



Localized irradiation of tumors prior to synthetic dsRNA therapy enhanced the resultant anti-tumor activity

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ABSTRACT

Background and purpose: Despite the potent tumoricidal activity of the synthetic dsRNA in culture, its *in vivo* anti-tumor activity has proven to be limited. We sought to devise and validate a new strategy to improve the *in vivo* anti-tumor activity by integrating localized irradiation into dsRNA therapy.

Materials and methods: Using a mouse lung cancer model and a mouse melanoma model in immuno-competent mice or athymic nude mice, we evaluated the combined anti-tumor activity using a synthetic dsRNA, polyinosine-cytosine (poly(I:C)).

Results: Localized irradiation of tumors prior to the poly(I:C) therapy significantly delayed the tumor growth as compared to monotherapies using the radiation or poly(I:C) alone. The poly(I:C) enhanced the tumor response to radiation with a dose modification factor as large as 20. The combined effect was synergistic only in immuno-competent mice with highly immunogenic tumors. The anti-tumor activity of the combination therapy was significantly impaired when the type I interferons in the mice were neutralized.

Conclusions: This combination modality may represent a promising approach to exploit synthetic dsRNA in cancer therapy and to enhance tumor response to radiation. T cell-mediated immunity was likely responsible for the combined synergistic effect. Type I interferons contributed significantly to the combined anti-tumor activity.

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Cancer is one of the leading causes of death in the U.S. New or improved approaches to control cancers are constantly sought. Double-stranded RNA can activate multiple pro-apoptotic mechanisms simultaneously [1]. It also induces apoptosis in certain tumor cells by interacting with the Toll-like receptor 3 (TLR3) [2]. Moreover, dsRNA is a potent inducer of type I interferons (IFNs) [3], which are pro-apoptotic, anti-proliferative, and anti-angiogenic [1,4–6]. Finally, both dsRNA and the type I IFNs induced by it are strongly immunostimulatory [7–10]. Previously, there had been numerous studies utilizing the poly(I:C) to control tumors in experimental animal models and clinical trials, and the anti-tumor activity was attributed to a large extent to the poly(I:C)'s ability to induce the production of type I IFNs. Overall, the anti-tumor activity of the poly(I:C) was limited and inconsistent *in vivo* [11–13], and higher doses of poly(I:C) were reported to be associated with adverse effects [14–16].

Recently, efforts to exploit the anti-tumor activities of synthetic dsRNA to kill tumor cells were revived [17–19]. *In vitro*, when

delivered into tumor cells using cationic liposomes, poly(I:C) inhibited the growth of a variety of tumor cells with IC₅₀ values in the nM range [19,20]. In a nude mouse model, Shir et al. (2006) reported that targeting the poly(I:C) into human glioblastoma cells or breast cancer cells over-expressing epidermal growth factor receptors by direct intratumoral injection led to the regression of pre-established tumors [17]. In a more recent study, we reported an alternative approach to improve the anti-tumor activity of the poly(I:C) by combining the dsRNA therapy with a chemotherapy agent, gemcitabine [19]. Our data showed that the poly(I:C) and gemcitabine synergistically delayed the tumor growth and prolonged the survival of the tumor-bearing mice [19]. The combination therapy also generated a strong and durable tumor-specific immune response [19]. This combination approach may help decrease the dose of the poly(I:C), and thus, minimize its adverse effects.

In the present study, we evaluated the feasibility of integrating radiotherapy into the poly(I:C) therapy to improve the anti-tumor activity of the poly(I:C). Ionizing radiation has a well-established ability to kill tumor cells [21]. We hypothesized that localized irradiation of the tumors prior to the poly(I:C) therapy will improve the resultant anti-tumor activity. Moreover, being a ligand to the TLR3, poly(I:C) is strongly immunostimulatory and can activate

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both innate and adaptive immune responses [9,10,22]. The tumor cells killed by the radiation are a good source of tumor antigens, and the dead tumor cells may be taken up by antigen-presenting cells, such as the dendritic cells, to induce T cell-mediated immunity [23,24]. Therefore, we further hypothesized that T cell-mediated immunity will contribute to the combined anti-tumor activity.

Using a highly immunogenic lung tumor model in immunocompetent mice, we demonstrated that localized irradiation of the tumors prior to the poly(I:C) therapy synergistically improved the resultant anti-tumor activity as compared to monotherapies using the poly(I:C) or the radiation alone. The poly(I:C) therapy reduced the radiation dose with a dose modification factor as large as 20. However, the combined effect was only additive when the combination therapy was applied to the same tumors in athymic nude mice or to immunocompetent mice with a poorly immunogenic model tumor, indicating that the T cell-mediated immunity was responsible for the synergistic effect from the combination therapy. Finally, our data showed that the type I IFNs induced by the poly(I:C) contributed significantly to the combined anti-tumor activity. When fully developed, it is expected that this combination therapy will represent a promising approach to more effectively take advantage of the anti-tumor activity of the synthetic dsRNA and to improve tumor response to radiation.

Materials and methods

Mice and cell lines

Female C57BL/6 mice, 6–8 weeks of age, were from Simonsen Laboratories, Inc. (Gilroy, CA). The Nu/Nu female nude mice (6–8 weeks) were from Charles River Laboratories, Inc. (Wilmington, MA). The TC-1 cells (ATCC #, CRL-2785), a highly immunogenic lung cancer cell line, were engineered by transforming primary C57BL/6 lung cells with human papillomavirus (HPV) type 16 E6 and E7 oncogenes and an activated H-ras [25]. They were grown in RPMI 1640 medium (Invitrogen). The poorly immunogenic or non-immunogenic B16-F10 mouse melanoma cells were from ATCC and were grown in DMEM. All media were supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin.

Preparation of poly(I:C)-liposome lipoplexes (pl:C/LP)

Cationic liposomes comprising of cholesterol, egg L- α -phosphatidylcholine, and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (4.6:10.8:12.9, m/m/m, all from Avanti Polar Lipids, Alabaster, AL) were prepared by the thin film hydration method. The final concentration of the DOTAP in the liposomes was 10 mg/mL. The pl:C/LP lipoplexes were prepared by mixing a pl:C solution (50 µg, Amersham Biosciences, Piscataway, NJ) and an equal volume of liposome suspension (8 µg of DOTAP) followed by gentle pipetting [19].

Animal studies

NIH guidelines for animal use and care were followed. The animal protocol was approved by our institutional IACUC. To establish tumor models in mice, tumor cells (TC-1 or B16-F10, 5×10^5 cells/mouse) were subcutaneously (s.c.) injected in the right hind upper leg of mice. The hair, if any, in the injection site was carefully trimmed before the injection. The size of the tumors and the values of tumor growth delay were calculated as previously described [26]. Tumor therapy was started when the diameter of the tumors reached 6–8 mm. To irradiate the tu-

mors, anesthetized mice were restrained in a jig with the tumor-bearing right hind legs extended in a row outside the jig. The tumors were irradiated with a desired dose of 6 Megavoltage X-rays (Varian Medical Systems, Palo Alto, CA). Adequate bolus was used under and over the irradiation area to minimize radiation dose inhomogeneity to less than 3% along the tumor depth. The X-ray field was 4.0×15.0 cm, and only the tumor-bearing legs were placed in the field. The X-ray dose for each batch of mice was verified using thermoluminescent dosimetry. Mice were allowed to rest for one day before the start of the dosing of the pl:C/LPs, which were injected peritumorally (p.t.) (or subcutaneously in a site distal to the tumors as where mentioned). The combined anti-tumor activity was evaluated as previously described [27]. A combination index of greater than 1 indicates the presence of a synergistic effect; an index of <1 or =1 indicates an additive or less than additive effect.

In vivo IFN- α/β blockade

The *in vivo* IFN- α/β blockade was completed as previously described with slight modifications [28]. Sheep anti-serum to murine C-Cell IFNs (NR-3087) and sheep anti-serum control for the NR-3087 were from the BEI Resources (Manassas, MD). Mice were intraperitoneally injected with 0.18 mL of the anti-serum on days –1, +2, and +4 with respect to the first pl:C/LP administration [28].

Quantification of IFN- α in tumors and blood

Tumor samples were homogenized in a microtube using a mini bead beater (BioSpec Products, Inc., Bartlesville, OK) and then centrifuged for 10 min. The supernatant was collected. The concentration of IFN- α was determined using a mouse IFN alpha (Mu-IFN- α) ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

Statistical analysis

Statistical analysis was completed using ANOVA followed by pair-wise comparisons using the Fisher's PLSD procedure. A *p*-value of <0.05 (2-tail) was considered statistically significant.

Results

Localized irradiation prior to the poly(I:C) therapy significantly delayed the growth of the highly immunogenic TC-1 tumors in immuno-competent mice

First, we confirmed that the poly(I:C) in the form of pl:C-liposome lipoplexes (pl:C/LP) significantly delayed the growth of the TC-1 tumors in mice (data not shown). Overall, the anti-tumor activity of the pl:C/LP lipoplexes was very limited. We then showed that localized irradiation of the TC-1 tumors significantly delayed the tumor growth (Fig. 1A), and that increasing the X-ray dose further prolonged the tumor growth delay (Fig. 1A). However, 40 Gy, the highest dose of X-rays we used, generated some observable adverse effects, such as hair loss on the right hind leg and a relatively shorter mouse survival time (data not shown). Thus, the dose of 20 Gy or less was used for further studies.

Data in Fig. 1B show that localized irradiation of tumors prior to the start of the injection of the pl:C/LP significantly further delayed the tumor growth as compared to monotherapies using the radiation or the pl:C/LP alone. The mean time it took for the tumors to reach 12–13 mm after the first dose of pl:C/LP was 4.0 , 4.8 ± 1.0 , 5.3 ± 1.0 (or 7.7 ± 1.0 when calculated after the regression period), and 21.0 ± 4.6 days for the control, pl:C/LP, irradiation (IR), and the combination (IR + pl:C/LP) groups, respectively (Fig. 1B). The value

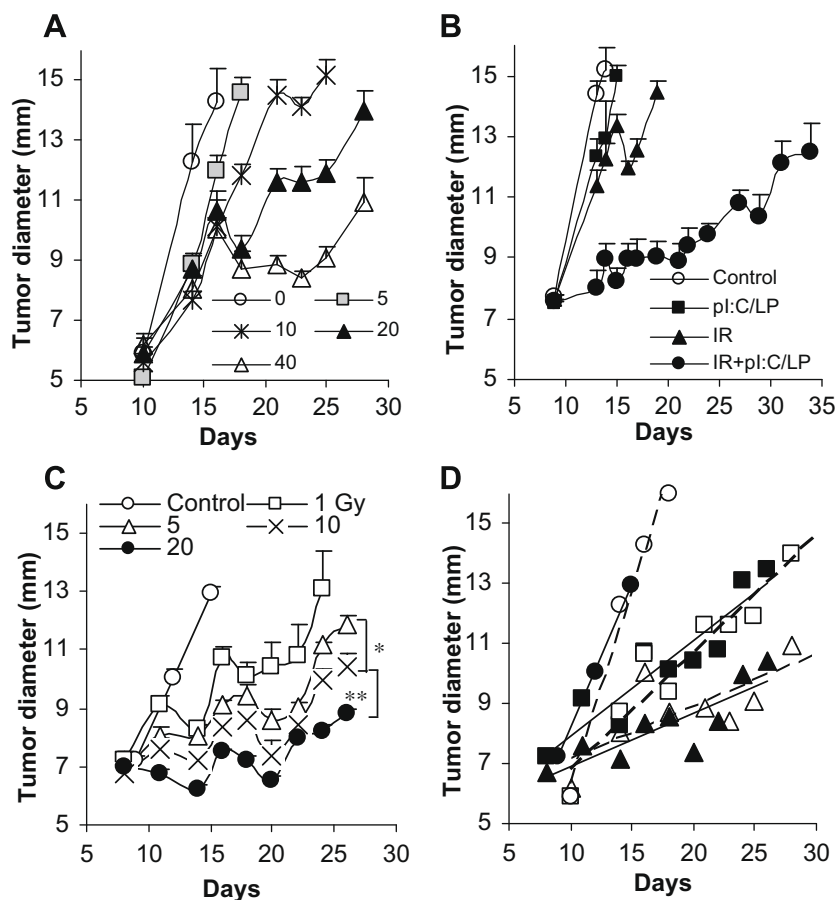


Fig. 1. Localized irradiation of tumors prior to the pl:C/LP therapy significantly delayed the growth of TC-1 tumors in C57BL/6 mice. (A) The effect of the dose of X-ray (Gy) on the growth of TC-1 tumors in mice. TC-1 cells were s.c. seeded in mice on day 0. On day 10, mice ($n = 5$) were locally irradiated. (B) Combination therapy using radiation followed by pl:C/LP significantly further delayed the growth of the TC-1 tumors. Ten days after tumor seeding (tumor diameter = 7.6 ± 0.3 mm), mice ($n = 9-10$) were locally irradiated with a single dose of X-rays (20 Gy). Starting on day 12, mice were dosed with pl:C/LP (50 μ g of pl:C) for 5 consecutive days. (C) Effect of the dose of the X-rays on the combined anti-tumor activity. Nine days after tumor seeding, the tumors were locally irradiated (1–20 Gy). Starting on day 11, mice ($n = 5-6$) were dosed with pl:C/LP (50 μ g pl:C) 3 times a week for two consecutive weeks. ANOVA revealed differences among those groups that received X-rays ($p = 0.003$ on day 26). $^*p = 0.02$ on day 26. $^{**}p = 0.007$ on day 26. (D) A re-plotting of some of the data in (A) and (C) showing that the tumor response to 20 Gy of X-ray alone was comparable to that to 1 Gy of X-ray followed by poly(I:C) (50 μ g). Similarly, the tumor responses to 40 Gy of X-ray alone and to 10 Gy of X-ray followed by the poly(I:C) were comparable. (●, ○) Control mice, (■) 1 Gy of X-ray followed by pl:C/LP, (□) 20 Gy of X-ray alone, (▲) 10 Gy of X-ray followed by pl:C/LP, (△) 40 Gy of X-ray alone. Data reported are means \pm SEM. SEM was not included in (D) for clarity.

of the tumor growth delay caused by the combination therapy was significantly larger than those of the monotherapies ($p < 0.05$).

Moreover, data in Fig. 1C show that increasing the dose of the X-ray from 5 to 20 Gy enhanced the resultant anti-tumor activity by the combination therapy. Interestingly, a simple comparison of the data in Fig. 1A and C indicated that the poly(I:C) therapy enhanced the tumor response to radiation. For example, it appeared that about 20 Gy of X-ray alone generated the same effect as 1 Gy of X-ray followed by the poly(I:C) therapy (50 μ g of pl:C, 3 times a week for two consecutive weeks) (Fig. 1D), suggesting a radiation dose modification factor of 20. Similarly, 10 Gy of X-ray followed by the poly(I:C) therapy generated the same effect as 40 Gy of X-ray alone (a dose modification factor of 4) (Fig. 1D).

The combination therapy was more effective than the monotherapies in athymic nude mice with highly immunogenic tumors and in immuno-competent mice with poorly immunogenic or non-immunogenic tumors

To understand whether the combination therapy was still effective in immuno-compromised mice or with poorly immunogenic tumors, we evaluated the anti-tumor activities in nude mice with the TC-1 tumors and in C57BL/6 mice with the poorly immuno-

genic B16-F10 melanoma. As shown in Fig. 2A, the combination therapy was still more effective than the monotherapies in delaying the growth of the TC-1 tumors in nude mice. The mean time it took for the tumors to reach 7–8 mm after the radiation was 3.3 ± 2.4 , 4.7 ± 1.4 , 9.4 ± 6.0 , and 19.1 ± 4.9 days for the control, pl:C/LP, IR, and IR + pl:C/LP groups, respectively (Fig. 2A, $p = 0.002$, IR + pl:C/LP vs. IR, $p = 3 \times 10^{-6}$, IR + pl:C/LP vs. pl:C/LP). Similarly, in the B16-F10 mouse melanoma model, the combination therapy also significantly delayed the tumor growth (Fig. 2B). The diameters of the tumors in mice that received the combination therapy were significantly smaller than those of the tumors in mice that received the monotherapies, starting 8 days after the first dose of pl:C/LP (e.g., on day 8, $p = 0.002$ vs. pl:C/LP, $p = 0.02$ vs. IR).

The immunogenicity of the tumors and the immuno-competency of the mice determined whether the combined activity was synergistic or additive

We calculated the combination index (CI) based on the diameter of the tumors after the combination therapy. As shown in Table 1, the combined effect was synergistic ($CI > 1.0$) only in the C57BL/6 mice with the TC-1 tumors, but was additive or less than additive

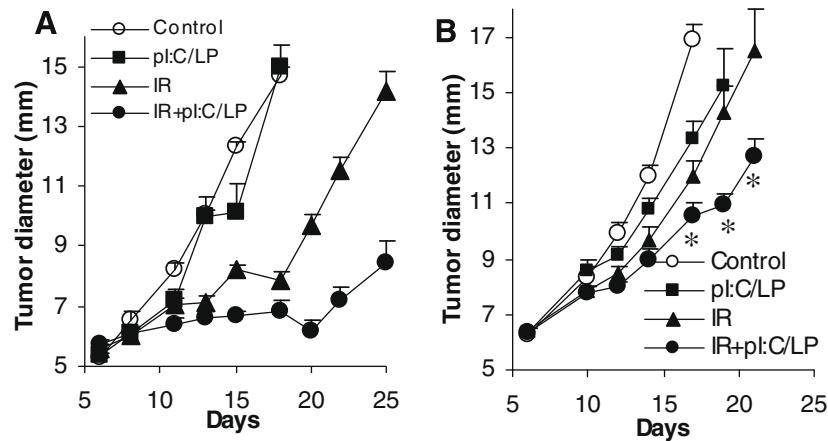


Fig. 2. The combination therapy was more effective than the monotherapies in athymic nude mice with highly immunogenic tumors and in immuno-competent mice with poorly immunogenic or non-immunogenic tumors. (A) Athymic nude mice with TC-1 tumors. TC-1 cells were seeded in mice on day 0. On day 6, tumors were irradiated (20 Gy). Starting on day 8, mice ($n = 7-10$) were dosed with pl:C/LP (37.5 μ g of pl:C) 3 times a week for 2 consecutive weeks. (B) C57BL/6 mice with B16-F10 tumors. Tumor cells were seeded in mice on day 0. On day 7, tumors were irradiated (15 Gy). Starting on day 9, mice ($n = 8-10$) were dosed with pl:C/LP (50 μ g of pl:C) 3 times a week for 2 consecutive weeks. () The values of the IR + pl:C/LP were significantly smaller than those of the others. Data reported are means \pm SEM.

Table 1

Fractional tumor diameter (FTD^a) relative to control mice in combination therapy using localized irradiation and poly(I:C).

Day ^b	pl:C/LP	IR	IR + pl:C/LP		Combination index ^d
			Observed	Expected ^c	
<i>TC-1 cells in C57BL/6 mice</i>					
13	0.85	0.79	0.56	0.68	1.2
14	0.91	0.81	0.59	0.74	1.3
<i>TC-1 cells in nude mice^e</i>					
8	0.93	0.92	0.93	0.86	0.9
11	0.88	0.86	0.78	0.75	1.0
13	0.99	0.71	0.66	0.71	1.1
15	0.82	0.67	0.54	0.55	1.0
<i>B16-F10 cells in C57BL/6 mice</i>					
10	1.03	0.94	0.94	0.97	1.0
12	0.92	0.85	0.80	0.78	1.0
14	0.91	0.81	0.75	0.73	1.0
17	0.79	0.71	0.62	0.56	0.9

^a FTD, fractional tumor diameter (mean tumor diameter experimental)/(mean tumor diameter of control). Values were calculated only after the start of the combination therapy and when the tumor diameter values for the control group were available.

^b Days after tumor seeding.

^c (Mean FTD of pl:C/LP) \times (mean FTD of IR).

^d Combination index (CI) = expected FTD/observed FTD.

^e CI was not calculated for day 17 because some mice were preemptively euthanized.

(CI < 1.0 or =1) in athymic nude mice with the TC-1 tumors and in the C57BL/6 mice with the poorly immunogenic B16-F10 melanoma.

The type I IFNs induced by the poly(I:C) contributed significantly to the anti-tumor activity of the combination therapy

Data in Fig. 3A showed that in the poly(I:C) monotherapy, when the pl:C/LP was injected into a site distal to the tumors, its anti-tumor activity was slightly weaker than when the pl:C/LP was injected peritumorally. Similarly, when combined with localized irradiation, the pl:C/LP was also less effective when injected into a site distal to the tumors than when injected close to the tumors (Fig. 3B).

As shown in Fig. 3C, peritumoral injection of the pl:C/LP induced a higher level of IFN- α in the tumor tissues than when the pl:C/LP was injected distal to the tumors, whereas the levels of

the IFN- α in the serum samples were comparable regardless of whether the pl:C/LP was injected close or distal to the tumors (Fig. 3D). The serum IFN- α level was measured 24 h after the injection of the pl:C/LP because the mouse serum IFN- α level peaked about 24 h after the pl:C/LP was subcutaneously injected into mice (Fig. 3E).

Finally, our data in Fig. 3F demonstrate that neutralization of the type I IFNs in the tumor-bearing mice significantly impaired the ability for the combination therapy to delay the tumor growth.

Discussions

Synthetic dsRNA has multiple anti-tumor mechanisms that may be exploited to control tumor growth. It is pro-apoptotic and immunostimulatory [1,2,9,10,22,29]. It is a potent inducer of type I IFNs, which are known to be pro-apoptotic, immunostimulatory, and anti-angiogenic [1,3–6]. Thus, it is not surprising that numerous studies have been carried out since the 1960s to materialize the tumor therapeutic potentials of the poly(I:C). Unfortunately, the results from previous studies including clinical trials have not been encouraging. In general, treatment with the poly(I:C) only slightly delayed the tumor growth, and the anti-tumor activity was inconsistent. Increasing the dose of the poly(I:C) is not a practical option to improve its efficacy because the poly(I:C) has serious dose-limiting adverse effects [14–16]. Nevertheless, data from some recent studies indicated that alternative approaches may be devised to improve the efficacy of the dsRNA therapy [17,19].

In the present study, we designed and validated an approach that can potentially improve the clinical efficacy of the dsRNA therapy. Ionizing irradiation is tumoricidal, which prompted us to locally irradiate the tumors prior to the dsRNA therapy. We hypothesized that the combination therapy will enhance the resultant anti-tumor activity, which was strongly supported by our data in Figs. 1B and 2A and B. In all these experiments, monotherapies using the poly(I:C) lipoplexes or the irradiation alone only slightly delayed the growth of the tumors. However, the tumor growth delay after the combination therapy was significantly longer. Just one round of therapy using a single dose of X-rays followed by a few doses of the poly(I:C) significantly delayed the growth of the two different and very aggressive tumors. For example, the growth of the TC-1 cells was delayed by 17 days (Fig. 1B), which is significant considering that the TC-1 tumor-bearing mice can only survive for

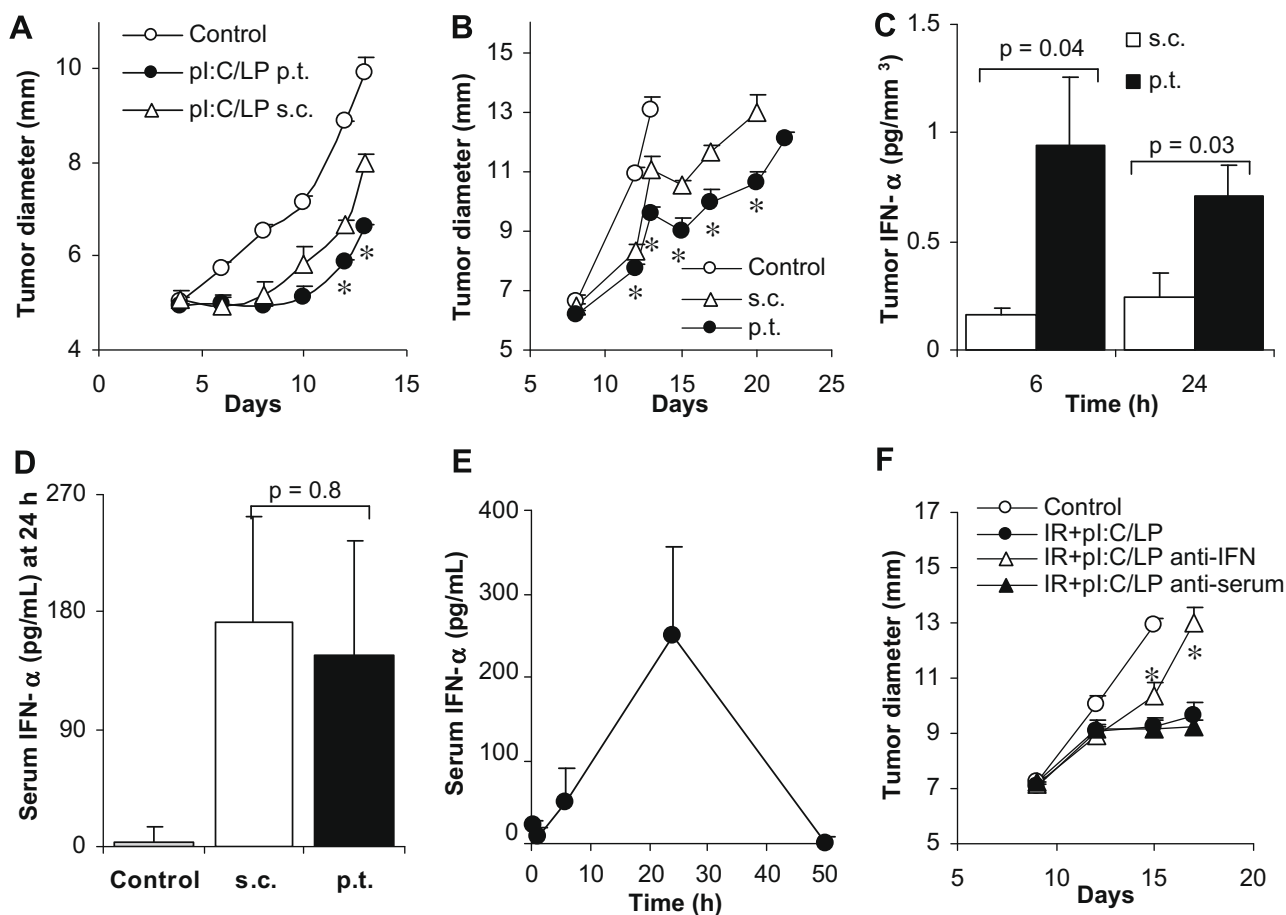


Fig. 3. The type I IFNs induced by the poly(I:C) contributed significantly to the anti-tumor activity generated by the combination therapy. (A) In poly(I:C) monotherapy, the pl:C/LP was more effective when injected peritumorally (p.t.) than when injected to a site distal to the tumors (s.c.). The poly(I:C) dose was 50 μ g, 3 times a week for one week. (*) p.t. vs. s.c. $p = 0.03$ on day 12, $p = 7.5 \times 10^{-5}$ on day 13. (B) In the combination therapy, the pl:C/LP injected close to the tumors was more effective in delaying the tumor growth. TC-1 tumors in C57BL/6 mice were irradiated (20 Gy) 9 days after tumor seeding. Starting on day 11, mice ($n = 6-7$) were injected with pl:C/LP (37.5 μ g of pl:C) peritumorally (p.t.) or distal to the tumors (s.c.) 3 times a week for 2 consecutive weeks. (○) Starting from day 12, the values of the p.t. and s.c. were different from each other ($p < 0.05$). (C and D) Peritumoral injection of the pl:C/LP induced a higher level of IFN- α in tumor tissues. TC-1 tumor-bearing mice were injected with pl:C/LP (37.5 μ g of pl:C) p.t. or in a site distal to the tumor (s.c.). The IFN- α level in the tumor (C) was measured 6 and 24 h later, and that in the blood (D) samples was measured 24 h later ($n = 3$). (E) Kinetics of IFN- α in the blood when tumor-free mice ($n = 3$) were injected once with the pl:C/LP (pl:C = 50 μ g) subcutaneously. (F) Neutralization of the type I IFNs decreased the anti-tumor activity of the combination therapy. TC-1 tumors were irradiated (15 Gy) 9 days after tumor seeding. Mice ($n = 5-6$) were injected (p.t.) with pl:C/LP (50 μ g) starting on day 11, three times a week for one week. One group of mice was injected i.p. with the anti-serum against IFN- α,β (anti-IFN), another group with control anti-serum (anti-serum). (○) The values in mice injected with the anti-type I IFN anti-serum were different from that in mice received the control anti-serum or received no anti-serum. Data reported are means \pm SEM.

about 20 days if left untreated [30]. The B16-F10 melanoma cells grew even more aggressively. When 5×10^5 of B16-F10 cells were injected into the C57BL/6 mice, mouse death was observed as early as 12 days later. When more than one round of the combination therapy is to be applied in the future, it is very likely that the tumor growth delay will be more extensive. A fractionated radiation regimen followed by poly(I:C) therapy is also expected to be more effective. In fact, tumor combination therapy using the poly(I:C) and localized radiation has been tested before [31–33], and the poly(I:C) was used as a radio-sensitizing agent and was given prior to the radiation. For example, it was shown that when Lewis lung carcinoma-bearing mice were dosed with poly(I:C) in the form of pl:C-poly-L-Lysine complexes 6 h before radiation (4 Gy), three times in 1.5 weeks, the combination treatment significantly delayed the tumor growth, although mechanistic studies were not reported [32]. However, the regimen of radiation followed by poly(I:C) therapy is likely preferred because a proper dose of localized radiation is expected to significantly decrease the tumor load so that the follow-up poly(I:C) therapy will become more effective. Moreover, there are data showing that ionizing radiation can improve the uptake of nucleic acids in liposomal carriers [34,35],

which was another reason to choose the schedule of radiation followed by the poly(I:C) therapy. Finally, our unpublished data suggested that radiation followed by the poly(I:C) therapy seemed to be more effective than when the radiation was administered after the completion of the poly(I:C) therapy.

One of the most important findings in the present study is that the poly(I:C) therapy apparently enhanced tumor response to radiation (Fig. 1C). In Fig. 1D, we compared the tumor growth curves when tumors were given different doses of X-ray alone and the curves when tumors were given different doses of X-ray but followed by the injections of pl:C/LP. A 20 Gy of X-ray alone generated roughly the same anti-tumor activity as 1 Gy of X-ray followed by 6 doses of pl:C/LP, suggesting a radiation dose modification factor of 20. Similarly, the anti-tumor activities by 40 Gy of X-ray alone and by 10 Gy of X-ray followed by pl:C/LP injections were comparable (dose modification factor of 4) (Fig. 1D). This finding has significant clinical implications because it indicated that a few doses of poly(I:C) in lipoplexes starting at a proper time after a radiotherapy are expected to help decrease the dose of the radiation.

Evidently the direct tumoricidal activity of the localized irradiation has contributed significantly to the anti-tumor activity from the combination therapy because a decreased dose of X-rays generated a decreased anti-tumor activity when the dose and dosing schedule of the poly(I:C) remained unchanged (Fig. 1C). Poly(I:C) is multi-functional, and we expected that multiple anti-tumor mechanisms would have contributed to the improvement of the anti-tumor activity. Poly(I:C) has direct tumor-killing activities, especially when delivered inside tumor cells [1,2,29]. Immunohistology data from our previous study showed that the peritumorally injected poly(I:C)-lipoplexes induced significant tumor cell apoptosis [19]. Thus, it is very likely that the direct pro-apoptotic activity of the poly(I:C) has contributed to some extent to the anti-tumor activity from the present combination therapy. The involvement of the poly(I:C) in the direct killing of the tumor cells was also partially supported by our data in Fig. 3A and B, which showed that the pl:C/LP was more effective in controlling the tumor growth when injected peritumorally than when injected in a site distal to the tumors. However, the stronger anti-tumor activity generated by the peritumorally injected poly(I:C) may be attributed to other reasons too. In the present study, we investigated primarily whether the T cell-mediated immunity and the type I IFNs induced by the poly(I:C) have contributed to the resultant anti-tumor activity.

Our data in Fig. 2A show that the combination therapy was still more effective than the monotherapies in delaying the growth of the TC-1 tumors in the athymic nude mice. This finding is interesting because data from recent studies indicated that functional T cells were indispensable in the anti-tumor activity generated by combining localized radiation with CpG oligos, because the combinational CpG-irradiation therapy was no more effective than the radiation alone in delaying the tumor growth in athymic nude mice or in whole-body irradiated mice [26,36,37]. CpG motifs are a ligand to TLR9 [38], and the poly(I:C) is a ligand to TLR3 [22]. Both CpG motifs and poly(I:C) are immunostimulatory. We have expected that the combinational poly(I:C)-radiation therapy would not be more effective than the radiation alone in the athymic nude mice. However, a more careful evaluation of the tumor growth revealed that while the combined effect from the localized irradiation followed by the poly(I:C) therapy was synergistic in the TC-1 tumors in immuno-competent C57BL/6 mice, it was only additive in the athymic nude mice with the same TC-1 tumors (Table 1). Therefore, it is likely that the T cell-mediated immunity contributed to the combined anti-tumor activity by rendering it synergistic. We have also shown that the combined effect from the localized irradiation and the poly(I:C) was only additive in the immuno-competent C57BL/6 mice when the highly immunogenic TC-1 tumors were replaced by the poorly immunogenic B16-F10 tumors (Fig. 2B and Table 1), again suggesting that the T cell-mediated immunity was responsible for the synergy observed in immuno-competent mice with highly immunogenic tumors. The fact that the radiation-pl:C/LP combination tended to be less effective in the B16-F10 in C57BL/6 mouse model than in the TC-1 in C57BL/6 mouse model also indicated the importance of T cell-mediated immunity. Recent data showed that TLR3 was expressed in the B16 cells, but treatment of the B16 cells with 'naked' poly(I:C) failed to generate a significant tumoricidal activity even *in vitro* [39], which agreed with previously published data [18]. However, it was reported that the poly(I:C) enhanced the tumor sensitivity to a protein synthesis inhibitor cycloheximide (CHX) *in vitro* [39]. It is unknown whether the poly(I:C) can enhance tumor sensitivity of radiation by interacting with TLR3. Finally, more experiments need to be carried out in the future to further identify the extent to which the T cells and natural killer (NK) cells have contributed to the combined anti-tumor activity. Poly(I:C) can activate NK cells, which are tumoricidal as well, and previous data documented the

activation of anti-tumor NK cells by poly(I:C) [40]. Nevertheless, this finding may also point out one of the advantages of using the poly(I:C) instead of the CpG oligos in the combination therapy. The TC-1 tumors in nude mice and the B16-F10 tumors in C57BL/6 mice resemble the real clinical situations. Many human tumors are poorly immunogenic or non-immunogenic, and the immune system of patients with advanced cancers is generally compromised.

The poly(I:C) is a potent inducer of type I IFNs, and the type I IFNs are pro-apoptotic, anti-proliferative, and anti-angiogenic [1,4–6]. Thus, it is very likely that the type I IFNs have contributed significantly to the anti-tumor activity of the combination therapy. For example, it was found that the concentration of the IFN- α in the tumor tissues was much higher when the pl:C/LP was injected locally than when injected into a site distal to the tumors (Fig. 3C). This finding may also explain the stronger anti-tumor activity observed when the pl:C/LP was peritumorally injected in the combination therapy than when it was injected into a site distal to the tumors (Fig. 3B), in agreement with a previous finding that the poly(I:C) was more efficacious when given locally [19,41,42]. However, more convincing evidence supporting that the type I IFNs contributed significantly to the combined anti-tumor activity is shown in Fig. 3F, in which the type I IFNs were neutralized in mice using anti-serum against IFN- α,β . The neutralization of the type I IFNs significantly compromised the anti-tumor activity of the combination therapy (Fig. 3F). The importance of type I IFNs in the anti-tumor activity of the poly(I:C) has been reported before. For example, Currie et al. (2008) showed that when AB1-HA tumor-bearing mice were treated with poly(I:C) in the presence of the polyclonal IFN- α,β -neutralizing anti-serum, the neutralization completely inhibited the poly(I:C)-driven tumor resolution [28]. However, there were cases where the anti-tumor activity of the poly(I:C) was found to be decoupled from its ability to induce the production of type I IFNs [11,13]. We suspect that how effectively the poly(I:C) is delivered into the tumor cells determines the extent to which the type I IFNs contribute to the resultant anti-tumor activity. When the poly(I:C) was injected distal to the tumors, its anti-tumor activity was thought to be related to its ability to induce type I IFNs. In contrast, consistent with our data in Fig. 3A and B, locally dosed poly(I:C) was shown to be more effective in suppressing tumor growth [17,41,42]. For example, it was reported that the growth of transplanted rat tumors was retarded when the cells were injected subcutaneously in admixture with poly(I:C), while systemic treatment of the same tumors with poly(I:C) failed to prevent the growth of a range of rat tumors [41]. The tumor regression observed by Shir et al. (2006) after the intratumoral injection of tumor cell-targeting poly(I:C) is another example [17]. Therefore, our future work should be directed towards improving the delivery of the poly(I:C) into tumors to further improve the combined anti-tumor activity. Our data in Fig. 3B show that subcutaneous injection of the poly(I:C) into a site distal to the tumors was less effective than when the poly(I:C) was given by peritumoral injection. Therefore, the peritumoral route was used in this study. Peritumoral or intratumoral injection is clinically feasible for some cancers, such as melanoma and certain head and neck cancers. It would be clinically difficult to inject other tumors peritumorally, however. We are currently investigating the feasibility of targeting the poly(I:C) into tumors after intravenous injections.

Finally, data from some previous studies indicated that the poly(I:C) caused dose-limiting adverse effects, especially to the liver [14–16], which significantly restricted the potential of poly(I:C) in cancer therapy. Our data suggested that the combination approach may help overcome or minimize this issue because the integration of the localized irradiation into the poly(I:C) therapy makes it possible to use less poly(I:C) and to dose it less frequently.

Moreover, our unpublished data also suggested that the potential liver toxicity issue of the poly(I:C) may be addressed using PEGylated poly(I:C)-liposome lipoplexes.

In conclusion, we reported that localized irradiation of tumors prior to synthetic dsRNA therapy significantly enhanced the resultant anti-tumor activity and that the poly(I:C) therapy enhanced tumor response to radiation. The combination therapy was effective regardless of the immunogenicity of the tumors or the immuno-competency of the host, although a synergistic effect was observed only in immuno-competent mice with highly immunogenic tumors. Finally, the type I IFNs induced by the dsRNA contributed significantly to the anti-tumor activity from the combination therapy. Improving the targeting of the synthetic dsRNA into tumor cells is expected to further improve the efficacy of this novel tumor therapeutic modality.

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