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Dietary polyphenol ellagic acid suppresses NF- κ B signaling pathway in human synovial fibroblasts by disrupting the binding of TNF- α to its receptors: Insights from molecular interactions

Zhe Jiang^{a,b,1}, Titi Liu^{a,b,1}, Qiangqiang Zhu^{b,c,1}, Dengyou Nie^{a,b}, Ziyang Yang^{b,c}, Yun Zhang^d, Yanli Shen^e, Haiyun Ding^e, Hongkang Zhu^f, Baiqing Tian^g, Jun Sheng^{b,c}, Chenxia Zhang^{b,c,*}, Chaoyi Xue^{b,c,*}, Huanhuan Xu^{a,b,*}

^a College of Science, Yunnan Agricultural University, Kunming 650201, China

^b Key Laboratory of Development and Utilization of Food and Medicinal Resources, Ministry of Education, Yunnan Agricultural University, Kunming 650201, China

^c College of Food Science and Technology, Yunnan Agricultural University, Kunming 650201, China

^d Beijing Yunpu Tea Industry Co., Ltd, Beijing 100000, China

^e Yuanjiang County Planting Industry Development Service Center, Yuxi 653300, China

^f Food Science and Engineering, Yangzhou University, Yangzhou 215000, China

^g Yunnan Liangdao Agricultural Technology Co., Ltd, Kunming 650000, China

ABSTRACT: Tumor necrosis factor- α (TNF- α) is a key proinflammatory cytokine that drives rheumatoid arthritis (RA) pathogenesis by sustaining synovial inflammation and joint destruction via binding to its receptors (TNFR1 and TNFR2), making this interaction a core therapeutic target. Ellagic acid (EA), a dietary polyphenol naturally abundant in pomegranates, berries, and nuts, is well known for its anti-inflammatory and antioxidant properties. In this study, we investigated whether EA targets the TNF- α pathway using a combination of molecular interaction analyses and cellular assays. Surface plasmon resonance (SPR) revealed that EA binds directly to TNF- α ($K_D = 3.588 \times 10^{-6}$ M), TNFR1 ($K_D = 6.488 \times 10^{-6}$ M), and TNFR2 ($K_D = 7.952 \times 10^{-6}$ M) with high affinity. Molecular dynamics simulations and competitive SPR assays demonstrated that EA disrupts the TNF- α –TNFR interaction. Functionally, EA (0.625–2.5 μ M) suppressed TNF- α -induced apoptosis in L929 cells and inhibited TNF- α -triggered NF- κ B activation in 293-TNF- α Res (NF- κ B) cells. In RA-relevant cell models using human synovial fibroblasts (HFLS and MH7A), EA attenuated TNF- α -stimulated NF- κ B signaling by reducing phosphorylation of IKK α / β , I κ B α , and p65, and by blocking p65 nuclear translocation. Notably, EA did not inhibit LPS-induced NF- κ B activation, indicating pathway selectivity. These findings demonstrate that EA inhibits NF- κ B activation by directly targeting the TNF- α –TNFR interaction, highlighting its potential as a food-derived functional ingredient for managing RA and related inflammatory conditions.

Keywords: Ellagic acid; TNF- α ; TNFRs; NF- κ B signaling; Molecular interactions

1. Introduction

¹ These authors contributed equally to this work.

* **Corresponding authors.**

zhangchenxia2017@163.com (Chenxia Zhang),
695485093@qq.com (Chaoyi Xue),
xuhuan1017@163.com (Huanhuan Xu).

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Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by persistent synovial inflammation, driven by the infiltration of inflammatory cells, dysregulated proinflammatory cytokine networks, and pathological angiogenesis within the joint cavity^[1]. These interconnected pathological processes culminate in progressive cartilage and bone erosion, leading to joint dysfunction and eventual disability^[2]. With an estimated global prevalence of approximately 1%, RA represents a significant health burden, particularly among the aging population^[3]. Despite significant advances in immuno-targeted therapies, a considerable therapeutic challenge remains. Approximately 40% of patients do not respond adequately to single-agent regimens, and 5–20% exhibit resistance to all currently available drugs^[4, 5]. This unmet clinical need underscores the urgency of identifying new molecular targets and developing alternative therapeutic strategies. In this context, dietary polyphenols have garnered increasing attention for their ability to modulate chronic inflammation, highlighting their potential in managing inflammatory conditions like RA^[6–9].

Tumor necrosis factor- α (TNF- α) stands as a master regulator of the inflammatory cascade in RA, playing a central role in both initiating and perpetuating synovitis and joint destruction^[10]. Its biological activity is mediated through binding to two distinct receptors, TNFR1 and TNFR2, which subsequently trigger downstream signaling pathways, most notably the nuclear factor-kappa B (NF- κ B) pathway^[11]. Consequently, disrupting the TNF- α –TNFR interaction is a validated and effective therapeutic strategy for RA^[4, 12]. This inhibition can be achieved through three primary mechanisms: directly binding to the native TNF- α trimer (as with biologic agents), inducing trimer dissociation into inactive forms (e.g., SPD304), or causing conformational changes that prevent receptor engagement^[10–13]. However, current approaches, including biologics and synthetic small molecules, are often limited by inadequate efficacy, adverse effects, or toxicity, prompting a search for safer alternatives^[4, 14].

Ellagic acid (EA), a naturally occurring dietary polyphenol abundantly found in pomegranates, berries, and nuts, has demonstrated considerable therapeutic potential due to its well-documented antioxidant and anti-inflammatory properties^[15, 16]. While its applications in oncology and metabolic diseases have been extensively studied^[17], its specific role and molecular mechanism in regulating proinflammatory signaling within synovial fibroblasts, the principal effector cells driving RA pathology^[18, 19], remain insufficiently explored. Previous studies have indicated that EA can alleviate symptoms in experimental models of RA and osteoporosis^[20, 21], with its anti-inflammatory effects linked to the suppression of the NF- κ B pathway^[22]. However, it is unknown whether EA directly interferes with TNF- α binding to its receptors in human synovial fibroblasts, the initiating step of its signaling cascade.

Given the inherent ability of polyphenols to modulate protein–protein interactions through their structural properties, we hypothesized that EA might inhibit the NF- κ B signaling pathway by directly disrupting the binding of TNF- α to its receptors. From a molecular interaction perspective, this study demonstrates that EA binds with high affinity (in the low micromolar range) to both TNF- α and its receptors (TNFR1 and TNFR2). This binding effectively disrupts the TNF- α –TNFR interaction, thereby inhibiting

TNF- α -induced apoptosis and NF- κ B activation in various cell models, including RA-relevant human fibroblast-like synoviocytes (HFLS) and MH7A cells. This research highlights EA's potential as a food-derived inhibitor of the TNF- α -TNFR interaction, providing novel mechanistic insights into its anti-inflammatory properties and establishing a theoretical foundation for the development of polyphenol-rich dietary interventions or EA-based nutritional supplements for managing inflammation-related disorders.

2. Materials and methods

2.1. Reagents and antibodies

EA (CAS No.: 476-66-4) was sourced from the Natural Compound Library of the Key Laboratory of Development and Utilization of Food and Medicinal Resources, Ministry of Education (Kunming, China). MTT, Actinomycin D (ACT-D), and LPS were provided by MedChemExpress (NJ, USA). DMEM, RPMI-1640 medium, and fetal bovine serum (FBS) were purchased from BioChannel Biotechnology Co., Ltd. (Nanjing, China). Primary antibodies against p-p65, p65, p-I κ B-kinase (IKK) α/β , IKK α , IKK β , p-I κ B α , and I κ B α were provided by Cell Signaling Technology (Beverly, MA, USA). Specific antibody against β -tubulin and secondary antibodies were provided by Sino Biological Inc. (Beijing, China). All other chemicals used are indicated.

2.2. Cell culture

L929, HFLS, and MH7A cells were sourced from a certified cell repository at the Chinese Academy of Sciences. All lines were examined negative for mycoplasma contamination. These cell lines were routinely cultured at 37°C in a 5% CO₂ humidified incubator (Binder). Specifically, both L929 and MH7A cells were grown in RPMI-1640 medium containing 10% FBS, whereas HFLS cells were propagated in DMEM containing 10% FBS. We performed experiments when the cells reached approximately 80% confluence.

2.3. MTT assay

Cell viability assays of EA against different cell lines were carried out following the established MTT method^[23]. In brief, cells were distributed in 96-well plates and incubated for 12 h. Subsequently, the cells were treated with the designated concentrations of EA for 9 or 24 h. MTT solution was introduced and the plates were kept at 37°C for 4 h. After removal of the supernatant, the formazan crystals were dissolved using DMSO. Finally, the OD values at 492 nm were detected.

2.4. Flow cytometry assay

L929 cells were incubated with EA at the stated concentrations for 9 h at 37°C. Annexin V-FITC was incubated with the harvested cells in 1 $\times$ binding buffer, and then PI was added to stain the cells in the dark for 15 min. Cell apoptosis was evaluated on a FACSCalibur flow cytometer, and the resulting data were interpreted with a FlowJo software.

2.5. Luciferase reporter analysis

Luciferase reporter assay was conducted using an established protocol^[24]. In brief, 293-TNF- α Res (NF- κ B) cells (Novoprotein) were distributed into 96-well plates and allowed to adhere overnight. Treatments included adalimumab (AD; Selleck Chemicals) or EA, with or without TNF- α (10 ng/mL) stimulation, for 9 h. Finally, NF- κ B reporter activity was quantified.

2.6. Immunoblotting analysis

The quantified cell lysates were boiled in 1 $\times$ SDS loading buffer, resolved by SDS-PAGE, and then transferred to membranes. Blocked membranes were incubated with specific primary antibodies at 4°C overnight, then with appropriate secondary antibodies for 1 h at room temperature. Bands were detected using an ultra-sensitive enhanced chemiluminescent substrate kit (Biosharp) and then analyzed by an AlphaView software.

2.7. Immunofluorescence staining and nuclear translocation assay

HFLS and MH7A cells were seeded into 12-well plates or 100-mm plates and allowed to adhere overnight. The cells were then treated with or without EA (1.25 μ M) in serum free medium containing TNF- α (10 ng/mL). After 30 min, immunofluorescence staining and nuclear translocation assay were performed by standard protocols as previously published^[24]. For fluorescence imaging, pictures were captured with a fluorescence microscope and subsequently combined using ImageJ software.

2.8. Molecular dynamics (MD) simulations

The crystal structures of TNF- α (PDB ID: 6OOY), TNFR1 (PDB ID: 7KP7), and TNFR2 (PDB ID: 3ALQ) were retrieved from the Protein Data Bank (PDB). Initial structural preparation, which involved deleting water molecules and incorporating hydrogen atoms, was executed with PyMOL and AutoDock Tools. The EA structure was obtained from PubChem and converted to the PDBQT format for docking using AutoDock. For molecular docking, a grid box was defined using the Grid module in AutoDock to fully encompass the protein structure. A global search algorithm was employed with 100 independent docking runs, utilizing AutoDock 4's genetic algorithm to identify optimal binding conformations. The resulting poses were ranked by binding energy, and the conformation with the lowest energy was used to subsequent simulation.

MD simulations were conducted using GROMACS 5.0 with the AMBER99SB-ILDN force field^[25]. The protein and protein-ligand complex were solvated in a TIP3P water box, ensuring a minimum 1.0 nm distance from the solute to the box perimeter. Neutralization of the system's charge was accomplished by incorporating Na⁺ and Cl⁻ ions. An energy minimization step was carried out via the steepest descent method, succeeded by equilibration in the NVT and NPT ensembles for 100 ps each. Temperature and pressure were regulated using the Berendsen thermostat and Parrinello-Rahman barostat, respectively. A production MD simulation was subsequently run for 50 ns. Analysis after the simulation included computation of binding free energies using the gmx_MMPBSA tool. Results were visualized with R and PyMOL for structural and energetic interpretation.

2.9. Surface plasmon resonance (SPR) assays

SPR studies were examined by a Biacore S200 system and by standard protocols as previously published^[24]. In brief, TNF- α , TNFR1, and TNFR2 (Sino Biological Inc.) were immobilized on a Series S CM5 sensor chip (Cytiva). The first flow cell was prepared to be presented as the reference channel. The EA sample was diluted in the running buffer (PBS-P supplemented with 5% DMSO) and injected for 90 s. Upon sample injection, the sensor surface was rinsed with running buffer for 90 s to observe dissociation. The kinetic data were assessed by a build-in evaluation software. Furthermore, solution competition assay was implemented based on the SPR “A-B-A” injection mode. In brief, the flanking solution (A) contained EA at concentrations of 0, 0.625, or 1.25 μ M, with pre- and post-sample contact periods each set to 180 s. The sample solution (B) consisted of 400 nM TNFR, either alone or mixed with EA (0.625 or 1.25 μ M), and was allowed a contact time of 180 s. The real-time SPR sensorgrams of TNF- α binding to TNFR1 and TNFR2 in the existence of varying EA concentrations were monitored.

2.10. Statistical analysis

Values are expressed as the mean $\pm$ standard error of the mean (SEM). The statistical significance of the data was analyzed from at least three independent experiments using One-way ANOVA with Tukey's *post hoc* test. Significant differences between two groups were considered to be $P < 0.05$.

3. Results and discussion

3.1. EA suppresses TNF- α -induced apoptosis in L929 cells

To investigate whether EA could functionally inhibit TNF- α , we first employed L929 cells, a model highly susceptible to TNF- α -induced apoptosis and thus ideal for screening TNF- α functional inhibitors^[26]. The chemical structure of EA is shown in Fig. 1A. Before evaluating its protective effects, we assessed the intrinsic cytotoxicity of EA. Treatment with EA alone (0.625–2.5 μ M) showed no significant effect on L929 cell viability compared to the control (Fig. 1B), confirming its inertness under these conditions and excluding potential interference from cytotoxic effects in subsequent experiments.

To induce cell apoptosis, we used a standard model combining ACT-D and TNF- α ^[24, 27, 28]. As shown in Fig. 1C, treatment with ACT-D + TNF- α significantly reduced cell viability compared to the ACT-D alone group. However, co-treatment with increasing concentrations of EA (0.625, 1.25, and 2.5 μ M) resulted in a significant and concentration-dependent recovery of cell viability. This indicates that EA can alleviate TNF- α -induced cell damage. Morphological observations under an optical microscope corroborated these quantitative data. Cells in the ACT-D + TNF- α group displayed typical necrotic changes, including membrane rupture, cell shrinkage, and reduced density. In contrast, these abnormalities were markedly alleviated in EA-treated groups, with morphology at 2.5 μ M EA closely resembling the normal control (Fig. 1D and E).

Quantitative analysis of apoptosis by flow cytometry further confirmed these findings. A marked increase in apoptotic cells was observed following ACT-D + TNF- α treatment compared to the control or ACT-D alone conditions. Upon EA treatment, the TNF- α -induced apoptosis rate decreased in a

concentration-dependent manner, with a particularly notable reduction in late apoptotic cells (Fig. 1F and G). Collectively, these data confirm that EA inhibits TNF- α -induced apoptosis in L929 cells.

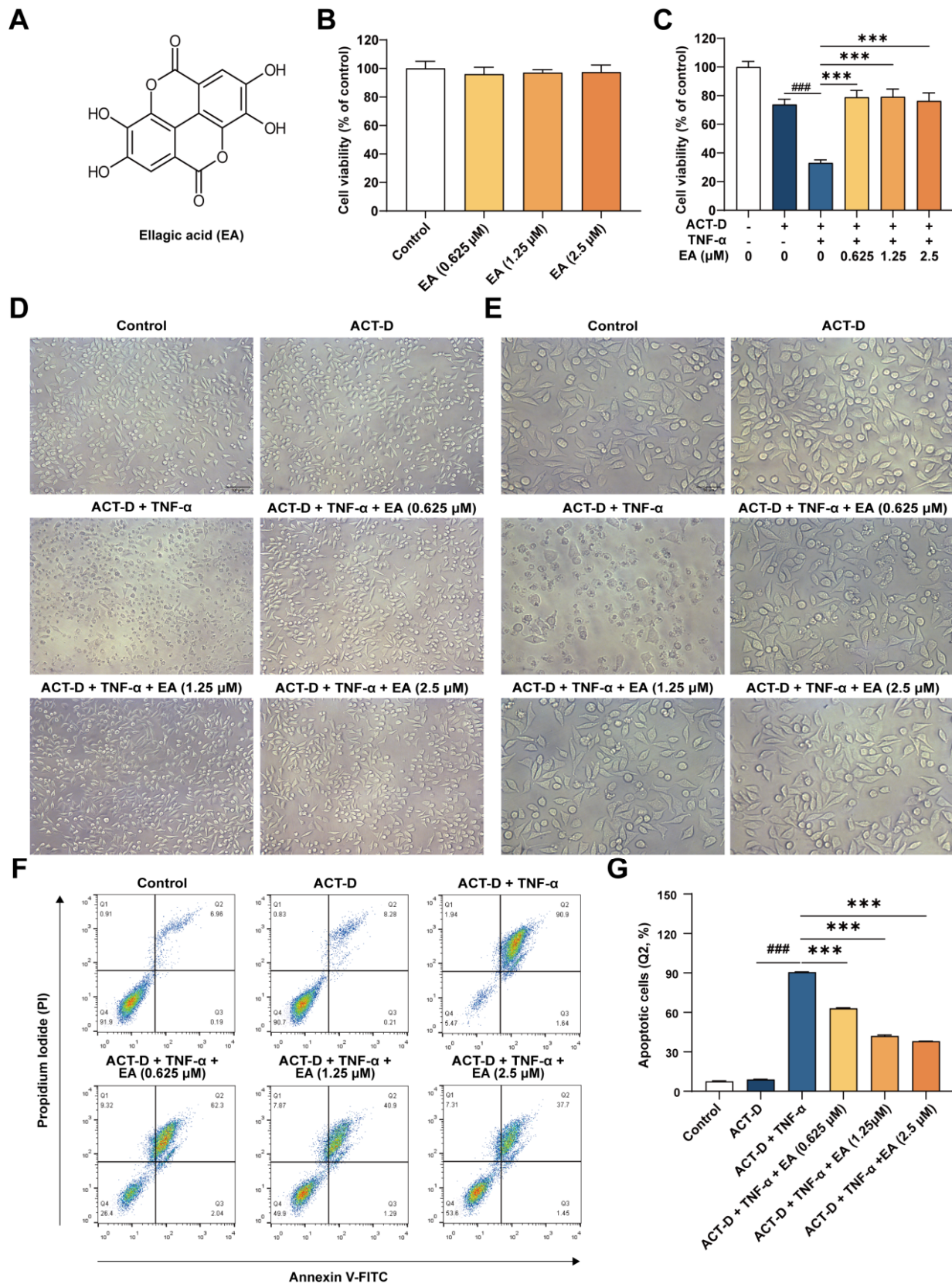


Fig. 1 EA mitigates TNF- α -induced apoptosis in L929 cells. (A) The chemical structure of EA. (B) Cytotoxicity assessment of EA in L929 cells. (C) EA protects L929 cells from TNF- α -induced apoptosis. (D, E) Morphological examination of L929 cells under a light microscope at 200 $\times$ (D, scale bar = 100 μ m) and 400 $\times$ (E, scale bar = 50 μ m) magnification. (F) Quantification of apoptotic cells by flow cytometry using Annexin V-FITC/PI staining. (G) The percentage of late apoptotic cells. ### $P < 0.001$; *** $P < 0.001$.

3.2. EA suppresses TNF- α -induced NF- κ B activation in 293-TNF- α Res (NF- κ B) cells

Given that apoptosis is frequently associated with dysregulated inflammatory signaling^[19], and NF- κ B is a core transcription factor mediating such processes^[23], we hypothesized that EA might exert its protective effects by modulating this pathway. To determine whether EA affects TNF- α -induced NF- κ B transcriptional activity, we performed a dual-luciferase reporter assay in 293-TNF- α Res (NF- κ B) cells^[15, 29]. As shown in Fig. 2A, treatment with EA alone at various concentrations did not significantly alter NF- κ B activity, indicating that EA itself does not activate this pathway. While TNF- α stimulation potently increased luciferase activity, this activation was significantly inhibited by co-treatment with either the TNF- α antagonist AD or increasing concentrations of EA.

Since NF- κ B transcriptional activation depends on p65 subunit phosphorylation (p-p65), we next examined the p-p65/p65 ratio. Western blot analysis revealed that TNF- α treatment markedly increased this ratio, confirming pathway activation (Fig. 2B). Consistent with the reporter assay, co-treatment with EA gradually reduced the p-p65/p65 ratio in a concentration-dependent manner. These results demonstrate that EA effectively blocks TNF- α -induced NF- κ B activation by downregulating p65 phosphorylation. Having confirmed this regulatory effect in a basic cell model, we proceeded to validate its applicability in disease-relevant models for RA.

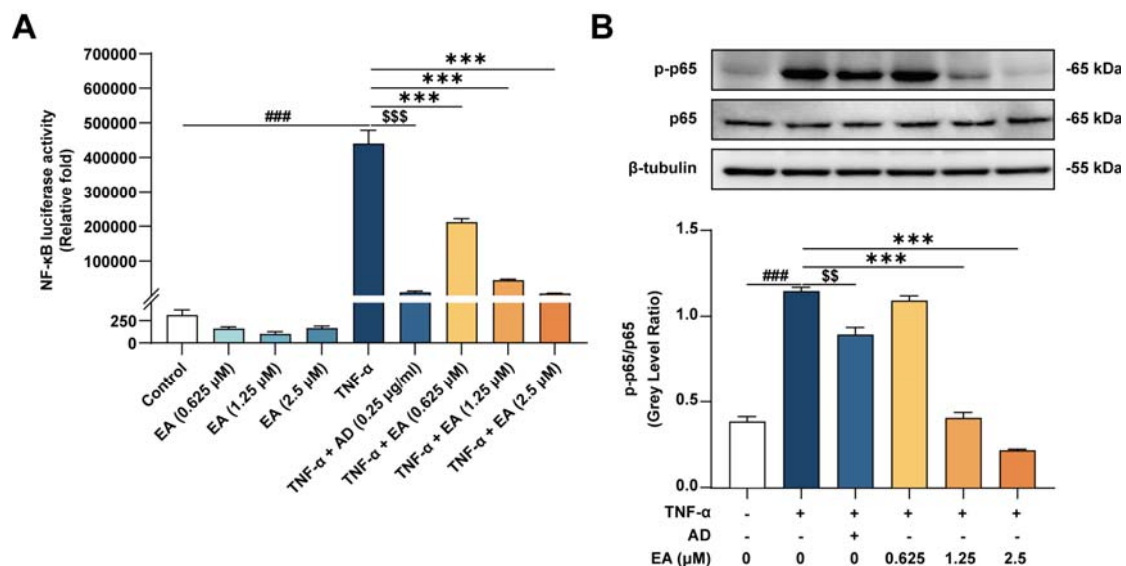


Fig. 2 EA blocks TNF- α -induced NF- κ B activation. (A) NF- κ B luciferase reporter assay. (B) Immunoblot analysis of p-p65 and total p65 in 293-TNF- α Res (NF- κ B) cells. ### $P < 0.001$; \$\$ $P < 0.01$, \$\$\$ $P < 0.001$; *** $P < 0.001$.

3.3. EA suppresses TNF- α -stimulated NF- κ B signaling pathway in synovial fibroblasts

Given that aberrant activation of synovial fibroblasts is a core driver of RA pathology^[2], we employed HFLS and MH7A cells to systematically evaluate EA's regulatory effect on the TNF- α -induced NF- κ B cascade^[30, 31]. Cytotoxicity assays first confirmed that EA (0.625–2.5 μ M) was non-toxic to both cell types (Fig. 3A and B). Based on this, a concentration of 1.25 μ M EA was selected for subsequent mechanistic studies.

To map the inhibitory nodes within the pathway, we examined the phosphorylation of key signaling molecules: IKK α / β , I κ B α , and p65^[15]. Western blot analysis showed that TNF- α treatment significantly

enhanced the band intensities of p-IKK α / β , p-I κ B α , and p-p65 in both HFLS and MH7A cells (Fig. 3C), indicating successful pathway activation. Notably, co-treatment with EA gradually attenuated these phosphorylation signals over time. Quantitative grayscale analysis in HFLS cells (Fig. 3D) revealed that EA significantly reduced the ratios of p-IKK α / β to IKK α / β (at 5, 15, and 30 min), p-I κ B α to I κ B α (at 5, 30, and 60 min), and p-p65 to p65 (at 15, 30, and 60 min). A similar inhibitory trend was observed in MH7A cells (Fig. 3E), where EA treatment led to significant reductions in the phosphorylation ratios of IKK α / β (at 30, and 60 min), I κ B α (at 0, 5, 15, 30, and 60 min), and p65 (at 5, 15, 30, and 60 min). These findings collectively indicate that EA can block TNF- α -induced NF- κ B signal transduction, thereby inhibiting the inflammatory activation of synovial cells.

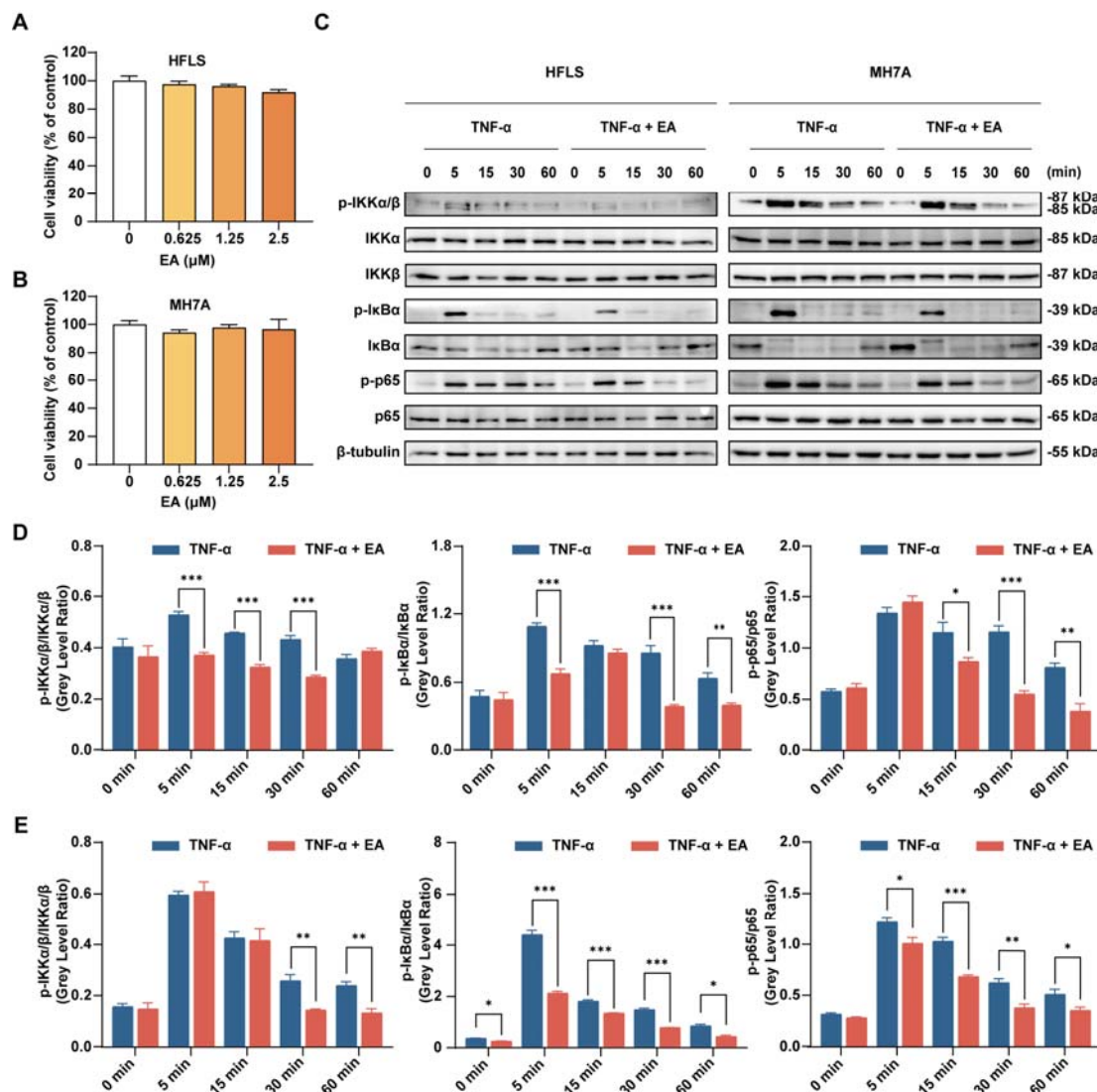


Fig. 3 EA suppresses TNF- α -stimulated NF- κ B signaling pathway in human synovial fibroblasts. Cytotoxicity of EA in HFLS (A) cells and MH7A (B) cells. (C) Immunoblot analysis of key NF- κ B signaling molecules. Quantitative grayscale analysis of these blots is shown for HFLS (D) and MH7A (E) cells. * P < 0.05, ** P < 0.01, *** P < 0.001.

3.4. EA does not suppress LPS-stimulated NF- κ B activation

To explore the selectivity of EA's inhibitory effect, we investigated its ability to suppress NF- κ B pathway activation triggered by lipopolysaccharide (LPS), which initiates inflammation through distinct receptor-mediated pathways (TLR4)^[24]. As shown in Fig. 4, western blot analysis revealed that LPS (10

ng/mL) treatment rapidly and significantly increased p65 phosphorylation in both HFLS and MH7A cells. In contrast to its effect on TNF- α signaling, EA treatment did not inhibit LPS-induced p65 phosphorylation. This lack of effect suggests that EA's action is selective for the TNF- α pathway, likely because it acts at the very proximal step of ligand-receptor interaction, a mechanism which is circumvented by LPS's activation of downstream signaling through its own receptor.

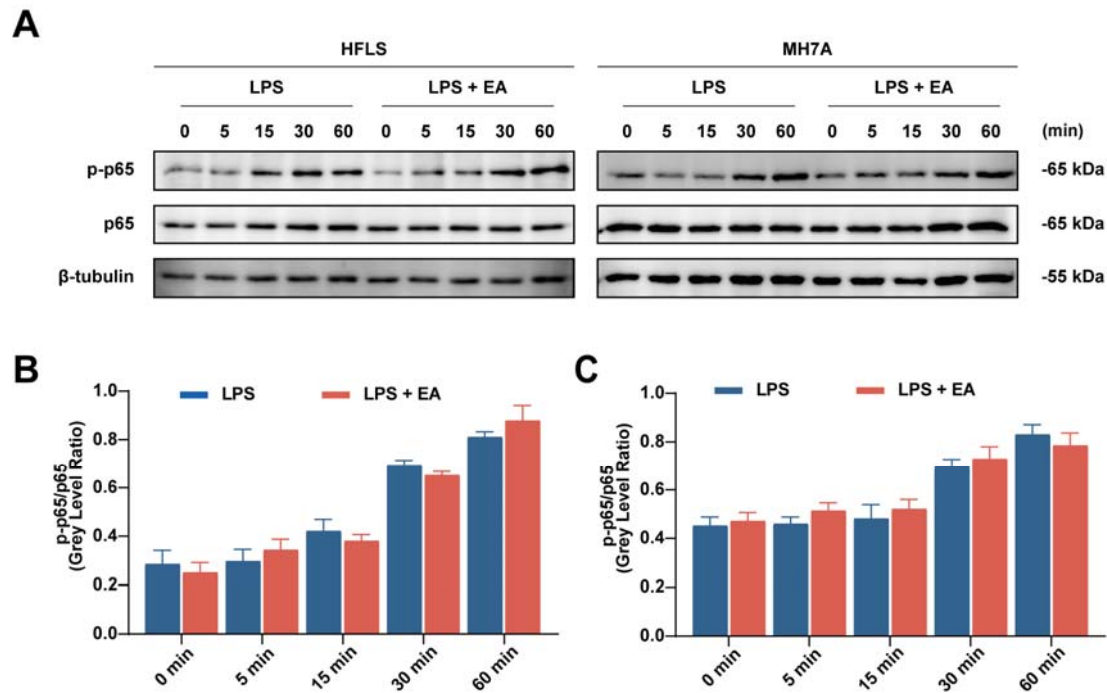


Fig. 4 EA does not inhibit LPS-induced NF- κ B activation. (A) Immunoblot analysis of p65 phosphorylation. Quantification of the p-p65/p65 ratio is shown for HFLS (B) and MH7A (C) cells.

3.5. EA suppresses TNF- α -stimulated p65 nuclear translocation

The phosphorylation and subsequent nuclear translocation of p65 represent the terminal critical event for NF- κ B pathway activation^[29]. To complete the mechanistic evidence chain, we assessed the impact of EA on TNF- α -induced subcellular redistribution of p65 using immunofluorescence and western blot.

Immunofluorescence analysis showed that TNF- α treatment significantly promoted the translocation of p65 from the cytoplasm to the nucleus in both HFLS and MH7A cells (Fig. 5A and B). However, in cells co-treated with EA, the nuclear fluorescence intensity of p65 was markedly reduced, indicating that EA effectively blocks this translocation event. These morphological observations were confirmed by western blot analysis of subcellular fractions. In HFLS cells (Fig. 5C), TNF- α stimulation significantly decreased cytoplasmic p65 while increasing nuclear p65. Co-treatment with EA significantly reversed this effect, restoring cytoplasmic p65 levels and reducing nuclear p65. An identical trend was observed in MH7A cells (Fig. 5D). These results demonstrate that EA effectively inhibits TNF- α -triggered p65 nuclear translocation, further supporting its blocking role at the terminal stage of NF- κ B signal activation.

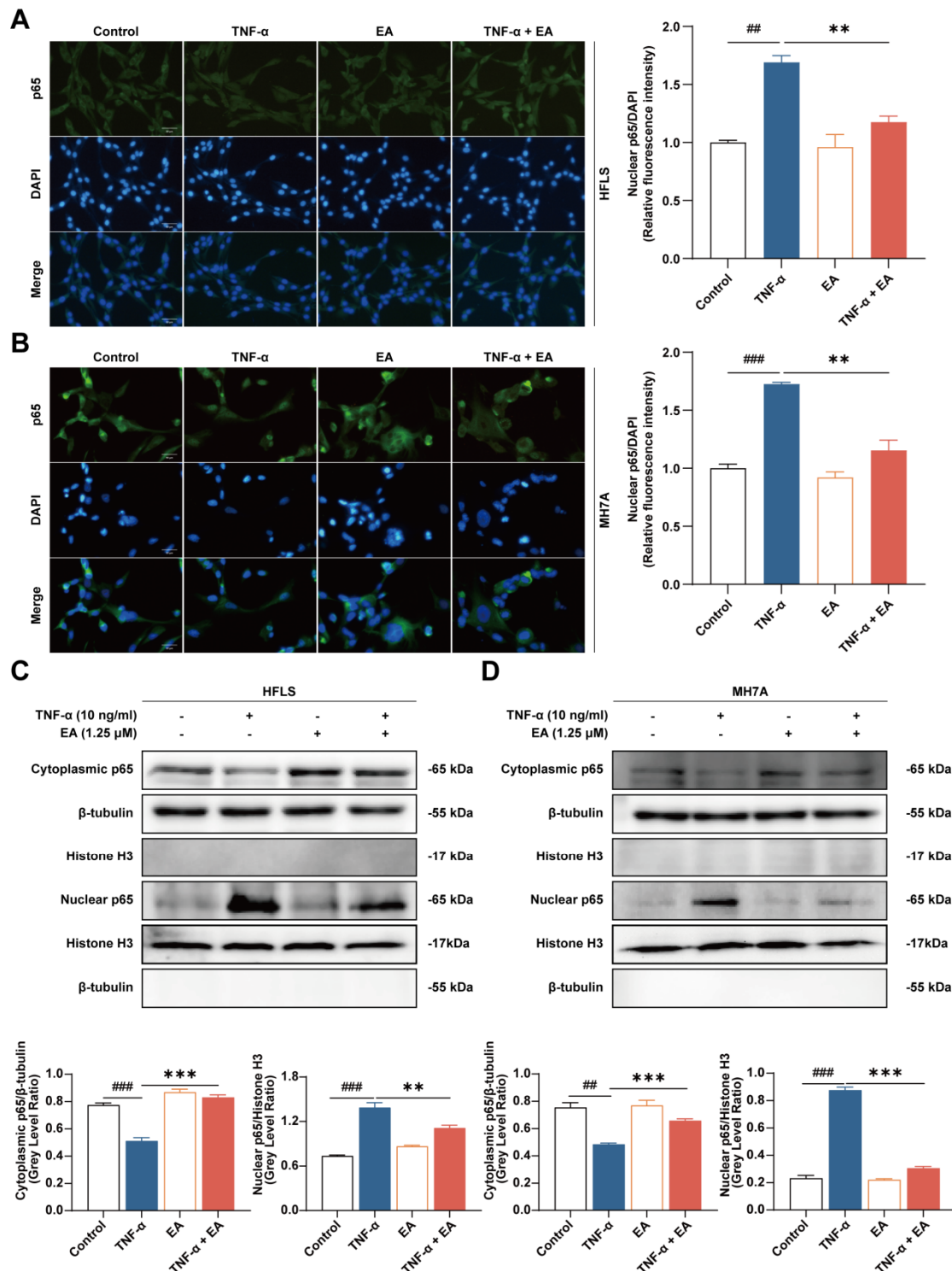


Fig. 5 EA blocks TNF- α -induced p65 nuclear translocation. (A, B) Immunofluorescence staining of p65 in HFLS (A) and MH7A (B) cells. Scale bar = 50 μ m. (C, D) Cytoplasmic and nuclear extracts were immunoblotted for p65 in HFLS (C) and MH7A (D) cells. β -tubulin and Histone H3 served as loading controls for the cytoplasmic and nuclear fractions, respectively. ## $P < 0.01$, ### $P < 0.001$; ** $P < 0.01$, *** $P < 0.001$.

3.6. EA blocks the interaction between TNF- α and TNFRs as revealed by MD simulations

To investigate whether EA disrupts the TNF- α -TNFR interaction at the pathway's initiation point, we employed computational molecular simulations^[32, 33]. Molecular docking indicated that EA could successfully bind to the TNF- α -TNFR complexes, forming stable ternary structures. To validate these results and analyze conformational dynamics, we performed 50-nanosecond (ns) MD simulations. Fig. 6A

and B display the final conformations of the TNF- α -TNFR1-EA and TNF- α -TNFR2-EA complexes. Throughout the simulation, EA remained stably bound within the interfacial cavity formed by TNF- α and the receptors, with no dissociation observed.

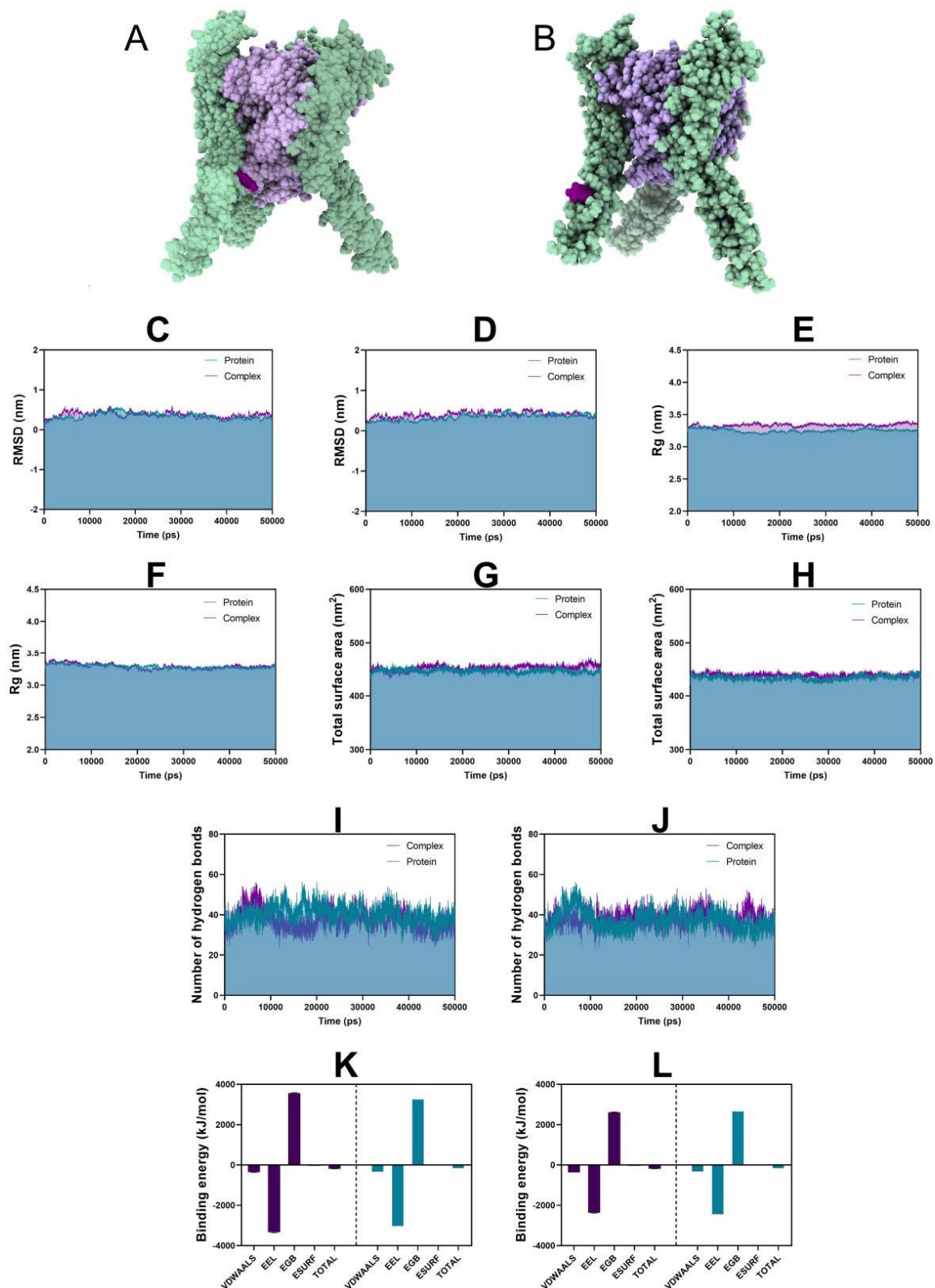


Fig. 6 MD simulations reveal EA disrupts TNF- α -TNFR interactions. Final conformations of the MD simulations for the TNF- α -TNFR1-EA (A) and TNF- α -TNFR2-EA (B) complexes. RMSD values of TNF- α -TNFR1-EA (C) and TNF- α -TNFR2-EA (D). Rg values of TNF- α -TNFR1-EA (E) and TNF- α -TNFR2-EA (F). Total surface area values of TNF- α -TNFR1-EA (G) and TNF- α -TNFR2-EA (H). Hydrogen bond counts of TNF- α -TNFR1-EA (I) and TNF- α -TNFR2-EA (J). Binding energies of TNF- α -TNFR1-EA (K) and TNF- α -TNFR2-EA (L).

The structural stability of the complexes was confirmed by several parameters. The root mean square deviation (RMSD) remained within a low range of 0–1 nm (Fig. 6C and D), the radius of gyration (Rg) remained stable between 3.0–3.5 nm (Fig. 6E and F), and the total surface area curves for the complexes and proteins almost overlapped with minimal fluctuation (Fig. 6G and H), indicating compact and well-folded structures. Compared to EA-free systems, the EA-bound complexes exhibited a higher and more stable number of hydrogen bonds (Fig. 6I and J), suggesting that EA enhances binding strength through multi-site interactions. Van der Waals forces and electrostatic interactions were also key contributors in the energy decomposition analysis (Fig. 6K and L). Critically, the introduction of EA reduced the absolute binding free energy between TNF- α and its receptors (from -202.24 ± 2.09 to -190.78 ± 2.83 kJ/mol for TNFR1, and from -197.55 ± 1.84 to -188.08 ± 1.74 kJ/mol for TNFR2). The decrease in negative value indicates that EA's intervention weakens the original affinity between TNF- α and TNFRs, effectively inhibiting their interactions.

3.7. EA directly binds to TNF- α and TNFRs with high affinity and disrupts their interactions as validated by SPR studies

We next used SPR technology to quantitatively validate the molecular interactions observed *in silico*^[34, 35]. Fig. 7A–C present SPR sensorgrams showing that EA binds to immobilized TNF- α , TNFR1, and TNFR2 in a dose-dependent manner, with response units (RU) increasing over 0–90 seconds and peaking at 25 μ M. This confirms the specific binding of EA to all three targets. Kinetic analysis revealed that EA exhibited a higher association rate constant (K_a) with TNF- α ($1.716 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) than with TNFR1 ($7.789 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$) or TNFR2 ($6.657 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$). The dissociation rate constants (K_d) were low for all complexes (ranging from 5.053×10^{-3} to $6.157 \times 10^{-3} \text{ s}^{-1}$), indicating high stability. The equilibrium dissociation constants (K_D), a measure of binding affinity, were calculated to be $3.588 \times 10^{-6} \text{ M}$ for TNF- α , $6.488 \times 10^{-6} \text{ M}$ for TNFR1, and $7.952 \times 10^{-6} \text{ M}$ for TNFR2. These low micromolar affinities confirm that EA binds with high affinity to both the cytokine and its receptors. Notably, EA's K_D for TNF- α is significantly lower than those reported for other natural compounds like emodin (26.49 μ M)^[26] and quercetin (61.76 μ M)^[36], highlighting its superior binding strength. Furthermore, its slightly higher affinity for TNFR1 over TNFR2 suggests a potentially stronger regulatory effect on TNFR1-mediated proinflammatory signaling.

To directly test whether EA competes for the TNF- α –TNFR interaction, we performed SPR competitive binding assays using the “A-B-A” injection mode. As shown in Fig. 7D, a strong binding response was observed when TNFR1 was injected over immobilized TNF- α (blue curve). However, when TNFR1 was pre-mixed with increasing concentrations of EA (0.625 or 1.25 μ M), the binding response was significantly reduced (red and green curves). A similar competitive inhibition was observed for the TNF- α –TNFR2 interaction (Fig. 7E). These results provide direct evidence that EA competitively inhibits the binding of TNF- α to its receptors.

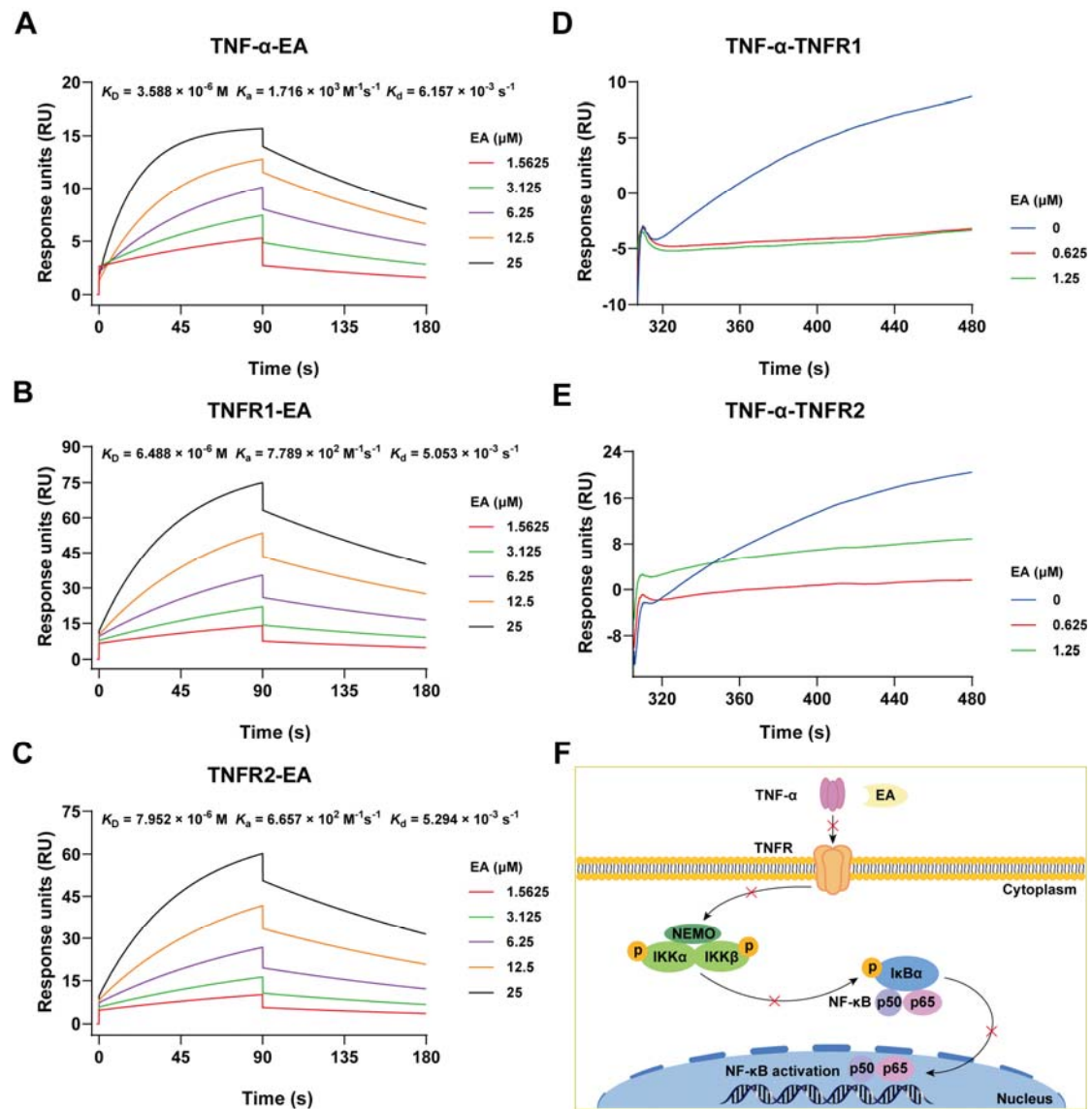


Fig. 7 EA directly binds to TNF- α and its receptors with high affinity and disrupts their interactions. (A–C) SPR sensorgrams and binding affinities of TNF α –EA (A), TNFR1–EA (B), and TNFR2–EA (A) interactions. (D, E) EA disrupts the TNF- α –TNFR interaction, as determined by the SPR-based “A-B-A” competition assay. (F) A working model for targeting the TNF- α –TNFR interaction with EA to block NF- κ B signaling in human synovial fibroblasts.

Based on the classification of TNF- α inhibition strategies^[12], our data suggest a mechanism whereby EA functions primarily through direct, high-affinity binding to TNF- α and TNFRs, rather than inducing trimer dissociation or major conformational changes. This molecular intervention at the pathway’s apex blocks downstream signaling, as evidenced by the inhibition of IKK/I κ B α /p65 phosphorylation and p65 nuclear translocation, ultimately exerting an anti-inflammatory effect relevant to RA (Fig. 7F). Notably, identifying the precise binding mode and specific interaction residues of EA within the TNF- α /TNFR complex would greatly strengthen the mechanistic understanding. However, such detailed structural elucidation typically requires co-crystallization studies, which will be a focus of our future investigations. Due to the unique chemical structure of EA, it may non-specifically interact with other cytokines such as IL-6, which warrants further investigation. Additionally, future studies employing co-culture systems or *in vivo* models could further elucidate the potential immunomodulatory effects of EA on T cells and other hematopoietic lineages.

As a natural polyphenol abundant in common foods like pomegranates, strawberries, and walnuts, EA's safety and bioactivity are well-established^[37,38]. A pharmacokinetic study in healthy volunteers showed that after consuming pomegranate extract, the maximum plasma concentration (C_{max}) of free EA reached approximately 0.36 μM ^[39]. This value is lower than the concentration range used in our *in vitro* experiments, which is influenced by many factors. First, regular dietary intake may lead to accumulation in tissues like the synovium, potentially reaching higher local concentrations. Second, EA is extensively metabolized by the gut microbiota into urolithins^[40], which possess potent anti-inflammatory properties and may contribute significantly to the *in vivo* efficacy. Our findings provide a mechanistic foundation for developing EA-based functional foods or dietary supplements, where higher, standardized doses could be formulated to achieve efficacious levels. This study not only reveals a novel mechanism of EA but also lays a theoretical basis for its application in nutritional strategies for managing inflammation-related disorders.

4. Conclusion

This study demonstrates that EA, a naturally occurring dietary polyphenol abundant in pomegranates, berries, and nuts, exerts potent anti-inflammatory effects by targeting the TNF- α signaling pathway at its initiation point. EA effectively inhibits TNF- α -induced apoptosis in L929 cells and suppresses NF- κ B activation in both 293-TNF- α Res (NF- κ B) cells and RA-relevant human synovial fibroblasts. Mechanistically, EA selectively blocks TNF- α -stimulated (but not LPS-induced) NF- κ B signaling by reducing the phosphorylation of IKK α/β , I κ B α , and p65, and by preventing p65 nuclear translocation. MD simulations and SPR assays further reveal that EA binds directly to TNF- α , TNFR1, and TNFR2 with high affinity, competitively disrupting the TNF- α –TNFR interaction. These findings establish EA as a selective inhibitor of the TNF- α /NF- κ B axis and provide a mechanistic basis for its potential application as a food-derived functional ingredient in the dietary management of RA and related inflammatory conditions. Future studies focusing on its *in vivo* efficacy, gut microbiota-derived metabolites (e.g., urolithins), and tissue-specific bioavailability will further support its translational relevance in nutrition and health.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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