Inflammation and immunomodulation in uveal melanoma

Ly, L.V.

Chapter 9

Peptide vaccination cooperates with tumor antigen-specific antibody for the treatment of established melanoma

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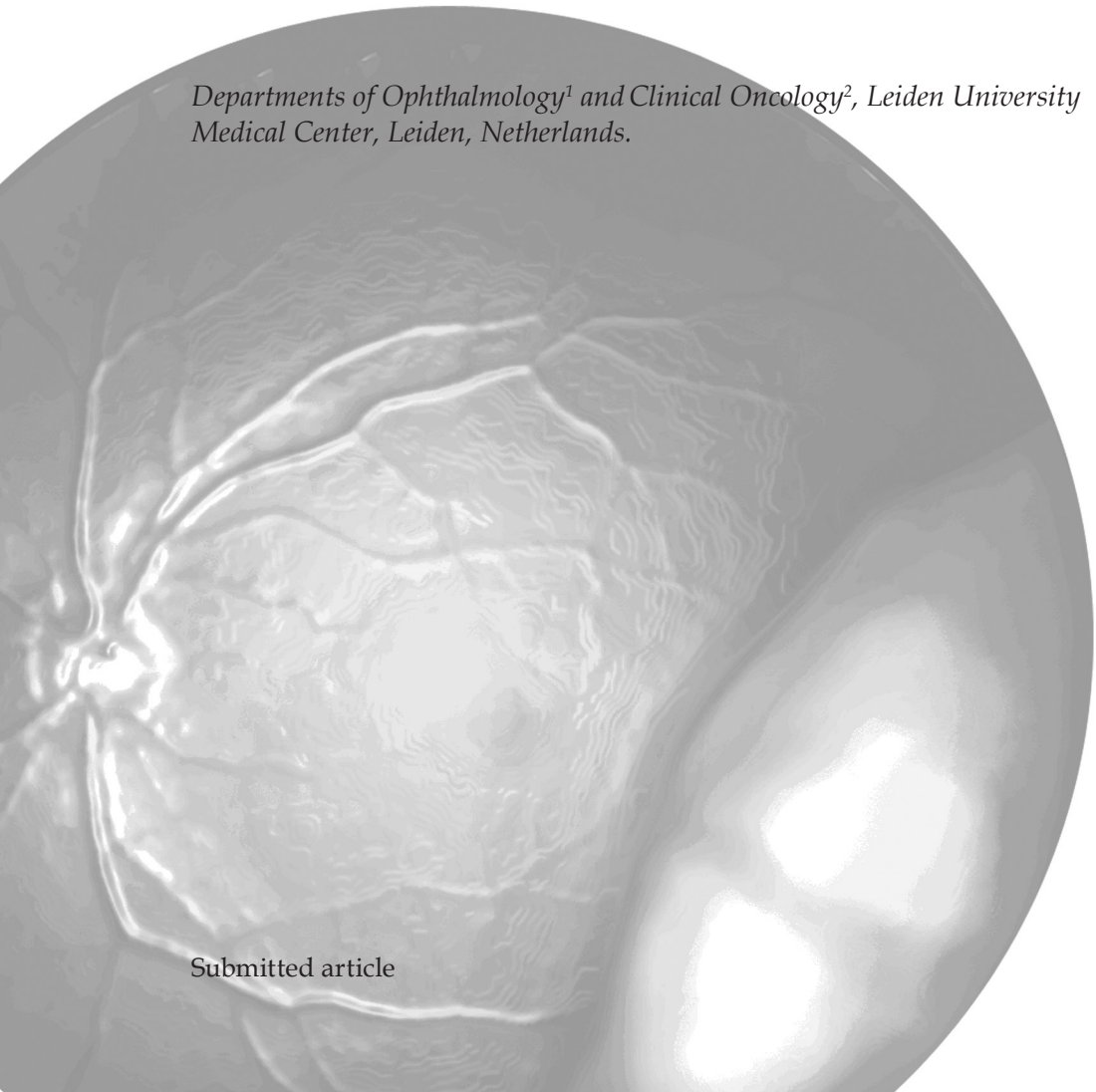

Submitted article

Abstract

Purpose: Monoclonal antibodies that bind tumor-associated surface antigens are therapeutically applied in a range of malignancies. T-cell based vaccination only recently met clinical success and the first cancer vaccine received FDA-approval this year. We tested combination treatment existing of a melanoma-specific antibody and 20-mer long peptide vaccines for the treatment of B16F10 skin melanoma.

Experimental Design: C57BL/6 mice were treated with monoclonal antibody TA99 directed against the TRP-1 (gp75) surface protein and peptide vaccines comprising the characterized TRP-$2_{181-188}$ and $gp100_{25-33}$ T cell epitopes. Prophylactic and therapeutic effects on tumor growth of separate and combination therapy were analyzed.

Results: Peptide vaccination induced high frequencies of TRP2/gp100-specific $CD8^+$ T cells that were capable to protect against a B16F10 tumor challenge, but failed to eradicate established skin melanomas. Addition of the TA99 monoclonal antibody resulted in strongly enhanced anti-tumor efficacy. The frequency of tumor-directed $CD8^+$ T cell responses of the combination group was comparable to that of the peptide vaccine alone group, suggesting that the complementary effect of the antibody did not act via boosting or broadening of T cell immunity.

Conclusions: These data support the concept of combination therapy in which passive transfer of monoclonal antibodies is supplemented with cancer peptide vaccines and indicate that optimal exploitation of the endogenous T cell repertoire directed against 'self' proteins can lead to eradication of established skin melanoma.

Introduction

Monoclonal antibodies have been successfully introduced in clinical practice as treatment for malignant diseases. Clinical efficacy varies between the antibodies, but some reached the stage of first-line treatment and outperform previously prescribed chemotherapeutics [1,2]. Antibodies are stable proteins with high binding capacity to three-dimensional conformations of target antigens. Among the FDA-approved therapeutic antibodies are those binding to tumor antigens present leukemias and lymphomas (CD20, CD22, CD33 and CD52), to signaling receptors on solid tumors as breast- and colorectal carcinomas (Her2/Neu and EGFR) and to (immuno)modulatory mediators in a variety of cancers (VEGF and CTL-A4) [1,2]. The mechanisms underlying the efficacy of antibody therapy are still poorly understood and include direct cytostatic effects, receptor blockade and immune-cell mediated. The fact that patients with the high affinity variant of the immunoglobulin receptor CD16 (FcγRIIIa) benefit significantly more from anti-CD20 antibody therapy than those that harbor the low affinity variant, convincingly demonstrates that immune components at least contribute to the working mechanism [3]. Fc receptors are largely expressed by myeloid immune cells of the innate arm and can mediate strong activation of these cells [4,5]. In addition to this direct involvement of the immune system, some data argue that antibody treatment can even lead to the induction of antigen-specific T cells via enhanced uptake of tumor antigens [3,6-9]

In contrast to monoclonal antibodies, therapeutic vaccination of cancer leading to tumor-directed T cell responses very recently reached FDA approval. The market introduction of an evidence-based anti-cancer vaccine is a cornerstone for immunotherapeutic treatment of cancer and most likely represent the forefront of novel effective vaccines [10]. Although hundreds of clinical trials with antigen-comprising vaccines failed to culminate in significant objective clinical responses in the past [11], we recently have demonstrated resolution of neoplastic lesions of the vulva using long peptide vaccines covering two Human Papillomavirus-derived proteins [12]. Pre-clinical data show that long peptide vaccines lead to preferred presentation by professional antigen-presenting cells like dendritic cells and prevent a detrimental vanishing T cell response sometimes seen with short peptides[13-15]. These results in virus-induced tumors prompted us to examine the efficacy of long peptide vaccines for the treatment of aggressive melanomas by targeting tumor differentiation antigens. Whereas HPV-induced neoplasias harbor viral proteins which are immunogenic antigens for the T lymphocyte population, the T cell pool specific for differentiation antigens is blunted by central tolerance [16-18]. Most melanoma differentiation antigens, including gp100, tyrosinase-related protein 2 (TRP-2) and TRP-1, are also expressed by normal melanocytes and in the thymus, leading to deletion of the high affinity T cells [16-18]. The residual T-cells in the natural repertoire are of moderate-to-low avidity and their cognate antigens are poor immunogens. One way to recruit a sufficient T cell response against such 'self' antigens is the replacement of specific amino acids in the peptide-epitopes to improve the binding-capacity and thereby immunogenicity of these peptides. Typically, amino acids are exchanged at positions that function as anchor residues for pockets in the groove of MHC class

I molecules [19-22], resulting in better and more stably binding peptides. In principle, these alterations do not influence the interface with T cell receptors, although recent studies revealed that the fine specificity of the recruited T cell repertoire might still be suboptimal for tumor-directed reactivity [23,24]. We recently described a novel approach for peptide improvement via changes at an unconventional site of the peptide, resulting in an alternative anchoring to class I MHC molecules [25]. This approach is potentially universal as the involved tyrosine residue (Y159) is present in most MHC class I alleles [25]. Our previous study showed that $CD8^+$ T cell responses to the D^b-presented $gp100_{25-33}$ epitope strongly enhanced by this approach.

Here we present data on the *in vivo* efficacy of altered peptide vaccines consisting of long immunogenic peptides comprising the melanoma differentiation antigens gp100 and TRP-2. Multiple rounds of immunization with this long peptide vaccine resulted in detectable frequencies of $CD8^+$ T cells against TRP-2 and very high frequencies (up to 40%) against gp100, able to kill peptide-loaded surrogate targets and the aggressively growing B16F10 melanoma *in vivo*. However, peptide vaccination failed to eradicate established B16F10 tumors. Importantly, addition of a monoclonal antibody (TA99) specific for the surface melanocyte protein TRP-1 (gp75) to the peptide vaccine resulted in a synergistic effect and was capable to control established melanomas. Administration of the antibody did not lead to strongly increased frequencies of tumor-specific T cells, but merely delayed initial tumor outgrowth and thereby created a time window for the generation of vaccine-induced T responses.

Material and methods

Mice

C57BL/6jico mice, 8 weeks old, were obtained from Charles River (France). TCR transgenic mice containing $gp100_{25-33}$/H-$2D^b$ specific receptors (designated as pmel) were a kind gift of Dr. N.P. Restifo (Bethesda) and were bred to express the congenic marker CD90.1. All animals were housed under Specific Pathogen Free conditions and cared for in accordance with the guidelines of the University Committee for the Humane Care of Laboratory animals (DEC) and NIH guidelines on laboratory animal welfare. Our research protocols were approved by the Committee for Animal Welfare, LUMC in Leiden.

Long peptide vaccination and TA99 antibody treatment

Mice were immunized by shaving part of the left flank and injecting s.c. 70 nmol of $gp100_{20-39}$ altered peptide (AVGALEG*P*RNQDWLGVPRQL) and 70 nmol TRP-$2_{175-193}$ peptide (QIANCSVYDFFVWLHYYSV) dissolved in Phosphate Buffered Saline (PBS). Immediately following peptide injection, 60 mg of Aldara™ cream (3M Heath care) containing 5% imiquimod was topically applied to the skin at the injection site. This immunization protocol was repeated for a second time after 7 days. Mice received two i.p. injections of recombinant human IL-2 ($6x10^5$

IU, Novartis) on the day of the second vaccination and on the consecutive day. Treatment with the TRP-1-specific monoclonal antibody TA99 (mouse IgG2a) was applied i.p. with 200 μg per injection. This antibody was purified from hybridoma HB-8704 obtained from Dr. A. Houghton (Memorial Sloan-Kettering Cancer Center, USA).

Intracellular cytokine staining

In order to determine the efficacy of our vaccination protocol, blood samples were collected from tail veins and analyzed for the frequency of IFNγ-producing T cells within the total population of $CD8^+$ T cells. Red blood cell lysis was performed for 10 minutes on ice and blood cells were then incubated overnight with 1 μg/ml wild type peptide gp100 (position 25-33: EGSRNQDWL) [26], TRP-2 peptide (position 180-188: SVYDFFVWL) [27] or TRP-1-peptide (position 455-463: TAPDNLGYA) [28] at 37°C in a 5% CO_2 atmosphere. Golgiplug (1 μg/ml) was added for preserving the cytokine intracellular. Next morning, cells were fixed, washed and stained with mAbs CD8α-PercP and IFN-γ-PE (BD Pharmingen), and analyzed on a Calibur flow cytometer (BD Biosciences).

In vivo cytotoxicity measurement

To analyze the cytotoxic efficacy of gp100- and TPR-2-reactive CTL *in vivo*, naïve C57BL/6 mice were vaccinated with long peptides and imiquimod as described above and injected i.v. with a mixture of peptide-pulsed CarboxyFluorescein diacetate Succinimidyl Ester (CFSE)-labeled splenocytes, as described before [25]. Briefly, splenic cells from CD90.1 congenic C57BL/6 mice were passed through nylon wool. Half of these cells was pulsed with wild type mouse gp100-peptide EGSRNQDWL or TRP-2 peptide SVYDFFVWL and labeled with 5 μM CFSE, while the other half was pulsed with control peptide from Ad5 E1a or OVA, and labeled with 0.5 μM CFSE. Target cell populations were washed, mixed and i.v. injected ($5x10^7$) into immunized recipient mice. The spleens of immunized recipient mice were harvested after two days, stained with CD3, CD8α and CD90.1-specific antibodies and analyzed with a Calibur flow cytometer (BD Biosciences). Percentage killing was calculated as the ratio between the number of gp100-pulsed targets and control peptide-pulsed targets.

Isolation of lymph nodes and TCR-transgenic T cells

After application of imiquimod, lymph nodes were removed and dissociated using 250 U/ml collagenase type 4 and 50 μg/ml DNase (Sigma-Aldrich) for 45 minutes at 37°C. Cell suspensions were pushed through a cell strainer (Gibco) to obtain single cells, washed twice and stained with antibodies to CD11c, CD86, CD40 and MHC II (Becton-Dickinson). Fc receptors were blocked by prior incubation with 2.4G2 antibody. Alternatively, single cells were plated in 24-well plates and incubated with CFSE-labeled TCR-transgenic T cells for four days. Percentage divided T cells was calculated by the ratio of undivided $CFSE^{high}$ cells to cells with lower CFSE intensity.

TCR-transgenic T cells specific for the $gp100_{25-33}$ epitope (pmel) [25] were obtained from spleen and lymph nodes of naïve CD90.1-positive pmel mice and enriched for T lymphocytes by nylon wool. These T cells were used for in vitro cultures or injected ($3x10^6$ specific T cells) into the tail vein of recipient mice. Percentage pmel T cells out of the total $CD8^+$ T cell pool was determined by flow cytometry using antibodies against CD90.1 and CD8 (Becton-Dickinson), as described in our previous publication[29].

Melanoma treatment

The B16F10 melanoma cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, CA) supplemented with 8% fetal calf serum (FCS, GIBCO), glutamine and 2% of penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were inoculated when growth status was 70% confluent. Subcutaneous melanomas were generated by s.c. injection of $5x10^4$ B16F10 tumor cells in the flank. Tumor size was measured twice a weak with a caliper and mice were sacrificed when tumors exceeded 1000 mm^3 in size.

Statistical analysis

Statistical analyses were performed using Graph Pad software. The used test for each data set is indicated in the figure legends.

Results

TLR-7L imiquimod drives local accumulation and maturation of dendritic cells.

Toll like receptors ligands are immunostimulatory compounds with activating ability for cells of the innate immune system. Previous data showed that the TLR-7L imiquimod outperformed other TLR ligands, like CpG oligo DNA, when applied with long peptides in saline solution [29,30,31]. Peptide formulations in saline were chosen instead of mineral oil, because the slow release of peptides from oil depots might lead to peptide presentation *in vivo* in the absence of innate immune activation [13,32,33]. Moreover, in this way we were able to analyze the effects of imiquimod without interfering stimulation by mineral oil suspensions. Imiquimod was applied in a crème at the skin of shaved flanks of mice and two days after application, the local draining lymph node and the contra lateral non-draining lymph node were excised, dissociated and stained with specific antibodies (Figure 1a). The frequency of dendritic cells (DC) selectively increased by imiquimod in the draining lymph nodes, as determined with the CD11c marker (Figure 1a). Frequencies of other leukocytes (B-cells, natural killer cells and T cells) did not change (data not shown). The expression of CD40, CD86 and MHC class II on DC were assessed to determine the maturation status of these antigen presenting cells. All three markers were clearly increased on the DC of draining lymph nodes, indicating a strong maturation process induced by imiquimod application. We also examined drainage, processing and presentation of 20-mer

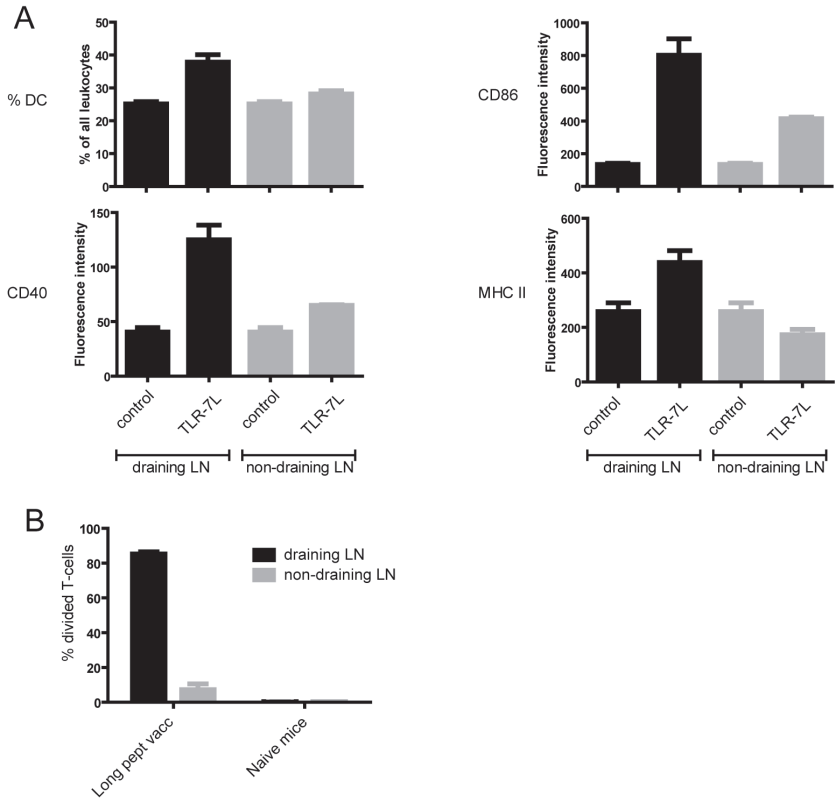

Figure 1. TLR-7L imiquimod drives local accumulation and maturation of dendritic cells.
(A) Imiquimod was topically applied as a crème on shaved flanks of mice. After two days, draining and contra lateral non-draining lymph nodes were excised, dissociated and analyzed by flow cytometry. Lymph node cells were stained with CD11c to determine dendritic cell frequencies. Activation markers CD40, CD86, and MHC II were analyzed on $CD11c^+$ cells. Data represent means and standard deviations of three mice from one out of two comparable experiments.
(B) Subcutaneously injection of 20-mer long gp100-peptide in saline shows selective drainage, processing and presentation in the local draining lymph node. One day after peptide injection and topical application of imiquimod, lymph nodes were excised, dissociated and used as antigen presenting cells for TCR-transgenic T cells. T cells were loaded with protein dye CFSE to determine percentage of divided cells in a four day culture. Means and standard deviation of three mice per group are depicted. Two independent other experiments gave comparable results.

long peptides after injection in saline (Figure 1b). A gp100-based peptide was subcutaneously injected and overlaid with imiquimod-containing crème at the injection site. *In vivo* presentation of the processed 9-mer epitope in the context of the MHC class I molecule H-$2D^b$ was determined using peptide-specific T cell receptor (TCR) transgenic T cells. Draining and non-draining lymph nodes were removed from the same animals and were used as antigen presenting cells to stimulate the T cells. Presentation of the minimal peptide-epitope was largely restricted to the local lymph node, indicating that peptide and adjuvant co-localize in the draining lymph node to induced T-cell responses.

Altered TRP-2 peptide fails to induce CD8$^+$ T cell responses against the natural epitope.

Our recent data on the gp100$_{25\text{-}33}$ peptide-epitope demonstrated that replacement of amino acids at positions 2 and 3 with respectively a glycine and a proline residue results in a highly immunogenic antigen with strong MHC class I binding capacity [25]. The same amino acid exchange in the mouse H-2K^b-binding peptide TRP-2$_{181\text{-}188}$ (VYDFFVWL) also resulted in strongly enhanced MHC binding [25]. We now tested if this altered TRP-2 peptide ('VGP') was able to recruit CD8$^+$ T cells from the endogenous repertoire that cross-react to the natural peptide. Mice were immunized with 19-mer long peptides comprising the natural TRP-2 sequence or the glycine/proline-replaced altered peptide together with topical application of the TLR-7L imiquimod. After the second immunization, blood samples were taken and analyzed for the presence of peptide-specific CD8$^+$ T cells (Figure 2a). Approximately 3% of the CD8$^+$ T cell pool responded to the short wild type peptide when mice were immunized with the long natural peptide. These T cells did, however, not respond to the short altered peptide. Likewise, activated T cells from mice that were immunized with the long altered peptide failed to efficiently recognize the wild type TRP-2 sequence, whereas they displayed strong reactivity to the altered variant (Figure 2a). To determine the specificity and cytolytic activity of the vaccine-induced T cells *in vivo*, we injected three different target cell populations loaded with different peptides into immunized mice (Figure 2b). These results underscored the *in vitro* data and showed that T cells raised against the altered peptide do not kill target cells loaded with the natural TRP-2 peptide-epitope (Figure 2c). We concluded that, although the binding capacity as well as the immunogenicity were increased, the altered TRP-2 peptide was not suitable for inducing tumor-specific T cells. The introduction of the glycine/proline residues at position 2 and 3 apparently induced a critical conformational change of the peptide/MHC complex that constrained the recruited T cell receptor repertoire to that particular fold. Further immunizations were based on long synthetic peptides comprising the natural TRP-2 sequence.

Peptide vaccine-induced T cells kill surrogate target cells but not solid B16F10 tumors.

The two melanoma antigens TRP-2 and gp100 were exploited in order to study the anti-tumor efficacy of 20-mer long peptide vaccination. As gp100 peptide, we used the altered variant containing a proline instead of the serine at position 3 of the minimal epitope, flanked with the natural mouse sequence. The TRP-2 peptide contained the natural sequence with natural mouse flanking amino acids. Immunization with these two long peptides and imiquimod resulted in low frequencies of peptide-specific T cells in the blood (Figure 3a). After the third immunization, strikingly high frequencies of gp100-specific CD8$^+$ T cells were found, whereas TRP-2-specific cells remained at frequencies around 2%. Importantly, the gp100-induced T cells were raised against the long altered peptide, but were tested against the natural gp100 short peptide. *In vivo* function of vaccination-induced T cells was studied by systemic injection of peptide loaded splenocytes as surrogate targets. An effective killing of these target cells

was observed for both antigens, demonstrating the capacity of the T cells to specifically recognize and kill antigen-positive targets (Figure 3b). Comparable killing percentages were observed for both antigens, indicating the sensitivity of this assay, which requires only low T cell frequencies. Vaccinated mice were then challenged with an inoculum of the aggressive melanoma B16F10 cell line, which results in formation and lethal outgrowth of solid tumors within three to four weeks (Figure 3c). Mice were vaccinated twice prior to tumor injection and after tumor establishment also two times with biweekly intervals. These prophylactic vaccinations had a clear impact on tumor growth and prevented the formation of tumors in half of the mice (Figure 3c). However, a therapeutic scheme of vaccination in which peptide vaccines started four days after tumor injection failed to show any delay in tumor grow (Figure 3d). These data demonstrate that long peptide vaccines were able to induce antigen-specific $CD8^+$ T cells with killing capacity, but that these immune effector cells failed to control established B16F10 tumors.

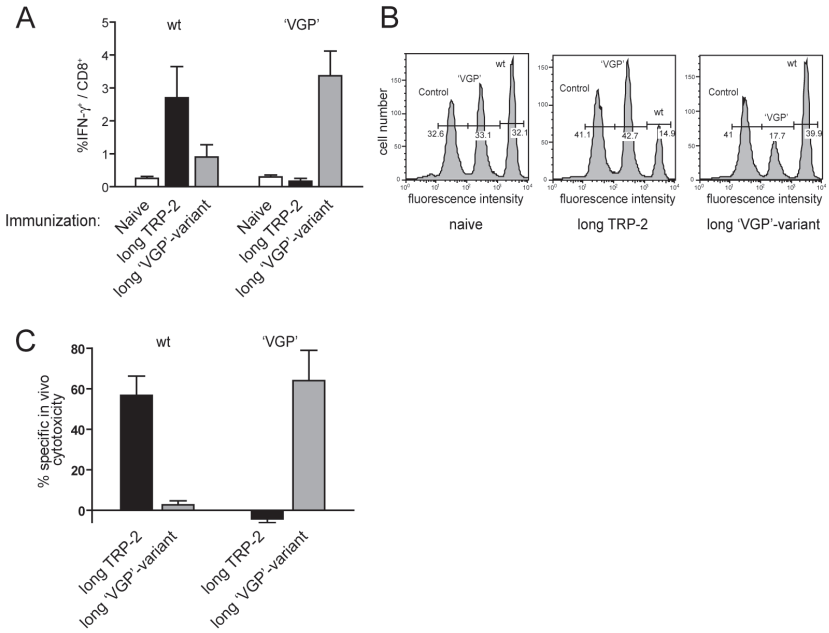

Figure 2. Amino acid replacement in the TRP-2 peptide-epitope does not lead to enhanced T cell priming.

Positions 2 and 3 in the TRP-$2_{181\text{-}188}$ (VYDFFVWL) peptide were replaced with a glycine and proline residue, respectively (VGPFFVWL), resulting in strongly enhanced binding to the MHC class I molecule K^b [25]. **(A)** Mice were twice immunized with 19-mer long peptides with the wild type or 'VGP' sequence. Five days after the last vaccination, frequencies of peptide-specific $CD8^+$ T cells was determined from blood cells by overnight incubation with short wild type peptide ('wt') or short altered peptide ('VGP') and intracellular staining of IFNg.

(B-C) Immunized mice were injected i.v. with CFSE-labeled and peptide-loaded splenocytes as surrogate target cells. Three populations of splenocytes were mixed: loaded with short control OVA peptide (low CFSE), 'VGP' altered peptide (intermediate CFSE) and wild type peptide (high CFSE). Two days after challenge, spleens of recipient mice were harvested and analyzed by flow cytometry for percentage killing. Summary of four mice per group is depicted in **(C)**. Means and standard deviations of one out of two comparable experiments are shown.

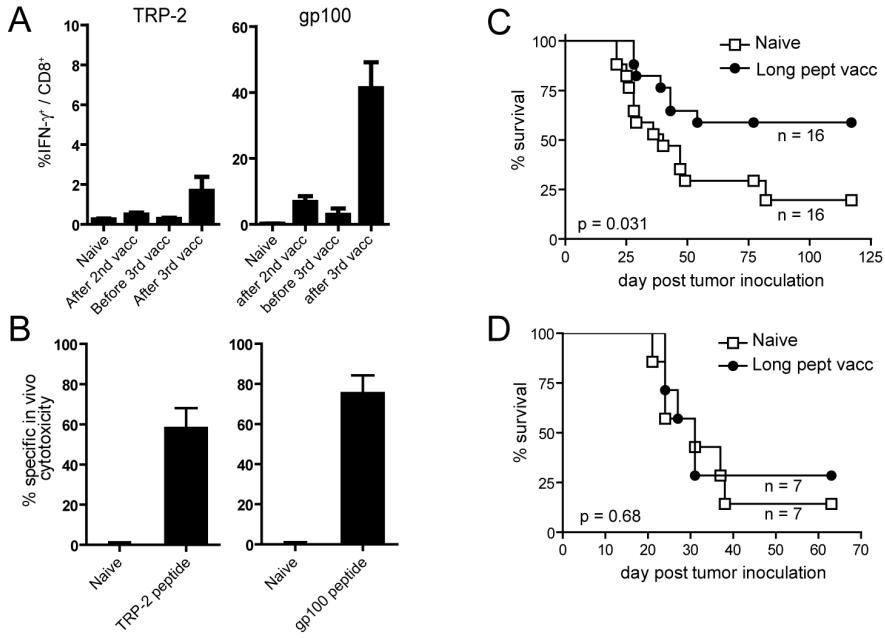

Figure 3. Vaccine-induced T cells protect outgrowth of B16F10 melanomas, but fail to eradicate established melanomas.
(A) Mice were three times vaccinated with 20-mer long peptides comprising the TRP-$2_{181-188}$ epitope and the $gp100_{25-33}$ epitope. Frequencies of peptide-specific $CD8^+$ T cells were determined from the blood after brief stimulation overnight with short, wild type peptides and intracellular IFNg staining. Blood samples were taken five days after the second vaccination, seven days before the third vaccination and five days after the third vaccination. **(B)** The killing capacity *in vivo* of immunized mice was tested with CFSE-labeled and peptide-loaded splenocytes, as in figure 2b. Wild type peptide sequences were used as target cells. **(C)** Vaccinated mice were challenged with a lethal dose of B16F10 tumor cells injected subcutaneously. Long TRP-2 and gp100 peptides were combined in the vaccine and mice received four vaccinations: two before tumor inoculation (day 0) and two after. Tumor growth was measured twice a week and mice were sacrificed when tumors reached 1000 mm^3. Data represent compiled data from two independent experiments. P-value is from statistical log rank test. **(D)** Therapeutic vaccination experiment in which a lethal dose of B16F10 tumor cells were first injected (day 0) and vaccinations started at day 4. One out of four experiments with similar outcome is shown. P-value is from statistical log rank test of Kaplan-Meier curves.

Addition of TA99 antibody to long peptide vaccines leads to effective melanoma treatment.

The vaccine-induced $CD8^+$ T cells with reactivity to TRP-2 and gp100 melanoma antigens were detected five days after vaccination onward and peaked at five days after the second immunization. This means that optimal effector T cell frequencies were found 16 days after tumor inoculation. We reasoned that this time window was too large to eradicate enough progressively growing B16F10 cells. Moreover, established B16F10 melanomas possess an immunosuppressive microenvironment, hampering local immune effector functions. The monoclonal antibody TA99 recognizes the TRP-1 (gp75) surface protein of melanoma cells and has been shown to mediate some anti-tumor potency [34-37]. TA99 was systemically applied at day 5 and 7 with the aim to slow down tumor outgrowth and create

a time-window to allow T cell priming. Indeed, established B16F10 melanomas were successfully eradicated with a combination treatment of long peptide vaccination and TA99 antibody (Figure 4a). No signs of immune pathology, like depigmentation, were observed, although the 'self'-reactive T cells and antibodies spread systemically. The application of the antibody therapy was tested at different time-points. Starting immediately at the day of tumor inoculation, the antibody alone completely prevented tumor formation (data not shown). When TA99 was injected at day 7, a slight delay of tumor growth was observed for the combination therapy, although the survival curves were not statistically different (Figure 4b). Starting at day 11 with antibody injections and at day 7 with peptide vaccination hardly influenced tumor growth (Figure 4c), even though multiple antibody treatments were provided. These data indicate that larger established tumors are resistant for these immune mediated treatments. We concluded that therapeutic vaccination with long peptides needs to be combined with a melanoma-directed monoclonal antibody in order to eradicate aggressively growing melanoma.

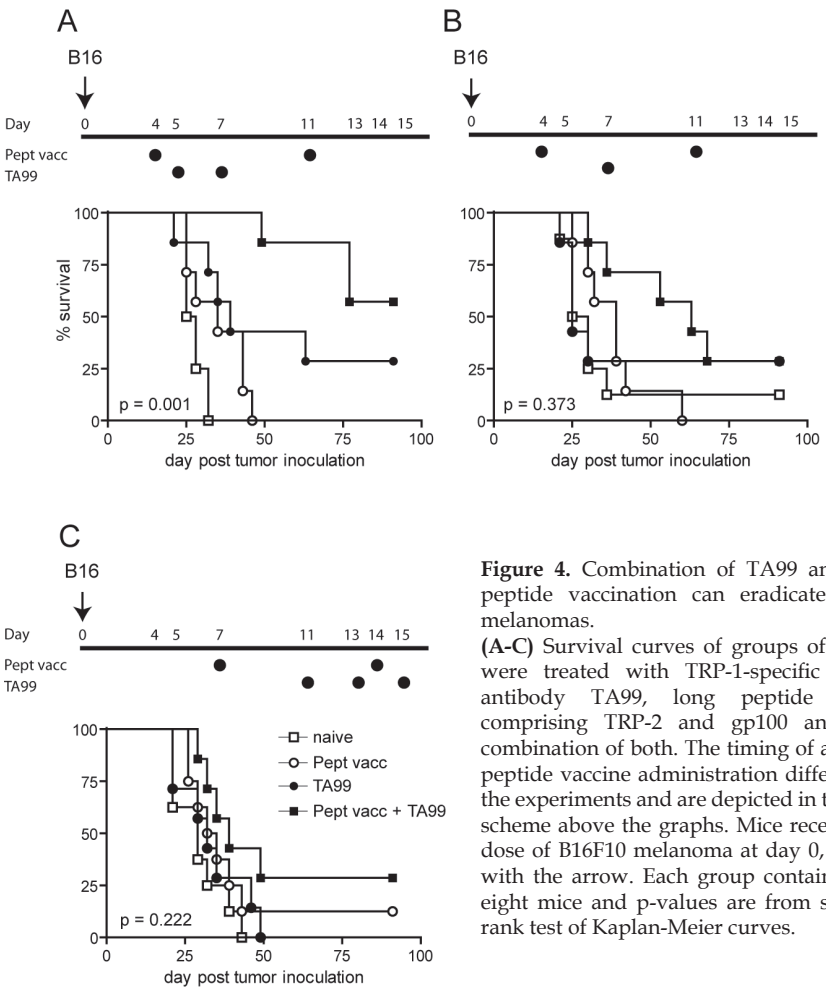

Figure 4. Combination of TA99 antibody with peptide vaccination can eradicate established melanomas.
(A-C) Survival curves of groups of mice which were treated with TRP-1-specific monoclonal antibody TA99, long peptide vaccination comprising TRP-2 and gp100 antigens or a combination of both. The timing of antibody and peptide vaccine administration differed between the experiments and are depicted in the treatment scheme above the graphs. Mice received a lethal dose of B16F10 melanoma at day 0, as indicated with the arrow. Each group contained seven to eight mice and p-values are from statistical log rank test of Kaplan-Meier curves.

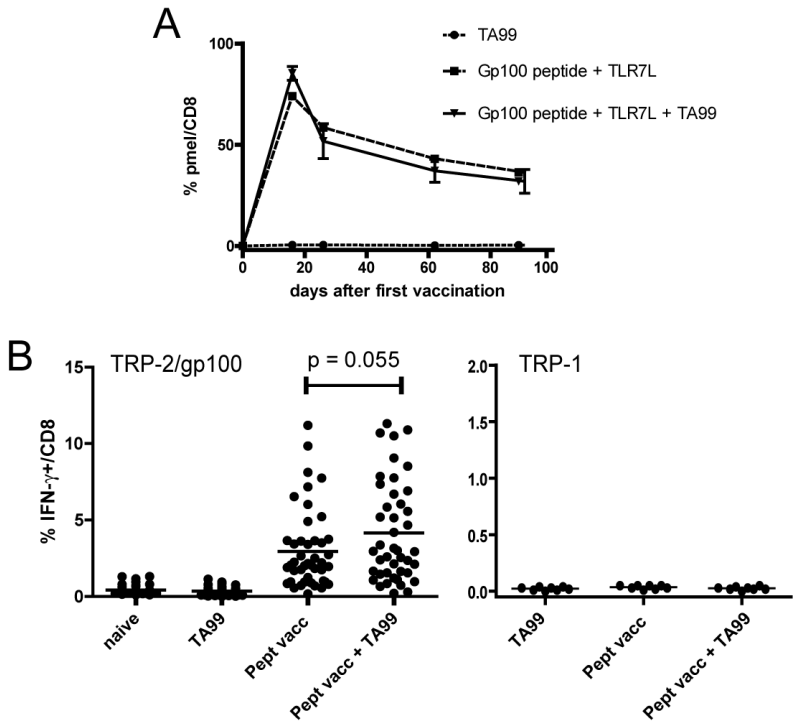

Figure 5. Mechanism of successful treatment by TA99 antibody and long peptide.
(A) The effect of TA99 antibody administration on the frequency of gp100-specific T cells was tested. Mice were injected with naïve TCR-transgenic cells from the pmel strain specific for the $gp100_{25-33}$ epitope. Mice were treated with TA99 antibody, long peptide vaccine or both and frequency of transferred pmel T cells was monitored over time from blood samples. Peptide vaccine was injected at days 0 and 7. TA99 antibody (100 ug) was provided i.p. every third day starting at day 7 for six times. **(B)** Addition of TA99 antibody slightly increased the frequency of endogenous TRP-2- and gp100-specific $CD8^+$ T cells, but not TRP-1-specific cells. Blood samples of mice that were challenged with B16F10 tumors and treated with long peptide vaccines and TA99 antibody were taken five days after the second vaccination, briefly stimulated *in vitro* with both $TRP\text{-}2_{180\text{-}188}$ and $gp100_{25\text{-}33}$ peptides (left panel) or $TRP\text{-}1_{455\text{-}463}$ peptide (right panel) and stained for IFNg. Data represent 36 animals per group (left panel) from four different experiments and 8 animals per group (right panel) from an independent experiment. Lines indicate the means. Difference between indicated groups was tested with Student T-test (p=0.055).

Mechanisms of action underlying the effective combination therapy.

We were interested in the mode of action of the combination therapy and if these two effector arms functionally depended on each other. Of note, the targeted TRP-1 antigen of this antibody is unrelated to the antigens comprised in the long peptide vaccine (TRP-2 and gp100). First, we analyzed if the presence of TA99 antibody led to higher frequencies of anti-tumor T cells. This mouse antibody is of IgG2a isotype and might liberate tumor antigens from the melanoma via complement activation or Fcγ receptors expressed by innate immune cells. TA99 was first injected in tumor-free mice that were treated with long peptide vaccination. T cell receptor transgenic T cells specific for the gp100 peptide-

epitope were supplied in order to facilitate the analysis of T cell expansion and activation status. No difference was seen in gp100-specific T cells over a period of 90 days when TA99 was present (Figure 5a). Apparently, addition of TA99 does not enhance the activation of gp100-specific T-cells.
Secondly, we studied the influence of TA99 on the priming of endogenous T cell responses to TRP-2 and gp100. Blood samples from tumor-challenged mice of experiments described in figure 4 were analyzed for frequencies of peptide-specific $CD8^+$ T cells. At the peak of the T cell response, five days after the second vaccination, the collective frequencies of TRP-2 and gp100-specific cells was modestly increased in the group with antibody treatment. However, the difference was borderline significant (Figure 5b, $p = 0.055$) and we consider it unlikely that this small difference could be responsible for the major improvement in tumor control as observed in Figure 4.
Thirdly, addition of the TA99 antibody might lead to T cell activation against other melanoma-derived antigens, especially the TRP-1 surface antigen. We analyzed this by examining the occurrence of T cells specific for the immunodominant epitope TRP-$1_{455\text{-}463}$ in the blood of treated mice [28]. No TRP-1 peptide specific T cells could be detected, suggesting that TA99 administration did not lead to broadening of the T cell response against B16F10 (Figure 5b). We concluded that TA99 antibody did not cause convincing enhancement or broadening of T cell immunity compared to long peptide vaccination alone. Therefore we hypothesize that the antibody has an independent role in the initial clearance of tumor cells via modulating FcR-expressing immune cells in the tumor micro-environment and that this results in a critical delayed tumor outgrowth necessary for the T cell response to cause action.

Discussion

Vaccination with long synthetic peptides results in robust immune responses, as demonstrated in several mouse models and clinical studies in Human Papillomavirus-induced malignancies [32]. The results in our current study is the first to show induction of high $CD8^+$ T cell frequencies directed to tumor associated antigens that are 'self' proteins. TRP-2 and gp100 are melanoma differentiation antigens and also expressed in normal melanocytes. The 20-mer long altered gp100 peptide used here harbors one amino acid change at position 3 of the minimal CTL epitope, from serine to proline, leading to dramatically increased binding capacity to the MHC presenting molecule and, importantly, preserving the overall structure so that vaccine-induced T cell receptors do cross-react to the natural epitope [25]. Our analysis of crystal structures confirmed that both peptide/MHC surfaces were nearly the same [25]. Strikingly, three vaccinations with this long altered peptide together with imiquimod adjuvant induced up to 50% gp100-specific $CD8^+$ T cells from the endogenous T cell repertoire, capable of killing peptide-loaded surrogate targets *in vivo* (see fig 3). Since we applied a weekly vaccination schedule, these strikingly high frequencies were only reached at day 19 when most tumors were already in progressive growth rate. Although these

CD8+ T cells are in principle self-reactive, no clear signs of depigmentation were detectable during the time of the experiments (up to three months). Similar amino acid exchange in the second epitope TRP-2 did not lead to cross-reactive T cells (see fig 2), most likely due to unaffordable conformational changes in the TCR contact residues of the peptide/MHC complex. In contrast to our previous experience on long peptides that comprise CD8+ T cell epitopes [13-15], the combined gp100 and TRP-2 responses failed to control outgrowth of established subcutaneous tumors. Two factors might explain this difference. First, the tumor antigens in our model constitute weak 'self' antigens, whereas our previous models included 'foreign' antigens like OVA and virus-derived antigens [13-15]. Second, the B16 tumor is a spontaneously arisen and very aggressive melanoma that efficiently exploits escape mechanisms, e.g. low MHC class I surface expression and creating an immunosuppressive microenvironment.

Successful immune interventions to eradicate pre-established B16 melanoma reported in literature include adoptive cell transfer, using fresh exogenous CD8+ T cells that were administered in lymphopenic hosts or at least in combination with strong immunomodulation (high dose IL-2, IFN-α, anti-CTL-A4 or depleting regulatory T cells)[7,25,38-41]. Indeed, we also published on the effective eradication of day 9 B16 tumors using transfer of TCR transgenic T cells which were *in vivo* activated by 20-mer long peptide vaccination [29]. The novelty of the current study lies in the fact that the endogenous T cell repertoire is exploited. The administration of the anti-TRP-1 monoclonal antibody TA99 was essential to reach successful tumor eradication; peptide vaccination alone was not sufficient. This mouse antibody is of IgG2a subtype and efficiently binds complement as well as FcγR on innate immune cells [34,36], quite similar to the FDA-approved human antibodies that target tumor surface antigens, e.g. CD20, CD33 and CD52. TA99 has been shown to prevent tumor formation in prophylactic setting [34,35,37], but its effect on established subcutaneous B16 tumors is unexplored to date.

One of the most intriguing questions concerns the mechanism of action of the combination therapy existing of peptide vaccination and monoclonal antibody. Some reports demonstrated enhancement of the tumor-specific T cell response after administration of monoclonal antibodies against tumor surface antigens [6,7,9]. Antibody binding leads to activation of the complement system and to cross-linking of FcγR on immune cells. This opsonisation of tumor debris and the formation of immune complexes strongly enhance the MHC-restricted presentation of tumor antigens [1-3] and, consequently, can activate antigen-specific T cells. By this means, antibodies mediate the recruitment of the adaptive immune arm, in addition to their direct cytolytic effect. In our study, however, we did not observe an enhanced nor broadened T cell response against melanoma antigens, most likely because our peptide vaccine already induced robust responses against gp100 and TRP-2. We hypothesize that administration of TA99 in our tumor model is responsible for initial tumor control by cytolytic effect, through complement membrane attack, ADCC and/or fagocytosis, and thereby creates a time window for vaccine-induced CD8+ T cell immunity to cope with the residual tumor. This idea is supported by the finding that late onset of antibody administration fails to control tumor outgrowth. Alternatively, the antibody might impact and influence the infiltrating myeloid cells of the tumor

microenvironment. Preliminary data on gene array analysis indeed suggest that the tumor associated macrophages are affected by the administration of TA99. We would like to support the concept of combination therapy, bridging the fields of T cell immunology and monoclonal antibodies, as some have previously advocated [2,41]. The concerted action of these two components is expected to bear superior anti-cancer efficacy and can be applied for human melanoma due to previously developed and clinically tested antibodies [42,43].

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