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Original Paper

Membrane-Bound CD40L Promotes Senescence and Initiates Senescence-Associated Secretory Phenotype via NF-κB Activation in Lung Adenocarcinoma

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Key Words
Cd40l mutant • Lung adenocarcinoma • Senescence • SASP • NF-κB

Abstract
Background/Aims: Cellular senescence acts as a barrier against tumorigenesis. The CD40L transgene, expressed in some tumor cells, not only becomes visible to antigen-presenting cells but also actively catalyzes its own termination. Here, we evaluated the effect of a membrane-bound mutant form of human CD40L (CD40L-M) on senescence and the senescence-associated secretory phenotype (SASP) in non-small cell lung cancer (NSCLC). ***Methods:*** CD40 expression levels in the NSCLC cell lines A549/TR, A549/DDP and H460 were examined by flow cytometry. Senescent cells and tissues were identified via SA-β-gal activity. Cell proliferation was visualized by EdU labeling. qRT-PCR, Western blotting, and immunofluorescence staining were conducted to assess mRNA and protein expression levels of CD40L, γ-H2A.X, p65, p-p65, IκBα, p53, p21 and p16. Cytokines secreted from transfected cells were tested by ELISA and cell migration assay. Capsid tyrosine-modified rAAV5-CD40L-M was packaged and carried out *in vivo*. ***Results:*** Overexpression of CD40L-M promoted senescence, inhibited proliferation, increased DNA damage-associated γ-H2A.X, and initiated the SASP in CD40-positive NSCLC cells. NF-κB signaling was activated by CD40L-M overexpression in these cells. Knockdown of NF-κB partially overcame senescence and failed to induce SASP. Furthermore, increased p53 and p21 protein levels induced by CD40L-M were also reduced following NF-κB suppression. ***Conclusions:*** These data showed that the membrane-bound CD40L mutant may promote cellular senescence and initiate the SASP of NSCLC cells in an NF-κB-dependent manner. Therefore, CD40L-M-induced senescence may be a potential approach to protect against lung adenocarcinoma.

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Introduction

Lung cancer is one of the deadliest diseases worldwide. Although treatment for advanced non-small cell lung cancer (NSCLC) is changing due to new emerging agents, the cisplatin-based doublet remains the foundational treatment for most patients with advanced NSCLC [1]. However, chemotherapy resistance stands as an obstacle to the therapeutic efficacy of treatments for lung cancer. To overcome lung cancer therapy resistance and facilitate prolonged anticancer effects of first-line therapies, combination chemotherapy regimens have been used; however, the overall survival benefits of these strategies in NSCLC is not yet satisfactory [2].

''Cellular senescence'' (or ''senescence'') is a distinct form of durable cell-cycle arrest that serves to prevent cancer in mammals [3]. The general biological purpose of senescence is to eliminate unwanted cells, which is conceptually similar to apoptosis. Senescence and apoptosis are the most important mechanisms for the elimination of damaged cells. This is particularly relevant in the fields of cancer and aging, which are both characterized by the accumulation of severe cellular damage. Consistent with this, senescence is a crucial barrier against cancer progression [4].

Senescent cells generally display an enlarged and flattened morphology with increased activity of senescence-associated beta-galactosidase (SA-β-gal). Other features of senescence include high levels of p21/WAF1 and p16/INK4a, the DNA damage response (DDR), as well as the senescence-associated secretory phenotype (SASP) [5]. Cellular senescence is not only broadly recognized to act as a potent barrier against tumorigenesis, it is also considered to contribute to the cytotoxicity of certain anticancer agents and influence chemotherapy outcomes [6].

The CD40 ligand (CD40L), a type II membrane-bound protein belonging to the tumor necrosis factor (TNF) superfamily, has been a target in cancer therapies due to its ability to trigger Th1-type immune responses that drive effector cells, which normalize the tumor microenvironment and directly suppress the growth of certain CD40-positive tumors; these include cytotoxic T lymphocytes, natural killer cells, and M1 macrophages [7]. Novel therapies based on CD40L, including infusion of recombinant CD40L protein, anti-CD40 antibodies, and CD40L gene therapy, potentially provide numerous anti-tumor approaches in one therapy, and offer attractive options for clinical trials [8, 9].

However, membrane-bound CD40L may be proteolytically cleaved to form soluble CD40L (sCD40L), which results in adverse effects. In our previous study, it was demonstrated that recombinant self-complementary adeno-associated virus 5 (scAAV5) efficiently delivered a non-cleavable human CD40L mutant (scAAV5-CD40L-M) to lung cancer cells. Transduction with scAAV5-CD40L-M resulted in effective expression of CD40L on the cell surface with low levels of cleaved sCD40L, which significantly reduced the percentage of viable cells through the caspase-3-dependent pathway *in vitro* and *in vivo,* with few side effects [10].

In the present study, we further explored multiple antitumor activities of membrane-bound CD40L in NSCLC. We first reported that the membrane-bound CD40L mutant promoted cell senescence and initiated the senescence-associated secretory phenotype in NSCLC. We also investigated the role of the NF-κB signaling in CD40L-induced senescent cells.

Materials and Methods

Cell culture and treatment

The human CD40-positive lung adenocarcinoma cell lines A549 and H460 were purchased from the Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). Cisplatin-resistant A549/DDP and paclitaxel-resistant A549/TR cell lines were kindly provided by Prof. Hongbing Shen (Nanjing Medical University, Nanjing, China). Human promyelocytic leukemia cells, differentiated along a neutrophil cell lineage (dHL60), were kindly provided by Prof. Jianyong Li (Nanjing Medical University, Nanjing, China). All cells were grown in DMEM medium containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin-

streptomycin and incubated at 37˚C with 5% CO_2 in a humidified incubator. To maintain drug resistance, A549/DDP and A549/TR were grown in DMEM containing 6.67 μM Cisplatin or 0.23 μM paclitaxel (Pure Chemistry Scientific Inc., USA) respectively, and then in drug free DMEM two days prior to experiments.

Plasmids and siRNA

The pcDNA3.1$^+$-CD40L plasmid was manufactured by Invitrogen Biotechnology (Shanghai, China) following current Good Manufacturing Practice guidelines, and the entire construct was sequenced to exclude the possibility of mutated nucleotides. The CD40L-M mutant containing 6 substitutions (Gln114 to Pro114, Lys115 to Arg115, Asp117 to Glu117, Gln118 to Glu118, Asn119 to Asp119, and Pro120 to Ser120) was constructed by Invitrogen Biotechnology (Shanghai, China). Then, pcDNA3.1$^+$-CD40L-M was produced and identified as reported above [10]. Small interfering RNAs (siRNAs) specific to NF-κB p65 and a negative control were obtained from GenePharma (Shanghai, China). The sense sequences of the siRNAs were as follows: NF-κB p65, 5'-AGAGGACAUUGAGGUGUAUTT-3'.

Flow Cytometry

Flow cytometry was performed by following a standard protocol. Briefly, cells were gently washed with PBS and collected using trypsinization, disaggregated to a single cell suspension and incubated with phycoerythrin (PE)-conjugated CD40 Ab. An appropriate PE-conjugated isotype control was used to measure background staining.

SA-β-gal assay

SA-β-gal activity was detected using the senescence β-galactosidase staining kit (Beyotime Institute of Biotechnology, Shanghai, China). Treated cells or tissues were fixed for 15 min at room temperature with 1 ml of fixative solution, washed three times with PBS and then incubated with X-gal staining solution mix overnight at 37˚C in the absence of CO_2. Cells or tissues were viewed using a phase contrast microscope. Senescent cells and tissues were identified via their blue staining.

EdU labeling

According to EdU kit instructions (KeyGEN Biotech, Nanjing, China), cells were inoculated into confocal dishes, incubated with 100 μl of 50 μM EdU solution for 2 h, fixed in 4% paraformaldehyde, decolorized with 50 μl of 2 mg/ml glycine solution, observed under LSM710 laser scanning confocal microscope (Carl Zeiss, Germany) and photographed. After the images were merged, the ratios of Apollo stained-positive cells to Hoechst stained-positive cells were calculated.

Cell viability assay

The IC50 and inhibition rate of tumor growth was determined by the MTT (3-[4, 5-dimethylthia-zol-2-yl]-2, 5-diphenyltetrazolium bromide) assay (Sigma Aldrich, St. Louis, MO, USA). Cancer cells were seeded into 96-well plates and treated with cisplatin or paclitaxel at different concentrations. After incubation, the media was replaced with 50 μl of MTT reagent (2 mg/ml), followed by further incubation for 2 h. Then, the media was removed and 150 μl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at 560 nm with a spectrophotometer.

Western blotting

Cells were lysed by ice-cold RIPA buffer containing proteasome inhibitor cocktail and phosphatase-inhibitor cocktail. The lysates were then centrifuged at 4˚C and the supernatant was collected. Protein samples were separated by SDS–PAGE, transferred onto a nitrocellulose membrane, and probed with an appropriate antibody. Antibodies to γ- H2A.X (#9718), p-p65 (Ser536, #3033), p65 (#4764), IκB-α (#9247), p53 (#2524), p21 (#2947), p16 (#4824) and GAPDH (#2118) and an HRP conjugated secondary antibody were obtained from Cell Signaling Technology (Beverly, MA, USA).

ELISA

Supernatants from transfected cells were collected and centrifuged at 400 × g for 5 min at 4˚C, the ELISA assay was performed using human IL-6 and IL-8 ELISA kits (KeyGEN Biotech, Nanjing, China) according to manufacturer's instructions. The absorbance was monitored at 450 nm using a microplate reader.

Cell migration assay

Conditioned medium from senescent cells was added to the bottom wells in 12-well Transwell inserts. dHL60 cells (human promyelocytic leukemia cells differentiated along a neutrophil cell lineage) were seeded in the chemotaxis chamber at a concentration of 2×10^5 and incubated at 37°C 5% CO_2 for 1 h. The transmigrated cells were then fixed with methanol, stained with 0.1% crystal violet, and counted under a light microscope (200 × magnification).

Virus packaging

Self-complimentary rAAV serotypes 5 and corresponding single tyrosine-to-phenylalanine (Y-F) mutants were packaged in HEK-293 cells as previously described [11]. The encapsidated rAAV genomes encoded self-complementary enhanced green fluorescent protein (EGFP). The integrity of the ITRs was confirmed before packaging. Titers of packaged rAAV were determined by real-time TaqMan amplification assay with vector-specific primers and probes.

Xenograft tumors in nude mice

The animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing University and performed according to the institutional guidelines and protocols. Male BALB/c athymic nude mice (4–6 weeks old, weighing 20–22 g) were provided by the Department of Laboratory Animals of Nanjing University and maintained in a laminar air-flow cabinet under specific pathogen-free conditions. To generate tumor growth *in vivo*, 1×10^6 H460 cells were subcutaneously injected into the mice. When the tumor volume reached 130 mm^3, the mice were randomly divided into five groups (six mice per group). The experiment was performed by intratumoral injection with AAV5-M-GFP, AAV5-M-CD40L-WT, and AAV5-M-CD40L-M vectors (at 1×10^{11} viral genomes [vgs]/mouse) weekly following intravenous injection with cisplatin (2 ml/kg) every three days. Tumor growth was subsequently monitored twice a week for six weeks by measuring tumor size with a caliper.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 software package for Windows (SPSS, Inc., Chicago, IL, USA). Comparisons between 2 groups were performed by the Student *t* test when data were normally distributed and group variances were equal. The Mann–Whitney rank sum test was used when data were not normally distributed or if group variances were unequal [12, 13]. Prism 6 software was used to generate graphs. All results were expressed as mean ± SD or median and percentiles. A P value < 0.05 was considered statistically significant.

Results

Overexpression of CD40L-M promotes senescence and inhibits proliferation in NSCLC cells

Previous research demonstrated that CD40 is expressed in a number of carcinoma types. Thus, we examined CD40 expression levels in the NSCLC cell lines A549/TR, A549/DDP, and H460 by fluorescence-activated cell sorting (FACS). The percentage of cells expressing CD40 was 63.52 ± 1.36%, 55.69 ± 6.02% and 87.72 ± 2.30% in A549/TR, A549/DDP, and H460 cells, respectively (Fig. 1A). We also stably overexpressed CD40L (CD40L-WT) and CD40L mutant (CD40L-M) in NSCLC cell lines. In the process, cells with CD40L-WT/CD40L-M became flattened and elongated in appearance after 48 h, suggesting morphological transformation related to cell senescence. Next, the SA-β-gal assay was performed. Results showed a greater number of positively stained CD40L-WT/CD40L-M cells than control cells in A549/TR, A549/DDP, and H460 cells (Fig. 1B, C). We then explored the effects of CD40L-WT/CD40L-M overexpression on DNA synthesis and cell proliferation, which are characteristics of cell senescence. The results showed that overexpression of CD40L-WT/CD40L-M inhibited DNA synthesis and cell proliferation (Fig. 1D).

Overexpression of CD40L-M increases γ-H2A.X and chemotherapy sensitivity

Another feature of senescence is the DNA damage response. We investigated whether CD40L-WT/CD40L-M overexpression induces DNA damage by examining γ-H2A.X by

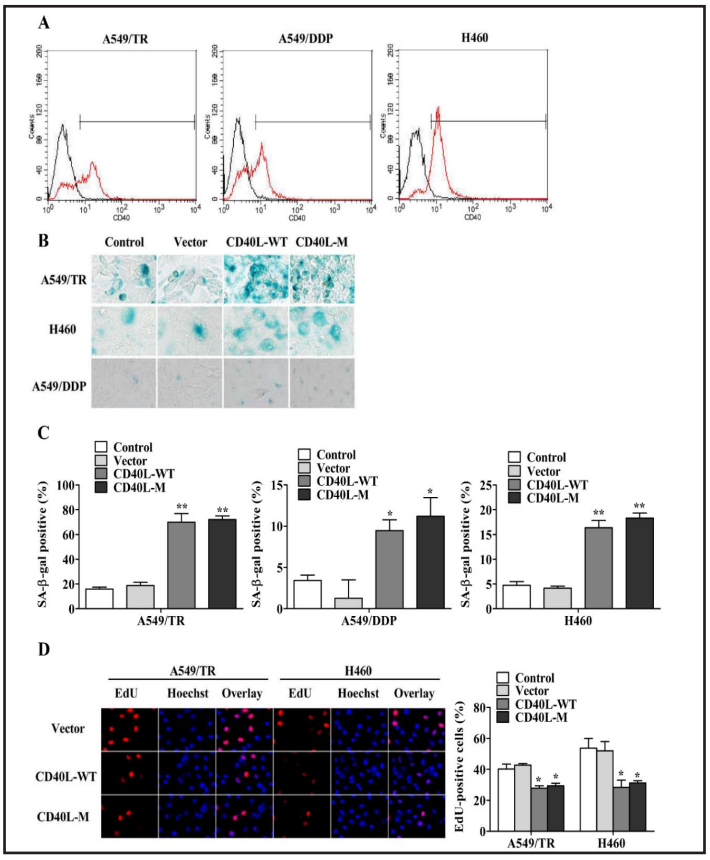

Fig. 1. CD40L-WT/CD40L-M promotes senescence and inhibits proliferation in NSCLC cells. Cells were transfected with an entry vector pcDNA3.1$^+$, pcDNA3.1$^+$-CD40L-WT or pcDNA3.1$^+$-CD40L-M and incubated for 48h. A. The levels of CD40 on non-small cell lung cancer cells were determined by Flow cytometric analysis. B. SA-β-gal-activity was measured using a light microscope (200 ×) in A549/TR, A549/DDP and H460 cells. C. Quantitative analysis of SA-β-gal positive cells. D. Representative micrographs of cell proliferation were visualized by EdU incorporation co-staining with Hoechst using a fluorescence microscope. Values are means ± SD of 3 to 5 independent experiments. * P<0.05 or ** P<0.01 versus control group, vector group.

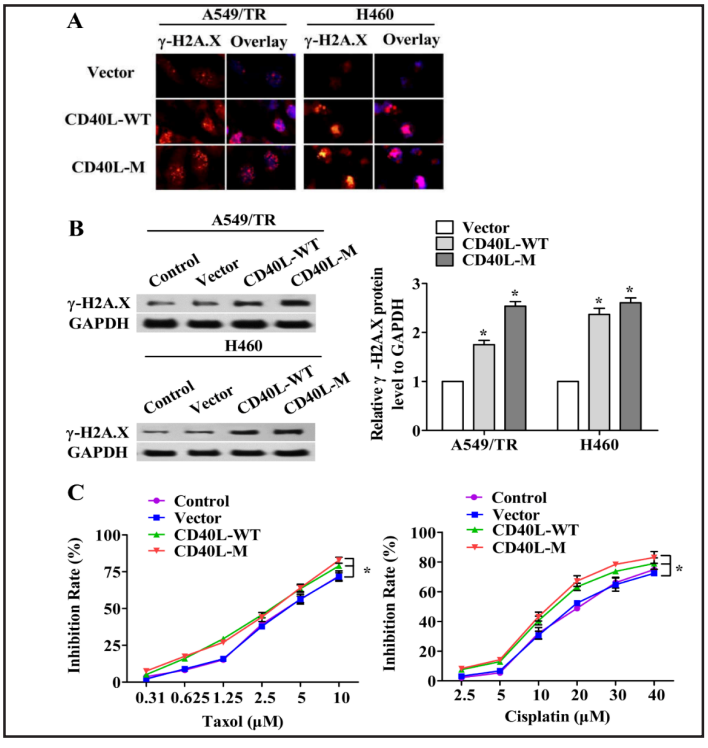

Fig. 2. CD40L-M increases DNA damage-associated γH2AX and chemotherapy sensitivity. A. Immunofluorescence staining of γ-H2A.X and Hoechst shows elevated γ-H2A.X levels 48h after transfection in A549/TR and H460 cells. B. The expression levels of γ-H2A.X were analyzed by Western blot using the indicated antibodies. C. Transfected cells were exposed to various doses of paclitaxel (in A549/TR) and cisplatin (in H460) for 48 h. The viability of cells was assessed by MTT assay as described in Methods. The inhibition rate was calculated according to the following formula: inhibition ratio (%) = [1 − (OD value in treatment group/OD value in control group)] × 100%. Values are median with 25th and 75th percentiles; Mann–Whitney rank sum test. * P<0.05 versus vector group.

immunofluorescence and Western blotting. DNA damage was confirmed by the formation of γ-H2A.X foci in the nuclei of CD40L-WT/CD40L-M overexpressed cells but not in

control cells (Fig. 2A), and the nucleoprotein also increased (Fig. 2B). Cytotoxic drugs act principally by damaging tumor-cell DNA. Therefore, we hypothesized that CD40L-WT/CD40L-M overexpression and treatment with chemotherapeutic reagents would result in either additive or synergistic effects on cytotoxicity. To test this hypothesis, NSCLC cells were incubated with paclitaxel or cisplatin in the presence or absence of CD40L-WT/CD40L-M overexpression and MTT assays were performed to assess cell survival. Compared with the control group, overexpression of CD40L-WT/CD40L-M in A549/TR cells sensitized the response to paclitaxel (Fig. 2C). The half maximal inhibitory concentration (IC50) values of A549/TR cells to paclitaxel in the negative control, CD40L-WT, and CD40L-M groups were 4.26 ± 0.18 μM, 2.97 ± 0.16 μM and 2.86 ± 0.16 μM, respectively. Meanwhile, overexpression of CD40L-WT/CD40L-M in H460 cells also sensitized the response to cisplatin.

Overexpression of CD40L-M initiates the SASP of NSCLC cells

Senescent cells undergo significant changes in the expression of genes that are predicted to influence the tissue microenvironment. These changes have been referred to as the SASP. We examined the levels of several key SASP cytokines and chemokines secreted into the media via ELISA. The results demonstrated that both IL-6 and IL-8 were increased in CD40L-WT- or CD40L-M-overexpressed A549/TR and H460 cells (Fig. 3A, B). Although components of the SASP were initially studied as markers of senescence, it is now clear that SASP components also actively participate in immune surveillance. To investigate whether CD40L-WT or CD40L-M-induced SASP resulted in recruitment of immune cells, we examined the migration of HL60 cells differentiated along the neutrophil lineage in response to conditioned media from CD40L-WT or CD40L-M-overexpressed cells. The data demonstrated that the conditioned media induced migration of dHL60 cells (Fig. 3C, D).

CD40L-M regulates SASP in NF-κB-dependent manner

Several studies have revealed that NF-κB transcription factors are involved in the regulation of secretory components of cellular senescence [3]. Meanwhile, others have reported that CD40L in airway epithelial cells triggered the activation of NF-κB signaling via IκB kinase. Therefore, phosphorylation of p65 on ser536, and its negative regulator IκBα, were analyzed by Western blotting (Fig. 4A, B). We then detected released NF-κB, which translocated to the nucleus following CD40L-WT or CD40L-M transfection. CD40L-WT or CD40L-M-overexpressed cells showed nuclear translocation of NF-κB p65 (Fig. 4C). CD40L-WT/CD40L-M overexpression significantly promoted nuclear localization of p65, whereas the total amount of p65 in the cytoplasm was slightly changed based on the ratio of p65/GAPDH (Fig. 4D, E). To address the functional significance of NF-κB for SASP induced by CD40L-WT/CD40L-M, we used siRNA against p65 to efficiently knock down p65 protein

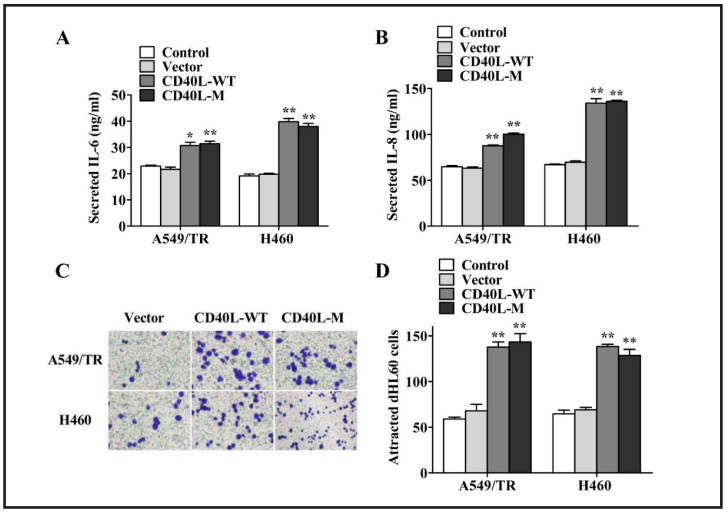

Fig. 3. Overexpression of CD40L-M initiates the SASP of NSCLC cells. A. Secreted IL-6 in the medium was detected by ELISA 48h after transfection in A549/TR and H460 cells. B. IL-8 detected after transfection. C. Representative microscope photographs of migration of HL60 cells in Transwell inserts stained by crystal violet visualized at 200 ×. D. Quantitative analysis of the migration of HL60 cells. Values represent means ± SD of 3 to 5 independent experiments. * P<0.05 or ** P<0.01 versus control group, vector group.

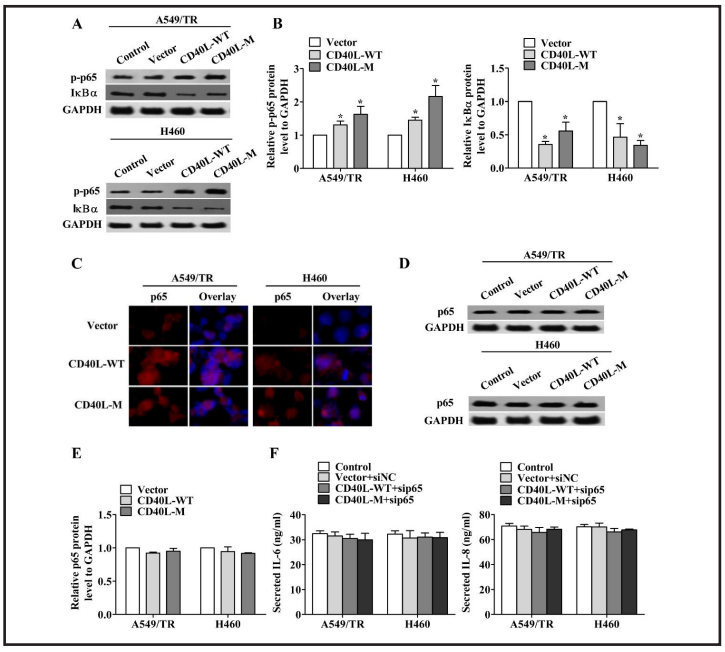

Fig. 4. CD40L-M regulates SASP in an NF-κB-dependent manner. A. Phosphorylation of p65 on ser536 and IκBα expression levels were analyzed by Western blotting after transfected A549/TR and H460 cells were incubated for 48 h. B. Relative p-p65 and IκBα protein levels to GAPDH. C. Nuclear translocation of p65 was detected by immunostained with anti-p65 antibodies and counterstained with Hoechst. D. The total p65 expression level in cells was tested by Western blot analysis. E. Total p65 protein levels relative to GAPDH. F. The levels of IL-6 and IL-8 were determined by ELISA after CD40L-WT/CD40L-M-expressed cells were transfected with siRNAs against p65. Student t test and Mann-Whitney rank sum test. Data are mean ± SD or median and percentiles from 3 to 5 independent experiments. * P<0.05 versus vector group.

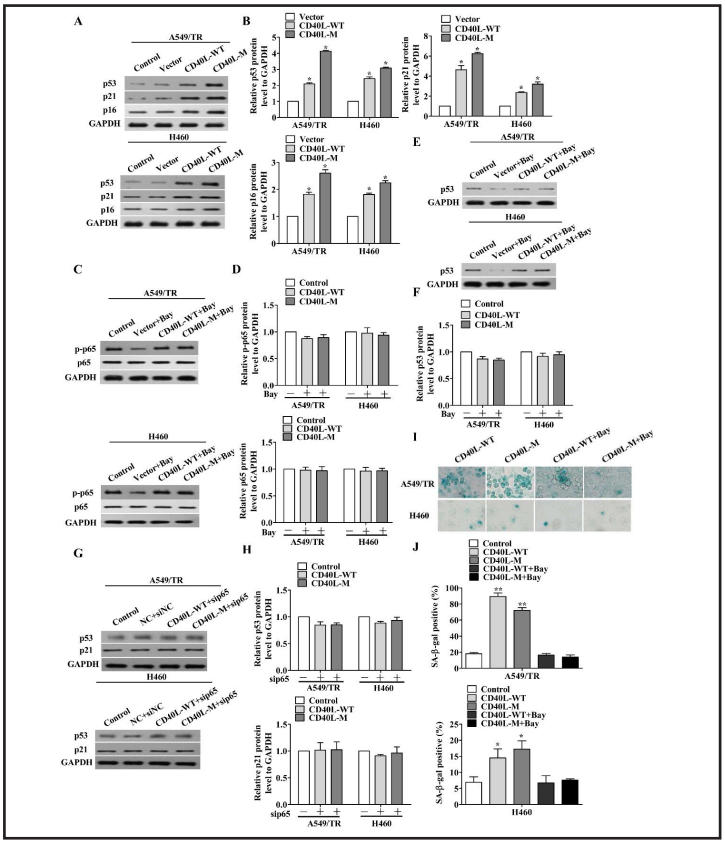

Fig. 5. NF-κB mediates the senescence program of CD40L-M-expressed NSCLC cells. A. The increased levels of p53, p21, and p16 were observed by Western blotting after transfected A549/TR and H460 cells were incubated for 48 h. B. p53, p21, and p16 protein levels relative to GAPDH. C. The levels of p-p65 and p65 were analyzed by Western blotting after transfected cells were treated with Bay11-7082. D. p-p65 and p65 protein levels relative to GAPDH. E. Western blotting analysis of p53 in transfected cells with or without Bay11-7082 treatment. F. p65 protein levels relative to GAPDH. G. p53 and p21 protein levels were analyzed by Western blotting after CD40L-WT/CD40L-M-overexpressed cells were transfected with p65 siRNAs. H. p65 and p21 protein levels relative to GAPDH. I. Representative images of SA-β-gal staining were shown in the graph. J. Quantitative analysis of SA-β-gal positive cells. GAPDH was used as a protein loading control. Values are median with 25th and 75th percentiles; Mann–Whitney rank sum test; * P<0.05 or ** P<0.01 versus control group, vector group.

levels in A549/TR and H460 cells. The results showed that the expression levels of IL-6 and IL-8 did not increase in response to CD40L-WT/CD40L-M overexpression after p65 knockdown (Fig. 4F).

NF-κB signaling mediates the senescence program of CD40L-M-expressing NSCLC cells

It has been shown that the tumor suppressor proteins p53 and pRB are involved in the appearance of cellular senescence. In the current study, analysis of senescence-associated proteins showed an increase of p53, p21, and p16 expression levels in CD40L-WT and CD40L-M-overexpressing NSCLC cells, and correspondingly weakened trends in control cells (Fig. 5A, B). To address the functional significance of NF-κB in the senescence program of CD40L-M-overexpressing NSCLC cells, transfected cells were treated with the NF-κB inhibitor Bay11-7082 (Fig. 5C, D). Results of this experiment showed a decrease in the expression of p53 (Fig. 5E). Further, NF-κB signaling was inhibited via p65 siRNA, and the protein levels of p53 and p21 were attenuated (Fig. 5F, G). SA-β-gal staining was also detected (Fig. 5H, I). The results indicated that senescence induced by CD40L might be mediated through an NF-κB-dependent pathway.

Effects of scAAV5-M-CD40L-M in animal model tumors

To extend our findings *in vivo*, we examined senescence (SA-β-gal staining), NF-κB activity (p-p65), DNA damage (γ-H2A.X), and CD40L in H460 xenograft tumors. Tumor tissues in the treatment groups exhibited greater accumulation of CD40L (Fig. 6A), p-p65, and γ-H2A.X (Fig. 6C, D) and positive β-galactosidase in comparison to the DDP group and DDP+Vector group, respectively (Fig. 6B) In addition, tumor growth

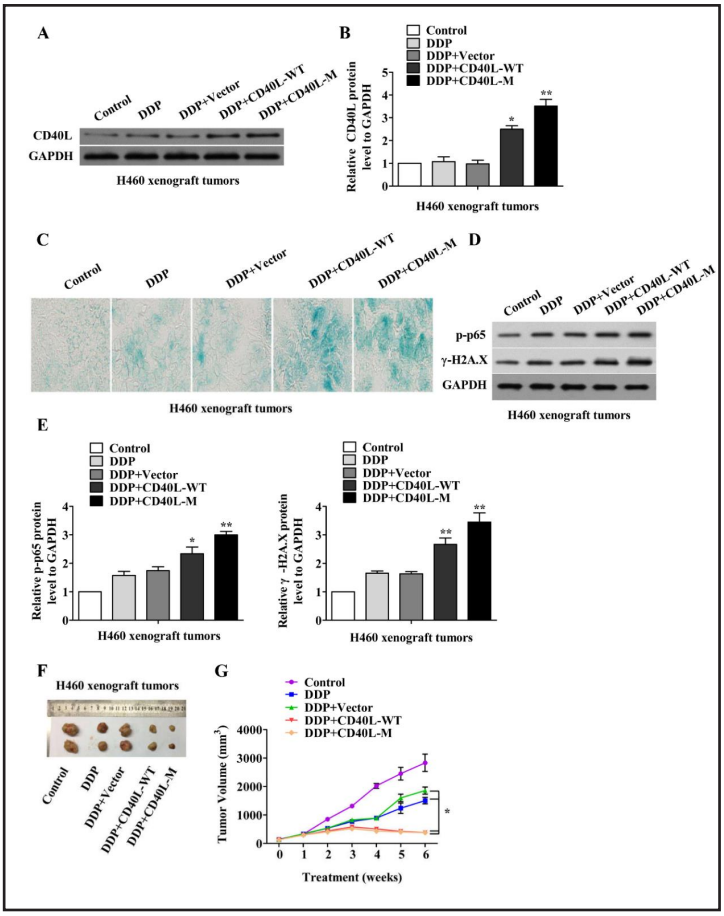

Fig. 6. Effects of csAAV5-M-CD40L-M in vivo. The experiment was performed by intratumoral injection with AAV5-M-GFP, AAV5-M-CD40L-WT, and AAV5-M-CD40L-M vectors (at 1 × 10^{11} vgs/mouse) weekly and following intravenous injection with cisplatin (2 ml/kg) every three days. A. The protein levels of CD40L in H460 xenograft tumors were evaluated by Western blotting. B. CD40L protein levels relative to GAPDH in vivo (median with 25th and 75th percentiles; Mann-Whitney rank sum test). C. SA-β-gal staining in H460 xenograft tumors (200 ×). D. The expression of p-p65 and γ-H2A.X was detected by Western blotting. E. p-p65 and γ-H2A.X protein levels relative to GAPDH in xenograft tumors (median with 25th and 75th percentiles; Mann-Whitney rank sum test). F. Tumor volume of each group after execution. G. Tumor growth curve. Values represent means ± SD of 6 independent experiments. * P<0.05 or ** P<0.01 versus DDP group, DDP + vector group.

was significantly suppressed in the mice receiving cisplatin or AAV5-M-CD40L/CD40L-M combined with cisplatin compared with the control group mice (Fig. 6E, F). The greatest tumor growth-inhibitory effect was observed in the combined treatment groups.

Discussion

Cellular senescence acts as a barrier against tumorigenesis. Overexpression of oncogenes and loss of tumor-suppressor genes triggers senescence in several *in vivo* tumor models where senescence is thought to play a fundamental role in restraining tumor growth and progression [5]. In the present work, we found that overexpression of CD40L-WT/CD40L-M in $CD40^+$ NSCLC cells increased SA-β-gal staining activity and inhibited DNA synthesis and cell proliferation. We demonstrated that senescent NSCLC cells secreted IL-6 and IL-8 *in vitro*, resulting in recruitment of dHL60 immune cells. Results also showed that CD40L-induced cellular senescence is partially dependent on the NF-κB signaling pathway, the most widely accepted downstream signal of CD40-CD40L molecules. Furthermore, our *in vivo* studies further verified the above results.

As opposed to a specific biomarker, senescent cells are characterized by the expression of a diversity of indicators that reveal irreversible proliferation arrest [14]. The DDR is a marker of senescence in some models. Many anti-neoplastic therapies typically rely on DNA damage, which engages potent DDR signaling pathways that culminate in apoptosis or growth arrest at checkpoints to allow for damage repair [15]. Some cells that escape apoptosis induced by chemotherapeutics and ionizing radiation can still undergo accelerated cellular senescence, and acquire the capability to bypass senescence, at a very low frequency. This innate ability of cancer cells to escape senescence has great significance for cancer recurrence and drug resistance [16]. There have been an increasing number of reports indicating that initiating a senescence program in solid tumors could be a potential therapeutic strategy to overcome drug resistance [17, 18]. A publication by Osman et al., has shown that senescence, rather than apoptosis, is the major mechanism of cisplatin-induced response in wild-type TP53 head and neck cancer cells, and that cisplatin resistance in TP53 null or high-risk mutant TP53 cells is due to a lack of senescence [17]. Further study by this group showed that Wee-1 kinase inhibition overcame the cisplatin resistance associated with high-risk TP53 mutations in head and neck cancers through mitotic arrest followed by senescence [18]. Consistent with prior studies, our data demonstrated that overexpression of CD40L-WT/CD40L-M promoted senescence, inhibited proliferation, increased DNA damage-associated γ-H2A.X, and enhanced chemotherapy sensitivity *in vitro* and *in vivo*.

New evidence supports a major role for cell-non-autonomous regulation of senescence in cancer. Senescent cells secrete a diversity of growth factors, cytokines, and proteases, known as SASP. Emerging data have revealed that NF-κB is the major signaling pathway that stimulates the appearance of SASP [19-22]. It is known that DNA damage induces NF-κB signaling via a variety of signaling complexes containing the essential NF-κB modifier NEMO, as well as via the activation of the p38MAPK [23] and MK2 [24] signaling pathways. Genomic instability evoked by cellular stress triggers epigenetic changes, e.g., the high mobility group box 2 (HMGB2) proteins which orchestrate the chromatin landscape of SASP gene loci [25], and upregulation of SIRT1 expression which deacetylates NF-κB to blunt its transcriptional activity [26]. Further, the two cyclin-dependent kinase inhibitors, p16INK4a and p14ARF, are effective inhibitors of NF-κB signaling. Here, we confirmed that CD40L mediated SASP in an NF-κB-dependent manner. SASP has been shown to modulate the recruitment and activation of the immune response at the tumor bed and in some cases, it may be pivotal for the clearance of senescent cells by phagocytosis [4]. Recent findings have revealed that tumor-infiltrating immune cell subsets can both induce senescence in the tumor by releasing pro-inflammatory cytokines, and clear senescent cancer cells [27]. Nevertheless, tumor-infiltrating myeloid cells have been shown to oppose senescence *in vivo* [5]. There may be heterogeneity of SASP as well as the resulting immune responses in a context-dependent manner.

It is widely accepted that in some tumor cells, CD40L transgene expression not only becomes visible to the antigen-presenting cells but also actively induces their eradication [9]. However, the cleaved product from membrane-bound CD40L, soluble CD40L, has been reported to enter systemic circulation and cause inflammatory diseases [28]. To reduce the adverse effects caused by sCD40L, we generated a human CD40L mutant (CD40L-M) that was not cleaved by MMPs and remained on the cell surface in a membrane-bound form. The CD40L-M mutant contains 6 substitutions which mimic the mouse CD40L to replace the cleaved sites [10]. Here, we further explored the multi-effect functions of the CD40L mutant. To deliver the CD40L gene efficiently, we previously evaluated the efficiency of transduction of different serotypes of AAV vectors in A549 cells and compared the transduction efficiency of a conventional AAV vector with that of scAAV vectors as well. These studies suggested that recombinant scAAV5 may provide an effective form of therapy for lung cancer [10]. In the present study, the site-directed mutagenesis of surface-exposed threonine (T) residues of scAAV5 was used as a potential vector because this optimization of surface-exposed tyrosine residues led to a marked enhancement of transduction both *in vitro* and *in vivo* [11].

In conclusion, these data indicate that membrane-bound CD40L mutant may promote senescence and initiate the SASP of NSCLC cells in an NF-κB-dependent manner. Therefore, CD40L-M-induced senescence may be a potential approach to protect against lung adenocarcinoma.

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Disclosure Statement

The authors declare that no conflict of interests exists.

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