Paclitaxel-exposed ovarian cancer cells induce cancer-specific CD4+ T cells after doxorubicin exposure through regulation of MyD88 expression

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Abstract. Ovarian cancer has the highest mortality rate among gynecological malignancies due to high chemoresistance to the combination of platinum with taxane. Immunotherapy against ovarian cancer is a promising strategy to develop from animal-based cancer research. We investigated changes in the immunogenicity of paclitaxel-exposed ovarian cancer cells following exposure to other chemotherapeutic drugs. Murine ovarian surface epithelial cells (MOSECs) showed some resistance to paclitaxel, a first-line therapy for ovarian cancer. However, MOSECs pre-exposed to paclitaxel died through apoptosis after incubation with doxorubicin or cisplatin for 2 h. Injected into mice, the paclitaxel-exposed MOSECs post-treated with doxorubicin induced more MOSEC-specific CD4+ T cells and extended survival for a greater time than MOSECs treated with paclitaxel alone; and bone marrow-derived dendritic cells (BMDCs) expressed higher levels of co-stimulatory molecules and produced IL-12 after co-culture with paclitaxel-exposed MOSECs treated with doxorubicin. We also observed that in paclitaxel-exposed MOSECs treated with doxorubicin, but not cisplatin, the expression of MyD88 and related target proteins decreased compared to paclitaxel-exposed MOSECs only, while in BMDCs co-cultured with these MOSECs the expression of myeloid differentiation primary response gene 88 (MyD88) increased. These findings suggest that paclitaxel pre-exposed cancer cells treated with doxorubicin can induce significant apoptosis and a therapeutic antitumor immune response in advanced ovarian cancer.

Introduction

Of the gynecological cancers, ovarian cancer imposes the highest mortality rate, even with surgery and adjuvant chemotherapy. Even with good responsive to primary therapy, ~80% of patients who present with advanced cancers will experience recurrence and succumb to the disease (1,2). In the absence of screening, the need for novel treatments that prevent disease progression following surgery, including combination and intraperitoneal chemotherapies, becomes a matter of some urgency. Compared with hematologic tumors and malignant melanoma, ovarian cancer may be less vulnerable to immunotherapy (3). Only recently have tumor-specific antigens been identified that could serve as targets for cytotoxic T-cell responses to ovarian cancer (4,5). New strategies are needed to generate and enhance immune responses against ovarian cancer, identify tumor-specific antigens and modulate immune-suppressive activities.

The combination of platinum and taxane is used currently as initial treatment for advanced epithelial ovarian cancer. Patients treated with paclitaxel and cisplatin respond better clinically and survive longer progression-free than with the previous standard of care (cisplatin plus cyclophosphamide) (6). Ovarian cancer is very sensitive to paclitaxel and cisplatin combination. However, acquired resistance to combined therapy with paclitaxel and a platinum-based drug, which may develop by one of several pathways, is a major reason for treatment failure and death in patients with ovarian cancer (7,8). The mechanisms responsible for the high resistance rate to paclitaxel are not well researched and methods to prevent or

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regulate the resistance using another drug or combination are not well studied either (9).

Toll-like receptors (TLRs) and ligand interaction trigger immune cells (10). Mice deficient in each TLR have demonstrated that each TLR has a distinct function in terms of pathogen recognition and immune responses (11). However, there is significant amount of evidence for the involvement of TLRs in disease largely from overexpression in multiple diseases, their activation causing enhanced disease in models (12). Despite marked differences in structure, paclitaxel and LPS share a receptor or signaling molecule and paclitaxel was thereby identified as a TLR4 ligand in murine macrophages (13,14). A previous study also reported doxorubicin, chemical TLR2 ligand, may play a role in the regulation of inflammatory and apoptotic mediators in the heart after administration (15). TLR4 and MyD88 were detected in some human cancer cell lines including ovarian cancer (16) and paclitaxel activated MyD88-dependent pathway and recruited NF-κB, leading anti-apoptotic molecule, to survive and resist drugs in cancer cells (17,18).

In patients previously treated with platinum, anthracyclines such as doxorubicin may be effective as single-agent treatments (19). Addition of doxorubicin to ovarian cancer regimens may significantly improve survival compared to platinum-based combinations without anthracyclines (20).

The use of single chemotherapeutic drug with high dose has shown some limitations due to development of drug resistance and high toxicity. However, attempts to delivery chemotherapeutic drugs simultaneously have shown many difficulties, such as different solubility in water. Following these results, we focused on the effect of sequential hydrophobic paclitaxel and hydrophilic doxorubicin treatment on survival of ovarian cancer cells and antitumor immune responses induced by drug-treated cancer cells concurrently in this study. Thus, tumor cells sequentially treated with an anticancer drug combination may open a new route to immune activation and disease management against advanced cancer.

Materials and methods

Cells, antibodies, anticancer drugs and mice. The MOSEC cell line was originally derived from murine ovarian surface epithelial cells (21). Antibodies against mouse CD4 (557308) and IFN-γ (554411) were purchased from BD Pharmingen; anti-CD40 (12-0401), CD80 (12-0801) and anti-CD86 (12-0862) were purchased from eBioscience. The polyclonal antibodies to cleaved caspase-3 were from Cell Signaling Technology; and female C57BL/6 mice, from the Chung-Ang Laboratory Animal Service (Seoul, Korea). Cisplatin (P4394), paclitaxel (T7191) and doxorubicin-HCl (D1515) were purchased from Sigma-Aldrich. Oregon Green 488-conjugated paclitaxel (P22310, Molecular Probes) were reconstituted with DMSO prior to use and diluted with RPMI-1640 to the required concentrations. The ethics committee of the College of Medicine, Chung-Ang University and College of Medicine, Inje University approved all protocols and procedures used in this study.

Drug uptake and the effect of anticancer drugs on MOSECs in vitro. On day 1, MOSECs ($5.0x10^6$/ml) were cultured in the presence of paclitaxel (50 μg/ml) at 37˚C for 2 h and then cultured in the absence of the drug for 24 h. MOSECs pre-exposed to paclitaxel were harvested and incubated with doxorubicin and cisplatin at low concentrations (10 μg/ml) for 2 h. An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, a yellow tetrazole) assay with Vybrant MTT cell assay kit (V-13154, Molecular Probes) for cell viability was performed with the drug-treated MOSECs after 24 and 48 h of incubation. Apoptosis was also measured using an Annexin V-FITC detection kit (556570, BD Pharmingen) according to the manufacturer's protocol. MOSECs were also cultured in the presence of Oregon green 488-conjugated paclitaxel (50 μg/ml) and/or doxorubicin (10 μg/ml) at 37˚C for 2 h to determine the drug uptake according to a previous study (22). Immunoblot assay of cleaved caspase-3 expression was performed in paclitaxel-treated MOSECs post-treated with doxorubicin or cisplatin and harvested at 3 and 20 h after last drug treatment. Primary antibodies used were; anti-cleaved caspase-3 (9662S, Cell Signaling) (1:1,000) and anti-β-actin (4967L, Cell Signaling) (1:2,000). Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit (7074S, Cell Signaling) (1:2,000).

Characterization of immune responses to the drug-treated tumor cells and the in vivo antitumor responses. Prior to inoculation, all groups of tumor cells ($1.0x10^6$/mouse) treated with single or sequential anticancer drug regimens were irradiated at 100,000 cGy/10 min. Mice were injected via the intraperitoneal route and boosted one week later with the same dose of cells treated by the same regimen. Immune responses were tested at one week or at day 50 after the last treatment. To harvest and collect the peritoneal exudate cells (PECs), 10 ml of cold sterile PBS was injected into the peritoneal cavity. After de-contamination to remove the RBC, the PECs were co-cultured with irradiated MOSECs for 16 h with complete medium in the presence of GolgiPlug (555028, BD Pharmingen). Cells stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD4 antibody and cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit (554714, BD Pharmingen). FITC-conjugated anti-IFN-γ was used for intracellular cytokine staining. The numbers of $CD4^+$ $IFN-\gamma^+$ double-positive T cells in $1.0x10^5$ PECs are calculated. To translate immune responses to antitumor effects, female C57BL/6 mice were challenged intraperitoneally with $1.0x10^6$ MOSECs per mouse. At day 3, tumor-bearing mice from each group (6-7 mice/group) were vaccinated twice at weekly intervals with the same dose of cells treated by the same regimen. At 40-50 days after tumor challenge, the general condition and weights of the mice were monitored twice weekly to assess the tumor burden and ascites accumulation resulting from progressive peritoneal carcinomatosis (23). The moribund animals were euthanized.

DC maturation and detection of cytokine secretion with RT-PCR and ELISA. Dendritic cells were generated from murine bone marrow cells as previously described with modifications (24). At day 6, MOSECs ($1.0x10^6$/mouse) pre-exposed to paclitaxel and then treated with doxorubicin or cisplatin were co-cultured with BMDCs at a ratio of 1:1 (MOSEC:DCs) in a 24-well plate. After 24 h of co-culture, cells were harvested

and the BMDCs were isolated using anti-CD11c antibody according to the manufacturer's protocol (130-052-001, Miltenyi Biotec). Cells were stained with antibodies to CD80, CD86 and CD40 for detecting BMDCs maturation and extracted RNA using TRIzol (15596-018, Life Technologies) to determine the mRNA level of IL-12p40, IL-6, TNF-α. Following primers were used for amplification: IL-12p40 (sense, 5'-CACCTGCCCAACTGCCGAGG-3'; and antisense, 5'-TAGCTCCCTGGCTCTGCGGG-3')/IL-6 (sense, 5'-ATGC TGGTGACAACCACGGCC-3'; and antisense, 5'-GGCATA ACGCACTAGGTTTGCCGA-3')/TNF-α (sense, 5'-AGCCC CCAGTCTGTATCCTT-3'; and antisense, 5'-CTCCCTTTGC AGAACTCAGG-3')/GAPDH (sense, 5'-GTGGAGTCTACT GGCGTCTT-3'; and antisense, 5'-GCCTGCTTCACCACC TTCTT-3'). The numbers of cycles and temperatures were used as previously determined (25). Cycling conditions for IL-12p40 were 30 sec at 95°C, 60 sec at 60°C, and 1 min at 72°C for 35 cycles; conditions for IL-6 were 30 sec at 95°C, 60 sec at 60°C and 1 min at 72°C for 35 cycles; conditions for TNF-α were 30 sec at 95°C, 60 sec at 53°C and 1 min at 72°C for 35 cycles and conditions for GAPDH were 30 sec at 95°C, 60 sec at 50.5°C and 1 min at 72°C for 35 cycles. PCR products were electrophoresed and analyzed. After 24 h of co-culture, culture media was collected and kept in -70°C to detect IL-12 protein level with ELISA. ELISA was conducted according to the manufacturer's instructions (900-M97, PeproTech).

Immunoblot analysis for MyD88 and downstream target proteins. MOSECs (1.0x10^6/mouse) treated with each condition of anticancer drug isolated according to the time schedule. BMDCs co-cultured with the drug-treated MOSECs for 24 h, cells were harvested and the BMDCs were isolated using anti-CD11c antibody according to the manufacturer's protocol (130-052-001, Miltenyi Biotec). All samples were lysed in Mammalian Protein Extraction Reagent (M-PER) (78501, Pierce). The protein transferred-membranes for western blotting were probed with an appropriate antibody. Primary antibodies used were: anti-mouse MyD88 (sc-74532), tumor necrosis factor receptor associated factor 6 (TRAF6) (sc-8409) and nuclear factor-κB (NF-κB) (sc-71675) from Santa Cruz Biotechnology (1:1,000). Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse (1:5,000-1:10,000). Enhanced chemiluminescence was performed with ECL-Plus (RPN2132, GE Healthcare). The bands were then quantified using Thermo Scion Image Analysis software (Scion Corp.).

Statistical analysis. All data are expressed as means ± standard error of the mean (SEM) and each value is representative of at least two different experiments. Comparisons between all individual data were made by analysis of variance (one-way ANOVA). Statistical significance was defined as p<0.05.

Results

The death rate of the paclitaxel-resistant MOSECs increases after treatment with doxorubicin and cisplatin through an apoptotic pathway. We first determined the single effect of paclitaxel, first-line chemotherapy for ovarian cancer, on MOSECs exposed to various doses of paclitaxel for a short

time (2 h). However, the high value on MTT assay showed a high level of viability in these MOSECs at 24 and 48 h after short time treatment (Fig. 1). We observed that the paclitaxel can be taken up by MOSECs and drug absorption rate did not affect reciprocally hydrophobic paclitaxel and hydrophilic doxorubicin after co-treatment. Most MOSEC cells co-expressed green (paclitaxel) and red (doxorubicin) fluorescence after treatment with paclitaxel and doxorubicin for 2 h (Fig. 1B). Doxorubicin was taken up by live paclitaxel-exposed MOSECs for a short time (2 h) on day 1 (Fig. 1C).

We next investigated the death rate of paclitaxel-exposed MOSECs after post-treatment with doxorubicin (10 µg/ml) or cisplatin (10 µg/ml) for 2 h. In these MOSECs, the MTT assay showed significant decreases in viability at 24 and 48 h after the second drug treatment (Fig. 2A). By cell-staining with FITC-conjugated Annexin V after post-treatment with doxorubicin, we showed that this decrease in viability occurred through apoptosis (Fig. 2B). We determined the extent of apoptotic death with immunoblot assay. Paclitaxel-exposed MOSECs post-treated with doxorubicin for short time showed greater caspase-3 activation than other groups at 20 h after finishing treatment with last drug (Fig. 2C). Thus our results suggest that MOSECs can absorb the paclitaxel after short incubation, but may be resistant. However, brief exposure to low-dose doxorubicin or cisplatin activated apoptosis in paclitaxel-exposed MOSECs.

MOSECs sequentially treated with paclitaxel and doxorubicin enhance MOSEC-specific CD4$^+$ T-cell immune responses and prolong survival in vaccinated mice. We determined whether paclitaxel-exposed MOSECs treated with doxorubicin or cisplatin killed by apoptosis were immunogenic *in vivo*. We observed that the mice vaccinated with paclitaxel-exposed MOSECs after post-treatment with doxorubicin induced highest MOSEC-specific CD4$^+$ T-cell immune responses (p<0.001) (Fig. 3). As a consequence of high immune responses, the survival rate of the mice vaccinated with paclitaxel-exposed MOSECs after post-treatment with doxorubicin was significantly higher at day 80 than the other groups (p<0.05) (Fig. 4A and B). Both the mice treated with irradiated-only MOSECs and with paclitaxel-exposed MOSECs increased in weight relatively early compared to those mice vaccinated with doxorubicin- or cisplatin-treated MOSECs and paclitaxel-exposed MOSECs post-treated with doxorubicin or cisplatin (p<0.017) (Fig. 4B and C). We also determined that the anticancer immune responses required CD4$^+$ T cells and NK cells based on antibody depletion experiments *in vivo* (data not shown). Our results suggest that MOSECs pre-exposed to paclitaxel and subsequently to doxorubicin induces antitumor immune response and prolong survival in tumor-bearing mice.

MOSECs pre-exposed to paclitaxel and post-treated with doxorubicin and cisplatin induce specific CD4$^+$ long-lasting T cells in vaccinated mice. At day 50 after last vaccination, the mice vaccinated with paclitaxel-exposed MOSECs post-treated with doxorubicin or cisplatin induced MOSEC-specific CD4$^+$ long-lasting T cells in greater numbers than the mice vaccinated with MOSECs exposed to a single anticancer drug only (p<0.001, Tax→MOSEC-Dox versus MOSEC-Tax; p<0.021, Tax→MOSEC-Dox versus MOSEC-Dox). We also
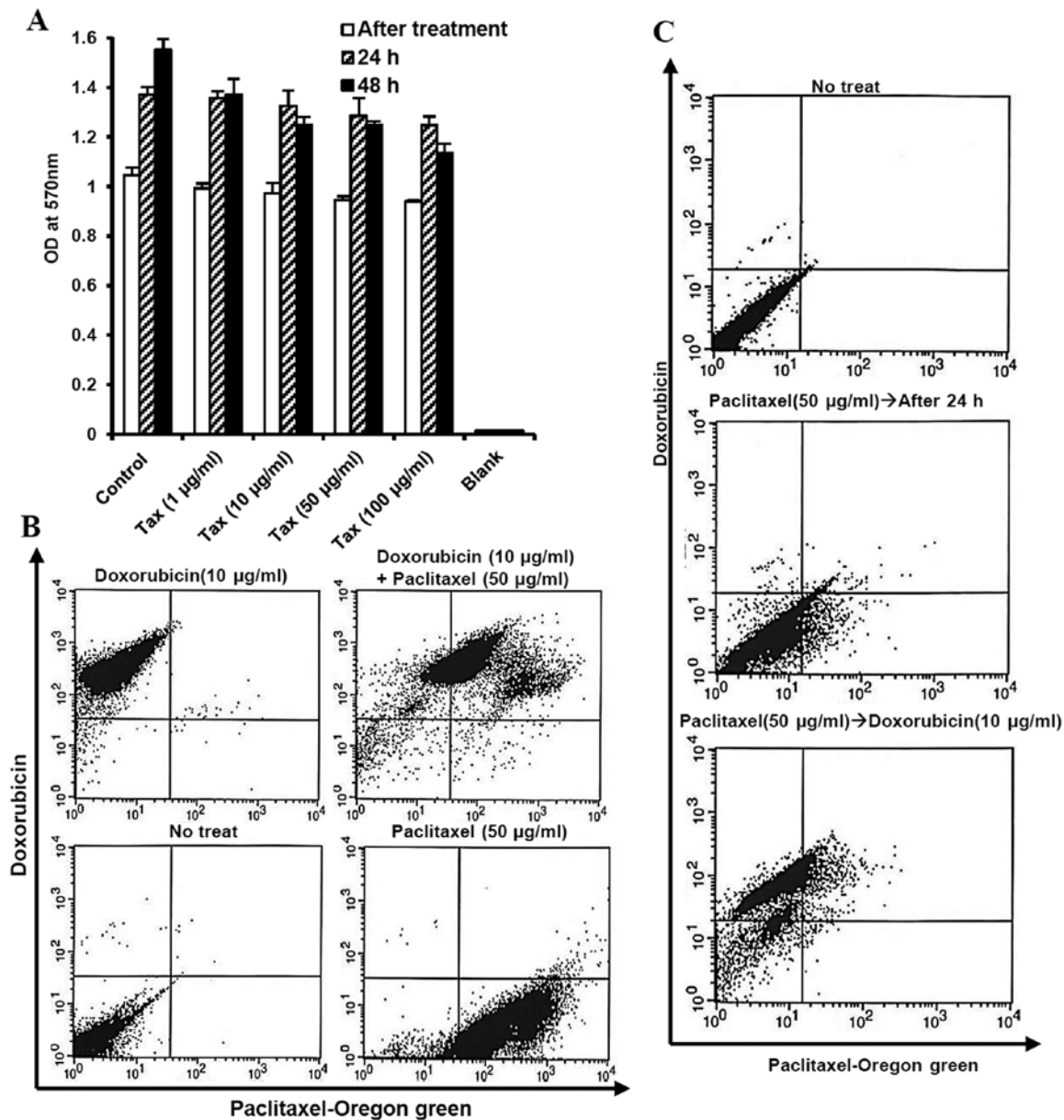
Figure 1. Viability after paclitaxel single treatment and drug uptake in paclitaxel-exposed MOSECs after treatment with doxorubicin *in vitro*. (A) MOSECs (1.0x10⁶) were incubated for 2 h in the presence of paclitaxel at graded concentrations (0, 1, 10, 50 and 100 µg/ml). Samples of MOSECs treated with paclitaxel for 2 h were incubated with medium lacking the drug for 24 h and an MTT assay was then performed. (B) Pools of MOSEC (1.0x10⁶) were incubated with Oregon green 488-conjugated paclitaxel (50 µg/ml) and doxorubicin (10 µg/ml), which has red auto-fluorescence, at 37˚C for 2 h. Flow cytometry showing uptake of paclitaxel and doxorubicin at single or co-treat condition by MOSECs. (C) MOSECs incubated for 2 h in the presence of Oregon-green 488 conjugated paclitaxel (50 µg/ml) were cultured without drug for 24 h. At 24 h of culture, paclitaxel-exposed MOSECs were cultured in the presence of doxorubicin for 2 h to determine the uptake of drug. The data are from one representative experiment that was performed three times.

observed that the mice vaccinated with MOSECs pre-exposed paclitaxel only did not generate MOSEC-specific CD4⁺ long-lasting T cells (Fig. 5). Thus, our data suggest that MOSECs pre-exposed to paclitaxel and treated briefly thereafter with a different anticancer drug induces and sustains an effective antitumor immune response against ovarian cancer.

MOSECs pre-exposed to paclitaxel and then treated with doxorubicin induce DC maturation and increase the number of IL-12-producing DCs in vitro. We investigated whether the apoptotic MOSECs from this treatment sequence could influence DC maturation. After co-culture with drug-treated

MOSECs and BMDCs for 24 h, BMDCs were harvested and isolated using anti-CD11c antibody. Interestingly, the expression of CD40 and CD86 in DCs co-cultured with paclitaxel-exposed MOSECs post-treated with doxorubicin was higher than in DCs co-cultured with paclitaxel-exposed MOSECs post-treated with cisplatin or MOSECs treated with a single anticancer drug (Fig. 6A). RT-PCR analysis was performed with isolated BMDCs for cytokines that promote or inhibit Th1 immune response. IL-12 (p40) mRNA levels were also significantly upregulated in BMDCs co-cultured with paclitaxel-exposed MOSECs post-treated with doxorubicin or cisplatin as much as in BMDCs co-cultured with MOSEC
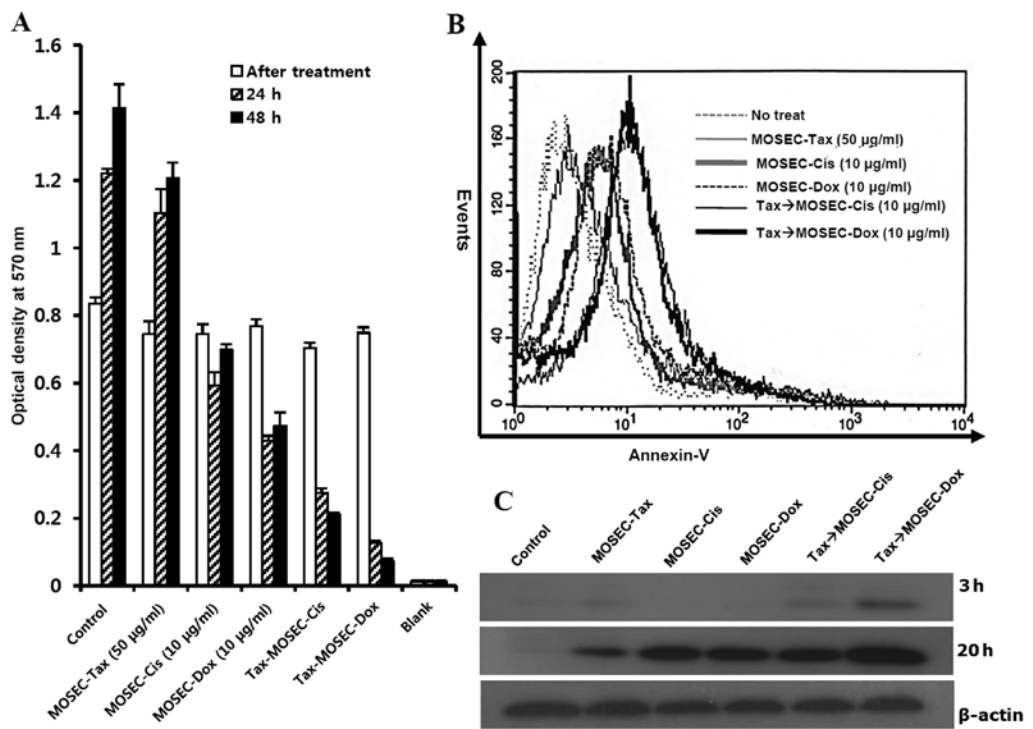
Figure 2. Viability and apoptosis in paclitaxel-treated MOSECs after subsequent treatment with doxorubicin or cisplatin. Paclitaxel-exposed (50 μg/ml) MOSECs (1.0x10^{6}) were incubated with doxorubicin (10 μg/ml) or cisplatin (10 μg/ml) at 37°C for 2 h. A pool of MOSECs treated with drugs was incubated in medium without drugs for 24 and 48 h and an MTT assay and Annexin V staining were then performed. (A) Representative MTT data show the viability of paclitaxel-exposed MOSECs after incubation with doxorubicin or cisplatin for 2 h. (B) Paclitaxel-exposed MOSECs were treated with doxorubicin or cisplatin for 2 h, and were then incubated with medium not containing drugs. The MOSECs collected after 20 h were reacted with Annexin V-FITC and analyzed by flow cytometry. (C) Quantitative immunoblot analysis of cleaved caspase-3 expression in the MOSECs treated with single anticancer drug or with paclitaxel followed by doxorubicin or cisplatin. The data are from one representative experiment that was performed twice.
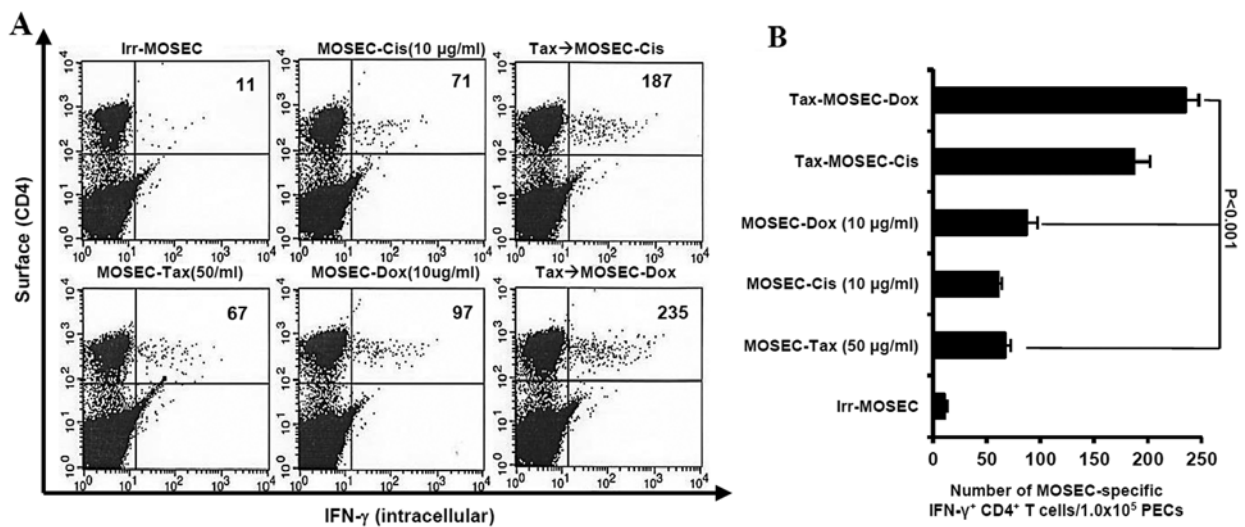

Figure 3. The effect of paclitaxel-exposed MOSECs treated subsequently with doxorubicin or cisplatin on immune responses. (A) C57BL/6 mice (n=5 per group) were immunized with paclitaxel-exposed (50 μg/ml) MOSECs post-treated with doxorubicin (10 μg/ml) or cisplatin (10 μg/ml) at 37°C for 2 h (1.0x10^{6} cells per mouse). One week after a boost vaccination, cells from the peritoneal cavity were harvested and stained with PE-conjugated anti-CD4 (L3T4) and stained for intracellular IFN-γ to characterize the MOSEC-specific CD4^{+} T cells after co-cultured with irradiated MOSECs for 16 h with complete medium in the presence of GolgiPlug. The numbers of CD4^{+} IFN-γ^{+} double-positive T cells in 1.0x10^{5} peritoneal exudate of cells (PECs, 1.0x10^{5}) are indicated in the upper right corner. (B) Comparisons between all individual data were made using one-way ANOVA. The bar graph represents the mean ± SEM numbers of antigen-specific IFN-γ-secreting CD4^{+} T-cell precursors per PECs (1.0x10^{5}). The data are from one representative experiment that was performed twice.

exposed to either LPS or paclitaxel alone. In contrast, IL-6 and TNF-α mRNA levels did not change significantly compared with BMDCs co-cultured with irradiated-only MOSECs as

control (Fig. 6B). A significantly high level of IL-12 concentration was also detected in culture media harvested after paclitaxel-exposed MOSECs post-treated with doxorubicin or
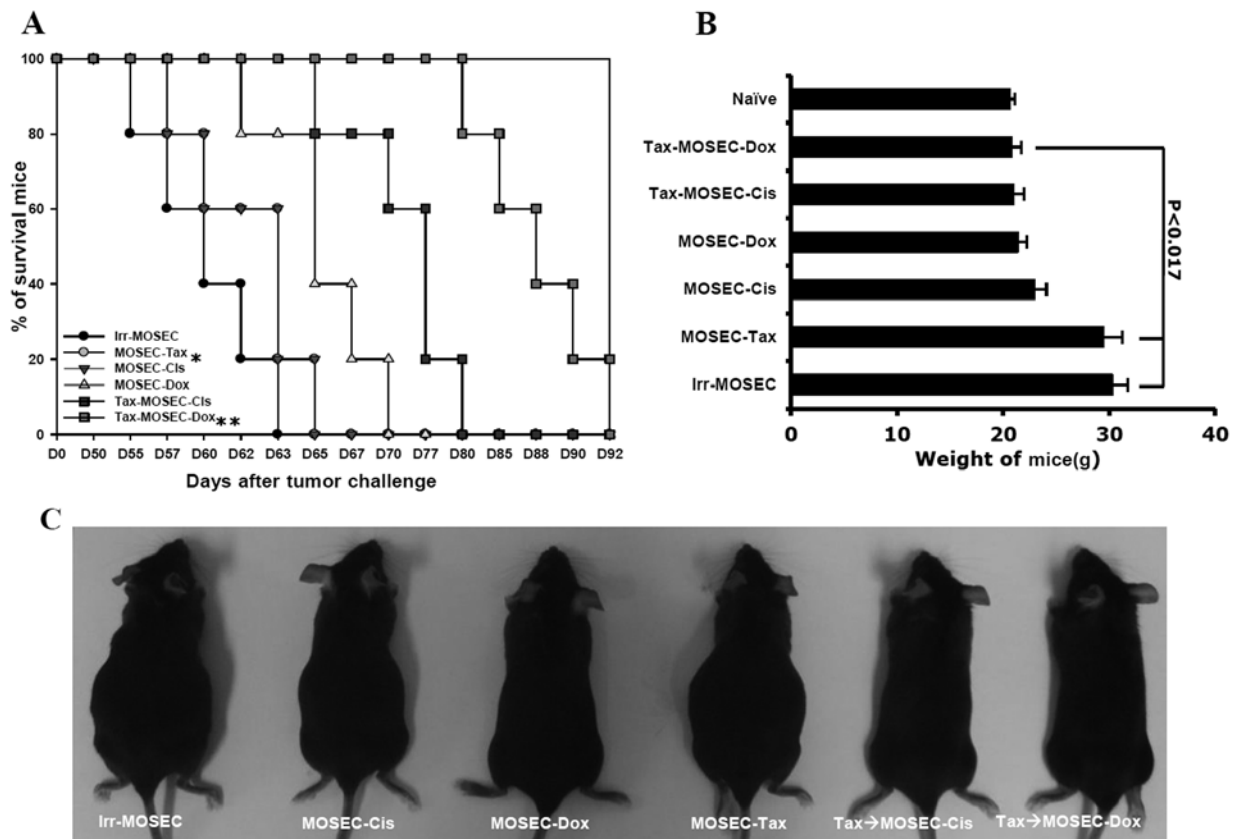
Figure 4. The effect of paclitaxel-exposed MOSECs treated subsequently with doxorubicin or cisplatin on antitumor immunity in vaccinated mice. C57BL/6 mice (n=6-7 per group) were initially challenged with 1.0×10^6 MOSECs per mouse by intraperitoneal injection. The mice were vaccinated with paclitaxel-exposed (50 µg/ml) MOSECs (1.0×10^6) that were subsequently incubated with doxorubicin (10 µg/ml) or cisplatin (10 µg/ml) at 37°C for 2 h. The mice were injected with the same regimen one week later. At 50 days after tumor challenge, the general condition and weight of each mouse were monitored twice weekly to assess the tumor burden and ascites accumulation. (A) Kaplan-Meier survival analysis for the tumor treatment categories (*p<0.001, Tax→MOSEC-Dox versus MOSEC-Tax; **p<0.05, Tax→MOSEC-Dox versus Tax→MOSEC-Cis). (B) Comparison of the animal weights at 50 days after tumor challenges (mean ± SEM) (*p<0.017, Tax→MOSEC-Dox versus MOSEC-Tax). (C) Representative images of tumor-bearing mice treated with various regimens at day 50 after tumor inoculation. Comparisons between all individual data were made using one-way ANOVA. The data are from one representative experiment that was performed twice.
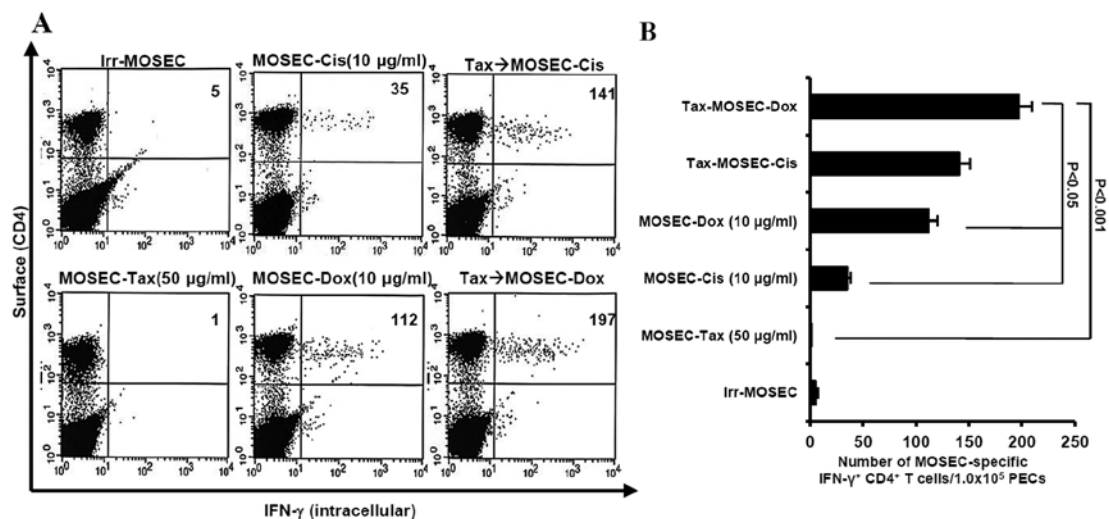

Figure 5. Generation of the long-term CD4+ T-cell immune response after immunization with paclitaxel-exposed MOSECs post-treated with doxorubicin or cisplatin. (A) C57BL/6 mice (n=5 per group) were immunized with paclitaxel-exposed MOSECs post-treated with doxorubicin or cisplatin. At 50 days after the boost vaccination, cells from the peritoneal cavity were harvested and stained with PE-conjugated anti-CD4 (L3T4) and stained for intracellular IFN-γ to characterize MOSEC-specific CD4+ T cells after co-cultured with irradiated MOSECs for 16 h with complete medium in the presence of GolgiPlug. The numbers of CD4+ IFN-γ+ double-positive T cells in 1.0×10^5 PECs are indicated in the upper right corner. (B) The bar graph represents the mean ± SEM numbers of antigen-specific IFN-γ-secreting CD4+ T-cell precursors per pool of PECs (1.0×10^5) (*p<0.001, Tax→MOSEC-Dox versus MOSEC-Tax; *p<0.05, Tax→MOSEC-Dox versus MOSEC-Dox, MOSEC-Cis). Comparisons between all individual data were made using one-way ANOVA. The data are from one representative experiment that was performed twice.
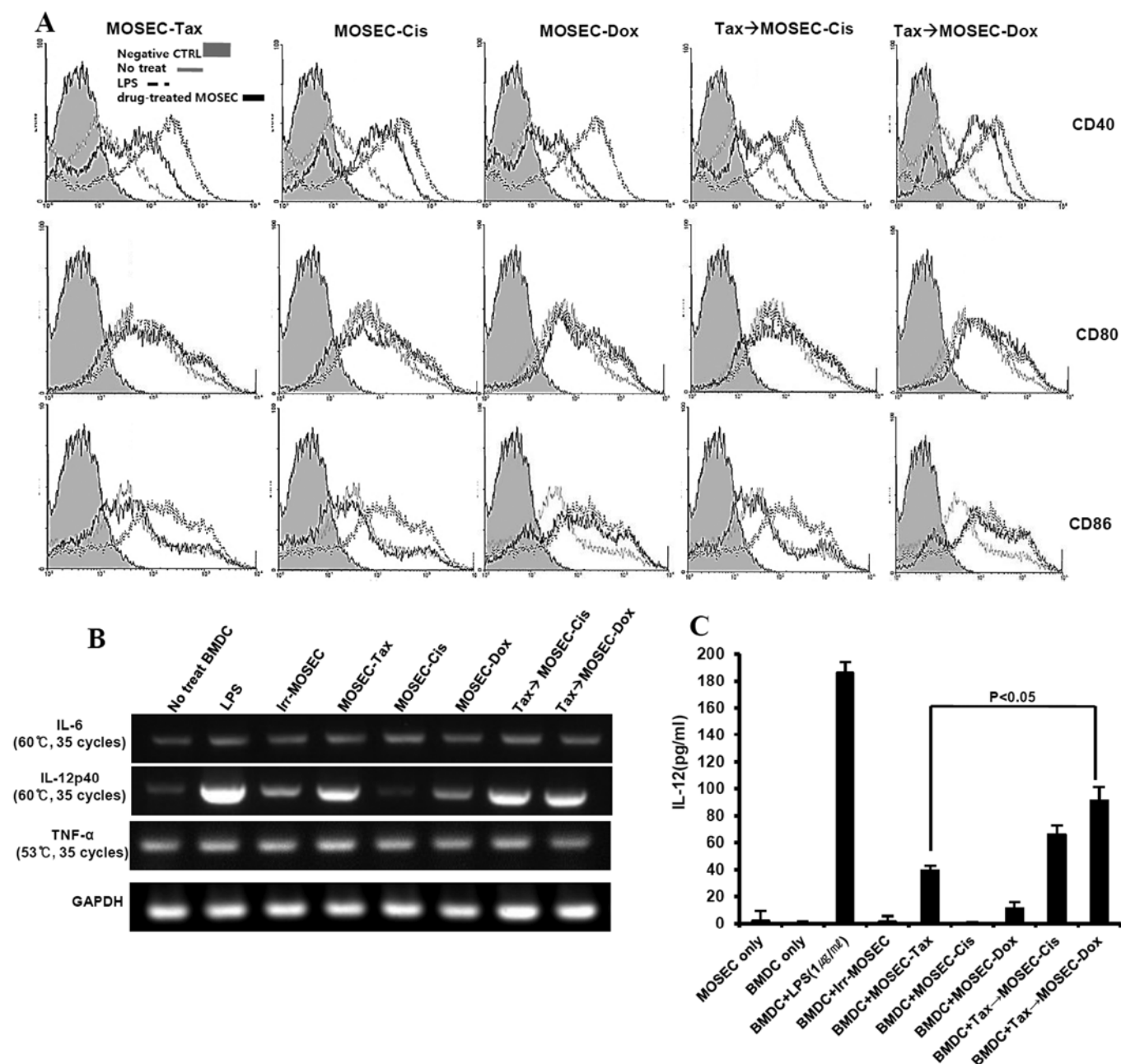
Figure 6. Maturation and the cytokine mRNA levels in bone marrow-derived DCs after co-culture with MOSECs treated with anticancer drugs. BMDCs derived from C57BL/6 mice were co-cultured with MOSECs treated with drugs as described in text at a ratio of 1:1 (MOSEC:DC) in a 24-well plate for 24 h. The DCs were isolated from co-culture using anti-CD11C antibody and immunostained to detec T cell surface markers and cytokines. (A) The cells contained medium, as untreated control; and LPS (800 ng/ml) as positive control. Co-stimulatory molecules were stained with anti-CD80, anti-CD86, and CD40. (B) RT-PCR analysis of IL-12, IL-6, and TNF-α expression. Data for LPS-treated BMDCs and for untreated BMDCs are presented as controls. (C) IL-12 secreted by BMDCs in the supernatants was measured by ELISA after BMDCs were co-cultured with MOSECs treated with drugs ($^*p<0.05$, Tax→MOSEC-Dox versus MOSEC-Tax). Comparisons between all individual data were made using one-way ANOVA. The data are from one representative experiment that was performed twice.

cisplatin for 24 h compared to that of paclitaxel alone (Fig. 6C). Our results suggest that the apoptotic MOSECs treated sequentially with paclitaxel and doxorubicin stimulate BMDCs to mature and to secrete cytokine to regulate Th1 cells.

MOSECs exposed to paclitaxel and doxorubicin in sequence downregulate MyD88 in cancer cells and upregulate MyD88 in DCs. LPS, a ligand for Toll-like receptor 4 (TLR4), shares with paclitaxel certain receptors and signaling molecules for immune cell activation (10). In patients with ovarian cancer,

tumor expression of MyD88, adaptor molecule for TLR4, may correlate negatively with survival (26). So, we first assessed the expression levels of TRAF6 and NF-κB, downstream of MyD88 signaling, in paclitaxel-exposed MOSECs post-treated with doxorubicin or cisplatin to explore the mechanism of resistance or susceptibility to the anticancer drug. We found that MOSECs constitutively expressed MyD88 and TRAF6, even sustained or upregulated expression of MyD88, TRAF6 and NF-κB at 20 h after exposure to paclitaxel. NF-κB was downregulated in MOSECs treated with doxorubicin or
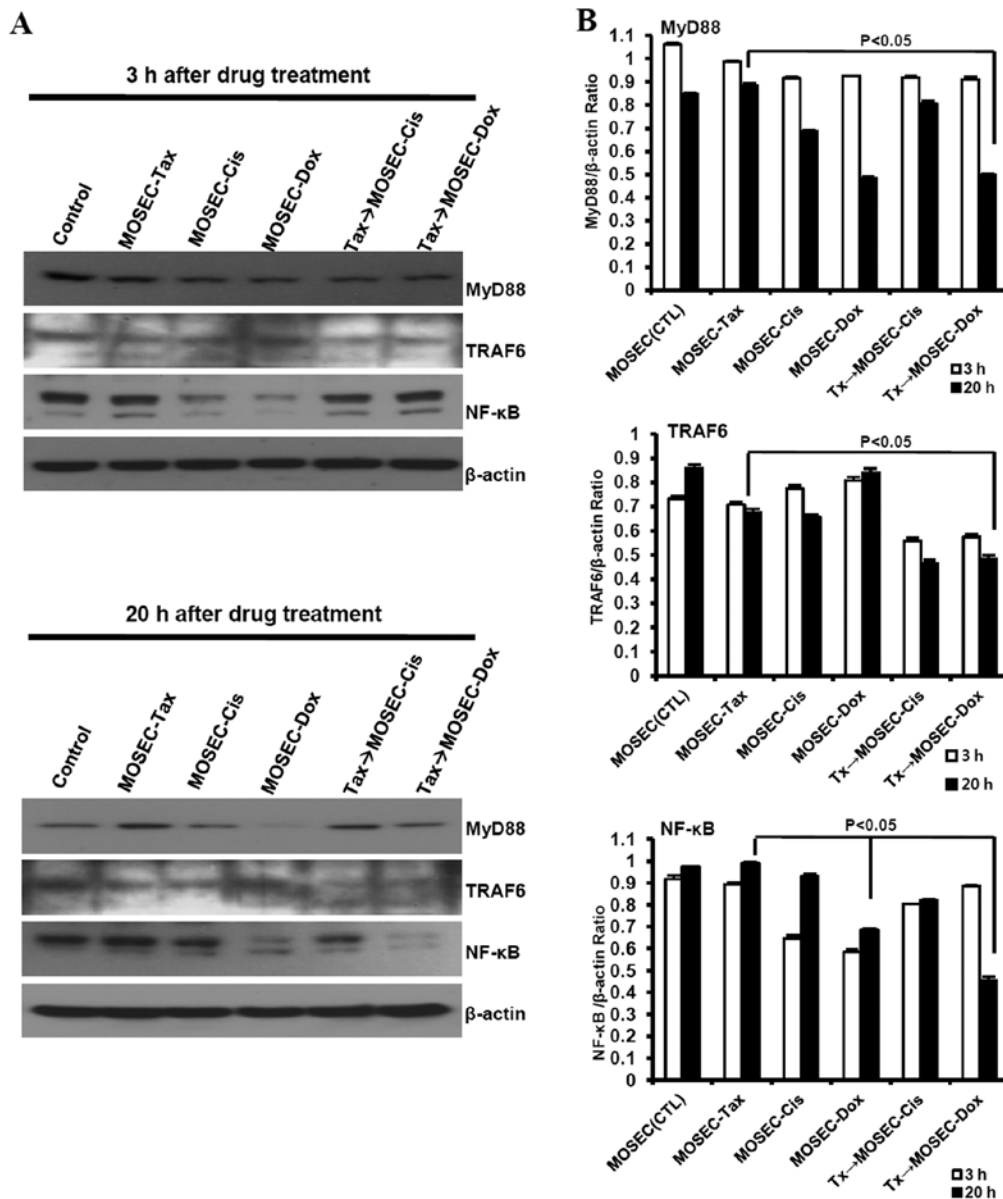
Figure 7. The expression of MyD88 and target proteins in paclitaxel-pre-exposed MOSECs treated with other cancer drugs. (A) Paclitaxel-exposed (50 μg/ml) MOSECs (5.0x10^6) were incubated in the presence of doxorubicin (10 μg/ml) or cisplatin (10 μg/ml) at 37˚C for 2 h and harvested at 3 h and 20 h after the end of last drug treatment. Cell lysates were prepared and blotted with anti-MyD88, TRAF6 and anti-NF-κB. (B) The densitometry values of target proteins were normalized to β-actin of the same sample. Comparisons between all individual data were made using one-way ANOVA. Data are expressed as mean value ± SEM. p-value compared MOSEC-Tax with Tax→MOSEC-Dox.

cisplatin only at 3 h after the end of treatment. The expression level of MyD88, TRAF6 and NF-κB was significantly decreased in paclitaxel-exposed MOSECs treated with doxorubicin compared to MOSECs pre-exposed paclitaxel only at 20 h after the end of treatment. Interestingly, we also observed a slight downregulation of TRAF6 expression, but not MyD88 and NF-κB, in paclitaxel-exposed MOSECs post-treated with cisplatin compared to MOSECs pre-exposed paclitaxel only at 20 h after the end of treatment (Fig. 7). Next, we examined the effect of paclitaxel-exposed MOSECs post-treated with doxorubicin on MyD88 signaling in BMDCs because previous data showed that BMDCs co-cultured with paclitaxel-exposed MOSECs post-treated with doxorubicin produced significant amount of IL-12. In BMDCs co-cultured with paclitaxel-exposed MOSECs post-treated with doxorubicin, the MyD88

and TRAF6 expression increased; this increase did not occur when the MOSECs were post-treated with cisplatin or were treated with paclitaxel only (Fig. 8). These findings suggest that MyD88 downregulation in paclitaxel-exposed MOSECs post-treated with doxorubicin increased susceptibility of these cells to apoptosis and that doxorubicin post-treatment of the MOSECs enabled them to induce MyD88-dependent BMDC maturation and IL-12 secretion to generate immune responses.

Discussion

Whole tumor cell vaccines may be easily prepared and administered directly by a physician without technical guidance. Early forms of whole cell vaccines usually consisted of killed tumor cells or lysates mixed with bacterial adjuvants (27,28).
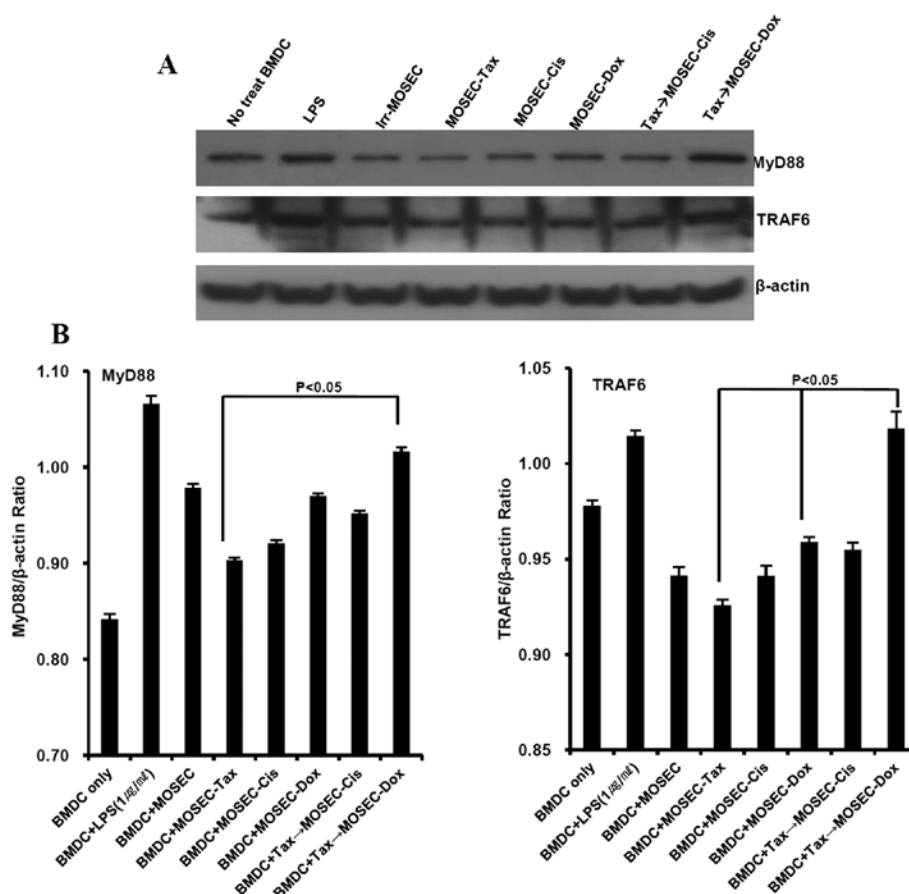
Figure 8. MyD88 expression in paclitaxel-exposed MOSECs and BMDCs. BMDCs derived from C57BL/6 mice were co-cultured with MOSECs treated with drug regimen at a ratio of 1:1 (MOSEC:DC) in a 24-well plate for 24 h. (A) The BMDCs were isolated by anti-CD11C antibody and lysed with RIPA buffer for immunoblot analysis for MyD88 and TRAF6. (B) The densitometry values of target proteins were normalized to β-actin of the same sample. Comparisons between all individual data were made using one-way ANOVA. Data are expressed as mean value ± SEM. p-value compared MOSEC-Tax or MOSEC-Dox with Tax→MOSEC-Dox.

However, the mechanisms of bacterial adjuvants are not understood very well, and their use may result in side-effects and inconsistent outcomes. In the present study we demonstrated an increase in the immunogenicity of paclitaxel-exposed ovarian cancer cells following brief exposure of the cells to a low dose of second anticancer drug (10 μg/ml), doxorubicin. From these results, we propose that tumor cells treated in this way may be used to produce a whole cell vaccine against ovarian cancer.

Chemotherapy for cancer is severely immune-suppressive, and is therefore very difficult to combine with immunotherapy. Acquired resistance to combined therapy with paclitaxel and cisplatin is also major reason for poor prognosis. Paclitaxel is reported to be a ligand to TLR4 (10). Paclitaxel-induced signaling activates NF-κB, leading anti-apoptotic molecule in cancer cells, through mediation by adaptor protein MyD88, which links with the cytoplasmic portion of TLR4. In mice, but not in humans, paclitaxel-induced NF-κB activation occurs through an LPS-mimetic pathway that also involves TLR4 (10). After receptor activation, a number of adaptor proteins which are involved MyD88 downstream signaling are recruited, such as IL-1 receptor-associated kinases (IRAK4), tumor necrosis factor receptor-associated factor 6 (TRAF6) and NF-κB. The kinase activity of IRAK-4 has also been shown to be essential for signaling, as overexpression of the kinase-dead form

of IRAK-4 resulted in a reduction in LPS-induced NF-κB activation (29). Recent studies also showed that TLR4/MyD88 signaling via TRAF6 and IRAK4 enhances invasiveness of human lung cancer cells through NF-κB and p38 MAPK pathway (30). We observed that MOSECs constitutively expressed MyD88 and TRAF6, even sustained or upregulated expression of MyD88, TRAF6 and NF-κB at 20 h after exposure to paclitaxel. However, our results also showed that MyD88, TARF6 and NF-κB expression in paclitaxel-exposed MOSECs post-treated with doxorubicin were all downregulated compared to MOSECs pre-exposed paclitaxel only. These results suggest that sequential drug combination might be one choice to overcome paclitaxel resistance in cancer cells. Another study also suggests that paclitaxel promotes cell survival by upregulation of the anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP) and of Akt phosphorylation (pAkt is inactive) through TLR4 ligation (26). The essential role of MyD88 in this sequence is supported by observation that tumor expression of MyD88 correlates negatively with patient survival in some studies (26).

TLR4/MyD88 signaling generates immune responses against cancer. Anthracycline drugs including doxorubicin induce rapid, pre-apoptotic translocation of calreticulin (CRT) to the cell surface and result in improved processing of tumor

cells by dendritic cells (31,32). However, synergistic effect of paclitaxel plus doxorubicin on enhancement the antitumor immunotherapy through an immune-modulatory action is not well investigated. Our results showed that paclitaxel-exposed MOSECs post-treated with doxorubicin induced CD4^{+} T-cell immune responses without the immune-suppression associated with chemotherapeutic drug treatment. Based on these results, paclitaxel and doxorubicin might also be especially effective as first-line chemotherapeutic drugs for MyD88-positive cancer in a situation where chemo- and immunotherapy are combined. Further investigation is needed, however, to fully understand the relationships between TLR4-MyD88 signaling and other immune-suppressive pathways, which may involve, for example, Stat3.

Previous studies show that MyD88^{-/-} BMDCs fail to upregulate IL-12 and IFN-α and -γ in response to viral particles and thus fail to induce Th1 immune responses and that DC activation by TLR4 ligands requires MyD88 (33,34). IL-12 produced by DC augments the cytotoxicity of T cells and NK cells and regulates IFN-γ production (35). Recently, another study also suggested that TLR4 and TLR2 play different roles in inflammation in a heart model (36). However, our results showed that BMDCs co-cultured with sequential paclitaxel and doxorubicin treatment activated MyD88 and TRAF6 signaling and result in generating significant IL-12p40 mRNA and IL-12 protein compared to other groups. Although MOSECs treated with paclitaxel only for a short time (2 h) also produced some IL-12, it might not be useful for clinical application because they showed resistance to paclitaxel. We also expect that little amount of paclitaxel and doxorubicin brought by MOSECs might play an important role to mature and activate DCs via TLR4 and TLR2 signaling. From these results, we need to further investigate and confirm the sequential combination with other cancer drugs.

Several recent studies showed that CD4^{+} T cells could eliminate tumors, even when the tumors expressed MHC class I, but not MHC class II, and this suggested that the CD4^{+} T cell responses could outperform the CD8^{+} CTLs in mediating an antitumor effector function (37,38). It has been reported that the CD4^{+} T-cell functions against cancer is maximized in the presence of NK cells (39). We might expect NK cells help to sustain the CD4 response through the activation of DCs or NK-DC interaction from the results that DCs produced the IL-12 after co-culture with paclitaxel-exposed MOSECs post-treated with doxorubicin. However, further investigation is required to find the specific bridge between NK cells and cancer-specific CD4^{+} T cells in antitumor immunotherapy. We also recognize the need to discover additional tumor-specific antigens expressed only on cancer cells that will induce CTLs against ovarian cancer, and also to optimize the strategies and conditions for using immunotherapy.

Taken together, our results using sequential treatment of ovarian cancer cells with paclitaxel and doxorubicin suggest a new model for overcoming cancer drug resistance and generating antitumor immune responses. However, we do not know exactly how much of each drug was delivered and the effects on the cancer cells. Further investigations may reveal other potentially effective combinations of drugs and through optimization of drug dosages and immunization schedules may lead to new clinical applications.

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