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Estrogen-receptor negative breast cancers exhibit a high cytokine content

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ABSTRACT

Introduction

Emerging hypothesis suggest that cytokines could play an important role in cancer as potential modulators of angiogenesis and leukocyte infiltration.

Methods

A novel multiplexed flow cytometry technology was used to measure the expression of 17 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α) at the protein level in 105 breast carcinoma. B lymphocytes, T lymphocytes and macrophages content was determined by immunohistochemistry.

Results

Fourteen of the 17 cytokines were expressed in breast carcinoma, whereas only 9 cytokines could be detected in normal breast. Most cytokines were more abundant in breast carcinoma than in normal breast, with IL-6, IL-8, G-CSF, IFN-γ, MCP-1 and MIP-1β being very abundant. IL-2, IL-6, IL-8, IL-10, IFN-γ, MCP-1, MIP-1β, TNF-α and to a lower extent IL-1β and IL-13 exhibited an expression inversely correlated to estrogen receptor (ER) and progesterone receptor status. Most cytokines were not correlated to the age of cancer diagnosis, the tumor size, the histologic type or the lymph node status. On the other hand, IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, MIP-1β were more abundant in high grade tumors compared to low grade tumors. In addition, IL-8 and MIP-1β were more expressed in HER2-positive than in HER2-negative patients. The expression of most of the studied-cytokines was correlated to AP-1 levels, which is known to regulate numerous cytokines. MCP-1, MIP-1β overexpression were linked to B lymphocyte, T lymphocyte and macrophage infiltration,

whereas high levels of IL-8 were correlated to a high macrophage content in tumor. Moreover, we showed that IL-8 positive tumors displayed an increased vascularization.

Conclusions

We show here that multiple cytokines were overexpressed in ER-negative breast carcinoma and that the three major cytokines were correlated to inflammatory cell component, which could account for the aggressiveness of these tumors.

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INTRODUCTION

Breast cancer represents the first leading cause of cancer death among women in developed countries [1]. Among the different prognostic factors, lack of estrogen receptor has been consistently associated with a poorer prognosis [2]. Most human breast cancers express ERα, and the presence of this receptor is generally considered as an indication of hormone-dependence [3]. In addition to ERα, cytokines are now emerging as potential factors involved in breast carcinogenesis [4, 5]. Cytokines constitute a diverse group of proteins comprising hematopoietic growth factor, interferons, lymphokines and chemokines [6].

Cytokines are produced by many cell populations, but the predominant suppliers are helper T cells (Th) and macrophages. Helper T cells have two important functions: to stimulate cellular immunity and inflammation, and to stimulate B cells to produce antibody. Two functionally distinct subsets of T cells (Th1 and Th2) secrete cytokines which promote these different activities. Th1 cells produce IL-2 and IFNγ, which activate cytotoxic lymphocytes (Tc) and macrophages to stimulate cellular immunity and inflammation [7]. Th2 cells secrete IL-4 and IL-5 which stimulate antibody production by B cells.

Moreover, it becomes evident that cancer tissues also produce cytokines [4, 8]. Numerous studies have analyzed the expression of diverse cytokines independently in breast cancer but only few have examined the profile of a group of different cytokines in the site of the tumor. In the present study, we have screened 17 members of the cytokine family (Interleukin-1β/ IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, Granulocyte Colony-Stimulating Factor/ G-CSF, Granulocyte Macrophage-Colony-Stimulating Factor / GM-CSF, IFN-γ, Monocyte Chemoattractant Protein-1/ MCP-1, Macrophage Inflammatory Protein-1-β/ MIP-1β, TNF-α) in breast cancer tissues. We found that most cytokines were overexpressed in breast cancer compared to normal tissues. IL-2, IL-6, IL-8, IL-10, INF-γ, MCP-1, MIP-1β, TNF, IL-1β and IL-13 were inversely correlated with

estrogen and progesterone receptor status. Most of the cytokines were not correlated with the age of patient, the tumor size, the histologic type or the lymph node status. IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1 and MIP-1β were abundantly expressed in high grade tumors. Moreover, IL-8 and MIP-1β were linked to HER2 expression. In addition, IL-8 positive tumors displayed a higher vascularization. IL-8, MCP-1 and MIP-1β robust expression was correlated to a strong inflammatory cell component.

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MATERIALS AND METHODS

Patients and samples.

Breast tumor surgical specimens were selected from the files of the Pathology Department tumor bank. Breast biopsies were surgical waste, that did not compromise the legal sample for diagnosis accuracy and which did not undergo genetic identification. The samples were selected by a pathologist on fresh surgical specimens immediately after resection. Mirror paraffin blocks were performed at the same time to ensure the tumoral nature of the tissue. When possible, normal breast tissues were also collected in the vicinity of the tumor. All the patients met the following criteria: primary unilateral, non-metastatic breast carcinoma, complete clinical, histological and biological information available. Clinical and pathological characteristics of the patients are reported in Supplemental Table S1. The tumors consisted of invasive carcinoma, mainly ductal and lobular and rare other subtypes (mixed ductal and lobular carcinoma, medullary carcinoma, apocrine carcinoma). The tumors were graded according to the modified Nottingham SBR grade [9] and categorized according the AJCC sixth edition for the pTNM staging. Estrogen receptor (ER)-α, progesterone receptor (PR) and HER2 status were determined at the protein level by immunohistochemistry. When equivocal, HER2 results were confirmed by fluorescence *in-situ* hybridization (FISH).

Immunohistochemistry and FISH analysis

Representative tissue sections from cases of invasive breast carcinomas were fixed in 10% formalin or in alcohol-formalin-acetic acid and embedded in paraffin. Sections from each case were deparaffinized, rehydrated and subsequently subjected to heat-induced antigen retrieval by immersing them, depending on the antibody, either in a water bath with a sodium citrate buffer (pH 6) or an EDTA buffer (pH 8). Immunohistochemistry (IHC) was performed using the Dako autostainer (Dako, Glostrup, Denmark). Then, the sections were incubated with the following primary antibodies: ERα (Novocastra, Newcastle Upon Tyne, U.K, clone 6F11, monoclonal), PR

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(DakoCytomation, clone PgR636, monoclonal) and HER 2 (DakoCytomation, A0485, polyclonal). They were respectively used at 1: 50, 1:100 and 1:1500 dilution with an incubation time of 30 min. Antibody was localized using the LSAB ® 2 Detection System (Dako). Diaminobenzidine (DAB, Dako) was used as the chromogen and the sections were lightly counterstained with hematoxylin. ER and PR expression was considered positive when at least 10 % of invasive tumoral cells showed a nuclear staining, whatever the intensity. For HER2, the IHC score was given according to the Herceptest ® scoring system, *i.e.* 0, absence of membranous staining or < 10% positive cells; 1+, > 10% stained cells with a weak and incomplete membranous staining; 2+, >10% stained cells with a weak or moderate complete membranous staining; 3+, >10% stained cells with a strong and complete membranous staining. A case was considered to be HER2 over-expressed if it scored 3+. A case scoring 2+ was considered HER2 over-expressed only if FISH analysis using the PathVysion HER-2 Probe kit (Vysis, Downer's grove, IL, USA) showed HER2 gene amplification.
Inflammatory cell component was evaluated on a subset of 10 tumors displaying high levels of IL8 and 10 tumors with low IL8 content using CD3 (T lymphocyte lineage), CD20 (B lymphocyte lineage) and CD68 (macrophages) antibodies. The intensity of each pattern of inflammatory infiltrate was graded as absent (0), minimal (1), moderated (2) or marked (3). The staining result for each antibody was scored by two investigators blinded for the IL8 status of the patients. Vessels were assessed within the invasive carcinoma in CD31-stained sections. The most vascular areas were selected by examination at low power (x40 magnification). The number of clusters or single cells stained for CD31 was recorded from the three most vascular x400 fields (0.18mm², each) and the vascular density (number of vessels/mm²) was calculated.

Protein extract preparation

Biopsies were first crushed in liquid nitrogen. The powder was then resuspended in TEG (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 10% glycerol) containing protease inhibitors (5 µg/ml aprotinin, leupeptin and pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride). The mixture was

then sonicated and the cellular debris were pelleted by centrifugation at 13,000g for 20 minutes at 4°C in microfuge tubes.

Cytokine multiplexed Bioplex assay

A panel of 17 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, MCP-1, MIP-1β, TNF-α) was measured in duplicate using the bioplex cytokine multiplexed assay (BioRad) according to the instructions of the manufacturer (see supplemental Methods). This novel multiplexed, particle based, flow cytometric assay uses anti-cytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes. 500 µg of total protein extracts of breast biopsies was used to measure cytokine concentration. For each cytokine, 8 standards ranging from 2 to 32,000 pg/ml were used, and the minimum detectable dose was < 10 pg/ml. The validation of the assay was done by comparing the results obtained by bioplex measure of IL-8 with a conventional ELISA measure of IL-8 (see Suppl. Fig. 1).

Gel shift assays

Gel shift assays were performed as previously described [10]. Briefly, 30,000 cpm of the [^{32}P]-labeled double-strand oligonucleotides were combined with 1 µg poly (dI-dC) and 5 µg of total cell extract. The reaction buffer contained 20 mM Hepes, pH 7.9; 1 mM DTT; 50 mM KCl; 10% glycerol; 2.5 mM $MgCl_2$. Protein-DNA complexes were separated from the free probe by non-denaturating gel electrophoresis with 4% polyacrylamide gels in 0.5 X TBE. Oligonucleotides used were: AP-1 cons: CGCTTGATGAGTCAGCCGGAA; The levels of shifted complexes were detected and quantified by counting with a Fujix-Bas 1000.

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Statistical analysis

Data was summarized by frequency for categorical variables and by median and range for continuous variables. Correlation between variables (cytokines) was evaluated with the Spearman correlation coefficient after LOG transformation. Associations between categorical variables were examined using $\chi 2$ analysis.

The median values of analyzed interleukins expression were compared using Kruskal-Wallis test according to the clinical data (Age, ER, PR, SBR, pT, pN) or analysis of variance (ANOVA) if possible.

For all statistical analysis, a p-value of less than 0.05 was considered statistically significant.

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RESULTS

Breast tumors overexpress cytokines

A total of 105 primary unilateral, non-metastatic breast carcinoma from patients (Supplemental Table S1) and 13 healthy breast biopsies were analyzed for cytokine expression at the protein level (Table 1). This was made possible by the use of a novel technology coupling Elisa to FACS analysis, which allows the detection in a single well of multiple proteins. We observed that healthy breast samples expressed very low levels of cytokines, except for IFN-γ and MIP-1β (Table 1). On the contrary, breast tumors displayed high levels of IL-6, G-CSF, IFN-γ and extremely high levels of IL-8, MCP-1 and MIP-1β. IL-5, IL-17, GM-CSF were not detected in both normal and tumor samples, whereas IL-1β, IL-2, IL-4, G-CSF and IL-10, were only detected in breast carcinoma. IL-6, IL-8, IL-12, IL-13, IFN-γ, MCP-1, MIP-1β and TNF-α were significantly more abundant in carcinoma compared to normal breast. The greatest differences were obtained for IL-8, MCP-1, MIP-1β which were 60 fold more abundant in carcinoma than in healthy breast, followed by IL-6 which about 14 fold overexpression in carcinoma. In summary, we demonstrate that breast tumors express higher levels of multiple cytokines compared to normal tissues.

Correlation between cytokines and Estrogen (ER) and Progesterone (PR) receptors

We next evaluated the possible correlation between cytokine expression and ER levels (Table 2). We observed that IL-4 and G-CSF were not correlated to ER status. On the other hand, IL-2, IL-6, IL-8, IL-10, IFN-γ, MCP-1, MIP-1β, TNF-α and to a lower extent IL-1β and IL-13 were significantly overexpressed in ER-negative tumors compared to ER-positive tumors, with the greatest differences observed for IL-8 and MCP-1.

We performed the same type of analysis to correlate cytokine levels with PR expression (Table 2). IL-2 and IL-13 were not correlated to PR status, whereas IL-1β, IL-6, IL-8, IL-10, INF-γ, MCP-1, MIP-1β, TNF-α and to a lower extent IL-4, IL-12 and G-CSF were more abundant in PR-negative

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tumors compared to PR-positive tumors. The greatest differences were obtained for IL-8, IL-6 and MCP-1.

The fact that some cytokines were correlated to either ER or PR, led us to analyze the distribution of all cytokines by combining both factors (Supplemental data Table S2). Three categories were designed, corresponding to true ER-negative (ER-/PR-) or ER-positive (ER+/PR+) compared to intermediate status (ER-/PR+ or ER+/PR-). This showed that IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IFN-γ, MCP-1, MIP-1β and TNF-α were inversely correlated to true ER status. The most abundant cytokines in true ER-negative breast carcinoma were IL-8, MCP-1 and MIP-1β.

Correlation with other clinical parameters

We then evaluated whether cytokine expression could be linked to clinical parameters such as tumor size, lymph node status, histological grade or the age of the patients. Cytokine profile was not linked to tumor size or to the histological type. We did not observe any correlation between the age of patients at cancer diagnosis and cytokine expression, except for IL-1β, which was inversely correlated to age: (≤ 50 years: 3.14 (0.2 - 194.2), 50-65 years: 2.13 (0 - 42.4), >65 years: 1.9 (0 - 260.6); p = 0.033). Lymph node status was not linked to any of the analyzed cytokine.

As ER-negative tumors are generally of higher grade than ER-positive tumors (which is confirmed in our samples, data not shown), we determined whether cytokine levels could be correlated to the grade. Most of the cytokines which were preferentially present in ER-negative tumors (IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, MIP-1β) were also more abundant in high grade tumors (Table 3).

Another factor which is frequently linked to the grade and to ER-status is the level of HER2. We observed a correlation between HER2 levels and IL-8 and MIP-1β, which were more expressed in HER2 positive patients (IL-8: HER2-negative: 50.8 (0 - 15,890); HER2-positive: 192.6 (23.7 - 8,357); p= 0.014; MIP-1β: HER2-negative: 514.9 (42.6 -14,439); HER2-positive: 976.1 (44.6 -

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4,978); p= 0.021). All these data suggest that high cytokine levels are associated with poor prognostic factors such as high grade, ER-negative status, and HER2 positive tumors.

AP-1 transcription factors are known to regulate the expression of many cytokines. To evaluate this issue, we performed gel shift assays measuring the global AP-1 binding to specific DNA binding sites. As reported by others [11], AP-1 was more abundant in ER-negative tumor samples compared to ER-positive tumors ($p<0.001$). There was also a higher HER2 level in AP-1-positive tumors ($p=0.025$). On the other hand, AP-1 status was not correlated to PR expression, to the grade, the size of the tumor and the lymph node status. Interestingly, high AP-1 levels correlated with high expression of several cytokines, including IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IFN-γ, MCP-1 and TNF-α (Table 4). Among these cytokines, the greatest differences were observed for IL-8 and MCP-1, which displayed respectively a 3.9 and 2.7 fold higher expression in AP-1-positive tumors compared to AP-1-negative tumors (Table 4).

Tumors expressing high levels of IL-8 display a high vascularization

We next focused our attention on the possible association between the high expression of IL-8 and vascularization. We analyzed 10 tumors displaying high levels of IL-8 and 10 tumors with a low IL-8 content. A microscopic analysis showed that tumors with a high expression of IL-8 were also more vascularized (median of 125 vessels/mm2 compared to 48 vessels/ mm2 in low IL-8 tumors; p= 0.0002) (Table 5). Overall, these results suggest that IL-8 expression is associated with a high neovascularization.

Leukocyte infiltration is correlated to high levels of cytokines

As IL-8, MCP-1 and MIP-1β, the most highly expressed chemokines in cancer tissues, are potent chemotactic molecules, we investigated whether their expression could be correlated to tumoral leukocyte infiltration. We performed a complete immunohistochemical analysis of our whole

collection of tumor using CD3 (B lymphocytes), CD20 (T lymphocytes) and CD68 (macrophages) antibodies. A representative picture is shown on figure 1. Normal breast displayed no to low staining whereas, breast cancer tissues exhibited a complete range of leukocyte infiltration from low to high levels. Our results show that high IL-8 levels were correlated to high macrophage infiltration, whereas strong expression of MCP-1 and MIP-1β was correlated to robust presence of B lymphocytes, T lymphocytes and macrophages (Table 6).

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DISCUSSION

It is well known that the interactions of tumor cells with their microenvironment may affect tumor growth and metastasis formation. Among these, inflammatory cells and cytokines were recently suggested to play a key role in breast carcinoma (for review, [4]).

A previous study of breast cancer patients had shown that IL-8 RNA was more abundant in the neoplastic compared to normal population, whereas no difference could be seen for IL-1α, IL-1β, IL-4, or IL-6 [12]. In addition, transcripts for IL-2, IL-4, IL-5, IL-7, TNF-α and IFNγ were not detected in either group [12, 13]. Increased seric levels of the IL-6, IL-8 and IL-10 have also been observed in patients suffering from breast cancer compared to healthy women [14, 15]. Among the 17 cytokines we analyzed, 8 were not detected in normal breast (IL-1β, IL-2, IL-4, IL-5, IL-10, IL-17, G-CSF, GM-CSF) and 3 were absent from breast carcinoma (IL-5, IL-17, GM-CSF). With the exception of IL-7, all cytokines which were present in both tissues, were significantly present at higher levels in breast carcinoma.

To date, the correlation between cytokine expression and clinical parameters remains elusive. We observed that IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, MIP-1β correlated with the grade. In the same line, IL-8 and MIP-1β were also linked to HER2 status, which is in agreement with reports demonstrating that IL-8 is regulated by HER2 in breast cancer cell lines [16]. In addition, IL-1β was inversely correlated to the age. IL-1β has been shown previously to be strongly expressed in high grade invasive breast carcinoma compared to in situ ductal carcinoma or benign lesions [17]. In contrast to these results, Green et al. did not observe any correlation between IL-1α, IL-1β, IL-4, IL-6, IL-8, TNFα, TNFβ, IL-2, IL-5, IL-7 and IFNγ and tumor histological grade, patient age or lymph node metastases in breast cancer patients, but this study analyzed only RNA levels [12].

Of particular note, our study shows an inverse correlation between the expression of ER and cytokines, which is in agreement with other studies [8, 18]. An inverse correlation between IL-8 and ER or PR in breast cancer biopsies has also been demonstrated [19]. Several studies have reported

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that IL-1α, IL-1β or IL-6 levels correlated inversely with ER levels [20-22]. This inverse correlation between cytokines and estrogen receptor status could not only reflect the higher aggressiveness of this subtype of breast tumors but could also be the result of a direct regulation of cytokine expression by ER. Several reports have demonstrated a direct down-regulation of cytokines by ER in different organs. This is not only the case for IL-6 [23, 24] and IL-8 [8, 18], but also for IL1-β (for review, [24]), IL-2 [25], IL-10 [26], IL-12 (for review, [27]), GM-CSF [24], IFN-γ [28, 29], MCP-1 [30, 31] and TNF-α [24]. PR is also known to down-regulate the expression of a number of cytokines, including IL-1β [32], IL-6 [30, 33], IL-8 [34], MCP-1 [32, 35] or TNF-α [32], which is in agreement with our results.

According to our work, Th1 cytokines (IL-2, IFN-γ) or Th2 cytokines (such as IL-4, IL-5) are not the major cytokines produced by the tumor. On the other hand, several cytokines produced by monocytes (IL-1β, IL-6, MCP-1) or macrophages such as (IL-1β, IL-6, IL-8, MIP-1β, TNF-α) are highly expressed by the tumor. We observed that tumors displaying high levels of IL-8 exhibited a significantly higher vascularization. The role of IL-8 in breast cancer angiogenesis has also been suggested by *in vitro* studies [18], but to our knowledge, our study is the first report of an association between IL-8 levels and angiogenesis in breast cancer samples.

We showed here that tumors expressing high levels of MCP-1 and MIP-1β exhibited a higher content in B lymphocytes, T lymphocytes and macrophages than tumors expressing low level of these chemokines. In the same line, robust expression of IL-8 was correlated to a strong macrophage infiltration. Our results are thus in agreement with previous studies which showed a correlation between the number of tumor associated macrophages (TAM) and IL-6, IL-8 or MCP-1 levels in breast and cervical cancers [36-38]. On the other hand, the correlation between MIP-1β and macrophage infiltration has not been reported to date, nor the association of high levels o, MCP-1 and MIP-1β to B and T lymphocytes presence in breast carcinoma. The precise role of leukocytes in

tumors remains to be elucidated in larger studies to determine if they contribute or not to tumor growth.

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CONCLUSIONS

In conclusion, the pattern of expression of multiple cytokines in breast cancer tissues that we report is the first step to understand the involvement of cytokines in breast cancer. Our data suggest that cytokines could be involved in the aggressiveness of ER-negative breast tumors. In addition, the robust expression of IL-8, MCP-1 and MIP-1β, the three major chemokines present in breast tumors, is correlated to strong leukocyte infiltration. We thus believe that our work could be the basis to study more precisely the role of these cytokines in breast cancer.

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LIST OF ABBREVIATIONS

ER: Estrogen Receptor, G-CSF: Granulocyte Colony-Stimulating Factor, GM-CSF: Granulocyte Macrophage-Colony-Stimulating Factor, IL-1β: Interleukin-1β, IL-2: Interleukin-2, IL-4: Interleukin-4, IL-5: Interleukin-5, IL-6: Interleukin-6, IL-7: interleukin-7, IL-8: interleukin-8, IL-10: Interleukin-10, IL-12: Interleukin-12, IL-13: Interleukin-13, IL-17: Interleukin-17, / IFN-γ: Interferon-γ, MCP-1: Monocyte Chemoattractant Protein-1, MIP-1β: Macrophage Inflammatory Protein-1-β, PR: Progesterone Receptor, TNF-α: Tumor-Necrosis-α

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

CC carried out the bioplex experiments and helped to draft the manuscript. FB interpreted the immunhistochemistry slides and revised the manuscript. SGB performed all stastical analysis and revised the manuscript. SB, SR and FB performed the immunohistochemistry experiments and FB revised also the manuscript. DL was involved in the bioplex experiments. GL conceived the study, supervised the work, drafted the manuscript, processed the biopsy samples, performed the Bioplex and gel shift experiments.

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Figure Legends

Fig. 1: Breast tumors display various levels of leukocyte infiltration.

Normal breast and breast carcinoma were stained for B lymphocytes B, T lymphocytes and macrophages using CD3, CD20 and CD68 immunostaining. Representative pictures of normal breast (A), tumor with weak leukocyte infiltration (B) and tumor with robust leukocyte infiltration (C) are shown here.

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Table 1 Cytokine are overexpressed in breast cancer

Biological Markers	Median fg/µg protein (range)		p
	Cancer (n=105)	Healthy breast (n=13)	(C vs. H)
IL-1β	2.7 (0 - 260)	0	<0.0001
IL-2	1.2 (0 - 42.6)	0	<0.0001
IL-4	1.7 (0 - 99.6)	0	<0.0001
IL-5	0	0	
IL-6	17.2 (4.4 - 26487)	1.2 (0 - 52.5)	<0.0001
IL-7	0.7 (0 - 8.5)	1.1 (0.7 - 16.9)	0.047
IL-8	68.1 (0 - 15890)	1 (0 - 78.5)	<0.0001
IL-10	0.3 (0 - 5.6)	0	<0.0001
IL-12 (p70)	2.3 (0 - 19.3)	1.4 (0.5 - 20.4)	0.005
IL-13	4.2 (1.6 - 394)	1.9 (1.2 - 21.6)	<0.0001
IL-17	0	0	
G-CSF	18.7 (5.3 - 110)	0 (0 - 49.2)	<0.0001
GM-CSF	0	0	
IFNγ	27.6 (15.5 - 1279)	16.6 (13.6 - 187)	<0.0001
MCP-1	121 (0 - 37117)	1.9 (0 - 45.9)	<0.0001
MIP-1β	559 (42.6 - 14439)	8.3 (5.0 - 142)	<0.0001
TNFα	7.2 (5.8 - 121)	1.6 (1.3 - 23.2)	<0.0001

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Table 2 Cytokines are present at high levels in ER-negative and PR-negative breast tumors

Median Biological Markers fg/µg protein (range)

	ER				
	Negative (n=45)		Positive (n=60)		p
IL-1β	3.7	(0 - 260)	2.2	(0 - 42.4)	0.031
IL-2	1.7	(0 - 42.6)	0.9	(0 - 11.0)	0.003
IL-4	3.8	(0 - 20.0)	1.1	(0 - 99.6)	NS
IL-6	25.4	(7.8 - 26487)	13.2	(4.4 – 619)	<0.001
IL-8	158	(5.0 - 15890)	40.5	(0 - 10298)	<0.001
IL-10	0.6	(0 - 5.6)	0.2	(0 - 2.86)	0.003
IL-12 (p70)	3.0	(0 -19.3)	2.0	(0 - 17.2)	0.029
IL-13	4.3	(3.4 - 52.6)	4.1	(1.6 – 394)	0.049
G-CSF	17.3	(7.1 - 110)	20.1	(5.3 - 87.3)	NS
IFNγ	32.6	(19.4 - 324)	25.6	(15.5 - 1279)	<0.001
MCP-1	210	(14.7 - 37117)	80.7	(0 - 1543)	<0.001
MIP-1β	748	(42.6 - 14439)	488	(87.2 - 10244)	0.005
TNFα	8.5	(6.1 - 80.0)	6.9	(5.8 - 120)	<0.001

	PR				
	Negative (n=30)		Positive (n=75)		p
IL-1β	5.3	(0 - 194)	2.3	(0 - 260)	0.008
IL-2	1.7	(0 - 6.6)	1.2	(0 - 42.6)	0.119
IL-4	4.6	(0 - 18.7)	1.1	(0 - 99.6)	0.040
IL-6	53.6	(9.9 - 26487)	13.7	(4.4 - 4312)	<0.001
IL-8	505	(7.3- 15890)	48.7	(0 - 11281)	<0.001
IL-10	0.8	(0 - 5.0)	0.2	(0 - 5.6)	<0.001
IL-12 (p70)	3.1	(0 - 7.3)	2.0	(0 -19.3)	0.024
IL-13	4.3	(3.6 - 35.5)	4.1	(1.6 - 394)	NS
G-CSF	24.1	(7.1 - 110)	17.4	(5.3 - 103)	0.056
IFNγ	33.8	(23.7 - 324)	25.6	(15.5 - 1279)	0.002
MCP-1	404	(17.7 - 37717)	96.3	(0 - 1543)	<0.001
MIP-1β	1121	(44.6 - 13287)	493	(42.6 - 14439)	0.001
TNFα	8.6	(6.2 - 37.2)	7.1	(5.8 - 121)	0.002

NS: non significative

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Table 3 Most cytokines are abundant in high grade tumors

Biological Markers fg/µg protein (range)							
	Grade I (n= 17)		Grade II (n=56)		Grade III (n= 32)		p
IL-1β	0.8	(0 - 260)	2.6	(0 - 102)	4.7	(0 - 194)	0.025
IL-2	0.9	(0 - 4.6)	1.2	(0 - 42.6)	1.4	(0 - 6.6)	NS
IL-4	3.4	(0 - 20.1)	0.6	(0 - 30.2)	3.9	(0 - 99.6)	0.037
IL-6	11.7	(7.8 - 4312)	14.1	(4.4 - 190)	30.6	(8.8 - 26487)	<0.001
IL-8	43.9	(9.1 - 11281)	35.4	(0 - 8358)	186	(0.3 - 15890)	<0.001
IL-10	0.2	(0 - 1.6)	0.2	(0 - 5.6)	0.7	(0 - 4.6)	0.030
IL-12 (p70)	1.4	(0 - 5.3)	2.1	(0 - 19.3)	2.9	(0.7 -17.2)	0.045
IL-13	4.1	(3.4 - 21.4)	4.2	(3.0 - 394)	4.2	(1.6 - 35.5)	NS
G-CSF	13.9	(7.1 - 87.3)	18.9	(5.3 - 110)	22.2	(9.9 - 79.2)	NS
IFNγ	26.8	(19.8 - 270)	25.6	(17.1 - 1279)	28.7	(15.5 - 324)	NS
MCP-1	96.3	(22.8 - 1208)	91.3	(0 - 22451)	201	(24.4 - 37117)	0.006
MIP-1β	451	(44.6 - 2674)	496	(42.6 - 14439)	776	(114 - 13287)	0.011
TNFα	7.15	(5.9 - 28.0)	7.1	(5.8 -120)	7.8	(6.2 - 37.2)	NS

NS: non significative

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Table 4 Cytokine levels are correlated to AP-1 expression

Median Biological Markers fg/µg protein (range)					
	AP-1				
	Negative (n=34)		Positive (n=69)		p
IL-1β	1.4	(0 - 11.5)	3.2	(0 - 260)	0.006
IL-2	0.4	(0 - 3.2)	1.4	(0 - 11)	0.001
IL-4	1.1	(0 - 99.6)	2.7	(0 - 30.2)	NS
IL-6	12.6	(4.4 - 70.7)	19.7	(7.8 - 26487)	0.001
IL-8	29.6	(0 -778)	115	(1.5 - 15890)	0.003
IL-10	0.2	(0 - 2.6)	0.3	(0 - 5.6)	0.004
IL-12 (p70)	1.8	(0 -17.2)	2.7	(0 - 7.3)	0.044
IL-13	4.2	(3.0 - 7.2)	4.2	(3.4 - 394)	NS
G-CSF	17.2	(5.3 - 87.3)	18.5	(7.1 - 110)	NS
IFNγ	25.4	(15.5 - 55.5)	28.7	(17.1 - 1279)	0.002
MCP-1	54.6	(0 - 1208)	148	(14.7 - 37117)	0.001
MIP-1β	503	(87.2 - 8778)	563	(42.6 - 14439)	NS
TNFα	6.9	(6.1 - 10.9)	7.8	(5.8 - 121)	0.002

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Table 5 IL-8 expression is correlated to angiogenesis

IL-8	Vessels/mm2
-	48
-	72
-	53
-	44
-	30
-	ND
-	57
-	46
-	63
-	20
+	244
+	128
+	78
+	279
+	116
+	209
+	124
+	94
+	81
+	359

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Table 6 MCP-1, MIP-1β and IL-8 expression and leukocyte infiltration

	staining	N	IL-8 median	IL-8 min	IL-8 max	p*
CD3	0	54	47.6	0	11281	0.17
	1	36	67.7	0.3	15890	
	2	11	289.7	27.6	10298	
	3	4	145.0	14.9	4408	
CD20	0	74	59.2	0	15890	0.42
	1	27	219.9	1.5	10298	
	2	3	165.2	14.9	4408	
	3	1	124.8	124.8	124.8	
CD68	0	5	23.6	0.6	146.7	0.0011
	1	59	48.6	0	15890	
	2	32	108.6	5.6	11281	
	3	9	289.9	14.9	10298	

	staining	N	MCP-1 median	MCP-1 min	MCP-1 max	p*
CD3	0	54	110.5	0	1543	0.0001
	1	36	107.9	23.3	8479	
	2	11	278.3	45.5	22451	
	3	4	3307	196.5	37117	
CD20	0	74	110.3	0	8478	0.0002
	1	27	192.9	23.35	22451	
	2	3	3185	196.5	37117	
	3	1	3439	3430	3430	
CD68	0	5	40.9	7.0	167.4	0.0016
	1	59	102.6	8.0	8479	
	2	32	137.9	0	37117	
	3	9	478.3	45.5	3430	

	staining	N	MIP1-β median	MIP-1β min	MIP-1β max	p*
CD3	0	54	472.5	42.6	12835	<0.0001
	1	36	560.7	44.6	10685	
	2	11	2702	274.5	14439	
	3	4	6757	1226	13287	
CD20	0	74	495.9	42.6	14439	<0.0001
	1	27	745.3	44.6	10685	
	2	3	4394	1226	9121	
	3	1	13287	13287	13287	
CD68	0	5	342.4	223.4	498.7	<0.0001
	1	59	458	42.6	12835	
	2	32	739.8	134.3	14439	
	3	9	3095	589.3	13287	

*p = ANOVA

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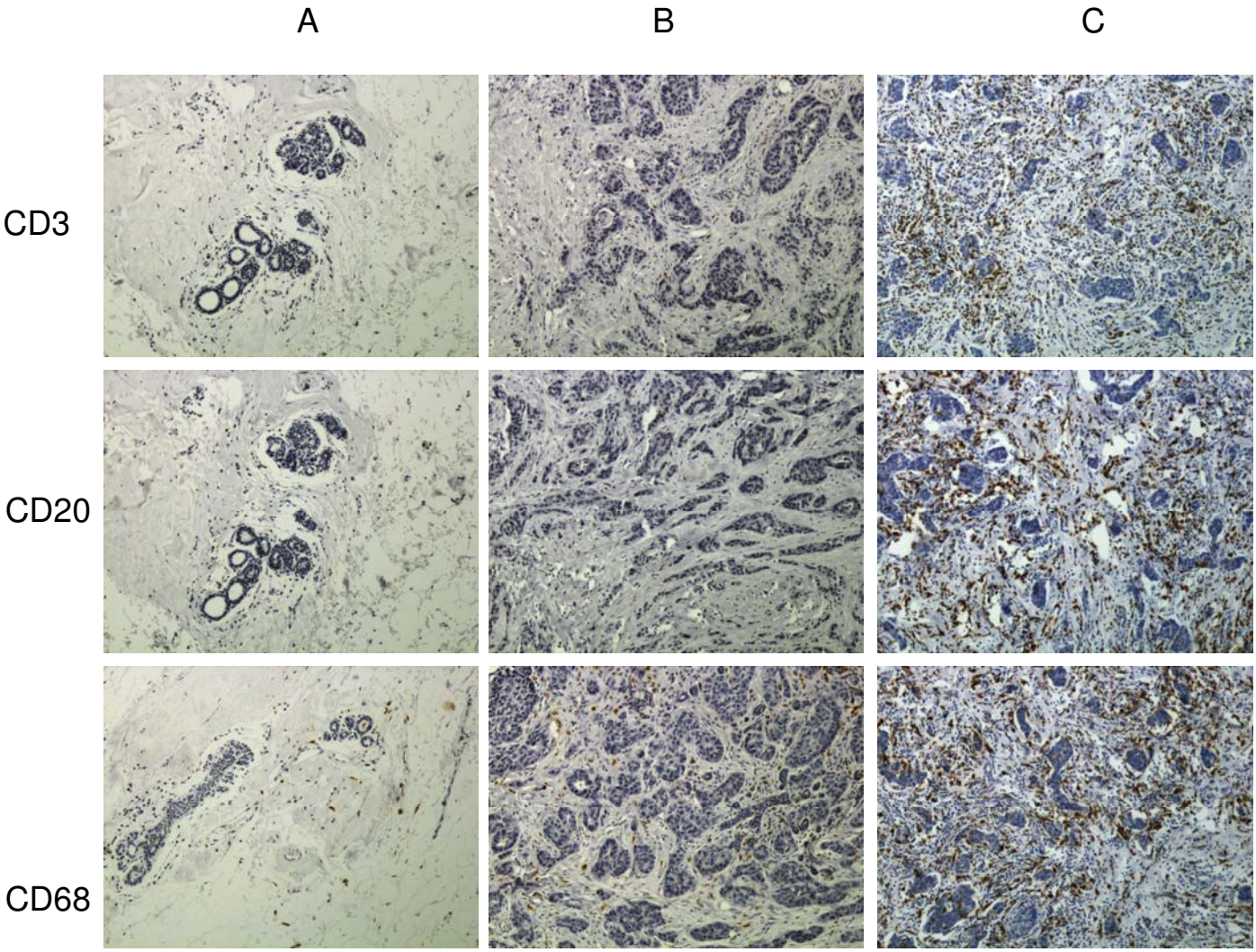


Fig. 1

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