



A biocompatible copper based therapeutic nanoplatform for CD44 specific tumor targeted therapy and potent immune reprogramming

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ARTICLE INFO

Keywords:

Copper-based therapeutic system
CD44-Mediated tumor targeting
Cuproptosis
cGAS-STING activation
Immune checkpoint blockade synergy

ABSTRACT

Despite advances in oncology, the dual challenge of achieving precise tumor-targeted therapy while simultaneously activating antitumor immunity remains a major clinical barrier. In this study, we engineered a biocompatible copper-based platform, hyaluronic acid (HA) modified Cu ions based therapeutic (B-Cu/HA), that integrates selective tumor targeting, intrinsic cytotoxicity, and immune activation within a single therapeutic system. Leveraging the HA-CD44 interaction, B-Cu/HA exhibited preferential accumulation and prolonged retention in CD44-overexpressing tumors, while maintaining an excellent biosafety profile. Across multiple cancer models, B-Cu/HA robustly inhibited tumor progression. Mechanistically, it induced cuproptosis through upregulation of FDX1 and aggregation of lipoylated DLAT, and triggered ROS-mediated activation of the cGAS-STING pathway, promoting immunogenic cell death. Transcriptomic analysis revealed activation of hypoxia and cytokine signaling pathways, aligning with enhanced CD8⁺ T-cell cytotoxicity and remodeling of the tumor immune microenvironment. The animal models studies demonstrated that B-Cu/HA significantly suppressed tumor growth without systemic toxicity, and synergistically enhanced the efficacy of the immune checkpoint inhibitors anti-TIGIT. Together, these findings establish B-Cu/HA as a multifunctional, immunomodulatory formulation that offers a clinically translatable strategy to enhance tumor immunotherapy and overcome resistance in CD44-overexpressing tumors.

1. Introduction

Cancer remains one of the leading causes of mortality worldwide. Despite substantial progress in treatment strategies, including surgery, chemotherapy, and immunotherapy, the prognosis for many cancer types remains unsatisfactory [1]. This is largely attributable to the ability of malignant cells to evade immune surveillance, acquire therapeutic resistance, and metastasize to distant organs. These challenges underscore the urgent need for synergistic anticancer strategies capable

of selectively targeting tumor cells, minimizing side effects, and enhancing therapeutic efficacy.

Hyaluronic acid (HA), as a natural polymer, exhibits excellent biocompatibility, biodegradability, and specific binding affinity for the CD44 receptor, making it an attractive candidate for targeted drug delivery in oncology [2,3]. Many solid tumors share conserved molecular hallmarks that represent exploitable therapeutic vulnerabilities [4]. Among these, CD44, a cell surface glycoprotein overexpressed in various malignancies, including hepatocellular carcinoma (HCC), colorectal

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cancer (CRC), cervical cancer, and ovarian cancer, has emerged as a compelling target for precision cancer therapy [3,5,6]. Accordingly, HA-based delivery systems have been extensively investigated as tumor-specific carriers to achieve selective cellular uptake and site-specific drug release.

Copper, an essential trace element, play a dual role in regulating cell proliferation and cell death [7,8]. Recent studies have identified a copper-dependent cell death mechanism—cuproptosis—driven by the loss of iron–sulfur cluster proteins and proteotoxic stress, offering a novel therapeutic avenue for cancer treatment [9,10]. Beyond their direct cytotoxicity, copper ions act as immunomodulators: mitochondrial reactive oxygen species (ROS) generated upon copper exposure trigger immunogenic cell death (ICD), characterized by the release of damage-associated molecular patterns (DAMPs) that activate dendritic cells and promote cytotoxic T lymphocyte infiltration, thereby reversing immunosuppressive tumor microenvironments [11–13]. Additionally, copper has been shown to regulate PD-L1 expression in tumor cells, providing a rationale for combining copper-based therapy with immune checkpoint inhibitors (ICIs) [14,15]. However, the therapeutic application of copper ions is limited by systemic toxicity and a lack of tumor specificity [10,16]. Thus, incorporating copper ions into HA-based nanocarriers offers a promising strategy to leverage both the tumor-targeting capacity of HA and the multifaceted anticancer effects of copper.

In this study, we developed a CD44-targeted copper ion delivery system (B–Cu/HA) by integrating copper ions with HA to selectively target CD44-overexpressing tumor cells, thereby enhancing both direct cytotoxicity and T cell-mediated antitumor immunity (Scheme 1). B–Cu/HA exhibited potent cytotoxic effects against multiple high-CD44

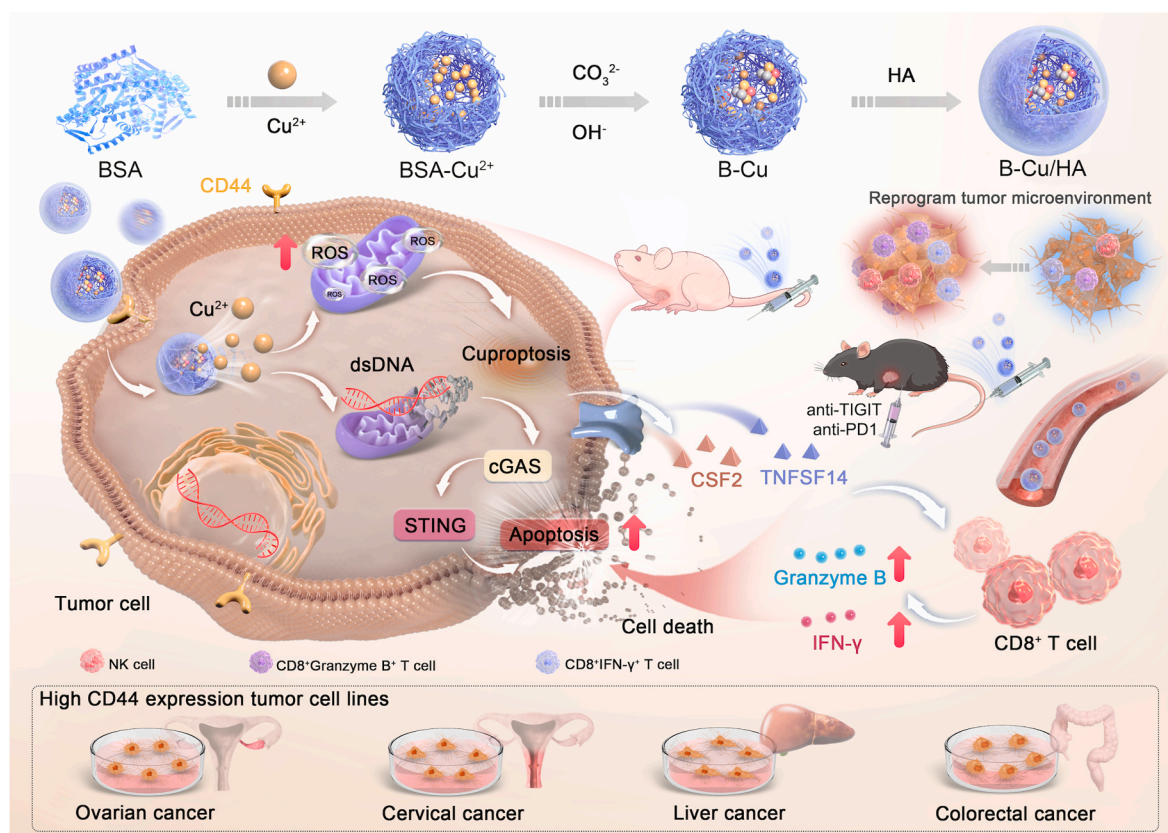
cancer cell lines, including HEY (ovarian cancer), HeLa (cervical cancer), SK-Hep-1 (liver cancer), and SW480 (colorectal cancer). Moreover, B–Cu/HA enhanced T-cell activation and cytotoxicity in tumor–immune co-culture systems. In vivo, B–Cu/HA demonstrated robust antitumor activity and synergistic effects when combined with ICIs (anti-TIGIT). These findings suggest that integrating Cu-based targeted therapy with immune checkpoint blockade represents a promising strategy to overcome resistance and improve outcomes in CD44-positive malignancies. This work provides comprehensive preclinical evidence supporting B–Cu/HA as a clinically translatable therapeutic platform for refractory cancers.

2. Results

2.1. Preparation and characterization of B–Cu/HA therapeutics

The targeted Cu-based therapeutics (B–Cu/HA) was synthesized via a BSA-templated biomineralization process, in which Cu^{2+} , OH^- , and CO_3^{2-} co-precipitated to form a Cu-coordinated BSA composite (B–Cu), followed by HA modification for CD44-specific targeting (Fig. 1A). Transmission electron microscopy (TEM) revealed that B–Cu/HA consisted of aggregated nanoparticles with diameters below 10 nm (~ 6 nm) (Fig. 1B and C). Dynamic Light Scattering (DLS) analysis showed an average hydrodynamic diameter of ~ 7 nm with a polydispersity index (PDI) of 0.189 (Fig. 1D), indicating a relatively narrow size distribution.

Elemental mapping confirmed the homogeneous distribution of C, O, N, S, and Cu (Fig. 1E), and the elemental composition was examined by energy-dispersive X-ray spectroscopy (EDS) (Fig. 1F). Zeta potential measurements demonstrated a surface charge shift after HA



Scheme 1. Schematic illustration for B–Cu/HA inducing CD44-targeted tumor therapy and immune reprogramming. In this study, we engineered a hyaluronic acid-modified copper-based platform (B–Cu/HA) that selectively targets CD44-overexpressing tumors. This system simultaneously induces cuproptosis through the upregulation of FDX1 and aggregation of lipoylated DLAT, and triggers ROS-mediated activation of the cGAS-STING pathway, thereby inhibiting tumor growth and remodeling the immune microenvironment. Combined with anti-TIGIT, B–Cu/HA exhibits synergistic antitumor efficacy, offering a translatable strategy for enhanced cancer immunotherapy.

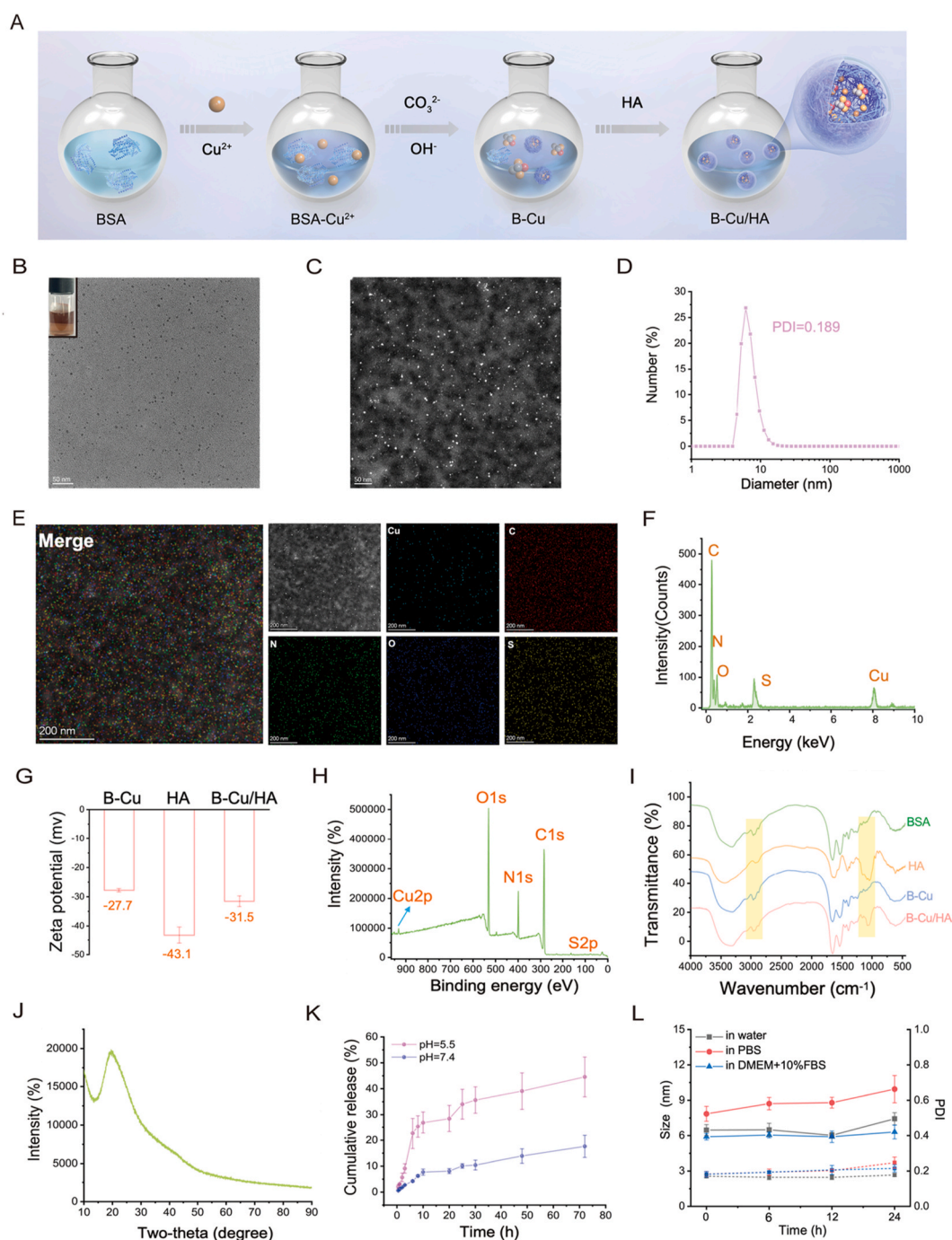


Fig. 1. Synthesis and characterization of B-Cu/HA. (A) Schematic illustration of the synthesis process for B-Cu/HA. (B, C) Representative bright-field and high-angle annular dark-field (HAADF) TEM image of B-Cu/HA. Scale bar = 50 nm. (D) DLS size distribution. (E) Elemental mapping of B-Cu/HA. Scale bar = 200 nm. (F) Energy dispersive X-ray (EDX) spectrum. (G) Zeta potential variation. (H) X-ray photoelectron spectroscopy (XPS) survey of B-Cu/HA. (I) Fourier transform infrared spectroscopy (FTIR) spectra. (J) X-ray diffraction (XRD) pattern of B-Cu/HA. (K) Cu ions release profiles of B-Cu/HA in buffer solution with different pH value. (L) Stability of B-Cu/HA in water, PBS, and DMEM + 10% FBS over time, showing hydrodynamic size (solid lines) and PDI (dashed lines).

conjugation, indicating successful modification (Fig. 1G). X-ray photoelectron spectroscopy (XPS) further confirmed the presence of C, N, O, S, and Cu, validating the incorporation of copper ions into the composite (Fig. 1H, Fig. S1). Fourier-transform infrared spectroscopy (FTIR) spectra revealed characteristic band shifts corresponding to each component, supporting successful assembly of B-Cu/HA (Fig. 1I).

X-ray diffraction (XRD) analysis showed a broad diffraction peak (Fig. 1J), indicative of an amorphous structure, which may facilitate physiological degradation. To assess pH-responsive copper release, B-Cu/HA was incubated in PBS at different pH values, and released

Cu^{2+} was quantified by ICP-MS. At pH 5.5 (mimicking the acidic tumor microenvironment or intracellular lysosomes), ~40% of Cu^{2+} was released at 48 h, whereas only ~13% was released at pH 7.4 (simulating normal blood circulation) (Fig. 1K), demonstrating the TME-responsive behavior of B-Cu/HA. As exhibited in Fig. 1L, negligible size variation was observed after incubation under various physicochemical conditions, indicating good stability that facilitates the safe delivery of the B-Cu/HA agent to the tumor site with minimal side effects.

2.2. Effects of B-Cu/HA on cellular uptake and proliferation inhibition in CD44-overexpressing cancer cells

To evaluate the effects of B-Cu/HA on different cancer cell types, we tested multiple cell lines, including ovarian cancer (HEY, OVCAR8), cervical cancer (HeLa, SiHa), liver cancer (SK-HEP-1, HCLLM3), and colorectal cancer (SW480, HCT116). Baseline expression of CD44, a cell surface marker associated with tumor proliferation and metastasis, was assessed by Western blot (Fig. 2A–D, Fig. S2). CD44 expression levels varied among cell lines, with HEY, HeLa, SK-HEP-1, and SW480 cells showing pronounced overexpression. Given HA's intrinsic binding affinity for CD44, these results suggested a potential for tumor-specific targeting by B-Cu/HA.

Cell viability was next assessed using the CCK8 assay (Fig. 2E). B-Cu/HA significantly reduced cell viability in HEY, HeLa, SK-HEP-1, and SW480 cells compared with B-Cu alone, indicating enhanced growth inhibition in CD44-overexpressing cancer cells. Immunofluorescence microscopy demonstrated efficient cellular uptake of B-Cu/HA, with maximal intracellular accumulation observed at 9 h post-treatment (Fig. 2F). Time-dependent CCK8 assays (24, 48, and 72 h) further confirmed sustained inhibition of cell survival in these cell lines following B-Cu/HA treatment, whereas controls (pure water, HA, or B-Cu) showed weaker effects (Fig. 2G). EdU incorporation assays revealed that B-Cu/HA markedly inhibited DNA synthesis in HEY, HeLa, SK-HEP-1, and SW480 cells (Fig. 2H). In addition, transwell migration and invasion assays demonstrated significant suppression of both cellular motility and invasiveness in B-Cu/HA-treated cells compared with control groups (Fig. 2I and Fig. S3).

Based on the analysis of scRNA-seq data from our previous study [17], we observed higher CD44 expression in ovarian cancer tissues compared with normal ovarian tissues (Fig. 3A). Notably, inhibition of CD44 attenuated the anti-tumor activity of B-Cu/HA (Fig. 3B and C), indicating that its tumor-targeting capability is mediated through the CD44–HA interaction.

2.3. B-Cu/HA induces cell death via cuproptosis and ROS-mediated activation of cGAS-STING signaling

Copper-based compounds have attracted considerable attention in tumor therapy due to their ability to trigger distinct forms of cell death, notably cuproptosis, characterized by the aggregation of lipoylated proteins and mitochondrial dysfunction [9,10]. In this study, we investigated the activation of cuproptosis by B-Cu/HA. We first examined morphological changes using transmission electron microscopy (TEM). B-Cu/HA treatment induced characteristic cuproptotic ultrastructural alterations, including mitochondrial swelling, loss of cristae, and membrane rupture (Fig. 4A). Consistent with this structural damage, a significant accumulation of mitochondrial ROS (mitoROS) was detected via MitoSOX staining, indicating severe mitochondrial stress (Fig. S4A). To elucidate the molecular mechanisms driving these mitochondrial impairments, we assessed the core regulators of the cuproptosis pathway. Western blot analysis showed markedly elevated FDX1 protein levels in B-Cu/HA-treated cells (Fig. 4B, Fig. S4B). Additionally, a significant increase in lipoylated DLAT (Lipo-DLAT) was observed following B-Cu/HA treatment (Fig. S4C). Quantitative PCR (qPCR) analysis revealed significant upregulation of critical cuproptosis regulators, including DLAT and FDX1 (Fig. S4D). These multidimensional findings, ranging from ultrastructural damage and oxidative stress to proteotoxicity and transcriptional regulation, strongly support that B-Cu/HA triggers cell death through the FDX1/DLAT-mediated cuproptosis pathway.

In addition to cuproptosis activation, B-Cu/HA treatment significantly increased intracellular reactive oxygen species (ROS). Flow cytometry demonstrated a robust elevation of ROS in the B-Cu/HA group, whereas HA or B-Cu alone elicited only mild increases (Fig. 4C). Elevated ROS levels are known to induce oxidative stress, resulting in

cellular damage and dysfunction. Furthermore, ROS serve as potent activators of the cGAS-STING pathway, a key regulator of innate immune responses triggered by cytosolic DNA and cellular stress [18,19]. To explore this, we assessed the activation of the cGAS-STING signaling axis. Western blot results showed that B-Cu/HA markedly upregulated cGAS and phosphorylated TBK1 (pTBK1) levels (Fig. 4D), indicating activation of this immune pathway. Consistent with the established role of the cGAS-STING pathway in promoting apoptosis and immunogenic cell death [20–22], B-Cu/HA-treated cells exhibited significantly higher apoptosis compared to all controls (Fig. 4E and Fig. S4E). To provide direct functional validation that the cGAS-STING axis drives these observed effects, we performed rescue experiments by silencing cGAS using siRNA in HEY and SK-HEP-1 cell lines (Fig. S4F). Crucially, the knockdown of cGAS significantly inhibited the cytotoxicity and apoptosis induced by B-Cu/HA, as evidenced by CCK-8 and apoptosis assays (Fig. 4F–G and Fig. S4G). Furthermore, to evaluate the downstream impact on immune activation, we conducted co-culture experiments with human T cells. Flow cytometry analysis revealed that silencing cGAS in B-Cu/HA-treated tumor cells decreased the percentages of active IFN- γ^+ and GZMB $^+$ CD8 $^+$ T cells (Fig. 4H and Figure. S4H). These findings functionally demonstrate that the activation of the cGAS-STING pathway is a critical mediator of both the direct anti-tumor effects and the subsequent T cell-mediated immune response triggered by B-Cu/HA. This dual mechanism, mitochondrial dysfunction via cuproptosis coupled with the enhancement of innate and adaptive immune responses through cGAS-STING, positions B-Cu/HA as a promising therapeutic candidate. Collectively, these findings reinforce the emerging potential of copper-based agents and oxidative stress inducers in cancer treatment [23–25].

2.4. B-Cu/HA enhances antitumor immunity by activating HIF signaling and cytokine pathways

To investigate the immunomodulatory effects of B-Cu/HA, we performed RNA sequencing (RNA-seq) to assess transcriptomic changes in four tumor cell lines following treatment. Differential gene expression analysis revealed substantial transcriptional alterations in HEY, HeLa, SK-HEP-1, and SW480 cells treated with B-Cu/HA compared to the water control, with 99, 936, 676, and 301 differentially expressed genes (DEGs), respectively (Fig. 5A). Volcano plots illustrating the distribution of DEGs for each cell line are presented in Fig. 5B. Subsequent pathway enrichment analysis identified significant upregulation of the hypoxia-inducible factor (HIF) signaling pathway and multiple cytokine-associated pathways (Fig. 5C–E), indicating a conserved cellular response across these tumor models.

To validate these findings, qPCR was conducted for 12 selected genes involved in the HIF signaling pathway (Fig. 5F). Notably, seven genes including EGLN1, VEGFA, HKDC1, SLC2A1, ENO2, HK2, and CDKN1A were consistently upregulated, confirming the activation of HIF-related signaling by B-Cu/HA. Furthermore, qPCR analysis of eight cytokines demonstrated significant upregulation of TNFSF14 and CSF2 in all cell lines (Fig. 5G), suggesting enhanced cytokine-mediated immune modulation.

To examine the functional impact of B-Cu/HA on antitumor immunity, tumor cells were co-cultured with T cells under different treatment conditions (Fig. 6A). Cell viability assays using CCK-8 revealed that the combination of B-Cu/HA and T cells elicited the greatest inhibition of tumor cell viability compared to monotherapies or controls across all four cell lines (Fig. 6B). Flow cytometric analysis of IFN- γ and granzyme B (GZMB) expression in CD8 $^+$ T cells after co-culture demonstrated a significant increase in cytotoxic T-cell activity in the B-Cu/HA group relative to pure water, HA, or B-Cu alone (Fig. 6C). Collectively, these data indicate that B-Cu/HA promotes antitumor immunity by concurrently activating the HIF signaling pathway within tumor cells and enhancing cytokine secretion, thereby potentiating CD8 $^+$ T-cell cytotoxic function.

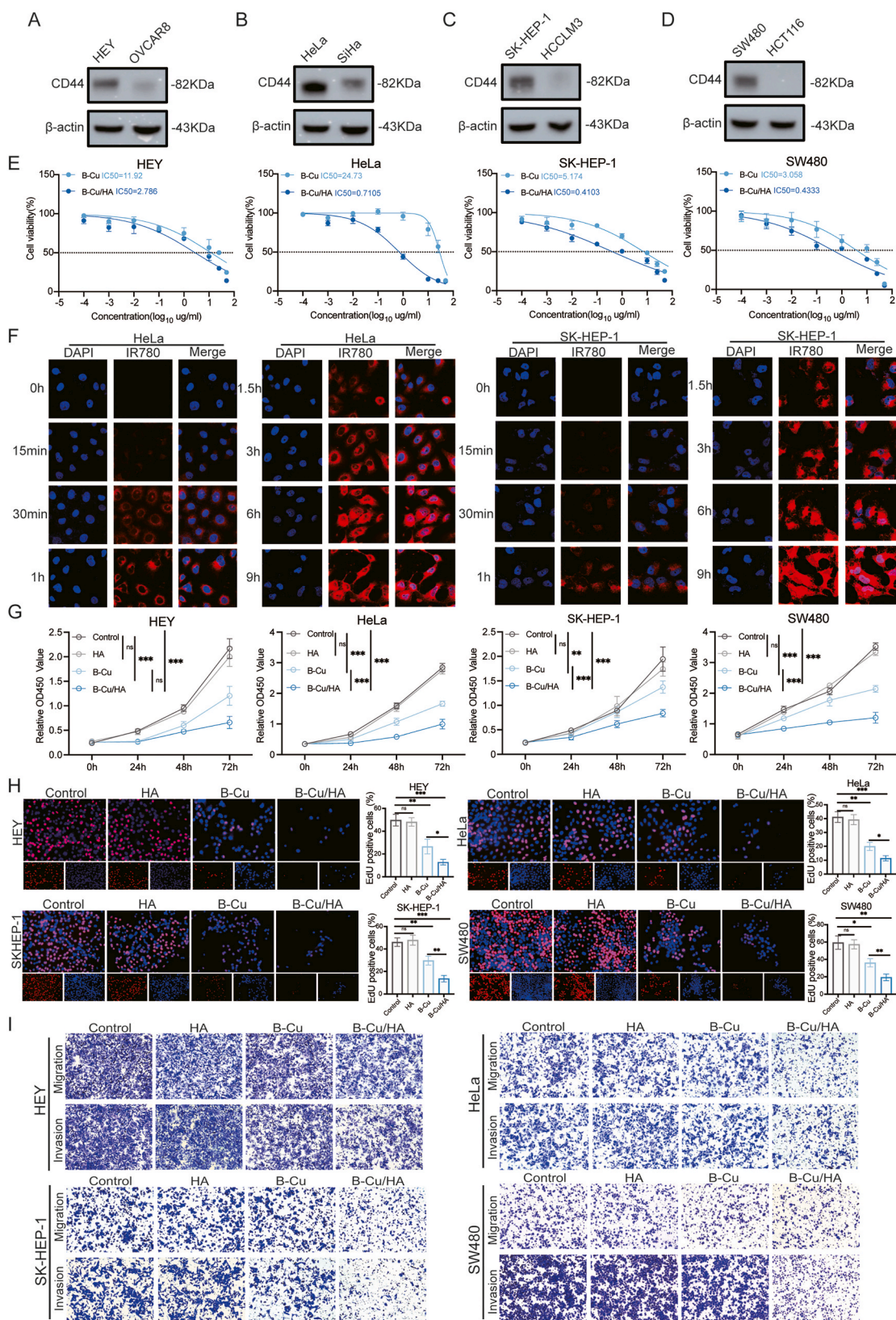


Fig. 2. B-Cu/H A inhibits the growth of multiple tumor cell lines. (A-D) CD44 protein expression in ovarian (HEY, OVCAR8), cervical (HeLa, SiHa), liver (SK-HEP-1, HCCLM3), and colorectal (SW480, HCT116) cancer cell lines, determined by Western blot. (E) Dose-response curves of B-Cu and B-Cu/H A in HEY, HeLa, SK-HEP-1, and SW480 cells, measured by the CCK-8 assay. (F) Fluorescence images showing the cellular uptake of IR780-labeled B-Cu/H A in HeLa and SK-HEP-1 cells at indicated time points. (G) Cell viability of HEY, HeLa, SK-HEP-1, and SW480 after treatment with pure water, HA, B-Cu, or B-Cu/H A for 24, 48, or 72 h, assessed by the CCK-8 assay. (H) Proliferation of HEY, HeLa, SK-HEP-1, and SW480 cells treated with pure water, HA, B-Cu, or B-Cu/H A, evaluated by the EdU incorporation assay. (I) Migration and invasion of HEY, HeLa, SK-HEP-1, and SW480 cells treated with pure water, HA, B-Cu, or B-Cu/H A, assessed by Transwell assays.

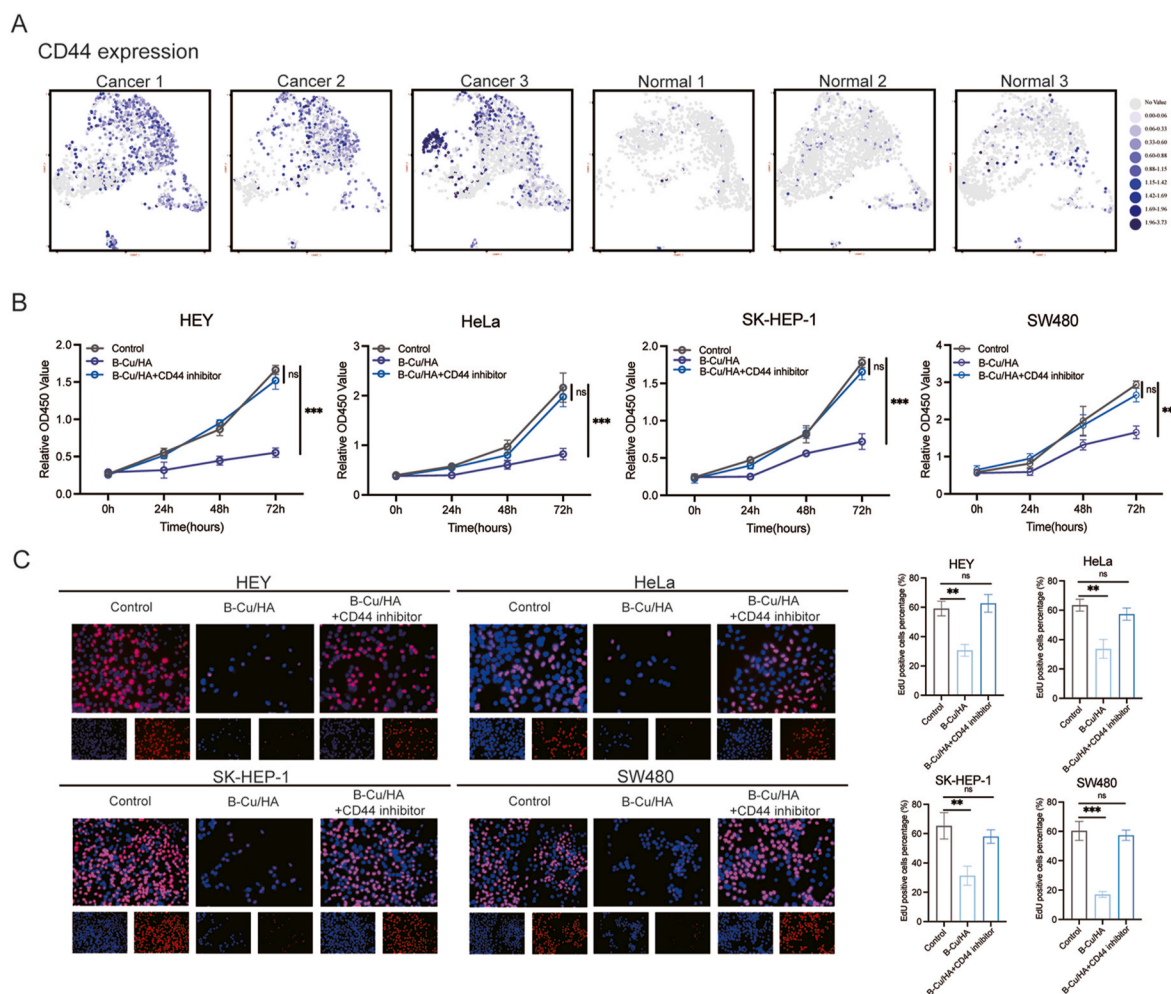


Fig. 3. B-Cu/HA exerts its targeting properties through CD44. (A) UMAP plot of the expression of CD44 in ovarian cancer and nonmalignant ovaries tissue revealed by single-cell RNA sequencing. (B) Cell viability of HEY, HeLa, SK-HEP-1, and SW480 treated with B-Cu/HA in the presence or absence of the CD44 inhibitor (Hermes-1; 10 µg/mL). (C) Cell proliferation of HEY, HeLa, SK-HEP-1, and SW480 after treatment with B-Cu/HA in the presence or absence of the CD44 inhibitor (Hermes-1; 10 µg/mL), assessed by the EDU assay.

2.5. Pharmacokinetics, biosafety, and biodistribution of B-Cu/HA

The pharmacokinetics, safety profile, and biodistribution of B-Cu/HA were systematically investigated. Following intravenous administration of B-Cu/HA to healthy Sprague-Dawley (SD) rats, blood Cu ion concentrations were measured at predetermined time points (0, 0.25, 0.5, 1, 2, 4, 8, 12, 18, and 24 h). The concentration–time curve exhibited a rapid distribution phase followed by a slower elimination phase, indicating favorable pharmacokinetic characteristics (Fig. 7A).

For safety assessment, healthy mice received tail-vein injections of pure water, HA, B-Cu, or B-Cu/HA (cumulative Cu dose: 60 mg/kg) over five consecutive administrations (Fig. 7B). Body weight monitoring revealed no significant differences among groups during the experimental period (Fig. 7C). Hematological and serum biochemical parameters remained within normal ranges in all groups (Fig. 7D and E). Histological examination of major organs (heart, liver, spleen, lung, kidney, brain, intestine, cervix, and ovary) using hematoxylin and eosin (H&E) staining showed no observable pathological alterations (Fig. 7F), confirming the biocompatibility of B-Cu/HA. Furthermore, we extended the observation period to 30 days to evaluate the long-term systemic toxicity of B-Cu/HA. Mice in the B-Cu/HA group exhibited normal behavior and body weight gain (Fig. S5A). Blood routine and blood biochemical analysis remained within healthy ranges (Fig. S5B–C). Additionally, histopathological analysis of major organs (heart, liver,

spleen, lungs, and kidneys) collected at day 30 showed no signs of chronic inflammation, necrosis, or structural abnormalities (Fig. S5D–E). These findings confirm that the B-Cu/HA possesses a favorable long-term safety profile.

Biodistribution was evaluated using IR780-labeled B-Cu/HA. In vivo fluorescence imaging revealed preferential tumor accumulation, with detectable signals at the tumor site within 1 h post-injection and peak fluorescence intensity at approximately 12 h, persisting up to 24 h (Fig. 7G and H). ICP-MS analysis of excised tissues demonstrated maximal Cu accumulation in tumors at 12 h post-injection, with sustained retention at 24 h (Fig. 7I). Ex vivo fluorescence imaging further confirmed high tumor retention and minimal off-target organ distribution (Fig. 7J and K).

Cytotoxicity toward normal ovarian IOSE-80 cells was assessed by CCK-8 assay, showing no significant reduction in cell viability (Fig. 7L). In healthy mice, serial bioluminescence imaging from 0 to 24 h post-injection of IR780-labeled B-Cu/HA illustrated its biodistribution and clearance pathway (Fig. 7M). Collectively, these data demonstrate that B-Cu/HA exhibits favorable pharmacokinetics, excellent tumor-targeting capability, minimal systemic toxicity, and good biocompatibility, supporting its potential for translational cancer nanotherapy.

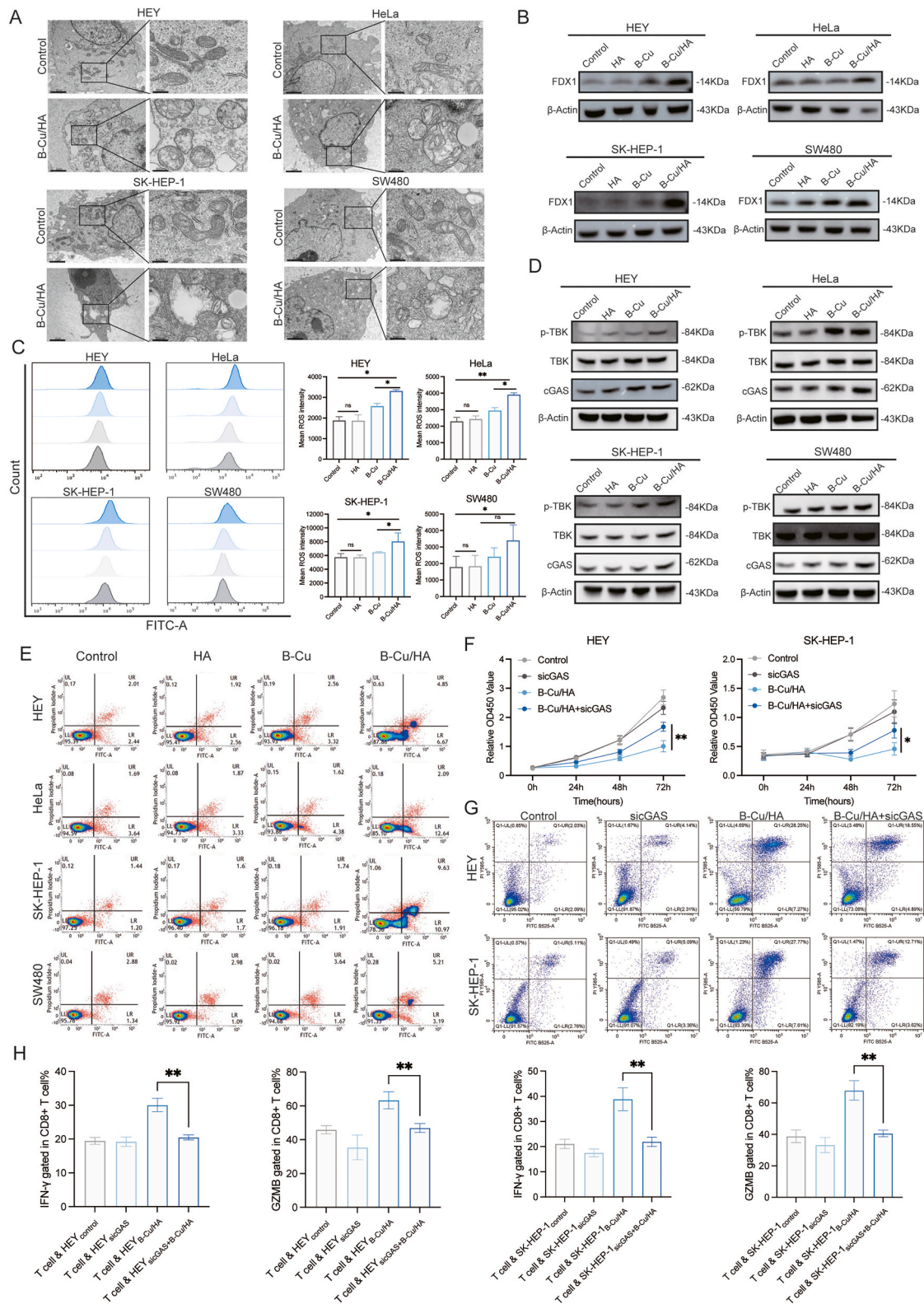


Fig. 4. B-Cu/HA triggers cell death via cuproptosis and cGAS-STING pathway. (A) Representative transmission electron microscopy (TEM) images showing mitochondrial ultrastructural changes in tumor cells after B-Cu/HA treatment. Scale bars are as indicated. (B) Western blot analysis of FDX1 protein levels in four cell lines following 72 h of treatment. (C) Intracellular reactive oxygen species (ROS) levels in tumor cells were analyzed by flow cytometry. (D) Western blot analysis of cGAS, TBK1, and p-TBK1 levels after 72 h of treatment. (E) apoptosis rates of HEY, HeLa, SK-HEP-1, SW480 cells after various treatment determined by flow cytometry. (F and G) Functional rescue experiments showing (F) cell viability (CCK-8 assay) and (G) apoptosis levels in tumor cells pre-transfected with cGAS siRNA followed by treatment with B-Cu/HA. (H) Flow cytometric quantification of the percentages of active IFN- γ ⁺ and GZMB⁺ CD8⁺ T cells in a tumor cell-T cell co-culture system following various treatment.

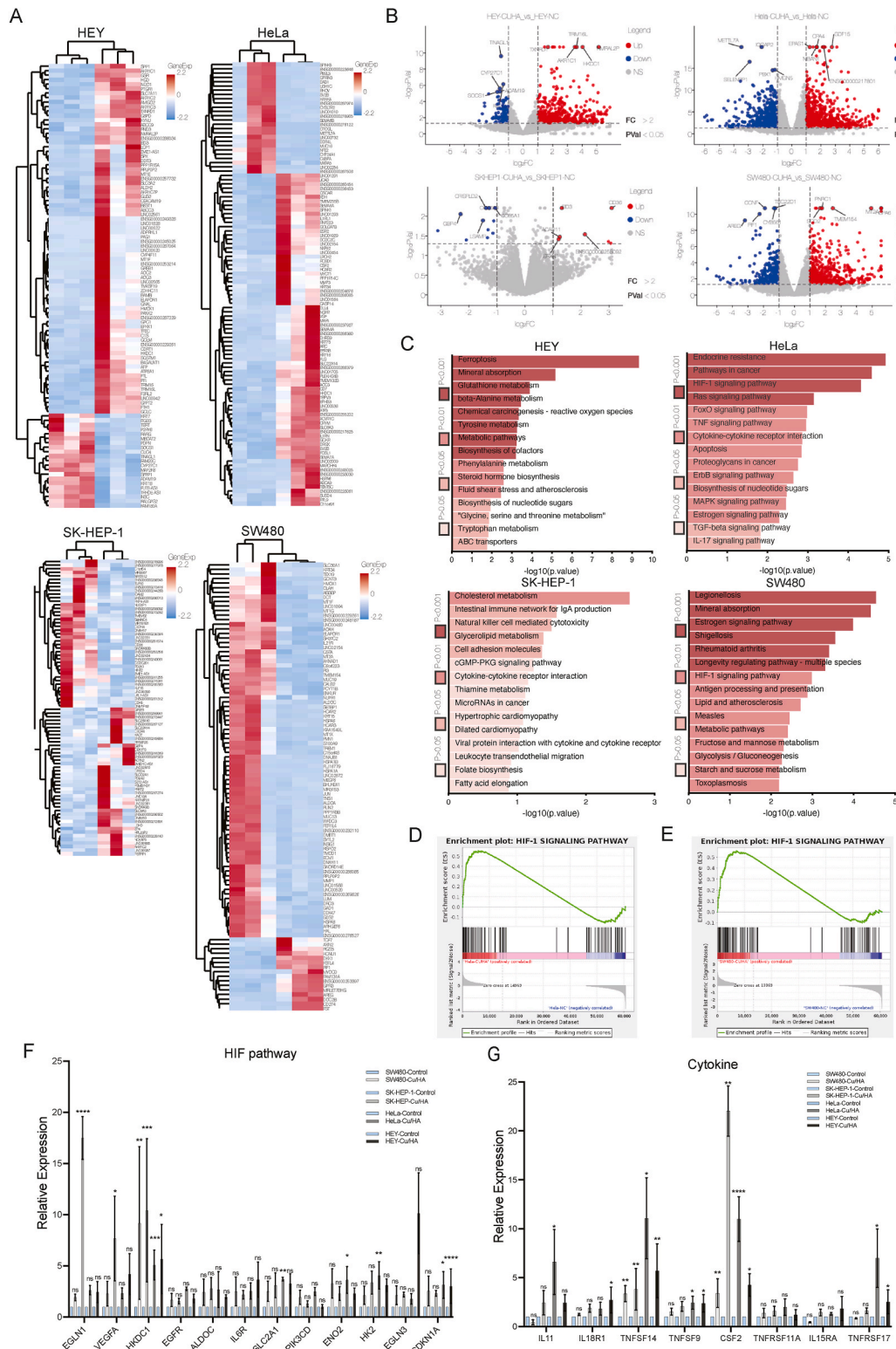


Fig. 5. RNA-Seq analysis demonstrated B-Cu/HA regulating HIF pathway and cytokine secretion. (A) Heatmap showing differentially expressed genes (DEGs) in HEY, HeLa, SK-HEP-1, and SW480 cells treated with pure water or B-Cu/HA. (B) Volcano plot displaying the differentially expressed genes treated with pure water or B-Cu/HA. (C) Pathway enrichment analysis of differentially expressed genes in HEY, HeLa, SK-HEP-1, and SW480 cells. (D and E) Gene Set Enrichment Analysis (GSEA) reveals the activation of the HIF-1 signaling pathway in HeLa and SW480 cells treated with B-Cu/HA. (F) qPCR validation of marker genes in the HIF signaling pathway in HEY, HeLa, SK-HEP-1, and SW480 cells. (G) qPCR analysis of differential cytokine expression in HEY, HeLa, SK-HEP-1, and SW480 cells.

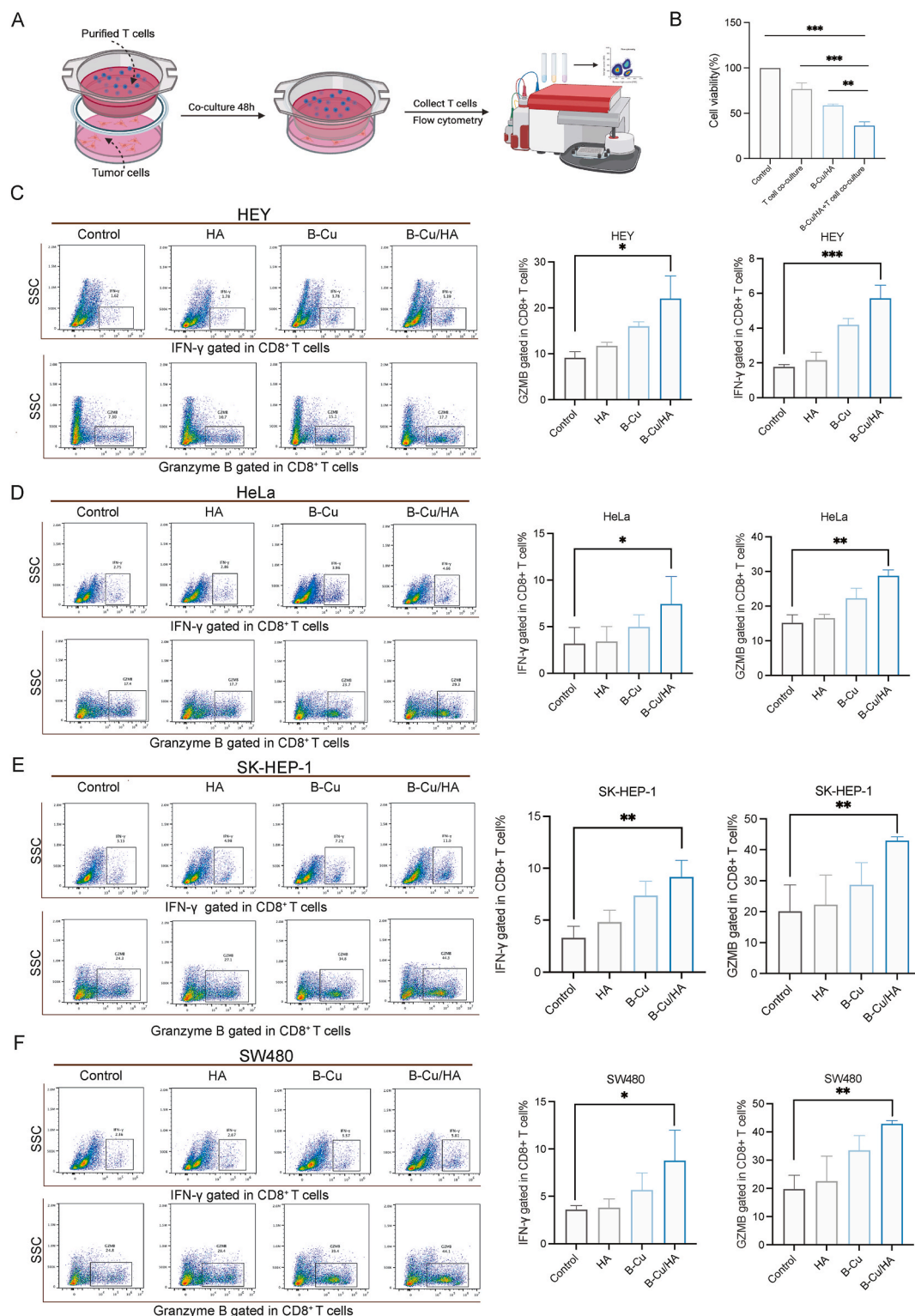
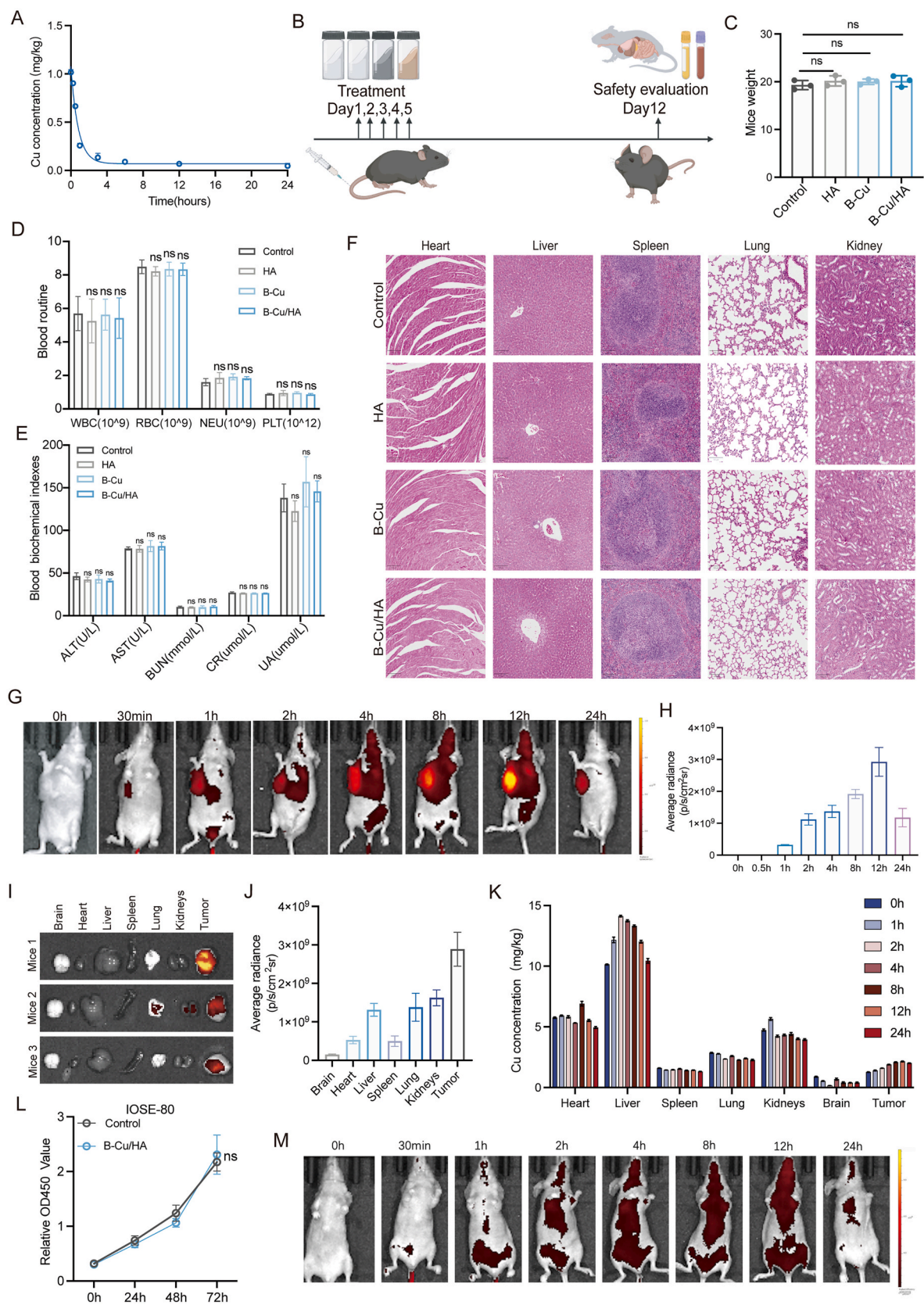


Fig. 6. B-Cu/HA enhance antitumor immunity in vitro. (A) Schematic illustration of the co-culture system using PBMC-derived T cells and tumor cells. (B) Cell viability of tumor cells in control group, T cell co-culture alone, B-Cu/HA treatment alone, or T cell co-culture combined with B-Cu/HA, determined by the CCK-8 assay. (C) Flow cytometric analysis of CD8⁺ T cell-mediated cytotoxicity following B-Cu or B-Cu/HA treatment.

2.6. Antitumor efficacy and safety of B-Cu/HA in xenograft mouse models

To investigate the antitumor efficacy of B-Cu/HA in vivo, HEY and SW480 xenograft mouse models were established. Tumor-bearing mice were administered pure water, HA, B-Cu, or B-Cu/HA (200 μL/mouse,

once daily for 5 consecutive days; n = 3 per group). The experimental design is schematically illustrated in Fig. 8A, emphasizing the comparative treatment analysis. Representative tumor images, along with tumor weight and growth curves (Fig. 8B–D), demonstrate a significant inhibition of tumor growth in the B-Cu/HA-treated group relative to controls. Immunohistochemical staining of tumor tissues revealed



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Fig. 7. Pharmacokinetics, biodistribution and biosafety of B-Cu/HA. (A) Blood circulation profile of copper ions in healthy SD rats after intravenous B-Cu/HA administration. (B) Schematic workflow for evaluating the safety evaluation in healthy mice. (C) Body weight changes of healthy mice intravenously injected with pure water, HA, B-Cu, or B-Cu/HA. (D, E) Hematological (D) and biochemical (E) parameters of healthy mice after intravenous injection of pure water, HA, B-Cu, or B-Cu/HA. (F) Hematoxylin and eosin (H&E) staining of major organs (heart, liver, spleen, lung, kidney, brain, intestine, cervix, and ovary) from healthy mice treated with pure water, HA, B-Cu, or B-Cu/HA. (G) Bioluminescence images of tumor-bearing mice at 0, 0.5, 1, 2, 4, 8, 12 and 24 h post-injection of IR780- B-Cu/HA. (H) The quantitative fluorescence analysis of the biodistribution profiles of IR780- B-Cu/HA in treated mice was provided, relative to Fig. 7G. (I) Biodistributions of Copper after intravenous administration B-Cu/HA at different time points. (J) Xenograft tumors and major organs (Brain, heart, liver, spleen, lung, kidneys) were obtained and examined for biodistribution quantification. (K) The quantitative fluorescence analysis of the xenograft tumors and major organs, relative to Fig. 7J. (L) Cell Viability of normal ovarian epithelial cells (IOSE-80) treated with pure water or B-Cu/HA, determined by the CCK-8 assay. (M) Bioluminescence images of healthy mice at 0, 0.5, 1, 2, 4, 8, 12 and 24 h post-injection of IR780- B-Cu/HA.

markedly reduced Ki67 expression in the B-Cu/HA group, indicating suppressed tumor cell proliferation (Fig. 8E). Furthermore, evaluation of organ morphology (Fig. 8G), blood biochemical parameters, and routine hematology (Fig. 8F) showed no significant abnormalities following B-Cu/HA treatment, supporting its favorable biocompatibility profile. Histopathological analysis of major organs via H&E staining (Fig. 8H) confirmed the absence of treatment-related toxicity. These results highlight the potent antitumor efficacy of B-Cu/HA coupled with an excellent safety profile.

2.7. Therapeutic evaluation of B-Cu/HA in combination with immune checkpoint inhibitors in tumor models

To evaluate the therapeutic efficacy and immunomodulatory effects of B-Cu/HA in combination with immune checkpoint inhibitors (anti-PD-1 and anti-TIGIT), we performed preclinical studies using the ovarian ID8 tumor mouse model. The experimental design is illustrated in Fig. 9A, where tumor-bearing mice were administered pure water (control), B-Cu/HA, anti-PD-1, anti-TIGIT, or combinations of B-Cu/HA with either anti-PD-1 or anti-TIGIT ($n = 3$ per group) over a defined treatment course. B-Cu/HA was given at 200 μ L/mouse once daily (QD) for 4 consecutive days, while immune checkpoint inhibitors were administered at 200 μ g/mouse QD for 4 days.

Tumor growth was monitored by *in vivo* intravital imaging (IVIS) and caliper measurements over time. Representative images and tumor weight data (Fig. 9B–D) revealed marked differences in tumor progression among groups. Notably, mice treated with B-Cu/HA combined with anti-TIGIT exhibited significantly reduced tumor burden compared to controls and monotherapy groups. However, while mice treated with B-Cu/HA + anti-PD-1 showed reduced tumor sizes compared to the control, this effect was not significantly superior to B-Cu/HA monotherapy. Tumor volume quantification at study endpoint (Fig. 9E) corroborated these findings, indicating a synergistic anti-tumor effect of B-Cu/HA with immune checkpoint blockade of TIGIT. To dissect the immune mechanisms underlying these observations, flow cytometry was performed on tumor and spleen tissues (Fig. 9F). Combination therapy with B-Cu/HA and anti-TIGIT significantly enhanced the activation and infiltration of CD8⁺ T cells within the tumor, as evidenced by increased frequencies of IFN- γ ⁺ CD8⁺ T cells, Granzyme B⁺ CD8⁺ T cells, and NK cells. Spleen immune profiling further supported systemic immunomodulation by B-Cu/HA and anti-TIGIT. IHC analysis of tumor sections confirmed the PD-1 and TIGIT expression following different treatments (Fig. S6A).

Safety evaluation included histopathological examination of major organs (heart, liver, spleen, lung, kidneys) (Fig. 9G, Fig. S6B), which showed no significant tissue damage across treatment groups. Furthermore, hematological analysis including blood biochemistry and routine parameters (Fig. 9H and I) indicated no overt systemic toxicity associated with B-Cu/HA and checkpoint inhibitor co-administration. These findings highlight the favorable safety profile of this combinatorial strategy, an important consideration for clinical translation. These findings support the potential of B-Cu/HA as a promising adjuvant to checkpoint blockade therapies, with the capacity to overcome immune evasion and improve therapeutic outcomes.

3. Discussion

Despite remarkable advances in targeted therapies and ICIs, durable clinical responses are often limited by the non-selective cytotoxicity of conventional chemotherapeutics and the immunosuppressive tumor microenvironment [26,27]. To address these barriers, we developed a HA-modified copper nanoplatfrom (B-Cu/HA) that integrates CD44-mediated tumor targeting with dual antitumor modalities: cuproptosis and ROS-dependent cGAS-STING activation. This combination not only achieved direct tumor cytotoxicity but also remodeled the TME toward enhanced CD8⁺ T-cell immunity, with further potentiation upon anti-TIGIT combination therapy.

HA binds CD44 with high affinity, enabling receptor-mediated endocytosis in a range of solid tumors [28,29]. By utilizing HA-CD44 affinity, B-Cu/HA achieved preferential accumulation and cytotoxicity across diverse CD44-overexpressing cancer models. While HA-based and copper-mediated therapies have been extensively reported [30,31], most existing studies are limited to evaluating cytotoxicity within a single cancer type, which restricts their broader clinical translation [32, 33]. In contrast, our study validates a multi-mechanistic immunotherapeutic strategy across multiple distinct tumor models. Additionally, rather than acting merely as a targeted agent, B-Cu/HA uniquely leverages copper-induced oxidative stress to bridge the gap between metabolic cell death and the activation of innate and adaptive immune responses, distinguishing it as a versatile, pan-cancer immunomodulatory therapy.

Mechanistically, B-Cu/HA disrupts copper homeostasis to induce cuproptosis, evidenced by the upregulation of the FDX1 and Lipo-DLAT expression [23]. The resulting mitochondrial proteotoxic stress generates high ROS levels, triggering intracellular DNA leakage and subsequent cGAS-STING activation [22,34]. Concurrently, this severe ROS-induced oxidative stress stabilizes HIF-1. Although HIF-1 typically promotes cell survival, severe oxidative stress can switch its role to drive apoptosis [35,36]. In our study, B-Cu/HA upregulated the HIF-1 signaling pathway. The sustained HIF-1 activation upregulates pro-apoptotic proteins, which ultimately trigger apoptosis observed in our assays. Beyond direct tumor killing, B-Cu/HA actively reprograms the TME. The coordinated upregulation of cytokines, such as TNFSF14 (LIGHT) and CSF2 (GM-CSF), enhances dendritic cell maturation and T-cell priming [37–40]. These changes suggest that B-Cu/HA actively reprograms tumor cells to secrete immune-stimulatory factors, further amplifying antitumor immunity.

ICIs targeting PD-1/PD-L1 have transformed cancer therapy, but efficacy in immunologically “cold” tumors, such as ovarian cancer, remains limited [41–43]. Our previous study has found that TIGIT is highly expressed on exhausted CD8⁺ T cells in ovarian carcinoma and has emerged as a dominant immune checkpoint in this context [17]. Consequently, combining B-Cu/HA with TIGIT blockade significantly enhanced CD8⁺ T-cell infiltration and effector function, yielding superior tumor control without added toxicity, supporting anti-TIGIT as a favorable synergistic partner for copper-based immunogenic therapies [44].

Despite the promising therapeutic efficacy, several limitations of this study should be acknowledged. First, to minimize animal usage in accordance with the 3R principles, a relatively small sample size was

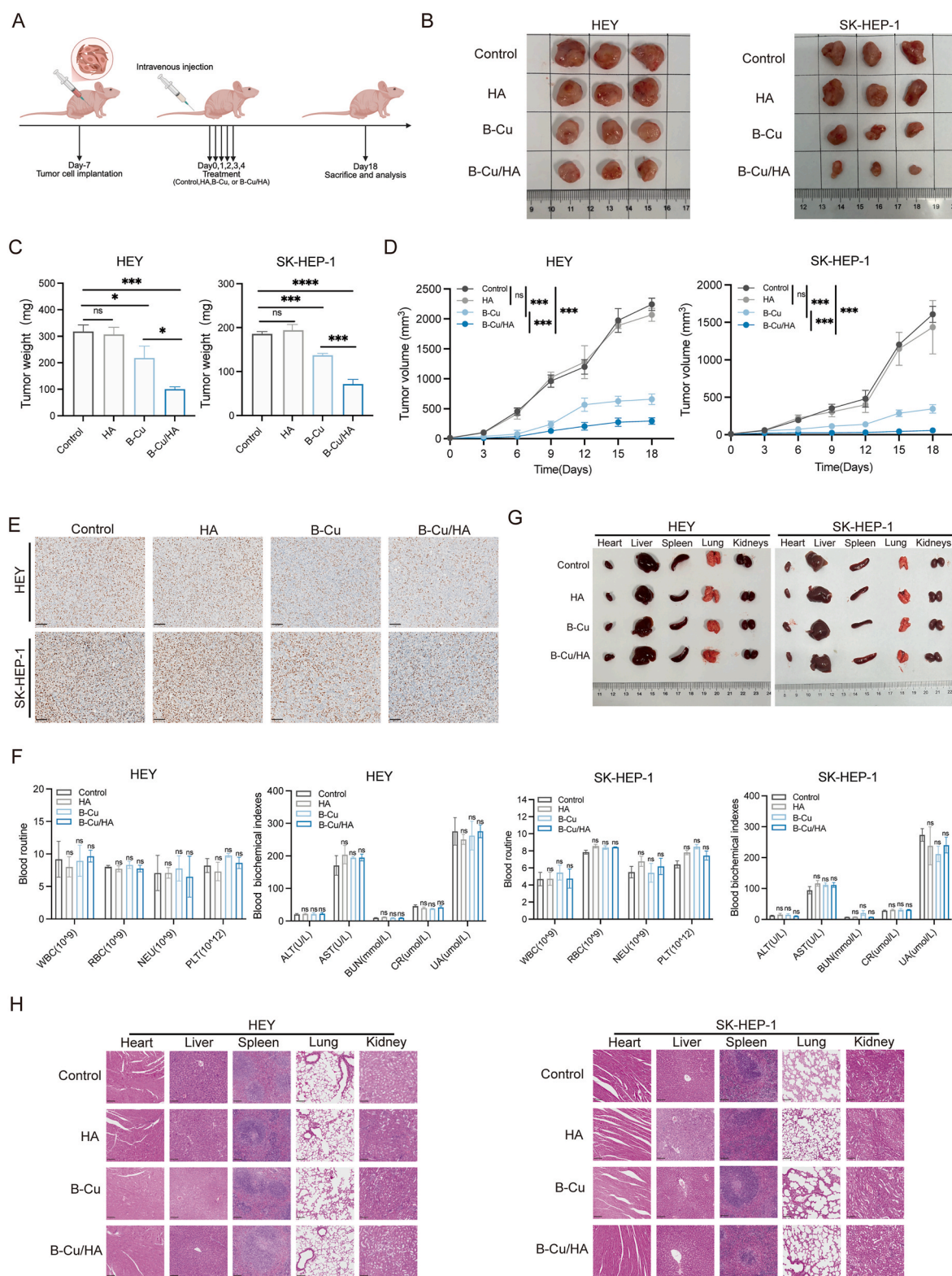
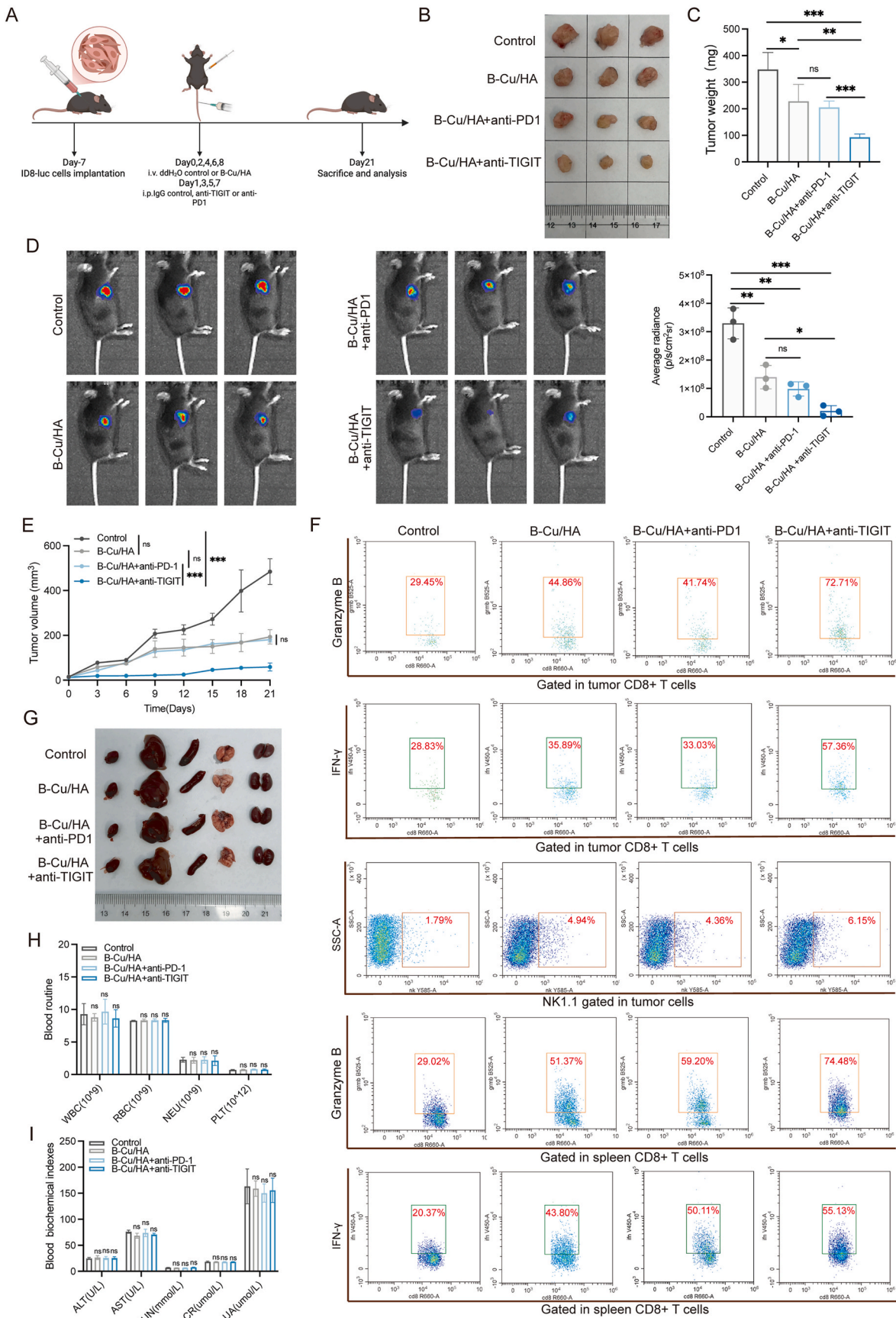


Fig. 8. In vivo antitumor efficacy of B-Cu/HA in HEY and SW480 xenograft models. (A) Schematic illustration of the experimental design for evaluating tumor growth inhibition by B-Cu/HA in HEY and SW480 xenograft mouse models. Mice were intravenously injected with pure water, HA, B-Cu, or B-Cu/HA (200 μ L/mouse, once daily for 5 days; n = 3 per group). (B) Representative images of xenograft tumors derived from HEY and SW480 cells. (C) Tumor weight of xenograft tumors derived from HEY and SW480 cells. (D) Tumor growth curves of xenograft tumors derived from HEY and SW480 cells. (E) Immunohistochemical staining for Ki67 of xenograft tumors from mice. (F) Hematological and biochemical parameters of mice treated with pure water, HA, B-Cu, or B-Cu/HA. (G) Representative images of major organs from mice in each treatment group. (H) H&E staining of major organs (heart, liver, spleen, lung, kidneys) from mice in each treatment group.



(caption on next page)

Fig. 9. Synergistic effect of B-Cu/HA and immune checkpoint inhibitors (ICI). (A) Experimental design for evaluating immune promotion by B-Cu/HA combined with ICI in ID8-bearing mice. Mice were treated with pure water, B-Cu/HA, anti-PD-1, anti-TIGIT, B-Cu/HA + anti-PD-1, or B-Cu/HA + anti-TIGIT ($n = 3$ per group). Treatment regimens: B-Cu/HA (200 μ L/mouse, intravenous injection once daily for 4 days); anti-PD-1 (200 μ g/mouse, intraperitoneal injection once daily for 4 days); anti-TIGIT (200 μ g/mouse, intraperitoneal injection once daily for 4 days). (B) Representative images of ID8 tumors from mice. (C) tumor weight of each group of xenograft mice. (D) Representative in vivo imaging system (IVIS) images of ID8 xenograft-bearing mice. (E) Tumor growth curves of xenograft tumors across each treatment group. (F) Flow cytometric analysis of tumor-infiltrating and splenic immune cells. (G) Representative images of major organs (heart, liver, spleen, lung, kidneys) from mice in each treatment group. (H, I) Hematological (H) and biochemical (I) parameters of mice with different treatments.

utilized for the in vivo experiments. While the consistent efficacy observed across three distinct tumor models supports the robustness of our findings, future studies will utilize larger animal cohorts to validate our results and further evaluate the translational potential of B-Cu/HA. Furthermore, our immune analysis mainly focused on CD8⁺ T cells. Since ROS and cGAS-STING activation also alter myeloid cells like TAMs and MDSCs, future studies are needed to evaluate the impact of B-Cu/HA on the entire immune microenvironment.

Our results establish B-Cu/HA as a multifunctional therapeutic platform that selectively targets CD44-high tumors, induces mitochondrial cuproptosis, activates ROS-dependent cGAS-STING immune signaling, promotes cytokine-mediated immune cell recruitment, and synergizes with TIGIT blockade to overcome immune resistance. By integrating targeted cytotoxicity with immunomodulation, B-Cu/HA addresses two central challenges in oncology: therapeutic selectivity and TME reprogramming. These mechanistic insights provide a strong rationale for further preclinical development, particularly in combination with anti-TIGIT immune checkpoint inhibitor, and support the broader concept of leveraging metal-based nanotherapeutics for dual-modality cancer treatment.

4. Methods

4.1. Preparation of HA-modified B-Cu nanoparticles (B-Cu/HA)

B-Cu/HA nanoparticles were synthesized based on a previously reported method [45,46], with slight modifications. Briefly, bovine serum albumin (BSA, 200 mg) was dissolved in 30 mL ultrapure water and stirred for 10 min. Copper chloride solution (3 mL, 30 mM) was then added dropwise under continuous stirring. After 5 min, sodium carbonate solution (0.6 mL, 0.45 M) was added dropwise until the solution turns blue. The mixture was maintained at 55 °C and stirred for 2 h, followed by ultrafiltration (30 kDa, 15 mL, Millipore) to obtain B-Cu nanoparticles.

For HA modification, HA (11 kDa) was dispersed in PBS (pH = 8.0), followed by the addition of EDC•HCl and NHS. The reaction was stirred for 2 h to activate the carboxyl groups. Subsequently, B-Cu nanoparticles were added to the activated HA solution and stirred for 24 h. The product was purified by ultrafiltration and washing to obtain HA-modified B-Cu nanoparticles (B-Cu/HA).

Copper ion release was assessed using the dialysis method. Briefly, 20 mL of B-Cu/HA suspension was placed into a dialysis bag (MWCO: 3.5 kDa) and immersed in 200 mL PBS (pH 5.5 or 7.4) at 37 °C. At predetermined time points, 20 mL of release medium was collected and replaced with fresh PBS. Samples were stored at 4 °C until analysis. Copper ion concentration was quantified using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700).

4.2. Analysis of scRNA-seq data

The single-cell RNA sequencing data used in this study were retrieved from our previous publication (GEO Accession No. GSE184880) [17]. We utilized the processed gene expression dataset to specifically investigate the expression of CD44. The cells were classified into ovarian cancer and normal groups based on the annotations established in the original study. The differential expression of CD44 between these two groups was analyzed and visualized using the NovolBrain Cloud Analysis Platform (www.novolbrain.com).

4.3. Cell apoptosis assay

Cell apoptosis was evaluated using the Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen, 40302 ES). HEY, HeLa, SK-HEP-1, and SW480 cells were harvested, centrifuged at $300 \times g$ for 5 min, and washed with PBS. Cells were resuspended in 100 μ L $1 \times$ binding buffer containing 5 μ L FITC and 10 μ L PI and incubated for 10–15 min at room temperature in the dark. Apoptosis was analyzed by flow cytometry within 1 h.

4.4. 5-Ethynyl-2'-deoxyuridine (EdU) assay

Cell proliferation was assessed using the Cell-Light™ EdU Apollo In Vitro Kit (RiboBio, C10310). HEY, HeLa, SK-HEP-1, and SW480 cells were seeded in 96-well plates and treated with control, HA, B-Cu, or B-Cu/HA for 48 h. For the competitive inhibition assay, cells were pre-incubated with 10 μ g/mL Anti-CD44 Antibody (Hermes-1; MCE, HY-P990859) for 2 h prior to the 48 h B-Cu/HA treatment. After treatments, medium was replaced with fresh medium containing EdU and incubated for 2 h. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS, and permeabilized with 0.5% Triton X-100 for 5 min at 4 °C. The reaction buffer was applied for 30 min in the dark, followed by nuclear staining with Hoechst 33342. Images were captured using an inverted fluorescence microscope (Olympus, FV1200).

4.5. Real-time PCR

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μ g total RNA using the HiScript III RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme Biotech Co., Ltd., China; Cat# R323-01). Quantitative PCR was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., China; Cat# Q711-02). Primer sequences are listed in the supplementary materials.

4.6. Intracellular ROS and mitoROS measurement

Intracellular ROS levels were determined using the DCFH-DA probe (MCE, HY-D0940), and mitoROS levels were detected using MitoSOX Red (MCE, HY-D1055). HEY, HeLa, SK-HEP-1, and SW480 cells were incubated with fresh medium containing 5 μ M DCFH-DA for 10 min at 37 °C, washed twice with PBS, and analyzed by flow cytometry (Beckman, CytoFLEX S).

4.7. Biodistribution

For in vivo biodistribution, IR780-labeled B-Cu/HA was intravenously injected into mice. Real-time imaging was performed at 0, 0.5, 1, 2, 4, 8, 12, and 24 h using an IVIS Spectrum system (PerkinElmer). At designated time points, mice were euthanized, and tumors and major organs (heart, liver, spleen, lungs, kidneys) were collected. Copper content was quantified by ICP-MS.

4.8. T cell isolation and stimulation

Peripheral blood from ovarian cancer patients was subjected to Ficoll density gradient centrifugation to isolate lymphocytes. CD3⁺ T cells

were purified using the MojoSort™ Human CD3 T Cell Isolation Kit. Purified T cells were stimulated with anti-CD3/CD28 (CD3/CD28 Streptamer® Kit, IBA Life Sciences, 6-8900-050).

4.9. Flow cytometry

CD3⁺ T cells were blocked with human Fc Block (BD Pharmingen™, 564220) and stained with fluorophore-conjugated antibodies against surface markers for 30 min at 4 °C. For intracellular staining, cells were treated with Cell Stimulation Cocktail (BD Pharmingen™, 550583) for 5 h at 37 °C, stained for surface markers, fixed, and permeabilized using the Fixation/Permeabilization Kit (BD Pharmingen™, 554714), followed by staining for intracellular proteins.

4.10. Animal experiments

Female and male BALB/c nude mice (3-5 weeks old) and male C57BL/6 mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All animal procedures were approved by the Laboratory Animal Welfare & Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (Approval No. AE20250027 and No. AE20250054).

For xenograft studies, female BALB/c nude mice were subcutaneously injected with 5×10^6 HEY cells, and male mice with 5×10^6 SW480 cells. Seven days later, tumor-bearing mice were randomly divided into four groups ($n = 3$) and intravenously injected once daily for 4 days with pure water, HA, B-Cu, or B-Cu/HA. Mice were sacrificed 18 days after tumor inoculation. Blood was collected for hematological analysis, and tumors and major organs were processed for H&E and IHC staining.

For immunotherapy combination studies, female C57BL/6 mice were subcutaneously injected with 5×10^6 ID8-luc cells. One week later, mice were randomized into four groups ($n = 3$) and treated with pure water, B-Cu/HA, B-Cu/HA + anti-PD-1, or B-Cu/HA + anti-TIGIT. Animals were sacrificed 21 days after tumor inoculation. Blood, tumors, and spleens were collected, and single-cell suspensions were prepared for flow cytometry.

Ethics approval

This study was performed following the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of Women's Hospital, School of Medicine, Zhejiang University (IRB-20200186-R). Written informed consent was obtained from each patient. All animal experiments were approved by Laboratory Animal Welfare & Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University (AE20250027 and AE20250054).

CRediT authorship contribution statement

Yifeng Fang: Conceptualization, Data curation, Writing – original draft. **Mengyan Tu:** Data curation, Writing – original draft. **Xinru Tu:** Data curation. **Hongbo Zhang:** Conceptualization, Writing – review & editing. **Yangyang Li:** Conceptualization, Data curation, Writing – review & editing. **Junfen Xu:** Conceptualization, Project administration, Supervision, Writing – review & editing.

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.

Acknowledgments

The study was supported by the Noncommunicable Chronic

Diseases-National Science and Technology Major Project (Grant No. 2025ZD0545603 to J.X.), the Key R&D Program of Zhejiang (Grant No. 2026C02A1110 to J.X.), the National Natural Science Foundation of China (Grant No. 82472891 to J.X.; 82372145 to H.Z.), the Zhejiang Province Natural Science Foundation of China (Grant No. LZ24H160001 to J.X.; LBD24H180001 to H.Z.), and Medical Interdisciplinary Innovation Program 2024, Zhejiang University School of Medicine, China (J. X.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2026.124373>.

Data availability

Data will be made available on request.

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