

Suppression of a functional CCR7 chemokine receptor inhibits the post-intravasation steps of metastasis in malignant murine mammary cancer cells

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Abstract. To study the role of the chemokine receptor CCR7 in the metastatic process, a murine CCR7 gene was transduced in two mammary cancer cell lines with different origins and molecular features; TS/A, derived from a spontaneous mammary cancer of BALB/c strain, and N202.1A, derived from a HER-2/neu transgenic mammary cancer (FVB background) and characterized by a high expression of HER-2/neu. Transduced CCR7 conferred to mammary cancer cells a chemotactic response towards CCL21 (a CCR7 ligand), but did not consistently affect *in vitro* growth properties. *In vivo*, CCR7-engineered cells gave rise to tumors in syngeneic hosts with growth rates similar to or slightly lower than the controls and with similar patterns of spontaneous metastases. When injected directly intravenously to study the late post-intravasation phases of metastasis, CCR7-engineered cells showed a strongly decreased lung colonizing ability. Such an effect was observed both with HER-2/neu-positive and -negative mammary cancer cells. When used as a prophylactic vaccine, CCR7-transduced cell vaccine succeeded in the long-term control of mammary tumorigenesis in 25% of the HER-2/neu transgenic females, suggesting an increased immunogenicity of CCR7-engineered cells.

Introduction

Metastasis is a hallmark of cancer (1) and is the main cause of death in cancer patients. It results from a multistage process, starting with invasion at the primary tumor site, followed by dissemination by the hematogenous or lymphatic route with arrest in distant organs, and ending with a distant-site

colonization, consisting in the conversion of disseminated single cancer cells into proliferating, proangiogenic secondary tumors. Each phase is likely governed by specific molecular mechanisms which are still incompletely understood (2,3).

Experimental models to evaluate the role played by candidate molecules in the metastatic process can take advantage of gene engineering to modify gene expression on the one hand (4), and several methods to induce metastasis on the other, for example the direct intravenous injection of cells that simulate the late, post-intravasation phases of metastasis (5).

Some chemokine receptors have been suggested to play a role in metastasis (6-9), CXCR4 and CCR7 being the most important candidates. Chemokine receptors are physiological mediators of the directed migration of leukocytes along a chemical gradient of ligand (10). An ectopic expression of the chemokine receptor CXCR4 was observed in a large series of human tumors with different histotypes and was, in some cases, correlated with lymphatic and distal dissemination; CCR7 expression was found to be correlated with metastasis in squamous-cell carcinoma and non-small-cell lung cancer (8). The potential role for CXCR4 and CCR7 receptors in the metastatic process has been confirmed by a few gene transduction studies (11,12), which used, as a recipient for gene transfer, non-epithelial tumor cells, such as T-cell hybridoma and melanoma. However in experiments studying the induction of metastatic ability by gene modifications, the histotype of origin is not irrelevant; in particular, mammary cells are profoundly different from melanocytes, the latter having a pro-metastatic gene expression machinery (13).

Breast cancer can show expression of CXCR4 and CCR7, and display a migratory response to their ligands (14). Functional demonstrations of the role played by CXCR4 in breast cancer metastasis were obtained through CXCR4 blocking by means of neutralizing antibodies, a selective synthetic polypeptide or siRNA (14-16). Upregulation of CXCR4 expression is essential for HER-2/neu-mediated tumor metastasis (17). On the contrary, the role of CCR7 in metastasis of mammary carcinoma still needs to be investigated through functional approaches.

In this study the role of the chemokine receptor CCR7 in mammary cancer metastasis was investigated through the

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transduction of a CCR7 gene. We used, as recipients, two mammary cancer cell lines with different origins and molecular features; TS/A, derived from a spontaneous mammary cancer of BALB/c strain (18), and N202.1A, derived from a mammary cancer arisen in the N#202 HER-2/neu transgenic line (FVB background) and characterized by the expression of HER-2/neu (19).

Materials and methods

CCR7 gene transduction. Two cell lines of murine mammary carcinoma were used for gene transduction; TS/A (18) and N202.1A (19). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS), 10% for TS/A and 20% for N202.1A, at 37°C in 5% CO₂ atmosphere. All media constituents were from Invitrogen, Milan, Italy. Expression vector pLNCX2-CCR7, carrying the murine cDNA gene for CCR7 (20), was kindly provided by Dr S. Hwang (National Cancer Institute, Bethesda, MD) (12). Cells were plated in 100-mm Petri dishes at 10⁶ cells/dish in 10 ml of culture medium. After 24 h, 2 µg/dish of pLNCX2-CCR7 or pLNCX2 (empty vector) was transfected by calcium phosphate precipitation (Invitrogen). Transduced cells were positively selected in the presence of G418 (Invitrogen) at a concentration of 800 µg/ml. After selection, cultures were routinely maintained in 500 µg/ml of G418.

Real-time RT-PCR. Total RNA was extracted from cell lines by TRIzol reagent. Total RNA (1 µg) was reverse-transcribed using reverse transcriptase in the presence of random hexamers. CCR7 expression was analysed by quantitative real-time RT-PCR using an ABI PRISM 5700 sequence detection system (Applied Biosystems, Applied, Milan, Italy), with CCR7 primers and under conditions reported in the literature (12). Relative quantification of the mRNA levels of CCR7 was determined using the $\Delta\Delta C_t$ method, and the results were expressed as fold difference in expression of CCR7-engineered cells relative to untransduced recipient cells.

Cytofluorometric studies. CCR7 membrane expression was studied through a fusion protein CCL19-IgG2b, kindly provided by Dr Krautwald, University of Schleswing-Holstein, Kiel, Germany (21). Harvested cells were incubated with 50 µl of CCL19-IgG2b at 4°C for 30 min, washed and incubated with 50 µl of 1:20 diluted rabbit anti-human IgG antiserum (Behring, Marburg, Germany) at 4°C for 30 min. After the final washings, cells were re-suspended in phosphate-buffered saline containing 1 µg/ml of ethidium bromide to gate out dead cells and were subjected to cytofluorometric analysis with a FACScan (Becton Dickinson, St. Jose, CA). HER-2/neu membrane expression was studied using a 1:20 dilution of the monoclonal antibody 7.16.4 recognizing rat neu antigen (Oncogene Research Products, San Diego, CA), and a secondary goat anti-mouse IgG antibody (Euroclone, Milano, Italy), diluted 1:20. Conditions and analysis were as above.

Migration assay. Migration assay was conducted using Transwell chambers (Costar, Cambridge, MA) with 8-mm pore size, polyvinylpyrrolidone-free polycarbonate filters.

DMEM supplemented with 20–400 nM CCL21 (Peprotech, London, UK) was placed in the lower compartment, whereas 5x10⁵ cells re-suspended in DMEM were seeded in the upper compartment and incubated overnight at 37°C in a 7% CO₂ atmosphere. Cells that migrated through the filter to reach the lower chamber were counted with an inverted microscope. The chemotaxis index was calculated as: (number of cells migrated toward CCL21 gradient/number of cells migrated in the absence of CCL21) x 100.

In vitro growth. To determine population doubling time of adherent cultures, 5x10⁵ viable cells were seeded in 25-cm² flasks, and growth curves were obtained by a direct count of cells harvested with trypsin-EDTA for 5 days after seeding. For anchorage-dependent clonogenicity, 200–6,400 cells were seeded in 60-mm tissue culture Petri dishes in DMEM + 20% FBS. After 14 days, colonies were fixed in methanol, stained with Giemsa and counted with an inverted microscope at low magnification. Anchorage-independent cloning efficiency was determined by suspending 10⁴ to 2x10⁵ cells in DMEM + 20% FBS containing 0.33% agar. Cell suspensions were then layered on a 5-ml base of 0.5% agar in 60-mm Petri dishes. Colony growth was monitored twice weekly and determined by counting at low magnification 14 days after seeding. To study the effect of CCL21 on cell proliferation, cells were seeded in 96-well Multiwell Falcon plates (10⁴ cells/well) in DMEM + 1% FBS. After 24 h, CCL21 was added at concentrations ranging from 60–240 ng/ml (corresponding to 5–20 nM). Cell growth was evaluated after a further 48- to 72-h incubation with the addition of 10 µl/well of WST-1 (Cell proliferation reagent, Roche, Monza, Italy), and by a reading of 450/620-nm absorbance in a Sunrise plate reader (Tecan, Switzerland). Results were expressed as % of control cultures performed in parallel in the absence of the chemokine.

In vivo studies. To study tumorigenicity TS/A and its transduced cells (5x10⁴) were injected subcutaneously (s.c.) into the right inguinal region of syngeneic 8- to 12-week-old BALB/cAnNCRL mice (Charles River Laboratories, Calco, Italy), whereas N202.1A and its transduced cells (10⁶ cells) were injected into healthy 8- to 16-week-old female syngeneic N#202 HER-2/neu transgenic mice (22) from our colony (23). Tumor incidence and growth were evaluated weekly. Neoplastic masses were measured with calipers; tumor volume was calculated as $\pi/6 \times [a \times b]^3$ in which *a* and *b* were two perpendicular major diameters. Experimental metastases were evaluated 30 days after the injection in a lateral tail vein of 5x10⁴ or 10⁵ cells for TS/A and N202.1A models, respectively. To evaluate lung metastases, lungs were contrasted with black India ink, and metastases were counted in dissected lung lobes under a stereoscopic microscope. For the evaluation of vaccine efficacy of 1A-CCR7-C11 cells, HER-2/neu transgenic mice with a BALB/c background (BALBneuT mice) from our colony were used (23). Starting at the sixth week of age, BALB-neuT mice entered the vaccination protocol, consisting of 2 weeks of bi-weekly intraperitoneal (i.p.) vaccinations with 2x10⁶ mitomycin C-treated engineered cells in 0.4 ml of PBS, followed by 1 week of five daily i.p. administrations (50 ng in the first course and 100 ng thereafter) of mouse rIL-12 (kindly provided by

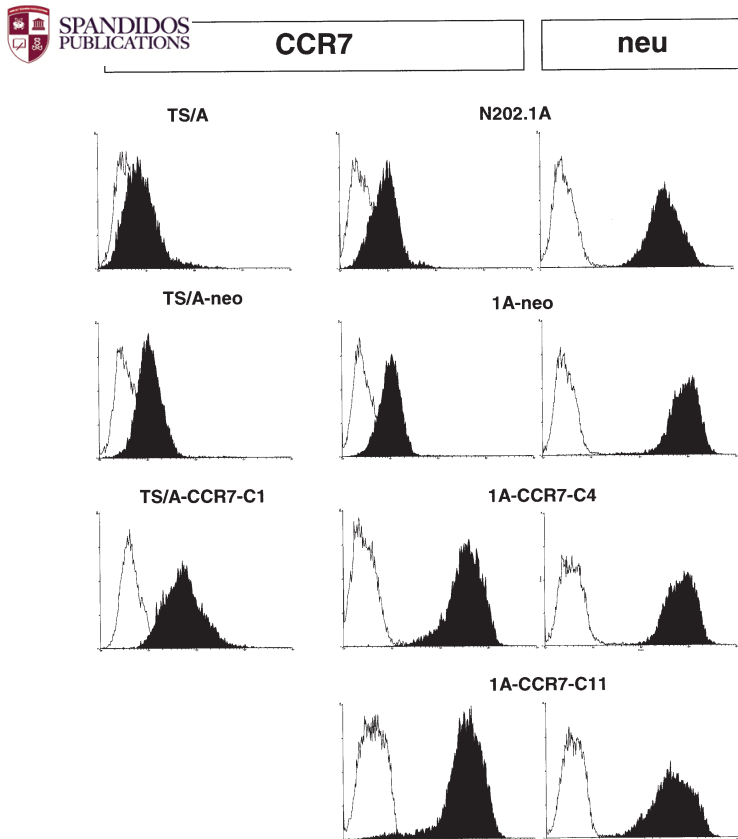


Figure 1. Flow cytometric analysis of CCR7 and neu membrane expression in transduced murine mammary carcinoma cells. In each panel the abscissa is fluorescence intensity on a logarithmic scale ranging from 10^0 to 10^4 , and the ordinate is the number of cells. Open profiles represent cells stained with secondary antibody alone, and dark profiles represent cells stained for the indicated antigen.

Dr S. Wolf, Genetics Institute, Andover, MA), and 1 week of rest. Vaccination cycles were repeated lifelong (24).

Results

CCR7 expression and function. TS/A and N202.1A cell lines were transduced with an expression vector carrying the cDNA

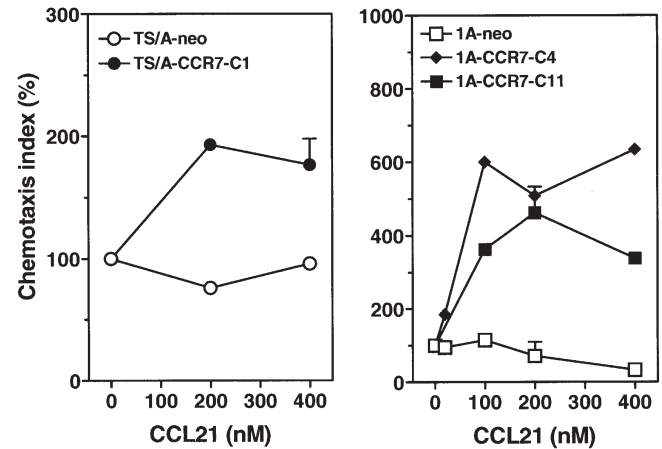


Figure 2. Chemotactic response of CCR7-engineered murine mammary carcinoma cells to different concentrations of CCL21. The chemotaxis index was calculated as: (number of cells migrated toward CCL21 gradient/number of cells migrated in the absence of CCL21) $\times 100$. The mean \pm SE of 2 replicates is shown.

for murine CCR7 or with the corresponding empty vector. A wide panel of clones were screened for CCR7 expression by real-time RT-PCR, and the highest expressors were chosen: for TS/A cell line, clone TS/A-CCR7-C1 expressed up to 10^5 -fold more CCR7 than parental cells or neo-resistant transduction controls, whereas for N202.1A cells, clones 1A.CCR7-C4 and 1A.CCR7-C11 expressed respectively ~ 1 and 3 million-fold more CCR7 than controls.

All the CCR7-engineered clones selected showed membrane expression of the chemokine receptor as seen by indirect immunofluorescence and cytofluorometric study (Fig. 1). CCR7-engineered N202.1A clones displayed the highest values of CCR7 membrane expression, while keeping unaltered a high HER-2/neu expression.

All the CCR7-engineered clones studied showed a migratory response to CCL21 (a CCR7 ligand) in a standard migration assay (Fig. 2); the higher migratory response observed with N202.1A-engineered clones paralleled the higher CCR7 membrane expression. Therefore transduced

Table I. *In vitro* growth properties of CCR7-engineered mammary cancer cells.

Cells	Gene modification	Doubling time (h)	Anchorage-dependent cloning efficiency (%)	Anchorage-independent cloning efficiency (%)	Proliferative response to CCL21 ^a (% of control)
TS/A	none	13.2 \pm 0.9	29.9 \pm 1.3	6.8 \pm 1.3	114.0 \pm 12.9
TS/A-neo	neo ^r	13.4 \pm 1.2	26.1 \pm 0.5	7.0 \pm 0.9	90.0 \pm 2.2
TS/A-CCR7-C1	neo ^r + mCCR7	13.7 \pm 1.0	28.2 \pm 0.7	11.8 \pm 1.3	100.6 \pm 4.7
N202.1A	none	26.1 \pm 1.2	8.1 \pm 1.3	0.6	104.0 \pm 7.7
1A-neo	neo ^r	23.7 \pm 1.5	2.8 \pm 0.4 ^b	1.0	96.3 \pm 2.5
1A-CCR7-C4	neo ^r + mCCR7	23.1 \pm 0.3	8.1 \pm 1.2	0.4	102.9 \pm 3.3
1A-CCR7-C11	neo ^r + mCCR7	37.5 \pm 1.5 ^b	4.6 \pm 0.3 ^b	0.1	96.4 \pm 17.1

^aData refer to 240 ng/ml CCL21. Similar results were obtained with lower CCL21 doses. ^b $p < 0.05$ vs corresponding untransduced cells (Student's t-test).

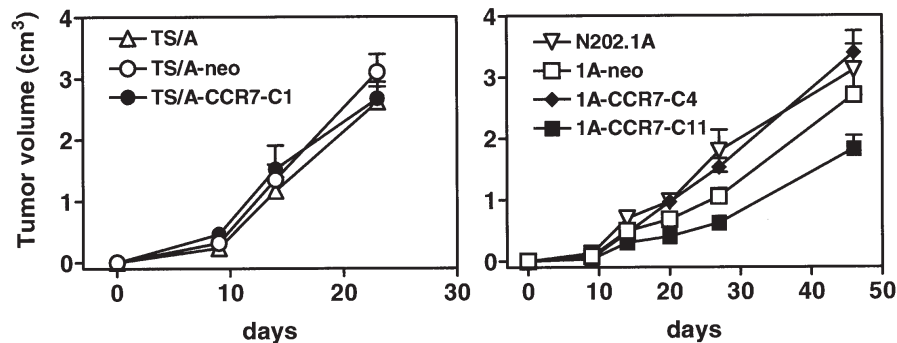


Figure 3. Tumorigenic ability of CCR7-engineered mammary cancer cells in syngeneic hosts. TS/A cells and derivatives were injected into BALB/c mice; N202.1A cells and derivatives were injected into N#202 HER-2/neu transgenic mice (FVB background). Each point represents the mean and SE (5 mice per group). From day 14 after cell injection onwards, tumor volumes induced by 1A-CCR7-C11 were significantly lower than those induced by N202.1A ($p < 0.05$, Student's *t*-test).

Table II. Metastatic ability of CCR7-engineered mammary cancer cells in syngeneic hosts.

Cells	Gene modification	Spontaneous lung metastases (tumor-bearing mice)			Experimental lung metastases (i.v. cell injection)		
		Incidence (%)	Median number	Range	Incidence (%)	Median number	Range
TS/A	none	5/5 (100)	20	1-48	5/5 (100)	117	54-136
TS/A-neo	neo ^r	5/5 (100)	17	5-69	5/5 (100)	45 ^a	30-88
TS/A-CCR7-C1	neo ^r + mCCR7	4/5 (80)	28	0-54	5/5 (100)	12 ^b	7-22
N202.1A	none	4/7 (57)	1	0-3	5/5 (100)	76	60-111
1A-neo	neo ^r	1/4 (25)	0	0-2	5/5 (100)	72	10-96
1A-CCR7-C4	neo ^r + mCCR7	4/5 (80)	2	0-6	n.d. ^c		
1A-CCR7-C11	neo ^r + mCCR7	1/5 (20)	0	0-1	1/5 (20)	0 ^b	0-1

^a $p < 0.05$ vs corresponding untransduced cells (non-parametric rank sum test). ^b $p < 0.01$ vs corresponding untransduced cells and vs transduction neo controls (non-parametric rank sum test). ^cn.d., not done.

CCR7 chemokine receptor was functional in murine mammary cancer cells.

In vitro growth of CCR7-engineered cells. We studied several parameters of *in vitro* growth of CCR7-engineered cells and controls (both parental cells and transduction controls), such as the doubling time, the cloning efficiency (both anchorage-dependent and -independent) and the growth in the presence of CCL21 (Table I). No parameter was consistently altered in association with CCR7, therefore CCR7 did not influence the *in vitro* growth of mammary cancer cells.

Tumorigenic and metastatic ability. All the CCR7-engineered clones of both origin (TS/A and N202.1A) maintained the ability to grow as subcutaneous tumors in syngeneic mice, with *in vivo* growth rates close or slightly lower than those of the respective controls (Fig. 3). Spontaneous metastatic ability of CCR7-engineered clones resembled that of the parental cell line (Table II); a very low ability to give rise to lung metastasis was observed with the N202.1A cell line and their CCR7-engineered cells, whereas TS/A and CCR7-transduced TS/A

cells displayed a high ability to metastasize spontaneously to the lungs.

However, when the late phases of the metastatic process were studied through cell injection directly into the blood stream, CCR7-engineered cells of both cell lines induced a significantly decreased number of lung metastases (Table II).

Immunogenicity of CCR7-engineered cells. HER-2/neu transgenic mice are a model for mammary carcinogenesis, since they show a progression from hyperplastic mammary gland to *in situ* carcinoma and then to invasive tumors resembling that observed in humans (23). In particular, HER-2/neu transgenic females with the BALB/c background (BALBneuT mice) developed mammary carcinomas in all 10 mammary glands, with the first tumor occurring between 20 and 25 weeks of age. We previously found that vaccination of tumor-free 6-week-old mice with allogeneic HER-2/neu-positive cells (such as N202.1A cells with FVB background) delayed the carcinogenic process, depending on the presence of appropriate adjuvant stimuli (24). To study the immunogenic ability of CCR7-engineered cells, we used 1A-CCR7-

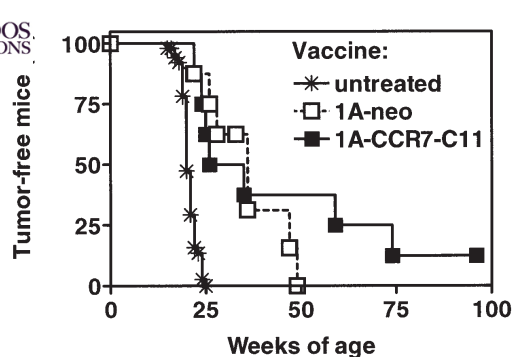


Figure 4. Vaccination with CCR7-engineered cells delayed mammary carcinogenesis in HER-2/neu transgenic mice. Both cell vaccines (1A-neo and 1A-CCR7-C11) induced a significant inhibition of carcinogenesis with respect to untreated mice ($p < 0.01$, Mantel-Haenzel test).

C11 cells as a preventive vaccine in BALB-neuT mice compared to the 1A-neo transduction control cells (Fig. 4). Both cell vaccines induced a significantly delayed carcinogenesis with respect to untreated mice. Prevention afforded by CCR7-engineered cells was not statistically different from that of the transduction controls. However two long-term tumor-free mice were present in the 1A-CCR7-C11-vaccinated group; one developed a mammary tumor at 59 weeks of age, while the other remained tumor-free for its entire life (>2 years).

Discussion

To study the role of the chemokine receptor CCR7 in the metastatic process of mammary cancer cells, we choose a gene-transduction approach and two experimental model systems with different etiology, molecular features and metastatic ability.

The TS/A cell line was derived from a mammary carcinoma that arose spontaneously in a retired breeder female of BALB/c strain (18). It is characterized by a high ability to give rise to spontaneous lung metastases from subcutaneously grown tumors.

The N202.1A cell line was derived from a mammary carcinoma that arose in HER-2/neu transgenic mice (19); these mice carry a rat HER-2/neu transgene driven by the MMTV-LTR promoter that causes HER-2/neu overexpression in the mammary gland and consequent mammary carcinogenesis (23). N202.1A cells maintain a high expression of HER-2/neu and are tumorigenic in the syngeneic hosts, with a low ability to give rise to spontaneous lung metastases. However N202.1A cells can give rise to lung metastases when injected directly into the blood stream, thus showing that they fail in the early phases of the metastatic process (such as local invasion), but that they can complete the late phases, such as arrest and growth in the pulmonary parenchyma.

The murine chemokine receptor CCR7 gene transduced in both cell lines was functional, since it mediated a migratory response to CCL21 (one of the two CCR7 ligands), within the same dose interval previously reported for human breast cancer cells (14). CCR7 expression did not consistently alter

any of the *in vitro* growth features examined, including proliferation in the presence of CCL21. A few non-leukocyte cell types expressing chemokine receptors were reported to have increased proliferation in the presence of the appropriate chemokine; CCR7-positive kidney mesangial cells showed a proliferative response to CCL21 (25,26), whereas CXCR4-positive human pancreatic tumor cells had enhanced proliferation in the presence of CXCL12 (27). Our data show that CCR7-expressing mammary cancer cells behave differently from other CCR7-expressing non-leukocyte cell types. This also suggests that the effects of a transduced CCR7 gene may depend on the cells chosen as recipients.

Some data reported in the literature on the role of chemokine receptors in the metastatic process were based on models which showed coexpression of CXCR4 and CCR7 (14). Both cell lines used as transduction recipients in this study (TS/A and N202.1A) expressed very low levels of CCR7 and were negative for CXCR4, as found in our PCR and microarray studies (unpublished results), thus constituting good models with which to study the effects of CCR7 alone in the absence of CXCR4 expression. We did not find significant effects of CCR7 expression on the tumorigenic and spontaneous metastatic ability, regardless of the HER-2/neu expression of the transduction recipient. The lack of effect was observed both with the TS/A model, which was able to metastasize spontaneously and therefore could efficiently complete early phases of the metastatic process, and with the N202.1A model, whose low metastatic ability could have easily allowed evaluation of the effects of a prometastatic candidate gene. Under our conditions we never observed locoregional metastatic lymph nodes at necropsy (data not shown), hence, even if the cells could be attracted to the lymph nodes, they did not appear to proliferate in that environment.

Transduced CCR7 expression was associated with a significant inhibition of the late post-intravasation phases of the metastatic process. A possible explanation for this phenomenon could be a better antigen presentation. CCR7 transduction was reported to increase the migratory ability of a dendritic cell vaccine into regional lymph nodes (28). Tumor cells are generally poor antigen-presenting cells. However, a study on different murine models has shown the crucial role played by tumor cell localization to secondary lymphoid organs in order to obtain a cytotoxic T cell response; the lack of expression of costimulatory molecules by tumor cells being almost irrelevant (29). To test this hypothesis we studied the immunogenic ability of CCR7-engineered cells in a prophylactic vaccine approach for prevention of mammary carcinogenesis. CCR7-transduced cell vaccine succeeded in the long-term control of mammary tumorigenesis in 25% of the HER-2/neu transgenic females, suggesting an increased immunogenicity of CCR7-engineered cells.

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