3. Materials and Methods

3.1. Cells and cell culture

MCF-7, T-47D, ZR75, BT-549, BT-474, MX-1, MDA-MB-231, and MDA-MB-468 were purchased from ATCC, USA. MCF-7 was cultured in EMEM media while T-47D, ZR75, BT-549, and BT-474 were maintained in the RPMI medium. MX-1 was obtained from CLS, Germany, and cultured in F12K media. MDA-MB-231 and MDA-MB-468 were cultured in DMEM media (HI-MEDIA, India). The media were supplemented with 10% FBS (Life Technologies, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Life Technologies, USA). MDA-MB-231 STING knockdown stable cell lines generated via puromycin selection (2-4 µg/mL). Cells were incubated at 37 °C, 5% CO2 in specified media. All cell lines were checked for mycoplasma contamination by Universal Mycoplasma detection kit (ATCC, USA).

3.2. Plasmids and reagents

STING cloned in pCMV6 ENTRY plasmid was a gift from Dr. Hong Bing Su (Wuhan University China), p65-shRNA and control shRNA were received from Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). STING-shRNA and IRF-3-shRNA were a generous gift from Dr. Peter Chumakov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences), STING-shRNA and IRF-3-shRNA were generous gifts from Dr. Peter Chumakov (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences). STING cloned in pCMV6 ENTRY plasmid was a gift from Dr. Hong Bing Su (Wuhan University China), p65-GFP, p65-shRNA, and control shRNA were received from Dr.Edurne Berra Ramirez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). Primary antibody against STING was purchased from Proteintech, USA, cGAS and HRP-conjugated secondary anti-rabbit and anti-mouse antibodies were purchased from Thermo Scientific, Anti-PD-L1 antibody and secondary Alexa Flour 594 tag antibody purchased from abcam USA. Antibodies against PARP, pSTAT3Y705, total STAT3, Histone3, and NF-κB p65 were purchased from Cell signaling, Inc. Prestoblue^{TM} cell viability reagent from Invitrogen, USA and Caspase-Glo® 3/7 Assay kit was purchased from Promega, USA, IL-6 ELISA R&D systems, USA.STAT3 inhibitor as HJC0152 purchased from selleckem, USA. Doxorubicin was purchased from Sigma, USA. USA, 2'3'-cGAMP and c-di-AMP from Sigma, Prestoblue^{TM} cell viability reagent from Invitrogen, USA and Caspase 3/7 luciferase reporter activity kit was purchased from Promega, USA

3.3. Generation of cGAS -sgRNA and p65-sgRNA clones

cGAS-sgRNA clones were generated using the protocol described by Ran et al. The guide-RNAs targeting the first exon of cGAS, p65 were designed using the GPP sgRNA Designer tool (Broad Institute) [213]. sg-RNA-top and sg-RNA-bottom strands were synthesized as described earlier [213]. Synthesized oligos were annealed and cloned into BbsI -linearized pSpCa9(BB)-2A-Puro (PX459) V2.0 vector. cGAS-sgRNA, p65-sgRNA clones were transformed into competent Stbl3 E. coli strain and transformants were screened by colony PCR using U6 sequencing primer and sg-RNA-bottom. Positive clones were finally confirmed by Sanger sequencing.

cGAS:

5′CACCGAGACTC GGTGGGATCCATCG′3.

5′AAACCGA TGG ATC CCA CCG AGTCTC′3.

p65

5’CACCGTCAATGGCTACACAGGACCA’3

5’AAACTGGTCCTGTGTAGCCATTGAC’3

3.4 Transfection

Different DNA constructs transfected in MCF-7, T-47D, ZR75, BT-474, BT-549, MX-1, and MDA-MB-231 using X-treame GENE (Sigma, USA) using the manufacturer’s protocol.

3.5 Collection of tissues

Human breast tumor and extra-tumoral tissue specimens were collected from patients undergoing surgery. Human studies were performed following the norms of the 1964 Declaration of Helsinki. Ethical approval from Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, UP, India ethical committee was taken before the collection of tissue. The prior consent of each patient was taken. Tissues were collected from the tumor zone (tissue within the tumor boundary), and normal zone (distal normal tissue at least 10 mm from the outer tumor boundary).

3.6 Quantitative analysis of gene expression

Total RNA was isolated using Tri Reagent (Life Technologies, USA) and was reverse transcribed to synthesize cDNA using Transcriptor First Strand cDNA synthesis kit (Roche, Germany) or SuperScript VILO cDNA Synthesis Kit (Life Technologies, USA) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex Taq TM (Takara, Japan) or SYBR mix (life technologies, USA) or Applied Biosystems as per the manufacturer's instructions. Specific primers of the genes are listed below.

STING: Fwd 5′-CGCCTCATTGCCTACCAG-3′;
Rev, 5′-ACATCGTGGAGGTACTGGG-3′;

cGAS: Fwd 5′-GGGAGCCCTGCTGTAACACTTCTTAT-3′;
Rev 5′-CCTTTGCATGCTTGGGTACAAGGT-3′;

β-Actin: Fwd 5′-TCGTGCGTGACATTAAGGGG-3′;
Rev 5′-GTACTTGCGCTCAGGAGGAG-3′;

GAPDH: Fwd 5′-AGAAGGCTGGGGCTCATTTG-3′;
Rev 5′AGGGGCCATCCACAGTCTTC 3′.

3.7 Western blot

Cells were plated at a density of 4.5 X 10^5 cells/well in the six-well plate and transfected with indicated expression plasmid or shRNA/sgRNA using X-tream GENE (Sigma, USA). After 48 h of transfection, cells were harvested, washed with ice-cold PBS, and lysed in buffer A (150 mM NaCl, 30 mM Tris-Cl, 10% Triton X-100, 10% Glycerol. Protease Inhibitor (Roche, Germany). The equal protein was loaded and resolved on 11% SDS-PAGE. Protein was electroblotted on the PVDF membrane at 110 V for 1 h at 4 °C. The membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) or 5% BSA (BSA (Sigma-Aldrich, USA), 0.1% Tween-20 in TBS-0.02 M Tris-Cl, 0.15 M NaCl) for 1 h at room temperature. The membrane was incubated overnight with a specific primary antibody and washed three times with TBS-T (TBS containing 0.1% Tween-20) and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and the signal was visualized by using an EZ-ECL chemiluminescence detection kit for HRP (Biological Industries, Israel) by exposing it to UVTEC gel documentation system.

For the western blotting from tissue samples, the tissue samples obtained from breast cancer patient was snap-frozen in liquid nitrogen. The tissue was homogenized to a fine powder in the presence of liquid nitrogen and lysed in RIPA lysis buffer (50 mM Tris [pH 7.4], 50 mM NaCl, 5mM EDTA, 1mM EGTA, 0.1%SDS, and 1% Triton-x100, 0.2% protease inhibitor cocktail, 1mM PMSF, 2mM NaF and 2.5 mM Sodium pyrophosphate). The lysates were freeze-thawed three times in liquid nitrogen. After 15 min of centrifugation (5000 RPM, 4°C), the supernatant was saved to use as a whole-cell lysate. The protein was analyzed by western blotting as described above.

3.8 NF-κB, IFN-β luciferase assay and IL-6 ELISA

To assess NF-κB activity, MCF-7, MDA-MB-231, and MX-1 cells were plated at a density of 1×10^5 cells/well in 24 well plates and treated with doxorubicin and luciferase assay was performed using Dual-Glo luciferase assay system (Promega, USA) [214].

MCF-7, MX-1, BT-474, and MDA MB 231 were seeded at a density of 1×10^5 cells in 24 well-plated, next-day co-transfected with IFN-β firefly luciferase or NF-kB firefly luciferase using gene X-tream GENE (Sigma) and treated with 200 µM c-di-AMP for 24 h IFN-β or NF-kb activity measured using as per manufacturer's instructions (Promega, USA) [225].

MCF-7 and MDA MB 231 were seeded at a density of 1×10^5 cells in 24 well-plated, next-day co-transfected with IFN-β firefly or co-transfected with IFN-β firefly luciferase using gene X-tream GENE (Sigma) and treated with $MnCl_2$ for 24 h IFN-β or NF-kb activity measured using as per manufacturer's instructions (Promega, USA) [225].

The same density of 1×10^5 cells/well cells was seeded and further treated with doxorubicin. After 24 hrs media supernatants were collected IL-6 levels were analyzed using ELISA as per the manufacturer's protocol (R&D systems USA).

3.9 Cell growth inhibition assay and clonogenic assay

All cell lines were seeded at a density of 5000 cells/well in 96 well plates and treated with different concentrations of c-di-AMP for 4 days. At the end of treatment, cell viability was measured using Presto blue cell viability reagent (Invitrogen, USA).

Cells were seeded at a density of 5000 cells/well in 96 well plates and treated with different concentrations of Doxorubicin and HJC0152 and a combination of both for 4 days. At the end of treatment, cell viability was measured using Presto blue cell viability

reagent (Invitrogen, USA). The clonogenic assay has been performed as described previously [215].

Cells were seeded at a density of 5000 cells/well in 96 well plates and treated with different concentrations of $MnCl_2$ and Paclitaxel and a combination of both for 4 days. At the end of treatment, cell viability was measured using Presto blue cell viability reagent (Invitrogen, USA). The clonogenic assay has been performed as described previously [215].

3.10 Molecular docking and cellular thermal shift assay

a) Binding of c-di-AMP with STING was further analyzed by Molecular Docking using Maestro ver 11.9, Schrodinger Suite. The crystal structure of STING [4KSY (Homo sapiens)] with a bound ligand having resolution 1.88 Å was retrieved from Protein Data Bank and prepared using the Protein Preparation Wizard and minimized using OPLS3e force field. All the heteroatoms and water molecules were removed except the conserved water molecules within 5 Å and the hydrogen atoms were added. Each structure was minimized for all-atom-constrained minimization using Ligprep module with OPLS3e force field.

b) Cellular thermal shift assay MDA-MB 231 cells were lysed in HBSS via three freeze-thaw cycles in liquid nitrogen. The total protein was quantified and an equal protein (Cell lysate) was aliquoted into PCR tubes and incubated with and without 30 µg/ml c-di-AMP for 1 h. After incubation suspension was transferred into PCR tubes subjected to specific temperature treatment for 3 min. Precipitated protein was separated using centrifugation at 17000×g for 20 min at 4 °C then the supernatant of each sample was collected and analyzed by western blot.

3.11 Analysis of tumor-induced T cell suppression and colony formation assay

Human PBMCs were isolated from the blood of healthy volunteers using the Ficoll gradient centrifugation method. Briefly, 24-well plates were coated overnight with 5 µg/ml anti-CD3 (BD Bioscience, USA), then washed twice with PBS. PBMCs were plated in complete DMEM medium (10% heat-inactivated fetal bovine serum, penicillin-streptomycin) and considered as Effectors cells. MDA-MB-231 control cell line and MDA-MB-231 shSTING stable cell lines plated in PBMC containing 24- well plates as

Targets with target-to-effectors ratios of 1:0, 1:4, and 1:16 in triplicates. Additionally, to analyze the PD-L1 mediated effect, the PD-L1 blocking antibody was also used in the same settings. After 4 days of co-incubation, 24-well plates wells were rinsed with PBS twice to harvest PBMCs. Further, the plate with survived adherent tumor cells were fixed and stained with Giemsa staining solution. The dried plates were scanned and quantified the intensity. PBMCs were stained for CD45, CD4, and CD8 first, followed by fixation, permeabilization, and intracellular staining of IFN-γ using fluorochrome-conjugated antibodies. 10,000 cells were acquired to assess the expression of IFN-γ by flow cytometer (BD FACS Calibur, Singapore).

3.12 Cell surface expression of PD-L1

To assess the expression of cell surface PD-L1, MDA-MB-231 and MDA-MB-231 STING KO stable cell line was generated. Cells were incubated with primary antibody (1:100) and washed with PBS and Followed with secondary antibody.10,000 cells were acquired to access the expression of PD-L1 by flow cytometer (BD FACS Calibur, Singapore).

3.13 Fluorescence microscopy

MDA-MB-231 cells were seeded and transfected with IRF3-GFP in an optical bottom dish. After overnight incubation treated cells were treated with c-di-AMP, and the cells were imaged using NIKON (Japan) Eclipse Ti2-E inverted fluorescence microscope.

MDA-MB-231, MCF-7 cells were seeded in an optical bottom dish and transfected with p65-GFP. After overnight incubation treated cells were treated with doxorubicin and imaged using NIKON (Japan) Eclipse Ti2-E inverted fluorescence microscope.

3.14 PARP Cleavage

MDA-MB-231 and MX-1 were seeded at density with $1x10^5$ cells, 24 well plates, and treated with doxorubicin and HJC0152 and a combination of both after 24 Hrs cells was lysed and PARP cleavage detected using as mentioned in previous sections western blot protocol.

3.15 3D spheroid inhibition assay

MDA-MB-231 and shRNA STING MDA-MB-231 cells 3D spheroids were generated as per the protocol of Thermo, 2000 cells seeded per well in Nunclon Sphera plate in 100 µL and the plate was centrifuged at 290×g for 3 minutes and placed in the incubator 100µl of

complete medium containing 6 µg/ml collagen-I was added to each well so that the final concentration of collagen in the medium is 3 µg/ml. The plate was centrifuged at 100 g for 3 minutes and placed back in the incubator. The next day different concentration of doxorubicin was added and at the end of the 7th day cell viability was measured as per CellTiter-Glo® 3D Cell Viability Assay Promega, USA.

3.16 Subcellular fractionation

MCF-7, MDA-MB-231, and MDA-MB-468 cells were re-suspended in buffer A (10 mM HEBES, 0.1 mM EDTA, 10 mM KCL, 0.4%NP40, 0.5mM DTT and 1mM PMSF) was added, and the cells were incubated for 20 min on ice. The lysates were centrifuged for 5 min at 200×*g* at 4 °C, and the supernatant was collected (cytosolic fraction). The nuclear fraction was re-suspended in buffer B (2 mM HEPS,400mM NaCl,1mM EDTA,1mM DTT,1mM PMSF) and incubated on ice for 20 min and centrifuged at 15000xg at 4 °C and the supernatant was collected.

3.17 Survival analysis

Breast cancer patients in The Cancer Genome Atlas (TCGA) database was ranked by chemotherapy (Adjuvant therapy) These groups were analyzed in a Kaplan-Meier survival plot to estimate the correlation between the gene's expression level and survival of patients. The KM plots for each probe along with the Hazard Ratio (HR) and P value were plotted [216].

3.18 TIMER Database Analysis

Tumor Immune Estimation Resource (TIMER) is a web server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells [217]. We used the "Correlation" module to get the expression scatterplots, Spearman's correlation, and estimated statistical significance between TMEM173 STING and CD274(PD-L1) expression, and also for correlation between CD274(PD-L1) and IL6 expression in breast cancer. Finally, TMEM173(STING) directly correlated with gene CD274 (PD-L1), ESR1(Estrogen receptor) and IL6. The partial correlation conditioned was adjusted to none.

3.19 Statistical analysis

Data are shown as mean ± SEM for no. of times the experiment was repeated. Comparisons of groups were performed using student t-test for repeated measurements to

determine the levels of significance for each group. The experiments were repeated independently and representation of Mean ±SD; *P < 0.05, **P < 0.01 and ***P < 0.001, based on a student's t-test was considered statistically significant. GraphPad Prism was used to perform all the statistical analyses.