Optimizing DC vaccination by combination with oncolytic adenovirus coexpressing IL-12 and GM-CSF

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Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Chae-Ok Yun

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This certifies that the doctoral dissertation of Song-Nan Zhang is approved

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이 감사의 글을 작성하면서 저는 머지않아 졸업을 하게 됩니다. 이 학위논문이 저의 4년의 박사과정을 전부 대변할 수 없고 또 학위논문을 완성하면서 아쉬운 점도 많았지만 그래도 최선을 다한 것 같아서 후회는 없었습니다. 이 자리를 빌어서 4년 동안 저에게 도움을 주셨던 그리고 많은 의지가 되었던 분들한테 감사의 말씀 올리고 싶습니다.

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항상 자애로우시고 우리 학생들을 잘 챙겨주시는 김주항 교수님, 병원 일에 바쁘시면서도 항상 시간만 나면 실험실에 들리셔서 학생들에게 힘을 북돋아 주셨던 교수님께 감사 드립니다. 그리고 저를 CSC의 세계로 이끌어주셨던 항상 친근하신 손주혁교수님한테도 감사의 말씀 드리고 싶습니다. 친누나처럼 항상 잘 챙겨주는 최혜진 선생님, 유쾌한 성격이 저한테에도 행복바이러스를 심어주시는 같아서 항상 즐거웠습니다.

실험실에서 저의 사부로 실험을 가르쳐 주셨던 성격 급하신 우리 지영누나와 항상

여유로우신 황경화 선생님, 많은 것을 아낌없이 가르쳐 주셨던 두분한테 고맙다는 말씀을 전해드리고 싶습니다. 실험실의 원로맴버이셨던 김재성 선배님, 실험실에서 같이 한 시간이 얼마 안되지만 선배님한테서 연구를 즐길수 있는 방법을 배우게 되었습니다. 실험실의 맏형으로서 모든 일에 책임적이고, 하는 말씀보다 마음씨가 더 따뜻한 김인욱 선배님, 항상 친절하고 맏누님으로서 모든 것을 떠맡고 계시는 민정누나, 졸업하고 다른곳에서 꿈을 펼치는 경주누나, 친절한 평환형, 덕, 지, 미를 겸비한 쿨한 성격의 아름누나, 푸근하고 친근한 동네형인 지훈형과 정우형, 자랑스러운 준모의 엄마가 된 정선누나, 우리 실험실 분위기 메이커이면서 실험에만은 누구보다도 진지한 내친구 일규, 항상 듬직한 태진이, 연구를 이제 막 시작하는 리연이, 항상 인사성고 있는 밝은 동생 오준이, 그리고 오준이와 함께 좋은 만남을 이어가고 있는 이쁜 지성이, 실험실 생활을 금방 시작한 햇병아리와 같은 성경과 언주, 그외에 바이러스로 인연을 맺게 된 하태영 선배, 착한 후배 민주, 성미 그리고 귀여운 동생 혜원이, 모두가 제가 한국에서 만나게 된 소중하고 고마운 인연들입니다. 모두들 항상 건강하시고 훌륭한 연구들을 계속해 나가시길 바랍니다.

저를 친아들처럼 아껴주시고 관심해주시는 저의 장인님과 장모님, 바쁘다는 핑계로 자주 찾아뵙지도 못했던 두분한테 감사의 마음을 전하고 싶습니다. 그리고 하나라도 더 챙겨줄려고 하는 처형 그리고 친형과도 같은 형님께도 고마운 마음을 전합니다. 같이 박사과정으로 입학하여 동기로 졸업하게 될, 앞으로 평생 동안 동고동락해야 할 사랑스러운 나의 아내에게 정말 고맙다는 말을 전합니다. 지난 힘들었던 시절에 옆에 있어주지 못해 항상 미안한, 지금 의젓한 석사생이 된 송철에게 감사의 마음을 전합니다. 그리고 가장의 의무를 다하기 위해 평생을 바친, 힘들었던 시절을 꿋꿋이 버텨나가셨던 존경하는 아버지에게 머리 숙여 감사의 마음을 전해드리고 싶습니다. 마지막으로 두 아들을 위해 평생을 고생하시다 돌아가신 어머님께 투병동안에도 곁을 지켜드리지 못하여 죄송한 마음, 보고싶은 그리움과 함께 이 논문을 삼가 드립니다.

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ABSTRACT

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(Directed by Professor Chae-Ok Yun)

Dendritic cell (DC)-based vaccination is a promising strategy for cancer immunotherapy. However, clinical trials have indicated that immunosuppressive microenvironments induced by tumors profoundly suppress antitumor immunity and inhibit vaccine efficacy, resulting in insufficient reduction of tumor burdens. To overcome these obstacles and enhance the efficiency of DC vaccination, we generated IL-12- and GM-CSF-coexpressing oncolytic adenovirus (Ad-ΔB7/IL12/GMCSF) as suitable therapeutic adjuvant to eliminate immune suppression and promote DC function. By treating tumors with Ad-ΔB7/IL12/GMCSF prior to DC vaccination, DCs elicited greater antitumor effects than in response to either treatment alone. DC migration to draining lymph nodes (DLNs) dramatically

increased in mice treated with the combination therapy. This result was associated with upregulation of $CCL21^+$ lymphatics in tumors treated with Ad-ΔB7/IL12/GMCSF. Moreover, the proportion of $CD4^+CD25^+$ T cells and VEGF expression was decreased in mice treated with the combination therapy. Furthermore, combination therapy using immature DCs also showed effective antitumor effects when combined with Ad-ΔB7/IL12/GMCSF. The combination therapy had a remarkable therapeutic efficacy on large tumors. Taken together, Oncolytic adenovirus coexpressing IL-12 and GM-CSF in combination with DC vaccination has synergistic antitumor effects and can act as a potent adjuvant for promoting and optimizing DC vaccination.

Key word: Cancer immunogene therapy; Adenovirus; Interleukin-12; Granulocyte-macrophage colony stimulating factor; Dendritic cell

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I. INTRODUCTION

Dendritic cells (DC) are potent antigen-presenting cells with a unique capability to induce primary immune responses to self or foreign antigens. DC-based vaccines are a promising strategy in cancer immunotherapy [1]. DCs pulsed with tumor-associated antigen can induce protective and therapeutic tumor-specific immune responses to various tumor types [2-4]. Recently, DC-based vaccine clinical trials have been performed with patients with prostate cancer, melanoma, lymphoma, and renal carcinoma [5-8]. However, beneficial patient responses were fewer than predicted. Although tumor-specific immune responses were seen in most of the patients, objective clinical responses were only observed in a few patients.

DCs have been reported to have significantly impaired biological functions in advanced cancer patients, which might be associated with the

failure of the DC vaccination in clinical trials [9-10]. Emerging evidence indicates that this effect might be mediated by various factors produced by tumor cells. Tumor tissues can produce immunosuppressive molecules, such as VEGF, TGF-β, and IL-10. These molecules induce an immunosuppressive microenvironment within the tumors. The function of tumor-associated DCs may also be suppressed under these conditions [11-12]. Therefore, DC vaccination efficacy may be improved by the addition of an adjuvant to reduce or eliminate tumor-induced immunosuppression.

Cytokine therapy has been reported to be an effective strategy for cancer therapy [13]. Ectopic cytokine expression in tumor tissues can induce strong antitumor immune responses, and can also convert the immunosuppressive tumor microenvironment to one with more antitumor characteristics. Interleukin (IL)-12 is an important cytokine in innate and adaptive immunity that is able to enhance Th1 immunity, increase CTL cytotoxicity, and inhibit angiogenesis [14]. Preclinical and clinical trials of IL-12-based immunotherapy have demonstrated strong antitumor immune responses [15-17]. Previous studies reported that immunosuppressive conditions can be reversed by induction of intratumoral IL-12 expression. These results were associated activation of effector T cell) , inducton of Treg cell apoptosis, and infiltration of CTL [18]. In addition, IL-12-induced IP-10 and Mig expression are strong inhibitors of

tumor angiogenesis [19]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a wide range of cell types and functions to stimulate proliferation, maturation, and function of antigen presenting cells (APCs) [20]. Previous studies have demonstrated an antitumor effect of GM-CSF in cancer immunotherapy, indicating that GM-CSF is a useful therapeutic agent in cancer therapy [21-22].

Recently, adenovirus (Ad) vectors as efficient gene delivery systems have been widely used in cancer gene therapy. Cytokine gene therapy can induce sustained antitumor effect via sustained expression of therapeutic dose of cytokine in the local tumor tissue and that can prolong the antitumor effect and reduce toxicity compared with treatment of recombinant cytokine protein [23-25]. Moreover, Ad vectors can be modified to allow for replication in only cancer cells [26]. Our previous studies have demonstrated that an oncolytic Ad can greatly amplify gene delivery efficacy in a cancer cell-restricted manner while they replicate [27-28]. In this report, we investigated the antitumor effects of an IL-12- and GM-CSF-coexpressing oncolytic Ad (Ad-ΔB7/IL12/GMCSF) in combination with DC vaccination in a murine melanoma model. This combination therapy elicited strong and synergistic antitumor effects, correlated with the elimination of immunosuppression in tumor tissues, and facilitated DC migration to draining lymph nodes (DLNs).

These preclinical results indicate that an oncolytic Ad coexpressing IL-12 and GM-CSF can act as a potent adjuvant for optimizing DC vaccination.

II. MATERIALS AND METHODS

Cell lines and cell culture

B16-F10 (murine melanoma) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Mice

C57BL/6 mice were obtained from SLC (Tokyo, Japan). Mice were used at 6-7 week of age, and all animal studies were performed according to institutionally approved protocols at Yonsei University College of Medicine.

Adenoviral vectors

To generate an Ad expressing IL-12 and GM-CSF at the E1 and E3 region, respectively, we first constructed an E3 shuttle vector expressing GM-CSF. The murine GM-CSF gene was excised from pCDNA3.1/GMCSF using NheI and XhoI, and sub-cloned into the Ad E3 shuttle vector, pSP72-E3, generating a pSP72/E3/GMCSF E3 shuttle vector. The newly constructed

pSP72/E3/GMCSF was then cotransformed with an Ad total vector (pvmdl324BstBI) into *Escherichia coli* BJ5183, yielding a pdl324ΔE3/GMCSF Ad plasmid. Structure of the resultant recombinant vector was confirmed by restriction enzyme digestion and PCR analysis. To construct an Ad E1 shuttle vector expressing IL-12, murine IL-12 gene excised from pCA14/IL12 [29] was subcloned into pXC1ΔB7 E1 shuttle vector [26], generating a pXC1ΔB7/IL12 E1 shuttle vector. The newly constructed pXC1ΔB7/IL12 E1 shuttle vector was then cotransformed with pdl324ΔE3/GMCSF into *E. coli* BJ5183 for homologous recombination, generating a pAd-ΔB7/IL12/GMCSF Ad vector (Figure 1A). In addition, we also constructed oncolytic Ad expressing either IL-12 (Ad-ΔB7/IL12) or GM-CSF (Ad-ΔB7/GMCSF) alone. Preliminary, we tested therapeutic efficacy of Ad-ΔB7/IL12/GMCSF, along with Ad-ΔB7, Ad-ΔB7/IL12 and Ad-ΔB7/GMCSF. The result showed significant enhanced antitumor effect was observed in Ad-ΔB7/IL12/GMCSF treated group compared with other groups (data not shown). According to this result, we selected only Ad-ΔB7/IL12/GMCSF as therapeutic agent for using in this study. All viruses were propagated in 293 cells and purified by CsCl density purification, dissolved in storage buffer (10 mmol/L Tris, 4% sucrose, 2 mmol/L $MgCl_2$), and stored at -80℃. Viral particle numbers were calculated from

measurements of absorbance at 260 nm (A_{260}), where 1 absorbency unit is equivalent to 10^{12} viral particles/mL.

Generation of bone marrow-derived dendritic cell

Bone marrow cells were harvested from flushed marrow cavities of femurs and tibias of C57BL6 mice under aseptic conditions. The cells were depleted of erythrocytes using RBC lysis buffer (Sigma, St Louis, MO) and were cultured in complete RPMI 1640 media (Gibco BRL) supplemented with 10% FBS, GM-CSF (10 ng/mL, ENDOGEN), and IL-4 (10 ng/mL, ENDOGEN), 2-mercaptoethanol 50 μmol, 100 IU/mL penicillin, and 100 μg/mL streptomycin. On day 2, the non-adherent cells were removed and the plates were replenished with fresh complete media containing GM-CSF and IL-4. On day 4, culture supernatant was collected and centrifuged, and the cell pellet was re-suspended in fresh media containing cytokines and returned to the plate. On day 6, the DCs were incubated with the tumor lysate (50 μg/mL) for 24 hr. LPS (1 μg/mL, Sigma) were added at day 7 for DCs maturation. After incubation for 24 hr, mature DCs were harvested and used in following studies. Immature DCs were cultured at same condition for 6 days as described above. But, the cell was not exposed to the tumor lysate and LPS.

Fluorescence-activated cell sorting (FACS) analysis

For the phenotypic analysis, the DCs were stained with surface molecules using immunofluorescence and analyzed by fluorescence-activated cell sorting (FACS) analysis. Cells were stained with anti-mouse CD11c, CCR7, CD40, CD80, CD86, or MHC I/II (Pharmingen, San Diego, CA) antibody at 4^{o}C for 45 min. After twice of PBS washing, the cells were incubated with FITC-conjugated goat anti-rat IgG secondary antibody at 4^{o}C for 15 min. For the assessment of $CD4^{+}CD25^{+}$ T cell population in the DLN, single cells were obtained from DLN after mechanical dissociation. The cells were stained with anti-mouse CD4 (PE) and anti-mouse CD25 (FITC) antibody at 4^{o}C for 45 min. All samples were analyzed on a BD Biosciences BD-LSR II Analytic Flow Cytometer, using FACSDiva software (BD Biosciences, San Jose, CA).

In vivo *antitumor effect*

B16-F10 cells (5×10^{5}) were injected subcutaneously (s.c.) into the right abdomen of 6 to 7 week-old male C57BL/6 mice. When the tumor volumes reached of around 120-130 mm^{3}, mice were sorted into groups with similar means of tumor volumes. Treatment groups included PBS-only control, Ad-

ΔB7/IL12/GMCSF only (5×10^9 VP/injection), DCs only (1×10^6 cells/injection), or a combination of DCs and Ad-ΔB7/IL12/GMCSF). In parallel, one group of mice was given a treatment regimen of a high dose of Ad-ΔB7/IL12/GMCSF (5×10^{10} VP/injection) in combination with DCs (1×10^6/injection). Tumor-bearing mice were intra-tumorally injected with three doses of Ad on days 0-2, followed by three injections of DCs on days 3-5. Tumor growth was monitored every other day using a caliper, and tumor volume was calculated by the following formula: volume = 0.523 $LW^{2\ 30}$, where L is length and W is width. Animals with tumors that were >3,000 mm^3 were killed for ethical reasons.

Expression of IL-12 and GM-CSF

IL-12 and GM-CSF expression were determined using an ELISA according to the manufacturer's instructions. B16-F10 melanoma cells were plated onto six-well plates at 1×10^4 per well and then infected with Ad-ΔB7/IL12/GMCSF at MOIs of 50, 100, and 500. At 48 hr after infection, supernatants were harvested and the level of IL-12 and GM-CSF was determined with conventional IL-12 ELISA kit (ENDOGEN, Woburn, MA) and GM-CSF ELISA kit (R&D systems, Minneapolis), respectively. For the assessment of cytokine expression in tumor tissue, tumor tissues were

removed from mice treated with Ad and/or DCs at 3 days after final treatment. Tissues were homogenized and liquefied in PBS containing protease inhibitor cocktail (Sigma). IL-12, GM-CSF, VEGF, and TNF-α level were measured by conventional ELISA kits (ENDOGEN, R&D systems). Each experiment was carried out three to four times with three replicates in each group.

Histology and immunohistochemistry

Tumor tissues were harvested from mice after 3 days of final treatment, and embedded in paraffin and sectioned at a thickness of 4 μm for hematoxylin and eosin (H&E) staining. For immunohistochemical staining, tumor tissues were snap-frozen and sectioned at a thickness of 7 μm. Tumor sections were blocked with 4% PBS–bovine serum albumin (Sigma) for 1 hr and incubated over night with appropriate dilution of anti-CD4 (purified rat anti-mouse CD4 monoclonal antibody; Pharmingen, San Diego, CA), anti-CD8 (purified rat anti-mouse CD8 monoclonal Ab; Pharmingen), anti-CD86 (purified rat anti-mouse CD86 monoclonal antibody; Pharmingen), anti-CD11c (purified hamster anti-mouse CD11c monoclonal antibody; Pharmingen), or anti-CCL21 (purified rat anti-mouse CCL21 monoclonal antibody; R&D systems) in antibody diluents (DAKO, Denmark). After overnight incubation, the sections were washed twice in PBS and incubated

with HRP-conjugated goat anti-rat or mouse anti-hamster antibody (Southern Biotechnology, Birmingham, AL) for 1 hr. Diaminobenzidine /hydrogen peroxidase (DAKO, Denmark) was used as the chromogen substrate. All slides were counterstained with Meyer's hematoxylin.

***Evaluation of DC migration* in vivo**

DCs were labeled with CellTracker Red CMTPX (Invitrogen, Carlsbad, CA) on day 6 of DC culture and were harvested on day 8. The tumor-bearing mice were intra-tumorally injected with DCs (1×10^6/time) alone three times every day or intra-tumorally injected with Ad-ΔB7/IL12/GMCSF (5×10^9 VP/time or 5×10^{10} VP/time) three times every day prior to DC injection. At 48 hr after final treatment, the DLNs were harvested and dissociated into single cells for FACS analysis.

Statistical analysis

The data was expressed as mean ± standard error (SE). Statistical analyses of the data were performed using the two-tailed Student *t* test (SPSS 13.0 software; SPSS, Chicago, IL). *P* values of less than 0.05 were considered statistically significant (*, $p < 0.05$; **, $p < 0.01$). Analysis of variance

(ANOVA) was used for multiple group comparison on antitumor effect examination.

III. RESULTS

Oncolytic Ad-mediated IL-12 and GM-CSF expression

Ad-ΔB7, an oncolytic Ad, was mutated in the retinoblastoma binding sites of E1A and had the E1B region deleted, as shown in Figure 1A. The cancer-specific viral replication and cytotocixity of Ad-ΔB7 has been previously confirmed [26]. To generate the Ad-ΔB7/IL12/GMCSF oncolytic Ad, murine IL-12 and GM-CSF genes were inserted into the E1 and E3 regions of the Ad-ΔB7 viral genome, respectively. Cultured murine melanoma B16-F10 cells were infected with Ad-ΔB7/IL12/GMCSF at MOIs of 50, 100, and 500. As shown in Figure 1B and C, a dose-dependent increase in secreted IL-12 and GM-CSF was observed in cells infected with Ad-ΔB7/IL12/GMCSF after 48 hr.

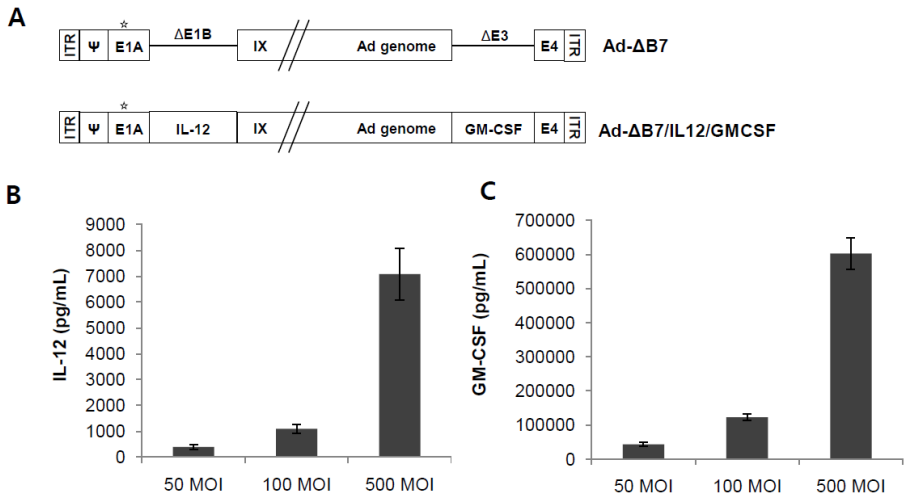


Figure 1. Characterization of oncolytic adenovirus coexpressing IL-12 and GM-CSF. (A) Schematic representation of the genomic structures of Ad-ΔB7 and Ad-ΔB7/IL12/GMCSF. Ad-ΔB7 contains mutated E1A (open star, mutation at Rb protein–binding site), but lacks E1B 19 and 55 kD (ΔE1B), and E3 region (ΔE3); the murine IL-12 and murine GM-CSF were inserted into E1 and E3 region of Ad genome, respectively. The level of IL-12 (B) and GM-CSF (C) expression was confirmed in B16-F10 cells after infection with Ad-ΔB7/IL12/GMCSF at different MOIs. Cell culture supernatants were collected at 48 hr after infection, and the level of IL-12 and GM-CSF was quantified by conventional ELISA kit. Data represent the mean ± SE of triplicate experiments, and similar results were obtained from at least three separate experiments.

Characterization of cultured DCs

To evaluate DC quality and phenotype, *in vitro* cultures of bone marrow-derived mature and immature DCs were assayed by flow cytometry. After the 8-day culture regimen, the cultures consisted of more than 80% DCs (Figure 2A). After antigen-pulsing and activation by lipopolysaccharide (LPS), the mature DCs showed markedly increased cell-surface expression of the co-stimulatory molecules CD40, CD80, CD86, and CC-chemokine receptor 7

(CCR7), as compared with immature DCs (Figure 2B and C).

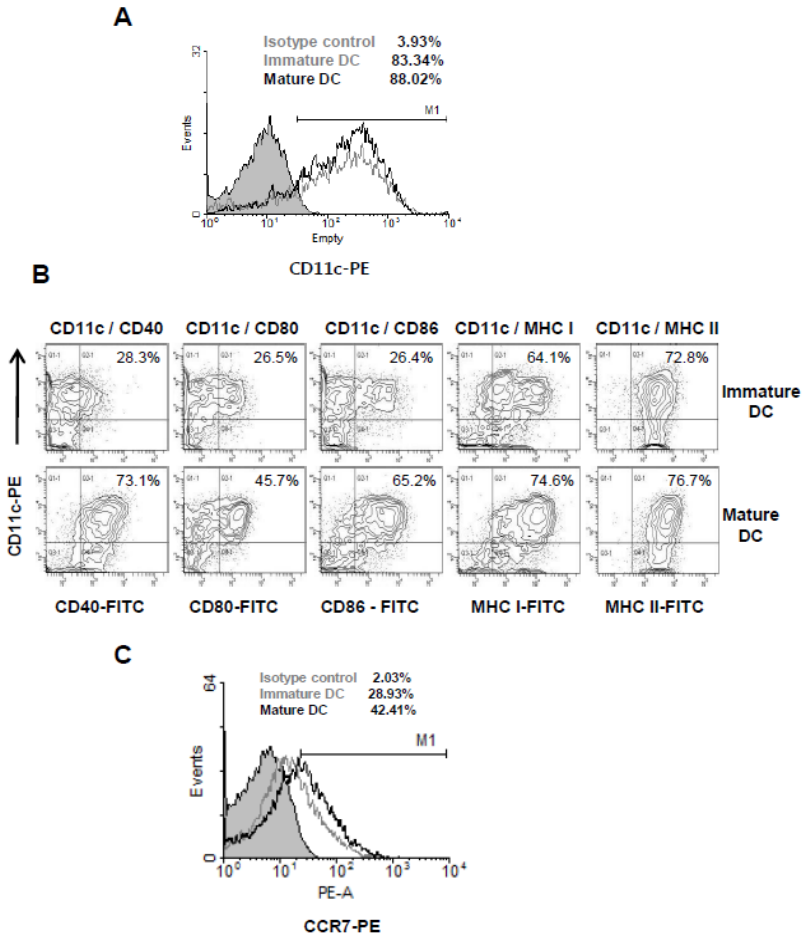


Figure 2. DC migration assay in vivo. (A) DCs were labeled with CMTPX in vitro. The CMTPX labeled DCs in fluorescence microscopic field (× 200 & × 400). The percentage of $CMTPX^+$ DCs (B) and $CD11c^+$ DCs (**C**) in the draining lymph nodes (DLNs) from mice treated with Ad-ΔB7/IL12/GMCSF and/or DC.

Therapeutic efficacy of Ad-ΔB7/IL12/GMCSF combined with DCs in an established murine melanoma model

To evaluate the therapeutic efficacy of Ad-ΔB7/IL12/GMCSF in combination with DCs *in vivo*, established B16-F10 melanoma tumors in C57BL/6 mice were intra-tumorally injected with three doses of Ad-ΔB7/IL12/GMCSF on days 0-2, followed by three injections of DCs on days 3-5. Treatment groups included PBS-only control, Ad-ΔB7/IL12/GMCSF only (5×10^9 VP/injection), DCs only (1×10^6/injection), or a combination of DCs and Ad-ΔB7/IL12/GMCSF, referred to as the combination therapy. In parallel, one group of mice was given a treatment regimen of a high dose of Ad-ΔB7/IL12/GMCSF (5×10^{10} VP/injection) in combination with DCs (1×10^6/injection), referred to as the high-dose combination therapy.

Control mice treated with PBS alone showed aggressive tumor growth and rapidly formed large tumors (over 2,500 mm^3) by day 8 (Figure 3A). In contrast, mice treated with Ad-ΔB7/IL12/GMCSF or DCs alone showed significant inhibition of tumor growth. On day 8, the mean tumor volumes in mice treated with DCs alone, Ad-ΔB7/IL12/GMCSF alone, the combination therapy, or the high-dose combination therapy were 1031 ± 443, 410 ± 170, 210 ± 24, and 177 ± 19 mm^3, respectively. These data correlate to 63, 85, 92, and 93% tumor growth inhibition, respectively, as compared to PBS alone

controls. Tumor growth inhibition was statistically significant in mice treated with the combination therapy as compared with individual treatment groups ($P < 0.05$ versus DCs or Ad-ΔB7/IL12/GMCSF). The high-dose combination therapy showed an even greater antitumor effect than combination therapy (on day 26, $P < 0.05$ versus combination therapy). In addition, the median survival of mice treated with Ad-ΔB7/IL12/GMCSF alone, DCs alone, the combination therapy, or high-dose combination therapy was 18, 18, 28, and over 90 days, respectively. All treatment groups showed significantly prolonged survival as compared to control mice ($P < 0.01$, Figure 3B). Importantly, four out of six mice treated with the high-dose combination therapy achieved complete remission and survived over 90 days. These results suggest that Ad-ΔB7/IL12/GMCSF in combination with DCs can induce a potent antitumor effect in the B16-F10 melanoma model.

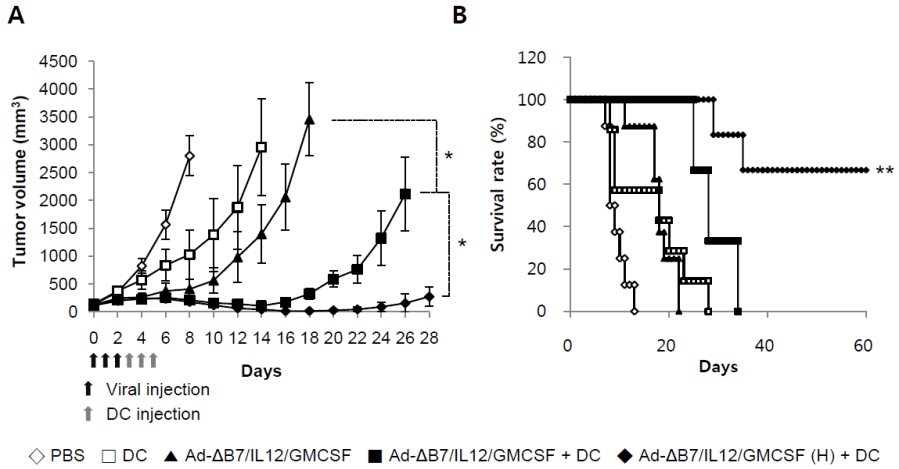


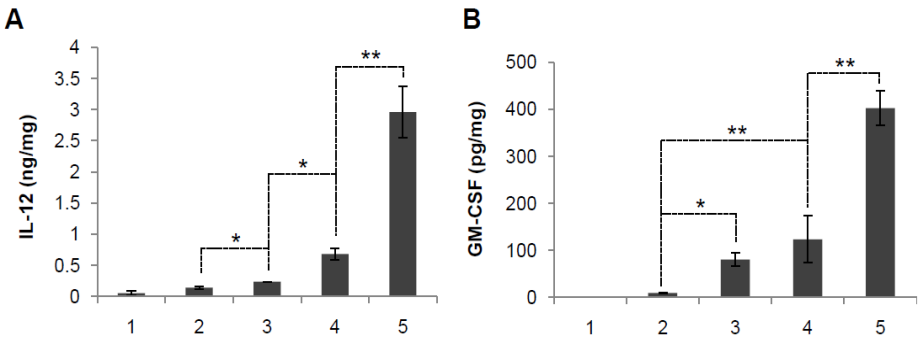
Figure 3. Antitumor effect of Ad-ΔB7/IL12/GMCSF in combination with DCs. (A) Pre-established B16-F10 tumor was injected with PBS (open diamonds), DCs (open squares), Ad-ΔB7/IL12/GMCSF (filled triangles), Ad-ΔB7/IL12/GMCSF plus DCs (filled squares), or Ad-ΔB7/IL12/GMCSF (H; High dose of Ad) plus DCs (filled diamonds). Tumor growth was monitored every day. (B) Survival percentage of tumor-bearing mice following treatment with Ad-ΔB7/IL12/GMCSF and/or DCs. Tumor size over 3,000 mm^3 was regarded as death. Data points represent the mean ± SE. All results in this figure represent results of at least seven mice per group. *$P < 0.05$.

IL-12 and GM-CSF expression and immune cell infiltration in treated tumor tissues

To examine the mechanisms of the observed antitumor effects, IL-12 and GM-CSF expression and immune cell infiltration in the tumor tissues were examined. Expression of IL-12 and GM-CSF was very minimal in tumors treated with PBS, but was present in tumors treated with Ad-ΔB7/IL12/GMCSF or DCs (Figure 4A and B). Tumors treated with the combination therapy showed markedly enhanced IL-12 and GM-CSF expression relative to tumors treated with Ad-ΔB7/IL12/GMCSF or DCs alone. This finding was even more dramatic in tumors treated with the high-

dose combination therapy.

Histological analyses revealed enhanced tumor necrosis in tumor tissues from mice treated with the combination therapy as compared with individual treatments. Significantly, nearly all tumor cells were eliminated from mice treated with the high-dose combination therapy. Immunohistochemical analyses using CD4-, CD8-, CD11c-, and CD86-specific antibodies were performed to identify the immune cells that infiltrated the tumor tissues. Significantly greater numbers of $CD4^+$ T cells, $CD8^+$ T cells, and $CD11c^+$ DCs were observed in tumors treated with the combination therapy than in tumors receiving individual treatments (Figure 4C). Correlating with the data in Figure 3, tumors treated with the high-dose combination therapy showed increased levels of tumor-infiltrating T cells and DCs. In addition, the expression of CD86 as a marker of activated APCs was significantly increased in tumors treated with the combination therapies as compared to the individual treatments.



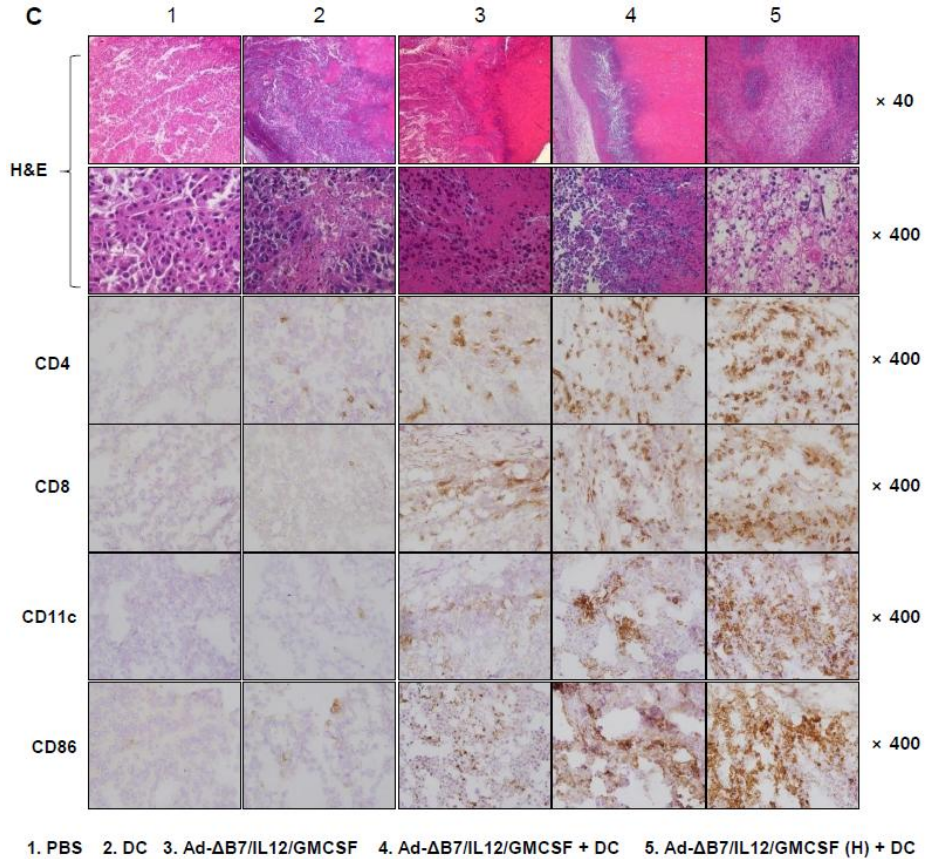


Figure 4. Generation of antitumor immune response by Ad-ΔB7/IL12/GMCSF in combination with DCs. The level of IL-12 (A) and GM-CSF (B) expression in tumor tissues treated with PBS, DCs, Ad-ΔB7/IL12/GMCSF, Ad-ΔB7/IL12/GMCSF plus DCs, or Ad-ΔB7/IL12/GMCSF (H) plus DCs. (C) Histological and immunohistochemical analysis in tumor tissues treated with Ad-ΔB7/IL12/GMCSF and/or DCs. Tumor tissues were collected from mice at 3 days after final treatment, and paraffin section of tumor tissue was stained with hematoxylin and eosin

(H&E) (top two rows, Original magnification: × 40 & × 400). Immune cells infiltrated in tumor tissues were examined by anti-CD4 antibody (third row), anti-CD8 antibody (fourth row), anti-CD11c antibody (fifth row) and anti-CD86 antibody (bottom row). Original magnification: × 400. Data points represent the mean ± SE of at least three mice per group, and similar results were obtained from at least three separate experiments. *$P < 0.05$; **$P < 0.01$.

VEGF expression and tumor angiogenesis is inhibited by combination therapy

IL-12 and E1A protein have been reported to inhibit tumor angiogenesis and downregulate vascular endothelial growth factor (VEGF) expression [31-32]. As shown in Figure 5A, VEGF expression was decreased in tumors from all treatment groups as compared to the PBS control, with a more robust reduction in the combination therapy group ($P < 0.05$ versus DCs alone). As expected, VEGF expression was decreased to the lowest levels in tumors treated with the high-dose combination therapy ($P < 0.05$ versus the combination therapy).

Tumor angiogenesis was examined by immunohistochemical analysis using a CD31-specific antibody. A reduced number of $CD31^+$ vessels were observed in mice treated with any of the individual or combination treatments

as compared to the PBS controls (Figure 5B and C). Consistent with the data above, the decrease in $CD31^+$ vessels was statistically greater in the combination-treated tumors than in tumors that received DCs or Ad-ΔB7/IL12/GMCSF alone ($P < 0.05$). Likewise, the high-dose combination therapy showed the greatest decrease ($P < 0.05$ versus the combination therapy) to nearly complete inhibition of angiogenesis.

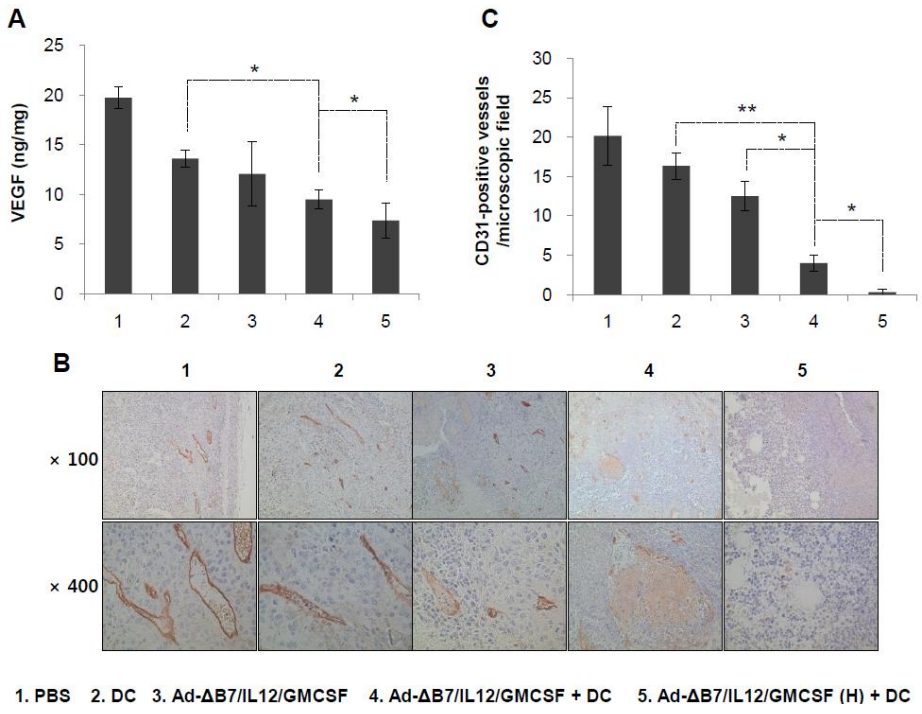


Figure 5. Decreased vascular endothelial growth factor (VEGF) expression and angiogenesis in tumors treated with combination therapy. Tumors were treated with PBS, DCs, Ad-ΔB7/IL12/GMCSF, Ad-ΔB7/IL12/GMCSF plus DCs, or Ad-ΔB7/IL12/GMCSF (H) plus DCs, and harvested from mice at 3

days after final treatment. (A) The level of VEGF expression in tumor tissues was quantified by conventional enzyme-linked immunosorbent assay. *P < 0.05. (B) $CD31^+$ microvessel immunohistochemistry in the tumor tissues treated with PBS, DC, Ad-ΔB7/IL12/GMCSF, Ad-ΔB7/IL12/GMCSF plus DCs, or Ad-ΔB7/IL12/GMCSF (H) plus DCs. Original magnification: × 100 & × 400. (C) The means of $CD31^+$ vessel density in microscopic field for each treatment group (× 200). Data points represent the mean ± SE of three mice per group, and similar results were obtained from at least two separate experiments. *P < 0.05; **P < 0.01.

Combination therapy promotes DC migration to draining lymph nodes

To explore whether DC migration to draining lymphnodes (DLNs) is promoted by combination therapy, DCs were labeled with a fluorescent probe, CMTPX (Figure 6A). A significantly increased number of $CMTPX^+$ DCs was observed in DLNs from mice treated with the combination therapy than from mice treated with DCs alone (P < 0.01). This finding was enhanced in mice treated with the high-dose combination therapy (P < 0.05 versus the combination therapy; Figure 6B and C). Moreover, combination therapy significantly augmented the proportion of $CD11c^+$ DCs in DLNs, implying that combination therapy also promotes migration of endogenous DCs to

DLNs (P < 0.05 versus Ad-ΔB7/IL12/GMCSF plus DCs; Figure 6D). Mice treated with the combination therapy showed significantly increased total cell numbers in DLNs as compared to mice treated with DCs alone (P < 0.01; Figure 6E). These results suggest that the combination therapy significantly promotes DC migration to DLNs and induces proliferation of immune cells in the DLNs.

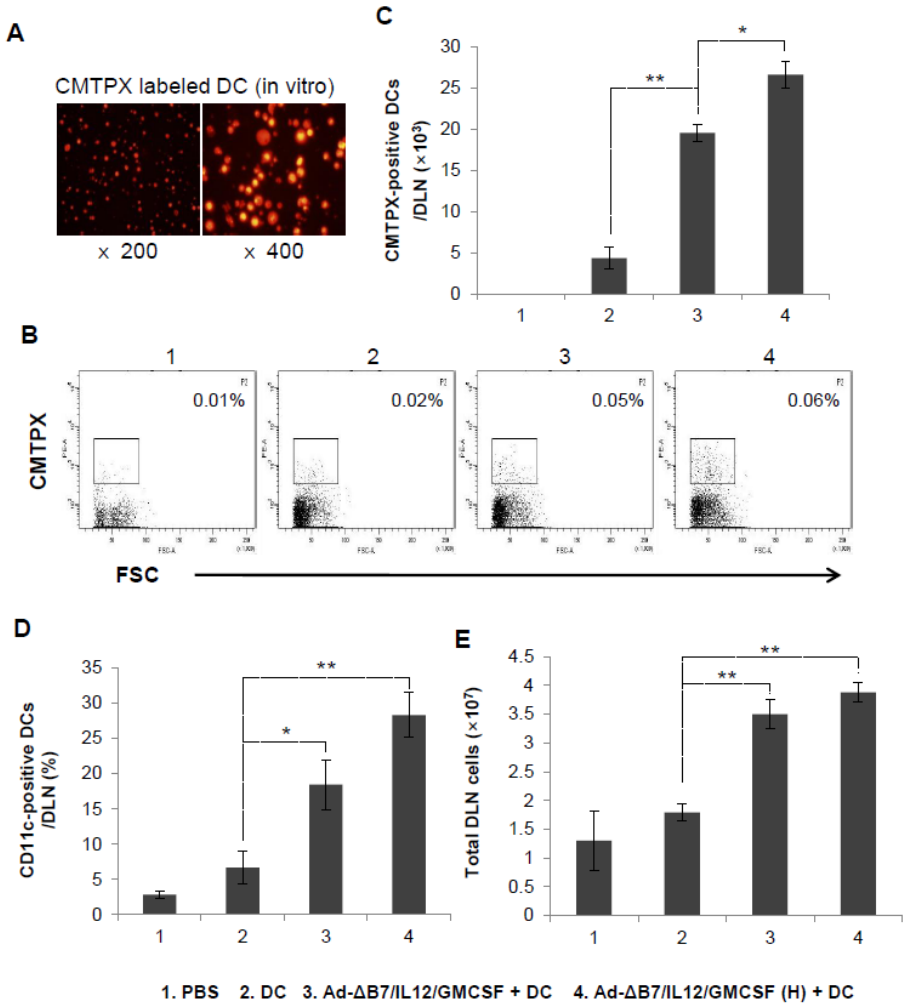


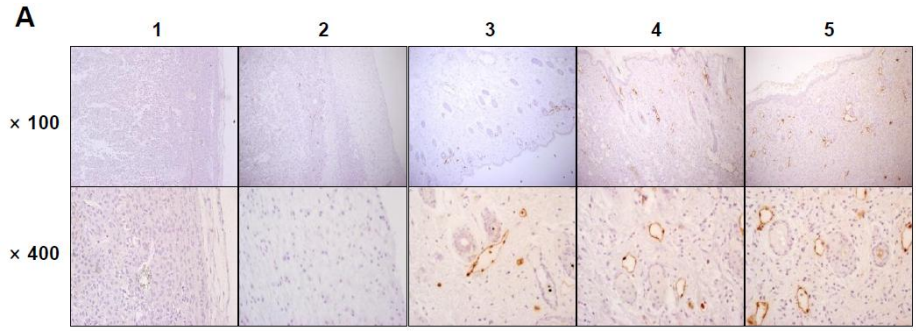
Figure 6. DC migration to the draining lymph nodes (DLNs). *Ex vivo* generated DCs were labeled with CMTPX (A). 2 days after final treatment, single cells were collected from DLNs and the migration was quantified by fluorescence-activated cell sorting analysis (B). (C) Total cell number of $CMTPX^+$ DCs in DLNs from mice treated with PBS, DC, Ad-ΔB7/IL12/GMCSF plus DCs, or Ad-ΔB7/IL12/GMCSF (H) plus DCs. (D) $CD11c^+$ DCs in the DLNs from mice treated with Ad-ΔB7/IL12/GMCSF and/or DCs. (E) Total cell number of DLNs from different groups of mice. Data points represent the mean ± SE of triplicate experiments. All results in this figure represent results of at least three mice per group, and similar results were obtained from at least two separate experiments. $*P < 0.05$; $**P < 0.01$.

Upregulation of $CCL21^+$ lymphatic vessels in tumor tissues treated with the combination therapy

A previous study reported that DC migration to DLNs depends on CCR7/CCL21 interaction and TNF-α regulation of $CCL21^+$ lymphatics within the tissue [33]. Tumors from mice treated with Ad-ΔB7/IL12/GMCSF alone showed a significantly increased number of $CCL21^+$ lymphatic vessels around the tumor tissues ($P < 0.01$ versus PBS and DCs alone; Figure 7A and B). Combination therapy greatly enhanced the number of $CCL21^+$ lymphatic

vessels as compared with the virus alone-treatment group ($P < 0.05$). Moreover, the high-dose combination therapy showed the greatest level of $CCL21^+$ lymphatics in the tumor tissues ($P < 0.01$ versus the combination therapy).

To better understand the mechanism of $CCL21^+$ lymphatic vessel upregulation in tumors treated with the combination therapy, expression of TNF-α in the tumors was measured by ELISA. TNF-α levels were augmented in the tumors treated with Ad-ΔB7/IL12/GMCSF or DCs alone and were significantly increased in the combination therapy group ($P < 0.05$ versus individual treatments; Figure 7C). Consistently, the high-dose combination therapy group had the highest levels of TNF-α expression ($P < 0.05$ versus combination therapy). These results indicate that increased TNF-α expression in tumor tissues treated with combination therapies might be the cause of the upregulation of $CCL21^+$ lymphatics.



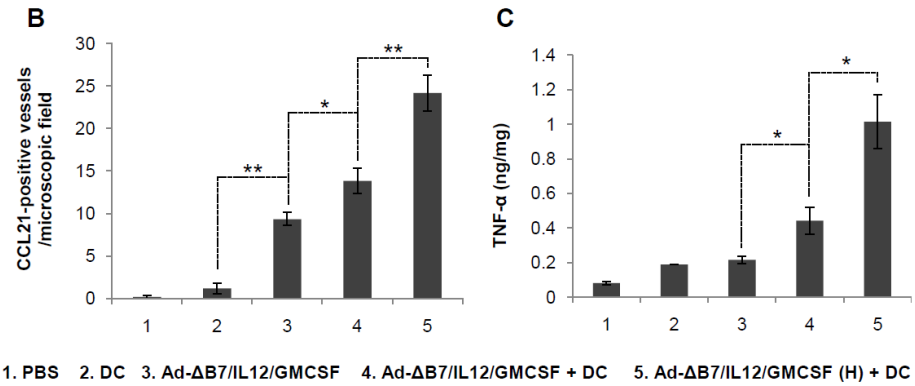


Figure 7. Visualization and quantification of $CCL21^+$ lymphatic vessels in tumors treated with Ad-ΔB7/IL12/GMCSF and/or DCs. Tumors harvested from mice treated with PBS, DC, Ad-ΔB7/IL12/GMCSF, Ad-ΔB7/IL12/GMCSF plus DCs, or Ad-ΔB7/IL12/GMCSF (H) plus DCs were embedded in paraffin and sectioned. (A) Immunohistochemical staining of $CCL21^+$ lymphatic vessels in tumor tissues. Original magnification: × 100 & × 400. (B) The means of $CCL21^+$ lymphatic vessel in microscopic field (× 200). (C) TNF-α expression in the tumor tissues was measured by conventional enzyme-linked immunosorbent assay. Data points represent the mean ± SE of triplicate experiments. All results in this figure represent results of at least three mice per group, and similar results were obtained from at least two separate experiments. *$P < 0.05$; **$P < 0.01$.

The $CD4^+CD25^+$ T cell population decreased in DLNs of mice treated with the combination therapy

$CD4^+CD25^+$ T cells are important immune suppressors whose development is partially regulated by VEGF. As described above, the expression of VEGF was markedly decreased in tumors treated with the combination therapy (Figure 5A). To evaluate changes in the $CD4^+CD25^+$ T cell population within the DLNs, lymphocytes were collected from DLNs of mice treated with PBS, DCs, Ad-ΔB7/IL12/GMCSF, or the combination therapy. The $CD4^+CD25^+$ T cell population from the DLNs was significantly decreased in mice treated with Ad-ΔB7/IL12/GMCSF as compared to mice treated with PBS or DCs ($P < 0.01$; Figure 8A and B). The decrease in the $CD4^+CD25^+$ T cell population was still greater in mice treated with the combination therapy ($P < 0.05$ versus Ad-ΔB7/IL12/GMCSF alone).

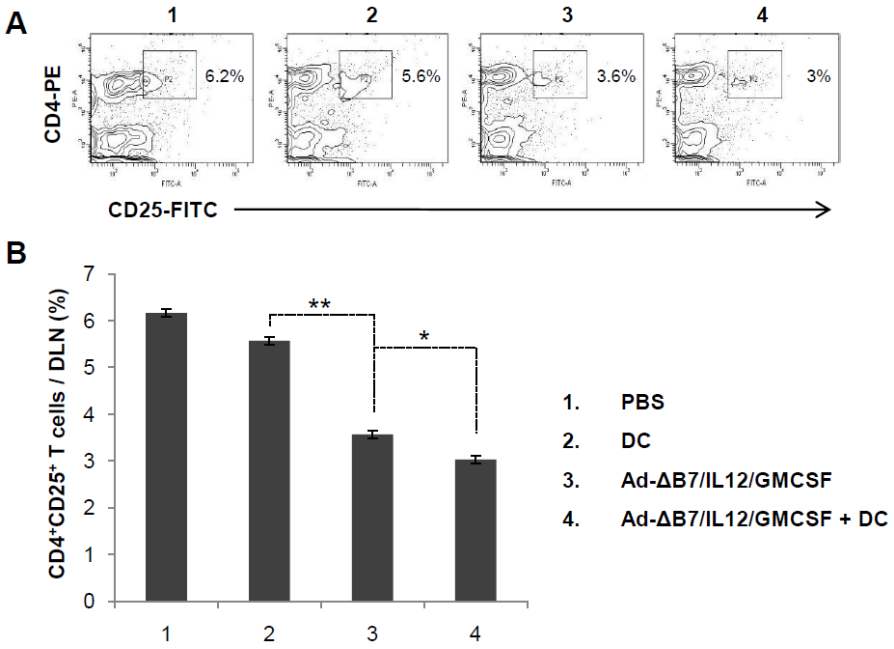


Figure 8. $CD4^+CD25^+$ T cells in draining lymph nodes (DLNs) from mice treated with Ad-ΔB7/IL12/GMCSF and/or DCs. DLNs were collected from mice treated with PBS, DC, Ad-ΔB7/IL12/GMCSF, Ad-ΔB7/IL12/GMCSF plus DCs, or Ad-ΔB7/IL12/GMCSF (H) plus DCs. Proportion of $CD4^+CD25^+$ T cells in the DLNs from different groups of mice were measured by fluorescence-activated cell sorting analysis. Data points represent the mean ± SE of three mice per group, and similar results were obtained from at least two separate experiments. *$P < 0.05$; **$P < 0.01$.

Antitumor effect induced by Ad-ΔB7/IL12/GMCSF in combination with immature DCs

Immature DCs differentiated from progenitor cells can actively uptake and process exogenous antigen. Following maturation, DCs downregulate their antigen acquisition and processing abilities and display increased immunogenicity. Immature DCs differ from mature DCs pulsed with tumor antigen *in vitro* in that immature DCs can take up *in situ* tumor antigens. B16-F10 melanoma models were used to evaluate the therapeutic effects of combination therapy using immature DCs in comparison to mature DCs. Similar antitumor effects were observed in combination therapies using mature or immature DCs (Figure 9A). This finding indicates that the

combinatorial strategy using the cytokine-expressing oncolytic Ad can promote maturation of immature DCs within the tumor tissue. Therefore, Ad-ΔB7/IL12/GMCSF-mediated expression of IL-12 and GM-CSF prior to DC injection might reverse the immunosuppressive microenvironment within the tumor, resulting in a microenvironment favorable to the maturation and function of immature DCs.

We further assessed the effect of the treatment schedule of oncolytic Ad and DCs on gross antitumor efficacy. The antitumor efficacy and survival rates were significantly improved in tumors treated with Ad-ΔB7/IL12/GMCSF prior to DC vaccination (Figure 9B and C; filled triangles) or in tumors treated with Ad-ΔB7/IL12/GMCSF and DCs on alternating days (open diamonds) as compared to tumors treated with Ad-ΔB7/IL12/GMCSF following DC vaccination (filled squares) ($P < 0.05$). Clearly, tumor treatment with Ad-ΔB7/IL12/GMCSF prior to DC vaccination has a higher antitumor efficacy.

The antitumor effect of combination therapies on large tumors representative of late-stage tumors was assessed. Mice were treated with high-dose combination therapy when tumor volumes reached three different sizes: 117.54 ± 14.29, 288.89 ± 39.96, and 573.66 ± 110.15 mm^3. Potent antitumor effects were induced by high-dose combination therapy even when the initial

tumor size reached over 500 mm^3. However, the therapeutic efficacy was significantly improved when the initial tumor sizes were less than 300 mm^3 (Figure 9D and E). These data suggest that combination therapy can elicit potent antitumor effects even when the tumor burden is large and in late-stage tumors.

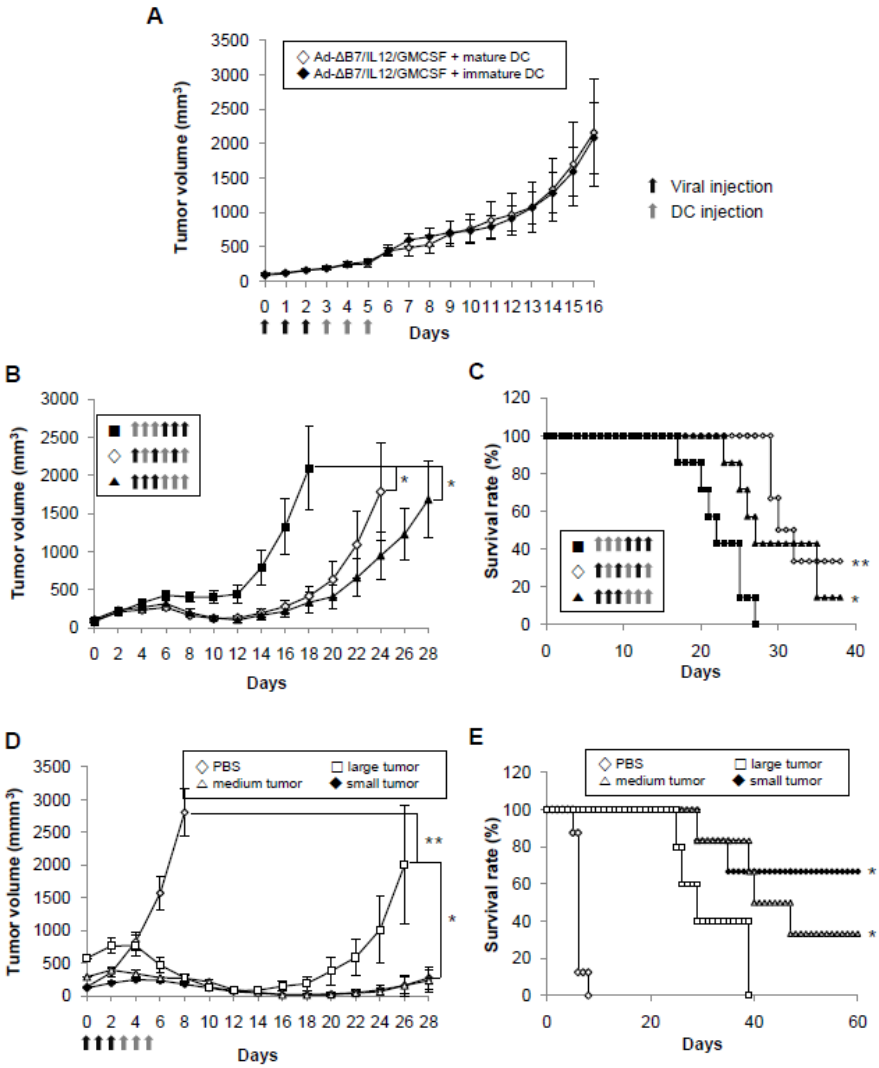


Figure 8. Therapeutic efficacy of combination therapy based on the DC maturation, treatment schedule, and tumor size. (A) Tumor-bearing mice were treated with Ad-ΔB7/IL12/GMCSF in combination with mature or immature DCs. Similar antitumor effects were observed in combination therapies using mature or immature DCs. (B, C) Effect of the treatment schedule of oncolytic adenovirus and DCs on antitumor efficacy. Tumors were treated with Ad-ΔB7/IL12/GMCSF prior to DC vaccination (filled triangles), Ad-ΔB7/IL12/GMCSF and DCs on alternating days (open diamonds), or Ad-ΔB7/IL12/GMCSF after DC vaccination (filled squares). Tumor treatment with Ad-ΔB7/IL12/GMCSF prior to DC vaccination induced the most potent antitumor efficacy. (D, E) Antitumor effect of combination therapies on large tumors. High-dose combination therapy induced potent antitumor effect on large tumors. Data points represent the mean ± SE. All results in this figure represent results of at least six mice per group. *$P < 0.05$; **$P < 0.01$.

IV. DISCUSSION

Tumor-induced immune suppression mediated by a variety of molecules, such as VEGF, IL-10, and TGF-β, is a major problem faced to cancer immunotherapy. These molecules produced by several tumor compartments not only impair the recruitment and maturation of DCs and T cells in the tumor tissue, but also induce abnormal systemic DC differentiation and activation. This immune modulation is a major obstacle in DC-based cancer vaccination [34-35]. Therefore, many recent studies have focused on optimizing DC vaccination protocols to overcome tumor-induced immune suppression.

IL-12 is a potent immune-stimulatory cytokine that can reverse tumor microenvironment immunosuppression [18]. DCs activated in the presence of IL-12, IL-18, and anti-CD40 antibody have been shown to potently improve antitumor immunity [36]. In addition, DCs transduced with the IL-12 gene and melanoma-associated antigen (gp100) were shown to induce more effective tumor-specific immune responses by up-regulating co-stimulatory molecules and by improving T cell stimulation [4]. Therefore, IL-12 is a good candidate for combination with DC vaccinations to improve the therapeutic efficiency. GM-CSF is associated with the function of antigen-presenting cells, but GM-CSF also plays an important role in the development of DCs *in vitro* and *in vivo* [37]. Recently, the therapeutic efficacy of DC vaccination for the treatment

of murine renal cell carcinomas was improved when combined with GM-CSF-secreting tumor cells [38]. To improve the efficacy of DC vaccination, IL-12 and GM-CSF were selected for use in combination therapies to activate and enhance host immune responses.

This study demonstrates that the combination of DC vaccination and oncolytic Ad coexpressing both IL-12 and GM-CSF greatly enhanced the antitumor effects and survival rates in a murine melanoma model, as compared with individual treatments (Figure 3A and B). Moreover, IL-12 levels were also markedly enhanced in tumor tissues that received the combination therapy (Figure 4A). IL-12 not only enhances Th1 immunity and CTL cytotoxicity, but also activates NK cells at high concentrations. Activated NK cells promote DC maturation, which induces a positive feedback loop in that the mature DCs secrete additional IL-12 [39]. In addition, GM-CSF secreted from Ad-ΔB7/IL12/GMCSF-infected tumor cells can also recruit and induce maturation of DCs within tumor tissues (Figure 4C). Taken together, these findings suggest that IL-12 and GM-CSF are potent stimuli that can activate and boost the host immune system, and can also synergistically promote DC vaccination efficacy.

VEGF not only plays a critical role in the development of tumor angiogenesis, but also inhibits the differentiation and maturation of DCs [40]. *In*

vivo administration of neutralizing VEGF-specific antibody can improve the function of DCs and increase the number of mature DCs in tumor-bearing mice [41]. IL-12 downregulated VEGF expression in a murine breast cancer model [42], and inhibited tumor angiogenesis [32]. Here, VEGF expression was inversely related to IL-12 expression. Specifically, VEGF expression was significantly reduced in tumors treated with the combination therapy as compared with control tumors or tumors that received individual treatments (Figure 5A). Likewise, enhanced anti-angiogenesis was observed in tumors that received the combination treatments (Figure 5B and C). These data suggest that IL-12-mediated anti-angiogenesis played a role in the combination therapy-induced antitumor effect. Importantly, reduction of VEGF expression due to the combination therapy might contribute to the reduction of the tumor microenvironment immunosuppression, therefore it might enhance enhancing DC vaccination efficacy.

Following maturation, DCs upregulate co-stimulatory molecules and acquire the ability to migrate to regional lymph nodes. Migration of DCs to regional lymph nodes is an essential step in the induction of immune responses and, therefore, is an important aspect of DC vaccination [34]. However**,** only a small proportion of injected DCs migrated to DLNs in cancer patients during clinical trials of DC vaccination [43-44]. To increase the

efficiency of DC vaccination, DC migration to DLNs must be enhanced. In this study, mice treated with the combination therapy showed significantly increased numbers of exogenously-injected and endogenous DCs that migrated to the DLNs (Figure 6B and C). This migration likely played a significant role in enhancing DC vaccination efficacy in tumor-bearing mice. This finding is in line with our previous study that indicated that DC migration can be promoted when DCs are combined with oncolytic Ad coexpressing IL-12 and 4-1BB ligand [45].

Migration of mature DCs to DLNs is driven by the interaction of high levels of CCR7 on mature DCs with its ligand, CC-chemokine ligand 21 (CCL21) in the lymphatics [46]. Mice treated with Ad-ΔB7/IL12/GMCSF alone or in combination with DCs showed a markedly increased number of CCL21-positive lymphatic vessels surrounding tumor tissues as compared with mice treated with PBS or DCs alone (Figure 7A and B). This suggests that the increased DC migration in the combination therapy group was due to upregulation of $CCL21^+$ lymphatic vessels in the tumor tissue. Martin-Fontecha *et al.* reported that TNF-α-induced tissue inflammation greatly increases DC migration to DLNs due to upregulation of CCL21 expression on lymphatic vessels *in situ* [33]. Therefore, TNF-α may be an inflammatory stimuli associated with the regulation of CCL21 expression on the endothelial

cells of lymphatics. Interestingly, combination therapy significantly increased the level of TNF-α in tumors (Figure 7C). TNF-α was likely secreted by tumor-infiltrating T cells, monocytes, and macrophages recruited to the $CCL21^+$ lymphatic vessels in the tumor tissues by IL-12 and GM-CSF expressed by Ad-ΔB7/IL12/GMCSF. However, it remains unclear whether IL-12 or GM-CSF directly regulates CCL21 expression on endothelial cells of the lymphatics.

Emerging evidence suggests that $CD4^+CD25^+$ T cell-mediated immune suppression is a crucial tumor immune evasion mechanism and is often a barrier for successful tumor immunotherapy. A previous study demonstrated that local IL-12 expression by tumor tissues can reverse the tumor immune suppression by inducing apoptosis of $CD4^+CD25^+Foxp3^+$ T cells [18]. In our study, the $CD4^+CD25^+$ T cell population was reduced in the DLNs of tumor-bearing mice treated with Ad-ΔB7/IL12/GMCSF alone or in combination with DCs as compared with the PBS and DCs-alone treatment groups. It is likely that the high level of IL-12 expressed in the tumor tissue contributed to $CD4^+CD25^+$ T cell elimination (Figure 8A and B). In addition, VEGF is associated with the development of $CD4^+CD25^+$ T cells in tumor-bearing mice and cancer patients [47-48]. Therefore, the decreased VEGF expression in the tumors from mice that received the combination therapy likely contributed

to reduction of $CD4^+CD25^+$ T cell population in the DLNs.

Interestingly, combination therapy using immature DCs had similar antitumor effects as the combination therapy using mature DCs (Figure 9A). IL-12 and GM-CSF secreted from Ad-ΔB7/IL12/GMCSF likely directly activated the immature DCs or induced an immune response that produced a number of immune-stimulating factors to circumvent the immunosuppressive microenvironment. Moreover, injected immature DCs can efficiently uptake tumor antigens *in situ* that were generated by oncolytic Ad-mediated oncolysis of cancer cells.

The order of virus and DC injection also affected the antitumor effects of the combination therapies (Figure 9B and C). These results indicate that injection of the cytokine-expressing oncolytic Ad prior to DC vaccination can reduce immune suppression and induce favorable immune response conditions within the tumor tissue, resulting in improved efficacy of DC vaccination. In addition, combination therapy also elicited notable antitumor effects in large tumors (Figure 9D and E), suggesting that combination therapy can overcome the limitation of DC vaccinations to reduce tumor burden in established solid tumors [49].

This study demonstrates synergistic antitumor effects between Ad-ΔB7/IL12/GMCSF and DCs in a murine melanoma model. The mechanism is

associated with the elimination of immune suppression in the tumor and promotion of DC maturation and migration to DLNs. These findings provide the basis for a feasible oncolytic Ad-based cytokine gene therapy in combination with DC vaccination for cancer treatment in the clinic.

V. CONCLUSION

In conclusion, DC vaccination combined with Ad-ΔB7/IL12/GMCSF effectively eliminates tumor-induced immunosuppressive microenvironment and promotes DC migration to draining lymph node. As a result, efficacy of DC vaccination was significantly improved by combined treatment with adenoviral vector co-expressing IL-12 and GM-CSF. Taken together, our preclinical approach provides feasible strategies for optimizing DC-based vaccination in the clinic.

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ABSTRACT (in Korean)

IL-12와 GM-CSF를 발현하는 아데노바이러스와의 병합투여에 의한 수지상세포 백신의 최적화 효과 검증

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장송남

수지상 세포를 이용한 항암면역치료방법은 항원제시세포로서는 유일하게 미성숙 T 세포의 활성화를 유도할 수 있는 수지상세포의 특성으로 인해 유망한 항종양 치료방법으로 대두되어 현재 많은 연구들이 진행되고 있다. 하지만 종양세포에 의해 발현 또는 분비되는 여러가지 면역억제분자에 의해 유도된 면역억제환경(immunosuppressive microenvironment)은 암환자를 대상으로 한 수지상 세포 백신의 임상치료 적용의 한계로 작용하여 결과적으로 만족할 만한 치료효과를 얻지 못하였다. 이러한 결과는 수지상 세포를 이용한 치료만으로는 효과적인 종양치료가 가능하지 않음을 의미하며 이러한 한계가 드러남에 따라 수지상 세포의 기능을 향상시킬 수 있는 다른 보조제의 필요성이 급부상 하고 있는 실정이다. 이에 본 연구에서는 IL-12 와 GM-CSF 를 동시에 발현하는

종양 선택적 살상가능 아데노바이러스(Ad-ΔB7/IL12/GMCSF)를 제작하여 수지상 세포 백신의 보조제로 사용하였으며 이를 통해 종양내 면역억제환경을 제거하고 수지상 세포 백신의 효능을 증가시키는 한편 수지상 세포와 Ad-ΔB7/IL12/GMCSF 의 병용투여에 의한 수지상 세포 백신의 최적화 조건을 찾고자 하였다.

실험 결과, 수지상 세포를 Ad-ΔB7/IL12/GMCSF 와 함께 병용투여하였을 때 단일 치료에 비해 월등한 치료효과를 나타내는 것을 확인하였다. 또한 병합치료에 의해 수지상 세포의 유출림프절로의 이동효율이 증가되었으며 이는 주로 Ad-ΔB7/IL12/GMCSF 치료에 의해 종양 조직내의 $CCL21^{+}$ 림프관의 증가에 의한 것임을 증명하였다. 뿐만 아니라 병합치료에 의해 $CD4^{+}CD25^{+}$T 림프구와 VEGF 의 발현이 마우스의 종양조직에서 감소함을 관찰하였다. 한편 Ad-ΔB7/IL12/GMCSF 와의 병합치료에 미성숙 수지상 세포를 사용한 경우에도 성숙 수지상세포세포를 사용한 경우와 유사한 항암효과를 보였으며 특히 종양의 크기가 큰 경우에도 병합치료가 효과적인 종양억제능을 나타내고 있음을 관찰 하였다.

결론적으로, 본 연구를 통하여 IL-12 와 GM-CSF 를 동시에 발현하는 종양 선택적 살상 가능 아데노바이러스와 수지상 세포 백신을 병합하여 항암치료에 이용함으로써 상승적인 항종양효과가 나타남이 증명되었으며 이는 IL-12 와 GM-CSF 를 동시에 발현하는 종양 선택적 살상 가능 아데노바이러스가 수지상 세포 백신을 이용한 항암치료의 강력한 보조제로 작용하여 수지상 세포 백신치료의 치료조건을 최적화하고 그 효능을 증가시킬 수 있음을 확인하였다.

핵심되는 말: 종양면역유전자치료; 아데노바이러스; IL-12; GM-CSF; 수지상 세포

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