TOLL-LIKE RECEPTOR AGONISTS AS THIRD SIGNALS FOR DENDRITIC CELL–TUMOR FUSION VACCINES

Edward I. Cho, MD, Chunrui Tan, BS, Gary K. Koski, PhD, Peter A. Cohen, MD, Suyu Shu, PhD, Walter T. Lee, MD

1 Head and Neck Institute, Cleveland Clinic, Cleveland, Ohio
2 Division of Otolaryngology–Head and Neck Surgery, Duke University Medical Center, Durham, North Carolina
3 Center for Surgery Research, Cleveland Clinic, Cleveland, Ohio
4 Hematology/Oncology, Mayo Clinic, Scottsdale, Arizona
5 Laboratory of Molecular and Tumor Immunology, Robert W. Franz Cancer Research Center, Earle A. Chiles Research Institute, Portland, Oregon
6 Division of Otolaryngology–Head and Neck Surgery, Duke University Medical Center, Durham, North Carolina. E-mail: walter.lee@duke.edu

Abstract: The aim of the present study was to evaluate the therapeutic efficacy of dendritic cell (DC)–tumor fusion hybrids with Toll-like receptor (TLR) agonists.

Methods: DC–tumor fusion hybrids were generated by electrofusion and injected into the inguinal lymph nodes of C57BL/6 mice with 3-day established pulmonary metastases. Paired TLR agonists polyinosine:polycytadilic acid [poly(I:C)] and cytosine–phosphate–guanine (CpG) were then injected intraperitoneally. Enzyme-linked immunosorbent assay (ELISA) was used to evaluate interleukin (IL)-12 production from the DC–tumor fusion hybrids in vitro.

Results: Fusion + TLR agonists (60 metastases) had significantly fewer metastases than did the untreated control (262 metastases, \( p = .0001 \)) and fusion alone (150 metastases, \( p = .02 \)). ELISA showed that the DC–tumor fusion hybrids yielded 90 pg of IL-12 after TLR stimulation compared with 1610 pg from dendritic cells alone.

Conclusions: CpG and poly(I:C) administered as a third signal with fusion hybrids as described significantly reduce melanoma metastasis compared with fusion hybrids alone. Fusion hybrids do not appear to be a significant source for IL-12 secretion.

Keywords: tumor immunology; dendritic cells; Toll-like receptor agonists; electrofusion; third signal

Despite advances in chemotherapy, radiation, and surgical therapies, treatment of advanced head and neck melanoma continues to be associated with high morbidity and mortality. As a result, other modalities of treatment have been
investigated. One particular area is that of immunotherapy, which seeks to use the immune system to recognize and eliminate cancer cells. Dendritic cells (DCs) play a central role in adaptive immunity because they are antigen-presenting cells involved in the initiation and modulation of the immune response. DC antigen presentation occurs when antigens are processed and presented on major histocompatibility complex (MHC) I or MHC II molecules. The MHC I molecule presentation is of particular interest because it activates cytotoxic CD8 T cells that can directly kill tumor cells. 1 There are currently a number of efforts centered on using DCs in immunotherapy, including a strategy that uses the DCs’ specialized antigen-presenting properties through DC–tumor fusion hybrids. Electrofusion is the process by which hybrids can be formed. By using a low-intensity alternating current (ac) followed by a short direct current (dc), cell membranes of tumors and DCs can coalesce to form a hybrid cell. This hybrid has the full complement of tumor antigens to present on the cell surface for immunologic activation of T cells. 2,3 In murine models, the DC–tumor fusion hybrids have been found to significantly reduce 3-day established pulmonary metastases of B16 melanoma, 4TI breast cancer, and MCA205 sarcoma. 2–4 However, these studies have also shown that DCs alone and a sham group (composed of a 50:50 mix of tumor and DC without electrofusion) do not have immunogenic activity or reduce metastases. 2,13 The mechanism of this antitumor effect is through a strong T-cell immune response by way of 3 signals. Signals 1 and 2 occur when antigen is presented by the MHC molecules of the DC to the T cell (signal 1) together with costimulatory molecules such as CD80, CD86, and CD40 (signal 2). Signal 3 is an additional inflammation signal that is a required immunological adjuvant for T-cell activation because of the intrinsically low immunogenicity of tumor-associated antigens. 5 A strong signal 3 adjuvant is interleukin (IL)-12, which polarizes T cells to the Th1 phenotype and allows for cytotoxic CD8 T-cell generation, function, and survival. The successful murine antitumor effects of the aforementioned DC–tumor fusion hybrids have required administration of intraperitoneal IL-12. However, because of the clinical toxicity of IL-12, research is needed to identify alternative third signals that are safe and available for clinical use. One potential class of agents consists of Toll-like receptor (TLR) agonists.

TLRs are “pattern recognition receptors” that recognize common molecular patterns on pathogens. When recognition occurs, TLR signaling then regulates the 3 signals necessary for a strong T-cell response. TLR agonists include lipopolysaccharide (LPS, TLR4), an endotoxin from the cell membrane of Gram-negative bacteria, cytosine–phosphate–guanine (CpG) DNA (CpG, TLR9) found mostly on bacterial and viral DNA, and polyinosine:polycytidylic acid [poly(I:C), TLR3], which is a double-stranded RNA found exclusively in viruses. 6 These TLR agonists have all been implicated in increasing antigen loading on MHC molecules (signal 1) and allowing for the upregulation of costimulatory molecules (signal 2). 7 Furthermore, these TLR agonists are also responsible for the induction of IL-12 p70, which is the bioactive form of IL-12. Research has found that CpG is a particularly powerful stimulus for IL-12 p70 in TLR9-expressing DC, 8 and that poly(I:C) allows for production of IL-12 in some human DCs. 9 Furthermore, recent studies have shown that stimulating DCs with select pairs of TLR agonists greatly enhances IL-12 p70 production. In particular, TLR agonists that coordinate stimulate the MyD88- (TLR9) and Toll/IL-1 receptor domain-containing adaptor-inducing interferon-beta (IFN-β) (TRIF, TLR3, and TLR4) pathways appear to have this effect on DCs. 10 Another benefit of TLR agonists is that some are relatively safe and nontoxic for clinical use. For example, imiquomod is a TLR agonist cream, available clinically and indicated for treating genital warts and basal cell carcinomas. 11 Therefore, the purpose of this study was to evaluate the efficacy of TLR agonists as DC–tumor fusion vaccine third signals on established pulmonary metastases in mice. Given prior numerous studies published with DC–tumor fusion hybrids and other third signals, we chose to focus our experimental design on the use of TLR agonists as a third signal. We hypothesized that intraperitoneal TLR agonists would be an effective third signal when administered with DC–tumor fusion hybrids. Second, we hypothesized that the mechanism of action of TLR agonists on DC–tumor fusion hybrids was through induced secretion of IL-12 p70, which we evaluated through an in vitro model.
MATERIALS AND METHODS

This study was approved by the Cleveland Clinic’s Institutional Animal Care and Use Committee. All animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Animals. Female C57BL/6 mice were purchased from the Biologic Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute (Frederick, MD). The animals were housed in a specific pathogen-free environment and used for experiments at the age of 8 to 12 weeks.

Tumor. D5LacZ melanoma is a clone of the B16 subline and syngeneic to the C57BL/6 mice. It was virally transduced with the LacZ gene, which codes for the antigenic protein, β-galactosidase. D5LacZ tumor cells were maintained in complete medium (CM) consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 μg/mL streptomycin, 100 μg/mL penicillin, 0.5 μg/mL fungizone, 50 μg/mL gentamycin (all from BioWhittaker, Walkersville, MD), and 5 × 10⁻⁵ M 2-mercaptoethanol (Sigma, St. Louis, MO). All cultured cells were maintained in a 37°C, 5% CO₂ incubator and harvested after a short incubation with a solution containing 0.25% trypsin (BioWhittaker).

Dendritic Cell Generation. Bone marrow DCs were harvested from the tibiae and femurs of C57BL/6 mice and depleted of B and T cells by negative selection using monoclonal antibody (mAb)-coated magnetic beads (Dynal Biotech, Carlsbad, CA). The cells were cultured in flasks at a concentration of 0.5 × 10⁶ cells/mL in CM supplemented with 10 ng/mL of granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL of IL-4 (Peprotech, Rocky Hill, NJ). On day 6, nonadherent cells were harvested and further cultured at a density of 1.0 × 10⁶ cells/mL with CM + GM-CSF + IL-4 for an additional 2 days.

Electrofusion. Cell fusion of DCs with irradiated (10,000 cGy) tumor cells was done using the electrofusion protocol developed in our lab.² Briefly, fusion was carried out in a circular (concentric) fusion chamber containing 15 × 10⁶ cells/cm² (DC:tumor cell ratio: 1 to 1). It was accomplished by 2 consecutive reactions. The first reaction uses an ac of 120 V/cm for 10 seconds to allow for a separation of charges and an oscillating dipole that allows for cells to be attracted to each other and form a chain-like arrangement. The second reaction is reversible membrane breakdown, which uses a dc pulse of 1100 V/cm for 25 microseconds and causes a temporary breakdown of the bilipid cell membrane. The cell suspension then underwent a tapering of the same ac current over 9 seconds to allow for spontaneous membrane rescaling and formation of chimeric hybrids. The cells were then cultured overnight, and the adherent cells were harvested after a brief exposure to trypsin and suspended in Hank’s balanced salt solution (HBSS) for in vivo use (Invitrogen, Carlsbad, CA). For analysis of successful electrofusion yield, tumor cells were stained green with intracellular carboxyfluorescein diacetate succinimidyl ester (CFSE) before fusion (Molecular Probes, Eugene, OR). Once the fusion was complete, adherent hybrids were labeled with red phycoerythrin (PE)-conjugated mAbs to cell markers found on DCs (CD80, CD86, ICAM-1, IAβ). Fluorescence-activated cell sorting (FACS) analysis was done, and cells that were successfully fused demonstrated both green and red fluorescence. After overnight culture, hybrids containing tumor cells (tumor–tumor and tumor–DC) and unfused tumor cells were mostly adherent to the flask, whereas unfused DC and hybrids not containing tumor (DC–DC) were substantially nonadherent.

Active Immunotherapy. Active immunotherapy using the DC–tumor fusion hybrids was used to treat 3-day D5LacZ pulmonary metastases. Pulmonary metastases were initiated by intravenous injections of 3 × 10⁵ tumor cells suspended in 1.0 mL of HBSS. Three days after tumor inoculation, fusion hybrids were delivered intranodally. This involved performing inguinal incisions in anesthetized mice to expose the superficial inguinal lymph nodes bilaterally. Unfractionated fusion hybrids were then injected intranodally on each side in 10 μL, and the incisions were closed using hemoclips (Weck Closure Systems, Research Triangle Park, NC). For the experimental groups, paired TLR agonists poly(I:C) (100 μg/0.5 mL) and CpG (50 μg/0.5 mL) were injected intraperitoneally on the
day of vaccination and days 3 and 7 postvaccination (Invivogen, San Diego, CA). The decision to use paired agents was based on a demonstration of powerful synergy in the MCA205 mouse sarcoma tumor.12 For the positive control group, IL-12 (0.2 μg/0.5 mL) was administered intraperitoneally on the day of vaccination and the 3 days following (Wyeth, Cambridge, MA). On days 21 to 23, mice were sacrificed and lung metastases were enumerated. Lungs were preserved in paraformaldehyde and surface metastases were counted. Furthermore, during the necropsy, other organs such as the spleen and liver were visualized to rule out metastases in other organs, of which there was none. There were 5 groups used in this study including control (no treatment), TLR agonists alone, fusion vaccine alone, fusion vaccine + TLR agonists, and fusion vaccine + IL-12. Previously published studies demonstrated that the use of IL-12 alone did not significantly alter tumor burdens.2,4 Also, previous studies have demonstrated that DC alone and a sham group (composed of a 50:50 mix of tumor and DC without electrofusion) did not have immunogenic activity or reduce metastases and were therefore not included as control groups.2,13

In collaboration with the Department of Biostatistics and Epidemiology, it was determined that 5 mice in each group were necessary to achieve sufficient power for analysis, with 1 overlap in the number of pulmonary metastases. It was also determined that 4 animals could be used in each group if there was no overlap in the number of pulmonary metastases. There were 11 mice in the control, TLR agonists alone, and fusion vaccine + TLR agonists groups; 10 mice in the fusion vaccine + IL-12 group; and 9 mice in the fusion vaccine alone group. The differences in numbers between groups reflect extra mice that were used because of mouse deaths that sometimes occurred immediately after surgery for intranodal injection (1 mouse died immediately after surgery for the fusion vaccine + IL-12 group and fusion vaccine alone group). One outlier was removed from the fusion vaccine alone group because it was 1 standard deviation below the median number of lung metastases. Outliers exist because we are using a biological model that cannot be fully controlled and can have rare experimental variance, and because several steps in the protocol can be operator dependent (ie, inadequate tail vein injection, morbidity from surgery). The mice used in the different groups represent data pooled from 2 separate experiments.

**Cytokine Analysis.** Analysis was done with the following cell groups: DC alone, tumor alone, sham group (composed of a 50:50 mix of tumor and DC without electrofusion), adherent fusion cell population, and nonadherent fusion cell population. The adherent fusion cell population included mostly hybrids containing tumor cells (tumor–tumor and tumor–DC) and unfused tumor cells. The nonadherent fusion cell population contained mostly hybrids without tumor (DC–DC) and unfused DC. All these cells were harvested and then counted and cultured overnight in 48-well plates containing CM (1 × 10⁶ cells/mL/well) and GM-CSF + IL-4. One day later, 1 group of cells were treated with paired stimulating agents administered 2 hours apart, whereas the other group was not. These agents included poly(I:C) (TLR3 agonist, 50 μg/mL), *E. coli* LPS (TLR4 agonist, 50 ng/mL), and ODN 1826 (synthetic CpG DNA, TLR9 agonist, 1 μM) (Invivogen). The decision to use these TLR agonists was based on preliminary results from our lab group (unpublished observations) that showed high levels of IL-12 p70 secretion from cultured DCs alone. Cells were incubated for 18 additional hours, at which time culture supernatants were harvested and analyzed by ELISA for murine IL-12 p70 heterodimer using OPT-EIA kits (BD/PharMingen, San Jose, CA). ELISA testing was performed in duplicate.

**Statistical Analysis.** Differences in the number of pulmonary metastases between groups were evaluated with the nonparametric, Wilcoxon rank-sum test (VassarStats, Poughkeepsie, NY). Median values of pulmonary metastases were also calculated. A value of *p* ≤ .05 was considered significant.

**RESULTS**

**Electrofusion of Tumor Dendritic Cells.** Successful electrofusion between D5LacZ melanoma cells and dendritic cells was shown through Giemsa-stained cytospin slides (Figure 1A) and through FACS analysis (Figure 1B). Cytospin showed multinucleated cells. FACS analysis routinely showed fusion yields ranging from 15% to 30%. Fusion hybrids appeared in the upper right
quadrant, indicating double-positive CFSE and CD80 staining. CD80 staining was accomplished through a PE-conjugated murine antibody specific for this DC surface marker, whereas CFSE staining was done through intracellular staining on the tumor cells before the fusion process.

**Treatment of 3-Day Pulmonary Metastases.** Three-day pulmonary metastases were established by tail vein injection, and the metastases were enumerated approximately 3 weeks later. The positive control, fusion vaccine + IL-12 (median of 10 metastases), had significantly fewer metastases than did the untreated negative control (262 metastases, \( p = .0001 \)) and fusion vaccine alone (150 metastases, \( p = .004 \)). Fusion vaccine + TLR agonists (60 metastases) had significantly fewer metastases than did the untreated control (262 metastases, \( p = .0001 \)), fusion alone (150 metastases, \( p = .02 \)), and TLR alone (128 metastases, \( p = .01 \)). No difference was seen between the fusion vaccine + TLR and fusion vaccine + IL-12 groups (60 vs 10 metastases, \( p = .15 \)). TLR alone (128 metastases, \( p = .01 \)) and fusion vaccine alone (150 metastases, \( p = .04 \)) were both significantly different from untreated control. These data are illustrated in Figure 2.

**In Vitro Analysis of IL-12 p70 Production in Fusion Hybrids.** After electrofusion, the adherent population, the nonadherent population, DC alone, tumor alone, and sham mix (tumor and DC mixed 50:50 without electrofusion) were either treated with paired TLR agonists or not at all. Hybrids containing tumor cells (tumor–tumor and tumor–DC) and unfused tumor cells were mostly adherent to the flask, whereas unfused quadrant, indicating double-positive CFSE and CD80 staining. CD80 staining was accomplished through a PE-conjugated murine antibody specific for this DC surface marker, whereas CFSE staining was done through intracellular staining on the tumor cells before the fusion process.

To summarize, the study demonstrates the efficacy of fusion vaccines in reducing pulmonary metastases, particularly when combined with TLR agonists. The data support the potential of this approach in cancer immunotherapy.
DC and hybrids not containing tumor (DC–DC) were mostly nonadherent. The adherent population contained 15% DC–tumor hybrids, whereas the nonadherent population contained 66% of DC alone or DC–DC fusion cells. Culture supernatants were taken 18 hours later and assayed for IL-12 p70 by ELISA. When TLR agonists were not added, IL-12 p70 was not detected. When the TLR agonists LPS (10 ng/mL) and CpG (3.3 μg/mL) were added, the adherent fusion hybrid population had 90 pg of IL-12 p70 production compared with 1610 pg in the DCs alone group. LPS and CpG were used as a positive control based on preliminary results from our lab group (unpublished observations) that showed high levels of IL-12 p70 secretion from cultured DCs alone. Tumor alone had 0 pg, the sham mix had 677 pg, and the nonadherent population had 12,296 pg of IL-12 p70 production. The results with paired poly(I:C) and CpG were similar, with 111 pg of IL-12 p70 produced in the adherent population and 10,278 pg in the nonadherent population (see Figure 3).

**FIGURE 3.** ELISA analysis for IL-12 p70 demonstrates minimal IL-12 production in the adherent population with fusion hybrids when treated with either LPS and CpG or poly(I:C) and CpG. The cells were plated at a concentration of 1 million cells per well. In this figure, “DC Alone” indicates dendritic cells alone, “Tumor Alone” indicates D5LacZ tumor cells alone, and “Sham Mix” indicates a 50:50 mix of DC and tumor cells. These first 3 groups of cells did not undergo fusion but were treated with paired TLR agonists LPS and CpG. “Nonadherent” indicates the fusion cell population that included mostly hybrids without tumor (DC–DC) and unfused DC. “Adherent” indicates the fusion cell population that included mostly hybrids containing tumor cells (tumor–tumor and tumor–DC) and unfused tumor cells. Note that these last 5 groups of cells were treated with either LPS and CpG, poly(I:C) and CpG, or no TLR agonists at all and are labeled accordingly. ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; poly(I:C), polyinosine:polycytadilic acid; CpG, cytosine (C) and guanosine (G) nucleosides separated by a phosphate (p).

**DISCUSSION**

Tumor immunotherapy promises to be a useful adjunct to existing therapies for head and neck melanoma. DC–tumor fusion has proven to be an efficient means to allow for the presentation of tumor-associated antigens. These fusion hybrids are efficiently formed through the process of electrofusion, given that our data demonstrate fusion yields of 15% to 30%, verified through FACS analysis and Giemsa staining. Fusion hybrids alone had an antitumor effect in our study that was statistically different from that of control (150 vs 262 metastases, $p = .04$). However, to induce a more potent antitumor effect, the treatment required a third signal, such as intraperitoneal IL-12. Once IL-12 was given, the tumor vaccine was able to more fully reduce 3-day established melanoma pulmonary metastases with a median of 10 metastases. Previously published studies demonstrated that the use of IL-12 alone did not significantly alter tumor burdens.2,4 However, because of the toxicity of IL-12 in clinical trials, other agents were investigated in this study as an alternative third signal. As a result of their ability to regulate signals 1, 2, and 3 of immune activation, our hypothesis was that TLR agonists would be an effective third signal when administered with DC–tumor fusion hybrids. In particular, the TLR agonists poly(I:C) and CpG have been found to result in IL-12 secretion from DCs.8,9 When paired TLR agonists poly(I:C) (100 μg/0.5 mL) and CpG (50 μg/0.5 mL) were injected intraperitoneally in fusion hybrid-treated mice, there was a significant difference in the number of pulmonary metastases (60 metastases) compared with fusion vaccination alone (150 metastases, $p = .02$) and TLR agonists alone (128 metastases, $p = .01$). This effect was not statistically different from the historical positive control, the fusion vaccine + IL-12 group (10 metastases, $p = .15$). To further investigate the mechanism of TLR agonists, our in vitro analysis assessed the production of IL-12 p70 in fusion hybrids when stimulated with paired TLR agonists. Our ELISA analysis showed that fusion hybrids alone combined with TLR agonists LPS and CpG produced decreased amounts IL-12 p70 (90 pg) compared with DCs alone with TLR agonists (1610 pg). The nonadherent cell population, composed of mostly unfused DCs, seemed to produce an even higher amount of IL-12 p70 (12,300 pg) compared with...
that of DCs alone (1610 pg). The ELISA results with poly(I:C) and CpG were similar. These data did not support our secondary hypothesis that the mechanism of action of TLR agonists on DC–tumor fusion hybrids was through induced secretion of IL-12 p70.

A publication by Zheng et al12 showed results similar to those of our study with near eradication of established pulmonary metastases using fusion vaccine and paired TLR agonists poly(I:C) and CpG in the mouse sarcoma line, MCA205. This study also used electrofusion to create DC–tumor fusion hybrids and had the same dosages and timings of the TLR agonists. Our in vivo results are consistent with those of the article by Zheng et al12 and further show the effectiveness of TLR agonists as a third signal in a different tumor cell line.

Furthermore, they proposed that TLR agonists exerted their effect through IL-12 secretion, and they tested this hypothesis in 2 ways. First, they tested for quantifiable changes in T-cell activity consistent with known effects of IL-12 in an in vitro model. They cocultured prepared lymph node cells treated with fusion vaccine and TLR agonists together with tumor cells and measured IFN-γ production. The high IFN-γ production indicated that these T cells were polarized toward a Th1 phenotype by IL-12. Second, they indirectly tested for the necessity of IL-12 by in vivo inhibition with an IL-12 neutralizing antibody. Combining this IL-12 neutralizing antibody with fusion vaccine and intraperitoneal TLR agonists resulted in an inhibition of the therapeutic effect of paired TLR agonists. These 2 results strongly suggest that the third-signal properties of TLR agonists are through IL-12 secretion, although there was no specification from their research whether this IL-12 comes from fusion hybrids or from endogenous immune cells.

An article by Pilon-Thomas et al14 suggests that IL-12 comes from bystander endogenous DCs. They also used the B16 melanoma line in C57BL/6 mice. However, rather than fusion, they used lysate-pulsed DCs and only a single TLR agonist, CpG. The CpG was initially used during the DC culturing process to allow for DC maturation. These CpG-matured DCs were proven to be effective antigen-presenting cells when pulsed with B16 lysate. This was tested by measuring IFN-γ production after coculture of these DCs with splenocytes from mice that had been vaccinated with lysate-pulsed DCs and rejected B16 tumor. The high IFN-γ production that resulted indicated that these CpG-matured DCs were able to stimulate CD8 T cells via IL-12. However, when these CpG-matured, lysate-pulsed DCs were used in an in vivo treatment model with established subcutaneous melanoma, no therapeutic benefit was seen. Instead, it was found that only CpG coadministered intraperitoneally with lysate-pulsed DCs was effective in causing a delay in tumor growth, leading the investigators to conclude their results may be secondary to CpG action on other host immune cells such as natural killer (NK) cells, B cells, monocytes, and endogenous DCs.

The aforementioned article by Zheng et al12 shows that TLR agonists exert their effect through IL-12 secretion. However, the actual source of IL-12 was not investigated. Our data suggest that DC–tumor fusion hybrids are not the source of IL-12 from TLR agonist stimulation; therefore, we suggest that the IL-12 may be coming from endogenous bystander immune cells. Further research is ongoing to determine how IL-12 is being secreted and which cells are responsible for it, although such experiments are beyond the scope of this present work and hypotheses.

Overall, there is a need for novel treatments for head and neck melanoma to improve rates of morbidity and mortality. TLR agonists may play a role in vaccine development because of their multiple effects on immune activity. In our model of melanoma pulmonary metastases, TLR agonists administered as a third signal with fusion hybrids significantly reduced melanoma pulmonary metastasis compared with fusion hybrids and TLR agonists alone. TLR agonists do not appear to induce IL-12 secretion from DC–tumor fusion hybrids. Further testing needs to be done to elicit the mechanism by which TLR agonists and fusion hybrids exert their therapeutic effect.

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