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Cancer Immunology Miniatures

Cancer Immunology Research

Short Peptide Vaccine Induces CD4$^+$ T Helper Cells in Patients with Different Solid Cancers

Stefanie Gross[1], Volker Lennerz[2], Elisa Gallerani[3], Nicolas Mach[4], Steffen Böhm[5], Dagmar Hess[5], Lotta von Boehmer[6], Alexander Knuth[6], Adrian Ochsenbein[7], Ulrike Gnad-Vogt[8], Ulf Forssmann[9], Thomas Woelfel[2], and Eckhart Kaempgen[1]

Abstract

Previous cancer vaccination trials often aimed to activate CD8$^+$ cytotoxic T-cell (CTL) responses with short (8–10mer) peptides and targeted CD4$^+$ helper T cells (T_H) with HLA class II–binding longer peptides (12–16 mer) that were derived from tumor antigens. Accordingly, a study of immunomonitoring focused on the detection of CTL responses to the short, and T_H responses to the long, peptides. The possible induction of concurrent T_H responses to short peptides was widely neglected. In a recent phase I vaccination trial, 53 patients with different solid cancers were vaccinated with EMD640744, a cocktail of five survivin-derived short (9- or 10-mer) peptides in Montanide ISA 51VG. We monitored 49 patients and found strong CD8$^+$ T-cell responses in 63% of the patients. In addition, we unexpectedly found CD4$^+$ T_H cell responses against at least two of the five short peptides in 61% (23/38) of the patients analyzed. The two peptides were recognized by HLA-DP4– and HLA-DR–restricted T_H1 cells. Some short peptide–reactive (sp)CD4 T cells showed high functional avidity. Here, we show that a short peptide vaccine is able to activate a specific CD4$^+$ T-cell repertoire in many patients, facilitating a strong combined CD4$^+$/CD8$^+$ T-cell response.
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Introduction

Different forms of antigens for anticancer vaccination have been used recently in numerous clinical trials: tumor-associated proteins, DNA or RNA encoding the antigens, or long (12–16 mer) or even "overlong" peptides (30–100 mer), but most trials so far have vaccinated with short (8–10 mer) peptides (1–5). Short (8–10 mer) peptides are known to bind to HLA class I and induce CD8$^+$ cytotoxic T-cell (CTL) responses, whereas longer peptides (12 or more amino acids) are required to activate CD4$^+$ helper T cells (T_H) via presentation on HLA class II (6). Accordingly, immunomonitoring in clinical vaccination trials focused on detecting the expected immune responses—i.e., CD8$^+$ CTL responses after vaccination with short peptides.

Here we report the follow-up of a phase I cancer vaccination trial with EMD640744, a cocktail of five survivin-derived short (9–10 mer) peptides in Montanide ISA 51 VG (7). The trial's primary objective was a comparison of three vaccine doses for immunologic efficacy. Its secondary objectives were safety, tolerability, clinical efficacy, and the overall CD8$^+$ T-cell responses detected by ELISpot and peptide-HLA (pHLA)-multimer assays. The study was remarkable, because of 49 patients eligible for immune monitoring, 31 (63%) showed vaccine-activated peptide-specific T-cell responses, as determined by IFNγ ELISpot assays and/or pHLA-multimer analyses: 16 of 49 (33%) *ex vivo* and up to 28 of 36 (78%) after short-term *in vitro* stimulation.

To obtain more information on the quality of the induced immune responses, we further analyzed samples of 38 patients by a function-based flow cytometric assay combining intracellular staining for different cytokines and degranulation [multifunctional T-cell assay (MFTC)]. Using this assay, we confirmed the majority of previously observed immune responses, detected three additional CD8$^+$ T-cell responses, and in a subgroup of 23 patients, found CD4$^+$ T-cell responses to two of the five short peptides that were originally shown to bind to HLA-A2 and HLA-A3.

[1]Department of Dermatology, University Hospital of Erlangen, Germany. [2]III. Medizinische Klinik und Poliklinik, University Medical Center of the Johannes Gutenberg University, Mainz, Germany. [3]IOSI Oncology Institute of Southern Switzerland, Bellinzona, Switzerland. [4]Clinical Research Unit of the Foundation Dr. Henri Dubois-Ferrière Dinu Lipatti, Oncology Center, Hôpitaux Universitaires de Genève (HUG), Genève, Switzerland. [5]Onkologie/Hämatologie, Kantonsspital, St. Gallen, Switzerland. [6]Department of Oncology, University Hospital Zurich, Zurich, Switzerland. [7]Klinik und Poliklinik für Medizinische Onkologie, Inselspital, Bern, Switzerland. [8]Merck KGaA, Darmstadt, Germany. [9]Merck Serono S.A., Geneva, Geneva, Switzerland, an affiliate of Merck KGaA, Darmstadt, Germany.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

S. Gross, U. Gnad-Vogt, U. Forssmann, and E. Kaempgen contributed equally to this article.

Current address for E. Gallerani: Multimedica Ospedale Castellanza, Varese, Italy; current address for U. Gnad-Vogt: CureVac GmbH, Frankfurt, Germany; current address for U. Forssmann: Genmab A/S, Copenhagen, Denmark; current address for E. Kaempgen: Dermatologikum, Berlin, Germany; current address for S. Böhm: Barts Cancer Institute, Cancer and Inflammation, Queen Mary University of London, UK; and current address for L. von Boehmer: The Rockefeller University, Molecular Immunology Lab, New York.

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Corresponding Author: Stefanie Gross, University Hospital Erlangen, Hartmannstr. 14, 91052 Erlangen, Germany. Phone: 49-9131-85-32730; Fax: 49-9131-85-32931; E-mail: Stefanie.Gross@uk-erlangen.de

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Materials and Methods

The reporting of the methods has been aligned with the MIATA (Minimal Information About T cell Assays) guidelines (8, 9). A detailed description of this section as a MIATA checklist can be found in the Supplementary Information.

Study design

The aim of the phase I trial was to determine the immunologic activity, safety and tolerability, and clinical activity of EMD640744 in Montanide ISA 51 VG in subjects with advanced solid tumors, conducted in five centers in Switzerland (Clinical trials.gov identifier nCt01012102). Details of the trial have been published (7).

Briefly, EMD640744 is a cocktail of Montanide ISA 51 VG with five short peptides based on the amino acid sequence of different regions of the survivin protein previously shown to bind to HLA-A1 (FTELTLGEF, Sur93-101/T2), HLA-A2 (LMLGEFLKL, Sur96-104/M2), HLA-A3 (RISTFKNWPK, Sur18-27/K10), HLA-A24 (STFKNWPFL, Sur20-28), or HLA-B7 (LPPAWQPFL, Sur6-14), respectively.

Planned treatment was to last for 11 weeks of initiation therapy (8 treatments), followed by 13 weeks of maintenance therapy (treatment every 4 weeks). Patients expressed at least one of the HLA-A1, -A2, -A3, -A24, and -B7 alleles and were suffering from metastatic or locally advanced survivin-expressing solid tumors for which no established therapy exists. The primary objective of this trial was to compare three doses of EMD640744 administered by subcutaneous injection in combination with Montanide ISA 51 VG with regard to immunologic efficacy. Secondary objectives of this trial comprised the assessment of the safety and tolerability and clinical efficacy of the three doses of study treatment.

Cells, peptides, and blocking antibodies

Peripheral blood mononuclear cell (PBMC) samples before and after vaccination were available for immunomonitoring from 49 of the 53 patients who started treatment. The monitoring with ELISpot and pHLA multimers, including all information about peptides, sample collection, and preparation, has been described (7).

In brief, PBMC samples were prepared at the five study sites by Ficoll density gradient centrifugation. Cells were frozen in aliquots in freezing medium (90% FCS with 10% DMSO) and shipped to the immunomonitoring laboratory under dry ice and upon arrival rapidly transferred to liquid nitrogen for storage until use.

EBV-transformed lymphoblastic cell lines (EBV-LCL) were generated as described elsewhere (10) and cultured in RPMI-1640 supplemented with 20% FCS (PAA) and gentamycin. For peptide loading, EBV-LCLs were washed twice in PBS and incubated in RPMI-1640 together with the corresponding peptide at 5 μg/mL for 1 hour at room temperature. Then cells were washed in MLPC-Medium and used for the assay at a ratio of 1:1.

Survivin peptides (Bachem) had a purity of >95%. For blocking of pHLA–T-cell receptor (TCR) interactions, the following antibodies were used at a concentration of 10 μg/mL: anti-HLA-ABC (BD, DX17), anti-HLA-DR/DP/DQ (BD, Tu39), anti-HLA-DR (Biolegend, L243), anti-HLA-DQ (Beckman Coulter, SPVL3), and anti-HLA-DP (Abcam, B7/21).

Multifunctional T-cell assay (MFTC)

Thawed cells were seeded in MLPC-Medium (RPMI-1640 with 10% pretested human pooled serum (Lonza), gentamycin, pyruvate, and nonessential amino acids) and stimulated with the corresponding peptide or the EMD640744 peptide mix (5–10 μg/mL). The next day, IL2 (5 U/mL, Roche) and IL7 (10 ng/mL, TEBU) were added. Half of the medium was replaced every 3 to 4 days with fresh MLPC-medium containing IL2 (5 U/mL). No IL2 was given to the *in vitro*–stimulated PBMCs in the last 2 days before the assay (days 12–15). On days 12 to 15, cells were restimulated overnight (37°C, 5% CO_2) in MLPC medium with or without the corresponding peptide and blocking antibodies (as indicated in Fig. 2) in the presence of BrefeldinA, Monensin, $CD107a^-$, and $CD154^-$ antibodies. The next day, cells were washed, stained with dead-cell stain Live/Dead aqua (Invitrogen) according to the manufacturer's instructions, and subsequently with surface-staining antibodies (CD8, CD4, and CD14). After washing, cells were fixed and permeabilized with fix/perm solution and perm/wash (both eBioscience) according to the manufacturer's instructions. Intracellular staining was performed in perm/wash with IL2, TNFα, and IFNγ antibodies for 30 minutes. Cells were then washed and resuspended in PBS.

For each sample, a negative buffer control (without peptide) was assessed. The values obtained from those negative controls were subtracted from the corresponding test samples. Criteria for a positive response were: results of individual cytokine-producing subsets at least 2 times higher than the corresponding subsets in the negative control (background), and the sum of all cytokine-producing subsets greater than 0.03%. Representative examples of data for an MFTC assay, including gating strategy, can be seen in Supplementary Fig. S1.

Samples were acquired on a FACS CANTO II flow cytometer with FACSDiVa software. The performance status of the FACS Canto II flow cytometer was checked with cytometer setup and tracking beads (BD) each day right before the sample acquisition. For data analysis, FlowJo version 9.7.5 was used.

Ethical considerations

This trial was conducted in accordance with the protocol and protocol amendments, the International Conference on Harmonization (ICH) guideline for Good Clinical Practice (GCP), applicable local regulations, and the Declaration of Helsinki, and was approved by independent ethics committees and by Swiss Medic. Written informed consent was received from participants prior to inclusion in the study.

Results

Induction of $CD4^+$ and $CD8^+$ T-cell responses after vaccination

From the 53 patients vaccinated and 49 eligible for primary response analysis, a subgroup of 38 patients had sufficient PBMCs for a more detailed analysis of immune responses by a function-based flow cytometric assay (MFTC). Compared with the ELISpot and pHLA-multimer analyses, the MFTC assay could characterize the cytokine profiles and polyfunctionality of the responding T cells, analyzing $CD8^+$ and $CD4^+$ T-cell subsets separately. Presuming activation of $CD8^+$ T-cell responses by vaccination, at first only the peptides matching the patients class I HLA types were tested. After discovering $CD4^+$ T-cell reactivity (Fig. 1A), the complete cocktail of the five peptides contained in EMD640744, and single peptides not matching patients' HLA, were tested for

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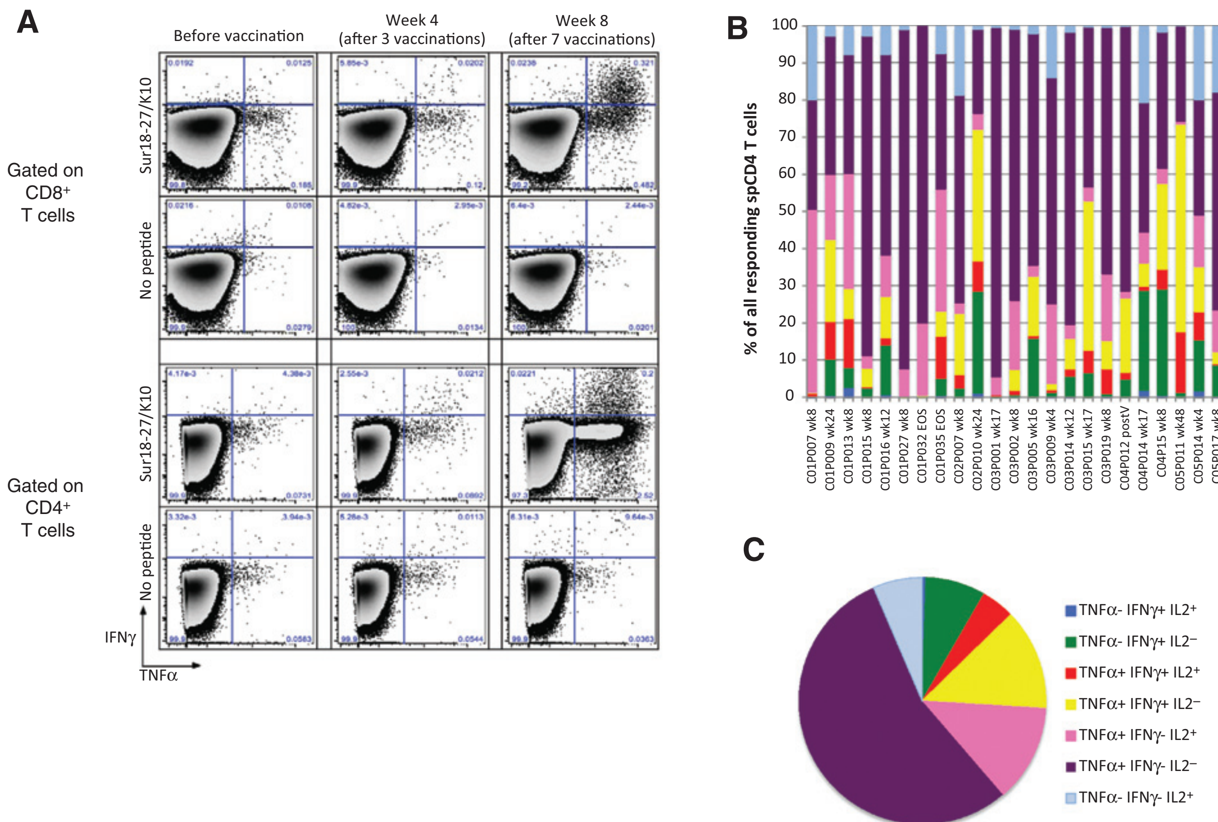

Figure 1.
spCD4 T cells. As shown by MFTC, vaccination with EMD640744 induces not only $CD8^+$ T-cell responses but also specific $CD4^+$ T-cell responses. A, representative example showing T-cell responses to peptide Sur18-27/K10 and the corresponding negative controls (without peptide) before and after vaccination in patient C03P002. B, cytokine expression pattern of all patients exhibiting a spCD4 T-cell response (different cytokine subsets as a percentage of all cytokine-producing $CD4^+$ T cells, background subtracted). For each patient, only one time point, the one with the highest frequency of spCD4 T cells, is shown. C, cumulated results from all patients/time points as shown in B; mean percentages of cytokine-producing subsets are shown.

the induction of $CD4^+$ T-cell responses (if sufficient PBMC material was available).

We detected three additional $CD8^+$ T-cell responses by MFTC, which raised the number of patients with a $CD8^+$ T-cell response to the vaccine to 34 out of 49 patients (69%, summarized in Table 1). In addition to the $CD8^+$ T-cell responses, the MFTC assay detected $CD4^+$ T cells responding to the short peptides contained in EMD640744 in 23 of the 38 patients (61%) after vaccination. Similar to the published $CD8^+$ T-cell responses (7), no differences in the $CD4^+$ T-cell responses were observed among the three different vaccine-dose groups (primary objective of the trial).

In 15 of the 38 patients, PBMC material collected before vaccination was available for analysis by MFTC. In only one of the 15 pre-vaccination samples a $CD4^+$ T-cell response was detected. However, in this patient (C03P015) the amount of responding $CD4^+$ T cells increased 30-fold from 0.1% at baseline to 3% analyzed 17 weeks after the start of vaccination (Supplemental Fig. S2). Of the remaining 14 patients, 7 developed a $CD4^+$ T-cell response after vaccination, indicating a *de novo* induction of spCD4 T-cell responses in at least those 7 patients.

In 17 of the 23 responding patients, we identified either the HLA-A2 or the -A3 binding peptides Sur96-104/M2 and Sur18-27/K10 as inducers of short peptide $CD4^+$ (spCD4) T-cell responses (e.g., in Fig. 1A). The remaining 6 patients had too little PBMC material available to test the single peptides, so only the cocktail EMD640744 was tested. In general, patients with a spCD4 response, tested with the single peptides and EMD640744, had a response to EMD640744 and either Sur96-104/M2 or Sur18-27/K10, indicating that only those two of the five peptides were capable of inducing spCD4 responses. The spCD4 T-cell responses were characterized by a pronounced production of TNFα alone or in combination with IFNγ and/or IL2, a clear T_H1-cytokine profile (Fig. 1B).

HLA restriction of short peptide (sp)CD4 T-cell responses

To further characterize the newly detected spCD4 responses, MFTC assays were repeated with or without pan-HLA class I– or class II–blocking antibodies. In all cases investigated, the HLA class II antibodies completely blocked the spCD4 T-cell responses, whereas HLA class I–specific antibody had no effect, indicating that spCD4 T cells recognize the survivin peptides in the HLA class II context (Fig. 2A).

These findings provided an explanation for the observation in patients C03P005, C04P012, and C04P015 where a spCD4T-cell

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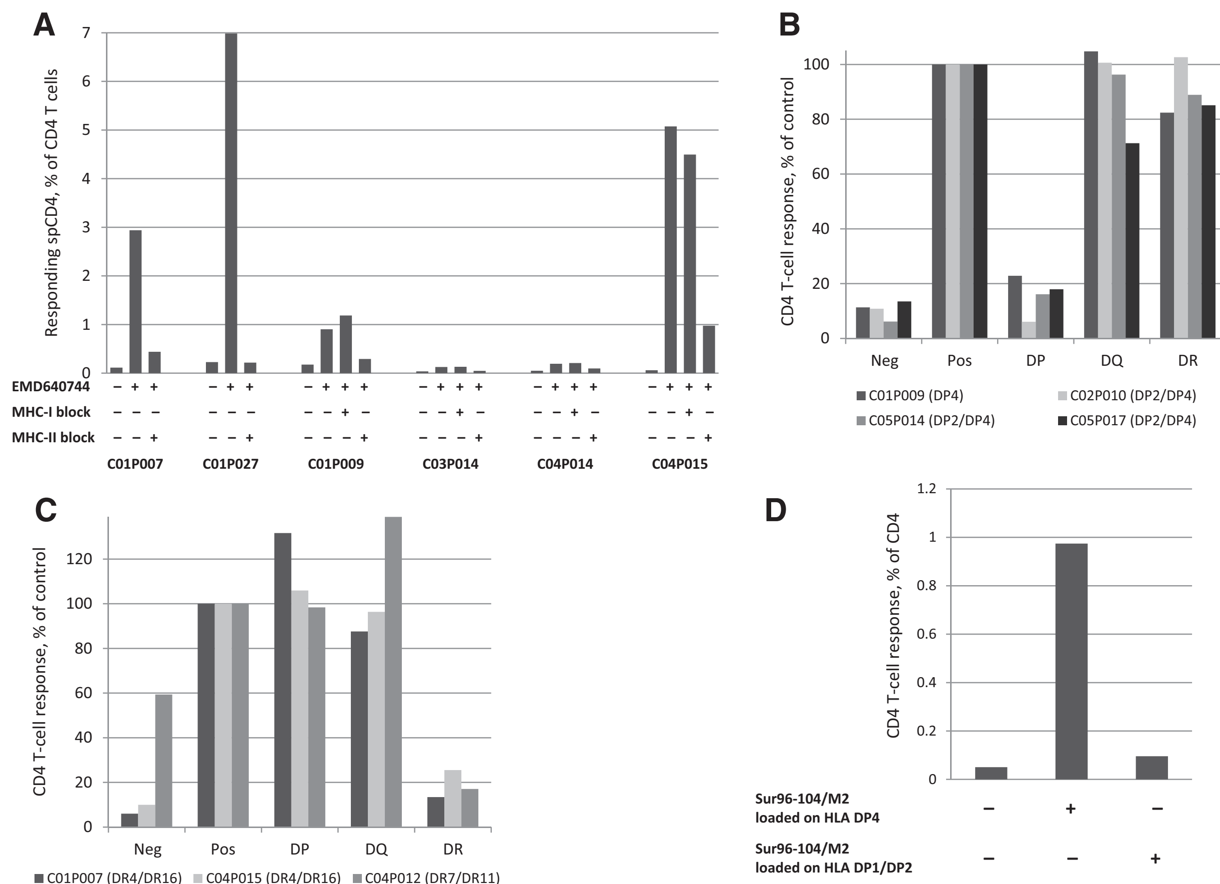

Figure 2.
HLA restriction of spCD4 T-cell responses. A, EMD640744-induced spCD4 responses were blocked with a pan-HLA class II–blocking (shown in 6 patients) but not a pan-HLA class I–blocking antibody (shown in 4 patients). B and C, HLA restriction of the two spCD4 T-cell response–inducing peptides, Sur96-104/M2 (B) and Sur18-27/K12 (C), was tested by stimulating T cells with the respective peptides in the presence of antibodies specifically blocking TCR interaction with HLA-DR, HLA-DQ, or HLA-DP. Each bar in a group represents one patient. Relevant HLA alleles of the corresponding patient are given below, behind the patient IDs. Values were normalized with the positive control (pos, stimulation with the respective peptide but without blocking antibodies) set to 100%. This was also done for the negative control (neg, stimulation without peptide and without antibodies). D, cells from patient C02P010 were tested as examples for reactivity to Sur96-104/M2 loaded either onto DP4 positive or onto DP1/DP2-expressing EBV-B–transformed lymphoblastic cell lines (EBV-LCL).

response against the A3-binding peptide was detected despite the fact that these patients were HLA-A3 negative.

To define the HLA class II–restriction, T cells were stimulated with the respective peptides in the presence of HLA class II subtype–specific antibodies to specifically block TCR interactions with HLA-DR, HLA-DQ, or HLA-DP, respectively. spCD4 responses to Sur96-104/M2 could be blocked with the HLA-DP–binding antibody in the 4 patients analyzed (Fig. 2B), whereas responses to Sur18-27/K10 were blocked by the HLA-DR–specific antibody in 3 patients analyzed (Fig. 2C). In one of those patients (C04P012) a very high background (without peptide) reactivity was observed; however, peptide reactivity was clearly stronger and blocking with HLA-DR antibody reduced the response to far below that of the observed background. Unfortunately, there were no cells left to repeat the experiment.

HLA typing of all responding patients for HLA-DR, HLA-DQ, and HLA-DP revealed that all patients reacting to Sur96-104/M2 were positive for HLA-DP4, and spCD4 cells only recognized peptide-loaded HLA-DP4, but not HLA-DP1/DP2–expressing EBV-transformed lymphoblastic cell lines (EBV-LCL; Fig. 2D), demonstrating that peptide Sur96-104/M2 was recognized in the HLA-DP4 context.

Patients reacting to the Sur18-27/K10 peptide were positive for HLA-DR4, -DR7, -DR11, or -DR16. Loading of the peptide onto different EBV-LCLs expressing only one of these alleles showed strong cross-reactivity to these closely related alleles (data not shown), indicating that Sur18-27/K10 exhibits a rather promiscuous binding to different HLA-DR alleles and that there are T cells recognizing the peptide in the context of various HLA alleles.

Functional avidity of Sur96-104/M2– and Sur18-27/K10–specific spCD4^{+} T cells

In 6 patients tested with different concentrations of the respective peptides, spCD4 T-cell responses were readily detectable at concentrations of 1 to 5 μg/mL. spCD4 T cells of patients C02P010 and C01P007 still showed weak responses at 0.1 μg/mL and spCD4 T cells of patient 0004-0015 even reacted to peptide Sur18-27/K10 at a concentration as low as 0.01 μg/mL (Fig. 3A).

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Table 1. Summary of all detected T-cell responses to EMD640744 (cocktail of five peptides) or the single peptides

		Pre-vaccination			Post-vaccination		
		Previously detected response by ELIspot or multimer assay[a]	MFTC assay		Previously detected response by ELIspot or multimer assay[a]	MFTC assay	
Patient	HLA		CD4	CD8		CD4	CD8
C01P002	A1, A3, B7	—	nd	nd	Sur18-27/K10	nd	nd
C01P007	A3	Sur18-27/K10	—	—	Sur18-27/K10	Sur18-27/K10	Sur18-27/K10
C01P008	A2	—	—	—	—	—	EMD
C01P009	A1, A2	—	nd	nd	Sur93-101/T2+Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C01P012	A2	—	nd	nd	Sur96-104/M2	—	Sur96-104/M2
C01P013	A2	Sur96-104/M2	nd	nd	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C01P015	B7	—	—	—	—	EMD	—
C01P016	A2	Sur96-104/M2	—	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C01P017	A1	—	—	—	—	—	—
C01P018	A1, A2	—	nd	nd	—	—	—
C01P024	A1	—	nd	nd	—	—	—
C01P027	A3	—	nd	nd	Sur18-27/K10	EMD	EMD
C01P028	A1, A3, B7	—	nd	nd	Sur18-27/K10	—	—
C01P029	A2, A3	—	nd	nd	—	—	Sur96-104/M2
C01P032	A2	—	nd	nd	Sur96-104/M2	EMD	—
C01P033	A2	—	—	—	—	—	—
C01P035	A2	—	—	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C01P037	A2	—	nd	nd	Sur96-104/M2	nd	nd
C01P040	A2	—	nd	nd	—	—	—
C02P003	A1, A2	—	—	—	—	—	—
C02P004	A3	—	nd	nd	Sur18-27/K10	—	Sur18-27/K10
C02P007	A3	—	—	—	Sur18-27/K10	(Sur18-27/K10)	Sur18-27/K10
C02P010	A2	—	nd	nd	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C03P001	A24, B7	—	nd	nd	—	EMD	—
C03P002	A3	—	—	Sur96-104/M2	Sur18-27/K10	Sur18-27/K10	Sur18-27/K10
C03P005	A2, B7	Sur96-104/M2	—	—	Sur96-104/M2	Sur96-104/M2+Sur18-27/K10	Sur96-104/M2
C03P009	A2, A24	Sur96-104/M2	—	—	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C03P012	A1, A24	—	nd	nd	—	nd	nd
C03P014	A2	Sur96-104/M2	nd	nd	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C03P015	A2	—	Sur96-104/M2	—	Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C03P016	A1, A2	—	—	—	Sur96-104/M2	—	—
C03P017	A1, A2	—	nd	nd	Sur93-101/T2 or Sur96-104/M2	nd	nd
C03P019	A2	—	nd	nd	Sur96-104/M2	EMD	—
C03P020	A1, A2	—	nd	nd	—	nd	nd
C03P021	A1	—	—	—	Sur93-101/T2	—	—
C04P004	A2, B7	—	nd	nd	—	nd	nd
C04P005	A2, A3, B7	—	nd	nd	Sur18-27/K10	—	EMD
C04P007	A3	—	nd	nd	Sur18-27/K10	nd	nd
C04P012	A2, A24	—	nd	nd	—	Sur18-27/K10	—
C04P013	A1, A3	—	nd	nd	—	—	EMD
C04P014	A2, A3, B7	Sur96-104/M2 + Sur18-27/K10	nd	nd	Sur96-104/M2+Sur18-27/K10	Sur18-27/K10	—
C04P015	A2	—	nd	nd	Sur96-104/M2	Sur18-27/K10	Sur96-104/M2
C04P016	A2	—	nd	nd	—	nd	nd
C05P002	A1, A3	—	nd	nd	—	nd	nd
C05P005	A1	—	nd	nd	—	nd	nd
C05P011	A24	Sur20-28	nd	nd	Sur20-28	EMD	EMD
C05P012	A1, A24, B7	—	nd	nd	Sur20-28	nd	nd
C05P014	A1, A2	—	nd	nd	Sur93-101/T2+Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
C05P017	A1, A2	—	nd	nd	Sur93-101/T2+Sur96-104/M2	Sur96-104/M2	Sur96-104/M2
No. of patients tested		49	15	15	49	38	38
No. of responders		8	1	3	31	23	23
Percentage positive of samples analyzed		**16.3**	**6.7**	**20.0**	**63.3**	**60.5**	**60.5**

NOTE: Responses detected by ELIspot and/or peptide HLA multimer staining have been published previously (7) and are summarized together for better comparison in one column. The pre-vaccination responses were derived from one time point, just before the first vaccination. The post-vaccination responses were measured in available samples (1–8 samples per patient) from different time points after vaccination [weeks 4, 8, 12, 16, 17, 24, 36, or end-of-study (EOS)]. Patients received at least four, usually more, vaccinations according to the vaccination schedule (7). A patient was scored positive if a T-cell response could be detected in at least one sample. MFTC assay for detection of spCD4 responses was performed in patients with available samples from pre-vaccination ($n = 15$) and post-vaccination ($n = 38$) time points. Abbreviations: EMD, positive response to the cocktail of five peptides contained in EMD640744, single peptides not tested; nd, assay not done; Sur96-104/M2, positive response to the A2-binding peptide Sur96-104/M2 and EMD (if tested); Sur18-27/K10, positive response to the A2-binding peptide Sur18-27/K10 and EMD (if tested); —, no response detected.

[a]Results have been published (7).

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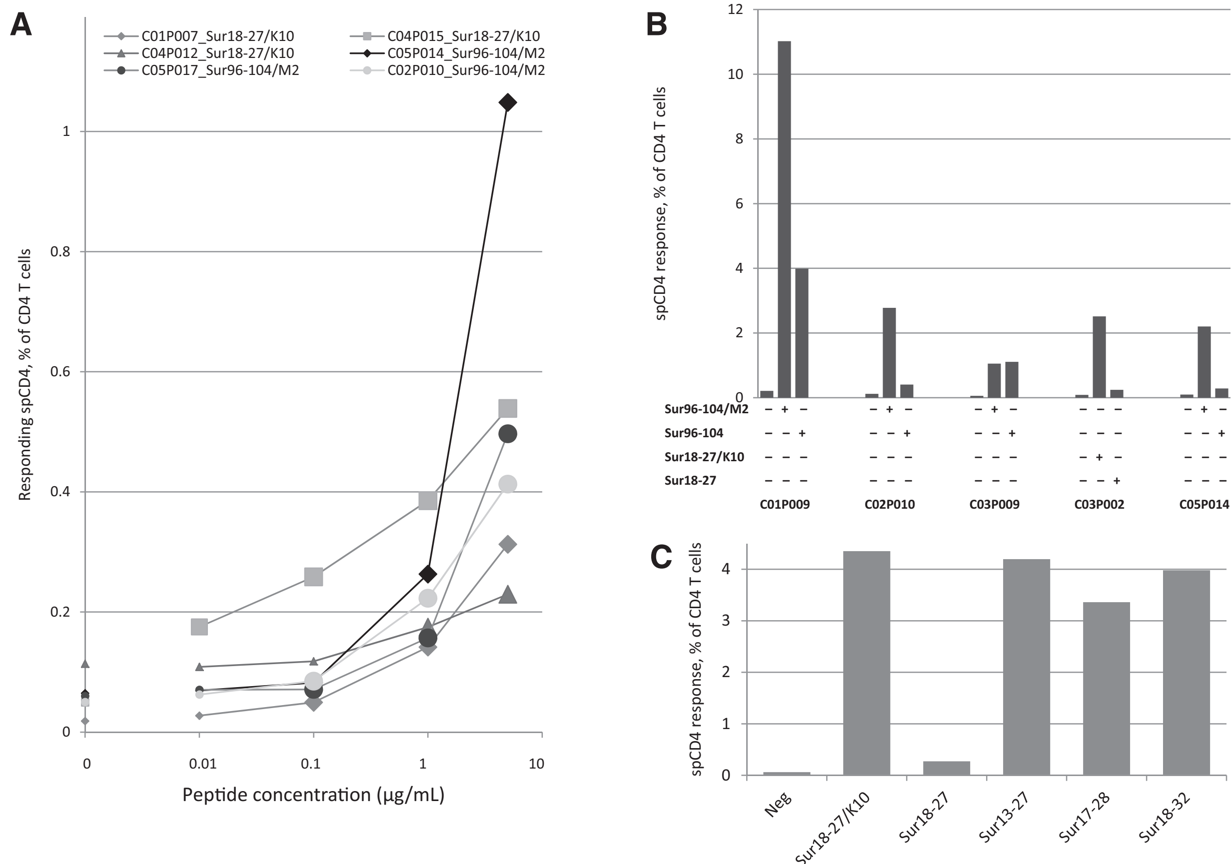

Figure 3.
Recognition of Sur96-104/M2 and Sur18-27/K10 and their native variants by spCD4 T cells. A, recognition of different concentrations of Sur96-104/M2 and Sur18-27/K10 by spCD4 T cells in 6 patients. The sum of all cytokine-producing spCD4 cells is shown as a percentage of total CD4 T cells. Corresponding background of each sample (stimulation without peptide) is marked on the axis as peptide dose of 0 μg/mL. Small symbols represent values that are below the 2-fold background, whereas large symbols represent values that are considered positive and are bigger than the 2-fold background. B, recognition of the native peptide variants Sur96-104 and Sur18-27 in comparison with the modified peptides Sur96-104/M2 and Sur18-27/K10 contained in EMD640744 was tested in 5 patients. C, in patient C04P015, who strongly reacts to the modified Sur18-27/K10 but only very weakly to the native Sur18-27, reactivity was also tested against three longer peptides containing the native sequence of Sur18-27: Sur13-27 (FLKDH**RISTFKNWPF**), Sur17-28 (H**RISTFKNWPF**L), and Sur18-32 (**RISTFKNWPF**LEGCA).

Recognition of native peptides by modified peptide-activated spCD4 T cells

Three peptides in EMD640744 (Sur96-104/M2 and Sur18-27/K10 among them) contained optimized anchor residue, i.e., are modified in one position compared with the native sequence. We and others have shown, at least for CD8$^+$ T cells in the HLA class I context, that such modified peptides can induce T cells that recognize their native counterparts as well (7, 11, 12).

Here, we demonstrate that the spCD4 T cells induced by vaccination with the modified peptides contained in EMD640744 could also recognize the native counterparts of Sur96-104/M2 (Sur96-104) and Sur18-27/K10 (Sur18-27), though responses to the native forms were weaker or almost absent in some cases (Fig. 3B). In patient C04P015, recognition was tested of longer variants of the native peptide (Fig. 3C). Despite the fact that spCD4 T cells induced by Sur18-27/K10 barely recognize the native sequence of Sur18-27, those cells do respond to the slightly longer peptide Sur17-28 almost as well as to Sur18-27/K10. Further lengthening of the peptide increases the reactivity only slightly, up to the maximum response seen with Sur18-27/K10.

Discussion

In this report, we show that the majority of patients undergoing active cancer immunotherapy with EMD640744 had combined CD4$^+$/CD8$^+$ T-cell responses to short peptides. In addition to the CD8$^+$ T-cell responses generated by EMD640744 vaccination and revealed by ELISpot- and pHLA-multimer staining assays (7), the MFTC assay detected prominent CD4$^+$ T-cell responses to EMD640744, in particular against two of the five short peptides. Thus, the EMD640744 vaccine can activate both CD8$^+$ and CD4$^+$ T-cell responses. In several patients, CD4$^+$ T-cell responses were even stronger (i.e., of higher frequency) than CD8$^+$ T-cell responses (representative example in Fig. 1A). In 8 of the 23

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patients with spCD4 T-cell reactivity, the spCD4 response was tested but not detectable in pre-vaccination samples, implying a vaccine-mediated *de novo* induction of responses. In only 1 patient was a pre-vaccination spCD4 T-cell response detected (C03P0015), which increased up to 30-fold after vaccination. This preexisting spCD4 T-cell response also points toward a role *in vivo*.

$CD4^+$ T-cell responses to short peptides have recently been reported in the context of influenza A– and mycobacterium tuberculosis–derived peptides (13, 14). The first such report in the context of tumors concerned a Melan-A/MART-1–derived decamer peptide resembling a minimal $CD4^+$ T cell epitope, as presented by Bioley and colleagues (15). The same group also found $CD4^+$ T cells specific for the short Melan-A/MART-1 peptide in patients after vaccination with the peptide (16). In addition, Harada and colleagues (17) reported a single case of spCD4 T cells that were induced by vaccination with a nonamer peptide derived from the ubiquitin-conjugated enzyme variant Kua (UBE2V).

Precisely how the short peptides are presented on HLA class II still remains unclear. It is likely that they represent minimal core peptides, having the correct anchor amino acid residues for binding to the respective class II alleles. Recognition of the core peptide by the TCRs of spCD4 T cells is sufficient to cause activation. The binding motif of the DP4 allele displays two main hydrophobic/aromatic anchors at position P1 (preferentially phenylalanine or leucine) and P6 (preference for phenylalanine) and an additional anchor at P9 favoring leucine (18). The DP4-binding Sur96-104/M2, with its sequence **L**M̲LGE**F**LK**L**, corresponds well to the DP4 binding motif. In contrast to this, the Sur18-27/K10 sequence RISTFKNWPK̲ does not fit in the described (19) peptide binding repertoires of common HLA-DR types. Only the isoleucine in position 2 might resemble a P1 anchor for HLA-DR4. Therefore, one could speculate that in the case of Sur18-27/K10, the presence of classical anchor residues might not be the primary reason for HLA class II binding properties, which may rely on other, yet unknown, features of the sequence. This is in line with our finding of promiscuous binding of this peptide to several HLA-DR alleles and associated cross-reactivity of spCD4 T cells. Of note is that the native variant of Sur18-27 is not, or is only very weakly, recognized by Sur18-27/K10–specific spCD4 T cells. However, Sur17-28 is merely two amino acids longer and is recognized almost equally well as Sur18-27/K10 (Fig. 3C). This indicates an important role for HLA-binding of the lysine at position 10 in the short peptide; however, the presence of the leucine at the neighboring position in the longer variant seems to compensate for the presence of the native phenylalanine at P10.

spCD4 responses were thought to be rare cases and rather anecdotal, because they have not been observed in other trials utilizing short nonamer peptides. A likely explanation for why spCD4 T-cell responses to short peptides are not observed more often is that standard immunomonitoring technologies such as ELISpot assays with PBMC cannot discriminate between $CD8^+$ and $CD4^+$ T cells and pHLA class I–multimer staining only detects T-cell responses in the HLA class I context. It was the implementation of the MFTC assay that enabled us to separate $CD4^+$ from $CD8^+$ T-cell responses and characterize the T_H subtype responding to the short target peptides. By MFTC we also detected three additional $CD8^+$ T-cell responses, indicating that in some cases the MFTC assay might be even more sensitive than other assays, though also consuming more cells.

The relevance of $CD4^+$ T cells for the promotion of productive $CD8^+$ T-cell responses has been shown by Janssen and colleagues (20) and other groups. Similarly, a long $CD4^+$ T-cell epitope derived from survivin, among several short $CD8^+$ epitopes, proved to be beneficial in a vaccination trial by Widenmeyer and colleagues (21).The promotion of specific $CD4^+$ T-cell responses to EMD640744 that we see in this trial may explain the high number of $CD8^+$ immune responses because activation of $CD8^+$ T cells is much more efficient if adequate $CD4^+$ T-cell help is available. In fact, of the 29 patients presenting a T-cell response in the MFTC assay, 16 (55%) showed a combined response of $CD4^+$ and $CD8^+$ T cells, whereas we found a "spCD4-only" response in only 6 patients. In the remaining 8 patients with a "$CD8^+$-only" response, some spCD4 responses might have been missed, because some patients were only tested with the single peptides matching patients' HLA, but not with the full cocktail. In addition, $CD4^+$ T-cell help may not only benefit the induction of $CD8^+$ T cells. Matsueda and colleagues (22) showed that antibody responses to short CTL epitopes are widely detectable and correlate with better overall survival. Because the induction of humoral responses is thought to depend on $CD4^+$ T-cell responses, it is also likely that some of the 31 different CTL epitopes used in their study served as spCD4 targets. Unfortunately, no serum samples were available in our trial to check for antibody responses to the short peptides from EMD640744. Because we only looked for classical T_H1 cytokines, such as TNFα, IFNγ, and IL2, we cannot rule out a (maybe even broader) T_H2 spCD4 response.

Within this small phase I trial it was not possible, and is not planned, to correlate the exceptionally high number of survivin-specific $CD8^+$ T-cell responses together with the unexpected spCD4 T-cell responses induced by EMD640744 with progression-free or overall survival, because the study population was too small and too heterogeneous, including a variety of different tumor types.

The physiologic relevance of the detected spCD4 T-cell responses still remains unclear. However, a phase II study using two of the five survivin peptides contained in EMD640744, with one of them being an inducer of spCD4 T-cell responses, showed a correlation of prolonged survival with the induction of $CD8^+$ T-cell responses as monitored by pHLA-multimer staining (23). In addition, Hunder and colleagues reported on the direct antitumor efficacy of $CD4^+$ T cells. They present a case study where a single infusion of NY-ESO-1–specific $CD4^+$ T-cell clones led to complete tumor regression and durable clinical remission (24). Hunder and colleagues primarily discuss the extensive cytokine production of the infused cells and subsequent activation of the patient's immune system as the mode of action of the infused $CD4^+$ T cells. However, it has also been reported that $CD4^+$ T cells can efficiently destroy tumor cells independently (25, 26) and, moreover, in a more recent study, that perforin and granzyme B secreting cytotoxic NY-ESO-1–specific $CD4^+$ T cells developed in melanoma patients after treatment with ipilimumab (27). In aggregate, $CD4^+$ T cells specific for small tumor peptides may play an important and hitherto unrecognized role in the immune concert fighting cancer cells and thus should be studied more extensively in future clinical trials.

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Disclosure of Potential Conflicts of Interest

U. Gnad-Vogt is Chief Medical Officer at CureVac GmbH. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Gross, L. von Boehmer, A. Knuth, A. Ochsenbein, U. Gnad-Vogt, U. Forssmann, T. Woelfel
Development of methodology: S. Gross, A. Knuth, A. Ochsenbein, T. Woelfel, E. Kaempgen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Gross, E. Gallerani, N. Mach, S. Böhm, D. Hess, L. von Boehmer, A. Knuth, A. Ochsenbein, E. Kaempgen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Gross, V. Lennerz, A. Knuth, U. Forssmann, E. Kaempgen
Writing, review, and/or revision of the manuscript: S. Gross, V. Lennerz, E. Gallerani, N. Mach, S. Böhm, D. Hess, L. von Boehmer, A. Knuth, A. Ochsenbein, U. Gnad-Vogt, U. Forssmann, T. Woelfel, E. Kaempgen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Gallerani
Study supervision: U. Gnad-Vogt, E. Kaempgen

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Cancer Immunology Research

Short Peptide Vaccine Induces CD4$^+$ T Helper Cells in Patients with Different Solid Cancers

Stefanie Gross, Volker Lennerz, Elisa Gallerani, et al.

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