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Advances in Brief

Depletion of CD25$^+$ CD4$^+$ T Cells and Treatment with Tyrosinase-related Protein 2-transduced Dendritic Cells Enhance the Interferon α-induced, CD8$^+$ T-Cell-dependent Immune Defense of B16 Melanoma

Julia Steitz, Jürgen Brück, Julia Lenz, Jürgen Knop, and Thomas Tüting[1]

Department of Dermatology, J. Gutenberg University, D-55101 Mainz, Germany

Abstract

Transduction of B16 melanoma cells with IFNα (B16-IFNα) enhances CD8$^+$ T-cell-dependent tumor immunity in mice, resulting in delayed outgrowth *in vivo*. Here we provide evidence that CD4$^+$ T cells down-regulate the IFNα-induced tumor immune defense. Importantly, depletion of regulatory CD25$^+$ CD4$^+$ T cells prevented growth of B16-IFNα in most mice and promoted long-lasting protective tumor immunity. Rejection of B16-IFNα could also be achieved with therapeutic injections of dendritic cells genetically engineered to express the melanoma antigen tyrosinase-related protein 2. These results support the development of novel strategies for the immunotherapy of melanoma using IFNα in combination with elimination of regulatory T cells or antigen-specific immunization.

Introduction

Recombinant IFNα has been used many years with limited success for the adjuvant treatment of patients with melanoma (1). Understanding its mechanism of action is a prerequisite for the development of more effective therapeutic strategies. Originally, it was thought that IFNα has a direct antiproliferative effect on tumor cells. However, the frequent induction of autoantibodies against thyroid antigens suggests that IFNα also modulates antigen-specific immunity. Therefore, IFNα might also act by enhancing immune responses to melanoma cells. Under physiological circumstances, IFNα is produced in large amounts during acute viral infections by bone marrow-derived plasmacytoid DCs[3] in blood and tissues (2). In this setting, plasmacytoid DCs are activated and migrate to inflamed lymph nodes, in which they present viral antigens to naive T cells in an immunostimulatory form (3). Evidence has accumulated that the presence of IFNα during T-cell stimulation profoundly affects the nature of the resulting T-cell response (4, 5). This was first demonstrated many years ago in a murine model of experimental contact allergy, in which adjuvant administration of Corynebacterium parvum enhanced cellular immunity against high doses of contact allergens via an IFNα-dependent mechanism (6). In a murine model of acute viral infection, it could be shown that IFNα is able to drive bystander T-cell proliferation and potentiate the clonal expansion and survival of CD8$^+$ T cells responding to specific antigens (7, 8). Furthermore, expression of IFNα by genetically modified, transplantable murine tumor cells enhanced tumor-specific CD8$^+$ T-cell-dependent immune responses (9–11). In the present study, we investigated the effect of *IFNα* gene therapy on cellular immune defense mechanisms in the B16 melanoma model of C57BL/6 mice. We present evidence that elimination of the regulatory CD25$^+$ subset of CD4$^+$ T cells enhances the IFNα-induced, CD8$^+$ T-cell-dependent tumor immune defense. Furthermore, local expression of IFNα in the tumor microenvironment promoted the therapeutic efficacy of a melanoma vaccine consisting of DCs genetically engineered to express the melanocytic self-antigen TRP2, which has recently been identified as a target for tumor-reactive cytotoxic T cells (12).

Materials and Methods

Animals and Cell Lines. C57BL/6 mice (H-2^b) were bred at the Central Animal Facility of the University of Mainz and were used for experiments at the age of 6–12 weeks. Nude C57BL/6 mice were purchased from M&B A/S (Ry, Denmark). B16 (H-2^b) is a murine melanoma cell line of C57BL/6 origin (a kind gift of Dr. S. Rosenberg, National Cancer Institute, Bethesda, MD) and was maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all reagents were from Life Technologies, Inc. GmbH, Eggenstein, Germany).

Retroviral Transduction. The construction and characterization of the DFG-mIFNα2 retroviral vector has been described previously (13). Ecotropic retroviral supernatant was generated by transient transfection of the plasmid DFG-mIFNα2 into Phoenix packaging cells using standard calcium phosphate transfection in the presence of chloroquine. Supernatant containing SAM-mB7.1-EN retrovirus was kindly provided by Dr. P. Hwu (National Cancer Institute, Bethesda, MD). For retroviral transduction, 10^6 B16 melanoma cells were seeded in 75-cm^2 flasks and were incubated with retroviral supernatants in the presence of Polybrene (8 μg/ml) at 37°C. The culture medium was replaced after 3 h, and antibiotic selection was applied after 48 h (0.75 mg/ml G418). Stable selection was completed after 7–14 days.

IFNα Bioassay. Production of biologically active IFNα was measured using the viral cytopathic effect inhibition assay of vesicular stomatitis virus on mouse L929 cells. Vesicular stomatitis virus and IFN-sensitive L929 cells were kindly provided by Dr. M. Ferrantini (Instituto Superiore di Sanità, Rome, Italy). Briefly, serial half-log dilutions of IFNα samples were performed in 96-well flat-bottomed microtiter plates and 2×10^4 L929 cells were added per well. After overnight incubation at 37°C, 2×10^3 plaque-forming units of vesicular stomatitis virus (multiplicity of infection, 0.1) were added to each well. The incubation was continued for an additional 48 h, after which, plates were washed and stained with crystal violet to observe residual viable cells. IFN activity is expressed in units/ml. The IFN activity was calibrated against the NIH reference standard for mouse IFNα/β. Antibody neutralization was performed using a polyclonal sheep antimouse IFNα/β antibody kindly provided by Dr. I. Gresser (CNRS, Villejuif, France).

Flow Cytometric Analysis. Expression of the costimulatory molecule B7.1 (CD80) was measured by flow cytometry. Cells were harvested, washed in ice-cold PBS supplemented with 2% FCS and 2 mM EDTA, and stained with phycoerythrin-conjugated mAb specific for CD80 or with corresponding isotype-matched control mAb (PharMingen, San Diego, CA). Surface expression was analyzed using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). Data were collected on 5000 viable cells and analyzed using CellQuest software.

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[2] To whom requests for reprints should be addressed, at Department of Dermatology, J. Gutenberg University, Langenbeckstrasse 1, D-55101 Mainz, Germany. Phone: 49-6131-177008; Fax: 49-6131-176614; E-mail: tueting@hautklinik.klinik.uni-mainz.de.

[3] The abbreviations used are: DC, dendritic cell; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; TRP2, tyrosinase-related protein 2; EGFP, enhanced green fluorescent protein; mAb, monoclonal antibody.

Tumor Challenge and Depletion of Lymphocytes. Mice were challenged by s.c. injection of 10^5 wild-type or genetically modified B16 melanoma cells in the flank. Tumor development was assessed two to three times weekly by palpation. Perpendicular diameters of tumors were measured using a Vernier caliper. All of the experiments included 4–6 mice per group. Depletion of T-cell subsets was performed by injections of anti-CD4, anti-CD8, or anti-CD25 mAb, purified from hybridoma supernatants (clones GK1.5, 2.43, or PC61, kindly provided by Dr. E. Schmitt, Institute of Immunology, University of Mainz). Anti-CD4 or anti-CD8 mAb (250 μg) was injected i.p. on days −1, +3, +7, +14, and +21 with respect to the day of tumor challenge. Anti-CD25 mAb (600 μg) was injected on day −1 and +2 with respect to the day of tumor challenge. Depletions were verified by flow cytometry 2 days after antibody injection.

Therapeutic Immunization with DCs. DCs were generated *in vitro* from bone marrow precursors in medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 1000 units/ml recombinant murine GM-CSF, 1000 units/ml IL-4 (a kind gift of Schering-Plough Research Institute, Kenilworth, NJ), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM HEPES, 50 μM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin. DC cultures were transduced on day 5 with recombinant adenoviruses expressing human TRP2 or the marker gene *EGFP*, and harvested for immunization on day 7 as described previously (14). For immunization, mice were injected i.v. with 2.5×10^5 adenovirus-transduced DC five times on a weekly basis starting 1 week after tumor challenge.

Statistical Analyses. Significant differences of tumor growth were assessed by Student's *t* test. The difference between groups was considered statistically significant when the *P* was lower than 0.05.

Results and Discussion

The Production of IFNα by Genetically Modified B16 Melanoma Cells Enhances Cellular Immune Defense. In previous studies, we found that murine MC38 adenocarcinoma cells that were genetically modified to produce murine IFNα2 displayed reduced tumorigenicity *in vivo*. Furthermore, expression of IFNα enhanced the growth and survival of tumor-specific $CD8^+$ T cells *in vitro* (11, 13). Here, we investigated how local expression of IFNα influences cellular immune responses in the poorly immunogenic B16 melanoma model. Using retroviral vectors, we genetically modified B16 melanoma cells to express murine IFNα2 or murine B7.1 (B16-IFNα or B16-B7) as described in "Materials and Methods." Expression of biologically active murine IFNα2 was verified by demonstrating the inhibition of the viral cytopathic effect of vesicular stomatitis virus on L929 fibroblasts. B16-IFNα cells produced ~1000 units of murine IFNα2 per 10^6 cells per 48 h, whereas B16-B7 or parental B16 cells did not produce any detectable murine IFNα2. Expression of murine B7.1 (CD80) was verified using flow cytometry. B16-B7 cells but not B16-IFNα or parental B16 cells homogeneously expressed murine B7.1 on their surface. *In vitro* growth rates of B16-IFNα, B16-B7, or parental B16 cells were measured by daily cell counts because IFNα may have suppressive effects on cell growth. However, B16-IFNα grew only insignificantly slower than B16-B7 or parental B16 cells in culture. To assess growth rates *in vivo*, 10^5 B16-IFNα, B16-B7, or parental B16 cells were injected into the flanks of syngeneic C57BL/6 mice. B16-IFNα cells grew with significant delay when compared with parental B16 cells (Fig. 1*A*). In a typical experiment, mice inoculated with parental B16 cells developed palpable tumors at approximately day 15; the tumors grew progressively and measured >10 mm in diameter by day 23, when mice were killed. Mice inoculated with B16-B7 cells displayed tumors with similar growth-kinetics. In contrast, mice inoculated with B16-IFNα cells developed palpable tumors around day 30 and then grew progressively so that these mice also had to be killed. These results confirmed published observations (15) and agree with our own data in the MC38 adenocarcinoma model (11, 13). We had also expected a reduced tumorigenicity of B16-B7 cells, because it has previously been reported that

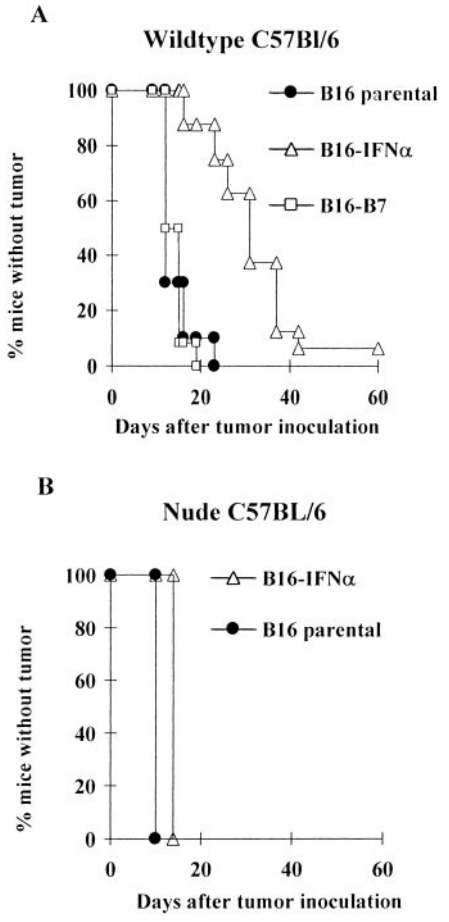

Fig. 1. Growth of B16 melanoma cells expressing IFNα is significantly delayed in wild-type but not in nude mice. In *A*, B16 melanoma cells (10^5), genetically modified to express murine IFNα2 (B16-IFNα) or murine B7.1 (B16-B7), as well as parental B16 melanoma cells (10^5) were injected s.c. into the flank of syngeneic C57Bl/6 mice. Tumor establishment was determined by palpation every other day. All of the animals with palpable tumors had eventually to be killed because of progressive tumor growth. Shown are cumulative results of five separate experiments expressed as the percentage of tumor-free mice ($n = 30$) at the indicated time points. Alternatively, 10^5 parental B16 or B16-IFNα melanoma cells were injected into T-cell-deficient nude C57Bl/6 mice (*B*).

expression of murine B7.1 enhanced cellular immune defense in several tumor models (16). However, the B16 melanoma cells used in our experiments are poorly immunogenic and expression of the co-stimulatory molecule B7.1 did not significantly alter their *in vivo* growth characteristics.

Next, we addressed whether T cells participate in the observed differences in tumor growth by injecting 10^5 B16-IFNα or parental B16 cells in T-cell-deficient C57BL/6 nude mice (Fig. 1*B*). Progressively growing tumors developed in all of the nude mice inoculated with parental B16 cells at day 10 and measured, on average, 20 ± 13 mm in diameter on day 17. All of the nude mice inoculated with B16-IFNα cells also developed progressively growing tumors at day 14 and measured, on average, 9 ± 6 mm in diameter on day 17. Thus, expression of IFNα caused only a slight delay in tumor growth in T-cell-deficient nude mice. These results suggested that the significantly delayed tumor growth of IFNα-producing B16 cells in wild-type mice requires the presence of T cells.

Depletion of $CD8^+$ T Cells Enhances, Whereas Depletion of $CD4^+$ T Cells Further Delays, Growth of IFNα-producing B16 Melanoma Cells. Because reports in the literature and our own data suggested that IFNα is able to stimulate tumor-specific $CD8^+$ T cells, we hypothesized that this subpopulation of lymphocytes was involved in the delayed *in vivo* growth of B16-IFNα. This was tested by *in vivo*

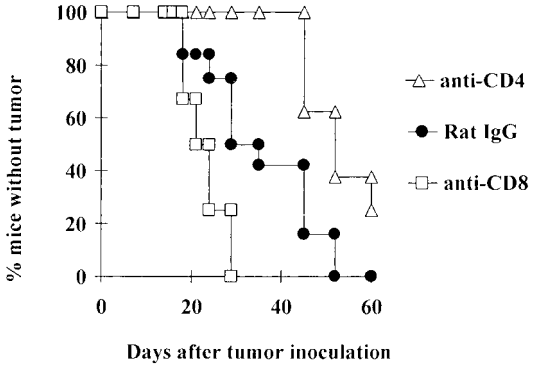

Fig. 2. The injection of anti-CD8 mAb enhances, whereas the injection of anti-CD4 mAb further delays, the growth of B16 melanoma cells expressing IFNα. Mice were given injections of 10^5 B16-IFNα s.c. in the flank and simultaneously treated with injections of purified anti-CD8 or anti-CD4 mAb or normal rat IgG as described in "Materials and Methods." Tumor growth was assessed by palpation every other day. Cumulative results of three separate experiments are expressed as the percentage of tumor-free mice ($n = 12$) at the indicated time points.

depletion of $CD8^+$ T cells with a cytotoxic anti-CD8 mAb injected immediately before and during tumor challenge as described in "Materials and Methods." Elimination of $CD8^+$ T cells *in vivo* enhanced the growth B16-IFNα cells in wild-type mice compared with control groups of mice receiving normal rat IgG (Fig. 2). This suggested that $CD8^+$ T cells participated in the IFNα-induced immune defense of the poorly immunogenic B16 melanoma. Surprisingly, depletion of $CD4^+$ T cells after injections of a cytotoxic anti-CD4 mAb further delayed growth of B16-IFNα cells, with 3 of 12 mice remaining tumor free at day 60 in three independent experiments (Fig. 2). Notably, most tumors started to grow after the depletion of $CD4^+$ T cells had been discontinued. These results supported the idea that $CD4^+$ T cells down-regulated the effect of IFNα on cellular tumor immune defense. Our results agree with published observations involving B16 melanoma cells genetically modified to produce IL-12, another Th1-biasing immunostimulatory cytokine that is normally secreted by activated DCs. B16 melanoma cells that expressed IL-12 were rejected by a significant number of mice only when $CD4^+$ T cells were simultaneously eliminated *in vivo* with a depleting anti-CD4 mAb (17).

Elimination of Regulatory $CD25^+$ $CD4^+$ T Cells Strongly Enhances the IFNα-induced Immune Defense of B16 Melanoma Cells. It has recently been reported that depletion of the negative regulatory $CD25^+$ subset of $CD4^+$ T cells enhances tumor immunity and autoimmunity in mice (18). We speculated that these cells were involved in inhibiting the IFNα-induced, $CD8^+$ T-cell-dependent immunity to B16 melanoma cells. To test this hypothesis, a cytotoxic anti-CD25 mAb was injected prior to tumor challenge. After the elimination of $CD25^+$ $CD4^+$ T cells, 10 of 12 mice rejected B16-IFNα cells in three independent experiments (Fig. 3*A*). To investigate the induction of long-lasting tumor immunity, we rechallenged six tumor-free mice in two independent experiments on day 60 with parental B16 melanoma cells. All six of the mice rejected unmodified B16 melanoma cells, which progressively grew in naive mice (Fig. 3*B*). From these experiments, we concluded that the elimination of regulatory $CD25^+$ $CD4^+$ T cells enhanced the IFNα-induced immune defense of B16 melanoma cells and promoted long-lasting tumor immunity.

While this article was under review, it has been reported (19) that B16 melanoma can be effectively treated with a vaccine consisting of irradiated B16 melanoma cells genetically modified to produce GM-CSF in combination with injections of anti-CD25 and anti-CTLA4 mAb. Importantly, this vaccine strategy was also able to break tolerance, promote the induction of autoreactive T cells specific for TRP2, and induce vitiligo-like fur depigmentation (19). Previously, we found that the induction of TRP2-specific immune responses after genetic immunization with recombinant adenoviruses was impaired by mechanisms of peripheral tolerance against self antigens. Immunization of mice with human TRP2 cDNA circumvented tolerance and stimulated TRP2-specific cellular and humoral immunity associated with the protection against challenge with B16 melanoma cells and autoimmune destruction of melanocytes resulting in fur depigmentation (20). Interestingly, we have not observed coat-color changes in our experiments involving the injection of anti-CD25 mAb and B16-IFNα, despite the induction of long-lasting protective immunity to B16 melanoma. However, immunization with DCs genetically engineered to express the melanoma antigen TRP2 after transduction with a recombinant adenovirus (Ad-TRP2-transduced DC) are also able to induce strong protective immunity against a subsequent challenge with B16 melanoma cells without any evidence of vitiligo-like fur depigmentation (14).

Local Production of IFNα in the Tumor Microenvironment Promotes the Efficacy of DCs Genetically Engineered to Express TRP2 in a Therapeutic Setting. When Ad-TRP2-transduced DCs were applied therapeutically on a weekly basis starting on day 7 after inoculation with B16 melanoma cells, we did not observe a significant impact on their *in vivo* growth (Fig. 4*A*). Thus, once established in the

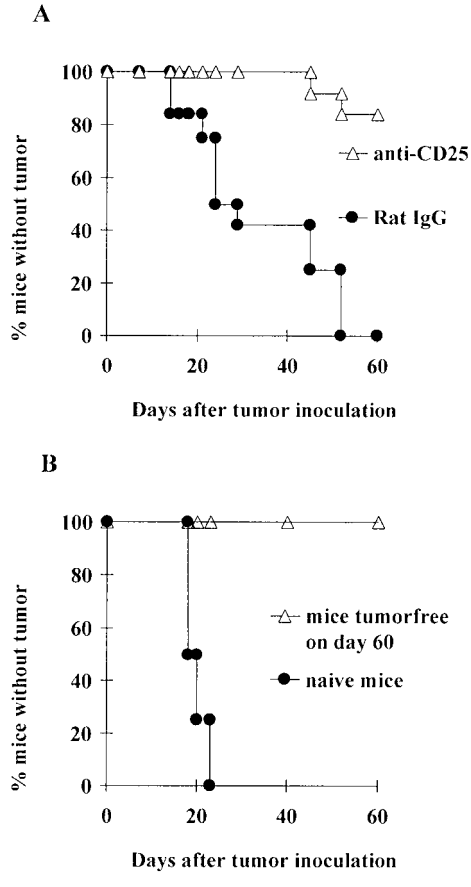

Fig. 3. The injection of anti-CD25 mAb prevents the growth of B16 melanoma cells expressing IFNα and promotes long-lasting tumor immunity. Mice were treated by injecting purified anti-CD25 mAb or normal rat IgG and challenged with 10^5 B16-IFNα melanoma cells s.c. in the flank as described in "Materials and Methods." Tumor growth was assessed by palpation every other day. Cumulative results of three separate experiments are expressed as the percentage of tumor-free mice ($n = 12$) at the indicated time points (*A*). Additionally, some tumor-free mice at day 60 ($n = 6$) and a control group of naive mice ($n = 10$) were rechallenged with 10^5 parental B16 melanoma cells in two independent experiments (*B*).

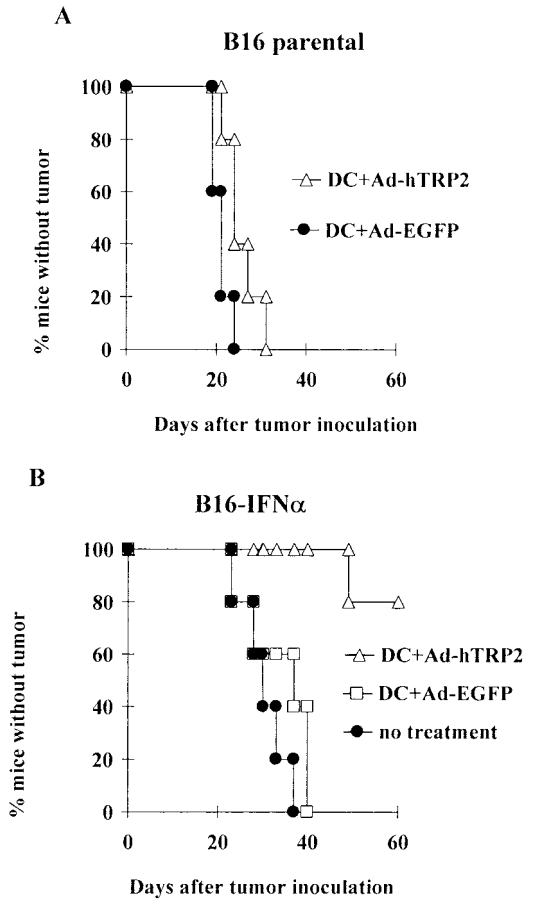

Fig. 4. Therapeutic immunization with Ad-hTRP2-transduced DCs prevents outgrowth of B16-IFNα but not parental B16 melanoma cells. B16 melanoma cells (10^5; *A*) or B16-IFNα melanoma cells (10^5; *B*) were inoculated s.c. on day 0. Starting on day 7, mice were therapeutically immunized on a weekly basis by i.v. injections with 2.5×10^5 DCs transduced with Ad-TRP2 or with the control adenovirus Ad-EGFP. Tumor growth was assessed by palpation every other day. Cumulative results of three separate experiments are expressed as percentage of tumor-free mice ($n = 15$) at the indicated time points.

skin, B16 melanoma cells grew progressively despite the induction of a tumor-protective antigen-specific immune response. Because IFNα is able to enhance $CD8^+$ T-cell-dependent tumor immune defense, we investigated in subsequent experiments whether local expression of IFNα would promote the therapeutic efficacy of Ad-TRP2-transduced DCs. Mice were challenged with B16-IFNα and again received injections weekly with Ad-TRP2-transduced DCs starting on day 7. Importantly, this treatment prevented outgrowth of B16-IFNα in 12 of 15 mice in three independent experiments (Fig. 4*B*). Enhancement of cancer vaccines with a variety of immunostimulatory cytokines has been demonstrated before. However, to our knowledge, this is the first report showing in an experimental murine model, that IFNα, which is in widespread clinical use for patients with melanoma, can augment the efficacy of melanoma antigen-specific immunization in a therapeutic setting. We hypothesize that IFNα supports effector functions of tumor-specific T lymphocytes in the tumor microenvironment by enhancing their recruitment, cytotoxicity, and survival. These issues will have to be directly addressed in future experiments.

In conclusion, our results suggest that local production of IFNα in the tumor microenvironment supports the induction of a $CD8^+$ T-cell-dependent tumor immune defense that is down-regulated by $CD25^+$ $CD4^+$ T cells. We present evidence that the elimination of these regulatory $CD25^+$ $CD4^+$ T cells enhances the efficacy of IFNα immunotherapy. Furthermore, we show that local expression of IFNα supports the therapeutic efficacy of melanoma antigen-transduced DCs. We believe that these important results, which reflect the biological effects of IFNα at the interface between innate and adaptive immunity, provide a scientific basis for the future clinical development of more effective strategies for the immunotherapy of melanoma, using IFNα in combination with the elimination of regulatory T cells or with active specific immunization against defined melanoma antigens.

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