Cancer Science
Japanese Cancer Association JCA
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Interleukin 6 augments lung cancer chemotherapeutic resistance via ataxia-telangiectasia mutated/NF-kappaB pathway activation

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Key words

Ataxia-telangiectasia mutated, chemotherapy, interleukin 6, multiple drug resistance, NF-kappaB

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Funding information

State Key Laboratory of Oncogenes and Related Genes (90-14-05). National Natural Science Foundation of China (81273203).

Received March 10, 2014; Revised June 13, 2014; Accepted June 27, 2014

Cancer Sci **105** (2014) 1220–1227

doi: 10.1111/cas.12478

Although it is known that ataxia-telangiectasia mutated (ATM) and interleukin 6 (IL-6) contribute to multiple drug resistance (MDR) in tumor chemotherapy, the exact role of ATM activation in MDR resulting from increased IL-6 expression is still unclear. In the present study, we demonstrate that the activation of the ATM-NF-kappaB pathway, resulting from increased IL-6 expression, plays a central role in augmented chemoresistance in lung cancer cell lines. This result was supported by the increased expressions of Bcl-2, Mcl-1, Bcl-xl, and the upregulation of MDR-associated protein ABCG2. The higher level of IL-6 reveals not only higher ATM/NF-kappaB activity but also increased expressions of ABCG2, Bcl-2, Mcl-1 and Bcl-xl. Most importantly, lung cancer cells themselves upregulated IL-6 secretion by activating the p38/NF-kappaB pathway through treatment with cisplatin and camptothecin. Taken together, these findings demonstrate that chemotherapeutic agents increase IL-6 expression, hence activating the ATM /NF-kappaB pathway, augmenting anti-apoptotic protein expression and contributing to MDR. This indicates that both IL-6 and ATM are potential targets for the treatment of chemotherapeutic resistance in lung cancer.

Chemotherapeutic resistance is closely associated with multidrug resistance (MDR). Although a relatively good response can be achieved in the initial stages of lung cancer chemotherapy, chemoresistance can develop quickly after initial chemotherapy, which clearly affects patients' survival.[1–3] Hence, chemotherapeutic resistance, especially MDR, is an important issue for chemotherapeutic failure and remains a challenge in the clinical treatment of lung cancer. Proinflammatory cytokines, such as interleukin 6 (IL-6), which are usually secreted by immune cells, have also been documented to be expressed by osteosarcoma, ovarian cancer cells and head and neck squamous cell carcinoma cells.[4–6] Further studies reveal that an elevated IL-6 level has a close relationship with poor clinical outcome of advanced lung cancer patients, indicating that IL-6 contributes to chemotherapeutic resistance in lung cancer.[7–9] Meanwhile, treatment with IL-6 reveals anti-apoptotic effects and promotes MDR.[6,10,11] However, until now, few studies have explored the role of IL-6 in chemotherapeutic agents-induced MDR.

The activation of protein kinase ataxia-telangiectasia mutated (ATM) is reported to be involved in DNA damage response and cell cycle checkpoints,[12] to increase MDR-associated protein expression, and to contribute to chemoresistance.[13,14] Treatment with chemotherapeutic agents triggers the phosphorylation of ATM and the export of IKK-gamma, which initiates the activation of TAK1-IKK-NF-kappaB.[15] The activation of ATM induces Ubc13-mediated TRAF6 polyubiquitin, promotes TAB 2-dependent TAK1 phosphorylation and increases the nucleus translocation of p65, indicating that ATM is the upstream kinase of the NF-kappaB pathway.[16,17] While ATM could be activated by chemotherapeutic agents-induced DNA double strand breaks (DSB),[12–14] the phosphorylation of ATM is clearly increased by treatment with hypoxia without apparent DNA damage.[18–20] Cisplatin treatment has been demonstrated to increase proinflammatory cytokines release,[21] and NF-kappaB activation initiates anti-apoptotic protein expression, augments proinflammatory cytokine secretion and contributes to MDR.[22] Chemotherapeutic agent-induced MDR raises the question of whether IL-6 secretion could be increased by treatment with chemotherapeutic agents, which, in turn, activate the ATM/NF-kappaB pathway, augment MDR-associated protein expression and contribute to MDR in lung cancer. However, little is known about the effect of ATM/NF-kappaB activation on IL-6-associated lung cancer chemotherapeutic resistance, which is clearly an important issue for treating lung cancer chemotherapeutic resistance.

The aim of the present study was to identify the functions of the ATM/NF-kappaB activation in IL-6-induced chemotherapeutic

Table 1. Primer sequence

Genes	F/R	Sequence
Beta-actin	F	5′-TCAAGATCATTGCTCCTCCTG-3′
Beta-actin	R	5′-CTGCTTGCTGATCCACATCTG-′
IL-6	F	5′-CCACACAGACAGCCACTCACC-3′
IL-6	R	5′-CTACATTTGCCGAAGAGCCCT-3′
ABCG2	F	5′-ACTGGCTTAGACTCAAGCACA-3′
ABCG2	R	5′-ATAGGCCTCACAGTGATAACCA-3′
Bcl-2	F	5′-GGAGGATTGTGGCCTTCTTT-3′
Bcl-2	R	5′-TCACTTGTGGCTCAGATAGGC-3′
Mcl-1	F	5′-TGCAGGTGTTGCTGGAGTAG-3′
Mcl-1	R	5′-CCTCTTGCCACTTGCTTTTC-3′
Bcl-xl	F	5′-GAGCTGGTGGTTGACTTTCTC-3′
Bcl-xl	R	5′-TCCATCTCCGATTCAGTCCCT-3′

resistance by studying changes in MDR-associated protein expression, and to examine their relationships with the ATM/NF-kappaB pathway activation. We found that IL-6 increased lung cancer cell chemotherapeutic resistance and enhanced the expressions of Bcl-2, Mcl-1, Bcl-xl and ABCG2 by activating the ATM/NF-kappaB pathway. The high level of IL-6 reveals not only higher activity of ATM/NF-kappaB but also increases the expression of ABCG2, Bcl-2, Mcl-1 and Bcl-xl. Importantly, treatment with cisplatin and camptothecin could increase IL-6 secretion in lung cancer cell lines by activating the p38/NF-kappaB pathway. These results indicate that both IL-6 and ATM are potential targets for the treatment of chemotherapeutic resistance in lung cancer.

Materials and Methods

Reagents. Recombinant Human IL-6 and Matched IL-6 Antibody Pairs were obtained from eBioscience (San Diego,

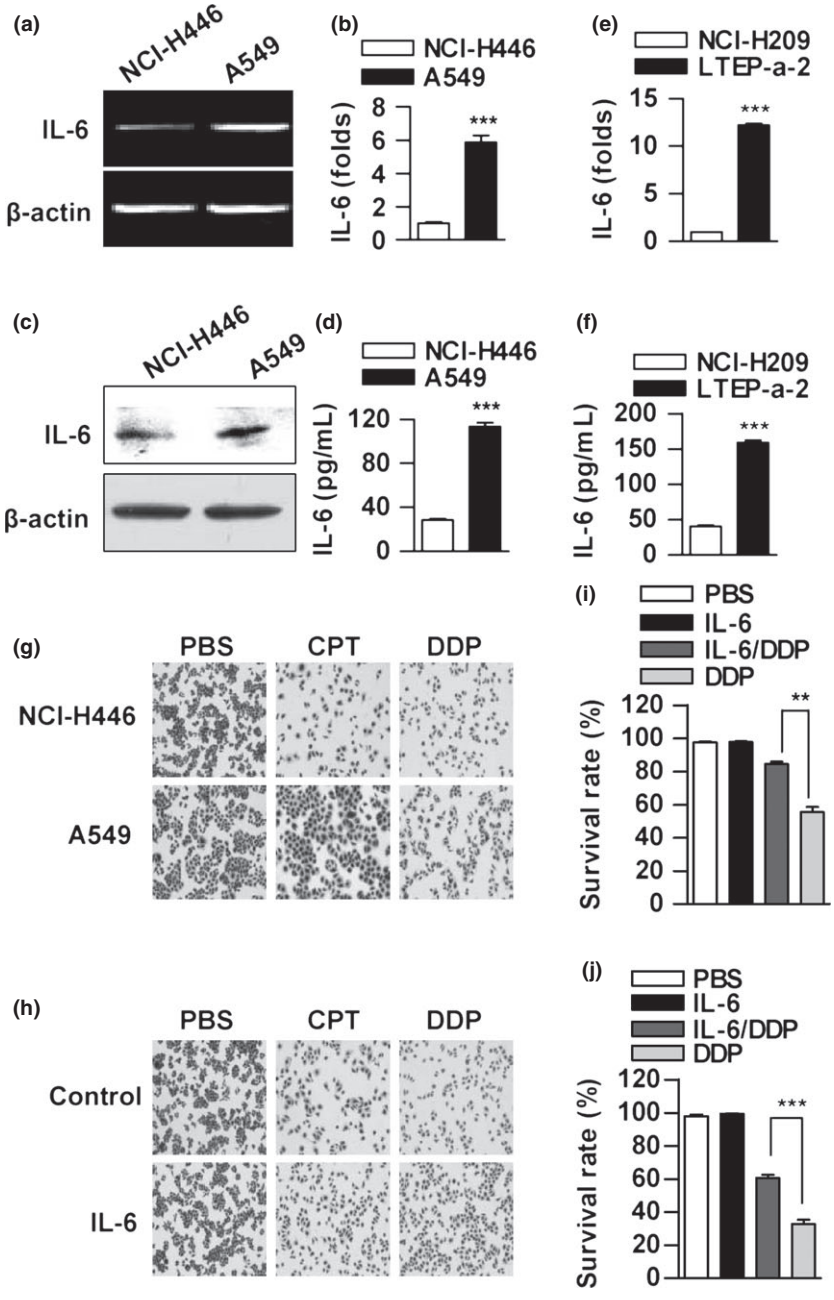

Fig. 1. Interleukin 6 (IL-6) treatment contributes to chemotherapeutic resistance in lung cancer cells. (a–f) The whole cellular RNA, protein and supernatant were prepared from cultured NCI-H446/A549 (a–d), NCI-H209/LTEP-a-2 (e, f) cells and the expression of IL-6 was determined by RT-PCR (a), real-time PCR (b,e), western blot (c) and ELISA (d, f) analyses. Beta-actin was used as an internal control. NCI-H446/A549 (g–i) and NCI-H209 cells (j) were pretreated with (2 ng/mL) IL-6 (h–j) or PBS (g) prior to cisplatin (DDP) (4 μg/mL) or camptothecin (CPT) (0.5 μg/mL) treatment and cell viabilities were determined by microscope (g, h), flow cytometry (i), and Trypan Blue cell count (j), analyses. The data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test or one-way ANOVA with the Newman–Keulspost test. One representative from three experiments is shown.

CA, USA). Camptothecin and cisplatin were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho and total kinase antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA). An Annexin V/PI Detection Kit was obtained from KeyGEN Biotech (Nanjing, China). DAPI was acquired from Vector Laboratories (Burlingame, CA, USA). The siRNA of IL-6, ATM, p65 and controls was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). RPMI-1640, DMEM and FBS were acquired from Hyclone (Logan, UT, USA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY, USA). SYBR Premix Ex Taq, Trizol and Prime-Script Reverse Transcriptase were obtained from TaKaRa Biotechnology (Dalian, China).

Cell lines. NCI-H446, NCI-H209, NCI-H1299, LTEP-a-2 cells and A549 cells, a kind gift from Professor GH Jin (Xiamen University), were grown in RPMI-1640 or DMEM media containing 10% FBS at 37°C under 5% CO_2. Cells were synchronized by serum starvation for at least 12 h before treatment.

Flow cytometric measurements. Cell apoptosis assay was assayed as described previously.[14] Briefly, cells were pretreated with IL-6 (2 ng/mL) for 5 h prior to cisplatin (4 μg/mL) treatment. Then, the cells were stained with Annexin V-FITC and propidium iodide for 20 min at room temperature. Flow cytometry was performed using a FACSCalibur Flow Cytometer, and the data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA).

ELISA. To determine the effect and mechanism of chemotherapeutic agent treatment on IL-6 release, 1×10^5 cells were treated with cisplatin or camptothecin for indicated periods, and the supernatant was collected. IL-6 concentration was determined via ELISA.[23]

Confocal immunofluorescence assays. The effects of IL-6 on ATM and p65 phosphorylation were investigated using immunofluorescence assays.[14] Briefly, cells were treated with IL-6 (2 ng/mL), fixed and permeabilized in 100% methanol. Then, the cells were blocked with 10% non-fat milk and incubated with primary antibodies overnight at 4°C, followed by staining with fluorescence-conjugated IgG (1:100 dilution). Images were captured using a confocal fluorescence microscope at 488 or 546 nm.

siRNA transfection. Cells plated at a density of 3×10^4 cells /well were transfected with relative siRNA using Lipofectamine 2000 (Invitrogen). The cells were collected for further experiments 48 h after transfection. The final concentration for each siRNA is 100 nM.

RT-PCR and quantitative PCR. The effects of IL-6 on MDR-related protein expression were investigated via RT-PCR and real-time PCR analyses, as described previously.[24] Briefly, whole cellular RNA was extracted, and reverse transcription was performed using PrimeScript Reverse Transcriptase (TaKaRa Biotechnology, Dalian, China). PCR amplification was conducted using the following conditions: 95°C for 3 min, followed by 30 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 60 s, and a final extension at 72°C for 10 min. Beta-actin was used as an internal control. To quantify gene amplification, real-time PCR analysis was performed using an ABI 7000 Sequence Detection System in the presence of SYBR Green (TaKaRa Biotechnology, Dalian, China). The cycling parameters were 95°C for 5 min, followed by 32 cycles of 95°C for 5 s, 55°C for 30 s and 72°C for 60 s, with a final extension at 72°C for 10 min; a melting curve analysis was subsequently conducted. The relative expression levels (defined as fold changes) of the target genes were normalized to the folds of the corresponding control cells. The primer sequences outlined in Table 1 were used in these assays.

Western blot analysis. The cells were treated with IL-6 (2 ng/mL) and the expression of related proteins was determined via western blot analysis.[14]

Statistical analysis. All experiments were repeated at least three times to confirm the results. The data are presented as the mean ± SEM. Student's *t*-test and one-way ANOVA with the Newman–Keulspost test were applied. Differences were considered significant at $P < 0.05$.

Results

Interleukin 6 treatment contributes to chemotherapeutic resistance in lung cancer cells. To explore the role of IL-6 in MDR

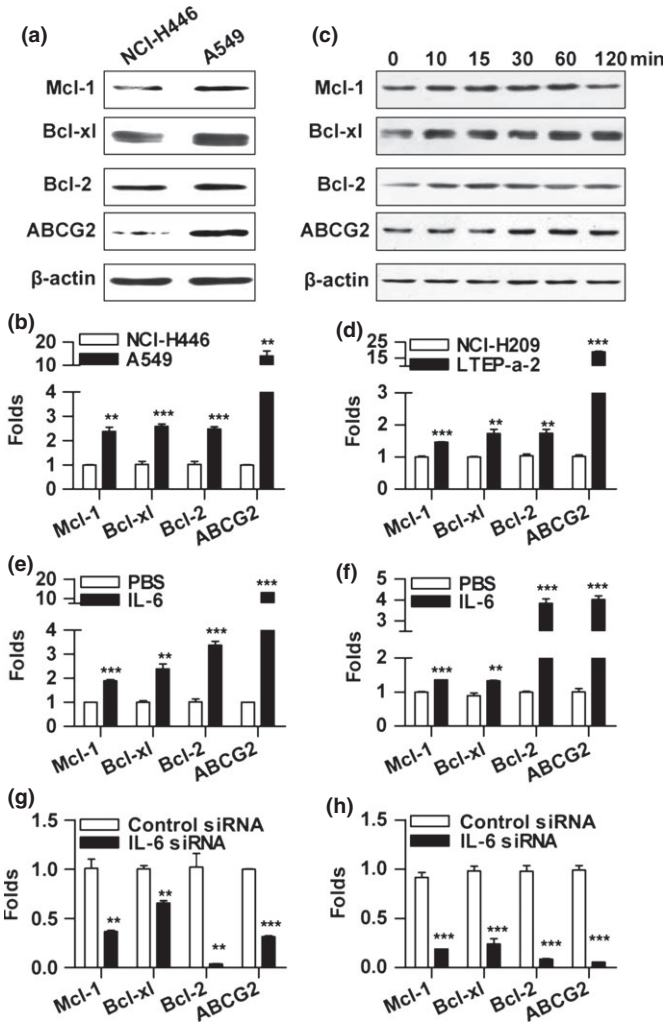

Fig. 2. Interleukin 6 (IL-6) increases ABCG2, Mcl-1, Bcl-xl and Bcl-2 expression in lung cancer cells. (a–d) The whole cellular protein, RNA, was extracted from NCI-H446/A549 (a, b) and NCI-H209/LTEP-a-2 (d) cells, and the expressions of ABCG2 and anti-apoptotic protein (Mcl-1, Bcl-xl and Bcl-2) were determined by western blot (a) and real-time PCR (b, d) analyses. NCI-H446 (c, e) or NCI-H209 (f) cells were treated with IL-6 (2 ng/mL) and the expressions of ABCG2, Mcl-1, Bcl-xl and Bcl-2 were determined by western blot (c) and real-time PCR (e, f) analyses. (g, h) A549 (g), LTEP-a-2 (h) cells were transfected with IL-6 or control siRNA and the expressions of ABCG2, Mcl-1, Bcl-xl and Bcl-2 were quantified by real-time PCR analyses. The data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t*-test. One representative from three experiments is shown. Beta-actin was used as the loading control.

in lung cancer, the relationship between IL-6 and lung cancer chemotherapeutic resistance was investigated. Compared with NCI-H446 and NCI-H209 cells, A549 (Fig. 1a–d) and LTEP-a-2 (Fig. 1e,f) cells clearly have higher IL-6 expression. Meanwhile, A549 (Fig. 1g) and LTEP-a-2 cells (Fig. S1a) have higher chemotherapeutic resistance to treatment with camptothecin and cisplatin. When IL-6 replenishment was performed, increased survival rates were achieved in NCI-H446 (Fig. 1i), NCI-H209 (Fig. 1j) and A549 cells (Fig. S1b). In addition, microscope observation confirmed that IL-6 replenishment increases cells' resistance to camptothecin or cisplatin treatment in both NCI-H446 (Fig. 1h) and NCI-H1299 cells (Fig. S1c–e). Taken together, these findings indicate that proinflammatory cytokines, such as IL-6, play an important role in lung cancer chemotherapeutic resistance.

Higher level of interleukin 6 promotes ABCG2, Mcl-1, Bcl-xl and Bcl-2 expression in lung cancer cells. To understand the role of IL-6 in lung cancer MDR, the relationships between IL-6 level and the expressions of Bcl-xl, Mcl-1, Bcl-2 and ABCG2 in lung cancer cells were investigated. Compared with NCI-H446 cells, A549 cells, which have higher IL-6 expression (Fig. 1a–d), clearly have higher expressions of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 at both translational (Fig. 2a) and transcriptional (Fig. 2b) levels. Transcriptional determination of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 in NCI-H209/LTEP-a-2 (Fig. 2d) or NCI-H1299/LTEP-a-2 cells (Fig. S2a) leads to similar conclusions. The IL-6 replenishment efficiently increased the expression of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 in NCI-H446 cells at both the protein (Fig. 2c) and RNA (Fig. 2e–f) levels in both NCI-H446 (Fig. 2c,e) and NCI-H209 cells (Fig. 2f). In addition, when IL-6 expression was knocked down by IL-6 siRNA transfection, the expressions of ABCG2, Bcl-xl, Mcl-1 and Bcl-2 were decreased in A549 (Fig. 2g), LTEP-a-2 cells (Fig. 2h) and NCI-H446 cells (Fig. S2b). Similar results were observed in the lung cancer cell line NCI-H1299 and (Fig. S2c). IL-6 administration obviously increased the Bcl-2 expression of adoptive transferred NCI-H446 cells and normal tissues (Fig. S2d). Taken together, these findings indicate that the level of IL-6 directly regulates ABCG2 and anti-apoptotic protein expression in lung cancer cells.

Interleukin 6 activates the ataxia-telangiectasia mutated and NF-kappaB pathways in lung cancer cells. Although ATM, which could be activated by DNA DSB, contributes to chemoresistance,[13,17,25] the phosphorylation of ATM can also be increased without apparent DNA damage.[18–20] Compared with NCI-H446 cells, A549 cells, which have a higher level of IL-6 (Fig. 1a–d), have higher levels of the phosphorylaed ATM and IκBα (Fig. 3a). The finding that IL-6 contributes to lung cancer MDR (Figs 1, 2) therefore raises the question of whether the ATM/NF-kappaB pathway could be activated by IL-6 treatment. Our results revealed that the phosphorylation of ATM at Ser1981 was clearly increased following IL-6 treatment (Fig. 3b), as were the levels of IkappaBalpha and p65 phosphorylation (Fig. 3b). The confocal microscopy results led to a similar conclusion that IL-6 treatment induces ATM and NF-kappaB activation in both NCI-H446 (Fig. 3c) and NCI-H1299 cells (Fig. 3d). Taken together, these findings indicate that IL-6 activates the ATM and NF-kappaB pathways in lung cancer cells.

Inhibition of ataxia-telangiectasia mutated and NF-kappaB activation abrogates the effects of interleukin 6 on ABCG2 and anti-apoptotic protein upregulation. To understand the role of ATM and NF-kappaB activation in augmented MDR through increased IL-6, inhibitors were used to inhibit related kinase activities. These inhibitors were invariably able to eliminate ATM or NF-kappaB kinase activity (Fig 4a,b, Fig. S3a). The inhibition of ATM or NF-kappaB activity clearly abrogated the effect of IL-6 on ABCG2 and anti-apoptotic protein

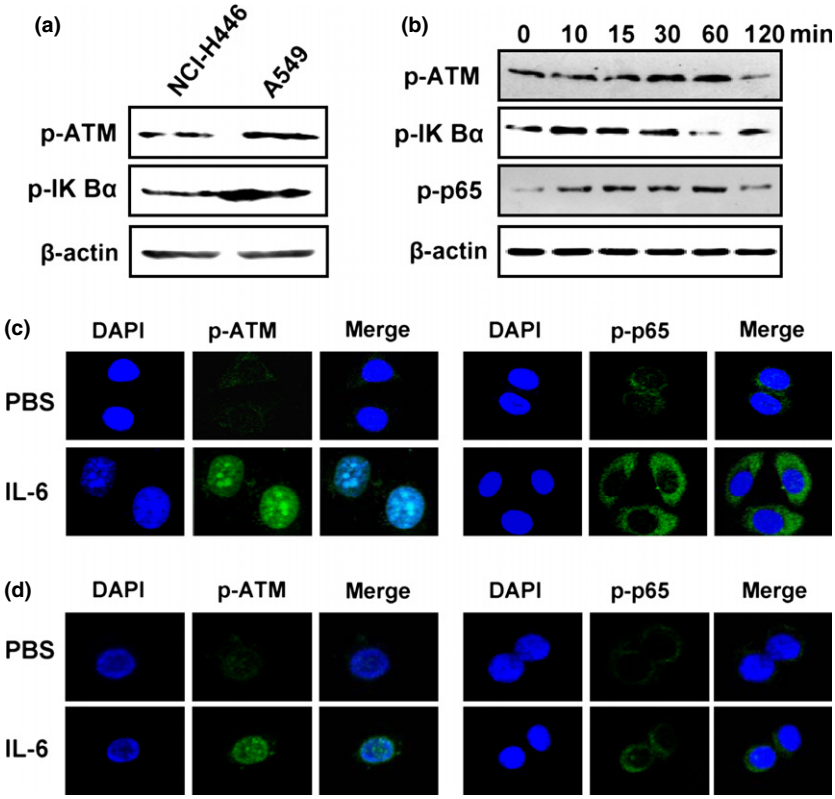

Fig. 3. Interleukin 6 (IL-6) activates the ataxia-telangiectasia mutated (ATM) and NF-kappaB pathways in lung cancer cells. (a) The whole cellular protein was extracted from NCI-H446 and A549 cells and the phosphorylation of ATM and IκBα was determined by western blot analyses. (b–d) NCI-H446 and NCI-H1299 cells were exposed to IL-6 (2 ng/mL). Whole cellular protein were extracted, and ATM, p65 and IκBα phosphorylation were determined by western blot (b) and confocal microscopy analyses (c, d). Beta-actin was used as an internal control. One representative from three experiments is shown.

expression at both the translational (Fig. 4c) and transcriptional (Fig. 4d) levels in NCI-H446 cells. Meanwhile, the ATM and p65 knockdown results of NCI-H446 cells led to a similar conclusion (Fig. 4e). Similar results were observed in the lung cancer cell line NCI-H209 (Fig. 4f), NCI-H1299 (Fig. S3b) and LTEP-a-2 cells (Fig. S3c). Taken together, the observation that inhibition of ATM or NF-kappaB activity eliminated the effect of IL-6 on ABCG2 and anti-apoptotic protein expression indicates that the ATM and NF-kappaB pathways are involved in IL-6-mediated MDR in lung cancer cells.

Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on interleukin 6 upregulation in lung cancer cells. It has been documented that treatment with chemotherapy increases IL-6 levels in advanced cancer patients.[7,8] To elucidate the mechanism of chemotherapy upregulating IL-6 expression, lung cancer NCI-H446 (Fig. 5a–d), NCI-H209 (Fig. 5e,f) and NCI-H1299 (Fig. S4a,b) cells were treated with camptothecin or cisplatin, and the expression of IL-6 was analyzed. The treatment with cisplatin (Fig. 5a,c,e) and camptothecin (Fig. 5b,d,f) increased IL-6 expression. The inhibition of p38 or NF-kappaB activity clearly abrogated the effects of cisplatin and camptothecin on IL-6 release at the translational levels (Fig. 5g,h) in NCI-H446 cells. Meanwhile, the p38 and p65 knockdown results of NCI-H446 cells led to a

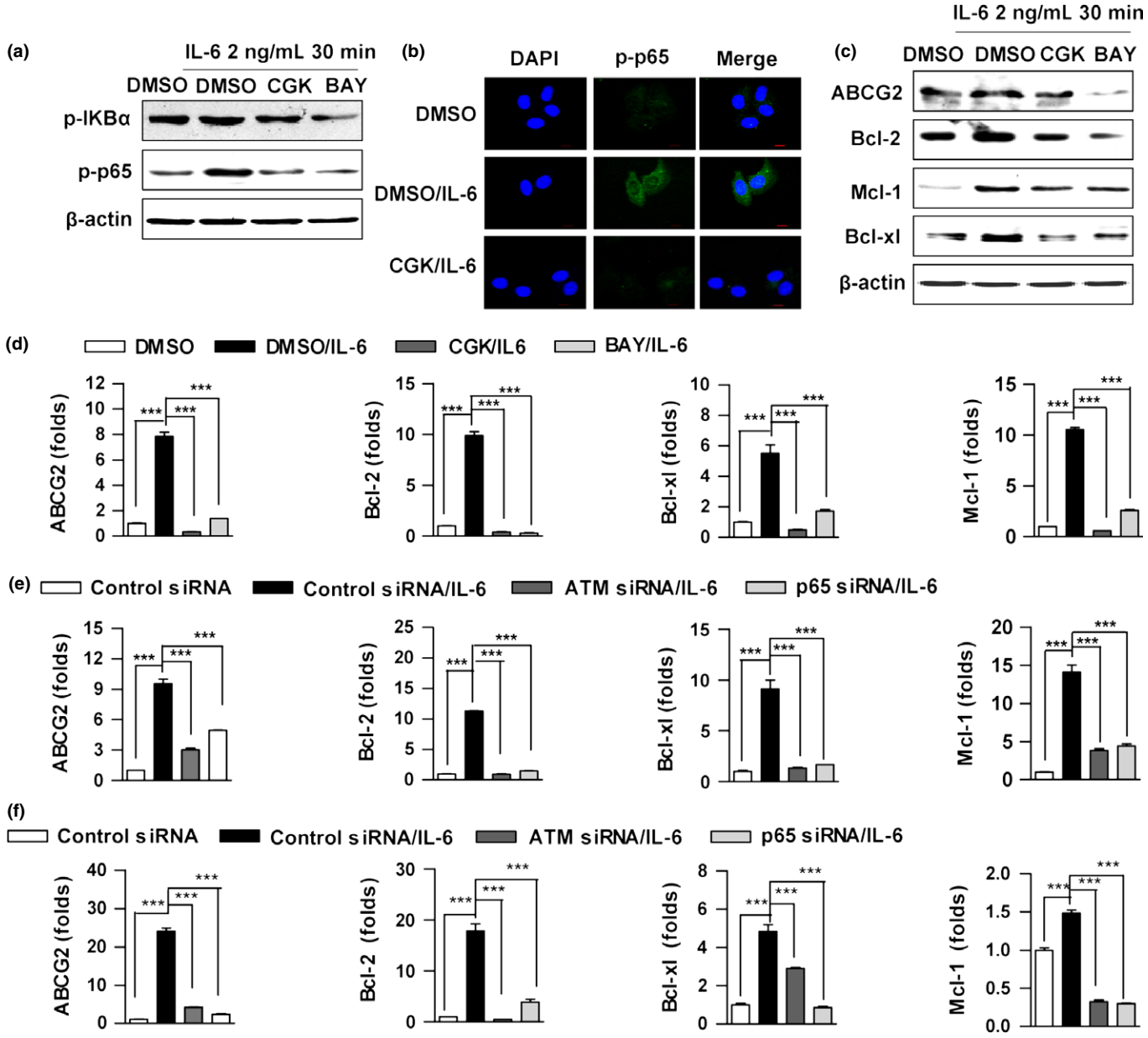

Fig. 4. Inhibition of ataxia-telangiectasia mutated (ATM) and NF-kappaB activation abrogates the effects of interleukin 6 (IL-6) on ABCG2 and anti-apoptotic protein upregulation. (a, b) NCI-H446 cells were pretreated with 20 μM CGK733 prior to IL-6 (2 ng/mL) treatment. The role of ATM phosphorylation in IL-6 induced NF-kappaB activation was determined by western blot (a) and confocal microscopy assays (b). Bay11-7082 was used as a negative control. (c–f) NCI-H446 (c–e) and NCI-H209 (f) cells were pretreated with 20 μM CGK733/Bay11-7082 (c, d) or ATM/p65 siRNA transfection (e, f) prior to IL-6 (2 ng/mL) treatment. The roles of ATM and NF-kappaB activation in IL-6 increased the expressions of ABCG2 and anti-apoptotic protein (Mcl-1, Bcl-xl and Bcl-2), which were determined via western blot (c) and real-time PCR assays (d–f). The data are presented as the mean ± SEM. ***$P < 0.001$, one-way ANOVA with the Newman–Keulspost test. One representative from three experiments is shown. Beta-actin was used as the loading control.

similar conclusion (Fig. 5i,j). Similar results were observed in the lung cancer cell line NCI-H209 (Fig. 5k,l) and NCI-H1299 cells (Fig. S4c,d). Taken together, the observation that inhibition of p38 or NF-kappaB activity eliminated the effects of cisplatin and camptothecin on IL-6 expression indicates that the p38 and NF-kappaB pathways are involved in chemotherapeutics agents-induced IL-6 expression, which, in turn, contributes to MDR in lung cancer.

Discussion

Chemotherapeutic agents can not only induce tumor cell apoptosis but also trigger inflammation in the tumor microenvironment.[26] Proinflammatory factors, such as IL-2, IL-7 and IL-6, are documented to be involved in MDR in head and neck squamous cells, glioma cells, prostate cancer, lung cancer and breast cancer.[10,27–30] Meanwhile, a higher level of circulating IL-6, increased by chemotherapeutic agent treatment, is associated with patients' survival in gastric carcinoma, prostate cancer, breast cancer and lung cancer,[7,8,31–33] indicating that MDR is influenced by the level of IL-6.[25,27,34] Hence, despite the higher level of vascular endothelial growth factor contributing to the upregulation of anti-apoptotic protein and ABCG2 (Fig. S5), IL-6 but not other proinflammatory cytokines was selected to be studied in this project. The present study demonstrated that a higher level of IL-6 upregulates ABCG2 and anti-apoptotic protein expression and augments chemotherapeutic resistance in lung cancer cells (Figs 1, 2). Moreover, inhibition of ATM-NF-kappaB activities abrogates the effect of IL-6 on ABCG2 and anti-apoptotic protein expression (Fig. 4), indicating that IL-6 and the ATM-NF-kappaB pathway are potential therapeutic targets in inflammation-associated lung cancer chemotherapeutic resistance. As tumor tissues are composed of tumor cells, fibroblasts and immune cells, the finding that fibroblasts and immune cells synthesize and secrete IL-6 [35,36] cannot exclude the possibility that other components of lung cancer tissues might contribute to the elevation of serum IL-6. In the present study, cancer cells were shown to secrete IL-6 under chemotherapeutic agent treatment conditions, indicating that IL-6 promotes MDR in both a paracrine and autocrine manner in lung cancer.

Ataxia-telangiectasia mutated, a nuclear serine-threonine kinase involved in DNA DSB repair and MDR, can be activated by chemotherapeutic drugs.[13,14] The phosphorylation of ATM can also be increased by hypoxia treatment without apparent DNA damage.[18,19] The present study demonstrated that IL-6 treatment clearly increases ATM phosphorylation (Fig. 3) without cell apoptosis (Fig. 1g), indicating that IL-6 activates ATM kinase in DNA damage in an independent manner. In the present study, both inhibition of ATM-NF-kappaB activities and gene silence eliminate the effect of IL-6 on ABCG2 and anti-apoptotic protein expression (Fig. 4), indicating that IL-6 and the ATM-NF-kappaB pathway are potential therapeutic targets for lung cancer chemotherapy. We also notice that constitutive activation of signal transducer and activator of transcription 3 (STAT3) upregulate P-gp expression and augment chemotherapeutic resistance.[37,38] Kim *et al.*[39] also found that afatinib activates IL-6R/JAK1

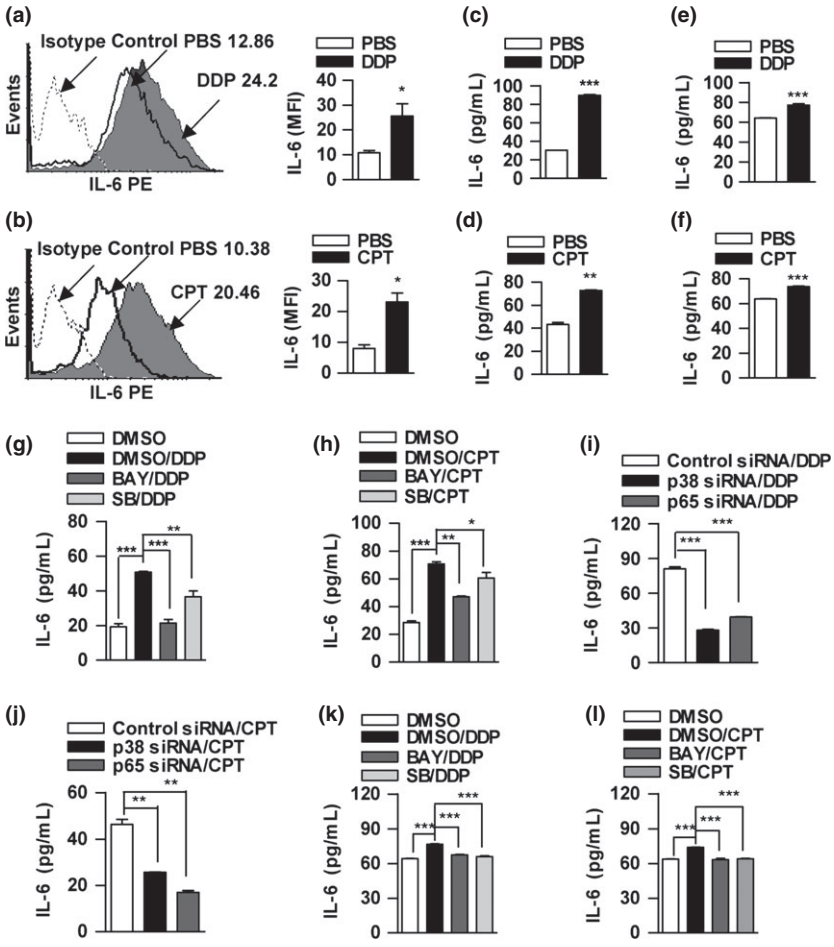

Fig. 5. Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on interleukin 6 (IL-6) upregulation in lung cancer cells. (a–f) NCI-H446 (a–d) and NCI-H209 cells (e, f) were exposed to 0.5 μg/mL cisplatin (DDP) or camptothecin (CPT) and IL-6 expression was determined by flow cytometry (a, b) and ELISA analyses (c–f), respectively. For the flow cytometry analysis, the numbers in the histogram indicate the geometric mean fluorescence (MFI) of the tested samples. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Student *t*-test. (g–l) NCI-H446 (g–j) and NCI-H209 cells (k, l) were pretreated with 20 μM inhibitor of SB203580 (20 μM)/Bay 11-7082 (20 μM) (g, h, k, l) or p38/p65 siRNA transfection (i, j) prior to cisplatin or camptothecin treatment. The roles of p38 and NF-kappaB activation in cisplatin or camptothecin-increased IL-6 expression were determined by ELISA analyses. Data are given as mean ± SEM, $n = 3$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, one-way ANOVA with post Newman–Keuls test. The data are presented as the mean ± SEM, *$P < 0.05$, **$P < 0.01$, one-way ANOVA with the Newman–Keulspost test. A representative of three independent experiments is shown.

/STAT3 signaling via autocrine IL-6 secretion. As IL-6 treatment clearly increases Jak2-Stat3 (Fig. S6) activation and the ATM phosphorylation is involved in Stat3 activation,[40] the exact interactions of ATM and Stat3 in IL-6 promoted chemotherapeutic resistance requires further exploration.

The treatment with chemotherapeutic agents triggers the export of ATM and sumoylated NEMO (IKK-γ), which activates XIAP-containing and ELKS-containing signalosome formation and results in TAK1-IKK-NF-kappaB activation.[15] In contrast, activated ATM induces Ubc13-mediated TRAF6 polyubiquitin, promotes recruitment of cIAP1, TAK1/TAB 2 and IKK through TRAF-binding motif.[22] The module of ATM-TRAF6-cIAP1 then stimulates TAB 2-dependent TAK1 phosphorylation, induces IKK activation and promotes the translocation of NF-kappaB (p65/p50) heterodimer to initiate target genes' transcription,[16,17] indicating that NF-kappaB, as a coordinator of inflammation and immune response, also plays a pivotal role in chemotherapeutic resistance.[11,41] In the present study, IL-6 treatment clearly activates ATM kinase (Fig. 3) without obvious cell apoptosis (Fig. 1g). Hence, the exact roles of XIAP and TRAF6 in NF-kappaB pathway activation as a result of increased IL-6 requires further exploration.

Tumor tissues are composed of tumor cells, fibroblasts and immune cells, and the finding that chemotherapeutic agent treatments such as cisplatin or camptothecin increase lung cancer cell synthesizing and secreting IL-6 (Fig. 5) cannot exclude the possibility that other components of lung cancer tissues might also contribute to the elevation of serum IL-6. As fibroblasts, endothelial cells and B cells express higher levels of IL-6 in LPS or afatinib treated patients,[39,42,43] the exact effects of chemotherapeutic agents on IL-6 expression of fibroblasts, endothelial cells and immune cells require further clarification.

Cancer stem cells are different from common cancer cells due to their ability to produce tumors and resist chemoradiation.[44] Apart from ABCB1,[45] ABCG2, CD44 and CD133 were recently recognized as lung cancer stem cell markers.[46–48] The present study reveals that IL-6 treatment increases ABCG2 expression at both the translational and transcriptional levels (Fig. 4), and contributes to chemotherapeutic resistance (Fig. 1), indicating that IL-6 treatment facilitates lung cancer cells acquiring cancer stem-like phenotypes. The exact effects and mechanism of IL-6 on lung cancer stem cell marker expression requires further investigation.

Taken together, our experiments reveal for the first time that IL-6 increases ABCG2, anti-apoptotic protein expression, and contributes to IL-6 augmented lung cancer chemotherapeutic resistance by activating the ATM and NF-kappaB pathways, indicating that ATM and IL-6 are potential targets for managing lung cancer chemotherapeutic resistance.

Acknowledgments

This work was supported by grants from the State Key Laboratory of Oncogenes and Related Genes (No. 90-14-05) and by grants from the National Natural Science Foundation of China (No. 81273203). F. G. Gao designed the research and copy-edited the paper. X. B. Huang conducted the western blot and flow cytometry analyses. H. Q. Yan conducted the western blot, ELISA and RT-PCR and real-time PCR analyses; S. Z. Ke and Y. N. Jiang conducted the confocal microscope assay; Y. N. Wang conducted the flow cytometry analyses and J. Li wrote the manuscript.

Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. *CA Cancer J Clin* 2010; **60**: 277–300.
2 Rodriguez E, Lilenbaum RC. Small cell lung cancer: past, present, and future. *Curr Oncol Rep* 2010; **12**: 327–34.
3 Jackman DM, Johnson BE. Small-cell lung cancer. *Lancet* 2005; **366**: 1385–96.
4 Wang TH, Chan YH, Chen CW *et al.* Paclitaxel (Taxol) upregulates expression of functional interleukin-6 in human ovarian cancer cells through multiple signaling pathways. *Oncogene* 2006; **25**: 4857–66.
5 Poth KJ, Guminski AD, Thomas GP *et al.* Cisplatin treatment induces a transient increase in tumorigenic potential associated with high Interleukin-6 expression in head and neck squamous cell carcinoma. *Mol Cancer Ther* 2010; **9**: 2430–9.
6 Duan Z, Lamendola DE, Penson RT, Kronish KM, Seiden MV. Overexpression of IL-6 but not IL-8 increases paclitaxel resistance of U-2OS human osteosarcoma cells. *Cytokine* 2002; **17**: 234–42.
7 Chang CH, Hsiao CF, Yeh YM *et al.* Circulating interleukin-6 level is a prognostic marker for survival in advanced non-small cell lung cancer patients treated with chemotherapy. *Int J Cancer* 2013; **132**: 1977–85.
8 Wojcik E, Jakubowice J, Skotnicki P *et al.* IL-6 and VEGF in small cell lung cancer patients. *Anticancer Res* 2010; **30**: 1773–8.
9 Nikiteas NI, Tzanakis N, Gazouli M *et al.* Serum IL-6, TNFalpha and CRP levels in Greek colorectal cancer patients: prognostic implications. *World J Gastroenterol* 2005; **11**: 1639–43.
10 Lee SO, Lou W, Johnson CS, Trump DL, Gao AC. Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. *Prostate* 2004; **60**: 178–86.
11 Domenech DJ, Oliva C, Rovira A *et al.* Interleukin 6, a nuclear factor-κB target, predicts resistance to docetaxel inhormone-independent prostate cancer and nuclear docetaxel antitumor activity. *Clin Cancer Res* 2006; **12**: 5578–86.
12 Shiloh Y, Ziv Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* 2013; **14**: 197–210.
13 Svirnovski AI, Serhiyenka TF, Kustanovich AM *et al.* DNA-PK, ATM and MDR proteins inhibitors in overcoming fludarabine resistance in CLL cells. *Exp Oncol* 2010; **32**: 258–62.
14 Ke SZ, Ni XY, Zhang YH, Wang YN, Wu B, Gao FG. Camptothecin and cisplatin upregulate ABCG2 and MRP2 expression by activating the ATM /NF-κB pathway in lung cancer cells. *Int J Oncol* 2013; **4**: 1289–96.
15 Wu ZH, Wong ET, Shi Y *et al.* ATM- and NEMO-dependent ELKS ubiquitination coordinates TAK1-mediated IKK activation in response to genotoxic stress. *Mol Cell* 2010; **40**: 75–86.
16 Miyamoto S. Nuclear initiated NF-kappaB signaling: NEMO and ATM take center stage. *Cell Res* 2011; **21**: 116–30.
17 Hinz M, Stilmann M, Arslan SC, Khanna KK, Dittmar G, Scheidereit C. A cytoplasmic ATM-TRAF6-cIAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-kB activation. *Mol Cell* 2010; **40**: 63–74.
18 Hammond EM, Kaufmann MR, Giaccia AJ. Oxygen sensing and the DNA-damage response. *Curr Opin Cell Biol* 2007; **19**: 680–4.
19 Bencokova Z, Kaufmann MR, Pires IM, Lecane PS, Giaccia AJ, Hammond EM. ATM activation and signaling under hypoxic conditions. *Mol Cell Biol* 2009; **29**: 526–37.
20 Olcina M, Lecane PS, Hammond EM. Targeting hypoxic cells through the DNA damage response. *Clin Cancer Res* 2010; **16**: 5624–9.
21 Kim HJ, Oh GS, Lee JH *et al.* Cisplatin ototoxicity involves cytokines and STAT6 signaling network. *Cell Res* 2011; **21**: 944–56.
22 Hadian K, Krappmann D. Signals from the nucleus: activation of NF-κB by cytosolic ATM in the DNA damage response. *Sci Signal* 2011; **4**: pe2. [doi: 10.1126/scisignal.2001712].
23 Jin HJ, Sui HX, Wang YN, Gao FG. Nicotine up-regulated 4-1BBL expression by activating Mek-PI3K pathway augments the efficacy of bone marrow-derived dendritic cell vaccination. *J Clin Immunol* 2013; **33**: 246–54.
24 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 2001; **25**: 402–8.
25 Heo JI, Oh SJ, Kho YJ *et al.* ATM mediates interdependent activation of p53 and ERK through formation of a ternary complex with p-p53 and p-ERK in response to DNA damage. *Mol Biol Rep* 2012; **39**: 8007–14.
26 Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010; **140**: 883–99.

27 Yao Z, Fenoglio S, Gao DC *et al.* TGF-beta IL-6 axis mediates selective and adaptive mechanisms of resistance to molecular targeted therapy in lung cancer. *Proc Natl Acad Sci USA* 2010; **107**: 15535–40.
28 Li D, Ronson B, Guo M *et al.* Interleukin 2 gene transfer prevents NKG2D suppression and enhances antitumor efficacy in combination with cisplatin for head and neck squamous cell cancer. *Cancer Res* 2002; **62**: 4023–8.
29 Cui L, Fu J, Pang JC *et al.* Overexpression of IL-7 enhances cisplatin resistance in glioma. *Cancer Biol Ther* 2012; **13**: 496–503.
30 Conze D, Weiss L, Regen PS *et al.* Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. *Cancer Res* 2001; **61**: 8851–8.
31 Liao WC, Lin JT, Wu CY *et al.* Serum interleukin-6 level but not genotype predicts survival after resection in stages II and III gastric carcimoma. *Clin Cancer Res* 2008; **14**: 428–34.
32 Nakashima J, Tachibana M, Horiguchi Y *et al.* Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin Cancer Res* 2000; **6**: 2702–6.
33 Salgado R, Junius S, Benoy I *et al.* Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. *Int J Cancer* 2003; **103**: 642–6.
34 Ammit AJ, Moir LM, Oliver BG *et al.* Effect of IL-6 trans-signaling on the pro-remodeling phenotype of airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2007; **292**: L199–206.
35 Lowe JM, Menendez D, Bushel PR *et al.* p53 and NF-κB coregulate proinflammatory gene responses in human macrophages. *Cancer Res* 2014; **74**: 2182–92.
36 O'Reilly S, Ciechomska M, Cant R, van Laar JM. Interleukin-6 (IL-6) trans signaling drives a STAT3-dependent pathway that leads to hyperactive transforming growth factor-β (TGF-β) signaling promoting SMAD3 activation and fibrosis via Gremlin protein. *J Biol Chem* 2014; **289**: 9952–60.
37 Zhang X, Xiao W, Wang L, Tian Z, Zhang J. Deactivation of signal transducer and activator of transcription 3 reverses chemotherapeutics resistance of leukemia cells via down-regulating P-gp. *PLoS ONE* 2011; **6**: e20965.
38 Grandis JR, Drenning SD, Zeng Q *et al.* Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. *Proc Natl Acad Sci USA* 2000; **97**: 4227–32.
39 Kim SM, Kwon OJ, Hong YK, Kim JH, Solca F *et al.* Activation of IL-6R/JAK1/STAT3 signaling induces de novo resistance to irreversible EGFR inhibitors in non-small cell lung cancer with T790M resistance mutation. *Mol Cancer Ther* 2012; **11**: 2254–64.
40 Zhang Y, Cho YY, Petersen BL *et al.* Ataxia telangiectasia mutated proteins, MAPKs, and RSK2 are involved in the phosphorylation of STAT3. *J Biol Chem* 2003; **278**: 12650–9.
41 Wang X, Jiang CM, Wan HY *et al.* CDA-2, a urinary preparation, inhibits lung cancer development through the suppression of NF-kappaB activation in myeloid cell. *PLoS ONE* 2012; **7**: e52117.
42 Chen WS, Xu XL, Bai L *et al.* Low-dose gamma-irradiation inhibits IL-6 secretion from human lung fibroblasts that promotes bronchial epithelial cell transformation by cigarette-smoke carcinogen. *Carcinogenesis* 2012; **33**: 1368–74.
43 Jin JF, Sundararaj KP, Samuvel DJ *et al.* Different signaling mechanisms regulating IL-6 expression by LPS between gingival fibroblasts and mononuclear cells: seeking the common target. *Clin Immunol* 2012; **143**: 188–99.
44 Chen J, Wang J, Chen D *et al.* Evaluation of characteristics of CD44+CD117+ ovarian cancer stem cells in three dimensional basement membrane extract scaffold versus two dimensional monocultures. *BMC Cell Biol* 2013; **14**: 7.
45 Grimm M, Krimmel M, Polligkeit J *et al.* ABCB5 expression and cancer stem cell hypothesis in oral squamous cell carcinoma. *Eur J Cancer* 2012; **48**: 3186–97.
46 Shi Y, Liu C, Liu X, Tang DG, Wang J. The microRNA miR-34a inhibits non-small cell lung cancer (NSCLC) growth and the CD44hi stem-like NSCLC cells. *PLoS ONE* 2014; **9**: e90022.
47 Sarvi S, Mackinnon AC, Avlonitis N *et al.* CD133+ cancer stem-like cells in small cell lung cancer are highly tumorigenic and chemoresistant but sensitive to a novel neuropeptide antagonist. *Cancer Res* 2014; **74**: 1554–65.
48 Niu Q, Wang W, Li Y *et al.* Low molecular weight heparin ablates lung cancer cisplatin-resistance by inducing proteasome-mediated ABCG2 protein degradation. *PLoS ONE* 2012; **7**: e41035.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. IL-6 treatment contributes to chemotherapeutics resistance in lung cancer cells.

Fig. S2. IL-6 increases ABCG2, Mcl-1, Bcl-xl and Bcl-2 expression in lung cancer NCI-H1299 and NCI-H446 cells.

Fig. S3. Inhibition of ATM and NF-kappaB activation abrogates the effects of IL-6 on ABCG2 and anti-apoptotic protein up-regulation in NCI-H1299 and LTEP-a-2 cells.

Fig. S4. Inhibition of p38 and NF-kappaB activation abrogates the effects of chemotherapeutic agents on IL-6 up-regulation in NCI-H1299 cells.

Fig. S5. Inhibition of ATM and NF-kappaB activation abrogates the effects of VEGF on ABCG2 and anti-apoptotic protein up-regulation.

Fig. S6. IL-6 induces Jak2 and Stat3 phosphorylation.