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**In breast cancer a high ratio of tumor-infiltrating intraepithelial CD8+ to FoxP3+ cells is characteristic for the medullary subtype**

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Running title: FoxP3+ in medullary breast cancer

Keywords: Medullary breast cancer, regulatory T cell, FoxP3, CCL22

**Abstract**

**Aims:** Medullary breast carcinoma (MBC) is a biologically distinct subtype of breast cancer characterized by prominent lymphocytic infiltrates and favorable clinical outcome. Tumor-infiltrating CD8<sup>+</sup> effector T cells may contribute to the good prognosis of this type of cancer, however certain subtypes of lymphocytes, such as FoxP3<sup>+</sup> regulatory T cells (Treg), can also suppress anti-tumor immunity. **Methods:** We determined tumor infiltration by FoxP3<sup>+</sup>, CCL22<sup>+</sup> and CD8<sup>+</sup> cells in paraffin-embedded sections of MBC and, as reference, in samples of **grade 3** ductal, lobular and mucinous breast cancer. **Results:** All analyzed MBC were strongly infiltrated by FoxP3<sup>+</sup> cells whereas only weak infiltrates were detected in ductal or lobular breast cancer. This finding was unexpected given the good prognosis of MBC. Strikingly, CD8<sup>+</sup> T cells exceeded the number of FoxP3<sup>+</sup> cells in MBC (CD8/FoxP3=2.6), whereas equal amounts of both cell types were found in ductal breast cancer (CD8/FoxP3=1.1). In both types of breast cancer we further detected cells expressing the Treg-attracting chemokine CCL22. **Conclusion:** In breast cancer, a predominance of tumor-infiltrating CD8<sup>+</sup> over FoxP3<sup>+</sup> cells **was observed in MBC**. Thus, the ratio of CD8 to FoxP3 rather than the absolute number of intratumoral FoxP3<sup>+</sup> cells may be predictive for the clinical outcome of cancer.

**Keywords:** breast cancer, medullary breast cancer, lymphocytes, regulatory T cells, cytotoxic T cells, CCL22, FoxP3.

## Introduction

Medullary breast carcinoma (MBC) is a particularly interesting subtype as it is characterized by less frequent lymph node metastasis<sup>1</sup> and good clinical outcome.<sup>2</sup> Histologically it consists of large sheets of poorly differentiated tumor cells appearing in a syncytial growth pattern and a prominent lymphocytic infiltration of the tumor stroma.<sup>3</sup> Several authors suggest that a high number of tumor-infiltrating lymphocytes may help to limit tumor burden and thus contribute to good prognosis in MBC.<sup>3-6</sup> Tumor-infiltrating lymphocytes however represent a heterogeneous group of immune cells composed of both effector cell subsets and immunosuppressive cells.

Regulatory T cells (Treg) represent a population of immunosuppressive cells capable of inhibiting both innate and adaptive immunity. A key function of Treg is the suppression of immune responses directed against self-antigens and therefore these cells are crucial for the prevention of autoimmunity.<sup>7</sup> Treg however also inhibit the cytotoxic activity of CD8+ T cells against malignant tumors and many studies have shown that Treg play a crucial role in tumor-induced immunosuppression.<sup>8</sup> The transcription factor FoxP3 is so far the most specific marker to identify human Treg, although it can also be expressed by other lymphocytes upon activation.<sup>9</sup> Infiltration of tumors by FoxP3+ Treg was observed in different types of human cancer and is associated with poor prognosis.<sup>10, 11</sup> The recruitment of Treg to the tumor tissue depends on specific mediators that attract these cells to the tumor site. The chemokine CCL22 represents one important factor<sup>12</sup> that can mediate migration of FoxP3+ Treg to the tumor site.<sup>10</sup>

Although the high number of intratumoral lymphocytes is constantly seen in MBC, infiltration by FoxP3+ Treg in this type of breast cancer has not been investigated. In this study we analyzed paraffin-embedded material of 13 MBC, 34 ductal breast cancers (DBC) and seven lobular or mucinous breast cancers for the presence of FoxP3+ and CD8+ lymphocytes and for CCL22-expressing cells in the tumor tissue. Interestingly, all analyzed MBC were strongly infiltrated by

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both FoxP3+ and CD8+ cells. CCL22 was detected in all tumors and was mainly expressed by tumor-infiltrating immune cells and in a few cases by the cancer cells themselves. We further determined the relative proportion of effector T cells to Treg in the tumors and found that intratumoral CD8+ cells clearly exceeded the number of FoxP3+ cells in MBC but not in DBC. The high CD8 to FoxP3 ratio may thus contribute to the relatively good prognosis of patients with MBC.

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## Material and Methods

### *Patients*

Paraffin-embedded material of 54 breast cancer patients, including 13 MBC, 34 DBC, four lobular breast cancers and three mucinous carcinomas was used for histological analysis (Table 1, Characteristics of patients and tumors). All cases were retrieved from the archives of the Institute of Pathology of the Ludwig Maximilian University, Munich, Germany. Age of patients ranged from 26 to 80 years. All patients were treated surgically between 2004 and 2008 at the same institution (Department of Obstetrics and Gynecology Maistrasse, University of Munich). Tumor typing and staging were performed according to the classification of the International Union against Cancer (UICC). Medullary breast carcinoma was diagnosed when the following five features were present: (1) well-circumscribed tumor borders, (2) **lymphocytic** reaction diffusely in the tumor and in the periphery, (3) syncytial pattern of tumor cell growing, (4) high nuclear grade and (5) high mitotic activity. **Ductal breast cancer tissues used in this study were all grade 3**. Tissue sections were reviewed by independent investigators at the Institute of Pathology of the LMU Munich.

### *Immunohistology*

All paraffin-embedded specimens were cut at 2–3  $\mu\text{m}$  and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany). After deparaffinization and rehydration immunohistochemical assays were performed by standard methods. The following antibodies were used: mouse-anti-human FoxP3 (1:100, Abcam, Cambridge, England), rabbit-anti-human CCL22 (1:350, Peprotech, Rocky Hill, NJ, USA) and rabbit-anti-human CD8 (1:50, Thermo Fisher Scientific, Fremont, CA, USA). Appropriate horseradish peroxidase- and alkaline phosphatase-conjugated secondary antibodies were used for detection. The amount of tumor-infiltrating FoxP3+, CCL22+ and CD8+ cells was evaluated semi-quantitatively by analyzing the complete surface of the tissue section. The following scoring system was used: -, no; + low

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numbers; ++, medium numbers; +++, high numbers of infiltrating cells. For the analysis of the CD8 to FoxP3 ratio, absolute cell numbers infiltrating the tumor epithelium were counted in digital images derived from CD8- and FoxP3-stained serial sections and the ratio was calculated for each pair. Digital images were obtained at 20- or 40-fold magnification (Axiovert Inverse Microscope, Carl Zeiss, Jena, Germany) and processed using Adobe Photoshop for adjustment of contrast and size.

#### *Cell culture and ELISA*

The human breast cancer cell lines MCF7 und MDA-MB-231 were maintained in DMEM medium supplemented with 10 % FCS, 1 % L-glutamine, 1 U/ml penicillin and 0.1 mg/ml streptomycin (all PAA Laboratories, Coelbe, Germany). Dendritic cells were generated by using PBMC from peripheral blood of healthy donors and by culturing the adherent fraction in the presence of GM-CSF (1000 U/ml) and IL-4 (500 U/ml) for 24 h. Supernatants were assayed for CCL22 by ELISA (R&D Systems, Minneapolis, MN, USA). For immunocytology, single cell suspensions of formalin-fixed cells were embedded in paraffin and stained as described.

#### *Statistical analysis*

Data are presented as mean in bar diagrams or as box plot and were analyzed by unpaired Student's t-test. Statistical analysis was performed using Graphpad Prism software.

## Results

### **Medullary breast carcinoma is strongly infiltrated by FoxP3+ cells**

To determine Treg infiltration in the tumor tissue of patients with MBC, we stained FoxP3+ cells in paraffin-embedded material of 13 individuals followed by semi-quantitative evaluation of the infiltration density. Interestingly, all analyzed MBC were strongly infiltrated by FoxP3+ cells (Figure 1A). All of these cells showed the typical nuclear staining pattern of the Treg-associated transcription factor FoxP3. In contrast, only few DBC were infiltrated by FoxP3+ cells with 30 of 34 analyzed tumors containing no or low amounts of cells. Low numbers of FoxP3+ cells were further observed in the four lobular breast cancers and the three mucinous carcinomas. In all breast cancer tissues two different types of infiltration by FoxP3+ cells were observed: first, infiltration into the tumor epithelium with direct contact to cancer cells (Figure 1B) and second, presence of FoxP3+ cells in the tumor stroma or in lymphoid clusters that were located either in or around the tumor tissue. Both patterns were observed in MBC and DBC, but lymphoid clusters were more frequent in MBC. In contrast to the tumor area, FoxP3+ cells were almost absent from the adjacent healthy breast tissue (Figure 1C). In conclusion, MBC are infiltrated by high numbers of FoxP3+ cells whereas other subtypes of breast cancer contain only few of these immunosuppressive cells.

### **The Treg-attracting chemokine CCL22 is expressed by tumor-infiltrating immune cells**

Recruitment of Treg to malignant tumors can be mediated through the chemokine CCL22.<sup>10</sup> To determine whether CCL22 may play a role in the migration of Treg to MBC, we analyzed the expression of this chemokine in the respective tumor tissues. CCL22 was mainly expressed by myeloid-shaped immune cells that infiltrated both the tumor stroma and epithelium (Figure 2A). CCL22-expressing immune cells were detected in all tumors and the adjacent normal breast tissue and no difference in the frequency of these cells was observed between MBC and DBC

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(Figure 2B). Of the four lobular breast cancer tissues all contained numerous CCL22+ cells. In some but not all cases CCL22-expressing cells were locally associated with accumulations of FoxP3+ cells (Figure 2A, example of DBC). In addition to the expression by immune cells, CCL22 was found to be expressed also by the cancer cells (Figure 3A). Whereas virtually none of these cells were found in MBC or lobular breast cancer, CCL22 expression by cancer cells was detected in 24 % of DBC (8 out of 34). Generally, CCL22-expressing cancer cells were not found in local association with FoxP3+ Treg. In some tumors, CCL22 was strongly expressed also by those cancer cells that infiltrated lymphatic vessels (lymphoangiosis carcinomatosa, Figure 3A). In contrast, no CCL22 secretion was detected in vitro from breast cancer cell lines (Figure 3B). In summary, CCL22+ immune cells were present in all analyzed breast cancer specimens whereas expression of this chemokine by cancer cells was less frequent and limited to DBC.

### **CD8+ T cells exceed the number of tumor-infiltrating FoxP3+ cells in medullary breast cancer**

The high levels of tumor-infiltrating FoxP3+ cells in MBC contrast with the relatively good prognosis of this cancer. It is generally accepted that tumor-infiltrating effector T cells, in contrast to Treg, are associated with favorable outcome.<sup>13, 14</sup> We therefore analyzed the MBC tissues for the presence of CD8+ effector T cells and further correlated these numbers with the infiltration by FoxP3+ lymphocytes. We detected a prominent infiltration of CD8+ T cells in all MBC (Figure 4A), which is in accordance with previous reports.<sup>6, 15</sup> In contrast, most DBC and all lobular breast cancers or mucinous carcinomas were infiltrated only weakly by CD8+ cells. In all tumors, CD8+ T cells were located not only in the tumor epithelium but also in the stroma and in lymphoid clusters. To determine the relation of CD8+ to FoxP3+ cells we analyzed serial sections of either CD8- or FoxP3-stained MBC tissues. Interestingly, we found important differences in the CD8 to FoxP3 ratio according to the position of both cell types in the tumor: in the tumor epithelium of MBC we observed a striking predominance of CD8+ T cells, whereas a variable CD8 to FoxP3

ratio was seen in lymphoid clusters and the tumor stroma (Figure 4B). To quantify the CD8 to FoxP3 ratio in the tumor epithelium of MBC and DBC we counted the number of both cell types in congruent visual fields using serial sections and calculated the mean of all ratios for both tumor subtypes (Figure 4C). Interestingly, a high CD8 to FoxP3 ratio was confirmed in the tumor epithelium of MBC (mean CD8/FoxP3 = 2.6), whereas a significantly lower ratio was found in DBC (mean CD8/FoxP3 = 1.1). Thus, in contrast to DBC, intratumoral CD8+ T cells in MBC clearly exceed the number of FoxP3+ cells.

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## Discussion

MBC constantly feature prominent lymphocytic infiltrates which are associated with good clinical outcome.<sup>3</sup> In general, grade 3 breast cancer with prominent lymphocytic infiltration was reported to have a more favorable prognosis.<sup>16</sup> We show here for the first time that MBC are strongly infiltrated by FoxP3+ cells, a population of lymphocytes with immunosuppressive function.<sup>7</sup> High numbers of FoxP3+ cells were observed in all analyzed MBC tissues and both the tumor epithelium and the surrounding stroma were infiltrated by these cells. Although tumor infiltration by FoxP3+ cells has so far not been investigated for MBC, intratumoral Treg in DBC or lobular breast cancer have previously been detected using histology,<sup>10, 17-19</sup> flow cytometry<sup>20</sup> or PCR.<sup>21, 22</sup> Two studies on DBC, using tissue microarrays, showed that the number of tumor-infiltrating FoxP3+ cells is significantly correlated with reduced survival.<sup>11, 23</sup> One recent study on invasive breast cancer however did not find a correlation between high Treg numbers and survival based on multivariate analysis.<sup>24</sup> The findings in DBC are in line with studies on other types of cancer where the number of tumor-infiltrating FoxP3+ cells is associated with poor prognosis.<sup>25, 26</sup> The strong infiltration of MBC by FoxP3+ cells together with the good prognosis of this tumor thus contrasts with the observations in other types of human cancer.

In addition to tumor-infiltrating FoxP3+ cells in MBC, we detected high levels of intratumoral CD8+ cells. This is in accordance with previous reports that describe tumor-infiltrating CD3+ and CD8+ cells in MBC.<sup>6, 15</sup> In contrast to Treg, tumor infiltration by T lymphocytes in general is thought to positively influence survival of cancer patients. In a study on patients with ovarian cancer, the five-year survival rate was 38 % among patients whose tumors contained CD3+ cells versus 4.5 % for patients without intratumoral T cells.<sup>27</sup> Similar findings, based on the examination of intratumoral T cells, were observed for other types of cancer such as colorectal carcinoma and melanoma.<sup>28, 29</sup> In the present study, MBC tumors were abundantly infiltrated by both CD8+ and FoxP3+ cells prompting us to determine the ratio of these T cell subpopulations.

Interestingly, we found a clear predominance of CD8+ T cells reflected by a mean CD8 to FoxP3 ratio of 2.6 in the tumor epithelium of MBC. In contrast, equal numbers of CD8+ and FoxP3+ cells were found in the tumor epithelium of DBC. Although the mean ratio of CD8+ to FoxP3+ cells showed a highly significant difference between both types of breast cancer, there was some overlap as few individual tumors escaped from this pattern. It would be interesting to analyze the clinical outcome of these patients. DBC tissues in our study had a relatively low lymphocytic infiltrate, it would therefore also be interesting to assess the CD8+ to FoxP3+ ratio between MBC and DBC with prominent inflammation. In summary, the high ratio of intratumoral CD8 to FoxP3 cells could contribute to the good prognosis of MBC.

The recruitment of effector T cells or Treg into malignant tumors is generally mediated by specific chemokines. CCR4 is a chemokine receptor expressed at higher levels on CD4+FoxP3+ cells than on other T cell populations in human blood.<sup>12</sup> One ligand for CCR4 is CCL22, a chemokine that has been detected in different human tumors.<sup>30, 31</sup> Indeed, tumor homogenates can induce CCL22-dependent migration of Treg<sup>32, 33</sup> and the role of CCL22 for the recruitment of Treg to tumors has also been demonstrated in vivo.<sup>10</sup> We investigated expression of this chemokine in different subtypes of breast cancer and found that all analyzed tumors and, in addition, the adjacent healthy breast tissues were infiltrated by CCL22-expressing myeloid-shaped immune cells. These cells were present in the tumor epithelium, the surrounding connective tissue and in some cases in lymphoid clusters where they were locally associated with FoxP3+ Treg. Unexpectedly, co-localization of CCL22+ with FoxP3+ immune cells was generally rare and limited to lymphoid accumulations. Further, no differences in the number of intratumoral CCL22-expressing cells were found between MBC and DBC, although MBC contained significantly higher numbers of FoxP3+ Treg. In addition, no correlation between the numbers of CCL22 and FoxP3-expressing cells was seen within the different DBC tissues analyzed in this study. This contrasts with a previous report based on rtPCR analysis that showed a positive correlation

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between CCL22 and FoxP3 mRNA transcripts in DBC.<sup>22</sup> Another study on Treg in DBC, based on tissue microarrays, described a positive correlation between CCL22 and FoxP3 expression only in lymphoid infiltrates but not in the tumor parenchyma, which is supported by our observations in DBC.<sup>23</sup> Thus, in breast cancer, CCL22-expressing immune cells may attract FoxP3+ Treg to lymphoid clusters surrounding the tumor bed, but the functional role of CCL22+ immune cells in the epithelium or the stroma of the tumors remains unclear.

Although it has been hypothesized that intratumoral CCL22 may not only be secreted by immune cells but also by the tumor cells themselves, conclusive data demonstrating CCL22 expression by cancer cells in solid tumors have not been available so far. Here, we could clearly show that, in breast cancer, CCL22-expressing tumor cells indeed exist. Whereas virtually no CCL22+ tumor cells were found in MBC, we detected those cells in 8 out of 34 DBC tissues. Interestingly, in some of these tumors, cancer cells with strong CCL22 expression were found in lymphatic vessels, suggesting that they might have a higher metastatic potential. As CCL22-expressing cancer cells were not detected in association with FoxP3+ or CD8+ cells it remains questionable whether these cells are involved in the recruitment of lymphocytes. It will be interesting to find out more about the significance of CCL22-expression by cancer cells.

In conclusion, our study shows that MBC, despite a good clinical prognosis, is strongly infiltrated by FoxP3+ cells. Intratumoral CD8+ cells clearly exceed the number of Treg in **most** MBC but not in other types of breast cancer. Thus, a predominance of tumor-infiltrating CD8+ over FoxP3+ cells is **typically observed in** the medullary subtype of breast cancer and may contribute to its favorable prognosis. This supports the hypothesis that the ratio of intratumoral CD8+ to FoxP3+ cells rather than the absolute number of regulatory T cells is predictive for the clinical outcome of cancer.

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### Disclosure

The authors declare no conflicts of interest.

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## Figure legends

### Figure 1. Breast cancer infiltration by FoxP3+ cells.

Tumor-infiltrating FoxP3+ lymphocytes were quantified in paraffin-embedded samples of human breast cancer that were double-stained with anti-human FoxP3 (red) and anti-human CCL22 (brown). (A) Infiltration by FoxP3+ cells was assessed semiquantitatively in medullary (MBC, black bars, n = 13) and ductal (DBC, white bars, n = 34) breast cancer specimens (-, no; +, low; ++, high; +++, very high infiltration). Bars indicate the percentage and numbers in below indicate the absolute number of tumor specimens. (B) Representative examples of infiltration by FoxP3+ cells are shown for the tumor epithelium of MBC and DBC, and (C) for the adjacent healthy breast tissue (40x magnification).

### Figure 2. Breast cancer infiltration by CCL22+ immune cells.

(A) Paraffin-embedded samples of human breast cancer were stained as in Figure 1 and representative examples for CCL22+ immune cells (brown) are shown for MBC, DBC and adjacent normal breast tissue (40x magnification). (B) Infiltration by CCL22+ myeloid-shaped cells was assessed semiquantitatively for MBC (n = 13, black bars), DBC (n = 34, white bars) and lobular breast cancer (LBC, n = 4, grey bars) (quantification score as in Figure 1).

### Figure 3. Expression of CCL22+ by breast cancer cells.

(A) Breast cancer samples were prepared as described in Figure 1. Expression of CCL22 by tumor cells (brown) is shown for examples of MBC, DBC and lymphangiosis carcinomatosa (lymphangiosis c.) within DBC. (B) CCL22 was measured in the supernatants of two breast cancer cell lines by ELISA and the supernatants of human dendritic cells were used as positive control.

**Figure 4. Relation of tumor-infiltrating CD8+ to FoxP3+ cells in breast cancer.**

Serial sections of paraffin-embedded breast cancer were stained with either anti-human CD8 (brown) or with anti-human FoxP3 (red) together with CCL22 (brown). (A) Infiltration by CD8+ cells was assessed semi-quantitatively for MBC (n = 13, black bars) and DBC (n = 34, white bars) specimens (quantification score as in Figure 1). Bars indicate the percentage and numbers in below indicate the absolute number of tumor specimens. (B) Congruent microscopic fields demonstrate the specific distribution of CD8+ and FoxP3+ cells in lymphoid clusters (LC) and the tumor epithelium (E) in tissue sections of MBC. (C) Intraepithelial CD8+ and FoxP3+ cells in MBC and DBC were quantified in congruent images of serial sections. The mean CD8 to FoxP3 ratios and the ranges for each type of tumor are shown in a box plot.

**Table 1 Patient and tumor characteristics**

Histological subtype	medullary	invasive ductal	lobular	mucinous
Number of samples	13	34	4	3
Age in years (range)	49.7 (32-71)	54.4 (26-80)	53.0 (41-73)	65.3 (58-78)
TNM Stage				
T1	3	9	1	1
T2	9	13	3	1
T3	1	8	0	1
T4	0	4	0	0
N0	8	12	1	1
N1	4	12	1	1
N2	0	7	1	0
N3	0	3	1	0
Nx	1	0	0	1
M1	0	17	0	0
Mx	13	17	4	3
Her2neu+	3	21	0	0
Tumor grade (SBR)				
2	1	0	4	3
3	12	34	0	0

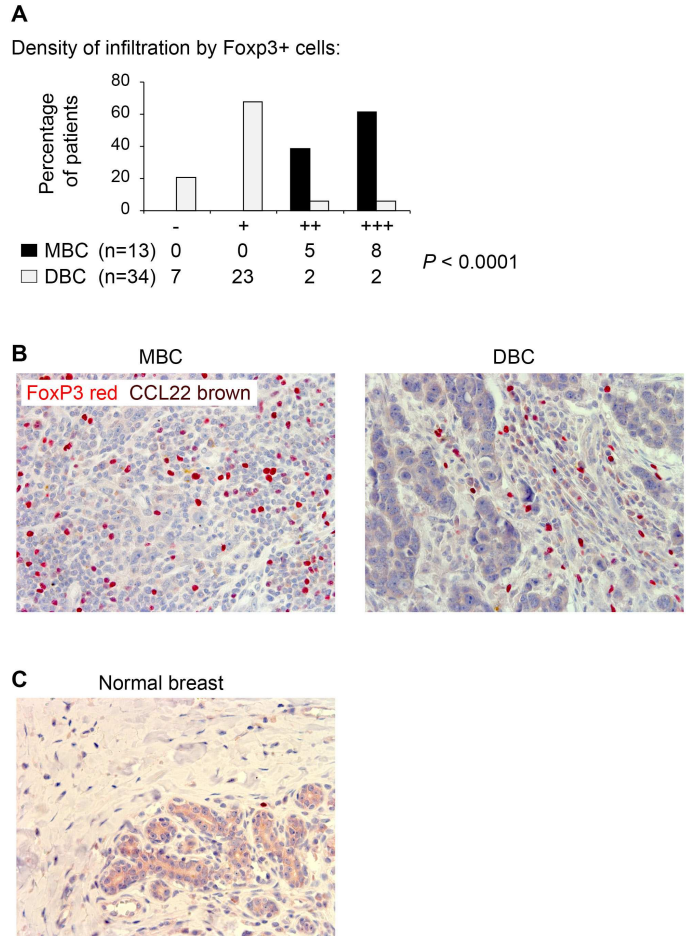


Figure 1

209x297mm (300 x 300 DPI)

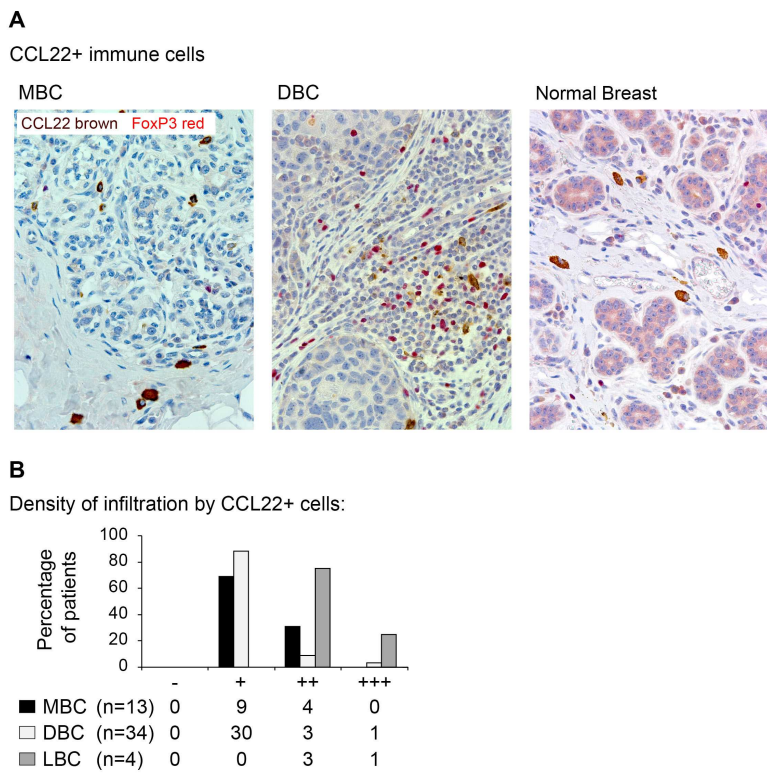


Figure 2

209x297mm (300 x 300 DPI)

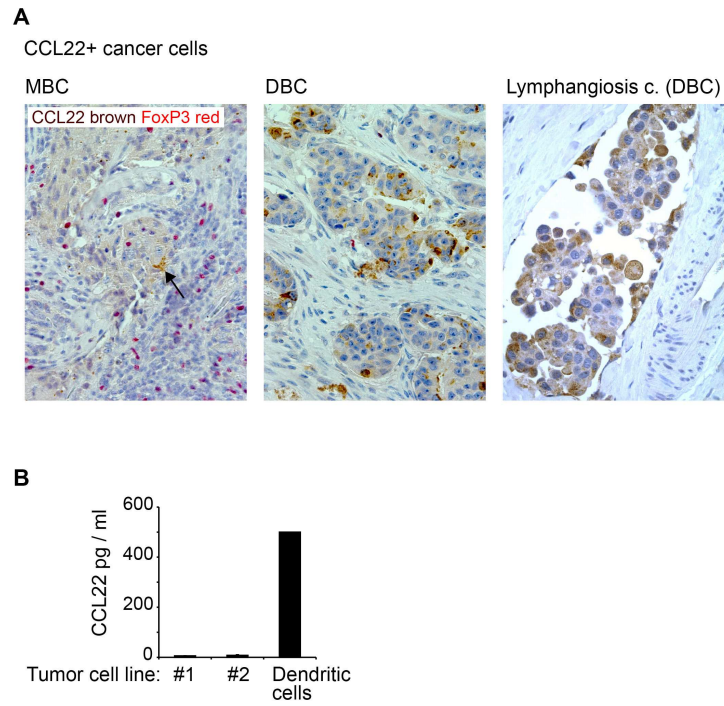


Figure 3

209x297mm (300 x 300 DPI)

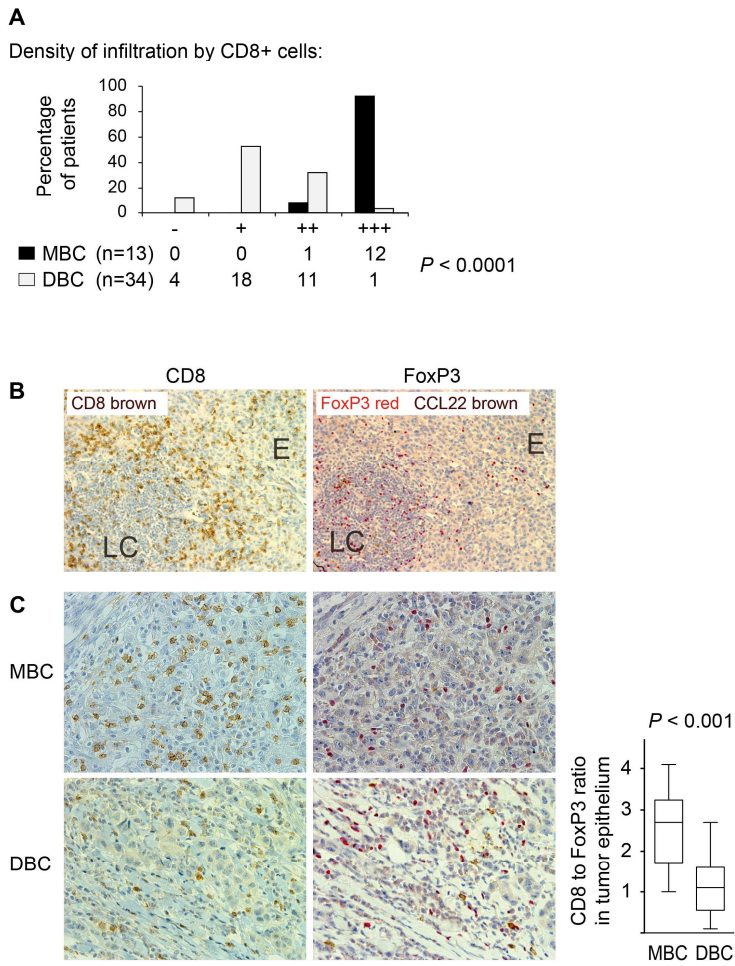


Figure 4

209x297mm (300 x 300 DPI)