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The TLR-7 Agonist, Imiquimod, Enhances Dendritic Cell Survival and Promotes Tumor Antigen-Specific T Cell Priming: Relation to Central Nervous System Antitumor Immunity

Robert M. Prins,2*† Noah Craft,¶ Kevin W. Bruhn,‡ Haumith Khan-Farooqui,* Richard C. Koya,‡ Renata Stripecke,‡ Jeff F. Miller,¶ and Linda M. Liau*§

Immunotherapy represents an appealing option to specifically target CNS tumors using the immune system. In this report, we tested whether adjunctive treatment with the TLR-7 agonist imiquimod could augment antitumor immune responsiveness in CNS tumor-bearing mice treated with human gp100 + tyrosine-related protein-2 melanoma-associated Ag-pulsed dendritic cell (DC) vaccination. Treatment of mice with 5% imiquimod resulted in synergistic reduction in CNS tumor growth compared with melanoma-associated Ag-pulsed DC vaccination alone. Continuous imiquimod administration in CNS tumor-bearing mice, however, was associated with the appearance of robust innate immune cell infiltration and hemorrhage into the brain and the tumor. To understand the immunological mechanisms by which imiquimod augmented antitumor immunity, we tested whether imiquimod treatment enhanced DC function or the priming of tumor-specific CD8+ T cells in vivo. With bioluminescent, in vivo imaging, we determined that imiquimod dramatically enhanced both the persistence and trafficking of DCs into the draining lymph nodes after vaccination. We additionally demonstrated that imiquimod administration significantly increased the accumulation of tumor-specific CD8+ T cells in the spleen and draining lymph nodes after DC vaccination. The results suggest that imiquimod positively influences DC trafficking and the priming of tumor-specific CD8+ T cells. However, inflammatory responses induced in the brain by TLR signaling must also take into account the local microenvironment in the context of antitumor immunity to induce clinical benefit. Nevertheless, immunotherapeutic targeting of malignant CNS tumors may be enhanced by the administration of the innate immune response modifier imiquimod.


Malignant tumors developing within the confines of the immune-privileged CNS present clinicians with few treatment options. Malignant melanoma is the third most common type of cancer that metastasizes to the brain (1), and expresses many well-characterized tumor-associated Ags (TAA).3 Patients with CNS melanoma metastases have not been recommended for immune-based therapies because these adjuvant treatments have historically failed to prevent relapses in the CNS clinically (1, 2). There is also a perceived potential for inducing CNS toxicity and experimental autoimmune encephalomyelitis (3). Together, these issues have limited enthusiasm for studies on CNS tumors. However, recent work has demonstrated that targeted therapies can induce antitumor immunity to tumors growing within the CNS in preclinical models and patients (reviewed in Ref. 4). These findings suggest that further investigation into TAA-specific immunotherapy may lead to more effective targeted treatments for CNS tumors.

An emerging strategy in the treatment of brain tumors involves the stimulation of an antitumor immune response (4–8). Immunotherapy is theoretically appealing because it offers the potential for a high degree of tumor specificity, while sparing normal brain structures (4, 7). Several different laboratories have demonstrated that effective immune responses within the CNS can be generated through the use of gene-modified tumor cell vaccines (9–16), the adoptive transfer of immune T cells (6, 17–22), or the use of dendritic cell (DC)-based vaccines (5, 23–31). These results imply that

3 Abbreviations used in this paper: TAA, tumor-associated Ag; DC, dendritic cell; PRR, pattern recognition receptor; MAA, melanoma-associated Ag; BM, bone marrow; CM, complete medium; h, human; m, murine; IRES, internal ribosomal entry site; eGFP, enhanced GFP; NP, nuclear protein; i.c., intracranial(iy); CMFDA, 5-chloromethylfluorescein diacetate; ROI, region of interest; BLI, bioluminescent imaging.
systemic immunity can enter the “immunologically privileged” CNS, selectively identify TAAs, and destroy brain tumor cells.

The innate immune system plays an important role in the body’s ability to mount adaptive immune responses (32). Innate immune system cells recognize pathogen-associated molecular patterns via a class of recently identified pattern-recognition receptors (PRRs) (32–34). TLRs comprise the main class of cell surface PRRs that are expressed on macrophages and DC subsets (33, 35). The intrinsic recognition of TLR ligands induces the maturation of APCs. TLR-7 is a recently identified TLR that is now known to recognize single-stranded RNA that is characteristic of common viruses such as HIV and influenza (36, 37). TLR-7 is expressed by plasmacytoid and myeloid DC, and these cells are able to secrete Th1-type cytokines in response to TLR-7 stimulation (38).

Imiquimod is an immunomodulatory, small-molecule compound in the imidazoquinoline family that displays both antiviral and antitumor effects (37, 39–42). Imiquimod was recently shown to exert its effects through TLR-7 (43). Topical treatment with imiquimod induces a variety of proinflammatory cytokines, such as IFN-α, TNF-α, and IL-12, from DC subsets (38, 39, 44). Imiquimod administration also facilitates the maturation of DC (45, 46) and can influence the priming of CD8+ T cells specific for completely foreign Ags (46, 47). This study was aimed at understanding the interaction between TLR stimulation and melanoma-associated Ag (MAA) peptide-pulsed DC vaccination for CNS tumor immunotherapy.

In this study, we demonstrated that imiquimod could augment the antitumor immune responses induced by MAA peptide-pulsed DC immunotherapy. These effects were associated with robust inflammatory responses occurring in and around tumors located within the CNS. Imiquimod administration significantly enhanced the survival and trafficking of s.c.-injected DC, as well as the priming of antitumor Ag-specific CD8+ T cells. We believe TLR agonists, such as imiquimod, may serve as potent adjuvants to the traditional DC-based immunotherapies.

Materials and Methods

Animals and cell lines

Female C57BL/6 mice were obtained from The Jackson Laboratory. Pmel-1 TCR mice were obtained from Dr. N. Restifo (National Cancer Institute/National Institutes of Health, Bethesda, MD) and bred at the University of California, Los Angeles (UCLA). The B16 murine melanoma cell line was obtained from the American Type Culture Collection and maintained in DMEM with 10% FCS, penicillin/streptomycin, and L-glutamine. B16 cells stably expressing firefly luciferase (B16-Fluc) were created as described elsewhere (48). Growth rates of B16-Fluc both in vitro and in vivo were similar to those of parental B16 cells.

Bone marrow (BM)-derived DC and peptide pulsing

The development of DC from murine BM progenitor cells was performed as previously published (24, 28, 49). BM cells were cultured overnight in DMEM, harvested, washed three times, and resuspended in PBS. For the lentiviral-mediated gene transfer, a class of recently identified pattern-recognition receptors (PRRs) are expressed on macrophages and DC subsets (33, 35). The intrinsic recognition of TLR ligands induces the maturation of APCs. TLR-7 is a recently identified TLR that is now known to recognize single-stranded RNA that is characteristic of common viruses such as HIV and influenza (36, 37). TLR-7 is expressed by plasmacytoid and myeloid DC, and these cells are able to secrete Th1-type cytokines in response to TLR-7 stimulation (38).

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Lentiviral-mediated gene transfer

Mouse DCs (5 × 106 cells) were produced from BM precursors in the presence of recombinant murine GM-CSF and mIL-4 for 7 days. DC were transduced with 5 μg of p24 equivalent/ml of the lentiviral vector pCMV-DVIRE-Luciferase-internal ribosome entry site (IRES)-enhanced GFP (mGFP), in a 6-well cluster plate. High-titer batches of lentiviral vectors were produced as previously described (50). This lentiviral construct was provided by Dr. J. Burton (UCLA). Protamine sulfate was added at a final concentration of 5 μg/ml and the transduction plates were incubated at 37°C, in 5% CO2 for 12–16 h. Cells were extensively washed with RPMI 1640 medium, counted, and resuspended in PBS for injection into mice. A total of 1 × 106 lentiviral-transduced DC were injected into each mouse s.c. for the imaging studies reported.

Basic experimental paradigm for DC vaccination and 5% imiquimod administration

Groups of mice were given two biweekly s.c. injections of either control NP or hgp10025–33 peptide-pulsed DC. The mice were then challenged with 1 × 106 B16-Fluc cells intracranially (i.c.) in the brain 1 wk after the second DC vaccination. Imiquimod (3M) was applied daily as a 5% cream to shaved skin at the flank before and after DC vaccination. Control mice were treated with vehicle control (3M). In selected groups, 5% imiquimod cream (provided by 3M) was administered to the s.c. DC vaccination site the day before, the day of, and the day after each DC vaccination. These mice then received 5% imiquimod every 3 days after tumor implantation until they developed tumor-associated symptoms and were euthanized. Control groups received a placebo cream (provided by 3M).

Pmel-1 adoptive transfer studies

For the adoptive transfer of Pmel-1 TCR transgenic T cells, splenocytes were aseptically obtained from Pmel-1 mice. The splenocytes were labeled with 5 μM 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes). A total of 5 × 106 cells were subsequently injected i.v. via the lateral tail vein into two groups of C57BL/6 mice. Both groups were immediately vaccinated with 1 × 106 hgp10025–33 peptide-pulsed DC s.c. Group 1 received 1 × 106 lentiviral-transduced DC s.c. or a placebo cream (3M) at the s.c. DC vaccination site every other day for a week. Seven days after the adoptive transfer and DC vaccination, the splenocytes and draining lymph node cells from both groups of mice were stained and analyzed by FACs.

Tetramer staining and FACS analysis

Spleens and lymph nodes were harvested from immunized mice and a single cell suspension was prepared in PBS by filtering through a mesh cell strainer. RBC were lysed with 1× PharmLyse (BD Pharmingen), and cells were washed, resuspended in Dulbecco’s PBS, and counted. A total of 1 × 106 splenocytes were then labeled with mAbs to CD11cPE (BD Pharmingen), CD80APC (Caltag Laboratories), TCRVβ13FITC, and/or CD44FITC and/or CD62LPE (all from BD Pharmingen) and multimeric, allophycocyanin-conjugated H-2Dp-gp100 or H-2Kk-TRP-2 (Coulter Immunomics). Cells were labeled for 30 min at room temperature, in the dark, followed by 15 min on ice. The cells were then washed twice, fixed, and analyzed. Stained cells were collected and analyzed on a FACScanLibur machine, using CellQuest software, and percentages of gated CD8+ lymphocytes that were tetramer+ were reported.

Tumor challenge and in vivo fluorescence imaging

Before tumor challenge, B16-Fluc cells were grown in supplemented DMEM, harvested, washed three times, and resuspended in PBS. For the i.c. implantation of B16-Fluc melanoma cells, animals were first anesthetized with ketamine/xylazine. The head was shaved and the skull exposed. Thereafter, the animal was positioned into a stereotactic frame (David Kopf Instruments) with small animal earbars. A burr hole was made using a Dremel drill — 1.5 mm lateral and 1 mm posterior from the intersection of the coronal and sagittal sutures (bregma). A total of 1 × 105 cells were injected using a Hamilton syringe at a depth of 3 mm in a volume of 2 μl. In vivo, bioluminescent imaging (BLI) was performed on intracranial tumor-bearing mice and mice vaccinated with Flucl-transduced DC. Before imaging, mice were anesthetized with a mixture of ketamine/xylazine (4:1) in PBS, injected i.p. with 100 μl of 30 mg/ml the luciferase substrate, β-Luciferin (Xenogen) in PBS, and shaved over the injection site to minimize the amount of light absorbed by black fur. A cooled charge-coupled device camera apparatus (IVIS, from Xenogen) was used to detect photon emission from tumor-bearing mice with an acquisition time of 2 min. Analyses of the images were performed as described previously (48) using...
Living Image software (Xenogen) and Igor Image analysis software (Wave Metrics) by drawing regions of interest (ROI) over the area and obtaining maximum values in photons per second per cm$^2$ per steradian or total flux values in photons per second. For i.c. B16-Fluc tumor size analysis, ROI were drawn to approximate the top of the skull and kept constant throughout each animal’s imaging. For s.c. DC-Fluc bioluminescent image analysis, regions of interest were drawn to encapsulate the vaccination site and kept constant for each mouse.

Statistical analysis

All error bars represent SEM. Continuous variables were compared using a paired Student $t$ test. The survival curves were determined using the Kaplan-Meier method. The log-rank test was used to compare curves between study and control groups. All statistical values were assessed by the Student $t$ test or ANOVA using Systat statistical software. Values of $p$ were two-tailed, and $p < 0.05$ was considered statistically significant.

Immunohistochemistry and cell counting

Immunohistochemical staining was performed as described previously (51). Briefly, frozen spleen and tumor tissues were immersed in OCT and snap-frozen in 2-methylbutane cooled by dry ice. Sections (20 μm) were cut on a cryostat (Zeiss), fixed in ice-cold acetone, and endogenous peroxidase activity was eliminated with 0.3% H$_2$O$_2$/PBS before staining. Sections were then incubated with primary Abs to CD3ε (500A2; BD Pharmingen), CD4 (RM4-5; BD Pharmingen), CD8α (53-6.7; BD Pharmingen), Ly49GH (4D11; BD Biosciences), CD11b (M1/70; BD Biosciences), or CD11c (HL3; BD Biosciences). The primary mAb incubation step was followed by a biotinylated secondary mAb (Vector Laboratories) and developed with a DAB substrate kit (Vector Laboratories). Negative controls consisted of isotype-matched rat or hamster IgG in lieu of the primary mAbs listed above. To provide semiquantitative data on the number of immunoreactive cells present within CNS tumors, cell counting was performed. A square grid, fitted to the microscope eyepiece, provided a defined field (area at ×200 magnification = 0.25 mm$^2$) to count immunoreactive cells per high-powered field. The number of positive cells per group was tabulated in eight fields. The average number of positive immunoreactive cells/0.25 mm$^2$ SEM is reported. The experiment was performed twice to verify the results.

Results

5% Imiquimod synergizes with melanoma Ag peptide-pulsed DC immunotherapy

We previously demonstrated that DC immunotherapy could provide significant protection against CNS tumor progression in murine CNS tumor models (28). In an effort to find adjunctive treatments that could synergize with our DC-based immunotherapy, we hypothesized that the administration of TLR agonists, such as imiquimod, might provide an inflammatory environment capable of eliciting enhanced antitumor immunity against malignant tumors. To test this idea, we vaccinated mice with hgp100 + TRP-2 MAA

FIGURE 1. Imiquimod enhances the antitumor effects of DC immunotherapy for CNS tumors. Mice were vaccinated with peptide-pulsed DC (irrelevant NP$_{396–404}$ or hgp100$_{25–33}$ + TRP-2$_{180–188}$ MAA peptide-pulsed DC) in the presence or absence of 5% imiquimod, and then challenged intracranially with $1 \times 10^5$ B16-Fluc cells. A, BLI of intracranial B16-Fluc tumor progression in representative mice treated with control NP peptide-pulsed DC (left), hgp100 + TRP-2 MAA peptide-pulsed DC (middle), and hgp100 + TRP-2 MAA peptide-pulsed DC + 5% imiquimod administration (right) at day 14. B, ROI were drawn to calculate the tumor burden and are represented graphically. *, $p = 0.036$; **, $p = 0.032$ by ANOVA repeated measures analysis. These panels are representative of one experiment that has been performed at least three times with similar findings.

FIGURE 2. Imiquimod administration does not enhance the survival of DC-vaccinated CNS tumor-bearing mice. Mice were treated as described in Fig. 1 and subsequently followed for survival. The graph depicts a standard Kaplan-Meier survival curve; $n = 4$ mice/group. The data are representative of three independent experiments.
mice the day before, the day of, and the day after DC vaccination; Imiquimod, or placebo cream, was applied to the skin of peptide-pulsed DC in the presence or absence of 5% imiquimod. hgp100 + TRP-2 MAA peptide-pulsed DC with and without 5% imiquimod. hgp100 + TRP-2 MAA peptide-pulsed DC vaccination, together with 5% imiquimod, resulted in consistently reduced CNS tumor burden compared with mice that had received irrelevant peptide-pulsed DC (with and without 5% imiquimod) and MAA peptide-pulsed DC treatment (Fig. 1). Unexpectedly, the survival of mice that received MAA peptide-pulsed DC vaccination and 5% imiquimod was not significantly extended beyond that of hgp100 + TRP-2 MAA peptide-pulsed DC vaccination alone (Fig. 2) as would have been expected given the differences in tumor burden. Histochemistry revealed large populations of brain-infiltrating leukocytes, small tumors and hemorrhage in mice treated with hgp100 + TRP-2 MAA peptide-pulsed DC and 5% imiquimod (Fig. 3 and data not shown). Immunolabeling and semi-quantitative cell counting confirmed that significantly greater numbers of CD11b+ cells were present in imiquimod-treated animals (p < 0.0004, Fig. 3, Table I). Clustering of CD3+ and CD8+ T cells was also observed (Fig. 3). Our results suggest that 5% imiquimod administration results in robust leukocytic infiltration into the brain and within CNS tumors in conjunction with DC vaccination.

5% Imiquimod administration results in sustained DC survival and enhanced trafficking into draining lymph nodes

To determine the mechanisms by which imiquimod enhances DC immunotherapy, we asked whether imiquimod administration affected the viability and trafficking of DC in vivo. It has previously been shown that murine DC injected into imiquimod-treated skin resulted in maturation of these DC (45). Thus, we tested whether imiquimod administration resulted in enhanced DC survival and trafficking using a combination of in vivo BLI and ex vivo FACS analysis. BM-derived DC were transduced with a lentiviral vector encoding firefly luciferase and eGFP (Fluc-IRES-eGFP) and injected into either placebo- or imiquimod-pretreated skin. As depicted in Fig. 4, the injection of transduced DC into imiquimod-treated skin resulted in dramatically enhanced survival of the injected cells (Fig. 4A, left panel) that persisted for at least 2 wk (Fig. 4B). Even though the relative expression of eGFP was dim, distinctly greater numbers of eGFP+ DC could be seen at the vaccination site (Fig. 4C), confirming the presence of these DCs at this site. Furthermore, enhanced trafficking of these DC into the draining inguinal lymph nodes was observed in mice pretreated with 5% imiquimod. This was demonstrated by in vivo BLI (Fig. 4A, right panel). The eGFP expression within DCs found in the draining lymph nodes was sufficiently dim to prevent ex vivo analysis. To

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Table 1. Increased number of CD11b+ cells within CNS melanomas in animals treated with MAA peptide-pulsed DC vaccination and 5% Imiquimod

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Cells/0.25 mm²</th>
<th>Expt. no. 1 (average ± SEM)</th>
<th>Expt. no. 2 (average ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control B16-Fluc tumor</td>
<td>9.5 ± 1.9</td>
<td>11.6 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>5% Imiquimod B16-Fluc tumor</td>
<td>45.5 ± 5.0</td>
<td>37.4 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Positive control spleen</td>
<td>127.7 ± 9.6</td>
<td>131.2 ± 5.6</td>
<td></td>
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</tbody>
</table>

* Imunohistochemistry was performed for CD11b in two independent experiments. The number of positively labeled cells was counted using a grid in eight locations within the tumor or spleen (+ control). Magnification, ×200. Area of grid, 0.25 mm².

*p < 0.0004, and 'p < 0.0005 (both control vs Imiquimod Tx using the paired t test).
circumvent this limitation, we labeled DCs with the fluorescent dye, CMFDA, and vaccinated mice in the presence or absence of 5% imiquimod treatment. Imiquimod administration resulted in a 4-fold increase in the percentage of CMFDA-labeled, CD11c+ DCs in the lymph nodes draining the vaccination site (Fig. 4D).

Consistent with our previous findings (52), persistent imiquimod administration in mice resulted in dramatic increases in CD11c+ DC populations in both the spleen and lymph nodes. Thus, we believe that imiquimod administration not only enhances the survival of DC, but additionally enhances their migration into the draining lymph nodes.

Imiquimod pretreatment enhances the priming and expansion of tumor-associated, self Ag-specific CD8+ T lymphocytes

Malignant melanoma, as well as CNS gliomas, are known to overexpress a variety of self TAA to which a significant amount of immunological tolerance exists (53). To test whether imiquimod administration, together with MAA peptide-pulsed DC vaccination, could enhance the priming of self tumor Ag-specific CD8+ T cells, we vaccinated mice with hgp100 + TRP-2 MAA peptide-pulsed DC with and without 5% imiquimod administration. The administration of imiquimod resulted in significantly elevated...
Imiquimod enhances the priming of self tumor Ag-specific CD8+ T cells. Groups of wild-type BL6 mice were adoptively transferred with 5 × 10^6 CMFDA-labeled Pmel-1 spleen cells i.v. and then immediately vaccinated with hgp100 peptide-pulsed DC. A control group was treated with a placebo cream while the experimental group was treated with 5% imiquimod every other day for a week. A. Seven days after the adoptive transfer and DC vaccination, the splenocytes and draining lymph node cells were removed and stained for CD3ε, CD8α, and H-2Db-gp100. Dot plots are gated from the CD3+CD8+ population. Annotated numbers reflect the percentage of CD3+CD8+gp100-specific T cells within the CD3+CD8+ population. B. Enhanced population CMFDA-low , gp100-specific CD8+ T cells in the draining lymph nodes of imiquimod-treated mice. Dot plots are gated from the CD8+ tetramer+ population and depict the CMFDA fluorescence in gp100-specific CD8+ T cells. Annotated numbers reflect the percentage of CD8+ T cells that are gp100-specific and fall within the low CMFDA fluorescence gate. The results shown are representative of one mouse in each group (n = 3 mice/group) that has been repeated twice with similar findings.

Discussion
In this study, we have demonstrated that the TLR-7 agonist imiquimod can be used as a vaccine adjuvant to potentiate the use of MAA peptide-pulsed DC vaccination against i.c. tumors. The topical use of 5% imiquimod, together with MAA peptide-pulsed DC vaccination, resulted in significantly greater protection against malignant brain tumor progression than either therapy used alone. This antitumor protection, however, was associated with a dramatic influx of leukocytes into the CNS tumor and surrounding brain parenchyma. Mechanistically, imiquimod administration induced remarkable changes in both the survival and trafficking of the injected DC. Additionally, topical imiquimod enhanced the priming of tumor-specific CD8+ T cells. Thus, we believe that the use of TLR agonists, such as imiquimod, may serve as potent innate immune response modifiers that may enhance the microenvironment and “danger signals” (55) critical for generating potent Th1-type immune responses and effective antitumor immunity.

Our findings also highlight the delicate nature of immune responses in the CNS. Imiquimod administration resulted in a dramatic decrease in CNS tumor growth, but an increase in what we believe was inflammation-induced mortality. Histology of the brains of CNS tumor-bearing mice treated with 5% imiquimod revealed robust leukocyte infiltration and inflammation. Thus, a delicate balance may exist between trying to induce localized antitumor immunity and averting inflammation-induced mortality in the CNS. Despite these findings, we believe that imiquimod administration may still be efficacious without inducing mortality in this model. We are currently testing different dosages, and at different time points, to examine whether the administration of imiquimod can be optimized. These findings highlight some of the important anatomical differences between the brain and other sites in the body where tumors grow. The confined anatomical locale of these malignant brain tumors, in which inflammation and swelling cannot be accommodated without significant potential morbidity in mice and patients, reminds us that the brain is distinct from other sites of the body, and immune-based therapies must be tailored to the specific microenvironment (4).

Ongoing experiments in our laboratory are aimed at elucidating the specific cellular signaling mechanisms by which TLR agonists, such as imiquimod, enhance DC vaccination. Like the natural agonist ssRNA, imiquimod is known to induce type 1 IFN release through activation of TLR7–8 on APCs (36, 37, 43). These effects are thought to differentially influence specific DC subsets based on their selective expression of various TLRs. However, whether imiquimod directly acts on the vaccination DC is still unknown. The expression of TLR7 on different DC subsets is controversial and appears to differ between mice and humans (38, 39, 44, 56). Because the activation of TLR3 (poly IC), TLR7/8 (imiquimod) and TLR9 (CpG) are all associated with the release of type I IFNs, we believe that the adjuvant use of these TLR agonists may represent logical future strategies for vaccines. Thus, future studies in our laboratory will be aimed at testing whether other TLR agonists show similar synergy in our model, and how the mechanisms of enhancement compare with imiquimod.

Finally, recent evidence suggests that persistent TLR signaling may help in bypassing regulatory T cell-induced tolerance (58), enhancing autoimmune T cell responsiveness (59), or even reversing regulatory T cell function (57). Our results suggest that persistent TLR7 activation with 5% imiquimod may be able to overcome tolerance to TAA in which peripheral T cell tolerance predominates. These effects may be an additional mechanism by which imiquimod synergizes with DC immunotherapy. Future studies are needed to determine the exact mechanisms by which imiquimod enhances DC immunotherapy, and to understand how
various TLR agonists can be used safely during CNS tumor immunotherapy.

Disclosures
The authors have no financial conflict of interest.

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