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

Matteo Bellone, Daniela Cantarella, Paola Castiglioni, Maria Cristina Crosti, Anna Ronchetti, Monica Moro, Maria Paola Garancini, Giulia Casorati and Paolo Dellabona

*J Immunol* 2000; 165:2651-2656; doi: 10.4049/jimmunol.165.5.2651

http://www.jimmunol.org/content/165/5/2651

**References** This article cites 31 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/165/5/2651.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Relevance of the Tumor Antigen in the Validation of Three Vaccination Strategies for Melanoma

Matteo Bellone, Daniela Cantarella, Paola Castiglioni, Maria Cristina Crosti, Anna Ronchetti, Monica Moro, Maria Paola Garancini, Giulia Casorati and Paolo Dellabona

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-2181-188 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 nonspecific, and induce vigorous responses due to the lack of tolerance. By contrast, most of the naturally expressed tumor-associated Ags (TAA) belong to self, and therefore may elicit an efficient 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 et al. identified in the sequence TRP-2181-200 a CTL epitope recognized within the Kb MHC class I molecule, and reported that passive transfer of TRP-2181-200-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).
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 xenogenic 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-2b) 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 melanoma Ag model 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′-CTGGGTACCAAAGGACAGCACATGACAC-3′ (5′ primer) and 5′-GCCGTTGAATCAGGGGAACACA-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.

In vitro culture

DC were prepared from bone marrow as described (24). On day 7–9 of 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^d 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 OVA257–264 (a gift of Dr. B. M. Conti-Fine, St. Paul, MN) or TRP-2_181–189 (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.

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.

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 staining 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 μM 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 ^51Cr 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 OVA257–264 (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 OVA257–264 pulsed DC elicits CTL, which specifically killed both B16-OVA melanoma cells and EL-4 cells pulsed with OVA257–264 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 presented are the cytotoxicity against B16 (Fig. 1, panels A-D), EL-4 (Fig. 1, panels E-H), and YAC-1 cells (Fig. 1, panels I-L). Values are expressed as percentage of tumor-free animals 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.

![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 x 10<sup>5</sup> 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 < 0.0028; 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<sub>181-188</sub> and tested in 4-h 51Cr release assays. Curves represent the cytotoxicity against B16 (□), TRP-2<sub>181-188</sub>-pulsed EL-4 (●), EL-4 (○), and YAC-1 cells (△). Values are expressed as percentage (average of triplicates) of specific 51Cr 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, all three vaccines induced B16-specific CTL. A single injection of TRP-2<sub>181-188</sub>-pulsed DC induced a vigorous CTL activity against both TRP-2<sub>181-188</sub>-pulsed EL-4 cells and B16 cells, and not against unpulsed EL-4 cells (Fig. 2A). 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. 2B). The finding that spleen cells from mice receiving three boosts of unpulsed DC never killed B16 cells (Fig. 2C) 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<sub>181-188</sub>-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 x 10<sup>4</sup> 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<sub>181-188</sub>-pulsed DC rejected the melanoma cells (Fig. 3), and the latency and survival time of vaccinated animals...
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\textsubscript{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\textsubscript{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 $5 \times 10^4$ of B16 cells (i.e., 10-fold the minimal tumorigenic dose). We previously documented that, 24 h after the s.c. injection of $5 \times 10^4$ 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>
<thead>
<tr>
<th>Vaccine</th>
<th>No. of Injections</th>
<th>Take (protection)</th>
<th>Latency (days)</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>4</td>
<td>10/10 (0)</td>
<td>10.1 ± 0.2</td>
<td>16.9 ± 0.5</td>
</tr>
<tr>
<td>DC</td>
<td>3</td>
<td>8/8 (0)</td>
<td>10.7 ± 1.2</td>
<td>16.1 ± 1.3</td>
</tr>
<tr>
<td>DC + TRP-2\textsubscript{181-200}</td>
<td>1</td>
<td>10/10 (0)</td>
<td>11.5 ± 0.4</td>
<td>17.1 ± 0.7</td>
</tr>
<tr>
<td>Vector</td>
<td>3</td>
<td>5/5 (0)</td>
<td>11.4 ± 1.4</td>
<td>17.8 ± 0.5</td>
</tr>
<tr>
<td>TRP-2 DNA</td>
<td>1</td>
<td>10/10 (0)</td>
<td>13.1 ± 1.2</td>
<td>20.1 ± 1.2</td>
</tr>
<tr>
<td>LTR72</td>
<td>2</td>
<td>7/7 (0)</td>
<td>15.0 ± 3.0</td>
<td>21.6 ± 3.1</td>
</tr>
<tr>
<td>LTR72 + TRP-2\textsubscript{181-200}</td>
<td>4</td>
<td>7/7 (0)</td>
<td>10.8 ± 0.8</td>
<td>16.6 ± 0.6</td>
</tr>
</tbody>
</table>

* Mice were injected s.c. in the right flank with 100 μl of one of the following vaccines: PBS, 2 × 10\textsuperscript{5} unpulsed, or TRP-2\textsubscript{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\textsubscript{181-200}(60 μg) mixture or to the LTR72 toxin only (6 μg). Vaccinated mice were challenged s.c. with $5 \times 10^3$ B16 cells 1 wk (for mucosal vaccination) or 2 wk (for the other vaccination procedures) after the last vaccine boost.
* Mice received the indicated number of vaccine boosts.
* 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.
* 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.
* 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\textsubscript{181-200}-pulsed DC gave the following result: $p < 0.0003$. In all other vaccination conditions, the comparison was not statistically significant.
of the survival curves of the two experimental conditions gave the following result: \( p < 0.0003 \).

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.

Acknowledgments

We thank G. Consogno for excellent technical assistance, M. P. Protti for critical reading of the manuscript, and B. M. Conti-Fine, C. Noppen, M. Gutttinger, N. Shastri, and M. Pizza for providing us reagents.

References


