MyD88 expression is associated with paclitaxel resistance in lung cancer A549 cells

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Abstract. The purpose of the present study was to investigate the relationship between myeloid differentiation primary response gene 88 (MyD88) expression and the resistance to paclitaxel of A549 lung cancer cells. In order to achieve MyD88 gene overexpression or knockdown in the A549 cell line, the cells were infected with lentivirus carrying MyD88 gene or shRNA to MyD88. MyD88 mRNA and protein expression was measured by RT-qPCR, immunohistochemistry and western blotting after infection for confirmation. Cell proliferation was detected by the WST-1 assay. Flow cytometry was used to measure the cell cycle and apoptosis. The transwell migration assay was used to observe the change of migration of transfected cells. The results showed that the overexpression of MyD88 increased the resistance of lung cancer A549 cells to paclitaxel, while the suppression of MyD88 increased the sensitivity of A549 lung cancer cells to paclitaxel. Following the paclitaxel treatment, a decreased apoptosis and G2 phase ratio, an increased cell migration ratio, and an increased production of IL-8 were found in MyD88-overexpressed A549 cells. The western blot analysis revealed that Myd88 overexpression resulted in an increased level of Bcl-2 but a decreased level of Bax in A549 cells. These findings suggested that the expression level of MyD88 is closely associated with paclitaxel resistance in A549 lung cancer cells. Thus, the downregulation of MyD88 in A549 cells increased its sensitivity to paclitaxel treatment, whereas the upregulation of MyD88 substantiates its paclitaxel resistance.

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Introduction

Lung cancer is one of the most common clinical cancer types and a leading cause of mortality in men and women worldwide (1). Chemotherapy is currently the basic therapy administered to advanced lung cancer patients. However, the resistance to various chemotherapy drugs is a major factor in the failure of chemotherapy in patients with lung cancer (2,3).

Paclitaxel is an effective antitumor drug against a variety of tumor cells by promoting microtubule polymerization and stable microtubule polymerization resulting in the buildup of microtubules in cells, arresting the cell division in the mitosis and blocking the division of normal cells. Studies have shown that paclitaxel has a broad spectrum of curative effect on ovarian, breast, lung and colorectal cancer, melanoma lymphoma and brain tumors. However, the appearance of paclitaxel resistance limits its long-term use (4).

Myeloid differentiation primary response gene 88 (MyD88) plays a key role in Toll-like receptor (TLR) signal transduction pathway. MyD88 protein comprises three domains: i) a death domain in its N-terminal component, ii) a central intermediate domain that enables MyD88 interaction with members of the IRAK kinase family, and iii) a toll-interleukin 1 receptor (TIR) domain in its C-terminal component that allows its interaction with TLRs (5). MyD88 has been found to be over-expressed in cancer tissues (such as colon and ovarian cancer), and was associated with tumor survival and chemoresistance. In colorectal cancer (CRC), a high expression of MyD88 was frequently detected in CRC patients with liver metastasis and associated with poor prognosis (6). Upregulation of MyD88 was also found in cancer tissues in ovarian cancer (7,8). Patients with ovarian cancer whose tumors did not express MyD88 had a statistically significant improved progression-free interval compared with patients whose tumors expressed MyD88 (7). Moreover, high expression of MyD88 conferred ovarian cancer cell resistance to chemotherapy (7,9). Patients bearing MyD88-positive tumors had a poor response to paclitaxel chemotherapy (9), suggesting MyD88 is a paclitaxel-resistant marker in cancer cells.

However, little is known regarding the relationship of MyD88 expression and paclitaxel chemoresistance in human lung cancer cells. In the present study, we constructed A549 human lung cancer cells with an overexpression or knockdown

of MyD88 by lentiviral vectors. The results showed that chemoresistance to paclitaxel was increased in the A549 cells with MyD88 overexpression, while it was decreased in the A549 cells with MyD88 knockdown. Further investigation showed that a decreased MyD88 expression level resulted in decreased cell proliferation, increased apoptosis, decreased cell migration and decreased IL-8 secretion in A549 cells. Consequently, the sensitivity of A549 cells to paclitaxel was increased.

Materials and methods

Cell line and maintenance. The human A549 lung cancer cell line was purchased from the Institute of Cell Biology (Shanghai, China) and cultured in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a humidified incubator containing 5% CO_2.

Construction of MyD88 lentiviral vectors. The MyD88 cDNA and siRNA sequences against human MyD88 gene were designed and synthesized by GenePharma (Shanghai, China). MyD88 cDNA was amplified by PCR and subcloned into EF1a-GFP/puro lentiviral plasmid vectors, designated as MyD88 and NC over (negative control), respectively. The siRNA (5'-GGTGGTGGTTGTCTCTGATGA-3') was inserted into the pGLV-h1-GFP/puro lentiviral vector. A control siRNA (5'-TTCTCCGAACGTGTCACGT-3') was used as a negative control (NC), designated as MyD88 and NC down, respectively. Lentiviruses encoded with MyD88 cDNA and siRNA against MyD88 and the control were produced by the co-transfection of 293T cells using Lipofectamine 2000 (Invitrogen Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. A549 cells ($5x10^4$) were infected with MyD88 lentivirus or NC vector at a MOI of ~100 for 72 h. The cells were then transferred into complete medium.

Immunocytochemistry. Expression of MyD88 protein was determined using immunocytochemistry. The day prior to the assay, a total of $1x10^4$ cells were seeded in a 96-well plate. After 24 h of incubation, the cells were fixed on slides using immune dyeing fixation reagent. The cells were permeabilized for 5 min with 0.1% Triton X-100 in phosphate-buffered saline (PBS) and blocked with blocking buffer (10% normal goat serum, 0.1% Triton X-100) for 30 min at room temperature. After blocking, the cells were washed with PBS and incubated overnight at 4˚C with the rabbit anti-human MyD88 antibodies (Abcam). On the following day, the cells were washed three times with PBS and incubated with Cy3-anti-rabbit IgG for 60 min at 37˚C. The cells were subsequently washed three times with PBS.

Quantitative RT-PCR (RT-qPCR). For RT-qPCR analysis, total RNA was isolated using the TRIzol reagent kit, and reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio., Inc., Shiga, Japan), according to the manufacturer's instructions. qPCR-based gene expression analysis was performed on a real-time PCR machine (7300; Applied Biosystems, Foster, CA, USA) using a standard SYBR-Green PCR kit. Reactions were conducted at 95˚C for 2 min, followed by 40 cycles of 95˚C for 15 sec, and 60˚C for 30 sec. The relative expression of each target gene normalized with GAPDH was calculated using the $2^{-\Delta\Delta ct}$ method. GAPDH served as a control. The primers used for GAPDH were: forward 5'-CCACTCCTCCACCTTTGAC-3' and reverse 5'-ACCCTGTTGCTGTAGCCA-3'.

Paclitaxel sensitivity analysis and cell proliferation. Cells ($0.8x10^4$/well) were seeded in a 96-well plate. After 24 h of incubation, 10, 20, 30, 40 and 50 μM paclitaxel (Sigma, St. Louis, MO, USA) was added and incubated for 48 h. After 48 h, the live cell population was analyzed using cell proliferation reagent WST-1 (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. Cell proliferation was assessed at different time points using WST-1.

Apoptosis analysis. The percentage of apoptotic cells was quantified using the Annexin V-PE kit (BD) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry within 30 min.

Cell cycle analysis. Cells ($3x10^5$) were pelleted by spinning for 5 min at 1000 rpm, 4˚C and resuspended in 1 ml of cold PBS. After fixation by adding 4 ml of absolute ethanol, the cells were centrifuged and resuspended in 1 ml of PBS. Subsequently, 100 μl of 200 μg/ml DNase-free RNase A was added to cell suspension and incubated for 30 min at 37˚C. The cells were stained with 100 μl of 1 mg/ml propidium iodide (light sensitive) and incubated for 5-10 min at room temperature prior to analysis.

Western blot analysis. The protein concentration of each sample was determined. Equal amounts of protein were loaded and separated discontinuously on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and subsequently transferred onto a PVDF membrane (Amersham Pharmacia, UK). The membrane was then incubated in TBST blocking solution (Tris-buffered saline including 0.1% Tween-20) containing 5% skim milk for 2 h at room temperature, followed by separate incubation with primary antibodies against MyD88 (Abcam), Bax, Bcl-2 (Cell Signaling), and β-actin (Beyotime, Jiangsu, China) overnight at 4˚C. After washing, the membrane was incubated with secondary antibodies, including HRP-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibodies for 2 h. After several washes, the immunoblot was detected with an enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

Cell migration assay. Cell migration was evaluated by the Transwell Permeable Support (Corning, Tewksbury, MA, USA) according to the manufacturer's instructions. Five 200-multiple microscopic fields were randomly selected to calculate the total count of the invaded or migrated cells. All assays were conducted three times.

Statistical analysis. For all the analyses, measurements obtained from the groups were expressed as the means ± SD for all data determined. Statistical analysis was performed using an unpaired Student's t-test followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant result.

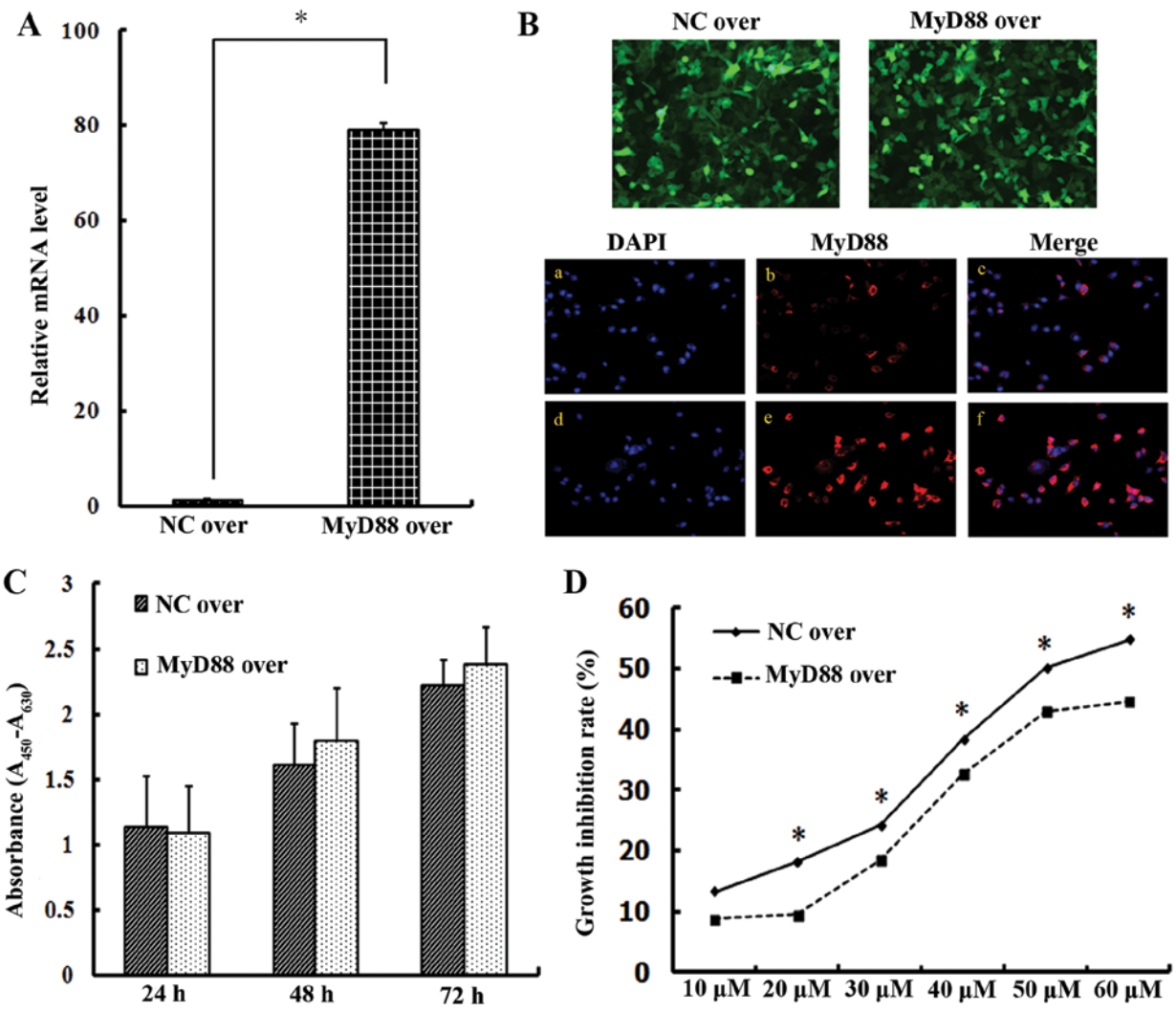

Figure 1. Inhibition of cell growth by paclitaxel in A549 cells after MyD88 gene overexpression. (A) The MyD88 mRNA level was measured using quantitative PCR in different groups. *P<0.001, experimental vs. control group. (B) The overexpression of MyD88 protein was confirmed by immunocytochemistry. (C) A549 cell proliferation was measured by the WST-1 assay following MyD88 gene overexpression. (D) The inhibition of cell growth by paclitaxel was determined in A549 cells following MyD88 gene overexpression. Paclitaxel treatment was carried out for 48 h with the indicated concentrations. MyD88, myeloid differentiation primary response gene 88. NC, negative control.

Results

Increased resistance to paclitaxel in A549 cells with overexpressed MyD88. To determine the functional consequences of elevated MyD88 expression in lung cancer, MyD88 was overexpressed in A549 lung cancer cells using a lentiviral vector. Overexpression was confirmed by qPCR and immunocytochemistry (Fig. 1A and B). The results showed that the proliferation of A549 cell was not altered following overexpression of MyD88 (Fig. 1C). However, MyD88-overexpressed cells were refractory to growth inhibition by paclitaxel when compared to the control (Fig. 1D).

Increased sensitivity to paclitaxel treatment in A549 cells with knockdown of MyD88 gene expression. A lentiviral vector containing shRNA was used to specifically target and stably knock down the expression of MyD88 in A549 lung cancer cells. qPCR analysis showed that the mRNA expression of MyD88 in cells transfected with shRNA to MyD88 was markedly lower than that of the control A549 cells (Fig. 2A). A decreased expression of MyD88 protein was observed following immunocytochemistry (Fig. 2B). The results showed that the cells did not exhibit altered growth activity at 24, 48 and 72 h after MyD88 gene silencing (Fig. 2C). In the presence of paclitaxel, cell proliferation was significantly inhibited (Fig. 2D), suggesting the downregulation of MyD88 had increased sensitivity of A549 cells to paclitaxel treatment.

Effects of MyD88 gene overexpression and knockdown on the cell cycle of A549 cells. Paclitaxel is known to arrest cell cycle at G2 phase. A DNA flow cytometric analysis was performed to determine the effect of paclitaxel on the cell cycle of A549 cells with different expression levels of MyD88. We found that MyD88 gene overexpression or knockdown did not affect the cell cycle of A549 cells (Fig. 3A and B). However, after treatment with paclitaxel, a G2 phase reduction was found in A549 cells with MyD88 gene overexpression, whereas MyD88 gene silencing resulted in the accumulation of cells in the G2 phase (Fig. 3C and D).

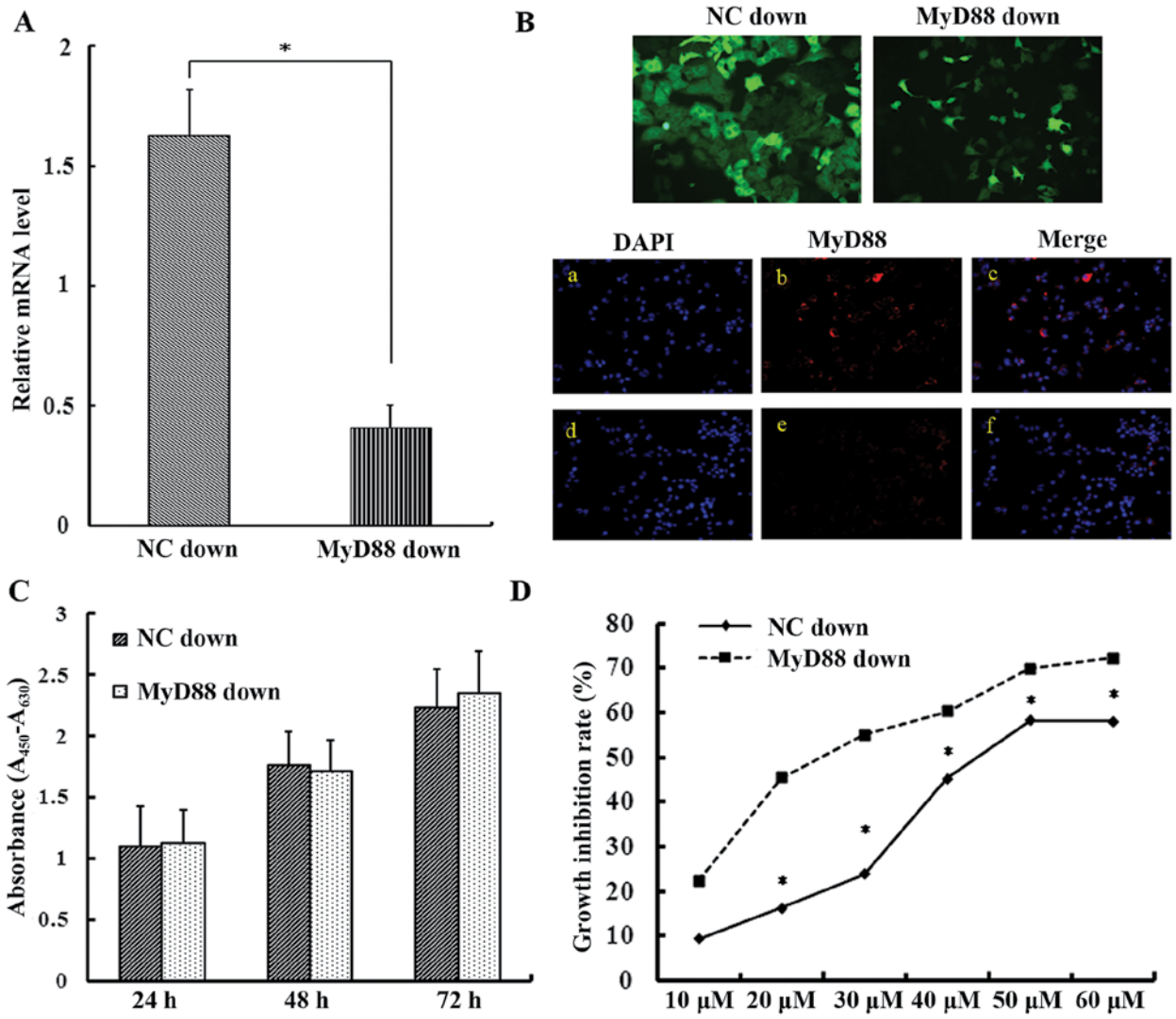

Figure 2. Inhibition of cell growth by paclitaxel in A549 cells after MyD88 gene knockdown. (A) The MyD88 mRNA level after RNAi treatment was measured using quantitative PCR in different groups. *P<0.005, experimental vs. NC control. (B) The expression of MyD88 protein after RNAi treatment was determined by immunocytochemistry. (C) A549 cell proliferation was measured by WST-1 assay after MyD88 gene knockdown. (D) The inhibition of cell growth by paclitaxel was determined in A549 cells after MyD88 gene knockdown. Paclitaxel treatment was carried out for 48 h with the indicated concentrations. MyD88, myeloid differentiation primary response gene 88. NC, negative control.

Regulation of cell apoptosis by MyD88 gene overexpression or knockdown in A549 cells. To analyze whether the change of MyD88 expression altered the resistance of A549 cells to paclitaxel treatment, MyD88 gene overexpression or knockdown was carried out in A549 cells. The cells were treated with paclitaxel for 48 h prior to the quantification of apoptotic cells with Annexin V. A significant decrease of the percentage of apoptotic cells was observed in MyD88-overexpressed A549 cells ($p<0.01$) (Fig. 4A-C), while an increase of the percentage of apoptotic cells was observed in MyD88 shRNA-transfected A549 cells ($p<0.01$) (Fig. D). These results suggested that the expression level of MyD88 in A549 cells may be associated with cell apoptosis induced by paclitaxel. In addition, the overexpression of MyD88 markedly decreased the expression of Bax in A549 cells and increased the expression of Bcl-2 (Fig. 4E and F). Collectively, these findings suggested that MyD88 overexpression or knockdown may alter cell resistance to paclitaxel through Bax and Bcl-2.

Enhanced MyD88 promotes migration in A549 cells. The role of MyD88 in tumor metastasis, which has been suggested from clinical data was investigated. A549 cells migrated more rapidly when MyD88 was overexpressed, as is evident by the percentage of migrated cells within a certain time (Fig. 5A and B). By contrast, the cells migrated gradually when MyD88 was knocked down (Fig. 5C and D). These data suggested that enhanced MyD88 may promote the motile abilities of A549 cells.

MyD88 expression was associated with IL-8 production in lung cancer cells. Previously, it was demonstrated that IL-8 was associated with paclitaxel resistance in human cancer cells (10). We examined the effect of MyD88 on IL-8 production in A549 lung cancer cells. Overexpression of MyD88 resulted in a significant increase in IL-8 production in a dose- and time-dependent manner ($p<0.01$) (Fig. 6A). By contrast, the knockdown of MyD88 by RNAi decreased

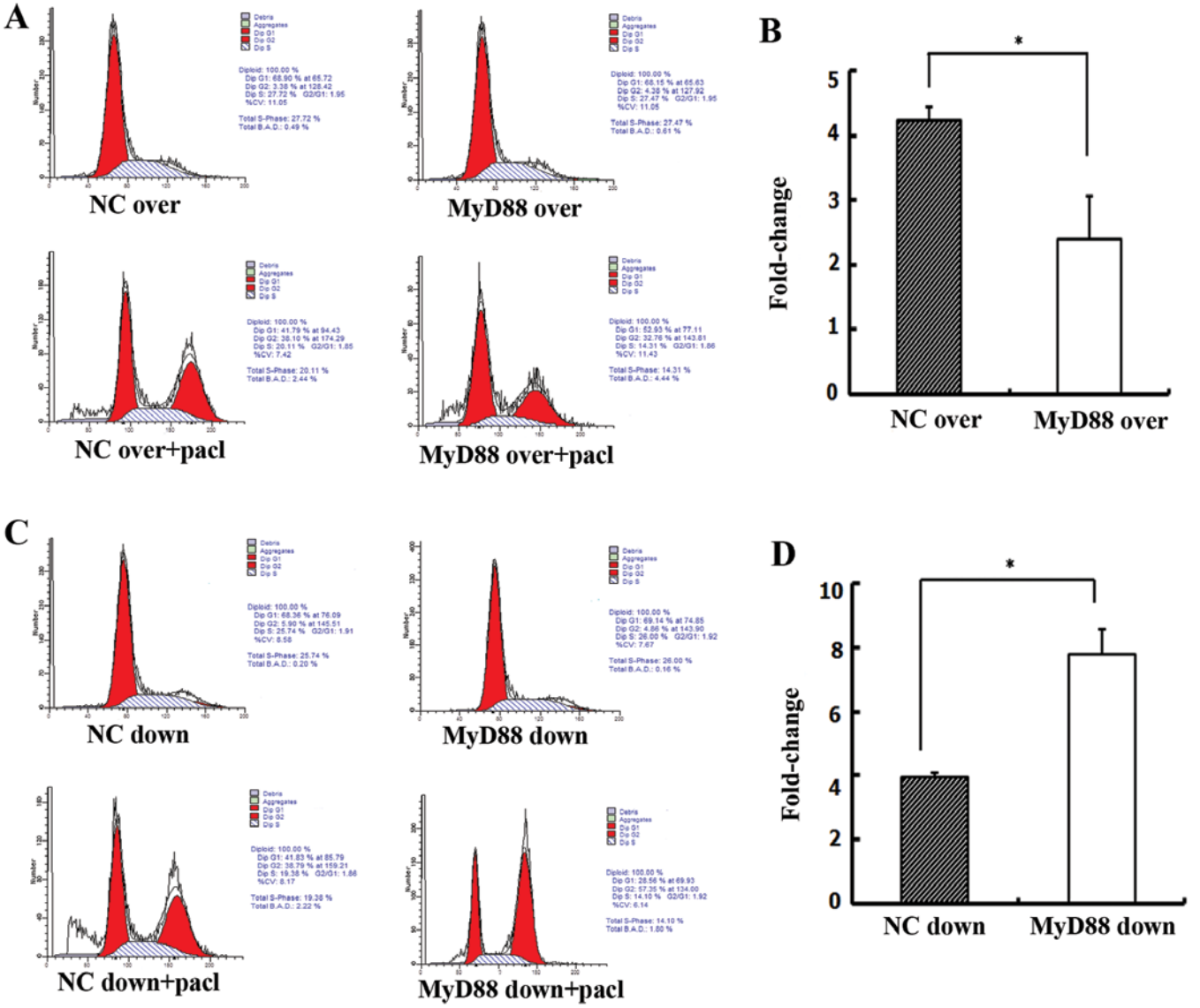

Figure 3. Cell cycle analysis of A549 cells treated with paclitaxel in relation to MyD88 gene overexpression or knockdown. (A) Flow cytometric analysis of cell cycle with overexpression of MyD88 and control A549 cells. (B) Quantitative analysis showing the fold-change of the G2 phase ratio following treatment of paclitaxel in the overexpression of MyD88 in A549 cells. (C) Flow cytometric analysis of cell cycle with knockdown of MyD88 and control A549 cells. (D) Quantitative analysis showing the fold-change of the G2 phase ratio following the treatment of paclitaxel in the knockdown of MyD88 in A549 cells. The cells were treated with the indicated concentration of paclitaxel for 48 h. MyD88, myeloid differentiation primary response gene 88. NC, negative control.

IL-8 production (Fig. 6B). These data suggested that MyD88 mediated-IL-8 production may be associated with paclitaxel sensitivity in lung cancer cells.

Discussion

MyD88 is a key adaptor molecule involved in the toll-like receptor (TLR) signaling pathway. Elevated MyD88 expression has been found in parenchymal cells in various types of cancer (6-8,11,12), although the mechanisms for MyD88 upregulation remain to be determined. Studies have shown that overexpression of the MyD88 level decreased sensitivity to paclitaxel in ovarian cancer and hepatocellular carcinoma cells (7,13). Results of recent studies (?) have shown the involvement of MyD88 in the development of lung cancer. However, little is known with regard to the relationship of MyD88 expression and paclitaxel chemoresistance in human lung cancer cells.

In the present study, MyD88 overexpression and knockdown was produced in A549 cells by lentiviral vectors. We found that there was no significant effect on the proliferation of A549 cells when MyD88 gene expression was altered. However, chemoresistance to paclitaxel was increased in MyD88 overexpressed A549 cells, while it was decreased in A549 cells after MyD88 knockdown.

Paclitaxel is an effective antitumor drug that acts against a variety of tumor cells (14). It exerts antitumor effects by forming stable bundles of microtubules, resulting in the arrest of the cell cycle at the G2/M phase transition, thus preventing the completion of mitosis (15,16). In the present study, we

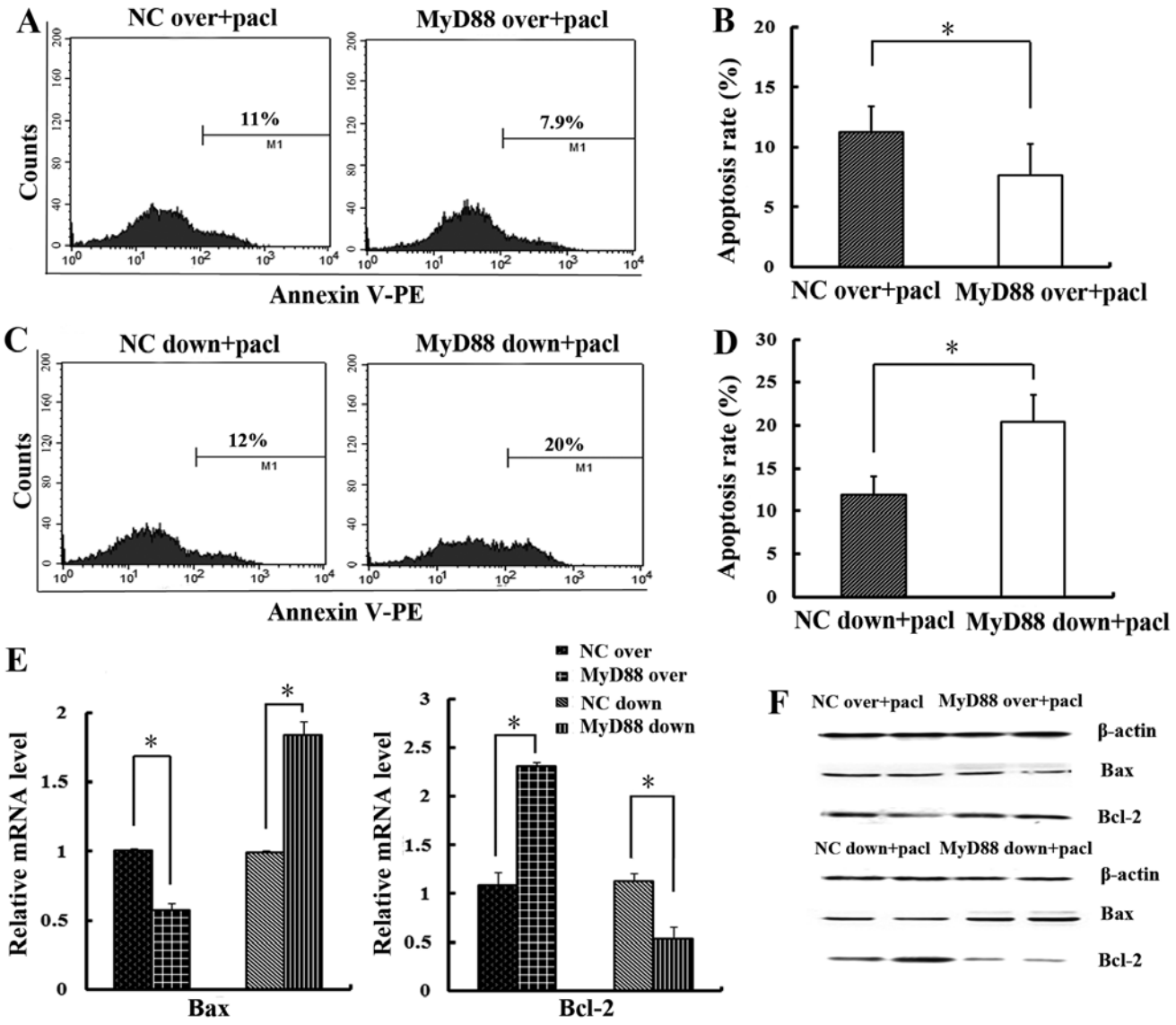

Figure 4. Analysis of cell apoptosis in A549 cells with MyD88 gene overexpression and knockdown. (A and B) The apoptosis of MyD88-overexpressed A549 cells was analyzed by Annexin V-PE staining following treatment with paclitaxel. Statistical analysis was carried out between the two groups. *P<0.05 between the compared groups. (C and D) The same analysis as described above was carried out on A549 cells after MyD88 gene knockdown. (E) qPCR analysis of Bax and Bcl-2 mRNA expression. The results were normalized to the amount of β-actin, which served as the internal control. Each value is the average of three independent experiments. (F) Western blot analysis of the Bax and Bcl-2 protein expression in each group. β-actin expression served as the loading control. MyD88, myeloid differentiation primary response gene 88. NC, negative control.

observed a cell cycle change in A549 cells treated with paclitaxel for 48 h after MyD88 overexpression and knockdown. The results showed that, the cell cycle at G2/M phase transition was decreased significantly in A549 cells with overexpression of MyD88 compared with the control group, whereas it was increased significantly in A549 cells with knockdown of MyD88. A role for MyD88 in the regulation of proteins controlling the passage is accompanied by a reduction in the expression of cyclins A and E (5). The reduction of cells in the G2 phase and an accumulation in the G0/G1 phase, suggests Myd88 is paclitaxel-resistant.

In addition, paclitaxel directly induces apoptosis of several tumor cell lines through a variety of mechanisms, such as phosphorylation of Bcl-2, activation of caspase 8 and mitogen-activated protein kinase (MAPK) signal transduction (17,18). The contribution of MyD88 to tumor progression is predominantly associated with its anti-apoptotic ability (7,12). To determine the change of apoptosis of A549 cells with a different expression level of MyD88, flow cytometric assays were used to detect the apoptosis of A549 cells. The results showed that the overexpression of MyD88 decreased cell apoptosis induced by paclitaxel when compared with the control group. Furthermore, the overexpression of MyD88 in A549 cells was able to enhance the expression of the anti-apoptotic protein Bcl-2, and inhibit the expression of pro-apoptotic protein Bax, which may lead to paclitaxel resistance. An increased level of protein Bcl-2 and a decreased level of protein Bax may be associated with the apoptotic pathway. Based on these data, we predicted that the MyD88 protein may be involved in the anti-apoptotic pathway, which affected drug resistance. Thus, more investigations are necessary to confirm this prediction.

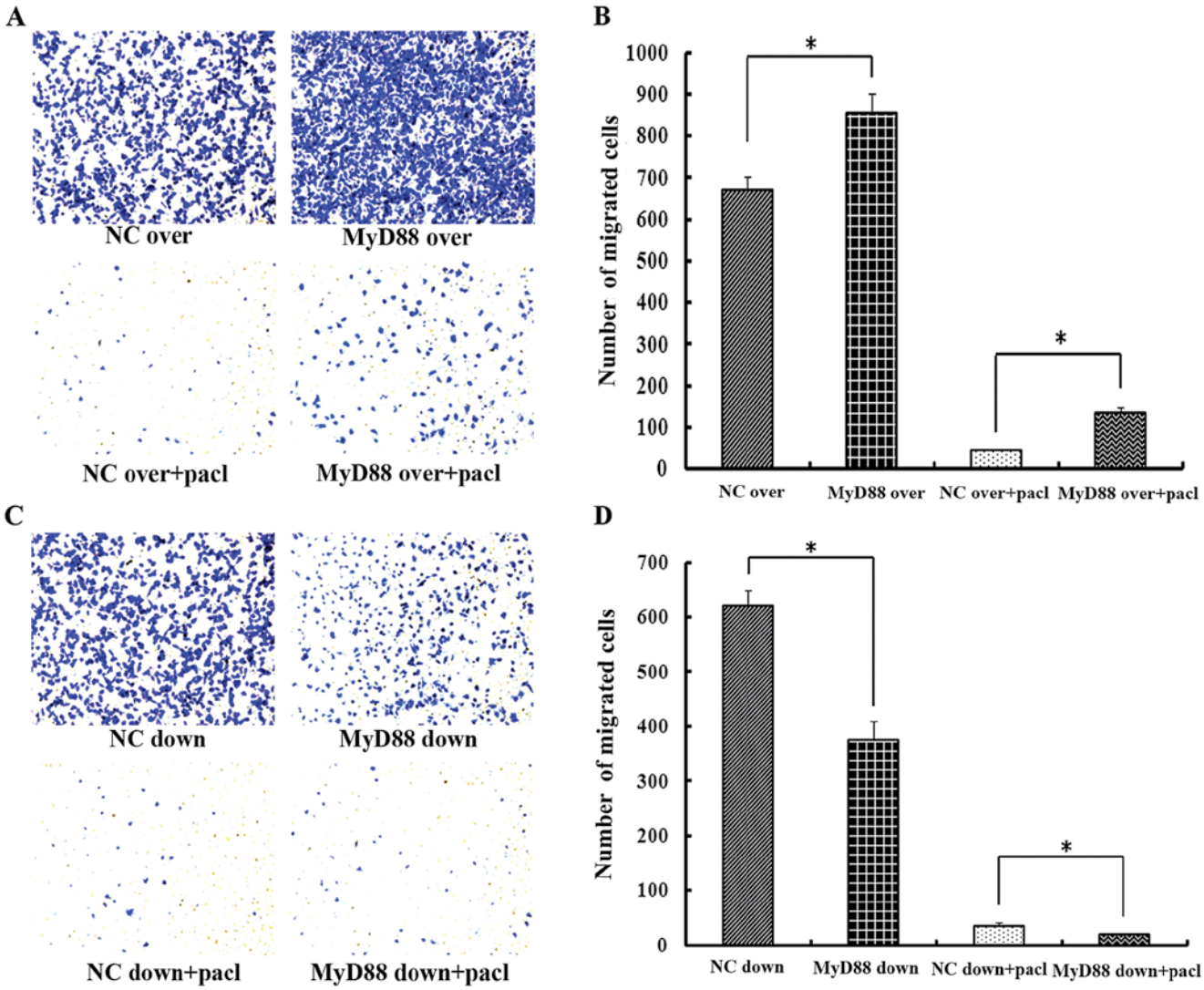

Figure 5. MyD88 promotes A549 lung cancer cell migration *in vitro*. Cell migration assays were performed on A549 cells with gene overexpression of MyD88 (A and B) or gene knockdown of MyD88 (C and D). The migrated cells were visualized by staining with hematoxylin and quantified by a cell counter. Data are presented as means ± SD from three independent experiments. MyD88, myeloid differentiation primary response gene 88. NC, negative control.

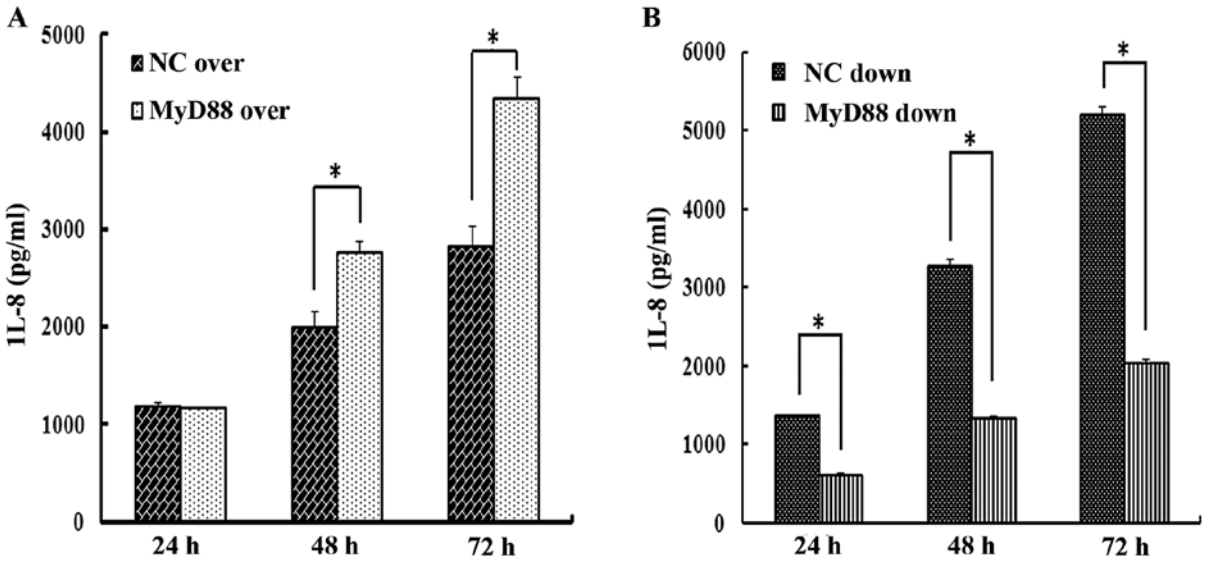

Figure 6. Overexpression of MyD88 promotes the production of IL-8. (A) The production of IL-8 was measured by ELISA in the culture supernatant of A549 cells after MyD88 overexpression. (B) The production of IL-8 was measured in the culture supernatant of A549 cells transfected with shRNA to MyD88. Statistical analysis was carried out between the groups as indicated ($^{*}p<0.01$). MyD88, myeloid differentiation primary response gene 88. NC, negative control.

Results of the present study have shown that apart from the ability to evade apoptotic stimulation, an elevated expression of MyD88 conferred A549 cells with enhanced abilities of migration. There are disagreements regarding the function of the MyD88 pathway regarding cell migration. Castoldi *et al* reported that TLR2, TLR4 and MYD88 signaling pathways are crucial for neutrophil migration in acute kidney injury induced by sepsis (19), while the results of Haley *et al* showed that the migration of Langerhans cells did not require MyD88-dependent signals (20). In the present study, we found that overexpression of MyD88 in A549 promoted cell migration compared with the control group.

Stress and drug-induced IL-8 signaling has been shown to confer chemotherapeutic resistance in cancer cells. Therefore, IL-8 signaling inhibition may be a significant therapeutic intervention in targeting the tumor microenvironment. IL-8 has been shown to be an important modulator of paclitaxel sensitivity in cancer cells. Duan *et al* found paclitaxel resistant-ovarian cancer cells produced increased IL-8 levels (10). Penson *et al* reported that increased IL-6 and IL-8 correlated with a poor initial response to paclitaxel in ovarian cancer patients (21). In lung cancer, it has been proved that IL-8 was associated with the tumorigenesis of human non-small cell lung cancer (22). In the present study, we found the knockdown of MyD88 increased paclitaxel sensitivity but decreased IL-8 production in A549 cells. Combined with these reported studies, our results suggest that IL-8 is involved in the response of lung cancer cells exposed to paclitaxel, and the importance of MyD88 in the regulation of the tumor microenvironment and tumor progression.

The specific roles of MyD88 in lung cancer cells merit further investigation. It is possible that MyD88 is important to the maintenance of cellular stability and that a loss of function may result in increased chemosensitivity to MyD88.

Acknowledgements

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