Advances in Brief

T-Cell Responses against Tyrosinase 368–376(370D) Peptide in HLA*A0201$^+$ Melanoma Patients: Randomized Trial Comparing Incomplete Freund's Adjuvant, Granulocyte Macrophage Colony-stimulating Factor, and QS-21 as Immunological Adjuvants[1]

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Abstract

We conducted a randomized trial in HLA*A0201$^+$ patients with American Joint Committee on Cancer stage III or IV melanoma immunized with tyrosinase 368–376(370D) peptide and gp100 209–217(210M) peptide to compare the potency of three different adjuvants. Patients received 3 monthly immunizations with 500 μg of each peptide either with incomplete Freund's adjuvant (IFA), QS-21, or granulocyte macrophage colony-stimulating factor (GM-CSF). The primary end point was induction of IFN-γ release by CD8$^+$ T cells against tyrosinase and gp100 peptides measured by enzyme-linked immunospot assays without *in vitro* prestimulation measured pretreatment, 2 and 8 weeks after the third vaccination. Four of 9 and 4 of 8 patients immunized using QS-21 and GM-CSF, respectively, developed increased frequencies of CD8$^+$ T cells against tyrosinase 370D peptide compared with 0 of 9 patients immunized using IFA ($P = 0.045$). T-cell responses against a gp100-related peptide showed similar results, but their relevance to T-cell reactivity against native gp100 209–217 is uncertain. These results show that: (*a*) QS-21 and GM-CSF are superior to IFA as immunological adjuvants for vaccination against tyrosinase 370D peptide; and (*b*) with appropriate adjuvants, increased frequencies of peptide-specific T cells after vaccination can be detected by enzyme-linked immunospot without prolonged prestimulation *in vitro*.

Introduction

Tyrosinase and gp100 are proteins involved in the melanocytic differentiation pathway. Several groups have demonstrated the presence of T cells recognizing peptides derived from tyrosinase and gp100 in melanoma patients and in healthy subjects (1–5). This has led to the idea of using these peptides to immunize melanoma patients with the goal of expanding T-cell responses that could have antitumor effects. Most of the work has focused on HLA-A2.1-restricted peptides because this allelotype is found in ~40% of patients.

Both tyrosinase 368–376(370D) peptide and gp100 209–217 peptide are HLA-A2.1-restricted peptides that can be processed, presented, and recognized by T cells in the immune repertoire. Alteration of the gp100 209–217 peptide at the second amino acid (changing threonine to a methionine) was found to increase markedly the affinity for HLA-A2.1 resulting in enhanced induction of T cells reactive to native gp100 (6). Immunization with this heteroclytic peptide, gp100 209–217(210M), with IFA[3] induced T cells against native gp100 209–217 peptide in 10 of 11 patients vaccinated (7). Immunogenicity of tyrosinase peptide appears to be lower. In the absence of adjuvant, 0 of 6 patients immunized with tyrosinase 368–376(370D) peptide developed a cytotoxic lymphocyte reaction to the peptide (1). However, immunization with tyrosinase peptide plus QS-21 can induce T-cell responses (5).

Because peptides derived from self proteins are minimally immunogenic alone (1, 8), they must be administered with a potent immunological adjuvant to induce detectable T-cell responses. Most trials have used IFA as an adjuvant (7, 9, 10). Another potent adjuvant is QS-21, a purified saponin (11), which can enhance both B-cell (12–14) and T-cell responses (15, 16). Multiple clinical studies have shown QS-21 to be well tolerated by patients (5, 13, 17). We have also been interested in GM-CSF as a vaccine adjuvant because of its role as one of the

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[3] The abbreviations used are: IFA, incomplete Freund's adjuvant; GM-CSF, granulocyte macrophage colony-stimulating factor; DC, dendritic cell; NCI, National Cancer Institute; PBMC, peripheral blood mononuclear cell; ELISPOT, enzyme-linked immunospot.

primary growth and maturation factors for DCs. We and others have shown that daily intradermal injections of GM-CSF for 10 days recruit DCs to the site of injection (18, 19) and, in 6 patients, administration of GM-CSF seemed to enhance the immunogenicity of peptides derived from melanosomal differentiation proteins (20). We reasoned that introduction of peptides into the area prepared by the daily GM-CSF injections could be an efficient method of loading DCs *in vivo* and immunizing the patient.

Despite the potentially critical role of adjuvants in vaccine design, we were aware of no data directly comparing adjuvants in patients receiving cancer vaccines. Therefore, we conducted a randomized trial in which patients were vaccinated with both the tyrosinase 368–376(370D) and gp100 209–217(210M) peptides using one of three different adjuvants: IFA, QS-21, or GM-CSF. The primary end point of the study was the induction of peptide-specific $CD8^+$ T cells.

Patients and Methods

Patients. All of the patients had American Joint Committee on Cancer stage III or IV melanoma either free of disease and fully recovered from surgery or with active disease. Patients with active disease had an expected survival of at least 5 months. Histology was confirmed by the Pathology Department at Memorial Sloan-Kettering Cancer Center. All patients were DNA typed and had to be HLA*A0201 positive as confirmed by the HLA Laboratory at Memorial Hospital. Eligibility criteria also included age ≥18 years, Karnofsky performance status ≥60%, WBC ≥3,500 cells/mm^3, platelets ≥75,000/mm^3, lactate dehydrogenase $< 2 \times$ the upper limit of normal, albumin ≥3.5 mg/dl, and ability to give written informed consent.

Patients were excluded if they had received chemotherapy, immunotherapy, or radiation therapy within the previous 4 weeks, or if they had a known immunodeficiency, previous splenectomy, or radiation therapy to the spleen. Patients could not have received prior vaccines containing IFA, tyrosinase, or gp100. Patients were also excluded if they had a serious underlying medical disease, active infection requiring antimicrobial drugs, or active bleeding. While on study, patients could not receive anti-inflammatory or antihistamine drugs. Women who were pregnant, <3 months postpartum, or lactating were not eligible. Patients with preexisting retinal or choroidal eye disease were also excluded.

Peptides. The vaccine consisted of tyrosinase 368–376(370D) peptide (YMDGTMSQV) and gp100 209–217(210M) peptide (IMDQVPFSV), which were prepared and administered separately. Both peptides are HLA-A2.1 restricted. The gp100 209–217(210 M) peptide was modified at the second position to increase the affinity of binding to HLA-A2.1 (6) and to enhance induction of T cells against the native peptide expressed by melanoma. Both peptides were provided by Cancer Treatment Evaluation Program/NCI under an Investigational New Drug application held by the NCI.

Adjuvants. IFA formulated as Montanide ISA-51 was manufactured by Seppic, Inc. and supplied by the NCI as single-use vials containing 3 ml of sterile adjuvant solution without preservative. QS-21 was prepared by Antigenics, Inc. (Woburn, MA) from *Quillaja saponaria Molina* tree bark by silica and reverse-phase chromatography (11, 16). The QS-21 was supplied in 0.5 mg/ml in PBS vials [(pH 6.8), volume 0.6 ml]. Human GM-CSF, made by Immunex Corporation (Seattle, WA), was supplied through the NCI.

Vaccine Preparation and Administration. The vaccine peptides were sterile and endotoxin-free. The tyrosinase 368–376(370D) peptide was supplied in vials containing 1.2 ml of a sterile 1 mg/ml solution for injection with 0.1 N HCl added to adjust the pH. The gp 100 209–217(210M) peptide was supplied in a 1 mg/ml solution in single use-vials containing 1 ml volume. Intact vials were stored frozen at −10°C to −20°C. Once thawed, the peptides were used within 3 h.

Treatment Plan. Patients were assigned by block randomization to receive IFA, GM-CSF, or QS-21 to ensure 9 patients in each adjuvant arm. Patients were stratified for age ([65 *versus* >65) and previous chemotherapy. All of the patients received 3 monthly vaccinations with both tyrosinase 368–376(370D) peptide and gp100 209–217(210M) peptide at a dose of 500 μg/peptide administered at separate sites. If patients failed to receive all three of the planned vaccinations, they were considered inevaluable for immunological response and were replaced. For patients randomized to the IFA arm, 500 μg of each peptide in aqueous solution at room temperature was mixed with Montanide in a ratio of 1:1 and injected s.c. For patients receiving QS-21, 500 μg of each peptide in aqueous solution was mixed with 100 μg of QS-21 and administered s.c. Patients randomized to GM-CSF received 40 μg of GM-CSF injected intradermally at a single skin site for 10 consecutive days for each vaccination. On day 7 of the GM-CSF treatment, 500 μg of each peptide was administered intradermally at separate sites within the erythematous reaction induced by the GM-CSF.

Physical examination, blood cell counts, and standard blood tests were performed before each immunization, and 4 and 8 weeks after the last immunization. Blood for T-cell studies was drawn pretreatment, and 2 weeks and 8 weeks after the last immunization.

The protocol was reviewed and approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center. All of the patients signed informed consent before participating in the clinical trial.

PBMCs. PBMCs were collected pretreatment 2 weeks and 8 weeks after the last vaccine. For each time point, 80 ml of heparinized blood were drawn, and PBMCs were isolated by centrifugation on a Ficoll-Paque gradient (Pharmacia, Piscataway, NJ) and stored in FCS/10% DMSO in aliquots of 5×10^6 cells at −180°C until needed.

Immunological Monitoring by ELISPOT. T-cell reactivity against tyrosinase 368–376(370D) peptide was measured by IFN-γ ELISPOT without previous *in vitro* stimulation. ELISPOT assays against gp100 (209–217) were carried out using a gp100-related peptide containing tyrosine at position 217 based on an early publication that contained an error in the gp100 peptide sequence (21). This results in a peptide with low avidity for HLA-A2 molecule compared with the native sequence, which contains valine at position 217. As a consequence, the significance of the gp100 ELISPOT results was difficult to interpret and these results [although very similar to the ELISPOT results with tyrosinase 368–376(370D) peptide] are not

reported here. PBMCs were thawed, and $CD8^+$ T cells were purified by magnetic microbeads (Miltenyi Biotec Inc., Auburn, CA) following the manufacturer's instructions, which yielded populations of >98% pure $CD8^+$ T cells.

HA-Multiscreen plates (Millipore, Burlington, MA) were coated with 100 μl of mouse antihuman IFN-γ antibody (10 μg/ml; clone 1-D1K; Mabtech, Nacka, Sweden) overnight at 4°C, washed, and blocked with human serum for 1 h at 37°C. $CD8^+$ T cells were plated in duplicate or triplicate (depending on the number of cells available) at 5×10^4/well with 1×10^4 irradiated T2 cells/well pulsed with 10 μg/ml β_2-microglobulin (Sigma Chemical Co., St. Louis, MO) and 10 μg/ml tyrosinase 368–376(370D) peptide. In some assays, peptide was added at lower concentrations (1 μg/ml) to detect high affinity T cells. Background activity was measured as $CD8^+$ T-cell reactivity against unpulsed T2 cells. Negative control wells were included in which T2 cells were pulsed with the irrelevant peptide KT-WGQYWQV, a HLA-A2.1-restricted peptide derived from the gp100 protein but completely distinct from the gp100 209–217 peptide, and not generally recognized by T cells from melanoma patients. Negative control wells were also included that contained only $CD8^+$ T cells (no T2 cells), only pulsed T2 cells (no T cells), and medium alone.

After incubation for 20 h at 37°C, plates were washed with PBS/0.5% Tween. Bound IFN-γ was detected by adding 100 μl of biotinylated antihuman IFN-γ (2 μg/ml; clone 7-B6-1; Mabtech). Spot development was performed by using avidin-biotin complexes as described (22), and spots were counted using an automated ELISPOT reader system (Carl Zeiss Vision, Göttinger, Germany) with KS ELISPOT 4.2 software. The number of specific spots was the number of spots counted in the wells containing $CD8^+$ T cells with T2 cells pulsed with the specific peptide of interest minus the number of the background activity spots ($CD8^+$ T cells with unpulsed T2 cells). In most experiments, a similar number of spots was observed in wells containing $CD8^+$ T cells and either unpulsed T2 cells or T2 cells pulsed with the irrelevant peptide. Negative control wells containing either $CD8^+$ T cells alone or pulsed T2 cells alone (no $CD8^+$ T cells) yielded no spots.

On the basis of intra-assay variability in healthy donors, a positive response to vaccination was defined as an increase in ≥12 spots compared with the prevaccination value (5).

Statistical Analysis. A Fisher's exact test was used to test for an overall association between adjuvant and immunological response to peptide. When an overall difference was observed, the QS-21 and GM-CSF arms were collapsed, and a Fisher's exact test was performed adjusted for these post-hoc comparisons.

Results

Patients Treated. A total of 31 patients were entered in this study. Four patients received fewer than three vaccines because of rapid progression of disease and were considered inevaluable. Nine evaluable patients were treated in each adjuvant group. The characteristics of the 27 evaluable patients are shown in Table 1. Eighteen patients were free of disease at the time of entry on the trial; 2 patients had unresectable stage III in-transit disease. Sites of metastatic disease among the 7 patients enrolled with active stage IV melanoma were: liver (5), lymph nodes (4), lung (3), heart (1), spleen (1), adrenal (1), pancreas (1), and abdominal wall (1). Each of the 7 patients had at least two known sites of metastases. Nineteen of the patients had received some form of systemic therapy before entering the study.

Table 1 Patient demographics

Characteristics	
Gender	
Male/female (no. of patients)	17/10
Median age (range)	54 (36–87)
Stage at entry (no. of patients)	
III, free of disease	10
III, active disease	2 (skin only)
IV, free of disease	8
IV, active disease	7
Median KPS (range)	90 (80–100)
Prior therapy (no. of patients)	
None	8
IFN-α	11
Chemotherapy	7
Interleukin-2	3
Vaccine	4
Levamisole	2
Intralesional DNCB	1
Tamoxifen	1

T-Cell Responses to Tyrosinase Peptide. T-cell responses against tyrosinase 368–376 (370D) peptide were evaluated by ELISPOT assay. Most assays were repeated a second time and patients were counted as responders if both assays showed a mean increase of ≥12 spots/well over pretreatment values (5). However, for 3 patients there were sufficient numbers of lymphocytes for only one round of assays. One patient, in the GM-CSF group, failed to return to clinic for PBMC collection and could not be assessed for T-cell response.

Overall, 8 of 26 patients (30%) developed T-cell responses to tyrosinase 368–370 (370D) peptide (Fig. 1). The response proportion in the QS-21 and GM-CSF groups (4 of 9 and 4 of 8, respectively) was significantly higher than the response proportion in the IFA group (0 of 9). An overall association between adjuvant and response was observed ($P = 0.045$). When the response rates of the QS-21 and GM-CSF groups were combined, the response rate of the IFA group was significantly lower ($P = 0.04$). At 8 weeks after immunization, the median number of T cells reactive to tyrosinase 368–370 (370D) peptide in the IFA, QS-21, and GM-CSF groups was 7, 32, and 22 per 10^5 $CD8^+$ T cells (1 of 14,000, 1 of 3,125, and 1 of 4,545 T cells), respectively (Fig. 2). Although 2 weeks after completing immunization the median number of T cells reactive to tyrosinase 368–370 (370D) peptide was not significantly increased in any of the adjuvant groups, we noted that at this time point 3 of 9 patients in the QS-21 group demonstrated >60 tyrosinase-reactive T cells/10^5 $CD8^+$ T cells (<1 of 1,700 T cells reactive with tyrosinase). Three of the 8 patients in whom antityrosinase T-cell responses were enhanced had received previous chemotherapy indicating that prior chemotherapy did not preclude an immunological response.

In 2 patients (QS-21 patient designated Q3 and GM-CSF

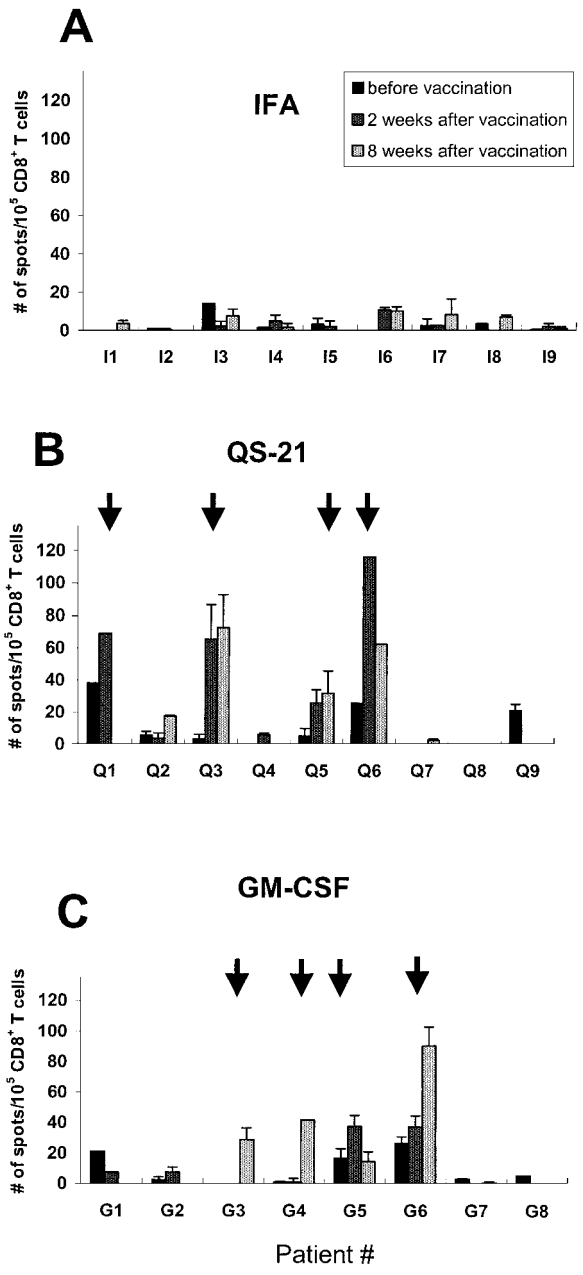

Fig. 1 T-cell responses against tyrosinase 368–376(370D) peptide by individual patient as measured by ELISPOT. Patients were immunized using either IFA (*A*), QS-21 (*B*), or GM-CSF (*C*) as adjuvants. The *Y* axis represents number of spots observed minus the number of spots in the negative control wells in which the APCs were not pulsed with peptide. The results for each patient are shown; *bars*, ± SD for triplicate wells. *Arrows* indicate patients considered to have shown a T-cell response to native tyrosinase 368–376 (370D) peptide as defined in "Materials and Methods." Reactivity against T2 cells alone showed a median of 11.8 spots/well (±14.3 SD).

patient designated G6), there were sufficient numbers of $CD8^+$ T cells to perform additional ELISPOT assays using a 10-fold lower concentration of peptide (1 μg/ml) to detect T cells with higher avidity for peptide. Reactivity against tyrosinase 368–376 (370D) peptide was detected in each patient under these more stringent conditions (data not shown) as well as under the standard conditions (Fig. 1). These results indicate that at least some of the patients developed responses consisting of T cells expressing T-cell receptors with relatively high avidity for the peptide.

Toxicity. All 31 of the patients entered were evaluable for toxicity. In general, vaccines were well-tolerated, and serious toxicity (≥grade 3) was not observed in any patient. One patient developed vitiligo.

Discussion

Successful clearance of infectious agents, such as virus, is associated with 100-5000-fold expansion of specific T cells (23). After donor leukocyte infusions to eradicate EBV-induced lymphoma in bone marrow transplant patients, T cells against EBV can constitute 2% of the peripheral T-cell population (24). It is not known how high a frequency of specific T cells is needed for cancer vaccines to induce reproducible anticancer effects, but it is clear that high T-cell frequency alone is not sufficient for tissue rejection. In transgenic mouse models in which up to 90% of naïve, nonactivated T cells recognize a specific autoantigen, autoimmunity is not observed unless the antigen is presented in an appropriately activated environment such as in the setting of viral infection (25, 26), immune adjuvant (27), or activated antigen-presenting cells (28). Therefore, one strategy to enhance immunogenicity is to administer the peptides with a potent immunological adjuvant to stimulate local inflammation. Most peptide trials to date have used a form of IFA (7, 9, 21, 29, 30), but other potent adjuvants are available. This paper reports the first cancer vaccine trial of which we are aware comparing adjuvants in a prospective randomized manner.

Our results confirm that tyrosinase 368–370 (370D) peptide is immunogenic and can be used to induce a T-cell response in patients. The principal finding was that QS-21 and GM-CSF were more effective adjuvants than IFA for inducing T-cell responses against tyrosinase peptide; patients immunized with peptide and either QS-21 or GM-CSF were more likely to develop a T-cell response to tyrosinase compared with patients immunized using IFA. Among the 9 patients immunized with IFA, none developed a detectable T-cell response against the tyrosinase peptide. Although the route of administration may play a role in the efficiency of immunization, it is unlikely that the immunological differences observed were attributable simply to the route of immunization, because vaccines given with QS-21 and IFA were both administered via the s.c. route

Our inability to detect reactivity against tyrosinase peptide in T cells isolated directly from patients immunized with peptide and IFA is consistent with results reported by other groups immunizing patients with other peptides and IFA (7, 9, 29–31). For example, among 7 patients immunized with gp100 209–217(210M) peptide and IFA at the Surgery Branch of the NCI, none had a detectable T-cell response to native gp100 209–217 peptide when T cells were tested immediately after being isolated from the patient (31). To detect T cells against native gp100 209–217 peptide after immunization with gp100 209–217(210M) peptide and IFA, it has been necessary to preincu-

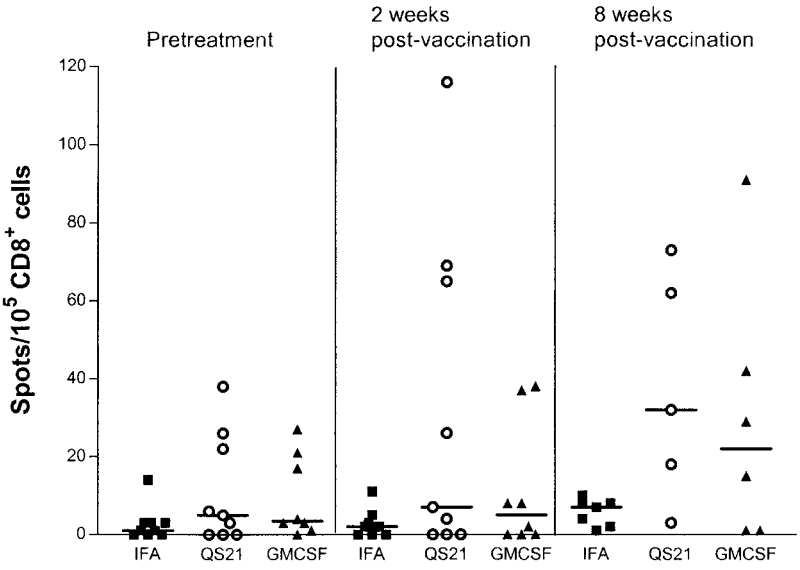

Fig. 2 T-cell responses against tyrosinase 368–376(370D) comparing adjuvants. Each point represents the mean number of T cells against tyrosinase 368–376(370D) peptide/10^5 $CD8^+$ T cells (after subtracting the number of spots in the negative control wells) in patients immunized with IFA (■), QS-21 (○), or GM-CSF (▲) as adjuvants. *Horizontal bars* represent median values.

bate PBMCs *in vitro* with target peptide for 10–12 days (with interleukin 2) before measuring T-cell response (7, 31). The requirement for *in vitro* expansion before detecting antipeptide T-cell responses has been observed in patients immunized with MART-1/MelanA and MAGE-3 peptides (9, 29, 30) presumably because of the low frequency of reactive T cells after immunization. This preincubation allows expansion of the specific T-cell population to a detectable level. In our study, $CD8^+$ T cells were assayed by ELISPOT directly from the patient with only overnight incubation with peptide because we hypothesized that assaying cells directly out of the patient more accurately reflects the status of the cells in the patient. Indeed, data from the NCI suggest that the ability to expand peptide-specific T cells *in vitro* does not correlate with antitumor effects (10).

This raises the important issue of how best to monitor T-cell responses in vaccine trials. A variety of techniques are used including limiting dilution assays, delayed-type hypersensitivity reactions, T-cell proliferation, ELISPOT, tetramers, cytokine secretion, or intracellular cytokine production. Each method has potential advantages and disadvantages. Some assays are quantitative but do not measure function (*e.g.*, tetramer assays). Other assays measure function but do not provide information about T-cell frequencies (*e.g.*, proliferation assays and delayed-type hypersensitivity reactivity). Limiting dilution assays measure function and are quantitative, but they are difficult to perform and can markedly underestimate T-cell frequencies.

In this study, we have used IFN-γ secretion by $CD8^+$ cells as measured by ELISPOT assay. The advantage of this assay technique is that it measures T-cell function and can provide a quantitative estimate of T-cell frequency. It is common to use a stimulatory peptide concentration of 10 μg/ml when performing the ELISPOT assay. However, it is possible that this relatively high peptide concentration may detect T cells of such low avidity that they have little clinical relevance. In 2 patients from this trial who showed T-cell responses to peptide in our standard ELISPOT conditions, we were able to test $CD8^+$ T cells using a 10-fold lower peptide concentration (1 μg/ml) and found we could still detect peptide-reactive T cells under these more stringent conditions. This suggests that at least some patients have T cells after immunization with relatively high avidity for peptide.

It has yet to be determined what magnitude of T-cell response is necessary for therapeutic effects and what is the best method to monitor T-cell responses in vaccine trials. We observed a maximum of ~100 antityrosinase T cells/10^5 $CD8^+$ T cells (Fig. 2), which corresponds to 0.1% of $CD8^+$ T cells. Until a vaccine is developed with clear and reproducible beneficial clinical effects, it will be difficult to know how best to monitor T-cell responses and what magnitude of T-cell response correlates best with efficacy. The fact that clinical responses have been observed in the absence of detectable T-cell responses (1, 7, 8) adds to the uncertainty. However, it seems likely that testing T cells directly from the patient is preferable to a technique using extensive *in vitro* prestimulation. On the basis of this assumption, our data suggest that QS-21 or GM-CSF is superior to IFA as an adjuvant for peptide vaccines.

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References

1. Jager, E., Bernhard, H., Romero, P., Ringhoffer, M., Arand, M., Karbach, J., Ilsemann, C., Hagedorn, M., and Knuth, A. Generation of cytotoxic T-cell responses with synthetic melanoma-associated peptides *in vivo:* implications for tumor vaccines with melanoma-associated antigens. Int. J. Cancer, *66:* 162–169, 1996.

2. Romero, P., Dunbar, P. R., Valmori, D., Pittet, M., Ogg, G. S., Rimoldi, D., Chen, J-L., Lienard, D., Cerottini, J-C., and Cerundolo, V. *Ex vivo* staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experi-

enced tumor-specific cytolytic T lymphocytes. J. Exp. Med., *188:* 1641–1650, 1998.

3. Bakker, A. B. H., Schreurs, M. W. J., de Boer, A. J., Kawakami, Y., Rosenberg, S. A., Adema, G. J., and Figdor, C. G. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. J. Exp. Med., *179:* 1005–1009, 1994.

4. Cox, A. L., Skipper, J., Chen, Y., Henderson, R. A., Darrow, T. L., Shabanowitz, J., Engelhard, V. H., Hunt, D. F., and Slingluff, C. L. J. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science (Wash. DC), *264:* 716–719, 1994.

5. Lewis, J. J., Janetzki, S., Schaed, S., Panageas, K. S., Wang, S., Williams, L., Meyers, M., Butterworth, L., Livingston, P. O., Chapman, P. B., and Houghton, A. N. Evaluation of $CD8^+$ T cell frequencies by the ELISPOT assay in healthy individuals and in patients with metastatic melanoma immunized with tyrosinase peptide. Int. J. Cancer, *87:* 391–398, 2000.

6. Parkhurst, M. R., Salgaller, M. L., Southwood, S., Robbins, P. F., Sette, A., Rosenberg, S. A., and Kawakami, Y. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. J. Immunol., *157:* 2539–2548, 1996.

7. Rosenberg, S. A., Yang, J. C., Schwartzentruber, D. J., Hwu, P., Marincola, F. M., Topalian, S. L., Restifo, N. P., Dudley, M. E., Schwarz, S. L., Spiess, P. J., Wunderlich, J. R., Parkhurst, M. R., Kawakami, Y., Seipp, C. A., Einhorn, J. H., and White, D. E. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med., *4:* 321–327, 1998.

8. Marchand, M., van Baren, N., Weynants, P., Brichard, V., Dreno, B., Tessier, M. H., Rankin, E., Parmiani, G., Arienti, F., Humblet, Y., Bourlond, A., Vanwijck, R., Lienard, D., Beauduin, M., Dietrich, P. Y., Russo, V., Kerger, J., Masucci, G., Jager, E., De Greve, J., Atzpodien, J., Brasseur, F., Coulie, P. G., van der Bruggen, P., and Boon, T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. Int. J. Cancer, *80:* 219–230, 1999.

9. Weber, J. S., Hua, F. L., Spears, L., Marty, V., Kuniyoshi, C., and Celis, E. A phase I trial of an HLA-A1 restricted MAGE-3 epitope peptide with incomplete Freund's adjuvant in patients with resected high-risk melanoma. J. Immunother., *22:* 431–440, 1999.

10. Lee, K.-H., Wang, E., Nielsen, M. B., Wunderlich, J., Migueles, S., Connors, M., Steinberg, S. M., Rosenberg, S. A., and Marincola, F. M. Increased vaccine-specific T cell frequency after peptide-based vaccination correlates with increased susceptibility to *in vitro* stimulation but does not lead to tumor regression. J. Immunol., *163:* 6292–6300, 1999.

11. Kensil, C. R., Patel, U., Lennick, M., and Marciani, D. Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex. J. Immunol., *146:* 431–437, 1991.

12. White, A. C., Cloutier, P., and Coughlin, R. T. A purified saponin acts as an adjuvant for a T-independent antigen. Adv. Exp. Med. Biol., *303:* 207–210, 1991.

13. Ragupathi, G., Meyers, M., Adluri, S., Howard, L., Musselli, L., and Livingston, P. O. Induction of antibodies against GD3 ganglioside in melanoma patients by vaccination with GD3-lactone-KLH conjugate plus immunological adjuvant QS-21. Int. J. Cancer, *85:* 659–666, 2000.

14. Helling, F., Zhang, S., Shang, A., Adluri, S., Calves, M., Koganty, R., Longenecker, B. M., Yao, T-J., Oettgen, H. F., and Livingston, P. O. GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. Cancer Res., *55:* 2783–2788, 1995.

15. Wu, J. Y., Gardner, B. H., Murphy, C. I., Seals, J. R., Kensil, C. R., Recchia, J., Beltz, G. A., Newman, G. W., and Newman, M. J. Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine. J. Immunol, *148:* 1519–1525, 1992.

16. Newman, M. J., Wu, J. Y., Gardner, B. H., Munroe, K. J., Leombruno, D., Recchia, J., Kensil, C. R., and Coughlin, R. T. Saponin adjuvant induction of ovalbumin-specific $CD8^+$ cytotoxic T lymphocyte responses. J. Immunol., *148:* 2357–2362, 1992.

17. Livingston, P. O., Adluri, S., Helling, F., Yao, T. J., Kensil, C. R., Newman, M. J., and Marciani, D. Phase 1 trial of immunological adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. Vaccine, *12:* 1275–1280, 1994.

18. Kaplan, G., Walsh, G., Guido, L. S., Meyn, P., Burkhardt, R. A., Abalos, R. M., Barker, J., Frindt, P. A., Fajardo, T. T., Celona, R., and Cohn, Z. A. Novel responses of human skin to intradermal recombinant granulocyte/macrophage-colony-stimulating factor: langerhans cell recruitment, keratinocyte growth, and enhanced wound healing. J. Exp. Med., *175:* 1717–1728, 1992.

19. Nasi, M. L., Lieberman, P. H., Busam, K. J., Prieto, V., Panageas, K. S., Lewis, J. J., Houghton, A. N., and Chapman, P. B. Intradermal injection of granulocyte-macrophage colony stimulating factor (GM-CSF) in patients with metastatic melanoma recruits dendritic cells. Cytokines Cell. Mol. Ther., *5:* 139–144, 1999.

20. Jager, E., Ringhoffer, M., Dienes, H. P., Arand, M., Karbach, J., Jager, D., Ilsemann, C., Hagedorn, M., Oesch, F., and Knuth, A. Granulocyte-macrophage-colony-stimulating factor enhances immune responses to melanoma-associated peptides *in vivo*. Int. J. Cancer, *67:* 54–62, 1996.

21. Salgaller, M. L., Marincola, F. M., Cormier, J. N., and Rosenberg, S. A. Immunization against epitopes in the human melanoma antigen gp100 following patient immunization with synthetic peptide. Cancer Res., *56:* 4749–4757, 1996.

22. Herr, W., Schneider, J., Lohse, A. W., Meyer zum Buschenfelde, K.-H., and Wolfel, T. Detection and quantification of blood-derived $CD8^+$ T lymphocytes secreting tumor necrosis factor α in response to HLA-A2.1-binding melanoma and viral peptide antigens. J. Immunol. Methods, *191:* 131–142, 1996.

23. Ahmed, R., and Gray, D. Immunological memory and protective immunity: understanding their relation. Science (Wash. DC), *272:* 54–60, 1996.

24. Lucas, K. G., Small, T. N., Heller, G., Dupont, B., and O'Reilly, R. J. The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. Blood, *87:* 2594–2603, 1996.

25. Ohashi, P. S., Oehen, S., Buerki, K., Pircher, H., Ohashi, C. T., Odermatt, B., Malissen, B., Zinkernagel, R. M., and Hengartner, H. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell, *65:* 305–317, 1991.

26. Oldstone, M. B., Nerenberg, M., Southern, P., Price, J., and Lewicki, H. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell, *65:* 319–331, 1991.

27. Goverman, J., Woods, A., Larson, L., Weiner, L. P., Hood, L., and Zaller, D. M. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. Cell, *72:* 551–560, 1993.

28. Garza, K. M., Chan, S. M., Suri, R., Nguyen, L. T., Odermatt, B., Schoenberger, S. P., and Ohashi, P. S. Role of antigen-presenting cells in mediating tolerance and autoimmunity. J. Exp. Med., *191:* 2021–2027, 2000.

29. Cormier, J. N., Salgaller, M. L., Prevette, T., Barracchini, K. C., Rivoltini, L., Restifo, N. P., and Rosenberg, S. A. Enhancement of cellular immunity in melanoma patients immunized with a peptide from MART-1/Melan A. Cancer J. Sci. Am., *3:* 37–44, 1997.

30. Wang, F., Bade, E., Kuniyoshi, C., Spears, L., Jeffery, G., Marty, V., Groshen, S., and Weber, J. Phase I trial of a MART-1 peptide vaccine in incomplete Freund's adjuvant for resected high-risk melanoma. Clin. Cancer Res., *5:* 2756–2765, 1999.

31. Pass, H. A., Schwarz, S. L., Wunderlich, J. R., and Rosenberg, S. A. Immunization of patients with melanoma peptide vaccines: immunologic assessment using the ELISPOT assay. Cancer J. Sci. Am., *4:* 316–323, 1998.

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