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ORIGINAL ARTICLE

Pitfalls of vaccinations with WT1-, Proteinase3- and MUC1-derived peptides in combination with MontanideISA51 and CpG7909

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Abstract T cells with specificity for antigens derived from Wilms Tumor gene (WT1), Proteinase3 (Pr3), and mucin1 (MUC1) have been demonstrated to lyse acute myeloid leukemia (AML) blasts and multiple-myeloma (MM) cells, and strategies to enhance or induce such tumor-specific T cells by vaccination are currently being explored in multiple clinical trials. To test safety and immunogenicity of a vaccine composed of WT1-, Pr3-, and MUC1-derived Class I-restricted peptides and the pan HLA-DR T helper cell epitope (PADRE) or MUC1-helper epitopes in combination with CpG7909 and MontanideISA51, four patients with AML and five with MM were repetitively vaccinated. No clinical responses were observed. Neither pre-existing nor naive WT1-/Pr3-/MUC1-specific $CD8^+$ T cells expanded in vivo by vaccination. In contrast, a significant decline in vaccine-specific $CD8^+$ T cells was observed. An increase in PADRE-specific $CD4^+$ T helper cells was observed after vaccination but these appeared unable to produce IL2, and $CD4^+$ T cells with a regulatory phenotype increased. Taken into considerations that multiple clinical trials with identical antigens but different adjuvants induced vaccine-specific T cell responses, our data caution that a vaccination with leukemia-associated antigens can be detrimental when combined with MontanideISA51 and CpG7909. Reflecting the time-consuming efforts of clinical trials and the fact that 1/3 of ongoing peptide vaccination trails use CpG and/or Montanide, our data need to be taken into consideration.

Keywords Vaccination · AML · MM · T cell · CpG · WT1

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Introduction

Several lines of investigation have provided conclusive evidence that epitopes derived from Wilms Tumor1 (WT1) and Proteinase3 (Pr3) are presented in the context of major histocompatibility complex (MHC) class I epitopes on solid cancer cells and/or leukemic blasts, and are potentially immunogenic. Molldrem et al. [1] identified $Pr3_{169–177}$, a nine-amino acid self-peptide derived from Pr3 that binds HLA-A*0201 as a leukemia-associated $CD8^+$ T cell antigen. $Pr3_{169–177}$-specific $CD8^+$ T cells have been isolated from healthy donors and from patients with chronic myeloid leukemia (CML), shown to kill CML and acute myeloid leukemia (AML) cells but not normal hematopoietic progenitors expressing low levels of these antigens, and to effectively inhibit the outgrowth of a panel of CML progenitors in proportion to the level of Pr3 overexpression [2]. WT1-derived HLA-A*0201-presented epitopes, including the HLA-A*0201-presented epitope $WT1_{126–134}$, have been shown to elicit epitope-specific $CD8^+$ T cells from healthy controls that can lyse myeloid leukemic blasts [3–5]. Recent clinical studies suggest that vaccination of patients with either the HLA-A*2402-presented natural or modified epitope $WT1_{235–241}$ [6], the HLA-A*0201-presented epitope $WT1_{126–134}$ alone [7], or a combination of $WT1_{126–134}$ with $Pr3_{169–177}$ [8] can indeed induce leukemia-reactive $CD8^+$ T cells that may contribute to control of solid tumor cells or leukemic blasts and induce clinical responses.

Another candidate tumor antigen is the mucin 1 protein (MUC1). Due to the aberrant glycosylation in the variable number of tandem repeat (VNTR) domain of MUC1-protein in cancer cells, non-glycosylated VNTR-derived peptides ($MUC1_{138–178}$) have been described as helper epitopes for the stimulation of $CD4^+$ T cells [9]. VNTR-derived peptides have been also described as targets for $CD8^+$ T cells, although these peptides appear unusual in not fully matching defined binding motifs for the respective HLA-molecules [10]. Preclinical studies have also identified multiple HLA-A*0201 epitopes derived from regions of MUC1 outside the VNTR including $MUC1_{79–87}$ [11]. MUC1-derived peptides have been shown to be presented not only by solid cancers [11], but also hematological malignancies including MM [12]. Initial clinical vaccination trials in patients with breast or ovarian cancer [11, 13] confirmed immunogenicity and lack of toxicity following vaccination with MUC1-derived immunogens.

In summary, WT1, Pr3, and MUC1-containing vaccines represent candidates for further clinical evaluation in patients with AML or MM. Therefore, we tested whether a vaccine composed of WT1 and Pr3 or MUC1-derived peptides can induce immunological and clinical responses.

Materials and methods

Clinical protocol

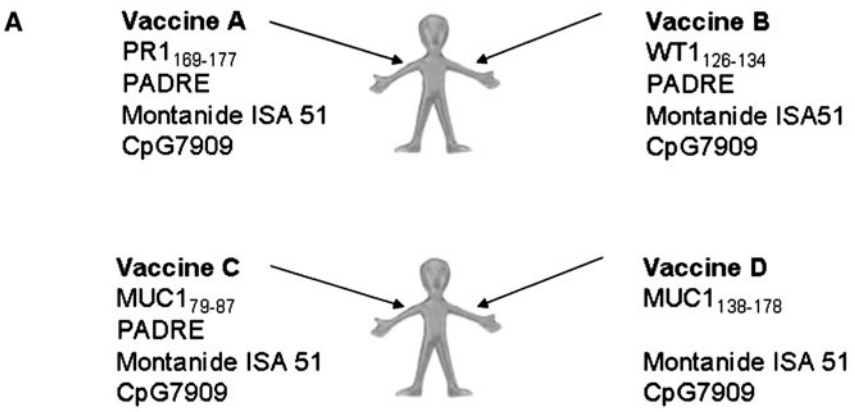
This was a non-randomized, non-blinded, controlled, bi-centric, open-label, adjuvant vaccination pilot study. Nine patients HLA*A0201 by genotyping were included. Inclusion criteria included confirmed primary or secondary AML or relapse of an AML, including RAEB/RAEB-T, IPSS score $\geq$1.5; and minimal residual disease (MRD) (defined as >5% $\leq$30% leukemic blasts in bone morrow). Patients with confirmed MM were also eligible if presenting with stage I or stable disease, or partial remission after cytoreductive chemotherapy. Further inclusion criteria were patients with older than ($\geq$) 18 years of age of either gender and of any race, life expectancy of at least 4 months, and adequate performance status (Karnofsky score $\geq$70%). Systemic corticosteroid or other immunosuppressive therapies were not allowed within the last 3 months or during the study. Prior chemotherapy or radiation therapy was allowed if at least 2 weeks had elapsed between the last dose of therapy and study entry and the patient had recovered from all treatment-related toxicities. Study reagents were injected subcutaneously on days 0, 14, 28, 42, 56, and 70. Patients were vaccinated simultaneously at different locations with two different types of vaccines. AML patients received a combination of pan HLA-DR T helper cell epitope (PADRE), CpG7909, MontanideISA51 and either $WT1_{126–134}$ (vaccine A) or $Pr3_{169–177}$ (vaccine B). MM patients received CpG7909, MontanideISA51, and either $MUC1_{79–87}$ and PADRE (vaccine C) or the oligomer $MUC1_{138–178}$ (vaccine D) (Fig. 1). The oligomer in vaccine D served as source for $CD4^+$ and $CD8^+$ T cell epitopes. Every patient received the study substances in a dose of 1.0 mg for each peptide, comparable to dose levels in other clinical trials [14–16], CpG7909 was administered at a final dose of 1 mg, again within the dose range of other successful clinical vaccination trials (range 0.5–8 mg) [17–21]. The final vaccine was freshly prepared from different compounds for each day of vaccination. PBMCs were collected at day −7 (range day −13/0) and every second week until day 84. Leukapheresis was performed at day 42 (range day 42/56) and 84 (range day 84/94) in order to harvest PBMCs and collected material processed and frozen immediately. The study was approved by the local ethics board, regulatory authorities and has been registered at the "Deutsche Krebstudienregister" (DKSR number 415 and 416; http://www.studien.de/includes/studien_suchen/studie.suchen.php?PIC_CASE=1&L).

Vaccine, peptides, pentamers, and antibodies

Clinical grade peptides were purchased from Clinalfa-Bachem (Weil am Rhein, Germany), and MontanideISA51 from Seppic (Köln, Germany). CpG7909 (VaxImmune^{TM})

Fig. 1 **a** Vaccine composition, **b** patient characteristics, and clinical responses after vaccination. CTCAE grading of side effects is indicated. Clinical responses were evaluated at the end of study (day 84). *DTH* delayed type hypersensitivity reaction, *SD* stable disease, *PD* progressive disease, *n.d.* not determined, *Pretreatment* treatment prior study entry

A



B

Number	Disease	% Blasts or IgG before/after vaccination	Pre-Treatment	Vaccine	Local erythema	other	Fever	DTH	Response
1	AML	20% / 20%	2	A+B	II	Fatigue I	-	-	SD
2	AML	3% / 70%	1	A+B	II-III	Fatigue I –II	III	-	PD
3	AML	30% / 30%	no	A+B	III	Fatigue II necrosis, infection III	III	-	SD
4	AML	20% / 55%	4	A+B	II	Dyspnoe III	-	-	PD
5	MM	10% / 50% 21,4 / 28,9g/l	>4	C+D	II	-	-	-	PD
6	MM	50% / 50% 26,9 / 27,1g/l	2	C+D	III	-	-	-	SD
7	MM	20% / 20% 15,4 / 18,1g/l	2	C+D	II-III	-	-	-	SD
8	MM	5% / 20% 10,4 /18,4g/l	2	C+D	II-III	-	-	-	PD
9	MM	CR / CR 12,7 / 14,1g/l	4	C+D	II	Fatigue I	I	-	SD

was kindly provided by Coley pharmaceuticals GmbH (Düsseldorf, Germany). Peptides $WT1_{126–134}$, $PR3_{169–177}$, $MUC1_{79–87}$, HIV-reverse transcriptase: $HIV_{RT\ 476–484}$, $HBVcore_{128–140}$, and $CMVpp65_{495–504}$, overlapping 15-mers for $MUC1_{138–178}$, PADRE, PE-labeled HLA-A*0201-presented $WT1_{126–134}$, $PR3_{169–177}$, $MUC1_{79–87}$, $HIV_{RT\ 476–484}$, and $CMVpp65_{495–504}$ pentamers were from ProImmune (Oxford, UK). Anti-CD3-Pacific Blue, anti-CD4-APC Alexa Fluor 750, anti-CCR7-PE-cy7, anti-CD27-APC Alexa Fluor 750, anti-PD-1-APC, anti-FoxP3-APC (including anti-human Foxp3 Staining Set), anti-TNFα-PE Cy7, anti-IL4-PE Cy7, anti-IL10-PE, were obtained from Invitrogen (Paisley, UK); anti-CD8-PercP, CD86-PE, anti-CD25-FITC, anti-CD127-PE Cy7, anti-IFNγ-FITC, and anti-IL2-APC from BD Biosciences (Erembodegem, Belgium); and anti-CD303-FITC (BDCA-2) from Miltenyi Biotec (Gladbach, Germany). Flow cytometry was performed on an LSR II flow cytometer (BD Biosciences) using FacsDiva software (BD Biosciences).

Pentamer staining and functional T cell assays

Pentamer staining and functional T cell assays were performed as described recently [4, 5, 22–24]. In brief, $\sim 1 \times 10^6$ PBMCs were incubated with pentamer for 30 min, then directly conjugated antibodies were added for 20 min at 4°C in order to specify T cell phenotype (CD3-Pacific Blue, CD27-APC-Alexafluo 750, PD-1 APC, and CD8-PercP, CCR7-PE-cy7). Cells were washed with PBS and resuspended in FACS buffer (0.1% BSA + 0.1% Na-azide in PBS) for FACS analysis. Gating for all pentamer stainings were standardized within individual samples to arrive at a fully comparative datasets. In order to test background staining for individual pentamers, 10^6 PBMCs derived from five HLA-A*0201-positive but CMV- and HIV-negative healthy individuals were co-incubated with individual pentamers and an anti-CD8 antibody and analyzed by flow cytometry. Unspecific staining was thereby determined for all pentamers as $\leq$0.05% of $CD8^+$ T cells

(data not shown). To assess the sensitivity of pentamer staining to detect vaccine-reactive T cells with intermediate avidity and low frequency, we took advantage of previous work from our laboratory with intermediate avidity $WT1_{126–134}$-specific T cells [4, 23]. These data and titration of a $WT1_{126–134}$-specific T cell clone into 10^6 $CD8^+$ T cells (Supplementary Fig. 4) demonstrate a highly reproducible and specific pentamer staining for intermediate avidity T cell lines and clones with frequencies ≥0.10%. Intracellular cytokine (ICC) assay was performed as described recently [4]. In brief, ~1 × 10^6 PBMCs were stimulated with the indicated peptide (10 μg/ml) and Brefeldin A (GolgiPlug™, BD) (1 μl/ml) for 6 h at 37°C. After 6 h, cells were washed and surface antibodies were added: anti-CD3-Pacific Blue, anti-CD4-APC Alexa Fluor 750, anti-CD8-PercP. Cells were incubated for 20 min at 4°C and washed with PBS. Cells were fixed and permeabilized with lysing and permeabilizing solution (BD FACS™). Next, anti-TNFα-PE Cy7, anti-IFNγ-FITC, and anti-IL2-APC or anti-IL4-PE Cy7 and anti-IL10-PE were added. Gating for all ICC assays was standardized within individual samples to arrive at a fully comparative datasets. Thereby, background signal for TNFα, IFNγ, and IL2 cytokine secretion was tested in 2 HLA-A*0201-positive HIV and CMV-negative healthy individuals. $CD4^+$- or $CD8^+$ cytokine-secreting cells were detected in <0.1% of cells (data not shown), thus the detection threshold was determined as 0.1%. In vitro expansion assays were performed as described recently [23]. In brief, 1 × 10^6 PBMCs/sample were stimulated with 1 μg/ml peptides $WT1_{126–134}$, $PR3_{169–177}$, $MUC1_{79–87}$, overlapping 15-mer $MUC1_{138–164}$, overlapping 15-mer $MUC1_{153–178}$, $HIV_{RT\ 476–484}$ or $CMVpp65_{495–503}$, together with 50 U/ml IL2 (Chiron, Emeryville, CA, USA), for 1 week at 37°C. After 1 week, a 6-h stimulation followed by an ICC assay was performed as described above. T cell clones with specificity for $WT1_{126–134}$ have been generated as described recently [4, 5, 23]. A twofold change in frequency of antigen-specific T cells was considered as vaccine-induced, an arbitrary threshold used in multiple vaccination studies to define vaccine-specific immune responses [7, 8].

Results

Study design and patient characteristics

The study was designed to induce leukemia-/MM-reactive T cells with a polyvalent vaccine composed of HLA-A*0201-presented peptides $WT1_{126–134}$ (vaccine A) and $Pr3_{169–177}$ (vaccine B) (AML patients) or $MUC1_{79–87}$ (vaccine C) and those derived from $MUC1_{138–178}$ (vaccine D) (MM patients) (Fig. 1). The vaccine included either a PADRE, which was engineered to bind common HLA-DR molecules with high affinity [25, 26], or a MUC1 oligomer [9] to induce $CD4^+$ helper T cells. To enhance immunogenic antigen-presentation, CpG7909 (VaxImmune™), a single-stranded oligodeoxynucleotide (ODN) that activates plasmacytoid dendritic cells through toll-like receptor (TLR) 9 triggering, and has been reported to increase tumor-reactive $CD8^+$ T cells upon vaccination in melanoma patients [17], was also included. Four AML (vaccine A + B) and 5 MM (vaccine C + D) patients were simultaneously vaccinated every 2 weeks until a total of six vaccinations (Fig. 1).

Clinical outcome and side effects

During the study observation period, two of four AML patients had progressive disease according to IWG criteria [27]; two of five MM patients progressed (EBMT, IBMTR and ABMTR criteria [28]); and, all other patients had stable disease at study day 84 (Fig. 1b). As all patients have not been progressive before vaccination, and also no late responses were observed, we concluded that no clinical responses were observed [7]. After vaccination, all patients had inflammation at the injection sites (grades II–III toxicity according to CTCAE: Cancer Therapy Evaluation Program, Common Terminology Criteria for adverse Events, Version 3.0). Four patients experienced mild fatigue (grades I–II) and three had fever (grades I–III) (Fig. 1b). To assess for vaccine-specific T cell responses, a delayed type hypersensitivity (DTH) test was performed with the following peptides from the vaccine at study days 2, 44, and 86: PADRE (all patients); $WT1_{126–134}$, $PR3_{169–177}$ (AML patients); and $MUC1_{79–87}$ and $MUC1_{138–178}$ (MM patients). None of the patients had a positive DTH test with any peptide at any time (Fig. 1b).

Vaccine-specific $CD8^+$ T cells (VST) cannot be recruited by vaccination and rather decline

To investigate if vaccination of the four AML patients with the HLA-A*0201-binding peptides $WT1_{126–134}$, $PR3_{169–177}$, or the five MM patients with the $MUC1_{79–87}$ peptide increased the frequency of VST, collected PBMCs were incubated with HLA-A*0201 restricted $WT1_{126–134}$ and $PR3_{169–177}$ pentamers if vaccinated with vaccines A and B (4 patients with AML) and $MUC1_{79–87}$ pentamers if vaccinated with vaccines C and D (5 patients with MM) [4, 5, 23]. As a negative control, an $HIV_{RT476–484}$ pentamer was used; and as a positive control, a $CMVpp65_{495–503}$ pentamer was used. In six of nine patients, $pp65_{495–503}$-specific T cells were detectable prior to vaccination and through study day 84 (range 0.15–3.30%) (CMVpos group) and frequencies did not significantly differ when

comparing patients before and after vaccination (Fig. 2a). Four of five MM patients had pre-existing $MUC1_{79–87}$ pentamer-positive $CD8^+$ T cells (range 0.15–0.30%) and one AML patient had pre-existing $WT1_{126–134}$ pentamer-positive $CD8^+$ T cells (0.15%, Supplementary Fig. 1) (defined as VST group). Thus, four patients had no VST prior vaccination (NoVST group). When comparing the frequency of VST in the group of patients with NoVST before and after vaccination, no increase in VST was observed. In contrast, a slight but significant decline in pentamer-positive VST (AML and MM patients, $p = 0.02$) was observed within the VST group ($n = 5$ patients) after vaccination while the frequency of $pp65_{495–503}$-specific T cells did not change significantly (Fig. 2a), e.g., in patient 4 with pre-existing $WT1_{126–134}$ pentamer-positive $CD8^+$ T cells (0.15%) the $WT1_{126–134}$ pentamer-positive $CD8^+$ T cells were no longer detectable at all other time points after vaccination including days 42 and 84 (Fig. 2a, Supplementary Fig. 1). In addition, no VST were observed in the bone marrow of patients before or 84 days after vaccination (data not shown).

To assess if the phenotype of persisting pentamer-positive $CD8^+$ T cells changed from naïve/central memory ($CD27^+/CCR7^+$) to effector memory/effector ($CD27^-/CCR7^-$) after vaccination, $CD8^+$ T cells were stained with pentamer, anti-CD27 and anti-CCR7 at day −7 and 42. Percentage of $CD27^-/CCR7^-$ pentamer-positive T cells was compared before and after vaccination and indeed only for $MUC1_{79–87}$ but not for $pp65_{495–503}$ pentamer-positive T cells a significant increase (*t* test $p = 0.03$) in the $CD27^-/CCR7^-$cell population was observed suggesting that naïve/central memory cells indeed had become effector memory or effector cells after vaccination (Fig. 2b). However, VST apparently failed to proliferate and were rather depleted (Fig. 2a). The pentamer-positive cells were also stained with anti-PD1, a marker of T cell exhaustion [29], but none of the pentamer-positive cells exhibited substantial PD1-expression (data not shown).

WT1- and MUC1-specific $CD8^+$ T cells are functionally impaired

The failure of pre-existing pentamer-positive WT1- and MUC1-specific T cells to expand after vaccination could be either a consequence of complete anergy, with an absolute inability to respond to antigen, or a selective inability to proliferate [30]. Therefore, PBMCs of patients within the VST group were stimulated for 6 h with $WT1_{126–134}$ and $PR3_{169–177}$ peptides (AML patients) and $MUC1_{79–87}$ peptide and overlapping 15-mers derived from the $MUC1_{138–178}$ oligomer (MM patients). Although, all tested patients had pre-existing $WT1_{126–134}$ or $MUC1_{79–87}$ pentamer-positive cells (Fig. 2a, b), we failed to detect specific TNFα, IFNγ, o rIL2 production by ICC in response to respective antigens before and after vaccination (all <0.1%), suggesting that the detectable WT1-/MUC1-pentamer-positive $CD8^+$ T cells were not functional in vivo prior to vaccination, and that function could not be rescued by vaccination. Also 7 days of stimulation of PBMCs from selected patients with $MUC1_{79–87}$, and two pools of overlapping 15-mers derived from $MUC1_{138–164}$ and $MUC1_{153–178}$ (MM patient), $pp65_{495–503,}$ and $HIV_{RT\ 476–484}$, did neither result in any pentamer-positive (<0.05%, data not shown) nor functional VST (Table 1) after vaccination, while $pp65_{495–503}$-specific T cells did not change in frequency (Table 1). Only in one AML patient (patient 1) who had no pre-existing vaccine-reactive T cells by pentamer staining, we could generate 0.4% $CD8^+$ T cells with specific IFNγ production against $WT1_{126–134}$ prior to vaccination which were, however, no longer detectable after vaccination (Table 1; Supplementary Fig. 2) in line with the observation that vaccination decreased the number of pentamer-positive VST (Fig. 2a).

Expansion of PADRE-specific $CD4^+$ T helper cells which do not substantially produce IL2

All vaccines contained a potential $CD4^+$ T helper epitope, either the 'pan HLA-DR T helper epitope' PADRE or $MUC1_{138–178}$, to induce $CD4^+$ T helper cells and potentially improve the generation and persistence of WT1-/Pr3-/MUC1-specific $CD8^+$ T cells. In five of eight patients, PADRE-specific $CD4^+$ T cells were detected prior to vaccination (range 0.20–0.40% of $CD4^+$ T cells) that produced either IFNγ or TNFα, but no IL2, IL4, or IL10 production was detectable (Fig. 2c and data not shown for IL4 and IL10). A significant increase in IFNγ-producing PADRE-specific $CD4^+$ T cell responses was detected after vaccination. Also higher amounts of TNFα-producing PADRE-specific T cells were observed, although not significant. T cells did not produce IL2 (Fig. 2c). In Patient 7, a two- to fourfold induction of TNFα producing $MUC1_{153–178}$-specific $CD4^+$ T cells was observed (<0.1% day −7, 0.4% day 42, 0.2% day 84), and, again, no IL2 production could be measured (Supplementary Table 1).

In order to test more thoroughly if PADRE stimulation could lead to induction of IFNγ and TNFα-secreting $CD4^+$ T cells that lack the ability to produce detectable amounts of IL2, PBMCs were stimulated for 1 week with helper peptides. In all four patients, PADRE-specific T cells were detectable prior to vaccination (frequency 0.2–2.0%) by ICC and produced either IFNγ or both IFNγ and TNFα. More importantly, in two patients they also produced IL2 (Table 2). After vaccination, the frequency of IFNγ- and/or TNFα-secreting cells increased twofold until day 42 or 84

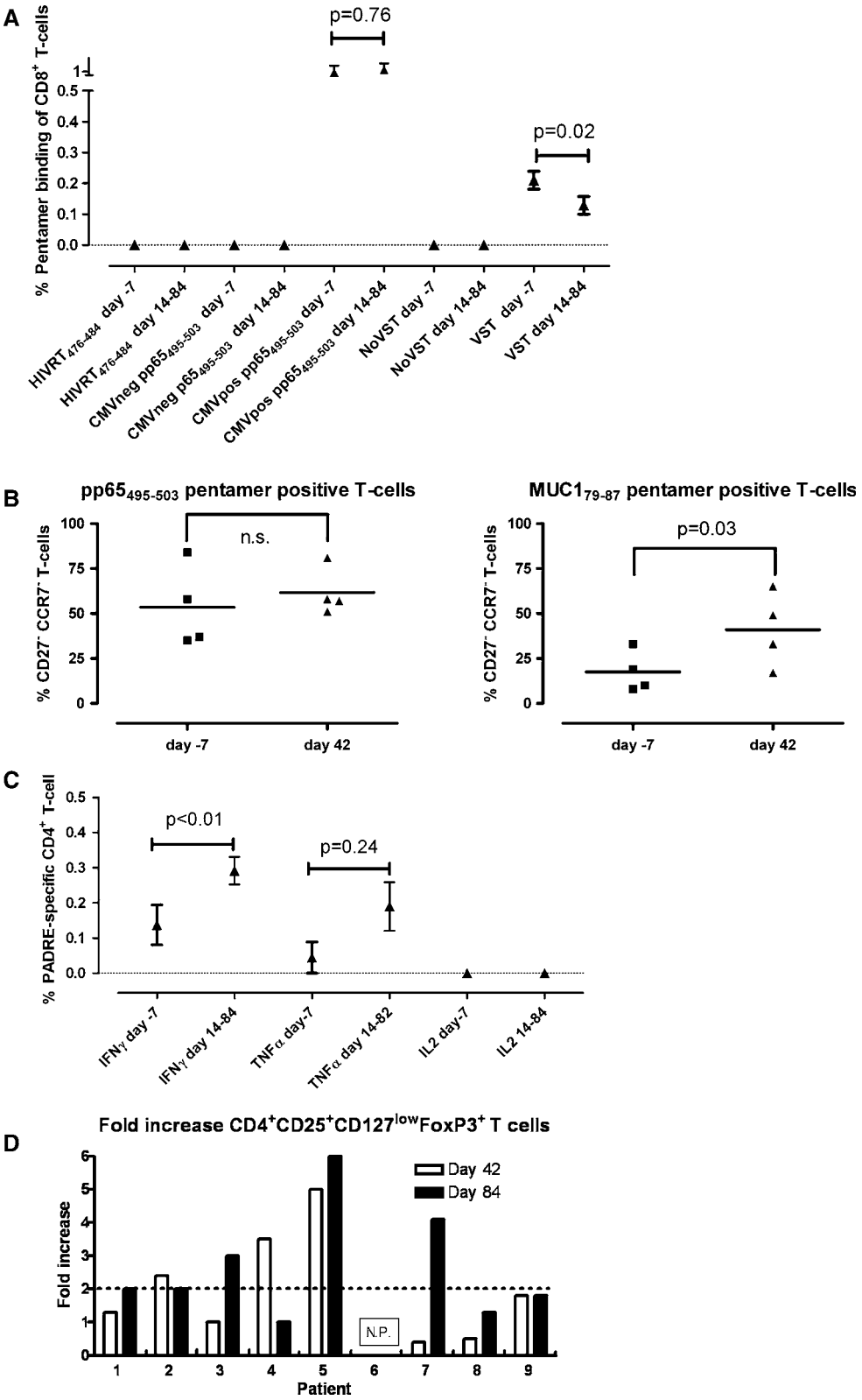


Fig. 2 Decrease in vaccine-specific T cells (VST) and increase in regulatory $CD4^+$ T cells after vaccination. **a** Pentamer-positive $CD8^+$ T cells before (day −7) and after (days 14–84) vaccination in all patients ($n = 9$). CMV sero-positive (CMVpos) group $n = 6$, sero-negative (CMVneg) group $n = 3$, VST before vaccination group (VST) $n = 5$, no VST before vaccination group (NoVST) $n = 4$, HIV-reverse-transcriptase-peptide (HIVRT). **b** Pentamer-positive $CD8^+$ T cells were gated and percentages of pentamer-positive effector memory/effector T cells ($CD27^-$/$CCR7^-$) calculated before vaccination and at day 42 after vaccination. **c** Percent cytokine producing PADRE-specific $CD4^+$ T cells as measured by ICC. **d** Flow cytometry analysis of patient-derived PBMC samples collected prior to vaccination and at day 42 and 84. Cells were incubated with anti-CD3, anti-CD8, anti-CD4, anti-CD25, anti-CD127, and anti-FoxP3. Regulatory phenotype is defined as: $CD25^+CD127^{low}FoxP3^+$ $CD4^+$ T cells. The *dotted line* represents the threshold of a more then twofold increase of $CD25^+CD127^{low}FoxP3^+$ $CD4^+$ T cells after vaccination. *n.p.* not possible

after vaccination (range 2.8–17.8-fold increase) (Table 2; Supplementary Fig. 3). Although the number of PADRE-specific IFNγ and/or TNFα producing $CD4^+$ T cells increased substantially after vaccination in all patients, pre-existing IL2 producing PADRE-specific $CD4^+$ T cells in patients were not found in greater numbers (Table 2).

Table 1 Cytokine production of in vitro expanded leukemia-/MM-/virus-specific $CD8^+$ T cells before and after vaccination

	After 7 days in vitro re-stimulation of $CD8^+$ T cells								
	Peptide	$HIV_{RT476-134}$		$pp65_{495-503}$		$WT1_{126-134}$		$Pr3_{169-177}$	
	Day	−7	42 & 84	−7	42 & 84	−7	42 & 84	−7	42 & 84
1	IFNg	<0.1	<0.1	**24.6**	**22.3**[a]	**0.4**	*<0.1*	<0.1	<0.1[a]
	TNFa	<0.1	<0.1	**4.0**	**3.9**[a]	<0.1	<0.1	<0.1	<0.1[a]
	IL2	<0.1	<0.1	**0.4**	**0.2**[a]	<0.1	<0.1	<0.1	<0.1[a]
2	IFNg	<0.1	<0.1[a]	**0.7**	**0.3**[a]	<0.1	<0.1[a]	<0.1	<0.1[a]
	TNFa	n.p.	<0.1[a]	n.p.	**0.3**[a]	n.p.	<0.1[a]	n.p.	<0.1[a]
	IL2	<0.1	<0.1[a]	**0.2**	<0.1	<0.1	<0.1	<0.1	<0.1[a]
3	IFNg	<0.1	<0.1[a]	<0.1	<0.1[a]	<0.1	<0.1[a]	<0.1	<0.1[a]
	TNFa	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	IL2	<0.1	<0.1[a]	<0.1	<0.1[a]	<0.1	<0.1[a]	<0.1	<0.1[a]
	After 7 days in vitro re-stimulation of $CD8^+$ T cells								
	Peptide	$HIV_{RT476-134}$		$pp65_{495-503}$		$MUC1_{79-87}$		$MUC1_{153-178}$ $MUC1_{138-164}$	
	Day	−7		42 & 84		−7		42 & 84	
9	IFNg	<0.1	<0.1	<0.1	<0.1[a]	<0.1	<0.1	<0.1	<0.1
	TNFa	<0.1	<0.1	<0.1	<0.1[a]	<0.1	<0.1	<0.1	<0.1
	IL2	<0.1	<0.1	<0.1	<0.1[a]	<0.1	<0.1	<0.1	<0.1
Pre-existing		**0/4**		**2/4**		**1/4**		**0/4**	
Induction			**0/4**		**0/4**		**0/4**		**0/4**
Loss			**0/4**		**0/4**		**1/4**		**0/4**

Intracellular cytokine production (ICC) 7 days after antigen-specific in vitro stimulation. Bold numbers indicate the specific detection of cytokine-secreting $CD8^+$ T cells; italic numbers indicate a twofold change when compared with day −7. If for day 42 & 84 only one value is indicated then measured values are identical

n.d. not determined due to lack of material

[a] Only one of the indicated time points was analyzed

Thus, IL2 production appeared to be impaired in PADRE-specific $CD4^+$ T cells after vaccination.

Increase of $CD4^+$ T cells with a regulatory phenotype after vaccination

It has been reported that CpG7909 can stimulate plasmacytoid dendritic cells and thereby increase the number of $CD4^+$ T cells with a regulatory phenotype and function in vitro [31] and in vivo [32] in mice, which could consequently inhibit the success of a vaccine [32, 33]. To investigate if the vaccination regimen influenced the frequency or maturation of plasmacytoid dendritic cells as reported recently (defined as CD303- and CD86-positive [21, 34]) or the frequency of regulatory T cells in the peripheral blood, patient-derived PBMC obtained at day −7, 42, and 84 were stained with anti-CD303 (BDCA-2) antibody, a single marker for plasmacytoid dendritic cells [35], anti-CD86, anti-CD25, anti-FoxP3, and anti-CD127, and analyzed by flow cytometry. The amount of both, immature ($CD86^-$) and mature ($CD86^+$) anti-CD303-positive (plasmacytoid dendritic) cells did not increase more then twofold over 84 days (range plasmacytoid dendritic cells <0.1–0.4% of total PBMCs) (Supplementary Table 2). However, five out of eight patients showed a more than twofold increase in $CD25^+$ $FoxP3^+$ $CD127^{low}$ $CD4^+$ T cells in one or both time points after vaccination (range 2.4–6.0-fold increase) (Fig. 2d).

Discussion

The main findings of our studies are that in contrast to multiple other vaccination phase I studies using similar antigens [7, 8, 11, 13, 36, 37] pre-existing WT1-/MUC1-specific T cells in advanced stage cancer patients (a) neither proliferated nor secreted cytokines upon peptide stimulation and (b) were rather depleted than rescued after vaccination.

Table 2 Cytokine production of PADRE-specific $CD4^+$ T cells before and after vaccination

	Day	After 7 days of PADRE-specific ex vivo expansion	
		−7	42 & 84
1 AML	IFNγ	**0.4**	***2.6*** & ***0.6***
	TNFα	**0.4**	***5.7*** & ***0.8***
	IL2	<0.1	<0.1
2 AML	IFNγ	**0.4**	***7.1*** & ***6.1***
	TNFα	**2.0**	***9.9*** & ***9.0***
	IL2	**0.3**	**0.3** & **0.2**
3 AML	IFNγ	**0.2**	***2.3***[a]
	TNFα	n.d.	n.d.
	IL2	<0.1	<0.1[a]
9 MM	IFNγ	**0.4**	**0.3** & ***1.1***
	TNFα	**0.2**	**0.3** & ***1.2***
	IL2	**0.3**	**0.3** & *<0.1*
Pre-existing		**4/4**	
Induction			**4/4** (TNFα and/or IFNγ)
Loss			**1/4** (IL2)

PADRE-specific cytokine production of $CD4^+$ T cells expression was measured by intracellular cytokine production assay (ICC) 7 days after PADRE-specific stimulation. Bold numbers indicate the specific detection of cytokine-secreting $CD4^+$ T cells; italic numbers indicate a twofold change when compared with day −7

n.d. not determined due to lack of material

[a] Only 1 of the indicated time points was analyzed

Non-functional but more importantly also functional WT1/MUC1-reactive T cells were observed in multiple patients before but not after vaccination. We also did not detect vaccine-reactive T cells in the bone marrow, a preferential homing side of memory T cells [38]. Thus, vaccination could have induced anergy or exhaustion of pre-existing VST. Anergy and exhaustion of tumor-reactive T cells in cancer patients can occur either from chronic or suboptimal antigen stimulation or from other immunosuppressive events supported by tumor cells or their microenvironment [39]. Exhaustion, anergy or deletion of reactive T cells has also been reported after repetitive injections of high doses of peptide i.p. [40], after a single low dose of peptide s.c. [41] or after introduction of antigens to immature dendritic cells through the endocytic receptor DEC-205 in mice [42].

The differences observed in our clinical study when compared with other vaccine trials might also reflect the type of underlying disease in vaccine recipients. Regulatory mechanisms potentially active in cancer patients that might be increasingly operative in patients with AML include myeloid suppressor cells, which can secrete inhibitory cytokines [43] or depletion of tryptophan by leukemic blasts [44]. However, recent clinical phase I studies suggest that vaccination of AML patients with WT1-derived peptides can induce leukemia-reactive $CD8^+$ T cells in patients with low and high leukemia load [6–8] making it unlikely that the type or stage of disease alone were responsible for the absence of immunogenicity of the vaccine tested in our study.

Epitope dominance and competition for HLA-class I-presented antigens on dendritic cells have been suggested as a mechanism, which can impair immune responses to certain antigens [45]. However, a successful combined vaccination with WT1 and Pr3 has been reported [8] making it unlikely that the combination of antigens hampered the success of our vaccine. Furthermore, HLA-class I-presented antigens were injected at different sites in order to circumvent the competition for antigen-loaded dendritic cells.

Multifunctional T cells are generally required to control viral infections or tumors [46]. Thus, T cells need to not only produce effector cytokines, e.g., TNFα, but also other cytokines such as IL2 that are essential for promoting T cell proliferation. The inability to detect IL2 production by PADRE-specific T helper cells could have impaired the induction of $CD8^+$ T cells or even supported depletion of MUC1/WT1-reactive $CD8^+$ T cells. It has indeed been reported that IL2 production of PADRE-specific $CD4^+$ T cells is crucial to induce potent $CD8^+$ T cell responses [47], and induction of IL2-producing PADRE-specific T cells has been selectively achieved when PADRE was directly loaded onto dendritic cells. This suggests that the presentation of the delivered helper antigen by professional antigen-presenting cells was suboptimal in our clinical trial.

To improve delivery and presentation of tumor antigens, clinical trials targeting MUC1, WT1 or Pr3 have taken advantage of peptide-pulsed dendritic cells [11] or administration/production of GM-CSF [7, 8, 13], although other studies have used no additional stimuli [6]. In all these studies, MHC class I-restricted vaccine-specific T cell responses could be enhanced. In our study, the vaccine contained CpG7909, which can activate plasmacytoid dendritic cells through TLR9 [48]. Successful vaccination trials with WT1 have been reported with myeloid dendritic cells [37] and the difference in the type of dendritic cell might have indeed hampered the immunological outcome of our trial. However, CpG7909 has been also reported to increase Melan-A- or NY-ESO-1-specific $CD8^+$ T cells in combination with a peptide vaccine in patients at dose levels 0.5–2.5 mg, which is comparable to the 1 mg dose used in our trial [17, 19, 20]. At higher dose levels of 6–8 mg without providing exogenous antigens, CpG7909 has been reported to boost pre-existing $CD8^+$ T cells with specificities against various melanoma-associated antigens, to induce an activated phenotype in plasmacytoid dendritic cells, and to enhance natural killer cell cytotoxicity [18,

21]. In our clinical trial, we could not detect an increase in activated phenotype of plasmacytoid dendritic cells, which could be a consequence of the lower CpG7909 dose used in our trial. An insufficient maturation of professional antigen-presenting cells resulted most likely in a suboptimal antigen-presentation and could thereby explain the observed partial deletion of pre-existing tumor-specific T cells after vaccination [42].

A reduced $CD8^+$ T cell response has also been observed in mice after vaccination with a TLR9 agonist [49]. We observed in a similar mouse model that although the combination of MontanideISA51 and CpG7909 has no negative impact on humoral immune responses, this combination has in particular with low CpG doses a negative impact on $CD8^+$ T cell responses (unpublished results RD Weeratna, A Vicari, HL Davis). Thus, the negative impact on $CD8^+$ T cell responses observed in the current study with this adjuvant combination is most likely due to the use of a low CpG concentration (1 mg when compared with 2.5–8 mg) [17–21] with suboptimal activation of antigen-presenting cells and a subsequently weakened Th1 response.

Finally, it has also been reported that CpG7909 can induce regulatory T cells in vitro [31] and that regulatory T cells can dampen an immune response after vaccination in men [50]. We did observe in our cohort an increase in T cells with a regulatory phenotype after vaccination. However, as three out of five patients with a twofold increase in regulatory T cells also had disease progression, we cannot entirely exclude that the increase in regulatory T cells was at least partially a consequence of disease progression or other physiological changes rather than a consequence of the vaccine [51]. An increase in regulatory T cells has also been observed in other vaccination trials [52] and a successful vaccination in AML or MM patients has been associated with a decrease in regulatory T cells [53]. Thus, regardless of the underlying mechanism, the increase in T cells with a regulatory phenotype might have further hampered the success of the vaccine, particular in the context that antigen delivery to professional antigen-presenting cell may have been sub-optimal.

In summary, we demonstrate that in contrast to our intention vaccination with WT1-, Proteinase3- and MUC1-derived peptides in combination with MontanideISA51 and CpG7909 rather hampers $CD8^+$ T cell responses in men. To our knowledge all so far published phase I studies with here used antigens [7, 8, 11, 13, 36, 37] included usually small numbers of patients (10–20) and reported the induction of VST after vaccination. This might partially reflect a strong bias towards the publication of positive clinical trials but could also reflect differences in the adjuvant used in our clinical trial. In this context, we speculate that the composition of the adjuvant can be of utmost importance for the success of a vaccine, e.g., suboptimal concentrations of CpG or combinations of different adjuvants with well defined immunogenic antigens can be detrimental, need to be avoided; and recently reported superior adjuvants [7, 8, 11, 13, 36, 37] should preferentially be used. However, we cannot entirely exclude confounding factors, e.g., due to the small sample size and physiological variations in immunological analyses of small patient cohorts, which are also a substantial problem for previously published clinical trials with a positive outcome. Considering that 1/3 (57 of 147) of ongoing peptide vaccination trails currently registered at clinicaltrials.gov use Montanide and that an increasing amount of trials take advantage of CpG or the combination of CpG and Montanide, our data need to be taken into consideration for the design of ongoing and planned immunotherapy protocols.

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