Original Article

Cytokine-driven modulation of WT1 and IL-10 in lung cancer progression

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Background: Lung cancer is driven by complex interactions between oncogenes and the inflammatory microenvironment. In particular, the cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), and their modulation of key regulators like Wilms' tumor 1 (WT1) and interleukin-10 (IL-10) remain underexplored. This study aims to investigate the role of these cytokines in WT1 and IL-10 regulation during lung cancer progression.

Methods: A total of 982 lung cancer patient samples from The Human Protein Atlas were analyzed. *In vitro*, RAW264.7 macrophages were transfected with a WT1 plasmid (pWT1) and treated with TNF-α, IL-1β, and lipopolysaccharide (LPS). WT1 and IL-10 expression was evaluated in A549, B16-F10, and J774.2 cell lines using reverse transcription polymerase chain reaction (RT-PCR), Western blotting, and enzyme-linked immunosorbent assay (ELISA). Immunofluorescence was employed to assess WT1 localization and phosphorylation, while immunohistochemistry was used to evaluate the correlation between WT1 and IL-10 in patient samples.

Results: WT1 expression progressively increased from stage I to IV lung cancer and positively correlated with IL-10 in stages II and IV. WT1 overexpression in RAW264.7 cells treated with LPS led to a 12.9-fold increase in IL-10 expression. Proinflammatory cytokines decreased WT1 in A549 and B16-F10 cells but increased it in J774.2 macrophages, leading to cytoplasmic localization and phosphorylation. Patient sample analysis revealed a positive correlation between WT1 and IL-10 in advanced stages.

Conclusions: These findings suggest that WT1 and IL-10 are modulated by inflammatory cytokines in a stage-dependent manner in lung cancer. WT1 upregulation is associated with increased IL-10 expression, particularly in advanced stages, highlighting potential therapeutic targets for modulating the immune response in lung cancer.

Keywords: Lung cancer; tumor necrosis factor-alpha (TNF-α); interleukin-1 beta (IL-1β); Wilms' tumor 1 (WT1); interleukin-10 (IL-10)

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Introduction

Background

Lung cancer is the leading cause of cancer death worldwide, accounting for 18.7% of all cancer deaths, with 1.8 million deaths, and is characterized by its aggressiveness and poor prognosis (1,2). Understanding the molecular mechanisms involved in the tumorigenesis of lung cancer is essential for the development of new effective therapeutic strategies (3). Multiple factors are involved in lung carcinogenesis, among which proinflammatory cytokines and oncogenes stand out (4,5).

Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) are known for their involvement in tumor development and metastasis by activating signaling pathways that promote cytoskeletal remodeling, extracellular matrix degradation, and cell adhesion (6-9). These cytokines can modulate the tumor microenvironment by recruiting and activating immune system cells, such as tumor-associated macrophages, which secrete growth factors and proteases that favor tumor cell survival and invasion (10-12). There are reports indicating the involvement of proinflammatory cytokines in the activation of vascular endothelial growth factor (VEGF), which promotes angiogenesis (13). They are also associated with immune evasion through the induction of immune checkpoint expression to inactivate T cells (14).

In lung carcinogenesis, proteins that function as tumor suppressors or oncogenes have been described. The Wilms' tumor 1 (WT1) gene was initially identified as a tumor suppressor gene in Wilms' tumor (15). However, its role as an oncogene has been characterized in various types of cancer, including lung cancer (16-18). Its function as an oncogene or tumor suppressor gene is context dependent and influenced by its interaction with other transcription factors. WT1 has been shown to induce the synthesis of various genes involved in processes such as angiogenesis, apoptosis, and metastasis in cancer (19-22).

WT1 can induce the synthesis of interleukin-10 (IL-10), an anti-inflammatory cytokine that plays an immunomodulatory role in the immune response, contributing to homeostasis by preventing the overexpression of proinflammatory cytokines and avoiding chronic inflammatory processes. In cancer, IL-10 has been shown to participate in immune evasion by tumors, contributing to the tumor microenvironment in processes such as immunosuppression (23-25).

Rationale and knowledge gap

In lung cancer, the relationship between inflammatory cytokines and WT1 is not fully understood.

Objective

Therefore, this study aims to elucidate the molecular mechanisms of proinflammatory cytokines (TNF-α and IL-1β) in the modulation of WT1 expression and localization and their impact on IL-10 modulation. Additionally, this study sought to evaluate the expression patterns and interactions between TNF-α, IL-1β, WT1, and IL-10 in lung cancer patients at different stages and establish correlations between the expression of these genes and the progression of this disease. We present this article

Highlight box

Key findings

- Therapeutic potential of targeting Wilms' tumor 1 (WT1) in lung cancer, expression patterns of cytokines and WT1 across different stages of lung cancer, correlation analysis between WT1 and interleukin-10 (IL-10), modulation of IL-10 synthesis by WT1, impact of proinflammatory cytokines on WT1 expression.

What is known and what is new?

- The role of cytokines in cancer, WT1 in cancer is well-established as an oncogene, IL-10 is known to contribute to immune evasion.
- Correlation between WT1 and IL-10 across different stages of lung cancer, impact of WT1 on IL-10 production, stage-specific expression patterns, influence of proinflammatory cytokines on WT1 in cancer.

What is the implication, and what should change now?

- The findings suggest that WT1 could be a viable therapeutic target in lung cancer treatment. By inhibiting WT1, it may be possible to disrupt the immunosuppressive microenvironment created by the WT1-IL-10 axis, potentially enhancing the effectiveness of existing immunotherapies and improving patient outcomes. Our study emphasizes the importance of understanding the interactions between inflammatory cytokines and oncogenic factors like WT1, highlighting the need for further research into the molecular mechanisms underlying the interactions between WT1, IL-10, and inflammatory cytokines for personalized medicine approaches.
- Future research should prioritize investigating WT1's role in lung cancer and other malignancies, particularly its interactions with immune cells and cytokines, there should be an emphasis on conducting clinical trials to assess the effectiveness of WT1 inhibitors that can modulate IL-10 expression in lung cancer patients.

in accordance with the STROBE and the MDAR reporting checklists (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-2024-1242/rc).

Methods

Expression analysis of TNF-α, IL-1β, WT1, and IL-10 in lung cancer patients

A meta-analysis was conducted using publicly available data from The Human Protein Atlas to assess the expression patterns of TNF-α, IL-1β, WT1, and IL-10 in lung cancer patients. This approach provides an overview of expression trends across different tumor stages, allowing for a comprehensive understanding of their potential involvement in disease progression. The expression data for TNF-α, IL-1β, WT1, and IL-10 were obtained from The Human Protein Atlas (https://www.proteinatlas.org). A meta-analysis was conducted and included 982 samples categorized by tumor stage: 510 for stage I, 277 for stage II, 163 for stage III, and 32 for stage IV. The expression of TNF-α, IL-1β, WT1, and IL-10 was quantified as fragments per kilobase of transcript per million mapped reads (FPKM). Correlations in expression levels were identified among patients classified in each tumor stage group.

Cell culture conditions

The murine melanoma cell line B16-F10 (CRL-6475), the human lung adenocarcinoma cell line A549 (CCL-185), and the murine macrophage lines J774.2 (CVCL_0357) and RAW264.7 (TIB-71) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F-12) (GIBCO®, Thermo Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO®, Mexico) and 1% antibiotic-antimycotic mixture (penicillin, streptomycin, and amphotericin B) (Sigma, St. Louis, MO, USA). The cell lines were maintained under specific conditions: at 37 ℃, with a relative humidity of ~85%, and in an atmosphere of 95% air and 5% CO_2.

Transfection of RAW264.7 cells with the WT1 plasmid

The RAW264.7 murine macrophage cell line was selected as a model due to its lack of endogenous WT1 expression and its high sensitivity to inflammatory stimuli. This characteristic makes it a suitable system for evaluating the effects of WT1 overexpression on IL-10 modulation in response to proinflammatory cytokines. RAW264.7 cells were transfected with a WT1 plasmid (pWT1) via polyethylenimine (PEI) as the transfection agent in the following quantities: 200 µL of 150 mM NaCl with 4 µL of PEI and 200 µL of 150 mM NaCl with 4 µg of pWT1. PEI solution was added to the pWT1 solution and incubated for 20 minutes before being added to the cells for transfection. After 48 hours, the transfected cells were selected with neomycin, resulting in a stable RAW264.7-pWT1 cell line.

RNA extraction and reverse transcription PCR

Total RNA was isolated from each cell line via the use of 1 mL of TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined by measuring the absorbance at 260/280 nm. Complementary DNA (cDNA) was synthesized from 5 µg of total RNA, 200 U of Superscript III, and 0.5 µg of oligo dT under the following conditions: 42 ℃ for 90 min and 70 ℃ for 10 min. The resulting cDNA was amplified via Taq DNA polymerase (Life Technologies) and the following primers: *WT1* forward: 5'-AACGCCCCTTCATGTGTGC-3' and reverse: 5'-GCTGGTCTGAACGAGAAAACCTTC-3' to amplify a 223-bp fragment; B-cell lymphoma 2 (*Bcl-2*) forward: 5'-TCATGTGTGTGGAGAGCGTCAA-3' and reverse: 5'-GTGTGTGTCTGTCTGTGTGTGTGA-3' to amplify a 408-bp fragment; and *IL-10* forward: 5'-TCTCCGAGATGCCTTCAGCAGA-3' and reverse: 5'-TCAGACAAGGCTTGGCAACCCA-3' to amplify a 126-bp fragment. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control, with the following primers: forward: 5'-GTGGGGCGCCCCAGGCACCA-3' and reverse: 5'-GTGGGGCGCCCCAGGCACCA-3', to generate a 425-bp product. The PCR products were analyzed via 0.8% agarose gel electrophoresis and visualized via ethidium bromide staining. All experiments were performed in triplicate to ensure reproducibility.

Western blot analysis of WT1

WT1 protein expression was determined in B16-F10 and A549 tumor cells. B16-F10 cells were used as a well-

established model for metastatic lung cancer in murine systems, while A549 cells were included as a representative human lung adenocarcinoma model. The use of B16-F10 cells also provides a foundation for potential *in vivo* studies in murine lung cancer models, ensuring translational relevance of the findings. The cells were cultured at a density of 1×10^5 cells/well in 6-well plates with 2 mL of DMEM/F-12 medium supplemented with 10% FBS and incubated overnight. Treatments with TNF-α (10 ng/mL), IL-1β (20 ng/mL), and LPS (100 ng/mL) were applied for 6 hours, after which the cells were collected. The cells were lysed in 100 µL of lysis buffer (1% Triton, 150 mM NaCl, and 25 mM Tris pH 7.6), and the protein concentrations were measured via a DC protein assay kit (Bio-Rad, Hercules, CA, USA). 50 µg of protein from each sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and immunodetected with antibody against WT1 (Santa Cruz Biotechnology Inc., Cat#SC-7385) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Antibody against GAPDH (Sigma-Aldrich cat#G8795) (Sigma-Aldrich Corp., St. Louis, MO, USA), was used for detection and normalization to the GAPDH protein. Proteins were visualized via the Western Lumi-Light System (Roche, Pleasanton, CA, USA) and all experiments were performed in triplicate.

Quantification of IL-10 by ELISA

IL-10 expression levels were quantified in tumor and macrophage lines via an ELISA kit. 200 µL of culture medium from each cell line (A549, B16-F10, and J774.2) treated with TNF-α (10 ng/mL), IL-1β (20 ng/mL), or LPS (100 ng/mL) for 6, 12, or 24 hours was used, all experiments were performed in triplicate to ensure reproducibility, following the manufacturer's protocol (PeproTech, Mexico City, Mexico).

Immunofluorescence assay for WT1 localization

WT1 localization was evaluated in A549, B16-F10, and J774.2 cells treated with TNF-α (10 ng/mL), IL-1β (20 ng/mL), and LPS (100 ng/mL) for 6 hours. The cells were fixed with methanol:acetone in a 1:1 ratio at –20 ℃, washed with phosphate-buffered saline (PBS) and permeabilized with permeabilization buffer (0.1% Triton with 1% sodium citrate in PBS), followed by three washes with PBS. The cells were blocked with 20% FBS in PBS for 30 minutes and incubated for 1 hour with antibody against WT1 (Santa Cruz Biotechnology Inc., Cat#SC-7385) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), antibody against phosphorylated WT1 at serine 363 (WT1-p363) (Santa Cruz Biotechnology Inc., Cat#SC-12933, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and antibody against phosphorylated WT1 at serine 393 (WT1-p393) (Santa Cruz Biotechnology Inc., Cat#SC-12934, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the samples were washed with PBS, anti-IgG-FITC rabbit (Santa Cruz Biotechnology Inc., Cat#SC-2012, Santa Cruz Biotechnology) and Texas Red Anti-mouse IgG1 goat (Santa Cruz Biotechnology Inc., Cat#SC-2979, Santa Cruz Biotechnology) antibodies were applied. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The slides were mounted and examined via confocal scanning microscopy. The signal intensity was expressed in pixels and analyzed with Image-Pro-Plus software, version 7.0 (Media Cybernetics, Rockville, MD, USA). WT1, WT1-p363, and WT1-p393 localization was determined via the Pearson correlation coefficient (PCC).

Patient samples and specimen collection

Samples for this retrospective analysis were obtained in 2010 from 27 lung cancer patients at the "Luz González Cosio" Regional Hospital of Zacatecas, Mexico. The samples were donated by Luz González Cosio Regional Hospital in 2010. The retrospective analysis was conducted under the ethics approval of Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas (UANL FCB; No. CEIBA-FCB-35/2022), where the experimental procedures were carried out. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. Individual consent for this retrospective analysis was waived. The sample size was determined by the availability of lung cancer patients diagnosed. Samples were categorized by tumor stage: stage I, stage II, stage III and stage IV to assess the relationship between WT1 and IL-10 modulation in lung cancer samples.

Immunohistochemistry for WT1 and IL-10 expression

WT1 and IL-10 expression in patient samples was determined via SC-7385 and SC-8438 antibodies, respectively (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Samples were anonymized and the sections were deparaffinized and permeabilized. Immunohistochemistry

was performed via a Universal Quick Kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. The samples were analyzed with a light microscope (Primo Star, Carl Zeiss, GmbH, Germany). All experiments were performed in triplicate to ensure reproducibility. The results obtained from patient samples provide a reference to compare *in vitro* WT1 and IL-10 expression patterns under experimental conditions.

Statistical analysis

Data from the Human Protein Atlas were analyzed via GraphPad Prism 10 (version 10.3; GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) and Tukey's post hoc test were used to compare expression levels across different stages. The correlations between TNF-α, IL-1β, WT1, and IL-10 expression in lung cancer patients were determined via the PCC, where values close to 1 indicate a positive correlation, –1 indicates a negative correlation, and values near 0 indicate no correlation. Bonferroni correction was applied to control for type I error risk. Changes in WT1 and IL-10 expression in cell lines treated with TNF-α, IL-1β, and LPS were analyzed via ANOVA and Tukey's post hoc test, with $P<0.05$ considered significant. WT1 localization in A549, B16-F10, and J774.2 cell lines treated with TNF-α, IL-1β, and LPS was calculated via the PCC, with nuclear localization values between 1 and 0.8, perinuclear values between 0.79 and 0.6, and cytoplasmic values less than or equal to 0.59.

Results

Differential expression of TNF-α, IL-1β, WT1, and IL-10 and their correlation in patients with different stages of lung cancer

To determine the relationships of the proinflammatory cytokines TNF-α and IL-1β with WT1 and IL-10 in lung cancer patients, data from 982 samples from the Human Protein Atlas categorized according to lung cancer stage were analyzed.

Figure 1A,1B show a trend toward decreased expression of TNF-α and IL-1β, whereas WT1 maintained high expression in stage IV patients (*Figure 1C*). Similarly, IL-10 expression was lower in stages II and III than in stage I but increased again in stage IV (*Figure 1D*). Pearson correlation analysis revealed a positive correlation between WT1 and IL-10 in stages II and IV and a negative correlation between WT1 and the proinflammatory cytokines TNF-α and IL-1β in stage IV (*Figure 1E*).

Modulation of IL-10 synthesis by WT1 in RAW264.7 macrophages

The RAW264.7 macrophage line does not express detectable levels of *WT1*, so the cells were transfected with a pWT1 plasmid to induce *WT1* expression and evaluate *IL-10* modulation. The results in pWT1-transfected RAW264.7 cells treated with TNF-α, IL-1β, and LPS as controls indicated modulation of WT1 function. Basal *IL-10* expression in RAW264.7 cells did not significantly increase when the cells were stimulated with TNF-α, IL-1β, or LPS, whereas in pWT1-transfected cells, a 2.6-fold increase in *IL-10* synthesis was induced. However, upon the addition of LPS, *IL-10* synthesis increased up to 12.9-fold (*Figure 2A*). The transcription factor capability of WT1 was analyzed by measuring the expression of *Bcl-2*, a gene modulated by WT1 (*Figure 2B*). The results in transfected RAW264.7 macrophages revealed high *Bcl-2* expression, similar to that observed in the MCF-7 cell line and lower than that in the 4T1 and B16-F10 cell lines (*Figure 2C*).

TNF-α and IL-1β modulate WT1 expression in tumor cells and macrophages

The effects of proinflammatory cytokines on *WT1* modulation were evaluated in tumor cells (A549 and B16-F10) and J774.2 macrophages cultured in the presence of TNF-α, IL-1β, and LPS, and the expression of these cytokines was quantified via RT-PCR and Western blotting.

The results indicate that various treatments (TNF-α, IL-1β, and LPS) significantly affect *WT1* gene expression in A549 and B16-F10 cells. In A549 cells, *WT1* expression decreased gradually over time and stabilized after 24 hours (*Figure 3A-3C*), whereas in B16-F10 cells, *WT1* expression decreased more markedly and continuously, especially with TNF-α expression at 6 hours, as shown by RT-PCR (*Figure 3D*) and Western blotting (*Figure 3E,3F*).

In contrast, in J774.2 macrophages, exposure to proinflammatory cytokines increased *WT1* expression, with TNF-α having the greatest effect, increasing *WT1* expression up to 7.8 times after 6 hours of exposure (*Figure 4A,4B*). This effect contrasts with the results observed in tumor cells.

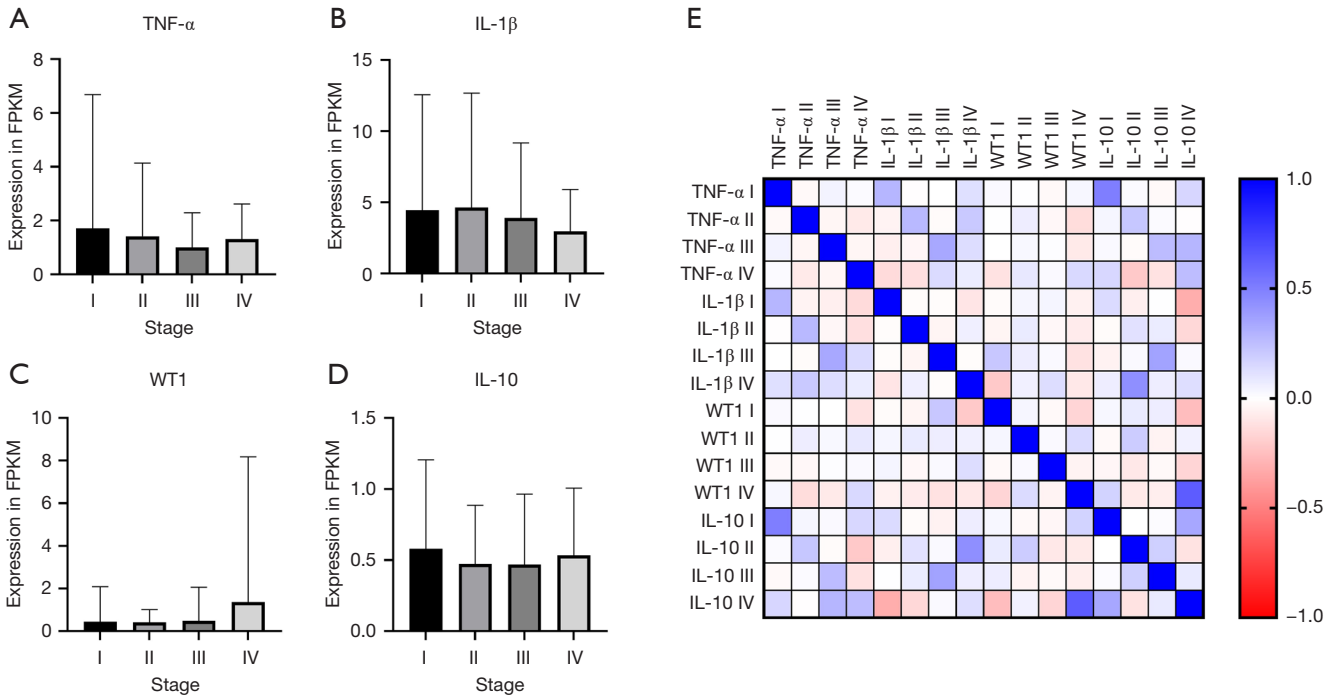


Figure 1 Gene expression and correlation of TNF-α, IL-1β, WT1, and IL-10 in lung cancer patients based on Human Protein Atlas. (A) Expression of TNF-α (data available from https://www.proteinatlas.org/ENSG00000232810-TNF/cancer/lung+cancer#IHC), (B) IL-1β (data available from https://www.proteinatlas.org/ENSG00000125538-IL1B/cancer/lung+cancer), (C) WT1 (data available from https://www.proteinatlas.org/ENSG00000184937-WT1/cancer/lung+cancer#IHC), and (D) IL-10 in FPKM from lung cancer patients at stages I, II, III, and IV (data available from https://www.proteinatlas.org/ENSG00000136634-IL10/cancer/lung+cancer#IHC). (E) Pearson correlation analysis showing a positive correlation for WT1 and TNF-α in stages II and IV, a positive correlation between WT1 and IL-1β in stage II but a negative correlation in stage IV, and a positive correlation between WT1 and IL-10 in stages II and IV. Statistical analyses were performed via ANOVA and Tukey's test at $P<0.05$, followed by Pearson's test. The bar on the far right indicates Pearson correlation values, with a gradient ranging from blue (strong positive correlation) to red (strong negative correlation), representing the degree of association between gene expression profiles. ANOVA, analysis of variance; FPKM, fragments per kilobase of transcript per million mapped reads; IL-1β, interleukin-1 beta; IL-10, interleukin-10; TNF-α, tumor necrosis factor alpha; WT1, Wilms' tumor 1.

Effects of proinflammatory cytokines on IL-10 synthesis in A549, B16-F10, and J774.2 macrophages

The effects of TNF-α and IL-1β on IL-10 expression in tumor cells and macrophages were analyzed by measuring IL-10 synthesis via ELISA. In the A549 cell line, treatment with TNF-α resulted in decreased IL-10 expression at 12 and 24 hours posttreatment, whereas IL-1β and LPS did not affect IL-10 expression (*Figure 5A*). In the B16-F10 cell line treated with TNF-α, IL-1β and LPS a significant reduction at 24 hours was observed (*Figure 5B*). In J774.2 macrophages treated with LPS, a significant increase in IL-10 expression was observed at 12 hours of exposure; however, at 24 hours, only TNF-α maintained an increase in IL-10 synthesis (*Figure 5C*).

Subcellular localization of WT1 in tumor cells and macrophages after exposure to proinflammatory cytokines

To determine whether the decrease in WT1 biological activity depends exclusively on expression or localization, an immunofluorescence assay was performed on A549 and B16-F10 tumor cells and J774.2 macrophages treated with TNF-α, IL-1β, and LPS.

In A549 cells, WT1 was localized primarily in the nuclear (43.6%) and perinuclear regions (40.3%). However, after treatment with TNF-α and IL-1β, WT1 shifted to the cytoplasm (63.6% and 52.1%, respectively) (*Figure 6A*). In the B16-F10 and J774.2 cell lines, WT1 was predominantly localized in the nucleus (61.7% and 80.9%, respectively), but after treatment, a shift toward the cytoplasmic region

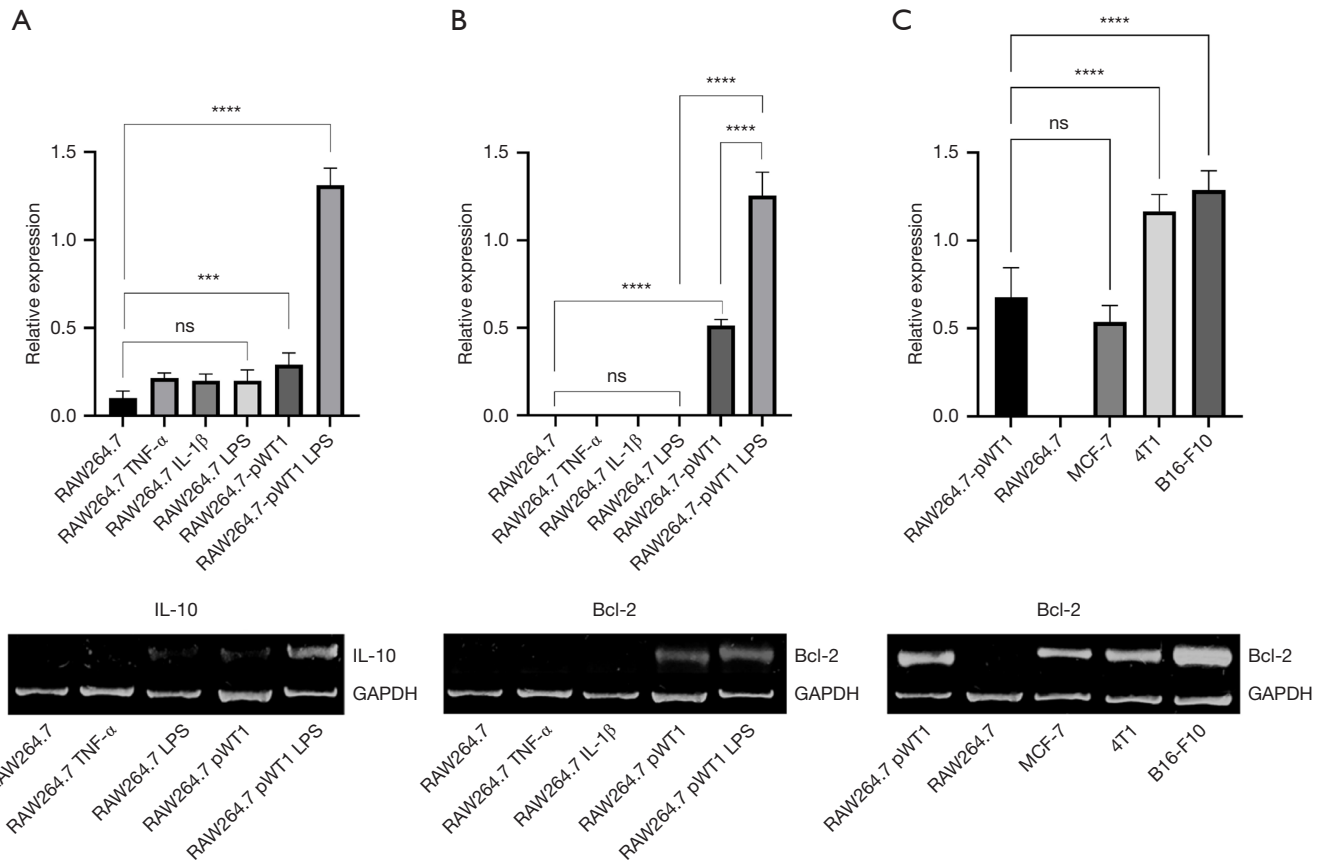


Figure 2 Expression analysis of Bcl-2 and IL-10 in RAW264.7 cells transfected with the WT1 expression plasmid. (A) Basal production of IL-10 is significantly increased when RAW264.7 cells express WT1, with a 12.9-fold increase observed when transfection is combined with LPS treatment. (B) Relative expression of Bcl-2 in RAW264.7 cells, with WT1 transfection causing Bcl-2 overexpression, which was further enhanced by LPS treatment. (C) Induction of Bcl-2 synthesis in RAW264.7 cells transfected for WT1 expression, with Bcl-2 levels similar to those in the MCF-7 cell line but lower than those in the 4T1 and B16-F10 cell lines. Statistical analyses were performed via ANOVA and Tukey's test (****, P<0.0001; ***, P<0.001; ns, not significant). Representative RT-PCR gel images for each condition are shown at the bottom, with GAPDH used as an endogenous control. ANOVA, analysis of variance; Bcl-2, B-cell lymphoma 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1 beta; IL-10, interleukin-10; LPS, lipopolysaccharide; pWT1, plasmid for WT1 expression; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha; WT1, Wilms' tumor 1.

was observed in both cell lines (*Figure 6B,6C*).

TNF-α and IL-1β induce WT1 phosphorylation

The immunofluorescence assay was used to analyze the relationship between phosphorylation and WT1 subcellular localization in tumor (A549 and B16-F10) and macrophage lines (J774.2) treated with TNF-α, IL-1β, and LPS. WT1, phosphorylated at Serines 363 and 393, was predominantly observed in the cytoplasmic region of the treated cells (*Figure 7A-7C*).

Clinical correlation between WT1 and IL-10 in lung cancer patients

Immunohistochemical analysis of WT1 in 27 lung cancer patients samples revealed increased WT1 expression in stages III and IV (19.66 and 45.00, respectively) compared with stages I and II (11.83 and 8.66, respectively), whereas IL-10 expression was lower in stage II than in stage I (22.00 and 29.33, respectively) but increased again in stages III and IV (31.17 and 41.17, respectively) (*Figure 8A-8C*). The PCC revealed a positive correlation between WT1 and IL-10 in stages II (0.73), III (0.37), and IV (0.48) (*Figure 8D*).

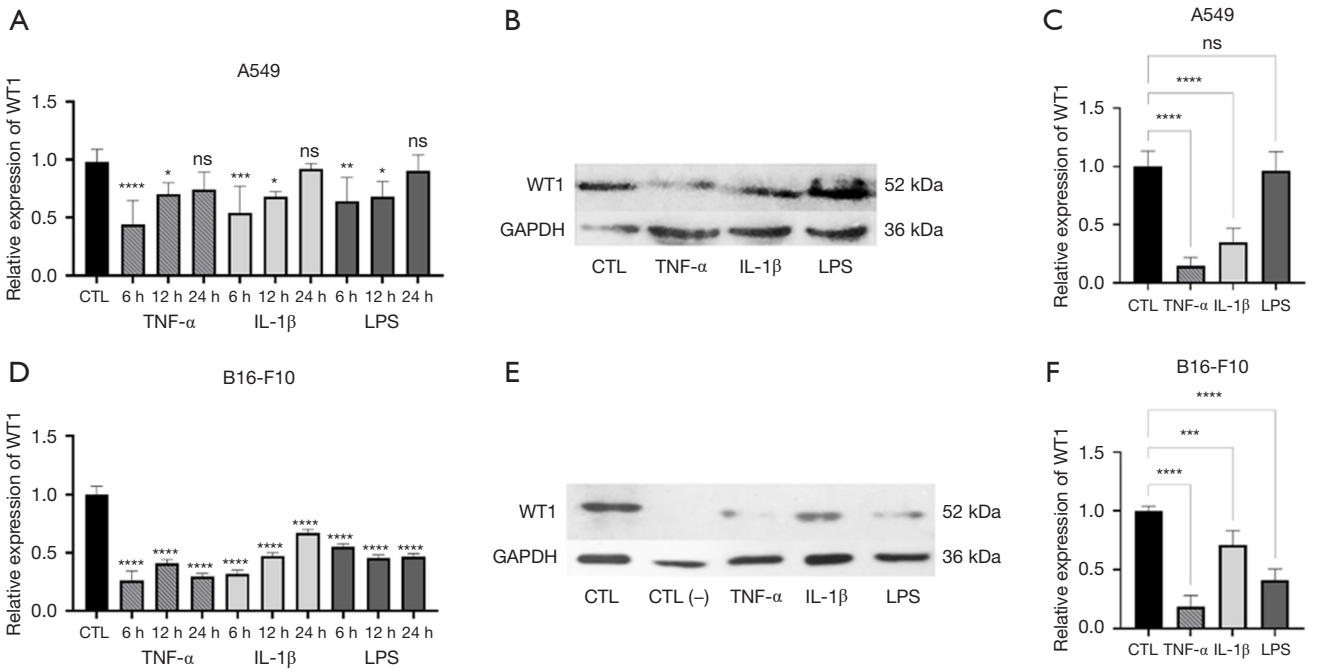


Figure 3 Modulation of WT1 expression in response to proinflammatory cytokines in tumor cells. (A) RT-PCR analysis of WT1 mRNA expression in A549 cells treated with TNF-α, IL-1β, and LPS for 6, 12, and 24 hours. (B) Western blot analysis for WT1 expression in A549 cells after 6 hours of treatment and (C) densitometric analysis. (D) RT-PCR assay of WT1 mRNA expression in B16-F10 cells after TNF-α, IL-1β, and LPS treatments for 6, 12, and 24 hours. (E) WT1 expression was analyzed by western blot, and (F) densitometric analysis was performed. GAPDH was used as an endogenous control. Statistical analyses were performed via ANOVA and Tukey's test, statistical significance is indicated as follows: ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. ANOVA, analysis of variance; CTL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor alpha; WT1, Wilms' tumor 1.

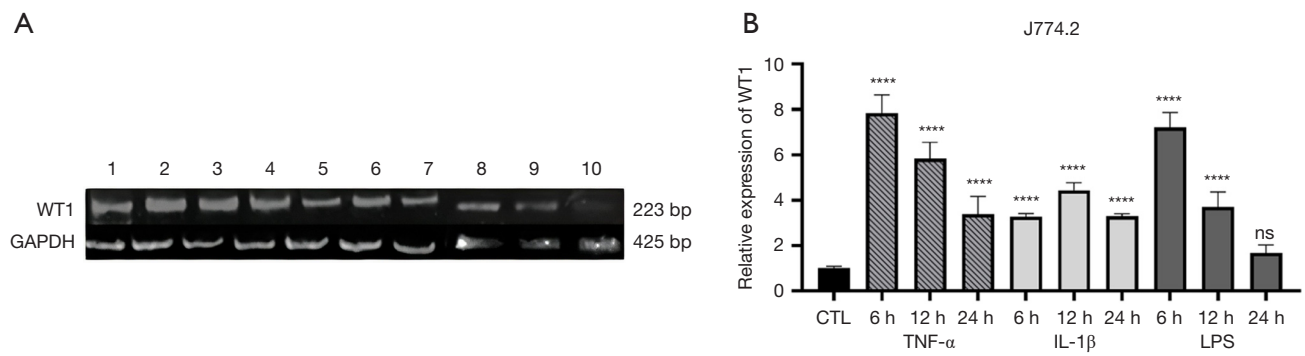


Figure 4 Modulation of WT1 synthesis in J774.2 cells. (A) RT-PCR assay for WT1 expression; lane 1 represents the untreated control, and lanes 2, 3, and 4 represent WT1 expression in J774.2 cells treated with TNF-α for 6, 12, and 24 hours, respectively. Lanes 5, 6, and 7 represent expression in cells treated with IL-1β for 6, 12, and 24 hours, respectively, and lanes 8, 9, and 10 under LPS treatment represent the inflammation control. (B) Densitometric analysis of WT1 expression in J774.2 cells, with GAPDH amplification used as an endogenous control. Statistical analyses were performed via ANOVA followed by the Tukey test (ns, not significant; ****, P<0.0001). ANOVA, analysis of variance; CTL, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha; WT1, Wilms' tumor 1.

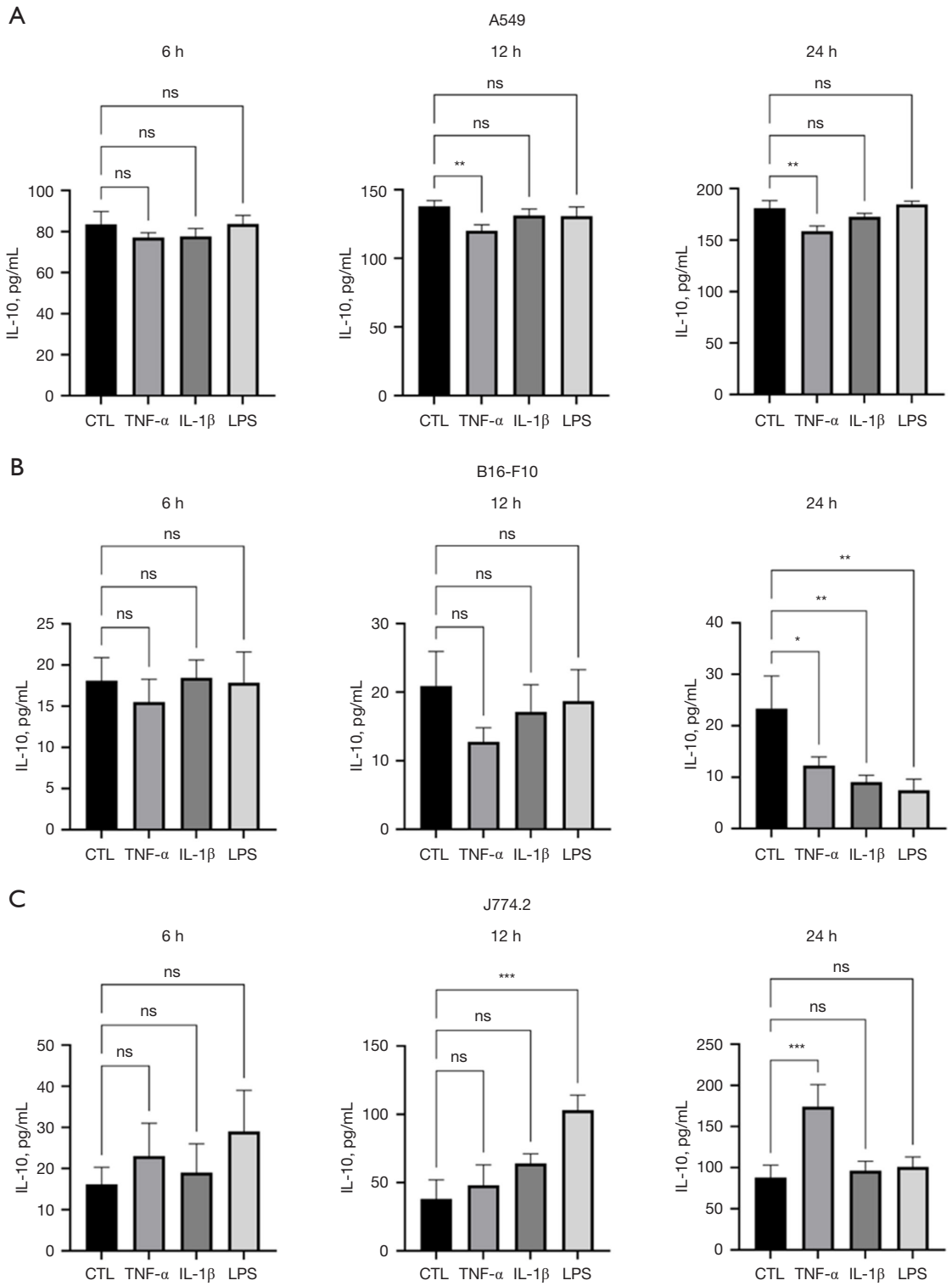


Figure 5 IL-10 expression in tumor and macrophages. IL-10 synthesis in (A) A549 cells and (B) B16-F10 and (C) J774.2 cells treated with TNF-α, IL-1β, and LPS for 6, 12, and 24 hours. Statistical analyses were performed via ANOVA and Tukey's test, statistical significance is indicated as follows: ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001. ANOVA, analysis of variance; CTL, control; IL-1β, interleukin-1 beta; IL-10, interleukin-10; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha.

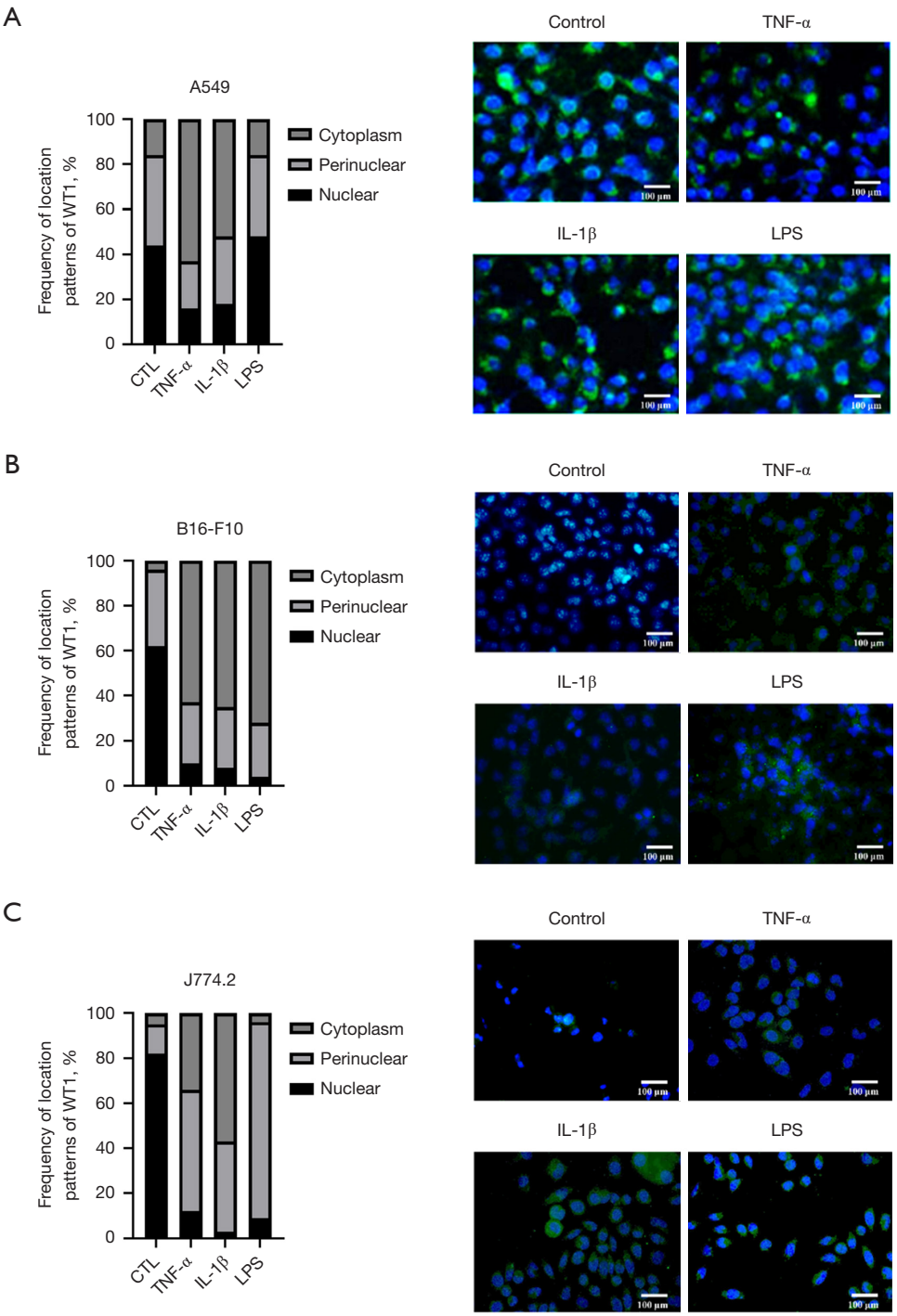
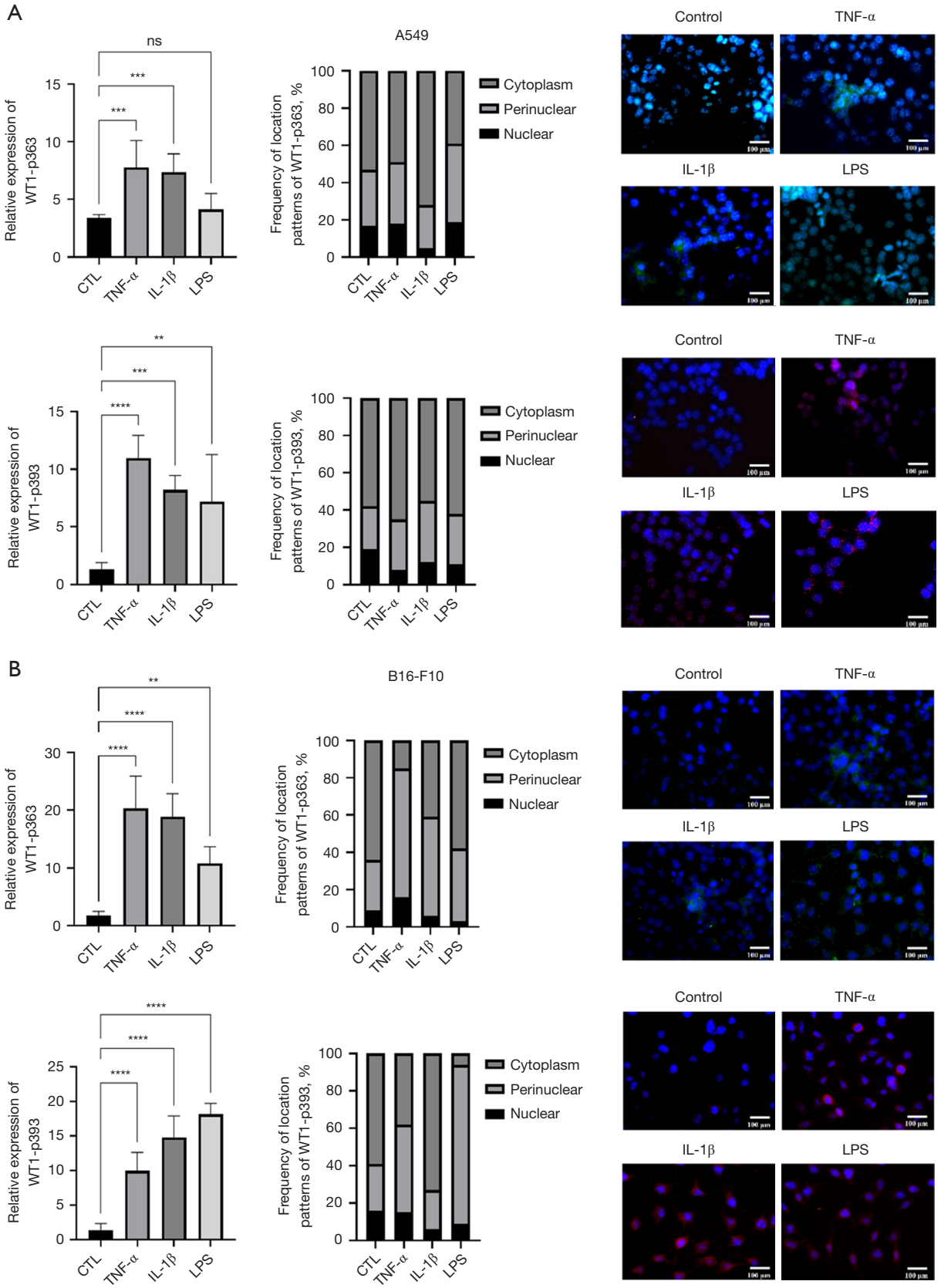


Figure 6 Localization of WT1 under TNF-α, IL-1β, and LPS treatments. (A) A549 cells were treated with TNF-α, IL-1β, and LPS for 6 hours, and WT1 (green) and DAPI (blue) were detected. (B) B16-F10 cells were treated with TNF-α, IL-1β, and LPS for 6 hours, and WT1 (green) and DAPI (blue) were detected. (C) J774.2 cells were treated with TNF-α, IL-1β, and LPS for 6 hours, WT1 (green) and DAPI (blue) were detected, and the predominant nuclear localization in the control for the 3 cell lines shifted to the cytoplasm under treatment effects. Localization analysis was carried out by calculating Pearson's correlation coefficient. CTL, control; DAPI, 4',6-diamidino-2-phenylindole; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha; WT1, Wilms' tumor 1.


A
A549
ns
***
***
Relative expression of WT1-p363
Frequency of location patterns of WT1-p363, %
Cytoplasm
Perinuclear
Nuclear
CTL
TNF-α
IL-1β
LPS
Control
TNF-α
IL-1β
LPS
**
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Relative expression of WT1-p393
Frequency of location patterns of WT1-p393, %
B
B16-F10
****
****
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****
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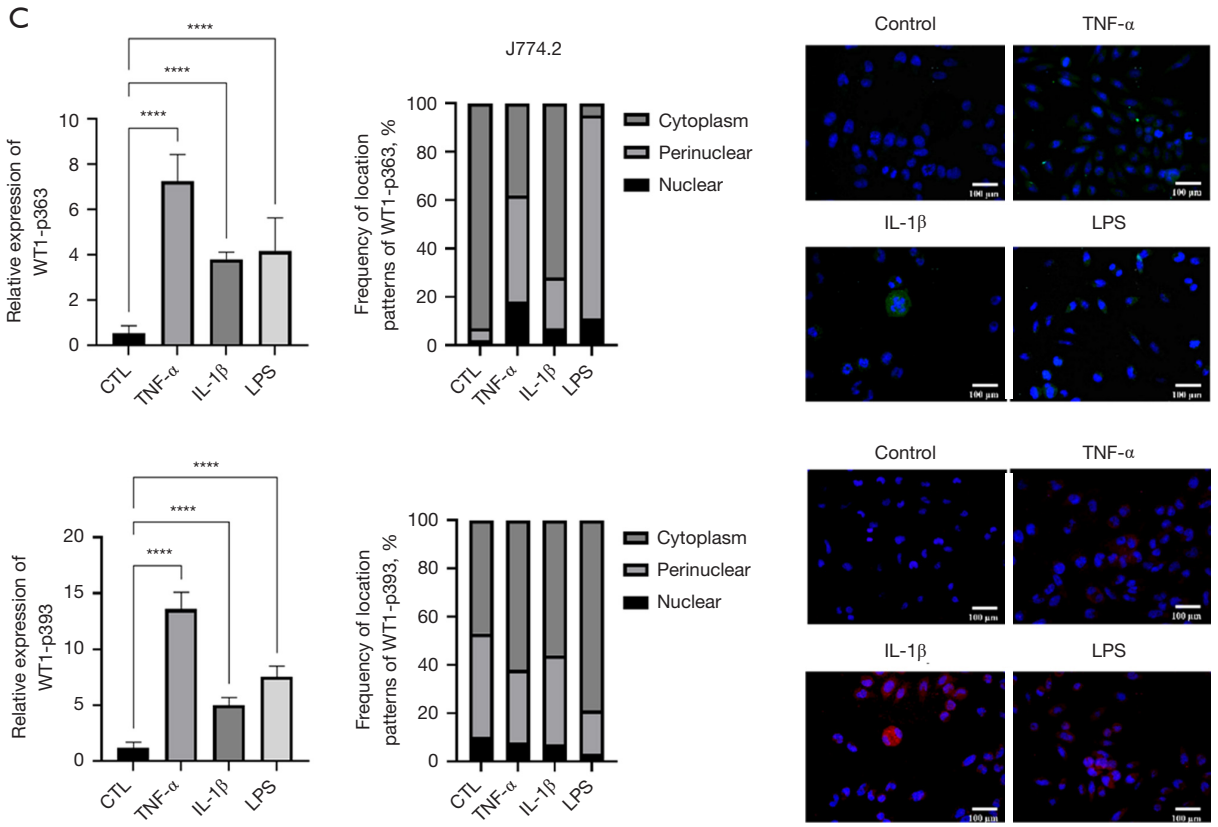

Figure 7 Phosphorylation of WT1 in A549, B16F10 and J774.2 cells treated with TNF-α, IL-1β, and LPS for 6 hours. (A) Detection of WT1-p363 (green) and DAPI (blue) and WT1-p393 (red) and DAPI (blue) in A549 cells. (B) Evaluation of WT1-p363 (green) and DAPI (blue) and WT1-p393 (red) and DAPI (blue) in B16-F10 cells. (C) Detection of WT1-p363 (green) and propidium iodide (red) and WT1-p393 (green) and propidium iodide (red) in J774.2 cells. Changes in phosphorylated WT1 expression were analyzed via ANOVA and Tukey's test (ns, not significant; **, P<0.01; ***, P<0.001; ****, P<0.0001). Localization was determined via Pearson's correlation analysis. ANOVA, analysis of variance; CTL, control; DAPI, 4',6-diamidino-2-phenylindole; IL-1β, interleukin-1 beta; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor alpha; WT1, Wilms' tumor 1.

Discussion

Key findings

This study highlights a strong connection between WT1 and the inflammatory process in lung cancer progression. Specifically, we found that WT1 expression increases significantly from stages I to IV, correlating with IL-10 expression in stages II and IV. The modulation of WT1 by inflammatory cytokines such as TNF-α and IL-1β demonstrated varying effects in tumor cells and macrophages, suggesting a differential role for WT1 in different cellular environments. Our *in vitro* experiments also revealed WT1 as a positive regulator of IL-10, especially under inflammatory conditions, which may promote an immunosuppressive tumor microenvironment conducive to cancer progression.

Strengths and limitations

The integration of patient sample analysis with *in vitro* experimentation, providing a comprehensive view of WT1's role in lung cancer. The use of multiple experimental approaches (RT-PCR, Western blotting, ELISA, and immunohistochemistry) allowed us to confirm the correlation between WT1 and IL-10 in lung cancer progression. However, while our study focuses on TNF-α

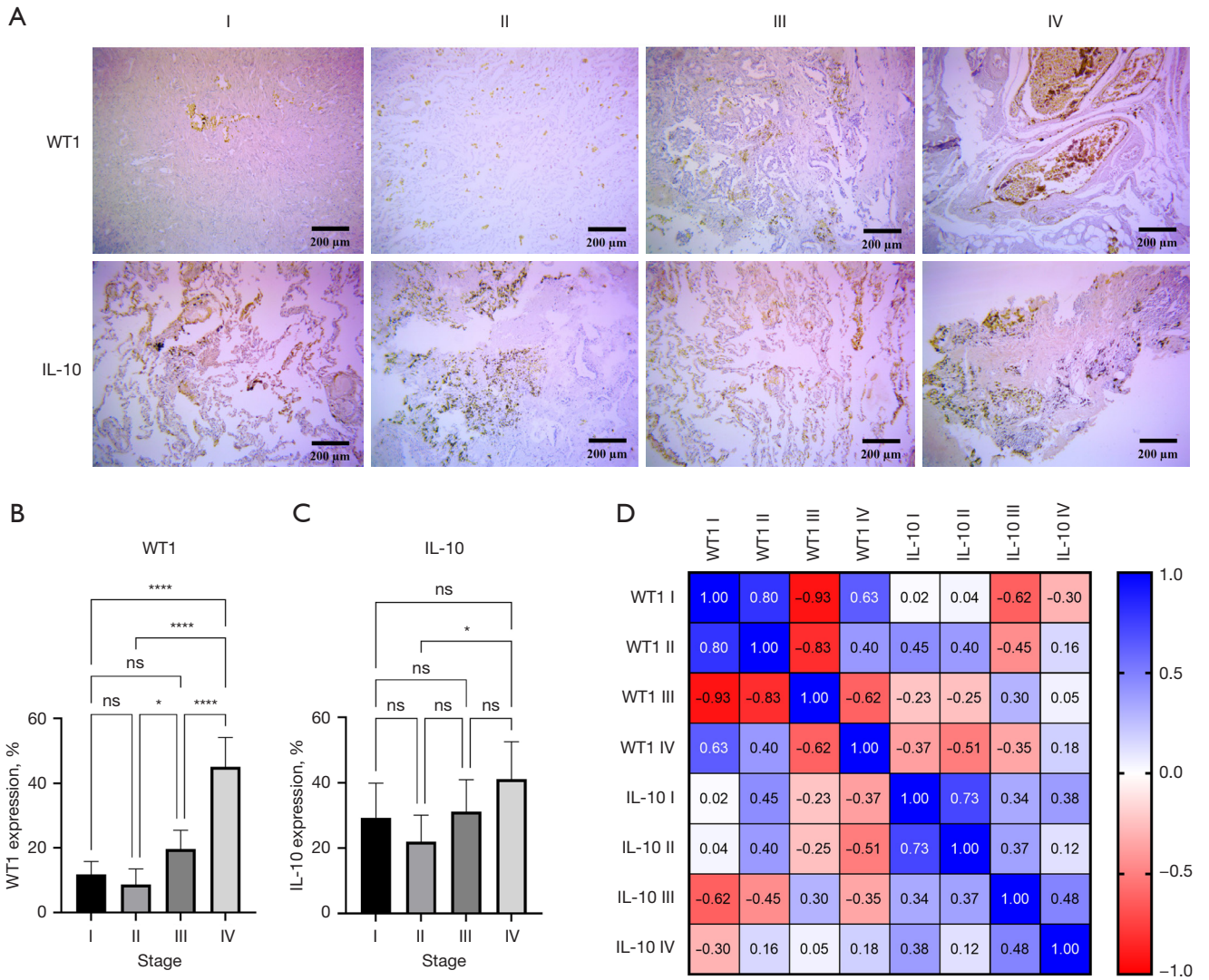

Figure 8 WT1 and IL-10 expression in lung cancer patients. (A) Immunohistochemistry for the detection of WT1 and IL-10 in patient samples divided into stages I, II, III, and IV. (B) Expression analysis of WT1, which tended to increase from stages I to IV. (C) IL-10 expression, which significantly decreased in stage II disease. (D) Pearson's correlation analysis revealed a positive correlation between WT1 and IL-10 in stages II, III, and IV. The bar on the far right indicates Pearson correlation values, with a gradient ranging from blue (strong positive correlation) to red (strong negative correlation), representing the degree of association between WT1 and IL-10 expression. Statistical significance is indicated as follows: ns, not significant; *, P<0.05; ****, P<0.0001. IL-10, interleukin-10; WT1, Wilms' tumor 1.

and IL-1β, other inflammatory cytokines might also contribute to WT1 regulation, and further exploration is necessary to clarify these pathways.

Comparison with similar research

Analysis of lung cancer patient samples obtained from The Human Protein Atlas revealed a decrease in the expression of the proinflammatory cytokines TNF-α and IL-1β with increasing tumor stage, suggesting a reduced inflammatory response in these patients (26). This may be related to the generation of a microenvironment that favors immunosuppression. This finding aligns with studies reporting that a decrease in proinflammatory cytokines promotes tumor progression and immune system evasion in advanced tumors (27,28).

The high expression of WT1 in advanced stages of lung cancer could be associated with processes such as resistance

to apoptosis, angiogenesis, and metastasis (29-31). In this study, we identified an increase in WT1 expression in stage IV patients and a positive correlation between WT1 and IL-10 expression in stages II and IV of lung cancer. IL-10, an anti-inflammatory cytokine, plays a crucial role in the anti-inflammatory response and the generation of a tolerogenic tumor microenvironment (25,32). The increased expression of IL-10 and WT1 in stage IV patients may reveal a mechanism through which the tumor evades the immune response, promoting an immunosuppressive microenvironment. This finding is consistent with research suggesting that oncoproteins can promote IL-10 synthesis to aid in tumor escape from the immune system (33-35).

To determine whether WT1 acts as a promoter or repressor of IL-10, we used RAW264.7 cells transfected to express WT1 as an exogenous expression model. Our results revealed a 2.6-fold increase in *IL-10* expression in RAW264.7 cells transfected with pWT1 compared with that in non-transfected cells. However, upon LPS treatment, RAW264.7 cells transfected with pWT1 presented an increase of up to 12.9-fold compared with the control, indicating that WT1 may act as a positive regulator of *IL-10*. These findings align with our results from lung cancer patient samples. To confirm the transcriptional modulation of WT1, we analyzed its effect on the expression of *Bcl-2*, a gene that WT1 has been documented to modulate transcriptionally in certain cell lines, demonstrating its oncogenic role in promoting a favorable microenvironment for tumor survival and maintenance (36,37).

The effects of the proinflammatory cytokines TNF-α and IL-1β on WT1 differ between tumor cells and macrophages. In A549 and B16-F10 tumor cells, exposure to TNF-α and IL-1β resulted in decreased WT1 expression, suggesting that the inflammatory process in tumors may suppress oncogenes. In contrast, in J774.2 macrophages, WT1 expression increased in response to TNF-α and IL-1β treatments, possibly as part of the macrophage response to promote tissue repair and resolve inflammation. WT1 has been described as promoting cell proliferation and inducing IL-10 synthesis to modulate the inflammatory profile (23,38).

Tumor cells and macrophages exhibited differences in the modulation of IL-10 expression in response to cytokine treatments. In A549 and B16-F10 cells treated with TNF-α, IL-10 expression decreases between 12 and 24 hours, suggesting that TNF-α may reduce IL-10 levels in tumor cells, contributing to a proinflammatory microenvironment and inhibiting tumor growth in the early stages (39). In contrast, J774.2 macrophages presented increased IL-10 synthesis after 6 hours of exposure, with TNF-α maintaining IL-10 overexpression for more than 24 hours. This highlights the modulatory role of TNF-α in inducing a sustained anti-inflammatory response in macrophages, a crucial step in resolving inflammation and promoting a microenvironment that facilitates cancer progression (40).

The proinflammatory cytokines TNF-α and IL-1β modulate WT1 expression and subcellular localization in both tumor cells and macrophages. Our results revealed the translocation of the protein from the nucleus to the cytoplasm in response to treatment, which may affect the role of WT1 in gene regulation. Distinct effects on IL-10 modulation were identified in tumor cells and macrophages due to WT1 downregulation and overexpression but similar effects on localization modulation, demonstrating that WT1 activity as a transcription factor depends on the proteins it interacts with in the promoter region. This has been described in a Bcl-2 promoter analysis, where WT1 binding to the promoter region determines whether Bcl-2 expression is activated or repressed (41).

Phosphorylation is a posttranslational modification that can modulate transcription factor function. In this study, we identified TNF-α and IL-1β as inducers of WT1 phosphorylation at positions 363 and 393. A significant increase in phosphorylated WT1 was observed after cytokine treatment in both tumor cell lines and macrophages, which may have altered WT1 localization and function. These findings suggest that inflammation plays a crucial role in WT1 posttranslational modifications, serving as a mechanism by which cells alter WT1 function in response to inflammatory signals (42).

The analysis of WT1 and IL-10 expression in lung cancer patients via immunohistochemistry corroborates our *in vitro* results, which revealed increased WT1 expression in stages III and IV, which is consistent with reports of the presence of WT1 in advanced tumor progression (32). Similarly, IL-10 decreases in stage II compared with stage I but increases again in stages III and IV. This pattern suggests a dynamic relationship between IL-10 and lung cancer progression, with a possible immunosuppressive role in advanced stages. These results are consistent with those of the Pearson correlation analysis, which revealed a positive correlation between WT1 and IL-10 in stages II, III, and IV, reinforcing the hypothesis that WT1 may promote an immunosuppressive microenvironment in lung cancer by inducing IL-10, contributing to immune evasion and tumor

progression. These data align with studies showing that IL-10 can be induced by oncoproteins, which play crucial roles in cancer immune evasion (43,44).

Explanations of findings

In this study, WT1 expression was found to progressively increase from stage I to stage IV in lung cancer patients, suggesting its pivotal role in tumor progression. This upregulation of WT1 may contribute to key processes such as resistance to apoptosis, enhanced angiogenesis, and metastasis, which are common in aggressive cancer states. As the disease advances, the elevated levels of WT1 likely promote tumor survival and growth, reinforcing its involvement in driving a more aggressive phenotype.

Furthermore, a significant positive correlation between WT1 and IL-10 expression in stages II, III, and IV highlights the complex interplay between these factors in fostering an immunosuppressive tumor microenvironment. The rise in IL-10 expression, known for its immune evasion properties, alongside WT1, suggests that WT1 may stimulate IL-10 production to enhance the tumor's ability to escape immune surveillance. The dynamic expression of IL-10, initially decreasing in stage II before rising again in later stages, indicates a shift from modulating early immune responses to establishing an immunosuppressive environment that favors tumor progression. Additionally, the modulation of WT1 by proinflammatory cytokines like TNF-α and IL-1β further emphasizes the role of the inflammatory microenvironment in regulating oncogenic factors. This interplay between inflammation and oncogenesis highlights the therapeutic potential of targeting the WT1-IL-10 axis, which could disrupt the immunosuppressive conditions and improve the efficacy of immunotherapies in lung cancer treatment.

Implications and actions needed

The results of this study suggest that targeting the WT1-IL-10 axis could offer new pathways for lung cancer treatment by disrupting the immunosuppressive tumor microenvironment. Therapeutic development should focus on creating WT1 inhibitors or IL-10 antagonists, as these could potentially enhance the immune system response against tumors.

For clinical translation, designed trials are needed to assess the safety and efficacy of WT1 inhibitors in lung cancer patients. Such trials should investigate not only different disease stages but also patient subgroups to determine the most beneficial timing and conditions for these therapies. The correlation between WT1 and IL-10 also presents an opportunity for biomarker identification. Future research should explore their utility as prognostic markers, potentially guiding personalized treatment approaches based on their expression patterns in lung cancer.

Further studies are crucial to understand the molecular mechanisms that underlie the relationship between WT1 and IL-10. This includes exploring the role of proinflammatory cytokines like TNF-α and IL-1β in modulating these interactions. This could lead to more refined and targeted therapies that aim to disrupting the tumor-promoting effects of the immune microenvironment, making the WT1-IL-10 axis a focal point in the development of next-generation cancer treatments.

Conclusions

This study has provided new insights into the relationship between inflammation and lung cancer progression through the modulation of WT1 and IL-10 synthesis. Our findings show that WT1 plays a key role in the regulation of IL-10, especially in the advanced stages of the disease, suggesting that the WT1-IL-10 axis may contribute to the creation of an immunosuppressive microenvironment. This environment promotes immune evasion and tumor progression. Furthermore, the observed differences in WT1 modulation between tumor cells and macrophages highlight the complexity of the interaction between proinflammatory cytokines and oncogenic factors. The results suggest that targeting WT1 from the perspective of the inflammatory process could be a promising therapeutic strategy to interfere with lung cancer progression.

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None.

Footnote

Reporting Checklist: The authors have completed the STROBE and MDAR reporting checklists. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-2024-1242/rc

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. The retrospective analysis was conducted under the ethics approval of Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas (UANL FCB; No. CEIBA-FCB-35/2022) and individual consent for this retrospective analysis was waived.

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