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

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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.

Abbreviations used in this article: BKA, below-the-knee amputation; CBCT, cone beam computed tomography; DLN, draining lymph node; FUS, focused ultrasound; HIFU, high-intensity FUS; IGRT, image-guided radiation therapy; LOFU, low-energy FUS; NDLN, nondraining cervical lymph node.

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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>−/−</sup> Tg<sup>B-W-Tg</sup>(TcrTcrb) 9R<sup>es<sup>es</sup></sup> (Tyrp1), and B6.Cg-Tg(TcrTcrb)425Cbnf1 (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 μl penicillin/streptomycin.

Generation of dendritic cells from bone marrow–derived cells
Bone marrow–derived cells were harvested from the femur and tibia of C57Bl/6 mice and were cultured in RPMI 1640 medium supplemented with 10% FBS in the presence of 20 ng/ml murine GM-CSF (Peprotech). Loosely adherent immature dendritic cells were harvested after 7 d of culture. If necessary, murine TNF-α (Peprotech) at a concentration of 100 ng/ml was added to the culture for the last 24 h to induce dendritic cell maturation.

Tumor models
A total of 3 × 10<sup>5</sup> B16-F1 melanoma cells suspended in HBSS (Invitrogen) was injected s.c. in the lumbar flanks of mice. Melanoma tumors were induced in the foot pads by injecting 2 × 10<sup>5</sup> B16-M1 cells in the dorsum of the right hind limb.

Tumor growth monitoring
Primary B16-M1 melanoma dorsal hind-limb tumors were measured three times per week with vernier calipers. Tumor volume was calculated using an ellipsoid formula: V = (π/6)(length × width × height). Primary dorsal hind-limb tumors exhibit Gompertzian growth, with a phase I volume of 30–50 mm<sup>3</sup>, phase II volume of 90–150 mm<sup>3</sup>, and phase III volume of 300–500 mm<sup>3</sup>. Therefore, treatment efficacy was determined by calculating the tumor growth delay to 90–150 mm<sup>3</sup>, in which the tumor is in the exponential phase II. Tumors that reach 300–500 mm<sup>3</sup>, in accordance with Institutional Animal Care and Use Committee–approved protocol.

ELISA
A total of 1.5–2.5 × 10<sup>5</sup> T cells was left rested or stimulated with either anti-CD4<sup>+</sup>-anti-CD28 Abs, T cell–depleted OVA peptide 323–339 (OVA<sub>323-339</sub>)–loaded splenocytes at a 1:5 T cell/spleenocyte 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).

Immuno-fluorescence 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- Tryptase (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/sample 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 exposed tumor resulting in tumor irradiation over the entire tumor volume. The ultrasound transducer was operated at 1.0 MHz, resulting in an ellipsoid focal spot <~1.5 mm in diameter and 12 mm in length (~6 dB of pressure), as measured along the ellipsoid axes. Ultrasound exposures were delivered to the tumor using a 1-mm grid pattern extending over the entire tumor volume. Before therapy, the tumor volume was required to calculate the grid size for the particular treatment. The ultrasound exposure duration per tumor was 1.5 s, after which the transducer was automatically positioned over the next grid point and the procedure repeated until the entire tumor volume was covered. Two layers of grid points were performed in each tumor. The therapeutic ultrasound device was operated in continuous-wave mode at a specific acoustic pressure/power regimen: acoustic power 3W, peak negative pressure ~2.93 MPA (80-mm focal length)/3.81 MPA (85-mm focal length), to provide nonablative LOFU. The resulting in situ intensity (<I<sub>90</sub>) at the focus was estimated to be 550 W/cm<sup>2</sup> at a depth of 4 mm in tissue. Total energy deposition to a tumor was ~900 J. For the ablative HIFU regimen, a similar setup was used with the following changes: 5.42 MPA peak negative pressure, 1.2-mm spacing between grid points, 4-s duration at each grid point at 75% duty cycle, generating an acoustic power of 12.5 W. Unlike the LOFU protocol, the HIFU was carried out at a single focal length of 80 mm.

In vivo hypofractionated CBCT IGR
All radiation was delivered using Xstrahl Limited’s Small Animal Radiation Research Platform to deliver a 10 Gy dose to a target tumor in 341 s. Anesthetized animals were placed on stage attached to a motorized platform, and the tumor-bearing right hind limb was extended, elevated, and secured to a 1.5-cm adhesive platform to minimize extraneous tissue exposure. Once secure, a CBCT was performed and the data opened in 3D Slicer for tissue segmentation and treatment planning. 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.
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, glucic acid acetic, 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.

Recurrence-free survival

The following events were scored 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 were processed as censored data: 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 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: actinb: F, 5′-GTGACCTTGACACCTGGAAGA-3′, R, 5′-CGCCGACCTCATGTACCTC-3′; Cblb: F, 5′-GGCGACATCGTCAATTCC-3′, R, 5′-ATGTCAGCTTGTCCTGGC-3′; Casp3: F, 5′-ATCGCAAGAGCT-3′, R, 5′-ACGCGCACTCTGGAGGAGG-3′; Foxp3: F, 5′-GCAGCATCATTGACCCTTTCA-3′, R, 5′-GCCGGACTGTGGCAGCTGAAGCTTTCCAATA-3′; Egr2: F, 5′-GCTTCTACTTGCAGCCCATC-3′, R, 5′-GTGTGGAGTCACCAGACCCT-3′; Ifnar: F, 5′-ACGCGCACAAGCTAGAATTT-3′, R, 5′-GCCGGACTGTGGCAGCTGAAGCTTTCCAATA-3′; Itch: F, 5′-CACAGCTAAG-3′, R, 5′-TCATGGTTTATGCACCACG-3′; Traf3: F, 5′-TGGCCAATGCAGCTCTCGG-3′, R, 5′-TGGCCAATGCAGCTCTCGG-3′; Tlr4: F, 5′-CAGGCAGCTCCGCGCTGGGAG-3′, R, 5′-CAGGCAGCTCCGCGCTGGGAG-3′; TLR7: F, 5′-ATGGATCTCAATGAGCAGATCC-3′, R, 5′-ATGGATCTCAATGAGCAGATCC-3′; TLR9: F, 5′-TGACATCCACAAAAGGGAGAAA-3′, R, 5′-TGACATCCACAAAAGGGAGAAA-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-CD10, and anti-CD45 (all from eBioscience). Anti-CD83 and anti-CD4 were purchased from Biolegend. 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 (Life Technologies). CD4+ and NDLN resident CD4+ T cells were isolated from both groups of C57BL/6 mice that were either left untreated or were treated with LOFU. Thirty-six hours after LOFU treatment, DLN T cells expressing a transgenic MHC class II–restricted TCR that recognizes the OVA253–329 peptide. T cells were collected from these mice as described earlier and were stimulated ex vivo using splenocytes loaded with OVA253–329 peptide. CD4+ T cells from the ipsilateral DLNs again produced significantly reduced amounts of IL-2 compared with cells from the contralateral DLNs 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-1113–125 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 reduced compared with cells harvested from contralateral DLNs 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/6 mice that were either left untreated or were treated with LOFU. Hundred 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 < 0.01, *p < 0.05). (G and H) Tumors were induced in C57BL/6 mice by s.c. injection of 3 × 10⁶ 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-γ 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 < 0.05).
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, to assess responses induced by endogenous tumor Ags. CD4+ T cells were isolated from the DLNs and NDLNs, and the expression of several anergy-associated genes was assessed. T cells derived from the DLNs showed varying degrees of upregulation of six of the seven anergy genes analyzed, including Rnf128, Itch, and Cblb and Egr2, as well as Tie4 and Casp3 (encoding for caspase 3) (Fig. 2B–H). Another transcription factor, Ikaros, which is also upregulated in several in vitro and in vivo T cell anergy models, was not upregulated in this melanoma model of tumor-induced anergy, and its levels remained largely similar in both the DLN- and the NDLN-derived T cells (Fig. 2G). Interestingly, when the tumors were treated with LOFU, the expression of four of those genes, Grai, Itch, Cblb, and Grg4, in T cells isolated from the DLN was significantly lower than in the T cells isolated from untreated mice (Fig. 2B–F), supporting that LOFU treatment prevented the induction of the expression of anergy-inducing genes in tumor Ag-specific CD4+ T cells.

**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).
ingly, lysates derived from LOFU-treated tumors did not only have no negative effect on the responses of OT-II cells to OVA\textsubscript{323-339} 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 cell

FIGURE 3. LOFU treatment of melanoma tumors potentiates dendritic cell–mediated priming of CD4\textsuperscript{+} T cells. (A) CD11c\textsuperscript{+} splenic dendritic cells were purified from C57BL/6 mice and cocultured with responder naive CD4\textsuperscript{+} T cells isolated from OT-II mice. B16-F1–OVA melanoma tumor lysates were prepared from untreated or LOFU-treated tumor-bearing mice and added to the respective cultures to drive dendritic cell–mediated T cell stimulation. In separate samples, exogenous OVA\textsubscript{323-339} peptide was also added along with tumor lysates. Supernatants were collected after 24 h, and IL-2 production was assessed by ELISA. Results are shown as mean±SEM from four independent experiments and analyzed with one-way ANOVA followed by a Tukey posttest (\(p < 0.05\), ***\(p < 0.001\)). (B) Bone marrow cells were isolated from the femur and tibia bones of C57BL/6 mice and differentiated in vitro into immature dendritic cells. Cells were further cultured for 24 more hours in the presence of either tumor lysate-free complete DMEM (No lys), lysates from untreated melanoma tumors (Untr lys), or lysates from LOFU-treated melanoma tumors (LOFU lys). CD11c\textsuperscript{+} cells were analyzed by flow cytometry for expressions of dendritic cell activation markers CD80, CD83, and CD86, and also for MHC-II molecules. Results are shown as average of mean fluorescence intensity (MFI) ± SEM and analyzed by one-way ANOVA with Tukey posttest (\(**p < 0.01\), ***\(p < 0.001\)). (C) B16-F1 melanoma tumors were left untreated or treated with LOFU. Tumor DLNs were isolated and depleted of T cells. DLN cells were then cocultured with naive Tyrp1 CD4\textsuperscript{+} 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 shown 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 (\(p < 0.05\)). (D) CD4\textsuperscript{+} T cells were isolated from the tumor DLNs of untreated or LOFU-treated B16-F1 melanoma tumor-bearing C57BL/6 mice, and stimulated with anti-CD3/anti-CD28 Abs in the presence or absence of tumor lysates prepared from untreated B16-F1 melanoma tumors. IL-2 outputs were measured by ELISA. Results are presented as mean±SEM from three different mice per condition. n.s., not significant.
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 naïve 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 melanoma results in the generation of APCs that are functionally more efficient at activating tumor Ag responder T cells. The effects of LOFU on tumors appeared to primarily target dendritic cells and not directly T cells. As expected (see Fig. 1), when T cells isolated from the DLNs of LOFU-treated, B16-bearing mice were activated with plate-bound anti-CD3 and anti-CD28 Abs, they produced higher amounts of IL-2 than cells obtained from untreated mice; however, this difference was not altered by coincubation with lysates prepared from untreated B16 tumors (Fig. 3D).

Activation of melanoma-specific T cells by dendritic cells is a crucial event in determining their fate. A successful Ag presentation event that is able to elicit an effect on T cell response is critically dependent on the activation state of dendritic cells, which would otherwise deliver tolerogenic stimuli. Our results indicate that treatment of melanoma tumors with LOFU resulted in increased CD4+ T cell activation as a consequence of inducing increased stimulatory activity on APCs, and thus hindering tumor-induced T cell tolerance. We did not observe any significant differences in the levels of infiltrating CD11b+CD11c+ dendritic cells or CD11b+Gr1+ myeloid-derived suppressor cells in LOFU-treated B16 melanomas, suggesting that the effect of LOFU was not being exerted on dendritic cell trafficking but likely on dendritic cell activation and Ag presentation (Fig. 4A). Trafficking of tumor Ags by molecular chaperones, including calreticulin and Hsp70, is crucial for the subsequent productive presentation of Ags to T cells (30–33). We therefore used both in vivo and in vitro approaches to detect changes in the expression of calreticulin and Hsp70 in untreated and LOFU-treated B16 melanoma tumors. Tumors, either left untreated or treated with LOFU, were harvested from tumor-bearing mice, made into single-cell suspensions, and stained with a live/dead marker to assess cell viability. We did not observe changes in cell viability in response to LOFU treatment, supporting that this low-energy form of FUS was not directly inducing tumor cell death (Fig. 4B). When B16 melanoma tumors were left untreated or exposed to LOFU treatment and tumor tissue sections were put on slides, LOFU-treated cells showed a change in the distribution of calreticulin, compared with untreated cells, which appeared to accumulate in discrete regions of the plasma membrane on B16 cells (Fig. 4C). Immunofluorescence analyses of LOFU-treated melanomas also indicated that LOFU induced increased expression of Hsp70 (Fig. 4D). To determine whether the increased Hsp70 expression also correlated with increased presence in the membrane of this protein, we stained nonpermeabilized CD45− TRP1+ B16 melanoma cells for Hsp70, and cell-surface expression after LOFU treatment was assessed by FACS. This analysis confirmed that LOFU treatment of B16 melanomas caused increased membrane presence of Hsp70 in tumor cells (Fig. 4E). B16 melanoma cells express very low levels of MHC-I molecules on their surfaces, leading to a very inefficient direct tumor Ag presentation by these cells to cytotoxic T lymphocytes, which further reduces the chance of elimination of malignant cells by the adaptive immune system. Treatment of melanoma tumors by LOFU also increased cell-surface MHC-I expression nearly 2-fold and, interestingly, also led to a similar enhancement of the expression of the tumor-specific Ag TRP1 (Fig. 4F).

Lysates from LOFU-treated B16 tumors allow dendritic cells to reanimate 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 populations 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 Ag-presenting dendritic cells that induced anergy in untreated melanomas 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 HIFU-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 naïve 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 \( \approx 50 \text{ mm}^3 \). 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 \( \approx 5 \text{ wk} \). Remarkably, the mice in the LOFU+IGRT group had a sustained response, with limited tumor growth for \( > 6 \text{ wk} \) 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 \( 75 \text{ mm}^3 \). 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–
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 $\approx 300$ mm$^3$ $\sim$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).

**FIGURE 5.** Distribution of T cell populations in DLNs and tumors in FUS-treated B16-bearing mice. (A and B) B16-F1 tumors were injected in the hind leg of mice and either left untreated (Ctrl) or treated with LOFU or HIFU. Thirty-six hours after FUS treatment, tumors and respective DLNs were collected. Percentages of CD8$^+$, CD4$^+$, and CD4$^+$Foxp3$^+$ T cells, as well as expression of CD69 in CD8$^+$ and CD4$^+$ cells, were analyzed by flow cytometry in the DLNs (A) and tumors (B). The data, shown as mean±SEM of three to four mice per condition, were analyzed by ANOVA with Tukey posttest ($^p = 0.05$).

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
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, Cblb, Grg4, 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

### 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

![FIGURE 6](http://www.jimmunol.org/)

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 < 0.001).
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.](image-url)
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 OX40 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 generation 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


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