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Original Article

RNF200 enhances radiotherapy sensitivity by modulating PD-L1 stability in tumor-associated macrophages of lung cancer

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Abstract: Radiotherapy is a cornerstone treatment for lung cancer; however, enhancing its efficacy and overcoming immune escape mechanisms - particularly those mediated by tumor-associated macrophages (TAMs) expressing programmed death-ligand 1 (PD-L1) - remain significant challenges. The E3 ubiquitin ligase RNF200 has been implicated in the regulation of PD-L1 expression, yet its role in the context of radiotherapy is not well understood. To address this, non-small cell lung cancer (NSCLC) tissue samples from patients with and without prior radiotherapy were analyzed for RNF200 and PD-L1 expression using quantitative RT-PCR and Western blotting. Additionally, RAW264.7 macrophages were subjected to ionizing radiation and genetically manipulated to assess the impact of RNF200 on PD-L1 expression and stability through co-immunoprecipitation and ubiquitination assays. Co-culture experiments with macrophages and lung cancer cells were performed to evaluate the influence of RNF200 on radiotherapy sensitivity. In NSCLC tissues and macrophages, radiotherapy was found to downregulate RNF200 expression while upregulating PD-L1 expression. Overexpression of RNF200 led to marked suppression of PD-L1 expression, whereas RNF200 knockdown produced the opposite effect. Co-immunoprecipitation and ubiquitination assays revealed that RNF200 physically interacted with PD-L1 and promoted its polyubiquitination and proteasomal degradation. Furthermore, co-culture studies demonstrated that macrophages overexpressing RNF200 enhanced the sensitivity of lung cancer cells to radiotherapy, as evidenced by reduced proliferation, increased necrosis, and decreased secretion of transforming growth factor beta TGF-β. Collectively, these findings indicate that RNF200 enhances radiotherapy sensitivity in lung cancer by regulating PD-L1 expression through ubiquitination. Targeting RNF200 may represent a promising strategy to improve the efficacy of radiotherapy in lung cancer treatment.

Keywords: Radiotherapy, tumor-associated macrophages, RNF200, programmed death-ligand 1, NSCLC

Introduction

Tumor-associated macrophages (TAMs) are a critical component of the tumor microenvironment (TME) and exert dual roles in tumor progression [1-3]. These macrophages are generally polarized into two phenotypic states: pro-inflammatory M1 macrophages, which exhibit anti-tumor activity, and immunosuppressive M2 macrophages, which support tumor growth and promote immune evasion [4, 5]. In most cancers, TAMs predominantly resemble the M2 phenotype and are associated with poor prognosis and resistance to therapy [6, 7]. In lung cancer, TAMs significantly influence the outcomes of radiotherapy by modulating the tumor microenvironment. Although radiotherapy remains a cornerstone treatment for non-small cell lung cancer (NSCLC), its therapeutic efficacy is often limited by TAM-mediated immune escape mechanisms, primarily driven by the expression of immune checkpoint molecules such as programmed death-ligand 1 (PD-L1) [4, 8, 9].

PD-L1 is a key immune checkpoint protein expressed on both tumor cells and TAMs. Its engagement with PD-1 receptors on T cells allows tumors to evade immune surveillance by inhibiting T-cell activation and cytotoxicity [10, 11]. In the context of radiotherapy, TAMs have been reported to upregulate PD-L1 expression,

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thereby facilitating immune escape and reducing the sensitivity of tumors to treatment [12]. However, the regulatory mechanisms that control PD-L1 expression, particularly under radiotherapy-induced conditions, remain incompletely understood. Emerging evidence indicates that post-translational modifications, such as ubiquitination, are critical determinants of PD-L1 stability and degradation [13, 14]. Although several E3 ubiquitin ligases have been implicated in this process, the specific ligase responsible for regulating PD-L1 under radiotherapy stress has not been fully elucidated.

RNF200, also known as constitutive photomorphogenesis protein 1 or ring finger and WD repeat domain 2, is an E3 ubiquitin ligase involved in regulating protein stability across diverse biological processes, including tumor progression [15, 16]. Recent studies have suggested that RNF200 may interact with immune regulatory molecules [17]; however, its role in lung cancer and its impact on radiotherapy sensitivity remain unclear. Preliminary evidence indicates that RNF200 expression is significantly downregulated in NSCLC tissues following radiotherapy, coinciding with an upregulation of PD-L1 [18]. This observation suggests a potential inverse regulatory relationship between RNF200 and PD-L1 expression.

In this study, we aimed to investigate the role of RNF200 in regulating PD-L1 expression in TAMs under radiotherapy conditions and to assess its impact on lung cancer sensitivity to radiotherapy.

Methods

Sample collection

Fifteen NSCLC tissue samples treated with radiotherapy (RT^+) and fifteen untreated (RT^-) were collected. Tissues were stored in RNAlater solution at -80°C until RNA and protein extraction. Ethical approval was obtained from the First Affiliated Hospital of Bengbu Medical University, and all patients provided informed written consent.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent, and cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara). qRT-PCR was conducted using SYBR Green Master Mix on a CFX96 Real-Time PCR system (Bio-Rad). The following primers were used: *RNF200 (human)*: Forward 5'-AATAGCACGTTAGCATCAAGACG-3', Reverse 5'-TGGCTTGCAGTTCGACTGTC-3'; *GAPDH (human)*: Forward 5'-ACAACTTTGGTATCGTGGAAGG-3', Reverse 5'-GCCATCACGCCACAGTTTC-3'; *RNF200 (mouse)*: Forward 5'-CAACGACTTCGTCTGTCCCA-3', Reverse 5'-GCCCATTGGTGCTACTCACT-3'; *PD-L1 (mouse)*: Forward 5'-GGCAGGAGAGGAGGACCTTA-3', Reverse 5'-TGCAGCTTGACGTCTGTGAT-3'; *GAPDH (mouse)*: Forward 5'-AGGTCGGTGTGAACGGATTTG-3', Reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *GAPDH* as the internal control.

Western blot

Protein lysates from tissue samples and cell cultures were prepared using RIPA buffer containing protease inhibitors. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% skim milk and incubated overnight with primary antibodies: RNF200 (ab70889, Abcam), PD-L1 (66248-1-Ig, Proteintech), GAPDH (10494-1-AP, Proteintech), anti-Myc tag antibody (A5968, Selleck), and anti-K48-linkage specific polyubiquitin antibody (#8081, Cell Signaling Technology). After washing, membranes were incubated with HRP-conjugated secondary antibodies and visualized using an ECL detection kit. Protein expression was quantified using ImageJ software.

Cell culture and irradiation

The mouse macrophage cell line RAW264.7 and lung cancer cell line LLC were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. For irradiation experiments, cells were exposed to X-ray radiation (6 Gy) using an RS-2000 Pro biological irradiator. Cells were collected at indicated time points for subsequent analysis.

Plasmid construction and siRNA transfection

The Myc-tagged RNF200 plasmid was constructed by cloning RNF200 cDNA into the pcDNA3.1 expression vector. For gene silencing, specific siRNAs targeting RNF200 were

synthesized (GenePharma). Transfections were performed using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. Cells were harvested 48-72 hours post-transfection for further analysis.

Co-Immunoprecipitation (Co-IP)

RAW264.7 cells were lysed in IP lysis buffer containing protease inhibitors. Lysates were incubated overnight at 4°C with primary antibodies, including anti-PD-L1 (66248-1-Ig, Proteintech) or anti-RNF200 (ab70889, Abcam). For the detection of ubiquitinated PD-L1, anti-Ub antibody (F0109, Selleck) was used. The antibody-protein complexes were captured using Protein G beads, washed, and analyzed by Western blot. For overexpression studies, RAW264.7 cells were transfected with Myc-tagged RNF200 plasmids, and anti-Myc tag antibody (A5968, Selleck) was used for Co-IP analysis.

Cycloheximide (CHX) chase assay

To assess protein stability, RAW264.7 cells overexpressing RNF200 or vector control were treated with 100 μg/mL CHX to block protein synthesis. Cells were collected at 0, 12, 24, and 36 hours post-treatment. Western blot was conducted to measure PD-L1 levels, and anti-Myc tag antibody was used to confirm RNF200 overexpression. Protein half-life was calculated based on band intensities.

Co-culture experiments

RAW264.7 macrophages and LLC cells were co-cultured at a 1:3 ratio in transwell chambers. After 24 hours of co-culture, cells were irradiated with 6 Gy X-ray. Proliferation and necrosis of LLC cells were assessed using EdU staining and Calcein AM/7-AAD assays, respectively. EdU (5-ethynyl-2'-deoxyuridine) staining was used to assess cell proliferation. EdU incorporates into newly synthesized DNA during the S-phase of the cell cycle. After incubation with EdU, cells were fixed, permeabilized, and reacted with fluorescent azide using a Click-iT reaction. Proliferating cells were then visualized under a fluorescence microscope. Calcein AM/7-AAD staining was used to evaluate cell viability and necrosis. Calcein AM is a membrane-permeable dye that fluoresces in live cells due to esterase activity, while 7-AAD (7-aminoactinomycin D) binds to DNA in necrotic cells with compromised membranes. After staining, cells were analyzed using flow cytometry to quantify live and necrotic populations. TGF-β secretion from macrophages was measured using ELISA kits (ab119557, Abcam).

Statistical analysis

All experiments were conducted at least in triplicate. Data are presented as mean ± SD. Statistical comparisons were performed using Student's t-test or one-way ANOVA with a post hoc test, with p-value of < 0.05 considered statistically significant.

Results

RNF200 expression is downregulated in NSCLC tissues following radiotherapy

Previous bioinformatic analysis using DegPred (www.degron.phasep.pro/detail/Q9NZQ7/) identified RNF200 as a potential E3 ubiquitin ligase targeting PD-L1. Despite this prediction, the role of RNF200 in lung cancer radiotherapy remains poorly understood. To investigate its function, a total of 30 NSCLC tumor tissue samples were collected, comprising 15 samples from patients who had received radiotherapy (RT^+) and 15 samples from untreated patients (RT^-). It was demonstrated that RNF200 mRNA levels were significantly lower in RT^+ tissues compared with RT^- tissues (**Figure 1A**). Consistently, Western blot analysis revealed a marked reduction in RNF200 protein expression accompanied by a pronounced increase in PD-L1 protein levels in RT^+ samples (**Figure 1B**). Densitometric quantification of the Western blots further confirmed the significant downregulation of RNF200 and upregulation of PD-L1 protein expression following radiotherapy (**Figure 1C** and **1D**). Collectively, these findings indicate that RNF200 expression is substantially suppressed, whereas PD-L1 is elevated in NSCLC tissues after radiotherapy, suggesting a potential role for RNF200 in modulating radiotherapy responses.

Radiation modulates PD-L1 and RNF200 expression in macrophages

To investigate the effects of radiation on PD-L1 and RNF200 expression in macrophages, RAW264.7 cells were exposed to increasing

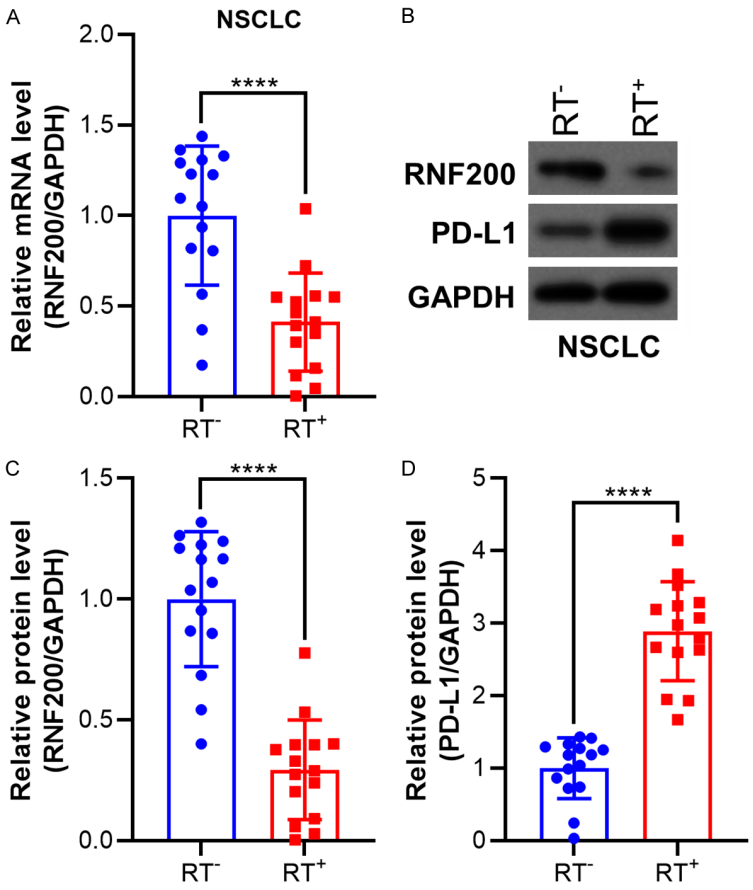

Figure 1. RNF200 is downregulated in tumor tissues of NSCLC patients following radiotherapy. (A) RNF200 mRNA expression levels were analyzed in NSCLC tumor tissues from untreated patients (RT^-) and patients treated with radiotherapy (RT^+) using qRT-PCR. (B) Representative Western blot images showing the protein expressions of RNF200 and PD-L1 in RT^- and RT^+ tissues. GAPDH was used as a loading control. Densitometric analysis of RNF200 (C) and PD-L1 (D) protein levels in RT^- and RT^+ tissues based on Western blot results. Data are shown as mean ± SD. $^{****}P < 0.0001$.

doses of X-ray irradiation. Western blot analysis revealed a dose-dependent increase in PD-L1 protein expression, accompanied by a corresponding decrease in RNF200 protein levels (**Figure 2A**). Densitometric quantification confirmed these trends, demonstrating significant upregulation of PD-L1 and downregulation of RNF200 in response to irradiation (**Figure 2B**). Similar patterns were also observed in lung cancer cells; however, the regulatory effects of irradiation in lung cancer cells appeared less pronounced compared to macrophages (Supplementary Figure 1).

In addition, quantitative RT-PCR analysis showed a dose-dependent reduction in RNF200 mRNA levels, while PD-L1 mRNA levels remained unchanged (**Figure 2C** and **2D**). Time-course experiments further demonstrated that PD-L1 protein levels significantly increased after irradiation, peaking at 24 hours and remaining elevated at 36 hours post-irradiation (**Figure 2E** and **2F**). In contrast, RNF200 protein levels exhibited a progressive decline over the same time period (**Figure 2E** and **2F**). Collectively, these results suggest that radiation inversely regulates PD-L1 and RNF200 expression in macrophages.

RNF200 regulates PD-L1 expression in macrophages

To determine whether RNF200 regulates PD-L1 expression, RAW264.7 cells were transfected with Myc-tagged RNF200 plasmids. Western blot analysis demonstrated that overexpression of RNF200 significantly reduced PD-L1 protein levels (**Figure 3A**), as confirmed by densitometric quantification (**Figure 3B**). Conversely, knockdown of RNF200 using specific siRNAs resulted in a marked increase in PD-L1 protein expression (**Figure 3C**), further supported by densitometric analysis (**Figure 3D**). Additionally, a rescue experiment reintroducing RNF200 established a direct regulatory relationship between RNF200 and PD-L1 expression (Supplementary Figure 2).

To investigate whether RNF200 modulates radiation-induced PD-L1 upregulation, RAW264.7 macrophages overexpressing RNF200 were exposed to 6 Gy of X-ray irradiation. In control macrophages transfected with empty vector, PD-L1 protein levels were significantly elevated following irradiation. In contrast, RNF200 overexpression markedly suppressed the radiation-induced increase in PD-L1 expression (**Figure 3E**). Densitometric analysis further quantified the reduction in PD-L1 levels in RNF200-

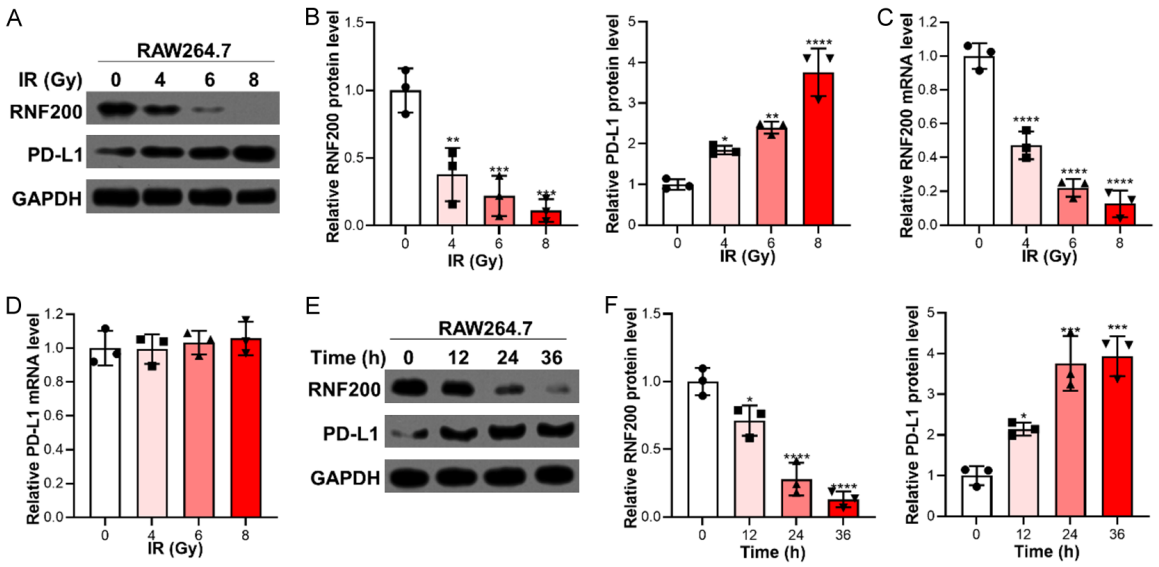

Figure 2. Radiation regulates PD-L1 and RNF200 expression in macrophages. (A) RAW264.7 cells were treated with different doses of X-ray radiation, and protein expression levels of PD-L1 and RNF200 were analyzed by Western blot. GAPDH was used as a loading control. (B) Densitometric analyses of RNF200 and PD-L1 expression in RAW264.7 cells following different doses of X-ray. RAW264.7 cells were treated with different doses of X-ray radiation, and mRNA levels of RNF200 (C) and PD-L1 (D) were analyzed by qRT-PCR. GAPDH was used as an internal control. (E) Time-course analysis of PD-L1 and RNF200 expression in RAW264.7 cells after 6 Gy of X-ray. Western blot images were collected at 0, 12, 24, and 36 hours post-irradiation. (F) Densitometric analysis of RNF200 and PD-L1 expression over time. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

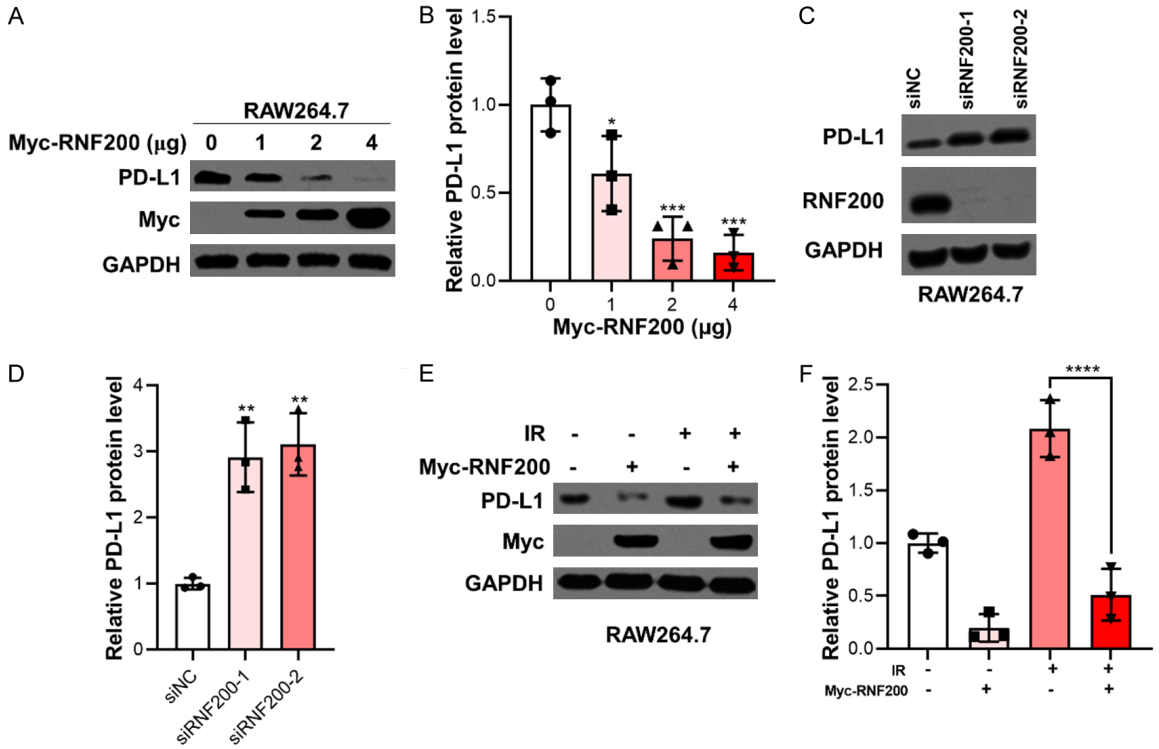

Figure 3. RNF200 regulates PD-L1 expression in macrophages. A. RAW264.7 cells were transfected with Myc-tagged RNF200 plasmids for 48 hours. PD-L1 expression was analyzed by Western blot. GAPDH was used as a loading control. B. Densitometric analysis of PD-L1 protein levels in RNF200-overexpressing macrophages. C. RAW264.7

cells were transfected with siRNAs targeting RNF200 for 72 hours, and PD-L1 levels were analyzed by Western blot. GAPDH was used as a loading control. D. Densitometric analysis of PD-L1 protein levels in RNF200-knockdown macrophages. E. RAW264.7 cells overexpressing RNF200 were exposed to 6 Gy of X-ray. PD-L1 expression was analyzed 24 hours post-irradiation by Western blot. F. Densitometric analysis of PD-L1 levels in RNF200-overexpressing macrophages after irradiation. Data are presented as mean ± SD. $^{**}P < 0.01$.

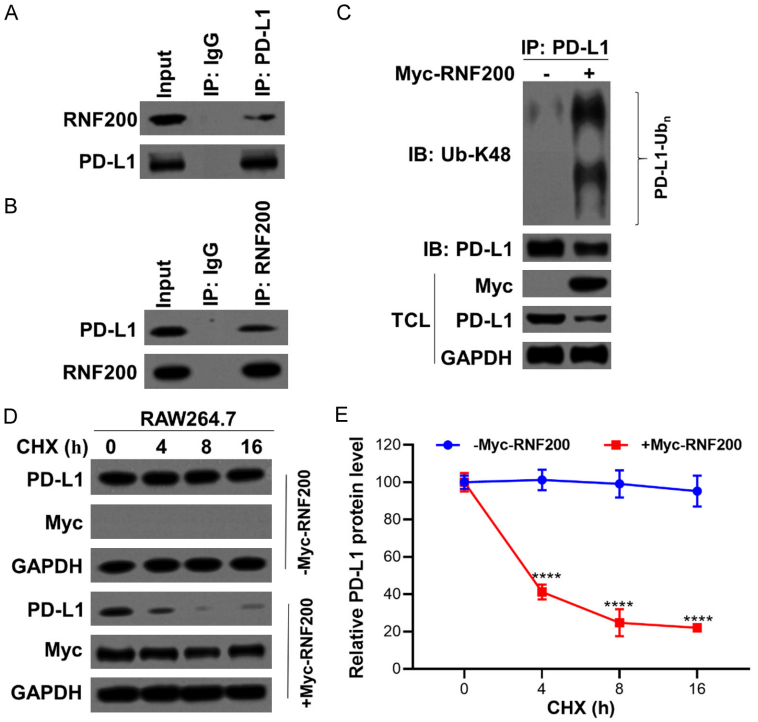

Figure 4. RNF200 binds to PD-L1 and promotes its ubiquitination and degradation. A. Co-immunoprecipitation (Co-IP) was performed using RAW264.7 cell lysates with anti-PD-L1 or anti-RNF200 antibodies. IgG was used as an isotype control. B. Reciprocal Co-IP confirmed the interaction between PD-L1 and RNF200. C. RAW264.7 cells overexpressing Myc-RNF200 were analyzed by Co-IP using anti-PD-L1 antibodies, and the polyubiquitination of PD-L1 was assessed by Western blot with an K48-linkage specific polyubiquitin antibody. D. Cycloheximide (CHX) chase assay was used to assess PD-L1 stability in RAW264.7 cells overexpressing RNF200 or empty vector. Western blot images were collected at 0, 12, 24, and 36 hours post-CHX treatment. E. Densitometric analysis of PD-L1 protein half-life in RNF200-overexpressing and control cells. Data are presented as mean ± SD. $^{**}P < 0.01$.

overexpressing cells compared to controls (**Figure 3F**). Collectively, these findings indicate that RNF200 counteracts the radiation-induced upregulation of PD-L1, suggesting its potential role in modulating macrophage-mediated immune responses during radiotherapy.

RNF200 promotes PD-L1 ubiquitination and degradation

To investigate whether RNF200 regulates PD-L1 stability, co-immunoprecipitation (Co-IP) assays were performed. RNF200 was found to physically interact with PD-L1 in RAW264.7 cells, as demonstrated by Co-IP using both anti-RNF200 and anti-PD-L1 antibodies (**Figure 4A** and **4B**). Overexpression of Myc-tagged RNF200 enhanced K48-linked ubiquitination of PD-L1, indicating that RNF200 promotes PD-L1 polyubiquitination (**Figure 4C**). CHX chase assays were conducted to assess the impact of RNF200 on PD-L1 protein stability. In cells overexpressing RNF200, the half-life of PD-L1 was significantly reduced compared to controls, as shown by Western blotting at various time points following CHX treatment (**Figure 4D**). Densitometric analysis confirmed the accelerated degradation of PD-L1 in RNF200-overexpressing cells (**Figure 4E**). Additionally, a rescue experiment using the proteasome inhibitor MG132 demonstrated that RNF200-mediated PD-L1 degradation occurred via the proteasome pathway (Supplementary Figure 3). Collectively, these results indicate that RNF200 regulates PD-L1 expression by promoting its ubiquitination and proteasomal degradation.

TAMs reduce radiotherapy sensitivity of lung cancer cells

The effect of TAMs on the sensitivity of lung cancer cells to IR was evaluated using a transwell co-culture system. Lung cancer cells co-cultured with RAW264.7 macrophages exhibited a significantly increased proliferative capacity following exposure to IR, as determined by EdU incorporation assays (**Figure 5A**). In addition to enhanced proliferation, the co-cultured lung cancer cells displayed reduced necrosis rates compared to non-co-cultured controls. This reduction in cell death was

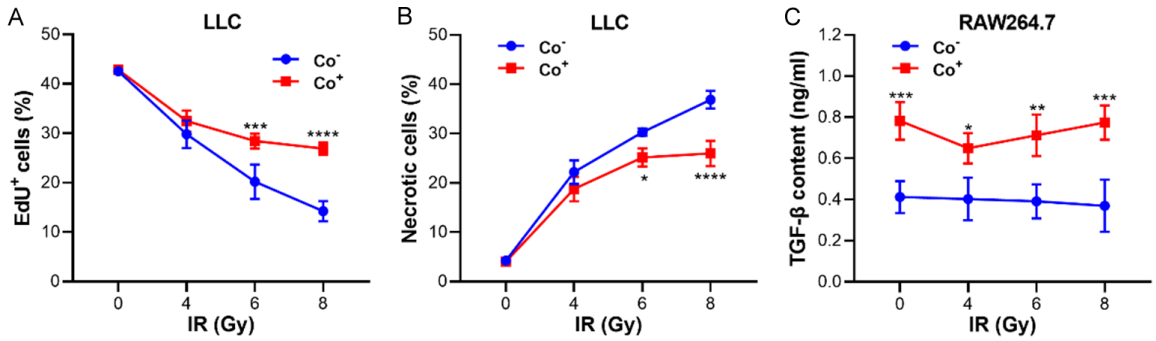

Figure 5. TAMs reduce radiotherapy sensitivity in lung cancer cells. A. RAW264.7 macrophages were co-cultured with LLC lung cancer cells at a 1:3 ratio. Proliferation of LLC cells was assessed after irradiation using EdU staining. B. Necrosis of LLC cells in co-culture was evaluated using Calcein AM/7-AAD staining. C. TGF-β secretion by RAW264.7 macrophages in co-culture was measured by ELISA. Data are shown as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

assessed by Calcein AM and 7-AAD staining, which distinguishes live and necrotic cells (**Figure 5B**). Furthermore, ELISA analysis demonstrated that macrophages co-cultured with lung cancer cells secreted substantially higher levels of TGF-β after irradiation relative to macrophages cultured alone (**Figure 5C**). These increased levels of TGF-β may contribute to the observed resistance of lung cancer cells to IR, as TGF-β has been implicated in promoting tumor cell survival and therapy resistance. Collectively, these findings indicate that TAMs can reduce the efficacy of radiotherapy in lung cancer cells by enhancing proliferation, decreasing necrotic cell death, and increasing secretion of immunosuppressive cytokines such as TGF-β.

RNF200 enhances radiotherapy sensitivity by modulating TAM function

To evaluate the role of RNF200 in TAM-mediated modulation of radiotherapy sensitivity, RAW264.7 macrophages were genetically modified to either overexpress or silence RNF200. In co-culture experiments with lung cancer cells, RNF200 overexpression in TAMs significantly reduced cancer cell proliferation following IR, as assessed by EdU staining (**Figure 6A**). Additionally, the necrosis rate of cancer cells was markedly increased in these co-cultures, as determined by Calcein AM/7-AAD staining (**Figure 6B**). ELISA measurements demonstrated that TGF-β secretion from TAMs was substantially decreased under RNF200 overexpression after IR (**Figure 6C**). Conversely, RNF200 knockdown in TAMs led to increased proliferation (**Figure 6D**), reduced necrosis (**Figure 6E**), and elevated TGF-β secretion (**Figure 6F**) in co-cultured lung cancer cells following IR. These findings suggest that RNF200 enhances radiotherapy sensitivity by modulating TAM function and reducing their immunosuppressive activity.

Discussion

This study provides comprehensive evidence that RNF200, an E3 ubiquitin ligase, plays a pivotal role in regulating radiotherapy sensitivity in lung cancer by modulating PD-L1 expression in TAMs. Through a series of experiments, we demonstrated that RNF200 expression is significantly downregulated following radiotherapy, which coincides with increased PD-L1 levels that facilitate immune escape. Mechanistic analyses revealed that RNF200 directly interacts with PD-L1, promotes its ubiquitination, and accelerates its proteasomal degradation, thereby suppressing the radiotherapy-induced upregulation of PD-L1.

Furthermore, functional studies using co-culture systems underscored the dual role of TAMs in modulating cancer progression and radiotherapy response, with RNF200 overexpression effectively reversing TAM-mediated immunosuppressive effects on lung cancer cells. Collectively, these findings substantially advance our understanding of immune regulation within the tumor microenvironment and highlight RNF200 as a promising therapeutic target to enhance the efficacy of radiotherapy in lung cancer.

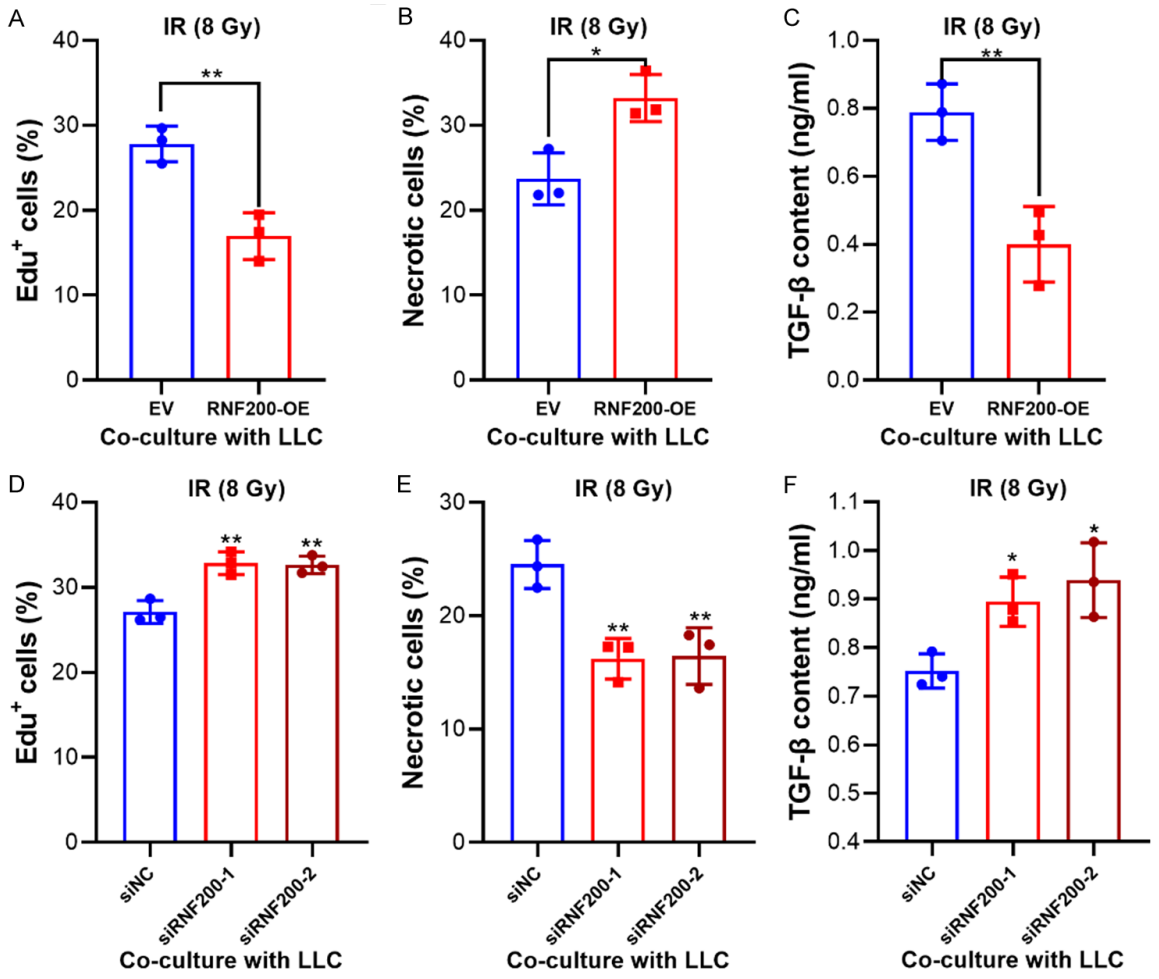

Figure 6. RNF200 enhances radiotherapy sensitivity by modulating TAM function. A. RAW264.7 macrophages overexpressing RNF200 were co-cultured with LLC cells at a 1:3 ratio. Proliferation of LLC cells was assessed after 8 Gy of irradiation using EdU staining. B. Necrosis of LLC cells in co-culture with RNF200-overexpressing macrophages was evaluated using Calcein AM/7-AAD staining. C. TGF-β secretion by RAW264.7 macrophages overexpressing RNF200 in co-culture was measured by ELISA. D. RAW264.7 macrophages with RNF200 knockdown were co-cultured with LLC cells at a 1:3 ratio. Proliferation of LLC cells was assessed after 8 Gy of irradiation using EdU staining. E. Necrosis of LLC cells in co-culture with RNF200-knockdown macrophages was evaluated using Calcein AM/7-AAD staining. F. TGF-β secretion by RAW264.7 macrophages with RNF200 knockdown in co-culture was measured by ELISA. Data are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Radiotherapy is a cornerstone treatment for NSCLC, yet its efficacy is often hindered by immune evasion mechanisms within the tumor microenvironment. PD-L1, an immune checkpoint molecule, plays a critical role in this process by inhibiting T-cell-mediated anti-tumor responses. Consistent with previous studies, we observed that radiotherapy induces PD-L1 upregulation, which is consistent with its role in fostering immune escape [19, 20]. However, our study advances the field by identifying RNF200 as a novel post-translational regulator of PD-L1. This discovery extends the current understanding, which has largely focused on transcriptional regulation of PD-L1 [21, 22]. By elucidating the role of RNF200 in ubiquitination-mediated PD-L1 degradation, we provide novel insights into the pathways that govern PD-L1 stability under radiotherapy conditions.

Our findings reveal that RNF200 acts as a suppressor of PD-L1 expression by promoting its polyubiquitination and subsequent degradation. Co-immunoprecipitation assays confirmed a direct interaction between RNF200 and PD-L1, while CHX chase experiments demon-

strated that overexpression of RNF200 significantly shortens the half-life of PD-L1. This observation highlights the importance of post-translational modifications in regulating immune checkpoints and suggests that targeting RNF200 may provide a therapeutic avenue to modulate PD-L1 levels and enhance radiotherapy efficacy. Notably, while other studies have explored the role of E3 ligases in immune regulation, the identification of RNF200 as a key player in PD-L1 degradation under radiotherapy conditions has not been previously reported, underscoring the novelty of this study.

In addition to elucidating the molecular mechanisms of RNF200 and PD-L1, we investigated the functional impact of TAMs on lung cancer cell responses to radiotherapy. TAMs are a major component of the tumor microenvironment and are known to exhibit both tumor-promoting and anti-tumor properties. Our co-culture experiments showed that TAMs significantly enhance lung cancer cell proliferation and reduce necrosis under radiotherapy conditions. These effects were accompanied by increased secretion of TGF-β, a cytokine known to promote immune suppression and tumor progression. These findings are consistent with prior studies that highlight the role of TAMs in fostering resistance to radiotherapy and immunotherapy [23, 24]. However, our work further demonstrates that RNF200 overexpression in TAMs reverses these immunosuppressive effects, reducing cancer cell proliferation, increasing necrosis, and suppressing TGF-β secretion. This underscores the potential of RNF200 as a regulator of TAM function and a therapeutic target for enhancing radiotherapy sensitivity.

Our results also provide important insights into the broader implications of RNF200's role within the tumor microenvironment. The ability of RNF200 to counteract radiotherapy-induced PD-L1 upregulation and TAM-mediated immunosuppression underscores its dual function in modulating both tumor cells and immune cells. This dual activity positions RNF200 as a promising candidate for combination therapies targeting both the tumor and its microenvironment. For example, combining RNF200-based strategies with immune checkpoint inhibitors could potentially enhance therapeutic efficacy by simultaneously reducing PD-L1 expression and reactivating T cell-mediated anti-tumor responses. Such approaches may be particularly valuable for overcoming the limitations of current radiotherapy protocols, which often fail to achieve durable responses in patients with advanced non-small cell lung cancer.

While our findings provide a strong foundation for understanding the role of RNF200 in radiotherapy sensitivity, this study has several limitations. First, most experiments were performed in vitro, and in vivo validation using animal models is needed to confirm the therapeutic relevance of targeting RNF200. In particular, generating macrophage-specific RNF200 knockout mice will help clarify its function within the tumor microenvironment. Although we showed that RNF200 promotes PD-L1 ubiquitination and degradation, the exact lysine residues on PD-L1 targeted by RNF200 remain unidentified. Future studies using site-directed mutagenesis will be necessary to pinpoint these sites. In addition, the specific E2 enzymes and regulatory cofactors involved in this process have not been determined. This study also did not explore possible compensatory mechanisms, such as other E3 ligases or deubiquitinases that may regulate PD-L1 when RNF200 is absent. Investigating these pathways will be important to fully understand PD-L1 regulation. Finally, we focused on PD-L1 as a single immune checkpoint molecule and did not assess whether RNF200 affects other immune regulators, such as PD-L2 or TIM-3. Future research should examine whether RNF200 broadly influences immune escape pathways. Addressing these questions will be essential to validate RNF200 as a therapeutic target and to develop strategies that enhance radiotherapy efficacy in lung cancer.

Conclusions

This study identifies RNF200 as a key regulator of PD-L1 stability and demonstrates its role in enhancing radiotherapy sensitivity by modulating the function of tumor-associated macrophages. By revealing a novel post-translational mechanism of immune checkpoint regulation, our findings provide important insights into the complex interactions between radiotherapy, immune evasion, and the tumor microenvironment. These results support the potential of targeting RNF200 to improve radiotherapy out-

comes in lung cancer. Future research, including in vivo studies and clinical trials, will be essential to translate these insights into effective therapeutic strategies for patients with non-small cell lung cancer.

Disclosure of conflict of interest

None.

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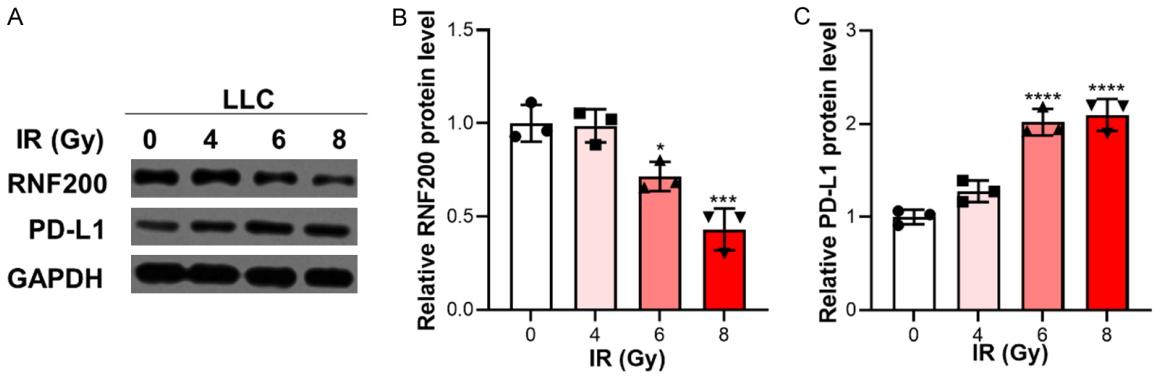
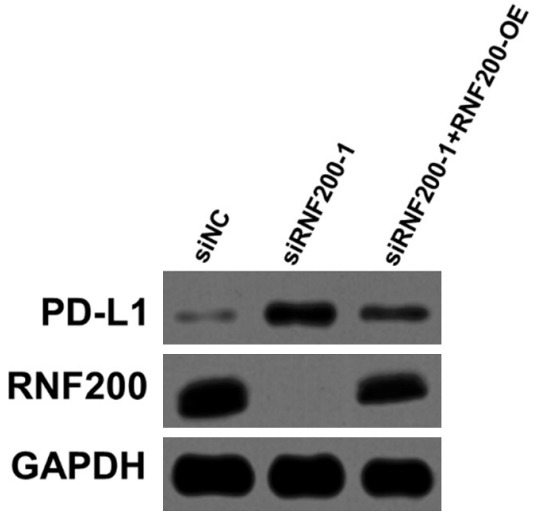
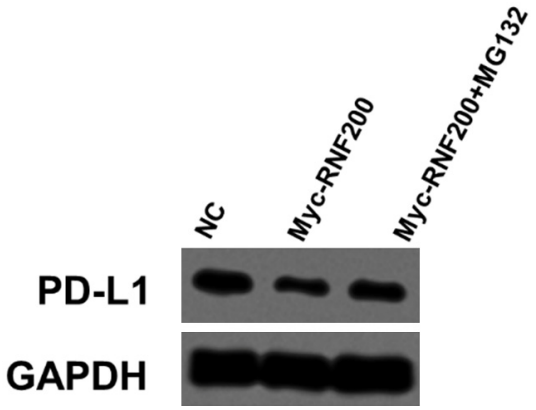


Supplementary Figure 1. Radiation regulates PD-L1 and RNF200 expression in lung cancer cells. (A) LLC cells were treated with different doses of X-ray radiation, and protein expression levels of PD-L1 and RNF200 were analyzed by western blot. GAPDH was used as a loading control. (B, C) Densitometric analyses of RNF200 (B) and PD-L1 (C) expression in LLC cells following different doses of X-ray. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.



Supplementary Figure 2. RNF200 regulates PD-L1 expression in macrophages. RAW264.7 cells were transfected with siRNAs targeting RNF200 (siRNF200-1) or RNF200 overexpressing plasmids (RNF200-OE) for 72 hours, and PD-L1 levels were analyzed by western blot. GAPDH was used as a loading control.



Supplementary Figure 3. RNF200-mediated degradation of PD-L1 is abolished by MG132. RAW264.7 cells were transfected with 2 μg Myc-tagged RNF200 plasmids (Myc-RNF200) for 24 hours, and then cells were incubated with 20 μM MG132 for 4 hours, followed by western blot to assess PD-L1 expression. GAPDH was used as a loading control.