

# The TLR-7 Agonist, Imiquimod, Enhances Dendritic Cell Survival and Promotes Tumor Antigen-Specific T Cell Priming: Relation to Central Nervous System Antitumor Immunity<sup>1</sup>

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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 peptide-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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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. *The Journal of Immunology*, 2006, 176: 157–163.

**M**alignant 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).<sup>3</sup> 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

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<sup>3</sup> 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(ly); 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- $\alpha$ , TNF- $\alpha$ , 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<sup>+</sup> 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 antiself tumor Ag-specific CD8<sup>+</sup> 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 RPMI 1640 (Invitrogen Life Technologies) with 10% FCS (Gemini Bio-Products) and 1% (v/v) penicillin, streptomycin, and fungizone (Gemini Bio-Products) in a petri dish. Nonadherent cells were replated on day 1 at  $2\text{--}3 \times 10^6$  cells/well in 24-well plates with murine GM-CSF (100 ng/ml; Amgen) and murine IL-4 (500 U/ml; R&D Systems). Cultures were maintained in RPMI 1640 medium containing 10% FCS and antibiotics (complete medium (CM)) at 37°C in 5% CO<sub>2</sub>. On day 4, nonadherent cells were removed by aspirating 80–90% of the media, and adherent cells were reseeded with an addition of 1 ml/well CM plus cytokines. DC were harvested as the loosely adherent cells from the day 8 cultures. DC were resuspended at  $5 \times 10^6$  cells/ml in serum-free RPMI 1640 and pulsed with 10  $\mu$ M control nuclear protein (NP)<sub>396–404</sub> or human (h) gp100<sub>25–33</sub> + TRP-2<sub>180–188</sub> MAA peptides for 2 h at 37°C and 5% CO<sub>2</sub>. A total of  $1 \times 10^6$  pulsed DC were injected s.c. per mouse.

### Lentiviral-mediated gene transfer

Mouse DCs ( $5 \times 10^6$  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  $\mu$ g of p24 equivalent/ml of the lentiviral vector CCL-CMV-firefly luciferase-internal ribosomal entry site (IRES)-enhanced GFP (eGFP) 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  $\mu$ g/ml and the transduction plates were incubated at 37°C, in 5% CO<sub>2</sub> for 12–16 h. Cells were extensively washed with RPMI 1640 medium, counted, and resuspended in PBS for injection into mice. A total of  $1 \times 10^6$  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 hgp100 + TRP-2 MAA peptide-pulsed DC. The mice were then challenged with  $1 \times 10^3$  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

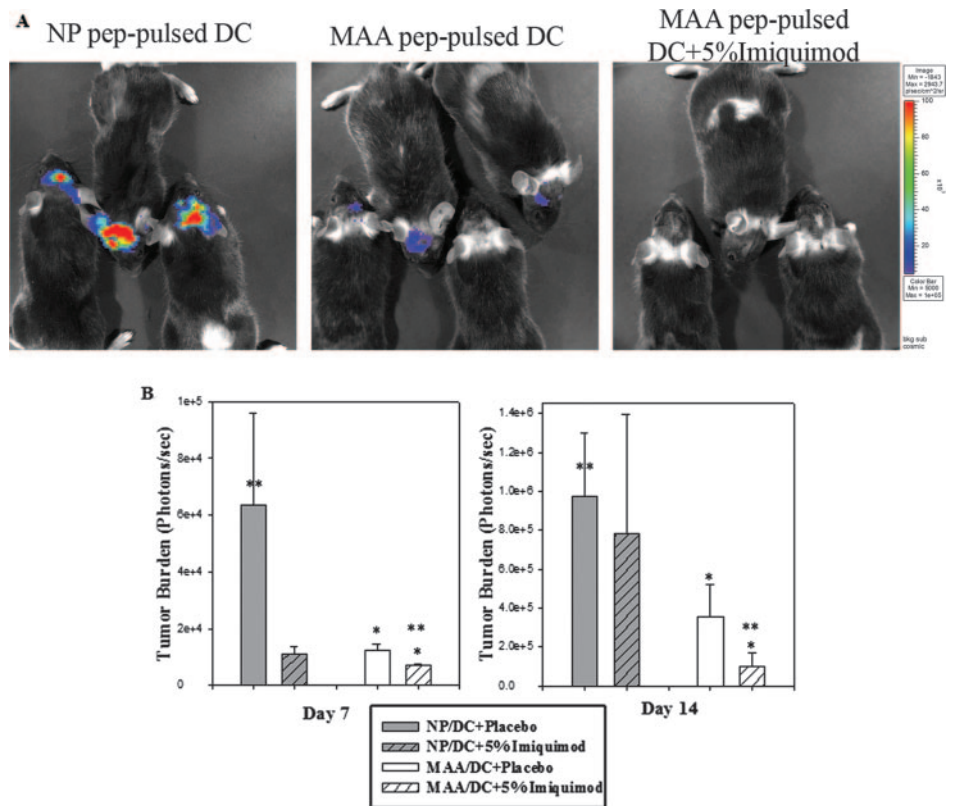
For the adoptive transfer of Pmel-1 TCR transgenic T cells, splenocytes were aseptically obtained from Pmel-1 mice. The splenocytes were labeled with 5  $\mu$ M 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes). A total of  $5 \times 10^6$  cells were subsequently injected i.v. via the lateral tail vein into two groups of C57BL/6 mice. Both groups were immediately vaccinated with  $1 \times 10^6$  hgp100<sub>25–33</sub> peptide-pulsed DC s.c. Groups ( $n = 3$  mice/group) were treated with either 5% imiquimod (3M) 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 $\times$  PharmLyse (BD Pharmingen), and cells were washed, resuspended in Dulbecco’s PBS, and counted. A total of  $1 \times 10^6$  splenocytes were then labeled with mAbs to CD3<sup>PerCP</sup> (BD Pharmingen), CD8 $\alpha$ <sup>PE</sup> (Caltag Laboratories), TCRV $\beta$ <sub>13</sub><sup>FITC</sup>, and/or CD4<sup>FITC</sup> and/or CD62L<sup>FITC</sup> (all from BD Pharmingen) and multimeric, allophycocyanin-conjugated H-2D<sup>b</sup>-gp100 or H-2K<sup>b</sup>-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 FACSCalibur machine, using CellQuest software, and percentages of gated CD8<sup>+</sup> lymphocytes that were tetramer<sup>+</sup> 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 stereotaxic frame (David Kopf Instruments) with small animal earbars. A burr hole was made using a Dremel drill  $\sim$ 1.5 mm lateral and 1 mm posterior from the intersection of the coronal and sagittal sutures (bregma). A total of  $1 \times 10^3$  cells were injected using a Hamilton syringe at a depth of 3 mm in a volume of 2  $\mu$ l. In vivo, bioluminescent imaging (BLI) was performed on intracranial tumor-bearing mice and mice vaccinated with Fluc-transduced DC. Before imaging, mice were anesthetized with a mixture of ketamine:xylazine (4:1) in PBS, injected i.p. with 100  $\mu$ l of 30 mg/ml the luciferase substrate, D-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



**FIGURE 1.** Imiquimod enhances the antitumor effects of DC immunotherapy for CNS tumors. Mice were vaccinated with peptide-pulsed DC (irrelevant NP<sub>396–404</sub> or hgp100<sub>25–33</sub> + TRP-2<sub>180–188</sub> MAA peptide-pulsed DC) in the presence or absence of 5% imiquimod, and then challenged intracranially with  $1 \times 10^3$  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.

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 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.

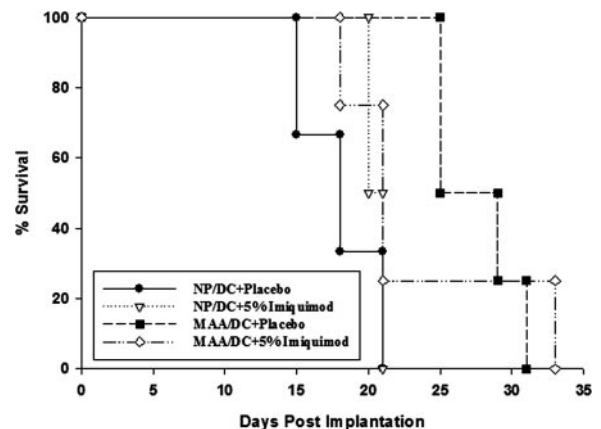
#### Immunohistochemistry and cell counting

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  $\mu\text{m}$ ) were cut on a cryostat (Zeiss), fixed in ice-cold acetone, and endogenous peroxidase activity was eliminated with 0.3%  $\text{H}_2\text{O}_2$ /PBS before staining. Sections were then incubated with primary Abs to CD3 $\epsilon$  (500A2; BD Pharmingen), CD4 (RM4-5; BD Pharmingen), CD8 $\alpha$  (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  $\times 200$  magnification = 0.25  $\text{mm}^2$ ) 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  $\text{mm}^2 \pm$  SEM is reported. The experiment was performed twice to verify the results.

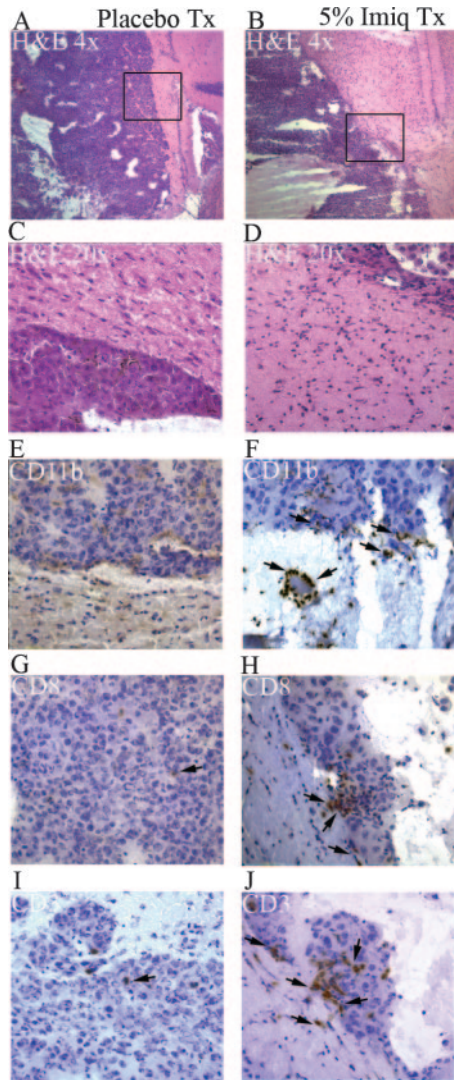
## Results

### 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 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.



**FIGURE 3.** Increased infiltration of immune cells into i.c. B16 tumors after imiquimod administration. Mice were vaccinated with either NP control or hgp100 + TRP-2 MAA peptide-pulsed DC ( $n = 4$ ) and then given a placebo cream or 5% imiquimod three times per week after i.c. B16-Fluc challenge. At day 21 postimplantation, mice were euthanized and their brains snap-frozen in isopentane cooled with dry ice. Sections were cut on a cryostat and were stained with H&E (A–D,  $\times 4$ – $20$  magnification) or stained immunohistochemically for CD11b (E and F), CD8 (G and H), and CD3 (I and J). Sections from representative stains are shown (placebo, A, C, E, G, and I; 5% imiquimod, B, D, F, H, and J).

peptide-pulsed DC in the presence or absence of 5% imiquimod cream. Imiquimod, or placebo cream, was applied to the skin of mice the day before, the day of, and the day after DC vaccination;

then subsequently three times per week after tumor implantation. The mice were challenged in the brain with a lethal dose ( $1 \times 10^3$ ) of B16-Fluc. Tumor burden was quantitated in mice with i.c. B16-Fluc tumors by BLI, as we have recently demonstrated (48). We then compared tumor progression in mice that had received control peptide-pulsed DC vaccination with mice that had received 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<sup>+</sup> cells were present in imiquimod-treated animals ( $p < 0.0004$ , Fig. 3, Table I). Clustering of CD3<sup>+</sup> and CD8<sup>+</sup> 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

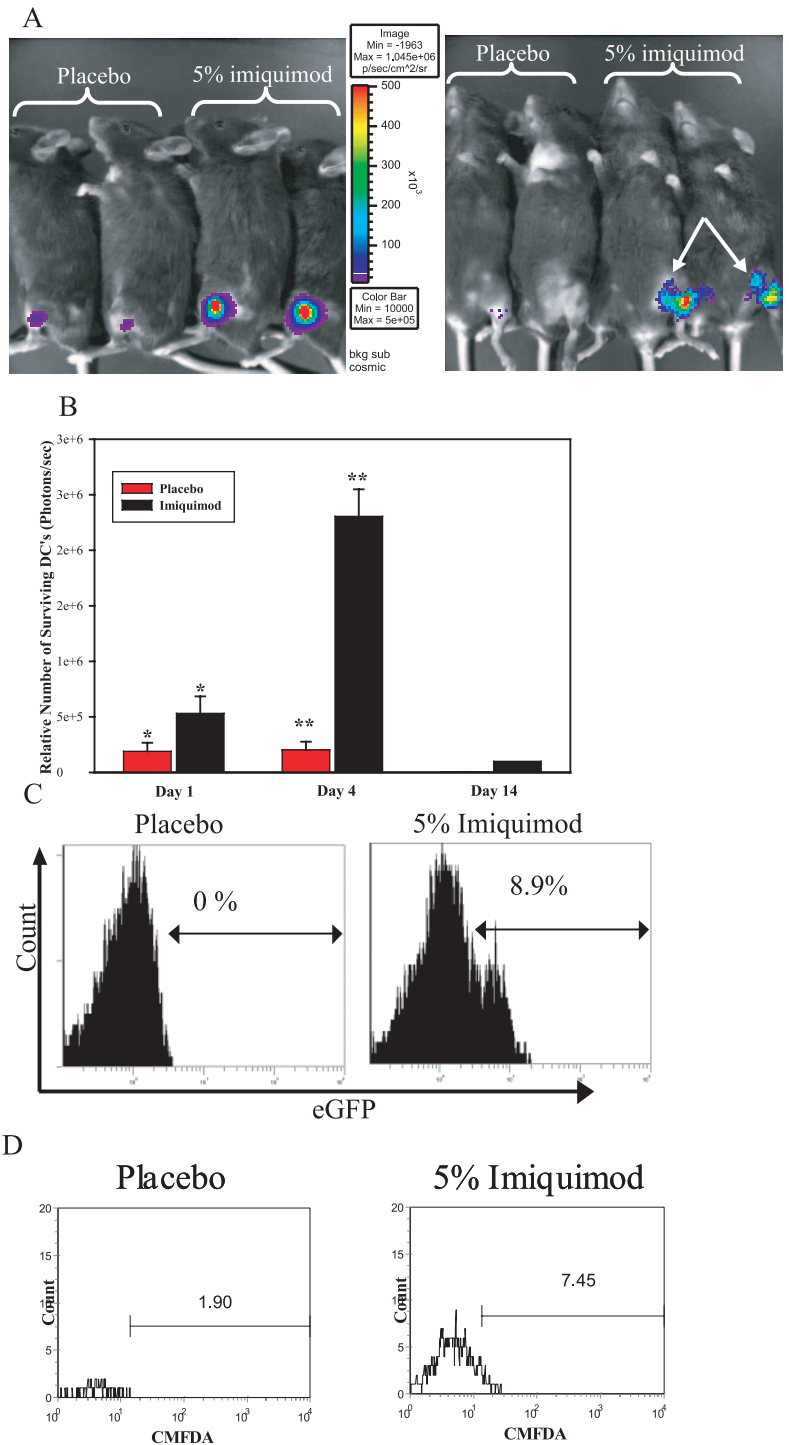
To determine the mechanisms by which imiquimod enhances DC immunotherapy, we asked whether imiquimod administration affected the viability and trafficking of DC in vivo. It has previously been shown that murine DC injected into imiquimod-treated skin resulted in maturation of these DC (45). Thus, we 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<sup>+</sup> 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

Table I. Increased number of CD11b<sup>+</sup> cells within CNS melanomas in animals treated with MAA peptide-pulsed DC vaccination and 5% Imiquimod<sup>a</sup>

Group	No. Cells/0.25 mm <sup>2</sup>	
	Expt. no. 1 (average $\pm$ SEM)	Expt. no. 2 (average $\pm$ SEM)
Control B16-Fluc tumor	9.5 $\pm$ 1.9 <sup>b</sup>	11.6 $\pm$ 1.8 <sup>b</sup>
5% Imiquimod B16-Fluc tumor	45.5 $\pm$ 5.0 <sup>c</sup>	37.4 $\pm$ 3.1 <sup>c</sup>
Positive control spleen	127.7 $\pm$ 9.6	131.2 $\pm$ 5.6

<sup>a</sup> Immunohistochemistry 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,  $\times 200$ . Area of grid, 0.25 mm<sup>2</sup>.

<sup>b</sup>  $p < 0.0004$ , and <sup>c</sup>  $p < 0.0005$  (both control vs Imiquimod Tx using the paired  $t$  test).

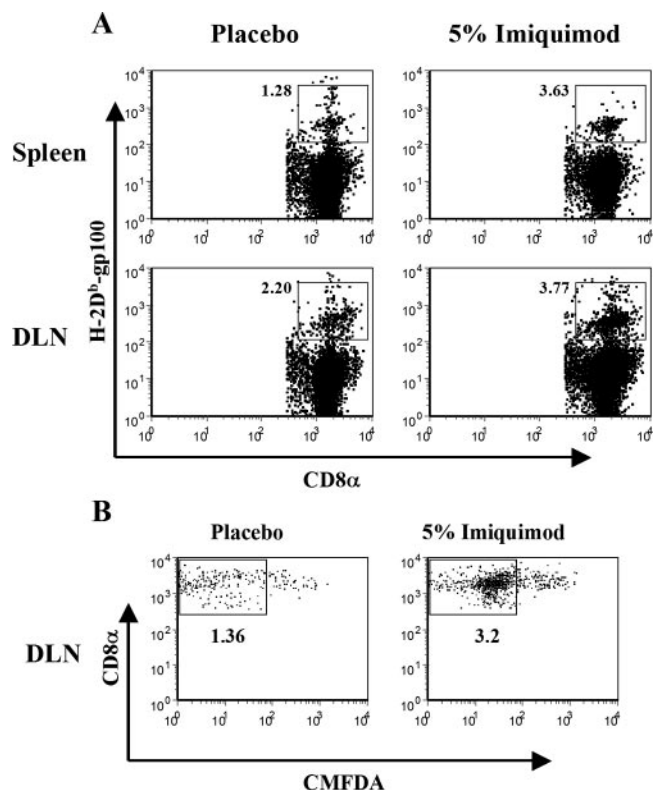


**FIGURE 4.** Imiquimod administration enhances both the survival and in vivo trafficking of DC. BM-derived DC were transduced with a lentiviral vector-encoding firefly luciferase and then injected s.c. into mice pretreated with either a placebo or 5% imiquimod. *A*, Real-time BLI of a representative experiment that depicts both the enhanced survival and trafficking of the DC. *B*, Quantitative assessment of DC survival in vivo in the presence or absence of 5% imiquimod administration. \*,  $p = 0.05$ ; \*\*,  $p = 0.00002$ . *C*, Analysis of ex vivo eGFP expression by the DC 4 days postinjection. *D*, Analysis of ex vivo CMFDA-labeled DC in the draining lymph nodes 4 days after s.c. injection. The CMFDA histogram is gated from the CD11c<sup>+</sup> DC population.

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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T lymphocytes*

Malignant melanoma, as well as CNS gliomas, are known to over-express 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<sup>+</sup> 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



**FIGURE 5.** Imiquimod enhances the priming of self tumor Ag-specific CD8<sup>+</sup> T cells. Groups of wild-type BL/6 mice were adoptively transferred with  $5 \times 10^6$  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 CD3 $\epsilon$ , CD8 $\alpha$ , and H-2D<sup>b</sup>-gp100. Dot plots are gated from the CD3<sup>+</sup>CD8<sup>+</sup> population. Annotated numbers reflect the percentage of CD3<sup>+</sup>CD8<sup>+</sup>gp100-specific T cells within the CD3<sup>+</sup>CD8<sup>+</sup> population. **B**, Enhanced population CMFDA-low<sup>+</sup>, gp100-specific CD8<sup>+</sup> T cells in the draining lymph nodes of imiquimod-treated mice. Dot plots are gated from the CD8<sup>+</sup>tetramer<sup>+</sup> population and depict the CMFDA fluorescence in gp100-specific CD8<sup>+</sup> T cells. Annotated numbers reflect the percentage of CD8<sup>+</sup> 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.

numbers of melanoma Ag-specific CD8<sup>+</sup> T cells in the spleen (data not shown). To improve the sensitivity of these findings, we adoptively transferred CMFDA-labeled, naive splenocytes from Pmel-1 gp100 TCR transgenic mice (54) into mice pretreated with placebo or 5% imiquimod. These mice were then vaccinated with hgp100<sub>25–33</sub> peptide-pulsed DC and the spleen and draining lymph node cells were then analyzed for gp100-specific CD8<sup>+</sup> T cells using tetramer analysis. As depicted in Fig. 5, imiquimod pretreatment resulted in an at least 2-fold increase in the percentage and numbers of gp100-specific CD8<sup>+</sup> cells isolated from the spleen and draining lymph nodes. Thus, the administration of imiquimod may help overcome tolerance to tumor-specific self Ags.

## 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<sup>+</sup> 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 I 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, enhanced cross-presentation by DC, and CTL activation (57), 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.

## References

- Tarhini, A. A., and S. S. Agarwala. 2004. Management of brain metastases in patients with melanoma. *Curr. Opin. Oncol.* 16: 161–166.
- Mitchell, M. S. 1989. Relapse in the central nervous system in melanoma patients successfully treated with biomodulators. *J. Clin. Oncol.* 7: 1701–1709.
- Bigner, D. D., O. M. Pitts, and C. J. Wikstrand. 1981. Induction of lethal experimental allergic encephalomyelitis in nonhuman primates and guinea pigs with human glioblastoma multiforme tissue. *J. Neurosurg.* 55: 32–42.
- Prins, R. M., and L. M. Liau. 2003. Immunology and immunotherapy in neurosurgical disease. *Neurosurgery* 53: 144–153.
- Liau, L. M., K. L. Black, R. M. Prins, S. N. Sykes, P. L. DiPatre, T. F. Cloughesy, D. P. Becker, and J. M. Bronstein. 1999. Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. *J. Neurosurg.* 90: 1115–1124.
- Plautz, G. E., D. W. Miller, G. H. Barnett, G. H. Stevens, S. Maffett, J. Kim, P. A. Cohen, and S. Shu. 2000. T cell adoptive immunotherapy of newly diagnosed gliomas. *Clin. Cancer Res.* 6: 2209–2218.
- Walker, P. R., T. Calzascia, N. de Tribolet, and P. Y. Dietrich. 2003. T-cell immune responses in the brain and their relevance for cerebral malignancies. *Brain Res. Brain Res. Rev.* 42: 97–122.
- Virasch, N., and C. A. Kruse. 2001. Strategies using the immune system for therapy of brain tumors. *Hematol. Oncol. Clin. North Am.* 15: 1053–1071.
- Giezeman-Smits, K. M., H. Okada, C. S. Brissette-Storkus, L. A. Villa, J. Attanucci, M. T. Lotze, I. F. Pollack, M. E. Bozik, and W. H. Chambers. 2000. Cytokine gene therapy of gliomas: induction of reactive CD4<sup>+</sup> T cells by interleukin-4-transfected 9L gliosarcoma is essential for protective immunity. *Cancer Res.* 60: 2449–2457.
- Graf, M. R., M. R. Jadas, J. C. Hiserodt, H. T. Wepsic, and G. A. Granger. 1999. Development of systemic immunity to glioblastoma multiforme using tumor cells genetically engineered to express the membrane-associated isoform of macrophage colony-stimulating factor. *J. Immunol.* 163: 5544–5551.
- Graf, M. R., R. M. Prins, and R. E. Merchant. 2001. IL-6 secretion by a rat T9 glioma clone induces a neutrophil-dependent antitumor response with resultant cellular, anti-glioma immunity. *J. Immunol.* 166: 121–129.
- Graf, M. R., R. M. Prins, W. T. Hawkins, and R. E. Merchant. 2002. Irradiated tumor cell vaccine for treatment of an established glioma. I. Successful treatment with combined radiotherapy and cellular vaccination. *Cancer Immunol. Immunother.* 51: 179–189.
- Okada, H., L. Villa, J. Attanucci, M. Erff, W. K. Fellows, M. T. Lotze, I. F. Pollack, and W. H. Chambers. 2001. Cytokine gene therapy of gliomas: effective induction of therapeutic immunity to intracranial tumors by peripheral immunization with interleukin-4 transduced glioma cells. *Gene Ther.* 8: 1157–1166.
- Herrlinger, U., C. M. Kramm, K. M. Johnston, D. N. Louis, D. Finkelstein, G. Reznikoff, G. Dranoff, X. O. Breakefield, and J. S. Yu. 1997. Vaccination for experimental gliomas using GM-CSF-transduced glioma cells. *Cancer Gene Ther.* 4: 345–352.
- Okada, H., J. Attanucci, K. M. Giezeman-Smits, C. Brissette-Storkus, W. K. Fellows, A. Gambotto, L. F. Pollack, K. Pogue-Geile, M. T. Lotze, M. E. Bozik, and W. H. Chambers. 2001. Immunization with an antigen identified by cytokine tumor vaccine-assisted SEREX (CAS) suppressed growth of the rat 9L glioma in vivo. *Cancer Res.* 61: 2625–2631.
- Friese, M. A., M. Platten, S. Z. Lutz, U. Naumann, S. Aulwurm, F. Bischof, H. J. Bühring, J. Dichgans, H. G. Rammensee, A. Steinle, and M. Weller. 2003. MICA/NGG2D-mediated immunogene therapy of experimental gliomas. *Cancer Res.* 63: 8996–9006.
- Kruse, C. A., K. O. Lillehei, D. H. Mitchell, B. Kleinschmidt-DeMasters, and D. Bellgrau. 1990. Analysis of interleukin 2 and various effector cell populations in adoptive immunotherapy of 9L rat gliosarcoma: allogeneic cytotoxic T lymphocytes prevent tumor take. *Proc. Natl. Acad. Sci. USA* 87: 9577–9581.
- Merchant, R. E., A. J. Grant, L. H. Merchant, and H. F. Young. 1988. Adoptive immunotherapy for recurrent glioblastoma multiforme using lymphokine activated killer cells and recombinant interleukin-2. *Cancer* 62: 665–671.
- Merchant, R. E., N. G. Baldwin, C. D. Rice, and H. D. Bear. 1997. Adoptive immunotherapy of malignant glioma using tumor-sensitized T lymphocytes. *Neur. Res.* 19: 145–152.
- Plautz, G. E., J. E. Touhalisky, and S. Shu. 1997. Treatment of murine gliomas by adoptive transfer of ex vivo activated tumor-draining lymph node cells. *Cell. Immunol.* 178: 101–107.
- Quattrocchi, K. B., C. H. Miller, S. Cush, S. A. Bernard, S. T. Dull, M. Smith, S. Gudeman, and M. A. Varia. 1999. Pilot study of local autologous tumor infiltrating lymphocytes for the treatment of recurrent malignant gliomas. *J. Neurooncol.* 45: 141–157.
- Sankhla, S. K., J. S. Nadkarni, and S. N. Bhagwati. 1996. Adoptive immunotherapy using lymphokine-activated killer (LAK) cells and interleukin-2 for recurrent malignant primary brain tumors. *J. Neurooncol.* 27: 133–140.
- Ashley, D. M., B. Faiola, S. Nair, L. P. Hale, D. D. Bigner, and E. Gilboa. 1997. Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. *J. Exp. Med.* 186: 1177–1182.
- Broder, H., A. Anderson, T. J. Kremen, S. K. Odesa, and L. M. Liau. 2003. MART-1 adenovirus-transduced dendritic cell immunization in a murine model of metastatic central nervous system tumor. *J. Neurooncol.* 64: 21–30.
- Insug, O., G. Ku, H. C. Ertl, and M. Blaszczak-Thurin. 2002. A dendritic cell vaccine induces protective immunity to intracranial growth of glioma. *Anticancer Res.* 22: 613–621.
- Okada, H., H. Tahara, M. R. Shurin, J. Attanucci, K. M. Giezeman-Smits, W. K. Fellows, M. T. Lotze, W. H. Chambers, and M. E. Bozik. 1998. Bone marrow-derived dendritic cells pulsed with a tumor-specific peptide elicit effective anti-tumor immunity against intracranial neoplasms. *Int. J. Cancer* 78: 196–201.
- Okada, H., T. Tsugawa, H. Sato, N. Kuwashima, A. Gambotto, K. Okada, J. E. Dusak, W. K. Fellows-Mayle, G. D. Papworth, S. C. Watkins, et al. 2004. Delivery of interferon- $\alpha$  transfected dendritic cells into central nervous system tumors enhances the antitumor efficacy of peripheral peptide-based vaccines. *Cancer Res.* 64: 5830–5838.
- Prins, R. M., S. K. Odesa, and L. M. Liau. 2003. Immunotherapeutic targeting of shared melanoma-associated antigens in a murine glioma model. *Cancer Res.* 63: 8487–8491.
- Yu, J. S., C. J. Wheeler, P. M. Zeltzer, H. Ying, D. N. Finger, P. K. Lee, W. H. Yong, F. Incardona, R. C. Thompson, M. S. Riedinger, et al. 2001. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. *Cancer Res.* 61: 842–847.
- Yu, J. S., G. Liu, H. Ying, W. H. Yong, K. L. Black, and C. J. Wheeler. 2004. Vaccination with tumor lysate-pulsed dendritic cells elicits antigen-specific, cytotoxic T-cells in patients with malignant glioma. *Cancer Res.* 64: 4973–4979.
- Liau, L. M., R. M. Prins, S. M. Kiertscher, S. K. Odesa, T. J. Kremen, A. J. Giovannone, J. W. Lin, D. J. Chute, P. S. Mischel, T. F. Cloughesy, and M. D. Roth. 2005. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. *Clin. Cancer Res.* 11: 5515–5525.
- Bendelac, A., and R. Medzhitov. 2002. Adjuvants of immunity: harnessing innate immunity to promote adaptive immunity. *J. Exp. Med.* 195: F19–F23.
- Barton, G. M., and R. Medzhitov. 2002. Toll-like receptors and their ligands. *Curr. Top. Microbiol. Immunol.* 270: 81–92.
- Medzhitov, R., and C. A. Janeway, Jr. 2002. Decoding the patterns of self and nonself by the innate immune system. *Science* 296: 298–300.
- Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5: 987–995.
- Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8. *Science* 303: 1526–1529.
- Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.
- Doxsee, C. L., T. R. Riter, M. J. Reiter, S. J. Gibson, J. P. Vasilakos, and R. M. Kedl. 2003. The immune response modifier and Toll-like receptor 7 agonist S-27609 selectively induces IL-12 and TNF- $\alpha$  production in CD11c<sup>+</sup>CD11b<sup>+</sup>CD8<sup>+</sup> dendritic cells. *J. Immunol.* 171: 1156–1163.
- Palamara, F., S. Meindl, M. Holcman, P. Luhrs, G. Stingl, and M. Sibilica. 2004. Identification and characterization of pDC-like cells in normal mouse skin and melanomas treated with imiquimod. *J. Immunol.* 173: 3051–3061.
- Lore, K., M. R. Betts, J. M. Brenchley, J. Kuruppu, S. Khojasteh, S. Perfetto, M. Roederer, R. A. Seder, and R. A. Koup. 2003. Toll-like receptor ligands modulate dendritic cells to augment cytomegalovirus- and HIV-1-specific T cell responses. *J. Immunol.* 171: 4320–4328.
- Wolf, I. H., L. Ceroni, K. Kodama, and H. Kerl. 2005. Treatment of lentigo maligna (melanoma in situ) with the immune response modifier imiquimod. *Arch. Dermatol.* 141: 510–514.
- Barnetson, R. S., A. Satchell, L. Zhuang, H. B. Slade, and G. M. Halliday. 2004. Imiquimod induced regression of clinically diagnosed superficial basal cell carcinoma is associated with early infiltration by CD4 T cells and dendritic cells. *Clin. Exp. Dermatol.* 29: 639–643.
- Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3: 196–200.
- Edwards, A. D., S. S. Diebold, E. M. Slack, H. Tomizawa, H. Hemmi, T. Kaisho, S. Akira, and C. Reis e Sousa. 2003. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 $\alpha^+$  DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33: 827–833.
- Nair, S., C. McLaughlin, A. Weizer, Z. Su, D. Boczkowski, J. Dannull, J. Vieweg, and E. Gilboa. 2003. Injection of immature dendritic cells into adjuvant-treated skin obviates the need for ex vivo maturation. *J. Immunol.* 171: 6275–6282.
- Thomsen, L. L., P. Topley, M. G. Daly, S. J. Brett, and J. P. Tite. 2004. Imiquimod and resiquimod in a mouse model: adjuvants for DNA vaccination by particle-mediated immunotherapeutic delivery. *Vaccine* 22: 1799–1809.
- Rechtsteiner, G., T. Warger, P. Osterloh, H. Schild, and M. P. Radsak. 2005. Cutting edge: priming of CTL by transcutaneous peptide immunization with imiquimod. *J. Immunol.* 174: 2476–2480.

48. Craft, N., K. W. Bruhn, B. D. Nguyen, R. Prins, L. M. Liao, E. A. Collisson, A. De, M. S. Kolodney, S. S. Gambhir, and J. F. Miller. 2005. Bioluminescent imaging of melanoma in live mice. *J. Invest. Dermatol.* 125: 159–165.
49. Ribas, A., L. H. Butterfield, B. Hu, V. B. Dissette, A. Y. Chen, A. Koh, S. N. Amarnani, J. A. Glaspy, W. H. McBride, and J. S. Economou. 2000. Generation of T-cell immunity to a murine melanoma using MART-1-engineered dendritic cells. *J. Immunother.* 23: 59–66.
50. Stripecke, R., R. C. Koya, H. Q. Ta, N. Kasahara, and A. M. Levine. 2003. The use of lentiviral vectors in gene therapy of leukemia: combinatorial gene delivery of immunomodulators into leukemia cells by state-of-the-art vectors. *Blood Cells Mol. Dis.* 31: 28–37.
51. Prins, R. M., F. Incardona, R. Lau, P. Lee, S. Claus, W. Zhang, K. L. Black, and C. J. Wheeler. 2004. Characterization of defective CD4<sup>+</sup>CD8<sup>+</sup> T cells in murine tumors generated independent of antigen specificity. *J. Immunol.* 172: 1602–1611.
52. Craft, N., K. W. Bruhn, B. D. Nguyen, R. Prins, J. W. Lin, L. M. Liao, and J. F. Miller. 2005. The TLR7 agonist imiquimod enhances the anti-melanoma effects of a recombinant *Listeria monocytogenes* vaccine. *J. Immunol.* 175: 1983–1990.
53. Mocellin, S., C. R. Rossi, D. Nitti, M. Lise, and F. M. Marincola. 2003. Dissecting tumor responsiveness to immunotherapy: the experience of peptide-based melanoma vaccines. *Biochim. Biophys. Acta* 1653: 61–71.
54. Overwijk, W. W., M. R. Theoret, S. E. Finkelstein, D. R. Surman, L. A. de Jong, F. A. Vyth-Dreese, T. A. Dellemijn, P. A. Antony, P. J. Spiess, D. C. Palmer, et al. 2003. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8<sup>+</sup> T cells. *J. Exp. Med.* 198: 1–13.
55. Matzinger, P. 2002. The danger model: a renewed sense of self. *Science* 296: 301–305.
56. Gorden, K. B., K. S. Gorski, S. J. Gibson, R. M. Kedl, W. C. Kieper, X. Qiu, M. A. Tomai, S. S. Alkan, and J. P. Vasilakos. 2005. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J. Immunol.* 174: 1259–1268.
57. Pulendran, B. 2004. Modulating vaccine responses with dendritic cells and Toll-like receptors. *Immunol. Rev.* 199: 227–250.
58. Yang, Y., C. T. Huang, X. Huang, and D. M. Pardoll. 2004. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat. Immunol.* 5: 508–515.
59. Lang, K. S., M. Recher, T. Junt, A. A. Navarini, N. L. Harris, S. Freigang, B. Odermatt, C. Conrad, L. M. Ittner, S. Bauer, et al. 2005. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat. Med.* 11: 138–145.