



Breast cancer subtypes express distinct receptor repertoires for tumor-associated macrophage derived cytokines

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ABSTRACT

Infiltration of the tumor microenvironment by macrophages is associated with poor outcomes in breast cancer and other solid tumors, however the identity and roles of many of the soluble factors these macrophages produce remains to be elucidated in detail. In addition to producing angiogenic factors (e.g. VEGF), proteases (e.g. MMP9) and immunomodulatory factors (e.g. IL10) which, by modifying the local microenvironment, likely contribute to progression in the majority of solid tumors, we have evaluated the extent to which macrophage cytokines may differentially affect distinct breast cancer subtypes. We identified 23 cytokines produced in a culture model of human tumor-associated macrophages and report that basal and luminal breast cancer cell lines express different repertoires of receptors for these cytokines. These data suggest that tumor-associated macrophages make specific contributions to different breast cancer subtypes and that understanding the importance of these interactions will be crucial to developing subtype-specific therapies targeting the macrophage component of the breast tumor microenvironment.

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1. Introduction

Breast cancer is a highly heterogeneous disease and gene expression profiling has revealed the existence of a number of distinct molecular subtypes which differ in their etiology, prognosis and response to therapy [1]. In addition to the neoplastic cells, breast tumors also contain a diverse range of non-neoplastic cells in their tumor microenvironment, including fibroblasts, myofibroblasts, endothelial cells and macrophages [2]. High levels of macrophage infiltration in breast cancer correlates with increased tumor cell invasion, increased angiogenesis and poor prognosis [3]. Tumor-associated macrophages express high levels of cytokines, growth factors and proteases and have a distinct immunophenotype from other tissue macrophages. In contrast with classically-activated “M1” macrophages, tumor-associated macrophages have been frequently described as “M2-polarized” although it is now apparent that such a rigid binarization may not be appropriate and a spectrum of tumor-associated macrophage phenotypes exists [4].

Despite long being thought to serve an anti-tumor role, tumor-associated macrophages are now recognized as contributing to tumor progression. However, the mechanisms by which tumor-associated macrophages promote breast carcinoma progression and the identity of the key cytokines which mediate these signals

are only beginning to be understood. In this study we hypothesized that the substantial differences in gene expression between the neoplastic cells of different breast cancer subtypes may result in a different repertoire of receptors for the cytokines produced by tumor-associated macrophages. To address this question, we used an antibody array based approach to evaluate the cytokines produced in a human tumor-associated macrophage model and then determined the subtype specificity of the receptors for these cytokines in a large panel of breast cancer cell lines.

2. Materials and methods

2.1. Cell culture

All reagents were purchased from Fisher Scientific unless otherwise noted. THP-1 (ATCC TIB-202) cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin–streptomycin, and 0.05 mM beta-mercaptoethanol. To induce differentiation, cells were treated with 5 ng/ml of PMA plus 20 ng/ml of IL-4 and 20 ng/ml of IL-13 in complete media for 24 h [5]. For the phagocytic activity assay, THP-1 cells were differentiated in Lab-Tek tissue culture chamber slides for 24 h. Phagocytosis was assessed by incubating with 1 µg/ml of FITC-dextran in complete media at 37 °C for 30 min. Cells were treated in parallel at 4 °C to control for non-specific binding. Cells were then washed in pre-chilled PBS, fixed with 4% paraformaldehyde, counterstained with Hoechst 33342 and analyzed by iCys cytometric analysis.

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2.2. Cytokine arrays

Cytokine levels in differentiated THP-1 conditioned media were measured using the RayBio Human Cytokine Antibody Array V (Raybiotech, Norcross, GA). To prepare conditioned media, 1×10^6 THP-1 cells were differentiated in six well plates as described above. After differentiation, cells were washed three times with PBS to remove PMA and cytokines and allowed to condition serum-free medium for 24 h. To quantify cytokine abundance, antibody membrane arrays were incubated with the conditioned medium and processed according to the manufacturer's instructions. Cytokine-antibody complexes were detected by chemiluminescence. Quantitation of four independent experiments was performed using NIH Image J (v1.42q) [6]. To allow comparison between experiments, data from non-saturated exposures were used for quantitation and data from individual arrays were normalized to the median signal intensity for that array prior to calculation of the average signal from all four arrays.

2.3. Gene expression analysis

We examined the relative gene expression levels of the receptors for the TAM-derived cytokines in a microarray dataset of 24 breast cancer cell line grown in 3D culture which we have previously described in detail [7]. Cell lines were assigned to either a "basal" or "luminal" subtype based on their gene expression profiles [8]. MIAME-compliant raw microarray data are available from ArrayExpress (E-TABM-244).

3. Results

3.1. Differentiation of THP-1 cells

Human THP-1 cells were differentiated in culture to a M2 tumor-associated macrophage like phenotype as previously described [5] by stimulation with 5 ng/ml phorbol myristol acetate, 20 ng/ml IL4 and 20 ng/ml IL13. To demonstrate that the THP-1 cells had indeed differentiated into macrophage-like cells, we performed a functional assay for phagocytic activity. As described in materials and methods, THP-1 cells were incubated with fluorescently-labeled FITC-Dextran (70 kDa molecular weight) to determine their ability to internalize this material. As a control, the

Table 1

Soluble cytokine production profile of differentiated THP-1 macrophages.

Cytokine	Average expression level \pm standard deviation ^a	Receptor/interacting protein
CCL4	3910 \pm 2960	CCR5/CCBP2
CCL5	2290 \pm 630	CCR1, CCR3, CCR5, CCBP2
CCL24	1140 \pm 190	CCR3
CSF1	410 \pm 90	CSF1R
GRO (CXCL1, CXCL2, CXCL3) ^b	7330 \pm 7140	CXCR2, DARC
CXCL7	1830 \pm 520	CXCR1, CXCR2, DARC
CXCL8 (IL8)	15160 \pm 9210	CXCR1, CXCR2, DARC
CXCL10	450 \pm 70	CXCR3
EGF	460 \pm 130	EGFR
FGF4	1210 \pm 250	FGFR1, FGFR2, FGFR3
GDNF	900 \pm 220	GFRA1, RET
HGF	710 \pm 200	MET
IGFBP1	640 \pm 210	IGF-1
IGFBP2	1220 \pm 270	IGF-2
IL10	890 \pm 270	IL10RA
IL16	530 \pm 80	CD4
LIF	460 \pm 20	LIFR
OSM	740 \pm 170	OSMR
PGF	680 \pm 120	FLT1
SPP1	7370 \pm 2450	CD44
TGFB2	1470 \pm 380	TGFBR1, TGFBR2
TIMP1	4610 \pm 2300	Various metalloproteinases
TIMP2	2130 \pm 860	Various metalloproteinases

^a Arbitrary luminescence units.

^b The GRO antibody detects each of these cytokines.

same treatments were performed at 4 °C, a temperature at which the phagocytic machinery of the cell has minimal activity. From these experiments (Fig. 1), we concluded that the PMA/IL4/IL13-treated THP-1 cells did indeed differentiate into a macrophage-like phenotype.

3.2. Analysis of tumor-associated macrophage derived cytokines

The levels of 79 cytokines were analyzed in differentiated THP-1 conditioned media using cytokine antibody arrays. We quantified

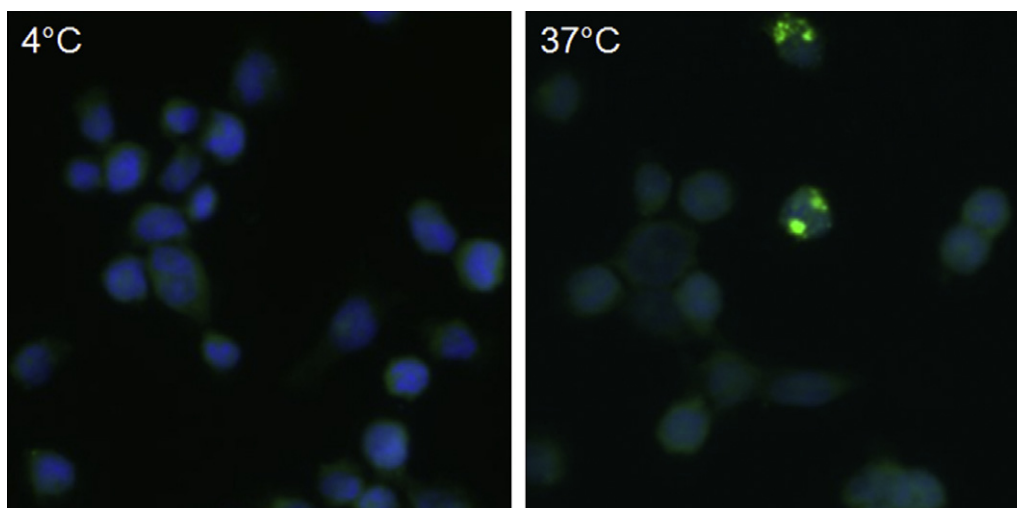


Fig. 1. FITC-Dextran phagocytosis assay. To determine whether differentiated THP-1 cells had phagocytic activity, a characteristic of macrophages, cells were incubated for 30 min with fluorescently-labeled FITC-dextran. One population was treated at 4 °C, a temperature at which the phagocytic machinery is minimally active. This control demonstrates the baseline level of fluorescence which results from binding of FITC-dextran to the cell surface. Clearly, at 37 °C, significant internalization of FITC-dextran has taken place over the same 30 min period.

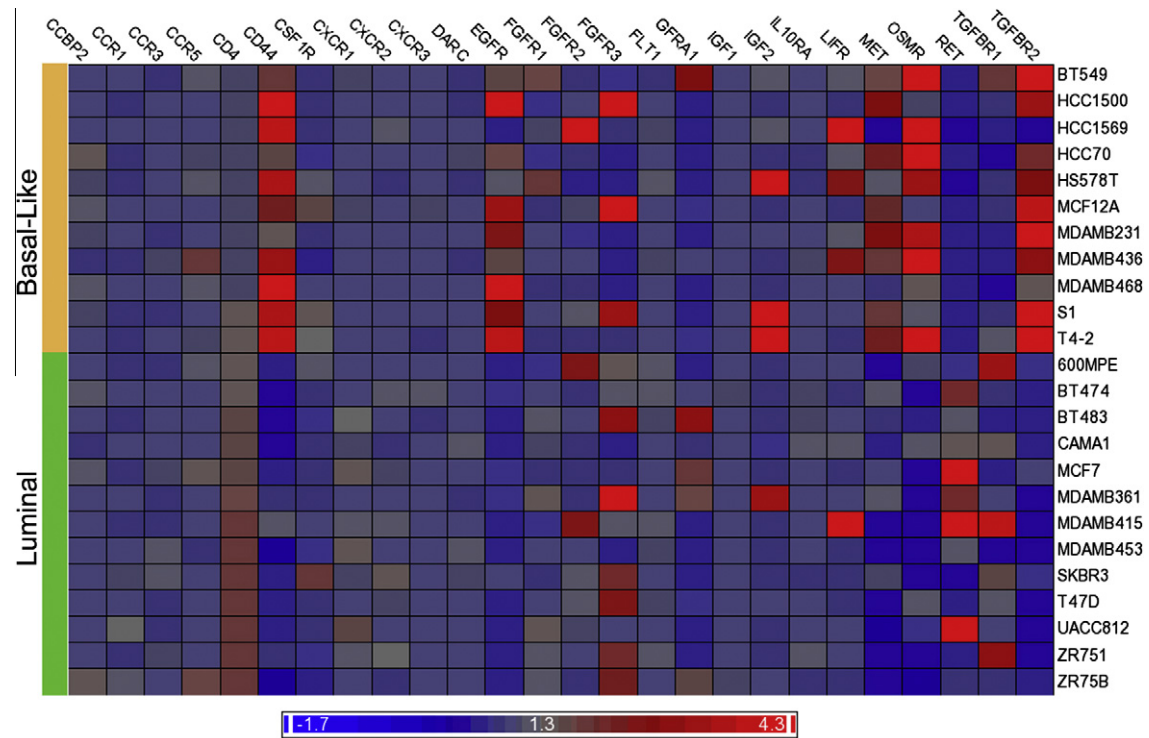


Fig. 2. Gene expression profile of receptors and interacting proteins for the cytokines detected in tumor-associated macrophage conditioned medium. Breast cancer cell lines were grown in 3D Matrigel culture for four days and gene expression was analyzed using Affymetrix microarrays. Cell lines are stratified into luminal and basal subtypes. Where more than one probe for a given gene was on the microarrays, and average value of all probes is presented.

each of the detected signals using densitometry and determined which of these factors were reproducibly detected in four independent experiments. The average expression levels of the 23 cytokines detected in differentiated THP-1 conditioned media are shown in Table 1. As anticipated from prior reports [9], we observed high levels of IL-10 and low levels of IL-12, which is the expected phenotype of M2-polarized macrophages.

3.3. Analysis of cytokine receptor expression in breast cancer cell lines

Although large studies have shown that macrophages are, in general, markers of poor prognosis, it is clear from the literature that there is significant heterogeneity in patient outcomes. We postulated that one potential cause of these differences may be that breast cancer cells of different molecular subtypes may express a different repertoire of cytokine receptors. To test this hypothesis, we examined the expression profile of the receptors for the predominant cytokines produced by differentiated THP-1 cells in a panel of breast cancer cell lines using Affymetrix gene expression microarray profiles as we have previously described [7]. Analysis of the expression of these cytokine receptors (Fig. 2) demonstrates that there are very significant differences (ANOVA, $P < 0.05$) between the luminal and basal-like subtypes. CD44, MET, TGFBR2, OSMR and EGFR were significantly enriched in the basal-like cell lines, while RET expression was significantly higher in the luminal cell lines.

4. Discussion

It is by now well established that tumor-associated macrophages make important contributions to tumor progression. By elaborating a diverse complement of cytokines, they can influence many cells of the tumor microenvironment. Cytokines such as VEGF

and PGF promote angiogenesis, while factors like IL10 are immunomodulatory and can result in an immunosuppressive environment [4]. In addition to these functions, which are likely operational in the majority of solid tumors, macrophages may also elicit tumor-specific effects, depending on the repertoire of cytokine receptors expressed by the neoplastic cells. In this study, we highlight the diversity that exists in the receptors for these cytokines among human breast cancer cell lines.

We found that the basal-like breast cancer cell lines expressed a broader range of receptors for macrophage-derived cytokines than luminal cell lines. It was particularly noteworthy that many of the most highly expressed cytokine receptors in the basal-like cell lines were those associated with tumor invasion and metastasis. This is consistent with the more aggressive clinical phenotype of basal-like breast cancer [10], which tends to be diagnosed at younger ages and has a more aggressive pattern of metastasis than the more indolent luminal tumors. The majority of these cell lines expressed EGFR, the receptor for Epidermal Growth Factor (Table 1) which has been reported to be secreted by breast tumor-associated macrophages [11]. In mouse models, macrophage-derived EGF has been reported to be a potent chemoattractant for MMTV-PyMT mammary tumor cells [12] which, like basal-like breast cancers, lack expression of the estrogen receptor at advanced stages. The basal-like cell lines also expressed high levels of CD44, the receptor for SPP1/Osteopontin which also promotes tumor cell invasion [13]. Similarly, these cell lines expressed high levels of the HGF receptor, MET, which promotes proliferation and metastasis via the Ras/MAPK and PI3K/Akt signaling pathways, with splice variants of CD44 participating in some of these functions [14]. Although it has been reported to inhibit proliferation of cancer cells from breast and other tissues [15], Oncostatin M has been found to promote the invasion of breast cancer cell lines and the expression of angiogenic factors such as VEGF [16]. TGFBR2 was also significantly enriched in the basal-like breast cancer cell lines,

which is consistent with reports indicating that expression of this receptor is correlated with particularly poor outcomes in estrogen receptor negative human breast cancer cases [17]. Like Oncostatin M, TGF-beta can have anti-proliferative effects, but is associated with metastatic progression in advanced disease [18].

The cytokine receptor most selectively expressed in luminal breast cancer cell lines was RET receptor tyrosine kinase. Although somatic mutations in RET are common in medullary thyroid carcinoma (40–50% of cases) [19], they are very rare in breast cancer [20] suggesting that ligand-driven activation of RET is likely to be the primary means of RET signaling in this tissue. The RET ligand, GDNF, which we have detected in macrophage conditioned medium, promotes the proliferation, scattering and survival of the RET-positive MCF7 cell line [21] and has been implicated in resistance to endocrine therapy [22] suggesting that macrophage-derived GDNF may contribute to these processes in ER+ luminal tumors.

Among the panel of receptors, FGFR3 showed the least subtype specificity with several luminal and basal-like cell lines expressing this receptor. These data are consistent with clinical reports which found no strong association between FGFR3 expression and other important clinicopathological parameters in breast cancer, although its expression was clearly associated with poor outcomes [23]. Collectively, almost all of the cell lines in the panel expressed modest levels of at least one FGF receptor, and signaling by this family of receptors is associated with both tumor cell invasion and proliferation [24].

Breast cancers have long been known to be heterogeneous for expression of key proteins such as ER, PR, and HER2 and more recently it has become clear that substantial gene expression differences exist between distinct breast cancer subtypes [1]. The present study highlights the differences that exist in the repertoires of receptors for cytokines produced by tumor-associated macrophages. Together, these data suggest that tumor-associated macrophages may elicit subtype-specific effects in human breast cancer and that identifying the key cytokine/receptor pairs in each breast cancer subtype will be essential to targeting macrophage-tumor cell interactions in the clinic.

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