

Induction of Potent Antigen-specific Cytotoxic T Cell Response by PLGA-nanoparticles Containing Antigen and TLR Agonist

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Previously we showed that biodegradable nanoparticles containing poly-IC or CpG oligodeoxynucleotide (ODN) together with ovalbumin (OVA) were efficient at inducing MHC-restricted presentation of OVA peptides in dendritic cells. The CTL-inducing activities of the nanoparticles were examined in the present study. Nanoparticles containing poly-IC or CpG ODN together with OVA were prepared using biodegradable polymer poly(D,L-lactic acid-co-glycolic acid), and then were opsonized with mouse IgG. The nanoparticles were injected into the tail vein of mice, and 7 days later the OVA-specific CTL activities were measured using an *in vivo* CTL assay. Immunization of mice with the nanoparticles containing poly-IC or CpG ODN together with OVA elicited potent OVA-specific CTL activity compared to those containing OVA only. In accordance with these results, nanoparticles containing poly-IC or CpG ODN together with OVA exerted potent antitumor activity in mice that were subcutaneously implanted with EG7.OVA tumor cells. These results show that encapsulation of poly-IC or CpG ODN together with antigen in biodegradable nanoparticles is an effective approach for the induction of potent antigen-specific CTL responses *in vivo*.

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Induction of robust cytotoxic T lymphocyte (CTL) responses is essential for the immunotherapy against cancers or viral infections. Naïve CD8 T cells become activated when their receptors recognize antigens presented by professional antigen presenting cells in the context of MHC-I molecules (1).

The cross-presentation pathway, which allows MHC-I-restricted presentation of exogenous antigen, appears to be an obligatory mechanism for the generation of CTL responses to antigens that are expressed only in nonprofessional antigen presenting cells (APCs) (2-6). In the absence of such a mechanism, viral or tumor antigens expressed in nonprofessional APCs could escape immunosurveillance because CTL responses can only be induced efficiently for the antigens presented via class I MHC molecules on professional APCs (2-6).

Delivery of antigens using nanoparticles prepared from biodegradable polymers such as poly(D, L-lactic acid-co-glycolic acid) (PLGA) into professional APCs is an efficient method for the induction of potent CTL responses. We and others have also shown that PLGA particle-mediated antigen delivery enhances and prolongs the MHC class I-restricted presentation of the exogenous antigens (cross-presentation) in dendritic cells (DCs) (7-10). PLGA-nanoparticles have also been shown to deliver antigens to APCs efficiently and generate Th1-type immune responses even against poor immunogens (11,12). In our hand, antigens encapsulated with PLGA were at least 100 times more effective in inducing MHC-I-restricted presentation of exogenous antigen in DCs (13). Another advantage of nanoencapsulation would also be the protection of the encapsulated antigens and the immunomodulators from degradation by serum enzymes (14).

Recently, we showed that nanoparticles containing poly-IC or CpG ODN together with ovalbumin (OVA) increases and prolongs both MHC class I- and class II-restricted presentation

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Abbreviations: APC, antigen presenting cell; DC, dendritic cell; ODN, oligodeoxynucleotide; OVA, ovalbumin

of OVA peptides in DCs (9). In the present study, we examined the capability of the nanoparticles to induced OVA-specific CTL responses in mice.

Nanoparticles containing poly-IC or CpG ODN together with OVA were prepared using a biocompatible/biodegradable polymer, PLGA, as described earlier (9). The poly-IC used in the present study was purchased from Invivogen (San Diego, CA, USA). Unmethylated CpG oligodeoxynucleotide (ODN), 5'-TCC ATG ACG TTC CTG ATG CT-3', was synthesized by the Bionics Co. Ltd (Seoul, Korea). The amounts of poly-IC and CpG DNA contained in the nanoparticles were 1.40 and 2.01 $\mu\text{g}/\text{mg}$ nanoparticles, respectively. The average content of OVA was 21.68 $\mu\text{g}/\text{mg}$ nanoparticles. For opsonization, OVA-specific mouse IgG (mIgG) or was attached covalently to the nanoparticles using (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) (EDC, Pierce, Rockford, IL, USA) as previously described (9).

The CTL inducing activities of the nanoparticles containing

OVA only (NP[OVA]), both OVA and poly-IC (NP[OVA+I : C]), or both OVA and CpG ODN (NP[OVA+CpG]) were compared in mice. In this experiment, the nanoparticles were injected intravenously into tail veins of mice (100 μg as OVA/mouse). Seven days later, an *in vivo* CTL assay was performed in the mice using CFSE-labeled syngeneic target cells, as described in detail in the earlier paper (9). Fig. 1A shows representative histograms of the cells isolated from the spleens. Addition of poly-IC or CpG to OVA-nanoparticles significantly increased their ability to induce OVA-specific CTLs in the spleens (Fig. 1B) and lymph nodes (Fig. 1C). Immunization of mice with both NP[OVA+I : C] and NP[OVA+CpG] further increased OVA-specific CTLs in the spleens and lymph nodes.

To confirm that the induction of OVA-specific CTL activity is sufficient to engender antitumor activity, mice were immunized with the nanoparticles containing bovine serum albumin (BSA) only (NP[BSA]), OVA only (NP[OVA]), both OVA

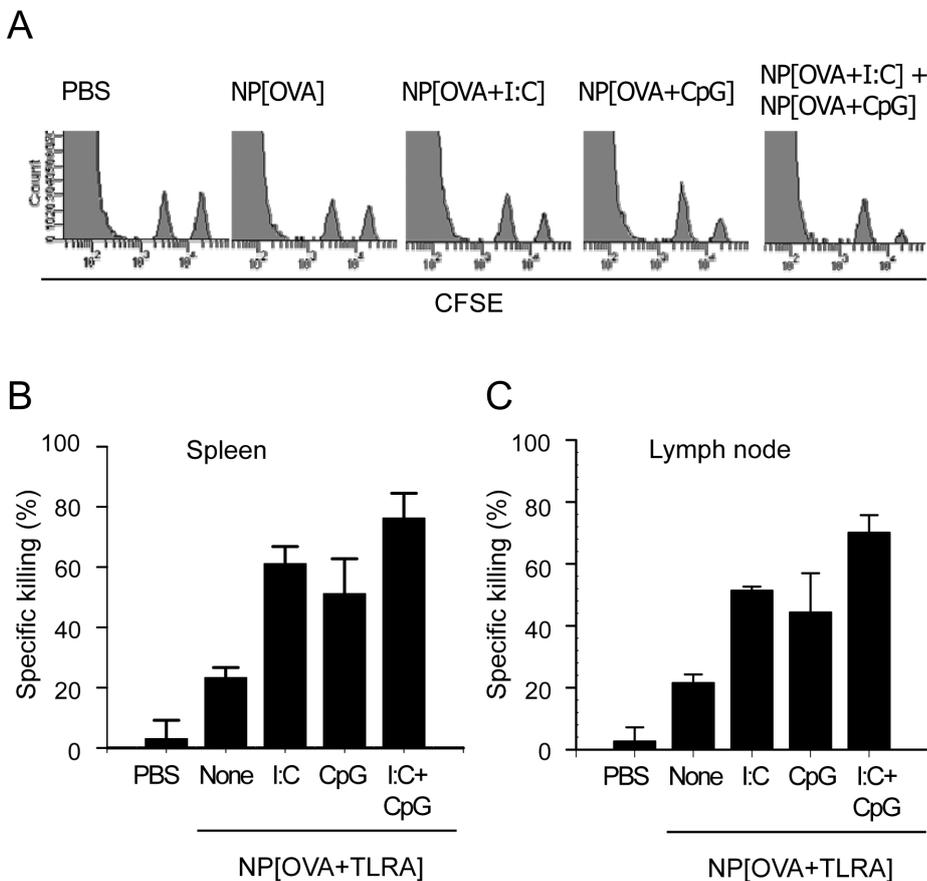


Figure 1. The CTL inducing activities of the nanoparticles. The nanoparticles containing OVA only (NP[OVA]), both OVA and poly-IC (NP[OVA+I : C]), or both OVA and CpG ODN (NP[OVA+CpG]) were injected intravenously into tail veins of mice. Seven days later, an *in vivo* CTL assay was performed in the mice using CFSE-labeled syngeneic target cells. (A) Representative histograms of the spleen cells of individual mice were shown. The percentages of specific killing of OVA[257-264] peptide-pulsed target cells in the spleens (B) and lymph nodes (C) were graphically represented.

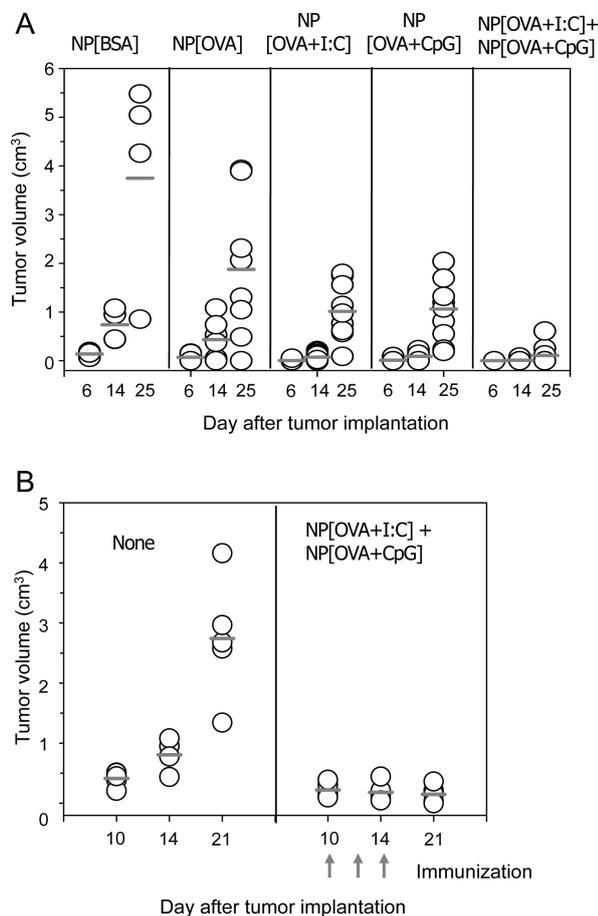


Figure 2. The antitumor activities of the nanoparticles. (A) Mice were immunized with the nanoparticles containing bovine serum albumin (BSA) only (NP[BSA]), OVA only (NP[OVA]), both OVA and poly-IC (NP[OVA+I : C]), or both OVA and CpG ODN (NP[OVA+CpG]), intravenously into tail veins of the mice. Seven days later, the mice were subcutaneously implanted with EG7.OVA tumor cells (5×10^5 /mouse). Two days later, the mice were again immunized with the same nanoparticles intravenously into tail veins of the mice. The tumor size was measured with a slide caliper and expressed as a tumor index, determined as the square root of (major axis \times minor axis). (B) Mice were subcutaneously implanted with the tumor cells, and then mixtures of NP[OVA+I : C] and NP[OVA+CpG] were injected into the tumor mass on 10, 12 and 14 days after the tumor implantation.

and poly-IC (NP[OVA+I : C]), or both OVA and CpG ODN (NP[OVA+CpG]), intravenously into tail veins of the mice (10 μ g as OVA or BSA/mouse). Seven days later, the mice were subcutaneously implanted with EG7.OVA tumor cells (5×10^5 /mouse), which is a mouse lymphoma expressing OVA. Two days later, the mice were immunized with the same nanoparticles intravenously into tail veins of the mice. The tumor size was measured with a slide caliper and expressed as a

tumor index, determined as the square root of (major axis \times minor axis). As shown in Fig. 2A, the growth of tumors was obvious from day 14 after tumor cell implantation, and reached to average size of 3.76 cm^3 at day 25 in the mice that were immunized with the nanoparticles containing an irrelevant protein, BSA. Immunization of the mice with NP[OVA] significantly reduced the size of the tumors. The average size of the tumors was 2.92 cm^3 at day 25 in the mice that were immunized with the NP[OVA]. Addition of poly-IC or CpG to OVA-nanoparticles significantly reduced the size of the tumors. Immunization of mice with both NP[OVA+I : C] and NP[OVA+CpG] almost completely reduced the development of the tumors.

The antitumor efficacy of the combined use of NP[OVA+I : C] and NP[OVA+CpG] was further confirmed in mice implanted with EG7.OVA tumor cells. In this experiment, mice were subcutaneously implanted with the tumor cells, and then both types of the nanoparticles were mixed in a 1 : 1 ratio, and injected into the tumor mass on 10, 12 and 14 days after the tumor implantation (20 μ g as OVA/mouse). As shown in Fig. 2B, intratumoral injection of both NP[OVA+I : C] and NP[OVA+CpG] completely inhibited the formation of tumor mass.

Because the EG7.OVA cells express only MHC-I molecules and not MHC-II molecules, it is reasonable to speculate that the antitumor activity shown by the nanoparticles is the reflection of the OVA-specific CTL activity (15). In addition, it is noteworthy to note that the TLR agonists, poly-IC and CpG, were entrapped inside the PLGA-nanoparticles. Encapsulation prevents not only the systemic effects of the TLR agonists, but also the enzymatic degradation of the TLR agonists (9,14-18).

Robust induction of CTL activity is important in the immunotherapy of tumors and viral infections. Recently, our laboratory has been involved in the development of strategies to enhance the MHC-I-restricted antigen presentation of exogenous antigen (8,9,19,20). We showed that nanoencapsulation of poly-IC or CpG together with OVA is an efficient approach to increase and prolong the MHC-restricted presentation of OVA peptides in dendritic cells (9). We also showed that IgG-opsonized PLGA-nanoparticles with a mean size of 1.1 μ m would be the choice of biodegradable carriers for the targeted-delivery of protein antigens for cross-priming *in vivo* (20). The present study confirms that nanoparticles containing poly-IC or CpG ODN together with OVA induce potent antitumor CTL activities in mice, and the OVA-specific

CTL activity is sufficient to inhibit the growth of EG7.OVA tumor cells in mice. Our study also shows that encapsulation of poly-IC or CpG ODN together with antigen in biodegradable nanoparticles is an effective approach for the induction of potent antigen-specific CTL responses.

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CONFLICTS OF INTEREST

The authors declare no financial or commercial conflicts of interest.

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