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Low-Intensity Focused Ultrasound Induces Reversal of Tumor-Induced T Cell Tolerance and Prevents Immune Escape

Sanmay Bandyopadhyay,* Thomas J. Quinn,† Lisa Scandiuzzi,‡ Indranil Basu,† Ari Partanen,‡ Wolfgang A. Tomé,† Fernando Macian,*‡ and Chandan Guha,*†,‡

Immune responses against cancer cells are often hindered by immunosuppressive mechanisms that are developed in the tumor microenvironment. Induction of a hyporesponsive state in tumor Ag-specific T cells is one of the major events responsible for the inability of the adaptive immune system to mount an efficient antitumor response and frequently contributes to lessen the efficacy of immunotherapeutic approaches. Treatment of localized tumors by focused ultrasound (FUS) is a minimally invasive therapy that uses a range of input energy for in situ tumor ablation through the generation of thermal and cavitation effect. Using a murine B16 melanoma tumor model, we show that a variant of FUS that delivers a reduced level of energy at the focal point and generates mild mechanical and thermal stress in target cells has the ability to increase immunogenic presentation of tumor Ags, which results in reversal of tumor-induced T cell tolerance. Furthermore, we show that the combination of nonablative low-energy FUS with an ablative hypofractionated radiation therapy results in synergistic control of primary tumors and leads to a dramatic reduction in spontaneous pulmonary metastases while prolonging recurrence-free survival only in immunocompetent mice. The Journal of Immunology, 2016, 196: 1964–1976.

Immune responses against cancer cells are frequently hampered by the immunosuppressive nature of the tumor microenvironment, which is also responsible for hindering the efficacy of cancer immunotherapy (1, 2). Several mechanisms have been identified underlying the ability of tumors to generate an immunosuppressive environment, including secretion of cytokines or other factors with inhibitory activity (3–5), recruitment of regulatory T cells and myeloid-derived suppressor cells (6–9), increased expression of ligands for coinhibitory receptors (10–13), or inhibition of dendritic cell maturation (14, 15). As a consequence of those mechanisms, T cells are often rendered unresponsive to tumor Ags (15). Induction of a hyporesponsive state to tumor Ags occurs both in CD4+ and in CD8+ T cell populations and is often responsible for the inability of the adaptive immune system to mount an efficient antitumor response (16–18). Decreased T cell responses to tumor Ags occur in both solid and hematological tumors and appear to be caused by inefficient presentation of Ags by dendritic cells, which results in the preferential activation of tolerogenic programs of gene expression that are dependent on the transcription factors NFAT and Egr2 (18–21). The important role of this process of tumor-induced T cell hyporesponsiveness is underscored by the fact that genetic mouse models where the induction of this tolerogenic gene expression program is prevented result in enhanced antitumor T cell responses and control of tumor growth (19, 21).

Treatment of localized tumors by focused ultrasound (FUS) is an image-guided minimally invasive therapy that uses a range of input energy for in situ tumor ablation (22, 23). The application of FUS to biological tissues is associated with the generation of thermal and cavitation effects, causing changes in target cell physiology, depending on the energy delivered. High-intensity FUS (HIFU) has been used clinically to thermally ablate localized tumors (23–26). The substantial thermal energy generated by that modality of FUS treatment causes rapid coagulative necrosis of the tissue at the targeted focal spots. Although several studies have reported some immunomodulatory effects, including increased lymphocyte infiltration, generation of IFN-γ-producing tumor-specific T cells in lymphoid organs, and dendritic cell maturation and migration into tumors (26–29), the thermally induced coagulative necrosis resulting from HIFU treatment can also attenuate the release of immunostimulatory molecules within the tumor microenvironment. Thus, although able to halt the progression of established primary tumors, HIFU might fail to protect against local and distal metastases arising from the surviving tumor cells.

In this report, we designed a variant of FUS that delivers a reduced level of energy at the focal point for a short time with limited tissue temperature elevation [(low-energy FUS (LOFU)]. Using a murine B16 melanoma tumor model, we show that LOFU treatment is capable of inducing a stress response in the tumor, which increases the expression of immunomodulatory factors enhancing efficient tumor Ag presentation and leading to reversal of tumor-induced tolerance, with increased effector cytokine production in tumor Ag-specific CD4+ T cells. Furthermore, the combination of LOFU with an ablative hypofractionated cone beam computed tomography (CBCT) image-guided radiation therapy (IGRT) results in synergistic control of primary tumors.
and a marked reduction in spontaneous pulmonary metastases with prolonged recurrence-free survival only in immunocompetent mice.

Materials and Methods

**Mice**

Six- to 8-wk-old C57BL/6, B6.Cg-Rag1<sup>–/–</sup>Tcr<sup>B+–/–</sup>Tg(TcrTcRb) 9E8/1 (Tyrp1), and B6.Cg-Tg(TcRtCerb)425Cbn/J (OT-II) mouse strains were purchased from The Jackson Laboratory. BALB/c/Nude mice were obtained from National Cancer Institute, distributed through Charles River. All mice were housed and maintained in pathogen-free facilities.

**Culture of B16 cell lines and primary CD4<sup>+</sup> T cells**

B16-F1 and B16-F10 melanoma cell lines were purchased from the American Type Culture Collection. A highly aggressive subclone of B16-F10 (B16-M1) was generated by isolating and expanding a metastatic clone that arose in a C57BL/6 mouse 6 wk after surgical removal of an established primary tumor. The B16-OVA melanoma cell line was kindly provided by E.M. Lord (University of Rochester Medical Center, Rochester, NY). The expression of OVA by B16-OVA cells was confirmed by real-time PCR. All melanoma cells were cultured in DMEM (Thermo Scientific) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 250 IU penicillin/streptomycin. CD<sup>4</sup><sup>+</sup> T cells were isolated using anti-CD4–conjugated magnetic Dynabeads (Life Technologies) according to the manufacturer’s protocol. Where indicated, CD<sup>4</sup><sup>+</sup> T cells were differentiated into Th1 cells by activation with plate-bound anti-CD3<sup>+</sup>anti-CD28 Abs, T cell–depleted OVA peptide 323–339 (OVA<sub>323-339</sub>–loaded splenocytes at a 1:5 cell/splenocyte ratio, or CD11c<sup>+</sup>purified dendritic cells (using CD11c–beads; Miltenyi Biotech) loaded with OVA<sub>323-339</sub> or melanoma tumor lysates at a 1:3 dendritic cell/T cell ratio. Culture supernatants were typically harvested 24 h after stimulation, and IL-2 or IFN-γ levels were measured by a sandwich ELISA (BD Biosciences).

**Immunofluorescence staining**

Tumor tissue was isolated, washed in PBS, and embedded in optimal cutting temperature compound (Electron Microscopy Sciences). Tissue sections (5 μm) were prepared and permeabilized with acetone for 5 min and incubated with goat serum for 30 min to block nonspecific protein–protein interactions. Tissue sections were incubated overnight with the following Abs: anti-Calreticulin (PA5-25992; Pierce), anti-CD11c (ab3312; clone TA99; Abcam), and anti-Hsp70 (NBPI-77455; Novus Biologicals). Appropriate secondary Abs were used for 30 min at room temperature. DAPI (Invitrogen) was used to detect nuclei. At least 10 fields/samples were blindly analyzed with an Inverted Olympus IX81 fluorescence microscope.

**FUS therapy system**

A therapy and imaging probe system (Philips Research North America, Briarcliff Manor, NY) was used for all ultrasound exposures. The system is capable of delivering focused and spatiotemporally controlled ultrasound energy and consists of a therapy control workstation, RF generators and control electronics, an 8-element spherical shell annular array ultrasound transducer (80-mm radius of curvature, 80-mm aperture), as well as a motion stage to allow for in-plane transducer movement and accurate positioning perpendicular to ultrasound beam axis. The FUS beam can also be steered approximately ±15 mm out-of-plane using electronic deflection of the focal point. The ultrasound beam propagates vertically into the target through a thin (25-μm) circular plastic membrane, with acoustic coupling provided by degassed water. During therapy, the system allows adjustments of acoustic output power, ultrasound exposure duration, duty cycle, and ultrasound frequency.

**In vivo FUS therapy**

Mice were anesthetized with a continuous flow 1.5% isoflurane in pure oxygen. To ensure proper acoustic coupling, we carefully shaved the tumor-bearing leg or lumbar flank. Once the animal was positioned for therapy, the tumor was acoustically coupled to the therapy and imaging probe system using degassed water and ultrasound gel. The center of the tumor was then placed at the focal length of 80 mm from the transducer. Ultrasound exposures were delivered to the tumor using a 1-mm grid pattern extending over the entire tumor volume. Two layers of grid points (spaced 5 mm apart) were performed in each treatment, resulting in two layers of ultrasound exposures at each grid point. LOFU was performed 2–4 h before CBCT. Ten Gray each was delivered for 3 successive days for a total hypofractionated dose of 30 Gy. In the combination therapy groups, LOFU was performed 2–4 h before CBCT.

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**References**

The Journal of Immunology 1965

The Journal of Immunology 1965

The Journal of Immunology 1965
Pulmonary metastasis evaluation

Lungs were isolated from animals that died spontaneously, were euthanized, or were sacrificed at the end of the 8-wk experiment. One milliliter Fekete’s solution (ethanol, glacial acetic acid, and formaldehyde-based bleaching fixative) was injected to insulate the lungs. The trachea was then clamped, and the entire lungs and heart were removed en bloc with washed PBS. The lungs were then placed in Fekete’s solution and allowed to bleach for 48 h before analysis. The left lung and the four lobes of the right lung were isolated and nodules counted with the aid of a dissecting microscope. Indistinct or fused nodules cannot be reliably enumerated; therefore, the lung was labeled as too numerous to count and assigned an arbitrary metastasis count of 250. Statistical analysis was performed using the nonparametric Kruskal–Wallis test, followed by the Dunn’s posttest for multiple comparisons.

Recurrent-free survival

The following scores were used as positive events in our recurrence-free survival analysis: spontaneous death with necropsy validation of tumor involvement, euthanasia because of extensive local metastasis to the draining popliteal or inguinal lymph nodes, or euthanasia because of moribund appearance indicating extensive systemic tumor burden. The following non-tumor-dependent deaths as processes were considered as censored; death within 24–48 h of amputation or sacrifice of any animals at the end of the 8-wk experiment. To prevent selective sacrifice of control or treated animals, we labeled cages using an alphanumeric code such that animal institute veterinarians were blinded from treatment and control groups. Recurrence-free survival was analyzed using a Mantel–Cox test, with statistical significance defined as p < 0.05.

Real-time PCR

Total RNA was extracted from cells using RNeasy Micro kit (Qiagen), and cDNA was synthesized using qScript cDNA supermix (Quanta Biosciences). The cDNA samples were subjected to real-time PCR using PowerSYBR (Applied Biosystems) as the reporter dye on a StepOnePlus real-time PCR system (Applied Biosystems). Expression of the transcripts studied was normalized to β-actin. The primer sets used are as follows: for catb: F, 5′-GTGACGTTGACATCCGTAAAGA, R, 5′-GTGTGGAGTCACCAGACCCT-3′; R, 5′-ATGTGACGTTGATGTTGCC-3′; Ref128: F, 5′-ATGCAAGAGCTCAACCAGGACG-3′, R, 5′-ATGCCAGCAGTAAGGCTTCAATA-3′; Ikaros: F, 5′-GCTGGCTCTCAGGAGGAG-3′, R, 5′-CGCACTGTTGACCTTACG-3′; Casp3: F, 5′-ACCGCACAAGCTAGAATTT-3′, R, 5′-CTTGGTGGGAAACTGTAGGATG-3′; Egr2, F, 5′-TATGGGTTGGGTATGACCAC-3′, R, 5′-CGCCAAGCTAAGGTTCCCTG-3′; Tbk1: F, 5′-TCACCTAAGTGGTCCACTGG-3′, R, 5′-CACAGCTAAGCACCAGTAT-3′; Itch, F, 5′-GTGTTGGAGTACACGAGGCCT-3′, R, 5′-GCTTCTTCATCGGCACACT-3′, Fosq, 5′-GGCCCTTCTCCAGGAGACGA-3′, R, 5′-GCTGATCATCGGCGTTTGT-3′.

Flow cytometry

Cells were preblocked with Fc block (CD16/CD32) Ab before immunostaining. The following fluorochrome-conjugated Abs were used: anti-CD80, anti-CD86, anti-MHC-I, anti-Gr1, anti-CD11b, anti-CD11c, anti-CD8, and anti-CD45 (all from eBioscience). Anti-CD93 and anti-CD4 were purchased from Biocolor. Anti-Hsp70 was from Novus Biologicals and anti-TRP1 was purchased from Abcam. Dead cells were detected by using a UV LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen). The immunostained cells were analyzed on an LSR-II Flow Cytometer (Becton Dickinson), and postacquisition analyses were carried out using the FlowJo software.

Animal study approval

All animal work and protocols were performed under an approved protocol designed following the guidelines set by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Results

Treatment of primary B16 melanoma with LOFU overcomes tumor-induced tolerance in CD4+ T cells

To determine how melanoma cells may modulate tumor-induced effector CD4+ T cell responses, we used three different mouse models. First, B16-F1 melanoma tumors were induced in C57BL/6J mice by s.c. injection of B16 cells in the lumbar flanks. Tumors were allowed to grow to a size of 70–80 mm3 and CD4+ T cells were then isolated from both the ipsilateral inguinal draining lymph nodes (DLNs) and the distal-contralateral nondenuding cervical lymph nodes (NDLNs). T cells were also obtained from control mice that did not harbor any tumors. Supporting previous reports of tumor Ag-specific T cell tolerance in murine melanoma (18, 21), CD4+ T cells isolated from the tumor DLNs produced significantly less IL-2 than cells isolated from the distal-contralateral NDLNs of the same mice, or from lymph nodes of control tumor-free mice, when stimulated ex vivo with anti-CD3 and anti-CD28 Abs (Fig. 1A). To confirm these data, we used a B16-F1 melanoma cell line that had been stably transfected to express OVA as a surrogate tumor Ag. These cells were s.c. injected into OT-II mice, a mouse strain with T cells expressing a transgenic MHC class II–restricted TCR that recognizes the OVA323–339 peptide. T cells were collected from these mice as described earlier and were stimulated ex vivo using splenocytes loaded with OVA323–339 peptide. CD4+ T cells from the ipsilateral DLNs again produced significantly reduced amounts of IL-2 compared with cells from the contralateral NDLNs or from tumor-free mice (Fig. 1B). These results were further corroborated in a third model using Tprp1 mice, which are deficient in tyrosinase-related protein 1 and bear T cells expressing a MHC class II–restricted TCR specific for the TRP-1115–123 peptide of this endogenous melanocyte differentiation Ag. Those mice were injected with B16-F1 cells. As in the previous two models, IL-2 production by CD4+ T cells harvested from the ipsilateral DLNs was significantly decreased compared with cells harvested from contralateral NDLNs or from tumor-free mice (Fig. 1C). Similar, but less pronounced, effects were also observed when IFN-γ expression was analyzed (Fig. 1D–F). Altogether, these results support that melanoma tumors induce hyporesponsiveness in tumor Ag-specific CD4+ T cells, which translates in a reduced capacity to produce effector cytokines upon restimulation.

HIFU is currently being used to predominantly cause tumor ablation through the generation of high amounts of heat inside the tumor tissue leading to coagulative necrosis. Although HIFU is a very effective, noninvasive ablative procedure to achieve local tumor control, it destroys the vasculature and tissue infrastructure almost instantaneously, thereby possibly limiting the ability of different immune cells to support Ag presentation and recognition. We therefore hypothesized that instead of HIFU, administering LOFU would induce a nonlethal thermal/mechanical stress in the tumor tissue that could generate novel tumor Ags and/or induce the expression of stress-induced proteins, which could increase the immunogenicity of the tumor and overcome tumor-induced tolerance of CD4+ T cells. To examine this possibility, we treated primary B16-F1 melanoma tumors grown on separate groups of C57BL/6J mice that were either left untreated or were treated with LOFU. Thirty-six hours after LOFU treatment, DLN and NDLN resident CD4+ T cells were isolated from both groups of mice and restimulated ex vivo with anti-CD3 and anti-CD28 Abs. CD4+ T cells from the DLNs of the LOFU-treated mice produced significantly more IL-2 compared with the cells obtained from the group of mice bearing untreated tumors. In contrast, T cells from the corresponding NDLNs produced comparable amounts of IL-2 in treated and untreated mice (Fig. 1G). Interestingly, whereas LOFU treatment led to increased capacity of CD4+ T cells to produce IL-2, T cells isolated from HIFU-treated tumors did not support a significant increase in IL-2 production compared with T cells isolated from untreated tumor-bearing mice (Supplemental Fig. 1). We also observed a similar, but less pronounced, effect on IFN-γ pro-
duction in LOFU-treated mice (Fig. 1H). Overall, these results indicated that LOFU treatments of B16 melanoma tumors appear to overcome the hyporesponsive state induced by the melanoma tumor microenvironment.

**LOFU treatment prevents the induction of anergy-associated genes in tumor-specific CD4\(^+\) T cells**

We have previously shown that melanoma tumors can induce an NFAT1-dependent program of gene expression that leads to the production of a set of proteins that interfere with TCR signaling and directly inhibit expression of cytokine genes, resulting in the establishment of functional anergy in CD4\(^+\) T cells (21). Based on the ability of LOFU to prevent tumor-induced T cell tolerance, we assessed the possibility that LOFU treatment could inhibit tumor-induced T cell tolerance by preventing anergy induction, which would account for the increased cytokine expression observed in the DLN resident CD4\(^+\) T cells after treatment with LOFU. We first monitored the expression of anergy-associated genes in CD4\(^+\) T cells isolated from mice treated as in (A), (B), or (C), respectively. Graphs show mean+SEM from four (A and B) or three (C–F) independent experiments. Results are shown as mean+SEM from three to five mice for each experiment. Data were analyzed using ANOVA with a Tukey posttest (***p < 0.001, **p < 0.01, *p < 0.05). (G and H) Tumors were induced in C57BL/6 mice by s.c. injection of 3 \(	imes\) 10\(^5\) B16-F1 melanoma cells in the lumbar flank. Tumors were left untreated or treated with LOFU. Thirty-six hours after LOFU treatment, CD4\(^+\) T cells were isolated from tumor DLNs or NDLNs and stimulated with anti-CD3 and anti-CD28 Abs. IL-2 and IFN-\(\gamma\) production were assessed by ELISA. The results (total cytokine production and ratio of the levels of cytokines produced by T cells from NDLN and DLN in each group) are presented as mean+SEM from three different mice per condition. Differences between cytokine production of DLN T cells in untreated or treated mice were analyzed using a two-tailed t test (**p < 0.01, *p < 0.05).
and the transcription factor Egr2 (Fig. 2A). We did not observe, however, any difference in the expression of Foxp3 between the DLN and NDLN T cells in tumor-bearing mice, suggesting that an increased presence of regulatory T cells was not likely contributing to the decreased CD4+ T cell responses under the conditions used in this study (Fig. 2A). We then determined whether treatment of B16 melanomas with LOFU would have an effect on the expression of those anergy-associated genes in T cells. B16 tumors growing on Tyrp1 mice were either left untreated or treated with LOFU. The expression of different anergy-associated genes was measured by RT-PCR in CD4+ T cells isolated from the DLNs and NDLNs. Expression of the anergy-associated genes is presented as fold induction (mean±SEM from five independent experiments; *p < 0.05 between untreated and LOFU-treated conditions, t test) over the values obtained in T cells from Tyrp1 mice bearing no tumor.

FIGURE 2. Treatment of melanoma tumors with LOFU prevents expression of anergy-associated genes in tumor-specific CD4+ T cells. (A) C57BL/6 mice were challenged with 3 × 10^5 B16 melanoma cells to induce tumors. After tumor development, total RNA samples were extracted from CD4+ T cells isolated from the DLNs and NDLNs of tumor-bearing mice and from tumor-free control mice. Expression of anergy-associated genes was measured by quantitative RT-PCR. Results are shown as fold induction of gene expression in the DLN or NDLN resident T cells in tumor-bearing mice compared with T cells isolated from tumor-free mice. Data represent mean±SEM from three independent experiments. (B–H) B16-F1 melanoma tumors were induced in Tyrp1 mice that were then left untreated or treated with LOFU. The expression of different anergy-associated genes was measured by RT-PCR in CD4+ T cells isolated from the DLNs and NDLNs. Expression of the anergy-associated genes is presented as fold induction (mean±SEM from five independent experiments; *p < 0.05 between untreated and LOFU-treated conditions, t test) over the values obtained in T cells from Tyrp1 mice bearing no tumor.

LOFU treatment of melanoma tumors potentiates dendritic cell–mediated activation of tumor-specific CD4+ T cells

To determine the possibility that the ability of LOFU treatment to prevent tumor-induced T cell tolerance might result from the production of immunomodulatory molecules that could increase the stimulatory capacity of dendritic cells, we directly tested whether lysates prepared from LOFU-treated tumors could elicit enhanced priming of Ag-specific T cells and consequently generate a more robust effector response. For this experiment, B16-F1–OVA melanoma cells were used to induce tumors in C57BL/6 mice. Lysates were prepared from untreated and LOFU-treated tumors. Splenic dendritic cells and responder naive CD4+ T cells were isolated from C57BL/6 and OT-II tumor-free mice, respectively, and were cocultured in the presence or absence of the different tumor lysates described earlier. Although the OVA-containing tumor lysates could act as a source of tumor Ag to prime responder T cells, we also added exogenous OVA323–339 peptide to ensure uniform loading of dendritic cells with this peptide in all conditions and more accurately determine the tolerogenic or activating nature of the different tumor lysates described earlier. Although the OVA-containing tumor lysates could act as a source of tumor Ag to prime responder T cells, we also added exogenous OVA323–339 peptide to ensure uniform loading of dendritic cells with this peptide in all conditions and more accurately determine the tolerogenic or activating nature of the different tumor lysates. As expected, control responder OT-II T cells, upon activation with dendritic cells loaded with OVA323–339 peptide, showed a strong response with elevated levels of IL-2 production. However, lysates obtained from untreated tumors markedly inhibited OT-II responses and resulted in a profound decrease in IL-2 production, even though exogenous OVA323–339 peptide was added to the culture (Fig. 3A). Interest-
ingly, lysates derived from LOFU-treated tumors did not only have no negative effect on the responses of OT-II cells to OVA<sub>323–339</sub> but were also able to elicit a strong activation of OT-II responder T cells even in the absence of exogenous peptide (Fig. 3A). Because the lysates prepared from LOFU-treated tumors seemingly exhibited a greater priming ability by dendritic cells of T cells, we proceeded to isolate bone marrow–derived cells containing the monocyte and dendritic cell progenitor population and differentiated them to dendritic cells. Immature dendritic cells thus obtained were then treated with lysates prepared from untreated or LOFU-treated tumors. Whereas B16-F1 lysates prepared from untreated tumors were able to increase CD86 surface expression in dendritic cells as much as lysates prepared from LOFU-treated tumors, only the latter were also able to induce a significant increase in the expression of CD80, MHC-II, and CD83 on dendritic cells (Fig. 3B). These results extended further support to our observation that LOFU treatment of B16 melanoma tumors prevents the negative effect on the T cell priming capacity of dendritic cells that normally occurs in the tumor microenvironment.

Our results prompted us to directly determine whether tumor DLN resident APCs would be functionally more efficient at activating target T cells after LOFU treatment of melanoma tumors. To that effect, B16-F1 melanomas were induced on C57BL/6 mice and were either left untreated or treated with LOFU. DLN cells were then cocultured with naive Tyrp1<sup>+</sup> CD4<sup>+</sup> T cells and stimulated with B16 melanoma tumor lysates. Supernatants were collected 24 h later and analyzed for IL-2 levels by ELISA. Data are presented as mean±SEM from three independent experiments. Differences between cytokine production in cultures using DLN cells from untreated or LOFU-treated mice were analyzed using a two-tailed t test (*p < 0.05).
suspensions were depleted of T cells and used to test the capacity and DLN APCs to activate tumor Ag-specific T cells. T cell-depleted DLN cells were cocultured for 24 h with naive Tyrp1-TCR CD4+ T cells and lysates were prepared from B16-F1 tumors. IL-2 production was measured by ELISA to monitor responder T cell priming. Cells isolated from the DLNs of LOFU-treated, tumor-bearing mice showed a significantly increased ability to activate Tyrp1 CD4+ T cells compared with cells isolated from untreated mice (Fig. 3C), supporting that LOFU treatment of B16 melanomas also indicated that LOFU induced increased expression of Hsp70 (Fig. 4D). To determine whether the increased presence of Hsp70 in tumor cells (Fig. 4E). confirmed that LOFU treatment of B16 melanomas caused increased membrane presence of Hsp70 in tumor cells (Fig. 4F).

Lysates from LOFU-treated B16 tumors also allow dendritic cells to reactivate anergic tumor Ag-specific T cells

Our results supported that tumor-induced T cell tolerance could be overcome after LOFU treatment. This observation was substantiated by the fact that after LOFU treatment of the tumor site, the expression of several anergy-associated genes in T cells from tumor DLNs was not increased compared with control T cells isolated from distal DLNs (Fig. 2), whereas activation-induced cytokine expression was also restored to levels close to those detected in T cells isolated from distal DLNs or in T cells isolated from control non-tumor-bearing mice (Fig. 1). FACS analyses revealed that those differences in the expression of anergy-associated genes did not result from differences in the distributions of T cells present in DLNs or in the tumor-infiltrating lymphocyte populations (Fig. 5A, 5B). This indicated that tumor-induced anergy did not result in decreased migration of T cells or in decreased number of contacts with APCs, which should elicit expression of the early activation marker CD69 even under conditions that would induce anergy in T cells (34). Rather, these data supported the existence of differences in the quality on the interaction between T cells and tumor Ag-presenting dendritic cells that induced anergy in untreated tumors but prevented the establishment of T cell tolerance in LOFU-treated mice. Further supporting this hypothesis, similar results were obtained when mice treated with HIFU, which did not prevent the induction of tolerance (Supplemental Fig. 1), were analyzed (Fig. 5). Notably, there was a significant increase in the presence of CD45+ cells in the LOFU-treated tumors, although it did not correlate with increased presence of T cells, suggesting that an influx of other inflammatory cells might occur in response to the extensive tissue damage caused by HIFU.

This prompted us to investigate whether LOFU might not only prevent the induction of tumor Ag-specific T cell anergy but also reverse established anergy and allow dendritic cells to generate a productive effector response even in previously tolerized T cells. To address this question, we isolated naive CD4+ T cells from spleen and lymph nodes of Tyrp1 mice, in vitro differentiated into Th1 cells and anergized by activating them through partial stimulation with anti-CD3 Abs in the absence of costimulation. As expected, T cells became hyporesponsive and showed a profound decrease in IL-2 production upon restimulation with anti-CD3 and anti-CD28 Abs (Fig. 6A). These anergic cells were then reactivated with dendritic cells loaded with lysates derived from either untreated or LOFU-treated melanoma tumors. As expected, anergic Tyrp1 T cells stimulated with dendritic cells loaded with tumor lysates from untreated B16-F1 melanoma produced negligible amounts of IL-2. However, when dendritic cells were loaded with tumor lysates prepared from LOFU-treated tumors, previously anergized T cells produced significantly more IL-2 than those activated with untreated lysates (Fig. 6B). These results support that LOFU treatment of melanoma tumors might result in the generation of immunogenic molecules that may enable dendritic cells to deliver activating signals that can breach tolerance, allowing otherwise anergic T cells to respond to Ag re-encounter to generate a productive response.

LOFU followed by ablation of tumor by hypofractionated IGRT results in enhanced T cell–mediated control of primary melanoma lesions

To further determine the consequences of our observation that LOFU therapy can modulate tumor immunogenicity and enhance antitumor immune responses, we performed a series of in vivo
treatment strategies evaluating primary tumor control using a combination of LOFU with or without tumor ablation using daily 10 Gy hypofractionated IGRT to a total dose of 30 Gy per mouse with established B16-M1 tumors located s.c. in the right dorsal hind limb. Treatment was initiated for all mice when tumor volume reached $\sim 50 \text{ mm}^3$. Tumor volumes in each group were then measured three times a week for up to 62 d (Fig. 7A). Untreated C57BL/6 or mice treated with LOFU alone continued to experience rapid primary tumor growth, reaching a volume of $300 \text{ mm}^3$ within 10 d of treatment, at which point a BKA was performed (Fig. 7A). In contrast, mice within the hypofractionated IGRT or LOFU+IGRT groups experienced significant growth delay for up to 3 wk posttreatment, after which mice treated with IGRT alone began to exhibit primary tumor regrowth, reaching a volume $\geq 300 \text{ mm}^3$ at $\sim 5$ wk. Remarkably, the mice in the LOFU+IGRT group had a sustained response, with limited tumor growth for $>6$ wk after treatment. The reduction in tumor volume in the group receiving LOFU+IGRT or IGRT when compared with the untreated or LOFU alone groups was statistically significant by day 25 ($p < 0.05$). Moreover, the reduction in tumor volume in the LOFU+IGRT group compared with IGRT alone was statistically significant by day 35 and remained statistically significant ($p < 0.05$) for the duration of the experiment (Fig. 7A). In addition, mice in the LOFU+IGRT group demonstrated regression of tumors from their baseline measurements, and a complete tumor-free response was seen in four of five mice.

In a separate set of experiments, we combined IGRT with LOFU or HIFU to assess their comparative effects. All tumors were induced s.c. in the right dorsal hind limb. To compare the effects of LOFU+IGRT or HIFU+IGRT on tumor growth in a more unbiased way, and to minimize exposure of normal nonmelanoma tissue to high temperatures generated by HIFU, we started FUS treatment with tumor sizes equaling or exceeding 75 mm$^3$. HIFU treatment led to quick thermal destruction of tissue. In nearly all animals tested, HIFU treatment of melanoma caused not only the loss of primary tumors but also of the tumor-bearing limb. Thus, only one-time 10 Gy doses of IGRT could be administered to HIFU-treated tumors. HIFU+IGRT administration ablated the tumors in the respective cohort within 48 h of treatment. From this point onward, appearance and growth of secondary tumors in the two experimental groups were monitored. Twenty days after treatment and ablation of primary tumors, secondary tumors exceeding 500--

**FIGURE 4.** LOFU treatment of melanoma tumors enhances expression of immunogenic markers in tumor cell. (A) Tumor cells isolated from untreated (Ctrl) or LOFU-treated mice were analyzed by flow cytometry for the presence and percentage representation of Gr1$^{+}$CD11b$^{hi}$ cells and CD11b$^{+}$CD11c$^{+}$ dendritic cells. Results are shown as mean+SEM from three to five mice for each experimental condition. (B) Representative FACS dot plot of B16 tumor cell suspension obtained from untreated or LOFU-treated mice were stained with a viability marker (Live/Dead Mk). Relative quantification of dead cells is reported. Box and arrow indicate dead cells (Live/Dead MK$^+$). (C and D) Immunofluorescence staining of B16-F1 tumor tissues isolated from untreated mice or from mice treated with LOFU. Tissue sections were stained with Abs to detect calreticulin (C) or Hsp70 (D) (green) and TRP1 (red). Nuclei were stained with DAPI. Original magnification $\times 60$. (E and F) Cells from tumors of LOFU-treated mice and untreated mice were stained for CD45 and for the expression of TRP1. CD45$^+$ TRP1$^+$ B16 cells were then analyzed for the expression of Hsp70 (E) or MHC-I (F). A representative histogram is shown. Gates and arrows indicate the selected population for the analysis. Data (mean+SEM from three mice) were analyzed using a two-tailed t test (**) $p < 0.05$. 

The Journal of Immunology 1971
1000 mm$^3$ in volume necessitated sacrifice of several mice in the HIFU+IGRT group (Supplemental Fig. 1B). Some of the mice in the LOFU+IGRT group also started to show palpable secondary tumors in the popliteal lymph nodes but continued to live well past the 40-d mark posttreatment, with two of five mice showing no apparent tumor growth despite having started off with a much larger primary tumor load than in the experiments shown in Fig. 7A.

To corroborate the immunomodulatory effect of LOFU, we performed experiments using the immunocompromised BALB/c nude model. In these mice, B16-M1 tumors grew much more rapidly, reaching ≥300 mm$^3$ ~1 wk earlier than C57BL/6 mice. The overall treatment response was similar, with untreated and LOFU alone resulting in no significant primary tumor control, whereas IGRT and LOFU+IGRT induced some delay in primary tumor growth (Fig. 7B), in both the IGRT and the LOFU+IGRT treatments, primary control was short-lived. In fact, BKA was required in the IGRT group <2 wk after starting treatment and in <3 wk in the LOFU+IGRT group. In addition, LOFU+IGRT in immunocompromised mice failed to result in statistically significant primary tumor control when compared with IGRT alone (Fig. 7B).

**LOFU followed by hypofractionated IGRT results in prolonged recurrence-free survival and reduced pulmonary metastasis**

Based on our data, we hypothesized that LOFU-induced enhanced antitumor responses might augment therapeutic IGRT, not only achieving better control of local disease but also of microscopic disease and distant metastases. Because B16-F10 is an aggressive cell line that rapidly grows to an unacceptable size if not treated, mice with primary tumors >300 mm$^3$ required BKA. Notably, by
FIGURE 6. Lysates from LOFU-treated B16-F1 melanoma tumors can reverse the hyporesponsive state of anergic T cells. (A) Naive CD4+ T cells were isolated from spleens and lymph nodes of Tyrp1 mice, and differentiated into Th1 cells. Cells were then either left untreated or treated with anti-CD3 alone for 16 h to induce anergy. Cells were then rested for 72 h in strict absence of IL-2 and restimulated with anti-CD3 and anti-CD28 Abs. IL-2 levels were measured by ELISA. The results are shown as mean±SEM from two independent experiments. (B) CD11c+ dendritic cells were isolated from spleens of tumor-free Tyrp1 mice. Anergic Th1 cells generated from Tyrp1 mouse-derived CD4+ T cells as described in (A) were cocultured with the dendritic cells and tumor lysates derived from untreated or LOFU-treated B16-F1 melanoma tumors. Supernatants were collected after 24 h and assayed for IL-2 by ELISA. Results are shown as mean±SEM from two independent experiments with three independent sets of tumor lysates used in each experiment. Data were analyzed using ANOVA with a Tukey posttest (**p < 0.01).

Discussion

The adaptive immune system constantly surveys for malignantly transformed cells. This is largely achieved by recognition of tumor-associated Ags that prime the appropriate T cell repertoire to mount antitumor immune responses. However, tumors also use diverse mechanisms to evade the adaptive immune system and thwart antitumor T cell responses (1). As a result, successful immunotherapy against cancer has to overcome the major obstacle of tumor-induced tolerance (35). Several mechanisms have been described to explain how tumors induce tolerance in different T cell subtypes, including defective presentation of tumor Ags and inadequate activation of APCs, signaling through coinhibitory receptors, immunosuppression by factors released within the tumor microenvironment, and local recruitment of suppressor cells (9, 15–17, 20, 36–38). For the immune response to become activated in a tumor Ag-specific way, a successful therapeutic approach must promote immunogenic cell death. The hallmarks of immunogenic cell death include the release of damage-associated molecular patterns, translocation of certain chaperone complexes to the cell surface, and increased susceptibility to dendritic cell–mediated cross-presentation of tumor-associated Ags (39). In this study, we sought to investigate whether a novel treatment for melanoma using nonablative LOFU would result in enhanced antitumor immune responses and prevention of tumor-induced tolerance.

Thermally ablative HIFU, although able to control primary tumors, is not always effective at preventing micrometastatic invasions in surrounding or distant tissues, suggesting that cell death caused by this FUS modality might fail to adequately prime an adaptive antitumor immune response. High thermal stress induced by HIFU is likely to destroy the vasculature and tissue infrastructure, limiting the ability of immune cells to reach the tumor site and support Ag presentation and/or recognition. Local or distal micrometastatic invasions should be prevented or ameliorated by an adequately primed immune system that could eliminate the relatively small tumor load of cells that escape initial ablative treatment. In this study, using a B16 murine melanoma model, we show that the use of nonablative LOFU treatment enhances priming activity of dendritic cells, which potentiates CD4+ T cell effector responses by overcoming the tolerizing effects of the tumor microenvironment and prevents local recurrence and distal metastases when administered before an ablative treatment.

Development of T cell hyporesponsiveness to tumor Ags has been described in T cells in several mouse tumor models and in human cancers (15, 18, 40). We have previously reported that tumor Ag-specific CD4+ T cells become anergic in tumor-bearing mice and express a series of anergy-associated genes that have been shown to hinder their ability to proliferate and produce effector cytokines (21, 41). Furthermore, prevention of the expression of those genes in mice that lack NFAT1 or Egr2, two transcription factors responsible for the expression of anergy-inducing genes (42–44), leads to inhibition of tumor Ag-specific T cell hyporesponsiveness and improved control of local tumor growth (19, 21). Using two different B16 mouse melanoma models, our data confirm that resident tumor Ag-specific CD4+ T cells in the tumor DLN upregulate the expression of anergy-associated genes, including Grail, Itch, Chib, Gr4, and Egr2. The activation of this program of gene expression was well correlated with a reduced ability to produce cytokines after ex vivo restimulation, supporting that B16 melanoma induces an intrinsic state of hyporesponsiveness in tumor Ag-specific CD4+ T cells. Importantly, treatment of the primary tumor with LOFU resulted in an increased ability of those tumor Ag-specific CD4+ T cells to produce cytokines upon restimulation. LOFU-induced restoration of the responsiveness to TCR engagement, in otherwise anergic cells, was accompanied by a reduction, to varying extents, of the expression of most anergy-inducing genes. The absence of changes in Foxp3 transcripts in DLN resident CD4+ T cells in...
tumor-bearing mice suggests, however, that LOFU did not affect Foxp3+ regulatory T cell migration or differentiation and supports that LOFU prevented tumor-induced tolerance by inhibiting the induction of T cell anergy.

Initial studies on tumor-induced T cell anergy identified the key role that APCs played in this process, and defective dendritic cell maturation has been defined as a major determinant of inefficient priming of tumor Ag-specific T cells (20, 45). Recently, it has been shown that unstable immunological synapses formed between T cells and dendritic cells presenting tumor Ags result in delayed nuclear export of NFAT and the activation of a tolerogenic NFAT-dependent program of gene expression that includes Egr2 (46). Increased T cell activation that follows LOFU treatment of B16 melanomas would potentially result from several different phenomena. First, treatment of the tumors with nonablative LOFU delivers both thermal and mechanical stress to the tumor cells. This stress could help generate novel unique "non-self" tumor Ags that, in turn, could make the tumor more immunogenic and less able to induce tolerance. Alternatively, stress-induced danger signals released by tumor cells could target dendritic cells and generate a tumor microenvironment that would be less conducive to the induction of tolerance in T cells. Stress-associated molecular chaperones, including heat shock proteins and calreticulin, have been implicated in dendritic cell maturation and enhanced antitumor immunity (30, 47–50). There is evidence that primary tumor lysates are rich in heat shock proteins that can trigger

**FIGURE 7.** LOFU followed by hypofractionated IGRT results in T cell–mediated, long-term primary tumor control and reduced distal metastases. (A) C57BL/6 mice with 50 mm³ s.c. dorsal right hind-limb tumors were separated into one of four treatment groups: untreated, LOFU, hypofractionated IGRT, or LOFU+IGRT, and tumor growth was monitored for 62 d or until primary tumor grew beyond 300 mm³. Graph shows mean ± SEM of tumor volume from one of two representative experiments (3–5 mice/group). Data were analyzed with either one-way ANOVA followed by a Bonferroni correction posttest (before day 29) or by two-tailed Student t test (after day 29). Significant differences (defined as p < 0.05) between untreated or LOFU-treated mice and IGRT- or LOFU+IGRT-treated mice (#) occurred after day 25, and between IGRT-treated and LOFU+IGRT-treated mice (*) after day 35. Individual graphs showing the distribution of tumor size at specific days are also shown. (B) Similar experiments as the ones described in (A) were performed in BALB/c nude mice. No significant differences were observed among the different groups at any time point. (C) C57BL/6 mice were monitored for primary tumor progression/recurrence, defined as either recurrence reaching a volume of 150 mm³ or the development of local metastasis to the popliteal or inguinal lymph nodes. In addition, animals that died spontaneously were scored as having recurrence or progression of disease. Recurrence-free survival data were analyzed using the Mantel–Cox test. (D) Lungs were harvested from animals that either died spontaneously, required euthanasia because of overwhelming tumor burden, or were sacrificed at the end of a 2-mo-long experiment. Lung metastases were then measured. Lungs with nodules that fuse into plaques or exceed 250 were deemed too numerous to count and assigned a maximal value of 250. A representative specimen is shown for each treatment group. Results are shown as mean ± SEM, with n = 3–5 mice/group, analyzed with a Kruskal–Wallis test, followed by Dunn’s posttest. *p < 0.05.
maturation signals in dendritic cells (51). Importantly, heat shock proteins are also capable of associating with and delivering antigenic peptides from tumor cells to dendritic cells; furthermore, their presence in the tumor cell plasma membrane has been associated with increased immune responses (52–54). Calreticulin has also been described to play an important role in antitumor response, and its translocation to the surface of tumor cells has been associated with increased phagocytosis of the tumor cell by dendritic cells and immune activation (55, 56). Previous studies using HIFU in murine adenocarcinoma models showed that this treatment significantly increased expression of costimulatory molecules on dendritic cells, which also produced higher levels of IL-12 and resulted in increased CTL activity (27, 28). Our data show that LOFU induces a redistribution of calreticulin in B16 cells and an increase in the expression of the inducible heat shock protein Hsp70, suggesting that cellular stress mediated by LOFU is capable of inducing changes in the expression of those stress-induced proteins. We also detected increased expression of MHC-II and B7 proteins, supporting that LOFU may lead to changes in the state of maturation or activation of dendritic cells that contribute to the potentiation of the efficient presentation of tumor Ags that is likely responsible for the enhanced tumor immunogenicity and for promoting T cell activation over anergy.

T cell tolerance induced by tumor Ags remains a major challenge in cancer immunotherapy. Effective reversal of tolerance in tumor-specific T cells is a key goal in clinical antitumor strategies. Our data find that preestablished anergy in T cells can be reversed by the activating effect on dendritic cells of lysates prepared from LOFU-treated melanoma tumors. This observation opens up the possibility that LOFU treatment of tumors could release novel immunogenic molecules from tumor cells that would not only prevent but also reverse preestablished tumor tolerance in T cells. Signaling through the IL-2R has long been known to prevent and reverse clonal anergy in T cells (57–59). However, we could not detect elevated amounts of IL-2 in any of our lysates, untreated or treated with LOFU (data not shown), making presence of IL-2 an unlikely candidate to have caused the reversal of anergy in our experiments. However, it is possible that other factors could contribute to this phenotype. In fact, T cell costimulation through the TNFR family member OX-40 ligand has also been shown to prevent and overcome T cell anergy in addition to increased effector response in both CD4+ and CD8+ T cells (60–62). Engagement of CD137, CD40, and blockade of PD1 have also been reported to prevent and reverse preestablished CD8+ T cell tolerance in vivo (63–65).

Whereas LOFU, a nonablative treatment, was not able to control the growth of an established B16 melanoma, which usually has a very aggressive behavior and a fast rate of growth, pretreatment of melanoma tumors with LOFU before performing ablative therapy using hypofractionated IGRT resulted in a significant delay in tumor growth, and in several cases, complete regression of tumors was observed only in immunocompetent mice. Recurrence-free survival in these mice was also markedly improved following that protocol. In addition, incidences of lung metastases were minimal in mice that received LOFU before tumor ablation compared with mice that received only ablative IGRT. Strikingly, LOFU failed to confer similar protection on T cell–deficient nude mice. This observation indicates that the protective effect of LOFU might not be restricted only to control primary tumor, but that it can also prevent the establishment of metastatic foci either locally or distally. This protection from metastasis may result from the enhancement of priming by dendritic cells of tumor Ag-specific T cells, causing prevention or reversal of T cell tolerance. LOFU pretreatment not only controlled tumor growth more effectively, but, as indicated before, likely resulted in the generalization of strongly immunogenic, IGRT-induced tumor death that provided protection from metastasis and ensured longer recurrence-free survival.

Prevention of T cell tolerance to endogenous tumor Ags is of paramount importance in anticancer immunotherapy. Our work shows that treatment of primary tumors with LOFU can accomplish that, making it a candidate therapy for the development of an in situ autologous tumor vaccine. LOFU treatment of solid tumors, in combination with an ablative approach, may thus result in increased efficacy of primary tumor eradication, as well as prevention of distal metastases.

Disclosures
A.P. is an employee of Philips Healthcare, Bethesda, MD. The other authors have no financial conflicts of interest.

References