Optimized dendritic cell vaccination induces potent CD8 T cell responses and anti-tumor effects in transgenic mouse melanoma models

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ABSTRACT

Despite melanoma immunogenicity and remarkable therapeutic effects of negative immune checkpoint inhibitors, a significant fraction of patients does not respond to current treatments. This could be due to limitations in tumor immunogenicity and profound immunosuppression in the melanoma microenvironment. Moreover, insufficient tumor antigen processing and presentation by dendritic cells (DC) may hamper the development of tumor-specific T cells. Using two genetically engineered mouse melanoma models (RET and BRAFV600E transgenic mice), in which checkpoint inhibitor therapy alone is not efficacious, we performed proof-of-concept studies with an improved, multivalent DC vaccination strategy based on our recently developed genetic mRNA cancer vaccines. The in vivo expression of multiple chimeric MHC class I receptors allows a simultaneous presentation of several melanoma-associated shared antigens tyrosinase related protein (TRP)-1, tyrosinase, human glycoprotein 100 and TRP-2. The DC vaccine induced a significantly improved survival in both transgenic mouse models. Vaccinated melanoma-bearing mice displayed an increased CD8 T cell reactivity indicated by a higher IFN-γ production and an upregulation of activation marker expression along with an attenuated immunosuppressive pattern of myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg). The combination of DC vaccination with ultra-low doses of paclitaxel or anti-PD-1 antibodies resulted in further prolongation of mouse survival associated with a stronger reduction of MDSC and Treg immunosuppressive phenotype. Our data suggest that an improved multivalent DC vaccine based on shared tumor antigens induces potent anti-tumor effects and could be combined with checkpoint inhibitors or targeting immunosuppressive cells to further improve their therapeutic efficacy.

Introduction

During the past decade, enormous improvements have been achieved in the treatment of metastatic melanoma. In particular, the immune checkpoint inhibitors, such as antibodies against cytotoxic T-lymphocyte-associated protein (CTLA)-4 and programmed cell death protein (PD)-1, have significantly improved the treatment efficacy in a subset of melanoma patients harboring wild type BRAF.1,2 In addition, BRAF and MEK-kinase inhibitors achieved profound clinical responses in melanoma patients with BRAFV600E mutations.3

Even with these advances, only a fraction of melanoma patients responds durably to immunotherapy.4 The therapy resistance was reported to be due to chronic inflammation and immunosuppression, tumor heterogeneity, as well as to lower numbers of somatic mutations encoding neo-antigens.5-8 Therefore, the attention of tumor immunologists has been shifted from shared tumor-associated antigens, to mutanome-encoded, patient specific neo-antigens.7 Nevertheless, tumor-associated antigens should not be forgotten since limitations in the neo-antigen expression could be overcome by boosting immune responses via targeting tumor-associated shared antigens.

We attempted to improve the presentation of shared melanoma-associated antigens (MAA) by dendritic cell (DC)-based immunotherapy since the clinical impact of such immunotherapies has been limited so far.9 Efficient major histocompatibility complex (MHC)-peptide expression on DC and their activation determines the degree and quality of the T cell response. DC-based immunotherapies require improvements regarding (i) the origin and polarization of DC, (ii) the maturation stimuli and (iii) the type and form of antigens to be loaded on DC.6 To overcome these limitations, we have developed earlier a novel genetic platform for the induction of CD8 T cell responses specific for MAA, human glycoprotein (hgp)100 and tyrosinase related protein (TRP)-2 by DC vaccination.10 We showed that an efficient peptide presentation through human beta 2 microglobulin (hβ2 m) can be
coupled with constitutive toll-like receptor 4 (TLR4) signaling through the polypeptide product of a single gene by mRNA electroporation into bone marrow-derived DC. This modality was highly efficient in breaking immune tolerance by stimulating the activation of DC and antigen-specific CD8 T cell responses, which inhibited tumor growth and improved the overall survival in melanoma-bearing mice.10,11

In this study, we broadened the repertoire of the hβ2 m-platform for CD8 T cell induction by including two additional MAA, TRP-1 and tyrosinase (TYR). Moreover, we utilized this chimeric mRNA construct system to examine whether multivalent DC immunization is more effective to inhibit melanoma progression than current vaccination approaches with long peptides or peptide-pulsed DC. Importantly, we test our mRNA-based DC vaccine in two different genetically engineered mouse models (GEMM) that develop de novo tumors in a natural immune–proficient microenvironment.12 Advanced tumors in RET-transgenic (RET-tg) and BRAFV600E mutated (BRAF) melanoma models closely mimic the histopathology and clinical development of their human counterparts as they are able to progress spontaneously toward metastatic disease.13,14 Since they are unresponsive to sole checkpoint inhibition they represent good preclinical model systems to test our vaccination strategy based on the presentation of shared MAA by DC.13-15 Finally, to target the immunosuppressive melanoma microenvironment we applied either anti-PD-1 antibodies or paclitaxel (Pac) at ultra-low doses, which was reported previously by us to reduce the production of chronic inflammatory mediators and to inhibit myeloid-derived suppressor cells (MDSC) in the RET-tg model.16

We found that the immunization with our improved DC vaccine induced multi-antigen-specific effector T cells and long-lasting memory T cells, leading to a significant prolongation of the survival of tumor-bearing RET-tg and BRAF mice. Moreover, both combined therapies with ultra-low dose paclitaxel or checkpoint inhibitor further improved the survival, induced stronger CD8 T cell activation and significantly attenuated an immunosuppressive pattern of MDSC and regulatory T cells (Treg). Our data suggest that mRNA-based DC vaccination with shared MAA showed a strong therapeutic effect in two melanoma GEMM and could be combined with other immunotherapeutic approaches to improve the efficacy of human melanoma treatment as an alternative to individualized neo–antigen vaccination.

Results

Chimeric β2-microglobulin molecule assembly

We have previously generated chimeric receptor constructs with MAA specific to human gp10025-33 and murine TRP-2180–188 (both H-2Dβ binder) and described their anti-tumor activity in melanoma-bearing mice.10,11 To broaden the clinical potential of the constructs we included additional MAA such as TRP-1455–463 (H-2Dβ binder) that was reported to confer anti-tumor immune responses12 and TYR360–368, which was predicted by SYFPEITHI prediction software as an H-2Dβ binder.18 Both peptides were assembled into the chimeric hβ2 m-platform with the TLR4 and Kb anchors (Supplementary Fig. 1 A, B) as previously described.19 The designation of new constructs is summarized in Supplementary Fig. S1 C.

DC present the MHC-I constructs on the cell surface and induce cytotoxic T cells

The kinetics of MHC-I construct expression on the cell surface of bone marrow-derived DC was monitored by flow cytometry with anti-hβ2m antibodies. All constructs were found to be expressed on the DC surface for at least 48 h, although TRP-1-Kb and TYR-Kb constructs were expressed at higher levels than TRP-1-TLR4 and TYR-TLR4 ones (Fig. 1 A, B), which is consistent with previous observations.10 We electroporated DC with mRNA-constructs containing Kb and TLR4 anchors for the same antigen at 1:1 ratio to ensure prolonged presentation via the Kb construct and DC maturation due to the constitutive intracellular TLR4 signaling. Upon electroporation, we observed a profound stimulation of IL-12 production by DC (similar to that induced by LPS) and no significant induction of IL-10 (Fig. 1 C). Moreover, electroporated DC displayed an upregulation of CD80, CD86 and HMC class II expression comparable to the stimulation with LPS (Fig. 1D–F). This marker profile and strong IL-12 secretion supports the DC maturation, which is in line with our previous data.10 In addition, the immunization with mRNA electroporated bone marrow-derived DC led to the induction of antigen-specific cytotoxic T lymphocytes (CTL), which specifically killed melanoma target cells in vitro (Fig. 1G, H). Thus, the new TRP-1/TYR-hβ2m-TLR4/Kb chimeric constructs electroporated into DC initiated a matured phenotype and induced antigen-specific CTL.

mRNA-electroporated DC induce stronger CTL responses than peptide-loaded DC

To evaluate if TRP-1/TYR-hβ2m-TLR4/Kb chimeric constructs are superior to conventional, peptide-loaded DC in inducing specific CTL killing in vivo, we transfected DC with mRNA of TRP-1-hβ2m-TLR4/Kb (TRP-1-EP), TYR-hβ2m-TLR4/Kb (TYR-EP) or with the mixture of them (Mix-EP; TRP-1/TYR-hβ2m-TLR4/Kb) at 1:1 ratio. In parallel, LPS matured DCs were loaded with TRP-1455–463 (TRP-1-PL), TYR360–368 (TYR-PL) peptide or peptide mixture (Mix-PL). Electroporated unloaded DC (control) and OVA257–264 peptide-loaded mature DC (SIINF-PL) served as controls. The induction of CTL responses was studied upon the vaccination of mice with respective DC preparations using CTL in vivo killing assay. As seen in Fig. 2 A, B, all three groups of mRNA-electroporated DC (EP) were able to elicit significantly stronger CTL responses than cells loaded with the respective peptides (PL). Moreover, mice vaccinated with mRNA-electroporated DC displayed significantly higher frequency of IFN-γ secreting and effector memory CD8 T cells in spleens upon in vitro restimulation than mice receiving peptide-loaded DC vaccines (Fig. 2 C, D).
closely resembles human melanoma regarding histopathology and clinical development. As seen in Fig. 3 A, the immunization with DC electrooporated by the mixture of TRP-1/TPR-hβ2m-TRL4/Kb mRNA (Mix-EP) resulted in a significantly improved survival as compared to mice treated with empty DC (control group). However, the survival of mice treated with DC containing a single antigen showed only a trend for the survival prolongation, which was not significant (Fig. 3 A). Since several clinical studies described promising effects with the long-peptide vaccination combined with adjuvants, we applied the vaccination with peptide loaded DC (Mix-PL) as well as with long peptide mix (LP, TRP-1448–472, TYR353–377) combined with anti-CD40 mAb and poly:IC for effective T helper cell-mediated
costimulation similar to our hβ2m-TLR4 constructs. Survival monitoring displayed a non-significant tendency for improved survival for the long peptide mix (LP+CD40+P: IC) vaccinated group (Fig. 3B). In contrast, vaccination with the mRNA-electroporated DC vaccine (Mix-EP, $P < 0.01$) showed a more profound and significantly higher survival prolongation than peptide-loaded DC (Mix-PL, $P < 0.05$; Fig. 3B). Consequently, the mRNA-electroporated DC vaccine (Mix-EP) showed an increased therapeutic efficiency as compared to all other vaccinated groups.

**Improved DC vaccine stimulates T cell-mediated immune responses**

Analyzing the activation status of tumor-infiltrating T lymphocytes upon mRNA-electroporated DC vaccination, we...
demonstrated an increased frequency of IFN-γ producing CD8 T cells in the group immunized with TRP-1/TYR group (Mix-EP) as compared to the control group (Fig. 3 C). Furthermore, the expression of T cell activation markers such as TCR z-chain (Fig. 3D), CD69 (Fig. 3E), PD-1 (Fig. 3 F) and the proliferation marker Ki67 (Fig. 3G) was significantly higher in the Mix-EP group. Moreover, these mice displayed an elevated frequency of CD44^CD62 L^¡ effector memory (EM) CD8 T cells compared to the control group (Fig. 3 H). Data are representative of two independent experiments. *P < 0.05, **P < 0.01.

Next, we characterized immunosuppressive cells MDSC (Fig. 4 A–C) and Treg (Fig. 4D–F) infiltrating skin tumors of treated mice. Although we observed no statistically significant differences in the MDSC frequency (data not shown), the level of nitric oxide (NO) production measured by the mean fluorescence intensity (MFI) showed a clear reduction in MDSC from the Mix-EP vaccinated group (Fig. 4B). In addition, the frequency of PD-L1^+ MDSC was significantly decreased (Fig. 4 C). Similar to MDSC, the frequency of Treg was not changed in the Mix-EP vaccinated group (Fig. 4D). However, we demonstrated a reduced expression of Ki67 and CD39 on these cells (Fig. 4E, F). This suggests that mRNA-based DC vaccination could stimulate powerful CD8 T cell responses without concomitant activation of immunosuppressive cell populations. We even detected a decrease in the immunosuppressive pattern of tumor-infiltrating MDSC and Treg.

**Combination of multivalent DC vaccination with ultra-low dose Pac stimulates anti-tumor responses in melanoma-bearing mice**

Our previous studies showed that treatment of melanoma-bearing RET-tg mice with Pac at non-cytotoxic, ultra-low doses reduced chronic inflammatory factors and immunosuppressive MDSC functions.16 In addition, it was suggested that the vaccination with multiple antigens leads to improved clinical outcomes.20,21 Therefore, we electroporated DC with a quadruple-antigen mRNA Mix (Q-Mix), containing TRP-1/TYR-hb2m-TLR4/Kb constructs and previously described hgp100/TRP-2-hb2m-TLR4/Kb constructs10,11 and...
treated melanoma-bearing mice from RET-tg and BRAF models in combination with ultra-low dose Pac (Supplementary Fig. 1 C,D).

Multivalent DC vaccination (Q-Mix) significantly prolonged mouse survival in both models (Fig. 5 A, B). As expected, therapeutic effects of vaccination were more profound when combined with ultra-low dose Pac (Q-Mix+Pac).

Analyzing immune cells in melanoma lesions, we demonstrated a significant increase in the frequency of IFN-γ+ CD8 T cells upon Q-Mix DC vaccination alone or in combination with Pac (Fig. 5 C and F). In addition, an elevated frequency of antigen-specific tumor-infiltrating CD8 T cells in treated RET-tg mice was detected by tetramer staining (Supplementary Fig. 2 A, B). Furthermore, Q-Mix DC vaccination alone or combined with Pac significantly increased the frequency of tumor-infiltrating CD8 T cells (Supplementary Fig. 3 A, G) as well as the expression of TCR ζ-chain (Fig. 5D, G), CD69 (Supplementary Fig. 3B, H) and PD-1 (Supplementary Fig. 3 C, I) on CD8 T cells. Interestingly, the frequency of EM (Fig. 5E, H) in both models and central memory (CM) T cells (Supplementary Fig. 3 J) in the BRAF model was predominantly increased upon Q-Mix+Pac treatment. No profound effects on the frequency of tumor-infiltrating MDSC in both models were observed (data not shown). However, we detected a remarkable decrease in the expression of Arg-1 (Fig. 6 A, E), PD-L1 (Fig. 6B, F) and NO (Supplementary Fig. 3E, K) in these cells. For tumor infiltrating Treg, we observed their decreased frequency in the RET-tg model (Fig. 6 C, G) as...
as a reduced expression of Ki67 (Fig. 6D, H) and CD39 (Supplementary Fig. 3 F, L). Interestingly, similar changes for effector T cells, MDSC and Treg were detected in spleens from RET-tg (Supplementary Fig. 4) and BRAF mice (Supplementary Fig. 5) and metastatic lymph nodes from these mice (data not shown), indicating a systemic effect of this combined treatment. Importantly, in tumor lysates of treated RET-tg mice, we detected also a significantly reduced concentration of cytokines and growth factors IL-10, TNF-α, IL-6 and VEGF (Supplementary Fig. 6) that are known to stimulate MDSC and Treg functions and expansion.

Analysis of memory T cells in tumor and spleen samples from surviving BRAF and RET-tg mice at day 82 post treatment start, showed an accumulation of EM and CM T cells upon the therapy with Q-Mix vaccine and Pac (Supplementary Fig. 7), indicating a systemic, long-lasting memory T cell induction upon combinatorial treatment.

**Combinatorial treatment with anti-PD-1 antibodies further increases anti-tumor efficiency**

Since anti-PD-1 antibodies are approved for the therapy of melanoma patients, we addressed the question whether the combination of checkpoint inhibition with our improved DC vaccine could enhance the anti-tumor effects. Indeed, the combinatorial treatment significantly prolonged the survival of tumor-bearing RET-tg mice as compared to Q-Mix vaccination or anti-PD-1 antibodies only (Fig. 7 A) This therapeutic effect was associated with a significant accumulation of CD8 T cells.
within the tumor (Fig. 7B) as well as with an increased expression of the TCR ζ-chain (Fig. 7C), Ki67 (Fig. 7D) and CD69 (Fig. 7E) in these cells. In addition, tumor-infiltrating CD8 T cells displayed an elevated frequency of EM T cells (Fig. 7F) and antigen-specific IFN-γ+ cells (Fig. 7G) upon combinatorial treatment as compared to single treatments.

Interestingly, similar to the combination with ultra-low dose Pac, DC vaccination combined with anti-PD-1 antibodies had profound inhibitory effects on tumor-infiltrating MDSC (Supplementary Fig. 8A-D). In addition, a similar impact was detected in spleens and metastatic lymph nodes (data not shown). Moreover, combination of the DC vaccine with anti-PD-1 antibodies led to a significant reduction of the frequency of intratumoral Treg (Supplementary Fig. 8E) associated with decreased frequencies of CD39+ and Ki67+ Tregs (Supplementary Fig. 8F, G). In addition, the amount of activated conventional CD4 T cells was significantly increased (Supplementary Fig. 8H). These results indicate that the combination of DC vaccination with chemo-modulating agents (such as ultra-low dose paclitaxel) or with the checkpoint inhibition could provide a significantly improved anti-tumor effect in preclinical melanoma GEMM.

**Figure 6.** Combined immunotherapy with vaccine and ultra-low dose paclitaxel downregulates MDSC and Treg activity markers. Tumor-infiltrating MDSC and Treg from RET (A-D, n = 8) and BRAF mice (E-H, n = 8) were analyzed by flow cytometry 10 days upon last vaccination. Arg-1 expression (A, E) by MDSC is presented as MFI. (B, F) PD-L1 expression is shown as the percentage of positive cells within total MDSC. (C, G) CD4+ CD25+ FOXP3+ Treg are presented as the percentage within total CD4 T cells. (D, H) Ki67 expression on Treg is shown as the percentage of respective subset among total Treg. Data are representative of two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Discussion

Limited mutation load and, therefore, reduced efficacy of neo-epitope based personalized immunotherapy along with profound immunosuppression in the tumor microenvironment may be major reasons of the resistance of a significant fraction of patients to current treatments with negative immune checkpoint inhibitors. Moreover, insufficient tumor antigen processing and presentation by DC may hamper the development of tumor-specific T cells. Many therapies aiming to boost T cell responses are now in use and CD8 T cells are the key players in anti-tumor immune responses since increased intra-tumoral infiltration of these cells was reported to correlate with a better patient outcome. Vaccinations using neo-antigens are now in the focus since they could be promising in breaking immune tolerance. However, approaches using the immunization with multiple shared tumor-associated antigens could be very efficient since the multivalent T cell response reduces the possibility of tumor escape in a larger cohort of patients. In addition, this vaccination strategy represents a promising approach for the treatment of tumors with low mutational load. Usually, immunotherapies include single agents that target individual steps in the anti-tumor immune response. However, such monotherapies are unlikely to overcome complex mechanisms that impede anti-tumor immunity in patients. Therefore new multivalent and combinatorial therapies are tested now in some ongoing clinical trials that include the targeting of several immune cell populations.

Figure 7. Enhanced anti-tumor effect of DC vaccine and anti-PD-1 antibodies in melanoma-bearing mice. RET-tg mice with established tumors were vaccinated i.p. three times in weekly intervals using DC electroporated with combination of mRNA encoding for hgp100 + TRP-2 + TRP-1 + TYR, h/2 m-Kb/TLR4 (Q-Mix). Some vaccinated mice additionally received 4 times i.p. injections of 250 μg anti-PD-1 antibody (aPD-1) or isotype control (Control) every 3 days. (A) Survival of mice is shown as a Kaplan-Meier curve (n = 6–12/group). Ten days after last vaccination, tumor infiltrating CD8 T cells were analyzed by flow cytometry (n = 8). Results are presented as the percentage of tumor-infiltrating CD8 T cells among total leukocytes (B), MFI of the TCR z-chain in CD8 T cells (C), the percentage of Ki67+ (D), CD69+ (E), CD62 L−CD44+ (F) and IFN-γ producing CD8 T cells (G) among total CD8 T cells. Data are representative of two independent experiments. "P < 0.05, ""P < 0.01, """"P < 0.001.
Here we applied immunization with shared MAA based on our recently developed genetic mRNA cancer vaccines, which was combined with chemo-modulation or checkpoint inhibition to tackle the immunosuppressive tumor microenvironment. We opted for mRNA-based DC vaccination since DC can stimulate both adaptive and innate anti-tumor immune responses and were already intensively used for cancer immunotherapy. Moreover, tumor-associated antigens could be delivered to DC by loading as peptides or recombinant proteins or by mRNA transfection. Importantly, mRNA cannot integrate permanently into the genome, and therefore, has no oncogenic potential. In addition, mRNA may act as an adjuvant by providing costimulatory signals, for example, via TLR, yet only few studies coupled constitutively active TLR4 for this purpose. We have previously shown that a universal MHC-I chimeric mRNA construct DC vaccine, which couples the presentation of MAA hgp100, TRP-2 and the stimulation of DC via constitutively active TLR4 domain, inhibited melanoma growth and improved mouse survival.

In this study, we designed two additional MAA MHC-I constructs to develop a multivalent (quadruple) DC vaccine for melanoma immunotherapy. We have characterized the newly designed constructs encoding for TRP1–455 and TYR360–368 as new MHC-I restricted MAA for this hβ2m-platform. We showed that the immunization with DC expressing the mix of TRP1/TYR-hβ2m-receptors resulted in stronger anti-tumor effects in the RET-tg melanoma mouse model as compared to TRP1/TYR long-peptide-mix vaccination with agonistic CD40 antibodies and the poly:IC adjuvant. Interestingly, the application of these adjuvants with mutated peptides was reported to induce a significant delay in the growth of MC-38 mouse colon adenocarcinoma. Furthermore, local intratumoral administration of adjuvants might be more effective, limiting systemic inflammation yet activating APCs locally. However, a direct comparison of different vaccination strategies is difficult, especially with regards to the adjuvant composition, the dose, and the application route (local versus systemic).

A better efficiency of our mRNA-based DC vaccination compared to long peptide vaccination could be due to the fact that peptides need protection from degradation in vivo to reach antigen presenting cells (APC) as recently demonstrated. Moreover, longer antigen presentation by DC in our system could influence the expansion of T cells and their differentiation to memory cells enhancing thereby the vaccination efficiency.

To address the question whether the quadruple antigen-mix vaccination could further enhance the therapeutic efficiency, we vaccinated mice from two melanoma GEMM (RET-tg and BRAF) with electroporated DC containing TRP-1-, TYR-, hgp100- and TRP-2-hβ2m-receptors. Indeed, we found a further increase in mouse survival associated with an enhanced accumulation of antigen-specific tumor-infiltrating CD8 T cells and a stronger induction of memory T cells in both GEMM. Since MAA were described to be overexpressed in melanoma patients, a high anti-tumor efficiency of the improved, multi-valent hβ2m-mRNA-based DC vaccine platform using shared MAA that induce potent tumor-infiltrating antigen-specific effector and memory CD8 T cells, without stimulating immunosuppressive cells like MDSC and Treg. The hβ2m-mRNA-based system is safe, modular and can be applied on multiple
tumor antigens in different cancers. In addition, when combined with approaches targeting the immunosuppressive tumor microenvironment (such as ultra-low dose Pac or anti-PD-1 antibodies) this strategy resulted in stronger anti-tumor effects associated with further CD8 T cell stimulation and reduction of immunosuppressive cells. Based on our preclinical data, we suggest that such combinatorial strategy could significantly increase the efficiency of existing immunotherapies of patients with melanoma and other tumors.

Materials and methods

Mice

C57 BL/6 mice expressing the human RET oncogene in melanocytes (RET-tg mouse model) were provided by I. Nakashima (Chubu University, Aichi, Japan). The C57BL/6 BRAFCA, TYR-CreER and PTENlox4-5 (BRAF) mice were kindly provided to R. Offringa by M. Bosenberg. C57BL/6 (H-2b) and B6.SJL (CD45.1/H-2b) were provided by the animal facility of German Cancer Research Center (DKFZ, Heidelberg, Germany). Mice were kept under pathogen-free conditions in the animal facility of DKFZ. Animal experiments were performed in accordance with government and institute guidelines and regulations.

Cell lines

B16-F10.9 is a mouse melanoma cell line (C57 BL/6 J background), which expresses relevant MAA. MO5-B16 is a mouse melanoma cell line transfected with ovalbumin (C57 BL/6 J background). 3LL-D122 Lewis lung carcinoma mouse cell line (C57BL/6) background) expressing no MAA was served as a negative control. All cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS, GibroBRL), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific). All cells were cultured at 37°C, 5%CO2.

Peptides and antibodies

The short MHC class I restricted peptides OVA257-264 (SIINFEKL), modified TRP-1455-463 (AAPDNLGYM), TYR360-368 (SSMHNALHI), hgp10025-33 (KVPRNQDWL), TRP-2180-188 (SVYDFFWVL), and the long (27/mer) peptides TRP-1447-473 (VTNTEMVFATPDNLGYA-YEVQWPQGEF) and TYR355-381 (ADPSQSMHALHFMTGNGSVMQSQRAS) were synthesized at DKFZ. The anti-mouse CD40 Ab 3/23 in mulg1 format was kindly provided by M. Glennie (Southampton, UK) to R. Offringa and D. Baumann in the context of the EU project IACT (Immunostimulatory Agonist Antibodies for Cancer Treatment). Anti-mouse directly conjugated monoclonal antibodies (mAbs) Gr1-PE-Cy7, CD11b-APC-Cy7, CD45.2-PerCP-Cy5.5, CD3-PerCP-Cy5.5, TCR-ζ chain (CD247)-Alexa Fluor® 647, CD4-PE-Cy7, CD8-APC-Cy7, CD25-APC-Cy7, CD69-APC, CD8-BV510, CD8-APC, CD4-BV421, TNF-α-APC, IFNγ-FITC, Ki67-PE were provided by BD Biosciences. Foxp3-FITC and Foxp3 fixation/permeabilization kit were from eBioscience. Anti-mouse PD-1-FITC, PD-L1-APC, CD39PE-Cy7, CD44-PE-Cy7, CD62 L-APC-Cy7, CD80-FITC, CD86-PE-Cy7, MHCII (IA/IE)-APC and β2-microglobulin-PE mAbs were provided by BioLegend. Streptavidin-PE-conjugated H-2-Kb mouse MHC-class I tetramers loaded with peptides derived from TRP-1455-463 (AAPDNLGYM), TYR360-368 (SSMHNALHI) were obtained from the NIH Tetramer Core Facility (Atlanta, USA).

Cloning of plasmids and expression vectors

pGEM-4Z 5′UT-eGFP-3′UT-A64 (pGEM-4Z) vector was kindly provided by Dr. Eli Gilboa (Miami, USA). This plasmid contains a 741-bp eGFP fragment from pepGFP-N1 (Clontech), flanked by the 5′ and 3′ UTRs of Xenopus laevis β-globin and 64 A-T bp. pGEM-4Z-hβ2mKb and pGEM-4Z-hβ2mTLR4 were generated previously by us and used as backbone vectors to exchange antigenic TYR360-368 and TRP1447-473 sequences by Restriction Free (RF) cloning method. Primers for RF-cloning were synthesized and PAGE purified (Sigma Aldrich). The sequences of the primers and the resulting vectors are summarized in the following. TRP1447-473-pGEM-4Z-hβ2mKb and -mTLR4 (forward primer 5′ TGTCCTCACTGACCGGCTTGG-TATGCTGCCGCCCGCTGACACCTGGGCTACATGGGAG GTGGCGATCGCCGGGAGTTGTGTT and reverse primer 3′ AACCACCTCGGATCGCCACCTCCATG-TAGCCCGAGGT GTCCGGGGCCGGCGACATACAGAGCCGGTC-AGTAGAC AC); TYR360-368-pGEM-4Z-hβ2mKb and -mTLR4 (forward primer 5′ TGTCCTCACTGACCGGCTTGG-TATGCTGCCGCCCGCTGACACCTGGGCTACATGGGAG GTGGCGATCGCCGGGAGTTGTGTT and reverse primer 3′ AACCACCTCGGATCGCCACCTCCATG-TAGCCCGAGGT GTCCGGGGCGACATACAGAGCCGGTC-AGTAGAC AC).

In vitro mRNA transcription

Template DNA cloned in the pGEM4Z-A64 vector was prepared using NucleoBond Xtra Maxi Plus DNA purification system (Macherey-Nagel) and linearized via the SpeI restriction enzyme site positioned at the 3′ end of the poly (A) tract of the vector. One μg of linear plasmid was used for in vitro mRNA transcription with T7mScript Standard mRNA Production System (CELLSCRIPT). The concentration and quality of the mRNA was assessed by spectrophotometry.

Generation of BMDC from murine bone marrow cells

Murine bone marrow-derived DC were generated as described with minor modifications. Briefly, bone marrow cells from femurs and tibiae of 4–5 weeks old C57 BL/6 female mice were cultured in BMDC growth medium, consisting of RPMI (Invitrogen) supplemented with 10% heat-inactivated FCS, 2 mmol/L glucose, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific) and 200 U/mL rmGM-CSF (Prospect). On day 8, non-adherent cells were harvested and cultured in fresh BMDC growth medium containing 100 U/mL recombinant...
mRNA electroporation of bone marrow-derived DC

2.5 × 10⁶ DC were washed twice and resuspended in OptiMEM medium containing 10–20 μg transcribed mRNA (5 μg mRNA/construct) and electroporated in a 2 mm cuvette using Gene Pulser Xcell™ Electroporation Systems (Bio-Rad) at 400 V, 0.9 ms, one pulse. Cells were resuspended in 5 mL BMDC growth medium with 100U/ml recombinant mGM-CSF and incubated for 6 h at 37°C, 5% CO₂ for β2m-mRNA-construct expression.

Therapy experiments

RET-tg mice that spontaneously develop skin melanoma were selected for the treatment at the age of 5–6 weeks when the mice displayed palpable skin tumors. BRAF mice were injected intradermally with 32 mM hydroxytamoxifen (4-HT). 21–28 days later, we initiated the therapy of animals, which developed highly pigmented, palpable melanoma skin lesions with the size of 5–10 mm². All melanoma-bearing mice were vaccinated with 0.5 × 10⁶ cells/in 200 μL cell suspension intraperitoneally (i.p) three times in weekly intervals using DC either electroporated with respective β2m-mRNA (5 μg/construct) or loaded with short peptides (30 μg/peptide). In some experiments, tumor-bearing mice were treated i.p. with ultra-low dose (1 mg/kg) Pac (Hexal). In other set of experiments, melanoma-bearing mice were injected i.p. four times every 3 days with 250 μg/mouse anti-PD-1 antibodies (clone 2A3) or isotype control antibodies (clone 2A3, both from BioXcell). Other tumor-bearing mice were treated subcutaneously 3 times in weekly intervals with mixture of 50 μg long peptide each (LP, TRP-1448–377), 30 μg anti-CD40 mIgG1 antibodies, and 100 μg poly:IC (InvivoGen) as adjuvants.

CTL in vitro killing assay

Ten days after the last vaccination mice were sacrificed and splenocytes were excised and sensitized with 50 μg/ml of respective peptide for 5 days. Afterwards lymphocytes were separated by Lympholyte-M gradient (Cedarlane) and seeded at concentrations of 5 × 10⁵–6 × 10⁵ cells/well. 5 × 10⁴ target cells (B16F10.9, B16MO5 and 3LL-D122 cell line expressing no MAA serving as a negative control) were labeled with ³⁵S methionine (Perkin Elmer) and co-cultured with sensitized lymphocytes in different target/effector cell ratios for 5 h at 37°C. Released ³⁵S-methionine in the cell culture supernatant was measured as counts per minute using Packard Matrix 96 direct β-counter and the percentage of specific killing was calculated as:

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\left( \frac{\text{Target}_{\text{measured}} - \text{Target}_{\text{spontaneous}}}{\text{Target}_{\text{total}} - \text{Target}_{\text{spontaneous}}} \right) \times 100
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CTL in vivo killing assay

Ten days following the last treatment, vaccinated mice were injected i.v. with 20 × 10⁶ splenocytes from B6.SJL (CD45.1⁺) mice labeled with CFSE (BioLegend) at low (0.3 μM) and high (3 μM) concentrations and loaded with respective peptides at 1:1 ratio. After 18 h, spleens of vaccinated mice were excised, stained with anti CD45.1 mAbs and analyzed by flow cytometry. The specific killing was calculated as:

\[
\left[ 1 - \left( \frac{\%\text{CFSE}_{\text{high}}(\text{day 1})}{\%\text{CFSE}_{\text{high}}(\text{day 0})} \right) \div \left( \frac{\%\text{CFSE}_{\text{low}}(\text{day 1})}{\%\text{CFSE}_{\text{low}}(\text{day 0})} \right) \right] \times 100
\]

Bio-Plex assay

Snap frozen skin tumor samples were mechanically disrupted and treated with lysis solution (Bio-Rad). Protein amount in lysates was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific). Inflammatory factors were measured by multiplex technology (Bio-Rad) according to the manufacturer’s instruction. Acquisition and data analysis was performed by bio-plex Manager™.

Preparation of single cell suspensions

Spleen, lymph nodes and tumor lesions were mechanically disintegrated by a plunger and filtered through a 40 μm cell strainer. After the depletion of the erythrocytes by ammonium chloride lysis, cells were washed with FACS buffer (PBS + 0.5% BSA + 0.1% Na-Azide) and resuspended in an appropriate buffer for further analysis.

Flow cytometry

After 10 min treatment with FcγRII/III block (eBioscience), cells were incubated for 30 min at 4°C with directly conjugated mAbs. To measure intracellular IFN-γ production, CD8 T cells were restimulated in vitro with 30 μg/mL specific peptide or 5 μg/mL irrelevant peptide (SIINFEKL). Stimulation with 50 ng/mL phorbol myristate acetate (PMA) and 750 ng/mL Ionomycin (Sigma Aldrich) were used as positive control. After 2 h of restimulation, 2 μM Brefeldin A and 3 μg/mL Monensin (both from eBioscience) were added. For fixation and permeabilization, the Foxp3 fixation/permeabilization kit was used followed by the treatment with FcγRII/III block (both from eBioscience) for 10 min. Then, cells were incubated with antibodies for flow cytometry for 30 min at 4°C. In some experiments, cells were stained for 2 h with streptavidin-PE-conjugated H-2-Kb mouse MHC-class I tetramers followed by anti-CD8-APC mAbs. To exclude dead cells, 7-AAD (BD Bioscience, San Jose, USA) was added. Acquisition was performed by FACSCanto II with FACS Diva 6.0 software (BD Bioscience). Flowjo software (Tree Star) was used to analyze at least 500,000 events.
Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data are presented as the mean ± SD. Statistical significance was assessed using the nonparametric Mann-Whitney test or Kruskal–Wallis according to data requirements. Bonferroni and Dunn’s posttests were followed according to their appropriate use. Survival curves were generated using the product-limit method of Kaplan and Meier, and comparisons were made using the log-rank test. P < 0.05 was considered as statistically significant.

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Disclosure of Potential Conflicts of Interests

The authors disclose no conflict of interest.

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