Telomerase (GV1001) vaccination together with gemcitabine in advanced pancreatic cancer patients

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Abstract. Telomerase is expressed in 85-90 % of pancreatic adenocarcinomas and might be a target for active cancer immunotherapy. A study was conducted to investigate safety and immunogenicity in non-resectable pancreatic carcinoma patients using a 16-amino acid telomerase peptide (GV1001) for vaccination in combination with GM-CSF and gemcitabine as first line treatment. Three different vaccine treatment schedules were used; [A (n=6), B (n=6) and C (n=5)]. Groups A/B received GV1001, GM-CSF and gemcitabine concurrently. Group C received initially GV1001 and GM-CSF while gemcitabine was added at disease progression. Group D (n=4) was treated with gemcitabine alone. Adverse events (AE) related to vaccination were mild (grades I-II). Grade III AEs were few and transient. An induced GV 1001-specific immune response was defined as an increase ≥2 above the baseline value in one of the assays (DTH, proliferation, ELISPOT and cytokine secretion assays, respectively). A telomerase-specific immune response was noted in 4/6 patients in group A, 4/6 patients in group B and 2/5 patients in group C. An induced ras-specific immune response (antigenic spreading) was seen in 5 of the 17 patients. The cytokine pattern was that of a Th1-like profile. A treatment induced telomerase or ras response was also noted in group D. All responses were weak and transient. A significant decrease in regulatory T-cells over time was noted in patients in groups A and B ($p<0.05$). Telomerase vaccination (GV1001) in combination with chemotherapy appeared to be safe but the immune responses were weak and transient. Measures have to be taken to optimize immune responses of GV1001 for it to be considered of clinical interest.

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Introduction

The majority (85-90%) of patients with pancreatic adenocarcinoma have unresectable disease at diagnosis. Gemcitabine is a palliative treatment option. Median survival of gemcitabine treated patients is only 6 months (1). In patients with resectable disease, postoperative gemcitabine therapy significantly delayed time to recurrence (2). However, irrespective of treatment regimens, survival of pancreatic cancer patients remains poor and new therapeutic strategies are needed.

During the past years introduction of targeted therapeutics has been tested in pancreatic adenocarcinoma (1,3,4). Therapeutic cancer vaccines (TCV) is such an approach. Chemotherapy in combination with TCV might add to the immunological and clinical effects of TCV. Gemcitabine may augment immune responses by increasing the amounts of antigens loaded onto antigen-presenting cells (APC) (5) and downregulate T-regulatory cells (6). Patients with pancreatic carcinoma receiving chemoradiotherapy were capable of mounting a humoral and cellular response to tetanus toxoid, pneumococcal and hemophilus vaccines indicating a functionally preserved immune system (7). Administration of gemcitabine did not significantly decrease the number of T and B cells or APC and enhanced the immune response against cancer vaccine (8).

Telomerase is expressed in 85-90% of pancreatic adenocarcinomas (9). GV1001 is a telomerase derived peptide vaccine (hTERT: 611–626) consisting of 16 amino acids (10). This multiepitope peptide vaccine binds to various DP, DR and DQ HLA class II molecules. GV 1001 vaccination in non-small cell lung-cancer patients induced a cellular immune response in 85% of the patients (11). In a dose escalation study of GV1001 in pancreatic cancer patients, the vaccine was safe and induced a telomerase specific T cell response in 63%. Patients mounting a specific immune response had a better survival than immune non-responders (12). In advanced melanoma, treatment with GV1001 in combination with temozolomide was safe. Those patients developing a GV1001 specific long-term T-cell memory response survived longer than those rapidly losing the T-cell immunity (10).

In this explorative investigation we studied immunogenicity and safety of GV1001 in combination with different schedules of GM-SCF and gemcitabine as first line treatment of patients with advanced pancreatic adenocarcinoma.

Materials and methods

Patients. The report includes the results of two studies. Study 1 consists of groups A and B, and study 2 of groups C and D (see below). In total 28 patients were enrolled. Evaluable patients for immunogenicity per protocol were those who had completed immune testing at weeks 0 and 6/7. Eligibility criteria included histologically confirmed diagnosis of non-resectable pancreatic adenocarcinoma and a life expectancy of at least three months. Patients were required to have a Karnofsky performance status ≥70 and adequate bone marrow, cardiac, renal and hepatic functions. Exclusion criteria included chemotherapy or other potentially immune-suppressive therapy within 4 weeks prior to start of treatment including chronic use of systemic anti-histamines, corticosteroids or high-doses of NSAIDs.

Prior to entry, a complete case history, physical examination and blood tests including haemoglobin, WBC with a differential and platelet counts, electrolytes, liver function tests, standard urine analysis and serum tumor marker (CA 19-9) as well as an abdominal CT or MRI scan was performed. Chest X-ray was done on clinical request. During the study, patients were checked at regular intervals for performance status, routine blood hematology and chemistry analyses, and the serum tumor marker.

Adverse events (AE) were assessed according to the National Cancer Institute Common Toxicity Criteria versions 2.0 (groups A/B) or 3.0 (groups C/D), respectively, and considered related to treatment if a relationship was reported as possible or probable. AEs related to gemcitabine were observed for 8 weeks in groups A/B, and in groups C/D at all administrations. The primary endpoint was induction of an immune response against the vaccine as well as safety evaluation.

Patients were treated according to the Declaration of Helsinki ethical principles for medical research involving human subjects. The trial was performed according to GCP guidelines and approved by the Regional Ethics Review Board in Stockholm, Sweden [dnr: 03-145 (groups A/B) and 2006/1491-32 (groups C/D)] and by the Medical Products Agency in Uppsala, Sweden [dnr; 151:2003/25888 (groups A/B) and 151:2006/45316 (groups C/D)]. All patients provided a signed informed consent prior to study entry.

Vaccine. The GV1001 peptide vaccine was supplied as a freeze-dried product in sterile vials. The 16-amino acid hTERT-peptide (EARPALLTSRLRFIPK) covers positions 611-626; 300 nmole (560 μg) of GV1001 in 0.10 ml saline (groups A/B) and in 0.20 ml saline (group C) was administered. The dose of GV1001 was based on studies in non-small cell lung cancer (11) and pancreatic carcinoma patients (12). Isopharma AS, Norway manufactured GV1001 and the supplier was GemVax AS, Norway for group A and B patients. GV1001 was manufactured by Laboratoire Elaiapharm, France and supplied by Penn Pharmaceutical Services Ltd, UK for group C patients.

Vaccination schedule. Group A patients received GV1001 (560 μg) intradermally (i.d) days 1, 3 and 5 during the first week followed by a once weekly schedule in weeks 2, 3, 4 and 6. At each vaccination, the patients also received 150 μg GM-CSF (Leukine, Berlex Laboratories, Seattle, WA, USA) i.d., 15 min prior to GV1001 at the site of vaccination. All injections were given in the lower abdominal wall. Group B received the vaccine schedule as in group A with the exception that GM-CSF (150 μg) was given i.d. for five consecutive days the first week (days 1-5), and in weeks 2, 3, 4 and 6, GM-CSF (150 μg) for four consecutive days starting on the day of the peptide vaccination. Gemcitabine 1,000 mg/m^2 was administered intravenously (i.v.) once weekly for seven consecutive weeks in both groups. When gemcitabine and vaccine were given the same day, the vaccine was administered first. In groups A/B, after the initial seven weeks, gemcitabine was continued until progression at the clinicians' discretion. In group C GV1001 (560 μg) plus GM-CSF (75 μg) i.d., (Leukine, Berlex Laboratories) was administered as in group A. Gemcitabine (1,000 mg/m^2) was added at the time of progression and continued at the clinicians discretion. In group D gemcitabine (1,000 mg/m^2) alone was given weekly for the first 7 weeks, and then in 4-week cycles with 3 consecutive weekly administrations of gemcitabine followed by a rest for 1 week.

Clinical evaluation criteria. Progressive disease (PD) was defined by radiological measurements, supplemented by serum tumor marker and/or at clinical progression as determined by the investigator. Computer tomography (CT) or magnetic resonance imaging (MRI) was performed at the end of vaccination to assess tumor burden in groups A/B and every 8th week in groups C/D to assess time to progression (TTP). Disappearance of all radiographic evidence was considered a complete response (CR), while 30% or more decrease in the size of the tumor was considered to be a partial response (PR). At least 20% increase or appearance of new lesions was considered progressive disease (PD). Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD was considered stable disease (SD). Level of serum CA19-9 was considered to be stable if the increase or decrease was ≤50%.

Immune tests. Collection of blood samples as well as delayed type hypersensitivity (DTH) test against GV1001 were done prior to start of vaccine treatment. During treatment and follow-up, DTH was performed at weeks 4 and 7 in groups A/B and at weeks 2, 3, 4, 6, 10 in group C.

Blood T-cell responses against GV1001 were evaluated at week 7 and then every 8th week until two consecutive negative tests were noted (groups A/B). The corresponding time points in groups C/D were weeks 6, 12, 20 and 28. T-cell phenotyping (flow cytometry) was performed at the same time points.

Delayed type hypersensitivity (DTH). GV1001 (0.112 mg) in 0.1 ml saline (groups A/B) was injected intradermally in the volar part of the forearm. GV1001 (0.105 mg) in 0.22 ml saline (group C) was injected intradermally in the lower abdominal wall. The skin test was read after 48 h by measuring the diameter of induration (mm). A positive DTH response was defined as ≥5 x 5 mm of induration.

In vitro immune responses. *In vitro* immune responses were analyzed against GV1001 (immunizing peptide) and a ras-derived peptide (KLVVVGAAGVGKSALTI) (manufactered by Merck KGaA, Darmstadt, Germany and supplied by Avencia LSM, UK) [>90% of the pancreatic carcinoma patients express the ras-oncoprotein (13)]. As a negative

control, a peptide corresponding to HIV reverse transcriptase (KEPIVGAETFYVDGA) (Thermo Fisher Scientific, Waltham, MA, USA) was used.

Proliferation assay. The proliferation assay (^{3}H-thymidine incorporation) has been described previously (14). Peripheral blood mononuclear cells (PBMC) were isolated. A total of 10^5 cells/well were incubated for 6 days with GV1001, the ras-peptide and the HIV-peptide (1 and 10 μg/ml), respectively. Phytohemagglutinin (PHA) (10 μg/ml) (Sigma) and purified protein derivative of tuberculin (PPD) (10 μg/ml) (Statens Seruminstitut, Copenhagen, Denmark) were used as controls.

A stimulation index (SI) was calculated by dividing mean radioactivity (cpm) of 6 replicates of experimental wells by that of the background value (cells in medium alone) (15). SI values (mean ± 2SD) of healthy donors (n=9) against GV1001, ras- and HIV-derived peptides were 1.12±0.75, 1.24±0.79 and 1.18±0.74, respectively. The cut-off level for a proliferative T cell response was set to ≥2.0.

A positive telomerase (T)/ras (R) proliferative T cell response was defined if all of the following criteria were met: i) an SI in experimental wells ≥2.0; ii) an SI of cells stimulated with the control peptide <2.0; iii) a vaccine induced SI at least twice that of the pre-vaccination value. All patients had a positive response to PPD and PHA prior to, during and after vaccination.

ELISPOT (IFN-γ). ELISPOT was performed as previously described (15). PBMC ($2x10^5$ cells/well) were cultured in 48-well plates with GV1001, ras and HIV peptides resp. (1 and 10 μg/ml), PHA (5 μg/ml) or PPD (2.5 μg/ml) for 5 days in 6 replicates. A millipore 96-well filter plate was coated with anti-IFN-γ antibody (10 μg/ml) (Mabtech, Stockholm, Sweden). Cultured PBMC were transferred to the coated plate and incubated for 20 h with the antigens as above. Cells were washed and incubated with a secondary biotinylated anti-IFN-γ antibody (1 μg/ml) (Mabtech, San Jose, CA, USA) for 2 h at room temperature. After washing, streptavidin-ALP conjugate (1:1,000) (Mabtech, San Jose, CA, USA) was added to the cells and incubated for 1 h at room temperature. Cells secreting IFN-γ were developed by adding substrate BCIP/NBT (Mabtech, San Jose, CA, USA) and incubated at room temperature for 5 min. The reaction was stopped at the appearance of dark spots. Spots were counted by an automatic ELISPOT assay reader (AID, Strassberg, Germany).

A vaccine induced IFN-γ response was defined if all of the following criteria were fulfilled: i) spot forming units (SFU) of stimulated (GV1001) cells significantly higher ($p<0.05$) than that of unstimulated cells (background) and at least twice that of the background; ii) SFU of cells stimulated with the control peptides not significantly ($p>0.05$) higher than that of the background; iii) SFU of a post-vaccination test at least twice that of the pre-vaccination test (15).

Cytokine secretion assay. Supernatants were collected (20 μl/well) after 24 and 120 h of incubation from the proliferation assay. The volume was replaced with complete medium.

In groups A/B, IL-4, IL-10, IFN-γ, TNF-α and GM-CSF were analyzed using the Luminex technology (LINCOplex Kit, Linco Research Inc., St. Charles, MO, USA) according to the manufacturer's instruction. In groups C/D, IL-4, IL-10, IFN-γ, TNF-α and GM-CSF were analyzed using a human 8-plex cytokine reagent kit (171-304000) and the Bio-Plex instrument (Bio-Rad, Hercules, CA, USA) according to manufacturer's instruction. Standard concentration curves were generated. The coefficient of variation of PHA stimulated cells (n=5) was 12±10% (mean ± SD).

Cytokine concentration (pg/ml) in supernatants of antigen stimulated cells divided by that of cells alone using the highest value at 24 or 120 h culture periods respectively was used. The post-vaccination ratio divided by pre-vaccination ratio at different time points is shown. A ratio ≥2 (relative increase) was considered an antigen-induced specific response (Table II). The absolute concentrations of the different cytokines over time are also shown.

A single time point-induced immune response (STIR) post-vaccination. A patient was considered to have a single time point-induced immune response if a response in one of the assays (DTH, proliferation, ELISPOT, cytokine secretion) was noted at one time point only.

A sustained immune response (SIR) post-vaccination. A patient was considered to be a sustained immune responder if an immune response was noted in at least one of the assays (DTH, proliferation assay, ELISPOT, cytokine secretion) at two time points or more.

Flow cytometry. Peripheral blood mononuclear cells were analyzed for T cell subsets by flow cytometry as previously described (16). Flurochrome-conjugated antibodies (CD3, CD4, CD8, CD25, CD45RA) (Becton Dickinson Biosciences, San Jose, CA, USA), CCR7 (R&D Systems, Minneapolis, MN, USA) and Foxp3 staining kit from e-Biosciences Inc. (San Diego, CA, USA) was used. A minimum of 20,000 events were collected using a FACSCalibur (BD) and analyzed by the Cellquest® Software (BD).

Statistical analysis. The non-parametric Mann-Whitney two-tailed rank sum test for comparison of independent variables and the two-tailed non-parametric Wilcoxon signed rank test for dependent observations, were applied.

Results

Patients. Twenty-one out of initially 28 enrolled patients completed immune testing at weeks 0 and 6/7 and were considered evaluable for an induced immune response. Clinical characteristics of the 21 patients are shown in Table I. Eight patients were initially enrolled in group A but two were withdrawn, one not fulfilling inclusion criteria and one with disease progression at week one. Two patients in group B withdrew informed consent at week 3 and 4, respectively. One was withdrawn due to progression at week 3. One patient in group B succumbed before study completion and was replaced. Six evaluable patients in group A and B, respectively, completed at least 7 weeks of study. All five patients enrolled in group C were immunologically evaluable. Five patients were enrolled in group D. One was withdrawn during the first week due to disease progression.

Table I. Clinical characteristics of pancreatic adenocarcinoma patients.

Patient no./group	Gender/age (years)	Tumor localization	Site of metastasis at inclusion	Previous treatment	Time from diagnosis to start of vaccination (weeks)	No. of immunisations	No. of Gemcitabine administrations (n)	Second line treatment	Time to progression[a] (weeks)	Overall survival[a] (weeks)
1/A	M/60	PAC	Liver, lung	None	10	7	18	None	33	35
3/A	F/79	LR	None	op Whipple	8	7	9	None	26	34
5/A	M/62	PAC	Liver	None	3	7	8	None	8	18
6/A	M/72	PAC	None	None	11	7	7	None	8	73
7/A	F/59	LR	Liver, lung	op Whipple	12	7	11	None	17	35
8/A	M/76	LR	None	op Whipple	7	7	10	None	43	49
10/B	M/60	PAC	Liver	None	4	7	6	None	10	12
11/B	M/72	LR	Lung, distant nodes	op Whipple	7	7	8	None	53	73
13/B	M/73	LR	Liver	op Whipple	6	7	9	None	17	20
14/B	M/73	PAC	Liver	None	5	7	9	None	23	42
16/B	M/75	PAC	None	None	8	7	11	5-Fu	104	166
18/B	F/66	PAC	None	None	8	7	6	None	32	36
19/C	F/70	PAC	Liver	None	12	7	3	None	8	13
20/C	F/57	PAC	None	None	5	6	0	None	10[b]	15
21/C	F/74	PAC	Lung, nodes	None	5	10	2	None	24	37
22/C	F/58	PAC	Liver	None	13	5	0	None	8	22
23/C	M/58	PAC	None	Gastroenterostomy	10	14	14	None	8	38
24/D	M/64	PAC	Lung, nodes	None	10	NA	22	None	32	33
25/Dc	M/52	PAC	Nodes	None	8	NA	21	5-Fu-Oxa	40	70
26/D	M/61	PAC	Liver	None	5	NA	19	None	32	36
27/D	M/66	PAC	Lung, nodes	None	4	NA	19	None	31	42

PAC, advanced pancreatic adenocarcinoma; LR, local recurrance; NA, not applicable. [a]From start of vaccine treatment. [b]Patient no. 20 was withdrawn after 5 weeks, due to hepatic dysfuntion liver metastasis. [c]After 12 weeks the patient decided to discontinue gemcitabine but to continue with 5-Fu/Oxa at which time point the disease was stable.

Table II. Treatment induced immune responses against telomerase (T)/ras (R).

Patient no./group	Week 6/7					Week 12/15					Week 20/21					Week 28[d]		DTH response at week	STIR response T/R	SIR response T/R
	Prolif.[a] (SI) T/R	Elispot[b] SFU/10^6 cells T/R	Cytokine secretion (ratio)[c] IFN-γ T/R	TNF-α T/R	GM-CSF T/R	Prolif.[a] (SI) T/R	Elispot[b] SFU/10^6 cells T/R	Cytokine secretion (ratio)[c] IFN-γ T/R	TNF-α T/R	GM-CSF T/R	Prolif.[a] (SI) T/R	Elispot[b] SFU/10^6 cells T/R	Cytokine secretion (ratio)[c] IFN-γ T/R	TNF-α T/R	GM-CSF T/R	Prolif.[a] T/R	Elispot[b] T/R			
1/A	-	-	**3.8/-**	**-/5.6**	-	-	-	**-/2.9**	-	-	-	-	-	-	-	-	-	-	+/+	-/+
3/A	-	-	**12/-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**4**	+/-	+/-
5/A	**-/2.5**	-	**13/-**	**3.5/-**	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	+/+	-
6/A	**-/3.7**	-	-	-	-	**-/30**	-	-	-	-	-	-	-	-	-	-	-	-	-/+	-/+
7/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8/A	-	-	**2.8/-**	**2.3/-**	**2.9/-**	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-
10/B	**6.1/-**	-	-	-	**2.3/-**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	+/-	-
11/B	-	-	-	-	-	-	-	-	-	-	-	-	**2.5/-**	-	-	-	-	-	+/-	-
13/B	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
14/B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**7**	+/-	-
16/B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18/B	**3.8/4.5**	-	-	**2.6/3.7**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/+	-
19/C	-	-	-	-	**8.8/-**	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-
20/C	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
21/C	-	-	-	-	-	-	**-/58**	-	-	-	-	-	-	-	-	-	-	-	-/+	-
22/C	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	-	-	-
23/C	-	-	-	-	-	-	**58/-**	-	-	-	-	-	-	-	-	-	-	-	+/-	-
24/D	-	-	-	-	-	-	-	-	-	-	-	-	**2.8/2.9**	-	-	ND	ND	ND	+/+	-
25/D	-	-	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-
26/D	-	-	-	-	-	-	-	-	**3.3/-**	-	-	-	-	-	-	ND	ND	ND	+/-	-
27/D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND	ND	-	-

Bold figures indicate a positive response. [a]Prolif., proliferation assay [stimulation index (SI) >2 was considered a positive response]. [b]Elispot, spot forming units (SFU)/10^6 cells. [c]A ratio between post and prevaccination cytokine secretion values was considered a positive response if the value was >2. No Th2 cytokines (IL-4, IL-10) were detected. [d]Cytokine secretion assay was not done at week 28. STIR, single time point immune response (see Materials and methods); SIR, sustained immune response (see Materials and methods); ND, not done.

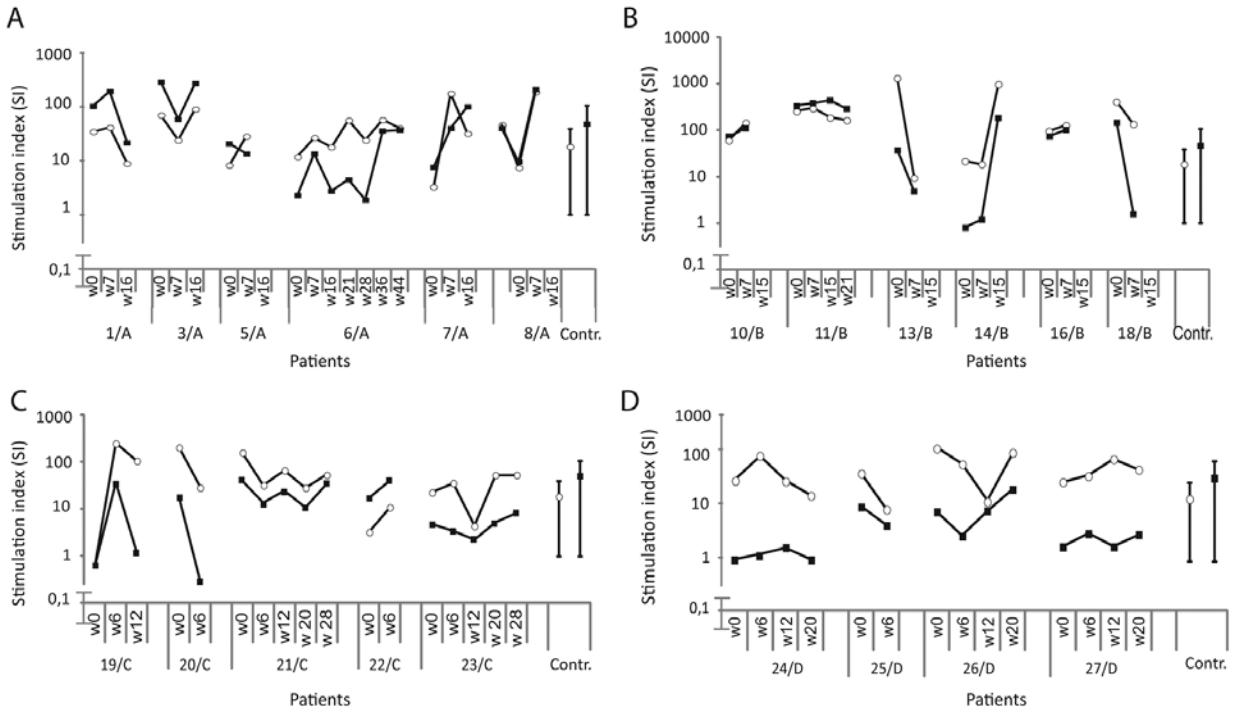

Figure 1. (A-D) Proliferative cellular response (SI, stimulation index) of individual patients against PHA (open symbols) and PPD (filled symbols) over time (w, weeks) in groups A, B, C and D patients. Healthy control donors (n=9) are shown as mean ± SD.

Immune responses

In vivo immune response.

Delayed type hypersensitivity (DTH). No patient had a DTH response prior to vaccination. One patient, no. 3 (group A), developed a DTH at week 4, which disappeared at week 7. Patient no. 14 (group B) showed a DTH response at week 7 (Table II).

In vitro immune responses.

Proliferation assay. Proliferative responses post-vaccination against telomerase (T) or ras (R) are shown in Table II. One patient (no. 3, group A) had a GV1001-specific proliferative response prior to vaccination but not at subsequent testing (data not shown). A GV1001-specific response was induced in two patients in group B. Two patients in group A, and one patient in group B had a ras-induced specific response at week 7. In one patient, the ras-induced immune response was sustained. No proliferative responses against telomerase or ras were detected in groups C and D. The proliferative response against PHA and PPD over time are shown in Fig. 1. The PHA and PPD responses of the patients were within the range of healthy donors.

ELISPOT (IFN-γ). Prior to vaccination no IFN-γ ELISPOT response was seen in any patient. No IFN-γ ELISPOT responses against telomerase or ras were induced at any time points in patients of groups A and B. A GV1001-specific IFN-γ response was evoked at week 12 in one patient (no. 23) in group C and a ras-specific response in patient 21 at week 12 (Table II). IFN-γ T cell responses against PHA and PPD over time for patients are shown in Fig. 2. The PHA and PPD responses of patients were within the range of healthy control donors.

Cytokine secretion assay. The relative increase of induced antigen specific cytokine secretion is shown in Table II. A telomerase response was noted in 4 patients in group A. A Th1-like (IFN-γ, TNF-α, GM-CSF) cytokine response pattern was seen but no Th2 cytokines (IL-4, IL-10) (data not shown). One patient had a ras response. In group B three patients mounted a telomerase response and one a ras response. Again, the response pattern was Th1 cytokines, but no Th2. One patient in group C developed a Th1-like cytokine response. In group D, two patients developed a telomerase Th1-like cytokine response and one a ras response. The absolute cumulative concentrations (pg/ml) of cytokines (IFN-γ, TNF-α, GM-CSF, IL-4, IL-10) for all individual patients are shown in Fig. 3.

Single time point immune responders and sustained immune responder. Four out of the 6 patients (67%) in group A developed a single time point induced immune response against telomerase and 3 (50%) against ras. In group B, 4 out of 6 (67%) patients mounted a telomerase response and one (17%) against ras. A telomerase response in group C was noted in 2/5 (40%) patients and a ras response in 1/5 (20%) patients. In group D, a telomerase response was recorded in 2/4 (50%) patients and a ras response in 1/4 (25%) patients.

A sustained induced immune response (an immune response at at least two different time points) was only seen in group A patients, in one patient against telomerase and in two patients against ras.

Lymphocyte subsets. There was no significant difference in T_{reg} cells at baseline comparing patients with healthy controls (n=9) (data not shown). A significant decrease in the frequency of T_{reg} ($CD4^+CD25^+Foxp3^+$) cells was noted for patients in groups A and B over time (Fig. 4), but no significant change in T_{reg} cells in groups C and D patients (data not shown). No

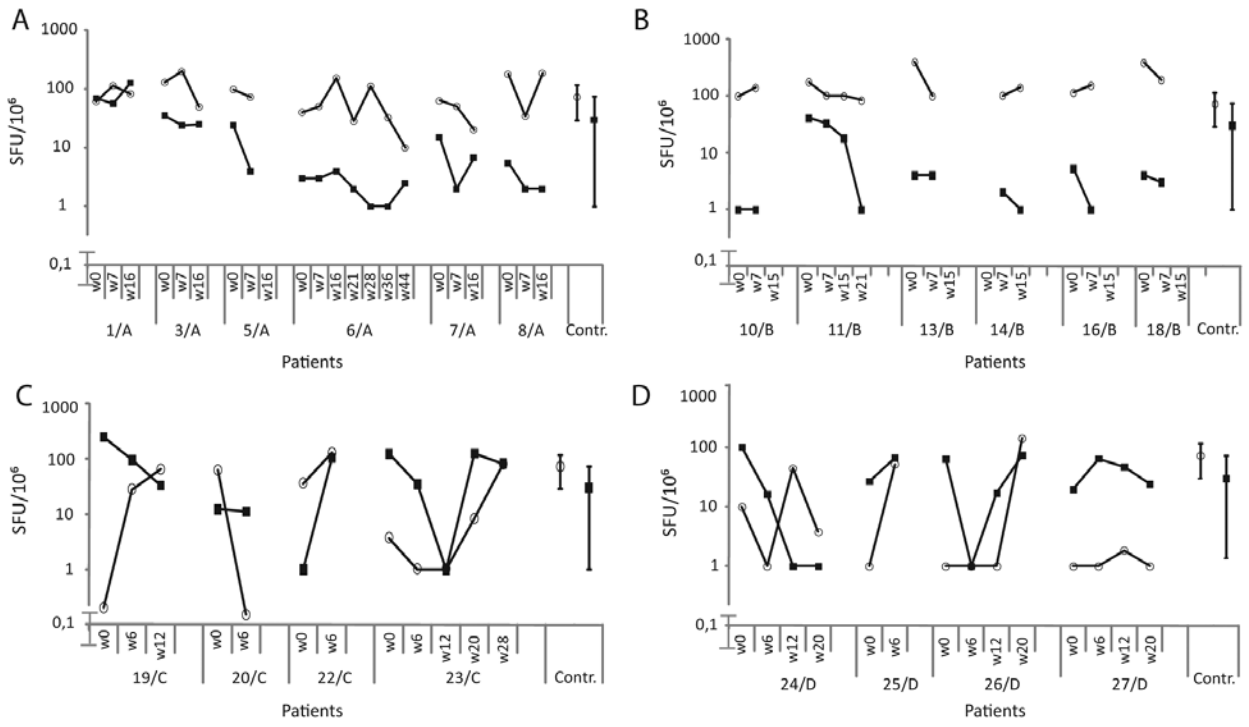

Figure 2. (A-D) IFN-γ T cell response (ELISPOT) against PHA (open symbols) and PPD (filled symbols) in patients over time (w, weeks) in groups A, B, C and D patients, respectively. Healthy control donors (n=9) are shown as mean ± SD.

Table III. Frequency of adverse events (AE) related to GV1001 and GM-CSF. Highest grade (1-5)[a] reported once for each patient.

	Group A (n=6)			Group B (n=6)			Group C (n=5)			Total (n=17)
Adverse events (AE)	Grade 1 no. of pts (%)	Grade 2 no. of pts (%)	Grade 3 no. of pts (%)	Grade 1 no. of pts (%)	Grade 2 no. of pts (%)	Grade 3 no. of pts (%)	Grade 1 no. of pts (%)	Grade 2 no. of pts (%)	Grade 3 no. of pts (%)	Grades 1-3 no. of pts (%)
Local AE										
Injection site reaction				3 (50)		3 (50)	4 (80)			10 (59)
Urticaria local					1 (17)					1 (6)
Pruritus					1 (17)					1 (6)
Systemic AE										
Fatigue	1 (17)	1 (17)		1 (17)	4 (67)		1 (20)		1 (20)	9 (53)
Chills	1 (17)			4 (67)			1 (20)			6 (35)
Fever	1 (17)		1 (17)	1 (17)	1 (17)		2 (40)			6 (35)
Leg pain				1 (17)	1 (17)					2 (12)
Myalgia				1 (17)	1 (17)					2 (12)
Elevated liver enzymes									2 (40)	2 (12)
Urticaria				1 (17)						1 (6)
Abdominal pain								1 (20)		1 (6)

No grade 4/5 AEs were seen. Pts, patients.

change over time was seen in $CD4^+$ or $CD8^+$ T cells or in NK cells in any of the groups (data not shown).

Adverse events. Immunization was done on an out-patient basis. AEs associated with GV1001 and GM-CSF was generally mild. No grades 4/5 AEs were seen. The most common AEs considered to be related to immunization are presented in Table III and were most commonly noted during the first 6 weeks (data not shown). Injection site reactions and influenza-like symptoms were most pronounced in group B. In group B, one injection of GM-CSF in two patients and two injections in two patients were omitted due to a strong skin reaction as well as one injection in one patient in group C.

Side-effects related to gemcitabine were as expected with predominantly hematological and infectious grade 1-4 AEs. The most frequent grade 3 (n=3) or grade 4 (n=5) AE was

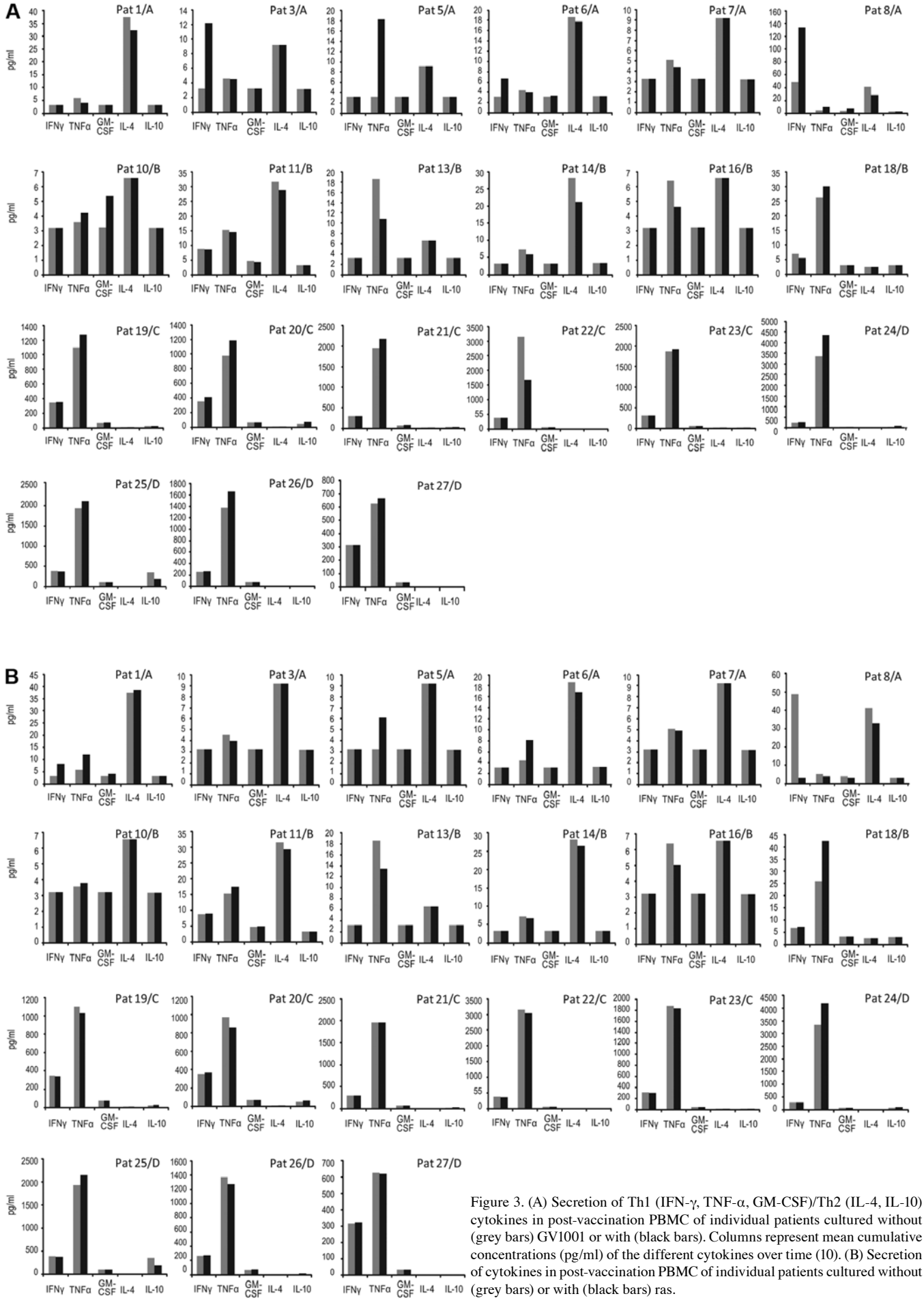

Figure 3. (A) Secretion of Th1 (IFN-γ, TNF-α, GM-CSF)/Th2 (IL-4, IL-10) cytokines in post-vaccination PBMC of individual patients cultured without (grey bars) GV1001 or with (black bars). Columns represent mean cumulative concentrations (pg/ml) of the different cytokines over time (10). (B) Secretion of cytokines in post-vaccination PBMC of individual patients cultured without (grey bars) or with (black bars) ras.

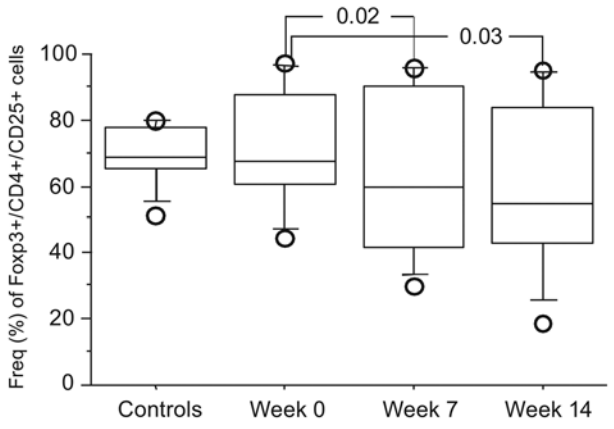

Figure 4. Frequency of T_{reg} cells ($CD4^+CD25^+Foxp3^+$) following immunisation with GV1001, GM-CSF in combination with gemcitabine in group A and B patients (n=8). The baseline frequency of T_{reg} cells in patients compared to controls (n=9) was statistically not significant. The box represents the 25th to 75th percentiles. The line in the middle represents the median. The top whisker is drawn from the value associated with the 75th to 90th percentile, and bottom from 25th to 10th percentile. Circles represent the outliers. P-values are indicated on the top.

neutropenia. Grade 3 infections were noted in two patients and trombocytopenia grade 3 and 4 in one patient, respectively. Four patients, respectively, had a grade 3 anorexia, nausea, fever or liver abscess (data not shown).

In total, 32 serious adverse events (SAE) were reported. In group A, 2 SAE in 2 patients; in group B, 6 SAE in 5 patients; in group C, 12 SAE in 4 patients; and in group D, 12 SAE in 2 patients. The majority was disease or gemcitabine related. One SAE was initially suspected to be related to GV1001 or GM-CSF in a patient in group C, who developed hepatic dysfunction (grade 3). Immunization was stopped. Liver metastasis obstructing the bile ducts was later diagnosed and considered to be responsible for the reported SAE.

Clinical efficacy. None of the vaccinated patients achieved CR or PR. In group A (n=6) four patients had SD and two patients PD at week 8 after start of treatment. A decline of >50% in CA 19-9 was observed in one patient in group A with a radiological SD (pat. no. 7). All patients in group B had SD at week 8 after start of treatment. One patient in group C who received only vaccination had SD for 24 weeks. The remaining patients in group C progressed rapidly. In group D, the four patients had SD for 31-40 weeks. In patient no. 25 gemcitabine was switched to 5-Fu/Oxa at week 12 due to the patient's own decision. There was no sign of progression until week 40.

Median time to progression (TTP) in group A was 22 weeks (range, 8-43), in group B 27 weeks (range, 10-104), in group C 8 weeks (range, 8-24) and in group D 32 weeks (range, 31-40). Median OS from start of study treatment to death was 35 weeks in group A (range, 18-73), 39 weeks in group B (range, 12-166), 22 weeks in group C (range, 13-38) and 39 weeks (range, 33-70) in group D (Table I).

Discussion

The combination of GV1001, gemcitabine and GM-CSF appeared to be safe and well tolerated. Vaccine related AEs were mild and transient. A higher dose of GM-CSF induced a higher frequency and severity of injection site reactions. Gemcitabine related side-effects were as expected (17) and without overlapping toxicity with the vaccine treatment.

Similar criteria for mounting a single time point induced immune response was applied as in the study of Bernhardt *et al* (12) immunizing non-resectable pancreatic carcinoma patients with GV1001 and GM-CSF but without gemcitabine where 75% of the patients mounted an immune response. In groups A and B, differing only in the dose of GM-CSF, a total of 67% of the patients showed an induced telomerase response. The results might indicate that concomitant treatment with gemcitabine may not hamper the induction of an immune response but delayed administration of gemcitabine might reduce the capacity to mount an immune response and may favor tumor progression (group C). The study might also support the notion that multiple immune read-out systems may increase the sensitivity to detect antigen specific immune responses (18,19).

In the cytokine secretion assay, IFN-γ and TNF-α were the most prevalent cytokines in post-vaccination T cell cultures, indicating the induction of a Th1-like response. Vetsika *et al* (20), also noted a TERT-specific Th1 skewed response in 69% of TERT vaccinated NSCLC patients. A Th-2-like response could not be detected in our study.

GV1001 is capable of binding to molecules encoded by multiple alleles of all the three loci of HLA class II (11) and also to be endogenously processed in APC of the skin (21) and as such able to induce $CD4^+$ and $CD8^+$ T cells (22). These characteristics of the hTERT peptide might enable all patients, irrespective of HLA-type, to present one or more GV1001 epitopes to immune effector cells. Moreover, cytokine secretion of activated CD4 T-cells may stimulate CD8 and NK cells to increased infiltration in the tumor as well as upregulation of MHC class I molecules, which might be downregulated in advanced cancer (4,23). Such an orchestration of cellular immune responses should be of therapeutic advantage.

In advanced colorectal cancer patients vaccinated with a multipeptide cancer vaccine containing both class I and II peptides, a class I response was noted in 43% of the patients, and a class II response in 65%. A total of 34% of the patients had both a class I and II response which was associated with a significantly higher disease control rate (24).

To improve the efficacy of cancer vaccines, adjuvants should be added. GM-CSF may be an appropriate choice. The dose and schedule of GM-CSF is however not clearly established. In a study by Faries *et al* (25), patients with resected melanoma received a melanoma vaccine with a high dose of GM-CSF (>300-400 μg/d for 5 days) compared to no GM-CSF. An enhanced antigen-specific response was seen in the GM-CSF group. However, early death and a trend towards worse survival was noted in the GM-CSF group. Slingluff *et al* (26) studied the effect of a low dose of GM-CSF (<20 μg/d once a week) together with a melanoma vaccine. There was a significantly lower rate of $CD8^+$ T-cell responses in the GM-CSF group (34%) compared to the control group (73%). We have previously analyzed the humoral and T-cell responses in CRC patients vaccinated with a recombinant CEA with or without concomitant GM-CSF (80 μg/d) for four consecutive days. GM-CSF significantly increased the humoral anti-CEA response as well as the T-cell response (27-29). In follicular lymphoma patients,

a customized idiotype vaccine together with GM-CSF induced an anti-idiotypic T-cell response. The dose of GM-CSF was important. A total of 50,000 units of GM-CSF were less effective than 10,000 units (30). Parmiani *et al* (31) showed that relatively low doses of GM-CSF (40-80 μg for 1-5 days together with a cancer vaccine) augmented a tumor specific immune response, while higher doses (100-500 μg) had no advantage, or even induced immune suppression. Doses exceeding 80 μg/day for more than 3-5 days have been shown to induce mobilization of myeloid-derived suppressor cells (32-34), which might inhibit tumor-specific and non-specific T-cell responses (35). In the present study, the different doses and schedules of GM-CSF did not permit firm conclusions due to the small number of patients but only in the low dose of GM-CSF group a sustained induced immune response was seen.

Chemotherapeutics may also augment a tumor vaccine specific cellular response by several mechanisms. The T cell repertoire might be skewed towards the tumor antigen during recovery from chemotherapy induced lymphopenia (36). Chemotherapeutics may augment a T cell response by reducing the number of T_{reg} cells (37). Gemcitabine has previously been shown to reduce the frequency of T_{reg} in man (6) and in an animal model to act synergistically with a tumor-vaccine to improve the therapeutic antitumor effect (5,38). The limited number of patient receiving gemcitabine only did not permit conclusions but in patients receiving gemcitabine and GV1001 concomitantly (groups A and B) a significant reduction in the number of T_{reg} cells was seen. Antigen presenting cells may cross-present tumor antigens induced by chemotherapy-mediated tumor lysis (36). We also observed induction of reactivity against a ras derived peptide. Epitope spreading could be due to tumor lysis induced by gemcitabine or by the vaccine as HER2 alone vaccinated breast cancer patients also showed antigenic spreading which was an independent predictor of survival (39).

Treatment with GV1001, GM-CSF and gemcitabine seemed to be safe. An immune response against telomerase in the best schedule was noted in approximately two thirds of the patients, similar to other studies but the immune response was weak and transient. It should be noted that the patients did not seem to be immune hyporesponsive as evaluated by PHA and PPD responses.

A multicenter study (Primovax) (http://www.clinicaltrials.gov/ct2/results?term=primovax &Search=Search) in advanced pancreatic cancer was early closed due to lack of effect. In another phase III study (Telovac study) chemotherapy ± GV1001 vaccination could not show a significant difference in overall survival (40). Based on the experience of the present study and those of others including immune responses and clinical efficacy, measures have to be taken to augment the magnitude and duration of the immune response to GV1001. Maybe, the GV1001 vaccine is not an optimal telomerase vaccine candidate, although it has been shown that immune responders to GV1001 vaccination survived longer than non-immune responders (12) and CLL patients exhibited spontaneously T cells recognizing GV1001, which T cells could lyse autologous telomerase expressing leukemic cells (41). Treatment strategies to downregulate immune suppression as anti-CTLA-4 or anti-PD1 monoclonal antibodies might be of importance to add (42,43). Advanced pancreatic carcinoma patients may not be a preferred clinical setting for vaccine treatment, as is the case for other TCVs in several other advanced tumors (44) but the combination of gemcitabine and a cancer vaccine may be a beneficial approach as shown in a pancreatic carcinoma animal model (45).

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