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Additive melanoma suppression with intralesional phospholipid conjugated TLR7 agonists and systemic IL-2

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Abstract

Objective—There remains a compelling need for the development of treatments for unresectable melanoma. Agents that stimulate the innate immune response could provide advantages for cell based therapies. However there are conflicting reports concerning whether Toll-like receptor (TLR) signaling controls tumor growth. The objective of this study was to evaluate the effect of the intralesional administration of a TLR7 agonist in melanoma therapy.

Methods—B16cOVA melanoma was implanted to TLR7^{-/-} mice to evaluate the roles of stromal TLR7 on melanoma growth. To capitalize on the potential deleterious effects of TLR7 stimulation on tumor growth, we injected melanoma tumor nodules with a newly developed and potent TLR7 agonist.

Results—B16 melanoma nodules expanded more rapidly in mice deficient in TLR7- and MyD88- compared to TLR9-deficient and wild type mice. Repeated injections with low doses of unconjugated TLR7 agonist were more effective at attenuating nodule size than a single high dose injection. To improve efficacy we conjugated the agonist to phospholipid or polyethylene glycol-phospholipid, which retained TLR7 specificity. The phospholipid conjugate was indeed more effective in reducing lesion size. Furthermore intralesional administration of the phospholipid TLR7 agonist conjugate enhanced the anti-melanoma effects of systemic IL-2 treatment and prolonged the survival of mice compared to IL-2 alone.

Conclusion—TLR7/MyD88 signaling in the stroma is involved in melanoma growth. Intralesional administration of a TLR7 agonist reduces the growth of melanoma nodules and enhances the anti-melanoma effects of IL-2.

Keywords

melanoma; innate immunity; TLR7 agonists; Conjugates; IL-2

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Introduction

Melanoma of the skin is one of the most clinically important skin and soft tissue malignancies. If properly diagnosed and treated in early stages, its prognosis and outcome are uniformly favorable. However, advanced melanoma has the highest per-death loss of years of potential life expectancy except for adult leukemia [1, 2]. Metastatic melanoma has been found to be susceptible to systemic immunologic therapy including adoptive cell therapy, immune check-point modulation and cytokine therapy. Systemic interleukin (IL)-2 therapy induces complete remission in a minority of patients and is approved for the treatment of unresectable melanoma, but has substantial morbidity that limits application to a select group of patients [2, 3]. Intralesional therapy with inflammatory stimulants, including Toll-like receptor agonists, has been variably successful [4].

Topical treatment with Toll-like receptor 7 agonist, imiquimod (R-837), was approved by the FDA in 1997 for use in treating viral warts and some nonmelanoma cutaneous tumors [5–7]. The TLRs are a key part of the innate immune system, and are expressed by diverse cell types including macrophages, dendritic cells, lymphocytes and epithelial cells [8, 9]. TLRs recognize both common ligands in microbes and endogenous host molecules with similar molecular structures. TLRs interact with lipid structures (TLR2 and TLR4) [10, 11], proteins (TLR4, TLR5 and TLR11)[12–14], and nucleic acids (TLR3, 7/8, and 9) [15, 16]. TLR7 recognizes naturally occurring single stranded RNA and synthetic low molecular weight ligands, including imidazoquinolines, and purine-like molecules [8, 17, 18]. All TLRs, except TLR3, signal through the myeloid differentiation primary response gene 88 (MyD88) adapter protein, resulting in activation of NF- κ B and increased transcription of the inflammatory cytokine genes that it regulates [19].

We previously reported a series of TLR7 agonists conjugated with phospholipids, or phospholipid-poly (ethylene glycol) (PEG), via a *N*-hydroxysuccinimide (NHS) activated ester group on amide linkage [20]. Here we demonstrate that TLR7 deficiency in the tumor microenvironment enhances growth of subcutaneous B16 melanoma nodules in mice, supporting the rationale that locally administering a TLR7 agonist might suppress tumor growth. Systemic administration of the phospholipid and phospholipid-PEG TLR7 conjugates induced prolonged elevations in serum proinflammatory cytokine levels, compared to the unmodified TLR7 agonist [20]. Prolonged delivery utilizing repeated intratumoral injections of the activated parent compound capable of in situ coupling to free amines, designated 1V199, into subcutaneous B16 melanoma nodules was more effective at limiting growth than a single injection or injection of a noncoupling compound. We hypothesized that the phospholipid (1V270) and phospholipid-PEG TLR7 (1V285) conjugates would have protracted activity between injections and enhanced efficacy. Intralesional administration of one phospholipid conjugate 1V270, significantly delayed tumor growth. We then tested for potential synergy between systemic administration of the T cell cytokine, IL-2, and local injection of the innate immune agonist, 1V270. Repeated intra-tumoral injections of 1V270 improved the anti-melanoma effect of concomitant systemic IL-2 treatment and prolonged host survival.

Materials and Methods

Animals

6 to 8 week old female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice genetically deficient for TLR7, TLR9 and MyD88, were gifts from S. Akira (Osaka University, Osaka, Japan) and were backcrossed ten generations onto the C57BL/6 background. All procedures and protocols were approved by the UCSD Institutional Animal Care and Use Committee.

Reagents

TLR7 agonists, 1V199 and 1V209, were synthesized as previously described [20]. 1V199, is an NHS ester derivative of 1V209 that can couple to molecules containing a primary amine group in situ. Ovalbumin (OVA, grade V, Sigma-Aldrich) 0.17 mg/ml was incubated with an excess amount of 1V199 (1.62mM) for 4 hours at 37 °C. Coupling chemistry was verified by a mobility shift from native OVA by SDS-PAGE. Phospholipid and phospholipid-PEG were conjugated to 1V209 as described [20]. Stock solutions were stored at 1mM in dimethyl sulfoxide (DMSO) at -20°C until use. IL-2 was obtained from the NCI Preclinical Repository (Frederick, MD). Endotoxin levels in all reagents and conjugates were <1pg/ng as measured by the QCL1000 end point chromogenic Limulus Amoebocyte Lysate (LAL) assay, (BioWhittaker, Walkerville, MD) or as otherwise described. H-2K^b-restricted OVA peptide (NH₂-SIINFEKL-COOH) was purchased from GeneScript (Piscataway, NJ). PE-conjugated H-2K^b OVA tetramer-SIINFEKL-PE (OVA-Tetramer) was obtained from the National Institutes of Health Tetramer Core Facility.

In vitro cytokine release assays

Bone marrow derived dendritic cells (BMDC) were prepared as described previously [21]. 5×10^4 BMDC were incubated with unconjugated TLR7 agonists or TLR7 agonist conjugates for 24h. Levels of IL-6 and IL-12 in the supernatant were measured by ELISA (BD Pharmingen, San Diego, CA).

Tumor models

B16 melanoma tumor cells expressing the transgene for cOVA (B16cOVA) were obtained from ATCC (Manassas, VA) and cultured in DMEM (Irvine Scientific, Irvine, CA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin/100 µg/mL streptomycin. 6 to 8 week old female C56BL/6 mice were subcutaneously (s.c.) injected with $1-2 \times 10^5$ B16cOVA in the flank. Primary tumors usually became palpable on day 7 and with an average diameter of 3 mm. On day 7, lesions were injected as indicated in the figure legends with a single dose or multiple doses of 50 µL solution containing TLR7 agonists or vehicle in DMSO. IL-2 (20,000 units per mouse) was intraperitoneally (i.p.) injected twice a day on days 7, 8, 9, 10, and 11. Five to twenty two mice per group were used. The number of mice in each individual study is described in the figure legends. Tumors were measured every other day. The tumor volumes were estimated according to the formula: volume (mm³) = (length) × (width)² × 0.5. Mice were followed for survival and sacrificed at a maximum tumor diameter of 1.0 cm.

Cytotoxic T cell (CTL) assay

CTL activity was assayed as previously described [22]. Briefly, 3.5×10^6 /ml effector splenocytes were restimulated in culture for 5 days with 3×10^6 /ml OVA peptide-pulsed stimulator splenocytes and 10 U/ml recombinant human IL-2 (Sigma) in RPMI1640 supplemented with 10% FBS. After restimulation, viable lymphocytes were recovered and washed. The recovered effector cells were incubated with target cells previously pulsed with OVA peptide or control cells. After 4 hours the supernatants were recovered and lysis was assayed by LDH release with the CytoTox 96 kit (Promega, Madison, WI) according to the manufacturer's instructions.

Fluorescent flow cytometry

A single cell suspension was prepared from spleens and draining lymph nodes using HBSS containing 5 µg/mL collagenase I and 20 µg/mL DNase I for one hour at room temperature. The cells were stained for CD4, CD8, or OVA-tetramer. Flow cytometry analysis was performed using FACSCalibur cytometer (BD Bioscience), and the data were analyzed using FlowJo software (Ashland, OR).

Histological Examination

Hematoxylin and eosin (H&E) and immunohistochemical staining were performed by UCSD Cancer Center Histology and Immunohistochemistry Shared Resources. Briefly subcutaneous tumors from sacrificed mice were removed, fixed in formalin, and embedded in paraffin. Sections 5 µm thick were stained with H&E. For immunohistochemical staining, the 5 µm frozen sections were incubated with biotinylated anti-CD11c or -F4/80 antibodies followed by alkaline phosphatase-conjugated streptavidin, Vector Blue substrate and nuclear fast red counterstain (Vector Laboratories, Inc., Burlingame, CA). The sections were examined under the microscope at $\times 100$ magnification.

Statistical Analysis

Data were plotted and fitted by nonlinear regression assuming a Gaussian distribution with uniform standard deviations between groups. The statistical differences for multiple comparisons were analyzed by two-way ANOVA with Bonferroni's *post hoc* or Dunnett's *post hoc* test. Kaplan-Meier survival curves were assessed by log rank tests. All analyses were performed using Prism software (version 4.0, GraphPad Software, Inc., San Diego, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Subcutaneous B16cOVA melanoma nodules expand rapidly in TLR7 deficient mice

Although pharmacologic activation of innate immunity by stimulating TLRs has been proposed for immunotherapy of melanoma [23, 24] there is little information regarding the importance of stromal TLRs in melanoma growth. To study the role of TLR7 in the tumor micro-environment, B16cOVA cells in the early logarithmic growth phase were subcutaneously implanted into wild type (WT) mice or mice that were genetically deficient for TLR7, TLR9, or MyD88. Nodule growth was monitored and mice were sacrificed at the

time the tumor reached 1 cm in any dimension, which was considered to be the survival endpoint. The median time to sacrifice for wild type, TLR7^{-/-}, TLR9^{-/-}, and MyD88^{-/-} mice was 20, 20, 22, and 19 days respectively (Figure 1A). Although both TLR7 and TLR9 signal through MyD88 the growth pattern for nodules diverged between these host strains. There was a trend toward faster tumor growth in the TLR7^{-/-} and MyD88^{-/-} mice, compared to the control mice, while tumor growth was slightly delayed in the TLR9^{-/-} mice. Notably, the tumor growth in TLR7^{-/-} mice was significantly faster than that for WT mice ($p < 0.05$).

As a part of innate immune function, the TLR-MyD88 signaling pathway activates macrophages and participates in their recruitment to sites of inflammation. Potentially the impaired TLR function in the genetically targeted mice could have reduced inflammatory cell recruitment that created a more permissive environment for tumor cell growth. On day 10 tumors were removed from each strain of mice when their size reached about 3–4 mm diameter and were examined histologically. Unexpectedly, the immune cell infiltration appeared similar in all groups of mice (Figure 2C).

Repeated intra-tumor injections with a TLR7 agonist reduces B16cOVA growth

The studies above indicated that the MyD88 pathway in the stromal or infiltrating inflammatory cells might play a role in suppressing the rapid expansion of tumor nodules. Accelerated nodule growth was seen in the TLR7^{-/-} mice, but not in the TLR9^{-/-} mice, implying that TLR7-specific stimulation in the tumor microenvironment might attenuate melanoma cell growth (Figure 2B). Hence we administered a TLR7 agonist, 1V199, directly into the tumor site using either a single high dose regimen or a repeated low dose protocol (Figures 3A and B). In the first protocol, 1V199 was injected into day 7 established subcutaneous tumors at a single high dose (11 nmoles/injection, High Single; Figure 3A); in the second, low doses (2.2 nmoles/injection) of 1V199 were administered every other day for two weeks (Low Multiple; Figure 3A). Repeated injections of low dose 1V199 significantly inhibited the tumor growth ($p < 0.03$ on day 17) compared to the vehicle-treated mice (Figure 3A) and improved survival of the recipients (28 days), compared to vehicle alone (19 days, $p < 0.025$, Figure 3B). In contrast, a single injection of high dose 1V199 (11 nmoles) did not significantly alter tumor growth nor survival.

The NHS ester on 1V199 can covalently bind to the primary amines on the solvent exposed surfaces of proteins under physiological conditions (Figure 1). We hypothesized that *in situ* conjugation to tumor associated antigens might enhance the anti-proliferative effects of this TLR7 agonist. To test this hypothesis, 1V209, the carboxylic acid derivative, which is not able to couple to amines, was synthesized [20]. Using the multiple low dose treatment strategy, B16cOVA nodule bearing mice received multiple intra-tumor injections of 1V199 or 1V209 (2.2 nmoles/injection) on days 7, 9, 11, 13, 15, 17, and 19 (Figure 3C). Median survival of mice treated with 1V209 was 20 days, similar to the vehicle treated control mice, whereas median survival of the mice treated with 1V199 was 28 days, significantly different from the control mice ($p = 0.016$ in Figure 3C).

Intralesional injection of a TLR7 agonist-phospholipid conjugate prolongs survival in mice with melanoma nodules

We previously reported that conjugation of lipids, phospholipids and PEG to TLR7 agonists could provide distinct immunostimulatory activities *in vitro* and *in vivo* [20]. These conjugates are TLR7 specific, and potently stimulate cytokine release from bone marrow derived macrophages and human peripheral blood mononuclear cells [20]. The use of PEG spacers between the TLR agonist and the phospholipid (1V285) improved aqueous solubility. In contrast, the phospholipid conjugate (1V270) acted as a depo, delivering the TLR7 activator over a more prolonged period [20]. Other prior reports suggested that the anti-tumor effects of topical TLR7 ligands could be mediated by infiltrating dendritic cells [25, 26]. Hence, we tested the ability of the conjugated compounds to stimulate the production of IL-6 and IL-12 by bone marrow derived dendritic cells (Figures 4A and B). The phospholipid- or PEG-phospholipid-TLR7 agonist conjugates (1V270 and 1V285) were 10 to 100 times more potent than unconjugated form (1V199) (Figures 4A and B).

We further tested the *in vivo* efficacy of these compounds in the melanoma model. Established subcutaneous melanoma nodules were injected with the unconjugated form of the TLR7 agonist (1V199), the phospholipid- TLR7 agonist (1V270) or the PEG-phospholipid TLR7 agonist (1V285) on days 7, 9, 11, 13, 15, 17 and 19. The survival times of animals treated with 1V199 or 1V270 were significantly better than the vehicle-treated animals ($p=0.01$ and $p=0.001$, respectively, Figure 4C). Treatment with 1V199 or 1V270 significantly inhibited tumor growth compared to vehicle-treated animals ($p<0.05$, on days 15 and 17). In contrast, the administration of the PEG-phospholipid TLR7 agonist (1V285) influenced neither the tumor growth nor survival (Figure 4C and 4D). The tumor growth curves were terminated on the day that the first mouse was sacrificed, in order to prevent skewing the data in favor of smaller tumor sizes in the surviving mice. The anti-tumor effects of 1V270 were abrogated in the MyD88^{-/-} mice, indicating that the effect of the treatment was dependent on the MyD88 signaling pathway (Figure 4E). The anti-melanoma effect of 1V270 was also observed in the mice bearing B16-F0 melanoma, which does not express an OVA transgene (data not shown). Collectively, the data indicate that the phospholipid conjugated TLR7 agonists suppressed melanoma growth and prolonged survival more effectively than the unconjugated TLR7 agonist, 1V199, in this mouse model.

Combination therapy with IL-2 and local treatment of 1V270 further improve the survival of the host compared to IL-2 single therapy

For advanced, unresectable melanoma, standard therapy with agents such as dacarbazine, temozolomide and IL-2 is associated with notoriously low response rates. Both MHC and non-MHC mechanisms of action have been proposed for IL-2 [27, 28]. We, therefore, tested the combination treatment of systemic high-dose IL-2 that may act on effector cells of the innate immune system and local intra-tumor TLR7 agonist (Figure 5A). Treatment with either IL-2 or phospholipid-TLR7 agonist conjugate (1V270) significantly reduced the tumor growth ($p<0.05$ compared to vehicle treated on day 15, respectively, Figure 5B). However, the median survival of mice treated with IL-2 and 1V270 were 19 and 21 days, respectively, similar to the vehicle treated group (17 days, Figure 5A). Combination therapy

suppressed tumor growth significantly on days 13 and 15 ($p<0.01$ and $p<0.001$, respectively), and extended survival to 26 days.

Combination therapy with IL-2 and local treatment of 1V270 enhances CD8⁺ T cell responses

To examine the inflammatory infiltrate in the mice treated as outline above, the tumor nodules were removed on Day16 and prepared for histological staining. The nodules were stained for dendritic cell (CD11c), macrophage (F4/80), and T cell (CD4 and CD8) markers. Qualitatively there were minimal differences in the staining patterns between all of the groups on Day 16. These data suggested that the treatment did not effect cellular recruitment into the tumor but had a separate effect on cellular function. We further studied the effects of combination therapy on cytotoxic T cell (CTL) expansion and activity. The cells isolated from draining lymph nodes were examined for CD8 and OVA-specific tetramer binding. Treatment with 1V270, or the combination of 1V270 and IL-2 increased the frequency of tetramer-specific CD8⁺ T cells (Figure 6B). Furthermore, the cytotoxic activity of OVA-specific T cells was examined using a CTL assay using *in vitro* restimulated splenocytes. Splenocytes harvested from mice that received the combination of 1V270 and IL-2 exhibited greater OVA specific cytotoxicity *in vitro* ($p<0.05$ compared to vehicle treated- or 1V270 treated group, Figure 6C).

Discussion

The current standard of care for patients with unresectable malignant melanoma is either single agent chemotherapy or high dose IL-2 [29]. Dacarbazine, the only FDA approved chemotherapy for melanoma, has a response rate of below 10% and lacks data showing an improvement in overall survival over the best supportive care [2]. Bolus high-dose IL-2 therapy can lead to long-term complete remission in a minority of patients [3]. The occasional dramatic response to cytokine therapy and the immune selection observed during disease progression has lead to the belief that melanoma may be treatable by immune therapy, and recent clinical trials with antibodies to the immunosuppressive molecule CTLA4 support this conclusion. Here the early suppression of growth in B16cOVA nodules in WT mice compared to MyD88^{-/-} mice suggested that enhancing innate immune function might work as an adjunct in immune based therapies.

To augment the efficacy of utilizing TLR7 agonists as an adjunct to chemotherapy or other immunologic therapies, we developed a series of compounds that were conjugated to phospholipids or PEG-phospholipids. In a prior report we demonstrated that these compounds retained their TLR7 specificity and stimulated macrophages and dendritic cells to release cytokines [20]. These compounds also stimulated release of inflammatory cytokines *in vivo* and are effective adjuvants. Although imiquimod is FDA approved and has some topical efficacy, we hypothesized that conjugation of a more potent TLR7 activator to a phospholipid would sustain innate immune activation. Alternatively, the conjugation of drugs to polyethylene glycol can increase water solubility and may improve delivery for some applications [30].

The phospholipid and phospholipid-PEG conjugated TLR7 agonists did stimulate cytokine release from cultured dendritic cells. However, the phospholipid-PEG conjugated drug did not suppress tumor growth *in vivo*, suggesting that increasing the water solubility of the compound reduced its subcutaneous efficacy. Conversely, the phospholipid conjugated drug was the most potent in reducing nodule growth, indicating possible prolonged TLR7 activation. It is likely that the conjugation prolonged the presence of the drug in the tissue, sustaining its activity in a depot form. Alternatively, conjugation to the phospholipid might have improved cellular permeability and delivery of the TLR7 activator to the endosome. This is biologically essential, as TLR7 and TLR9 are only active after proteolytic cleavage and transport to the endosome [31, 32]. Hence, the intracellular uptake and trafficking of the compound to the endosome would be key to sustaining the activity.

Although IL-2 therapy has the potential to induce a prolonged remission in melanoma, the side effects of the agent are often not well tolerated [3]. Adjunctive therapy that would allow lower doses of IL-2 to achieve remission would be valuable. The addition of an innate immune activator, however, has the potential for additive systemic cytokine associated toxicity. Systemic administration of a TLR7 agonist has been shown to be well tolerated in humans, and in a small number of patients with chemotherapy refractory melanoma there was stabilization of disease [33]. Hence a continued optimization of lead candidates in this area is warranted.

Although melanoma cells can express TLR7, it is not likely that the anti-tumor effect was mediated solely by direct activation of the tumor cells [34]. The stromal cells in the periphery and local innate immune cells were likely also stimulated by the drug, enhancing the activity. Topical TLR7 activation recruits dendritic cells and increases the capacity of antigen-presenting cells to induce reactive T cells. Here we demonstrated that the combination of systemic IL-2 and local TLR7 agonist increased the frequency of antigen specific T cells and enhanced CTL activity. There have recently been reports that topical TLR7 can act as an adjuvant in enhancing anti-tumor responses [7, 25, 33, 35, 36]. Although the adjuvanticity of the TLR7 conjugates was not formally tested in this report, we previously found that agonists conjugated to albumin or to phospholipid caused rapid elevation of IgG2a antibodies [20]. Mice immunized with the phospholipid conjugates and antigen developed sustained antigen-specific IgG2a levels and IFN γ splenocyte responses without further boosting [20]. Hence, conjugation of a TLR7 agonist to phospholipid moieties is a promising strategy for topical or intralesional application, which could augment antigen specific immune responses. Future experiments will investigate the potential for the phospholipid conjugates to act as an adjuvant for melanoma directed immunotherapy.

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Abbreviations

TLR Toll-like receptor

IL	Interleukin
PEG	Polyethyleneglycol
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa B
NHS	<i>N</i> -hydroxysuccinimide

Figure 1. Structures of TLR7 agonists and conjugates

(A) 1V199; TLR7 agonist with an NHS ester group. (B) 1V209; TLR7 agonist without an NHS ester group. (C) 1V270; 1V209 conjugated to a phospholipid (D) 1V285; 1V209 conjugated to PEG-phospholipid. (E) Molecular weight shift of OVA after conjugation with 1V199. OVA 0.17mg/mL was incubated with vehicle, 1V199 or 1V209 (1.62mM) at 37 C° for 4 hours. The products were separated by SDS-PAGE and stained with Coomassie. 1V199 conjugated to ovalbumin increased the molecular weight, but 1V209 did not. The molecular masses (M) of the standards are shown in kDa.

Figure 2. Rapid progression of B16cOVA melanoma nodules in TLR7 deficient mice
 1×10^5 B16cOVA were injected subcutaneously into wild type C57BL/6 mice ($n=17$), MyD88^{-/-} ($n=9$), TLR7^{-/-} ($n=18$), and TLR9^{-/-} ($n=17$). The nodules were serially assessed and mice were sacrificed on the day the nodules measured 1 cm in any dimension. **(A)** The survival curves are shown. Median survivals of C57BL/6 (WT), TLR7^{-/-}, TLR9^{-/-}, and MyD88^{-/-} mice were 20, 20, 22, and 19 days, respectively. **(B)** Tumor growth in WT, MyD88^{-/-}, TLR7^{-/-}, and TLR9^{-/-} mice. The tumor sizes are expressed as described in the material and methods. Data shown are means $\pm$ SEM, pooled from two independent experiments. *, denotes $p < 0.05$ of TLR7^{-/-} mice compared to wild type mice by two-way ANOVA with Bonferroni's post hoc test. **(C)** Representative tumor sections from WT, TLR7^{-/-}, TLR9^{-/-}, or MyD88^{-/-} mice. The tumors were harvested on day 10 and processed for H&E staining. Original magnification is $\times 200$. The scale bar indicates 100 μm .

Figure 3. Intra-tumor injection of TLR7 agonist delays B16cOVA growth in wild type C57BL/6 mice

1×10^5 B16cOVA were injected s.c. into WT mice ($n=10$ per group). (A) Mice received intra-tumor injections of 1V199, either 11 nmoles on day 7 (High Single) or 2.2 nmoles on days 7, 9, 11, 13, 15, 17, and 19 (Low Multiple). Vehicle-injected mice served as the control. Data shown are means $\pm$ SEM, representative of two independent experiments. *, $p < 0.03$, by two-way ANOVA followed by Dunnett's post hoc test compared to the vehicle-treated mice. (B) Kaplan-Meier survival curves of melanoma injected mice treated with different schedules of a TLR7 agonist. Median survival were 19, 28, and 20 days in the mice treated with vehicle alone, multiple administration of low dose 1V199 (2.2 nmoles per mouse) and single administration of high dose 1V199 (11 nmoles per mouse), respectively. Data shown are representative of two independent experiments. *, denotes $p < 0.025$ assessed by Kaplan-Meier survival curves and log rank tests. (C) Kaplan-Meier survival curves of melanoma bearing mice that received intra-tumor injections of 1V199 (2.2 nmoles) ($n=8$) or 1V209 (2.2 nmoles) ($n=10$) on days 7, 9, 11, 13, 15, 17, and 19. Median survival of the mice injected with 1V199 and 1V209 were 29 and 20 days, respectively. Data shown are representative of two independent experiments.

Figure 4. Phospholipid-TLR7 agonist conjugates slow tumor growth and prolong survival
(A and B) Comparison of in vitro immunostimulatory potencies of 1V199, 1V270, and 1V285. 1×10^5 BMDC were cultured with graded concentrations of 1V199, 1V270, or 1V285 for sixteen hours and the levels of IL-6 and IL-12 in the culture supernatants were determined by ELISA. Data shown are means $\pm$ SEM, representative of two independent experiments. **(C)** Kaplan-Meier survival curves of mice with melanoma nodules treated intralesionally with TLR7 agonist conjugates. 2×10^5 B16cOVA cells were injected s.c. into mice to establish nodules. The nodules were injected with vehicle, 2.2 nmoles or equivalent doses of 1V199 (n=20), 1V270 (phospholipid conjugate) (n=8) or 1V282 (PEG- conjugate) (n=9), on days 7, 9, 11, 13, 15, 17, and 19. Vehicle-treated mice served as controls (n=15).

Median survival time of mice treated with vehicle alone, 1V199, 1V270, and 1V285 were 20, 25, 32.5 and 22 days respectively. Survival of mice treated with 1V199 or 1V270 was significantly prolonged compared to the control animals ($p=0.01$ and $p=0.001$, respectively). Data shown are pooled from two independent experiments. (C) Suppression of tumor growth by TLR7 agonist conjugates. Mice were allowed to engraft tumor nodules as above and were similarly treated with intralesional injections 1V199 ($n=22$), 1V270 ($n=10$) or 1V282 ($n=10$). The lesions were serially measured in two dimensions and the volumes were calculated. Data shown are mean $\pm$ SEM, pooled from two independent experiments. * denotes $p<0.05$ of 1V270 and 1V199 treated groups on days 15 and 17 compared to vehicle treated mice by two-way ANOVA and Bonferroni post hoc testing. (E) TLR7 agonist treatment does not inhibit melanoma growth in MyD88^{-/-} mice. 2×10^5 B16cOVA cells were injected s.c. into MyD88^{-/-} mice ($n=5$) mice to establish nodules. On day 7, 9, 11, 13, 15, and 17, were intralesionally injected with 1V270 2.2 nmoles or vehicle alone. The lesions were serially measured in two dimensions and the volumes were calculated. Data shown are mean $\pm$ SEM.

Figure 5. Concomitant intralesional 1V270 injections synergize with systemic IL-2 treatment
Mice with B16cOVA melanoma nodules received systemic IL-2, 1V270 (2.2 nmoles per injection) or combination treatment. **(A)** Treatment schedule of single agent and combination therapy. IL-2 (20,000 units per mouse) was intraperitoneally injected twice a day on days 7, 8, 9, 10, and 11. Intra-tumor injections of 1V270 (2.2 nmoles) were performed on days 7, 9, 11, 13, 15, and 17. **(B)** Kaplan-Meier survival curves of melanoma implanted mice treated with single agent or combination therapy. Median survival of mice treated with IL-2 (n=5), 1V270 (n=5), or a combination of IL-2 plus 1V270 (n=13) were 19, 21, and 26 days, respectively. Median survival time of the vehicle-treated control group (n=13) was 17 days. Data shown are representative of two independent experiments. **(C)** Inhibition of tumor growth in the mice treated with combination therapy. The mice bearing melanoma were treated with IL-2 (n=14), 1V270 (n=9), or a combination of IL-2 plus 1V270 (n=18) as described above. The tumor nodules were measured in two dimensions and the volumes calculated. Data shown are mean $\pm$ SEM, pooled from two independent experiments. * denotes $p < 0.01$ compared to vehicle treated mice by two-way ANOVA with Bonferroni's *post hoc* testing.

Figure 6. Concomitant intralesional 1V270 injections synergize with systemic IL-2 treatment
Mice with B16cOVA melanoma nodules received systemic IL-2, 1V270 (2.2 nmoles per injection) or combination treatment as in Figure 5(A). Mice (n=5/group) were sacrificed on day 16 and the nodules were removed for histological sectioning and immunostaining. Shown are representative immunostains for each group 100x magnification. **(B)** Combination therapy increased CD8⁺ T cells binding to OVA-specific tetramers. Cells pooled from the lymph nodes of mice implanted with tumor nodules and treated with vehicle (veh), 1V270, IL-2, or IL-2 plus 1V270 were analyzed for CD8 expression and tetramer binding by flow cytometry (n=5/group). Data shown are the frequencies of tetramer positive cells in the CD8⁺ population. Data are representative of two independent experiments yielding similar results. **(C)** The combination therapy enhances OVA peptide-specific CTL activity. The splenocytes from the mice in (B) were restimulated with OVA peptide and IL-2 for five days. CTL activity was measured against target cells pulsed with OVA peptide or control cells at an effector to target ratio of 1:25. * denotes p<0.05 using one-way ANOVA with Bonferroni's *post hoc* testing.