

Intradermal DNA electroporation induces survivin-specific CTLs, suppresses angiogenesis and confers protection against mouse melanoma

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Abstract Survivin is an intracellular tumor-associated antigen that is broadly expressed in a large variety of tumors and also in tumor associated endothelial cells but mostly absent in differentiated tissues. Naked DNA vaccines targeting survivin have been shown to induce T cell as well as humoral immune responses in mice. However, the lack of epitope-specific CD8⁺ T cell detection and modest tumor protection observed highlight the need for further improvements to develop effective survivin DNA vaccination approaches. Here, the efficacy of a human survivin DNA vaccine delivered by intradermal electroporation (EP) was tested. The CD8⁺ T cell epitope surv_{20–28} restricted to H-2 Db was identified based on in-silico epitope prediction algorithms and binding to MHC class I molecules. Intradermal DNA EP of mice with a human survivin encoding plasmid generated CD8⁺ cytotoxic T lymphocyte (CTL) responses cross-reactive with the mouse epitope surv_{20–28}, as determined by intracellular IFN- γ staining, suggesting that self-tolerance has been broken. Survivin-specific CTLs

displayed an activated effector phenotype as determined by CD44 and CD107 up-regulation. Vaccinated mice displayed specific cytotoxic activity against B16 and peptide-pulsed RMA-S cells in vitro as well as against surv_{20–28} peptide-pulsed target cells in vivo. Importantly, intradermal EP with a survivin DNA vaccine suppressed angiogenesis in vivo and elicited protection against highly aggressive syngeneic B16 melanoma tumor challenge. We conclude that intradermal EP is an attractive method for delivering a survivin DNA vaccine that should be explored also in clinical studies.

Keywords Survivin · DNA vaccine · Intradermal electroporation · CTL · Tumor immunology · Angiogenesis

Introduction

Survivin, a member of the inhibitor of apoptosis (IAP) family, regulates important pathways implicated in cell cycle progression, cell proliferation and cell death [1]. This intracellular protein is broadly expressed during embryonic development [2], mostly absent in differentiated cells, but then strongly up-regulated in human cancer cells of different origins [3, 4]. Survivin expression is associated with enhanced tumor cell viability and resistance to cancer therapies [5]. Accordingly, knock-down of survivin expression in tumor cells has highly detrimental effects on cancer cell viability and tumor progression [6]. Survivin is also up-regulated in endothelial cells during tumor associated angiogenesis [7]. Widespread expression in several types of human cancers and tumor stroma, general absence in normal adult tissues and a requirement for tumor cell survival essentially identify survivin as an almost ideal “universal” tumor associated antigen (TAA).

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Spontaneous cellular, as well as humoral immune responses against survivin have been detected in patients with several kinds of cancer [8, 9], further validating survivin as a TAA that can be exploited for therapeutic purposes. Importantly, CD8+ and CD4+ T cell responses restricted to different human and mouse MHC molecules have been characterized and several relevant survivin epitopes were identified. All together, these features make survivin a highly attractive target for T cell based immune strategies against cancer. Dendritic cell- and gene-based vaccines targeting survivin have been tested in preclinical settings where induction of T cell responses and tumor protection were observed [10, 11].

DNA vaccination is an attractive immunotherapeutic strategy that appears to mimic natural immunity and is able to induce long-lasting T cell- and antibody-mediated tumor protection [12]. Naked DNA vaccines have several advantages over other vaccination methods including that they are easy to generate, the desired response can be readily modified, they are cost-effective to produce and purify at large-scale, the same production platform can be employed for any antigen, native protein antigen is generated in vivo with no need for expensive purification schemes, the antigen is processed and presented by host cells independent of haplotype restriction, plasmid DNA contains “in-built” adjuvant danger signals that activate innate immunity, they are highly stable allowing long-term storage and finally, they are considered safe, since they are non-infectious and virtually no genomic integration has been observed. Despite high efficacy in preclinical models, naked DNA vaccines need to be improved in order to overcome the low efficacy which so far has been achieved in clinical trials. Recently, in vivo DNA electroporation (EP) has emerged as an efficient delivery method that allows efficient DNA uptake, leads to long-term and high levels of antigen expression as well as the induction of several cytokines and chemokines, thereby increasing the potency of DNA vaccines [13]. Immunization through the skin is an attractive approach for clinical applications, as skin is readily accessible and a high number of antigen presenting cells are present, e.g. Langerhans cells [14]. A novel intradermal (i.d.) DNA EP method has been developed for DNA vaccine delivery that ensures high levels of antigen expression as well as improved and protective anti-tumor cellular immunity [15, 16].

Naked DNA vaccines targeting survivin have been shown to induce T cell as well as humoral immune responses in animal models [17–19]. However, the induction of epitope-specific responses has not been conclusively demonstrated. Indeed, even the lack of detectable CD8+ cytotoxic T cell (CTL) responses restricted to previously published mouse epitopes has been reported [19]. Moreover, in cases where significant reduction of tumor growth

was shown as a result of survivin DNA vaccination, modest or no long-term protection was observed. Therefore, further optimization and improvement of survivin DNA vaccine strategies are required that will lead to the induction of measurable survivin-specific T cell responses and efficient tumor protection. The present study demonstrates in a pre-clinical model the efficacy of a novel i.d. DNA EP approach in inducing anti-survivin CTL responses and long-term tumor protection in prophylactic as well as therapeutic settings.

Materials and methods

Mice and cell lines

C57BL/6 mice were bred and maintained at the animal facilities of the Microbiology and Tumor Biology Center at the Karolinska Institute. All animal studies have been reviewed and approved by the Swedish National Board for Laboratory Animals. B16 is a spontaneously raised murine melanoma and RMA-S is a transporter associated protein-deficient murine T cell lymphoma [20]. Cell lines were cultured in IMDM and RPMI media, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin (complete medium) (GibcoBRL, Life Technologies, Grand Island, NY, USA) in a humidified incubator at 37°C with 5% CO₂. Mouse lymphocytes were cultured in RPMI complete medium supplemented with 1% non-essential amino acids and 1% sodium pyruvate (GibcoBRL).

Plasmids

Full-length human survivin cDNA was cloned into pcDNA3.1 as previously described [18]. The cloned fragment containing survivin cDNA was excised and then inserted into the pVAX vector (Invitrogen, Carlsbad, CA, USA) by using the compatible restriction sites *Hind*III and *Xho*I. *E. coli* (TOP10, Invitrogen) carrying the pVAX constructs were cultured in Luria-Bertani medium containing 50 mg/l Kanamycin and endotoxin-free plasmids used for immunizations were purified using the GigaPrep Endofree Kit (Qiagen GMBH, Hilden, Germany).

Intradermal DNA electroporation

C57BL/6 mice were anesthetized with isoflurane and injected intradermally with 40 µg (40 µl of PBS) of plasmid DNA at two sites (20 µg each) near the base of the tail using a 29-gauge insulin-grade syringe (Micro-Fine U-100, BD Consumer Healthcare, Franklin Lakes, NJ, USA). Immediately, a parallel needle array electrode (two rows of

four 2-mm pins (1.5 × 4 mm gaps) was placed over the injection blebs, and electric pulses (two 1,125 V/cm pulses followed by eight 275 V/cm pulses) were applied using the Derma Vax™ DNA vaccine Skin Delivery System (provided by Cyto Pulse Sciences Inc.). Mice were immunized two times either at days –21 and –7 (early setting) or at days +10 and +17 (late setting) with respect to tumor challenge, referred as day 0.

B16 melanoma tumor challenge

Mice were challenged with a lethal dose of syngeneic B16 melanoma cells. B16 cells in the logarithmic growth phase ($\leq 75\%$ confluent) were harvested and a single cell suspension was prepared in PBS. Mice were injected subcutaneously in the left flank with 100 μ l containing 10^5 B16 cells. Tumor growth was evaluated twice a week by measuring perpendicular tumor diameters with calipers, and the mean diameter was recorded for 100 days. Mice were sacrificed when they became moribund or when the mean tumor diameter reached 10 mm, the limit permitted according to the approved ethical protocol.

Epitope prediction and peptide synthesis

Full-length mouse and human survivin amino acid sequences were screened to identify potential H-2 Db- and Kb-restricted 9-mer epitopes using the following web-based prediction sites: The Bioinformatics & Molecular Analysis Section (BIMAS) [21]; the Promiscuous MHC Class-I Binding Peptide Prediction Server (ProPred) [22]; and the SYFPEITHI database for MHC ligands and peptide motifs [23]. The score and ranking obtained using these three different web-sites were taken into account. The mouse-derived peptides; ATFKNWPFL (surv_{20–28}), ACTPERMAE (surv_{32–40}), FIHCPTENEP (surv_{43–52}), CPTENEPDL (surv_{46–54}), AFLTVKKQM (surv_{85–93}), LDRQRAKNKI (surv_{104–113}), AKETNNKQKE (surv_{114–123}) as well as human survivin peptide STFKNWPFL (surv_{20–28}), control TRP2 peptide SVYDFVWL (trp_{2180–188}) and mouse VEGFR2 peptide FSNSTNDILI (flk_{1615–624}) were all obtained from UFPeptides (Ferrara, Italy) at $>90\%$ purity.

Antibodies and flow cytometry

Monoclonal antibodies anti-mouse CD107a (clone 1D4B), anti-mouse CD44 (clone IM7), anti-mouse CD8 α (clone 53-6.7), anti-mouse H-2 Kb (clone AF6-88.5), anti-mouse H-2 Db (clone KH95), anti-mouse IFN- γ (clone XMG1.29) conjugated to FITC, PE, PerCP, or APC fluorochromes were used for flow cytometry analysis (Becton Dickinson Biosciences). Non-specific binding was blocked by adding

unconjugated rat anti-mouse CD16/CD32 antibody (mouse BD Fc block, clone 2.4G2) prior to the surface staining procedure. The samples were analyzed with a FACSCalibur cytometer and the data obtained were processed using CellQuest Pro software (Becton Dickinson Biosciences).

MHC class I stabilization assay

RMA-S cells in logarithmic growth phase were harvested, kept at room temperature during 1.5 h and then seeded (2×10^5 cell/well) in 96-well plates (U-bottom) in complete RPMI medium containing different concentrations of H-2 Db- and Kb-restricted peptides. Cells were cultured during 16 h at 37°C, 5% CO₂. Then, cells were stained for the detection of surface levels of H-2 Db and Kb MHC class I molecules and analyzed by flow cytometry.

Intracellular cytokine staining

Lymphocytes from immunized mice were isolated from peripheral blood. Mice were bled from the tail vein and blood samples collected in tubes containing heparin. Red blood cells (RBCs) were lysed using hypotonic buffer PharmLyse (Becton Dickinson Biosciences, Mountain View, CA, USA). Lymphocytes were seeded in U-bottom 96 wells plates and stimulated during 8 h with MHC class I-restricted peptides (10 μ g/ml each). Anti-mouse CD107a antibody was added at this point and degranulation of cytotoxic granules was evaluated. CD8+ T cells producing IFN- γ were detected by intracellular cytokine staining following addition of GolgiPlug (Becton Dickinson Biosciences) during the last 6 h of stimulation. Staining was performed using Cytofix/Cytoperm™ Fixation/Permeabilization Solution set (Becton Dickinson Biosciences) according to the manufacturer's instructions.

In vitro cytotoxicity assay

Single cell suspensions from spleen and lymph nodes were isolated from immunized mice and RBCs were lysed using hypotonic buffer PharmLyse. Lymphocytes were cultured for 5 days in the presence of 2 mM of surv_{20–28} peptide and 50 U/ml of recombinant human IL-2. After 5 days of stimulation, viable lymphocytes were counted by trypan blue exclusion and seeded in V-bottom 96-well plates. B16 and peptide-pulsed RMA-S target cells were labeled with ⁵¹Cr (Na₂CrO₄, Perkin Elmer) 1 h at 37°C, washed and 5,000 of target cells were cocultured with mouse lymphocytes at different effector: target ratios (100:1, 50:1, 25:1) in quadruplicates during 6 h at 37°C, 5% CO₂. Release of ⁵¹Cr in culture supernatants was measured by a γ -counter (Wallac Sverige AB, Stockholm, Sweden) and the percentage of specific lysis was determined using the equation: $100 \times$

[(sample release – spontaneous release)/(maximum release – spontaneous release)].

In vivo cytotoxicity assay

Target cell populations were prepared from naive C57BL/6 mice. RBC-lysed splenocytes (10^8 cells/ml PBS 0.5% FBS) were stained with 0.2 or 2 μ M carboxyfluorescein succinimidyl ester (CFSE) for 5 min. Ten volumes of PBS 10% FBS were subsequently added to stop the labeling. Target cells were pulsed (60 min at 37°C) with antigen-specific or irrelevant peptide as an internal control. Cells (6×10^6) from each population were then mixed together in equal proportions and injected intravenously into empty or survivin DNA vaccinated C57BL/6 mice. Spleens and lymph nodes were removed 20 h later and single-cell suspensions were generated before analysis on a FACSCalibur cytometer (Becton Dickinson Biosciences). CFSE+ donor target splenocytes were distinguished from host cells and the percentage of target cell killing was determined as follows: $100 - [(\text{percentage of surv}_{20-28} \text{ peptide-pulsed targets in pSURV vaccinated recipients} / \text{percentage of control targets in pSURV vaccinated recipients}) / (\text{percentage of surv}_{20-28} \text{ peptide-pulsed targets in pEMPTY vaccinated recipients} / \text{control targets in pEMPTY vaccinated recipients}) \times 100]$.

In vivo matrigel plug angiogenesis assay

Ten days after the last immunization, mice were injected subcutaneously in the midventral abdominal region with 250 μ l of matrigel (Becton Dickinson Biosciences) containing 200 ng of human bFGF (PeproTech, Rocky Hill, NJ). Seven days later matrigel plugs were removed, fixed overnight in 10% formalin-PBS and stored in 30% sucrose-PBS. Then, plugs were washed, embedded in OCT compound (Tissue-Tek, Histolab, Sweden) and stored frozen at -20°C until further analysis. The glass slides were dried at room temperature for 1 h, then an ice cold mixture of methanol and acetone (1:1) was applied to fix samples and remove residual OCT. Subsequently, samples were permeabilized in 0.3% Triton X-100. After blocking with 5% BSA, samples were then stained with anti-mouse CD31 (MEC 13.3 Becton Dickinson Pharmingen) antibody overnight at 4°C and then with the Texas Red-conjugated secondary anti rat immunoglobulin antibody (712-075-153, Jackson ImmunoResearch Laboratories Inc., Newmarket, Suffolk, UK) during 2 h at room temperature. Slides were mounted with Fluorescein-FragEL Mounting media (Calbiochem, Nottingham, UK) and visualized using a Zeiss axioplan 2 microscope (Carl Zeiss GmbH, Oberkochen, Germany). Photos were taken with an AxioCam HRm camera and processed using Zeiss Axiovision 4.5 software. The presence of vessels was evaluated in ten random visual

fields per sample at 20 \times magnification. Vascular density was expressed as the number of vessels per field.

Statistical analysis

Statistically significant differences between different groups of data were evaluated using a two tailed *t* test. Statistical significance for comparison of survival curve was evaluated with the log rank test. Differences were considered significant when a *P* value less than 0.05 was obtained (**P* < 0.05).

Results

Predicted epitope surv_{20–28} binds to H-2^b molecules

The primary aim of our study was to investigate whether i.d. EP with a human survivin encoding plasmid could induce efficient survivin-specific CD8+ T cell and mediate tumor protection. To this end, we first sought to identify a mouse survivin epitope which T cells would recognize following immunization with human full-length survivin plasmid enabling us to measure CD8+ T cell responses. Candidate epitopes restricted to H-2 Db and Kb molecules were identified in the amino acid sequence of mouse survivin antigen using three different web-based algorithms; BIMAS [21]; ProPred [22]; and SYFPEITHI [23]. The search focused on the identification of 9-mer peptides restricted to H-2 Db and Kb MHC class I molecules with conserved sequences between mouse and human survivin proteins. Epitopes with a single mouse/human amino acid difference, not expected to interfere with T cell Receptor (TCR) recognition, were also considered.

High scoring peptides have better probabilities of binding to and being presented by MHC class I molecules. The peptide surv_{20–28} was identified as a candidate epitope with high scores in each of the three prediction algorithms, ranking in first and fifth positions for H-2 Db and Kb, respectively (Table 1). Since mouse and human surv_{20–28} peptides only differ in the first amino acid (alanine in mouse, serine in human), which is located outside the TCR recognition area, the likelihood that immunization with the gene encoding for human survivin would lead to the induction of CTLs that recognize the mouse epitope was considered to be high. Both mouse and human surv_{20–28} peptides yielded exactly the same scores in the epitope prediction analysis. We tested the ability of this peptide and other mouse survivin peptides previously published [24] to bind to H-2 Db and Kb molecules in the MHC class I stabilization assay [25] using RMA-S cells [20]. Interestingly, surv_{20–28} was the only survivin peptide that could efficiently stabilize H-2 Db molecules at the surface of RMA-S cells (Fig. 1a),

Table 1 Epitope prediction analysis for surv_{20–28} peptide

Sequence	Specie	Bimas Db		Bimas Kb		ProPred I Db		ProPred I Kb		SYFPEITHI Db	
		Rank	Score	Rank	Score	Rank	Score	Rank	Score	Rank	Score
ATFKNWPFL	Mouse	1	264.000	5	2.640	1	0.990	3	0.950	1	24
STFKNWPFL	Human		264.000		2.640		0.990		0.950		24

The full-length mouse survivin amino acid sequence was screened to identify potential H-2^b-restricted epitopes using web-based prediction analysis Bimas, ProPed I and SYFPEITHI. The score and ranking obtained using these three different web sites for the candidate 9-mer peptide surv_{20–28} are summarized. The same analysis was done with the corresponding human survivin sequence

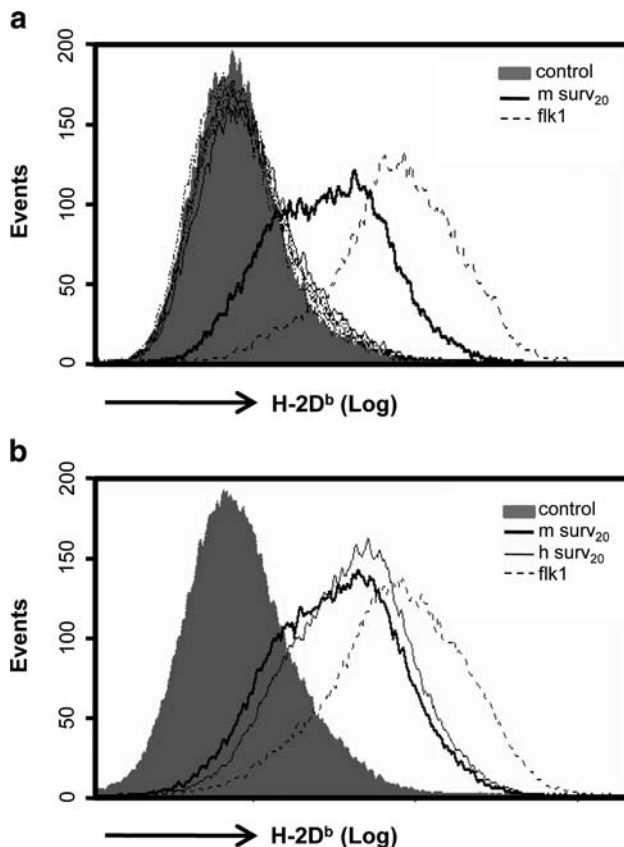


Fig. 1 Surface stabilization of H-2 Db molecules by predicted epitope surv_{20–28}. RMA-S cell were incubated with 100 μ M of different MHC class I-restricted peptides. Then surface levels of H-2 Db molecules were detected by flow cytometry analysis and compared with those observed after incubation without peptide (control, filled gray histograms) or with the flk1_{615–624} peptide (flk1, fragmented line histograms) employed as negative and positive controls, respectively. **a** Histograms representing H-2 Db levels after incubation with individual mouse survivin-derived peptides are shown, including the predicted epitope surv_{20–28} (thick line) as well as surv_{32–40}, surv_{43–52}, surv_{46–54}, surv_{85–93}, surv_{104–113}, surv_{114–123} (dotted lines) that could not stabilize H-2 Db molecules under these conditions. **b** Histograms representing H-2 Db levels after incubation with mouse (thick line) and human (thin line) surv_{20–28} peptides are shown

although to a lesser extent compared to a high affinity H-2 Db-restricted peptide derived from the mouse VEGFR2 protein (flk1) [26]. Importantly, the mouse and human

surv_{20–28} peptides bound MHC class I molecules with similar efficiency as measured by the RMA-S stabilization assay (Fig. 1b). Taken together, these data indicated that the predicted surv_{20–28} peptide fulfilled the desired features of a potential CTL epitope.

Intradermal electroporation with a survivin DNA vaccine induces surv_{20–28}-specific CD8⁺ T cell responses

The efficacy of i.d. DNA EP in inducing survivin-specific CD8⁺ T cell responses was evaluated by intracellular IFN- γ staining. Peripheral lymphocytes from vaccinated mice were stimulated *ex vivo* with trp2_{180–188} peptide as negative control, a pool of six H-2^b-restricted survivin-derived peptides, and mouse or human surv_{20–28} peptides. The percentage of CD8⁺ T cells producing IFN- γ was determined by flow cytometry (Fig. 2a). Approximately, 1% of all CD8⁺ T cells were shown to specifically produce IFN- γ when stimulation was performed with surv_{20–28} peptide in mice immunized with survivin encoding plasmid, while only low background levels were detected in mice vaccinated with empty plasmid or when T cells were stimulated with the trp2 control peptide instead. Almost identical responses were observed stimulating with either mouse or human surv_{20–28} peptides in accordance with the induction of cross-reactive CD8⁺ T cells able to recognize both peptides. Importantly, the observed induction of CD8⁺ T cell responses against the mouse epitope indicates that i.d. EP with the human survivin plasmid is able to break self-tolerance to the mouse survivin TAA. No detectable CD8⁺ T responses were obtained when T cells were stimulated with a pool of previously described H-2^b-restricted survivin peptides [24], suggesting that the predicted surv_{20–28} epitope is immunodominant among the peptides tested. The cytotoxic potential of surv_{20–28}-specific CD8⁺ T cells was evaluated by extracellular staining of the late endosome/lysosome marker CD107a (Lysosome-Associated Membrane Protein 1, LAMP-1) and the activation marker CD44. A higher mean fluorescence intensity (MFI) for CD107 staining was observed in surv_{20–28}-specific CD8⁺ T cells (IFN- γ (+)) compared to the overall non-specific CD8⁺ T cell population (IFN- γ (-)) (Fig. 2b). A higher MFI is indicative of

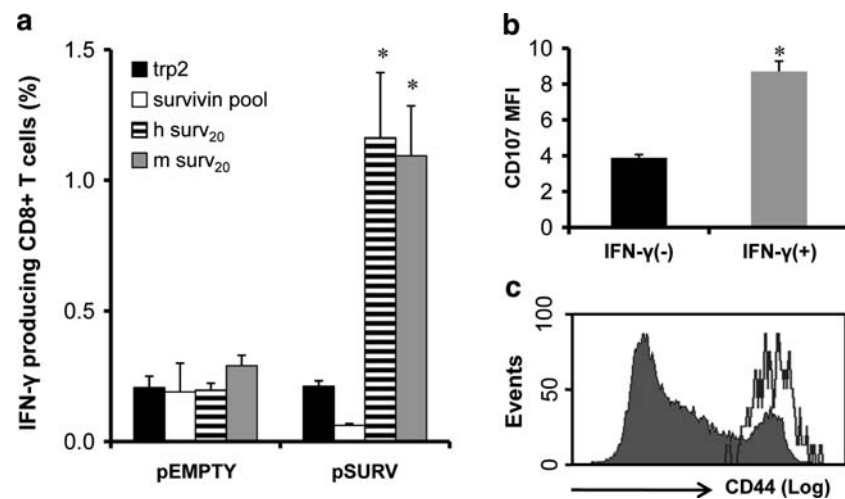


Fig. 2 Suriv_{20–28}-specific IFN- γ production, CD44 and CD107 up-regulation in peripheral CD8+ T cells induced by intradermal survivin DNA electroporation. **a** Lymphocytes from mice vaccinated with empty (pEMPTY, $n = 3$) or survivin (pSURV, $n = 6$) encoding plasmids were isolated from peripheral blood and stimulated during 8 h with MHC class I-restricted peptides, including: trp_{2180–188} (trp2); a pool of surv_{32–40}, surv_{43–52}, surv_{46–54}, surv_{85–93}, surv_{104–113}, surv_{114–123} (survivin pool); mouse surv_{20–28} (*m* surv₂₀); and human surv_{20–28} (*h* surv₂₀). CD8+ T cells producing IFN- γ were detected by intracellular staining and flow cytometry analysis. The percentages of IFN- γ positive cells in

CD8+ population are shown (mean + SEM). **b** Peripheral lymphocytes from mice vaccinated with pSURV ($n = 9$) were stimulated with mouse surv_{20–28} peptide and CD107 levels were analyzed. Mean fluorescence intensity (MFI) in IFN- γ negative (–) and positive (+) CD8+ T cell populations are shown (mean + SEM). **c** Peripheral lymphocytes from mice vaccinated with pSURV were stimulated with mouse surv_{20–28} peptide and CD44 expression was analyzed. Representative histograms displaying CD44 levels in IFN- γ negative (–) (filled histogram) and positive (+) (empty histogram) CD8+ T cell populations are shown

CD107 exposure at the cell surface and represents an indirect measure of cytotoxic vesicle degranulation. Additionally, surv_{20–28}-specific CD8+ T cells displayed high levels of the activation marker CD44, a key regulator of intratumoral CTL migration that determines the ability of T cells to kill cancer cells [27]. High levels of CD44 (CD44^{high} phenotype) were observed in surv_{20–28}-specific CD8+ T cells (IFN- γ (+)) when compared to the broad distribution (CD44^{–/low/high}) observed on the overall non-specific CD8+ T cell population (IFN- γ (–)) (Fig. 2c). Taken together, these results demonstrate that i.d. EP with survivin DNA efficiently induced surv_{20–28}-specific CTL responses.

Intradermal DNA electroporation induces survivin-specific CTLs with killing activity in vitro and in vivo

To evaluate whether surv_{20–28} specific CTLs induced by survivin DNA EP displayed functional killing activity, standard chromium release assays were performed. Cytotoxic activity of lymphocytes cultured in vitro for 5 days in the presence of surv_{20–28} peptide was determined. As shown in Fig. 3a, specific cytotoxic activity was observed with effector cells from mice vaccinated with pSURV against surv_{20–28}-pulsed but not against control-pulsed RMA-S targets. No detectable cytotoxic activity was observed in control vaccinated mice. These results show the potential of surv_{20–28} specific CTLs to efficiently kill tumor cells presenting surv_{20–28} epitope at the cell surface. The ability of

surv_{20–28} specific CTLs to recognize endogenously processed epitope and kill B16 cells in vitro was also evaluated (Fig. 3b). Data from individual mice vaccinated with pSURV show that two out of five mice displayed detectable cytotoxic activity against B16 cells (Fig. 3b, right panel). No cytotoxic activity was detected in mice vaccinated with control pEMPTY plasmid. These results indicate that the surv_{20–28} epitope is naturally processed and presented at the surface of B16 cells and potentially accessible to CTLs.

To further evaluate whether surv_{20–28} specific CTL killing occurred in vivo, spleen cells from naïve mice were simultaneously stained with either a high or a low concentration of CFSE and pulsed with surv_{20–28} or control peptide, respectively. Then both populations were mixed in equal proportions and adoptively transferred to immunized mice by i.v. injection. Specific killing of the surv_{20–28}-pulsed population (CFSE^{high}) relative to the internal control population (CFSE^{low}) was analyzed by flow cytometry. Spleens and lymph nodes from DNA vaccinated recipient mice were analyzed 20 h after target cell inoculation. Essentially the same number of surv_{20–28}-pulsed (CFSE^{high}) and control (CFSE^{low}) target cells were detected in mice vaccinated with empty plasmid (Fig. 3c, left panel), while a selective decrease of surv_{20–28}-pulsed (CFSE^{high}) population with respect to control (CFSE^{low}) target cells (Fig. 3c, right panel) was observed in mice vaccinated with survivin plasmid. An overall surv_{20–28}-specific in vivo killing of 17% was observed when the spleens of these mice

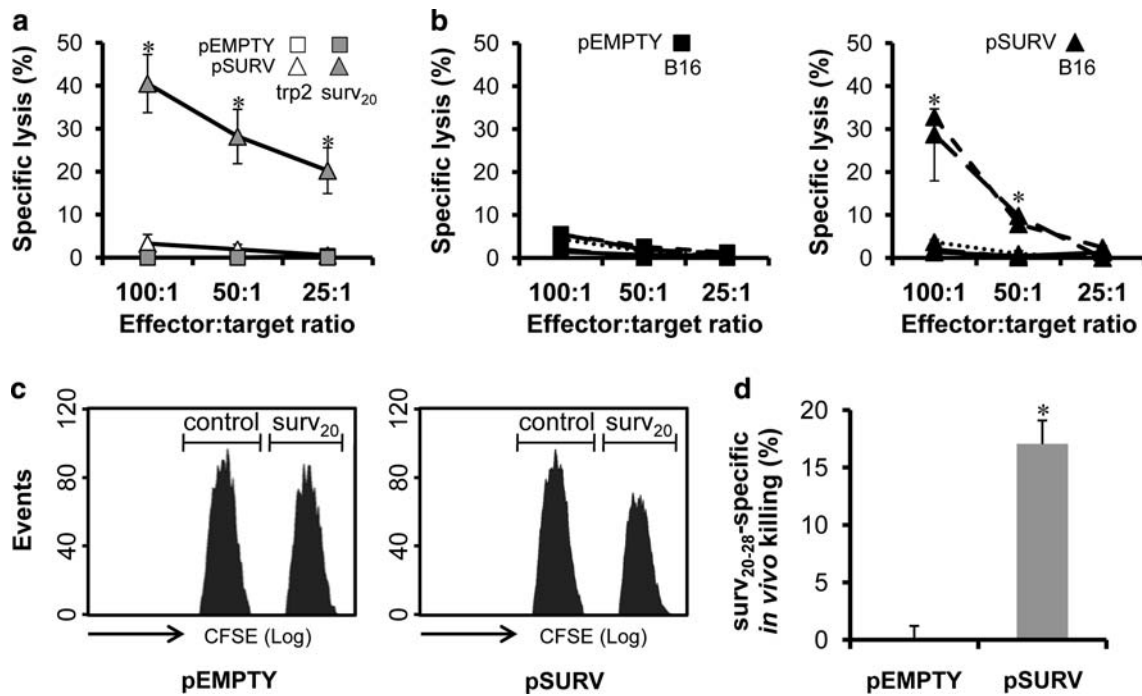


Fig. 3 Surviv_{20–28}-specific CTL killing induced by intradermal survivin DNA electroporation. Mice were immunized two times at a 2-week interval and 12 days after the last immunization *in vitro* as well as *in vivo* cytotoxicity assays were performed. Effector cells were obtained from mice vaccinated with empty (pEMPTY, squares, $n = 5$) or survivin (pSURV, triangles, $n = 5$) plasmids and stimulated for 5 days with surviv_{20–28} peptide. **a** Lymphocytes were co-cultured with ⁵¹Cr-labeled RMA-S cells pulsed with trp2_{180–188} (white symbols) or surviv_{20–28} (gray symbols) peptides at different effector: target ratios. Release of ⁵¹Cr was measured in culture supernatants and the percentage of specific lysis was determined (mean + SEM). **b** The same experiment as described above but using B16 cells as targets (black symbols). Data

(pSURV) were analyzed (Fig. 3d). Interestingly, *in vivo* cytotoxicity was even higher (25%) when pooled inguinal lymph nodes were analyzed instead of individual spleens (data not shown). These results demonstrated that surviv_{20–28} specific CTLs were able to mediate specific *in vivo* killing.

Survivin DNA electroporation suppresses angiogenesis *in vivo*

The efficacy of *i.d.* DNA EP in targeting survivin-expressing endothelial cells forming new blood vessels (angiogenesis) was evaluated by the *in vivo* matrigel assay. Mice were vaccinated twice at a 2-week interval with either empty (pEMPTY) or survivin (pSURV) encoding plasmids. Ten days after the last immunization, mice were *s.c.* inoculated with matrigel containing bFGF, an angiogenic factor that strongly up-regulates survivin expression in endothelial cells [7]. Matrigel plugs were removed 1 week later, fixed and immunofluorescence analysis was performed (Fig. 4a). Representative images of matrigel sections showing nuclei (top panels) and endothelial cells (middle panels)

from individual mice are shown (mean + SEM). **c** Target splenocytes from naive mice were stained with 0.2 or 2 μ M CFSE and pulsed with irrelevant trp2 (control, CFSE^{low}) or mouse surviv_{20–28} (surv₂₀, CFSE^{high}) peptides, respectively. Cells from each population were then mixed together in equal proportions and injected *i.v.* into empty (pEMPTY, $n = 4$) or survivin (pSURV, $n = 4$) DNA vaccinated mice. Spleens were removed 20 h later and single-cell suspensions were analyzed by flow cytometry. Representative histograms (gated on CFSE positive cells) displaying CFSE^{low} and CFSE^{high} populations detected in vaccinated recipient mice are shown. **d** The percentage of surviv_{20–28}-specific killing detected in vaccinated mice is shown (mean + SEM)

from each group vaccinated with either empty (pEMPTY, left panels) or survivin (pSURV, right panels) encoding plasmids. Merged fluorescent images are also shown (lower panels). Vascular density, here expressed as the number of blood vessels per visual field, was quantified and shown to be decreased by nearly 50% in mice vaccinated with survivin encoding plasmid (pSURV) when compared with empty plasmid (pEMPTY) vaccinated mice (Fig. 4b). These results indicate that the formation of new blood vessels, a key process during tumor progression, can be targeted by *i.d.* EP with a survivin DNA vaccine.

Survivin DNA electroporation confers tumor protection

The anti-tumor efficacy of *i.d.* survivin DNA EP was tested by evaluating the protection against the aggressive B16 melanoma tumor model. Mice were immunized twice at a 2-week interval with either empty (pEMPTY) or survivin (pSURV) encoding plasmids and 1 week after the last immunization mice received a *s.c.* tumor challenge with a lethal dose of B16 cells (early setting). In this early

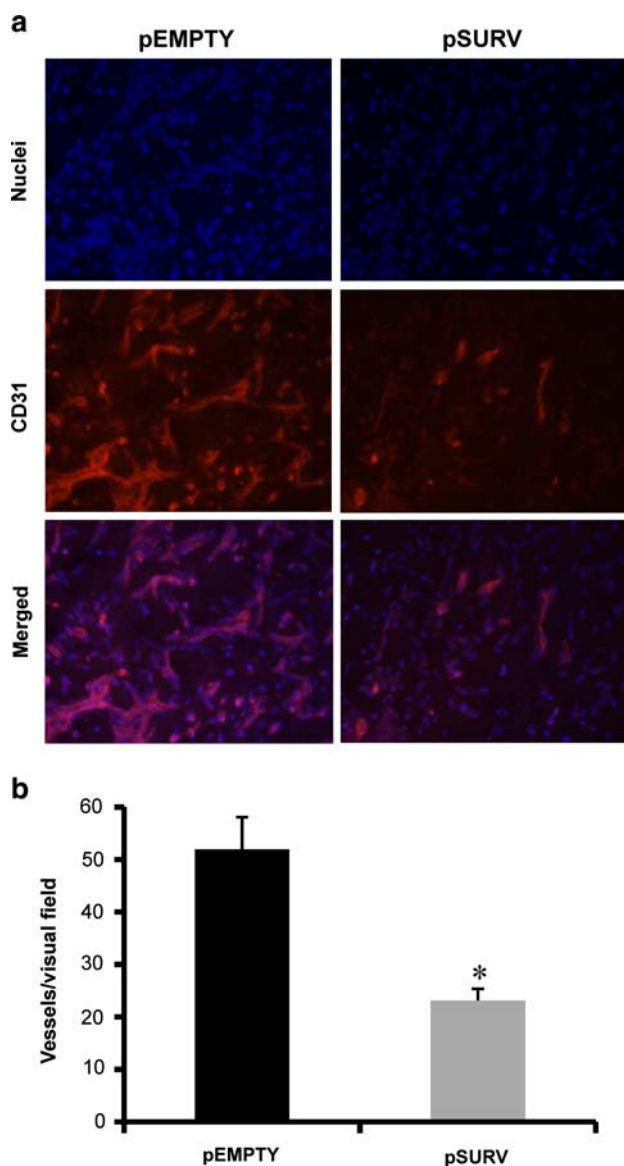


Fig. 4 Suppression of in vivo angiogenesis induced by intradermal survivin DNA electroporation. Mice were immunized two times with empty (pEMPTY, $n = 4$) or survivin (pSURV, $n = 4$) plasmids and 10 days after last immunization matrigel was s.c. inoculated. One week later, matrigel plugs were removed, fixed and immunofluorescence analysis was performed. **a** Representative pictures for each group are shown. Cell nuclei in the matrigel are shown in blue (top panels). The presence of endothelial cells was detected by CD31 staining (red, middle panels). Merged fluorescent images are also shown (lower panels). **b** Blood vessel density was quantified as described in the material and methods section. Data plotted from both groups are shown (mean + SEM)

vaccination setting, nearly 40% of mice vaccinated with pSURV were protected from tumor development and remained tumor-free during the entire period of the experiment (Fig. 5a), in contrast to mice vaccinated with pEMPTY which all succumb to the tumor. Additionally, a very similar proportion of mice (40%) were protected when the vaccine was administered in a “late setting” twice at a

1-week interval starting 10 days after the mice had received the tumor challenge (Fig. 5b). However, tumor growth data from individual mice showed that none of the survivin immunized mice bearing a palpable tumor underwent tumor regression (Fig. 5c), why we cannot conclude that this late vaccination setting can be considered as therapeutic. We therefore surmise that i.d. EP with a survivin DNA vaccine can confer tumor protection to mice when delivered either before or after challenge with B16 melanoma cells.

Discussion

We have shown that a naked DNA vaccine encoding the human survivin antigen delivered by i.d. EP could efficiently induce CTL responses against a newly described mouse survivin self-epitope. Also, in vivo anti-angiogenic effects as well as anti-tumor protection against the highly aggressive B16 melanoma tumor challenge were generated. To the best of our knowledge, this is the first report showing that a naked DNA vaccine targeting the survivin antigen elicits CTL responses against a defined self-epitope, efficiently suppresses angiogenesis and, most importantly, yields long-lasting tumor protection.

Previously, we and others have shown in various mouse experimental models that naked survivin DNA vaccination is able to induce cellular as well as humoral responses against survivin [17–19]. In these studies, however, no epitope specific CTL responses were reported. We have here extended our studies by testing a more efficient DNA delivery approach and also by improving the detection of survivin-specific CTL responses, which is of considerable importance when targeting an intracellular antigen, such as survivin. T cell epitopes for survivin have been described in preclinical studies using mouse models, but it was not until after the initiation of our studies that epitopes restricted to H-2^b (C57BL/6 background) were described [24, 28]. For this reason, we began our study by searching for potential epitopes in the mouse survivin sequence and then evaluated whether a CTL response against the relevant mouse self-antigen could be induced. Since a plasmid containing the full-length human survivin cDNA was used for the immunization, the search for potential mouse epitope candidates was restricted to peptides that were either absolutely conserved between mouse and human sequences or that contained a single mouse/human amino acid difference not expected to interfere with TCR recognition. The peptide surv_{20–28} showed the highest scores and ranked top in the analysis for candidate epitopes restricted to H-2 Db and also yielded relatively good scores for the H-2 Kb allele. We hypothesized that DNA vaccination using the human survivin antigen could generate a CTL response against the predicted epitope surv_{20–28} because the difference between

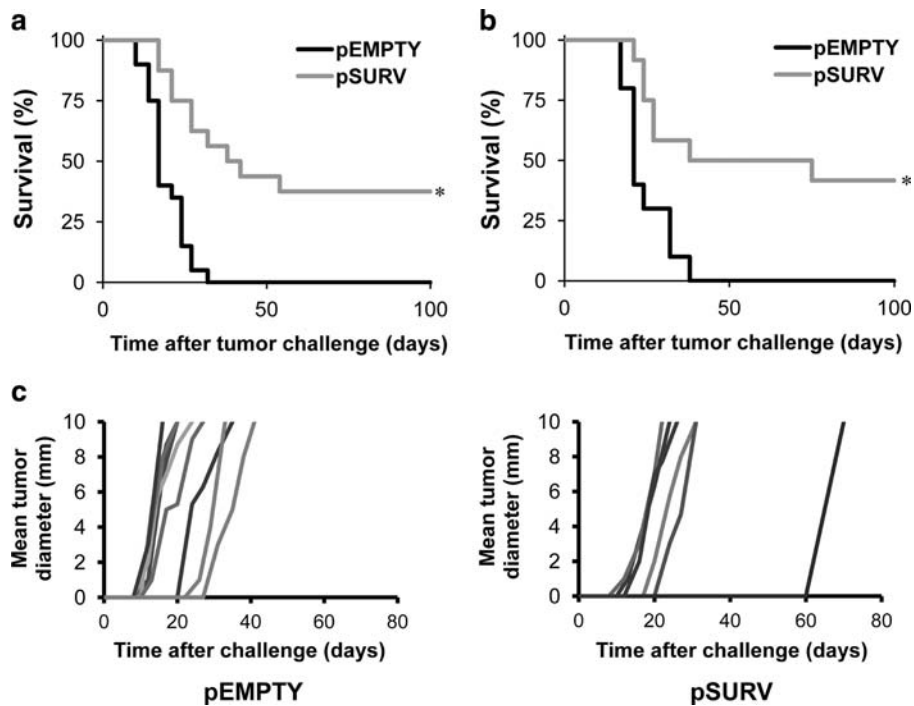


Fig. 5 Survival of mice immunized by intradermal survivin DNA electroporation following challenge with B16 melanoma cells. **a** Mice were immunized two times with empty (pEMPTY, $n = 20$) or survivin (pSURV, $n = 16$) plasmids at days -21 and -7 and then challenged with a lethal dose of syngeneic B16 melanoma cells at day 0 (early setting). Data are shown as the percentage of survival after tumor challenge from two independent experiments. **b** Mice were s.c. challenged with a lethal dose of syngeneic B16 melanoma cells at day 0 and then

immunized two times with empty (pEMPTY, $n = 10$) or survivin (pSURV, $n = 12$) encoding plasmids at days $+10$ and $+17$ (late setting). Data are shown as the percentage of survival after tumor challenge from two independent experiments. **c** Tumor growth in mice treated with the late vaccination setting (described above) was registered as the mean tumor diameter. Data from individual mice immunized with empty (pEMPTY, $n = 10$, left panel) or survivin (pSURV, $n = 12$, right panel) encoding plasmids are shown

mouse (alanine) and human (serine) peptides is in first amino acid and was not expected to affect the interaction between MHC/peptide complex and the TCR. Moreover, both peptides showed exactly the same scores in all the epitope prediction algorithms employed (Table 1) and bound to H-2 Db molecules with the same efficiency (Fig. 1b), therefore we anticipated the induction of cross-reactive CTL responses. We further compared the ability to bind H-2 Db and Kb of our newly identified candidate epitope as well as others survivin-derived epitopes previously described in the literature [24]. Rather surprisingly, the surv_{20-28} peptide was the only one able to bind and stabilize H-2 Db (Fig. 1a) and, to a lesser extent, Kb (data not shown) molecules on RMA-S cells, indicating that it has a higher probability of being presented than the other peptides.

Recently, i.d. EP has been described as an efficient delivery method for DNA vaccines that is able to induce enhanced cellular responses against human TAAs in mice [16, 29]. Here we showed that i.d. survivin DNA EP efficiently induced CTL responses against the mouse as well as human epitope surv_{20-28} measured by intracellular IFN- γ staining. This indicates that self-tolerance to this

antigen has been overcome. We also observed that individual mice responded to the same extent against both peptides (data not shown), supporting the notion that a cross-reactive CTL response has been induced. No detectable responses were observed when lymphocytes were stimulated with a pool of previously described survivin epitopes (Fig. 2a). These results suggest that the surv_{20-28} is an immunodominant epitope among the peptides tested. Surv_{20-28} -specific CTLs were found to be functionally activated after peptide stimulation as measured by IFN- γ production (Fig. 2a) and up-regulation of the cytotoxic vesicle degranulation marker CD107 (Fig. 2b). Interestingly, surv_{20-28} -specific CTLs also showed high expression levels of the activation marker CD44 (Fig. 2c), recently described as a key regulator of intratumoral CTL infiltration that determines the ability of T cells to kill cancer cells and ultimately efficiently reject tumor [27]. Importantly, surv_{20-28} CTLs displayed specific functional cytotoxic activity against tumor cells as well as peptide-pulsed targets in vitro and in vivo (Fig. 3). Moreover, surv_{20-28} CTLs could recognize and kill B16 cells that endogenously process and present survivin-derived epitopes in 40% of the tested mice (Fig. 3b). Although, this is the first study to report surv_{20-28} as a relevant TAA epitope

in mice, the human peptide was previously described as a CTL epitope restricted to HLA-A24 [30], a very frequent allele expressed especially in the Asian population. The presence of surv_{20–28} CTLs in cancer patients supports the notion that this peptide is processed and presented by cancer cells. Therefore, surv_{20–28} is a relevant survivin-derived epitope that has been demonstrated to be immunogenic in both preclinical models and cancer patients.

One of the outstanding advantages of survivin as a TAA is that it is also expressed in endothelial cells forming blood vessels during tumor associated angiogenesis and molecular targeting of survivin results in suppression of angiogenesis [31]. Therefore, i.d. DNA EP-induced survivin CTLs were expected to target tumor as well as endothelial cells. The later cells represent an attractive target population because they are genetically more stable than tumor cells and angiogenesis is essential for tumor growth. Here we showed efficient suppression of angiogenesis induced by i.d. EP with the survivin encoding vaccine (Fig. 4) reinforcing the therapeutic potential of this vaccination approach.

Previous efforts targeting tumors using naked survivin DNA vaccines delivered by classical intramuscular injection have shown anti-tumor effects in vivo [17, 19]. In these studies, however, modest or no long-lasting tumor protection was reported, motivating us to use a more efficient vaccination approach. Here we show, in accordance with the observed efficient induction of CTL responses and suppression of angiogenesis, that i.d. survivin DNA EP protected mice from challenges with highly aggressive B16 melanoma cells, a tumor model that is hard to target with DNA vaccine approaches [32] and that has not been previously targeted using survivin-based vaccines. Our vaccination approach prolonged survival and completely protected 40% of the mice immunized either before (early setting, Fig. 5a) or after (late setting, Fig. 5b) tumor challenge, when small tumors were developing in some but not all of the mice. Tumor protection observed upon the late immunization setting cannot be considered strictly therapeutic because no tumor regression was registered in mice immunized with the survivin encoding plasmid (Fig. 5c, right panel), and probably reflects the protection of mice that had not developed tumors. Accordingly, whereas some of the mice had palpable tumors after 10 days, others did not develop palpable tumors until 20 days or more. Moreover, once tumors become palpable a highly aggressive growth was observed in all mice regardless the treatment received, reaching the size limit (mean tumor diameter, 10 mm) within 2 weeks. Considering the aggressive nature of the B16 tumor model, the therapeutic window using this model is very small for testing our vaccination approach. Importantly, it should be noted that mice which were protected from tumor development following survivin vaccination remained tumor-free during the entire one hundred day experimentation period.

Intriguingly, the proportion of mice protected against B16 tumor challenge in vivo coincides with the proportion of mice that showed detectable cytotoxicity against B16 cells in vitro (Fig. 3b). This observation indicates that while the majority of the surv_{20–28}-specific CTLs are able to recognize peptide-pulsed cells (Fig. 3a), much fewer can recognize naturally processed peptides on B16 tumors, as commonly is the case for CTLs specific for tumor-derived peptide epitopes known to be naturally processed [33].

To what extent potential direct anti-tumor or antiangiogenic effects contribute to the observed tumor protection and whether CTLs against surv_{20–28} or other survivin-derived epitopes mediate the anti-tumor responses remains to be established. The anti-angiogenic effects observed upon id EP with pSURV could, at least in part, account for the anti-tumor effects of the vaccination but also could potentially lead to the generation of autoimmune side effects. In theory, targeting angiogenesis could interfere with physiological processes that require the formation of new blood vessels. Although all vaccinated mice appeared healthy, further studies are needed to carefully evaluate this possibility. Survivin gene-based vaccination has, however, previously been shown to induce appropriate responses in the absence of apparent signs of angiogenesis-related autoimmunity [10]. In the aforementioned report, survivin vaccination suppressed angiogenesis in vivo and elicited CTL-mediated killing of murine endothelial cells in vitro without impairing either wound healing or fertility.

Therapeutic as well as prophylactic tumor protection using survivin gene-based vaccine have previously been reported [10]. In these studies, a far more complex vaccine, consisting of an attenuated *S. typhimurium* carrying a plasmid encoding a mutant ubiquitin-survivin fusion in combination with the chemokine CCL21, was used. Both anti-angiogenic and anti-tumor effects in prophylactic as well as therapeutic settings were demonstrated using a pulmonary metastasis model of non-small cell lung carcinoma. However, specificity of CTL responses against survivin-derived epitopes was not conclusively shown. Furthermore, although anti-tumor efficacy was evident, protection was not evaluated for longer than 4 weeks after i.v. challenge. Importantly, the delivery of both the survivin DNA vaccine and the encoded CCL21 chemokine adjuvant by attenuated *S. typhimurium* were required to achieve effective tumor-protective immunity and the oral vaccine encoding only the survivin antigen was markedly less efficient.

With respect the EP method, we have previously shown a potent induction of human prostate-specific antigen (PSA)- and human epidermal growth factor receptor 2 (HER2)-specific CTLs [16, 29]. However, PSA is an antigen that is not expressed in mice and thus this study did not evaluate the ability to overcome tolerance against a self-antigen. In the case of HER2, CTLs against shared

HLA-A2-restricted epitopes between mouse and human sequences were induced although tumor protection was evaluated using cells transfected with human HER2, which is a foreign antigen for mouse immune system. In another study, i.d. EP with a DNA vaccine encoding for the immature laminin receptor protein (OFA-iLRP), an embryonic self-antigen expressed by a variety of tumors, failed to induce anti-tumor responses [15]. CTL-mediated tumor protection was achieved only when OFA-iLRP was fused to CCR6 ligands that targeted the antigen to antigen-presenting cells.

I.d. delivery of DNA vaccines has a great clinical potential, not only because skin is readily accessible and injections are better tolerated, but also because of the unique immunological properties of the skin [14]. EP seems to combine high levels of antigen expression and adequate activation of both innate and adaptive immunity along with safety, tolerability, reproducibility and clinically acceptable administration [13]. Thus, our studies extend upon former reports by showing that long-lasting tumor protection can be achieved by i.d. EP with a plasmid encoding for the human survivin antigen. We anticipate that the simplicity of our vaccination strategy will greatly facilitate the clinical application of survivin DNA vaccines with a considerable therapeutic potential.

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Conflict of interest statement The authors declare that they have no conflict of interest. AKR is an employee at Cyto Pulse Sciences Inc.

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