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Clinical Trial Results

A Phase I Open-Label Clinical Trial Evaluating the Therapeutic Vaccine hVEGF26–104/RFASE in Patients with Advanced Solid Malignancies

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Key Words. Angiogenesis inhibitors • Peptite vaccine • Vascular Endothelial Growth Factor A

Trial Information

- **ClinicalTrials.gov Identifier:** NCT02237638
- **Sponsor:** Immunovo
- **Principal Investigator:** Henk M.W. Verheul
- **IRB Approved:** Yes

Lessons Learned

- The novel therapeutic vaccine hVEGF$_{26–104}$/RFASE was found to be safe and well tolerated in patients with cancer.
- hVEGF$_{26–104}$/RFASE failed to induce seroconversion against native hVEGF$_{165}$ and, accordingly, neither a decrease in circulating vascular endothelial growth factor (VEGF) levels nor clinical benefit was observed.
- Remarkably, hVEGF$_{26–104}$/RFASE induced VEGF$_{165}$-neutralizing antibodies in a nonhuman primate model. The absence of seroconversion in human calls for caution in the interpretation of efficacy of human vaccines in nonhuman primates.

Abstract

Background. Targeting vascular endothelial growth factor-A (VEGF) is a well-established anticancer therapy. We designed a first-in-human clinical trial to investigate the safety and immunogenicity of the novel vaccine hVEGF$_{26–104}$/RFASE.

Methods. Patients with advanced solid malignancies with no standard treatment options available were eligible for this phase I study with a 3+3 dose-escalation design. On days 0, 14, and 28, patients received intramuscular hVEGF$_{26–104}$, a truncated synthetic three-dimensional (3D)-structured peptide mimic covering the amino acids 26–104 of the human VEGF$_{165}$ isoform, emulsified in the novel adjuvant Raffinose Fatty Acid Sulphate Ester (RFASE), a sulpholipopolysaccharide. Objectives were to determine safety, induction of VEGF-neutralizing antibodies, and the maximum tolerated dose. Blood was sampled to measure VEGF levels and antibody titers.

Results. Eighteen of 27 enrolled patients received three immunizations in six different dose-levels up to 1,000 μg hVEGF$_{26–104}$ and 40 mg RFASE. No dose-limiting toxicity was observed. Although in four patients an antibody titer against hVEGF$_{26–104}$ was induced (highest titer: 2.77 ^{10}log), neither a reduction in VEGF levels nor neutralizing antibodies against native VEGF$_{165}$ were detected.

Conclusion. Despite having an attractive safety profile, hVEGF$_{26–104}$/RFASE was not able to elicit seroconversions against native VEGF$_{165}$ and, consequently, did not decrease circulating VEGF levels. Deficient RFASE adjuvant activity, as well as dominant immunoreactivity toward neoepitopes, may have impeded hVEGF$_{26–104}$/RFASE's efficacy in humans.

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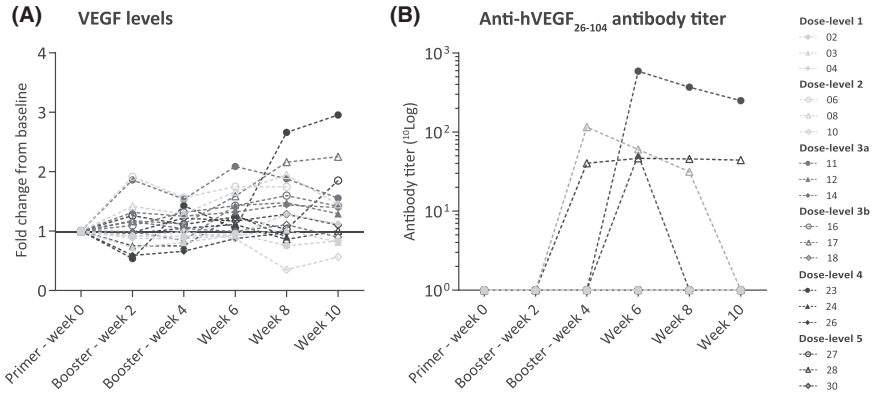

Figure 1. VEGF levels and anti-hVEGF$_{26–104}$ antibody titers, shown per patient, per dose level. VEGF levels in serum are shown relative to baseline in **(A)**. Antibody titers measured in serum are shown for hVEGF$_{26–104}$ in **(B)**. Titers are in ^{10}log scale: 2.06 for patient 08, 2.77 for patient 23, 1.71 for patient 24, and 1.67 for patient 28, respectively.
Abbreviations: VEGF, vascular endothelial growth factor-A.

Discussion

Inhibition of vascular endothelial growth factor-A (VEGF) is a well-established anticancer approach in combination with cytotoxic agents. Nonetheless, the observed clinical benefit is usually rather modest, and long-term treatment can be burdensome because of repeated intravenous administration. Therefore, neutralization of VEGF by active immunization could be an attractive alternative treatment strategy. It would offer the advantage of continuous and a potentially more pronounced inhibition of VEGF without the need for repeated antibody administrations. Here, we describe the results of a phase I trial of the novel therapeutic vaccine hVEGF$_{26–104}$/RFASE [1, 2] in patients with advanced solid malignancies.

The vaccine comprised a truncated synthetic 3D-structured peptide mimic covering the amino acids 26–104 of the human VEGF$_{165}$ isoform, emulsified in the novel adjuvant Raffinos Fatty Acid Sulphate Ester (RFASE), a sulpholipopolysaccharide [3, 4]. Dose-limiting toxicities, including related grade ≥ 3 adverse events (AEs), were not observed. None of the AEs could be associated with VEGF inhibition. No significant reduction in serum VEGF levels was found (Fig. 1A) and no clinical benefit was observed. Interestingly, in four patients, an antibody titer against hVEGF$_{26–104}$ was measured (highest titer 2.77 ^{10}log), peaking four to six weeks after the first immunization (Fig. 1B). Nevertheless, cross-reactive antibodies against native VEGF$_{165}$ were not detected.

There might be several explanations for the poor immunogenicity of hVEGF$_{26–104}$/RFASE in humans. First, the capped N- and C-terminal sequence of human VEGF$_{165}$ and the dimerization-domain (in which two cysteines were replaced by alanines) that became solvent-exposed in the monomeric hVEGF$_{26–104}$ represent potential neoepitopes. These epitopes could conceivably elicit dominant immunoreactivity and thereby interfere with reactivity to native VEGF. The fact that cross-reactivity to native VEGF was observed in cynomolgus monkeys [2] may be related to interspecies B- or T-cell receptor repertoire differences. Second, lower VEGF levels in nonhuman primates might make them more susceptible to breaking self-tolerance. The hVEGF$_{26–104}$ dose in humans might still have been below the threshold for breaking immune tolerance, since antigen-dosing in vaccination strategies is generally not linearly correlated with the desired immune response but rather has an "on–off' effect. Finally, RFASE adjuvant might not have been sufficiently potent to induce an immune response against a self-antigen like VEGF, especially in the context of cancer-related immunosuppression. Proven clinically active Toll-like receptor agonists, like Poly I:C (polyinosinic:polycytidylic acid) or CpG oligodeoxynucleotides, might stimulate a more potent immune response. However, adjuvant substitution would require not only altering the drug composition but also additional preclinical testing for drug-combination safety, as well as conducting a new phase I trial. In view of these considerable hurdles, it was decided to terminate further development and testing of the vaccine at this point.

In conclusion, the therapeutic vaccine hVEGF$_{26–104}$/RFASE displayed an attractive safety profile, but did not elicit an immune response strong enough to convey clinical benefit for patients with advanced solid malignancies.

Trial Information	
Disease	Advanced cancer/solid tumor only
Stage of Disease/Treatment	Metastatic/advanced
Prior Therapy	No designated number of regimens
Type of Study	Phase I, 3+3
Primary Endpoints	Safety, tolerability, maximum tolerated dose
Secondary Endpoint	Efficacy

Additional Details of Endpoints or Study Design

Trial design: Patients with advanced solid malignancies with no standard treatment options available were eligible for this phase I study with a 3 + 3 dose-escalation design. Patients were enrolled in six different dose levels (Table 1). The study medication consisted of 1.0 mL $hVEGF_{26–104}$ (in escalating doses of 62.5 μg, 125 μg, 250 μg, 500 μg, 1,000 μg, 2000 μg, and 4,000 μg) combined with 1.0 mL RFASE (20 mg in dose-levels 1, 2, and 3a and 40 mg in dose-levels 3b, 4, and 5). The total volume that was administered was therefore 2.0 mL. Injections were administered in a split-dose contralateral fashion, in either the left and right deltoid or gluteal muscles. The starting dose of 62.5 μg $hVEGF_{26–104}$ equaled one-eighth of the maximal dose given in animals. The starting dose of 20 mg RFASE equaled half of the maximal dose given in animals. On days 0, 14, and 28, patients received $hVEGF_{26–104}$/RFASE intramuscularly, followed by an observation period of six weeks. To assess potential toxicity of RFASE, three patients enrolled in the first cohort of the study received 1.0 mL RFASE (20 mg) as a single agent 14 days prior to the first immunization with $hVEGF_{26–104}$/RFASE. Another booster injection could be administered to patients showing response or stable disease on imaging without (prior) VEGF neutralization in serum at first evaluation (10 weeks).

Study endpoints: The coprimary outcome measures of this study were the safety and tolerability profile of $hVEGF_{26–104}$/RFASE and the effective dose of $hVEGF_{26–104}$/RFASE required to neutralize VEGF in serum. Secondary outcome measures were the anti-$VEGF_{165}$ and anti-$VEGF_{26–104}$ antibody titers induced by $hVEGF_{26–104}$/RFASE immunization and clinical benefit, defined by at least no signs of progression at first evaluation.

Safety profile: Toxicity was graded by the National Cancer Institute CTCAE version 4.0 and recorded using electronic case record forms. Serious adverse events were reported to the Dutch Central Committee on Research Involving Human Subjects (CCMO) through the web portal "ToetsingOnline." Dose-limiting toxicity (DLT) was defined as any one of the following toxicities considered by the investigator to be related to $hVEGF_{26–104}$/RFASE and occurring during the DLT assessment window (day 0 of week 0 to day 7 of week 9): any-grade ≥ 3 hematological toxicity or any-grade ≥ 3 nonhematological toxicity that was not attributable to disease progression or another clearly identifiable cause, excluding grade 3 diarrhea that responded to standard-of-care therapy; grade 3 nausea or vomiting, in the absence of premedication, that responded to standard-of-care therapy; or grade 3 infusion reaction, in the absence of premedication that responded to standard-of-care therapy. Patients were observed for DLTs for a minimum of 42 days after their last dose of $hVEGF_{26–104}$/RFASE before any patient in the next higher dose cohort received treatment, except in cases in which there was no VEGF neutralization observed 14 days after the third and last dose of hVEGF26–104/RFASE.

VEGF serum levels: VEGF protein concentration was measured in serum, frozen at the day the material was received, and stored at −80°C until analysis, using a commercially available human enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Abingdon, U.K.) according to manufacturer's instructions. Absorbance was measured using a Bio-Tek Synergy HT plate reader with an optical density (OD) of 450 nm. VEGF levels were measured every 2 weeks in the DLT period, and VEGF neutralization was defined as a VEGF level below 9 pg/mL.

VEGF serological responses: Anti-VEGF antibody titers were measured in serum, frozen the day the material was received, and stored at −80°C until analysis, using an in-house developed ELISA. Microplates were coated with 100 μL recombinant $hVEGF_{165}$ (1 μg/mL; BioLegend, San Diego). After washing, the plates were blocked with 200 μL 4% horse serum (Sigma-Aldrich, St. Louis, MO). Hereafter, the plates were incubated with 100 μL 1:30 diluted serum. Horseradish peroxidase (HRP)–conjugated rabbit antihuman immunoglobulin G antibodies (1:8,000 dilution; Sigma-Aldrich, St. Louis) were applied to detect bound antibodies in the microplate wells. In the presence of chromogenic substrate TMB (R&D Systems, Abingdon, UK) color was developed by the enzymatic reaction of HRP. Absorbance was measured using a BioTek Synergy HT plate reader at an OD of 450 nm. If the OD was above a predetermined cut-off (mean + 3 SDs of all patient serum baseline OD levels), a relevant antibody response was suspected and a dilution series was performed. The antibody titer was defined as the ^{10}logarithm of the highest dilution which resulted in a signal above the predetermined cut-off. A similar ELISA was performed on all samples to measure antibodies recognizing $VEGF_{26–104}$.

Tumor response assessment: Tumor response was assessed according to RECIST 1.1 at baseline, 10 weeks after start of treatment and every eight weeks during the follow-up period in case of response and/or a repeated booster administration.

Cytokine release assay: Peripheral blood mononuclear cells from healthy donors were isolated by standard Ficoll-Hypaque density centrifugation. Cells were cultured for 24 hours (1×10^6 cells per mL per well) with lipopolysaccharide (1 μg/mL) or RFASE (1 μg/mL) (without squalene-in-water component, originally tested in a range from 0.5 to 5 μg/mL) in culture medium (Iscove's Modified Dulbecco's Media (IMDM), 10% Fetal Calf Serum (FCS), pen/strep). Dimethyl sulfoxide (DMSO) 0.1% served as negative control. A cytokine release assay for interleukin (IL)-1β, IL-6, IL-10, IL-8, and TNF-α (CBA Human Inflammatory Cytokines Kit, Becton Dickinson, CA) was performed following the manufacturer's instructions with cell culture supernatants collected after 24 hours and temporarily stored at -20°. Data acquisition was performed on a FACS-Calibur flow cytometer (Becton Dickinson, CA). Quantity (picograms per milliliter of the respective cytokines was calculated using FCAP array software (Soft Flow Hungary Ltd.).

Statistical analysis: Statistical analyses were performed using IBM SPSS Statistics for Windows (Version 25.0; SPSS, Armonk, NY). Kaplan-Meier analysis was performed to determine overall survival and progression free survival. Means of cytokine release assays were compared with a student t-test. Median C-reactive protein and WBC levels were compared using a Wilcoxon matched-pairs signed rank test. A p value of <.05 was considered statistical significant

Investigator's Analysis Drug tolerable, efficacy indeterminant

Drug Information

Drug 1	
Generic/Working Name	$hVEGF_{26–104}$/RFASE
Trade Name	$hVEGF_{26–104}$/RFASE
Company Name	Immunovo
Drug Type	Vaccine
Drug Class	Angiogenesis - VEGF
Dose	Variable per
Route	Other
Schedule of Administration	Intramuscular vaccination on day 0, 14 and 28

Dose-Escalation Table

Dose level	Dose of drug: hVEGF26–104/RFASE	Number enrolled	Number evaluable for toxicity
1	62.5 μg/20 mg	4	4
2	125 μg/20 mg	3	3
3A	250 μg/20 mg	4	4
3B	250 μg/40 mg	4	4
4	500 μg/40 mg	8	7
5	1,000 μg/40 mg	4	4

Patient Characteristics

Number of Patients, Male	19
Number of Patients, Female	11
Stage	Patients with advanced solid malignancies with no standard treatment options available were eligible
Age	Median (range): 65 (40–78) years
Number of Prior Systemic Therapies	Median (range): 3 (0–9)
Performance Status: ECOG	0 — 1 1 — 24 2 — 2 3 — 0 Unknown — 3
Other	One patient had both esophageal as well as oropharynx cancer, hence 31 instead of 30 cases are listed in the histologic diagnoses itemized below.
Cancer Types or Histologic Subtypes	Urothelial, 1; Neuroendocrine tumor (pancreas and unknown primary), 2; Squamous cell carcinoma (unknown primary), 1; Salivary duct, 1; Ovarian, 2; Pancreas, 1; Colorectal, 7; Gastric, 2; Pleiomorphic adenoma, 1; Metaplastic carcinoma, 1; Glioblastoma, 1; Tungbase, 1; Tonsil, 1; Oropharynx, 1; Esophageal, 2; Hepatocellular, 2; Hypopharynx, 1; Breast, 2; Prostate, 1.

Primary Assessment Method

Title	Response evaluation week 10
Number of Patients Screened	30
Number of Patients Enrolled	27
Number of Patients Evaluable for Toxicity	26
Number of Patients Evaluated for Efficacy	26

Evaluation Method	RECIST 1.1
Response Assessment CR	*n* = 0 (0%)
Response Assessment PR	*n* = 0 (0%)
Response Assessment SD	*n* = 5 (19%)
Response Assessment PD	*n* = 13 (50%)
Response Assessment OTHER	*n* = 8 (31%)
(Median) Duration Assessments PFS	70 days, CI: 69–71
(Median) Duration Assessments TTP	69 days, CI: 55–85
(Median) Duration Assessments OS	157 days, CI: 117–197
(Median) Duration Assessments Duration of Treatment	70 days
Outcome Notes	Other category specified: Early death from malignant disease (*n* = 3) Early death from other cause (*n* = 1) Not assessable (withdrew consent) (*n* = 2) Not assessable (rapid clinical deterioration) (*n* = 1) Not assessable (off-study after infections) (*n* = 1)

Adverse Events

All Dose Levels, All Cycles

Name	NC/NA	1	2	3	4	5	All Grades
Injection site reaction	38%	62%	0%	0%	0%	0%	62%
Fatigue	57%	31%	12%	0%	0%	0%	43%
Fever	65%	27%	8%	0%	0%	0%	35%
Nausea	88%	4%	8%	0%	0%	0%	12%
Flu like symptoms	88%	12%	0%	0%	0%	0%	12%
Weight loss	92%	4%	4%	0%	0%	0%	8%
General disorders and administration site conditions, malaise	92%	4%	4%	0%	0%	0%	8%
Pain in extremity	92%	8%	0%	0%	0%	0%	8%
Neck pain	96%	4%	0%	0%	0%	0%	4%
Anorexia	92%	4%	4%	0%	0%	0%	8%
Bone pain	96%	0%	4%	0%	0%	0%	4%
Skin and subcutaneous tissue disorders, erythema	96%	4%	0%	0%	0%	0%	4%
Aspartate aminotransferase increased	96%	0%	4%	0%	0%	0%	4%
Dyspnea	96%	4%	0%	0%	0%	0%	4%
Myalgia	96%	0%	4%	0%	0%	0%	4%
Skin and subcutaneous tissue disorders, rash	96%	4%	0%	0%	0%	0%	4%
Dizziness	96%	4%	0%	0%	0%	0%	4%
Edema limbs	96%	4%	0%	0%	0%	0%	4%
Alkaline phosphatase increased	96%	4%	0%	0%	0%	0%	4%
Blood and lymphatic system disorders, venous stasis	96%	4%	0%	0%	0%	0%	4%
Blood bilirubin increased	96%	4%	0%	0%	0%	0%	4%
Diarrhea	96%	4%	0%	0%	0%	0%	4%
Headache	96%	4%	0%	0%	0%	0%	4%

All adverse events listed are possible, probable, or certainly related. See also Table 5.
Abbreviation: NC/NA, no change from baseline/no adverse event

Serious Adverse Events		
Name	**Grade**	**Attribution**
Fever	1	Probable
Fever	2	Possible
Fever	2	Possible
Pain in extremity	3	Unrelated
Pain in extremity	3	Unlikely
Tumor pain	3	Unrelated
Anemia	3	Unrelated
Confusion	2	Unrelated
Urinary tract infection	3	Unrelated
Sepsis	4	Unrelated
Somnolence	3	Unrelated
Thromboembolic event	3	Unrelated
Abdominal pain	2	Unlikely
Upper GI hemorrhage	3	Unrelated
Malaise	2	Possible
Nausea	2	Possible
Vomiting	2	Unrelated

See also Table 6. Abbreviation: GI, gastrointestinal.

Dose-Limiting Toxicities				
Dose level	**Dose of drug: hVEGF26–104/RFASE**	**Number enrolled**	**Number evaluable for toxicity**	**Number with a dose-limiting toxicity**
1	62.5 μg/20 mg	4	3	0
2	125 μg/20 mg	3	3	0
3A	250 μg/20 mg	4	4	0
3B	250 μg/40 mg	4	4	0
4	500 μg/40 mg	8	7	0
5	1,000 μg/40 mg	4	4	0

Assessment, Analysis, and Discussion	
Completion	Study terminated before completion
Terminated Reason	Company stopped development
Investigator's Assessment	Drug tolerable, efficacy indeterminant

Vascular endothelial growth factor-A (VEGF) is an angiogenic growth factor involved in normal physiology (such as embryogenesis) and disease (such as cancer) [5]. VEGF is produced by several cell types in the human body, including cancer cells and megakaryocytes [6]. Four isoforms are detected in the human body, of which $VEGF_{165}$ and $VEGF_{121}$ circulate and are detectable by the VEGF enzyme-linked immunosorbent assay (ELISA) as used. VEGF in serum is largely derived from platelets, which secrete VEGF upon wounding and in the tumor vasculature to stimulate angiogenesis (i.e., the growth of new blood vessels from preexisting capillaries) [7]. Upon treatment with the anti-VEGF monoclonal antibody bevacizumab, VEGF is neutralized and no longer exerts biological activity [8].

Antiangiogenic therapy is mostly combined with cytotoxic agents, although there is mounting interest to combine it with other forms of anticancer treatment, such as immunotherapy and radiotherapy [9–11]. Nonetheless, the clinical benefit observed from antiangiogenic therapy is usually modest, and treatment withdrawal has been associated with rebound growth, possibly due to compensatory pathways activated by other proangiogenic factors and cytokines [12, 13]. Therefore, neutralization of VEGF by active immunization could be an attractive alternative [14]. VEGF inhibition might not only be more durable but also more pronounced due to the induction of a polyclonal antibody response, resulting in higher avidity binding. Furthermore, tumor-associated plasma cells might ensure that endogenous antibodies have a better tumor-penetrating capacity, as compared with

exogenously administered antibodies [15]. In addition, continued VEGF suppression beyond progressive disease might convey a survival benefit, as demonstrated in metastatic colorectal cancer [16, 17]. Finally, active immunization could lead to a notable reduction in hospital visits and treatment costs, as compared with monoclonal antibody therapy.

hVEGF$_{26–104}$ is a three-dimensional (3D)-structured truncated peptide antigen derived of the endogenous protein human VEGF$_{165}$ that perfectly mimics the 3D structure of the cysteine knot motif of VEGF$_{165}$. Immunization with hVEGF$_{26–104}$ is thus expected to result in antibodies that can cross-react with and neutralize VEGF$_{165}$. Biological activity of the (monomeric) peptide hVEGF$_{26–104}$ itself is prohibited by the substitution of two cysteines, vital for the formation of the VEGF$_{165}$ homodimer and consequent receptor binding capacities, for alanines. hVEGF$_{26–104}$ is mixed 1:1 with RFASE adjuvant, a sulpholipopolysaccharide in a squalane-in-water emulsion with polysorbate 80 as emulsifier [3, 4]. Immunization of nonhuman primates with hVEGF$_{26–104}$/RFASE resulted in an RFASE dependent antibody titer against hVEGF$_{26–104}$ and cross-reactive antibodies against VEGF$_{165}$ 28 days after primer immunization. Anti-VEGF$_{165}$ antibodies were able to inhibit the binding of bevacizumab with VEGF$_{165}$ in a competition ELISA. Moreover, the biological activity of VEGF$_{165}$ could be inhibited by the addition of immunized monkey serum in a VEGF-specific bioassay [18].

In the current phase I clinical trial, 18 out of 27 enrolled patients received all three immunizations and completed the dose-limiting toxicity observation period (Table 2); reasons for not completing treatment are listed in Table 3. Comparison of median difference in C-reactive protein (CRP) and white blood cell (WBC) count before and after vaccination suggests a possible, but very weak, innate immune response (Fig. 2). In four patients a body temperature of 38.5°C or higher was observed (Fig. 2); in only one of these patients an antibody titer against hVEGF$_{26–104}$ was detected. However, a correlation between dosage and these parameters was not observed. In 44% of all administrations a grade 1 local reaction was observed, mostly warmth, pain, and swelling (Table 4). Neither clinically significant toxicity (Table 5, 6) nor clinical benefit was observed at any of the dose-levels. At a first evaluation by computed tomography scan, stable disease (SD) was observed in five patients. Nevertheless, four of these patients had clinical progression (progressive disease; PD) and went off-study. One patient in the first dose level received an additional booster vaccine after 10 weeks (optional for patients with SD and no signs of VEGF suppression); she progressed at evaluation 10 weeks later. In total, 13 patients showed PD. Four patients succumbed before first evaluation; three because malignant disease and one because of pneumonia. Finally, four patients were not assessable. Median overall survival was 157 days (95% confidence interval [CI], 117–197), and median progression free survival was 70 days (95% CI, 69–71; Table 3).

Despite the encouraging results in nonhuman primates, hVEGF$_{26–104}$/RFASE did not elicit the formation of VEGF$_{165}$-cross-reactive antibodies in patients with cancer. The lack of seroconversion against native VEGF calls for caution in the interpretation of human vaccine efficacies in nonhuman primates. There might be several explanations for the apparent poor antigenicity of hVEGF$_{26–104}$/RFASE in humans. Besides the earlier mentioned possibilities of diversion of the immune response by dominant neoepitopes in the hVEGF$_{26–104}$ domain, suboptimal dosing or insufficient adjuvant potency provided by RFASE in humans might have played a role.

To break immunosuppression and self-tolerance, a powerful adjuvant is a key component of any cancer vaccine. Most cancer peptide vaccines have relied on adjuvants such as incomplete Freund's adjuvant (IFA) or Montanide ISA-51, both water-in-oil emulsions with the antigen forming a depot for slow release purpose. RFASE is an oil-in-water emulsion designed to function as an antigen depot and to induce local inflammation and activation of Toll-like receptor (TLR)-4 signaling. Interestingly, evidence is emerging that Toll-like receptor ligands, such as CpG oligonucleotides (TLR-9 agonist) [19] and Poly I:C polyinosinic:polycytidylic acid; (TLR-3 agonist) [20] used as vaccine adjuvants, show more effective immune responses after peptide vaccination as compared with IFA or Montanide ISA-51 [21–23]. In our in vitro models, stimulation of healthy control human peripheral blood mononuclear cells with lipopolysaccharide (LPS) showed a significant increase in (inflammatory) cytokine release, whereas stimulation with RFASE failed to induce any detectable cytokine release over background levels (Fig. 3). This is a clear indication that RFASE, which is related to LPS, does not have the capacity for induction of an immune response in humans.

Notwithstanding promising activity in nonhuman primate studies, hVEGF$_{26–104}$/RFASE did not elicit cross-reactive neutralizing antibodies against native VEGF and did not show any hint of clinical activity in patients with advanced solid malignancies. We propose that in future studies, addition or substitution of RFASE by an alternative adjuvant with proven efficacy should be considered to break self-tolerance, induce cross-reactive antibodies against VEGF$_{165}$, and consequently, decrease VEGF serum levels.

Disclosures

Hans J. van der Vliet: CSO Lava Therapeutics (E), Lava Therapeutics (IP), Lava Therapeutics, Glycostem (RF), Lava Therapeutics (OI); **Tanja D. de Gruijl:** DCPrime BV (C/A), Idera Pharmaceuticals (RF). The other authors indicated no financial relationships.

(C/A) Consulting/advisory relationship; (RF) Research funding; (E) Employment; (ET) Expert testimony; (H) Honoraria received; (OI) Ownership interests; (IP) Intellectual property rights/inventor/patent holder; (SAB) Scientific advisory board

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Figures and Tables

Table 1. Dose-levels

Dose-level	$hVEGF_{26-104}$, µg	RFASE, mg
Dose-level 1[a]	62.5	20
Dose-level 2	125	20
Dose-level 3A	250	20
Dose-level 3B	250	40
Dose-level 4	500	40
Dose-level 5	1,000	40

[a]The patients in dose-level 1 received a first immunization with 20 mg RFASE alone to study the potential adverse effects of the adjuvant.
Abbreviation: RFASE, raffinose fatty acid sulphate ester.

Table 2. Baseline characteristics

Dose-Level	Sex	Age	ECOG status	Prior systemic therapies	Tumor type	Enrolled	Completed DLT period
1	F	51	1	2	Urothelial	Yes	No
1	M	77	1	3	NET[a] of pancreas	Yes	Yes
1	F	49	1	3	SCC[b] of unknown primary	Yes	Yes
1	M	70	1	2	Salivary duct	Yes	Yes
2	M	56	NA	7	NET[a] of unknown primary	No	Screen failure
2	F	56	1	4	Ovarian	Yes	Yes
2	F	59	NA	1	Pancreas	No	Screen failure
2	M	69	1	0	Colorectal	Yes	Yes
2	M	59	NA	0	Gastric	No	Screen failure
2	M	70	1	4	Gastric	Yes	Yes
3A	F	67	1	3	Pleiomorphic adenoma	Yes	Yes
3A	F	68	1	4	Metaplastic carcinoma	Yes	Yes
3A	M	68	2	3	Glioblastoma	Yes	No
3A	M	67	1	3	Tongue base	Yes	Yes
3B	F	59	1	2	Colorectal	Yes	No
3B	M	60	1	6	Colorectal	Yes	Yes
3B	F	78	1	2	Colorectal	Yes	Yes
3B	M	55	1	3	Tonsil	Yes	Yes
4	M	54	1	1	Colorectal	Yes	No
4	M	64	2	3	Oropharynx and esophageal	Yes	No
4	F	62	1	1	Ovarian	Yes	No
4	M	66	1	6	Hepatocellular	Yes	No
4	M	70	1	1	Colorectal	Yes	Yes
4	M	63	1	1	Hypopharynx	Yes	Yes
4	F	40	1	3	Breast	Yes	No
4	M	77	1	5	Esophageal	Yes	Yes
5	M	69	1	9	Hepatocellular	Yes	Yes
5	M	60	0	2	Colorectal	Yes	Yes
5	M	71	1	3	Prostate	Yes	No
5	F	72	1	3	Breast	Yes	Yes

Abbreviations: ECOG, Eastern Cooperative Oncology Group; F, female; M, male; NA, not applicable; NET.
[a]neuroendocrine tumor.
[b]squamous cell carcinoma.

Table 3. Response evaluation

	Dose-level 1	Dose-level 2	Dose-level 3a	Dose-level 3b	Dose-level 4	Dose-level 5	All dose-levels (95% CI)
Screened	4	6	4	4	8	4	30
Enrolled	4	3	4	4	8	4	27
Evaluable for toxicity	4	3	4	4	7[a]	4	26
Evaluable for efficacy[b]	4	3	4	4	7[a]	4	26
Stable disease	2	0	1	2	0	0	5
Progressive disease	1	2	2	1	4	3	13
Other[c]	1	1	1	1	3	1	8
Median PFS, (days)	140	70	70	NA	68	69	70 (69–71)
Median TTP, (days)	112	68	70	69	62	69	69 (55–85)
Median OS, (days)	146	151	500	125	137	174	157 (117–197)
Median response duration, (days)	NA	NA	NA	NA	NA	NA	NA
Median treatment duration, (days)	84	70	69	74	34	77	70

[a]One patient in dose-level 4 did not commence treatment because of pulmonary embolism and was therefore excluded from efficacy and toxicity evaluations.
[b]First response evaluation (wk 10) using RECIST 1.1.
[c]Other category specified per dose-level (DL): DL 1: early death from malignant disease (1×); DL 2: not assessable (rapid clinical deterioration) (1×); DL 3A: early death from malignant disease (1×); DL 3B: not assessable (withdrew consent) (1×); DL 4: early death from malignant disease (1×), early death from other cause (1×), not assessable (off-study after infections) (1x); DL 5: not assessable (withdrew consent) (1×).
Abbreviations: CI, confidence interval; OS, overall survival; NA, not available; PFS, progression-free survival; TTP, time to progression.

Table 4. Local injection site reactions

	Dose-level 1	Dose-level 2	Dose-level 3A	Dose-level 3B	Dose-level 4	Dose-level 5	All dose-levels, *n* (%)
Reactions[a]	3	6	6	9	3	1	28 (44)
Primer	1	1	3	3	2	0	10 (40)
First booster	1	2	3	3	0	1	10 (50)
Second booster	1	3	0	3	1	0	8 (42)
Type[b]							
Abscess	0	0	0	0	0	0	0 (0)
Cellulitis	0	0	0	0	0	0	0 (0)
Nodule	0	0	1	0	0	0	1 (4)
Induration	0	4	0	2	0	0	6 (21)
Swelling	2	4	1	5	0	0	12 (43)
Pain	2	0	3	7	1	1	14 (50)
Erythema	1	1	0	0	1	0	3 (11)
Warmth	1	4	4	6	2	0	17 (61)

[a]Number of local injection site reactions observed in 64 vaccine administrations in 26 patients.
[b]Specification of local reaction type (multiple reaction types possible per reaction).

Table 5. Adverse events

Adverse event[a]	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total	NC/NA, %
Injection site reaction	16	0	0	0	0	16	38.5
Fatigue	8	3	0	0	0	11	57.7
Fever	7	2	0	0	0	9	65.4
Nausea	1	2	0	0	0	3	88.5
Flu like symptoms	3	0	0	0	0	3	88.5
Weight loss	1	1	0	0	0	2	92.3
Malaise	1	1	0	0	0	2	92.3
Anorexia	1	1	0	0	0	2	92.3
Pain in extremity	2	0	0	0	0	2	92.3
Neck pain	1	0	0	0	0	1	96.2
Bone pain	0	1	0	0	0	1	96.2
Erythema	1	0	0	0	0	1	96.2
Aspartate aminotransferase increased	0	1	0	0	0	1	96.2
Dyspnea	1	0	0	0	0	1	96.2
Myalgia	0	1	0	0	0	1	96.2
Rash	1	0	0	0	0	1	96.2
Dizziness	1	0	0	0	0	1	96.2
Edema limbs	1	0	0	0	0	1	96.2
Alkaline phosphatase increased	1	0	0	0	0	1	96.2
Venous stasis	1	0	0	0	0	1	96.2
Blood bilirubin increased	1	0	0	0	0	1	96.2
Diarrhea	1	0	0	0	0	1	96.2
Headache	1	0	0	0	0	1	96.2
Total	51	13	0	0	0	64	

[a]Listed adverse events are possible, probable, or certainly related.
Abbreviation: NC/NA, no change from baseline/no adverse event.

Table 6. Serious adverse events

SAE	Grade 1	Related	Grade 2	Related	Grade 3	Related	Grade 4	Related	Grade 5	Total
Fever	1	Probable	2	Possible	0		0		0	3
Pain in extremity	0		0		2	Unlikely 1×; Unrelated 1×	0		0	2
Tumor pain	0		0		1	Unrelated	0		0	1
Anemia	0		0		1	Unrelated	0		0	1
Confusion	0		1	Unrelated	0		0		0	1
Urinary tract infection	0		0		1	Unrelated	0		0	1
Sepsis	0		0		0		1	Unrelated	0	1
Somnolence	0		0		1	Unrelated	0		0	1
Thromboembolic event	0		0		1	Unrelated	0		0	1
Abdominal pain	0		1	Unlikely	0		0		0	1
Upper GI hemorrhage	0		0		1	Unrelated	0		0	1
Malaise	0		1	Possible	0		0		0	1
Nausea	0		1	Possible	0		0		0	1
Vomiting	0		1	Unrelated	0		0		0	1
Total	1		7		8		1		0	17

Abbreviations: GI, gastrointestinal; SAE, serious adverse event.

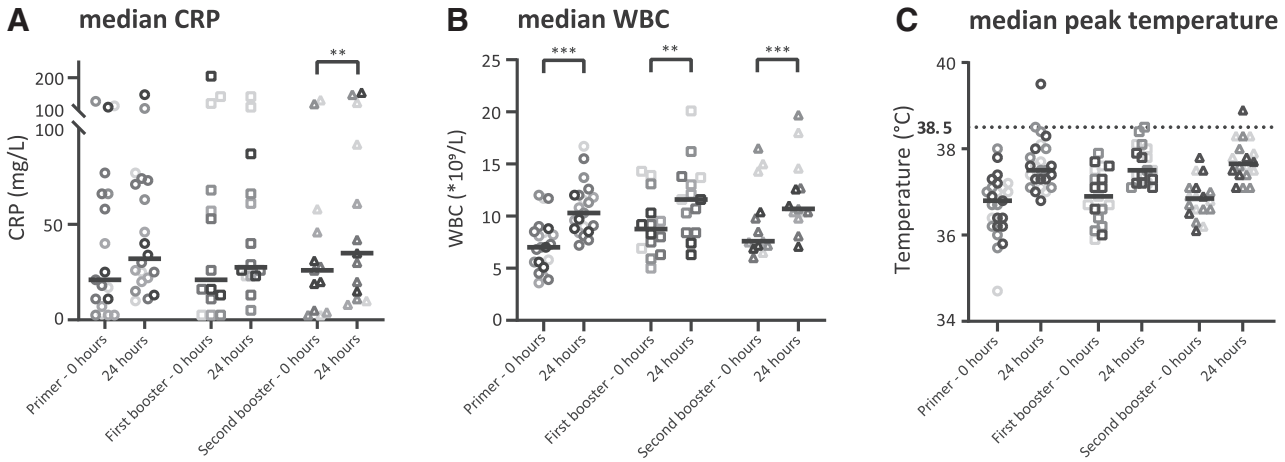

Figure 2. Median rise in CRP **(A)** and WBC **(B)** 24 hours after vaccination as compared with baseline was 7.91 mg/l (95% confidence interval [CI], 0.78–11.55, p = .030) and 2.87*109/L (95% CI, 2.30–3.83, p < .001), respectively. Median peak body temperature within 24 hours after vaccination (C) was 0.90°C (95% CI, 0.67–1.22), 0.50°C (95% CI, 0.32–1.07), and 0.95°C (95% CI, 0.52–1.28) higher as compared with baseline, for primer, first, and second booster, respectively. In four cases, a body temperature of 38.5°C or higher was observed. *P < 0.05, **P < 0.01, ***P < 0.001.
Abbreviations: CRP, C-reactive protein; WBC, white blood cell.

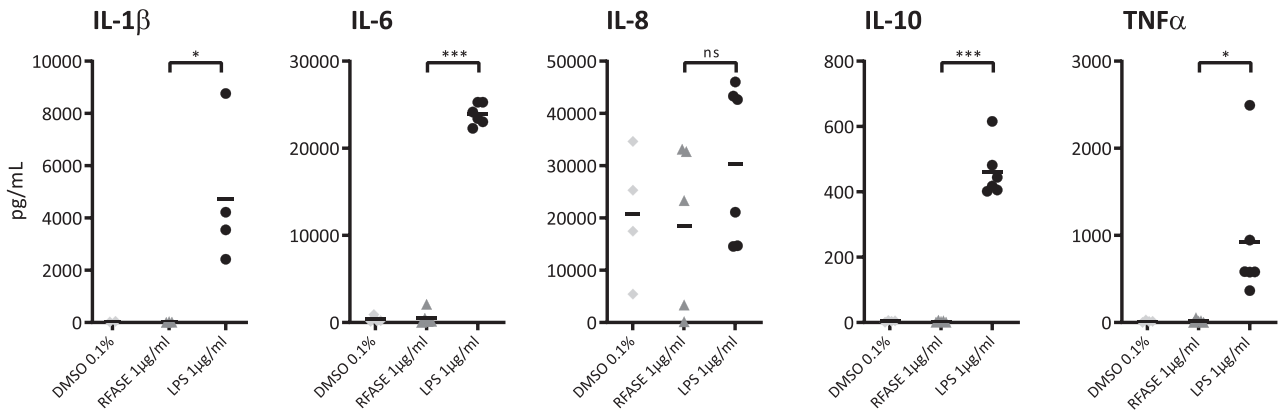

Figure 3. Cytokine levels of IL-1β, IL-6, IL-8, IL-10, and TNFα in human peripheral blood mononuclear cell supernatants of healthy controls are shown after incubation for 24 hours with either RFASE (1 µg/mL) or LPS (1 µg/mL). DMSO 0.1% served as negative control. *P < 0.05, **P < 0.01, ***P < 0.001.
Abbreviations: DMSO, Dimethyl sulfoxide; IL, interleukin; LPS, lipopolysaccharide; ns, not significant; RFASE, raffinose fatty acid sulphate ester; TNF, tumor necrosis factor.