

Antitumor Activity and Immune Response Induction of a Dual Agonist of Toll-Like Receptors 7 and 8

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Abstract

Viral and synthetic single-stranded RNAs are the ligands for Toll-like receptors 7 and 8 (TLR7 and TLR8). We have reported a novel class of synthetic oligoribonucleotides, referred to as stabilized immune-modulatory RNA compounds, which act as agonists of TLR7, TLR8, or both TLR7 and TLR8 depending on the sequence composition and the presence of specific chemical modifications. In the present study, we evaluated the antitumor activity of a dual TLR7/8 agonist in tumor-bearing mice with peritoneal disseminated CT26.CL25 colon and 3LL-C75 lung carcinomas. Peritoneal administration of dual TLR7/8 agonist in mice bearing CT26.CL25 colon carcinomas had potent dose-dependent antitumor activity, which was associated with a marked decrease in CD4⁺CD25⁺Foxp3⁺ T regulatory cells and a significant increase in tumor antigen-specific IFN- γ -secreting effector cell responses in splenocytes and local tumor-infiltrating cells. In 3LL-C75 lung carcinoma, dual TLR7/8 agonist induced strong immune responses and antitumor effects in C57BL/6 and TLR9^{-/-} mice, but not in TLR7^{-/-} and MyD88^{-/-} mice, indicating that the agonist induces immune responses via TLR7 and through the MyD88-dependent signaling pathway. TLR8 is not functional in mice. Additionally, s.c. administration of TLR7/8 agonist effectively prevented lung metastasis of tumors in the CT26.CL25 pulmonary metastasis model. These studies show that the dual TLR7/8 agonist induced Th1-type immune responses and potent antitumor activity in mice via TLR7 and through the MyD88-dependent pathway. *Mol Cancer Ther*; 9(6); 1788–97. ©2010 AACR.

Introduction

Toll-like receptors (TLR) recognize specific molecular signatures called pathogen-associated molecular patterns that are present in pathogens. Among the 10 TLRs identified in humans, TLR3, TLR7, TLR8, and TLR9 are expressed in endosomal compartments and detect nucleic acid molecular patterns of viruses and bacteria (1–3). Other TLRs are expressed on the cell surface and recognize cell wall constituents of extracellular pathogens. Recent studies suggest that pathogen-associated molecular patterns evoke different immune responses by targeting distinct TLRs and their intracellular adaptor molecules. Agonists of TLR7 and TLR8 induce Th1-type immune responses (4, 5). Although bacteria or bacterial-derived molecules such as BCG have long been used as anticancer immunotherapeutic agents (6), recent understanding of TLR-mediated modulation of immune responses has

led to novel immunotherapeutic opportunities for the treatment of cancers (7).

TLR7 and TLR8 are the receptors for viral and synthetic single-stranded RNAs. TLR7 is expressed in human B cells and plasmacytoid dendritic cells (4) and TLR8 is expressed in human monocytes and myeloid dendritic cells (8, 9). Wide use of synthetic RNA as agonists of TLR7 and TLR8 has been hampered by their susceptibility to nuclease degradation (10–12), and lipid carriers have been needed for *in vitro* and *in vivo* studies (8, 11–13). We have reported a novel class of synthetic oligoribonucleotides, referred to as stabilized immune-modulatory RNA (SIMRA) compounds (14–17). We have shown that SIMRA compounds act as TLR7, TLR8, and dual TLR7/8 agonists, depending on the sequence composition and specific chemical modifications present in them (14–17). SIMRA compounds induce potent immune responses in cell-based assays and *in vivo* in mice and nonhuman primates without requiring formulation with lipid carriers (14–17).

In the present study, we evaluated the antitumor activity of a dual TLR7/8 agonist in two murine syngeneic tumor models, CT26.CL25 colon carcinoma and 3LL-C75 lung carcinoma. We also studied the ability of the dual TLR7/8 agonist to alter the tolerogenic tumor microenvironment, especially CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg), and favor tumor-specific T-cell activation. Studies in knockout mice suggest that TLR7 and MyD88, but not TLR9, are required for TLR7/8 agonist-induced immune responses and antitumor activity.

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org>).

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Materials and Methods

Reagents

Synthesis and purification of oligoribonucleotide-based dual TLR7/8 agonist, 5'-UG1CUG1CUUCUG1-X-G1UCUUCG1UCG1U-5', wherein G1 and X represent 7-deazaguanosine and 1,2,3-propanetriol linker, respectively, were carried out as described previously (14, 15). The TLR7/8 agonist was synthesized with phosphorothioate backbone modification. After synthesis, oligoribonucleotide purity and molecular integrity were determined by ion-exchange high-performance liquid chromatography, capillary gel electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectral analysis. The compound was tested for endotoxin by LAL test and the levels of endotoxin were <1 endotoxin unit/mg. The synthetic peptides, β -gal, TPHPARIGL (representing the naturally processed H-2 L^d restricted epitope spanning amino acids 876–884 of β -gal; refs. 18, 19), AH1, SPSYVYHQF (containing a CTL determinant from CT26 tumor cells; ref. 20), and OVA_{257–264} (control peptide), were synthesized by New England Peptide, Inc., to a purity of >99% as determined by high-performance liquid chromatography.

Mice and cell lines

Female BALB/c and C57BL/6 mice, 5 to 8 weeks of age, were purchased from Charles River Laboratory. TLR9 (TLR9^{-/-}), TLR7 (TLR7^{-/-}), and MyD88 (MyD88^{-/-}) knockout mice were obtained from Oriental Bioservice. IL-12 (IL-12^{-/-}), IL-6 (IL-6^{-/-}), and IFN- γ (IFN- γ ^{-/-}) knockout mice were purchased from The Jackson Laboratory. Animal study protocols were approved by the Idera Pharmaceutical Institutional Animal Care and Use Committee. CT26.CL25 (American Type Culture Collection) is a subclone of CT26 colon carcinoma that has been transduced with *Escherichia coli* β -gal gene, which has been shown to be equally as lethal as the parental clone, CT26.WT, in normal mice. 4T1 (American Type Culture Collection) is a mammary adenocarcinoma cell line. 3LL-C75, a highly immunogenic variant of 3LL Lewis lung carcinoma, was obtained from Dr. E. Gorelik (University of Pittsburgh, Pittsburgh, PA; ref. 21). Cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals), 2 mmol/L L-glutamine, 100 μ g/mL streptomycin, and 100 units/mL penicillin (Mediatech). CT26.CL25 was maintained in the same medium supplemented with 400 μ g/mL G418 sulfate (Life Technologies).

Assessment of serum cytokine/chemokine levels

Blood collected by retro-orbital bleeding at 2 hours after the first administration of dual TLR7/8 agonist was used to determine serum levels of IL-12 by sandwich ELISA (PharMingen) as described previously (19). Selected serum samples were further evaluated for cytokine/chemokine profiles using Mouse 20-Plex Panel according to the manufacturer's recommendations (Invitrogen).

Tumor models

Peritoneal disseminated tumor models were established by i.p. administration of 3×10^5 CT26.CL25 cells or 5×10^6 3LL-C75 cells in BALB/c or C57BL/6 mice, respectively. TLR7/8 agonist (10–100 mg/kg) was administered i.p. three times per week starting on day 0 for a total of nine times for CT26.CL25 and six times for 3LL-C75 tumor-bearing mice. Systemic treatment effects were evaluated in the CT26.CL25 lung metastasis model: BALB/c mice were inoculated i.v. with 4×10^5 tumor cells and were then administered s.c. with 50 mg/kg dual TLR7/8 agonist three times per week for 2 weeks. Mice were monitored twice a week for tumor growth and survival.

Mice in the metastasis model study were euthanized on day 14. Lungs were harvested and pulmonary tumor nodules were counted following X-gal staining (Promega) and then photographed.

Secondary tumor challenge in tumor-free mice

Long-term surviving mice bearing CT26.CL25 peritoneal tumors following dual TLR7/8 agonist treatment were rechallenged with 3×10^5 parental tumor cells by i.p. inoculation on day 65 and the mice were not further treated with agonist.

Adoptive immune cell transfer

Syngeneic splenocytes (5×10^6) from naïve BALB/c mice and TLR7/8 agonist-treated, long-term surviving mice were adoptively transferred i.p. to two groups of naïve BALB/c mice. The mice (five per group) were then cross-challenged with 5×10^5 CT26.CL25 cells by i.p. or s.c. administration or with 5×10^5 4T1 cells by i.p. administration on day 3.

IFN- γ ELISPOT assay

Splenocytes and ascites fluid from tumor-bearing mice treated with 50 mg/kg TLR7/8 agonist or PBS control ($n = 3$) were collected on day 20. T cells from splenocytes were purified using T-cell enrichment columns (R&D Systems). Purified T cells or cells from ascites fluid (2.5×10^5) were stimulated with 2.5×10^5 mitomycin C (50 μ g/mL; Sigma)-treated and β -gal, AH1, or OVA peptide-pulsed syngeneic spleen cells for 24 hours. The frequency of T cells specific for the AH1 and β -gal peptides expressed by CT26 and CT26.CL25 tumor cells, respectively, was determined by IFN- γ ELISPOT assay according to the manufacturer's directions (R&D Systems). Spots were enumerated electronically (Zellnet).

Flow cytometric analysis of Tregs

Ascites fluid was collected from tumor-bearing mice treated with 50 mg/kg TLR7/8 agonist or PBS control ($n = 3$) on day 20 after tumor implantation. Mice in the PBS control group developed large volume of ascites and the average number of cells collected was $13.8 \pm 2.8 \times 10^6$. Mice treated with TLR7/8 agonist had smaller ascites

and the average number of cells collected was $3.7 \pm 1.03 \times 10^6$. The frequency of Tregs was determined by flow cytometry using Treg staining kit (eBioscience). Briefly, about 10^6 cells were resuspended in 100 μ L of PBS containing 0.5% bovine serum albumin and incubated with FITC-conjugated rat anti-mouse CD4 (clone RM4-5) and allophycocyanin-conjugated rat anti-mouse CD25 (clone PC 61.5). Then, the cells were treated with fixative/permeabilization buffer and incubated with purified anti-mouse CD16/CD32 (2.4G2) monoclonal antibody to block nonspecific binding of antibody to Fc receptors. Intracellular staining was done using phycoerythrin-conjugated anti-mouse/rat Foxp3 antibody (clone FJK-16s). Data were collected on a FC500 MPL cytometer (Beckman Coulter) and analyzed using FlowJo software (TreeStar). A small lymphocyte gate was created based on forward and side scatter properties, followed by a CD4⁺ gate. The frequency of Tregs was then determined by plotting CD25 versus Foxp3.

Statistics

Differences in measured variables between groups at each time point were assessed by two-tailed, unpaired Student's *t* test. Survival curves were analyzed with the log-rank test. Data are expressed as mean \pm SEM where applicable. Statistical significance was accepted at $P < 0.05$.

Results

Treatment of CT26.CL25 colon carcinoma-bearing mice with TLR7/8 agonist led to dose-dependent induction of cytokines and increase in survival

TLR7/8 agonist treatment of mice that received i.p. administration of CT26.CL25 cells (Fig. 1A) resulted in a dose-dependent induction of IL-12 production 2 hours after the first dose of TLR7/8 agonist (Fig. 1B). We further analyzed serum cytokine levels from mice that received 50 mg/kg TLR7/8 agonist or PBS by multiplex analyses. TLR7/8 agonist induced Th1-type cytokines/chemokines, including IL-2, IL-12, IFN- γ -inducible protein-10 (IP-10), monokine induced by IFN- γ , and tumor necrosis factor- α , but not Th2-type cytokines such as IL-4 (Fig. 1C).

Treatment with TLR7/8 agonist produced a dose-dependent increase in survival of tumor-bearing mice (Fig. 1D). The mean survival time of tumor-bearing mice in the PBS-treated and 10, 50, and 100 mg/kg TLR7/8 agonist-treated groups was 23 ± 1.1 , 43.5 ± 4.4 , 53 ± 4.1 , and 65 ± 1.9 days, respectively. These results show that TLR7/8 agonist induced Th1-type immune responses and prolonged survival of treated tumor-bearing mice.

Treatment of CT26.CL25 tumor-bearing mice with TLR7/8 agonist led to tumor antigen-specific IFN- γ -secreting effector cell responses

To examine if the treatment of tumor-bearing mice with TLR7/8 agonist resulted in the development of tumor

antigen-specific IFN- γ -secreting effector cell responses, three mice in each of the PBS and 50 mg/kg TLR7/8 agonist treatment groups were euthanized on day 20. T cells isolated from splenocytes or ascites cells were stimulated with mitomycin C-treated and β -gal, AH1, or OVA peptide-pulsed syngeneic spleen cells for 24 hours. As shown in Fig. 2, a significant increase in tumor antigen-specific IFN- γ -secreting effector cell responses to the H-2^d-restricted model antigen β -gal₈₇₆₋₈₈₄ as well as the CT26 colon carcinoma endogenous antigen AH1 was observed in mice treated with TLR7/8 agonist. Tumor antigen-specific IFN- γ -secreting effector cell responses were observed using T cells purified from splenocytes (Fig. 2A) and more strongly with T cells from the tumor site (Fig. 2B).

Treatment of CT26.CL25 tumor-bearing mice with TLR7/8 agonist induced an immunologic memory response in surviving mice

To study if the treatment of tumor-bearing mice with TLR7/8 agonist would lead to memory responses in the surviving mice, we rechallenged mice from the above experiment ($n = 3$ and $n = 6$ in the 50 and 100 mg/kg dose groups, respectively) or naïve mice with i.p. administration of 3×10^5 CT26.CL25 tumor cells. No further treatment was given. Mice that had survived the initial tumor burden following TLR7/8 agonist treatment rejected the same tumor cell rechallenge. Two of three mice from the 50 mg/kg dose group and six of six from the 100 mg/kg dose group survived up to 100 days after CT26.CL25 tumor cell rechallenge ($P < 0.001$, versus the PBS group, log-rank test; Fig. 3A). Naïve mice that received tumor cell challenge died of tumor burden by day 27 (Fig. 3A).

When splenocytes derived from TLR7/8 agonist-treated mice were adoptively transferred into naïve mice, challenge with CT26.CL25 cells either i.p. or s.c. resulted in protection against tumor growth and subsequent death (Fig. 3B and C). This protection was tumor specific, and naïve mice that received splenocytes from TLR7/8 agonist-treated mice were not protected from syngeneic, non-organ-related 4T1 mammary carcinoma cell challenge (Fig. 3D). These results suggest that CT26.CL25 tumor-bearing mice treated with TLR7/8 agonist developed tumor-specific immunologic memory.

Treatment of CT26.CL25 tumor-bearing mice with TLR7/8 agonist significantly reduced the frequency of Tregs

Tregs suppress primary and memory immune responses and activation of self-reactive T cells, which can promote tumor growth (22). To determine the effect of TLR7/8 agonist treatment on Tregs at the tumor site, we collected ascites fluid on day 20 from mice treated with TLR7/8 agonist or PBS. CD25 and Foxp3 expression on CD4⁺ cells was assessed by flow cytometry. Representative dot plots showing the distribution of

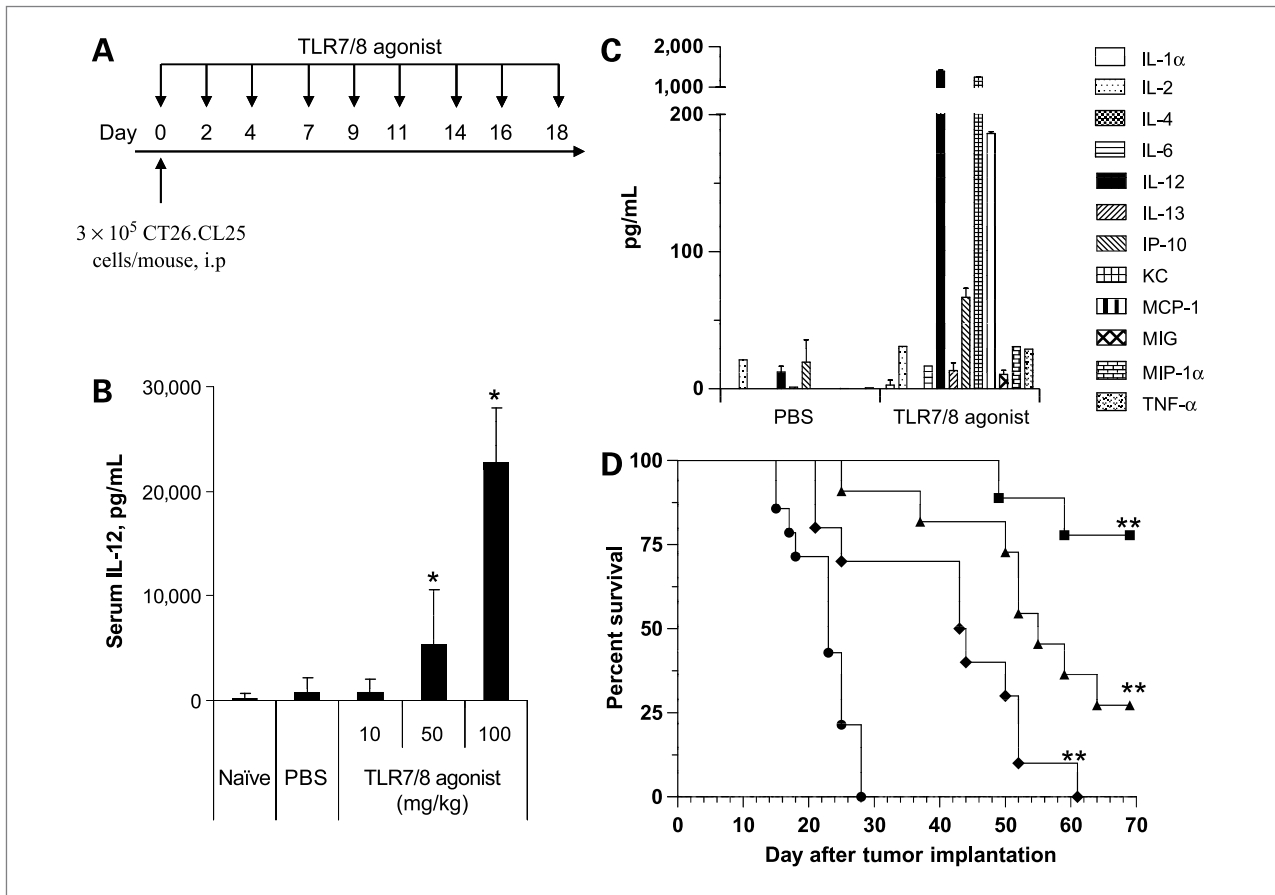


Figure 1. Dose-dependent IL-12 induction and antitumor activity of TLR7/8 agonist in the peritoneal disseminated CT26.CL25 colon carcinoma model. A, study protocol. B, serum IL-12 levels in tumor-bearing mice 2 h after TLR7/8 agonist administration as determined by ELISA (n = 5). C, serum cytokines and chemokines of tumor-bearing mice treated with 50 mg/kg TLR7/8 agonist and PBS control (n = 5) as evaluated by multiplex assays with Luminex xMAP system. D, BALB/c mice implanted with 3×10^5 CT26.CL25 cells by i.p. administration were treated with TLR7/8 agonist at 10 mg/kg (diamonds), 50 mg/kg (triangles), and 100 mg/kg (squares) three times a week for 3 wk. PBS-administered mice served as control (circles). Treatment with TLR7/8 agonist increased the survival of tumor-bearing mice in a dose-dependent manner. **, $P < 0.001$, versus the PBS group (log-rank test). Each group had 10 mice.

CD25⁺, Foxp3⁺, or CD25⁺Foxp3⁺ (Treg) cells in the peritoneal cavity of individual mice treated with PBS or TLR7/8 agonist are shown in Fig. 4A and B, respectively. The frequency of CD25⁺Foxp3⁺ cells was significantly reduced in TLR7/8 agonist-treated mice compared with control mice (Fig. 4C). PBS-treated mice had $36.6 \pm 2.2\%$ Tregs (Fig. 4A), whereas mice treated with TLR7/8 agonist had fewer Tregs at the tumor site (Fig. 4B and C; $8.0 \pm 6.4\%$, $P < 0.01$). These results suggest that TLR7/8 agonist reduced the number of Tregs in the tumor microenvironment, allowing the host immune system to fight tumor growth and prolong the survival of mice.

TLR7/8 agonist-mediated immune responses and antitumor activity via TLR7- and MyD88-dependent signaling pathways

To study the mechanism of action of TLR7/8 agonist, we administered 3LL-C75 tumor cells i.p. to C57BL/6

wild-type (B6.WT), TLR9^{-/-}, TLR7^{-/-}, and MyD88^{-/-} mice, and then treated the mice with TLR7/8 agonist or PBS as shown in Fig. 5A. Serum cytokine levels were measured 2 hours after the first administration of TLR7/8 agonist. TLR7^{-/-} and MyD88^{-/-} mice had levels of cytokines similar to those seen in untreated (naïve) mice (Fig. 5B). By contrast, administration of TLR7/8 agonist induced strong serum cytokines, including IL-12 and IP-10, in both B6.WT and TLR9^{-/-} mice (Fig. 5B).

Treatment of B6.WT mice bearing murine 3LL-C75 lung carcinomas with TLR7/8 agonist resulted in the survival of 70% of mice with a mean survival time of 95.2 ± 45.1 days, compared with 10% survival of mice with a mean survival time of 35.5 ± 31.4 days in the control group (Fig. 5C; Supplementary Table S1). Treatment of TLR9^{-/-} mice bearing 3LL-C75 tumors with TLR7/8 agonist resulted in 100% survival of mice with a mean survival time of >123 days compared with no mice

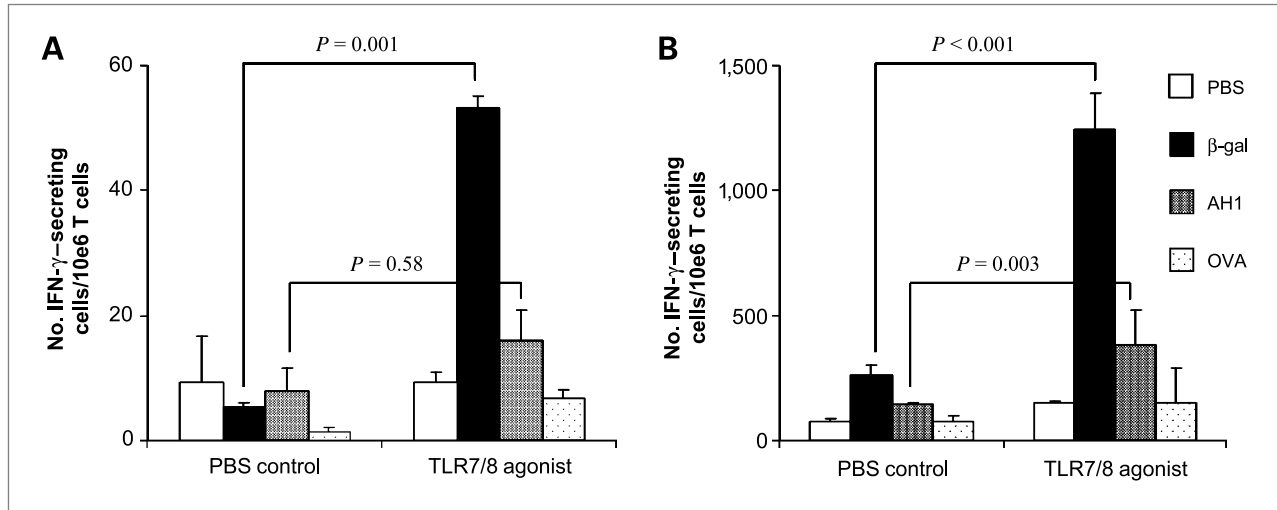


Figure 2. TLR7/8 agonist treatment of tumor-bearing mice elicits tumor antigen-specific IFN- γ -secreting effector cell responses in splenocytes and peritoneal-infiltrating lymphocytes. Splens (A) and peritoneal exudates (B) from mice treated with 50 mg/kg TLR7/8 agonist or PBS ($n = 3$) were collected on day 20 after tumor implantation. Splenocytes or tumor ascites cells (2.5×10^5) were stimulated with 2.5×10^5 mitomycin C-treated and β -gal, AH1, or OVA peptide-pulsed syngeneic spleen cells for 24 h. T cells specifically responding to stimulation with CT26.CL25 tumor antigens, β -gal and AH1, were enumerated by IFN- γ ELISPOT. Results are representative of two similar experiments.

surviving with a mean survival time of 26.6 ± 5.8 days in the PBS control group (Fig. 5C; Supplementary Table S1). In contrast, treatment of TLR7^{-/-} mice bearing 3LL-C75 tumors with TLR7/8 agonist did not result in increase in survival (0% survival of mice with a mean survival time of 23.5 ± 3.3 days) compared with control group (0% survival of mice with a mean survival time of 21.3 ± 0.5 days; Fig. 5C; Supplementary Table S1). Similarly, treatment of 3LL-C75 tumor-bearing MyD88^{-/-} mice

with TLR7/8 agonist did not result in increase in survival (0% survival with a mean survival time of 33.0 ± 9.3 days) compared with control group (0% survival with a mean survival time of 25.3 ± 2.1 days; Fig. 5C; Supplementary Table S1). These results are consistent with the acute serum cytokine induction data in these mice following TLR7/8 agonist administration. Together, these results suggest that the TLR7/8 agonist activates immune responses via TLR7 through a MyD88-dependent signaling pathway in

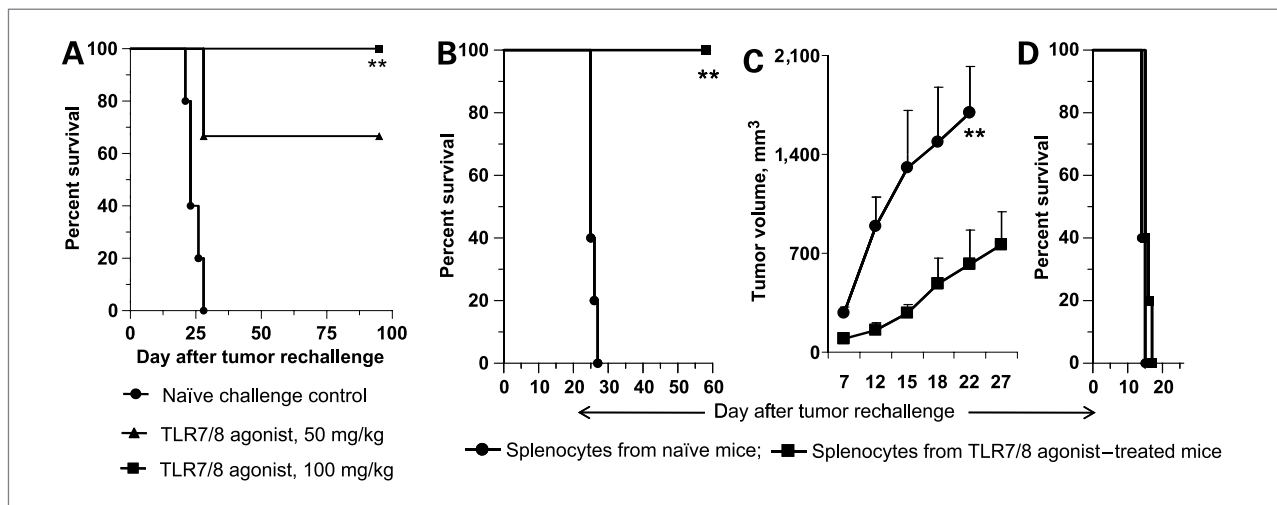


Figure 3. Tumor-bearing mice treated with TLR7/8 agonist develop tumor-specific memory responses. A, naïve mice and mice surviving on day 70 following TLR7/8 agonist (50 and 100 mg/kg groups) treatment (Fig. 1D) were challenged with CT26.CL25 tumor cells and followed for survival. B, syngeneic splenocytes (5×10^6) were adoptively transferred (i.p.) into BALB/c mice from naïve BALB/c mice or TLR7/8 agonist-treated long-term survivors bearing CT26.CL25 tumors (five per group). The mice were then challenged with 5×10^5 CT26.CL25 i.p. or s.c. (C) or with 5×10^5 4T1 breast tumor cells i.p. (D) on day 3. Each group had five mice. **, $P < 0.01$, compared with splenocytes from naïve mice.

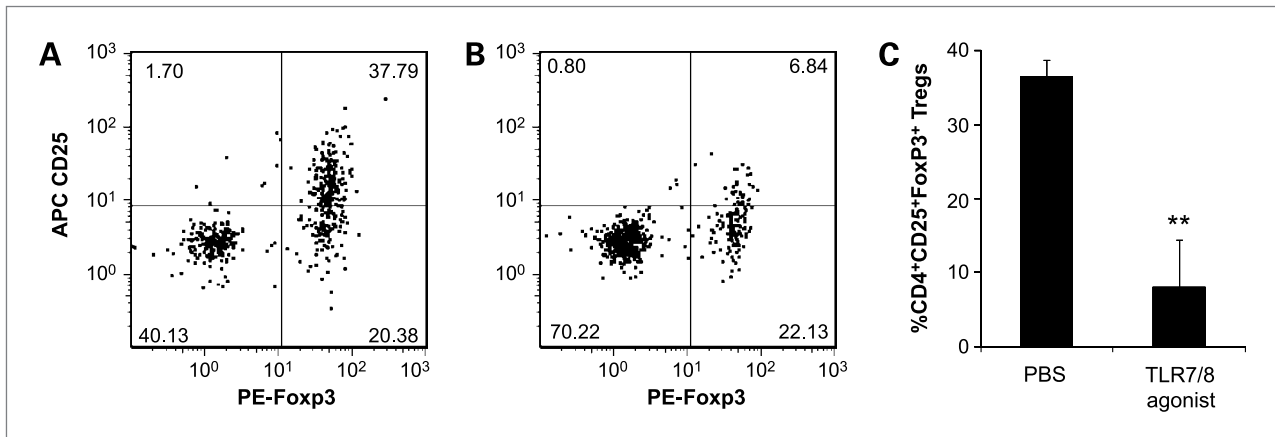


Figure 4. Identification of Tregs at the tumor site. Tumor ascites cells were collected from the peritoneal cavity 20 d after CT26.CL25 i.p. implantation from mice treated with 50 mg/kg TLR7/8 agonist or PBS. Expression of CD25 and Foxp3 on CD4⁺ cells was assessed by flow cytometry. Representative staining from a mouse in the PBS-treated (A) and TLR7/8 agonist-treated (B) groups. The percentages of CD25⁺ and Foxp3⁺ cells of gated CD4⁺ T cells are indicated in each quadrant of the dot plot. PE, phycoerythrin. C, plot showing the average frequency of Tregs from three individual mice. **, $P < 0.01$, compared with PBS control.

mice. TLR8 is not functional in mice (2), and thus the agonist activity observed in mice is primarily from TLR7 activation.

IFN- γ was required for TLR7/8 agonist-mediated antitumor activity

The data shown above, including cytokine induction profiles and tumor antigen-specific IFN- γ -secreting effector cell responses, indicated a clear induction of Th1-type responses following administration of TLR7/8 agonist. We examined the antitumor effects of TLR7/8 agonist in B6.WT, IL-12^{-/-}, IL-6^{-/-}, and IFN- γ ^{-/-} mice bearing 3LL-C75 murine lung carcinomas. The treatment protocol is shown in Fig. 6A. Treatment of WT mice bearing 3LL-C75 tumors with TLR7/8 agonist resulted in 80% survival of mice with a mean survival time of 103.8 ± 7.6 days compared with 0% survival of mice with a mean survival time of 36.4 ± 8.5 days in the control group (Fig. 6B; Supplementary Table S1).

Similarly, treatment of IL-12^{-/-} mice bearing 3LL-C75 tumors with TLR7/8 agonist resulted in 78% survival of mice with a mean survival time of 102.7 ± 5.8 days, compared with 11% survival of mice with a mean survival time of 42.1 ± 30.4 days in the control group (Fig. 6B; Supplementary Table S1). Treatment of IL-6^{-/-} mice bearing 3LL-C75 tumors with TLR7/8 agonist resulted in 75% survival of mice with a mean survival time of 98.6 ± 7.7 days, compared with 0% survival of mice with a mean survival time of 37.9 ± 9.1 days in the control group (Fig. 6B; Supplementary Table S1). In contrast, TLR7/8 agonist treatment had no effect on the survival of IFN- γ ^{-/-} mice bearing the same tumors (0% survival of mice with a mean survival time of 31.0 ± 4.6 days) compared with the PBS control group (0% survival with a survival time of 25.2 ± 6.8 days; Fig. 6B; Supplementary Table S1), suggesting that IFN- γ was required for the

TLR7/8 agonist-induced antitumor activity in the 3LL-C75 tumor model.

Antitumor activity of TLR7/8 agonist in the CT26.CL25 lung metastasis model

Further, to determine the induction of systemic antitumor responses and potential to prevent tumor metastasis, we studied TLR7/8 agonist activity in the CT26.CL25 pulmonary metastasis model. The incidence of pulmonary metastases increased with i.v. administration of CT26.CL25 cells to BALB/c mice. These mice were treated with 50 mg/kg TLR7/8 agonist or with PBS on days 0, 1, 3, and 6. On day 14, mice were euthanized and the metastatic burden in their lungs was assessed by counting the number of pulmonary tumor nodules. A significant reduction in the progression of tumor metastases was observed in the lungs of TLR7/8 agonist-treated mice compared with the PBS control group ($P = 0.0001$; Fig. 7A). Data shown in Fig. 7 are combined from two independent experiments. Figure 7B shows one representative lung from the PBS and TLR7/8 agonist-treated groups.

Discussion

Viral single-stranded RNAs are the natural ligands for TLR7 and TLR8 (2, 23). Synthetic small molecules such as imidazoquinolines and certain nucleoside analogues activate TLR7 and/or TLR8 (3, 24). Imiquimod, a TLR7 agonist, has been approved as a topical agent for the treatment of superficial basal cell carcinoma and actinic keratosis (25, 26). Imidazoquinoline-based compounds cause side effects following systemic administration, however, and are commonly used only by topical application (27). Imiquimod is effective only when administered

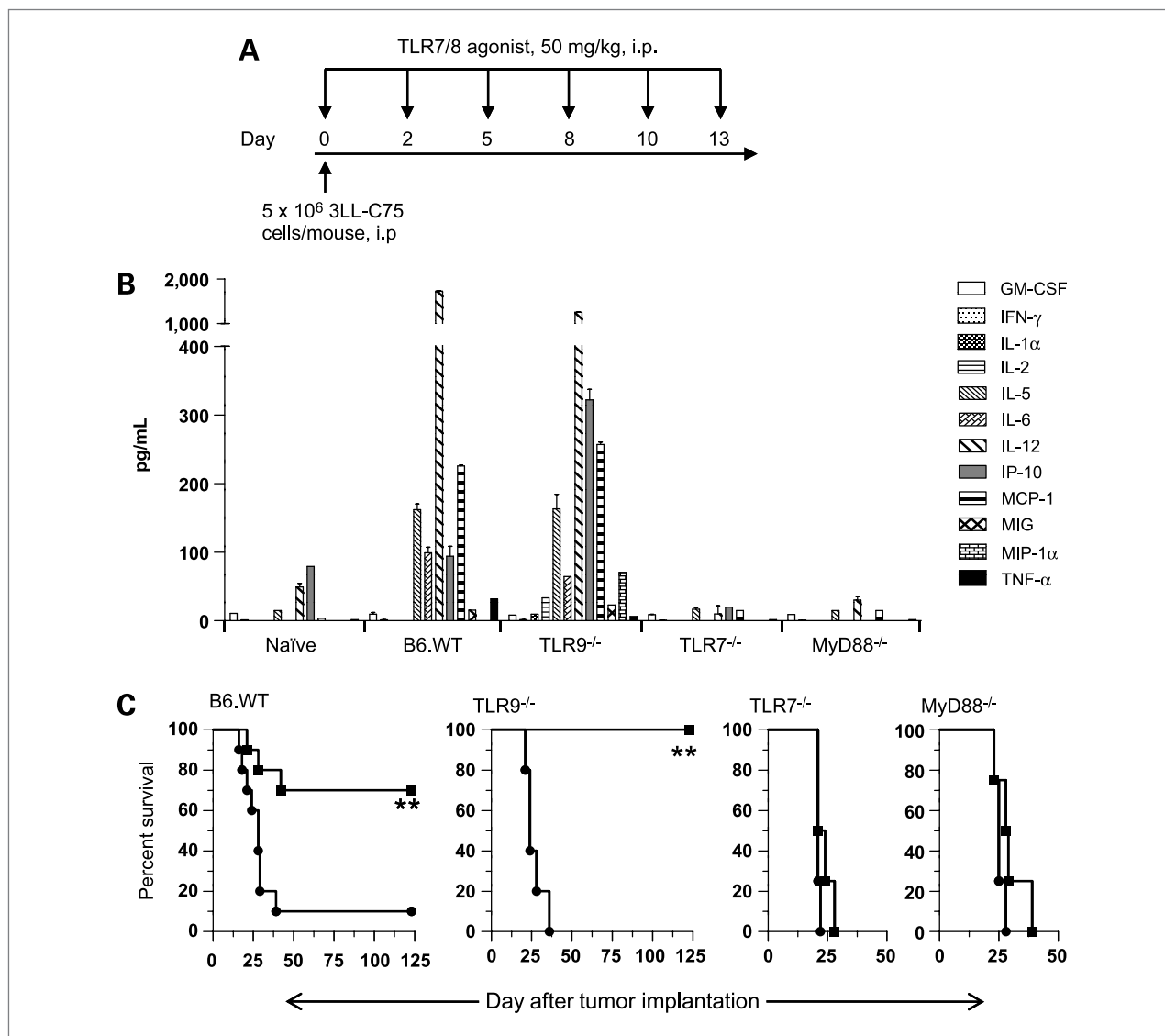


Figure 5. TLR7/8 agonist elicits antitumor activity via TLR7- and MyD88-dependent signaling pathways. **A**, TLR7^{-/-}, TLR9^{-/-}, MyD88^{-/-}, and B6.WT mice were inoculated with 5×10^6 3LL-C75 cells by i.p. administration on day 0. The mice were then treated with 50 mg/kg TLR7/8 agonist by i.p. administration three times a week. Each group had five mice except for the B6.WT group, which had 10 mice. **B**, blood samples were collected 2 h after the first agonist administration, and serum samples were evaluated by multiplex assays with Luminex xMAP system. **C**, survival of B6.WT, TLR9^{-/-}, TLR7^{-/-}, and MyD88^{-/-} mice following treatment. Filled squares and circles represent TLR7/8 agonist- and PBS-treated mice, respectively. **, $P < 0.01$, compared with PBS control.

locally; tumors located distally do not respond significantly to treatment (28). Although imidazoquinolines activate TLR7 and/or TLR8, no direct interaction with these receptors has been shown. Imidazoquinolines also interfere with the adenosine and opioid growth factor receptor signaling pathways independent of TLR7 and TLR8 (29, 30)

Recent studies have shown that synthetic RNAs formulated with lipid carriers also activate TLR7 and TLR8 in a sequence-dependent manner (2, 23). However, single-stranded RNA is highly susceptible to nuclease degradation, and the use of lipid carriers has been required to

stabilize RNAs against nucleases (12, 31–36). We have designed RNAs linked through their 3'-ends to prevent 3'-exonuclease degradation, referred to as SIMRA compounds, which act as agonists of TLR7, TLR8, or both TLR7 and TLR8, depending on the nucleotide sequence and chemical modifications incorporated (14–17). SIMRA compounds induce Th1-type immune responses without lipid formulation *in vitro* and *in vivo*, including in non-human primates (14). In the present study, we analyzed the antitumor potential of a SIMRA compound that acts as a dual TLR7/8 agonist without lipid formulation in preclinical tumor models in mice.

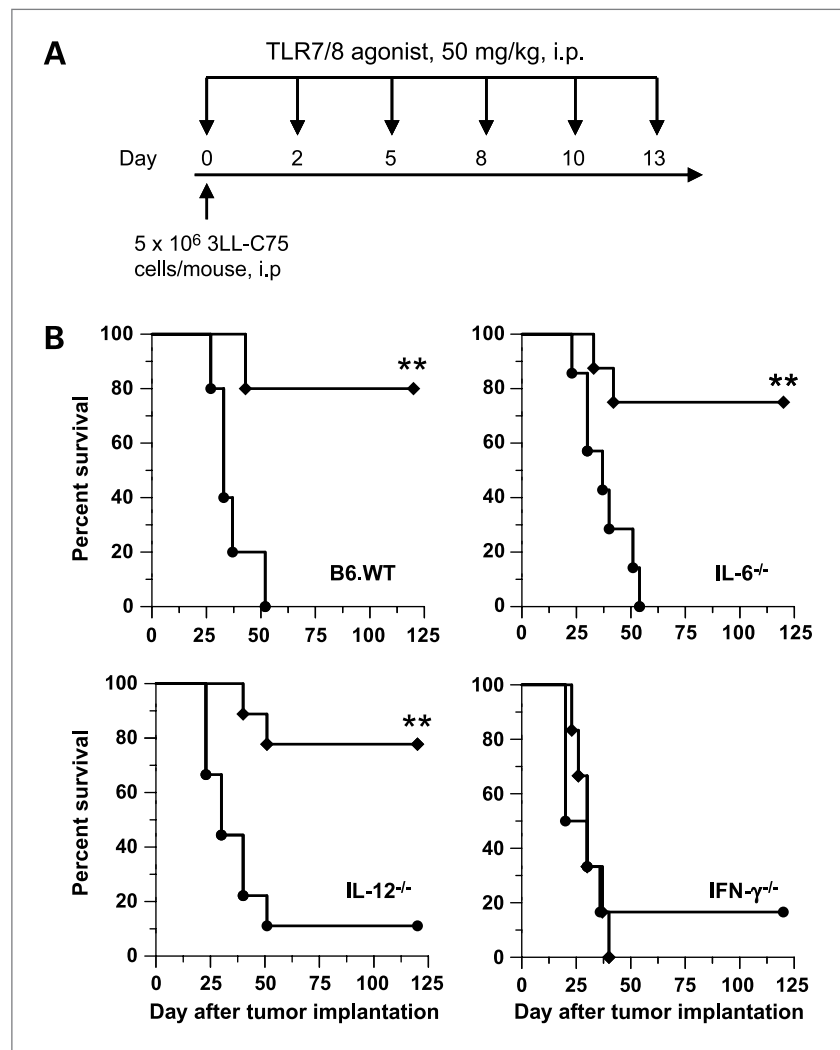
The TLR7/8 agonist studied here induced the production of Th1-type cytokines/chemokines, including IL-2, IL-12, IP-10, monokine induced by IFN- γ , and tumor necrosis factor- α , in mice. Although it has been well demonstrated that many tumors carry immunogenic epitopes that can be recognized by the host immune system, in most cases, host defenses fail to mount an appropriate response, causing uncontrolled tumor growth in cancer patients (37, 38). The failure of the host immune system to elicit defense against tumor cells could be related to the low immunogenic nature of tumor antigens and defects in the host immune system itself (39, 40). Tumor-associated immune suppression, including myeloid-derived suppressor cells and Tregs, plays a key role in causing and maintaining such defects, the reversal of which is vitally important for the success of immunotherapy (41, 42). The ability of a TLR7/8 agonist to induce a potent Th1-type immune response and decrease the number of Tregs could lead to a tumor microenviron-

ment more favorable for the host immune system to respond strongly against the tumor, as indicated by induction of tumor antigen-specific IFN- γ -secreting effector cell responses both systemically and locally.

Our previous studies using transfected cell lines showed that the TLR7/8 agonist used herein activates both TLR7 and TLR8 (14–17). In the present study, a TLR7/8 agonist induced potent immune and antitumor activities in TLR9 $^{-/-}$, C57BL/6 WT, and BALB/c WT mice. The TLR7/8 agonist did not induce cytokine production in or increase the survival of tumor-bearing TLR7 $^{-/-}$ and MyD88 $^{-/-}$ mice, indicating that it mediates immune activation and antitumor activity via TLR7 and through the MyD88-dependent signaling pathway.

Moreover, s.c. administration of TLR7/8 agonist significantly inhibited lung metastasis of CT26.CL25 tumor in mice. Also, tumor-bearing mice that survived following TLR7/8 agonist treatment were protected against

Figure 6. IFN- γ is required for TLR7/8 agonist-induced antitumor activity in the 3LL-C75 tumor model. A and B, IL-12 $^{-/-}$, IL-6 $^{-/-}$, IFN- γ $^{-/-}$, and B6.WT mice bearing 3LL-C75 murine lung carcinomas were treated with 50 mg/kg TLR7/8 agonist as shown in the protocol (A) and monitored for survival (B). Filled diamonds and circles represent TLR7/8 agonist- and PBS-treated mice, respectively. **, $P < 0.01$, compared with PBS control.



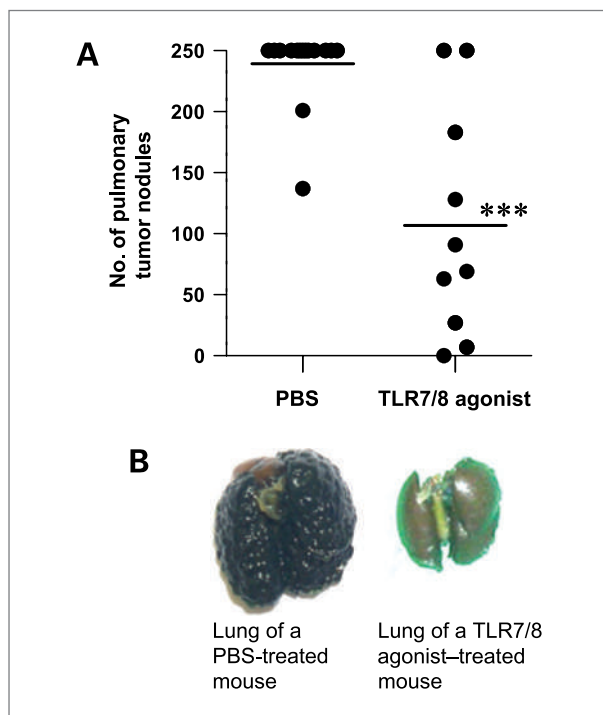


Figure 7. Administration of TLR7/8 agonist by s.c. route resulted in the development of systemic antitumor effects against CT26.CL25 lung metastases. BALB/c mice were administered with 4×10^5 CT26.CL25 cells i.v. and treated with 50 mg/kg TLR7/8 agonist ($n = 10$) or PBS ($n = 15$) by s.c. administration on days 0, 1, 3, and 6. The lungs were collected on day 14. A, the number of pulmonary tumor nodules was enumerated following staining with X-gal. Data combined from two independent experiments. $***, P = 0.0001$, compared with PBS control. B, photographs of representative lungs of mice in each group stained with X-gal.

subsequent challenge with the same tumor cells, suggesting development of tumor-specific memory responses. These tumor-specific memory cells were adoptively transferred into naïve mice and provided protection against tumor cells administered either i.p. or s.c. The

same mice were not protected from syngeneic, non-organ-related 4T1 mammary carcinoma cell challenge.

TLR7 and TLR8 are expressed on different immune cells in humans. TLR7 is expressed in human B cells and plasmacytoid dendritic cells (4) and TLR8 is expressed in human monocytes and myeloid dendritic cells (8, 9). Because these receptors are expressed in different types of immune cells, they produce different immune response profiles. However, TLR8 is not functional in mice. The immunologic and antitumor activities observed with TLR7/8 agonist in these tumor models in mice are a result of TLR7 activation. Antitumor activity resulting from TLR8 activation is largely unknown in mice. Evaluation of TLR7/8 agonist in other animal models would enable assessment of the full potential of these compounds. The TLR7/8 agonist evaluated in the present study was previously shown to induce IFN- α and IP-10 in plasma and activate T lymphocytes, natural killer cells, and monocytes in nonhuman primates (14).

Taken together, in this article we showed that dual TLR7/8 agonist induced potent Th1-type immune responses in mice and antitumor activity via TLR7 and through the MyD88-dependent signaling pathway. Administration of TLR7/8 agonist to tumor-bearing mice resulted in a marked decrease in CD4⁺CD25⁺Foxp3⁺ Tregs and a significant increase in tumor antigen-specific IFN- γ -secreting effector cell responses in splenocytes and local tumor-infiltrating cells. The TLR7/8 agonist effectively prevented lung metastasis of tumors in the CT26.CL25 pulmonary metastasis model.

Disclosure of Potential Conflicts of Interest

All authors are employees of Idera Pharmaceuticals and hold company stock.

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