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The TLR7 Agonist Imiquimod Enhances the Anti-Melanoma Effects of a Recombinant Listeria monocytogenes Vaccine

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Activation of innate immune cells through TLR triggers immunomodulating events that enhance cell-mediated immunity, raising the possibility that ligands to these receptors might act as adjuvants in conjunction with T cell activating vaccines. In this report, topical imiquimod, a synthetic TLR7 agonist, significantly enhanced the protective antitumor effects of a live, recombinant listeria vaccine against murine melanoma. This tumor protective effect was not dependent on direct application to the tumor and was associated with an increase in tumor-associated and splenic dendritic cells. Additionally, the combination of imiquimod treatment with prior vaccination led to development of localized vitiligo. These findings indicate that activation of the innate immune system with TLR ligands stimulates dendritic cell activity resulting in a bypass of peripheral tolerance and enhanced antitumor activity. The results of these studies have broad implications for future designs of immunotherapeutic vaccines against tumors and the treatment of metastatic melanoma.

Many cancer immunotherapies are centered on the activation of CD8 CTL that recognize specific tumor Ags (1). Tissue specific differentiation Ags, expressed in both tumors and normal tissues, are commonly the chosen targets. Therapies for melanoma in both animal and human studies have included the Ags MART-1, gp100, tyrosinase, tyrosinase-related protein (TRP)α-1, and TRP-2 (2–6). Overcoming self tolerance is central to the success of this type of immunotherapy because the target Ags represent immunologic self. The possibility for success is suggested by prior evidence showing that T cells recognizing melanocyte-specific differentiation Ags escape thymic deletion and persist in the periphery (7). One therapeutic strategy is to enhance the function of APCs such as dendritic cells (DCs) by providing them with tumor Ags along with immunostimulatory signals that induce maturation and augment the APC ability to activate T cells (8–10).

CTL-mediated immunity can also be induced using live bacterial vectors to stimulate the immune system and simultaneously deliver Ags (11). Listeria monocytogenes is a Gram-positive, facultative, intracellular bacterium that is commonly used in studies of cell-mediated immunity (12). Listeria infects many cell types, including macrophages and other professional APCs (13, 14). Ags expressed by Listeria can access both MHC class I and class II processing pathways, and are presented to both CD8 and CD4 T cells. Additionally, stimulation of TLR (15) on the surface of APCs and the activation of internal pattern recognition molecules such as nucleotide-binding oligomerization domain proteins (16) may contribute to the immunostimulatory effects of Listeria. Activation of these pathways leads to induction of inflammatory cytokines such as IL-12, IFNs, and TNF-α (17–22) resulting in enhanced innate and adaptive responses and the promotion of cell-mediated immunity to infection (23).

Our group and others have shown that L. monocytogenes can induce protective cell-mediated immunity against heterologous Ags. Recombinant L. monocytogenes (rLM) expressing viral proteins can induce specific antiviral CD8 T cell responses (24–28) and protect against virally induced tumors in animal models (29–31). Additionally, the use of heterologous Ags as pseudo-tumor leads to rejection of tumors following rLM immunization (32, 33). Immunization with Listeria expressing the lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) led to s.c. rejection of a glioma cell line expressing heterologous NP (34). Interestingly, animals clearing these tumors were subsequently immune to rechallenge with glioma cells that did not express NP, suggesting that epitope spreading had occurred (34). Recently, our group demonstrated that rLM expressing the melanoma-associated Ag TRP2 (LM-TRP2) was capable of inducing a TRP-2-specific CTL response in BL/6 mice, and immunization with rLM expressing TRP-2 led to partial protection from B16 challenge (35, 36). LM-TRP2 could also be used therapeutically to treat established B16 tumors in naive mice (37). Strains that have been attenuated by deletion of the virulence gene, actA, are also capable of generating a protective immune response (37) and treatment of mice with antibiotics 24 h following therapeutic immunization with nonattenuated rLM did not inhibit the induction of antitumor immunity (37). Additionally, strains that have been attenuated by deletion of both the inlB and the actA virulence genes produce a robust and
protective immune response with limited associated toxicity, thus improving their potential utility as cancer vaccines in humans (38).

Imiquimod is an immunomodulatory compound in the imidazoquinoline family that displays both antiviral and antitumor effects (39, 40). Approved for the treatment of genital warts and actinic keratoses, imiquimod has been used clinically for a wide range of infectious and neoplastic skin disorders. A recent trial of 30 patients showed topical imiquimod to be effective in clearing stage 0 melanoma (lentigo maligna) (41). The exact mechanisms by which imiquimod mediates its effects in human melanoma are currently unknown, although it is known to activate TLR7 (42). Additionally, topical treatment with imiquimod induces a variety of proinflammatory cytokines including IFN-α, TNF-α, and IL-12 (17–19) and facilitates the maturation and migratory capabilities of DCs (43). Topical imiquimod treatment of mice with melanoma led to accumulation of plasmacytoid DCs (pDCs) and partial clearance of s.c. melanoma in the M3 DBA/2 mouse melanoma model (44). Thus, we sought to determine the effectiveness of imiquimod as an adjuvant during immunization with a live rLM vaccine expressing TRP-2. In this study, we found that the use of topical imiquimod, when used in combination with the rLM vaccine expressing TRP-2, led to profound enhancement of the anti-melanoma protective response and to localized vitiligo. These effects were not dependent on application of imiquimod directly to the tumor, suggesting use of these compounds as vaccine adjuvants may be relevant to metastatic melanoma immunotherapies as well.

Materials and Methods

Bacterial and mouse strains

Female C57BL/6 (H-2b MHC) mice were purchased from The Jackson Laboratory and were between 6 and 10 wk of age and age-matched before initiation of experiments. L. monocytogenes 10403S (45) (serotype 1/2a, obtained from D. Hinrichs (Veterans Affairs Medical Center, Portland, OR) via D. Portnoy (University of California, Berkeley, CA)) was the virulent parental bacterial strain used for all recombinant constructs, and was grown and maintained in brain-heart infusion broth or on agar plates with streptomycin (100 μg/ml) selection. Recombinant LM strains Lm-NP and LM-TRP2-NP expressing the LCMV NP604–640 epitope (both strains) and TRP2180–188 (only LM-TRP2-NP) under control of the L. monocytogenes hly promoter and containing the N1C signal sequence were created as previously described (36). A defined, in-frame deletion was made in the actA gene by allelic replacement as previously described (25) to create the ΔactA strain.

Immunization of mice

C57BL/6 mice (6 to 10 wk old) were inoculated in the tail vein with 0.1 LD50 of each rLM strain in 200 μl of PBS, using 28-gauge needles. Boosting immunizations were given 2–3 wk later at a dose of 1.0 LD50. All studies were conducted with the approval of the University of California, Los Angeles Animal Research Committee (ARC).

Intracellular cytokine staining

Intracellular cytokine staining of splenocytes was performed as previously described (36). Briefly, 1–2 × 106 splenocytes were stimulated in 96-well flat-bottom plates with peptide or medium alone, plus brefeldin A (BD Pharmingen) and 50 U/ml IL-2, for 6 h at 37°C in 5% CO2. Cells were washed with staining buffer (PBS with 3% FBS and 0.09% sodium azide), pretreated with anti-FcR Ab for 10 min, and then stained with anti-CD4 FITC, anti-CD8 PE, and anti-CD44 CyChrome (BD Pharmingen) at 1/100 final concentration, on ice for 20–30 min. Cells were then permeabilized and fixed with Cytofix/CytoPerm (BD Pharmingen), then stained for intracellular IFN-γ with anti-IFN-γ FITC or a FITC-labeled isotype control mAb (1/100).

Tumor challenge and in vivo fluorescence imaging of tumors

The B16 murine melanoma cell line was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine. B16 cells stably expressing firefly luciferase (B16-Fluc) were created as described elsewhere (35). Growth rates of B16-Fluc both in vitro and in vivo were similar to those of parental B16 cells. Before tumor challenge, B16-Fluc cells were grown in supplemented DMEM, harvested, washed three times, and resuspended in PBS. For s.c. tumors, mice were anesthetized, shaved on the flank, and injected s.c. with 1 × 106 tumor cells in 50 μl of PBS and 50 μl of Matrigel (BD Biosciences). For metastatic (lung) tumors, mice were immobilized and injected i.v. with 1 × 106 tumor cells in 200 μl of PBS into the tail vein. Before imaging, mice were anesthetized with a mix of ketamine to xylazine (4:1) in PBS, injected i.p. with 100 μl of 30 mg/ml luciferase substrate, α-luciferin (Xenogen) in PBS, and shaved over the injection area (s.c.) or chest (lung metastases) to minimize the amount of light absorbed by black fur. A cooled charge coupled device camera apparatus (IVIS; Xenogen) was used to detect photon emission from tumor bearing mice with an acquisition time of 2 min. Analysis of the images was performed as previously described (46, 47) using Living Image software (Xenogen) and Image analysis software (Wave Metrics) by drawing regions of interest over the tumor region and obtaining maximum values in photons/second/cm2/stereadian. Imiquimod (3M) was applied daily as a 5% cream to shaved skin at the tumor site or to the flank. In experiments in which imiquimod was applied to a distant site, tumors were placed s.c. on the abdomen and imiquimod 5% cream was applied to the abdomen or to the back. Control mice were treated with vehicle control.

Statistical analysis

All error bars represent SEM. Significant differences of tumor growth, intracellular staining, splenocyte weight, and number of splenocytes were assessed by Student’s t test. The difference between groups was considered statistically significant when value p < 0.05.

Immunohistochemistry

Immunohistochemical staining was performed as previously described (48). Primary Abs used were against CD3ε (145-2C11; BioDesign International), CD11c (GR1.5; BioDesign International), CD8α (T75; BioSource International), or CD11c (HL3; BD Biosciences). The primary mAb incubation step was followed by a biotinylated secondary mAb (Vector Laboratories) and developed with a diaminobenzidine or Nova Red substrate kit (Vector Laboratories). Negative controls consisted of isotype-matched rat or hamster IgG in lieu of the primary mAbs as listed.

Bacterial recovery assay and survival curve

Infected mice were sacrificed and liver and spleen were homogenized in 1% Triton X-100/PBS. Serial dilutions of homogenates were plated on brain-heart infusion/streptomycin agar plates and colonies were counted after growth at 37°C for 24 h. For survival curves, infected mice were monitored daily for signs of systemic illness. Moribund animals were sacrificed according to ARC guidelines.

Results

Topical imiquimod leads to a partial antitumor response

Imiquimod has recently been shown to be partially effective as a therapy against melanoma in situ in humans (30). To determine whether imiquimod is effective in treating B16 melanoma tumors, we s.c. challenged mice with B16 cells expressing firefly luciferase (B16-Fluc) and treated the site daily with topical 5% imiquimod cream. Tumors were monitored by bioluminescent imaging, and results shown in Fig. 1A represent tumor volume 2 wk after implantation. In two separate experiments, a partial response was noted, but the effects were variable and were not statistically significant. However, histological examination of the tumor sites revealed destruction of tumors in mice that received imiquimod compared with animals that did not (Fig. 1B). A similar response to lung metastatic tumors was also noted in mice challenged with B16 cells i.v. and treated topically with imiquimod alone (data not shown). These observations demonstrate that imiquimod treatment alone induces partial and variable tumor destruction, both locally and via systemic immunomodulation.

Topical imiquimod induces systemic immunomodulation

During experimental dissections, we noted marked splenomegaly in imiquimod-treated mice, but normal spleens in naive animals or animals with tumor alone. Previous studies have demonstrated that imidazoquinolines can stimulate splenocyte proliferation ex vivo.
Other studies have shown that imiquimod causes an increased migratory capacity of DCs to draining lymph nodes (43). A more recent study demonstrated that imiquimod induces splenomegaly and an accumulation of "pDC-like" cells in the spleen (44). Thus, we hypothesized that application of topical imiquimod is likely responsible for producing the enlarged spleens. Daily treatment of tumor bearing or naive mice with topical imiquimod caused a marked and reproducible increase in splenic weight (Fig. 2A) and total number of splenocytes (data not shown). The presence of tumor did not affect the induced splenomegaly. Spleens were characterized by a loss of germinal center architecture, a massive increase in larger monocytic cells, and the appearance of many multinucleate giant cells compared with spleens in controls (Fig. 2B). No significant changes were observed in the numbers of CD8<sup>+</sup> or CD4<sup>+</sup> T cells, or NK cells (CD3<sup>−</sup>, NK1.1<sup>+</sup>) by FACS analysis (Fig. 3A). There was a moderate increase in the number of B220<sup>+</sup> cells. However, these B220<sup>+</sup> cells likely represent a subset of pDC-like cells based on recent reports of imiquimod effects in other mouse strains (44). Increases in splenic NK-T cells (CD3<sup>−</sup>, NK1.1<sup>+</sup>), myeloid lineage cells (Gr-1<sup>+</sup>, CD11b<sup>+</sup>), and DCs (CD11c<sup>+</sup>) were most pronounced (Fig. 3). The striking splenic phenotype in imiquimod-treated animals accompanied by the substantive influx of immune cells demonstrates that imiquimod applied topically to the skin results in potent effects both systemically as well as locally. Because DCs are professional APCs and are capable of T cell activation, we hypothesized that imiquimod would enhance the antitumor protection induced by an anti-melanoma vaccine. To

![Image of graph](https://example.com/graph1.png)

**FIGURE 1.** Imiquimod treatment leads to partial protection from B16 s.c. tumor challenge in naive mice. A. Groups of seven naive mice were challenged with 1 × 10<sup>4</sup> B16-Fluc cells s.c. and treated daily with vehicle control or with 5% imiquimod cream over the implantation site. Tumor volume was monitored with real-time bioluminescent imaging as described. Graph represents mean ± SE of tumor size at 2 wk and is representative of other time points. Similar results were seen in a separate experiment (not statistically significant). B. Mice received either nothing (left) or topical imiquimod every day (right) to the tumor site. Loss of tumor architecture and increased tumor necrosis are seen in tumors treated with imiquimod as shown in representative frozen sections from day 18 tumors (H&E stained).

![Image of graph](https://example.com/graph2.png)

**FIGURE 2.** Topical imiquimod treatment induces splenomegaly and an influx of DCs. A. Groups of four mice each with and without B16 tumors were treated daily with topical imiquimod for 18 days. Spleens were harvested and weighed and mean ± SE is shown. Topically applied imiquimod led to a significant increase in the splenic weight in both the unchallenged and tumor bearing mice. B. Groups of six mice each were treated daily for 18 days with topical imiquimod in the absence of B16 tumors or rLM. Histological analysis of the spleens shows a loss of typical germinal center architecture and a massive influx of macrophages and monocytes as well as multinucleate giant cells. Staining with CD11c Ab demonstrates the majority of these larger cells are DCs.
test this hypothesis we used a recombinant *L. monocytogenes* vaccine expressing the melanoma Ag TRP-2.

**Imiquimod treatment leads to increased susceptibility to infection with *Listeria***

Infection of mice with rLM expressing the melanoma self-Ag TRP-2 generates Ag-specific CD8<sup>+</sup> CTL and confers partial protection against B16 melanoma progression (36). We hypothesized that the addition of imiquimod during vaccination would enhance antitumor protection resulting from rLM immunization. To test this hypothesis, we treated groups of C57BL/6 mice with topical 5% imiquimod cream for 3 days preceding bacterial challenge. LM-TRP2-NP or the control strain (LM-NP) was used. Both strains also express the immunodominant H-2K<sup>b</sup> restricted NP<sub>396-404</sub> epitope from LCMV as a readily measurable, internal, non-self epitope control. Imiquimod was applied daily to the shaved flank of mice for 3 days before vaccinating with either rLM strain in the tail vein, and treatment was continued daily following vaccination. Surprisingly, in comparison to untreated mice, 90% of imiquimod-treated mice died 2–4 days following rLM injection (Fig. 4A). To determine bacterial loads in imiquimod-treated animals, we counted CFUs in the liver and spleen 48 h following injection (Fig. 4B). Topically applied imiquimod clearly inhibited the ability to control a normally sublethal infection of LM-NP or LM-TRP2-NP. To determine whether death was dependent upon bacterial growth and spread, we repeated the experiment using a mutant *Listeria* strain (ΔactA) that was unable to polymerize host cell actin and spread from cell to cell (50). This attenuated strain did not cause death in mice pretreated with imiquimod, when given at the same dose as wild-type rLM (data not shown), suggesting that mice were dying as a result of bacterial growth and spread, and not simply due to exposure to the initial inoculum. Additionally, when LM-TRP2-NP was inoculated at nonlethal doses in combination with imiquimod, no augmentation of the CD8<sup>+</sup> T cell response was noted (data not shown). Given the lethality of this combined treatment and the apparent lack of augmentation of the T cell response, we sought to determine the effects of imiquimod on tumor protection in the context of an already existing memory T cell population.

**FIGURE 3.** Topical imiquimod treatment induces splenic influx of myeloid and NKT cells. *A*, Immunostaining and FACS analysis demonstrated no significant change in the number of CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes, or NK cells after imiquimod treatment. There is a modest increase in the number of B220<sup>+</sup> (likely B220<sup>+</sup> DCs) and NKT cells in the spleens of imiquimod treated mice. *B*, In imiquimod-treated mice there is a marked increase in the number of CD11b<sup>+</sup> and CD11c<sup>+</sup> DCs as well as Gr-1<sup>+</sup> myeloid cells. Data represent individual mice (symbols) and the mean is shown by thick bars. *, *p* < 0.05; ***, *p* < 0.01.

**FIGURE 4.** Imiquimod pretreatment increases the susceptibility to primary *Listeria* infection. *A*, Groups of four to six mice each were treated with nothing or with topical imiquimod daily for 4 days. Then, on day 0, mice were infected with 0.1 LD<sub>50</sub> of various strains of rLM as indicated and observed. Mice that received imiquimod treatment continued to receive daily application of topical 5% imiquimod cream. Topical imiquimod led to decreased survival after infection with virulent rLM strains LM-NP and LM-TRP2-NP. *B*, Groups of six mice each were treated with nothing or with topical imiquimod daily for 4 days. Mice were then infected with LM-NP, spleens and livers were excised after 48 h, and bacterial counts were determined per organ. Treatment with imiquimod was associated with decreased ability to clear *Listeria* as shown by increased bacterial counts in the organs from imiquimod-treated mice. Data are mean ± SE.
Imiquimod enhances vaccine-induced anti-melanoma immunity

Prior studies in our laboratory demonstrated that immunization with LM-TRP2-NP is capable of inducing a specific CTL response against TRP-2 and that immunity is associated with protection from challenge with B16 melanoma cells (36, 37). We hypothesized that treatment of tumors with imiquimod in the context of vaccine-induced memory T cells would enhance the antitumor response. To determine the effects of imiquimod as a vaccine adjuvant in this setting, we administered imiquimod to tumors in previously vaccinated mice. C57BL/6 mice were immunized and boosted with LM-TRP2-NP or LM-NP. Two weeks later, mice were s.c. challenged with B16-Fluc cells. Mice were then treated with imiquimod 5% cream daily to the tumor implantation site. The protection normally conferred by the LM-TRP2-NP vaccine strain was significantly enhanced by the application of topical imiquimod (Fig. 5A). Imiquimod conferred only partial and variable antitumor protection when applied to mice immunized with the control LM-NP strain (Fig. 5A) or to naive mice (data not shown). In several individual experiments the effects of imiquimod alone were variable. In contrast, the combination of rLM immunization and topical imiquimod led to profound and highly reproducible tumor rejection in this model.

To determine whether the synergistic effects of imiquimod were dependent on application directly to the tumor, we repeated these experiments with imiquimod cream applied to a site distant from the tumor. Mice that had imiquimod applied to a distant site were almost as equally protected as mice that had imiquimod applied directly to the tumor (Fig. 5B). This finding suggests that topically applied imiquimod induces systemic antitumor immunity. Although imiquimod clearly increased the efficiency of tumor rejection, it did not result in a quantitative increase in TRP-2 specific CD8+ T cells in the spleen (Fig. 5C).

To further probe the ability of imiquimod to enhance the systemic effects of the rLM vaccine, we immunized and boosted mice with either LM-TRP2-NP or LM-NP and then challenged the mice 2 wk later with i.v. delivered B16-Fluc cells. Imiquimod or placebo control cream was applied to the shaved flank daily. Mice were sequentially imaged with bioluminescent imaging to monitor tumor growth in the lungs. Mice that received either imiquimod alone or vaccine alone were partially protected from lung tumor development and resulting morbidity (Fig. 6A). However, mice that received LM-TRP2-NP and topical imiquimod were profoundly protected from lung tumor development (Fig. 6A) and none had died by day 31 posttumor challenge. As was noted in the spleen, mice treated with imiquimod showed an accumulation of CD11c+ DCS in lungs bearing tumors as measured by flow cytometry (Fig. 6B). This finding was independent of prior immunization with LM-TRP2-NP.

To determine the long term outcome of the antitumor protection caused by the combination of imiquimod and a pre-existing rLM-induced anti-TRP-2 CTL response, we continued one experiment with s.c. B16-Fluc challenge past the 3 wk when all other control increased tumor protection when applied directly to the tumor or to the back of the mouse away from the tumor. Graph is a representative experiment with six mice per group (mean ± SE). C, Splenocytes were harvested from mice with or without B16 tumors and after vaccination with LM-TRP2-NP, application of topical imiquimod, or both. Presence of intracellular IFN-γ secretion was determined after stimulation with various peptide epitopes in vitro using flow cytometry. Percentage of IFN-γ-positive cells is shown as a mean of four mice per group ± SE. Topical imiquimod did not alter the percentage of CD8+ Ag-specific epitopes in either naive or vaccinated mice.
groups had large tumors necessitating euthanasia. Surprisingly, by 6 wk, six of seven mice in the group that received LM-TRP2-NP and imiquimod were still alive and four of the seven animals had no detectable tumors. Lastly, the same four mice without tumors also developed localized vitiligo over the site of tumor implantation and imiquimod application (Fig. 7). This finding had not been observed in prior experiments with imiquimod or rLM vaccine alone and indicates that although the effects of imiquimod can be seen systemically, they are most potent in the skin under the application site and clearly represent the ability to bypass peripheral tolerance.

**Discussion**

We have demonstrated the ability of the synthetic TLR7 agonist imiquimod to potentiate the antitumor effects of a recombinant *L. monocytogenes* vaccine against melanoma. Complete protection of mice from the aggressive B16 murine melanoma is a challenge not met by many therapies. This effect is most powerful locally, as exemplified by the localized vitiligo where imiquimod was applied and represents a clear breaking of peripheral tolerance. However, the potentiation of prior vaccination is not dependent on application directly to the tumor. This observation has broad implications for immunotherapy against metastatic disease.
One hypothesis to explain the synergistic effects of topical imiquimod with LM-TRP2-NP vaccination is that imiquimod treatment results in an expanded population of vaccine-induced CD8$^+$ Ag-specific CTLs. We did not observe a quantitative change in the number of TRP-2-specific CD8$^+$ T cells in the spleen. However, imiquimod treatment alone led to a significant DC response in both the tumor and the spleen, but did not lead to reproducible antitumor protection in the absence of a vaccine-induced memory T cell population. An alternate hypothesis is that imiquimod-induced DCs may enhance the vaccine-induced antitumor response by stimulating the cytokine function of vaccine-specific CD8$^+$ T cells at the tumor site. This hypothesis is supported by the fact that TLR7 agonists can synergize with CD40L to stimulate CD8 responses (51) and can uniquely induce IL-12 and TNF-α from CD11c$^+$, CD11b$^+$, CD8$^+$ DCs (52). IL-12 pretreatment is known to potentiate the antitumor effects of IFN-α in the B16 mouse melanoma model (53). Lastly, in the setting of established tolerance, recent evidence suggests that persistent TLR signaling is required for bypassing regulatory T cell-induced tolerance (54). Indeed, after 3 wk of daily imiquimod application in the presence of a pre-existing CTL response against the melanocyte-associated Ag TRP-2, localized vitiligo was induced, clearly demonstrating a break of peripheral tolerance to self-Ags.

The increase of DCs in the spleen and at the tumor site raises the possibility that these cells are involved in imiquimod’s mechanism of action. The molecular phenotypes of these cells have recently been characterized (44), but their role in tumor clearance is still unclear. Because the number of DCs is increased in animals that have not received the rLM vaccine and are not protected from tumor challenge, other factors are likely involved. We propose a two-step model to explain our observations. First, LM-TRP2-NP immunization leads to expansion of TRP-2-specific CD8$^+$ T cells that home in on and partially destroy the tumor. Application of imiquimod then leads to enhanced activation and migration of DCs to the tumor site. These DCs are better able to process tumor debris and present other tumor Ags to naïve circulating T cells. This phenomenon, termed “determinant spreading,” has been shown to correlate highly with melanoma regression responses in human vaccine trials (55). We have previously shown that vaccination with rLM expressing a pseudo-tumor Ag can lead to functional determinant spreading in a rat glioma model (56). The contribution of determinant spreading to the augmented protection observed in our model is currently being assessed.

The observation that imiquimod treatment leads to increased susceptibility to bacterial infection is somewhat contrary to the idea that TLR agonists can enhance DC maturation and function (57). Indeed, imiquimod has been demonstrated to enhance clearance of another intracellular organism, Leishmania, both in vivo and in vitro in macrophages (58). However, although activated DCs are adept at presenting Ag and inducing adaptive immune responses, they are not as effective as macrophages at directly killing intracellular bacteria (59, 60), and may be less susceptible to T cell-mediated killing than other cell types (61, 62). Therefore, the large numbers of DCs induced by imiquimod may serve as a protected reservoir for Listeria to replicate undisturbed. Alternatively, recent findings by our group (63) show that mice deficient in type I IFN signaling are protected from Listeria-induced splenic apoptosis. Additionally, type I IFNs induced by injection of poly (I:C), a TLR3 agonist, led to increased susceptibility to Listeria infection in wild-type mice, but not in mice defective in type I IFN signaling. These findings suggest that imiquimod-induced susceptibility to Listeria might be due to splenic apoptosis dependent upon increased type I IFNs, which are known to be induced by imiquimod (19).

Although the natural ligand for TLR7 was recently determined to be ssRNA (64), synthetic TLR7 and TLR8 agonists are also potent TLR activators. In this study we demonstrate that a vaccine-induced antitumor T cell response can be augmented systemically by topical application of imiquimod. This novel finding provides evidence that TLR activation can lead to enhanced systemic immunity and may lead to improved immunotherapies for metastatic disease. Additionally, our observation that enhanced tumor protection is associated with an inability to clear an intracellular bacterial infection shows that the delicate balance between innate and adaptive immunity will be an important consideration when using these agents clinically, especially in immunocompromised patients. Further studies on the exact mechanisms of tumor protection and bacterial susceptibility are warranted to assess the potential of imiquimod for treating neoplastic disease.

Disclosures

The authors have no financial conflict of interest.

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