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NLRP4–ER Stress–CCL5 axis as a translational target for NK cell-based immunotherapy in lung cancer

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Abstract

Background Effective infiltration of natural killer (NK) cells into tumors remains a major bottleneck limiting the success of immunotherapy in lung cancer. Identifying the molecular mechanisms controlling NK cell tropism is therefore critical for improving NK cell-based therapies.

Methods We assessed NLRP4 expression and immune cell infiltration in human lung cancer tissues. Co-immunoprecipitation coupled with mass spectrometry identified NLRP4-interacting proteins, including the endoplasmic reticulum (ER) chaperone BIP. In vivo studies were conducted using NLRP4-overexpressing murine and human lung cancer cells in nude, NSG humanized, and C57BL/6 mouse models, with or without NK cell depletion or BIP inhibition via HA15. NK cell migration was evaluated using Transwell co-culture assays and CCL5 ELISA. Tumor growth and immune composition were analyzed by flow cytometry, immunofluorescence, and RNA sequencing.

Results NLRP4 expression was downregulated in human lung tumors relative to adjacent normal tissues and positively correlated with NK cell infiltration. NLRP4 overexpression suppressed tumor growth in both murine and humanized models, accompanied by enhanced NK cell recruitment. Mechanistically, NLRP4 interacted with BIP, and pharmacological inhibition of BIP with HA15 synergistically enhanced NK cell-dependent tumor suppression without altering CD8⁺ T cell abundance. NK cell depletion completely abrogated the antitumor effects of NLRP4, confirming NK cell dependency. Furthermore, NLRP4 overexpression increased CCL5 secretion, which selectively promoted NK cell migration and activation in vitro, establishing a chemokine-driven positive feedback loop in innate antitumor immunity.

Conclusion NLRP4 enhances NK cell tumor tropism through ER stress-mediated chemokine regulation, establishing a positive feedback loop within the tumor microenvironment. Targeting the NLRP4–ER Stress–CCL5 axis to augment NK cell-mediated immunity represents a promising strategy to overcome current limitations in lung cancer immunotherapy.

Keywords

NLRP4, NK cells, NSCLC, ER stress, CCL5, BIP, immunotherapy

Introduction

In recent years, cancer immunotherapies that target the patient's own immune system have achieved major clinical breakthroughs. These include immune checkpoint inhibitors (ICIs) directed against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death-ligand 1 (PD-L1) [1, 2]. Yet, in advanced lung cancer, ICIs still display limited objective response rates (ORRs) in routine practice [3]. Given the limited clinical efficacy of T-cell-based immunotherapy in “cold” non-small cell lung cancer (NSCLC), enhancing endogenous natural killer (NK) cell infiltration and cytotoxicity has emerged as an attractive alternative therapeutic strategy to overcome tumor immune evasion and improve patient outcomes. NK cells are key innate immune effectors that control tumor growth [4]. Indeed, NK-cell products have shown impressive activity in hematologic cancers: in a phase 1/2 trial for B-cell malignancies, 18 of 37 patients (48.6%) achieved an objective response [5], and in another study of CAR-NK cells

59 for follicular lymphoma, a complete response (CR) rate of 85 % was observed, with responses
60 persisting for nearly 17 months [6].

61 Unfortunately, NK-cell therapy remains largely ineffective against solid tumors. The complex tumor
62 microenvironment (TME), characterized by hypoxia, nutrient deprivation, and immunosuppressive
63 cells, impairs NK-cell infiltration and cytotoxicity [7], thereby limiting the clinical benefit of
64 adoptively transferred NK cells. Hence, a critical unmet clinical need is to enhance the tumor tropism
65 and functional persistence of cytotoxic NK cells in solid tumors. Identifying strategies to achieve this
66 goal in lung cancer is the overarching scientific question addressed by the present study.

67 Cellular immunotherapy shows limited efficacy in solid tumors such as lung cancer, primarily due to
68 poor immune cell infiltration [2, 8]. Enhancing tumor tropism of effector cells is therefore critical for
69 improving cell-based therapies.

70 NK cells are promising for adoptive immunotherapy, but their therapeutic potential is constrained by
71 inadequate tumor trafficking [8]. Chemokines such as CCL5 (RANTES) play a key role in recruiting
72 NK cells to tumor sites by binding to CCR1/CCR3/CCR5 receptors [9]. However, the upstream
73 mechanisms regulating CCL5 expression in tumor cells remain poorly defined.

74 We focused on NLRP4 based on our preliminary findings that NLRP4 overexpression in tumor cells
75 upregulates multiple chemokines, including CCL5, promotes NK cell infiltration, cytotoxicity, and
76 suppresses tumor growth. While NLRP4 was initially characterized as a negative regulator of innate
77 immunity [10], emerging evidence suggests context-dependent pro-inflammatory functions [11, 12].
78 The molecular basis for NLRP4-mediated chemokine induction is unknown.

79 We hypothesized that NLRP4 engages endoplasmic reticulum (ER) stress responses to regulate
80 chemokine production. Binding immunoglobulin Protein (BIP/GRP78), the master ER chaperone,
81 functions beyond protein folding to modulate immune signaling and can translocate to the cytosol or
82 cell surface under stress conditions [13]. BIP's role in linking ER stress to chemokine expression has
83 not been explored.

84 Here, we elucidate how the NLRP4-BIP axis regulates chemokine expression and enhances NK cell
85 tumor tropism. Using AAV-mediated NLRP4 delivery, we further explore improving CAR-NK
86 immunotherapy efficacy in lung cancer. Our findings reveal a positive feedback anti-tumor loop
87 mediated by NLRP4-overexpressing tumor cells that may overcome current barriers in solid tumor
88 cell therapy.

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Methods

Cell Culture and Reagents

Human non-small cell lung cancer (NSCLC) cell line NCI-H1975 and mouse NSCLC cell line Lewis lung carcinoma (LLC) were obtained from the American Type Culture Collection (ATCC, USA). Stable NLRP4-overexpressing NCI-H1975 and LLC cell lines were established via lentiviral transduction. NCI-H1975 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), whereas LLC cells were maintained in DMEM containing 10% FBS. All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO₂. HA15 (#HY-100437) was obtained from MedChemExpress (Monmouth Junction, NJ, USA).

Western Blotting (WB)

Total protein was extracted from cells using RIPA lysis buffer supplemented with a phosphatase and protease inhibitor cocktail (Beyotime Biotechnology, #P1006) and was denatured at 100°C for 30 minutes. Equal amounts of protein were separated on 4-20% SDS-PAGE gels (Bio-Rad) at 80 V initially, which was increased to 130 V after the protein marker fully migrated (Thermo Fisher, #26616). Proteins were transferred onto nitrocellulose membranes using the eBlot™ L1 rapid wet transfer system, with transfer time adjusted according to protein molecular weight. Membranes were blocked with 5% non-fat milk in TBS-T (0.1% Tween-20) for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies (Table S1), including PERK (Selleck, #F0027), BIP (Selleck, #F0221), p-eIF2 α (Selleck, #F0257), IRE1 α (CST, #3294S), XBP-1s (CST, #40435S), eIF2 α (CST, #5324T), NALP4 (Novus Biologicals, #NB100-56156), DDDDK-Tag (ABclonal, #AE169PM), and XBP-1u (Proteintech, #25997-1-AP), all diluted 1:1000 unless otherwise specified. Loading controls included β -Actin (Proteintech, #66009-1-Ig), α -Tubulin (Proteintech, #66031-1-Ig), and GAPDH (Proteintech, #60004-1-Ig). After washing three times with TBS-T, membranes were incubated with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, #115-035-003) or goat anti-rabbit IgG (Jackson ImmunoResearch, #111-035-003) secondary antibodies (1:5000) for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate and imaged on an Amersham Imager 600 system.

RNA Isolation and Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from cultured cells using TRIzol Reagent (Invitrogen, #15596018). Cells were lysed and mixed with ethanol before loading onto spin columns; columns were first centrifuged, then washed, dried briefly, and RNA was eluted in a low-volume buffer. Then reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit (Seville Biotechnology, # G3337). Quantitative real-time PCR was performed with SYBR Green (Seville Biotechnology, #G3326) detection chemistry on an ABI 7500 Real-Time PCR System (Applied Biosystems). Each gene was assayed in triplicate; any replicate whose Ct value differed by more than one cycle from the other two was excluded. Relative gene expression was normalized to the internal reference gene GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method. qPCR primers were designed based on NCBI reference sequences: Mouse Gapdh (NM_001411843.1) was amplified using the following primers: forward 5'-CATCACTGCCACCCAGAAGACTG-3' and reverse 5'-ATGCCAGTGAGCTTCCCGTTCAG-3'. Mouse Ccl5 (NM_013653.3) was amplified using forward 5'-CCTGCTGCTTTGCCTACCTCTC-3' and reverse 5'-ACACACTTGGCGGTTTCCTTCGA-3'. Mouse Cxcl2 (NM_009140.2) was amplified using forward 5'-GCCAGACAGAAGTCATAGCCA-3' and reverse 5'-CAGTTAGCCTTGCCTTTGTTCA-3'.

Co-Immunoprecipitation (Co-IP) and Mass Spectrometry (MS)

Cells were washed twice with $1\times$ PBS, scraped into 1.5 mL tubes, and lysed with NP-40 lysis buffer (Beyotime Biotechnology, #P0013F) supplemented with protease inhibitors (Beyotime Biotechnology, #P1006) at a ratio of 20-30 μ L per 1×10^5 cells. Lysates were incubated on ice for 10 minutes and centrifuged at $14,000\times g$ for 10 minutes at 4°C . Supernatants were collected, with 500 μ L used for immunoprecipitation and 80 μ L reserved as input. Anti-DYKDDDDK magnetic beads (25 μ L) were pre-washed twice with 500 μ L NP-40 lysis buffer by gentle pipetting and magnetic separation. The pre-washed beads were incubated with 500 μ L of lysate overnight at 4°C on a rotator. After incubation, beads were separated on a magnetic rack and washed four times with 500 μ L NP-40 lysis buffer until the OD280 of the wash supernatant was below 0.05. For elution, beads were resuspended in 80-100 μ L $1\times$ SDS-PAGE loading buffer and heated at 100°C for 10 minutes. After cooling, tubes were placed on the magnetic rack, and the supernatant was collected for SDS-

PAGE analysis. Input samples (80 μ L) were mixed with 20 μ L 5 $\times$ SDS-PAGE loading buffer and were similarly denatured prior to electrophoresis.

The immunoprecipitated protein complexes obtained from Co-IP were subsequently subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Samples underwent sequential pretreatment steps including protein disulfide bond reduction, alkylation of free thiols, tryptic digestion, peptide extraction, and desalting. The processed peptides were dissolved in 0.1% formic acid (FA) and separated using an EASY-nLC 1000 ultra-high performance liquid chromatography system (Thermo Fisher Scientific). Chromatographic separation was performed on a specific analytical column with the flow rate precisely controlled at 500 nL/min. Full mass spectrometry (MS) scans were acquired at a resolution of 60,000, covering a mass-to-charge ratio (m/z) range of 350-1800. The automatic gain control (AGC) target was set at 4×10^5 , with an isolation window of 0.7 Da and a maximum ion injection time of 50 ms. Tandem mass spectrometry (MS/MS) fragmentation was performed using higher-energy collisional dissociation (HCD) mode with the collision energy optimized to 30%. The MS/MS scan resolution was set at 15,000, with an AGC target of 1×10^5 and a maximum ion injection time of 22 ms. All mass spectrometry data were processed using the Proteome Discoverer (v2.4) platform.

Flow Cytometry Assay

Single-cell suspensions were isolated from mouse subcutaneous tumor tissues or spleens. Cells were incubated with Mouse TruStain FcX™ antibody (BioLegend, #156604) at 4°C for 10 minutes to block Fc receptor-mediated nonspecific binding. Subsequently, cells were stained with the following fluorochrome-conjugated antibodies (Table S1) at appropriate dilutions for 40 minutes at 4°C in the dark: Zombie NIR™ Fixable Viability Kit (BioLegend, #423105), Zombie UV™ Fixable Viability Kit (BioLegend, #423108), anti-CD45-APC-Cy7 (BD Biosciences, #557659), anti-CD45-PE-Cy7 (eBioscience, #25-0451-82), anti-CD3e-PE-Cy7 (BD Biosciences, #552774), anti-F4/80-BV421 (BD Biosciences, #565411), anti-CD11b-AF700 (BioLegend, #101222), anti-CD11b-FITC (BioLegend, #101205), anti-CD45R/B220-BV510 (BioLegend, #103248), anti-CD86-FITC (BioLegend, #105006), anti-CD11c-BUV496 (BioLegend, #750483), anti-Ly6G/Ly6C-BV711 (BioLegend, #108443), anti-Ly6C-BV605 (BioLegend, #128036), anti-Ly6G-APC (eBioscience, #17-9668-82), anti-PD-L1-BV785 (BioLegend, #124331), anti-TIGIT-PE (BioLegend, #142104), anti-CD49b-

178 PE/Dazzle™594 (BioLegend, #108924), anti-H-2Kd/H-2Dd-APC (BioLegend, #114714), anti-
179 TCRβ-PE-Cy7 (BioLegend, #109221), anti-CD4-PE (BioLegend, #100511), anti-CD8a-AF700
180 (BioLegend, #100730), and anti-NK-1.1-BB700 (BD Biosciences, #566502), anti-I-A/I-E-PE
181 (BioLegend, #107608), anti-CD11c-PE/Cy7 (BioLegend, #117317), anti-NK-1.1-BV421
182 (BioLegend, #108731).
183 Following surface staining, cells were fixed at room temperature for 30 min in the dark using IC
184 Fixation Buffer (eBioscience, #00-8222-49) and then permeabilized with Permeabilization Buffer
185 (10×) (eBioscience, #00-8333-56). Cells were subsequently stained at 4 °C in the dark for 40 min
186 with anti-CD206-PerCP/Cy5.5 (BioLegend, #141715). For nuclear antigen staining, cells were fixed
187 and permeabilized using a working solution prepared from Fixation/Permeabilization Concentrate
188 (eBioscience, #00-5123-43) and Fixation/Permeabilization Diluent (eBioscience, #00-5223-56)
189 according to the manufacturer's instructions, and then stained at 4 °C in the dark for 40 min with
190 anti-IFN-γ-PE (BioLegend, #505807), anti-Granzyme B-AF647 (BioLegend, #515406), and anti-
191 TNF-α-FITC (BioLegend, #506303). Data were acquired on a BD FACS Fortessa (BD Biosciences,
192 San Jose, CA, USA) and analyzed using FlowJo v10.

193

194 **Animal Studies**

195 All animal experiments were approved by the Animal Care and Use Committee of the Shanghai
196 Chest Hospital Institutional Animal Care and Use Committee (Approval No. KS21005), and were
197 conducted in strict accordance with the guidelines of the Association for Assessment and
198 Accreditation of Laboratory Animal Care International (AAALAC). Mice were group-housed
199 (maximum 5 per cage) in specific pathogen-free (SPF) facilities at the Shanghai Institute of Materia
200 Medica, using individually ventilated cages (GM500; Tecniplast). Animals had free access to water
201 and food. Environmental conditions were maintained at 22 ± 2°C temperature, 55 ± 10% relative
202 humidity, and a 12-h light/12-h dark cycle. Humane endpoints, including moderate clinical
203 symptoms, signs of distress or discomfort, or body weight loss exceeding 15%, were strictly
204 observed and were not exceeded in any experiment.
205 For tumor inoculation, LLC-Nlrp4-Vector Ctrl or LLC-Nlrp4-OE cells (5×10^5 cells per mouse)
206 were subcutaneously injected into the right inguinal region of 4-week-old C57BL/6N mice. H1975-

207 NLRP4-Vector Ctrl or H1975-NLRP4-OE cells (5×10^7 cells per mouse) were subcutaneously
208 injected into the right dorsal flank of 4-week-old BALB/c nude or NSG mice.

209 To establish humanized cell-derived xenograft (CDX) models, human peripheral blood mononuclear
210 cells (PBMCs) were isolated and intravenously injected into immunodeficient NSG mice one day
211 prior to tumor cell inoculation at a dose of 5×10^7 cells per mouse. Subsequently, H1975-NLRP4-
212 NC or H1975-NLRP4-OE cells (5×10^7 cells per mouse) were subcutaneously inoculated into the
213 right dorsal flank of recipient mice. When tumors reached a volume of approximately 80-100 mm³,
214 mice were randomized into treatment groups balanced by tumor burden. Tumor volumes were
215 measured every 2-3 days using a Vernier caliper and were calculated with the ellipsoid formula: V
216 (mm³) = $\frac{1}{2} \times \text{length (mm)} \times \text{width}^2$ (mm²).

217 Experimental groups included: vehicle control (corn oil, MCE, HY-Y1888), HA15 treatment (30
218 mg/kg, MCE, HY-100437), adenovirus-mediated NLRP4 knockdown (Adenovirus-NLRP4-OE,
219 HBAAV2/9-h-NLRP4-3xflag-Null, #90040913, Hanbio, Shanghai, China; 5×10^{11} viral particles),
220 and combination therapy (Adenovirus-NLRP4-OE, 5×10^{11} viral particles, plus HA15 30 mg/kg).
221 HA15 or corn oil was administered once daily via intraperitoneal injection. Additionally, AAV-
222 shRNA (5×10^{11} viral particles) was administered via multi-site intratumoral injections (three sites
223 per tumor, 10 μ L per site) on days 1, 5, 10, and 15 after grouping.

224 For in vivo NK cell depletion, mice received anti-mouse NKG2D antibody (BioXCell, #BE0111) at
225 100 μ g per mouse via intraperitoneal injection (i.p.), starting 2 days before tumor implantation (day
226 -2) and continuing twice weekly for the duration of the study.

227

228 **Collection of Tumor Interstitial Fluid and ELISA**

229 Freshly excised tumors were briefly rinsed with pre-chilled PBS, minced, and placed on a 100- μ m
230 cell strainer mounted over a 50-mL conical tube. Samples were centrifuged at $100 \times g$ for 10 min at
231 4 °C, and tumor interstitial fluid (TIF) collected at the tube bottom was stored at -80 °C.

232 Ccl5 levels in TIF were measured using a mouse Ccl5 ELISA kit (MultiSciences, #EK2129).

233 Microplates were pre-washed with $1 \times$ wash buffer. Standards (100 μ L, two-fold serial dilutions) and
234 blanks (100 μ L diluent) were added to designated wells, and samples (80 μ L detection buffer + 20 μ L
235 TIF) were added to sample wells. Detection antibody (50 μ L) was added, and plates were incubated
236 at 25 ± 3 °C for 1.5 h with shaking at 100–300 rpm. Plates were washed six times, followed by

incubation with 100 μ L streptavidin solution for 30 min at 25 ± 3 °C. After washing, 100 μ L substrate solution was added for color development in the dark for 5–30 min, and the reaction was stopped with 100 μ L stop solution. Absorbance was measured at 450 nm within 30 min, with 570 nm used for background correction.

241

242 **Immunofluorescence**

243 Mouse tumor tissues were fixed with 4% paraformaldehyde (Meilunbio, #MA0192-2) at room
244 temperature for 10 min. Tissue sections were outlined with a hydrophobic barrier pen (Fuzhou
245 Maixin Biotechnology Development Co., Ltd., PEN-0002). Sections were permeabilized with 0.1%
246 Triton® X-100 (BioFroxx, 1139ML100) for 15 min and washed three times with TBS buffer (Hubei
247 Baiao Biotechnology Co., Ltd., BP0152). Non-specific binding was blocked with 10% donkey serum
248 (Southern Biotech, 0100-01) at 37°C for 30 min.

249 BIP antibody (iBtek, A11366) was diluted 1:100 in TBS and applied overnight at 4°C. Sections were
250 then incubated with Alexa Fluor® 594 donkey anti-rabbit IgG (H+L) (Thermo Fisher Scientific,
251 A21207; 1:400 in TBS) at 37°C for 45 min, followed by washing. LLC cells stably overexpressing
252 NLRP4 fused to GFP were visualized in the 488 nm channel. Nuclei were counterstained with DAPI
253 (Solarbio, C0060; 1:500) for 5 min in the dark. Sections were mounted with fluorescent mounting
254 medium (Southern Biotech, 0100-01) and stored at 4°C.

255 Images were acquired using a BX53 fluorescence microscope (Olympus) and the Panoramic SCAN
256 II system (3D HISTECH). Nuclei appeared blue (DAPI), BIP was detected in the red channel (Alexa
257 Fluor® 594), and NLRP4-FITC 488 was visualized in green.

258

259 **In vitro NK cell migration assay**

260 NK cell migration was assessed using 6.5 mm Transwell inserts with an 8 μ m pore size in 24-well
261 plates (Corning, Cat#3422). Recombinant mouse CCL5 (100 ng/mL; Abcam, #ab9916) was added to
262 the lower chambers containing 600 μ L of serum-free RPMI-1640 medium. Single-cell suspensions
263 prepared from C57BL/6J mouse spleens were adjusted to the desired concentration, and 5×10^5
264 cells were seeded into the upper chambers. After incubation at 37°C for 12 h, migrated cells were
265 harvested from the lower chambers, stained with antibodies against CD45, TCR β , CD4, CD8,
266 NK1.1, CD11b, Ly6C, Ly6G, and F4/80 (Table 1), and analyzed by flow cytometry. NK cells were

distinguished from other lymphocyte subsets, and the number of migrated immune cells was quantified. The chemotactic effect of CCL5 was determined by comparing migration between CCL5-treated (100 ng/mL) and untreated control groups.

RNA Sequencing

Total RNA was extracted using TRIzol Reagent (Invitrogen, #15596018) according to the manufacturer's instructions and dissolved in RNase-free water. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer ($OD_{260/280} = 1.8-2.2$), and RNA integrity and quality were assessed using an Agilent 5300 system with the RNA Nano 6000 Assay Kit ($RIN > 6.5$). mRNA was enriched using Oligo(dT) magnetic beads, fragmented, and reverse-transcribed into cDNA, followed by end repair and adaptor ligation. The ligation products were purified with AMPure XP beads ($1.0\times$) and amplified by PCR. The resulting cDNA libraries were sequenced on an Illumina NovaSeq Xplus platform with paired-end 150 bp reads (PE150). Raw sequencing reads were quality-controlled to remove adapters and low-quality reads. Clean reads were aligned to the mouse reference genome (GRCm39/mm10) using HISAT2, and transcripts were assembled and quantified with StringTie. Differentially expressed genes (DEGs) were identified using DESeq2 with thresholds of $|\log_2FC| > 1$ and $FDR < 0.05$. Tumor-infiltrating immune cell composition was profiled using CIBERSORT, and functional enrichment analyses were performed using Gene Set Enrichment Analysis (GSEA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Lentiviral Transduction and Generation of Stable Cell Lines

Mouse and human NLRP4-OE lentiviral plasmids were designed and synthesized by Hanbio (Shanghai, China). The gene ID for human NLRP4 was 147945, and for mouse NLRP4 was 446099. The NLRP4-OE plasmid used was pHBLV-CMV-NLRP4-3FLAG-EF1-ZsGreen-T2A-PURO, and the corresponding negative control plasmid (NCOE) was pHBLV-CMV-MCS-3FLAG-EF1-ZsGreen-T2A-PURO. Lentivirus was produced by co-transfecting 293T cells with the NLRP4-OE or control plasmids, the packaging plasmid pSPAX2 (Addgene, #12260), and the envelope plasmid pMD2.G (Addgene, #12259) using Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen, #15338030) according to the manufacturer's instructions. After 24 h, the culture supernatant containing viral particles was collected and filtered through a 0.45 μm membrane. Target cells were

infected with lentivirus in the presence of 10 µg/mL polybrene (Genomeditech, #GM-040901A) for 24 h. Stable overexpressing clones were subsequently selected using 2 µg/mL puromycin (Yeasten, #60210ES).

AAV and Plasmid Construction

AAV vectors for NLRP4-OE and the corresponding negative control were synthesized by Shanghai Hanbio Biotech. The viruses were administered to mice via multi-site intra-tumoral injections. The vector used was pHBAAV-CMV-MCS-3FLAG. The primers for the NLRP4 insert were designed as follows: forward primer AAV-h-NLRP4-B/K-F: TGACCTCCATAGAAGACACCGGGATCCGCCACCATGGCAGCCTCTTTCTTC; reverse primer AAV-h-NLRP4-B/K-R: TCCTTGTAGTCGTTAATTAAGGTACCGATCTCTACCCTTGTGATTG.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software). Data were presented as mean ± standard deviation (SD) from at least three independent experiments. Tumor volume data were expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were performed using a two-tailed Student's t-test or Wilcoxon test, as specified. For comparisons involving multiple groups or conditions, one-way or two-way analysis of variance (ANOVA) was applied. Correlation analyses among immune cell fractions were performed using Spearman's rank correlation coefficient (Spearman's rho) or Pearson correlation coefficient. Correlation matrices were visualized using the R package (version 4.4.1). P values < 0.05 were considered statistically significant. Statistical significance was indicated as follows: NS, P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Results

NK cell infiltration was associated with NLRP4 overexpression.

We observed that human lung cancer tissues exhibited reduced expression of NLRP4 compared to adjacent normal controls (Fig. 1A). Notably, tissues with high NLRP4 expression exhibited pronounced NK cell infiltration (Fig. 1B), and NLRP4 abundance positively correlated with NK cell numbers, whereas NKT cell infiltration showed no correlation with NLRP4 expression (Fig. 1B).

Orthotopic implantation of LLC murine lung cancer cells engineered to overexpress NLRP4 markedly inhibited tumor growth (Fig. 1C) and was accompanied by robust infiltration of NK cells (Fig. 1D, left) and macrophages (Fig. 1D, middle), whereas CD8⁺ and CD4⁺ T-cell infiltration remained unchanged (Fig. 1D, right). Meanwhile, NLRP4-OE in the human lung cancer cell line H1975 markedly inhibited tumor growth in vivo (Fig. S1A). Flow cytometric profiling of the tumor microenvironment revealed increased infiltration of dendritic cells (DCs) and macrophages (Fig. S1B), indicating that NLRP4 primarily modulated innate immune cell recruitment. Furthermore, bulk RNA sequencing of these NLRP4-overexpressing cells revealed significant transcriptional upregulation of NK cell-related gene signatures (Fig. 1E, Fig. 1F) (Table S2). Estimation of immune cell fractions in the tumor microenvironment using CIBERSORT also indicated increased NK cell abundance in NLRP4-overexpressing LLC tumors (Fig. 1G and Fig. 1H). Pathway enrichment analysis of differentially expressed genes from RNA-seq revealed that ER stress-related pathways, including the Calcium signaling pathway, were significantly upregulated in the NLRP4 overexpressing group (Fig. S2).

NLRP4 interaction with BIP

To elucidate the mechanism by which NLRP4 modulates downstream NK-cell responses, we performed Co-IP coupled with mass spectrometry. Specifically, proteins interacting with NLRP4 were enriched from LLC cell lysates using an anti-NLRP4 antibody, followed by mass-spectrometric identification of the interaction network (Fig. 2A). The endoplasmic reticulum (ER)-resident chaperone BIP was identified as a key candidate among the NLRP4-interacting proteins (Table S3). Subsequent Co-IP assays confirmed the NLRP4-BIP interaction (Fig. 2B). We still observed XBP1s expression decreased, and we also observed Co-IP in XBP1s (Fig. 2B and Supplementary Fig. S3A). While the database analysis showed the BIP (Fig. 2C), XBP1, and PERK (Fig. S3B) increased in tumor tissue. The BIP-selective ATPase inhibitor HA15 dose-dependently increased CCL5 expression (Fig. 2D). These results confirm that the interaction between NLRP4 and BIP, in conjunction with up-regulated CCL5 expression, promotes NK cell infiltration. Immunofluorescence microscopy further verified co-localization of NLRP4 and BIP (Fig. 2E).

Synergistic anti-tumor effect of AAV-NLRP4 overexpression and BIP inhibition in immunodeficient and humanized mouse models

To evaluate the functional impact of BIP modulation in vivo, we employed an AAV-mediated intratumoral delivery system. In nude mice, combined administration of AAV-NLRP4 and HA15 markedly inhibited tumor growth (Fig. 3A–C) without affecting body weight (Fig. S4A). Analysis of the tumor microenvironment revealed increased NK cell infiltration in the combination group, whereas the abundance of myeloid cells, macrophages, dendritic cells, CD45⁺TIGIT⁺, and CD45⁺PD-L1⁺ cells remained unchanged compared with controls (Fig. 3D, Fig. S4C). In NSG mice reconstituted with human immune components, implantation of human tumor cells confirmed that NLRP4-mediated immunomodulation significantly suppressed tumor outgrowth (Fig. 3E–F) without affecting body weight (Fig. S4B). Combination therapy again enhanced NK cell infiltration, while macrophages, dendritic cells, myeloid cells, and CD45⁺PD-L1⁺ cells showed no significant differences (Fig. 3H, Fig. S4D). These results collectively indicate that the antitumor effect of NLRP4 overexpression, particularly in combination with HA15, is primarily mediated through NK cell recruitment rather than modulation of other immune cell subsets.

NLRP4 overexpression synergizes with BIP inhibition to suppress tumor growth in an NK cell-dependent manner

To further validate the NK cell-dependent antitumor activity of NLRP4 overexpression, we conducted NK cell-depletion experiments in C57BL/6 mice bearing LLC-Nlrp4-OE tumors. Consistent with observations in H1975 models, Nlrp4 overexpression markedly suppressed tumor growth ($P < 0.001$) (Fig. 4A and C). Notably, NK cell depletion completely abrogated the tumor-suppressive effect of Nlrp4 overexpression, indicating that this antitumor activity is critically dependent on NK cells (Fig. 4A and C). Notably, the combination of Nlrp4-OE with HA15 treatment further potentiated tumor inhibition ($P = 0.0478$) without affecting body weight (Fig. 4A–C). Moreover, flow cytometric analysis of the tumor microenvironment revealed that Nlrp4-OE combined with HA15 significantly enhanced NK cell infiltration and increased the proportion of cytotoxic GZMB⁺/IFN- γ ⁺ NK cells (Fig. 4D). Following NK1.1-mediated depletion, CD4⁺ and CD8⁺ T cells were increased in the LLC-OE + anti-NK1.1 group compared with LLC-Vector controls (Fig. 4E–F). However, HA15 treatment did not significantly alter T cells, macrophage subsets, or dendritic

cells in either vector or NK-depleted conditions, nor in combination with Nlrp4 overexpression (Fig. 4E–F and Fig. S5).

NLRP4 promoted CCL5-dependent NK cell migration and suppressed tumor growth

To demonstrate whether Ccl5 chemoattracted NK cells in our previous publications [11], we measured Ccl5 levels in LLC-Nlrp4-Vector/OE tumor tissues by ELISA. The results confirmed significantly elevated Ccl5 secretion in OE tumors compared with vector controls (Fig. 5A–B). Further, we established a co-culture Transwell assay (Fig. 5C). Recombinant Ccl5 significantly enhanced the migration of murine NK cells (Fig. 3D–E) and concomitantly upregulated activation markers on both NK and CD8⁺ T cells (Fig. 5D–E), suggesting that Ccl5 not only attracted NK cells but also potentiated their effector functions. Conversely, neutrophils, monocytes, B cells, double-negative T cells, and double-positive T cells did not exhibit significant migration in response to Ccl5 (Fig. 5E), indicating a selective chemotactic effect. Collectively, these results implied that Nlrp4 established a chemokine-mediated positive feedback loop, promoting NK cell tumor tropism and activating innate anti-tumor immunity.

Discussion

The NLR family represents a crucial class of cytoplasmic pattern-recognition receptors (PRRs) that detect exogenous pathogen-derived or endogenous danger signals and trigger the assembly of multiple protein complexes, including inflammasomes, which activate immune-related signaling cascades, thereby initiating innate immunity and inflammatory responses [14]. Unlike other NLR members, such as NLRP3, previous studies have shown that NLRP4 exerts negative regulation on innate immune responses [12]. Our group and others have demonstrated that NLRP4 negatively modulates the type I interferon pathway. Specifically, NLRP4 forms a protein complex, the NLRP4 signalosome, with TBK1 and USP38 and serves as a scaffold to orchestrate downstream signaling [10].

Intriguingly, our preliminary data reveal that NLRP4-overexpressing human lung-tumor tissues exhibit NK-cell infiltration, with NLRP4 expression positively correlating with NK-cell abundance (Fig. 1B). Overexpression of NLRP4 in the murine LLC line significantly suppresses tumor growth

in vivo (Fig. 1C) and is accompanied by robust infiltration of NK cells and macrophages, whereas CD8⁺ T-cell infiltration remains unchanged (Fig. 1D). Multiplex cytokine profiling revealed that conditioned medium from NLRP4-OE LLC cells exhibited markedly elevated levels of the chemokines CXCL2, CXCL10, and CCL5 compared with control cells, as we previously reported [11]. Chemokines are considered key “messengers” that orchestrate crosstalk between tumor and immune cells and are essential for directing T- and NK-cell trafficking into tumors [15]. CCL5 recruits NK cells into the TME, and its expression positively correlates with NK-cell infiltration [16]. Our data in the LLC tumor module (Fig. 4) also demonstrate it. Thus, the first central scientific question we aim to address is the molecular mechanism by which NLRP4 overexpression promotes CCL5 expression. To this end, we performed an interactome screen for NLRP4-binding partners, and mass-spectrometry analysis revealed a physical interaction between NLRP4 and the ER-stress chaperone BIP (Fig. 2A), suggesting that NLRP4 participates in the regulation of ER stress. Despite species origin differences between LLC (mouse-derived) and H1975 (human-derived) lung cancer cell lines, NLRP4 overexpression consistently promoted CCL5 secretion, enhanced NK-cell recruitment, and suppressed tumor growth in both models. Our previous work established that CCL5 is required for NK-cell-mediated antitumor activity in this system [11], and independent studies confirm its essential role in NK-cell recruitment in vivo [17]. Together, these data not only position NLRP4 as an upstream regulator of this CCL5-dependent pathway, but also underscore the generality and cross-species conservation of the NLRP4–CCL5–NK cell axis.

ER stress is triggered by the accumulation of unfolded or misfolded proteins, disrupting ER homeostasis and activating the unfolded protein response (UPR) to restore equilibrium and promote cell survival [18]. When stress is irresolvable, the UPR switches to pro-apoptotic and/or autophagic programs. The UPR sensors IRE1 α (inositol-requiring enzyme 1 α), PERK, and ATF6 α are maintained in inactive monomeric states through association with BIP. BIP’s intrinsic ATPase activity drives conformational cycling between an ADP-bound “open” state and an ATP-bound “closed” state, dictating its affinity for unfolded proteins. Binding of unfolded proteins to BIP’s substrate-binding domain induces allosteric release of UPR sensors, relieving BIP-mediated inhibition of IRE1 α and other pathways [19]. The IRE1–XBP1 axis has been shown to promote CCL5 (RANTES) expression via XBP1 mRNA splicing. Kang et al. demonstrated IRE1 α -dependent

CCL5 induction in erythroid cells [20], and Izumi et al. reported IRE1 α -driven IL-1 β production in macrophages during infection [21]. Consistently, our data show that HA15, a small-molecule inhibitor of BIP's ATPase activity, augments CCL5 secretion (Fig. 2D).

Current studies on ER stress–tumor crosstalk mainly focus on direct regulation of tumor cells or TME-resident immune cells by UPR sensors (IRE1 α , PERK, ATF6 α). However, whether tumor-intrinsic ER stress–IRE1 α signaling modulates immune-cell function by controlling chemokine secretion remains largely unexplored. Yang and colleagues first revealed that tumor-cell XBP1 signaling shapes an immunosuppressive microenvironment by regulating myeloid-derived suppressor cells (MDSCs) [22], whereas Mattias showed that ER stress enhances NK-cell tumoricidal capacity [23]. Notably, we found that NLRP4 overexpression directly boosts NK-cell GZMB/IFN- γ production (Fig. 4D) [11], implying that tumor-cell ER stress can modulate NK-cell function. Activated NK cells secrete IFN- γ and other cytokines to kill tumor cells, recruit macrophages and dendritic cells (DCs) [24], and establish a positive-feedback anti-tumor loop via macrophage-derived CCL5, thereby synergizing with ACT.

Integrating our findings with this conceptual framework, we propose a novel, NLRP4-centered axis that links tumor cell-intrinsic ER stress to NK cell-mediated anti-tumor immunity. We hypothesize that NLRP4-overexpressing tumor cells activate the BI pathway through ER stress, upregulate chemokines such as CCL5, and exploit endogenous chemotactic gradients in the TME to recruit and activate NK cells and other immune effectors. This dynamically reshapes the immune microenvironment and enhances the efficacy of NK cell therapy through a chemokine-cytokine positive-feedback loop (Fig. 6 and Fig. S6).

The ER-stress regulatory network is a central hub linking tumor-cell survival mechanisms to immune-microenvironment remodeling; targeting this pathway could achieve both direct tumoricidal effects and potent anti-tumor immunity. Thus, deciphering the NLRP4-BIP/ER-stress axis as a novel mechanism governing chemokine expression and NK-cell trafficking may overcome the bottleneck of poor immune-cell infiltration in lung cancer immunotherapy, improving the efficacy of NK cell therapy in solid tumors. Conceptual validation is first performed within this study.

476

477 In summary, to address the clinical challenge of limited immune-cell infiltration in solid tumors, we
478 integrate our previous finding that tumor-intrinsic NLRP4 remodels the local microenvironment and
479 propose the central hypothesis that “the NLRP4 signalsome modulates BIP ATPase activity to
480 orchestrate ER stress, thereby promoting NK-cell tumor tropism and enhancing cellular
481 immunotherapy.” Using in vitro and in vivo models, we will dissect the mechanisms by which
482 NLRP4 drives NK-cell trafficking, with particular emphasis on the NLRP4-BIP/ER-stress circuit that
483 governs CCL5 expression and establishes a positive-feedback anti-tumor loop. These studies will
484 provide direct mechanistic insight into CCL5-mediated NK-cell recruitment, break the technical
485 bottleneck of ACT in lung cancer and other solid tumors, and expand the patient population
486 benefiting from cellular immunotherapy.

487

488 **Limitations of the study**

489 Although dissecting the NK-cell infiltration machinery in the tumor microenvironment (TME) has
490 become a central theme in cancer immunology, the precise molecular logic whereby the innate
491 pattern-recognition receptor NLRP4 sculpts NK-cell function remains largely uncharted. Building on
492 our preliminary data, we have now extended these findings toward a translational setting and provide
493 proof-of-concept that ex vivo-expanded peripheral NK cells can be synergistically combined with
494 NLRP4-targeted modulation, thereby broadening the therapeutic portfolio of onco-immunotherapy. A
495 limitation of the present work is the absence of high-resolution structural information; acquiring
496 cryo-electron microscopy snapshots of the native NLRP4 signalsome may be critical to fully
497 decode its conformational dynamics. Prospective studies are additionally required to validate
498 whether NLRP4 over-expression (NLRP4-OE) serves as an “ignition switch” that converts an
499 immunologically “cold” TME into an inflamed, NK-cell-permissive niche.

500 **Conclusion**

501 This study identifies NLRP4 as a critical regulator of NK cell-mediated antitumor immunity in lung
502 cancer. NLRP4 overexpression drives NK cell recruitment and activation via BIP-dependent
503 upregulation of CCL5, establishing a chemokine-mediated positive feedback loop that selectively
504 enhances NK cell infiltration without affecting other immune subsets. Combined NLRP4
505 overexpression and BIP inhibition synergistically suppress tumor growth in murine and humanized

506 models in an NK cell-dependent manner, underscoring the mechanistic specificity of this axis. These
 507 findings uncover a previously unrecognized NLRP4–BIP–CCL5 pathway as a potential
 508 immunotherapeutic target for potentiating innate antitumor responses, offering a promising strategy
 509 to enhance NK cell-mediated tumor clearance.

510

Abbreviations

AAV	Adeno-associated virus
ACT	Adoptive cell-transfer therapies
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
CDX	Cell-derived xenograft
Co-IP	Co-immunoprecipitation
Co-IP-MS	Co-immunoprecipitation followed by mass spectrometry
CR	Complete response
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GSEA	Gene Set Enrichment Analysis
NSCLC	Human non-small cell lung cancer
ICIs	Immune-checkpoint inhibitors
KEGG	Kyoto Encyclopedia of Genes and Genomes
LLC	Lewis lung carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
NK	Natural killer
ORRs	Objective response rates
OE	Overexpression
PBMCs	Peripheral blood mononuclear cells
RT-qPCR	Real-time quantitative PCR

TME	Tumor microenvironment
WB	Western blotting

Supplemental information

The data supporting the findings of this study are available in this paper and its supplementary information files. Any additional information required to reanalyze the data reported in this paper is available from the Lead Contact upon reasonable request.

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Author contributions

HW and YL performed experiments, analyzed data, created the figures, and wrote the manuscript. SL and HW conceived and designed the article. ZL and YW reviewed and revised the manuscript. All authors critically read and approved the final manuscript.

Data availability

- The RNA-seq data have been deposited to Zenodo (<https://doi.org/10.5281/zenodo.17592045>) and are publicly available as of the date of publication.
- The mass spectrometry data have been deposited in Zenodo (<https://doi.org/10.5281/zenodo.17669232>) and are publicly available as of the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from

539 the lead contact upon request.

540

541 **Declarations**

542 **Ethics approval and consent to participate**

543 This study was conducted in compliance with the Declaration of Helsinki. The study protocol was
544 reviewed and approved by the Ethics Committee of Shanghai Chest Hospital (Approval No.
545 KS21005). Written informed consent was obtained from all participants before enrollment. All
546 animal experiments were performed following the institutional regulations for the ethical treatment
547 of animals and were approved by the Shanghai Chest Hospital Institutional Animal Care and Use
548 Committee (Approval No. KS21005).

549 **Consent for publication**

550 Not applicable.

551 **Competing interests**

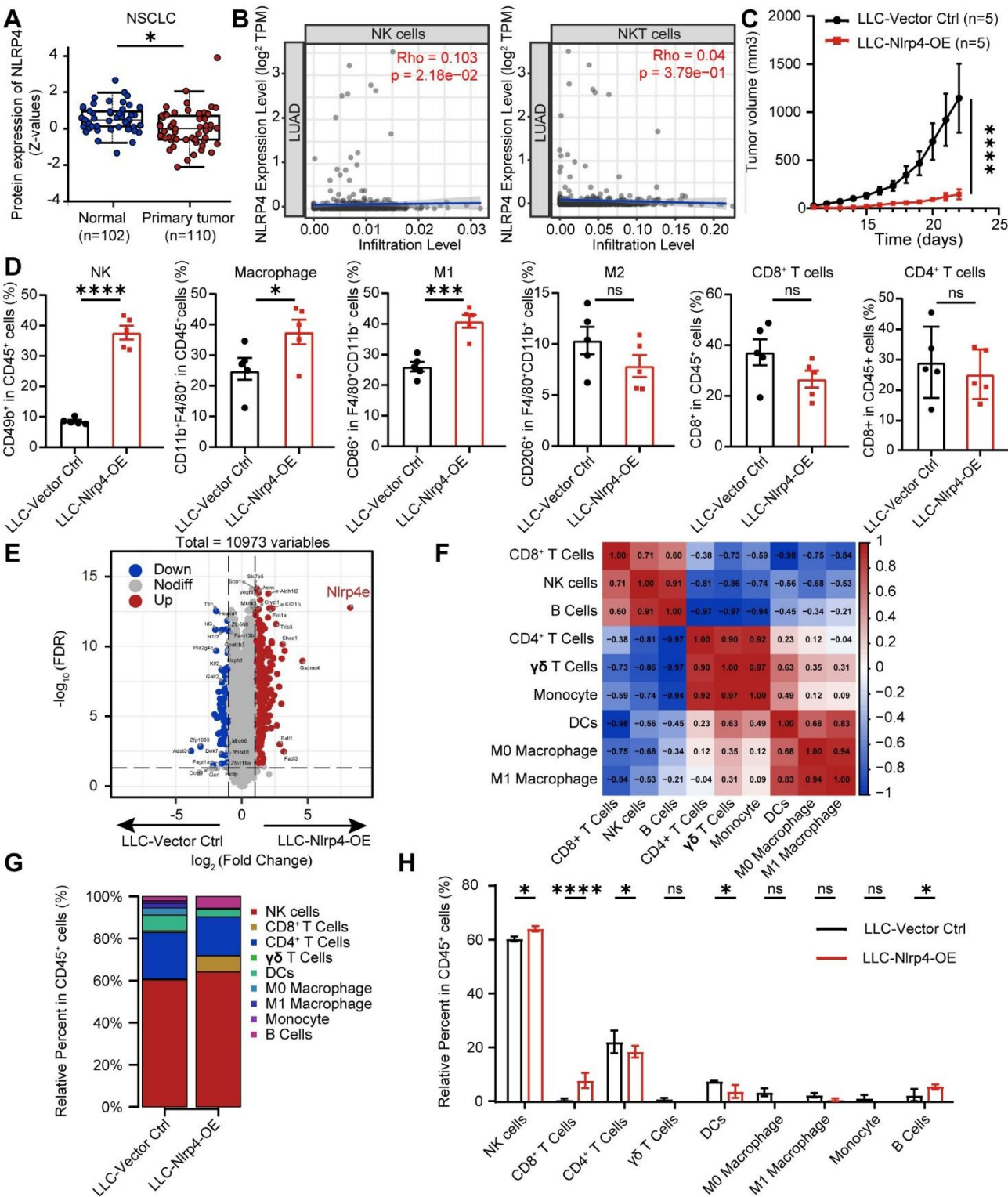
552 The authors declare no competing interests.

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622 **Fig. 1 Expression of NLRP4 in NSCLC and its association with NK cell infiltration.** (A) Bar
623 chart showing the relative protein expression of NLRP4 in normal tissues and NSCLC patient
624 samples. Data were presented as mean ± SEM (bars) with individual data points (dots). Z-values

625 represent standard deviations from the median within each cancer type. Data were obtained from the
626 CPTAC (Clinical Proteomic Tumor Analysis Consortium), normalized within each sample and across
627 samples. **(B)** Correlation between NLRP4 expression and infiltration of NK or NKT cells.
628 Correlation coefficients were calculated using purity-adjusted Spearman's rho. Data were obtained
629 from TIMER2.0 (<http://timer.cistrome.org/>). **(C)** Tumor growth curves of LLC-Vector control (n = 5)
630 and LLC-Nlrp4-OE (n = 5) in C57BL/6J mice bearing subcutaneous tumors. Data were presented as
631 mean $\pm$ SEM, analyzed by two-way ANOVA. **(D)** Flow cytometric analysis of NK cells,
632 Macrophages, CD8⁺ and CD4⁺ T-cell infiltration in subcutaneous tumors from LLC-Vector Ctrl (n =
633 5) and LLC-Nlrp4-OE (n = 5) C57BL/6J mice. Data were presented as mean $\pm$ SD, analyzed using a
634 two-tailed Student's t-test. **(E)** Volcano plot showing differential expression analysis of RNA-seq
635 data from subcutaneous tumors of C57BL/6J mice in the LLC-Vector Ctrl (n = 5) and LLC-Nlrp4-
636 OE (n = 5) groups. DEGs were identified using DESeq2 with thresholds of $|\log_2FC| > 1$ and FDR <
637 0.05. **(F)** Estimation of immune cell fractions in the tumor microenvironment based on RNA-seq
638 data using CIBERSORT, followed by correlation analysis using Pearson's correlation coefficient. **(G)**
639 Stacked bar chart representing the proportion of different immune cell populations. **(H)** Grouped bar
640 chart showing immune cell infiltration levels. Data were presented as mean $\pm$ SD, analyzed by two-
641 way ANOVA. LUAD, Lung adenocarcinoma.

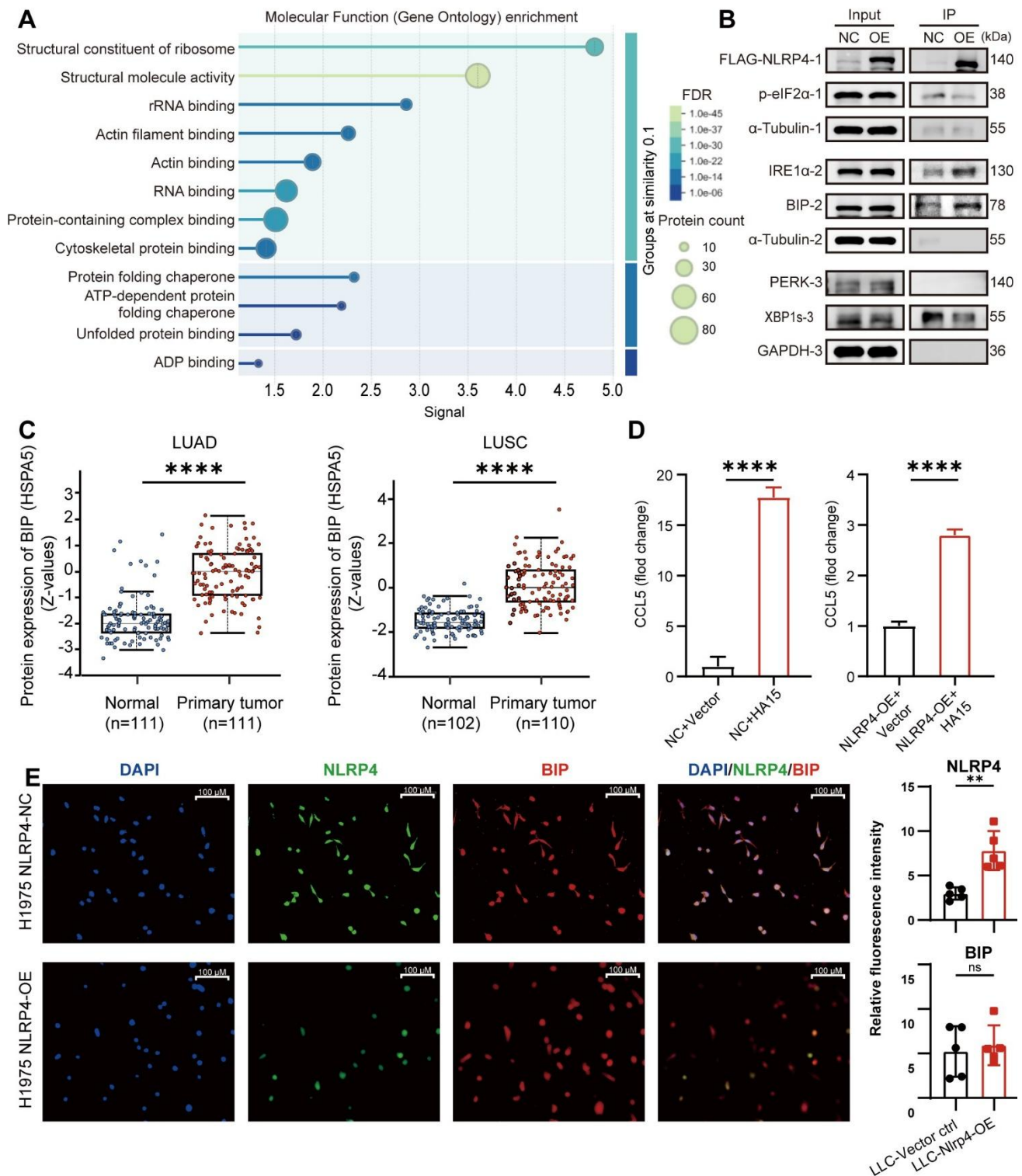


Fig. 2 NLRP4 regulated ER stress and downstream signalling pathways. (A) Lollipop plot showing enriched pathways from Co-IP-MS analysis of NLRP4-interacting proteins. Proteins co-immunoprecipitated with NLRP4 in LLC cells were subjected to enrichment analysis. Each lollipop (bubble) represents a significantly enriched pathway. The size of each bubble indicates the number of

648 associated genes, and the color reflects the adjusted p-value (FDR). Enrichment analysis was
649 performed using the STRING database and visualized with R 4.4.1 software. **(B)** Co-IP of LLC-
650 Vector Ctrl and LLC-Nlrp4-OE cells, with WB detection of ER stress-related proteins. Original
651 Western blot images and the corresponding unexposed ECL films were provided in Fig. S2. **(C)** Bar
652 chart showing relative BIP protein expression in normal tissues and NSCLC patient samples. Data
653 were presented as mean $\pm$ SD (bars) with individual data points (dots). Z-values represent standard
654 deviations from the median. Data were obtained from CPTAC and normalized within and across
655 samples. **(D)** qPCR analysis of CCL5 expression in LLC cells treated with the BIP inhibitor HA15
656 (concentration to be determined). Data were presented as mean $\pm$ SD, analyzed using a two-tailed
657 Student's t-test. **(E)** Immunofluorescence detection of NLRP4 and BIP in subcutaneous tumors from
658 LLC-Vector Ctrl and LLC-Nlrp4-OE. Nuclei were stained with DAPI (blue), BIP was detected in the
659 red channel (Alexa Fluor® 594), and NLRP4 in the green channel (FITC 488). Representative
660 images were shown, and fluorescence intensity was quantified using ImageJ software. Data were
661 presented as mean $\pm$ SD and analyzed using a two-tailed Student's t-test.

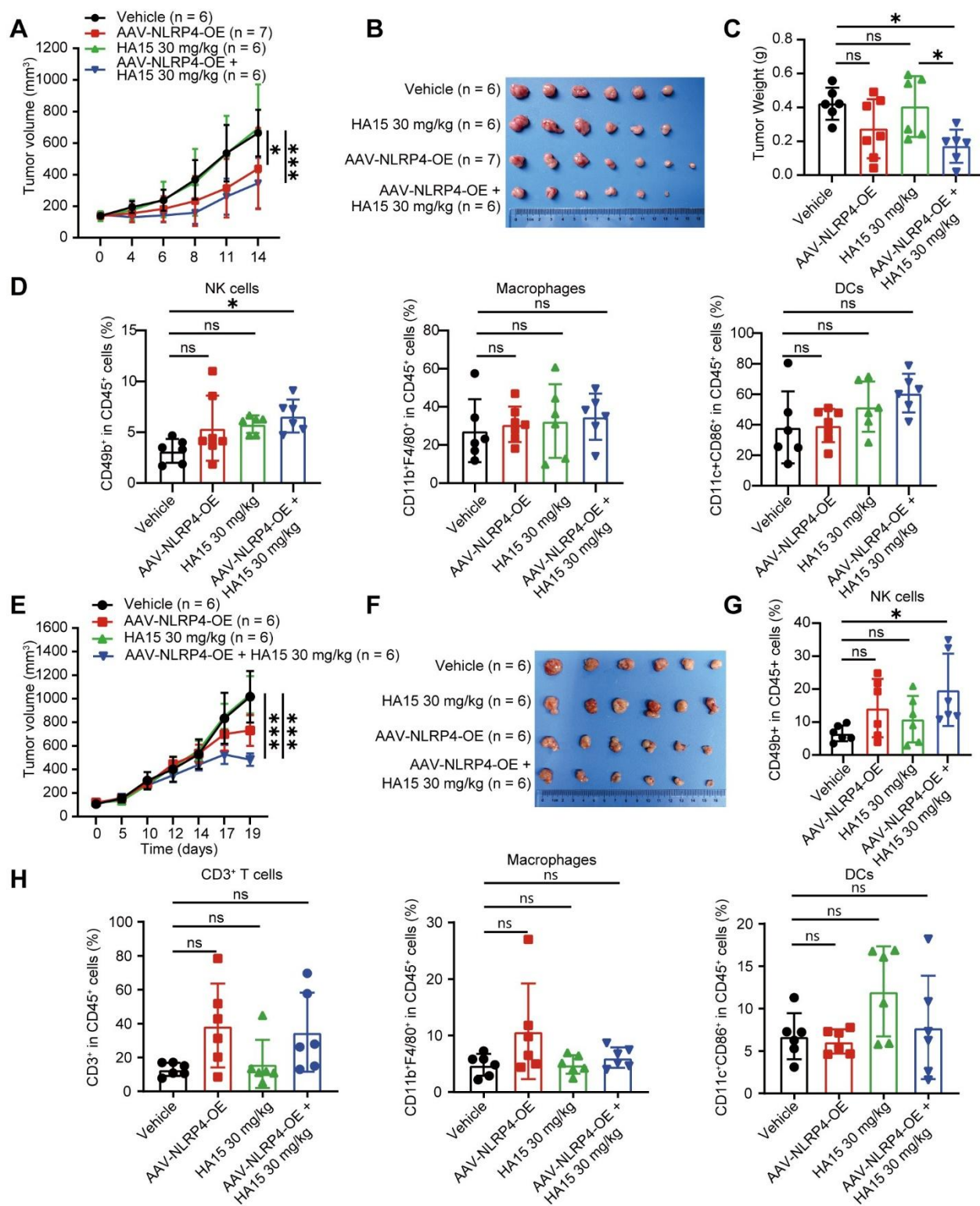


Fig. 3 Synergistic anti-tumor effect of AAV-NLRP4 overexpression and BIP inhibition in immunodeficient and humanized mouse models. (A–D) BALB/c nude mice: Subcutaneous H1975-Vector control or H1975-NLRP4-OE tumors were treated with vehicle control (n = 6), HA15 (30 mg/kg, n = 6), AAV-NLRP4-OE (n = 7), or the combination of AAV-NLRP4-OE + HA15 (30

668 mg/kg, n = 6) for 14 days. Tumor volume over time (A), endpoint tumor size (B), and tumor weight
669 at day 14 (C) were measured. Flow cytometric analysis of tumor-infiltrating immune cells was
670 performed and shown in (D). Data were presented as mean $\pm$ SEM for growth curves and mean $\pm$ SD
671 for tumor weight and immune cell analyses. Statistical significance was determined by one/two-way
672 ANOVA or two-tailed unpaired Student's t-test.

673 **(E–H)** humanized NSG mice: Subcutaneous H1975-Vector control or H1975-NLRP4-OE tumors
674 were treated with vehicle control (n = 6), HA15 (30 mg/kg, n = 6), AAV-NLRP4-OE (n = 6), or AAV-
675 NLRP4-OE + HA15 (30 mg/kg, n = 6) for 19 days. Tumor volume over time (E), endpoint tumor
676 size (F), and flow cytometric analysis of tumor-infiltrating immune cells (G, H) were measured. Data
677 were presented as mean $\pm$ SEM for growth curves and mean $\pm$ SD for immune cell analyses.
678 Statistical significance was determined by two-way ANOVA (tumor growth) or one-way ANOVA
679 (immune cell infiltration).

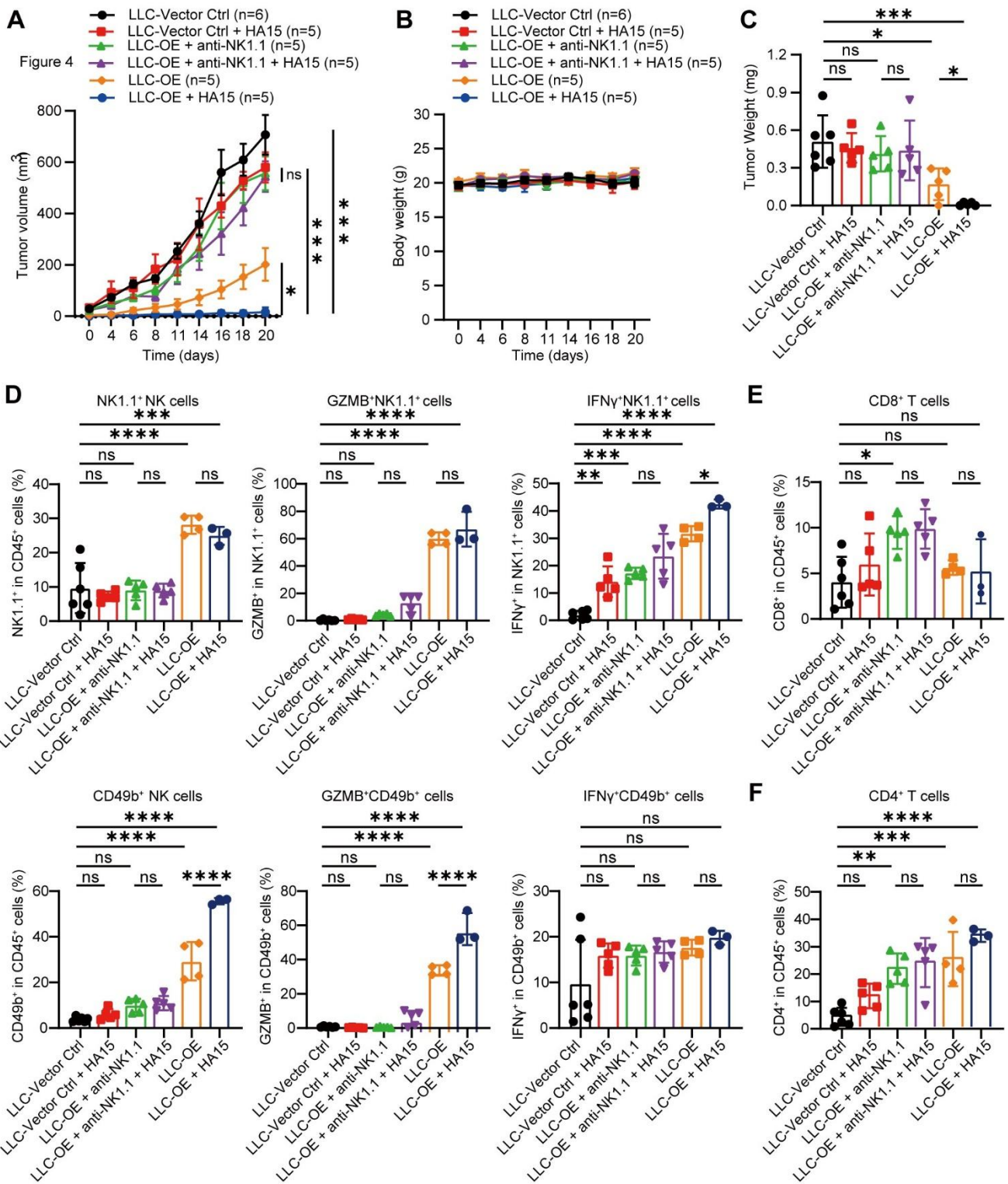
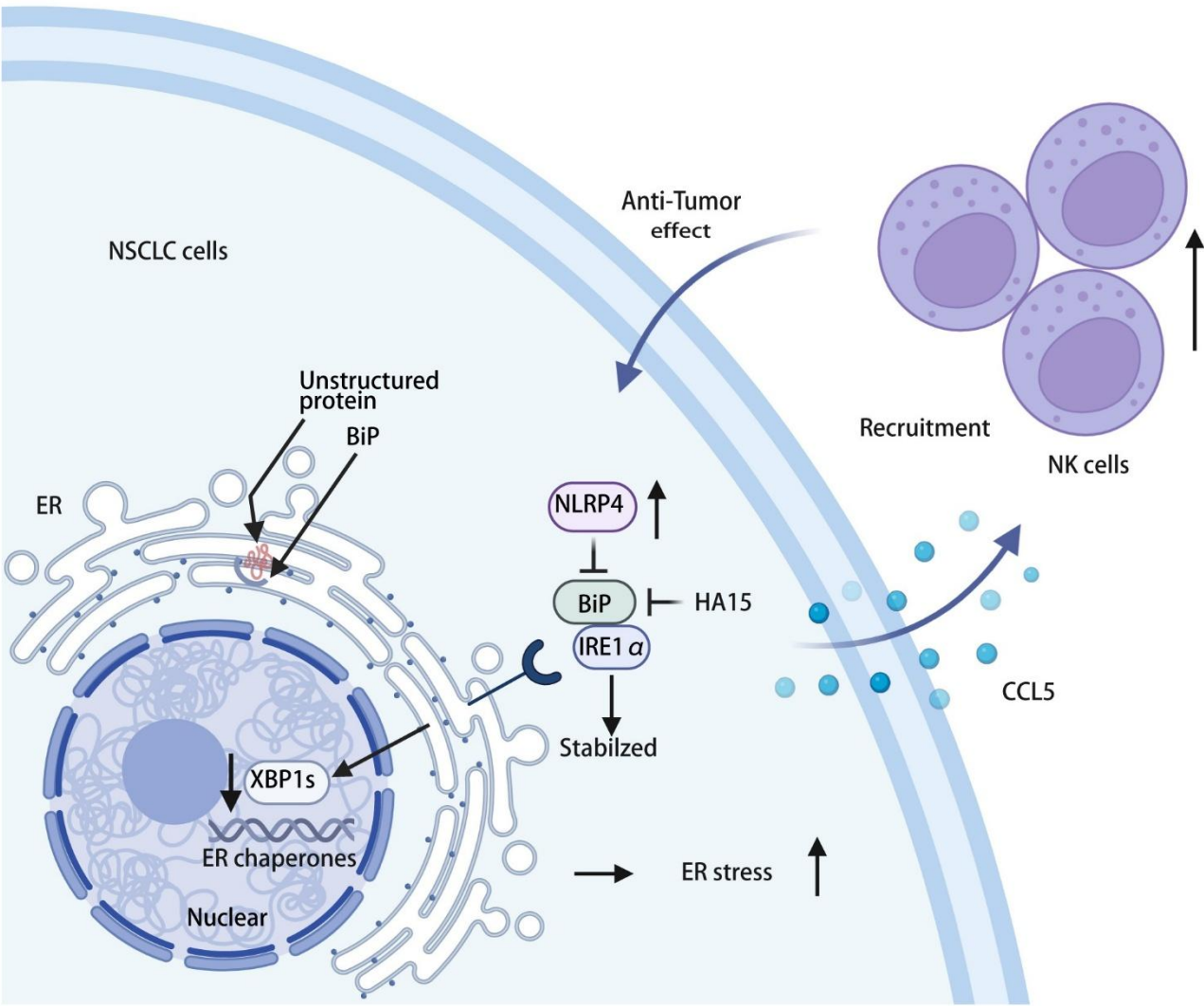


Fig. 4 Combined Nlrp4 overexpression and HA15 suppressed tumor growth in an NK cell-dependent manner. C57BL/6J mice bearing subcutaneous tumors from LLC-Vector control or LLC-Nlrp4-OE cells were divided into six groups: LLC-Vector control (n = 6), LLC-Vector control + HA15 (30 mg/kg, n = 5), LLC-Nlrp4-OE (n = 5), LLC-Nlrp4-OE + HA15 (n = 5), LLC-Nlrp4-OE +

686 anti-NK1.1 (n = 5), and LLC-Nlrp4-OE + anti-NK1.1 + HA15 (n = 5). **(A-B)** Tumor volume (A) and
687 body weight (B) during the treatment period. Data were presented as mean $\pm$ SEM and analyzed by
688 two-way ANOVA. **(C)** Tumor weight at day 20. Data were presented as mean $\pm$ SD and analyzed
689 using one-way ANOVA with Tukey test for multiple comparisons. **(D)** Flow cytometric analysis of
690 immune cell infiltration in subcutaneous tumors. Data were presented as mean $\pm$ SD and analyzed
691 using one-way ANOVA with Tukey test for multiple comparisons.

697 $\pm$ SD, analyzed using a two-tailed Student's t-test. (C) Transwell assay showing the effect of Ccl5 on
 698 the migration of splenic immune cells isolated from C57BL/6J mice. Upper chamber: 5×10^5 cells in
 699 100 μ L 1640 medium; lower chamber: serum-free 1640 medium containing 100 ng/mL Ccl5.
 700 Migrating cells were quantified by flow cytometry. (D-E) Quantification of NK (D) and other
 701 immune cells (E) in the lower chamber. Data were presented as mean $\pm$ SD, analyzed by two-tailed
 702 Student's t-test.



703
 704

705 **Figure S6** Proposed mechanism by which NLRP4 regulates endoplasmic reticulum stress and
 706 promotes NK cell recruitment through CCL5. This study demonstrated that NLRP4 interacted with
 707 BiP to regulate ER stress, leading to downregulation of XBP1s, which subsequently promoted CCL5
 708 secretion and enhanced NK cell recruitment into the tumor microenvironment (TME).

709

Supplementary Files

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- [TableS2.xlsx](#)
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