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Molecular Evaluation of the Therapeutic Potential of DNA-Based Vaccines Against Experimentally Induced Lung Cancer

S.E. Hassab El-Nabi[1], Abdel-Aziz A. Zidan[2], Nourhan K. El. Ghayesh[2], Karolin K. Abdel-Aziz[2] and EL-Hassan M. Mokhamer[2]

[1]Zoology Department, Faculty of Science, Menoufia University, Egypt.

[2]Zoology Department, Faculty of Science, Damanhur University, Egypt.

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Abstract

Lung cancer, or lung carcinoma, is a malignant lung tumor characterized by uncontrolled cell growth in lung tissue. In Egypt, it has high morbidity and mortality rates. Polyinosinic-polycytidylic acid (poly (I: C)) is a double-stranded RNA analog, a potent immunostimulatory agent that activates multiple components of the immune system, and cyclophosphamide is an alkylating cytotoxic drug that inhibits DNA synthesis to kill tumor cells. Transforming Growth Factor Beta 1 (TGF-β1) plasmid vaccine aims to neutralize or downregulate the immunosuppressive effects of TGF-β1, enhancing immune system activity against cancer cells. This study investigated the impact of the TGF-β1 plasmid vaccine, alone or in combination with cyclophosphamide and poly (I: C), on lung metastasis of B16/F10 murine melanoma cells in syngeneic C57BL/6 mice. The results demonstrated overexpression of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) proteins in tumor-bearing or cyclophosphamide-treated mice. In contrast, the vaccine ameliorated the overexpression of EGFR and VEGF.

These findings suggest that the TGF-β1 plasmid vaccine, especially in combination with poly (I: C), effectively mitigates lung cancer progression in mice by inhibiting tumor growth, enhancing cell survival, and reducing the chemotherapy side effects. This study highlights the potential of the TGF-β1 plasmid vaccine as a promising therapeutic strategy for lung cancer.

Keywords: Lung cancer, B16/F10 melanoma cells, Poly(I:C), cyclophosphamide, TGF-β1 plasmid vaccine, EGFR.

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1. Introduction:

Lung cancer is a very aggressive and highly prevalent disease worldwide, with a high morbidity and mortality rate [1]. The transformation from a normal to malignant lung cancer phenotype is thought to arise in a multistep fashion, through a series of genetic and epigenetic alterations, ultimately evolving into invasive cancer by clonal expansion [2]. Following the development of primary cancer, the continued accumulation of genetic and epigenetic abnormalities, acquired during clonal expansion, influences the processes

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of invasion, metastasis, and resistance to cancer therapy [3]. The identification and characterization of these molecular changes are of critical importance for improving disease prevention, early detection, and treatment.

The B16/F10 descends from the B16 cell line which was established from a spontaneous melanoma in the ear of a C57BL/6 mouse [4]. Intravenous (i.v.) inoculation with B16/F10 cells, therefore, leads to the formation of lung tumors [5].

Cyclophosphamide (CTX) is an antineoplastic alkylating agent extensively used for treating various malignancies, including lymphoma, leukemia, multiple myeloma, ovarian cancer, lung cancer, and sarcoma [6]. The drug is not cell-cycle phase-specific and metabolizes to an active form capable of inhibiting protein synthesis through DNA and RNA crosslinking [7]. However, CTX has some serious side effects including leukopenia, immunosuppression, gastrointestinal damage, and disruption of gut microbiota [8], [9]. Currently, new schedules are developed to overcome immunosuppression in advanced cancer.

Cancer treatment has progressed from surgical resection, radiation therapy, chemotherapy, and targeted drug therapy. Polyinosinic-polycytidylic acid (poly(I:C)) is a synthetic analog of double-stranded RNA, and its application in tumor immunotherapy has been well explored for several decades. Poly(I: C) activates the human innate immune system, with subsequent regulation of adaptive immunity [10], leading to alterations in the tumor microenvironment and a striking suppression of tumor growth [11]. Moreover, poly(I:C) can directly trigger cancer cells to undergo apoptosis [12]. These direct anticancer effects of poly(I:C) are mediated by the upregulation of antitumor genes and the induction of cell apoptosis by activation of its main related receptors, such as toll-like receptor 3 (TLR3), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) [13].

DNA vaccines use plasmids to deliver genes encoding tumor antigens, stimulating the adaptative immune response toward cancer cells [14]. Exploring Transforming Growth Factor Beta 1 (TGF-β1) as a potential component in cancer-treating vaccines represents a rapidly advancing area of research. The focus is generally on modifying the immune response to better target and eliminate cancer cells. A TGF-β1 vaccine might be designed to modulate the immune response, enhancing the ability of the body's immune cells to recognize and attack cancer cells. This can involve increasing the activity of cytotoxic T-cells (CTLs) and decreasing the immunosuppressive environment created by the tumor [15]. On the other hand, cancer cells often exploit TGF-β1 to create a microenvironment that supports tumor growth and immune evasion. A TGF-β1 vaccine could aim to disrupt this microenvironment, making it less conducive to tumor growth and more susceptible to immune attack. TGF-β1 plays a role in the progression and metastasis of certain cancers. By targeting TGF-β1 through vaccination, it might be possible to inhibit these processes, thereby slowing down or preventing the spread of cancer [16].

2. Materials and methods:

Preparation and Culture of B16/F10 Melanoma Cell Line:

B16 cells, were derived from a $gp100^{+}$ spontaneous murine melanoma cell line, and were obtained from

the cell culture department, NAWAH Scientific Center (Cairo, Egypt). B16/F10 melanoma cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and incubated at 37°C with 5% CO_2. Cells were passaged at 70-80% confluency using trypsin-EDTA for detachment. A trypan blue exclusion assay was used to determine cell viability. Cells were mixed with 0.4% trypan blue solution, loaded into a hemocytometer, and counted live cells under a microscope.

TGF-β1 Plasmid Vaccine Preparation:

The TGF-β1 gene was optimized for mammalian codon usage to ensure efficient expression in mouse cells. The pD2529-CAG vector backbone was purchased from Addgene company, containing a strong CAG promoter, and was used to drive high-level expression of the TGF-β1 gene in mammalian cells. The TGF-β1 gene was amplified using Standard PCR and inserted into the pD2529-CAG vector through restriction enzyme digestion and ligation, followed by transformation in competent *E. coli* cells for plasmid amplification, followed by purification to obtain endotoxin-free DNA suitable for this study. The purified plasmid was dissolved in PBS and adjusted to the required concentration for mice intramuscular injection (i.m). TGF-β1 plasmid vaccine was provided by Dr/ Marcela Diaz Montero's laboratory at the Sylvester Comprehensive Cancer Center, University of Miami, Florida, United States of America.

Reagents

CTX [CAS NO: 6055-19-2] (500mg pack size) and Poly (I:C) [CAS No:42424-50-0] (25mg pack size) were purchased from (Sigma, St. Louis, MO), prepared under aseptic conditions and dissolved in PBS acquired from Thermo Fisher Scientific US company.

Ethics Statement

This study was approved by the Ethics Committee and Institutional Review Board of the Faculty of Science, Damanhur University, Egypt; No. DMU-SCI-CSRE- 22 11 02.

Experimental protocol:

The experiment was done on 56 healthy adult female C57BL/6 mice (Age, 6-7 weeks), weighing 22g-24g. Animals were obtained from the animal house of the National Research Center (Dokki, Cairo, Egypt). Also, the model mouse of metastatic lung tumor was prepared by the methods of [17]. At the beginning of the experiment, 56 female C57BL/6 mice were divided into two groups, the first group (gp1) included 6 mice that only served as a normal control group, and the second group included 50 mice that were injected intravenously (iv) via caudal vein with 0.1ml of B16/F10 cells which collected in PBS to an appropriate concentration (5×10^6 cells/mL) [18], the mice were left for 25 days until lung foci developed. After 25 days, two mice were dissected and confirmed for the presence of lung tumor, and then the second group was divided into 8 sub-groups (2-9), each group containing 6 mice to receive the treatment as follows:

Group 1 (gp1): mice were administrated with only PBS and served as a normal control group.

Group 2 (gp2): Served as lung cancer group without any treatment.

Group 3 (gp3): mice were treated with CTX drug through intraperitoneal injections (i.p) (4mg/mouse)

once a week for three weeks [19].

Group 4 (gp4): mice were treated with poly (I:C) drug (i.p) (200 µg/mouse) [20] once a week for three weeks.

Group 5 (gp5): mice were treated with TGF-β1 plasmid vaccine through intramuscular injections (i.m) (50µg/mouse) once a week for three weeks.

Group 6 (gp6): mice were treated with CTX drug (i.p) (4mg/mouse) and poly (I:C) drug (i.p) (200 µg/mouse) once a week for three weeks.

Group 7 (gp7): mice were treated with CTX drug (i.p) (4mg/mouse) and TGF-β1 plasmid vaccine (i.m) (50µg/mouse) once a week for three weeks.

Group 8 (gp8): mice were treated with poly (I:C) drug (i.p) (200 µg/mouse) and TGF-β1 plasmid vaccine (i.m) (50µg/mouse) once a week for three weeks.

Group 9 (gp9): mice were treated with CTX drug (i.p) (4mg/mouse) plus poly (I:C) drug (i.p) (200 µg/mouse) and TGF-β1 plasmid vaccine (i.m) (50µg/mouse) once a week for three weeks.

Epidermal growth factor receptor (EGFR) Protein Expression:

EGFR protein expression was assessed by immunohistochemistry (IHC) using the Diagnostic BioSystems kit and carried out on paraffin sections and a monoclonal antibody clone 31G7 was used in the IHC staining. The staining procedures were performed according to the antibody manufacturer's recommendations of [21]. IHC stained sections were examined using a Leica microscope (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland).

Evaluation of immunohistochemical results "Area Percentage"

The areas exhibiting positive brown immunostaining were selected for evaluation, regardless of the staining intensity, using the Leica scoring program. EGFR immunostaining was quantified as a percentage of the area in a standard measuring frame across six representative fields in all groups, using 400x magnification via light microscopy displayed on the screen. Scoring results were subjected to statistical analysis to evaluate the difference between all groups.

Molecular Biology Investigations:

Gene expression analysis for NF-κB, STAT1, and GAPDH (internal control) was conducted by quantitative real-time PCR (qRT-PCR) using TaqMan probes.

The RNeasy Mini Kit from Qiagen (Hilden, Germany; Catalog no. 74104) was used to purify total RNA from mouse tissue in the Physiology Laboratory at the Faculty of Science, Damanhour University, Egypt. The quantity and quality of the isolated RNA were immediately assessed using the NanoPhotometer® NP80 in the central laboratory at the Faculty of Science, Damanhour University. RNA purity was evaluated by measuring the absorbance ratios at 260 nm and 280 nm (A260/A280 ratio). Pure RNA preparations should have ratios ranging from 1.8 to 2.0.

The ability to reverse the transcription process is a crucial aspect of modern molecular biology. This process is facilitated by the enzyme RNA-

dependent DNA polymerase, commonly known as reverse transcriptase. By reversing messenger RNA, researchers can produce complementary DNA (cDNA), which can be detected or quantified using RT-PCR assays. For this study, the High-Capacity cDNA Reverse Transcription Kit (Catalog no: 4368814) from Applied Biosystems, Invitrogen, United States, was used in the IMCERT lab located in the central laboratories building at Damanhour University, Egypt. The resulting cDNA was amplified using the Applied Biosystems StepOne™ Real-Time PCR system with specific TaqMan primers and then analyzed with StepOne™ software in the same lab. Specific primers and probes were designed for NF-κB, STAT1, and GAPDH using the Custom TaqMan® Assay Design Tool from Applied Biosystems to quantify their expression levels in mouse tissues.

Western blotting for VEGF, Caspase-3, and β-actin proteins:

To investigate the effects of various treatments, including the CTX drug, Poly (I:C) drug, and TGF-β1 plasmid vaccine, on the VEGF, Caspase-3, and β-actin proteins in a lung cancer model in mice at Global Research Labs, Medical Centre II, Naser City, Cairo, Egypt, lung tissues were homogenized for protein extraction. Subsequently, samples were prepared for SDS-PAGE. Proteins were separated based on molecular size using SDS-PAGE, transferred onto membranes, and probed with specific antibodies.

Statistical Analysis:

Statistical analysis was done using Graph Pad Prism 9 software (GraphPad, La Jolla, CA). A one-way analysis of variance (ANOVA) with a Tukey post-hoc test was used to analyze the significance of differences between groups. Values were considered significantly different at $p \leq 0.05$.

3. Results:

The Phenotypic Characteristics of Lung Morphology of Mice in all Studied Groups after the End of the Experiment

The phenotypic characteristics of lung morphology were examined using a stereomicroscope after the experiment for all studied groups. Normal mice exhibited typical left and right lungs (Figure 1.A). In contrast, all mice injected with B16/F10 cells displayed a significant number of metastatic nodules, which manifested as large nodules on the lung surface, complicating counting and measurement (Figure 1.B). The lungs of mice treated with the CTX drug after B16/F10 cell injection (gp3) showed structural changes similar to those of the tumor group, with a slight reduction in metastatic nodule size (Figure 1.C). Mice injected with B16/F10 cells and treated with a TGF-β1 plasmid vaccine combined with the CTX drug (gp7) (Figure 1.G) or the poly(I:C) drug (gp8) (Figure 1.H), as well as those treated with CTX in combination with poly(I:C) and the TGF-β1 plasmid vaccine (gp9) (Figure 1.I), exhibited improvements in lung structure with no metastatic nodules present.

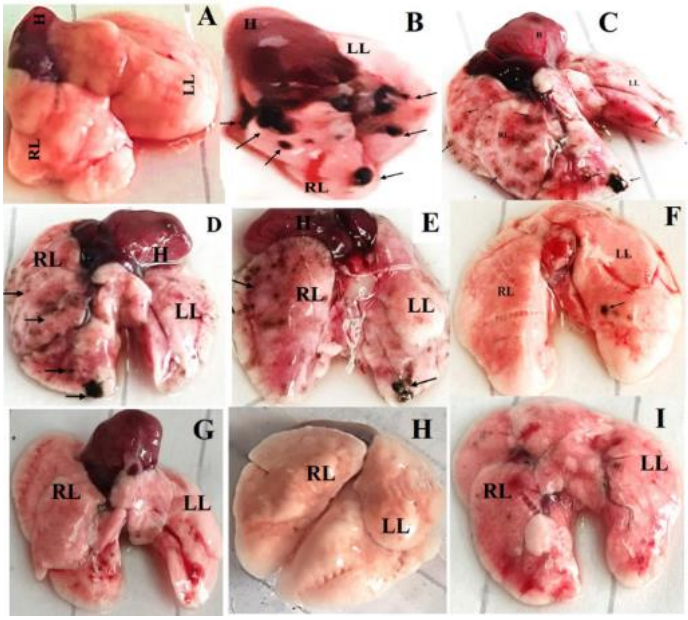

Figure1. Effect of B16/F10 cells, CTX, Poly (I:C) individually or in combination with TGF-β1 plasmid vaccine on lung metastasis formation *in vivo*. Representative photos, A) lungs in negative control group (gp1), B) lungs in tumor group (gp2), C) lungs in Tumor + CTX group (gp3), D) lungs in Tumor + poly(I:C) group (gp4), E) lungs in Tumor + vaccine group (gp5), F) lungs in Tumor + CTX + poly(I:C) group (gp6), G) lungs in Tumor + CTX+ vaccine group (gp7), H) lungs in tumor + poly(I:C) + vaccine group (gp8), I) lungs in tumor + CTX + poly(I:C) + vaccine group (gp9). Abbreviation left lung (LL), right lung (RL), Heart (H), metastatic nodules (arrow).

Immunohistochemical Examination

Expression of Epidermal Growth Factor Receptor (EGFR) protein

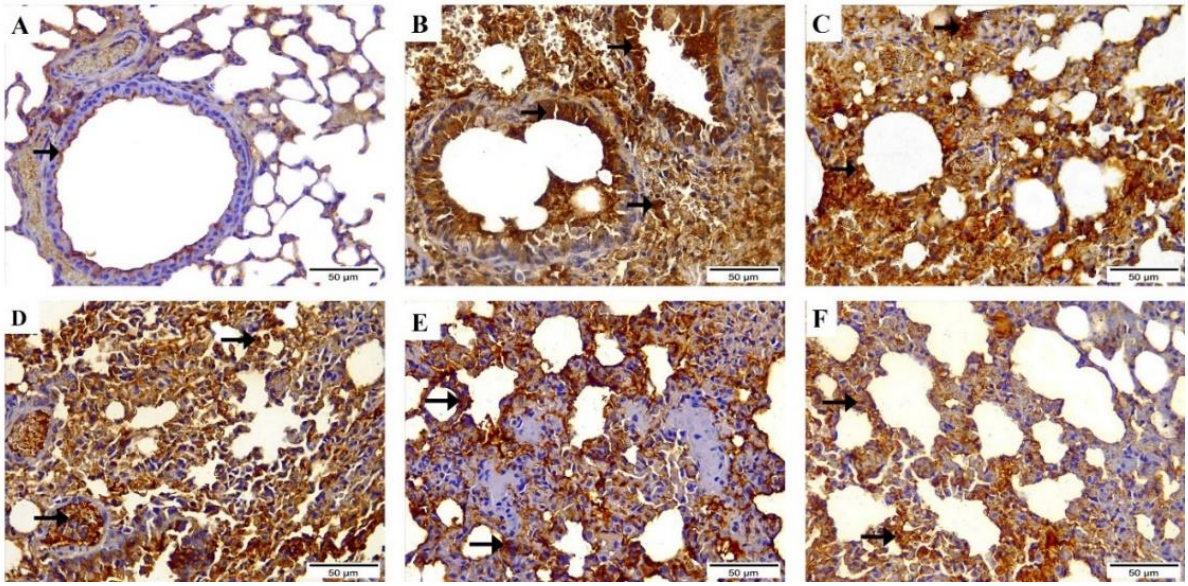

Figure2. Photomicrographs displayed the expression of EGFR protein along lung tissue in all studied groups at the end of the experiment (EGFR Antibody, Magnification Power=x400 & Scale Bar=50µm): A) Lung section from Negative Control Group (gp1) existed with standard reaction as scarce EGFR positive expression (arrow). B) Lung section from Tumor Group (gp2) highlighted the strongest positive EGFR cytoplasmic expression along metaplastic epithelium and interstitial lung tissue (arrows). C) Lung section from Tumor + CTX Group (gp3) revealed great positive cytoplasmic EGFR reactivity (arrows). D) Lung section from Tumor + Poly (I:C) Group (gp4) exhibited high positive cytoplasmic EGFR expression along lung tissue (arrows). E) Lung section from Tumor + TGF-β1 plasmid vaccine Group (gp5) presented moderate positive cytoplasmic EGFR reactivity along lung tissue (arrows). F) Lung section from Tumor + CTX + Poly (I:C) Group (gp6) demonstrated moderate positive cytoplasmic EGFR expression along lung tissue (arrows).

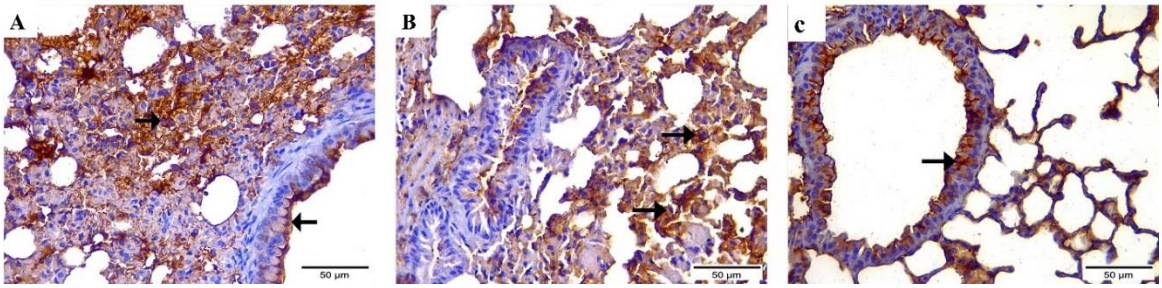

Figure3. Photomicrographs displayed the expression of EGFR protein along lung tissue in all studied groups at the end of the experiment (EGFR Antibody, Magnification Power=x400 & Scale Bar=50µm): A) Lung section from Tumor + CTX + TGF-β1 plasmid vaccine Group (gp7) displayed positive cytoplasmic EGFR reactivity along lung tissue (arrows). B) Lung section from Tumor + Poly (I:C) + TGF-β1 plasmid vaccine Group (gp8) revealed few positive cytoplasmic EGFR expressions along lung tissue (arrows). C) Lung section from Tumor + CTX + Poly (I:C) + TGF-β1 plasmid vaccine Group (gp9) presented the lowest expression of cytoplasmic EGFR along lung tissue (arrow).

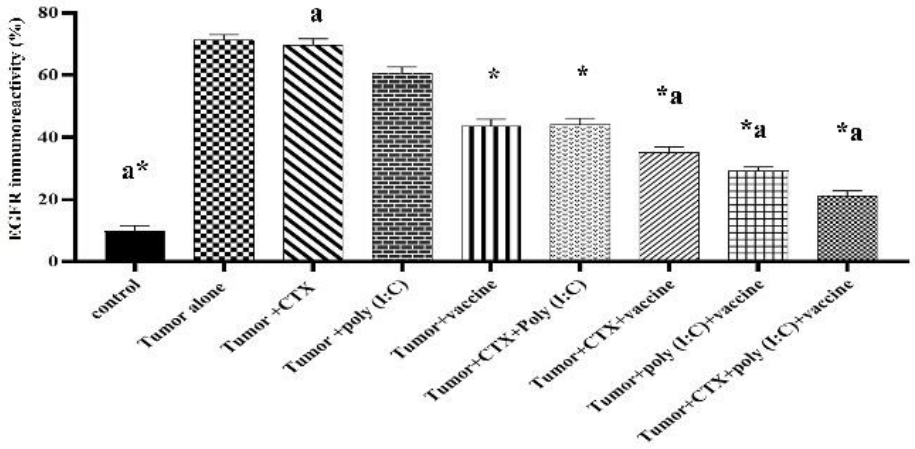

Figure4. EGFR immunoreactivity (%) in the lung tissue of all studied groups at the end of the experiment, Data are expressed as mean± S.D ($p \leq 0.05$). *(*)*: A significant difference in comparison with the tumor group (gp2), *(a)*: A significant difference compared to the tumor group treated with the vaccine (gp5).

Molecular Biology Investigations

Relative gene expression of NF-κB, STAT1, and GAPDH genes using qRT-PCR.

Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and the control sample by comparing gene expression levels in different groups after the end of the experiment by using RT-PCR. The data are normalized to GAPDH (internal control). The mRNA expression of the NF-κB gene in lung tissue for the tumor group (gp2) showed a pronounced increase in the mean value compared with the normal control group (gp1). On the other hand, mice induced tumor and treated with CTX drug alone (gp3) showed insignificant changes in mRNA expression of the NF-κB gene compared to tumor group (gp2) and a significant decrease in mRNA expression of the NF-κB gene compared with TGF-β1 plasmid vaccine treated group (gp5).

Mice-induced tumors treated with TGF-β1 plasmid vaccine (gp5) alone or in combination with CTX drug (gp7) or in combination with poly(I:C) drug (gp8) showed a significant decrease in mRNA expression of the NF-κB gene compared to tumor group (gp2).

The mRNA expression of the STAT1 gene in lung tissue for the tumor group (gp2) showed a highlighted increase compared with the normal control group (gp1). On the other hand, mice-induced tumors treated with CTX alone (gp3) showed a significant decrease in the mRNA expression of the STAT1 gene when compared to the tumor group (gp2). Mice-induced tumors treated with TGF-β1 plasmid vaccine (gp5) alone or in combination with CTX drug (gp7) showed a significant decrease in mRNA expression of the STAT1 gene compared to the tumor group (gp2). Also, mice-induced tumors treated with TGF-β1 plasmid vaccine in combination with poly(I:C) drug (gp8) or in combination with poly(I:C) drug and CTX drug (gp9) showed a significant decrease in mRNA expression of the STAT1 gene compared to tumor group (gp2).

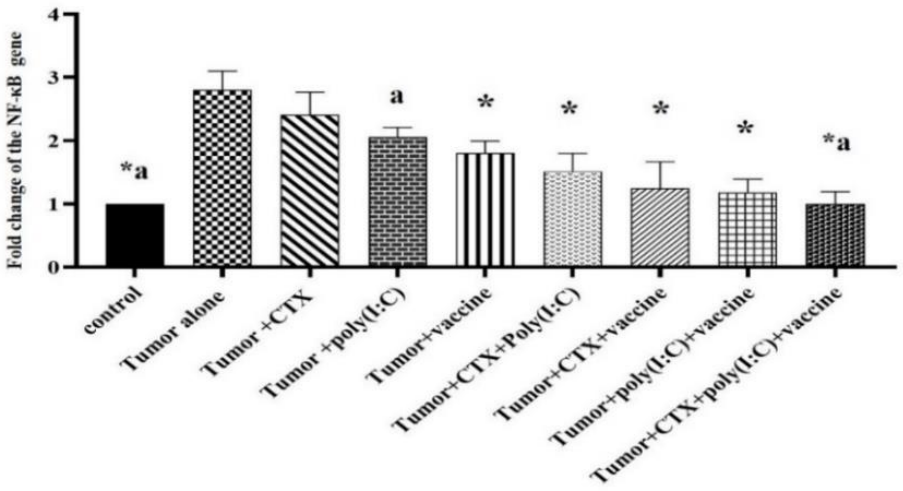

Figure5. Relative expression of NF-κB gene in the lung tissues of all studied groups. Data are expressed as mean± S.D ($p \leq 0.05$). (*): A significant difference in comparison with the tumor group (gp2), (a): A significant difference in comparison with the tumor group treated with vaccine (gp5).

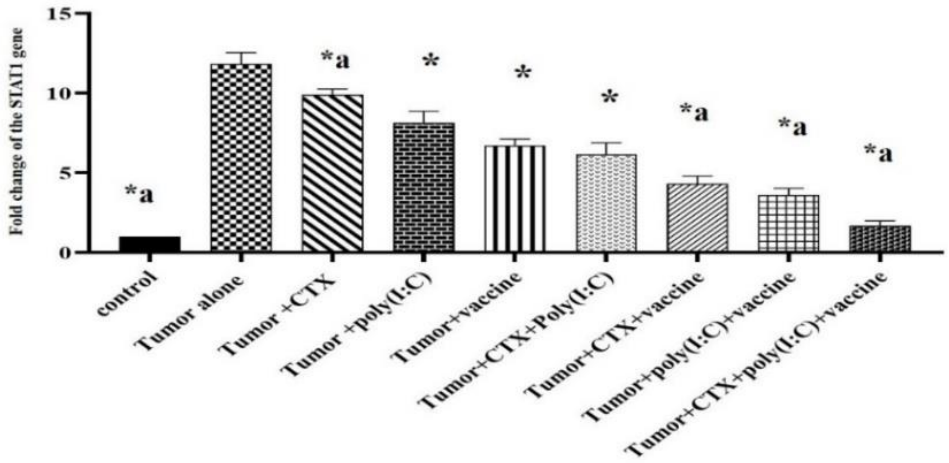

Figure6. Relative expression of STAT1 gene in the lung tissues of all studied groups. Data are expressed as mean± S.D ($p \leq 0.05$). (*): A significant difference in comparison with the tumor group (gp2), (a): A significant difference in comparison with the tumor group treated with the vaccine (gp5).

Expression of VEGF, Caspase-3, and β-actin (internal control) proteins using the western blot Technique

VEGF, Caspase-3, and β-actin protein expression levels obtained from Western blot, in mice lung tissue after different treatments shown in figure 11. Moreover, VEGF and Caspase-3 expression were quantified by densitometry, normalized by β-actin levels, and expressed as a percentage of the model groups. Mice-induced tumor (gp2) showed a drastic increase in VEGF protein expression compared to the normal control group (gp1). On the other hand, mice-induced tumors treated with CTX drug alone (gp3) or in combination with poly (I:C) (gp6) showed a significant decrease in VEGF protein expression compared to the tumor group (gp2). Mice-induced tumors treated with TGF-β1 plasmid vaccine alone (gp5) or in combination with CTX (gp7) showed a significant decrease in VEGF protein expression compared to the tumor group (gp2).

Mice-induced tumor group (gp2) showed a pronounced increase in Caspase-3 protein expression compared to the normal control group (gp1). Mice-induced tumors treated with poly (I:C) (gp4) showed a significant increase in Caspase-3 protein expression when compared to the tumor group (gp2). On the other hand, mice-induced tumors treated with the TGF-β1 plasmid vaccine alone (gp5) or in combination with CTX (gp7) showed a significant increase in Caspase-3 protein expression when compared to the tumor group (gp2).

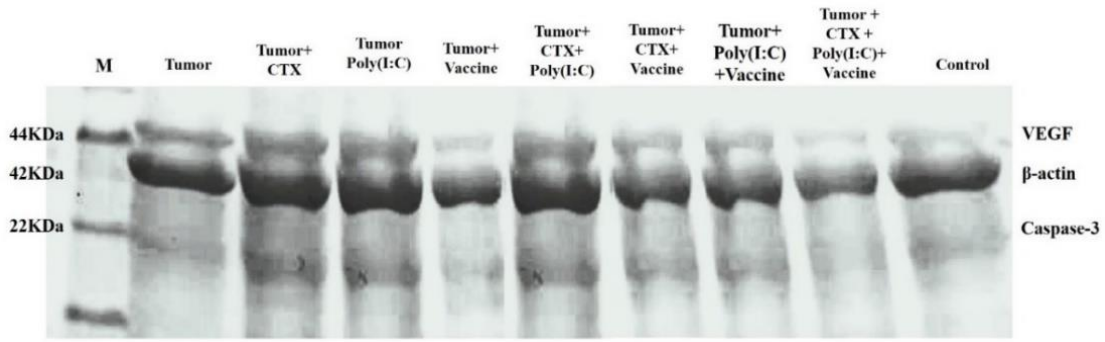

Figure7. Western blot analysis showed VEGF and Caspase-3 protein concentrations in mice lung tissues (30µg of cell lysate). The membrane was visualized at wavelength 340 nm using the UVP Transilluminator gel documentation system, Analytik Jena, USA.

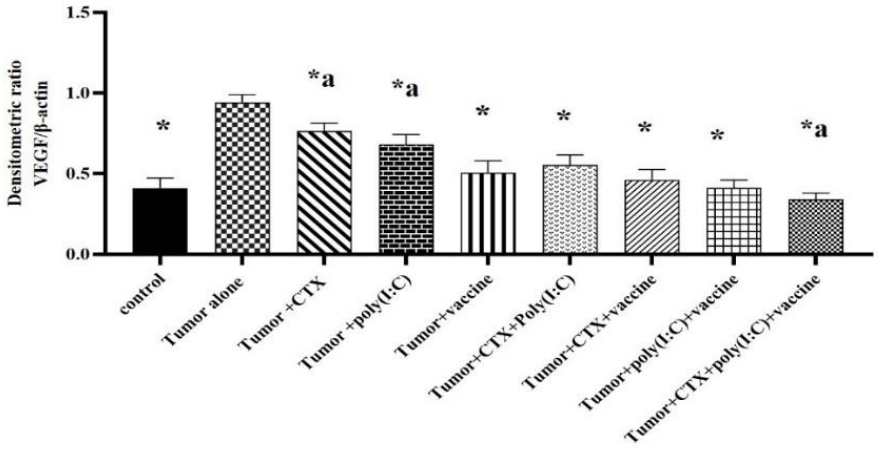

Figure8. Densitometric analysis of immunoblotting for

a VEGF relative to β-actin in the lung tissues of all studied groups at the end of the experiment. Data are expressed as mean± S.D ($p \leq 0.05$). (*): A significant difference in comparison with the tumor group (gp2), (a): A significant difference in comparison with the tumor group treated with vaccine (gp5).

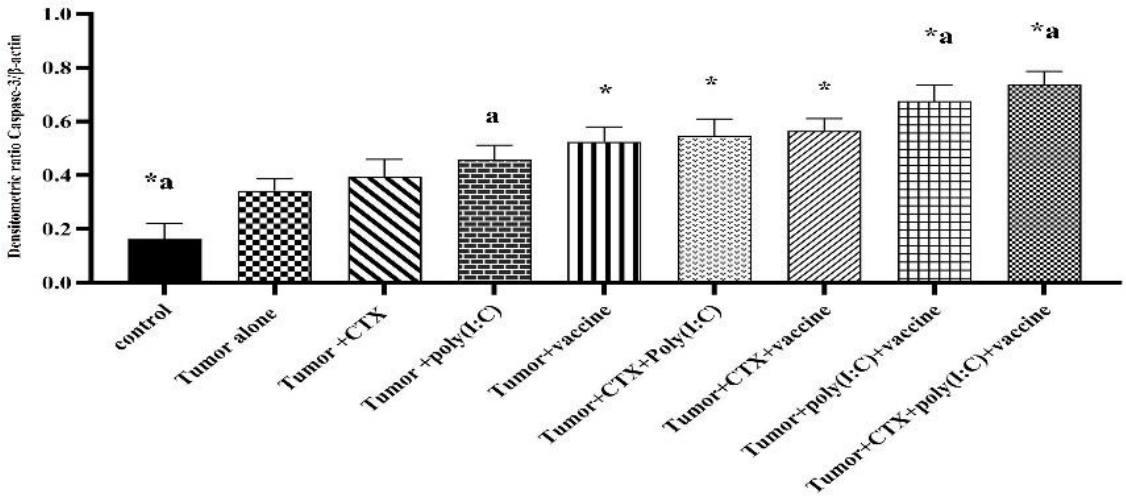

Figure 9. Densitometric analysis of immunoblotting for a Caspase-3 relative to β-actin in the lung tissues of all studied groups at the end of the experiment. Data are expressed as mean± S.D ($p \leq 0.05$). (*): A significant difference in comparison with the tumor group (gp2), (a): A significant difference in comparison with the tumor group treated with vaccine (gp5).

4. Discussion:

The NF-κB pathway plays a crucial role in regulating inflammation, tumor growth, and metastasis. In the melanoma metastasis lung cancer group (gp2), NF-κB expression was significantly elevated, which aligns with its knowing function in promoting melanoma progression and immune evasion [22]. NF-κB contributes to lung cancer primarily by driving the secretion of inflammatory cytokines such as TNF-α, IL-1, and IL-6, which create a tumor-promoting inflammatory microenvironment [23]. While these cytokines are vital for immune defense, cell recruitment, and tissue repair, their dysregulation can lead to chronic inflammation. Additionally, they sustain NF-κB activity through a feedback loop, enhancing the expression of anti-apoptotic genes (e.g., Bcl-2, survivin), angiogenic factors (e.g., VEGF), and proteases (e.g., MMPs), thereby supporting tumor survival, angiogenesis, and invasion [24,25]. Furthermore, reactive oxygen species (ROS) produced in the lung microenvironment further activate NF-κB by promoting the degradation of IκB [25]. In contrast, the CTX-treated group (gp3) exhibited reduced NF-κB expression, consistent with evidence that CTX suppresses NF-κB through its cytotoxic and immunomodulatory effects [26]. Similarly, treatment with Poly (I:C) (gp4) and the TGFβ1 plasmid vaccine (gp5) downregulated NF-κB, likely by inhibiting pro-inflammatory signaling pathways. This is supported by findings that Poly (I:C) suppresses NF-κB signaling via activation of Toll-like receptor 3 (TLR3), leading to reduced tumor progression [27]. Inhibition of TGFβ1 also decreases NF-κB activity, thereby curbing tumor growth and inflammation [28].

Combination therapy groups (gp6, gp7, gp8, and gp9), particularly the triple therapy group (gp9), demonstrated the most significant reduction in NF-κB expression, indicating a synergistic effect in

suppressing inflammation and tumor progression. This observation is consistent with studies showing that combination therapies targeting NF-κB can enhance antitumor efficacy [29].

The upregulation of STAT1 in melanoma and its potential role in lung cancer metastasis is a complex topic. While STAT1 is primarily known for its tumor-inhibiting properties, its expression can vary based on the tumor microenvironment and the stage of the disease [30]. The current results revealed that the B16/F10 murine melanoma-induced metastatic lung cancer group 2, has shown an increased STAT1 expression which may result from immune responses and inflammation within the tumor microenvironment. Melanoma cells activate immune cells, such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, prompting them to produce interferons (IFNs), particularly IFN-γ. This production activates the JAK-STAT signaling pathway, leading to the upregulation of STAT1 [31]. Additionally, pro-inflammatory cytokines like TNF-α and IL-6 enhance STAT1 expression through NF-κB and JAK-STAT signaling, promoting tumor cell survival and resistance to apoptosis [32-34]. In contrast, decreased STAT1 expression in the treatment groups (Groups 3–9) reflects the therapeutic effects of cyclophosphamide (CTX), polyinosinic-polycytidylic acid (poly(I:C)), and the TGF-β1 vaccine. CTX reduces IFN-γ production and inflammation by depleting regulatory T cells (Tregs) and enhancing CTL and NK cell activity, which downregulates STAT1[35]. While poly(I:C) initially activates STAT1 through TLR3-mediated IFN production, it also triggers negative feedback mechanisms, such as the upregulation of SOCS1(Suppressor of Cytokine Signaling 1), which inhibits JAK-STAT signaling and decreases STAT1 expression [36]. The TGF-β1 vaccine neutralizes TGF-β1, a cytokine that promotes immune suppression and STAT1 activation, thereby restoring immune surveillance and reducing STAT1 levels [37]. Furthermore, the combination therapies in groups (gp6-gp9) synergistically enhance these effects by further reducing inflammation, oxidative stress, and IFN production. This leads to significant downregulation of STAT1 and improved anti-tumor responses [38]. Collectively, these treatments suppress STAT1 expression, demonstrating their therapeutic potential in treating melanoma lung metastasis.

VEGF, a key mediator of angiogenesis, plays a critical role in promoting tumor growth and metastasis. In the melanoma metastasis lung cancer group (gp2), VEGF was highly expressed, reflecting its function in facilitating tumor vascularization. In contrast, the CTX-treated group (gp3) showed decreased VEGF expression, consistent with research indicating that CTX suppresses angiogenesis by lowering VEGF levels [39]. Similarly, treatment with Poly (I:C) (gp4) and the TGFβ1 vaccine (gp5) significantly reduced VEGF expression, likely due to the inhibition of angiogenic signaling pathways [40],[41]. Supporting evidence shows that Poly (I:C) downregulates VEGF by activating immune-mediated anti-angiogenic mechanisms [42], while TGFβ1 inhibition reduces VEGF expression and tumor-driven angiogenesis [43].

The combination therapy groups (gp6–gp9), particularly the triple therapy group (gp9), achieved the most pronounced reduction in VEGF levels, indicating a synergistic suppression of angiogenesis. This observation aligns with studies

demonstrating that combined therapies targeting VEGF amplify anti-angiogenic effects [44].

Caspase-3, a key executioner in the apoptosis pathway, is crucial for programmed cell death. In the melanoma metastasis lung cancer group (gp2), caspase-3 expression was altered, indicating impaired apoptotic mechanisms. However, the CTX-treated group (gp3) exhibited increased caspase-3 expression, consistent with findings that CTX induces apoptosis via DNA damage and p53 activation [45]. Similarly, treatment with Poly (I:C) (gp4) and the TGFβ1 vaccine (gp5) significantly upregulated caspase-3, likely through the activation of both intrinsic and extrinsic apoptotic pathways. Studies have shown that Poly (I:C) promotes apoptosis by inhibiting anti-apoptotic signaling pathways, such as PI3K/AKT and NF-κB, which are upstream regulators of caspase-3, thereby enhancing its activity [46]. Likewise, TGFβ1 inhibition facilitates caspase-3 activation and tumor cell death [43].

The TGFβ1 vaccine also boosts anti-tumor immunity by reversing TGFβ1-mediated immune suppression, restoring the activity of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, further contributing to caspase-3 activation [47]. Combination therapy groups (gp6–gp9), especially the triple therapy group (gp9), demonstrated the highest levels of caspase-3 upregulation, underscoring a synergistic effect in promoting tumor cell death.

EGFR, a receptor tyrosine kinase, plays a critical role in tumor cell proliferation, migration, resistance to apoptosis, and angiogenesis [48]. In this study, lung tissue from the tumor group (gp2) displayed strong cytoplasmic EGFR expression and significantly increased immunoreactivity. This aligns with findings that approximately 89% of lung cancer cases exhibit EGFR overexpression at the protein level, which is associated with poor survival rates and reduced chemosensitivity [49]. EGFR overexpression often results from gene mutations or amplification, with mutation hotspots in exons 18-21. Deletions in exon 19 and the L858R mutation in exon 21 account for 85-90% of EGFR mutations [50], [51]. Enhanced EGFR signaling contributes to apoptosis evasion, promoting tumor aggressiveness [52].

The CTX-treated group (gp3) showed reduced EGFR expression, consistent with research indicating that CTX suppresses EGFR signaling through its cytotoxic effects [14]. Similarly, treatment with Poly (I:C) (gp4) and the TGFβ1 vaccine (gp5) downregulated EGFR expression, likely by inhibiting downstream pathways such as MAPK/ERK and PI3K/AKT. Studies support that Poly (I:C) reduces EGFR expression by activating immune-mediated tumor suppression mechanisms [13], while TGFβ1 inhibition decreases EGFR expression and tumor growth.

Combination therapy groups (gp6–gp9), particularly the triple therapy group (gp9), exhibited the most significant EGFR downregulation, highlighting a synergistic effect in suppressing tumor progression. This observation is consistent with evidence that combination therapies targeting EGFR enhance antitumor efficacy [53].

5. Conclusion and future perspectives:

The current study shows that the TGF-β1 plasmid vaccine has great therapeutic potential in reducing the progression of lung cancer in a mouse model of metastasis, especially when combined with polyinosinic-polycytidylic acid (poly(I:C)). Even

though the results are encouraging, more investigation is needed to fully comprehend the underlying processes of TGF-β1's interaction with the immune system and how it contributes to the increased therapeutic effectiveness of cyclophosphamide and poly(I:C).

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7. Abbreviations:

CTX	Cyclophosphamide
EGFR	Epidermal Growth Factor Receptor
RIG-I	Retinoic acid-Inducible Gene I
STAT1	Signal Transducer and Activator of Transcription 1
TGF-β1	Transforming Growth Factor Beta 1
TLRs	Toll-Like Receptors
MDA-5	Melanoma differentiation-associated protein 5
NFκB	Nuclear factor kappa B
PBS	Phosphate Buffer Saline
poly(I:C)	polyinosinic-polycytidylic acid
VEGF	vascular endothelial growth factor

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