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J Immunol published online 30 November 2012
http://www.jimmunol.org/content/early/2012/11/30/jimmunol.1200135

Supplementary Material http://www.jimmunol.org/content/suppl/2012/11/30/jimmunol.1200135.DC1

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Effective Cooperation of Monoclonal Antibody and Peptide Vaccine for the Treatment of Mouse Melanoma

Long V. Ly,* Marjolein Sluijter,† Sjoerd H. van der Burg,† Martine J. Jager,* and Thorbald van Hall‡

M onoclonal antibodies have been introduced successfully in clinical practice as treatment for malignant diseases. Clinical efficacy varies among the Abs, but some reached the stage of first-line treatment and outperform previously prescribed chemotherapeutics (1, 2). Abs are stable proteins with high binding capacity to three-dimensional conformations of target Ags. Among the U.S. Food and Drug Administration (FDA)–approved therapeutic Abs are those binding to tumor Ags present on leukemias and lymphomas (CD20, CD22, CD33, and CD52), to signaling growth receptors on solid tumors such as breast and colorectal carcinomas (Her2/Neu and EGFR), and to (immuno) modulatory mediators in a variety of cancers (VEGF and CTLA-4) (1, 2). The mechanisms underlying the efficacy of Ab therapy are still poorly understood and include direct cytostatic effects, receptor blockade, and immune cell recruitment. The fact that patients with the high-affinity variant of the IgR CD16 (FcγRIIIa) benefit significantly more from anti-CD20 Ab therapy than do those who harbor the low-affinity variant convincingly demonstrates that immune components contribute to the mechanism (3). FcRs are primarily expressed by myeloid immune cells of the innate arm and can mediate strong activation of these cells (4). In addition to this direct involvement of the immune system, some data argue that Ab treatment can even lead to the induction of Ag-specific T cells via enhanced uptake of tumor Ags (3, 5–9).

In addition to mAbs, therapeutic vaccination as a treatment modality for cancer was recently approved by the FDA. The introduction of the first evidence-based anticancer vaccine to the market represents a cornerstone for immunotherapeutic treatment of cancer and is at the forefront of novel effective vaccines (10, 11). Although most clinical trials with peptide vaccines failed to culminate in significant objective immune and clinical responses (12), we recently demonstrated durable resolution of neoplastic human papilloma virus (HPV) lesions of the vulva using long-peptide vaccines (13). Preclinical data showed that long-peptide vaccines lead to preferred presentation by professional APCs, such as dendritic cells, and prevent the detrimental vanishing T cell response that is sometimes seen with short peptides (14–17). 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 Ags, such as gp100, tyrosinase-related protein (TRP)-2, and TRP-1. Although HPV-induced neoplasia harbor viral proteins that are immunogenic Ags for the T lymphocyte population, the T cell pool specific for differentiation Ags is blunted by central tolerance (18–20). Most melanoma differentiation Ags are also expressed by normal melanocytes and in the thymus, leading to deletion of the high-affinity T cells (18–20). The residual T cells in the natural repertoire are of low to moderate avidity, and their cognate Ags are poor immunogens.

In this article, we present data on the in vivo efficacy of a combination immunotherapy, consisting of a long-peptide vaccine and a mAb. We found that vaccination with the 20-mer synthetic long peptide containing the Dβ-presented epitope gp10025–33 elicited tumor-reactive CD8 T cells but that an altered peptide of the same length was much more immunogenic (21). Multiple rounds of immunization with this altered long-peptide vaccine resulted in recruitment of gp100-specific CD8 T cells from the endogenous repertoire at frequencies of up to 40%. However, this vaccination protocol required ≈3 wk and, therefore, failed to eradicate estab-

*Department of Ophthalmology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands; and †Department of Clinical Oncology, Leiden University Medical Center, 2333 ZA Leiden, The Netherlands

Received for publication January 13, 2012. Accepted for publication November 1, 2012.

This work was supported by The Netherlands Organization for Scientific Research (Mozaiek Grant 017.003.059 to L.V.L.) and foundations Stichting Blinden-Penning, Gratama, and Leids Universiteits Fonds.

Address correspondence and reprint requests to Dr. T. van Hall, Department of Clinical Oncology, K1-P, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. E-mail address: T.van_Hall@lumc.nl

The online version of this article contains supplemental material.

Abbreviations used in this article: FDA, U.S. Food and Drug Administration; HPV, human papillomavirus; LUMC, Leiden University Medical Center; TRP, tyrosinase-related protein.
lished and rapidly growing B16F10 tumors. Addition of a mAb (TA99) specific for the surface melanocyte protein TRP-1 (gp75) to this peptide vaccine led to effective control of these melanomas. Our data indicate that tissue-resident FcγR+ immune cells control initial tumor growth via activation by the mAb and, thereby, create a window of time in which vaccine-induced T cell responses can be generated.

Materials and Methods

Mice

C57BL/6jisco mice. 8 wk old, were obtained from Charles River (Lille, France). TCR-transgenic mice containing gp10025–33/H-2d-specific receptors (designated as pmel) were a kind gift of Dr. N.P. Restifo (National Cancer Institute, Bethesda, MD) and were bred to express the congenic marker CD90.1. C3+ mice were provided by Dr. M. Daha (Leiden University Medical Center [LUMC]), and Fcγ chain-deficient mice were provided by Dr. S. Verbeek (LUMC). 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 (Dier Experimenten Commissie) and National Institutes of Health guidelines on laboratory animal welfare. Our research protocols were approved by the Committee for Animal Welfare, LUMC.

Peptide vaccination and TA99 Ab treatment

The B16F10 melanoma cell line was cultured in IMDM (Life Technologies), supplemented with 8% FCS (Life Technologies), glutamine, and 2% penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Subcutaneous melanomas were generated by s.c. injection of 3 × 104 to 5 × 104 B16F10 tumor cells in the flank. Tumor size was measured twice a week with a caliper, and mice were sacrificed when tumors exceeded 1000 mm3 in size. Mice were immunized at indicated time points by shaving part of the flank and injecting s.c. 70 nmol 9-mer gp10025–33 peptide EGSRNQDWL or an altered variant EGSRNQDVGVPRQL. The TRP-2–derived peptide was also injected in a 70-nmol dose (TRP-2175–193 peptide QIANCSVYDFFVWL-A VGALEG). All peptides were dissolved and injected in PBS. Immediately following peptide injection, 60 mg Aldara cream (3M Health Care) was injected in the subcutaneous site. Tumors were measured twice a week with a caliper, and mice were sacrificed when tumors exceeded 1000 mm3 in size.

Mice were immunized at indicated time points by shaving part of the flank and injecting s.c. 70 nmol 9-mer gp10025–33 peptide EGSRNQDWL or an altered variant EGSRNQDVGVPRQL, or the altered 20-mer peptide AVGALEGSRNQDVLGPVRQL. Target cell populations were used for in vitro cultures or injected (3 × 106) into the tail vein of recipient mice, as previously described (25).

Immunohistochemistry

Formaldehyde-fixed and paraffin-embedded tissues were cut in 4-μm sections and stained for T cells against CD3 with a polyclonal rabbit anti-human/mouse CD3 (clone A 0452; Dako, Glostrup, Denmark) or for macrophages using mAb F4/80 (clone C1:A3-1; IgG2b, Serotec). Subsequently, biotinylated swine anti-rabbit IgG Ab (clone E0431; Dako) or rabbit anti-rat IgG (clone E0467; Dako), respectively, was used as the secondary Ab. Stainings were visualized with alkaline phosphatase–strepavidin (AK-5000: Vector Laboratories, Burlingame, CA) and developed with Fast Red (Sctek, Logan, Utah) in a naphthol-phosphate buffer (Sctek) with 50 mM levamisole (Dako). The slides were counterstained with Mayer’s hematoxylin. Control sections were incubated with secondary Ab alone.

Statistical analysis

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

Results

Peptide vaccination with altered gp100 epitope and TLR-7 ligand imiquimod induces high frequencies of self-reactive CD8 T cells

Our previous work revealed that the altered gp100 peptide vaccine induced CD8 T cells capable of recognizing the natural gp100 sequence presented by B16 melanoma cells (21). The inclusion of the TLR-7 ligand imiquimod in the vaccine was essential for this immune-activating effect, and this ligand outperformed other TLR ligands, such as Cpg oligonucleotides (25–27). Peptide was formulated in saline, because the slow release of peptides from oil depots might lead to peptide presentation in vivo in the absence of innate immune activation, leading to a vanishing and tolerant T cell response (17). Moreover, in this way we were able to analyze the effects of imiquimod without the potential interference of stimulation by mineral oil suspensions. Topical application of imiquimod cream on the shaved flanks of mice led to an increase in dendritic cells in the local draining lymph nodes, but not in the contralateral nondraining lymph nodes in the opposite flanks (Supplemental Fig. 1), indicating that imiquimod predominantly acted on innate immune cells in its local environment. The dendritic cells in the local lymph nodes were strongly activated, as reflected by the increased expression of CD86 (Fig. 1A), CD40, and MHC class II molecules (Supplemental Fig. 1A).

We also examined the biodistribution, processing, and MHC class I presentation of the 20-mer-long peptide after s.c. injection in saline and observed that the processed form of this long peptide was predominantly found in the same local draining lymph node (Fig. 1B). The minimal peptide–epitope, consisting of 9 aa, was detected at a much greater extent in the nondraining lymph node in the opposite flank, indicating a much broader biodistribution for
this short peptide (Fig. 1B). Both peptide-length variants promoted the priming and expansion of Ag-specific TCR-transgenic T cells in a similar way, as measured 18 d after vaccination (Fig. 1C). These experiments were performed with the altered gp100 peptide with a strongly improved MHC class I–binding capacity (21), and we wondered whether elongation of the natural peptide–epitope was able to break tolerance to this self-Ag, because synthetic long peptides outperformed shorter variants in previous mouse models (14–17). C57BL/6 mice were immunized with 9-mer or 20-mer peptides containing the natural gp100 sequence EGSRNQDWL together with imiquimod. The frequencies of vaccine-induced CD8 T cells were low, but detectable, in both of these groups (Fig. 1D). Elongation of the peptide vaccine did not lead to convincingly enhanced frequencies, in contrast to alteration of the peptide sequence. One amino acid substitution (S → P at position 3) resulted in 6-fold greater vaccine-induced CD8 T cell responses (Fig. 1D), irrespective of the peptide length. Furthermore, tumor-protection experiments with B16F10 melanoma cells corroborated these in vitro findings (Supplemental Fig. 1B, 1C): the natural peptide offered as a long-peptide vaccine failed to control tumor outgrowth, and, moreover, the altered peptide mediated tumor protection, irrespective of peptide length. Together, these data showed that, using these vaccine formulations, elongation of a weakly immunogenic self-peptide is not able to break tolerance in the T cell compartment.

Finally, we aimed at improving the immunogenicity of other melanoma Ags and observed previously that substitution of amino acids at positions 2 and 3 of the mouse H-2Kb–binding peptide TRP-2181–188 (VYDFFVWL) resulted in strongly enhanced MHC binding (21). Therefore, we tested whether a 19-mer-long variant of this altered TRP-2 peptide (VGP) was able to recruit CD8+ T cells from the endogenous repertoire that are capable of cross-reacting with the natural peptide sequence. Unfortunately, the very immunogenic altered TRP-2 peptide largely activated CD8 T cells with TCRs incapable of interacting with the natural TRP-2/Kb complexes (Supplemental Fig. 2). We concluded that the altered TRP-2 peptide was not suitable for inducing tumor Ag–specific T cells. Therefore, further immunizations were based on long-synthetic peptides comprising the natural TRP-2 sequence and the altered gp100 peptide.
munization with these two long peptides with topically applied imiquimod resulted in detectable frequencies of peptide-specific T cells in the blood (Fig. 2A). After the third immunization, strikingly high frequencies of self-reactive gp100-specific CD8+ T cells were found: up to 40% of the total CD8 T cell pool (Fig. 2C). The lower frequencies seen after only two vaccinations were able to selectively clear peptide-loaded surrogate targets in the animals (Fig. 2B) and, moreover, largely prevented the outgrowth of the aggressive B16F10 melanoma in a prophylactic setting (Fig. 2C). However, a therapeutic scheme of vaccination in which peptide injections started 4 d after tumor injection failed to delay tumor growth (Fig. 2D). Importantly, palpable B16F10 melanoma nodules were present at day 12 after inoculation, and we needed to sacrifice the mice from day 20 onwards because of large tumor volumes, although high frequencies of tumor-reactive CD8 T cells were detectable at this late stage of disease. These data demonstrate that long-peptide vaccines were able to induce Ag-specific CD8+ T cells with killing capacity, but these immune effector cells arose too late to control the rapidly growing and immunosuppressive B16F10 tumors.

**Addition of TA99 Ab to long-peptide vaccines leads to effective melanoma treatment**

The mAb TA99 recognizes the TRP-1 (gp75) surface protein of melanoma cells and was shown to mediate some antitumor effect during early tumor growth (28–31). Injection of TA99 at days 0, 4, and 5 of B16F10 inoculation completely prevented the outgrowth of melanomas (Supplemental Fig. 3A), indicating that this mAb also possessed therapeutic potency in this s.c. melanoma model. Therefore, we combined peptide vaccination with passive injections of the TA99 mAb for the treatment of established melanoma and began Ab treatment on day 5. In this scheme, treatment with the peptide vaccine or TA99 alone did not lead to overt eradication of melanomas, whereas the combination delayed tumor outgrowth and even resulted in long-term survival of >50% of the animals (Fig. 3). The application of the Ab was then postponed in the treatment protocol to days 7 and 11, but this resulted in a clear decrease in tumor-free surviving mice (Supplemental Fig. 3B, 3C). Importantly, the specificity of TA99 was crucial for the eradication of established tumors, because treatment with isotype-control mAb was not effective (Supplemental Fig. 3D). We concluded that therapeutic vaccination with long peptides cooperates with a melanoma-directed mAb and can eradicate fast-growing small melanomas.

**Mechanisms of action underlying the effective combination therapy**

We were interested in understanding the mode of action of the combination therapy, knowing that the targeted TRP-1 Ag of this Ab is unrelated to the Ags contained in the long-peptide vaccine (TRP-2 and gp100). First, we analyzed whether the presence of TA99 Ab led to higher frequencies of tumor Ag–specific T cells. This mouse Ab is of the IgG2a isotype and might activate the complement system, leading to direct killing of the tumor cells and spread of tumor-associated Ags. However, treatment of B16F10 tumors in mice genetically deficient for the central complement pathways did not result in a significant increase in the frequency of TA99-reactive T cells. Therefore, we hypothesized that TA99 bound to tumor Ags and engaged the immune system, which in turn activated effector mechanisms of the adaptive immunity. The results of these studies demonstrate that combination therapy with long peptides and a melanoma-directed mAb can lead to efficient elimination of established melanomas in a syngeneic s.c. tumor model.
factor C3 was at least as effective as in wild-type mice (Fig. 4A, 4B). The seemingly better treatment efficacy in C3 knockouts might relate to the tumor-promoting role that was described for the complement system (32). In contrast, mice deficient for the common \( \gamma \)-chain of the activating Fc\( \gamma \)Rs completely failed to mount a tumor-rejection response, whereas vaccine-induced T cells were as common as in wild-type mice (Fig. 4C, 4D). These data suggested that Fc\( \gamma \)R-expressing innate immune cells, not the complement system, are responsible for the initial growth arrest of melanomas via TA99. In line with previous findings in experimental tumor models (33, 34), we hypothesize that tissue-resident macrophages can mediate tumor rejection in the early phase of tumor growth upon TA99-induced triggering of Fc\( \gamma \)Rs.

Second, we examined whether this initial tumor control by TA99 led to enhanced liberation of tumor Ags from killed melanoma cells, because previous reports suggested that this enhanced cross-presentation constituted the mode of action of mAb therapy (6, 8). TA99 was injected in tumor-bearing mice that were treated with long-peptide vaccination. TCR-transgenic T cells specific for gp100 were supplied as reporter cells to carefully analyze expansion and activation of these cells. No difference was seen in the population of transgenic gp100-specific T cells over a period of 90 d when TA99 was provided (Fig. 5A). In addition, compilation of blood T cell frequencies from mice treated in the several tumor challenge experiments shown in Fig. 3 and Supplemental Fig. 3 revealed only a slight increase in peptide-specific CD8 T cell percentages in mice receiving TA99 (Fig. 5B); this difference was not statistically significant. We concluded that the addition of TA99 does not strongly impact the activation of gp100-specific T cells. Furthermore, we assessed the influence of TA99 on the priming of endogenous T cell responses to other melanoma-derived Ags, in particular the TRP-1\( \text{455-463} \) epitope (24). No TRP-1 peptide-specific T cells could be detected (Fig. 5B), suggesting that TA99 administration did not lead to broadening of the T cell response against B16F10. Finally, we analyzed immune cell infiltrates of melanomas treated with peptide vaccination versus combination therapy, using flow cytometry and immunohistochemistry. Untreated B16F10 tumors predominantly contained F4/80\(^+ \) macrophages and T cells of both CD4 and CD8 subsets. Peptide vaccination resulted in significantly increased CD8 T cell counts compared with CD4 T cells or macrophages (Fig. 5C), suggesting that tumor-specific CD8 T cells properly homed to the tumor sites. Comparable enhanced CD8

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**FIGURE 3.** Combination therapy consisting of TA99 Ab and peptide vaccination can eradicate established melanomas. Tumor growth curves (A) and Kaplan–Meier survival curves (B) from four groups of mice that were not treated or were treated with TRP-1–specific mAb TA99, long peptide vaccination comprising TRP-2 and gp100 Ags, or a combination of both. One line in (A) represents one mouse, and the numbers in the graph refer to the number of long-term-surviving mice. Peptide vaccines were provided on days 4 and 11. Injections of 200 \( \mu \)g TA99 Ab (i.v.) were given on days 5 and 7. Statistical \( p \) value is from log-rank test of Kaplan–Meier curves.

**FIGURE 4.** Complement factor C3 is dispensable, but Fc\( \gamma \)Rs are critical for effective melanoma treatment. Combination therapy was performed in complement factor C3-knockout mice (A, B) and in the FcR common \( \gamma \)-chain knockout mice (C, D). Blood samples from immunized mice revealed normal induction of peptide-specific CD8 T cells in both knockout mice (A, C). Survival curves of groups of mice are plotted in Kaplan–Meier graphs (B), \( n = 10 \) and (D), \( n = 7 \). The timing of Ab and peptide vaccine administration was the same as in Fig. 3. The \( p \) values are from statistical log-rank test of Kaplan–Meier curves.
Peptide vaccination alone was not sufficient in our model to provide durable clearance of melanoma cells, and although TA99 was shown to prevent melanoma formation in prophylactic settings (29–31), it failed to eradicate established s.c. tumor nodules. The TA99 mAb is of the IgG2a subtype, and it efficiently binds complement and FcγR on innate immune cells (28, 30), quite similar to the FDA-approved human Abs that target tumor surface Ags (e.g., CD20, CD33, and CD52). Of note, TRP-1 is a metalloenzyme involved in pigment formation but is not an oncogenic growth factor, excluding the potential direct effect of growth inhibition by Ab binding. Interestingly, complement factor C3 was dispensable for effective treatment, whereas genetic deficiency of FcγR completely abolished tumor control (Fig. 4). We anticipate that the mAb activates local innate immune cells and, thereby, creates a window of opportunity needed for the antitumor CD8 T cells to emerge in higher frequencies. Tissue-resident macrophages display activating FcγR and are capable of controlling tumor outgrowth (33, 34).

Vaccination with long synthetic peptides results in robust immune responses, as demonstrated in several mouse models and clinical studies (17, 43). In the current study, we show induction of high CD8 T cell frequencies directed to tumor-associated Ags that are self-proteins. However, alteration of the peptide sequence was critical to break tolerance to the gp100 Ag, which is expressed in melanocytes. Elongation of the peptide, as such, was not enough to break tolerance. The 20-mer-long altered gp100 peptide used in this study contains 1 aa change, from serine to proline, which causes major complications, can be avoided by using peptide vaccines that strongly activate adoptively transferred T cells in vivo (25, 42). The novelty of the current study lies in the fact that the endogenous T cell repertoire is exploited in combination with passive injections of the anti–TRP-1 mAb TA99. Therefore, we concluded that the TRP-1-binding TA99 mAb exerts its function in early resistance to melanoma cells via local FcγR-expressing innate immune cells, which provides a critical delay in tumor outgrowth that is necessary for the emergence of antitumor T cell immunity.

Discussion

Successful immunotherapy of established B16F10 melanoma reported in the literature includes adoptive T cell transfer with activated CD8 T cells administered in lymphopenic hosts or with strong immunomodulatory compounds (high-dose IL-2, IFN-α, anti-CTLA-4, or depleting regulatory T cells) (8, 21, 38–41). We and other investigators showed that the need for lymphodepletion, which causes major complications, can be avoided by using peptide vaccines that strongly activate adoptively transferred T cells in vivo (25, 42). The novelty of the current study lies in the fact that the endogenous T cell repertoire is exploited in combination with passive injections of the anti–TRP-1 mAb TA99. Peptide vaccination alone was not sufficient in our model to provide durable clearance of melanoma cells, and although TA99 was shown to prevent melanoma formation in prophylactic settings (29–31), it failed to eradicate established s.c. tumor nodules. The TA99 mAb is of the IgG2a subtype, and it efficiently binds complement and FcγR on innate immune cells (28, 30), quite similar to the FDA-approved human Abs that target tumor surface Ags (e.g., CD20, CD33, and CD52). Of note, TRP-1 is a metalloenzyme involved in pigment formation but is not an oncogenic growth factor, excluding the potential direct effect of growth inhibition by Ab binding. Interestingly, complement factor C3 was dispensable for effective treatment, whereas genetic deficiency of FcγR completely abolished tumor control (Fig. 4). We anticipate that the mAb activates local innate immune cells and, thereby, creates a window of opportunity needed for the antitumor CD8 T cells to emerge in higher frequencies. Tissue-resident macrophages display activating FcγR and are capable of controlling tumor outgrowth (33, 34).
previously (44, 45). However, we cannot exclude that a vaccine with short Trp-2 peptides would generate more potent responses, as suggested by other investigators (46, 47). Although these CD8 T cells are in principle self-reactive, no clear signs of depigenation were detectable during the experiments (up to 3 mo). In contrast to our previous experience with long peptides that make up foreign CD8 T cell epitopes (14−17), the combined responses to gp100 and TRP-2 failed to control the outgrowth of established s.c. tumors. Two factors might explain this difference. First, the tumor Ags in our model constitute weak self-Ags, whereas our previous models included viral and model Ags, such as HPV and OVA (14−17). Second, the B16 tumor is a spontaneous and very aggressive melanoma that efficiently exploits escape mechanisms (e.g., low MHC class I surface expression and creation of an immunosuppressive microenvironment).

One of the most intriguing questions concerns the mechanism of action of the combination therapy consisting of peptide vaccination and mAb. Some reports demonstrated enhancement of the tumor-specific T cell response after administration of mAb against tumor surface Ags (5, 6, 8). Ab binding leads to activation of the complement system and to cross-linking of FcγR on immune cells. This opsonization of tumor debris and the formation of immune complexes strongly enhance the MHC-restricted presentation of tumor Ags and, consequently, can activate Ag-specific T cells (1–3). By this means, Abs mediate the recruitment of the adaptive immune arm. However, we did not observe such an enhanced or broadened T cell response against melanoma Ags, most likely because our peptide vaccine already induced robust responses against gp100 and TRP-2 (Fig. 2).

We advocate the concept of combination therapy, bridging the fields of T cell immunology and mAb, as other investigators suggested (1, 48). The concerted action of these two components is expected to bear superior anticancer efficacy and can be applied for human melanoma because of previously developed and clinically tested Abs (48, 49).

Acknowledgments

We thank Drs. Ferry Ossendorp, John Haanen, and Kees Melief for critical reading of this manuscript and Jurgen van Heemst for providing control IgG2a Ab.

Disclosures

The authors have no financial conflicts of interest.

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Legends of the supplementary figures

Supplementary Figure 1. Effective generation of CD8 T cells to the gp100 ‘self’ peptide from the endogenous repertoire.

(A) A long peptide vaccine formulation of 20-mer synthetic peptide and TLR-7 ligand imiquimod as adjuvant elicits gp100-specific CD8 T cells from the endogenous repertoire. Imiquimod was topically applied as a crème on shaved flanks of mice on top of the peptide injection site. After two days, draining and contra lateral non-draining lymph nodes were excised, dissociated and analyzed by flow cytometry. CD11c-positive dendritic cells were enumerated (% DC) and their activation status determined by expression levels of CD40 and MHC class II. Data represent means and standard error of the mean of three mice from one out of two comparable experiments.

(B-C) Prophylactic vaccination with gp100 peptide vaccines. Natural peptide sequence (‘EGS’) was compared to altered peptide (‘EGP’) as synthetic long peptide vaccines (B) and short altered peptide was compared to long altered peptide (C). After two vaccinations, mice were challenged with a lethal dose of B16F10 tumor cells injected subcutaneously. Protective capacity was mediated by altered peptides, irrespective of length. Mice were sacrificed when tumors reached 1000 mm³.

Supplementary Figure 2. Amino acid replacement in the TRP-2 peptide-epitope induced CD8 T cells that fail to cross-react against the wild type sequence.
Positions 2 and 3 in the TRP-2\textsubscript{181-188} (VYDFFVWL) peptide were replaced with a glycine and proline residue, respectively (VGPFFVWL), resulting in strongly enhanced binding to the presenting MHC class I molecule K\textsuperscript{b} (20). (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\textsuperscript{+} 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 IFN\textgamma.

(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.

**Supplementary Figure 3.** *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 in different treatment schemes. (D) Survival curves of groups of mice treated with peptide vaccine in combination with isotype control antibody (mouse IgG2a). The timings 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 nine mice and p-values are from statistical log rank test of Kaplan-Meier curves.

**Supplementary Figure 4.** *Treated B16F10 tumors comprise a high immune cell infiltrate.*

Tumor-infiltrating immune cells were stained by immunohistochemistry for macrophages (F4/80) and T cells (CD3) and visualized with Fast Red dye. Four times the lethal dose of B16F10 was injected ($10^5$) to force the growth of melanomas despite the treatment protocols. Mice were sacrificed and tumors were removed when tumors reached 125 mm$^3$. Tissues were fixed in formalin, embedded in paraffin and counterstained with Mayer’s hematoxylin. Representative tissues are depicted from six tumors analyzed.
Supplementary figure 1

A

% DC
% of all leukocytes

CD40
Fluorescence intensity

MHC II
Fluorescence intensity

control TLR-7L control TLR-7L
draining LN non-draining LN

B

% survival
day post tumor inoculation

naive long EGS long EGP

C

% survival
day post tumor inoculation

naive short EGP long EGP
Supplementary figure 3

A

B

C

D

Percent survival

naive

Pept vacc

TA99

Pept vacc + TA99

naive

Pept vacc

Pept vacc + TA99

Pept vacc + control mAb

p = 0.0005

p = 0.373

p = 0.222

p = 0.005

Day post tumor inoculation

Day post tumor inoculation

Day post tumor inoculation

Day post tumor inoculation

0 25 50 75 100

0 25 50 75 100

0 25 50 75 100

0 25 50 75 100

0 25 50 75 100

0 25 50 75 100

0 25 50 75 100

0 25 50 75 100
Supplementary Figure 4

F4/80

CD3

non-treated B16F10

treated with pept vacc

treated with pept vacc and TA99