Research Article

Senescent bladder cancer cells promote acquired cisplatin resistance by facilitating macrophage infiltration

Zhiwen Xie†, Wenhao Wang†, Tiewen Li, Yichen Zhang, Di Cui, and Bangmin Han*

Department of Urology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200080, China
†These authors contributed equally to this work.

Abstract

Background: Cisplatin-based chemotherapy represents the standard first-line treatment for muscle-invasive bladder cancer. However, the mechanisms underlying acquired resistance remain incompletely understood. **Objective:** This study explored the role of chemotherapy-induced senescence in shaping a tumor microenvironment that fosters resistance. **Methods:** Cisplatin-induced cellular senescence in bladder cancer cells was investigated by activating the nuclear factor κ-light-chain-enhancer of activated B cells signaling pathway and assessed by β-galactosidase staining, enzyme-linked immunosorbent assay, reverse transcription-quantitative polymerase chain reaction, and Western blotting. **Results:** Transwell migration assays and bioinformatic analyses revealed that these senescent cells secreted factors that enhanced macrophage recruitment. Mechanistically, senescence led to the downregulation of the androgen receptor (*AR*) gene, which in turn facilitated macrophage infiltration. Intriguingly, the recruited macrophages subsequently upregulated AR expression in cancer cells, ultimately diminishing their sensitivity to cisplatin, as shown in Cell Counting Kit-8 assays and molecular profiling. The findings reveal a novel "senescence–macrophage–chemoresistance" axis in bladder cancer, wherein cisplatin-induced senescent cells drive treatment failure by promoting macrophage-dependent AR signaling and chemoresistance. **Conclusion:** These findings provide valuable insights for the development of novel therapeutic strategies for overcoming cisplatin resistance in muscle-invasive bladder cancer.

Keywords: Bladder cancer, Cisplatin, Macrophages, Androgen receptor, Nuclear factor κ-light-chain-enhancer of activated B cells signaling pathway

1. Introduction

Bladder cancer (BC) is the second most common urological malignancy worldwide, posing a significant clinical challenge due to its high recurrence rate and substantial healthcare burden.[1] Pathologically, BC is classified, in terms of tumor invasion depth, into the muscularis propria, non-muscle-invasive BC (NMIBC), which is confined to the mucosal or submucosal layers, and muscle-invasive BC (MIBC), which invades the detrusor muscle or beyond. Although NMIBC accounts for most initial diagnoses, MIBC is considerably more aggressive and represents approximately 25% of all BC diagnoses.[2-4] Cisplatin-based chemotherapy is the standard treatment for MIBC; however, acquired resistance frequently develops during therapy.[5] Therefore, elucidating the mechanisms of acquired cisplatin resistance in BC is of significant importance.

Growing evidence suggests that cisplatin resistance involves multiple biological processes, including enhanced drug efflux, improved DNA damage repair, and suppression of apoptotic signaling.[2,6-8] Resistance may also arise from alterations in key genes, particularly the androgen receptor (*AR*).[9,10] Studies have linked various AR-regulated mechanisms to cisplatin resistance. While AR inhibition shows promise for treating cisplatin-resistant patients,[11-13] the factors regulating AR expression in BC cells remain unclear.

In a study using a BC mouse model, cisplatin has been shown to trigger a robust immune response by activating the

*Corresponding author:
Bangmin Han (hanbm@sjtu.edu.cn)

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cyclic guanosine monophosphate–adenosine monophosphate synthase/stimulator of interferon genes signaling pathway, leading to suppressed tumor proliferation and increased infiltration of $CD8^+$ T cells and dendritic cells in the tumor microenvironment.[14] Recent studies further indicated that cisplatin chemotherapy enhances the infiltration of both $CD8^+$ T cells and macrophages into the tumor microenvironment. This immunological remodeling may help explain the variable outcomes of clinical trials combining chemotherapy with immune checkpoint inhibitors in BC.[15] Consequently, strategies to overcome cisplatin resistance have incorporated a combination of irradiation and targeted therapy against myeloid-derived suppressor cells.[16] These findings underscore the importance of investigating the relationship between immune cells and cisplatin treatment in BC.

In this study, we demonstrated that cisplatin induced senescence in BC cells through activation of the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. Senescent BC cells subsequently promote macrophage infiltration by reducing AR expression. Importantly, the findings reveal that macrophages enhance cisplatin resistance by upregulating AR expression. These results provide new insights into the interplay between AR signaling, tumor-associated macrophages (TAMs), and cisplatin resistance in BC.

2. Materials and methods

2.1. Data collection and bioinformatic analysis

The gene expression dataset GSE165767, containing transcriptomic profiles of cisplatin-treated and untreated BC cells, was obtained from the Gene Expression Omnibus database. Differential expression analysis was performed using the "limma" package in R (3.60.0, Gordon K. Smyth, Australia), with significantly dysregulated genes defined as those with $|\log2(\text{fold change})| > 1$ and an adjusted $p<0.05$. Gene ontology enrichment analysis was conducted on the differentially expressed genes (DEGs) to identify significantly enriched biological processes.

2.2. Cell culture

The human monocytic cell line human acute monocytic leukemia cell line-1 (THP-1) and the BC cell line UMUC3 were obtained from the Cell Bank of the Shanghai Academy of Chinese Sciences. Cells were maintained in RPMI-1640 medium (BIOAGRIO, USA) supplemented with 10% fetal bovine serum (BIOAGRIO, USA) and 1% penicillin–streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin; XA4122, BIOAGRIO, USA). All cultures were incubated at 37°C in a 5% CO_2 humidified incubator.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from BC cells using TRIzol reagent. RNA (1 μg) was reverse-transcribed into complementary DNA using the HyperScript III RT SuperMix, and RT-qPCR was performed according to the manufacturer's instructions. Gene expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the internal reference. All reagents were from EnzyArtisan, China, and experiments were independently repeated 3 times. Primer sequences are listed in Table S1.

2.4. Western blotting

Total protein was extracted using radioimmunoprecipitation lysis buffer containing a protease inhibitor cocktail (Solarbio, China). Proteins were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, membranes were incubated overnight with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Protein signals were detected using an enhanced chemiluminescence kit (New Cell & Molecular Biotech, China), and band intensities were quantified by utilizing ImageJ software (1.8.0, Wayne Rasband, USA). Primary antibodies included anti-GAPDH (1:100,000; 60004-1-Ig; Proteintech, USA) and anti-AR (1:2,000; 5153; Cell Signaling Technology, USA). Each experiment was independently repeated at least 3 times.

2.5. Senescence assay

Cellular senescence was detected using a β-galactosidase staining kit (RG0039, Beyotime, China). UMUC3 cells, when reaching a 80–90% confluence, were treated with 1 μg/mL cisplatin for 24 h, following the manufacturer's protocol. Stained cells were imaged under an inverted fluorescence microscope (Leica, Germany).

2.6. Immune cell infiltration analysis

Immune cell infiltration levels were predicted using the CIBERSORTx algorithm, which deconvolutes transcriptome data to estimate the relative proportions of different cell types in heterogeneous tissue samples. Patients were stratified into high- and low-AR–expression groups based on the median *AR* expression for subsequent analysis.

2.7. Cell counting kit-8 assay (CCK-8)

Cell viability was assessed using CCK-8 (C0037, Beyotime, China) according to the manufacturer's instructions.

Briefly, UMUC3 cells were seeded into 96-well plates at 2×10^4 cells/mL per well in 100 μL medium. After 24 h, 10 μL of CCK-8 reagent was added to each well and incubated for 2 h. Absorbance was measured on a microplate reader (BioTek, USA), and the proliferation rate was expressed as the relative absorbance of treated groups compared to the mock-treated control.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were collected from cisplatin-treated and untreated cells. Levels of interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) were quantitatively measured using commercial ELISA kits (U48-1510E and U48-3716E, YOBIBIO, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm, and cytokine concentrations were determined against a standard curve.

2.9. Cell migration assay

Cell migration was evaluated by employing a transwell chamber system. Cells were washed twice with serum-free RPMI-1640 medium before cell seeding. A total of 5×10^4 cells/mL were plated into the upper chamber, while the lower chamber contained supplemented RPMI-1640 medium as a chemoattractant. After 24 h of incubation (37°C, 5% CO_2), migrated cells on the lower membrane surface were counted in 10 random fields at 100× magnification. The migration rate was calculated as the ratio of migrated cells in the treatment group to the control. All experiments were performed with three technical replicates and independently repeated 3 times. Cell counting was conducted by two independent researchers blinded to the experimental grouping.

2.10. Statistical analysis

All quantitative analyses were performed by investigators blinded to the experimental conditions. Experiments were independently repeated 3 times. Data are presented as mean ± standard deviation and analyzed using GraphPad Prism software (9.0, GraphPad, USA). Differences between two groups were assessed using Student's *t*-test, while one-way analysis of variance with Tukey's *post hoc* test was used for multiple comparisons. A $p<0.05$ was considered statistically significant.

3. Results

3.1. Cisplatin induces senescence in bladder cancer cells

To investigate the effect of cisplatin on cellular senescence in BC, we first examined the expression of senescence-associated genes. Analysis of the GSE165767 dataset revealed that cisplatin treatment significantly upregulated *IL6*, *TNFA*, and *CXCL1* expression in BC cells (Figure 1A). We then treated UMUC3 cells with 1 μg/mL cisplatin to validate these findings. Consistent with the bioinformatic data, cisplatin markedly increased the expression of *IL6*, *TNF*, and *CXCL1* genes (Figure 1B), as well as p21 protein levels (Figure 1C), in UMUC3 cells. In addition, cisplatin enhanced the secretion of IL-6 and TNF-α from BC cells (Figure 1D). Importantly, β-galactosidase staining provided direct visual confirmation of cisplatin-induced cellular senescence (Figure 1E and F). Together, these results indicate that cisplatin effectively induces senescence in BC cells.

3.2. Cisplatin induces senescence by activating the NF-κB signaling pathway

To explore the mechanism of cisplatin-induced senescence, we first assessed NF-κB pathway activation in cisplatin-treated BC cells using the GSE165767 dataset. Our analysis revealed significant activation of the NF-κB signaling pathway (Figure 2A), along with increased phosphorylation of NF-κB in treated cells compared to control cells (Figure 2B). To further investigate NF-κB's role, we used BAY117082, a specific NF-κB inhibitor. Co-treatment with cisplatin and BAY117082 significantly reduced the expression of senescence-associated genes (e.g., *IL6*, *TNF-α*, and *CXCL1*) compared to cisplatin alone (Figure 2C). Similarly, cisplatin-induced upregulation of p21 was attenuated by NF-κB inhibition (Figure 2D). In addition, BAY117082 suppressed cisplatin-induced secretion of IL-6 and TNF-α (Figure 2E). Consistent with these findings, β-galactosidase staining demonstrated that NF-κB inhibition conspicuously reduced cisplatin-induced senescence (Figure 2F and G). Collectively, these results indicate that cisplatin promotes BC cell senescence through the NF-κB signaling pathway.

3.3. Senescent bladder cancer cells exhibit reduced AR expression

Although AR is known to promote cisplatin resistance in BC, the effects of cisplatin on AR expression remain unclear. Analysis of the GSE165767 dataset revealed that cisplatin significantly reduced AR expression in BC cells (Figure 3A). This finding was validated by using RT-qPCR and western blotting on UMUC3 cells. Both AR mRNA and protein levels were significantly downregulated in cisplatin-treated cells compared to controls (Figure 3B and C). Further mechanistic analyses revealed that TNF-α–mediated NF-κB activation contributed to AR suppression (Figure 3D and E). Notably, co-treatment with cisplatin and BAY117082 restored AR expression (Figure 3F). These findings suggest that cisplatin-

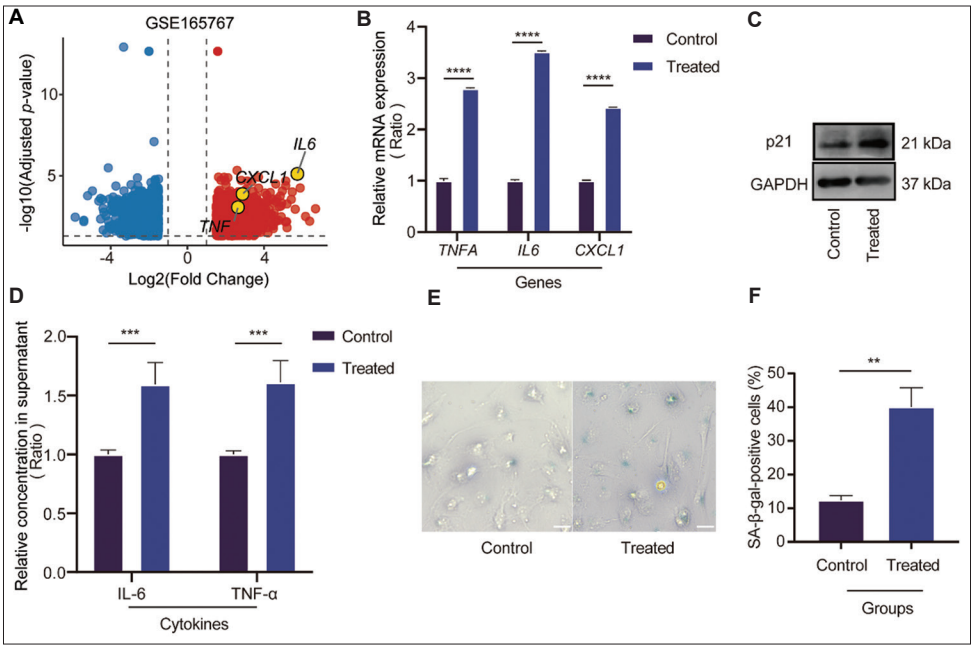

Figure 1. Cisplatin induces senescence in bladder cancer cells. (A) Volcano plot illustrating the differentially expressed senescence-associated genes in the control group and the cisplatin-treated group, with blue dots indicating downregulated genes after cisplatin exposure and red dots indicating upregulated genes. (B) Reverse transcription-quantitative polymerase chain reaction results showing relative messenger RNA expression of senescence-associated genes in cisplatin-treated and untreated UMUC3 cells. (C) Western blot analysis of p21 expression in cisplatin-treated and untreated UMUC3 cells. (D) Concentration of IL-6 and TNF-α in the supernatants of cisplatin-treated and untreated UMUC3 cells. (E) SA-β-gal staining of treated and untreated UMUC3 cells (scale bar: 50 μm; magnification: 20×). (F) Quantitative analysis of cellular senescence. **p<0.01, ***p<0.001, and ****p<0.0001.

Abbreviations: CXCL1: C-X-C motif chemokine ligand 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL-6: Interleukin 6; SA-β-gal: Senescence-associated β-galactosidase; TNF-α: Tumor necrosis factor-α.

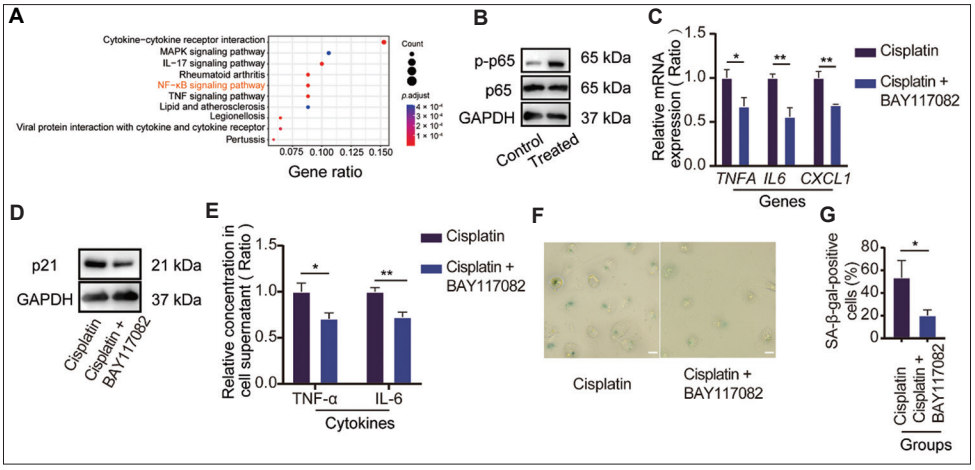

Figure 2. Cisplatin induces senescence in bladder cancer cells by activating the NF-κB signaling pathway. (A) Dot plot illustrating the most enriched pathways among upregulated genes in the cisplatin-treated group compared to the control group based on the GSE165767 dataset. (B) Western blot analysis of p65 and p-p65 expression in cisplatin-treated and untreated UMUC3 cells. (C) Reverse transcription-quantitative polymerase chain reaction results showing relative messenger RNA expression of senescence-associated genes in UMUC3 cells treated with cisplatin alone and cisplatin + BAY117082. (D) Western blot analysis of p21 expression in UMUC3 cells treated with cisplatin alone and cisplatin + BAY117082. (E) Concentration of IL-6 and TNF-α in the supernatants of UMUC3 cells treated with cisplatin alone and cisplatin + BAY117082. (F) SA-β-gal staining of UMUC3 cells treated with cisplatin alone and cisplatin + BAY117082 (scale bar: XX μm; magnification: XX×). (G) Quantitative analysis of cellular senescence. *p<0.05 and **p<0.01.

Abbreviations: CXCL1: C-X-C motif chemokine ligand 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; p-p65: Phospho p65; SA-β-gal: Senescence-associated β-galactosidase; TNF: Tumor necrosis factor.

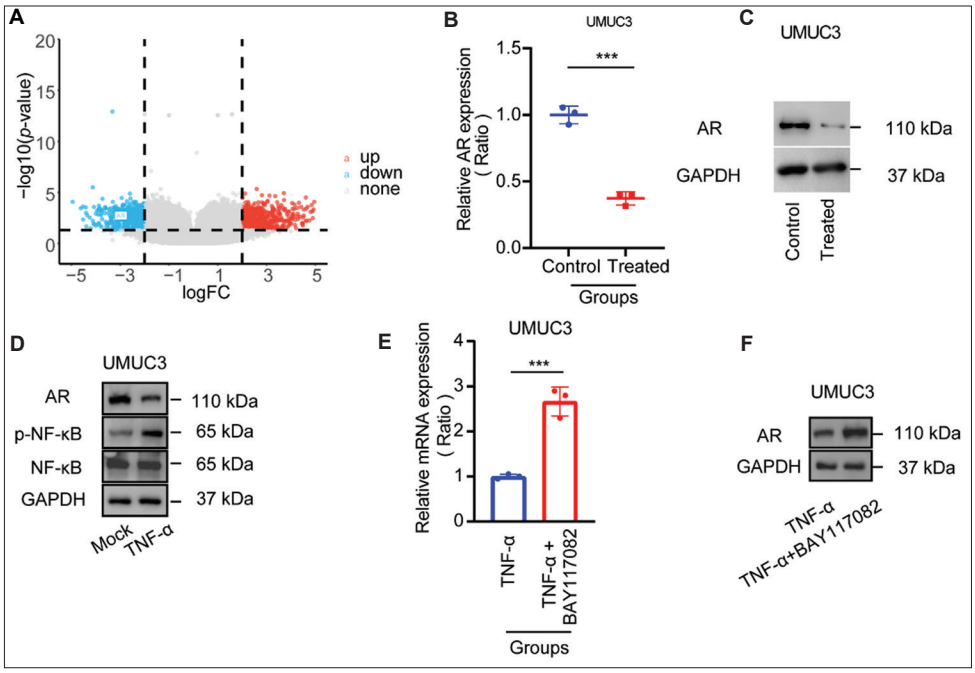

Figure 3. Senescent bladder cancer cells reduce AR expression by activating the NF-κB signaling pathway. (A) Volcano plot illustrating the differentially expressed genes in the cisplatin-treated group compared to the control group, with blue dots indicating downregulated genes after cisplatin exposure and red dots denoting upregulated genes. (B) RT-qPCR results showing *AR* messenger RNA expression in cisplatin-treated and untreated UMUC3 cells. (C) Western blot analysis of AR expression in cisplatin-treated and untreated UMUC3 cells. (D) Western blot analysis of p-p65, p65, and AR expression in mock- and TNF-α–treated UMUC3 cells. (E) RT-qPCR results showing *AR* mRNA expression of UMUC3 cells treated with TNF-α alone and TNF-α + BAY117082. (F) Western blot analysis of AR expression of UMUC3 cells treated with TNF-α alone and TNF-α + BAY117082. ***$p<0.001$.
Abbreviations: AR: Androgen receptor; FC: Fold change; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NF-κB: Nuclear factor κ-light-chain-enhancer of activated B cells; p-p65: Phospho p65; TNF-α: Tumor necrosis factor-α; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

induced senescence reduces AR expression through the TNF-α/NF-κB signaling pathway.

3.4. Senescent bladder cancer cells promoted macrophage infiltration

Given the immunomodulatory role of AR, we investigated its association with immune cell infiltration in BC using the CIBERSORTx algorithm on The Cancer Genome Atlas (TCGA) datasets. Our analysis revealed that macrophage infiltration was significantly elevated in bladder tumor tissues compared to adjacent normal tissues (Figure 4A). Notably, AR expression negatively correlated with macrophage infiltration in BC (Figure 4B). Further analysis using the cBioPortal database indicated that AR expression was more prevalent in MIBC (Figure 4C), and an inverse correlation between AR levels and macrophage infiltration was particularly pronounced in MIBC patients (Figure 4D). Mechanistically, senescent BC cells (induced by cisplatin) showed enrichment in cell migration-related pathways (Figure 4E), suggesting a link between AR downregulation and immune microenvironment remodeling. In addition, the analysis demonstrated that senescent BC cells enhance macrophage infiltration (Figure 4F). In summary, decreased AR expression in BC is associated with increased macrophage infiltration, highlighting AR's potential immunoregulatory role in tumor–immune interactions.

3.5. TAMs reduce cisplatin efficacy

To examine the impact of TAMs on cisplatin-treated BC cells, we first performed a CCK-8 assay, revealing that macrophage infiltration significantly promoted cancer cell proliferation after cisplatin exposure (Figure 5A). Co-culture with macrophages also reduced expression of γ-H2A histone family member X, a DNA damage marker (Figure 5B). Furthermore, TAM co-culture downregulated pro-apoptotic proteins and upregulated anti-apoptotic proteins in cisplatin-treated cells (Figure 5C). β-galactosidase staining demonstrated that macrophages attenuated cisplatin-induced senescence (Figure 5D and E). Given AR's role in cisplatin resistance, we evaluated AR expression and found that macrophage co-culture substantially increased AR expression levels at both mRNA and protein levels (Figure 5F and G). Together, these results indicate that macrophage infiltration contributes to cisplatin resistance in BC cells.

4. Discussion

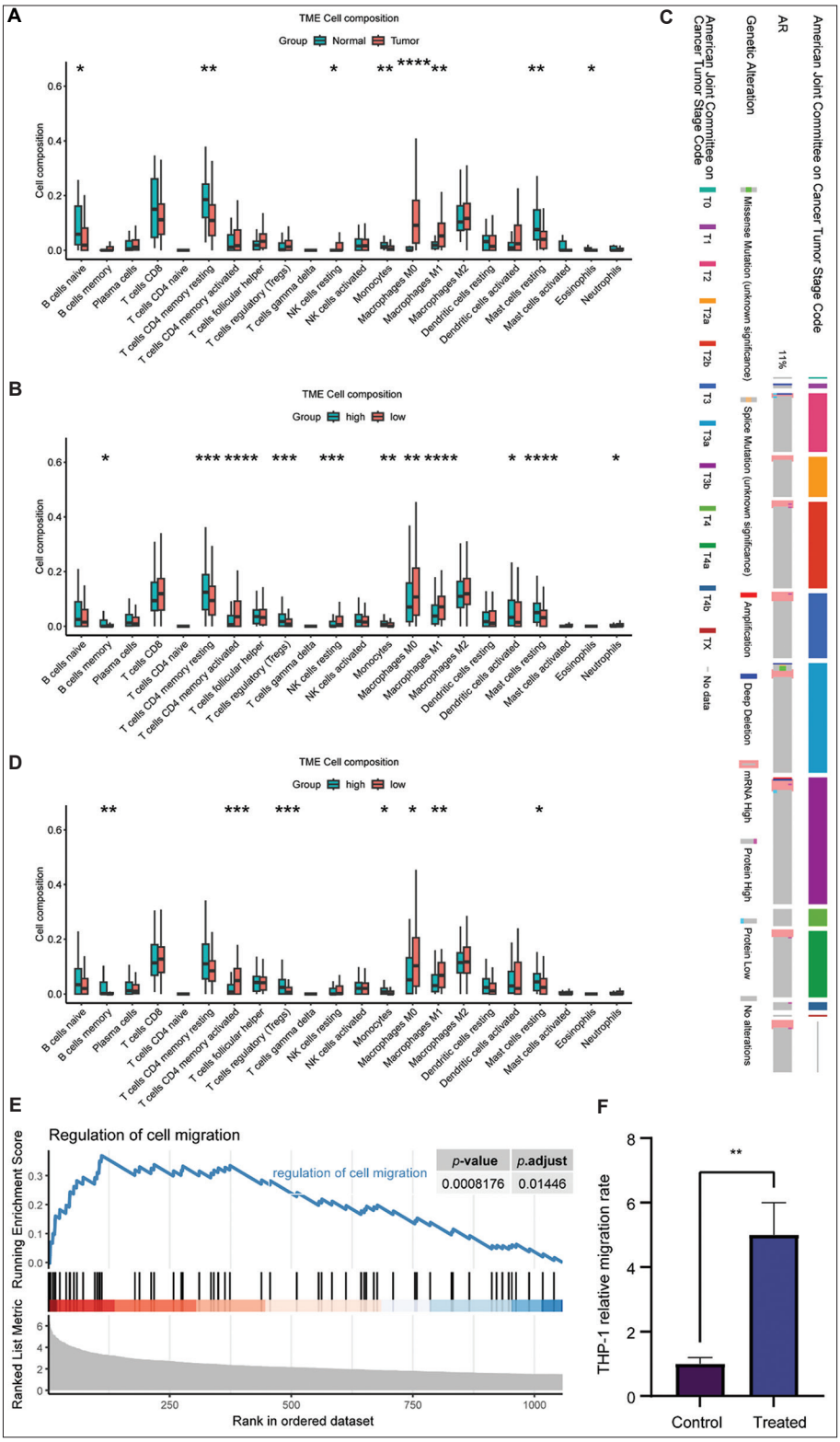

Figure 4. Senescent bladder cancer cells promote macrophage infiltration. (A) Box plot illustrating differential immune cell infiltration in bladder cancer tissues compared to adjacent normal tissues in the TCGA–BLCA dataset. (B) Box plot illustrating differential immune cell infiltration in high-AR expression tissues compared to low-AR expression ones in the TCGA–BLCA dataset. (C) CIBORSORTx analysis showing the AR is commonly expressed in MIBC. (D) Box plot illustrating differential immune cell infiltration in high-AR expression tissues as compared to their low-AR expression counterparts in the TCGA–BLCA–MIBC dataset. (E) GSEA plot illustrating that regulation of the cell migration signaling pathway was significantly enriched in senescent bladder cancer cells. (F) Bar chart showing that senescent bladder cancer cells enhance macrophage infiltration. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Abbreviations: AR: Androgen receptor; BLCA: Bladder urothelial carcinoma; CD: Cluster of differentiation; GSEA: Gene set enrichment analysis; MIBC: Muscle-invasive bladder cancer; NK: Natural killer; TCGA: The Cancer Genome Atlas; TME: Tumor microenvironment.

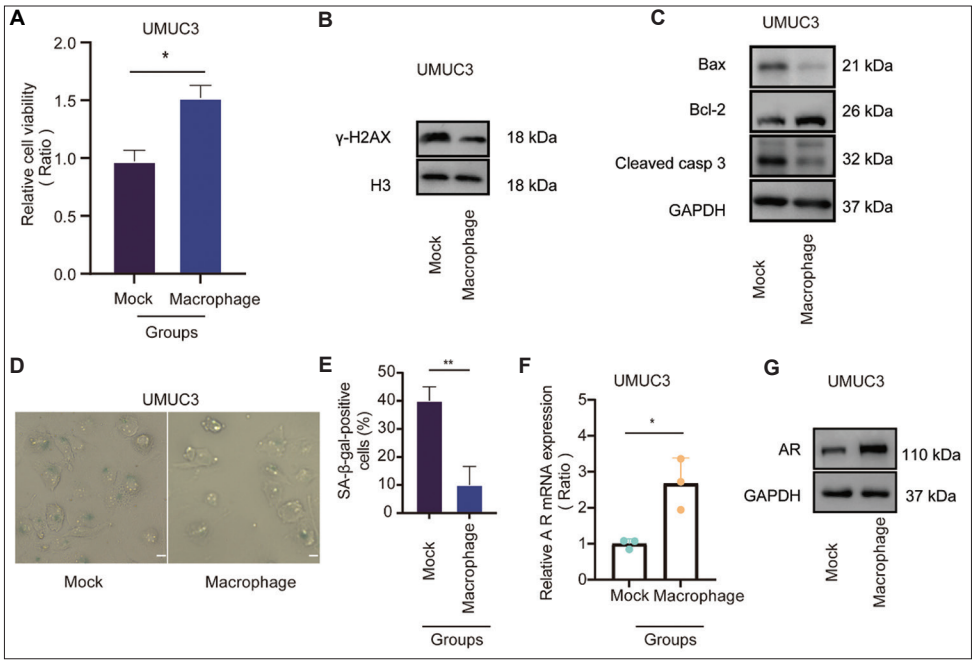

Figure 5. The reduced outcome of cisplatin treatment was caused by TAMs. (A) Bar plot showing the relative cell viability of UMUC3 cells co-cultured with macrophages. (B) Western blot analysis of γ-H2AX expression in UMUC3 cells co-cultured with macrophages. (C) Western blot analysis of Bax, Bcl-2, and cleaved casp 3 expression in UMUC3 cells co-cultured with macrophages. (D) SA-β-Gal staining of UMUC3 cells co-cultured with macrophages (scale bar: 50 μm; magnification: 20×). (E) Quantitative analysis of cellular senescence. (F) Reverse transcription-quantitative polymerase chain reaction results showing *AR* messengerRNA expression in UMUC3 cells co-cultured with macrophages. (G) Western blot analysis of AR expression in UMUC3 cells co-cultured with macrophages. *$p<0.05$ and **$p<0.01$.
Abbreviations: γ-H2AX: γ-H2A histone family member X; AR: Androgen receptor; casp 3: Caspase 3; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; SA-β-gal: Senescence-associated β-galactosidase; TAM: Tumor-associated macrophage.

Cisplatin-based chemotherapy remains the standard treatment for advanced BC. However, its efficacy is often hampered by the development of resistance, resulting in a 5-year overall survival rate of <60% for MIBC patients.[17] Understanding the mechanisms underlying acquired cisplatin resistance is therefore a critical research priority.

Although cisplatin is known to induce cellular senescence, its role in BC has been poorly characterized. Our study provides the first evidence that cisplatin triggers senescence in BC cells through NF-κB activation. This finding aligns with previous work by Jing *et al*., who reported that cisplatin-induced senescence in vascular endothelial cells was mediated by NF-κB.[18] In contrast, Sun *et al*. demonstrated that cisplatin induced senescence in melanoma A375 cells through the p53/p21 pathway, but not in A549, HeLa, or MDA-MB-231 cells.[19] These discrepancies underscore the context-dependent nature of cisplatin-induced senescence and highlight the need for caution and care in the interpretation across different cancer types.

While elevated AR expression has been linked to cisplatin resistance,[12,13] we observed that cisplatin treatment significantly reduced AR levels in BC cells. Mechanistically, cisplatin activated the NF-κB pathway and increased TNF-α secretion. Since TNF-α–mediated NF-κB activation can suppress stromal AR expression,[20] we looked into whether NF-κB inhibition could restore AR levels. The findings revealed that co-treatment with BAY117082 and cisplatin significantly increased AR expression compared to cisplatin alone. These findings are coincident with those of Shi *et al*., who reported that cisplatin downregulated AR in hepatocellular carcinoma in a dose-dependent manner,[21] suggesting a broader role for cisplatin in modulating AR signaling across cancers.

Emerging evidence indicates that AR expression influences immune regulation. To explore the immunotherapeutic effects of AR in BC cells, the CIBERSORTx algorithm was applied to the TCGA–bladder urothelial carcinoma (BLCA) dataset to analyze the correlation between AR expression and tumor microenvironment composition in the TCGA-BLCA dataset. The analysis exhibited significantly greater macrophage infiltration in the dataset's bladder tumor samples compared to normal tissue samples. Notably, high-AR expression cases in both BC and MIBC showed substantially higher macrophage infiltration than their low-AR expression counterparts.

Enrichment analysis of RNA sequencing data from cisplatin-treated T24 cells revealed an enhanced leukocyte migration capacity upon cisplatin treatment. *In vitro* functional assays confirmed that cisplatin promoted macrophage migration when co-cultured with BC cells. Mechanistically,

IL-6 was identified as a potential mediator, in line with its known roles in macrophage migration.[22] Cisplatin treatment increased IL-6 secretion, providing a plausible explanation for the enhanced macrophage migration.

To assess the influence of macrophages on AR expression, we established a co-culture system. AR expression was significantly upregulated in BC cells co-cultured with macrophages compared to those cultured alone, which was in consistency with previous studies reporting that macrophages increased AR expression in BPH1 cells.[23] In addition, AR expression was inversely correlated with immune cell infiltration in human epidermal growth factor receptor 2-positive breast cancer.[24] Macrophage co-culture not only enhanced BC cell viability but also conferred increased cisplatin resistance.[25,26] This resistance-promoting effect was particularly evident in the co-culture system, suggesting that TAMs may contribute to chemotherapy resistance in BC.

Despite these mechanistic and translational insights into cisplatin resistance and AR-related immune regulation in BC, several limitations remain in our study. Our work lacks clinical and animal experiments for further validation, and the potential off-target effects of BAY117082 beyond its NF-κB inhibitory activity should be explored.

5. Conclusion

Our study reveals a bidirectional regulatory loop between cisplatin treatment and the tumor microenvironment in BC. Cisplatin-induced downregulation of AR expression promotes macrophage infiltration, while TAMs upregulate AR expression in BC cells, thereby fostering cisplatin resistance. These findings provide new insights into the involved interplay between chemotherapy, AR signaling, and immune modulation in BC progression and the development of resistance to treatment.[27,28] Consequently, integrated therapeutic strategies, such as NF-κB inhibitors (BAY117082), AR antagonists (enzalutamide), or macrophage-targeting therapies,[29-32] may enhance cisplatin efficacy in BC. However, due to the limitations of this study, these findings should be interpreted with caution. Therefore, further studies should validate the proposed "senescence–macrophage–AR" axis in tumor xenografts, examine the correlation between macrophage infiltration and AR expression in clinical biopsies harvested before and after cisplatin treatment, and investigate potential off-target effects of BAY117082 beyond its NF-κB inhibitory activity.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Bangmin Han
Formal analysis: Tiewen Li
Investigation: Yichen Zhang
Methodology: Di Cui
Writing–original draft: Zhiwen Xie, Wenhao Wang
Writing–review & editing: Zhiwen Xie, Wenhao Wang

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability statement

Not applicable.

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