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**Cripto-1 Plasmid DNA Vaccination Targets Metastasis and Cancer Stem Cells in Murine Mammary Carcinoma**

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(Article begins on next page)

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

3

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13

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15

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46 **Statement of translational relevance**

47 Despite the wide range of therapies approved for treatment of breast cancer,  
48 mortality of patients due to metastatic spread has not been yet been addressed.

49 The development of metastasis targeting treatments is essential in decreasing  
50 breast cancer related deaths in long term. Here we describe a vaccine based  
51 therapeutic approach targeting tumor antigen Cripto-1 expressed on tumor cells.

52 We show that vaccination with Cripto-1 encoding DNA elicits an anti-Cripto-1  
53 directed immune response that consequently controls metastasis. Cripto-1  
54 expression has also been found on cancer stem cell like cells. Cancer stem cells  
55 are highly resistant to chemo and radiotherapy. They can be the cause for  
56 relapse and metastases due to their persistence after standard treatment. The  
57 anti-Cripto-1 directed immune response was able to eliminate cancer stem cells.

58 Taken together, our data shows great potential of targeting tumor associated  
59 antigen Cripto-1 in controlling metastasis and eliminating cancer stem cells.

60

61

62 **Abstract**

63 **Purpose:** Metastatic breast cancer is a fatal disease responding poorly to  
64 classical treatments. Cancer vaccines targeting antigens expressed by metastatic  
65 breast cancer and cancer stem cells have the potential to become potent anti-  
66 cancer therapies. Cripto-1 is an onco-fetal protein frequently overexpressed in  
67 invasive breast cancer and cancer-initiating cells. In this study, we explored the  
68 potential of a Cripto-1 encoding DNA vaccination to target breast cancer in  
69 preclinical models.

70 **Experimental Design:** BALB/c mice and BALB-neuT mice were treated with a  
71 DNA vaccine encoding for mouse Cripto-1 (mCr-1). Mice were challenged with  
72 murine breast cancer 4T1 cells or TUBO spheres, or spontaneously developed  
73 breast cancer in the BALB-neuT model. Tumor growth was followed in all mouse  
74 models and lung metastases were evaluated. In-vitro assays were performed to  
75 identify the immune response elicited by vaccination.

76 **Results:** Vaccination against mCr-1 reduced primary tumor growth in the 4T1  
77 metastatic breast cancer model and significantly reduced lung metastatic  
78 burden. The primary tumors in the BALB-neuT model are Cripto-1 negative.  
79 Consequently, we did not observe protection regarding the primary tumors.  
80 However, vaccination significantly reduced lung metastatic burden in this model.  
81 Spheroid cultured TUBO cells, derived from a BALB/neuT primary tumor, obtain  
82 cancer stem cell like phenotype and upregulate m-Cr-1. We observed reduced  
83 tumor growth in vaccinated mice after challenge with TUBO spheres.

84 **Discussion:** Our data indicates that vaccination against Cripto-1 results in a  
85 protective immune response against mCr-1 expressing and metastasizing

86 tumors. Targeting Cripto-1 by vaccination is a promising potential  
87 immunotherapy for treatment of metastatic breast cancer.

88

89

## 90 **Introduction**

91 Breast cancer is the most common cancer among women in western countries  
92 and incidence rates have been rising in developing countries in the last years (1).  
93 Breast cancer is a heterogeneous disease and understanding molecular  
94 dysregulations has resulted in identification of novel therapeutic targets. The  
95 development of kinase inhibitors and Her2 targeting monoclonal antibodies led  
96 to increased survival rates among breast cancer patients, in particular in patients  
97 with local disease (2). However, relapse and metastases remain a hurdle to  
98 therapy and are the most common causes of death among women with breast  
99 cancer (3). Metastases derive from disseminated tumor cells, where epithelial  
100 mesenchymal transition (EMT) is a required process for the occurrence of  
101 metastasis at distant sites (4). Which cells in particular undergo this process and  
102 have greater potential to metastasize is not fully understood. Cancer stem cells  
103 (CSC) have been proposed to be one source of metastasis in breast cancer, and  
104 circulating tumor cells in patients with metastatic breast cancer express EMT  
105 markers and display a stem cells phenotype (5,6).

106 In recent years, immunotherapy has become of interest in cancer therapy and  
107 has been successfully used to treat metastatic disease (7). The term  
108 immunotherapy summarizes diverse modalities of immune-based treatments,  
109 including checkpoint blockade, vaccines and adoptive transfer of immune cells.  
110 Checkpoint blocking antibodies targeting PD-1 and CTLA-4 are currently in

111 clinical trials (NCT02129556, NCT02892734) for metastatic breast cancer.  
112 CTLA-4 and PD-1 blockade exhibits two distinct mechanisms of action with PD-1  
113 blockade restoring function of anergic T cells and CTLA-4 expanding the T cells  
114 repertoire (8).

115 Until now, therapeutic vaccines in cancer have been less successful. The success  
116 of antitumor vaccines is highly dependent on the choice of antigen and co-  
117 stimulating agents as well as mode of delivery (9). Vaccines have the great  
118 potential to boost pre-existing anti-tumor immunity, and to activate tumor  
119 eliminating effector cells. For breast cancer, several different vaccines targeting  
120 Her2 are currently in clinical trials (NCT01570036, NCT01152398,  
121 NCT02276300, NCT00194714), and we have conducted a pilot trial with a full  
122 length non-transforming Her2 DNA (10). For treatment of metastatic breast  
123 cancer, it is of particular interest to target antigens expressed on CSC and  
124 metastasizing cells.

125 Cripto-1 (Cr-1) is an onco-fetal protein re-expressed in the majority of human  
126 tumors, including breast cancer (11). In breast cancer, Cr-1 expression in tumor  
127 cells is negatively correlated with survival (12). Cr-1 is a GPI-anchored cell  
128 surface protein essential in embryonic development. The protein co-localizes  
129 with several receptors and is involved in Nodal, TGF $\beta$ , and Wnt/ $\beta$ catenin  
130 signaling among others (13). In tumors, Cr-1 has been shown to be involved in  
131 cell proliferation and migration, EMT and angiogenesis (14). In addition, Cr-1  
132 plays an important role in the maintenance of embryonic stem cells and is a  
133 target gene of the transcription factors Nanog and Oct4 in stem cells. Indeed, Cr-  
134 1-positive cells were found to be Nanog- and Oct4-positive and able to form  
135 spheres in vitro (15). Studies on CSC in melanoma and prostate cancer have

136 shown that Cr-1 expression is associated with an undifferentiated phenotype  
137 (15,16). The expression of Cr-1 on CSC together with its role in intracellular EMT  
138 signaling makes it a potential antigen for metastasis and CSC targeting in breast  
139 cancer.

140 We have previously shown that vaccination against Cr-1 elicits a protective  
141 immune response in C57BL/6 mice and results in reduced tumor burden upon  
142 subcutaneous challenge with murine melanoma B16F10 cells. Intravenous (i.v.)  
143 challenge with B16F10 in mice vaccinated with plasmids encoding murine Cr-1  
144 (pmCR) resulted in significant reduction of lung metastatic foci (17).

145 Here we describe that vaccination induced an anti-Cr-1 directed humoral  
146 response that protects from metastasis burden in the aggressive orthotopic 4T1  
147 and the spontaneous BALB-neuT breast cancer mouse models. Further, we show  
148 Cr-1 specific clearance of breast CSC *in vivo*. Anti-Cr-1 vaccination could  
149 potentially be of great benefit for patients with breast cancer, reducing the risk  
150 of relapse and disease progression.

151

## 152 **Material and methods**

### 153 **Cell lines**

154 4T1 luciferase expressing cells (4T1) TS/A and D2F2 cell lines was maintained in  
155 RPMI 1640 supplemented with L-glutamine and 10% heat-inactivated FBS (Life  
156 technologies). TUBO cell line (18) was maintained in DMEM supplemented with  
157 20% FBS (Sigma-Aldrich). Murine Cripto-1(mCr-1)-expressing 4T1 (4T1mCr-1)  
158 cells were generated by transducing 4T1 cells with lentiviral particles (Amsbio).  
159 mCr-1-expressing cells were FACS sorted, see Flow cytometric analysis, and  
160 further selected with Geneticin (Life technologies).

### 161 **Spheroid culture**

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

### 169 **Mice**

170 BALB/c mice were either purchased from ScanBur and maintained at the  
171 Department of Microbiology, Tumor and Cell Biology (Karolinska Institutet,  
172 Stockholm, Sweden) or bred and maintained at the Molecular Biotechnology  
173 Center (University of Torino, Torino, Italy). BALB-neuT mice were bred and  
174 maintained at the Molecular Biotechnology Center (University of Torino, Torino,  
175 Italy). Mice were handled in accordance to regional Animal ethics committees  
176 (Stockholms Norra Djurförsöksetiska Nämnd Avdelning 2, Sweden N426/11,

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178 837/2015-PR).

#### 179 **Plasmid**

180 Mouse Cr-1(NM\_011562.2) encoding plasmid was generously donated by Bianco  
181 C et al., (NCI NIH Bethesda) (19) and the coding sequence was subsequently  
182 cloned into the pVAX11 vector (Invitrogen) to obtain pmCr-1. pmCr-1 and  
183 pVAX11 were expanded in *E.coli* (TOP10, Invitrogen) grown in LB medium  
184 containing Kanamycin selection (50 µg/ml). Plasmids were purified using  
185 GigaPrep Endofree Kit (Qiagen).

#### 186 **4T1mCr-1 orthotopic model**

187 BALB/c mice were vaccinated at 8 and 10 weeks of age by intradermal injection  
188 of 40 µg of plasmid in PBS followed by electroporation with plate electrodes  
189 (IGEIA). Electroporation protocol has been previously described (17). In week 12,  
190  $2 \times 10^5$  4T1mCr-1 cells diluted in 50 µl PBS were injected into the mammary fat  
191 pad. Tumors were measured by palpation twice per week and tumor volume was  
192 calculated using the formula  $(\pi/6) \times L \times W \times H$  (20). Mice were sacrificed 3 weeks  
193 after tumor challenge and primary tumors were excised and weighed. Tumors  
194 were snap frozen in OCT. For lung colony formation assay, single-cell  
195 suspensions were prepared from harvested lungs, seeded in 15 cm dishes and  
196 cultured in RPMI supplemented with L-glutamine, 10% FBS, 1% PenStrep, 6-  
197 Thioguanine (Sigma Aldrich). Medium was changed every 3-4 days. Upon colony  
198 formation, cells were fixed with 4% formaldehyde and stained with hematoxylin.  
199 Colonies were evaluated by counting.

#### 200 **BALB-neuT model**

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

### 212 **TUBO P3 model**

213 BALB/c mice were vaccinated at 8 and 10 weeks of age by intramuscular  
214 injection of 50 µg plasmid in saline. The injection was followed by  
215 electroporation using IGEA array needle electrode. Two weeks after the second  
216 vaccination mice were challenged subcutaneously (s.c.) with  $2 \times 10^4$  TUBO P3  
217 spheroids as described (21). Mice were inspected weekly for the presence of the  
218 tumor, whose dimension was reported as mean tumor diameter. Overall survival  
219 was reported as the time required by the tumor to reach the threshold of 10 mm  
220 mean tumor diameter, according to ethical guidelines.

### 221 **Antibodies**

222 See supplemental table 1.

### 223 **Serum**

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

### 227 **Western Blot**

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

### 240 **Flow cytometric analysis**

241 For flow cytometric analysis, single cell suspensions were prepared and  $2 \times 10^5$   
242 cells were stained per sample. Cr-1 specific antibodies in serum of pmCr-1  
243 vaccinated mice were detected by cell surface staining of 4T1mCr-1 with serum  
244 from pmCR-1 vaccinated mice. For FACS sorting, transduced 4T1 cells were first  
245 stained with pmCr-1 serum and then with anti-mIgG-PE. pVAX1 serum was used  
246 as a negative control staining. For IgG subclass analysis, 4T1mCr-1 binding  
247 serum derived antibodies were detected with anti-mIgG-FITC, anti-mIgG1-FITC,  
248 anti-IgG2a-FITC and anti-IgG2b-FITC. For unstained control, cells were only

249 stained with secondary antibodies. Percentage of IgG1, IgG2a and IgG2b were  
250 calculated by dividing mean fluorescent intensities (MFIs) by the sum of MFI for  
251 IgG1, IgG2a and IgG2b after subtraction of MFI of unstained cells. All samples  
252 were acquired either on LSRII (BD) or Novocyte (ACEA) and analyzed using  
253 FlowJo (Tree Star).

#### 254 ***In vivo* imaging**

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

#### 261 **Lung colony assay**

262 Lungs from 4T1mCr-1 bearing mice were harvested and kept in cooled PBS  
263 supplemented with 10% FBS. Lungs were individually mechanically and  
264 enzymatically digested in RPMI supplemented with 5% FBS, 2 mg/ml Dispase,  
265 100 µg/ml DNase I, 200 µg/ml Collagenase IV for 30 min at 37°C. Cell suspension  
266 was filtered using a 70 µm filter (Fisher Scientific). Removal of red blood cells  
267 was done using RBC lysis buffer (BioLegend) and followed by suspension in  
268 supplemented RPMI-1640 media containing 6-Thioguanine (60 µM) and seeded  
269 in 150 mm cell culture dishes (Corning). After 10 days, cells were washed with  
270 PBS, followed by formaldehyde fixation and Hematoxylin Harris (VWR, 351945S)  
271 staining for 5 minutes. Primary tumors were excised and weighed. To evaluate  
272 lung metastasis, colonies were enumerated and metastatic index was calculated,  
273 MI = number of colonies/primary tumor weight.

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

275 4T1mCr-1 and 4T1 cells were harvested and labeled with <sup>51</sup>Cr (Perkin Elmer).  
276 After labeling, target cells were incubated for 10 minutes at 4°C with 10 µl of  
277 serum from pmCr-1 or pVAX1 vaccinated mice. 5x10<sup>3</sup> cells per well were then  
278 plated in 96-well plates without washing. wt BALB/c mice were sacrificed and  
279 splenocytes isolated. NK cells were purified with magnetic beads by DX5-positive  
280 selection (Miltenyi Biotech). NK cell fraction and negative fraction were titrated  
281 onto target cells. 25 µl of co-culture supernatant were harvested after 4 and 16 h  
282 onto LUMA plates (Perkin Elmer). Radioactivity was detected in beta-counter  
283 (Perkin Elmer).

284 **Statistical analysis**

285 Data was analyzed with Prism 7 (GraphPad software). All in vivo data is shown  
286 as mean ± SD. Tumor growth, metastatic index and tumor growth rate were  
287 compared using Mann-Whitney test. Tumor weights were compared with  
288 unpaired t-test. Survival data were compared with log rank test. For NK cell  
289 cytotoxicity, 5 independent experiments are displayed and compared with  
290 paired t-test. p-values < 0.05 were considered statistically significant.

291

292 **Results**

293 Vaccination with mouse Cripto-1-encoding DNA plasmid reduces metastatic  
294 burden and primary tumor growth in 4T1 metastasis model

295 We aimed to understand if vaccination with pmCr-1 would elicit a protective  
296 immune response in a model of murine metastatic breast cancer. We screened  
297 four mouse mammary carcinoma cell lines on BALB/c background for Cr-1

298 expression by western blot. Weak bands of Cr-1 were found in 4T1, TUBO and  
299 TS/A, while D2F2 was negative for mCr-1 expression (Supplemental Fig. 1). As a  
300 first approach to establish the protective potential of mCr-1 vaccination-induced  
301 immune responses over the dissemination of mammary cancer cells in BALB/c  
302 models, we generated a stable mCr-1 expressing 4T1 transfectant (4T1mCr-1),  
303 which was used as a model for spontaneous lung metastasis (Supplemental Fig.  
304 1). BALB/c mice were vaccinated with pmCr-1 or control pVAX1 plasmids prior  
305 to implantation of 4T1mCr-1 cells into the mammary fat pad. Primary tumor  
306 growth was evaluated by *in vivo* luciferase activity detection at day 14 (Fig. 1A)  
307 and twice per week through palpation (Fig. 1B). At day 23 after tumor  
308 inoculation, mice were sacrificed and primary tumor weight measured (Fig. 1C).  
309 Primary tumor size and weight were significantly reduced in pmCR-1- compared  
310 to pVAX1-vaccinated mice. Furthermore, pmCR-1 vaccination greatly reduced  
311 spontaneous metastasis to the lungs as evaluated by a colony formation assay  
312 (Fig. 1D). Cr-1 vaccination results in anti-tumor immunity capable of controlling  
313 tumor growth and inhibiting metastatic spread.

#### 314 Cripto-1 specific humoral response

315 It was previously shown that DNA vaccination in BALB/c mice can elicit a  
316 humoral response (22). We therefore evaluated the humoral response after  
317 vaccination with pmCR-1(23,24). Serum of pmCR-1-vaccinated mice was found  
318 to contain antibodies that stained specifically mCr-1 expressing 4T1 cells (Fig.  
319 2A), while no signal was observed on 4T1 cells. We found that the majority of  
320 these antibodies belonged to IgG2a and IgG2b subclasses (Fig. 2B). In mice, these

321 subclasses are responsible for mediating ADCC by NK cells, macrophages and  
322 neutrophils.

323

#### 324 Cripto-1 directed antibody dependent cellular cytotoxicity

325 NK cells play a major role in the success of antibody-based immunotherapy. For  
326 several clinically successful therapeutic antibodies, including anti-Her2, anti-  
327 EGFR and Anti-CD20, NK cells mediated cytotoxicity is a known mechanism of  
328 action (25).

329 To confirm that Cr-1 specific antibodies can mediate ADCC, we tested if serum  
330 from pmCr-1-vaccinated mice increases cytotoxicity by NK cells. NK cells were  
331 purified from BALB/c splenocytes with magnetic bead selection and co-cultured  
332 with 4T1mCr-1 or 4T1 cells in the presence of pmCr-1 or pVAX1 serum. We  
333 found that pmCr-1 serum significantly increased lysis of 4T1mCr-1 cells by NK  
334 cells (Fig. 2C, D). No cytotoxic activity was detected by splenocytes depleted of  
335 NK cells (data not shown). To show that ADCC is Cr-1 specific we co-cultured NK  
336 cells with 4T1 cells in presence of serum from pmCr-1 and pVAX1 vaccinated  
337 mice. No difference in 4T1 lysis by NK cells was observed in presence of pmCr-1  
338 serum compared to pVAX1 serum (Fig. 2E).

339

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

341 We additionally wanted to test if pmCr-1 vaccination has therapeutic effect in a  
342 more clinically relevant model (26). The BALB-neuT mouse model is genetically  
343 engineered to develop spontaneous cancerous lesions in the mammary tissue.  
344 We evaluated Cr-1 expression in the breast tumors of the model and only found  
345 low expression in tumors of 8 mm mean diameter (Suppl. Fig. 2) with no Cr-1

346 expression in smaller tumors. Mice were vaccinated at 10 and 12 weeks of age,  
347 but this did not result in difference in tumor outgrowth (data not shown) nor did  
348 it affect tumor incidence in this mouse model (Fig. 3A). Consequently, we did not  
349 observe survival benefits (Fig. 3B) until mice were sacrificed according to the  
350 ethical regulations. At sacrifice, lungs were evaluated for the presence of  
351 metastasis. Micrometastases derived from the primary tumors can be found in  
352 the lungs within 8 weeks of primary tumor occurrence (27).

353 Lungs from pmCR-1- and pVAX1-vaccinated mice were sectioned, stained with  
354 hematoxylin and eosin and metastatic foci enumerated. We found that  
355 metastatic burden was significantly reduced in pmCR-vaccinated BALB-neuT  
356 mice (Fig. 3C). We observed that both the number of foci as well as metastatic  
357 size was reduced (Fig. 3C, D).

358

### 359 Vaccination results in protective immune response targeting cancer stem cells

360 Since targeting Cr-1 inhibits metastases, which can be caused by CSC, and Cr-1  
361 expression has previously been associated with CSC in melanoma, colon and  
362 breast cancer CSC (5,28-30). We therefore wanted to evaluate if Cr-1 vaccination  
363 elicits a protective immune response against Cr-1 expressing CSC. It has been  
364 shown that the murine mammary carcinoma cell line TUBO acquires CSC  
365 phenotypic markers when passaged 3 times as spheres (P3 TUBO cells) (21,31).  
366 Over the three passages in spheroid culture of TUBO, we observed a gradual  
367 increase in expression of Cr-1 (Fig. 4A). These TUBO P3 cells were s.c. injected in  
368 vaccinated BALB/c mice. We observed a decreased growth rate as a result of  
369 pmCR-1 vaccination. The time to reach the mean tumor size of pmCr-1 group (4  
370 mm in diameter) was significantly longer in pmCr-1- compared to pVAX1-

371 vaccinated mice (Fig. 4B). In addition, we found that 3 out of 11 mice in the  
372 pmCr-1 group were completely tumor free more than 60 days after tumor  
373 inoculation (Fig. 4C). In comparison, all mice in pVAX1 treatment group  
374 developed tumors within 47 days. Vaccination targeting Cr-1 also resulted in a  
375 trend towards improved survival ( $p=0.078$ ) (Fig. 4D).

376

## 377 **Discussion**

378 The metastatic process of tumors is complex and until today not fully  
379 understood. Two critical cellular processes are crucial for the occurrence of  
380 metastasis, which are EMT and mesenchymal-epithelial transition (MET) (32).  
381 EMT enables cells to survive without cell-cell contact, to migrate and to  
382 extravasate from the primary tumor. At the site of distant metastasis MET is  
383 required for cells to establish metastatic colonies and grow out. Cr-1 is  
384 expressed in cells undergoing EMT and higher expression of this protein has  
385 been found in more aggressive types of human breast cancer (12,33).

386 We have previously reported that Cr-1 is an immunogenic antigen and that  
387 vaccination against Cr-1 results in protective anti-tumor immune responses  
388 against murine melanoma. In this model, a strong protective effect against  
389 pulmonary metastases was observed upon i.v. challenge with metastatic B16F10  
390 cells (17). It is of considerable importance to study the vaccine in a model  
391 recapitulating the complete metastatic cascade from tumor cells undergoing  
392 EMT at the primary tumor site to MET at the site of metastasis. We therefore  
393 chose to study this process in the 4T1 orthotopic breast cancer model and in  
394 Her2 transgenic BALB-neuT mice. When 4T1 cells are orthotopically injected  
395 into the mammary fat pad, they spontaneously metastasize (34,35). Similarly, the

396 BALB-neuT mice develop autochthonous mammary tumors that early  
397 metastasize and colonize the lungs (27). These models enable the study of EMT  
398 and MET *in vivo*. Due to low endogenous Cr-1 expression, we overexpressed  
399 murine Cr-1 in 4T1 cells (Suppl. Fig. 1). We observed that Cr-1 vaccination  
400 reduced metastatic burden in both the orthotopic 4T1 and the spontaneous  
401 BALB-neuT breast cancer model (Fig.1D and 3C, D). Control of the primary  
402 tumor was only seen in the Cr-1 overexpressing 4T1 model. This is in line with  
403 the lack of Cr-1 expression in the primary tumors of the BALB-neuT model  
404 (Suppl. Fig. 2).

405 We observed that the pmCr-1 vaccination induced an anti-mCr-1 humoral  
406 response in the BALB/c mouse model, while we were not able to identify anti-  
407 mCr-1 antibodies in the pVAX1 vaccinated mice (Fig. 2A). Further did the  
408 majority of Cr-1 targeting antibodies belong to the IgG2a subclass (Fig. 2B), able  
409 to bind murine activating Fcγ receptors with relatively high affinity. In view of  
410 these results, we aimed at understanding the role of NK cells in Cr-1 vaccination-  
411 induced tumor control. Collectively, our data pointed at a critical role for NK  
412 mediated ADCC in pmCr-1-vaccinated mice (Fig. 2). These results are  
413 reminiscent of our earlier findings, where we have shown that Her2-vaccination  
414 in BALB/c mice initiated a humoral anti-Her2 immunity and consequently killing  
415 of Her2-positive tumor cells by NK cells (22). *In vitro* cytotoxicity data  
416 demonstrated that lysis of Cr-1 expressing cells by NK cells was increased in the  
417 presence of serum from pmCr-1-vaccinated mice (Fig. 2C and D), pointing at a  
418 major role for ADCC in the tumor elimination. Hereby we were able to show one  
419 mechanism of vaccination-induced tumor elimination by NK cells.

420 In a previous study we have shown that anti-Cr-1 vaccination in C57Bl/6 mice  
421 induced an *in vitro* detectable cytotoxic T cell response (17). After vaccination,  
422 Cr-1 specific cytotoxic T cells have not been detected *in vitro* in BALB/c  
423 splenocytes (data not shown). Although these results do not entirely rule out a  
424 possible role for T cells in the observed *in vivo* tumor protection, they argue for a  
425 difference in immune response between BALB/c and C57Bl/6 mice upon DNA  
426 vaccination. In a study performed by Radkevich-Brown et al. Her2 DNA  
427 vaccination elicited a humoral immune response in Her2 transgenic BALB/c  
428 mice. In a direct comparison, Her2 vaccination induced significantly lower levels  
429 of Her2-specific antibodies in C57Bl/6 mice than in the BALB/c mice (36). The  
430 differences observed are to be explained with the genetic differences of the mice  
431 strains and can be translated to our findings in the BALB/c and C57Bl/6 mice  
432 after Cr-1 DNA vaccination (17).

433 Cr-1 expression is potentially limited to CSC, a few cells undergoing EMT in the  
434 primary tumor, and metastasizing cells. De Castro et al. recently described Cr-1  
435 expression in EMT-like areas in the JygMC(A) breast cancer model. In contrast,  
436 no Cr-1 expression was detected in metastatic lesions in the lung (37).  
437 Vaccination against Cr-1 could potentially interrupt the metastatic process at an  
438 early stage and thereby prevent the establishment of metastases at distant sites.  
439 In CSC of several tumor types, Cr-1 expression has been confirmed (15,38,39).  
440 We have found that spheroid cultures of murine breast cancer cells, which are  
441 considered to be enriched in CSC, upregulate Cr-1 expression (Fig 4A)  
442 (21,30,31). Subcutaneously injected TUBO P3 cells grew out in all BALB/c mice  
443 within 6 weeks after injection. After vaccination against Cr-1, 27% of mice did  
444 not develop tumors (Fig. 4C). In the remaining mice, we observed a reduced

445 tumor growth rate (Fig. 4B and C). Immune responses induced by Cr-1  
446 vaccination specifically target Cr-1-positive CSC and control tumor burden.

447 In patients, high levels of Cripto-1 expression in the tumor have been associated  
448 with decreased survival and could be correlated to advanced disease (12). In  
449 addition, Cripto-1 has been found in the serum of breast cancer patients,  
450 suggesting its potential function as a biomarker (40). For lung cancer, it was  
451 reported that serum levels of Cripto-1 correlated with tumor stage (41). These  
452 reported clinical findings associate increase expression of Cripto-1 with  
453 metastasis and worse survival in breast cancer patients.

454 It is crucial for patient survival to eliminate tumor cells that can cause relapse  
455 and metastasis to potentially prolong patient survival. New therapeutic  
456 strategies, which can specifically target both CSC and metastases, have the ability  
457 to reduce the risk of relapse and disease related death in cancer patients.

458 Immune targeting therapies have shown a great potential in treatment of  
459 metastatic diseases (42). It is crucial to identify novel immunogenic antigens that  
460 can be targeted by immunotherapies. We propose that Cripto-1 is a suitable  
461 candidate for immunotherapy in breast cancer patients, targeting a different  
462 subset of breast cancer cells than in our previous Her2 DNA vaccine clinical trial  
463 (10). We have shown that targeting Cripto-1 in breast cancer mouse models  
464 reduced metastasis and targeted CSC. For patients, a DNA vaccine targeting  
465 Cripto1 could potentially translate into increased disease free and overall  
466 survival.

467

468

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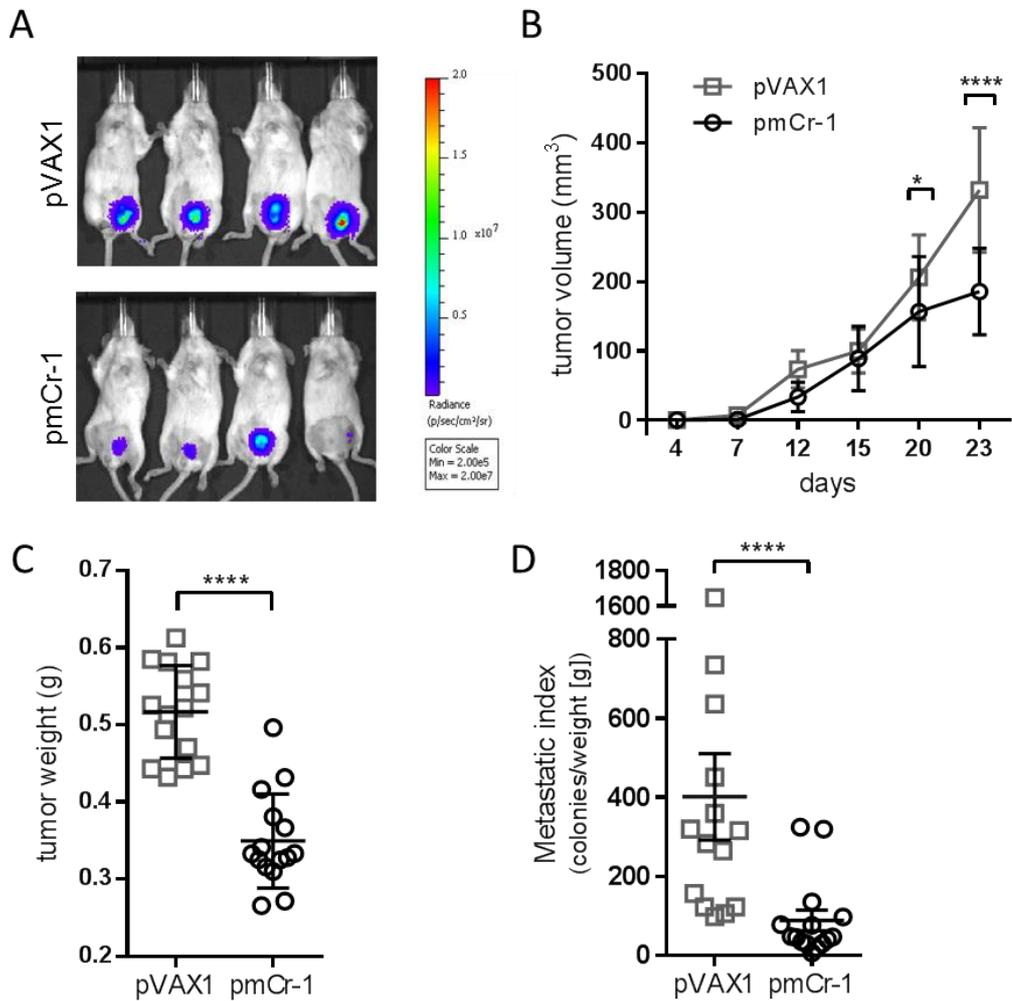
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617  
618  
619  
620

621 **Figure 1**



622

623

624 *Tumor growth and metastatic spread in orthotopic 4T1mCr-1 breast cancer model*

625

626 Orthotopic injection of  $2 \times 10^5$  4T1mCr-1 cells in pmCr-1- or pVAX1- vaccinated

627 BALB/c mice. Mice were sacrificed on day 23 after tumor inoculation. **A**,

628 Luciferase expression at day 14 after tumor inoculation. 4 representative mice

629 are displayed. **B**, Volume of primary tumors. Mice in pVAX1 (n=5) and pmCr-1

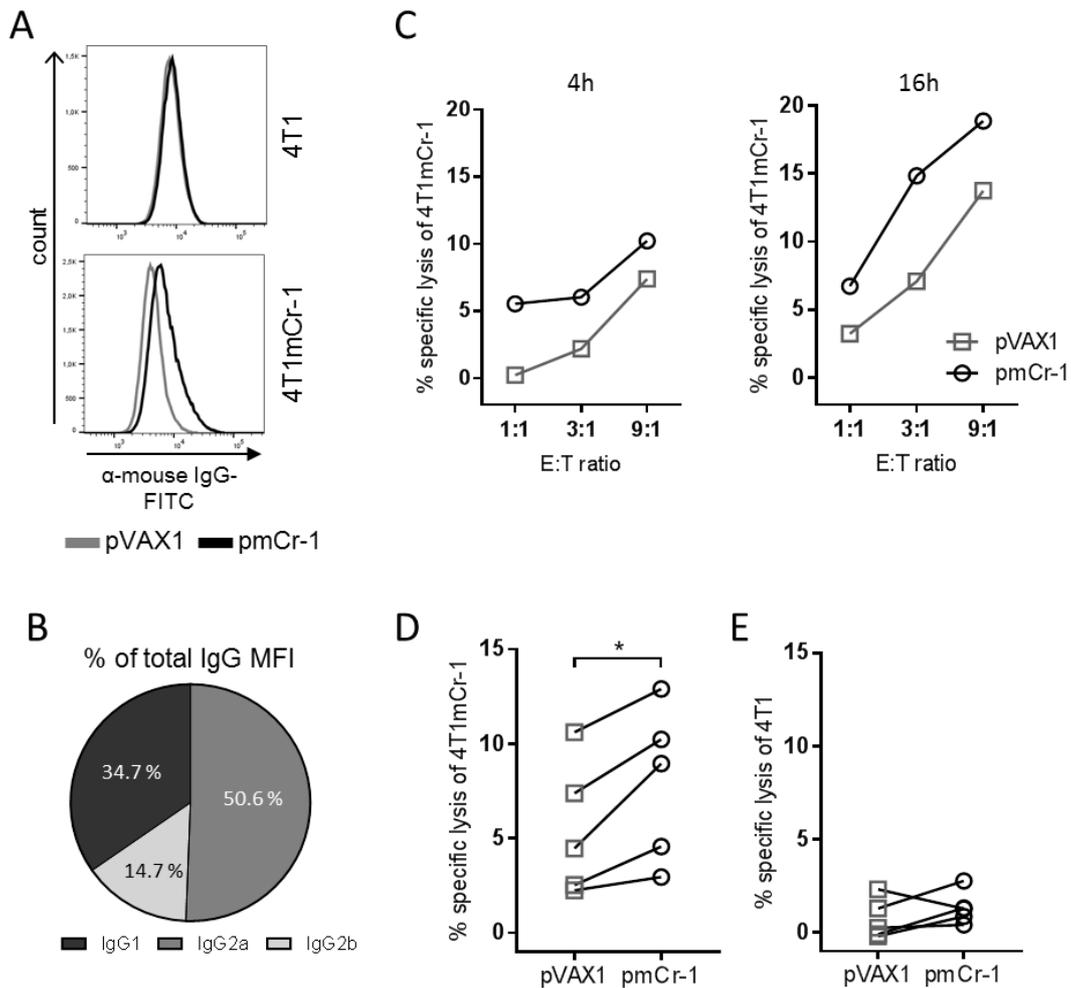
630 (n=5) group were palpated twice per week until experimental endpoint on day 23.

631 Error bars represent standard deviation; \*  $p=0.0321$ , \*\*\*\*  $p<0.0001$  (Mann-

632 Whitney test). **C**, Primary tumor weight at day 23. Error bars represent standard  
633 deviation; \*\*\*\*  $p < 0.0001$  (unpaired t-test). **D**, Single cell suspension of lung  
634 tissue was seeded in petri dish and cultured in selection medium. At day 10,  
635 colonies were fixed and counted. Metastatic index (MI) was calculated by MI=  
636 number of colonies/primary tumor weight. Error bars represent standard  
637 deviation; \*\*\*\*  $p < 0.0001$  (Mann-Whitney test).

638

639 **Figure 2**



640

641 *Humoral response induced by pmCr-1 vaccination in BALB/c mice*

642

643 BALB/c mice were vaccinated with pmCr-1 or pVAX1. Two weeks after the boost

644 vaccination, serum was collected for analysis. **A**, 4T1mCr-1 and 4T1 cells were

645 incubated with serum from pmCr-1 and pVAX1. Surface binding serum

646 antibodies were detected with anti-mIgG-FITC antibody. Cells were analyzed on

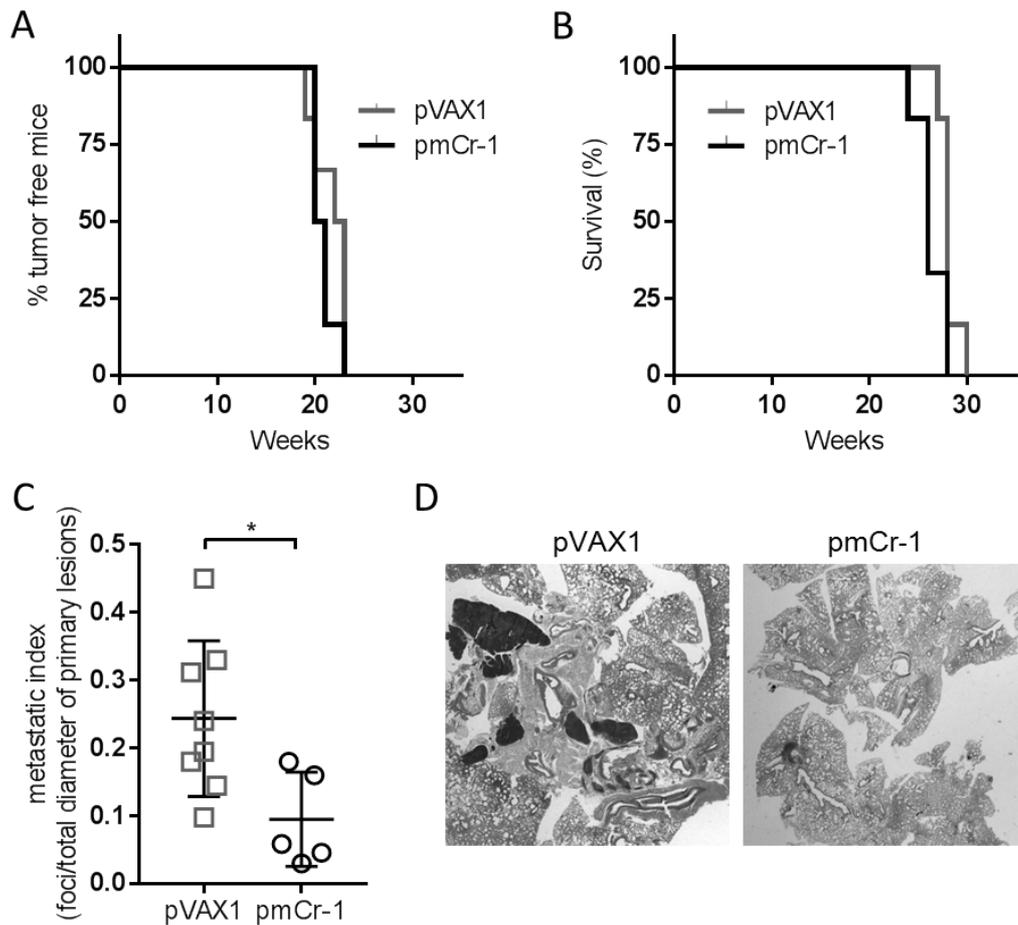
647 flow cytometer. **B**, Subclasses of antibodies in pmCr-1 serum binding Cr-1 were

648 detected with secondary anti-mIgG1-FITC, anti-mIgG2a-FITC, anti-mIgG2b-FITC.

649 Cells were analyzed by flow cytometry. **C**, NK cells cytotoxicity against 4T1mCr-

650 1 cells in the presence of pmCr-1 or pVAX serum. Assay supernatants were  
651 harvested after 4h and 16h for analysis. **D**, NK cell cytotoxicity against 4T1mCr-1.  
652 Summary of 5 individual experiments after 4h co-culture at 9:1 effector to target  
653 ratio; \* p=0.0158 (Paired t test). **E**, NK cell cytotoxicity against 4T1. Summary of  
654 5 individual experiments after 4h co-culture at 9:1 effector to target ratio.  
655

656 **Figure 3**



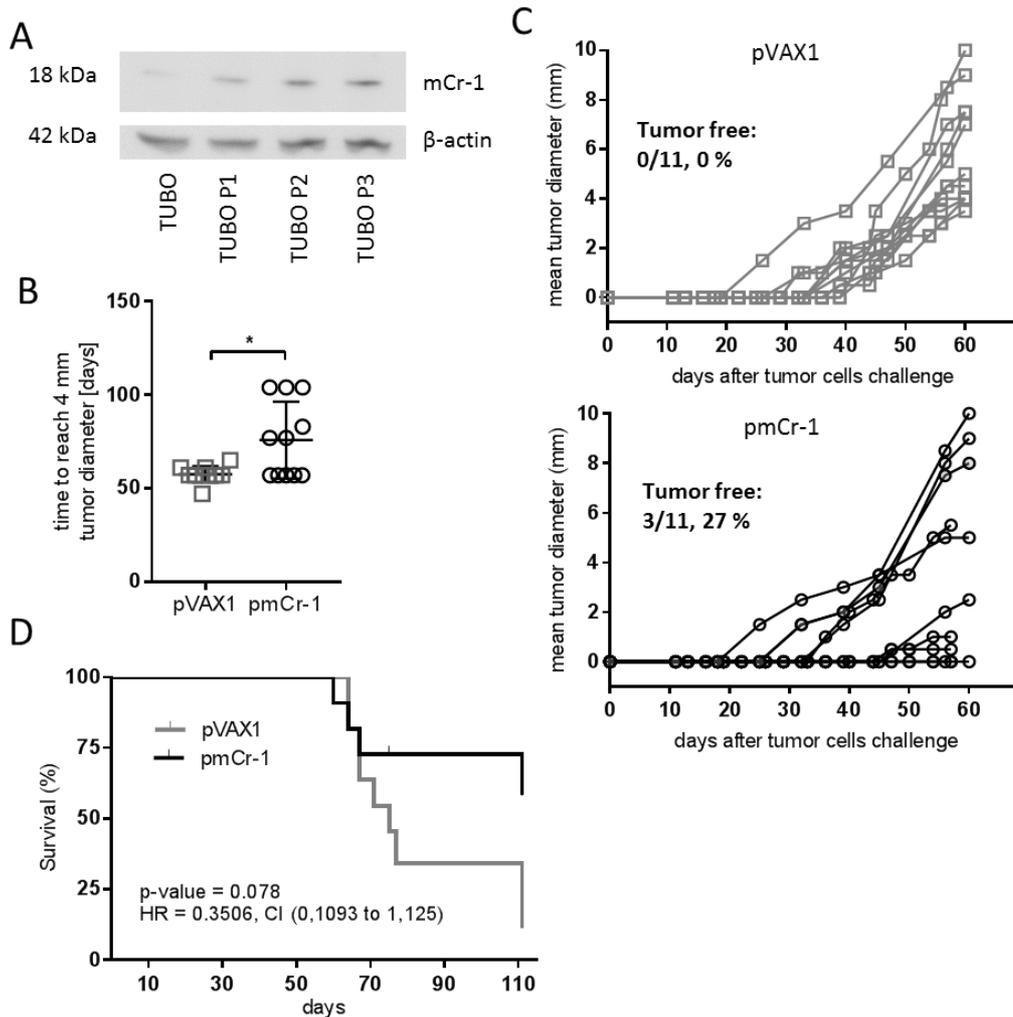
657

658 *Metastatic spread in Her2/neu driven spontaneous breast cancer model BALB-*  
659 *neuT*

660 BALB-neuT mice were vaccinated at 10 weeks and 12 weeks with pmCr-1 or  
661 pVAX1. Mice were followed over time and sacrificed upon ethical endpoint. **A**,  
662 Tumor incidence in pVAX1 (n=6 ) and pmCr-1 (n=6) vaccinated BALB-neuT  
663 mice. **B**, Survival of pVAX1 (n=6) or pmCr-1 (n=6) vaccinated BALB-neuT mice.  
664 Mice were sacrificed upon ethical endpoints. **C**, Metastatic burden in the in  
665 pVAX1 (n=5) and pmCr-1 (n=5) mice. Metastatic index is calculated by  
666  $MI = \text{number of foci} / \text{sum of the diameter of all primary lesions}$ . Error bars  
667 represent standard deviation; \* p=0.021 (Mann-Whitney test). **D**, Light

668 microscopy image of the lung sections after hematoxylin and eosin staining, 10x  
669 magnification.  
670

671 **Figure 4**



672

673 *Vaccination induced immune response is targeting breast cancer stem cells*

674 P3 TUBO cells were s.c. injected in pmCr-1 or pVAX1 immunized BALB/c mice. **A**,

675 Western Blot for Cr-1 in spheroid passaged TUBO cell line. **B**, Tumor growth rate

676 in pmCr-1 (n=11) and pVAX (n=11) vaccinated mice. Error bars represent

677 standard deviation; \* p=0.0453 (Mann-Whitney test). **C**, Individual tumor growth

678 curves for pmCr-1 and pVAX until day 61. **D**, Survival curves for mice immunized

679 with pmCr-1 or pVAX1 after s.c. challenge with TUBO P3. p=0.078 (Mantel-Cox

680 test).

681