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# Extracts of *Larix Leptolepis* effectively augments the generation of tumor antigen-specific cytotoxic T lymphocytes via activation of dendritic cells in TLR-2 and TLR-4-dependent manner

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**Key words: Dendritic cells, Toll-like receptor, Adjuvant, Cytotoxic T lymphocytes, Type-1 immunity, Cancer immunotherapy**

**Abstract**

Type-1 immunity plays a crucial role in host defense against various tumors and infectious diseases. Here, we first demonstrated that extract of *Larix Leptolepis* (ELL), one of the most popular timbers at Hokkaido area in Japan, strongly activated Type-1 immunity. ELL induced production of Type-1 cytokines such as IL-12 and TNF-$\alpha$ from bone marrow-derived dendritic cells (BMDCs) in TLR2- and TLR4-dependent manner and remarkably up-regulated the expression of MHC and co-stimulatory molecules. In addition, antigen-specific CTLs were significantly augmented by the combined administration of ELL, antigen and BMDCs. Finally, we revealed that combination therapy using ELL, antigen and BMDCs significantly inhibited the growth of established tumor in mouse model. Thus, these findings suggested that ELL would be a novel adjuvant for inducing an activation of Type-1-dependent immunity including activation of BMDCs and induction of tumor-specific CTLs, which is applicable to the therapy of cancer and infectious diseases. [146 words]

## 1. Introduction

Immune system is one of the most important machineries for maintenance of our health by elimination of “non-self” pathogens, such as bacteria, viruses, fungi, parasites, and tumors. However, chronic infection, tumor-bearing condition, aging, and malnutrition occasionally cause dysfunction of the immune system [1-4]. An immunological adjuvant, which nonspecifically activates the innate immunity and subsequently induces antigen-specific immune responses, is one of the most popular methods for overcoming immunosuppressive conditions [5]. Type-1 and Type-2 immunity is involved in the regulation of cellular and humoral immunity, respectively [6]. Type-1 immunity is crucial for the rejection of tumors and the elimination of pathogens such as viruses and bacteria [7-9]. Generally, cytotoxic T lymphocytes (CTLs) play a pivotal role as effector cells for the elimination of tumor cells and intracellular pathogen [7, 10, 11]. Thus, it is of great important to develop a novel adjuvant for enhancing Type-1 immunity-dependent CTL-generation, essential for preventing cancer and infectious diseases. Many investigators have searched good adjuvants to activate antigen-presenting cells (APCs) [5, 12-14].

Dendritic cells (DCs), one of the most powerful APCs, have a pivotal role for initiating immune responses [15-17]. It has been well-known that DCs take up extracellular antigens, process them into peptides, and promptly load them on major histocompatibility (MHC) class II molecules, which activate $CD4^+$ T cells. DCs also have a unique function, called “cross-presentation”, which induces protective immunity by the antigen-specific cytotoxicitic $CD8^+$ T lymphocytes (CTLs) against tumors and intracellular infectious microbes [18]. For example, systemic chronic infection of malaria significantly reduced T cell responses, but the antigen-loaded DCs could break the immune-suppressive condition [19]. In addition, adoptive cell transfer with tumor-antigen-loaded DCs is one of the most powerful methods for the therapy of

cancers [15, 20, 21].

*Larix Leptolepis* (*Larix kaempferi*), deciduous and conifer tree planting at subarctic zone, is useful as architectural material in Japan. We have carried out research to seek novel functional materials from the sawdust of *Larix Leptolepis*. In the present work, we found that extract of *Larix Leptolepis* (ELL) from Japan remarkably activated DCs and effectively enhanced the generation of tumor antigen-specific CTLs, suggesting it will become a novel promising adjuvant to potentiate Type-1-dependent cellular immunity.

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## 2. Materials and methods

### 2.1. Mice

Wild-type (WT) C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). C57BL/6-background TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR9$^{-/-}$ mice were kindly provided by Dr. S. Akira (Department Host defense, Research Institute for Microbial Disease. Osaka University, Osaka, Japan). $OVA_{323-339}$-specific I-$A^b$-restricted T cell receptor transgenic mice (OT-II) on the C57BL/6 background were kindly donated by F.R. Carbone (University of Melbourne, Victoria, Australia). All mice were maintained in specific pathogen-free conditions according to the guideline of animal department at Hokkaido University and used at 6–8 weeks of age.

### 2.2. Antibodies and reagents

APC-conjugated anti-CD11c monoclonal antibody (mAb) (HL3), and anti-CD4 mAb (GK1.5), PE-conjugated anti-IL-12p40/70 mAb (C15.6), and anti-IFN-$\gamma$ mAb (XMG1.2), and anti-IL-4 mAb (11B11), FITC-conjugated anti-TNF-$\alpha$ mAb (MP6-XT22), and anti-CD8$\alpha$ mAb (AF6-88.5) were purchased from BD bioscience (San Diego, USA). 7-amino-actinomycin D (7AAD) was purchased from Beckman coulter (Miami, USA). OVA protein was purchased from Sigma–Aldrich Japan (Tokyo, Japan). H-2K$^b$ OVA tetramer-SIINFEKL-PE (OVA tetramer) was purchased from MBL (Nagoya, Japan). IL-2 was kindly supplied by Takuko Sawada (Shionogi Pharmaceutical Institute Co. Ltd., Osaka, Japan).

### 2.3. Preparation of ELL

*Larix Leptolepis* was crushed to sawdust and beated up with pure water for 1 hour. Next, to remove low molecular weight components, the extract was dialyzed extensively

against water and lyophilized. The final LPS contents in the ELL was less than 6.25 pg / mg detected by the Toxicolor test (Seikagaku Kogyo Co., Tokyo, Japan).

**2.4. Generation of bone marrow-derived dendritic cells (BMDCs).**

Bone marrow (BM) cells were prepared from WT, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR9$^{-/-}$ mice. BMDCs were generated from BM cells with RPMI medium (Sigma Aldrich, Germany) containing 10% FCS (BD Bioscience) in the presence of GM-CSF (20 ng/ml) as described previously [22]. Loosely adherent clustering cells were harvested on day 6-8. CD11c$^{+}$ BMDCs were isolated with anti-CD11c micro beads and used in the present experiments.

**2.5. Flow cytometry**

For analysis of cell surface molecules, BMDCs were stimulated with or without ELL (2, 5, and 10 mg/ml) for 24 hrs and stained with 7AAD, fluorescence-labeled anti-CD11c, anti-MHC class I (H-2Kb), anti-CD86, anti-MHC class II (I-Ab), and anti-CD40 mAbs. For intracellular cytokine staining, BMDCs were stimulated with or without ELL (0.5, 2, and 10 mg/ml) for 24 hrs and Brefeldin A was added at 6 hr. CD4$^{+}$ T cells were stimulated with plate-bound anti-CD3$\varepsilon$ mAb (2 μg/ml) in 96-well flat-bottom plates for 6 hr and Brefeldin A was added at 4 hr. Then, cells were harvested and stained with 7AAD and APC-conjugated anti-CD11c or anti-CD4 mAb. After fixation and permeabilization, the cells were stained with PE-conjugated anti-IL-12p40/70, anti-TNF-$\alpha$, anti-IFN-$\gamma$ or anti-IL-4 mAbs. Data were acquired on a FACSCalibur or FACSCanto (BD Bioscience) and the percentages of cytokine-producing cells were analyzed by CellQuest software (BD Bioscience) or FlowJo software (Tree Star).

**2.6. ELISA**

BMDCs were stimulated with or without ELL (10 mg/ml) for 12 hrs. IL-12p70 and TNF-α levels in the culture supernatants were measured by OptEIA™ mouse IL-12 and TNF-α ELISA set (BD Bioscience) according to the manufacturer's instructions, respectively.

### 2.7. Coculture of BMDC and naïve CD4$^+$ T cells

CD45RB$^+$ naïve CD4$^+$ T cells were isolated from spleen cells of OT-II mice by FACSAria (BD Bioscience). The cells ($2x10^5$ cells) were stimulated with mitomycin C-treated BMDCs, $OVA_{323–339}$ peptide (0.5 μg/ml) and IL-2 (10 U/ml) in the presence or absence of ELL (10 mg/ml). After 4 days, IFN-γ and IL-4 production were determined by flow cytometric analysis.

### 2.8. Antigen-specific CTL induction

WT C57BL/6 mice were immunized in the footpad with saline, OVA protein (200 μg), ELL (5 mg) plus OVA protein, BMDCs ($5x10^5$) plus OVA protein, or BMDCs plus OVA protein plus ELL twice at 5 days intervals. Five days after the last immunization, lymphocytes were prepared from popliteal lymph nodes and stained with FITC-conjugated anti-CD8α mAb and PE-conjugated OVA tetramer for analysis of OVA-specific CTLs.

### 2.9. Cytotoxicity assay

The cytotoxicity mediated by tumor-specific CTLs was measured by a 6 hr $^{51}Cr$-release assay, as described previously [13]. Briefly, $^{51}Cr$-labeled target cells were cocultured with various numbers of effector cells for 6 hr in V-bottomed microtiter plates. Released $^{51}Cr$ in the culture supernatants were measured by gamma counter (Packard Cobra II gamma counter, Meriden, CT, USA) and the percentage of specific lysis was calculated.

### 2.10. Immunotherapy for transplanted tumor

OVA-expressing EG7 cells ($2x10^6$) were intradermally (i.d.) inoculated into WT C57BL/6 mice. When the tumor mass became palpable (6–7 mm), the tumor-bearing mice were treated with saline, OVA protein (200 μg) plus BMDCs ($2x10^6$), OVA protein plus ELL (10 mg), BMDCs plus ELL, or OVA protein plus BMDCs plus ELL. The antitumor activity was determined by measuring the tumor size in perpendicular diameters. Tumor volume was calculated by the following formula: tumor volume = 0.4 x length (mm) x $[\text{width (mm)}]^2$.

**2.11. Statistical analyses**

All experiments were repeated at least three times. Mean values and SDs were calculated for the data from representative experiment and shown in the figures. Significant differences in the results were determined by the Student's t-test. The $p<0.05$ was considered as significant in the present experiments.

## 3. Results

### 3.1. ELL induces production Type-1 cytokines from BMDCs.

Type-1 cytokines such as IL-12 and TNF-α are closely related with activation of Type-1 immunity [23, 24]. To evaluate the immunomodulating potential of ELL, we examined whether ELL induce production of IL-12 and TNF-α from BMDCs. BMDCs prepared from WT C57BL/6 mice were cultured in the presence or absence of ELL for 12 hrs and cytokine producing BMDCs were detected by flow cytometry. As a result, the percentages of IL-12- or TNF-α-producing DCs were increased by stimulation with ELL in a dose dependent manner (Fig. 1A). Here, we confirmed that ELL has no significant toxicity against thymocytes. (data not shown). In addition, BMDCs were stimulated with ELL for 24 hrs and cytokine levels in the culture supernatants were determined by ELISA. Consistent with the intracellular staining analysis, we confirmed that ELL strongly induced IL-12 and TNF-α production from BMDCs (Fig. 1B). IL-10 and IL-4 were not detected by intracellular staining. However, IL-10 was detected by ELISA in culture supernatants though its levels are greatly low (data not shown). These findings suggested that ELL directly stimulated dendritic cells and activated Type-1 immunity.

### 3.2. ELL induces activation of BMDCs in TLR2- and TLR4-dependent manners.

It has been well demonstrated that BMDCs express various pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), which is critical for host defense against bacteria and viruses [25]. To address the activation mechanisms of BMDC by ELL, we examined the contribution of TLRs (TLR2, TLR4 and TLR9) to the cytokine production by BMDCs after stimulation with ELL. BMDCs prepared from WT, $TLR2^{-/-}$, $TLR4^{-/-}$ and $TLR9^{-/-}$ mice were stimulated with ELL for 24 hrs. We found that IL-12 and TNF-α production was markedly decreased in TLR2- or TLR4-deficient BMDCs in

comparison with WT or TLR9-deficient BMDCs (Fig. 2). These data clearly demonstrated that TLR2 and TLR4 are involved in the activation of BMDCs after ELL stimulation.

### 3.3. ELL significantly causes maturation of BMDCs.

Stimulation signals from TLRs induce maturation of DCs and augment expression of MHC class I and class II molecules [26]. In addition, costimulatory molecules are highly expressed on the matured DCs, which are indispensable for fully activation of T cells. It has been indicated that CD86 and CD40 are especially important molecules for activation of Type-1 immunity [27-29]. Therefore, we examined the expression levels of MHC class I, MHC class II, CD86, and CD40 on DCs after BMDCs were cultured with ELL for 24 hrs. As a result, ELL-stimulated DCs exhibited elevated expression of these molecules compared with non-stimulated DCs (Fig. 3A). We further stimulated BMDCs with various concentration of ELL and confirmed that ELL enhanced expression levels of MHC class I, MHC class II, CD86, and CD40 molecules on the cell surface of BMDCs in a dose-dependent manner (Fig. 3B). In addition, we examined that effect of ELL in Th1/Th2 differentiation. Naïve OT-II cells were stimulated with OVA-peptide in the presence or absence of ELL. In Day4, these cells were stimulated with immobilize anti-CD3 mAb. As a result, ELL strongly enhances efficiency of Th1 cells and suppressed of Th2. (Fig. 3C). Thus, these data suggested that ELL would activate T cell-mediated Type-1 immune responses via maturation of DCs.

### 3.4. Administration of ELL with BMDCs efficiently induces antigen-specific CTLs *in vivo.*

To investigate whether ELL act as an adjuvant to induce antigen-specific CTLs *in vivo*, we immunized in the footpad of C57BL/6 mice with ELL, BMDCs and ovalbumin (OVA)

twice 5 days intervals. The lymphocytes were prepared from popliteal lymph nodes 5 days after final immunization to examine the generation of OVA-specific CTLs by staining with OVA tetramer. In the mice treated with DCs+OVA+ELL, the percentages and total numbers of OVA tetramer$^{+}$ CD8$^{+}$ CTLs were significantly increased compared with other immunization groups (Fig. 4A, 4B). Moreover, we examined the cytotoxic activity of CTL against EG7. As a result, administration of ELL strongly enhanced CTL function (Fig. 4C). Therefore, these findings indicated that ELL would be a useful adjuvant for vaccine therapy for cancer.

### 3.5. DC vaccine therapy combined with ELL effectively inhibits tumor growth

Finally, we examined the antitumor effects of vaccination with ELL in a therapeutic tumor model. EG7 tumor cells, expressing OVA as a model tumor antigen, were intradermally injected into C57BL/6 mice. When the tumor mass became palpable (6-7 mm), the tumor-bearing mice were treated by intradermal injection with OVA, BMDCs and ELL near the DLN of the tumor mass. Combination therapy with DCs+OVA+ELL significantly inhibited the growth of established tumor (Fig. 5). Although the mice treated with DCs+OVA and OVA+ELL also exhibited antitumor effects, DC vaccine therapy combined with ELL (DCs+OVA+ELL) were most potent therapeutic effects than other groups. We also confirmed that high percentages of OVA tetramer$^{+}$ CTLs infiltrated into tumor tissue of mice treated with DC+OVA+ELL (data no shown). Taken together, our data strongly indicated that ELL is a promising adjuvant for cancer immunotherapy.

## Discussion

In this article, we have shown that ELL is a potent adjuvant that can selectively induce Type-1 immunity through DCs activation. ELL strongly induced IL-12 and TNF-$\alpha$, which play a crucial role in DCs-mediated cancer immunotherapy [7, 30, 31] . Especially, IL-12 directly enhances proliferation of CTL and immunological synapse formation between CTL and target cells [32, 33]. In addition, IL-12 and TNF-$\alpha$ synergistically induces IFN-$\gamma$ positive CTL and effective antitumor activity [34]. Moreover, IL-12 and TNF-$\alpha$ also activate NK, NKT and Th1 cells, which also play an important role in tumor rejection [35-37].

TLRs, which are mainly expressed on DCs and macrophages, are critically important receptors in host defense against bacteria and viruses infection. Interestingly, here we first demonstrated that ELL acted as a TLR2 and TLR4 ligand and induced activation of DCs (Fig. 2). TLRs were sensors of microbe specific compounds, however it has been demonstrated that hyaluronun, heat shock protein (HSP), high mobility group B-1 (HMGB-1), saturated fatty acids, Versican are identified as the endogenous ligands of TLR2 and/or TLR4 [38, 39-42]. It is poorly understood how TLR2/4 recognized various endogenous and exogenous ligands. TLR2/4 interacts with other molecules (e.g., CD36, TLR1, TLR6 and CD14 etc.) and each complex recognizes different ligands, respectively [43-45]. These post-transcriptional regulations might be responsible for recognition of components of ELL by TLR2/4. TLR2 and TLR4 use MyD88 as adaptor molecules as well as almost TLRs, except TLR3 [25]. Moreover, TLR2 and TLR4 use TIRAP in contrast to other TLRs [46]. TLR4, but not TLR2 use TRIF/TICAM pathway, which induces Type1 interferon as well as TLR3. Thus, TLR2 and TLR4 have been considered to act cooperatively when they recognize various ligands [38, 47, 48]. Therefore, it is possible to speculate that ELL partially stimulates innate immunity by two signal pathways mediated by TLR2 and TLR4. HPLC analysis showed that ELL

consisted mainly of arabinose, galactose and glucose. In addition, phenol-sulfuric acid assay revealed that ELL consisted entirely of sugars (data not shown). These results suggest that some complex of Galactose, Arabinose and/or Gulucose might be novel ligands for TLR2 or TLR4. Indeed, arabinogalactan, lipoarabinomannan and galactomannan were reported as stimulator of DCs [49-51]. Previously, it is reported that arabinogalactan have an adjuvant activity for human PBMC. Arabinogalactan is included in bacterial cell wall, for example BCG. However, Arabinogalacton in BCG did not activate BMDCs [47]. It is noteworthy that structure of polysaccaride is different among species even if similar ingredients. Now, we are planning to identify the active components of ELL.

Because of severe immune suppression in cancer patient, it is a great research target in cancer immunotherapy to overcome the immune defects through activation of Type-1 immune responses. Although adoptive transfer of DCs loaded with tumor antigen is one of the most prominent strategies for cancer therapy, usage of adjuvants, inducing activation of DCs, is a promising strategy for developing effective cancer immunotherapy. In this work, we demonstrated that ELL contained plant-derived TLR ligands, which effectively induced Type-1 cytokine production by DCs, and adoptive transfer of DCs with ELL and tumor antigen remarkably inhibited tumor growth in the tumor-bearing mice, indicating that ELL would be a potent adjuvant to activate Type-1 immune responses (Fig. 5). So far, taxol, AILb-A and polysaccharide of safflower have been reported as plant-derived TLR ligands [52-54]. We also previously revealed that particular soybean had TLR2 and TLR4 ligands and induced Type-1 immune responses via IL-12 production from DCs in both mouse and human systems [55]. Thus, it is possible that functional screening of various plants might lead to find novel and effective adjuvants. Indeed, we already demonstrated that some extracts from various plant samples exhibited induction of Type-1 immunity. ELL is unique adjuvant because it is

derived from plant. Previously, we revealed that liposome-CpG, which effectively activates TLR9, is strong adjuvant [13]. Most of adjuvants are pure ligands, however, ELL includes various components. In our experiment, lower doses of ELL could not induce the production of TNF-$\alpha$ and IL-12 (data not whown). This may be because ELL contains little amount of component recognized by TLRs. Because ELL activity is less than CpG, to potentiate adjuvant activity as well as CpG and other adjuvants, we have to identify and purify the critical components of ELL, before application to clinical practices.

Cross-presentation of exogenous antigen by DCs is an important mechanism in activation of $CD8^+$ T cell-mediated immunity [56, 57]. Several reports demonstrated that TLR ligand promoted endosomal acidification, proteasomal activity, and TAP translocation in DCs, resulting in the efficient presentation of exogenous antigen and enhancement of CTL activation [58-61]. Therefore, promotion of cross-presentation would be involved in CTL induction by vaccination with ELL.

It is known that stimulation with multiple TLRs effectively activates DCs and subsequently induces strong T cell responses [62]. For example, the yellow fever vaccine YF-17D, one of the most successful empiric vaccines, activates dendritic cells via multiple TLRs [63]. In the present work, we finally revealed that adoptive transfer of DCs with ELL remarkably inhibited tumor growth in the tumor-bearing mice (Figure 5). The marked antitumor effects of ELL might be due to the synergistic impact of TLR2 and TLR4 ligand in ELL. Previously we demonstrated that in vivo administration of TLR9 ligand, CpG-ODN, up-regulated Type-1 immunity, also remarkably caused tumor regression [13, 64]. Thus, combination therapy of CpG-ODN and novel TLR ligands in ELL would be useful for therapeutic strategies of tumor and infectious diseases via induction of antigen-specific CTL induction.

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**Figure legends**

**Figure 1. ELL induces production Type-1 cytokines from BMDCs.**

BMDCs ($1x10^6$ cells), generated from bone marrow cells of WT mice, were stimulated with ELL. (A) Intracellular stainings with anti-IL-12p40/p70 or anti-TNF-$\alpha$ mAbs were performed for the BMDCs cultured with or without ELL (0.5, 2, and 10 mg/ml) for 12 hrs. The stained cells were then analyzed by flow cytometry, and the representative profiles of three independent experiments are indicated in the figure. The number in each profile indicates the percentages of cytokine-producing $CD11c^+$ BMDCs in 7AAD-negative populations. (B) Twenty-four hrs after ELL stimulation, IL-12p70 and TNF-$\alpha$ levels in the culture supernatants were determined by ELISA. Means and SDs were calculated from the representative data of five independent experiments and indicated in the figure.

**Figure 2. ELL induces activation of BMDCs in TLR2- and TLR4-dependent manners.**

BMDCs ($1x10^6$ cells) were generated from bone marrow cells of WT, $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR9^{-/-}$ mice. The BMDCs were cultured in the presence of ELL (10 mg/ml) for 24 hrs. IL-12p70 (A) and TNF-$\alpha$ (B) levels in the culture supernatants were determined by ELISA. Means and SDs were calculated from the representative data of three independent experiments indicated in the figure. Asterisks (*, **) mean $p < 0.05$ or $p < 0.01$, respectively, and show significant differences against the data of WT BMDCs**.**

**Figure 3. ELL significantly causes maturation of BMDCs.**

BMDCs ($1x10^6$ cells) were generated from bone marrow cells of WT mice and stimulated with or without ELL (2, 5, and 10 mg/ml) for 24 hrs. Expression levels of MHC

class I, MHC class II, CD86, or CD40 molecules on $CD11c^+$ $7AAD^-$ BMDCs were analyzed by flow cytometry. (A) The representative FACS profiles of three independent experiments are indicated in the figure. Dashed and bold lines mean control and ELL (10 mg/ml) treated groups, respectively. (B) The mean fluorescence intensity (MFI) of MHC class I levels and percentages of MHC class II-, CD86-, or CD40-expressing BMDCs were analyzed by flow cytometry. (C) OT-II naïve $CD4^+$ cells were isolated and cocultured with BMDCs, peptide (0.1 μg/ml) and IL-2 (10 U/ml) in the presence or absence of ELL (10 mg/ml). After 4 days, IFN-γ and IL-4 production were determined by Flow cytometry. The representative FACS profiles of three independent experiments are indicated in the figure. The means and SDs were calculated from the representative data of three independent experiments and indicated in the figure.

**Figure 4. Administration of ELL with BMDCs efficiently induces antigen-specific CTLs *in vivo.***

BMDCs were generated from bone marrow cells of WT mice. WT C57BL/6 mice were immunized with saline (Control), OVA alone (200 μg) (OVA), BMDCs ($5x10^5$) plus OVA (DC+OVA), OVA plus ELL (5 mg) (OVA+ELL), or BMDCs plus OVA plus ELL (DC+OVA+ELL) twice at 5 days intervals. Five days after the last immunization, lymphocytes were prepared from popliteal lymph nodes. The percentages of OVA-tetramer positive popiulations in $CD8^+$ T cells were determined by flow cytometry. (A) The representative profiles of three independent experiments are indicated in the figure. The numbers in the FACS profile indicated the percentages of OVA-$tetramer^+$ cells in $CD8^+$ T cells. (B) The percentages and absolute numbers of OVA-$tetramer^+$ cells were calculated from the data of three independent experiments, and the means and SDs are indicated in the figure. Asterisks mean $p < 0.05$ and show significant differences against the data of DC+OVA+ELL. (C) The cytotoxicity against EG7 and EL4

was measured by $^{51}$Cr-release assay. The means and SDs were calculated from the representative data of three independent experiments and indicated in the figure. Asterisks mean $p < 0.05$ show significant differences against the data of DC+OVA (**), OVA (*).

**Figure 5. DC vaccine therapy combined with ELL effectively inhibits tumor growth.**

OVA-expressing EG7 cells ($2x10^6$) were intradermally inoculated into WT C57BL/6 mice. BMDCs were generated from bone marrow cells of WT mice. (A) When the tumor mass became large (7–8 mm), the tumor-bearing mice were treated with saline (□; Control), BMDCs ($2x10^6$) plus ELL (20 mg) (▲; DC+ELL), OVA (200 μg) plus ELL (△; OVA+ELL), OVA plus BMDCs (●; OVA+DC), or BMDCs plus OVA and ELL (○; DC+OVA+ELL). The tumor sizes were measured in perpendicular diameters for 27 days and the means and SDs were indicated in the figure (n=4). Similar results were obtained in two independent experiments. Asterisks (*) mean $p < 0.05$ and show significant differences against the data of DC+OVA+ELL**.** (B) The representative photographs of the treated mice at Day 21 were indicated in the figures.

**Figure 1. Koizumi SI., et al.**

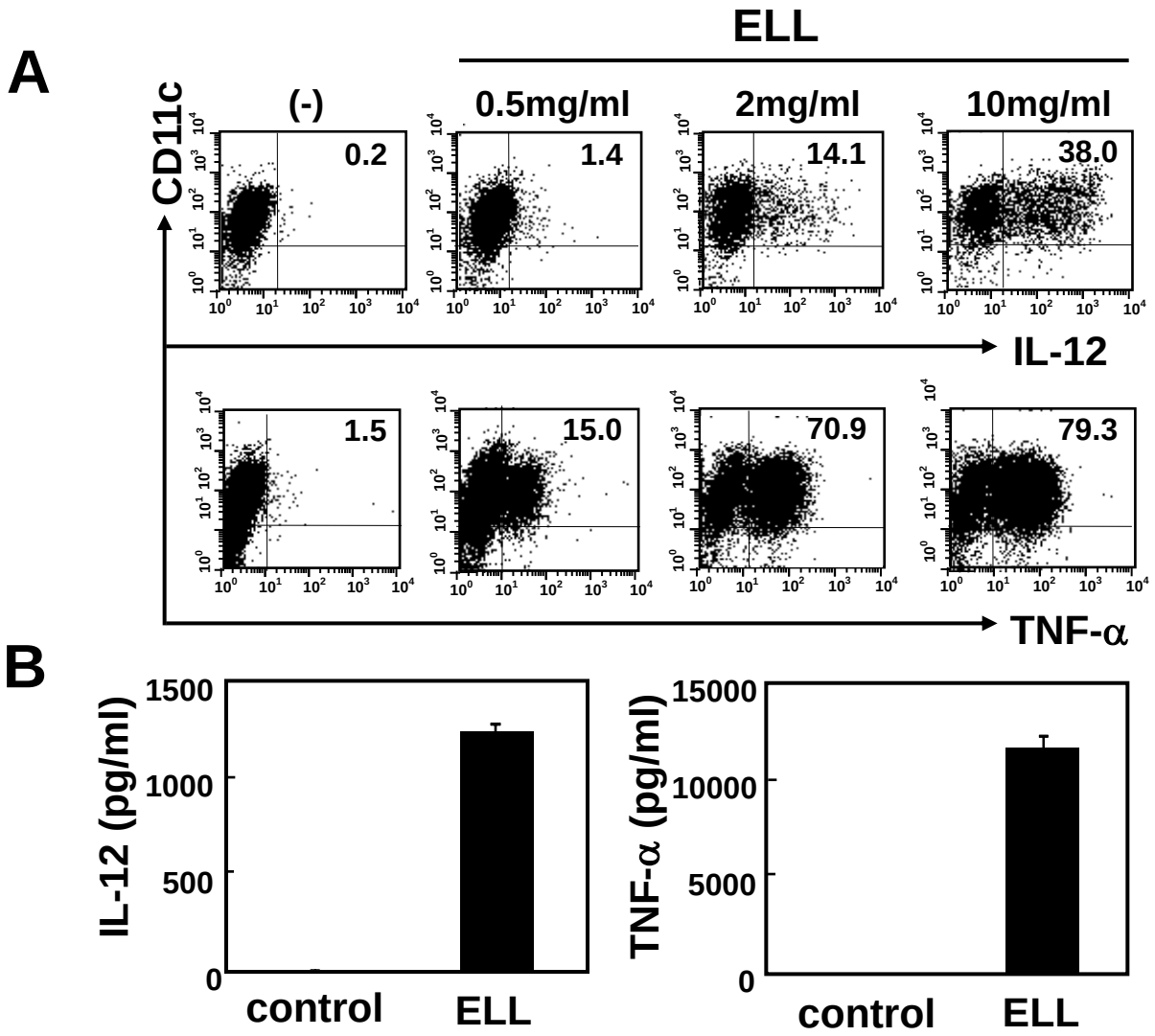


**Figure 2. Koizumi SI., et al.**

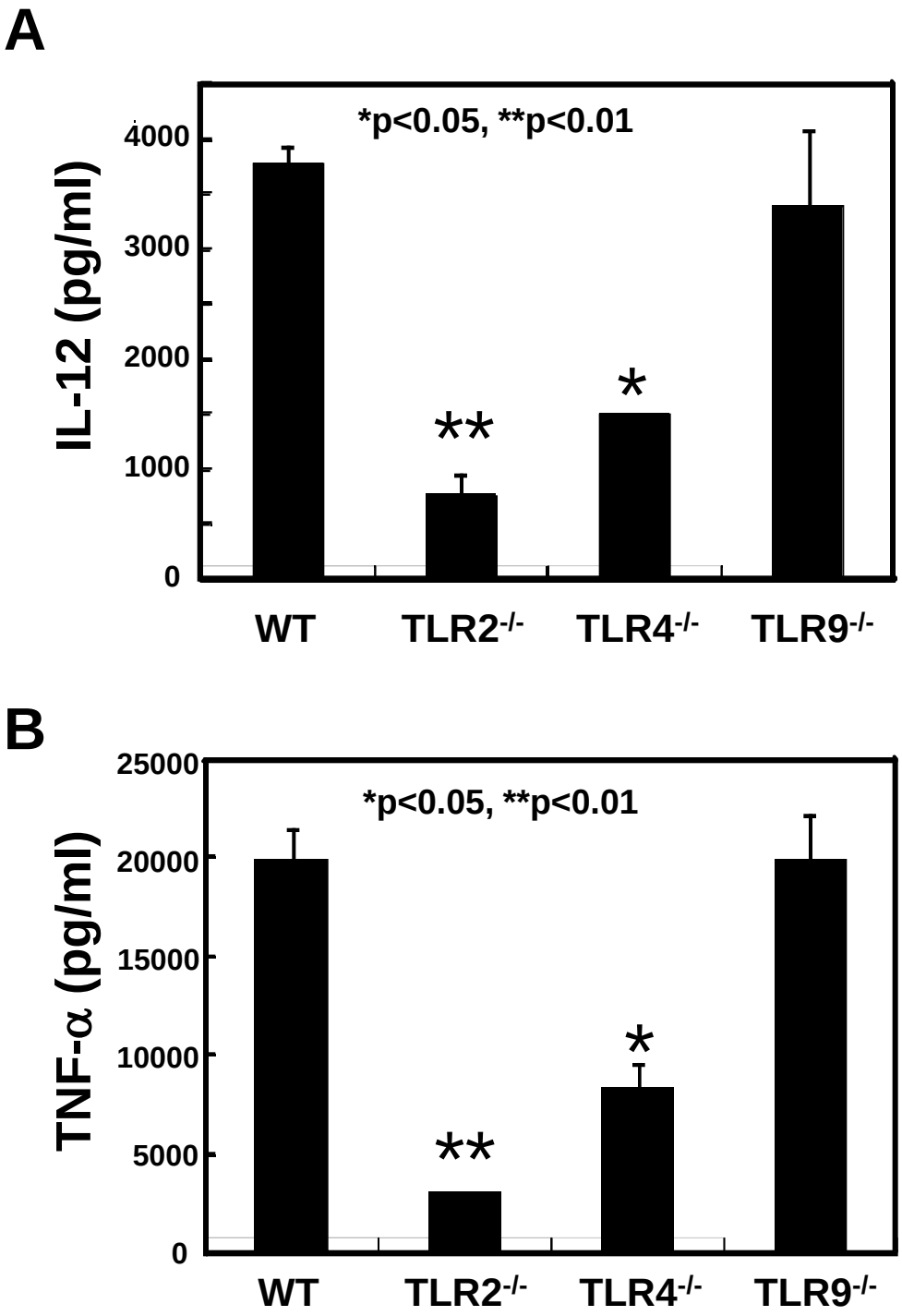
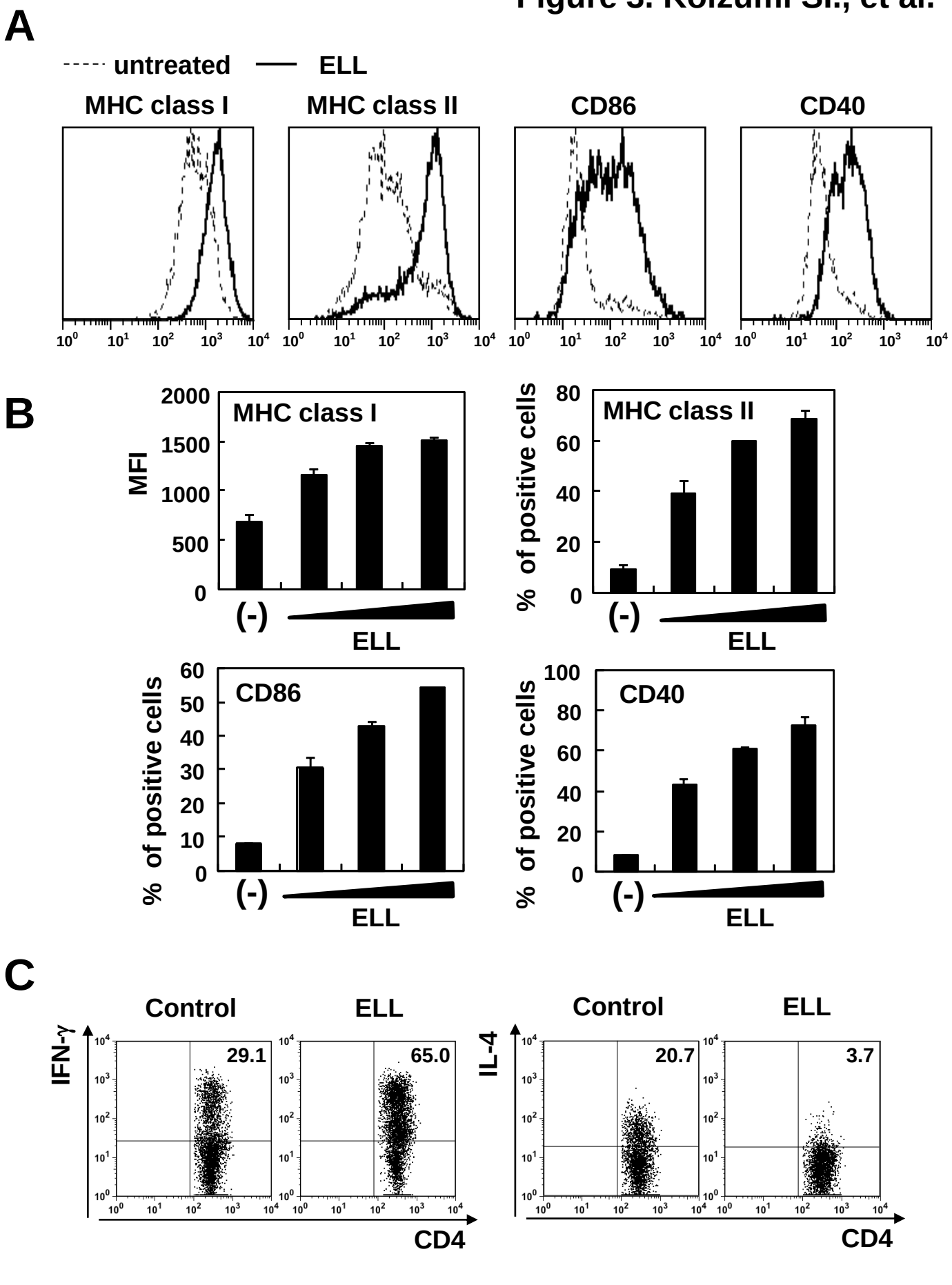


Figure 3. Koizumi SI., et al.



# Figure 4. Koizumi SI., et al.

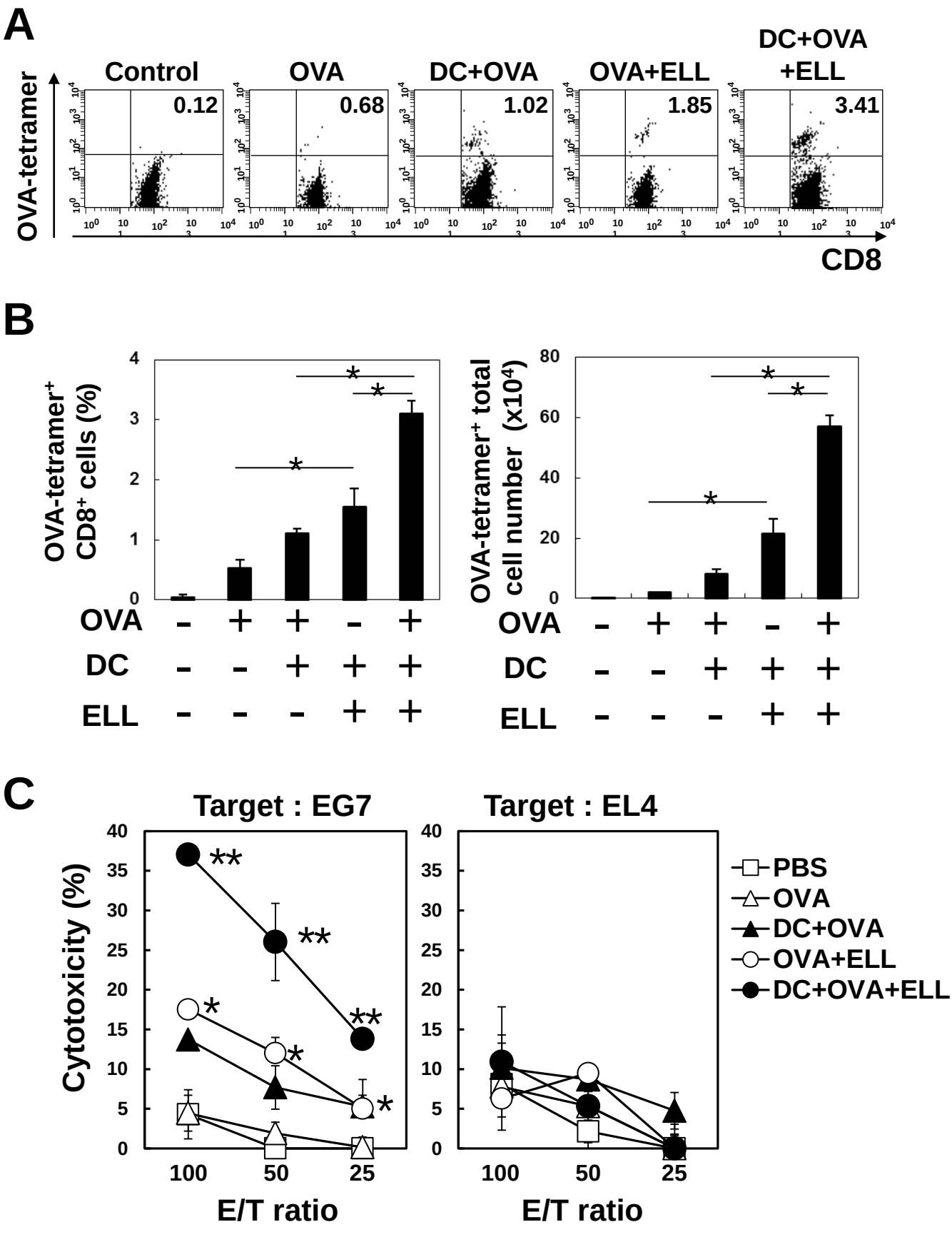
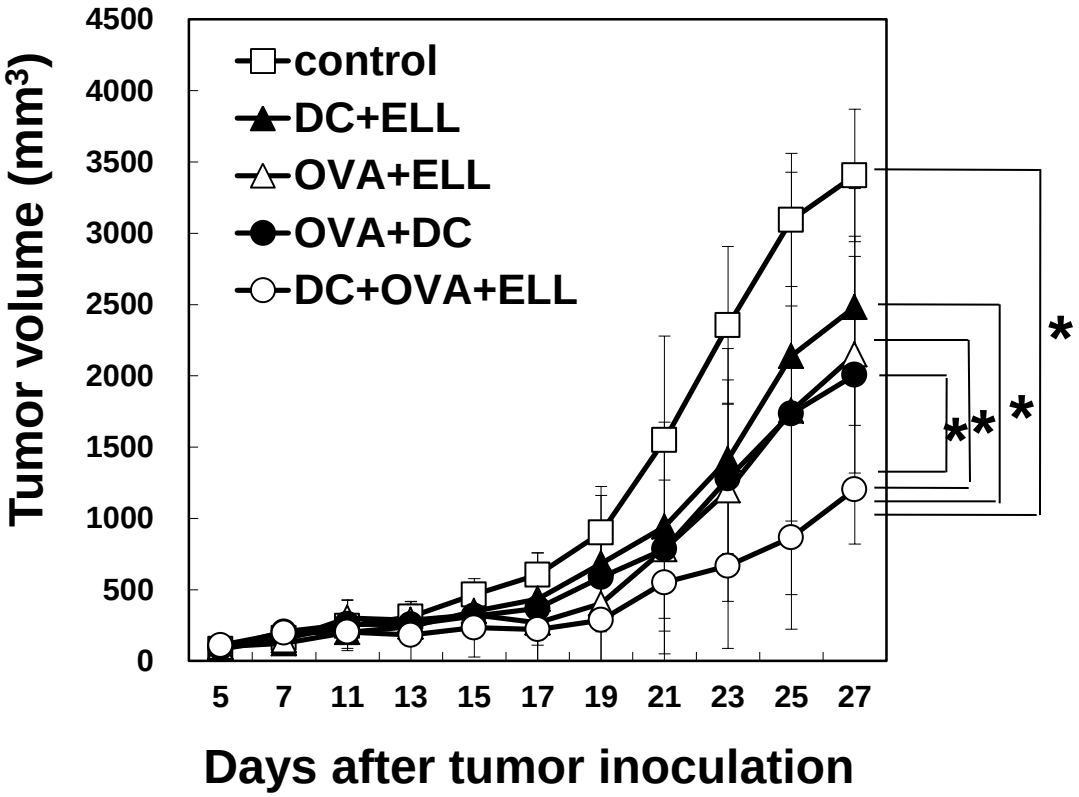
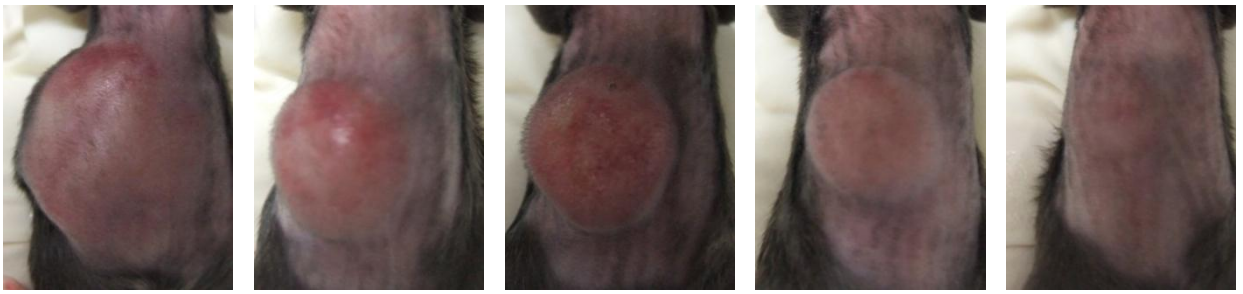


**Figure 5. Koizumi SI., et al.**

**A**




**B**



Control DC+ELL DC+OVA OVA+ELL DC+OVA +ELL