Phase I/II Combined Chemoimmunotherapy with Carcinoembryonic Antigen–Derived HLA-A2–Restricted CAP-1 Peptide and Irinotecan, 5-Fluorouracil, and Leucovorin in Patients with Primary Metastatic Colorectal Cancer

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Abstract

Purpose: We conducted a phase I/II randomized trial to evaluate the clinical and immunologic effect of chemotherapy combined with vaccination in primary metastatic colorectal cancer patients with a carcinoembryonic antigen–derived peptide in the setting of adjuvants granulocyte macrophage colony-stimulating factor, CpG-containing DNA molecules (dSLIM), and dendritic cells.

Experimental Design: HLA-A2–positive patients with confirmed newly diagnosed metastatic colorectal cancer and elevated serum carcinoembryonic antigen (CEA) were randomized to receive three cycles of standard chemotherapy (irinotecan/high-dose 5-fluorouracil/leucovorin) and vaccinations with CEA-derived CAP-1 peptide admixed with different adjuvants [CAP-1/granulocyte macrophage colony-stimulating factor/interleukin-2 (IL-2), CAP-1/dSLIM/IL-2, and CAP-1/IL-2]. After completion of chemotherapy, patients received weekly vaccinations until progression of disease. Immune assessment was done at baseline and after three cycles of combined chemoimmunotherapy. HLA-A2 tetramers complexed with the peptides CAP-1, human T-cell lymphotrophic virus type I TAX, cytomegalovirus (CMV) pp65, and EBV BMLF-1 were used for phenotypic immune assessment. IFN-γ intracellular cytokine assays were done to evaluate CTL reactivity.

Results: Seventeen metastatic patients were recruited, of whom 12 completed three cycles. Therapy resulted in five complete response, one partial response, five stable disease, and six progressive disease. Six grade 1 local skin reactions and one mild systemic reaction to vaccination treatment were observed. Overall survival after a median observation time of 29 months was 17 months with a survival rate of 35% (6 of 17) at that time. Eight patients (47%) showed elevation of CAP-1–specific CTLs. Neither of the adjuvants provided superiority in eliciting CAP-1–specific immune responses. During three cycles of chemotherapy, EBV/CMV recall antigen–specific CD8+ cells decreased by an average 14%.

Conclusions: The presented chemoimmunotherapy is a feasible and safe combination therapy with clinical and immunologic efficacy. Despite concurrent chemotherapy, increases in CAP-1–specific T cells were observed in 47% of patients after vaccination.

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Despite advances in occult blood screening (1), colonoscopy (2), surgery (3), radiotherapy, and adjuvant (4–6) chemotherapy, colorectal cancer is still the second leading cause for cancer-related mortality in industrialized countries in men and the third leading cause in women. Palliative chemotherapy regimens have been improved by adding topoisomerase-I inhibitors and platin-based cytotoxic drugs. Currently, the majority of patients with newly diagnosed metastatic colorectal cancer are treated with irinotecan or oxaliplatin together with high-dose 5-fluorouracil (5-FU) and leucovorin (7, 8). However, even with therapy, patients with Unio Internationale Contra Cancrum stage IV disease reach an average overall survival time of 15 to 20 months (9, 10).

In this setting, immunotherapy offers a potential complementary approach to chemotherapy, especially in the setting of minimal residual disease and limited prior chemotherapy that may adversely affect immune responsiveness. Because

vaccination strategies result in slower times to response than cytotoxic chemotherapy, high tumor burdens may interfere with vaccination strategies by limiting survival time and also by hampering the immune system and decreasing specific T-cell functions as reported in hematologic malignancies (11). However, vaccination approaches require the choice of multiple factors such as epitopes, antigen source (peptide, peptide pool, protein, vector, lysed tumor cells, and whole tumor cells), antigen presentation (dendritic cells, transfected tumor cells, and CD40-activated B cells), adjuvants, route of administration (s.c., i.m., i.v., and intradermal), and vaccination schedule.

This study was designed to answer two critical questions in the field of tumor immunity. Because many patients with metastatic colorectal cancer are currently treated with irinotecan/high-dose 5-FU/leucovorin chemotherapy, the first aim of the study was to determine whether concurrent immunization at the time of irinotecan/high-dose 5-FU/leucovorin chemotherapy resulted in a measurable immune response. With this approach, we were able to immunize patients who had had limited prior chemotherapy and excellent performance status. We monitored the effect of chemotherapy on the cellular immune system with viral recall antigens. As an immunotherapeutic target, we selected CAP-1, which is the immunodominant MHC class I HLA-A2–restricted nonamer epitope of the carcinoembryonic antigen (CEA), a 180 kDa protein which is expressed by over 90% of colorectal tumors (12). CTL lines specific for CAP-1 have been generated from patients immunized with a recombinant CEA vaccine and are able to kill CEA-expressing tumor cells *in vitro* (13). CAP-1 has been successfully used before in CEA-positive cancer patients to generate immune responses (14).

The second aim of the study was to directly compare the immunologic efficacies of granulocyte macrophage colony-stimulating factor (GM-CSF), bacterial DNA sequences with nonmethylated CpG motifs (CpG-ODN or dSLIM molecules), and dendritic cells in a randomized fashion. Recent studies are controversial on the relative efficacy of GM-CSF, CpG-containing dSLIM molecules, interleukin-2 (IL-2), and dendritic cells as vaccine adjuvants (15–20). Additionally, we determined feasibility and toxicities of this combination regimen.

Patients and Methods

Patients. This was an open-label prospective randomized clinical phase I/II trial of HLA-A2–positive patients with primary metastatic colorectal cancer. The protocol was approved by the institutional review committee and by the German Drug Administration. All patients signed informed consent. Inclusion criteria required an age of between 18 and 75 years, positive HLA-A2 status, elevated serum CEA (>5μg/L) and/or CEA-positive tumor, untreated metastatic disease, chemotherapy-free interval after adjuvant treatment of at least 6 months, Karnofsky index >70%, life expectancy of at least 3 months, sufficient bone marrow and liver function, HIV and hepatitis B and C negativity, absence of central nervous system metastases, no immunosuppressant medication, and negative pregnancy test. Between December 1999 and May 2001, a total of 70 consecutive patients with primary metastatic colorectal cancer were screened according to the inclusion criteria. Fifty-three patients were excluded, of whom 21 were HLA-A2 negative, 15 had been pretreated for their metastatic disease, 1 was older than 75 years, 2 had a normal serum CEA, 3 patients refused informed consent, 2 suffered from renal impairment, 3 had rapid progressive tumors and needed immediate chemotherapy, 2 had no measurable lesions, 3 had a low Karnofsky score, and 1 had a change of histology (non-adeno). Seventeen patients were eligible and enrolled in the study. They had a median age of 60 years (range: 32-75 years); 8 were male and 9 were female; 13 had been diagnosed with colon cancer and 4 with rectal cancer. The mean serum CEA level was 266 ± 453 μg/L.

Study design. All patients received a complete clinical examination including computed tomography scans of chest and abdomen/pelvis, electrocardiogram, echocardiography, pulmonary function test, as well a laboratory workup. At baseline and after three cycles of chemoimmunotherapy, patients underwent a leukapheresis to obtain peripheral blood mononuclear cells for diagnostic and therapeutic purposes. Toxicities were continuously documented and graded according to the WHO criteria. Patients were randomized to receive CAP-1, GM-CSF and IL-2 or CAP-1, dSLIM and IL-2 or CAP-1, and IL-2 alone. In addition, patients were randomized to be vaccinated with or without CAP-1–pulsed autologous dendritic cells as the primary vaccine. Two vaccinations were given 2 and 1 week before the first cycle of chemotherapy. Alternating with the three cycles, two vaccinations were given in the chemotherapy-free interval with a 1-week pause. After the third cycle, patients were vaccinated weekly until progressive disease. The study design is illustrated in Fig. 1.

Chemotherapy. Patients were treated with 80 mg/m^2 irinotecan, 2,000 mg/m^2 high-dose 5-FU, and 500 mg/m^2 leucovorin for three cycles (six weekly administrations) as described before (21, 22), if progressive disease did not occur during therapy.

Vaccine production and administration. Double stem-loop immunomodulators (dSLIM-30L1), which are covalently closed dumbbell-shaped DNA molecules containing unmethylated CpG motifs, were produced from 5′-CCTAGGGGTTACCACCTTCATTGGAAAACGTTCTT-CGGGGCGTTCTTAGGTGGTAACC-3′ oligodeoxynucleotides (ODN) under conditions resembling good manufacturing practice as described (20, 23). CAP-1 peptide (YLSGANLNL) was synthesized under good manufacturing practice–like conditions as well. Clinical grade molgramostim (GM-CSF) was purchased from Novartis Pharma GmbH (Nürnberg, Germany). Clinical grade aldesleukin (IL-2) was purchased from Chiron GmbH (Marburg, Germany). Vaccine preparations were mixed under sterile conditions and frozen at −80°C. Shortly before administration, vaccines were thawed at room temperature and given s.c. at altering sites of the upper arm. Depending on the randomization, the first vaccine consisted of an average of 1×10^7 CAP-1–pulsed dendritic cells or 50 μg CAP-1 with 250 μg dSLIM, or 50 μg CAP-1 with 50 μg GM-CSF, or 50 μg CAP-1 alone. The second and all following vaccinations contained 50 μg CAP-1 together with 1×10^6 IU IL-2 and either 250 μg dSLIM, 50 μg GM-CSF, or no adjuvant.

Generation and maturation of dendritic cells. CD14-positive cells were isolated from leukapheresis material by immunomagnetic enrichment technique using the CliniMACS device (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the guidelines of the manufacturer. Purified CD14 cells (purity grade >97%) were cultured in serum-free CellGenix medium (CellGenix, Freiburg, Germany) containing Glutamax I (Life Technologies, Inc., Karlsruhe, Germany) in the presence of 800 units/mL GM-CSF and 500 units/mL IL-4 (R&D Systems GmbH, Wiesbaden, Germany). On days 3 and 6, 50% fresh medium with cytokines was added. Cells were separated by Ficoll centrifugation on day 6 to purge them from dead cells. On day 7, cells were transferred to medium containing Glutamax I, 800 units/mL GM-CSF, 500 units/mL IL-4, 1 μg/mL prostaglandin E2 (Sigma-Aldrich, Deisenhofen, Germany), 20 ng/mL tumor necrosis factor (Sigma-Aldrich), 1,000 units/mL IL-6 (R&D Systems), and 10 μg/mL anti-CD40 antibody (BD Biosciences, Hamburg, Germany). Mature dendritic cells were harvested, washed, and characterized by flow cytometry on day 10. Dendritic cells were incubated with 50 μg CAP-1 peptide in 500 μL 0.9% NaCl for 2 hours at 37°C. Afterwards, dendritic cells were washed and resuspended in 1 mL of 0.9% NaCl solution for s. c. administration.

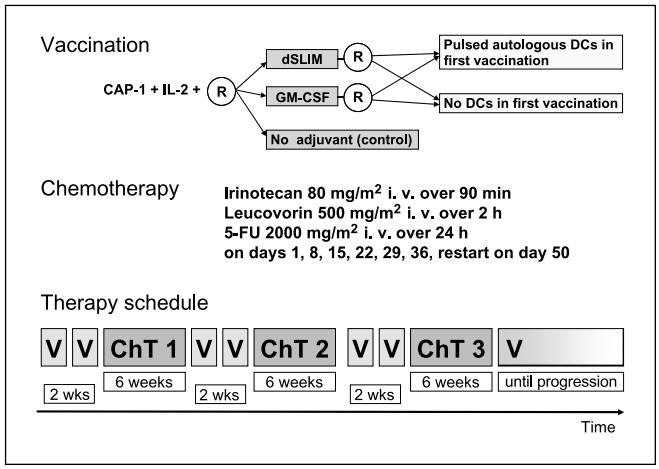

Fig. 1. Patients were first randomized (*circled R*) to receive CAP-1 and IL-2 with different adjuvants (dSLIM, GM-CSF, or none). Subsequently, they were randomized to receive their first vaccination with or without pulsed autologous dendritic cells. Vaccinations (*V*) and chemotherapy (*ChT*) were given in an alternating schedule, starting with two vaccinations.

Clinical assessment of response. Patients were restaged after each cycle of chemoimmunotherapy and evaluated for response after complete treatment by computed tomography of involved sites and extensive laboratory workup. Patients were monitored monthly for serum CEA levels to assess the clinical efficacy of immunotherapy after completion of chemotherapy and while undergoing weekly vaccinations. A complete response (CR) was defined as the disappearance of all tumor signs, a partial response (PR) as the regression of all lesions by at least 30% in diameter, and progressive disease as an increase of at lease 20% in diameter or occurrence of new lesions. Stable disease was if neither PR nor progressive disease criteria were met.

Immune assessment. State-of-the-art immune assessment (24) including MHC class I tetramer analysis and intracellular cytokine assay was done on all samples. Assays were applied according to Dana-Farber Cancer Institute Immune Assessment Laboratory standard operating procedures, which were developed by thorough research and testing. Daily quality assessment and quality control was applied to all involved reagents and machines. Peripheral blood mononuclear cells from leukapheresis were isolated by Ficoll centrifugation and frozen in liquid nitrogen before performance of assays. In case of progressive disease, 50 mL of blood were drawn from the patient after the last treatment instead of leukapheresis. Cells were phenotypically evaluated by flow cytometry for monocyte, T-cell, and B-cell markers (CD14, CD3, CD4, CD8, CD14, CD19, CD20, CD45, CD45RA, and CD45R0). Peptides for *in vitro* use were purchased from New England Peptides (Gardner, MA).

Flow cytometry. Uncompensated digital acquisition was done on a five-color flow cytometer (Beckman-Coulter FC500, Miami, FL). Data were compensated and analyzed with FlowJo for Macintosh software version 4.3 (Treestar, Ashland, OR).

MHC class I tetramer analysis. Biotinylated HLA-A2 monomers were synthesized in association with different peptides and β2-microglobulin as previously described (25). Monomers were multimerized with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). Peripheral blood mononuclear cells, 2×10^6 to 4×10^6, were stained with 2 μg tetramer and anti-CD27 FITC, anti-CD45RA ECD, anti-CD8 PC7, anti-CD4 PC5, anti-CD14 PC5, and anti-CD19 PC5 (all antibodies from Beckman-Coulter). For quantitation of vaccine-specific CD8+ T cells, CAP-1 tetramers were used. Human T-cell lymphotrophic virus type I TAX (LLFGYPVYV) tetramers served as negative control. To evaluate recall antigen – specific memory CTL, peripheral blood mononuclear cells were stained with cytomegalovirus (CMV) pp65 (NLVPMVATV) and EBV BMLF-1 (GLCTLVAML) tetramers. Cells were gated on lymphocyte population, CD4/CD14/CD19-negative cells ("bin gate," as described before; ref. 24), and CD8. The limit of detection for CAP-1 – specific CTL was 0.02% as defined by staining peripheral blood mononuclear cells from 12 HLA-A2 – positive healthy volunteers.

IFN-γ intracellular cytokine assay. Thawed peripheral blood mononuclear cells were incubated at 37°C and 5% CO for 1 hour with either 10 μg/mL CAP-1 peptide, 10 μg/mL HIV RT-POL

Table 1. Characteristics and clinical results of all enrolled patients

Patient ID	Sex	Age (y)	Primary tumor	Sites of metastases	Serum CEA (μg/L)
P01	F	48	Rectum	Liver, lung, bone, adrenal gland	12
P02	M	66	Colon	Liver	23
P03	F	44	Colon	Liver	602
P04	F	70	Colon	Liver	142
P05	F	69	Colon	Liver, lung, mediastinum	196
P06	F	32	Colon	Liver, spleen, ovaries, pelvis, peritoneum	11
P07	F	60	Colon	Liver	792
P08	M	57	Rectum	Lung	16
P09	M	44	Rectum	Liver	19
P10	M	64	Colon	Liver, lung	881
P11	F	38	Colon	Liver	21
P12	F	72	Colon	Liver, spleen, lung, mediastinum	1690
P13	F	43	Rectum	Liver, bone, pararectal, paraaortal lymph nodes	21
P14	M	61	Colon	Liver	23
P15	M	75	Colon	Liver	44
P16	M	62	Colon	Peritoneum	22
P17	M	55	Colon	Liver (primary resection)	<5*

Abbreviations: DC, dendritic cells; SD, stable disease; PD, progressive disease; FFTF, freedom from treatment failure.
*P17 had a CEA-positive tumor.

(ILKEPVHGV) as negative control, or 2 μg/mL staphylococcal enterotoxin B as positive control. To block secretion of cytokines, 10 μg/mL brefeldin A (Sigma, St. Louis, MO) was added. Cells were incubated for 5 hours at 37°C and 5% CO_2. Afterwards, cells were stained with CAP-1 tetramers as described above for 20 minutes at room temperature and later fixed and permeabilized. To detect intracellular cytokines, cells were stained with anti–IFN-γ FITC (BD Biosciences), anti-CD69 ECD, anti-CD4/14/19, and anti-CD8 PC7 (Beckman-Coulter) for 20 minutes at room temperature and analyzed by flow cytometry.

In vitro *peptide stimulation of T cells.* To expand CAP-1–specific T cells, thawed peripheral blood mononuclear cells were incubated with 1 μg/mL of CAP-1 peptide in a 96-well plate (2×10^6 cells/mL) in the presence of 20 IU/mL of IL-2 (Chiron Corp., Emeryville, CA) at 37°C and 5% CO_2 for 8 days. IL-2 was refreshed on day 4 (20 IU/mL). Cells were further phenotypically and functionally analyzed by tetramer staining and intracellular cytokine assays as described above.

Generation of a CAP-1–specific T-cell line. CD40-activated B cells generated from peripheral blood mononuclear cells of patients and healthy donors were used to stimulate autologous CD8+ T cells (26). CTLs were cultured in T-cell medium in 24-well plates in the presence of 10 ng/mL IL-7 (Endogen, Inc., Woburn, MA) on day 0 and 20 IU/mL IL-2 on days 1 and 4. Restimulation of T cells was done with irradiated, 1 μg/mL CAP-1–pulsed CD40-activated B cells every week. Cells were repeatedly tested with CAP-1 tetramers for specific CTL. Functional analysis was done with the Europium release assay as previously described (27).

Statistics. All statistical tests were done with the statistics software SPSS V11.0 for Windows (SPSS, Inc., Chicago, IL). A Student's *t* test was used to calculate differences between pre- and post-therapeutic CAP-1–specific CD8+ cell frequencies. The same test was applied to changes in recall antigen CD8+ cell frequencies before and after one and three cycles of chemotherapy, respectively. Calculations of time to progression and overall survival were done with a Kaplan-Meier analysis. Differences in survival between treatment groups were calculated with a log-rank test. Probabilities (*P* values) smaller than 0.05 (below 5%) were considered as statistically significant.

Results

Patient treatment

Seventeen patients (13 colon and 4 rectal cancers) were enrolled in this trial. Twelve patients received the planned three cycles of chemoimmunotherapy, two patients received only one cycle (six administrations of irinotecan/5-FU/leucovorin), and three patients discontinued before the first cycle had been finished because of progressive disease. Two of the 17 patients were randomized to receive CAP-1, GM-CSF, and IL-2; six received CAP-1, dSLIM, and IL-2; and five patients received CAP-1 and IL-2 only. CAP-1–pulsed dendritic cells were administered during the first vaccination to four patients, three of whom received CAP-1, dSLIM, and IL-2 in the consecutive vaccinations and one was randomized to continue with CAP-1, GM-CSF, and IL-2. The study was discontinued after 17 patients, as it became apparent that neither of the adjuvants provided superiority in eliciting a high CAP-1–specific immune response and enough data had been acquired to analyze the other objectives of the study.

Toxicity

Chemotherapy was generally well tolerated. There were no treatment delays due to blood count, nausea/vomiting, or pain. WHO grade 3 diarrhea occurred in four cases, which did not require hospitalization. Vaccinations caused mild local reactions (swelling, induration, or erythema) at the sites of injection in six cases. One patient reported transient chills and a slight increase in temperature (38°C) that resolved within a couple of hours.

Clinical response

Clinical response to combined modalities. Five of 17 patients (29%) achieved a CR (one patient with curative liver metastasis

Table 1. Characteristics and clinical results of all enrolled patients (Cont'd)

Primary vaccine	Boost	No. cycles of three planned	Clinical response	FFTF (mo)	Survival (mo)
CAP-1	CAP-1 + IL-2	0.3	PD	2	3
CAP-1 + GM-CSF	CAP-1 + GM-CSF + IL-2	3.0	PR	15	34
CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	SD	5	21
CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	SD	12	32
DC-CAP-1	CAP-1 + GM-CSF + IL-2	0.5	PD	2	2
DC-CAP-1	CAP-1 + dSLIM + IL-2	3.0	CR	12	26
CAP-1	CAP-1 + IL-2	3.0	SD	11	15
CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	SD	7	17
CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	CR	12	28
DC-CAP-1	CAP-1 + dSLIM + IL-2	3.0	SD	10	28
DC-CAP-1	CAP-1 + dSLIM + IL-2	1.0	PD	3	10
CAP-1	CAP-1 + IL-2	0.5	PD	2	2
CAP-1 + GM-CSF	CAP-1 + GM-CSF + IL-2	3.0	PD	6	13
CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	CR	9	11
CAP-1	CAP-1 + IL-2	0.5	PD	1	6
CAP-1	CAP-1 + IL-2	3.0	CR	13	21
CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	CR	24	24

resection and two patients with metastatic disease of the peritoneum), one (6%) a PR, and five (29%) patients had stable disease combining for a general response rate of 35% (CR + PR). Six (36%) patients showed progressive disease, five during the first cycle and one at the end of the third.

Of the five patients who were treated without any adjuvants, one achieved a CR, one had stable disease, and three patients progressed (response rate 1/5; 20%). Of four patients who had received CAP-1–pulsed dendritic cells, one achieved a CR, one had stable disease, and two patients progressed. The group of 13 patients without dendritic cell–based vaccines showed four CR, one PR, four stable disease, and four progressive disease. In the GM-CSF group with three patients, one PR and two progressive disease occurred. dSLIM vaccines (n = 9) showed four CR, four stable disease, and only one progressive disease (response rate 4/9; 44%) response rate. Comparisons of dSLIM-receiving patients (n = 9; response rate 44.4%) with patients who did not receive dSLIM (n = 8; response rate 25%) indicated a favorable response to dSLIM treatment although the numbers were too small to perform statistical analysis.

After a median observation time of 29 months (range: 22-34 months), 11 of 17 patients had died due to their disease and 5 patients had relapsed. One patient remained free of disease. He had a stage IV cancer with elevated serum CEA and underwent curative resection of two liver metastases before start of therapy. The median time to progression of relapsed/progressive patients was 8 months (range: 1-15 months) and the median survival 17 months (range: 2-34 months). A log-rank test did not reveal any statistically significant differences in time to progression or survival between the groups dendritic cell/nondendritic cell and adjuvant/no adjuvant (data not shown). Table 1 summarizes patient characteristics and clinical results.

Clinical response to immunotherapy. During the three cycles, it was not possible to discriminate clinical effects of treatment modalities because vaccinations and chemotherapy were given simultaneously. After completion of chemotherapy, vaccinations were the only treatment for the patients. Thus, clinical response to immunotherapy could be evaluated. Of 12 patients who completed three cycles, all but one showed increasing serum CEA levels within 1 month after chemotherapy was discontinued. The patient with decreasing serum CEA levels had undergone a curative resection of liver metastases. He had a measurable increase of CAP-1–specific CTL. Figure 2 shows the course of serum CEA in all evaluable patients.

Immunologic response

Peptide/MHC class I tetramers. Peptide/MHC class I tetramers were used to detect CAP-1–specific CD8+ T cells, which were analyzed either freshly thawed or after a weeklong *in vitro* CAP-1 stimulation to enhance sensitivity. At baseline, CAP-1–specific CD8+ cells were observed in unstimulated samples of 8 patients (47%; CD8+ specific frequency: 0.045 ± 0.035%) and in 11 patients (65%) after *in vitro* stimulation (0.110 ± 0.085%). An immunologic response, defined as an increase of specific CD8+ cells after three cycles (or fewer in progressive patients), was found in unstimulated samples of 4 patients (24%; 0.045 ± 0.024% to 0.171 ± 0.077%; P > 0.05). After an *in vitro* stimulation with CAP-1 peptide, 8 patients (48%) showed a significant increase of specific

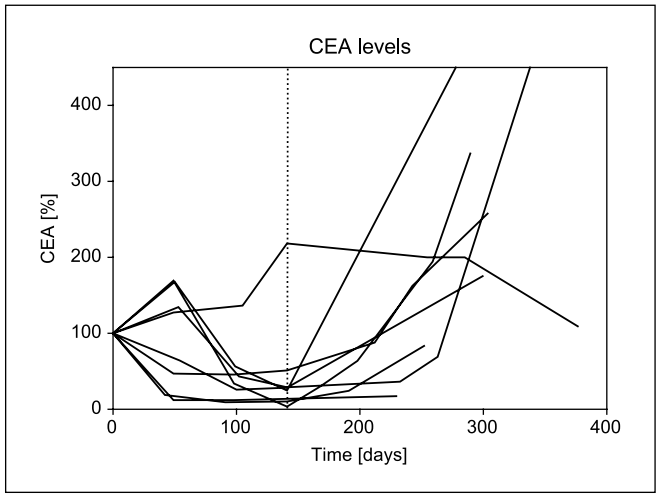

Fig. 2. Normalized CEA levels of eight patients who received weekly booster vaccinations after three cycles of immunochemotherapy. All but one patient showed increasing levels after discontinuation of cytostatic drugs (*dotted line*).

CD8+ from pre- to post-vaccination (0.058 ± 0.031% to 0.204 ± 0.139%; P = 0.04). Responders included four patients who had received CAP-1/dSLIM/IL-2 as adjuvants, one of the CAP-1/GM-CSF/IL-2 arm, and three patients who received CAP-1 and IL-2 only (all progressive disease within first cycle). Two of eight patients had received dendritic cell in their first vaccine. Figure 3 shows examples of two patients responding to vaccination therapy with an increase of CAP-1–specific CD8+ cells.

IFN-γ intracellular cytokine assay. Samples were processed freshly thawed and after a 1-week CAP-1 peptide *in vitro* stimulation to increase sensitivity of this assay. At baseline, T cells of two patients (12%; CD8+ specific frequency: 0.27 ± 0.03%) showed an increased IFN-γ secretion to CAP-1 peptide and in three patients (18%; CD8+ frequency: 0.39 ± 0.19) after *in vitro* stimulation. Immunologic response to CAP-1 from prevaccination to postvaccination was detected in two unstimulated patient samples (12%; from 0.00 to 0.03 ± 0.003; P > 0.05) and in four stimulated patient samples (24%; from 0.00 ± 0.00 to 0.27 ± 0.15; P = 0.05). Among responders were two patients vaccinated with CAP-1/dSLIM/IL-2, one with CAP-1/GM-CSF/IL-2 (three cycles completed) and one with CAP-1/IL-2 (not completed). One patient had received dendritic cell in the first vaccine. The results from tetramer analysis and IFN-γ intracellular cytokine assay showed no correlation. Table 2 summarizes the immunologic results.

Impact of chemotherapy on T cells. The cytotoxic effects of three cycles of irinotecan/high-dose 5-FU/leucovorin on the cellular immunity were evaluated by CD3+/CD4+ and CD3+/CD8+ frequencies as well as the responses to viral recall antigens before and after completion of treatment. In addition, peripheral blood mononuclear cells of patients who had received only one cycle due to progressive disease were evaluated. Twelve patients completed all three cycles. Their average CD4+ and CD8+ cell frequencies were not affected by chemotherapy (at baseline: 45 ± 14% and 26 ± 13%; after completion of therapy: 43 ± 14% and 26 ± 11%). Five patients who underwent only one or fewer cycles of chemotherapy showed similar average frequencies (CD4+ and CD8+ at baseline: 41 ± 18% and 21 ± 5%; after vaccination: 43 ±

19% and 23 ± 16%). CMV pp65– and EBV BMLF-1–specific CD8+ cell frequencies were assessed in all patients at baseline and after therapy. All but one patient had detectable CD8+ responses to the recall antigens. The average recall antigen CD8+ frequency at baseline was 0.64 ± 0.70%, which was similar to that of 12 healthy donors (0.61 ± 0.89%). Patients who were treated with three cycles (n = 12) showed an average decrease of recall antigen CD8+ frequency from 0.65 ± 0.68% to 0.47 ± 0.51%, which was statistically significant (P = 0.017). This is an average decrease of recall antigen–specific CD8+ cells by 14% if baseline values were set to 100%. In contrast, five patients with a therapy of one cycle (6 weeks) or fewer had an increase in recall antigen CD8+ from 0.62 ± 0.73% to 1.81 ± 2.14%. This was compared with an average increase of 310%, if baseline frequencies were set to 100%. Due to the small numbers, these results were not statistically significant. The recall antigen tetramer results of four patients from each group are depicted in Fig. 4.

Generation of a CAP-1–specific T-cell line. A CAP-1–specific T-cell line (>1% specificity) could not be generated after multiple stimulations with CD40-activated B cells in seven different donors (patients and healthy controls). The maximum frequency that could be achieved was 0.5% specific CD8+ cells in three of six patients. However, when CAP-1 was substituted by the heteroclitic peptide CAP1-6D, peptide stimulations gave rise to a specific T-cell line in one healthy donor (Fig. 5).

Discussion

The presented combination of vaccination-based immunotherapy and irinotecan/high-dose 5-FU/leucovorin chemotherapy is a feasible combination regimen for primary metastatic colorectal cancer patients. The side effects were well tolerated and the treatment could be administered as planned.

The clinical overall response rate to the combination regimen was 35%. Only one patient showed a continuously decreasing CEA level after completion of chemotherapy, indicating that the vaccination strategies did not have a clinical effect after the third cycle in the rest of the patients. However, it cannot be ruled out that the vaccinations had a transient effect during the first 5 months of combination therapy.

It is known that immune responses to viral vaccines during chemotherapy are lower than expected in healthy people (28). Therefore, it was an important aim of this trial to determine whether the irinotecan/high-dose 5-FU/leucovorin chemotherapy regimen had an adverse effect on the memory CTL repertoire. Our data clearly show that three cycles of irinotecan/high-dose 5-FU/leucovorin reduce the relative number of EBV- or CMV-specific CTL by an average 14% without having an effect on the absolute CD8+ cell count. It is likely that chemotherapy eliminates antigen-specific memory CTLs, which do not recover as quickly as other CD8+ subsets. One intriguing observation is that 0.5 to 1 cycle of irinotecan/high-dose 5-FU/leucovorin *increased* recall antigen–specific CTL frequencies, although this was not statistically significant due to the small patient group. We speculate that a short administration of irinotecan/high-dose 5-FU/leucovorin chemotherapy shuts off regulatory immunologic mechanisms such as CD4+/CD25+ T cells and thereby increases antigen-specific responses. This is supported by recent observations in mice, which were treated with chemotherapy-modulated vaccinations (29). Cyclophosphamide and doxorubicin could enhance a tumor-specific CTL response by abrogating the suppression of CTL by CD4+/CD25+ T cells.

Our tetramer results indicate that CEA is not a neoantigen for most of the patients. Nagorsen et al. (30) reported that one third of HLA-A2–positive patients with colorectal cancer show T-cell responses to the CAP-1 peptide by IFNγ ELIspot. Although these cells can be detected by highly sensitive methods, they remain ineffective for tumor control. Almost 50% of our patients showed an immunologic response to vaccination (increase of CAP-1–specific CTL). IFNγ secretion was observed in T cells of 24% of the patients after *in vitro*

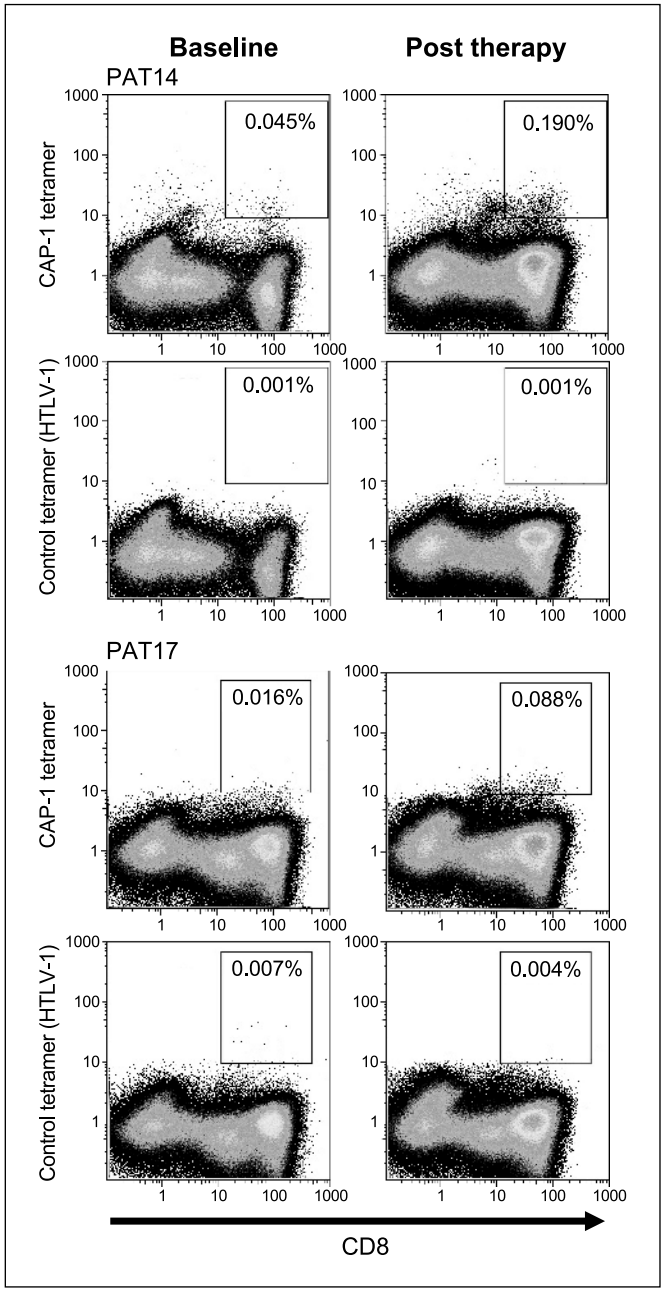

Fig. 3. Example of two patients (PAT14 and PAT17) responding to three cycles of immunochemotherapy with an increase of CAP-1–specific CTLs (CD8+). PAT14 showed a significant CAP-1 response at baseline (0.045% CD8+). As a control tetramer, TAX HLTV-1 was used (*bottom*).

Table 2. Immunologic results of all enrolled patients

Patient ID	Primary vaccine	Boost	No. cycles of three planned	Tet CD8+ CAP-1 frq pre	Tet CD8+ CAP-1 frq post	Tetramer increase pre/post	ICC CD8+ CAP-1 frq pre	ICC CD8+ CAP-1 frq post	ICC increase pre/post
P01	CAP-1	CAP-1 + IL-2	0.3	0.08	0.13	+	0.65	0.00	–
P02	CAP-1 + GM-CSF	CAP-1 + GM-CSF + IL-2	3.0	0.00	0.01	–	0.00	0.41	+
P03	CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	0.01	0.02	–	0.00	0.04	+
P04	CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	0.07	0.04	–	0.00	0.00	–
P05	DC-CAP-1	CAP-1 + GM-CSF + IL-2	0.5	0.14	0.00	–	0.00	0.00	–
P06	DC-CAP-1	CAP-1 + dSLIM + IL-2	3.0	0.00	0.00	–	0.01	0.22	+
P07	CAP-1	CAP-1 + IL-2	3.0	0.09	0.04	–	0.21	0.20	–
P08	CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	0.09	0.04	–	0.30	0.02	–
P09	CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	0.04	0.01	–	0.00	0.00	–
P10	DC-CAP-1	CAP-1 + dSLIM + IL-2	3.0	0.01	0.15	+	0.00	0.00	–
P11	DC-CAP-1	CAP-1 + dSLIM + IL-2	1.0	0.05	0.15	+	0.05	0.00	–
P12	CAP-1	CAP-1 + IL-2	0.5	0.02	0.09	+	0.00	0.00	–
P13	CAP-1 + GM-CSF	CAP-1 + GM-CSF + IL-2	3.0	0.01	0.06	+	0.00	0.00	–
P14	CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	0.09	0.31	+	0.02	0.01	–
P15	CAP-1	CAP-1 + IL-2	0.5	0.05	0.07	+	0.00	0.39	+
P16	CAP-1	CAP-1 + IL-2	3.0	0.00	0.00	–	0.05	0.02	–
P17	CAP-1 + dSLIM	CAP-1 + dSLIM + IL-2	3.0	0.01	0.13	+	0.00	0.00	–

NOTE: Frequencies (%) of CAP-1 – specific CTL (CD8+ CAP-1 frq) were obtained from tetramer staining at baseline (Tet pre) and after the last therapy cycle (Tet post) as well as from intracellular cytokine assays (ICC pre/post) in prestimulated samples. Background CD8+ frequencies were subtracted from CAP-1 CD8+ frequencies. A response was defined as more than 0.02% tetramer-positive CAP-1 – specific CD8+ CTLs.

stimulation, which is lower than the result of the tetramer analysis. This could be due to nonfunctional T cells or to the lower sensitivity of the IFNγ intracellular cytokine assay, which has been reported before (24, 31).

We did not find any striking differences in immunologic response between adjuvants (CAP-1/GM-CSF/IL-2 versus CAP-1/dSLIM/IL-2 versus CAP-1/IL-2) or between the use of the primary vaccine with or without dendritic cells. Therefore, we

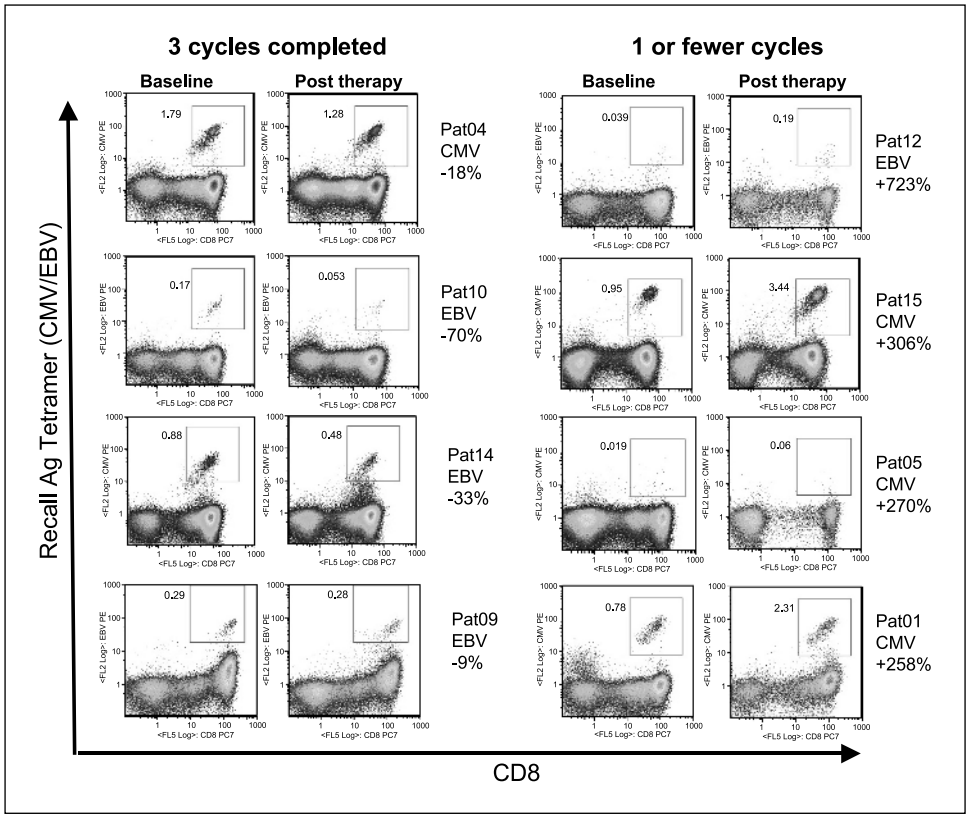

Fig. 4. Flow cytometric CMV or EBV peptide tetramer analysis in 4 of 11 patients who completed three cycles (*two left columns*) and 4 of 5 patients who received only one or fewer cycles (*two right columns*). Patients with three cycles showed a significant decrease of CMV/EBV-specific CD8+ frequencies after therapy, whereas an increase was observed in patients with one or fewer cycles.

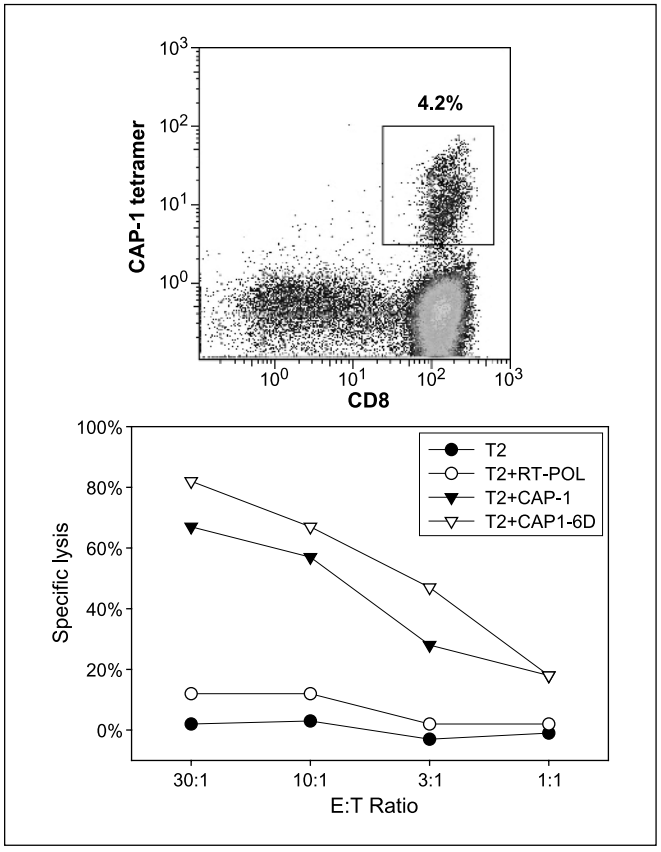

Fig. 5. Tetramer analysis of a CAP-1 – specific CTL line (*top*) generated from peripheral blood mononuclear cells by multiple stimulation with the heteroclitic peptide CAP1-6D, which was able to lyse CAP-1 – and CAP1-6D – pulsed T2 cells in a Europium release cytotoxicity assay (*bottom*).

discontinued the trial after 17 enrolled patients. Although the immunologic response of our trial looks convincing (50% patients with increase of CAP-1 tetramer – positive CTL), the clinical efficacy does not. Unfortunately, this has been observed in numerous tumor vaccination studies (14, 29, 32). In our and other phase I/II studies, we are trying to immunize an already "infected" host, who is overwhelmed by an exponential growth of billions of destructive tumor cells. This has not worked with vaccines against already existing infectious diseases. To break the T-cell ignorance towards the tumor, it is necessary to elucidate questions about optimal vaccine preparation, timing and schedule, dosage, and adjuvants. We speculate that we will not experience consistent clinical responses in tumor patients if we do not reach tumor antigen – specific CTL frequencies comparable to those against virus antigens. It may be necessary to increase dosage of peptide and/or adjuvants. Whereas the role of GM-CSF as an adjuvant is still controversial (15, 16, 18), DNA molecules with unmethylated CpG motifs (CpG-ODN or dSLIM) are promising candidates in preclinical immunologic studies (17, 19, 20, 33, 34). This is one of the first studies to administer CpG-ODN/dSLIM as an adjuvant in cancer patients. The phosphorodiester-based dSLIM was well tolerated and did not cause any systemic side effects at a dosage of 250 μg.

CAP-1 has been used in other vaccination trials before. In a phase I study, Morse et al. (14) vaccinated 21 HLA-A2 – and CEA-positive cancer patients with CAP-1 – pulsed dendritic cells. Patients were treated in three dose escalation groups (1×10^7, 3×10^7, and 1×10^8 dendritic cells, i.v.) weekly or biweekly with a maximum of four immunizations. No major toxicities could be observed. Two patients showed clinical responses (one minor and one stable disease); all other had progressive disease. Immunologic response was measured by skin delayed-type hypersensitivity measurements. Two patients had new delayed-type hypersensitivity responses to CEA. Five patients showed a CEA delayed-type hypersensitivity before treatment, confirming our results that CEA is not a neoantigen in many cancer patients.

After we had started our trial, CAP1-6D, a heteroclitic peptide of CAP-1, was synthesized and reported to enhance the sensitization of CTL by 100 to 1,000 times (35, 36). Fong et al. (37) isolated peripheral dendritic cells after systemic administration of Flt3 ligand, pulsed the cells with CAP1-6D, and administered the vaccine to 12 colon and non – small-cell lung cancer patients with abnormal serum CEA. Tumor regression could be documented in two patients, and one patient experienced a mixed response. Seven patients developed CAP1-6D – specific CTL as evaluated by tetramer analysis. The patients achieved specific CTL frequencies of over 1% in contrast to our patients who showed weaker responses. This is probably due to the higher potency of CAP1-6D.

In conclusion, the presented chemoimmunotherapy is a feasible and safe combination therapy that shows clinical and immunologic efficacy. Neither of the adjuvants provided a superior CTL immune response although the numbers were too small for statistical analysis. In addition, the use of peptide-pulsed dendritic cells in the primary vaccine did not enhance immunity to CAP-1 compared with peptide/adjuvants alone. Irinotecan/high-dose 5-FU/leucovorin chemotherapy only slightly affected antigen-specific CTLs. In addition, our data suggest that a limited administration of chemotherapy could enhance specific CTL responses and increase the efficacy of future vaccination strategies.

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