1　**Cripto-1 plasmid DNA vaccination targets metastasis and cancer**

2　**stem cells in murine mammary carcinoma**

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36   **Conflict of interest:**

37   R.Kiessling is in the Scientific Advisory Board for the companies Immunicum AB,

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Statement of translational relevance

Despite the wide range of therapies approved for treatment of breast cancer, mortality of patients due to metastatic spread has not been yet been addressed. The development of metastasis targeting treatments is essential in decreasing breast cancer related deaths in long term. Here we describe a vaccine based therapeutic approach targeting tumor antigen Cripto-1 expressed on tumor cells. We show that vaccination with Cripto-1 encoding DNA elicits an anti-Cripto-1 directed immune response that consequently controls metastasis. Cripto-1 expression has also been found on cancer stem cell like cells. Cancer stem cells are highly resistant to chemo and radiotherapy. They can be the cause for relapse and metastases due to their persistence after standard treatment. The anti-Cripto-1 directed immune response was able to eliminate cancer stem cells. Taken together, our data shows great potential of targeting tumor associated antigen Cripto-1 in controlling metastasis and eliminating cancer stem cells.

Abstract

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

Experimental Design: BALB/c mice and BALB-neuT mice were treated with a DNA vaccine encoding for mouse Cripto-1 (mCr-1). Mice were challenged with murine breast cancer 4T1 cells or TUBO spheres, or spontaneously developed breast cancer in the BALB-neuT model. 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.

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

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

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86 tumors. Targeting Cripto-1 by vaccination is a promising potential

87 immunotherapy for treatment of metastatic breast cancer.

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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, TGFβ, and Wnt/βcatenin

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

6

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 melanomaB16F10 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

Material and methods

Cell lines

4T1 luciferase expressing cells (4T1) TS/A and D2F2 cell lines was maintained in RPMI 1640 supplemented with L-glutamine and 10% heat-inactivated FBS (Life technologies). TUBO cell line (18) 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).

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 6×10^4 cells/ml in ultra-low attachment plates (Corning). The resulting spheroids were monitored daily and passed using enzymatic and mechanical dissociation every 3-5 days. Cells were re-seeded at 6×10^4. 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 to regional Animal ethics committees (Stockholms Norra Djurförsoksetiska Nämnd Avdelning 2, Sweden N426/11,

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177   N239/14; University of Torino ethical committee authorization number

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   (IGEA). Electroporation protocol has been previously described (17). In week 12,

190    $2x10^5$ 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 ($\pi/6$) x L x W x 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 2×10^4 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**

10

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 $2x10^5$

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.

12

Antibody dependent cellular cytotoxicity (ADCC) assay

4T1mCr-1 and 4T1 cells were harvested and labeled with ^{51}Cr (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. 5×10^3 cells per well were then

plated in 96-well plates without washing. wt 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 beta-counter

(Perkin Elmer).

Statistical analysis

Data was analyzed with Prism 7 (GraphPad software). All in vivo data is 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, 5 independent experiments are displayed and compared with

paired t-test. p-values < 0.05 were considered statistically significant.

Results

Vaccination with mouse Cripto-1-encoding DNA plasmid reduces metastatic

burden and primary tumor growth in 4T1 metastasis model

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 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

14

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

322 neutrophils.

323

324 <u>Cripto-1 directed antibody dependent cellular cytotoxicity</u>

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 <u>Reduced lung metastasis after vaccination in the BALB-neuT mouse model</u>

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   <u>Vaccination results in protective immune response targeting cancer stem cells</u>

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-

16

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 pat, they spontaneously metastasize (34,35). Similarly, the

17

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.

18

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.

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Analysis and interpretation of data: K. Witt, M.A. Ligtenberg, L. Conti, S. Lanzardo, R. Ruiu, A. Lladser, A. Lundqvist, R. Kiessling

Writing, review and revision of the manuscript: K. Witt, M.A. Ligtenberg, A. Lundqvist, R. Kiessling

Administrative, technical, or material support: C. Rolny, A. Lladser, A. Lundqvist

Study supervisor: F. Cavallo, R. Kiessling

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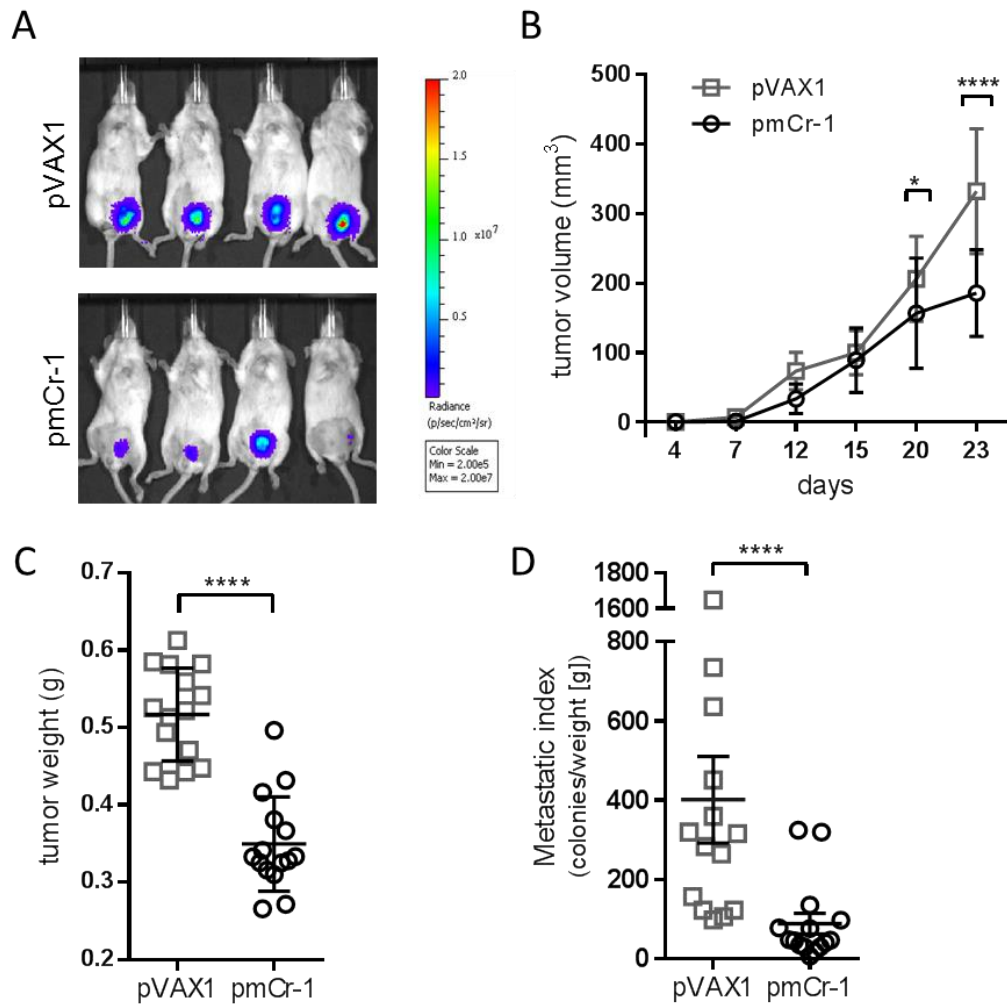
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624 *Tumor growth and metastatic spread in orthotopic 4T1mCr-1 breast cancer model*

625

626 Orthotopic injection of $2x10^5$ 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 palped 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

Figure 2

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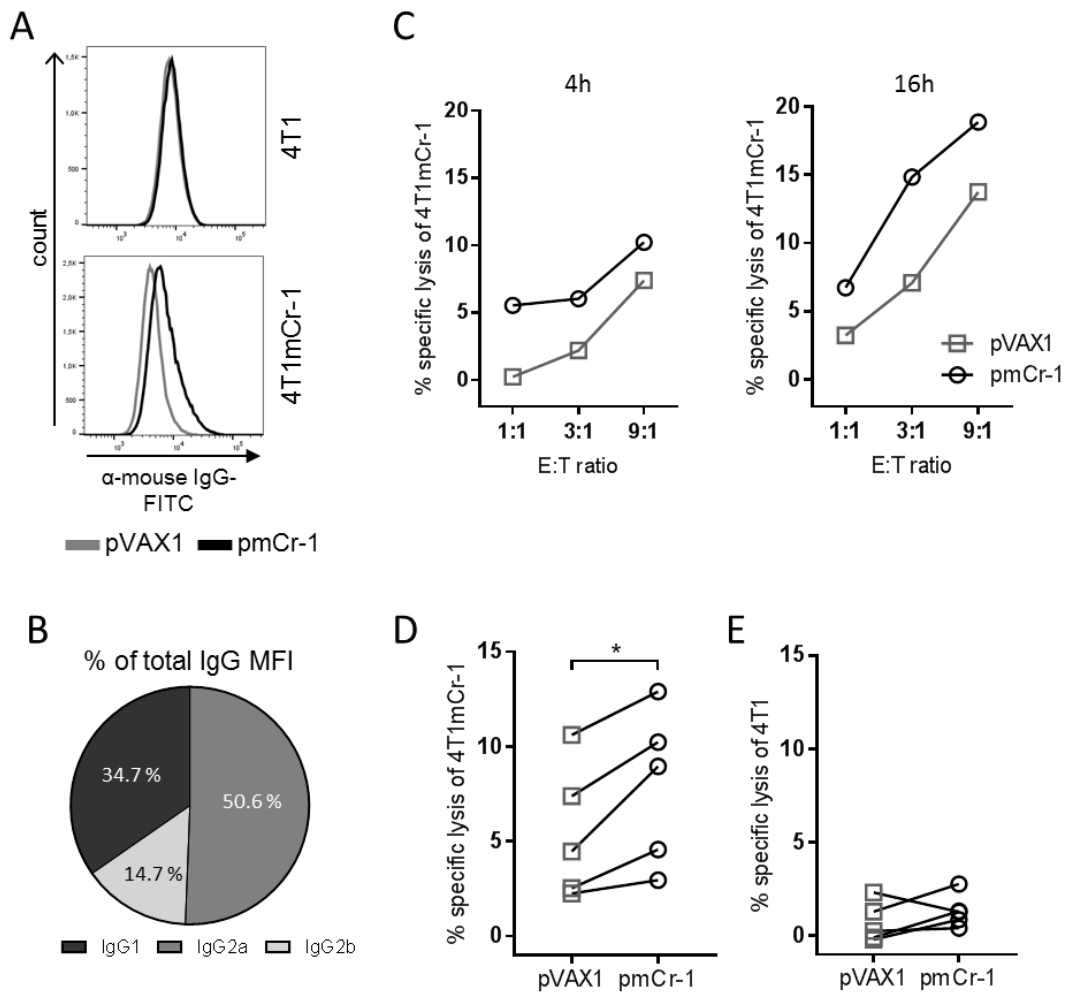
Humoral response induced by pmCr-1 vaccination in BALB/c mice

642

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

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

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

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

flow cytometer. **B**, 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. **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

Figure 3
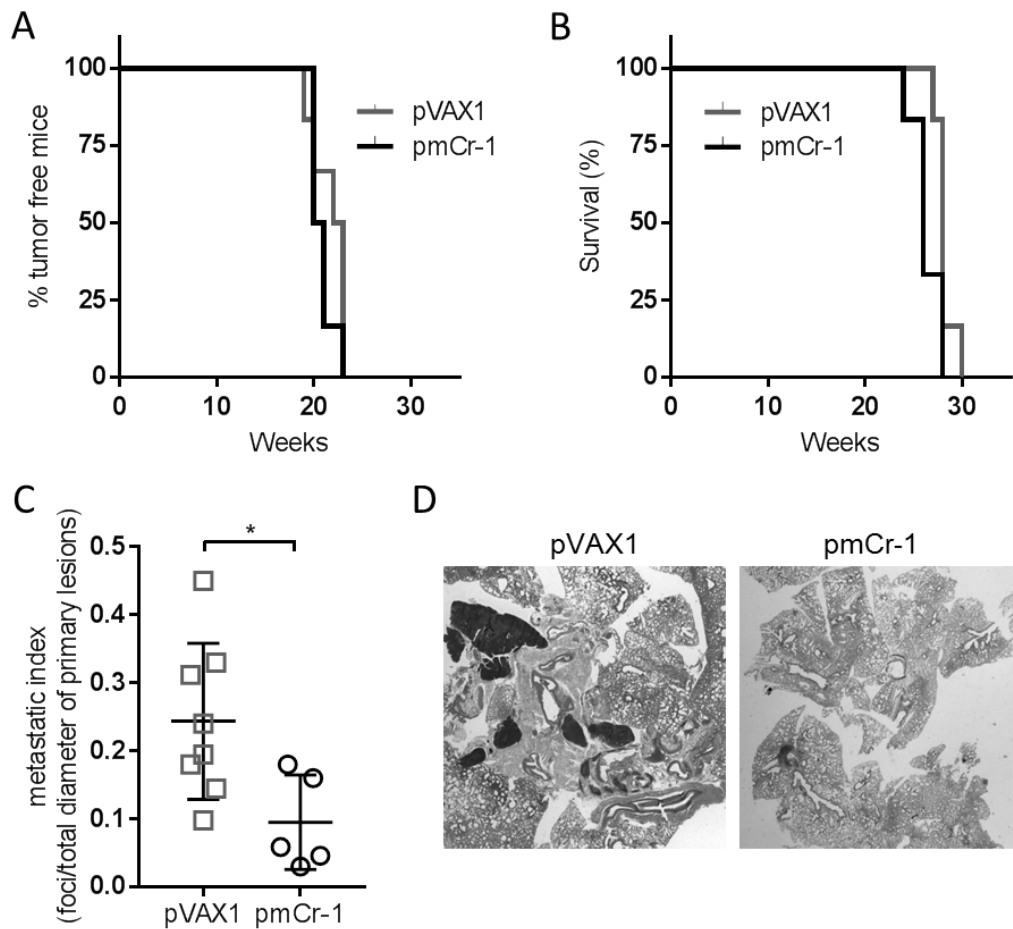
Metastatic spread in Her2/neu driven spontaneous breast cancer model BALB-

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 upon ethical endpoints. **C**, Metastatic burden in the in

pVAX1 (n=5) and pmCr-1 (n=5) mice.   Metastatic index is calculated by

MI=number of foci/sum of the diameter of all primary lesions. Error bars

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

Figure 4

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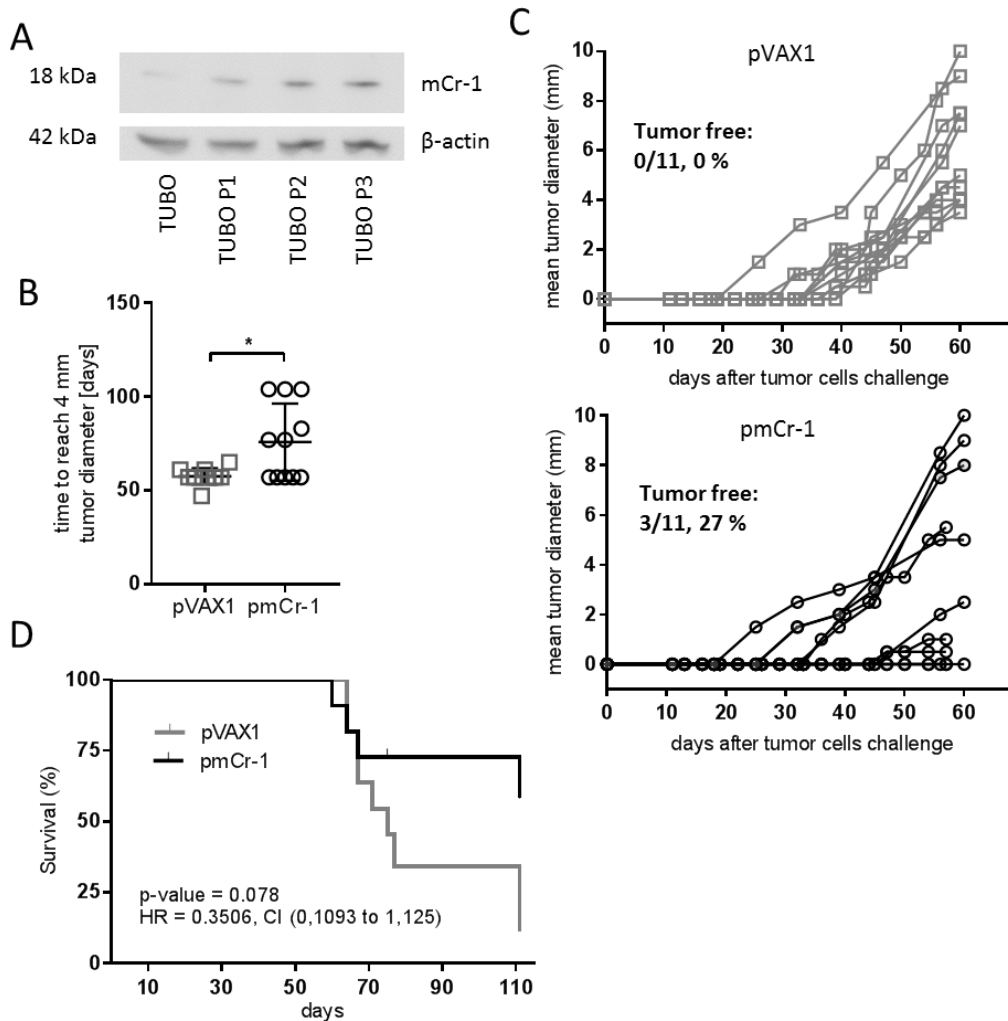
Vaccination induced immune response is targeting breast cancer stem cells

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

Western Blot for Cr-1 in spheroid passaged 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. p=0.078 (Mantel-Cox

test).

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