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

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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 demonstrate 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 neoplasmia 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-
lished and rapidly growing B16F10 tumors. Addition of a mAb (TA99) specific for the surface melanocyte protein TRP-1 (gp75) to the peptide vaccine lead 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 gp10025–33/9-2A2–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 70 nmol 9-mer gp100 25–33 peptide EGRSNQDWL or an altered variant EGPRNQDWL or the altered 20-mer peptide AVGAELEGPNQDQLVPQRL. The TRP-2–derived peptide was also injected in a 70-nmol dose (TRP-2 175–193 peptide QIANCSVYDFFVWLHYYSV). All peptides were dissolved and injected in PBS. Immediately following peptide injection, 60 mg Aldara cream (3M Health Care) was applied onto the vaccinated area. 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 gp100 25–33 peptide EGRSNQDWL or an alternated variant EGPRNQDWL to preserve the intracellular cytokine. On the following day, treatment with the TRP-1–specific mAb TA99 (mouse IgG2a) was provided via a 200-μg i.p. injection. This Ab was purified from hybridoma HB-7804 obtained from Dr. A. Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY). Isotype-control Ab (clone SPV3L) against human HLA class II was obtained from Dr. R. Toes (LUMC).

Intracellular cytokine staining

The frequency of IFN-γ-producing T cells was determined from blood samples. After RBC lysis, lymphocytes were incubated overnight with 1 μg/ml wild-type peptide gp100 (position 25–33: EGRSNQDWL) (22), TRP-2 peptide (position 180–188: SVYDFFFWLV), or TRP-1 peptide (position 455–463: TAPDNLGYA) (24) at 37°C in a 5% CO2 atmosphere. GolgiPlug (1 μg/ml) was added to preserve the intracellular cytokine. On the following morning, cells were fixed, washed, stained with mAbs CD8a-PE and IFN-γ–allophycocyanin (BD Pharmingen), and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

In vivo cytotoxicity measurement

To analyze the cytotoxic efficacy of gp100- and TPR-2–reactive CTL in vivo, naive C57BL/6 mice were vaccinated with long peptides and imiquimod, as described above, and injected i.v. with a mixture of peptide-pulsed CFSE-labeled splenocytes, as described previously (21). Briefly, splenic cells from CD90.1 congenic C57BL/6 mice were passaged through nylon wool. Half of these cells was pulsed with wild-type mouse gp100- peptide EGSRNQDWL or TRP-2 peptide SVYDFFFWL and labeled with 5 μM CFSE, whereas 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 injected i.v. (1 × 107) into immunized recipient mice. The spleens of immunized recipient mice were harvested after 2 d, stained with CD3-, CD8a-, and CD90.1-specific Abs, and analyzed with a FACSCalibur flow cytometer (BD Biosciences). The percentage killing was calculated as the ratio between the number of gp100-pulsed targets and control peptide–pulsed targets.

Isolation of immune cells from lymph nodes and tumors

Lymph nodes and tumors were removed and dissociated using 250 U/ml collagenase type 4 and 50 μg/ml DNase (Sigma-Aldrich) for 45 min at 37°C. Cell suspensions were pushed through a cell strainer (Life Technologies) to obtain single cells, washed twice, and stained with Abs to CD3, CD4, CD8, F4/80, CD11b, CD11c, CD86, CD40, and MHC class II (all from BD Biosciences). FcRs were blocked by prior incubation with 2.4G2 Ab. All samples were measured with a FACSCalibur flow cytometer (BD Biosciences) and analyzed using Flowjo software (TreeStar). Alternatively, single cells were plated in 24-well plates and incubated with CFSE-labeled TCR-transgenic T cells for 4 d. The percentage of divided T cells was calculated as the ratio of undivided CFSElow cells to cells with lower CFSE intensity. TCR-transgenic T cells specific for the gp100 25–33 epitope (pmel) were obtained from spleen and lymph nodes of naive CD90.1+/− mice and enriched for T lymphocytes by nylon wool. These T cells 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 E431; Dako) or rabbit anti-rat IgG (clone E0467; Dako), respectively, was used as the secondary Ab. Stainings were visualized with alkaline phosphate–strepavidin (AK-5000: Vector Laboratories, Burlingame, CA) and developed with Fast Red (Sctytek, Logan, Utah) in a naphthol-phosphate buffer (Sctytek) with 50 mM levamisole (Dako). The slides were counterstained with Mayer’s hema- toxylin. Control sections were incubated with secondary Abs 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. 1A), 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). 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.

Peptide vaccine–induced T cells fail to eradicate established B16F10 melanomas

The two melanoma Ags TRP-2 and gp100 were exploited for the therapeutic protocol to treat established B16F10 melanomas. Im-

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**FIGURE 1.** Effective generation of CD8 T cells to the gp100 self-peptide from the endogenous repertoire. A long-peptide vaccine formulation of 20-mer synthetic peptide and the TLR-7 ligand imiquimod as adjuvant elicits gp100-specific CD8 T cells from the endogenous repertoire. (A) Imiquimod was applied topically as a cream on shaved flanks of mice. After 2 days, draining and contralateral nondraining lymph nodes were excised, dissociated, and analyzed by flow cytometry. CD11c+ dendritic cells were enumerated, and their activation status was determined by expression levels of CD86 (see Supplemental Fig. 1 for other activation markers). Data represent means and SEM of three mice from one of two comparable experiments. (B) A total of 70 nmol of short (9-mer) or long (20-mer) versions of the altered gp100 peptide was dissolved in saline and injected s.c. In vivo drainage, processing, and MHC class I presentation of the Ags after 1 d was determined by excision of lymph nodes (LN), dissociation, and coculture of single cells with gp100-specific TCR-transgenic T cells (pmel) in vitro. T cells were loaded with protein dye CFSE to determine the percentage of divided cells in a 4-d coculture. Mean and SEM of three mice/group is depicted. Three other independent experiments gave comparable results. (C) TCR-transgenic CD8 T cells specific for the gp100/D complex (pmel) were injected in peptide-vaccinated mice as reporter cells to analyze priming and expansion. Eighteen days after two vaccinations with short or long peptides, blood samples were examined for the percentage of TCR-transgenic CD8 T cells using the congenic marker CD90.1. Frequencies of peptide-specific T cells are expressed as percentage of the total pool of CD8 T cells. Results are from five mice/group, and comparable results were obtained in three independent experiments. (D) Length variants (9-mer and 20-mer) of the natural gp100 peptide sequence (EGS) fail to elicit high frequencies of vaccine-induced T cells from the endogenous repertoire. The altered peptide (EGP) harbors one amino acid substitution at position 3 (from serine to proline) and efficiently breaks tolerance to the natural Ag (21). A total of 70 nmol of synthetic peptide was injected in saline together with topical application of imiquimod. At the indicated time points, peptide-reactive CD8 T cell frequencies were determined in blood using ex vivo measurement of cytokine production. There were five animals per group, and comparable results were obtained in two other experiments.
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. 2A). 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

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**FIGURE 2.** Vaccine-induced T cells protect outgrowth of B16F10 melanomas but fail to eradicate established melanomas. (A) Mice were vaccinated three times with 20-mer-long peptides comprising the TRP-2181–188 epitope and the altered gp10025–33 peptide. Frequencies of peptide-specific CD8 T cells were determined from the blood after brief stimulation overnight with short, natural peptides and by intracellular IFN-γ staining. Blood samples were taken before vaccination, 5 d after the second vaccination (day 12), and 5 d after the third vaccination (day 19). (B) The in vivo killing capacity of immunized mice was tested with CFSE-labeled and peptide-loaded splenocytes as surrogate targets after two peptide vaccinations. Immunized mice were injected i.v. with a mix of splenocytes pulsed with short control peptide (low CFSE) and TRP-2 or natural gp100 peptide (high CFSE). Two days after inoculation, spleens of recipient mice were harvested and analyzed by flow cytometry for percentage killing. Summary of four mice/group is depicted from one of three comparable experiments. (C) Prophylactic vaccination with 20-mer-long TRP-2 and gp100 peptides induces protective immunity to B16F10 melanoma tumors. After two vaccinations, mice were challenged with a lethal dose of B16F10 tumor cells injected s.c. Mice were sacrificed when tumors reached 100 mm3. Data represent compiled data from two independent experiments. The p value is from statistical log-rank test of Kaplan–Meier curves. (D) Therapeutic vaccination experiment in which a lethal dose of B16F10 tumor cells was injected at day 0, and two vaccinations were provided starting at day 4. One of four experiments with similar outcome is shown. The p value is from statistical log-rank test of Kaplan–Meier curves.
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 γ-chain of the activating FcγRs 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 FcγR-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 FcγRs.

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-1455–463 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
FIGURE 5. Mechanism of successful treatment by TA99 Ab and long peptide. The effect of TA99 Ab administration on the frequency and specificity of CD8 T cells was examined. (A) Mice were injected s.c. with a lethal dose of B16F10 cells; naive gp100-specific TCR-transgenic cells (pmel) were infused 3 d later. Peptide vaccine and TA99 were provided on the same days as indicated in Fig. 3. At several time points, the frequencies of TRP-transgenic T cells in the total CD8 T cell pool were measured in blood samples using the congenic CD90.1 marker. (B) Frequencies of gp100- and TRP-2–specific CD8 T cells from the endogenous repertoire were determined from blood samples from animals used in tumor experiments shown in Fig. 3 and Supplemental Fig. 3. The addition of TA99 Ab to the treatment scheme increased the frequency of these TRP-2– and gp100-specific CD8+ T cells only slightly (p = 0.055, Student t test). TRP-1–specific CD8+ T cells were not observed in treated animals. Blood samples were taken 5 d after the second vaccination, briefly stimulated in vitro with both TRP-2_180–188 and gp100_25–33 peptides (left panel) or TRP-1_155–163 peptide (right panel), and stained for IFN-γ. Data are from 36 animals/group (left panel) from four different experiments and 8 animals/group from an independent experiment (right panel). The horizontal line indicates the means of each group. (C) Tumor-infiltrating immune cells were counted by flow cytometry from treated B16F10 melanomas that were mechanically and enzymatically dispersed in vitro. Four times the lethal dose was injected (10^6) to force the growth of melanomas, despite the treatment protocols. Mice were sacrificed when tumors reached 125 mm^3. T cells were stained with CD3+, CD4+, and CD8+ Abs, and macrophages (Mf) were stained with Ab F4/80. Percentages of infiltrating subsets of the total pool of CD45+ hematopoietic cells were determined, and ratios were calculated for each tumor. The horizontal line indicates the mean of each group (n = 6). Comparable results were obtained in one additional independent experiment.

T cell numbers were found in tumors treated with the combination of peptide vaccination and TA99, corroborating our notion that TA99 did not lead to enhanced cellular immunity. Furthermore, immunohistochemistry revealed that treated melanomas displayed a denser immune infiltrate, which can be interpreted as an active immune rejection response that may be counterbalanced by tumor-resistance mechanisms (Supplemental Fig. 4). Finally, gene-expression profiling for tumor-infiltrating macrophages (35) in large established tumors did not reveal differences between tumors treated with peptide vaccines alone or the combination, suggesting that TA99 did not polarize the macrophages into another differentiation direction (36, 37).

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).

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 normal 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, near the minimal CTL epitope, leading to dramatically increased binding capacity to the MHC class I–presenting molecule and, importantly, preserving the overall structure so that vaccine-induced TCRs cross-react with the natural epitope (21). Our analysis of crystal structures confirmed that both peptide/MHC surfaces were nearly the same. Strikingly, three vaccinations with this long altered peptide together with imiquimod induced up to 40% gp100-specific CD8+ T cells from the endogenous T cell repertoire (Fig. 2). Frequencies of Trp-2–reactive T cells were much lower, but were comparable to those reported...
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 depigmentation 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 adaptor molecules and, consequently, can activate Ag-specific T cells 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 against gp100 and TRP-2 in a wide variety of tumor types.

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