Imiquimod Enhances the Systemic Immunity Attained by Local Cryosurgery Destruction of Melanoma Lesions

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Melanoma lesions can be frozen *in vivo,* resulting in necrotic death of malignant cells and in tumor antigen release suitable for cross-presentation by professional antigen-presenting cells. Imiquimod is a small molecule with adjuvant pro-inflammatory effects that can be topically delivered as a cream. Local cryosurgery of B16/ovalbumin (OVA)-derived subcutaneous tumor nodules leads to curative destruction of the lesions. If imiquimod is repeatedly applied on the cryo-treated lesion, a conspicuous, leukocyte-rich inflammatory infiltrate appears during the days following treatment. Mice treated by cryosurgery plus imiquimod rejected rechallenges of B16/OVA in 90% of the cases, whereas cryosurgery alone failed to prevent tumor grafting in 70% of the cases. The combination treatment of B16/OVA tumors was also able to protect 60% of the mice against outgrowth of a lethal dose of non-transfected B16 tumor cells. Addition of imiquimod to cryosurgery results in increases of the cellular immune response against tumor antigens as measured by *in vitro* IFN-γ production and T-cell proliferation in response to OVA. The potent memory response is not only directed against the OVA epitope, but also toward a broader range of B16 antigens. Our data indicate that these combined treatments turn the treated tumor lesion into an autologous tumor vaccine, which is even able to cause vitiligo in several cases. These preclinical data and the simplicity of the procedures warrant the design of a pilot clinical trial.

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INTRODUCTION

In situ tumor destruction with several techniques has received increasing attention as a minimally invasive option for local management of cancer. As a result of *in situ* cancer cell destruction, large amounts of tumor debris are released and they could be potentially taken up by immune-system cells. For long it has been discussed whether this tumor debris is able to induce a systemic immune response and it has been demonstrated that maturation of antigen-presenting cells, especially dendritic cells (DCs), is a prerequisite for the induction of adaptive immunity (De Vries *et al.*, 2003).

Cryoablation, or destruction of tissue by freezing, has long been recognized as a potential tool in the treatment of cancer (Gage *et al.*,1998). Cryosurgery has been successfully used for the ablation of skin, prostate (Cohen, 2004), kidney (Gill *et al.*, 2005), bone (Bickels *et al.*, 1999), and liver tumors (Seifert and Junginger, 2004), as well as for treatment of locally advanced breast malignancies (Kaufman and Rewcastle, 2004) and even fibroadenomas (Littrup *et al.*, 2005). Besides local destruction of the primary tumor, it has been claimed that cryoablation is sometimes able to induce systemic antitumor immune responses. Several case reports mention a reduction of metastatic disease after cryoablation of the primary tumor (Gage, 1969; Soanes *et al.*, 1970). Moreover, together with its local destructive effect, cryosurgery also leads to an inhibition of secondary and metastatic tumor growth compared with surgical excision (Joosten *et al.*, 2001).

Tumor cryoablations release large amounts of tumor antigens in the form of necrotic tumor cells and cellular debris, and enhance migration of DCs from the tumor site to the draining lymph nodes (Sauter *et al.*, 2000; Gazzaniga *et al.*, 2001). Cryoablation induces both a tumor-specific T-cell response in the tumor draining lymph nodes and an increased systemic natural killer (NK) cell activity, which correlates with rejection of tumors on rechallenge. This immunologic response is clearly limited and it may be necessary to augment it by adding an adjuvant (Sabel *et al.*, 2005). A recent paper has shown the systemic antitumor immune effect of tumor cryosurgery combined with subsequent *in situ* injection of autologous unmodified immature DCs (Machlenkin *et al.*, 2005). According to other reports, it

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Abbreviations: DC, dendritic cell; OVA, ovalbumin; pDC, plasmacytoid DC; TLR, toll-like receptor

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is useful to either administer a blocking monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4 (den Brok *et al.*, 2004), the toll-like receptor (TLR)9-ligand CpG-ODN (den Brok *et al.*, 2006), or an immunotheraphy with IL-2 and GM-CSF (Ito *et al.*, 2003) at the time of local tumor destruction.

Imiquimod is an immunomodulatory, small-molecule compound in the imidazoquinoline family that displays both antiviral and antitumor effects through TLR-7 (Hemmi *et al.*, 2002). As a TLR-7 agonist, imiquimod stimulates immature plasmacytoid DCs (pDCs). Through the induction of the surface expression of costimulatory molecules such as CD40, CD80, and CD86, as well as through the production and release of specific cytokines, especially IFN-α, TNF- α, and IL-12, imiquimod-treated DCs induce type-1 T-helper immunity. Imiquimod in the treatment of primary (melanoma lentigo maligna) or metastatic melanoma has been used previously (Ahmed and Berth-Jones, 2000; Steinmann *et al.*, 2000).

In this report, we tested whether adjunctive treatment with the TLR-7 agonist imiquimod could augment antitumor immune responsiveness in B16/ovalbumin (OVA) tumor mice treated with cryosurgery. Mice that received either imiquimod alone or cryosurgery alone were partially protected from tumor development. Strikingly, mice that received cryosurgery and topical imiquimod were protected even from a subsequent tumor cell challenge.

RESULTS

Development of an *in situ* tumor ablation model

To investigate the induction of antitumor immune responses following *in situ* tumor destruction, we developed a mouse B16/OVA model in which cryosurgery was used to destroy established tumors. On day 10 after tumor inoculation on the flank, B16/OVA tumors were treated either by cryosurgery or excision. Non-treated mice developed large tumor burdens by day 20 of tumor growth, at which time the mice were killed for analysis. Efficacy of cryosurgery was judged by the occurrence of local recurrence during all further experiments described. Local recurrence after cryosurgery was observed only in 5% of the mice (Figure 1), compared with 20% of recurrences observed after tumor excision.

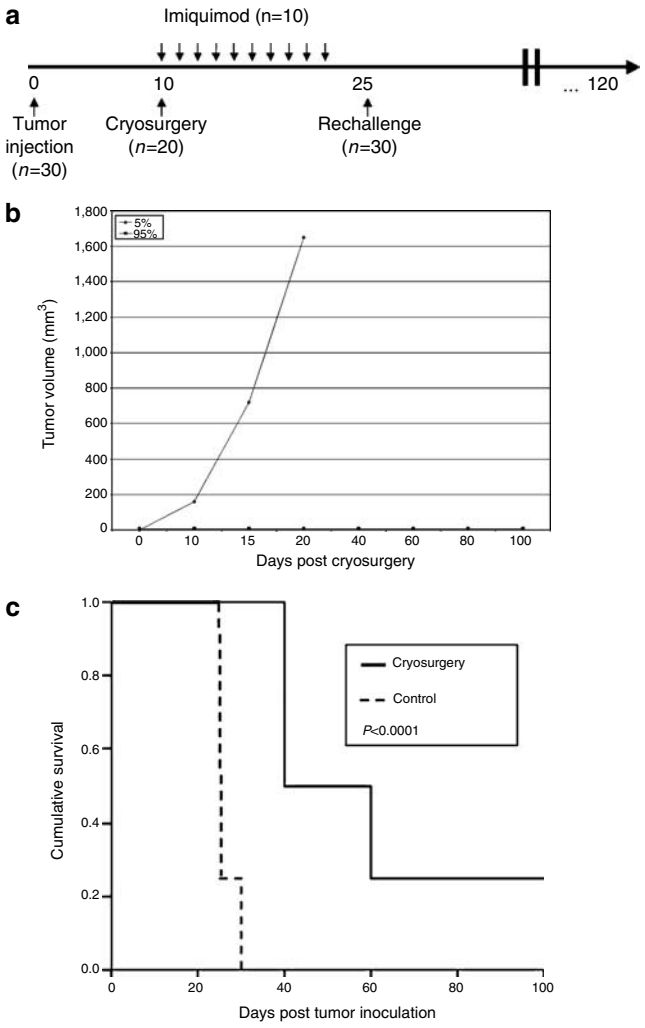

Figure 1. Experiment design, local recurrence, and survival. (**a**) Time schedule outlining the different treatments used in the experiments. Ten days after tumor injection, B16/OVA melanoma tumors (6–8 mm) were treated with cryosurgery. Fifteen days after cryosurgery mice were rechallenged. (**b**) Local recurrence of B16/OVA tumors after cryosurgery. (**c**) Cryosurgery of B16/OVA tumors resulted in a clear delay in the outgrowth of tumors after rechallenge with B16/OVA tumor and in a low level of protection (25% of the mice), compared with either mice undergoing surgical excision or control naïve mice.

Effect of cryosurgery and tumor excision

The effect of cryosurgery of the primary tumor on the growth rate of the second tumor in the flank was determined and compared with excisional treatment. A detailed time schedule of the different treatments is given in Figure 1. Rechallenge was given 15 days after cryosurgery. In some experiments, because of the occurrence of local recurrences in several mice treated with surgical excision, age-matched, untreated naïve mice were used as control. As shown in Figures 1 and 2, cryosurgery of B16/OVA resulted in a clear delay in the outgrowth of B16/OVA tumor cells and in a low level of protection (25–30% of the mice). On the contrary, a rechallenge with the less immunogenic B16F10 melanoma cell line (3×10^4) 15 days after cryosurgery resulted in all mice developing tumors, with some delay compared with controls, but with no protection whatsoever (data not shown).

Effect of topical imiquimod

Topical imiquimod treatment without cryosurgery was not sufficient to eradicate either the primary tumor or a tumor rechallenge. The clinical response of the melanoma to imiquimod depends on the size of the tumors at the beginning of treatment. When tumor size at the beginning of the experiment was $>150\,mm^3$, all mice treated with imiquimod showed progressive disease. A partial response was noted instead when topical imiquimod was begun at the same time of tumoral cell injection (data not shown).

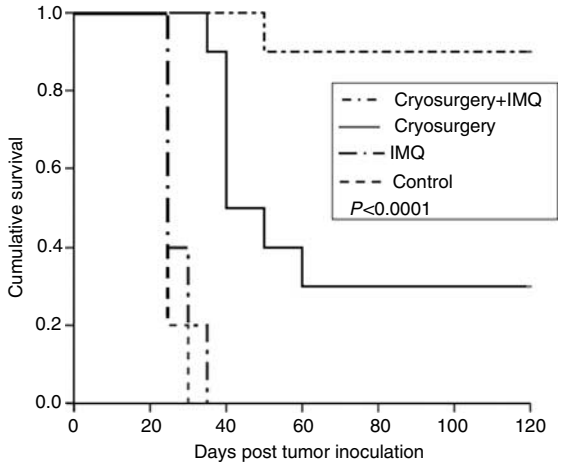

Figure 2. Kaplan-Meier Survival. The same time schedule of Figure 1 was used. Mice with B16/OVA tumors (6–8 mm) were treated with cryosurgery. At the same time, mice were topically treated with imiquimod or vehicle cream for 10 days. Fifteen days later, a rechallenge with 3×10^4 B16/OVA cells was given in the contralateral flank. Survival curve shows 90% protection of melanoma outgrowth after combined treatment with cryosurgery and imiquimod ($P<0.0001$) ($n=8$–10). Five age-matched untreated naïve mice were used as a control. One representative experiment of three independent experiments is shown. Mice in the topical imiquimod group were killed as soon as primary tumor volume reached 1,500 mm^3, with no sign of protection against rechallenge, and died with an average survival time overlapping to that of the control group (data not shown).

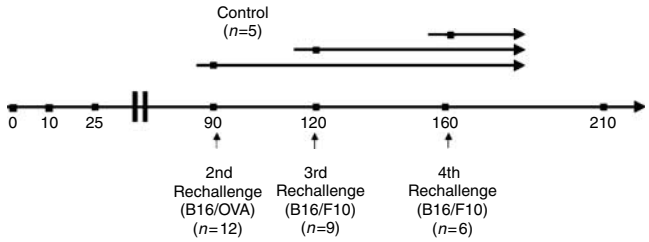

Figure 3. Survivors of experiments of Figure 2 after several rechallenges, according to the time schedule outlining the different treatments as used in the experiments. Five age-matched, untreated naïve mice were used as control for each rechallenge. To test the induced tumor resistance, 12 of the cured mice were repeatedly rechallenged (up to three times) with different tumor dose levels, (day 90, second rechallenge with 10^5 B16/OVA; day 120, third rechallenge with 3×10^4 B16F10; day 160, fourth rechallenge with 2×10^5 B16F10, data not shown). Seventy-five percent protection was observed in mice rechallenged with B16/OVA (cryosurgery + imiquimod group). Surviving mice were rechallenged with 3×10^4 B16F10 cells on the controlateral flank, with a 66% protection rate at day +160 for mice initially treated with the combination of cryosurgery and imiquimod.

Topical application of imiquimod induced skin inflammation with increased number of inflammatory cells in the dermis. Histochemistry systemically revealed a large subcutaneous population of infiltrating leukocytes (data not shown). Mice that had been treated daily with imiquimod for 10 days presented a pronounced splenomegaly, compared with the normal spleens of either naive or untreated mice with tumor (data not shown).

Effect of cryosurgery and topical imiquimod

Following the same time schedule of Figure 1, we explored whether *in situ* tumor destruction in combination with topical imiquimod could enhance the antitumor immunity in our model. Imiquimod or placebo cream was applied once a day to the skin of the mice the day of and during 9 days after cryosurgery. Fifteen days later, mice were challenged in the contralateral back with a lethal dose (3×10^4 cells) of the B16/OVA tumor. The combination of cryosurgery and imiquimod treatment resulted in the protection against such a lethal dose in 80–90% of the mice (Figure 2). Mice treated with cryosurgery + imiquimod that had rejected the first B16/OVA tumor challenge were completely protected against a second B16/OVA tumor rechallenge (10^5 cells) 90 days later. In comparison, all cryosurgery-treated mice developed second tumors (Figure 3).

To study the immune response against antigens other than OVA protein, we used the poorly immunogenic B16F10 melanoma cell line. We investigated whether the cryosurgery + imiquimod-treated mice that had rejected the previous rechallenge with B16/OVA tumor were protected against a third rechallenge with a lethal dose of B16F10 (3×10^4). Six out of 12 mice from the group that underwent local cryosurgery and topical imiquimod treatment were alive at day 160 following three rechallenges, whereas no mice from any other group survived at that time point (Figure 3). A fourth rechallenge with 2×10^5 B16F10 cells was ultimately lethal to all survivors, although a delayed outgrowth of the B16F10 tumor was also observed compared with controls (data not shown).

In summary, only mice that were treated *in situ* by cryosurgery and topical imiquimod had a statistically significant survival advantage ($P<0.0001$) in comparison with untreated. This combination treatment induces a potent memory response, which is not only directed against the OVA epitope, but also toward a broader range of B16 antigens.

Inflammatory infiltrates in the treated and rejected tumors

The pathology study of primary tumors showed no differences between cryosurgery + imiquimod-treated mice and cryo-treated mice or between imiquimod-treated mice and controls. An intense infiltrative response consisting of polymorphonuclear leukocytes around the tumor was detected 24 hours after cryosurgery. At day 7, tumor tissue was completely replaced by necrotic masses and fibrosis (Figure 4).

The pathology study of secondary tumors growing after rechallenge also showed no relevant differences among all mice groups. In a subsequent experiment, we indelibly marked the administration site of B16/OVA tumor rechallenge and 48 hours later it was analyzed from a histological standpoint in all mice groups (i.e., those respectively treated with imiquimod, cryosurgery, the combination of both, and controls). Remarkably, as no infiltrating neutrophils were seen in controls, such an infiltration was conspicuously more pronounced in mice treated with combination treatment, compared with either therapy option alone. Indeed, only in

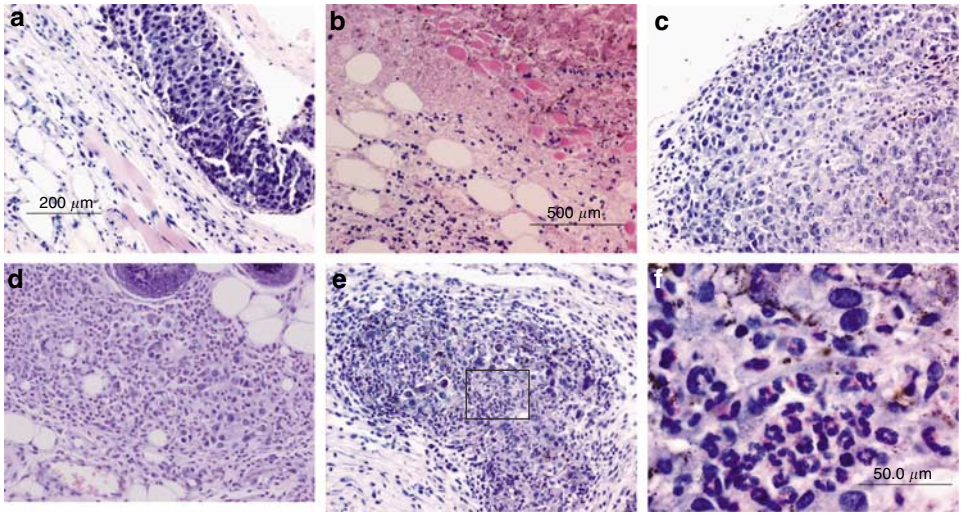

Figure 4. Hematoxylin–eosin staining of representative mice. (**a**) Untreated tumor control with well-defined tumor areas, at day 2. (**b**) Tumor treated with cryosurgery. Extensive intratumoral necrotic area and surrounding fibrotic tissue with a massive leukocyte infiltration at day 7. (**c**) Neutrophil infiltration around a tumor nest in a cryosurgery-treated mouse. (**d**) Massive chemotaxis and B16/OVA tumor cell destruction in a mouse treated with the combination of cryosurgery and imiquimod. (**e**) Massive chemotaxis and B16F10 tumor cell destruction in a mouse treated with the combination of cryosurgery and imiquimod. (**f**) Higher magnification of the area boxed in (**e**), showing an intense neutrophil infiltrate. (**c–f**) are based on a rechallenge removed 48 hours after its implantation. (**a**, **c**, **d**, and **e**) original magnification ×200; (**b**) original magnification ×100; (**f**) original magnification ×1000.

lesions from mice treated with the combination of cryosurgery and imiquimod we were able to detect a massive neutrophil infiltrate, even with signs of tumor cell lysis (Figure 4). To establish whether such an inflammatory response may be specifically directed against the OVA protein, we repeated this experiment following a rechallenge with B16F10 cells. The results were virtually identical, proving that leukocyte infiltration secondary to combination treatment also targets B16 cell line antigens different from OVA protein.

Vitiligo

Cryosurgery induced localized vitiligo at the site of tumor implantation in 80% of mice. Cryosurgery associated with topical imiquimod induced localized and generalized vitiligo in several mice (Figure 5). This finding had not been observed in experiments with imiquimod alone and indicates that the effects of imiquimod can be seen systemically when used as an adjuvant to cryosurgery.

Proliferation assay and measurement of cytokine production

To analyze the immune response induced after either tumor cryoablation or cryosurgery plus imiquimod treatment, we performed a proliferation assay. Lymphocytes were plated in 96-well plates alone or with 10 μM of OVA.

In Figure 6a, the stimulation index is comparatively given according to treatment groups. Lymphocytes from control animals did not proliferate in the presence of OVA protein. *Vice versa*, lymphocytes from treated animals proliferated in response to OVA protein, although with a relatively weak stimulation index. Moreover, the stimulation index of lymphocytes from mice treated with cryosurgery was lower than that of those treated with cryosurgery + imiquimod.

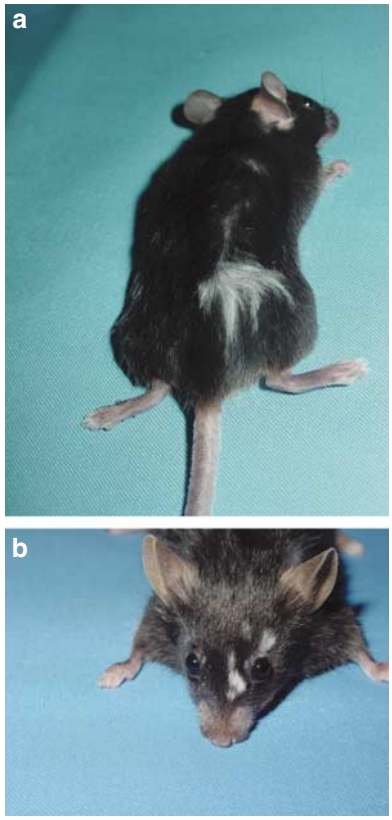

Figure 5. Vitiligo in treated mice. (**a**) Cryosurgery induced localized vitiligo at the application site. (**b**) In several mice, cryosurgery associated with topical imiquimod induced both localized and distant site vitiligo.

In Figure 6b, data on IFN-γ production when lymphocytes are stimulated *in vitro* with either OVA protein or culture medium as a control are comparatively given according to treatment groups. IFN-γ production is lower in mice treated

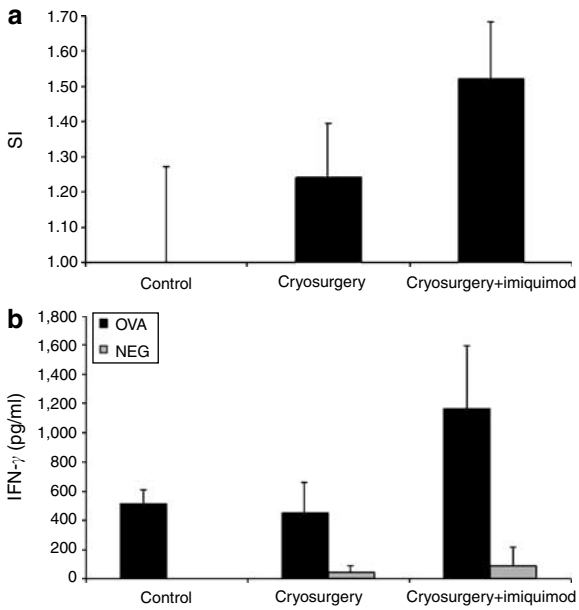

Figure 6. Characterization of immune response induced after different treatments. Animals from different groups were killed and lymph node removed. Lymphocytes were stimulated *in vitro* alone or with OVA protein. Supernatants were collected to measure IFN-γ production and cells pulsed with [^{3}H] thymidine for proliferation assay. Thymidine incorporation was determined and the stimulation index calculated. (**a**) The stimulation index is comparatively given according to treatment groups. (**b**) Data on IFN-γ production when lymphocytes are stimulated *in vitro* with either OVA protein or culture medium as a control.

with cryosurgery than in those treated with cryosurgery + imiquimod.

Assay of NK cell activity and CTL responses

Neither NK cell activity nor CTL responses were observed in any mice treatment group (data not shown).

DISCUSSION

Metastatic melanoma of the skin is extremely difficult to treat. Despite a variety of available options including surgery, immunotherapy, chemotherapy, X-ray therapy, regional hyperthermia, and carbon dioxide laser ablation treatment is often unsuccessful. DC vaccination has been shown to evoke tumor-specific responses in several patients with melanoma (Nestle *et al.*, 1998), although in many cases these *ex vivo*-generated DC are ineffective to cure patients with established melanoma. Melanocytes are sensitive to low temperatures and cryosurgery has been proposed as an alternative to melanoma surgery. However, recurrence rates up to 50% are reported (Gaspar and Dawber, 1997); this fact may be partly explained by extension of malignant melanocytes down hair follicles and other dermal appendages, where they are relatively protected from the effects of cryosurgery. Cryosurgery exerts damage effect on tumor cells, including but not limited to disruption of metabolic pathways, conversion of the lipid layers of the cell membrane into semisolids, breakdown of the cytoskeletal microtubular networks, exposure of the cells to a hyperosmotic environment, and the grinding action of irregular ice formations. In our hands, although a tumor-specific immune response was observed when leaving the tumor frozen, mice were weakly protected against a tumor rechallenge (25–30%).

To increase antitumor immunity following cryosurgery, we explored the stimulation of TLR7 by imiquimod. Imiquimod (1-(2-methylpropyl)-1*H*-imidazol (4,5-c)quinolin-4-amine) is an imidazoquinoline compound that is currently approved for the treatment of genital human papillomavirus infection, basal cell carcinoma, and actinic keratoses. Recently, significant interest has developed in the use of imiquimod for lentigo maligna and metastatic malignant melanoma (Ahmed and Berth-Jones, 2000; Steinmann *et al.*, 2000). Several patients with skin melanoma metastases or *in situ* melanoma have been treated with imiquimod under different protocols, with complete or almost complete clinical and pathological remission in all cases (Steinmann *et al.*, 2000; Vereecken *et al.*, 2003; Wolf *et al.*, 2003). However, contradictory results have also been reported (Ugurel *et al.*, 2002; Fisher and Lang, 2003). Imiquimod induces apoptosis in malignant melanoma cells in a rather tumor-selective manner both *in vitro* and *in vivo* (Schon *et al.*, 2004). The proapoptotic activity of imiquimod is independent of membrane-bound death receptors, whereas it depends on Bcl-2 expression, as demonstrated by overexpression of Bcl-2 in melanoma cells. The application of imiquimod in modifying the expression of metalloproteinases, promoting the expression of their inhibitors and modifying the expression of genes involved in angiogenesis, could potentially create a local microenvironment unfavorable for tumor expansion (Hesling *et al.*, 2004). Despite the striking clinical effect in our experiments, we did not see any specific action of imiquimod directly involving tumor biology. However, when the cryoablation of established B16/OVA tumors was combined with topical imiquimod administration, potent antitumor immune responses were induced. Intriguingly, the combination treatment of B16/OVA tumors was also able to protect 60% of the mice against outgrowth of a lethal dose of B16F10 tumor. These results indicate the simultaneous induction of responses against multiples epitopes and not only against the OVA protein. The combination of sharp excision with imiquimod was not examined because the hypothesis holds that only cryo-induced tissue destruction would make tumor antigens sufficiently available for an enhanceable immune response.

Theoretically, the mechanism responsible for the induction of antitumor immunity following combination treatment with cryosurgery and imiquimod should be related to DCs. In human skin, epidermal Langerhans' cells and dermal DCs continuously monitor the antigenic environment and any encounter with damaged tissue induces their activation and migration to the draining lymph nodes. The expression of TLR7 in mice is considerably different from humans, allowing direct dermal DCs and Langerhans' cell activation in the mouse. Topical cutaneous application of the TLR7 imiquimod can induce the maturation and migration of cutaneous

DCs. It has recently been shown that topical imiquimod leads to Langerhans' cell maturation and their migration to draining lymph nodes (Burns *et al.*, 2000; Suzuki *et al.*, 2000).

In humans, the molecule first acts through pDC. Palamara *et al.* (2004) showed an increase of pDCs in spleen and skin of both normal mice and mice with melanoma treated with imiquimod. The number of these cells correlates well with the clinical response of the tumors to the drug, being higher in responders. They proposed that the antitumor properties of imiquimod are related, at least in part, to the recruitment of pDCs and to the release of IFN-α by these cells. Human pDCs are found in blood and secondary lymphoid organs (Yang *et al.*, 2005), express only TLR-7 and TLR-9 (Kadowaki *et al.*, 2001), and appear to have a role in the clinical response to imiquimod. pDCs are recruited to cutaneous epithelial and lymphoproliferative tumors treated with imiquimod and they are the primary source of IFN-α in skin lesions in response to imiquimod treatment (Urosevic *et al.*, 2005).

Topical application of imiquimod following tumor cryosurgery may thus be aimed at enhancing DC activation, but also at activating other cells, such as neutrophils. In particular, although apparently documenting only a modest involvement of direct T-cell immunity, our studies demonstrate an essential role for neutrophils during the first 48 hours after treatment, at least when it comes to efficaciously counter rechallenges. Furthermore, inflammatory infiltration features were virtually identical when B16F10 was used to rechallenge, proving that the overall response is not limited to OVA. Although the exact meaning of such an infiltration remains elusive, it has to be considered as sufficiently efficacious as to impede tumor development after rechallenge. Probably it will require gene microarray analysis to understand better how the inflammatory process differs between sharp excision, thermal damage, and thermal damage plus TLR agonism.

In a recent paper, DCs were activated *in vivo* by injecting immature cells into a cutaneous site that had been treated with topical imiquimod (Nair *et al.*, 2003). Imiquimod may enhance immune response to vaccination (Shackleton *et al.*, 2004) and should be considered in the design of future clinical trials that seek to optimize DC-based immunotherapy. Recent evidence suggests that persistent TLR signaling may help in bypassing regulatory T-cell-induced tolerance (Yang *et al.*, 2004), enhancing autoimmune T-cell responsiveness (Lang *et al.*, 2005), or even reversing regulatory T-cell function (Pulendran, 2004; Prins *et al.*, 2006).

Clinical interest in the *in situ* destruction of melanoma has resurfaced (Ito *et al.*, 2003; den Brok *et al.*, 2004, 2006; Machlenkin *et al.*, 2005). Tumor cells are weakly immunogenic and even produce factors that suppress cellular immunity. Methods to enhance the immunogenicity of tumor cells might improve the clinical efficacy of cryosurgery. If the cryoablation of cutaneous melanoma associated with topical imiquimod can induce an antitumor immune response capable of reducing both local and distant recurrence, then this approach may be even superior to surgical excision. Further studies are warranted to assess the potential of this combination for treating melanoma.

MATERIALS AND METHODS

Mice

C57BL6/J mice were purchased from the Jackson laboratory (Charles River laboratories, Barcelona, Spain). Animal care was carried out in accordance with our institutional guidelines. For tumor experiments, female 7–9-week-old mice with an average weight of approximately 20 g were used. Mice were acclimatized and after 1 week under observation they were caged in groups of five or 10 and their back was shaved. The B16-F10 cell line was obtained from the American Type Culture Collection. B16-F10 cells transfected to express chicken OVA have been described previously (B16/OVA) (Dobrzanski *et al.*, 1999). All experiments were done according to the guidelines for animal care of the Center for Applied Pharmacological Research (University of Navarra, Pamplona, Spain).

Tumors

Mice were injected subcutaneously in the middle of the right flank with 3×10^5 B16/OVA cells. Cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies, Renfrew, UK), supplemented with 10% fetal calf serum, harvested and injected in 100 μl of phosphate-buffered saline. Tumor growth was monitored every 3 days. Tumors were measured with a dial-caliper and volumes were determined using the formula $(\text{width})^2 \times \text{length} \times 0.52$. Tumors were selected for cryosurgery when their diameter measured 6–8 mm. In tumor rechallenge experiments, mice were killed when tumors reached a volume of 1500 mm^3.

Cryosurgery

Mice were anesthetized by intraperitoneal injection of ketamine-HCl (100 mg/kg). Cryosurgery was performed using an intermittent liquid nitrogen spray (Cry-A-C device, Brymill, Ellington, CT). The spray was emitted from a distance of 2–3 cm from the tumor at a 90° angle. A three-cycle 30-second freeze/thaw regimen was performed to ensure maximum tumor cell death. After the procedure, mice were kept on a heating pad until they recovered from anesthesia. This point in treatment was taken as day 0. Excisional treatment of the tumor consisted of complete removal of the lesion with part of the underlying muscle.

Imiquimod treatment

Immediately after cryosurgery, during 10 days, mice received topical treatment with imiquimod. Imiquimod was obtained as a 5% cream (Aldara™, 3 M, Barcelona, Spain) in packets containing 12.5 mg of imiquimod. The drug was applied daily to shaved skin at the tumor site. Four mice per group were treated with a single packet of imiquimod so that each mouse received approximately 25% of the total active imiquimod agent present (3.125 mg). Control mice were treated with vehicle control.

Rechallenge

Fifteen days after cryosurgery of tumors, mice were challenged by subcutaneous injection on the contralateral flank with 3×10^4 B16/OVA cells in 100 μl of phosphate-buffered saline. Some mice that rejected the first rechallenge received a second, third, and even a fourth rechallenge with B16OVA or B16F10 cells, inoculated on days 90, 120, and 160 (Figure 3).

Statistics

Overall survival after rechallenges was estimated according to the Kaplan–Meier method. Comparison of group survival was calculated using the log-rank test.

Tissue and sera processing

Spleen and lymph nodes were collected and cut into pieces. For every group, tumors were dissected and specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4–7 μm, and stained with hematoxylin–eosin.

Proliferation assay and measurement of cytokine production

Animals were killed and lymph node or spleens removed. Spleen and lymph node cells were triturated and then plated in 96-well plates at 8×10^5 cells/well with culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-β-mercaptoethanol) alone, or with 10 μM of OVA in a final volume of 0.25 ml. Supernatants were collected after 48 hours of culture to measure IFN-γ production. Then, cells were pulsed with 0.5 μCi/well of [^{3}H]-thymidine for 18 hours and harvested. Thymidine incorporation was determined in a scintillation counter (Topcount; Packard, Meridan, CT). Finally, the stimulation index was calculated as the ratio of response to OVA protein over mean response in the absence of protein.

IFN-γ production was measured by ELISA (Pharmingen, San Diego, CA) according to the manufacturer's instructions.

NK cell activity assay

NK cell activity of freshly harvested spleen cells was measured by a chromium-release assay using YAC-1 and EL-4 as control targets. YAC-1 and EL-4 were labeled with 50 μCi of $Na_2^{51}CrO_4$ for 1 hour. Spleen cells with different ratios of target cells were plated in a round-bottom 96-well plate. Cells were incubated for 4 hours. Spontaneous release was determined by incubating only target cells and maximum release was measured by standing target cells in medium containing 1% Triton X-100. Supernatants were collected, and the radioactivity released during incubation from lysed target cells was measured. The percentage of specific lysis was calculated using the following formula:

$$\text{specific lysis}\,(\%) = (\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release}) \times 100$$

Measurement of CTL response

Spleen cells (8×10^6) were plated in 24-well plates and stimulated with 2 ml of culture medium containing OVA protein (10 μM). Cytotoxic activity was measured 5 days after initial culture using the ^{51}Cr-release assay. B16-OVA and EL-4 (as control target) were radiolabeled with 50 μCi of $Na_2^{51}CrO_4$ for 1 hour, washed three times and incubated for 4 hours at different ratios. Spontaneous release was determined by incubating only target cells and maximum release was measured by standing target cells in medium containing 1% Triton X-100. Supernatants were collected, and the radioactivity released during incubation from lysed target cells was measured. The percentage of specific lysis was calculated using the following formula:

$$\text{specific lysis}\,(\%) = (\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release}) \times 100$$

CONFLICT OF INTEREST

The authors state no conflict of interest.

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