A phase 1 trial extension to assess immunologic efficacy and safety of prime-boost vaccination with VXM01, an oral T cell vaccine against VEGFR2, in patients with advanced pancreatic cancer

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A phase 1 trial extension to assess immunologic efficacy and safety of prime-boost vaccination with VXM01, an oral T cell vaccine against VEGFR2, in patients with advanced pancreatic cancer

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ABSTRACT

VXM01 is a first-in-kind orally applied tumor vaccine based on live attenuated Salmonella typhi carrying an expression plasmid encoding VEGFR2, an antigen expressed on tumor vasculature and a stable and accessible target for anti-angiogenic intervention. A recent randomized, placebo-controlled, phase I dose-escalation trial in advanced pancreatic cancer patients demonstrated safety, immunogenicity and transient, T-cell response-related anti-angiogenic activity of four priming vaccinations applied within one week. We here evaluated whether monthly boost vaccinations can be sustained increased frequencies of vaccine-specific T cells.

Patients with advanced pancreatic cancer were randomly assigned at a ratio of 2:1 to priming with VXM01 followed by up to six monthly boost vaccinations, or placebo treatment. Vaccinations were applied orally at two alternative doses of either 10^5 colony-forming units (CFU) or 10^7 CFU, and concomitant treatment with standard-of-care gemcitabine during the priming phase, and any treatment thereafter, was allowed in the study. Immunomonitoring involved interferon-gamma (IFNγ) ELISPOT analysis with long overlapping peptides spanning the entire VEGFR2 sequence.

A total of 26 patients were treated. Treatment-related adverse events preferentially associated with VXM01 were decreases in lymphocyte numbers in the blood, increased frequencies of neutrophils and diarrhea. Eight out of 16 patients who received at least one boosting vaccination responded with pronounced, i.e. at least 3-fold, increase in VEGFR2-specific T cell frequencies compared with placebo treatment.

In conclusion, prime/boost vaccination with VXM01 was safe and immunogenic and increased vaccine specific T cell responses compared with placebo treatment.

Introduction

In the past decade, advances in immuno-oncology and the unprecedented clinical success of immune checkpoint inhibition have paved the way for the development of adoptive transfer of genetically modified lymphocytes and cancer vaccination.1-6

Pancreatic cancer is expected to become the third leading cause of cancer-related deaths in the United State.7 More than 85% of the cases are pancreatic ductal adenocarcinomas (PDAC). The poor 5-yr survival rate of 8% is largely due to the mostly advanced, non-resectable situation at clinical presentation, and to marked resistance to chemotherapy.8-11 Treatment options are limited compared with other cancers, and since 2011, only one drug has been approved by the FDA.12 Several approaches to immunotherapy for pancreatic cancer have been explored with little success in early clinical trials including immune checkpoint inhibitors,13,14 therapeutic vaccines,15-19 adoptive T cell therapy including chimeric antigen receptor transduced T cells,20 monoclonal antibodies, and antibody-drug conjugates directed against various antigens, as well as adjuvant and cytokine therapies.21 Clinical success of pancreatic cancer immunotherapy is limited by the peculiar microenvironment, which is characterized by the presence of a low...
number of tumor-specific CD8<sup>+</sup> T cells, predominance of TH-2 polarized CD4<sup>+</sup> T helper cells, impaired NK cell activity and increased frequencies of immune suppressive regulatory T cells, myeloid-derived suppressor cells, and M2 macrophages. The dense and complex pancreatic tumor stroma, involving an anergic tumor vasculature not only supports tumor growth and promotes angiogenesis and metastasis, but also serves as a physical barrier that is not readily permeable to immune effector cells and drugs. Thus, targeting such barriers will be instrumental for efficient pancreatic cancer immunotherapy. Current efforts focus on combination therapies using the immunogenic response generated by chemotherapy or radiation therapy, and immune checkpoint blockade, to profoundly improve clinical responses and survival benefits in patients with pancreatic cancer.

Oral vaccination is a novel approach in cancer immunotherapy that exploits the large surface area and the immunologic contexture of the intestine for immune stimulation. Oral *Salmonella* typhimurium-mediated DNA vaccination has shown consistent anti-angiogenic and antitumor activity in different murine tumor models. Moreover, an analogous human-specific carrier strain *Salmonella* typhi Ty21a has been thoroughly studied and is widely used as a vaccine for the prevention of typhoid fever. The oral T cell vaccine VXM01 consists of live, attenuated *Salmonella* typhi carrying a eukaryotic expression plasmid that encodes for full length vascular endothelial growth factor receptor 2 (VEGFR2). VEGFR2 is a high-affinity receptor for vascular endothelial growth factor-A (VEGF-A) and is the main mediator of the VEGF-A-related endothelial growth and survival signals. VEGFR2 is highly expressed on tumor vasculature as well as on certain tumor and immune cells. Moreover, the modulation of tumor growth with anti-VEGF-A and VEGFR2 antibodies and small-molecule VEGFR2 inhibitors in cancer patients has established VEGFR2 as a therapeutic target in several cancer indications including pancreatic cancer. We have recently reported safety, tolerability, immune responses, and preliminary efficacy data of the first-in-human oral antitumor vaccine VXM01 during a dose-escalation study. We demonstrated that four priming vaccinations with VXM01 applied throughout one initial week resulted in overall increased VEGFR2-specific, polyclonal mixed CD8<sup>+</sup> and CD4<sup>+</sup> effector T cell responses and correlated with reduced tumor perfusion. VEGFR2-specific T cell responses peaked after 21 d but rapidly declined within 3 mo to levels comparable to those before vaccination. We therefore conducted an extension trial to assess the safety and tolerability of a continuous boosting treatment with VXM01, and to explore whether this regimen can maintain elevated VEGFR2-specific T cell responses after the initial prime vaccination.

### Results

#### Patients and treatment

Between August 2013 and February 2014, 27 patients with locally advanced, inoperable, or stage IV pancreatic cancer were enrolled and randomly assigned. One patient did not receive treatment due to serious pre-treatment adverse event (AE) leading to withdrawal from the study. Therefore, the analysis included 18 patients treated with VXM01, and 8 patients treated with placebo (isotonic sodium chloride). There were no statistical differences in the demographic baseline disease characteristics of the patients between the two groups, except for the gender distribution (Table 1). Moreover, the placebo group had a lower proportion of patients with metastatic disease, a Karnofsky performance status <90 or increased CA19-9 levels (both elevated and >1,000 U/mL). The only notable difference between the two dose groups of patients treated with VXM01

### Table 1. Patient demographics and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo N = 8</th>
<th>VXM01 N = 18</th>
<th>VXM01 10&lt;sup&gt;6&lt;/sup&gt; CFU N = 12</th>
<th>VXM01 10&lt;sup&gt;7&lt;/sup&gt; CFU N = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>6 (75)</td>
<td>7 (39)</td>
<td>5 (42)</td>
<td>2 (33)</td>
</tr>
<tr>
<td>Women</td>
<td>2 (25)</td>
<td>11 (61)</td>
<td>7 (58)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>Race, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>8 (100)</td>
<td>18 (100)</td>
<td>12 (100)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Karnofsky performance status, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1 (12.5)</td>
<td>1 (5.6)</td>
<td>1 (8.3)</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>5 (62.5)</td>
<td>6 (33.3)</td>
<td>4 (33.3)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>80</td>
<td>2 (25.0)</td>
<td>11 (61.1)</td>
<td>7 (58.3)</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td>Extent of disease, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locally advanced</td>
<td>2 (25.0)</td>
<td>2 (11.1)</td>
<td>2 (16.7)</td>
<td>0</td>
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<tr>
<td>Metastatic</td>
<td>6 (75.0)</td>
<td>16 (88.9)</td>
<td>10 (83.3)</td>
<td>6 (100.0)</td>
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<tr>
<td>Time from diagnosis, months (range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>7.5 (2–20)</td>
<td>6 (0–28)</td>
<td>6.5 (0–28)</td>
<td>6 (2–16)</td>
</tr>
<tr>
<td>Level of CA19–9, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>4 (50.0)</td>
<td>3 (16.7)</td>
<td>2 (16.7)</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Elevated, &lt; 1,000 U/mL</td>
<td>2 (25.0)</td>
<td>9 (50.0)</td>
<td>4 (33.3)</td>
<td>5 (83.3)</td>
</tr>
<tr>
<td>Elevated, &gt; 1,000 U/mL</td>
<td>2 (25.0)</td>
<td>6 (33.3)</td>
<td>6 (50.0)</td>
<td>0</td>
</tr>
<tr>
<td>Previous therapy other than gemcitabine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1 (12.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Folfoxirin</td>
<td>0</td>
<td>1 (5.6)</td>
<td>1 (8.3)</td>
<td>0</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>1 (12.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CFU, colony-forming units; CA19–9, carbohydrate antigen 19–9; Folfoxirin, folinic acid, 5-fluorouracil, irinotecan and oxaliplatin.
was the frequency of patients with CA19-9 levels higher than 1,000 U/mL (50% and 0% in the 10^6 and 10^7 CFU group, respectively).

Treatment consisted of four priming vaccinations on days 1, 3, 5, and 7, followed by six monthly boost vaccinations beginning 1 mo after the last priming vaccination (Fig. 1). Vaccinations were orally given as a drink solution buffered with bicarbonate at two alternative doses of either 10^6 colony-forming unit (CFU, (N = 12)) or 10^7 CFU (N = 6) of VXM01. Concomitant treatment with standard-of-care gemcitabine up to day 38, and any treatment thereafter, was allowed in the study (Fig. 1). All the 26 enrolled patients entered the priming part and received all four priming doses of VXM01 (n = 18) or placebo treatment (n = 8). In total, 24 patients entered the boosting part and 22 patients received at least one boosting dose of VXM01 (n = 15) or placebo (n = 7). Finally, 11 out of 15 patients having entered the VXM01 boosting phase received all six boosting doses. The study was prematurely discontinued by 11 patients (n = 7 in the VXM01 arms, and n = 4 in the placebo arm), 3 patients during the priming part, and 8 patients during the boosting part due to death (n = 5), AEs (n = 1), deterioration of state of health (n = 1), or consent withdrawal (n = 4) as detailed in Fig. S2. The doses for boosting were identical to the doses administered for initial priming except for three patients in the 10^7-dose group who received a reduced dose, i.e., 10^5 CFU, due to the fecal excretion of VXM01 (see below). One patient in the VXM01 10^6 CFU arm was not treated with boosting administrations due to the fecal excretion of VXM01 and discontinued the study due to the withdrawal of consent.

Adverse events (AE)

In total, 576 treatment-emergent adverse events (TEAEs) were reported, 369 TEAEs (160 after prime and 209 after boosting doses) in 18 patients after VXM01 administration, and 207 TEAEs (95 after prime and 112 after boosting doses) in 8 patients after placebo administration. Most of the TEAEs were of mild and moderate severity (Table 2). Severe AEs were reported in six patients after prime (n = 3 in the VXM01 arms, n = 3 in the placebo arm) and 14 patients after boost vaccination (n = 10 in the VXM01 arms, n = 4 in the placebo arm).

The most frequent TEAEs of any grade skewed toward the VXM01 treatment group after the prime vaccination were decreases in platelets (44.4% vs. 12.5%) and in lymphocytes (27.8% vs. 12.5%), nausea (16.7% vs. 0%), and diarrhea (22.2% vs. 12.5%) (Table 2), confirming the findings of the previous study.41 Treatment-related TEAEs preferentially associated with boosting doses of VXM01 were decreased in lymphocytes (22% vs. 0%), and increased in neutrophil count (16.7% vs. 0%) and diarrhea (22% vs. 0%). There were no marked differences in nature, frequency, and severity of TEAEs between the two VXM01 doses tested (data not shown).

Excretion in fecal samples was observed up to day 38 in 2 out of 12 patients (16.7%) in the lowest dose group and in 4 out of 6 patients (66.7%) in the highest dose group after the administration of VXM01. Both patients who excreted VXM01 at a dose of 10^6 CFU had a gastric bypass. No such anatomic changes were present in patients who excreted VXM01 after administration of 10^7 CFU. Subsequent stool cultures were negative without any antibiotic intervention. Neither VXM01 nor plasmid DNA was found or excreted in any other body fluids in any patient throughout the study (data not shown).

Two patients, both from the highest dose VXM01 treatment arm, were screened positive, i.e., with anti-LPS antibody indexes >1.1, for IgG antibodies against the carrier bacterium at day 0 and 38, but none of the patients were screened positive for anti-LPS IgM (Fig. S3). Seroconversion was not detected in any of the patients up to 9 mo after the first administration, except for one patient in the lower dose VXM01 treatment arm who experienced two episodes of diarrhea between months 3 and 6, presumably due to food-borne Salmonella infection, with a positive result at months 6 and 7. Moreover, we did not observe any seroconversion upon further follow-up of the patients up to 24 mo.

T cell responses

Assessment of VEGFR2-specific T cell responses was conducted by interferon gamma (IFNγ) enzyme-linked immunosorbent spot (ELISpot) analysis.41 T cells were stimulated by autologous dendritic cells (DC) (T cell:DC ratio 5:1) that were

![Figure 1](image-url). Study design including vaccination schedule and immunomonitoring time points. During the prime phase of the trial, patients received four prime doses of VXM01 or placebo as add-on to their standard chemotherapy with gemcitabine on days 1, 3, 5, and 7. Boosting vaccination with VXM01 or placebo was started 1 mo after the last prime administration and performed following a 1× per month administration schedule, as add-on to their background therapy, i.e., gemcitabine or investigator choice on days 38 (Month 1; M1), 60 (M2), 90 (M3), 120 (M4), 150 (M5), and 180 (M6). T cell response was measured during the priming phase on days 0, 4, 14, 21, and 38, and during the boosting phase on days 48 (M1+10 d), 100 (M3+10d), 190 (M6+10d), 270 (M9), and 360 (M12); IMM, immunomonitoring.
pulsed with (i) a pool of long overlapping synthetic peptides spanning the entire VEGFR2 sequence, (ii) equal amounts of human IgG that served as negative control antigen, or (iii) with a mixture of viral recall antigens derived from CMV and adenovirus as positive control. The respective original mean IFN-γ T cell responses in vaccinated patients in both treatment arms (6 out of 26) showed the presence of preexisting VEGFR2-specific T cells already before the vaccination, i.e., a significant (p < 0.05) difference between the VEGFR2 and control spot count mean values and a mean absolute spot count at D0 1.5 times higher in VEGFR2-containing wells than in control wells (data not shown). The course of VEGFR2-specific T cell responses in each individual patient was recorded as either (i) the difference between mean IFN-γ T cell responses after priming and boosting, (ii) the fold increase of VEGFR2-specific gamma spot counts in VEGFR2-containing wells and in negative control wells (Fig. 2A), (iii) the fold increase of mean spot counts in VEGFR2-containing wells over those in negative control wells (Fig. S4B). In total, 66.7% (8 out of 12) of the patients in the lower dose group and 75% (3 out of 4) of the patients in the higher dose group showed a clear increase, i.e., >1.8-fold, of VEGFR2-specific T cell responses after priming or boosting. The magnitude and time course of the IFN-γ T cell response displayed distinct response profiles encompassing immune responses (a) remaining at low baseline levels throughout the study, (b) peaking rapidly after the priming phase and returning to baseline levels, (c) peaking early after the initiation of the boosting phase and declining before the end of the boosting phase, and (d) rising gradually during and after the boosting phase. In the higher dose group, T cell responses in responding patients consistently peaked after one to three boosting vaccinations, and in all cases vaccine-specific T cells declined after the last vaccination, reaching baseline levels after 1 y in the remaining two patients.

Altogether, mean VEGFR2 T cell responses in vaccinated patients exceeded those in placebo patients (Fig. 2B–E). Due to the large heterogeneity in individual response patterns including non-responding patients, the mean difference between the placebo group and the lower VXM01 dose group was small and statistically not significant. However, VEGFR2 responses were significantly increased in the higher VXM01 dose group compared with both the placebo group and the lower dose group (Fig. 2B). Adjusted p values for group-wise comparisons of mean VEGFR2-specific IFN-
gamma spots conducted for all time points revealed respective statistical significance for increased spot counts in the high-dose group at day 90 (Fig. 2C). A statistical trend toward an increased T cell response was further detectable in this group compared with the placebo group and the lower dose group at day 60 and in comparison to the lower dose group also at day 120.

In the lower dose group, the mean VEGFR2-specific IFNγ T cell response reached a peak already after the priming phase (81 ± 155 spot-forming units (SFU) per 10^5 T cells at day 15). Subsequently, vaccine-specific T cells declined slowly over time because only a minority of the patients in this group showed a clear response to the boosting vaccination (Fig. 2B). In contrast, patients receiving the high-dose VXM01 vaccination showed an overall strong anti-VEGFR2 response particularly after the first boosting vaccination, reaching a peak value of 299 ± 180 SFU per 10^5 T cells at day 100, and remaining higher than 100 SFU per 10^5 T cells up to day 270. This strong increase of the
vaccine-specific T cell response was observed in three patients out of four (75%) that entered the boosting phase (Fig. S4A). In the placebo group, VEGFR2-specific T cell responses declined over time and were below the baseline levels beyond day 100. In contrast in both VXM01 arms vaccine-specific T cells remained elevated at 1.9 and 4.4 times the baseline level up to day 180 (Fig. S4B) and 1.1- and 16.9-times the baseline level up to day 360 in patients vaccinated with 10^6 CFU and 10^7 CFU VXM01, respectively (Fig. 2D). Due to these differences in T cell response kinetics, we compared the overall strength of VEGFR2-specific T cell response between the groups not only in regard to individual time points, but also by integrating response values at all time points as the “area under the curve.” Again, respective cumulative data shown in Fig. 2E demonstrate significantly increased VEGFR2-specific T cell responses in the high-dose group. Despite increased T cell responses in several patients there was no overall significant increase in VEGFR2-specific T cell responsiveness in the lower dose group.

For a more systematic comparison of T cell responses in vaccinated and placebo-treated patients, we graded the increase of VEGFR2-specific T cell responses throughout the observation period according to the study protocol using prefixed criteria, which had been previously determined in the frame of the previously reported VXM01 trial,41 as detailed in the Patients and Methods section. We detected increases in VEGFR2-specific T cell response grade throughout and early after the priming phase in both placebo and vaccinated patients (Fig. 2F). These were also observed in placebo-treated patients of the previous clinical trial and thus potentially related to concomitant treatment regimens applied to both patient groups. For example, gemcitabine has previously been shown to exert immune stimulatory effects in cancer patients.42,43 VEGFR2-specific T cell responses vanished over time in the placebo group, with median VEGFR2 T cell response below grade 1 after day 48 (Fig. 2F, blue line). In contrast, strong immune responses persisted in several of VXM01-vaccinated patients and the median VEGFR2-T cell response grades remained higher or equal to 1, and higher or equal to 2 up to day 270, in the low and high-dose VXM01 groups, respectively (Fig. 2F). Altogether, we observed in the boosting period pronounced, i.e., grade 2 and 3, T cell responses in 5 out of 11 patients who received at least one boosting vaccination and who had evaluable ELIspot results.

Regarding clinical outcome, we did not detect statistically significant differences between placebo and VXM01-treated patients. No clear trend was observed for VEGFA or collagen IV serum levels, and no significant difference was observed when comparing the tumor marker CA19-9 between the different treatment arms during the study. The tumor perfusion, as evaluated with DCE-MRI,44,45 only increased slightly in patients treated with 10^6 CFU VXM01 after the boosting period. No alteration in the blood pressure levels was observed in the different treatment groups in this study (data not shown).

**Discussion**

We show here that a prime-boost regimen with VXM01, a first-in-kind, oral T cell vaccine, based on recombinant live attenuated *Salmonella* typhi vector targeting VEGFR2-expressing cells, can be safely administered and can elicit strong and sustained T cell responses against VEGFR2. Side effects were mostly mild and in accordance to those reported previously for a VXM01 priming-only regimen.41 Importantly, the frequency of drug-related TEAEs were comparable after prime and boost doses indicating that further dosing with VXM01 did not increase the nature, severity, and frequency of TEAEs.

The aim of the study was not to characterize the polyfunctional nature of induced vaccine-specific T cells, nor the correlation with anti-angiogenic and clinical activities, because these issues had been already addressed in detail in the preceding VXM01 main study.41 The main objective of this study was rather to explore whether repetitive administration of VXM01 after an initial priming period could maintain higher frequencies of VEGFR2-specific T cells. We were also interested in exploring a potential dose-effect of VXM01, although the cohort size of the highest dose group was kept small because we had not observed such an effect in the VXM01 main study.41

In contrast to the main study, the results obtained from the extension study suggested an improved T cell response in the highest dose group. This might be due to the fact that the study cohorts of both trials differed from each other in regard to the levels of pre-existing VEGFR2-specific T cells that were frequent in the main study but rare in the extension study. Spontaneous T cell responses against tumor cell-associated antigens have been previously detected by us and others in a broad variety of different tumor entities, including pancreatic cancer.46-51 It appears possible that the reactivation of pre-existing T cells in the main study was efficiently induced already by lower doses of the vaccine, while higher doses may be particularly effective in eliciting and maintaining new T cell responses against the vaccination antigen. Nevertheless, data from the extension trial correspond very well to the subgroup of patients in the main study who presented with low numbers of pre-existing VEGFR2-specific T cells.41 Likewise, in both studies, the placebo groups revealed a transient early increase of VEGFR2-specific T cells during the first 2 weeks, possibly caused by concomitant immunosuppressive chemotherapies.52-54

Vaccination of pancreatic cancer patients against tumor-stroma-associated antigens such as VEGFR2 is hampered by central thymic and peripheral tolerance against auto-antigens and by the well-established strong immunosuppressive tumor microenvironment in this entity.49-51 Our study is further limited by the overall small study group, high inter-individual variations in T cell response patterns, and tumor burden among vaccinated and placebo patients and by the potentially confounding effect of the preceding gemcitabine treatment. Nevertheless, our cumulative data provide evidence for the capacity of VXM01 prime-boost regimens to induce, boost, and maintain throughout the vaccination period VEGFR2-specific T cells at elevated levels in highly advanced pancreatic cancer patients. This was particularly and consistently apparent in the higher dose group. It may be important to note that after peaking during the boosting period, VEGFR2-specific T cell responses tend to decline in most patients despite continued vaccination although their levels remained elevated as compared with baseline levels. T cell responses fully declined to baseline levels only after discontinuation of the boosting regimen. Thus, the continuation of the vaccinations might be suitable to maintain VEGFR2-specific T cells at elevated levels even in advanced
stage pancreatic cancer patients. We assume that the reduction of VEGFR2-specific T cells after the peak was either due to reduced immunogenicity of the vaccine, to tolerogenic mechanisms or exhaustion, and/or may have been related to a mobilization of induced T cells into VEGFR2-expressing target tissues.41

The intestinal mucosa represents a highly immunologically active, large, and orally accessible interface, and thus represents an attractive organ for vaccination. Not only can high numbers of T cells be activated in this mucosal compartment and the related mesenteric lymph nodes,35,36 but also are those T cells prone to acquire the cellular machinery for homing, accumulation, and proper cytotoxic activation within the gastrointestinal tract, governing gastrointestinal and mucosal tumors such as pancreatic, gastric, or colonic cancer or, potentially, lung cancer.37

In conclusion, this study in patients with highly advanced pancreatic cancer demonstrated safety and excellent tolerability of a prime-boost regimen for VXM01 as well as promising specific T cell activity, in particularly when administered at the highest doses of $10^7$ CFU. Based on the observed immunologic response and the favorable safety profile, VXM01 is being further explored in colorectal cancer and in glioblastoma, and its combination with anti-PD-L1 immune checkpoint inhibition blockade is under development for the treatment of colorectal cancer and squamous cell carcinoma of the head and neck.

**Patients and methods**

**Patients**

Eligible patients had inoperable, locally advanced, or stage IV pancreatic cancer, completed at least one cycle of gemcitabine treatment, were chemotherapy naïve within 60 d before the screening visit except gemcitabine treatment, had a Karnofsky index above 70 and an anticipated life expectancy of $> 3$ mo, and an adequate bone marrow reserve, hepatic, and renal function at the start of the trial. Exclusion criteria included autoimmune disease; major surgery or recent significant trauma; uncontrolled hypertension or arterial thromboembolic events in the prior 6 mo, congestive heart failure $\geq$grade III, serious ventricular arrhythmia, peripheral arterial occlusive disease $>\text{grade 2b}$, hemoptysis, or gastrointestinal bleeding or other relevant conditions; and concomitant treatment or treatment in the prior 2 weeks with antibiotics, anti-angiogenic anticancer therapy, systemic corticosteroids, or any other immunosuppressive agents.

**Study design**

The monocentric study (EudraCT No 2015-003068-34) was a randomized, placebo-controlled, phase I trial consisting of a double-blind priming period (four oral doses) and an open-label boosting period (up to six single monthly doses). This is an extension and bridging study to the phase I dose escalation study completed previously.41 Patients were randomly assigned to VXM01 or placebo at a ratio of 2:1 with a computer-generated random list, such that in both dose groups ($10^6$ and $10^7$ CFU), patients were also assigned to placebo. Randomization was concealed so that neither patients, nor the investigator, nor the sponsor knew which agent was being administered. To maintain masking, a sodium bicarbonate-containing drinking solution with either VXM01 or isotonic sodium chloride (placebo) was prepared by personnel not otherwise involved in the study. The data safety monitoring board (DSMB) had access to the random list. All patients were included in the safety analysis. Only minor protocol violations were recorded.

The primary objective of the study was to assess safety and immunologic responses of the prime-boost regimen. The study was reviewed by the responsible institutional review board (Ethics Committee of the Medical Faculty of Heidelberg University) and approved by the federal authority (Paul-Ehrlich-Institut, Langen, Germany). The trial was conducted according to the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference of Harmonisation. All participants provided written informed consent before entering the study.

**Treatment**

During the first part of the extension study, eligible patients were hospitalized from day 0 to day 10. The patients received the prime doses of VXM01 ($N = 18$) or placebo ($N = 6$) as add-on to their standard chemotherapy gemcitabine on days 1, 3, 5, and 7 (Fig. 1). The tested doses were $10^6$ CFU for the first dose group, and $10^7$ CFU for the second dose group. Boosting re-vaccination with up to six single oral doses of VXM01 or placebo was started 1 mo after the last prime administration and performed following a once-per-month administration schedule, as add-on to their background therapy, i.e., gemcitabine or investigator choice after day 38 (Fig. S1) on days 38 (Month 1; M1), 60 (M2), 90 (M3), 120 (M4), 150 (M5), and 180 (M6) (Fig. 1). The boosting doses were identical to the doses administered for initial vaccination (priming) as long as no reduction of dose was required due to positive fecal excretion results. Patients treated with $10^7$ CFU during prime treatment with positive excretion results received a reduced dose of $10^6$ CFU in the boosting part. Detailed activities during the prime and boosting parts of the study are provided in the flowchart (Fig. S2).

**Clinical laboratory and safety evaluation**

Hematology, clinical chemistry, coagulation, and urinalysis were measured up to M18. Medical and surgical history, cancer history, status and anticancer treatment, physical examination, vital signs, electrocardiogram, and blood pressure were also documented. AEs and TEAEs were graded according to the National Cancer Institute’s CTCAE criteria (version 4.03). In addition, VXM01 distribution and shedding was investigated in blood, saliva, tears, urine, and stool samples on days 0, 2, 4, and 8.

Anticarrier antibodies (anti-LPS IgG and IgM) as well as anti-VEGFR2 antibodies (IgG and IgM) were analyzed by a validated enzyme-linked immunosorbent assay (ELISA) according to GLP regulations on days 0, 38 and months 3, 6, and 9, by
Aurigon (Tutzing, Germany) and ATRC (Dunakeszi, Hungary) after assay transfer.

Serum levels of the angiostatic biomarkers collagen IV and VEGF-A were analyzed by commercial ELISA assays (Human Collagen IV ELISA, Serum, KT-035; Kamiya Biomedical Company, Seattle, US, and Quantikine Human VEGF-A Immunoassay; DVE00, R&D Systems GmbH, Wiesbaden, Germany, respectively) according to GLP regulations on days 0, 38 and months 3, 6, and 7 by Aurigon (Tutzing, Germany) and ATRC (Dunakeszi, Hungary) after assay transfer. The level of the tumor marker CA19-9 was measured in serum samples on days 0, 38 and months 6 and 7.

Tumor perfusion was analyzed by dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) on a 1.5 Tesla System (Siemens Avanto, Erlangen, Germany) at day 0, day 38, and months 3, 6, and 7.

**Immunologic assessment of the T cell response**

Immunomonitoring of VEGFR2-specific T cells was performed on days 0, 4, 14, 21, 38, 48 (M1+10 d), 100 (M3+10 d), 190 (M6+10 d), 270 (M9), and 360 (M12) (Fig. 1) in peripheral-blood lymphocytes isolated from cryopreserved peripheral-blood mononuclear cells (PBMCs) from all patients included in this study (Table 1) by IFNγ ELISPOT assay. PBMCs were isolated by the NCT laboratory, Heidelberg, Germany.

Whole blood was obtained by venipuncture using lithium heparin blood collection tubes (Sarstedt). Transportation of blood was done under ambient conditions to the Monitoring Laboratory. Processing of PBMCs was performed using density gradient centrifugation (Biochrome, Berlin, Germany) in 50 mL Falcon tubes (with frill) filled with ca. 18 mL of blood and ca. 15 mL of RPMI medium. PBMCs were processed and cryopreserved within a median time of 4 h after blood draw. The average cell yield, determined with trypsin blue manual count after processing, was 1.1 × 10^6 PBMCs per mL of whole blood.

For cryopreservation, slow freezing method was performed using freezing medium composed of 70% fetal calf serum (Biochrom), 20% X-VIVO 20 medium (Lonza), and 10% DMSO (Sigma Aldrich). Aliquots of 1.0 to 1.2 × 10^7 cells/mL/vial were stored in slow freezing containers (Nalgene) at −80°C for up to 3 d, and then transferred to liquid nitrogen for long-term storage.

After thawing, the average cell viability was 90% and the mean cell yield of viable cells was 70%, as determined by Trypan blue (Sigma Aldrich) viability test. As cells underwent a 1-2 day cell culture before being plated for ELISpot, no additional cell count was performed.

Autologous dendritic cells were used as antigen-presenting cells. PBMCs were thawed and cultured for 4 h, and non-adherent cells were washed off and cultured in X-VIVO 20 medium (Lonza) supplemented with 100 U/mL IL-2 and 60 U/mL IL-4 (Miltenyi Biotec, Bergisch Gladbach, Germany). For dendritic cell generation, adherent cells were cultured with 560 U/mL of recombinant human GM-CSF and 500 U/mL IL-4 (Miltenyi). After 7 d of cell culture, cells were cultured in cytokine free media (X-VIVO 20; Lonza) for 24 h and purified. Dendritic cells were enriched by negative selection using the Pan Mouse IgG Dynabeads® (Invitrogen, Darmstadt, Germany), coupled to mouse anti-human CD3, CD19 (both LifeTechnology, Oslo, Norway), and CD56 (clone: C218, Beckman Coulter, Krefeld, Germany) antibodies. T cell purification was performed using the Untouched™ Human T Cells system (Invitrogen).

The quantitative evaluation of antigen-specific T cells secreting IFNγ was conducted as described previously. ELISPOT plates (MAHA S4510; Millipore, Ireland) were coated with 1 μg of anti-IFNγ antibody (clone 1-D1K; Mabtech, Nackastrand, Sweden) per well, incubated overnight at 4°C, washed and blocked with complete RPMI medium (10% human AB serum). 2 × 10^4 dendritic cells were plated in 100 μL per well in triplicate, and loaded with 10 μg/mL of antigenic peptides. A pool of 20-mer synthetic peptides #1-135 derived from human VEGFR2 with 10 amino acids overlap (omitting #21+22+76+77) (Proimmune, Oxford, UK) was used as test peptides. A pool of 15-mer synthetic peptides covering the complete sequence of pp65 protein (CMV pp56 PepTivator) with 11 amino acids overlap, mixed with a pool of 15-mer synthetic peptides covering the complete sequence of the hexon protein of human adenovirus 5 with 11 amino acids overlap (AdV5 Hexon PepTivator), as well as SEB (Staphylococcus enterotoxin B), was used as a positive control. Human Normal Immunoglobulin (KIOVIG, Baxter) was used as a negative control. After 14 h of antigen pulsing, 1 × 10^5 purified T cells (DynaBeads) were added to each well for an additional 40-h co-culture. IFNγ spots were developed using an enzyme-coupled detection antibody system using an anti-IFNγ antibody coupled to biotin (clone 7_B6-1 diluted 1:1000, Mabtech), streptavidin ALP (diluted 1:1000, Mabtech), and NCT/BCIP substrate kit (BioRad, Munich, Germany). Plates were analyzed using an automated ELISPOT reader and ImmunoSpot V 5.0.9 Smart Count Software (CTL, Bonn, Germany) using the following spot definition settings: Size: 100%; Size Count: normal; Maximum Spot Size: 9.6250 mm^2; Minimum Spot size 0.0051 mm^2; Spot Separation: 3; Diffuse Processing: normal; Background Balance: 0–80.

The strength of the VEGFR2-specific T cell response after vaccination was graded for each time point in patients matching the acceptance criterion of a minimum mean of 10 SFU at day 0 according to the following rules: (a) grade 0: non-significant difference between test wells and negative control wells, as evaluated with t-test, or no increased difference of absolute spot numbers in test and negative control wells compared with day 0; (b) grade 1–3: significant difference between test and control wells, as evaluated using unpaired t-test, and increased ratio of absolute spot numbers in VEGFR2 to negative control wells compared with day 0 lower than three-fold (grade 1), higher than or equal three-fold and less than five-fold (grade 2), and higher than or equal five-fold (grade 3). Grading definition criteria were pre-defined in the statistical analysis plan.

The laboratory has not fully established good clinical laboratory practice conditions. Sample reception, PBMC processing, PBMC slow freezing for long-term storage, thawing of PBMCs, and cell counting were performed according to established laboratory protocols that were then approved as standard operation procedures. ELISPOT was performed according to established laboratory protocols. The study was performed
using general research investigative but pretested assays. For all experiments, raw data can be provided upon request.

**Statistical analysis**

Data were analyzed by two-tailed t-test (ELIspot), and one-way ANOVA with Tukey’s multiple comparison tests. All statistical analyses, including the area under curve analysis, were performed using GraphPad Prism v.7.01 (GraphPad Software, Inc., LaJolla, CA, USA).

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AE</td>
<td>adverse event</td>
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<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DSMB</td>
<td>data safety monitoring board</td>
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<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IMM</td>
<td>immunomonitoring</td>
</tr>
<tr>
<td>PDAC</td>
<td>pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>SFU</td>
<td>spot-forming unit</td>
</tr>
<tr>
<td>TEAE</td>
<td>treatment-emergent adverse event</td>
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<tr>
<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2</td>
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**Disclosure of potential conflicts of interest**

FHSW, MWB, WEH and PB are advisors to VAXIMM AG. HL is an officer of VAXIMM GmbH, and HL and MS own stock options of VAXIMM AG. SW and AVK are employees of VAXIMM. KB is a director of VAXIMM AG. All other authors and MWB declare that they have no conflicts of interest.

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**Author contributions**

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**Provision of study materials or patients:** FHSW, TS, NH, TF, GM, MWB, RK, CK, CS, PK, LG, WEH, and PB

**Collection and assembly of data:** FHSW, TS, LP, NH, TF, HL, MS, SW, KB, GM, MWB, AVK, RK, CS, PK, MB, LG, WEH, and PB

**Data analysis and interpretation:** FHSW, TS, LP, NH, TF, HL, MS, SW, KB, GM, MWB, AVK, RK, CS, PK, MB, LG, WEH, and PB

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