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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).

mAbs binding to tumor-associated surface Ags are therapeutically applied in a range of malignancies. Therapeutic vaccination only recently met with clinical success, and the first cancer vaccine received U.S. Food and Drug Administration approval last year. To improve current protocols, we combined peptide vaccines with mAb to the tyrosinase-related protein (TRP)1 surface Ag for the treatment of B16F10 skin melanoma. Vaccine formulations with synthetic long peptides failed to elicit strong CD8 T cell responses to self-differentiation Ags gp100 and TRP-2, whereas altered peptide sequences recruited gp100-specific CD8 T cells from the endogenous repertoire with frequencies of 40%. However, these high frequencies were reached too late; large, progressively growing melanomas had already emerged. Addition of the TRP-1–directed mAb TA99 to the treatment protocol mediated eradication of s.c. lesions. The mode of action of the Ab did not depend on complement factor C3 and did not lead to improved Ag presentation and CD8 T cell immunity; rather, it recruited FcγR-bearing innate immune cells during early tumor control, thereby creating a window of time for the generation of protective cellular immunity. These data support the concept of combination therapy, in which passive transfer of mAbs is supplemented with cancer peptide vaccines. Moreover, we advocate that tumor Ag–specific T cell immunity directed against self-proteins can be exploited from the endogenous repertoire. The Journal of Immunology, 2013, 190: 000–000.

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Abbreviations used in this article: FDA, U.S. Food and Drug Administration; HPV, human papillomavirus; LUMC, Leiden University Medical Center; TRP, tyrosinase-related protein.

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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-2Db–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. C57+ mice were provided by Dr. M. Daha (Leiden University Medical Center [LUMC]), and FcRγ-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 5% 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 × 106 to 5 × 106 B16F10 tumor cells in the flank. Tumor size was measured twice a week with a caliper, and mice were sacri\-ficed when tumors exceeded 1000 mm3 in size.

Mice were immunized at indicated time points by shaving part of the hind flank and injecting s.c. 70 nmol 9-mer gp10025–33 peptide EGSQRNQDWL or an altered variant EGQRNQDL or a 20-mer altered 9-mer peptide AVAGELEGQRNQDLFWLPQRL, or the 20-mer altered peptide AVGAVLEGQRNQDLFWLPQRL was injected in a 70-nmol dose (TRP-121-39 peptide QIANCSVYDFFWLV-HYYSV). All peptides were dissolved and injected in PBS. Immediately following peptide injection, 60 mg Aldara cream (3M Health Care) containing 5% imiquimod was topically applied to the skin at the injection site. This immunization protocol was repeated 7 d later. Mice received two i.p. injections of recombinant human IL-2 (6 × 105 IU; Novartis, Breda, The Netherlands) on the day of the second vaccination, as well as 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-8704 obtained from Dr. A. Houghton (Memorial Sloan-Kettering Cancer Center, New York, NY), isotype-control Ab (clone SPVL3) against human HLA class II was provided by Dr. M. Daha (Leiden University Medical Center [LUMC]), and FcRγ/CD90.1+ pmel mice and enriched for T lymphocytes by nylon wool. These CD90.1+ pmel 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 E4313; Dako) or rabbit anti-rat IgG (clone E0467; Dako), respectively, was used as the secondary Ab. Stainings were visualized with alkaline phosphate–streptavidin (AK-5000; Vector Laboratories, Burlingame, CA) and developed with Fast Red (Sctyet, Logan, Utah) in a naphthol-phosphate buffer (Sctyet) 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 TLR7 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 nondondraining 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 nondondraining lymph node in the opposite flank, indicating a much broader biodistribution for

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This text is a natural representation of the document provided, focusing on the key aspects related to the combination therapy of Ab and peptide vaccine, including the experimental setup, methods, and results.
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.

**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-
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

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 1000 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 \( \gamma \)-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\( _{455-463} \) 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.
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, at position 3 of 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 depignification 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).

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