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ORIGINAL ARTICLE

Inhibition of induced nitric oxide synthase enhances the anti-tumor effects on cancer immunotherapy using TLR7 agonist in mice

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Abstract Toll-like receptor (TLR) agonists have been shown to have anti-tumor activity in basic research and clinical studies. However, TLR agonist monotherapy in cancer treatment dose not sufficiently eliminate tumors. Activation of the innate immune response by TLR agonists and other pathogen-associated molecular patterns is effective for driving adaptive immunity via interleukin (IL)-12 or IL-1, but is counteracted by the simultaneous induction of immunosuppressive cytokines and other molecules, including IL-10, tumor growth factor-β, and induced nitric oxide synthase (iNOS). In the present study, we evaluated the anticancer effect of the TLR7 agonist, imiquimod (IMQ), in the absence of iNOS. The administration of IMQ in iNOS-knockout (KO) mice implanted with tumor cells significantly suppressed tumor progression as compared to that in wild-type mice and improved the survival rate. Moreover, injection with IMQ enhanced the tumor antigen-specific Th1 response in iNOS-KO mice with tumors. The enhancement of the antigen-specific Th1 response was associated with an increase in IL-2 and IL-12b expressions in the tumor-draining lymph nodes. Combination therapy with IMQ and an iNOS inhibitor also significantly inhibited tumor growth in the established tumor model. Finally, our results indicated that the enhancement of iNOS expression through the administration with TLR agonists impairs host anti-tumor immunity, while the inhibition of iNOS could enhance the therapeutic efficacy of TLR agonists via the increase in Th1 immune response.

Keywords Cancer immunotherapy · Toll-like receptor agonists · Induced nitric oxide synthase · Th1

Abbreviations

Ab	Antibody
DC	Dendritic cell
DLN	Draining lymph node
IFN	Interferon
IL	Interleukin
IMQ	Imiquimod
iNOS	Inducible nitric oxide synthase
KO	Knockout
L-NAME	N^G-nitro-L-arginine methyl ester
MDSCs	Myeloid-derived suppressor cells
NO	Nitric oxide
OVA	Ovalbumin
TLR	Toll-like receptor
WT	Wild-type

Introduction

Cancer therapy using Toll-like receptor (TLR) agonists has been examined in both basic research and clinical studies. The administration of TLR agonists activates the innate and adaptive host immunity [1]. This activation has the potential to suppress tumor progression. In particular, the adaptive immune response stimulated by TLR agonists can induce dendritic cell (DC) maturation and the tumor antigen-specific Th1 immune response. Although the TLR7 agonist, imiquimod (IMQ), is clinically used in the treatment of superficial basal cell carcinoma, other TLR

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agonists have limited success in treating human cancers [2]. This points to the dual nature of the responses induced by TLR agonists; both pro-inflammatory and anti-inflammatory molecules, including cytokines and chemokines, are produced by TLR agonists. This activity, combined with a tumor suppressive environment in which the expression of anti-inflammatory molecules is enhanced, has limited clinical application of TLR agonists in cancer therapy.

Inducible nitric oxide synthase (iNOS) is an enzyme that catalyzes the production nitric oxide (NO). In tumor microenvironment, NO promotes angiogenesis, metastasis, and immunosuppression [3]. Various tumor cells can induce NO production via the up-regulation of iNOS expression, and iNOS expression related the prognosis of the patient with any cancer [4, 5]. Recent studies demonstrated that myeloid-derived suppressor cells (MDSCs) also produce NO and suppress the host immune response in tumor microenvironment [6]. Thus, NO production deeply contributes to the progression of tumor.

The expression of iNOS in various cells is also enhanced by the administration of various TLR agonists directly or indirectly via several cytokines [Interferon (IFN)-γ, tumor necrosis factor-α, and interleukin (IL)-1β] [7–9]. Therefore, there is a possibility that NO production is enhanced during cancer therapy using TLR agonists and that anti-tumor immunity induced by TLR agonists is impaired by the enhancement of NO production. In the present study, we addressed the hypothesis that the inhibition of iNOS activity during cancer therapy with a TLR7 agonist will stimulate the tumor antigen-specific host immune response to inhibit tumor growth. We were able to show that the administration of IMQ and simultaneous inhibition of iNOS activity promote the tumor antigen-specific Th1 response, leading to the suppression of established tumor growth in vivo.

Materials and methods

Mice

Female C57BL/6 J wild-type (WT) mice and BALB/c mice (age 8–10 weeks; weight 25–30 g) were purchased from Japan SLC Inc. (Shizuoka, Japan). iNOS-knockout (KO) mice with a C57BL/6J background were obtained from Jackson Laboratory (Bar Harbor, ME). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and with the guidelines for the care and use of animals established by the Animal Care and Use Committee of Gifu University.

Cell lines and reagents

The EG7 cells [EL-4 cells expressing ovalbumin (OVA)] and colon carcinoma CT26 cells used in this study were generously provided by Hidekazu Shirota (Laboratory of Experimental Immunology, Cancer and Inflammation Program, National Cancer Institute, Frederick, MD). The cells were cultured in suspension in RPMI 1640 (Invitrogen Ltd., Paisley, UK) containing 10 % heat-inactivated FCS (PAA Laboratories, GmbH, Linz, Austria), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 g/ml).

In vivo tumor studies

Mice were challenged with EG7-OVA cells (1×10^6 EG7-OVA cells in C57BL/6 mice) and CT26 carcinoma cells (3×10^5 CT26 carcinoma cells in BALB/c mice). These tumor cell lines formed solid tumors when the tumor cells were subcutaneously inoculated into the flank. Tumor cells were implanted into the flank of WT or iNOS-KO mice on day 0. After the tumors became palpable (>5 mm in diameter), the mice were intratumorally administered with IMQ (20 μg/mouse). Tumor growth curves were generated using 6–9 mice per group, and all results were derived by combining data from 2 to 3 independent experiments. As previous study, tumor size was calculated using the following formula: (length × width × height)/2 [10]. When tumor exceeded a diameter of 2.0 cm, any animals were immediately euthanized as per ACUC protocol.

ELISPOT assay

The ELISPOT assay was performed as described previously [11]. In brief, single-cell suspensions were prepared from the spleen and draining lymph node (DLN) of tumor-baring mice treated with IMQ. A total of 2.0×10^5 cells per well were stimulated for 14–16 h with 0, 0.005, or 0.05 μg/ml of ova SIINFEKL peptide in 96-well MultiScreen filter plates (Millipore, Billerica, MA) previously coated with monoclonal rat anti-IFN-γ antibody (Ab) (R4-6A2) (BD Biosciences, San Jose, CA). The plates were washed and treated with biotinylated polyclonal goat anti-IFN-γ Ab (R&D Systems, Minneapolis, MN) followed by streptavidin alkaline phosphatase. Staining was visualized thorough the addition of a 5-bromo-4-chloro-3-indolyl phosphatase solution (Sigma-Aldrich, St. Louis, MO) and counted manually under 40× magnification. A single-blind reviewer counted the number of cytokine-secreting cells, and all data were generated by analyzing three separate wells per sample.

Flow cytometric analysis

Tumor-infiltrating lymphocytes were isolated from tumor-bearing mice treated with IMQ. For flow cytometry, tumor-infiltrating lymphocytes were stained according to a standard protocol. The following antibodies (Abs) were used: APC-labeled anti-mouse CD4 mAb (clone GK1.5; eBiosciences, San Diego, CA), PE-Cy7-labeled anti-mouse CD8 mAb (clone 53-6.7; eBiosciences, San Diego, CA), PE-Cy7-labeled anti-mouse CD11b mAb (clone M1/70; eBiosciences), VioBlue-labeled anti-mouse CD11c mAb (clone N418; Miltenyi Biotec, Bergisch Gladbach, Germany), FITC-labeled anti-mouse Ly-6G mAb (clone RB6-8C5; BD Biosciences), and PE-labeled anti-mouse CD49b mAb (clone DX-5; Biosciences). Samples were acquired using a FACSCanto ll flow cytometer, and data analysis was performed using FACSDiva software (BD Biosciences).

Real-time reverse transcription (RT)-PCR

The real-time RT-PCR was performed as described previously [12]. In brief, total RNA was isolated and transcribed into complementary DNA (cDNA) using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) and a high-capacity cDNA transcription kit (Applied Biosystems, Foster City, CA). The cDNA was used as a template for real-time RT-PCR along with primer–probe sets for iNOS, IL-2, IL-12b, FasL, IFN-γ, CCL2, CXCL10, and 18S (TaqMan Gene Expression Assays; Applied Biosystems) and TaqMan universal PCR master mix (Applied Biosystems) according to the manufacturer's recommendations. Real-time RT-PCR was carried out using a Light-Cycler 480 system (Roche Diagnostic Systems, Basel, Switzerland).

Isolation of CD11c+ and CD11b+ cells

DLN cells or tumor-infiltrating lymphocytes were sorted by CD11c+, or CD11b+ status using magnetic beads conjugated with an anti-CD11c, or anti-CD11b antibody (Miltenyi Biotec) as described in our previous study [13]. The magnetically labeled cells were purified using quadroMACS system (Miltenyi Biotec).

Statistics

Values are expressed as means ± standard errors of the mean (SEMs). Differences between experimental and control groups were analyzed by the Kruskal–Wallis test followed by Scheffe's F test. The Wilcoxon's test of Kaplan–Meier plots was used to analyze differences in animal survival. Significance was established at $P < 0.05$.

Results

Effect of intratumoral injection with a TLR7 agonist on established tumors in WT and iNOS-KO mice

TLR7 agonists, in particular IMQ, have been recently used for cancer therapy in basic and clinical researches. Although several TLR agonists enhance the host immune response, these agonists simultaneously induce immunosuppressive factors, including iNOS. We first examined the iNOS expression in tumor-draining lymph nodes (DLNs) after intratumoral injection with IMQ. As shown in Fig. 1a, iNOS mRNA expression was enhanced in the DLNs of WT mice after IMQ injection. Moreover, iNOS-expressing cells after IMQ injection were mainly CD11c+ cells in DLNs (Fig. 1b). In tumor site, the administration of IMQ enhanced iNOS mRNA expression in CD11c+ and CD11c−/CD11b+ cells. Next, we examined the effect of IMQ treatment on tumor growth inhibition in WT and iNOS-KO mice. EG7 cells were inoculated into the right flank of WT and iNOS-KO mice, and IMQ was peritumorally administrated when the tumor grew to 5 mm in length. IMQ administration impaired the growth of established tumors in vivo (Fig. 1c). The efficacy of IMQ on tumor growth inhibition markedly increased in iNOS-KO mice. Moreover, the median survival rate was significantly increased in iNOS-KO mice treated with IMQ (Fig. 1d).

Induction of OVA-specific Th1 response in WT and iNOS-KO mice after IMQ injection

To clarify the mechanism by which IMQ injection promotes anti-tumor immunity in WT and iNOS-KO mice, cells were isolated from the spleen and tumor DLN of EG7 tumor-bearing mice. The cells were cultured ex vivo with the OVA SIINFEKL peptide, which is a CD8-restricted epitope expressed by EG7. Splenocytes and DLN cells from tumor-bearing WT and iNOS-KO mice treated with IMQ responded to stimulation by OVA peptide by secreting IFN-γ. As shown in Fig. 2a, the number of spot strongly increased by the stimulation with OVA peptide in splenocytes and DLN cells from iNOS-KO mice treated with IMQ. Next, we measured mRNA levels for IFN-γ and FasL in DLNs from WT and iNOS-KO mice after IMQ administration (Fig. 2b). IFN-γ and FasL mRNA expressions in iNOS-KO mice were increased at day 3 after IMQ administration. These data also indicated that Th1 immune response was enhanced in iNOS-KO mice after IMQ injection. IL-2 and IL-12b mRNA expressions also were up-regulated in DLN of iNOS-KO mice treated with IMQ (Fig. 2b). These cytokines have critical role for the induction of

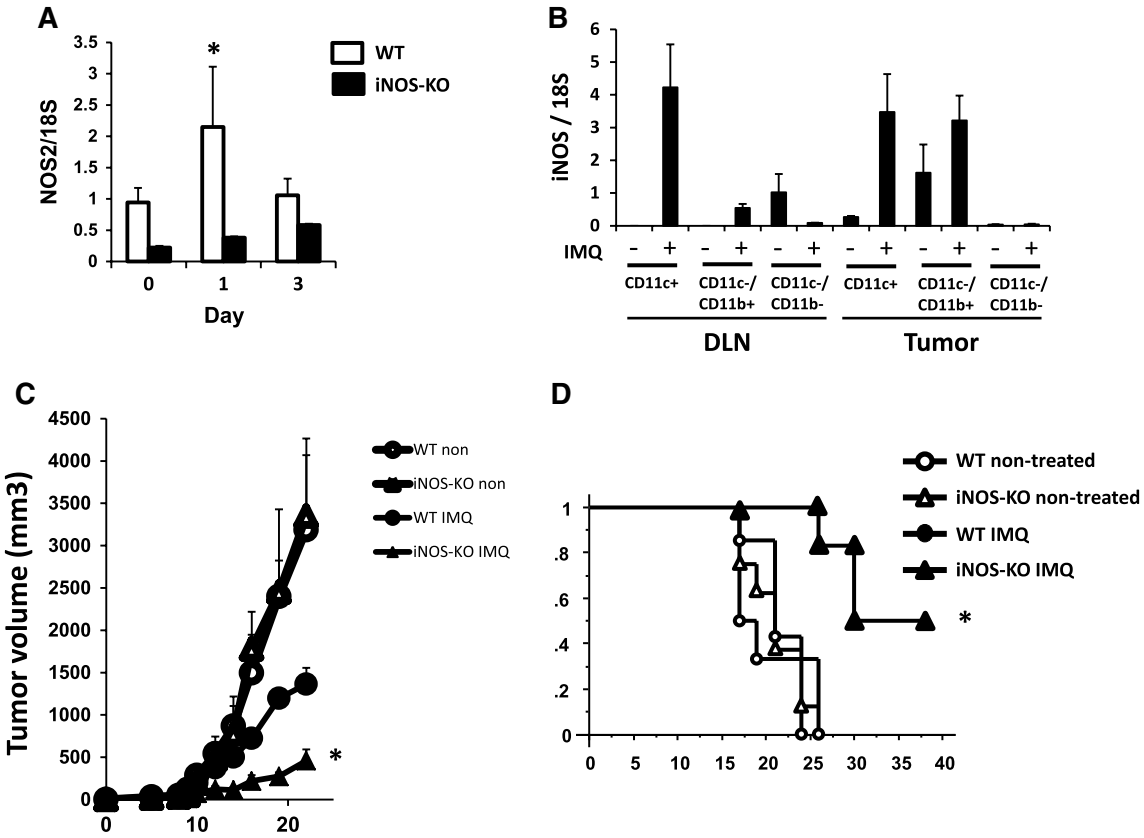

Fig. 1 Inhibition of iNOS enhances the anti-tumor effect of a TLR7 agonist in an established tumor in vivo. EG7 cells (1×10^6/mouse) were inoculated into the flank of WT and iNOS-KO mice on day 0. After the tumors became palpable, the mice were intratumorally injected with IMQ (20 μg/mouse). **a** The relative expression levels of iNOS mRNA in the draining lymph node (DLN) of tumor-bearing WT and iNOS-KO mice were measured by real-time RT-PCR. The results were normalized to the expression of 18S rRNA. Each value is shown as mean and SEM for three mice. **b** CD11c+, CD11c−/CD11b+, and CD11c−/CD11b− cells in DLNs were isolated using immunomagnetic separation system 1 day after the IMQ injection, and total RNA was extracted from these cells. The relative expression levels of iNOS mRNA in CD11c+, CD11c−/CD11b+, and CD11c−/CD11b− cells were measured by real-time RT-PCR. The results were normalized to the expression of 18S rRNA. **c** Data show the mean ± SEM increase in tumor size of 6–9 mice per group, from two experiments. **d** Survival plots of tumor-bearing mice are shown. Similar results were obtained from two independent experiments. *Statistically significant differences

Th1 response. Therefore, this enhancement of IL-2 and IL-12b contributes to the induction of tumor antigen-specific Th1 response.

Effect of IMQ treatment on the phenotype of tumor-infiltrating lymphocytes in WT and iNOS-KO mice

We measured the frequency of CD4+, CD8+, DX5+, CD11c+, and CD11b+/Ly6G+ cells in tumor-infiltrating lymphocytes of WT and iNOS-KO mice at day 3 after IMQ administration. As shown in Fig. 3, the frequency of CD8+ and CD11c+ cells was significantly enhanced in iNOS-KO mice treated with IMQ. However, there was no difference between WT and iNOS-KO mice treated with IMQ in the ratio of CD4+, DX5+, and CD11b+/Ly6G+ cells.

Effect of combination therapy with IMQ and N^G-nitro-L-arginine methyl ester (L-NAME) on established tumors

L-NAME is a iNOS inhibitor; hence, we used this agent to substantiate data obtained from iNOS-KO mice. WT mice were administered L-NAME orally at 0 or 2 mg/ml in drinking water for 2 day before IMQ injection. The co-administration of IMQ and L-NAME significantly slowed the growth of established tumors in EG7 tumor-bearing mice (Fig. 4a). In established tumor models using CT26 colon carcinoma cells, combination therapy with L-NAME and IMQ also impaired tumor growth. ELISPOT assay revealed that the number of spot after the stimulation with OVA peptide in DLN cells was significantly increased by the treatment with IMQ and L-NAME (Fig. 4b). These results indicated that the co-treatment with IMQ and L-NAME enhanced the anti-tumor cellular immunity.

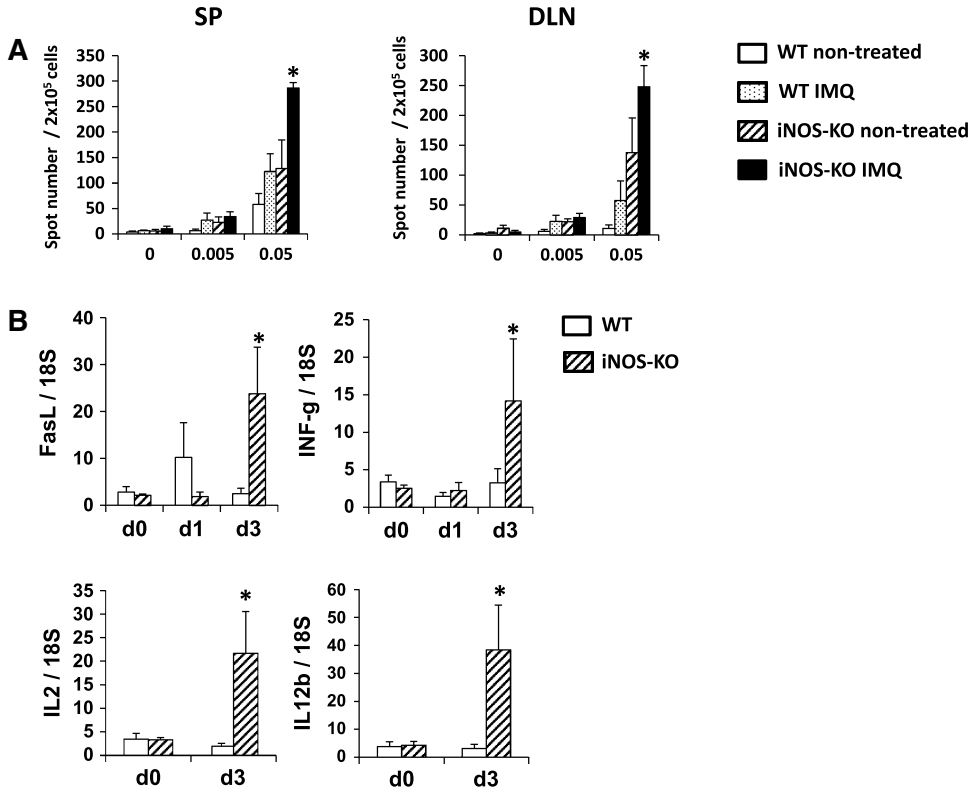

Fig. 2 Induction of tumor antigen-specific Th1 immunity by the administration with TLR7 agonist to tumor-bearing WT mice and iNOS-KO mice. **a** DLN cells and spleens in tumor-bearing WT and iNOS-KO mice were isolated 7 days after IMQ injection. These cells were stimulated with OVA peptide in vitro and monitored for IFN-γ production using ELISPOT assay. Results represent the mean ± SEM of 4–5 mice/group. **b** The relative expression levels of IFN-γ, FasL, IL-2, and IL-12b mRNA in the DLNs of tumor-bearing mice were measured using quantitative real-time RT-PCR. The results were normalized to the expression of 18S rRNA. Each data point and error bar represents the mean and SEM, respectively, of data from triplicate samples. Similar results were obtained from two independent experiments. *Statistically significant differences

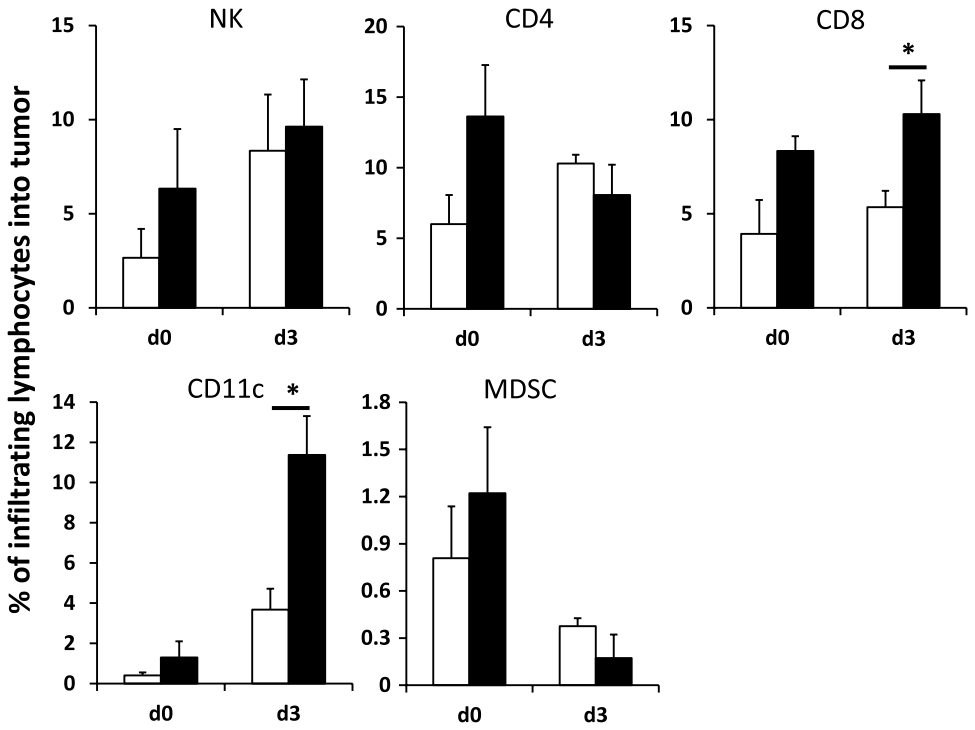

Fig. 3 Phenotype of lymphocytes in the tumor sites of WT and iNOS-KO mice after IMQ injection. The tumor-infiltrating lymphocytes from tumor-bearing WT and iNOS-KO mice were isolated 3 days after IMQ injection. Data show the percentage of CD4+, CD8+, DX5+, CD11c+, and CD11b+/Ly6G+ cells. Each data point and error bar represents the mean and SEM, respectively, of data from triplicate samples. Similar results were obtained from two independent experiments. *Statistically significant differences

Discussion

TLRs are types of pattern recognition receptors and recognize molecules that are widely shared by various pathogens. TLR1, TLR2, TLR4, TLR5, and TLR6 are presented on the surface of the cells and recognize bacterial and fungal components [1]. In contrast, TLR3, TLR7, and TLR9 intracellularly work by the recognition of nucleic

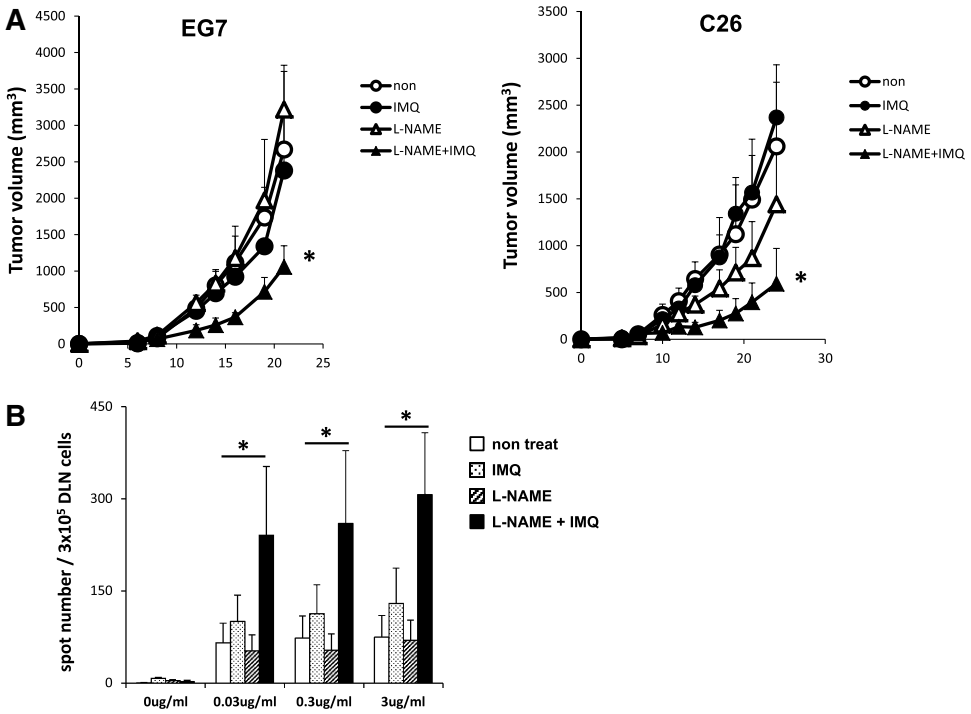

Fig. 4 The anti-tumor effect of a TLR7 agonist and iNOS inhibitor, L-NAME, on tumor-bearing WT mice. EG7 cells (1×10^6/mouse) and C26 cells (3×10^5/mouse) were inoculated into the flank of mice on day 0. After the tumors became palpable (>5 mm in diameter at approximately 10 days), the mice were orally administered L-NAME at a dose of 2 mg/mL in drinking water and intratumorally administered TLR7 agonist 2 days later. **a** Data show the mean ± SEM increase in tumor size of 4–6 mice per group. **b** DLNs cells in tumor-bearing WT mice treated with IMQ and/or L-NAME were isolated 7 days after IMQ injection. These cells were then stimulated by OVA peptide in vitro and monitored for IFN-γ secretion by ELISPOT assay. Results represent the mean ± SEM of 4–5 mice/group. Similar results were obtained from two independent experiments. *Statistically significant differences

acid from pathogens [14]. Many reports have evaluated the effect of TLR agonists on various cancer models [15, 16]. In particular, TLR2, TLR4, TLR7, and TLR9 agonists were used in basic and clinical studies for several cancer models. TLR7 agonist has been clinically used for patients with skin cancers. However, monotherapy by TLR agonists failed to induce complete tumor regression. Thus, anti-tumor therapy with TLR agonists has also had limited success for human cancers [2]. Our findings have shown that the administration of TLR7 agonist in iNOS-KO mice induced the tumor antigen-specific Th1 response and significantly suppressed tumor growth (Figs. 1, 2). Moreover, FasL and IFN-γ mRNA expressions in iNOS-KO mice were enhanced by the administration with IMQ. These data supported the enhancement of cellular immunity in iNOS-KO mice treated with IMQ. Flow cytometry analysis revealed that CD11c+ cells significantly increased at the tumor site in iNOS-KO mice after the treatment. Combination therapy with a TLR7 agonist and iNOS inhibitor is markedly effective for this established tumor model.

Although the activation of host immunity by TLR agonists and others is effective on the induction of adaptive immunity, this activation counteracted the adaptive immunity by simultaneous induction of immunosuppressive cytokines and molecules, including IL-10, tumor growth factor-β, indoleamine-2, 3-dioxygenase, and iNOS [17, 18]. These inhibitory factors may impair the therapeutic effect of TLR agonists on cancer. Therefore, inhibition of these immunosuppressive factors in cancer therapy using TLR agonists may induce stronger cancer immunity, resulting in the elimination of tumor. In the present study, we evaluated the effect of IMQ, TLR7 agonist, using an established primary tumor model in iNOS-KO mice, because IMQ is clinically used in the treatment of genital warts and superficial basal cell carcinomas [19]. The injection of IMQ significantly increased the expression of iNOS mRNA in DLNs (Fig. 1a). In DLNs, the enhancement of iNOS expression was observed in CD11c+ cells (Fig. 1b). On the other hand, in tumor site, both of CD11c+ and CD11c−/CD11b+ cells significantly expressed iNOS mRNA after the administration of IMQ. These results indicated that iNOS-expressing cells after IMQ injection were CD11c+ cells in DLNs, and both of CD11c+ and CD11b+ cells in tumor site. The administration of IMQ significantly impaired primary cancer growth in iNOS-KO mice compared with WT mice (Fig. 1c). Moreover, the survival rate

for iNOS-KO mice treated with IMQ was improved compared with WT mice (Fig. 1d). These data indicate that the inhibition of iNOS activity significantly enhanced the anti-tumor effect of IMQ. iNOS inhibitor, L-NAME, also facilitated the anti-tumor effect of IMQ on EG-7-bearing WT mice (Fig. 4a). Even in other established primary cancer (CT26) models, an increased inhibitory effect on tumor development was observed in WT mice treated with L-NAME and IMQ. These results demonstrate that the increased anti-tumor effect of L-NAME and IMQ was not dependent on the type of cancer cell line or mice. In general, the induction of tumor antigen-specific Th1 immune response is critical in cancer therapy to potentiate the anti-tumor effect. Many reports have evaluated the induction of tumor antigen-specific immune response in various animal cancer models and clinical studies [20–22]. In the present study, the tumor antigen-specific Th1 immune response in DLN was enhanced in iNOS-KO mice treated with IMQ (Fig. 2a). Moreover, the co-administration of L-NAME and IMQ significantly increased the number of IFN-γ producing cells after the stimulation with tumor antigen (Fig. 4b). In the DLN, the mRNA expression of FasL and IFN-γ in iNOS-KO mice increased after administering with IMQ compared with WT mice (Fig. 2b). These results indicated that tumor antigen cellular immune response in DLN was augmented by IMQ and the suppression of iNOS activity. IL-2 and IL-12b expressions in DLN were increased in iNOS-KO mice after IMQ injection. These cytokines are critical on the induction of antigen-specific immune response [17, 23]. Therefore, the increase of IL-2 and IL-12b expression may be involved in the tumor antigen-specific Th1 immune response in DLN. Moreover, the number of CD11c+ cells increased in the tumor site of iNOS-KO mice after treatment with IMQ (Fig. 3). The increase in CD11c+ cells in tumor site may be involved in the enhancement of tumor antigen-specific immune response. The chemokine mRNA level in CD11b+ cells after IMQ injection tended to be higher in iNOS-KO mice compared with WT mice (Supplementary Figure S1). These enhancements of chemokine expression may affect the increase in CD11c+ cells in tumor site. Moreover, the ratio of CD8+ T cells increased in the tumor site of iNOS-KO mice after treatment with the TLR7 agonist (Fig. 3). Our study showed that the increase in tumor antigen-specific immune response and CD8+ T cells in the tumor may significantly prevent the progression of tumor growth in iNOS-KO mice.

TLR7 agonist has already been legally approved for the treatment of HPV-related warts and skin keratosis [24]. Although the administration of TLR7 agonist enhances anti-tumor immunity in host, immunosuppressive molecules are induced by TLR7 agonist. A recent study on cancer therapy demonstrated that the excessive inflammation induced by TLR7 agonist results in self-immunosuppression via IL-10 and that blocking IL-10 could enhance the therapeutic efficacy [25]. In the present study, iNOS expression in tumor-bearing mice was enhanced after IMQ administration (Fig. 1a). The up-regulation of iNOS expression leads to the enhancement of NO production. High levels of NO impair signal transduction in T cells by nitration of tyrosine and cysteine residues and reduce the production of IL-2 and granzyme B [26]. Therefore, cancer therapy via TLR7 agonists can induce the tumor antigen-specific host immune response while simultaneously enhancing the expression of iNOS, which work to suppress the host immune system. Although monotherapy using TLR agonist seemed to fail to induce complete tumor rejection, the inhibition of immune-suppressive factors induced by TLR agonists serves as a new method in cancer therapy using TLR agonists.

In conclusion, we demonstrated that the TLR7 agonist enhances the expression of iNOS in tumor-bearing mice and inhibits the tumor antigen-specific Th1 response in an established cancer model. The inhibition of iNOS expression after TLR7 agonist administration could enhance the therapeutic efficacy of TLR agonists via the strong induction of the Th1 response.

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Conflict of interest The authors declare no competing financial interests.

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