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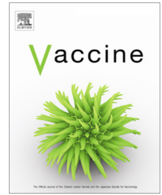

# An improved conjugate vaccine technology; induction of antibody responses to the tumor vasculature

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ABSTRACT

The induction of an antibody response against self-antigens requires a conjugate vaccine technology, where the self-antigen is conjugated to a foreign protein sequence, and the co-application of a potent adjuvant. The choice of this foreign sequence is crucial as a very strong antibody response towards it may compromise the anti-self immune response. Here, we aimed to optimize the conjugate design for application of vaccination against the tumor vasculature, using two different approaches. First, the immunogenicity of the previously employed bacterial thioredoxin (TRX) was reduced by using a truncated from (TRXtr). Second, the *Escherichia coli* proteome was scrutinized to identify alternative proteins, based on immunogenicity and potency to increase solubility, suitable for use in a conjugate vaccine. This technology was used for vaccination against a marker of the tumor vasculature, the well-known extra domain B (EDB) of fibronectin. We demonstrate that engineering of the foreign sequence of a conjugate vaccine can significantly improve antibody production. The TRXtr construct outperformed the one containing full-length TRX, for the production of anti-self antibodies to EDB. In addition, efficient tumor growth inhibition was observed with the new TRXtr-EDB vaccine. Microvessel density was decreased and enhanced leukocyte infiltration was observed, indicative of an active immune response directed against the tumor vasculature.

Summarizing, we have identified a truncated form of the foreign antigen TRX that can improve conjugate vaccine technology for induction of anti-self antibody titers. This technology was named ImmunoBoost (I-Boost). Our findings are important for the clinical development of cancer vaccines directed against self antigens, e.g. the ones selectively found in the tumor vasculature.

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## 1. Introduction

Several recent reports mark the current interest in cancer vaccination based on targeting the tumor vasculature [1–4], because angiogenesis is a hallmark of tumor growth [5–8]. Importantly, there is an immense interest in the induction of antibody responses, due to the eminent success of antibody-based drugs. Cancer vaccines aim to generate an immune response against a determinant of the tumor. Such determinants can be viral antigens, mutated self-proteins or specifically expressed self-antigens. Due to several central and peripheral tolerance mechanisms, it is extremely challenging to induce an immune response to cancer-expressed self-antigens. Central tolerance includes the deletion of autoreactive T cells during embryonic development in the thymus and the selection of B cells in the bone marrow [9,10]. In addition, there are several peripheral tolerance mechanisms, e.g. the requirement for involvement of different co-stimulatory molecules to ensure proper T cell and B cell activation [11].

In previous work we have shown that it is possible to break self-tolerance in mice, and to induce antibody responses against the tumor vascular markers extra domain A (EDA) and B (EDB) of the extracellular matrix molecule fibronectin [12,13]. This was achieved by conjugating the self-protein (EDA/EDB) to a foreign antigen, the bacterial thioredoxin (TRX). The presence of antibodies inhibited primary tumor growth as well as metastasis formation.

Although this approach worked relatively well, it was observed by us and others [14–16] that not all mice developed antibodies to the self-antigen. In addition, the antibody production against the bacterial domain of the conjugate protein was relatively high, as

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compared to the amount of antibodies produced against the self-part of the molecule. It was hypothesized that a too strong anti-TRX response can suppress the response to the self-antigen by a phenomenon that is called epitope suppression [14,17]. In such case there is a preferred stimulation of TRX-specific B cells since no immune tolerance needs to be broken to stimulate these cells [15]. An additional disadvantage of a high anti-TRX antibody response is the rapid clearance of the fusion protein after vaccination, leaving less fusion protein available for antigen presentation by dendritic cells or B cells [16].

To address these issues and to improve the antibody response towards the self-antigen we followed two approaches. First, a fusion protein was generated with a truncated form of TRX (TRXtrunc, TRXtr), composed of the C-terminal 58 amino acids of the full-length TRX protein, to reduce the TRX immunogenicity. In a second approach we scrutinized the *Escherichia coli* (*E. coli*) proteome to identify proteins similar in size to TRX, suitable to be used in the fusion protein instead of TRX. Proteins were further selected based on their predicted immunogenicity and their potency to increase solubility of the fusion protein. Also, a chimeric molecule was engineered, containing different domains of these *E. coli* proteins. A series of new vaccines was tested for antibody production against the tumor vasculature and anti-tumor efficacy. The results show the generation of one significantly improved conjugate vaccine. This technology shows to have impact for the development of new vaccines for treatment of cancer patients.

## 2. Results

### 2.1. Design and production of engineered conjugate vaccines

To test the hypothesis that a conjugate vaccine, consisting of a self-antigen fused to bacterial thioredoxin (TRX uniprot#P0AA25, 109 aa, 11.8 kDa), can be improved by optimized design of the non-self fusion partner, two approaches were followed. The first approach aimed at reducing the anti-TRX response in favor of the anti-self response, by truncating the TRX sequence in the vaccine. We designed TRXtrunc (TRXtr), a 6.2 kDa variant of the full-length protein, consisting of the C-terminal 58 amino acids of the molecule. In a second approach, we scrutinized the *E. coli* proteome (strain K12; uniprot proteome ID#UP000000625) to identify proteins with a similar size as TRX and with comparable physicochemical properties, i.e. with a comparable percentage of bulky hydrophilic and charged amino acids (Table 1) [18]. This resulted in the selection of type-1 fimbrial protein, A chain (TFP, uniprot#P04128; 13 kDa). For TFP we generated a variant where a number of alanine and glycine residues were replaced by lysine or aspartic acid residues, to increase immunogenicity (TFPv, Table S1, Fig. 1B). In addition, a protein was designed consisting of several stretches of predicted highly soluble peptide sequences of TFP and two other *E. coli* proteins, i.e. the cell division protein ZapB (uniprot#P0AF36.1; 9.6 kDa) and the small heat shock protein IbpA (uniprot#P0C054; 15.8 kDa). This designer protein was referred to as the chimeric designer peptide (CDP) (Fig. 1B).

**Table 1**
Molecular characteristics of the different non-self fusion partners.

| | Bulky hydrophilic/charged aa[a] | Total # aa[b] | Ratio[c] | Percentage[d] |
|---|---|---|---|---|
| TRX | 31 | 109 | 0.40 | 28% |
| TRXtr | 14 | 59[e] | 0.31 | 24% |
| TFP | 23 | 132 | 0.21 | 17% |
| TFPv | 27 | 132 | 0.26 | 20% |
| CDP | 10 | 61 | 0.20 | 16% |

[a] Amino acids.
[b] Total number of amino acids in the protein.
[c] Ratio of bulky hydrophilic/charged amino acids and remaining amino acids.
[d] Percentage bulky hydrophilic/charged in the total protein.
[e] Total 59 aa (58 aa + methionine; start site codon).

Fusion genes (Fig. 1A) for parent proteins and designer peptides with the self-antigen EDB (Fig. 1B) were produced in the *E. coli* strain Rosetta gami DE3. SDS-Page analyses showed that all proteins were efficiently produced (Fig. 1C). Purified proteins were subsequently used for induction of antibody responses in mice. Comparisons were made with the previously used EDB vaccine (TRX-EDB), that contains full-length TRX [12], as well as with a vaccine containing only TRX. Unconjugated EDB and TRX were prepared for ELISA based detection of antibodies against both domains.

### 2.2. Novel non-self fusion partners are superior to TRX in the production of anti-self antibodies

We investigated whether reducing the size of TRX, and thereby its immunogenicity, can enhance the antibody response to the self-antigen EDB. C57BL/6 mice were vaccinated with purified proteins in the presence of Freund's Complete Adjuvant (FCA). Subsequent booster vaccinations were given in the presence of Freund's Incomplete Adjuvant (FIA), according to the vaccination scheme shown in Fig. 2A. Blood was drawn one week after each vaccination and antibody titers against EDB and TRX were measured by ELISA. Booster vaccinations are required to induce a proper immune response against EDB (Fig. 2B). We found that the alternative non-self fusion partners outperformed full-length TRX at inducing antibodies to the self-antigen EDB (Fig. 2C; time-point S4; $^{***}P < 0.0001$ for TRXtr-EDB and TFP-EDB, $^{**}P < 0.001$ for TFPv-EDB and not significant (ns) $P = 0.1049$ for CDP-EDB). In direct comparisons TRXtr- and TFP-conjugates showed higher antibody titers against EDB than TFPv and CDP-conjugates (Supplementary Fig. S1). To investigate the importance of the non-self antigenic part, antibody titers against TRX and EDB were measured in the sera of mice vaccinated with TRX alone, TRX-EDB and TRXtr-EDB. Interestingly, while the TRXtr conjugate induced lower antibody titers against TRX, as compared to the full-length TRX conjugate (Supplementary Fig. S2), the response to the self-antigen EDB was much higher after vaccination with the TRXtr conjugate (Fig. 2D; $^{***}P < 0.0001$).

In preclinical experiments using T241 fibrosarcoma tumors implanted in C57BL/6 mice, TRXtr-EDB outperformed TRX-EDB at inhibiting tumor growth (Fig. 3A; $^{*}P < 0.05$). Body weights of the mice remained stable, demonstrating absence of toxicity (Fig. 3B).

The same technology was also applied for vaccination against murine lysyl oxidase (LOX, UniProtKB/Swiss-Prot: P28301; LYOX_-Mouse). After treatment with the vaccine TRXtr-LOX tumor growth was significantly inhibited by 38% in an immunocompentent subcutaneous CT26 colon carcinoma model as compared to the TRXtr alone used as control vaccine (Supplementary Fig. S3).

Vaccination against the tumor vascular marker EDB reduced tumor microvessel density (Fig. 3C and D). Also in these experiments TRXtr-EDB outperformed TRX-EDB at suppression of vessel density. In experiments investigating the enhanced immunity by the vaccines, it was observed that both the TRXtr-EDB and the TRX-EDB vaccines increased the number of tumor infiltrating leukocytes (Fig. 3E, $^{**}P < 0.005$ (TRX-EDB group); $^{**}P < 0.001$ (TRXtr-EDB group)).

## 3. Discussion

In order to break tolerance and induce antibodies against a self-antigen, it is possible to vaccinate with a conjugate of the self-protein with a foreign antigen. The current report presents an improved conjugate vaccine technology, referred to as Immuno-Boost (I-Boost), based on modulation of the antigenicity of the

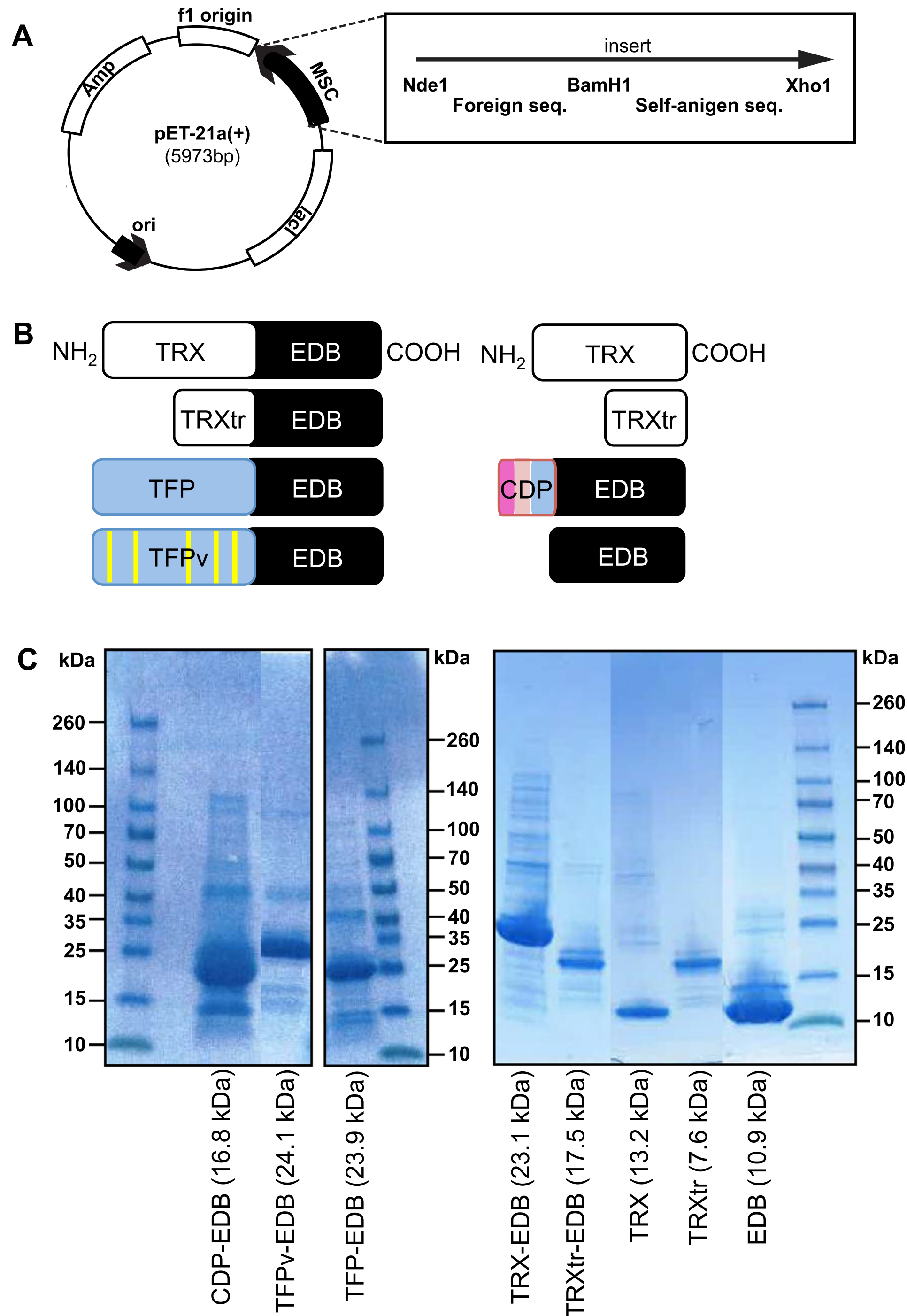


**Fig. 1.** Fusion protein constructs. (A) The pET21a(+) expression vector for production of the different fusion protein constructs. Protein expression is under the control of the IPTG-inducible T7*lac* promoter. MSC, multiple cloning site; lacI, lacI gene; Amp, ampicillin resistance gene. The DNA sequences of the different protein constructs (insert) were inserted between the restriction sites Nde1, BamH1 and Xho1 of the MSC. (B) The engineered protein constructs. The fusion proteins TRX-EDB (20 kDa), TRXtr-EDB (17.5 kDa), TFP-EDB (23.9 kDa), TFPv-EDB (24.1 kDa) and CDP-EDB (16.8 kDa) were used for vaccination. Amino acid replacements in TFPv are indicated in yellow. CDP is composed of peptide stretches derived from ZapB (indicated in pink), IbpA (salmon colored) and TFP (blue). The TRX (13.2 kDa) and TRXtr (7.6 kDa) protein. The EDB (10.9 kDa) protein was used for detection of antibodies in ELISA. (C) Identification of the proteins used for vaccination on reducing SDS-PAGE after purification. The gel was stained with colloidal Coomassie blue solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

non-self part, in favor of the antibody response to the self-antigen. We show this principle by generating antibody responses against the tumor vascular antigen EDB in the preclinical T241 fibrosarcoma cancer model. These results provide impact for translation of this improved technology into the clinic.

Breaking the humoral immune tolerance for self-antigens is still a major challenge that requires a conjugate vaccine composed of the self-antigen linked to a foreign immunogen and a potent adjuvant. Often, bacterial thioredoxin (TRX) is used as the foreign antigen, because of its small size and capacity to increase the solubility of the conjugate protein. The efficiency of such a vaccine is dependent on the amount of antibodies induced against the chosen tumor vascular self-antigen. We have previously shown that the anti-self antibody response against EDB is decreased as the amount

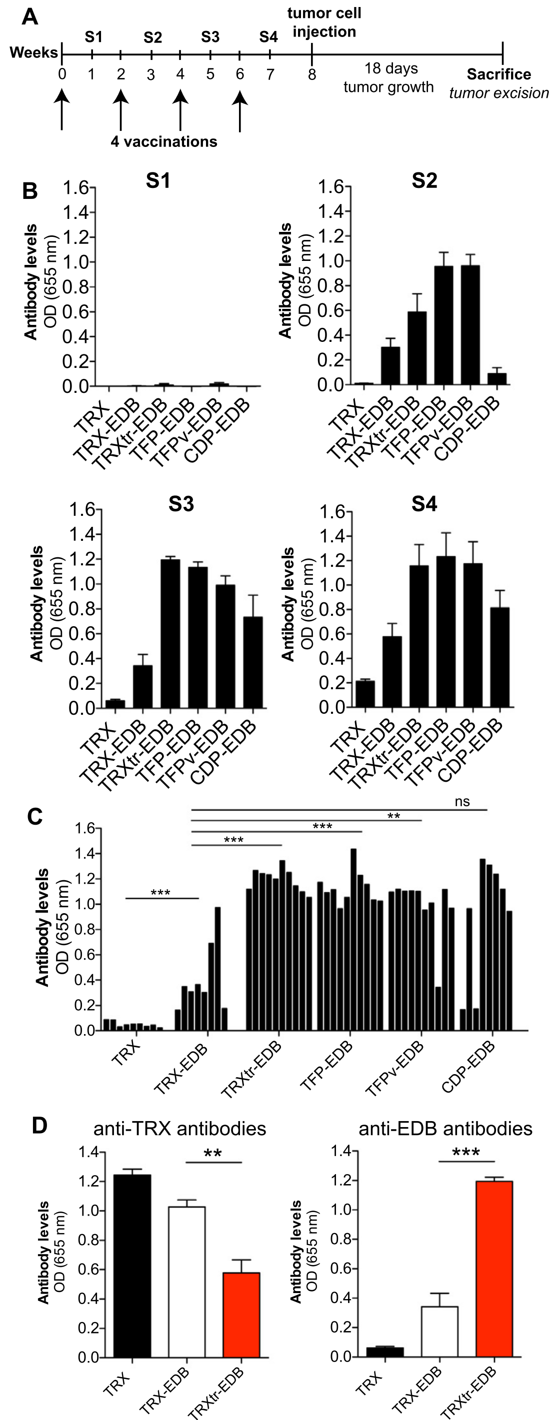


**Fig. 2.** Novel fusion partners are superior to TRX in inducing antibodies to the self-antigen EDB. (A) Schematic overview of the vaccination and tumor growth schedule. Mice are vaccinated 4 times with 2-week intervals (arrows). One week after each vaccination blood samples (S1-S4) are taken. One week after the last booster vaccination tumor cells are injected subcutaneously into the flank and tumor growth is monitored. (B) Anti-EDB antibody levels per treatment group (n = 8–10 mice/group) and blood sampling time-point (S1-S4). Each bar represents one treatment group. Antibodies were assayed at a serum dilution of 1:150. (C) ELISA was used to analyze the level of anti-EDB antibodies at time-point S4 in the serum of the mice (n = 8–10 mice/group) vaccinated with the different constructs (TRX; TRX-EDB; TRXtr-EDB; TFP-EDB; TFPv-EDB and CDP-EDB). Serum of mice vaccinated with TRX served as a control (***P < 0.0001 for TRXtr-EDB and TFP-EDB, ** P < 0.001 for TFPv-EDB and ns P = 0.1049 for CDP-EDB). Each bar represents an individual mouse at serum dilution 1:150. D) Antibody levels in the differently vaccinated mice time-point S4 towards TRX (left panel, serum dilution 1:1000, ***P < 0.0005) and the self-antigen EDB (right panel, serum dilution 1:150, *** P < 0.0001). Data are represented as mean ± SEM (n = 8–10 mice/group).

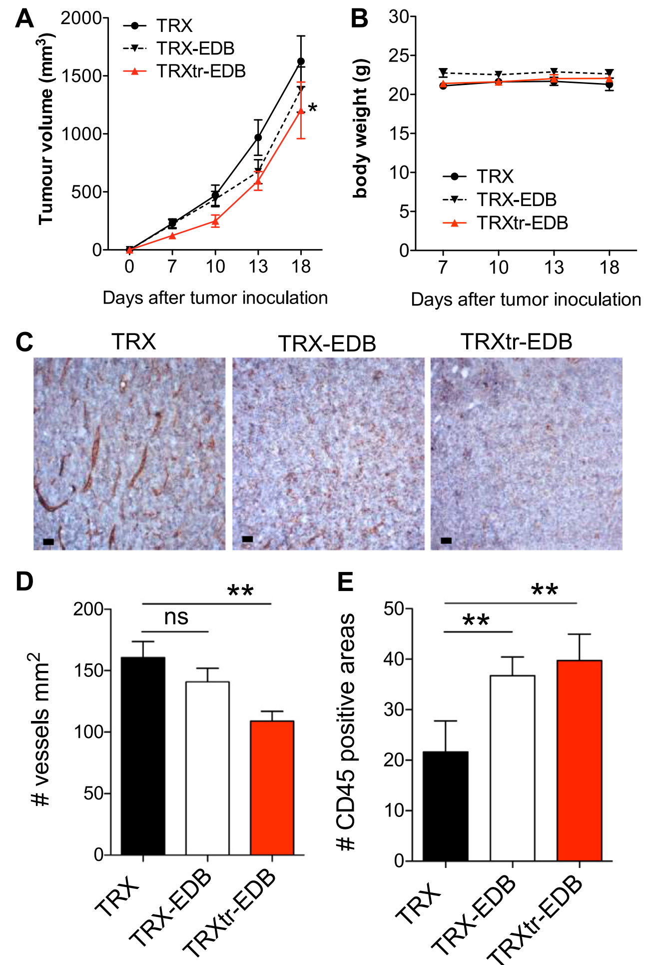


**Fig. 3.** Vaccination against EDB inhibits angiogenesis and induces leukocyte infiltration. (A) T241 fibrosarcoma tumor growth curves of the different treatment groups displayed as mean ± SEM (n = 8–10 mice/group). In the TRXtr-EDB group tumor growth was significantly (* P < 0.05) inhibited compared to control vaccinated mice (TRX group). B) Body weight curves of the mice after tumor cell inoculation. (Mean ± SEM, n = 8–10 mice/group). (C) Representative images of T241 tumors, CD31 staining, scale bar 35 μm. (D) Microvessel density in tumors of control (TRX, black bars) and TRX-EDB (white bars) or TRXtr-EDB (red bars) vaccinated mice; 3 fields/tumor were analyzed in 8–10 tumors/group. (E) T241 tumors of mice vaccinated against EDB show an increased infiltration of leukocytes (TRX-EDB ** P<0.005; TRXtr-EDB ** P<0.001); 3 fields/tumor were counted and 8–10 tumors/group analyzed. Data are presented as mean ± SEM.

of TRX is increased [16]. This can be explained by the fact that IgG antibody responses are regulated by epitope density and CD21 mediated co-stimulation [19]. This observation and the fact that not all mice developed antibodies against conjugate vaccines containing full-length TRX, led to the hypothesis that the antibody response is dependent on the non-self to self-ratio in the vaccine fusion protein. Therefore, we truncated the TRX sequence or replaced TRX by a different non-self antigen with similar characteristics to improve the conjugate vaccination approach. Indeed, TRX truncation efficiently enhanced the amount of antibodies against EDB, through decreasing the antigenicity of the TRX sequence. The necessity of the requirement of the foreign antigen TRX to be conjugated to the self-antigen has been demonstrated before [16]. In this paper we show that the TRX-part is absolutely required to induce an immune response against EDB and that vaccination with EDB alone does not induce an antibody response against EDB.

The results presented on the used vaccines are obtained from a preventive setting rather than a therapeutic setting, where vaccination is preformed after formation of an established tumor. Subcutaneous models as used in this paper do not allow a therapeutic setting, because of the fact that tumors develop very rapidly and mice die within a period of 3–4 weeks. To address this issue, we have previously used the MMTV-PyMT transgenic model of breast cancer. Since EDB is not expressed in this model we approached the question with a vaccine against the extra domain A of fibronectin (EDA). It was observed that the vaccine attenuated the progression and metastasis formation in this model [13].

An interesting result of the current study is the finding of enhanced numbers of immune cells infiltrating the tumor tissue. This demonstrates that at least part of the efficacy of the vaccine is through cell based effector mechanisms, presumably antibody dependent cellular cytotoxicity (ADCC). However, complement dependent cytotoxicity (CDC) cannot be excluded, neither can a direct cellular effect on endothelial (and tumor) cells by the ligation of EDB. This may also explain why no difference in the amount of CD45+ cell infiltrate was observed between the mice treated with the TRX-EDB or TRXtr-EDB vaccine. We have looked into more detail at the immune response induced with the TRX-EDB vaccine [12] and found that the major subclass induced by the vaccine was IgG1, which is characteristic of a Th2-response, thus an antibody-mediated immune response in mice. Furthermore, we found macrophages trying to engulf the tumor vessels in the tumors of mice vaccinated with TRX-EDB, increased fibrinogen leakage and increased neutrophil infiltration in these tumors, which are all indicative of ADCC and CDC [12].

The lower titers against EDB observed following vaccination with TRX-EDB may be explained by considering epitope suppression or increased clearance of the fusion protein from the injection site because many antibodies against TRX are present in the circulation (Supplementary Fig. S2). Indeed, as hypothesized, with the designed novel non-self fusion partners (TRXtr, TFP, TFPv and CDP) higher antibody titers against the self-antigen EDB can be induced, which is likely the result of less epitope suppression. Thus, the immunogenicity of the novel non-self fusion partners was indeed reduced compared to TRX. Unfortunately, the amount of antibodies against the self-antigen EDB was not directly correlated to the anti-tumor response, when TFP, TFPv and CDP were used, which made TRXtr the preferred antigen for fusion.

The current study demonstrates that several alternative non-self fusion partners in conjugate vaccines can elicit strong and specific antibody responses against self-antigens. We also showed here that this technology allows us to generate vaccines against tumor vasculature to inhibit tumor growth. Previous data show that the immune response generated with TRX-EDB combined with Freund's adjuvant lasts for about 7 months [20], but that longevity of the response is dependent on the adjuvant used. We have no such data for TRXtr-EDB, but we expect longevity of the response to be similar to the response induced with TRX-EDB, since Freund's was used as an adjuvant here. Although Freund's was used as an adjuvant here, for translation into the clinic the adjuvant can be replaced by the biodegradable adjuvant Montanide ISA 720, in combination with a CpG B oligonucleotide, a TRL9 agonist [20]. The adjuvant Montanide ISA 720/CpG showed a comparable immune response towards EDB as the response induced with Freund's adjuvant.

Our findings suggest that the new vaccination technology against the angiogenic tumor vasculature is safe and can be applied in combination with other innovative or conventional anti-cancer treatment regimens. This technology was named Immuno-Boost (I-Boost) and can also be used for the induction of an antibody response against other self-antigens in different preclinical models.

## 4. Experimental procedures

### 4.1. Non-self fusion partners

The following proteins were identified from the *E. coli* proteome (strain K12; uniprot proteome ID#UP000000625) with a similar size or smaller than bacterial thioredoxin (TRX, uniprot#P0AA25, 109 aa, 11.8 kDa; [21]) and similar physicochemical properties: Type-1 fimbrial protein, A chain (TFP; 18 kDa; acc no. UniProtKB/Swiss-Prot P04128), cell division protein ZapB (9.6 kDa; acc no. UniProtKB/Swiss-Prot P0AF36.1) and the small heat shock protein IbpA (15.8 kDa; acc no. UniProtKB/Swiss-Prot P0C054). Protein sequences including the modifications as described are given in the Supplementary material. The immunogenicity and solubility of TRX was determined by identifying the number of amino acids with bulky hydrophilic side chains and the number of amino acids with charged side chains, defined as: glutamine (Gln, Q), arginine (Arg, R), histidine (His, H), lysine (Lys, K), aspartic acid (Asp, D) and glutamic acid (Glu, E). The ratio of bulky hydrophilic/charged amino acids and remaining amino acids within the TRX protein is 0.40 (Table 1).

In order to decrease the immunogenicity of TRX, a truncated form of thioredoxin, named TRXtr (TRXtrunc) was designed. For the TRXtr the C-terminal part of the full-length thioredoxin sequence (aa 51–109) was used, thus including the last 58 C-terminal amino acids of the protein. Compared to full-length thioredoxin, the TRXtr has a ratio of bulky hydrophilic/charged amino acid and remaining amino acids of 0.31 (Table 1). Of the full-length TFP protein only the protein sequence without the signaling peptide sequence was used (aa 24–182; 13 kDa). The ratio of bulky hydrophilic/charged amino acids and remaining amino acids within the TFP protein is 0.21. We also generated a TFPvariant (TFPv) protein by mutating a number of alanine (Ala, A) or glycine residues (Gly, G) to lysine or aspartic acid, (Table 1; Supplementary Experimental Procedures); and thereby a TFP protein with higher immunogenicity and solubility.

To generate a protein with high solubility, peptide stretches predicted to have high solubility were selected from the TFP, ZapB and IbpA protein sequence (Supplementary Experimental Procedures). The ratio of hydrophilic and hydrophobic amino acids within CDP is 0.09. To increase the immunogenicity of CDP some alanine or glycine residues were mutated to lysine or aspartic acid residues to obtain a ratio of 0.20 (Table 1; Supplementary Experimental Procedures). Since CDP was artificially generated we checked if no identical proteins were present within the mouse or human proteome. To this end a protein BLAST using the NCBI BLASTP tool 2.6.1 [22,23], was performed. The CDP protein sequence was compared to the reference proteins (refseq_protein) and Protein Data Bank proteins (pdb) of Mus musculus (taxid:10090) and Homo sapiens (taxid:9606). No significant similarity was found between proteins present in the mouse or human proteome.

### 4.2. Fusion protein constructs

The expression vector pET21a TRX-EDB was constructed as previously described [12]. The pET21-TRX vector was a kind gift of Dr. Anna-Karin Olsson (Uppsala University, Uppsala, Sweden). A schematic representation of the plasmid construct is depicted in Fig. 1A. The TRX sequence in the pET21a TRX-EDB vector was replaced with a PCR-amplified TRXtr sequence (192 bp). The following primers were used: *forward primer* 5′-TATCATATGGG CAAACTGACCGTTGCAAAACTGA-3′ and *reverse primer* 5′-AGCG GATCCGCTACCAGAACCAGAACCGGCCAG-3′. The resulting vector construct was named pET21a TRXtr-EDB. To construct the

pET21a-TRXtr plasmid, the TRX-EDB sequence was restricted from the plasmid by Nde1 and Xho1 restriction enzyme (Thermo Scientific, Waltham, MA, USA) cleavage and replaced by the TXRtr sequence. The sequences for the Type-1 fimbrial protein, A chain (aa 24–182) (TFP; 393 bp; acc no. UniProtKB/Swiss-Prot P04128), TFPvariant (TFPv; 393 bp) or chimeric designer peptide (CDP; 183 bp) were ordered from Genscript (Piscataway, NJ, USA) and inserted in frame upstream of the EDB domain, in the pET21a expression vector.

### 4.3. Expression and purification of the different fusion-proteins

Proteins were expressed and purified as previously described [12]. Briefly, overnight cultures of *E. coli* Rosetta gami (DE3) (Novagen; EMD Chemicals) were diluted 1:2 or 1:3 and grown until $OD_{600}$ (optical density) 0.5. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Invitrogen, Life Technologies, CA, USA) for 4 h at 37 °C or at 22 °C for 16 h. Bacterial pellets were dissolved in PBS, 5 M urea/PBS or 8 M urea/PBS (Acros Organic-Thermo Fisher Scientific, Geel, Belgium), depending on the protein to be purified. Proteins were released from the bacterial pellets by sonication on ice for 12 cycles (30 s on and 30 s off) or 15 × 20 s on and 30 s off on ice (Soniprep 150 MSE, amplitude 18–26 μm). Bacterial debris was pelleted by centrifugation at 4500 rpm (3584g) (Hettich Rotina 420R, Geldermalsen, The Netherlands) and the supernatant was collected. The supernatant was then mixed with Ni-NTA agarose slurry (Qiagen, Hilden, Germany) and incubated "end-over-end" for 3 h at 4 °C. For purification of the TRX protein 10 mM imidazole was added during the Ni-agarose incubation step. The Ni-NTA agarose was pelleted, washed and transferred to a column, a syringe with a glass filter (Satorius Stedim Biotech GmBH, Göttingen, Germany). Depending on the protein purified the column was washed 10 mM or 20 mM imidazole (J.T. Baker, Avantor Performance Materials B.V., Deventer, The Netherlands) in 20 mM Tris (pH 8.0)/0.1 M NaCl to remove non-specific/background protein binding (Supplementary material). Final protein was eluted in four fractions of 200 mM imidazole. TRX-EDB protein was produced and purified as previously described [12]. Protein containing fractions of TRX-EDB, TRXtr-EDB, TFP-EDB and TFPv-EDB were pooled and dialyzed against PBS. Final protein concentration was estimated by comparison with BSA fraction V (Roche Diagnostics, Mannheim, Germany) standard on SDS-PAGE stained with colloidal Coomassie blue solution and by a protein quantification assay (Micro BCA Protein Assay, Pierce Biotechnology, Rockford, IL, USA). Protein identity was confirmed by mass-spectrometry. Recombinant EDB protein was a kind gift from Dr. Anna-Karin Olsson (Uppsala University, Uppsala, Sweden).

### 4.4. Cell culture

T241 fibrosarcoma cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in DMEM (cat no. BE12-604F, Lonza Biowhittaker, Leusden, The Netherlands) supplemented with 2 mM L-glutamine (cat no. 17-605C, Lonza Biowhittaker), 100 U/ml pen/strep (cat. no. DE17-602E Lonza Biowhittaker) and 10% FCS (cat. no. S1810-500, Biowest, Nuaillé, France).

### 4.5. Animal experiments

All animal experiments were approved by the local animal ethics board of the VU University Medical Center (Dierexperimentencommissie (DEC), registration no. AngL 14-01) and were in accordance with Dutch guidelines and law on animal experimentation. Approximately 8-weeks old female C57BL/6 (ENVIGO, Horst, The Netherlands) (n = 8–10 per group) were vaccinated 4 times in 2-weeks intervals with 50 μl vaccine in each groin (total injection volume 100 μl). Fusion proteins were mixed 50:50 with either Freund's complete adjuvant (F-5881, Sigma-Aldrich, Zwijndrecht, The Netherlands) for primer vaccinations, or with Freund's incomplete adjuvant (F-5506, Sigma-Aldrich) for booster vaccinations. The amount of protein administered/injected per vaccination was determined by the size/Mw of the protein (Table S1). One week after each vaccination blood was drawn from the tail vein. Two weeks after the last booster vaccination all mice were inoculated in their right flank with $0.5 \times 10^6$ T241 fiborsarcoma cells (C57BL/6 background) in a volume of 100 μl. Tumor volume was measured with a caliper and calculated according to the formula $width^2 \times length \times \pi/6$. Mice were sacrificed 18 days after tumor cell inoculation.

### 4.6. ELISA

Detection of antibodies by ELISA was performed as previously described [16,24]. Briefly, microtiter plates (Nunc, Maxisorp, cat. no. 44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 8 μg/ml recombinant EDB or TRX protein in PBS for 1 h at 37 °C. Plates were blocked with horse serum (H1138, Sigma-Aldrich) for 1 h at 37 °C. For detection of EDB antibodies sera were assayed at a dilution of 1:150 or a dilution series (1:100, 1:300, 1:900, 1:2700, 1:8100 and 1:24,300) was made. For detection of TRX antibodies sera were assayed at a dilution of 1:1000 or as a dilution series (1:1000, 1:3000, 1:9000, 1:27,000, 1:81,000). Sera were incubated for 45 min at 37 °C. For detection, plates were incubated with a biotinylated polyclonal goat anti-mouse antibody (E0433, DAKO, Agilent Technologies, Glostrup, Denmark), diluted 1:2000 in PBS-T 0.01%, 45 min at 37 °C, followed by incubation with Streptavidin-HRP (P0397, DAKO A/S, Hervelee, Belgium), diluted 1:2000 (concentration 0.4 μg/ml) in PBS-T 0.01%, 30 min at 37 °C. Plates were developed with TMB (T8665, Sigma-Aldrich) and absorbance was read at 655 nm with a Biotek Synergy HT plate reader (BioTek, Bad Friedrichshall, Germany).

### 4.7. Immunohistochemistry

T241 tumor tissues were paraffin embedded and sectioned (5 μm) with a Leica RM 2135 microtome (Leica, Nieuw-Vennep, The Netherlands). Sections were deparaffinized with xylene (VWR International) followed by 100% (Nedalco), 96% and 70% ethanol and rehydration in phosphate buffered saline (PBS). Endogenous peroxidase was blocked by incubating the slides in 0.3% $H_2O_2$/PBS for 15 min at room temperature (RT) (Hydrogen peroxide 30%, BDH Prolabo, VWR International, Amsterdam, The Netherlands). Antigens were retrieved in 10 mM sodium citrate buffer pH 6.0 and autoclaving. After cooling down, sections were blocked with 3% Bovine Serum Albumin Fraction V (BSA, Roche Diagnostics, Penzberg, Germany)/PBS for 1 h at RT and incubated with CD31 (PECAM-1) rat anti-mouse monoclonal antibody IgG2a (DIA310M, clone SZ31, Lot#1053, 0.2 mg/ml, Dianova GmbH, Hamburg, Germany) diluted 1:50 in 0.5% BSA/PBS overnight at 4 °C. Then tissue sections were incubated with donkey biotinylated anti-rat IgG antibody (product code: 712-067-003, Lot#126489, 1.3 mg/ml, Jackson ImmunoResearch laboratories, Baltimore, PA, USA) diluted 1:500 in 0.5% BSA/PBS for 45 min at RT. For staining of CD45 sections were incubated with CD45 rabbit anti-human/mouse polyclonal antibody (ab10558, Lot#GR269008-1, 1 mg/ml, Abcam, Cambridge, UK) diluted 1:100 in 0.5% BSA/PBS overnight at 4 °C. As secondary antibody swine biotinylated anti-rabbit polyclonal antibody (E0353, Lot#00095809, 0.51 g/l, Dako A/S, Heverlee, Belgium) diluted 1:500 in 0.5% BSA/PBS for 45 min at RT was used. This was followed by incubation with Streptavidin-HRP (P0397,

Lot#00077168, Dako) diluted 1:200 for 30 min at RT and 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB) staining (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands). Sections were counterstained with Mayer's hematoxylin (Klinipath, Duiven, The Netherlands) for 30 s and the reaction was stopped under running tap water for 10 min. Finally, sections were dehydrated in 70% ethanol, 96% ethanol and 100% ethanol for 2 min consecutively and mounted with Quick D mounting medium (Klinipath).

Microvessel density was assessed by manual counting of tumor tissue stained for CD31. In total 3 fields/tumor (100× magnification) and 8–10 tumors per experimental group. Pictures were captured with an Olympus BX50 microscope (Olympus Optical Co. GmbH, Hamburg, Germany) equipped with a CMEX DC 5000C camera (Euromex microscopes, Arnhem, The Netherlands). Leukocyte (CD45) positive patches were counted manually in 3 fields/tumor (100× magnification; 8–10 tumors per experimental group).

### 4.8. Statistical analysis

Means were compared with a Mann-Whitney *U* test or two-tailed student's *t* test, if Gaussian distribution could be assumed. For comparison of tumor growth a two-way ANOVA with Bonferroni post-test was used for repeated measurements at different time points. Values of $P < 0.05$ were considered statistically significant.

## Authorship contributions

EJH designed research, performed experiments, analyzed data and wrote the manuscript; CTL and SL performed experiments, analyzed data and edited the manuscript; PNS, JvB and KHM designed research, AWG designed research and wrote the manuscript.

## Conflict of interest

The authors declare to have no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.03.064.

## References

[1] Wentink MQ, Huijbers EJM, de Gruijl TD, Verheul HMW, Olsson A-K, Griffioen AW. Vaccination approach to anti-angiogenic treatment of cancer. Biochim Biophys Acta 2015;1855:155–71.
[2] Wentink MQ, Hackeng TM, Tabruyn SP, Puijk WC, Schwamborn K, Altschuh D, et al. Targeted vaccination against the bevacizumab binding site on VEGF using 3D-structured peptides elicits efficient antitumor activity. Proc Natl Acad Sci USA 2016;113:12532–7.
[3] Huijbers EJM, Griffioen AW. The revival of cancer vaccines – the eminent need to activate humoral immunity. Hum Vaccine Immunother 2017;13:1112–4.
[4] Zhuang X, Ahmed F, Zhang Y, Ferguson HJ, Steele JC, Steven NM, et al. Robo4 vaccines induce antibodies that retard tumor growth. Angiogenesis 2015;18:83–95.
[5] Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995;1:27–31.
[6] Griffioen AW, Molema G. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 2000;52:237–68.
[7] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
[8] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
[9] Boehm T. Thymus development and function. Curr Opin Immunol 2008;20:178–84.
[10] Busslinger M. Transcriptional control of early B cell development. Annu Rev Immunol 2004;22:55–79.
[11] Abbas AK, Lichtman AHH, Pillai S. Cellular and molecular immunology. Elsevier Health Sciences; 2011.
[12] Huijbers EJM, Ringvall M, Femel J, Kalamajski S, Lukinius A, Abrink M, et al. Vaccination against the extra domain-B of fibronectin as a novel tumor therapy. FASEB J 2010;24:4535–44.
[13] Femel J, Huijbers EJM, Saupe F, Cedervall J, Zhang L, Roswall P, et al. Therapeutic vaccination against fibronectin ED-A attenuates progression of metastatic breast cancer. Oncotarget 2014;5:12418–27.
[14] Sad S, Gupta HM, Talwar GP, Raghupathy R. Carrier-induced suppression of the antibody response to a 'self' hapten. Immunology 1991;74:223–7.
[15] Schutze MP, Deriaud E, Przewlocki G, LeClerc C. Carrier-induced epitopic suppression is initiated through clonal dominance. J Immunol 1989;142:2635–40.
[16] Saupe F, Huijbers EJM, Hein T, Femel J, Cedervall J, Olsson A-K, et al. Vaccines targeting self-antigens: mechanisms and efficacy-determining parameters. FASEB J 2015;29:3253–62.
[17] Herzenberg LA, Tokuhisa T. Epitope-specific regulation. I. Carrier-specific induction of suppression for IgG anti-hapten antibody responses. J Exp Med 1982;155:1730–40.
[18] Onda M, Beers R, Xiang L, Nagata S, Wang Q-C, Pastan I. An immunotoxin with greatly reduced immunogenicity by identification and removal of B cell epitopes. Proc Natl Acad Sci USA 2008;105:11311–6.
[19] Jegerlehner A, Storni T, Lipowsky G, Schmid M, Pumpens P, Bachmann MF. Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. Eur J Immunol 2002;32:3305–14.
[20] Huijbers EJM, Femel J, Andersson K, Björkelund H, Hellman L, Olsson A-K. The non-toxic and biodegradable adjuvant Montanide ISA 720/CpG can replace Freund's in a cancer vaccine targeting ED-B – a prerequisite for clinical development. Vaccine 2012;30:225–30.
[21] Holmgren A. Thioredoxin. Annu Rev Biochem 1985;54:237–71.
[22] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[23] Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, et al. Protein database searches using compositionally adjusted substitution matrices. FEBS J 2005;272:5101–9.
[24] Saupe F, Reichel M, Huijbers EJM, Femel J, Markgren P-O, Andersson CE, et al. Development of a novel therapeutic vaccine carrier that sustains high antibody titers against several targets simultaneously. FASEB J 2017;31:1204–14.