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Relevance of the Tumor Antigen in the Validation of Three Vaccination Strategies for Melanoma[1]

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Many preclinical studies of cancer immunotherapy are based on the testing of a single vaccination strategy in several tumor models. Moreover, most of those studies used xenogeneic Ags, which, owing to their high immunogenicity, may not represent realistic models for the validation of cancer immunotherapies. To address these issues, we compared the vaccination efficacy of three well established strategies (i.e., naked DNA; peptide-pulsed dendritic cells (DC), or a mixture of peptide and the *Escherichia coli* toxin LTR72) using the xenogeneic OVA or the naturally expressed tyrosinase-related protein 2 (TRP-2) tumor Ag in the B16 melanoma model. C57BL/6 mice received one to three s.c. injections of peptide-pulsed DC or DNA, or one to four mucosal administrations of peptide-toxin mixture. One to 2 wk later, the animals were challenged s.c. with B16 or B16 cells expressing OVA (B16-OVA). Vaccination of mice with OVA induced in all cases melanoma-specific CTL and protection against B16-OVA. When TRP-2 was used, all three vaccines elicited B16-specific CTL, but only DC pulsed with the immunodominant T cell epitope TRP-$2_{181–188}$ allowed protection against B16. Even more importantly, a vaccination regimen with TRP-2-pulsed DC, started 24 h after the injection of a lethal number of B16 cells, caused a therapeutic effect in 60% of the challenged animals. Our results strongly emphasize the relevance of the tumor Ag in the definition of immunotherapeutic strategies for cancer, and support the use of peptide-pulsed DC as cancer vaccine in humans. *The Journal of Immunology,* **2000, 165: 2651–2656.**

Several experimental evidences strongly suggest to add immunotherapy to the more conventional combination of surgery, radiotherapy, and chemotherapy for cancer (1). Many preclinical studies of cancer immunotherapy evaluated the therapeutic efficacy of a single vaccine at a time (1). Moreover, they were often produced using artificial xenogeneic Ags, generated by the transfection of well-characterized Ags into tumor cells (e.g., chicken OVA or bacterial β-galactosidase (e.g., Refs. 2 and 3)). These Ags are nonself, and induce vigorous responses due to the lack of tolerance. By contrast, most of the naturally expressed tumor-associated Ags (TAA)[3] belong to self, and therefore may elicit an inefficient immunity owing to preexisting tolerance. Therefore, more realistic experimental models (i.e., spontaneous mouse tumors expressing TAA similar to the ones found in human neoplasms) should be used to validate and compare new and already existing immunotherapeutic approaches.

B16 is a spontaneous and poorly immunogenic melanoma, which nevertheless contains Ags able to activate a specific CTL response (4, 5). The tyrosinase-related protein 2 (TRP-2) is a tissue differentiation Ag expressed by normal and malignant melanocytes both in humans and mice (6, 7). Several epitopes recognized by human $CD8^+$ T cells have been identified on the TRP-2 protein (8), and TRP-2-specific T cells have been found in melanoma infiltrating lymphocytes, which determined clinical tumor regression (6). Bloom at al. identified in the sequence TRP-$2_{181–200}$ a CTL epitope recognized within the K^b MHC class I molecule, and reported that passive transfer of TRP-$2_{181–200}$-specific T cells into C57BL/6 mice reduced the number of B16 lung metastases (7).

Dendritic cells (DC) are powerful APC, able to generate primary T cell responses (9), and become efficient tumor vaccines when pulsed with synthetic or natural tumor peptides, tumor-derived RNA, or transduced with vectors encoding different proteins, or directly fused to or incubated with tumor cells (reviewed in Ref. 10). Clinical trials based on the use of peptide-pulsed DC in melanoma and renal carcinoma patients have indeed been reported (reviewed in Ref. 11).

Naked DNA vaccination is an efficient means of induction of Ag-specific immunity (reviewed in Ref. 12). It presents several potential advantages when compared with the more conventional vaccination strategies: multiple Ags or one chimeric DNA encoding for a fusion protein can be contained in a single vaccine. Moreover, its large scale production and storage is cheaper and easier than protein-based vaccines (12).

DNA can be administered either alone or complexed with different carriers, and may follow different routes, such as i.d., s.c., or i.m. (12). Physical administration of DNA by gene gun- or aerosol-based systems appears to elicit consistent response in mice (12). Gene gun delivery of the TRP-2 DNA indeed resulted in activation of TRP-2-specific CTL and delayed outgrowth of B16 melanomas, although no animals were cured by such treatment (13).

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[3] Abbreviations used in this paper: TAA, tumor-associated Ags; DC, dendritic cells; B16–0VA, B16 cells expressing OVA; LT, heat-stable *Escherichia coli* enterotoxin; LTR72, mutant of LT; TRP-2, tyrosinase-related protein 2.

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DC exert a predominant role in DNA vaccination (14), although other tissues may capture the injected DNA and express the encoded protein for several weeks after vaccine boost (15). Therefore, an ideal target organ for DNA vaccination is the skin, where resident professional APC uptake the plasmid DNA and migrate to the lymph nodes (9, 16). Furthermore, s.c. administration of naked DNA requires neither pretreatment of the tissue, nor gene gun delivery (17).

Mucosal vaccination is a noninvasive strategy to elicit both humoral and cellular immune responses (18). Oral, nasal, and vaginal routes of delivery have been used successfully (18). Mucosal vaccination requires the coadministration of a toxin, whose function is not yet clarified (18). DC seem to be the APC involved also in the presentation of CTL epitope peptides following intranasal immunization (19). One of the drawbacks in the use of toxins as adjuvants is their toxicity, which precludes their use in humans (18). Giuliani et al. (20) recently reported that a site-specific mutagenesis on the heat-stable *Escherichia coli* enterotoxin (LT) originated a new toxin, LTR72, which is markedly less toxic than wild-type LT, although maintaining most of its adjuvant effect.

We compared the three aforementioned vaccines in the B16 melanoma model, using as TAA the naturally expressed TRP-2 melanoma Ag, or the xenogeneic surrogate OVA, expressed in a nonsecreted form in B16 cells (B16-OVA).

We show that the results, in terms of CTL induction and protection against a challenge with live melanoma cells, depend on the immunogenicity of the Ag model.

Our results strongly emphasize the relevance of the experimental model in the definition of immunotherapeutic strategies for cancer, and identify peptide-pulsed DC as the most powerful cancer vaccine.

Materials and Methods

Mice and cell lines

C57BL/6 (H-2^b) female mice, 8–10 wk old, were purchased from Charles River Breeding Laboratories (Calco, Italy), housed in a pathogen-free animal facility, and treated in accordance with the European Community guidelines. The in vivo experiments were approved by the Ethical Committee of the Istituto Scientifico H. San Raffaele. TRP-2 expression in the H-2^b B16F1 (B16) line (American Type Culture Collection, Manassas, VA) was confirmed by RT-PCR (data not shown). A truncated form of OVA lacking the leader sequence was obtained from the full-length cDNA (21) by PCR using the following oligonucleotides: 5′-CTGGGTAC CAAAGACAGCACCATGACAC-3′ (5′ primer) and 5′-GCCGTGGAAT TCAGGGGAAACACA-3′ (3′ primer). The cDNA was cloned into the expression vector pcDNA3-ct myc (Ref. 22, kindly provided by Dr. M. Guttinger, Dibit, Milan, Italy), and used to transfect B16 melanoma cells by electroporation, using a Gene Pulser apparatus (Bio-Rad, Richmond, CA) set at 250 V and 960 mF. Clones surviving selection by 1 mg/ml G418 (Boehringer Mannheim, Mannheim, Germany) were screened to express OVA cDNA by PCR, and to present the K^b-restricted SIINFEKL epitope (OVA$_{257–264}$) to the T cell hybridoma B3Z (kindly obtained from Dr. N. Shastri, Berkeley, CA). B16-OVA clones inducing the highest production of IL-2 by B3Z were selected. RMA-OVA cells were obtained from the transfection of the truncated OVA cDNA into the C57BL/6-derived T cell lymphoma line RMA (23). The minimal tumorigenic doses for B16F1 and B16 expressing OVA are 5×10^3 and 10^4, respectively.

DC vaccination

DC were prepared from bone marrow as described (24). On day 7–9 of culture with recombinant GM-CSF and IL-4 (1000 U/ml; PharMingen, San Diego, CA), nonadherent cells were used for in vitro phenotypic analysis as well as for mice immunization. For phenotypic analysis, DC were incubated with normal mouse serum for 30 min at 4°C, double stained with the PE-conjugated anti-CD11c mAb and one of the following FITC-conjugated mAbs: CD3, CD4, CD8, CD19, CD40, CD80, CD86, NK/2B4, K^b, D^b, or I-A^b (PharMingen), and analyzed by flow cytometry. On day 7 of the in vitro culture, most of the nonadherent cells showed the morphology and phenotype of DC with at least 50% of the cells expressing high levels of I-A^b and CD86 molecules (data not shown). For immunization experiments, DC were resuspended in PBS at 5×10^6/ml and incubated for 60 min at 37°C with 5 μg/ml of the synthetic peptides OVA$_{257–264}$ (a gift of Dr. B. M. Conti-Fine, St. Paul, MN) or TRP-2$_{181–188}$ (a gift of Dr. C. Noppen, Basel, Switzerland). DC were irradiated (3000 rad), washed, and resuspended at 2×10^6/ml in PBS. Mice received one to three s.c. injections of 100 μl DC suspension every 2 wk. In parallel, groups of mice were injected with unpulsed DC. Two weeks after the last boost, mice were challenged on the opposite flank with 5×10^4 B16 cells or 10^5 B16-OVA cells. Tumor size was evaluated by measuring two perpendicular diameters by a caliper twice a week. Animals were scored positive when the mean tumor diameter was >2 mm. Mice with no visible or palpable tumor 60 days after tumor challenge were scored negative. Animals were killed when the mean tumor diameter was >10 mm. To evaluate the efficacy of the vaccine to prevent the growth of small preestablished melanomas, naive mice ($n = 20$) were injected s.c. with 5×10^4 B16 cells. One day later, mice were randomly assigned to either one of the following two treatments: three weekly controlateral injections of unpulsed or TRP-2-pulsed DC (2×10^5/injection). Animals were followed thereafter as described above.

Mucosal vaccination

Mice were lightly anesthetized and immunized on days 0, 7, 14, and 21 with a 50-μl vol per nostril of a mixture composed by 60 μg of either one of the synthetic peptides and 6 μg of LTR72 toxin (I.R.I.S. Chiron s.p.a., Siena, Italy) in PBS, or with LTR72 alone. One week after the last boost, mice were challenged in the left flank with B16 or B16-OVA cells as described above.

DNA vaccination

Endotoxin-free plasmid preparations were obtained using the Qiagen Endo-free giga kit (Qiagen, Hilden, Germany). Purified DNA was resuspended in 0.9% sodium chloride at the final concentration of 1 mg/ml. The amount of endotoxin in these preparations was estimated to be <0.06 EU/mg by *Limulus* amebocyte lysate Pyrogent (BioWhittaker, Walkersville, MD). Mice received one to three s.c. injections of 100 μg DNA (recombinant plasmids or mock vector) in the footpad. Two weeks after the last boost, mice were challenged in the left flank with B16 or B16-OVA cells as described above.

In vitro CTL induction

Animals were killed 2 wk after the last boost of vaccine, and their spleens were removed and processed individually. A single cell suspension was obtained by smashing and filtering the organ through a sterile cell strainer (70 μm; Becton Dickinson, Franklin Lakes, NJ). Thirty million splenocytes were resuspended in 10 ml RPMI 1640 containing 10% heat inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (culture medium) and cultured in a T25 flask with 3×10^6 mitomycin-c-treated (25) B16-OVA cells, or 1 μM TRP-2 synthetic peptide in 10 ml culture medium. After 4 days, blasts were isolated on a lympholyte-M gradient (Cedarlane, Hornby, Ontario, Canada), cultured for an additional day in medium supplemented with 20 IU/ml human rIL-2, and tested for cytolytic activity in a 4-h ^{51}Cr release assay (25).

Statistical analysis

Statistical analyses were performed using the log-rank and the Wilcoxon tests. Comparison of survival curves was considered statistically significant for $p < 0.05$.

Results

CTL play a major role in the rejection of immunogenic tumors (26). OVA is a well characterized Ag and generates the immunodominant CD8$^+$ T cell epitope OVA$_{257–264}$ (27). Therefore, OVA represents an ideal Ag to compare the relative immunization potential of peptide-pulsed DC and naked DNA injected s.c., or synthetic peptides mixed with the LTR72 toxin given intranasally.

As previously reported (24), a single injection of OVA$_{257–264}$-pulsed DC elicited CTL, which specifically killed both B16-OVA melanoma cells and EL-4 cells pulsed with OVA$_{257–264}$, and not unpulsed EL-4 cells or the NK cell target YAC-1 (data not shown). For both DNA and mucosal vaccination, at least two boosts of the vaccine were required for in vitro detection of OVA-specific CTL,

which increased after the third and fourth boosts, respectively (data not shown).

To evaluate the functional activity in vivo of the immune response induced by the three vaccinations, 2 wk after the last boost of DNA (three injections), or peptide-pulsed DC (one injection only) or 1 wk after the fourth nasal dose of peptide-LTR72 vaccine, animals were challenged s.c. with 10^5 B16-OVA cells. The three vaccines induced a comparable protective effect, with 60% of the animals rejecting the tumor challenge (Fig. 1). Therefore, all three vaccines were effective in inducing tumor-specific CTL and exerted similar protective effects against B16-OVA.

To verify the efficacy of the three vaccines at inducing B16-specific CTL, animals were immunized with TRP-$2_{181–188}$-pulsed DC, TRP-2 DNA, or TRP-$2_{181–188}$-LTR72 mixture, and sacrificed 1 (for mucosal vaccination) or 2 wk (for the other two vaccination strategies) after the last boost. Spleen cells from individual animals were restimulated in vitro with the synthetic peptide corresponding to the epitope sequence TRP-$2_{181–188}$ and tested in a standard cytotoxicity assay 5 days later. As depicted in Fig. 2, where the data

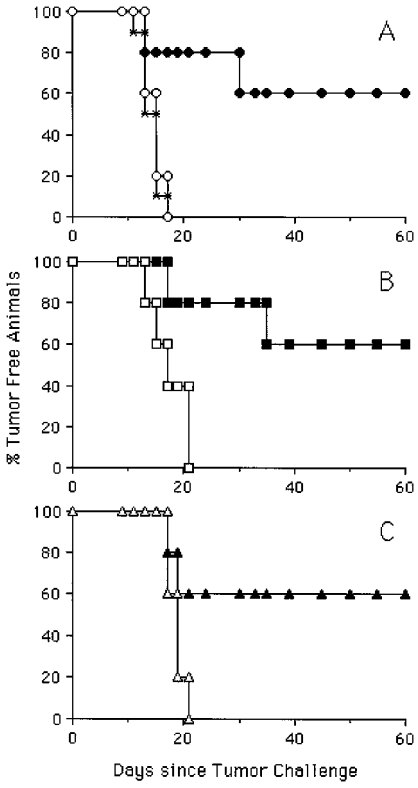

FIGURE 1. Comparable protective efficacy of $OVA_{257–264}$-pulsed DC, naked OVA DNA, and $OVA_{257–264}$-LTR72 mucosal vaccination. C57BL/6 mice were immunized by (*A*) a single s.c. injection of 2×10^5 irradiated $OVA_{257–264}$-pulsed DC ($n = 5$, ●), unpulsed DC ($n = 5$, ○), or PBS ($n = 10$, ∗); (*B*) three s.c. injections of 100 μg of OVA DNA ($n = 5$, ■) or mock DNA ($n = 5$, □); and (*C*) four intranasal doses of a $OVA_{257–264}$ (60 μg)-LTR72 (6 μg) mixture ($n = 5$, ▲), or LTR72 toxin only ($n = 5$, △). One week after mucosal vaccination, or 2 wk after the last boost of peptide-pulsed DC or naked DNA, mice were challenged s.c. in the left flank with 10^5 B16-OVA cells (i.e., 10-fold the minimal tumorigenic dose). Animals were scored positive when the mean of the two perpendicular diameters of the tumor was >2 mm. Values are expressed as percentage of tumor-free animals at the indicated time after tumor challenge. Statistical comparison, conducted by the Log-Rank test, of the survival curves gave the following results: $OVA_{257–264}$-pulsed DC vs unpulsed DC, $p < 0.0228$; OVA DNA vs mock DNA, $p < 0.0185$; $OVA_{257–264}$-LTR72 mixture vs LTR72, $p < 0.038$.

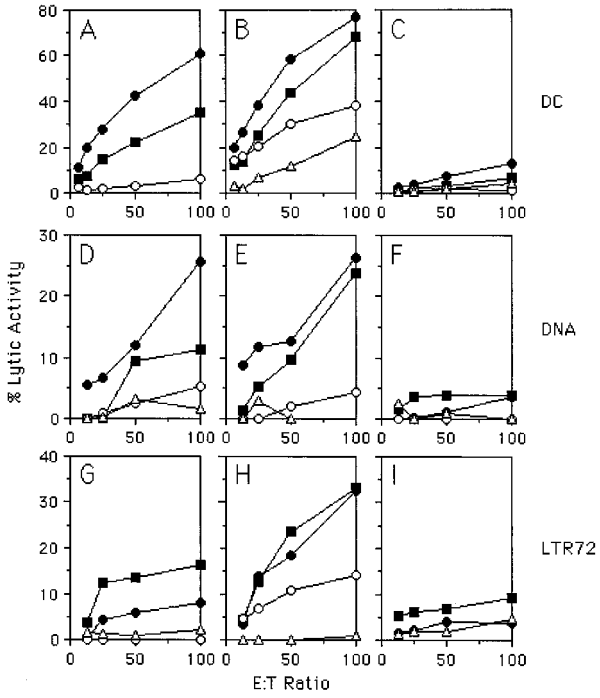

FIGURE 2. TRP-$2_{181–188}$-pulsed DC, naked OVA DNA, and TRP-$2_{181–188}$-LTR72 vaccination induce B16-specific CTL. Mice were immunized with 1× (*A*), or 3× (*B*) TRP-$2_{181–188}$-pulsed DC, 3× unpulsed DC (*C*), 1× (*D*), or 3× (*E*) TRP-2 DNA, or 3× mock DNA (*F*), 2× (*G*), or 4× (*H*) TRP-$2_{181–188}$-LTR72 mixture, or 4× LTR72 toxin only (*I*). Two weeks later, spleen cells from each animal were cultured for 5 days in the presence of 1 μg/ml TRP-$2_{181–188}$ and tested in 4-h ^{51}Cr release assays. Curves represent the cytotoxicity against B16 (■), TRP-$2_{181–188}$-pulsed EL-4 (●), EL-4 (○), and YAC-1 cells (△). Values are expressed as percentage (average of triplicates) of specific ^{51}Cr release (% Lytic Activity) at the indicated E:T ratios. The SD of the triplicate determinations for each point of the curves was <10%, and spontaneous release never exceeded 25%. The data shown represent the best level of cytotoxic activity detected in more than three independent experiments.

shown represent the best level of cytotoxicity detected in more than three independent experiments, all three vaccines induced B16-specific CTL. A single injection of TRP-$2_{181–188}$-pulsed DC induced a vigorous CTL activity against both TRP-$2_{181–188}$-pulsed EL-4 cells and B16 cells, and not against unpulsed EL-4 cells (Fig. 2*A*). After three injections of the vaccine, the specific CTL activity further increased, with the appearance also of an unspecific cytolytic activity, as demonstrated by the lysis of unpulsed EL-4 and YAC-1 cells (Fig. 2*B*). The finding that spleen cells from mice receiving three boosts of unpulsed DC never killed B16 cells (Fig. 2*C*) rules out the possibility of CTL induction either by an unspecific DC-mediated effect, or by in vitro priming with the synthetic peptide.

Also, DNA (Fig. 2, *D* and *E*) and mucosal vaccinations (Fig. 2, *G* and *H*) induced melanoma-specific CTL, although with a lower cytolytic potential. Three injections of TRP-2 DNA and four doses of TRP-$2_{181–188}$-LTR72 mixture were needed to detect a significant lysis against B16. No lysis was obtained with spleen cells from animals immunized with mock DNA vector or LTR72 toxin (Fig. 2, *F* and *I*).

Vaccinated mice were challenged with 5×10^4 B16 cells to compare the ability of the three vaccines to induce a protective immune response against B16. Almost 40% of the animals vaccinated with TRP-$2_{181–188}$-pulsed DC rejected the melanoma cells (Fig. 3), and the latency and survival time of vaccinated animals

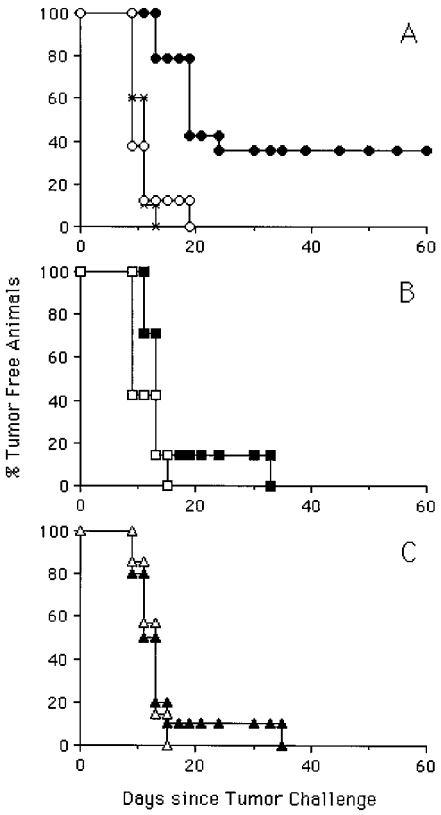

FIGURE 3. Only TRP-$2_{181–188}$-pulsed DC elicit protection against B16. C57BL/6 mice were immunized by one of the following vaccines: (*A*) three s.c. injections of TRP-$2_{181–188}$-pulsed ($n = 14$, ●), or unpulsed DC ($n = 8$, ○) or PBS ($n = 10$, ∗); (*B*) two s.c. injections of TRP-2 ($n = 7$, ■) or mock DNA ($n = 5$, □); (*C*) four intranasal doses of a TRP-$2_{181–188}$-LTR72 mixture ($n = 5$, ▲), or LTR72 toxin only ($n = 5$, △) by intranasal route. One week after mucosal vaccination, or 2 wk after the last boost of peptide-pulsed DC or naked DNA, mice were challenged s.c. in the left flank with 5×10^4 B16 cells (i.e., 10-fold the minimal tumorigenic dose). Animals were scored positive when the mean of the two perpendicular diameters of the tumor was >2 mm. Values are expressed as percentage of tumor-free animals at the indicated time after tumor challenge. Statistical comparison, conducted by the Log-Rank test, of the survival curves gave the following results: TRP-$2_{181–188}$-pulsed DC vs unpulsed DC, $p < 0.0002$; TRP-2 DNA vs mock DNA, $p < 0.1094$; TRP-$2_{181–188}$-LTR72 mixture vs LTR72, $p < 0.1518$.

were significantly prolonged when compared with the controls (Table I). However, at least three injections of the vaccine were required to obtain a prophylactic effect.

In contrast, immunization with TRP-2 DNA or TRP-$2_{181–188}$-LTR72 mixture failed to induce a protective immunity (Fig. 3, and Table I). Animals receiving two injections of TRP-2 DNA or four doses of TRP-$2_{181–188}$-LTR72 mixture showed only some delay in the appearance of the tumor and in the animal survival, which, however, were not statistically significant.

Prophylactic experiments in mice are a very useful means to compare different vaccination strategies against cancer, but they may have a limited clinical relevance in man. Therefore, we tested our most efficient vaccination strategy (i.e., TRP-2-pulsed DC) in tumor-bearing mice. Because many of the reports in cancer immunotherapy suggest that the target of a specific immunotherapy would be the cure of patients carrying minimal tumor-residual diseases (e.g., Refs. 10 and 11), and because of the high aggressiveness of the B16 melanoma, we started the therapeutic regimen 24 h after s.c. implantation of 5×10^4 of B16 cells (i.e., 10-fold the minimal tumorigenic dose). We previously documented that, 24 h after the s.c. injection of 5×10^4 of B16 cells, melanoma cells were clearly visible and proliferating (several mitosis) at the site of injection (4). A therapeutic regimen of three weekly s.c. injections of TRP-2-pulsed DC caused the delay in the appearance of the tumor in 4/10 challenge mice, and permanent (>60 days) melanoma rejection in 2/10 animals, while all 10 control animals vaccinated with unpulsed DC developed the tumor by day 14 (Fig. 4). Moreover, the difference of the survival curves of the two groups was statistically significant ($p < 0.0003$).

Discussion

Our findings demonstrate that the Ag used in preclinical models of vaccination has a relevant impact on the therapeutic outcomes and therefore biases the validation of immunotherapeutic strategies.

All three vaccines used in this study were effective when the strongly immunogenic Ag OVA was used, even though peptide-pulsed DC showed a more favorable ratio between the number of boosts and the induction of a specific immune response. Indeed, a single immunization with peptide-pulsed DC was as protective as three injections of OVA DNA or four doses of peptide-toxin mixture. A more quantitative comparison cannot be done because the

Table I. *TRP-2-pulsed DC are the most efficient vaccination strategy against the B16 melanoma*

Vaccine[a]	No. of Injections[b]	Take (protection)[c]	Latency (days)[d]	Survival (days)[e]
PBS	4	10/10 (0)	10.1 ± 0.2	16.9 ± 0.5
DC	3	8/8 (0)	10.7 ± 1.2	16.1 ± 1.3
DC + TRP-$2_{181–200}$	1	10/10 (0)	11.5 ± 0.4	17.1 ± 0.7
	3	9/14 (36)	18.7 ± 1.4	23.2 ± 1.1
Vector	3	5/5 (0)	11.4 ± 1.4	17.8 ± 0.5
TRP-2 DNA	1	10/10 (0)	13.1 ± 1.2	20.1 ± 1.2
	2	7/7 (0)	15.0 ± 3.0	21.6 ± 3.1
	3	7/7 (0)	11.4 ± 0.3	18.0 ± 0.9
LTR72	4	7/7 (0)	10.8 ± 0.8	16.6 ± 0.6
LTR72 + TRP-$2_{181–200}$	4	10/10 (0)	14.2 ± 3.6	21.3 ± 3.7

[a] Mice were injected s.c. in the right flank with 100 μl of one of the following vaccines: PBS, 2×10^5 unpulsed, or TRP-$2_{181–188}$-pulsed DC, 100 μg TRP-2 DNA, or vector only. For mucosal vaccination, mice were intranasally exposed to 100 μl of the LTR72 (6 μg)-TRP-$2_{181–200}$ (60 μg) mixture or to the LTR72 toxin only (6 μg). Vaccinated mice were challenged s.c. with 5×10^4 B16 cells 1 wk (for mucosal vaccination) or 2 wk (for the other vaccination procedures) after the last vaccine boost.

[b] Mice received the indicated number of vaccine boosts.

[c] Numbers represent tumor-bearing animals/total number of animals at day 60 after the challenge. The percentage of tumor-free animals is reported in parenthesis.

[d] Numbers indicate the arithmetic mean ± SE of the time of appearance of the tumor. Results of the statistical analysis are reported in the legend to Fig. 2.

[e] Numbers indicate the arithmetic mean ± SE of the survival time of the challenged animals. Statistical comparison, carried out by the log-rank test, of the survival curves of mice injected three times with DC or TRP-$2_{181–200}$-pulsed DC gave the following result: $p < 0.0003$. In all other vaccination conditions, the comparison was not statistically significant.

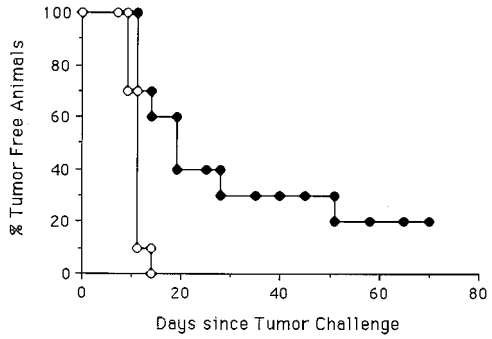

FIGURE 4. Treatment of B16 melanoma-bearing mice with TRP-2-pulsed DC induces tumor rejection. Mice were challenged s.c. with 5×10^4 B16 cells. One day later, mice were immunized with three s.c. injections of TRP-$2_{181–188}$-pulsed ($n = 10$, ●) or unpulsed DC ($n = 10$, ○). Animals were scored positive when the mean of the two perpendicular diameters of the tumor was >2 mm. Values are expressed as percentage of tumor-free animals at the indicated time after tumor challenge. Statistical comparison, conducted by the log-rank test, of the survival curves of the two experimental condition gave the following result: $p < 0.0003$.

amount of Ag used was different for the three vaccines (i.e., 5 μg/ml of synthetic peptide for DC pulsing in vitro; 100 μg DNA; and 60 μg of synthetic peptide for mucosal vaccination). Moreover, the DNA used for vaccination contains CD8 and CD4 epitopes, therefore allowing activation of both T cell populations. Nevertheless, the data obtained with OVA demonstrated that, under the conditions tested, the three vaccines have similar effects in terms of Ag-specific CTL induction and tumor protection.

When TRP-2 was used as a more realistic tumor model, all three vaccines induced B16-specific CTL. However, at variance with the OVA model, only peptide-pulsed DC elicited a protective response against B16. Moreover, TRP-2-pulsed DC were effective in preventing the growth of 24-h preestablished melanomas. These data demonstrate the higher immunogenic potential of peptide-pulsed DC and confirm that there is no direct correlation between the in vitro finding of a tumor-specific CTL response and the in vivo functional activity against a growing tumor (11). However, it is particularly intriguing that this discrepancy was found only when the self TRP-2 Ag was used. A testable hypothesis is that B6 mice bear more CTL precursors for the OVA than for the TRP-2 determinant. OVA is a nonself Ag, hence the anti-OVA T cell repertoire should not be subject to the rules of central and peripheral tolerance (28). OVA vaccination should elicit both high- and low-avidity CTL. At variance, TRP-2 is a self Ag, and could delete or tolerize most of the high-avidity CTL repertoire, while sparing the low-avidity one (29). However, high-avidity CTL for the TRP-2 epitope can be found in B6 animals, primed in vivo with the very immunogenic B16 melanoma cells expressing GM-CSF (7, 30). Therefore, it can be hypothesized that all three vaccinations elicited low-avidity OVA-specific CTL, but only three injections of TRP-$2_{181–188}$-pulsed DC also allowed activation of high-avidity TRP-2-specific CTL. Given that high-avidity CTL exert a much stronger antitumor activity in vivo than the low-avidity ones, only vaccination with DC can disclose protection against the challenge with living tumor cells (30).

The amount of Ag displayed by the target cells might also influence the clinical outcome. Indeed, B16 cells express very low amounts of MHC class I molecules on the cell surface (4). K^b-TRP-$2_{181–188}$ complexes on the target cell might not be enough in vivo for triggering the killing machinery of low-avidity CTL.

Finally, the higher immunogenic potential of the OVA antigenic determinant may reside in its MHC binding affinity (31). Indeed, K^b binding and stabilization assays performed on RMA-S cells showed that TRP-$2_{181–188}$ has a 3- to 4-fold lower binding affinity than $OVA_{257–264}$ (not shown). Therefore, the resulting K^b-TRP-$2_{181–188}$ complex on DC in vivo, involved in DNA and mucosal vaccinations (14, 19), should be much less stable, disfavoring the sustained interactions with specific TCR necessary to prime naive T cells (32). It is conceivable that only injection of relatively high numbers of DC, pulsed in vitro with a high amount of peptide, can directly supply T cells with both the appropriate Ag density and the whole repertoire of costimulatory signals (9) to elicit an optimal protective CTL response against the weakly immunogenic B16 melanoma.

Collectively, our results also show that, in this model and under the experimental conditions tested, peptide-pulsed DC are the most powerful vaccine. A deeper analysis of the vaccination properties of these strategies is required. Indeed, several reports have been published aimed at improving each one of the vaccination procedures adopted in this work. As an example, it has been reported that generation of tumor immunity by DC correlates with their maturation stage (33). DNA vaccination efficacy can increase by the preparation of plasmids containing several cytokines, costimulatory molecules, or a recall Ag (34). Finally, different doses and sites of Ag exposure could be used for mucosal vaccination.

The finding that, depending on the Ag model used, dramatically different results can be obtained in terms of therapeutic activity against cancer, underscores the need to rigorously choose the most realistic experimental model for validation of new immunotherapies.

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