Chapter-1

To characterize the modulatory role of TRIM34 expression in lung cancer cell lines.

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INTRODUCTION

Lung cancer is a prevalent and life-threatening disease, ranking among the top causes of cancer-related deaths worldwide. The GLOBOCAN 2020 database, which provides global cancer statistics, highlights the significant impact of lung cancer on public health[26]. Despite advancements in medical science and technology, lung cancer's complex and diverse nature presents significant challenges in both diagnosis and treatment. Traditional treatment options for lung cancer include surgery, radiation therapy, and chemotherapy[223,224]. While these methods have shown some success, they often come with substantial side effects and may not be equally effective for all patients, particularly those with advanced or metastatic lung cancer. In recent years, immunotherapy has emerged as a groundbreaking approach to treating various types of cancer, including lung cancer. Unlike traditional therapies that directly target cancer cells, immunotherapy focuses on empowering the patient's immune system to recognize and attack cancer cells more effectively[225]. This approach aims to boost the immune response against cancer cells, enhancing the body's natural ability to eradicate malignant cells.

One of the key players in immunotherapy for cancer is Interferon (IFN). Interferons are signaling proteins produced by the body's immune cells in response to the presence of viruses, bacteria, or abnormal cells like cancer cells[207,226]. Acting as messengers, Interferons activate various immune responses to counter the threat. In the context of cancer, Interferons play a crucial role in inhibiting tumor growth, promoting cancer cell death, and improving the recognition of cancer cells by immune cells, thereby facilitating their destruction[206,207,226,227]. Tripartite Motif (TRIM) proteins have also garnered significant attention in the field of cancer immunotherapy. TRIMs constitute a large family of proteins involved in diverse cellular processes, including immunity, cellular signaling, and protein degradation[228,229]. In response to Interferons, specific TRIM proteins are induced and actively participate in orchestrating the immune response against cancer cells. One of the key functions of TRIM proteins is acting as ubiquitin E3 ligases in the process of ubiquitination[182,183,230–232]. Ubiquitination involves attaching ubiquitin molecules to target proteins, thereby marking them for degradation by proteasomes or modulating their activity[97,116,118]. This regulatory mechanism allows TRIM proteins to influence the behavior of various cellular proteins, thereby impacting critical pathways involved in cancer development and progression[50,130,231]. In lung cancer, alterations in the expression and activity of TRIM protein have been observed. Some TRIM proteins act as tumor suppressors, helping to control the growth and proliferation of cancer cells, while others function as oncogenes, promoting tumor growth and survival[125,127,189,233–248].

Dysregulation of TRIMs can lead to disturbances in cellular homeostasis, contributing to the initiation and progression of lung cancer[128]. Among the TRIM proteins, TRIM34 has drawn attention due to its association with Interferon levels. Increased expression of TRIM34 has been linked to the presence of Interferons, suggesting its involvement in mediating Interferon's anticancer effects. Further research into the specific role of TRIM34 in the context of lung cancer and immunotherapy may uncover new therapeutic targets and strategies to enhance treatment outcomes. The field of immunotherapy for lung cancer is rapidly evolving, and ongoing research is exploring combinations of immunotherapeutic agents, personalized treatment approaches, and novel targets like TRIM proteins[130].

As our comprehension of the intricate interactions between the immune system and cancer cells deepens, there is optimism for the development of more effective and less toxic treatments, providing improved prospects for individuals worldwide battling with lung cancer. However, it remains crucial to stay up-to-date with the latest advancements in medical research, as the landscape of cancer treatment continues to evolve and progress.

This investigation yields valuable insights into the complex interplay involving interferon and TRIM34 expression in lung cancer cells, potentially opening up a new avenue for impeding lung cancer metastasis through the induction of cell death. The understanding of TRIM34 regulation by Interferons treatment might lay the groundwork for pioneering targeted therapies, contributing to the ongoing progress in treating lung adenocarcinoma. However, further research is necessary to fully elucidate the underlying molecular mechanisms and validate the therapeutic potential of targeting TRIM34 in lung cancer treatment. As the realm of cancer immunotherapy undergoes continuous evolution, the exploration of novel and effective strategies to combat lung cancer remains essential for enhancing patient outcomes and alleviating the global burden of this challenging disease.

MATERIAL AND METHODS

Human Cell lines: NCI-H23, A549, and NCI-H522 cells were procured from NCCS and maintained according to the procedure provided in the material and methods. We have chosen these three cell lines for our study due to their distinct mutations associated with lung cancer. The NCI-H23 cell line exhibits a K-ras 12 mutation, alongside a mutation occurring at codon 246 of the p53 gene [249]. Conversely, the NCI-H522 cell line possesses a mutation at codon 191 of the TP53 gene [250]. The A549 cell line, however, demonstrates wild-type status about various common mutations associated with lung cancer, including those affecting EGFR, PIK3CA, TP53, ALK, and PTEN [251].

Detailed methodology is described in the material and methods section.

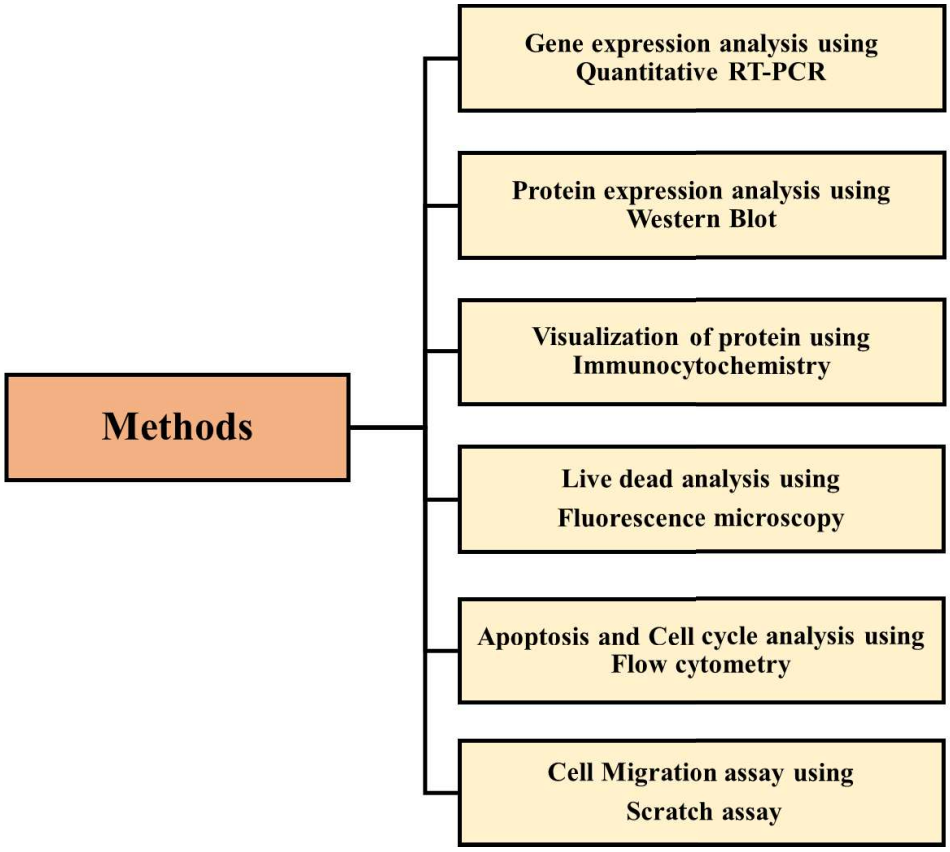

Figure 1.1: Illustrates the experimental methods conducted in the present investigation to achieve the proposed objective.

RESULTS

Basal mRNA level of *TRIM34* in Lung adenocarcinoma cells.

Immunotherapy, a treatment approach that utilizes Interferon, aims to increase the expression of TRIM34, a specific gene believed to enhance cell apoptosis or programmed cell death. To accurately assess the baseline expression level of *TRIM34*, it is crucial to measure it in the absence of Interferon treatment. This enables us to understand the impact of Interferon-induced TRIM34 expression and its significance in promoting cell apoptosis in the context of Immunotherapy. For that, we compared the basal expression of *TRIM34* in three different lung adenocarcinoma cell lines: A549, NCI-H23, and NCI-H522. The obtained results, as shown in Fig. 1.2, revealed that that NCI-H23 and NCI-H522 cells have lower expression levels of *TRIM34* compared to A549 cells indicating that *TRIM34* may play a role in the development or progression of lung adenocarcinoma. The downregulation of *TRIM34* in these cell lines suggests that it could be involved in processes that contribute to the pathogenesis of lung cancer.

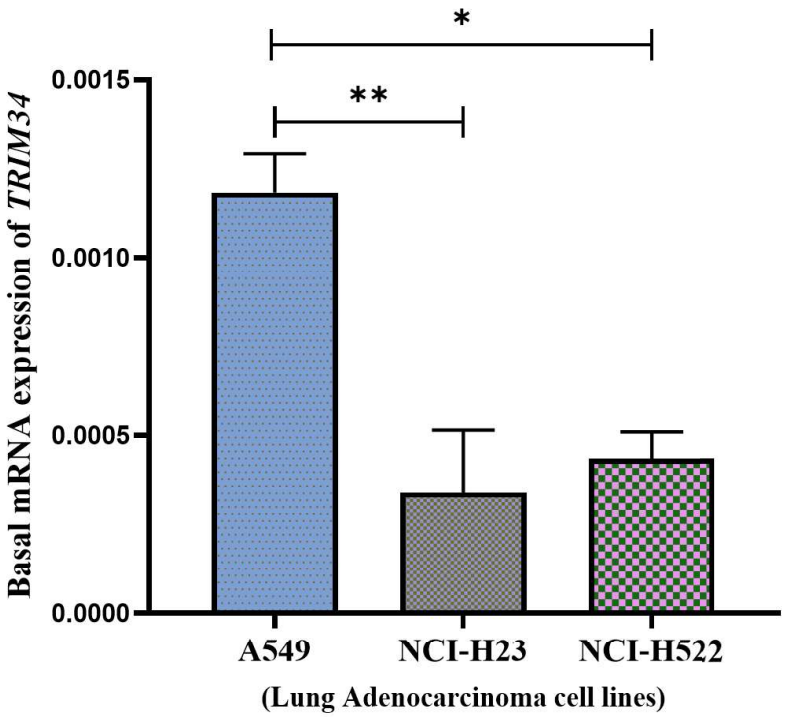

Figure 1.2: Quantification of basal mRNA level of *TRIM34* gene in lung adenocarcinoma cell lines. Cells were harvested, and total RNA was isolated and reverse-transcribed. Relative mRNA expression of *TRIM34* was measured by qRT-PCR and normalized to *GAPDH* gene expression. Graphs are plotted as Mean ± SEM, n=6. Statistical significance is denoted by *, and **, to indicate $p \leq 0.05$, and $p \leq 0.01$ respectively.

***TRIM34* expression is upregulated by three different types of Interferons.**

We investigated if IFNs exposure to lung cancer cell line alters the expression of *TRIM34* in lung adenocarcinoma. For that, we have used three different types of Interferons: Type I (IFN-α), Type II (IFN-γ), and Type III (IFN-λ).

Interferon Alpha induces the *TRIM34* expression in NSCLC.

To find out an effective dose of IFN-α that upregulates *TRIM34* expression, various dosages i.e.10, 50, 100, 200, and 500 IU/ml were used. The investigation involved determining the temporal dynamics of *TRIM34* expression following exposure to IFN-α. We have seen that *TRIM34* reaches maximal expression levels at 6 hours post-interferon exposure in A549 cells and at 8 hours post-exposure in NCI-H23 and NCI-H522 cells. It has been observed that IFN-α exposure increased the expression of *TRIM34*. It was observed that expression of *TRIM34* (8.4-fold) was statistically increased ($p \leq 0.01$) in NCI-H23 cells (Fig. 1.3A) on exposure of 200 IU/ml (241 ng/ml) whereas in NCI-H522 cells (Fig. 1.3C), it was estimated 2.8-fold upregulated with significance level of ($p \leq 0.0001$) at 500 IU/ml (602 ng/ml) after 8 hours of treatment. Similarly, in A549 cells (Fig. 1.3B), *TRIM34* expression was

upregulated by 3.1-fold ($p \leq 0.01$) at 100 IU/ml (121 ng/ml) after 6 hours of treatment. These findings indicate that *TRIM34* expression is significantly upregulated in lung cancer cells when exposed to IFN-α.

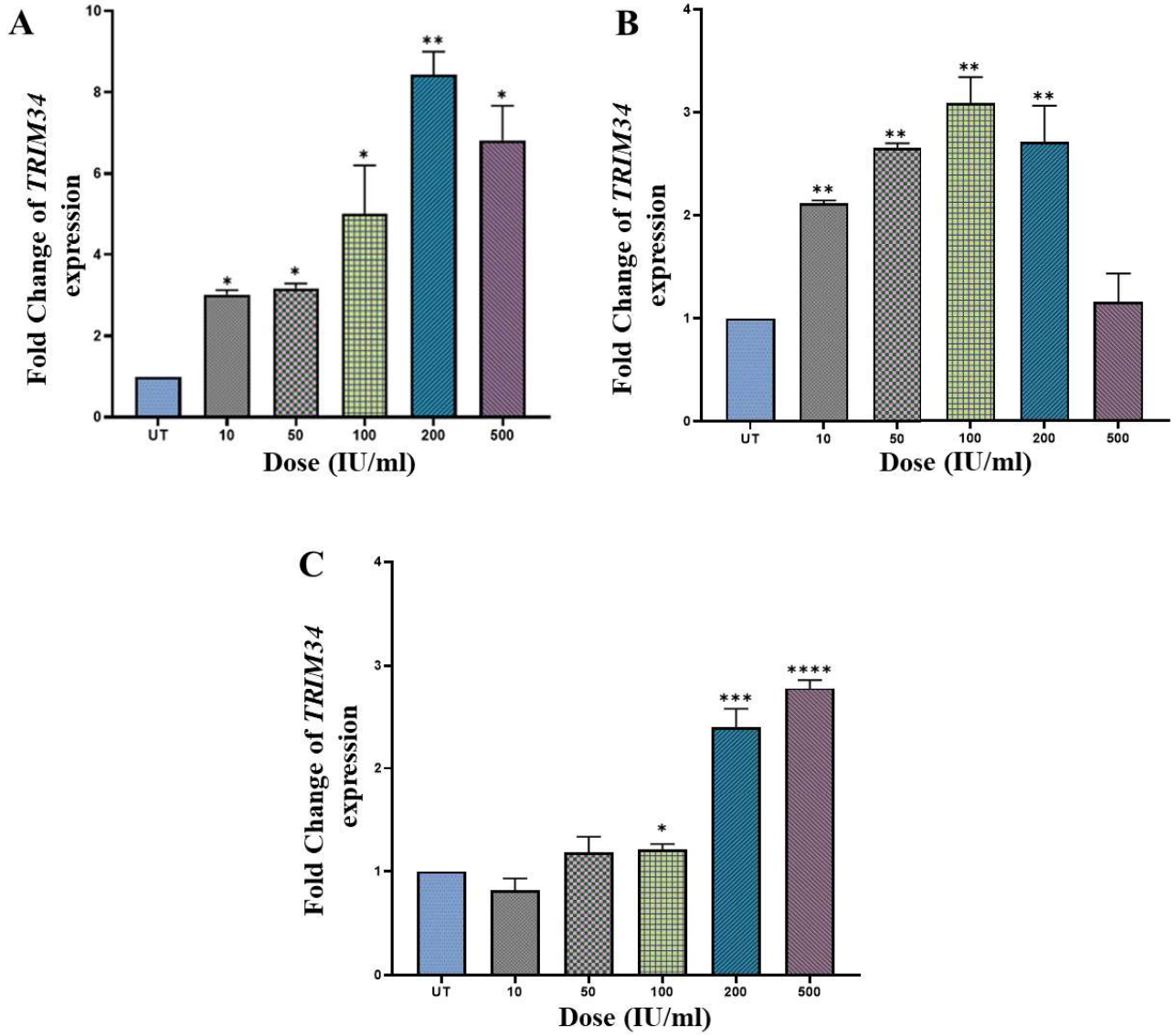

Figure 1.3: Effect of Interferon Alpha on *TRIM34* gene expression in a Dose-dependent manner in different lung adenocarcinoma cells. Cells were stimulated with various doses of Interferon Alpha for 6 hours in A549 and 8 hours in NCI-H23 and NCI-H522. *TRIM34* gene expression level was measured by qRT-PCR and normalized by *GAPDH*. **A.** NCI-H23, **B.** A549, and **C.** NCI-H522 cells. Data was calculated as Mean ± SEM (n=3). Statistical significance is denoted by *, **, ***, and **** to indicate $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$ respectively.

Interferon Gamma induces the *TRIM34* expression in NSCLC.

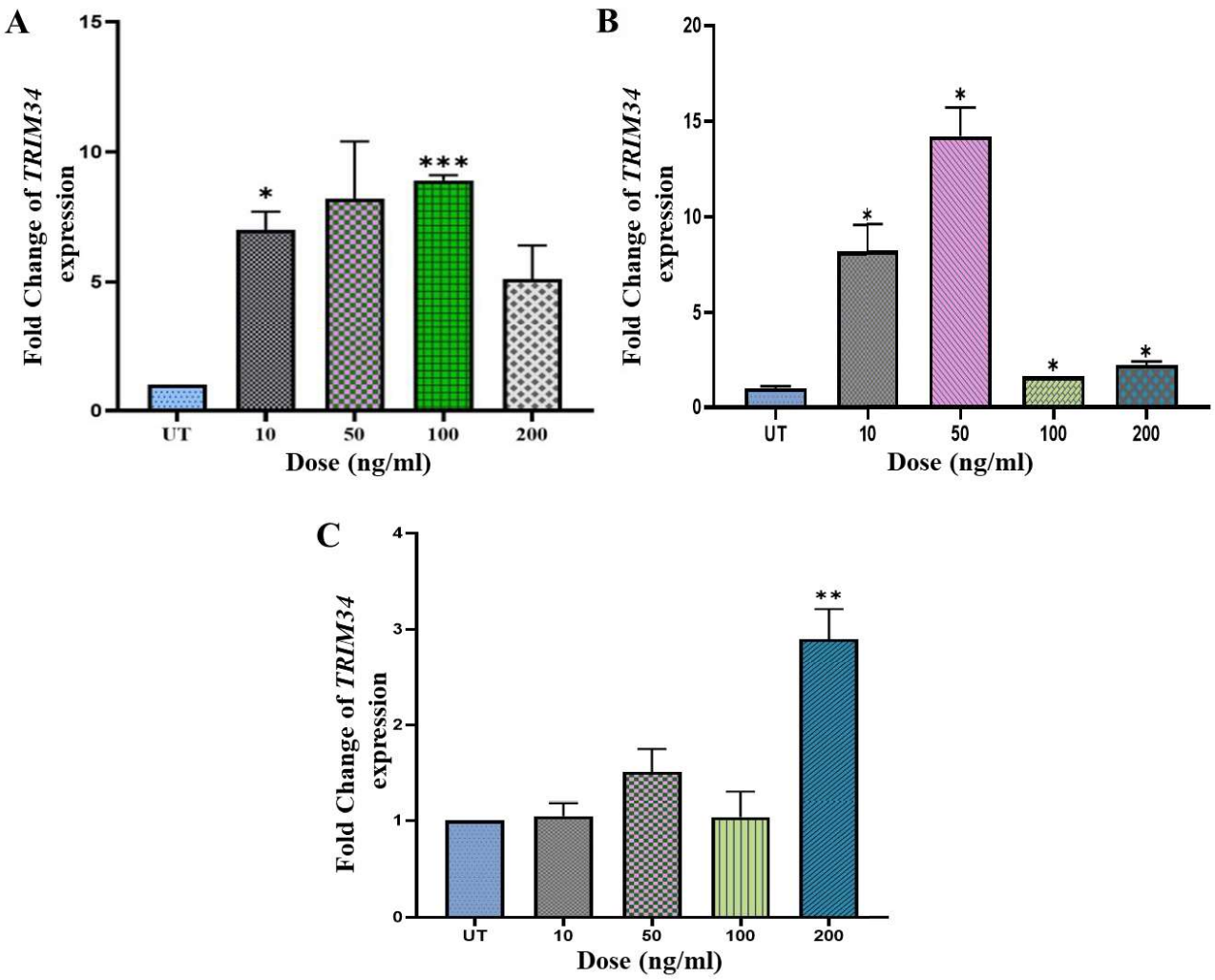

Figure 1.4: Dose-dependent expression of *TRIM34* on exposure of Interferon Gamma in lung adenocarcinoma cells. A549, NCI-H23, and NCI-H522 cells were stimulated by different doses of interferon-gamma for 10 hours. In **A.** NCI-H23, **B.** A549, and **C.** NCI-H522 cells, the expression of the *TRIM34* gene was quantified by qRT-PCR and normalized with *GAPDH* gene. Data was calculated as Mean ± SEM (n=3). Statistical significance is denoted as *, **, and *** to indicate $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ respectively.

TRIM34 mRNA expression was measured using qRT-PCR after 10 hours of various doses (10, 50, 100, and 200 ng/ml) of interferon-gamma (IFN-γ) treatment in NCI-H23, NCI-H522, and A549 cells. IFN-γ enhanced *TRIM34* mRNA expression. In NCI-H522 cells (Fig. 1.4C), *TRIM34* overexpression was estimated 2.9-fold which was statistically significant ($p \leq 0.01$) after 10 hours of treatment of IFN-γ at 200 ng/ml. Upregulation of *TRIM34* was observed 14.2-fold with a statistically significant ($p \leq 0.05$) at 50 ng/ml of IFN-γ in A549 cells (Fig. 1.4B). NCI-H23 cells (Fig. 1.4A) demonstrated significantly upregulated ($p \leq 0.001$) *TRIM34* expression (8.9-fold) on 100 ng/ml of IFN-γ exposure. These findings suggest that treatment of IFN-γ significantly enhances *TRIM34* expression in lung cancer cells. The observed upregulation of *TRIM34* highlights the potential involvement of IFN-γ in

modulating the expression of *TRIM34* and its potential implications in the pathogenesis or therapeutic response of lung cancer.

Interferon Lambda induces the *TRIM34* expression in NSCLC.

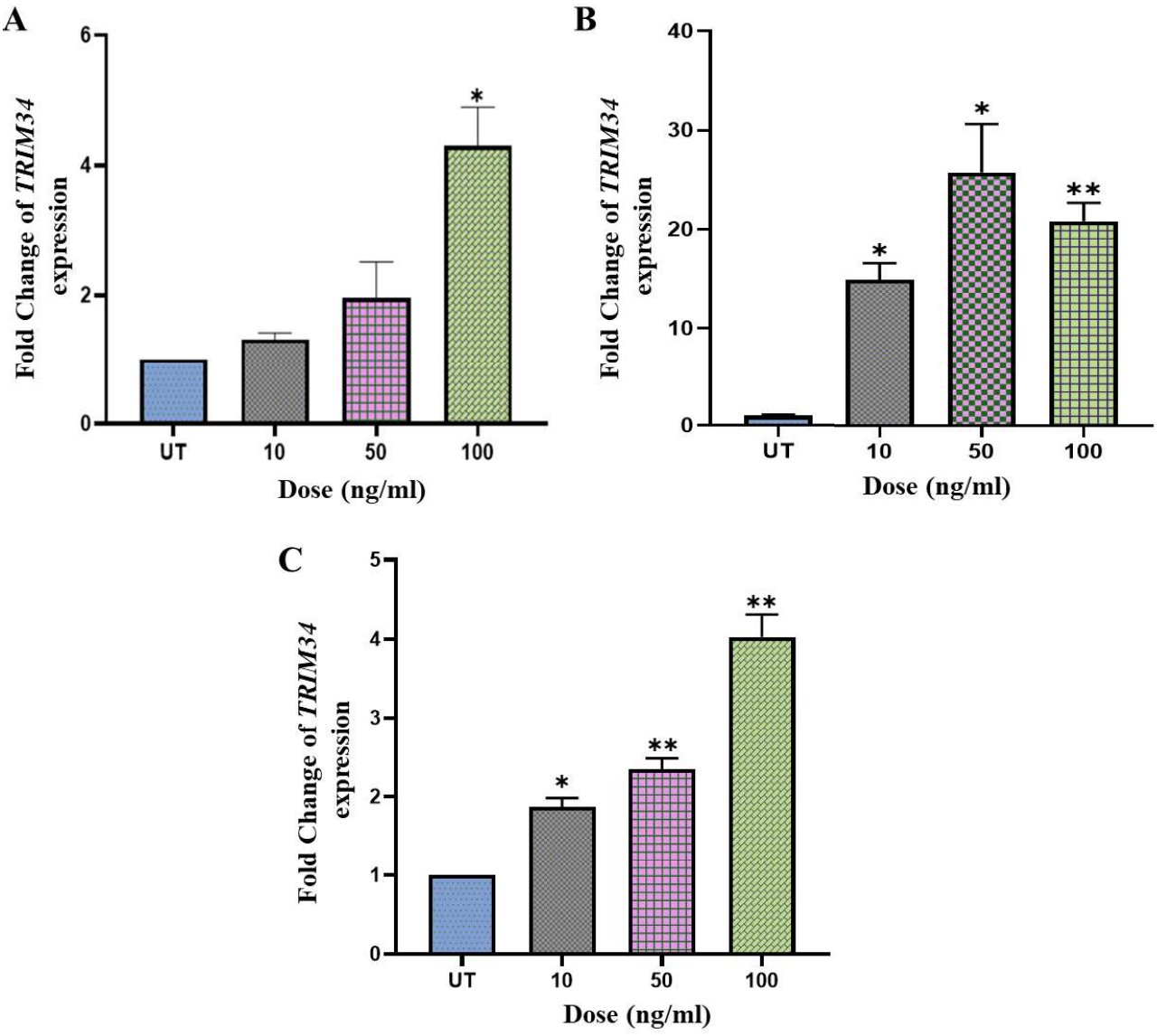

Figure 1.5: Dose-dependent expression of *TRIM34* on exposure of Interferon Lambda in lung adenocarcinoma. The expression of the *TRIM34* gene was measured by qRT-PCR and normalized to *GAPDH* gene. **A**. NCI-H23, **B.** A549, and **C.** NCI-H522 cells. Data was calculated as Mean ± SEM (n=3). *, and ** stands in for $p \leq 0.05$, and $p \leq 0.01$ to indicate statistical significance, respectively.

TRIM34 mRNA expression was measured in NCI-H23, NCI-H522, and A549 cells with various doses (10,50,100 ng/ml) of interferon-Lambda (IFN- λ). In NCI-H23 cells (Fig. 1.5A), *TRIM34* overexpression was found to increase by 4.3-fold which was statistically significant ($p \leq 0.05$) after 10 hours of treatment on 100 ng/ml of IFN-λ. *TRIM34* expression was significantly upregulated *($p \leq 0.05$)* by 25.7-fold in A549 cells (Fig. 1.5B) on 50 ng/ml of IFN-λ at 8 hours of treatment. On exposure of 100 ng/ml of IFN-λ to NCI-H522 cells, upregulation of *TRIM34* was found 4-fold with statistical

significance ($p \leq 0.01$) (Fig. 1.5C). These findings suggest that IFN-λ treatment can significantly elevate *TRIM34* expression in lung cancer cells. The observed upregulation of *TRIM34* highlights the potential involvement of IFN-λ in modulating *TRIM34* expression.

In conclusion, our research findings demonstrate that IFN-α, IFN-γ, and IFN-λ induce overexpression of *TRIM34* in lung cancer cells. The observed upregulation of *TRIM34* indicates its potential functional implications in cellular processes related to lung cancer.

***TRIM6-34* mRNA expression in response to IFN-α treatment.**

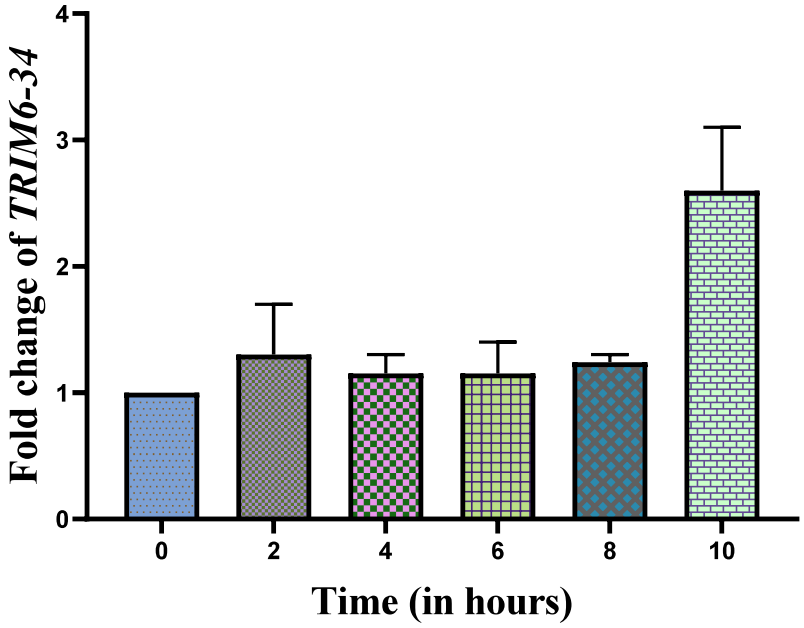

Figure 1.6: Effect of Interferon Alpha on *TRIM6-34* gene in a Time-dependent manner in A549 cells. Cells were stimulated with Interferon Alpha (100 IU/ml) for 2, 4, 6, 8, and 10 hours in A549. *TRIM6-34* gene expression level was measured by qRT-PCR and normalized to *GAPDH* gene expression in A549 cells. Graph plotted as Mean ± SEM. (n=3). non-significance (ns).

We investigated the gene expression of *TRIM6-34*, which is a variant of the *TRIM34* gene that has undergone splicing. Our primary focus was to determine whether there were any noticeable alterations in the expression of *TRIM6-34* in A549 cells following a 2,4,6,8, and 10-hour treatment with IFN-α. We observed that there were no significant changes in the expression levels of *TRIM6-34* in the A549 cells subjected to IFN-α treatment. This implies that the presence of IFN-α did not elicit any notable effects on the expression of the TRIM6-34 variant.

Wroblewski *et al.* (2001) found that NCI-H23 cells are known to be sensitive to IFN- γ signaling, resulting in the upregulation of Major Histocompatibility Complex (MHC) Class I molecules. This event consequently triggers the initiation of an immune response[252]. Subsequently, based on this literature and our result, further study on TRIM34 was conducted on NCI-H23 cells with IFN- γ.

TRIM34 protein expression on IFN- γ exposure.

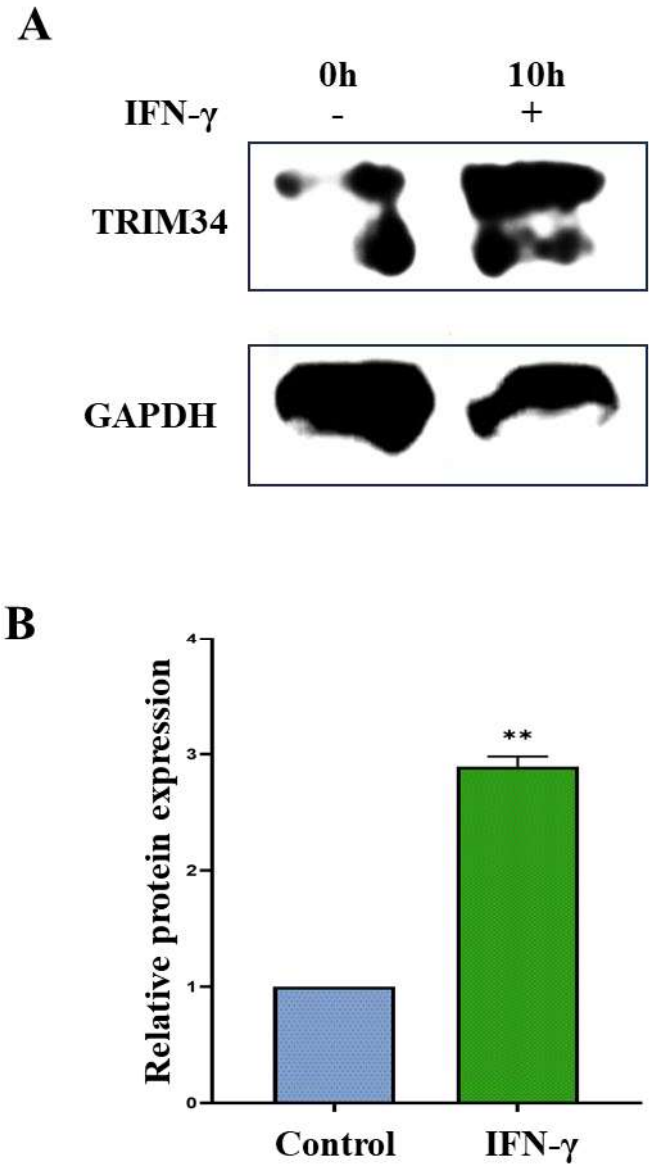

Figure 1.7: Protein level expression of TRIM34 using western blot after 10 hours of Interferon gamma. **A.** Blot of TRIM34 and GAPDH protein expression in NCI-H23 cells. TRIM34 expression was normalized by GAPDH. **B**. Graph represents densitometry analysis of blot using ImageJ software. (**; $p \leq 0.01$), (n=3).

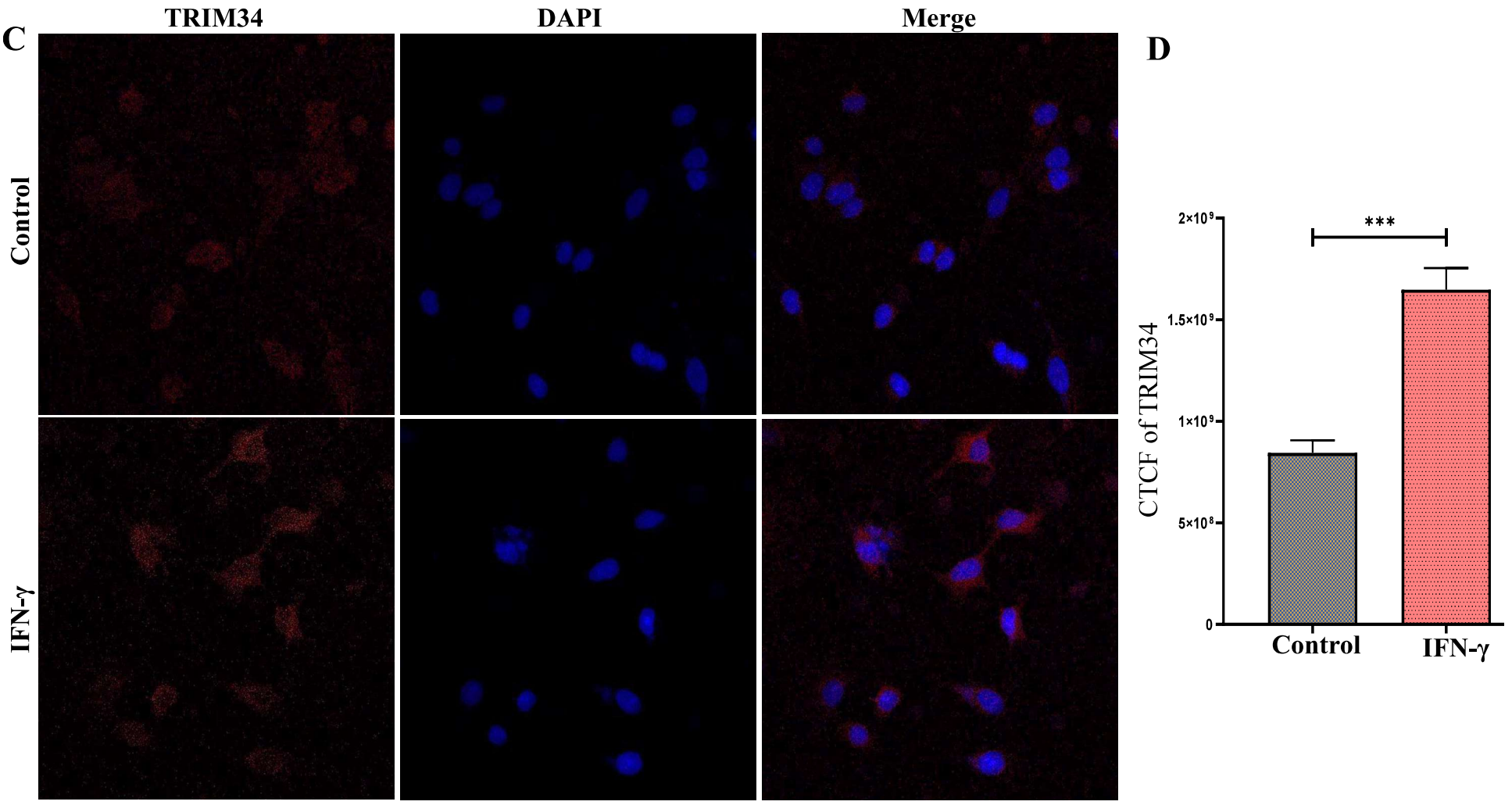

Figure 1.7: Protein level expression of TRIM34 using Immunocytochemistry after 10 hours of Interferon gamma **C.** TRIM34 localization. TRIM34 protein (red) and Nucleus (Blue). **D**. Graphs represent the corrected total cell fluorescence (CTCF) of TRIM34. 63X magnification. (***; $p \leq 0.001$)

TRIM34 protein level expression was measured by Western blot in NCI-H23 after 10 hours of exposure to IFN-γ (100 ng/ml) (Fig. 1.7A). The blot analysis of TRIM34 was carried out by ImageJ software and protein expression was normalized with GAPDH. Results suggest that significantly higher (2.8 folds; $p \leq 0.01$) TRIM protein level expression was observed with IFN-γ treatment (Fig. 1.7B). These findings suggest that IFN-γ treatment resulted in the amplification of TRIM34 protein in NCI-H23 cells.

Western blot results were further supported by TRIM34 immunocytochemistry in NCI-H23 cells after IFN-γ (100 ng/ml) treatment. The mean fluorescence intensity was determined by CTCF analysis (Fig. 1.7D). TRIM34 localization was observed (red color intensity, Fig. 1.7C). statistically significant ($p \leq 0.001$) in NCI-H23 after IFN- γ exposure. IFN- γ in the lung adenocarcinoma cells may increase the activity of TRIM34 which may decrease cell proliferation and induce cell death.

TRIM34 overexpression decreases cell viability of NCI-H23 cells.

To observe the effect on cell viability after Interferon-gamma treatment for 10 hours in NCI-H23 cells, the percent of live & dead cells was calculated. For the cell viability experiment, Calcein AM and Ethidium homodimer-1 (EthD-1) staining were used to differentiate between living and dead cells. Upon treatment with 100 ng/ml of IFN-γ, a significant increase in red fluorescence was observed in the treated group compared to the untreated group (Fig. 1.8A-B). This observation suggests that the overexpression of TRIM34, induced by IFN- γ treatment, significantly ($p \leq 0.01$) promotes cell death of NCI-H23 cells (Fig. 1.8B-C). The increased red fluorescence, indicative of dead cells, further supports the notion that TRIM34 may play a role in mediating apoptosis in response to IFN-γ treatment. These findings highlight the potential pro-apoptotic effects of TRIM34 and its involvement in the cellular response to IFN treatment in NCI-H23 cells.

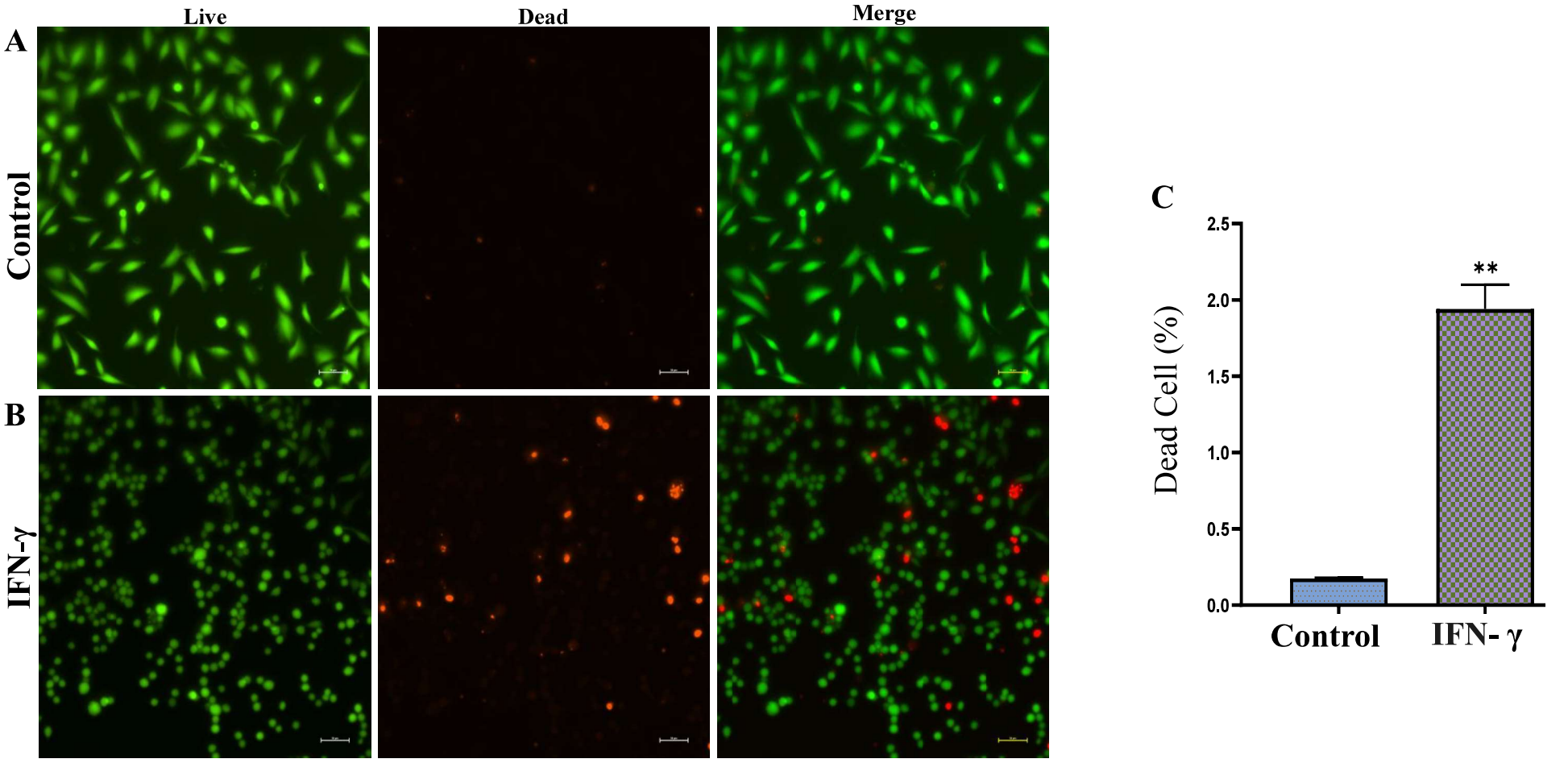

Figure 1.8: Overexpression of TRIM34 induces cell death in NCI-H23 cells after 10 hours of treatment of IFN-γ. Calcein AM stains for live cells in green, and EthD-1 stains for dead cells in red. Images were seen with a fluorescence microscope at 20X objectives. **A**. Control (Untreated) Cells, **B.** Cells were treated with IFN-γ (100 ng/ml) to observe the cell viability in NCI-H23 cell. **C** Calculation of Dead cell (%) by ImageJ Software. ** stands for $p \leq 0.01$ to indicate statistical significance.

IFN-γ and IFNs decrease the cell viability of NCI-H23 cells.

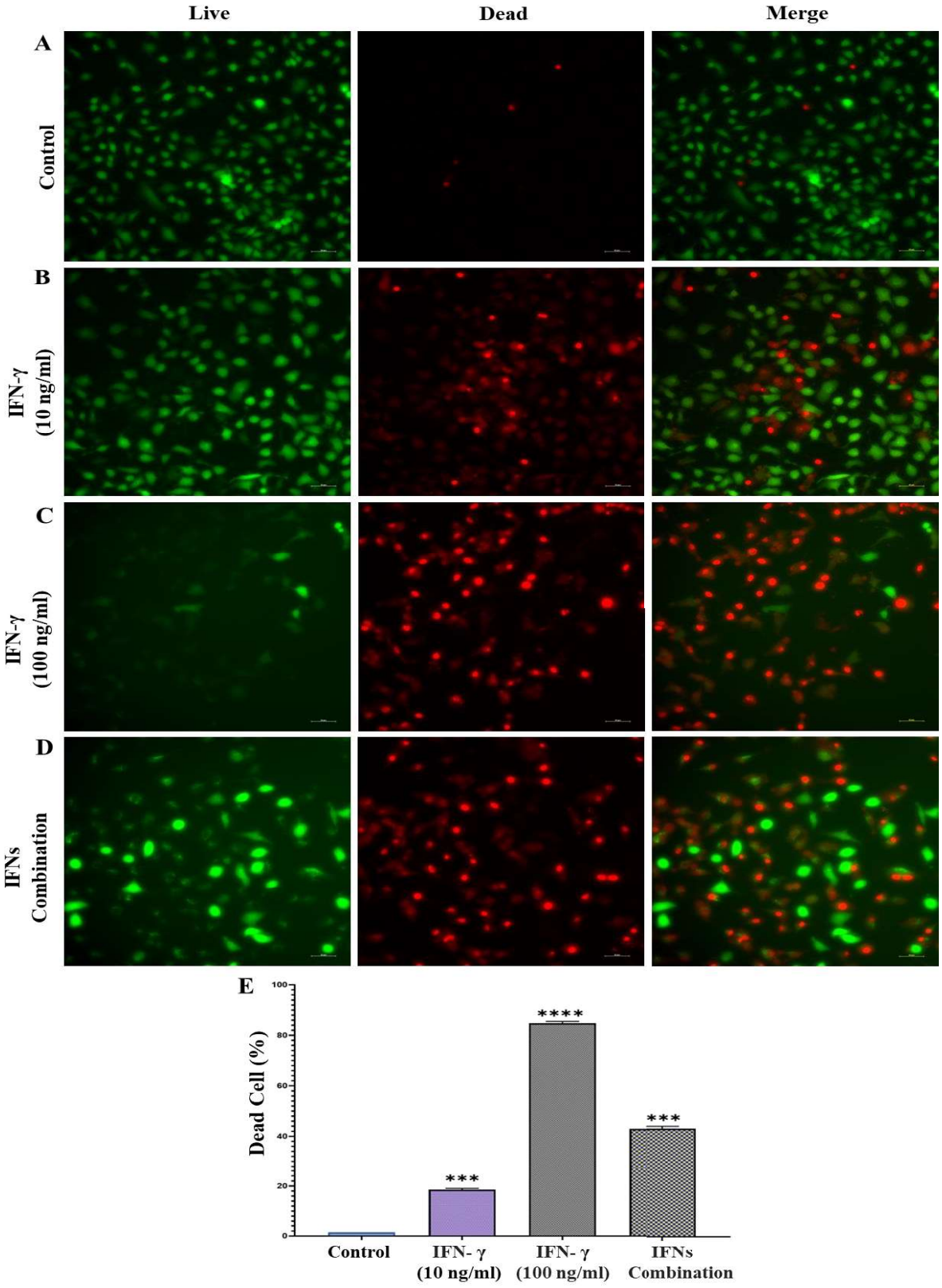

Figure 1.9: Cell death analysis using calcein AM and EthD-1 staining method. Calcein AM stains for live cells in green, and EthD-1 stains for dead cells in red. Cell viability of NCI-H23 cells for **A.** Control, **B.** IFN-γ (10 ng/ml), **C.** IFN-γ (100 ng/ml), and **D.** IFN Combination (10 IU/ml IFN-α+10 ng/ml IFN-γ+10 ng/ml IFN-λ) treatment for 24 hours. **E.** Calculation of Dead cell (%) by ImageJ Software. Images were seen with a fluorescence microscope at 20X objectives. Mean ± SEM. (n=3) (***; $p \leq 0.001$, and ****; $p \leq 0.0001$).

The findings indicate that the treatment of Interferon (IFN) can trigger cell death, as demonstrated in the data obtained. Our result indicated that treatment with IFN-γ at a concentration of 10 ng/ml led to cell death by 14% ($p \leq 0.001$). Similarly, at a higher concentration of 100 ng/ml, IFN-γ resulted in a significantly higher ($p \leq 0.0001$) percentage of cell death, i.e. 80%. Moreover, when all IFNs were used in combination, 56% of cell death was measured ($p \leq 0.001$). This finding suggests that IFN-γ treatment induces cell death in lung adenocarcinoma cells after 24 hours of treatment.

Cell death analysis by apoptotic marker by qRT-PCR.

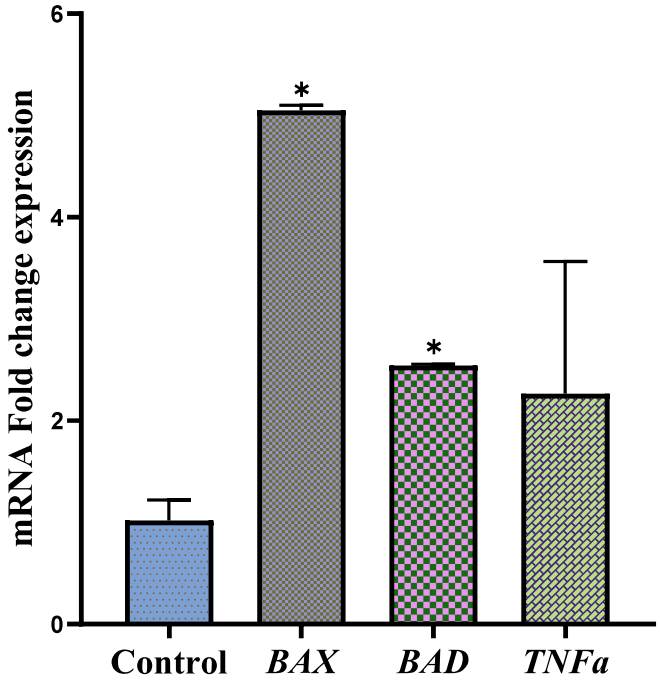

Figure 1.10: Gene expression of cell death markers. NCI-H23 cells were treated with IFN-γ for 10 hours. Evaluation of apoptosis regulating gene expression after IFN-γ exposure. The expression of the *BAX, BAD,* and *TNF-α* genes was measured by qRT-PCR and normalized with *GAPDH* gene expression in NCI-H23 cells. Mean ± SEM. (n=3) (*; $p \leq 0.05$).

We assessed the gene expression levels of *BAX, BAD*, and *TNF-α* following treatment with IFN-γ (100 ng/ml). Our experimental findings reveal a substantial five-fold upregulation in the expression of the *BAX* ($p \leq 0.05$) compared to untreated cells, as illustrated in Fig. 1.10. Similarly, after 10 hours of IFN-γ treatment, the *BAD* gene exhibits a significant 2.5-fold upregulation ($p \leq 0.05$). Additionally, the expression of the TNF-α gene exhibited a 2.2-fold increase post-treatment. Nevertheless, this observed elevation did not reach statistical significance when compared to the control group. The concurrent upregulation of *BAX, BAD*, and *TNF-α* gene expression suggests that elevated levels of *TRIM34* induce apoptosis. These findings imply that the overexpression of *TRIM34* induces apoptosis in lung cancer cells.

Apoptosis analysis after IFNs exposure.

On the treatment of IFNs, percent apoptosis was examined in NCI-H23 cells using FACS. Treatment was given by three different types of Interferon. FACS results were evaluated and represented in Figure 1.11. After 10 hours of exposure to IFN-Alpha (100 IU/ml), IFN-Gamma (100 ng/ml), and IFN-Lambda (100 ng/ml), the apoptotic percentage of NCI-H23 cells was calculated. Among all IFNs, IFN-γ was effective inducing apoptosis in NCI-H23 cells. In comparison to the untreated cells, percent apoptosis was observed to increase in NCI-H23 cells after IFN-γ treatment. These findings indicate that IFN-Gamma treatment with overexpression of TRIM34 had a more pronounced effect in inducing apoptosis in NCI-H23 cells.

Cell Cycle Analysis Using FACS.

The results of the cell cycle analysis demonstrate the impact of various Interferon treatments on the distribution of cells in different phases of the cell cycle. The NCI-H23 cells, in a control group (Fig. 1.12A), exhibited a cell cycle distribution of 47.5% in the G1/G0 phase, 14.6% in the S phase, and 12.1% in the G2/M phase. Upon treatment with 100 IU/ml IFN-Alpha (Fig. 1.12B), the cell cycle distribution in NCI-H23 cells exhibited 48.2% of cells in the G1/G0 phase, 17% in the S phase, and 11.4% in the G2/M phase. Similarly, treatment with 100 ng/ml IFN-Gamma (Fig. 1.12C) led to a cell cycle distribution of 45.3% in the G1/G0 phase, 16.3% in the S phase, and 15.4% in the G2/M phase for NCI-H23 cells. Furthermore, treatment with 100 ng/ml IFN-Lambda (Fig. 1.12D) resulted in a cell cycle distribution of 42.8% in the G1/G0 phase, 16.5% in the S phase, and 13.9% in the G2/M phase for NCI-H23 cells. It is observed that after a treatment duration of 10 hours, no significant changes in the cell cycle phases of NCI-H23 cells.

Scratch Assay to analyze cell migration of NCI-H23 cells.

The scratch assay was performed to assess the effect of IFN-γ (100 ng/ml) treatment with overexpression of TRIM34 on cell migration (Fig. 1.13). The results showed that the rate of cell closure was lower in the treatment group compared to the control group (Fig. 1.13A-B). After 24 hours, the percentage of closure was 38% in the treatment group, whereas it was 55% in the control group (Fig. 1.13C). Furthermore, after 48 hours, the closure rate was 63% in the treatment group, significantly low in comparison to untreated cells (95% closure rate) (Fig. 1.13C). These findings suggest that IFN-γ treatment inhibits the migration of cells, leading to a slower closure of the scratch area. This result suggested that IFN-γ treatment induces overexpression of TRIM34 which reduces the cell proliferation and migration.

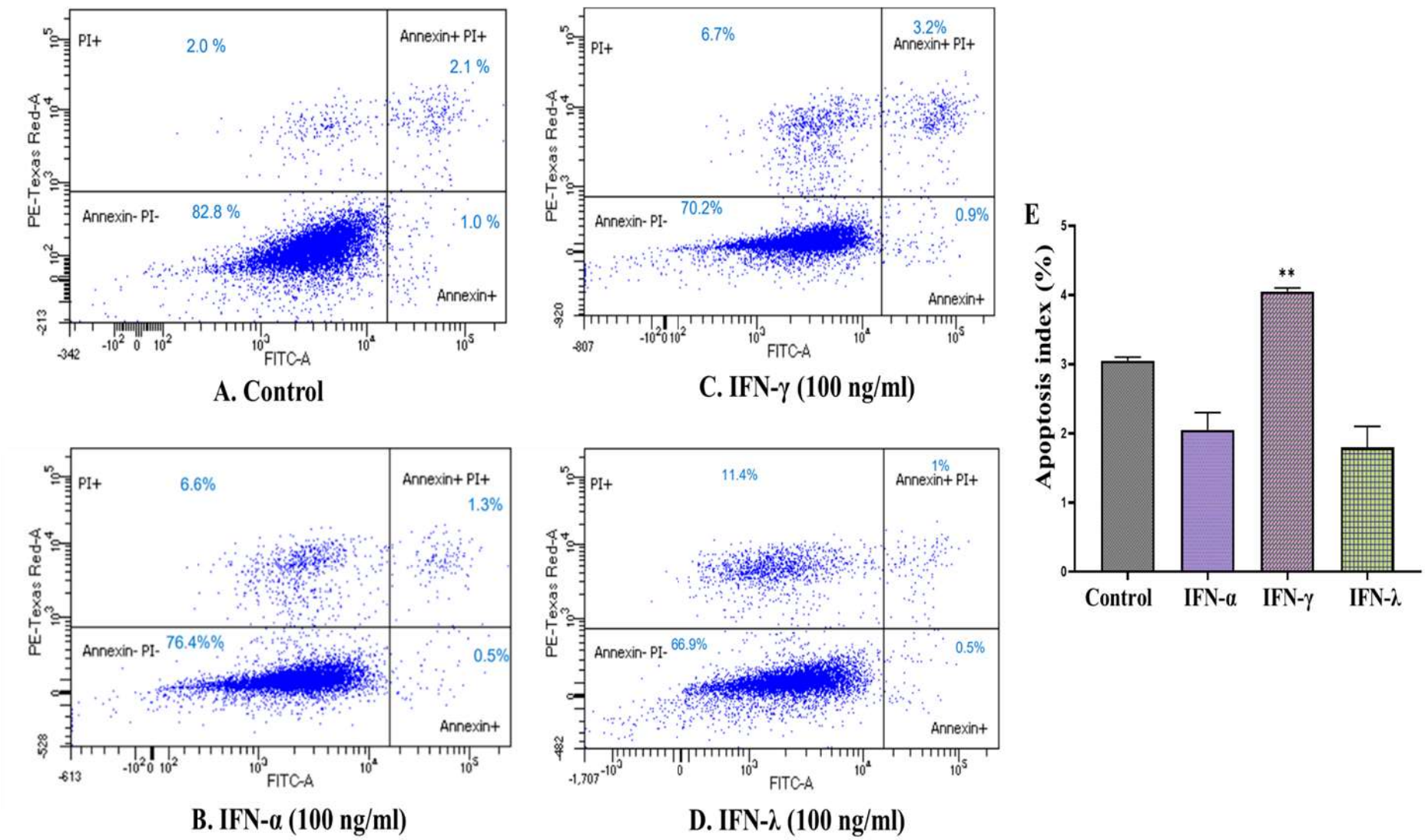

Figure 1.11: Cell death analysis using FACS on IFN treatment for 10 hours to NCI-H23 Cells. **A.** Untreated, **B.** Interferon Alpha (100 IU/ml), **C.** Interferon Gamma (100 ng/ml), and **D.** Interferon Lambda (100 ng/ml) treatment to NCI-H23 Cells. Percent apoptosis measured using **E.** Apoptosis index percentage. Mean ± SEM. (n=3). * Stand for $p \leq 0.05$ to indicate statistical significance.

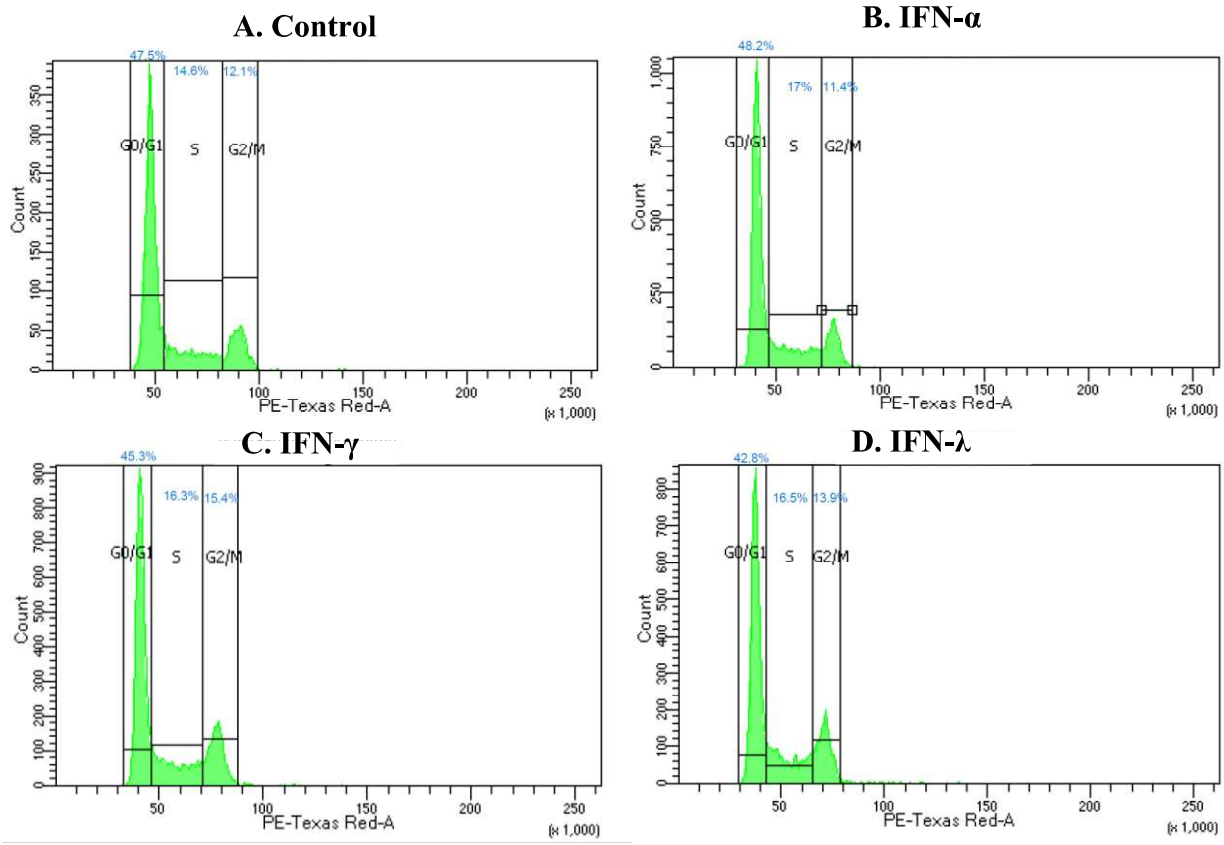

Figure 1.12: Cell cycle analysis by using FACS in NCI-H23 cells after the interferon treatment targeting through upregulating TRIM34. Quantification of the cell cycle in **A.** Untreated, **B.** Interferon Alpha (100 IU/ml), **C**. Interferon Gamma (100 ng/ml), and **D.** Interferon Lambda (100 ng/ml) treatment to NCI-H23 Cells.

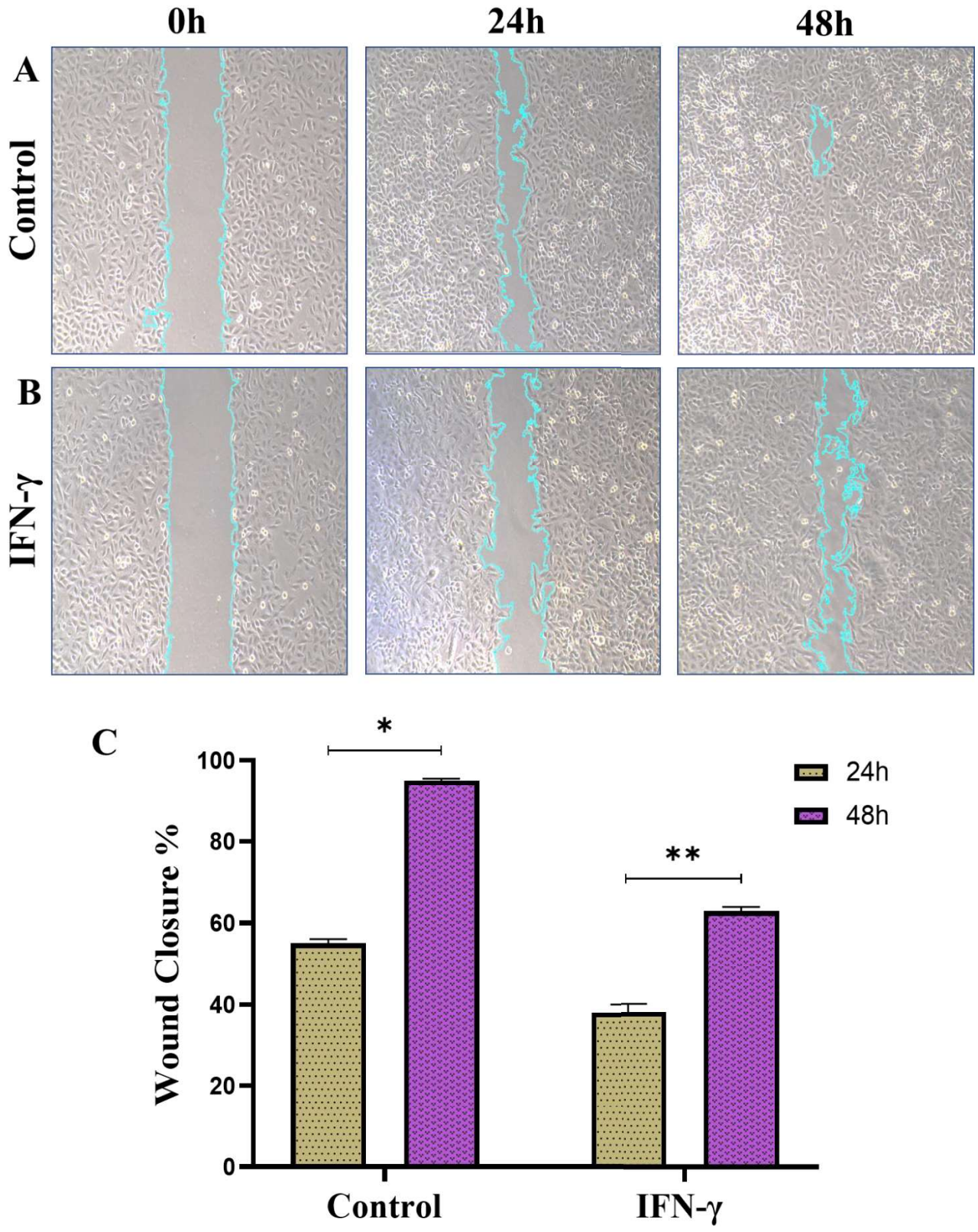

Figure 1.13: Scratch assay to analyze cell migration after Interferon-gamma treatment of 24 and 48 hours. **A.** Untreated, **B**. Interferon Gamma (100 ng/ml) treatment to NCI-H23 Cells. **C.** Percent wound closure measured by ImageJ software. * and ** stands for $p \leq 0.05$ and $p \leq 0.01$ to indicate statistical significance respectively.

DISCUSSION

Lung cancer is the most often diagnosed type and a major cause of cancer deaths worldwide (18.4% of all cancer fatalities) [1, 2]. Smoking is responsible for causing more than 80% of deaths related to lung cancer. Other factors that increase the risk of developing lung cancer include exposure to radon and asbestos, prolonged and repeated exposure to air pollution containing polycyclic aromatic hydrocarbon (PAH) emissions, as well as a personal or family history of lung cancer [3, 4]. The World Health Organization (WHO) recognizes two main types of lung cancer: non-small cell lung cancer (NSCLC), and small cell lung cancer (SCLC) [5,6,7]. Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma are other subtypes of NSCLC. Many categories can be subcategorized under each class depending on the molecular targetable genetic profile [8]. It turns out that the 5-year survival rate for both NSCLC and SCLC kinds of metastatic lung cancer is only approximately 4% [9, 10].

Numerous treatment options are available for individuals suffering from advanced-stage lung cancer. These therapies range from single-agent immunotherapy to quadruple therapy, which combines immunotherapy with chemotherapy and anti-VEGF medications[253]. To manage advanced lung cancer, the FDA has authorized immunotherapy drugs for use as standalone treatments or in conjunction with existing immunotherapies and chemotherapy[254]. Recent years have seen remarkable advancements in cancer immunotherapy. IFNs are the first human immunotherapeutic for cancer to be licensed by the US FDA[206]. Interferons (IFNs) are chemical messengers that make the nearby normal cells resistant to an infection of a similar sort. Because they contain anti-tumor action and immunomodulatory properties, interferons are a great alternative for treating cancer[255]. Tripartite motif (TRIM) proteins operate as an E3 ubiquitin ligase in the ubiquitin-proteosome system and some can be upregulated by interferon [256,257]. The TRIM expression has been implicated in the regulation of cell processes, including apoptosis [257].

One interesting prospect in the present research is to find the potential role of the *TRIM34* gene, which is known to have tumor suppressant activity in colon cancer[218]. Investigators have previously reported overexpression of TRIM proteins such as TRIM5α[258], TRIM21[259], TRIM22[260] in response to IFN-γ. Human TRIM34 is a known paralog of TRIM5α, and they share approximately 57% amino acid identity[214,261]. We thus made a speculation that Interferon can induce TRIM34 expression as well, in the lung adenocarcinoma cells. We used different Interferons in varied dosages and at various times to enhance the expression of *TRIM34*. IFN treatment was able to significantly upregulate TRIM34 expression in the lung adenocarcinoma cells. For in vitro studies, our observations have revealed that all three types of interferons (IFN-α, IFN-γ, and IFN-λ) can upregulate TRIM34. NCI-H23 cells exhibit sensitivity to interferon signaling, particularly IFN-γ, leading to increased expression of Major Histocompatibility Complex (MHC) Class I molecules and initiation of an immune response[252]. The study prompted further research on these cells, indicating a focus on

understanding their immunological implications and potential in disease contexts. A concentration of 100 ng/ml after 10 hours of exposure of IFN-γ, results in a sustained high expression of TRIM34 in NCI-H23 cells. Other investigators have also used 100 ng/ml IFN-γ dose to study various phenomena in NSCLC cells [262] and in human gastric cancer cell lines [263].

The role of Interferon (IFN) in regulating cancer immunity has been extensively studied, but the precise cellular and molecular effects of IFN-γ induced TRIM34 in cancer are not known [227]. Apoptosis, or programmed cell death, is a fundamental mechanism for eliminating damaged or abnormal cells, and its dysregulation can contribute to cancer development and progression. In non-small cell lung cancer (NSCLC), high doses of IFN-γ were shown to activate the JAK1-STAT1-caspase pathway, resulting in apoptosis. However, low doses of IFN-γ induced cancer stemness characteristics through the intercellular adhesion molecule-1 (ICAM1)-PI3K-Akt-Notch1 axis [262].

IFN-γ can induce apoptosis in lung cancer through various mechanisms. Yin *et al.* (2019) have shown the formation of RNase L homodimers, which can cleave ribosomal subunits and generate rRNA segments that trigger mitochondrial death. Furthermore, our gene expression analysis is supported by previously done studies, wherein IFN-γ was found to induce the production of Bak homodimers and Bax-Bak heterodimers in the mitochondria, which ultimately can lead to apoptosis through the activation of caspase-9, caspase-3, and PARP cascades in lung cancer [264]. In addition to its broad impact on tumor cells, IFN-γ has been found to induce apoptosis in lung adenocarcinoma cells through the overexpression of TRIM34. TRIM34 is a protein that is upregulated in response to IFN-γ stimulation and has been implicated in apoptosis induction.

To assess apoptosis, the exposure of phosphatidylserine on the outer leaflet of the plasma membrane was examined, as this modification occurs in apoptotic cells [265]. Annexin V and propidium iodide assays by FACS are used to detect phosphatidylserine exposure and distinguish between live, apoptotic, and necrotic cells. The results of the apoptosis assay showed increased phosphatidylserine exposure on the outer leaflet of the plasma membrane in the IFN-γ induced overexpressed TRIM34 groups, indicating enhanced apoptosis induction. The overexpression of TRIM34 for 10 hours was shown to stimulate the expression of key apoptotic genes, namely BAD and BAX. BAD and BAX are well-known regulators of apoptosis and are crucial for the initiation of the apoptotic process. BAD promotes apoptosis by binding and inhibiting anti-apoptotic proteins, allowing pro-apoptotic proteins to carry out their function. BAX, on the other hand, acts as a pro-apoptotic protein and triggers the release of cytochrome c from mitochondria, leading to the activation of caspases and subsequent cell death. The findings suggest that overexpression of TRIM34 in lung adenocarcinoma cells can initiate apoptosis by upregulating the expression of BAD and BAX (Fig. 1.10). Our findings are consistent with Song. M. *et al.* (2019) who have demonstrated that 100 ng/ml of IFN-γ is highly effective in promoting apoptosis and cancer regression in lung cancer cells [262]. Furthermore, our observation

indicates that IFN-γ stimulates TNF-α expression, but post-treatment TNF-α levels did not reach statistical significance compared to the control group. This observation was also supported by Vila-del Sol V *et al.* (2008) in murine macrophage cells [266]. In addition, Boutsikou E *et al.* (2018) showed that TNF-α upregulation can improve immunotherapy and longer survival of NSCLC patients [267].

Fang *et al.* (2021) investigated the impact of IFN-γ on lung cancer cells and found that it activates the JAK1/2-STAT1 and AKT-mTOR signaling pathways, leading to the endoplasmic reticulum (ER) stress and unfolded protein response (UPR). Their study also demonstrated that IFN-γ induced ER stress is associated with cell cycle arrest and apoptotic cell death [268]. As the role of TRIM34 in ER stress-induced cell death is still not known, it can be speculated that TRIM34 overexpression in response to IFN-γ mediates cell death through a similar mechanism. This assumption is based on the fact that other TRIMs, such as TRIM13, are known to induce cell death by regulating ER stress [269]. Although ample data on the anti-viral role of TRIM34 is available, data on other biological functions of TRIM34 is scarce which warrants further research. Besides, little is currently known about the subcellular localization of TRIM34 [270].

Extracellular Trap cell death (ETosis) is a distinctive cell death pathway in which extracellular traps (ETs) are generated by neutrophils and mast cells as part of innate immunity by trapping microbes using chromatin-DNA, antimicrobial agents, and enzymes. Stimulation triggers reactive oxygen species (ROS) production, leading to ET formation [271]. IFN-γ triggers an ETosis in lung cancer cells that exhibit apoptotic and nonapoptotic responses. Surprisingly, nonapoptotic cells undergo ETosis, controlled by caspase-3 and autophagy activated by GTPase protein M1 and transcription factor 6. IFN-γ influences ETosis through autophagy and Fas-associated protein with death domain-mediated caspase-8/-3 activation. This triggers caspase-mediated lamin degradation, followed by DNA damage-induced ATR/ATM-regulated ETosis in lung cancer cells [272].

Additionally, our study delved into the critical aspect of cell migration, which is central to cancer metastasis. Lung adenocarcinoma is characterized by its aggressive nature and high metastatic potential[273]. Intriguingly, scratch assay results demonstrated that IFN-γ had a profound inhibitory impact on the migration ability of these cancer cells. This finding is particularly noteworthy, as it contrasts with some previous research reporting IFN-γ-induced epithelial-mesenchymal transition (EMT), which is associated with increased migratory capacity[274]. However, it should be noted that the research group used a lower dose of IFN-γ (100 IU/ml). It is interesting to note that in NSCLC, high doses (≥100 ng/ml) of IFN-γ enhance cancer regression but low doses (≤0.2 ng/ml) of IFN-γ enhance the metastatic character of these cancer cells[262]. TRIM34, as previously discussed, is a protein that is upregulated in response to IFN-γ stimulation and has been implicated in apoptosis induction. The results of these studies indicate that IFN-γ can significantly attenuate the migrating ability of lung adenocarcinoma cells (Fig. 1.13). Understanding the molecular mechanisms underlying

the impact of TRIM34 overexpression on cell migration in lung adenocarcinoma is crucial for gaining insights into the regulation of tumor metastasis and potentially identifying new targets for therapeutic intervention.

In the present study, we demonstrate that IFN-γ induces an increase in TRIM34 expression in human non-small cell lung cancer (NSCLC) cells. While our findings suggest a potential association between IFN-γ stimulation and TRIM34-mediated apoptosis, further investigation is required to establish a direct causal relationship. The implications of our study potentially offer novel therapeutic strategies for lung adenocarcinoma. The link between TRIM34 and IFN-γ underscores the significance of interferon-based immunotherapy in lung cancer, where the activation of TRIM34 can be harnessed to induce apoptotic pathways while simultaneously inhibiting the metastatic potential of cancer cells. Understanding the cellular and molecular mechanisms underlying the effects of IFN-γ in tumor cells, such as its ability to promote TRIM34-dependent apoptosis, is crucial for developing targeted and effective immunotherapy strategies. However, further studies are necessary to validate and deduce the mechanism for our observed in vitro phenomena.