

Carboplatin inhibits NSCLC growth and is associated with alterations in HMGB1 expression and macrophage polarization

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Research Article

Keywords: HMGB1, chemotherapy, carboplatin, RAGE, NSCLC

Posted Date: August 28th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10614554/v1>

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Additional Declarations: No competing interests reported.

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16 **Abstract:**

17 **Background:** Platinum resistance remains a major obstacle to the effective treatment
18 of non-small cell lung cancer (NSCLC). High mobility group box 1 (HMGB1), a
19 multifunctional damage-associated molecular pattern molecule, has been implicated in

various biological processes involved in NSCLC progression, including tumor cell proliferation, invasion, and apoptosis. However, the potential relationship between HMGB1 and carboplatin responsiveness in NSCLC has not been fully elucidated. Extracellular HMGB1 can interact with the receptor for advanced glycation end products (RAGE), thereby activating multiple downstream signaling pathways, including NF- κ B and MAPK, which are associated with inflammatory and immune responses. Tumor-associated macrophages (TAMs) are important components of the tumor microenvironment and can generally be classified into the anti-tumor M1 phenotype and the pro-tumor M2 phenotype. Increasing evidence suggests that TAM polarization contributes to NSCLC progression and therapeutic response. Nevertheless, whether carboplatin influences TAM polarization through modulation of the HMGB1/RAGE axis and thereby contributes to NSCLC progression remains to be further investigated.

Methods: First, the optimal treatment conditions of carboplatin in NSCLC cells were determined using the MTT assay. HMGB1 expression was evaluated by quantitative real-time PCR (RT-qPCR), ELISA, and western blotting. Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8) and colony formation assays. Macrophage polarization was evaluated by flow cytometry based on phenotype-associated markers. For in vivo studies, an LLC tumor-bearing C57BL/6 mouse model was established to evaluate the effects of HMGB1 modulation and carboplatin treatment on tumor growth. Histopathological changes were assessed by hematoxylin and eosin (H&E) staining,

apoptosis was evaluated using the TUNEL assay, and Ki67 expression was analyzed by immunofluorescence. Immunofluorescence staining combined with fluorescence co-localization analysis was performed to examine the spatial association between HMGB1 and RAGE. All the animals were purchased from Beijing Sibefo Biotechnology Co., Ltd. All animal procedures were conducted in accordance with the guidelines of the Tianjin Medical Experimental Animal Protection Center and were approved by the Animal Ethics Committee of Yishengyuan Gene Technology (Tianjin) Co., Ltd. (Animal Use Permit No. SYXK(Jin)2021-0003; Approval No. YSY-DWLL-2025014).

Results: HMGB1 expression was elevated in NSCLC cells. Functional assays showed that HMGB1 overexpression was associated with increased proliferation and reduced apoptosis of A549 cells, whereas HMGB1 knockdown exerted the opposite effects. In addition, HMGB1 silencing and carboplatin treatment were associated with an increased proportion of M1-like macrophages and attenuated the effects induced by RAGE agonist stimulation. Collectively, these findings suggest that carboplatin treatment and HMGB1 knockdown are associated with reduced NSCLC cell proliferation, enhanced apoptosis, and alterations in macrophage polarization. In the LLC tumor-bearing mouse model, carboplatin treatment partially attenuated the tumor-promoting effects associated with HMGB1 overexpression.

Conclusion: The present study suggests that HMGB1 may contribute to NSCLC progression and influence the cellular responses to carboplatin treatment. HMGB1

knockdown was associated with reduced tumor cell proliferation, enhanced apoptosis, and alterations in macrophage polarization. These findings support a potential role for the HMGB1/RAGE axis in the interaction between NSCLC cells and the tumor immune microenvironment. Further studies are required to clarify the underlying mechanisms and to determine whether targeting HMGB1/RAGE may improve therapeutic outcomes in NSCLC.

Keywords: HMGB1, chemotherapy, carboplatin, RAGE, NSCLC

Introduction :

Lung cancer remains a major global public health challenge and is one of the leading causes of cancer-related morbidity and mortality worldwide. According to the GLOBOCAN 2022 estimates, approximately 2.48 million new lung cancer cases and 1.82 million lung cancer-related deaths were reported globally, accounting for 12.4% of all newly diagnosed cancers and 18.7% of all cancer deaths, respectively. These statistics highlight the substantial disease burden associated with lung cancer and underscore the urgent need for improved therapeutic strategies ¹. Despite substantial advances in early detection, surgical techniques, targeted therapy, and immunotherapy, lung cancer continues to be associated with poor clinical outcomes and high mortality rates. Therefore, the identification of novel therapeutic targets and the development of more effective treatment strategies remain important research priorities. Based on histopathological characteristics, lung cancer is broadly classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC accounting for

approximately 85% of all lung cancer cases². NSCLC comprises several histological subtypes, including lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and large cell carcinoma. Among these, LUAD and LUSC represent the predominant subtypes and together account for the vast majority of NSCLC cases. Due to their high incidence and clinical significance, considerable research efforts have focused on elucidating the molecular mechanisms underlying their development, progression, and therapeutic resistance³.

Current treatment strategies for NSCLC include surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy. Despite remarkable advances in molecularly targeted therapies and immune checkpoint inhibitors, platinum-based chemotherapy remains a cornerstone of treatment for patients with advanced NSCLC. Among platinum-containing agents, cisplatin and carboplatin are widely used as first-line chemotherapeutic drugs. However, the clinical efficacy of platinum-based chemotherapy is often limited by treatment-related toxicities and the development of drug resistance. Cisplatin, in particular, has been associated with a range of adverse effects, including nephrotoxicity, ototoxicity, neurotoxicity, and myelosuppression, which may compromise treatment tolerance and clinical outcomes. Consequently, a better understanding of the molecular mechanisms underlying the response to platinum-based chemotherapy is essential for improving therapeutic efficacy in NSCLC ⁴. Compared with cisplatin, carboplatin is generally associated with a more favorable safety profile and improved tolerability, making it a widely used alternative in the

treatment of NSCLC ⁵. Nevertheless, the therapeutic efficacy of carboplatin is frequently compromised by intrinsic or acquired resistance. Therefore, identifying molecular determinants of carboplatin responsiveness and developing combination strategies to enhance its antitumor activity remain important areas of investigation. Such approaches may contribute to improving treatment outcomes in patients with NSCLC.

High mobility group box-1 (HMGB1) is a non-histone nuclear protein. Depending on its subcellular localization, it exhibits different functions ⁶. The biological functions of HMGB1 are highly dependent on its subcellular localization. Within the nucleus, HMGB1 acts as a chromatin-binding protein that participates in DNA organization, transcriptional regulation, and DNA repair. Following cellular stress or stimulation, HMGB1 can translocate from the nucleus to the cytoplasm, where it has been implicated in the regulation of autophagy and secretion. Once released into the extracellular milieu through either active secretion or passive release from damaged cells, HMGB1 functions as a prototypical damage-associated molecular pattern (DAMP). Extracellular HMGB1 can interact with pattern recognition receptors, including the receptor for advanced glycation end products (RAGE), thereby triggering downstream signaling cascades involved in immune regulation, inflammation, and tumor progression ^{7,8}. It is expressed abnormally in multiple types of cancer, and is mostly expressed at a high level ⁹. Accumulating evidence indicates that HMGB1 is aberrantly expressed in lung cancer, with elevated expression observed in both clinical specimens and experimental models. Increased HMGB1 expression has been associated with

multiple malignant characteristics, including chemoresistance, tumor invasion, metastasis, and poor clinical prognosis. These findings suggest that HMGB1 plays an important role in the progression of lung cancer and may represent a potential therapeutic target ¹⁰. Previous studies have reported that HMGB1 depletion is associated with increased tumor cell apoptosis and reduced metastatic potential in lung cancer. These findings further support a role for HMGB1 in tumor progression. The receptor for advanced glycation end products (RAGE) is a multiligand pattern recognition receptor belonging to the immunoglobulin superfamily. RAGE recognizes a broad spectrum of structurally diverse ligands, including HMGB1, members of the S100 protein family, and amyloid- β peptides. The interaction between HMGB1 and RAGE has been implicated in the regulation of inflammatory responses, immune modulation, and tumor progression¹¹. The HMGB1/RAGE signaling axis has been implicated in the activation of innate immune cells, including dendritic cells and macrophages, thereby facilitating immune responses associated with immunogenic cell death (ICD). However, the biological effects of HMGB1/RAGE signaling appear to be highly context-dependent. For example, in colorectal cancer ¹², activation of this pathway has been reported to promote the recruitment of myeloid-derived suppressor cells (MDSCs), leading to suppression of T-cell activity and the establishment of an immunosuppressive tumor microenvironment. These findings suggest that the functional consequences of HMGB1/RAGE signaling may vary according to the tumor type and immune context ¹³. Overall, the biological functions of HMGB1 are highly context-dependent and cannot be simply categorized as either tumor-promoting or

tumor-suppressive. Nevertheless, accumulating clinical evidence indicates that elevated HMGB1 expression is generally associated with poor prognosis and reduced overall survival in patients with lung cancer. Tumor-associated macrophages (TAMs) are major components of the tumor microenvironment and are broadly classified into two functional phenotypes: classically activated M1 macrophages, which exhibit antitumor properties, and alternatively activated M2 macrophages, which are generally associated with tumor progression and immunosuppression ¹⁴. These macrophage subsets exhibit distinct biological functions, with M1 macrophages generally exerting antitumor effects, whereas M2 macrophages promote tumor progression and immune evasion. At both primary and metastatic tumor sites, M2-TAMs facilitate cancer progression through extracellular matrix remodeling, angiogenesis, leukocyte recruitment, and the establishment of an immunosuppressive tumor microenvironment. Accumulating evidence further indicates that increased M2 macrophage infiltration is associated with poor prognosis and reduced overall survival in patients with NSCLC ¹⁵.

In the present study, a combination of in vitro and in vivo experiments was performed to investigate the potential role of HMGB1 in the antitumor effects of carboplatin in NSCLC ¹⁶. Specifically, we examined whether HMGB1 expression was associated with macrophage polarization through the HMGB1/RAGE axis and evaluated its relationship with tumor cell proliferation, apoptosis, and the cellular response to carboplatin treatment. By integrating cellular and animal models, this study aimed to provide further insight into the potential involvement of the HMGB1/RAGE

pathway in the interaction between NSCLC cells and the tumor immune microenvironment¹⁷. In terms of animal experiments, the focus was on observing the effect of HMGB1 on the proliferation of NSCLC and its relationship with RAGE.

Materials and methods

Cell culture

Human non-small cell lung cancer cells (A549, BNCC337696), mouse lung cancer cells (LLC, BNCC100069), and human acute monocytic leukemia cells (THP-1, BNCC358410) were used in this study. A549 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. LLC cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 0.05 mM β -mercaptoethanol, and 1% penicillin/streptomycin. All cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Lentivirus production and infection

Cells were seeded into 6-well plates at a density of $1-3 \times 10^5$ cells/well in 2–4 mL of complete culture medium and incubated overnight at 37°C in a 5% CO₂ incubator. Under sterile conditions, 2 μ g of plasmid DNA was diluted in 100 μ L of serum-free medium, and 25 μ L of Lipofectamine transfection reagent was diluted separately in 100 μ L of serum-free medium. The two solutions were then combined, gently mixed, and incubated at room temperature for approximately 30 min. When the cells reached

approximately 80% confluence, they were washed twice with serum-free medium, followed by the addition of 1 mL of serum-free medium to each well. The transfection mixture was added dropwise to each well with gentle mixing to ensure even distribution, and the cells were incubated at 37°C in a 5% CO₂ incubator. The HMGB1-overexpressing stable LLC cell line was purchased from Hanheng Biotechnology (Shanghai) Co., Ltd.

Cell co-culture assay

A cell co-culture system was established using a 6-well plate equipped with cell culture inserts. THP-1 cells were seeded into the lower chamber (growth area: 9.6 cm²) at a density of 1.0×10^6 cells/well in 2.6 mL of complete culture medium. A549 cells were seeded into the upper insert (growth area: 4.67 cm²) at a density of 5.0×10^5 cells/insert in 1.5 mL of complete culture medium. When THP-1 cells reached approximately 40–50% confluence, the insert containing A549 cells was placed into the corresponding well to establish the co-culture system. The cells were then co-cultured under standard culture conditions for the indicated period before subsequent experiments.

Cell treatment

A549 cells were transfected with si-NC, si-HMGB1-1, si-HMGB1-2, si-HMGB1-3, oe-NC, or oe-HMGB1 for 24 h. Subsequently, cells were treated with carboplatin at concentrations of 0, 5, 10, 20, 40, and 80 µg/mL for 24, 48, or 72 h. To evaluate the effect of HMGB1 on the cellular response to carboplatin, A549 cells transfected with si-HMGB1-2 or oe-HMGB1 were incubated for 6 h, after which the culture medium

was replaced with fresh complete medium containing 20 µg/mL carboplatin, followed by an additional 72 h of incubation.

Quantitative real-time polymerase chain reaction (RT-qPCR)

First-strand cDNA was synthesized using the FastKing one-step reverse transcription kit (TIANGEN). Quantitative real-time PCR (RT-qPCR) was performed using 2× SYBR Green qPCR Master Mix (Servicebio). The relative mRNA expression level of HMGB1 was calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH serving as the internal control.

基因名称	序列信息
HMGB1	Forward 5'-TTGTCGGGAGGAGCATAA-3'
	Reverse 5'-GGGCGATACTCAGAGCAGAA-3'
GAPDH	Forward 5'-AATCCCATCACCATCTTCC-3'
	Reverse 5'-GAGTCCTTCCACGATACCAA-3'

基因名称	序列信息
SI-HMGB1-1	5'-CCGTTATGAAAGAGAAATGAA-3'
SI-HMGB1-2	5'-GCAGATGACAAGCAGCCTTAT-3'
SI-HMGB1-3	5'-CCCAGATGCTTCAGTCAACTT-3'

Western blotting

Total protein was extracted using RIPA lysis buffer supplemented with PMSF (BiOrigin). Protein concentrations were determined using a BCA Protein Assay Kit (Utibody). Equal amounts of protein were separated by 5–10% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and incubated overnight at 4°C with primary antibodies against HMGB1 (1:1000), Bcl-2 (1:1000), Caspase-3 (1:2000), and β -actin (1:2000). After washing, the membranes were incubated with HRP-conjugated secondary antibodies (1:8000) at room temperature for 1 h. Protein bands were visualized using a hemiScope e6100 chemiluminescence imaging system (Clinx). The expression levels of the target proteins were quantified by normalizing their band intensities to that of β -actin.

Immunofluorescence, IF

Tissue sections were immersed in xylene for 10 min, followed by a second immersion in fresh xylene for an additional 10 min. After rehydration, antigen retrieval was performed in citrate buffer using a pressure cooker for 15 min. The sections were then permeabilized with 0.5% (w/v) Triton X-100 for 10 min, blocked with bovine serum albumin (BSA), and incubated at room temperature for 1h. The sections were subsequently incubated overnight at 4°C with primary antibodies against HMGB1 (1:1000), RAGE (1:500), and Ki67 (1:1000). After three washes with PBS, the sections were incubated with the secondary antibody (1:200) for 2 h at room temperature in the dark, followed by three additional PBS washes. The nuclei were stained with DAPI for 20 min at room temperature in the dark. After a final three washes with PBS, the sections were examined and imaged using a fluorescence microscope. Fluorescence images were semi-quantitatively analyzed using ImageJ software to determine the mean

244 fluorescence intensity.

245 **Cell counting Kit-8 (CCK-8) assay**

246 Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Beyotime). A549 cells
247 were seeded into 96-well plates at a density of 4×10^3 cells per well and incubated
248 overnight at 37°C. Cells were then treated according to the designated experimental
249 groups. Subsequently, 10 µL of CCK-8 solution was added to each well, followed by
250 incubation at 37°C for 2h. The absorbance was measured at 450 nm using a microplate
251 reader.

252 **Colony formation assay**

253 Colony formation assays were performed following transfection or drug treatment.
254 A549 cells were seeded into 12-well plates at a density of 600 cells per well and cultured
255 for 7 days. The colonies were then fixed and stained with 0.1% crystal violet for 3 min.
256 Images were captured using a digital camera, and colonies exhibiting positive crystal
257 violet staining were counted.

258 **Apoptosis detection by flow cytometric analysis**

259 Cell apoptosis was evaluated using an Annexin V-FITC/PI Apoptosis Detection Kit
260 (LiankeBio) according to the manufacturer's instructions. A549 cells were stained with
261 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) at room temperature in
262 the dark for 20 min. Apoptosis was analyzed using a FACSCalibur™ flow cytometer
263 (BD Biosciences).

Animal experiments

Male C57BL/6 mice (6–8 weeks old) were purchased and maintained under specific pathogen-free (SPF) conditions, with a controlled temperature of 20–26°C, relative humidity of 40–70%, and a 12h light/dark cycle. Mice were randomly divided into four groups: control (NC), carboplatin treatment, HMGB1 overexpression, and HMGB1 overexpression combined with carboplatin treatment groups (n = 4 per group). LLC cells (1×10^6) were subcutaneously injected into the right axilla of each mouse to establish the tumor model. Carboplatin was administered intraperitoneally at a dose of 15 mg/kg, twice or three times per week depending on experimental design. Tumor growth was monitored weekly for 5 consecutive weeks, and tumor volume was calculated using the formula: $\text{volume} = (\text{length} \times \text{width}^2 \times 0.5)$. After 4 weeks of treatment, mice were anesthetized with 1% isoflurane inhalation and euthanized by cervical dislocation. Tumors were excised, weighed, and either snap-frozen and stored at -80°C or fixed in 4% paraformaldehyde for subsequent analyses.

Enzyme-linked immunosorbent assay (ELISA)

HMGB1 secretion was quantified using a human HMGB1 ELISA kit (Thermo Fisher Scientific, EEL047) according to the manufacturer's instructions. THP-1 cells were differentiated into macrophages by treatment with 185 ng/mL phorbol 12-myristate 13-acetate (PMA) for 6h and subsequently co-cultured with A549 cells or incubated with conditioned medium under the indicated experimental conditions. Cells were assigned to the following groups: control, HMGB1 knockdown, RAGE agonist (D-ribose, 10

mM; MedChemExpress, HY-W018772) treatment, carboplatin (20 µg/mL) treatment, and the corresponding combination groups. After the indicated treatments, culture supernatants were collected and centrifuged at 1,200 rpm for 10 min to remove cellular debris. HMGB1 concentrations in the supernatants were determined by ELISA according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader, and HMGB1 concentrations were calculated from the standard curve.

Hematoxylin and Eosin (H&E) staining

Tumor tissues were harvested from mice, fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned into 4-µm-thick sections. Following deparaffinization, the sections were subjected to hematoxylin and eosin (H&E) staining. Whole-slide images were acquired using a PANNORAMIC digital slide scanner (3DHISTECH), and representative images were captured using ×20 and ×40 objectives.

TUNEL assay

TUNEL staining was performed using a commercial kit (Servicebio) according to the manufacturer's instructions. Paraffin-embedded tumor sections were deparaffinized with xylene and incubated with 100µL of proteinase K (20 µg/mL) at 37°C for 20 min, followed by incubation with 50µL of equilibration buffer for 30 min. Subsequently, the sections were incubated with 56µL of TUNEL reaction mixture (recombinant TdT enzyme: CF488-dUTP labeling mixture: equilibration buffer = 1:5:50) at 37°C for 1h. The nuclei were counterstained with DAPI for 20 min at room temperature in the dark, followed by three washes with PBS. Finally, the stained sections were scanned using a

PANNORAMIC digital slide scanner (3DHISTECH, Budapest, Hungary).

Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD). All experiments were performed with three independent biological replicates. Statistical analyses were conducted using GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA). Differences among three or more groups were analyzed using one-way analysis of variance (ANOVA). A *P* value < 0.05 was considered statistically significant.

Results

Screening carboplatin concentration and upregulation of HMGB1 expression in NSCLC

Carboplatin was selected for subsequent experiments based on preliminary studies. To determine the optimal treatment concentration, A549 cells were exposed to different concentrations of carboplatin, and the half-maximal inhibitory concentration (IC₅₀) was determined at 24, 48, and 72h. As shown in Fig. 1A–B, the lowest IC₅₀ value was observed after 72h of treatment. Therefore, a concentration of approximately 20 $\mu\text{g/mL}$, corresponding to the 72-h IC₅₀, was selected for subsequent experiments. The mRNA expression of HMGB1 following carboplatin treatment at different concentrations was evaluated by RT-qPCR. In addition, HMGB1 knockdown and overexpression vectors were constructed, and their transfection efficiencies were confirmed by RT-qPCR. HMGB1 expression was markedly reduced in all knockdown groups, with the greatest reduction observed in the si-HMGB1-3 group (Fig. 1C). Therefore, si-HMGB1-3 was selected for subsequent experiments. In contrast, HMGB1 expression was significantly

increased in the HMGB1-overexpression group.

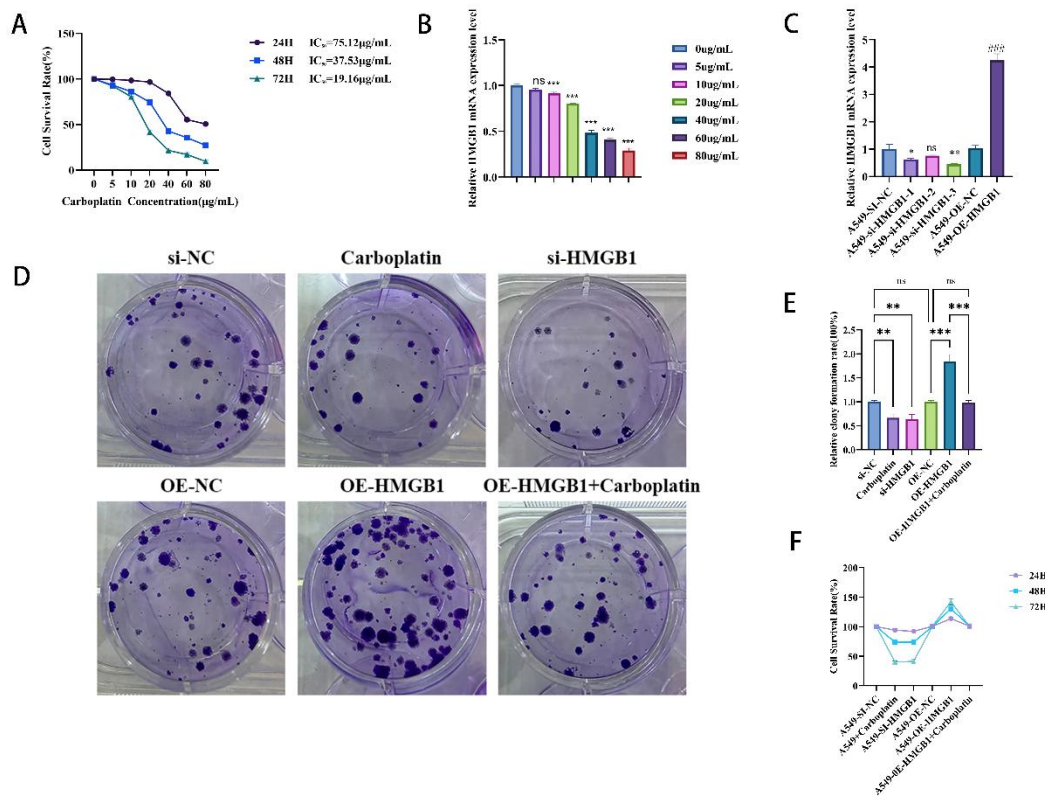


Figure 1: The expression of HMGB1 is inhibited by carboplatin, and overexpression of HMGB1 promotes tumor formation. A: The CCK-8 experiment measured the survival rate of A549 cells at different concentrations at 24, 48, and 72h. B: qPCR determined the expression efficiency of HMGB1 at different carboplatin concentrations. C: qPCR detected the efficiency of knockdown and overexpression of HMGB1. D: Colony formation experiment measured the formation ability of A549 cells in different groups. E: The overexpression of HMGB1 in the colony formation experiment group promoted tumor formation, while the addition of carboplatin inhibited tumor formation. F: The CCK-8 detection showed that knockdown of HMGB1 inhibited tumor proliferation, overexpression promoted tumor cell proliferation, and the addition of carboplatin could inhibit this process.

Overexpression of HMGB1 enhances the proliferation ability of NSCLC cells

Western blot analysis showed that HMGB1 expression was significantly increased in the HMGB1-overexpression (OE-HMGB1) group (Fig. 2C–D). Carboplatin treatment markedly reduced HMGB1 expression, including in OE-HMGB1 cells. The effects of HMGB1 on the proliferative capacity of A549 cells were further evaluated in six experimental groups. CCK-8 assays demonstrated that both carboplatin treatment and HMGB1 knockdown significantly inhibited cell proliferation (Fig. 1F), whereas HMGB1 overexpression enhanced cell proliferation. Notably, the proliferative effect induced by HMGB1 overexpression was attenuated by carboplatin treatment. Consistent with the CCK-8 results, colony formation assays showed that HMGB1 overexpression significantly increased the colony-forming ability of A549 cells, whereas carboplatin treatment effectively suppressed this effect (Fig. 1D–E).

HMGB1 inhibits the apoptotic level of NSCLC

Flow cytometry and Western blot analyses were performed to evaluate apoptosis in the different experimental groups. Flow cytometric analysis showed that both HMGB1 knockdown and carboplatin treatment significantly increased apoptosis, whereas HMGB1 overexpression reduced apoptosis and partially attenuated the pro-apoptotic effect of carboplatin (Fig. 2A–B). Consistent with these findings, Western blot analysis demonstrated that HMGB1 overexpression increased Bcl-2 expression while decreasing Caspase-3 expression (Fig. 2C–F)¹⁹.

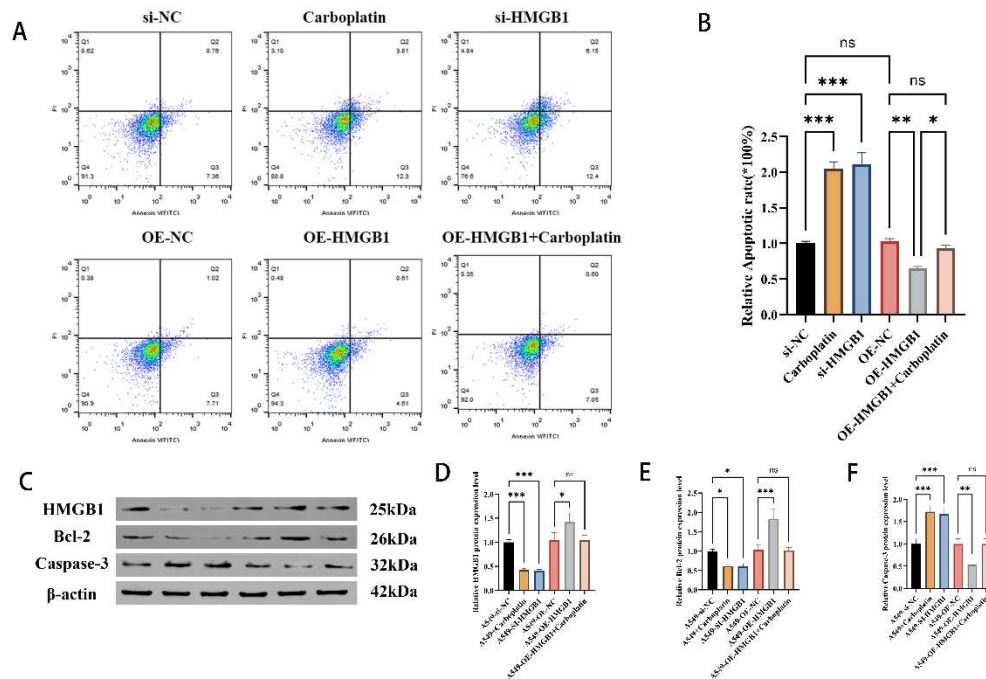


Figure 2: Expression of HMGB1 affects the apoptosis level of cells. A, B: Flow cytometry was used to verify the apoptosis rate of cells in different groups. Adding carboplatin or knocking down HMGB1 promoted the apoptosis of tumor cells, while overexpression inhibited tumor cell apoptosis. During this process, adding carboplatin could partially restore the apoptosis level. C-F: Western Blot results showed that overexpression of HMGB1 led to an increase in BCL-2 expression and a decrease in Caspase-3 expression, further indicating that HMGB1 affects cell apoptosis.

HMGB1-RAGE regulates the polarization level of tumor-associated macrophages (TAMs)

THP-1 cells were differentiated into resting macrophages (M0) using phorbol 12-myristate 13-acetate (PMA) and subsequently assigned to either the control M0 group or the HMGB1-knockdown group²⁰. Flow cytometric analysis demonstrated that HMGB1 knockdown significantly increased CD86 expression (Fig. 3A–C), whereas CD206 expression was markedly decreased (Fig. 3D–F)²¹. Morphological observation

revealed that macrophages in the HMGB1-knockdown group predominantly exhibited a polygonal morphology with irregular cell shapes (Fig. 3G). Immunofluorescence analysis showed partial co-localization of HMGB1 and RAGE (Fig. 3H–J), suggesting that HMGB1-mediated regulation of macrophage polarization may be associated with RAGE signaling²².

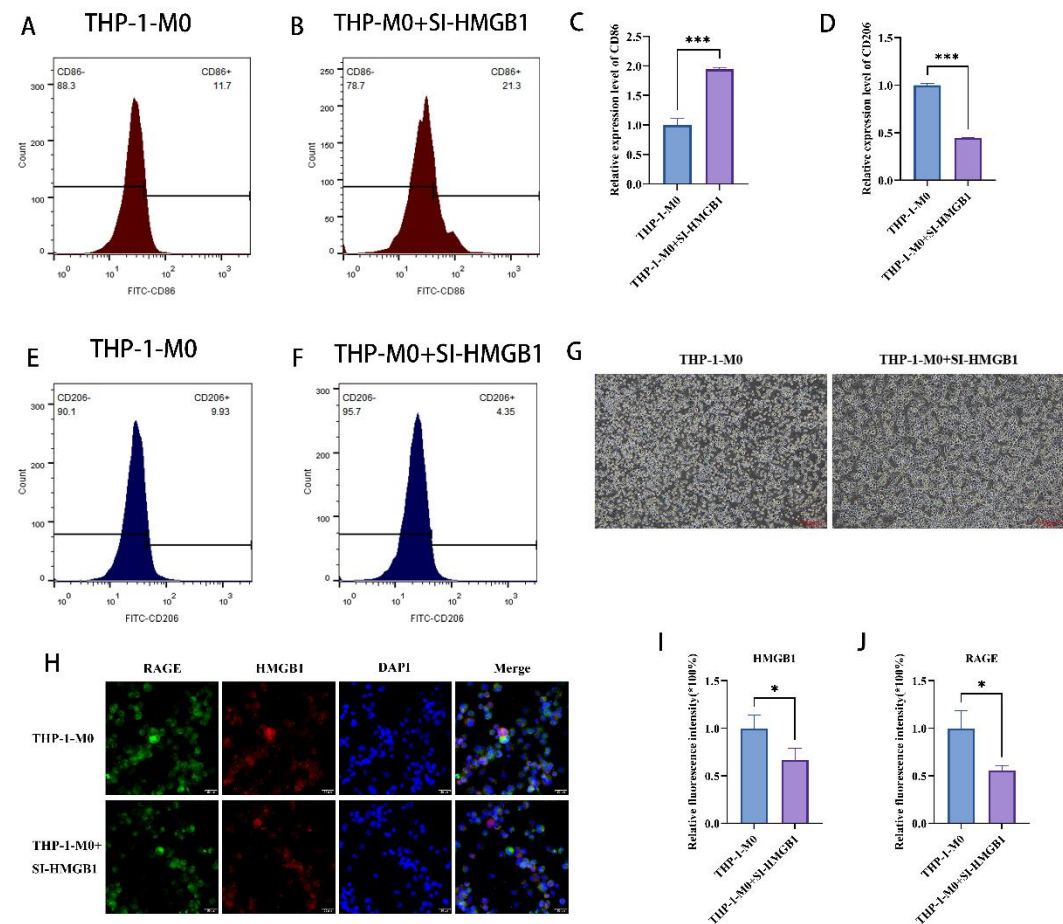


Figure 3: THP-1 cells were induced to become THP-1-M0, where CD86 is the marker for M1-type macrophages and CD206 is the marker for M2-type. Under the microscope, different morphologies of the polarized cells can be observed. A-C: Flow cytometry shows that knockdown of HMGB1 increases the expression of CD86 and promotes the polarization of cells to M1-type macrophages; D-F: Flow cytometry shows that HMGB1 inhibits the expression of CD206 and promotes the polarization of cells to M2-type macrophages; G: Under the microscope, the flat shape of the cells is irregular, consistent with the morphology of M1-type cells; H: Observation of cell fluorescence

shows the binding degree of HMGB1 and RAGE; I, J: In the fluorescence staining, the relative fluorescence intensity of HMGB1 and RAGE can reflect the expression in the HMGB1 knockdown group and the expression of RAGE in the knockdown group is also reduced.

RAGE agonist intervention on the inhibitory effect of carboplatin on NSCLC

Immunofluorescence staining showed that HMGB1 was predominantly localized in the nucleus (Fig. 3H). HMGB1 knockdown was accompanied by a reduction in RAGE expression. To further investigate the potential involvement of RAGE in HMGB1-mediated macrophage polarization, THP-1-derived macrophages were treated under the six experimental conditions shown in Fig. 4. Flow cytometric analysis demonstrated that treatment with the RAGE agonist decreased CD86 expression (Fig. 4A–B) and attenuated the promotive effect of carboplatin on M1 macrophage polarization. Similarly, although HMGB1 knockdown increased CD86 expression, this effect was partially reversed following RAGE agonist treatment. In contrast, RAGE agonist treatment increased CD206 expression (Fig. 4C–D) and partially reversed the inhibitory effect of carboplatin on M2 macrophage polarization. The reduction in CD206 expression induced by HMGB1 knockdown was also attenuated after RAGE agonist treatment. Morphological observation showed that macrophages in the RAGE agonist group exhibited a spindle-shaped morphology with elongated pseudopodia (Fig. 4E). ELISA analysis further demonstrated that RAGE agonist treatment increased HMGB1 secretion, whereas this increase was reduced following HMGB1 knockdown. In contrast, carboplatin treatment decreased HMGB1 secretion (Fig. 4F). Collectively,

these findings suggest that carboplatin suppresses HMGB1 expression, whereas RAGE activation is associated with increased HMGB1 expression and macrophage polarization. The observed effects are consistent with the involvement of HMGB1–RAGE signaling in regulating macrophage polarization.

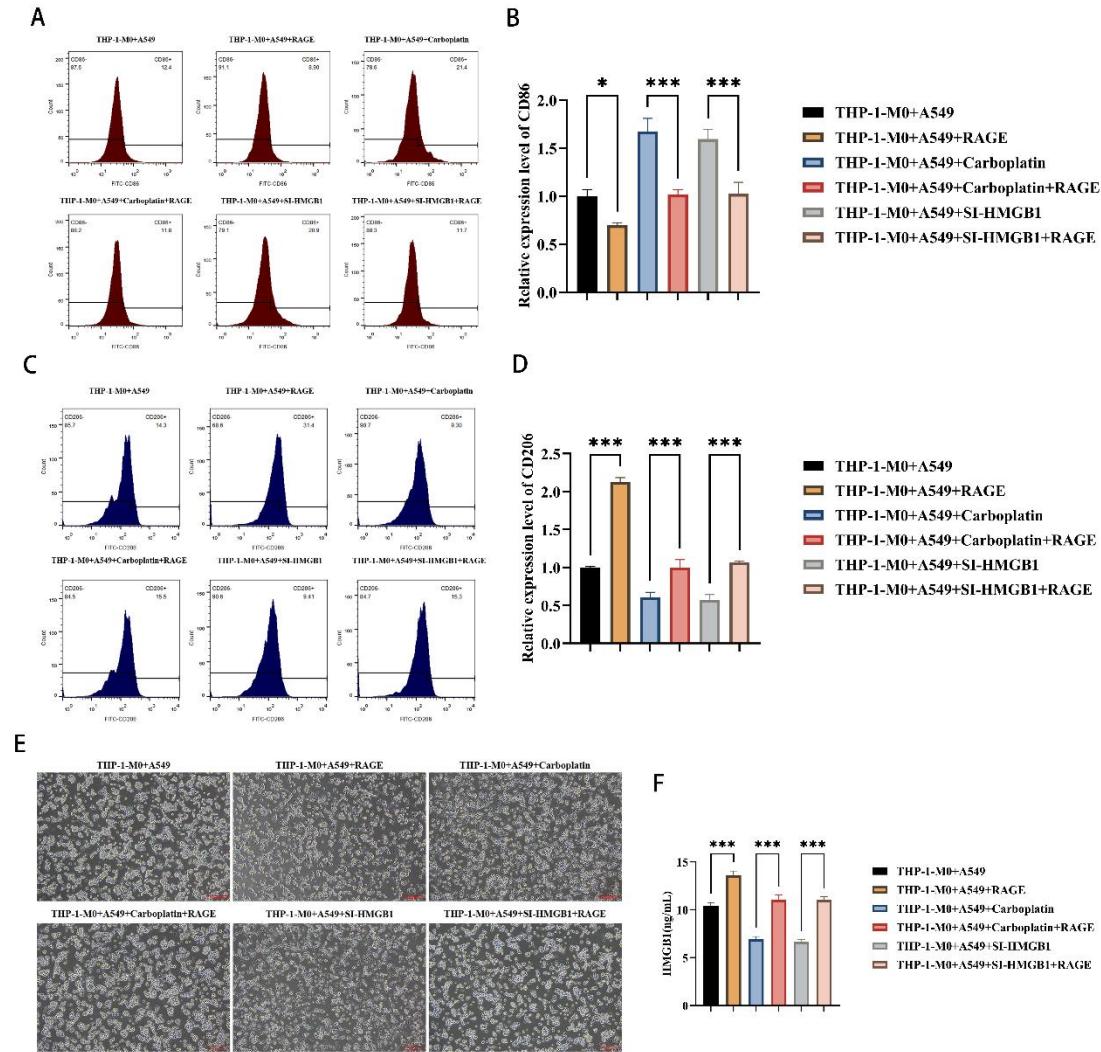


Figure 4: The expressions of CD86 and CD206 vary under different groups, and the morphological changes can also indicate the influence of HMGB1 on A549 cells. A, B: The expression of CD86 in different groups was measured by flow cytometry, which reflects the polarization level of M1-type macrophages. Under the action of RAGE agonist, the M1-type cells decreased, and after knockdown

of HMGB1, the effect of RAGE weakened; C, D: The expression of CD206 in different groups was measured by flow cytometry, which reflects the polarization level of M2-type macrophages. Under the action of the RAGE agonist, the M2-type cells increased, and the inhibitory effect of carboplatin on M2-type polarization weakened. E: Under the microscope, the cell morphologies of different groups were diverse. Cells in the RAGE group had long and obvious pseudopodia, while cells in the carboplatin group were flat and had a polygonal shape; F: ELISA was used to measure the expression levels of HMGB1 in different groups. After adding RAGE, the expression of HMGB1 also increased, and carboplatin inhibited this effect.

In vivo experiments have verified that the intervention of HMGB1 binding to RAGE on the therapeutic effect of carboplatin, and it promotes the proliferation ability of NSCLC

An LLC xenograft model was established in C57BL/6 mice to evaluate the effects of carboplatin and HMGB1 overexpression on tumor growth (Fig. 5A). Tumor growth curves and tumor weights showed that carboplatin treatment significantly inhibited tumor growth, whereas HMGB1 overexpression partially reversed this inhibitory effect (Fig. 5B–C). H&E staining further demonstrated increased tumor cell density in the HMGB1-overexpression group compared with the carboplatin-treated group (Fig. 5D). Consistent with these findings, TUNEL staining revealed that HMGB1 overexpression significantly reduced tumor cell apoptosis compared with carboplatin treatment alone (Fig. 5E–F), indicating attenuation of the pro-apoptotic effect of carboplatin. Collectively, the in vivo findings were consistent with the in vitro results, suggesting

that HMGB1 overexpression antagonizes the antitumor effects of carboplatin. The observed effects are consistent with the involvement of HMGB1–RAGE signaling in NSCLC progression.

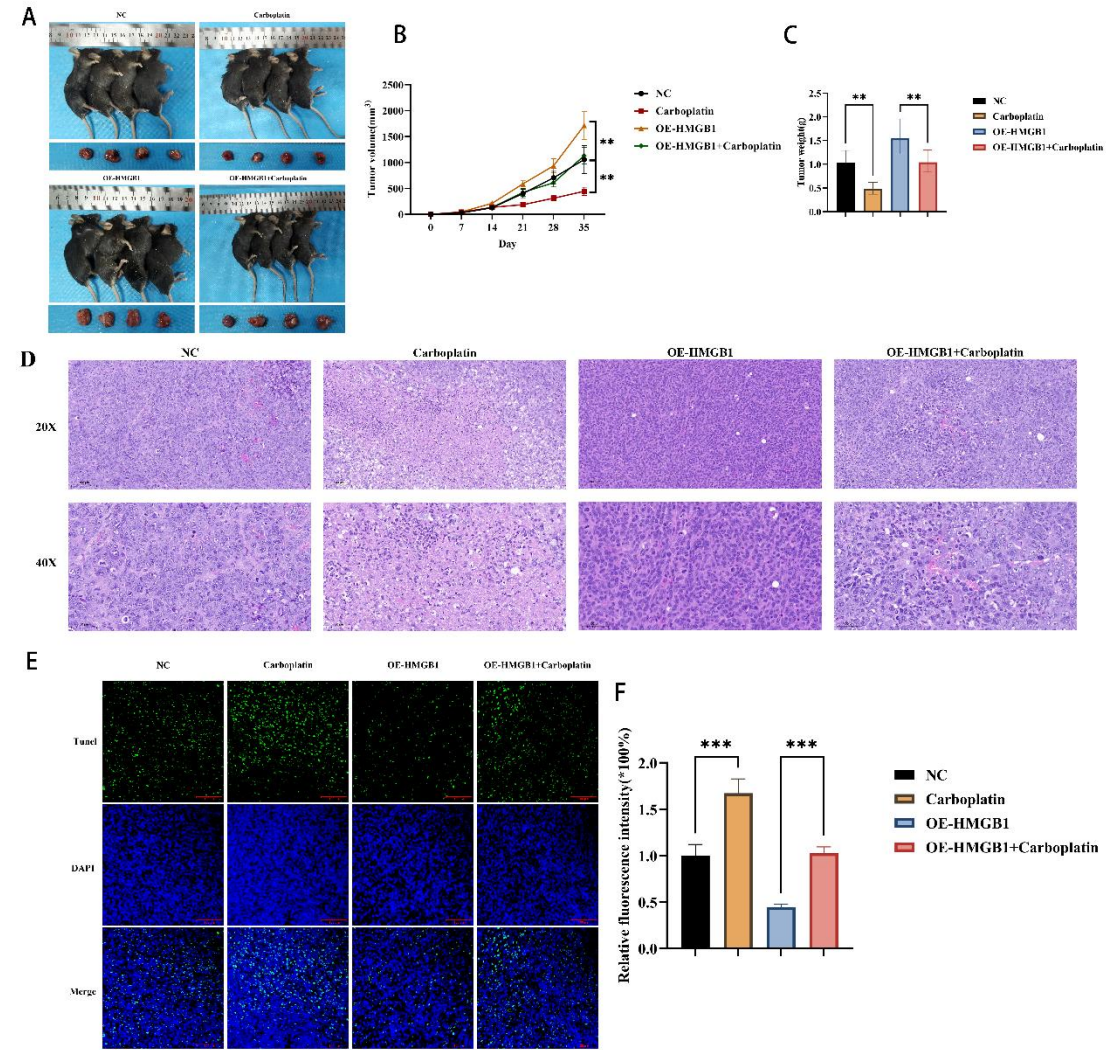


Figure 5: The expression of HMGB1 in in vivo experiments is influenced by carboplatin and RAGE. A: Experiment using C57 mice, tumor tissues were collected after 35 days of culture; B: Tumor volume growth curve, it can be seen that the tumor volume in the overexpressed HMGB1 group increased significantly, and this process can be inhibited by carboplatin; C: Changes in tumor weight, the tumor weight in the overexpressed group increased significantly, and the

change was similar to the tumor volume curve; D: HE staining to observe the pathological state of cells, the pathological changes in the overexpressed group were significant; E: TUNEL staining shows that carboplatin can promote apoptosis in the overexpressed HMGB1.

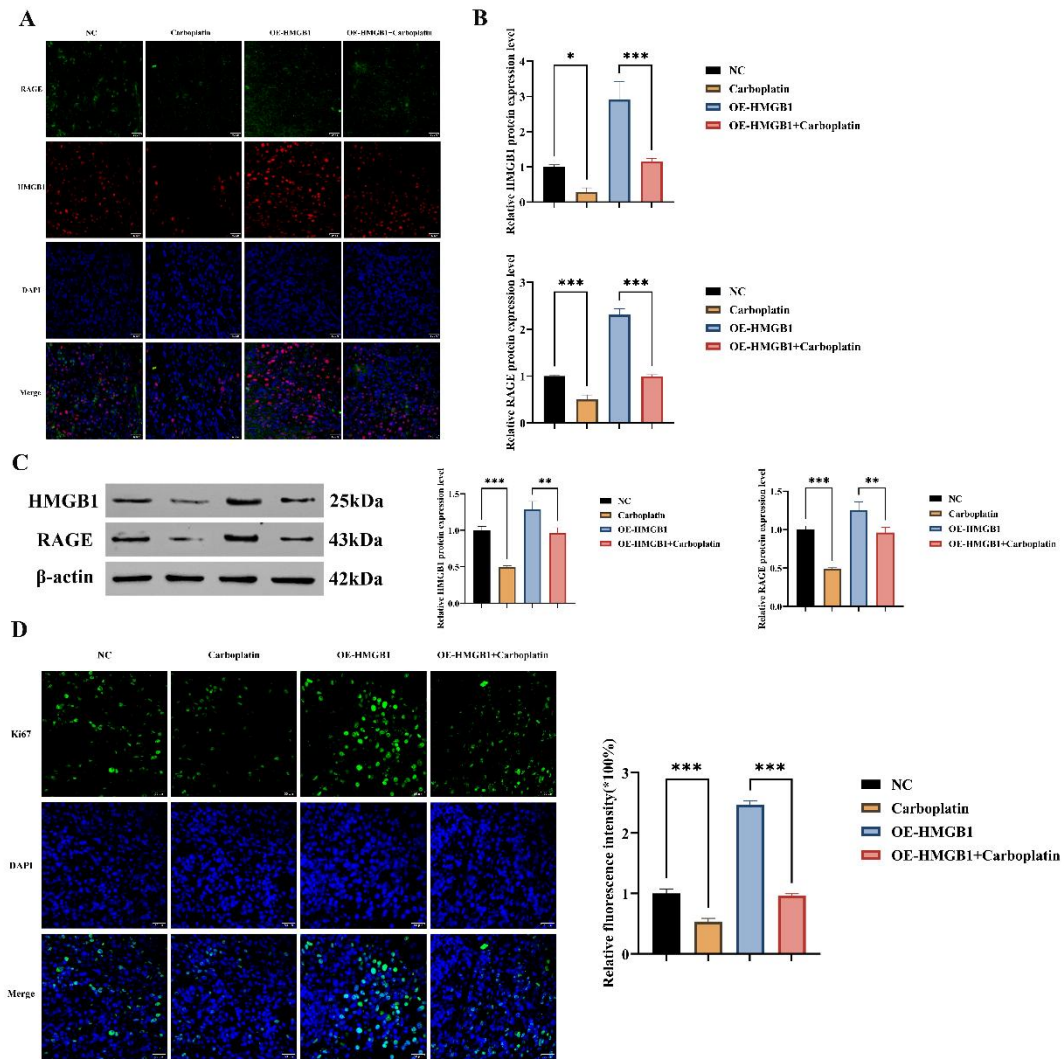


Figure 6: The expression of HMGB1 is correlated with the expression of RAGE. A, B: Cell fluorescence shows the expression of HMGB1 and RAGE in different groups. The carboplatin group significantly inhibited the expression of HMGB1 and RAGE; C: WB again verified the expression levels of HMGB1 and RAGE; D: Cell fluorescence verified the expression level of Ki67 in different groups, which reflects the proliferation ability. The cells in the overexpressed HMGB1 group had

the strongest proliferation ability, and carboplatin could inhibit its proliferation.

Discussion :

Tumor-associated macrophages (TAMs) play a critical role in the progression of NSCLC. In the present study, HMGB1 expression was markedly increased in NSCLC. Functional analyses demonstrated that HMGB1 overexpression promoted the proliferation of NSCLC cells and inhibited apoptosis in vitro, while also enhancing tumor growth in vivo. These findings suggest that the HMGB1–RAGE axis may contribute to the crosstalk between chemotherapy-induced tumor cell stress and the tumor immune microenvironment²³. Following carboplatin treatment, NSCLC cells released HMGB1 into the extracellular space. Previous studies have suggested that, in the oxidative tumor microenvironment, extracellular HMGB1 predominantly exists in its disulfide form¹¹, which exhibits pro-inflammatory activity and may bind to the RAGE receptor on TAMs to activate downstream signaling pathways.

HMGB1 is predominantly localized in the nucleus, whereas RAGE, one of its best-characterized receptors, has been widely implicated in chronic inflammation and the tumor microenvironment²⁴. In the present study, immunofluorescence analysis showed that RAGE agonist treatment was associated with increased HMGB1 expression. Moreover, HMGB1 overexpression reduced the sensitivity of NSCLC cells to carboplatin, whereas HMGB1 knockdown produced the opposite effect. Previous studies have suggested that the interaction between HMGB1 and RAGE can activate downstream signaling pathways, including NF- κ B and MAPK, thereby amplifying

inflammatory responses (Fig. 7). These signaling events have also been reported to promote STAT1 activation while suppressing STAT3 signaling, potentially contributing to macrophage polarization and tumor progression²⁵. This coordinated signaling is thought to contribute to TAM polarization and the transition from an M2- to an M1-like phenotype. M2 macrophages are characterized by the production of anti-inflammatory mediators, including IL-10 and TGF- β , and typically express CD206.²⁶ In contrast, M1 macrophages exhibit enhanced antitumor activity and are associated with increased expression of TNF- α , IL-12, iNOS, and CD86²⁷. In the present study, flow cytometric analysis demonstrated that carboplatin treatment increased the proportion of CD86-positive macrophages while reducing CD206 expression, suggesting a shift toward an M1-like phenotype. Previous studies have shown that M1 macrophages not only exert direct cytotoxic effects against tumor cells but also enhance adaptive antitumor immunity through interactions between the co-stimulatory molecule CD86 and CD28 expressed on T cells, thereby promoting T-cell activation and proliferation²⁸. Based on our findings together with previous reports, we speculate that carboplatin-induced HMGB1 release may alter the tumor immune microenvironment through RAGE-associated signaling. Previous studies have suggested that extracellular disulfide HMGB1 can activate the NF- κ B and MAPK pathways following RAGE binding, thereby modulating STAT1 and STAT3 signaling. These signaling events may contribute to macrophage polarization and promote the transition from an M2- to an M1-like phenotype²⁹. Previous studies have shown that CD86 expressed on M1 macrophages interacts with CD28 on T cells to provide co-stimulatory signals, thereby

promoting T-cell activation and contributing to antitumor immunity. This mechanism
 may partly explain the inhibitory effects on NSCLC proliferation and invasion observed
 following macrophage reprogramming. Nevertheless, several limitations should be
 acknowledged. First, although the present study demonstrated the antitumor effects of
 carboplatin and HMGB1 modulation in vivo, the polarization status of TAMs within
 tumor tissues was not directly evaluated. Second, the present study primarily focused
 on RAGE-associated signaling, whereas the potential contributions of other HMGB1
 receptors, such as TLR2 and TLR4, were not investigated. Finally, the biological
 functions of HMGB1 are highly dependent on its redox state and subcellular
 localization. Future studies are therefore warranted to further elucidate the roles of
 different redox isoforms of HMGB1 and their intracellular and extracellular distribution
 in regulating macrophage polarization and the therapeutic response of NSCLC.

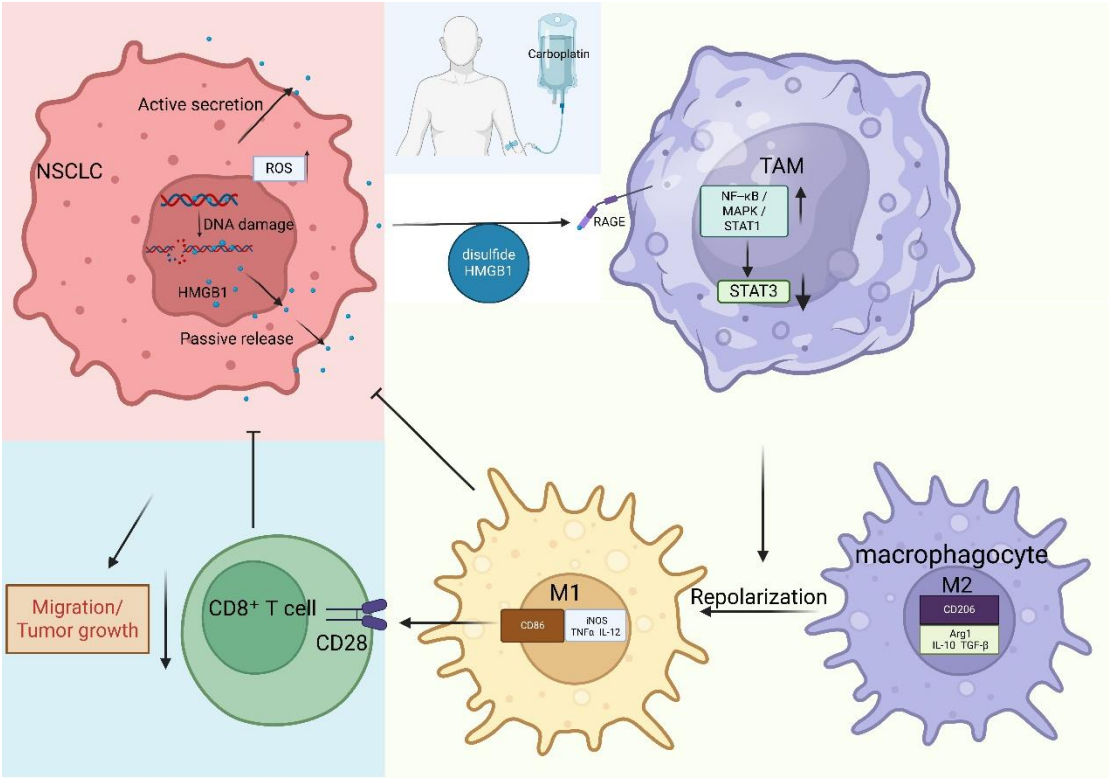


Figure 7: Schematic illustration of carboplatin-induced regulation of TAM polarization and NSCLC progression via the HMGB1–RAGE axis. Following carboplatin treatment, NSCLC cells experience DNA damage, leading to both passive release and active secretion of HMGB1. In the tumor microenvironment, extracellular HMGB1 is predominantly present in its disulfide form, which is considered to possess pro-inflammatory activity. HMGB1 can bind to the RAGE receptor expressed on tumor-associated macrophages (TAMs), thereby contributing to the activation of downstream signaling pathways, including NF- κ B, MAPK, and STAT1, as reported in previous studies. These signaling events are associated with enhanced STAT1 activity and suppression of STAT3 signaling, which may contribute to the polarization of TAMs toward an M1-like phenotype. M1 macrophages are characterized by high expression of CD86, iNOS, TNF- α , and IL-12, among which CD86 functions as a co-stimulatory molecule that can interact with CD28 on T cells to support T-cell activation. In contrast, M2 macrophages are characterized by the expression of CD206, Arg1, IL-10, and TGF- β , which are suppressed during this process. Overall, M1-like macrophage polarization is associated with inhibition of NSCLC cell proliferation and invasion, either directly or through modulation of antitumor immune responses.

In conclusion, the HMGB1–RAGE axis appears to function as a context-dependent regulatory mechanism in NSCLC, linking chemotherapy-induced tumor cell stress to macrophage polarization. The biological effects of HMGB1 are closely associated with its subcellular localization and redox state, which may influence downstream signaling pathways and contribute to its pleiotropic functions³⁰. However, this study has several limitations, including the lack of investigation into the redox

status of HMGB1 and the potential involvement of other receptors beyond RAGE. Future studies are warranted to further explore these aspects and to evaluate the therapeutic potential of combining carboplatin with immune checkpoint-based strategies targeting this pathway, which may provide new insights for NSCLC treatment.

Conclusion:

In conclusion, HMGB1 was found to be significantly upregulated in NSCLC and plays an important role in promoting cell proliferation and inhibiting apoptosis. In addition, our results suggest that HMGB1 may influence TAM polarization through the HMGB1–RAGE signaling axis, thereby contributing to altered sensitivity to carboplatin. These findings indicate that HMGB1 may serve as a potential therapeutic target for improving chemotherapy efficacy in non-small cell lung cancer, providing new insights into the molecular mechanisms underlying tumor progression and chemotherapeutic response.

Abbreviations

HMGB1: High mobility group box-1; RAGE: Advanced glycosylation end products; NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; TAM: Tumor-associated macrophage; LUSC: Lung squamous cell carcinoma; LUAD: Lung adenocarcinoma; ICD: Immunogenic cell death; DAMP: Damage-Associated Molecular Pattern; MDSC: Myeloid-Derived Suppressor Cell;

Declarations

Ethical Statement

All the animal experiments complied with the guidelines of the Tianjin Medical Experimental Animal Care, and animal protocols were approved by the Institutional Animal Care and Use Committee of Yi Shengyuan Gene Technology(Tianjin) Co., Ltd. (protocol number YSY-DWLL-2025014).

Author's contributions

Conceptualization: Haoyuan Li, Erdun E, Shuguang Bao Data curation: Haoyuan Li, Shuguang Bao, Erdun E Formal analysis: Haoyuan Li, Shuguang Bao, Yanqing Gao Methodology: Haoyuan Li, Shuguang Bao, Yanqing Gao Writing-original draft: Haoyuan Li Software: Haoyuan Li Bao Wen Xinze Liu, Yanqing Gao Validation: Erdun E Xin Wang Yanqing Gao Visualization: Erdun E Shuguang Bao Funding acquisition: Bateer Han Project administration: Bateer Han Supervision: Bateer Han Resources: Bateer Han Writing-review&editing: Bateer Han All authors read and approved the final version to be published.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Funding

1.Natural Science Foundation Project of Inner Mongolia Autonomous Region,Project (2025MS08151)

575 2.The General Project of Inner Mongolia Medical University,Project (YKD2025MS009)

576 3.Science and Technology Projects for the Construction of High-level Clinical Specialties,

577 Project (2024YNZD001)

578 4.Science and technology projects for the construction of high-level clinical specialties, Project

579 (2024YNQN003)

580 5.Public hospital research joint fund science and technology, project (2025GLLH0247)

581 6.Public hospital research joint fund science and technology, project (2024GLLH0049)

582 7.Public hospital research joint fund science and technology, project (2023GLLH0141)

583 **Consent to participate**

584 Not applicable

585 **Acknowledgements**

586 Not applicable

587 **Consent for publication**

588 Not applicable.

589 **Competing interests**

590 The author declares no competing interests.

591 **Conflict of interest**

592 No conflicts of interest to report.

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