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Treatment Failure of a TLR-7 Agonist Occurs Due to Self-Regulation of Acute Inflammation and Can Be Overcome by IL-10 Blockade

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Multiple TLR agonists have been shown to have antitumor effects in animal models. However, the therapeutic efficacy of TLR agonist monotherapy in cancer treatment has been limited, and the mechanisms of failure remain unknown. We demonstrate that topical treatment with a TLR-7 agonist, imiquimod, can elicit significant regression of spontaneous breast cancers in neu transgenic mice, a model of human HER-2/neu† breast cancer. However, tumor growth progressed once imiquimod therapy was ended. Gene expression analysis using tumor-derived RNA demonstrated that imiquimod induced high levels of IL-10 in addition to TNF-α and IFN-γ. Elevated levels of circulating IL-10 were also detected in sera from imiquimod-treated mice. Elevated serum IL-10 appeared to be derived from IL-10 and dual cytokine secreting (IFN-γ+ and IL-10+) CD4+ T cells rather than CD4+CD25+Foxp3+ T regulatory cells, which were also induced by imiquimod treatment. Blockade of IL-10, but not TGF-β, enhanced the antitumor effect of imiquimod by significantly prolonging survival in treated mice. These data suggest that the excessive inflammation induced by TLR agonists may result in a self-regulatory immunosuppression via IL-10 induction and that blocking IL-10 could enhance the therapeutic efficacy of these agents. The Journal of Immunology, 2010, 184: 000–000.

The use of TLR agonists as novel agents in cancer treatment has been extensively studied over the last several years in both the laboratory and the clinic. Activating the immune system via TLR can result in the generation of both innate and adaptive immune responses, either of which have the potential to significantly impact tumor growth. Presumably, adaptive immunity stimulated via TLR-activated APCs would control systemic metastatic disease. Although TLR ligation has been shown to result in a marked systemic or local inflammatory response when used as monotherapy (1, 2) such treatment has yet to be associated with significant clinical responses in most human malignancies.

One of the most commonly used TLR agonists is imiquimod—a TLR-7 specific agent, which was originally approved by the U.S. Food and Drug Administration for the treatment of genital warts. The antitumor effects of imiquimod have been shown in multiple animal models (3–6). Although antiangiogenesis and apoptosis induction have also been suggested as the mechanism of the antitumor action of imiquimod, its major mechanism is believed to be mediated via activation of dendritic cells (DCs) and promoting the generation of Ag- specific CTLs (7–9). Imiquimod has been shown to be a potent adjuvant for cancer vaccine (3, 8, 10) and to the potential clinical utility and mechanisms of action of TLR-7 ligation in a locally advanced disease setting.

Materials and Methods

Animals

A colony of neu-transgenic mice (strain name, FVB/N-TgN [MMTVneu]-202Mul) was established in our animal facilities from breeding pairs obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained under strict inbreeding conditions. All of the procedures described in this study were performed in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines. The mice harbor nonmutated, nonactivated rat neu under the control of the mouse mammary tumor virus (MMTV) promoter. Expression of neu under the MMTV promoter results in amplified expression of the protein in the breast epithelium (13). Spontaneous neu mediated breast cancers developed in the animals between 25 and 40 wk old.

Reagents

Five percent imiquimod (Aldara) and a vehicle control cream were supplied by 3M Pharmaceuticals (St. Paul, Minnesota). FBS was obtained from Gemini Bioproducts (Woodland, CA). RPMI 1640, PBS, penicillin-streptomycin, and l-glutamine were obtained from Life Technologies-BRL (Grand Island, NY). Flourochrome-conjugated Abs targeting CD3, CD4, and CD8 were obtained from BD Pharmingen and eBiosciences (San Diego, CA). The primer and probes for real-time PCR and Taqman Universal PCR Master Mix were obtained from Applied Biosystems (Foster City, CA). The H-2Dβ/RNEU120–429 (PDSLRLDSVF) tetramer was
obtained from the National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA). In general, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Topical treatment of mammary tumors with the TLR-7 agonist

Mice that developed palpable spontaneous mammary tumors were randomly assigned to be treated with imiquimod or a control cream. A thin film (25 μl) of either 5% imiquimod cream or control cream was applied daily to shaved skin over the tumor site on three consecutive days followed by 4 d of rest, which was defined as one cycle. The treatment duration was six cycles (6 wk). Tumor size was measured twice per week using Vernier calipers and calculated as the product of length × width × height × 0.52. Tumor size was compared between groups using the Student’s t test (two-tailed). For some experiments, tumors were depleted of specific lymphocytes using mAbs (clone GK1.5 for CD4+ T cells, clone 2.43 for CD8+ T cells, and clone PK1.36 for NK cells). The mAb (100 μg for CD8+ T cells and NK cells, 300 μg for CD4+ T cells) was given three times during the week before imiquimod treatment and twice per week during the treatment. The depletion efficiency (>95%) was monitored by flow cytometry. For the IL-10 and TGF-β blocking experiments, mice received i.p. injection of 100 μg antimurine IL-10 mAb (clone JES052A5; R&D Systems, Minneapolis, MN) or antimurine TGF-β (clone 1D11; R&D Systems) (14) twice per week during imiquimod treatment.

Flow cytometry

The staining of splenocytes was performed as we have previously described (15). Splenocytes were stained with CD3-FITC/RNeu420, tetramer-PE/CD8-PerCP/CD4-APCs. Cells derived from TDLNs were stained with CD80-FITC/CD86-PE/CD11c-PC5 and CD3-FITC/tetramer-PE/CD8-PerCP/CD4-APCs. Cells derived from TDLNs were stained with CD3-FITC/RNeu420–429, CD4-FITC/RNeu420, CD8-FITC/RNeu420, CD3-APC/RNeu420, CD4-PE/RNeu420, CD8-APC/RNeu420,γδ TCR-APC/RNeu420, and CD11c-PC5. Splenocytes were stained with CD3-FITC/RNeu420–429, CD4-FITC/RNeu420, CD8-FITC/RNeu420, CD3-APC/RNeu420, CD4-PE/RNeu420, CD8-APC/RNeu420,γδ TCR-APC/RNeu420, and CD11c-PC5. Splenocytes were stained with CD3-FITC/RNeu420–429, CD4-FITC/RNeu420, CD8-FITC/RNeu420, CD3-APC/RNeu420, CD4-PE/RNeu420, CD8-APC/RNeu420,γδ TCR-APC/RNeu420, and CD11c-PC5. Tumor cell suspensions were stained with purified anti-neu Ab (Ab-4; Calbiochem, San Diego, CA), biotinylated H2-Dq, or biotinylated H1-Aq (BD Pharmingen), followed by secondary staining using goat anti-mouse FITC or streptavidin-PE. For intracellular cytokine analysis, cells were activated with the leukocyte stimulation (13). Lung metastasis was observed in 30% of controls (n = 10; Fig. 1B) but in none of the imiquimod-treated mice (n = 21; p = 0.03), suggesting that imiquimod has a systemic antitumor effect in addition to directly inhibiting tumor growth.

Evaluation of TILs showed a significant increase in the ratio of CD8+ T cells, induced by TLR-7 ligation, significantly inhibit the growth of spontaneous breast cancers in neu transgenic mice

Topical imiquimod or a control cream was applied at the onset of palpable spontaneous tumors (average tumor size = 93 mm3). As shown in Fig. 1A, tumor growth was significantly inhibited in the group of mice that received imiquimod. After two cycles of therapy (day 15), imiquimod-treated tumors were significantly smaller than vehicle-treated tumors (125.3 ± 27.6 mm3 versus 302.8 ± 62.5 mm3; p = 0.03). After six treatment cycles, the average tumor size was 73 ± 5 mm3 in the imiquimod group versus 136 ± 262 mm3 in controls (p = 0.01; Fig. 1A). At the end of 6 wk of treatment, we evaluated the animals for the presence of spontaneous lung metastasis that is well described in these animals (13). Lung metastasis was observed in 30% of controls (n = 10; Fig. 1B) but in none of the imiquimod-treated mice (p = 0.03; Fig. 1B), suggesting that imiquimod has a systemic antitumor effect in addition to directly inhibiting tumor growth.

Evaluation of TILs showed a significant increase in the ratio of CD8+ T cells in imiquimod-treated mice (0.78 ± 0.17 in control tumors versus 2.62 ± 0.54 imiquimod-treated mice; p = 0.007; Fig. 1C). Tumor cells derived from imiquimod-treated mice expressed higher levels of MHC I (mean fluorescence intensity, 88.5 ± 0.5 in control versus 237.0 ± 4.0 in treated tumors; p = 0.01; Fig. 1D). The expression of MHC II was not changed by the treatment (p = 0.21). To examine whether the observed MHC I upregulation on the surface of tumor cells was a direct effect of imiquimod, we treated MMC—a tumor cell line derived from syngeneic tumors in these mice—with imiquimod. Results demonstrate that there was no upregulation of MHC I after direct stimulation (p = 0.37; imiquimod versus PBS-treated cells; Supplemental Fig. 1), indicating that the effect on MHC I after in vivo treatment was probably indirect via induction of proinflammatory cytokines. Furthermore, we found that these tumor cells did not express TLR-7 (Supplemental Fig. 2).

Evaluation of rat neu Ag-specific CD8+ T cells among splenocytes using tetramer staining demonstrated a significant increase in imiquimod-treated mice (0.99 ± 0.1% in control versus 3.3 ± 0.7% after 1 wk imiquimod treatment; p = 0.007), which persisted throughout treatment (Fig. 1E). To further determine the role of CD8+ T cells in mediating the antitumor effect of imiquimod, mice were selectively depleted of CD4+, CD8+ T cells, or NK cells during treatment. As shown in Fig. 1F, the antitumor effect of TLR-7 ligation was completely abrogated by CD8+ T cell depletion.
but not by CD4+ T cell or NK cell depletion. Histologic examination of the tumors after 6 wk of treatment showed hemorrhage and fibrin deposition as well as cholesterol clefs, suggesting excessive inflammation induced by the imiquimod treatment (Fig. 1G).

Once imiquimod treatment was stopped, however, tumor growth progressed again. Fig. 1H shows the regrowth of tumors in mice after 3 wk imiquimod treatment (Fig. 1H). Tumors grew back similarly even after longer treatment (data not shown). To determine the potential mechanisms of failure of the induced adaptive immune response, we examined serial gene expression changes in the tumors during imiquimod treatment.

**TLR-7 ligation elicits the upregulation of both pro- and anti-inflammatory cytokine genes in the tumor as well as elevated serum levels of IL-10**

Gene expression analysis demonstrated that after one cycle of imiquimod treatment, there was significant change in the expression of 646 genes among the total of 22,000 examined genes (p < 0.05). GSEA exploring all KEGG mouse pathways showed that multiple immune response pathways were activated in the tumor, including TLR signaling pathways and cytokine-cytokine receptor pathways (Supplemental Table I). Both proinflammatory (e.g., IL-2, IFN-γ, TNF) as well as anti-inflammatory (e.g., IL-10) genes were upregulated in the cytokine receptor pathway after imiquimod treatment (Fig. 2A). Validation of expression of TNF-α, IFN-γ, TGF-β, and IL-10 (Fig. 2B–E) was performed by quantitative PCR. Although the levels of TNF-α and IFN-γ increased to 4.4 fold and 8.0 fold of control after 1 wk imiquimod treatment (p = 0.03 and 0.02, respectively; Fig. 2B, 2C), the level of IL-10 was increased to 42.6-fold of control (p = 0.006; Fig. 2D). This increase was maintained at 2 wk of treatment. Of note, the level of TGF-β gene expression in the tumor was not changed by imiquimod treatment; the expression level at week 1 was 0.74-fold of control (p = 0.24; Fig. 2E). We also examined the expression level of IL-13, another immunosuppressive
FIGURE 2. TLR-7 ligation elicited the upregulation of both proinflammatory and anti-inflammatory cytokine genes in the tumor as well as elevated serum levels of IL-10. A, Heat map of genes in the cytokine-cytokine receptor pathway, which was shown to be upregulated by GSEA. Red indicates high expression level, and blue indicates low expression level. The four columns on the left are tumor samples from the control group, and the four columns on the right are tumor samples from imiquimod-treated mice. All genes treated tumors and tumors from 1 wk or 2 wk of IL-10. B–E, The relative expression levels (mean ± SEM) of TNF-α (B), IFN-γ (C), IL-10 (D), and TGF-β (E) in control untreated tumors and tumors from 1 wk or 2 wk imiquimod-treated mice. All genes are normalized to β-actin. *p < 0.05, from control. F, The level of serum IL-10 from control (○) or imiquimod-treated (●) mice before treatment or at 1, 2, 3, 4, and 5 wk after treatment. Each data point represents the value from an individual mouse. The horizontal bar represents the mean in each group. The experiment was repeated twice with similar results.

Both CD4+Foxp3+ Treg and IL-10 secreting regulatory Th1 cells are induced by TLR-7 ligation.

It is known that CD4+CD25+Foxp3+ Treg cells can exert their inhibitory effect partially via secretion of IL-10 (23), so we examined whether Foxp3+ expressing T cells were the source of elevated serum IL-10. As shown in Fig. 3, imiquimod treatment resulted in elevated levels of CD4+CD25+Foxp3+ Treg cells in spleen (6.19 ± 0.33% versus 10.70 ± 0.76%; p = 0.0006; Fig. 3A), TDLNs (4.41 ± 0.32% versus 7.48 ± 0.72%; p = 0.0046; Fig. 3B), and TILs (1.55 ± 0.41% versus 3.01 ± 0.25%; p = 0.026; Fig. 3C). The ratio of CD8+ T cells to Foxp3+ Treg cells decreased after imiquimod treatment in spleen (3.04 ± 0.14 to 1.78 ± 0.28; p = 0.004), TDLNs (4.48 ± 0.40 to 2.96 ± 0.21; p = 0.010), and TILs (12.35 ± 2.73 to 4.38 ± 0.55; p = 0.038). However, simultaneous staining of IL-10 and Foxp3 showed that IL-10 is secreted mainly from Foxp3-negative T cells, indicating that Foxp3+ Treg cells are not the major source of elevated serum IL-10 (Fig. 3D, 3E).
Intracellular cytokine staining using splenocytes from control and imiquimod-treated mice demonstrated that imiquimod treatment increased T cells secreting IFN-γ (3.45 ± 0.37% versus 9.49 ± 0.93%; p = 0.0003; Fig. 4A) and IL-10 (0.30 ± 0.05% versus 1.79 ± 0.64%; p = 0.04; Fig. 4B). Furthermore, imiquimod induced a population of CD4+ T cells secreting both cytokines (Fig. 4C, 4D). The dual cytokine-secreting cells, which are rarely found in T cells from control tumor-bearing mice (0.11 ± 0.01%), increased more than 10-fold to 1.53 ± 0.52% in splenocytes derived from imiquimod-treated mice (p = 0.02, compared with control).

IL-10 blockade augments the antitumor effect of imiquimod and results in long-term survival of imiquimod-treated mice

To determine whether imiquimod-induced IL-10 secretion by T cells was operative in treatment failure, we treated mice with an anti–IL-10 mAb. An anti–TGF-β mAb was also evaluated to counteract TGF-β secretion by the elevated levels of Treg cells. As shown in Fig. 5, the control untreated mice died at 32 ± 8 d after the start of the experiment. Imiquimod alone (6 wk treatment) enhanced the survival time to 54 ± 5 d (p = 0.0004 from control). Blockade of TGF-β did not prolong the survival time (52 ± 3 d; p = 0.26 from imiquimod alone). Blockade of IL-10 significantly enhanced the
antitumor effect of imiquimod and significantly prolonged survival ($p = 0.002$, from imiquimod alone).

**Discussion**

Imiquimod is commonly used in the treatment of genital warts and superficial basal cell carcinomas as well as other noninvasive cancers (24, 25). When topical imiquimod was evaluated as a treatment for invasive melanoma, however, sustained remissions were rarely observed. Recent evidence suggests that only metastatic lesions located in the dermis will respond to the topical therapy (26). Using a mouse model of invasive breast cancer, we have found that TLR-7 ligation by imiquimod results in acute tissue inflammation and significant inhibition of spontaneous mammary tumor growth in neu-transgenic mice. However, tumors grew back after treatment withdrawal. These studies demonstrate that imiquimod therapy not only stimulates proinflammatory cytokines and augments Ag-specific CD8$^+$ T cells, but also stimulates the proliferation of Foxp3$^+$ regulatory T cells, IL-10 secreting CD4$^+$ T cells, and dual secreting IFN-γ+IL-10+CD4$^+$ T cells, all of which have been shown to be involved in self regulation of inflammation. IL-10, but not TGF-β blockade, significantly enhanced the antitumor effect of imiquimod, resulting in markedly prolonged survival of treated mice.

The observation that acute inflammation can lead to tumor destruction has been previously reported for a variety of local treatments, such as intratumoral bacterial/viral injection, cytokine injection, and cryoablation (27–29). All of these treatments have been shown to induce intratumor inflammation that favors cross-priming of tumor Ags and upregulation of MHC I. The activation of TLR might be the common underlying mechanism for many of these local treatments. Our study demonstrated that imiquimod not only induces local inflammation, but also has a systemic effect, as evidenced by the absence of lung metastasis in treated mice and the systemic increase in Ag-specific CD8$^+$ T cells, which seems to mediate the antitumor effect of imiquimod. This finding is consistent with previous reports that topical imiquimod as a vaccine adjuvant can augment CTL response to protein, peptide, or DC vaccine (4, 8, 10, 30). The potential mechanism that can account for the failure of TLR agonists in eradicating cancer, however, is not well defined. Our study demonstrated that imiquimod can activate the self-regulatory feedback, including Treg cells and IFN-γ/IL-10–secreting CD4$^+$ T cells, and blockade of IL-10 can augment the antitumor effect of imiquimod.

The interplay of TLR ligation with DC activation and Treg cell stimulation is complex. Both DCs and Treg cells have been shown to express functional TLRs, and activation via the receptor can have an immune-stimulating or -suppressing effect (31). Using RT-PCR, we found that the expression of TLR-7 on Treg cells (CD4$^+$CD25$^+$), although much lower than that on DCs, is significantly higher than TLR-7 on CD4$^+$CD25$^+$ cells and CD8$^+$ T cells (data not shown). Whether this relatively higher expression of TLR-7 on Treg cells is related to the observed imiquimod-induced increase in Treg

**FIGURE 4.** IL-10–secreting CD4$^+$ T cells are induced by TLR-7 ligation. A–C, Summary graph showing the percentages of CD4$^+$ T cells secreting IFN-γ (A), IL-10 (B), or both IFN-γ and IL-10 (C) in control and imiquimod-treated (6 wk) mice. Each data point represents data from an individual mouse. The horizontal bar represents the mean in each group. D, Representative flow graphs showing cytokine-positive cells among all CD4$^+$ spleenocytes in control and imiquimod-treated mice. Similar results were obtained from two independent experiments.

**FIGURE 5.** IL-10 blockade augments the antitumor effect of imiquimod and results in prolonged survival of imiquimod-treated mice. Mice with spontaneous breast tumors received control cream (○), topical imiquimod (●), imiquimod plus anti–mTGF-β Ab (▲), imiquimod plus anti–mIL-10 Ab (▲), imiquimod plus rat IgG (◇), or anti–IL-10 Ab and anti–mTGF-β Ab without imiquimod (□) for 3 wk. Tumor growth was continuously monitored for an additional 2 mo after treatment withdrawal (4–6 mice per group). Two independent experiments showed similar results.
cells, both systemically and in the tumor, remains to be investigated. Previous studies have reported contradicting effects of imiquimod on Treg cells (32, 33). In a study on the use of imiquimod to treat squamous cell carcinomas of the skin in immunosuppressed organ transplant patients in whom Treg are abundant (32), investigators demonstrated that imiquimod treatment reduced the numbers of Foxp3-expressing Treg cells in treated lesions. Moreover, evaluation of the infiltrating Treg cell population suggested that TLR-7 ligation resulted in decreased expression of Foxp3, IL-10, and TGF-β in those cells (32). A more recent publication by Forward et al. (33) showed that TLR-7 agonists including imiquimod enhance the immunosuppressive activity of murine CD4+CD25+ Treg cells. Whether the controversial data are due to different host system, mouse versus human, or different treatment method, remains to be investigated in future studies.

Although Treg cells have been shown to secrete IL-10 (23, 34), we found that the Treg cells induced by imiquimod expressed little IL-10, leading us to assume that the CD4+CD25+Foxp3+ population did not substantially contribute to elevated serum IL-10 levels observed in treated animals. Intracellular cytokine staining analysis of different immune subsets showed that IL-10 was produced mainly by CD4+ T cells, but not by DCs (CD11c+), monocytes (CD14+), macrophages (F4/80+), or CD8+ T cells (data not shown). Our finding is consistent with a recent publication on the role of IL-10 blockade in controlling murine viral infection, which shows that CD4+ T cells produce copious amounts of IL-10 (35). Although it has been shown that tumor cells can also secrete IL-10 (36–38), one can assume that tumor cells are not the major source of IL-10 in the current mouse model, based on the observation that tumors are much smaller in imiquimod-treated mice (Fig 1A), yet the serum IL-10 level is higher in imiquimod-treated mice.

Two types of CD4+ T cells secreting IL-10 were significantly elevated in imiquimod-treated animals compared with controls: IL-10+ Th2 and dual-secreting IFN-γ/IL-10+CD4+ T cells. It is well established that Th2 cells are anti-inflammatory and a necessary response to pathologic inflammation (39). In situations of tissue destructive inflammation, such as that seen in autoimmunity disease, Th1 can also become self-regulatory by cosecreting IFN-γ and IL-10. These cells have been shown to suppress maturation of DCs and the further differentiation of Th1 in response to Ag (40). Investigations have suggested that IFN-γ secretion enhances IL-10 secretion, particularly in disease conditions in which the host has already been primed to Ag, such as in chronic infection or cancer (41). Ag-experienced T cells appear to require IFN-γ to further enhance IL-10 secretion for the inhibition of Ag-specific T cell responses, especially CD8+ T cells (41). In the studies presented, the demonstration of enhanced treatment efficacy with IL-10 blockade underscores the potential importance of IL-10–secreting CD4+ T cells as a mediator of treatment failure after TLR-7 ligation. The inhibitory effect of IL-10 on Ag-specific T cells has been well documented. Viral infections in mice have been shown to result in upregulation of IL-10, inhibiting effector T cell function and leading to chronic infection (42). Serum containing soluble IL-10 has been shown to inhibit granzyme B release from EBV specific T cell clones (43). Blocking IL-10 receptor restores effector function and results in rapid elimination of virus (42, 44). Interestingly, it has been shown that the TLR-9 agonist CpG can also induce IL-10, potentially via activation of p38 MAPK, and IL-10 blockade, or inhibiting the p38 MAPK signaling in DC can augment the antitumor effect of CpG (45, 46).

Our work suggests that a mechanism of treatment failure with TLR-7 ligation is the host self-regulatory response to induced inflammation. Moreover, IL-10 secretion appears to be an important mediator of immunosuppression, and IL-10 blockade greatly enhanced the antitumor effect of TLR-7 ligation. Topical use of imiquimod in breast cancer with inflammatory breast cancer or cutaneous metastasis of breast cancer is currently tested in a clinical trial in our group. Whether IL-10 blockade is feasible in patients with cancer needs to be further investigated, because this study did not address the potential of developing immune-related side effects. A recent publication on patients with cancer receiving anti–CTLA-4 therapy reported that decreased levels of IL-10 coincided with the patient’s immune-related adverse events (47). This finding indicates that caution needs to be taken before the IL-10 blockade approach can be translated into cancer treatment.

Disclosures
The authors have no financial conflicts of interest.

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