An indispensable role of type-1 IFNs for inducing CTL-mediated complete eradication of established tumor tissue by CpG-liposome co-encapsulated with model tumor antigen

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Abstract

We have evaluated the capacity of a novel, nanoparticle-based tumor vaccine to eradicate established tumors in mice. C57BL/6 mice were intradermally (i.d.) inoculated with ovalbumin (OVA)-expressing EG-7 tumor cells. When the tumor size reached 7–8 mm, the tumor-bearing mice were i.d. injected near the tumor-draining lymph node (DLN) with liposomes encapsulated with unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotides (CpG-ODN) (CpG-liposomes) co-encapsulated with OVA. This vaccination protocol markedly prevented the growth of the established tumor mass and ~50% of tumor-bearing mice became completely cured. Tumor eradication correlated with the generation of OVA/H-2Kb-tetramer+ CTLs in the tumor DLN and at the tumor site with specific cytotoxicity toward EG-7 cells. Interestingly, tetramer+ CTLs failed to be induced in lymph node-deficient Aly/Aly mice. Thus, tetramer+ CTLs appeared to be generated in the tumor DLN and subsequently migrated into the tumor site. In vivo antibody blocking experiments revealed that CD8+ T cells, but not CD4+ T, NK or NKT cells, were the major effector cells mediating tumor eradication. CTL induction was also inhibited when vaccinated tumor-bearing mice were treated with both anti-IFN-α and anti-IFN-β mAbs but not with anti-IFN-α or anti-IFN-β mAb alone. Neither IFN-γ−/− nor IL-12−/− mice showed impaired induction of tetramer+ CTLs. Thus, these findings revealed that CpG-ODN-induced IFN-α/β, but not IL-12 or IFN-γ, is critical for the generation of tumor-specific CTLs in response to vaccination. These results highlight the potential utility of CpG-liposomes co-encapsulated with protein tumor antigens as therapeutic vaccines in cancer patients.

Introduction

Antigen-presenting cells (APCs), especially dendritic cells (DCs), play a pivotal role in bridging innate and acquired immunities. These cellular interactions are essential for inducing antigen-specific protective immunity against infectious diseases and malignant tumors (1, 2). DCs interact with pathogens using Toll-like receptors (TLRs). Engagement of TLRs induces the production of IL-12, IL-18, IFN-γ, IFN-α/β and/or IL-10, which influences the activation of NK cells, NKT cells and conventional T cells (3, 4). For example, unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotides (CpG-ODNs), which represent ligands of TLR9, can induce the production of IFN-α/β, IFN-γ and IL-12 from DCs, which are essential for activation of type-1 innate and acquired effector cells (3–8). Several studies have shown that activation of type-1 immune responses by CpG-ODN inhibits tumor growth (8–10). Moreover, CpG-ODN, when administered together with tumor antigens, demonstrate potent adjuvant activity, resulting in the generation of tumor-specific CTLs and complete inhibition of tumor growth when mice were vaccinated prior to or a few days after tumor
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challenge (11–14). Thus, the anti-tumor activities of CpG-ODN and CpG-ODN-based tumor vaccines are only effective during tumor immunophylaxis but not for immunotherapy of an established tumor mass. One approach that has been utilized to circumvent this limitation is to combine the vaccination of antigenic tumor peptides with CpG-ODN and Flt3-L (12, 13). Here, we have utilized an alternative approach to develop tumor vaccines that are based on the adjuvant activities of CpG-ODN.

In a previous study, we have demonstrated that CpG-ODN co-encapsulated with a model tumor antigen ovalbumin (OVA) is superior to unmodified CpG-ODN plus tumor antigen for inducing IFN-γ-producing NK cells, NKT cells and antigen-specific CTLs (6). Here we have applied this powerful liposomes co-encapsulated with unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotides and ovalbumin [(CpG + OVA)-liposome]-based tumor vaccine to the therapy of mice bearing a large established tumor mass. We addressed whether (CpG + OVA)-liposomes can completely cure tumor-bearing mice. We also evaluated the molecular and cellular mechanisms that are responsible for the complete rejection of an established tumor mass by CpG-ODN-based tumor vaccination therapy.

It has been reported that IL-12, produced by CpG-ODN-stimulated DC, is a critical cytokine for inducing IFN-γ-producing NK, NKT, type-1 helper T cells (T H1) and type-1 cytotoxic T cells (Tc1) cells, which orchestrate type-1 immunity (5, 6, 15). However, recent results have also emphasized an important role of type-1 IFNs (IFN-α/β), which are produced by most cells upon virus infection (16, 17). It has also been reported that activation of plasmacytoid dendritic cell (pDC) with agonists of TLR7 or TLR9 induces robust production of IFN-α/β, which is crucial for the generation of antigen-specific CTLs and for the activation of NK cells (18–21). However, these experiments have been carried out in mice that were either tumor free or that had a small tumor burden. As reported previously, immune surveillance mechanisms are strongly suppressed in tumor-bearing mice because of various immunosuppressive conditions (22–25). Immunoregulatory cells, including APCs and T cells, obtained from tumor-bearing mice have diminished immune functions compared with tumor-free animals. Therefore, it has remained unclear whether CpG-ODN-induced IFN-α/β can overcome the strong immunosuppression in tumor-bearing mice and induce the CTL-mediated rejection of established tumors.

To investigate the capacity of CpG-ODN-based vaccines to eradicate established tumors, we developed a tumor therapy model by vaccination with liposomes encapsulated with unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotides (CpG-liposomes) co-encapsulated with a model tumor antigen, OVA. Using this model, we demonstrate here that (CpG + OVA)-liposome-induced IFN-γ, but not IL-12 and IFN-γ, is indispensable for inducing tetramer+ tumor-specific CTLs that can completely cure mice from established tumors.

It is well established that IFN-γ-producing T H1 play a crucial role in the induction of tumor-specific CTLs in tumor-bearing mice. Potent T H1 responses are required to overcome the strong immunosuppression that is mediated by immunoregulatory T cells or immunosuppressive immature myeloid cells (26–29, 30). However, here we demonstrate that CD4+ T cells, NKT cells or NK cells are not required for inducing tetramer+ tumor-specific CTLs that can completely cure tumor-bearing mice in response to vaccination with (CpG + OVA)-liposomes. We also provide evidence that intradermal vaccination with (CpG + OVA)-liposomes near the draining lymph node (DLN) of the tumor is required for inducing tetramer+ tumor-specific CTLs that can eradicate an established tumor mass.

Thus, the present paper establishes that (i) tumor vaccination with (CpG + OVA)-liposomes can induce tetramer+ tumor-specific CTLs in tumor-bearing mice that can completely eradicate an established tumor mass, (ii) intradermal administration of (CpG + OVA)-liposomes near the tumor DLN is essential for inducing tumor-specific CTLs at the local tumor site (DLN and tumor site) and (iii) this vaccination protocol induces tumor-specific CTLs in an IFN-α/β-dependent and CD4+ T H1-independent manner. Collectively, our findings reveal that nanoparticles of a tumor vaccine and the adjuvant CpG-ODN exhibit superior anti-tumor activities that could be exploited for the development of effective therapeutic tumor vaccines for human patients.

Methods

Mice

C57BL/6 mice were obtained from Charles River Japan (Yokohama, Japan). Alymphoplasia mutant mice (Aly/Aly mice), which are characterized by the systemic absence of lymph nodes (LNs) and Peyer’s patches (31), were purchased from CLEA Japan (Tokyo, Japan). IL-12p40-deficient C57BL/6 mice were purchased from The Jackson Laboratory. IFN-γ-deficient C57BL/6 mice were kindly provided by Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). All mice were female and were used at 6–8 weeks of age.

Antigen, mAb and tetramer

OVA protein was purchased from Sigma–Aldrich Japan (Tokyo, Japan). FITC-CD8 mAb was purchased from PharMingen (San Diego, CA, USA). Anti-CD8 mAb-conjugated microbeads for the MACS system were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD8, anti-CD4 and anti-NK1.1 mAbs for cell depletion were produced by 53-6.7.2, GK1.5 and PK136 hybridoma clones, respectively. Anti-IFN-α and anti-IFN-β mAbs for cytokine neutralization were purchased from PBL Biomedical Laboratories. H-2K b OVA tetramer-SIINFEKL-PE (OVA tetramer) and H-2K b tetramer-SIYRYGGL-PE (control tetramer) were purchased from MBL (Nagoya, Japan).

CpG-ODN and liposomes

Phosphorothioate-stabilized CpG-ODN 1668 (5′-TCCATGACGTTCTCGATGCT-3′) was synthesized by Hokkaido System Science (Sapporo, Japan). Cationic liposomes were purchased from NOP Corporation (Tokyo, Japan).

Tumor immunotherapy model

OVA-expressing EG-7 cells (2 × 10⁶) were intradermally (i.d.) inoculated into C57BL/6 mice. When the tumor mass became...
large (7–8 mm), the tumor-bearing mice were treated with saline, liposome-encapsulated OVA protein (200 µg), liposome-encapsulated CpG-ODN (50 µg) or liposome-encapsulated CpG-ODN plus OVA protein. The anti-tumor activity was determined by measuring the tumor size in perpendicular diameters. Tumor volume was calculated by the following formula: tumor volume = 0.4 × length (mm) × width (mm)^2. Tumor-bearing mice that survived for >60 days after therapy were considered completely cured.

**Antibody staining and flow cytometry**

For analysis of OVA-specific CTL frequencies, lymphocytes from the DLN and tumor tissue were stained with FITC-conjugated anti-CD8 mAb and PE-conjugated OVA tetramer or control tetramer. Data were acquired on a Becton Dickinson FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed using CellQuest software (Becton Dickinson).

**Cytotoxicity assay**

The cytotoxicity mediated by tumor-specific CTLs was measured by a 6-h ^51^Cr-release assay, as described previously (32). Tumor-specific cytotoxicity was determined using EG-7 cells (OVA gene-transfected EL-4 cells) as target cells. Parental EL-4 cells were used as control target cells. To confirm the antigen specificity of H-2K^b^-restricted CTLs, ^51^Cr-labeled target cells were incubated with CD8^+^ CTLs pre-treated with OVA tetramer, which can block the recognition of H-2K^b^-restricted target peptide (OVA257–264) recognition by CTLs. CD8^+^ T cells were enriched by MACS system according to the manufacturer’s protocol. The percent cytotoxicity was calculated as described previously (32).

**Results**

**Eradication of an established tumor mass by vaccination with CpG-ODN and tumor antigen co-encapsulated in liposomes**

The anti-tumor effect of vaccination with CpG-ODN and tumor antigen (OVA) co-encapsulated in liposomes was examined in a therapeutic tumor model. We encapsulated the CpG-ODN together with a model protein tumor antigen (OVA) into cationic liposomes [(CpG + OVA)-liposomes]. C57BL/6 mice were i.d. inoculated with OVA-expressing EG-7 tumor cells. When the tumor mass became palpable (7–8 mm), the tumor-bearing mice were treated by intradermal injection of (CpG + OVA)-liposomes near the DLN of the tumor. As shown in Fig. 1, vaccination of tumor-bearing mice with (CpG + OVA)-liposomes caused profound growth inhibition of the established tumor mass and ~50% tumor-bearing mice were completely cured from the tumor (Fig. 1B). Although both treatments with CpG-liposomes and treatment with ovalbumin encapsulated in liposomes (OVA-liposomes) showed a significant therapeutic effect against EG-7-bearing mice compared with control (Fig. 1A and C–E), (CpG + OVA)-liposomes treatment was most effective than these two groups. Instead, all tumor-bearing mice treated with CpG-liposomes or OVA-liposomes died within 50 days after tumor inoculation (Fig. 1B).

**Generation of tumor-specific CTLs in tumor-bearing mice by vaccination with (CpG + OVA)-liposomes**

To investigate the induction of tumor-specific CTLs during EG-7 eradication by treatment with (CpG + OVA)-liposomes, we analyzed the frequency of CTL induction in tumor-bearing mice.
mice 6 days after vaccination. Lymphocytes were prepared from tumor DLN or tumor mass to examine the generation of tumor antigen (OVA)-specific CTLs by staining with OVA/H-2Kb-tetramers. In the mice vaccinated with (CpG + OVA)-liposomes, tetramer+ CD8+ cells were significantly increased at the DLN (1.3%) and at the tumor mass (30.8%), as compared with mice treated with CpG-liposomes or OVA-liposomes alone (Fig. 2A, a–h). The increased percentage of tetramer+ cells was not due to non-specific binding of OVA/H-2Kb-tetramers because <0.1% and <1.8% of all CD8+ Tcells at the DLN and the tumor tissue, respectively, were stained with control tetramer reagent (Fig. 2A, i–l). We also demonstrated that total lymphocyte population isolated from DLN exhibited specific cytotoxicity against EG-7 but not EL-4 tumor cells (Fig. 2B). Moreover, CD8+ T cells purified from the DLN exhibited strong cytotoxicity against EG-7 cells and this cytotoxicity was strongly blocked by OVA/H-2Kb-tetramer (Fig. 2C). These results revealed that vaccination with (CpG + OVA)-liposomes effectively induced OVA/H-2Kb-tetramer+ CD8+ CTLs in tumor-bearing mice that were able to specifically
lyse EG-7 tumor cells by recognizing the H-2K\(^b\)-binding OVA\(_{257-264}\) peptide antigen.

**Critical role of the tumor DLN for generation and accumulation of tumor-specific CTLs in tumor tissue following vaccination with (CpG + OVA)-liposomes**

As shown in Fig. 2(A), tetramer\(^+\) CD8\(^+\) CTLs were highly enriched at the tumor mass, as compared with the DLN. This result indicated that CD8\(^+\) CTLs might be induced at the DLN first, then rapidly migrate and accumulate in the tumor mass. To test this working hypothesis, we compared the anti-tumor effect of (CpG + OVA)-liposomes between wild-type and LN-deficient (Aly/Aly) mice bearing an established EG-7 tumor mass. In contrast to tumor-bearing wild-type mice, the therapeutic effect of (CpG + OVA)-liposomes vaccination was markedly decreased in tumor-bearing Aly/Aly mice and no mice treated with the vaccine were cured from the tumor (Fig. 3A–C). We further evaluated the critical role of tumor DLN for tumor eradication by (CpG + OVA)-liposomes vaccine therapy. Consistent with the results shown in Fig. 1, the tumor growth was completely blocked when (CpG + OVA)-liposomes were injected i.d. into tumor-bearing mice at the ipsilateral (right) side near the tumor mass, and this correlated with increased induction of tetramer\(^+\) tumor-specific CTLs. However, when tumor-bearing mice were vaccinated with (CpG + OVA)-liposomes at the contralateral (left) side distant from the tumor, the therapeutic effect of CpG-ODN-based vaccination was greatly decreased, no mice were cured from the tumor, and this correlated with reduced generation of tetramer\(^+\) CTLs (Fig. 4). These results suggested that tumor-specific CTLs were first induced in the tumor DLN, but not distal LN, spleen or tumor tissue, and then migrated into the tumor site.

**Tumor eradication by treatment with (CpG + OVA)-liposomes is dependent upon CD8\(^+\) CTLs induced in the absence of CD4\(^+\) T cell help**

The cellular mechanisms underlying complete tumor eradication by (CpG + OVA)-liposomes vaccination was examined by blocking experiments using various mAbs recognizing anti-tumor effector cells (Fig. 5). The anti-tumor effect induced by treatment with (CpG + OVA)-liposomes was completely abolished in tumor-bearing mice injected with anti-CD8 mAb (Fig. 5A). However, tumor-bearing mice treated with either anti-CD4 mAb or anti-NK1.1 mAb showed the same anti-tumor effect as vaccinated tumor-bearing mice. These results indicated that the final effector cells during tumor eradication were CD8\(^+\) CTLs. This finding was confirmed by examining the effect of mAb treatment on the generation of OVA/H-2K\(^b\)-tetramer\(^+\) CTLs in tumor-bearing mice by vaccination with (CpG + OVA)-liposomes. As shown in Fig. 5(B), depletion of NK1.1\(^+\) (NK and NKT) cells and CD4\(^+\) T cells had no significant effect on the induction of OVA tetramer\(^+\) CTLs. Thus, only the induction of tetramer\(^+\) CTLs correlated directly with the capacity of the (CpG + OVA)-liposomes vaccine to induce strong anti-tumor responses in tumor-bearing mice. From these results, we conclude that OVA/H-2K\(^b\)-tetramer\(^+\) CD8\(^+\) CTLs, induced independently of NK, NKT and T\(_h\) cells, are the final effector cells essential for complete tumor eradication in vivo.

**Type-1 IFNs (IFN-\(\alpha\) or IFN-\(\beta\)), but not IL-12 and IFN-\(\gamma\), are required for the generation of OVA/H-2K\(^b\)-tetramer\(^+\) CTLs in response to vaccination of tumor-bearing mice with (CpG + OVA)-liposomes**

To determine which cytokines are critical for the generation of OVA/H-2K\(^b\)-tetramer\(^+\) CTLs in response to vaccination with (CpG + OVA)-liposomes.
Fig. 4. Vaccination with (CpG + OVA)-liposomes near the tumor DLN, but not at the opposite side of the tumor, induces potent anti-tumor immunity. (A) C57BL/6 mice were i.d. inoculated with EG-7 cells at the right flank. When the tumor mass became palpable (7–8 mm), the tumor-bearing mice were injected i.d. with saline (open triangle), (CpG + OVA)-liposomes near the DLN (right: filled circle) or (CpG + OVA)-liposomes at the LN at the opposite side of the tumor (left: open circle). The anti-tumor effect was determined as in Fig. 1. Six days after vaccination, lymphocytes were prepared from the DLN and the tumor tissue, and the percentage of OVA/H-2Kb-tetramer+ CD8+ cells was determined (B). The numbers in the top right quadrant represent the percentage of tetramer+ cells among total CD8+ cells.

Fig. 5. Blockade of the anti-tumor effect of (CpG + OVA)-liposomes by in vivo administration of mAb against CD8, but not CD4 or NK1.1. C57BL/6 mice were i.d. inoculated with EG-7 tumor cells and injected with (CpG + OVA)-liposomes as described in Fig. 1. Tumor-bearing mice were also injected intravenously with saline (filled circle), anti-NK1.1 mAb (filled triangle), anti-CD4 mAb (filled square), or anti-CD8 mAb (open circle) at days 5, 6, 9, 12, 15, 18 and 21 after tumor inoculation. As a control, C57BL/6 mice inoculated with EG-7 tumor cells were injected with saline instead of (CpG + OVA)-liposomes (open triangle) (A). Six days after (CpG + OVA)-liposome treatment lymphocytes were prepared from the DLN or the tumor mass to determine the percentage of OVA/H-2Kb-tetramer+ CD8+ cells (B). The numbers in the top right quadrant represent the percentage of tetramer+ cells among total CD8+ cells. Similar results were obtained in three different experiments.
(CpG + OVA)-liposomes, we utilized IFN-α- and IFN-β-specific neutralizing mAbs and IL-12p40 and IFN-γ gene-deficient mice. The OVA/H-2Kb-tetramer+ CTLs were induced in a normal manner in EG-7-bearing IL-12p40−/− and IFN-γ−/− mice treated with (CpG + OVA)-liposomes, indicating that IL-12 and IFN-γ are not required for the induction of OVA/H-2Kb-tetramer+ CTLs (Fig. 6A, a–h). In addition, when either anti-IFN-α mAb or anti-IFN-β mAb is injected in tumor-bearing mice, the induction of OVA/H-2Kb-tetramer+ CTLs in the DLN and at the tumor site was similar to that of saline-injected mice (Fig. 6A, i–l). However, when the vaccinated, tumor-bearing mice were treated with both anti-IFN-α and anti-IFN-β mAbs, the induction of OVA/H-2Kb-tetramer+ CTLs was greatly diminished (13.8%), as compared with control mice (38.7%) (Fig. 5A, m and n). Thus, these findings demonstrate that type-1 IFNs (IFN-α and IFN-β) are critical for the induction of tumor-specific CTLs in response to treatment with (CpG + OVA)-liposomes, and that IFN-α and IFN-β play mutually redundant roles in this process. Consistent with the therapeutic effect of (CpG + OVA)-liposomes vaccination, high levels of tumor-specific cytotoxicity was detected in tumor-bearing IL-12−/− and IFN-γ−/− mice and in tumor-bearing mice treated with either anti-IFN-α or anti-IFN-β antibody, whereas tumor-specific cytotoxicity was reduced in tumor-bearing mice injected with both anti-IFN-α and IFN-β antibodies (Fig. 6B). These results indicate that vaccination of tumor-bearing mice with (CpG + OVA)-liposomes induces fully activated tumor-specific CTLs in a type-1 IFN-dependent manner.

Discussion

In this article, we have demonstrated that CpG-liposomes co-encapsulated with antigenic tumor protein can induce tumor-specific CTLs that are capable of completely eradicating established tumors in mice (Figs 1 and 2). Although it has been reported that CpG-ODN and tumor antigen can induce potent anti-tumor activities, in these studies CpG-ODN was injected prior to, or a few days after, tumor inoculation (9–14). Induction of anti-tumor immunity in normal mice or in mice bearing a small tumor burden is relatively easy. In sharp contrast, however, inducing anti-tumor immunity in mice that bear a large tumor mass is very challenging because of the development of an immunosuppressive environment concomitant with tumor growth (22–25). Here, we have evaluated whether CpG-liposome-based vaccination can induce the complete eradication of a large established tumor (Fig. 1). We

![Fig. 6. Critical role of IFN-α/β for the generation of OVA/H-2Kb-tetramer+ CTLs in response to vaccination with (CpG + OVA)-liposomes. (A) C57BL/6 wild-type (c and d), IL-12−/− mice (e and f) and IFN-γ−/− mice (g and h) were i.d. inoculated with EG-7 tumor cells and vaccinated with (CpG + OVA)-liposomes as described in Fig. 1. Anti-IFN-α (i and j), anti-IFN-β (k and l) or anti-IFN-α + anti-IFN-β (m and n) were intravenously injected into tumor-bearing wild-type mice at days 5, 6, 9 and 12 after tumor inoculation. As a control, wild-type mice inoculated with EG-7 cells were injected with saline instead of (CpG + OVA)-liposomes (a and b). Six days after (CpG + OVA)-liposomes injection, lymphocytes were prepared from the DLN and the tumor mass and stained with OVA/H-2Kb-tetramer. The numbers in the top right quadrant represent the percentage of tetramer+ cells among total CD8+ cells. (B) Six days after (CpG + OVA)-liposomes injection, lymphocytes were prepared from the DLN of the wild-type, IFN-γ−/− and IL-12−/− mice, and from wild-type mice treated with anti-IFN-α or IFN-β antibodies. Cytotoxicity against EG-7 or parental EL-4 cells was measured by a 6-h 51Cr-release assay. Similar results were obtained in three different experiments.](https://academic.oup.com/intimm/article-abstract/18/3/425/675262/ by guest on 25 January 2019)
found that vaccination of tumor-bearing mice with (CpG + OVA)-liposomes resulted in tumor eradication in ~50% of the animals. Tumor eradication correlated with a high frequency (about >25%) of OVA/H-2K^Kb-tetramer^+ CTLs among tumor-infiltrating CD8^+ T cells (Figs 1 and 2). These results suggest that robust expansion of tumor-specific CTLs is required for the complete eradication of a large tumor mass. Soluble CpG-ODN or OVA were unable to induce such strong anti-tumor activities (data not shown). There are two possible explanations for the superior anti-tumor activities of the (CpG + OVA)-liposomes vaccine as compared with the unmodified CpG + OVA vaccine. First, uptake of CpG-ODN and tumor antigen by DCs may be accelerated by the encapsulated form of CpG-ODN and tumor antigen. This may enhance the subsequent induction of anti-tumor innate and acquired immunities (6, 33). Second, encapsulation may extend the persistence of CpG-ODN and antigen in vivo. Liposomes promote the deposition of CpG-ODN and antigen at the injection site, which may be beneficial for long-lasting persistence of CpG-ODN and antigen in vivo. Thus, (CpG + OVA)-liposomes may support the simultaneous activation of TLR9-expressing DCs and promote the generation of CTLs in tumor-bearing mice.

It has been demonstrated that the migration of antigen-presenting DCs into DLN and their interaction with precursor CTLs are essential for inducing antigen-specific CTLs in vivo (34–37). Consistent with these results, tumor DLN is required for inducing tumor-specific CTLs in our tumor vaccine protocol because OVA/H-2K^Kb-tetramer^+ CTLs were not induced in LN-deficient Aly/Aly mice (Fig. 3). We also demonstrated that injection of the vaccine near the tumor DLN is required for efficient induction of CTLs and anti-tumor activities (Fig. 4). However, the anti-tumor effect was greatly reduced when the vaccine was injected into a site distal from the tumor mass (Fig. 4). These results suggest that tumor DLN is the most critical lymphoid organ, which spatiotemporally controls the generation and migration of tumor-specific CTLs at the local tumor site. T, B, NK and NKT cells, DCs and macrophages are also dramatically increased in the tumor DLN after vaccination with (CpG + OVA)-liposomes (data not shown). This enhanced migration of various immunoregulatory cells into the tumor DLN may be due to production of certain chemokines that are up-regulated by T_h1-dominant immune responses.

It has been reported that CD4^+ T cells, especially T_h1 cells, are crucial for the induction of effective anti-tumor immunity in tumor-bearing mice (26–29). However, it has also been reported that, in certain CpG-ODN-based vaccine protocols, CD4^+ T cells are not essential for the induction of anti-tumor immunity (10, 12, 13). Consistent with these findings, we demonstrate that (CpG + OVA)-liposomes vaccination induces tumor-specific CTLs and completely cures mice of established tumors in the absence of CD4^+ T cells (Fig. 5). Using CD4-deficient mice, we also confirmed that the anti-tumor effects of (CpG + OVA)-liposomes are intact in the absence of CD4^+ T cells (data not shown). This may be due to depletion of CD4^+ CD25^+ regulatory T cells (Treg), which can potently inhibit CD8^+ T cell responses. Numbers of Treg increase during tumor growth (22–25), and depletion of Treg cells may effectively relieve tumor-specific CTLs of the suppressive effects of these cells. We also demonstrated that induction of tumor-specific CTLs and anti-tumor activity are induced in tumor-bearing mice depleted of NK1.1^+ cells, despite previous reports indicating that CpG-ODN administration results in the activation of cytotoxicity and IFN-γ-production by NK and NKT cells (6). However, in our vaccine model, NK and NKT cells are not the major effector cells during tumor eradication. Instead, our findings indicate that CD8^+ T cells are both required and sufficient for the observed anti-tumor activity (Fig. 5).

CpG-ODN induces robust IFN-α/β production from pDC via TLR9-MyD88-IRF7 signaling. Recently, Honda et al. (21) reported that IFN-α/β produced by stimulation with CpG-ODN plus OVA is essential for inducing OVA-specific CTLs in normal mice. Thus, the molecular mechanisms of CTL induction by treatment with CpG-ODN and OVA have been demonstrated in normal mice. However, we have shown that unmodified CpG + OVA cannot completely cure established tumors in mice, whereas (CpG + OVA)-liposomes vaccination completely cures mice of established tumors (data not shown). Therefore, it will be important to investigate how (CpG + OVA)-liposomes induce anti-tumor immunity in tumor-bearing mice. Our results in Fig. 6 demonstrate that IFN-α/β, but not IL-12 and IFN-γ, is critically important for inducing tumor-specific CTLs in tumor-bearing mice. It has been reported that IL-12 and IFN-γ are essential cytokines for the induction of anti-tumor immunity (26–29, 30). However, our results reveal a critical role of IFN-α or IFN-β for overcoming immunosuppression in tumor-bearing mice in response to (CpG + OVA)-liposomes vaccination therapy (Fig. 6). Since depletion of type-1 IFN-producing pDCs resulted in a substantial reduction in the induction of CTLs in response to treatment with CpG-ODN plus anti-CD40 mAb (21), it is possible that pDCs represent the most critical source of IFN-α/β for the induction of tumor-specific CTLs in tumor-bearing mice though they (5%) exhibited lower phagocytic activity to uptake (CpG + OVA)-liposome compared with mDC (30%) (data not shown). In this context, it has been reported that IFN-α/β up-regulates the expression of p53 in the tumor cells (38). Therefore, IFN-α/β may inhibit tumor growth not only by directly activating tumor-specific CTLs, but also by inhibiting tumor growth via p53-mediated apoptosis of tumor cells. IFN-α/β has also been reported to stimulate the production of IL-15, which is a pivotal cytokine for maintenance of CD8^+ memory T cells (39–41). Therefore, IFN-α/β may be involved not only in direct activation of tumor-specific CTLs but also in the long-term maintenance of CTL memory in vivo. We are currently investigating this issue using IL-15^−/− mice.

Based on our data, we conclude that CpG-liposomes co-encapsulated with tumor antigens are powerful nanoparticle-based tumor vaccines. This vaccine can induce tumor-specific CTLs and completely cure tumor-bearing mice. Although intravenous treatment with CpG-ODN induced adverse side effects, recent clinical trials demonstrated that CpG-ODN treatment by the subcutaneous route showed no significant side effects in >500 tumor patients (7, 42–44). Therefore, it will be possible to apply CpG-liposomes co-encapsulated with tumor protein antigens to tumor immunotherapy. Although EG-7 expressing model tumor antigen (OVA) were used in this work, we are now investigating the therapeutic activity of liposome-CpG co-encapsulated with endogenous tumor antigen to demonstrate the applicability of this vaccination therapy to human cancer therapy.
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Abbreviations

- APC: antigen-presenting cell
- CpG-liposomes: liposomes encapsulated with unmethylated CpG-ODN unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotide
- CpG-ODN: unmethylated cytosine-phosphorothioate-guanine containing oligodeoxynucleotide
- (CpG + OVA)-liposomes: liposomes co-encapsulated with CpG-ODN and ovalbumin
- DC: dendritic cell
- DLN: draining lymph node
- i.d.: intradermally
- LN: lymph node
- OVA: ovalbumin
- OVA-liposomes: liposomes encapsulated with ovalbumin
- pDC: plasmacytoid dendritic cell
- TLR: Toll-like receptor
- Treg: CD4+ CD25+ regulatory T cell

References

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