Synergy of Topical Toll-like Receptor 7 Agonist with Radiation and Low-Dose Cyclophosphamide in a Mouse Model of Cutaneous Breast Cancer

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Abstract

Purpose: This study tested the hypothesis that topical Toll-like receptor (TLR) 7 agonist imiquimod promotes antitumor immunity and synergizes with other treatments in a model of skin-involving breast cancer.

Experimental Design: TSA mouse breast carcinoma cells were injected s.c. into syngeneic mice. Imiquimod 5% or placebo cream was applied topically on the shaved skin overlying tumors three times/wk. In some experiments, local ionizing radiation therapy (RT) was delivered to the tumor in three fractions of 8 Gy, given on consecutive days. Cyclophosphamide was given intraperitoneally (i.p.) in one dose of 2 mg/mouse. Mice were followed for tumor growth and survival.

Results: Treatment with imiquimod significantly inhibited tumor growth, an effect that was associated with increased tumor infiltration by CD11c+ cells, and abolished by depletion of CD8+ cells. Administration of imiquimod in combination with RT enhanced significantly tumor response compared with either treatment alone (P < 0.005), and 11% to 66% of irradiated tumors completely regressed. Importantly, the addition of topical imiquimod also resulted in growth inhibition of a secondary tumor outside of the radiation field. Low-dose cyclophosphamide given before start of treatment with imiquimod and RT further improved tumor inhibition and reduced tumor recurrence. Mice that remained tumor-free rejected a tumorigenic inoculum of TSA cells, showing long-term immunologic memory.

Conclusions: Topical imiquimod inhibits tumor growth and synergizes with RT. Addition of cyclophosphamide further increases the therapeutic effect and induces protective immunologic memory, suggesting that this combination is a promising strategy for cutaneous breast cancer metastases.

Introduction

Breast cancer usually arises from the mammary ducts, which are ectodermal appendages. This may account for its predilection for cutaneous metastases (epidermotropism). While some locally advanced tumors may directly infiltrate the adjacent skin, true cutaneous metastases are defined as cancers involving dermal or subcutaneous tissue that is not contiguous with the primary tumor. Skin metastases occur in up to 10% of metastatic tumors, with breast cancers having the second highest incidence/prevalence of skin involvement after melanoma (1). A retrospective analysis of 420 patients with cutaneous metastases identified primary breast carcinoma in 212 patients (30%), a meta-analysis reported an incidence of 24% (457 of 1,903; refs. 1, 2). These metastases frequently occur in the vicinity of the original tumor, such as at the chest wall or its overlying skin following a mastectomy or on the skin of the residual breast tissue after a segmental mastectomy. Initially, skin lesions are generally managed by surgical resection and postoperative radiation, but they frequently recur. Chest wall recurrences of breast cancer often herald or are associated with metastatic disease in vital organs. Overall, the prognosis is relatively poor with a mean survival of 31 months from the diagnosis of skin metastases (1). Importantly, chest wall lesions often progress to become ulcerated, with common bleeding and infection. These complications have a detrimental impact on quality of life and particularly warrant novel therapies. Toll-like receptor (TLR) agonists are included in the ranked National Cancer Institute list of...
immunotherapeutic agents with the highest potential to treat cancer (3, 4). Among them, the TLR7 agonist imiquimod is U.S. Food and Drug Administration–approved as a topical treatment for premalignant and early skin cancers and genital warts. Imiquimod is a very effective treatment for superficial basal cell carcinoma, achieving complete tumor eradication in the majority of patients, mediated by the activation of innate and adaptive immune responses (5, 6). Activation of antitumor immune responses by imiquimod is mediated via TLR7 signaling which enhances dendritic cell (DC) maturation and antigen presentation ability. Imiquimod also promotes T-helper (TH)1 skewing and increased T-cell homing to the tumor, in part, by the indirect effect of type I IFN derived from plasmacytoid DC (pDC) activated by imiquimod (7–9). In addition, direct pro-apoptotic effects on cancer cells that could contribute to tumor responses have been described (10).

The success of imiquimod in treating superficial skin cancers has prompted its testing in other tumors which are amenable to topical therapy, such as primary and metastatic melanoma, with some encouraging but limited results (11–13). Because of their accessibility, unresectable breast cancer skin metastases provide the ideal experimental setting to test novel combinations of local treatments and offer the opportunity to test immune-mediated effects on systemic disease.

The combination of TLR stimulation with other treatments is an area of active investigation. Synthetic agonists of TLR9 were shown to synergize with local ionizing radiation therapy (RT) by inducing antitumor immune responses in mice models and early clinical trials (14–16). The combination of TLR9 agonist and cyclophosphamide (CY) has also been shown to improve survival compared to each treatment alone in a model of embryonal rhabdomyosarcoma (17). Cyclophosphamide was also tested in combination with 2 TLR7/8 agonists designed for systemic rather than topical administration and showed additive effects in a mouse model of colon carcinoma (18). The combination of topical imiquimod with local RT and/or cyclophosphamide for skin metastases of breast or other cancers has not been tested before.

Here, we evaluated the antitumor efficacy of imiquimod in the syngeneic and poorly immunogenic TSA mouse model of breast cancer. We show that topical imiquimod directly applied to established tumors is an effective local treatment for TSA cells, which are TLR7-negative, and can synergize with local RT. Moreover, addition of low-dose cyclophosphamide, a strategy that has been shown to have immunomodulatory function in both preclinical and clinical studies (19–21), increased the strength and durability of the antitumor immune response.

Materials and Methods

Mice

Six to 8-week-old BALB/c mice were obtained from Tacó Animal Laboratory and maintained under pathogen-free conditions in the animal facility at New York University Langone Medical Center (New York, NY). All animal experiments were carried out according to protocols approved by the Institutional Animal Care and Use Committee of New York University.

Cells and reagents

TSA is a BALB/c mouse–derived poorly immunogenic mammary carcinoma cell line (22). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen Corporation) with 2 mmol/L glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 × 10⁻⁵ mol/L 2-mercaptoethanol, and 10% FBS (Gemini Bio-Products; complete medium), and routinely monitored and found to be free of contamination by mycoplasma by the Mycoplasma Detection Kit (Roche Diagnostics). TLR7 expression was determined by reverse transcriptase PCR (RT-PCR) using primers specific for mouse TLR7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (23). Five percent imiquimod (Aldara) and a placebo control cream were supplied by Graceway Pharmaceuticals. Cyclophosphamide monohydrate (Sigma-Aldrich) was dissolved in water at 20 mg/ml and prepared fresh before use.

In vitro toxicity assay

TSA (1.5 × 10⁴ per well) cells were cultured in a 96-well plate in complete medium with reduced serum (1% FBS) in the presence of imiquimod or vehicle control, and viability was measured at 24, 48, or 72 hours using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.).

Tumor challenge and treatment

BALB/c mice were injected s.c. with 1 × 10⁵ TSA cells in the right flank (primary tumor) on day 0. On day 10, when tumors were palpable, animals were randomly assigned to placebo or imiquimod treatment. A small amount (~35 μL)
of cream was applied on the shaved skin over the tumor (corresponding to ~87.5 mg/kg of the active compound) 3 times/wk and gently rubbed in until completely adsorbed. In some experiments, TSA cells were injected in the left flank on day 2 (secondary tumor) as previously described (24). Perpendicular tumor diameters were measured with a Vernier caliper, and tumor volumes were calculated as length \( \times \) width\(^2 \times 0.52\). In some experiments, mice were followed until the tumor reached an average diameter of 12 mm (corresponding to ~5% body weight), according to institutional guidelines.

RT was administered as previously described (24, 25). Briefly, all mice (including mice receiving mock radiation) were lightly anesthetized by intraperitoneal injection of Avertin (240 mg/kg) and positioned on a dedicated plexiglass tray. Radiotherapy was delivered in 3 fractions of 8 Gy given on days 12, 13, and 14 to a field including the tumor with 5 mm margins using a Clinac 2300 C/D Linear Accelerator (Varian Medical Systems) fitted with a 25-mm RadioSurgery conical collimator (BrainLAB AG), which is designed to deliver very sharp and limited radiation dose fields. Superflab bolus (1.5-cm tissue equivalent material) was placed over the tumor, and a source-to-skin distance (SSD) of 100 cm was set. RT was delivered at 600 cGy/min (corresponding to SSD of 100 cm) and a 1.67 W\( \times \)€\( \text{SSD} \) of 100 cm was set. RT was delivered at 600 cGy/min with 6 MV X-rays. Cyclophosphamide was given i.p. at 2 mg/mouse on day 9.

**In vivo T-cell subset depletion**

Depletion of T-cell subsets was achieved by injecting anti-mouse CD4 GK1.5 mAb or anti-mouse CD8 2.43 mAb (BioXCell) i.p. at 100 \( \mu \)g/mouse on day 7, 8, and 9 post-tumor cell inoculation. The depletion was maintained by repeated weekly injections of mAb. Depletion was confirmed by testing spleen cells for the presence of CD4\(^+\) and CD8\(^+\) T-cells using non–cross-reactive FITC-RMA4-4 and PE-anti-CD8\(\beta\) mAb (BD PharMingen).

**Immunostaining of tumor sections**

Tumors were harvested at day 26 postinoculation, fixed for 1 hour at 4°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in optimum cutting temperature (OCT) medium. Sections (8 \( \mu \)m) were stained as previously described (24) with phycoerythrin–Texas Red–conjugated rat anti-mouse CD11c or PE-conjugated rat anti-mouse CD8\(\alpha\) (Caltag) or PE-conjugated Hamster anti-mouse CD11c (BD Pharmingen) and counterstained with 5 \( \mu \)g/mL 4',6-diamidino-2-phenylindo (DAPI; Sigma). Images were obtained with the use of a Nikon Eclipse 800 deconvolution microscope. The numbers of CD4\(^+\), CD8\(^+\) T cells and CD11c\(^+\) DCs were counted in 3 randomly selected fields (\( \times 20 \)) in each tumor.

**Measurements of interleukin-10 by ELISA**

Tumors, serum, tumor-draining lymph node (TDLN), and spleen were harvested at day 26 from TSA tumor-bearing mice (\( n = 6 \)/group) treated with topical placebo or imiquimod. Tumor lysates were prepared by mincing tumors in lysis buffer containing 10 mmol/L Tris (pH 8.0, 0.5% Nonidet P-40, 250 mmol/L NaCl, 10 mmol/L sodium orthovanadate, 100 \( \mu \)mol/L phenylmethylsulfonyl fluoride, 1 \( \mu \)g/mL leupeptin, 1 \( \mu \)g/mL pepstatin, and 1 \( \mu \)g/mL aprotonin (all from Sigma-Aldrich). After sonication and incubation on ice for 30 minutes, lysate supernatants were obtained by centrifugation at 13,000 rpm for 20 minutes at 4°C. Spleen and TDLN cells (4 \( \times \)10\(^6\) cells/mL) were cultured in 96-well tissue culture plates for 24 hours in T-cell medium [RPMI-1640 supplemented with 2 mmol/L L-glutamine, 100 \( \mu \)U/mL penicillin, 100 \( \mu \)g/mL streptomycin, 5 \( \times \)10\(^{-5}\) mol/L 2-mercaptoethanol, 10% FBS, and 10 U/mL human recombinant interleukin (IL)-2] in the presence of phorbol-12-myristate 13-acetate (PMA; 5 ng/mL) and ionomycin (500 ng/mL). Cell-free supernatants were collected and stored at ~80°C. IL-10 was measured by ELISA (R&D Systems). Samples from 2 mice were pooled to obtain 3 independent samples of the 6 mice analyzed in each treatment group.

**Flow cytometry**

Single-cell suspensions were obtained by digestion of treated and untreated tumors with 0.2 mg/mL DNase and 1.67 WünschU/mL Liberase (Roche) as previously described (26). The Fixable Viability Dye eFluor 660 (ebioscience) was used to distinguish live and dead cells. Aliquots of 10\(^6\) cells were incubated with anti-mouse CD16/32 (Fc block) for 10 minutes followed by staining for 30 minutes with Pacific Blue-anti-CD45 mAb, PE-anti-H2-K\(^b\), FITC-anti-H2-L\(^a\), and FITC-anti-ICAM-1 (BD Pharmingen). Lymphocytes isolated from spleen and peripheral blood were stained with FITC-anti-CD4 mAb, PE-Cy-5-anti-CD25, and PE-anti-FoxP3 mAb using the Fixation/Permeabilization Concentrate and Diluent Kit (ebioscience). All samples were analyzed using a LSRII flow cytometer and FlowJo version 7.6.3 (Tree Star).

**Tumor-specific production of IFN-\(\gamma\) by TDLN cells**

TDLN cells (0.5 \( \times \)10\(^6\)) were cultured in 48-well tissue culture plates with 1 \( \mu \)mol/L AH1 peptide (SPSYVYHQF), a CD8 T-cell epitope known to be an immunodominant antigen in TSA cells (22), or control peptide derived from MCMV (YPHFMPITNL; GenScript) for 72 hours in T-cell medium. IFN-\(\gamma\) concentration was measured in cell-free supernatant of duplicate well using flowcytomix kit (ebioscience).

**Statistical analysis**

The Student t test was used to compare the effect of imiquimod versus vehicle control in in vitro toxicity assays, levels of IL-10 and surface molecules in placebo versus imiquimod-treated mice. Mixed model ANOVA was used to compare treatment arms with respect to mean tumor volumes at each day of measurement. The dependent variable was composed of the tumor volumes observed for all animals at all time points of observation. The model included treatment and time as fixed classification factors. The Tukey multiple comparison procedure was used to compare treatment arms with respect to tumor volume at
each day of measurement while keeping the family-wise type I error rate for the set of comparisons below the nominal 5% level. Random coefficients regression was used to assess the effect of treatment on tumor growth. The dependent variable was the natural log of tumor volume at all available time points; log volumes were used in place of observed volumes as the change in log volume over time was well approximated as linear. For both the ANOVA and random coefficients analyses, the correlation structure was modeled by assuming observations to be correlated only when acquired from the same animal with the strength of correlation between observations inversely dependent on the elapsed time between the observations. Treatment arms were compared in terms of survival, defined as time to death due to disease or sacrifice, using log-rank tests. All reported P values are 2-sided and were declared statistically significant at the 5% level. The statistical computations were carried out using SAS for Windows, version 9.3 (SAS Institute).

Results

Imiquimod toxicity in vitro is minimal for TLR7-negative TSA mouse breast cancer cells

TLRs are expressed by many epithelial tumors, including breast cancer (27). Expression of TLR7 has been reported in some human breast cancer cell lines, although the functional consequences remain undefined (28). To determine whether imiquimod has therapeutic activity against cutaneous metastases of breast cancer, we used a mouse model of poorly immunogenic breast cancer, which mimics the situation of patients with cancer with chest wall recurrence as TSA tumor cells form subcutaneous nodules that invade the dermis and ulcerate through the skin. By RT-PCR analysis, expression of TLR7 was not detected in TSA cells (data not shown). TSA cells did not show a significant reduction in viability at 24 hours when cultured in the presence of imiquimod (Supplementary Fig. S1). However, a modest but significant toxicity (P < 0.005) was seen at 48 hours in the presence of 10 μg/mL of imiquimod, and at 48 hours (P < 0.05) in the presence 5 μg/mL of imiquimod. Overall, results suggest that imiquimod is toxic for TSA cells only at relatively high concentrations and this effect is independent from TLR7 expression, consistent with the previously reported pro-apoptotic effect of imiquimod in some human epithelial cancer cells (10).

Topical imiquimod inhibits TSA tumor growth in vivo by a CD8-dependent mechanism

To determine the effect of imiquimod in vivo, imiquimod or placebo cream was applied onto the skin over TSA tumors starting at day 10 postinjection of tumor cells, when tumors became palpable (Fig. 1A). Imiquimod significantly inhibited tumor growth (P < 0.0001 compared with placebo) but did not induce any complete tumor regression (Fig. 1B, top). Analysis at day 26 showed increased infiltration by CD11c+ DC and CD4+ and CD8+ T cells in imiquimod-treated tumors (Fig. 1C and D). The antitumor effect of topical imiquimod was abrogated by depletion of CD8+ cells (Fig. 1B, bottom), indicating that it was mediated by activation of antitumor CD8+ T cells and/or CD8+ pDCs (29). Conversely, CD4+ T cells may play a suppressive role as their depletion improved significantly (P < 0.005) tumor inhibition. A marked increase in the levels of the immunosuppressive cytokine IL-10, produced by CD4+ T cells, was recently reported in the spontaneous tumors of neu-transgenic mice upon topical imiquimod treatment (30). Measurements of IL-10 levels showed no significant change in the tumor and TDLNs of imiquimod-treated mice (Fig. 1E). However, a modest but statistically significant increase was detected in the serum, as well as the supernatants of ex vivo stimulated spleen cells, consistent with the increased serum levels reported in neu-transgenic mice (30). Overall, in the TSA model, there was no evidence of IL-10 induction at the site of topical imiquimod treatment, possibly because IL-10 was already elevated in placebo-treated tumors (Fig. 1E). However, increased IL-10 production in ex vivo stimulated spleen cultures indicates that a population of IL-10–producing T cells is expanded in imiquimod-treated mice.

Topical imiquimod synergizes with local radiotherapy in inhibition of the irradiated tumor and of a tumor outside of the radiation field

We have previously shown that RT of established TSA tumors given as a single dose of 20 Gy, 3 fractions of 8 Gy, or 5 fractions of 6 Gy caused a similar tumor growth inhibition, but no tumor eradication was achieved by any of these radiation regimens, and there was no effect on a secondary tumor outside of the radiation field (24). Importantly, only fractionated RT showed synergy with anti-CTLA-4 antibody in inducing antitumor immune responses leading to eradication of the majority of the irradiated tumors and of tumors outside of the radiation field (abscopal effect), with the best synergy seen with 8 Gy × 3 (24). Therefore, the regimen of 8 Gy × 3 was chosen for testing in combination with imiquimod. In the first experiment, mice bearing a single tumor were treated with topical imiquimod starting at day 10, and RT was delivered to the tumor at days 12, 13, and 14 (Fig. 2A). As expected, RT and imiquimod as monotherapy delayed tumor growth but did not induce complete regression. In contrast, the majority of tumors receiving imiquimod + RT had completely regressed between days 25 and 30 (Fig. 2B and C). Random coefficient regression analysis confirmed a significant interaction between RT and imiquimod (P = 0.0115), consistent with a synergistic effect of this combination.

To determine whether the antitumor effect of imiquimod, alone or in combination with RT, is localized to the treated area or could extend to other skin/chest wall sites involved by tumor, TSA cells were injected at 2 separated sites, defined as “primary” and “secondary” tumors (Fig. 3A and B). At start of treatment both tumors were palpable, but the secondary tumors were smaller than primary tumors (mean volume, 8.1 ± 4.7 vs. 21.8 ± 7.5 mm3) as TSA cells were injected with 2-day delay. RT was delivered only to the primary tumor, and we have previously shown that RT alone does not affect growth of the secondary tumor outside of the radiation field (24). Imiquimod applied to the
primary tumor caused a significant tumor inhibition that was enhanced by administration of RT, leading to complete regression of 30% of the primary tumors by day 25 (Fig. 3C), confirming data shown above (Fig. 2). Treatment of the secondary tumor with imiquimod did not alter the effect of imiquimod alone or with RT at the primary tumor. Interestingly, treatment of the primary tumor with imiquimod inhibited the growth of the secondary tumor, an effect that was not further enhanced by treating also the secondary tumor with imiquimod (Fig. 3D). However, application of imiquimod to the secondary tumor significantly enhanced inhibition of the secondary tumor when the primary tumor was treated with imiquimod + RT. Overall results suggest that imiquimod has both direct (i.e., at the site of application) and indirect antitumor effects. Part of the direct effects of imiquimod may result in sensitization of a tumor outside of the radiation field to antitumor immune responses triggered by concomitant use of RT and imiquimod at another tumor site. To determine whether the expression of molecules known to facilitate tumor rejection by T cells was increased in tumors treated with topical imiquimod, TSA cells freshly isolated from placebo and imiquimod-treated mice were analyzed ex vivo by flow cytometry. Imiquimod induced a statistically significant increase in the levels of 2 MHC class I alleles and of intercellular adhesion molecule-1 (ICAM-1; Fig. 4A). Analysis of TDLNs from mice treated with imiquimod + RT confirmed the development of T-cell responses to the CTL epitope AH1, which is derived from an immunodominant antigen in TSA cells and presented by H2-Ld (ref. 22; Fig. 4B). These data support the hypothesis that imiquimod induces immune-mediated tumor inhibition by promoting both priming and effector phases of antitumor T-cell responses.
Low-dose cyclophosphamide improves local control and reduces recurrence of tumors treated with RT and topical imiquimod

The inhibition of tumor growth by imiquimod treatment alone was reported to be only temporary in neu-transgenic mice as tumors resumed growth after topical imiquimod was stopped (30). To determine whether the complete tumor regression observed after treatment with the combination of imiquimod + RT was durable, mice from the experiment in Fig. 2 were followed over time. All tumors recurred between 20 and 30 days after discontinuation of imiquimod treatment at day 35, with the longest tumor-free period of 40 days, indicating that the immune response triggered by treatment was unable to eliminate all cancer cells and not potent and/or durable enough to prevent tumor regrowth. An attractive strategy to improve antitumor immune responses is the use of low-dose cyclophosphamide, a chemotherapy drug that has been shown to have immunomodulatory function in both preclinical and clinical studies (19–21). The optimal dose and schedule of cyclophosphamide to enhance the effect of vaccination have been previously defined by Machiels and colleagues to be 100 mg/kg administered 1 day before vaccination in a mouse breast cancer model (19). At this dose, cyclophosphamide caused a significant reduction of regulatory T (Treg) cells detectable in the spleen and peripheral circulation 3 days after administration to tumor-bearing mice (Fig. 5A and B). Therefore, we tested the effect of cyclophosphamide administered at 100 mg/kg 1 day before the first dose of imiquimod. Cyclophosphamide by itself caused a significant tumor growth delay (P < 0.0005 cyclophosphamide + placebo vs. placebo) and enhanced tumor growth inhibition by imiquimod, although the difference was not significant (P > 0.05; imiquimod + cyclophosphamide vs. imiquimod), and no complete tumor regression was achieved (Fig. 5C). Interestingly, IFN-γ responses to the tumor-specific CTL epitope AH1 were the highest in TDLNs of mice treated with the combination of imiquimod + cyclophosphamide than single treatment with cyclophosphamide or imiquimod, alone (Fig. 5D). The increase showed a trend to significance when compared with imiquimod alone (P = 0.087). A single dose of cyclophosphamide had no effect on serum levels of IL-10 but diminished significantly IL-10 production induced by imiquimod treatment in ex vivo stimulated spleen cells (Fig. 5E and F). Overall, data suggest that cyclophosphamide mitigates at least some of the immunosuppressive mechanisms resulting in improved antitumor T-cell responses.

Next, the effect of cyclophosphamide was tested in mice receiving RT with or without imiquimod (Fig. 6A). Mice receiving cyclophosphamide in combination with RT and imiquimod showed a significant improvement in tumor inhibition compared to all other groups (P < 0.0005; cyclophosphamide + imiquimod + RT vs. placebo and imiquimod + RT, and P < 0.0005 vs. cyclophosphamide + RT; Fig. 6B). Median survival was significantly increased in all treated mice compared with placebo (P < 0.0005) and was 31 days for placebo, 52 days for imiquimod + RT, 49 days for cyclophosphamide + RT, and 73 days for cyclophosphamide + imiquimod + RT (Fig. 6C). Complete tumor regression occurred between days 24 and 31 and was seen in 50% of mice treated with cyclophosphamide + imiquimod + RT, 30% of mice treated with cyclophosphamide + RT, and 11% of mice treated with imiquimod + RT. Although some tumors continued regressing after termination of imiquimod administration at day 24 posttumor inoculation, tumor recurrence was eventually seen in all
mice except 3 mice in cyclophosphamide + RT group and 3 mice in cyclophosphamide + imiquimod + RT group, which remained tumor-free 65 days after termination of imiquimod administration. Mice that remained tumor-free at day 90 were rechallenged with a tumorigenic inoculum of TSA cells together with a group of naïve mice. While all naïve mice developed tumors by day 10 postchallenge, all mice...
that had survived tumor-free after treatment with cyclophosphamide + imiquimod + RT and 2 of 3 mice treated with cyclophosphamide + RT rejected the challenge and remained healthy until the end of the follow-up period 2 months after re-challenge.

Collectively, these results indicate that a single low-dose cyclophosphamide promotes the development of long-term protective immunity when combined with RT and that addition of imiquimod improves local tumor control and survival in mice treated with cyclophosphamide + RT, supporting further testing of combinations of TLR7 agonists with chemotherapy and radiation.

Discussion

In this study, we show in an experimental breast cancer model of skin metastasis that a local immune response modifier can be combined with local radiotherapy and systemic low-dose chemotherapy to induce effective antitumor immunity with the potential to eliminate the tumor. The appeal of this approach is that it exploits the accessibility of breast cancer skin metastases to convert a difficult therapeutic problem into an opportunity for in situ vaccination of the patients against the tumor.

Topically applied imiquimod inhibited TSA tumor growth. This effect was associated with increased tumor infiltration by DC and T cells and was abolished by depletion of CD8+ cells. Similar CD8 dependence of the therapeutic effect of topical imiquimod was previously described by Lu and colleagues in the neu-transgenic model of breast cancer (30). In the latter, tumor-specific CD8+ T cells were increased in imiquimod-treated mice, and local tumor control was associated with decreased

Figure 5. Low-dose cyclophosphamide reduces Treg cells and IL-10 production and enhances tumor-specific IFN-γ production by TDLNs. A and B, mice were injected s.c. with TSA cells on day 0. Cyclophosphamide was administered i.p. on day 9 in a single dose of 100 mg/kg, and spleen (A) and blood (B) were analyzed on day 12 for the presence of Treg cells by staining for CD4, CD25, and FoxP3. Data are the mean ± SD of 3 mice/group and are representative of 2 experiments. C, tumor growth delay in mice treated with PLA (open squares), IMQ (closed triangles), cyclophosphamide + placebo (PLA; open circles), and cyclophosphamide + IMQ (closed squares). PLA and IMQ were administered as in Fig. 1. Cyclophosphamide was given once at day 9. Data are the mean ± SEM of 7 mice/group. D, production of IFN-γ by TDLN cells harvested at day 18 and stimulated with the tumor-specific CTL epitope AH1 (full circles) or control peptide (empty circles). Each symbol represents one mouse. Horizontal lines indicate the mean of AH1 (solid line) or control (dotted line) peptide-stimulated cultures. E and F, IL-10 concentration measured at day 18 in supernatants of ex vivo stimulated spleen cells (E) and serum (F). Each symbol represents one mouse. Data are representative of 2 experiments. * P < 0.0005; ** P < 0.005; *** P < 0.05.
spontaneous lung metastases, an effect that could result from decreased seeding from the treated tumor and/or inhibition by antitumor T cells that are active systemically. However, recent evidence by Drobits and colleagues indicates that the antitumor effect of topical imiquimod, at least within the first 1 or 2 weeks, can be entirely mediated by pDCs that acquire CD8 and lytic function (29). In a mouse melanoma model, they showed that imiquimod induced increased tumor infiltration by CD8+ T cells and natural killer cells but neither was required for tumor inhibition. It is, therefore, possible that CD8+ pDCs play a role in inhibition of the TSA tumors. However, evidence that imiquimod applied to the primary tumor leads to inhibition of a secondary tumor that is not topically treated (Fig. 3) is consistent with development of an adaptive immune response. Importantly, the long tumor-free interval (up to 40 days) seen in mice with complete tumor regression after treatment with the combination of imiquimod + RT, and the development of long-term protective memory in mice that received cyclophosphamide + imiquimod + RT indicate that imiquimod can be used as an adjuvant to improve the therapeutic efficacy and pro-immunogenic effects of RT and low-dose cyclophosphamide (31).

Improved tumor inhibition observed in mice treated with imiquimod and CD4+ T-cell depletion suggests that CD4+ T cells have a regulatory or suppressive role (Fig. 1B). Induction of IL-10 secretion by a CD4+ T-cell subset different from Treg cells in mice treated with imiquimod was previously shown to be a mechanism of suppression of the antitumor immune response (30). However, in our model, imiquimod treatment did not lead to increased levels of IL-10 in tumor or TDLNs. A significant increase was noted in the serum and in supernatants of ex vivo stimulated spleen cells, suggesting that some induction of IL-10 production by T cells may represent a negative feedback reaction to the increased immune activation caused by imiquimod. It remains to be determined whether this increased polarization of T cells toward IL-10 production is responsible for tumor recurrence. Elevated levels of IL-10 were present in tumors regardless of treatment and may be an impediment to tumor rejection. Interestingly, administration of cyclophosphamide 1 day before the start of imiquimod treatment decreased significantly IL-10 produced by spleen cells (Fig. 5E) and showed a trend to increase IFN-γ produced by tumor-specific CD8 T cells (Fig. 5D). Although cyclophosphamide treatment did not significantly enhance the therapeutic effect of imiquimod (Fig. 5C), in combination with RT and imiquimod + RT, cyclophosphamide promoted the development of a durable antitumor immune response able to prevent tumor recurrence and reject a tumor challenge over 2 months after the end of treatment (Fig. 6). The best characterized immunomodulating effect of cyclophosphamide at non-lymphodepleting doses is its ability to selectively deplete and/or disable Treg cells (32, 33) and uncover the therapeutic effect of immunotherapy (19, 20, 34). In mice treated at day 9 with cyclophosphamide, there was a significant decrease in Treg cells at day 12 in the spleen and peripheral circulation (Fig. 5A and B). The ability of cyclophosphamide given before RT to induce complete tumor regression and protective tumor-specific memory in a fraction of TSA tumor–bearing mice indicates that cyclophosphamide can also uncover the ability of RT to generate an in situ tumor vaccine (35). The relative contribution of Treg cells reduction, induction of an immunogenic cell death, or other pro-immunogenic activities of cyclophosphamide to this effect remains to be determined (36, 37). Importantly, the addition of topical imiquimod led to further improvement in the local tumor control achieved by cyclophosphamide + RT and a trend to

**Figure 6.** Low-dose cyclophosphamide improves tumor inhibition by IMQ + RT. A, treatment schema. Tumor growth delay (B) and survival (C) in mice treated with placebo (PLA; n = 17), IMQ + RT (n = 9), cyclophosphamide + PLA + RT (n = 13), and cyclophosphamide + IMQ + RT (n = 14), as indicated. Only a subset of mice in the control group was followed for survival. The arrow indicates the day of rechallenge of mice that had remained tumor-free. Data show the mean ± SEM of 9 to 17 mice/group and are from 2 independent experiments combined. ***, P < 0.0005; **, P < 0.005.
improved survival (Fig. 6B and C). About 42% of mice with complete tumor regression following treatment with cyclophosphamide + imiquimod + RT remained tumor-free, whereas mice with complete regression after imiquimod + RT in different experiments all developed recurrent tumors. As cyclophosphamide was given only once before imiquimod and RT treatments started, it is likely that the strength of the initial antitumor response induced is what determines the long-term tumor control, whereas the cyclophosphamide-mediated reduction in IL-10-secreting T cells induced by imiquimod may contribute to the persistence of the response (30).

In different experiments, the percentage of mice with complete tumor regression following treatment with imiquimod + RT varied from 11% to 66%, with the largest percentage seen in mice receiving imiquimod up to day 35 (Fig. 2). Therefore, prolonged treatment with imiquimod may be required to achieve optimal effects.

Whether imiquimod is needed to sustain the immune response or to sensitize tumor cells to immune rejection remains to be established. The enhanced expression of MHC class I and ICAM-1 molecules induced by imiquimod on tumor cells (Fig. 4) suggests that the improved response of the secondary tumor to the abscopal antitumor response triggered by treatment of the primary tumor with imiquimod + RT can be due to better recognition of tumor by T cells. However, other effects of imiquimod could contribute to this sensitization, including angiogenesis inhibition (38), and increased T-cell recruitment and function (39). We are currently conducting a comprehensive analysis of the changes induced by imiquimod in TSA tumors as well as in patients with breast cancer (40) to address these questions.

Overall, the data presented indicate that topical imiquimod is a valuable adjuvant for use in the treatment of breast cancer metastases involving the skin. Imiquimod can improve antitumor immune responses by itself and in concert with RT. The addition of low-dose cyclophosphamide further enhanced these effects. The complexity of the microenvironment of a given tumor and the preexist-
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