Chapter III

Material and Methods

3. Material and Methods:

3.1. Cell Lines and Culture Conditions:

The **human NK cell line, "NK92", (CRL-2407**) was purchased from ATCC (American Type Culture Collection (Rockville, MD, USA). NK92 cells were maintained every 2-3 days in complete media composed of; RPMI-1640 media (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 10% Horse serum (Gibco, New Zealand) and 10 IU/mL of recombinant human interleukin-2 (IL-2) (Sigma-Aldrich, USA), and 0.02mM of folic acid (Sigma-Aldrich, China), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, Germany), 1% antibiotic-antimycotic (Gibco by life technologies, USA).

The **breast cancer cell line "MDA-MB-231"** was a kind gift from Dr. Rupak Mukhpadhyay and the cells were maintained in RPMI-1640 media supplemented with 10% of FBS (Gibco, USA) and 1% antibacterial-antimycotic (Gibco by life technologies, USA). The passage of MDA MB-231 cells was conducted upon 80% confluency. The cell lines were cultured at 37°C in a 5% CO_2 humidified incubator (Thermo Fisher Scientific, USA).

3.1.1. Cell Viability:

Cell counting and viability were assessed by the trypan blue method for maintaining cultures. Cell viability was 80-90% before experimental use. Identification of NK92 live cells in the experiments was assessed by the parameter of forward scatter versus side scatter gating (FSC vs SSC) intensity. In addition, microscopic observation was conducted to evaluate NK92 cell granularity and aggregation.

3.1.2. Chemical Compounds:

Two forms of folate were used in this study; **Folic Acid (FA)** (Sigma-Aldrich, India) and **5-Methyl tetrahydrofolic acid disodium salt (5-MTHFA)** (Sigma-Aldrich, USA). The used **succinate** form was "sodium succinate hexahydrate" (Himedia, India). The stock solution of Folic acid was dissolved in 1M of sodium bicarbonate; NaHCO3, while the stock solutions of 5-MTHFA and succinate were dissolved in sterilized deionized water (dH2O). All solutions were sterilized by filtration using 0.22mm filters before being added to the cell culture complete media.

Measure the concentration of folate in folate-free complete media:

Folate concentration in folate-free complete media for NK92 was measured using the FA/VB9 ELIZA kit (ImmunoTag. ITEU0381) as directed by the manufacturer.

3.2. The Experimental Conditions:

For experimental purposes; triplicates per each treatment condition were counted in three independent experiments for each further assay.

3.2.1. Study the effect of Folate and succinate treatments on NK-92 cell line in mono-culture conditions:

To study the effect of folate, 2.5×10^5 of NK 92 cells were cultured in *free-folic acid RPMI-1640* complete media (REF:27016-021, Gibco, USA) supplemented with different concentrations of folate; 0.02mM, and 0.2mM of either folate forms (FA or 5-MTHFA) or without folate supplementation (NoF). Similarly, in order to investigate the effect of succinate, complete media was supplemented with different concentrations of succinate; 50µM, 100µM, and 500µM. The complete media supplemented with 0.02mM of FA was considered as control.

3.2.2. Study the effect of folate and succinate treatments on NK92 cells in a co-culture system with MDAMB231 breast cancer cells:

NK92 cells were co-cultured with MDAMB231 for 72 hours in transwell plates (Nunc™ Polycarbonate Cell Culture Inserts in Multi-Well Plates (insert membrane pore size 0.4 µm, Thermo Scientific, USA) with (1:1) Effector: Target ratio. The NK92 was seeded in the wells while MDAMB231 was in the inserts. The cell lines were seeded separately and kept overnight before effector-target co-culture. The co-cultured cells were grown in the complete *free-FA RPMI1640* media supplemented with the following folate concentrations; no folate supplementation; NoF, 0.2mM of either FA or 5-MTHFA. In the line of study the succinate effect on NK92 cells in co-cultured conditions, complete media were supplemented with succinate concentrations (50µM and 500µM). The complete media supplemented with 0.02mM of FA was considered as control.

3.3. Flow cytometry analysis:

Treated NK92 cells were harvested from mono-culture (after 24hrs, 48hrs, and 72hrs) and co-culture (after 72hrs) conditions to analyze the expression levels of the surface adhesive marker CD56, and intracellular proteins, HIF1α and IFNγ cytokine. Surface staining was briefly performed as follows; 2.5×10^5 of NK92 cells were collected immediately from culture, washed and resuspended in 1X PBS and then incubated with APC-conjugated anti-CD56(BD Biosciences, USA) for 30 min at RT in the dark, washed twice with 1X PBS then immediately analyzed by flow cytometry (BD FACS-Lyric, USA).

Intracellular staining for HIF1α and IFNγ cytokine was performed with APC-conjugated anti Hu-IFNγ and PE-conjugated anti Hu/Mo-HIF1α antibodies (eBioscience, USA) as the following; treated NK92 cells were collected, washed with 1X PBS and then resuspended in fixation buffer (1% paraformaldehyde in 1X PBS) and incubated at RT for 10 minutes. Then, fixed cells were washed with 1X PBS and resuspended in permeabilization buffer (0.2% Tween20 in 1x PBS) and incubated for 15 min at RT. Following, the cells were washed and resuspended in the intracellular staining buffer (0.1% Tween20 in 1X PBS), and incubated with the antibodies, anti-HIF1α and anti-IFNγ, for 30 min at RT in the dark. Then cells were washed twice with 1X PBS and immediately examined by the flow cytometry.

The Expression level of the analyzed proteins was assessed and presented by the mean fluorescence intensity (MFI) of the antibodies-conjugated fluorophores. Ten thousand (10,000) events were counted per sample for each protein or marker and obtained the percentage of positive NK92 cells and MFI values. For all staining experiments, unstained controls for each treatment were included.

3.3.1. Gating strategy:

NK92 cells in mono-culture or co-culture conditions treated with different treatments of folate or succinate were firstly gated in FSC-A and FSC-H axes to get the “Singlets” population to exclude cell aggregates and debris (Figure 1a). Then, the Singlets population was subgated in FSC-A and SSC-A axes to get the “Live cells” population (Figure 7b). The Live cells gate was examined for the presence and quantities of the markers in the treated NK92 cells (represented by the value of MFI), and the percentage of positive cells for the studied surface or intracellular markers (Figure 7c, 7d).

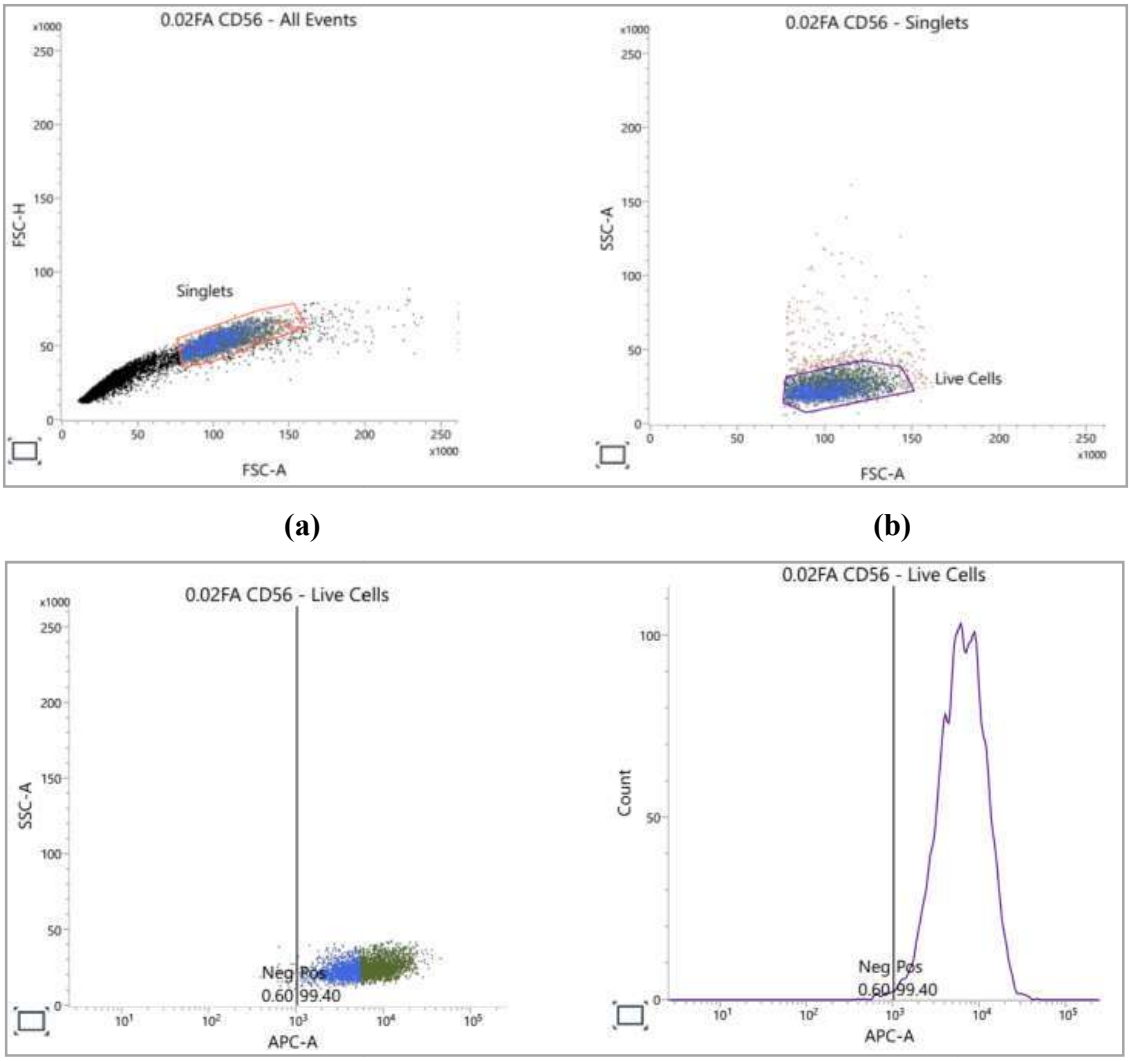

Figure (7): Representative flow plots showed the employed gating strategy.

3.4. Cytokines and Chemokines analysis:

The supernatants media of the co-cultures experimental conditions were collected and stored at -80^{o}C to quantify the secreted cytokines and chemokines by BD® Cytometric Bead Array (CBA) kits according to the manufacturer's instructions (BD Biosciences, United States). The Mean Fluorescence Intensity (MFI) values were analyzed by the FCAP array software (version3, BD Biosciences, United States) to obtain the cytokines and chemokines concentrations (Pg/mL).

3.5. Gene expression study (RNA preparation and Quantitative qRT-PCR):

To investigate the expression of NK cell function-related genes, folate metabolism-related genes, and key genes associated with the mTOR pathway, one million of treated mono-cultured NK92 cells were collected after 72 hrs. The harvested cells were centrifuged and discarded the supernatant then lysed with TRIzol reagent. The total RNA was extracted by the (Ambion, Thermo Fisher Scientific, USA) kit according to the manufacturer's instructions. cDNA synthesis was prepared by Reverse Transcriptase kit (Invitrogen, Applied Biosystems, Foster City, USA) from 3 µg RNA as described in the manufacturer's protocol. RNA and cDNA samples were qualified and quantified by the ratio of absorption at 260 nm and 280 nm with Nano-VueTM plus spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom).

RT-qPCR was performed using an SYBR green-based PCR assay kit (Applied Biosystems, USA) on QuantStudio3 Time PCR System (Applied Biosystems, USA) according to the manufacturer's recommendations using the primers shown in (Table 1). RT-PCR reactions were carried out in a volume of 10 µL containing 1X SYBR green PCR master mix, 0.4 µM of each primer, 100 ng of cDNA and dH_2O (distilled water) to adjust the volume. For all genes, a two-step real-time PCR protocol was conducted. The protocol consisted of a denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 sec, and a subsequent step at 60°C for 1 min. Relative gene expression of the target genes was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) and all RT-PCR experiments were performed in duplicates. *GAPDH* gene (Glyceraldehyde 3-phosphate) was used as an internal reference control for normalization (RT^2 qPCR Primer (Qiagen, Hilden, Germany)).

Primers for target genes were designed manually using the primer blast tool on NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and Geneious software and checked by UCSC-insilico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) and OligoAnalyzer tool on IDT website (https://sg.idtdna.com). The used primers sequences are listed in Table (1).

Table (1): The genes and sequences of primers used in RT-PCR.

Gene	Gene Full name	Primer pair sequence 5'→3'
NK cell function-related genes		
IFNγ	Interferon Gamma	RT^2 qPCR Primer (Qiagen, Hilden, Germany)
GZMB	Granzyme B	F-GCTTCCTGATACGAGACGAC
		R-TTTCACAGGGATAAACTGCTGG
PRF1	Perforin 1	F- ACTGTTTTTCAGGGAGGTGGC
		R- TTGAGAATGGCGGAGGGCTTAG
Folate metabolism-related genes		
RFC	Reduced Folate Carrier (SLC19A1)	F-CCTCGTGTGCTACCTTTG
		R- GTGATCTCGTTCGTGACC
DHFR	Dihydrofolate reductase	F-TAAACTGCATCGTCGCTGTGT
		R-AGGTTGTGGTCATTCTCTGGAAA
MTHFR	Methylenetetrahydrofolate reductase	F-TCCCGTCAGCTTCATGTTCT
		R-TGTCGTGGATGTACTGGATGA
mTOR pathway-related genes		
mTOR	mammalian target of rapamycin	F-TTTAGCGGTCATGTCAATGG
		R-GTCATAGCAACCTCAAAGCA
Raptor	Regulatory-associated protein of mTOR	F-GCTCCAATGCTGGCTTGATC
		R-GTGAGGCAGGAGGTGAATAGG
Rictor	rapamycin-insensitive companion of mTOR protein	F-CCAAACAGCTCACGGTTGTAG
		R-CCATGAGGGTGGCAAGAAAG
RPS6KB1	ribosomal protein S6 kinase B1	F-TCGCCACCTGTTCTTACACC
		R-ACCTTGCCGACCACAGTATG
RPS6KB2	Ribosomal Protein S6 Kinase B2	F-TCGCCACCTGTTCTTACACC
		R-CGATCCAGTGAGCATGTCGTAC
ATF3	Activating transcription factor 3	F-GTGGAGAGATGTTTGGAAAC
		R-CAGTGTGAGTGACTTCTC

Gene	Gene Full name	Primer pair sequence 5'→ 3'
ATF4	Activating transcription factor 4	F-CTCCAGCGACAAGGCTAAGG
		R-GGCATGGTTTCCAGGTCATC
AMPKa1	Protein Kinase AMP-Activated Catalytic Subunit Alpha 1	F- GGATCAGTTAGCAACTATCG
		R-GTTAGGTCAACAGGAGAAG
HIF1α	hypoxia-inducible factor 1alpha	F- TTTTGGCAGCAACGACACAG
		R-TTCAGCGGTGGGTAATGGAG
SREBP1	sterol regulatory element-binding protein 1	F- GCAACACAGCAACCAGAAAC
		R-GTGTCCTCCACCTCAGTCTTC
MYC	Master Regulator of Cell Cycle Entry and Proliferative Metabolism (BHLH Transcription Factor)	F-TCCCAACCACCACCATCCCTGTTTG
		R-CCTTCTCACCTGCCTTCTGCCATTC

3.6. Protein Analysis:

Total cellular protein was extracted from mono-cultured NK92 cells in folate conditions; NoF, 0.2mM of FA or 5-MTHFA, and 50 µM and 500 µM of succinate. The treated cells were grown for 72 hrs, harvested, centrifuged, and then washed with 1X PBS. Following, the pelleted cells were lysed in 1X RIPA buffer which contained; 25 mM Tris•HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS, and protease-phosphatase inhibitors cocktail (Thermo Scientific, USA) for 30 minutes incubation on ice, and sonicate the lysates for 2 min 3 times on ice to shear genomic DNA and cellular components, and then centrifuged at 12,000 xg for 10 min at 4°C and collected the supernatants for downstream protein analysis (western blotting or ELISA). The protein concentration for each cell lysate was determined by the Nano-VueTM plus spectrophotometer at 280 nm wavelength. Protein samples were aliquoted and stored at -80°C until use for protein analysis methods, western blotting and indirect ELISA.

The protein analysis was conducted for SREBP1 and the phosphorylated proteins in the mTOR pathway, including phosphorylated Raptor at the Ser863 and Ser792 sites, as well as phosphorylated P70S6K at the Thr389 site.

3.6.1. Western blotting analysis:

Equal amounts of total protein lysate (500 µg) per sample were loaded and separated by SDS-PAGE on a 10%-12% gel. Molecular weight markers ranging from 10 to 250 kDa (Precision Plus Protein Dual Color Standards, BioRad, California, USA) were included. Proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane by semi-dry blotting. The membrane was blocked for 1 hour at RT and then incubated with the corresponding primary antibody (Invitrogen, USA) (Table 2) overnight (16 hours) at 4°C. After washing, blots were incubated for one hour with the corresponding HRP-conjugated secondary antibodies (1:5000 dilution) at RT. The PVDF membranes were incubated with the chromogenic substrate (TMB) for 5-7 min and visualized in the GelDoc Imaging System (BioRad, California, USA).

3.6.2. Enzyme-linked Immunosorbent Assay (ELISA):

The expression levels of the proteins mentioned in Table (2) were measured using indirect ELISA methods in the protein lysates of the treated NK92 cells in the pre-mentioned treatment conditions. The method was performed briefly as follows; protein lysate samples were diluted in coating buffer (100ng/ml) and added to the ELISA plate wells then incubated at 4^{o}C overnight in addition to negative control. After incubation and washing steps with 1X PBS, a blocking buffer (Casein for SREBP1 or BSA for the phosphorylated proteins) was added and incubated for 3 hours at RT. Then, the primary antibody was added with the dilution (1:200) and incubated on shaker for 1-2 hr. at RT. After washing steps, the corresponding secondary antibody conjugated with horseradish peroxidase (HRP) was added. Following another round of incubation and washing, a substrate solution containing tetramethylbenzidine (TMB) was added, which produced a colorimetric reaction in proportion to the amount of the target protein present in the samples. Optical density (OD) values were measured at 450 nm using a microplate reader (Multiskan GO. Version 100.40, Thermo Scientific, USA). Each sample was assayed in duplicate readings. The concentrations of the protein in the samples were determined by comparing their OD values. The mean OD plus three standard deviations of the 3 negative controls was used as the cutoff reading. Protein levels were expressed as ratios (Arbitrary Units; AUs) which were calculated by dividing the mean optical density of a test sample by the mean optical density plus 3 standard deviations for the 3 negative controls.

Table (2): The primary and secondary antibodies and the used dilutions for western blotting.

Primary antibody	The used dilution or concentration	Corresponded Secondary antibody (1:5000 dilution)
SREBP-1 Monoclonal Antibody (Invitrogen, MA5-11685).	1µg/ml	Goat anti-mouse HRP conjugated (Invitrogen, BD Biosciences, Foster City, USA)
Phospho-P70S6Kinase p-Thr389 (Invitrogen, MA5-15202).	1:1000 dilution	
Phospho-Raptor p-Ser863 (Invitrogen, PA5-64849).	1:1000 dilution	Goat anti- Rabbit IgG, HRP conjugated (Invitrogen, Applied Biosystems, USA)
Phospho-Raptor p-Ser792 (Invitrogen, PA5-17116).	1:1000 dilution	
β-actin (Invitrogen, Applied Biosystems, USA) as internal control.	1:1000 dilution	Goat anti-mouse HRP conjugated (Invitrogen, BD Biosciences, Foster City, USA)

3.7. Statistical Analysis:

GraphPad Prism Software (version 9.5.1) was used for statistical analysis and graphing. Initially, the data distribution was examined, and subsequently, statistical analysis was performed to compare the different treatment groups. For normally distributed data, ordinary one-way ANOVA or two-way ANOVA with Tukey's or Mann-Whitney post-corrections were employed. Non-normally distributed data were analyzed using a non-parametric one-way ANOVA test along with the Kruskal-Wallis post-hoc test with Dunn's correction for multiple comparisons. The data were presented as mean ± SEM (standard error of the mean) and *p*-values less than 0.05 were considered statistically significant.