Cancer Therapy: Clinical

Telomerase Peptide Vaccination Combined with Temozolomide: A Clinical Trial in Stage IV Melanoma Patients

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Abstract

Purpose: The study is a proof-of-principle trial evaluating toxicity, immune response, and clinical response in melanoma patients after combined therapy with temozolomide and the telomerase peptide vaccine GV1001. Our previous GV1001 trials showed immune responses in approximately 60% of lung or pancreatic cancer patients.

Experimental Design: Twenty-five subjects with advanced stage IV melanoma (M1B or M1C) received concomitant temozolomide and GV1001. Temozolomide was administered 200 mg/m² orally for 5 days every fourth week, and GV1001 as eight injections over 11 weeks. Immune response was evaluated by delayed type hypersensitivity, T-cell proliferation, and cytokine assays. The immunologic responders continued monthly vaccination.

Results: The treatment was well tolerated. A GV1001-specific immune response was shown in 18 of 23 evaluated subjects (78%). Patients developing long-term T-cell memory survived more than those rapidly losing their responses. The immune response exhibited several characteristics of possible clinical significance including high IFNγ/IL-10 ratios, polyfunctional cytokine profiles, and recognition of naturally processed antigens. Survival compared favorably with matched controls from a benchmark meta-analysis (1 year: 44% vs. 24%, 2 years: 16% vs. 6.6%). The clinical responses developed gradually over years, contrary to what is expected from chemotherapy. Five patients developed partial tumor regression and six more recorded stable disease. One patient has no remaining disease on fluorodeoxyglucose positron emission tomography scans after 5 years.

Conclusions: The immunologic response rate is considerable compared with previous GV1001 trials without concomitant chemotherapy, although low toxicity is retained. The results warrant further studies of GV1001/temozolomide treatment and support the general concept of combining cancer vaccination with chemotherapy.

Introduction

Cancer chemotherapy had been to a large extent ineffective until the development of broad regimes combining different agents. The limited efficacy of monotherapy reflects tumor heterogeneity and genetic instability, favoring escape of resistant tumor subpopulations. Cancer vaccination offers a different angle of attack, as compared with cytostatics, and may therefore be particularly effective in combinatory regimes. However, most chemotherapeutic agents are immunosuppressive, and it was long assumed that vaccines should therefore not be combined with chemotherapy. More recent knowledge has challenged this conventional wisdom (1, 2). In particular, it is discussed whether chemotherapy may be utilized to counter tumor protection from regulatory T-cells (Tregs), while prevailing the immunocompetence necessary for vaccine response (3–5).

Here we report a proof-of-principle trial (phase I/II) of combined therapy with temozolomide and telomerase peptide vaccination in 25 stage IV melanoma patients. The primary objectives were toxicity and immunologic response. Tumor response was a secondary study objective. Current prognosis for metastatic melanoma is dismal, with median survival of 4 to 9 months in patients with visceral involvement (6, 7). Dacarbazine has been considered standard treatment, but the tumor response rate is only 6% to 15%, mostly partial responses, and there is no documented effect on survival (7). The oral drug temozolomide is metabolized to dacarbazine in vivo and carries a potential advantage in penetration of the blood–brain...
barrier. In phase III trials, temozolomide and dacarbazine have produced similar response rates (8, 9).

Telomerase represents a possible target for universal cancer vaccines (10, 11). The enzyme maintains telomere length in dividing cells and is considered essential for tumor growth. Telomerase activity has been shown in all studied cancer forms, including stem cell–like tumor cells (12–14). Interesting results have been reported from phase I/II studies with 3 telomerase peptide vaccines (15–20). Peptide GV1001 represents a 16 amino acid sequence (EARPALLTSRLRFIPK) from the active site of human telomerase reverse transcriptase (hTERT; refs. 21, 22). The peptide was selected on the basis of computer algorithms predicting strong human leukocyte antigen (HLA) class II binding properties and multiple nested HLA class I binding motifs. The GV1001 vaccine may thus recruit both CD4+ T helper cells and CD8+ cytotoxic T cells. We have previously conducted 2 phase I/II trials with GV1001, in pancreatic and lung cancer patients (19, 20). Both studies were conducted without chemotherapy. The results indicated no serious adverse effects, a GV1001-specific immune response in approximately 60% of patients and an association between immune response and survival. T-cell clone studies showed both T helper and cytotoxic responses (19, 20, 23). In the present trial, we hypothesized that temozolomide may enhance the immune response by inducing antigen release or modulating tumor tolerance. The study, to our knowledge, is the first clinical trial evaluating combinatorial therapy with temozolomide and a cancer vaccine.

### Patients and Methods

#### Patients

Twenty-five subjects with stage IV melanoma were enrolled between January 2004 and November 2005. Inclusion criteria: histologically confirmed melanoma, nonresectable metastatic disease (AJCC stage IV), any HLA genotype, measurable tumor, age between 18 and 75 years, Eastern Cooperative Oncology Group (ECOG) performance status 0–2, adequate hematologic, renal, and hepatic function. Exclusion criteria: previous chemotherapy, clinical signs of brain metastases, severe cardiac disease, severe active infections, and need for immunosuppressive medication. Screening for brain metastases was not done.

The trial was approved by the Norwegian Medicines Agency, the Regional Committee for Medical Research Ethics, and the Hospital Review Board. It was carried out in compliance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all patients.

#### Treatment

The treatment schedule is shown in Appendix Figure A1 (see supplementary data online). Temozolomide was administered according to standard recommendations for malignant melanoma, 200 mg/m² orally on 5 consecutive days every 28 days. GV1001 was given as 3 injections during week 2 (Monday, Wednesday, Friday), 2 injections during week 3 (Monday, Friday), and single injections at weeks 6, 7, and 11. Immune responders were given booster vaccines at 4-week intervals. GV1001 (300 nmol peptide in 125 μl saline) and adjuvant granulocyte macrophage colony stimulating factor (GM-CSF; 75 μg) was administered by intradermal injection in the right paraumbilical area.

The duration of treatment depended on the clinical and immunologic response, according to the protocol: patients with objective tumor response or stable disease (SD) at week 12 and immunologic response received additional cycles with temozolomide and GV1001, with response evaluation at every third cycle. Patients with objective response/SD without immunologic response received additional cycles with temozolomide only. Patients with progressive disease (PD) and immunologic response discontinued temozolomide, but were offered booster vaccination with GV1001 every 4 weeks. All patients completing the first 7-week sequence of vaccines (23 of 25 patients) were considered evaluable for immunologic response.

#### Clinical evaluation

Tumor response was assessed by computed tomography (CT) scans and clinical examination at 12-week intervals, and classified according to the Response Evaluation Criteria in Solid Tumors (RECIST; ref. 24). Tumor response and survival was evaluated by intention-to-treat analysis.

Blood screening and assessment of adverse drug reactions were conducted at each visit. Adverse events were graded according to the National Cancer Institute common
toxicity criteria (NCI-CTC) version 3.0 and considered related to treatment if the relationship was reported as probable or suspected.

**Immunologic evaluation**

Peripheral blood mononuclear cells (PBMC) were obtained at weeks 0, 5, 9, and 12 and at every third booster vaccination. Pre- and postvaccination samples were analyzed in parallel for proliferation response to GV1001 peptide stimulation ($^{3}H$-Thymidine assays). T-cell responses were considered GV1001-specific when the stimulatory index (SI; response with antigen divided by response without antigen) was more than 2. Specific immune responses were further characterized by cytokine assays and T-cell clone experiments (HLA restriction, specificity for truncated peptides, response to recombinant hTERT protein). Materials and methods for immunologic assays, delayed type hypersensitivity (DTH) recording, flow cytometry, and production of peptides and recombinant hTERT protein are described in Appendix 1 (see supplementary data online).

**Statistics**

The study reports overall survival (OS) and progression-free survival (PFS), calculated from start of study treatment. OS for the intention-to-treat population, calculated by the Kaplan–Meier method, was compared with predicted survival based on a meta-analysis of clinical trials including 2,100 patients (6). The meta-analysis was conducted by Korn and colleagues for providing survival benchmarks for the intention-to-treat population, calculated by the Kaplan–Meier method, was compared with predicted survival by use of Student’s $t$ test. The DTH reactions were negative during standard study treatment in all subjects whose recordings were obtained, including 11 immune responders in T-cell assays. Interestingly, 3 patients turned positive after omission of temozolomide and continuous booster vaccination. In our previous GV1001 trials without temozolomide (19, 20), the DTH reactions were positive in a substantial number of patients (26 of 62). These observations may point to a modulating effect of temozolomide on the GV1001 response. DTH reactions have been associated with Th-1 profiles, but cytokine analyses showed that the present DTH negativity did not reflect low-level Th1 cytokines (see the following text).

**Development of long-term T-cell memory**

Most cancer vaccine trials have for practical reasons been limited to short-term vaccination and immunomonitoring, whereas clinical efficacy probably requires long-term T-cell responses (25, 26). Here, immune responders were offered continued monthly vaccination. Long-term follow-up revealed that 10 of 12 patients continuing vaccination developed durable GV1001-specific T-cell activity. Strikingly, all the 10 patients with durable T-cell responses at month 6 survived more than the 2 patients rapidly losing their responses (Table 1). Moreover, the survivors exhibited retained responses in follow-up samples obtained at later time points, ranging from week 36 to week 258 (Fig. 1B; Table 1; data not shown).

**Tumor response**

All 25 patients enrolled had PD at study entry and belonged to the most advanced categories within stage IV (64% M1C, 36% M1B, 0% M1A). Interestingly, 5 subjects developed partial tumor regression (partial response;
PR). Their tumor volumes decreased by 65% to 96% (Fig. 2A). An additional 6 patients experienced disease stabilization, whereas 14 subjects had continued PD. All 5 patients with PR developed GV1001-specific T-cell responses, and the immune responses prevailed throughout their tumor regression periods (Fig. 1B). Interestingly, the clinical responses developed far more gradually than expected from chemotherapy, with only 1 reaching PR at week 12, 3 more within week 36, and the fifth at week 48.

Patient 19 (P19) had disseminated MIC melanoma at study entry, including multiple visceral metastases. After start of vaccination, no new lesions appeared. The total tumor diameter started decreasing gradually, reached PR at week 48, and has continued to regress over a period exceeding 5 years (Fig. 2B). Remarkably, the patient has no symptoms of tumor disease, and all residual CT-lesions are negative on fluorodeoxyglucose positron emission tomography (FDG-PET) scans. As melanomas are highly PET-sensitive, the patient may have a complete response (CR). She currently receives booster vaccines at 3-month intervals and retains a strong GV1001-specific immune response (Fig. 1B).

Table 1. Disease stage, immune response, and clinical outcome

<table>
<thead>
<tr>
<th>Patient</th>
<th>AJCC stage</th>
<th>T-cell response</th>
<th>SI after vacc</th>
<th>Long-term T-cell memory</th>
<th>Tumor response</th>
<th>PFS (days)</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>IV; M1C</td>
<td>NE</td>
<td>NE</td>
<td>NA</td>
<td>PD -</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>IV; M1C</td>
<td>POS</td>
<td>14</td>
<td>POS (&gt;6 mo)</td>
<td>SD 168</td>
<td>344</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>IV; M1C</td>
<td>POS</td>
<td>11</td>
<td>NA</td>
<td>PD -</td>
<td>257</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>IV; M1C</td>
<td>NE</td>
<td>NE</td>
<td>NA</td>
<td>PD -</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>IV; M1C</td>
<td>POS</td>
<td>122</td>
<td>NA</td>
<td>PD -</td>
<td>583</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>IV; M1B</td>
<td>POS</td>
<td>35</td>
<td>POS (&gt;9 mo)</td>
<td>SD 168</td>
<td>379</td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>IV; M1B</td>
<td>POS</td>
<td>26</td>
<td>POS (&gt;9 mo)</td>
<td>PD -</td>
<td>317</td>
<td></td>
</tr>
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<td>P8</td>
<td>IV; M1C</td>
<td>POS</td>
<td>3.7</td>
<td>NEG (&lt;6 mo)</td>
<td>SD 140</td>
<td>289</td>
<td></td>
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<tr>
<td>P9</td>
<td>IV; M1C</td>
<td>POS</td>
<td>39</td>
<td>NEG (&lt;6 mo)</td>
<td>PD -</td>
<td>283</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td>IV; M1C</td>
<td>POS</td>
<td>12</td>
<td>NA</td>
<td>PD -</td>
<td>194</td>
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</tr>
<tr>
<td>P11</td>
<td>IV; M1B</td>
<td>POS</td>
<td>11</td>
<td>POS (&gt;25 mo)</td>
<td>PR 585</td>
<td>1,134</td>
<td></td>
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<td>P12</td>
<td>IV; M1B</td>
<td>POS</td>
<td>8.0</td>
<td>NA</td>
<td>PD -</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>P13</td>
<td>IV; M1B</td>
<td>POS</td>
<td>79</td>
<td>POS (&gt;14 mo)</td>
<td>PR 502</td>
<td>563</td>
<td></td>
</tr>
<tr>
<td>P14</td>
<td>IV; M1C</td>
<td>NEG</td>
<td>0.8</td>
<td>NA</td>
<td>PD -</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>P15</td>
<td>IV; M1C</td>
<td>NEG</td>
<td>1.3</td>
<td>NA</td>
<td>PD -</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>P16</td>
<td>IV; M1C</td>
<td>POS</td>
<td>15</td>
<td>POS (&gt;10 mo)</td>
<td>PR 385</td>
<td>795</td>
<td></td>
</tr>
<tr>
<td>P17</td>
<td>IV; M1B</td>
<td>NEG</td>
<td>1.3</td>
<td>NA</td>
<td>PD -</td>
<td>1,998+</td>
<td>(alive)</td>
</tr>
<tr>
<td>P18</td>
<td>IV; M1C</td>
<td>POS</td>
<td>17</td>
<td>NA</td>
<td>PD -</td>
<td>239</td>
<td></td>
</tr>
<tr>
<td>P19</td>
<td>IV; M1C</td>
<td>POS</td>
<td>61</td>
<td>POS (&gt;60 mo)</td>
<td>PR 1,905+</td>
<td>1,905+</td>
<td>(alive)</td>
</tr>
<tr>
<td>P20</td>
<td>IV; M1C</td>
<td>POS</td>
<td>24</td>
<td>POS (&gt;9 mo)</td>
<td>SD 168</td>
<td>590</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>IV; M1B</td>
<td>POS</td>
<td>29</td>
<td>POS (&gt;13 mo)</td>
<td>PD -</td>
<td>413</td>
<td></td>
</tr>
<tr>
<td>P22</td>
<td>IV; M1C</td>
<td>POS</td>
<td>122</td>
<td>POS (&gt;14 mo)</td>
<td>PR 248</td>
<td>594</td>
<td></td>
</tr>
<tr>
<td>P23</td>
<td>IV; M1B</td>
<td>NEG</td>
<td>1.1</td>
<td>NA</td>
<td>SD 168</td>
<td>312</td>
<td></td>
</tr>
<tr>
<td>P24</td>
<td>IV; M1C</td>
<td>POS</td>
<td>116</td>
<td>NA</td>
<td>PD -</td>
<td>418</td>
<td></td>
</tr>
<tr>
<td>P25</td>
<td>IV; M1B</td>
<td>NEG</td>
<td>1.1</td>
<td>NA</td>
<td>SD 154</td>
<td>169</td>
<td></td>
</tr>
</tbody>
</table>

0 M1A 18/23 10/12 5 PR Mean: 497
9 M1B 6 SD Median: 317
16 M1C 14 PD

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| aDisease stage and metastasis category (within stage IV) at study entry.
| bPOS, positive, that is, T-cell proliferation specific for GV1001 (SI > 2); NEG, negative (no specific response); NE, not evaluable, that is, first round of vaccination (7 injections) not completed and postvaccination samples not available.
| cSI (response with GV1001 divided by response without GV1001) after vaccination (vacc).
| dLong-term T-cell response. POS, GV1001-specific response > 6 months (last available and positive sample is indicated in brackets); NEG, turning negative within 6 months; NA: not applicable (because no initial T-cell response or no available long-term samples).
| eTumor response (RECIST). Best response during vaccination (compared with baseline, evaluated at 12-week intervals).
| fPFS from start of study treatment. CT scan at study entry was used as reference.
| gSurvival from start of study treatment.
| hP17 was included in a different phase I study and developed clinical response.
| iP19 has some residual lesions on CT, but these lesions are negative on FDG-PET.
Figure 1. GV1001-specific T-cell responses. PBMCs were obtained prior to start of therapy, at weeks 5, 9, and 12 and at every third booster vaccination. The PBMCs were stimulated once in vitro and tested for proliferation against irradiated PBMCs with or without peptide GV1001. A, pre- and postvaccination T-cell response from all evaluated patients. Columns represent mean SI, that is, response with antigen (GV1001) divided by response without antigen. Responses with SI > 2 were considered GV1001-specific. B, long-term T-cell memory in clinical responders. Columns represent mean cpm of triplicates. Tumor response periods are indicated with horizontal bars below charts. The assays show durable GV1001-specific T-cell responses in 5 patients with PR and 1 with SD.
Figure 2. Tumor response. A, PR in 5 patients. Top, columns represent total volume of all measurable lesions at study entry and minimum volume after vaccination. Bottom, response of individual tumor lesions for each patient. Location of lesion: LH, liver hilus; LN, lymph node; Ma, mammary glands; Med, mediastnum; Mu, muscle; P, pulmonal; SC, subcutaneous V visceral. B, tumor response in patient 19. Top, total diameter of all measurable lesions (corresponding to RECIST) at time points for CT evaluation. P19 stopped temozolomide at month 8, but still receive booster vaccines at 3-month intervals. Middle, response of individual lesions at different time points. All residual CT lesions are negative on FDG-PET scans, suggesting that no viable tumor tissue may remain. PET scans have not been repeated after month 42. Bottom, abdominal CT scans at study entry and after 61 months, showing regression of 3 metastatic lesions (CR = complete regression; PR = partial regression). The month 61 scan was taken without contrast due to allergy.
Patient 11 (P11) had multiple metastases to lung and mammary glands at study entry. CT evaluations showed a 29% regression in total tumor diameter at week 12, improving to 74% at week 36. All 5 metastases regressed partly or completely [Fig. 2A; Appendix Fig. A3 (see supplementary data online)]. We recorded PD at week 84 due to a new subcutaneous lesion, but continued vaccination and temozolomide. The patient stayed in good general health for 3 years, before developing brain metastases. Subjects P13, P16, and P22 also responded with gradual tumor regression, accompanied by durable hTERT-specific T-cell responses (Figs. 1B and 2A). CT scans showed regression of 6 of 8, 4 of 4, and 7 of 7 pre-study lesions, respectively [Fig. 2A; Appendix Figs. A2 and A3 (see supplementary data online)].

Progression-free and overall survival

Table 1 lists PFS and OS, calculated from start of study treatment. By intention-to-treat analysis (n = 25), median OS was 10.4 months (range, 2.2–66 months) and mean OS 16.3 months (M1B 20 months; M1C 14 months). Immune responders had increased median OS and PFS compared with nonresponders (median OS 396 days vs. 250 days; median PFS 112 days vs. 84 days). These differences did not reach statistical significance (log-rank test; P > 0.05), reflecting the low number of subjects. Advanced melanoma patients usually progress further within weeks or few months (6, 8). It is therefore of interest that the 5 PR patients exhibited extended PFS ranging from 8 to more than 62 months, as well as OS ranging from 19 to more than 62 months.

Trial subjects may differ from other patients, for example, due to selection processes determining which individuals are referred to trial units. Korn and colleagues have conducted a meta-analysis of 2,100 trial patients and suggested that survival in phase I/II melanoma studies is compared with their data set (6). They also outlined a method for calculating predicted survival, correcting for prognostic factors. Applying this method, we calculated a survival advantage for this study treatment, bordering 95% statistical significance (Fig. 3; intention-to-treat analysis). The apparent advantage was relatively stable at different time points (1 year: 44% vs. 24%; 2 years: 16% vs. 6.6%; 3 years: 12% vs. 3.7%). Moreover, one may note that the "control group" was not based on untreated subjects, but on trial patients receiving potentially active therapy. Korn and colleagues suggested declaring a treatment worthy of further study if the 1-year survival was better than predicted, with a P value less than 0.10. This criterion was met both by intention-to-treat and per protocol analysis (P = 0.059 and P = 0.032, respectively). The interpretation of these findings is still complex, as discussed in the following text.

T-cell clones from clinical responders

We generated 8 GV1001-specific T-cell clones from 2 clinical responders (P11 and P22). The clones were HLA class II DR restricted (Fig. 4A; data not shown). Taken together with our data from other studies (refs. 19, 23; unpublished data), we find that GV1001 is recognized on a series of DP, DR, and DQ molecules. The HLA promiscuity of GV1001 suggests that the vaccine is applicable to the general patient population, without a need for HLA typing.

A diverse response in terms of HLA restriction and fine specificity may reduce the risk of tumor escape (25). Here, we determined the fine specificity of T-cell clones by experiments with truncated peptides spanning the GV1001 amino acid sequence. The 4 analyzed clones from subject P22 recognized 3 different core motifs from the N-terminal part of GV1001 (Fig. 4B). One of the motifs was shared by a P11 clone (Fig. 4D). In previous studies, we have observed that multiple motifs from the central and C-terminal parts of peptide GV1001 may also be recognized (21).

Vaccine-specific T-cell clones may be of sparse relevance in vivo if they fail to recognize naturally processed antigens. Here, we observed recognition of naturally processed hTERT epitopes (Fig. 4C and D). Most clones responded more vigorously to hTERT protein than to pure GV1001 peptide (Fig. 4C; data not shown). We also noted strong responses against low concentrations of truncated peptides (Fig. 4C and D). These observations suggest that the clones may have higher affinity for naturally processed epitopes than for the vaccine peptide. The data also point to a simple mechanism for epitope spreading, as the cross-reacting natural epitopes may stretch beyond the GV1001-sequence.

Cytokine profiles

The cytokine profiles for 14 of 18 immunologic responders were investigated in Bioplex assays by using panels measuring up to 27 cytokines. The results showed GV1001-specific secretion of multiple cytokines in all 14 patients [Fig. 5; Appendix Table A2 (see supplementary data online)]. According to the Th1/Th2-paradigm, a Th1-like pattern is desirable for cancer eradication (27).
Figure 4. GV1001-specific T-cell clones from clinical responders. T-cell clone (T) proliferation after stimulation with irradiated EBV-transformed cells (EB) with or without peptide GV1001 or hTERT protein. Columns represent mean cpm of triplicate wells. A, patient 22. HLA restriction was determined by blockage with monoclonal antibodies against DP, DQ, or DR molecules. B, patient 22. Fine specificity analysis by stimulation with truncated peptides covering the GV1001 sequence (amino acid sequences given in right text box). The 4 P22 clones recognize 3 different core sequences, annotated in text boxes below the chart. C, patient 22. Recognition of naturally processed antigens. T-cell clones were stimulated with EBV-transformed cells incubated with recombinant hTERT protein. The assay indicates response to naturally processed protein in clones 5, 10, 18 (not 45). D, patient 19. Fine specificity and recognition of naturally processed antigens. T-cell clones were stimulated with truncated peptides or EBV-transformed cells incubated with recombinant hTERT protein. The core sequence recognized is highlighted in red below the chart.
Figure 5. Secretion of Th1/Th2 cytokines in postvaccination T-cell cultures. Cytokine assays were carried out in 14 of 18 immune responders, that is, all subjects were sufficient samples were obtained. T cells were stimulated with irradiated PBMCs with or without peptide GV1001. Supernatants were analyzed in duplicates or triplicates by Bioplex cytokine assays. Columns represent mean concentration (pg/mL). Data for non-Th1/Th2 cytokines are shown in Appendix Table A2 (see supplementary data online).
However, we have previously observed that human responses frequently do not follow a Th1/Th2 delineation (23, 28, 29). The present responses comprised high levels of key Th1-cytokines IFNγ and TNFα, but also of hallmark Th2 cytokines, interleukin 5 (IL-5) and IL-13. This applied both to the T-cell bulk cultures (Fig. 5) and to the T-cell clones (ref. 23; data not shown). Collectively, our present and previous results indicate that cytokine profiling should not rely on a Th1/Th2 dichotomy. Of note, we detected only low levels of IL-4 and IL-10 (Fig. 5). The responses would therefore easily be designated “Th1” with commonly used panels measuring only IFNγ, TNFα, IL-4, and/or IL-10. Interestingly, the high IFNγ/IL-10 ratios may reflect a favorable balance between immunity and tolerance, as IL-10 is considered to promote regulatory T cells and “tolerogenic” dendritic cells.

The analyses further showed GV1001-specific secretion of a broad range of proinflammatory cytokines and chemokines [Appendix Table A2 (see supplementary data online)], including IL-1β, IL-6, IL-8, GM-CSF, and macrophage inflammatory protein-1β. This polyfunctional cytokine profile was observed in all evaluated patients, both in T-cell bulk cultures and T-cell clones (Appendix Table A2 (see supplementary data online); ref. 23; data not shown). We also noted that the GV1001-specific responses included IL-17. A subset of activated T-cell clones may thus belong to the Th17 lineage (30, 31).

Finally, we asked whether temozolomide had affected the cytokine profile of the GV1001 response and compared the cytokine patterns to data from patients in our other GV1001 trials (Appendix Fig. A4 (see supplementary data online); refs. 20, 23; data not shown). The results suggested no substantial difference in the range of cytokines secreted or the ratio between key cytokines.

Discussion

The concept of combining cancer vaccination with chemotherapy is attractive, and also challenging. Because of the long-standing assumption that chemotherapy would preclude immunization, there is sparse experience on how these modalities interact. Here, we combined temozolomide with an hTERT peptide vaccine. The lack of increased toxicity is of particular interest, as temozolomide is bone marrow toxic and telomerase is expressed by hematologic stem cells. There is also no evidence of long-term toxicity in subjects receiving up to 5 years booster vaccination, in spite of durable immune responses. Interestingly, the immunologic response rate of 78% is higher than that in any previous GV1001 trial (19–22, 32). Follow-up studies of 12 immune responders revealed that 10 patients with durable GV1001 responses all survived more than the 2 subjects rapidly losing their T-cell activity. The clinical evaluation showed objective tumor responses in 5 subjects. OS was extended compared with matched controls from a benchmark meta-analysis.

The immune response rate of 78% indicates that temozolomide did at least not preclude immunization. Most subjects had normal lymphocyte counts at time of vaccination (data not shown). This may be related to the temozolomide-free intervals in the dosage regime for melanoma. Temozolomide is known to affect T-cell counts, but mostly if administered continuously (33–36). The considerable immune response rate may, moreover, reflect a beneficial effect of temozolomide. Several studies in mice have reported enhanced immunization after combining vaccination with temozolomide, or that temozolomide suppresses Treg function (4, 36–40). Chemotherapy may also suppress myeloid-derived suppressor cells (5).

The immunologic evaluation per protocol did not include Treg analyses, but we made an attempt to investigate the influence of temozolomide on Treg counts. Flow cytometry analyses showed that CD4+CD25high cells with a Treg phenotype were present in peripheral blood after temozolomide treatment and after the subsequent vaccine injections (data not shown). We did not obtain sufficient pretreatment samples to determine whether temozolomide had still affected Treg frequency and can therefore not make any conclusions about the effect of temozolomide on T-cell populations. In our ongoing melanoma vaccine trial (NCT00961844), this issue is addressed. Interestingly, preliminary data suggest that temozolomide induces decreased Treg counts (unpublished data). The cytokine data reported earlier in the text indicated that temozolomide had not substantially altered the cytokine profiles compared with previous GV1001 trials. Nevertheless, temozolomide is known to induce apoptosis of melanoma cells and is likely to induce inflammation and cross-priming of tumor-associated antigens. We hypothesize that this process may synergize with CD4+ T helper cells recruited through GV1001 vaccination. T helper cells may in particular engage antigen presenting cells (APC) presenting antigens from apoptotic tumor and induce epitope spreading (27). In ongoing studies of long-term survivors after GV1001 vaccination, we have detected responses against telomerase antigens not included in the vaccine (unpublished data).

Although most peptide vaccines have represented short CTL epitopes, the use of longer peptides recruiting T helper cells may yield advantages in vivo (1, 25, 27). A T helper response may more effectively synergize with apoptosis and inflammation induced by radiation or chemotherapy, as depicted earlier in the text. The durable memory responses reported herein for GV1001 point to another possible advantage; several studies have suggested that T helper activity is necessary for the development of CTL memory (41, 42). Long peptides may, moreover, be processed by endogenous APCs and recruit T-cell clones with diverse specificities, as observed for GV1001. Several long peptides, including GV1001, have furthermore been shown to elicit cytotoxic responses against nested short epitopes (1, 19, 43–45).

The present GV1001 responders developed polyfunctional cytokine profiles with mixed Th1/Th2 patterns and a high IFNγ/IL-10 ratio [Fig. 5; Appendix Table A2 (see supplementary data online)]. The broad and proinflam-
tory cytokine profile may mobilize the adaptive and the innate immune system. Interestingly, polyfunctional cytokine profiles have been associated with protective immunity in patients surviving HIV or Leishmania (46–48). Polyfunctional cytokine patterns may be particularly important in a cancer vaccine setting, where there is a need to overcome established tumor tolerance and transform the inflammatory milieu.

Six patients in the present trial harbored T-cell responses against GV1001 prior to vaccination, and others have reported spontaneous GV1001-specific activity in subjects with chronic lymphocytic leukemia (49). Furthermore, we show that GV1001-specific T-cell clones recognize endogenous APCs pulsed with hTERT protein. Both the pre-vaccination reactivity and the hTERT protein responses suggest that GV1001-associated epitopes are naturally processed and immunogenic in cancer patients. This study did not include screening of tumors for telomerase expression and hence did not address whether quantitative differences in expression level between patients may have influenced the vaccine response.

We report objective tumor regression in 5 patients and temporary disease stabilization in an additional 6 subjects. Several observations suggest that the regressions are unlikely to be caused only by temozolomide. First, all clinical responders belonged to the immunologic response group and developed long-term GV1001-specific memory. Second, the response rate is relatively high compared with the approximately 13% rate reported in phase III temozolomide trials (8, 50). Third, chemotherapy responses rarely occur in patients such as P19 with widespread visceral metastases outside of lung. Fourth, P19 developed most of her tumor regression after omitting temozolomide at month 8 and continuing vaccination (Fig. 2B). Fifth, responses to temozolomide are frequently short-lived, whereas 4 of 5 responders here exhibited PFS more than 1 year. Finally, responses to chemotherapy usually materialize shortly after start of treatment. By contrast, 4 of 5 responses in the present trial fell short of PR criteria at month 3, and all responses gradually improved over months or years. This pattern is in accordance with vaccine responses, depending on mobilizing the immune system and overcoming tumor tolerance. Taken together, these observations suggest a role of GV1001 in the development of clinical responses. There is, however, a need to conduct a randomized study before making any conclusions on clinical efficacy. The slow development of tumor responses shows that a conventional 3-month evaluation on the basis of RECIST may be misleading in cancer vaccine trials and supports the concept of developing adjusted response criteria, as suggested by others (26).

The group of study patients survived more than expected, compared with predicted survival as calculated from Korn’s meta-analysis. This finding should be interpreted with utmost caution, but is in line with possible clinical benefits suggested by the tumor regression and PFS data. Subjects with clinical signs of brain metastases were excluded from our trial. On the basis of the high frequency of asymptomatic brain involvement among patients with disseminated melanoma, it is still likely that a considerable fraction carried brain metastases. Because screening for cerebral involvement was not done, we calculated predicted survival on the basis of Korn’s data from studies not excluding brain metastases. Of note, patients with clinical signs of brain metastasis are rarely enrolled in trials, and the calculation takes into account the generally good performance status in our patients. Survival in our trial was calculated from the start of study treatment, whereas Korn’s meta-analysis gives survival from a possibly earlier point (“time of registration”). The latter difference suggests that our survival advantage may be underestimated. In all, these issues illustrate that any comparison between different trials carries considerable uncertainty. The apparent survival benefit of GV1001/temozolomide is interesting, but need to be reproduced with a randomized control group.

We conclude that combined therapy with temozolomide and GV1001 is well tolerated in advanced melanoma patients, and that standard temozolomide dosage for melanoma does not preclude development of vaccine responses. The immunologic responses exhibit several features of possible clinical significance, including durable T-cell memory, recognition of naturally processed antigens, high IFNγ/IL-10 ratios and polyfunctional cytokine profiles. Collectively, present and previous trials show that peptide GV1001 may recruit a diverse spectrum of T-cell clones with different HLA restrictions and fine specificity. Regarding clinical outcome, we observed objective tumor responses in 5 long-term immune responders. OS was extended compared with predicted survival from a meta-analysis. There have also been signs of a possible clinical activity in our previous GV1001 studies (19–21). However, both clinical and immunologic response rates are higher in the present trial, whereas the low toxicity from GV1001 monotherapy is retained. In our opinion, the results warrant further studies of combinatorial therapy with GV1001 and temozolomide. Our findings also support the general concept of combining cancer vaccination with chemotherapy.

Disclosure of Potential Conflicts of interest

G. Gaudernack is a member of the advisory board for Kael-Gemvax (patent holders for GV1001). The other authors declare no conflict of interest.

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Telomerase Peptide Vaccination Combined with Temozolomide: A Clinical Trial in Stage IV Melanoma Patients

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