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# *In Situ* Tumor Ablation Creates an Antigen Source for the Generation of Antitumor Immunity

**Martijn H. M. G. M. den Brok,[1,2] Roger P. M. Sutmuller,[1] Robbert van der Voort,[1] Erik J. Bennink,[1] Carl G. Figdor,[1] Theo J. M. Ruers,[2] and Gosse J. Adema[1]**

*[1]Department of Tumor Immunology, Nijmegen Center for Molecular Life Sciences, Nijmegen, the Netherlands, and [2]Department of Surgery, University Medical Center Nijmegen, Nijmegen, the Netherlands*

## ABSTRACT

**Tumor-destructing techniques, like radiofrequency ablation (RFA), allow eradication of large tumors. Potentially, *in situ* tumor destruction also can provide the immune system with an antigen source for the induction of antitumor immunity. Antigen-presenting cells could take up antigens in the periphery after which they induce specific immune responses. Recent data show that especially antigen-presenting dendritic cells are crucial for the induction of potent immune responses. However, virtually nothing is known regarding the induction of immune responses after *in situ* tumor destruction in mice or humans. We used the well-defined murine B16-OVA melanoma cell line to develop a novel tumor model to explore: (*a*) the immunologic consequences of *in situ* tumor destruction; and (*b*) the efficacy of a combination approach of tumor destruction and immunostimulation. Applying this model system we demonstrate that following RFA, a weak but detectable immune response develops, directed against OVA, but also against a broader range of B16 antigens. Adoptive transfer experiments further indicate that antitumor reactivity can be transferred to naïve mice by splenocytes. To augment the response observed, we administered a blocking monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4 at the time of tumor destruction. Interestingly, this strongly enhanced antitumor immunity, resulting in long-lasting tumor protection. These results illustrate that *in situ* tumor destruction can provide a useful antigen source for the induction of antitumor immunity, provided that additional immunostimulatory signals are coadministered.**

## INTRODUCTION

*In situ* tumor ablation with a thermal energy source, such as radiofrequency ablation (RFA), laser, microwave, or cryoablation, has received increasing attention during the past decades as a minimally invasive technique for management of focal cancer, and encouraging results have been obtained. RFA treatment is widely used for local ablation of liver tumors with a size of up to 4 cm (1, 2). RFA has been proven to be a safe procedure with a complication rate $<10\%$. The technique can be performed during open surgery or as a minimally invasive procedure when applied percutaneously or via laparoscopy. RFA has been used successfully for the management of bone tumors (3), lung tumors (4), renal cancer (5), and primary or metastatic liver tumors (1, 2, 6, 7). Although RFA treatment is not applicable to every patient, RFA and comparable techniques require fewer resources, result in faster recovery, and, in most cases, offer reduced morbidity and mortality compared with surgical resection. Unfortunately, many of these patients will die from multiple metastases that remain untreated. Therefore, the addition of a relevant systemic therapy would be highly valuable.

On tumor ablation *in situ*, large amounts of tumor debris are released that could potentially be taken up by the immune system. For long it has been discussed whether ablated tumor debris is able to induce a systemic immune response; however, a systematic analysis has not been reported and therefore convincing evidence is lacking. Few studies report on an occasional patient with spontaneously regressing metastases and reduced numbers of developing secondary foci postablation (8, 9). However, based on the results of vaccination studies with large amounts of irradiated autologous tumor cells or tumor lysates, it is not likely that a large amount of tumor debris by itself is sufficient to induce a potent antitumor response (10, 11). Moreover, recent insights in the requirements for the induction of an effective immune response demonstrate that maturation of antigen-presenting cells, especially dendritic cells, is a prerequisite for the induction of adaptive immunity (12, 13). The importance of immune activation for the induction of antitumor immunity has been well established. For example, expression of the B7 molecules appeared to be sufficient to induce T-cell-mediated rejection of a variety of tumors (14, 15). Likewise, local injections of stimulating antibodies against the costimulatory molecule CD40 led to enhanced systemic antitumor responses in mice (16). Similar results have been obtained by antibody triggering of other stimulatory members of the tumor-necrosis factor receptor family: OX40, 4–1BB, CD27, and CD30 (reviewed in Ref. 17). In addition to these stimulatory pathways, blockade of inhibitory receptors [*e.g.*, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)] also has been applied successfully to induce tumor rejection (18). In recent years, *ex vivo* generated dendritic cells loaded with tumor antigens have been studied and shown to evoke tumor-specific responses in cancer patients (19, 20). The tumor debris generated after *in situ* tumor destruction potentially can be used as an antigen source for the immune system. Combining *in situ* tumor destruction with immune-potentiating strategies may represent a relatively simple way of *in situ* immune response induction.

We report the development of a mouse tumor model that allowed us to explore the potential of *in situ* tumor destruction alone or in combination with immunomodulatory approaches. The results demonstrate that following RFA a weak but detectable immune response is induced. Furthermore, administration of CTLA-4-blocking antibodies that lower the threshold for T-cell activation potentiates the immune response leading to increased tumor protection.

## MATERIALS AND METHODS

**Animals.** Male and female C57BL/6n mice were purchased from Charles River Wiga (Sulzfeld, Germany) and kept under specified pathogen-free conditions in the Central Animal Laboratory, Nijmegen University (Nijmegen, the Netherlands). All of the experiments were performed according to the guidelines for animal care of the Nijmegen Animal Experiments Committee. For ablations and tumor experiments, 9–11-week-old mice were used.

**Tumors.** Mice were injected s.c. in the middle of the right femur with $5 \times 10^5$ cells of the OVA-transfected murine melanoma cell line B16-F10 (B16-OVA, clone MO5), which was provided by Dr. Kenneth Rock (Dana-Farber Cancer Institute, Boston, MA; Ref. 21) or with the murine thymoma cell line EL4 (American Type Culture Collection, Manassas, VA). Cells were cultured as described previously, harvested, and injected in a 1:3 mixture of

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**Requests for reprints:** Gosse J. Adema, Department of Tumor Immunology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, Postbox 9101, 6500 HB Nijmegen, the Netherlands. Phone: 31-24-361-7600; Fax: 31-24-354-0339; E-mail: g.adema@ncmls.kun.nl.

Matrigel (BD Biosciences, Alphen a/d Rijn, the Netherlands) and PBS in a total volume of 50 $\mu$l as used previously (22). Evaluation of tumor size was performed every 3 days using calipers. Tumor volumes were scored with the formula $(A \times B^2) \times 0.4$, in which A is the largest and B is the shortest dimension. Tumors were selected for ablation when their diameter measured 5–7 mm. Mice carrying macroscopic satellite lesions (2 of 50 mice) were excluded from the experiments because complete ablation of such lesions appeared difficult. In tumor rechallenge experiments, mice were killed when tumors reached a volume of $\pm 850$ mm$^3$.

**RFA.** Animals were anesthetized by isoflurane inhalation and properly shaved at the tumor area and on the contralateral flank. After placement and proper attachment of the contralateral side onto an electricity-conducting pad (grounding pad), the tumor area was disinfected with alcohol. An RFA needle with active tip of 8 mm (SMK-15; Cotop, Amsterdam, the Netherlands) was inserted s.c. and placed in the middle of the tumor. After placement of the RFA needle, impedance could be evaluated on the RF lesion generator system (Model RFG-3B; Radionics, Burlington, MA). Treatment then was started by delivering RFA energy. During two treatment cycles of 80 s, temperature could be monitored using a thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 75–80°C could be reached.

**Rechallenges.** Forty days after ablation of tumors, mice were challenged by s.c. injection on the contralateral femur with $15 \times 10^3$ B16-OVA cells, $10 \times 10^3$ B16-F10 cells (provided by I. J. Fidler), or $15 \times 10^3$ EL4 cells. The amount of tumor cells was based on titration experiments demonstrating that this number of cells yielded a solid 100% tumor take while leaving an appropriate experimental window to study the effects of RFA. Some mice that rejected the first rechallenge received a second rechallenge. These were inoculated on the left and the right flank. Injections were performed in PBS in a total volume of 100 $\mu$l. Statistical analysis for all of the rechallenges was performed using the log-rank Kaplan-Meier estimation.

**Adoptive Transfers.** At day 35 after ablation, mice were boosted with an s.c. challenge of $25 \times 10^3$ B16-OVA cells to enhance an active immune response. Control mice also received $25 \times 10^3$ B16-OVA cells. Ten days afterward, serum and spleens were isolated, and single-cell suspensions were obtained by crushing and passing through nylon mesh. Lymphocytes were concentrated by density gradient centrifugation (Lympholyte-M; Cedarlane Laboratories, Sanbio, Uden, the Netherlands). Recipient naïve mice then received $35 \times 10^6$ lymphocytes or 200 $\mu$l serum i.v. in the tail vein from RFA-treated or control donor mice. These mice were challenged with $15 \times 10^3$ B16-OVA cells 3 days later.

**CTLA-4 Treatment.** The hamster hybridoma 9H10 was cultured, and using standard isolation procedures, IgG was collected (23). Total hamster IgG was used as control antibody (Jackson ImmunoResearch, West Grove, PA). Directly after RFA, at days 3 and 6 mice received an i.p. injection of 200 $\mu$g anti-CTLA-4 or hamster IgG in a total volume of 200 $\mu$l.

**Tetramer Stainings.** A T-cell culture was obtained from splenocytes and draining lymph nodes of mice 10 days after ablation of a B16-OVA tumor. Cells obtained from naïve age-matched mice were used as controls. Stimulation of these cells ($1 \times 10^5$) was performed by addition of irradiated IFN-$\gamma$-treated B16-OVA cells ($5 \times 10^4$) in interleukin 2-supplemented culture medium (10 Cetus units/ml). At days 5 and 10 (before staining), cells were collected, after which dead cells were removed by a Ficol-Hypaque gradient. The OVA-specific cytotoxic T cell (CTL) clone OVA-2 was cultured as described previously (24) and used as positive readout (data not shown). At day 10 of culture, cells were stained for 15 min at room temperature by OVA-tetramers (H2Kb) conjugated to allophycocyanin, which were a gift from S. H. van der Burg (LUMC, Leiden, the Netherlands). Cells subsequently were counterstained for CD8b-FITC (PharMingen, San Diego, CA) and propidium iodide (Sigma, St. Louis, MO) and analyzed on a FACS-Calibur system with CELLQuest software (both from Becton Dickinson Immunocytometry Systems, San Diego, CA). Values are presented as percentages of tetramer-positive cells within the total CD8b$^+$ population.

**IFN-$\gamma$ ELISA.** The same bulk cultures as described for the tetramer stainings were used to collect supernatant 24 h after stimulation. Capture and biotinylated detection antibodies directed to mouse IFN-$\gamma$ were purchased from PharMingen, and using standard ELISA procedures, IFN-$\gamma$ concentration was measured in 50 $\mu$l supernatant. Data were analyzed for statistical significance by Student's *t* test.

## 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 RFA was used to destruct established tumors. Initial experiments with the RFA equipment indicated a tight balance between incomplete ablation of B16-OVA tumors, resulting in recurrences and too severe ablation of the tumor-surrounding tissue. Therefore, we optimized the size of the tumor at the moment of destruction, duration of the ablative cycles, and the impedance. Two consecutive treatment cycles of 80 s with an impedance of 400 ohms, together covering the whole tumor area (7 mm in diameter), yielded the best results (data not shown; Fig. 1*B*). Using this treatment regimen, postoperative survival rates of

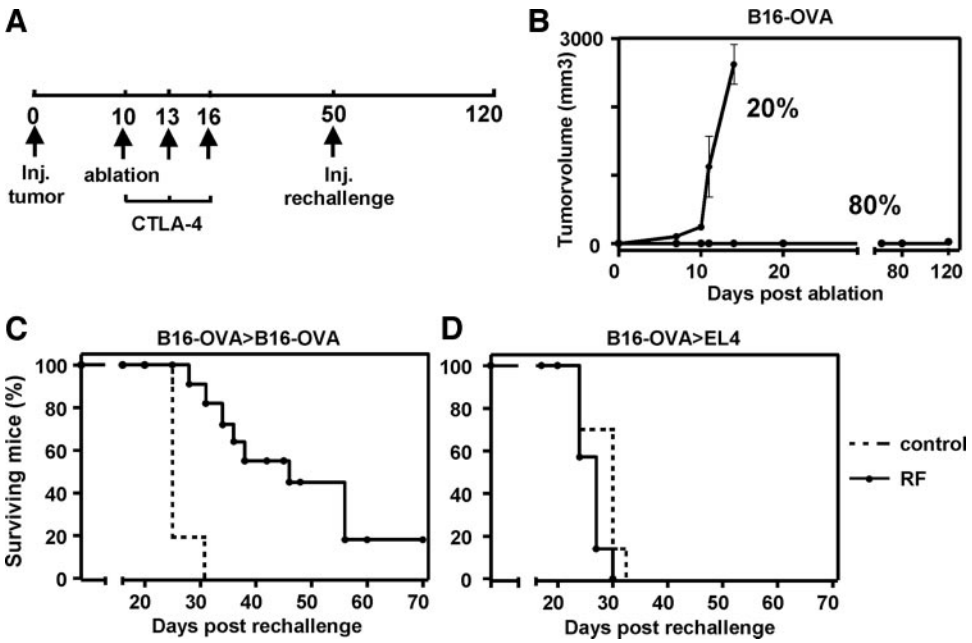


Fig. 1. Induction of a tumor-specific immune response following radiofrequency ablation (RFA). *A*, time schedule outlining the different treatments as used in the experiments. Ten days after tumor inoculation the B16-OVA melanoma tumors (7 mm) were ablated by RFA. Forty days after RFA mice were rechallenged. *B*, the overall survival of animals after ablation; T = 0 corresponds to the time of ablation. *C* and *D*, 40 days after ablation $15 \times 10^3$ B16-OVA cells (*C*) or $15 \times 10^3$ EL4 mouse thymoma cells (*D*) were injected s.c. in the contralateral leg (●). Normal growth was monitored by injection of $15 \times 10^3$ B16-OVA cells into naïve mice (*dotted lines*); T = 0 corresponds to the time of injection of the tumor rechallenge. $P < 0.005$ for *C* ($n$ = 7–11); one representative experiment of three independent experiments is shown.

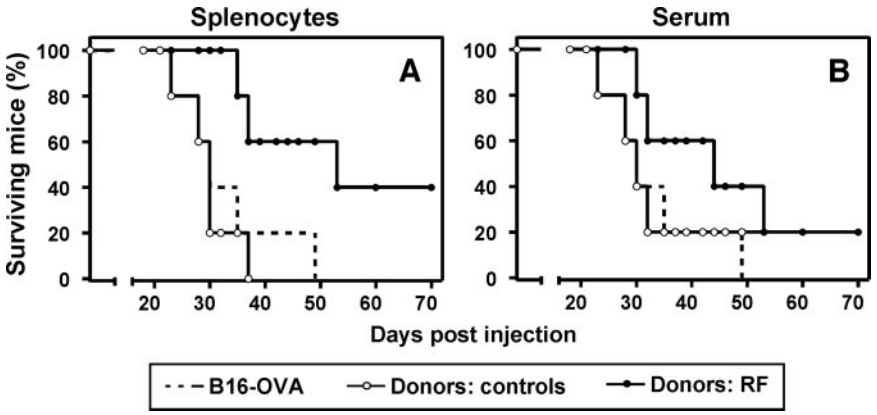


Fig. 2. Adoptive transfer of immune reactivity. Forty days after radiofrequency ablation (RFA), mice were injected with $25 \times 10^3$ B16-OVA cells to boost the response. Ten days later, splenocytes (*A*) or serum (*B*) of these mice were harvested and transferred to naïve mice. Three days later, recipient mice received a challenge with $15 \times 10^3$ B16-OVA cells (●). Control transfers were performed using spleen cells and serum from mice that received $25 \times 10^3$ B16-OVA 10 days before the isolation of spleen and serum (○). Normal growth was monitored by injection of $15 \times 10^3$ B16-OVA cells into naïve mice (*dotted lines*); T = 0 corresponds to the time of injection of the tumor challenge. $P < 0.02$ for RFA *versus* control donor in *A* ($n = 5$ per group); one of two independent experiments is shown.

100% were obtained with a recurrence rate of 20% (Fig. 1*B*). We observed that whenever recurrences occurred a few days after treatment, they appeared at the edge of the treated area and progressed rapidly. Wounds healed completely in 20–30 days.

**Immune Responses after RFA.** Using the aforementioned model, we determined whether specific antitumor reactivity could be detected after RFA. B16-OVA tumor-bearing mice were RFA treated and then rechallenged with either B16-OVA cells or nonrelated EL4 thymoma cells. A detailed time schedule of the different treatments is given in Fig. 1*A*. Rechallenges were given 40 days after ablation to exclude direct effects of the RFA treatment on the tumor rechallenge. Because surgical excision of the established tumor was not possible because of the occurrence of local recurrences in the majority of these mice, age-matched, untreated naïve mice were used as controls. As shown in Fig. 1*C*, RFA of B16-OVA resulted in a clear delay in the outgrowth of B16-OVA tumor cells and a low level of protection (20% of the mice). In contrast, no delay in outgrowth of the nonrelated EL4 mouse thymoma was observed (Fig. 1*D*). These data imply that a weak but tumor-specific immune response had developed after *in situ* tumor destruction by RFA.

**Adoptive Transfer of Specific Immunity.** To further demonstrate the involvement of the immune system, we investigated whether the observed effects on tumor growth and protection could be transferred from RFA-treated mice to naïve mice via serum or splenocytes. Interestingly, transfer of $35 \times 10^6$ splenocytes of RFA-treated mice resulted in delayed tumor outgrowth and partial protection against a lethal B16-OVA challenge (Fig. 2*A*). Transfer of serum of the same mice only resulted in a minor delay in tumor outgrowth (Fig. 2*B*). No delay in tumor growth was observed after transfer of control splenocytes or serum from mice that received B16-OVA cells 10 days before the isolation of splenocytes and serum (Fig. 2, *A* and *B*). These results indicate that the observed immune response is mainly cell mediated. The finding that splenocytes derived from mice that carry nonablated B16-OVA tumors are unable to transfer antitumor reactivity demonstrates that specific immunity is not simply induced by a growing B16-OVA tumor but is in addition dependent on ablation of the tumor.

**Long-Lasting Immunity against B16-OVA.** We investigated whether the RFA-treated mice that had rejected the initial rechallenge were protected against a second rechallenge with B16-OVA and wild-type B16-F10, its less immunogenic counterpart. Because only a limited number of surviving mice were available, groups of three or four mice were rechallenged with a lethal dose of B16-OVA in one flank, whereas B16-F10 was given contralaterally. Interestingly, mice that had rejected the first B16-OVA challenge were completely protected against a second B16-OVA rechallenge 70 days later (Fig. 3). This observation demonstrates that immunologic memory was present in these mice. Furthermore, a delayed outgrowth of the wild-type B16-F10 tumor also was observed compared with controls (Fig. 3). These data indicate that the immune response is not only directed against the immunodominant OVA epitope but also toward a broader range of B16 antigens.

**CTLA-4 Blockade Enhances Immunity against B16-OVA and Induces Specific CTL.** Administration of blocking monoclonal antibodies against CTLA-4 has been used previously to facilitate the induction of T-cell responses by disrupting the negative regulatory function of CTLA-4 (23). Therefore, we explored whether *in situ* tumor destruction in combination with CTLA-4 blockade could enhance the antitumor immunity in our model. Blocking anti-CTLA-4 antibody 9H10 or a control antibody was administered on days 0, 3, and 6 after RFA and mice were rechallenged 40 days later. As shown in Fig. 4, the combination of RFA and CTLA-4 treatment resulted in an increase in protection against a lethal B16-OVA injection from 25–75% of the mice. No increase in protection was observed when control IgG was administered after RFA (Fig. 4). Furthermore, anti-CTLA-4 treatment without RFA was not sufficient to eradicate either the primary tumor or a tumor challenge given 40 days after antibody injection (data not shown).

To determine whether antigen-specific T cells are induced after

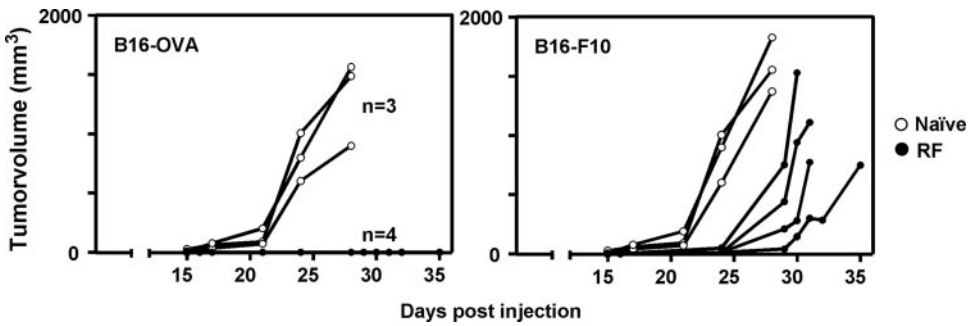


Fig. 3. Memory and anti-B16 immune response. Mice that had rejected a B16-OVA rechallenge after radiofrequency ablation (RFA) treatment of a B16-OVA tumor (see Fig. 1) received a second set of rechallenges (●). A total of $15 \times 10^3$ B16-OVA cells (*left panel*) and $10 \times 10^3$ B16-F10 wild-type cells (*right panel*) were injected on the left flank and on the right flank, respectively. The same tumor cell injections were given to naïve mice (○). T = 0 corresponds to the time of injection of the second tumor rechallenge ($n = 3$–4 per group); one of two independent experiments is shown.

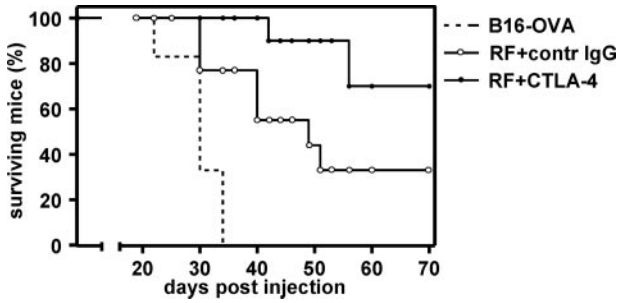


Fig. 4. Coadministration of blocking cytotoxic T-lymphocyte-associated antigen 4 (*CTLA-4*) monoclonal antibodies enhances the antitumor effect. At days 0, 3, and 6 after RFA of B16-OVA, mice received 200 $\mu$g anti-CTLA-4 antibody (●) or control IgG (○). Forty days after ablation, a rechallenge with $15 \times 10^3$ B16-OVA cells was given in the contralateral leg. Normal tumor growth was monitored by injection of the same amount of cells into naïve mice (*dotted line*); T = 0 corresponds to the time of injection of the tumor rechallenge. $P < 0.05$ for both lines ($n$ = 7–11 per group); one of two independent experiments is shown.

RFA with or without CTLA-4 blockade, we analyzed the presence of OVA-specific $CD8^+$ T cells by MHC-tetramer staining after a single restimulation of spleen and lymph node cells. As shown in Fig. 5*A*, a low number of OVA-specific CTLs were detected 10 days after RFA treatment plus control IgG. However, in RFA mice that also received CTLA-4 treatment, a 20-fold increase in OVA-specific CTL was observed (Fig. 5*A*). No OVA tetramer-positive cells were discerned in naïve mice.

Analysis of IFN-$\gamma$ production in response to B16-OVA tumor cells confirmed the increased antitumor reactivity in RFA plus CTLA-4-treated mice and further demonstrated that these specific CTLs are functional (Fig. 5*B*).

These findings collectively demonstrate that *in situ* tumor destruction can provide a useful antigen source for the induction of antitumor immunity. Weak antitumor T-cell responses are generated after RFA-mediated tumor ablation that can be significantly enhanced by coadministration of blocking CTLA-4 antibodies.

## DISCUSSION

RFA is a minimally invasive therapy for local tumor destruction that generates large amounts of tumor debris (2). Using a newly developed *in situ* tumor destruction model, we now demonstrate that following RFA, a weak, but tumor-specific, immune response is induced, resulting in protection against a lethal tumor rechallenge in 20% of the mice. The antitumor reactivity can be transferred to naïve mice by splenocytes and is directed against multiple tumor antigens. Moreover, the response induced by RFA can be potentiated by coadministration of blocking CTLA-4 antibodies.

For long it has been recognized that heating or freezing of tumors is an effective way of tumor destruction *in situ*. In recent years, technical developments in equipment and monitoring devices have strongly increased the applicability of RFA (1, 2, 5, 6, 25, 26). RFA currently is widely used for destruction of tumors, particularly for the management of inoperable liver metastases. Tumor debris remaining after *in situ* tumor destruction is a potential antigen source for the induction of antitumor immunity. However, little is known regarding the induction of antitumor immune responses following RFA. Preliminary studies in pig and rabbit revealed an influx of immune cells in the periphery of the coagulation area directly after RFA, together with an increased T-cell proliferation (27, 28).

We have demonstrated in a mouse B16-OVA tumor model that *in situ* tumor ablation results in the induction of immunity against a lethal tumor rechallenge given 40 days after tumor ablation. The time between tumor ablation and tumor rechallenge excludes any direct or nonspecific immune effect of the RFA treatment on the growth of the tumor rechallenge. Our finding that ablation of 5–7-mm B16-OVA tumors resulted in a delay in tumor growth and partial protection against a subsequent B16-OVA but not EL4 tumor rechallenge is indicative for the involvement of a tumor-specific immune response. Adoptive transfer experiments of splenocytes of ablated mice further demonstrated for the first time that the protective antitumor response observed after RFA is predominantly mediated via the cellular arm of the immune system. The finding that no delay in tumor growth or protection was observed after transfer of splenocytes from mice that carry B16-OVA tumors for 10 days without RFA demonstrates that the effect is at least in part dependent on RFA treatment of the primary tumor.

The mechanism by which RFA induces or enhances immune responses is still poorly understood. Ablative treatments are known to release tumor debris, and an increase in the tumor-associated "carcinoembryonic antigen" has been detected following ablation of colorectal liver metastasis (our personal observations). A limited number

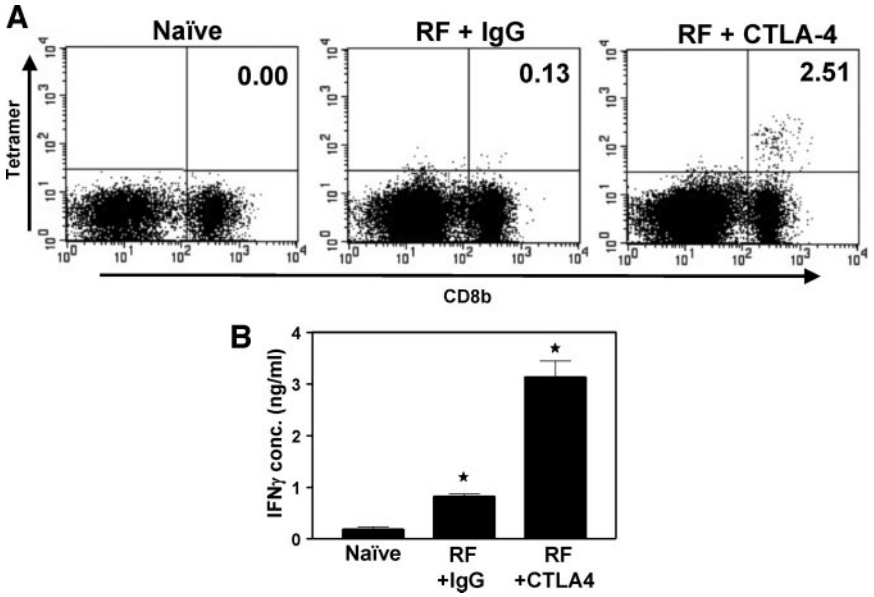


Fig. 5. Increased OVA-specific cytotoxic T cell numbers and IFN-$\gamma$ production following cytotoxic T-lymphocyte-associated antigen 4 (*CTLA-4*) blockade. *A*, at day 10 after ablation, a mix of lymph node and spleen cells was obtained from mice treated with radiofrequency ablation (RFA) and control IgG, mice treated with RFA and anti-CTLA-4 antibodies, or naïve mice. T cells were harvested and cultured in the presence of irradiated B16-OVA cells and interleukin 2 for 10 days, followed by staining with OVA-Kb tetramers and anti-CD8b. Depicted numbers are percentages of tetramer-positive cells within the $CD8b^+$ population. *B*, IFN-$\gamma$ production by the stimulated T-cell cultures indicated previously was analyzed using ELISA. $*P < 0.01$ when compared with naïve as determined by Student's $t$ test. Mean values with SD from triplicates are shown from one of two independent experiments.

of studies also reported an increase in C-reactive protein, interleukin 6, soluble type I tumor necrosis factor receptor, and interleukin 2 after cryoablation of tumor tissue (29, 30), but little is known about the cytokine release after RFA. Scavenging of the tumor debris by immune phagocytes in combination with the release of inflammatory cytokines potentially could be responsible for the observed weak immune responses after RFA. The experiments now indicate that low, but detectable, amounts of specific $CD8^+$ T cells are induced following RFA, which likely also requires $CD4^+$ T cells. In combination with CTLA-4 blockade (to be discussed), these $CD8^+$ T cells further expand. The involvement of natural killer cells and elevated cytokine levels in the delayed outgrowth of tumors can formally not be excluded. However, we consider this unlikely because of the time span of 40 days between ablation and tumor rechallenge. Moreover, the absence of antitumor effects on a rechallenge with EL4 cells following ablation of a B16-OVA tumor and *vice versa* is indicative for the induction of a tumor-specific immune response (Fig. 1; data not shown). Whether the effect of RFA is solely based on the induction of a *de novo* immune response, occurs as a consequence of the elimination of the suppressive effect of the B16OVA tumor on T-cell function, or both, remains to be determined.

Rechallenge experiments in surviving mice revealed that once mice had rejected B16-OVA, they were completely protected against a new tumor challenge. These data indicate that in the few mice surviving the first challenge, a *bona fide* systemic memory response was generated. A clear delay in the outgrowth of the wild-type B16-F10 tumor also was observed in the surviving mice. We note that a direct rechallenge with wild-type B16-F10 after ablation of B16-OVA only resulted in a small delay in outgrowth (data not shown). These data indicate that the immune response induced by RFA is directed against multiple B16 antigens. However, they also suggest that on rejection of B16-OVA tumors the pool of B16-F10-reactive T cells is expanded or further broadened by epitope spreading to obtain the delay in B16-F10 outgrowth in the surviving mice (31).

The aforementioned data indicate that in our B16-OVA model, a weak, but specific, antitumor response can be achieved following RFA in a limited number of mice. Data on antitumor responses in patients after local ablative treatment have only been reported incidentally (8, 9). On the basis of our current knowledge on immune response induction in general, and the unique requirement for mature professional antigen-presenting cells in particular, the aforementioned results imply that immune activation following RFA is suboptimal. Similarly, vaccination with irradiated tumor cells or tumor cell lysates alone does not result in the induction of a potent antitumor immune response in mice or humans (10, 11).

Several strategies have been reported to augment an immune response, including the administration of stimulatory antibodies to CD40, 4–1BB, or blocking monoclonal antibodies against CTLA-4. Repetitive administration of blocking CTLA-4 monoclonal antibody has been shown to cure mice from established tumors (32, 33), and in combination with antitumor vaccination, CTLA-4 blockade significantly enhanced the potency of the vaccine (34, 35). In our *in situ* tumor destruction model, we demonstrate that the antitumor effect is markedly improved if ablation was accompanied by CTLA-4 blockade. Because CTLA-4 blockade by itself could not abrogate the outgrowth of the tumor, these data indicate that the ablation of the tumor and the anti-CTLA-4 treatment act synergistically (data not shown). CTLA-4 previously has been shown to maintain the threshold for a T cell to proceed to full activation and to limit the proliferative ability of activated antigen-specific T cells (18). On combining CTLA-4 blockade with RFA, increased numbers of IFN-$\gamma$-producing and OVA-specific CTLs were detected. Recent reports also suggest an important role of CTLA-4 in the function of $CD4^+CD25^+$ regulatory T cells (36, 37). Therefore, involvement of these regulatory T cells in the tumor destruction model cannot be excluded and is currently under investigation.

In the clinical setting, RFA has been successfully applied for management of colorectal liver metastases (7). Encouraging results also have been obtained more recently in the treatment of breast cancer patients (25). However, these studies are mainly focused on the successful eradication of the treated tumor foci, whereas little or no clinical benefits have been reported for untreated lesions. Furthermore, when RFA is applied for the destruction of large-sized tumors (>4 cm), local recurrence rates increase strongly. Therefore, an efficient systemic antitumor therapy in addition to ablation could be highly beneficial. The results described here indicate that RFA in combination with immunomodulation can induce a systemic immune response and therefore might enhance the efficacy of RFA treatment and protection against local recurrences and the development of metastasis. Therefore, RFA treatment may offer interesting novel possibilities in combination with existing immunotherapy strategies. The data in this article suggest that tumor debris is a useful antigen source for the immune system, provided that additional immunostimulatory signals are coadministered.

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