Predicting Tumor Outcome following Cancer Vaccination by Monitoring Quantitative and Qualitative CD8$^+$ T Cell Parameters[1]

Antonio Rosato,[2,3]* Alessia Zoso,[3,4]* Silvia Dalla Santa,* Gabriella Milan,† Paola Del Bianco,‡ Gian Luca De Salvo,‡ and Paola Zanovello[2]*

Identification of reliable surrogate predictors for evaluation of cancer vaccine efficacy is a critical issue in immunotherapy. We analyzed quantitative and qualitative CD8$^+$ T cell parameters in a large pool of BALB/c mice that were DNA-vaccinated against P1A self tumor-specific Ag. After immunization, mice were splenectomized and kept alive for a subsequent tumor challenge to correlate results of immune monitoring assays with tumor regression or progression in each individual animal, and to assess the prognostic value of the assays. The parameters tested were 1) percentage of in vivo vaccine-induced tumor-specific CD8$^+$ T cells; 2) results of ELISPOT tests from fresh splenocytes; 3) percentage of tumor-specific CD8$^+$ T cells in culture after in vitro restimulation; 4) in vitro increase of tumor-specific CD8$^+$ T cell population expressed as fold of expansion; and 5) antitumor lytic activity of restimulated cultures. Except for the ELISPOT assay, each parameter tested was shown by univariate statistical analysis to correlate with tumor regression. However, multivariate analysis revealed that only in vitro percentage of Ag-specific CD8$^+$ T cells was an independent prognostic factor that predicted tumor outcome. These findings should be considered in the design of new immune monitoring systems used in cancer immunotherapy studies. ***The Journal of Immunology,*** **2006, 176: 1999–2006.**

Tumor-associated Ags (TAA)[5] recognized by T cells have been used in active immunization aimed at treating cancer patients (1–3). The overall success rate of these cancer vaccine trials has been quite low and the criteria used to define their efficacy have relied too heavily on subjective endpoints rather than objective clinical data. In fact, the current endpoint of most vaccination studies is evaluation of immune response by standardized assays that provide a biological surrogate predictor for vaccine efficacy (4). However, the correlation of these immunoassays and/or bioactivity endpoints to actual clinical benefit and success of therapy is largely unknown. Sensitive techniques such as tetramer or ELISPOT assays have been used to demonstrate the generation of antitumor T cells in vaccinated patients, but the scarcity of clinical response in these patients has made it difficult to validate the utility of these assays. Indeed, no study to date has firmly established the association between clinical response and antivaccine T cell response (4, 5). Therefore, identifying a laboratory assay that can function as a surrogate predictor for the assessment of clinical benefit is critical to the development of valid anti-cancer immunotherapeutic strategies (6, 7).

Most preclinical studies in mice that have attempted to correlate T cell responses and antitumor efficacy did not evaluate the clinical benefit of vaccination and T cell activity in the same mice. Moreover, T cell activity is often assessed in cultures of pooled splenocytes from a group of vaccinated mice, thus precluding the emergence of individual response variability that can be observed in single mice. Several reports have clearly indicated that even within the same genetic background, the individual capacity to mount an immune response may greatly vary (8–11).

In an attempt to fill this gap, we reproduced, in an experimental mouse model, what is currently done in clinical studies of patients undergoing antitumor vaccination, in which the response is analyzed individually. As more recent vaccination protocols against human cancers tend to enroll patients with low or minimal tumor burden, we decided to perform our study in a prophylactic setting, thus avoiding immune system perturbations induced by the tumor itself. Under these controlled conditions, it was possible to define the validity of various immune assays in monitoring cancer vaccine efficacy, calculating the statistical correlation of each parameter with tumor outcome, and assessing whether select assays may be ultimately regarded as reliable surrogate predictors or correlates of protection.

Although this approach is obviously easier in a preclinical experimental model, as well as not being biased by clinical variables, the lack of multiple physiological TAA produced from genes not expressed in normal tissues and comparable to those previously identified in humans remains a drawback. One of the few identified mouse tumor Ags that originates from a normal gene is P1A. Our previous studies showed that vaccination of BALB/c mice with a

*Department of Oncology and Surgical Sciences and †Department of Medical and Surgical Sciences, University of Padova, Padova, Italy; and ‡Clinical Trials and Biostatistics Unit, Istituto Oncologico Veneto, Padova, Italy

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[2] Address correspondence and reprint requests to Dr. Antonio Rosato or Dr. Paola Zanovello, Department of Oncology and Surgical Sciences, University of Padova, Via Gattamelata 64, I-35128 Padova, Italy. E-mail addresses: antonio.rosato@unipd.it or paola.zanovello@unipd.it

[3] A.R. and A.Z. contributed equally to this study.

[4] Current address: Department of Pathology and Medicine, The Johns Hopkins School of Medicine, Baltimore, MD 21205.

[5] Abbreviations used in this paper: TAA, tumor-associated Ag; ROC, receiver operating characteristic; AUC, area under the curve; MLPC, mixed leukocyte-peptide culture.

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DNA plasmid coding for P1A Ag strongly induced CTL that recognized the P1A$_{35-43}$ epitope in the context of the L^d MHC class-I molecule. However, only ~50% of vaccinated mice were protected against challenge with J558 plasmacytoma tumor cells expressing the relevant Ag (9, 11). This situation closely resembles the human setting where objective clinical responses are often lacking despite documentation of immune responses (4, 12–14).

To identify which surrogate predictor of antitumor immune response would correlate with progression or regression of tumors, we investigated quantitative and qualitative CD8$^+$ T cell parameters in a large pool of mice immunized against the P1A self tumor-specific Ag, and subsequently challenged with a syngeneic Ag-expressing tumor.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/c mice (H-2^d) were purchased from Charles River Laboratories. Procedures involving animals and their care conformed with institutional guidelines that comply with national and international laws and policies and were reviewed by the local ethical committee.

Cell lines and CTL clone

J558 is a myeloma cell line induced in BALB/c mice by mineral oil injection and positive for P1A Ag. IR5P1A$^-$ is a P1A-loss variant isolated from a P815 tumor (9). LDA-5, a P1A-specific CTL clone, was obtained by limiting dilution assay of splenocytes derived from a DNA-immunized mouse (15). Culture media and conditions have been reported previously (11, 15).

Expression vector and DNA-based immunization protocol

The P1A-expressing vector pBKCMV-P1A and its preparation have been described previously (9). For DNA-based immunization, mice were anesthetized by a mix of ketamine and xylazine (100 and 10 mg/kg, respectively) and then injected i.m. with 100 μl of cardiotoxin (2.5 μM in saline solution; lot no. 407.000; Latoxan) in each tibialis anterior muscle. Five days later, 50 μl of saline solution containing 25 μg of plasmid DNA was injected into each tibialis anterior muscle for a total of 50 μg/mouse. Control mice were injected with pBKCMV empty plasmid.

Synthetic peptides and mixed leukocyte-peptide cultures (MLPC)

The MHC class I L^d-restricted peptide, corresponding to aa 35–43 of P1A protein (LPYLGWLVF), was synthesized and purified by Tecnogen. Fourteen days after plasmid DNA inoculation, mice were anesthetized and underwent splenectomy. MLPC cell cultures were conducted as described previously (16), and after 5 days of incubation, cells were analyzed by flow cytometry and tested for their lytic activity in a ^{51}Cr-release assay.

Magnetic cell separation (MACS)

Immunomagnetic separation of CD8$^+$ cells was conducted with a MiniMACS system (Miltenyi Biotec), according to the manufacturer's specifications.

IFN-γ-detecting ELISPOT assay

The ELISPOT assay was conducted using the R&D Systems reagents (R&D Systems). Splenocytes were resuspended at a concentration of 5 × 10^6 per ml in DMEM 10% FBS, and added with P1A peptide, at a concentration of 1 μM, and rIL-2 (20 IU/ml). One hundred microliters of cells was plated in duplicate on microtiter plates and incubated at 37°C and 5% CO_2 for 24 h. For color development, the ELISPOT Blue Color Module (R&D Systems) was used. Spots were quantified using an automated ELISPOT reader (Transtec 1300; Amplimedical Bioline).

Synthesis of MHC/peptide tetrameric complexes

A detailed description of tetramer construction has been reported elsewhere (15). The MHC-peptide complex was enzymatically biotinylated with the BirA enzyme (Avidity), following the manufacturer's instructions, and separated from free biotin by gel-filtration chromatography. PE-labeled tetramers were produced by mixing the biotinylated complexes with Extravidin-PE (Sigma-Aldrich) and validated by staining CTL clones with the appropriate specificity. Each tetramer batch was titrated and used at the optimum concentration (5 μg/ml) of L^d H chain.

Cell staining and flow cytometry analysis

For in vivo tetramer staining, fresh splenocytes (3 × 10^6 per sample) from each mouse were resuspended in 100 μl of cytometry buffer (0.9% NaCl solution containing 2% BSA and 0.02% NaN_3; both from Sigma-Aldrich), and incubated with 2 μl of anti-mouse FcγR 2.4G2 mAb ascites (ATCC HB-197) for 10 min at room temperature to reduce nonspecific staining. After washing, cells were resuspended in 50 μl of cytometry buffer and labeled with L^d-P1A$_{35-43}$ tetramer-PE (P1A-tet, 5 μg/ml) for 20 min at room temperature. Each sample was then stained with rat anti-mouse CD8-Tri-color (0.1 μg/10^6 cells, clone CTCD8α; Caltag Laboratories) and with hamster anti-mouse CD3-FITC (1 μg/10^6 cells, clone 145-2C11; Caltag Laboratories). For in vitro tetramer staining, restimulated spleen cells (10^6 per sample) were incubated with 2.4G2 Ab and with P1A-tet, as describe above. Cells were then incubated with rat anti-mouse CD8-FITC mAb (0.1 μg/10^6 cells, clone CTCD8α; Caltag Laboratories) for 20 min at 4°C. LDA-5 CTL clone was used as a positive control, whereas negative controls were splenocytes derived from pBKCMV-injected mice. Every sample was also stained with a control tetramer-PE (Ctr-tet, L^d-gp70) according to the described protocols (15). Cytofluorimetric analyses were performed on a flow cytometer FACSCalibur (BD Biosciences), and data analysis was conducted using the CellQuest software (BD Biosciences). To calculate the "fold of expansion" parameter, we assumed that all primed cells underwent proliferation without considering concomitant event of T cell death and used the following formula: fold of expansion = *ln* (*n* P1A-specific CD8$^+$ T cells after in vitro restimulation) − *ln* (*n* P1A-specific CD8$^+$ T cells before in vitro restimulation).

Chromium release assay

Cytolytic activity of different MLPC was measured as described elsewhere (16). J558 tumor cells and IR5-P1A$^-$ cells pulsed or not with P1A peptide were used as target cells. For peptide pulsing, 10^6 ^{51}Cr-labeled target cells per milliliter were incubated with the relevant peptide (1 μM final concentration) for 30 min at 37°C, and then washed twice before use. After 4 h of incubation at 37°C, supernatants were harvested and radioactivity was counted in a microplate scintillation counter (Top-Count; Packard Instruments). Cytotoxicity was expressed either as percentage of lysis or as LU_{30}. One lytic unit was defined as the number of effector cells capable of killing 30% of the target cells, and results were expressed as LU_{30}/culture (17).

Tumor protection assay

Three weeks after DNA immunization, mice were challenged s.c. with 2.5 × 10^6 J558 tumor cells, and tumor growth was monitored twice a week by caliper measurements for a total of 60 days after tumor inoculation. Tumor volume (TV) was calculated using the formula: TV (mm^3) = $D \times d^2/2$, where D and d are the longest and the shortest diameters, respectively. Animals with tumors growing progressively or undergoing regression were referred to as progressor or regressor mice, respectively. Mice injected with pBKCMV empty plasmid served as negative controls. Splenectomy after vaccination and before tumor inoculation did not alter the response to tumor challenge, as survival rates of vaccinated mice undergoing splenectomy were not significantly different from those of animals having intact spleen (data not shown and Ref. 11). The in vivo tumor growth experiments were conducted according to the United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (18), and animals were sacrificed by CO_2 overdose.

Statistical analysis

The association of CD8$^+$ T cell parameters with tumor outcome was evaluated by both univariate and multivariate analyses. The Mann-Whitney U test was used to compare the distribution of surrogate predictors between control and vaccinated animals, and between progressor and regressor mice. Medians were calculated and reported with their range and p value. All variables entered a multiple logistic regression model. A stepwise variable selection procedure was used to identify the factors that contributed additional prognostic information to the multivariate model for survival. The accuracy of factors to discriminate between survival status of mice was verified by the area under the receiver operating characteristic (ROC) curve (area under the curve (AUC)). A rough guide for classifying the accuracy of a diagnostic test is a traditional point system: 0.90–1, excellent; 0.80–0.90, good; 0.70–0.80, fair; 0.60–0.70, poor; and 0.50–0.60, fail (19). Survival curves and probabilities were estimated using the Kaplan-Meier technique. Log-rank test for comparisons was used when required. Analyses of data were performed using the SAS statistical package (SAS, release 8.02).

Results

Antitumor activity of plasmid DNA vaccination against the P1A tumor-specific Ag

DNA-based immunization against P1A tumor Ag was performed in 84 BALB/c mice by i.m. inoculation of pBKCMV-P1A plasmid, whereas a control group of 11 mice received the empty plasmid. To obtain splenocytes for the in vivo and in vitro immune monitoring assays, 2 wk after the immunization procedure, all treated mice underwent splenectomy and were kept alive for subsequent tumor challenge. One week after splenectomy, mice were injected s.c. with J558 tumor cells. Tumor growth and survival were monitored for 2 mo after the vaccination. As reported in Fig. 1*A*, J558 tumors grew rapidly and progressively in all control mice, reaching, in around 3 wk, an average volume of almost 600 mm^3. In P1A-vaccinated mice, a striking dichotomy soon became apparent, as one group of mice ($n = 44$, progressors) showed a kinetics and a tumor growth curve that strictly overlapped those observed in control mice, whereas in the second group of mice ($n = 40$, regressors), J558 tumors grew slowly for 7–12 days and then underwent complete regression. At the end of observation period (60 days), no sign of tumor recurrence was present in regressor mice and the overall survival rate of P1A-vaccinated mice (47.6%) significantly differed from that of control mice (0%, $p < 0.0001$, Mantel-Haenzel test, Fig. 1*B*).

These data strictly parallel those we previously reported (9, 11) and closely resemble results of many clinical vaccination trials, in which immunization is poorly associated with tumor regression. Therefore, this experimental model could be instrumental in investigating the prognostic relevance of surrogate predictors of $CD8^+$ T cell activity.

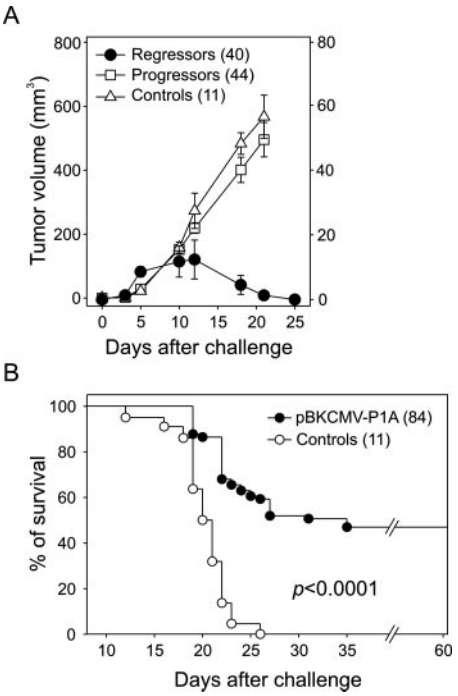

FIGURE 1. Effect of vaccination with plasmid DNA encoding the P1A tumor-specific Ag on tumor growth. *A*, Kinetics of tumor growth in BALB/c mice immunized with pBKCMV-P1A plasmid and then inoculated s.c. with J558 tumor cells expressing the relevant Ag. Mice injected with the empty plasmid served as negative controls. Tumor growth was monitored by caliper measurements and reported as mean volume ± SE. Scale on the *left* refers to tumors of control (△) and progressor mice (□), whereas the scale on the *right* refers to tumors of regressor mice (●). *B*, Cumulative survival of vaccinated (●) and control (○) mice reported in *A*. Immunization with pBKCMV-P1A increased the survival rate after tumor challenge ($p < 0.0001$, Mantel-Haenzel test). The cumulative number of mice used in the experiment is indicated in parentheses in each plot.

Correlation of P1A-specific $CD8^+$ T cells induced in vivo by vaccination with tumor outcome

One criterion that documents anti-vaccine T cell response is the increase in the number of Ag-reactive T cells after vaccination. We first quantified the number of P1A-specific $CD8^+$ T cells in fresh splenocytes obtained from vaccinated and control mice before tumor challenge. Cytofluorimetric analyses with P1A tetramers were performed on immunomagnetically purified $CD8^+$ T cells isolated from each mouse. At the end of the observation period, percentages of P1A-tet^+/$CD8^+$/$CD3^+$ T cells were plotted as a function of tumor growth. As shown in Fig. 2*A*, the percentage of P1A-specific $CD8^+$ T cells in the spleen of control mice was very low (median, 0.05; range, 0.02–0.12) and significantly differed from that observed in vaccinated mice when evaluated as a group (median, 0.14; range, 0.02–1.39; $p = 0.0004$). Conversely, within the group of vaccinated mice, regressors displayed a significantly higher increase in the percentage of P1A-tet^+/$CD8^+$/$CD3^+$ T cells (median, 0.16; range, 0.04–1.39) than progressor mice (median, 0.11; range, 0.02–0.55; $p = 0.0071$). Values detected in vaccinated mice, although still very low, reflected a real increase in the Ag-specific $CD8^+$ T cell population, because staining with an irrelevant tetramer was low and produced results similar to those of control mice. These features are illustrated in Fig. 2*B*, which displays an exemplificative animal for each group selected according to the proximity of its staining to the group median.

With these data, we constructed a ROC curve for "in vivo tetramer staining" parameter (Fig. 2*A*, *inset*) and found that the AUC value was poor, at 0.675. Thus, direct in vivo quantification of Ag-specific $CD8^+$ T cells following vaccination did significantly discriminate between progressor and regressor mice, but was not an accurate diagnostic test.

The ELISPOT assay: a poor predictor of prognosis

The ELISPOT assay, which can directly quantify ex vivo cytokine-releasing cells upon antigenic stimulation, is regarded as a sensitive and reproducible measure of cell-mediated response to vaccination (20). One advantage of this test is very brief restimulation time, which limits potential biases that could be introduced with a prolonged restimulation in vitro. However, the prognostic value of the ELISPOT assay had not been established. To this end, splenocytes from individual mice were seeded in an ELISPOT test and spots were quantified after 24 h of P1A peptide restimulation (Fig. 3). Spot number in the spleen of control mice (median, 26.5; range, 11–61) was affected by a nonspecific response, because a similar number of spots was detected in parallel cultures restimulated with an irrelevant peptide (data not shown). Conversely, splenocytes from vaccinated mice displayed a significantly greater spot number (median, 48; range, 13–179) than control animals ($p = 0.0172$). Yet, when this parameter was plotted as a function of tumor outcome, no significant difference in spot number was observed between regressor ($n = 38$; median, 46.5; range, 20–168) and progressor mice ($n = 43$; median, 48; range, 13–179; $p = 0.4490$). Accordingly, the ROC curve constructed for the ELISPOT parameter had a failed AUC value of 0.509 (Fig. 3, *inset*).

Thus, it appears that the ELISPOT assay has mainly qualitative value, as it can reliably identify vaccinated vs normal mice, but is not prognostically relevant because it cannot discriminate between regressor and progressor mice.

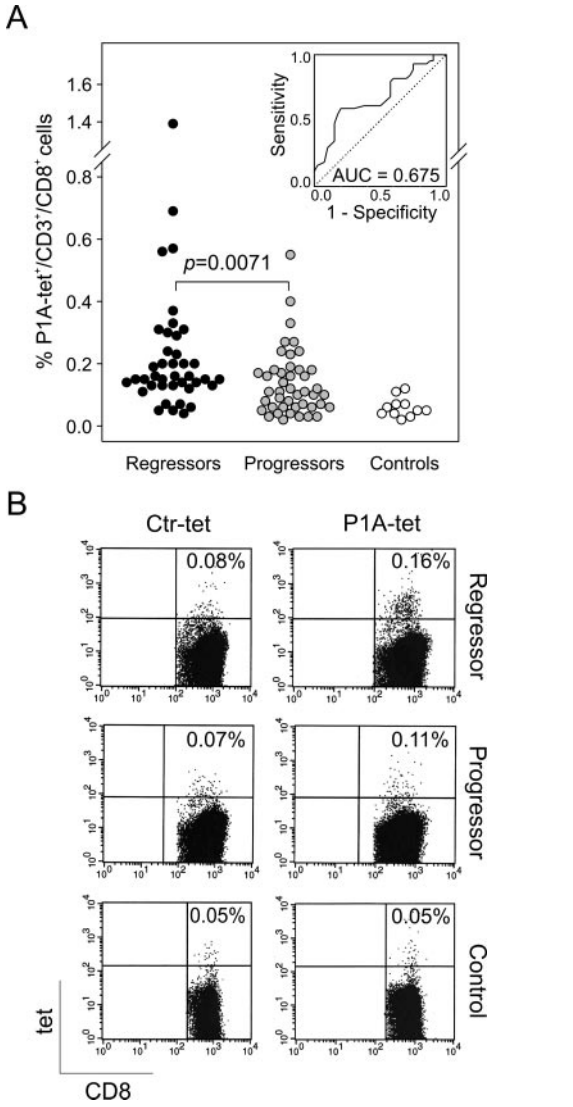

FIGURE 2. In vivo quantification of P1A-specific CD8$^+$ T cells induced by pBKCMV-P1A plasmid immunization and correlation with tumor outcome. *A*, Vaccinated and control mice underwent splenectomy at day 14 after DNA inoculation and were challenged with J588 cells 1 wk later. The percentages of P1A-tet$^+$/CD8$^+$ T cells calculated within the CD3$^+$ population were plotted as a function of progression or regression to tumor challenge. Statistical analysis of data using the Mann-Whitney *U* test disclosed a significant difference ($p = 0.0071$) between regressor (●) and progressor mice (◍). ○, Control mice. The *inset* reports the ROC curve and the relative AUC value for this test. *B*, Representative cytometry analysis of a regressor, a progressor, and a control mouse, respectively, whose percentages of P1A-specific CD8$^+$ T cells were the closest to the median of the respective group. Plots on the *right* refer to staining with the specific tetramer (P1A-tet), whereas plots on the *left* report the background staining obtained with an irrelevant tetramer (Ctr-tet).

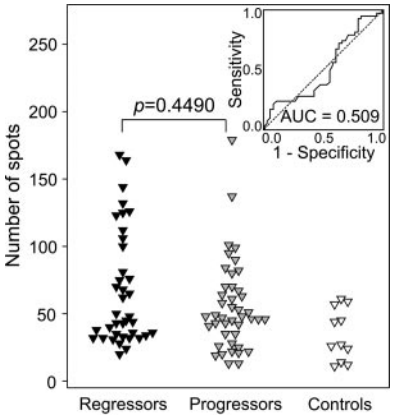

FIGURE 3. Quantification and ELISPOT functional analysis of CD8$^+$ T cells induced by vaccination. Concomitantly with the in vivo tetramer staining assay, splenocytes from immunized and control mice were restimulated with the P1A peptide for 24 h in ELISPOT. After tumor challenge, results of the test were plotted in accordance to tumor regression or progression. Statistical analysis of data using the Mann-Whitney *U* test demonstrated that the ELISPOT assay had no prognostic value ($p = 0.4490$). Regressor, progressor, and control mice are depicted (▼, ▽, ▽) respectively. The *inset* reports the ROC curve and the relative AUC value for this test.

P1A-specific CD8$^+$ T cells after in vitro restimulation

When the number of anti-vaccine T cells is low, a round of in vitro restimulation is commonly performed to expand the population under study. To this end, splenocytes from vaccinated and control mice were restimulated with the P1A peptide, and the percentage of Ag-specific CD8$^+$ T cells was evaluated by tetramer staining. Results were then correlated with tumor outcome after challenge. Fig. 4*A* shows that the percentage of P1A-tet$^+$/CD8$^+$ T cells detected in control mice cultures was negligible (median, 0.40; range, 0.10–0.60) and significantly different from that of the vaccinated mice group (median, 4.15; range, 0.1–39.98; $p < 0.0001$). Cytofluorimetric analysis of a representative animal for each group, selected for the proximity of its staining to the median value of the group, is illustrated in Fig. 4*B*. In control mice, staining with an irrelevant tetramer overlapped that with P1A tetramer, thus indicating that in vitro addition of peptide did not activate unprimed cells. In contrast, in the group of vaccinated mice, regressors presented a striking and statistically significant increase in the percentage of P1A-tet$^+$/CD8$^+$ T cells (median, 9.82; range, 0.4–39.98) compared with that of progressors (median, 2.05; range, 0.10–16.00; $p < 0.0001$). Notably, staining with an irrelevant tetramer always produced a background signal, indicating that the expanded population was effectively P1A-specific (Fig. 4*B*).

When the accuracy of the test was calculated using the ROC curve (Fig. 4*A*, *inset*), we found that the AUC value was good (0.804). Thus, quantifying tumor-specific CD8$^+$ T cells after in vitro Ag-driven amplification constitutes a powerful tool for accurately discriminating between progressor and regressor mice.

The fold of expansion parameter and its correlation with tumor outcome

In vitro restimulation with Ag was followed by expansion of the tumor-specific vaccine-primed T cell population. We assumed that all primed cells underwent stimulation and proliferated without considering the potential concomitant phenomena of T cell death. Based on this assumption and on the results of in vivo and in vitro tetramer staining, we calculated the input and output number of P1A-specific CD8$^+$ T cells at the beginning and end of in vitro cultures. Then, using the equation reported in *Materials and Methods*, we extrapolated a further parameter that was called fold of expansion, which indicated how many expansion cycles Ag-specific CD8$^+$ T cells underwent in culture under selective proliferative pressure of Ag. At the end of challenge, results were plotted and correlated to tumor outcome. As shown in Fig. 5, collective data from vaccinated mice (median, 3.54; range, −0.73–6.57) significantly differed from those of control mice (median, 1.44; range, −0.34–3.36; $p = 0.0003$). Moreover, this parameter effectively discriminated between regressor (median, 4.18; range, 0.23–6.57)

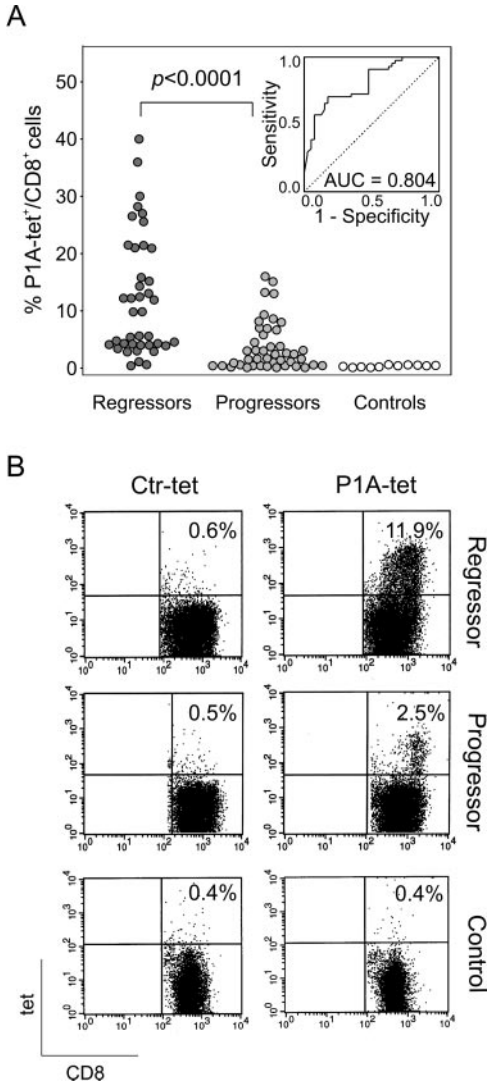

FIGURE 4. Quantifying P1A-specific $CD8^+$ T cells after in vitro restimulation. *A*, Splenocytes from vaccinated and control mice were restimulated in vitro with the P1A peptide. The percentages of P1A-$tet^+/CD8^+$ T cells were plotted as a function of progression or regression to tumor challenge. Statistical analysis of data using the Mann-Whitney *U* test revealed a striking difference ($p < 0.0001$) between regressor (●) and progressor mice (◍). ○, Control mice. The *inset* reports the ROC curve and the relative AUC value for this test. *B*, Representative cytometry analysis of a regressor, a progressor, and a control mouse, respectively, whose percentages of P1A-specific $CD8^+$ T cells after in vitro restimulation were the closest to the median of the respective group. Plots on the *right* refer to staining with the specific tetramer (P1A-tet), whereas plots on the *left* report the background staining obtained with an irrelevant tetramer (Ctr-tet).

and progressor mice (median, 2.78; range, −0.73–5.34; $p < 0.0001$) and was also fairly accurate as a diagnostic test (AUC value, 0.785; Fig. 5, *inset*).

Although determination of fold of expansion is an artificial parameter that does not consider a number of potential biases due to in vitro conditions, it might be a useful marker for predicting the clinical outcome of cancer vaccination.

Analysis of cytotoxic activity

To test whether the in vitro expanded P1A-specific $CD8^+$ T cells were also functional, we evaluated the cytotoxicity of cultures. Fig. 6*A* shows that lytic activity of cell cultures from control mice was low against Ag-negative P1A peptide-pulsed target cells. Lytic activity of cell cultures from progressor mice was greater than controls, but still lower than that of regressor mice. Notably, regressor mice displayed a cytotoxicity similar to that detected for an anti-P1A CTL clone.

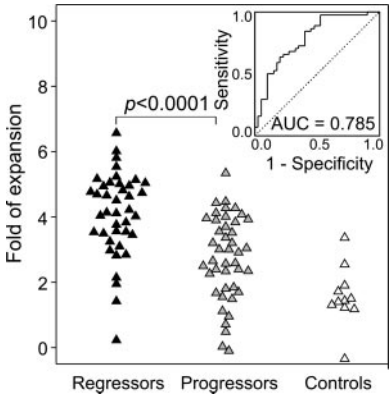

FIGURE 5. The fold of expansion parameter and its correlation with tumor outcome. Details for calculation of this parameter have been given in the text. When tumor challenge was completed, results for each mouse were correlated to tumor outcome. Data showed that there was a significant difference ($p < 0.0001$) between regressor (▲) and progessor mice (◬). △, Control mice. The *inset* reports the ROC curve and the relative AUC value for this test.

Expression of cytotoxicity as lytic units provides a powerful mathematical tool to maximize differences, as the algorithm used takes into account the shape of the curve, and allows extrapolation that numerically quantifies the lytic activity of a culture (17). We calculated the cytolytic activity of each culture as LU_{30}, and plotted the results as a function of tumor outcome. Data are reported in Fig. 6*B*, and refer to the lytic activity against P1A-expressing J558 plasmocytoma, the same cells used for challenge and that harbor P1A endogenously. No LU_{30} could be calculated in cultures from control mice, which were significantly different from vaccinated mice (cumulative data, median, 72.3; range, 0–1540.5; $p = 0.0006$). A striking lytic capacity was observed only in cultures from regressor mice (median, 200.7; range, 0–1540.5); this value was significantly different from that of progressor mice (median, 0; range, 0–546; $p < 0.0001$).

The ROC curve calculated for the "cytotoxicity" parameter (Fig. 6*B*, *inset*) had a fair AUC value (0.781), thus indicating that this traditional approach for monitoring the efficacy of a vaccination protocol can still be considered sufficiently accurate to provide prognostic results.

Multivariate statistical analysis

The high number of mice studied allowed us to also carry out a multivariate analysis of data. All parameters were considered together (except the ELISPOT, which was previously shown to not be significant and, therefore, was excluded) to assess whether individual correlations with tumor regression, calculated using univariate analysis, were maintained. As reported in Table I, only in vitro tetramer staining parameter was significantly associated with tumor survival; all other parameters lost their significance. We can then conclude that this variable is an independent prognostic surrogate predictor of immune response that reliably predicts the efficacy of $CD8^+$ T cells in counteracting tumor growth.

Discussion

In this paper, we attempted to transpose in a controlled preclinical mouse model a plural approach to immune monitoring of

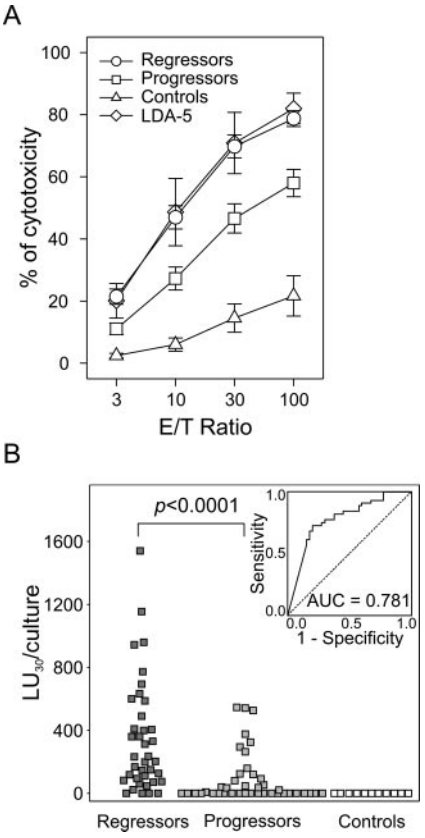

FIGURE 6. Predictive power of the cytotoxicity test. *A*, Splenocytes were restimulated in MLPC with the P1A antigenic peptide and the Ag-specific cytotoxicity (mean ± SE) was evaluated 5 days later against P1A peptide-pulsed IR5P1A$^-$ target cells. Effectors were MLPC from regressor (○), progressor (□), and control mice (△), and P1A-specific LDA-5 CTL clone (◇). *B*, The cytolytic activity, reported as LU_{30}/culture against J558 target cells, of each vaccinated mouse was plotted as a function of progression or regression to tumor challenge. Statistical analysis of data using the Mann-Whitney *U* test revealed a significant difference ($p < 0.0001$) between regressor (■) and progressor mice (▨). □, Control mice. The *inset* reports the ROC curve and the relative AUC value for this test.

anti-tumor vaccine efficacy to establish the correlation of several commonly used immune parameters to clinical outcome, assessing with statistical means their predictive value. This was conducted in a large pool of mice that were immunized, individually tested for anti-P1A CD8$^+$ T cell response after splenectomy, and kept alive for a subsequent challenge with Ag-expressing tumor cells. This allowed for collection of both in vitro and in vivo data for each individually tested animal, reflecting what occurs in a clinical study.

For any monitoring method to serve as a surrogate predictor of treatment status or outcome, a strong association between the immune parameter and clinical efficacy needs to be categorically established (6, 7, 21–23). When this was calculated for the in vivo parameters investigated, namely the quantification of P1A-specific CD8$^+$ T cells by tetramer staining and their quantification/functional analysis by ELISPOT assay, results were quite disappointing. Indeed, in the first case, the test was shown to have a low accuracy, although it was able to discriminate between progressor and regressor mice. This is likely related to the very low number of P1A-specific CD8$^+$ T cell precursors present before vaccination. Although these cells might have undergone several expansion cycles after vaccine administration, they did not demonstrate detectable levels due to the limited sensitivity of the system. In fact,

Table I. *Multivariate statistical analysis of biomarkers studied*

Variable	*p*	Odds Ratio	Confidence Interval 95%
Percentage of tet-P1A$^+$ in vivo	0.6625		
Percentage of tet-P1A$^+$ in vitro	<0.0001	0.841	0.766 ± 0.922
Fold of expansion	0.0993		
LU_{30}/culture	0.3848		

tetramer staining conducted ex vivo can only detect fairly large expansions of specific T cells, with frequencies of >1% of the CD8$^+$ T cells, which can be observed only during anti-viral responses (24–26). Accordingly, the use of tetramers in a clinical setting for the immune monitoring of antitumor vaccine responses and correlation with clinical outcome has generated little statistically validated data (27). A notable exception was found in a recent report showing a correlation between levels of tetramer positivity and clinical response after vaccination with WT1 (Wilms' tumor) peptide (28). However, this appears uniquely linked to the high immunogenicity of the WT1 protein, which is capable of inducing natural responses, resulting in higher levels of tetramer-positive CD8$^+$ T cell precursors than other TAA, and thus rendering this technique more accurate, sensitive and informative.

Surprisingly, the accuracy of ELISPOT was even lower, although this assay did provide functional data. In particular, two aspects are worth noting. First, there was no correlation between data obtained by tetramer staining and ELISPOT results (data not shown). As previously reported, this may indicate that Ag-specific cells are either overestimated by tetramers, or that not all of them are fully functional and able to respond to the antigenic restimulation analyzed by ELISPOT (28–31). A second important point is that ELISPOT completely failed to discriminate between progressor and regressor mice in our experimental conditions, and yet it has been previously proposed as a valid test for immune monitoring of clinical trials due to its reliability and practicality (20). This apparent paradox is actually consistent with previously published data because this assay has been extensively used to assess the immunogenicity of vaccines, i.e., the quantification of specific T cells in immunized vs not immunized patients, but has proven of little use for predictive purposes. In fact, data from antitumor vaccination clinical trials have shown that ELISPOT did not detect an increase in tumor-specific CD8$^+$ T cells after vaccination (31), or generated only qualitative results in a subgroup of patients (32–39). In only two studies were the levels of tumor Ag-specific CD8$^+$ T cells assessed by ELISPOT shown to be related to clinical response (40, 41). Thus, it appears that direct ex vivo quantification of vaccine-induced tumor-specific CD8$^+$ T cells is beyond the limit of detection of currently available immunological assays, including tetramers. Results are insignificant probably due to the low sensitivity and accuracy of the tests used.

Results remarkably improved when the frequency of specific CD8$^+$ T cells was increased through in vitro restimulation. Although initially it might appear preferable to define immune status without introducing potential in vitro biases, results indicated that culture expansion of P1A-specific CD8$^+$ T cells ultimately produced more accurate and predictive results for tumor outcome. This was certainly the case for the in vitro percentage of tetramer-positive P1A-specific CD8$^+$ T cells obtained after culture. This test had the highest accuracy and was the only one that retained a predictive value in multivariate analysis. The high accuracy of this surrogate predictor is likely due to the enormous proliferative pressure primed CD8$^+$ T cells are subjected to in vitro, leading to

potent expansion of Ag-specific populations. This phenomenon might amplify the differences already present in vivo, which cannot be accurately discriminated due to the limits of the detection system. In this regard, recent results have shown that a correlation between $CD8^+$ T cell response and objective clinical response in patients vaccinated with MAGE-3 Ag could be disclosed only after in vitro restimulation of PBMC under limiting dilution conditions and cloning, as direct ex vivo evaluation of vaccine-specific $CD8^+$ T cells was not feasible due to their low frequency, which was below the detection limits of ELISPOT or tetramer staining (42, 43). As an independent prognostic factor, the capacity of $CD8^+$ T cells to expand in vitro may reflect an analogous ability to expand in vivo during the response to tumor challenge, although evidence for this is still lacking. The strength of the immune reaction has been shown to be critical in achieving an anti-tumor effect, although simply measuring the immune response does not directly reflect this reactivity (44).

We previously reported a direct correlation between the in vitro cytolytic activity of anti-P1A $CD8^+$ T cells and tumor outcome in vaccinated animals (11). In the present work, this observation has been confirmed and expanded, thus reinforcing the concept that $CD8^+$ T cells play a fundamental role in tumor destruction. This feature of the $CD8^+$ T cell response does not have a clinical counterpart yet, because lytic activity has always been used phenomenologically to demonstrate the functionality of expanded cells and has not been considered per se as a potential surrogate predictor. We are aware that cytotoxicity is certainly not the only effector function by which $CD8^+$ T cells mediate antitumor effects, as other molecules, such as IFN-γ, are involved in tumor rejection (45). Indeed, this analysis of P1A-specific cytotoxicity does not attempt to explain antitumor effects, but is presented simply as a tool for evaluating the immune process.

Finally, data reported in the present study indicate that the surrogate predictors analyzed do not completely reflect the impact of the vaccine-induced immune response on the tumor. In fact, discrepancies can still be observed between mice with high biomarker activity and with tumors in progression and mice with low biomarker activity and tumors in regression. This dichotomy is likely dependent on the fact that we have not completely elucidated the effector mechanisms that play a critical role in mediating the antitumor effects of Ag-specific $CD8^+$ T cells. Moreover, further mechanisms, involving both tumor resistance and lymphocyte quiescence, might account for the unexpected and paradoxical coexistence of tumor-specific T cells and tumor growth observed not only in preclinical studies but also in clinical trials (13, 46).

Overall, the general accuracy of the assays used in immune monitoring appears collectively low and must certainly be improved to provide more reliable data. Nonetheless, the correlations we have reported between in vitro surrogate predictors of $CD8^+$ T cell activity and the antitumor response in vivo, in particular the in vitro percentage of tetramer-positive tumor-specific $CD8^+$ T cells, appears to be a promising approach that could be considered for immune monitoring to cancer vaccines and clinical testing.

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Disclosures

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