Cripto-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma

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Running title: Protective vaccine against metastatic breast cancer and CSCs

Keywords: cancer immunotherapy, DNA vaccine, metastatic breast cancer, cancer stem cells, tumor associated antigen

Financial Support:
This work was supported by the Swedish Cancer Society (C. Rolny, CAN 2016/825; A. Lundqvist, CAN 2015/421; R. Kiessling, 160645), the Cancer
Society in Stockholm and The King Gustaf V's Jubilee Foundation (R. Kiessling; 164073), the Swedish Medical Research Council (R. Kiessling; 521-2003-4100), Stockholm City Council Project Grant (R. Kiessling; ALF Medicin, 201520140036), Knut and Alice Wallenberg Foundations (R. Kiessling), the Italien Association for Cancer Research (F. Cavallo, IG 16724), Fondazione Ricerca Molinette Onlus (F. Cavallo), the University of Torino (F. Cavallo), Cancer Research Foundations of Radiumhemmet (A. Lundqvist, 161192), CONICYT-AFB-170004 from 'Comisión Nacional de Investigación Científica y Tecnológica de Chile' (A. Lladser), grant FONDECYT-1171703 from 'Fondo Nacional de Desarrollo Científico y Tecnológico de Chile' (A. Lladser), Swedish Research Council (C. Rolny, 2013/5982), and Fondazione Umberto Veronesi (L. Conti).

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**Conflict of interest:**

R.Kiessling is in the Scientific Advisory Board for the companies Immunicum AB, Idogen Inc, Glactone Pharma AB and RXi Pharma, and a board member of Clinical Laserthermia Systems AB, and has received compensation for arranging courses for Bristol Myers Squibb and a research grant from Moderna Therapeutics. The other authors declare no conflict of interest.

**word count:** 3442
total number of figures: 4 original, 7 supplementary figures, 1 supplementary table
Abstract

Metastatic breast cancer is a fatal disease that responds poorly to treatment. Cancer vaccines targeting antigens expressed by metastatic breast cancer cells and cancer stem cells could function as anticancer therapies. Cripto-1 is an oncofetal protein overexpressed in invasive breast cancer and cancer-initiating cells. In this study, we explored the potential of a Cripto-1–encoding DNA vaccine to target breast cancer in preclinical mouse models. BALB/c mice and BALB-neuT mice were treated with a DNA vaccine encoding mouse Cripto-1 (mCr-1). BALB/c mice were challenged with murine breast cancer 4T1 cells or TUBO spheres; BALB-neuT mice spontaneously developed breast cancer. Tumor growth was followed in all mouse models and lung metastases were evaluated. In vitro assays were performed to identify the immune response elicited by vaccination. Vaccination against mCr-1 reduced primary tumor growth in the 4T1 metastatic breast cancer model and reduced lung metastatic burden. In BALB-neuT mice, because the primary tumors are Cripto-1 negative, vaccination against mCr-1 did not affect primary tumors but did reduce lung metastatic burden. Spheroid cultured TUBO cells, derived from a BALB/neuT primary tumor, develop a cancer stem cell like phenotype and express mCr-1. We observed reduced tumor growth in vaccinated mice after challenge with TUBO spheres. Our data indicate that vaccination against Cripto-1 results in a protective immune response against mCr-1 expressing and metastasizing cells. Targeting Cripto-1 by vaccination has promise as an immunotherapy for treatment of metastatic breast cancer.
Introduction

Breast cancer is the most common cancer among women in western countries and incidence rates have been rising in developing countries (1). Breast cancer is a heterogeneous disease and insight into its molecular dysregulations has resulted in identification of therapeutic targets. The development of kinase inhibitors and Her2 targeting monoclonal antibodies led to increased survival rates among breast cancer patients, in particular in patients with local disease (2). However, relapse and metastases remain the most common causes of death among women with breast cancer (3). Metastases derive from disseminated tumor cells that have undergone an epithelial mesenchymal transition (EMT) (4). Why certain cells undergo EMT and have greater potential to metastasize is not understood. Cancer stem cells (CSCs) may be one source of metastatic cells in breast cancer. Indeed, circulating tumor cells in patients with metastatic breast cancer express EMT markers and display a stem cell phenotype (5,6).

Immunotherapy has been successfully used to treat metastatic disease (7). Immunotherapy includes diverse modalities of immune-based treatments, including checkpoint blockade, vaccines and adoptive transfer of immune cells. Checkpoint blocking antibodies targeting PD-1 and CTLA-4 are in clinical trials (NCT02129556, NCT02892734) for treatment of metastatic breast cancer. CTLA-4 and PD-1 blockade exhibits two distinct mechanisms of action: PD-1 blockade restores function of anergic T cells and CTLA-4 expands the T-cell repertoire (8). Therapeutic vaccines in cancer have been less successful. The success of antitumor vaccines is dependent on the choice of antigen and co-stimulating agents as well as mode of delivery (9). Vaccines can boost pre-existing antitumor immunity as well as activate tumor eliminating effector cells. For breast
cancer, several vaccines targeting Her2 are in clinical trials (NCT01570036, NCT01152398, NCT02276300, NCT00194714), and we have conducted a pilot trial with a full length non-transforming Her2 DNA (10).

For treatment of metastatic breast cancer, it is useful to target antigens expressed on CSCs and metastasizing cells. Cripto-1 (Cr-1) is an oncofetal protein re-expressed in the majority of human tumors, including breast cancer (11). In breast cancer, Cr-1 expression in tumor cells is negatively correlated with survival (12). Cr-1 is a GPI-anchored cell surface protein essential in embryonic development. The protein co-localizes with several receptors and is involved in Nodal, TGFβ, and Wnt/βcatenin signaling among others (13). In tumors, Cr-1 is involved in cell proliferation and migration, EMT, and angiogenesis (14). In addition, Cr-1 plays an important role in the maintenance of embryonic stem cells. The Cr-1 gene is a target of the transcription factors Nanog and Oct4 in stem cells. Indeed, Cr-1-positive cells are Nanog- and Oct4-positive and able to form spheres in vitro (15). Studies on CSCs in melanoma and prostate cancer have shown that Cr-1 expression is associated with an undifferentiated phenotype (15,16). The expression of Cr-1 on CSCs together with its role in intracellular EMT signaling makes it a potential target for intervention in metastatic breast cancer.

We have previously shown that vaccination against Cr-1 elicits a protective immune response in C57BL/6 mice and results in reduced tumor burden upon subcutaneous challenge with murine melanomaB16F10 cells. Intravenous (i.v.) challenge with B16F10 in mice vaccinated with plasmids encoding murine Cr-1 (pmCR) resulted in fewer lung metastatic foci (17).
Here we describe that vaccination with pmCR induced a humoral response against Cr-1 that protects against metastasis in the aggressive orthotopic 4T1 and the spontaneous BALB-neuT breast cancer mouse models. Further, we show Cr-1 specific clearance of breast CSCs in vivo. Anti-Cr-1 vaccination could potentially benefit patients with breast cancer, reducing the risk of relapse and disease progression.

**Material and methods**

**Cell lines**

The 4T1 luciferase expressing mouse mammary carcinoma cells (4T1) were originally derived from a single spontaneous tumor that arose in a BALB/cfC3H mouse (18) and were purchased 2012 from the Karmanos Cancer Institute at Wayne State University. D2F2 cells were originally derived from a spontaneous murine mammary tumor that arose from the BALB/c hyperplastic alveolar nodule line D2 and kindly provided by Dr. Wei-Zen Wei at Karmanos Cancer center in 2011 (19). The TS/A cell line is derived from a BALB/c mouse mammary carcinoma (20). 4T1, D2F2 and TS/A cells were maintained in RPMI 1640 supplemented with L-glutamine and 10% heat-inactivated FBS (Life technologies). The TUBO cell line was established from a spontaneous mammary tumor in a BALB-neuT mouse(21) was maintained in DMEM supplemented with 20% FBS (Sigma-Aldrich). Murine Cripto-1 (mCr-1)-expressing 4T1 (4T1mCr-1) cells were generated by transducing 4T1 cells with lentiviral particles (Amsbio). mCr-1-expressing cells were FACS sorted (see Flow cytometric analysis) and further selected with Geneticin (Life Technologies). The cell lines have not been
tested for mycoplasma and were not authenticated. All cell lines were kept in culture for less than a month.

**Spheroid culture**

TUBO and 4T1 single-cell suspensions were seeded in DMEM-F12 supplemented with 20 ng/ml EGF, 20 ng/ml FGF, 5 μg/ml insulin, 0.4 % BSA (Peprotech, Sigma Aldrich) at a concentration of 6 x 10^4 cells/ml in ultra-low attachment plates (Corning). The resulting spheroids were monitored daily and passaged using enzymatic and mechanical dissociation every 3-5 days. Cells were reseeded at 6 x 10^4/ml. Spheroid cultures were passaged 3 times and passage 1 (P1), 2 (P2) and 3 (P3) were collected for further experiments.

**Mice**

BALB/c mice were either purchased from ScanBur and maintained at the Department of Microbiology, Tumor and Cell Biology (Karolinska Institutet, Stockholm, Sweden) or bred and maintained at the Molecular Biotechnology Center (University of Torino, Torino, Italy). BALB-neuT mice were bred and maintained at the Molecular Biotechnology Center (University of Torino, Torino, Italy). Mice were handled in accordance with an Institutional Animal Care and Use Committee and regional Animal ethics committees (Stockholms Norra Djurförsoksetiska Nämnd Avdelning 2, Sweden N426/11, N239/14; University of Torino ethical committee authorization number 837/2015-PR).

**Plasmid**

Mouse Cr-1(NM_011562.2) encoding plasmid was generously donated by Bianco C et al., (NCI NIH Bethesda) (22) and the coding sequence was subsequently cloned into the pVAX1 vector (Invitrogen) to obtain pmCr-1. pmCr-1 and pVAX1 were expanded in *E.coli* (TOP10, Invitrogen) grown in LB medium containing...
Kanamycin (50 μg/ml). Plasmids were purified using GigaPrep Endofree Kit (Qiagen).

**4T1mCr-1 orthotopic model**

BALB/c mice were vaccinated at 8 and 10 weeks of age by intradermal injection of 40 μg of plasmid in PBS followed by electroporation with plate electrodes (IGEA). Electroporation protocol has been previously described (17). In week 12, 2x10^5 4T1mCr-1 cells diluted in 50 μl PBS were injected into the mammary fat pad. Tumors were measured by palpation twice per week and mean tumor diameter was calculated using the formula (L + W)/2. Mice were sacrificed 3 weeks after tumor challenge and primary tumors were excised and weighed. Tumors were snap frozen in OCT. For lung colony formation assay, single-cell suspensions were prepared from harvested lungs, seeded in 15 cm dishes and cultured in RPMI supplemented with L-glutamine, 10% FBS, 1% PenStrep, 6-Thioguanine (Sigma Aldrich). Medium was changed every 3-4 days. Upon colony formation, cells were fixed with 4% formaldehyde and stained with hematoxylin. Colonies were evaluated by counting.

**BALB-neuT model**

BALB-neuT mice were vaccinated with prime and boost at 10 and 12 weeks of age, respectively, by intramuscular injection of 50 μg of plasmid in saline. The injection was followed by electroporation using IGEA array needle electrode. Mice were inspected weekly for the presence of tumors, the dimensions of which were reported as mean tumor diameter. When mice reached a total number of 10 mammary tumors, or a tumor reached a threshold size of 10 mm mean tumor diameter, mice were culled, lungs were harvested and fixed in paraffin followed by staining with hematoxylin and eosin. Lung metastases were counted on a
Nikon SMZ1000 stereomicroscope (Mager Scientific). The metastatic index was calculated by dividing the number of metastatic foci by the sum of the diameter of all primary lesions.

**TUBO P3 model**

BALB/c mice were vaccinated at 8 and 10 weeks of age by intramuscular injection of 50 μg plasmid in saline. The injection was followed by electroporation using IGEA array needle electrode. Two weeks after the second vaccination, mice were challenged subcutaneously (s.c.) with 2x10^4 TUBO P3 spheroids as described (23). Mice were inspected weekly for the presence of the tumor, the dimension of which was reported as mean tumor diameter. Overall survival was reported as the time required by the tumor to reach the threshold of 10 mm mean tumor diameter, according to ethical guidelines.

**Antibodies**

See Supplementary Table S1.

**Serum**

Serum was collected for analysis and *in vitro* studies 2 weeks after the second vaccination. Sera from all mice in each group within one experiment were pooled.

**Western Blot**

Cell lysates were prepared from fresh cell culture or snap frozen cell pellets stored at -80°C with 1 M RIPA buffer (150mM NaCl, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 50mM Tris-HCl ph 7.4) and 1x protease (Roche). Protein concentrations were determined with Pierce BCA protein assay (Thermo Scientific) prior to loading onto gel. 20 μg protein lysates were reduced with 1x NUPAGE reducing agent (Invitrogen) and 1x NuPage LDS...
Sample Buffer (Invitrogen) and loaded on 10% NuPAGE Bis-Tris acrylamide gels (Invitrogen). Proteins were transferred to PVDF membrane with methanol wet-transfer. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1h at RT. Membranes were developed using Pierce ECL Western Blotting Substrate reagent kit. Luminescence was detected using LAS-1000 CCD camera system (Fujifilm, Tokyo, Japan).

**Flow cytometric analysis**

For flow cytometric analysis, single cell suspensions were prepared and $2 \times 10^5$ cells were stained per sample. Cr-1 specific antibodies in serum of pmCr-1 vaccinated mice (pmCr-1 serum) were detected by cell surface staining of 4T1mCr-1 with serum from pmCR-1 vaccinated mice. For FACS sorting, transduced 4T1 cells were first stained with pmCr-1 serum and then with anti-mIgG-PE. Serum from pVAX1-injected mice (pVAX1 serum) was used as a negative control staining. For blocking of staining, 500 ng recombinant mouse Cr-1 protein (rmCr-1) (R&D) were add to staining with pmCr-1 or pVAX1 serum. For IgG subclass analysis, 4T1mCr-1 binding serum derived antibodies were detected with anti-mIgG-FITC, anti-mIgG1-FITC, anti-IgG2a-FITC and anti-IgG2b-FITC. For unstained control, cells were only stained with secondary antibodies. Percentage of IgG1, IgG2a and IgG2b were calculated by dividing mean fluorescent intensities (MFIs) by the sum of MFI for IgG1, IgG2a and IgG2b after subtraction of MFI of unstained cells. All samples were acquired either on LSRII (BD) or Novocyte (ACEA) and analyzed using FlowJo (Tree Star).

**In vivo imaging**

*In vivo* imaging was done with IVIS SpectrumCT (PerkinElmer) using D-Luciferin (Life Technologies). 5 μg D-Luciferin per gram mouse was injected i.p. and
allowed to disseminate in the mouse for two minutes followed by anesthesia with Isoflurane at 3% for three minutes prior to transfer onto the heated, 37°C, SpectrumCT platform (Perkin Elmer) for imaging and analysis using Living Image Software (Perkin Elmer).

**Lung colony assay**

Lungs from 4T1mCr-1 bearing mice were harvested and kept in cooled PBS supplemented with 10% FBS. Lungs were individually mechanically and enzymatically digested in RPMI supplemented with 5% FBS, 2 mg/ml Dispase, 100 μg/ml DNase I, 200 μg/ml Collagenase IV for 30 min at 37°C. Cell suspension was filtered using a 70 μm filter (Fisher Scientific). Removal of red blood cells was done using RBC lysis buffer (BioLegend), followed by resuspension in supplemented RPMI-1640 media containing 6-Thioguanine (60 μM) and seeded in 150 mm cell culture dishes (Corning). After 10 days, cells were washed with PBS, followed by formaldehyde fixation and Hematoxylin Harris (VWR, 351945S) staining for 5 minutes. Primary tumors were excised and weighed. To evaluate lung metastasis, colonies were enumerated and metastatic index was calculated, MI = number of colonies/primary tumor weight.

**Antibody dependent cellular cytotoxicity (ADCC) assay**

4T1mCr-1 and 4T1 cells were harvested and labeled with 51Cr (Perkin Elmer). After labeling, target cells were incubated for 10 minutes at 4°C with 10 μl of serum from pmCr-1 or pVAX1 vaccinated mice. 5x10³ cells per well were then plated in 96-well plates without washing. Wild-type BALB/c mice were sacrificed and splenocytes isolated. NK cells were purified with magnetic beads by DX5-positive selection (Miltenyi Biotech). NK cell fraction and negative fraction were titrated onto target cells. 25 μl of co-culture supernatant were harvested after 4
and 16 h onto LUMA plates (Perkin Elmer). Radioactivity was detected in a gamma counter (Perkin Elmer). NK cells were pre-activated by i.p. injection of 150 µg Poly I:C (Invivogen) in wt BALB/c mice 24 h prior the harvest of splenocytes.

Statistical analysis

Data were analyzed with Prism 7 (GraphPad software). All in vivo data are shown as mean ± SD. Tumor growth, metastatic index and tumor growth rate were compared using Mann-Whitney test. Tumor weights were compared with unpaired t-test. Survival data were compared with log rank test. For NK cell cytotoxicity, results from 7 mice were compared with paired t-test. p-values < 0.05 were considered statistically significant.

Results

mCR-1-encoding DNA plasmid reduced metastases and tumor growth

We aimed to understand if vaccination with pmCr-1 would elicit a protective immune response in a model of murine metastatic breast cancer. We screened four mouse mammary carcinoma cell lines on the BALB/c background for Cr-1 expression by western blot. Cr-1 was expressed in 4T1, TUBO and TS/A, but not in D2F2 (Supplementary Fig. S1). To determine whether mCr-1 vaccination could induce a protective immune responses against metastasis of mammary cancer cells in BALB/c models, we generated a stable mCr-1 expressing 4T1 transfectant (4T1mCr-1) mouse as a model for spontaneous lung metastasis (Supplementary Fig. S1). BALB/c mice were vaccinated with pmCr-1 or control pVAX1 plasmids prior to implantation of 4T1mCr-1 cells into the mammary fat
pad. Primary tumor growth was evaluated by in vivo luciferase activity detection at day 14 (Fig. 1A) and twice per week through palpation (Fig. 1B). At day 23 after tumor inoculation, mice were sacrificed and primary tumor weight measured (Fig. 1C). Primary tumor size and weight were significantly reduced in pmCR-1–vaccinated mice as compared to pVAX1-vaccinated mice. Furthermore, pmCR-1 vaccination reduced spontaneous metastasis to the lungs as evaluated by a colony formation assay (Fig. 1D). Thus, Cr-1 vaccination results in anti-tumor immunity capable of controlling tumor growth and inhibiting metastatic spread.

**Cripto-1 specific humoral response**

DNA vaccination in BALB/c mice can elicit a humoral response (24). We therefore evaluated the humoral response after vaccination with pmCR-1(25,26). Serum of pmCR-1-vaccinated mice was found to contain antibodies that stained mCr-1 expressing 4T1 cells (Fig. 2A, B), whereas no signal was observed on 4T1 cells. The presence of recombinant mCr-1 protein reduced staining of cells in the 4T1mCr-1 cell line with pmCr-1 serum to control levels as seen with pVAX1 serum, but had no effect on staining with the pVAX1 control serum (Fig. 2B). Most of the antibodies in pmCr-1 serum belonged to IgG2a and IgG2b subclasses (Fig. 2C). In mice, these subclasses are responsible for mediating ADCC by NK cells, macrophages and neutrophils.

**Cellular cytotoxicity dependent on Cr-1 antibody**
Several clinically successful therapies, including anti-Her2, anti-EGFR and anti-CD20, depend on NK cell mediated ADCC (27), which also functions in DNA vaccine induced protection against Her2 expressing mouse tumors (22).

To confirm that Cr-1 specific antibodies can mediate ADCC, we tested if serum from pmCR-1-vaccinated mice increases NK cell cytotoxicity. NK cells were purified from BALB/c splenocytes with magnetic bead selection and co-cultured with 4T1mCr-1 or 4T1 cells in the presence of pmCr-1 or pVAX1 serum. We found that pmCr-1 serum increased lysis of 4T1mCR-1 cells mediated by these NK cells, although cytotoxicity in general was low (Supplementary Fig. S2). We therefore in vivo pre-activated NK cells with poly I: C, which mimics NK cell activation in tumor bearing mice (28). We found that in vivo pre-activated NK cells showed increased cytotoxic activity against 4T1mCr-1 cells. Further, lysis of 4T1mCr-1 cells by pre-activated NK cells was significantly increased in the presence of pmCr-1 serum (Fig. 2D, Supplementary Fig. S2), providing support for the role of ADCC in the vaccine induced resistance. No cytotoxic activity was detected by splenocytes depleted of NK cells. We did not observe increased lysis of 4T1 by pre-activated NK cells in presence of pmCr-1 serum compared to pVAX1 serum (Supplementary Fig. S3).

**Reduced lung metastasis after vaccination in the BALB-neuT mouse model**

We then tested whether pmCr-1 vaccination has therapeutic effect in a more clinically relevant model (29). The BALB-neuT mouse model is genetically engineered to develop spontaneous cancerous lesions in mammary tissue. We evaluated Cr-1 expression in the mammary tumors of the model and only found low expression in tumors of 8 mm mean diameter (Supplementary Fig. S4) with
no Cr-1 expression in smaller tumors. Vaccination at 10 and 12 weeks of age had no effect on tumor multiplicity (Supplementary Fig. S5) or tumor incidence in this mouse model (Fig. 3A). Survival of these mice up to the point at which they were sacrificed according to ethical regulations was unaffected (Fig. 3B).

Micrometastases derived from the primary tumors can be found in the lungs of HER-2 transgenic mice within 8 weeks of primary tumor occurrence (30). Since Cr-1 mRNA was increased (5.6 fold) as detected by qPCR in the lungs of BALB-neuT compared to wt BALB/c mice (Supplementary Fig. S6), we asked whether pmCR-1 vaccine had an effect on metastasis. Lungs from pmCR-1- and pVAX1-vaccinated mice sampled at the time of sacrifice were sectioned, stained with hematoxylin and eosin, and metastatic foci counted. We found that both the number of metastatic foci normalized to tumor size and the metastatic size were reduced in pmCR-vaccinated BALB-neuT mice (Fig. 3C, D).

**Vaccination results in a protective immune response against cancer stem cells**

Cr-1 expression has previously been associated with CSCs in melanoma, colon and breast cancer (5,31-33). We therefore evaluated whether Cr-1 vaccination elicits a protective immune response against Cr-1 expressing CSCs. The murine mammary carcinoma cell line TUBO acquires CSC phenotypic markers when passaged three times as spheres (P3 TUBO cells) (23,34). Over three passages in spheroid culture of TUBO, we observed a gradual increase in expression of Cr-1 (Fig. 4A). We confirmed surface Cr-1 expression in 3 out of 4 spheroid cultures using pmCr-1 (Supplementary Fig. S7). These TUBO P3 cells were s.c. injected into vaccinated BALB/c mice. We observed a decreased growth rate as a result of pmCR-1 vaccination. The time to reach the mean tumor size of the pmCr-1 group
(4 mm in diameter) was significantly longer in pmCr-1- compared to pVAX1- vaccinated mice (Fig. 4B). In addition, we found that 3 out of 11 mice in the pmCr-1 group were tumor free more than 60 days after tumor inoculation (Fig. 4C). In comparison, all mice in pVAX1 treatment group developed tumors within 47 days. Vaccination targeting Cr-1 also resulted in a trend towards improved survival (p=0.078) that was not quite statistically significant (Fig. 4D).

Discussion

The metastatic process of tumors is complex and remains poorly understood. Two cellular processes crucial for the occurrence of metastasis are EMT and mesenchymal-epithelial transition (MET) (35). EMT enables cells to survive without cell-cell contact, to migrate and to extravasate from the primary tumor. At the site of distant metastasis MET is required for cells to establish metastatic colonies and proliferate. Cr-1 is expressed in cells undergoing EMT; higher expression of Cr-1 is found in more aggressive types of human breast cancer (12,36).

We have previously reported that Cr-1 is an immunogenic antigen and that vaccination against Cr-1 results in protective anti-tumor immune responses against murine melanoma. In this model, a protective effect against pulmonary metastases was observed upon i.v. challenge with metastatic B16F10 cells (17). We studied the pmCR-1 vaccine in the 4T1 orthotopic breast cancer model and in Her2 transgenic BALB-neuT mice, which recapitulate the metastatic cascade from tumor cells undergoing EMT at the primary tumor site to MET at the site of metastasis. When 4T1 cells are orthotopically injected into the mammary fat pad, they spontaneously metastasize (37,38). Similarly, the BALB-neuT mice develop...
autochthonous mammary tumors that metastasize and colonize the lungs (30). The tumor development and progression mimics human breast cancer (39). Both models enable the study of EMT and MET in vivo. Due to low endogenous Cr-1 expression, we overexpressed murine Cr-1 in 4T1 cells. We observed that Cr-1 vaccination reduced metastatic burden in both the orthotopic 4T1 and the spontaneous BALB-neuT breast cancer model. Control of the primary tumor was only seen in the Cr-1 overexpressing 4T1 model. This is in line with the lack of Cr-1 expression in the primary tumors of the BALB-neuT model. We observed that the pmCr-1 vaccination, but not pVAX1 vaccination, induced an anti-mCr-1 humoral response in the BALB/c mouse model. mCr-1 specificity of these antibodies was confirmed by blocking the staining with recombinant mCr-1 protein.

Most of the Cr-1 targeting antibodies belonged to the IgG2a subclass, which are able to bind murine activating Fcγ receptors with relatively high affinity. In view of these results, we aimed at understanding the role of NK cells in the Cr-1 vaccination-induced tumor control. Our data show that protection in pmCr-1-vaccinated mice depends on NK mediated ADCC. These results are reminiscent of our earlier findings, where we showed that Her2-vaccination in BALB/c mice initiated humoral anti-Her2 immunity and killing of Her2-positive tumor cells by NK cells (24). In vitro cytotoxicity data demonstrated that lysis of Cr-1 expressing cells by NK cells was increased in the presence of serum from pmCr-1-vaccinated mice, showing that tumor elimination depends on vaccine induced ADCC. Monoclonal antibodies targeting human Cr-1 inhibit cell proliferation by blocking binding of Cr-1 to members of the TGF-β family (40). Tumor bearing mice may have a reduced humoral anti-Cr-1 response caused by soluble mCr-1,
since Cr-1 can be shed (41) and found in sera of patients with breast- and non-small cell lung cancer (42,43).

In a previous study we have shown that anti-Cr-1 vaccination in C57BL/6 mice induced a cytotoxic T cell response (17). After vaccination, Cr-1 specific cytotoxic T cells have not been detected \textit{in vitro} in BALB/c splenocytes. Although these results do not entirely rule out a role for T cells in \textit{in vivo} tumor protection, they argue for a difference in immune response between BALB/c and C57BL/6 mice upon DNA vaccination. Her2 DNA vaccination elicited a humoral immune response in Her2 transgenic BALB/c mice(44). In a direct comparison, Her2 vaccination induced less Her2-specific antibodies in Her2 transgenic mice on C57BL/6 background than on BALB/c background (44). The differences observed in the BALB/c and C57BL/6 mice after Cr-1 DNA vaccination may be explained by the genetic differences between the mouse strains.

Cr-1 expression is potentially limited to CSCs, a few cells undergoing EMT in the primary tumor, and metastasizing cells. Cr-1 is expressed in EMT-like areas in the JygMC(A) breast cancer model but not in metastatic lesions in the lung (45). Vaccination against Cr-1 could interrupt the metastatic process at an early stage and thereby prevent the establishment of metastases at distant sites.

In CSCs of several tumor types, Cr-1 expression has been confirmed (15,46,47). We have found that spheroid cultures of murine breast cancer cells, which are considered to be enriched in CSCs (23,34), express Cr-1. Subcutaneously injected TUBO P3 cells grew out in all BALB/c mice within 6 weeks after injection. After vaccination against Cr-1, 27% of mice did not develop tumors. In the remaining mice, we observed a reduced tumor growth rate. Immune responses induced by Cr-1 vaccination could target Cr-1-positive CSCs and control tumor burden.
In patients, high Cripto-1 expression in the tumor has been associated with decreased survival and could be correlated to advanced disease (12). In addition, Cr-1 has been found in the serum of breast cancer patients, suggesting its potential function as a biomarker (43). For lung cancer, serum levels of Cr-1 correlated with tumor stage (42). These clinical findings associate increased expression of Cr-1 with metastasis and worse survival in breast cancer patients. Survival of patients depends on elimination of tumor cells that could cause relapse and metastasis. New therapeutic strategies, which can target both CSCs and metastases, have the ability to reduce the risk of relapse and disease related death in cancer patients. Immune targeting therapies have shown a great potential in treatment of metastatic diseases (48). Immunogenic antigens that can be targeted by immunotherapies must be identified. We propose that Cr-1 is a candidate target for immunotherapy in breast cancer patients and would target a different subset of breast cancer cells than our Her2 DNA vaccine clinical trial(10). Here we have shown that targeting Cr-1 in mammary carcinoma mouse models reduced metastasis and targeted CSCs. For patients, a DNA vaccine targeting Cr-1 could translate into increased disease free and overall survival.
References


Figure legends

Figure 1
Tumor growth and metastatic spread in an orthotopic 4T1mCr-1 breast cancer model. Orthotopic injection of 2x10^5 4T1mCr-1 cells in pmCr-1- or pVAX1-vaccinated BALB/c mice. Mice were sacrificed on day 23 after tumor inoculation. **A**, Luciferase expression at day 14 after tumor inoculation. 4 representative mice from each group (pVAX1, n=11 and pmCr-1, n=11) are displayed. **B**, Mean tumor diameter of primary tumor. Mice in pVAX1 (n=15) and pmCr-1 (n=15) group were palpated twice per week until experimental endpoint on day 23. Error bars represent standard error of the mean;* p=0.0045, ** p=0.0007, **** p<0.0001 (Mann-Whitney test, α=0.01). **C**, Primary tumor weight at day 23. Error bars represent standard deviation; **** p<0.0001 (unpaired t-test). **D**, Single cell suspension of lung tissue was seeded in petri dishes and cultured in selection medium. At day 10, colonies were fixed and counted. Metastatic index (MI) was calculated by MI = number of colonies/primary tumor weight. Error bars represent standard deviation; **** p<0.0001 (Mann-Whitney test). All data are representative of two independent experiments.

Figure 2
Humoral response induced by pmCr-1 vaccination in BALB/c mice. BALB/c mice were vaccinated with pmCr-1 or pVAX1. Two weeks after the boost vaccination, serum was collected for analysis. **A**, Histogram plots of 4T1mCr-1 and 4T1 cells stained with pmCr-1 and pVAX1 serum. Surface binding serum antibodies were detected with anti-IgG-FITC. Cells were analyzed on flow cytometer. Data are...
representative of three independent experiments. B, Summary of 4 independent stainings of 4T1mCr-1 with pmCr-1 or pVAX1 serum in the presence or absence of 500 ng recombinant mCr-1 protein. 4T1mCr-1 pVAX1 vs pmCr-1, * p=0.0286 (Mann-Whitney test). 4T1mCr-1 pmCr-1 vs pmCr-1 + 500 ng rmCr-1, * p=0.0286 (Mann-Whitney test). C, Subclasses of antibodies in pmCr-1 serum binding Cr-1 were detected with secondary anti-mIgG1-FITC, anti-mIgG2a-FITC, anti-mIgG2b-FITC. Cells were analyzed by flow cytometry. Data are representative of three independent experiments. D, Cytotoxicity of Poly I:C in vivo pre-activated NK cells against 4T1mCr-1 cells in the presence of pmCr-1 or pVAX1 serum. Assay supernatants were harvested after 4h of co-culture for analysis. Results of spleen cells from 7 individually tested mice in two independent experiments were summarized and cytotoxicity in the presence of pmCr-1 vs pVAX serum was compared at all E:T ratios. 3:1, * p = 0.0156; 9:1, * p= 0.0156 (non-parametric paired t-test).

Figure 3

Metastatic spread in Her2/neu driven spontaneous breast cancer model BALB-neuT. BALB-neuT mice were vaccinated at 10 weeks and 12 weeks with pmCr-1 or pVAX1. Mice were followed over time and sacrificed upon ethical endpoint. A, Tumor incidence in pVAX1 (n=6) and pmCr-1 (n=6) vaccinated BALB-neuT mice. B, Survival of pVAX1 (n=6) or pmCr-1 (n=6) vaccinated BALB-neuT mice. Mice were sacrificed according to ethical endpoint guidelines. C, Metastatic burden in pVAX1 (n=5) and pmCr-1 (n=5) mice. Metastatic foci were enumerated and normalized to the sum of the diameter of all primary lesions. Error bars
represent standard deviation; * p=0.021 (Mann-Whitney test). D, Light microscopy image of the lung.

Figure 4
Vaccination induced immune response is targeting breast cancer stem cells. P3 TUBO cells were s.c. injected in pmCr-1 or pVAX1 immunized BALB/c mice. A, Western Blot for Cr-1 in spheroid passed TUBO cell line. B, Tumor growth rate in pmCr-1 (n=11) and pVAX (n=11) vaccinated mice. Error bars represent standard deviation; * p=0.0453 (Mann-Whitney test). C, Individual tumor growth curves for pmCr-1 and pVAX until day 61. D, Survival curves for mice immunized with pmCr-1 or pVAX1 after s.c. challenge with TUBO P3. Mice were sacrificed according to ethical endpoint guidelines. p=0.078 (Mantel-Cox test). All data are representative of two independent experiments.
Crypt-1 plasmid DNA vaccination targets metastasis and cancer stem cells in murine mammary carcinoma

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