregated from blood components on the upper cell layer after centrifugation for 45 minutes at 500xg at RT. Three layers were obtained after centrifugation (Foggy, gleamy and yellow from top to down). The first two layers were removed and the yellow required layer (50ml) was transferred to conical tube. It was again centrifuged for 5 minutes at 500xg each time. After 2-3 washes, the supernatant became foggy. PBMC in the form of pellet was transferred to T-75 culture flask with 20mL media (RPMI 1640) with FBS 10%, 1% streptomycin/penicillin, and 1 g/mL PHA. Incubated for 1 hour in 5% CO2 and 37°C.

It is recommended to utilize RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin without adding with PHA if a short incubation (1 hour) is used at this step. This step was performed to separate the monocytes that attached to the flask surface from the lymphocytes that remained suspended. After that media was removed from the flask, and added to a 50 mL tube, and centrifuged for 5 min at 500xg. The cells from the pellet were re-suspended in media (which primarily contained lymphocytes) and were transferred to a new T-75 flask containing 25 mL media (RPMI 1640) containing FBS 10%, 1% streptomycin/penicillin, and 1 g/mL PHA, incubated for 3 days at 37°C. After 24 hours of growth, 15 ml fresh media was added to the T- 175 flask and was incubated for 3 days. Then media was removed and suspended lymphocytes from the flask and transfer to a 50 mL tube. It was centrifuged for 5 min at 500xg and resuspended the cells were and transferred cells to a new T-75 or T-175 flask containing 25 mL (T-75) or 50 mL (T-175) RPMI 1640 with 10% FBS, 1% streptomycin/penicillin, and 20 ng/mL human IL-2 or IL-15. The lymphocytes were grown for 5 days.

3.6.1 Cryopreservation of T Lymphocytes

In these experiments human T cells were immediately used after isolation and also preserved the leftover cells for the repetition of the experiments and reproducibility of the results. For the preservation of PBMCs in liquid nitrogen at low temperatures (ultra-low), DMSO (cryo-protectant) was used to prevent cells damage with

ice crystals formation. DMSO at higher temperatures is toxic to cells. Therefore, once the cells are thawed, DMSO was washed immediately. To freeze freshly isolated PBMCs, they were mixed in freezing medium (5x10^6cells/ml) containing DMSO 10-20% and FBS 40% in RPMI-1640 medium. Container with the mixture was placed at -80°C for gradual cooling overnight followed by its transfer to liquid nitrogen for long term preservation. For this purpose, protocol was adopted from Efthymiou et al [252] with a small modification.

3.6.2 Exosomes Isolation from H1975 Cells

Exosomes required for identifying their effects on markers expression responsible for T cells activities were isolated with the similar techniques mentioned as above in section 3.4 from H1975 cells (from MGC and NGC). The adopted EVs classification system divides these membrane structures into three groups: Exosomes (size range upto 150 nm), ectosomes (size range upto 1000 nm, referred here as microvesicles, MVs), and apoptotic bodies (size range upto 5 μm) [253]. In this study the purity of Exosomes was confirmed from NTA (Almost all particles were in the range of Exosomes, below 200 nm) and Western Blot results with Exosomes Specific Biomarkers (CD9 and CD63).

3.6.3 Effects of Cancer Exosomes on IFN-γ Expression in CD4 and CD8-T Cells

The effects of NGC and MGC derived NSCLC Exosomes on the expression of IFN-γ in CD4 and CD8 T cells was analyzed by treating the human T- cells in six-well plates with counted cells number (1x10^6cells/ml) and quantified Exosomes (200ul) as shown in Table 3.3. After treatment, plates were incubated at 37°C for 24 hours. The expression of human T cells was observed by using the Flow cytometer analysis [254].

Four groups were categorized based on treatments:

TABLE 3.3: Plate designed for the treatment experiments

Well number	Experiment
1	Human T cells
2	T Human T cells and PHA/PMA
3	Human T cells and Exosomes (derived from NGC grown H1975 Cells)
4	Human T cells and Exosomes (derived from MGC H1975 Cells)

In the first well, only Human T cells were added to media, while in the second well Human T cells were added 1 μl PHA/PMA. Similarly, in the third well, human T cells were added with 200μl Exosomes isolated from H1975 cells under NGC. The fourth well Human T cells and 200μl of the isolated Exosomes from MGC were added. Well 3 and 4 were also added with 1μl PHA/PMA. The plate was incubated for 24 hours at 37°C.

3.6.4 Flow Cytometer Samples Preparation and Analysis

After 24 hours of incubation, four centrifuge tubes were labeled accordingly and the media containing human T cells were transferred into these tubes. All tubes were centrifuged for 5 mints at 1500 xg. The cells were collected in the pellets form. 1XPBS was used for pellet wash and re-centrifuged with the same protocol used before. Each washed pellet of cells was re-suspended into 100ul cell surface markers like Anti-CD45 antibody, (ab40763), CD3 Monoclonal Antibody, eBioscience^{TM} (Catalog # 11-0032-82), Anti-CD4 antibody (ab269349) and CD8 Monoclonal Antibody (Catalog # MHCD0801). Marker solution was prepared according to suppliers' recommendations. The samples were incubated for 30 mints at 4°C and were centrifuged for 5 mints at 1500 xg, the supernatants were removed and 100μl membrane breaking buffer was added and mixed. These tubes were incubated at 4°C overnight and after 24 hours' tubes were centrifuged at 1500 xg for 5 min. Pellets were washed with 1XPBS. Each washed pellet of cells was re-suspended into 100μl Intracellular markers stain e.g. IFN-/*gamma* Monoclonal Antibody, eBioscience^{TM} (Catalog # 53-7319-42) to analyze CD4-IFN-γ, CD8-IFN-γ). The

tubes were incubated for 30 mints at RT and were run through a Flow cytometer. Samples preparation protocol was adopted from Holmes etal., 2001 [251]. After the Flow cytometer was completed, data was analyzed.

3.7 Mouse Models Tumorigenesis and their Exosomes Isolation for Treatment with Healthy Mouse Spleen Isolated Immune Cells

3.7.1 Animal Facilities

The effects of tumor mice derived Exosomes on normal mice spleen isolated T cells were analyzed. The main concept of this part of the study is summarized in figure 3.4. Selected C57BL/6 mice (age: 8 weeks, number was 200) were housed in

FIGURE 3.4: Schematic diagram of animal models study.

specific pathogen-free facilities in ventilated cages, with normal water food supply

and regular cages cleaning on daily basis. The C57BL/6 mice strain is particularly useful in cancer research as they have low mammary tumor incidences. The mice could acclimatize for 7 days following delivery to the animal house prior to tumor induction. These experiments were approved by the Macau University of Science and Technology Bioethics committee. Aseptic precautions and standard operating procedures were implemented in the animal facility. These precautions included the practice of hand washing and the use of disposable gowns, gloves, and hats before entering the facility. The laminar flow hoods and gloves were regularly cleaned with 70% ethanol to prevent cross-contamination between animals. Additionally, all instruments and consumables were autoclaved before being used in the hood.

This in vivo experiment was established to produce and isolate induced tumor's Exosomes. H1975 cells were cultured to obtain adequate quantity. Two groups with tumor induced group (200) and un-treated healthy mice (40) were categorized and housed in different cages in the same environment. H1975 cells ($1x10^6$ cells/ml) with 0.2 ml FBS-free media containing 50% matrigel were injected subcutaneously, followed the protocol described by Graham et al [255] and Ahsan et al [256]. All tumor induction procedures were performed in aseptic environment to prevent infection. The cells were injected using a 24-gauge needle and 1 ml syringe. Animals were examined and weighed twice weekly to ensure general health and to monitor tumor growth. After the tumor production started in mice, these were observed with the tumor size and when the tumor size reached to 70-100 mm3, Blood was collected from the retro-bulbar sinus from both groups separately. Whole blood was collected in tubes and were processed for serum separation [257].

3.7.2 Serum Preparation

Blood was allowed to clot for 15-30 minutes at room temperature and then centrifuged for 10 minutes at 1000xg. Serum was collected in separate tubes and were processed for Exosomes isolation immediately. Blood from five mice yielded approximately 4-7 ml serum.

3.7.3 Exosomes Isolation

According to a previously described method [258] the serum samples were added into 50 ml tubes, the large cell's fragments or debris were eliminated by centrifugation for 20 minutes at 2000xg on 4°C. Further microvesicles and apoptotic bodies were removed from the collected supernatant by centrifugation at 4°C for 45 minutes at 12000xg. The supernatant was passed through 0.22μm filter and

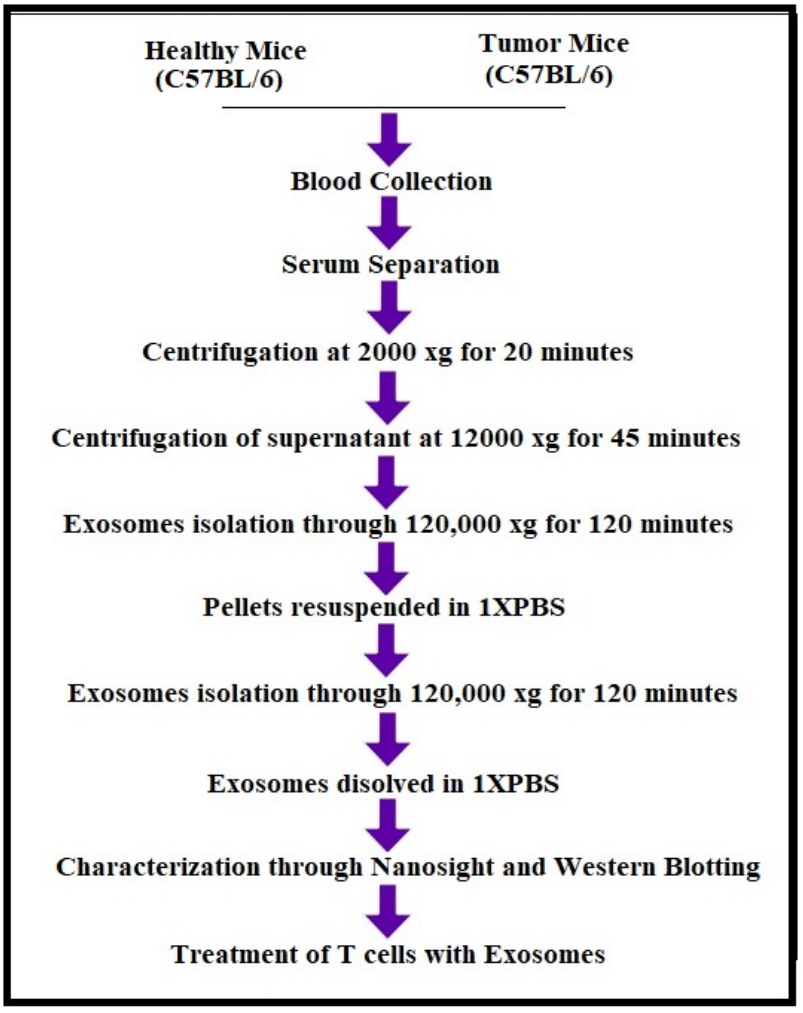

FIGURE 3.5: Experimental flow of Exosomes isolation from healthy and tumor mice through serial centrifugation and their characterization through Nanosight and western blot analysis.

was transferred into new ultracentrifuge tubes. The centrifuge machine was pre-cooled to 4°C and the tubes were centrifuged for 120 minutes at 120,000xg. The pellets were re-suspended in PBS and then filtered with a 0.22μm. Centrifugation was repeated (120 minutes at 120,000xg) and the pellets (Exosomes) were

re-suspended in 200μl PBS. Nanosight analysis and western blotting were used for the characterization and quantification of the Exosomes. After confirmation, samples were processed for further experiments.

3.7.4 T cells Isolation from Normal Mice Spleen

The process of spleenocytes isolation was carried out using aseptic techniques and sterile medium as discussed earlier by Grosjean et al. [259]. This was accomplished by euthanizing a normal mouse and collecting the spleen via dissection of the abdominal cavity using autoclaved scissors and other instruments. Blood remnants and other debris were rinsed out of the spleen with PBS (5 times washes). The spleen was then transferred to 10% FBS and 1% penicillin-streptomycin supplemented RPMI 1640 media. Then it was placed in a 70-micron cell-strainer covered 50ml tube and was gently sliced into small pieces with sterile scissors. A small quantity of media was added to avoid drying of spleen. The fragments of spleen tissue were gently forced through the strainer with a 1 ml syringe plunger by adding media. Gentle approaches were used during the procedure to avoid cellular damage and death. The spleen tissues finally turned into milky appearance, indicating that the majority of the cells were removed and collected in tube. 25-30 ml media was added to the collected cells and were centrifuged at 2000 xg for 5 min at 4°C. The cells pellet was disaggregated because retaining the cells in the pellet for an extended period of time can cause them to lose viability. Then the cells pellet was re-suspended in 1X RBC (Red Blood Cells) Lysis Buffer (supplied by eBioscienceTM, Catalog number: 00-4333-57) and then tubes were incubated on ice for 5 min to lyse RBCs. After incubation, cells were washed in cold 1XPBS or medium up to 25 mL and were centrifuged at 4°C at 2000xg for 5 mints. The pellet was disaggregated before being re-suspended in 5ml of medium and mixed with pipetting. Filtration of the cell suspension using a 70-micron cell strainer removed clumps of dead cells and debris. Cells were counted using a hemocytometer at a 1:10 dilution with Trypan blue after being incubated on ice for 10 min. The lymphocytes appeared to be spherical and glowing. The viability rate was found to be 90%. The cells were then diluted to the desired concentration (5 million

cells/ml) for the subsequent operations. Cells were plated directly on media and incubated with 5% CO_2 at 37°C.

3.7.5 Treatment of Mice T Cells with Tumor and Healthy Mice Isolated Exosomes

Spleen isolated cells were taken out of the incubator and a counted number of cells were diluted to $1x10^6$ cells/ml. A six well plat was taken and wells were added with 2ml media containing cells and were slowly mixed in wells through a gentle pipetting or swirling the plate. The normal mice spleen isolated T cells were treated with purified Exosomes in different sets of experiments as shown in Table 3.4, included Mice T cells only, Mice T cells + LLC (Lewis lung carcinoma cell line), Mice T cells + Normal mice Exosomes, Mice T cells + LLC + Normal mice Exosomes, Mice T cells + Tumor mice Exosomes, and Mice T cells + LLC + Tumor mice Exosomes.

Six groups were categorized based on treatments: The reason behind the use of

Table 3.4: Plate designed for the treatment experiments

Well number	Experiment
1	Mice T cells only
2	Mice T cells + LLC
3	Mice T cells + Normal mice Exosomes
4	Mice T cells + LLC + Normal mice Exosomes
5	Mice T cells + Tumor mice Exosomes
6	Mice T cells + LLC + Tumor mice Exosomes

LLC was to analyze the expression of the selected markers in the presence of mice tumor cells (LLC). The plate was incubated for 24 hours at 37°C. The plate was taken out and the media containing cells were collected in separate tubes and were centrifuged for 5 min at 1500xg. The cell pellets were processed for Flow cytometer analysis. Samples were prepared for the Flow cytometer analysis as mentioned earlier. The antibodies used were Tumor Necrosis Factor alpha (TNF-α) (Anti-TNF alpha antibody Catalog #ab8097), Natural killer T (NKT) cells

(NK1.1 Monoclonal Antibody (PK136), eBioscience^{TM} Catalog # 14-5941-82), Proliferation marker Ki-67 (Anti-Ki67 antibody Catalog # ab16667), Interferon-gamma (IFN-γ) (Anti-Interferon gamma antibody Catalog # ab23637).

3.8 Analysis of miRNA Differential Expression in NGC and MGC Derived Exosomes (H1975 Cells)

The miRNAs previously studied in NSCLC were selected for their differential expression analysis in the isolated Exosomes (NGC and MGC). The major aim of this experiment was to observe any change in their expression in our MGC which mimic the cancer microenvironment e.g. hypoxia, acidic environment and starvation stress. Exosomes were isolated from the H1975 cells (NGC and MGC) and total RNA was isolated. The RNA samples were converted into cDNA and by using quantitative real time PCR (qRT-PCR), the expression was analyzed.

3.8.1 Total RNA Isolation from Exosomes

Total RNA was isolated by Trizol method [260]. Two separate vials containing 200ul Exosomes (NGC and MGC) were added with 200ul TRIZOL reagent. For the complete dissociation of complexes of proteins, the homogenized samples were incubated at room temperature for 5 minutes and centrifugation was done to remove debris. The supernatants were transferred into new labeled tubes and chloroform (50ul) was added. The samples were mixed through vertex machine for 15 seconds and incubated for 5 min at RT followed by centrifugation at 4°C at 12000xg for 15 min. Three layers were observed. The upper transparent aqueous phase contained RNAs, the lower phases were separated proteins, DNA and other contaminants. The aqueous phase solution was collected into the new labeled tubes and were added with 0.1ml isopropyl alcohol. The samples were incubated for 10 min at 4°C followed by centrifugation at 12000xg for 10 min at 4°C. The RNA pellets were re-suspended in 200ul 75% ethanol. The samples were mixed

and centrifuged at 7500xg at 4°C for 5 min. The RNA pellets were air dried after removing supernatant. A complete drying of the RNA pellet was avoided as it decreases its solubility. The RNA pellets were dissolved in grade RNAase free water and were mixed slowly through pipetting.

3.8.2 Quantification of Isolated RNA through Nano-Drop

The dissolved RNA samples and a vail of RNAase-free water were placed on ice. The Nano drop sample reading eye was cleaned and dried with molecular grade water through a Kim wipe. Then 1ul of RNAase-free water only was loaded into Nano drop to record a blank. The water was wiped out and reading eye was dried. 1ul of each RNA sample was added and analyzed their quantity (ng/ul) as well as the A260/A280 and A260/A230 ratios were also recorded. The sample reader was cleaned and dried with the use of a clean and dry Kim wipe and grade water [261].

3.8.3 RNA Reverse-transcription to cDNA (Complementary DNA)

Total RNA of each sample was converted to cDNA through the provided protocol of TaqMan MicroRNA Reverse Transcription Kit (applied Biosystems). All procedures were carried out in designated reverse transcription/PCR vented hoods. Prior to and after usage, UV light was turned on inside the hood for 15 minutes. The hood was also thoroughly cleaned before and after use. For cDNA synthesis 2μg RNA was calculated by using the Table 3.5. The data given below is from one of the repeated experiments. For RNA analysis, there were two groups of the reaction NGC and MGC Exosomes. Each reaction was run in triplicates along with a negative control (RNAase free water instead of RNA) was used. Partial protocol was adopted from Green et al [262].

Note: C1-Concentration obtained from the nanodrop, C2-Concentration required for PCR, V1-Volume needed to be calculated and taken from RNA isolated, V2-standard.

C1V1=C2V2

TABLE 3.5: Calculation for RNA to cDNA synthesis.

S#	Sample ID	Cl (ng/l)	C2	V2	C2V2	V1 OF RNA (ul)	H_2O(ul)	Total Volume (ul)
1	NGC Exosomes	347	2000	1	2000	5.76	7.14	12.9
2	MGC Exosomes	510	2000	1	2000	3.92	8.98	

V1=C2xV2/C1

NGC RNA to be taken=2000x1/347=5.76 μl + 7.14 μl H_2O

MGC RNA to be taken=2000x1/510=3.92 μl + 8.98 μl H_2O

Two tubes of RNA dilutions (12.9 μl having the same quanity of RNA 2000ng from both group) were prepared and were placed on ice until further use. The cDNA master mix was prepared as given in Table 3.6. Total 17.1μl master mix was added to 12.9μl RNA and the total volume of one RNA sample prepared was 30μl.

TABLE 3.6: Calculation for cDNA PCR reaction

	Reagents	Stock	Working	1 reaction (µl)	2 reaction (μl)
for 30 µl final volume	Water +RNA	-	-	12.9	-
	First strand buffer	5X	1X	6	12
	dNTPs	10mM	0.2mM	0.6	1.2
	Oligo dT	10UM	2.5UM	7.5	15
	DTT	0.1M	0.005M	1.5	3
	M-MLV RT	(200 units/ ul)	1U	1.5	3
	Total Volume			30	-

The tubes were placed in PCR machine and by using the following conditions (Table 3.7), the reaction was run. After the PCR run was completed, all the cDNA samples were preserved at -80°C.

TABLE 3.7: Thermo cycle conditions for cDNA synthesis.

Temperature °C	**Time**
25	10 min
42	90 min
94	5 min
4	∞

After the PCR run was completed, all the cDNA samples were kept at -80°C.

3.8.4 qRT-PCR Analysis

Samples of cDNA were taken out from -80°C and were allowed to thaw prior to use. A pre-mix product based reactions consisting calculated components are shown in Table 3.8. 18ul master mix was added in each PCR tubes and a 2ul of the cDNA was added [263]. Total volume of the reaction was 20ul. The tubes were placed in qRT-PCR and program was run with the temperature conditions given in Table 3.9. The miRNAs including miRNA-122-5p, miRNA- 20b-5p, miRNA- 132-3p, miRNA-451a, miRNA-200b, miRNA-665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p, miRNA-30d-5p, miRNA-338-3p and miRNA-139- 5p were analyzed in the both groups of exosomes. The forward and reverse primers for these miRNAs are shown in Table 3.10.

3.8.5 Relative Quantification and Data Analysis

Relative quantification was done to compare the expression of the target miRNAs with the endogenous control miRNA U6 (RNU6-1). For the miRNAs of interest and the endogenous control miRNA, reactions were run in triplicates. CFX Maestro Software was used for data analysis obtained from CFX Real-Time PCR Instruments.

Table 3.8: Required components for qRT-PCR volume.

Reagents	**Stock**	**Working**	**1 reaction(μl)**	**2 reaction(μl)**
Water	-	-	7.2	14.4
Master Mix	-	-	10	20
F. Primer	(10 μM)	0.2mM	0.4	0.8
R. Primer	(10 μM)	0.2mM	0.4	0.8
cDNA	-	-	2	-
Total Volume	-	-	20	-

Table 3.9: Conditions for qRT-PCR.

Temperature °C	**Time**
94	5 min
94	1 min
Annealing Temperature of primers	1 min
72	1 min
72	7 min
4	∞

Table 3.10: Forward and reverse primers sequences for the selected miRNAs.

miRNAa	**Forward and Reverse Primers**
miRNA-122-5p	F-5'-TGTGACAATGGTGTTTGGTCG-3' R-5'-TGTCGTGGAGTCGGCAATTG-3
miRNA-20b-5p	F-5'-CAAAGTGCTC ATAGTGCAG GTAG-3' R-5'-GCAAAGTGCT CATAGTGCAGG-3'
miRNA-132-3p	F-5'-GCGCGCGTAACAGTCTACAGG-3' R-5'-TCGTATCCAGTGCAGGGTCC-3'
miRNA-451a	F-5'-ACCGTTACCATTACT-3' R-5'-CTCACACGACTCACGA-3'
miRNA-200b	F-5'-GTTTATGGGAGTTTAGGGGAT-3' R-5'-AAACTCRCCTTACAAAAAACAATAC-3'
miRNA-665	F-5'-GCCGAGACCAGGAGGCTGA-3' R-5'-CTCAACTGGTGTCGTGGA-3'
hsa-miR-29a-5p	F-5'-CGCGGATCC TGGATTTAGTAAGATTTGGGC-3' R-5'-CCG GAATTCACATGCAATTCAGGTCAGTG-3'
miRNA-20a-5p	F-5'-GTAAAGTGCTTATAGTGCAG-3' R-5'-GTCGTATCCAGTGCGTGTCG-3'
miRNA-30b-5p	F-5'- ATCGCTGTAAACATCCTACAC-3' R-5-GTCGTATCCAGTGCAAGGGTCCGAGG
miRNA-30d-5p	F-5'- CCTGTTGGTGCACTTCCTAC-3' R-5'- TGCAGTAGTTCTCCAGCTGC-3'
miRNA-338a-3p	F-5'-TGCGGTCCAGCA TCAGTGAT-3' R-5'-CCAGTGCAGGGT CCGAGGT-3'
miRNA-139-5p	F-5'-UCUACAGUGCACGUGUCUCCAGU-3' R-5'-UGGAGACACGUGCACUGUAGAUU-3'
U6	F-5'-CTCGCT TCGGCAGCACA-3' R-5'-AACGCTTCAC GAATTTGCGT-3'

3.8.6 Prediction of miRNAs Target Genes and Pathway Analysis by using Bioinformatics Tools

From the miRNAs expression analysis, seven miRNAs with more or less up regulation in MGC samples were observed in comparison with NGC. Those miRNAs were further analyzed through bioinformatics tools to identify their targeted genes, functional enrichment analysis, analysis of protein-protein Interactions, identification of Hub genes in Networks, identification of Hub genes roles in cell proliferation negative regulation, expression analysis of involved genes in cell proliferation negative regulation and finally prognostic values for overlapping genes were also identified.

3.8.7 Prediction of Target Genes for the Identified miRNAs

MiRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/), a user-friendly database that includes predicted data obtained by a machine learning algorithm, focuses on consistency, accessibility, user-friendly architecture, and often up-to-date content [264], was used to identify the target genes of selected miRNAs. Furthermore, two other well-known and promising miRNA-target prediction methods miRDB: http://mirdb.org/, and Targetscan 7.2: http://www.targetscan.org/vert 72/), were also used to cross-checked the results obtained through MiRWalk database. For further investigation, the target genes predicted by all prediction programs were selected. TBtools and R package "UpSetR" were used to explore and simulate the interactions between these target sets [265, 266].

3.8.8 Functional Enrichment Analysis

The selected genes were then uploaded to Metascape (metascape.org/gp/index.html) for Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The Metascape database is an online tool for gene annotation and interpretation, that helps the users to decipher one or

more gene lists [267]. The Metascape database was used in this analysis to look at overlapping genes' GO annotations and KEGG pathways. A significance level of $p < 0.05$ was used. In addition, the KOBAS database was employed to conduct enrichment analyses. Results were further crosschecked using Reactome ($p < 0.01$), KEGG disease ($p < 0.05$), NGHRI GWAS Catalog ($p < 0.05$), and PANTHER pathway ($p < 0.01$). The findings of the GO annotation and KEGG pathway enrichment study were visualized using the R programming language. To prevent key details being missed by just considering the intersection of predicted targets, miRWalks 3.0 predicted targets (with score > 0.95 and binding sites 3'UTR) was subjected to their own GO annotation and KEGG pathway enrichment analysis.

3.8.9 Analysis of Protein-Protein Interactions

The Network analyst tool created a protein-protein interaction (PPI) network with interacting genes. The web interface for NetworkAnalyst was built using the PrimeFaces library and JavaServer Faces Technology (v10.0). About 300 R functions are used to do the rest of the back-end calculations (v4.02). JavaScript was used to create the interactive visualization. The PPI network was constructed using a medium trust parameter (minimum necessary interaction score > 0.400), and disconnected nodes were omitted from the network. Cytoscape software (version 3.7.1) was used to visualize the list of PPI pairs for further study. The Molecular Complex Detection (MCODE) plugin in Cytoscape was used to locate possible clusters in the PPI network based on topology, which may aid in the identification of most probable primary target genes. The MCODE process set the degree cut-off value to 2 and the node score cut-off value to 0.2.

3.8.10 Identification of Hub Genes in Networks

The integrity of fit for a power-law distribution was resolved using the coefficient of certainty (R2) after each network's degree distribution was determined. Few genes with a large R2 were considered to be the core hub genes. Modularity analysis, the Power-law degree distribution measure, and centrality analysis were used to

analyze the configuration of each network. Different metrics, such as betweenness centrality, were used in centrality analysis to identify the hub genes in each network.

3.8.11 Identification of Hub Genes Roles in Negative Regulation of Cell Proliferation

Enrichnet server was used to further evaluate the function of hub genes in negative regulation of cellular proliferation. The KEGG database was used as the search parameter, with FDR P-value change, 0.05 significance level, and Median community cut-off to measure the involvement of gene in negative regulation of cellular proliferation. The findings were shown in the form of a table that included the most important genes.

3.8.12 Expression Analysis of Genes Involved in Negative Regulation of Cell Proliferation

The Gene Expression Profiling Interactive Analysis (GEPIA) platform was used to compare the expression of genes (http://gepia.cancer- pku.cn/) in lung cancer which were playing role in Negative Regulation of Cell proliferation. GEPIA combined TCGA and the Genotype-Tissue Expression (GTEx) project's mRNA sequencing re- sults, allowing for personalized functionalities such as differential expression anal- ysis, profiling plotting, and patient survival analysis [268, 269]. Using the R kit pheatmap, the Lung cancer expression of hub genes was visualized as a heatmap.

3.8.13 Statistical Analysis

All data are given as means ± standard error. Statistical comparisons were made by using the t-test (unpaired, one-tailed). All analyses were performed with Graphpad Prism 8.0. A p value of <0.05 (CI: 95%) was considered to indicate statistical significance. A p-value less than 0.05, it was flagged with one star (*).

If a p-value was less than 0.01, it was flagged with 2 stars (**). If a p-value is less than 0.001, it was flagged with three stars (***).

Chapter 4

Results

The quantified exosomes from the NGC and MGC were observed to determine the level of change in production due to differences in growth conditions. Two models of T cells, Jurkat cells and human blood isolated T cells, were treated with these exosomes to identify the effects on proliferation and their markers expression. Flow cytometry results revealed a decrease in the number of Jurkat cells treated with MGC-derived exosomes compared to NGC-derived exosomes. Further analysis showed a reduction in the expression of IFN-γ in human blood isolated T cells when treated with MGC-derived exosomes, as opposed to NGC-treated cells.. In animal model experiments, tumor mice isolated exosomes were treated with normal mice T cells, resulting in a decrease in the expression of IFN-γ, Ki-67, and TNF-α. Similarly, the presence of MGC-isolated exosomes led to a decrease in the number of NKT cells among the T cell sub-population. A comparison of selected miRNAs in NGC and MGC-derived exosomes revealed differential expression. Bioinformatics analysis suggested that these miRNAs may target important genes involved in cellular proliferation and survival. In conclusion, exosomes play a crucial role in cell-to-cell communication and the maintenance of cellular environment. However, in the context of cancer, the cargo carried by abnormal exosomes secreted by cancer cells not only facilitates metastasis but also manipulates immune cells to create a favorable environment for cancer progression and development.

4.1 Exosomes Isolation and Quantification

The Nanosight results confirmed the presence and the size range (100-150 nm) of NGC and MGC derived Exosomes as shown in Figures 4.1 & 4.2. These exper-

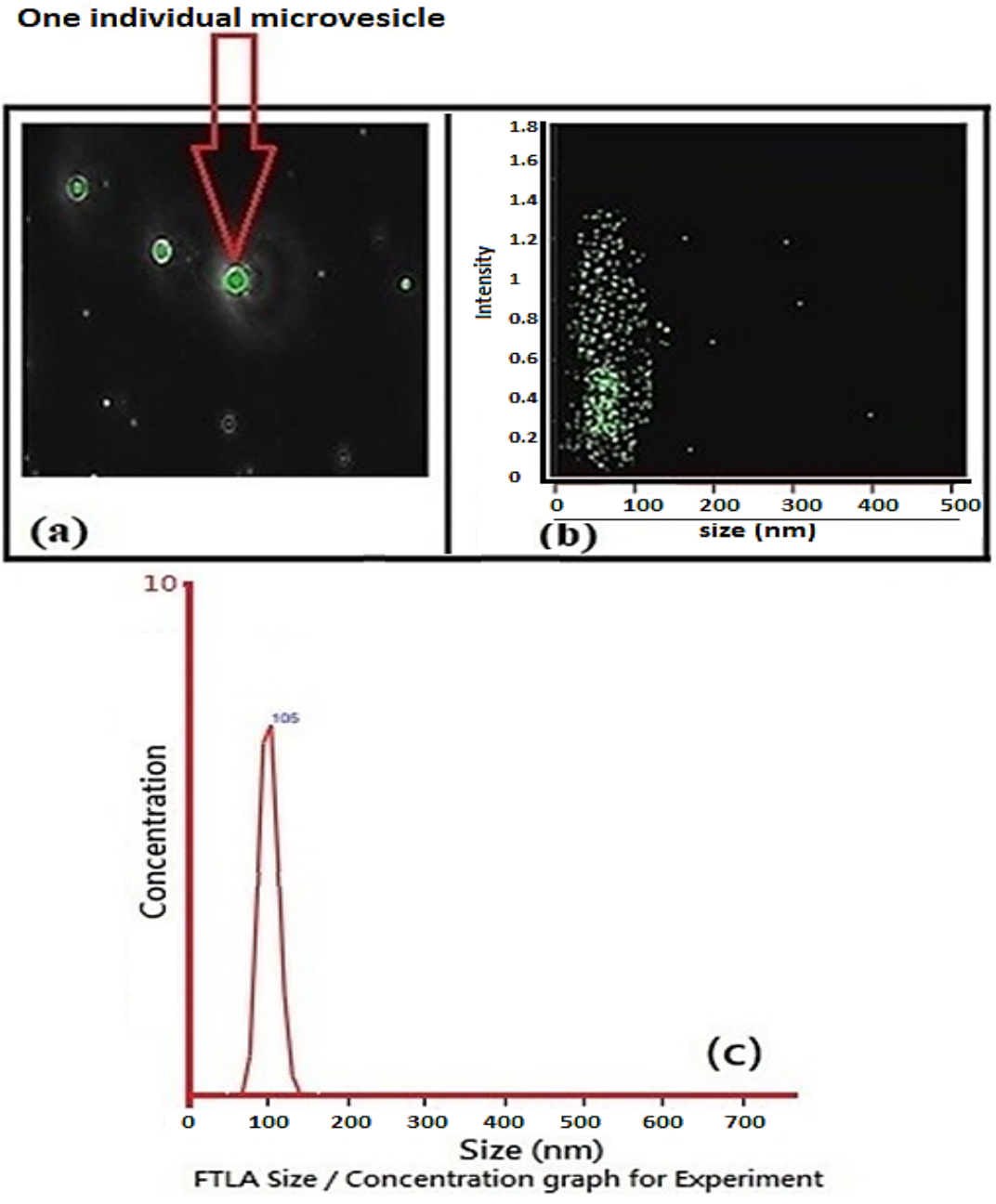

FIGURE 4.1: Confirmation of NGC Exosomes through Nanosight Tracking analysis (NTA). (a) Real-time detection of Exosomes (b) Exosomes particles detection by Nano Sight machine, each dot rep- resents each particle. (c) Graphical representation of Exosomes based on their size range.

iments were optimized with several attempts and the conclusive confirmed data is shown in this chapter. The statistical analysis revealed significant disparity in

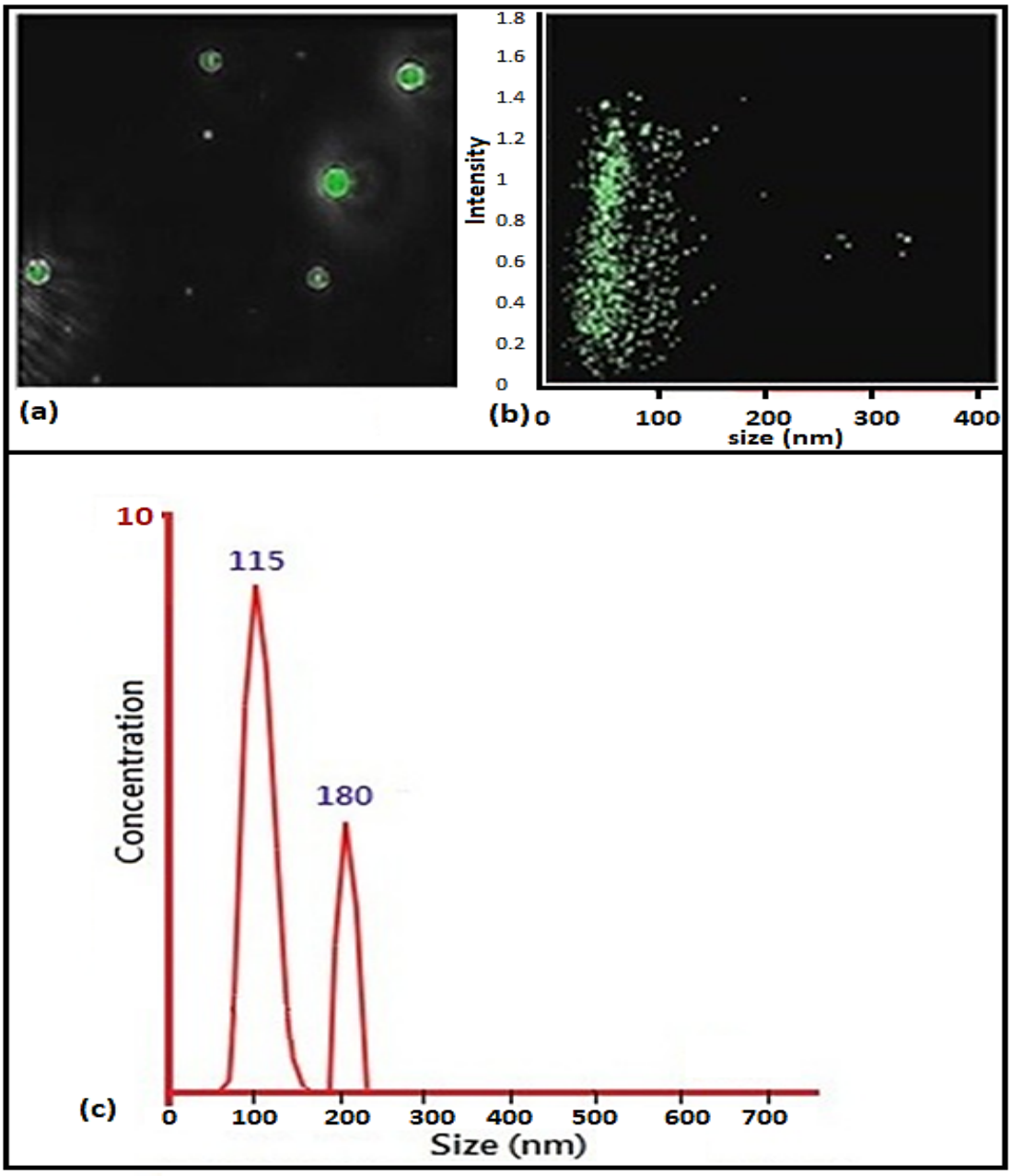

FIGURE 4.2: Confirmation of MGC Exosomes through Nanosight analysis (NTA). (a) Real-time detection of Exosomes particles (b) Exosomes particles detection by Nano Sight machine, each dot represents each particle. (c) Graphical representation of Exosomes based on their size range.

the p value (0.0167) between the mean quantities of NGC (6.7 x10^8) and MGC (8.8 x 10^8) Exosomes. This difference in means ± SEM was calculated as 2.100 ± 0.6600, with a confidence interval spanning from 0.2676 to 3.932 as shown in figure 4.3. The hypothesis of changes in growth conditions may increase the Exosomes production was achieved. The Nano sight technique can detect Exosomes particles on the basis of their size, but each particle in the size ranges from 30-150 nm

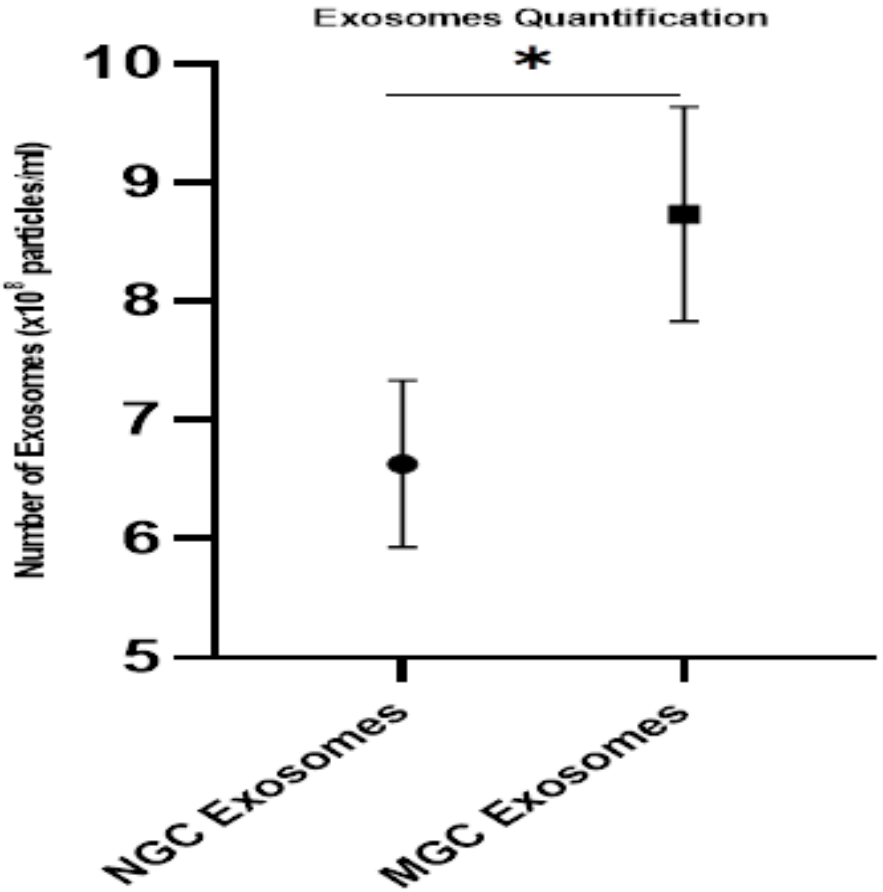

FIGURE 4.3: Graphical representation of the analyzed data obtained from the Nanosight for the quantification of Exosomes production in NGC and MGC

may not be Exosomes. Therefore, validation of Exosomes through surface markers (CD9 and CD63) was performed by using SDS PAGE and western blot technique with three repeats. The SDS PAGE shown in Figure 4.5 (a) where a protein precision marker was loaded to mark the possible location of all target protein and also the banding pattern of NGC and MGC Exosomes carrying protein were loaded. For the confirmation of Exosomes, CD63 and CD9 markers antibodies were used as the bands confirmed the presence and quantity of Exosomes among the two samples through Western blot analysis. These tetraspanins, CD63 [270], and CD9 [271] have been used as markers of Exosomes for the last two decades. The isolated Exosomes were confirmed, and the variation in bands size indicated a difference in Exosomes production in NGC and MGC as shown in figure 4.5 (b).

For the statistical analysis, the MGC Exosomes protein expressions were compared with NGC Exosomes. The data of each protein shown here is normalized with Tubulin protein (loading control). The CD9 markers expression was highly significant with a p value <0.0001, with a difference between means (MGC-NGC Exosomes) ± SEM of 0.3443 ± 0.001 and 95% CI was 0.3415 to 0.3471. Similarly, the CD63 marker expression was also highly significant in MGC Exosomes in comparison with NGC Exosomes with a p value <0.0001 with a difference between means (MGC-NGC Exosomes) ± SEM of 0.08285 ± 0.0003 and 95% CI was 0.08188 to 0.08382. The other cancer protein analyzed from these two groups were also significantly overexpressed in MGC Exosomes in comparison with NGC Exosomes. HVEM results demonstrated a p value <0.0001 with a difference between means (MGC-NGC Exosomes) ± SEM of 0.1128 ± 0.006638 and 95% CI was 0.09441 to 0.1313. CEACAM1 comparison between the two group had also a p value <0.0001that shows a high difference of its expression in NGC Exosomes in comparison with MGC Exosomes. A difference between means (MGC-NGC Exosomes) ± SEM was 0.1027 ± 0.0006459 and 95% CI was found to be from 0.1009 to 0.1044. The p value of PDL-1 was also <0.0001 with a difference between means (MGC-NGC Exosomes) ± SEM of 0.2102 ± 0.0007509 and 95% CI was found to be from 0.2081 to 0.2123. Calnexin (endoplasmic reticulum marker) was used as negative control, which was found absent in both NGC and MGC Exosomes. Overall these results shows a increased expression of our selected protein. From the previouse studies it was found that tumor cells up-regulate the expression of the PD1 ligands (PDL-1), which induce T cells exhaustion [272]. Similarly, Sienel et al. confirmed the expression of CEACAM1 (Carcino-embryonic antigen-related cell adhesion molecule 1) in NSCLC [273] and CEACAM1 ligation on T cells triggers a signal transduction pathways that inhibits T cells cytokine secretion [274]. The H1975 cells-derived Exosomes were analyzed with these selected proteins, which are actively involved in T cells dysfunction. It was found that the PDL-1, CEACAM1, HVEM were present with a bigl difference in expression between the two groups derived Exosomes as shown in figure 4.5. TCR is phosphorylated by oligomerization of TCR/CD3 chains in response to foreign antigens presented by MHC on the surface of APC or tumor cells. This is followed by the recruitment

of activated Lck and Zap-70 to the phosphorylated ITAM (tyrosine motifs) of the TCR tail, which starts the downstream cascade of TCR-signaling. When PD-1 binds with its ligands during TCR cross-linking, SHP-2 (and potentially SHP-1) is recruited to ITSM when two tyrosine residues on PD-1s cytoplasmic tail become phosphorylated.). As a result, Lck and Zap- 70 are dephosphorylated. The PI3K/Akt/mTOR and Ras/MAPK/Erk pathways are inhibited by PD-1 ligation in T cells, which causes a decrease in glycolysis and amino acid metabolism and an increase in fatty acid oxidation. This al- teration in T cells metabolic reprograming may have an impact on how T cells development proceeds, decreasing the differentiation of effector and memory T cells while boosting the differentiation of T regulatory cells and exhaustion of T cells. The presence of the HVEM protein in Exosomes derived from H1975 cells was also found. This might be an agent to play a role in T cells dysfunction and proliferation. HVEM is an important immune check- point in cancer detection [275] which is expressed on cancer cells membranes, interferes with immune cell signaling pathways by binding to B and T lymphocyte attenuator (BTLA) and suppressing T cells as shown in figure 4.4 [276, 277].

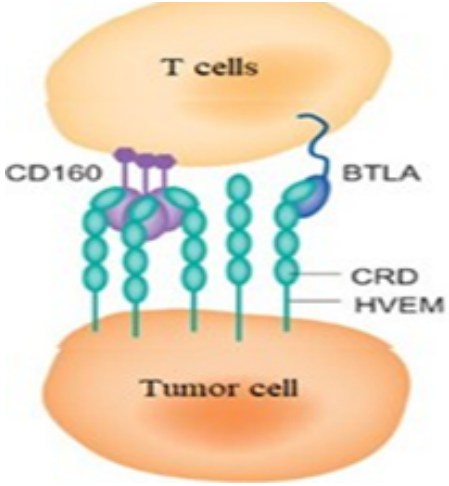

FIGURE 4.4: Binding mechanism of HVEM expressed on tumor cell to BTLA expressed on T cells, thus leading to T cells inhibitory effects.

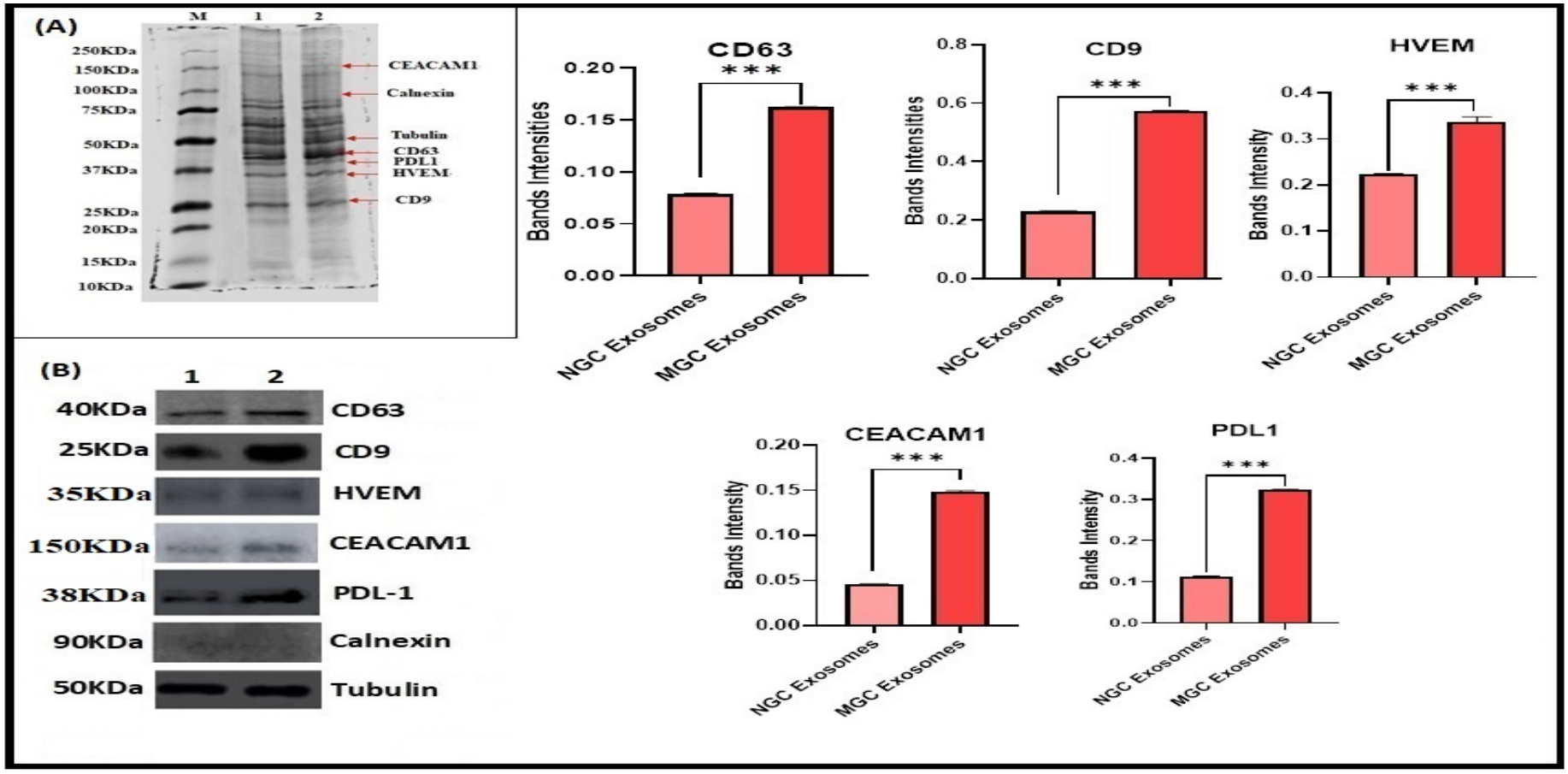

FIGURE 4.5: (a) SDS PAGE of Exosomes, M=Marker of 250KDa; Lane 1=NGC Exosomes; Lane 2=MGC Exosomes. The red arrows represent the possible positions of the target protein. (b) quantification and confirmation of NGC and MGC Exosomes through western blot analysis with CD9 and CD63 markers (Exosomes surface protein). HVEM, CEACAM1, PDL-1 were selected cancer-associated protein involved in T cells dysfunction/exhaustion. Graphical analysis is a mean of three replicated experiments. Lane 1=NGC Exosomes; Lane 2=MGC Exosomes. Calnexin was used as negative control for Exosomes, Tubulin was used as loading control and all protein were normalized with Tubulin. The graphs represent the protein bands intensities marked with SEM.

Another protein detected in H1975 Exosomes was CEACAM1, this protein was also found with a higher expression in MGC as compared with the NGC derived Exosomes. CEACAM1 is expressed in the cancer and its binding site on T cells is the inhibitory molecule TIM-3, also known as HAVCR2 (T cell immune-globulin domain and mucin domain-3) that has been demonstrated to trigger T cell depletion in malignancies [278].

4.2 Treatment of Jurkat Cells with H1975 Cells Derived Exosomes

Jurkat cells were treated with the H1975 cells derived Exosomes from NGC and MGC. The Jurkat cells were collected from the media after 24 hours and were stained with CD69 and CD25 markers, the Flow cytometer analysis revealed that there was an altered expression of both markers, indicating the suppressive role of cancer-derived Exosomes on Jurkat cells. Jurkat cells treated with MGC Exosomes were found decreased as compared to NGC Exosomes treatments. Following statistical analysis, utilizing Jurkat cells alone as the control group (mean=21.85%), the investigation observed alterations in CD69 expression across three additional treatment cohorts. The cohort comprising Jurkat cells treated with PHA/PMA (mean=77.95%) exhibited a notably significant divergence from the control group, with a p value of 0.0001. The disparity between means (Jurkat cells + PHA/PMA-control) ± SEM was 56.10 ± 0.9192. Moreover, the group treated with Jurkat cells + PHA/PMA + NGC Exosomes (mean= 59.15%) also displayed a substantial dissimilarity relative to the control group, showing a p value of 0.0006. The difference between means (Jurkat cells +PHA/PMA+NGC Exosomes-control) ± SEM was 37.30 ± 1.275. Likewise, the cohort treated with Jurkat cells + PHA/PMA + MGC Exosomes (mean= 39.90%) evidenced a significant distinction from the control group, yielding a p value of 0.0013. The difference between means (Jurkat cells + PHA/PMA+MGC Exosomes-control) ± SEM was 18.05 ± 0.9394. as shown in the Flow cytometer results given in Figure 4.6. The flow cytometer graphs shown

here for both markers are the results of one experiment while the graphs are the mean of two replicates of the experiments.

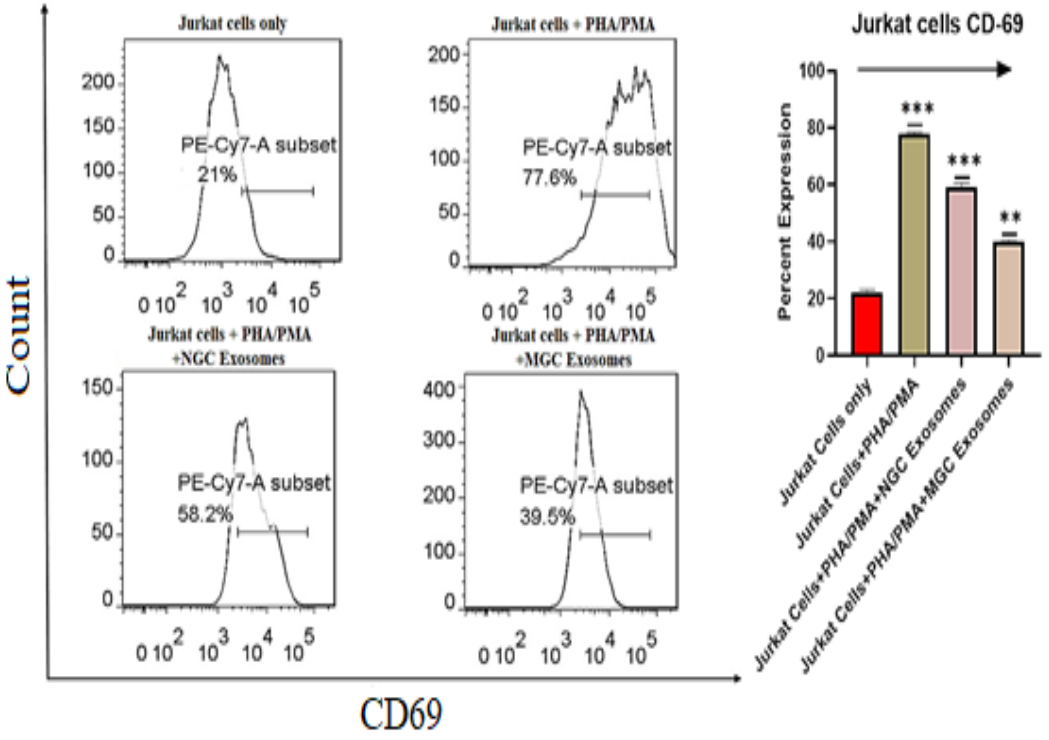

FIGURE 4.6: Flow cytometer analysis of CD69 labeled Jurkat cells after treatment with Exosomes

Similarly, The Jurkat cells were assessed by quantifying CD25 expression, after treatment with both groups of Exosomes. To identify the effects on Jurkat cells, another marker (CD25) expression was determined and analyzed statistically. In this experiment, Jurkat cells only (mean= 37.50%) was also used as control group and the changes in expression of CD25 on Jurkat cells was observed in the rest of the three treatment groups. The Jurkat cells + PHA/PMA (mean= 92.35%) was found with a significant difference in comparison with the control group with a p value = 0.0011. Difference between means (Jurkat cells + PHA/PMA-control) ± SEM was 54.85 ± 2.599. The second treatment group, Jurkat cells + PHA/PMA + NGC Exosomes (mean= 79.15%) was also having a high significant difference in comparison with the control group with a p value= 0.0007. Difference between means (Jurkat cells + PHA/PMA + NGC Exosomes-control) ± SEM was 41.65 ± 1.553 with a 95% CI of 34.97 to 48.33.Similarly, the third group, Jurkat cells + PHA/PMA + MGC Exosomes (mean= 47.75%) group had also a significant

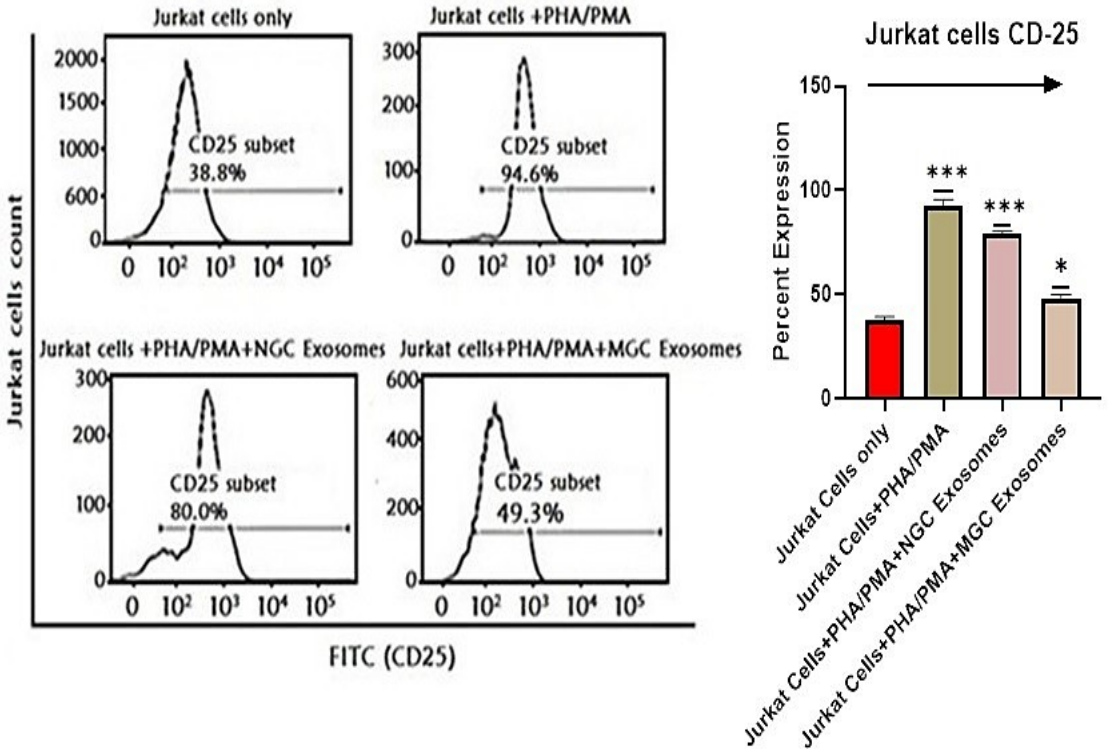

FIGURE 4.7: Flow cytometer analysis of CD25 labeled Jurkat cells after treatment with Exosomes

difference in comparison with the control group with a p value= 0.0184. Difference between means (Jurkat cells + PHA/PMA + MGC Exosomes-control) ± SEM was 10.25 ± 2.023 as shown in figure 4.7. Both the markers in stimulated (with PHA/PMA) Jurkat cells were found reduced when treated with cancer-derived Exosomes. The effects were seen more in MGC Exosomes in comparison with NGC-derived Exosomes treatments. The hypothesis that the cancer Exosomes may affect the T cells and their markers expression was validated.

4.3 Effects of H1975 Cells (NGC & MGC Grown) Derived Exosomes on IFN-γ Expression in Healthy Human T cells

The effects of cancer-exosomes on the IFN-γ expression in healthy human fresh blood isolated T cells (CD8 and CD4 T cells) was also analyzed. The Interferon gamma expression was assessed in both CD4 and CD8 T cells, after treatment

with both groups of Exosomes. Here PHA/PMA was also used to stimulate the T cells. To identify the effects on the expression of IFN-γ in both types of T cells,

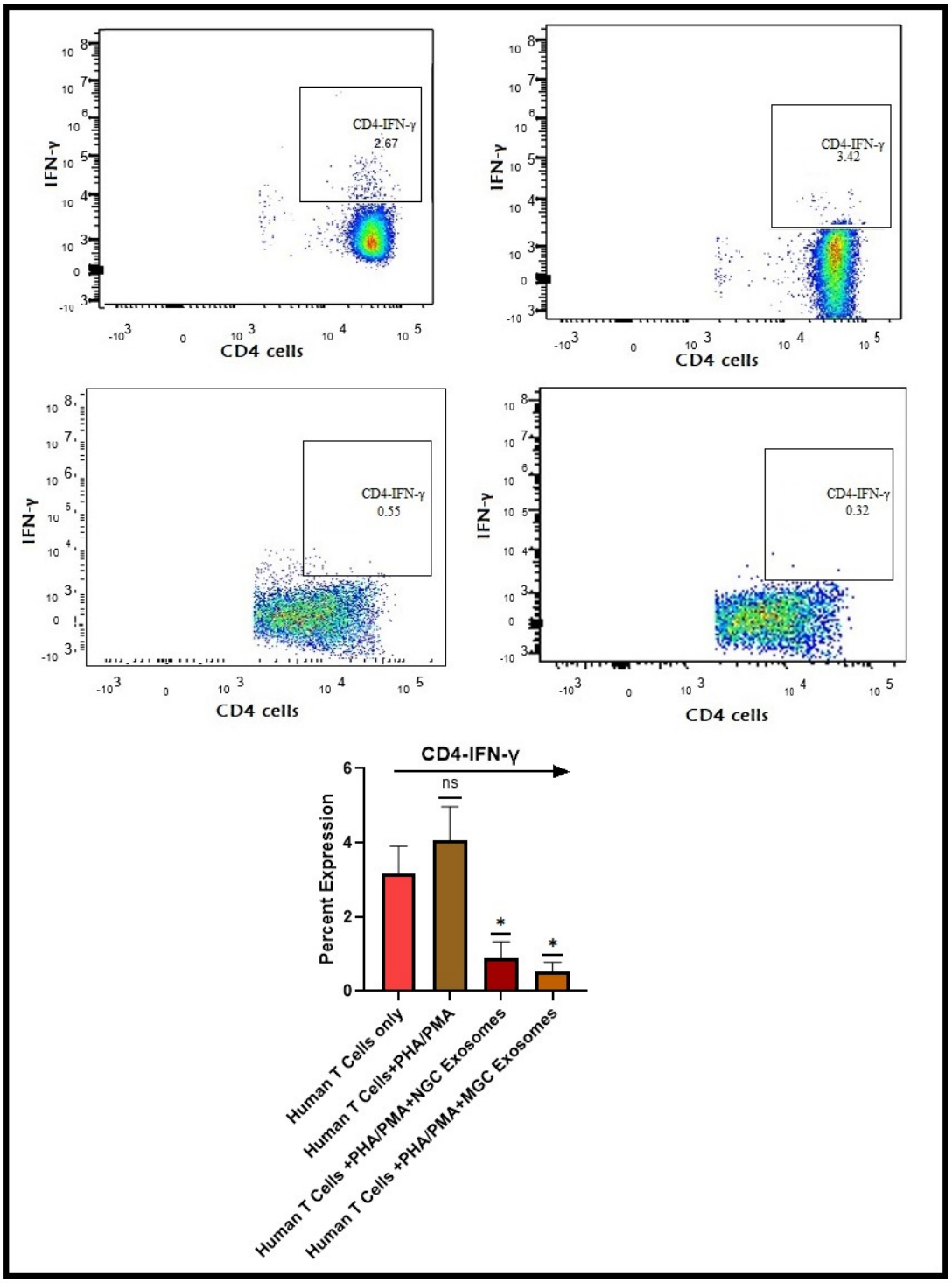

FIGURE 4.8: Flow cytometer analysis of CD4-IFN-γ labeled Human T cells grown with Exosomes.

statistically analysis of the data revealed that among the four groups, where Human T cells only (mean=3.180%) were used as a control for IFN-γ expression in

CD4 T cells. With this control group the other three groups were compared. The first treatment group was Human T cells+PHA/PMA (mean= 4.060%) was found none significant in comparison with the control group with a p value of 0.1974 and difference between means (Human T cells + PHA/PMA-Control) ± SEM was 0.8800 ± 0.8184 with a 95% CI of -2.641 to 4.401. The second treatment group, Human T cells+PHA/PMA+NGC Exosomes (mean= 0.87%) was also having a significant difference in comparison with the control group with a p value= 0.0312. Difference between means (Human T cells+PHA/PMA+NGC Exosomes - Control) ± SEM was -2.305 ± 0.6048 with a 95% CI of -4.907 to 0.2970. Similarly, the third group, Human T cells+PHA/PMA +MGC Exosomes (mean= 0.51%) group had also a significant difference in comparison with the control group with a p value= 0.0196.

Difference between means (Human T cells +PHA/PMA+MGC Exosomes - Control) ± SEM was -2.670 ± 0.5442 with a 95% CI of -5.012 to -0.3283 as shown in figure 4.8. While the expression analysis of IFN-γ in CD8 T cells among the four groups, where Human T cells only (mean= 2.44%) were also used here as a control for IFN-γ expression in CD8 T cells. With this control group the other three groups were compared. The first treatment group was Human T cells+PHA/PMA (mean= 8.82%) was found highly significant in comparison with the control group with a p value of 0.0022 and difference between means (Human T cells +PHA/PMA - Control) ± SEM was 6.385 ± 0.4245 with a 95% CI of 4.558 to 8.212. The second treatment group, Human T cells + PHA/PMA + NGC Exosomes (mean= 6.95%) was also having a high significant difference in comparison with the control group with a p value= 0.0080. Difference between means (Human T cells+PHA/PMA+NGC Exosomes - Control) ± SEM was 4.510 ± 0.5763 with a 95% CI of 2.030 to 6.990. Similarly, the third group, Human T cells + PHA/PMA + MGC Exosomes (mean= 3.07%) group was found with a none significant difference in comparison with the control group with a p value= 0.1305. Difference between means (Human T cells +PHA/PMA+MGC Exosomes - Control) ± SEM was 0.6350 ± 0.4094 with a 95% CI of -1.127 to 2.397 as shown in Figure 4.9.

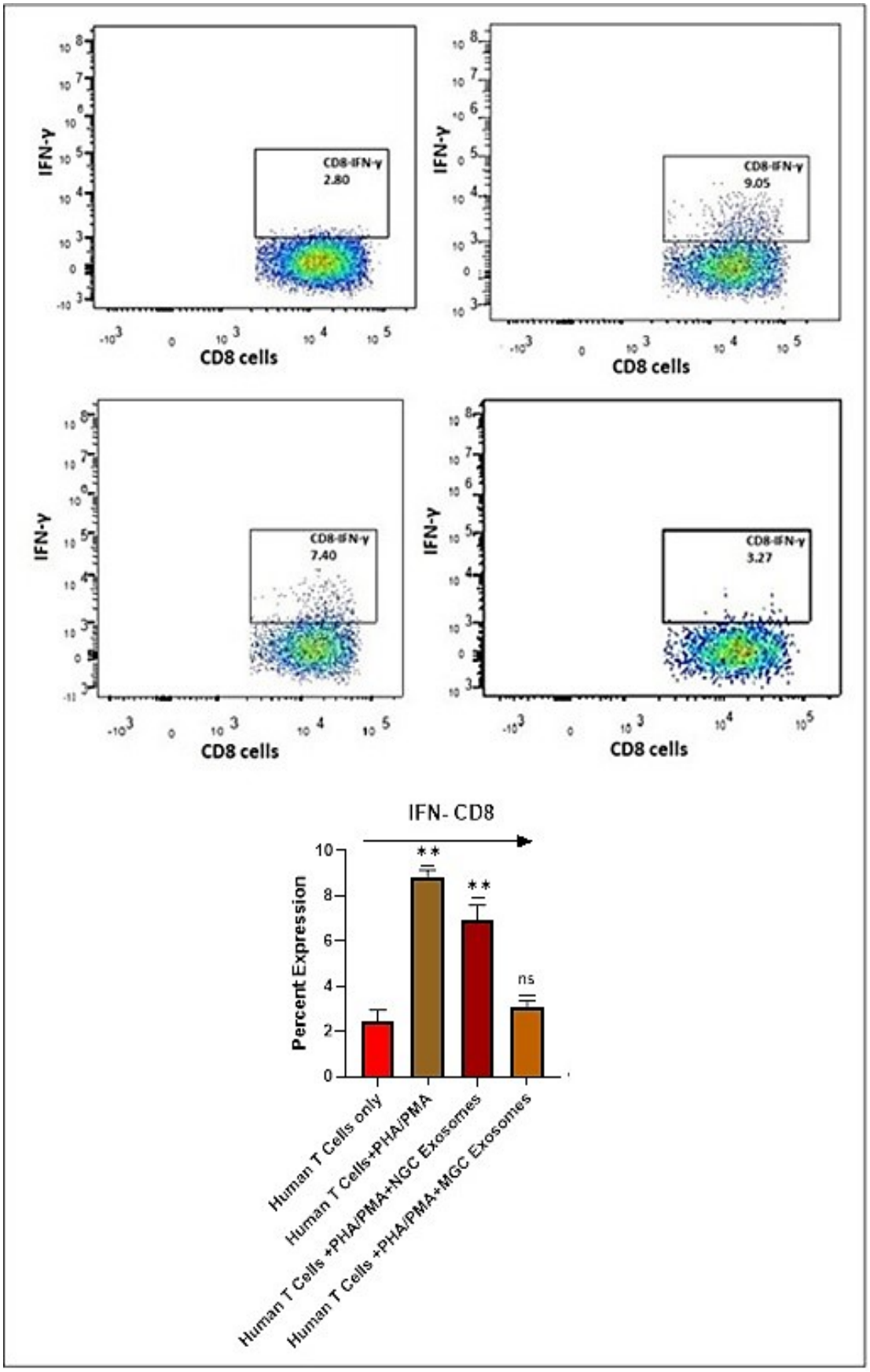

FIGURE 4.9: Flow cytometer analysis of CD8-IFN-γ.

These results confirmed that H1975 derived Exosomes alters the expression of IFN-γ in both types of T cells.

IFN-γ and cancer have a complex interaction that varies based on the type of cancer, its stage, and the microenvi- ronment within the tumor. IFN- levels can sometimes fall, while rarely they may rise or remain the same. The ability of the tumor to escape the immune system, the presence of immune-suppressive cells, and the general inflammatory state of the tumor microenvironment all have an impact on the response of IFN in cancer [279]. This result supports a previous study that cancer Exosomes can contain molecules that inhibit the signaling pathways of IFN-γ. This can hinder the immune response, as interferon gamma is a key player in activating immune cells like T cells and natural killer cells to target cancer cells [280].

4.4 Effects of Tumor Induced Mice Derived Exosomes on Normal Mice T Cells Markers Expression

Exosomes were isolated from both tumors induced mice and healthy mice groups and were analyzed through Nanosight and western bloting for quantification. All the particles in both groups were in the range (20-150 nm), but some larger particles with a small number were also observed as shown in figure 4.10 (a-I and a-II). The SDS PAGE shown in Figure 4.10 (b-I) where a protein precision marker was used to mark the possible location of all target protein and also the banding pattern of healthy and tumor isolated Exosomes carrying protein. For the confirmation of Exosomes from both groups, CD63 and CD9 markers antibodies were used and the bands confirmed the presence and quantity of Exosomes among the two samples through Western blot analysis as shown in 4.10 (b-II and b-III). For the statistical analysis, the tumor mice Exosomes protein expressions were compared with healthy mice Exosomes. The data for each protein shown here has been normalized using Tubulin protein as a loading control. The CD9 markers

expression was highly significant with a p value <0.0001, with a difference between means (tumor mice Exosomes- healthy mice Exosomes) ± SEM of 2.067 ± 0.01783 and 95% CI was 2.017 to 2.116. Similarly, the CD63 marker expression was also highly significant in tumor mice Exosomes in comparison with healthy mice Exosomes with a p value <0.0001 with a difference between means (tumor mice Exosomes- healthy mice Exosomes) ± SEM of 0.7871 ± 0.01762 and 95% CI was 0.7382 to 0.8360. Calnexin was used as negative control, which was found absent in both groups. The tumor induced mice Exosomes were treated with normal mice spleen isolated T cells. The rationale for incorporating the Lewis lung carcinoma cell line (LLC) in this phase of the experiments was to examine the expression of the chosen markers in the context of tumor cells.

After 24 hours of treatment incubation, the T cells collected from the wells and staining was done through FACS antibodies, and was analyzed through a flow cytometer. NKT-Cells and also the expression of IFN-γ, Ki-67, and TNF-α was analyzed. The expression of IFN-γ was analyzed in CD4 T cells in the first set of experiments. Activated lymphocytes (CD4 T cells and CD8 T cells, are the principal producers of IFN-γ. When treated with tumor mouse-derived Exosomes, a decrease in CD4-IFN-γ expression was observed. In contrast, when T cells were treated with normal mouse Exosomes, IFN-γ expression was somehow increased, as seen in figure 4.11. The flow cytometry results of IFN-γ expression in CD4 T cells were statistically analyzed, The Mice T cells (mean= 11.75%) was used as control and the changes in expression of CD4-IFN-γ was observed in the rest of the five treatment groups. The Mice T cells+LLC (mean= 10.20%) had a none significant difference in comparison with the control group with a p value= 0.0777. Difference between means (Mice T cells+LLC – Control) ± SEM was -1.550 ± 0.6946 with a 95% CI of -4.539 to 1.439. The Mice T cells+Normal Exosomes (mean= 9.7%) was had a significant difference in comparison with the control group with a p value=0.0392. Difference between means (Mice T cells+Normal Exosomes – Control) ± SEM was -2.050 ± 0.6103 with a 95% CI of -4.676 to 0.5760. Similarly, the Mice T cells+LLC+Normal Ex- osomes (mean= 8.35%) had a highly significant difference in comparison with the control group with a p value= 0.0053. Difference between means (Mice T cells+LLC+Normal Exosomes

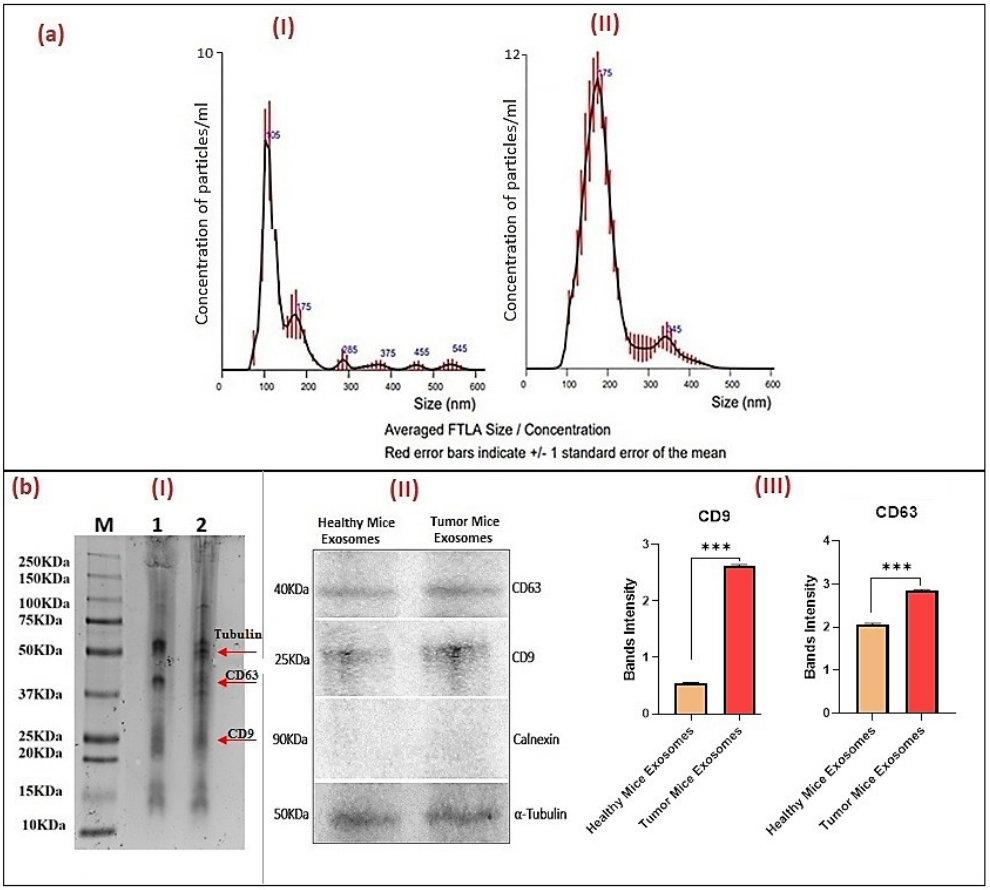

FIGURE 4.10: (a) Quantification of Exosomes derived from healthy (a-I) and tumor mice (a=II) blood through NTA. (b-I) SDS PAGE of healthy and tumor mice blood isolated Exosomes, M=Marker of 250KDa; Lane 1= healthy mice Exosomes; Lane 2= tumor mice Exosomes. The red arrows represent the possible positions of the CD63, CD9 and Tubulin protein. (b-II) quantification and confirmation of healthy and tumor mice blood isolated Exosomes through western blot analysis with CD9 and CD63 markers (Exosomes surface protein). Calnexin was used as negative control for Exosomes, Tubulin was used as loading control. The graphs represent the protein bands intensities analyzed with p values for significance.

– Control) ± SEM was -3.400 ± 0.3536 with a 95% CI of -4.921 to -1.879. Then the Mice T cells+Tumor Exosomes (mean= 3.95%) had a high significant difference in comparison with the control group with a p value= 0.0012. Difference between means (Mice T cells+Tumor Exosomes – Control) ± SEM was -7.800 ± 0.3808 with a 95% CI of -9.438 to -6.162. The last group Mice T cells+LLC+Tumor Exosomes (mean= 2.20%) had a significant difference in comparison with the control group with a p value= 0.0009. Difference between means (Mice T cells+LLC+Tumor Exosomes – Control) ± SEM was -9.550 ± 0.4031 with a 95% CI of -11.28 to -7.816. IFN-γ expression was also examined in CD8 T cells, and both healthy

and tumor mice Exosomes as shown in figure 4.12. The flow cytometry results of IFN-γ expression in CD8 T cells were also statistically analyzed, The Mice T cells (mean= 10.05%) was used as control and the

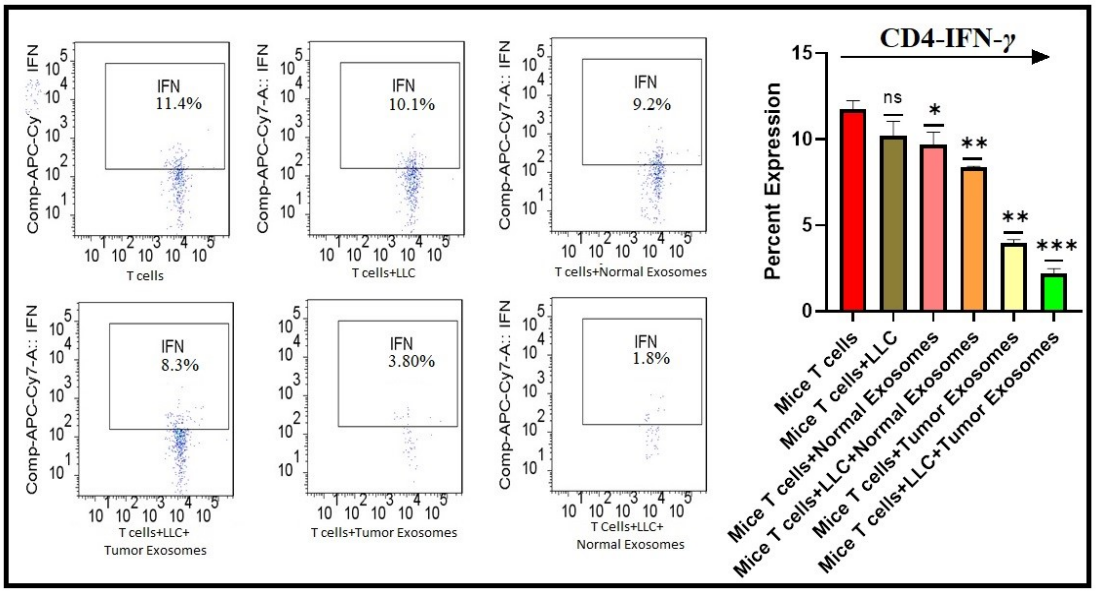

FIGURE 4.11: Flow cytometer analysis of CD4-IFN-γ labeled in normal mice T cells treated with Exosomes.

changes in expression of CD8-IFN-γ was observed in the rest of the five treatment groups. The Mice T cells+LLC (mean= 11.65%) had a none significant difference in comparison with the control group with a p value= 0.1274. Difference between

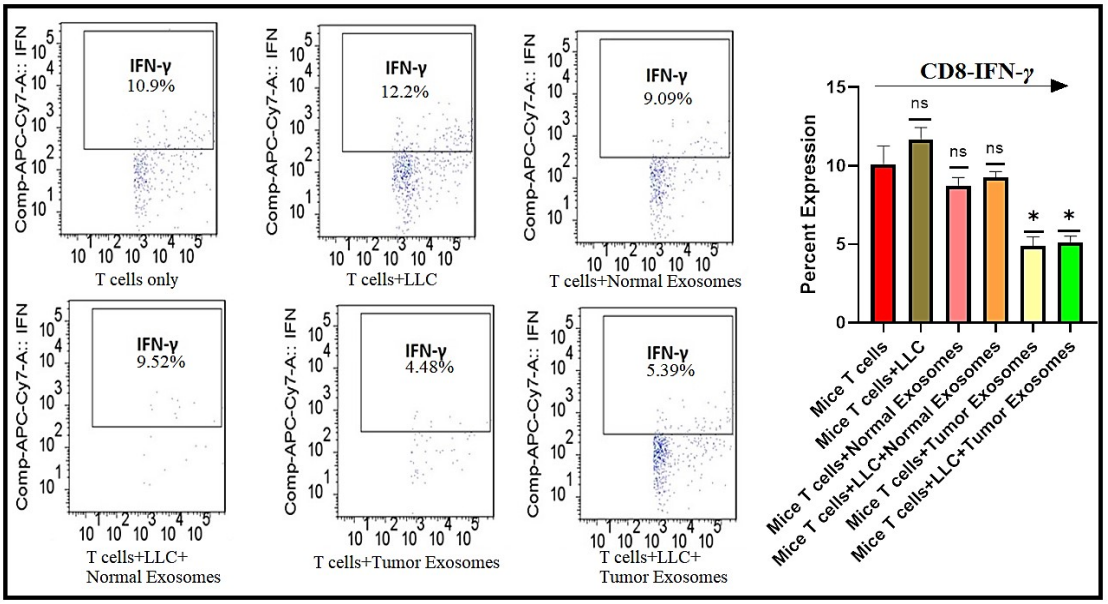

FIGURE 4.12: Flow cytometer analysis of CD8-IFN-γ labeled in normal mice T cells

means (Mice T cells+LLC - Control) ± SEM was 1.600 ± 1.012 with a 95% CI of -2.756 to 5.956. The Mice T cells+Normal Exosomes (mean= 8.69%) had a none significant difference in comparison with the control group with a p value= 0.1426. Difference between means (Mice T cells+Normal Exosomes – Control) ± SEM was -1.355 ± 0.9373 with a 95% CI of -5.388 to 2.678. Similarly, the Mice T cells+LLC+Normal Exosomes (mean= 9.26%) had a none significant difference in comparison with the control group with a p value= 0.2340. Difference between means (Mice T cells+LLC+Normal Exosomes – Control) ± SEM was -0.7900 ± 0.8889 with a 95% CI of -4.615 to 3.035. Then the Mice T cells+Tumor Exosomes (mean= 4.89%) had a significant difference in comparison with the control group with a p value= 0.0159. Difference between means (Mice T cells+Tumor Exosomes – Control) ± SEM was -5.160 ± 0.9437 with a 95% CI of -9.220 to -1.100. The last group Mice T cells+LLC+Tumor Exosomes (mean= 5.09%) had a significant difference in comparison with the control group with a p value= 0.0157. Difference between means (Mice T cells+LLC+Tumor Exosomes – Control) ± SEM was -4.955 ± 0.8997with a 95% CI of -8.826 to -1.084. As a result, tumor Exosomes may influence the anti-proliferative, pro-apoptotic, and anticancer pathways associated with IFN-γ in the tumor microenvironment.

4.5 Effects of Cancer Exosomes on Tumor Necrosis Factor Alpha (TNF-α) in CD8 T cells

The flow cytometry results of TNF-α expression in CD8 T cells were statistically analyzed as shown in figure 4.13, the Mice T cells (mean= 51.25%) was used as control and the changes in expression of CD8-TNF-α was observed in the rest of the five treatment groups. The Mice T cells+LLC (mean= 65.65%) had a high significant difference in com- parison with the control group with a p value= 0.0015. Difference between means (Mice T cells+LLC - Control) ± SEM was 14.40 ± 0.7906 with a 95% CI of 11.00 to 17.80. The Mice T cells+Normal Exosomes (mean= 87.8%) had a higher significant difference in comparison with the control group with a p value= 0.0002. Difference between means (Mice T cells+Normal

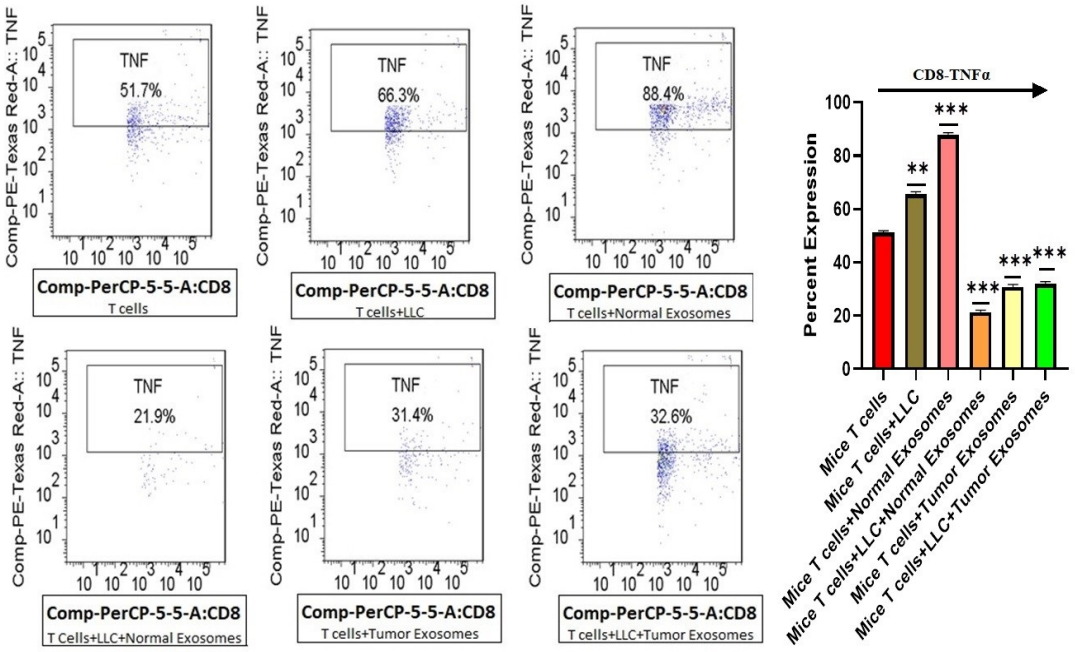

FIGURE 4.13: Flow cytometer analysis of CD8-TNF labeled in normal mice T cells

Exosomes - Control) ± SEM was 36.55 ± 0.7500 with a 95% CI of 33.32 to 39.78. Similarly, the Mice T cells+LLC+Normal Exosomes (mean= 21.30%) had a higher significant difference in comparison with the control group with a p value= 0.0003. Difference between means (Mice T cells+LLC+Normal Exosomes - Control) ± SEM was -29.95 ± 0.7500 with a 95% CI of -33.18 to -26.72. Then the Mice T cells+Tumor Exo- somes (mean= 30.75%) had a higher significant difference in comparison with the control group with a p value= 0.0007. Difference between means (Mice T cells+Tumor Exosomes - Control) ± SEM was -20.50 ± 0.7906 with a 95% CI of -23.90 to -17.10. The last group Mice T cells+LLC+Tumor Exosomes (mean= 32.05%) had a higher significant difference in comparison with the control group with a p value= 0.0007. Difference between means (Mice T cells+LLC+Tumor Exosomes - Control) ± SEM was -19.20 ± 0.7106 with a 95% CI of -22.26 to -16.14.

4.6 Effects of Cancer Exosomes on Ki-67 Expression in T Cells

The impact of cancer derived Exosomes on expression of the Ki67 marker in both CD4 and CD8 T cells was also investigated as shown in figure 4.14. The flow cytometry results of Ki67 expression in CD4 T cells were statistically analyzed, The Mice T cells (mean= 27.90%) was used as control and the changes in expression of CD4-Ki67 was observed in the rest of the five treatment groups. The Mice T cells+LLC (mean= 41.45%) had a high significant difference in comparison with the control group with a p value= 0.0025. Difference between means (Mice T cells+LLC - Control) ± SEM was 13.55 ± 0.9552 with a 95% CI of 9.440 to 17.66. The Mice T cells+Normal Exosomes (mean= 42.40%) had a high significant difference in comparison with the control group with a p value= 0.0020. Difference between means (Mice T cells+Normal Exosomes - Control) ± SEM was 14.50 ± 0.9220 with a 95% CI of 10.53 to 18.47. Similarly, the Mice T cells+LLC+Normal Exosomes (mean= 43.20%) had a high significant difference in comparison with the control group with a p value= 0.0041. Difference between means (Mice T cells+LLC+Normal Exosomes - Control) ± SEM was 15.30 ± 1.389 with a 95% CI of 9.323 to 21.28. Then the Mice T cells+Tumor Exosomes (mean= 40.90%) had a high significant difference in comparison with the control group with a p value= 0.0044. Difference between means (Mice T cells+Tumor Exosomes - Control) ± SEM was 13.00 ± 1.221 with a 95% CI of 7.748 to 18.25. The last group Mice T cells+LLC+Tumor Exosomes (mean= 24.35%) had a significant difference in comparison with the control group with a p value= 0.0327. Difference between means (Mice T cells+LLC+Tumor Exosomes - Control) ± SEM was -3.550 ± 0.9552 with a 95% CI of -7.660 to 0.5601. The flow cytometry results of Ki67 expression in CD8 T cells were statistically analyzed, The Mice T cells (mean= 12.65%) was used as control and the changes in expression of CD8-Ki67 was observed in the rest of the five treatment groups. The Mice T cells+LLC (mean= 8.060%) had a high significant difference in comparison with the control group with a p value= 0.0016. Difference between means

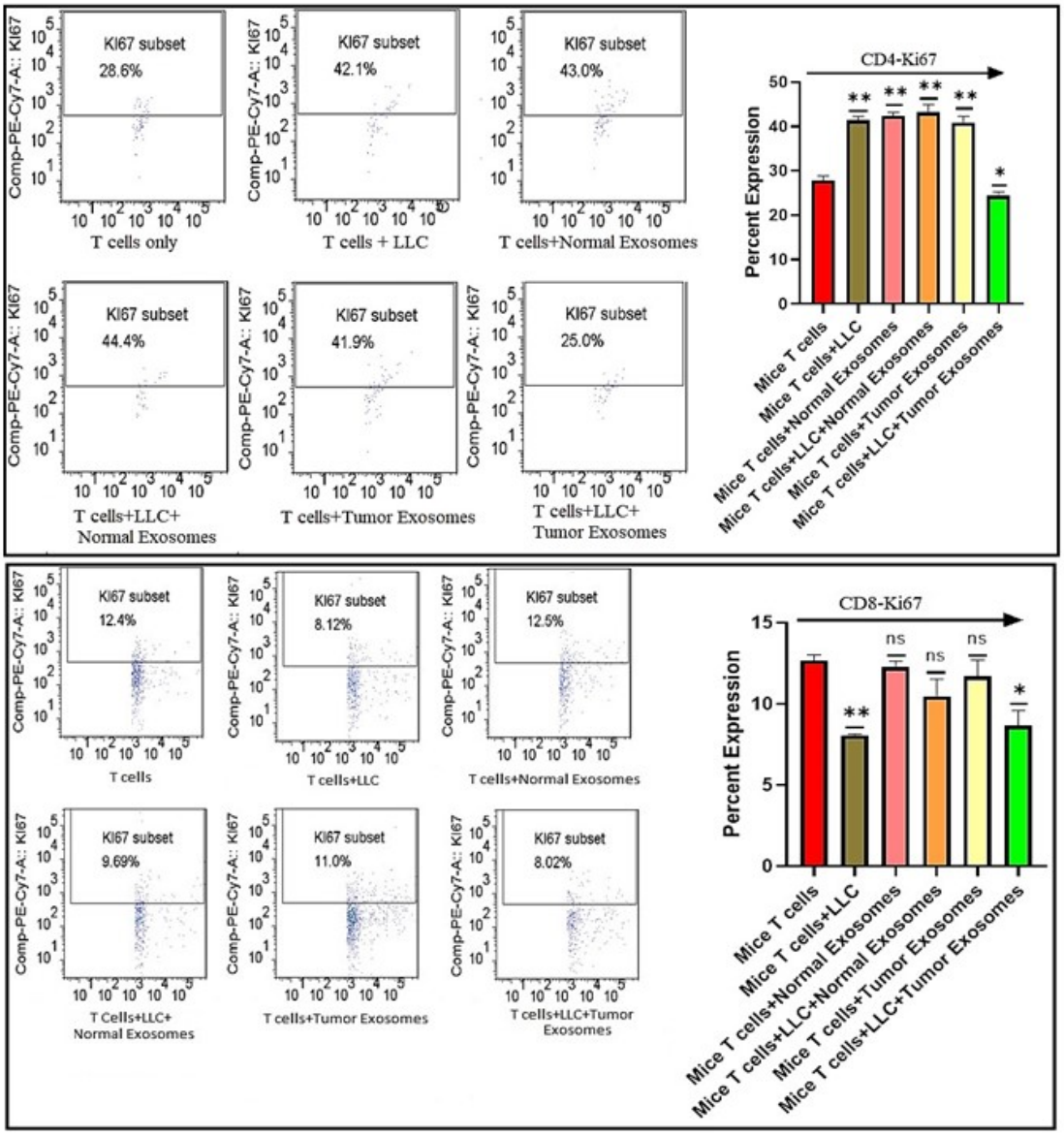

FIGURE 4.14: Flow cytometer analysis of KI67 expression in mice CD4 and CD8 T cells treated with Exosomes.

(Mice T cells+LLC - Control) ± SEM was -4.590 ± 0.2571 with a 95% CI of -5.696 to -3.484. The Mice T cells+Normal Exosomes (mean= 12.25%) had a none significant dif- ference in comparison with the control group with a p value= 0.1877. Difference between means (Mice T cells+Normal Exosomes - Control) ± SEM was -0.4000 ± 0.3536 with a 95% CI of -1.921 to 1.121. Similarly, the Mice T cells+LLC+Normal Exosomes (mean= 10.45%) had a none significant difference in comparison with the control group with a p value= 0.0546. Difference between means (Mice T cells+LLC+Normal Exosomes - Control) ± SEM was -2.205 ± 0.7953 with a 95% CI of -5.627 to 1.217. Then the Mice T cells+Tumor Exosomes

(mean= 11.70%) had a none significant difference in comparison with the control group with a p value=0.1648. Difference between means (Mice T cells+Tumor Exosomes - Control) ± SEM was -0.9500 ± 0.7433 with a 95% CI of -4.148 to 2.248. The last group Mice T cells+LLC+Tumor Exosomes (mean= 8.66%) had a significant difference in comparison with the control group with a p value= 0.0142. Difference between means (Mice T cells+LLC+Tumor Exosomes - Control) ± SEM was -3.990 ± 0.6871 with a 95% CI of -6.946 to -1.034.

Ki67 is a tumor antigen present in developing, dividing cells but not in cells that are still growing. It may be necessary to evaluate different CD8 T cells subgroups, also including active and growing CD8+ cells, to acquire clinically meaningful information. This can be accomplished by using the Ki67 protein. In the G1, S, G2, and M stages of the cell cycle, Ki67 is expressed throughout all proliferating cells. As a result, it has been argued that the Ki67 growing CD8 T cells subset represents an active CD8 T cells subset. The function of CD8+ Ki67 T cells may differ depending on the type of tumor.

4.7 Effects of Cancer Exosomes on NKT-Cells Population

From the statistical analysis of the data obtained from flow cytometry analysis of NKT cells as shown in figure 4.15, The Mice T cells (mean= 17.80%) was used as control and the changes in number of NKT cells was observed in the rest of the five treatment groups. The Mice T cells+LLC (mean= 18.25%) had a none significant difference in comparison with the control group with a p value= 0.3047. Difference between means (Mice T cells+LLC- Control) ± SEM was 0.4500 ± 0.7500 with a 95% CI of -2.777 to 3.677. The Mice T cells+Normal Exosomes (mean=19.75%) was also having a none significant difference in comparison with the control group with a p value= 0.1009. Difference between means (Mice T cells+Normal Exosomes - Control) ± SEM was 1.950 ± 1.040 with a 95% CI of -2.527 to 6.427. Similarly, the Mice T cells+LLC+Normal Exosomes (mean= 46.20%) had a highly

significant difference in comparison with the control group with a p value= 0.0003. Difference between means (Mice T cells+LLC+Normal Exosomes - Control) ± SEM was 28.40 ± 0.7211 with a 95% CI of 25.30 to 31.50. Then the Mice T cells+Tumor Exosomes (mean= 28.40%) had a significant difference in comparison with the control group with a p value= 0.0027. Difference between means (Mice T cells+Tumor Exosomes - Control) ± SEM was 10.60 ± 0.7810 with a 95% CI of 7.240 to 13.96. The last group Mice T cells+LLC+Tumor Exosomes (mean= 12.45%) had a significant difference in comparison with the control group with a p value= 0.0131. Difference between means (Mice T cells+LLC+Tumor Exosomes - Control) ± SEM was -5.350 ± 0.8846 with a 95% CI of -9.156 to -1.544. Natural killer T cells are a type of specialized T cells that comes in a variety of shapes and

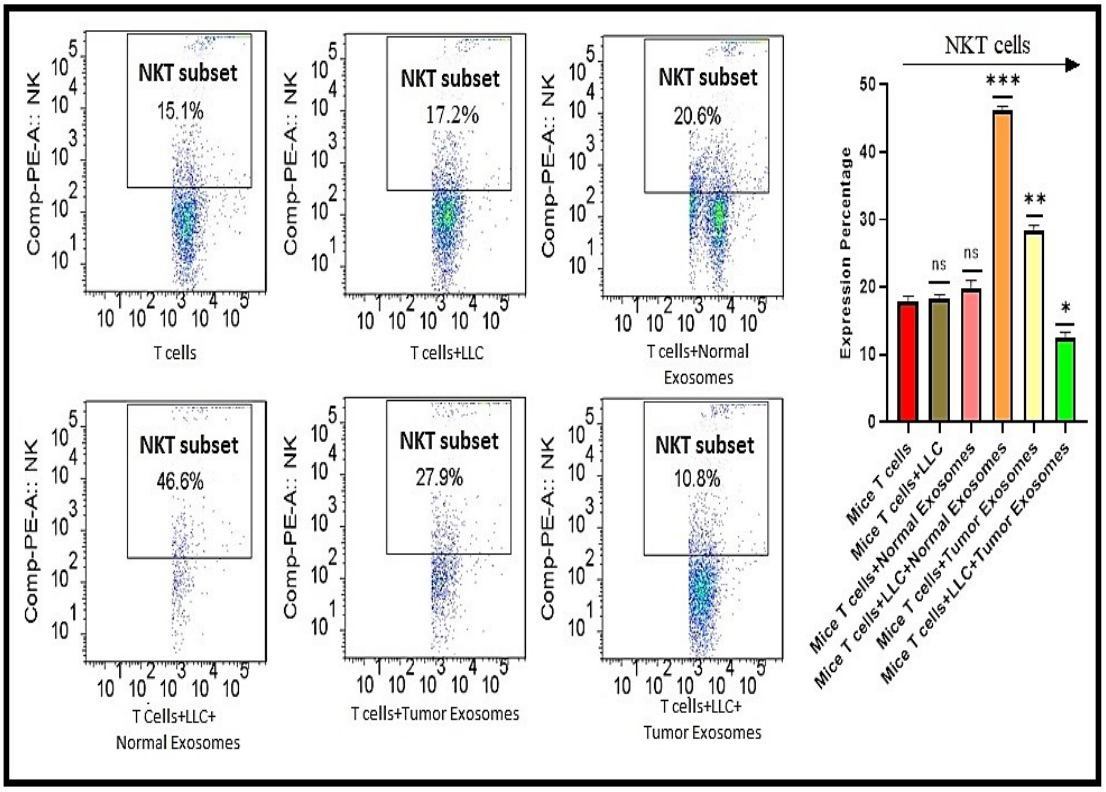

FIGURE 4.15: Flow cytometer analysis of NKT-Cells population grown with or without LLC cells and normal and tumor Exosomes.

sizes. These cells have an innate cell-like ability to respond quickly to antigenic exposure, as well as adaptive cell precision in antigenic recognition and a wide range of effector responses. NKT-Cells, like ordinary T cells have a TCR to identify antigens. TCR produced by NKT-Cells, on the other hand, detect lipid antigens presented by the non-polymorphic and conserved MHC-1 similar molecule CD1-d,

unlike normal T cells. NKT-Cells produce cytokines can influence host immune responses to cancer by modulating various immune-cells in the TME.

4.8 Expression Analysis of Selected miRNAs in NGC and MGC Derived Exosomes

The differential expression analysis of selected miRNA cargo from Exosomes derived from NGC and MGC was conducted. The total RNA was quantified using Nano drop, and the results are shown in Table 4.1. MGC yielded a higher amount of RNA compared to NGC. Therefore, for the differential expression analysis, 2ug of RNA was used from both groups.

Table 4.1: Quantification analysis of Exosomes isolated RNA.

S. No.	Sample-ID	Nucleic Acid (ng/uL)
1.	NGC Exosomes	347
1.	MGC Exosomes	510

The total RNA was converted to cDNA immediately by conventional PCR to avoid degradation by RNases contamination. The cDNA was used with specific primers in RT-qPCR, and the data was analyzed through Biorad CFX 3.0 software. The results demonstrated (Figure 4.16) that among twelve selected miRNAs (miRNA-122-5p, miRNA-20b-5p, miRNA-132-3p, miRNA-451a, miRNA-200b, miRNA-665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p, miRNA-30d-5p, miRNA-338-3p and miRNA-139-5p), there was the expression of nine miRNAs (miRNA-122-5p, miRNA-20b-5p, miRNA-132-3p, miRNA-451a, miRNA-200b, miRNA-665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p), were found in H1975 derived Exosomes in both conditions, while the three miRNAs (miRNA-30d-5p, miRNA-338-3P and miRNA-139-5p) were not shown any result/expression in any group.

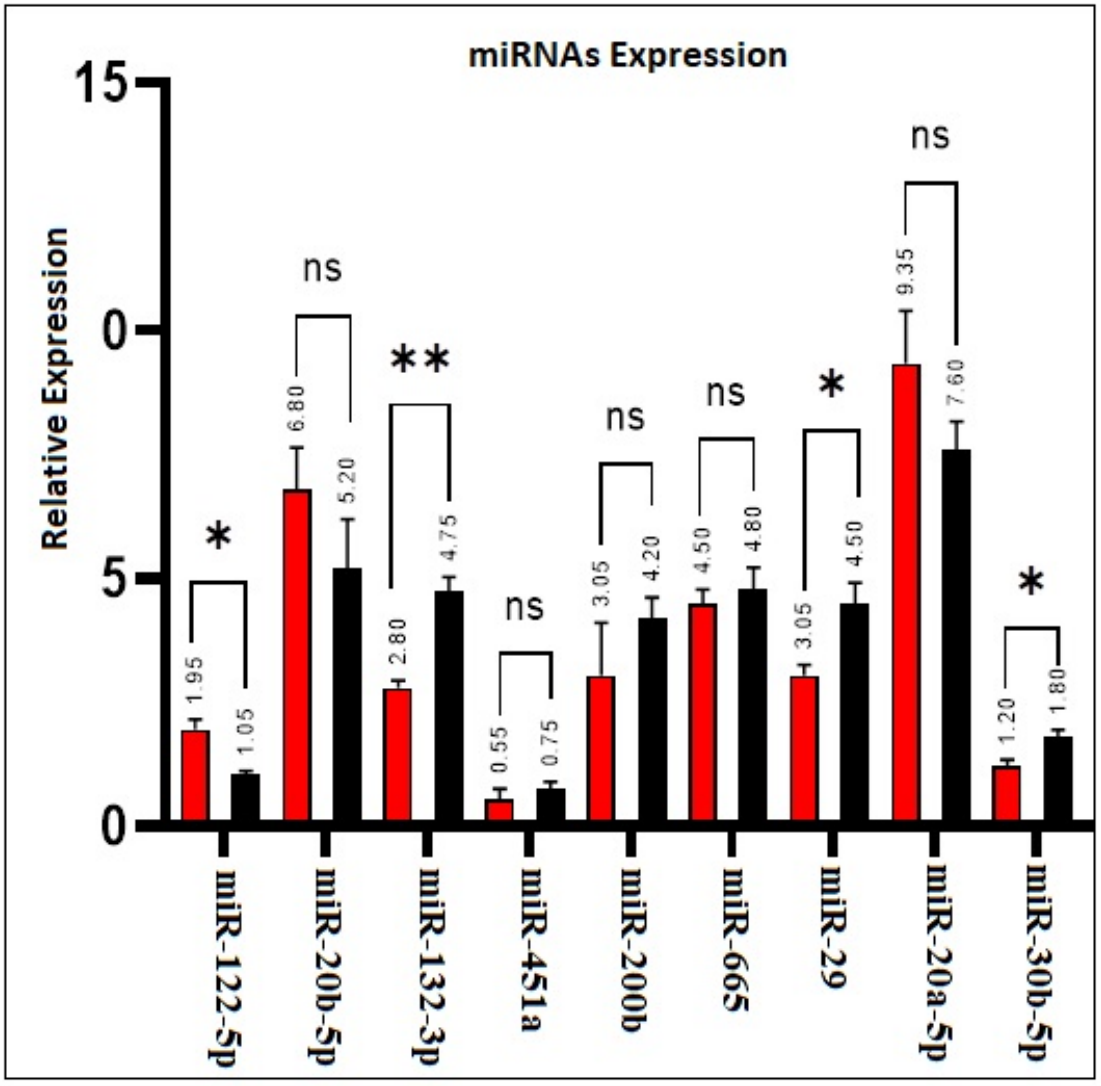

FIGURE 4.16: Relative expression of miRNAs in NGC and MGC isolated Exosomes from H1975 cells

The comparative expression of those nine miRNAs was analyzed and a more or less difference in the expression of selected miRNAs between NGC and MGC derived Exosomes was observed. The statistical analysis of the data from both NGC and MGC Exosomes was done where the expression of miRNA-122-5p was found with a significant difference with a p value of 0.0148. it was reduced in MGC Exosomes in comparison with the NGC Exosomes. The expression of miRNA-20b-5p had a none significant difference with a p value of 0.1124 among the two groups. While miRNA-132-3p expression was found with a higher significant difference in MGC Exosomes in comparison with NGC Exosomes with a p value of 0.0064. Expression of miRNA-451a was observed as none significant in MGC Exosomes with a p value of 0.1914. The expression of miRNA-200b was also none significant with

a p value of 0.1453. Similarly, the miRNA-665 expression had a none significant difference with a p value of 0.2465. There was a significant difference observed in the expression of miRNA-29 with a p value of 0.0248. The expression of miRNA-20a-5p had a none significant difference with a p value of 0.0879 among the two groups. There was a significant difference observed in the expression of miRNA-30b-5p with a p value of 0.0248. This provides an evidence that cancer cells secrets Exosomes filled with deregulated miRNAs, which affects the target cells by regulating their genes and may lead to several changes in cellular morphology and behavior.

4.9 Bioinformatics Analysis

To better understand how particular miRNAs interact in different biological processes, their potential targets were discovered using miRWalk 3.0 and total 929 genes were found to be overlapped by the three approaches suggests that they may be potential targets for particular miRNAs. To gain a deeper understanding of the function and regulatory patterns of the aforementioned miRNAs at the cellular level, GO annotation and KEGG pathway enrichment of the 929 overlapping target genes of expressed miRNAs were performed using the web-based functional enrichment tool Metascape and KOBAS. According to the results, GO Biological functions contain a lot of terms related to plasma membrane-bounded cell projection morphogenesis (10.10%), actin filament-based process (9.84%), and negative control of cellular component organization (9.33%) (Figure 4.17, Table 4.2).

TABLE 4.2: GO Biological functions of the targeted genes of expressed miRNAs.

GO	**Category**	**Description**	**Count**	**%**	$\log_{10}$**(p)**	$\log_{10}$**(q)**
GO:0120039	GO Biological Processes	plasma membrane bounded cell projection morphogenesis	39	10.10	-13.65	-9.81
GO:0030029	GO Biological Processes	actin filament-based process	38	9.84	-10.61	-7.15

GO	**Category**	**Description**	**Count**	**%**	$\log_{10}$**(p)**	$\log_{10}$**(q)**
GO:0051129	GO Biological Processes	negative regulation (cellular component organization)	36	9.33	-9.53	-6.25
GO:0120035	GO Biological Processes	Regulates plasma membrane	33	8.55	-8.99	-5.80
GO:0048729	GO Biological Processes	tissue morphogenesis	29	7.51	-7.65	-4.64
GO:0014706	GO Biological Processes	striated muscle tissue development	21	5.44	-7.64	-4.64
GO:0016311	GO Biological Processes	Dephosphorylation	25	6.48	-7.59	-4.63
GO:0060021	GO Biological Processes	roof of mouth development	11	2.85	-7.56	-4.62
RHSA,112315	Reactome (Gene Sets)	Chemical Synapses Transmission	18	4.66	-7.36	-4.48
GO:0045596	GO Biological Processes	negative regulation of cell differentiation	31	8.03	-7.31	-4.44
GO:0001501	GO Biological Processes	development of skeletal system	24	6.22	-7.08	-4.27
ko04728	KEGG Pathway	Dopaminergic synapse	12	3.11	-6.60	-3.89
R-HSA-199991	Reactome Gene Sets	Membrane-Trafficking	27	6.99	-6.60	-3.89
GO:0045936	GO Biological -Processes	negative regulation of phosphate metabolic process	26	6.74	-6.50	-3.83
GO:0070848	GO Biological -Processes	response to growth factor	29	7.51	-6.35	-3.72
GO:1905114	GO Biological -Processes	cell surface- receptor signaling pathway involved in cell-cell signaling	26	6.74	-6.33	-3.72
GO:0034330	GO Biological -Processes	cell junction organization	18	4.66	-6.32	-3.72
GO:0060485	GO Biological-Processes	development of mesenchyme	17	4.40	-6.28	-3.70

GO	Category	Description	Count	%	$\log_{10}$(p)	$\log_{10}$(q)
GO:0045786	GO Biological -Processes	cell cycle negative regulation	26	6.74	-6.15	-3.61

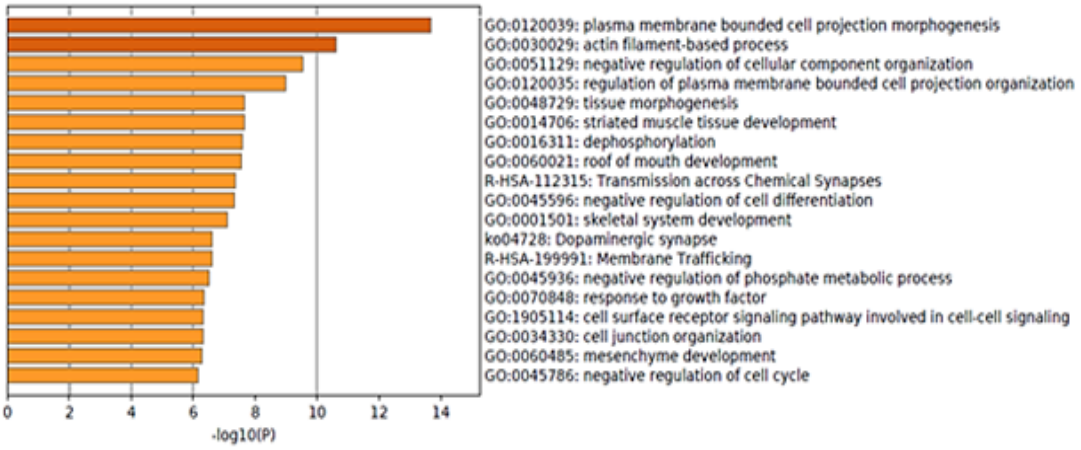

FIGURE 4.17: The bar graph of GO biological processes of targeted genes

Similarly, it was found that GO molecular activities were abundant in Plasma membrane bound cell projection morphogenesis, actin filament-based processes, negative regulation of cellular components organization, and negative regulation of cell cycle (Table 4.3).

The EnrichNet server, which has the most recent version of the pathway gene sets' search module, was used to perform functional enrichment analysis on all predicted target genes. To avoid the lack of important information that results from only taking into account the predicted targets' intersection, 929 target genes with a score >0.95 were all used as input to the EnrichNet server (Table 4.4). The data showed that just 74 genes shared overlap with several GO terms, significantly differentiating the GO keywords from the enrichment findings of the 929 genes. Only the 74% of genes that overlapped were chosen for further study.

TABLE 4.4: The functional enrichment analysis of 929 genes through EnrichNet server

Annotation (process/pathway)	Significance of network distance distribution (XD-Score)	Significance of overlap (Fisher-test, q-value)	Overlapped Genes
Long-term potentiation	0.78823	1.6e-04	7

Annotation (process/pathway)	**Significance of network distance distribution (XD-Score)**	**Significance of overlap (Fisher-test, q-value)**	**Overlapped Genes**
Melanogenesis	0.67994	2.5e-05	9
Amyotrophic lateral sclerosis (ALS)	0.56764	2.1e-02	4
Long-term depression	0.52352	1.1e-02	5
Vasopressin-regulated water reabsorption	0.47540	8.8e-02	3
Pathogenic Escherichia coli infection	0.38099	1.2e-01	3
Apoptosis	0.37900	2.1e-02	5
Gastric acid secretion	0.36880	6.4e-02	4
Vascular smooth muscle contraction	0.34390	1.1e-02	6
Shigellosis	0.31176	1.6e-01	3
TGF-beta signaling pathway	0.29033	8.8e-02	4
Wnt signaling pathway	0.28176	1.1e-02	7
Gap junction	0.28036	8.8e-02	4
Prostate cancer	0.27555	8.8e-02	4
Chemokine signaling pathway	0.24886	1.1e-02	8
Chagas disease	0.21127	1.3e-01	4
Regulation of actin cytoskeleton	0.20791	1.1e-02	8
Hypertrophic cardiomyopathy (HCM)	0.19103	3.0e-01	3
Salivary secretion	0.18706	3.0e-01	3
Inositol phosphate metabolism	0.18319	5.4e-01	2
Hedgehog signaling pathway	0.18319	5.4e-01	2
Calcium signaling pathway	0.17390	6.5e-02	6
Dilated cardiomyopathy	0.16513	3.5e-01	3
Colorectal cancer	0.15208	6.1e-01	2
Ether lipid metabolism	0.14301	1.0e+00	1
Axon guidance	0.14301	2.4e-01	4

Annotation (process/pathway)	**Significance of network distance distribution (XD-Score)**	**Significance of overlap (Fisher-test, q-value)**	**Overlapped Genes**
GnRH signaling pathway	0.13727	4.0e-01	3
Regulation of autophagy	0.13449	1.0e+00	1
Insulin signaling pathway	0.13042	2.7e-01	4
Bacterial invasion of epithelial cells	0.12647	6.7e-01	2
Amoebiasis	0.12647	4.3e-01	3
Pancreatic cancer	0.11890	6.7e-01	2
SNARE interactions in vesicular transport	0.11890	1.0e+00	1
Prion diseases	0.11890	1.0e+00	1
Oocyte meiosis	0.11410	4.5e-01	3
Adherens junction	0.11176	6.7e-01	2
(ARVC)Arrhythmogenic-right ventricular-cardiomyopathy	0.10834	6.7e-01	2
B-cell receptor signaling-pathway	0.10500	6.7e-01	2
Cardiac muscle contraction	0.10500	6.7e-01	2
VEGF signaling pathway	0.10176	6.7e-01	2
Phosphatidylinositol signaling system	0.10176	6.7e-01	2
MAPK signaling pathway	0.10130	8.9e-02	7
Alzheimer's disease	0.08817	3.9e-01	4
Progesterone-mediated oocyte maturation	0.07605	7.4e-01	2
Huntington's disease	0.06985	4.4e-01	4
ErbB signaling pathway	0.06866	7.7e-01	2
Malaria	0.04926	1.0e+00	1
Endocytosis	0.04733	5.4e-01	4
Taste transduction	0.04543	1.0e+00	1

Annotation (process/pathway)	**Significance of network distance distribution (XD-Score)**	**Significance of overlap (Fisher-test, q-value)**	**Overlapped Genes**
mTOR signaling pathway	0.03823	1.0e+00	1
NSCLC	0.03484	1.0e+00	1
Vibrio cholerae infection	0.03484	1.0e+00	1
Protein (endoplasmic reticulum)	0.03373	7.2e-01	3
TCR signaling pathway	0.02843	1.0e+00	2
Basal cell carcinoma	0.02540	1.0e+00	1
Leukocyte transendothelial migration	0.02105	1.0e+00	2
Parkinson's disease	0.00692	1.0e+00	2
Glioma	0.00462	1.0e+00	1
Pathways in cancer	0.00151	6.9e-01	5
Tight junction	0.00022	1.0e+00	2
Natural killer cell mediated cytotoxicity	0.00022	1.0e+00	2
Focal adhesion	-0.00118	1.0e+00	3
Antigen processing and presentation	-0.00188	1.0e+00	1
Ubiquitin mediated proteolysis	-0.00290	1.0e+00	2
Adipocytokine signaling pathway	-0.00391	1.0e+00	1
Melanoma	-0.00589	1.0e+00	1
Renal cell carcinoma	-0.00967	1.0e+00	1
Viral myocarditis	-0.00967	1.0e+00	1
Chronic myeloid leukemia	-0.01148	1.0e+00	1
ECM-receptor interaction	-0.02981	1.0e+00	1
Small cell lung cancer	-0.03110	1.0e+00	1
Hematopoietic cell lineage	-0.03479	1.0e+00	1
Fc gamma R-mediated phagocytosis	-0.03934	1.0e+00	1

Annotation (process/pathway)	**Significance of network distance distribution (XD-Score)**	**Significance of overlap (Fisher-test, q-value)**	**Overlapped Genes**
Lysosome	-0.06324	1.0e+00	1
Neurotrophin signaling pathway	-0.06566	1.0e+00	1
Cell adhesion molecules (CAMs)	-0.06954	1.0e+00	1
Neuroactive ligand-receptor interaction	-0.07133	1.0e+00	2
Phagosome	-0.07574	1.0e+00	1
Purine metabolism	-0.08128	1.0e+00	1
Cytokine-cytokine receptor interaction	-0.10376	1.0e+00	1
Olfactory transduction	-0.11303	1.0e+00	1
Metabolic pathways	-0.11331	1.0e+00	3

Melanogenesis, Vasopressin-regulated water reabsorption, Long-Term Potentiation, and Amyotrophic Lateral Sclerosis (ALS) were considerably enriched in terms of biological processes, pointing to a direct connection between particular miRNAs and the cancer process.

All of the miRNAs may play a role in carcinogenesis and unfavorable cell proliferation, according to the pathway enrichment analysis, which enriched terms like TGF-beta signalling pathway, Wnt signaling pathway, VEGF signalling pathway, mTOR signalling pathway, ERBb signalling pathway, T-Cells receptor signalling pathway, and MAPK signalling pathway. Based on 74 overlapping genes, the network analyst constructed a protein-protein interaction network (Figure 4.18).

The genes in the PPI network were analysed for centrality to find hub genes. Only 5 genes were selected as hub genes (BCL2, TGFA, MITF, SMAD1, BCL2), which may be important in the negative regulation of cellular processes by the discovered miRNAs.

TABLE 4.3: GO Molecular functions of the targeted genes of expressed miRNAs.

GO	**Description**	$\log_{10}$**(p)**
GO-0030029	Actin-filament based process	-10.5
GO-0051493	cytoskeleton organization regulation	-9.5
GO-0030036	actin cytoskeleton organization	-8.5
RHSA111885	Opioid Signalling	-12.1
hsa04928	parathyroid hormone synthesis, secretion, and action	-11.5
hsa04725	Cholinergic synapse	-11.4
RHSA983168	Proteasome degradation & Ubiquitination (Antigen processing)	-11.7
RHSA983169	MHC I mediated antigen presentation & processing	-11.1
RHSA8951664	Neddylation	-10.2
RHSA8856828	Clathrin mediated endocytosis	-13.8
RHSA8856825	Cargo recognition-clathrin-mediated endocytosis	-11.4
RHSA199991	Membrane-Trafficking	-9.9
GO-1901699	cellular response- nitrogen compound	-3.6
GO-0048589	developmental growth	-4.4

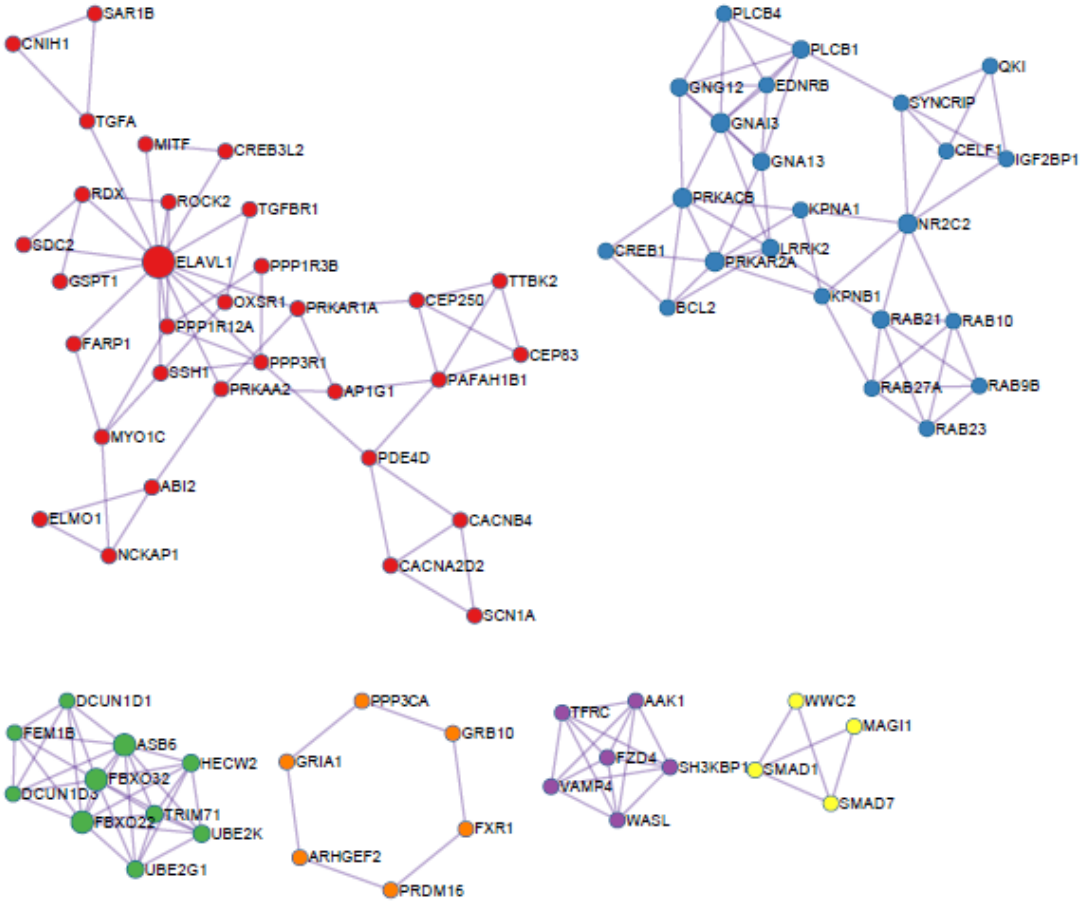

FIGURE 4.18: Protein-protein interaction networks of overlapped genes

The expression patterns (5 hub genes) was examined in normal and cancer tissues in the TCGA database to better understand their involvement in diverse malignancies. Which exhibit matrix of expression heat map based-on the supplied hub genes list. The heat map revealed that the hub genes displayed unique patterns of expression in various malignancies. Each significantly differentially expressed gene, such as BCL2 TGFA MITF, SMAD1, BCL2, suggests a strong link between this gene and the associated malignancy. The role of hub genes in the negative regulation of cell proliferation was identified through the EnrichNet tool given in Table 4.5.

TABLE 4.5: The role of miRNAs target hub genes in negative regulation of cell proliferation.

Role	**Significance of network-distance distribution (XD-Score)**	**Significance of overlap, (Fisher-test, q-value)**	**Dataset size, (uploaded gene set)**	**Dataset size, (pathway gene set)**	**Dataset size, (overlap)**	**Related-miRNA**
Negative B cell proliferation	0.59677	0.72016	74	13	BCL2	miRNA-200b
Negative regulation of epithelial cell proliferation	0.05700	1.00000	74	59	TGFA	miRNA-451a
Negative regulation of cell proliferation	0.02691	0.93698	74	147	MITF, SMAD1, BCL2	miRNA-132-3p and miRNA-29

Chapter 5

Discussion

5.1 Discussion

Tumor cells suppress cellular immunological responses in the host with the help of a variety of pathways and mechanisms. Although lung malignancies have tumor antigens that the immune system may identify, the antigen presentation is frequently poor [279]. Tumor cells make, release, and utilize Exosomes in order to promote tumor growth. Exosomes generated from tumors are currently being studied to see how they can help the tumor. Exosomes carries information and genetic messages from cancer cells to normal or aberrant cells (surrounding or distant). All bodily fluids contain Exosomes produced by malignancies. They change the phenotypic and functional characteristics of recipients when they enter target cells, reprogramming them to be active contributors to angiogenesis, thrombosis, metastasis, and immunosuppression. It is not clear how precisely cancer Exosomes contribute to the development of lesions or the advancement of cancer in target tissues due to a complicated mechanism. But however some studies have suggested that cancer Exosomes may contain proteins that promote cell migration, invasion, and angiogenesis. Cancer Exosomes may also help the microenvironment of the target tissue for metastasis by facilitating interactions between cancer cells and the cells of the target tissue, creating a favorable niche for cancer cell colonization and growth [281]. Another phenomenon might be that Exosomes derived from

cancer cells could carry factors that promote the formation of new blood vessels (angiogenesis) in the target tissue. This supports the nutrient and oxygen supply required for the growing cancer cells to establish a lesion. Induction of Epithelial-Mesenchymal Transition (EMT) is also one of the studied mechanism via which mesenchymal traits are acquired by epithelial cells, allowing for greater motility and invasiveness [282]. It's possible that compounds found in cancer Exosomes cause EMT in target tissue cells, promoting lesion development and cancer cell invasion [283]. The genetic materials in Exosomes may also affect target tissue cells' gene expression, which may lead to modifications that promote the development of lesions. some enzymes and other biological factors found in cancer Exosomes that promote remodeling of the extracellular matrix in the target tissue can also facilitate cancer cell invasion and migration [284].

Thus these all possible phenomenon initially change and create favorable environment, form niches and thus leads to tumor formation which are all steps of metastasis. Cancer Exosomes are believed to have a role in the metastatic process, but it's crucial to understand that there are many variables at play in this intricate phenomena [285]. The multi-step process of metastasis entails the migration of cancer cells from the main tumor to other body tissues or organs. Although they are not the only source of metastasis, cancer Exosomes are thought to be involved in several phases of this process [286]. The main areas of research where currently scientists are trying to identify the accurate mechanisms of "how cancer Exosomes identify where to transfer cargo from the cancer cells" however Selective Cargo Loading, Receptor-Mediated Uptake, Homotypic Attraction, Chemotaxis and Homing Signals are somehow studied to be an important possible reasons for the target specificity [287].

Tumor-produced Exosomes carry cargos that are similar to the contents of parent cells and could be used as non-invasive cancer diagnostics. For cancer therapy, their role in suppressing host antitumor responses and mediating drug resistance is critical. Exosomes produced by tumors may interfere with cancer treatment, but they could also be used as adjuvants and antigenic components in anticancer vaccines [165].

Tumor cells and tumor-associated cells can use Exosomes to transmit macromolecules and cellular constituents whether as donors or receivers [288]. Different cellular stress and/or disease circumstances influence Exosomes biogenesis and secretion [87]. Exosomes may have a role in tumor development, migration, vasculature, inflammation, immunologic re-modeling, and therapeutic actions, suggesting the importance of Exosomes-mediated host-recipient interaction. Exosomes play a vital role in modulating tumor immunity by mediating cross-talk between the microenvironment and tumor cells. The functions of Exosomes in cancer has been investigated extensively over the last decade, but there are still various research gaps that need to be addressed to link several reports on the role of Exosomes in different cancers, including NSCLC. Experimental approaches are very much necessary for understanding the true biological pathways of cancer. For example, providing the same natural environment and conditions that the tumor has in the body is a difficult subject of concern when studying the exact phenomena of the tumor microenvironment. Most previous researches designed to identify exosomal mechanisms in cancer used cell lines that were cultured in conditions that do not match the biochemical and physiological parameters of the cancer microenvironment. Exosomal productivity and the expression of exosomal contents such as nucleic acids and proteins are affected by environmental influences. Such results may misguide the scientific findings.

In the current project, the first aim was to compare the productivity of the Exosomes in the normal growth conditions (NGC), which are usually practiced in the labs to culture the cancer cells, where the normal oxygen saturation is provided to the media supplemented with FBS and normal pH of the human body is provided in the form of media. So those conditions are not the true representations of the natural tumor environment. Therefore the growth conditions were altered with a term as modified growth conditions (MGC) in this project where the cell line source of Exosomes were cultured in media with low pH, without adding the FBS and antibiotics and the oxygen saturation was also kept low in the incubator (hypoxia). The low pH and hypoxic conditions of cancer microenvironment [289] were mimicked here to provide the cancer cells with the proper environment where they naturally grow. The reason for not adding the FBS and antibiotics was that the

tumor surrounding is deficient with most of the supplements and the antibiotics may affect the survival and physiology of the Exosomes.

Tumors have microenvironment physiology that is dissimilar from that of normal tissues. Hypoxia, anoxia (O_2 depletion), energy and glucose deprivation, elevated extracellular acidosis and lactate levels are only a few of the factors that are distributed inside the tumor mass. The aberrant tumor vasculature and diverse microcirculation play a big role in creating this hostile microenvironment. Hypoxia and other unfavorable micro-environmental factors have been shown to impart irradiation resistance, resulting in therapy failure. The fixation of DNA damage (radiation-induced) is less efficient under hypoxia. Proliferation restrictions, gene expression alterations, proteome modifications (e.g., increased functioning of resistance-related proteins, enhanced transcription of growth factors and DNA-repair enzymes), and genomic- modifications are all indirect approaches (genomic instability leading to clonal heterogeneity and selection of resistant clonal variants). These alterations, which are brought on by the hostile microenvironment, might encourage tumor development and acquired treatment resistance, both of which lead to a poor clinical outcome and prognosis [290]. Hypoxia is a common hallmark of solid tumors, and it may promote tumor development, angiogenesis, and metastasis via exosomes-mediated signaling. When 3 distinct breast cancer cell lines were subjected to mild (1% O_2) and severe (0.1% O_2) hypoxia, the amount of exosomes present inside the conditioned medium increased dramatically [291].

With this entire hypothesis, the experiments were conducted and the Exosomes were produced in both NGC and MGC grown H1975 cells and were isolated through ul- tracentrifugation. The quantification to compare the production of the Exosomes was done through nanosight and western blotting, wherein in both conditions (NGC & MGC), we observed a prominent change in the number of Exosomes. That supported the hypothesis of increased production of Exosomes in the tumor environment in comparison with the normal conditions provided. Then the next part of this research was to analyze the impact of these two groups derived Exosomes on Jurkat cells through treatment experiments and the results were analyzed through a flow cytometer. From all the markers (CD25 and CD69)

of Jurkat cells, it was demonstrated that the MGC derived Exosomes had a reduced Jurkat cells number in comparison with the other parameters discussed in detail in our results chapter. Activated proliferating lymphocytes are recognized to exhibit a variety of molecules on their surface, including CD25 and CD69, which are typically expressed at low levels or even absent on quiescent cells [292]. Their downregulation can have implications for T cell proliferation, decreased expression of CD25 may result in reduced responsiveness to IL-2, potentially limiting the ability of T cells to receive signals for growth and division. While CD69 downregulation itself may not directly affect T cell proliferation, it is associated with the resolution of the early activation phase, during which T cells are actively dividing. So the reduced expression of these two markers may have a negative effects on T cells proliferation [293, 294]. Similarly, the validation of these results was extended to study IFN-γ expression in CD4 and CD8 T cells, which were isolated from human blood. So, the flow cytometer results demonstrated a decreased IFN-γ expression in both types of T cells, the CD4 T cells had comparatively more reduced IFN-γ. These results indicate that if the tumor cells secreted Exosomes may affect the CD4-IFN-γ secretion then that may lead to inhibiting their direct anti-tumor activity, following activation and polarization into the TH1 phenotype. This is obvious that IFN-γ has a major role in the tumor-host retort and reduced IFN-γ expression is frequently detected in lung cancer [295]. IFN-γ plays a multifaceted role in the immune system, including influencing T cell proliferation, it can promote T cell proliferation by acting as a growth factor, which provides signals that stimulate the cell cycle progression of T cells, leading to their division and expansion. A nother mechanism of IFN-γ is to enhance the ability of APCs, such as macrophages and dendritic cells, to present antigens to T cells. This improved antigen presentation can, in turn, stimulate T cell activation and proliferation [296]. Thus the reduced expression of IFN-γ in the experiments could be a source to inhibit the T cells devision. One of the reasons behind the decreased secretion of IFN-γ might be the Exosomes, which are produced by tumor cells and may suppress the IFN-γ secretion in the source cells. Based on the above evident results, animal models were further used to confirm these effects.

In the current study initially a single cell line was used for Exosomes production,

isolation and analyzing their effects on T cells as a starting point and proof of concept. This approach was chosen to lay the foundation for next part of the project, offering the flexibility. The rationale for utilizing a single cell line was twofold. Firstly, it allowed for a controlled experimental environment, enabling us to scrutinize the specific impact of Exosomes derived from the H1975 cell line without the introduction of variability associated with multiple cell line sources. Secondly, it was driven by practical considerations. The results obtained with this single cell line served as a valuable preliminary insight that guided us to proceed for patient-derived xenografts (PDX).

Consequently, the scope of the study was expanded by incorporating tumor in mice models that is generally known as PDX. PDX involves the transplantation of human tumor tissue or cells, acquired from a patient, into mice or other laboratory animals [297]. These mice then serve as hosts for the tumor, allowing researchers to study the behavior of the cancer in a more in vivo-like environment. PDX models are valuable tools for studying cancer biology, testing potential treatments, and predicting how a patient's tumor may respond to specific therapies, making them an important aspect of cancer research [298]. Due to these qualities of PDX, in the current study, mice were injected human cancer derived commercially available lung cancer cell line (H1975), which was isolated in 1988 from the lungs of a nonsmoking female with non-small cell lung cancer. The tumor induced mice was actually a PDX in the current project.

Exosomes were isolated from normal and tumor mice serum (tumors were generated for these experiments as mentioned in previous chapters). The impact of the Exosomes was investigated on mice spleen isolated T lymphocytes, after the quantification as well as quality of Exosomes was validated using NTA and Western blotting. In healthy mice isolated CD4 T cells exhibiting reduced IFN-γ expression, similar to the earlier findings was observed. Cancer exosomes also decreased IFN-γ expression in healthy mice isolated CD8 T cells. In comparison CD4-IFN-γ expression was more reduced than CD8-IFN-γ. Along with these expressions, the Ki67 and TNF-α expression was also decreased, a reduction in Ki-67 levels in T cells signals a potential slowdown in the cell cycle and a shift towards a

non-dividing or arrested state. This suggests a regulatory mechanism controlling T cell proliferation. Ki-67's involvement in DNA replication implies a diminished ability for T cells to undergo division, while its sensitivity to environmental cues implies a context-dependent modulation of T cell proliferation, especially in response to differentiation signals or immunosuppressive conditions [299]. Similarly the reduction in TNF-α levels can diminish inflammatory signaling, impacting T cell activation and proliferation. TNF-α serves as a co-stimulatory signal, so lower levels may impair T cell responsiveness to proliferative cues. Additionally, TNF-α influences T cell differentiation, and its reduction may alter the balance between T cell subsets with implications for their proliferative capacity [300]. TNF has been utilized to treat certain types of cancer by inducing cell death in malignant cells. TNF is thought to have an oncogenic role in a variety of cancers on the other side, including NSCLC. The transcription factor NF-kB is activated by TNF. Inflammation-induced cancer is exacerbated by NF-kB, which is a critical effector of TNF. TNF could be a biomarker in NSCLC, according to data from The Cancer Genome Atlas database, and TNF and its receptors have a complicated role in NSCLC. TNF is rapidly elevated in NSCLC in response to EGFR suppression, according to recent research, and this overexpression leads to NF-kB activation. TNF may mediate immunotherapy's harmful adverse effects and alter immune checkpoint inhibitor resistance. TNF-inhibiting drugs are commonly used to treat a variety of inflammatory and rheumatologic disease, and they could be particularly useful when used in conjunction with targeted therapy for NSCLC and other malignancies [301]. IFN-γ is cytokine (inflammatory) plays a main role as antitumor immune response, according to common wisdom. Less IFN-γ expression in the TME upsurge the hazard of tumor metastasis during immunotherapy, although it has been utilized clinically to treat a range of cancers. Although there is mounting evidence that IFN-γ can promote cancer progression, the mechanisms underpinning this contentious involvement in tumor development remain unknown [302]. IFN-γ is frequently regarded as a crucial anticancer cytokine that is largely produced by natural killer (NK), NKT, and activated T cells [303, 304]. IFN-γ signaling has been proposed to increase carcinogenesis and metastasis [305] by

activating a cascade of downstream signaling processes, particularly classical JAK-STAT signaling, and the transcription of several IFN-γ-inducible genes, both of which cause cell-cycle arrest and apoptosis in tumor cells, inducing inflammatory responses immunosuppression, or other unknown pathways [306]. Sustained low-level IFN promoted the growth of numerous tumor types in mice models [304]. Despite the fact that IFN has been used in multiple anticancer clinical trials, it has been demonstrated that low-level IFN production at the tumor site enhances the likelihood of tumor metastasis during immunotherapy [307]. In most of the cancer types including NSCLC, beyond genetic research, epigenetic studies, particularly microRNA research, have been explored in both quantity and quality during the last decade. This has increased our knowledge of basic cancer biology and opened up new avenues for therapeutic development [308]. MiRNA dysregulation is a common finding in NSCLC and has been connected to the onset, development, and metastasis of the illness through influencing their target genes. [309]. Exosomes, which are supplied to target cells via extracellular fluid, contain miRNAs [310]. Due to their importance in the modulation of cell function and post-transcriptional/translational gene regulation in a number of malignancies, these RNA molecules, which make up a sizeable amount of Exosomes' cargo, are likely the ones that have received the most research [311]. These miRNAs are therefore highly focused by researchers to be used as biomarkers in NSCLC. Poroyko et al. discovered that the exosomal payload of miRNAs differs between SCLC and NSCLC patients. It was demonstrated that thirteen miRNAs in particular are useful for differentiating the two histologies. For differentiating between SCLC and NSCLC patients, three of them (miRNA-331-5p, miRNA-451a, and miRNA-363-3p) showed the best specificity and sensitivity [312]. Additionally, miRNA-203 was previously identified by Rabinowits et al. as a lung ADC biomarker in blood-derived Exosomes, and it is effective at differentiating between NSCLC and SCLC cases (sensitivity 80%, specificity 100%) [313]. Exosomal miRNAs released by NSCLC are used as biomarkers in addition to interfering with target cells, such as immune cells, and suppressing tumors. In this investigation, the expression of a subset of miRNAs was examined that are often released in NSCLC and some of which have been linked to the inhibition of immune cells.

In this study NGC and MGC exosomes were assessed for their effects on the expression of selected miRNAs. Total 12 miRNAs (miRNA-122-5p, miRNA-20b-5p, miRNA-132-3p, miRNA-451a, miRNA-200b, miRNA- 665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p, miRNA-30d-5p, miRNA-338-3p and miRNA-139-5p) were analyzed, there was the expression of nine miRNAs (miRNA-122-5p, miRNA-20b-5p, miRNA-132-3p, miRNA-451a, miRNA-200b, miRNA-665, miRNA-29, miRNA-20a-5p, miRNA-30b-5p), found in H1975 derived Exosomes in both conditions, while the three miRNAs (miRNA-30d-5p, miRNA-338- 3P and miRNA-139-5p) were not shown any result/expression in our experiments. Then the comparative expression of those nine miRNAs was performed, which demonstrated that there was a more or less difference in the expression of seven miRNAs between NGC and MGC derived Exosomes.

miRNA-122-5p, a type of short noncoding RNA, was previously found to be deregulated in Exosomes produced from NSCLC cells [314]. The level of miRNA-20b-5p expression in NSCLC tissues was found to be considerably greater than in normal neighboring tissues. Furthermore, the expression level of miRNA-20b-5p in NSCLC cell lines was explored, and it was discovered that the expression level of miRNA-20b-5p in the NSCLC cell lines A549 and H1299 was much higher than in the normal 16HBE cell line [315]. The function of miRNA-132 is complicated because it can be oncogenic in endocrine pancreatic tumors, squamous cell carcinoma of the tongue, breast cancer, and colorectal cancer [316, 317], or a tumor suppressor (prostate cancer, osteosarcoma, NSCLC, and ovarian cancer [318]. Down regulation of miRNA-126 was found in NSCLC Exosomes, which increased NSCLC cell cycle progression, cell proliferation, invasion, and cell migration. NSCLC cell proliferation, colony formation, migration, and invasion were all inhibited by miRNA-126 loaded NSCLC Exosomes, whereas apoptosis and cell cycle arrest were promoted [319]. Low tumor miRNA200 expression has been linked to poor differentiation, a higher risk of lymph node metastasis, and docetaxel and gefitinib resistance, underlining the link between low tumor miRNA-200 expression and poor prognosis in NSCLC patients [320]. Huang, Chu, et al. assessed miRNA-665 expression level in NSCLC cell lines and tissues and found that its upregulation may have a major function in NSCLC [288]. MiRNA-20a

could be used as a biomarker for NSCLC [321]. MiRNA-30b expression is reduced in primary tumors of NSCLC, and it suppresses NSCLC invasion, cell proliferation, and migration by targeting Cthrc1, Rab18, and EGFR [322]. In NSCLCs, miRNA-30d-5p has been shown to act as either cancer promoters or suppressors [323].

Other than miRNAs, many proteins may be loaded in cancer cells in Exosomes and are secreted, which upon entrance/ attachment to the surface of the target cells initiate several biological changes. It was identified that some of the proteins which may inhibit or exhaust T cells from the literature and their expression was identified in H1975 Exosomes. The Exosomes isolated from both conditions were analyzed for selected proteins that are expressed in cancer and may lead to T cells suppression. Among our two groups, three proteins (CEACAM1, HVEM, and PD L1) were found over expressed. In the previous studies Zhang, Xinwen, et al. found expressed in the H1975 cells derived Exosomes and among the two conditions, the expression of CEACAM1 was observed overexpressed in MGC, while the expression of HVEM and PDL1 was the same in both conditions Exosomes. Along with these three proteins it was also identified that the presence of other two T cells suppressive protein CD48 and CD155, but there were no bands observed. In the previous studies Zhang, Xinwen, et al. found that CEACAM5 was shown with increased expression in NSCLC cells and tissues. CEACAM expression was also linked to clinic-pathological parameters in patients with NSCLC, including T division, lymph invasion, and histological grade. In vitro experiments revealed that CEACAM5 deletion increased p38-Smad2/3 signalling, which in turn reduced NSCLC cell proliferation and migration. It was discovered that CEACAM5 is necessary for the inhibition of NSCLC tumor growth in mice [324]. The bulk of studies have demonstrated that CEACAM1 inhibits T cell growth. A signal cascade that reduces T cell cytokine generation and proliferation is set off by CEACAM1 ligation on T cells [274, 325]. T cell stimulation in vitro with cytokines including IL2, IL7, and IL15 results in immediate and significant CEACAM1 up-regulation that lasts for days. CEACAM1 is triggered by CEACAM1, it's ligand [326]. Similarly, HVEM, which is expressed on tumor cell membranes and triggers immune

cell signaling pathways, leads to either activity inhibition or restriction of function BTLA. HVEM has main function in tumor T cells suppression. However, the therapeutic significance of its dynamic alterations in distinct T cells subsets in peripheral blood in cancer patients is mainly unknown [327]. Other than these two proteins the most important protein, PD-1 located on T cells interacts with PD-L1, located on or secreted by cancer cells. This linkage inhibits T cells, which then can lead to outgrow of the tumor [328]. The adaptive immunity is modulated by PD-1. Cytokine production, T cell proliferation and cyto-lytic activity are all repressed whenever the ligands PDL1 and PDL2 bind to PD1. While a lot about PD1's physiologic roles in controlling the primary immune response and T cell fatigue are known but there is a deficiency about that how PD-1 ligation impacts signaling pathways. The ligation of cytotoxic T lymphocyte antigen-4, a comparable inhibitory molecule, seems to affect more signaling cascades than PD-1 ligation that is recognized to decrease membrane-proximal T cell signaling events [329].

Based on these findings, more research into the influence of cancer Exosomes on T lymphocytes and their cytokines, as well as other types of immune cells, both in vitro and in vivo is recommended. Cancer cells may use Exosomes to modulates the immune cells, causing additional problems and severity. Because each component of the immune system has a unique biological effect, down-regulating or deactivating any of them will result in a reduction in the immune system's ability to combat cancer. Exosomes from tumors are being discovered as new immune-regulatory agents. The ability to communicate and send inhibitory or stimulatory impulses to immune effector cells is impressive and intriguing. The tumor microenvironment's cellular composition and the cell types targeted by Exosomes could be the key to tumor Exosomes' dual-functional capability. Tumor Exosomes-driven contacts can be implicit or explicit, depending on whether immune recipient cells are present in the tumor microenvironment. To disable antitumor effector cells, immune cells entering tumors have a significant incentive to create immunosuppressive tumor Exosomes. Immunotherapies that disrupt suppressive pathways in immunological recipient cells induced by tumor Exosomes

transmitting juxtacrine or paracrine signals could be effective against these cancers. Tumor Exosomes are largely employed in the tumor microenvironment to promote tumor escape by inhibiting antitumor immunity controlled by invading activated T cells. Immunotherapies are unlikely to be helpful in this case, as tumor exosomes are dedicated to directly support tumor progression. Instead, traditional tumor-suppressing medications should be considered. At the same time, it's vital to remember that all cells in tumor cells produce Exosomes, and that immune-stimulatory signaling caused by tumor Exosomes can alter the environment to encourage immune activities rather than tumor growth, whether directly or indirectly. The equilibrium of many of these intracellular linkages in the TME will decide the fate of the tumor, as well as the activity of the regional immune response. To inhibit tumor Exosomes activity in the prospective, molecular, genomic, or immunological therapies may be applied. Currently, research is focused on better understanding the molecular processes that drive tumor Exosomes discharge, ingestion by recipient cells, and the transcriptional and translational modifications that these Exosomes cause. Such research could indicate to the likelihood that cancer Exosomes affect immune system responses, thereby increasing metastasis. Exosomes have a distinct set of mRNAs, miRNAs, and proteins that has been extensively studied. Tumor cell Exosomes can be transferred to immune cells, causing changes in gene expression and cell behavior. Galectin-1, a exosomal protein tumor-derived, induced suppressor-phenotype in T cells, implying that it could be a feasible therapeutic-target for limiting T cells dysfunction and increasing anti-tumor immune-responses. The immune system is manipulated by cancer Exosomes to establish an immune-suppressive milieu. CD8+ T cells are the main immune cells that target the tumor because tumor Exosomes influence cytokine secretion patterns and gene expression. This could be due to the contents accumulating in tumor Exosomes. Cancer cells secret a big number of Exosomes, and their payload is essential for cancer progression. Understanding the particular biochemical and molecular process that leads to immune escape of tumor Exosomes and their components (RNA and protein) in vitro and in vivo is critical for establishing future therapeutic options, given the heterogeneity and complexity of cancer.

Keeping the importance in mind it would be unfair not to discuss the therapeutic applications of Exosomes. Several cancer therapies are currently being developed, including (1) Exosomes from immune cells are being used to suppress cancer cells in a natural way [330], (2) preventing cancer-derived Exosomes from being released, (3) using Exosomes as gene carriers [331], and (4) using Exosomes as anti-cancer drug carriers [332].

Exosomes produced by cancer cells are thought to accelerate cancer pathogenesis by helping to build the pre-metastatic microenvironment, tumor growth and progression, angiogenesis, immune evasion, treatment resistance and anti-apoptotic signaling [284, 333, 334]. As a result, inhibiting the production, release, and absorption of cancer cell-derived Exosomes could be an effective cancer therapy. Bobrie et al. [335] discovered that blocking Rab27a, a critical facilitator of Exosomes production, reduced primary tumor development and lung dissemination of metastatic cancer (4T1) cells using a mouse model.

Exosomes have a lot of potential in cancer therapy, but using natural Exosomes is difficult and rarely produces the desired therapeutic response. Engineered Exosomes carrying specific proteins, RNAs, or medicines, on the other hand, have been proven to have a lot of promise for cancer treatment [336].

Endogenous miRNAs binds to targeted mRNAs leads to gene expression regulation. As a result, miRNAs may prove to be an effective cancer therapeutic technique. MiRNAs are quickly damaged in vivo, and miRNA delivery to key target cells, tissues, and systems is a major challenge. Because Exosomes are persistent small vesicles that may carry functional bioactive substances over long distances with a significant level of target selectivity, they have been viewed as a promising carrier for miRNAs in treatment of cancer [284]. In last decade many researchers focused on exosomal-based transportation of miRNAs and miRNA inhibitors for treatment of cancer. Exosomes loaded with the anti-glioma miRNA (miRNA-146b) have been shown to inhibit glioma growth in vitro and glioma xenograft growth in rats [337].

Many scientists have recently started working on an exosomal-based cancer vaccine [184]. TNF-alpha-related-apoptosis-inducing-ligand (TRAIL), for example, is a cytokine that acts as a ligand to cause cell death [338]. TRAIL-armed Exosomes could trigger apoptosis in cancer cells and limit tumor development in vivo, according to Rivoltini et al. Furthermore, IL-18 enriched Exosomes increase the production of Th1 cytokines and the proliferation of PBMC, implying that IL-18 enriched Exosomes have a greater ability to promote specific anti-tumor immunity since they elicit a larger immune response [339].

Anti-tumor chemotherapeutic medications can successfully kill fast-growing tumor cells. These drugs, on the other hand, can cause substantial negative effects by harming normal, healthy cells that are quickly growing. Furthermore, several hydrophobic medications have difficulty targeting tumor cells with any degree of selectivity. As a result, an effective drug carrier is essential. Because of their naturally occurring origin and stable lipid bilayer structure, Exosomes have the potential to be an effective carrier for chemotherapeutic medicines. Tang et al. [340] published a study in 2012 that demonstrated the use of tumor cell-derived microparticles as chemotherapeutic drug carriers. They discovered that chemotherapeutic medications placed onto micro-particles had a powerful anti-tumor effect in vivo and in vitro.

In the past, extracorporeal techniques have been employed to get rid of immunosuppressive components associated with cancer. One of the most well-known instances is a Lentz's group study in which 16 patients with metastatic cancer received ultra-pheresis treatment, which involves removing specific blood fractions connected to immune suppression. A second biopsy after treatment showed a considerable rise in lymphocyte infiltration and tumor necrosis. When immunological energy was reversed, Karnofsky's health would sometimes get better. The mean cross-sectional diameters of all detectable lesions were reduced by 50% or more in 6 of the 16 subjects [341]. This work demonstrated proof-of-principle that immune suppressant extracorporeal clearance can be utilized to activate cancer immunological responses in the clinic, but it has serious flaws: Because it is: a)

non-selective for particular inhibitors; b) theoretically could lead to the loss of immune-stimulatory cytokines; and c) not widely applicable.

Aethlon Medical, a biotechnology company in San Diego, has developed a novel hollow-fiber cartridge called the Hemopurifier that works with the current dialysis technique. Recent clinical trials have demonstrated its safety in patients with hepatitis. HIV particles, which resemble Exosomes in size, were successfully eliminated in a number of situations [342]. A proprietary lectin-based resin that the Hemopurifier uses as an affinity substrate has a significant attraction for highly glycosylated viral surface proteins and may selectively deplete circulating virus, leading to virus buildup in the cartridge. Tumor cell membranes and produced immunosuppressive microvesicles are more glycosylated in compared to non-malignant cells, which results in a preferential affinity for lectins [343]. Patients with cancer have much more circulating microvesicles than healthy individuals. In contrast to 0-0.5 ug in healthy volunteers, microvesicles in cancer patients vary from 2,000 to 5,000 ug/ml protein/ml blood [344].

The clear higher concentration of circulating microvesicles and the potential for selective depletion due to glycosylation variations make the Hemopurifier a promising method for removing immunosuppressive microvesicles released by cancer in their current state. The number of Exosomes removal targets is increased by the resin in the Hemopurifier cartridge's capacity to bind a range of molecules, including antibodies. To diminish microvesicles that express the HER2 protein, for example, the Hemopurifier resin could be used with therapeutically important antibodies like Herceptin. Proteins like FasL, MHC I, MHC II, CD44, placental alkaline phosphatase, TSG-101, MHC I-peptide complexes, and MHC II-peptide complexes have been discovered on cancer-secreted micro vesicles. The antibodies to these proteins are widely available and simple to include in the Hemopurifier cartridge [345].

T cells that are malignant have a number of cellular states and characteristics. Both prolonged TCR stimulation and T cell domestic and foreign cues can influence the fate of malfunctioning T cells [345]. Although the characteristics of T cells

malfunction in malignancy are comparable to T cells dysfunction in persistent viral infection, other factors in the complex TME augment immunosuppression and can alter T cells malfunctioning in cancer further. From the current study, it can be concluded that exosomes specificity in cancer cells is lost and also it effects the function of the associated receptors. It has been reported that in the normal physiology of the cell different mechanisms like originating cell identity, surface proteins and molecules, cargo sorting, intercellular communication and receptor-mediated uptake helps the Exosomes to deliver the cargo among different cells/tissues [346]. Exosomes has a controlled receptor mediated signal transduction mechanisms for target specificity e.g. tetraspanins and integrins are two examples of particular surface proteins and molecules found on Exosomes that serve as indicators of their origin and composition. These surface proteins may act as markers to help Exosomes find their intended target cells. Furthermore, certain ligands on the Exosome's surface can interact with receptors on target cells to help them bind to and be taken up by those cells [347]. In general, there are three pathways through which Tumor-Derived Exosomes (TEXs) transfer information, 1) TEXs convey intercellular signals by binding to receptors on recipient cells, 2) TEXs fuse with the recipient cell's membrane and release their cargo and 3) Recipient cells can phagocytose and internalize TEXs. The interaction between TEXs and T cells remains a subject of debate. Muller et al [348] have suggested that T cell subsets can internalize a small number of PKH26-labeled TEXs after co-incubation for 48–72 hours, indicating that receptor-ligand interactions alone can affect T cell functions. This suggests that internalization of TEXs may not be necessary for signal delivery leading to changes in gene expression. Conversely, Vignard and colleagues [349] have found that T cell functions can still be impaired by internalizing TEXs, even though T cells may face greater difficulty in internalizing TEXs compared to other immune cells. Studies have shown that resting or activated CD8+ T cells can internalize melanoma-derived Exosomes within as early as 5 hours of exposure, as observed through electron microscopy or confocal microscopy, consistent with previous research [350]. Moreover, the nucleic acids contained in TEXs, especially mRNA and miRNAs, also contribute to functional changes in T cells, suggesting that TEXs can reprogram recipient T cells through

internalization [351]. For example, TEXs have been shown to down-regulate inhibitory genes in CD4+ T cells, resulting in the loss of CD69 expression on T cell surfaces. Additionally, when transfected into normal T cells, RNA extracted from TEXs can alter T cell function. In summary, TEXs can influence T cell functions through both receptor-ligand binding and internalization pathways [352].

T cells that are dysfunctional due to cancer may differ from some of those found in chronic sites of infection over time. However, distinct forms of dysfunctional T lymphocytes can still be seen in various cancers and even within tumors. Immune checkpoint inhibition and accompanying com- bination therapy have been utilized to restore anti-cancer immunity and reverse dysfunctional T cells. Several concerns, such as how to sustain lengthy effectiveness and how to choose the most effective adjuvant therapy, remain unanswered. Furthermore, understanding the processes that produce and maintain these diverse defective T cells phenotypes is crucial. Continued technology improvements will help attain this goal, supporting the development of personalized tactics for targeting dysfunctional T cells in cancer patients for precision treatment.

Chapter 6

Conclusion and Recommendation

6.1 Conclusion

Among several other factors, over secreted Exosomes are the key player of the cancer microenvironment to facilitate tumor by spreading the aberrant/cancerous biomolecules to distant or nearby surroundings. This was shown in the current study that by providing cancer microenvironment conditions (MGC) to the cancer cell line, up regulated the Exosomes production and secretion. These cancers derived Exosomes play a prominent role in effecting the normal cellular proliferation especially the immune cells, which was revealed when Jurkat cells, human isolated fresh blood T cells and mouse T cells were treated with cancer derived Exosomes and its population was found decreased. This is also revealed that the cancer Exosomes effect the proliferation directly or indirectly by effecting the T cells surface or internal markers, thus leading to T cells death or dysfunction. Which are the active agents in triggering the cancer cells. The internal loaded cargo in the form of miRNAs and proteins are the factors that makes the cancer Exosomes aggressive and competent. These are taken from the originating cancer cells and are delivered to different sites of the body leading to enhance the metastasis. The deregulated miRNAs and proteins in cancer Exosomes target the recipient cell by modifying their normal physiology by disturbing their targeted genes important

in different cellular activities and survival. The tumor cells up regulate the Exosomes secretion, resulting in a flood of oncosomes in the blood, which not only aid tumor metastasis but also modulate the immune system. Immune suppression is a well-known cancer strategy, but how tumor cells target and effect immune system, in particular, is not yet known [353]. The tumor produced Exosomes have a direct effect on T cells proliferation and markers expression, suggesting that they may play a role in T cells dysfunction and death. These findings need to be confirmed in vitro and in vivo to fully understand the molecular mechanisms underlying these effects. While examining tumor Exosomes and their cargo (RNA, Protein, etc.), the same physiological conditions as those exists in the tumor microenvironment should be used. Because these factors affect not only the Exosomes production but also the expression of numerous biomolecules in the originating cells that are loaded in the Exosomes. To put it another way, cancer cells of the tumors inside the body may have different Exosomes and biomolecule expression than tumor cells Exosomes generated in 2-D culture in the lab. As a result, the scientific community may be misled or misguided by this disparity.

6.2 Future Directions

Exosomes produced from tumors are important for guiding NSCLC targeted therapy, and Exosomes themselves can be a therapeutic target.

1. Such studies will help identify the pathogenic role of cancer Exosomes in immune cell dysfunction and suppression as well as point out potential therapeutic avenues. For biological applications such targeted drug delivery, gene therapy, cancer diagnosis and treatment, vaccine development, and tissue regeneration, Exosomes have favorable features that make them a viable manipulation platform. Natural Exosomes have a variety of uses; however, they do have substantial limitations when used in clinical settings.

2. Recent efforts to alter Exosomes and produce designer Exosomes have been sparked by these limitations. In order to get beyond Exosomes' inherent limitations on drug delivery to wounds, neurons, and the cardiovascular system for damage healing, designer Exosomes are being produced. These biological components are intriguing. According to newly announced promising research and advancements, Exosomes may be able to replace the majority of current T cell therapy. Following the release of an article by Lai et al. [354], the Exosomes produced by MSCs, which attracted considerable attention, prompted thousands of other Exosomes investigations in the field of regenerative medicine.

3. Exosomes manufacturing and designer Exosomes have advanced to a new stage. Researchers' interest in Exosomes engineering led to the earlier stage of the process. In recent years, biological therapies have been actively pursued, and various businesses have been established to produce modified Exosomes medicines. According to multiple prior studies, some of the negative traits of natural Exosomes seem to prevent their expansion to medicinal uses. Exosomes production and purification on a large scale, as well as their heterogeneity, are difficulties that must be addressed in this context.

4. Thanks to the development of large-scale Exosomes production technologies and engineered cell lines for the synthesis of enhanced tailored Exosomes, the creation of therapeutic and diagnostic platforms associated with EVs is predicted to be viable in the near future.Designer Exosomes have shown to have a great potential, whereas Exosomes engineering methods for improving the efficacy of natural Exosomes are rapidly emerging. Because undifferentiated cytotoxicity affects both tumor and healthy cells, chemotherapy drugs, which are frequently used to treat cancer, are associated with substantial side effects. Designer Exosomes that have been specifically targeted, have distinctive targeting moieties on their surface, and are loaded with anti-cancer drugs in their lumen provide a flexible platform that can address several difficulties at once in this situation.

5. Designer Exosomes have shown to have a great potential, whereas Exosomes engineering methods for improving the efficacy of natural Exosomes are rapidly emerging. Because undifferentiated cytotoxicity affects both tumor and healthy cells, chemotherapy drugs, which are frequently used to treat cancer, are associated with substantial side effects. Designer Exosomes that have been specifically targeted, have distinctive targeting moieties on their surface, and are loaded with anti-cancer drugs in their lumen provide a flexible platform that can address several difficulties at once in this situation.

6. Another intriguing use of modified Exosomes is in the creation of vaccines. Studies on the generation of vaccines show that host cells infected with parasites, viruses, or bacterial pathogens release Exosomes containing numerous antigens of the pathogens. Cocktail and multi-epitopic vaccines (MEVs) were found to be effective in preventing pathogens from evading the immune system because of antigenic escape, according to vaccine development experiments.

With these last few sentences, it is concluded that additional in-depth research is required to determine the specifics and processes of Exosomes produced by tumors that invade immune cells by concentrating on specific biological components and render them unable to fight tumor cells. These goals might be reached by reducing the Exosomes storm in cancer or by creating different methods to protect immune cells from cancer Exosomes attacks.

Bibliography

[1] J. Ferlay, M. Colombet, I. Soerjomataram, C. Mathers, D. M. Parkin, M. Piñeros, A. Znaor, and F. Bray, "Estimating the global cancer incidence and mortality in 2018: Globocan sources and methods," *International journal of cancer*, vol. 144, no. 8, pp. 1941–1953, 2019.

[2] A. Ali, M. F. Manzoor, N. Ahmad, R. M. Aadil, H. Qin, R. Siddique, S. Riaz, A. Ahmad, S. A. Korma, W. Khalid, *et al.*, "The burden of cancer, government strategic policies, and challenges in pakistan: A comprehensive review," *Frontiers in nutrition*, vol. 9, p. 940514, 2022.

[3] H. Lemjabbar-Alaoui, O. U. Hassan, Y.-W. Yang, and P. Buchanan, "Lung cancer: Biology and treatment options," *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, vol. 1856, no. 2, pp. 189–210, 2015.

[4] A. C. Society, "Cancer facts & figures 2022," 2022.

[5] Asbestos.com, "Lung cancer statistics," 2023. 2023.

[6] C. Zappa and S. A. Mousa, "Non-small cell lung cancer: current treatment and future advances," *Translational lung cancer research*, vol. 5, no. 3, p. 288, 2016.

[7] Z. Chen, C. M. Fillmore, P. S. Hammerman, C. F. Kim, and K.-K. Wong, "Non-small-cell lung cancers: a heterogeneous set of diseases," *Nature Reviews Cancer*, vol. 14, no. 8, pp. 535–546, 2014.

[8] J. Domagala-Kulawik and A. Raniszewska, "How to evaluate the immune status of lung cancer patients before immunotherapy," *Breathe*, vol. 13, no. 4, pp. 291–296, 2017.

[9] D. Zamarin, R. B. Holmgaard, S. K. Subudhi, J. S. Park, M. Mansour, P. Palese, T. Merghoub, J. D. Wolchok, and J. P. Allison, "Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy," *Science translational medicine*, vol. 6, no. 226, pp. 226ra32–226ra32, 2014.

[10] J. Domagala-Kulawik, "The role of the immune system in non-small cell lung carcinoma and potential for therapeutic intervention," *Translational lung cancer research*, vol. 4, no. 2, p. 177, 2015.

[11] S. Ilyas and J. C. Yang, "Landscape of tumor antigens in t cell immunotherapy," *The Journal of Immunology*, vol. 195, no. 11, pp. 5117–5122, 2015.

[12] Y. Lian, L. Meng, P. Ding, and M. Sang, "Epigenetic regulation of mage family in human cancer progression-dna methylation, histone modification, and non-coding rnas," *Clinical epigenetics*, vol. 10, no. 1, pp. 1–11, 2018.

[13] S. Kelderman and P. Kvistborg, "Tumor antigens in human cancer control," *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, vol. 1865, no. 1, pp. 83–89, 2016.

[14] T. K. Garg, S. M. Szmania, J. A. Khan, A. Hoering, P. A. Malbrough, A. Moreno-Bost, A. D. Greenway, J. D. Lingo, X. Li, S. Yaccoby, *et al.*, "Highly activated and expanded natural killer cells for multiple myeloma immunotherapy," *haematologica*, vol. 97, no. 9, p. 1348, 2012.

[15] Y. Lavin, A. Mortha, A. Rahman, and M. Merad, "Regulation of macrophage development and function in peripheral tissues," *Nature Reviews Immunology*, vol. 15, no. 12, pp. 731–744, 2015.

[16] T. Kitamura, B.-Z. Qian, and J. W. Pollard, "Immune cell promotion of metastasis," *Nature Reviews Immunology*, vol. 15, no. 2, pp. 73–86, 2015.

[17] H. Gonzalez, C. Hagerling, and Z. Werb, "Roles of the immune system in cancer: from tumor initiation to metastatic progression," *Genes & development*, vol. 32, no. 19-20, pp. 1267–1284, 2018.

[18] M. Tamminga, T. J. N. Hiltermann, E. Schuuring, W. Timens, R. S. Fehrmann, and H. J. Groen, "Immune microenvironment composition in non-small cell lung cancer and its association with survival," *Clinical & translational immunology*, vol. 9, no. 6, p. e1142, 2020.

[19] V. Finisguerra, G. Di Conza, M. Di Matteo, J. Serneels, S. Costa, A. Thompson, E. Wauters, S. Walmsley, H. Prenen, Z. Granot, *et al.*, "Met is required for the recruitment of anti-tumoural neutrophils," *Nature*, vol. 522, no. 7556, pp. 349–353, 2015.

[20] M. Donadon, K. Hudspeth, M. Cimino, L. Di Tommaso, M. Preti, P. Tentorio, M. Roncalli, D. Mavilio, and G. Torzilli, "Increased infiltration of natural killer and t cells in colorectal liver metastases improves patient overall survival," *Journal of Gastrointestinal Surgery*, vol. 21, no. 8, pp. 1226–1236, 2017.

[21] D. E. Speiser, P.-C. Ho, and G. Verdeil, "Regulatory circuits of t cell function in cancer," *Nature Reviews Immunology*, vol. 16, no. 10, pp. 599–611, 2016.

[22] N. S. De Silva and U. Klein, "Dynamics of b cells in germinal centres," *Nature reviews immunology*, vol. 15, no. 3, pp. 137–148, 2015.

[23] M. C. Svensson, C. F. Warfvinge, R. Fristedt, C. Hedner, D. Borg, J. Eberhard, P. Micke, B. Nodin, K. Leandersson, and K. Jirström, "The integrative clinical impact of tumor-infiltrating t lymphocytes and nk cells in relation to b lymphocyte and plasma cell density in esophageal and gastric adenocarcinoma," *Oncotarget*, vol. 8, no. 42, p. 72108, 2017.

[24] F. Petitprez, A. de Reyniès, E. Z. Keung, T. W.-W. Chen, C.-M. Sun, J. Calderaro, Y.-M. Jeng, L.-P. Hsiao, L. Lacroix, A. Bougoüin, *et al.*, "B cells are associated with survival and immunotherapy response in sarcoma," *Nature*, vol. 577, no. 7791, pp. 556–560, 2020.

[25] F. Galli, J. V. Aguilera, B. Palermo, S. N. Markovic, P. Nisticò, and A. Signore, "Relevance of immune cell and tumor microenvironment imaging in the new era of immunotherapy," *Journal of Experimental & Clinical Cancer Research*, vol. 39, no. 1, pp. 1–21, 2020.

[26] B. Stankovic, H. A. K. Bjørhovde, R. Skarshaug, H. Aamodt, A. Frafjord, E. Müller, C. Hammarström, K. Beraki, E. S. Bækkevold, P. R. Woldbæk, *et al.*, "Immune cell composition in human non-small cell lung cancer," *Frontiers in immunology*, vol. 9, p. 3101, 2019.

[27] F. Castro, A. P. Cardoso, R. M. Gonçalves, K. Serre, and M. J. Oliveira, "Interferon-gamma at the crossroads of tumor immune surveillance or evasion," *Frontiers in immunology*, vol. 9, p. 847, 2018.

[28] N. Karachaliou, M. Gonzalez-Cao, G. Crespo, A. Drozdowskyj, E. Aldeguer, A. Gimenez-Capitan, C. Teixido, M. A. Molina-Vila, S. Viteri, M. De Los Llanos Gil, *et al.*, "Interferon gamma, an important marker of response to immune checkpoint blockade in non-small cell lung cancer and melanoma patients," *Therapeutic advances in medical oncology*, vol. 10, p. 1758834017749748, 2018.

[29] D. A. Almiron Bonnin, M. C. Havrda, and M. A. Israel, "Glioma cell secretion: A driver of tumor progression and a potential therapeutic target-glioma secretion drives tumor progression," *Cancer research*, vol. 78, no. 21, pp. 6031–6039, 2018.

[30] B. Ghesquiere, N. Colaert, K. Helsens, L. Dejager, C. Vanhaute, K. Verleysen, K. Kas, E. Timmerman, M. Goethals, C. Libert, *et al.*, "In vitro and in vivo protein-bound tyrosine nitration characterized by diagonal chromatography," *Molecular & Cellular Proteomics*, vol. 8, no. 12, pp. 2642–2652, 2009.

[31] M. Kanada, M. H. Bachmann, and C. H. Contag, "Signaling by extracellular vesicles advances cancer hallmarks," *Trends in cancer*, vol. 2, no. 2, pp. 84–94, 2016.

[32] S. W. Tse, C. F. Tan, J. E. Park, J. Gnanasekaran, N. Gupta, J. K. Low, K. W. Yeoh, W. J. Chng, C. Y. Tay, N. E. McCarthy, *et al.*, "Microenvironmental hypoxia induces dynamic changes in lung cancer synthesis and secretion of extracellular vesicles," *Cancers*, vol. 12, no. 10, p. 2917, 2020.

[33] R. Bajaj, S. T. Kundu, C. L. Grzeskowiak, J. J. Fradette, K. L. Scott, C. J. Creighton, and D. L. Gibbons, "Impad1 and kdelr2 drive invasion and metastasis by enhancing golgi-mediated secretion," *Oncogene*, vol. 39, no. 37, pp. 5979–5994, 2020.

[34] C. Wang, S. Zhang, J. Liu, Y. Tian, B. Ma, S. Xu, Y. Fu, and Y. Luo, "Secreted pyruvate kinase m2 promotes lung cancer metastasis through activating the integrin beta1/fak signaling pathway," *Cell Reports*, vol. 30, no. 6, pp. 1780–1797, 2020.

[35] M. P. Zaborowski, L. Balaj, X. O. Breakefield, and C. P. Lai, "Extracellular vesicles: composition, biological relevance, and methods of study," *Bioscience*, vol. 65, no. 8, pp. 783–797, 2015.

[36] M. Yáñez-Mó, P. R.-M. Siljander, Z. Andreu, A. Bedina Zavec, F. E. Borràs, E. I. Buzas, K. Buzas, E. Casal, F. Cappello, J. Carvalho, *et al.*, "Biological properties of extracellular vesicles and their physiological functions," *Journal of extracellular vesicles*, vol. 4, no. 1, p. 27066, 2015.

[37] M. P. Bebelman, M. J. Smit, D. M. Pegtel, and S. R. Baglio, "Biogenesis and function of extracellular vesicles in cancer," *Pharmacology & therapeutics*, vol. 188, pp. 1–11, 2018.

[38] G. Palmisano, S. S. Jensen, M.-C. Le Bihan, J. Laine, J. N. McGuire, F. Pociot, and M. R. Larsen, "Characterization of membrane-shed microvesicles from cytokine-stimulated β-cells using proteomics strategies," *Molecular & cellular proteomics*, vol. 11, no. 8, pp. 230–243, 2012.

[39] H. Sonoda, N. Yokota-Ikeda, S. Oshikawa, Y. Kanno, K. Yoshinaga, K. Uchida, Y. Ueda, K. Kimiya, S. Uezono, A. Ueda, *et al.*, "Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury," *American Journal of Physiology-Renal Physiology*, vol. 297, no. 4, pp. F1006–F1016, 2009.

[40] L. M. Doyle and M. Z. Wang, "Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis," *Cells*, vol. 8, no. 7, p. 727, 2019.

[41] R. Kalluri *et al.*, "The biology and function of exosomes in cancer," *The Journal of clinical investigation*, vol. 126, no. 4, pp. 1208–1215, 2016.

[42] G. Raposo and W. Stoorvogel, "Extracellular vesicles: exosomes, microvesicles, and friends," *Journal of Cell Biology*, vol. 200, no. 4, pp. 373–383, 2013.

[43] S. J. Gould and G. Raposo, "As we wait: coping with an imperfect nomenclature for extracellular vesicles," *Journal of extracellular vesicles*, vol. 2, no. 1, p. 20389, 2013.

[44] B. Shen, N. Wu, J.-M. Yang, and S. J. Gould, "Protein targeting to exosomes/microvesicles by plasma membrane anchors," *Journal of Biological Chemistry*, vol. 286, no. 16, pp. 14383–14395, 2011.

[45] J. Kowal, G. Arras, M. Colombo, M. Jouve, J. P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach, and C. Théry, "Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes," *Proceedings of the National Academy of Sciences*, vol. 113, no. 8, pp. E968–E977, 2016.

[46] C. Kahlert, S. A. Melo, A. Protopopov, J. Tang, S. Seth, M. Koch, J. Zhang, J. Weitz, L. Chin, A. Futreal, *et al.*, "Identification of double-stranded genomic dna spanning all chromosomes with mutated kras and p53 dna in the serum exosomes of patients with pancreatic cancer," *Journal of Biological Chemistry*, vol. 289, no. 7, pp. 3869–3875, 2014.

[47] L. Balaj, R. Lessard, L. Dai, Y.-J. Cho, S. L. Pomeroy, X. O. Breakefield, and J. Skog, "Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences," *Nature communications*, vol. 2, no. 1, pp. 1–9, 2011.

[48] S. Mathivanan, C. J. Fahner, G. E. Reid, and R. J. Simpson, "Exocarta 2012: database of exosomal proteins, rna and lipids," *Nucleic acids research*, vol. 40, no. D1, pp. D1241–D1244, 2012.

[49] D.-K. Kim, B. Kang, O. Y. Kim, D.-s. Choi, J. Lee, S. R. Kim, G. Go, Y. J. Yoon, J. H. Kim, S. C. Jang, *et al.*, "Evpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles," *Journal of extracellular vesicles*, vol. 2, no. 1, p. 20384, 2013.

[50] A. V. Vlassov, S. Magdaleno, R. Setterquist, and R. Conrad, "Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials," *Biochimica et Biophysica Acta (BBA)-General Subjects*, vol. 1820, no. 7, pp. 940–948, 2012.

[51] X. Huang, T. Yuan, M. Tschannen, Z. Sun, H. Jacob, M. Du, M. Liang, R. L. Dittmar, Y. Liu, M. Liang, *et al.*, "Characterization of human plasma-derived exosomal rnas by deep sequencing," *BMC genomics*, vol. 14, no. 1, pp. 1–14, 2013.

[52] M. Mittelbrunn, C. Gutiérrez-Vázquez, C. Villarroya-Beltri, S. González, F. Sánchez-Cabo, M. Á. González, A. Bernad, and F. Sánchez-Madrid, "Unidirectional transfer of microrna-loaded exosomes from t cells to antigen-presenting cells," *Nature communications*, vol. 2, no. 1, pp. 1–10, 2011.

[53] A. Waldenström and G. Ronquist, "Role of exosomes in myocardial remodeling," *Circulation research*, vol. 114, no. 2, pp. 315–324, 2014.

[54] C. Hewson, D. Capraro, J. Burdach, N. Whitaker, and K. V. Morris, "Extracellular vesicle associated long non-coding rnas functionally enhance cell viability," *Non-coding RNA research*, vol. 1, no. 1, pp. 3–11, 2016.

[55] A. Conigliaro, V. Costa, A. Lo Dico, L. Saieva, S. Buccheri, F. Dieli, M. Manno, S. Raccosta, C. Mancone, M. Tripodi, *et al.*, "Cd90+ liver cancer cells modulate endothelial cell phenotype through the release of exosomes containing h19 lncrna," *Molecular cancer*, vol. 14, no. 1, pp. 1–11, 2015.

[56] T. Liu, X. Zhang, S. Gao, F. Jing, Y. Yang, L. Du, G. Zheng, P. Li, C. Li, and C. Wang, "Exosomal long noncoding rna crnde-h as a novel serum-based biomarker for diagnosis and prognosis of colorectal cancer," *Oncotarget*, vol. 7, no. 51, p. 85551, 2016.

[57] L. Qu, J. Ding, C. Chen, Z.-J. Wu, B. Liu, Y. Gao, W. Chen, F. Liu, W. Sun, X.-F. Li, *et al.*, "Exosome-transmitted lncarsr promotes sunitinib resistance in renal cancer by acting as a competing endogenous rna," *Cancer cell*, vol. 29, no. 5, pp. 653–668, 2016.

[58] J. Song, D. Kim, J. Han, Y. Kim, M. Lee, and E.-J. Jin, "Pbmc and exosome-derived hotair is a critical regulator and potent marker for rheumatoid arthritis," *Clinical and experimental medicine*, vol. 15, no. 1, pp. 121–126, 2015.

[59] U. Gezer, E. Özgür, M. Cetinkaya, M. Isin, and N. Dalay, "Long non-coding rnas with low expression levels in cells are enriched in secreted exosomes," *Cell biology international*, vol. 38, no. 9, pp. 1076–1079, 2014.

[60] Y. Li, Q. Zheng, C. Bao, S. Li, W. Guo, J. Zhao, D. Chen, J. Gu, X. He, and S. Huang, "Circular rna is enriched and stable in exosomes: a promising biomarker for cancer diagnosis," *Cell research*, vol. 25, no. 8, pp. 981–984, 2015.

[61] X. Dai, C. Chen, Q. Yang, J. Xue, X. Chen, B. Sun, F. Luo, X. Liu, T. Xiao, H. Xu, *et al.*, "Exosomal circrna_100284 from arsenite-transformed cells, via microrna-217 regulation of ezh2, is involved in the malignant transformation of human hepatic cells by accelerating the cell cycle and promoting cell proliferation," *Cell death & disease*, vol. 9, no. 5, pp. 1–14, 2018.

[62] N.-S. Tan, N. S. Shaw, N. Vinckenbosch, P. Liu, R. Yasmin, B. Desvergne, W. Wahli, and N. Noy, "Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription," *Molecular and cellular biology*, vol. 22, no. 14, pp. 5114–5127, 2002.

[63] P. de Medina, M. R. Paillasse, G. Ségala, F. Khallouki, S. Brillouet, F. Dalenc, F. Courbon, M. Record, M. Poirot, and S. Silvente-Poirot, "Importance of cholesterol and oxysterols metabolism in the pharmacology of tamoxifen and other aebs ligands," *Chemistry and physics of lipids*, vol. 164, no. 6, pp. 432–437, 2011.

[64] L. Zakharova, M. Svetlova, and A. F. Fomina, "T cell exosomes induce cholesterol accumulation in human monocytes via phosphatidylserine receptor," *Journal of cellular physiology*, vol. 212, no. 1, pp. 174–181, 2007.

[65] B.-T. Pan, K. Teng, C. Wu, M. Adam, and R. M. Johnstone, "Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes.," *The Journal of cell biology*, vol. 101, no. 3, pp. 942–948, 1985.

[66] H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee, and J. O. Lötvall, "Exosome-mediated transfer of mrnas and micrornas is a novel mechanism of genetic exchange between cells," *Nature cell biology*, vol. 9, no. 6, pp. 654–659, 2007.

[67] D. D. Taylor and C. Gercel-Taylor, "Microrna signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer," *Gynecologic oncology*, vol. 110, no. 1, pp. 13–21, 2008.

[68] C. Falker, A. Hartmann, I. Guett, F. Dohler, H. Altmeppen, C. Betzel, R. Schubert, D. Thurm, F. Wegwitz, P. Joshi, *et al.*, "Exosomal cellular prion protein drives fibrillization of amyloid beta and counteracts amyloid beta-mediated neurotoxicity," *Journal of neurochemistry*, vol. 137, no. 1, pp. 88–100, 2016.

[69] A. L Isola and S. Chen, "Exosomes: the messengers of health and disease," *Current neuropharmacology*, vol. 15, no. 1, pp. 157–165, 2017.

[70] X. Wu, T. Zheng, and B. Zhang, "Exosomes in parkinson?s disease," *Neuroscience bulletin*, vol. 33, no. 3, pp. 331–338, 2017.

[71] A. Hartmann, C. Muth, O. Dabrowski, S. Krasemann, and M. Glatzel, "Exosomes and the prion protein: more than one truth," *Frontiers in neuroscience*, vol. 11, p. 194, 2017.

[72] W. Liu, X. Bai, A. Zhang, J. Huang, S. Xu, and J. Zhang, "Role of exosomes in central nervous system diseases," *Frontiers in molecular neuroscience*, vol. 12, p. 240, 2019.

[73] J. Shen, C.-K. Huang, H. Yu, B. Shen, Y. Zhang, Y. Liang, Z. Li, X. Feng, J. Zhao, L. Duan, *et al.*, "The role of exosomes in hepatitis, liver cirrhosis and hepatocellular carcinoma," *Journal of cellular and molecular medicine*, vol. 21, no. 5, pp. 986–992, 2017.

[74] G. Bellin, C. Gardin, L. Ferroni, J. C. Chachques, M. Rogante, D. Mitrečić, R. Ferrari, and B. Zavan, "Exosome in cardiovascular diseases: a complex world full of hope," *Cells*, vol. 8, no. 2, p. 166, 2019.

[75] C. Lee, S. A. Mitsialis, M. Aslam, S. H. Vitali, E. Vergadi, G. Konstantinou, K. Sdrimas, A. Fernandez-Gonzalez, and S. Kourembanas, "Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension," *Circulation*, vol. 126, no. 22, pp. 2601–2611, 2012.

[76] L. Zhong, D. Liao, J. Li, W. Liu, J. Wang, C. Zeng, X. Wang, Z. Cao, R. Zhang, M. Li, *et al.*, "Rab22a-neof1 fusion protein promotes osteosarcoma lung metastasis through its secretion into exosomes," *Signal transduction and targeted therapy*, vol. 6, no. 1, pp. 1–16, 2021.

[77] T. B. Steinbichler, J. Dudás, S. Skvortsov, U. Ganswindt, H. Riechelmann, and I.-I. Skvortsova, "Therapy resistance mediated by exosomes," *Molecular cancer*, vol. 18, no. 1, pp. 1–11, 2019.

[78] M. A. Rahman, J. F. Barger, F. Lovat, M. Gao, G. A. Otterson, and P. Nana-Sinkam, "Lung cancer exosomes as drivers of epithelial mesenchymal transition," *Oncotarget*, vol. 7, no. 34, p. 54852, 2016.

[79] R. Kalluri and V. S. LeBleu, "The biology, function, and biomedical applications of exosomes," *Science*, vol. 367, no. 6478, p. eaau6977, 2020.

[80] D. W. Greening, S. K. Gopal, R. Xu, R. J. Simpson, and W. Chen, "Exosomes and their roles in immune regulation and cancer," in *Seminars in cell & developmental biology*, vol. 40, pp. 72–81, Elsevier, 2015.

[81] J. Skog, T. Würdinger, S. Van Rijn, D. H. Meijer, L. Gainche, W. T. Curry, B. S. Carter, A. M. Krichevsky, and X. O. Breakefield, "Glioblastoma microvesicles transport rna and proteins that promote tumour growth and provide diagnostic biomarkers," *Nature cell biology*, vol. 10, no. 12, pp. 1470–1476, 2008.

[82] J.-L. Qu, X.-J. Qu, M.-F. Zhao, Y.-E. Teng, Y. Zhang, K.-Z. Hou, Y.-H. Jiang, X.-H. Yang, and Y.-P. Liu, "Gastric cancer exosomes promote tumour cell proliferation through pi3k/akt and mapk/erk activation," *Digestive and liver disease*, vol. 41, no. 12, pp. 875–880, 2009.

[83] E. Ristorcelli, E. Beraud, S. Mathieu, D. Lombardo, and A. Verine, "Essential role of notch signaling in apoptosis of human pancreatic tumoral cells mediated by exosomal nanoparticles," *International journal of cancer*, vol. 125, no. 5, pp. 1016–1026, 2009.

[84] P. Friedl and S. Alexander, "Cancer invasion and the microenvironment: plasticity and reciprocity," *Cell*, vol. 147, no. 5, pp. 992–1009, 2011.

[85] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer," *cell*, vol. 100, no. 1, pp. 57–70, 2000.

[86] D. F. Quail and J. A. Joyce, "Microenvironmental regulation of tumor progression and metastasis," *Nature medicine*, vol. 19, no. 11, pp. 1423–1437, 2013.

[87] A. Becker, B. K. Thakur, J. M. Weiss, H. S. Kim, H. Peinado, and D. Lyden, "Extracellular vesicles in cancer: cell-to-cell mediators of metastasis," *Cancer cell*, vol. 30, no. 6, pp. 836–848, 2016.

[88] H. Peinado, H. Zhang, I. R. Matei, B. Costa-Silva, A. Hoshino, G. Rodrigues, B. Psaila, R. N. Kaplan, J. F. Bromberg, Y. Kang, *et al.*, "Pre-metastatic niches: organ-specific homes for metastases," *Nature Reviews Cancer*, vol. 17, no. 5, pp. 302–317, 2017.

[89] H. Peinado, M. Alečković, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M. Hergueta-Redondo, C. Williams, G. García-Santos,

C. M. Ghajar, *et al.*, "Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through met," *Nature medicine*, vol. 18, no. 6, pp. 883–891, 2012.

[90] L. K. Ferrarelli, "Exosomes prep the metastatic site," *Science Signaling*, vol. 8, no. 380, pp. ec150–ec150, 2015.

[91] L. Zhang, S. Zhang, J. Yao, F. J. Lowery, Q. Zhang, W.-C. Huang, P. Li, M. Li, X. Wang, C. Zhang, *et al.*, "Microenvironment-induced pten loss by exosomal microrna primes brain metastasis outgrowth," *Nature*, vol. 527, no. 7576, pp. 100–104, 2015.

[92] A. Hoshino, B. Costa-Silva, T.-L. Shen, G. Rodrigues, A. Hashimoto, M. Tesic Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, *et al.*, "Tumour exosome integrins determine organotropic metastasis," *Nature*, vol. 527, no. 7578, pp. 329–335, 2015.

[93] I. Keklikoglou, C. Cianciaruso, E. Güç, M. L. Squadrito, L. M. Spring, S. Tazzyman, L. Lambein, A. Poissonnier, G. B. Ferraro, C. Baer, *et al.*, "Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models," *Nature cell biology*, vol. 21, no. 2, pp. 190–202, 2019.

[94] A. Ribas and J. D. Wolchok, "Cancer immunotherapy using checkpoint blockade," *Science*, vol. 359, no. 6382, pp. 1350–1355, 2018.

[95] J. Street, E. Koritzinsky, D. Glispie, R. Star, and P. Yuen, "Urine exosomes: an emerging trove of biomarkers," *Advances in Clinical Chemistry*, vol. 78, pp. 103–122, 2017.

[96] Y. Yagi, T. Ohkubo, H. Kawaji, A. Machida, H. Miyata, S. Goda, S. Roy, Y. Hayashizaki, H. Suzuki, and T. Yokota, "Next-generation sequencing-based small rna profiling of cerebrospinal fluid exosomes," *Neuroscience letters*, vol. 636, pp. 48–57, 2017.

[97] T. Machida, T. Tomofuji, D. Ekuni, T. Maruyama, T. Yoneda, Y. Kawabata, H. Mizuno, H. Miyai, M. Kunitomo, and M. Morita, "Micrornas in

salivary exosome as potential biomarkers of aging," *International journal of molecular sciences*, vol. 16, no. 9, pp. 21294–21309, 2015.

[98] W. Huang, Y. Yan, Y. Liu, M. Lin, J. Ma, W. Zhang, J. Dai, J. Li, Q. Guo, H. Chen, *et al.*, "Exosomes with low mir-34c-3p expression promote invasion and migration of non-small cell lung cancer by upregulating integrin $\alpha 2\beta 1$," *Signal transduction and targeted therapy*, vol. 5, no. 1, pp. 1–13, 2020.

[99] B. Sandfeld-Paulsen, N. Aggerholm-Pedersen, R. Baek, K. Jakobsen, P. Meldgaard, B. Folkersen, T. Rasmussen, K. Varming, M. Jørgensen, and B. Sorensen, "Exosomal proteins as prognostic biomarkers in non-small cell lung cancer," *Molecular oncology*, vol. 10, no. 10, pp. 1595–1602, 2016.

[100] L. Niu, X. Song, N. Wang, L. Xue, X. Song, and L. Xie, "Tumor-derived exosomal proteins as diagnostic biomarkers in non-small cell lung cancer," *Cancer science*, vol. 110, no. 1, pp. 433–442, 2019.

[101] K. B. Johnsen, J. M. Gudbergsson, M. N. Skov, L. Pilgaard, T. Moos, and M. Duroux, "A comprehensive overview of exosomes as drug delivery vehicles?endogenous nanocarriers for targeted cancer therapy," *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, vol. 1846, no. 1, pp. 75–87, 2014.

[102] A. Von Schulze and F. Deng, "A review on exosome-based cancer therapy," *Journal of Cancer Metastasis and Treatment*, vol. 6, p. 42, 2020.

[103] S.-i. Ohno, M. Takanashi, K. Sudo, S. Ueda, A. Ishikawa, N. Matsuyama, K. Fujita, T. Mizutani, T. Ohgi, T. Ochiya, *et al.*, "Systemically injected exosomes targeted to egfr deliver antitumor microrna to breast cancer cells," *Molecular Therapy*, vol. 21, no. 1, pp. 185–191, 2013.

[104] Z. Xunian and R. Kalluri, "Biology and therapeutic potential of mesenchymal stem cell-derived exosomes," *Cancer Science*, vol. 111, no. 9, pp. 3100–3110, 2020.

[105] C. Liu, N. Li, and G. Liu, "The role of micrornas in regulatory t cells," *Journal of Immunology Research*, vol. 2020, 2020.

[106] A. M. Al-Sadi, F. A. Al-Oweisi, S. G. Edwards, H. Al-Nadabi, and A. M. Al-Fahdi, "Genetic analysis reveals diversity and genetic relationship among trichoderma isolates from potting media, cultivated soil and uncultivated soil," *BMC microbiology*, vol. 15, no. 1, pp. 1–11, 2015.

[107] R. H. Blackwell, K. E. Foreman, and G. N. Gupta, "The role of cancer-derived exosomes in tumorigenicity & epithelial-to-mesenchymal transition," *Cancers*, vol. 9, no. 8, p. 105, 2017.

[108] X. Zhang, X. Yuan, H. Shi, L. Wu, H. Qian, and W. Xu, "Exosomes in cancer: small particle, big player," *Journal of hematology & oncology*, vol. 8, no. 1, pp. 1–13, 2015.

[109] J. D. Rabinowitz and H. A. Coller, "Cancer metabolism: Partners in the warburg effect," *Elife*, vol. 5, p. e15938, 2016.

[110] Y. Hsu, J. Hung, W. Chang, Y. Lin, Y. Pan, P. Tsai, C. Wu, and P. Kuo, "Hypoxic lung cancer-secreted exosomal mir-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein zo-1," *Oncogene*, vol. 36, no. 34, pp. 4929–4942, 2017.

[111] R. J. Dilley and W. A. Morrison, "Vascularisation to improve translational potential of tissue engineering systems for cardiac repair," *The international journal of biochemistry & cell biology*, vol. 56, pp. 38–46, 2014.

[112] T. F. Gajewski, H. Schreiber, and Y.-X. Fu, "Innate and adaptive immune cells in the tumor microenvironment," *Nature immunology*, vol. 14, no. 10, pp. 1014–1022, 2013.

[113] W. H. Fridman, C. Sautès-Fridman, J. Galon, *et al.*, "The immune contexture in human tumours: impact on clinical outcome," *Nature Reviews Cancer*, vol. 12, no. 4, pp. 298–306, 2012.

[114] A. Bobrie, M. Colombo, G. Raposo, and C. Théry, "Exosome secretion: molecular mechanisms and roles in immune responses," *Traffic*, vol. 12, no. 12, pp. 1659–1668, 2011.

[115] C. Théry, M. Ostrowski, and E. Segura, "Membrane vesicles as conveyors of immune responses," *Nature reviews immunology*, vol. 9, no. 8, pp. 581–593, 2009.

[116] T. Moroishi, T. Hayashi, W.-W. Pan, Y. Fujita, M. V. Holt, J. Qin, D. A. Carson, and K.-L. Guan, "The hippo pathway kinases lats1/2 suppress cancer immunity," *Cell*, vol. 167, no. 6, pp. 1525–1539, 2016.

[117] S. Yu, C. Liu, K. Su, J. Wang, Y. Liu, L. Zhang, C. Li, Y. Cong, R. Kimberly, W. E. Grizzle, *et al.*, "Tumor exosomes inhibit differentiation of bone marrow dendritic cells," *The Journal of Immunology*, vol. 178, no. 11, pp. 6867–6875, 2007.

[118] Y. Liu, X. Xiang, X. Zhuang, S. Zhang, C. Liu, Z. Cheng, S. Michalek, W. Grizzle, and H.-G. Zhang, "Contribution of myd88 to the tumor exosome-mediated induction of myeloid derived suppressor cells," *The American journal of pathology*, vol. 176, no. 5, pp. 2490–2499, 2010.

[119] B. Y. Nabet, Y. Qiu, J. E. Shabason, T. J. Wu, T. Yoon, B. C. Kim, J. L. Benci, A. M. DeMichele, J. Tchou, J. Marcotrigiano, *et al.*, "Exosome rna unshielding couples stromal activation to pattern recognition receptor signaling in cancer," *Cell*, vol. 170, no. 2, pp. 352–366, 2017.

[120] M. Fabbri, A. Paone, F. Calore, R. Galli, E. Gaudio, R. Santhanam, F. Lovat, P. Fadda, C. Mao, G. J. Nuovo, *et al.*, "Micrornas bind to toll-like receptors to induce prometastatic inflammatory response," *Proceedings of the National Academy of Sciences*, vol. 109, no. 31, pp. E2110–E2116, 2012.

[121] Y. Liu, Y. Gu, Y. Han, Q. Zhang, Z. Jiang, X. Zhang, B. Huang, X. Xu, J. Zheng, and X. Cao, "Tumor exosomal rnas promote lung pre-metastatic niche formation by activating alveolar epithelial tlr3 to recruit neutrophils," *Cancer cell*, vol. 30, no. 2, pp. 243–256, 2016.

[122] Y. Zhou, C. Wu, G. Lu, Z. Hu, Q. Chen, and X. Du, "Fgf/fgfr signaling pathway involved resistance in various cancer types," *Journal of Cancer*, vol. 11, no. 8, p. 2000, 2020.

[123] M. Musella, G. Manic, R. De Maria, I. Vitale, and A. Sistigu, "Type-i-interferons in infection and cancer: Unanticipated dynamics with therapeutic implications," *Oncoimmunology*, vol. 6, no. 5, p. e1314424, 2017.

[124] S. R. Baglio, M. A. van Eijndhoven, D. Koppers-Lalic, J. Berenguer, S. M. Lougheed, S. Gibbs, N. Léveillé, R. N. Rinkel, E. S. Hopmans, S. Swaminathan, *et al.*, "Sensing of latent ebv infection through exosomal transfer of 5? ppprna," *Proceedings of the National Academy of Sciences*, vol. 113, no. 5, pp. E587–E596, 2016.

[125] A. Anel, A. Gallego-Lleyda, D. de Miguel, J. Naval, and L. Martínez-Lostao, "Role of exosomes in the regulation of t-cell mediated immune responses and in autoimmune disease," *Cells*, vol. 8, no. 2, p. 154, 2019.

[126] L. X. Liu, N. P. Lee, V. W. Chan, W. Xue, L. Zender, C. Zhang, M. Mao, H. Dai, X. L. Wang, M. Z. Xu, *et al.*, "Targeting cadherin-17 inactivates wnt signaling and inhibits tumor growth in liver carcinoma," *Hepatology*, vol. 50, no. 5, pp. 1453–1463, 2009.

[127] D. Hang, J. Zhou, N. Qin, W. Zhou, H. Ma, G. Jin, Z. Hu, J. Dai, and H. Shen, "A novel plasma circular rna circ farsa is a potential biomarker for non-small cell lung cancer," *Cancer medicine*, vol. 7, no. 6, pp. 2783–2791, 2018.

[128] C. Roth, S. Kasimir-Bauer, K. Pantel, and H. Schwarzenbach, "Screening for circulating nucleic acids and caspase activity in the peripheral blood as potential diagnostic tools in lung cancer," *Molecular oncology*, vol. 5, no. 3, pp. 281–291, 2011.

[129] C. Rolfo, "Exosomal proteins in lung cancer: the last frontier in liquid biopsies," *Journal of Thoracic Oncology*, vol. 11, no. 10, pp. 1609–1611, 2016.

[130] J. Wan, X. Ling, B. Peng, and G. Ding, "mir-142-5p regulates cd4+ t cells in human non-small cell lung cancer through pd-l1 expression via the pten pathway," *Oncology reports*, vol. 40, no. 1, pp. 272–282, 2018.

[131] H. Wang, R. Peng, J. Wang, Z. Qin, and L. Xue, "Circulating microrna as potential cancer biomarkers: the advantage and disadvantage," *Clinical epigenetics*, vol. 10, no. 1, pp. 1–10, 2018.

[132] N. Nizyaeva, G. Kulikova, A. Shchyogolev, and V. Zemskov, "The role of microrna in regulation of the body?s immune responses," *Biology Bulletin Reviews*, vol. 6, no. 6, pp. 473–482, 2016.

[133] A. Mehta and D. Baltimore, "Microrna as regulatory elements in immune system logic," *Nature Reviews Immunology*, vol. 16, no. 5, pp. 279–294, 2016.

[134] S. Heublein, D. Mayr, A. Meindl, A. Kircher, U. Jeschke, and N. Ditsch, "Vitamin d receptor, retinoid x receptor and peroxisome proliferator-activated receptor γ are overexpressed in brca1 mutated breast cancer and predict prognosis," *Journal of Experimental & Clinical Cancer Research*, vol. 36, no. 1, pp. 1–11, 2017.

[135] U. Testa, E. Pelosi, G. Castelli, and C. Labbaye, "mir-146 and mir-155: two key modulators of immune response and tumor development," *Non-coding RNA*, vol. 3, no. 3, p. 22, 2017.

[136] S. Hirschberger, L. C. Hinske, and S. Kreth, "Mirnas: dynamic regulators of immune cell functions in inflammation and cancer," *Cancer letters*, vol. 431, pp. 11–21, 2018.

[137] J. C. Dudda, B. Salaun, Y. Ji, D. C. Palmer, G. C. Monnot, E. Merck, C. Boudousquie, D. T. Utzschneider, T. M. Escobar, R. Perret, *et al.*, "Microrna-155 is required for effector cd8+ t cell responses to virus infection and cancer," *Immunity*, vol. 38, no. 4, pp. 742–753, 2013.

[138] J. Davidson-Moncada, F. N. Papavasiliou, and W. Tam, "Micrornas of the immune system: roles in inflammation and cancer," *Annals of the New York Academy of Sciences*, vol. 1183, no. 1, pp. 183–194, 2010.

[139] Z. Qin, P.-Y. Wang, D.-F. Su, and X. Liu, "mirna-124 in immune system and immune disorders," *Frontiers in immunology*, vol. 7, p. 406, 2016.

[140] S. D. Alipoor, E. Mortaz, J. Garssen, M. Movassaghi, M. Mirsaeidi, and I. M. Adcock, "Exosomes and exosomal mirna in respiratory diseases," *Mediators of inflammation*, vol. 2016, 2016.

[141] K. Yakimchuk, "Exosomes: isolation and characterization methods and specific markers," *Mater Methods*, vol. 5, p. 1450, 2015.

[142] N. P. Hessvik and A. Llorente, "Current knowledge on exosome biogenesis and release," *Cellular and Molecular Life Sciences*, vol. 75, no. 2, pp. 193–208, 2018.

[143] T. Janas, M. M. Janas, K. Sapoń, and T. Janas, "Mechanisms of rna loading into exosomes," *FEBS letters*, vol. 589, no. 13, pp. 1391–1398, 2015.

[144] B. J. Crenshaw, B. Sims, and Q. L. Matthews, "Biological function of exosomes as diagnostic markers and therapeutic delivery vehicles in carcinogenesis and infectious diseases," in *Nanomedicines*, pp. 1–33, IntechOpen London, UK, 2018.

[145] R. Sullivan, G. Maresh, X. Zhang, C. Salomon, J. Hooper, D. Margolin, and L. Li, "The emerging roles of extracellular vesicles as communication vehicles within the tumor microenvironment and beyond," *Frontiers in Endocrinology*, vol. 8, p. 194, 2017.

[146] C. Théry, "Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles," *Annu. Rev. Cell Dev. Biol*, vol. 30, pp. 255–89, 2014.

[147] S. C. Abreu, D. J. Weiss, and P. R. Rocco, "Extracellular vesicles derived from mesenchymal stromal cells: a therapeutic option in respiratory diseases?," *Stem cell research & therapy*, vol. 7, no. 1, pp. 1–10, 2016.

[148] M. Colombo, C. Moita, G. Van Niel, J. Kowal, J. Vigneron, P. Benaroch, N. Manel, L. F. Moita, C. Théry, and G. Raposo, "Analysis of escrt functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles," *Journal of cell science*, vol. 126, no. 24, pp. 5553–5565, 2013.

[149] W. Stoorvogel, "Resolving sorting mechanisms into exosomes," *Cell research*, vol. 25, no. 5, pp. 531–532, 2015.

[150] B. N. Hannafon and W.-Q. Ding, "Intercellular communication by exosome-derived micrornas in cancer," *International journal of molecular sciences*, vol. 14, no. 7, pp. 14240–14269, 2013.

[151] C. Subra, K. Laulagnier, B. Perret, and M. Record, "Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies," *Biochimie*, vol. 89, no. 2, pp. 205–212, 2007.

[152] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Bru?gger, and M. Simons, "Ceramide triggers budding of exosome vesicles into multivesicular endosomes," *Science*, vol. 319, no. 5867, pp. 1244–1247, 2008.

[153] S. Gettinger, L. Horn, D. Jackman, D. Spigel, S. Antonia, M. Hellmann, J. Powderly, R. Heist, L. V. Sequist, D. C. Smith, *et al.*, "Five-year follow-up of nivolumab in previously treated advanced non–small-cell lung cancer: results from the ca209-003 study," *Journal of Clinical Oncology*, vol. 36, no. 17, pp. 1675–1684, 2018.

[154] N. B. Leighl, N. Rekhtman, W. A. Biermann, J. Huang, M. Mino-Kenudson, S. S. Ramalingam, H. West, S. Whitlock, and M. R. Somerfield, "Molecular testing for selection of patients with lung cancer for epidermal growth factor receptor and anaplastic lymphoma kinase tyrosine kinase inhibitors: American society of clinical oncology endorsement of the college of american pathologists/international association for the study of lung cancer/association for molecular pathology guideline," *Journal of Clinical Oncology*, vol. 32, no. 32, p. 3673, 2014.

[155] E. Izumchenko, X. Chang, M. Brait, E. Fertig, L. T. Kagohara, A. Bedi, L. Marchionni, N. Agrawal, R. Ravi, S. Jones, *et al.*, "Targeted sequencing reveals clonal genetic changes in the progression of early lung neoplasms and paired circulating dna," *Nature communications*, vol. 6, no. 1, pp. 1–13, 2015.

[156] M. Macías, E. Alegre, A. Díaz-Lagares, A. Patiño, J. L. Pérez-Gracia, M. Sanmamed, R. López-López, N. Varo, and A. González, "Liquid biopsy: from basic research to clinical practice," *Advances in clinical chemistry*, vol. 83, pp. 73–119, 2018.

[157] P. Garrido, E. Conde, J. De Castro, J. J. Gómez-Román, E. Felip, L. Pijuan, D. Isla, J. Sanz, L. Paz-Ares, and F. López-Ríos, "Updated guidelines for predictive biomarker testing in advanced non-small-cell lung cancer: a national consensus of the spanish society of pathology and the spanish society of medical oncology," *Clinical and Translational Oncology*, vol. 22, no. 7, pp. 989–1003, 2020.

[158] R. B. Corcoran and B. A. Chabner, "Application of cell-free dna analysis to cancer treatment," *New England Journal of Medicine*, vol. 379, no. 18, pp. 1754–1765, 2018.

[159] R. Chen, X. Xu, Z. Qian, C. Zhang, Y. Niu, Z. Wang, J. Sun, X. Zhang, and Y. Yu, "The biological functions and clinical applications of exosomes in lung cancer," *Cellular and Molecular Life Sciences*, vol. 76, no. 23, pp. 4613–4633, 2019.

[160] W. Yu, J. Hurley, D. Roberts, S. Chakrabortty, D. Enderle, M. Noerholm, X. Breakefield, and J. Skog, "Exosome-based liquid biopsies in cancer: opportunities and challenges," *Annals of Oncology*, vol. 32, no. 4, pp. 466–477, 2021.

[161] Y. Wang, M. Guo, D. Lin, D. Liang, L. Zhao, R. Zhao, and Y. Wang, "Docetaxel-loaded exosomes for targeting non-small cell lung cancer: preparation and evaluation in vitro and in vivo," *Drug delivery*, vol. 28, no. 1, pp. 1510–1523, 2021.

[162] L. A. Mulcahy, R. C. Pink, and D. R. F. Carter, "Routes and mechanisms of extracellular vesicle uptake," *Journal of extracellular vesicles*, vol. 3, no. 1, p. 24641, 2014.

[163] W. Xu, Z. Yang, and N. Lu, "From pathogenesis to clinical application: insights into exosomes as transfer vectors in cancer," *Journal of Experimental & Clinical Cancer Research*, vol. 35, no. 1, pp. 1–12, 2016.

[164] R. Zhang, Y. Xia, Z. Wang, J. Zheng, Y. Chen, X. Li, Y. Wang, and H. Ming, "Serum long non coding rna malat-1 protected by exosomes is up-regulated and promotes cell proliferation and migration in non-small cell lung cancer," *Biochemical and biophysical research communications*, vol. 490, no. 2, pp. 406–414, 2017.

[165] T. L. Whiteside, "Tumor-derived exosomes and their role in cancer progression," *Advances in clinical chemistry*, vol. 74, pp. 103–141, 2016.

[166] R. S. Herbst, D. Morgensztern, and C. Boshoff, "The biology and management of non-small cell lung cancer," *Nature*, vol. 553, no. 7689, pp. 446–454, 2018.

[167] Y. Zhang, M. Li, and C. Hu, "Exosomal transfer of mir-214 mediates gefitinib resistance in non-small cell lung cancer," *Biochemical and biophysical research communications*, vol. 507, no. 1-4, pp. 457–464, 2018.

[168] S. Wu, M. Luo, K. K. To, J. Zhang, C. Su, H. Zhang, S. An, F. Wang, D. Chen, and L. Fu, "Intercellular transfer of exosomal wild type egfr triggers osimertinib resistance in non-small cell lung cancer," *Molecular cancer*, vol. 20, no. 1, pp. 1–17, 2021.

[169] T. Brabletz, R. Kalluri, M. A. Nieto, and R. A. Weinberg, "Emt in cancer," *Nature Reviews Cancer*, vol. 18, no. 2, pp. 128–134, 2018.

[170] S. He, Z. Li, Y. Yu, Q. Zeng, Y. Cheng, W. Ji, W. Xia, and S. Lu, "Exosomal mir-499a-5p promotes cell proliferation, migration and emt via mtor signaling pathway in lung adenocarcinoma," *Experimental cell research*, vol. 379, no. 2, pp. 203–213, 2019.

[171] F. Yang, Y. Yan, Y. Yang, X. Hong, M. Wang, Z. Yang, B. Liu, and L. Ye, "Mir-210 in exosomes derived from cafs promotes non-small cell lung cancer

migration and invasion through pten/pi3k/akt pathway," *Cellular signalling*, vol. 73, p. 109675, 2020.

[172] J. Kim, T. Y. Kim, M. S. Lee, J. Y. Mun, C. Ihm, and S. A. Kim, "Exosome cargo reflects tgf-β1-mediated epithelial-to-mesenchymal transition (emt) status in a549 human lung adenocarcinoma cells," *Biochemical and biophysical research communications*, vol. 478, no. 2, pp. 643–648, 2016.

[173] L. Yin, X. Liu, X. Shao, T. Feng, J. Xu, Q. Wang, and S. Hua, "The role of exosomes in lung cancer metastasis and clinical applications: an updated review," *Journal of Translational Medicine*, vol. 19, no. 1, pp. 1–16, 2021.

[174] D.-X. Gan, Y.-B. Wang, M.-Y. He, Z.-Y. Chen, X.-X. Qin, Z.-W. Miao, Y.-H. Chen, and B. Li, "Lung cancer cells-controlled dkk-1 production in brain metastatic cascade drive microglia to acquire a pro-tumorigenic phenotype," *Frontiers in cell and developmental biology*, vol. 8, p. 591405, 2020.

[175] S. Taverna, M. Pucci, M. Giallombardo, M. A. Di Bella, M. Santarpia, P. Reclusa, I. Gil-Bazo, C. Rolfo, and R. Alessandro, "Amphiregulin contained in nsclc-exosomes induces osteoclast differentiation through the activation of egfr pathway," *Scientific reports*, vol. 7, no. 1, pp. 1–14, 2017.

[176] L. Mashouri, H. Yousefi, A. R. Aref, F. Molaei, S. K. Alahari, *et al.*, "Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance," *Molecular cancer*, vol. 18, no. 1, pp. 1–14, 2019.

[177] Y. Liu, F. Luo, B. Wang, H. Li, Y. Xu, X. Liu, L. Shi, X. Lu, W. Xu, L. Lu, *et al.*, "Stat3-regulated exosomal mir-21 promotes angiogenesis and is involved in neoplastic processes of transformed human bronchial epithelial cells," *Cancer letters*, vol. 370, no. 1, pp. 125–135, 2016.

[178] Z. Li, C. Zeng, Q. Nong, F. Long, J. Liu, Z. Mu, B. Chen, D. Wu, and H. Wu, "Exosomal leucine-rich-alpha2-glycoprotein 1 derived from non-small-cell lung cancer cells promotes angiogenesis via tgf-β signal pathway," *Molecular Therapy-Oncolytics*, vol. 14, pp. 313–322, 2019.

[179] F. Chalmin, S. Ladoire, G. Mignot, J. Vincent, M. Bruchard, J.-P. Remy-Martin, W. Boireau, A. Rouleau, B. Simon, D. Lanneau, *et al.*, "Membrane-associated hsp72 from tumor-derived exosomes mediates stat3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells," *The Journal of clinical investigation*, vol. 120, no. 2, pp. 457–471, 2010.

[180] S.-h. Huang, Y. Li, J. Zhang, J. Rong, and S. Ye, "Epidermal growth factor receptor-containing exosomes induce tumor-specific regulatory t cells," *Cancer investigation*, vol. 31, no. 5, pp. 330–335, 2013.

[181] G. Berchem, M. Z. Noman, M. Bosseler, J. Paggetti, S. Baconnais, E. Le Cam, A. Nanbakhsh, E. Moussay, F. Mami-Chouaib, B. Janji, *et al.*, "Hypoxic tumor-derived microvesicles negatively regulate nk cell function by a mechanism involving tgf-β and mir23a transfer," *Oncoimmunology*, vol. 5, no. 4, p. e1062968, 2016.

[182] G. Chen, A. C. Huang, W. Zhang, G. Zhang, M. Wu, W. Xu, Z. Yu, J. Yang, B. Wang, H. Sun, *et al.*, "Exosomal pd-l1 contributes to immunosuppression and is associated with anti-pd-1 response," *Nature*, vol. 560, no. 7718, pp. 382–386, 2018.

[183] X. Zhou, W. Zhang, Q. Yao, H. Zhang, G. Dong, M. Zhang, Y. Liu, J.-K. Chen, and Z. Dong, "Exosome production and its regulation of egfr during wound healing in renal tubular cells," *American Journal of Physiology-Renal Physiology*, vol. 312, no. 6, pp. F963–F970, 2017.

[184] N. Chaput, N. E. Schartz, F. André, J. Taïeb, S. Novault, P. Bonnaventure, N. Aubert, J. Bernard, F. Lemonnier, M. Merad, *et al.*, "Exosomes as potent cell-free peptide-based vaccine. ii. exosomes in cpg adjuvants efficiently prime naive tc1 lymphocytes leading to tumor rejection," *The Journal of Immunology*, vol. 172, no. 4, pp. 2137–2146, 2004.

[185] F. André, N. Chaput, N. E. Schartz, C. Flament, N. Aubert, J. Bernard, F. Lemonnier, G. Raposo, B. Escudier, D.-H. Hsu, *et al.*, "Exosomes as potent cell-free peptide-based vaccine. i. dendritic cell-derived exosomes transfer functional mhc class i/peptide complexes to dendritic cells," *The Journal of Immunology*, vol. 172, no. 4, pp. 2126–2136, 2004.

[186] S.-H. Kim, E. R. Lechman, N. Bianco, R. Menon, A. Keravala, J. Nash, Z. Mi, S. C. Watkins, A. Gambotto, and P. D. Robbins, "Exosomes derived from il-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis," *The Journal of Immunology*, vol. 174, no. 10, pp. 6440–6448, 2005.

[187] A. J. Abusamra, Z. Zhong, X. Zheng, M. Li, T. E. Ichim, J. L. Chin, and W.-P. Min, "Tumor exosomes expressing fas ligand mediate cd8+ t-cell apoptosis," *Blood Cells, Molecules, and Diseases*, vol. 35, no. 2, pp. 169–173, 2005.

[188] T. Whiteside, "Tumour-derived exosomes or microvesicles: another mechanism of tumour escape from the host immune system?," *British journal of cancer*, vol. 92, no. 2, pp. 209–211, 2005.

[189] D. D. Taylor, Ç. Gerçel-Taylor, K. S. Lyons, J. Stanson, and T. L. Whiteside, "T-cell apoptosis and suppression of t-cell receptor/cd3-ζ by fas ligand-containing membrane vesicles shed from ovarian tumors," *Clinical cancer research*, vol. 9, no. 14, pp. 5113–5119, 2003.

[190] L. Frängsmyr, V. Baranov, O. Nagaeva, U. Stendahl, L. Kjellberg, and L. Mincheva-Nilsson, "Cytoplasmic microvesicular form of fas ligand in human early placenta: switching the tissue immune privilege hypothesis from cellular to vesicular level," *Molecular human reproduction*, vol. 11, no. 1, pp. 35–41, 2005.

[191] G. Van Niel, J. Mallegol, C. Bevilacqua, C. Candalh, S. Brugiere, E. Tomaskovic-Crook, J. K. Heath, N. Cerf-Bensussan, and M. Heyman, "Intestinal epithelial exosomes carry mhc class ii/peptides able to inform the immune system in mice," *Gut*, vol. 52, no. 12, pp. 1690–1697, 2003.

[192] S. Östman, M. Taube, and E. Telemo, "Tolerosome-induced oral tolerance is mhc dependent," *Immunology*, vol. 116, no. 4, pp. 464–476, 2005.

[193] M.-T. Hsu, Y.-K. Wang, and Y. J. Tseng, "Exosomal proteins and lipids as potential biomarkers for lung cancer diagnosis, prognosis, and treatment," *Cancers*, vol. 14, no. 3, p. 732, 2022.

[194] J. Wu and Z. Shen, "Exosomal mirnas as biomarkers for diagnostic and prognostic in lung cancer," *Cancer medicine*, vol. 9, no. 19, pp. 6909–6922, 2020.

[195] L. Zhou, T. Lv, Q. Zhang, Q. Zhu, P. Zhan, S. Zhu, J. Zhang, and Y. Song, "The biology, function and clinical implications of exosomes in lung cancer," *Cancer letters*, vol. 407, pp. 84–92, 2017.

[196] H. Mirzaei, A. Masoudifar, A. Sahebkar, N. Zare, J. Sadri Nahand, B. Rashidi, E. Mehrabian, M. Mohammadi, H. R. Mirzaei, and M. R. Jaafari, "Microrna: A novel target of curcumin in cancer therapy," *Journal of Cellular Physiology*, vol. 233, no. 4, pp. 3004–3015, 2018.

[197] D. J. Clark, W. E. Fondrie, A. Yang, and L. Mao, "Triple silac quantitative proteomic analysis reveals differential abundance of cell signaling proteins between normal and lung cancer-derived exosomes," *Journal of proteomics*, vol. 133, pp. 161–169, 2016.

[198] M. P. Bard, J. P. Hegmans, A. Hemmes, T. M. Luider, R. Willemsen, L.-A. A. Severijnen, J. P. van Meerbeeck, S. A. Burgers, H. C. Hoogsteden, and B. N. Lambrecht, "Proteomic analysis of exosomes isolated from human malignant pleural effusions," *American journal of respiratory cell and molecular biology*, vol. 31, no. 1, pp. 114–121, 2004.

[199] T. L. Whiteside, "Tumor-derived exosomes and their role in tumor-induced immune suppression," *Vaccines*, vol. 4, no. 4, p. 35, 2016.

[200] L. Melton, "Proteomics in multiplex," *Nature*, vol. 429, no. 6987, pp. 105–107, 2004.

[201] K. R. Jakobsen, B. S. Paulsen, R. Bæk, K. Varming, B. S. Sorensen, and M. M. Jørgensen, "Exosomal proteins as potential diagnostic markers in advanced non-small cell lung carcinoma," *Journal of extracellular vesicles*, vol. 4, no. 1, p. 26659, 2015.

[202] O. Fortunato, P. Gasparini, M. Boeri, and G. Sozzi, "Exo-mirnas as a new tool for liquid biopsy in lung cancer," *Cancers*, vol. 11, no. 6, p. 888, 2019.

[203] C. Freitas, C. Sousa, F. Machado, M. Serino, V. Santos, N. Cruz-Martins, A. Teixeira, A. Cunha, T. Pereira, H. P. Oliveira, *et al.*, "The role of liquid biopsy in early diagnosis of lung cancer," *Frontiers in Oncology*, vol. 11, p. 634316, 2021.

[204] X. Wang, X. Jiang, J. Li, J. Wang, H. Binang, S. Shi, W. Duan, Y. Zhao, and Y. Zhang, "Serum exosomal mir-1269a serves as a diagnostic marker and plays an oncogenic role in non-small cell lung cancer," *Thoracic cancer*, vol. 11, no. 12, pp. 3436–3447, 2020.

[205] Z.-J. Zhang, X.-G. Song, L. Xie, K.-Y. Wang, Y.-Y. Tang, M. Yu, X.-D. Feng, and X.-R. Song, "Circulating serum exosomal mir-20b-5p and mir-3187-5p as efficient diagnostic biomarkers for early-stage non-small cell lung cancer," *Experimental Biology and Medicine*, vol. 245, no. 16, pp. 1428–1436, 2020.

[206] H. Dejima, H. Iinuma, R. Kanaoka, N. Matsutani, and M. Kawamura, "Exosomal microrna in plasma as a non-invasive biomarker for the recurrence of non-small cell lung cancer," *Oncology letters*, vol. 13, no. 3, pp. 1256–1263, 2017.

[207] J. Lin, Y. Wang, Y.-Q. Zou, X. Chen, B. Huang, J. Liu, Y.-M. Xu, J. Li, J. Zhang, W.-M. Yang, *et al.*, "Differential mirna expression in pleural effusions derived from extracellular vesicles of patients with lung cancer, pulmonary tuberculosis, or pneumonia," *Tumor Biology*, vol. 37, no. 12, pp. 15835–15845, 2016.

[208] A. R. Halvorsen, M. Bjaanæs, M. LeBlanc, A. M. Holm, N. Bolstad, L. Rubio, J. C. Peñalver, J. Cervera, J. C. Mojarrieta, J. A. López-Guerrero, *et al.*,

"A unique set of 6 circulating micrornas for early detection of non-small cell lung cancer," *Oncotarget*, vol. 7, no. 24, p. 37250, 2016.

[209] H. Tamiya, A. Mitani, A. Saito, T. Ishimori, M. Saito, H. Isago, T. Jo, Y. Yamauchi, G. Tanaka, and T. Nagase, "Exosomal microrna expression profiling in patients with lung adenocarcinoma-associated malignant pleural effusion," *Anticancer research*, vol. 38, no. 12, pp. 6707–6714, 2018.

[210] M. Kuchuk, C. L. Addison, M. Clemons, I. Kuchuk, and P. Wheatley-Price, "Incidence and consequences of bone metastases in lung cancer patients," *Journal of bone oncology*, vol. 2, no. 1, pp. 22–29, 2013.

[211] X.-R. Yang, C. Pi, R. Yu, X.-J. Fan, X.-X. Peng, X.-C. Zhang, Z.-H. Chen, X. Wu, Y. Shao, Y.-L. Wu, *et al.*, "Correlation of exosomal microrna clusters with bone metastasis in non-small cell lung cancer," *Clinical & experimental metastasis*, vol. 38, no. 1, pp. 109–117, 2021.

[212] D. Yuwen, B. Sheng, J. Liu, W. Wenyu, and Y. Shu, "Mir-146a-5p level in serum exosomes predicts therapeutic effect of cisplatin in non-small cell lung cancer," *Eur Rev Med Pharmacol Sci*, vol. 21, no. 11, pp. 2650–2658, 2017.

[213] Q. Zheng, H. Ding, L. Wang, Y. Yan, Y. Wan, Y. Yi, L. Tao, and C. Zhu, "Circulating exosomal mir-96 as a novel biomarker for radioresistant non-small-cell lung cancer," *Journal of Oncology*, vol. 2021, 2021.

[214] X.-X. Peng, R. Yu, X. Wu, S.-Y. Wu, C. Pi, Z.-H. Chen, X.-C. Zhang, C.-Y. Gao, Y. W. Shao, L. Liu, *et al.*, "Correlation of plasma exosomal microrna s with the efficacy of immunotherapy in egfr/alk wild-type advanced non-small cell lung cancer," *Journal for Immunotherapy of Cancer*, vol. 8, no. 1, 2020.

[215] L. H. Gray, A. Conger, M. Ebert, S. Hornsey, and O. Scott, "The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy," *The British journal of radiology*, vol. 26, no. 312, pp. 638–648, 1953.

[216] J. Brown, "Exploiting tumour hypoxia and overcoming mutant p53 with tirapazamine," *British Journal of Cancer*, vol. 77, no. 4, pp. 12–14, 1998.

[217] D. Hanahan and R. A. Weinberg, "Hallmarks of cancer: the next generation," *cell*, vol. 144, no. 5, pp. 646–674, 2011.

[218] V. Petrova, M. Annicchiarico-Petruzzelli, G. Melino, and I. Amelio, "The hypoxic tumour microenvironment," *Oncogenesis*, vol. 7, no. 1, p. 10, 2018.

[219] J. R. Molina, Y. Sun, M. Protopopova, S. Gera, M. Bandi, C. Bristow, T. McAfoos, P. Morlacchi, J. Ackroyd, A.-N. A. Agip, *et al.*, "An inhibitor of oxidative phosphorylation exploits cancer vulnerability," *Nature medicine*, vol. 24, no. 7, pp. 1036–1046, 2018.

[220] M. G. Vander Heiden, L. C. Cantley, and C. B. Thompson, "Understanding the warburg effect: the metabolic requirements of cell proliferation," *science*, vol. 324, no. 5930, pp. 1029–1033, 2009.

[221] X. L. Zu and M. Guppy, "Cancer metabolism: facts, fantasy, and fiction," *Biochemical and biophysical research communications*, vol. 313, no. 3, pp. 459–465, 2004.

[222] M. Molls, M. S. Anscher, C. Nieder, and P. Vaupel, *The impact of tumor biology on cancer treatment and multidisciplinary strategies*. Springer, 2009.

[223] S. Walenta and W. F. Mueller-Klieser, "Lactate: mirror and motor of tumor malignancy," in *Seminars in radiation oncology*, vol. 14, pp. 267–274, Elsevier, 2004.

[224] P. Sonveaux, F. Végran, M. W. Dewhirst, and O. Feron, "Targeting lactate exchanges in tumours: from basic characterization to new therapeutic applications," *Radiotherapy & Oncology*, vol. 96, p. S57, 2010.

[225] M. Stubbs, P. M. McSheehy, J. R. Griffiths, and C. L. Bashford, "Causes and consequences of tumour acidity and implications for treatment," *Molecular medicine today*, vol. 6, no. 1, pp. 15–19, 2000.

[226] L. E. Gerweck, "Tumor ph: implications for treatment and novel drug design," in *Seminars in radiation oncology*, vol. 8, pp. 176–182, Elsevier, 1998.

[227] A. Németh, N. Orgovan, B. W. Sódar, X. Osteikoetxea, K. Pálóczi, K. É. Szabó-Taylor, K. V. Vukman, Á. Kittel, L. Turiák, Z. Wiener, *et al.*, "Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated dna," *Scientific reports*, vol. 7, no. 1, pp. 1–16, 2017.

[228] L. A. Beninson and M. Fleshner, "Exosomes in fetal bovine serum dampen primary macrophage il-1β response to lipopolysaccharide (lps) challenge," *Immunology letters*, vol. 163, no. 2, pp. 187–192, 2015.

[229] E. Eitan, S. Zhang, K. W. Witwer, and M. P. Mattson, "Extracellular vesicle–depleted fetal bovine and human sera have reduced capacity to support cell growth," *Journal of extracellular vesicles*, vol. 4, no. 1, p. 26373, 2015.

[230] J. Li, Y. Lee, H. J. Johansson, I. Mäger, P. Vader, J. Z. Nordin, O. P. Wiklander, J. Lehtiö, M. J. Wood, and S. E. Andaloussi, "Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles," *Journal of extracellular vesicles*, vol. 4, no. 1, p. 26883, 2015.

[231] R. Kornilov, M. Puhka, B. Mannerström, H. Hiidenmaa, H. Peltoniemi, P. Siljander, R. Seppänen-Kaijansinkko, and S. Kaur, "Efficient ultrafiltration-based protocol to deplete extracellular vesicles from fetal bovine serum," *Journal of extracellular vesicles*, vol. 7, no. 1, p. 1422674, 2018.

[232] C. Théry, S. Amigorena, G. Raposo, and A. Clayton, "Isolation and characterization of exosomes from cell culture supernatants and biological fluids," *Current protocols in cell biology*, vol. 30, no. 1, pp. 3–22, 2006.

[233] E. O. Mahgoub, E. Razmara, A. Bitaraf, F.-S. Norouzi, M. Montazeri, R. Behzadi-Andouhjerdi, M. Falahati, K. Cheng, Y. Haik, A. Hasan, *et al.*, "Advances of exosome isolation techniques in lung cancer," *Molecular Biology Reports*, vol. 47, no. 9, pp. 7229–7251, 2020.

[234] T. Skotland, K. Sandvig, and A. Llorente, "Lipids in exosomes: Current knowledge and the way forward," *Progress in lipid research*, vol. 66, pp. 30–41, 2017.

[235] R. E. Lane, D. Korbie, W. Anderson, R. Vaidyanathan, and M. Trau, "Analysis of exosome purification methods using a model liposome system and tunable-resistive pulse sensing," *Scientific reports*, vol. 5, no. 1, pp. 1–7, 2015.

[236] B. Li, X. Dong, J. Zhu, T. Zhu, X. Tao, D. Peng, and Q. Li, "Crosstalk between h1975 tumor cells and platelets to induce the proliferation, migration and tube formation of vascular endothelial cells," *Oncology Letters*, vol. 22, no. 3, pp. 1–10, 2021.

[237] A. Haghighitalab, M. M Matin, F. Khakrah, A. Asoodeh, and A. R. Bahrami, "Cost-effective strategies for depletion of endogenous extracellular vesicles from fetal bovine serum," *Journal of Cell and Molecular Research*, vol. 11, no. 2, pp. 42–54, 2020.

[238] A. Doyle and J. B. Griffiths, "Cell and tissue culture: laboratory procedures in biotechnology," *(No Title)*, 1998.

[239] Y. Zhang, X. Feng, T. Li, E. Yi, and Y. Li, "Metformin synergistic pemetrexed suppresses non-small-cell lung cancer cell proliferation and invasion in vitro," *Cancer medicine*, vol. 6, no. 8, pp. 1965–1975, 2017.

[240] N. M. Anderson and M. C. Simon, "The tumor microenvironment," *Current Biology*, vol. 30, no. 16, pp. R921–R925, 2020.

[241] C. Coughlan, K. D. Bruce, O. Burgy, T. D. Boyd, C. R. Michel, J. E. Garcia-Perez, V. Adame, P. Anton, B. M. Bettcher, H. J. Chial, *et al.*, "Exosome isolation by ultracentrifugation and precipitation and techniques for downstream analyses," *Current Protocols in Cell Biology*, vol. 88, no. 1, p. e110, 2020.

[242] A. S. P. H. P. Carnell-Morris, D. Tannetta and R. Dragovic, "Analysis of extracellular vesicles using fluorescence nanoparticle tracking analysis,," *Extracellular Vesicles*, pp. 153–173, 2017.

[243] B. Carr and M. Wright, "Nanoparticle tracking analysis," *Innovations in Pharmaceutical Technology*, vol. 26, pp. 38–40, 2008.

[244] D. Bachurski, M. Schuldner, P.-H. Nguyen, A. Malz, K. S. Reiners, P. C. Grenzi, F. Babatz, A. C. Schauss, H. P. Hansen, M. Hallek, *et al.*, "Extracellular vesicle measurements with nanoparticle tracking analysis–an accuracy and repeatability comparison between nanosight ns300 and zetaview," *Journal of extracellular vesicles*, vol. 8, no. 1, p. 1596016, 2019.

[245] Y. Weng, Z. Sui, Y. Shan, Y. Hu, Y. Chen, L. Zhang, and Y. Zhang, "Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling," *Analyst*, vol. 141, no. 15, pp. 4640–4646, 2016.

[246] R. J. Simpson, "Sds-page of proteins," *Cold Spring Harbor Protocols*, vol. 2006, no. 1, pp. pdb–prot4313, 2006.

[247] Y. Wu, W. Deng, and D. J. Klinke II, "Exosomes: improved methods to characterize their morphology, rna content, and surface protein biomarkers," *Analyst*, vol. 140, no. 19, pp. 6631–6642, 2015.

[248] M. Le, C. Fernandez-Palomo, F. E. McNeill, C. B. Seymour, A. J. Rainbow, and C. E. Mothersill, "Exosomes are released by bystander cells exposed to radiation-induced biophoton signals: Reconciling the mechanisms mediating the bystander effect," *PloS one*, vol. 12, no. 3, p. e0173685, 2017.

[249] T. Mahmood and P.-C. Yang, "Western blot: technique, theory, and trouble shooting," *North American journal of medical sciences*, vol. 4, no. 9, p. 429, 2012.

[250] H. Ando, T. Matsushita, M. Wakitani, T. Sato, S. Kodama-Nishida, K. Shibata, K. Shitara, and S. Ohta, "Mouse-human chimeric anti-tn igg1 induced

anti-tumor activity against jurkat cells in vitro and in vivo," *Biological and pharmaceutical bulletin*, vol. 31, no. 9, pp. 1739–1744, 2008.

[251] K. Holmes, L. M. Lantz, B. Fowlkes, I. Schmid, and J. V. Giorgi, "Preparation of cells and reagents for flow cytometry," *Current protocols in immunology*, vol. 44, no. 1, pp. 5–3, 2001.

[252] A. Efthymiou, N. Mureanu, R. Pemberton, S. Tai-MacArthur, D. Mastronicola, C. Scottà, G. Lombardi, K. H. Nicolaides, and P. Shangaris, "Isolation and freezing of human peripheral blood mononuclear cells from pregnant patients," *STAR protocols*, vol. 3, no. 1, p. 101204, 2022.

[253] R. Szatanek, M. Baj-Krzyworzeka, J. Zimoch, M. Lekka, M. Siedlar, and J. Baran, "The methods of choice for extracellular vesicles (evs) characterization," *International journal of molecular sciences*, vol. 18, no. 6, p. 1153, 2017.

[254] H. Svensson, A. Johannisson, T. Nikkilä, G. Alm, and B. Cederblad, "The cell surface phenotype of human natural interferon-α producing cells as determined by flow cytometry," *Scandinavian journal of immunology*, vol. 44, no. 2, pp. 164–172, 1996.

[255] B. Graham, J. Curry, T. Smyth, L. Fazal, R. Feltell, I. Harada, J. Coyle, B. Williams, M. Reule, H. Angove, *et al.*, "The heat shock protein 90 inhibitor, at 13387, displays a long duration of action in vitro and in vivo in non-small cell lung cancer," *Cancer science*, vol. 103, no. 3, pp. 522–527, 2012.

[256] A. Ahsan, S. G. Ramanand, C. Whitehead, S. M. Hiniker, A. Rehemtulla, W. B. Pratt, S. Jolly, C. Gouveia, K. Truong, C. Van Waes, *et al.*, "Wild-type egfr is stabilized by direct interaction with hsp90 in cancer cells and tumors," *Neoplasia*, vol. 14, no. 8, pp. 670–IN1, 2012.

[257] A. C. Teilmann, A. Nygaard Madsen, B. Holst, J. Hau, B. Rozell, and K. S. P. Abelson, "Physiological and pathological impact of blood sampling by retro-bulbar sinus puncture and facial vein phlebotomy in laboratory mice," *PLoS One*, vol. 9, no. 11, p. e113225, 2014.

[258] K. Gao, J. Jin, C. Huang, J. Li, H. Luo, L. Li, Y. Huang, and Y. Jiang, "Exosomes derived from septic mouse serum modulate immune responses via exosome-associated cytokines," *Frontiers in immunology*, vol. 10, p. 1560, 2019.

[259] C. Grosjean, J. Quessada, M. Nozais, M. Loosveld, D. Payet-Bornet, and C. Mionnet, "Isolation and enrichment of mouse splenic t cells for ex vivo and in vivo t cell receptor stimulation assays," *STAR protocols*, vol. 2, no. 4, p. 100961, 2021.

[260] D. C. Rio, M. Ares, G. J. Hannon, and T. W. Nilsen, "Purification of rna using trizol (tri reagent)," *Cold Spring Harbor Protocols*, vol. 2010, no. 6, pp. pdb–prot5439, 2010.

[261] M. Gangwar, A. Shukla, V. K. Patel, P. Prakash, and G. Nath, "Assessment of successful qrt-pcr of sars-cov-2 assay in pool screening using isopropyl alcohol purification step in rna extraction," *BioMed Research International*, vol. 2021, 2021.

[262] M. R. Green and J. Sambrook, "Quantification of rna by real-time reverse transcription-polymerase chain reaction (rt-pcr).," *Cold Spring Harbor Protocols*, vol. 2018, no. 10, 2018.

[263] L. Moldovan, K. Batte, Y. Wang, J. Wisler, and M. Piper, "Analyzing the circulating microrna in exosomes/extracellular vesicles from serum or plasma by qrt-pcr," *Circulating MicroRNAs: Methods and Protocols*, pp. 129–145, 2013.

[264] C. Sticht, C. De La Torre, A. Parveen, and N. Gretz, "mirwalk: an online resource for prediction of microrna binding sites," *PloS one*, vol. 13, no. 10, p. e0206239, 2018.

[265] C. Chen, H. Chen, Y. Zhang, H. R. Thomas, M. H. Frank, Y. He, and R. Xia, "Tbtools: an integrative toolkit developed for interactive analyses of big biological data," *Molecular plant*, vol. 13, no. 8, pp. 1194–1202, 2020.

[266] J. R. Conway, A. Lex, and N. Gehlenborg, "Upsetr: an r package for the visualization of intersecting sets and their properties," *Bioinformatics*, 2017.

[267] Y. Zhou, B. Zhou, L. Pache, and M. Chang, "0000–0002–4526–489x ao, khodabakhshi ah, et al," *Metascape provides a biologistoriented resource for the analysis of systems-level datasets. Nat Commun*, vol. 10, no. 1, p. 1523, 2019.

[268] Z. Tang, B. Kang, C. Li, T. Chen, and Z. Zhang, "Gepia2: an enhanced web server for large-scale expression profiling and interactive analysis," *Nucleic acids research*, vol. 47, no. W1, pp. W556–W560, 2019.

[269] Z.-c. Xie, T.-t. Li, B.-l. Gan, X. Gao, L. Gao, G. Chen, and X.-h. Hu, "Investigation of mir-136-5p key target genes and pathways in lung squamous cell cancer based on tcga database and bioinformatics analysis," *Pathology-Research and Practice*, vol. 214, no. 5, pp. 644–654, 2018.

[270] J.-M. Escola, M. J. Kleijmeer, W. Stoorvogel, J. M. Griffith, O. Yoshie, and H. J. Geuze, "Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human b-lymphocytes," *Journal of Biological Chemistry*, vol. 273, no. 32, pp. 20121–20127, 1998.

[271] C. Théry, A. Regnault, J. Garin, J. Wolfers, L. Zitvogel, P. Ricciardi-Castagnoli, G. Raposo, and S. Amigorena, "Molecular characterization of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73," *The Journal of cell biology*, vol. 147, no. 3, pp. 599–610, 1999.

[272] E. J. Wherry and M. Kurachi, "Molecular and cellular insights into t cell exhaustion," *Nature Reviews Immunology*, vol. 15, no. 8, pp. 486–499, 2015.

[273] W. Sienel, S. Dango, U. Woelfle, A. Morresi-Hauf, C. Wagener, J. Brummer, W. Mutschler, B. Passlick, and K. Pantel, "Elevated expression of carcinoembryonic antigen-related cell adhesion molecule 1 promotes progression of non-small cell lung cancer," *Clinical Cancer Research*, vol. 9, no. 6, pp. 2260–2266, 2003.

[274] T. Nagaishi, H. Iijima, A. Nakajima, D. Chen, and R. S. Blumberg, "Role of ceacam1 as a regulator of t cells," *Annals of the New York Academy of Sciences*, vol. 1072, no. 1, pp. 155–175, 2006.

[275] S. Ren, Q. Tian, N. Amar, H. Yu, C. J. Rivard, C. Caldwell, T. L. Ng, M. Tu, Y. Liu, D. Gao, *et al.*, "The immune checkpoint, hvem may contribute to immune escape in non-small cell lung cancer lacking pd-l1 expression," *Lung cancer*, vol. 125, pp. 115–120, 2018.

[276] J. Kaye, "Cd160 and btla: Lights out for cd4+ t cells," *Nature Immunology*, vol. 9, no. 2, pp. 122–124, 2008.

[277] N. Watanabe, M. Gavrieli, J. R. Sedy, J. Yang, F. Fallarino, S. K. Loftin, M. A. Hurchla, N. Zimmerman, J. Sim, X. Zang, *et al.*, "Btla is a lymphocyte inhibitory receptor with similarities to ctla-4 and pd-1," *Nature immunology*, vol. 4, no. 7, pp. 670–679, 2003.

[278] X. Chen, L. Feng, Y. Huang, Y. Wu, and N. Xie, "Mechanisms and strategies to overcome pd-1/pd-l1 blockade resistance in triple-negative breast cancer," *Cancers*, vol. 15, no. 1, p. 104, 2022.

[279] S. M. Dubinett, R. K. Batra, P. W. Miller, and S. Sharma, "Tumor antigens in thoracic malignancy," *American Journal of Respiratory Cell and Molecular Biology*, vol. 22, no. 5, pp. 524–527, 2000.

[280] Q.-H. Xie, J.-Q. Zheng, J.-Y. Ding, Y.-F. Wu, L. Liu, Z.-L. Yu, and G. Chen, "Exosome-mediated immunosuppression in tumor microenvironments," *Cells*, vol. 11, no. 12, p. 1946, 2022.

[281] K. Stefanius, K. Servage, and K. Orth, "Exosomes in cancer development," *Current Opinion in Genetics & Development*, vol. 66, pp. 83–92, 2021.

[282] R. Sahebi, H. Langari, Z. Fathinezhad, Z. Bahari Sani, A. Avan, M. Ghayour Mobarhan, and M. Rezayi, "Exosomes: New insights into cancer mechanisms," *Journal of cellular biochemistry*, vol. 121, no. 1, pp. 7–16, 2020.

[283] J. Banyard and D. R. Bielenberg, "The role of emt and met in cancer dissemination," *Connective tissue research*, vol. 56, no. 5, pp. 403–413, 2015.

[284] M. Osaki and F. Okada, "Exosomes and their role in cancer progression," *Yonago acta medica*, vol. 62, no. 2, pp. 182–190, 2019.

[285] H. Chen, V. Chengalvala, H. Hu, and D. Sun, "Tumor-derived exosomes: Nanovesicles made by cancer cells to promote cancer metastasis," *Acta Pharmaceutica Sinica B*, vol. 11, no. 8, pp. 2136–2149, 2021.

[286] U. H. Weidle, F. Birzele, G. Kollmorgen, and R. Rueger, "The multiple roles of exosomes in metastasis," *Cancer Genomics & Proteomics*, vol. 14, no. 1, pp. 1–15, 2017.

[287] M. Zöller, "Exosomes in cancer disease," *Cancer gene profiling: methods and protocols*, pp. 111–149, 2016.

[288] H. Schwarzenbach and P. Gahan, "Exosomes in immune regulation. noncoding rna 2021, 7, 4," 2021.

[289] P. Vaupel, F. Kallinowski, and P. Okunieff, "Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review," *Cancer research*, vol. 49, no. 23, pp. 6449–6465, 1989.

[290] P. Vaupel, "Tumor microenvironmental physiology and its implications for radiation oncology," in *Seminars in radiation oncology*, vol. 14, pp. 198–206, Elsevier, 2004.

[291] H. W. King, M. Z. Michael, and J. M. Gleadle, "Hypoxic enhancement of exosome release by breast cancer cells," *BMC cancer*, vol. 12, no. 1, pp. 1–10, 2012.

[292] A. Caruso, S. Licenziati, M. Corulli, A. D. Canaris, M. A. De Francesco, S. Fiorentini, L. Peroni, F. Fallacara, F. Dima, A. Balsari, *et al.*, "Flow cytometric analysis of activation markers on stimulated t cells and their correlation with cell proliferation," *Cytometry: The Journal of the International Society for Analytical Cytology*, vol. 27, no. 1, pp. 71–76, 1997.

[293] J. Yates, F. Rovis, P. Mitchell, B. Afzali, J.-S. Tsang, M. Garin, R. Lechler, G. Lombardi, and O. Garden, "The maintenance of human cd4+ cd25+

regulatory t cell function: Il-2, il-4, il-7 and il-15 preserve optimal suppressive potency in vitro," *International immunology*, vol. 19, no. 6, pp. 785–799, 2007.

[294] L. S. Taams, J. Smith, M. H. Rustin, M. Salmon, L. W. Poulter, and A. N. Akbar, "Human anergic/suppressive cd4+ cd25+ t cells: a highly differentiated and apoptosis-prone population," *European journal of immunology*, vol. 31, no. 4, pp. 1122–1131, 2001.

[295] F. Wang, J. Xu, Q. Zhu, X. Qin, Y. Cao, J. Lou, Y. Xu, X. Ke, Q. Li, E. Xie, *et al.*, "Downregulation of ifng in cd4+ t cells in lung cancer through hypermethylation: a possible mechanism of tumor-induced immunosuppression," *PLoS One*, vol. 8, no. 11, p. e79064, 2013.

[296] T. Yamazaki, H. Akiba, A. Koyanagi, M. Azuma, H. Yagita, and K. Okumura, "Blockade of b7-h1 on macrophages suppresses cd4+ t cell proliferation by augmenting ifn-γ-induced nitric oxide production," *The Journal of Immunology*, vol. 175, no. 3, pp. 1586–1592, 2005.

[297] E. De Thaye, K. Van de Vijver, J. Van der Meulen, J. Taminau, G. Wagemans, H. Denys, J. Van Dorpe, G. Berx, W. Ceelen, J. Van Bocxlaer, *et al.*, "Establishment and characterization of a cell line and patient-derived xenograft (pdx) from peritoneal metastasis of low-grade serous ovarian carcinoma," *Scientific Reports*, vol. 10, no. 1, p. 6688, 2020.

[298] G. J. Yoshida, "Applications of patient-derived tumor xenograft models and tumor organoids," *Journal of hematology & oncology*, vol. 13, no. 1, pp. 1–16, 2020.

[299] G.-J. Huang, A. L. Smith, D. H. Gray, C. Cosgrove, B. H. Singer, A. Edwards, S. Sim, J. M. Parent, A. Johnsen, R. Mott, *et al.*, "A genetic and functional relationship between t cells and cellular proliferation in the adult hippocampus," *PLoS biology*, vol. 8, no. 12, p. e1000561, 2010.

[300] T. Calzascia, M. Pellegrini, H. Hall, L. Sabbagh, N. Ono, A. R. Elford, T. W. Mak, P. S. Ohashi, *et al.*, "Tnf-α is critical for antitumor but not antiviral t

cell immunity in mice," *The Journal of clinical investigation*, vol. 117, no. 12, pp. 3833–3845, 2007.

[301] K. Gong, G. Guo, N. Beckley, Y. Zhang, X. Yang, M. Sharma, and A. A. Habib, "Tumor necrosis factor in lung cancer: Complex roles in biology and resistance to treatment," *Neoplasia*, vol. 23, no. 2, pp. 189–196, 2021.

[302] M. Song, Y. Ping, K. Zhang, L. Yang, F. Li, C. Zhang, S. Cheng, D. Yue, N. R. Maimela, J. Qu, *et al.*, "Low-dose ifnγ induces tumor cell stemness in tumor microenvironment of non–small cell lung cancerlow-dose ifnγ induces tumor stemness in nsclc," *Cancer research*, vol. 79, no. 14, pp. 3737–3748, 2019.

[303] C. S. Tannenbaum and T. A. Hamilton, "Immune-inflammatory mechanisms in ifnγ-mediated anti-tumor activity," in *Seminars in cancer biology*, vol. 10, pp. 113–123, Elsevier, 2000.

[304] Y.-F. He, X.-H. Wang, G.-M. Zhang, H.-T. Chen, H. Zhang, and Z.-H. Feng, "Sustained low-level expression of interferon-γ promotes tumor development: potential insights in tumor prevention and tumor immunotherapy," *Cancer Immunology, Immunotherapy*, vol. 54, no. 9, pp. 891–897, 2005.

[305] M. Ayers, J. Lunceford, M. Nebozhyn, E. Murphy, A. Loboda, D. R. Kaufman, A. Albright, J. D. Cheng, S. P. Kang, V. Shankaran, *et al.*, "Ifn-γ–related mrna profile predicts clinical response to pd-1 blockade," *The Journal of clinical investigation*, vol. 127, no. 8, pp. 2930–2940, 2017.

[306] M. Xiao, C. Wang, J. Zhang, Z. Li, X. Zhao, and Z. Qin, "Ifnγ promotes papilloma development by up-regulating th17-associated inflammation," *Cancer research*, vol. 69, no. 5, pp. 2010–2017, 2009.

[307] W. Gong, G.-M. Zhang, Y. Liu, Z. Lei, D. Li, Y. Yuan, B. Huang, and Z.-H. Feng, "Ifn-γ withdrawal after immunotherapy potentiates b16 melanoma invasion and metastasis by intensifying tumor integrin αvβ3 signaling," *International journal of cancer*, vol. 123, no. 3, pp. 702–708, 2008.

[308] K.-L. Wu, Y.-M. Tsai, C.-T. Lien, P.-L. Kuo, and J.-Y. Hung, "The roles of microrna in lung cancer," *International journal of molecular sciences*, vol. 20, no. 7, p. 1611, 2019.

[309] X. Zhu, M. Kudo, X. Huang, H. Sui, H. Tian, C. M. Croce, and R. Cui, "Frontiers of microrna signature in non-small cell lung cancer," *Frontiers in Cell and Developmental Biology*, vol. 9, p. 643942, 2021.

[310] J. D. Arroyo, J. R. Chevillet, E. M. Kroh, I. K. Ruf, C. C. Pritchard, D. F. Gibson, P. S. Mitchell, C. F. Bennett, E. L. Pogosova-Agadjanyan, D. L. Stirewalt, *et al.*, "Argonaute2 complexes carry a population of circulating micrornas independent of vesicles in human plasma," *Proceedings of the National Academy of Sciences*, vol. 108, no. 12, pp. 5003–5008, 2011.

[311] A. Thind and C. Wilson, "Exosomal mirnas as cancer biomarkers and therapeutic targets," *Journal of extracellular vesicles*, vol. 5, no. 1, p. 31292, 2016.

[312] C. Huang, W. Yue, L. Li, S. Li, C. Gao, L. Si, L. Qi, C. Cheng, M. Lu, and H. Tian, "Microrna-665 regulated cell proliferation, migration and apoptosis by targeting nemo-like kinase in non-small cell lung cancer," 2020.

[313] G. Rabinowits, C. Gerçel-Taylor, J. M. Day, D. D. Taylor, and G. H. Kloecker, "Exosomal microrna: a diagnostic marker for lung cancer," *Clinical lung cancer*, vol. 10, no. 1, pp. 42–46, 2009.

[314] C. Li, F. Qin, W. Wang, Y. Ni, M. Gao, M. Guo, and G. Sun, "hnrnpa2b1-mediated extracellular vesicles sorting of mir-122-5p potentially promotes lung cancer progression," *International Journal of Molecular Sciences*, vol. 22, no. 23, p. 12866, 2021.

[315] L. Peng, S. Li, Y. Li, M. Wan, X. Fang, Y. Zhao, W. Zuo, D. Long, and Y. Xuan, "Regulation of btg3 by microrna-20b-5p in non-small cell lung cancer," *Oncology Letters*, vol. 18, no. 1, pp. 137–144, 2019.

[316] A. J. Schetter, S. Y. Leung, J. J. Sohn, K. A. Zanetti, E. D. Bowman, N. Yanaihara, S. T. Yuen, T. L. Chan, D. L. Kwong, G. K. Au, *et al.*, "Microrna expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma," *Jama*, vol. 299, no. 4, pp. 425–436, 2008.

[317] L. Yang, N. Belaguli, and D. H. Berger, "Microrna and colorectal cancer," *World journal of surgery*, vol. 33, no. 4, pp. 638–646, 2009.

[318] Y.-W. Chung, H.-S. Bae, J.-Y. Song, J. K. Lee, N. W. Lee, T. Kim, and K.-w. Lee, "Detection of microrna as novel biomarkers of epithelial ovarian cancer from the serum of ovarian cancer patient," *International Journal of Gynecologic Cancer*, vol. 23, no. 4, 2013.

[319] M. Li, Q. Wang, X. Zhang, N. Yan, and X. Li, "Exosomal mir-126 blocks the development of non-small cell lung cancer through the inhibition of itga6," *Cancer cell international*, vol. 20, no. 1, pp. 1–11, 2020.

[320] Z. Liu, L. Yao, B. Tan, L. Li, and B. Chen, "Detection of microrna-200b may predict the inhibitory effect of gefitinib on non-small cell lung cancer and its potential mechanism," *Oncology Letters*, vol. 12, no. 6, pp. 5349–5355, 2016.

[321] P. Chaniad, K. Trakunran, S. L. Geater, W. Keeratichananont, P. Thongsuksai, and P. Raungrut, "Serum mirnas associated with tumor-promoting cytokines in non-small cell lung cancer," *PloS one*, vol. 15, no. 10, p. e0241593, 2020.

[322] S. Chen, P. Li, R. Yang, R. Cheng, F. Zhang, Y. Wang, X. Chen, Q. Sun, W. Zang, Y. Du, *et al.*, "microrna-30b inhibits cell invasion and migration through targeting collagen triple helix repeat containing 1 in non-small cell lung cancer," *Cancer cell international*, vol. 15, no. 1, pp. 1–10, 2015.

[323] R. Cui, W. Meng, H.-L. Sun, T. Kim, Z. Ye, M. Fassan, Y.-J. Jeon, B. Li, C. Vicentini, Y. Peng, *et al.*, "Microrna-224 promotes tumor progression in nonsmall cell lung cancer," *Proceedings of the National Academy of Sciences*, vol. 112, no. 31, pp. E4288–E4297, 2015.

[324] X. Zhang, X. Han, P. Zuo, X. Zhang, and H. Xu, "Ceacam5 stimulates the progression of non-small-cell lung cancer by promoting cell proliferation and migration," *Journal of International Medical Research*, vol. 48, no. 9, p. 0300060520959478, 2020.

[325] C.-J. Chen and J. E. Shively, "The cell-cell adhesion molecule carcinoembryonic antigen-related cellular adhesion molecule 1 inhibits il-2 production and proliferation in human t cells by association with src homology protein-1 and down-regulates il-2 receptor," *The Journal of Immunology*, vol. 172, no. 6, pp. 3544–3552, 2004.

[326] A. Donda, L. Mori, A. Shamshiev, I. Carena, C. Mottet, M. H. Heim, C. Beglinger, F. Grunert, C. Rochlitz, L. Terracciano, *et al.*, "Locally inducible cd66a (ceacam1) as an amplifier of the human intestinal t cell response," *European Journal of Immunology*, vol. 30, no. 9, pp. 2593–2603, 2000.

[327] J. Liu, J. Li, M. He, G.-L. Zhang, and Q. Zhao, "Distinct changes of btla and hvem expressions in circulating cd4+ and cd8+ t cells in hepatocellular carcinoma patients," *Journal of immunology research*, vol. 2018, 2018.

[328] S. Lantuejoul, J. Adam, N. Girard, M. Duruisseaux, A. Mansuet-Lupo, A. Cazes, I. Rouquette, L. Gibault, S. Garcia, M. Antoine, *et al.*, "Pd-l1 testing in non-small cell lung carcinoma: Guidelines from the pattern group of thoracic pathologists," in *Annales de Pathologie*, vol. 38, pp. 110–125, 2018.

[329] J. L. Riley, "Pd-1 signaling in primary t cells," *Immunological reviews*, vol. 229, no. 1, pp. 114–125, 2009.

[330] H. Liu, L. Chen, Y. Peng, S. Yu, J. Liu, L. Wu, L. Zhang, Q. Wu, X. Chang, X. Yu, *et al.*, "Dendritic cells loaded with tumor derived exosomes for cancer immunotherapy," *Oncotarget*, vol. 9, no. 2, p. 2887, 2018.

[331] Q. Lin, M. Qu, B. Zhou, H. K. Patra, Z. Sun, Q. Luo, W. Yang, Y. Wu, Y. Zhang, L. Li, *et al.*, "Exosome-like nanoplatform modified with targeting

ligand improves anti-cancer and anti-inflammation effects of imperialine," *Journal of controlled release*, vol. 311, pp. 104–116, 2019.

[332] J. E. Pullan, M. I. Confeld, J. K. Osborn, J. Kim, K. Sarkar, and S. Mallik, "Exosomes as drug carriers for cancer therapy," *Molecular pharmaceutics*, vol. 16, no. 5, pp. 1789–1798, 2019.

[333] W. Sun, J.-d. Luo, H. Jiang, and D. D. Duan, "Tumor exosomes: a double-edged sword in cancer therapy," *Acta Pharmacologica Sinica*, vol. 39, no. 4, pp. 534–541, 2018.

[334] N. Dilsiz, "Role of exosomes and exosomal micrornas in cancer," *Future science OA*, vol. 6, no. 4, p. FSO465, 2020.

[335] A. Bobrie, S. Krumeich, F. Reyal, C. Recchi, L. F. Moita, M. C. Seabra, M. Ostrowski, and C. Théry, "Rab27a supports exosome-dependent and-independent mechanisms that modify the tumor microenvironment and can promote tumor progression," *Cancer research*, vol. 72, no. 19, pp. 4920–4930, 2012.

[336] M. Bruschi, S. Ravera, L. Santucci, G. Candiano, M. Bartolucci, D. Calzia, C. Lavarello, E. Inglese, A. Petretto, G. Ghiggeri, *et al.*, "The human urinary exosome as a potential metabolic effector cargo," *Expert review of proteomics*, vol. 12, no. 4, pp. 425–432, 2015.

[337] M. Katakowski, B. Buller, X. Zheng, Y. Lu, T. Rogers, O. Osobamiro, W. Shu, F. Jiang, and M. Chopp, "Exosomes from marrow stromal cells expressing mir-146b inhibit glioma growth," *Cancer letters*, vol. 335, no. 1, pp. 201–204, 2013.

[338] S. R. Wiley, K. Schooley, P. J. Smolak, W. S. Din, C.-P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith, *et al.*, "Identification and characterization of a new member of the tnf family that induces apoptosis," *Immunity*, vol. 3, no. 6, pp. 673–682, 1995.

[339] S. Dai, X. Zhou, B. Wang, Q. Wang, Y. Fu, T. Chen, T. Wan, Y. Yu, and X. Cao, "Enhanced induction of dendritic cell maturation and hla-a*

0201-restricted cea-specific cd8+ ctl response by exosomes derived from il-18 gene-modified cea-positive tumor cells," *Journal of molecular medicine*, vol. 84, no. 12, pp. 1067–1076, 2006.

[340] K. Tang, Y. Zhang, H. Zhang, P. Xu, J. Liu, J. Ma, M. Lv, D. Li, F. Katirai, G.-X. Shen, *et al.*, "Delivery of chemotherapeutic drugs in tumour cell-derived microparticles," *Nature communications*, vol. 3, no. 1, pp. 1–11, 2012.

[341] M. R. Lentz, "Continuous whole blood ultrapheresis procedure in patients with," *J Biol Response Mod*, vol. 8, no. 5, 1989.

[342] R. H. Tullis, R. P. Duffin, M. Zech, and J. L. Ambrus Jr, "Affinity hemodialysis for antiviral therapy. i. removal of hiv-1 from cell culture supernatants, plasma, and blood," *Therapeutic Apheresis*, vol. 6, no. 3, pp. 213–220, 2002.

[343] A. Litynska, M. Przybyuulo, E. Pochec, D. Hoja-uuLukowicz, D. Ciouulczyk, P. Laidler, and D. Gil, "Comparison of the lectin-binding pattern in different human melanoma cell lines," *Melanoma research*, vol. 11, no. 3, pp. 205–212, 2001.

[344] D. D. Taylor, S. Akyol, and C. Gercel-Taylor, "Pregnancy-associated exosomes and their modulation of t cell signaling," *The Journal of Immunology*, vol. 176, no. 3, pp. 1534–1542, 2006.

[345] T. E. Ichim, Z. Zhong, S. Kaushal, X. Zheng, X. Ren, X. Hao, J. A. Joyce, H. H. Hanley, N. H. Riordan, J. Koropatnick, *et al.*, "Exosomes as a tumor immune escape mechanism: possible therapeutic implications," *Journal of translational medicine*, vol. 6, no. 1, pp. 1–7, 2008.

[346] M. Simons and G. Raposo, "Exosomes–vesicular carriers for intercellular communication," *Current opinion in cell biology*, vol. 21, no. 4, pp. 575–581, 2009.

[347] Y. Zhang, Y. Liu, H. Liu, and W. H. Tang, "Exosomes: biogenesis, biologic function and clinical potential," *Cell & bioscience*, vol. 9, no. 1, pp. 1–18, 2019.

[348] L. Muller, M. Mitsuhashi, P. Simms, W. E. Gooding, and T. L. Whiteside, "Tumor-derived exosomes regulate expression of immune function-related genes in human t cell subsets," *Scientific reports*, vol. 6, no. 1, p. 20254, 2016.

[349] V. Vignard, M. Labbé, N. Marec, G. André-Grégoire, N. Jouand, J.-F. Fonteneau, N. Labarrière, and D. Fradin, "Micrornas in tumor exosomes drive immune escape in melanoma," *Cancer immunology research*, vol. 8, no. 2, pp. 255–267, 2020.

[350] G. N. Shenoy, J. Loyall, O. Maguire, V. Iyer, R. J. Kelleher Jr, H. Minderman, P. K. Wallace, K. Odunsi, S. V. Balu-Iyer, and R. B. Bankert, "Exosomes associated with human ovarian tumors harbor a reversible checkpoint of t-cell responses," *Cancer immunology research*, vol. 6, no. 2, pp. 236–247, 2018.

[351] F. Fanini and M. Fabbri, "Cancer-derived exosomic micrornas shape the immune system within the tumor microenvironment: State of the art," in *Seminars in cell & developmental biology*, vol. 67, pp. 23–28, Elsevier, 2017.

[352] B. T. Maybruck, L. W. Pfannenstiel, M. Diaz-Montero, and B. R. Gastman, "Tumor-derived exosomes induce cd8+ t cell suppressors," *Journal for immunotherapy of cancer*, vol. 5, pp. 1–15, 2017.

[353] A. Marcus, B. G. Gowen, T. W. Thompson, A. Iannello, M. Ardolino, W. Deng, L. Wang, N. Shifrin, and D. H. Raulet, "Recognition of tumors by the innate immune system and natural killer cells," *Advances in immunology*, vol. 122, pp. 91–128, 2014.

[354] R. C. Lai, F. Arslan, M. M. Lee, N. S. K. Sze, A. Choo, T. S. Chen, M. Salto-Tellez, L. Timmers, C. N. Lee, R. M. El Oakley, *et al.*, "Exosome secreted by msc reduces myocardial ischemia/reperfusion injury," *Stem cell research*, vol. 4, no. 3, pp. 214–222, 2010.