Chapter V: A study on the effect of high dietary salt on the crosstalk between cancer cells and macrophages / TAMs in vitro

5.1 Introduction

Monocytes are a type of white blood cell that circulates in the bloodstream after being released from the bone marrow. Once they migrate into various tissues throughout the body, they differentiate into macrophages and dendritic cells. In the context of cancer, monocytes constantly infiltrate the tumor microenvironment (TME) and replace the existing tumor-resident macrophages [1]. Infiltrating macrophages in TME, undergoes a transformation into tumor-associated macrophages (TAMs), playing crucial roles in regulating tumor growth, immune responses, and chemoresistance [2]. However, macrophage displays a diverse functional phenotype based on environmental cues. Normally TME is highly immunosuppressive, under the influence of which macrophages, T-cells, NK, and dendritic cells cannot carry out their normal function of tumor suppression [3]. Immunosuppressive TME, therefore, poses a formidable challenge in overcoming cancer progression. However, it is evident from ^{23}Na MRI that sodium content in malignant tissue is significantly higher compared to surrounding tissue [4,5]. Also, recent studies have pointed out that salt (NaCl) induces the release of pro-inflammatory cytokines in macrophages and activates the anti-tumor function of T-cells [6,7]. This leads to the question: "What is the effect of high salt on restoring the pro-inflammatory state from the immunosuppressive state in tumor microenvironment?". Also, the effect of such high salt on inducing anti-tumor effects in TME has not yet been studied. Therefore, in this study, we have explored the role of high salt in inducing inflammation in THP-1 macrophage cells (representing naïve infiltrating macrophage cells) and THP-1 derived TAM cells (representing tumor resident macrophage cells) by *in vitro* experiments. We also studied the effect of high salt on the crosstalk between macrophages/TAMs with breast cancer cell lines using various studies like proliferation, migration, invasion and adhesion. To delve deeper, we further explored the global transcriptomics changes of the MDA MB-231 cell line cocultured with high salt-treated TAM cells. DEG's from RNA sequencing analysis showed enrichment in pathways involved in ribosome biogenesis, oxidative phosphorylation, and ROS generation. Go ontology term showed enrichment of DEG's in important pathways involved in cellular respiration, ATP synthesis and mitochondrial electron transport chain.

5.2 Results

5.2.1 Standardizing conversion of THP-1 monocytes to macrophage-like cells

THP-1 monocytes into macrophage-like cells conversion were carried out using the standardized protocol (**Figure 5.1A**). THP-1 monocytes were treated with PMA (5 ng/ml) for 48 hours. Following this treatment, the PMA-containing RPMI1640 medium was discarded, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS), and then incubated for an additional 24 hours in complete RPMI1640 medium. Phase-contrast bright-field imaging of the PMA-treated cells revealed a significant number of adherent cells with star-shaped appendices, characteristic of macrophage-like cells **(Figure 5.1B)**. Monocyte-to-macrophage conversion was confirmed using qPCR analysis of macrophage-specific markers, such as *CD11b* and *CD14*. Our data suggested that PMA-treated cells showed a significant increase in *CD11b* and *CD14* mRNA levels compared to untreated THP-1 monocytes **(Figure 5.1C)**. This observation suggested that PMA treatment successfully converted THP-1 monocytes into macrophage-like cells.

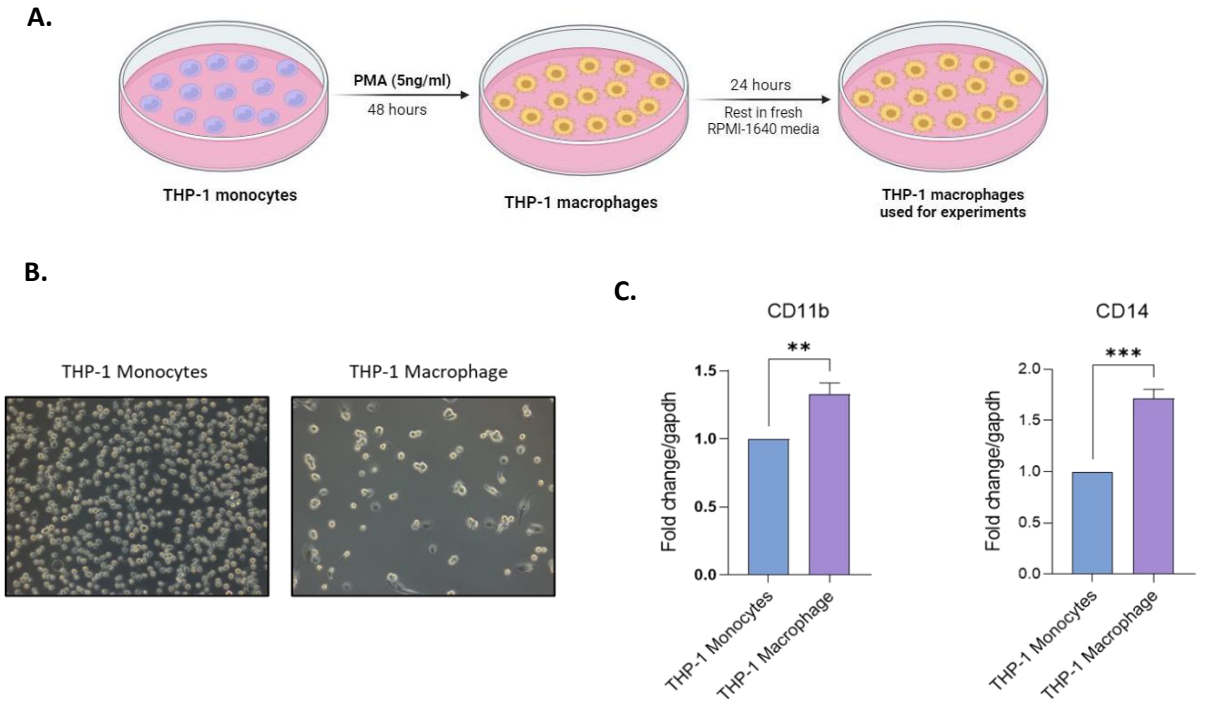


Figure 5.1: Conversion of THP-1 monocytes to macrophage-like cells. A. Schematic representation of the conversion of THP-1 monocytes into macrophage-like cells **B.** Bright-field phase-contrast microscopic

images of THP-1 monocytes (left) and macrophages (right), scale bar=50 µm **C.** Relative mRNA expression of *CD11b* and *CD14* genes in THP-1 monocytes and PMA-treated THP-1 cells (macrophages) using qPCR (n=3). Unpaired Student's t-test was performed between control vs. treatment, representing statistical significance (**p<0.005 and ***p<0.0001).

5.2.2 Standardization of THP-1 to TAM conversion and study of the impact of high salt on TAM polarization

Tumor-associated macrophages (TAMs) were generated by using conditioned media of cancer cells (MDA MB-231) [8-10]. Briefly, MDA-MB-231 cells were cultured to 80% confluency, at which point DMEM was replaced with RPMI. After 24 hours, the conditioned media (CM) was centrifuged to remove cellular debris and then added to PMA-differentiated macrophage cells, which were then cultured for 48 hours in a 1:1 ratio of CM to Fresh RPMI. TAM so generated in transwell inserts was used for co-culture studies with MDA MB-231 breast cancer cells. The process has been represented with a schematic diagram (Figure 5.2A). ^{23}Na MRI has shown that on average, the total sodium concentration (TSC) in malignant lesions was increased by more than 60% of the level in normal tissue *i.e.* TSC of malignant tissue is ~ 53 ± 16 mmol/l higher compared to normal tissue [4]. To understand the effect of high salt on TAM polarization, 50 mM NaCl was added during the addition of conditioned media from MDA MB-231 cells, and THP-1 cells were incubated for 48 hours. Bright-field microscopy revealed that high salt suppressed the polarization of THP-1 cells to TAM cells, as evidenced by morphological changes. Specifically, TAM cells treated with high salt exhibited a smooth oval shape similar to normal macrophage cells, in contrast to TAM cells treated only with conditioned media, which showed elongated protrusions and surface projections **Figure 5.2B.** Gene expression analysis of TAM markers such as *Arginase 1 (Arg1), Frizzled-like 1 (Fizz1), CD163*, and *CD206* showed high expression in cells treated with conditioned media from MDA MB-231, confirming TAM formation. However, cells treated with high salt exhibited decreased expression of these markers, indicating that high salt suppresses the polarization of macrophage cells into TAMs **Figure 5.2C**.

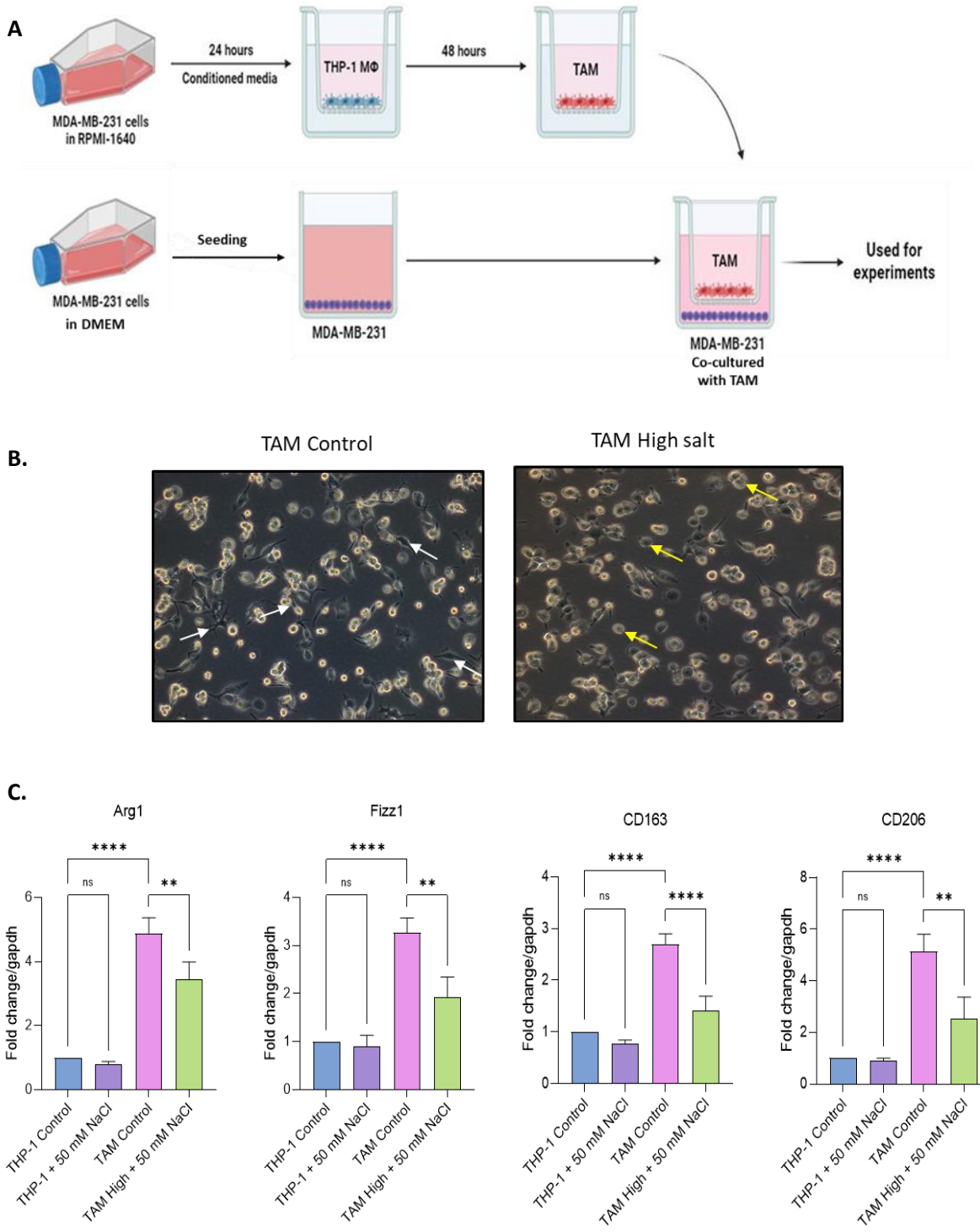


Figure 5.2: Conversion of THP-1 monocytes to macrophage-like cells. A. Schematics of conversion of THP-1 monocytes into macrophage-like cells **B.** Bright-field phase-contrast microscopic images of TAM (left) and TAM treated with 50 mM NaCl (right), scale bar=50 µm. White arrows (left) highlight the typical finger-like projections of TAMs, while yellow arrows (right) indicate a more rounded, macrophage-like morphology of TAMs under high salt conditions **C.** Gene expression of PMA-treated THP-1 cells (macrophages) and THP-1 differentiated TAM cells treated with and without 50mM NaCl (n=3). One-

way analysis of variance (ANOVA) was performed between the samples for statistical significance (**p<0.01 and ****p<0.0001).

5.2.3 High salt induces pro-inflammatory state in THP-1 macrophage cells

Macrophages are a heterogeneous population of immune cells that serve essential functions in development, homeostasis, tissue repair and immunity. Macrophage activation plays a pivotal role in the initiation and resolution of inflammation under a variety of pathological conditions including cancer. Macrophages can exist either in a pro-inflammatory state (M1) or an anti-inflammatory state (M2). However, under the influence of tumor microenvironment, macrophages are converted to the M2 state which further aids in tumor progression [11]. To understand the effect of high salt on macrophage cells. Cells were treated with 50mM NaCl in cancer cells for 24 hours followed by gene expression using qPCR. High salt-treated macrophages showed up-regulation of pro-inflammatory markers CXCL10, CCL11, MCP-1, TNFα, IL-6, IL-8 and IL-34 in PMA-treated THP-1 cells **(Figure 5.3)**. The expression of anti-inflammatory marker CCR7 was downregulated in these cells.

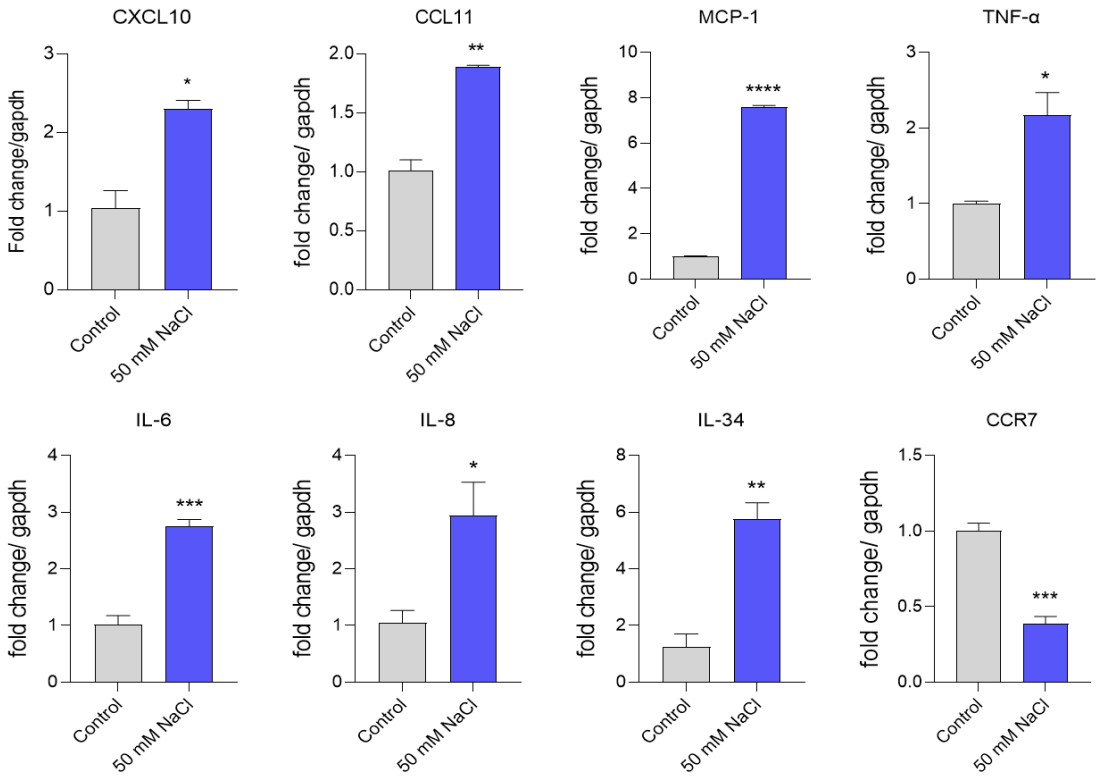


Figure 5.3: High salt induces pro-inflammatory gene expression in THP-1 macrophage cells. qPCR analysis of pro-inflammatory markers gene in control and high salt treated THP-1 macrophage cells

(n=3). Unpaired Student's t-test was performed between control vs. treatments or between two treatments representing statistical significance (*p<0.05, **p<0.005, ***p<0.0005).

5.2.4 High salt induces pro-inflammatory genes involved in anti-tumor function in macrophages and TAM cells

Although the function of macrophage cells is significantly influenced by their surrounding microenvironment, certain pro-inflammatory cytokines and interleukins play a crucial role in orchestrating the immune response by inducing the infiltration of immune cells at the sites of injury or in tumors. These molecules can have dual roles, acting as either pro-tumor or anti-tumor agents, depending on the context. However, cytokines and interleukins such as IL-23, TNF-α, IL-17, IL-1β, IL-12, and MCP-1 primarily function as pro-inflammatory agents in the tumor microenvironment. They facilitate the recruitment and activation of various immune cells, such as T cells and macrophages, enhancing the local inflammatory response. This can lead to the inhibition of tumor growth by promoting anti-tumor immune activities [12].

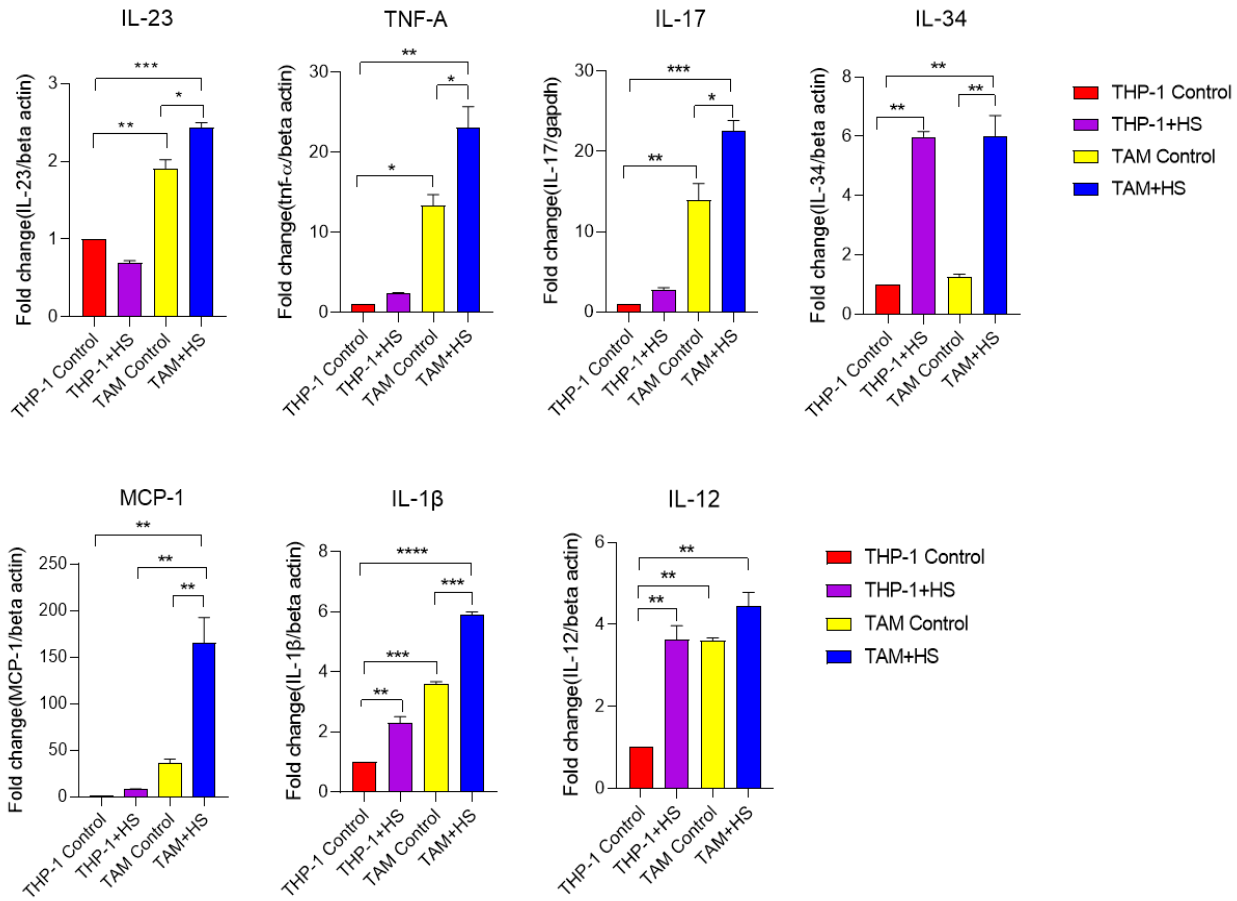


Figure 5.4: High salt induces pro-inflammatory genes involved in anti-tumor function in macrophages and TAM cells. qPCR analysis of pro-inflammatory markers gene in control and high salt treated THP-1 macrophage and TAM cells (n=3). One-way analysis of variance (ANOVA) was performed between control vs. treatments or between two treatments representing statistical significance (*p<0.05, **p<0.005, ***p<0.0005).

To investigate the effect of high salt on THP-1 macrophage and TAM, the cells were seeded in 6-well plates and treated with high salt for 24 hours. qPCR was then performed to analyse the expression of the aforementioned pro-inflammatory markers. High salt treatment induced significant changes in the expression of these genes in both THP-1 macrophage and TAM, with levels notably higher compared to untreated cells **(Figure 5.4).**

5.2.5 High salt-treated breast cancer cells induce chemotaxis of THP-1 monocytes

Chemotaxis is a directional movement of cells toward the site of a chemical stimulus, it is a crucial process in the immune response, particularly in the recruitment of monocytes towards the sites of infection, injury, or tumor. In order to study the effect of high salt-treated breast cancer cells on the infiltration of circulating monocytes towards the site of tumor, we treated MDA MB-231 and MCF-7 cell lines with high salt (50mM NaCl) for 24 hours. A transwell migration assay was conducted with 10% Fetal bovine serum (FBS) as the positive control using a transwell insert of 5.0µM pore size. Monocytes exhibited significant movement towards the positive control, validating the assay, and demonstrated increased migration towards both MDA MB-231 and MCF-7 cells. A schematic representation of the experimental set-up is shown in **Figure 5.5A**. Notably, high salt-treated cancer cells induced significantly higher monocyte migration compared to their untreated counterparts (**Figure 5.5B**). The number of monocytes that migrated through the pore was counted and shown in **Figure 5.5C**. qPCR analysis revealed high expression levels of the chemotactic gene, MCP-1 in both MCP-1 and MDA MB-231 cell lines, under high salt conditions. These results suggested that high salt-induced secretion of MCP-1 in cancer cells possibly promotes monocyte migration towards these cancer cells. Hence, high salt conditions in the TME may facilitate monocyte infiltration, potentially suppressing tumor progression through the enhanced recruitment of immune cells.

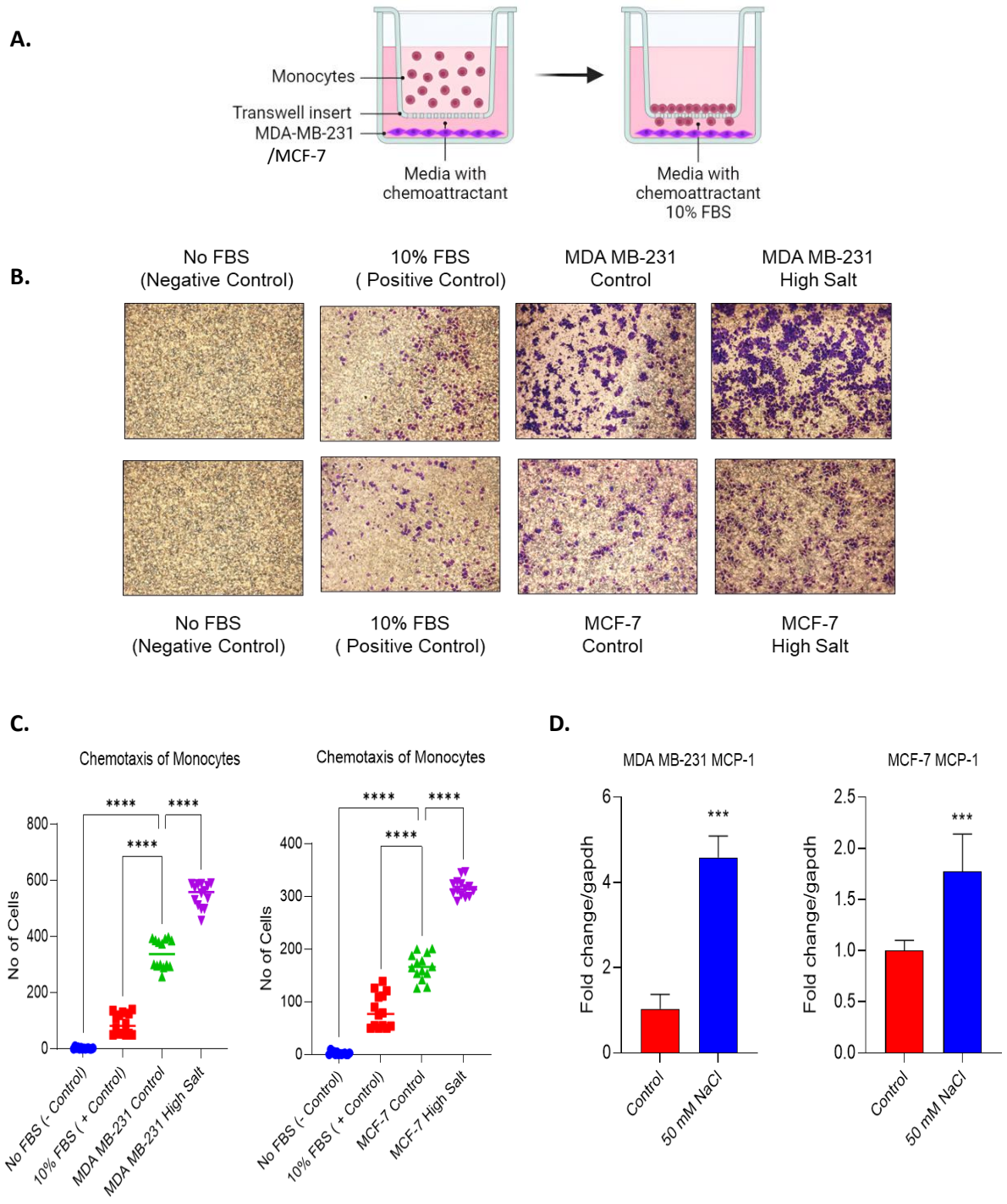


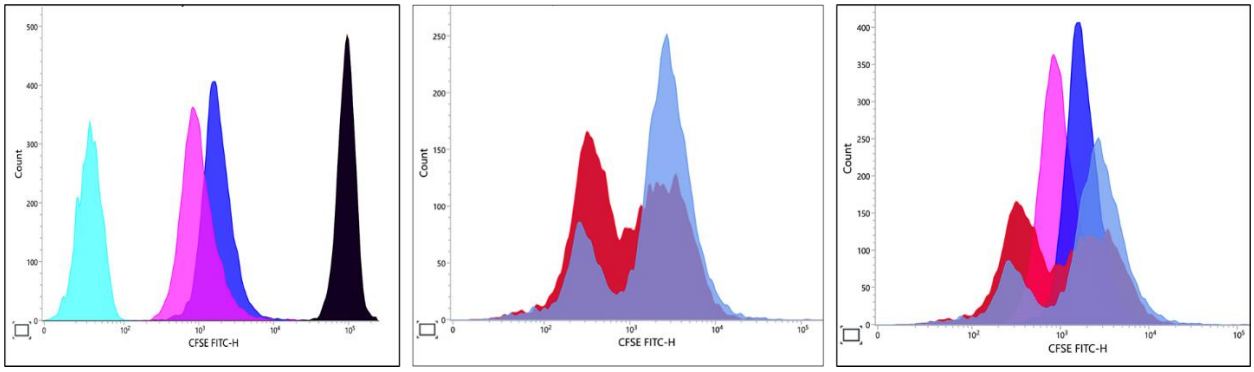
Figure 5.5: High salt-treated breast cancer cells induce chemotactic infiltration of THP-1 monocytes through MCP-1. A. Schematics of chemotaxis assay showing monocyte chemotaxis in presence of chemoattractant **B.** Chemotaxis assay of THP-1 monocytes using transwell insert 5.0µM pore size, using 10% FBS as positive control. High salt treated MDA MB-231 and MCF-7 cells showed increased infiltration of monocytes towards breast cancer cells (n=3 each) **C.** Quantitative representation of no of monocyte cells infiltrated through the transwell membrane. Cells were stained with 0.5% crystal violet

and counted manually **D.** qPCR analysis of MCP-1/CCL-2 gene in control and high salt treated MDA MB-231 and MCF-7 cells (n=3). One-way analysis of variance (ANOVA) was performed between control vs. treatments or between two treatments representing statistical significance (*p<0.05, **p<0.005, ***p<0.0005). Unpaired Student's t-test was performed for qPCR gene expression analysis between the control vs. treatment sample (***p<0.0005).

5.2.6 Macrophage and tumor-associated macrophage (TAM) suppresses MDA MB-231 cell proliferation under the high salt condition

In a previous experiment, we showed that high salt-treated breast cancer cells induced chemotaxis of the monocytes. Here, we investigated the effect of macrophages and TAMs on the proliferation of MDA MB-231 cells under high salt treatment. THP-1 cells were seeded in the upper chamber of a Boyden chamber and differentiated using PMA, as previously described. To convert these macrophages into TAM-like cells, cells were cultured with MDA MB-231 cell-conditioned media for 48 hours. After differentiation, the Boyden chamber was transferred to a 6-well plate pre-seeded with CFSE-stained MDA MB-231 cells. The cells were then treated with high salt and co-cultured for 7 days. The cells were trypsinized and analysed using flow cytometry. Compared to the control group, high salt-treated cells exhibited slower proliferation as shown by higher residual CFSE staining. Notably, MDA MB-231 cells co-cultured with high salt-treated macrophages showed the greatest inhibition of cell proliferation, compared to cells treated with high salt alone or TAMs **(Figure 5.6)**. This study indicated that high salt treatment induces macrophages possibly secreted pro-inflammatory markers leading to suppression of the proliferation of cancer cells.

A.



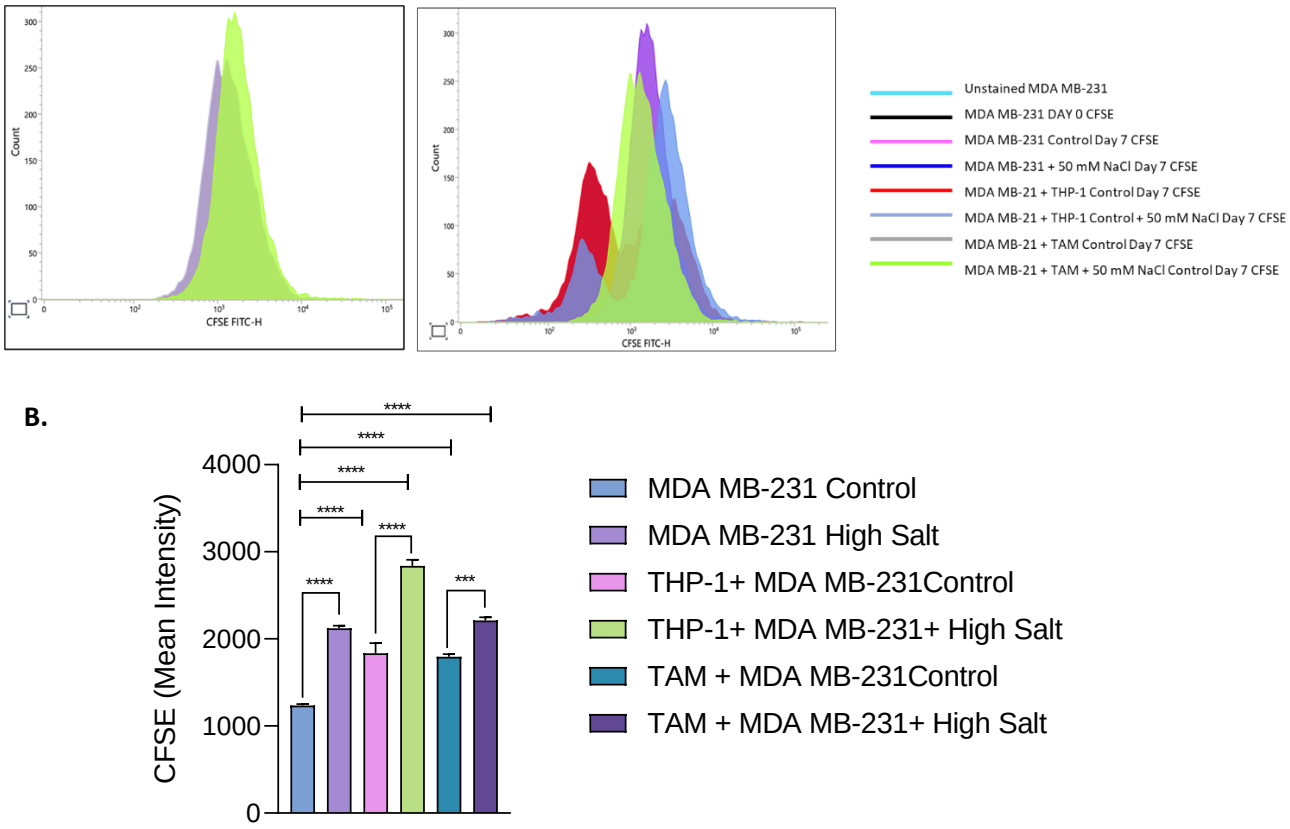


Figure 5.6: CFSE-based flow cytometry analysis showed high salt-treated macrophage and Tumor associated macrophage (TAM) cells suppress MDA MB-231 cell proliferation. A. Cell proliferation assay of MDA MB-231 breast cancer cell line under high salt stress condition using CFSE dye dilution method in flow cytometry **B.** Quantitative representation of CFSE intensity compared to DAY 0 control of MDA MB-231 (statistical analysis by one-way ANOVA test. ** p<0.05, *** p<0.005, ****p<0.0005).

5.2.7 High salt-treated macrophage and TAMs suppressed MDA MB-231 migration under the co-culture condition

To investigate the impact of high salt-treated macrophages and TAMs on the migration of MDA MB-231 cells under co-culture conditions, the wound-healing assay was conducted as previously described. In the co-culture setup, transwell inserts containing macrophages or TAMs were positioned as the upper compartment of a 6-well plate containing MDA MB-231 cells. Remarkably, cancer cells cocultured with macrophages and TAMs under high salt conditions exhibited significantly higher inhibition **(Figure 5.7)**. The inhibition was more prominent in the case of macrophage cells, however, TAMs under similar environment also inhibited the migration of a highly metastatic cell line, MDA MB-231. This suggests that macrophages/TAMs could potentially abrogate metastatic properties of breast cancer cells in the presence of high salt.

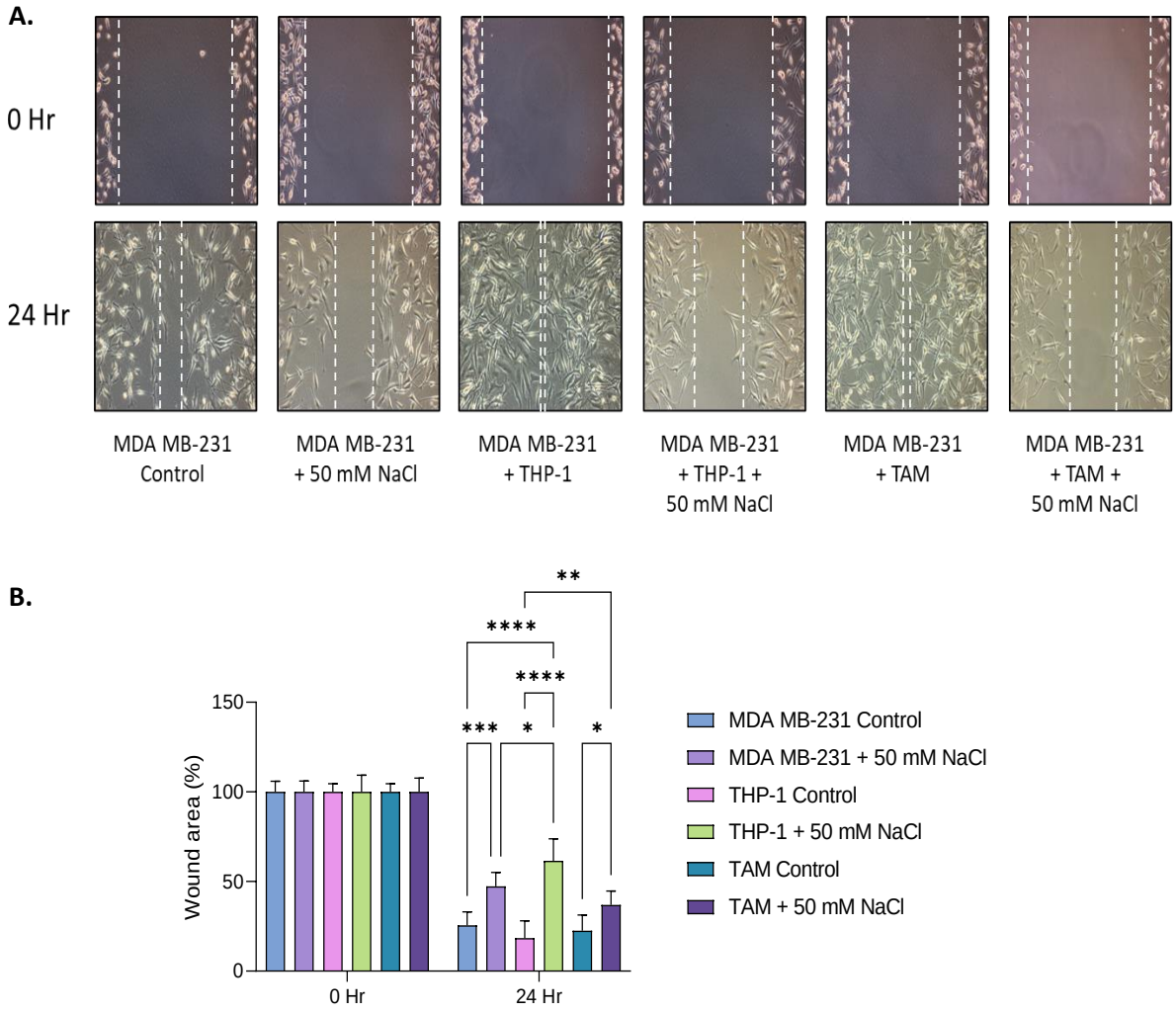


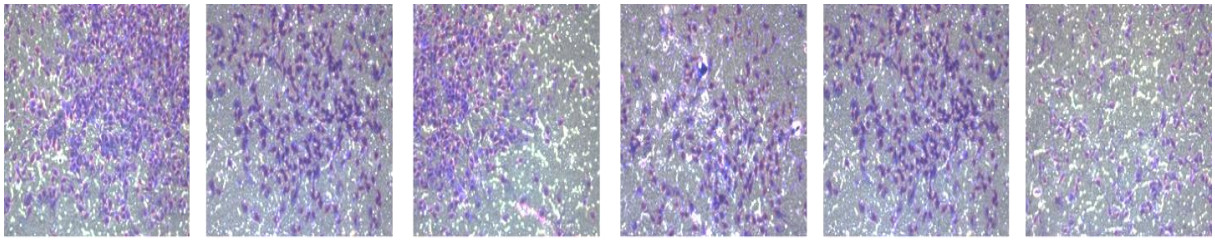
Figure 5.7: High salt treated macrophage and tumor-associated macrophage (TAM) cells suppress MDA MB-231 migration. A. Wound healing assay of MDA MB-231 co-cultured with THP-1 and TAM under high salt condition **B.** Quantitative representation of percent wound area remaining calculated using MRI wound healing tool, ImageJ software, NIH at 0 hrs and 24 hrs (statistical analysis by two-way ANOVA test. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$).

5.2.8 High salt-treated macrophage and TAMs suppressed MDA MB-231 invasion

The invasion of the extracellular matrix is one of the key hallmarks of metastatic cancer cells. To study the effect of high salt on macrophage/TAM-mediated invasion of MDA MB-231 cells, a co-culture set-up was established where, MDA MB-231 cells were co-cultured with THP-1 macrophage and TAM cells, under the influence of high salt for 24 hours in transwell insert of

0.4µM. At the end of the treatment period, MDA MB-231 cells were trypsinized seeded on extracellular matrix (ECM) coated Boyden Chamber of 8.0-micron pore size and were allowed to invade the extracellular matrix for 18 hrs. At the end of the incubation periods, all the content from the transwell insert was carefully removed and cells non-invaded cells were scrapped using cotton buds gently. Inserts were washed with 1X PBS carefully and fixed with 4% paraformaldehyde followed by permeabilization with 100% methanol, cells were then stained with 0.5% crystal violet to visualize the cells. Invading cells on the lower side of transwell inserts were counted manually. High salt treatment decreases the number of invading cells compared to the control **(Figure 5.8)**. Interestingly TAMs in the presence of high salt showed the highest inhibition of MDA MB-231 invasion compared to macrophages.

A.



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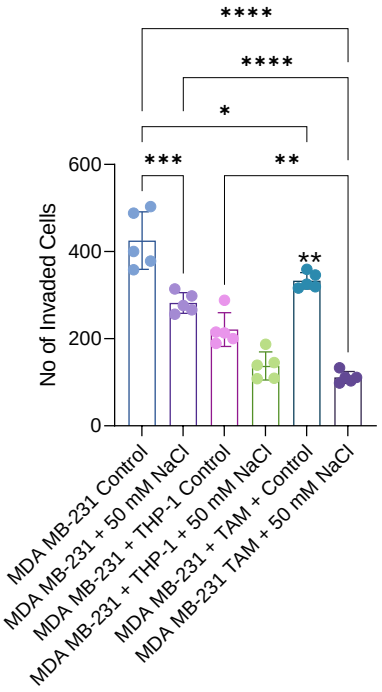


Figure 5.8: High salt treated macrophage and Tumor-associated macrophage (TAM) cells suppress MDA MB-231 invasion in ECM. **A.** Invasion assay of MDA MB-231 co-cultured using THP-1 and TAM under high salt condition **B.** Quantitative representation of Invaded cells at 0 hrs, 24 hrs photographed using a microscope (statistical analysis by two-way ANOVA test. * p<0.05, ** p<0.005, ***p<0.0005).

5.2.9 High salt-treated macrophage and TAMs suppressed MDA MB-231 adhesion to extracellular matrix under co-culture condition

The ability of cancer cells to adhere to the extracellular matrix is a crucial trait of metastatic behaviour. Metastatic cells adhere to new sites of metastasis through interactions between cell surface adhesion molecules and the extracellular matrix or endothelial cells at the target tissue. This adhesion is crucial for the successful colonization and growth of metastatic tumors in distant organs. Upon high salt treatment, both macrophages and TAMs co-cultured with MDA-MB-231 cells demonstrated a significant reduction in adhesion to collagen I and IV-coated wells **(Figure 5.9)**. Interestingly, co-culture with TAMs increased adhesion of the breast cancer cell, however, treatment with high salt led to suppression of adhesion of the cells to collagen I and IV.

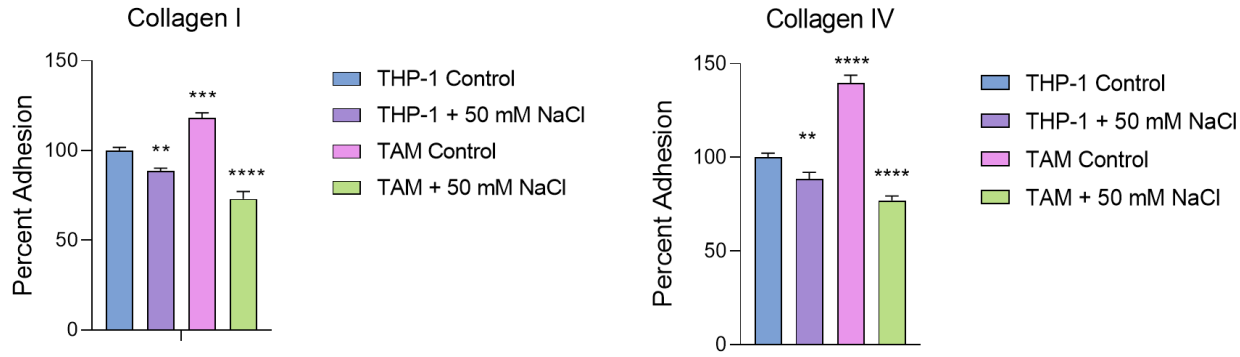
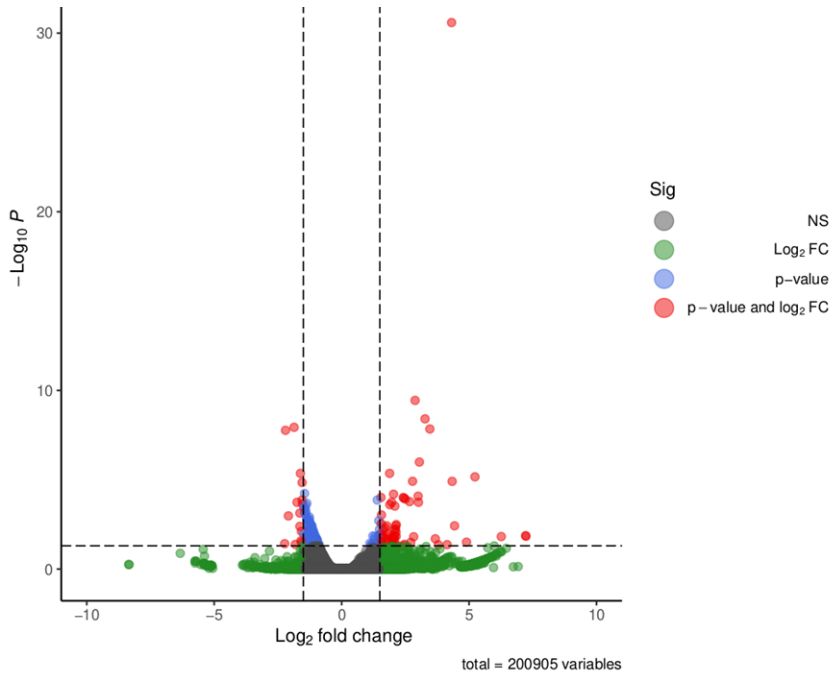


Figure 5.9: High salt-treated macrophage and Tumor associated macrophage (TAM) cells suppress MDA MB-231 Invasion in ECM. Quantitative representation of percent of MDA MB-231 cells adhered to collagen I and Collagen IV coated extracellular matrix under the influence of high salt treated macrophage and TAM cells (n=3) (statistical analysis by one-way ANOVA test. ** p<0.01, ***p=0.0001, ****p<0.0001).

5.2.10 Effect of high salt on global transcriptome profile of MDA MB-231 cells co-cultured with TAM

As described earlier, TAMs are the most abundant immune cells in tumor microenvironment. Hence the effect of high salt treated TAM co-cultured with MDA MB-231 was performed, after the treatment cell line was subjected to RNA-Sequencing analysis for studying the global transcriptomic changes. We identified 214 no of differentially expressed genes (DEGs) with p-value <0.05 and an absolute Log2 fold change of 1.5, which is represented as a volcano plot **(Figure 5.10A)**. Counts per million of the top 50 up and down-regulated genes sorted by P value were plotted as a heatmap, followed by Z-score normalization **(Figure 5.10B)**. Out of the total 215 DEGs, 51 genes were upregulated and 164 genes were downregulated. To assess the potential functions of the 215 genes, overrepresentation analysis (ORA) using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) were conducted **(Figure 5.10 C and D)**. Differentially expressed genes (DEGs) were categorized into different groups based on their potential roles in apoptosis, cell cycle regulation, metastasis, and proliferation, as determined by a literature review **(Table 5.1)**.

A.



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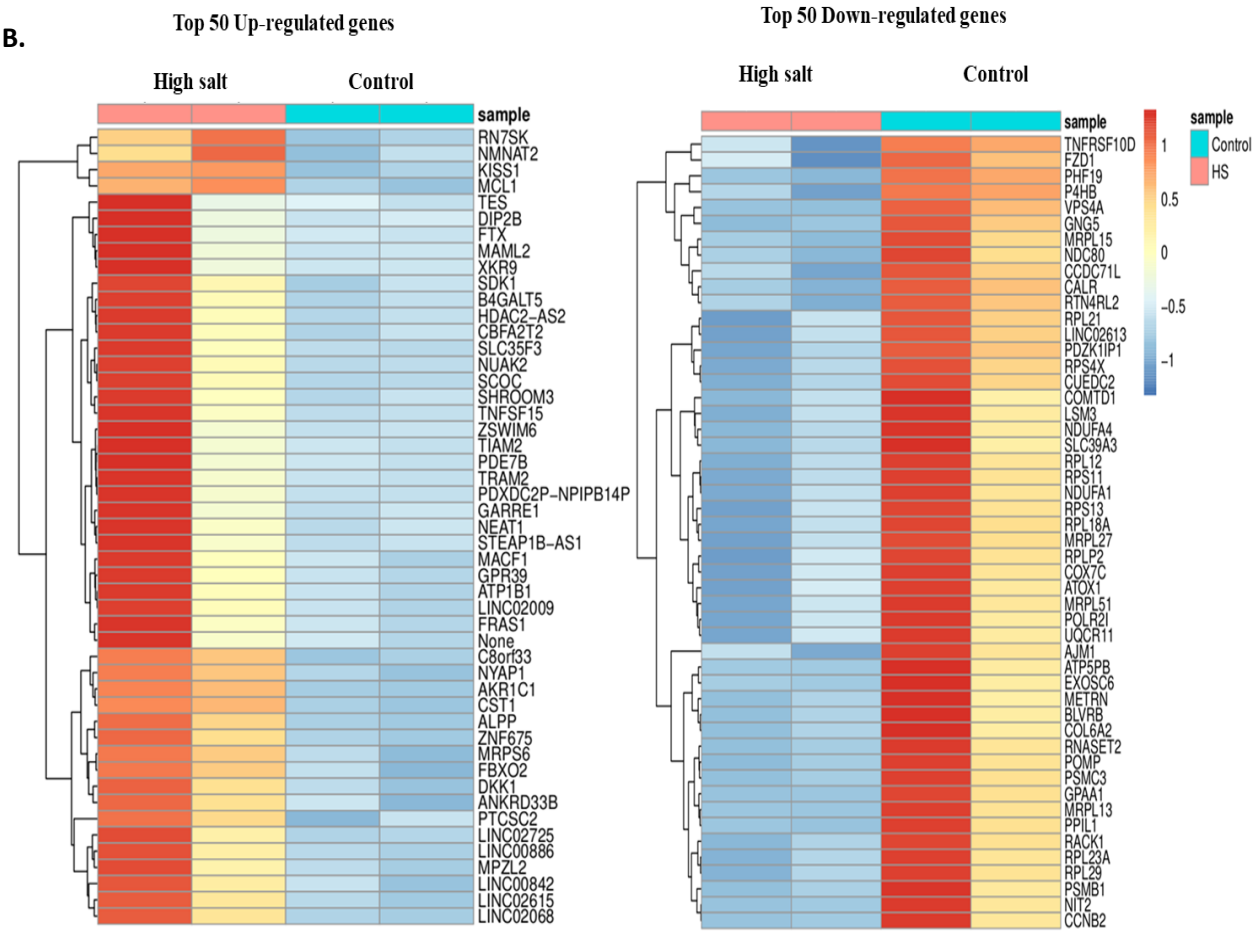
Top 50 Up-regulated genes
High salt
Control
sample
RN7SK
NMNAT2
KISS1
MCL1
TES
DIP2B
FTX
MAML2
XKR9
SDK1
B4GALT5
HDAC2-AS2
CBFA2T2
SLC35F3
NUAK2
SCOC
SHROOM3
TNFSF15
ZSWIM6
TIAM2
PDE7B
TRAM2
PDXDC2P-NPIPB14P
GARRE1
NEAT1
STEAP1B-AS1
MACF1
GPR39
ATP1B1
LINC02009
FRAS1
None
C8orf33
NYAP1
AKR1C1
CST1
ALPP
ZNF675
MRPS6
FBXO2
DKK1
ANKRD33B
PTCSC2
LINC02725
LINC00886
MPZL2
LINC00842
LINC02615
LINC02068
Top 50 Down-regulated genes
High salt
Control
sample
TNFRSF10D
FZD1
PHF19
P4HB
VPS4A
GNG5
MRPL15
NDC80
CCDC71L
CALR
RTN4RL2
RPL21
LINC02613
PDZK1IP1
RPS4X
CUEDC2
COMTD1
LSM3
NDUFA4
SLC39A3
RPL12
RPS11
NDUFA1
RPS13
RPL18A
MRPL27
RPLP2
COX7C
ATOX1
MRPL51
POLR2I
UQCR11
AJM1
ATP5PB
EXOSC6
METRN
BLVRB
COL6A2
RNASET2
POMP
PSMC3
GPAA1
MRPL13
PPIL1
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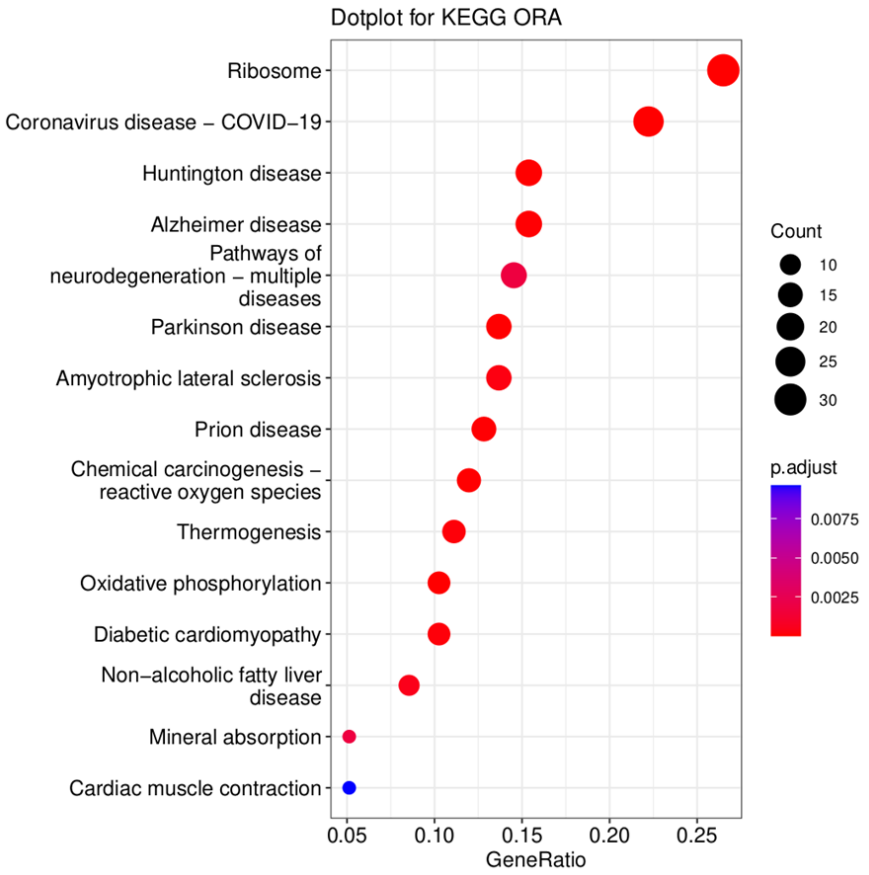
Dotplot for KEGG ORA
Ribosome
Coronavirus disease – COVID–19
Huntington disease
Alzheimer disease
Pathways of neurodegeneration – multiple diseases
Parkinson disease
Amyotrophic lateral sclerosis
Prion disease
Chemical carcinogenesis – reactive oxygen species
Thermogenesis
Oxidative phosphorylation
Diabetic cardiomyopathy
Non–alcoholic fatty liver disease
Mineral absorption
Cardiac muscle contraction
0.05
0.10
0.15
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GeneRatio
Count
10
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25
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p.adjust
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D.

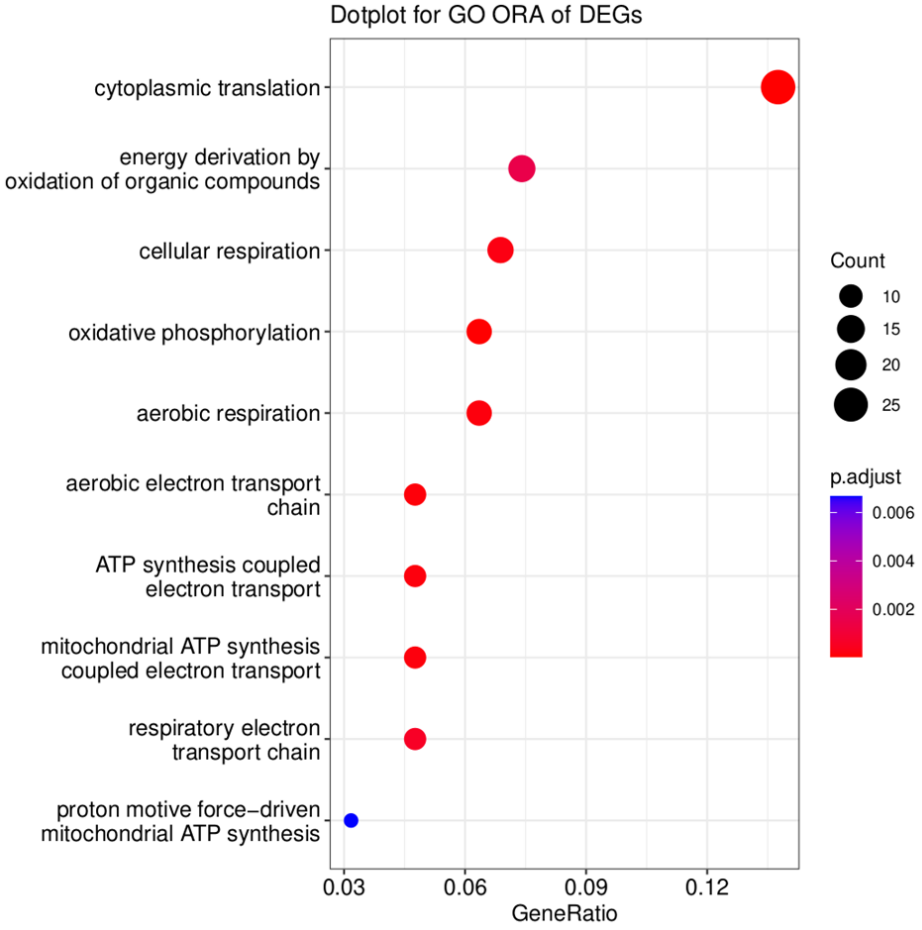
Dotplot for GO ORA of DEGs
cytoplasmic translation
energy derivation by oxidation of organic compounds
cellular respiration
oxidative phosphorylation
aerobic respiration
aerobic electron transport chain
ATP synthesis coupled electron transport
mitochondrial ATP synthesis coupled electron transport
respiratory electron transport chain
proton motive force–driven mitochondrial ATP synthesis
0.03
0.06
0.09
0.12
GeneRatio
Count
10
15
20
25
p.adjust
0.006
0.004
0.002

Figure 5.10: High salt induces TAM-mediated global transcriptomics changes in MDA MB-231 cells. A. Log2fold change and p-value of genes shown as a volcano plot. Red points represent differentially expressed genes that have p-value < 0.05 and absolute log2foldchange > 1.5 **B.** Z-score normalized counts per million reads of top 50 up- and downregulated genes sorted by the p-value represented as heatmap. Samples are sorted by hierarchical clustering **C.** Significant KEGG pathways that are enriched in differentially expressed genes **D.** Gene ontology overrepresentation (GO-ORA) analysis of differentially expressed genes (DEGs). The X axis represents gene ratio and; the Y axis represents enriched gene ontology terms.

Table 5.1: DEGs enriched from RNA sequencing data of MDA-MB-231 cells co-cultured with tumor-associated macrophages (TAM) under high salt conditions were categorized by biological function, suggesting their potential involvement in the observed experimental effect

Biological Functions	Genes
Apoptosis	CCDC71L, NIT2, RPL35, CCDC34, LAGE3, MT1X, CCDC167, TES
Cell Cycle	RPS13, MRPL15, NIT2, RPL35, CCDC34, MT1X
Metastasis	KISS1, DKK1, RAC2, SRM, HSP90B1, MRPL13, GPAA1, CCDC71L, NDC80, PDZK1IP1, PHF19, NUP37, RPL35, CCDC34, RPL23, LAGE3, LGALS1, FABP5, IGFBP1, NPM3, MMP7, HES1, TES
Proliferation	RPL4, SRM, RACK1, MRPL13, CCDC71L, NDC80, PHF19, NIT2, NUP37, RPL35, CCDC34, RPL23, LAGE3, IER5L, EIF4EBP1, LGALS1, FABP5, NPM3, HES1

5.2.11 Validation of genes differentially regulated in RNA sequencing analysis

For validation of differentially regulated genes (DEG's) in RNA Sequencing analysis. DEG's real-time primers were designed for a few genes randomly from the top 50 up and down-regulated genes using NCBI, Primer blast tool. mRNA expressions of 6 DEG's TNFSF15, KISS1, DKK1, CCDC167, TES and MMP-7 gene were studied using qPCR (n=3). mRNA expressions of all these genes were in agreement with the RNA sequencing data.

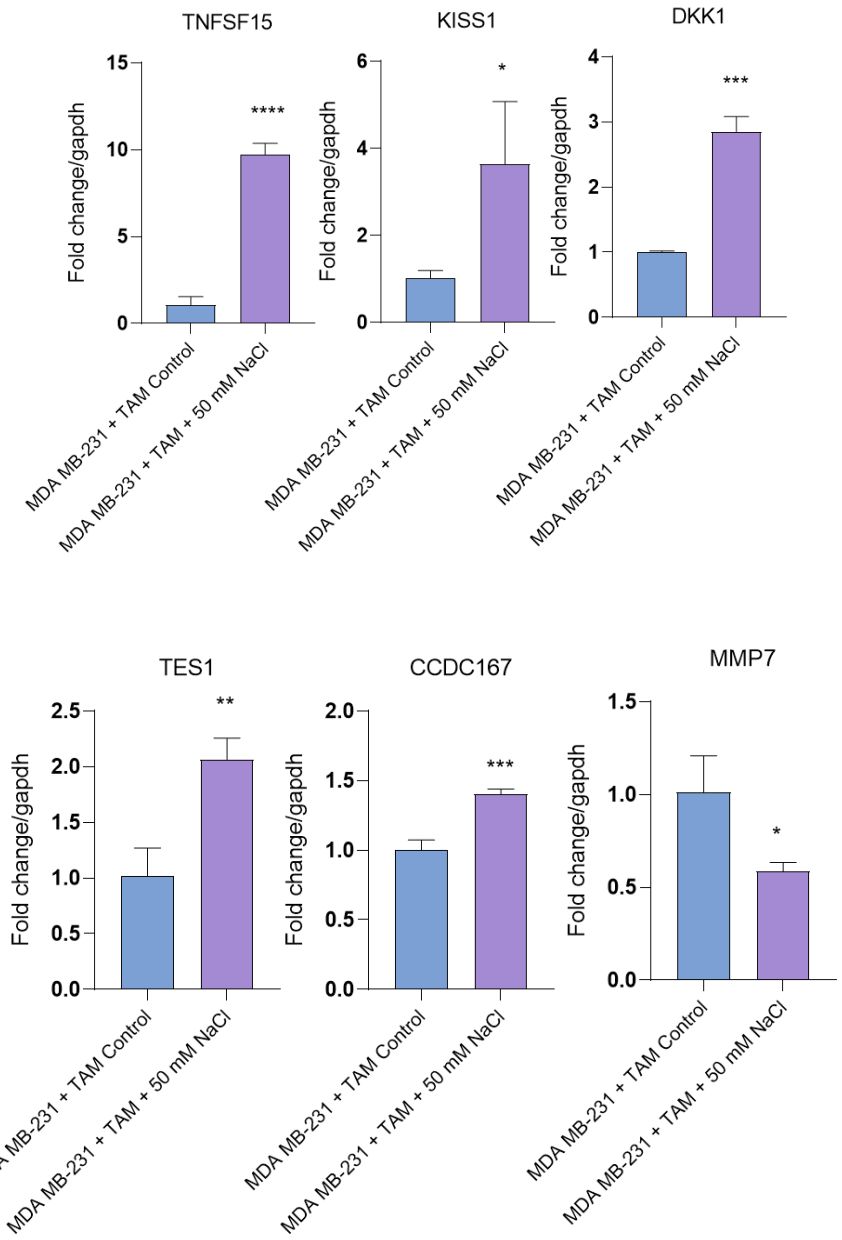

Figure 5.11: Validation of selected DEG's from high salt treated TAM co-cultured with MDA MB-231 cells.

Real-time PCR-based validation of up and down regulated genes from RNA-Sequencing data in MDA MB-231 cell line under high salt stress conditions. Unpaired Student's t-test was performed between control vs. treatment, representing statistical significance, (* p=0.05, ** p<0.005, *** p<0.001, ****p<0.0001).

Table 5.2: List of selected top 50 DEGs with expression profile and anti-cancer function in different types of cancer *

Sr. No	Gene Name	Up/Down regulated in RNA-Sequencing analysis	Function as per literature	References
1.	TNFSF15	Up regulated	TNFSF15 facilitates differentiation and polarization of macrophages toward M1 phenotype to inhibit tumor growth	[13]
2.	KISS1	Up regulated	KiSS-1 has been identified as a putative human metastasis suppressor gene in melanomas and in breast cancer cell lines.	[14] [15]
3.	DKK1	Up regulated	DKK1 overexpression dramatically inhibits breast cancer cell migration and invasion through suppression of β-catenin/MMP7 signalling pathway	[16]
4.	TES	Up regulated	High expression of TES gene correlate with decreased levels of the anti-apoptotic proteins such as Bcl-2, survivin and increased levels of pro-apoptotic proteins i.e. p53, Puma, Bax.	[17]
5.	RBX1	Down regulated	RBX1 is probably a new biomarker of TNBC carcinogenesis, thus suggesting that targeting the RBX1/FBXO45/TWIST1 axis may be an underlying strategy for TNBC treatment.	[18]
6.	HSP90B1	Down regulated	High HSP90B1 expression is associated with bone metastasis in renal cell carcinoma.	[19]
7.	PA2G4	Down regulated	The long PA2G4-p48 isoform has numerous roles in cancer, including inhibiting the tumor-suppressing activity of p53	[20]
8.	PSMB1	Down regulated	The proteasome beta1 subunit plays a novel role in tumorigenesis by degrading p27Kip1. PSMA1 mediates tumor progression and poor prognosis of gastric carcinoma by deubiquitinating and stabilizing TAZ	[21] [22]
9.	POMP	Down regulated	In cancer cells, NRF3 upregulates assembly of the 20S proteasome by directly inducing gene expression of the 20S proteasome maturation protein POMP.	[23]
10.	RPS13	Down regulated	RPS13 could promote the growth and cell cycle progression of gastric cancer cells at least through inhibiting p27(kip1) expression.	[24]
11.	RACK1	Down regulated	RACK1 increases β-catenin stability and promotes breast cancer proliferation.	[25]

12.	RPS4X	Down regulated	RPS4X was speculated as a gene commonly expressed in most CRCs, and its expression level was negatively correlated with tumor prognosis.	[26]
13.	MRPL13	Down regulated	MRPL13 Promotes Tumor Cell Proliferation, Migration and EMT Process in Breast Cancer Through the PI3K-AKT-mTOR Pathway	[27]
14.	GPAA1	Down regulated	GPAA1 facilitates EGFR-ERBB2 dimerization, which further contributes to tumour growth and metastasis and to cancer progression	[28]
15.	MRPL15	Down regulated	MRPL15 plays a role in ovarian cancer through pathways such as the cell cycle, DNA repair, and mTOR 1 signalling.	[29]
16.	GNG5	Down regulated	GNG5 is highly expressed in invasive ductal carcinoma of the breast where it regulates the secretion of E-cadherin through the Wnt-signalling pathway.	[30]
17.	RPLP2	Down regulated	RPLP2 is highly expressed and associated with poor prognosis in various cancer types such as, breast, colon and lung cancers by affecting the translation of specific cellular mRNAs.	[31]
18.	RPS11	Down regulated	RPS11 is a stress-response markers of glioblastoma stem cells, are novel predictors of poor prognosis in glioblastoma patients	[32]
19.	CCDC71L	Down regulated	Overexpressed CCDC71L countervailed the anti-tumor influence of LINC00514 knockdown on TNBC cell proliferation, migration, invasion and apoptosis.	[33]
20.	CUEDC2	Down regulated	Higher level of CUEDC2 in ERα+ve breast cancer corresponded to poorer disease prognosis. It additionally influences mitotic progression.	[34]
21.	COX7C	Down regulated	COX7C upregulation strongly correlates with VTE in colon cancer, which implicates its role as a biomarker and therapeutic target of VTE in colon cancer.	[35]
22.	NDC80	Down regulated	NDC80 promotes the proliferation and metastasis of colon cancer cells	[36]
23.	PDZK1IP1	Down regulated	PDZK1IP1 (MAP17) expression promotes the horizontal propagation of EMT and metastasis by transferring the MAP17 protein between subsets of neoplastic cells.	[37]
24.	PHF19	Down regulated	PHF19 expression was positively associated with GBM progression, including cell proliferation, migration, invasion, chemosensitivity, and tumorigenesis.	[38]

25.	MRPL27	Down regulated	MRPL27 might contribute to the carcinogenesis progression in human cancers including cholangiocarcinoma.	[39]
26.	NIT2	Down regulated	The depletion of NIT2 markedly inhibited colon cancer cell proliferation and colony formation and induced cell cycle arrest in the G0/G1 phase. It triggered the apoptosis of colon cancer cells through the caspase-3 and poly (ADP-ribose) polymerase (PARP) pathways.	[40]
27.	ZIC2	Down regulated	ZIC2 promotes the tumorigenesis and anoikis resistance of NSCLC by transcriptionally inhibiting the Src/FAK signalling pathway.	[41]
28.	NUP37	Down regulated	NUP37 proliferation, migration, and invasion of gastric cancer through activating the PI3K/AKT/mTOR signalling pathway	[42]
29.	CFDP1	Down regulated	CFDP1 enhanced the malignancy of HCC via the NEDD4-mediated PTEN/PI3K/AKT pathway.	[43]
30.	CSTB	Down regulated	CSTB silencing CSTB inhibited the HCC progression via the ERK/AKT/mTOR signalling pathway	[44]
31.	RPL35	Down regulated	RPL35A knockdown could significantly suppress cell proliferation, migration, enhance apoptosis and arrest cell cycle.	[45]
32.	SAP30	Down regulated	SAP30 enhanced chromatin accessibility and RNA polymerase II occupancy at promoters in breast cancer cells, acting as a coactivator for genes involved in cell motility, angiogenesis, and lymph-angiogenesis, thereby driving tumor progression.	[46]
33.	CCDC34	Down regulated	The knockdown of CCDC34 significantly suppressed bladder cancer cells proliferation and migration, and induced cell cycle arrest at G2/M phase and increased apoptosis in vitro.	[47]
34.	RPL23	Down regulated	RPL23 depletion inhibited HCC cell proliferation, migration and invasion, and distant metastasis.	[48]
35.	RBM3	Down regulated	RBM3 upregulates the N6-methyladenosine (m6A) methylation on the mRNA of catenin beta 1 (CTNNB1) in a manner dependent on methyltransferase 3 (METTL3), an N6-adenosine-methyltransferase complex catalytic subunit.	[49]
36.	TXNDC17	Down regulated	TXNDC17, through participation of BECN1, induces autophagy and consequently results in paclitaxel resistance in ovarian cancer.	[50]

37.	LAGE3	Down regulated	LAGE3 facilitated the cell proliferation, migration, and invasion, and suppressed the apoptosis of HCC cells in vitro.	[51]
38.	IER5L	Down regulated	IER5L modulation of the cell cycle, proliferation, resistance to thermal stress, and cell survival under ionizing irradiation	[52]
39.	MMP14	Down regulated	Upregulation of MMP14 is associated with poor prognosis in breast cancer patients	[53]
40.	EIF4EBP1	Down regulated	Overexpression of 4EBP1 drives proliferation of luminal breast cancer cells by mechanisms involving cell cycle regulators such as cyclin D1 and the cdk inhibitor p27	[54]
41.	LGALS1	Down regulated	NCAPG to promote the proliferation, migration, invasion, and metastasis in NSCLC.	[55]
42.	FABP5	Down regulated	FABP5 promotes cell proliferation, progression and invasion of tumors via fatty acid uptake oxidation and several signalling such as PPAR β/δ and HIF-1α	[56]
43.	MT1X	Down regulated	MT1X knockdown inhibits cell growth, induces apoptosis, arrests cells in the S cell cycle, and inhibits the wound healing proportion in ccRCC.	[57]
44.	SERTAD1	Down regulated	SERTAD1/ p34SEI-1 affects cancer cell survival and tumorigenesis by inducing ubiquitination and degradation of PTEN in a NEDD4-1-dependent manner	[58]
45.	IGFBP1	Down regulated	IGFBP-1 can function by activating cell-surface receptors directly in an IGF-independent way, which can enhance the migration or adhesion ability for cancer cells	[59]
46.	NPM3	Down regulated	NPM3 promotes lung adenocarcinoma progression by promoting cell proliferation and migration	[60]
47.	CCDC167	Down regulated	Suppresses growth of MCF7 breast cancer	[61]
48.	DDIT4	Down regulated	lncRNA DDIT4-AS1-mediated activation of autophagy promotes progression and chemoresistance of TNBC	[62]
49.	MMP7	Down regulated	Its expression is associated with tumor invasion, metastasis, and survival in a variety of cancers including breast cancer.	[63]
50.	HES1	Down regulated	Hes1 promotes cell proliferation and migration by activating Bmi-1 and PTEN/Akt/GSK3β. Also known to promote aerobic glycolysis via IGF2BP2-mediated GLUT1 m6A modification	[64]

*The screening criteria was based on the lowest False Discovery Rate (FDR) and fold changes exceeding 1.5-fold

5.3 Discussion

It is well accepted in the literatures that tumor-associated macrophages (TAMs) are the major component of tumor microenvironment (TME) and constitute up to 50% of tumor cells in tumor microenvironment [65]. Macrophages in TME are of two origins, infiltrating monocytes and tissue-resident macrophages [66,67]. Macrophages exhibit a diverse range of phenotypic states based on the cues from their surrounding microenvironment. According to the classical concept, macrophages can either exist in two opposing states tumor killing/ pro-inflammatory (M1) state or tumor promoting/ anti-inflammatory (M2) state. However, new studies have shown that macrophages can show a spectrum of phenotypes based on the source of activation [68]. These macrophages are typically recruited to the tumor site under the influence of growth factors and cytokines from cancer and other cells in tumor microenvironment, where they adopt a pro-tumorigenic phenotype (M2). These TAMS then promote the progression of cancer by secreting factors like hepatocyte growth factor, vascular endothelial growth factor, epithelial growth factor, platelet-derived growth factor, TGF-β1 etc [69,70]. These factors are responsible for metastasis, invasion, angiogenesis and immune suppression by inhibiting T cell-mediated anti-tumor immune responses. However, TAM are highly plastic cells and switch between M1 and M2 states depending upon the surrounding milieu [71]. Modulating tumor immune cells within the tumor microenvironment (TME) to target cancer cells is a promising new strategy for cancer treatment. New macrophage-centric approaches are being explored, such as reprogramming TAMs to an antitumor 'M1-like' state and using antibody-dependent cell-mediated cytotoxicity (ADCC) to enhance macrophage-mediated cancer-killing, antigen presentation, and phagocytosis [72,73]. M1 macrophages exert their anti-tumor effects by identifying and killing tumor cells through the release of molecules like ROS and NO, or by secreting antibodies in the ADCC process [71].

To mimic the cross-talk between TAMs and breast cancer cells, THP-1 monocyte cells were used for generating an in vitro model of TAMs using cell-conditioned media of breast cancer cells. TAMs were then co-cultured with breast cancer cells using a transwell insert system of 0.4µM pore size to facilitate the exchange of cytokines and other factors. TAM generation was confirmed using morphological changes and gene expression markers. TAM cells exhibited elongated shape, larger size, and prominent surface projections, in contrast to the round or oval shape of normal macrophages [74]. TAM cells express typical M2 gene markers such as

Arg1 (arginase 1), Fizz1/RELM beta (Human resistin-like beta), and surface markers CD163 and CD206 [8,75]. qPCR analysis of these M2 markers confirmed the successful differentiation of TAMs from THP-1 macrophages. Notably, high salt treatment significantly reduced the expression of TAM markers, indicating that high salt impedes the transition of THP-1 macrophages (M0 phenotype) to TAMs (M2 phenotype). Our result corroborated with another study that found high salt suppressed IL-4 and IL-13 mediated M2 transition [76]. The tumor microenvironment is consistently infiltrated by immune cells, particularly circulating monocytes, T cells, and NK cells. Understanding the impact of high salt on these immune cells is crucial to predicting their overall behaviour and outcome of cancer. To investigate this, we studied the effect of high salt on monocyte infiltration using a transwell insert assay. THP-1 monocytes migrated in response to a chemoattractant (10% FBS). Migration significantly increased when cultured with MDA MB-231 cells and 10% FBS. However, when MDA MB-231 cells were treated with high salt for 24 hours, monocyte infiltration drastically increased. This suggests that high salt cause breast cancer cells to secrete chemotactic agents, enhancing the infiltration of circulating monocytes to the tumor site. Chemokines such as CCL2 (MCP-1) and CCL5, along with cytokines like CSF-1 and CXCL12 play a crucial role in recruiting circulating monocytes to the tumor site [77] [1]. Such infiltration monocytes play an important role in cancer. To investigate the chemokines involved in monocyte infiltration, we performed a gene expression analysis of CCL2/MCP-1.

Normally, once at the tumor site, monocyte-derived cells infiltrate and reside in the tumor core where they acquire a TAM phenotype [67]. These TAMs promote tumor progression by creating an immunosuppressive microenvironment, secreting growth factors that facilitate cancer cell metastasis and invasion, and inducing angiogenesis. Nevertheless, targeting TAM for fighting against cancer cells is a promising new approach, among which metabolic reprogramming of TAM has shown to be an effective tool to fight cancer [78]. New research has shown that high salt in tumor microenvironment induces a pro-inflammatory state in immune cells including macrophages, TH17 and TH2 cells while inhibiting the pro-tumor function of M2 and TH1 cells [6,7,79]. M1-like macrophages are known carry out anti-tumor functions by secreting the release of proinflammatory cytokines and chemokines like TNF-α, IL-1β, and CXCL10 etc. To study the effect of high salt on macrophage cells, we studied the gene expression study of pro-inflammatory genes using qPCR. Our result showed that pro-inflammatory mediators like

CCL11, TNF-α, IL-6, IL-8, CXCL10, IL-34 and MCP-1 were upregulated whereas, CCR7 which is involved in cancer cell proliferation was downregulated. High salt-induced macrophage-dependent MCP-1 secretion further helps in macrophage recruitment towards the site of cancer [80]. CCL11/eotaxin, helps in the recruitment of eosinophils at tumor site, inducting an anti-tumor function in breast cancer [81]. TNF-alpha (Tumor Necrosis Factor-alpha) is a cytokine that plays a multifunctional role in inflammation and apoptosis. It induces inflammatory and immune responses, as well as cause tumor cell apoptosis or necrosis [82]. IL-6, in combination with TGF-β, induces the proliferation of pathogenic Th17 cells. IL-6 promotes anti-tumor immunity, contributing to its anti-tumor function [83]. IL-34 enhances the differentiation of immunosuppressive cells towards a pro-tumor state in TME [84]. CXCL10 produced by macrophages are responsible for tumor antigen-presenting cell CD8+ T cells in TME. It also functions in the chemotaxis of immune cells and it also functions in the chemotaxis of immune cells and induction of apoptosis in cancer cells [85,86]. IL-8 secreted from macrophages stimulates the migration of neutrophils and monocytes/macrophages and M1 polarization in TME [87]. CCR7 an important cytokine that plays a role in the activation of naïve $CD8^+$ T cells that promote the proliferation of cancer cells was found to be downregulated [88].

Further, we explored the effect of high salt on TAMs, focusing on key mediators such as IL-1β, IL-12, TNF-α, and IL-23, which are known to be secreted by TAMs in the tumor microenvironment and play crucial roles in mediating anti-tumor immunity [89]. Using gene expression analysis, we discovered that the expression of these anti-tumor mediators was significantly upregulated in THP-1-derived TAMs. Under the influence of high salt, this upregulation was further enhanced. These findings suggest that high salt conditions led TAMs to secrete these anti-tumor mediators, thereby helping to restore the anti-tumor function of immune cells.

In order to understand, how high salt influences the cross-talk between TAMs and cancer cells, we established a co-culture system between TAM and triple-negative invasive breast cancer cell line MDA MB-231 using transwell insert, which allows free cross-talk of cytokines and interleukins between the cells. During development macrophages are known to maintain tissue homeostasis by trophic activities *e.g.* they have the ability to provide support and growth factors for the development of progenitor stem cells [90]. This ability of macrophages is exploited by cancer cells for their own proliferation. Under normal circumstances, TAMs secrete

EGF, PDGF, and VEGF that can stimulate the self-renewing capacity of cancer stem cells, which induces cancer proliferation. However, under high salt stress conditions, this ability to shelter tumor cells is altered and restored to a normal (M1) pro-inflammatory state, thereby inhibiting cancer progression. We investigated the effect of high salt treatment on TAMs and their influence on the proliferation of MDA-MB-231 cells using a flow cytometry-based CFSE proliferation assay. We found that high salt treatment induced macrophages to secrete factors that significantly suppressed the proliferation rate of MDA-MB-231 cells compared to cells treated only with high salt. However, the effect of high salt-treated TAMs on the proliferation of MDA-MB-231 cells was moderate. Macrophage cells are also responsible for the invasion and migration of cancer cells by mediating matrix remodelling, promoting angiogenesis and immune suppression by secretion of colony-stimulating factor 1 (CSF-1) and epidermal growth factor (EGF), and Matrix metalloproteinase-9 (MMP-9) [91]. Targeting these molecules has shown promising results in managing metastatic breast cancer [92].

Our study investigated the impact of high salt-treated macrophages and TAMs on the migration and invasion capacities of MDA-MB-231 cells in a co-culture system. Results demonstrated that high salt-treated macrophages and TAMs more effectively suppressed cancer cell migration compared to MDA-MB-231 cells treated with high salt alone. In invasion assays, high salt-treated TAMs exhibited the highest inhibition of MDA-MB-231 cell invasion into the extracellular matrix, surpassing the effects of high salt-treated macrophages and MDA-MB-231 cells exposed to high salt. These findings indicate that high salt conditions possibly modulate the secretion of growth factors like CSF-1, EGF and VEGF by TAMs, consequently influencing the migratory and invasive behaviours of MDA-MB-231 cells. Similar results were obtained in a study performed to understand the effect of high salt-treated macrophage and TAM cells on the adhesion property of MDA MB-231 to Collagen-I and Collagen IV, the most abundant component of extracellular matrix. Cancer cell metastasis is the biggest challenge in the treatment of cancer. MMPs are crucial facilitators of cancer metastasis by breaking down barriers, modulating the tumor microenvironment, influencing the immune response, creating niches for cancer stem cells, and aiding in the establishment of metastatic sites. They degrade ECM components such as collagen, elastin, gelatin, and proteoglycans, which are essential for allowing cancer cells to invade surrounding tissues and migrate to distant locations [93]. The most prominent metalloproteases involved in breast cancer metastasis are MMP-1

(Collagenase-1), MMP-2 (Gelatinase A), MMP-3 (Stromelysin-1), MMP-7 (Matrilysin), MMP-9 (Gelatinase B), MMP-11 (Stromelysin-3), MMP-13 (Collagenase-3) As previously mentioned, RNA sequencing of MDA MB-231 cells co-cultured with TAM under high salt conditions also showed downregulation of the MMP-7 gene, which plays a crucial role in cancer cell metastasis. This was evident from our experimental results, including the invasion of ECM and collagen I and IV adhesion assays.

Finally, we investigated the global transcriptomic changes in MDA MB-231 cells co-cultured with TAM under high salt conditions to understand the cross-talk between TAM and breast cancer cells. Our analysis revealed that DEGs are significantly enriched in multiple KEGG pathways. Notably, many of the enriched pathways were involved in cancer, such as Ribosome, ROS, oxidative phosphorylation, mineral absorption etc. Gene ontology ORA of DEGs showed genes involved in cytoplasmic translation, cellular respiration, electron transport chain mitochondrial ATP synthesis etc. High salt treatment showed suppression of ribosome genes, respiration and genes involved in cellular energetics. Ribosomes are core to the protein synthesis machinery of cells, since tumor cells proliferate rapidly the function of ribosomes becomes critical. A recent study has also shown the non-canonical function of many ribosomes in cancer. Our study revealed many ribosomal proteins *e.g.* RPS12, RPS27L, RSL1D1, RPS13, RPS4X, RPL21 and RPL23 were downregulated [94-97]. The function of these proteins in anti-cancer function is already known. However, the effect of TAM on cellular respiration, mitochondrial and ribosome function remains to be explored further. From our study, we identified differentially expressed genes (DEGs) relevant to tumorigenesis. The DEGs identified in our study were further classified into different categories based on their functions, such as apoptosis, cell cycle, metastasis, and proliferation **(Table 5.1).** A comprehensive literature review was performed to study the top 50 DEGs for their association with cancer which are listed in **Table 5.2.** It is interesting to note that the expression of the majority of the top DEGs was downregulated suggesting the efficacy of high salt in regulating pro-tumorigenic factors. For validation of RNA-Sequencing data, mRNA expressions of 6 DEG's e.g. TNFSF15, KISS1, DKK1, TES, CCDC167 and MMP-7 were studied using qPCR. mRNA expressions of all these genes were in agreement with the RNA sequencing data.

Normally TNFSF15, functions to enhance the bacterial clearance role in macrophages by interacting with death receptor 3 (DR3) and inducing ROS, NO and promoting autophagy [98].

TNFSF15 also functions in inhibiting vasculogenesis in cancer cells [99], making it a potential target for anti-tumor therapy. Under the influence of high salt, TAM cells induce cancer cells to secrete TNFSF15, which works in an autocrine/ paracrine fashion in promoting tumor clearance function of macrophage cells. However, further study is warranted to establish this using knockdown or over-expression studies. KISS1 genes are tumor suppressor genes that are known for their anti-metastatic effect in breast cancer cells by suppressing MMP-9 activity, which plays an important role in the remodelling of ECM for promoting cancer invasion [15]. Our RNA sequencing data revealed the downregulation of KISS1 and MMP-7 gene, which was also validated using a qPCR gene expression study. Thus, under the influence of high salt TAM cells suppressed the expression of the KISS1 gene and inhibited MDA MB-231 migration and invasion as evident from our experimental data. The Dickkopf-1 (DKK1) gene encodes a secretory protein that is significantly upregulated in the serum of cancer patients and serves as a prognostic marker. It functions by inhibiting the β-catenin/MMP7 signalling pathway, which is responsible for cancer invasion and migration [16,100]. Gene expression analysis revealed significant upregulation of DKK1, suggesting that high salt conditions may induce TAM-mediated upregulation of DKK1 and downregulation of MMP-7, leading to decreased cell migration and invasion. The testin (TES) gene is a cytoplasmic protein expressed in normal human tissues and functions as a tumor suppressor [101]. It forms complexes with other cytoskeleton proteins, contributing to cell motility, adhesion, and cell cycle regulation. High TES expression correlates with inhibition of migration, invasion and increased apoptosis [17]. The expression of TES in patients with breast cancer is reduced. However, our data from gene expression analysis revealed that high salt-treated TAM cells induce the expression of TES which might be involved in the suppression of MDA MB-231 migration and invasion. Although CCDC167, expression was downregulated in RNA sequencing data, our result from gene expression analysis showed opposing upregulation of CCDC167 gene. However, there are only a few reports on the function of CCDC167 in cancer where it was found to enhance breast cancer cell apoptosis and inhibit cell cycle synergistically with known cancer treatment drugs [61] .

In conclusion, high salt treatment in macrophages and TAM cells induces the secretion of pro-inflammatory cytokines and interleukins, potentially helping to restore the immunosuppressive tumor microenvironment to its anti-tumor function. Additionally, high salt influences the crosstalk between TAM and MDA-MB-231 cells, positively impacting the anti-tumor function

by suppressing the proliferation, invasion, migration, and adhesion properties of MDA-MB-231 cells. RNA sequencing analysis revealed differentially expressed genes (DEGs) that can be exploited to further enhance the anti-tumor function of tumor suppressor genes and immune cells in the tumor microenvironment. Although these findings need further validation through gene knockdown and overexpression studies and further confirmed with animal experiments, this study critically demonstrated the anti-tumorigenic role of high salt in breast cancer cells which may be exploited as a tool for therapeutic interventions.

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