Material and Methods

Reagents and antibodies

RPMI-1640 medium (cat. no. AL162S) was procured from Himedia (India), while Fetal Bovine Serum (cat. no. 10270106), Trypsin-EDTA (cat. no. 25200056), and Penicillin-Streptomycin solution (cat. no. 15140122) were obtained from Gibco (Carlsbad, CA, USA). Universal type I Interferon (cat. no. 11200-1), Recombinant Human Interferon Gamma Protein (cat. no. 285-IF-100) were obtained from R & D Biosystems (Minneapolis, Minnesota, USA), and Recombinant Human IL-29/IFN-lambda 1 Protein (cat. no. NBP2-34996) from Novus Biological (Englewood, Colorado, USA). The TRIM34 antibody was procured from Thermo Fisher Scientific (Waltham, MA, USA), while the GAPDH antibody and the anti-rabbit antibody conjugated with horseradish peroxidase (HRP) were sourced from Santa Cruz (Dallas, CA, USA). Additionally, Alexa Fluor 488-labeled goat anti-rabbit secondary antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture

Lung adenocarcinoma cell lines, including NCI-H23, A549, and NCI-H522 were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. These cell lines are widely used as models to investigate lung adenocarcinoma, a type of lung cancer [219–221]. The lung adenocarcinoma cell lines were cultured in RPMI-1640 (w/ 1mM Sodium pyruvate, 2mM L-Glutamine, 4.5gms Glucose per liter, 10mM HEPES buffer, and 1.5gms per liter Sodium bicarbonate) medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic penicillin-streptomycin.

To maintain optimal cell growth conditions, the cell lines were incubated at 37°C in a CO_2 incubator set at 5% CO_2 level.

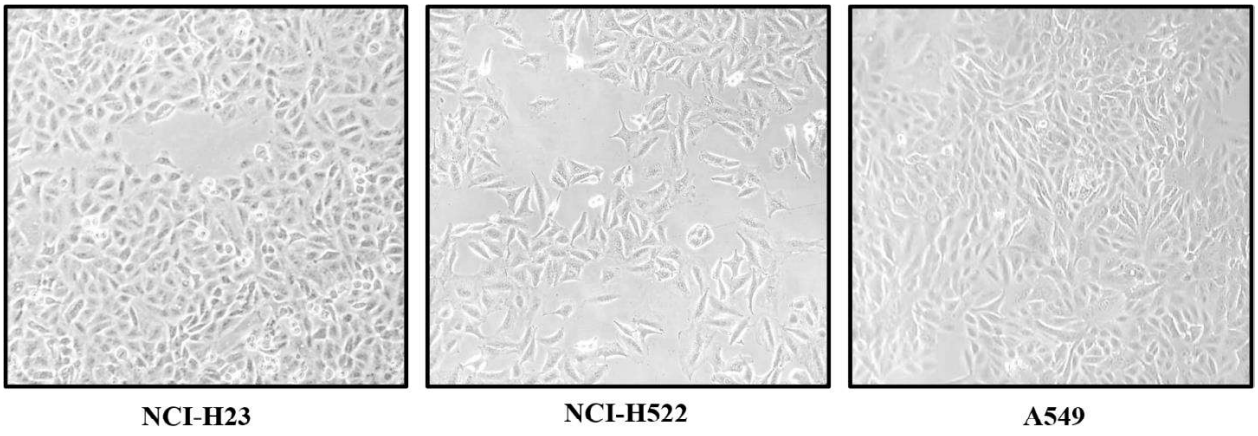

Figure N: Photograph showing morphology of NSCLC cells used for the study.

Dose-dependent Study of Interferon Treatment

In this experiment, a total of 0.1 x 10^6 cells were seeded in each well of a 12-well cell culture plate. The cells were allowed to adhere and grow for the required confluence state. Cells were exposed to Interferon Alpha (10,50,100,200, and 500 IU/ml), Interferon Gamma (10,50,100, and 200 ng/ml), and Interferon Lambda (10,50, and 100 ng/ml).

RNA isolation

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 15596018). TRIzol is a widely used reagent that facilitates the isolation of RNA from cells or tissues. Cells were washed and 500 µl of TRIzol was added to each well. Following a brief scraping of the plate, the TRIzol/cell lysate was collected using a pipette and transferred into a 1.5 ml tube. 200 µl of chloroform (Himedia, India, cat. no. MB109) was added to the solution, followed by vigorous shaking. After 5-minute of incubation at room temperature, the mixture underwent centrifugation at 4 °C and 12,000 rpm for 15 minutes. Supernatant was collected and three volumes of isopropanol (Himedia, India, cat. no. MB063) was added, and incubated at room temperature for 10 minutes. Samples were centrifuged at 4 °C and 12,000 rpm for 15 minutes. The pellet was washed twice with 500 µl of 75% ethanol and centrifuge at 4 °C, 7500 rpm for 5 minutes and pellet was suspended in 500 µl of 100% ethanol. After ethanol evaporation, pellet was suspended in appropriate volume of RNase/DNase-free DEPC-treated water (Himedia, India, cat. no. ML024) to dissolve the RNA pellet.

Quantitative and qualitative analysis of RNA

The estimation of total RNA concentration was performed using the BioSpec-NanoDrop spectrophotometer by measuring the absorbance ratio at 260/280 nm. A ratio of ~2.0 was considered indicative of RNA purity. The quality of the RNA was assessed through electrophoresis on a 1.2% agarose gel. Visualization of the RNA band was achieved using an iBright CL1000 Imaging system (Invitrogen, Waltham, MA, USA). The relative intensity of the two primary components, 28S, and 18S bands, was used to assess the RNA's integrity.

cDNA Preparation by Reverse transcription

To synthesize complementary DNA (cDNA), a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA, cat. no. 4374966) was used. For the RNA reverse transcription (RT) step, 10 µl of 2X RT master mix was pipetted into each tube according to manufacturer instruction. 1 µg RNA concentration of each sample was used for cDNA preparation.

Table 1: According to the manufacturer's instruction, each reaction mix was prepared with the following composition.

Sr. No.	Component	Volume /Reaction (µl)
1	10X RT Buffer, 1.0 mL	2.0
2	10X RT Random Primers, 1.0 mL	2.0
3	25X dNTP Mix (100 mM)	0.8
4	Multiscribe Reverse Transcriptase, 50 U/ µl	1.0
5	RNase Inhibitor, 100 µl	1.0
6	Nuclease free Water	Variable
7.	RNA Sample	Variable
	Total per Reaction	**20.0**

The cDNA synthesis reaction was carried out according to Table 2 PCR reaction parameter in a total volume of 20 µl of volume of each sample. cDNA samples were stored at -20°C for further experiments.

Table 2: Program of thermal cycler conditions used in Eppendorf Mastercycler machine.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Primer Designing

To design primers that are incorporated in the proposed study, Primer3 software was used. The Primer length was carefully considered and set within the range of 18 to 22 base pairs to ensure optimal binding efficiency and specificity. Moreover, some important parameters were taken into account during the primer design process. The primers were designed to have a GC content between 55% to 60%, as this range is known to help stable primer-target interactions. Furthermore, the primers were designed to yield amplicons of approximately 100-200 bases in length. This length range allows for accurate quantification and detection of the target DNA molecule. To confirm the specificity of the primers, the self-complementarity of both the forward and reverse primers was carefully analyzed. For the experimental process, the designed primers were procured from Eurofins Genomics India Pvt. Ltd. A comprehensive list of the designed primers was prepared and utilized for subsequent experimental procedures. A list of primers used in research is given in Table 3.

Table 3: List of the primers used for the study. Sequence 5′-3′ (F: forward; R: reverse)

Gene	Accession number	Sequence
TRIM34-F	NM_021616.6	AAGCAGCTGTCCTGTGTGTG
TRIM34-R		CTCACAAAGCCAGCAAATGA
TRIM6-34-F	NM_001003819.4	ACTCCAGGCAGTCCTCAAGA
TRIM6-34-R		TCCTCAGCCTCTGCAAACTT
BAX-F	NM_001291428.2	GATGCGTCCACCAAGAAGCT
BAX-R		CGGCCCCAGTTGAAGTTG
BAD-F	NM_032989.3	CGGAGGATGAGTGACGAGTT
BAD-R		GATGTGGAGCGAAGGTCACT
TNFa-F	NM_000594.4	AACCTCCTCTCTGCCATCAA
TNFa-R		CCAAAGTAGACCTGCCCAGA
GAPDH-F	NM_001357943.2	GAGTCAACGGATTTGGTCGT
GAPDH-R		TTGATTTTGGAGGGATCTCG

Quantitative Real-time polymerase chain reaction (qRT-PCR)

The qRT-PCR reaction was prepared using Powerup SYBR Master Mix (Applied Biosystems, Waltham, MA, USA, cat. no. A25742), which contains SYBR Green dye for fluorescent detection of the PCR products. The SYBR Green dye binds to double-stranded DNA, enabling real-time monitoring of the amplification process.

Table 4: Reaction master mix and lists of all the kit components.

Sr. No.	Component of kit	Volume (10µl/well)
1	PowerUp SYBR Green Master Mix (2X)	5 µl
2	Forward primer	0.5 µl
3	Reverse primer	0.5 µl
4	cDNA template	1 µl
5	Nuclease-free water	3 µl
	Total per Reaction	10 µl

The appropriate volume of each reaction was transferred to the respective wells of MicroAmp Fast Optical 96-well Reaction plate (Applied Biosystems, Waltham, MA, USA, cat. no. 4346907). *GAPDH* was used as a housekeeping gene for normalization. To maintain sample integrity, the plate was sealed using a MicroAmp Optical Adhesive Film (Applied Biosystems, Waltham, MA, USA, cat. no. 4311971). The qRT-PCR reactions were carried out on a QuantStudio 12K Flex qRT-PCR system (Applied Biosystems, Waltham, MA, USA).

Table 5: The thermal cycling conditions.

Step	Temperature (°C)	Time	Cycles
UDG activation	50	2 min	1
Activation (Dual-Lock DNA polymerase)	95	2 min	1
Denature	95	15 sec	40
Anneal	55-60	15 sec	
Extend	72	1 min	

The expression level of the target genes was quantified using the $2^{-\Delta\Delta Ct}$ method[222]. This method involves normalizing the expression levels of the target genes to an internal control gene.

Western Blot Analysis

Cells were harvested after treatment of Interferon and lysed using Pierce RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 89900). The RIPA buffer disrupts the cell membrane and releases cellular contents, including proteins. To quantify the amount of protein in the lysates, Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 23227) was used. This assay is based on the reaction between proteins and a BCA (bicinchoninic acid) reagent, which forms a color complex. The intensity of the color was measured at 562 nm in a Synergy H1 multi-mode Microplate Reader and it is proportional to the protein concentration, allowing for accurate quantification. The samples were prepared utilizing NuPAGE LDS sample buffer (4X) (Invitrogen, Waltham, MA, USA, cat. no. NP0007), along with 2-Mercaptoethanol (SRL, India, cat. no. 83759(1324196)).

Protein concentration of 35 µg was loaded per well. For protein separation, polyacrylamide gel electrophoresis (PAGE) was performed using NuPAGE Bis-Tris precast polyacrylamide gels (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. NP0323). PAGE separates proteins based on their size and charge, with smaller proteins migrating faster through the gel. Following electrophoresis, the proteins were transferred from the gel onto a methanol-activated polyvinylidene difluoride (PVDF) membrane using a Power Blotter XL system (Invitrogen, Waltham, MA, USA). This transfer process allows the proteins to be immobilized on the membrane, preserving their spatial arrangement from the gel. PVDF membrane was incubated for 2 hours in 3% BSA blocking buffer to prevent the non-specific binding of primary antibodies. The membrane was incubated with TRIM34 primary antibody (Invitrogen, Waltham, MA, USA, cat. no. PA5-41621) for overnight at 4°C. Followed by washing, the membrane was incubated for 2 hours with a secondary antibody (Santa Cruz, Dallas, CA, USA, cat. no. sc-2357) that was tagged with horseradish peroxidase (HRP). After incubation, protein bands on the membrane were visualized using the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. UB278521). This substrate reacts with the

HRP enzyme, generating a luminescent signal that correlates with the amount of target protein present on the membrane. The protein bands were observed and image was captured using the iBright CL1000 Imaging system (Invitrogen, Waltham, MA, USA). This imaging system allows for the visualization and documentation of the luminescent signals emitted by the protein bands on the membrane. Densitometric analysis of the blots was carried out using ImageJ software. GAPDH was used as an internal control.

Cell Apoptosis Assay

NCI-H23 cells were seeded at a density of 3 x 10^5 cells per well in a 6-well plate. After treatment, the cells were harvested using 500 µl of 0.25% Trypsin solution and centrifuged at 500 g for 5 minutes at 4 °C. The cell pellet was washed with 1X cold phosphate-buffered saline. The cells were suspended in 100 µl of 1X annexin binding buffer. The cells were then treated with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions of the Cell Apoptosis Kit (Invitrogen, Waltham, MA, USA, cat. no. V13241). The cells were incubated at room temperature for 15 minutes. After the incubation period, 400 µl of 1X annexin binding buffer. Following the staining procedure, the sample was kept on ice and the cells were subjected to analysis using BD FACSAria Flowcytometry (BD Biosciences, Franklin Lakes, NJ, USA). Fluorescence emission at 530 nm and 575 nm with excitation at 488 nm was selected for the analysis. The analysis included the following samples: 1) Unstained cells (normal cells without any dye), 2) Positive control cells (cells treated with 400 mM H_2O_2 for 30 minutes), 3) Control cells (stained with dye), and 4) Treated cells (stained with dye).

Flow cytometry allows for the detection and quantification of different cellular populations based on their fluorescence properties. In this case, the fluorescence signals emitted by Annexin V-FITC and propidium iodide (PI) were measured by the flow cytometer. Annexin V-FITC binds to phosphatidylserine, a phospholipid that is externalized on the outer leaflet of the plasma membrane during the early stages of apoptosis. Propidium iodide (PI) is a DNA-binding dye that can enter cells with compromised plasma membranes, such as late-stage apoptotic or necrotic cells. By detecting the fluorescence emitted by Annexin V-FITC and PI, the flow cytometer can distinguish between live cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic or necrotic cells (Annexin V-FITC positive, PI positive), and dead cells (Annexin V-FITC negative, PI positive).

Cell Cycle Analysis

NCI-H23 cells were seeded in a 6-well plate at a density of 3 x 10^5 cells per well. After treatment, the cells were detached using 500 µl of 0.25%Trypsin solution and centrifuged at 2000 rpm for 7 minutes at 4 °C. Cell pellet underwent a thorough washing step using 1X cold phosphate-buffered saline (PBS).

Cells were fixed in 70% ethanol. Cells were centrifuged twice with 3 ml of PBS at 300 g for 5 minutes at 4 °C. For further analysis, the cells were treated with 50 µl of a 100 µg/ml RNase solution in each sample. Following that, Cells were incubated in 200 µl of a 50 µg/ml Propidium Iodide (PI) solution at 4 °C for 15 minutes. After completing the staining procedure, BD FACSAria Flow Cytometry system (BD Biosciences, Franklin Lakes, NJ, USA) was used to analyzed cell cycle. The protocol included the following samples: 1) Unstained cells (normal cells without any dye), 2) cells with PI only, 3) Control cells (stained with dye), and 4) Treatment cells (stained with dye).

Flow cytometry measured the fluorescence intensity of DNA-binding dyes to determine the amount of DNA in individual cells. The analysis of DNA content helped characterize different phases of the cell cycle, such as Sub-G1, G1, S, and G2/M. In cell cycle analysis by FACS, quadrants were formed by plotting DNA content (X-axis) against cell count (Y-axis). Preset gates divided the histogram into four quadrants: Q1 (apoptotic cells), Q2 (G1 phase), Q3 (S phase), and Q4 (G2/M phase). This allowed to quickly assess and quantify the distribution of cells in various cell cycle stages.

Cell Viability Assay

3 x 10^4 NCI-H23 cells were seeded in each well of an 8-well Lab-Tek II chambered slide (Thermo Fischer Scientific, Waltham, MA, USA, cat. no. 154534). The Live-Dead Assay was performed to assess cell viability using a Cytotoxicity kit (Invitrogen, Waltham, MA, USA, cat. no. L3224). After treatment, the cells were stained using 2 µM Calcein AM and 4 µM Ethidium homodimer-1 (EthD-1) for 30 min according to the manufacturer's protocol provided with the Cytotoxicity kit. Calcein AM is a cell-permeant dye that is converted to a fluorescent form (Calcein) by live cells with intact plasma membranes. EthD-1, on the other hand, is a DNA-binding dye that can only enter cells with compromised plasma membranes, such as dead or dying cells.

Cell imaging was performed using a Nikon-Ti2E Fluorescence microscopy system (Tokyo, Japan). Image analysis was conducted using ImageJ Software. The percentage of live and dead cells can be determined through image analysis based on the fluorescence signals emitted by Calcein and EthD-1. This experimental procedure provides a standardized approach to assess cell viability and cytotoxicity following treatment in NCI-H23 cells using the Live-Dead Assay.

Immunocytochemistry (ICC)

NCI-H23 cells were seeded in each well of an 8-well Lab-Tek II chambered slide (Thermo Fischer Scientific, Waltham, MA, USA, cat. no. 154534). Following the treatment, the cells were fixed with methanol. After the fixation, the cells were subjected to a blocking step to prevent nonspecific binding. Cells were incubated overnight at 4°C with a primary TRIM34 antibody. The primary antibody recognizes and binds to the target protein within the cells. After incubation, the cells were washed to

remove any unbound primary antibodies.

Cells were treated with a fluorescently labeled Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor™ 488 (Invitrogen, Waltham, MA, USA, cat. no. R37116) for 2 hours in the dark. After incubation in the secondary antibody, the cells were incubated with 300 nM DAPI (Himedia, India, cat. no. MB097), a DNA-specific fluorescent dye, for 2 minutes. To preserve the stained cells and facilitate observation, the slide was mounted using a mounting media. The mounted slide was then subjected to imaging using LSM 710 Confocal microscopy (Carl Zeiss, Germany). The acquired images were analyzed using ZEN 3.6 software, which is commonly used for image analysis in confocal microscopy. The mean intensity value of the fluorescence signal was measured using the software. Additionally, the fluorescence signal was quantified using the Corrected Total Cell Fluorescence (CTCF) formula, Mean of Corrected total cell fluorescence (CTCF) = Integrated density – (Area of selected cell x Mean fluorescence of Background). This calculation helps to normalize the fluorescence intensity based on the size of the selected cell and the background fluorescence.

Scratch Assay

A cell culture consisting of 3 x 10^5 cells was seeded in a 6-well culture dish and allowed to grow until reaching 100% confluence, where the cells form a monolayer and cover the entire well surface. To create a scratch in the cell monolayer, a pipette tip was used to physically scrape the cells. After creating the scratch, cells were washed. Then cells were incubated with (100 ng/ml) or without Interferon-gamma (IFN-γ). At different time interval, such as 0, 24, and 48 hours, images of the scratched area were captured. The closure of wound was analyzed. The wound area was measured using ImageJ software. By measuring the wound area at different time points, the extent of wound closure can be determined. To express the wound closure as a percentage, the following formula was used: Wound Closure % = (Area on day 0 - Area on day n) / Area on day 0 × 100. This formula calculates the relative reduction in wound area compared to the initial (day 0) area.

Statistical Analysis

The results were presented as the Mean ± SEM. Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software, Inc). Student's t-test was performed for data analysis. A result was deemed statistically significant if the value was $p < 0.05$ (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** $p \leq 0.0001$).