Complete Regression of Advanced Primary and Metastatic Mouse Melanomas following Combination Chemoimmunotherapy

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Abstract

The development of therapeutic strategies which induce effective cellular antitumor immunity represents an important goal in cancer immunology. Here, we used the unique features of the genetically engineered Hgf-Cdk4R24C mouse model to identify a combination chemoimmunotherapy for melanoma. These mice develop primary cutaneous melanomas which grow progressively and metastasize in the absence of immunogenic foreign proteins such as oncogenes or antigens. Primary and metastatic tumors evade innate and adaptive immune defenses, although they naturally express melanocytic antigens which can be recognized by antigen-specific T cells. We found that primary melanomas continued to grow despite infiltration with adaptively transferred, in vivo-activated, tumor-specific CD8+ T cells. To promote tumor immune defense, we developed a treatment protocol consisting of four complementary components: (a) chemotherapeutic preconditioning prior to (b) adoptive lymphocyte transfer and (c) viral vaccination followed by (d) adjuvant peritumoral injections of immunostimulatory nucleic acids. Lymphocyte ablation and innate antiviral immune stimulation cooperatively enhanced the expansion and the effector cell differentiation of adaptively transferred lymphocytes. The efficacy of the different treatment approaches converged in the tumor microenvironment associated with complete regression of melano-ma cells. This combination chemoimmunotherapy caused complete regression of advanced primary melanomas in the skin and metastases in the lung with minimal autoimmune side effects. Our results in a clinically highly relevant experimental model provide a scientific rationale to evaluate similar strategies which unleash the power of innate and adaptive immune defense in future clinical trials. [Cancer Res 2009;69(15):6265–74]

Introduction

Powerful new strategies for the antigen-specific immunotherapy of cancer have been developed in experiments with transplanted mouse tumor cells. CD8+ T lymphocytes capable of specifically recognizing and destroying tumor cells in vivo can be effectively stimulated using a number of different vaccines including synthetic MHC class I-binding tumor peptides, peptide-pulsed dendritic cells, recombinant viral vectors, and genetically modified tumor cells expressing immunostimulatory cytokines (1–4). However, despite encouraging results in experimental models, each of these vaccine approaches by itself showed only limited success in clinical trials (5–11). Subsequent studies in mice using TCR-transgenic mice and antigen-transfected transplantable tumor cell lines revealed that self-tolerance (12), ignorance (13), and tumor-induced immune suppression (14–16) could prevent immune cell–mediated recognition and destruction of tumors and likely present barriers for effective cancer immunotherapy in patients.

In order to increase the efficacy of antigen-specific tumor immunotherapy, vaccines have been combined with other treatment modalities such as immunostimulatory monoclonal antibodies and toll-like receptor agonists (17–20). An adoptive transfer of tumor-specific T lymphocytes also potently enhances antitumor immunity (21, 22). The identification of appropriate combination treatment protocols which can be successfully translated into the clinic has become one of the major challenges in the field. Experiments with transplanted mouse tumor cells have only been of limited predictive value. This is best illustrated by the fact that tumor vaccines with synthetic peptides and dendritic cells were highly effective against transplanted tumors in mice (1, 2), but not against primary tumors in patients (5, 6). Transplanted mouse tumor cell lines vary greatly in their immunogenicity and interact with the immune system in a different way when compared with primary tumors evolving autochthonously in an immunocompetent host.

To overcome the experimental limitations of tumor transplantation, we established the genetically engineered Hgf-Cdk4R24C mouse model for melanoma, a tumor entity of considerable interest to tumor immunologists (23). This experimental system imitates the expected clinical situation because individual Hgf-Cdk4R24C mice sporadically develop primary cutaneous melanomas due to the cooperative activity of deregulated receptor tyrosine kinase signaling and impaired p16-dependent cell cycle arrest, two characteristic pathogenetic features of human melanoma (23–28). Here, we used the unique features of the Hgf-Cdk4R24C mouse melanoma model to develop a highly effective combination chemoimmunotherapy protocol. The sequential application of four complementary therapeutic approaches, i.e., chemotherapeutic preconditioning prior to adoptive lymphocyte transfer and viral vaccination followed by adjuvant peritumoral injections of immunostimulatory nucleic acids, was able to promote strong cytotoxic inflammation in the tumor microenvironment associated with complete regression of advanced primary melanomas in the skin, disappearance of metastases in the lungs, and only minimal autoimmune side effects.

Materials and Methods

Mice and carcinogen treatment. C57BL/6 mice (H-2b) were purchased from Charles River. Hgf-Cdk4R24C C57Bl/6 mice were bred as described
previously (20). TCR-transgenic pml-1 mice (21) were obtained from The Jackson Laboratory. To enhance melanomagenesis, newborn mice were painted once at day 4 of life with 40 μg of 7,12-dimethylbenz(a)anthracene (DMBA) in 10 μL of acetone. Animal experiments were performed at the Central Animal Facility of the University Hospital Bonn in adherence to the standards of German law for the care and use of laboratory animals.

**Analysis of primary melanomas.** Mice were inspected and photographed weekly. Growing melanocytic tumors >2 mm in diameter were considered primary melanomas. Their sizes were measured using a vernier calliper and recorded as mean diameter (in millimeters). Mice were sacrificed when progressively growing melanomas exceeded 10 mm or when signs of illness were observed. Autopsy was performed on all mice. Regional lymph nodes and internal organs were inspected for visible metastases and tumors harvested.

**Tumor transplantation experiments.** B16 cells were maintained in DMEM supplemented with 10% FCS, 2 mmol/L of l-glutamine, 50 μmol/L of 2-mercaptoethanol, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen). Syngeneic C57BL/6 (H-2b) mice were challenged intracutaneously with 105 B16 melanoma cells. The tumor size was calculated according to the formula: volume = (length × width2)/2 (in mm2). Mice with tumors >100 mm2 were sacrificed. Experiments were performed in groups of five mice and repeated at least twice.

**Tumor treatment with adoptively transferred lymphocytes and recombinant adenovirus.** Pmel-1 TCR-transgenic mice recognize the H-2Db-binding human gp100 (amino acids 25–33) peptide KVPRNQDWL. Effector T cells transduced with the mouse gp100 (amino acids 23–33) peptide EGRSRQDWL (21). Pmel-1 mice expressed Thy1.1 to facilitate T-cell tracking. For adoptive transfer, lymphocytes were isolated from lymph nodes and spleens. The number of CD8+/V13+Thy1.1+ TCR-transgenic (pmel-1) T cells was determined by flow cytometry. Each treated mouse received 2 × 106 pmel-1 T cells i.v. T cells were activated in vivo by one i.p. injection of 5 × 105 plaque-forming unit recombinant adenovirus expressing human gp100. Ad-gp100 was generated as described previously (29), propagated on 293 cells, purified by cesium chloride density gradient centrifugation and subsequent dialysis according to standard protocols.

**Chemotherapeutic preconditioning and adoptive immune stimulation.** Chemotherapy was performed by injecting mice i.p. with 2 mg (100 μg/kg) of cyclophosphamide. The synthetic phosphothioate-stabilized CpG oligodeoxynucleotide 1826 with the sequence 5'-TCCATGACGTTCCT-3' ("Cpg") was purchased from TIB Molebi, Polynosinic-polycytidylic acid ("pIC") was purchased from Sigma-Aldrich. Treatment of mice was performed by injecting 50 μg of CpG DNA and 50 μg of pIC dissolved in 100 μL of PBS peritumorally every 3 to 4 d.

**Histopathology, immunohistochemistry, and immunofluorescence.** Tumor samples were immersed in a zinc-based fixative (DAKO), embedded in paraffin, and stained with H&E. Immunostaining was performed with the rabbit pAb Pep7 against TRP1 (a kind gift from Vincent Hearing, NIH, Bethesda, MD) or the rat monoclonal antibodies against CD45 (BD PharmMingen) and CD3 (Seroclone) followed by appropriate fluorochrome-conjugated or biotin-conjugated secondary antibodies and the LSAB-2 color development system (DAKO). Heavily pigmented tumors were bleached before staining (20 min at 37°C in 30% H2O2 and 0.5% KOH, 20 s in 1% acetic acid, and 5 min in TRIS buffer).

**Quantitative reverse transcription-PCR.** Tumor samples were immediately snap-frozen in liquid nitrogen. Total RNA was isolated using TRI Reagent (Sigma) and purified using RNeasy columns (Qiagen). RNA was reverse-transcribed using Superscript II and oligo-dT18 primers (Invitrogen). PCR was performed with TaqMan assays [Rat Ctn Mm00060793_s1, Si (gp100) Mm00489896_m1, Igfb Mt03024053_m1, Cxcl2 Mm00436450_m1, and Cxcl10 Mm00445253_s1] or with fast SYBR Green Master Mix (ABI) using the following primers (from 5' to 3'): Bfng forward AGGCAGAG- CATCACCTTGAGG, reverse CCACTACACCAGAACAGGACCA; Ifng forward CTCTTTCTGATCTGCTGAG, reverse CCGTATGTCTTCCTCAGGTCCT; Gzmb forward CTCAAATGACATCAGTGC, reverse TGGCCT- TCACATTGACATTG; Ppif forward TGGAAGACCTATCAGGACCC, reverse AAGGATGGGGAATGGAGG; Tbet forward CAACACCCCTCTTGGC- CAAGG, reverse TCCCCAAGCTGTTGACAGT; and Foxp3 forward CCACG- GAAAGACAGACCTT, reverse TCTTCAACAGGCCACTTGTG.

**Flow cytometry.** Tumor tissue, spleens, and lymph nodes were harvested, dissociated mechanically, incubated with 1 mg/mL of collag- nase D (Roche) in PBS with 5% fetal bovine serum (Biochrom) for 30 min at 37°C and filtered through 70-μm cell strainers (BD Biosciences). Staining was performed with fluorochrome-conjugated antibodies against CD45, B220, CD8a, CD3, V/J13, CD90, Gr-1, CD11b, CD11c, H2-Kb (all from BD Pharmingen), and IFN-γ (eBioscience). For intracellular staining, Golgistop and Golgiplug (BD Biosciences) were used according to standard protocols. Data were acquired with a FACS Canto flow or a FACS LSRII flow cytometry system (FACS Core Facility, Bonn, Germany) and analyzed with FACSDiva (BD Biosciences) or FlowJo (TreeStar, V8.7.1) software.

**Statistical analyses.** Statistically significant differences were calculated with the nonparametric Mann-Whitney U test using the SPSS 11 computer program.

**Results**

**Primary Hgf-Cdk4R24C melanomas avoid recognition and destruction by tumor-specific T lymphocytes.** We previously reported that a treatment regimen consisting of recombinant adenosine vaccination against the melanomaspecific protein TRP2 in combination with adjuvant peritumoral injections of TLR agonists was only able to delay the growth of primary carcinogen-induced melanomas in Hgf-Cdk4R24C mice (20). To further enhance cellular antitumor immunity, we performed adoptive lymphocyte transfer experiments using TCR-transgenic CD8+ T cells derived from pml-1 mice. Pmel-1 T cells recognize an H-2Db-binding peptide epitope derived from the human melanosomal protein gp100. Effector T cells cross-reacts with the slightly different mouse gp100 epitope, which is naturally expressed by melanocytes and melanoma cells (21). In our experiments, we took advantage of the early and synchronous development of primary melanomas in Hgf-Cdk4R24C mice following epicutaneous application of DMBA shortly after birth. At an age of 70 days, all DMBA-exposed Hgf-Cdk4R24C mice carry between 5 and 15 macroscopically visible primary cutaneous melanomas with the largest tumors measuring between 4 and 6 mm in diameter. Tumor-bearing mice were randomly assigned to treatment and control cohorts. The treatment cohort received an adoptive transfer of pml-1 T lymphocytes and a simultaneous vaccination with recombinant Ad-gp100 (lymph/vacc, LV). The treatment was repeated in surviving mice at an age of 91 days. Flow cytometric analyses of splenocytes and circulating peripheral blood mononuclear cells in individual LV-treated tumor-bearing mice confirmed expansion and effector cell differentiation of transferred pml-1 T lymphocytes in vivo (Fig. 1A). Despite the presence of large numbers of circulating tumor-specific effector T lymphocytes in LV-treated mice, we observed that primary Hgf-Cdk4R24C melanomas continuously grew similar to untreated control mice (Fig. 1B).

Next, we investigated how primary tumors avoid recognition and destruction by tumor-specific T lymphocytes. We sacrificed Hgf-Cdk4R24C mice bearing primary tumors 10 days after the first LV treatment. Immunofluorescence and flow cytometric analyses of primary tumors from untreated control mice revealed only very few infiltrating immune cells. In contrast, tumors from LV-treated mice showed diffuse infiltration with CD45+ immune cells (Fig. 1C).
Histomorphologic, immunohistochemical, and flow cytometric analyses of LV-treated tumors showed large numbers of infiltrating CD3+ T lymphocytes in close contact with melanoma cells, including a significant percentage of CD8-Thy1.1-Vβ13+ (pmel-1) effector cells (Fig. 1D). Thus, primary Hgf-Cdk4R24C melanomas were principally accessible for tumor-specific T lymphocytes but escaped their cytotoxic effector functions in the tumor microenvironment. The majority of immune cells within primary tumors showed a Gr1+CD11b+ phenotype, Gr1+CD11b+ or CD4+CD25+ cells could only very rarely be found. This suggested that tumor tolerance may involve the activity of myeloid-derived suppressor cells but not of tumor-associated macrophages or of regulatory T cells (data not shown).

**Enhancement of cellular antitumor immunity using combination treatment approaches.** Next, we investigated the ability of pmel-1 T cells to recognize primary Hgf-Cdk4R24C melanoma cells in vitro. Primary Hgf-Cdk4R24C melanoma cells are heavily pigmented and reverse transcription-PCR (RT-PCR) analyses show...
strong expression of the melanocyte-specific protein gp100. Short-term cultures of primary melanoma cells showed both spindle-shaped and epithelioid morphologies which recapitulated their growth pattern in vivo (Fig. 2A). These melanoma cells only expressed very low levels of MHC class I molecules on their cell surface which could be up-regulated following exposure to recombinant IFN-α (Fig. 2B). IFN-γ ELISPOT assays showed that cultured melanoma cells could be recognized by pmel-1 effector T lymphocytes in vitro as effectively as cultured B16 melanoma cells. Tumor cell recognition could be significantly improved following incubation with recombinant IFN-α (Fig. 2C).

Based on our previous experience with adjuvant injections of the TLR agonists, CpG and pIC (20), we reasoned that the stimulation of viral pattern recognition receptors which activate the type I IFN system should promote the antitumor efficacy of adoptively transferred pmel-1 T lymphocytes (30, 31). We initially evaluated this hypothesis in parallel experiments using the transplanted B16 melanoma model in a rigorous 10-day treatment model in which tumors were already macroscopically visible (Fig. 3A). Adoptively transferred pmel-1 T lymphocytes and viral vaccination with Ad-gp100 (LV) do not affect the progressive growth of advanced B16 melanomas in this experimental setting (Fig. 3B). The antitumor efficacy of adoptive lymphocyte transfer and viral vaccination could be boosted by persistent stimulation of innate immunity through adjuvant peritumoral injections of CpG and pIC (LV + I) leading to considerably prolonged survival (Fig. 3B).

We previously showed that chemotherapeutic preconditioning with cyclophosphamide enhances the efficacy of melanoma vaccines (18). Cyclophosphamide also facilitates the expansion of adoptively transferred T lymphocytes (32, 33). Strikingly, a treatment protocol consisting of cyclophosphamide treatment before adoptive lymphocyte transfer and viral vaccination followed by adjuvant injections of CpG and pIC (C + LV + I) achieved complete regression of large B16 melanomas (Fig. 3B). This combination treatment protocol was much less effective without innate immune stimulation (C + LV) or without adoptive lymphocyte transfer (C + V + I), indicating that all four components were required (Fig. 3B). Mice in the C + LV + I–treated groups remained tumor-free for several months and showed only limited destruction of melanocytes indicating preferential recognition and destruction of malignant over normal melanocytic cells (data not shown).

In subsequent experiments, we treated established transplanted Hgf-Cdk4<sup>R24C</sup> melanomas generated by serial transplantation of primary tumors (Fig. 3C). Groups of mice with macroscopically visible transplanted Hgf-Cdk4<sup>R24C</sup> melanomas received an adoptive transfer of pmel-1 T lymphocytes and recombinant adenoaviral vaccination (LV) on day 14 or were left untreated. Established transplanted Hgf-Cdk4<sup>R24C</sup> melanomas were not affected by the LV treatment similar to advanced transplanted B16 melanomas (Fig. 3D). However, treatment according to the C + LV + I protocol also resulted in complete regression and long-term cure, demonstrating unequivocally that pmel-1 T cells are able to recognize and destroy Hgf-Cdk4<sup>R24C</sup> melanoma cells (Fig. 3D).

Complete regression of primary cutaneous as well as metastatic Hgf-Cdk4<sup>R24C</sup> melanomas. Having shown the efficacy of our combination chemoimmunotherapy protocol against transplanted Hgf-Cdk4<sup>R24C</sup> melanoma, we also treated mice with primary Hgf-Cdk4<sup>R24C</sup> melanomas. DMBA-exposed Hgf-Cdk4<sup>R24C</sup> mice were again randomly assigned to different cohorts at an age of 70 days at which time they carried multiple, macroscopically visible primary melanomas. Strikingly, two consecutive C + LV + I treatment cycles were able to cause the complete regression of primary cutaneous melanomas (Fig. 4A and B). In a total of 30 mice treated in 8 independent experiments, we observed significantly prolonged survival (Fig. 4C). Control cohorts of tumorbearing mice received either cyclophosphamide and CpG/pIC (C + I) or adoptive lymphocytes and viral vaccination (LV) only, both of which slightly delayed tumor progression (Fig. 4C).
Next, we determined the effect of the C + LV + I treatment protocol on metastatic disease. DMBA-exposed Hgf-Cdk4R24C mice already carried spontaneous lung metastases at an age of 70 days when treatment was started (Fig. 4D). In a cohort of five C + LV + I–treated mice which showed complete regression of their DMBA-induced primary melanomas in the skin at an age of 135 days, we did not find any metastases in the lungs. In contrast, lung metastases were found in almost all LV-treated and untreated mice at the time of death (Fig. 4D). Thus, our combination chemoimmunotherapy not only caused the regression of primary cutaneous melanomas but also of spontaneous metastases in the lungs.

Cytotoxic inflammation in the tumor microenvironment. Next, we investigated the effect of chemotherapeutic preconditioning and adjuvant innate immune stimulation on the number and functional status of adoptively transferred pmel-1 T cells in tumor-bearing mice. Cyclophosphamide treatment before adoptive lymphocyte transfer and viral vaccination (C + LV) enhanced the expansion of adoptively transferred pmel-1 T lymphocytes (Fig. 5A). Additional adjuvant peritumoral injections of CpG/pIC after adoptive lymphocyte transfer and viral vaccination (C + LV + I) promoted their survival and supported their effector cell differentiation as evidenced by an increase in the percentage of lymphocytes capable of producing IFN-γ (Fig. 5A). Experiments with groups of mice bearing transplanted B16 melanoma analyzed at different time points confirmed that chemotherapy and innate immune stimulation cooperatively increased expansion, survival, and effector differentiation of adoptively transferred pmel-1 T lymphocytes in vivo (*, P < 0.05; Fig. 5B).

The combined activities of the C + LV + I protocol converge in the microenvironment of primary tumors. Quantitative RT-PCR analyses 10 days after adoptive lymphocyte transfer in C + LV + I–treated mice showed significantly increased expression of mRNA coding for IFN-γ, CXCL-10, perforin, and granzyme B (*, P < 0.05; Fig. 5C) when compared with LV-treated mice. This correlated with enhanced immune cell infiltration in C + LV + I–treated mice (data not shown). Increased expression of the transcription factor T-bet characteristic for a Th1-biased cytotoxic immune response and decreased expression of the transcription factor Foxp3 characteristic for regulatory T cells was observed in both LV-treated and C + LV + I–treated tumors. Histopathologic and immunofluorescence analyses revealed massive tumor cell destruction associated with dense infiltration of CD3+ T cells (Fig. 5D). Collectively, these results show that the C + LV + I treatment strongly promotes cytotoxic inflammation in primary melanomas.

Development of new primary melanomas in successfully treated Hgf-Cdk4R24C mice. Subsequently, we monitored cohorts
of C + LV + I–treated mice which showed complete tumor regression for longer time periods. Starting at an age of 150 days, ~2 months after the second C + LV + I treatment cycle, these mice developed new pigmented melanomas in their carcinogen-treated skin (Fig. 6A). These new primary melanomas in successfully C + LV + I–treated mice at an age of 200 days histomorphologically resembled those observed in untreated mice at an age of 90 days with very little immune cell infiltrate. Quantitative RT-PCR analyses of tumors revealed increased expression of transforming growth factor-β and CXCL-2 relative to IFN-γ and CXCL-10, as well as decreased expression of perforin, granzyme B, and T-bet (Fig. 6B). Interestingly, we did not observe an increase in the expression of Foxp3 or an increase in CD4+CD25+ T cells in these tumors which would indicate

do the local activity of regulatory T cells (Fig. 6B and C). Again, most immune cells within tumors showed a Gr1+CD11b+ phenotype which corresponded to enhanced infiltration with granulocytes, frequently in association with superficial ulceration (Fig. 6C; data not shown).

Importantly, we were able to detect a small but significant population of CD8+Thy1.1+Vα13+ (pmel-1) T cells not only in the spleens but also in the new pigmented cutaneous melanomas in all investigated mice at an average age of 200 days, i.e., 3 months after the second treatment cycle. These pmel-1 T cells could be expanded to large numbers following a booster vaccination with recombinant Ad-gp100 (Fig. 6D). Thus, adoptively transferred pmel-1 T cells in C + LV + I–treated mice acquire memory status, persist for an extended time, and can be efficiently restimulated
in vivo. Future experiments will have to reveal whether these reactivated T cells are again capable of causing tumor regression.

**Discussion**

In our work, we identified a highly effective combination chemoimmunotherapy which can reverse immune tolerance in the tumor microenvironment and cause complete regression of advanced primary and metastatic melanomas in a novel genetically engineered tumor model. This model avoids the use of immunogenic foreign proteins as oncogenes or antigens (i.e. SV40 large T antigen or Her-2/neu; refs. 34–41). Instead, tumors arise autochthonously in the skin, grow progressively, and metastasize spontaneously to draining lymph nodes and lungs because of two nonimmunogenic genetic changes also observed in patients. Although tumor cells express several melanocyte-specific lineage differentiation antigens which can be potentially recognized by T lymphocytes, they express low levels of MHC class I molecules and effectively evade innate as well as adaptive immune defense. Using an adoptive T-cell transfer approach, we found that tumors continued to grow even in the presence of large numbers of in vivo–activated melanoma-specific T lymphocytes. To overcome tumor

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**Figure 5.** Combination chemoimmunotherapy promotes strong cytotoxic inflammation in the tumor microenvironment of primary melanomas. **A,** T-cell expansion and effector cell differentiation 10 d after adoptive transfer in tumor-bearing Hgf-Cdk4R24C mice. Top row, flow cytometric dot plots quantifying Thy1.1+Vβ13+ splenocytes in the CD8+ gate of mice treated as indicated. Bottom row, corresponding histograms for antigen-specific intracellular IFN-γ expression in CD8+Thy1.1+Vβ13+ T cells. **B,** time course of T-cell expansion and effector cell differentiation in mice bearing established B16 melanomas. Left, mean percentage (±SE) of Thy1.1+Vβ13+ splenocytes in the CD8+ gate. Right, percentage of CD8+Thy1.1+Vβ13+ T cells able to produce IFN-γ (points, mean; bars, SE). Data are from six individual mice in three separate experiments for each time point (*, P < 0.05). **C,** molecular characterization of the microenvironment in treated primary tumors. Results of quantitative RT-PCR for the indicated genes calculated as mean fold-change of expression in five individual samples of mice from the LV-treated and the C + LV + I–treated group relative to the untreated control group (columns, mean; bars, SE; *, P < 0.05; **, P < 0.01). **D,** cytotoxic immune cell infiltration. Left, H&E stain of a C + LV + I–treated primary melanoma (magnification, ×200). Right, corresponding immunohistochemical stain for CD3+ T lymphocytes (magnification, ×400).
immune tolerance, we combined adoptive lymphocyte transfer and viral vaccination with adjuvant peritumoral injections of the immunostimulatory nucleic acids, CpG and pIC, which activate viral pattern recognition receptors of the toll receptor family (23, 30, 42). Persistent innate immune stimulation strongly enhanced the therapeutic efficacy of adoptive lymphocyte transfer and adenoviral vaccination. Chemotherapeutic preconditioning with cyclophosphamide, which has facilitated tumor treatment with adoptively transferred lymphocytes for many years (43), further increased antitumor efficacy leading to complete and long-term regression of advanced, macroscopically visible transplanted as well as primary melanomas. All four complementary components of our treatment protocol were required to achieve complete regression and long-term remission of large primary and transplanted melanomas.

Cyclophosphamide pretreatment and adjuvant injections of immunostimulatory nucleic acids cooperatively enhanced both the expansion as well as the cytotoxic effector cell differentiation of adoptively transferred pmel-1 T lymphocytes and induced a strong cytotoxic inflammation in the tumor microenvironment. Cyclophosphamide has pleiotropic effects including the promotion of T-cell homeostatic expansion, inhibition of regulatory T-cell function, damage to the tumor vasculature, and functional alteration of tumor-infiltrating immune cells (32, 33, 44). CpG and pIC activate the type I IFN system through viral pattern recognition receptors (31). IFNs may be involved in resurrecting the barriers to cancer development on several levels. We show that IFN-α increases MHC class I expression on Hgf-Cdk4R24C melanoma cells and supports recognition by pmel-1 T cells. In addition, IFNs can also promote tumor cell apoptosis, impair angiogenesis, counteract immunosuppression, prolong T-cell survival, and enhance cytotoxic effector functions in the tumor microenvironment (45–48).
Of the potential mechanisms for tumor immune tolerance in primary Hgf-Cdk4C/C mice, we could rule out the local activity of regulatory T cells or tumor-associated macrophages as these cells were rarely found within primary tumors. Instead, myeloid-derived suppressor cells may be involved because the majority of tumor-infiltrating immune cells show a Gr1+CD11b+ phenotype. In addition, we speculate that the well-known immunosuppressive activity of hepatocyte growth factor and the distinct immunologic properties of melanocytes might participate in inhibiting T-cell effector functions in the tumor microenvironment.

All successfully C + LV + I-treated mice with complete tumor regression eventually developed new primary DMBA-induced melanomas which morphologically resembled those observed in untreated mice. A detailed molecular and cellular analysis of the tumor microenvironment indicated that the balance of cytokines and chemokines had shifted back in favor of tumor progression. Tumor growth occurred despite the presence of melanoma-specific (pmel-1) memory T cells, which could be found both in secondary lymphoid organs as well as in the tumor itself. These potentially tumor-specific T cells could be efficiently reactivated in vivo with a recombinant adenovirus booster immunization. The newly arising primary melanomas apparently evaded the surveillance function by pmel-1 memory T cells, presumably because they do not adequately activate the innate immune system. The effect of the vaccine-induced T-cell memory response on tumor growth will have to be determined in future experiments.

Interestingly, treated mice with complete tumor regression showed only limited vitiligo-like fur depigmentation predominantly at the site of tumors, although pmel-1 T cells can principally recognize and kill all cells of melanocytic origin which express the melanosomal protein gp100. This suggests that our combination chemoinmunotherapy promotes preferential recognition and destruction of malignant over normal pigment cells. The relative tumor selectivity may be conferred by combining the antigen-specificity of T cells with the nonspecific action of chemotherapy and innate immune stimulation on both immune and tumor cells. This may be an advantage over the use of high-dose interleukin 2, which promotes the antitumor efficacy of adoptively transferred lymphocytes with significant auto-reactivity against pigment cells (49).

Treatment protocols combining chemotherapy, total body irradiation, adoptive lymphocyte transfer and vaccine strategies are currently evaluated in a large number of clinical trials. Our results suggest that adjuvant innate immune stimulation using immunostimulatory nucleic acids can also be a powerful approach to enhance the therapeutic efficacy of adoptive lymphocyte transfer and viral vaccination. The use of TLR agonists may even replace total body irradiation because it has recently been reported that this treatment modality depends on TLR triggering (50). The unique experimental features of the genetically engineered Hgf-Cdk4C mouse model, which faithfully portrays the biology of melanoma in man, may help to facilitate the successful clinical translation of effective strategies for combination chemoinmunotherapy against this deadly disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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