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Th1 and Th2 Cytokine Gene Expression in the Peripheral Blood of Breast Cancer Patients Compared to Controls

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Abstract

Background: Cytokines produced by different subsets of T cells are the key mediators to adjust the quality of immune responses. Despite evidence of induction of an immune reaction against tumors such as breast cancer, not all responses are protective. In this study we attempt to evaluate the expression profile of several cytokines from the T helper 1 and 2 subsets in blood cells from patients with breast cancer.

Methods: We recruited 100 recently diagnosed patients with confirmed pathological reports. Peripheral blood samples were taken and the expression of the transcripts for IL-2, IL-12A, IL-12B, IFN-γ, IL-4, IL-10, IL-13 and IL-13Rα2 were measured by quantitative real time PCR. We correlated the results to clinical findings and compared the results to those from 64 healthy individuals.

Results: Among the studied cytokines, we observed a significant increase in the expression of T helper 1 pro-inflammatory cytokines IL-12B, IL-2 and IFN-γ compared to healthy controls. Elevated expression of those cytokines was associated with high stage and/or high grade tumors but there was no association with other clinical determinants. Simultaneously, blood cells showed high expression of IL-10, but not the other studied T helper 2 cytokines.

Conclusion: The mixed and complex cytokine profile of T helper 1 and 2 immunity in blood cells may show an immunological imbalance which makes the overall system favor cancer. This may be a potential target for immunotherapy approaches, however more comprehensive results are needed.

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Introduction

Exciting discoveries in immunology over the previous two decades have made it possible to take advantage of cells and mediators of the immune system for therapeutic approaches and customize immune responses against cancers. Adoptive transfer of immune cells or utilization of cytokines in different types of clinical trials has shown the extensive capability of immunotherapy to boost responses that initially failed against cancers.[1,2] In an immune response, effector cells exert their action either by direct cell-cell contact or by production of soluble cytokines. The ultimate outcome of responses depends on the types of recruited cells and the profile of cytokines they produce. Results show that cytokines function in very complex inter-relationship network with multiple positive and negative feedback signals. The effects of one cytokine can be amplified or suppressed by others or a cytokine may trigger a cascade of a series of actions.[3]

T lymphocytes are the main source of cytokines in an adaptive immune response and different subsets of T helper cells (Th) produce various arrays of cytokines to regulate other immune cells and possibly receive back-signals from other cells. Therefore, in the niche of a solid tumor such as breast cancer, an immune response against tumor

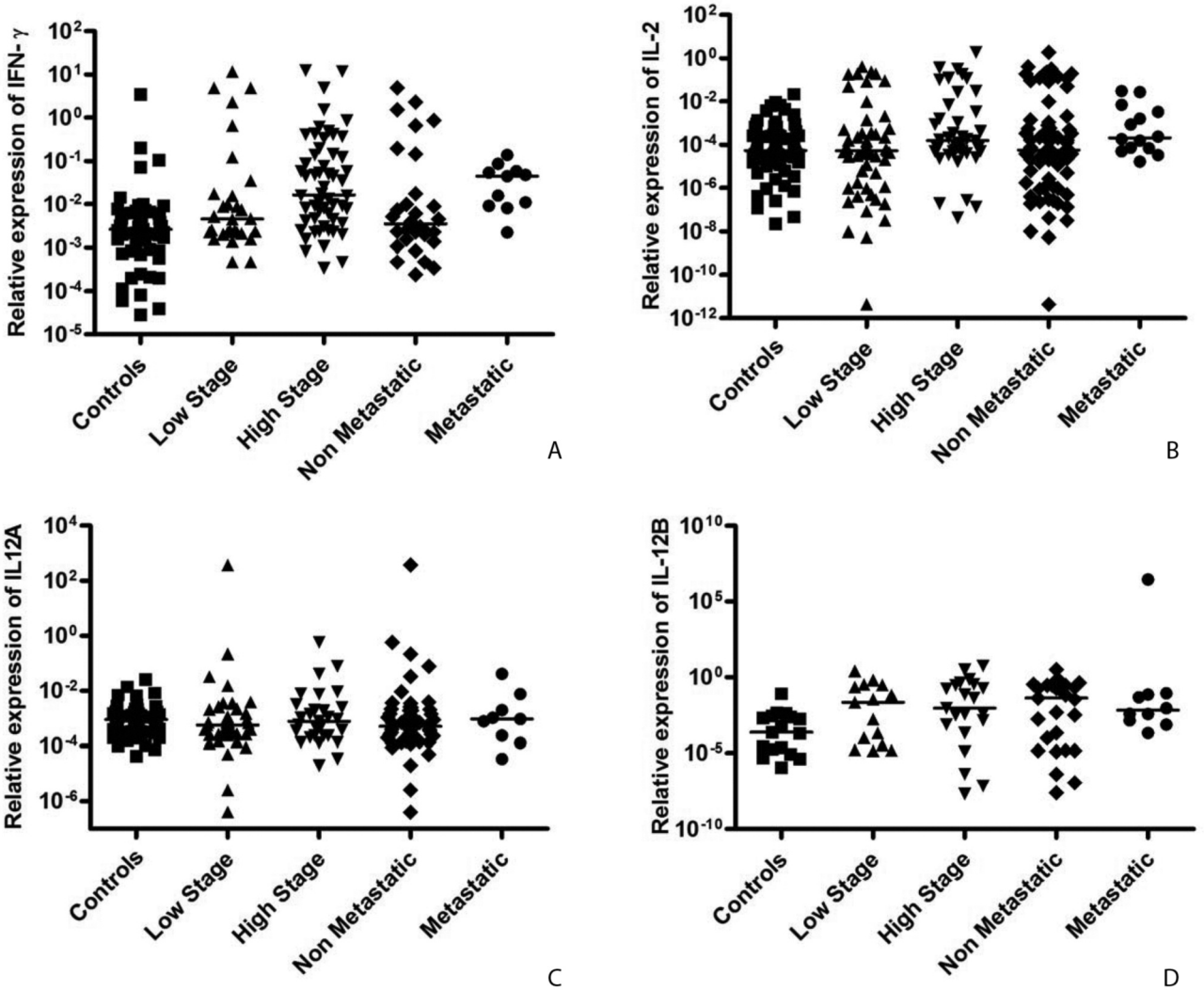

Figure 1. Blood cells from breast cancer patients showed different patterns of Th1 cytokine expression. The levels of mRNA transcripts in the peripheral blood cells from the patients and the healthy controls were assessed by relative quantitative real time PCR against b-actin as housekeeping gene. IFNγ (A) and IL-2 (B) had significantly higher expression levels in blood cells from the patients diagnosed with high stage and metastatic breast cancer (P<0.05). Although the expression of IL-12A was not different in the patients and control groups (C) (P=0.5), IL-12B had significantly higher expression in all tested patients (D) (P<0.001). Data were analyzed by Mann Whitney test.

cells needs orchestrated cellular interactions and production of the correct cytokines to shape a winning immune response. Several Th cells including Th1, Th2, Th17, Th9, Th22, follicular Th, and regulatory T cells have been described based on the profile of cytokines they produce.[4] The balance between these subsets or their cytokines plays major role in the quality of immune response. Two main subsets of Th cells, Th1 (believed to initiate cellular immunity and cytotoxicity against targets) and Th2 (mostly counteract Th1 and facilitate humoral immunity) were originally identified in mice by Mosmann et al. Subsequent evidence has demonstrated a similar dichotomy in humans.[5] In spite of this functional categorization, there is increasing evidence that particular components of the Th2-mediated immune response may act against the original grouping. It has been shown that the presence of Th2 derived IL-4 decreases tumor growth, initiates anti-tumor activity and even rejects tumors in mice.[6] Similarly, IL-10 can co-stimulate natural killer (NK) cells or enhance the proliferation of certain subsets of CD8+ T cells.[7] Many other reports show evidence regarding adverse effects of inflammation and presence of pro-inflammatory cytokines on immune responses against tumors. Such results show great potential complexity of cytokines' network. Several lines of evidence indicate an association of cytokines with adverse disease outcomes, including breast cancer. Although most researches have focused on the measurement of pro-inflammatory cytokines, the results are inconsistent.[8] More systematic evaluations that take into consideration the opposing arms are needed to draw conclusive results. Therefore, in this study we assess the expression of a panel of cytokines which include IL-2 in addition to a number of Th1- (IL-12A, IL-12B and IFN-γ) and Th2-related (IL-4, IL-10, IL-13, IL-13Rα2) cytokines in peripheral blood cells from a series of patients with different stages of breast cancer compared to normal controls.

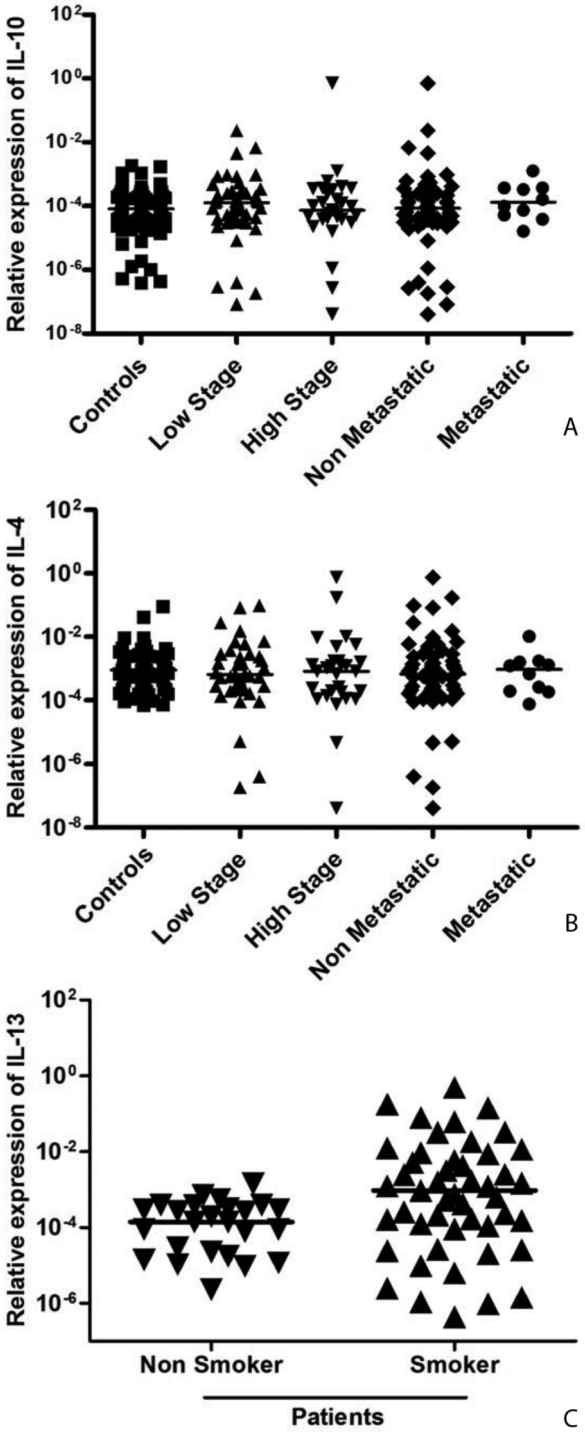

Figure 2. The profile of T helper 2 cytokines gene expression was inconsistent in the patients. Expression of IL-10 (A) and IL-4 (B) in blood cells of the patients and the controls were evaluated by relative quantitative real time PCR against β-actin as housekeeping gene. Patients at high stage of the disease or metastatic tumors had higher expression of IL-10 (P<0.001) while IL-4 expression was not different among patients and controls (P=0.4). Expression of IL-13 transcripts in the patients with smoking habit was significantly higher than non-smoker patients (C) (P<0.001). Results were analyzed by Mann Whitney test.

Materials and Methods

Patients and sampling

This case-control study was performed at the Institute for Cancer Research, Shiraz University of Medical Sciences during 2011-2012. We recruited 100 patients from the Breast Cancer Clinic of the University with definite pathological diagnoses of breast cancer. Table 1 shows the characteristics of the studied patients. Sampling was carried out before any clinical intervention such as chemotherapy, radiotherapy and surgery. In addition to the patients, 64 healthy volunteers participated in the study as the control group. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences and performed in accordance with the 1964 Declaration of Helsinki. All participants gave their informed consent prior to study enrollment. Peripheral blood was drawn by venipuncture with EDTA as anticoagulant and samples were processed immediately.

RNA isolation

Total RNA was extracted from peripheral blood samples by the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 2 ml of peripheral blood was transferred to a 50 ml conical tube and the RBCs were removed by NH4Cl lysis. RNA from the WBC pellet was extracted by 1 ml of TRIzol reagent and eluted in 40 µl DEPC-treated water. We assessed the quantity of extracted RNA by spectrophotometry and the quality by gel electrophoresis.

Quantitative real-time PCR (qRT-PCR)

cDNA was synthesized from 5 µg of a DNase-treated aliquot of total RNA, utilizing the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the manufacturer's instructions. Gene specific primers were designed by Primer-BLAST online software. Table 2 lists the sequences of the gene specific primers for IL-2, IL-12A, IL-12B, IFNγ, IL-4, IL-10, IL-13, IL-13Rα2 and β-actin (as the housekeeping gene). Quantitative real-time PCR (q-RT-PCR) reactions were set up in a final volume of 20 µl that contained 0.5 µg cDNA product, 150 nM of each primer and 1x SYBR Green-I master mix (Applied Biosystems, USA). Gene expressions were determined using Chromo4 Real-time PCR Detector (Bio-Rad, USA). Thermal cycling for all genes was set up as: a denaturation step at 95°C for 10 min, followed by 40 cycles that included denaturation at 95°C for 15 s, annealing at 56°C for 30 s and extension at 60°C for 60 s. Acquisition of a fluorescence signal was programmed at the end of the extension step. Accuracy of the amplifications was confirmed by performing melting curve analyses after each run. The Pfaffl model was used to combine gene quantification and normalization into a single calculation.[9]

Statistical analysis

We used the nonparametric Mann-Whitney U-test with SPSS software v. 15.0 (IBM SPSS, USA) to evaluate the differences in target gene

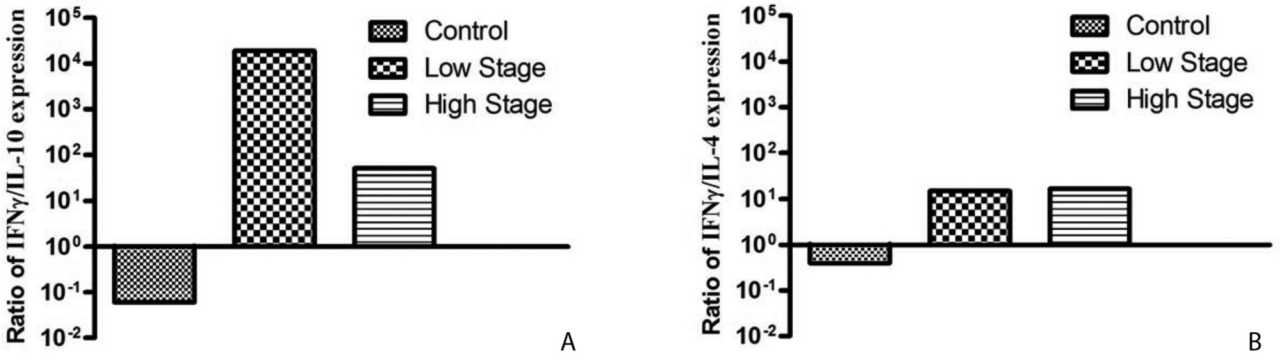

Figure 3. The IFN-γ/IL-4 and IFN-γ/IL-10 ratios in the breast cancer patients were inconsistent. The significant increased expression of IL-10 (A) ($P< 0.05$) was not in accord with the levels of IL-4 expression (B) ($P= 0.2$) in the patients. This variation resulted in different magnitude of changes in the ratios of IL-10 and IL-4 cytokines with respect to IFN-γ expression.

expressions among different groups. To demonstrate cytokine over-expressions, the 75-percentile of expression of each gene in the control group was defined as the base level of transcription. Data were processed and illustrated by Prism 5 software (Graphpad Prism, USA). In all statistical analyses, $P<0.05$ was considered as significant.

Results

In this study we used qRT-PCR to investigate the expression of a panel of Th1 and Th2 cytokines in blood cells from 100 recently diagnosed patients with breast cancer (Table 1) and compared the results with findings in 64 healthy controls.

Th1 cytokine gene expression profile

As the representative cytokines of Th1 cells, gene expressions of IFN-γ, IL-12A and IL-12B were determined in peripheral blood cells from patients with breast cancer and compared with healthy controls. Figure 1 shows the relative expressions of those cytokines and IL-2 where β actin was considered as an internal housekeeping gene. Results demonstrated a 24-fold increase in the expression of IFN-γ transcripts in blood cells from patients (Figure 1A, $P<0.001$). There was significantly higher expression of IFN-γ in patients with high disease stages or those with metastatic breast cancer. There were similar findings regarding IL-2 expression. We observed significantly higher expression of IL-2 in patients with higher stages of the disease and those with metastatic breast cancer ($P<0.05$, Figure 1B). When we measured the expression of IL-12A, we noted no significant difference between patients and healthy controls (Figure 1C). However, blood cells from patients contained up to 9-fold more IL-12B transcripts than normal controls ($P<0.05$), which was statistically significant in all patient groups (Figure 1D).

The statistical association studies also showed a significant relation between over-expression of IL-2 and disease grade in the tested patients. Similarly, 60% of patients with stage III breast cancer showed over-expression of IL-12B in their

Table 1. Histopathologic and demographic information of the patients.

Factor	Frequency	Percentage
Her2/neu		
Negative	47	47
1+	31	31
2++	4	4
3+++	18	18
Estrogen Receptor		
Negative	26	26
Positive	74	74
Progesterone Receptor		
Negative	34	34
Positive	66	66
Smoking		
Positive	81	81
Negative	19	19
Familial History		
Positive	5	5
Negative	95	95
Clinical stage		
I	16	16
II	38	38
III	27	27
IV	19	19
Metastasis		
Non-metastasis	81	81
Metastatic	19	19
Tumor grade		
I	21	21
II	46	46
III	33	33
Side of tumor		
Right	44	44
Left	56	56
Size of tumor		
T1	31	31
T2	54	54
T3	9	9
T4	6	6
Tumor necrosis		
Positive	71	71
Negative	29	29
Tumor calcification		
Positive	80	80
Negative	20	20

Table 2. Sequences of primer pairs used in the study.

Primer	Forward 5' → 3'	Reverse 5' → 3'
β-actin	GGCGGCACCACCATGTACCC	GGAGGGGCCGGACTCGTCAT
IL-2	ACCTCAACTCCTGCCACAAT	GCCTTCTTGGGCATGTAAAA
IL-12A	ACCAGGTGGAGTTCAAGACC	TGGCACAGTCTCACTGTTGA
IL-12B	CATGGGCCTTCATGCTATTT	TGATGTACTTGCAGCCTTGC
IFN-γ	TCCCATGGGTTGTGTGTTTA	AAGCACCAGGCATGAAATCT
IL-4	AGCAGTTCCACAGGCACAAG	CTGGTTGGCTTCCTTCACAG
IL-10	TGGTGAAACCCCGTCTCTAC	CTGGAGTACAGGGGCATGAT
IL-13	GTACTGTGCAGCCCTGGAAT	TTTACAAACTGGGCCACCTC
IL-13Rα2	TCCCTATTTGGAGGCATCAG	TGCTGGAATAGGTCCCAAAG

peripheral blood cells. Other histopathological findings that included tumor calcification, tumor necrosis, lymph node resection (number of positive lymph nodes), as well as ER, PR and Her2 expressions had no statistically significant correlations with the expression levels of the studied Th1 cytokines (Table 1).

Th2 cytokine gene expressions

Gene expressions of IL-10, IL-4, IL-13 and IL-13Rα2 of Th2 immunity were assessed in the blood cells of patients and control individuals. As shown in Figure 2A, we observed significantly higher expression of IL-10 in peripheral blood cells from patients compared to controls (1.9-fold, $P<0.05$). However, the change in IL-4 expression was not statistically significant ($P=0.4$, Figure 2B). Expressions of IL-13 and IL-13Rα2 genes were weak in peripheral blood cells of most patients and controls. Low levels of IL-13 expression were weakly detectable in 60 patients and in 5 controls. Also, 2 patients and 6 controls poorly expressed IL-13Rα2 in their peripheral blood cells. Interestingly, those patients who smoked showed significantly higher levels of IL-13 transcripts (6.7-fold) compared to non-smoker patients ($P<0.05$, Figure 2C). The statistical tests for association showed that gene expressions of IL-10, IL-4, IL-13 and IL-13Rα2 in the studied patients were not associated with expressions of ER, PR, Her2, tumor size, and familial history of cancer (Table 1).

Inconsistent IFN-γ/IL-4 and IFN-γ/IL-10 ratios

As seen in Figures 1A and 2A, there were significantly higher expressions of IFN-γ (the main cytokine of Th1 immunity) and IL-10 (one of the main suppressive cytokines) transcripts in the peripheral blood cells of patients compared to controls. There was no significant difference between patients and controls regarding IL-4 expression (the main cytokine of Th2 immunity). Thus, at least in patients with lower grades of the disease, the ratio of IFN-γ/IL-10 (Figure 3A) changed at larger magnitudes than the ratio of IFN-γ/IL-4 ($P<0.05$; Figure 3B).

Discussion

In the present study we assessed the abundance of transcripts of some major cytokines related to the Th1 and Th2 arms of the immune system in the peripheral blood cells from patients with breast cancer. To this end and to study multiple cytokines simultaneously, we used qRT-PCR and measured the expressions of IL-2, IL-12A, IL-12B, IL-4, IL-10, IL-13 and IL-13Rα2 cytokines based on the presence of their mRNA transcripts in blood cells. We also used data from the patients' records to correlate our findings with clinicopathological results. We identified significantly elevated expressions of IL-2, IFN-γ and IL-12B pro-inflammatory cytokines in patients' blood cells. A literature search showed similar observations for the over-expression of IL-2 in serum and blood cells of cancer patients and its possible adverse effect in the course of the disease.[10-12] In addition to T cells, high expressions of IL-2 might support

the development of tumors by enhancing cell proliferation and/or inhibition of apoptosis.[13] The presence of pro-inflammatory cytokines in tumor micro-environments and serum from cancer patients have been shown in several studies.[14-16] Therefore, in addition to immune enhancing roles, they may cause adverse effects and contribute to cancer progression and metastasis.[17] Our patients had higher expressions of these cytokines which was more prominent in patients with high stage tumors. Among those, IFN-γ had the highest levels of elevation. Recently, it was found that elevated serum levels of IFN-γ in uveal melanoma patients correlated with the spread of metastasis and represented a negative prognostic marker.[18] Also, other studies showed that relapse or disease progression was associated with strong CD8+ T cell infiltration in breast cancer patients.[19] It was also observed that during tumorigenesis, the detection of tumor cells by innate effectors (monocytes and NK cells) led to IFN-γ secretion with subsequent recruitment of regulatory T cells to evade the antitumor immune response.[20] Overexpression of IFN-γ correlated with up regulation of programmed death-1-ligand 1 (PD-L1) in breast cancer cells, an important mechanism of tumor immune evasion.[21] In addition, there was a direct correlation between the HER2/neu-specific T cell responses and nuclear localization of IFN-γ receptor in the tumors. Possibly, these patients are at risk of developing HER2/neu negative breast cancer. The results of these studies emphasize a potentially critical role for an inflammatory type of anti-tumor immune response such as IFN-γ that can facilitate tumor antigen loss and relapse of more invasive tumors.[22] Data exists that associate genetic polymorphisms of the gene and high production of IFN-γ to breast cancer susceptibility.[23,24] However, several *in vitro* and *in vivo* studies have shown effective immune enhancement by IFN-γ.[25,26] Therefore high concentrations of IFN-γ alone are not enough to mount a protective response; additional immune accessories are needed to exert its effects. We have also assessed IL-12B (p40) expression. In addition to its homodimer form, IL-12B binds to p35 (IL-12A) to form heterodimeric IL-12p70 or combines with IL-23A to form IL-23 as potent pro-inflammatory cytokines. Despite this, an increased expression of IL-12B has been found among patients with either high or low grade tumors. Interestingly, IL-12B has genetic polymorphisms and there are at least two allelic isoforms for IL-12B. The A allele has been recognized as predisposing factor for breast cancer and is the allele which confers additional cytokine production.[27,28] Concurrent with high expression of pro-inflammatory cytokines, we have observed an increased level of IL-10 transcripts in blood cells of the studied patients. IL-10 functions as one of the major immune regulators. High expression of IL-10 in tumor tissues or stromal areas has been associated with higher mortality rates in cancer patients.[29,30] Therefore, one could say high expression of IL-10 in the immune system of patients abolished immune activation and hampered the response. However, recent findings could show that IL-10 might defy its cliché roles. Experiments showed that IL-10 could induce the activation of tumor-specific cytotoxic CD8 T cells inside tumors, enhance the expression of IFN-γ and granzyme cytokines and intra-tumoral antigen presentation.[31] In addition, other reports failed to show any differences in plasma IL-10 levels between breast cancer patients and healthy individuals.[32-34] Our analysis of IL-4 expression has shown no significant difference between patients and controls. IL-4 from Th2 cells functions as a potent suppressor of Th1 immunity. Apart from T cells, results show that tumors and adipose derived stem cells isolated from breast cancer tissue can produce IL-4 and has been associated with the expression of regulatory molecules on T cells and a lessened immune response.[35] However, it seems tumor specific immune suppression in our patients was not IL-4 dependent.

The complexity of the cytokine network in the process of a tumor immune response expands when data from other entities, such as tumors by themselves, other immune cells (Th17, regulatory T cells), and innate immune cells are added to the

scenario.[36] In addition to the direct effect of tumors, it seems that activation of Tregs and possibly Th17 cells can turn protective anti-tumor immune responses in favor of tumors and tumor progression.[35,37,38] In that regard we previously have shown a significant increase in expression of CTLA4 and FoxP3 (foot prints of Tregs), IL-17, IL-23 and IL-27 (Th-17 cell-related cytokines) transcripts in the peripheral blood cells of patients with breast cancer. High expressions were positively correlated with disease stage in most cases.[35,39]

Our findings, along with similar results from other investigators regarding a mixed cytokine reaction may highlight the presence of an immunological imbalance in cancer patients. However, it seems that we only see the tip of the iceberg of the complex network for immune responses against tumors, such as breast cancer. Therefore for future research, it will be useful to compare these cytokine profiles before and after surgery. This may enable us to find the right approach for these patients. Immunotherapy approaches are attractive strategies to manipulate and adjust the response. However more comprehensive information is still needed to make us fully capable of attaining that goal.

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Conflict of Interest

No conflict of interest is declared.

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