Role of tumor-derived exosomal miRNAs in M2-macrophage reprogramming during colorectal cancer progression

M.Sc. Thesis

By

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DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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Role of tumor-derived exosomal miRNAs in M2-macrophage reprogramming during colorectal cancer progression

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **'Role of tumor derived exosomal miRNAs in M2-macrophage reprogramming during Colorectal cancer progression'** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from August 2021 to May 2023 under the supervision of Dr. Mirza S. Baig, Associate professor, IIT Indore.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Neha Singh 15/05/2023

Signature of the student with date

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This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

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NEHA SINGH

DEDICATED

TO

MY PARENTS

AND

FAMILY

Abstract

Colorectal cancer is one of the leading causes of death worldwide. Its incidence and mortality have significantly increased during the past few years. Colorectal cancer cells cross-talk with other cells through exosomes in their tumor microenvironment. One of the ways of cross-talk is through exosomes. Exosomes play a significant role in facilitating cross talk between macrophages and colorectal cancer cells. In this study, exosomes produced by colorectal cancer cells were separated and their impact on the polarization of macrophages was determined. This revealed that early on, these exosomes responsible for pro-inflammatory macrophages, and later, anti-inflammatory macrophages. Anti-inflammatory macrophages are in charge of tumor growth, metastasis, and angiogenesis. Furthermore, CRC exosomes are responsible for increased expression of Programmed death ligand-1, which results in immunosuppression; thus, to inhibit exosomal mediated PD-L1 expression, a small inhibitor that blocks the PD-L1 interaction with PD-1 was discovered.

LIST OF PUBLICATIONS

1. K. Wadhonkar, **N. Singh**, F. M. Heralde, S. P. Parihar, N. Hirani, and M. S. Baig, "Exosome-derived miRNAs regulate macrophage-colorectal cancer cell cross-talk during aggressive tumor development," *Colorectal Cancer*, vol. 12, no. 1, p. CRC40, Mar. 2023, doi: 10.2217/crc-2022-0012
2. Atre, R., Sharma, R., Vadim, G., Solanki, K., Wadhonkar, K., **Singh, N.**, Patidar, P., Khabiya, R., Samaur, H., Banerjee, S., & Baig, M. S. (2023). The indispensability of macrophage adaptor proteins in chronic inflammatory diseases. *International immunopharmacology*, *119*, 110176. Advance online publication. https://doi.org/10.1016/j.intimp.2023.110176
3. *Khandu Wadhonkar* [1], ***Neha Singh*** [1] *Mirza S Baig* [1], Identification of small inhibitor against PD-L1 to develop an anticancer therapy. **(Manuscript prepared)**

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NOMENCLATURE

ml	millilitre
mM	millimolar
ng	nanogram
nM	nanomolar
ns	nanosecond
µL	microlitre
µM	micro-molar
° C	degree centigrade

ACRONYMS

AKT	Ak strain transforming
ARG-1	Arginase 1
CD	Cluster of Differentiation
cDNA	Complimentary DNA
CIN	Chromosomal instability
CM	Condition media
Colo205	Colon cancer cell line
CRC	Colorectal Cancer
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cells
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
EVs	extracellular vesicle
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDAC11	Histone deacetylase 11
HSP	Heat shock protein
IL	Interleukin
JAK	Janus activated Kinases
MAPK pathway	Mitogen-activated protein kinase pathway
MHC	Major histocompatibility complex
miRNA	microRNAs
MLH protein	MutL protein
MMR	Mismatch repair
MSH protein	MutH protein
MSI	Microsatellite instability
MVs	Micro-vesicles

NFW	Nuclease free water
NK cells	Natural killer cells
NTA	Nanoparticle tracking Analysis
ORO	Oil Red O
OxLDL	Oxidized LDL
PBS	Phosphate-buffered saline
PD-1	Programmed death-1
PDBQT	Protein Data Bank, Partial Charge (Q), & Atom Type (T)
PDGF	Platelet-Derived Growth Factor
PD-L1	Programmed death ligand-1
PFA	Paraformaldehyde
PI3K	Phosphoinositide-3-kinase–protein kinase
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative Real-Time PCR
RAB	Ras-related protein
RPMI	Roswell Park Memorial Institute
SDF	Structure Data File
SEM	Scanning electron microscopy
SOCS	Suppressor of cytokine signaling 3
STAT	Signal Transducer and Activator of Transcription
TEM	Transmission electron microscope
TGF-β	Transforming Growth Factor beta
ThP1	human leukemia monocytic cell line
TME	Tumor microenvironment
TNF-α	Tumor Necrosis Factor-alpha

1. Chapter 1: Introduction

1.1. Colorectal Cancer

Colorectal cancer (CRC), as its name it occurs in the colon and rectum region. In the world, colorectal cancer (CRC) is known for the third most frequently diagnosed cancer, which accounts for a total of 10% of all cancers, and leads the second rank in terms of total mortality with an estimate of 935,000 deaths in the year 2020 report **(Figure 1.1.1.)** [1]. It is the second most common cancer in women and the third most common in men. The occurring and mortality in women around 25% less compare to men. The chance of incidence also showed increase with the increasing age. Earlier it was also predicted that by the end of 2035 around 2.5 million new cases increase [2], [3]. The rates are also depend on the geographical region such as the incidence are much higher in the developed country compared to the developing county due to different lifestyle, diet and other factors [4].

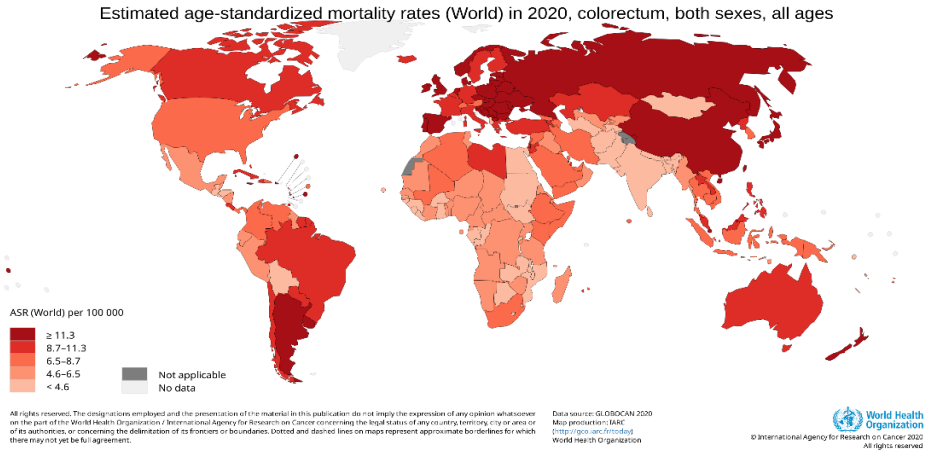


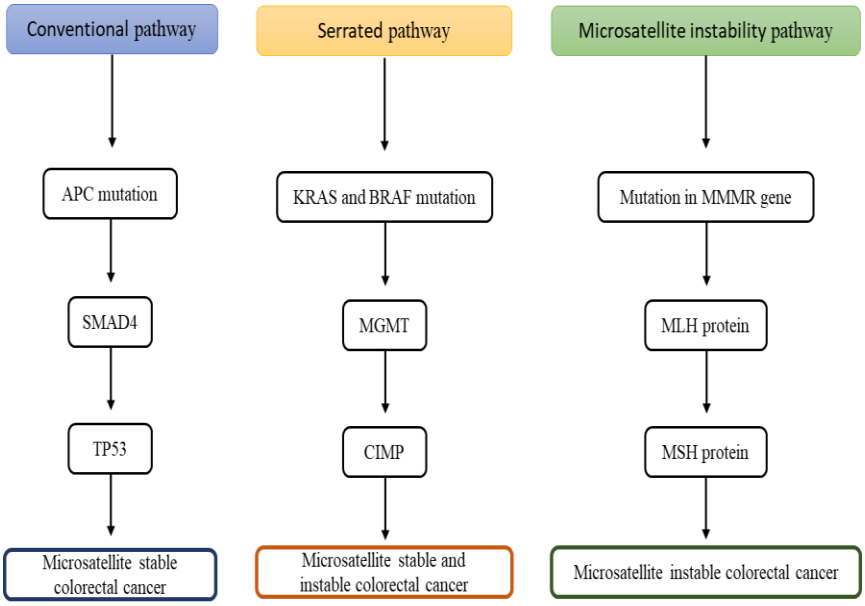
Figure 1.1.1.: Map showing the estimated age standard mortality rate of CRC in the world 2020, for both the sex and all age group. (Map was reproduce form GLOBOCAN https://gco.iarc.fr/).

The risk factor includes hereditary, dietary habits, any life history of adenomatous polyps, inflammatory disease or chronic inflammation [5]. Family history is responsible for around 10-20% for colorectal cancer in all patients, with varying risk depending upon the age and degree of affective individual in family [6], [7]. Although several cancer susceptibility genes are also responsible for colorectal cancer risk but the factors causing hereditary cancer are still unclear and needed to further explore [8].

Colon cancer development takes more than 10-15 years. It starts with the first appears epithelium transforms into a hyperproliferative epithelium leading to advance cancer stages [9]. Sporadic, genetic, and colitis-associated are the three primary form of CRC [10]. There are four stages during the development of the colorectal cancer 1) Initiation 2) Promotion 3) Progression and 4) Metastasis. The development of CRC starts with the hyperproliferation in healthy colon and form non-cancerous outgrowth which is localized in the intestinal mucosa. Of these outgrowths only 10% of the polyps progress to early adenoma which invade the muscularis propria of intestine. Followed by the growth of tumor into the serosa and visceral peritoneum. Then, the tumor enlarged more and starts moving (metastasis) and fully developed into the carcinoma condition (**Figure 1.1.2.**). The liver is the primary site of metastasis in case of CRC then followed by lungs and bone [11].

Figure 1.1.2: Colorectal cancer can development and progression: CRC begins in the healthy colon as a benign tumor in the form of polyps that expand in size and invade the muscularis propria, followed by metastasis to develop into cancer.

Globally, the progression of adenomas happens through three distinct molecular pathways depending on the several genetic and epigenetic events: The chromosomal instability (CIN), serrated neoplasia pathway and microsatellite instability (MSI). Chromosomal instability (CIN) pathway traditionally termed as the Adenomas-carcinoma pathway, account for 70-90% of the CRC. This pathway is characterized by structural chromosomal abnormalities (aneuploidy) follows various mutation in oncogenes or tumor suppressor genes such as the APC mutation followed by RAS activation or TP53 loss of function [12]. In contrast, the serrated neoplasia account for 10-20% of CRC and characterized by activation of MAPK pathway (RAS and RAF mutations) and methylation CpG island mutation. The third is MSI



which occur due to dysfunction of DNA mismatch repair (MMR) such as MMR in MLH proteins or MSH proteins [5] as shown in **Figure 1.1.3**.

Figure 1.1.3: Molecular pathways in colorectal cancer: (A) The most common pathway responsible for the CRC development is the conventional pathway which progress by continuous accumulation of genetic mutations and results in chromosomal instability which associated with the high level of the CIN and microsatellite stability tumors. (B) the serrated neoplasmic pathway is the 2nd most common pathway in which the genetic mutations

occur in BRAF or KRAS gene and progress through the methylation of tumor suppressing genes. (C) microsatellite instability (MSI) occurs due to dysfunction in mismatch repair genes such as gene encoding for the MSH and MLH proteins.

Symptoms: CRC suspected when there is some form of abnormalities present in the lower gastrointestinal region. There are guidelines published by National Institute for health and professional excellence to manage by the health practitioner may identify that patient may have high risk of CRC. The symptoms which are associated with the CRC are rectal bleeding, abdominal pain, abdominal mass, unexplained weight loss, iron-deficiency or anemia and change in bowel habits but CRC is widely an asymptomatic disease until it reaches to the severe stage. In spite of that, the confirmation of CRC may require additional assessment for clarification of condition [13]. According to several clinical guidelines, the colonoscopy is the method of choice for finding and removing CRC polyps. For the successful treatment, the patient who diagnosis with the early stage has better chances of survival. So, any sign or symptom is important and the patient should be encouraged for urgent medical attention and CRC diagnosis test.

For the diagnosis, the patient first undergoes the physical examination of abdomen and analyses of family history for cancer. If the patient suspected it transferred for gastroenterology clinic where doctor should consult patient based on the family history, assessing risk factor, age etc. and according to that the diagnostic test should be decided. Most of the diagnosis method which are currently used are Guaiac fecal occult blood test (gFOBT), Fecal immunochemical test for hemoglobin (FIT), optical colonoscopy (OC), flexible sigmoidoscopy (FS) and digital rectal exam (DRE). Colonoscopy is the widely used test. It’s better for advanced stage lesion compared to the early stage mucosal lesions [14].

1.2. Colorectal cancer and inflammation

In the CRC, the mutation plays major role but the tumor also show evolution during the period of cancer due to various interactions in the surrounding. Around the tumor there are various growth factors, molecules, cell etc. are present which together makeup the environment termed as tumor microenvironment (TME). In the TME, various soluble factors, exosomes, micro-vesicles etc. are released. Majorly, there are three types of the cells present in the TME are: vascular cells, cancer associated fibroblast and infiltrating immune cells. All these cells interact with each other and they are responsible for both positively or negatively monitoring the tumor growth, invasion, energy metabolism, angiogenesis [15]. The TME promotes more aggressive tumor cell proliferation by recruiting immunosuppressive cells, renewing extracellular matrix, and increasing angiogenesis, eventually promoting CRC proliferation and metastasis. In colorectal cancer, cell-to-cell communication is very essential for physiological development and pathology and communication with each other is mediated by synthesis of soluble compounds like cytokines, chemokines, and growth factors into the surrounding microenvironment [16]. During the cancer progression, there is deficiency occur in host immune cells. Immune cells work in all aspect of the cancer from tumorigenesis to the inhibition through immunotherapy. The immune cells phenotype can make the cancer condition ‘inhibiting’ or ‘promoting’. Both the innate and the adaptive immune cells are present in the TME. Innate immune cells include macrophages, neutrophils, mast cells, dendritic cells (DCs), NK-cells, and myeloid-derived suppressor cells. T and B lymphocytes are examples of adaptive immune cells. Immune cells involved in colorectal cancer (CRC) are:

1. **Dendritic cells:** They are widely known for the antigen presentation to the B and T cells. DCs represent the antigen on their surface receptor and interact with the T-cytotoxic cells to activates the adaptive immune response and thus works against the tumor [17].
2. **NK-cells:** They are known for the cytotoxic effect and IFN-γ production but in the case of CRC, there is reduction in NK-cells, thus the cytolytic activity reduce, IFN-γ downregulated [18].
3. **T-cells:** T-cytotoxic cell $CD8^+$, $CD4^+$ and the T-helper cells, they all lead to cancer cell death. They recognize the antigen present on dendritic cell or macrophages and activates their cytotoxic role and leads to the cancer cell reduction. They also release the IFN-γ which is responsible for activation of JAK/STAT pathway and provokes the immune system for cancer cell killing [17].
4. **B-cells:** They perform a variety of functions, including antibody generation, antigen presentation, and immunological suppression. In the case of CRC, B-cell subtypes are seen in the peripheral blood, mesenteric lymph nodes, and the primary tumor site, with varying morphologies. The tumoral B-cells are activated form with the memory phenotype. They interact with tumoral T-cells and activates the cancer cells killing [17].
5. **Tumor associated macrophages:** These cells constitute the major part in tumor condition in all stages of CRC. They can be produced from circulating monocytes or tissue resident macrophages and they can infiltrate the TME or tumor mass. When activated, macrophages release various are activated, they release a variety of inflammatory cytokines, growth factors, proteolytic enzymes, and other substances that play an important role in tumor progression and metastasis. There are two categories based on activation -

- M1 phenotype: 'Classical activated' cells play a part in pathogen or undesirable cell clearance, and as a result, they have a high

inflammatory response and release a variety of pro-inflammatory cytokines such as IL-12, IL-23, IL-1β, TNF-α etc.

- M2 phenotype: 'Alternative activated' cells account for around 80% of tumor mass and are responsible for the secretion of anti-inflammatory cytokines such as IL-10, ARG-1, TGF-β, and others. [19] **(Figure 1.2.).**

Macrophages can acquire M1 or M2 like phenotype based on the stimuli present in TME. In the early stage of tumor, macrophage behave more like M1 which shows the proinflammatory effect and try to inhibits the tumor growth. In contrast, later stage tumor has more density of M2 like macrophage and thus, aid in the tumor growth and metastasis.

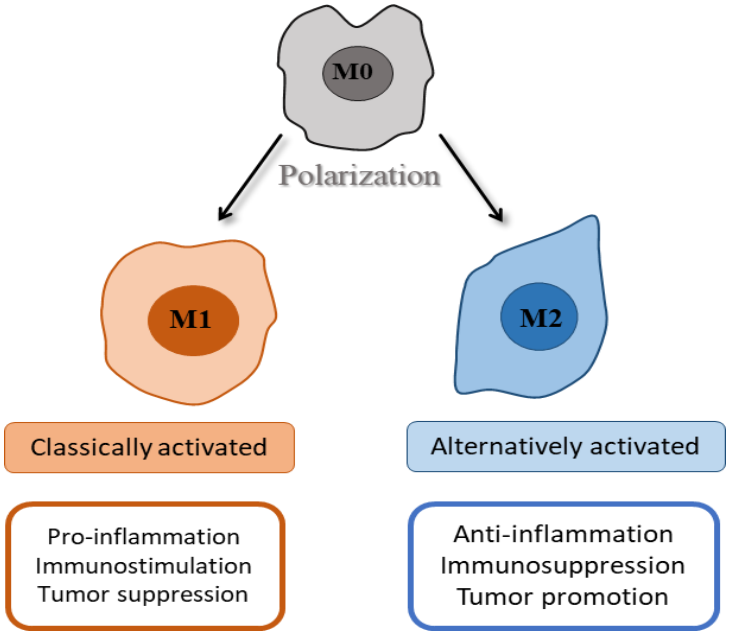


Figure 1.2.: Polarization of macrophage in the classically activated macrophages and alternatively activated macrophages in the presence of stimuli. The pro-inflammatory macrophages are responsible for the immune stimulation where the anti-inflammatory macrophages are for the immunosuppression.

2. Chapter 2: Literature Review

2.1. Role of exosomes

In Colorectal cancer, the surrounding environment is tumor microenvironment (TME) have various cells, growth factors, cytokines which are released by different cells and effect each other to stimulate pr suppresses cancer condition. The cancer cells also release various soluble factors, small molecules, growth facto etc., which also plays major role. It has been found that the condition media which have soluble factor are responsible for the polarization of macrophage in more M2 like phenotype and have anti-inflammatory effect [20]. CRC cells also secretes small molecules such as extracellular vesicles. These extracellular vesicle (EVs) contains genetic information-bearing macromolecules including among others protein, nucleic acid mRNA, and microRNAs (miRNA) [21]. EVs are lipid bilayer-bound vesicles and are naturally secreted from most cell types and released into the extracellular space. Micro-vesicles (MVs), exosomes, and apoptotic bodies are the three primary subgroups of EVs, and they are distinguished by their biogenesis, release mechanisms, size, composition, and action [22].

Exosomes have recently been discovered to play a crucial impact in cancer and they serve as primary mediators between the tumor cell and surrounding cells. The size of the exosome is around 30-100 nm and originated from the endosome [23]. The role of exosomes has been reported in many diseases and shows an impact during immune response maintenance. Exosomes are useful because studies have shown that the bioactive cargoes, they carry reflect their cellular origin, can influence recipient cells and disease status, hence showing their biological importance. They are highly-enriched with tetraspanins, a family of transmembrane proteins like CD81, CD63, and CD9 which interact with many interacting proteins like MHC molecule and

integrin, and reflect on their endosomal origins [24]. They also contain heat shock proteins (HSP70 and HSP90), cytosolic proteins involved in the endocytic pathway including annexin II, RAB5/RAB7, and tumor-susceptibility protein TSG101 which are involved in the endocytic pathway. Exosomes can be isolated through various techniques like ultracentrifugation, differential centrifugation, density gradient centrifugation, and precipitation, and are identified and characterized by transmission electron microscope (TEM), flow-cytometry and western blot analysis [25], [26]. Exosome research is a newly emerging field that is rapidly advancing. The development of new approaches will make it easier to translate exosome research into therapeutic applications especially cancer cell-secreted miRNAs which are mainly carried by exosomes and have been studied recently for their role in post-transcriptional regulation of gene expression via mRNA targeting.

Function of exosomes constituents:

1. ***MicroRNA:*** MicroRNAs plays important role in the cancer condition. these are type of non-coding RNA with a short length made up of 18-25 nucleotides [27]. They are involved in post-transcriptional alterations, which play a crucial function in inflammatory response regulation They interact with messenger RNA (mRNA) 3'-end untranslated regions to control gene expression through translational repression or mRNA degradation. Many cells express miRNAs, which regulate both pro-and anti-inflammatory responses [28]. Such as by controlling the expression of Suppressor of cytokine signaling 3(SOCS3) and Histone deacetylase 11(HDAC11) at the post-transcriptional level miR-203 and miR-145 respectively involved in the polarization of macrophages toward M2 phenotype which suppress the immune response and support metastasis of CRC [29]–[31].

2. ***Proteins:*** Exosomal proteins play important roles during cancer stages such as angiogenesis, epithelial-mesenchymal transition (EMT), extracellular matrix remodeling, tumor-related immunological modulation, premetastatic behaviours, and treatment resistance.

2.1.1. CRC-derived exosomal in the regulation of macrophage polarization

Over the past few years, several evidence has been accumulating that macrophages have a tumor-promoting function in tumor microenvironment (TME). The majority of leukocytes that infiltrate solid tumors are tumor associated monocytes/macrophages (TAMs). Most of them come from circulating monocytes [32]. TAMs, which are transformed as pro-tumorigenic, are widely distributed in both the main and metastatic locations of CRC consequential of these immune responses and are seen throughout all cancer stages [33]. Macrophages provide cytokines, chemokines, and growth factors to the CRC, resulting in a supportive TME. Although exosomes have been firmly linked to cellular communication between CRC cells and macrophages or other TME cells, the exact mechanism is yet unknown. CRC exosomes miRNAs are known for reprogramming macrophages to develop into an anti-inflammatory, pro-tumorigenic M2 phenotype. M1 macrophages act as troops attacking cancer cells by releasing high levels of pro-inflammatory cytokines (IL-12, IL-1, and TNF-α), whereas M2 macrophages act as anti-inflammatory producing cytokines like IL-4, IL-10 and growth factors like TGF-, PDGF, with an excess M2 phenotype resulting in immune suppression and supporting TME [34]. Consequently, the modification and migration of TAMs at the tumor site are

crucial. Then, it would necessary to concentrate on the mechanisms and purposes of exosomes in reprogramming of macrophages in colorectal cancer.

2.2. Programmed Death 1 (PD-1) and Programmed Death-Ligand-1 (PD-L1) interaction

The exosomal miRNAs widely known for the macrophage polarization towards M2 phenotype **(Table 2.1).** There are two miRNAs, miR-21-5p and miR-200a which have very important role in macrophage reprogramming towards M2 along with this, they are also responsible for the higher PD-L1 expression. The higher PD-L1 interact with the PD-1 receptor and thus inhibits the immune response.

Table 2.1: Known miRNAs for their role in macrophage polarization.

miRNA	Target	Refrences
miR-934	PTEN	(Zhao et al., 2020)
miR-25-5p miR-130b-3p miR-425-5p	PTEN	(D. Wang et al., 2020; Xu et al., 2022)
miR-21-5p miR-200a miR-29b	PTEN	(Patel et al., 2015; Shao et al., 2018; Yin et al., 2022)
miR-203	SOCS3	(Takano et al., 2017)
miR-145	HDAC11	(Shinohara et al.,2017)
miR-1246	-	(Cooks et al., 2018; Qian et al., 2020)
Let-7d	CCL7	(Noh et al., 2020)

2.2.1. Significance of PD-1 and PD-L1 interaction in colorectal cancer

There are several modes of the treatment available for CRC such as surgery, radiotherapy, chemotherapy. The second-line of treatment may include as immunotherapy where different immune cells help in fighting the cancer. The immunotherapy is currently getting great attention and have effective result in various type of cancers. One of them is PD-1 and PD-L1 interaction-based immunotherapy.

PD-1 is an immune checkpoint protein, transmembrane receptor which expressed on T-cells and interact which the ligand PD-L1 and PD-L2. The interaction between the receptor and ligand responsible for the immune suppression via inactivating T-cytotoxic cells. In normal condition this interaction is important for inhibiting the exaggerated immune response but in the case of the cancer. Higher expression of the ligands and interaction with the PD-1 leads T cells dysfunction and apoptosis. Thus makes the cancer more aggressive **(Figure 2.2.1.)** [35].

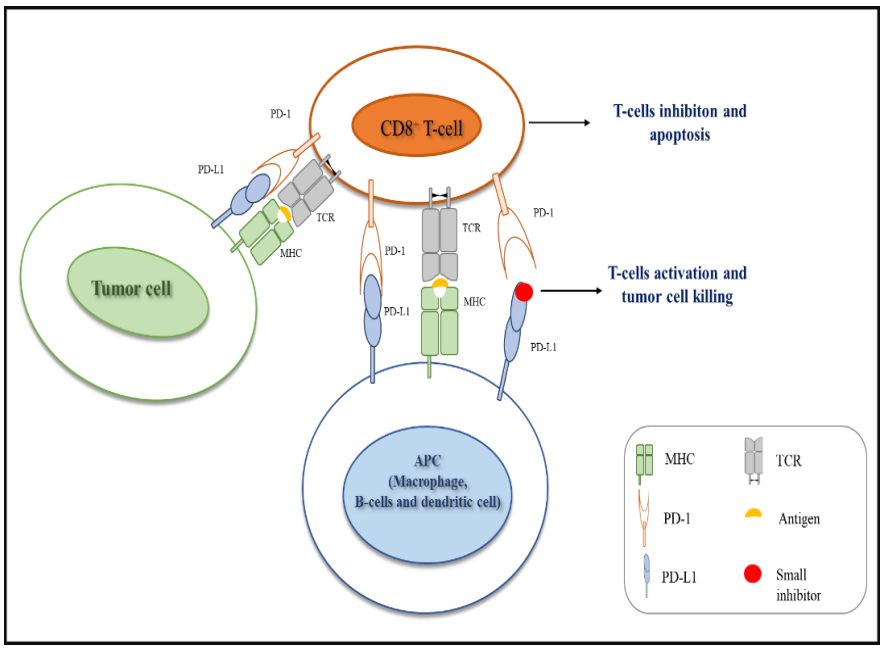


Figure 2.2.1: The role of immune check point (PD-L1 AND PD-1) in the T-cell activity: The PD-1/PD-L1 checkpoint monitor the immune response and keep in check to avoid overexpression. In case of the cancer the iinteraction lead to the suppression of immune system and T-cell inactivation. By preventing PD-L1 from attaching to PD-1 with immune checkpoint inhibitor T cells are able to destroy tumor cells.

2.2.2. Therapeutic targeting of PD-L1

There are various PD-1 or PD-L1 based immunotherapies are available for various type of cancer but most the therapies are based on the large antibody inhibitors. These antibodies bind to either PD-1 or PD-L1 which enable the activated T-cells and thus help in the cancer inhibition. In the case of the CRC, currently 5 FDA approved antibodies are present against PD-1/PD-L1 interactions are nivolumab, pembrolizumab, atezolizumab, durvalumab, and avelumab [36]. These antibodies are very costly and required multiple dosage which is not suitable for most of the individuals. Identifying the small inhibitor against PD-1/PD-L1 can be cost effective and easy intake.

3. Chapter 3: Plan of thesis

This thesis broadly aims to identify the effect of colorectal cancer cell derived exosomes on macrophages and macrophage derived exosomes effect on the cancer cell. In the CRC development, there is tumor grows in colon and rectum region which release various soluble factors, cytokines, small molecules etc., which makeup the tumor microenvironment. Earlier it was found that the condition media are responsible for the macrophage polarization towards M2-like phenotype which have the anti-inflammatory effect and thus, helps in progression of the cancer. But the effect of CRC cell exosomes on macrophages is still need to be explored.

The exosomes are extracellular vesicles which act as genetic cargo which transfer various constituent of one cell to another cell and those constituents may have effect on the recipient cell.

Here, our aim is to identify the effect on exosomes on macrophage, how they start responding due to the exosomes or its constituent where they polarized to pro-inflammatory on anti-inflammatory phenotype. This is the on side of the hypothesis. Along with this, where there any effect of macrophage exosomes on cancer cell, whether they able to affect the cancer condition positively or negatively is need to be identified. Along with this, we are interested in develop a therapeutic strategy to inhibit the effect of these exosomes on both cancer cells and the macrophage. For this, we are purposing a small inhibitor-based immunotherapy against the PD-1/PD-L1 interaction, so that the immune cells especially T-cells remain active and have cytotoxic effect and thus, tumor cell apoptosis take place (Figure 3.1.).

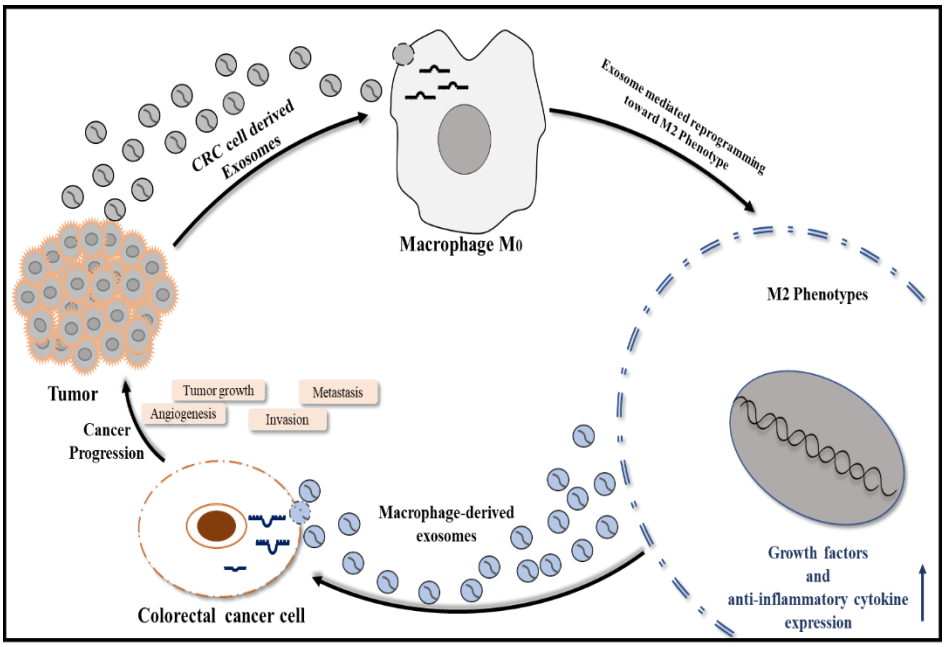


Figure 3.1.: The Disease Model for Colorectal Cancer: In CRC the tumor release exosomes in the TME which moves toward the macrophages and lead to their exosomes mediated reprogramming of macrophage toward anti-inflammatory macrophage. The M2-like macrophage in response release growth factors, anti-inflammatory cytokines along with exosomes which may be responsible for the cancer progression through various functions such as tumor growth, invasion, metastasis and angiogenesis.

Broadly the following objective has been covered in this thesis are:

AIM 1 - Determine to which extent CRC cell derived exosomes impacts macrophage polarization.

For this aim, we will cover the first path from tumor to macrophages. we will first isolate the exosomes from CRC cell condition media and characterized them via size distribution method along with the concentration of exosomes. Then, we will examine the effect of CRC cell derived exosomes on macrophage, the morphological changes and the

cytokines expression. Along with this we will examine the phagocytic activity of exosome treated macrophages.

AIM 2- To develop a therapeutic approach against novel/known miRNA.

In this aim, we will firstly check the expression of PD-L1 ligand on both macrophage as well as colorectal cancer cell via in-vitro method. Secondly, we will develop the immunotherapy against PD-L1 ligand to block its interaction with the receptor PD-1.

4. Chapter 4: Material and Methodology

4.1. Material

Cell Culture : ThP1 (human monocyte cell line) and Colo205 (Colon cancer cell line) was obtained from National Centre for Cell Science (NCCS), both the cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Catalogue No. A1049101, Gibco, California, U.S.) media, heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Catalogue No. 15140122, Invitrogen, California, U.S.), β-mercaptoethanol (Catalogue No. M3148, Sigma, St. Louis, Missouri, U.S.), phorbol 12-myristate 13-acetate (PMA) (Catalogue No. P8139, Sigma, St. Louis, Missouri, U.S.), LPS (Catalogue No. L2630, Sigma, St. Louis, Missouri, U.S.), Human IL-4, OxLDL, DMSO, hemocytometer and trypan blue dye (Catalogue No. 15250061, Thermo Fisher, M.A, U.S), CO_2 incubator, water bath, microscope.

Chemicals: Chloroform, Iso-propanol, 75% Ethanol, Phosphate-buffered saline (PBS), Nuclease free water (NFW), Phorbol 12-myristate 13-acetate (PMA), TRIzol reagent (Invitrogen), TaKaRa PrimeScript 1st strand cDNA Synthesis kit (Catalogue No. 6110A), Appliedbiosystems PowerUp SYBR Green Master Mix, ORO (Oil Red O) (Sigma, Catalogue No. # 01391), 4% Paraformaldehyde (PFA), DAPI (Thermo Scientific).

4.2. Methodology

4.2.1. Cell culture

I. **ThP1 cell line:** THP-1 cell line is a human monocyte cell line (suspension) and was purchased from National Centre for Cell Science (NCCS), Pune, India. THP-1 was cultured in RPMI MEDIA along with 10% heat inactivated FBS (Fetal bovine Serum), antibiotic 1% 100 U/ml penicillin and 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. To differentiate the THP-1 into the

macrophage, cell was treated with the 25 ng of phorbol 12-myristate 13-acetate (PMA) in the culture media for 48 hours. Thereafter, the PMA treated media changed with the fresh complete media before treating with condition media or Colo cell exosomes. Then, cells were incubated in CO_2 incubator (5%) at 37°C. As the doubling time of THP-1 is more than 24 hours, it takes around 48 hours to get confluence in 90 mm plate. Before seeding, the cells were counted via a hemocytometer.

II. **COLO205 cell line:** Colo205 is a colon cancer cell line. It was received from National Centre for Cell Science (NCCS), Pune, India. Colo205 were grown in the RPMI media, along with 10% heat inactivated FBS (Fetal bovine Serum), antibiotic 1% 100U/ml penicillin and 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. After passaging, the cells were incubated in humidified incubator with 5% CO_2 at 37°C. As the doubling time of Colo205 is around 24 hours, it takes around 48hr to get confluence in 90 mm plate.

4.2.2. Preparation of Condition media

For the preparation of Colo205 (Cancer cell) Condition media, the Colo205 cell passaged many times and after 80-90% confluency of the cells. The cells were centrifuged at 800 rpm for 5 min., supernatant was discarded and the cell pellet was again re-suspended in the media and seed it in 90 mm plate. Post 48 hours (After 2 days), the cell supernatant was collected in sterile 15 ml tube and again centrifuged to pellet down the cellular debris, followed by filtration through 0.2 μm sterile filter. The collected condition media was stored it in -20°C or -80°C for further use depending upon the need. If the media was needed to use in one week, then it was stored in the -20°C and if I we needed the media after more than one week, then it was stored it in 80°C.

4.4.3. Preparation of Oxidized LDL (OxLDL)

The human LDL was purchase (Catalogue No. Pro562, Prospec) and diluted up to 0.5 mg/ml with the autoclaved PBS (Phosphate Buffer Saline) (PBS composition: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4; pH 7.4). For oxidation the filtered copper sulfate of 10 μM (Catalogue No. 102784, Merck) was used. The oxidation was done for 24 hours at 37°C. The oxidation was stopped by adding 1 mM EDTA (ethylenediaminetetraacetic acid). Further the oxidized LDL was dialysis with the PBS to remove out the EDTA and store at 4°C until required to use.

4.2.4. Thp1 cell treated with the cancer cell condition media

The cells were seeded in 6-well culture plates with the density of 1×10^5 cells in each well and allow them to settle then after one day, the cells were treated with PMA for their differentiation into M0. After 24 hours treatment with the PMA, the media was changed with condition media, and control had fresh media. The morphological changes were monitored via microscope and captured the images for each time point 24, 48 and 72 hours. After the treatment completion, the cells were washed with 1X PBS and lysed in the TRIzol reagent. The trizol lysed sample were kept in -80°C, till further proceed.

4.2.5. Isolation of exosomes via Ultracentrifugation

For isolation of the exosomes from cancer cell (Colo205), the condition media generated similar to the above discussed. To isolate the exosome first the media was thawed in water bath at 37°C. The media was centrifuged three time for differential centrifugation at 300 g (5 min.), 1200 g (20 min.) and 10000 g (30 min.) and then filtered to remove debris and then

ultracentrifuged at 1,00,000 g for 3 hours at 4°C [37]. The exosomes pellet was resuspended in media and stored in -80°C for further use **(Figure 4.2.5.).**

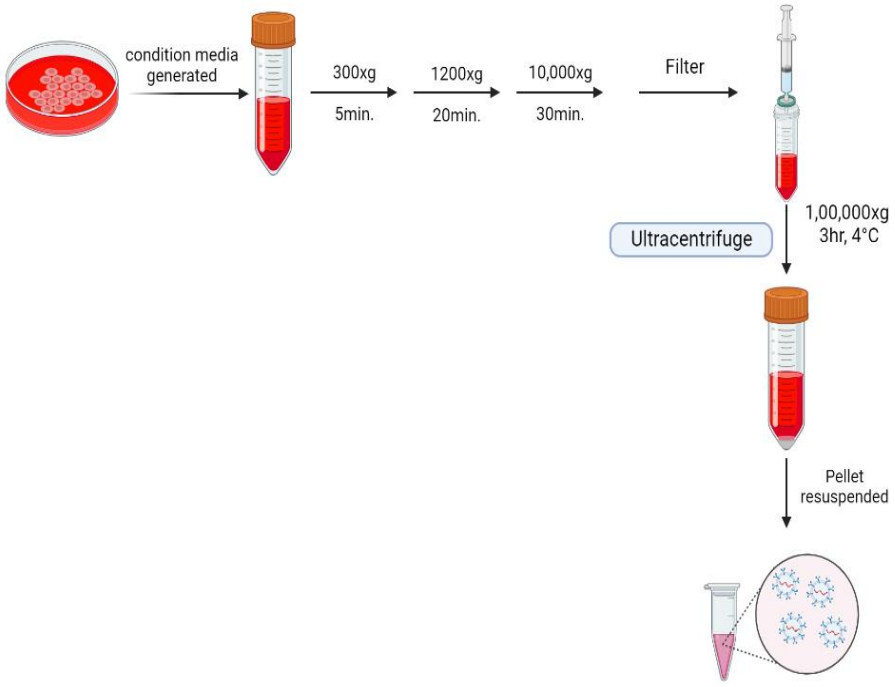


Figure 4.2.5.: Exosome isolation protocol via Ultracentrifugation method: The condition media of cells generated which undergoes the differential centrifugation followed by the ultracentrifugation at 1,00,000 g for 3 hours at 4°C. The isolated exosomes were stored in -80°C until use.

4.2.6. Characterization of exosomes

a. Exosome visualization via Scanning electron microscopy (SEM)

- Pellet of healthy cell's exosome vortexed and resuspended into 0.2-1 ml DPBS.
- The exosome was fixed in 2% formaldehyde aqueous solution.
- Then the exosomal sample were diluted in distilled water in serial dilution.

- Add 2-5 µl vesicle mixture on to the cleaned silicon chips and the vesicle drying done under ventilation hood.
- The samples on silicon chips were mounted on SEM stage via carbon paste.
- The SEM was performed for visualization of exosome morphology.
- To get the best result, isolated the fresh exosome and make slide for SEM and visualize within 7 days.

b. Characterization of Exosomes via Nanoparticle tracking Analysis (NTA)

To determine the size distribution and the particles concentration of Colo205 cancer cell derived exosomes were diluted with the PBS and analyzed by nanoparticle tracking analysis (NTA) using NTA 3.4 Build 3.4.003 equipped with a laser (Blue405). The Colo205 exosomes were diluted in PBS between 20 and 80 particles per frame the scattered and illuminated by the laser beam. The Brownian motion of exosomes were captured for 20 seconds each at the camera level.

The video was analyzed via NTA 3.2 software. Size distribution profiles are replicated from each video to provide a representative size distribution of exosomes averaged.

4.2.7. Thp1 cells treated with cancer cell exosomes

To determines the effect of the Colon cancer exosomes, Thp1 cell seeded in 6-well plate of approximately 1×10^5 cell in each well. For the differentiation of monocyte to M0, the cells were treated with the PMA 25 ng/ml for 24 hours. After M0 differentiation the media was changed. Cell was treated with the cancer cell exosomes for time point 24, 72 and 96 hours. The morphological changes were monitored via microscope and captured the images for each time point. After the treatment completion, the

cell was washed with 1X PBS and lysed in the TRIzol reagent. The trizol lysed sample were kept in -80°C, till further proceed.

4.2.8. RNA isolation

To check the expression of various genes, the ThP1 lysed 6-well plate was thawed on ice and RNA isolation procedure was followed:

- Each sample ere pulverized with pipette for few mins till the stickiness gets disappeared.
- The sample were transferred in 1.5 ml tube.
- Add equal volume of chloroform in each tube and mix it vigorously by hand only.
- Then put the tubes into centrifuge at 13,000 rpm for 30 minutes.
- Observe the 3 separate layers (top layer is aqueous phase, middle layer is protein as whitish layer and bottom layer is RNAiso/Trizol).
- Collect the top layer aqueous layer in the separate tube and add equal volume of Iso-propanol in each tube. Mix it well.
- Put the sample for spin at 13,000 rpm for 30 min.
- After centrifuge, see the pellet and discard the supernatant.
- Add the 500 ml 75% ethanol in each tube and spin again at 13,000 rpm for 30 min.
- Discard the supernatant.
- Place the tubes upside down on the tissue paper and leave it to dry until ethanol gets evaporate.
- Add 10-20 µl of nuclease free water in each tube and mix the pellet and give the short spin.
- Then put the tubes on dry water bath at 50-60°C for 5-10 minutes.
- Proceed for RNA quantification by Nanodrop instrument and note down the RNA concentration for future cDNA synthesis.

4.2.9. cDNA Synthesis

After RNA quantification, the cDNA synthesis was done by TaKaRa PrimeScript 1st strand cDNA Synthesis kit, following protocol.

- For 1ug of RNA, the master mix 1 is prepared:

Reagent	volume
Oligo dT Primer (50uM)	1 µl
dNTP mixture	1 µl
Template RNA	1 µg
RNase free dH_2O	X µl
Total	10 µl

- After the making sample tube incubated at 65°C for 5 min. in water bath then immediately transfer on ice.
- Prepared the master mix 2 and transfer 10 µl in each sample tubes.

Reagent	volume
Template RNA prime mixture (from initial step)	10 µl
5X primescript buffer	4 µl
RNase inhibitor	0.5 µl
Primescript RTase	1 µl
RNase free dH_2O	4.5 µl
Total	20 µl

- Mix the sample gently.
- Incubate the reaction mixture at 30°C for 10 min., 50°C for 60 min. and 70°C for 15 min. in the PCR machine.
- After the cDNA synthesis, the cDNA is diluted with 180 µl of nuclease free water and store the cDNA in -20°C.

4.2.10. Quantitative Real-Time PCR (qRT-PCR)

PCR Polymerase Chain reaction is the one of the powerful technology in the field of molecular biology which is used for the amplification if the target DNA or cDNA into thousand to millions of copies by the use of specific oligonucleotide sequence, heat stable DNA polymerase and thermal cycle. One of the variants of PCR is the Real time Quantitative RT-PCR which is commonly used for the detection of RNA expression.

RT-PCR is reverse transcription-based PCR method which used to convert RNA into cDNA followed by the amplification of the cDNA using specific primers and fluorescent dye. During the amplification as the fluorescent signal increase with increase in the template and shows the directly proportional relation. The fluorescent intensity generated due to the intercalation or the breakdown of dye labelled probe during the amplification of the target gene. The change in the fluorescent intensity is measured by the thermal cycle instrument with fluorescent detecting feature. The RT-PCR instrument generate the amplification plot between the fluorescent change and no. of cycle. In the PCR cycles, when the fluorescence signal becomes higher than the background noise, it is termed as the CT value and the difference between two values represented as the (ΔCT) of one cycle which termed as the doubling amount of the target gene. The CT values are plotted against then plot against the standard target quantities, Calibration curve and target amount in unknown samples then it is interpolated from the diagram.

We use SYBR Green as fluorescent dye (Applied Biosystems). RT-PCR run in the Thermo Fisher StepOne plus and amplification was detected. CT values and analyzed. The primers set which are used are mentioned in **Figure 7.1.** The GAPDH was used as the target gene for normalization.

4.2.11. Confocal Microscopy for phagocytosis assay

The cell was cultured and seed into 12-well plate over the coverslip (19 mm diameter) with the density of 1×10^4 cells in each well. The cells were treated with PMA 25 ng/ml for 24 hours for their differentiation into M0 phenotype. Cell further treated with the cancer cells exosomes (for 24 and 72 hours), LPS, IL-4 for 24 hours. time point. Cells were treated with OxLDL for 6hr. The media were aspirated and cells were washed 2 times with PBS. Followed by the treatment, the macrophage was stained with 0.3% freshly prepared Oil Red O for 20 minutes and placed it in dark or in incubator. The stained cells were washed with 1X PBS 3 times to excised the unbound dye. Cells was fixed with 4% paraformaldehyde for 20 minutes followed by washing again. Further the cells were placed on slide along with the counter staining of nucleus with DAPI (1 μg/ml). stained cells were visualised via an Olympus confocal laser scanning microscope (FV100), and images were captured at 20, 40, and 60 X magnification.

4.2.12. Computational Virtual Screening

a. Retrieval of structural data, preparation, and analysis of interacting residues

   The crystalline structures of PD-1 and PD-L1 were obtained in pdb format from Protein Data Bank (PDB) (https://www.rcsb.org/) and prepared for molecular docking. Only chain A of PD-L1 was used for virtual screening, and the other chains were removed in Discovery Studio. The water molecules were removed and polar hydrogen atoms were added to the structures to prepare them for molecular docking. In UCSF Chimaera, the structure was energetically minimised prior to docking. PD-1 and PD-L1 were docked, and interacting residues were examined at 3 Å to determine the interacting key residues in the UCSF Chimaera [38].

Only FDA-approved drug libraries of natural chemicals were downloaded in SDF (Structure Data File) format for virtual screening. The SDF files were converted to PDBQT format for docking using Open babel version 3.0 [39] and screened against PD-L1 structure.

b. In-silico Molecular docking and Virtual screening

A total of 30,000 chemicals were obtained in SDF format from the database. Open Babel then translated the resulting compounds to pdbqt [39]. Following that, the ligands were used for docking in Schrodinger and AutoDock Vina v1.2.0 [40], [41]. Docking was accomplished using the Schrodinger suite's Glide module [42]. The PD-L1 crystal structure (PDB 4ZQK; Chain A) was obtained from the RCSB PDB (https://www.rcsb.org/). The Protein Preparation Wizard of the Schrodinger suite was used to prepare the protein structure for docking [43]. The structure was then improved and optimised in PROPKA at pH 7.0 [44] before being minimised using the OPLS4 force-field [45]. The receptor grid was then created around the receptor, covering the entire receptor. The Schrodinger suite's LigPrep module was used to prepare ligands for docking in Schrodinger [46]. For docking in AutoDockVina, the polar hydrogen atoms and Kolmann charges were introduced to the crystal structure of PD-L1 in AutoDock 4 [47]. In AutoDockVinav1.2.0, a grid box was generated around the receptor, covering the entire molecule, and ligands were docked with the receptor.

The compounds were docked with the PD-L1 Chain A and the top most compounds were analysed. The high dock score compounds in both the platform were selected and compared. The no. of interacting critical residues used for further analysis. The type of interaction between the compounds and the PD-L1 were visualised in the Schrödinger and BIOVIA Discovery Studio Visualizer. Top most 6 drug was selected as small repurposed drug with highest dock score and interacting residues.

4.2.12. ImageJ analysis

ImageJ enables users to measure picture properties such as intensity, size, form, and texture. It also includes tools for conducting statistical analysis on image data.

It was utilised to analyse confocal and morphological images in this case. The fluorescence intensity of confocal images was measured after splitting the pictures into relevant channels. For the scale bar in the morphological image. The values were then analysed and plotted in GraphPad PRISM 7.0.4.216 (GraphPad Software. lnc, 1992-2017).

4.2.13. Statistical analysis

Statistical analysis was performed using Microsoft excel, GraphPad and ImageJ software. The significance was assessed using ANOVA two-way test. All the data represented were performed in three independent experiments and the error denotes the standard deviation.

5. Chapter 5 Results and Discussion

5.1. Effect of Cancer cell condition media on macrophages

In the CRC condition, the macrophages play a central role as inflammatory effect due to its relatively high density in tumor mass or TME. These macrophages are termed as tumor associated macrophage (TAM) which effect by the various stimuli in the surrounding. The macrophage either show the polarization toward M1 or M2-like phenotype. M1-like macrophages are responsible for the immune activation and thus have role in tumor cell death. In contrast, M2-like macrophages have role in immunosuppression and thus leads to the tumor progression.

Earlier it was identified that the CRC condition media (CM) have the effect on THP-1 cell (monocytes), it reduced the proliferation of monocytes cell and arrest them in G1-stage of cell cycle and also confirmed that the monocytes differentiated into more like M2-phenotype compared to M1 type. Along with that, it was confirmed that CRC condition media induce the production of anti-inflammatory cytokines and chemokines [20]. In one of the study it was also show that CRC CM responsible for the higher expression of pro-inflammatory cytokines (IL-6, IL-12β, and IFN-ɣ) and decreased expression anti-inflammatory cytokines (IL-4 and IL-10) [48]. However, it was unclear whether they are responsible for M1 or M2 like macrophage or they have the mixed colony of both the time. It's also needed to investigate whether the macrophage polarization is time dependent process or not.

To determine the response of macrophage due to CRC CM is time dependent or not. First, we differentiate the monocyte into macrophages (M0) and treated with cancer cell CM to check both the morphological changes and cytokines expression but in time dependent manner. We incubate cell for 24-, 48- and 72-hours time point and monitor the

morphological changes. Treatment which cancer cell CM showed the morphological changes from 24 to 72 hours and compared. M0, M1 and M2 like macrophages are differ in the morphology. M0 is more like the large, rounded cells where M1 is elongated spindle shaped and the M2 appears as flattened, round with the elongated filopodia [49]. We compared the changes in our case and it was found that in early time i.e., 24 hours have the M1 like population, at 48 hours there is the mixed colony of both M1 and M2 macrophage but in the later time 72 hours the macrophages are more like M2 like flattened cells. These morphological changes that the macrophages are affected by CRC condition media may be the time dependent manner thus, at different time point they have different phenotype (**Figure 5.1.1)**.

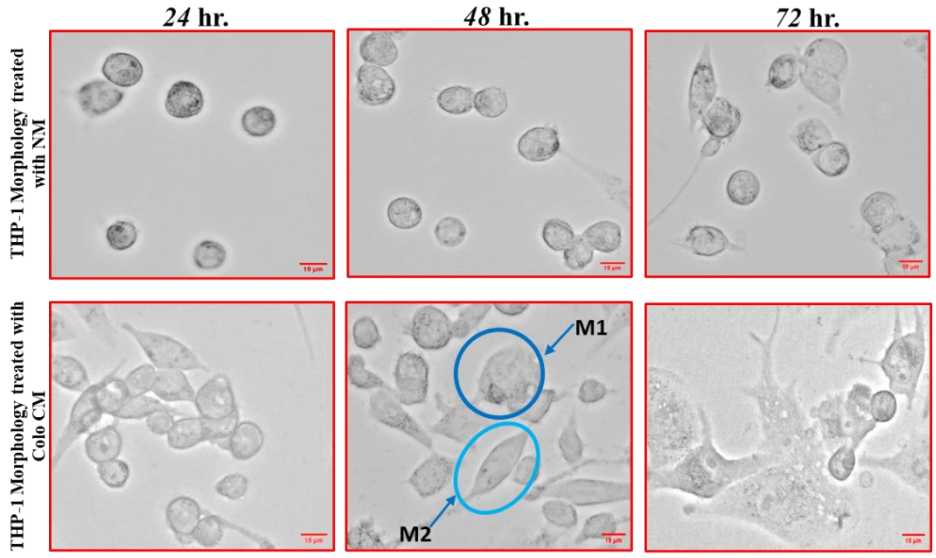


Figure 5.1.1.: The morphological changes in macrophage (ThP-1) treated with the cancer cell exosome (Colo205): macrophage polarization in M1 and M2 macrophage, in 24 hours the morphology of macrophage is more like M1 where in 48 hours there was mixed colony of M1 and M2 like macrophages and in 72 hours the macrophage started flattened with multiple branching and converted into M2 phenotype. PMA treatment of THP-1 25 ng/ml for 24 hours, 20x, Scale bar- 15 μm (Image captured by fluorescence microscope, THERMOFISHER EVOS M500).

To further validate these morphological changes, we check the expression of various pro-inflammatory and anti-inflammatory markers such as IL-6, TNF-α, IL-1β and ARG-1. In the early time point, the expression of the pro-inflammatory markers was found significantly higher compare to late time point. However, the later time point shows the high anti-inflammatory cytokine expression compare to early time point, which proofs that macrophage polarization due to soluble factors is a time dependent process and have visual morphological changes **(Figure 5.1.2.).**

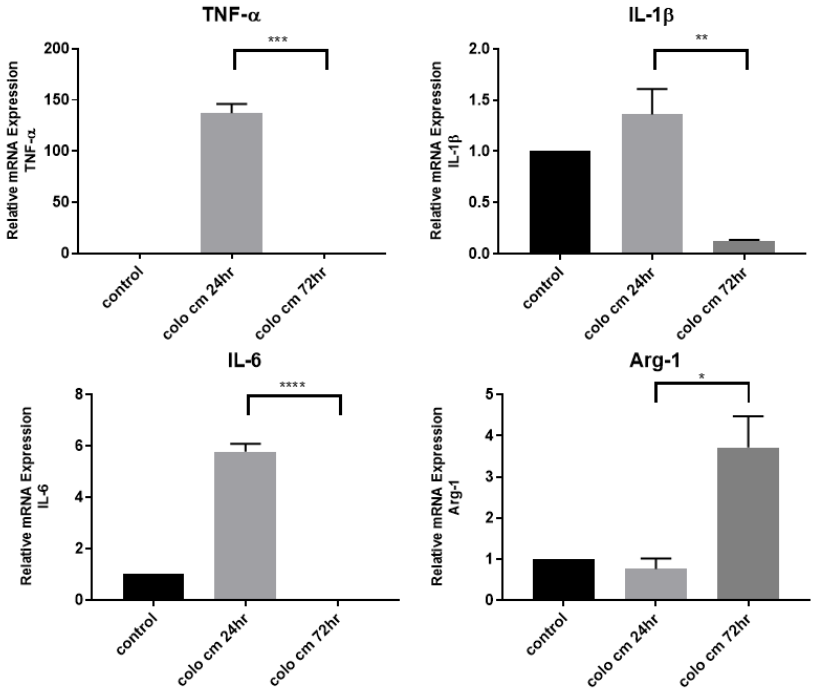


Figure 5.1.2.: Effect on pro-inflammatory and anti-inflammatory cytokines expression in macrophage treated with the colorectal cancer cell condition media: ThP-1 macrophage treated with PMA (25 ng/ml) for 24 hours for differentiation of monocyte into macrophage. Further Condition media treated macrophage shows the higher expression of pro-inflammatory cytokines TNF-α, IL-6, IL-1β at 24 hour and less at 72 hours. The expression of anti-inflammatory cytokine Arg-1 had higher expression at 72 hours instead of 24 hours. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005.

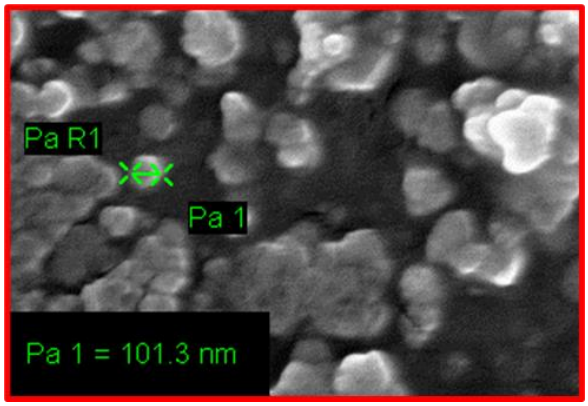
5.2. Characterization of exosomes

In the TME, the cancer cells release various soluble factors, small molecules such as extracellular vesicles. Majorly, the extracellular vesicles have two subclasses depending upon the size: 1) Micro-vesicles which are larger in size range of 100-1000 nm and 2) exosomes which are very small in size range of 30-2 00 nm. We are focusing on the exosomes which serves as vehicles and moves from one cell to another cell. These exosomes have significant role in effecting surrounding cells and the environment.

To isolate the exosomes, we first generated the condition media of Colo205 and proceed to isolate the exosomes via ultracentrifugation method. To characterized the exosomes, we performed the scanning electron microscopy. To identify he structure and shape of cancer cell exosomes SEM was performed. The SEM images revealed that the exosomes are spherical in shape and ranged from 90-120 nm in diameter and validate the clear distinguished lipid bilayer structure as expected.

Next, to analyze the size and concentration of exosomes we measured the particle concentration and size distribution using NTA. The results shows that the size is around 125 nm and concentration of 4.29×10^{8} particles/ml These results suggests that the isolated form are exosomes.

(A)



(B)

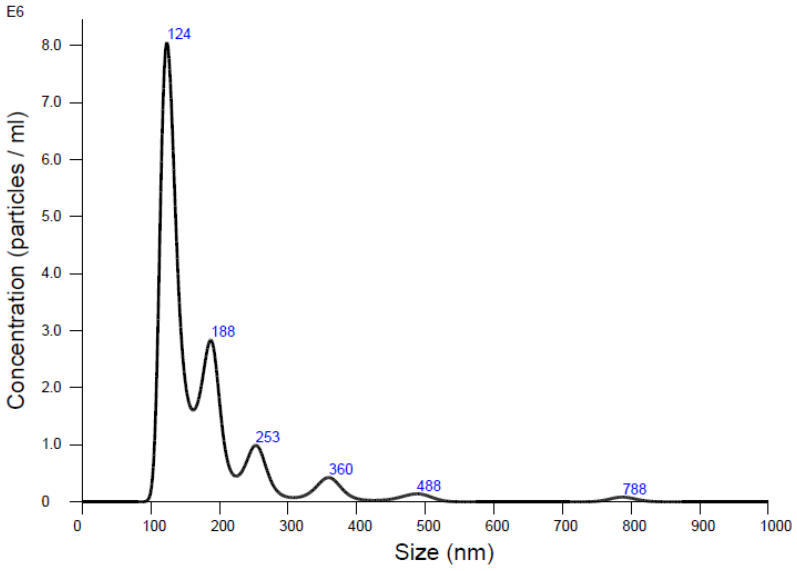


Figure 5.2.: Characterization of colorectal cancer cell derived exosome: **(A)** Representative SEM image of CRC derived exosomes. (B) Representative graph of particle concentration and size distribution of CRC derived exosome measured by the nanoparticle tracking analysis (NTA).

5.3. Effect of Cancer cell exosomes on macrophages

In the TME, the exosomes play a very significant role as these small molecules can make a great change in the surrounding cells such as immune cell, fibroblast cell or may circulate to the other site to effect other organs as a metastasis site. Each constituent inside the exosomes has some role such as miRNAs, proteins, lncRNA etc. they are known to individual affect the cells and lead to change in cancer condition. There several miRNAs known for reprogramming of macrophage toward M2 phenotype but the role as whole exosomes on macrophages and to which fold the cytokines expression affects is still needed to explore.

To check the morphological change, first it needed to understand that when the exosomes transfer from cancer cell to macrophage, in the initial stage they behave as foreign molecule for the macrophage thus in initial interaction, the macrophage shows response to as pro-inflammatory macrophage which tries to removes the foreign molecules from its surrounding environment but during internalization, the exosomes release its constituent which have different role such as miRNA which can target the gene and inhibit or activates the signaling which can lead to polarization of macrophage toward M2 phenotype. In several type of cancer, it was found that cancer cell derived exosomes give rise to M2 like phenotype such as lung tumor derived-exosome promote macrophage polarization in M2 phenotype [50], Additionally, immunosuppressive macrophage polarization was discovered in cases of bladder cancer [51] but not in case of CRC.

To identify the morphological changes, we treated the macrophage with the colo205 cell exosomes and monitors the changes in macrophages. It was found that the M0 macrophage treated with exosomes starts elongated in size and in the 24 hours we had distinct M1 type of macrophage where in 72 hours the macrophages start reprogramming

and have the mixed colony of M1 and M2 phenotype. In contrast as we moved further for next time point i.e., 72 hours the macrophages had the morphology of 72 and up to 96 hours the macrophage get fully flattened with the outgrow branches and remain in M2 type macrophages. However, the control where the cells were not treated by exosomes, their morphology remains round and they didn't get polarized due to the absence of any stimuli **(Figure 5.3.1.).**

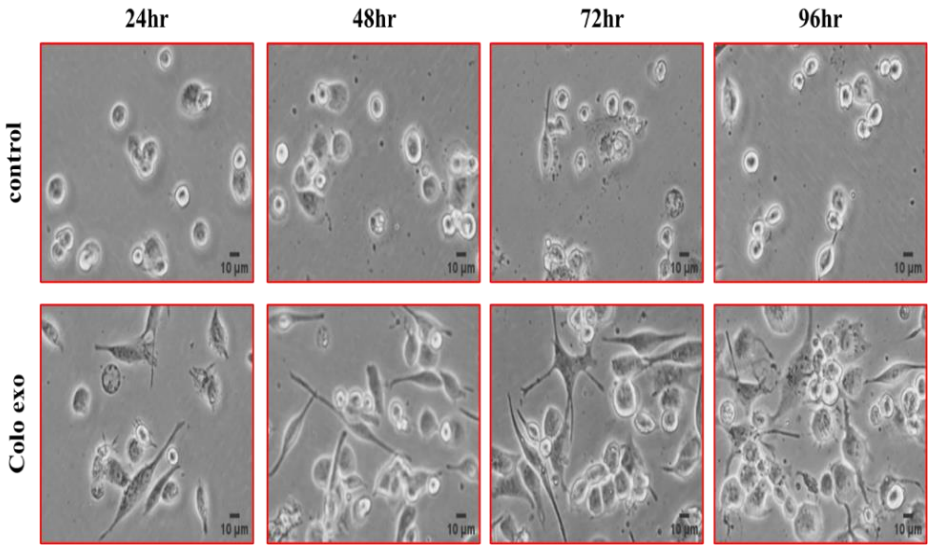


Figure 5.3.1.: The morphological changes in macrophage (ThP-1) treated with the cancer cell exosome (Colo205): macrophage polarization in M1 and M2 macrophage, in 24 hours the morphology of macrophage is more like M1 where in 48 hour there is mixed colony of M1 and M2 like macrophages and in 72 and 96 hours the macrophage starts flattened with multiple branching and converted into M2 phenotype PMA treatment of THP-1- 25 ng/ml for 48 hours. Images were captured at a magnification of 20x, Scale bar- 10µm via fluorescence microscope with mercury lamp, TS100 eclipse in brightfield.

To further validate these morphological changes, we checked the expression of various pro-inflammatory cytokines like IL-23, IL-12, TNF-α and anti-inflammatory cytokines such as IL-10, ARG-1, PDGF in the absence and presence of cancer cell exosomes for 24 hours, 72 hours and 96 hours. It was observed that the expression level was also co-relating with the morphological changes. The pro-inflammatory cytokines had the higher expression at 24 hours which starts dropping in 72 and 96 hours. The expression of IL-23 showed very high expression which around the 45-times compare to control and dropped till 20-times in 96 hours' time where the TNF-α and IL-12 shows only14, 7-times increase at 24 hours which get reduced up to 10, 2 times respectively. The anti-inflammatory cytokines showed the kinetic increase from 24 hours to 96 hours compared to control which that in the early stage the macrophages are M1 like but later they reprogrammed into the M2 like phenotype. The results represented were from three independent experiment (**Figure 5.3.2.)**.

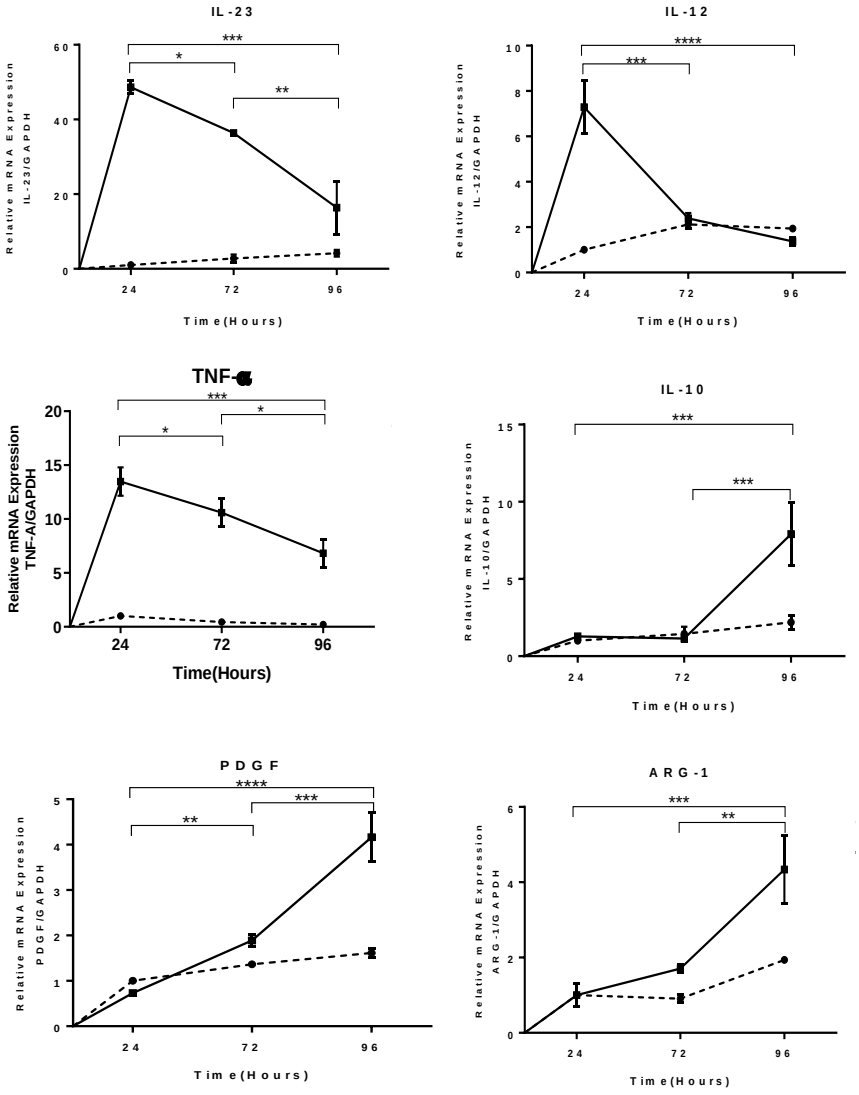


Figure 5.3.2.: Effect on pro-inflammatory and anti-inflammatory cytokines expression in macrophage treated with the colorectal cancer cell condition media: ThP-1 macrophage treated with PMA (25 ng/ml) for 24 hours for differentiation of monocyte into macrophage. Further exosome treated macrophage shows the higher expression of pro-inflammatory cytokines IL-23, IL-12 and TNF-α at 24 hour and less at 72 hours. The expression of anti-inflammatory cytokine IL-10, PDGF, ARG-1 had higher expression at 72 hours instead of 24 hours. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005.

5.4. Phagocytosis assay

Macrophage are professional phagocytes both the pro-inflammatory and anti-inflammatory macrophages shows the phagocytosis. In some case, the M1-like macrophages shows more phagocytosis compare to M2-like macrophage but to check the phagocytosis activity due to exosomes, we started with measuring the phagocytic activity in an in-vitro assay. We seeded the ThP-1 cell in 12-well plate over the coverslip and treated with PMA for their differentiation form monocyte to M0 macrophages. The cells were treated with the exosomes for 24 hour and 72 hour and LPS and IL-4 for 24 hours. As M1-like macrophages (pro-inflammatory) are known for more phagocytic activity compared to the M2-like macrophage (anti-inflammatory). We here checked their phagocytic activity through OxLDL. OxLDL act as indicator for the uptake by phagocytic cells thus, macrophage with more OxLDL uptake will have the more phagocytic activity.

To determine this relation of phagocytosis with OxLDL we used fluorescence method where Oil Red O stains the OxLDL and thus shows its uptake by the macrophages. To compare our treated cell with exosomes, we took two positive controls i.e., LPS for pro-inflammatory macrophage and IL-4 for anti-inflammatory macrophages. As we hypothesized our data show a significant increase in Oil Red O expression in LPS treated cells and exosome treated cell for 24 hours. However, the treatment with IL-4 and exosome treatment for 72 hours showed the less expression and thus less phagocytic activity. In case of control, the M0 macrophage showed very less stain of Oil Red O which means they have very less phagocytic activity compared to pro-inflammatory and anti-inflammatory macrophages as shown in **Figure 5.4**.

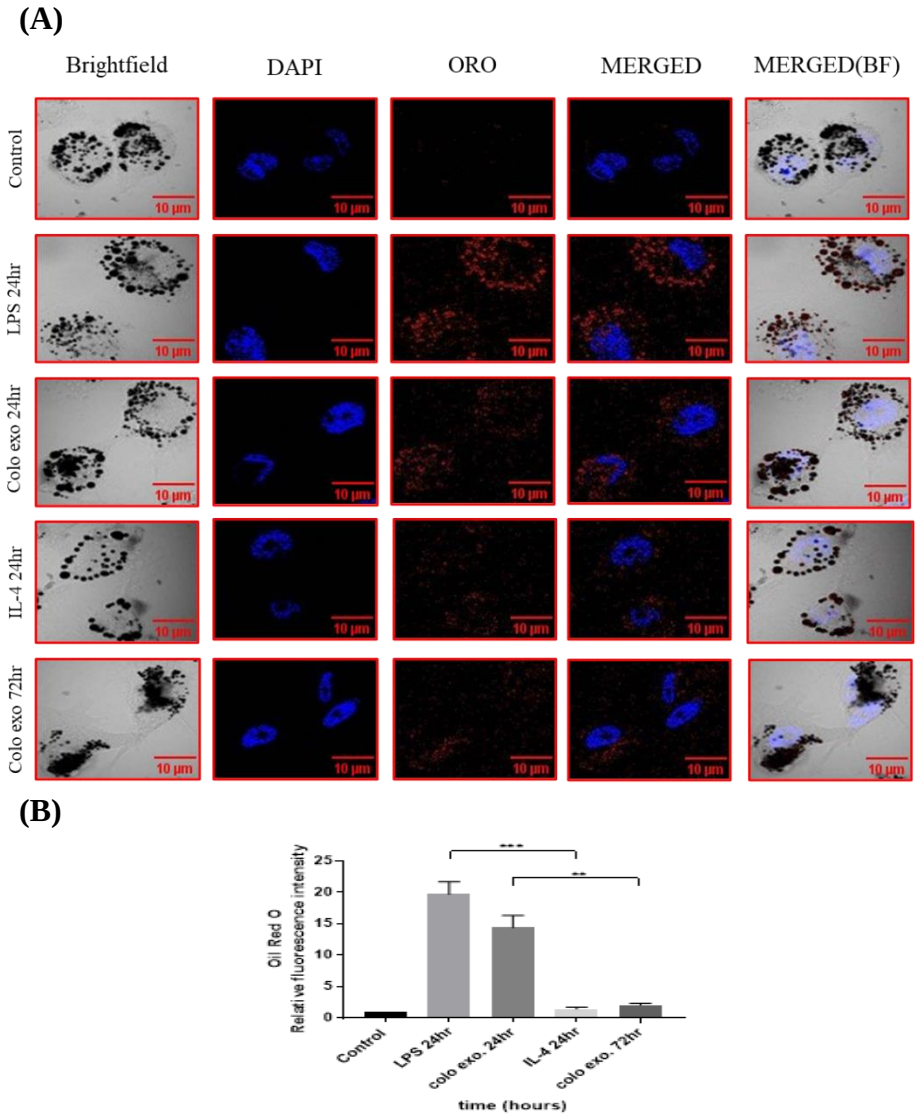


Figure 5.4.: Phagocytosis activity of macrophage: (A) phagocytosis activity was demonstrated in ThP-1 after differentiation with PMA (25 ng/ml) for 24 hours and stimulated with LPS, IL-4 and Colo205 exosome followed by OxLDL treatment to detect the uptake. Cells were fixed and stain with Oil Red O (Red) and nucleus counterstain with DAPI and representative image from 3 independent experiment are shown. Image was captured via Olympus confocal laser scanning microscope (FV100) at magnification of X100, scale bar was set at 10 µm in zoomed images. (B) Quantification of relative fluorescence intensity, *p<0.05, **p<0.005, ***p<0.0005.

5.5. Expression of PD-L1 in cancer cells and macrophages

PD-L1 is the programmed death ligand which is known to be present to be present on macrophage and cancer cell. In healthy individual, the PD-1/PD-L1 immune checkpoint regulates the immune response but in case of CRC it was found that the CRC cell responsible for the up-regulation of PD-L1 in TAM macrophage which can help immune suppression.

To determine the expression of PD-L1 in both cancer cell as well as macrophage we treated macrophage with colorectal cancer cell derived exosomes and colorectal cancer cell with the macrophage derived exosome. We found out that in both the case the higher expression of PD-L1 was found compare to control condition which means the exosomes form both the cell effect the PD-L1 expression.

Recently, it was identified that CRC exosomal miR-21-5p and miR-200a were responsible for higher PD-L1 expression in Tumor associated macrophage. These two miRNAs target the PTEN and SOCS1 genes and thus activates the PI3K/AKT and JAK/STAT pathway in macrophage which promotes the PD-L1 expression in macrophage [52]. Thus, the higher expression of PD-L1 in macrophage in our case also is due to the activation of either individual pathways or both the pathways.

(A) (**B)**

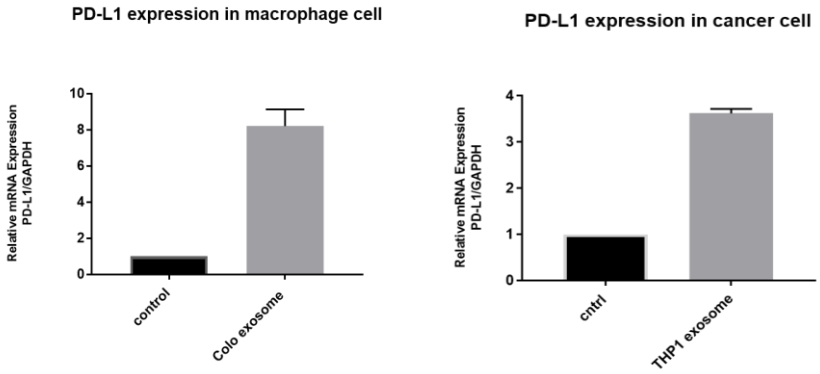


Figure 5.5.: Quantitative RT-PCR analysis of PD-L1 expression in macrophage and CRC cells: (A) ThP-1 macrophage treated with PMA (25 ng/ml) for 24 hours and then treated with Colo205 exosomes. (B) Colo205 treated with the macrophage exosome.

5.6. In-silico virtual screening against PD-L1

I. Structure of PD-1 and PD-L1, critical interacting residues

Molecular docking was performed with the crystalline structure of PD-1 and PD-L1 complex solved by X-ray diffraction method at the resolution of 2.45 Å. PD-1 presumes as a β-sandwich immunoglobulin variable Ig-V type topology with the dislfide bond between Cys54 and Cys123. The structure of PD-1 is well defined by electron density. Similarly, the ligand PD-L1's N-terminus domain also have Ig-V type topology. The complex of PD-1 and PD-L1 has the well-defined electron density and binding of PD-1 and PD-L1 in the complex is in 1:1 ratio.

To determine the critical interacting residues, we performed the protein-protein docking which revealed the 9 residues: F19, Y56, Q66, R113, A121, D122, Y123, K124 and R125 for PD-L1 and 7 residues for PD-1 N66, Y68, Q75, T76, K78, A132 and E136 for binding of PD-1/PD-L1 complexes shown in table 5.6.1. To show the critical residues in the spherical form, we used the binding site feature in BIOVIA Discovery Studio by selecting critical residues of PD-L1 in **(Figure 5.6.).**

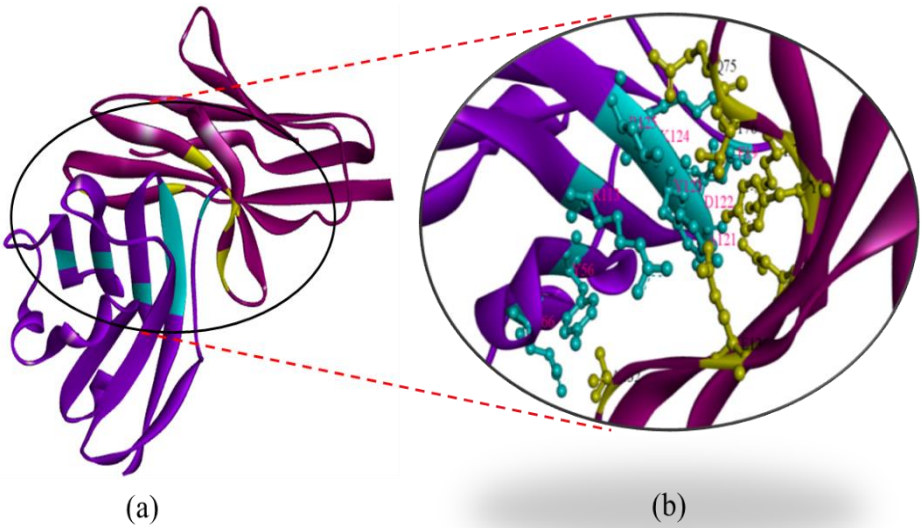


Figure 5.6.: Complex of PD-L1 and PD-1: a) The complex structure of PD-L1 and PD-1 (4ZQK) displayed as a flat ribbon representation, where PD-1 in magenta and PD-L1 in purple-colored ribbons. The molecular interaction at PD-1 and PD-L1 are highlighted in green and yellow color respectively. b) The molecular interactions at each of the interface where in the interacting residues are shown in stick type with color code same as in the respective structure shown in (a).

Table 5.6.1.: Analysis of interface and interacting residues of PD-L1 and PD-1 complex within 3Å region from Chimera

Crystal Structure	Interacting Residues and its position in PD-L1 and PD-1 complex		No of Residue
PD-1 PD-L1 Complex (4ZQK)	PD-L1	F19,Y56,Q66,R113,A121, D122,Y123,K124,R125	9
	PD-1	N66,Y68,Q75,T76,K78, A132,E136	7

II. Molecular docking of PD-L1 with small inhibitors

Virtual screening of drug was performed with the crystal structure of the PD-L1 target molecule. The crystalline structure 4zqk was used for PD-L1 from PDB database. The chains of PD-L1 were processed in Discovery studio by deleting the duplicating chains [53]. Chain B of PD-L1 has no role in interaction with PD-1 thus, it was also removed and only single chain of PD-L1 'chain A' was used for molecular docking purpose.

Further, structure was prepared by removing the water molecules, any heteroatom or any co-crystalized molecule followed by addition of polar hydrogen atoms in Discovery Studio and structure was energetically minimized in UCSF Chimera [53], [38].

The site-specific molecular docking was carried out with the FDA approved compound along with the prepared crystal structure of the target PD-L1 both in Schrödinger and AutoDock Vina to screen out the compounds [41], [46]. The screening of compound was based on their interaction at the interface.

For docking results, in Schrodinger only single pose was selected thus, only best binding pose of the compound was appeared as result where in AutoDock Vina, the output results have the 10 poses thus, the binding poses of all docked compound were analyzed in the Pymol and Discovery Studio which results in 2,000 compounds against PD-L1. The selection of screening compound was based on their interacting residues, ligand efficiency, high dock score. Out of those 1,000 compounds were selected which have the dock score greater than -6.00.

Further, to filter out only best compounds, we compared the results of both platform and make a list of those compound which have both high dock score in both platform and they are interacting at the interface.

After selection process, we got the 6 compounds with high dock score as well as interacting with critical residues.

The selected drugs were involved both in conventional and carbon hydrogen bonding along with various electrostatic interaction such as Van Der Waal forces, Pi-alkyl bonding which was analyzed in Maestro and BIOVIA Discovery Studio Visualizer protein ligand interaction tool.

As most of the PD-L1 inhibitors are antibodies which have larger molecular weight, size and very costly to purchase for multiple dosage. These small inhibitors may use against the PD-L1 as it will be cost effective. The blockage of PD-L1 with the small inhibitor has showed the effective result in in-silico however, further validation through in vitro experiment is still required before clinical use.

Table 5.6.2.: Docking scores of the selected compounds in different docking platform

Compounds	Docking Score (Schrodinger)	Docking Score (Autodock Vina)	Interacting residue
RD-1	-8.881	-6.6	**Y 56**, R113, A121, D122, Y123, K124, R125
RD-2	-8.449	-6.4	F19, **Y56**, R113, A121, D122, Y123, K124, R125
RD-3	-8.449	-7	F19, **Y56**, R113, A121, D122, Y123, K124, R125
RD-4	-6.701	-6.7	**Y56**, Q66, R113
RD-5	-6.598	-6.3	F19, **Y56**, Q66, A121, R113, D 122, Y123
RD-6	-6.471	-6.3	R113, D122, Y 123, K124, R125

6. Chapter 6 Conclusion and future scope

Colorectal cancer is known for the 3rd most diagnostic cancer in the world which occur in the colon and rectum region due to several genetic mutations either in chromosome, in microsatellite gene or in DNA repair genes. CRC caused due to lifestyle, diet, family history, inflammatory bowel disease and chronic inflammation. As is closely associated with the chronic inflammation, there are various immune cells are involved such as B cell, T cell, NK cells, DCs and specially macrophages. Earlier it was found that the in the TME or in tumor mass majority of immune cells are tumor associated macrophages (TAM). The relation between TAM and tumor is very strong as the TAM can contribute in the progression or inhibition of tumor in accordance to the stimuli present in TME. There is different form of stimuli are released by cells such as growth factors, cytokines, chemokines and extracellular vesicles. The smallest type of extracellular vesicles are exosomes. Though they are very small still they serve as vehicle for transportation of various constituents form one cell to another. Especially the exosomes release from cancer cells and macrophage, the exosomes of cancer cell can cause the macrophage polarization or reprogramming in pro- and anti-inflammatory macrophage and in response to that exosomes release by these macrophages can promote or inhibit the CRC.

There are some evidences which shows that the condition media of CRC responsible for macrophage differentiation in M1 or M2 type but it was not dissected into when macrophage polarize into M1 type or reprogram in M2 type. Thus, this study aims firstly to dissect the macrophage polarization. In this context, we first started with the condition media and the data obtained from this study showed that the macrophage polarization is more like time dependent process where in early stage there are more like M1 type of macrophages where in later stage they reprogram toward M2 type. Further we were interested into the response of macrophage treated only with

exosome instead of condition media and there we come to know two interesting things. Firstly, the soluble factors are less responsible for macrophage reprogramming towards M2-phenotype. The macrophage shows polarization due exosome very fast and mostly in M2-phenotype. secondly is that phagocytic activity of macrophage is higher in pro-inflammatory i.e., M1 type of macrophage compare to the M2 type. We able to dissect the macrophage polarization due to exosomes but as exosome have various constituents such as miRNAs, lncRNA, proteins, DNA etc. which constituent responsible for the macrophage polarization or reprogramming is still need to explore. There are various miRNAs which are known in CRC for reprogramming of macrophage to M2 type but what the components of exosomes responsible for M1 type is still need to study.

Further we study about the expression of PD-L1 ligand expression. PD-1/PD-L1 is the immune check point which helps in regulation of immune activity through T-cytotoxic cells (Tc). During our study, we determine that the exosomes are responsible for the higher expression of PD-L1 in both macrophages as well as colorectal cancer cell. The high expression of PD-L1 can suppress the immune response to promote the cancer. Thus, to develop a therapeutic strategy against the PD-L1 expression we identified the novel small drug inhibitor via in-silico method. Using the computational tools we identified the compounds against PD-1/PD-L1 interaction. Further studies are required to check these compounds in the in-vitro and in-vivo experiments. Earlier there are various antibody-based immunotherapy being develop against the PD-1/PD-L1 interaction but the small drug inhibitor was not available. Other than this, to develop a potential therapeutic strategy we can target the specific constituent of exosome which is responsible for the PD-L1 expression such as there are two miRNAs known in case of CRC miR-21-5p and miR-200a which led to the expression of PD-L1 in macrophage. So, instead of the indirect target the PD-L1 expression we can block these two miRNAs through anti-miRNA-based therapy. Anti-miRNA is a complementary strand to the miRNA so binding to the miRNA block

the interaction of miRNA with its target gene and thus there will be no overexpression of PD-L1. Targeting through anti-miRNA can be made more effect in two ways either the transportation of Anti-miRNA in encapsulated exosomes or by targeting multiple miRNAs via single long anti-miRNA which have complementarity for 2 or more miRNAs. Further, there is no single component of exosome which responsible for cancer thus study of various component still required

7. Annexure

7.1. List of primer

Table 7.1. Primers used for quantification of RT-PCR

S. No.	Gene Target	Sequence (5' → 3')
1.	h-GAPDAH-F	AATCCCATCACCATCTTCCA
2.	h-GAPDAH-R	TGGACTCCACGACGTACTCA
3.	h-IL-6-F	TTCAATGAGGAGACTTGCCTGG
4.	h-IL-6-R	CTGGCATTTGTGGTTGGGTC
5.	h-TNF-a-F	CCCATGTTGTAGCAAACCCTC
6.	h-TNF-a-R	TGAGGTACAGGCCCTCTGAT
7.	h-IL-1b-F	AGGGACATCATCAAACCTGACC
8.	h-IL-1b-R	GCTGAGGTCTTGTCCGTGAA
9.	h-Arg-1-F	ACTTAAAGAACAAGAGTGTGATGTG
10.	h-Arg-1-R	CACCAGGCTGATTCTTCCGT
11.	h-IL-23-F	CCCAAGGACTCAGGGACAAC
12.	h-IL-23-R	TGGAGGCTGCGAAGGATTTT
13.	h-IL-12B-F	GACCTTGGACCAGAGCAGTG
14.	h-IL-12B-R	GCAGGAGCGAATGGCTTAGA
15.	h-IL-10-F	AAGACCCAGACATCAAGGCG
16.	h-IL-10-R	AGGCATTCTTCACCTGCTCC
17.	h-PDGF-F	CACACCTCCTCGCTGTAGTATTTA
18.	h-PDGF-R	GTTATCGGTGTAAATGTCATCCAA
19.	h-PD-L1-F	ATGGTGGTGCCGACTACAAG
20.	h-PD-L1-R	GGAATTGGTGGTGGTGGTCT

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