

Human lung adenocarcinoma CD47 is upregulated by interferon-g and promotes tumor metastasis

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Tumor cells can evade attack by phagocytes by upregulating the self-marker CD47. The mechanisms underlying tumor CD47 upregulation, however, remain unclear. Here, we report that human lung adenocarcinoma CD47 is upregulated by interferon-g (IFN-g), the level in the tumor microenvironment of which is markedly increased after tumor metastasis and chemotherapy. The IFN-g receptor is expressed in various human lung adenocarcinoma tissues regardless of the CD47 protein expression, and lung adenocarcinoma CD47 expression is significantly enhanced following tumor metastasis or chemotherapy treatment. In line with this, CD47 expression in various lung cancer cells is markedly increased by IFN-g treatment. Mechanistically, IFN-g promotes CD47 expression by activating interferon regulatory factor-1 (IRF-1), which binds to an IRF-1-binding domain within the CD47 promoter region and increases CD47 transcription. Functionally, IFN-g-enhanced CD47 expression facilitates human lung cancer cell invasion both in vitro and in vivo, whereas IFN-g-induced CD47 upregulation and cancer metastasis are blocked by mutating the IRF-1-binding site within the CD47 promoter. Our results reveal IFN-g-enhanced CD47 expression as a novel mechanism promoting human lung adenocarcinoma progression.

INTRODUCTION

Metastasis is the primary underlying cause of mortality in lung cancer patients. For a primary lung tumor to successfully metastasize, it must possess two important characteristics: the ability to avoid destruction by the immune system and a considerable migratory capacity. Previous studies have demonstrated that CD47 is an important molecule that can modulate both events, promoting immune evasion by initiating signal regulatory protein a (SIRPa)-mediated inhibitory signaling in immune cells¹ and facilitating tumor cell migration as an adhesion molecule.² On the one hand, as a new immune checkpoint molecule,³ CD47 binds to SIRPa on the phagocyte surface and initiates inhibitory signaling to prevent phagocytosis of tumor cells by phagocytes.^{4,5} Exploiting CD47-SIRPa signaling has been proven to be an effective way for cancers to evade immune attack

by phagocytes. Blockade of CD47 alone via antibodies or fusion proteins or in combination with other antitumor reagents shows great potential in the treatment of various cancers.^{6–8} On the other hand, CD47 is also an essential component in cell migration and transmigration.^{9–11} CD47 blockade significantly alters the interactions of CD47 with integrins and inhibits leukocyte cell migration and transmigration.¹² Supporting the roles of CD47 in facilitating both tumor immune evasion and metastasis, CD47 expression is upregulated in various tumor cells and positively linked to tumor malignancy.^{13–15} Given the upregulation of CD47 in metastatic lung cancer, a new strategy that explores the use of chimeric antigen receptor T cells (CAR-T) targeting CD47 to treat lung cancer cell metastasis in nude mice has been developed.¹⁶ However, the mechanism underlying CD47 upregulation in tumor cells remains incompletely understood. Zhang et al.¹⁷ recently showed that hypoxia-inducible factor 1a (HIF-1a) upregulates CD47 expression in breast cancer cells to promote phagocytosis evasion. However, that study failed to explain the inflammatory cytokine-stimulated upregulation of CD47 expression in other cancers or cultured cancer cells under normoxic conditions. Although a previous study by Betancur et al.¹⁸ implied that a CD47-associated super-enhancer may link proinflammatory signaling to CD47 upregulation in breast cancer, only a few kinds of cancer possess CD47-associated super-enhancers. Comparing

Received 13 September 2021; accepted 29 April 2022;
<https://doi.org/10.1016/j.omto.2022.04.011>.

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CD47 with another immune checkpoint molecule, PD-L1, Casey et al.¹⁹ reported that the expression of both CD47 and PD-L1 could be regulated by MYC. Given that the MYC oncogene encodes a transcription factor that is overexpressed in many human cancers, their study may explain the upregulation of CD47 expression in cancers with high MYC activity. Recent evidence has demonstrated that CD47 expression can also be promoted by nuclear factor kappa B (NF- κ B) through direct binding to a specific response element in the CD47 promoter and that the upregulation of CD47 expression may serve as part of the mechanism of tumor chemoresistance.²⁰ Given that NF- κ B signaling is often associated with inflammatory cytokines, their study links CD47 upregulation in tumor cells to certain inflammatory conditions during chemotherapy treatment.

As a type II interferon, IFN- γ has long been regarded as an antitumor factor that suppresses tumor growth via immune activation.²¹ Given that IFN- γ is secreted mainly by T cells, its level in the tumor microenvironment (TME) is generally increased following T cell infiltration during chemotherapy or radiotherapy.^{22,23} However, the understanding of the role of IFN- γ in anticancer immunity has been evolving over the last decade. It has been shown that IFN- γ plays both antitumorigenic and protumorigenic roles in different phases of the immunoeediting process.^{24,25} Increased IFN- γ expression can induce CD4⁺ T cell apoptosis, alter the CD4:CD8 ratio, and subsequently impair secondary antitumor immune responses.²⁶ In addition, recent studies suggest that IFN- γ can upregulate the expression of programmed cell death protein 1 ligand (PD-L1), a membrane-bound immune checkpoint molecule on the surface of tumor cells. As the binding of PD-L1 to programmed cell death protein 1 (PD-1) on activated CD8⁺ T cells leads to T cell apoptosis, IFN- γ signaling may facilitate tumor cell escape from antitumor CD8⁺ T cell cytotoxicity, promote tumor cell survival, and induce adaptive resistance.²⁷ Given that CD47 is also a membrane-bound immune checkpoint molecule like PD-L1, we postulate that IFN- γ may play protumorigenic roles by upregulating tumor cell CD47 expression.

In the present study, we report that CD47 expression in various human lung adenocarcinomas and cells can be induced by IFN- γ and that CD47 upregulation induced by IFN- γ is positively associated with tumor metastasis. Our study identifies an IFN regulatory factor-1 (IRF-1)-binding domain within the CD47 promoter region and shows that IFN- γ , whose level in the TME is elevated following tumor metastasis or chemotherapy, can promote CD47 expression by activating IRF-1. The promotive effects of IFN- γ on human lung adenocarcinoma CD47 expression and cancer metastasis were further validated in athymic BALB/c nude mice implanted with human lung cancer cells. Our results reveal that human lung adenocarcinoma can exploit IFN- γ to upregulate CD47 expression during metastasis and chemotherapy treatment.

RESULTS

Upregulation of lung adenocarcinoma CD47 expression following cancer metastasis or chemotherapeutic treatment
To explore the expression and function of CD47 in human lung adenocarcinoma progression, we collected tissue samples from 120 lung

adenocarcinoma patients at Drum Tower Hospital, Nanjing University School of Medicine (Nanjing, China). Examination of CD47 distribution in lung adenocarcinoma tissue sections via immunohistochemistry found that, to our surprise, a considerable number of tumor tissue samples (30 of 120) expressed little or no CD47 (Table S1). The level of CD47 in lung adenocarcinoma displayed no significant difference among different age and sex groups. The differential CD47 expression levels in lung cancer samples agreed with the data in The Human Protein Atlas database, which showed no or very low levels of CD47 in a certain number of tumor types, even at advanced stages (<https://www.proteinatlas.org/ENSG00000196776-CD47/pathology>). However, a significant increase in the number of CD47⁺ cells was observed in lung adenocarcinoma at an advanced pathological stage, which correlated with the possibility of tumor metastasis.³ The observation of metastatic lung adenocarcinoma expressing high levels of CD47 (CD47⁺ rate, 68.3%, 41/60 versus 81.7%, 49/60) suggests that the upregulation of CD47 expression may be associated with tumor metastasis.

To validate this hypothesis, we obtained tumor tissue samples from 14 lung adenocarcinoma patients who had been diagnosed with tumor lymph node metastasis. Analysis of CD47 levels in the paired primary lung adenocarcinoma and lymph node-metastatic lung adenocarcinoma tissue samples showed that the CD47 levels in lymph node-metastatic lung adenocarcinoma tissue were significantly higher than those in primary lung adenocarcinoma tissue (Figures 1A and 1B). Screening tissue samples in cancer tissue bank at GuLou Hospital, we also found five lung adenocarcinoma biopsy samples that were collected before and after the primary lung adenocarcinoma metastasis to the liver. Tumor tissues from the same patients were dissected to examine CD47 levels. As shown in Figures 1C and 1D, the CD47 levels in liver-metastatic cancer tissue were significantly higher than those in primary lung adenocarcinoma tissue. We also examined the expression level of CD47 in lung adenocarcinoma before and after chemotherapy with sorafenib and found that the expression level of CD47 was markedly increased after sorafenib treatment (Figures S1A and S1B).

Upregulation of lung adenocarcinoma CD47 expression by IFN- γ
To explore the mechanisms that govern CD47 upregulation in lung adenocarcinoma following tumor metastasis or chemotherapy treatment, we analyzed the TME before and after tumor metastasis or chemotherapy treatment. We found that following tumor metastasis or chemotherapy treatment, the TME exhibited a common phenomenon: increase in IFN- γ levels. As shown in Figure S2, immunohistochemical (IHC) staining showed that IFN- γ levels in the TME were markedly increased following tumor metastasis or chemotherapy treatment. Given that the elevation of IFN- γ levels in the TME occurred after both tumor metastasis and chemotherapy treatment, and that IFN- γ can promote the expression of similar immune checkpoint molecules, such as PD-L1,²⁸ we postulate that IFN- γ may play a role in upregulating lung adenocarcinoma CD47 expression during the process of tumor metastasis or chemotherapy. Supporting this hypothesis, we found that lung adenocarcinoma tissue samples, regardless of the CD47 protein level, expressed the IFN- γ receptor, IFN- γ R (Figure S3).

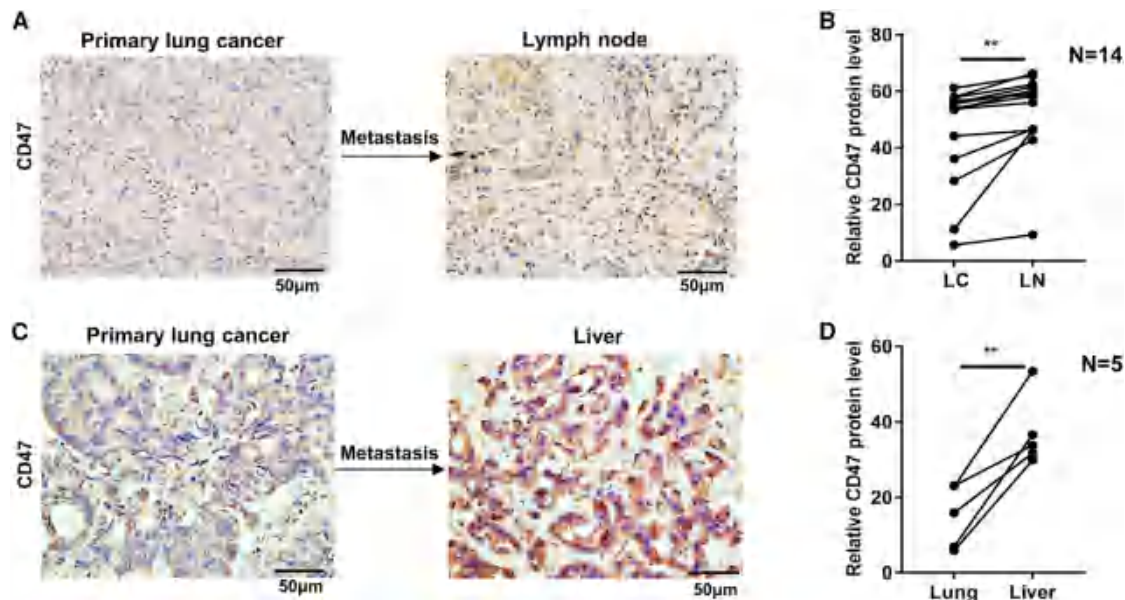


Figure 1. CD47 levels in human lung adenocarcinoma cells were increased following tumor metastasis

(A and B) Comparison of CD47 expression between primary lung adenocarcinoma and lung adenocarcinoma metastases in the lymph nodes. (C and D) Comparison of CD47 expression between primary lung adenocarcinoma and lung adenocarcinoma metastases in the liver. (A) and (C) showed representative IHC images for CD47 staining from 14 to 5 lung adenocarcinoma tissue samples, respectively. Data were presented as means \pm SDs. ** $p < 0.01$. Scale bar, 50 μ m.

To test whether IFN- γ can promote CD47 expression in lung adenocarcinoma, we treated various human lung cancer cells with IFN- γ and monitored CD47 levels by flow cytometry, immunofluorescence labeling, and western blot (WB) analysis. As shown in Figure 2, flow cytometry analysis of CD47 surface expression clearly indicated an increase in the CD47 level upon IFN- γ treatment in all lung cancer cell lines (Figure 2A). The upregulation of CD47 expression on the cell surface of various lung cancer cell lines was confirmed by immunofluorescence labeling (Figure 2B). Western blot analysis showed that the total CD47 level in various lung cell lines was also markedly increased by IFN- γ treatment (Figure 2C). In line with these findings, qRT-PCR analysis indicated that the transcriptional level of CD47 in various lung cancer cell lines was also enhanced upon IFN- γ treatment (Figure 2D).

Primary lung cancer cells were also derived from lung adenocarcinoma patients and validated for IFN- γ -induced CD47 expression. In this experiment, we isolated cancer cells from tumor tissue samples collected from three lung adenocarcinoma patients. The primary cancer cells were treated with IFN- γ for 24 h, and CD47 levels were monitored by flow cytometry. As shown in Figure S4, the CD47 levels on the cell surface of all three isolated cancer cell populations were markedly increased by IFN- γ treatment.

IFN- γ upregulates CD47 expression via activating IRF-1-mediated signaling

To determine how IFN- γ increases CD47 expression in cancer cells, we treated A549 cells with IFN- γ and then monitored the expression alterations in genes in the IFN signaling pathway. As shown in Fig-

ure 3A, qRT-PCR analysis showed that the mRNA levels of the transcription factors IRF-1, IRF-9, STAT1, IRF-7, STAT2, and NF- κ B2 were significantly increased in A549 cells following IFN- γ treatment. In particular, the expression of IRF-1 was increased nearly 20-fold. To evaluate whether transcription factors were involved in the IFN- γ -dependent regulation of the CD47 mRNA, we silenced these potential genes by transfecting A549 cells with different sets of siRNAs (Figure S5). Then, we treated cells with or without IFN- γ and monitored the activity of the CD47 reporter. After normalization against control luciferase activity, we found that knockdown of each of these genes reduced CD47 reporter activity, confirming the involvement of the IFN signaling pathway in CD47 upregulation induced by IFN- γ in human lung cancers. Among the molecules along the IFN signaling pathway, IRF-1 made the greatest contribution, and silencing IRF-1 expression inhibited more than 60% of IFN- γ -induced CD47 expression in A549 cells (Figure 3B). We further tested the IRF-1 protein level in various lung cancer cells with or without IFN- γ treatment. Supporting the role of IRF-1 in IFN- γ -induced lung cancer CD47 expression, WB analysis showed that the IRF-1 protein levels in various lung cancer cells were significantly increased by IFN- γ treatment (Figure 3C).

To further explore how IRF-1 induction by IFN- γ can upregulate CD47 expression in lung cancer cells, we performed a bioinformatic analysis of the CD47 gene to determine whether IRF-1 can interact with the promoter region of CD47 using the JASPAR database (<https://jaspar.genereg.net/>).²⁸ As shown in Figure 4A (left), a putative binding site of IRF-1 within the promoter region of CD47 was predicted. We next constructed a reporter system consisting of the

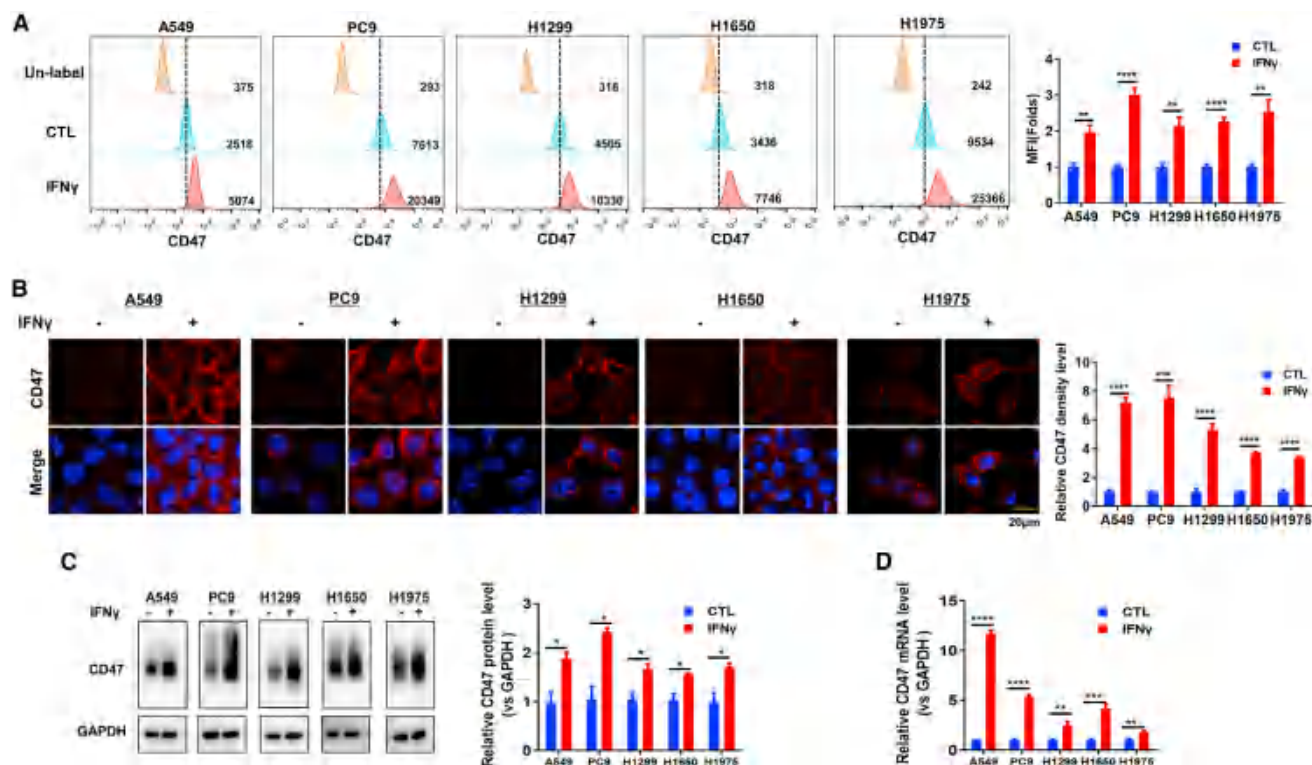


Figure 2. Induction of CD47 by IFN-g in human lung cancer cells

(A) Flow cytometry analysis of CD47 surface expression in human lung cancer cell lines upon incubation with recombinant IFN-g (100 ng/mL, 24 h). Right: Representative image; left: quantitative analysis. (B) Immunofluorescence analysis of CD47 expression in human lung cancer cell lines upon incubation with recombinant IFN-g (100 ng/mL, 24 h). Scale bar, 20 mm. (C) Western blot analysis of CD47 expression in human lung cancer cell lines after IFN-g treatment. (D) qRT-PCR analysis of CD47 mRNA in human lung cancer cell lines upon incubation with recombinant IFN-g (100 ng/mL, 24 h). Left: Representative image; right: quantitative analysis. Data from more than 3 independent experiments were presented as means \pm SDs. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

wild-type (WT) IRF-1-binding site or deleted IRF-1-binding sequence within the CD47 promoter region and transfected A549 cells with these constructs. A reporter assay demonstrated that deletion of the IRF-1 binding site within the CD47 promoter region (CD47 Prom DIRF1) via site-directed mutagenesis completely blocked the induction of CD47 by IFN-g (Figure 4A, right), confirming the role of IRF-1 binding to the CD47 promoter region in IFN-g-induced CD47 expression. To confirm the binding of the predicted factors to the specific sequence sites, we carried out chromatin immunoprecipitation (ChIP) assays. As shown in Figure 4B, the binding of IRF-1 to the CD47 promoter induced by IFN-g treatment was much stronger than the binding to an immunoglobulin G (IgG) control. Specific binding of IRF-1 with the CD47 promoter region was further validated in another lung cancer cell line, PC9 cells (Figure S6). Next, IRF-1 mRNA and protein levels detected by qRT-PCR and WB assays, respectively, confirmed the successful knock-down of IRF-1 expression via IRF-1-specific small hairpin RNA (shRNA) (Figures 4C and 4D). Various assays, including qRT-PCR (Figure 4E), flow cytometry (Figure 4F), immunofluorescence labeling (Figure 4G), and WB analysis (Figure 4H) showed that IRF-1 knockdown abolished the induction of CD47 at the mRNA and protein levels by IFN-g treatment. Taken together, our results suggest

that IFN-g induces CD47 expression by increasing the expression of IRF-1, which binds to the promoter region of CD47 and promotes CD47 expression.

Role of IFN-g-enhanced CD47 expression in promoting tumor metastasis

Previous reports have indicated that CD47 is involved in regulating cell migration.¹² To explore the function of IFN-g-induced CD47 in cancer cells, we generated CD47-knockout (KO) A549 cells by the CRISPR-Cas9 system (Figures S5A and S5B). As shown in Figures S7C–S7E, various assays, including flow cytometry (Figure S7C), WB analysis (Figure S7D), and immunofluorescence labeling (Figure S7E), showed that CD47 was knocked out completely with and without IFN-g treatment. Subsequently, A549 cells and CD47-KO A549 cells were treated with IFN-g for 24 h. The cell migration was monitored by cell monolayer scratch and transwell assays, respectively. As shown in Figure 5, IFN-g treatment markedly enhanced A549 cell migration to repair wounds (Figure 5A) and infiltration through transwell filters (Figure 5B). Knockout of CD47 expression in A549 cells via the CRISPR-Cas9 system largely abolished the enhanced cell migration (Figures 5A and 5B) induced by IFN-g, suggesting that CD47 is a critical component in

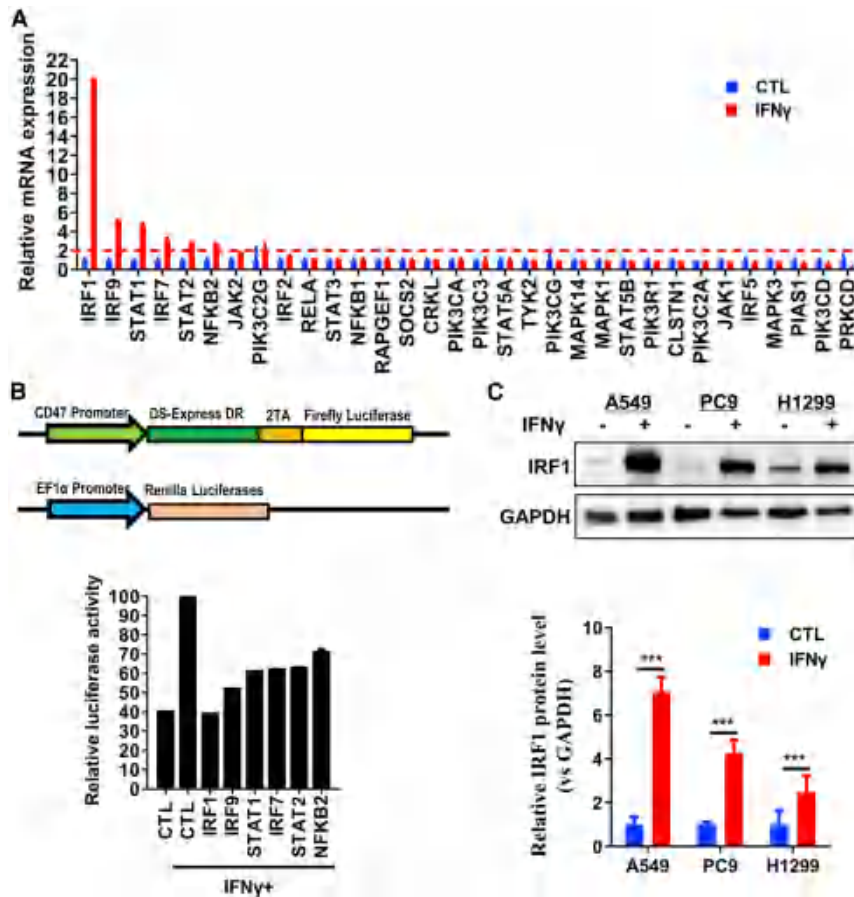


Figure 3. Identification of genes involved in the IFN signaling pathway that upregulates CD47 expression

(A) qRT-PCR analysis of various genes involved in the IFN signaling pathway in A549 cells upon incubation with recombinant IFN-g (100 ng/mL, 24 h). (B) Normalized luciferase reporter expression of A549 cells transduced with different sets of lentiviral shRNA constructs. Upper: Schematic representation of the CD47-Prom-Firefly Luciferase-EGFP-Renilla luciferase constructs used to generate the reporter cell lines. Lower: Quantitative analysis. (C) IRF1 protein levels in human lung cancer cells upon incubation with recombinant IFN-g (100 ng/mL, 24 h). Upper: Representative image; lower: quantitative analysis. Data from more than 3 independent experiments were presented as means \pm SDs. ***p < 0.001.

liver (Figure 6C). In contrast, CD47 KO blocked the growth and liver metastasis of CD47-KO A549 tumors induced by IFN-g. These results strongly argue that IFN-g promotes human lung cancer metastasis by enhancing tumor CD47 expression.

DISCUSSION

Previous studies have shown the upregulation of CD47 in various tumors and the positive correlation between CD47 level and tumor malignancy.^{15,30} However, the mechanisms underlying the tumor cell CD47 upregulation remain incompletely understood. In fact, there are

many established tumors even at advanced stages expressing no or very low CD47 (<https://www.proteinatlas.org/ENSG00000196776-CD47/>) pathology. In this study, through screening over 120 human lung adenocarcinoma tissues and various lung cancer cells, we find that CD47 levels are markedly increased following tumor metastasis or chemotherapy treatment, and both events correlate with the elevation of IFN-g levels in the TME.

Given that CD47 upregulation occurs in many different metastatic cancers,^{31–33} the mechanism underlying tumor CD47 upregulation should be a common mechanism shared by various cancers. Although previous studies have reported that CD47 expression in breast cancer cells can be enhanced by HIF-1a or CD47-associated super-enhancers,^{17,18} these mechanisms may not apply to other tumor cells or breast cancer cells under various conditions. In contrast, as a major inflammatory cytokine released by T cells, IFN-g has been widely detected in the TME, and its level is generally increased following cancer metastasis or chemotherapy treatment.^{22,34,35} Therefore, an increase in CD47 expression induced by IFN-g may serve as a common mechanism underlying the upregulation of cancer CD47 expression. In line with this, the upregulation of CD47 by the IFN-g/JAK1/STAT1 pathway was reported by Ye et al. in NCI-H1975 and A549 cells.³⁶ Supporting the role of IFN-g-induced CD47 in tumor metastasis,

IFN-g-promoted tumor cell migration. This finding was also confirmed in PC9 cells (Figure S8).

To determine the effect of IFN-g-mediated upregulation of tumor cell CD47 on tumor metastasis in vivo, we established a lung cancer cell metastasis model using immunodeficient BALB/c-nude mice.²⁹ A549 cells and CD47-KO A549 cells were directly injected into the lungs of the BALB/c-nude mice (Figure 6A). Three weeks after the orthotopic injection, the mice which were injected A549 cells or CD47-KO A549 cells were randomly divided into two groups (Figure 6A). As shown, one group was injected IFN-g (10 ng/mouse, once every 2 days), and the other group served as a control without IFN-g injection. After 5 weeks, the mice were sacrificed to analyze tumor growth and metastasis. The distribution of A549 tumor cells in various mouse organs was examined by both IHC staining and immunolabeling using an anti-human CD47 antibody. As anti-human CD47 antibody only detected human A549 tumor cells but not mouse cells, labeling of the mouse lungs and other organs with the anti-human CD47 antibody would clearly reflect human lung cancer cell growth and metastasis in mice. As shown by both IHC staining and immunolabeling of human CD47 (Figures 6B and 6C), IFN-g treatment markedly promoted not only A549 tumor growth in the mouse lungs (Figure 6B) but also A549 tumor metastasis from the mouse lungs to the mouse

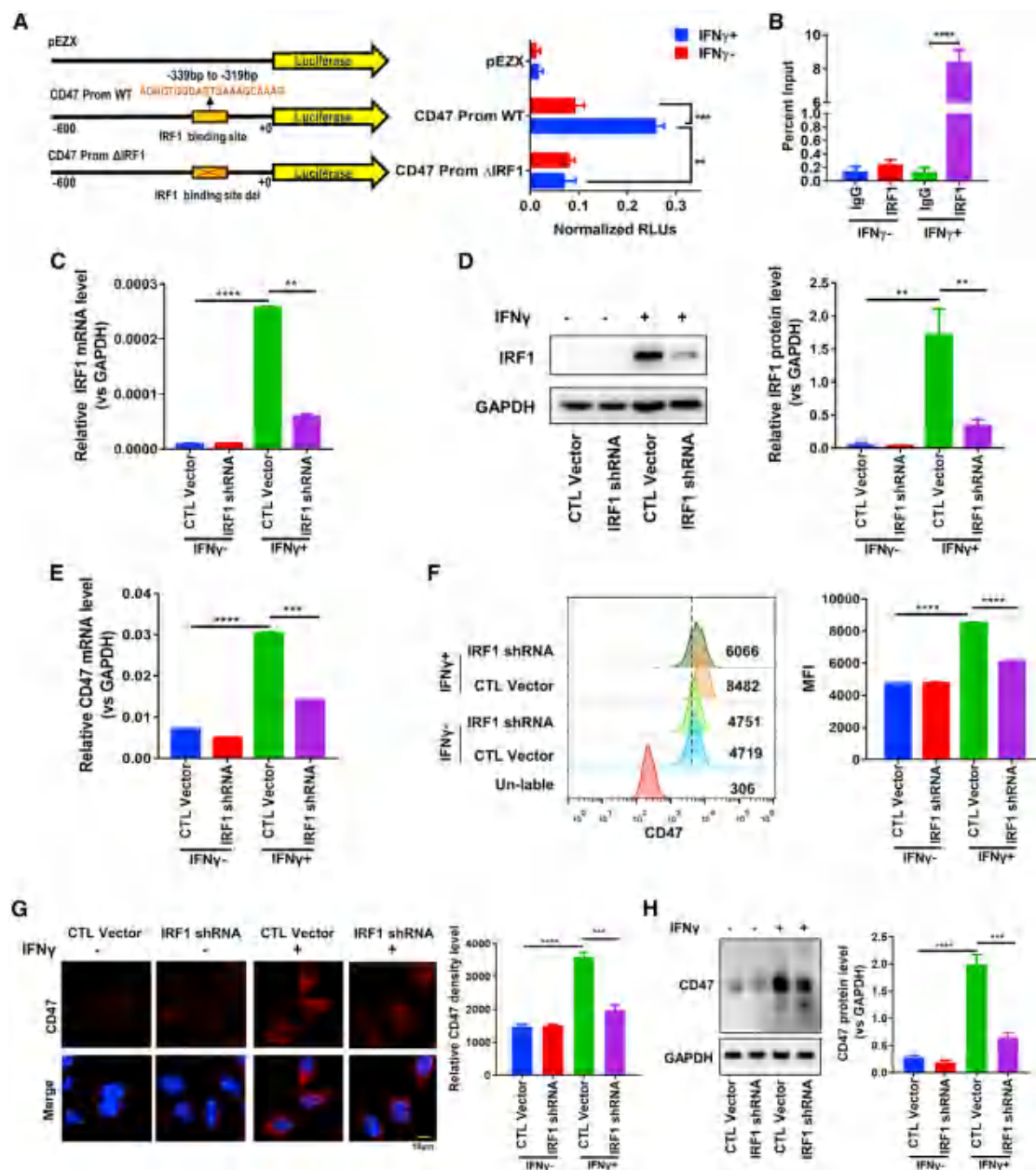


Figure 4. IFN-g induces CD47 expression in lung cancer cells through IRF-1

(A) Sequence of the CD47 promoter showing the position of the putative IRF-1-binding site. (B) Reporter assay for putative IRF-1 binding. The results were normalized to relative luciferase units (RLUs). (C) ChIP assay analyzing the CD47 promoter in A549 cells. The results were normalized to the input. (D and E) IRF-1 mRNA (D) and protein (E) levels in A549 cells transfected with IRF-1-specific shRNA or scramble shRNA after IFN-g treatment.

(F and G) CD47 level in A549 cells transfected with IRF-1-specific shRNA or scramble shRNA after IFN-g treatment detected by flow cytometry (F) and immunofluorescence (G). Scale bar, 10 mm. (H) Western blot analysis of CD47 levels in A549 cells transfected with IRF-1-specific or scramble shRNA. Left: Representative image; right: quantitative analysis. Data from 3 independent experiments were presented as means \pm SDs. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

several investigators had also reported that IFN-g and CD47 could promote metastasis.^{9,37–39} As IFN-g can also induce the expression of PD-L1 in tumor cells,⁴⁰ the coordinate induction of CD47 and PD-L1 by IFN-g may underlie the pro-tumorigenic role of IFN-g during antitumor immunotherapy. Supporting the effect of IFN-g

on cancer cell CD47 induction, we found that various human lung cancers, regardless of the CD47 protein expression pattern, expressed IFN-g receptors (Figure S3). Our finding that IFN-g enhances cancer cell CD47 expression, which in turn promotes cancer metastasis, suggests the possible involvement of IFN-g in tumor metastasis. These

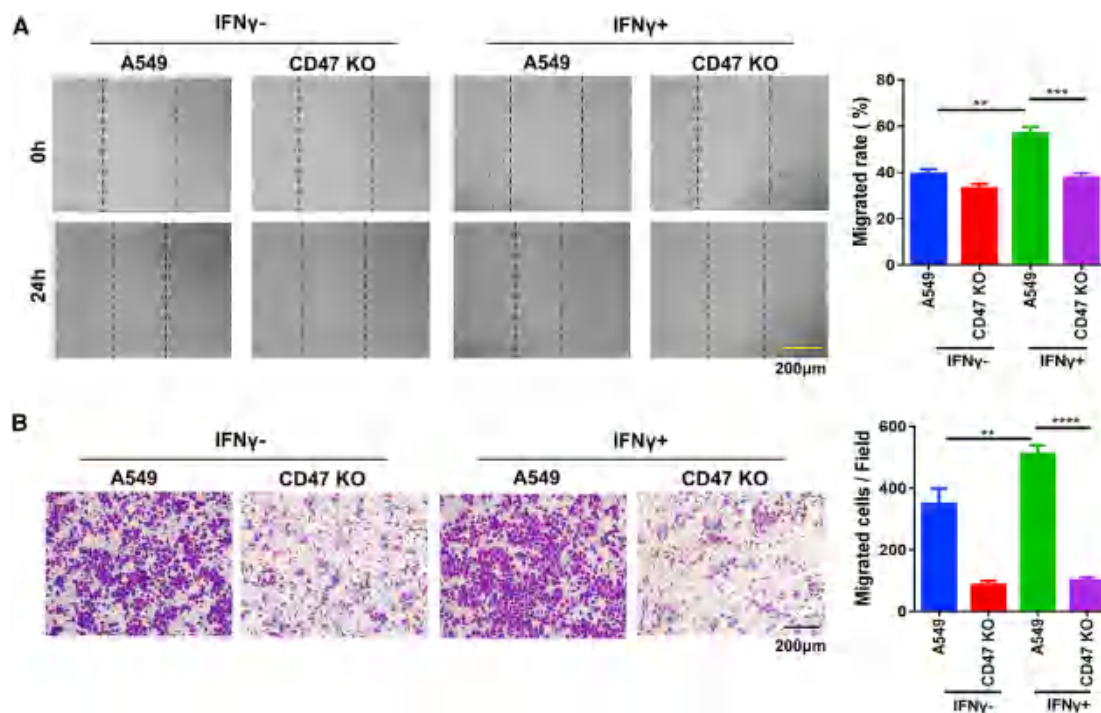


Figure 5. IFN-g promotes lung cancer cell metastasis by upregulating CD47 expression in vitro

(A) Scratch-wound healing assay in A549 cells (A549-WT) and CD47-knockout A549 cells (A549-CD47-KO) were treated with/without IFN-g. (B) Transwell assay in A549 cells (A549-WT) and A549-CD47-KO cells were treated with/without IFN-g. Data from 3 independent experiments were presented as means \pm SDs. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bar, 200 μ m.

results agree with the conclusion that IFN-g plays a dual role, promoting or suppressing tumor progression during the different phases of the immunoeediting process, such as elimination, equilibrium, and escape.^{41–43}

In addition to serving as a self-recognition molecule to ligate phagocyte SIRP α , CD47 is an adhesion molecule associated with integrin.⁴⁴ CD47 has been shown to participate in cell migration and transmigration processes.⁴⁵ Blockade of CD47 via antibodies or functional fusion proteins can inhibit leukocyte transmigration across collagen-coated transwell filters.¹² These studies strongly argue that in addition to ligating phagocyte SIRP α to prevent cell phagocytosis by phagocytes,⁴⁶ CD47 can bind to other adhesion molecules or initiate independent signaling pathways to facilitate cell adhesion and migration. Although the mechanism underlying CD47-mediated cell migration remains unclear, it may involve the lateral interactions of CD47 with cell surface integrin or initiation of various signaling pathways, including calcium homeostasis, cyclic nucleotide signaling, nitric oxide and hydrogen sulfide biosynthesis, and stem cell transcription factor activities.⁴⁷ In the present study, we used both in vitro and in vivo systems to examine the role of CD47 induced by IFN-g in regulating human lung cancer cell migration and metastasis. As CD47 KO largely abolished IFN-g-promoted tumor cell migration in vitro (Figure 5) and metastasis of human lung adenocarcinoma from the lungs to the liver in nude

mice (Figure 6), our results suggest an effect of CD47 on promoting tumor cell migration. In summary, the present study demonstrates that human lung adenocarcinoma CD47 expression is upregulated by IFN-g through activating IRF-1-induced CD47 transcription, and upregulated CD47 expression by IFN-g promotes human lung cancer metastasis.

MATERIALS AND METHODS

Human tissues

In total, 120 lung adenocarcinoma and paired adjacent normal lung tissue samples, 5 pairs of lung adenocarcinoma tissue samples collected before and after tumor metastasis to the liver, 4 pairs of lung adenocarcinoma tissues collected before and after chemotherapy treatment, and 14 lung adenocarcinoma with lymph node metastasis tissue samples were collected from patients who underwent surgical resection at the Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China). After surgery, the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C . The clinical features of the patients are detailed in Table S1. All of the patients signed consent letters, and all of the experiments were performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the guidelines of Nanjing University and approved by the ethics committee of Nanjing University Medical School (2020-158-01).

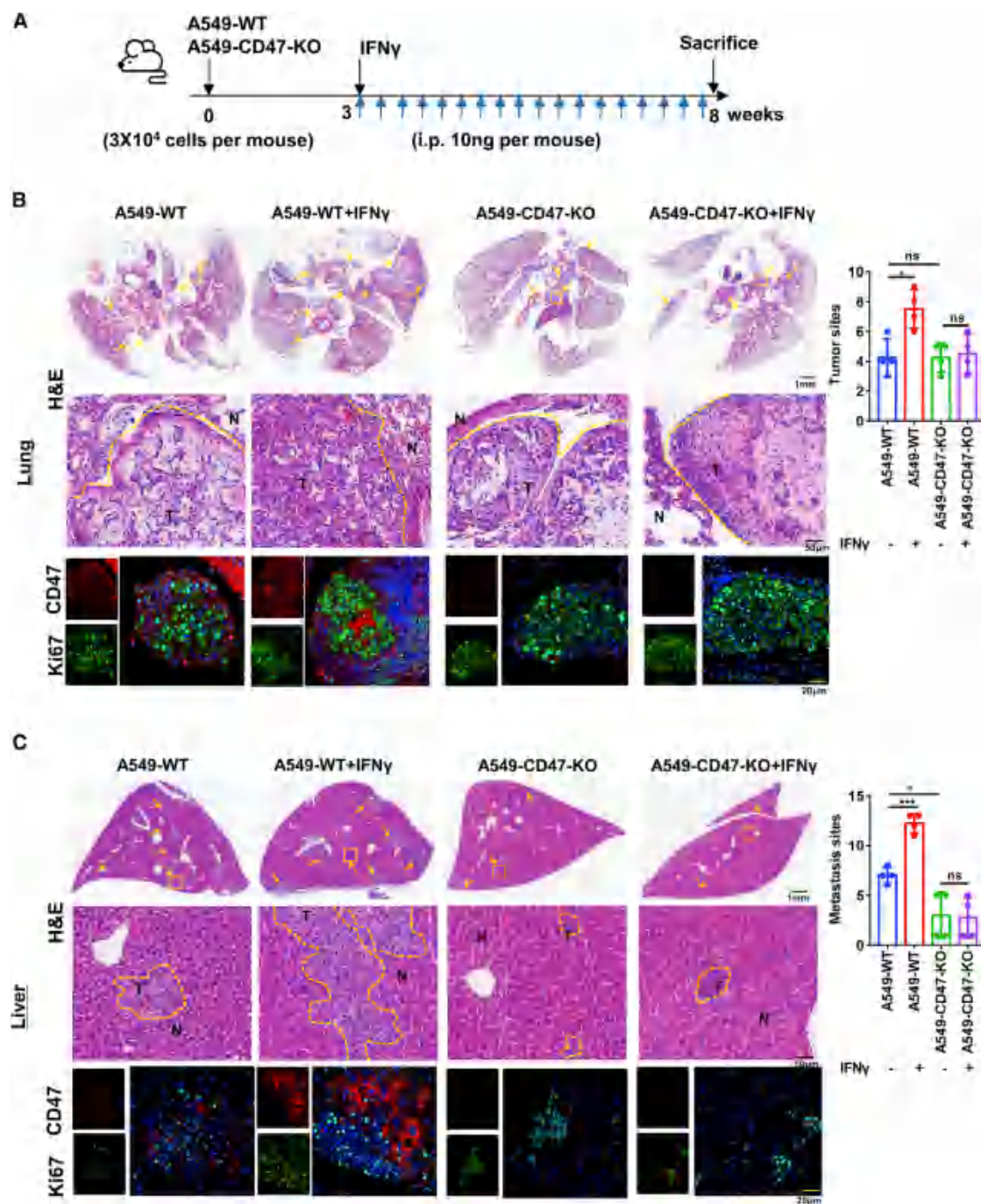


Figure 6. IFN-g promotes human lung cancer cell metastasis in immunodeficient mice by upregulating CD47

(A) A549 cells (A549-WT) and A549-CD47-KO were engrafted in the lungs of the BALB/c-nude mice. Three weeks post-engraftment, the mice were randomly divided into 2 groups. One group was administered with IFN-g (10 ng/mouse, injected once every 2 days) (A549-WT + IFN-g; A549-CD47-KO + IFN-g), and the other group without IFN-g injection was served as control (A549-WT; A549-CD47-KO). After 5 weeks, the mice were sacrificed to analyze tumor growth and metastasis. (B and C) H&E staining and human CD47 immune staining in mouse lungs (B) and livers (C). In both (B) and (C), left: representative images; right: quantification of tumor nodules. Data were presented as means \pm SDs. * $p < 0.05$, *** $p < 0.001$. NS, no significance.

Cell lines

Five human lung cancer cell lines (A549, PC9, H1299, H1650, and H1975) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). A549, PC9, H1299, and H1650 were maintained in DMEM (Gibco, Carlsbad, CA, USA). H1975 was maintained in 1640 medium (Gibco). Cell culture medium was supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco). All of the cells were cultured in a humidified incubator at 37°C with 5% CO₂.

Lung cell isolation from human lung cancer tissue

Lung cancer cell suspensions were obtained from human tumor samples by enzymatic and mechanical digestion using the gentle MACS Dissociator (Miltenyi, Bergisch-Gladbach, Germany). Briefly, tumor specimens were minced into small pieces under sterile conditions and digested for 30 min at 37°C following the manufacturer's protocol (Miltenyi). The resulting cell suspension was filtered through a 70-μm mesh (BD Biosciences, San Jose, CA, USA), the red blood cells were lysed, and the cell suspension was washed with DMEM. Cells were seeded in 6-well plates and incubated overnight. The following day, the cells were washed with 10 mM PBS twice and then exposed to 100 ng/mL IFN-γ (PeproTech, Cranbury, NJ, USA) for 24 h. On day 3, the cells were collected for flow cytometry analysis.

IHC

IHC was performed on 5-mm sections of tissue specimens following the manufacturer's protocol. Briefly, tissues were fixed in 4% paraformaldehyde and washed with 10 mM PBS. An isotype-matched monoclonal antibody (mAb) (Iso, anti-rabbit IgG mAb, Equitech-Bio, Kerrville, TX, USA) with no specific affinity to proteins of interest was used as the negative control. Antibodies against CD47 (ab175388, Abcam, Cambridge, UK) and IFN-γ (15365-1-AP, Proteintech, Wuhan, China) as well as the isotype control mAbs were diluted in 5% bovine serum albumin at 25 mg/mL and immobilized on tissue slides at 4°C overnight. For defining the CD47 expression stage, intensity of staining was categorized as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong); the percentage of chromatin cells as 0 (none), 1 (1%–10%), 2 (11%–50%), 3 (51%–80%) and 4 (>80%). Multiply these 2 numbers: 0–2 is considered (–); 3–4 (+); 5–8 (++) and 9–12 (+++). A range of 0–1+ was considered as negative expression, while 2–3+ was positive expression.⁴⁸ Staining was evaluated by two pathologists in a blinded fashion. The average integrated optical density (IOD) of CD47 and IFN-γ IHC staining in cancer and normal tissue sections were calculated with Image-Pro Plus software (version 5.0, Media Cybernetics, Rockville, MD, USA). Five fields were analyzed per slide.

Flow cytometry analysis

Cancer cell lines were seeded in six-well plates. Then, the cells were exposed to 100 ng/mL IFN-γ (PeproTech) for 24 h after the cells reached 70%–80% confluence. On day 3, the cells were trypsinized and washed with 10 mM PBS twice. Then, the cells were stained with a fluorescein isothiocyanate (FITC)-conjugated anti-CD47 antibody (sc-21786, Santa Cruz Biotechnology, Dallas, TX, USA) on ice

for 30 min and analyzed by flow cytometry using an LSRII (Beckman Coulter Danaher, Washington, DC, USA). Data were analyzed using FlowJo software. Experiments were performed at least three times for each cell line.

Immunofluorescence

Immunofluorescence microscopy was performed to identify the expression of CD47 on the surface of cancer cells. Cells were cultured on four-well chamber slides. At the time of harvest, cells were fixed with 4% paraformaldehyde and subsequently probed with antibodies against CD47 (sc-21786, Santa Cruz), IFN-γR (10808-1-AP, Proteintech), or PD-L1 (#13684, Cell Signaling Technology, Danvers, MA, USA) followed by incubation with fluorophore-tagged secondary antibodies (488 and 594 nm). All of the samples were treated with DAPI for nuclear staining (358 nm). For confocal microscopy, a Leica (Wetzlar, Germany) confocal microscope was used.

Plasmid and siRNA transfection

siRNAs designed to specifically silence were purchased from GenePharma (Shanghai, China). A scrambled siRNA served as a control. The siRNA sequences are listed in Table S3. Human shRNA plasmids designed to specifically repress the expression of IRF-1 were purchased from GeneCopoeia (Guangzhou, China). An empty plasmid served as a negative control (control plasmid). For the reporter analyses, CD47 promoter WT plasmid containing the IRF-1 binding site was generated by inserting the fragment (600 bp, obtained from the University of California, Santa Cruz [UCSC] database, <http://genome-asia.ucsc.edu>), from A549 genomic DNA into the multicloning site (MCS) of the PGL3 Basic Vector (Promega, Madison, WI, USA). The CD47 promoter DIRF1 plasmid was carried out using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA), cloned by PCR amplification with primer pairs that flank the region to be deleted from the CD47 promoter WT plasmid. The clonal paired primers used were as follows: CD47 prom WT XhoI-F: CCCTCGAGGAAGAGCAGAGCGAGTA; CD47 prom WT HindIII-R: CCAAGCTTGCGCACTCGGCCCCG CCCCAGCA; Q5CD47 prom DIRF1-F: AGGAGAAAAGTAGAGAG AG; and Q5CD47 prom DIRF1-R: CGCGCTTCAGGGGCCGGGT. The plasmid and siRNAs were transfected into A549 cells using Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Total RNA and protein were isolated 36 h after transfection and assessed by qRT-PCR and western blotting, respectively.

WB analysis

Western blotting was performed following the manufacturer's protocol. Primary antibodies against CD47 (AF4670, R&D Systems, Minneapolis, MN, USA), PD-L1 (#13684, Cell Signaling Technology) and IRF1 (11335-1-AP, Proteintech) were used. Immunoreactivity was visualized with an ECL-Plus kit (Thermo Fisher Scientific, Waltham, MA, USA) using the ChemiDoc MP system (Bio-Rad, Hercules, CA, USA). For WB analysis of CD47, cell lysates were supplemented with NaIO₄ (10 mM) to enhance CD47 detection before

mixing with a nonreducing SDS-PAGE sample buffer and heating for 5 min at 60°C.

Total RNA extraction with TRIzol and gene expression qPCR assays

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific) and stored at -80°C according to the manufacturer's protocol. The isolated RNA was reverse transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) in a 20-mL reaction volume. mRNA expression was determined on a LightCycler 96 qPCR system (Roche Diagnostics, Mannheim, Germany). The mRNA levels of genes were measured by qPCR using SYBR Mix (Vazyme) following the vendor's specifications. Briefly, the reactions were performed in a volume of 20 mL (10 mL of SYBR mix, 0.5 mL each of forward primer and reverse primer, 2 mL of cDNA, and 7 mL of diethylpyrocarbonate [DEPC] water). The reactions were started with an incubation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The qPCR primers are listed in Table S2. All of the experiments were performed in duplicate, and then the median cycle threshold (Ct) was calculated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.

Transient luciferase reporter assays

A549 cells were seeded in 24-well plates for 24 h and then transfected in triplicate using Lipofectamine 3000 according to the manufacturer's manual with 0.5 mg of each experimental plasmid and 0.5 mg of the Renilla pRL-SV40P plasmid (used for normalization) per well. After 24 h of culture, relative luciferase units (RLUs) were measured in non-treated and IFN-treated cells (3 h, 100 ng/mL IFN- γ) using the Dual-Glo Luciferase Assay System and GloMax 96 Microplate Luminometer (Promega) according to the manufacturer's instructions.

ChIP

Formaldehyde-cross-linked chromatin was prepared from 2×10^7 A549 cells, and ChIP was performed using the SimpleChip Plus Enzymatic Chromatin IP Kit (Magnetic Beads 9005) from CST according to the manufacturer's instructions. Antibodies were purchased from CST (normal rabbit IgG) and Abcam (anti-IRF1, ab26109). To calculate DNA enrichment in the ChIP assays, real-time qPCR was performed on a Light Cycler 96 qPCR system (Roche). The primers for the CD47 promoter were as follows: CD47-promoter forward: 5'-GGGTCGGTTCCTGCCCTTTC-3' and CD47-promoter reverse: 5'-GAGGGTGGGAGTGAAAGCAAA-3'.

Generation of CD47 KO cells with CRISPR-Cas9

The CRISPR-Cas9 system was used to generate CD47 KO A549 cells. Single-guide RNA (sgRNA)-targeting human CD47 was designed and synthesized in Tsing (Nanjing, China). The primers for the CD47 gRNA plasmid were as follows: h-CD47-gRNA-F, CACCGTACTGAAGTATACGTAAAG; h-CD47-gRNA-R, AAACCTTTACGTACTTTCAGTAC. Subsequently, the sgRNA was cloned into the all-in-one LentiCRISPR V2 vector and generate lentiviruses in 293T cells according to the manufacturer's protocol. To generate CD47

KO A549/PC9 cells, A549/PC9 cells were incubated with lentiviruses for 48 h in the presence of polybrene (10 mg/mL). Then, single cells were seeded in 96-well plates and selected with puromycin (5 mg/mL) to generate stable CD47 KO A549 cells.

Cell migration assay

Cell migration was assessed using transwell plates (Corning Incorporated, Corning, NY, USA) containing an 8-mm pore membrane. Cells were harvested 24 h after transfection and suspended in FBS-free DMEM. The cells were then added to the upper chamber (2×10^4 cells/well), and 0.5 mL DMEM plus 20% FBS was added to the lower compartment. The plates were incubated for 24 h in an incubator. After the incubation, the cells that had reached the lower surface of the filter membrane were fixed with 4% paraformaldehyde for 20 min at room temperature. The membrane was washed 3 times with PBS and stained with 0.1% crystal violet in methanol for 15 min at room temperature. The cells remaining on the upper surface of the filter membrane (nonmigratory) were gently removed with a cotton swab. The lower surfaces (with migrated cells) were captured by photomicroscope (BX51 Olympus, Tokyo, Japan), and the cells were blindly counted (five fields per chamber).

Tumor xenografts in mice

Four-week-old male BALB/c-nude mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and maintained under specific pathogen-free conditions at Nanjing University. A549 WT and A549 CD47 KO cells were injected into the lungs of mice (3×10^4 cells in 0.02 mL PBS per mouse). The needle was inserted into the left side of the armpit, midway down and 5 mm deep at a 45° angle. After 2 weeks, the mice injected with same cells were each randomly divided into 2 groups. One group of the mice was injected with 10 ng of IFN- γ /mouse every other day and the other group was the control. All of the mice were sacrificed 28 days after the first injection to remove the lungs and liver. The tissues were fixed in 4% paraformaldehyde for 24 h. The tissue was processed for hematoxylin and eosin (H&E) staining or immunohistochemical staining for CD47 or Ki67. All of the animal handling and experiments were approved by the institutional review board of Nanjing University (IACUC-2007008).

Statistical analysis

All of the images shown for WB and migration assays are representative of at least three independent experiments. Luciferase reporter, qRT-PCR, and proliferation assays were performed in triplicate, and each individual experiment was repeated several times. Data from at least three independent experiments are presented as the means \pm SDs. Differences between groups were analyzed by one-way ANOVA and performed by GraphPad Prism (version 9.0 for Windows). The significance of the difference between CD47 expression and several clinical and pathological variables was assessed by the χ^2 test or the Mann-Whitney U test performed using SPSS (version 21.0 for Windows). The statistical analysis was carried out as indicated and significance determined as follows: ns, $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omto.2022.04.011>.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Science and Technology of the People's Republic of China (2018YFA0507100), the National Nature Science Foundation of China (31801088, 31670917, and 31770981), the China Postdoctoral Science Foundation (2021M703595), the Natural Science Foundation of Jiangsu Province (BK20170076), the Six Talent Peaks Project in Jiangsu Province, and the Fundamental Research Funds for the Central Universities (020814380095 and 020814380082).

AUTHOR CONTRIBUTIONS

K.Z., H.L., and Tao Wang designed the experiments; S.Q., Z.J., G.L., J.X., Ting Wang, and B.Y. performed the research and analyzed the data; J.W., Y.Y., X.Y., and Tao Wang collected the patients' samples; K.Z. and H.L. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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