

# Immunological factors influencing clinical outcome in lung cancer patients after telomerase peptide vaccination

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**Abstract** We have previously reported two trials in non-small cell lung cancer (NSCLC) evaluating vaccine therapy with the telomerase peptide GV1001. The studies demonstrated considerable differences in survival among immune responders, highlighting that an immune response is not necessarily beneficial. In the present study, we conducted long-term clinical follow-up and investigated immunological factors hypothesized to influence clinical efficacy. Peripheral blood mononuclear cells from 33 NSCLC trial patients and 15 healthy donors were analyzed by flow cytometry for T regulatory cells (Tregs, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup>FOXP3<sup>+</sup>) and two types of myeloid-derived suppressor cells (MDSCs, *HLA-DR<sup>low</sup>CD14<sup>+</sup>* or *Lin<sup>-/lo</sup>HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup>*). T cell cultures were analyzed for 17 cytokines. The results demonstrated that immune responders had increased overall survival (OS,  $p < 0.001$ ) and progression-free survival ( $p = 0.003$ ), compared to subjects without immunological response. The mean OS advantage

was 54 versus 13 months. Six patients were still alive at the last clinical update, all belonging to the immune responders. No serious toxicity had developed (maximum observation 13 years). Most patients developed a polyfunctional cytokine profile, with high IFN $\gamma$ /IL-4 and IFN $\gamma$ /IL-10 ratios. Low Treg levels were associated with improved OS ( $p = 0.037$ ) and a favorable cytokine profile, including higher IFN $\gamma$ /IL-10 ratios. High CD33<sup>+</sup> MDSC levels were associated with poorer immune response rate ( $p = 0.005$ ). The levels of CD14<sup>+</sup> MDSC were significantly higher in patients than in healthy controls ( $p = 0.012$ ). We conclude that a randomized GV1001 trial in NSCLC is warranted. The findings suggest that Tregs and MDSCs are associated with a tolerogenic cytokine milieu and impaired clinical efficacy of vaccine responses.

**Keywords** Lung cancer · Myeloid-derived suppressor cells · Regulatory T cells · Cytokines · Cancer vaccine · Survival

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## Abbreviations

MCP	Monocyte chemoattractant protein
MDSC	Myeloid-derived suppressor cell
MIP	Macrophage inflammatory protein
NSCLC	Non-small cell lung cancer
OS	Overall survival
PFS	Progression-free survival
Th	T helper
Treg	T regulatory cell

## Introduction

Lung cancer is the most prevalent form of cancer and the leading cause of cancer-related death worldwide [1]. The

finding that PD-1 blockade produces clinical responses in non-small cell lung cancer (NSCLC) [2] has yielded increased interest for cancer vaccines and their possible combination with checkpoint inhibitors [3]. GV1001 is a 16-aa telomerase peptide vaccine designed to activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells [4]. We have conducted a series of trials evaluating GV1001 therapy in patients with NSCLC, pancreatic cancer, or melanoma [5–7]. Other investigators have performed GV1001 trials in pancreatic cancer and hepatocellular carcinoma patients [8, 9].

Telomeric DNA provides stability to chromosomes [10], and normal somatic cells can undergo a limited number of cell divisions because the telomeres are shortened at each mitosis. Tumor cells reset this biological clock by expressing the enzyme telomerase that synthesizes new telomere units [11]. Telomerase is expressed in most human cancers, including lung cancer [12, 13], and is considered as an attractive target for a universal cancer vaccine [14–16].

The GV1001 peptide is recognized on multiple HLA class II molecules encoded by DP, DQ, and DR subloci [5, 7, 17, 18]. This promiscuous HLA-binding profile suggests that the GV1001 vaccine may be applicable to the general patient population and may elicit a broad T helper response within each individual. Further, GV1001 includes nested HLA class I epitopes, facilitating recruitment of CD8<sup>+</sup> cytotoxic T cells.

In the GV1001 trials, we have observed an association between immune response and survival [5–7]. However, we have also noted a considerable difference in survival within the group of immune responders. This observation highlights the issue that an immune response, though tumor specific, is not necessarily beneficial. We therefore decided to investigate the GV1001 vaccine response with regard to key immunological factors hypothesized to be important for clinical efficacy: myeloid suppressor cells, T regulatory cells, and cytokine profile.

A T helper (Th) 1-dominated cytokine profile with high levels of INF $\gamma$  and TNF $\alpha$ , but low levels of IL-4 and IL-10, is considered desirable in cancer vaccine therapy. Numerous studies have demonstrated that Th1 cytokines promote cytotoxic cellular responses and anti-tumor activity [19]. By contrast, a Th2-dominated response with high IL-4 and IL-10 levels may inhibit Th1 differentiation [19]. Regulatory T cells (Tregs) with a CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>lo</sup><sup>w/-</sup>FOXP3<sup>+</sup> phenotype have been extensively studied in animal models and are considered to counter anti-tumor immunity even in humans [20]. However, it is not known whether Treg levels can serve as biomarkers for clinical efficacy of cancer vaccination. Myeloid-derived suppressor cells (MDSCs) have also been shown to suppress immunity against tumor [21–23]. In mice, MDSCs are characterized by cell surface markers Gr1 and CD11b and have a granulocytic or monocytic morphology [22, 23]. In humans,

MDSCs constitute a diverse group of cells, the common denominator being their suppressive function and myeloid origin [21]. The most extensively studied MDSC phenotype in humans is defined as CD33<sup>+</sup>CD11b<sup>+</sup> HLA-DR<sup>-</sup> cells lacking expression of markers of mature myeloid and lymphoid cells [22, 24, 25]. Another key MDSC subset has been identified as CD14<sup>+</sup>HLA-DR<sup>low</sup> [26–28].

In the present article, we report long-term clinical data from the two GV1001 vaccine trials (CTN-2000 and CTN-2006) in NSCLC patients. We further report the results of our investigations of cytokine profiles, Tregs, and MDSCs in the vaccinated patients and how these factors relate to clinical outcome.

## Materials and methods

### Patients, protocols, and PBMC collection

Patient characteristics are given in Tables 1 and 2. The patients were included in protocols CTN-2000 or CTN-2006, which have been reported previously [6, 17]. Briefly, in the CTN-2000 protocol, 27 subjects with advanced NSCLC were vaccinated with two telomerase peptides (GV1001 and I540) [6, 17]. The majority (21/27) had stage IV disease. All CTN-2000 patients were considered incurable and had completed standard therapy before inclusion into the protocol. In the CTN-2006 trial, 23 inoperable stage III patients received radiotherapy (2 Gy  $\times$  30) and weekly docetaxel, followed by GV1001 vaccination [6]. These patients were previously untreated. The last update on clinical data was performed June 05, 2015, and is included in the present report.

In both CTN-2000 and CTN-2006, PBMCs were obtained prior to vaccination, week 6, week 10, and at later booster vaccinations. PBMCs were isolated from blood by gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway) and frozen for storage in liquid nitrogen, as previously described [29]. T cell proliferation (<sup>3</sup>H-thymidine) assays were used to determine the immune response to GV1001. T cell responses were considered Ag specific when the stimulatory index (response with Ag divided by response without Ag) was above 2. Materials and methods for the CTN-2000 and CTN-2006 trials are described in the respective trial articles [6, 17]. In the CTN-2000 trial, 13/24 evaluable subjects developed a GV1001 response. In CTN-2006, a GV1001-specific response developed in 16/20 evaluable patients.

In the extended immunological studies reported below, we included all 33 patients whose samples were available after completion of standard immune-monitoring, i.e., 33/42 patients who completed the first 6 weeks of vaccination. We mostly used patient PBMCs collected between

study months 2 and 7. In addition, 15 healthy blood donors were used as controls. These controls were not age/sex matched. The patients available for this immunological follow-up study represented 22/23 subjects in CTN-2006 and 27/30 patients with locally advanced disease (stage IIb/III). Among stage IV patients, samples were only available from 7 patients, and these were not representative for all stage IV subjects regarding clinical outcome. We therefore decided not to perform statistical analyses relating Treg/MDSC values with clinical outcome for stage IV patients.

### Ethical concerns

The trials were approved by the Norwegian Medicines Agency, the Regional Committee for Medical Research Ethics and the Hospital Review Board. Signed informed consent was obtained from all patients. The study was performed in compliance with the World Medical Association Declaration of Helsinki.

### Peptides

The vaccine peptide GV1001 (EARPALLTSRLRFIPK) was supplied by Pharmexa (Horsholm, Denmark).

### Bioplex assay

Thawed PBMCs were plated in 24-well plates ( $1.5\text{--}2 \times 10^6$  c/w) and stimulated once in vitro with the GV1001 peptide, as described earlier [5, 17]. The T cells were then restimulated in 96-well plates with irradiated PBMCs  $\pm$  GV1001 peptide. Bioplex cytokine analyses were performed on supernatants harvested 48 h after T cell stimulation, according to the manufacturer's protocol (BioRad Laboratories, Hercules, CA, USA). Supernatants were analyzed in duplicate, each parallel kept separate through T cell stimulation and Bioplex assays. Supernatant levels of IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, G-CSF, GM-CSF, INF $\gamma$ , MCP-1, MIP-1b, TNF $\alpha$  were measured. The T cells used for Bioplex assays were also tested in T cell proliferation assays, performed in triplicate as previously described [29]. Both proliferation and cytokine responses were considered Ag specific when the stimulatory index was above 2.

### Flow cytometry

#### *Analysis of HLA-DR<sup>low</sup>CD14<sup>+</sup> MDSCs*

PBMC from patient samples ( $n = 28$ ) and healthy controls ( $n = 11$ ) were obtained as described above. Thawed cells were stained with anti-CD19-APC, anti-CD14-PE (Becton Dickinson, Trondheim, Norway), anti-HLA-DR-e450

(eBioscience, San Diego, CA, USA), or e450-isotype control (eBioscience).

#### *Analysis of Lin<sup>-lo</sup>HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs (CD33<sup>+</sup> MDSCs)*

PBMCs from patient samples ( $n = 24$ ) and healthy controls ( $n = 10$ ) were obtained as described above. Thawed cells were stained with anti-lineage antibodies (anti-CD4/CD8/CD19/CD57-e450, eBioscience) and anti-CD14-PerCP-Cy5 (eBioscience). The samples were further stained with either anti-HLA-DR-FITC, anti-CD33-APC, and anti-CD11b (Becton Dickinson) or FITC-isotype, APC-isotype, and PE-isotype (Becton Dickinson).

#### *Analysis of CD4<sup>+</sup>CD127<sup>low</sup>Foxp3<sup>high</sup>CD25<sup>high</sup> regulatory T cells*

PBMCs from patients samples ( $n = 31$ ) were obtained as described above. Thawed cells were stained with anti-CD4-e450 (eBioscience), anti-CD25-AF700 (BioLegend, San Diego, CA, USA), anti-CD127-PE, and anti-FoxP3-AF488 (Becton Dickinson). Isotype controls were stained with anti-CD4-e450 (eBioscience), PE-isotype, AF488-isotype (Becton Dickinson), and AF700-isotype (BioLegend). Intracellular staining with anti-FoxP3 or AF488-isotype was done using Foxp3 Staining Buffer Set (eBioscience).

The flow cytometry was performed using a BD LSRII flow cytometer and FlowJo software. The quantities of MDSCs or Tregs were calculated as percentage of total PBMCs or CD4<sup>+</sup> T cells, respectively.

### Statistical analysis

#### *Descriptive*

Data were described with proportions (percentage) for categorical data and with median, mean, and range for continuous data.

#### *Univariate analyses*

Mann–Whitney *U* test was used for comparing patients and healthy controls with regard to distribution of Lin-CD33<sup>+</sup> MDSCs or HLA-DR<sup>low</sup>CD14<sup>+</sup> MDSCs.

#### *Survival analyses*

Overall survival (OS) was defined as time from the start of vaccination to death or end of study (June 05, 2015), whichever came first. Progression-free survival (PFS) was

defined as time from the start of vaccination to progression, death, or end of study.

OS and PFS were analyzed with Kaplan–Meier analysis and compared between immune responders and non-responders using log-rank test. In accordance with the criteria in protocols CTN-2000 and CTN-2006, the patients were classified as immune responders if and only if they exhibited a GV1001-specific T cell response in proliferation assays with a stimulatory index above 2. Kaplan–Meier analyses with log-rank tests were also

performed to compare PFS between the patients with high versus low levels of Tregs or MDSCs. For this purpose, the patients were dichotomized into a “high” and “low” group for Tregs or MDSC, based on the median Treg or MDSC level as cutoff.

All tests were two sided. We consider this study an exploratory analysis. Therefore, we did not perform any correction for multiple testing, and  $p$  values  $<0.05$  were considered statistically significant. All analyses were performed using SPSS, version 18 (SPSS Inc., USA).

**Table 1** CTN-2000

Patient <sup>a</sup>	Age	Sex	Stage	Tumor response <sup>b</sup>	Immune response <sup>c</sup>	Progression-free survival (months)	Survival (months)	% Tregs	% CD14 <sup>+</sup> MDSC	% CD33 <sup>+</sup> MDSC
701	60	M	IIIB	SD	–	10	11	ns <sup>f</sup>	ns	ns
702	69	M	IV	PD	NE	<3	4	ns	ns	ns
703	68	F	IV	PD	+	<3	15	7.07	0.03	0.58
704	60	F	IV	SD	+	8	18	ns	ns	ns
706	60	M	IV	PD	+	<3	3	ns	ns	ns
707	59	M	IV	PD	–	<3	3	ns	ns	ns
708	39	M	IV	SD	+	4	7	10.13	ns	ns
709	63	F	IV	PD	–	<3	3	ns	ns	ns
710	55	M	IIIA	CR <sup>d</sup>	+	>141 <sup>d</sup>	>159 <sup>Alive</sup>	3.98	0.27	0.25
711	48	M	IV	PD	+	<3	25	2.97	ns	ns
712	49	F	IV	SD	+	16	71	3.11	0.04	0.72
713	76	F	IIIB	PD	+	<3	19	ns	ns	ns
714	68	M	IV	PD	+	<3	32	4.01	0.08	1.00
715	45	F	IV	PD	NE	<3	5	ns	ns	ns
716	72	M	IV	PD	–	<3	2	ns	ns	ns
717	56	M	IV	PD	–	<3	3	ns	ns	ns
718	57	F	IV	PD	–	<3	4	ns	ns	ns
719	44	M	IV	PD	–	<3	4	ns	ns	ns
720	57	F	IV	NE	NE	<3	9	ns	ns	ns
721	63	F	IV	PD	+	<3	6	4.27	ns	ns
722	60	F	IV	PD	–	<3	4	ns	ns	ns
723	53	M	IV	PD	+	<3	17	3.84	0.05	ns
724	60	F	IV	PD	–	<3	10	3.15	0.6	1.05
725	55	F	IIB	SD	+	19	46	4.1	0.81	0.66
726	49	M	IV	PD	–	<3	4	ns	ns	ns
727	57	M	IIIB	NED <sup>e</sup>	+	>144 <sup>e</sup>	>144 <sup>Alive</sup>	ns	2.03	0.92
728	50	M	IIIB	PD	–	<3	18	8.66	1.57	1.62

CR complete response, SD stable disease, PD progressive disease, NE not evaluable, NED no evidence of disease

<sup>a</sup> 12/26 patients did not complete the vaccination period (8 vaccines) prior to first clinical evaluation at week 12, due to early disease progression. Patient 728 was enrolled after the scheduled inclusion period and was not included in the trial paper

<sup>b</sup> Best tumor response after vaccination (RECIST)

<sup>c</sup> GV1001-specific immune response, as demonstrated in T cell assays ( $n = 10$ ) and/or delayed-type hypersensitivity skin reactions ( $n = 9$ )

<sup>d</sup> Patient 710 had a durable complete response at last clinical follow-up (month 141) and is still alive per June 05, 2015 (month 159)

<sup>e</sup> Patient 727 has residual fibrosis, with no detectable disease (144 months of observation)

<sup>f</sup> ns = no samples available for testing

## Results

### Long-term clinical follow-up

Tables 1 and 2 show patient characteristics, immune responses, and updated clinical data (June 2015) for CTN-2000 and CTN-2006, respectively. The long-term survivors have been continuously followed at the study hospitals. Serious adverse events have not been observed in either trial. The patients received no further cancer therapy until disease progression. For most CTN-2000 subjects, further therapy was not warranted even after progression, as they had already completed standard therapy. No study subjects from either protocol have received immunological checkpoint inhibitors.

Two patients from the CTN-2000 study are still alive, 12 and 13 years after the start of vaccination, respectively. Neither of them has evidence of cancer relapse. From the CTN-2006 protocol, four patients are still alive, all without evidence of relapse (observation time 7–8 years). All six survivors in the two protocols belong to the immunological vaccine responders and have retained durable

GV1001-specific T cell responses as far as monitored. None of the patients have developed evidence of adverse effects. The six survivors have not received any additional NSCLC therapy.

### Immune responders had prolonged survival

We observed GV1001-specific T cell responses in 29/45 evaluable subjects in the two protocols taken together [17]. Kaplan–Meier/log-rank analyses on the updated data (June 2015) confirmed our previous observation that immune responders, compared to non-responders, had increased progression-free survival (PFS) and overall survival (OS). All patients tested for immune response were included in the analyses. The OS and PFS advantages applied to both protocol CTN-2000 and CTN-2006 (Tables 1 and 2), though statistically significant only for CTN-2000 when the protocols were analyzed separately (OS<sub>CTN2000</sub>  $p < 0.001$ , PFS<sub>CTN2000</sub>  $p = 0.026$ , OS<sub>CTN2006</sub>  $p = 0.10$ , PFS<sub>CTN2006</sub>  $p = 0.08$ ). Taking both trials together, log-rank test demonstrated a statistically significant advantage for immune responders, both for PFS ( $p = 0.003$ ) and for

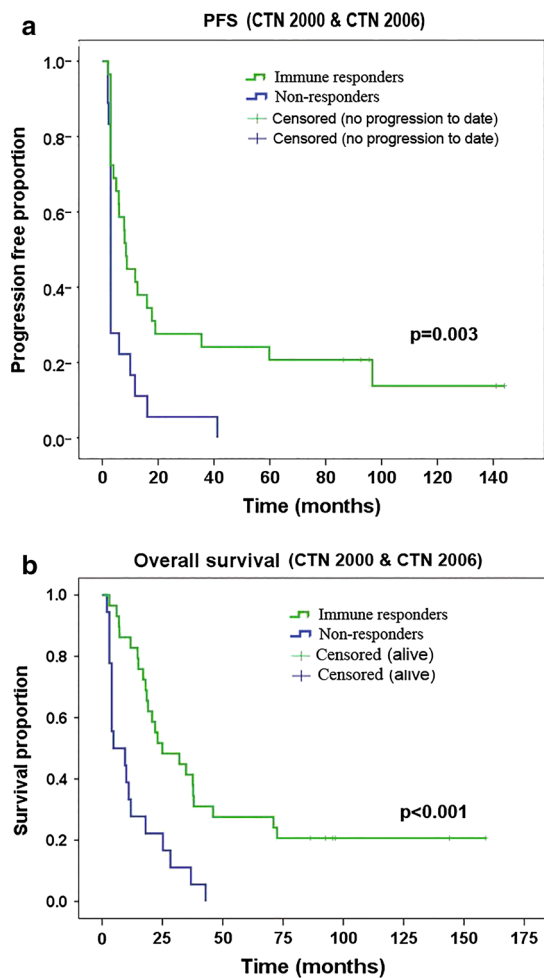
**Table 2** CTN-2006

Patient	Age	Sex	T cell response	Relapse-free survival (days)	Survival (days)	% Tregs	% CD14 <sup>+</sup> MDSC	% CD33 <sup>+</sup> MDSC
101	66	M	POS	359	1147	4.36	ns	ns
102	62	M	NEG	70	288	5.26	0.13	4.74
104	57	F	POS	238	705	6.38	0.13	1.99
105	50	M	POS	267	630	7.25	0.92	1.44
106	53	M	POS	179	446	5.16	0.19	1.00
107	57	F	POS	>2818 <sup>b</sup>	>2818 <sup>Alive</sup>	4.82	0.04	ns
108	54	M	POS	151	220	6.12	2.49	0.88
110 <sup>a</sup>	49	F	NEG <sup>a</sup>	63	144	ns <sup>c</sup>	ns	ns
111	59	M	POS	>1819 <sup>b</sup>	2204	5.32	0.07	ns
113	63	F	POS	63	564	4.91	0.03	0.51
115	58	F	POS	259	1059	5.54	0.15	0.17
117	52	M	NEG	357	860	10.80	0.22	10.94
202	63	F	NEG	1251	1304	5.32	0.06	5.03
203 <sup>a</sup>	74	M	NEG <sup>a</sup>	93	1120	3.45	0.02	3.36
204 <sup>a</sup>	63	M	NEG <sup>a</sup>	491	766	ns	ns	ns
205	60	F	POS	382	667	7.99	0.11	1.76
301	70	F	NEG	182	361	ns	0.11	3.38
302	51	M	POS	>2944 <sup>b</sup>	>2944 <sup>Alive</sup>	3.37	0.26	0.68
303	78	M	POS	>2909 <sup>b</sup>	>2909 <sup>Alive</sup>	11.30	0.01	ns
304	56	M	POS	1079	1143	2.69	0.04	ns
305	58	M	POS	540	1155	3.35	ns	0.70
306	69	F	POS	183	360	5.17	0.25	0.62
307	46	F	POS	>2622 <sup>b</sup>	>2622 <sup>Alive</sup>	3.24	0.08	1.01

<sup>a</sup> Not evaluable per protocol (included in intention-to-treat population)

<sup>b</sup> No relapse per June 05, 2015

<sup>c</sup> ns = no samples available for testing



**Fig. 1** Survival versus immune response. Kaplan–Meier analysis comparing progression-free survival (PFS, **a**) or overall survival (**b**) between patients with/without a GV1001-specific T cell response. Log-rank  $p$  values are displayed

OS ( $p < 0.001$ ) (Fig. 1). The mean PFS advantage was 35 versus 6.7 months, and mean OS advantage was 54 versus 13 months. We also performed Kaplan–Meier analysis on all subjects with locally advanced disease ( $n = 29$ ). Again, immune responders had significantly extended PFS ( $p = 0.02$ ) and OS ( $p = 0.01$ ). There was a 4.6-fold increase in mean PFS (50 vs. 11 months) and a 3.3-fold OS advantage (69 vs. 21 months).

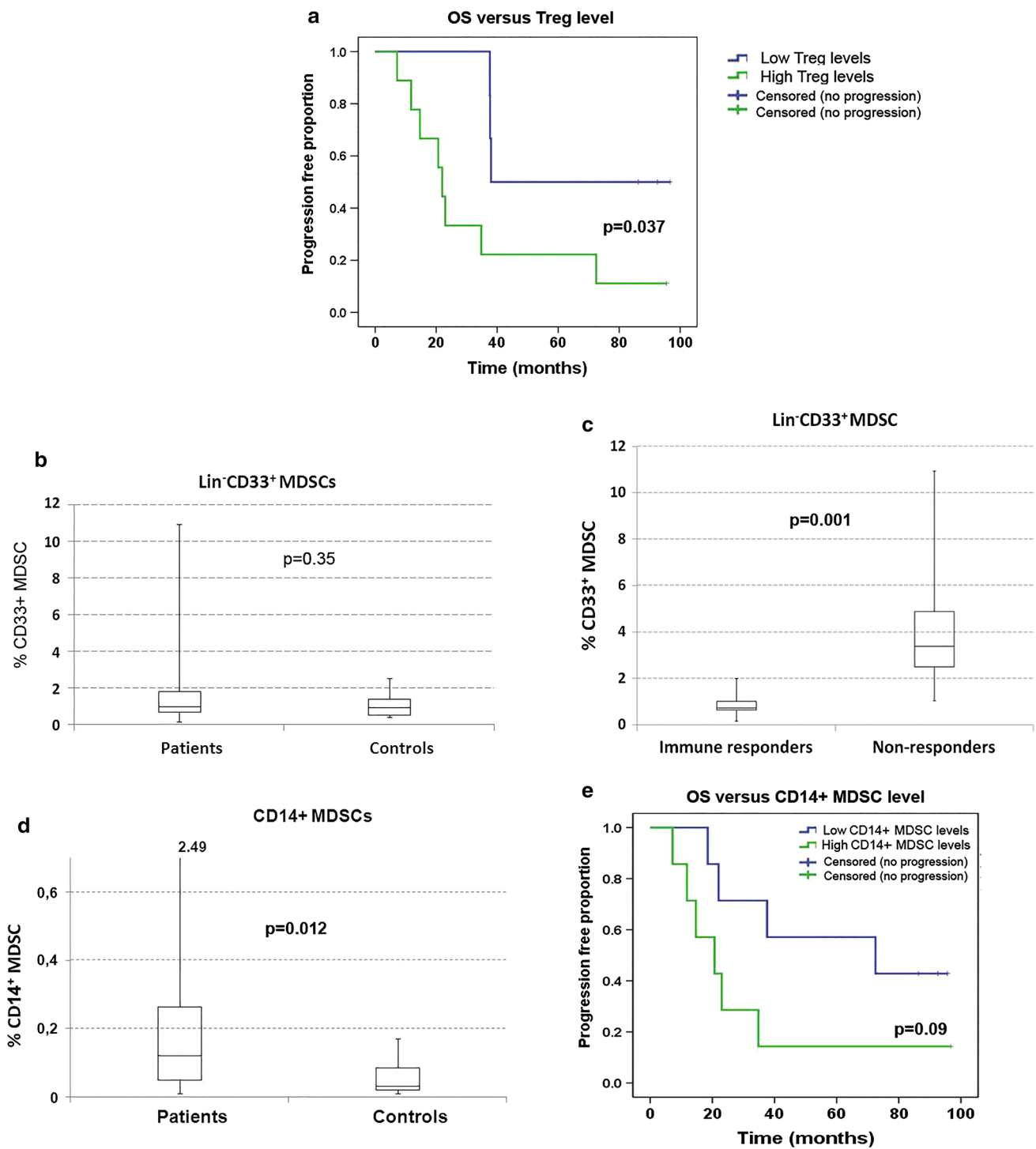
The observed association between immune response and clinical outcome was most evident for the “tail” of the PFS/OS curves (Fig. 1), representing long-term survivors. All six patients still alive were immune responders. On the other hand, a considerable subpopulation of immune responders had short survival. This variability points to the need to study factors that influence whether an immune response translates to clinical benefit. In the studies of Tregs and MDSCs reported below, we investigated whether these regulatory cells represent biomarkers of clinical outcome.

**Fig. 2 a** Treg level versus survival. PBMCs from 31 GV1001 trial patients were analyzed by flow cytometry for T regulatory cells (Tregs,  $CD4^+CD25^+CD127^{low/-}FOXP3^+$ ), and the percentage of Tregs in  $CD4^+$  T cells was calculated. The patients were dichotomized into a  $Treg^{high}$  and a  $Treg^{low}$  group, based on the median Treg percentage as cutoff. Overall survival (OS) for CTN-2006 immune responders was compared between the  $Treg^{high}$  and  $Treg^{low}$  groups by Kaplan–Meier analysis. The log-rank  $p$  value is displayed. **b**  $CD33^+$  MDSC levels in NSCLC patients compared to healthy controls. PBMCs from 24 trial patients plus 10 healthy controls were analyzed by flow cytometry for  $CD33^+$  MDSCs ( $Lin^{-/lo}HLA-DR^+CD33^+CD11b^+$ ). The percentage of  $CD33^+$  MDSCs in PBMCs was calculated. The  $CD33^+$  MDSC levels were not increased in NSCLC patients compared to healthy controls (median 1.0 versus 0.95 %,  $p = 0.35$ , Mann–Whitney  $U$  test). **c**  $CD33^+$  MDSC levels versus immune response. The figure shows the  $CD33^+$  MDSC percentage in PBMCs among immune responders and non-immune responders, indicating median (line), 25/75 % quartiles (boxes), and range. The level of  $CD33^+$  MDSCs was significantly lower among immune responders (Mann–Whitney  $U$  test,  $p = 0.001$ ). **d**  $CD14^+$  MDSC levels in NSCLC patients compared to healthy controls. PBMCs from 28 trial patients and 11 healthy controls were analyzed for the presence of  $CD14^+$  MDSCs by flow cytometry. The percentage of  $CD14^+$  MDSCs in PBMCs was calculated. The  $CD14^+$  MDSC levels were increased in NSCLC patients compared to healthy controls ( $p = 0.012$ , Mann–Whitney  $U$  test). **e**  $CD14^+$  MDSC level versus survival. The patients were divided into a  $CD14^+$  MDSC<sup>high</sup> and a  $CD14^+$  MDSC<sup>low</sup> level group, based on the median value as cutoff. Overall survival (OS) for CTN-2006 immune responders was compared between the MDSC<sup>high</sup> and MDSC<sup>low</sup> level groups by Kaplan–Meier analysis. Log-rank  $p$  value is displayed

### Immune responders with low Treg levels recorded prolonged PFS

Regulatory T cells are hypothesized to impair the development of anti-cancer vaccine responses and to counter their clinical effectiveness, but there is sparse evidence from clinical studies. Here, we performed Treg flow cytometry analysis on PBMCs from all 31 patients with available samples. Tregs were defined as  $CD4^+CD127^{low}Foxp3^{high}CD25^{high}$  cells (Suppl Fig. 1). The patients were divided into a  $Treg^{high}$  or  $Treg^{low}$  group, using as cutoff the median Treg value (4.91 %) for all 31 patients. There was no significant difference between the  $Treg^{low}$  and  $Treg^{high}$  group with regard to the frequency of immune responders.

We addressed whether Tregs may counter the clinical effectiveness of vaccine responses by comparing OS and PFS for the two Treg groups. The statistical analysis was performed on the CTN-2006 population, as expected survival was for other reasons (stage and pre-treatment) substantially different in CTN-2000 patients. Interestingly, immune responders with low Treg levels recorded significantly increased OS ( $p = 0.037$ , Fig. 2a) and PFS ( $p = 0.046$ ). The median PFS for immune responders in CTN-2006 was 61 months in the  $Treg^{low}$  group compared to 8.5 months in the  $Treg^{high}$  group.



**Lin<sup>-</sup>CD33<sup>+</sup> MDSCs were associated with immune response rate**

To determine the level of Lin<sup>-</sup>HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs (CD33<sup>+</sup> MDSCs), PBMCs from NSCLC patients (*n* = 24) and healthy controls (*n* = 10) were stained for expression of CD14, HLA-DR, CD33, CD11b, and lineage markers (CD4/CD8/CD19/CD57). The gating strategy is

shown in Suppl. Figure 2. We found no significant difference between NSCLC patients and healthy controls in levels of CD33<sup>+</sup> MDSCs (Fig. 2b).

Next, we investigated whether levels of CD33<sup>+</sup> MDSCs were associated with PFS or immune response rate. The patients were divided into a high or a low CD33<sup>+</sup> MDSC level group using the median value (1.00 %). We observed no association between PFS and CD33<sup>+</sup> MDSC level

(not shown). However, there was a striking difference in immune response rate between the CD33<sup>+</sup> MDSC<sup>low</sup> and the CD33<sup>+</sup> MDSC<sup>high</sup> group. Whereas all (12/12) CD33<sup>+</sup> MDSC<sup>low</sup> patients were immune responders, only 5/12 CD33<sup>+</sup> MDSC<sup>high</sup> patients developed an immune response ( $p = 0.005$ , Fisher's exact test). Figure 2c shows the recorded CD33<sup>+</sup> MDSC distribution among immune responders and non-responders.

### CD14<sup>+</sup> MDSCs were present only in NSCLC patients and associated with poorer PFS

To investigate the levels of HLA-DR<sup>low</sup>CD14<sup>+</sup> MDSCs (CD14<sup>+</sup> MDSCs), we stained PBMCs from 28 NSCLC patients and 11 healthy controls (Suppl. Figure 3). The measured values among patients ranged from 0.01 to 2.49 % (median 0.12), compared to 0.01–0.17 for healthy controls (median 0.03) (Fig. 2d). This difference was statistically significant (Mann–Whitney  $U$  test,  $p = 0.012$ ). Finally, we asked whether the level of CD14<sup>+</sup> MDSCs may influence clinical outcome among immunological vaccine responders. To address this question, we used the median CD14<sup>+</sup> MDSC level as cutoff and compared OS and PFS for CTN-2006 immune responders with low versus high CD14<sup>+</sup> MDSC levels. The median PFS was 60 versus 7.8 months, in favor of the group with low CD14<sup>+</sup> MDSC levels ( $p = 0.08$ ). Similarly, OS was extended in the CD14<sup>+</sup> MDSC<sup>low</sup> group (median 73 versus 21 months, Fig. 2e).

### Polyfunctional cytokine profiles with high IFN $\gamma$ /IL-4 and IFN $\gamma$ /IL-10 ratios

Samples from 15 NSCLC patients (8 from CTN-2006 and 7 from CTN-2000) were tested in Bioplex cytokine assays. These patients represented all immune responders with available samples for Bioplex assays. We demonstrated a GV1001-specific cytokine response in 15/15 subjects (stimulatory index  $>2$ , Suppl. Table 1). Moreover, GV1001-specific T cell proliferation was observed for all samples in parallel <sup>3</sup>H-thymidine assays (stimulatory index  $>2$ , data not shown).

A polyfunctional GV1001-specific cytokine pattern was detected for most patients (Fig. 3a). Applying an arbitrary cutoff value of 30 pg/ml, a majority of patients showed substantial secretion of at least 7 cytokines. There is an increasing evidence that Th17-cells, secreting IL-17, is a key player in shaping immune responses and may enhance anti-tumor activity [30, 31]. Here, we demonstrated secretion of IL-17 in cultures from 9/15 patients (Fig. 3b).

The Th1/Th2 cytokine pattern for a representative patient is shown in Fig. 3c. In general, the levels of Th1 cytokines IFN $\gamma$  and TNF $\alpha$  were high, while levels of IL-4 and IL-10 were low. As shown in Fig. 3d, e, the IFN $\gamma$ /

**Fig. 3** Cytokine profile. The cytokine profile of T cell cultures from 15 NSCLC patients was determined in Bioplex assays. T cells were stimulated with irradiated PBMCs  $\pm$  peptide GV1001. Supernatants were harvested after 48 h and analyzed as duplicates in Bioplex assays, measuring 17 cytokines. The duplicates were kept separate from start of T cell stimulation and throughout the Bioplex assays. **a** Polyfunctionality. The sectors represent the fraction of patients producing the given number of cytokines, at concentrations above the chosen cutoff value ( $>30$  pg/ml). **b** IL-17 secretion was demonstrated in cultures from 9/15 patients, at concentrations above the chosen cutoff value ( $>30$  pg/ml). Columns represent mean concentration (pg/ml) from duplicates. **c** Mixed Th1/Th2 cytokine profile (patient 307). The diagram depicts a representative cytokine pattern, with high levels of IFN $\gamma$  (Th1), TNF $\alpha$  (Th1), IL-5 (Th2), and IL-13 (Th2), amid low levels of IL-4 (Th2) and IL-10 (Tr1/Th2). Columns represent mean concentration (pg/ml) from duplicates. **d–g** Secretion of IFN $\gamma$ , IL-4, IL-10, IL-5, and IL-13. T cells from all patients tested secreted IL-5 and IL-13, while IFN $\gamma$  was detected for all except patient 205. Most patients showed high IFN $\gamma$ /IL-4 and IFN $\gamma$ /IL-10 ratios (**d**, **e**). The levels of Th2 cytokines IL-5 (**f**) and IL-13 (**g**) were still generally high, suggesting that the cytokine patterns did not follow a Th1/Th2 delineation. Patient 205 was the only subject not secreting IFN $\gamma$ , IL-4, or IL-10

IL-4 and IFN $\gamma$ /IL-10 ratios were high across both protocols. This pattern may suggest a favorable balance between immunity and tolerance. The high IFN $\gamma$ /IL-4 ratios, however, did not reflect a classic Th1 profile as the key Th2 cytokines IL-5 and IL-13 were both high (Fig. 3f, g). We have previously observed a similar breach of Th1/Th2 delineation at a clonal level [18, 32].

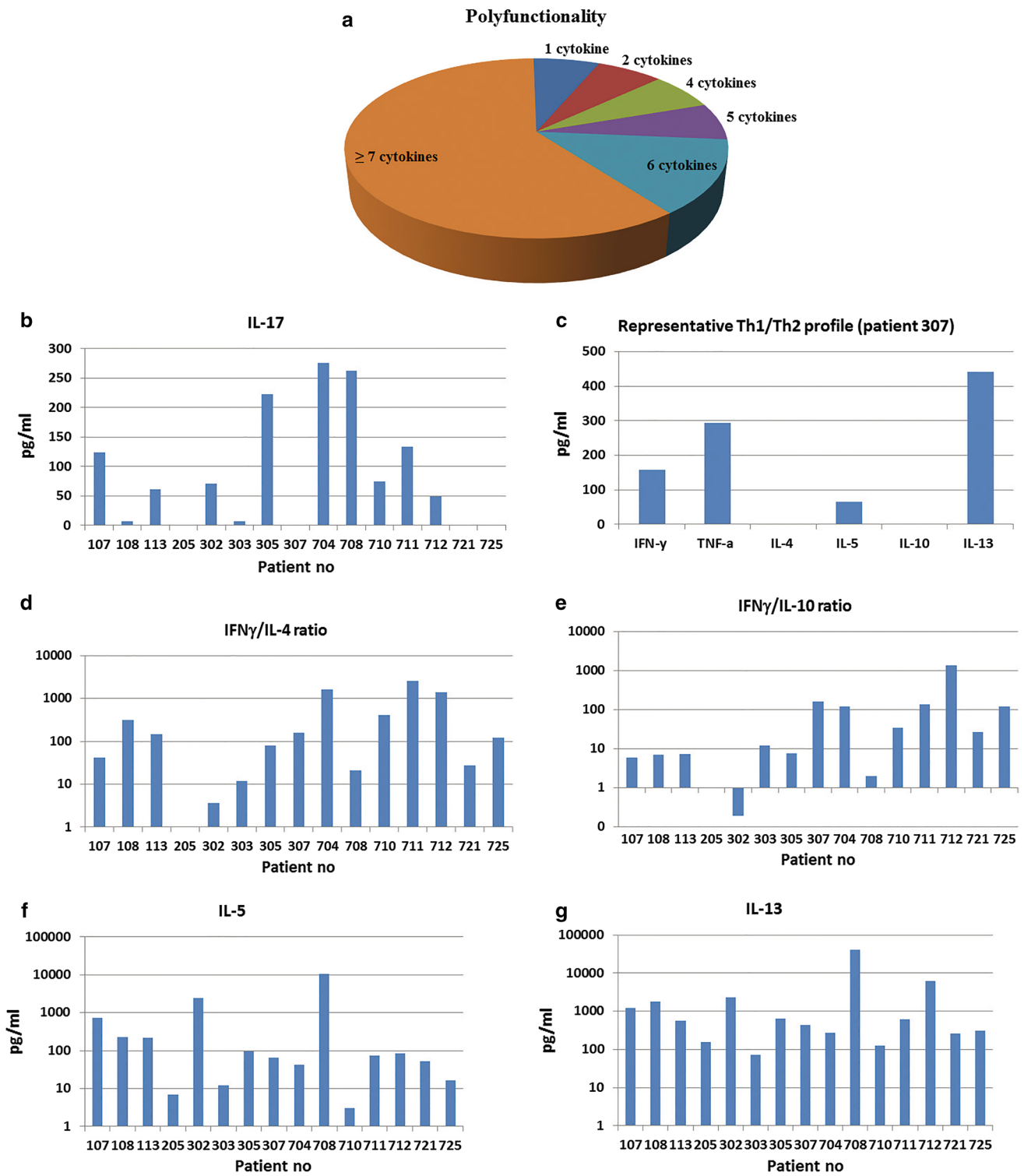
### Patients with low Treg levels recorded higher IFN $\gamma$ /IL-10-ratios

IL-10 is known to be secreted by tolerogenic cells including type 2 macrophages and T regulatory type 1 (Tr1) cells and is considered to promote tumor tolerance [33]. We hypothesized that high levels of Foxp3<sup>+</sup> Tregs, presumably reflecting a tolerogenic environment, may correlate with elevated IL-10 levels as compared to IFN $\gamma$ . This hypothesis was assessed by comparing the Treg<sup>high</sup> and Treg<sup>low</sup> groups (defined above) with regard to IFN $\gamma$ /IL-10 ratio ( $n = 13$ ). Interestingly, we found that the Treg<sup>high</sup> group had markedly decreased IFN $\gamma$ /IL-10 ratios (Fig. 4a). We next asked whether the Treg<sup>low</sup> group had a more Th1-driven response. Indeed, the IFN $\gamma$ /IL-5 and IFN $\gamma$ /IL-13 ratios were substantially higher in the Treg<sup>low</sup> group, suggesting a more Th1-weighted pattern (Fig. 4b). The mean IFN $\gamma$ /IL-5 ratio was increased 16-fold (22 vs. 1.33).

### CTN-2000 patients had a more Th1 - weighted cytokine profile compared to CTN-2006

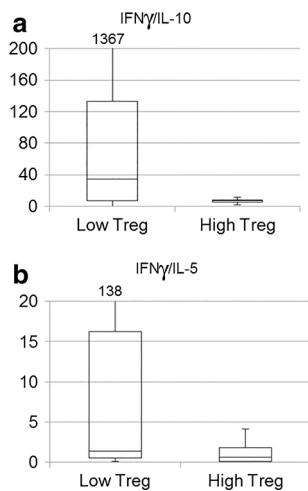
The patients in protocol CTN-2006 differed considerably from protocol CTN-2000 in terms of disease stage and prior treatment. Further, the CTN-2006 group received vaccination as adjuvant therapy, at a point where the immune





system would be strongly influenced after 6 weeks of chemoradiation. We investigated whether these differences resulted in different cytokine profiles between patients in the CTN-2000 versus the CTN-2006 protocol. The results showed that the levels of IFN $\gamma$  as well as the IFN $\gamma$ /IL-13

and IFN $\gamma$ /IL-5 ratios were considerably higher in CTN-2000, suggesting a more Th1-like profile (Suppl. Figure 4). This observation may reflect how chemoradiation influences the cytokine profile, but needs to be confirmed in a new study.



**Fig. 4** Treg level versus cytokine profile. The cytokine profile of samples from 15 NSCLC patients was determined in Bioplex assays. Their Treg levels were determined by flow cytometry analysis (Tregs: CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>low/-</sup>FOXP3<sup>+</sup>). The patients were dichotomized into a Treg<sup>high</sup> and a Treg<sup>low</sup> group, based on the median Treg value as cutoff. The *box plots* in **a** and **b** indicate median (*line*), 25/75 % quartiles (*boxes*), and range. **a** Lower Treg levels in patients with high IFN $\gamma$ /IL-10 ratios. The Treg<sup>high</sup> group had decreased IFN $\gamma$ /IL-10 ratios compared to the Treg<sup>low</sup> group (median 7.0 vs 34.4). **b** Th1-like response in Treg<sup>low</sup> group. The IFN $\gamma$ /IL-5 (**b**) and IFN $\gamma$ /IL-13 (not shown) ratios were higher in the Treg<sup>low</sup> group, suggesting a more Th1-weighted pattern

## Discussion

We observed a statistically significant association between survival and immune response in the GV1001 trials. We also noted a considerable diversity in survival within the group of immune responders and investigated what separates the clinically useful from the pointless immune response. This question is as challenging as it is important. Cytokine profiles, Tregs, and MDSCs may be decisive factors. However, it has proven difficult to establish whether the clear effects of these factors reported in animal models apply in a complex human setting. Further, we performed in-depth cytokine profiling of the GV1001 response. The results support the notion that high levels of Tregs and MDSCs impair cancer vaccine responses and suggest that these cell types are associated with lower IFN $\gamma$ /IL-10 ratios and reduced progression-free survival among immune responders.

A randomized trial would be needed to determine whether the superior survival for immune responders is caused by the vaccine, but the observed strong association is of interest. The clinical follow-up data reported herein suggest that the OS and PFS advantages for immune responders are maintained long term, without development of side effects. One may argue that patients with less advanced disease may be more likely to respond

immunologically to vaccination. However, the survival advantage appeared not to be explained by disease stage and held firm in separate analyses on stage III patients. These observations support the rationale for further development of the GV1001 vaccine.

Increased levels of Tregs are found in many cancer types [20, 34], including NSCLC [35, 36]. The detrimental effect of increased numbers of Tregs is supported in this study. We find that immune responders with low levels of Foxp3<sup>+</sup> Tregs have increased PFS and a more favorable cytokine profile, including higher IFN $\gamma$ /IL-10 ratios, compared to those with high Treg levels. These findings are in line with compelling evidence from animal studies indicating that Tregs counter anti-tumor immune responses, as well as some clinical studies suggesting that increased Treg levels are negative prognostic factors [20, 37, 38].

In the CTN-2006 trial, we combined vaccination with chemoradiation. The observations in our study may reflect that a Treg-/IL-10-dominated tumor milieu counters the efficacy of T cell responses induced by chemoradiation. This hypothesis is in line with current understanding of immunogenic cell death [33] and of particular relevance for regimes combining vaccination with chemotherapy or radiation. Our data further suggest that Treg levels influence the quality of the immune response, but not the frequency of immune responders. Possibly, only patients with low Treg levels are able to benefit from a GV1001 vaccine response. This represents a possible explanation as to why some immune responders experience long-term survival, while others do not. The mechanism may be related to epitope spreading. We have previously observed that long-term survivors after GV1001 vaccination exhibit increased epitope spreading compared to immune responders without evidence of clinical benefit [39].

We observed differences in cytokine profiles both between patients with different Treg levels and between the CTN-2000 and CTN-2006 protocols. The limited number of patients did not allow for statistical analysis of whether these factors were independent or influenced each other. This point illustrates the exploratory nature of our study and the need to confirm the findings in new studies.

There is increasing evidence that MDSCs are potent suppressors of anti-tumor T cell responses. The phenotypes of these cells are heterogenous in humans. We evaluated two categories of MDSCs considered to be highly suppressive of human anti-tumor responses [22, 24, 26–28]. The levels of Lin<sup>-/lo</sup>HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs have been reported to be increased in many forms of cancer [25, 40], including one study of NSCLC [41]. However, we found no significant increase in NSCLC patients, compared to healthy controls. Moreover, the levels did not correlate with progression-free survival. We did not have sufficient material to perform functional studies with sorted MDSCs, and

circulating levels of MDSCs may not reflect the MDSCs activity in lymph nodes and tumor. The results should therefore be interpreted with caution. However, biomarker analyses in clinical practice can hardly rely on functional assays or tumor-derived MDSCs. Hence, our data suggest that Lin<sup>-lo</sup>HLA-DR<sup>-</sup>CD33<sup>+</sup>CD11b<sup>+</sup> MDSCs may not be a suitable biomarker in NSCLC.

We identify substantial levels of HLA-DR<sup>low</sup>CD14<sup>+</sup> MDSCs only in NSCLC patients. Cells with this phenotype have also been detected in cancer patients in other studies [26–28, 38]. In our study, the minimal levels of such cells (0.01–0.17 %) in healthy controls suggest that the HLA-DR<sup>low</sup>CD14<sup>+</sup> cells are associated with NSCLC development and may be a candidate biomarker. Our observation that OS and PFS were inferior in subjects with HLA-DR<sup>low</sup>CD14<sup>+</sup> MDSCs is in line with other studies, including data from NSCLC patients who had received chemotherapy [42] and the IMA901 peptide vaccine trial in renal carcinoma patients [38]. Interestingly, an experimental agent for countering MDSC has been reported to enhance the immune response after vaccination in NSCLC patients [43].

Cancer vaccines do not enter a virgin environment but need to overcome established tumor tolerance. Compelling evidence from *in vitro* studies and animal models suggests that Foxp3<sup>+</sup> Tregs interact with other regulatory cell types, including tolerogenic DCs, type 2 macrophages, and Tr1 cells [20]. This network theory is supported by our observation that patients with low levels of Foxp3<sup>+</sup> Tregs develop vaccine responses with decreased IFN $\gamma$ /IL-10 ratios. Moreover, the association with lower IFN $\gamma$ /IL-5 and IFN $\gamma$ /IL-13 ratios supports the notion that a Treg-/Tr1-driven environment counters Th1 differentiation, yielding a muted and more Th2-like response (Fig. 4). Our data support the notion that GV1001 and other cancer vaccines may need to be combined with drugs countering established tolerance. We have previously observed that GV1001 can safely be combined with temozolomide [7], which has been shown to reduce Treg levels in several studies [44]. Regarding the clinical importance of Tregs, it is of interest that the melanoma breakthrough drug ipilimumab, which was until recently considered mainly to work on effector T cells, has been shown to efficiently deplete Tregs from tumor [45, 46].

The Th1/Th2 paradigm was established based on elegant studies in mouse models [47] and has led to widespread use of IFN $\gamma$ /IL-4 assays to determine a Th1 or Th2 profile. Our data from the present and previous studies suggest that this practice may be misleading, as an IFN $\gamma$ /IL-4 response may include high levels of other Th2 cytokines (IL-5, IL-13) as well as IL-10. Here, we have demonstrated this in T cell bulk cultures. Previously, we have found this in T cell

clones [18, 32]. In our experience, the breach of Th1/Th2 dichotomy is observed across different cancer forms, disease stages, and vaccine strategies [7, 18, 48]. These observations suggest that it may be wise to avoid Th1/Th2-labels and rather focus on polyfunctionality or the ratio between key cytokines, like IFN $\gamma$ /IL-10.

The GV1001 cancer vaccine has been approved in Korea for treatment of pancreatic cancer (personal communication, Dr. M Kim, CEO, Kael-Gemvax). A randomized GV1001 trial in NSCLC patients has been prepared (NCT01579188). Such a trial may present an opportunity to follow up the leads from the present study, in particular by comparing cytokine patterns and levels of Tregs and MDSCs with clinical outcome. The extensive cytokine panel used in the present study was useful for our purpose, but in a large-scale trial, it is important to limit the panel. Based on the close association between IL-5 and IL-13, one may omit one of these cytokines. It is probably more important to retain IL-17 and chemokines like MIP-1a and MIP-1b to assess polyfunctionality. The polyfunctional profile observed in most patient samples in the present study may be beneficial, as suggested from animal studies and data from humans with HIV and other chronic infections [49, 50]. We have not studied the cytokine pattern at single-cell level, but have previously observed that the polyfunctionality is retained in GV1001-specific T cell clones confirmed to be monoclonal by TCR mapping [18].

We conclude that a randomized GV1001 trial in NSCLC patients is warranted, based on a high immune response rate, favorable cytokine profiles, low toxicity after long-term observation, and superior survival for immune responders. The development of effective cancer vaccines depends on identifying factors influencing whether an immune response is clinically effective. Putative biomarkers like cytokine profiles, Tregs, or MDSCs can be addressed one by one in elegant animal studies, but in patients, the role of each factor is difficult to clarify. Because large patient cohorts are rarely available, we consider it important to perform in-depth studies of material from small-scale trials and accumulate data from different studies. The present investigation contributes to this collective bank of knowledge and provides leads as to which factors may be important. Our findings need to be confirmed in new patient cohorts, but suggest that Tregs, CD33<sup>+</sup> MDSCs, and CD14<sup>+</sup> MDSCs may represent biomarkers for prognosis after cancer vaccination and serve to identify patients not benefitting from vaccine therapy. The results also support the notion that vaccine responses with an IFN $\gamma$ <sup>high</sup>/IL-10<sup>low</sup>/IL-4<sup>low</sup> and polyfunctional cytokine profile may counter cancer. Finally, the data indicate that HLA-DR<sup>low</sup>CD14<sup>+</sup> MDSCs are associated with development of NSCLC.

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#### Compliance with ethical standards

**Conflict of interest** J.A. Kyte has received research support (≈10,000 Euro) from Kael-Gemvax. G. Gaudernack is a member of Kael-Gemvax advisory board. The other authors declare that they have no conflict of interest.

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