Therapeutic Efficacy of Antigen-Specific Vaccination and Toll-Like Receptor Stimulation against Established Transplanted and Autochthonous Melanoma in Mice

Damia Tormo, Aleix Ferrer, Pilar Bosch, Evelyn Gaffal, Etiena Basner-Tschakarjan, Jörg Wenzel, and Thomas Tüting

Laboratory of Experimental Dermatology, Department of Dermatology, University of Bonn, Bonn, Germany

Abstract

Malignant melanoma is an attractive model disease for the development of antigen-specific immunotherapy because many antigens recognized by tumor-specific T cells have been identified. In C57BL/6 mice, genetic immunization with recombinant adenovirus encoding xenogeneic human tyrosinase-related protein 2 (Ad-hTRP2) induces protective but not therapeutic cellular immunity against growth of transplanted B16 melanoma cells. Here, we additionally applied CpG DNA and synthetic double-stranded RNA, which activate the innate immune system via Toll-like receptors (TLR). Both adenoviral vaccination and peritumoral injections of TLR ligands were required for rejection of established B16 melanoma in the skin. To more closely mimic the clinical situation in patients with melanoma, we evaluated this combined immunotherapeutic strategy in genetically modified mice, which overexpress hepatocyte growth factor (HGF) and carry an oncogenic mutation in the cyclin-dependent kinase 4 (CDK4)^{R24C}. HGF $\times$ CDK4^{R24C} mice rapidly develop multiple invasive melanomas in the skin following neonatal carcinogen treatment, which spontaneously metastasize to lymph nodes and lungs. Vaccination with Ad-hTRP2 followed by injections of TLR ligands resulted in delayed growth of autochthonous primary melanomas in the skin and reduction in the number of spontaneous lung metastases but did not induce tumor regression. Carcinogen-treated HGF $\times$ CDK4^{R24C} mice bearing multiple autochthonous melanomas did not reject transplanted B16 melanoma despite treatment with Ad-hTRP2 and TLR ligands, suggesting the development of tumor immunotolerance. Further investigations in our novel genetic melanoma model may help to better understand the role of the immune system in the pathogenesis and treatment of this life-threatening disease. (Cancer Res 2006; 66(10): 5427-35)

Introduction

Antigen-specific immunotherapy for melanoma has been primarily investigated for many years using the transplantable B16 melanoma cell line in C57BL/6 mice (1). Vaccination strategies targeting melanocyte-specific proteins, such as the tyrosinase enzyme family or the gp100 protein, which are naturally expressed by melanoma cells, have shown the existence of peripheral immunotolerance against these weakly immunogenic self-antigens. Use of dendritic cells, immunogenic viruses, or linked helper determinants as vaccine adjuvants are required for the induction of protective cellular immunity against this clinically relevant category of antigens (2–5). However, immunomediated destruction of established B16 melanoma has thus far been difficult to achieve with vaccine approaches. IFN-α secreted locally in the tumor microenvironment by genetically engineered transplanted tumor cells supports the induction as well as the therapeutic efficacy of cellular tumor immunity (6–9). Synthetic immunostimulatory oligonucleotides, such as CpG-containing DNA and double-stranded RNA (dsRNA), are able to strongly activate innate immunity via Toll-like receptors (TLR) and induce the production of type I IFNs as well as other proinflammatory cytokines and chemokines. Peritumoral injections of CpG DNA and polyinosinic acid:poly-CMP (polyI:C), which engage TLR9 and TLR3, respectively, have been shown to promote tumor immune responses in several tumor models (10–13). These TLR agonists therefore seem promising candidates to enhance the efficacy of antigen-specific immunotherapy.

Genetically modified mice are currently being generated, which recapitulate the molecular pathogenesis of melanoma and develop autochthonous primary tumors in the skin. These experimental models may be more suitable for the evaluation of novel therapeutic strategies. Previously, we evaluated a candidate vaccine approach against autochthonous primary melanoma in mice, which carry an oncogenic mutation in the cyclin-dependent kinase 4 (CDK4)^{R24C} mice, a protein critically involved in cell cycle regulation (14, 15). This CDK4^{R24C} mutation is of particular immunologic interest because it was originally identified with melanoma-specific cytolytic T cells and could subsequently also be detected in the germ line of some families with hereditary melanoma (16, 17). To further improve this experimental model, we crossed mice overexpressing the hepatocyte growth factor (HGF) with CDK4^{R24C} mice. Deregulated receptor tyrosine kinase signaling is frequently observed in human melanoma and supports the development of spontaneous and UV-inducible cutaneous melanoma in HGF mice (18–20). The CDK4^{R24C} mutation strongly promotes growth of melanoma in HGF mice. Neonatal carcinogen treatment of HGF $\times$ CDK4^{R24C} mice results in a dramatic tumor phenotype characterized by many primary melanomas in the skin, which grow rapidly within the first 3 months of life and spontaneously metastasize to the draining lymph nodes and lungs.[1] This new experimental mouse model is ideally suited to evaluate new treatment modalities. Here, we show that both

Requests for reprints: Thomas Tüting, Laboratory of Experimental Dermatology, Department of Dermatology, University of Bonn, Sigmund Freud Strasse 25, 53105 Bonn, Germany. Phone: 49-228-287-9257; Fax: 49-228-287-9393; E-mail: thomas.tueting@ukb.uni-bonn.de.

doi:10.1158/0008-5472.CAN-06-0399

[1] D. Tormo et al., submitted for publication.

adenoviral vaccination against the model melanocytic self-antigen tyrosinase-related protein 2 (TRP2) and peritumoral injections of synthetic CpG DNA and dsRNA are required for rejection of established B16 melanoma in the skin. Application of this immunotherapeutic regimen in carcinogen-treated HGF × $CDK4^{R24C}$ mice bearing multiple autochthonous primary melanomas resulted in delayed tumor growth and reduction in the number of spontaneous lung metastases but did not induce tumor regression. Importantly, engrafted B16 melanoma cells grew progressively in carcinogen-treated HGF × $CDK4^{R24C}$ mice with primary cutaneous melanomas despite vaccination with recombinant adenovirus encoding human TRP2 (Ad-hTRP2) and application of TLR ligands, suggesting the induction of tumor immunotolerance.

Materials and Methods

Mice. C57BL/6 mice (H-2^b) were purchased from Charles River Laboratories (Sulzfeld, Germany). $CDK4^{R24C}$ mice were originally generated on a mixed 129SV × CD1 background using a knock-in strategy ensuring physiologic regulation of the mutant $CDK4^{R24C}$ protein during the cell cycle (21). $CDK4^{R24C}$ mice used in the experiments reported here were crossed back with C57BL/6 mice for more than eight generations. HGF mice were originally generated on the FVB background using a transgenic strategy by placing HGF under the control of the metallothionein promoter (22). HGF mice used in the experiments here were crossed back with C57BL/6 mice for at least five generations and mated with $CDK4^{R24C}$ C57BL/6 mice.[1] Overexpression of HGF on the C57BL/6 background leads to a "chocolate point phenotype" due to melanocytes in the epidermis. HGF × $CDK4^{R24C}$ mice can easily be distinguished from wild-type (WT) littermates by their dark noses, ears, and paws. All animal experiments were done at the Central Animal Facility of the University of Bonn (Bonn, Germany) in adherence to the standards of the German law for the care and use of laboratory animals.

Engraftment of B16 melanoma in the skin or the lungs. B16 (H-2^b) is a spontaneous murine melanoma and was maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 IU/mL penicillin, and 100 μg/mL streptomycin (all reagents were from Life Technologies GmbH, Eggenstein, Germany). Engraftment of melanoma in the skin was done by intracutaneous injection of 10^5 B16 melanoma cells. Tumor growth was assessed twice weekly by palpation. Tumor size was measured using a caliper and calculated as the maximal product of two bisecting diameters in mm^2. Mice with tumors >100 mm^2 were sacrificed. Engraftment of melanoma in the lungs was done by i.v. injection of 4×10^5 B16 melanoma cells. The number of macroscopically visible melanoma metastases on the surface of the lungs was counted with the help of a dissecting microscope 14 days after challenge. Experiments were done in groups of five mice and repeated two to four times.

Induction of primary autochthonous melanoma in the skin. Litters of newborn mice were painted once at day 4 of life with either 20 or 200 μg 7,12-dimethylbenz(*a*)anthracene (DMBA). Two weeks later, tumor growth was promoted by treatment with 5 μg 12-*O*-tetradecanoylphorbol-13-acetate (TPA) twice weekly for a total of 5 weeks. Development of melanocytic neoplasms as well as other skin tumors was carefully recorded on a weekly basis after completion of TPA treatment. Nevi and melanomas were counted, and the size of the largest tumor was measured using a caliper. Tumor sizes were calculated as the maximal product of two bisecting diameters in mm^2. Additionally, mice were photographed with a digital camera. When progressively growing melanomas exceeded 100 mm^2, mice were sacrificed. Occasionally, mice also had to be sacrificed because of weight loss and apparent sickness. Autopsy was done in all mice. Lungs were retrieved, and the number of black nodules on their surface indicating metastatic spread of melanoma was counted.

Histopathologic and immunohistochemical analyses. Samples of skin, lymph nodes, and lungs were obtained when mice were sacrificed. Tissue specimens were in part fixed in 10% buffered formalin, embedded in paraffin, and routinely stained with H&E. For immunohistopathologic investigations, a zinc-based fixative was used as an alternative to formalin (DAKO, Hamburg, Germany). To confirm the melanocytic origin of tumor cells, sections were immunostained with the TRP2-specific polyclonal rabbit antibody Pep8 (a kind gift from Vincent Hearing, NIH, Bethesda, MD) followed by a biotin-conjugated anti-rabbit secondary antibody and the LSAB-2 color development system (both from DAKO). To determine the proliferative activity of tumor cells, a Ki67-specific polyclonal rabbit antibody was used.

Plasmids, recombinant adenoviruses, and genetic immunization. Plasmid DNA was grown in *Escherichia coli* strain DH5α and purified using Qiagen Plasmid Maxi kits (Qiagen, Hilden, Germany). E1- and E3-deleted adenoviral vectors were propagated on 293 cells, purified by cesium chloride density gradient centrifugation and subsequent dialysis according to standard protocols, and stored at −70°C. Genetic immunization with recombinant adenovirus was done by i.p. injection of 5×10^8 plaque-forming unit recombinant adenovirus resuspended in 100 μL PBS. Genetic immunizations with corresponding expression plasmids using the gene gun was done by transfecting the skin of the shaved abdomen *in vivo* by two shots with the Helios gene gun (Bio-Rad, Munich, Germany) resulting in the delivery of ~2 μg plasmid DNA as described previously (3).

Treatment with TLR ligands. The synthetic phosphothioate-stabilized CpG oligodeoxynucleotide 1826 with the sequence 5′-TCCATGACGTTCCTGACGTT-3′ was purchased from TIB Molbiol (Berlin, Germany). Synthetic polyI:C was purchased from Amersham Biosciences (Freiburg, Germany). Treatment of mice was done by injecting 50 μg CpG DNA and 50 μg polyI:C dissolved in 100 μL PBS in and around cutaneous melanomas. Alternatively, CpG DNA and polyI:C were injected intracutaneously in the flank for the treatment of lung metastases.

Detection of antigen-specific T cells and antibodies. The induction of peptide-specific $CD8^+$ T cells was measured using the IFN-γ enzyme-linked immunospot (ELISPOT) technique with commercially available antibodies (PharMingen, Heidelberg, Germany) as described previously (3). The H-$2K^b$-binding peptide SVYDFFVWL ($TRP2_{aa180-188}$) derived from TRP2 and the H-$2K^b$-binding peptide ICPMYARV ($\beta\text{-gal}_{aa497-504}$) derived from β-galactosidase were purchased from Genosphere (Paris, France). The induction of humoral immunity to β-galactosidase was determined using the ELISA technique with recombinant *E. coli*–derived protein as a solid-phase antigen. The induction of humoral immunity to TRP2 was assessed using the Western blot technique with lysates of B16 melanoma cells or 293 cells infected with Ad-TRP2 as described previously (3).

Statistical analyses. The significance of differences in the number of lung metastases was assessed with the nonparametric Mann-Whitney *U* test using the SPSS 11 computer program.

Results

Both antigen-specific vaccination and peritumoral injection of synthetic CpG DNA and polyI:C are required for rejection of established B16 melanoma in the skin. In our previous studies, we observed that genetic immunization with Ad-hTRP2 was able to provide protective immunity against subsequent transplantation of B16 melanoma cells. The xenogeneic human TRP2 protein is 80% homologous to the murine sequence. It contains the H-$2K^b$-binding peptide SVYDFFVWL ($TRP2_{aa180-188}$) recognized by melanoma-reactive CTL and provides linked immunogenic CD4 helper determinants in the divergent amino acid sequences. However, genetic immunization with Ad-hTRP2 is not effective against established B16 melanoma in the skin or the lungs. To promote the therapeutic efficacy of vaccine-induced antigen-specific cellular immunity, we additionally did peritumoral injections of immunostimulatory CpG DNA and polyI:C, which mimic a viral infection and strongly activate innate immunity via TLRs. Groups of five mice were first challenged intracutaneously with B16 melanoma

cells and subsequently vaccinated with Ad-hTRP2 or Ad-β-gal either alone or in combination with local injections of TLR ligands according to the schedule depicted in Fig. 1*A*. Mice with either Ad-hTRP2 alone or Ad-β-gal and TLR ligands showed a slight delay in tumor growth compared with the control group of mice receiving Ad-β-gal alone (Fig. 1*A* and *B*). Growth of melanoma in the skin was not affected by injections of Ad-β-gal as evidenced in experiments with mice, which were either left untreated or treated with CpG and polyI:C only (data not shown). Importantly, vaccination with Ad-hTRP2 followed by treatment with TLR ligands induced tumor rejection in a significant percentage of mice with a total of 18 of 30 animals being tumor-free after 50 days. Induction of TRP2-specific cellular and humoral immunity could be verified in this experimental group (data not shown). In selected experiments, mice who rejected B16 melanoma were followed for up to 120 days. Some of these mice developed vitiligo-like fur depigmentation. In alternative experiments, we assessed the efficacy of our combined immunotherapeutic strategy against B16 melanoma lung metastases. Groups of five mice were challenged i.v. with B16 melanoma cells, vaccinated with recombinant adenovirus, and treated with TLR ligands according to the schedule depicted in Fig. 1*C*. Metastatic growth of B16 melanoma cells was prevented in the lungs of all mice receiving TLR ligands (Fig. 1*C*).

Genetic vaccination of HGF × CDK4^{R24C} C57BL/6 mice induces effective cellular antimelanoma immunity. Before investigating the efficacy of our combined immunotherapeutic strategy against carcinogen-induced autochthonous primary melanoma in the skin, we wanted to confirm the ability to induce antigen-specific immunity against the melanocytic self-antigen TRP2 in HGF × CDK4^{R24C} C57BL/6 mice. Groups of HGF × CDK4^{R24C} mice were injected with Ad-hTRP2 or Ad-β-gal and subsequently shot five times at weekly intervals with the corresponding plasmid DNA pCI-hTRP2 or pCI-β-gal using the gene gun. Similar to WT mice, this strategy led to *in vivo* induction of TRP2$_{aa180-188}$ peptide–specific T cells and TRP2-reactive antibodies associated with vitiligo-like fur depigmentation in HGF × CDK4^{R24C} mice immunized against TRP2 (Fig. 2*A*). Subsequently, groups of five HGF × CDK4^{R24C} mice were challenged intracutaneously with B16 melanoma cells, vaccinated with Ad-hTRP2 or Ad-β-gal, and injected with TLR ligands according to the schedule depicted in Fig. 1*A*. As shown in Fig. 2*B* and *C*, treatment with

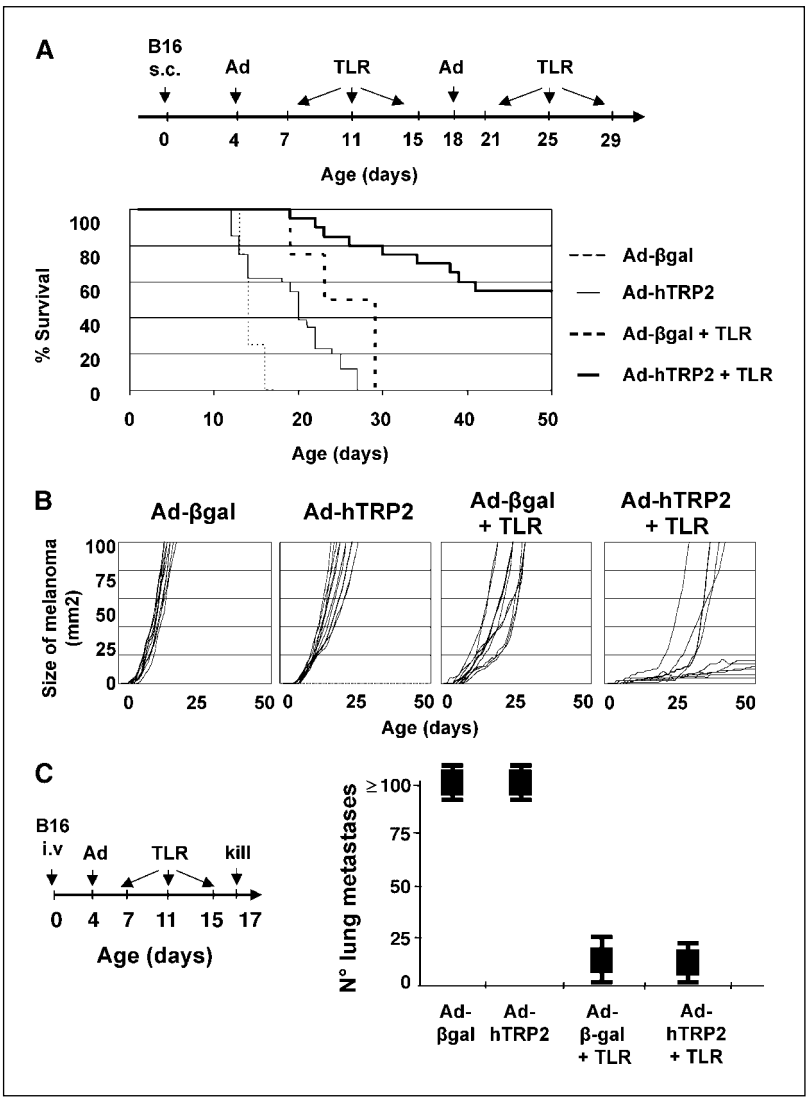

Figure 1. Peritumoral injections of CpG and polyI:C enhance the therapeutic efficacy of a genetic melanoma vaccination with recombinant adenovirus. *A,* groups of five C57BL/6 mice were engrafted intracutaneously with 10^5 B16 melanoma cells. Four days later, mice were therapeutically vaccinated with Ad-hTRP2 or Ad-β-gal. Synthetic polyI:C and CpG were injected peritumorally on days 7, 11, and 15 to enhance the vaccine efficacy. Vaccination was repeated on day 18 followed by injections with TLR ligands on days 21, 25, and 29. Tumor growth was assessed twice weekly by multiplying the largest perpendicular tumor diameters, indicating the tumor area in mm^2. Routinely, mice were sacrificed when the tumor area exceeded 100 mm^2. Kaplan-Meier graph representing cumulative survival of mice in the indicated treatment groups from four independent experiments with five mice per group. *B,* size of cutaneous B16 melanoma in individual mice of the indicated treatment groups over time expressed as tumor area in mm^2. Results of two representative experiments with five mice per group. The experiment was repeated several times with similar results. *C,* groups of five C57BL/6 mice were engrafted i.v. with 4×10^5 B16 melanoma cells. Four days later, mice were therapeutically vaccinated with Ad-hTRP2 or Ad-β-gal. Synthetic polyI:C and CpG were injected intracutaneously on days 7, 11, and 15 to enhance the vaccine efficacy. Mice were sacrificed on day 17, and the number of B16 metastases on the lung surfaces were counted. *Points,* average number of lung metastases in the different treatment groups in three independent experiments with five mice per group; *bars,* SE.

Ad-hTRP2 and TLR ligands was able to promote rejection of established B16 melanoma in the skin of HGF × CDK4^{R24C} mice. However, the percentage of tumor-free mice on day 50 was lower in HGF × CDK4^{R24C} mice compared with WT mice. Alternatively, groups of HGF × CDK4^{R24C} mice were challenged i.v. with B16 melanoma cells, vaccinated with Ad-hTRP2 or Ad-β-gal, and treated with TLR ligands according to the schedule depicted in Fig. 1*C*. Growth of B16 melanoma metastases in the lungs was significantly reduced in all mice receiving TLR ligands. Again, therapy was less effective in HGF × CDK4^{R24C} mice compared with WT mice (Fig. 2*D*).

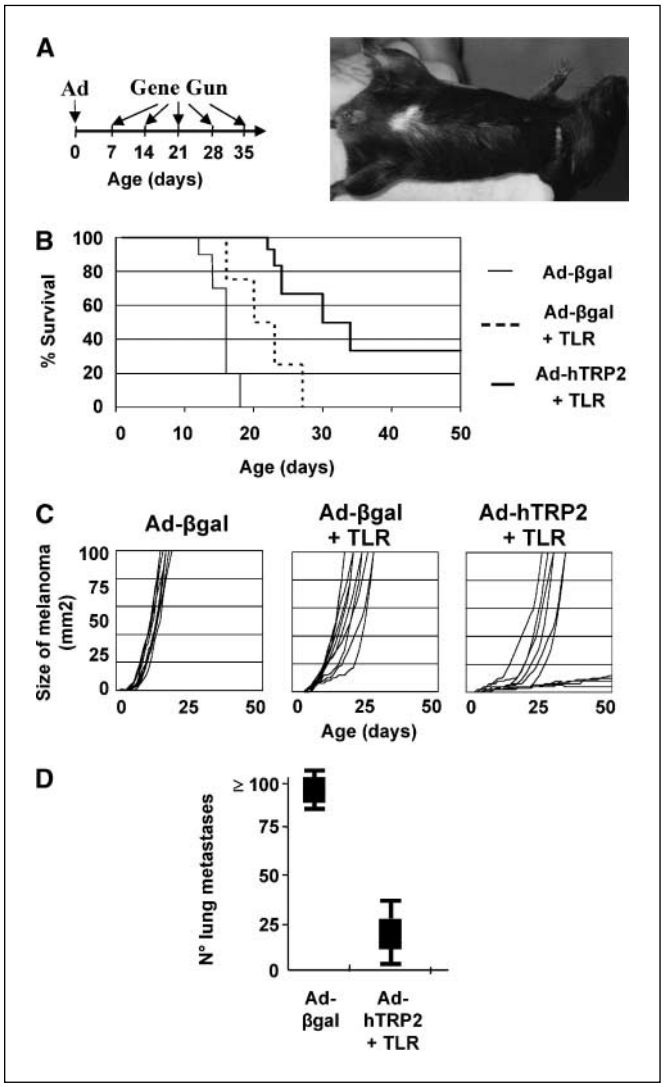

Figure 2. Combined vaccination and TLR stimulation can treat engrafted B16 melanoma in the skin of HGF × CDK4^{R24C} mice. *A,* HGF × CDK4^{R24C} mice were genetically immunized by priming with Ad-hTRP2 or Ad-β-gal and boosting with the corresponding plasmid DNA using the gene gun. After 8 weeks, vitiligo-like fur depigmentation was observed in all mice receiving Ad-hTRP2 and pCMV-hTRP2. Representative mouse of cohort. *B,* groups of five HGF × CDK4^{R24C} C57BL/6 mice were engrafted intracutaneously with 10^5 B16 melanoma cells and treated with Ad-hTRP2 or Ad-β-gal in combination with synthetic TLR ligands as described in Fig. 1*A*. Kaplan-Meier graph representing survival of mice in the indicated treatment groups. *C,* size of cutaneous B16 melanoma in individual mice of the indicated treatment groups over time expressed as tumor area in mm^2. Results of two representative experiments with five mice per group. The experiment was repeated several times with similar results. *D,* groups of HGF × CDK4^{R24C} C57BL/6 mice were engrafted i.v. with 4 × 10^5 B16 melanoma cells and treated with Ad-hTRP2 or Ad-β-gal in combination with synthetic polyI:C and CpG as described in Fig. 1*C*. *Points,* average number of lung metastases in the different treatment groups in two independent experiments with five mice per group; *bars,* SE.

Carcinogen treatment induces autochthonous primary melanomas in the skin of HGF × CDK4^{R24C} mice, which spontaneously metastasize in the lymph nodes and lungs. Previously, we observed that neonatal application of 200 μg DMBA on the skin of HGF × CDK4^{R24C} mice followed by TPA resulted in rapid growth of ~50 autochthonous cutaneous melanomas in every single mouse within the first 3 months of life.[1] To reduce the number of tumors, we compared carcinogen treatment with 200 and 20 μg DMBA followed by TPA. Cohorts of HGF × CDK4^{R24C} mice were treated according to the schedule depicted in Fig. 3*A*. All mice in both treatment groups developed melanocytic tumors in the skin within the first weeks of life while still under TPA treatment. Primary cutaneous melanomas grew progressively, and all mice had to be sacrificed when the size of the largest tumor exceeded 100 mm^2 in tumor area. As shown in Fig. 3*A*, mice treated with 200 μg DMBA survived up to >100 days, whereas mice treated with only 20 μg DMBA survived up to ≥50 days. Representative pictures of mice are shown in Fig. 3*B*. On average, melanomas grew slightly slower in the 20 μg DMBA cohort (Fig. 3*B*). Treatment with 200 μg DMBA induced between 40 and 50 melanomas at the time of sacrifice, whereas mice treated with only 20 μg DMBA developed between 10 and 20 melanomas (Fig. 3*C*). Skin papillomas were not observed in HGF × CDK4^{R24C} mice treated with 20 μg DMBA and only very rarely in mice treated with 200 μg DMBA. All mice developed spontaneous metastases in the draining lymph nodes and lungs. The number of lung metastases was similar in both groups (Fig. 3*D*). The homogenous development of rapidly growing primary melanomas in all HGF × CDK4^{R24C} mice and the spontaneous metastatic spread to lymph nodes and lungs provide an ideal experimental system for the evaluation of novel therapeutic strategies.

Treatment of carcinogen-induced autochthonous primary melanoma in HGF × CDK4^{R24C} mice with genetic vaccination and synthetic TLR ligands delays tumor growth but does not lead to tumor regression. One of the most important aims of our study was to evaluate the therapeutic efficacy of adenoviral vaccination in combination with TLR ligands against autochthonous primary melanomas in the skin. Litters of newborn HGF × CDK4^{R24C} mice were treated with 20 μL DMBA followed by TPA. Melanocytic lesions appeared under TPA treatment during the first 5 weeks of life. Their number and sizes were recorded from day 56 on. Cohorts of 15 melanoma-bearing mice were randomly assigned to two treatment groups at ages 4 to 6 weeks when they were separated to avoid any bias by the selection of mice. One group was therapeutically vaccinated starting at around day 74 of life with Ad-hTRP2 followed by peritumoral injections with TLR ligands according to the schedule shown in Fig. 4*A*. The second group received two injections of Ad-β-gal only. Treatment with Ad-hTRP2 and TLR ligands delayed growth of autochthonous melanomas in the skin and prolonged survival up to 50 days compared with the control group (Fig. 4*A* and *B*). We did not observe tumor regression or vitiligo-like fur depigmentation in any of the mice. At the time of sacrifice, the total number of primary cutaneous melanomas did not differ between the two treatment groups (Fig. 4*C*). However, 'the number of lung metastases in mice receiving Ad-hTRP2 and TLR ligands was significantly lower (Fig. 4*D*). ELISPOT assays done at the time of sacrifice confirmed the induction of TRP2$_{180\text{-}188}$ peptide–reactive memory T cells *in vivo* (data not shown).

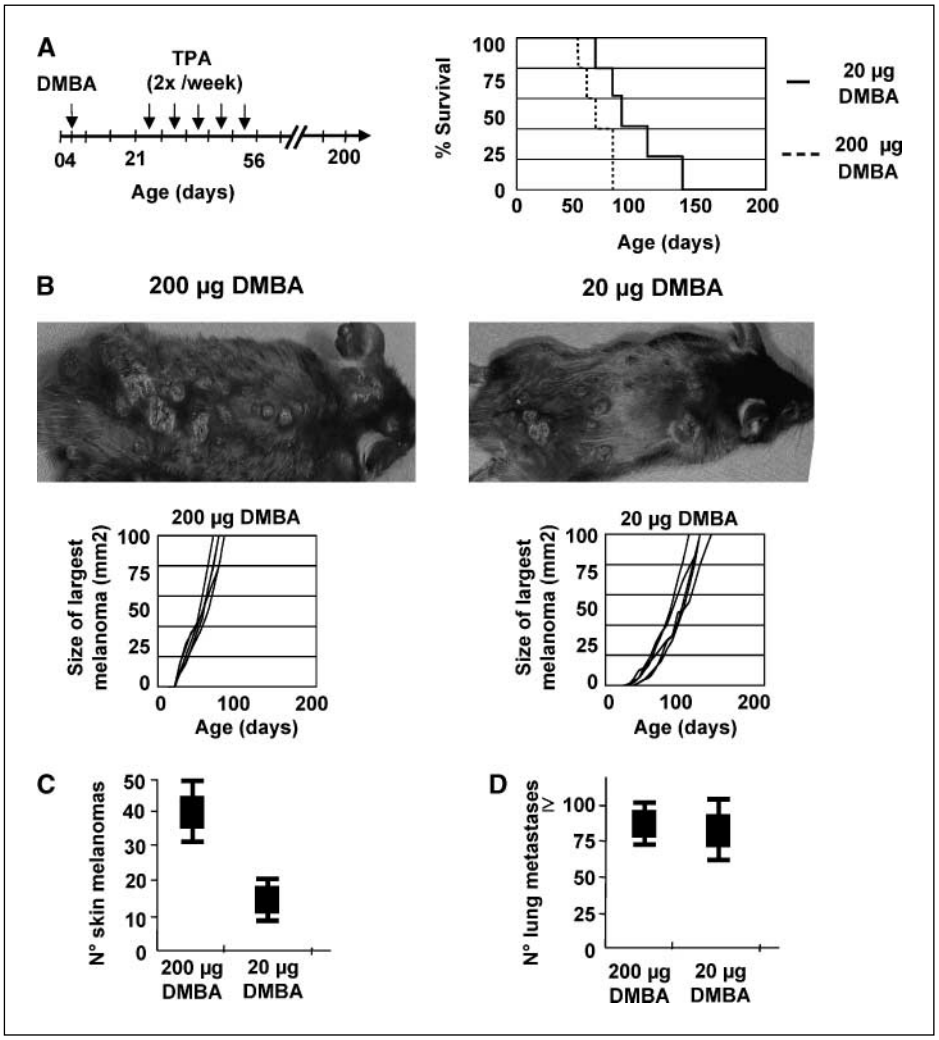

Figure 3. Carcinogenic treatment of HGF × CDK4^{R24C} mice leads to rapid development of multiple invasive melanomas in the skin and spontaneous metastases in the lungs. *A,* cohorts of HGF × CDK4^{R24C} mice were treated with either 200 or 20 μg DMBA at day 4 after birth followed by twice weekly application of 5 μg TPA from week 3 to 8. Kaplan-Meier graph representing survival of five carcinogen-treated mice in each of the two cohorts. Routinely, mice were sacrificed when melanomas grew progressively and tumor area exceeded 100 mm^2. *B,* representative pictures of carcinogen-treated HGF × CDK4^{R24C} mice with autochthonous primary melanoma at 12 weeks of age and sizes of the largest primary cutaneous melanoma in representative groups of five individual mice over time expressed as tumor area in mm^2. Mice had to be sacrificed because of progressive and disseminated growth of melanoma in the skin and systemic signs of illness. *C, points,* number of primary cutaneous melanomas at the time of sacrifice; *bars,* SE. *D, points,* number of spontaneous lung metastases at the time of sacrifice; *bars,* SE.

Histopathologic investigations revealed areas of lymphocytic infiltrations in and around heavily pigmented primary melanomas only in mice injected with TLR ligands. Tumors from both treatment groups consistently expressed the melanocytic antigen TRP2, excluding antigen loss as a potential tumor escape mechanism (data not shown). Taken together, these experimental results show that our candidate immunotherapeutic strategy shows therapeutic efficacy against autochthonous primary melanoma and spontaneous lung metastases but does not induce tumor regression.

Comparison of growth kinetics and histopathologic appearance between engrafted B16 melanoma and carcinogen-induced autochthonous primary melanoma in the skin. In direct comparison, engrafted B16 melanoma in the skin grew much more rapidly than carcinogen-induced autochthonous primary melanomas in HGF × CDK4^{R24C} mice. Specifically, B16 melanomas reached a size of 100 mm^2 within a little more than 2 weeks after injection, whereas autochthonous melanomas took >2 months to develop the same size (Fig. 5*A*). B16 melanoma cells predominantly established in the deep dermis and subcutis with little or no contact to the upper dermis or epidermis, whereas carcinogen-induced primary melanomas in HGF × CDK4^{R24C} mice involved the dermo-epidermal junction and grew horizontally in the epidermis and vertically into the dermis and subcutis. The architecture of primary melanoma in HGF × CDK4^{R24C} mice was reminiscent of the histomorphology of human melanoma (Fig. 5*B, 1*). B16 melanoma showed a rather uniform cellular phenotype with prominent cellular atypia as well as many mitoses, whereas primary melanomas in HGF × CDK4^{R24C} mice were composed of a mixture of epitheloid and spindle-shaped cells arranged in strands and nests with only occasional cellular atypia (Fig. 5*B, 2*). B16 melanoma was always surrounded by a fibrotic capsule and an inflammatory infiltrate similar to a foreign body reaction, whereas primary melanomas in HGF × CDK4^{R24C} mice did not show an inflammatory infiltration or a mesenchymal tissue reaction (Fig. 5*B, 3*). Both transplanted as well as autochthonous melanoma grew invasively in underlying s.c. fat and muscle and induced neoangiogenesis (Fig. 5*B, 4*). As expected, B16 melanoma and primary melanomas in HGF × CDK4^{R24C} mice both expressed the melanocytic antigen TRP2, which represents our candidate vaccine target (Fig. 5*B, 5*). In agreement with the macroscopically visible growth kinetic and mitotic index, B16 melanoma cells were highly positive for the proliferation marker Ki67. In contrast, primary melanomas in HGF × CDK4^{R24C} mice only showed occasional spindle-shaped cell staining positive for Ki67 (Fig. 5*B, 6*). These observations documented the profound differences of the two experimental models.

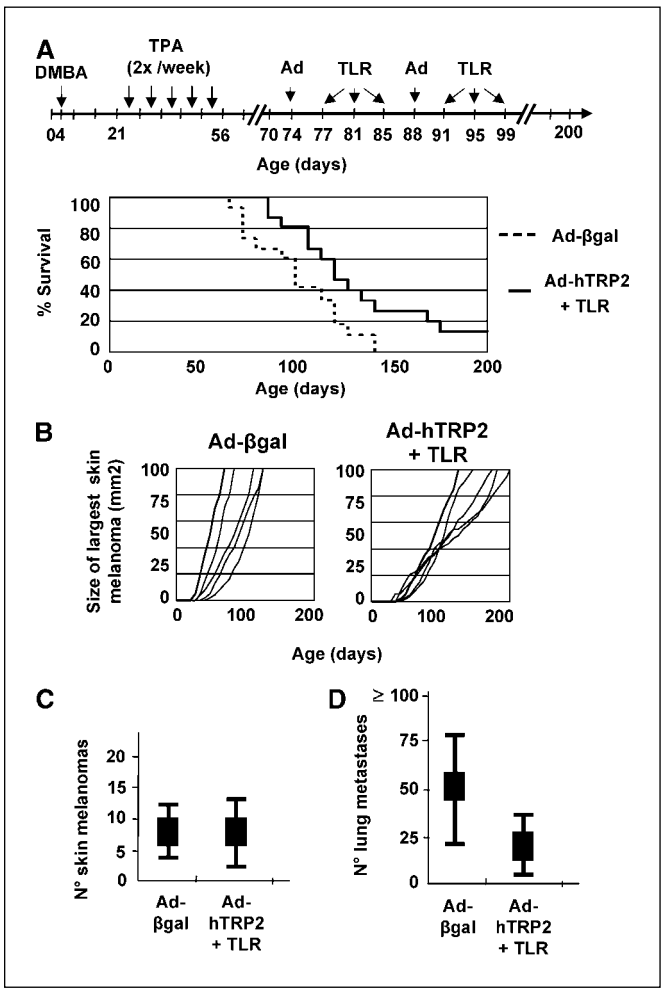

Figure 4. Treatment of carcinogen-induced HGF × CDK4^{R24C} mice with recombinant adenovirus and TLR ligands delays growth of autochthonous primary melanomas in the skin and spontaneous metastases in the lungs. *A,* cohorts of HGF × CDK4^{R24C} mice were treated with 20 μg DMBA at day 4 after birth followed by twice weekly application of 5 μg TPA from week 3 to 8. Starting at around day 74, mice bearing autochthonous primary melanoma were therapeutically vaccinated with Ad-hTRP2 in combination with synthetic TLR ligands as described in Fig. 1*A*. A control cohort of carcinogen-treated HGF × CDK4^{R24C} mice received Ad-β-gal only. Kaplan-Meier graph representing survival of carcinogen-treated HGF × CDK4^{R24C} mice in the two treatment cohorts. Routinely, mice were sacrificed when melanomas grew progressively and tumor area exceeded 100 mm^2. *B,* size of the largest primary cutaneous melanoma in representative groups of five individual mice over time expressed as tumor area in mm^2. *C, points,* number of primary cutaneous melanomas at the time of sacrifice; *bars,* SE. *D, points,* number of spontaneous lung metastases at the time of sacrifice; *bars,* SE.

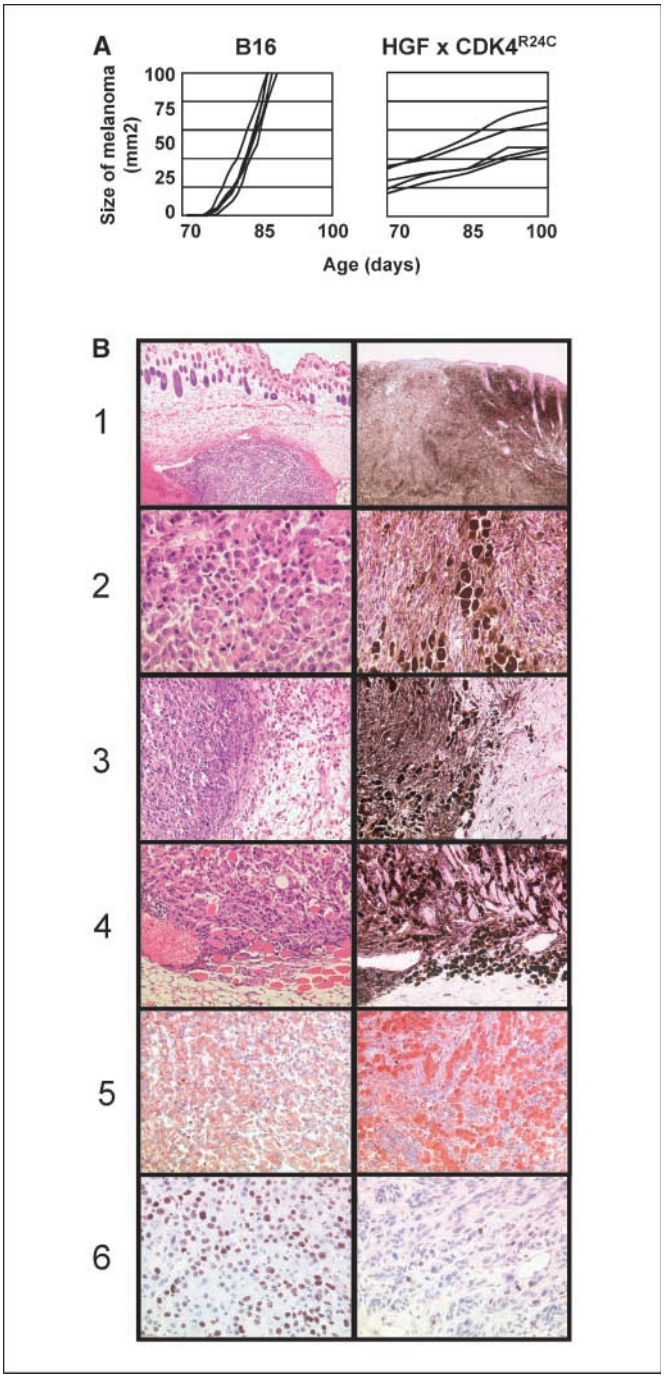

Figure 5. Growth kinetics and histopathologic appearance of engrafted B16 melanoma and carcinogen-induced autochthonous primary melanoma in the skin differ considerably. *A,* growth kinetics of 10^5 B16 melanoma cells engrafted in the skin of WT mice versus autochthonous primary melanoma in the skin of HGF × CDK4^{R24C} mice treated neonatally with 20 μg DMBA and TPA as described above. Sizes of B16 melanomas or the largest primary melanoma in a representative group of individual mice over time expressed in mm^2. *B,* histologic and immunohistologic comparison of engrafted cutaneous B16 melanoma and primary autochthonous melanoma in the skin of carcinogen-treated HGF × CDK4^{R24C} mice. *1,* representative H&E-stained skin sections at low-power magnification (×25). *2,* cellular morphology at high-power magnification (×200). *3,* inflammatory infiltrate and mesenchymal reaction at the tumor border at high-power magnification (×200). *4,* angiogenesis and invasion at the deep tumor front at high-power magnification (×200). *5,* immunohistochemical staining for TRP2 confirming the melanocytic origin of the tumor cells and expression of the antigenic target at high-power magnification (×200). *6,* immunohistochemical staining for the proliferation marker Ki67 at high-power magnification (×200).

Evidence for tumor immunotolerance in carcinogen-treated HGF × CDK4^{R24C} mice bearing autochthonous melanomas in the skin. Finally, we investigated the ability of carcinogen-treated HGF × CDK4^{R24C} mice bearing autochthonous primary melanomas to reject transplanted B16 melanoma. Cohorts of HGF × CDK4^{R24C} mice were treated neonatally with DMBA followed by application of TPA as described in Fig. 3*A*. At an age of ~70 days, groups of five mice with an average of ~10 autochthonous primary melanomas in the skin were additionally engrafted either intracutaneously or i.v. with B16 melanoma cells, vaccinated with Ad-hTRP2, and injected with TLR ligands according to the schedule depicted in Fig. 6*A*. Control groups of mice received Ad-β-gal only. As shown in Fig. 6*B* to *D*, B16 melanoma cells grew

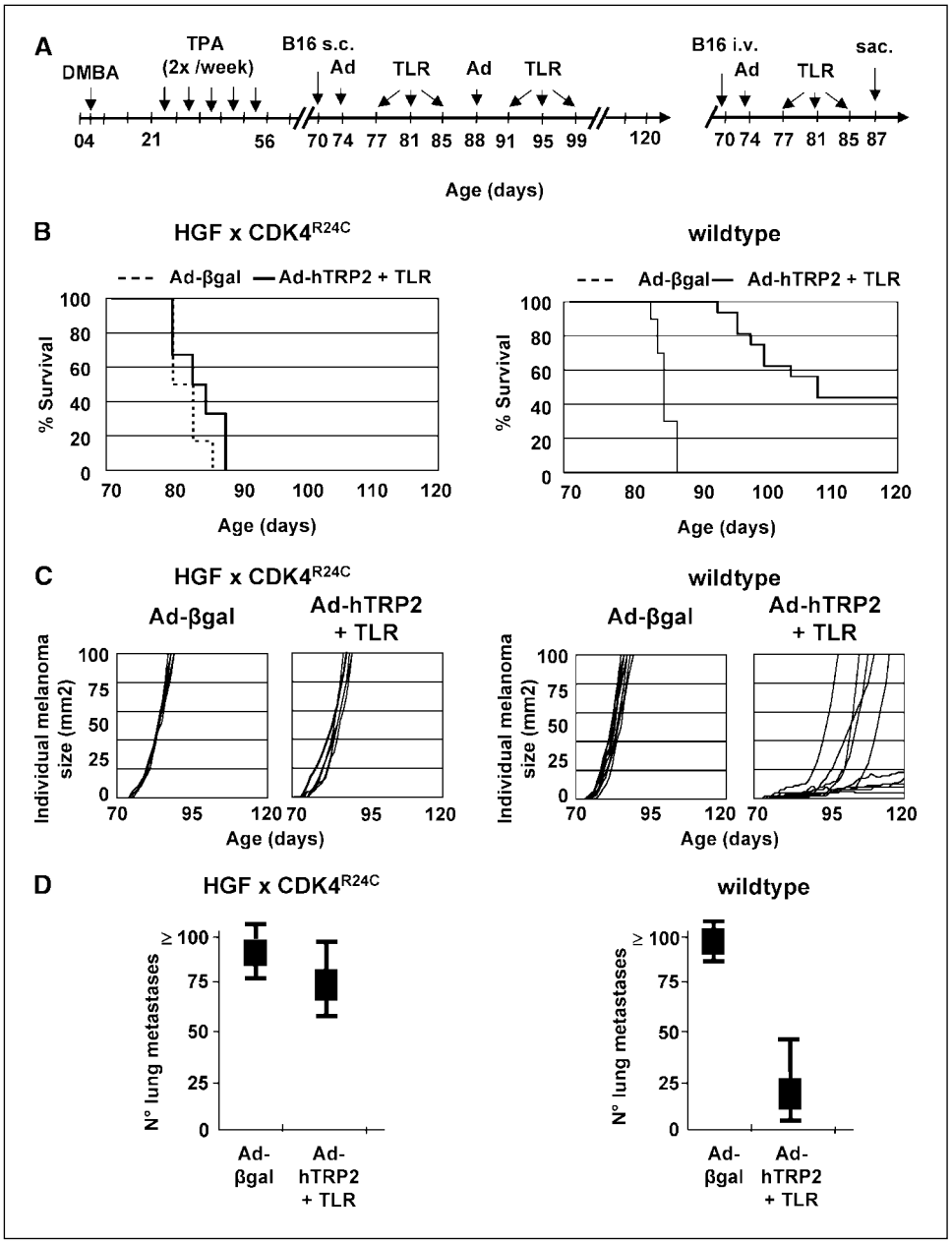

Figure 6. B16 melanoma engrafted in the skin grows progressively in carcinogen-treated HGF × CDK4^{R24C} mice bearing autochthonous cutaneous melanoma despite treatment with Ad-hTRP2 and TLR ligands. *A,* HGF × CDK4^{R24C} mice and, as a control, either WT or CDK4^{R24C} mice were treated with DMBA at day 4 after birth followed by twice weekly application of TPA from week 3 to 8. Around day 70, mice were engrafted intracutaneously or i.v. with B16 melanoma cells and therapeutically vaccinated with Ad-hTRP2 in combination with synthetic TLR ligands as described in Fig. 1*A*. Control cohorts of mice received Ad-β-gal only. *B,* Kaplan-Meier graphs representing survival of therapeutically vaccinated carcinogen-treated WT, CDK4^{R24C}, or HGF × CDK4^{R24C} mice. Routinely, mice were sacrificed when melanomas grew progressively and tumor area exceeded 100 mm^2. *C,* size of cutaneous B16 melanoma in representative groups of five individual mice over time expressed as tumor area in mm^2. *D, points,* average number of B16 melanoma lung metastases in the different treatment groups from two independent experiments with five mice per group; *bars,* SE.

rapidly in the skin and lungs of both groups. To rule out that carcinogen treatment could have caused general immunosuppression, we engrafted groups of five WT mice, which were also treated neonatally with DMBA and TPA either intracutaneously or i.v. with B16 melanoma cells at an age of 70 days and treated them with Ad-hTRP2 and TLR ligands as shown in Fig. 6*A*. Again, control groups received Ad-β-gal only. A significant percentage of mice treated with Ad-hTRP2 and TLR ligands were able to control growth of established B16 melanoma metastases in the skin or lungs (Fig. 6*B-D*). Similar results were obtained in carcinogen-treated CDK4^{R24C} mice (data not shown). Collectively, we concluded from the results of these experiments that the development of autochthonous primary melanomas in the skin of carcinogen-treated HGF × CDK4^{R24C} mice induces a state of immunotolerance at an age of ~10 to 12 weeks.

Discussion

In our investigations, we evaluated the efficacy of an immunotherapeutic strategy combining adenoviral vaccination with injections of CpG DNA and dsRNA against engrafted B16 melanoma and carcinogen-induced autochthonous primary melanoma. Our rationale was to induce tumor-reactive CD8$^+$ T cells and support their effector function in the tumor tissue. We chose TRP2 as a clinically relevant, CTL-defined model self-antigen because it is naturally expressed by melanoma cells as well as melanocytes both in mouse and man. By using CpG DNA and polyI:C as ligands for TLR9 and TLR3, respectively, we sought to activate both MyD88- and TRIF-dependent signal transduction cascade and optimally stimulate the production of type I IFNs and other proinflammatory cytokines, which are able to enhance tumor immunity. In agreement with published data, we observed that injections of

TLR ligands alone were able to significantly inhibit growth of established B16 melanoma lung metastases (13). In contrast, combined stimulation of the innate and adaptive arm of the immune system was required to control growth of established B16 melanoma in the skin. Injections of CpG DNA alone only slowed the growth of cutaneous B16 melanoma as reported previously (12). Interestingly, vitiligo-like fur depigmentation was observed in mice, which rejected B16 melanoma following treatment with Ad-hTRP2 and TLR ligands, supporting the notion that effective immunity against melanoma is frequently associated with the induction of melanocyte-specific autoimmunity (1, 23). Future studies will have to show how the combined stimulation of the innate and adaptive immune system synergistically enhances antimelanoma immunity. An attractive hypothesis is the potential reciprocal interaction of activated natural killer (NK) and dendritic cells, which support the stimulation and the effector functions of antigen-specific $CD8^+$ CTL.

The most important goal of our study was to evaluate the efficacy of our candidate immunotherapeutic strategy in a novel genetic mouse model against autochthonous primary melanoma, which more closely represents the expected clinical situation. We used HGF $\times$ $CDK4^{R24C}$ mice, which rapidly develop primary cutaneous melanoma following neonatal treatment with DMBA and TPA. This model is very attractive because primary melanoma spontaneously metastasizes to lymph nodes and lungs in the absence of other tumor types. Our experiments showed that melanoma vaccination in combination with TLR ligand injections is able to delay growth of autochthonous primary melanomas and reduce the number of spontaneous lung metastases of genetically melanoma-prone HGF $\times$ $CDK4^{R24C}$ mice. However, regression of primary melanoma and visible vitiligo-like fur depigmentation could not be achieved despite the induction of antigen-specific cellular immunity. This result reflects the experience in clinical trials for melanoma vaccines where the induction of tumor-specific T cells is occasionally associated with prolonged survival but only very rarely with complete tumor regression (24).

The development of autochthonous primary melanoma in carcinogen-treated HGF $\times$ $CDK4^{R24C}$ mice could, in principle, lead to spontaneous induction of melanocyte-specific immune responses associated with concomitant immunity to transplanted melanoma cells (25). This has been reported in the melanoma-prone MT/ret mice when crossed onto the C57BL/6 background (26). However, we found that carcinogen-treated HGF $\times$ $CDK4^{R24C}$ mice bearing autochthonous primary melanomas were unable to reject B16 melanoma engrafted at an age of $\sim$70 days even when treated with Ad-hTRP2 and TLR ligands. The apparent inefficacy of our immunotherapeutic strategy in melanoma-bearing HGF $\times$ $CDK4^{R24C}$ mice was not due to the carcinogenic treatment with DMBA plus TPA because established B16 melanomas were rejected by a significant percentage of WT and $CDK4^{R24C}$ mice under the same experimental conditions. Furthermore, untreated HGF $\times$ $CDK4^{R24C}$ mice were able to mount cellular immunity against TRP2 associated with therapeutic efficacy against established B16 melanoma metastases as well as vitiligo-like fur depigmentation. These results suggest that carcinogen-treated HGF $\times$ $CDK4^{R24C}$ mice bearing autochthonous primary melanoma develop tumor tolerance. This agrees with a similar observation recently reported in another spontaneous mouse model (27). We hypothesize that injections of TLR ligands are able to partially overcome the intrinsic barrier to immune cell infiltration and effector function of the tumor in melanoma-bearing HGF $\times$ $CDK4^{R24C}$ mice as has been shown in another autochthonous mouse tumor model (10). Eventually, however, tumor growth wins the battle against the immune system.

In our experiments, we show that a vaccine combination that is effective against tumors engrafted in the skin of normal mice may not be as effective against autochthonous primary tumors. In our opinion, this observation is of great importance because the majority of concepts in tumor immunology stem from experiments based on tumors engrafted in syngeneic recipients. We compared the growth kinetics and the histopathologic appearance of engrafted B16 melanoma and autochthonous melanoma in HGF $\times$ $CDK4^{R24C}$ mice in detail to show the fundamental difference. Autochthonous melanomas slowly develop for many weeks, which may allow for the establishment of a unique tumor microenvironment characterized by tolerogenic dendritic cells, high concentrations of immunosuppressive cytokines, such as interleukin-10 (IL-10) and tumor growth factor-β (TGF-β), ineffective T-cell cytotoxicity, and high frequency of regulatory T cells. In future experiments, the immunotolerance mechanisms preventing effective tumor immune defense in HGF $\times$ $CDK4^{R24C}$ mice will have to be characterized to optimize our immunotherapeutic strategy. It is conceivable that mice develop tumor antigen–specific tolerance as a result of T-cell deletion, anergy, or regulation. However, nonspecific mechanisms impairing immunologic effector mechanisms, such as the cytotoxicity of T cells and NK cells in the tumor tissue, will also have to be considered. In our experimental model, transgenic overexpression of HGF may itself be involved in suppressing tumor immunity because HGF has recently been shown to inhibit the function of antigen-presenting dendritic cells and induce the expression of IL-10 and TGF-β (28, 29). The potential immunosuppressive effect of HGF could also explain why treatment of established B16 melanoma with Ad-hTRP2 and TLR ligands was consistently less effective in HGF $\times$ $CDK4^{R24C}$ mice compared with WT mice. Thus, HGF may represent a link between oncogenic transformation and suppression of antitumor immune responses, which have recently achieved increasing attention and may support the development of melanoma in our model (30, 31). In addition to the stimulation of TLR, it might be helpful to attract more dendritic cells into tumor tissue and counteract immunoregulatory as well as immunosuppressive factors, such as IL-10 or TGF-β (32, 33). Alternatively, elimination of regulatory T cells and adoptive transfer of *ex vivo* activated effector T cells may be required to more effectively circumvent tumor-induced immunotolerance (34, 35).

In our opinion, investigations in genetic mouse models, which are based on molecular changes observed in the pathogenesis of hereditary and sporadic human melanoma, in principle, represent the expected clinical situation much more adequately than models involving transplantation of tumor cell lines. We firmly believe that further investigations in genetic mouse models will improve our understanding of the role of the immune system in the pathogenesis of melanoma and will facilitate the optimization of treatment regimens, which might be effective in the future treatment of melanoma patients. The HGF $\times$ $CDK4^{R24C}$ experimental mouse melanoma model provides unique possibilities for further studies because melanoma develops autochthonously in the skin and spontaneously metastasizes to lymph nodes and lungs in the absence of other tumor types.

Acknowledgments

Received 1/31/2006; revised 3/3/2006; accepted 3/10/2006.

Grant support: Deutsche Forschungsgemeinschaft grant Tu 90/3-2+3 (T. Tüting).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Petra Speuser and Stefanie Büchs for expert technical assistance and Dr. Bernd Arnold and Dr. Glenn Merlino for helpful discussions.

References

1. Ramirez-Montagut T, Turk MJ, Wolchok JD, Guevara-Patino JA, Houghton AN. Immunity to melanoma: unraveling the relation of tumor immunity and autoimmunity. Oncogene 2003;22:3180–7.
2. Bowne WB, Srinivasan R, Wolchok JD, et al. Coupling and uncoupling of tumor immunity and autoimmunity. J Exp Med 1999;190:1717–22.
3. Steitz J, Bruck J, Steinbrink K, Enk A, Knop J, Tuting T. Genetic immunization of mice with human tyrosinase-related protein 2: implications for the immunotherapy of melanoma. Int J Cancer 2000;86:89–94.
4. Steitz J, Bruck J, Gambotto A, Knop J, Tuting T. Genetic immunization with a melanocytic self-antigen linked to foreign helper sequences breaks tolerance and induces autoimmunity and tumor immunity. Gene Ther 2002;9:208–13.
5. Leitner WW, Hwang LN, deVeer MJ, et al. Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. Nat Med 2003;9:33–9.
6. Belardelli F, Ferrantini M. Cytokines as a link between innate and adaptive antitumor immunity. Trends Immunol 2002;23:201–8.
7. Tüting T, Gambotto A, Baar J, et al. Interferon-α gene therapy for cancer: retroviral transduction of fibroblasts and particle-mediated transfection of tumor cells are both effective strategies for gene delivery in murine tumor models. Gene Ther 1997;4:1053–60.
8. Hiroishi K, Tüting T, Lotze MT. IFN-α-expressing tumor cells enhance generation and promote survival of tumor-specific CTLs. J Immunol 2000;164:567–72.
9. Steitz J, Bruck J, Lenz J, Knop J, Tüting T. 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. Cancer Res 2001;61:8643–6.
10. Garbi N, Arnold B, Gordon S, Hammerling GJ, Ganss R. CpG motifs as proinflammatory factors render autochthonous tumors permissive for infiltration and destruction. J Immunol 2004;172:5861–9.
11. Heckelsmiller K, Rall K, Beck S, et al. Peritumoral CpG DNA elicits a coordinated response of CD8 T cells and innate effectors to cure established tumors in a murine colon carcinoma model. J Immunol 2002;169:3892–9.
12. Kawarada Y, Ganss R, Garbi N, Sacher T, Arnold B, Hammerling GJ. NK- and CD8(+) T cell-mediated eradication of established tumors by peritumoral injection of CpG-containing oligodeoxynucleotides. J Immunol 2001;167:5247–53.
13. Whitmore MM, deVeer MJ, Edling A, et al. Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. Cancer Res 2004;64:5850–60.
14. Sotillo R, Garcia JF, Ortega S, et al. Invasive melanoma in Cdk4-targeted mice. Proc Natl Acad Sci U S A 2001;98:13312–7.
15. Steitz J, Büchs S, Tormo D, et al. Evaluation of genetic melanoma vaccines in cdk4-mutant mice provides evidence for immunological tolerance against authochthonous melanomas in the skin. Int J Cancer 2006;118:373–80.
16. Wölfel T, Hauer M, Schneider J, et al. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 1995;269:1281–4.
17. Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat Genet 1996;12:97–9.
18. Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Res 2003;63:756–9.
19. Otsuka T, Takayama H, Sharp R, et al. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. Cancer Res 1998;58:5157–67.
20. Noonan FP, Recio JA, Takayama H, et al. Neonatal sunburn and melanoma in mice. Nature 2001;413:271–2.
21. Rane SG, Dubus P, Mettus RV, et al. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in β-islet cell hyperplasia. Nat Genet 1999;22:44–52.
22. Takayama H, LaRochelle WJ, Sharp R, et al. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A 1997;94:701–6.
23. Dudley ME, Wunderlich JR, Robbins PF, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 2002;298:850–4.
24. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific $CD8^+$ T cells in patients with melanoma. J Immunol 2005;175:6169–76.
25. Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S, Houghton AN. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med 2004;200:771–82.
26. Lengagne R, Le Gal FA, Garcette M, et al. Spontaneous vitiligo in an animal model for human melanoma: role of tumor-specific $CD8^+$ T cells. Cancer Res 2004;64:1496–501.
27. Willimsky G, Blankenstein T. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. Nature 2005;437:141–6.
28. Yamaura K, Ito K, Tsukioka K, et al. Suppression of acute and chronic rejection by hepatocyte growth factor in a murine model of cardiac transplantation: induction of tolerance and prevention of cardiac allograft vasculopathy. Circulation 2004;110:1650–7.
29. Okunishi K, Dohi M, Nakagome K, et al. A novel role of hepatocyte growth factor as an immune regulator through suppressing dendritic cell function. J Immunol 2005;175:4745–53.
30. Pardoll D. Does the immune system see tumors as foreign or self? Annu Rev Immunol 2003;21:807–39.
31. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;5:263–74.
32. Guiducci C, Vicari AP, Sangaletti S, Trinchieri G, Colombo MP. Redirecting *in vivo* elicited tumor infiltrating macrophages and dendritic cells towards tumor rejection. Cancer Res 2005;65:3437–46.
33. Thomas DA, Massague J. TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell 2005;8:369–80.
34. Antony PA, Piccirillo CA, Akpinarli A, et al. $CD8^+$ T cell immunity against a tumor/self-antigen is augmented by $CD4^+$ T helper cells and hindered by naturally occurring T regulatory cells. J Immunol 2005;174:2591–601.
35. Hwang LN, Yu Z, Palmer DC, Restifo NP. The *in vivo* expansion rate of properly stimulated transferred $CD8^+$ T cells exceeds that of an aggressively growing mouse tumor. Cancer Res 2006;66:1132–8.

Therapeutic Efficacy of Antigen-Specific Vaccination and Toll-Like Receptor Stimulation against Established Transplanted and Autochthonous Melanoma in Mice

Damia Tormo, Aleix Ferrer, Pilar Bosch, et al.

Cancer Res 2006;66:5427-5435.