# The role of macrophages in the chronic activation of cancer cells and tumour progression

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## Abstract

Tumour microenvironment cells play important roles in tumour growth and progression. Tumour-associated macrophages (TAM) are one of the most abundant inflammatory leukocytes in the tumour microenvironment and known drivers of carcinogenesis.

Therefore, investigating how *in vitro* differentiated macrophages affect cancer cells' biological behaviour in different co-culturing conditions would help in understanding cancer biology and offer insight into novel treatment strategies.

Human monocytic Thp-1 cells differentiated by PMA were used as an *in vitro* alternative model for human peripheral-blood macrophages in this project. As several studies used the co-culture model to test the roles of immune cells in cancer cells, we used PMA-differentiated macrophages to be co-cultured with A549 lung cancer cells under different conditions. Those published papers used co-culturing techniques to investigate cancer cells' behaviour upon short-term co-culturing with macrophages, which differs from *in vivo* tumour biology that arises from several weeks of interactions with tumour microenvironment cells. In the first part of this project, we assessed gene expression and functional changes in A549 cells co-cultured with macrophages for a short period of time (3 days). The results showed that our differentiated macrophages in the co-cultured transwell model system induced gene expression, proliferation, and epithelial-mesenchymal transition in A549 cells.

The second part of this project was established to test the hypothesis that long term crosstalk of cancer cells with macrophages could cause chronic changes in cancer hallmarks that remained after removing the stimuli (macrophages) due to information stored in cancer cell memory about responding to the stimulus. To investigate this hypothesis, we rested A549 cells in single culture after 3 days or 30 days of co-culturing with macrophages.

Our results show that coculturing A549 cells with macrophages for a long time resulted in a sustained mesenchymal transition state and induced proliferation after removing macrophages in long-programmed A549 cells. However, resting A549 cells in single culture after short-term co-culture with macrophages returned them to an epithelial state, while proliferation remained induced in short-programmed cells. Further RNA sequencing data analysis revealed the

similarities between "long-programmed" and "CO 3 days" cells in their differential gene expression pattern and the activation of cancer progression related pathways. However, no significant differences were found between short-programmed and parental A549 cells except for proliferation-related genes expression, which was remained-induced in short programmed A549 cells which reflected in their proliferation functional assays.

# Table of Contents

## List of Figures

# List of Tables

# Chapter 1 Introduction

## 1.1 Lung cancer

### 1.1.1 Lung cancer incidence and mortality

Worldwide, lung cancer (LC) is the leading cause of cancer related death. According to estimates from Cancer Research UK (CRUK), lung cancer is the third most common cancer in the UK. In 2017, more than half of the population, or 48,000 cases, were diagnosed with lung cancer at a rate of 130 cases per day. Around 75–80% of cases of LC are of Non-Small Cell Lung Cancer (NSCLC), which makes up the majority of registered cases globally (CRUK, 2022). Among all cancers in the UK, LC had the highest mortality rates. There were 34,800 LC deaths, 95 every day, accounting for 21% of all cancer deaths (2017-2019). Most of these deaths occurring in people aged 85 to 89 years of age and older (CRUK, 2022).

In terms of LC survival, CRUK records from 2013–2017 in England show that 9.5% of LC patients have a survival of 10 years or more, compared to 16.2% of cancer patients who have a survival of five years or more and 40.6% of people with LC who have a survival of one year or more. Females with LC have higher survival rates than males at one, five, and ten years, and patients under 40 have a better survival, according to reports from 2009 to 2013. In the past 40 years, there has not been a noticeable improvement in the survival rate for LC patients in the UK because of late diagnosis of the advanced stage disease (CRUK, 2022). Although it has been found that patients with lung cancer have a higher survival rate for five years or longer when diagnosed at early stage, the survival rate is only 67%, which is lower than the survival rate for other solid malignancy diagnosed at an early stage (Mountain, 1997; Sant et al., 2003).

### 1.1.2 Histopathological types of lung cancer

Primary epithelial lung cancer carcinoma classified according to their microscopical characteristics into two major types: non-small cell lung cancer (NSCLC) accounting for 80 % of lung cancer cases, and small cell lung carcinoma (SCLC) for the remaining 20% of lung cancer patients (CRUK, 2022). According to Mitchell, NSCLC has subclassified into three types: adenocarcinoma (ADC),

large cell carcinoma (LCC), and squamous cell carcinoma (SCC) (Mitchell et al., 2011).

The microscopical features of the SCC type are characterised by large malignant cells with a high cytoplasmic to nucleus ratio and the presence of intracellular or scattered intercellular keratin surrounded by malignant cells. While ADC types are characterised by glandular formation with mucin production, which can be subclassified according to their molecular features into preinvasive and minimally invasive types (Travis et al., 2013). LCC type has different features and cell origin from SCC and ADC and is characterised by sheets of large round cells with plate-staining cytoplasm and vesicular nuclei (Mitchell et al., 2011).

## 1.2 Tumour microenvironment

### 1.2.1 Macrophages phenotypes and polarization

Macrophages are heterogeneous group of cells with different roles depending on the location where they developed. They are components of tissue microenvironment change their functions and phenotypic characters in response to many diseases, including autoimmune disease, chronic inflammatory disease, and cancer (Gordon, 2007). It is known that there are two distinct populations of macrophages in normal tissue states, blood monocyte derived macrophages (BM-DMs) and tissue resident macrophages. BM-DMs differentiate from hematopoietic stem cells in the bone marrow and start to self-renew their populations once placed in adult tissue. However, tissue resident macrophages maintain themselves in tissues separately from bone marrow precursors (Figure 1). In response to inflammation, BM-DMs developed to macrophages once recruited to inflamed tissue and cooperate with resident macrophages to maintain tissue repair (Yona et al., 2013). Some studies revealed that BM-DMs in lung have different gene expression from tissue resident macrophages, suggesting that they have different roles in tissue repair (Gibbings et al., 2015). However, bone marrow transplant studies have shown that BM-DMs and tissue macrophages have common characteristics (van de Laar et al., 2016).

The most commonly used classification of macrophages emerged in the 1990s, when Stein and his team found that macrophages had distinct gene expression in response to different stimuli. They classified macrophages into two groups: M1, which polarized in response to bacterial molecules such as lipopolysaccharide ,and M2, which activated in response to T helper 2 cells cytokines interleukin such as IL-4 and IL-10 (Stein et al., 1992). For validation of this classification, Gordon and Taylor found that each type differs in the cytokines production, M1 macrophages produce pro inflammatory cytokines that induce T helper 1 (Th1) cells' responses with anti-tumor activity. However, M2 macrophages secrete cytokines that stimulate Th2 cells responses with pro-tumorigenic and anti-inflammation activity (Gordon and Taylor, 2005).

In 2014, Murray and his research group observed the plasticity and complexity of macrophages in the tumor microenvironment (TME), and found that macrophages are likely to exist in heterogenous activation states driven by microenvironmental signals at any time rather than following M1/M2 classification (Murray et al., 2014). This observation contradicts the old M1/M2 classification, which is generally classified as outdated and inconsistent with the heterogenous theory of macrophages within TME (Hao et al., 2012). According to this, researchers in the field suggest using different methods of macrophage classification. Some researchers used cytokine stimulation conditions to classify macrophages; for example, M (LPS) refers to activated macrophages by LPS stimuli, and M (IL4) refers to the M2 phenotypic activation state stimulated by IL4 cytokine (Noy and Pollard, 2014; Martinez and Gordon, 2014). However, this classification has not been used in all publications, and M1/M2 nomenclature is still being applied in new publications. Most cancer studies that used the M1/M2 classification mentioned the method of polarization, which is a nomenclature guideline followed when using M1/M2 classification. In most cancer studies, this classification is used as an indication of tumor progression behavior results from macrophage polarization to M2 tumor progression phenotype when co cultured with cancer cells.

### 1.2.2 Tumor associated Macrophages (TAMs)

TAMs are the dominant leukocyte population found in the tumor microenvironment in all tumor types; their roles have been extensively investigated in the past and are still under investigations. In 1970, Evans and Alexander reported that TAMs role in cancer is to kill cancer cells by cytokine activation (Evans and Alexander, 1970). However, further studies have reported that TAMs actively promote all aspects of tumor initiation and development (Mantovani et al., 1979). In 2001 animal studies, Lin and his team found that TAMs have a significant role in enhancing tumor growth and metastasis in breast cancer (Lin et al., 2001).

The source of TAMs in TME is a mixture of tissue resident macrophages and BM-DMs; this precursor heterozygosity is reported in many solid tumors, including glioma and pancreatic cancer (Figure 1) (Feng et al., 2015; Zhu et al., 2017). In pancreatic ductal adenocarcinoma (PDAC) study, they indicated that the precursor of macrophages influences tumor development, while BM-DMs macrophage deletion had no effect on PDAC development, the ablation of tissue resident macrophages reduced tumor progression, which reflects the importance of determining TAMs source in the treatment of cancer (Zhu et al., 2017). As macrophages affect tumor phenotype, other cellular components influence TAMs phenotype in TME.

It has been reported that TAMs could exist in both protumor and antitumor phenotypes in the same TME which is an effect of the signals and secretions of tumor and immune cells present within the TME (Guo et al., 2016). Although heterogeneous populations of TAMs are reported in many tumors, animal based studies in addition to clinical data support the hypothesis that the pro-tumorigenic phenotype is the dominant phenotype of TAMs in TME (Zaynagetdinov et al., 2011; Gordon and Mantovani, 2011). In clinical data reviewed by Bingle and his team for many cancer types, they found that there is a strong correlation between TAMs density and poor prognosis in breast, prostate, bladder, and NSCLC in human cancer tissue, which represents 80% of cancer studies (Bingle et al., 2002). Moreover, in animal model study, Lin and his team found that injecting mice with macrophages enhanced breast tumor growth and metastasis (Lin et al., 2001).

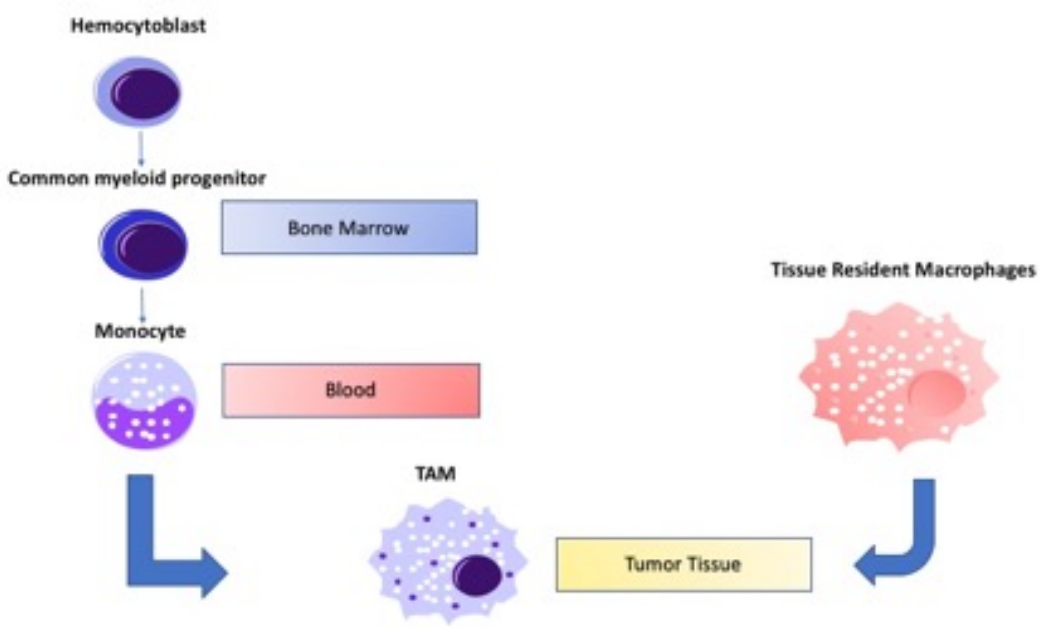



**Figure 1 Precursors of TAM in TME**

Monocytes are continuously produced in bone marrow by hematopoietic stem cells. Precursors of TAM in cancer are monocytes from blood and local tissue-resident macrophages.

## 1.3 The Crosstalk between Tumour associated macrophages and cancer cells

Accumulating data indicate that the crosstalk between tumour and macrophages mediates phenotypic and functional changes in both cell types. As the interaction between macrophages and cancer cell is driven by cytokines released from both cells, the recruitment of macrophages into TME are controlled by chemokines and growth factors, as well as their survival and infiltration within TME (Solinas et al., 2009)(Figure 2). Roca and his research group found that CCL2 chemokine (monocyte chemoattractant protein-1, MCP) recruits the circulating blood monocyte into tumour site and maintain their survival of pro tumorigenic M2 phenotype (Roca et al., 2009)(Figure2). CCL2 is produced by tumour cells and macrophages, and clinical data in NSCLC indicate that macrophages infiltration is associated with high expression of chemokine receptors on tumour cells and poor prognosis in lung cancer patients (Wu et al., 2008). Other cytokine families such as CCL3 were also found to participate in the recruitment and infiltration of macrophages in the murine lung progression process (Wu et al., 2008).

Several studies have investigated how the tumour promoting activity of TAMs achieved by studying cancer cell signalling and underlying mechanisms that influence macrophage phenotypes (Hagemann et al., 2006; (Voss et al., 2017). Most of these studies have used the co-culture model to mimic the *in vitro* crosstalk in TME between two cells. In co cultured macrophages with ovarian cancer cells, it has been observed that tumour secretions upregulate cytokine expression in macrophages such as CSF-1, IL-12, IL-6, and CCL2 which induce the M2 tumorigenic phenotype (Hagemann et al., 2006). Furthermore, Voss and his team found that apoptotic lymphoma cells enhance the protumour activation state of TAMs and inhibit antitumor activity (Voss et al., 2017). Another study found that the hypoxic tumour environment induced Neuropilin 1 (NRP1) expression, a co receptor for VEGF, in cervical cancer, which enhanced the polarization of M2 protumour TAM phenotype (Chen et al., 2019). In addition to cancer cells apoptotic and hypoxic effects on TAMs polarization, a study done by Wenes and his team found that tumour cells mediate the metabolic changes in macrophages to induce M2 like phenotype (Wenes et al., 2016). Regarding cancer cell signalling that controls cytokine production in TAMs, it has been found that dysregulation of nuclear factor Kappa B (NF-KappaB) signalling in tumour cells maintains the immunosuppressive phenotype of TAMs through activation of interleukin (IL)-1R and MyD88 (Hagemann et al., 2008). All these mechanisms enhance the theory that inflammatory macrophages M1 is active during the early stages of cancer, but as the cancer progresses, this M1 macrophage transforms into the alternative tumour- promoting M2 phenotype (Lin et al., 2001). Later on, in the dynamic interactions of activated TAMs with cancer cells, these activated pro tumorigenic TAMs enhance all aspects of the cancer hallmarks.

### 1.3.1 The role of Tumour associated macrophages (TAMs) in cancer development and progression

In the process of cancer progression, malignant cells acquire several oncogenic mutations that enhance their growth, proliferation, metastasis, and migration to other parts of the body. In addition, it has been found that the immune system plays a major role in providing a mutagenic inflammatory environment during cancer progression, which supports cancer hallmarks (Qian and Pollard, 2010). According to this, in the past few years, many researchers have shifted their

research from investigating cancer cells alone into studying the roles of TME components in tumour progression (Yuan et al., 2016). In chronic inflammation, macrophages play an important role in maintaining the inflammatory response to the constant exposure to the causative stimulus that predisposes the tissue to cancer (Balkwill et al., 2005). This type of chronic inflammation raised in response to long term exposure to stimuli is associated with an increased risk of cancer in infected tissue called smouldering inflammation (Mantovani and Sica, 2010). In the lung, it has been found that long term infection with the Haemophilus influenzae virus causes chronic obstructive pulmonary disease (COPD), which increases the risk of lung cancer. In animal model, it has been found that injecting the lysate into mice caused COPD which induced the progression of lung cancer by increasing the number of lung surface tumours (Moghaddam et al., 2009).

In investigation the reasons for developing cancer in chronically inflamed tissue, researchers have found that DNA damage occurred in mice infected with chronic ulcerative is driven by reactive oxygen species production and a deficiency of DNA repair mechanisms, which predispose tissue to cancer (Meira et al., 2008). In addition to genetic instability, the interaction between tumour and macrophages cells results in the production of cytokines and chemokines, which play an important role in the proliferation of mutant epithelial cells and modulating the signalling of cancer cells (Sarode et al., 2020).

Considering the accumulating clinical data that indicate the association of high density of TAMs with poor prognosis in lung cancer patients and other solid tumours, researchers have started to investigate the nature of this connection at the molecular level (Takanami et al., 1999; Chen et al., 2005). In 2001, Lin and his team found that TAMs not only promote cancer growth in breast tumour but also enable the progression of cancer. Based on the finding that macrophages colony stimulating factor 1 (CSF 1) is associated with poor prognosis in human breast carcinoma, they developed mice with heterozygous mutations of CSF1 and observed that these mice developed tumours earlier than mice with full loss of macrophages (Lin et al., 2001). It has also been found that CSF1 promotes the infiltration of TAMs in the primary tumour site, which later stimulates the vascular endothelial growth factor (VEGF) secretion that controls cancer cell angiogenesis (Lin et al., 2007).

In the first study that investigated the role of TAMs in tumour angiogenesis *in vitro*, Bingle and his colleagues found that transplantation of breast tumours spheroids with macrophages into mice results in upregulation of VEGF release and more blood vessel formation than the tissue from the spheroids without macrophages (Bingle et al., 2006). This reflects the importance of TAMs in angiogenesis, which is considered the basic step in tumour progression that allows the access of malignant cells to enter the blood circulation and migrate to distant metastatic sites (Sherwood et al., 1971).

### 1.3.2 The Role of tumour associated macrophages in cancer invasion and metastasis

Metastasis is the process of acquiring invasion properties by tumour cells, which enable their migration and motility from the primary site of cancer to a distant site. It has been known for all tumour types that metastasis is the leading cause of death in cancer patients (Steeg, 2016). Therefore, many researchers have studied the process of metastatic cancer to enhance the development of new treatment strategies that could improve the poor survival outcome.

The first step of malignant cells to metastasize from the primary site is the destruction of the national barrier of the extracellular matrix (ECM), which contains the basement membrane and the interstitial tissue. Then, tumour cells enter the bloodstream and lymphatic system to colonise at distant organ. Paget (1889), who proposed the "soil and seed" hypothesis in explaining how the metastasis process works with the contribution of the TME, hypothesised that tumour cells represent the seeds that are colonising the soil, which represents the metastatic environment in breast cancer (Paget, 1989).

The epithelial to mesenchymal transition (EMT) process is the extensively studied mechanism that proposed for explaining the soil and seed theory. It is defined by the loosening of cell-cell junction and deregulation of adhesion molecules in epithelial cancer cells in order to acquire a malignant phenotype. While this process is regulated by a complex network of signalling pathways and transcription factors, it has been found that loss of E-cadherin is the main regulator and initiator of mesenchymal transition in cells adherent junctions (Birchmeier and Behrens, 1994; Schneider and Kolligs, 2015). The progressive

loss of E-cadherin in epithelial tissue is associated with increased gene expression of several transcription factors, including zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2, SNAIL1, SNAIL2, and TWIST family members (Craene and Berx, 2013). The activation of these transcription factors following the loss of E-cadherin induces the intracellular signalling such as MAPK, PI3K, ERK and Ras in epithelial cells (Tse and Kalluri, 2007).

While E-cadherin is downregulated during EMT, N-cadherin is upregulated, which called “cadherin switch” that mediates invasive tumorigenesis (Araki et al., 2011). It has been found that high expression of N-cadherin correlated with cancer metastasis in many cancer types, such as NSCLC and prostate cancer (Jennbacken et al., 2010; Hui et al., 2013). N-cadherin forms weak adherent junctions in non-epithelial malignant tissue, lead to the activation of several signalling pathways, including MAPK, ERK and platelet-derived growth factor receptor (PDGFR) that enhance malignant cell survival and metastasis (Loh et al., 2019).

Following EMT as the key mechanism that has drawn researchers’ interest, the interaction between tumour and TME has lately emerged as the second mechanism that helps in understanding the severity of metastasis (Tse and Kalluri, 2007). In several tumour types, it has been approved that TAMs support the metastasis process in cancer cells (Zhang et al., 2015). It has been found that there is a bidirectional crosstalk resulting from the co culturing of cancer cells with M2 macrophages, which leads to the release of the same factors from both cells to promote tumour progression and metastasis (Schmall et al., 2015). IL-6 is one of the cytokines that mainly secreted by macrophages and play an important role in EMT process (Mauer et al., 2015). In clinical data, it has been found that high level of IL6 correlates with poor prognosis in lung cancer patients (Pine et al., 2011). In an *in vitro* study done by Dehai and his team, they co cultured the A549 cell line with Thp-1 differentiated macrophages and observed increased IL-6 expression, which correlated with the induction of EMT in lung cancer cells (Dehai et al., 2014). Additionally, it has been found that macrophages produced many proteolytic enzymes within TME, such as matrix metallopeptidases (MMPs), which help in breaking down the extracellular matrix and allow cancer cells invasion and metastasis (Chanmee et al., 2014). In TAMs isolated from human lung cancer tissue, MMP9 and VEGF mRNA gene expressions were correlated

with increased lung cancer cell migration and invasion (Wang et al., 2011). In addition to these factors, it has been found that TAMs promote tumour metastasis by secreting Wnt family member (WNT) 5A, epidermal growth factor (EGF) and IL-1β, which are essential factors in establishing tumour growth and metastasis (Hagemann et al., 2004; Coffelt et al., 2009).

In breast cancer, the most frequently referenced study approved the important role of CSF-1 in the induction of tumour metastasis by observing that the PyMT mice model with CSF-1 homozygous mutations has a lower rate of cancer metastasis than wild type mice with overexpressed CSF 1 (Lin et al., 2001). The same observation has found in pancreatic cancer when Mitchem and his colleagues found that metastasis rate decreased in mouse model by depletion of macrophages through CSF1 inhibition (Mitchem et al., 2013). In addition to the roles that TAMs play in the induction of cytokines that promote the EMT process in TME, it has been shown that co culturing M2 macrophages with lung cancer cells upregulates the proteins involved in cell proliferation and motility. A study done by Guo and his teams found that αB-Crystallin protein (CRYAB) expression is upregulated in co cultured lung cancer cells by M2 macrophage signals, which induce EMT and lung cancer metastasis (Z. Guo et al., 2019). In clinical data of many cancer types including breast cancer, laryngeal carcinoma, renal carcinoma, and hepatocellular carcinoma show a correlation between CRYAB overexpression and tumour metastasis with poor prognosis (Moyano et al., 2006; Stegh et al., 2008; Huang et al., 2013).

As many studies have indicated that TAMs cause cancer cell invasion, it has been shown that TAMs also enhance tumour survival in blood circulation and promote the generation of tumour niche at the distant site (Nielsen and Schmid, 2017).

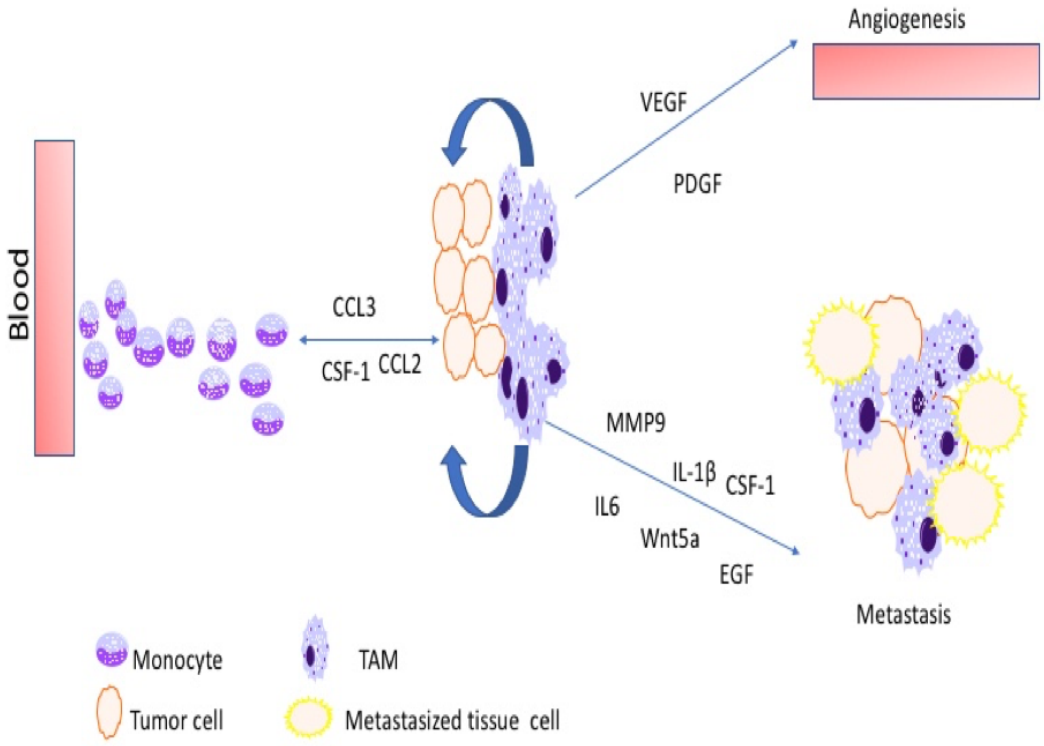



**Figure 2** Crosstalk between TAM and cancer cells induces macrophages recruitment and the release of chemokines and cytokines from both cells that promote tumour angiogenesis and metastasis (adapted from (Hao et al., 2012)

### 1.3.3 Oncogenes and TME

It has been known that the genomic components of tumour play an important role in shaping the landscape of tumour microenvironment. This enhanced by the genetic information of the Cancer Genome Atlas (TCGA) which were correlated with RNA data of the immune system cells activity. They found that tumour signalling pathways activated through gain of functions in oncogenes or loss of functions in tumour suppressor genes, which also enhance cancer cells immune evasion through activation of anti-immune response of TME (Spranger and Gajewski, 2018). Moreover, Rooney and colleagues found that high density of immunosuppressive components of TME specifically macrophages were correlated with activation of TP53 tumour suppressor gene and PIK3C oncogenes (Rooney et al., 2015).

In investigation the mechanisms of cancer cells immune evasion to understand cancer resistance to anti-immune therapies and improve cancer treatment, researchers found that immune evasion mechanisms of cancer activated by

tumour suppressor genes or oncogenes mainly impair T cell functions by different methods. These methods include suppression the tumour antigens elements that present tumour to immune system or recruitment of anti-immune cells to the tumour site by upregulation of chemoattractant cytokines (Wellenstein and de Visser, 2018).

The most mutated tumour suppressor gene in cancer is Trp53, and its roles in cancer have been widely investigated. It has been found that silencing of TrP53 resulted in the reduction of immune cells infiltration and immune evasion of cancer cells (Xue et al., 2007). However, restoration of Trp53 resulted in tumour regression and increased expression of IL-1β, IL-12β and IL-15 cytokines that mediate immune cells infiltration (Iannello et al., 2013). In investigation the mechanisms underlie P53 loss in promoting of tumour, recently Wellenstein and colleagues found that silencing of P53 in mouse breast cancer caused the release of WNT ligands, which in turn promote tumour-associated macrophages to release IL-1β , causing neutrophilic inflammation and promote cancer metastasis (Wellenstein et al., 2019).

In addition to P53 mutations, liver kinase B1 (LKB1), a tumour suppressor gene, found mutated in 30 % of NSCLC patients associated with mutations in K-RAS gene and bad prognosis of patients (Ji et al., 2007). In mouse model, LKB1 deletion mutations found to be associated with induction of neutrophil infiltration and reduction of T cells and increased proinflammatory cytokines production (Koyama et al., 2016). Loss of LKB1 led to increased expression of immunosuppressive IL-6 and CXCL7 cytokines, whilst blocking of IL-6 caused lkb1-defecient tumours response to anti-tumour therapy (Koyama et al., 2016). In addition, Phosphatase and tensin homolog (PTEN) gene is another tumour suppressor gene found highly mutated in lung cancer and other tumour types which its loss found to be associated with increased expression of immunosuppressive cytokines such as VEGF and CCL2 (Peng et al., 2016).

On the other hand, MYC gene is an oncogene found highly expressed in aggressive tumours phenotypes. It is a transcription factor caused the upregulation of many genes involved in cell differentiation and proliferation (Dang, 2012). In mouse model, it has been observed that MYC inactivation resulted in remodelling the tumour microenvironment through activation of $CD4^{+}$T cells which enhances anti-tumour immune response (Sodir et al., 2011). Another

immunosuppressive role of MYC has been reported by Kortlever and his team, they observed that MYC activation resulted in macrophages recruitment through CCL9 and IL-23 secretions which impaired T cells function and induced the immune evasion of cancer cells (Kortlever et al., 2017).

Furthermore, EGFR is one of the ErbB family of receptor tyrosine kinases, acts as oncogene when overexpressed in cancer. The phosphorylated form of EGFR causes the activation of signalling pathways involved in cancer cells proliferation and differentiation. In NSCLC, it has been found that EGFR overexpression enhances the cancer cell with multiple binding sites of EGF which activates the signalling pathways involved in cancer metastasis and chemoresistance (Hynes and Lane, 2005). In molecular analysis of NSCLC patients' samples, it has been found that patients developed resistance to Gefitinib, a tyrosine kinase inhibitor, when they acquired secondary EGFR T790M mutation which interfere with inhibitor binding (Pao et al., 2005; Kumar et al., 2008). Additionally, K-RAS gene is another oncogene found highly mutated in lung cancer and other epithelial cancer types. It is mutations found in 20-30 % of lung adenocarcinoma patients and associated with chemoresistance and poor prognosis (Califano et al., 2012). K-RAS gene encodes GTPase membrane-bound protein, which can be found in two forms: GTP bound (active) or GDP bound (inactive). The GTP bound state activates the downstream intracellular signalling pathways that mediate tumorigenesis in NSCLC such as MEK and Raf (Ferrer et al., 2018). In investigation the effects of KRAS signalling on the immune microenvironment, Sparmann and Bar reported that the expression of mutant Ras led to increase the pro-tumorigenic CXCL8 transcription in human cells (Sparmann and Bar-Sagi, 2004). Also, the expression of mutant Ras in human kidney cells reported to increase IL-6 secretions which promotes tumour vascularisation (Ancrile et al., 2007).

## 1.4 Cellular reprogramming during cancer progression

The multiple ways in which TAMs affect tumour development highlight the significance of immune cells in the carcinogenesis of many cancer types. As being mentioned, the interaction between cancer and immune cells through cytokines and chemokines released in TME is what drives the dual function of macrophages, which either promote or eliminate cancer cells. As long as the TAM M2 pro-tumorigenic phenotype is activated during tumour growth, cancer cells are continually exposed to cytokines and chemokines, which promote aggressive metastasis and more therapy-resistant malignant cells. When cells permanently change their behaviour in response to stress, this is known as cellular reprogramming, and it has been considered as a therapeutic target in cancer research studies. This is related to a biological process called “cellular memory,” which defined as “persistence in cell state acquired overtime and can be triggered by a transient stimulus or environmental signals”(Berenguer and Celià-Terrassa, 2021). The resulting state of cells is called “cellular reprogramming” because cells change their identity in response to stimuli, and the changes produced persist after the stimulus withdrawal.

In normal conditions, cellular reprogramming occurs when cells respond to some extracellular signals during development, such as enzyme secretion, which causes permanent changes in cells that persist after the disappearance of the extracellular signals. These changes occur due to the self-activating memory system of cells, which developed later to induce gene transcription further along a signaling pathway (Alberts et al., 2002). In cancer, this phenomenon has been observed and referred to as one of the causes of cancer recurrence in some cases due to the cancer cells' memory ability of storing the information of responding to stimulus in a long-lasting manner (Hudson, 2021). Furthermore, recent research suggests that transitions between epithelial and mesenchymal states in cancer are controlled by cellular memory processes that regulate gene regulatory networks in cancer cells (Wang et al., 2009; Thiery et al., 2009).

Regarding the reprogramming of cancer cells in TME, several studies have investigated the interaction between tumours and macrophages using a co-culture model, but the results were used to promote prognosis rather than identify the molecular mechanisms that influence cancer cells’ malignancy (Fukuda et al., 2006). Additionally, whereas tumours result from prolonged interactions with

immune cells, most *in vitro* co culture studies incubated cells for short term(3-5 days), which is different from the biology of cancer development (Fukuda et al., 2006). Furthermore, it has been reported that genetic reprogramming of immune cells happens when they are exposed to stimuli for a long time, which affects their responses to subsequent changes within TME (Dominguez-Andres and Netea, 2019). Given these findings, we hypothesise that long-term co-culture systems reprogram the biological activity of tumour cells as well as the immune system.

### 1.5 Targeting Tumor associated macrophages in cancer treatment

Due to the association of TAMs infiltration with poor prognosis in many cancer types, many researchers have investigated the pathways of TAMs polarization and their involvement in tumor progression to develop new strategy of immunotherapy targeting TAMs (Zaynagetdinov et al., 2011; Gordon and Mantovani, 2011). As it has been known that TAMs are recruited form blood monocytes by cytokines released from tumor and polarized to TAM protumor phenotype, a study done by Tang and his team mentioned that one of the methods of targeting macrophages in cancer is the inhibition of macrophages recruitment to tumor cite (Tang et al., 2013). This could be achieved by inhibition of C-C motif chemokine ligand 2 (CCL2) or Macrophages- colony stimulating factor (M-CSF), which are found overexpressed in tumors with high density macrophages and associated with poor survival (Gazzaniga et al., 2007; Zhu et al., 2011; Qian et al., 2011; Zhang et al., 2014). An examples of monocyte recruiting inhibitors molecules are: Trabectedin which block CCL2, and JNJ-28312141 targeting M-CSF. Both drugs are found to be effective in decreasing TAMs density and supressing tumour growth (Allavena et al., 2005; Kubota et al., 2009). In addition, inhibition of VEGF and CCL5 is another way of reduction of macrophages density because they are chemoattractant that recruit macrophages into tumour site to enhance tumour progression (Roland et al., 2009; Simon, 2010). Another way of decreasing macrophages density in tumour tissue is inducing their apoptosis by chemical reagents such as Bisphosphonates (Rogers and Holen, 2011).

Taking an advantage of macrophages plasticity, some treatment strategies target macrophages polarisation pathways to enhance tumoricidal M1 phenotypes or block M2 phenotypes of TAMs in TME (Tang et al., 2013). Hagemann and his

team polarised M2 TAMs into anti-tumour phenotype (Hagemann et al., 2008). In addition, *in vivo* study on animal model indicates that TME in lung cancer changes their phenotype by ablation of chemokines factors that results in transforming M2 pro metastasis macrophages into M1 tumoricidal macrophages, which provide promising method of lung cancer therapy (Schmall et al., 2015).

Example of the pathways that involve in M1 phenotype activation is the nuclear factor- ΚB (NF-ΚB) pathway, the upregulation of this pathway results in stimulation of T helper type 1 cytokines to induce the M1 phenotype (Biswas and Lewis, 2010). The Ginsenoside Rh2 (G-Rh2) is a monomeric compound has approved to have anti-tumour effects by regulating (NF-KB) and reducing metastasis in lung cancer cells (Li et al., 2015). In addition, the signalling transducer and activator of transcription (STAT) pathways have been targeted in enhancing tumoricidal TAMs phenotypes by upregulation of STAT1, which is found to be associated with M1 phenotypes (Sica and Bronte, 2007). Eubank and his team reported that GM-CSF is an effective drug upregulate STAT1 and enhance the anti-tumour M1 phenotype in TME (Eubank et al., 2009).

On the other hand, STAT3 and STAT6 are associated with M2 phenotypes, blocking them by specific dugs lead to stop the tumorigenic activity of M2 TAMs phenotype (Sica and Bronte, 2007). Examples of these drugs are WP1066 for STAT3 inhibition and AS1517499, leflunomide, and TMC-264 for STAT6 blocking (Hussain et al., 2007; Weisser et al., 2011).

**Aims and objectives**

**Aim 1:** Generate PMA based differentiated macrophages by determining the optimal PMA concentration for Thp-1 treatment.

**Aim2:** Evaluate the crosstalk effects and functional roles of macrophages on A549 cells upon short time co culturing.

**Aim 3:** Evaluate the pro tumorigenic roles of macrophages on lung cancer cells clone after short and long term co culture with macrophages to evaluate whether long term (30 days) co culture will cause chronic changes in cancer cells genes expression and functional behaviour compared with those A549 cells exposed shortly to macrophages.

**Aim4:** Conduct RNA-seq analysis to identify gene expression profile in A549 lung cancer cells after short-term and long-term co-culture with macrophages to evaluate whether long-term (30 days) co-culture will chronically up-down-regulate gene expression in tumour cells compared with those rested after co-cultured with macrophages for short-term (3 days) incubation. This data compared to gene expression profile of A49 cells co culture with macrophages for 3 days without resting and parental A549 cells.

# Chapter 2 Materials and Methods

## 2.1 Cell culture

### 2.1.1 Cell maintenance

A549 cell lines were obtained from cell signaling and cultured in Ham's F-12K media purchased from Thermo Fisher Scientific (Life Technologies). Thp-1 were derived from cell signaling and cultured in RPMI purchased from Sigma Aldrich.

Both RPMI and F-12K media were supplemented with 10% Fetal bovine serum (FBS) (Sigma Aldrich), 1% Penicillin-Streptomycin (Thermo Fisher Scientific), 0.1% β-mercaptoethanol and 1% Glutamine while Ham's F-12K was ready supplemented with Glutamine. Both cells were cultured at 37˚C of 5% CO2 incubator with humidified atmosphere. They were maintained at 70% confluence and split in a 1:2 dilution factor.

For rinsing cells, Dulbecco's Phosphate Buffered Saline (DPBS) from Gibco™ life technologies were used to wash adherent cells. For adherent cells trypsinization, cells were treated with 1x trypsin-EDTA 0.05% (Thermo Fisher Scientific) and incubated for 2-3 minutes to dissociate adherent cells from the flask and pass them to new one. All cells were subculture every 3-4 days.

### 2.1.2 Trypan Blue method for cell counting

For cell proliferation assessment, cell suspension was mixed with trypan blue solution (Sigma Aldrich) at a 1:1 ratio and cell numbers were counted under an inverted phase contrast microscope by a hemocytometer. Trypan blue stained dead cells blue, while bright cells are counted. The sum of living cells count in the 4 quadrants was calculated and the average of 4 was multiplied by the dilution factor and $10^6$ factor of slide. The resulted number is the number of cells in the dimension of million cells per ml.

### 2.1.3 Cryopreservation of cells

To freeze cells for long term storage in -80 ˚C cell suspension was centrifuged at 1200 rpm for 5 minutes at room temperature and the pellet was resuspended in cold freezing media contains media for frozen cells and 10% Dimethyl Sulfoxide

(DMSO). Then, cells were directly aliquoted as 1 ml aliquots into 2 ml cryotubes and stored in a freezing container at -80 ˚C for long term storage.

### 2.1.4 Generation of single cell clone

In order to get a single cell clone, A549 cells were cultured in F-12K media and trypsinised when they reached 80-90% confluence. Cells were counted at 1x106 cells/ml as a final volume, and by using multichannel pipette 175 µl of fresh media were pipetted into all columns of 96 well plates from A to G. Then, 50 µl of cell suspension were added into the first column from A1 to G1. 175 µl from first column were taken and added into the second column (A2-G2) and another 175 µl were taken from second column and added to the third column (A3-G3). The third, fourth, and A11-G11 were reached by repeating this. Each time cells were mixed up and down 3 times to obtain sequential dilution. Another 96 well plate was used to get another dilution and 175 µl were taken from the last column of the first plate (A11-G11) and added to the first column of the second plate (A1-G1) which already contains 175 µl of fresh F-12K media. The same serial dilution applied to all columns until the last column of second plate. The two plates were cultured in 37 $^0$C incubator with regular checking to ensure each well contains healthy growing single cell. Once cells in 96 wells plates began to divide and proliferate, 5 different clones of A549 cells were selected, washed 3x with PBS and trypsinised with 10 µl trypsin. Then, cells in each well transferred into new well of 12 wells plates, cultured until they were confluent and transferred into 6 well plate. For the last cell expansion, cells were transferred into T25 ml flask and kept for one week to reach the required confluency. Then, each cells clone was trypsinised and frozen in -80 ˚C labelled with clone numbers for further use.

### 2.1.5 Phorbol-12-myristate-13-acetate (PMA) based Thp-1 differentiation into macrophages

### 2.1.6 Optimization of lowest PMA concentration for Thp-1 differentiation

Thp-1 monocyte were plated at density of 0.5 X $10^6$ in 6 wells plate. They were treated with different concentrations of PMA 25, 50 and 100 ng/ml to determine the low optimal concentration. The selection of these concentrations and 48 hrs

duration of treatment with PMA are based on most common protocol from the literature (Starr et al., 2018; Pinto et al., 2020). Then, cells were washed 3x with PBS and trypsinised with 1x trypsin and collected for gene expression analysis of macrophages differentiation markers which reflects the differentiated status of PMA based differentiated macrophages. Primer's list of macrophages differentiation markers in table 1.

### 2.1.7 Determining resting time for differentiated Thp-1 post PMA removal

After Thp-1 monocyte treatment with 50 ng/ml for 2 days, cells were washed 3x with PBS and supplemented with fresh media to rest for 2 or 4 days. This is called resting time and cells were labeled 2 days rest (Condition B) and 4 days rest (condition C) in this experiment compared to 2 days treatment without rest (Condition A). After completion of determined resting time, each well was washed with PBS to remove detached cells and trypsinised attached cells. To assess cells adherence, hemocytometer technique was used to count and calculate the percentage of the attached cells in each resting time. RNA was extracted from attached cells for PCR analysis of macrophages differentiation markers gene expression.

### 2.1.8 Assessment of macrophages differentiation in co-culture system with lung cancer

Thp-1 monocytes were seeded in 6 well plates at density of $0.5x10^6$ and treated with 50 ng/ml PMA for 48 hrs incubation. After 48 hrs, cells were washed 3x with PBS and rested for 48hrs with fresh media as previously determined resting time for differentiated macrophages. After resting period, $0.5x10^6$ A549 lung cancer cells were plated in Polyethylene Terephthalate (PET) Trans-well membrane inserts (pore size 0.4 mm) of 6 well plates 2 hrs before the co -culture experiment to allow cells monolayer adherence and inserted into macrophages 6 well plate. The same conditions of single macrophages culture applied for co cultured macrophages in double culture, condition B (2 days rest) and condition C (4 days rest), which compared to single culture conditions. After each resting time of co cultured macrophages, A549 cells in inserts plate were removed and macrophages were washed with PBS to remove detached cells, trypsinised and counted by hemocytometer technique. Also, the same experiment was done and

macrophages in double cultured were lysed by TRIzol for RNA extraction and RT-PCR assessment of macrophages markers expression in each resting time.

| Gene | Forward primer | Reverse primer | Annealing temperature ($C^0$) | Cycles |
|---|---|---|---|---|
| CD11b | TTTCTGCCCTTCT TTGCTTTGG | TAGTCGCACTGGTAGAG GCT | 58 | 28 |
| CD68 | ACAGGGAATGAC TGTCCTC | ACGTGTAGTCTCCAAGG TC | 58 | 28 |
| TLR4 | AGAATGCTAAGGT TGCCGCT | CGGAGTCTGAAAGCTCT GGG | 58 | 28 |
| IL-1B | TGTACGATCACTG AACTGCA | GAAGTCAGTTATATCCT GGC | 58 | 28 |
| GAPDH | TCCCATCACCATC TTCCA | CATCACGCCACAGTTTC C | 58 | 28 |

**Table 1** List of primers and conditions used for RT-PCR analysis to assess Thp-1 differentiation into macrophages

## 2.2 Characterization of A549 lung cancer cells in co-culture model

### 2.2.1 Short term co-culture of A549 cells with macrophages and generation of short reprogrammed A549 cells

Thp1 differentiated macrophages of 0.5 x $10^6$ cells density were plated in 6 wells plate with 2mls of RPMI medium, and the same density of A549 cells (1:1 ratio) were plated in 6 trans-well insert in 2mls of Ham's F-12K medium. A549 cells were plated in Polyethylene Terephthalate (PET) Trans-well membrane inserts (pore size 0.4 mm) of 6 well plates (corning) 2hrs before the experiment to allow cells to adhere and make a monolayer. Then, A549 cells trans-well were inserted into 6 wells plate containing 2 days rested macrophages as described before, and the whole 6 well plate with the inserts were incubated for 72 hrs days without changing media. In parallel, 0.5 x$10^6$ A549 cells plated in trans-well inserted into empty 6 wells plate containing F-12K media, 2 ml top of trans-well and 2 ml below as a control of A549 cells co cultured experiment under the same condition (Figure 3). Then, A549 cells co cultured with macrophages and A549 cultured alone were collected after 3 days for further RNA and protein analysis for assessment of A549 co cultured with macrophages for short term period (3 days).

The same 3 days co-culture experiment were done on different single cell clones of A549 with macrophages. After 3 days of co-culture, lung cancer cells were maintained in fresh F-12K media and expanded in T25 flask for one month without macrophages. After one month, some cells were lysed with TRIzol for RNA extraction and labelled as “short reprogrammed” A549 cells. The rest of cells were collected for protein analysis and other were frozen in -80 $^0$C for future experiment. Primers used for upregulated genes amplification in co cultured A549 cells are listed in table 2. Antibodies used for western immunoblotting are listed in table 3.

### 2.2.2 Long term co-culture A549 cells with macrophages and generation of long re programmed A549 cells

For long term co-culture of macrophages with A549 cells, single cell clones of A549 were plated in T25 ml flask until they reach 80-90 % confluent. Then, A549 cells were trypsinised and plated in 6 well corning insert at 0.5 x$10^4$ density for each well two hours before co-culture with macrophages. In parallel, Thp-1 were treated for 48 hrs with 50 ng/ml PMA in 6 well plate, washed with PBS and rested for another 48 hrs. A549 cells insert were placed on top of rested macrophages and incubated for first 4 days of long co-culture 30 days experiment. During the resting time of the first plate of differentiated Thp-1, another Thp-1 were plated in 6 well plate for differentiation to replenish macrophages at the fourth day of long co-culture experiment. Each time of macrophages replenishment, A549 cells were split and transferred to another 6 well corning insert until day 30. This was repeated every 3 days until the end of 30 days, A549 cells were trypsinised and plated in T25 flask with fresh media for one month post macrophages removal, which labelled as long reprogrammed lung cancer cells. The rest of cells were collected for further immunoblotting and RNA analysis and other were frozen in -80 $^0$C for future experiments. Primers used for upregulated genes amplification in co-cultured A549 cells are listed in table 2.

| Gene | Forward Primer | Reverse Primer | Annealing temperature ($C^0$) | cycles |
|---|---|---|---|---|
| SERPINB3 | AAATTGATGGAATGGACAAG | AGAGGATGCTGTTGGTCTTA | 58 | 36 |
| P2RX4 | CTCAAGTCGTGCATTTATGA | TGCTCGTAATCTTCCACATA | 58 | 36 |
| PTGS2 | CCCGCAGTACAGAAAGTATC | TTCACGTTATTGCAGATGAG | 58 | 36 |
| NPC1 | ACGAACAGTACCTGACCATC | GATGGCCCTATGTAACTGAG | 58 | 36 |
| FSTL1 | GCAGTAATGGCAAGACCTAC | CAGACAGTTCAATGAGAGCA | 58 | 36 |
| PLAUR | CTTACCGAGGTTGTGTGTG | GCAGTCAATGAGGAAAGTCT | 58 | 36 |
| GAPDH | TCCCATCACCATCTTCCA | CATCACGCCACAGTTTCC | 58 | 36 |

**Table 2** List of primers used for PCR to assess the upregulated genes in A549 lung cancer cells to investigate the pro tumorigenic effects of macrophages on A549 cells

### 2.2.3 Generation of conditioned medium (CM)

After two days treatment of Thp-1 with 50 ng/ml PMA, macrophages were washed 3x with PBS, replenished with fresh RPMI media and rested for two days. This media was collected after 48 hrs and centrifuged at 1500 rpm for 5 minutes to remove cells debris. Then, the centrifuged media was used for plating 3x $10^6$ A549 cells in T25 flask for 3 days and thus enriched with macrophages secreted factors (Figure 3).

For generating co cultured CM which contain both macrophages and A549 cells secreted factors after 3 days of co culturing (Figure 3). After three days of co culturing macrophages in 6 wells plate with A549 cells in trans-well, media from both 6 well plate and the inserts were collected and centrifuged to remove cells debris and obtain filtered CM. Then, co cultured CM either used directly for culturing 3x$10^6$ A549 for three days in T25 flask or stored in -20 $^{\circ}$C until required. Then, cells trypsinised and collected for RNA extraction. In this experiment, A549 cells plated in F12-K serum free media as a control in parallel to A549 cells cultured in CM for three days. Then, cells were collected and analyzed in comparison to A549 cells in CM.

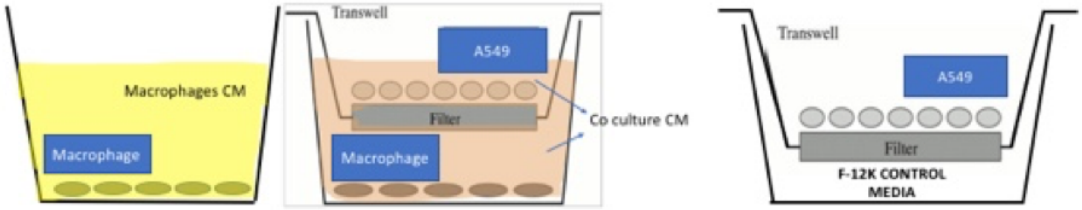



**Figure 3** Macrophages and A549 cells co-culture experiment

### 2.2.4 *In vitro* scratch wound assay (wound healing assay)

According to Liang, Park and Guan, established the *in vitro* scratch assay method which enable measuring the rate of cell migration (Liang et al., 2007). Both Long- and short- programmed A549 cells rested in single culture for one month after co cultured with differentiated macrophages. To assess Long- and short-programmed A549 cells migration properties, this experiment was performed in 3 days after the end of one month resting of both cells. Cells were seeded in 6 wells plate 2 hours before the experiment conduction. Then, sterile 200 ul pipette tip were used to scratch the centre of the well. After the wounds were created, cells within the wound area were washed 3x with PBS to remove unattached cells and replenished with fresh F12-K media to allow cells migration. In parallel, wound created in the well of parental A549 cells with fresh F12-K media as control for this experiment. Photographs of the wound area were taken at the 0 hrs of wound creation and in the first 24 hrs, 48 hrs and 72 hrs of co- culture incubation time. The wound wide was measured by ImageJ (V. 1.38), software.

## 2.3 Gene expression analysis by Polymerase Chain Reaction (PCR)

### 2.3.1 RNA extraction and cDNA generation

200 µl of QIAzol$^{TM}$ (Cat. No. 79306) were used for 1X10$^{6}$ cells. Cells in T25 or T75 flasks were trypsinised and collected for RNA extraction while cells in 6 wells plate or corning inserts the TRIzol were added directly to cells in plate. Then, 10% of TRIzol volume were used for phenol-chloroform, 80 µl chloroform for 200 µl TRIzol. 15 seconds of vigorous shaking for the mix was applied until the sample becomes homogenous, and samples were centrifuged at 4 $^{0}$C maximum speed

13.2 g for 30 minutes. Three layers were resulted after centrifugation: the upper aqueous phase contains RNA, the intermediate phase contains DNA, and the lower red organic phase contains protein. The upper phase was transferred to a new sterile Eppendorf tube and the equal volume of isopropanol was added 180 - 200 µl. Samples were mixed and stored on ice for 15 minutes and centrifuged at 13.2 g for 15 minutes at 4 $^0$C. Centrifugation were resulted in precipitation of RNA pellet and the supernatant was removed. Then, pellets were washed in 500 µl of 75% ethanol and spin at 13.2 for 5 minutes. After washes with ethanol, RNA pellet was left for 6-10 minutes with lid open to air dry. RNA pellets were dissolved in RNA-ase free water (20 µl) and heated at 60 $^0$C on the Eppendorf Thermal Cycler for 6 minutes to help re dissolve the pellet.

### 2.3.2 Quantification of RNA purity by Nanodrop

Following RNA extraction, RNA purity and quantity was analyzed using the NanoDrop ND 1000 UV-Vis spectrophotometer (Thermofisher, USA). After cleaning Pedestal surfaces by distilled water, the blank measurement was performed by 2 µl of RNA-ase free water before samples quantification. Then, 2 µl of each sample were pipetted onto the lower optical lens, both samples and blank were measured at 260 and 230 nm absorbance.

The pure and contaminant RNA was determined by 260/280 and 260/230 ratios, and RNA concentration was calculated by the software in ng/ µl. 260/280 range between1.8 and 2.0 indicates pure RNA, of the ratio is lower than 2, it may indicate the presence of contaminants which absorb at 280 nm such as proteins or phenol. 260/230 ratio of pure RNA should be in the range of 2.0-2.2 and if it is lower than expected indicate the presence of contaminants absorb at 230 nm. Also, total RNA was run on 0.8% gel to determine intact RNA subunits.

### 2.3.3 Complementary DNA (cDNA) synthesis

To reverse RNA to cDNA, the reverse transcription kit Omniscript® RT (QIAGEN) was used for 2 ug of total purified RNA diluted in RNA-ase free water.

Reverse transcriptase (RT) master mix was prepared in 20 µl total containing: 1 µl of random hexamers ,2 µl of deoxyribonucleotides triphosphate (dNTP), 2 µl of 10X buffer reagent and 1 ul of Reverse Transcriptase (4 U/µl). Components were

mixed by gentle pipetting, added to 2 ug of RNA sample in dH2O (14 µl) and incubated at 37 $^{0}$C in the water bath for 2 hours. After incubation, tubes were centrifuged for 30 seconds and diluted with 180 µl sterile dH2O. The final cDNA concentration was 1 µg/100µl which used for RT-PCR or frozen in -20 for later use.

### 2.3.4 Primer design

Designing primers for semi quantitative reverse transcription polymer chain reaction (RT-PCR) analysis was done by Primer 3 online program (http://primer3.ut.ee/) version 0.4.0 using the codon region of each gene. The standard settings were modified as following: the length size of primer 350-500bp, annealing temperature of primer (TM) was set at 58-60 $^{0}$C and max self-complimentary at 5. All primers listed in table 1 and 2 were purchased from Sigma-Aldrich and diluted in nuclear free water at 100 µM concentration and stored in -20 $^{0}$C until use.

### 2.3.5 Polymerase Chain Reaction

Master mix of PCR for each sample in 0.5 ml microcentrifuge tube contains: 10 µl RNase free water, 0.25 µl of 100 µM stock for a final concentration of 1 µM for both reverse and forward primers and 12.5 µl Tag DNA polymerase. Then, 23 µl of master mix was vortexed and loaded into PCR tube and 2 µl of cDNA sample was added to master mix for PCR cycles. The sequences of primers and number of cycles for each gene are indicated in the corresponding table 1 and 2.

Biometra T3000 Thermocycler machine was used for PCR reaction under the following condition: polymerase activation on 95 $^{0}$C for 15 minutes, denaturation phase on 94 $^{0}$C for 5 seconds, annealing phase at 58 $^{0}$C and the final phase is the extension at72 $^{0}$C for 90 seconds. The final amplified PCR products were visualised on Agarose Gel Electrophoresis.

### 2.3.6 Evaluating gene expression on gel electrophoresis

1.3% of agarose gel was used for DNA samples by weighting 1.3 g of agarose in 100 ml of Tris-borate-EDTA x10 (TBE) (Cat. No.AM9863). Then, the solution was mixed and microwaved for 2 minutes with interval swirl until the agarose was dissolved and get a homogenous mixture. After the solution cooled down, 10 µl of Ethidium Bromide (EtBr) (SIGMA-ALDRICH E1510-10ML) was added for DNA

visualization. The solution was swirled and poured slowly into gel casting apparatus with combs and kept in air dry for 15-20 minutes until the gel solidified. For gel running, 10x TBE running buffer was poured to the level which covers the gel surface in the gel box. 12 µl of DNA sample was loaded into gel wells. The power supply of gel electrophoresis was set to 100 V for 13-15 minutes and DNA leader was separated in parallel to samples as an indicator for gel running. Finally, the gel was exposed to UV light by using ChemiDoc$^{TM}$ imaging system (Cat. No. 17001402). Bands intensity were measured by using image J (V. 1.38).

## 2.4 Western Immunoblotting

### 2.4.1 Protein lysate preparation

To prepare cells for protein quantification, cells were collected and washed 3x with cold PBS. Then, cells were re-suspended in Complete Radioimmunoprecipitation Lysis (RIPA) buffer prepared by combining 10 µl sodium orthovanadate solution and 10-20 µl protease inhibitor cocktail solution per 1 ml RIPA lysis buffer. 1 ml complete RIPA was added for 2.0 x $10^7$ cells in suspension following the provider's instructions (Santa Cruz Biotechnology. Dallas, Texas, U. S) to break up the pellet and lysed cells. Samples were incubated on ice for 30 minutes with occasional mixing and centrifuged at 1200 RPM for 10 minutes at 4 $^0$C to collect supernatant in 1.5 ml Eppendorf tubes. Bradford assay was used to determine protein concentration in samples supernatant with BSA standards were prepared at the following concentrations: 2, 4 ,8 ,10, and 12 µg/µl. The absorbance of each sample was measured in triplicates using spectrophotometer plate reader at 595 nm. After protein quantification was calculated against BSA protein control, samples were prepared for western blotting by addition of calculated quantities of protein lysate, 2X SDS buffer (Novogene) and d $H_2O$. The samples were heated at 95 $^0$C for 10 minutes for protein denaturation and stored in -20 $^0$C for use in protein gel electrophoresis.

### 2.4.2 Western Blot analysis

Protein samples (15µl) were loaded to SDS-PAGE on a 10% polyacrylamide gel for 5 minutes at 80 volts until samples are through the stacking gel layer and lined up evenly at the edge of the separating gel. Then, the volts were set at 120 for one hour and check protein ladder for bands separation. Proteins were transferred onto nitrocellulose membrane using the wet-transfer protocol (Bio-Rad Laboratories). After protein transfer, membrane was blocked in 5% skimmed milk in TBS-0.1%Tween-20 for 1hr at room temperature under gentle shaking. Membrane was incubated overnight with primary antibodies (Table 3) in 5 % BSA, TBS-0.1% Tween-20 at 4 °C under gentle shaking, and then the membrane washed 3x with TBS-0.1% Tween-20 for 5-10 minutes each under shaking. Following primary antibodies, the secondary antibodies were prepared in 5% milk, TBS-0.1 %Tween-20 and added to the membrane for 1 hr incubation at room temperature under gentle shaking. Finally, membrane was washed 3x for 5 minutes each with wash buffer. Membrane detection was performed by chemiluminescence ECL system using the SuperSignal WestPico Enhanced chemiluminescence Kit and visualized on BioRad Transilluminator.

| Protein | Antibody Species | Company | Primary Ab dilution | Secondary Ab dilution | Molecular weight (kDa) |
|---|---|---|---|---|---|
| N-cadherin(13A9) | Mouse | SANTA CRUZ | 1:1000 | goat anti-mouse 1:30000 | 130 |
| E-cadherin (67A4) | Mouse | SANTA CRUZ | 1:1000 | goat anti-mouse 1:30000 | 120-135 |
| GAPDH | Mouse | SANTA CRUZ | 1:50000 | goat anti-mouse 1:5000 | 37 |

**Table 3** Antibodies used to assess the EMT status of A549 cells

## 2.5 Next Generation RNA Sequencing

A549 single cell clones were co cultured with macrophages in triplicate at 1:1 ration, and with 0.5 x $10^6$ density for both macrophages and A549 lung cancer cells. RNA sequencing applied for samples labelled as following: A549 single cell clone co cultured with macrophages for short term (3 days) were labelled as (co 3 days), A549 single cell clone co cultured with macrophages for 3 days and allowed to grow individually in fresh media for further 30 days in single culture labelled (short programmed), A549 single cell clone co-culture with macrophages for 30 days followed by culturing in single culture without macrophages for another 30 days labelled (long programmed), and parental A549 cultured in fresh media were labelled as (control).

RNA was extracted from all cells by previously mentioned RNA extraction method. Then, RNA purity was assessed by spectrophotometer. Furthermore, RNA integrity was assessed by ribosomal subunits bands visualization on 1.0% agarose gel. 1µg RNA was mixed with Ethidium Bromide and loaded on the gel and visualized on UV illuminator ChemiDoc imager.

### 2.5.1 Differential gene expression analysis, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes

First, to obtain clean data, raw FASTQ data were processed after removing low-quality reads. Then, all the downstream analyses performed on the clean data with high quality. Genes with P-value <=0.05 found by DESeq2 and foldchange of 2 were set as threshold for significantly differential expression. Gene ontology and KEGG analysis were performed with considering genes with P value less than 0.05 classified as differential expressed genes. Analysis of number of up and down regulated genes was performed for 3 biological replicates for each sample.

# Chapter 3 Generation and assessment of PMA based macrophages differentiation

## 3.1 Introduction

Macrophages play an important role in the innate immune system's maintenance through phagocytic activity and antimicrobial secretions, which regulate the immune system response in many diseases. In addition, macrophages maintain tissue haemostasis and are a major component in tumour microenvironment, which has been widely investigated to understand their functions in tumour growth and treatment. TAMs are monocytes derived from blood circulation and migrated to the tumour site where they differentiated into TAMs (Mosser and Edwards, 2008). However, other studies have indicated that some TAM are transformed in cancer tissue from resident macrophages as it is reviewed by Gordon (Gordon, 2007).

The aim of this part of project is to generate Thp-1 differentiated macrophages and co-culture them with lung cancer cells to investigate the effects of TAM on cancer cells. Thp-1 monocytes are leukemic cell line have been used by many researchers because they are easily available to use in the lab and have a simple and standard protocol for maintenance and differentiation. Furthermore, the difficulty in obtaining primary human peripheral blood macrophages which needs an invasive technique of continuous bleeding from human with limited cells number encourages researchers to use PMA Thp-1 differentiated macrophages as a model for *in vitro* cancer studies. The protocol of PMA based Thp-1 differentiation has optimized to obtain macrophages closely mimic the peripheral macrophages criteria.

## 3.2 Results

### 3.2.1 Determining optimal PMA concentration required for macrophage differentiation

Because there are differences in the concentration and duration of Thp-1 treatment with PMA between published PMA-based differentiation protocol (Park et al., 2007; Baxter et al., 2020), the aim of this part of study is to determine the concentration and period of Thp-1 treatment and resting by a selected dose. This dose should not be toxic to Thp-1 cells, and the macrophages produced are similar to monocytes derived macrophages (MDMs) in their morphology and adherence properties (Park et al., 2007; Starr et al., 2018). In addition, the selected PMA concentration should not upregulate genes in a way that will mask other stimuli influence (Pinto et al., 2020), such as co culturing with lung cancer cells in the next aim of our study. With all of the PMA concentration criteria used for thp-1 differentiation, it is important to mention that high PMA concentrations are toxic to cells and cause cell death due to upregulation of genes related cells death (Starr et al., 2018). However, low PMA concentrations induce Thp-1 differentiation while limiting undesirable effects.

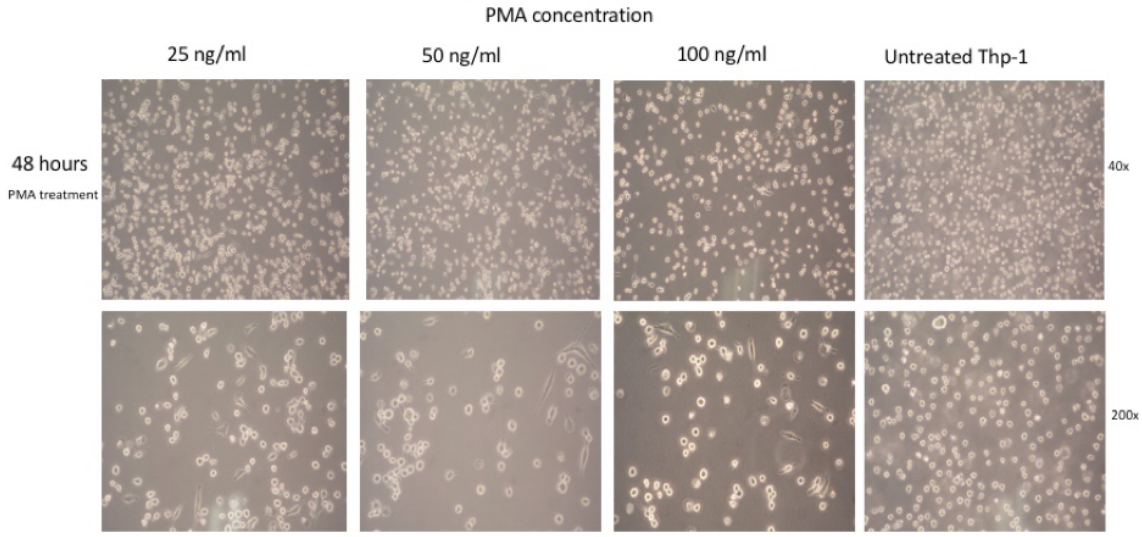
As mentioned earlier, choosing optimal conditions of PMA concentration and incubation times is important for obtaining macrophages model to achieve the aims of this study. Different concentrations ranging from 5 to 400 ng/ml have been reported in the literature, with 1 to 5 days incubation times with PMA are associated with different differentiation level and functional changes (Auwerx, 1991; Kohro et al., 2004; Tedesco et al., 2018). To determine the optimal PMA concentration in this project, taking into consideration recommended (the most common protocol from literature and our lab protocol) (Park et al., 2007; Daigneault et al., 2010), Thp-1 cells were treated with 25, 50 and 100 ng/ml for 48 hrs and differentiation properties were tested in all concentration to determine the optimal one.

In terms of morphological changes, after removing unattached cells, the attached differentiated macrophages treated with those concentrations successfully showed morphological characters of differentiated macrophages. As shown in figure 4A, cells become elongated in comparison to Thp-1 untreated cells with round single-cell morphology. Furthermore, after washing cells twice with PBS to

remove detached cells and trypsinising adherent cells, the percentage of the attached cells were calculated. Of note, the same density of Thp-1 cells ($0.5x10^6$) were treated with 25, 50 and 100 ng/ml in separate 6 well plates for 48 hrs as described in methods. We found that cells adherence ratio was different by counting adherent cells as cells treated with 25 ng/ml PMA showed weak attachment with only 20% of cells attached in comparison to strong attached Thp-1 cells treated with 50 and 100 ng/ml with 90% of adherent cells (Figure 4B). According to this observation, 25 ng/ml were excluded as optimal PMA concentration for Thp-1 differentiation.

In addition to morphological changes, gene expression of macrophages markers levels was assessed in 25 ng, 50 ng, and 100 ng Thp1 treated cells. CD11b, CD14, CD68, TLR4 and IL-1B were selected to differentiate between monocyte and differentiated macrophages. CD14 is a monocyte marker that used for recognizing undifferentiated monocytic Thp-1 cells (COHN and BENSON, 1965). As shown in figure (5), we found CD14 expressed in untreated Thp-1 cells but not in differentiated macrophages treated with 25, 50 and 100 ng/ml concentrations. However, CD11B, CD68, TL4 and IL-1B are macrophages markers were increased significantly during differentiation in comparison to untreated Thp1 cells. The weak expression of specified genes such as CD68 and TLR4 in cells treated with 25 ng/ml in comparison to cells treated with 50 and 100 ng/ml, and in accordance with the adherence criteria in previous results indicated that 25 ng/ml is not the optimum concentration for Thp-1 differentiation. However, the indicated macrophages markers increased in the same extent in cells treated with high concentration 50 and 100 ng/ml which supported the choice of 50 ng/ml as the lowest optimal concentration to induce an effective macrophage differentiation (Figure 5).

**A**




**B**

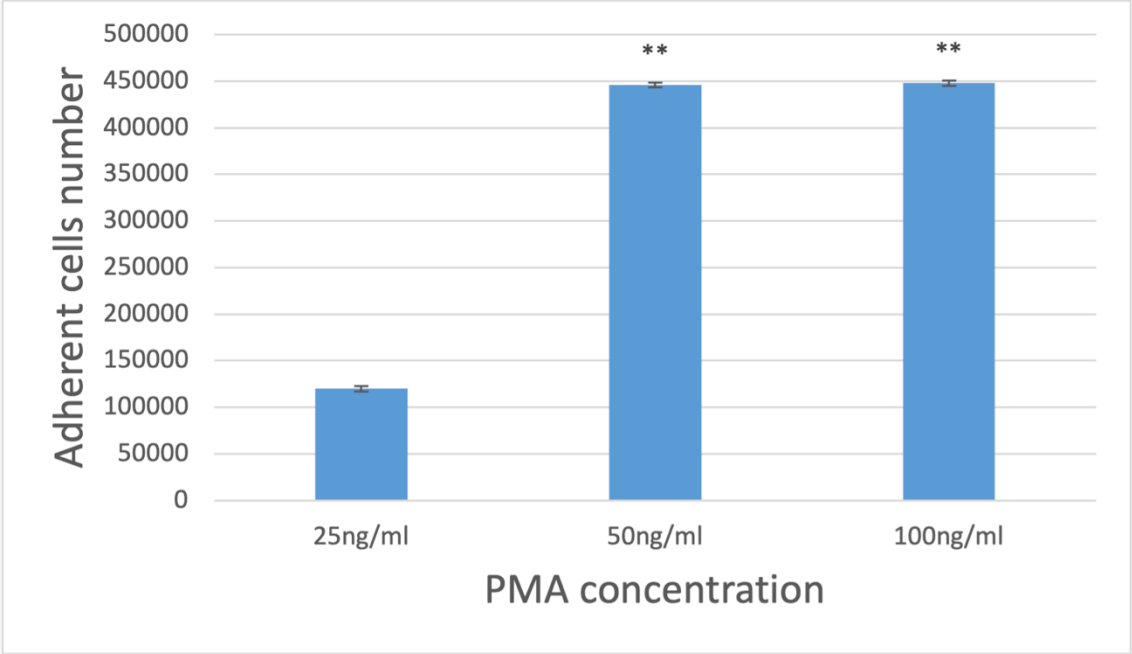



**Figure 4** A-morphology of differentiated Thp-1 cells treated for 48 hrs with 25, 50 and 100 ng/ml PMA. B-Adherent cells count of differentiated Thp-1cells treated for 48 hrs with 25, 50 and 100 ng/ml PMA. Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples presented in the figure.

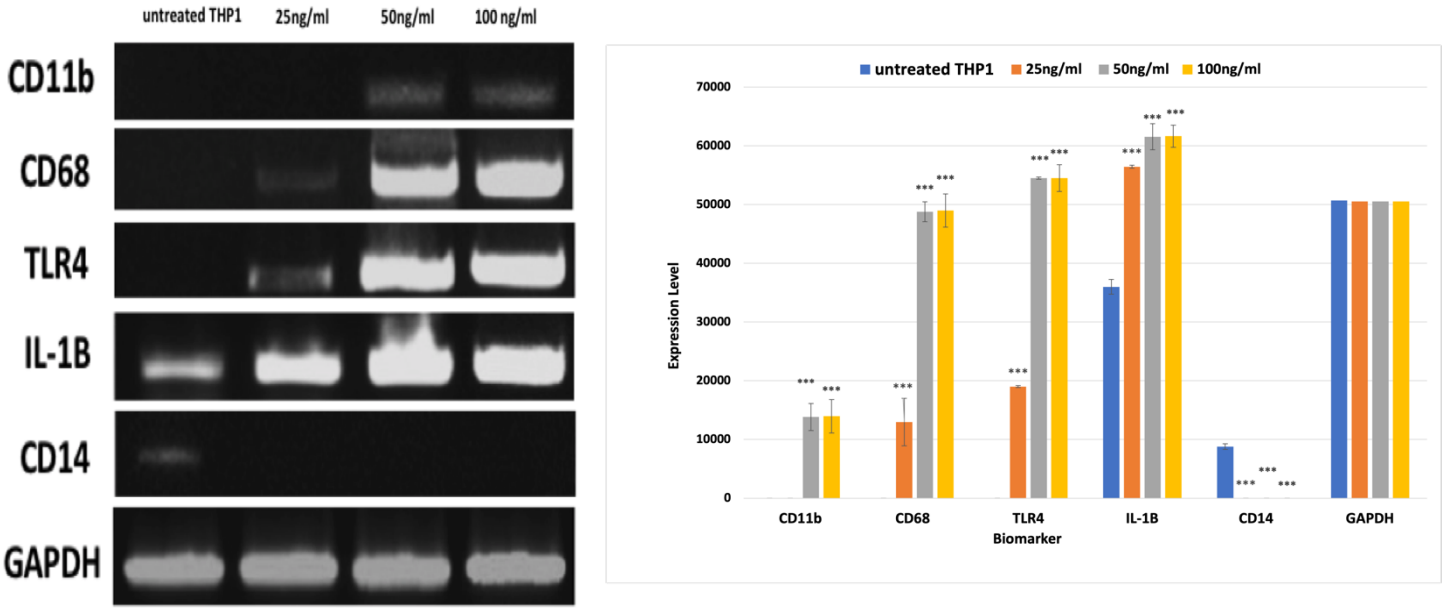



**Figure 5 Gene expression of macrophages differentiation markers in macrophages-like differentiated Thp-1 treated with 25, 50 and 100 ng/ml PMA for 48hrs.** The expression level of the indicated genes was tested in different PMA concentration using GraphPad Prism V 8.0. Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples.

### 3.2.2 Determining of macrophage resting period after PMA removal

PMA removal after treatment should be applied to differentiated macrophages cells because 1- in the next aim of investigating crosstalk between macrophages and lung cancer cells, remaining PMA will mask gene activation in cancer cells and conceal macrophages activation effects on lung cancer cells which is the main aim of this project. 2- presence of PMA induces growth arrest in NSLC by activation of PKC pathway and Kruppel-like transcription factor 6 (KLF6) which associated with increase of the cyclin-dependent kinase inhibitors (CDKIs) (Tahara et al., 2009).

To determine how long Thp-1 differentiated macrophages maintain their adherence and expression of differentiation markers following PMA removal in single culture, they were rested for 2, 4 and 6 days after washing twice with PBS and replenishment with fresh media. For assessing morphological changes of rested cells in single culture, three conditions have applied to macrophages,

condition A: 2 days treatment and 2 days rest. Condition B: 2 days treatment, 4 days rest. Condition C: 2 days treatment, 6 days rest. All these conditions were compared to Thp-1 untreated cells and 2 days treated cells without resting (WO/R).

Cell adherence of 2 and 4 days rested macrophages was tested by counting attached cells after washing 2 times with PBS and trypsinization. In earlier experiment of 50 ng/ml 2 days treatment cells without resting (WO/R), the attached cells presented 90% of cells which is very similar to condition A of 2 days rest with keeping macrophages morphology (Figure 6A, B). However, the attached cells presented macrophages morphology count has decreased to 60 % at day 4 rest post PMA removal (condition B). In condition C, significantly 80% of cells detached and floated which reflects cells death and macrophages loss as shown in figure (6A, B).

In addition to cells adherence, expression of macrophages genes markers was assessed in all conditions to determine the resting time that Thp-1 maintain their macrophages criteria after PMA removal. When macrophages rested for 2 days after PMA removal, all macrophages' markers expressed at the same level of cells treated with 50 ng PMA without rest (Figure 6-C). For macrophages rested for 4 days (condition B), gene expression level of TLR4 and IL-1B have decreased significantly compared to condition A, while CD11B and CD68 expressed at the same level of cells rested for 2 days after PMA removal. However, macrophages markers have not been analysed in condition C because the cells have died and lost their macrophages criteria with only 20% of the cells adhered.

Taking together, gene expression of macrophages markers and cells adherence indicate that macrophages maintain their phenotype in culture after PMA removal for a maximum of 2 days period in single culture. These findings of reduction of some macrophage's gene markers indicate that leaving macrophages for more than 2 days rested in fresh media will not generate reliable macrophages model for our study.

A.

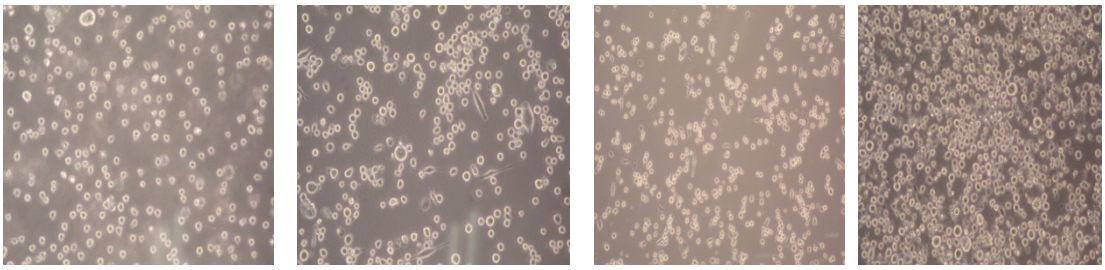
Macrophages alone -Single culture

Untreated Thp-1.

Condition A 2 days rest

Condition B 4 days rest

Condition C 6 days rest



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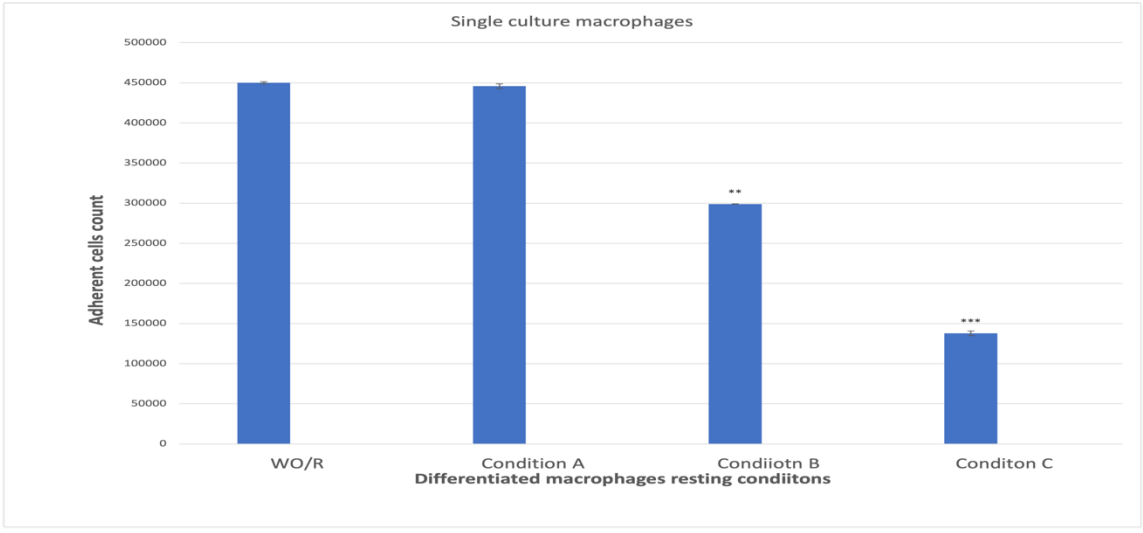

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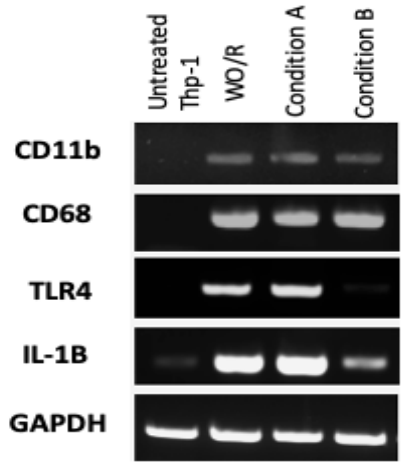


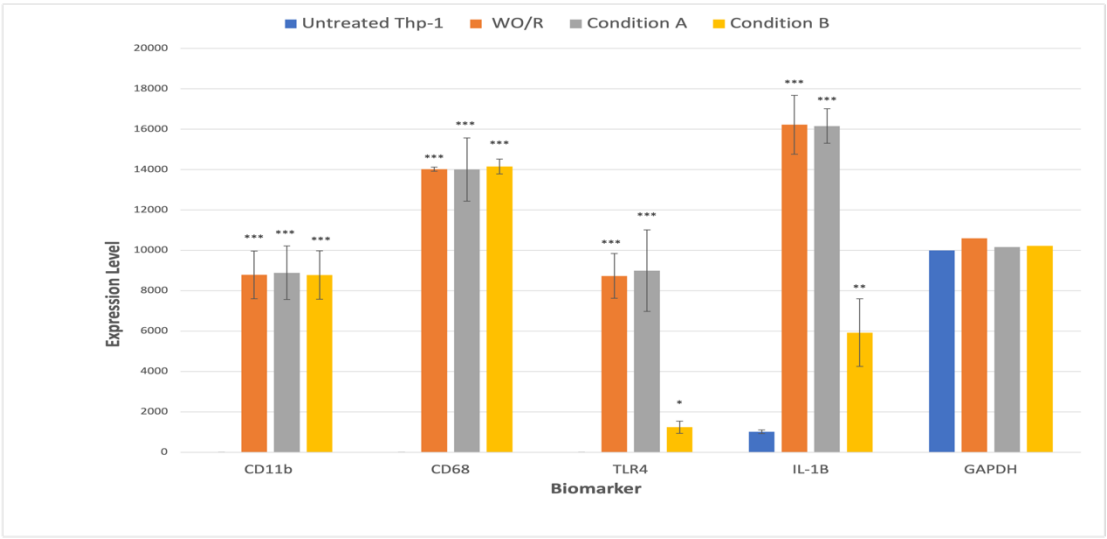




**Figure 6 Assessment of resting conditions of differentiated macrophages in single culture.** A-Morphology of differentiated Thp-1 cells rested for 2 or 4 or 6 days after 2 days treatment with 50 ng/ml PMA. B- Adherent cells count in resting conditions. C-Gene expression of macrophages markers in resting conditions. Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples.

### 3.2.3 Macrophages differentiation promoted by co culturing with lung cancer cells in comparison to single culture

Following the selection and assessment of macrophages model in single culture without any external stimuli as reported in earlier experiments, differentiated macrophages criteria were evaluated in co-culture system with lung cancer cells. The purpose of this experiment is to evaluate how long the macrophages can keep their phenotypes during co-culture system, which will be used to determine the duration of macrophages replenishment in the further aim of long-term lung cancer cells incubation with macrophages.

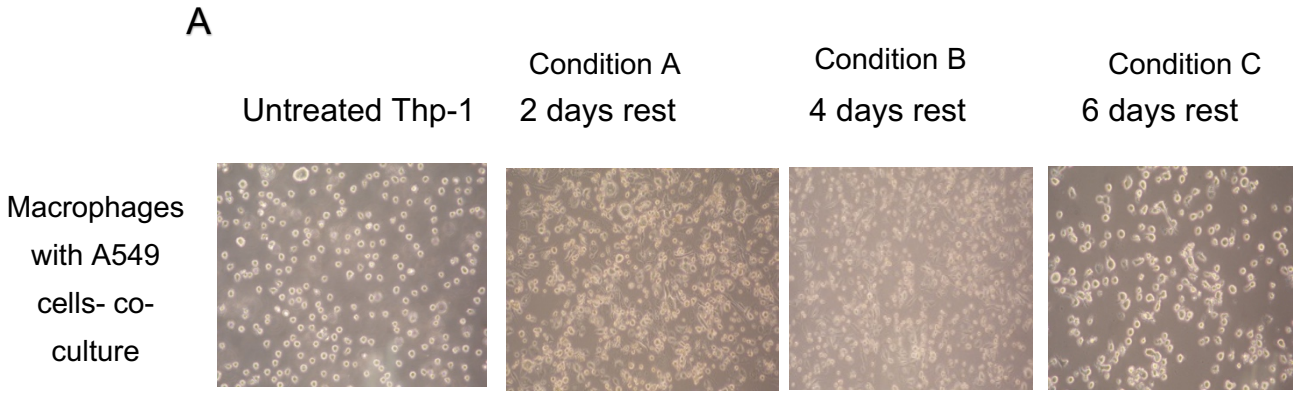
In order to generate TAMs *in vitro*, it is reported that conditioned media from breast cancer cells and lymphoma cell lines used to drive monocyte into macrophages *in vitro* (Grugan et al., 2012). Other researchers used co-culture system of macrophages with cancer cells to transform macrophages into M2 or TAMs *in vitro* (Z. Guo et al., 2019; Benner et al., 2019). This indicated that the presence of cancer cells enhances macrophages differentiation and maintain their differentiated criteria for a longer time in comparison to macrophages cultured alone. This observation reported by Poh and Ernst, they reported that TME cancer cells promote macrophages infiltration and survival through the activation of survival signals (Poh and Ernst, 2018).

To investigate macrophages stability, the morphological phenotype and genes expression of macrophages markers have assessed in previous conditions A, B and C in co-culture with A549 lung cancer cells compared to single culture resting conditions. The morphology of differentiated macrophages showed flat elongated cells with pseudopods observed in condition A and B, and cells in condition C also had macrophages morphology at day 6 rest (Figure 7A). Furthermore, as mentioned early, macrophages adherence was assessed by counting the attached cells which showed that 80% of macrophage adhered to the plate at day 4 of condition B in the presence of A549 cells (Figure 7B). This is close to condition A in single culture macrophages. Surprisingly, at day 6 rested macrophages adherent cells count increased to 50 % in double culture compared to 20% in monoculture macrophages as shown in figure (7B).

In support of the role of cancer cells in promoting macrophages differentiation, gene expression profile of co-culture macrophages was performed in A and B resting conditions and compared to two days treatment without rest (WO/R) and

untreated Thp-1 cells. In accordance with the percentage of attached macrophages in double culture, macrophages significantly maintained their gene expression of all those differentiation markers in condition B at the level similar to 2 days rested macrophages (condition A) and 2 days treated without resting cells (Figure 7-C). These results indicate that macrophages are still stable in our double culture model until day 4, which support that replenishing macrophage will be between day 3 and 4 in the next aim of generating long co culturing A549 cells with macrophages. These observations along with published study support that certain cytokines secreted from tumor cells as a result of crosstalk with macrophages in TME enhance macrophages stability and survival (S. et al., 2015).

A

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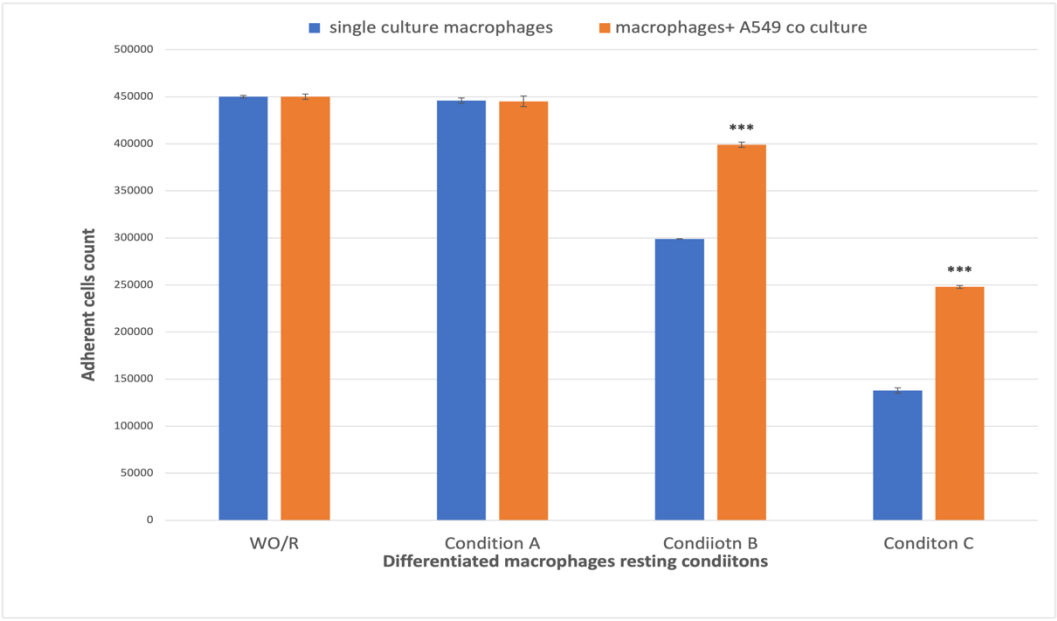

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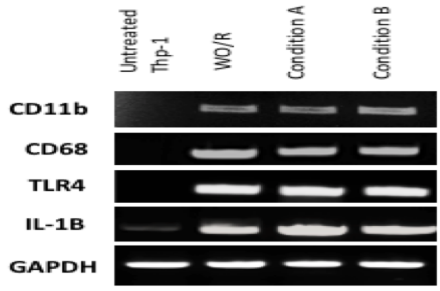


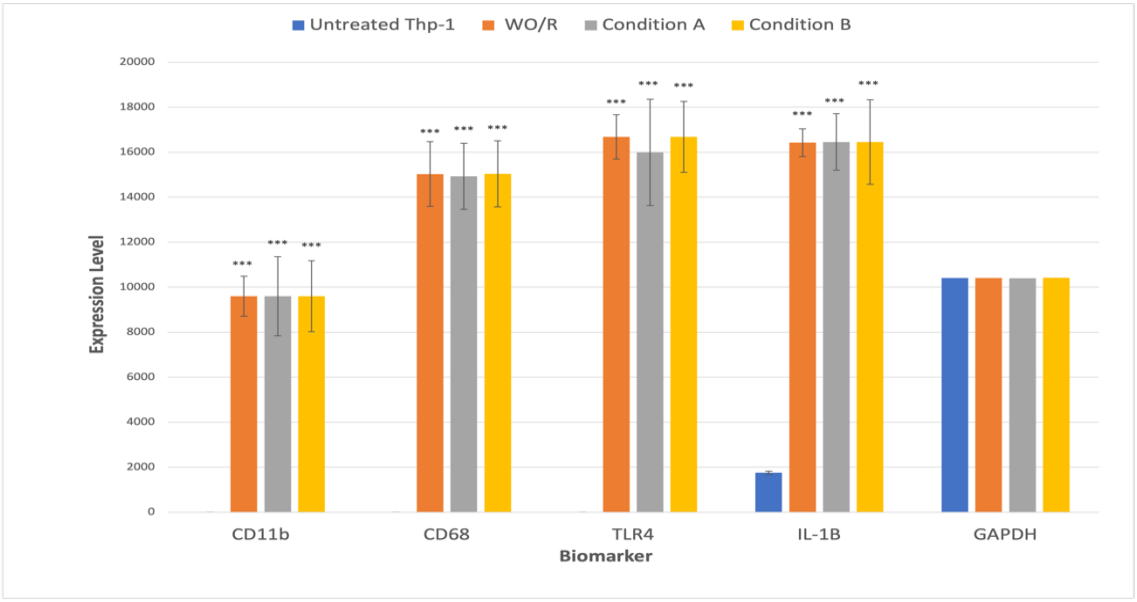




**Figure 7 Assessment of resting conditions of differentiated macrophages co cultured with A549 lung cancer cells.** A-Morphology of differentiated Thp-1 cells rested with A549 lung cancer cells for 2 or 4 or 6 days after 2 days treatment with 50 ng/ml PMA. B-Adherent cells count of differentiated macrophages co cultured with lung cancer cells in A, B and C resting conditions compared to single culture differentiated macrophages. C-Gene expression of macrophages markers in differentiated macrophages resting conditions co cultured with lung cancer A549 cells. Data are displayed as mean values ± standard error of 2 independent experiments. The analysis were performed and plotted in Prism V8.0 (GraphPad software). A p-value of ≤ 0.05 was considered as statistical significance between the samples presented in the figure.

## 3.3 Discussion

Most *in vitro* cancer studies used Thp-1 cell lines for PMA macrophages differentiation to mimic primary macrophages. To evaluate differentiated macrophages, they assess the adhesion and morphology phenotypes, macrophages differentiation markers and cytokines production after PMA treatment (Park et al., 2007). As it is reported by Starr and colleagues the morphological changes of Thp1 treated with PMA were dependent on the concentration, duration of incubation and period of rest, the process of PMA treatment has been done in two steps to optimize macrophages for our study model (Starr et al., 2018).

The first step is determining the lowest PMA concertation done by treated cells with different concentration and selected the lowest one that induce cell adhesion and gene expression of differentiation markers. In the literature, Pinto and colleagues reported that 50 ng is the lowest optimal concentration based on quantitative proteomics measurements of PMA based differentiated macrophages which reduce the alterations in gene expression in response to second stimuli (Pinto et al., 2020).

The second step is the recovery of single culture differentiated macrophages in PMA-free medium and evaluating macrophages phenotype during recovery time. It has been determined that PMA inhibits Thp-1 cells proliferation before induction of differentiation into macrophages by inhibition Thp-1 cells at G1-phase of the cell cycle and modulation the expression of cell cycle regulators (Traore et al., 2005). Thus, cells adherence properties and the gene expression of attached differentiated cells were the criteria of assessing macrophages differentiation after PMA recovery. Regarding the time of cells recovery from PMA and in coincidence with our condition B data t in single culture, Spano and his team reported that differentiated macrophages after 72 hrs withdrawal of PMA by resting cells in PMA free media resulted in cells detachment and death by apoptosis of cells that did not differentiate (Spano et al., 2013). On the other hand, macrophages infiltration has been observed in double culture with macrophages as it is previously reported that cancer cells enhance macrophages polarisation into M2 pro tumour phenotypes.

# Chapter 4 The Pro-tumorigenic effects of macrophages on lung cancer cells upon short time crosstalk process

## 4.1 Introduction

Several data have been published in supporting the role of TAMs in the development of many cancer hallmarks across a wide range of tumor types including NSCLC (Ruffell and Coussens, 2015; Z. Guo et al., 2019; Wei et al., 2019). In this project, we have used A549 NSCLC cells in co-culture model to assess the impact of macrophages on the expression of selected genes, cell proliferation, and metastasis. Utilizing the transwell co-culture method, we investigate A549 gene expression changes caused by short time interaction with macrophages. Furthermore, the same genes were examined in A549 cells post short time incubation with macrophages to investigate the lasting effects of crosstalk on A549 cells genes activation. Analysis the genes that shown activated in published paper in our A549 cancer cells models will give us indications of the validity of our differentiated macrophages and transwell system used in this study to achieve the crosstalk between macrophages and lung cancer cells.

Moreover, we aim to assess the conditioned media effects on A549 cells, as it has been discussed in section1.3, the crosstalk between macrophages and cancer cells mediated by cytokines secretion which result in phenotypical and functional changes in both cells. However, different cytokines have been identified to induce EMT in lung cancer and other cancer types (Gal et al., 2008; Bonde et al., 2012; Kawata et al., 2012). This may suggest that macrophages effects on cancer hallmarks are variable, and it is influenced by cancer tissue and pathogenesis of malignant cells. Several methods have been published to investigate EMT in cancer cells, the cadherin protein switch, a sign of EMT in metastatic cancer, is often the first step to be looked at when testing the EMT in cancer cells. After investigations genes expression changes, we aim to assess lung cancer cells proliferation and EMT state upon short term co culturing with macrophages. This aim also established to plan for the long term co culturing for the next chapter.

## 4.2 Results

### 4.2.1 Gene expression changes of lung cancer cells upon short term co culturing with Thp-1 differentiated macrophages

It has been reported that Thp-1 differentiated macrophages used as a model to mimic primary macrophages roles in cancer progression studies by analysing the gene expression profile which have similar macrophages markers expression to primary macrophages (Genin et al., 2015). This supports that *in vitro* differentiated macrophage mimic TAMs in TME, which enhanced using differentiated macrophages as a tool in co-culture studies to investigate the effects of macrophages on cancer cells progression (Genin et al., 2015). It has been reported that cancer cells polarized macrophages to pro tumorigenic phenotype (Hagemann et al., 2006), in this part of project the effects of macrophages on lung cancer cells have been investigated by analysing the gene expression changes upon three days co-culture of NSCLC A549 cells with macrophages in one-to-one ratio.

The most up-regulated 15 genes of co cultured A549 cells analysed by RT-PCR were selected based on a study done by Guo et al, they reported the GO ontology and quantitative proteomic analysis of NSCLC after co-culture with Thp-1 PMA differentiated macrophages in 1:1 ratio for 72 hrs (Z. Guo et al., 2019). We co cultured A549 cells with differentiated macrophages for 3 days. After RNA extraction of co-culture A549 cells, PCR analysis results shown 6 genes out of 15 were upregulated in lung cancer cells in comparison to parental A549 cells as control. These genes (SERPINB3, PTGS2, P2RX4, NPC1, FSTL1 and PLAUR) were significantly upregulated in 3x biological repeats of co culturing lung cancer cells with Thp-1 macrophages as shown in figure (8). We may conclude that macrophages activate indicated genes because of crosstalk with A549 lung cancer cells based on these findings as macrophages is the only stimuli presents in this co-culture experiment by removing any other external stimuli (PMA) that may alter lung cancer gene expression in transwell co-culture system. According to this observation and to demonstrate the activation of lung cancer cells genes mediate by independent interaction between two cells through secreted factors, conditioned media were generated from different culture and used for A549 culturing to assess the expression pattern of the same genes.

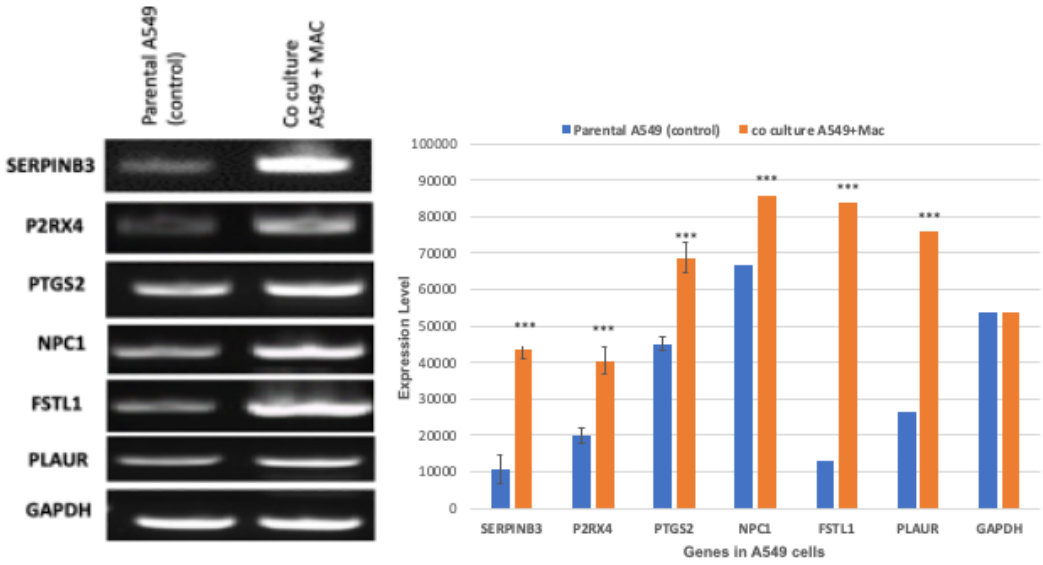



**Figure 8 PCR analysis of up regulated genes in A549 lung cancer cells co cultured with macrophages for 3 days.** Bands intensity was quantified using image J (V.1.38). Data are displayed as mean values ± standard error of 3 independent experiments. The analysis were performed and plotted in Prism V8.0 (GraphPad software). A p-value of ≤ 0.05 was considered as statistical significance between the samples presented in the figure.

### 4.2.2 Assessment of gene expression in A549 cells in 1- and 3-days post co culturing with macrophages

In the previous experiment, we tested genes expression in A549 cells which showed up regulation in these genes after 3 days of co-culture with macrophages relative to lung cancer cells in single culture. In this experiment, we tested the hypothesis that when the interaction with the stimuli (Thp-1 like macrophages) stopped, the activated genes will turn off. To achieve this, after 3 days co cultured, A549 cells were removed from the double culture system, washed 3x with PBS, trypsinised and plated in new insert in fresh media. These cells were rested for one day, and three days in another plate, then cells were collected for PCR to analyse the expression of the 6 genes.

Our data from three independent experiments demonstrate that gene expression start to decrease slightly on the first day culturing without macrophages for all 6 genes, while after three days the expression decreased significantly compared to

co cultured A549 as shown in figure (9). All the 6 genes had a reduction in expression in the way that become similar to A549 parental lung cancer cells (Figure 9). These findings suggest that the duration of the interaction with macrophages during the co-culture affects gene expression changes in A549 cells, which may suggest that prolonging the co-culture with macrophages may lead to the permanent overexpression of these genes. Of note, while short term co-culture result in the activation of these genes, their effects are not long lasting. This observation indicates that gene expression changes in A549 cells are affected by time of exposure to macrophages through the interaction between two cells and suggest that long term co culturing with macrophages may result in permanent upregulation of selected genes.

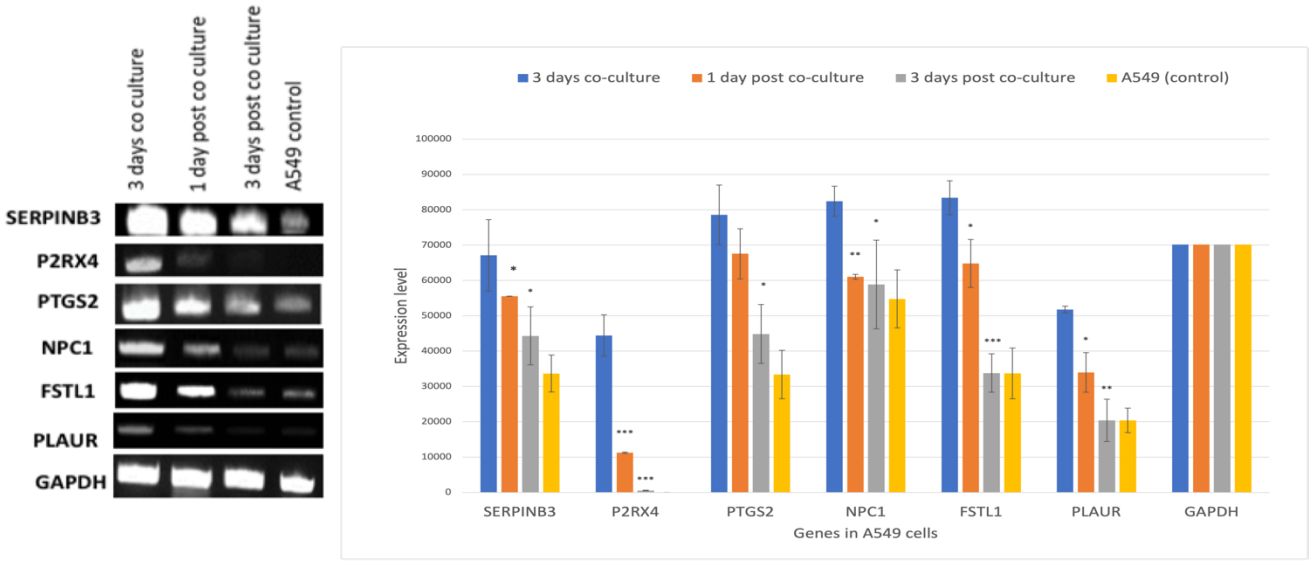



**Figure9 PCR analysis shows the expression levels of SERPINB3, P2RX4, PTGS2, NPC1, FSTL1, and PLAUR genes in A549 cells post co cultured with macrophages.** Bands intensity was quantified using image J (V. 1.38). Data are displayed as mean values ± standard error of 2 independent experiments. The analysis were performed and plotted in Prism V8.0 (GraphPad software). A p-value of ≤ 0.05 was considered as statistical significance between the samples presented in the figure.

### 4.2.3 Conditioned media effects on lung cancer progression

As previous results showed that upon co culturing of lung cancer with macrophages, 6 genes are upregulated in A549 cells. In order to understand the mechanism that induce genes activation, we hypothesized that either macrophages secreted cytokines as functional response to co culturing with lung cancer cells or macrophages has already secreted cytokines during differentiation which caused upregulation of lung cancer genes. To examine these two hypotheses, conditioned media (CM) collected from differentiated macrophages in single culture and from co-culture system as described in methods used to culture A549 cells for 3 days.

To investigate the effects of conditioned media on lung cancer cells, gene expression level of previously described 6 activated genes in co cultured A549 were analyzed in A549 plated in each one of two generated conditioned media for 3 days as detailed in methods. As shown in figure (10), in comparison to A549 cultured in fresh media, all previous activated genes were upregulated in both co-

culture conditioned media and macrophages CM. However, the expression level of SERPINB3, P2RX4 and NPC1 genes in co-culture CM are distinct from other genes, they are strongly induced in comparison to weak activation of these genes in A549 incubated with macrophages CM. For other genes (PTGS2, FSTL1 and PLAUR), there are no significant differences in the level of activation of these genes when cultured in conditioned media from macrophages in comparison to A549 plated in conditioned media generated from co cultured system. A slight increase in expression of some genes observed in A549 cells cultured in macrophages CM in comparison to A549 in fresh media including PTGS2, NPC1, FSTL1 and PLAUR.

As it has been known that conditioned media contains secreted factors include interleukins, growth factors and cytokines which mediate phenotypical and functional changes in cancer cells. According to this observation, many researchers have been tried to identify cancer progression related factors and their mechanisms in conditioned media. Our findings show that macrophage conditioned media contains sufficient secreted components to up-regulate genes in A549 cells. However, SERPINB3, P2RX4, and NPC1 genes, require crosstalk communication between macrophages and lung cancer cells resulting in high level expression of these three genes in comparison to other genes.

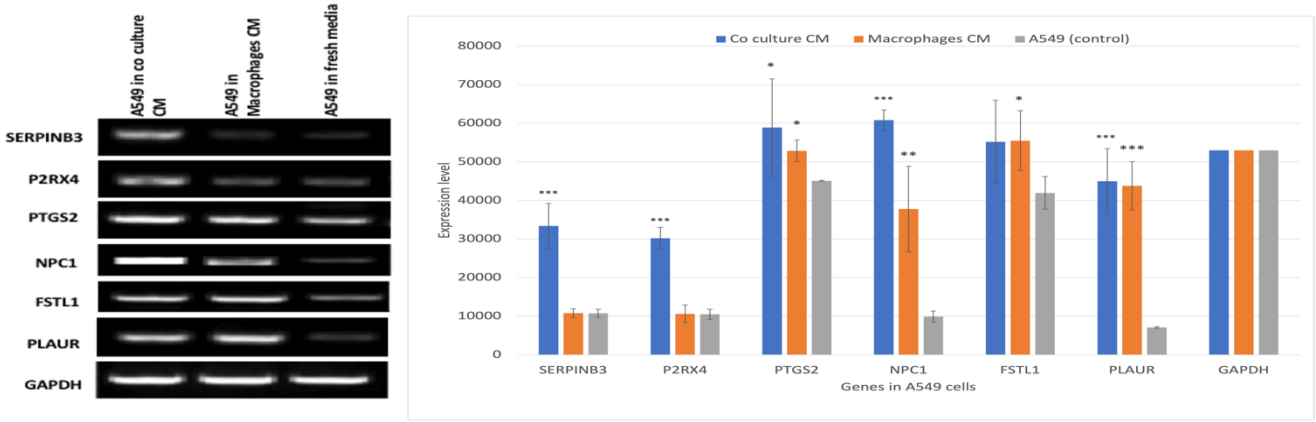



**Figure 10 PCR analysis shows the expression levels of SERPINB3, P2RX4, PTGS2, NPC1, FSTL1, and PLAUR genes in A549 cells incubated with conditioned media**. Bands intensity was quantified using image J (V. 1.38). Data are displayed as mean values ± standard error of 2 independent experiments. The analysis were performed and plotted in Prism V8.0 (GraphPad software). A p-value of ≤ 0.05 was considered as statistical significance between the samples presented in the figure.

### 4.2.4 Thp-1 Differentiated macrophages induce proliferation and epithelial mesenchymal transition (EMT) in short term co cultured A549 lung cancer cells

In addition to genes expression changes in co-cultured A549 cells, the proliferation of lung cancer cells has been tested to investigate the pro-tumorigenic roles of TAM in 3 days co-culture as a confirmatory hallmark that supports the crosstalk between TAMs and cancer cells in our co-culture model. We hypothesized that the short-term co-culture of lung cancer cells with macrophages may promote cancer cell proliferation as well as it activates genes expression. In order to assess this hypothesis, $0.5x10^6$ A549 lung cancer cells were co cultured for 3 days with the same density of Thp-1 differentiated macrophages as stated in methods. For each 24 hrs, A549 cells were counted by using hemocytometer and the results are cells count in 24, 48 and 72 hrs incubation period. In parallel, $0.5x10^6$ A549 lung cancer cells were plated in single culture with fresh media as a control. Our results showed that the macrophages significantly promoted A549 cell proliferation by 30% on day 1 and by 50% - 51% on days 2 and 3 compared to A549 in single culture (Figure 11). Notably, the higher proliferation in co-cultured A549 cells on days 2 and 3 compared to day 1 may point to an increase in the secreted cytokines that promote cancer cell proliferation by increasing the time of interaction between two cells.

In published data of the role of macrophages in tumor progression, many researchers reported that macrophages promote tumor progression in many cancer types because of crosstalk between two cells (Green et al., 2009; Zhang et al., 2013). In support of clinical data indicating that macrophages density is associated with poor prognosis, *in vitro* studies using co-culture system of differentiated macrophages with cancer cells found that macrophages play pro tumorigenic roles by enhancing cancer cells migration and transition to the mesenchymal state (EMT) (Sheshadri et al., 2014; Wei et al., 2019). These findings of published data along with our data of SERPINB3 and P2X4 upregulation in cocultured A549 cells, imply that examining the morphological and functional changes of 3 days co cultured A549 cells may help in understanding the effects of macrophages on lung cancer metastasis in 3 days short term crosstalk process.

In terms of morphological changes, it was noted that co cultured A549 cells acquired spindle elongated shape compared to single culture A549 in fresh media, which has epithelial pebble shape as illustrated in figure (12A). This observation suggests that 3 days independent interaction between macrophages and lung cancer cells enhance the mesenchymal transformation of lung cancer cells. These morphological changes were confirmed to be transition from an epithelial to a mesenchymal state by immunoblot analysis of mesenchymal associated proteins of co cultured A549 cells. Cells pellets were collected, and proteins were quantified in co-culture A549 cells and A549 cells in single culture as a control. As shown in figure (12B), co cultured A549 lost their epithelial state, as represented by low expression of E-cadherin, and adopted mesenchymal state, as reflected by high expression of N-cadherin in comparison to A549 control. These findings indicate that lung cancer cells cultured with Thp-1 differentiated macrophages display an EMT phenotype as a result of macrophage-lung cancer cell crosstalk. Although the mechanism of cancer EMT induction is not fully understood, it has been reported that cytokines secretion from macrophages activate JAK-STAT signaling pathway in cancer cells which enhance metastasis and cancer progression (Zhou et al., 2019). Moreover, the published study indicated that TAMs enhance malignant cells migration by cytokines secretion using co-culture model to produce cell to cell communication in *in vitro* environment which support our results of transition of A549 epithelial cells to mesenchymal state upon 3 days co culturing with macrophages.

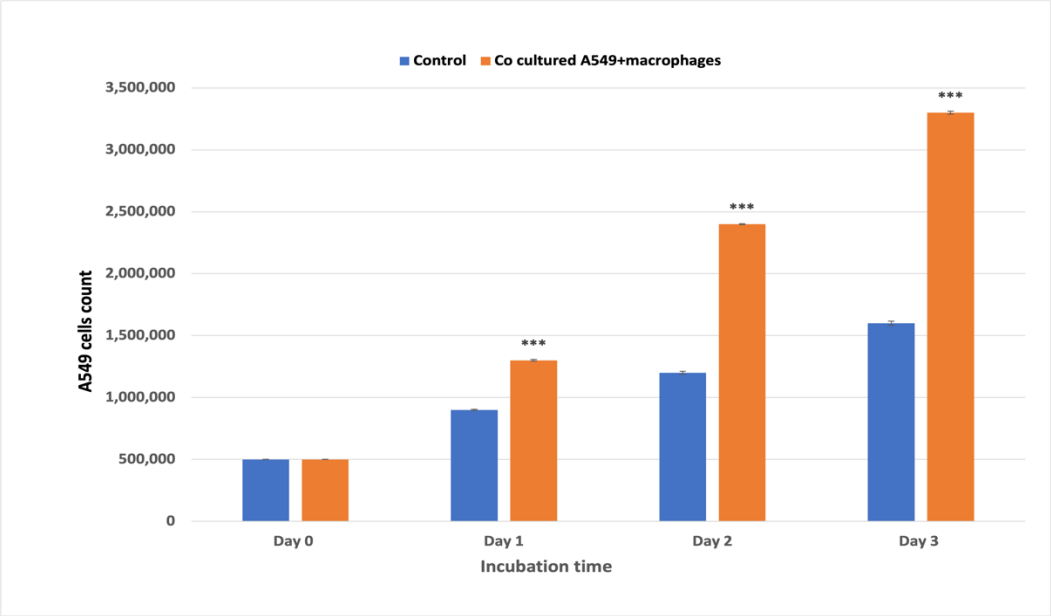
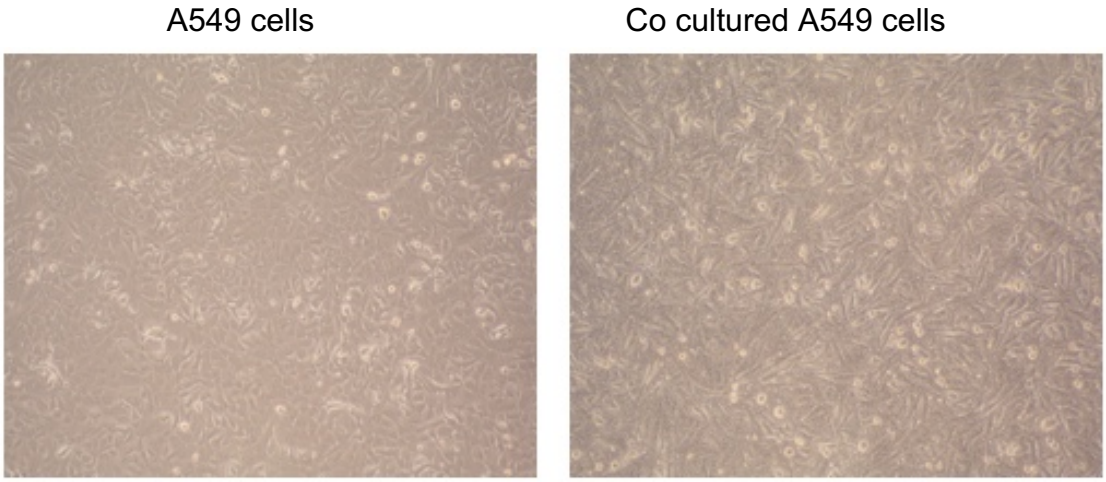



**Figure 11 Proliferation of A549 cells co cultured with macrophages for 3 days.** The analysis were performed and plotted in prism V8.0(GraphPad software). Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples compared to control group presented in the figure.

**A**

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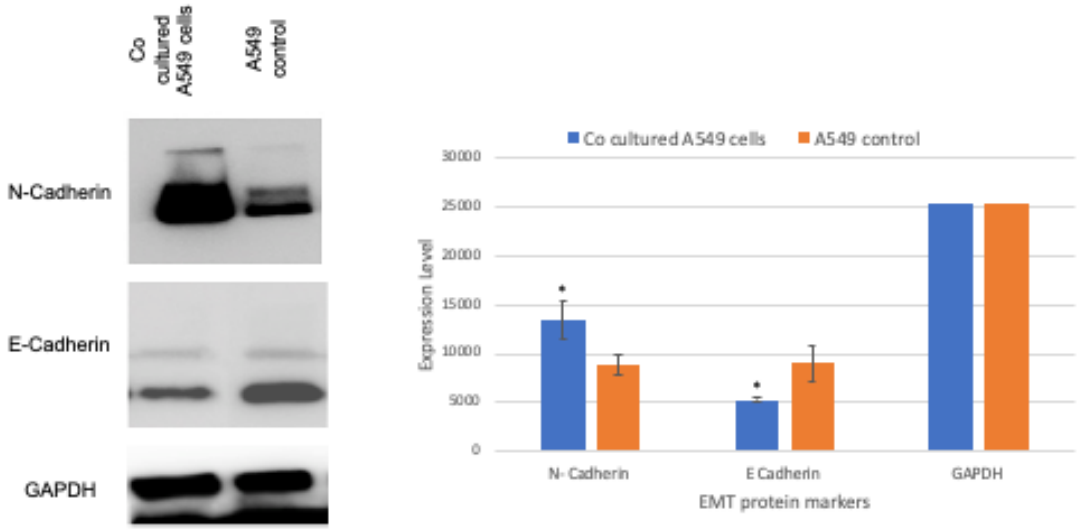


**Figure 12 Morphological changes and EMT markers expression in co-cultured A549 cells.** A-Morphological changes of A549 co-cultured with differentiated macrophages for 3 days. B- Expression of EMT markers in A549 cells co-cultured with differentiated macrophages for 3 days as assessed by western blot. The quantitative analysis of N-cadherin and E-cadherin proteins expression were performed and plotted in Prism V8.0 (GraphPad software). Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples compared to control group presented in the figure.

## 4.3 Discussion

Following macrophages differentiation and optimization for our model study, we assess gene expression in A549 cells upon co-culture with macrophages as initial step to investigate the effects of Thp-1 differentiated macrophages in changing the phenotypic and functional properties of cancer cells. It has been reported that co culturing macrophages with cancer cells affect cancer cells invasiveness and functional characteristics (Zhang et al., 2013; Yang et al., 2018; Sarode et al., 2020). We evaluate gene expression changes upon co-culturing macrophages with cancer cells due to the importance of significant differences in mRNA expression between conditions in biological activity of genes and novel diagnostic of cancer in addition to the association of poor prognosis of cancer patients with increased transcription of some genes such as P2RX4 in prostate cancer and SERPINB3 in many advanced cancer (Turato et al., 2012; Di Virgilio et al., 2016).

In a study done by Guo et al, they assessed the invasion phenotypes in lung cancer cells by investigating the differentially expressed proteins in co cultured A549 cells. They focus on the effectiveness of microfluidic-based device to mimic the *in vivo* microenvironment of lung carcinoma invasion without considering the time factor of co culturing macrophages with cancer cells in the experiment parameter (Z. Guo et al., 2019). However, the main objective of this study is to examine the long-lasting effects of macrophages on lung cancer gene expression activation upon short- and long-term incubation with macrophages. And the aim of investigating cancer cells at different time point is to mimic how chronic exposure to macrophages in *in vivo* tumour cells enhance cancer cells development and metastasis. As it is reported in the literature that cancer cells exposure to IL-6 secreted in TME maintain the positive feedback loop which mediate the chronic inflammation and EMT in breast cancer cells (Sansone et al., 2007).

Interestingly, we found that some of those activated genes in our 3 days co cultured A549 cells play an important roles in cancer metastasis and invasion including SERPINB3 ,which identified in many tumor types to induce the epithelial mesenchymal transition (EMT) (Quarta et al., 2010) Also, SERPINB3 overexpression found to be highly expressed in many cancer types including lung, head and hepatocellular carcinoma and it is overexpression associated with aggressive malignant tumor (Vidalino et al., 2009; Sheshadri et al., 2014).

Additionally, P2X4 gene which belong to ATP-gated nonselective ionotropic channels and one of the most sensitive and ubiquitously expressed P2X receptors has reported to induce invasiveness and epithelial to mesenchymal transition in prostate cancer (He et al., 2020). Likewise in Breast cancer , P2x4 has been shown to induce invasiveness of malignant cells by promoting the autophagy and cell survival (Chadet et al., 2022). The third selected gene in our study is the prostaglandin-endoperoxide synthase-2(PTGS2), which codes for the COX enzyme. Several studies indicates that it is overexpression has been linked to increased angiogenesis, metastasis, and chemotherapy resistance in patients with NSCLC (Huang et al., 1998; Hida et al., 2000; Dohadwala et al., 2001). The PLAUR gene was found to induce mesenchymal transition in glioblastoma cells and metastasis in *in vivo* mouse model (Gilder et al., 2018). The roles of other genes in cancer including FSTL1 and NPC1 still under investigations.

In this study, we cultured A549 cells in the conditioned media for two reasons. First, we wanted to validate that the changes in A549 cells were caused by independent crosstalk between two cells in our co-culture transwell and mediated by secretion factors in the conditioned media. The second reason is to identify if the crosstalk of cancer cells with macrophages may increase the release of cytokines or growth factors from both cells and activate new signalling pathway that affect cancer cells invasion and other hallmarks. However, identifying these cytokines in both media is needed to obtain an accurate overview of the broad range of short-term crosstalk effects on cytokines secretion and their receptors activation. In gastric cancer, it has been identified that co culturing macrophages with gastric cancer cells induce TNF-a secretion from gastric cancer cells which induce the release of CXCL1 and CXCL5 from macrophages and promoting gastric cancer cells migration through the activation of CXCR2/STAT3 feed forward loop (Zhou et al., 2019) . The emerging studies direct their work into understanding the mechanism of cytokines in promoting tumour migration which provide therapeutic targets for cancer cells metastasis.

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# Chapter 5 Characterization of lung cancer cells after long- or short- term co-culture with macrophages

## 5.1 Introduction

Several studies designed their co-culture model in order to mimic cells interaction *in vitro* and evaluate genetic and functional changes of cancer cells upon incubation with tumour microenvironment cells (Hadjidaniel et al., n.d.; Li et al., 2018; Wei et al., 2019; Z. Guo et al., 2019). However, these studies evaluate genetic and functional changes of co cultured cells for short time (3-5days) (Z. Guo et al., 2019; Wei et al., 2019), which may interfere with the accuracy of findings because tumours develop following prolonged interactions of cancer cells with macrophages and other tumour microenvironment cells that last for weeks or months. In our previous results, we assessed gene expression and functional changes of heterogeneous lung cancer cells that incubated with macrophages for 3 days. In this part of study, we addressed our questions based on the two hypotheses of cancer development and cell memory: first, tumour biology *in vivo* results from cancer cells interaction with the surrounding environment, including TAM, over a period of several weeks/months. Second, the phenomenon of cancer cells biological memory that stores information about a stimulus in chronic inflammation, which causes cancer recurrence possibility in the absence of stimulus (Hudson., 2021). According to these two hypotheses, we addressed our question whether lung cancer cells gene expression and functional changes that result from long time interaction with macrophages will persist after removing macrophages due to storing information of responding to macrophages secreted factors (the stimulus) in lung cancer memory. Furthermore, we asked how these changes in A549 cells after long time incubation with macrophages will differ from A549 cells rested after exposed shortly to macrophages during short time incubation and from A549 cells cultured with macrophages without resting.

In order to investigate these questions, first, we performed our experiments in this part of study on a homogenous A549 cell population by generating single cell clones from A549 lung cancer cells as described in methods, and the same clone was used for all the experiments. This was done in order to prove that any gene expression, phenotypic or functional changes in lung cancer cells that will occur are the common changes in heterogeneous lung cancer which will make homogenous lung cancer population respond similarly. Then, lung cancer cells

co cultured with macrophages for 3 days or 30 days and rested in single culture after macrophages removal for one month. These two cells were compared with co cultured A549 cells which incubated with macrophages for 3 days without resting in single culture and parental A549 cell. Furthermore, gene expression of previous identified 6 genes and functional analysis of lung cancer cells mesenchymal state, migration and proliferation were performed to assess changes in A549 cells upon different co culturing conditions. In addition, RNA-seq analysis were performed in our samples to identify gene expression changes that will be paired with behavioural changes in cell mesenchymal transition state and proliferation of lung cancer cells.

To clarify samples labelling and A549 culture conditions used in this part of project, clones from long term co-culture with macrophages and rested for 1 month after macrophages removed labelled as long programmed cells, cells from short term co-culture with macrophages and rested for one month post macrophages removal termed short programmed, cells co-culture with macrophages for 3 days labelled 3 days co cultured, and parental cells were labelled as control. Of note, all A549 cells used in the experiments of this part of project are single clones of A549 generated as described in methods.

## 5.2 Results

### 5.2.1 Assessment of gene expression in lung cancer after long- or short co-culture with macrophages

In our previous experiment of gene assessment in heterogenous A549 cells co cultured with macrophages for 3 days relative to parental lung cancer cells in single culture, the data showed upregulation of 6 genes including SERPINB3, P2RX4, PTGS2, NPC1, FSTL1 and PLAUR. In addition, the previous results showed that the expression of these genes decreased in 3 days after removing macrophages and cultured A549 in single culture. Here we evaluate the expression of those 6 genes in A549 lung cancer single cell clone incubated with macrophages for 3 days and compared to parental A549 single clone as a control. Following RNA extraction and PCR analysis of 6 genes, we found that all 6 genes

are upregulated in co cultured A549 single clone compared to parental as shown in figure (13). The upregulation of those 6 genes in A549 single cell clone confirmed that incubation of macrophages with lung cancer cells for short time resulted in intrinsic activation of these genes, which was the common response of heterogenous A549 cell population.

Furthermore, we hypothesised that long term incubation of lung cancer cells with macrophages could result in persistent activation of those 6 genes in cancer cells after removing macrophages. This could happen because of chronic (long-term, 30 days) interaction of A549 cells with macrophages and long-time exposure to secreted factors in co-culture system, which may result in storing information in A549 cells memory of responding to stimulus (macrophages secreted factors). However, we hypothesised that the activation of those genes will be reduced after short time incubation with macrophages (3 days) due to short time exposure of cells to secreted cytokines. To perform this, $0.5x10^6$ A549 cells clone were incubated with same density of macrophages for short term (3 days) or long-term (30 days). Post 3 days or 30 days incubation with macrophages, lung cancer cells were washed 3 times with PBS and rested in single culture for one month after macrophages removal and labelled short programmed and long programmed cells respectively. To test the changes of gene expression level in long-programmed and short-programmed clones, they were compared to single clone of A549 co cultured with macrophages for 3 days without resting (co 3 days) and parental A549 clone cultured in single culture (control), which performed in parallel to long and short programmed clones. Then, RNA was extracted from each sample including 3 days co-culture, short-programmed, long-programmed, and parental (control) cells, and RT-PCR performed for 6 genes that shown upregulated in previous experiment of A549 3 days co cultured cells.

As shown in figure (13), when long programmed clones were compared to parental, we found that all 6 genes were significantly upregulated in long programmed clones. In comparison of long programmed to 3 days co cultured A549 clones, genes including SERPINB3, P2RX4, PTGS2, NPC1 and PLAUR had higher expression level in long programmed than co cultured A549, while FSTL1 genes had lower expression level than co cultured clone (Figure13).

These data indicate that, similar to A549 clone which co cultured with macrophages for 3 days without resting, the expression level of those genes in long programmed clone were consistently upregulated after 30 days incubation with macrophages and resting A549 in single culture without macrophages. Of note, the higher expression levels of some of those genes in long programmed than co cultured clone may reflect that prolonged incubation time of A549 with macrophages increased the expression of those genes and they remined in this high level after macrophages removal.

Furthermore, when long programmed were compared to short programmed A549 clones, we found all of 6 genes were dramatically upregulated in long programmed clone, except for FSTL1 gene which had a slightly higher expression level in long programmed than short- programmed clones (Figure 13). We observed the downregulation of all 6 genes in short- programmed clones to the level similar to parental A549 clone, which indicate that removing macrophages after 3 days of co culturing with A549 cells was insufficient to persist the upregulations of those genes to the high level seen in long programmed and 3 days co cultured cells. In other words, three days of A549 cells exposing to secreted cytokines in crosstalk process are not causing permanent changes in the biological memory of A549 response to secreted cytokines that caused the upregulation of 6 genes in co cultured and long programmed clones.

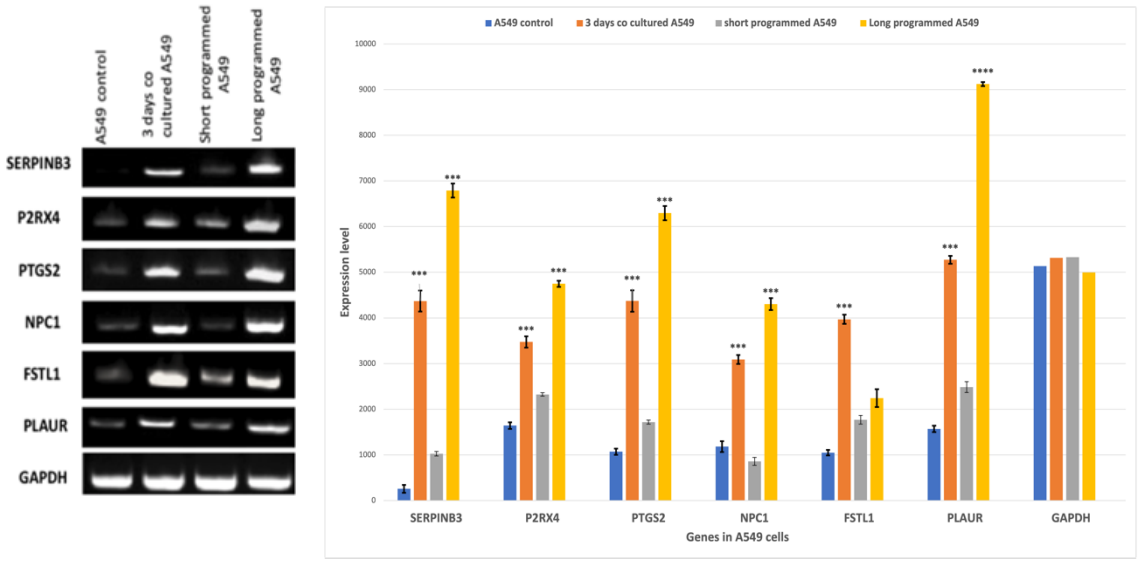


**Figure 13 RT-PCR analysis of gene expression level in 3 days co cultured, short programmed, long programmed and control A549 clones.** Bands intensity was quantified using image J (V. 1.38). Data are displayed as mean values ± standard error of 2 independent experiments. The analysis were performed and plotted in Prism V8.0 (GraphPad software). A p-value of ≤ 0.05 was considered as statistical significance between the samples presented in the figure.

### 5.2.2 Epithelial mesenchymal transition (EMT) and migration assessment in A549 after long- or short- time incubation with macrophages

Our previous data have shown that heterogenous 3 days co cultured lung cancer cells underwent transition to mesenchymal state upon short term co culturing with macrophages. This suggests that these morphological and functional changes may have resulted from the heterogeneity of lung cancer cells, or it was the common phenotypes of a heterogenous A549 population that occurred upon exposure to macrophages secreted factors during co culturing that promote EMT in A549 cells. To confirm that mesenchymal transition occurred in heterogenous A549 population was the common phenotypes resulted from crosstalk with macrophages, we used single clone of A549 cells to assess the mesenchymal state of lung cancer cells co-cultured with macrophages for 3 days. We did this by investigation of cells morphology and expression of cadherin proteins in co cultured A549 clones. As seen in figure (14B,15), single clone of A549 cells co cultured with macrophages for 3 days had an elongated spindle shape compared to parental and immunoblot analysis of mesenchymal and epithelial markers

showed increased N-cadherin and decreased E-cadherin expression. These observations show two indications, first, single A549 clone that co cultured with macrophages for 3 days underwent mesenchymal state. Second, this mesenchymal transition occurred in A549 single clone was the common phenotype of heterogenous A549 cell resulted in response to macrophages' secreted factors during 3 days of crosstalk process between two cells.

Moreover, we hypothesised that long-term incubation of lung cancer cells with macrophages could result in a permanent transition to a mesenchymal state after removing macrophages and resting cells for one month in single culture. This could happen as a result of A549 cells being exposed to macrophages secreted factors for prolonged period during long-term co-culturing with macrophages. Which may enhance A549 cells' memory in the continuation of cellular changes that persist the mesenchymal state after macrophages removal. However, we expect that removing macrophages after short term incubation with A549 cells will not be sufficient to sustain lung cancer cells in mesenchymal state, as was the case with the reduction of the expression of 6 genes in A549 short-programmed clone.

To achieve this, $0.5x10^6$ A549 clone were co cultured with the same density of macrophages for 3 days (short-term) or 30 days (long-term). Then, both 3 days co cultured and 30 days co cultured cells clones washed, trypsinised and plated in single culture for one month after removing macrophages with regular weekly split. Then, cells labelled (short programmed) after short term co-culture and (long programmed) after long term co-culture. After one month resting, both A549 cells collected for further mesenchymal analysis. Moreover, they were compared to 3 days co cultured and parental A549 clones. Of note, in long term incubation of A549 cells, macrophages were replenished every 3 days and A549 cells were washed, trypsinised and transferred to new transwell insert as described in methods section.

In terms of morphological changes, our data showed that long programmed A549 clone acquired elongated spindle shape as in 3 days co cultured clone, whereas short programmed had morphological appearance similar to parental A549 cells which described as pebble-like shape of epithelial cells (Figure 14D). These findings suggest that long term incubation of lung cancer cells with macrophages result in persistent of mesenchymal morphological changes after macrophages

removal which similar to co cultured A549 cells morphology that occurred in the presence of macrophages. However, in short-programmed clone, the elongated mesenchymal morphology of 3 days co cultured cells disappeared following macrophages removal, suggesting that A549 cells' short time exposure to macrophages secreted factors caused temporary mesenchymal phenotypic changes that turn cells to epithelial shape after removing macrophages and resting A549 cells in single culture for several weeks.

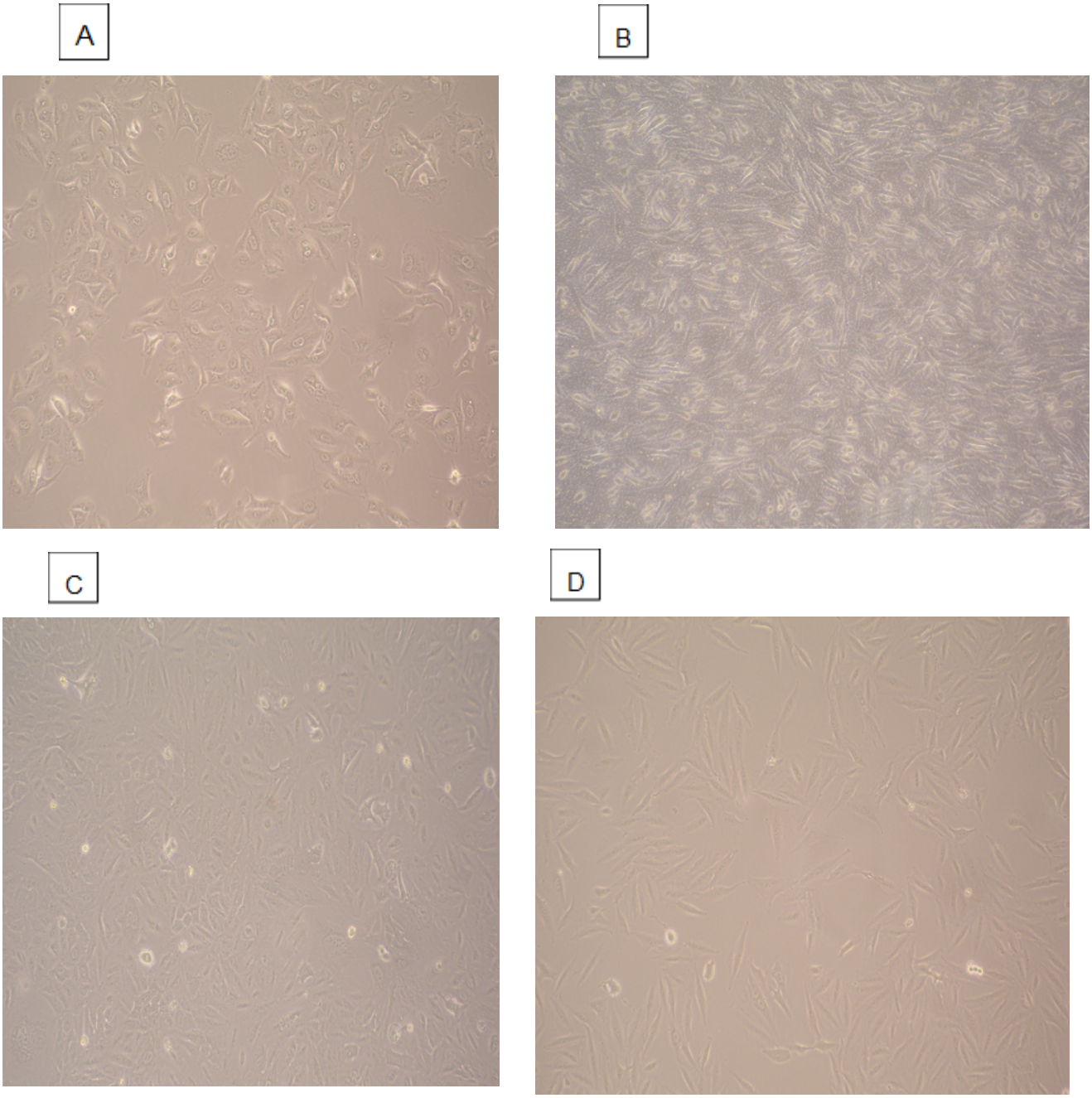


**Figure 14** Morphological changes of A549 cell clones. A- parental A549 (control). B- 3 days co-cultured A549 clone. C- short programmed A549 clone. D- long programmed A549 clone.

Furthermore, in addition to morphological changes, we confirmed the assessment of mesenchymal characteristics of long- and short- programmed clones of lung cancer cells by immunoblot analysis of epithelial and mesenchymal markers proteins expression. Cells pellet of long and short programmed clones were collected, and proteins quantified for N-cadherin and E-cadherin expression analysis.

As shown in figure (15), long programmed cells expressed high level of N-cadherin while E-cadherin dramatically decreased or not expressed in comparison to short-programmed cells. This is similar to cadherin expression pattern in 3 days co cultured clone, with increased N-cadherin and decreased E-cadherin expression (Figure 15). However, short-programmed clone had cadherin expression level similar to those of parental A549 cells, with increased epithelial marker expression (E-cadherin) and decreased mesenchymal marker expression (N-cadherin) (Figure 15). These observations, along with morphological changes, confirmed that co culturing single clone of A549 for long time with macrophages resulted in permanent switch of epithelial A549 to mesenchymal state, which continued after removing macrophages and was similar to transition state of co cultured A549 in presence of macrophages. However, the removal of macrophages after short time incubation with A549 single cell clone, led lung cancer cells to return to an epithelial state, in which E-cadherin protein expression increased and N-cadherin decreased.

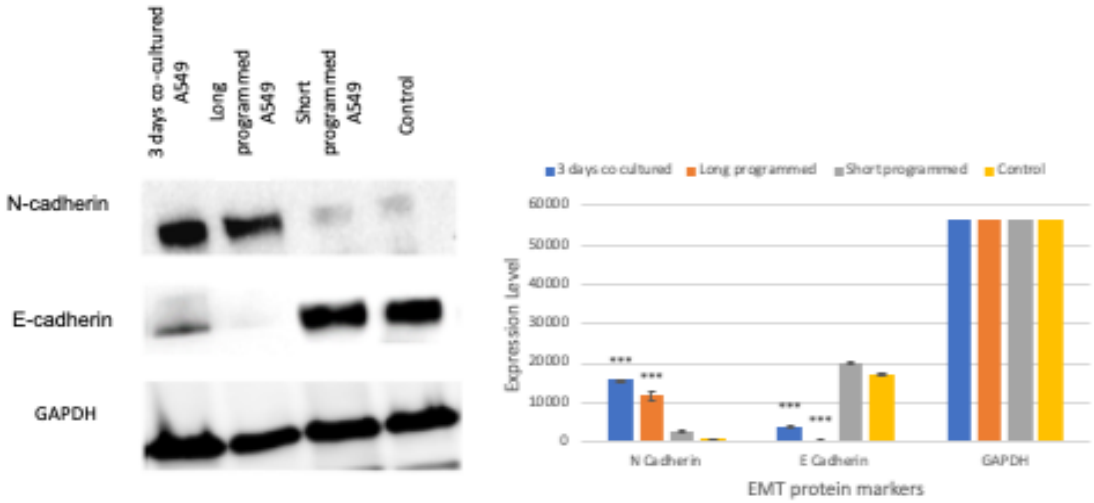


**Figure 15 EMT protein expression in 3 days co-cultured, long-programmed, short-programmed, control A549 cell clones assessed by western blot.** The quantitative analysis of N-cadherin and E-cadherin proteins expression were performed and plotted in Prism V8.0 (GraphPad software). Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples compared to control group presented in the figure.

Additionally, it has been known that lung cancer cells acquire the ability to migrate when co cultured with macrophages by transition to a mesenchymal state, which gives cancer cells the ability to metastasize and invade the distant tissues (Tse and Kalluri, 2007). It has been reported that short time co culturing of A549 lung cancer cells with macrophages promoted migration abilities of lung cancer cells compared to parental (Z. Guo et al., 2019). According to these studies, we assessed the migration abilities of long-and short-programmed cells following EMT assessment to validate the effects of macrophages on the induction of A549 cells migration after long or short time co culturing with macrophages. As earlier results showed the permanent mesenchymal state following 30 days incubation with macrophages, we expect that migratory abilities of long-programmed cells will be enhanced by mesenchymal transition but not in short-programmed cells, which returned to epithelial state after short time incubation with macrophages.

To assess the migration abilities, the wound healing assay was conducted in long- and short- programmed cells along with control (parental) A549 cells. This assay was performed by plating long programmed A549 as well as short-programmed cells in 6 wells plate with 0.5x $10^6$ density and wound created in

A549 cells' plate after one hour of plating and washing unattached cells as described in methods. In parallel to long- and short- programmed cells, parental A549 cells plated in 6 well plate with the same density as a control. Of note, we established this experiment of wound healing assay in 3 days for long programmed cells after 30 days of co culturing with macrophages and 30 days resting in single culture, and in 3 days of short-programmed cells after 30 days of co culturing with macrophage and 30 days resting in single culture. Then, the ability of cells to migrate was measured by the wound width during the 3 days. In order to compare the wound width measurements between long- and short-programmed cells, the wound width was measured on day 0 of culturing cells and compared to day 1,2 and 3.

As shown in figure (16A, B) long programmed cells started to migrate into the wound area on day 1 of culturing cells, but short programmed cells showed no significant change in wound width as in parental cells. On the second day, more cells were moved toward wound scratch in long programmed cells' plate with decreased wound width, while there was no difference observed in short-programmed cells (Figure 16A, B). The movement ability increased significantly with time periods in long programmed cells as the wound entirely closed at day 3, whereas the wound width in short-programmed cells did not show changes as in parental cells (Figure 16A, B). These findings indicate that even after the removal of macrophages following 30 days co culturing with A549 single cell clone, the effects of macrophages on promoting cancer cell migration along with mesenchymal transition persist in lung cancer cells. However, migration abilities along with mesenchymal transition state disappeared in A549 cells after removing macrophages following 3 days co culturing with lung cancer cells. These observations, which are in line with the expression of epithelial and mesenchymal proteins in both long - and short- programmed cells, support the hypothesis that prolonged exposure of A549 cells to macrophages secreted cytokines during long time crosstalk process resulted in chronic acquisition of migration ability in lung cancer cells. In other words, long term crosstalk of lung cancer cells with macrophages enhanced lung cancer cells' memory to continue responding to macrophages even when they were absent. However, this chronic changes in A549 cells did not occur after removing macrophages from 3 days co

culturing. Furthermore, these changes will be confirmed with RNA genes expression analysis in RNA sequencing data section.

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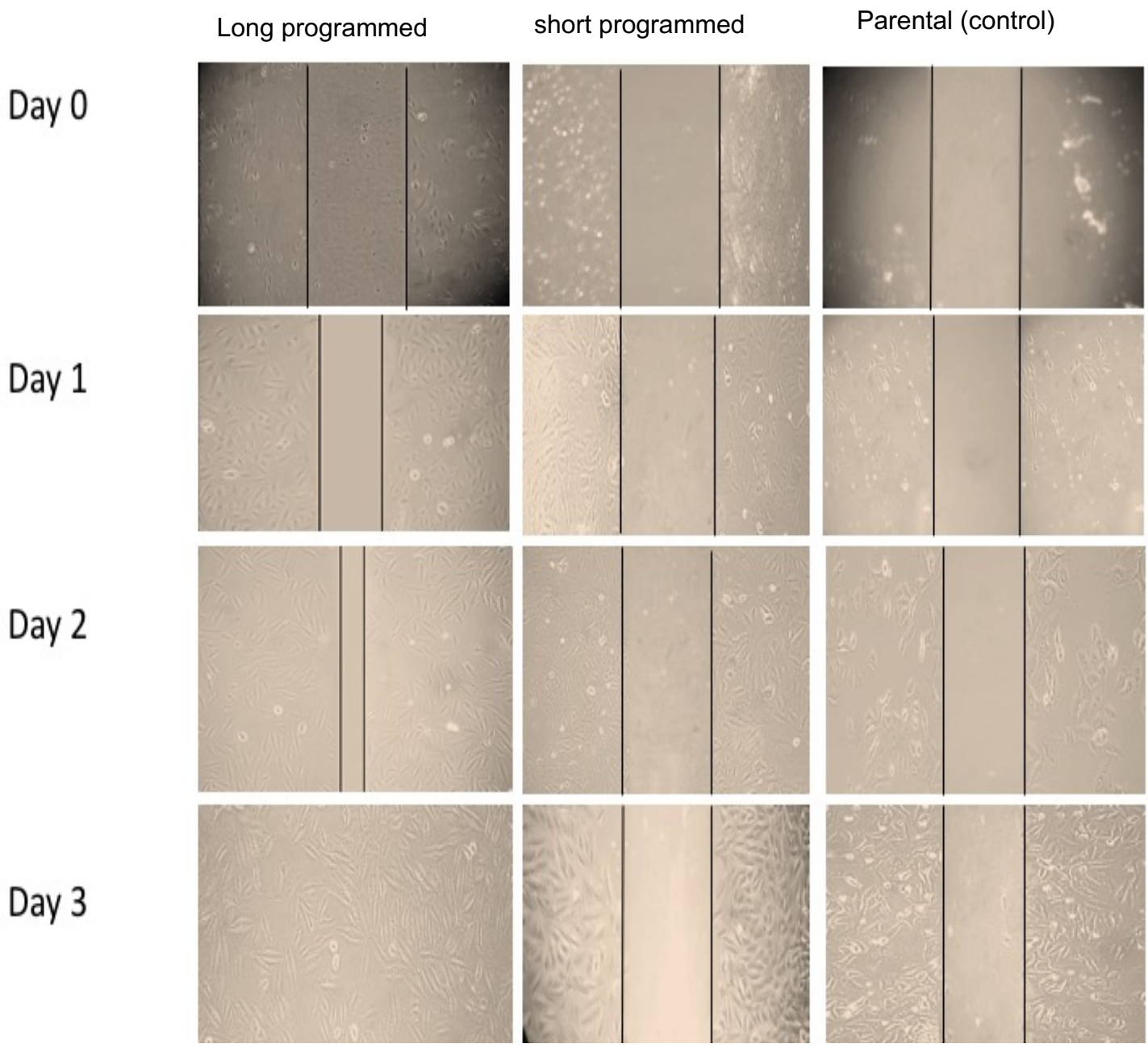


**B**

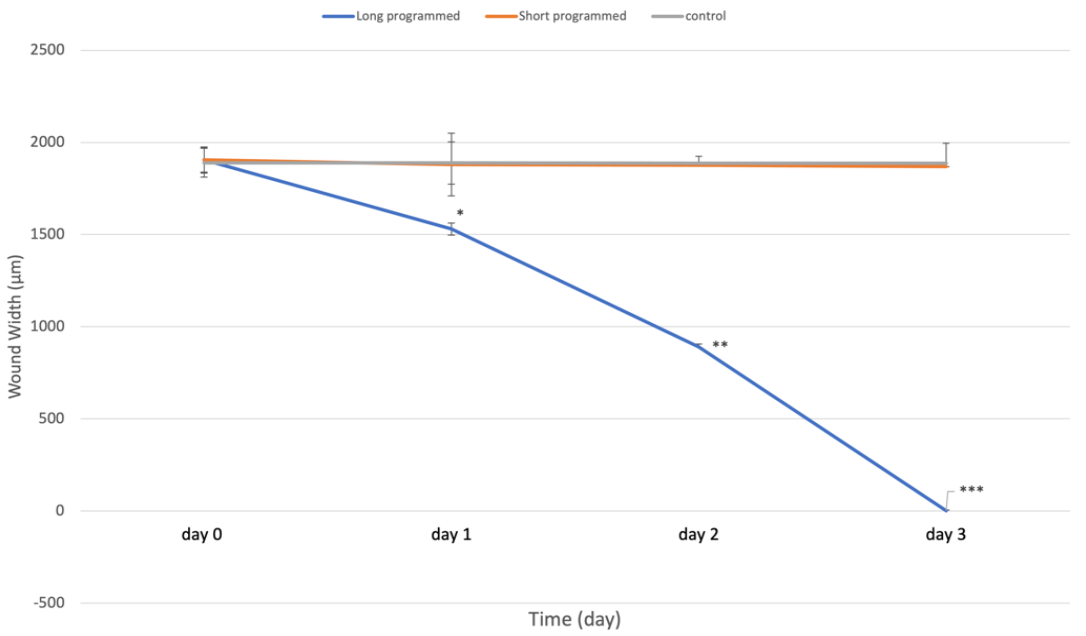



**Figure 16 Representative images of A549 clone wound healing during 3 days**. A-wound closure of long programmed, short programmed, control A549 cells during 3 days. B-The rate of wound closure between 2 intact zones over 3 days in A549 clones. Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples.

### 5.2.3 Macrophages induced lung cancer proliferation after short- or long- term incubation with macrophages

In our previous data we assessed cell proliferation of heterogenous A549 co cultured with macrophages for 3 days and the results showed high proliferation rate of co cultured cells relative to parental cell in single culture (Figure 11). In this part of study, firstly, we aim to evaluate lung cancer cells proliferation in single clone of A549 cells co cultured with macrophages to ensure that increased in proliferation rate was due to intrinsic changes of lung cancer cells following incubation with macrophages and not because of heterogenous mixed population of A549 cells. Secondly, in line with previous aims of investigating macrophages effects on cancer hallmarks in long – and short- programmed cells, here we aim to investigate proliferation of A549 cell after long- or short-time incubation with macrophages and resting lung cancer cells in single culture for month. We expect that long time incubation of macrophages with lung cancer cells will result in sustain increased of A549 cells proliferation while short time incubation will not have chronic effects on lung cancer cells after removing macrophages as it is happened in gene expression and mesenchymal transition properties.

To perform the first aim, single clone of A549 cells were generated as described in methods and co cultured with macrophages for three days, with $0.5x10^6$ cells density for macrophages and A549 cells. Then, daily cells count was performed in A549 cells during the three days of co culturing. As shown in figure (17), cell proliferation increased in co cultured A549 cells at day 1 (50 % increase), with significant increased at day 2 and 3 (70-87%) in comparison to control. This confirmed that incubation of macrophages with lung cancer cells for 3 days resulted in intrinsic changes of cancer cells that increased A549 cells proliferation.

To investigate the second aim, after 30 days incubation of A549 single cell clone with macrophages followed by resting A549 cells in single culture without macrophages, A549 cells were plated in 6 wells plate and daily count was performed for three days for cells labelled (long programmed). The same steps were done after 3 days of A549 cells co culturing with macrophages and resting cells in single culture without macrophages, daily count was performed for three days for cells labelled (Short programmed).

As shown in figure (17), cell proliferation rate increased in similar rate in both long- and short- programmed cells compared to control. The rate of proliferation ranged between 35% to 25 % for the day 1 and 3, while it appears that both cells are proliferating steadily on day 2. However, in comparison to 3 days co cultured cells, both long - and short- programmed cells show less proliferation rate when culturing without macrophages. These findings indicate three things: first, macrophages acutely educated lung cancer cells to divide rapidly as co cultured single cell clone showed high proliferation rate relative to parental and programmed cells. Second, the effects of macrophages on inducing lung cancer cell proliferation sustain after long- or short-time incubation with macrophages in absence of macrophages, but at slow rate than in presence of macrophages. Third, macrophages-induced proliferation effects remain even after short time incubation with macrophages which is different from previous tested cancer hallmarks that persist in long programmed but not in short-programmed cells.

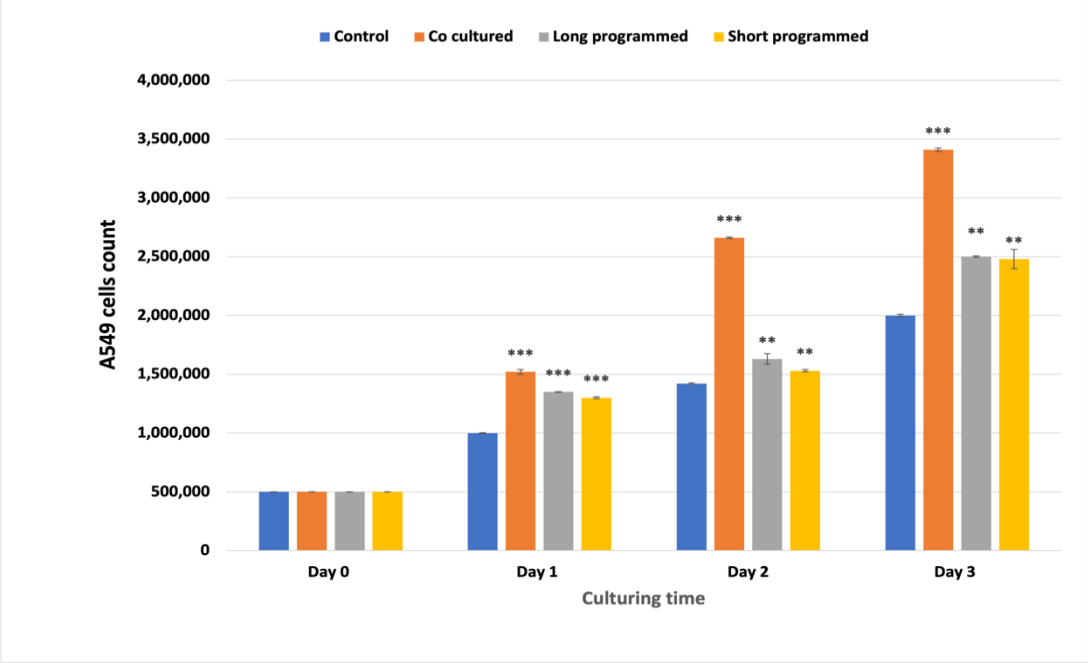



**Figure 17 cell proliferation of A594 single cell clones.** single cell clone co cultured with macrophages for 3 days (co cultured A549), single cells clone co cultured with macrophages for 30 days and cultured in single culture for 30 days (Long programmed), single cells clone co cultured with macrophages for 3 days and cultured in single culture for 30 days (short programmed). Data are displayed as mean values ± standard error of 2 independent experiments. A p-value of ≤ 0.05 was considered as statistical significance between the samples.

### 5.2.4 Next Generation RNA sequencing

In general, RNA-seq is a technique widely used in cancer research studies to classify the gene expression level known as “transcriptome” to up- or down-regulated between different cultures or treatment conditions of cells, such as comparing cancer cells treated with a specific drug to non-treated cells. In addition, RNA sequencing is utilized to investigate the pathogenesis of cancer by analyzing the downstream biological pathways that affect the cellular processes in order to develop new drug for cancer treatment. It is a powerful technique based on using next generation sequencing (NGS) to identify various RNA populations, such as messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

The main aim of this part of study is to identify the pro tumorigenic effects of macrophages on lung cancer cells cultured under different conditions with macrophages by comparing the differential expression of genes (DEGs), the downstream system biology analysis provided by gene ontology (GO), and the analysis of changes in cellular processes and enrichment of biological pathways provided by the Kyoto Encyclopedia of genes and genomes (KEGG).

Based on the biology of cancer development *in vivo*, which requires weeks to months of interaction with the surrounding environment, we hypothesized that long time incubation of A549 cells with macrophages may result in chronic changes of A549 cells that persist after macrophages removal. We compare changes in gene expression of A549 cells cultured without macrophages for one month after long time (30 days) co cultured with macrophages to A549 cells cultured alone for one month after short time (3 days) co cultured with macrophages, and both of which were compared to A549 cells co cultured with macrophages without resting in single culture. RNA was extracted for each clone and quality was assessed as described in methods, the expression profile for three biological repeats of each sample were performed at Novogene Europe (Cambridge Science Park, Cambridge, United Kingdom) using NGS (next generation sequencing). For clarity, samples labelling and A549 culture conditions used for RNA-seq are as following: clones from A549 co cultured with macrophages for long term (30 days) and rested for 1 month after macrophages removed labelled as long programmed (long prog), cells from short term co culture (3 days) with macrophages and rested for one month post macrophages

removal termed short programmed (short prog), cells co-culture with macrophages for 3 days without resting labelled (co 3 days ) or (co-culture), and parental cells were labelled as (control).

#### 5.2.4.1 Hierarchical clustering of differentially expressed genes

Based on the hypothesis of this part of study to identify the differences of genes expression between our samples model of A549 cells cultured under different conditions illustrated in previous sections, the hierarchical grouping of the index-coded samples of A549 were conducted for genes clustering. In order to identify differentially expressed genes between control, co 3 days, long programmed and short programmed cells, the genes with similar expression patterns in three biological replicates of each sample are clustered together in the heatmap of figure (18). The high expressed genes represented in red, and the low expressed genes are in green with threshold of fold change >2 and p value <0.05.

Based on these adjustments, the gene expression pattern in the heatmap indicates that long programmed cells are similar to co-culture cells with some differences in upregulated genes clustering. On the other hand, upregulated and downregulated genes clustering in short-programmed cells are remarkably identical to control cells. These results indicate that the long programmed A549 cells which have been cultured in the absence of macrophages for several weeks (30 days) still retain a gene profile that is similar to activated co cultured A549 cells by macrophages without resting. However, the short-programmed cells which have been cultured in the absence of macrophages following short time (3 days) incubation with macrophages have a gene profile that is similar to parental lung cancer cells that have not been co cultured with macrophages.

Furthermore, the significant up and down regulated genes was calculated as described in the methods. In terms of significant gene expression differences, in comparison to control, we found that there were more upregulated (5001) and downregulated (4539) significantly differentially expressed genes in co-culture cells (co 3 days), while in long programmed there were less dysregulated genes, 3311 upregulated and 1812 downregulated (Figure 19A-B). Interestingly, short-programmed cells showed very few upregulated genes (365) and no downregulated genes expressed in comparison to parental cells (Figure 19-C), suggesting that both cells have similar changes in genes expression. This

indicates that the gene expression pattern of short-programmed cells returned to the pattern of parental cells after macrophages removal from 3 days co culturing. On the other hand, in comparison to 3 days co cultured cells, we found 1948 upregulated and 1549 downregulated genes were differentially expressed in long programmed cells relative to co-culture cells, while in short programmed were 1258 genes significantly upregulated and 1223 downregulated (Figure 19D, E).

The significant up- and down- regulated genes expression pattern in these samples confirm the similarities between co cultured (co 3 days) and long programmed cells as well as the similarities between short- programmed and control cells. This supports our hypothesis that long-term exposure of lung cancer cells to macrophages-secreted factors during the crosstalk process could result in a pattern of gene expression changes sustained after macrophages removal that is similar to the gene profile seen in presence of macrophages in co cultured A549 cells. However, removing macrophages after short time of incubation with macrophages returned genes expression to the pattern similar to parental A549 cells. Furthermore, the detailed enrichment of GO and KEGG pathways were analysed in A549 clones to find the similarities and commonalities between A549 clones cultured under different conditions.

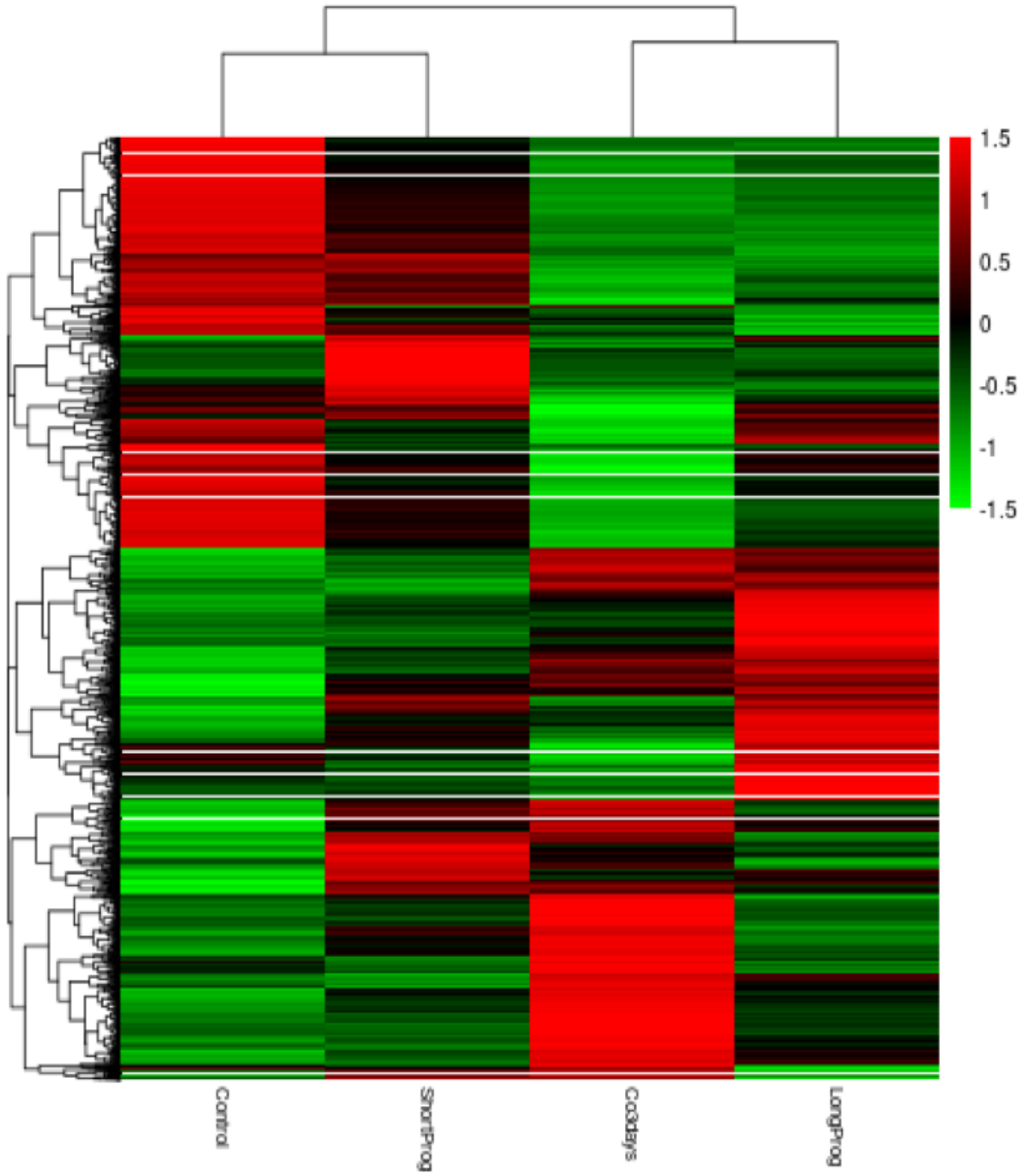
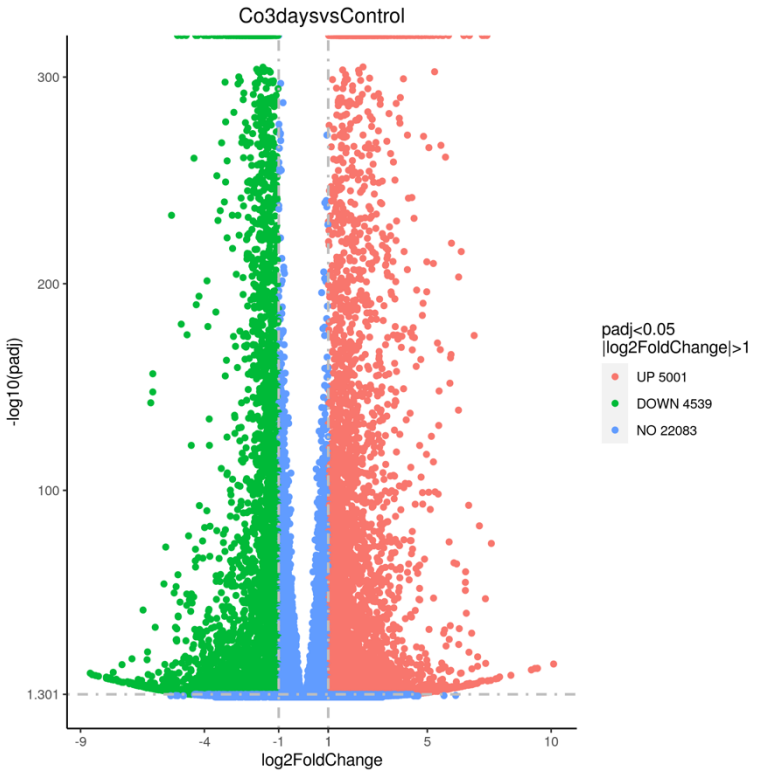



**Figure 18** Gene expression pattern of A549 clones represented by the heat map. Parental A549 (control), short programmed (short prog), co-culture cells for 3 days (co 3 days), long programmed (long prog). Data is representative from 3 independent experiments.

A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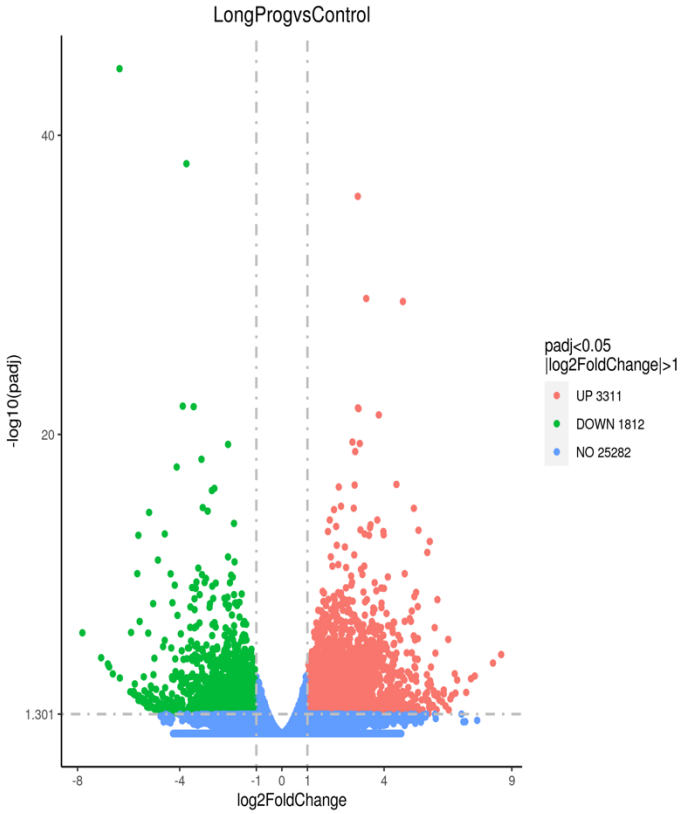
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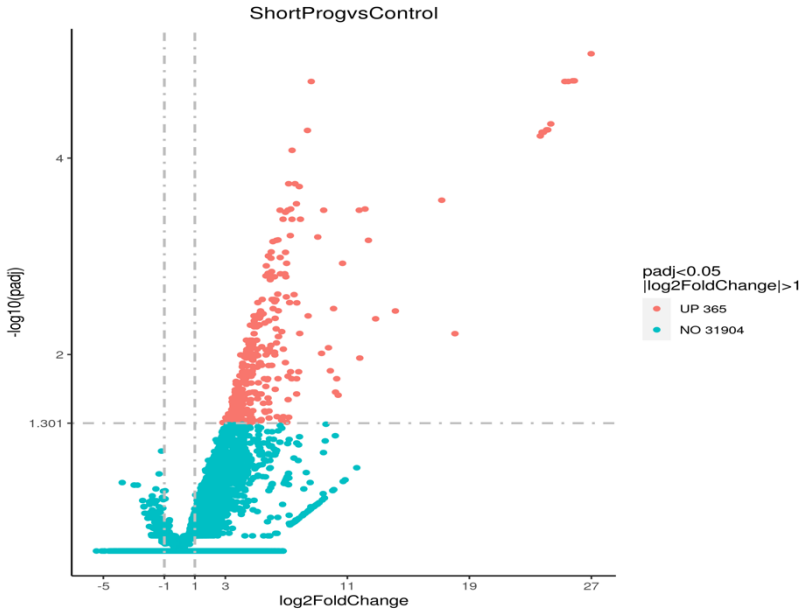
-8
-4
-1
0
1
4
9
log2FoldChange
padj<0.05
|log2FoldChange|>1
UP 3311
DOWN 1812
NO 25282

C


ShortProgvsControl
4
2
1.301
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-5
-1
1
3
11
19
27
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UP 365
NO 31904

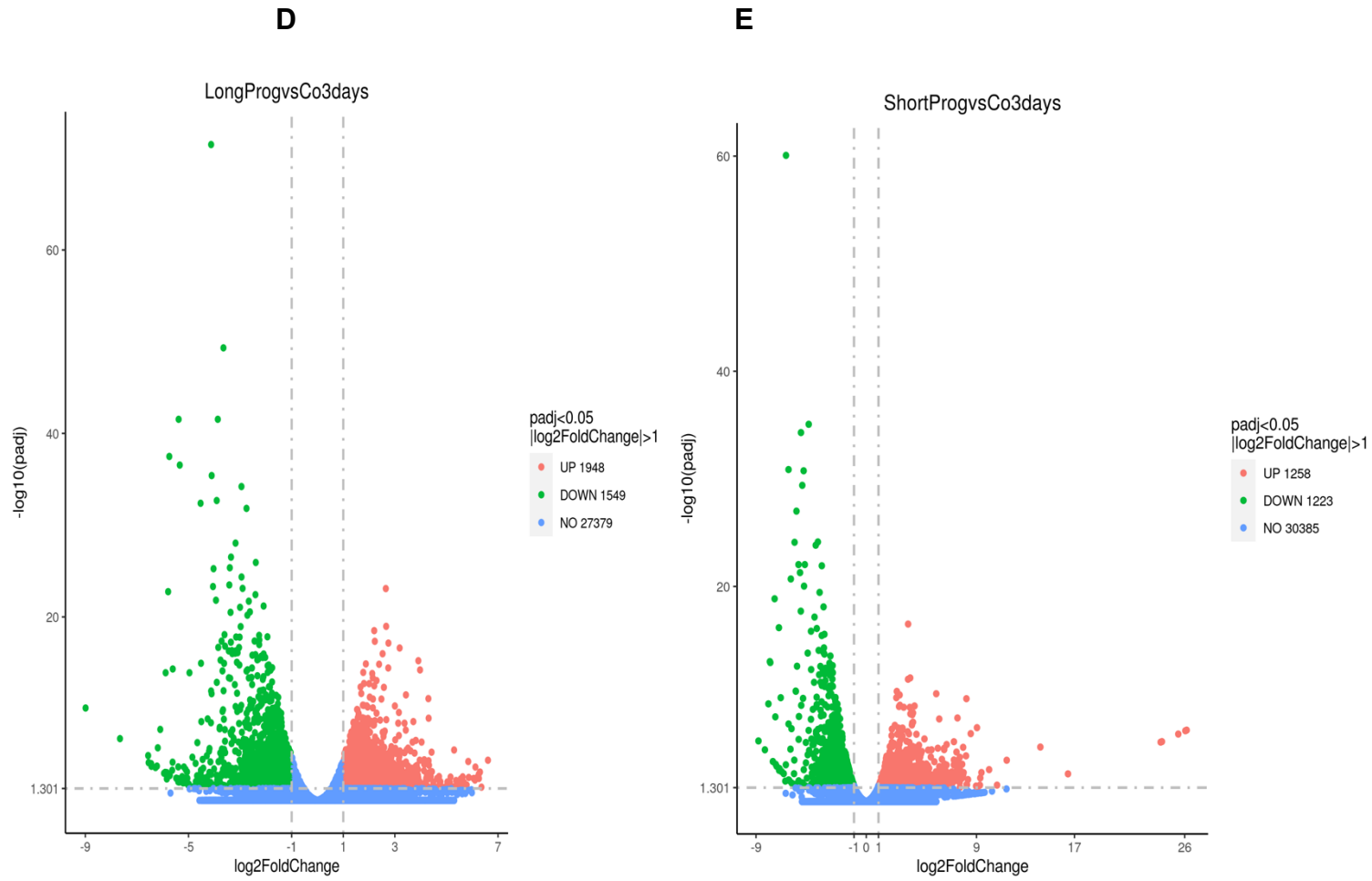



**Figure 19** Volcano plots of identified differentially expressed genes between A- 3 days co-culture vs control B- long programmed vs control C-short- programmed vs control D-long programmed vs co 3 days E- short-programmed vs co 3days

#### 5.2.4.2 Gene ontology (GO) enrichment analysis of differential expressed genes

Gene ontology analysis helps us to identify the functional properties of the differential expressed genes and classify those genes into a category of biological process based on their functions. Additionally, we will be able to compare the upregulated and downregulated genes functions in A549 clones cultured under different conditions.

In this part of RNA sequencing analysis, data of up-regulated and down-regulated genes were analysed in A549 lung cancer cells clones cultured under different conditions. Gene ontology enrichment analysis was conducted in A549 lung cancer cells including co 3 days, long-programmed and short-programmed cells compared to control samples, which could help us to understand the roles of the significant differential expressed genes in our samples. Among all samples, the upregulated and downregulated differentially expressed genes were classified into three groups: biological processes (BP), molecular function (MF) and cellular component (CC). As shown in figure (20), the gene ontology shows the top 10 significant enriched upregulated and downregulated annotations for each sample compared to control with genes count in each enrichment. Based on the bioinformatic analysis, we found common enrichment of the up regulated genes in both co-culture and long-programmed cells in “extracellular matrix organisation” and “proteinaceous extracellular matrix” but those were not identified in the up regulated genes of short- programmed cells. It has been reported that the activation of genes involved in the extracellular matrix molecular process was shown in the progression of the non-invasive bladder cancer to the invasive type (Zhang et al., 2020) , which suggests that co-cultured and long-programmed lung cancer cells transformed into an invasive state by presence of macrophages in short -term co-culturing and in the absence of macrophages after long time incubation in long programmed cells. However, removing macrophages after short- term co-culturing process reversed A549 cells into non-invasive state (Figure 20A, C, E). On the other hand, the enrichment of the downregulated genes in comparison to control showed some similarities between co-cultured and long-programmed cells, including mRNA and splicing processing, but their oncogenic potential in lung cancer has not yet been determined. However, there was no significant enrichment of the down-regulated genes in short-programmed

cells in comparison to control (Figure 20- B, D). This may indicate that the same biological processes that went down in short-programmed cells after removing macrophages are similar to parental cells.

Furthermore, in comparison to co 3 days (co-culture) cells, long programmed and short programmed cells enrichment of up-regulated and down-regulated genes shown in figure (21). In long-and short-programmed cells, the up regulated genes have not significantly enriched in processes related to carcinogenesis that we found in previous comparison of co cultured and long programmed cells relative to control (Figure 21A, C). We found that “ribosome biogenesis” and other ribosome-related genes were upregulated in long-programmed clones compared to co- cultured A549 cells. This finding highlights the important role of ribosomes in the regulation of gene expression, and increased ribosome biogenesis leads to increased protein synthesis, which is the main factor in maintaining carcinogenesis (Chatziantoniou and Zaravinos, 2022) (Figure 21 A,C).

On the other hand, for the downregulated genes of long and short-programmed compared to co-cultured cells, we found the enrichment in "extracellular matrix organization" and "proteinaceous extracellular matrix" downregulated in both long and short programmed cells (Figure 21B, D). These genes were upregulated in co-cultured and long-programmed cells relative to control, which may indicate that the expression of genes involved in the biological process of cancer cells invasion were significantly reduced in long- and short- programmed cells in the absence of macrophages compared to lung cancer cells incubated with macrophages (Co 3 days) (Figure 21B, D). In addition, "chemokine activity", "chemokine receptor binding", "growth factor binding" and "CXCR chemokine receptor binding" were found to be enriched in downregulated genes of both long- and short-programmed A549 cells, which reflects those macrophages are the major source of most cytokines secreted in the process of crosstalk with cancer cells. Therefore, compared to A549 cells co cultured with macrophages without resting, the absence of macrophages when programmed A549 cells rested in single culture led to a decrease in the secreted growth factors and cytokines, which in turn decreased the expression of their receptors in lung cancer cells. Furthermore, CXCR chemokine receptors genes were also found to be downregulated in long and short programmed cells after macrophages removal. It has been reported that the CXCR chemokine receptor is highly selective for

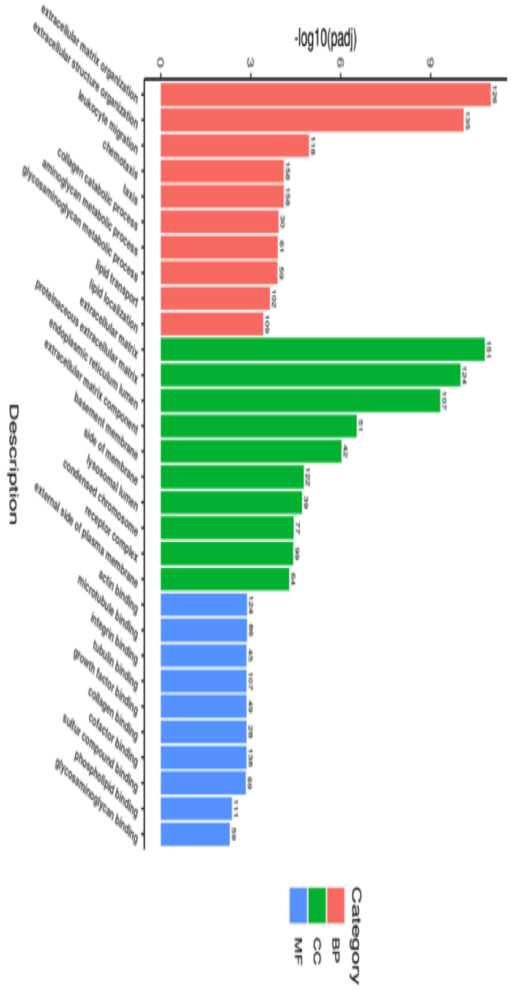
CXCL8 chemokines, which are mainly secreted by immune cells and mediate tumour progression of different cancers (Spaks et al., 2016; Chen et al., 2018).

In conclusion, in terms of investigating the effects of macrophages on cancer processes in lung cancer cells, GO enrichment analysis suggests that the incubation of A549 cells with macrophages for short time induced the expression of genes involved in several processes that promote cancer progression in comparison to parental A549 cells. Additionally, these genes were also upregulated in long programmed cells, which indicate that the long exposure of lung cancer cells to macrophages caused chronic changes in gene expression that persisted after the macrophages removed and were similar to those that occurred in co cultured cells (co 3 days). However, the expression of genes involved in cellular processes that play important roles in promotion of cancer cells, which are induced by presence of macrophages in co cultured A549 cells such as CXCR chemokine receptor binding, were decreased in long- and short programmed compared to co 3 days cells.

Co 3 days vs control

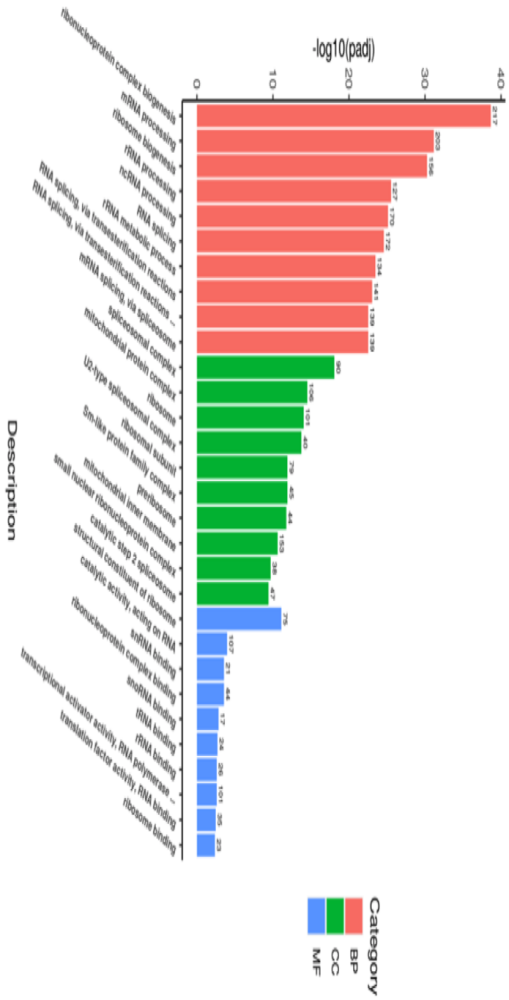
A

Up



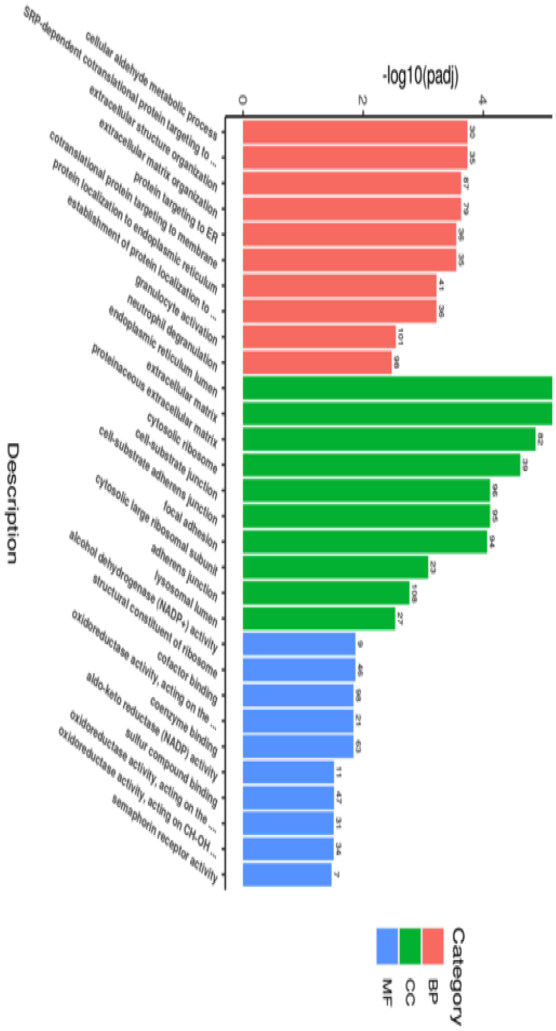

B

Down

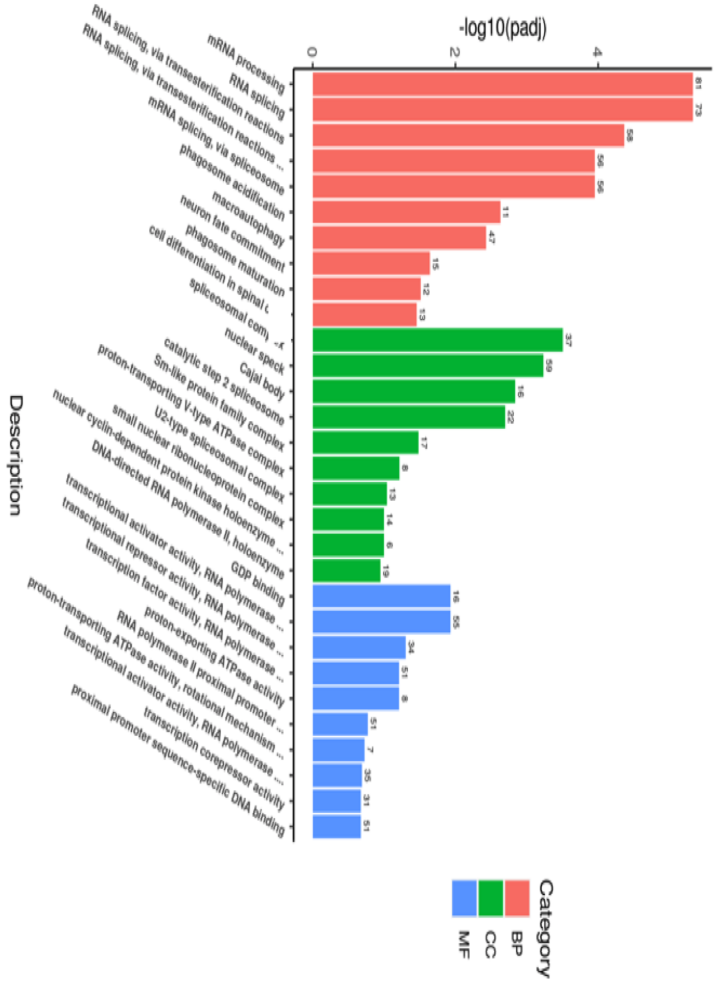


Long programmed vs control

C Up




D Down



short programmed vs
control

E

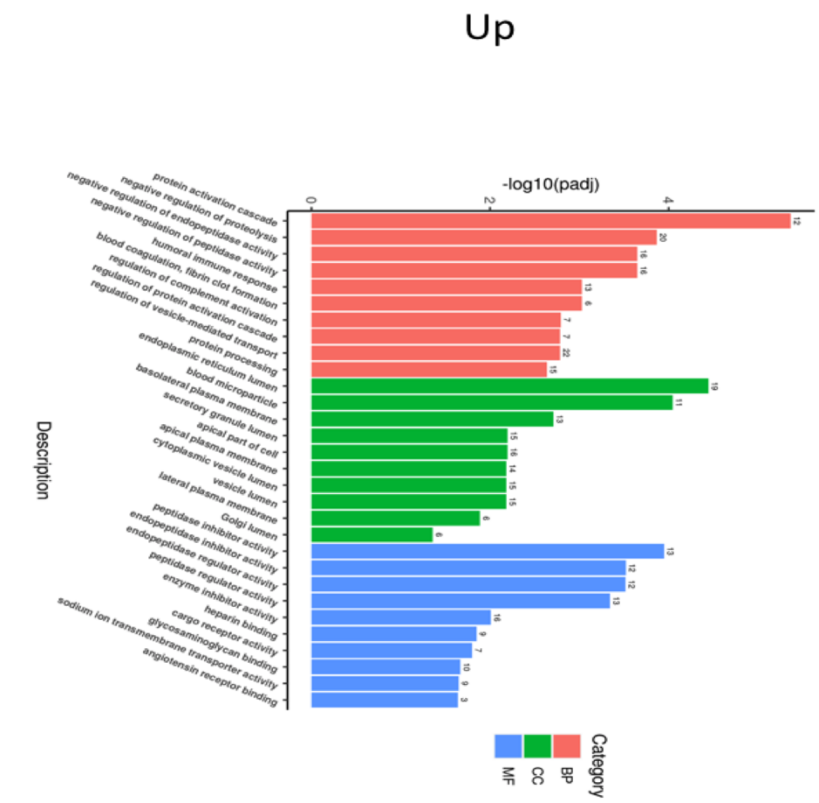
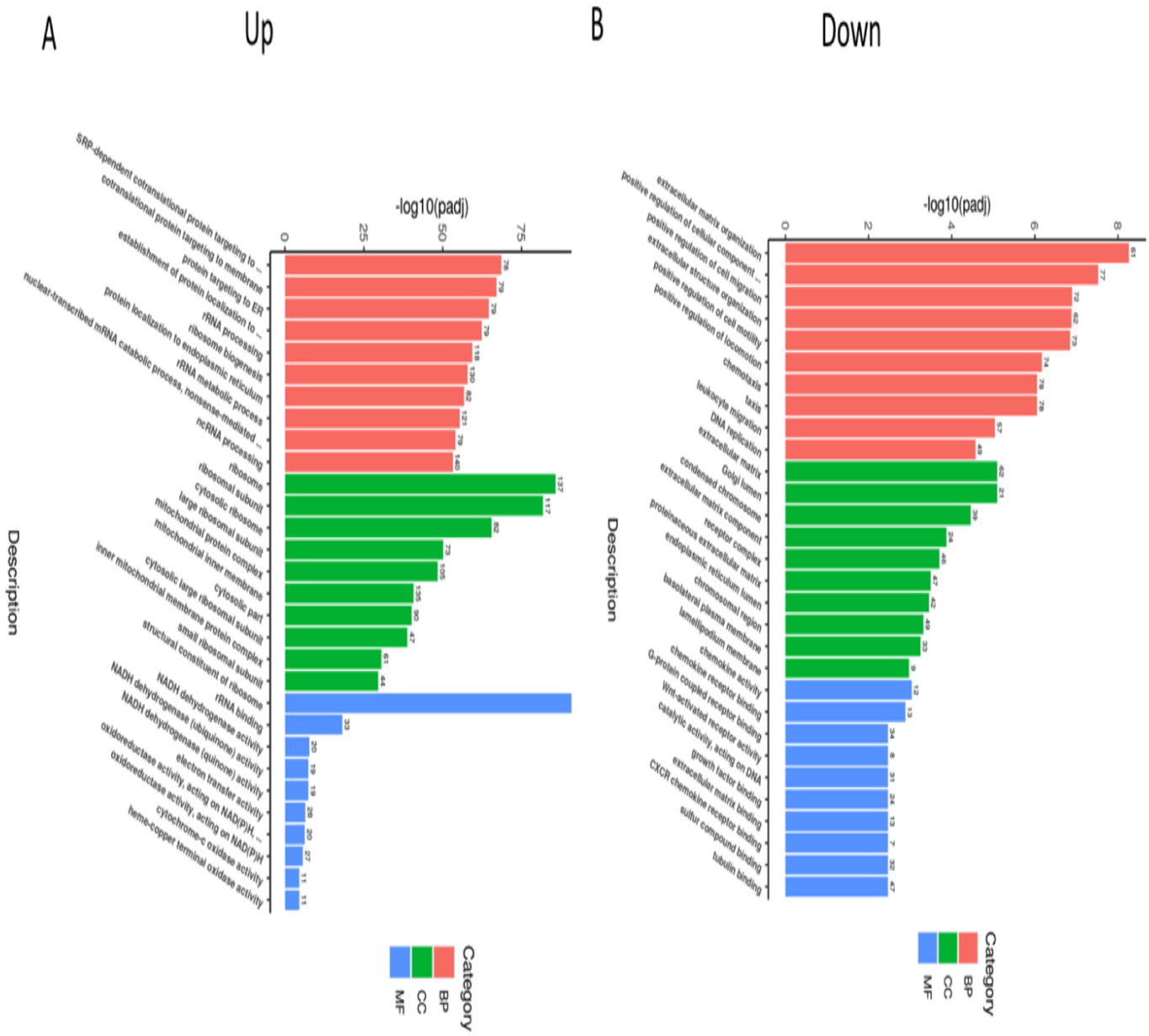



**Figure 20** top 10 functional enrichment analysis of gene ontology (GO). Analysis was performed by comparing gene expression level in A-up regulated co 3 days vs control. B-downregulated co 3 days vs control. C-up regulated long programmed vs control. D- down regulated long programmed vs control. E-up regulated short programmed vs control

Long programmed vs co 3 days



Short-programmed vs co 3 days

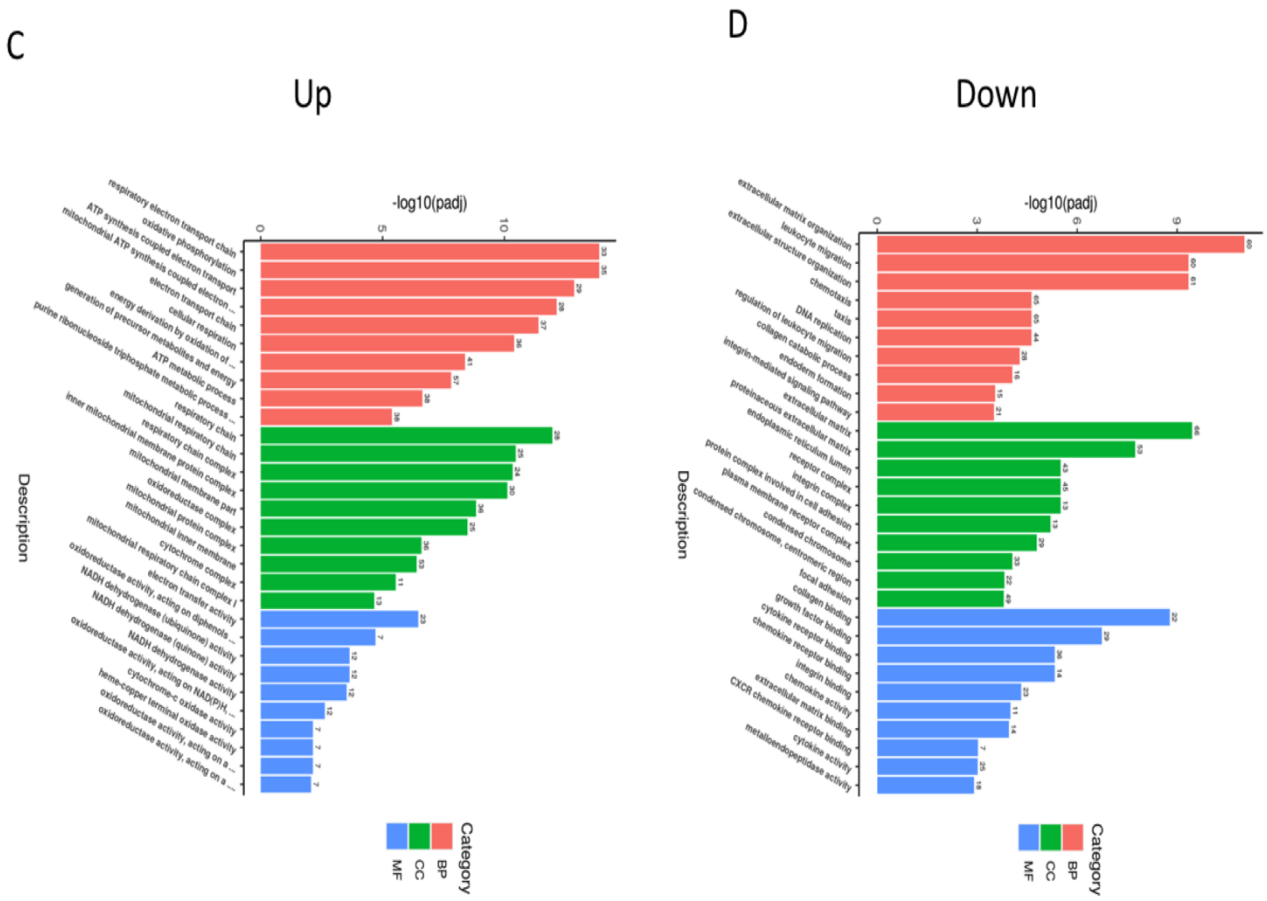



**Figure 21** Top 10 functional enrichment analysis of Gene ontology (GO). Analysis was performed by comparing gene expression level in A- up regulated genes in long programmed vs co 3 days. B-downregulated genes in long programmed vs co 3 days. C-upregulated genes in short-programmed vs co 3 days. D- downregulated genes in short-programmed vs co 3 days.

#### 5.2.4.3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

KEGG pathway analysis was performed to find the major enrichment pathways across samples in order to examine the impact of macrophages on the gene expression of lung cancer cells in presence of macrophages and on single culture of programmed A549 cells after macrophages were removed. Moreover, analysis of the significant enriched pathways may help in identifying the molecular processes underlying the phenotypic and functional changes of lung cancer cells underwent different culture conditions. Samples labels are the same labels used in previous section of gene ontology analysis, clones from A549 co cultured with macrophages for long term and rested for 1 month after macrophages removed labelled as long programmed (long prog), cells from short term co-culture with macrophages and rested for one month post macrophages removal termed short programmed (short prog), cells co-culture with macrophages for 3 days labelled (co 3 days), and parental cells were labelled as (control).

In comparison to parental A549 cells, 20 significant pathways of upregulated genes were arranged from most to least significant in co-cultured, long-programmed, and short-programmed A549 cells. The most significantly enriched pathways in co cultured cells were ‘extracellular matrix (ECM)-receptor interaction’, ‘IL-17 signalling pathway’, ‘Malaria’, ‘Arrhythmogenic right ventricular cardiomyopathy’, ‘Viral protein interaction with cytokine and cytokine receptor’ and ‘TNF signalling pathway’. Of these pathways, we emphasise on ECM-receptor interaction, IL-17 signalling pathway and TNF signalling pathways which are in close association with tumorigenesis (Figure22A and table 4A).

The long-programmed cells shared 3 significant upregulated pathways with co-culture cells which are IL-17 signalling, TNF signalling and ECM-receptor interaction (Figure 22B and table 4B). The activation of the ECM-receptor interaction genes reflects the important roles of the ECM structural changes that promote lung cancer metastasis and malignancy persistence (Chatziantoniou and Zaravinos, 2022). As several studies indicate that ECM-receptor interaction play an important roles in cancer progression and metastasis (Stankevicius et al., 2016; Maltseva and Rodin, 2018). In addition, some studies investigate the expression of specific genes in ECM receptor interaction pathway as a biomarker for the assessment of colorectal cancer prognosis (Boudjadi et al., 2013; Gong

et al., 2019). For the enrichment of IL-17 signalling pathway in co cultured and long programmed cells, it has been reported that the reason of the activation of this pathway in tumour cells because of its association with tumour inflammation as IL-17 acts as pro inflammatory cytokines that enhance lung cancer metastasis (Han et al., 2014; Wu et al., 2016).

These common pathways between co cultured and long programmed cells may imply that long-term incubation of lung cancer cells with macrophages resulted in persistent activation of these pathways even after macrophages removal. This suggests that these pathways are important for the development of cancer treatment strategies and targeting of macrophages in cancer. However, compared to control cells, those pathways are not activated in short- programmed cells, and the most two significant enriched upregulated pathways are not related to tumorigenesis process which are “Complement and coagulation cascades” and “Staphylococcus aureus infection” (Figure 22C and table 4C).

On the other hand, the downregulated pathways in co 3 days, long programmed and short programmed cells in comparison to control are shown in supplemental figure (1A, B). We found the pathways enrichment that significantly downregulated in both co cultured and long programmed clones is Herpes simplex virus 1 infection whereas no significant downregulated pathways detected in short-programmed cells relative to control.

In line with previous GO analysis, co 3 days and long programmed cells shared some of the pathways enrichment related to carcinogenesis progression in comparison to control. This suggests that the same carcinogenic molecular mechanisms occurred by exposure of lung cancer cells to macrophages secreted factors in co 3 days cells or after long exposure to macrophages in long programmed clones.

**Long- and short-Programmed cells vs co 3 days up-regulated and down-regulated KEGG pathways enrichments**

In order to find the differences in programmed A549 cells activated pathways after removing macrophages from long- and short-term co-culture transwell system, both programmed cells were compared to co-culture KEGG pathways enrichment. Interestingly, among the top 20 up regulated pathways, we found

that Huntington and other neurodegenerative disease pathways were significantly upregulated in short and long programmed A549 cells relative to co 3 days (Figure 23A, B and table 5-A, B). In accordance with these results, Valera and Garcia reviewed the oncogenic pathways that altered in neurodegenerative disease in order to understand the role of cancer pathways in neurodegenerative disease pathogenesis, they found that cancer and neurodegenerative disease shared the alterations of the same molecular mechanisms which result in survival and proliferation of cancer cells while cell death and apoptosis in neurodegenerative disease (Varela and Garcia-Rendueles, 2022). In our model, both programmed cells were exposed to macrophages secreted factors that may induce and persist the proliferation and survival of A549 cells in single cultures for a month following long-or short-term incubation with macrophages.

Furthermore, we found that the “Ribosome” pathway was significantly upregulated in long programmed but not identified in short-programmed cells. The reason for the activation of genes involved in ribosome pathway may refer to the continuation of EMT state in long programmed cells as it has been reported that EMT is a multi-step process, and the increased of ribosome biogenesis is a marker for continuation of EMT state in cancer cells, while the inhibition of rRNA synthesis ended the process (Prakash et al., 2019). These suggest that long-term interactions between lung cancer cells and macrophages result in a continuous mesenchymal state even after macrophages removal, which is consistent with the previous experiment's findings of increased N-cadherin and decreased E-cadherin proteins expression in long programmed cells.

On the other hand, compared to co 3 days cells, among the significant reduced pathways in long programmed cells, “MicroRNA” was the one related to cancer pathogenesis (Figure 23C, table 5A). The roles of microRNA in cancer have been reviewed by Zhu and his team and Bartel, they reported important tumour suppressor or oncogenic roles of microRNA in cancer by targeting various mRNA encoding oncogenes or tumour suppressor genes (Bartel, 2009; Zhu et al., 2021). In addition, abnormal miRNA profile was identified as an important biomarker for NSCLC diagnosis and prognosis (Iorio and Croce, 2012). Some of the downregulated miRNA targets identified in our long-programmed clones, such as TIMP3, a tumour suppressor gene targeted by miR-222 (Table 5A). In line with this, Garofalo and his team found that miR-222 enhance tumorigenesis in

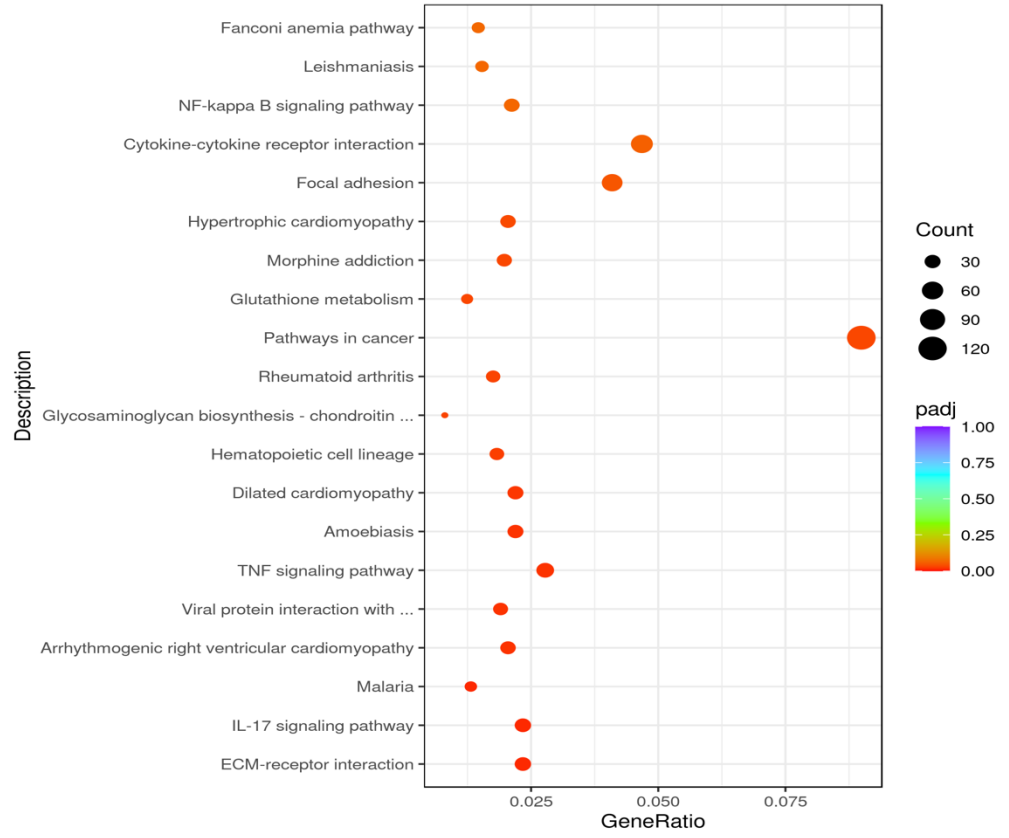
aggressive NSCLC by targeting TIMP3 (Garofalo et al., 2009). These findings indicate the persistence of pro tumorigenic effects of macrophages in lung cancer cells by downregulation of tumour suppressor genes in long programmed cells after macrophages removal.

In short -programmed cells relative to co-culture, the top 20 pathways were significantly downregulated shown in (Figure 23D, table 5B). Among these pathways," TNF signalling", "ECM-receptor interaction", "Rheumatoid arthritis", and "IL-17 signalling", which are related to cancer progression were found downregulated in short-programmed cells relative to co-culture. To emphasise, culturing A549 clone in single culture after short time incubation with macrophages resulted in the significant decrease of those pathways that were found up regulated in co 3 days and long programmed cells relative to control as shown in previous section. This also indicates the decrease of carcinogenic processes related to those pathways in short-programmed cells after removing macrophages.

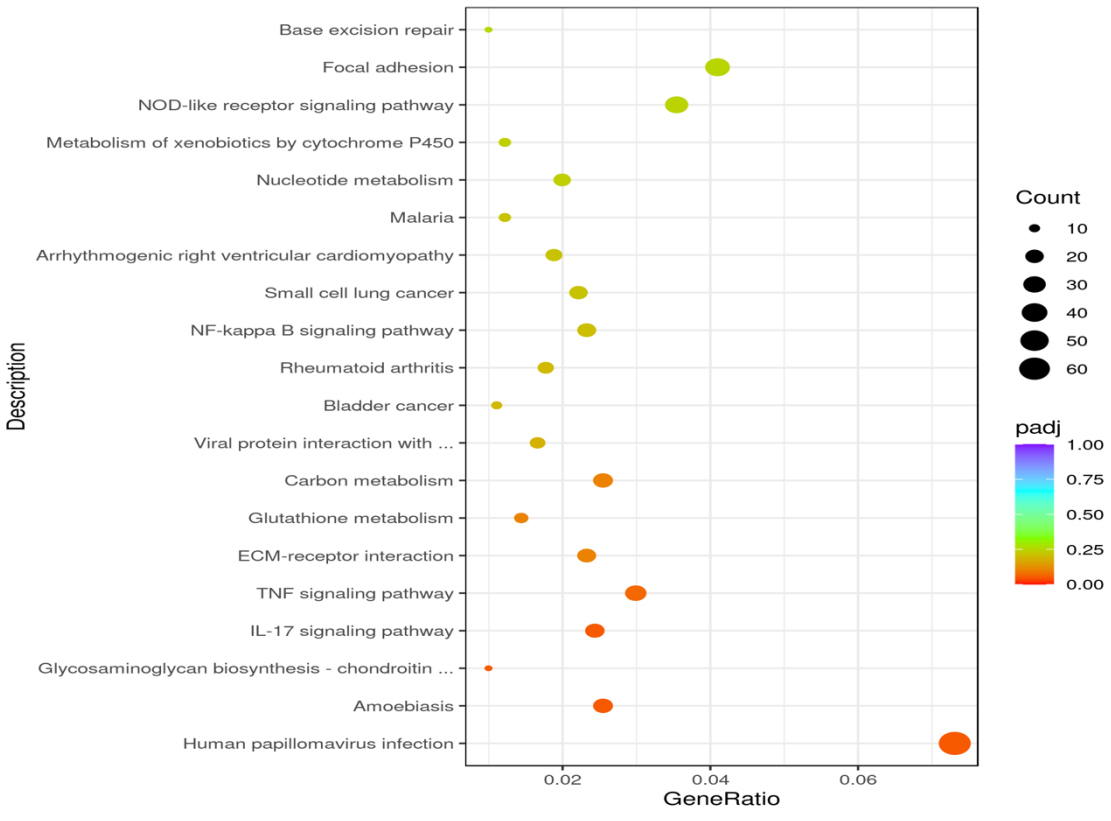
Lastly, in comparison of two programmed cells, short-programmed vs long, only two significant upregulated pathways were identified and not related to tumorigenesis, which are "Complement and coagulation cascades" and "Maturity onset diabetes of the young". However, more down regulated pathways were identified in short programmed compared to long programmed cells including "ECM-receptor interaction", "Cytokine-cytokine receptor interaction", "Rheumatoid arthritis", "IL-17 signalling pathway" and "PI3K-Akt signalling pathway" (supplemental Figure 2A, B and table1).

In conclusion, all these KEGG enrichment data showed the persistence of pro tumorigenic effects of macrophages in A549 cells co cultured with macrophages for long time followed by macrophages removal, which is similar to carcinogenesis effects that occurred in presence of macrophages in co 3 days cells. This emphasises on the effects of long-time exposure of A549 cells to macrophages on the biological memory of lung cancer cells, which allows cancer cells to continue the pro tumorigenic impacts of macrophages even after they were no longer present.

**A**


Fanconi anemia pathway
Leishmaniasis
NF-kappa B signaling pathway
Cytokine-cytokine receptor interaction
Focal adhesion
Hypertrophic cardiomyopathy
Morphine addiction
Glutathione metabolism
Pathways in cancer
Rheumatoid arthritis
Glycosaminoglycan biosynthesis - chondroitin ...
Hematopoietic cell lineage
Dilated cardiomyopathy
Amoebiasis
TNF signaling pathway
Viral protein interaction with ...
Arrhythmogenic right ventricular cardiomyopathy
Malaria
IL-17 signaling pathway
ECM-receptor interaction
Description
0.025
0.050
0.075
GeneRatio
Count
30
60
90
120
padj
1.00
0.75
0.50
0.25
0.00

**B**


Base excision repair
Focal adhesion
NOD-like receptor signaling pathway
Metabolism of xenobiotics by cytochrome P450
Nucleotide metabolism
Malaria
Arrhythmogenic right ventricular cardiomyopathy
Small cell lung cancer
NF-kappa B signaling pathway
Rheumatoid arthritis
Bladder cancer
Viral protein interaction with ...
Carbon metabolism
Glutathione metabolism
ECM-receptor interaction
TNF signaling pathway
IL-17 signaling pathway
Glycosaminoglycan biosynthesis - chondroitin ...
Amoebiasis
Human papillomavirus infection
Description
0.02
0.04
0.06
GeneRatio
Count
10
20
30
40
50
60
padj
1.00
0.75
0.50
0.25
0.00

C

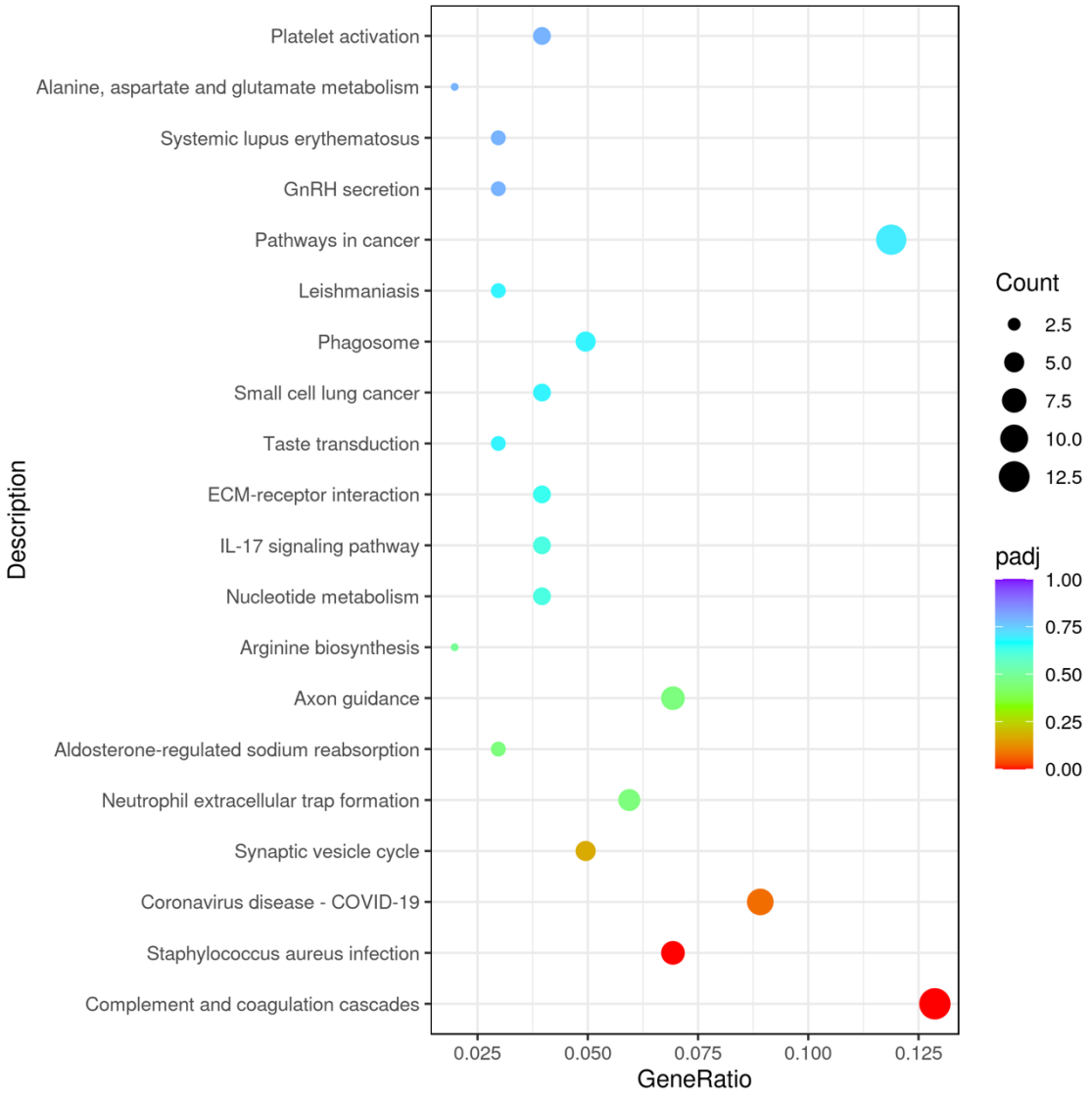



**Figure 22** Top 20 functional enrichment analysis of upregulated KEGG pathways. A-KEGG pathways of co 3 days vs control. B- KEGG pathways of long programmed vs control. C-KEGG pathways of short programmed vs control

| KEGG description | Gene Ratio | Padj | Genes |
|---|---|---|---|
| ECM-receptor interaction | 32/1368 | 0.01179429 | ITGA3/CD44/ITGB5/AGRN/LAMB1/FN1/DAG1/ITGA2/HSPG2/LAMA3/ITGB4/VTN/THBS1/ITGA11/COL6A2/COL6A1/COL4A6/ITGA5/ITGA10/COL4A4/THBS3/LAMB3/ITGA1/ITGB3/COL4A3/ITGB8/ITGA4/LAMC3/ITGA2B/AC092153.1/COL6A3/LAMA4 |
| IL-17 signalling pathway | 32/1368 | 0.01179429 | CXCL5/NFKBIA/CXCL8/PTGS2/LCN2/TNFAIP3/CCL2/TRAF3/CXCL2/IKBKE/CXCL3/MAPK11/TRAF2/TRADD/IL17RC/IL6/MAPK3/USP25/IL1B/CXCL6/FOS/MMP1/-/S100A9/CSF3/CSF2/AC015813.5/MUC5B/MUC5AC/SLC12A5-AS1/S100A8/CCL7 |
| TNF signalling pathway | 38/1368 | 0.0179378 | CXCL5/TNFRSF1A/BCL3/CSF1/NFKBIA/PTGS2/BIRC3/MAP3K8/TNFAIP3/CFLAR/JUNB/CCL2/TRAF3/AKT1/CXCL2/MAP2K6/CXCL3/IRF1/MAPK11/TRAF2/VEGFC/TRADD/CREB3L4/ICAM1/IL6/MAPK3/TRAF1/IL15/MLKL/IL1B/CXCL6/FOS/CX3CL1/-/CSF2/-/CREB3L3/SLC12A5-AS1 |

Table 4-A KEGG significant upregulated pathways in co 3 days vs control

| KEGG description | Gene Ratio | Padj | Genes |
|---|---|---|---|
| IL-17 signalling pathway | 22/903 | 0.05095548 | CXCL8/IL6/TRAF2/CCL2/IKBKE/MAPK11/IL17RC/PTGS2/MMP1/TRAF3/TRADD/TNFAIP3/LCN2/CXCL2/CSF2/CXCL3/CXCL5/-/MAPK3/NFKBIA/JUND/MUC5B |
| TNF signalling pathway | 27/903 | 0.06790707 | IL6/TRAF1/TRAF2/ICAM1/CCL2/MAPK11/CSF1/TNFRSF1A/PTGS2/BIRC3/TRAF3/TRADD/LIF/TNFAIP3/JUNB/CXCL2/CREB3L4/CSF2/CXCL3/-/CXCL5/MLKL/BCL3/IRF1/MAPK3/CREB3L3/NFKBIA |
| ECM-receptor interaction | 21/903 | 0.1037307 | LAMB3/ITGB3/AGRN/COL4A6/ITGA11/COL6A1/LAMB1/ITGA3/CD44/LAMA3/THBS3/ITGA2/COL6A2/ITGB5/LAMA5-AS1/ITGB4/LAMC2/COL4A4/ITGA4/LAMA4/VTN |

Table 4-B: KEGG significant upregulated pathways in long programmed vs control

| KEGG description | Gene Ratio | Padj | Genes |
|---|---|---|---|
| Complement and coagulation cascades | 13/101 | 3.81E-09 | FGA/FGB/CFH/F5/F7/FGG/VTN/CFI/C3P1/F2/-/BDK |
| Staphylococcus aureus infection | 7/101 | 0.00013693 | -/CFH/FGG/CFI/C3P1/-/C5 |

Table 4-C: KEGG significant upregulated pathways in short-programmed vs control

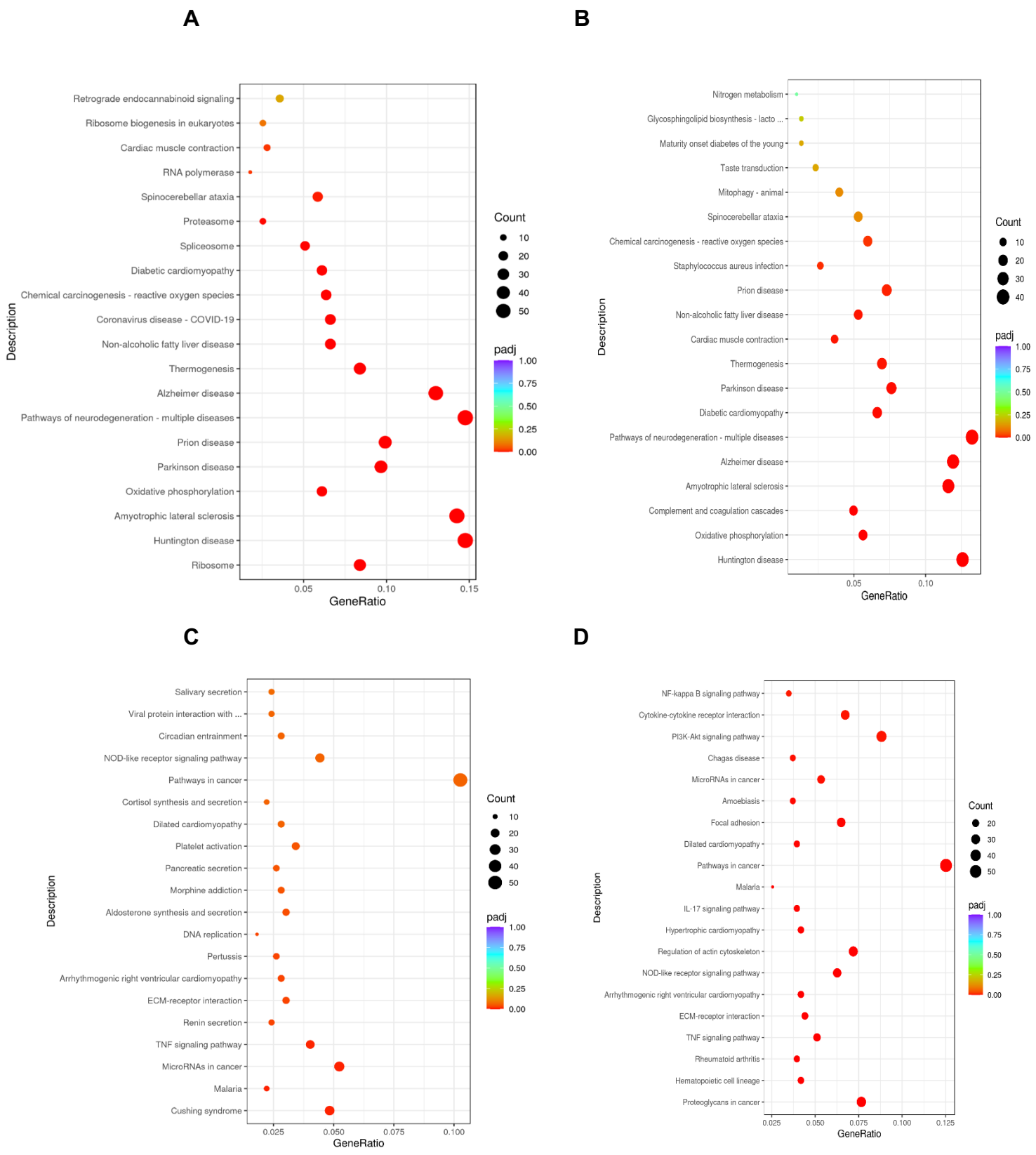



**Figure 23** Top 20 functional enrichment analysis of upregulated and downregulated KEGG pathways. A- Upregulated KEGG pathways of long programmed vs co 3 days cells. B- Upregulated KEGG pathways of short programmed vs co 3 days. C- Downregulated KEGG pathways of long programmed vs co 3 days. D- Downregulated KEGG pathways of short programmed vs co 3 days.

| KEGG description | Dysregulation | Gene Ratio | Padj | Genes |
|---|---|---|---|---|
| Ribosome | Up regulated | 33/393 | 1.36E-17 | MRPL27/MRPL24/MRPL36/AC073861.1/RPL26/MRPL14/-/RPL24P8/-/AC009245.1/-/-/RPL35P1/RPS28/MRPL12/-/MRPL15/MRPL34/FO393411.1/RPL8/RPL3P4/AC016739.1/MRPL1/MRPS15/-/-/MRPL28/RPS27/MRPL4/MRPS14/RPL4/-/AL450998.1 |
| Huntington disease | Up regulated | 58/393 | 4.26E-15 | /NDUFA4L2/NDUFAB1/NDUFS4/PSMB7/CLTB/UQCRH/-/BBC3/POLR2F/SEM1/ATG101/-/PSMB1/COX6C/ATP5PF/CREB3/NDUFA9/UQCRHL/BAX/PSMA7/POLR2H/COX8A/NDUFA11/NDUFA8/UQCR11/-/COX7A2/PSMA1/UQCRFS1/NDUFA1/-/TRAF2/NDUFC2/POLR2G/NDUFB7/POLR2J/CYC1/-/POLR2L/PSMB4/-/-/STX1A/-/CREB5/NDUFS8/ATP5F1D/AP2S1/-/-/DNAH17-AS1/AL354836.1/PSMC4/-/DNAH14/NDUFV3/- |
| Amyotrophic lateral sclerosis | Up regulated | 56/393 | 6.58E-11 | /NDUFA4L2/NDUFAB1/NDUFS4/PSMB7/NXT1/UQCRH/-/SEM1/ATG101/-/PSMB1/COX6C/ATP5PF/ATF4/DDIT3/NDUFA9/UQCRHL/BAX/PSMA7/COX8A/PRPH/NDUFA11/NDUFA8/UQCR11/-/COX7A2/PSMA1/UQCRFS1/NDUFA1/-/TRAF2/NDUFC2/NDUFB7/CYC1/-/PSMB4/-/RAB39B/-/-/NDUFS8/ATP5F1D/-/AF106564.1/NRG4/-/DNAH17-AS1/AL354836.1/-/TOMM40/PSMC4/-/DNAH14/NDUFV3/- |
| Cushing syndrome | Down regulated | 24/497 | 0.00854631 | NR4A1/FZD7/WNT7B/CCNE2/ADCY9/PBX1/ADCY6/E2F1/PDE11A/CDKN2C/CACNA1D/TCF7/RASD1/FZD1/ITPR3/WNT5B/CACNA1I/ADCY7/FZD5/GNAI1/ITPR1/-/FZD8/ITPR2 |
| Malaria | Down regulated | 11/497 | 0.00854631 | CXCL8/ICAM1/ITGB2/IL6/THBS1/CSF3/MYD88/CCL2/IL1B/LRP1/PECAM1 |
| MicroRNAs in cancer | Down regulated | 26/497 | 0.00854631 | PTGS2/TP63/CYP24A1/DNMT3B/CDC25C/PDGFB/SOX4/CCNE2/RECK/E2F1/BRCA1/EFN |

| | | | | |
|---|---|---|---|---|
| | | | | A2/KIF23/THBS1/BCL2L11/ERBB3/DNMT1/BMF/TIMP3/BMPR2/MTOR/EFNA1/CDCA5/NOTCH3/RDX |

Table5-A: KEGG significant upregulated and downregulated pathways in long programmed vs co 3 days cells

| KEGG description | Dysregulation | Gene Ratio | Padj | Genes |
|---|---|---|---|---|
| Huntington disease | Up regulated | 38/302 | 8.04E-07 | PPARGC1A/-/CACNA1B/NDUFS4/COX6C/-/-/SEM1/PLCB1/ATP5PF/UQCRFS1/KLC3/-/-/NDUFAB1/-/NDUFA1/COX7A2/UQCRH/POLR2H/CLTB/-/NDUFA11/NDUFA9/UQCRHL/UQCR11/DNALI1/SDHD/-/PSMA1/COX8A/DNAH14/DCTN6/- |
| Alzheimer disease | Up regulated | 36/302 | 0.000492164 | -/NDUFS4/COX6C/-/-/DDIT3/SEM1/ATP2A1/PLCB1/-/-/-/-/ATP5PF/UQCRFS1/KLC3/-/-/NDUFAB1/-/NDUFA1/COX7A2/UQCRH/FZD8/-/NDUFA11/NDUFA9/SLC39A5/NOX1/UQCRHL/UQCR11/SDHD/-/PSMA1/COX8A/- |
| Pathways of neurodegeneration - multiple diseases | Up regulated | 40/302 | 0.00095416 | -/CACNA1B/NDUFS4/COX6C/GPR37/-/-/DDIT3/SEM1/ATP2A1/PLCB1/-/-/-/-/ATP5PF/UQCRFS1/KLC3/-/-/NDUFAB1/-/NDUFA1/COX7A2/UQCRH/FZD8/-/NDUFA11/NDUFA9/NOX1/UQCRHL/UQCR11/DNALI1/SDHD/-/PSMA1/COX8A/DNAH14/DCTN6/- |
| TNF signalling pathway | Down regulated | 22/431 | 5.58E-05 | CCL2/ICAM1/IL6/TNFAIP3/CXCL6/IL1B/CXCL5/CXCL2/CX3CL1/NFKBIA/BIRC3/CSF1/-/CXCL3/MAP2K6/CSF2/TRAF3/JUNB/IRF1/VEGFC/TRAF2/CFLAR |

| | | | | |
|---|---|---|---|---|
| ECM-receptor interaction | Down regulated | 19/431 | 5.58E-05 | ITGB3/ITGA11/ITGA2/ITGA10/COL6A1/LAMA3/THBS1/ITGA3/COL6A2/ITGA1/COL4A6/ITGB5/AC092153.1/ITGA4/CD44/COL6A3/ITGA5/ITGAV/FN1 |
| IL-17 signalling pathway | Down regulated | 17/431 | 0.00028348 | CXCL8/CCL2/IL6/TNFAIP3/CXCL6/IL1B/CXCL5/CXCL2/MMP1/NFKBIA/S100A9/CXCL3/CSF3/CSF2/TRAF3/IKBKE/TRAF2 |

Table 5-B**:** KEGG significant upregulated and downregulated pathways in short-programmed vs co 3 days cells.

#### 5.2.4.4 Identification of epithelial mesenchymal transition (EMT) associated genes

Epithelial mesenchymal transition is one of the hallmarks of cancer which indicate the invasiveness and poor prognosis of tumour. It has been known that cancer cells transition to mesenchymal state enable the migratory ability of cancer cells which lead to the acquisition of invasiveness and metastasis characteristics. Our previous results characterized the EMT state of A549 lung cancer cells under different conditions. We found that long term incubation of lung cancer cells resulted in chronic mesenchymal state transition post macrophages removal which is similar to A549 cells co cultured with macrophages without resting in co 3 days cells. However, short-programmed cells did not show phenotypic and mesenchymal markers expression as shown in earlier experiments. For further investigation of EMT in A549 cells after removing macrophages in short- and long- programmed and co cultured A549 cells, we performed NGS (RNA-seq) to identify the activated molecular players involved in EMT and metastasis. To categorize the up- regulation of gene expression level, the control A549 cells were used as a baseline for short term co cultured (co 3 days), long programmed and short programmed A549 cells. The up-regulated genes were grouped with 3-fold changes or more for a significant expression. Based on computational analysis, Kim and his team identified the gene expression signature for specific stages in multi cancer types (Kim et al., 2010). According to this and other

studies, we compared our data to the mesenchymal cells signature contains several EMT markers (Their, 2002; Jechlinger et al., 2003; Taube et al., 2010). We found that 7 EMT markers are significantly up regulated in co cultured and long programmed but not in short programmed A549 cells (Table 6). Some of these markers expressed higher in co 3 days than long programmed cells such as SPARC, MMP2 and GREM1 while COL6A2, TWIST1 and INBHA are highly expressed in long programmed than co cultured cells (Table 6). In line with previous functional assays results, high expression of these genes in long programmed cells indicate the continuation of EMT mechanism in A549 cells after long term incubation with macrophages even post macrophages removal. However, the down regulations of EMT markers in short-programmed cells indicate the discontinuation of EMT process when macrophages removed after short time incubation with A549 cells. This also suggests that the role of macrophages in initiating EMT process is occurred by long time interaction with macrophages, but short time crosstalk between macrophages and cancer cells not resulted in persistence of EMT process.

| **Gene name** | **Function** | **Co 3 days FC** | **Long programmed FC** | **Short-programmed FC** |
|---|---|---|---|---|
| **SPARC** (**Secreted Protein Acidic and Cysteine Rich)** | -Encodes extracellular matrix glycoprotein<br>-Activates EMT-inducing transcription factor (SNAIL) through activation of MEK signalling pathway in NSCLC A549 cells which induce cell migration and invasion (Grant et al., 2014) | **112.77** | **15.408** | **0.653** |
| **COL6A2 (Collagen Type VI Alpha 2 Chain)** | -Encodes extracellular matrix protein<br>-Induce EMT in malignant cells through activation of the Akt–GSK-3b–b-catenin–TCF/LEF axis (Chen et al., 2013). | **5.97** | **14.06** | **0.565** |

| | | | | |
|---|---|---|---|---|
| **FBN1(Fibrillin 1)** | - Encodes extracellular matrix protein<br>- Promotes ovarian cancer metastasis through p53-and SLUG associated signalling (Wang et al., 2015) | **3.075** | **3.923** | **0.713** |
| **MMP2 (Matrix Metalloproteinase 2)** | -Disrupt the basal lamina and initiate renal tubular EMT through Transforming growth factor- β1 (Cheng and Lovett, 2003). | **5.166** | **2.680** | **0.606** |
| **GREM1(Gremlin1)** | -Encodes bone morphogenetic protein (BMP) antagonist<br>-Promotes lung metastasis of breast cancer through MMP13 oncogene expression and STAT3 activation (Sung et al., 2020) | **130.029** | **20.506** | **1.550** |
| **TWIST1** | -Transcription factor belongs to the evolutionarily conserved basic-helix-loop-helix (bHLH) family.<br>-Mediate EMT in cancer through E-cadherin repression and activation of mesenchymal markers (Yang et al., 2004) | **1.744** | **5.635** | **0.539** |
| **INBHA (Inhibin Subunit Beta A)** | - Acts as oncogene in cancer<br>-Induce EMT in breast cancer through activation of TGF-β signalling pathway (Yu et al., 2021) | **3.712** | **6.271** | **0.501** |

**Table 6:** EMT markers fold change expression in co 3 days, long programmed and short programmed A549 clones compared to control.

#### 5.2.4.5 Identification of genes-related cell proliferation

Cancer cell proliferation is one of the hallmarks of tumor that causes cancer cells to evade the programmed apoptosis and controlled growth to divide uncontrollably through the activation of proliferative signaling.

In our previous data, we investigated the proliferation of A549 lung cancer cells after long- or short-term incubation with macrophages by proliferation assay, and the results showed increased the proliferation rate in both long and short programmed cells compared to control. However, relative to co culture cells (co 3 days), the rate of proliferation decreased after short- or long- term co culture with macrophages when macrophages were removed and resting A549 cells in single culture for a month.

In this experiment, we investigated the signature of genes related to cell proliferation in co cultured (co 3 days), long programmed and short programmed A549 cells using RNA sequencing data. To identify the up- regulated genes, we used parental (control) A549 cells as a baseline for other cells in order to analyze the proliferation associated genes expression changes between these samples. The significance in gene expression was adjusted with 3-fold change in our analysis. Based on these adjustments, as shown in table7, proliferation genes found to be upregulated in all A549 cells with significant higher expression in co 3 days (co cultured) compared to long and short programmed cells, which have similar gene expression fold change of 13 proliferation related genes. These genes have identified in lung adenocarcinoma as key regulators of cell proliferation, and some of them are transcription factors that regulate the cell proliferation differential expressed genes such as E2F1 which known as common markers for cancer proliferation (Wang et al., 2021). Another example of the up-regulated genes is MCM4 which identified in NSCLC as a marker for proliferation and resistance to therapy (Kikuchi et al., 2011). Furthermore, in mouse model, high expression of CDK1 associated with lung cancer progression and considered a prognostic biomarker for lung tumor (Li et al., 2020). Based on these findings, we conclude that long- and short- term co-culture of macrophages with A549 lung cancer cells increased cell growth via up regulated genes related cells proliferation, and these effects persisted after removing macrophages while increased in presence of macrophages as shown in co-culture genes signature profile of expression of these genes.

| Gene name | Co 3 days FC | Long programmed FC | Short-programmed FC |
|---|---|---|---|
| AURKB | 60.33 | 15.50 | 15.42 |
| KIF2C | 23.50 | 9.05 | 5.60 |
| CDKN3 | 4.45 | 2.26 | 3.113 |
| CENPF | 6.81 | 1.44 | 2.95 |
| PLK1` | 25.10 | 6.11 | 7.11 |
| MYBL2 | 36.66 | 5.86 | 9.07 |
| FOXM1 | 10.24 | 2.31 | 2.9 |
| NDC80 | 35.5 | 9.94 | 12.088 |
| CDK1 | 16.9 | 5.34 | 8.22 |
| CDC20 | 6.64 | 4.97 | 1.60 |
| CDCA5 | 15.06 | 5.78 | 3.41 |
| E2F1 | 26.63 | 6.21 | 9.20 |
| MCM4 | 4.83 | 1.39 | 1.6 |

**Table7:** The fold change gene expression of up regulated genes related cell proliferation in co 3 days, long programmed and short programmed A549 clones compared to control

## 5.3 Discussion

Cells co-culture techniques have been used widely in cancer research in order to investigate the biological mechanisms that underlie the interaction between cancer and microenvironment cells (Miki et al., 2007). The main aim of most of these studies is to assess cancer cells hallmarks upon co culturing process with stromal cells without consideration of incubation time as a parameter for their studies. However, cancer cells arise from weeks or months interaction with stromal cells *in vivo* which make the *in vitro* short time incubation studies different from *in vivo* cancer biology. In light of this, in this part of study, we investigate the effects of macrophages on lung cancer cells after 30 days of incubation with macrophages considering macrophages replenishment after 3 days to keep lung cancer cells exposure to healthy differentiated macrophages secreted factors continuous for 30 days.

In addition, in this part of study we aim first to assess the role of our differentiated macrophages on lung cancer cells and assess this role in presence of macrophages and how long these effects persist in the absence of macrophages. To bear in mind that we labeled our cells as programmed based on the theory that the long communication between tumor cells and their surroundings, which is macrophages in our model, results in continuous cellular changes in cancer cells known as “cellular reprogramming”.

Our results coincide with many published studies as it has been reported that macrophages affect cancer cells characteristics and hallmarks differently (L. Gong et al., 2019). Furthermore, some studies reported that the pro tumorigenic effects of immune cells after long time interaction with cancer cells resulted in continuous cellular changes of cancer cells that make tumors more aggressive and therapy resistance (Blussé Van Oud Alblas and Van Furth, 1979; Dominiak et al., 2020). In this project, we have found that long time communication of lung cancer cells with macrophages resulted in permanent phenotypic and genes expression changes persist after removing macrophages.

We investigated the proliferation and epithelial mesenchymal transition state in our programmed cells as we did in heterozygous co cultured cells of previous section based on studies indicate that macrophages affect cancer cells hallmark differently. Our results support this hypothesis and suggest that some of cancer characteristics that changed in presence of macrophages may persist or reverse

after removing macrophages. We found that the mesenchymal state of cancer cells after short time incubation with macrophages disappeared and returned to epithelial cells state when lung cancer cells rested in single culture. This also supports the study indicates that EMT is reversible in cancer and plasticity in cancer phenotypic characteristics occurred (Tarin, 2005; Christiansen and Rajasekaran, 2006). Interestingly, and our results suggest that differentiated macrophages- induced EMT in lung cancer after short time crosstalk is reversible and requires the presence of macrophages in short incubation or long-time exposure to macrophages secreted factors that lead to irreversible chronic EMT in lung cancer cells. Which shown in our long-programmed cells that displayed the mesenchymal state after long-time incubation with macrophages and rested cells in single culture. In addition, the high migration rate of long programmed cells supports the study indicates that long time exposure of lung cancer cells to macrophages results in more invasive cancer cells as the migration of cancer cells is a marker of cancer cells invasiveness

On the other hand, interestingly, the functional assessment of proliferation indicates that macrophages-induced proliferation persists after either short or long-time incubation with macrophages. These findings suggest that further investigation of underlying mechanisms is needed because cancer proliferation is the hallmark that responsible for developing resistance to cancer therapy and tumor recurrence (Gao et al., 2015). According to both proliferation assay and the expression of proliferation related genes in long and short programmed cells, we may suggest that the pro tumorigenic roles of macrophages on increasing A549 cells proliferation is irreversible and persist after removing macrophages.

RNA sequencing data allow us to analyze the differential gene expression profile that could help in identification the distinct gene signatures paired with our A549 cells morphological and functional changes. Regarding cell proliferation, it has been known that regulated by cell cycle associated genes, cell division associated genes and cell proliferation associated genes. In our samples model, we found that some of these genes including CDK1, CDKN3, AURKB, KIF2C, PLK1 and CENPF are associated with all three processes. This suggests the biological significance of these genes in the roles of macrophages that responsible for abnormal cell proliferation in our co-culture and programmed cells. Further investigations of the functions of these genes may help in

understanding the role of macrophages in activation the signaling pathways that lead to abnormal cell proliferation. Furthermore, understanding the mechanism of these genes could be a promising immunotherapeutic target for lung cancer (Zhu et al., 2021). These proliferation genes were identified in lung adenocarcinoma (LUAD), and we found them in NSCLC, which reflect the importance of identifying the molecular signatures of lung cancer by analyzing RNA expression of proliferation common markers for further investigation of their functions. As it has been reported that epigenetic silencing of E2F1 by long coding RNA suppressed tumor growth in lung adenocarcinoma patients (X. Guo et al., 2019).

The high expression of EMT transcription factors in long programmed and co cultured cells is correlated with loss of E-cadherin and increase N-cadherin which usually used to support the significance of EMT in cancer research. These EMT transcription factors in addition to their function of direct suppression of E-cadherin and promoting N-cadherin, they also suppress several additional junctional proteins, such as claudins which enhance cells invasiveness and metastasis of cancer cells.

As the main goal of this study is to evaluate the pro-tumorigenic effects of macrophages on lung cancer cells cultured under different conditions, we focused on the analysis of carcinogenesis related pathways of KEGG enrichment. However, we found other pathways up regulated in our models of cancer cells that related to infection such as COVID-19 and malaria. It has been reported that there is molecular association between COVID-19 disease and lung cancer reflected by the enrichment of COVID-19 pathway related genes (Soon Nan et al., 2021). It indicates that the lung susceptibility to COVID-19 is the common pathogenic basis shared by lung cancer and COVID-19, and the data investigating the possibility of developing lung cancer after COVID-19 infection is being considered. (Chatziantoniou and Zaravinos, 2022).

The enrichment of ECM in up regulated genes of co cultured and long programmed vs control and in downregulated genes of short-programmed vs co cultured cells support the opinion that the interaction between ECM and tumor cells lead tumor cells to infiltrate the tumor microenvironment cells. (Chatziantoniou and Zaravinos, 2022). In our model, it may suggest that in short term incubation with macrophages and after long term incubation of

macrophages, A549 genes that infiltrate macrophages were activated, but not after short time incubation with macrophages.

# Chapter 6 Discussion

Multiple studies have recently used the human monocytic Thp-1 cell line as an alternative model for peripheral blood macrophages. However, the optimal PMA concentration used for Thp-1 treatments has not been properly discussed in these studies. Therefore, in this study, we targeted a range of PMA concentrations ranging between 25 and 100 ng/ml by assessing the gene expressions of surface markers and cell adhesion in differentiated macrophages. The lowest optimal concentration was chosen to treat Thp-1 cells according to differentiation criteria, considering the next aim of co-culturing macrophage-like cells with lung cancer cells. As the high concentrations of PMA have proven to mask the effects of differentiated thp-1 and cause undesired gene expression (Park et al., 2007), the low concentration of 25 was too low as it showed weak expression of CD11b, CD68, and TLR4. We found that there was no difference between 100 and 50 ng/ml in the expression level of surface marker genes; therefore, 50 ng/ml was chosen as the minimal effective concentration. A study done by Pinto and his colleagues supported the finding that 50 ng/ml is the lowest optimal concentration based on quantitative proteomics measurements of PMA based differentiated macrophages (Pinto et al., 2020)., which reduce the alterations in gene expression in response to second stimuli. Following these findings, our research suggests that selecting the optimal PMA concentration for Thp-1 differentiation is important for observing successful Thp-1 differentiation into macrophages.

Following the determination of optimal PMA concentration, we have investigated the resting period of differentiated macrophages to grow in the absence of PMA to allow cellular recovery before inserting lung cancer cells in co-culture system to avoid the negative effects of PMA on lung cancer cells. Therefore, the resting period was determined by assessing cell adhesion and gene expression levels of macrophage surface markers. We found that differentiated macrophages in single culture start to lose their adherence criteria at day 4 as well as gene expression of surface markers compared to those rested for 2 days. In support of our findings, it has been reported that differentiated macrophages that rested for long period of time in single culture lost their phenotype (Spano et al., 2013).

However, the presence of cancer cells was found to enhance macrophage differentiation in co-culture system by increasing adherent cell number and inducing gene expression of macrophage markers.

As previously reported, soluble factors such as cytokines and growth factors secreted in the CM mediate the interaction between cancer and the surrounding TME cells. One of the aims of this study is to compare the effects of co-culture CM on lung cancer gene expression with those of macrophage CM. The findings show that some genes, including SERPINB3 and P2RX4, are induced by co culture CM but not macrophages CM, whereas others, such as PTGS2, NPC1, FSTL1, and PLAUR, are induced by both macrophages CM and co culture CM. It has been reported that upregulated SERPINB3 and P2RX4 are responsible for EMT in many cancer types (Quarta et al., 2010; He et al., 2020), which supports our results that co-culturing lung cancer cells with macrophages resulted in cytokines release that induced EMT genes in lung cancer cells.

Most of the published papers employed short-term incubation of macrophages with cancer cells ranging between 3 and 5 days to study the impact of macrophages on cancer progression (Z. Guo et al., 2019; Wei et al., 2019; Castellaro., 2019.). However, these studies demonstrate that when cancer cells co-cultured with macrophages, they migrate faster than when they are cultured alone in a single culture under short-term conditions (Z. Guo et al., 2019; Zhang et al., 2019). We hypothesised that long-term incubation of lung cancer cells with macrophages (30 days) would test whether macrophages caused chronic alterations in cancer cells that remained for 30 days after macrophages were removed. We found that long-programmed A549 clone migrated faster than short-programmed cell clone, implying that our differentiated macrophages caused persistent changes in lung cancer cells that impact the signalling system underpinning cancer cell metastasis and proliferation. This also indicates that our differentiated macrophage model polarised to M2 pro-tumorigenic phenotype based on the influence of macrophages on lung cancer cell proliferation and EMT state of lung cancer cells. However, it has been reported that macrophages may act as anti-tumor cells throughout the early stages of tumour development due to the activity of other immune system cells (Gordon., 2011). This highlights the

necessity of addressing the complexity of the tumor microenvironment *in vivo* that influences macrophage behaviour via cytokines and signals produced by other cell types present in the TME.

Our RNA-seq data confirmed the proliferation and EMT changes that occurred in co cultured A549 and in programmed A549 cells. As it has been shown, gene expression patterns in long programmed cells are similar to those in co-cultured cells, while short-programmed cells return to the pattern similar to that of parental A549 cells. This may reflect the fact that macrophages can influence gene expression in cancer cells, and their long interactions with cancer cells can result in chronic activation of specific genes via cytokines and growth factor secretions. In addition, studies have demonstrated that these factors secreted by TAM can induce epigenetic changes that influence gene expression patterns in cancer cells (Lamouille et al., 2014; Yuan et al., 2010). Furthermore, this interaction has the potential to influence cancer hallmarks, including proliferation and metastasis, which are important for cancer progression.

In this thesis, *in vitro* experiments were used to investigate the effects of macrophages on lung cancer cells at different time points. However, it should be noted that there are limitations in applying these results to lung cancer patients tumours for many reasons, including the complexity of TME, which is difficult to mimic through an *in vitro* model. In the first instance, the immune system responds through the secretion of several cytokines that could be driving the pro-tumorigenic phenotype. In addition, the findings of this thesis are based on independent cross-talk between macrophage-like and lung cancer cells, which is different from patient tumour samples where cell-cell interaction occurred and affected cell phenotypes and biological behaviour. This suggests that extending *in vitro* experiments to include direct co-culture of macrophages and lung cancer cells is necessary to confirm the findings with the role of juxtracrine signalling.

## Summary of findings and future directions

Human monocytic Thp-1 cells have been used as an alternative model for peripheral blood macrophages in cancer research that investigate the biological behavior of cancer cells. However, PMA optimal concentration for treating Thp-1 cells has not been the focused of these studies as well as the period of treatment time. In this study we tested the 25,50, and 100ng/ml PMA concentrations for Thp-1 treatment over 48hrs to determine the optimal PMA concentration based on the differentiated cells adherence properties and gene expression level of differentiated macrophages cell surface markers. 50 ng/ml was chosen as lowest optimal PMA concentration for the reason that high concentration could mask gene expression changes or toxicity of lung cancer cells as the main reason of generating differentiated macrophages is to investigate the effects of macrophages on lung cancer cells upon co culturing. As it has been reported that treated monocytes with high concentration results in upregulation of some genes in differentiated macrophages in the way that masks the effects of other stimuli in induction of gene expression (Park et al., 2007). The assessment of macrophages cells adherence and gene expression upon co culturing with A549 lung cancer cells showed that lung cancer cells promoted macrophages adherence and survival in comparison to macrophages in single culture.

On the other hand, for the effects of macrophages on A549 cells, we first found that genes chosen based on previous study are activated in our heterogenous A549 lung cancer model upon short time (3 days) incubation with macrophages. These genes are (SERPINB3, P2RX4, PTGS2, NPC1, FSTL1 and PLAUR). This was the initial results that showed us our differentiated macrophages and transwell system worked successfully to achieve the cells independent crosstalk. In addition, the co-culture conditioned media effects on activation of those genes in A549 cells confirmed that the soluble secreted factors (cytokines) are mediated the crosstalk between two cells in our co-culture transwell model. However, the upregulation of some of these genes by macrophages conditioned media suggests that some cytokines secreted by macrophages only while others secreted upon co culturing with cancer cells. Further investigations of lung cancer cells hallmarks showed us the pro tumorigenic roles of macrophages on the

significant induction of proliferation and epithelial mesenchymal transition upon short time incubation with macrophages.

The investigation of long- and short-programmed A549 cells was initially done by RT-PCR gene expression analysis of the 6 genes which were upregulated in long programmed A549 cells as in 3 days co cultured A549 cells. However, the expression of those genes was decreased in short-programmed cells resting in single culture after short incubation with macrophages. Interestingly, the long-programmed cells showed the mesenchymal transition state continuation after removing macrophages while short-programmed cells returned to epithelial state after short exposure to macrophages. Surprisingly, we found that A549 cells proliferation rate induced in short-programmed cells as well as in long programmed cells at the similar rate. However, co cultured cells proliferation rate was higher in presence of macrophages than programmed cell.

Furthermore, RNA sequencing data revealed the similarities between "long-programmed" and "CO 3 days" cells in their differential gene expression pattern and the activation of cancer progression related pathways. However, no significant differences were found between short-programmed and parental A549 cells except for proliferation-related genes expression, which was remined-induced in short programmed A549 cells which reflected in their proliferation functional assays.

Future directions:

- Use primary macrophages in co-culture transwell model with lung cancer cells to test the effects of macrophages in short and long crosstalk incubation with A549 cells.

- A recommended step in establishing generalized findings of the impacts of macrophages on lung cancer cells hallmarks is to conduct the same experiment on additional lung cancer cell lines, such as the NCI-H1975 cell line, and culturing them under the similar conditions used in this thesis.

- Based on the findings that some genes in A549 cells were activated by macrophages conditioned media while other genes were stimulated by co-culture conditioned media. It is recommended to investigate the major cytokines that drive lung cancer cells invasion and metastasis in A549 cells occurred upon crosstalk with macrophages. This could be done by cytokines array screening or ELISA for specific cytokines in macrophages and co-culture conditioned media to investigate the new cytokines that were secreted upon co culturing process of macrophages with lung cancer cells. We might be able to learn more about the signalling pathways through which these cytokines contribute to the invasion and metastasis of cancer cells by reducing or blocking these cytokines in our long-programmed cells. Another method of analysing the cytokines is to examine the expression of cytokine receptors and their ligands in cancer cells. As it has been reported by Zhou and colleagues, CXCR2 chemokine receptors were found overexpressed in gastric cancer patients and correlated with metastasis and poor prognosis. They found that CXCL1 and CXCL5 cytokines, which are produced by macrophages, can activate the CXCR2/STAT3 pathway in gastric cancer cells by analysing the phosphorylation of STAT3 (Zhou et al., 2019).

- Lung cancer highly metastasizes to the bone, brain, and liver, resulting in poor prognosis (Reck et al., 2013). EMT is one of the cancer phenotypic changes that causes cancer metastasis and invasion (Campbell, 2018). The finding of a permanent EMT transition in long programmed A549 cells as well as in co 3 days A549 cells that had been grown for three days in the presence of macrophages could give insight to the underlying mechanisms that mediate EMT in lung cancer cells. Selecting the target marker for the process of examining the underlying mechanisms may benefit from gene ontology analysis to identify the most significant upregulated genes or proteins involved in EMT process. In addition to EMT related genes found upregulated in our model samples, some studies reported proteins involved in multiple processes such as cytoskeleton organization involved in metastasis in lung cancer (Cheung et al., 2016; Safadi et al., 2016). Knockdown or silencing of these markers in lung cancer cells and assessment of the associated EMT markers, phenotypic

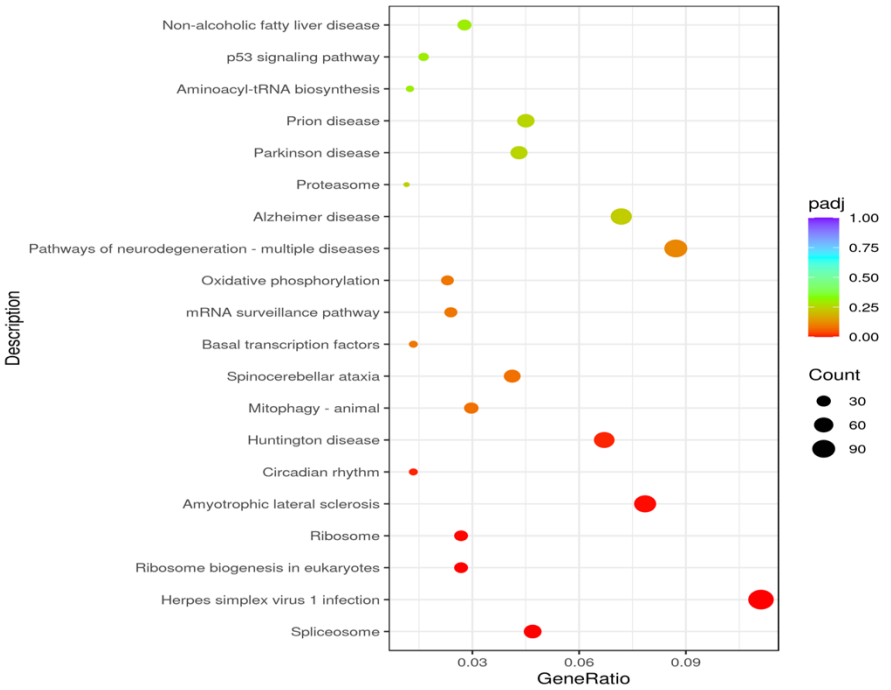
changes, and activation of the downstream common cancer cells signalling may help in identifying the mechanism underlying EMT and metastasis in cancer. This could be applied to long programmed A549 cells to examine whether those cells will be associated with clinicopathological metastasis in *in vivo* model.

- Epigenetic modifications are an important phenomenon that can increase the expression of genes involved in cancer cell EMT, migration, and invasion, which are required for the formation of metastatic tumours. The investigation of these epigenetic changes in our long programmed and co 3 days A549 cells may help to identify the underlying mechanisms that caused EMT and invasion properties of lung cancer cells. Epidermal growth factor (EGF) and transforming growth factor-beta (TGF-) are two soluble growth factors known to be secreted by tumour-associated macrophages and necessary for the activation of EMT in cancer cells (Williams et al., 2019). As a result, TGF-SMAD3, WNT-catenin, and Notch signalling are activated and induced EMT-related transcription factors such as Snail, Slug, and Twist (Lamouille et al., 2014). The mRNA expression of these EMT-TFs is controlled by DNA methylation and histone modifications. For example, Snail down regulated E-cadherin expression by recruiting histone lysine-specific demethylase 1 (LSD1) at the CDH1 promoter (Lin et al., 2010). In pancreatic cancer cells, a genome-wide elevation in H3K36me2 was shown to regulate the expression of nearly all master EMT-TFs, providing a more unifying mechanism for EMT (Yuan et al., 2020). All of these published studies given an insight of investigations the epigenetic modifications that affected targeted EMT-markers expression in our generated A549 cells models.

# Appendixes

## Supplemental figures and tables

A




B

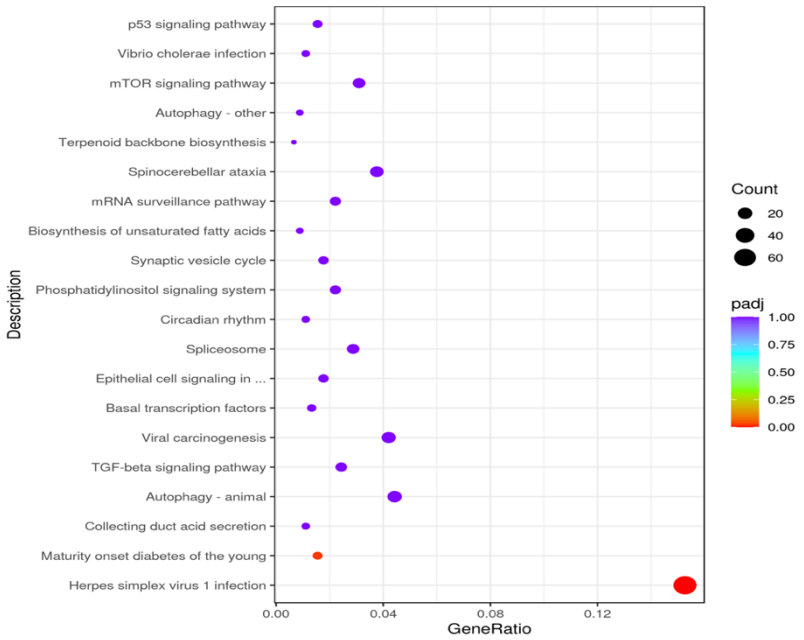
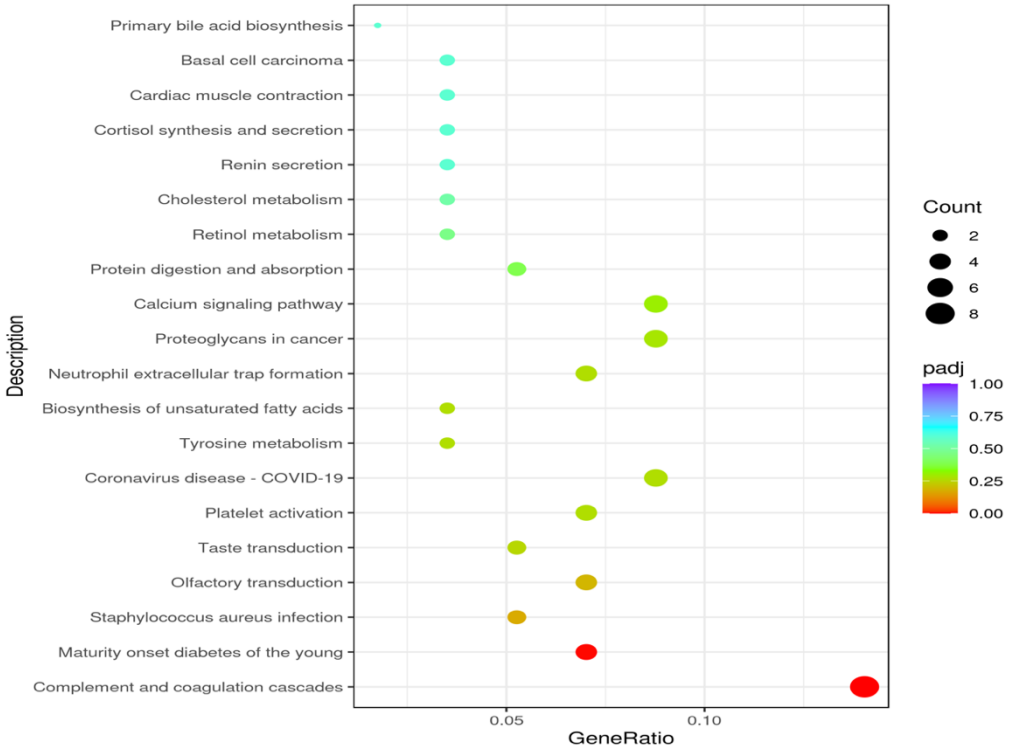



Figure 1 Top 20 functional enrichment analysis of downregulated KEGG pathways. A- KEGG pathways of co 3 days vs control. B-KEGG pathways of long programmed vs control.

A




B

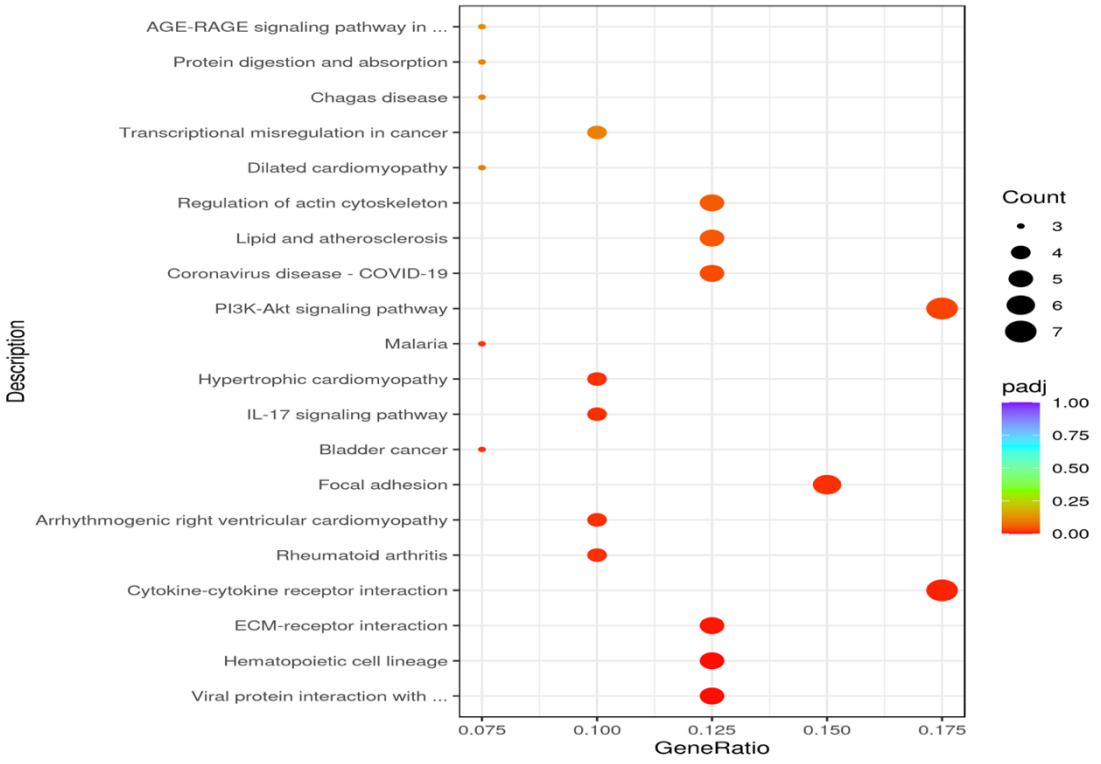



**Figure 2** Top 20 functional enrichment analysis of upregulated and downregulated KEGG pathways. A-upregulated KEGG pathways of short-programmed vs long programmed. B- downregulated KEGG pathways of short - programmed vs Long programmed

| KEGG description | Dysregulation | Gene Ratio | Padj | Genes |
|---|---|---|---|---|
| Complement and coagulation cascades | Up regulated | 65/6023 | 1.69E-05 | F7/F5/F2/FGG/FGA/FGB/CFH/C5 |
| Maturity onset diabetes of the young | Up regulated | 18/6023 | 0.00130926 | HNF1A/HNF4A/FOXA3/IAPP |
| ECM-receptor interaction | Down regulated | 74/6023 | 0.00490501 | ITGB3/COL6A1/COL6A2/ITGA11/ITGA2 |
| Cytokine-cytokine receptor interaction | Down regulated | 198/6023 | 0.00885118 | IL6/IL34/IL24/CXCL8/CCL2/CRLF2/EBI3 |
| IL-17 signalling pathway | Down regulated | 71/6023 | 0.01505775 | IL6/MMP1/CXCL8/CCL2 |
| PI3K-Akt signalling pathway | Down regulated | 293/6023 | 0.02986665 | IL6/FGF5/ITGB3/COL6A1/COL6A2/ITGA11/ITGA2 |

Table 1: KEGG significant upregulated and downregulated pathways in short-programmed vs long programmed cells

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