

Anti-inflammatory effects of recombinant TGF- α zyme protein on endotoxin-inflamed lung cell lines

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ABSTRACT

Background and Objectives: Pulmonary inflammation is central to the pathogenesis of various respiratory diseases, driving the need for novel therapeutics. Lipopolysaccharide (LPS) is a potent inflammatory trigger that induces proinflammatory cytokine synthesis via distinct signaling cascades. The objectives of this study were to evaluate the cytotoxic and anti-inflammatory effects of recombinant TGF-Arazyme fusion protein on LPS-stimulated human lung A549 and MRC-5 cell lines.

Materials and Methods: A gene construct fusing Arazyme with a non-mitogenic TGF α fragment was expressed, purified via Ni-NTA chromatography, and validated enzymatically. Cytotoxicity was assessed by MTT assay. Inflammatory cytokine levels (TNF- α , IL-1 β , IL-6, IL-10) were quantified by RT-PCR and ELISA. Anti-inflammatory efficacy was further validated in a murine (BALB/c) air pouch model infected with *E. coli*.

Results: At 20 μ g/mL, the chimeric protein showed no significant toxicity in MRC-5 cells but dose-dependently reduced A549 viability ($p < 0.01$). Co-treatment with LPS and the chimeric protein significantly downregulated proinflammatory cytokines (TNF- α , IL-1 β , IL-6) at both mRNA and protein levels ($p < 0.01$, $p < 0.001$), while increasing IL-10 ($p < 0.05$). In the air pouch model, TGF-Arazyme reduced leukocyte counts by 48.3% ($p < 0.01$), comparable to dexamethasone (51.2%).

Conclusion: TGF-Arazyme exhibits both anti-inflammatory and selective cytotoxic properties, positioning it as a promising candidate for inflammatory lung diseases and lung cancer.

Keywords: Lung inflammation; Metalloproteases; TGF- α ; Lipopolysaccharides; A549 cells

INTRODUCTION

The pathophysiology of several acute and chronic respiratory conditions—including lung cancer, cystic fibrosis, COPD, and idiopathic pulmonary fibrosis—is profoundly influenced by lung inflammation (1, 2). Inflammatory responses, characterized by

vasodilation, increased capillary permeability, and immune cell infiltration, impair gas exchange and worsen disease severity (3).

LPS, a component of the gram-negative bacterial cell wall serves as a major inflammatory trigger that activates immune pathways such as the nucleotide-binding oligomerization domain (NOD)-like

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receptor protein 3 (NLRP3) inflammasome and nuclear factor-kappa B (NF- κ B). The NLRP3 inflammasome is composed of the NOD-like receptor, the NLRP3 protein, procaspase-1, and apoptosis-associated speckle-like protein with a caspase-binding domain. This complex is capable of recognizing a variety of caspase-associated proteins (3). Additionally, LPS has been shown to activate key immune pathways including the NLRP3 inflammasome and NF- κ B, promoting oxidative stress, ROS generation, and production of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α). Persistent inflammation drives apoptosis and fibrosis, further compromising pulmonary function (4-9).

Current therapeutic strategies—corticosteroids, NSAIDs, and biologics—are limited by side effects, resistance, and incomplete resolution. Thus, identifying novel anti-inflammatory agents with enhanced efficacy and minimal toxicity remains critical.

Arazyme, a metalloprotease from *Serratia* species, has demonstrated anti-inflammatory and immunomodulatory potential (10-12). This 51.5 kDa enzyme, produced by *Aranicola proteolyticus* (syn. *Serratia proteomaculans*), is isolated from the gut of the spider *Nephila clavata* (10-13).

The targeting domain TGF α L3 was selected based on two principles: (i) EGFR, a tyrosine kinase receptor, is overexpressed in numerous malignancies including lung adenocarcinoma (A549 cells); and (ii) use of a non-mitogenic TGF α L3 fragment (KD3.2) avoids proliferative signaling and associated side effects. Molecular docking studies confirm stable EGFR binding. Moreover, this fusion protein targets two distinct receptors: EGFR (via TGF α L3) and TLR4 (via Arazyme) on innate immune cells.

MATERIALS AND METHODS

A gene construct was designed using a specific linker to fuse Arazyme (a metalloprotease from *Serratia proteomaculans*) with the non-mitogenic fragment of TGF α (TGF α L3). The chimeric gene was synthesized and cloned into a suitable expression vector by Biomatik (Canada). The construct contained an N-terminal hexahistidine (6 $\times$ His) tag to facilitate purification. The recombinant protein was expressed and purified through Ni-NTA chromatography, and its enzymatic activity was confirmed via the Folin method. All stages of the study were approved by the

local Ethics Committee of Tehran University of Medical Sciences (Code: IR.BMSU.REC. 1398.417).

Gene expression and optimization. *E. coli* BL21 (DE3) cells harboring the recombinant plasmid were cultured in Luria-Bertani (LB) medium containing appropriate antibiotics at 37°C with shaking (180 rpm) until reaching an optical density (OD 600) equivalent to 0.5 McFarland units. Protein expression was induced by adding 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and cultures were incubated for 4 hours at 37°C with shaking (130 rpm).

Following induction, cells were harvested by centrifugation (10,000 rpm, 10 min, 4°C), and the pellet was resuspended in PBS at a ratio of 5 mL per gram of wet cell weight. Cell disruption was achieved by sonication (6 cycles of 30 seconds at 70 W) in an ice-water bath. After centrifugation (10,000 rpm, 10 min, 4°C), the insoluble pellet containing inclusion bodies was solubilized in 8 M urea and incubated overnight at 4°C with gentle agitation. The soluble fraction was separated by centrifugation and stored at 4°C for subsequent purification.

To maximize soluble protein yield while minimizing IPTG and incubation time, expression was tested using various IPTG concentrations (0.25, 0.5, 0.75, and 1 mM) and incubation durations (2, 4, and 18 hours). Protein expression in both soluble (supernatant) and insoluble (pellet) fractions was analyzed by SDS-PAGE on 10% polyacrylamide gels at 100 V for 1 hour.

Purification of the TGF α -AraA and AraA chimera proteins. Recombinant proteins (Arazyme: 51.5 kDa; Arazyme-linker-TGF α L3: 59.3 kDa) were purified using Ni-NTA affinity chromatography, *ProteinIso*® Ni-NTA Resin (TransGen Biotech, China). The Ni-NTA column was equilibrated with 4 mL of denaturing binding buffer (8 M urea, 50 mM NaH₂PO₄, 300 mM NaCl, pH 7.8; (TransGen Biotech, China). The solubilized protein sample was loaded onto the column and incubated for 4 hours at room temperature to allow binding.

The column was washed sequentially with 4 mL denaturing wash buffer (8 M urea, 50 mM NaH₂PO₄, 300 mM NaCl, pH 6.0) once and 8 mL native wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 25 mM imidazole, pH 8.0; TransGen Biotech company, China) three times. Bound proteins were eluted with 8 mL native elution buffer (50 mM NaH₂PO₄, 300 mM NaCl,

250 mM imidazole, pH 8.0) repeated three times. Remaining proteins were removed by washing with 4 mL of 0.5 mM MES buffer (TransGen Biotech company, China). Protein concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The purified proteins were dialyzed against PBS (0.01 M, pH 7.4) using a 30 kDa cutoff dialysis membrane at 4°C overnight to remove imidazole.

Protease activity evaluation. Proteolytic activity was assessed using the Folin-Ciocalteu method with a tyrosine standard curve. The standard curve was generated using tyrosine concentrations of 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1 mM. For each standard, 700 µL of sample was mixed with 700 µL of 500 mM sodium carbonate and 175 µL of Folin-Ciocalteu reagent (BioAssay, Germany). After incubation at 37°C for 10 minutes, absorbance was measured at 660 nm (Fig. 1.B).

For protease activity measurement, 500 µL of purified protein was mixed with 250 µL of casein phosphate substrate and incubated at 40°C for 15 minutes. The reaction was terminated by adding 250 µL of 10% trichloroacetic acid, followed by centrifugation (10,000 rpm, 10 min). Subsequently, 700 µL of supernatant was mixed with 700 µL sodium carbonate and 175 µL Folin-Ciocalteu reagent, incubated at 37°C for 10 minutes, and absorbance measured at 660 nm. Enzyme activity was calculated from the tyrosine standard curve equation. Enzyme activity was calculated using the tyrosine standard curve equation (14).

Cell culture and cytotoxicity assay. Human lung fibroblast MRC-5 and epithelial adenocarcinoma A549 cells (Pasteur Institute of Iran) were cultured in RPMI 1640 medium (pH 7.2, supplemented with 10% fetal bovine serum and 40 µg/mL gentamicin) at 37°C in a humidified atmosphere containing 5% CO₂ until reaching 80% confluence.

For cytotoxicity assessment, cells (1×10^5 /well) were seeded in 96-well plates and treated with various concentrations of recombinant proteins (5-50 µg/mL) for 24 hours. Following treatment, MTT reagent (Merck, USA) was added and incubated for 3 hours at 37°C (Fig 2A). Formazan crystals were dissolved in DMSO (Invitrogen, USA), and absorbance was measured at 570 nm using an ELISA reader. All experiments were performed in triplicate (15). To establish the inflammatory model, cells were treated with

LPS (1-25 µg/mL; InvivoGen, USA) for 24 hours, and cell viability was assessed by MTT assay. Based on preliminary results, 10 µg/mL LPS was selected for subsequent experiments as it induced significant but non-lethal inflammatory responses.

Cytokine quantification. ELISA: Cells (1×10^5 /well) cultured in 24-well plates were treated with 10 µg/mL LPS for 24 hours, followed by 10 µg/mL recombinant proteins (Arazyme or TGF-Arazyme) for an additional 24 hours. Culture supernatants were collected after centrifugation (4,000 rpm, 1 min, 4°C). TNF- α , IL-1 β , IL-6, and IL-10 levels (pg/mL) were measured using commercial ELISA kits (BosterBio & RayBiotech companies, USA) according to the manufacturer's instructions. Absorbance was read at the recommended wavelength using an ELISA microplate reader.

RT-PCR. Total RNA was extracted from treated cells using a RNA extraction kit (Invitrogen, USA) following the manufacturer's protocol (16).

cDNA was synthesized from 5 µL of RNA using an oligo(dT) primer and cDNA synthesis kit (biotechrabbit, Germany). Reactions were incubated at 52°C for 60 minutes, followed by 95°C for 5 minutes to terminate the reaction (Table 1).

Quantitative real-time PCR was performed using an ABI StepOne™ thermocycler with SYBR Green qPCR Master Mix (Ampliqon, Denmark) and gene-specific primers. Thermal cycling conditions: 95°C for 2 minutes (initial denaturation), followed by 40 cycles of 95°C for 5 seconds and 60°C for 40 seconds (annealing/extension). A melt curve analysis was performed from 55°C to 95°C. Gene expression was normalized to β -actin, and fold changes were calculated using the $\Delta\Delta C_t$ method (16) (Table 2).

In vivo air pouch model. All animal procedures were approved by the Ethics Committee of Baqiyatallah University of Medical Sciences (ethical approval code: IR.BMSU.REC.1398.417). Fifteen female BALB/c mice (8 weeks old, 20-25 g) were housed under standard conditions (25°C, 12-hour light/dark cycle, free access to food and water).

Air pouch induction. On days 0 and 3, 3 mL of sterile air was injected subcutaneously into the interscapular region to create and maintain the air pouch. On day 6, inflammation was induced by injecting 1

Table 1. Ingredients used for cDNA synthesis

Contents	Final concentration	Volume (µl)
dNTP Mix (10 mM each)	1 mM (each dNTP)	2 µl
RNase Inhibitor, 40 U/µl (optional)	1 U/µl	0.5 µl
Oligo (dT)12–18 (10 µM)	1.25 µM	1 µl
5× cDNA Synthesis Buffer	1×	4 µl
RevertUP™ II Reverse Transcriptase	10 U/µl	1 µl
PCR Grade Water	-	Variable
RNA Template	500 ng	Variable
Total volume	-	20 µl

Table 2. Time schedule used to perform real-time PCR

Program	Temperature	Time	Cycles
Hold	95°C	2 min	1
Cycling (2 steps)	95°C	5 sec	40
	60°C	40 sec	
Melt	Ramp up 55°C to 95°C		1

mL of *E. coli* suspension with 10⁶ Colony Forming Unit/ml (CFU/ml) into the established air pouch.

Experimental groups (n=3 per group):

- Group 1: *E. coli* (infection control)
- Group 2: Sterile air (negative control)
- Group 3: *E. coli* + Arazyme-linker-TGFαL3 (30 µg/mL)
- Group 4: *E. coli* + Arazyme (30 µg/mL)
- Group 5: *E. coli* + Dexamethasone (1 µM; positive control)

Assessment of inflammatory parameters. Mice were sacrificed 6 days post-infection. Air pouches were lavaged with 5 mL of sterile PBS containing 1 mM EDTA. Lavage fluid was collected, and the total volume recorded. Total leukocyte counts were determined using a hemocytometer. Differential cell counts were performed on Giemsa-stained cytospin slides, with approximately 100 cells counted per slide under a light microscope (×40 magnification).

Statistical analysis. All experiments were performed in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 19 and GraphPad Prism software. Comparisons between groups were performed using one-way ANOVA followed by post-hoc

tests or Student's t-test for two-group comparisons. A p-value < 0.05 was considered statistically significant.

Ethical approval. The Ethical code of this project is IR.BMSU.REC.1398.417.

RESULTS

Successful expression and purification of recombinant proteins. The PCR products were sequenced and confirmed using the construct provided by Biomatik (data not shown). Expression of Arazyme-linker-TGFαL3 and Arazyme in *E. coli* BL21 was optimized at 37°C for 4 hours with 1 mM IPTG. Both proteins were purified from 1 L of culture using Ni-NTA affinity chromatography, yielding concentrations of 880 mg/mL and 2 mg/mL, respectively. SDS-PAGE analysis revealed distinct bands at the expected molecular weights: 59.3 kDa for Arazyme-linker-TGFαL3 and 51.5 kDa for Arazyme (Figs. 1A and B).

Proteolytic activity of purified proteins. Enzymatic activity was determined using a tyrosine standard curve. The results demonstrated that the chimeric protein retained proteolytic activity comparable to native Arazyme, with no significant difference between the two proteins. However, a significant increase in enzymatic activity was observed in purified proteins compared to unpurified forms (p < 0.05; Fig. 1D).

Selective cytotoxicity against lung cancer cells. The cytotoxicity of recombinant proteins was evaluated by MTT assay (Fig. 2A). Native Arazyme exhibited similar cytotoxicity across both cell lines,

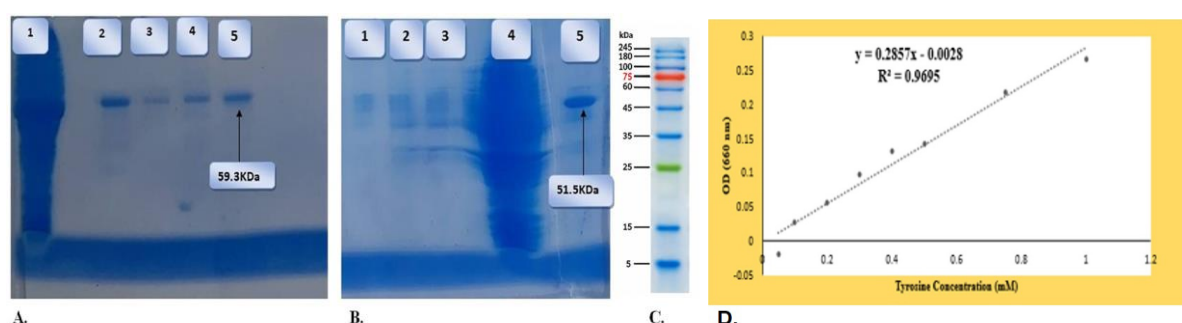


Fig. 1. SDS-PAGE purification of Arazyme-linker-TGF α L3 proteins A: 1: Denaturing wash buffer (pH=6); 2 and 3: Native wash buffer (pH=8); 4: Unpurified protein dissolved in 8 M urea; 5: (pH=8) Native elution buffer B: SDS-PAGE purification of Arazyme proteins; 1: Unpurified protein dissolved in 8 M urea; 2: Denaturing wash buffer (pH=6); 3: Native wash buffer (pH=8); 4 and 5: (pH=8) Native elution buffer C: LADDER D: Standard absorption chart at 660 nm for different concentrations of tyrosine

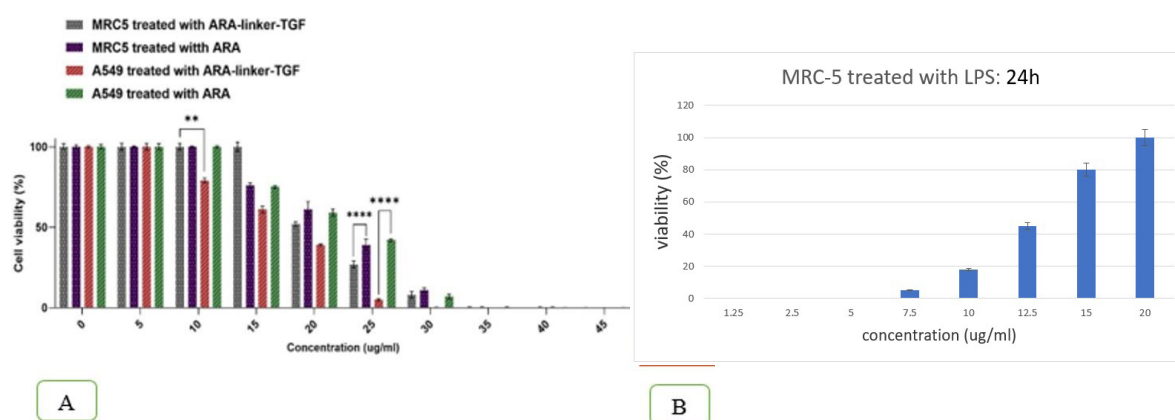


Fig. 2. A: MTT assay results for the cytotoxicity of Arazyme-linker-TGF α L3 and Arazyme on the MRC-5 and A549 cell lines B: Effect of LPS on MRC-5 Cell Viability

This figure depicts the impact of LPS on MRC-5 cell viability, as measured via the MTT assay. The graph highlights the suppression of cell viability, which is correlated with increased expression of inflammatory mediators (TNF- α , IL-6, IL-1 β , and IL-10), providing a baseline for evaluating the anti-inflammatory effects of recombinant proteins.

with no significant difference in IC50 values (MRC-5: 16.15 μ g/mL; A549: 15.89 μ g/mL). In contrast, Arazyme-linker-TGF α L3 demonstrated significantly greater cytotoxicity against A549 cells (IC50: 10.35 μ g/mL) compared to MRC-5 cells (IC50: 16.21 μ g/mL; $p < 0.01$). At concentrations >20 μ g/mL, the chimeric protein showed greater cytotoxicity in both cell lines compared to native Arazyme ($p < 0.0001$). Based on these findings, a concentration of 10 μ g/mL was selected for subsequent experiments (Table 3).

LPS reduces MRC-5 cell viability in a dose-dependent manner. Treatment of MRC-5 cells with increasing LPS concentrations (1-25 μ g/mL) for 24

hours resulted in a dose-dependent suppression of cell viability (Fig. 2B). The concentration of 10 μ g/mL LPS was selected for subsequent experiments, as it caused significant reduction in cell viability while maintaining sufficient cell populations for assessing inflammatory responses.

Modulation of inflammatory gene expression at transcriptional level: in MRC-5 cells. LPS treatment alone significantly upregulated TNF- α , IL-1 β , and IL-6 expression. Co-treatment with Arazyme-linker-TGF α L3 significantly reduced expression of all three pro-inflammatory cytokines compared to LPS alone (TNF- α : $p < 0.00001$; IL-1 β :

Table 3. Experimental groups

Row	Group	Drug/Injection Material	Amount
1	Control	Sterile air	3 and 6 ml
2	-	Bacteria	10 ⁶ cells
3	Arazyme -linker-TGF α L3 treated	Arazyme -linker-TGF α L3 and Bacterial suspension	30 μ g/ml and 10 ⁶ cells
4	Arazyme treated	Arazyme and Bacterial suspension	30 μ g/ml and 10 ⁶ cells
5	Positive Control	Dexamethasone and Bacterial suspension	1 μ M and 10 ⁶ cells

$p < 0.00001$, 55% reduction; IL-6: $p < 0.00001$). The chimeric protein demonstrated greater inhibition of IL-1 β compared to native Arazyme ($p < 0.001$). IL-10 expression was significantly increased in the Arazyme-linker-TGF α L3 + LPS group compared to LPS alone ($p < 0.001$) (Figs. 3. A-D).

In A549 cells. LPS treatment significantly increased TNF- α expression, which was dramatically suppressed by Arazyme-linker-TGF α L3 ($p < 0.00001$) and to a lesser extent by native Arazyme ($p < 0.0001$). IL-1 β expression was significantly reduced by both recombinant proteins ($p < 0.00001$). IL-6 expression was similarly decreased by both proteins ($p < 0.00001$). Notably, IL-10 expression showed a significant increase in the Arazyme-linker-TGF α L3 + LPS group ($p < 0.0001$), whereas native Arazyme and dexamethasone showed no significant effect (Figs. 3. A-D).

Suppression of pro-inflammatory cytokine secretion at protein level. ELISA results confirmed the qPCR findings with some minor differences (Figs. 4. A-D).

IL-1 β secretion: MRC-5: LPS significantly increased IL-1 β ($p < 0.0001$). Dexamethasone significantly reduced it ($p < 0.0001$), while neither recombinant protein showed significant effect.

- A549: LPS increased IL-1 β ($p < 0.05$). Arazyme-linker-TGF α L3 significantly reduced IL-1 β ($p < 0.05$), whereas native Arazyme and dexamethasone showed no significant effect.

IL-6 secretion: MRC-5: LPS significantly increased IL-6 ($p < 0.05$). Both Arazyme ($p < 0.05$) and dexamethasone ($p < 0.05$) significantly reduced IL-6, with Arazyme showing greater inhibition.

- A549: LPS increased IL-6 ($p < 0.05$). Both recombinant proteins significantly inhibited this increase ($p < 0.01$), with Arazyme-linker-TGF α L3 showing

greater reduction than native Arazyme.

TNF- α secretion: MRC-5: LPS significantly increased TNF- α . Both recombinant proteins significantly reduced TNF- α (Arazyme-linker-TGF α L3: $p < 0.00001$; Arazyme: $p < 0.05$), with the chimeric protein showing superior inhibition.

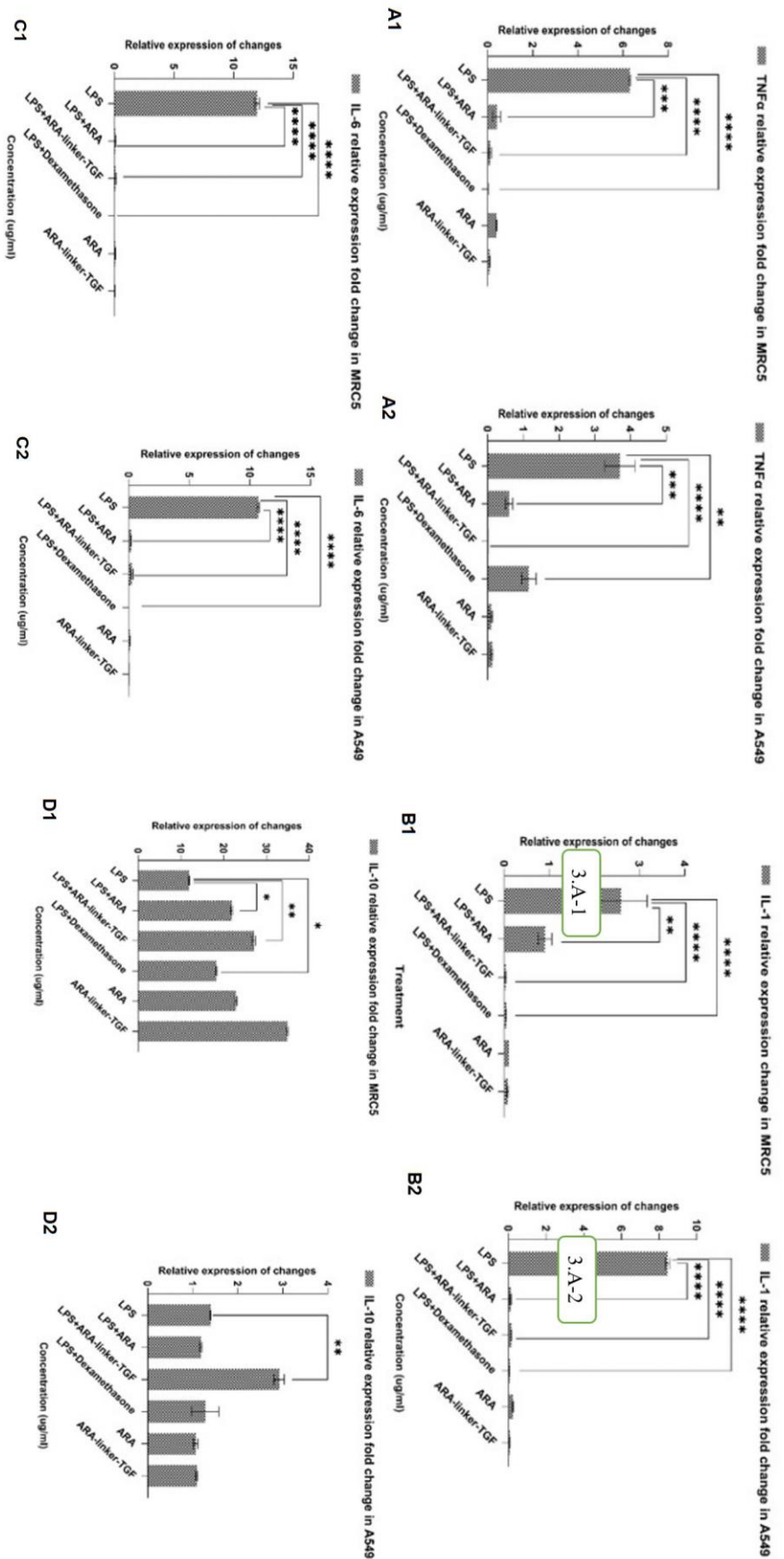
- A549: Although both recombinant proteins reduced TNF- α compared to LPS alone, the reductions were not statistically significant. Dexamethasone significantly reduced TNF- α ($p < 0.05$).

IL-10 secretion: MRC-5: LPS significantly increased IL-10. Both recombinant proteins significantly reduced IL-10 compared to LPS alone (chimeric: $p < 0.001$; Arazyme: $p < 0.05$), with dexamethasone showing the greatest reduction.

- A549: LPS markedly increased IL-10. Arazyme-linker-TGF α L3 significantly altered IL-10 secretion ($p < 0.05$), while native Arazyme and dexamethasone showed no significant effect.

In vivo efficacy in the air pouch model. In the mouse air pouch model, *E. coli* injection significantly increased leukocyte infiltration compared to the sterile air control (Figs. 5A and B). Treatment with Arazyme-linker-TGF α L3 reduced total leukocyte counts by 48.3% ($p < 0.01$) compared to the *E. coli* control group. This reduction was comparable to dexamethasone (51.2% reduction; $p = 0.32$, no significant difference between treatments). Both recombinant protein-treated groups showed significantly lower inflammation than the bacteria-only group, with the chimeric protein demonstrating efficacy comparable to the standard anti-inflammatory drug.

Exudate volume analysis revealed that bacteria-infected pouches exhibited significantly greater inflammation than sterile pouches ($p < 0.05$). Moreover, inflammation was significantly lower in Arazyme- and Arazyme-linker-TGF α L3-treated groups compared to the dexamethasone-treated group ($p < 0.005$).



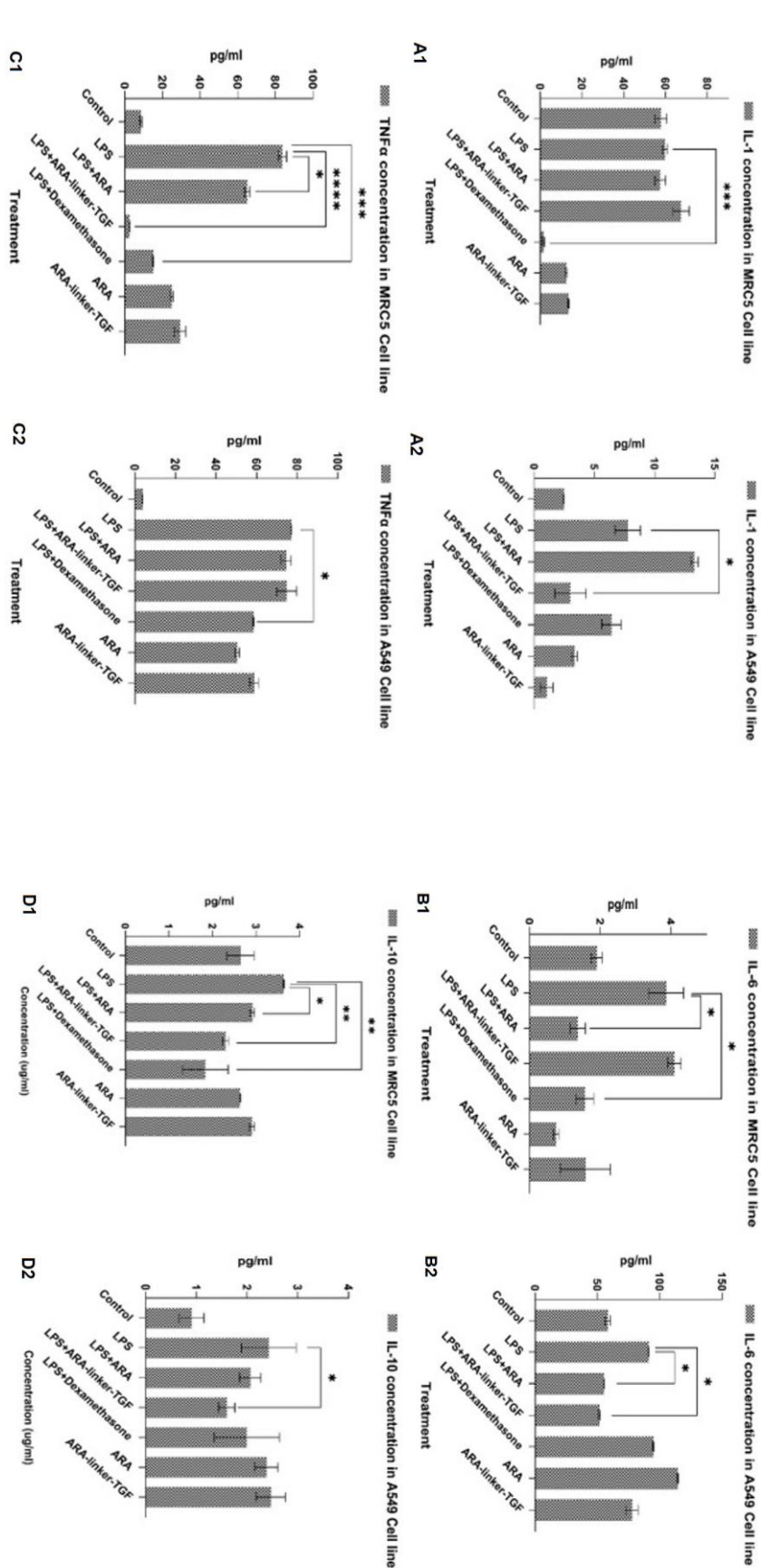


Fig. 4. A) A1: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the MRC-5 cell line
 **** indicates $p < 0.0001$

A2: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the A549 cell line
 * indicates $p < 0.05$

B) B1: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the MRC-5 cell line
 * indicates $p < 0.05$, ** indicates $p < 0.01$

B2: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the A549 cell line
 * indicates $p < 0.05$, ** indicates $p < 0.01$

C) C1: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the MRC-5 cell line
 * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$

C2: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the A549 cell line
 * indicates $p < 0.05$

D) D1: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the MRC-5 cell line
 * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$

D2: Evaluation of the effect of recombinant proteins Arazyme-linker-TGF α 3 and Arazyme at a concentration of 20 μ g/ml and comparison of targeted binding to EGFR and also after the addition of LPS at a concentration of 10 μ g/ml using ELISA in the A549 cell line
 * indicates $p < 0.05$

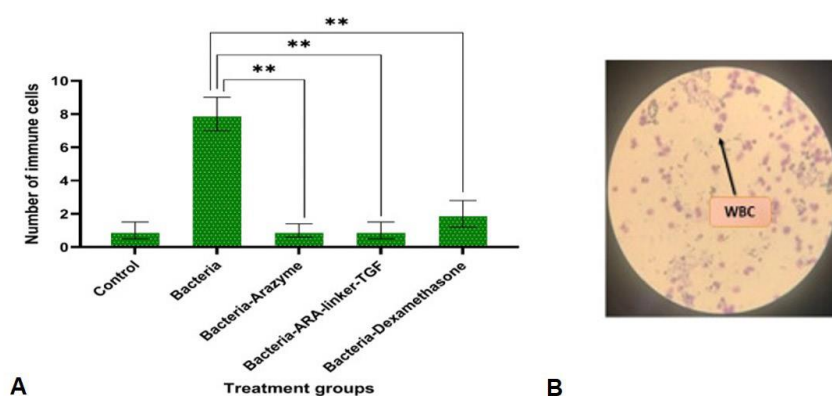


Fig. 5. A) Groups included: 1) Negative control: receiving sterile air (2 cc). 2) Positive control (inflammation): receiving *Escherichia coli* bacterial suspension (10^6 cells). 3) Chimeric protein treatment: receiving bacteria (10^6 cells) and Arazyme-linker-TGF α L3 protein at a concentration of 30 μ g/ml. 4) Arazyme treatment: receiving bacteria (10^6 cells) and Arazyme enzyme at a concentration of 30 μ g/ml. 5) Positive drug control: receiving bacteria (10^6 cells) and the standard drug dexamethasone at a concentration of 1 μ M. 24 hours after the injection of stimuli, the animals were sacrificed and air sac exudate was collected. The total number of inflammatory cells migrating into the air sac (including neutrophils and other leukocytes) was counted using a Neobar slide and under a light microscope. The results are presented as the mean number of cells per milliliter of exudate. The significant decrease in the number of cells in the groups treated with recombinant proteins and dexamethasone compared to the positive control group (bacteria alone) indicates the effective inhibition of the inflammatory response by these compounds. ($P < 0.01$).

B) The cells were observed under a 40x microscope.

DISCUSSION

Anti-inflammatory superiority and protein engineering. The present study demonstrates that the engineered Arazyme-linker-TGF α L3 fusion protein possesses significantly enhanced anti-inflammatory properties compared to native Arazyme. In LPS-stimulated MRC-5 cells, the chimeric protein markedly suppressed the major pro-inflammatory cytokines IL-1 β , TNF- α , and IL-6 while simultaneously upregulating IL-10. Native Arazyme showed weaker effects and, in some instances, exacerbated inflammatory responses in the presence of LPS.

The superior activity of Arazyme-linker-TGF α L3 is likely attributable to its dual-targeting capability. The TGF α L3 moiety enables specific binding to EGFR, which is overexpressed on activated inflammatory cells and many tumor cells, while the Arazyme component can independently interact with TLR4 on innate immune cells (14). This strategic protein engineering approach aligns with current trends in developing targeted biologics with improved therapeutic profiles (17, 18).

Selective cytotoxicity and EGFR dependency. A

key finding of this study was the selective cytotoxicity of Arazyme-linker-TGF α L3 against A549 lung tumor cells, which overexpress EGFR. The recombinant protein showed significantly greater cytotoxicity toward A549 cells (IC₅₀: 10.35 μ g/mL) compared to MRC-5 cells (IC₅₀: 16.21 μ g/mL). This cell type-specific activity underscores the importance of EGFR expression in determining therapeutic responsiveness and highlights the potential of this recombinant construct as a targeted anticancer agent (18, 19). The IC₅₀ value of 10.35 μ g/mL falls within a biologically active range for recombinant therapeutic proteins, supporting its potential for future optimization and therapeutic development (20).

Correlation between real-time PCR and ELISA data. Minor discrepancies were observed between mRNA expression and protein secretion levels for certain cytokines, particularly IL-1 β and IL-6 in A549 cells. Such differences are biologically expected and may be explained by several mechanisms: (i) post-transcriptional regulation via AU-rich elements in the 3' UTR of cytokine mRNAs; (ii) differences in protein half-lives (TNF- α : ~20 min; IL-6: ~15 h); and (iii) inherent differences in assay sensitivity (21-23).

Despite these minor variations, the overall trends were consistent across both analytical methods in both cell lines, validating the anti-inflammatory efficacy of Arazyme-linker-TGF α L3.

Preclinical validation. The in vivo effects were confirmed using a mouse air pouch model of *E. coli*-induced inflammation. Both Arazyme-linker-TGF α L3 and Arazyme significantly reduced leukocyte infiltration and exudate accumulation. Notably, Arazyme-linker-TGF α L3 reduced leukocyte infiltration by 48.3%, comparable to dexamethasone (51.2% reduction; $p=0.32$). This demonstrates that the recombinant protein is as effective as a standard anti-inflammatory drug in a localized pathophysiological setting. The enhanced efficacy observed in vivo compared to in vitro may be attributed to the presence of a diverse population of immune cells (neutrophils, macrophages, lymphocytes) in the mouse model. Many of these cells express TLR4, the primary receptor for LPS, making them responsive to modulation by Arazyme-based recombinant proteins (24, 25).

Position in targeted therapies and future directions. Our findings align with the growing body of evidence supporting EGFR-targeted fusion proteins for inflammatory diseases and cancer. The dual functionality—anti-inflammatory and cytotoxic—distinguishes Arazyme-linker-TGF α L3 as a unique candidate for EGFR-positive malignancies with inflammatory components. This represents an advantage over conventional single-function therapeutics. Future investigations should focus on: (i) clarifying the precise molecular mechanism of action, including EGFR interaction and downstream signaling pathways; (ii) comprehensive in vivo toxicology and pharmacokinetic studies to establish safety profiles; and (iii) dose-response optimization for clinical translation (26). The limitations of this study include five main points: first, the precise mechanism of action on the EGFR receptor has not been directly proven, as no experiments using specific EGFR inhibitors or inhibition assays were performed; second, the in vivo investigation was very limited, employing only a single dose (30 μ g/mL) at a single time point (6 days post-infection), whereas dose-response and time-course studies are clearly needed; third, the animal sample size in this pilot study was only three per group, while larger groups are required to detect

small effect sizes; fourth, the non-zero cytotoxicity observed in normal cells indicates that at high concentrations (50 μ g/mL), the TGF-Arazyme fusion protein reduced the viability of MRC-5 cells by approximately 40%, suggesting that further optimization of the linker or targeting domain is necessary; and finally, no pharmacokinetic data including half-life, biodistribution, and serum stability were evaluated for this recombinant protein.

CONCLUSION

In summary, the engineered Arazyme-linker-TGF α L3 fusion protein exhibits enhanced anti-inflammatory activity in normal lung cells and selective cytotoxicity against EGFR-overexpressing lung cancer cells compared to native Arazyme. Its efficacy in reducing tissue inflammation in a preclinical mouse air pouch model further supports its therapeutic potential. The dual functionality of this chimeric construct highlights the value of rational protein engineering in designing multifunctional biologics with improved specificity and efficacy. These results suggest that Arazyme-linker-TGF α L3 represents a promising dual-purpose therapeutic candidate for the treatment of lung cancer and inflammatory lung disorders. However, further mechanistic, pharmacokinetic, and toxicological investigations are required before clinical application can be considered.

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