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Report

Immune dysfunction and micrometastases in women with breast cancer

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Summary

Cytokines produced by T lymphocytes are critical to the efficacy of a given immune response and dysregulation of immune responses may play a role in cancer progression. We assessed the intracellular cytokine profiles of T cells in the peripheral blood of women with breast cancer and explored the relationship of these responses with the presence of cancer in lymph nodes and bone marrow. Peripheral blood lymphocytes from 84 patients and 26 healthy volunteers were analyzed by 4-color flow cytometry for surface markers and for intracellular cytokines. Bone marrow samples from some of these patients were also collected and analyzed for the presence of epithelial cells (micrometastases) by flow cytometry. The percentages of both $CD4^+$ and $CD8^+$ cells producing type1 (IL-2, IFN-γ or TNF-α) and type 2 (IL-4) were significantly lower in patients with breast cancer compared to healthy controls. These results indicate a general immune dysfunction in these patients as opposed to a shift in the balance of type1 and type2 cells. These dysregulated T cell responses did not correlate with age, stage of disease, or nodal status. However, we did observe a correlation between number of micrometastases in the bone marrow and T cell responsiveness.

Introduction

One of the most common questions that patients with cancer ask their physicians relates to the relationship of the immune system to the development of cancer. It is now possible, with the advent of new immunological assays, to evaluate immune function in cancer patients, even at the earliest stages. Understanding the immunological responsiveness in cancer patients is the first step to determining whether it plays a role in tumor progression and is also essential for developing effective immunotherapeutic strategies.

T cells play important immunoregulatory roles and recent studies have suggested that an imbalance of the normal ratio of type 1 and type 2 T cells (for example, a decrease in type 1 cells and/or an increase in type 2 cells) may result in impaired cell-mediated immunity in cancer patients and other pathological disorders [1–7]. Type 1 cytokines such as interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) tend to drive cell-mediated immune responses while type 2 cytokines such as IL-4, IL-6, and IL-10 tend to drive humoral immune responses [8–12]. $CD4^+$ helper T cells (T_H) have been categorized as type 1 (T_H1) or type 2 (T_H2) on the basis of the cytokines they produce. Similarly, type 1 (Tc1) and type 2 (Tc2) CD8 + T cytotoxic cells have been described. Analysis of type 1 and type 2 T cell responses in patients with breast cancer allows exploration of the potential role of shifts in cellular and humoral responses within the immune system and their impact in this disease.

The measurement of immune function both general and specific has been a challenge. Proliferative assays can tell us whether there are T cells present that can proliferate in response to a particular immune stimulus, but they generally do not yield information as to the nature of the T cells responding, (i.e. their phenotype (CD4, CD8, etc.) or function (type 1 or type 2)). Although altered cytokine production in cancer patients can be measured from sera or stimulated bulk cultures of peripheral blood mononuclear cells (PBMCs) using ELISA assays [1–4, 13–15], the phenotypes of the T cells producing the cytokines of interest are generally not determined. However, the recently developed flow cytometry technique of intracellular cytokine staining and analysis allows the determination of phenotype (e.g. $CD4^+$ vs $CD8^+$) and function (type of cytokine produced) at the single cell level [16–21]. We have applied this technique to characterize the immune function of women presenting for primary surgical treatment of breast cancer.

This is the first large study of breast cancer patients characterized using the intracellular cytokine assay. We assessed the cytokine profiles of $CD4^+$ T_H cells and $CD8^+$ T_C cells in the peripheral blood of women with breast cancer prior to their first surgical resection, and compared the results to healthy volunteers. We also analyzed the relationship between standard prognostic

features and cytokine profiles. Finally, this study was conducted in conjunction with an analysis of bone marrow micrometastases, and correlations between immune function and the presence of micrometastases will also be presented.

Materials and methods

Patients and healthy subjects

Patients undergoing definitive surgery for breast cancer were invited to participate in a clinical study to assess immune function and bone marrow micrometastases. This study protocol was approved by the UCSF Committee on Human Research and patients enrolled signed an informed consent form. All patients between the age of 20 and 85, with a known diagnosis of breast cancer were invited to participate in this study. While samples were obtained from patients who had undergone neoadjuvant therapy, these patients are not included in the present analysis as we did not want to confound the results with prior exposure to hormonal and/or chemotherapy.

Bone marrow was also obtained for the analysis of circulating epithelial cells (CEC) by flow cytometry as part of the research protocol. Immediately after induction of anesthesia, patients were turned on their hip and an 11 gauge bone marrow aspirate needle was used to withdraw a sample of 5 cc each from the posterior iliac crest from 2 separate but adjacent sites. Eighty four patients with newly diagnosed breast cancer are included in this analysis. Table 1 lists the characteristics of these patients and their tumors. Peripheral blood was also obtained from 26 healthy volunteers (23 female, 3 male; age: 26–59) to establish a ''normal'' range of T cell responsiveness.

Table 1. Patient characteristics

Characteristic	No. patients
Age (N = 84)	
≤ 50	35 (42%)
> 50	49 (58%)
Node Status (N = 83)	
Node (+)	37 (45%)
Node (−)	46 (55%)
0 nodes	46 (55%)
1–3 nodes	23 (28%)
≥ 4 nodes	14 (17%)
Stage (N = 83)	
DCIS	12 (14%)
Stage I	30 (36%)
Stage II	31 (37%)
Stage III	10 (12%)
Grade (N = 77)	
Grade 1	22 (29%)
Grade 2	30 (39%)
Grade 3	25 (32%)
Size (N = 68)	
< 2 cm	43 (63%)
≥ 2 cm	25 (37%)
Estrogen receptor (N = 74)	
ER+	56 (76%)
ER−	18 (24%)
Progesterone receptor (N = 74)	
PR+	45 (61%)
PR−	29 (39%)

Lymphocyte stimulation and analysis of cytokine producing cells by flow cytometry

Peripheral blood was obtained from patients with breast cancer prior to their first surgery as well as from several healthy volunteers. Aliquots of heparinized whole blood were incubated at 37 °C with PMA + ionomycin to stimulate T lymphocytes or were left unstimulated. After 1 h, brefeldin A (10 μg/ml) was added to block secretion of cytokines. The samples were incubated for another 5 h at 37 °C, then cooled to 4 °C and held there overnight. Red blood cells were lysed and the cells were fixed with FACS lysing solution (BD Biosciences). Finally the cells were frozen in 10% DMSO + 1% BSA in PBS at −80 °C in a freezing chamber (Stratagene). It should be noted that T cell activation was performed before freezing and that cryopreserving these activated cells allowed concomitant staining and analysis of several patients' samples.

Frozen cells were thawed, washed with PBS, and resuspended in FACS permeabilizing solution 2 (BD Biosciences) for 10 min. The cells were washed again, then incubated on ice with directly conjugated mAbs for 30 min. After staining, the cells were washed, refixed in 1% paraformaldehyde and then analyzed on a FACS Calibur flow cytometer (BD Biosciences). The following monoclonal antibodies from BD Biosciences were used: APC-conjugated anti-CD3, PerCP-conjugated anti-CD8, PerCP-conjugated anti-CD4, PE-conjugated anti-CD69, PE-conjugated anti-IL-2, and FITC-conjugated anti-IFN-γ, anti-TNF-α, and anti-IL-4.

A viable lymphocyte gate was set using forward and side scatter. Cells within this gate were then examined for CD3 and CD8 positivity. Since PMA + ionomycin stimulation results in down-modulation of the CD4 antigen, we defined $CD3^+/CD8^-$ cells as our CD4 T cell subset. Gates were then set on either the $CD3^+/CD8^+$ cells or the $CD3^+/CD8^-$ cells, and cells within these gates were examined for cytokine expression. Analyses were performed using CELLQuest software (BD Biosciences).

Analysis of micrometastases in bone marrow by flow cytometry

Bone marrow aspirates were collected from patients at the time of breast surgery. Attending surgeons used 11G Jamshidi needles to aspirate from two unilateral sites in the posterior iliac crest, and collected 3–6 ml total aspirate into EDTA-containing tubes for analysis within 24 h. Initial processing consisted of dilution in one-half

volume of Cell Buffer (Immunicon) in a 7 ml glass tube (BD Labware). Samples then underwent a magnetic enrichment for epithelial cells using anti-EpCAM (MJ37)-coated immunomagnetic particles; a second MAb, anti-EpCAM (EBA-1), was also added for subsequent detection. The tube was subjected to a magnetic field for 15 min in a magnetic separator (Immunicon), and the bone marrow was aspirated from the tube. The tube was removed from the magnet and cells remaining in the tube were resuspended in 2 ml of Cell Buffer. The resuspended cells were transferred to a 12 × 75 polystyrene tube (BD Labware) and subjected to magnetic separation for 5 min. The fluid in the tube was aspirated and the cells were resuspended in 150 μl of Cell Buffer. CD45 PerCP-Cy5.5 and nucleic acid dye in 20 μl were added along with 5 μl of anti-her-2 APC (BD Biosciences). The sample was incubated in the dark for 15 min, then fixed by the addition of 350 μl of 1% paraformaldehyde and transferred to a TruCOUNT tube (BD Biosciences). Samples were analyzed on a FACS Calibur (BD Biosciences) with 4 color option, until 35,000 bead events were acquired. A threshold on both Anti-EpCAM (EBA-1) and nucleic acid dye was used for acquisition.

Statistical analyses

Student's t-test was used to measure the differences between the T cell responses from patients and those from healthy subjects. Correlations between the data were analyzed using the Pearson correlation test. The level of significance was set at $p < 0.05$.

Results

T cell subsets and cytokine production

T cell subset analyses ($CD4^+$ and $CD8^+$) and functional analyses (cytokine production) were performed on peripheral blood obtained from women with breast cancer prior to any therapy. We assessed the ability of $CD4^+$ and $CD8^+$ T cells to produce IL-2, IL-4, IFN-γ, or TNF-α using a 4-color intracellular cytokine flow cytometric technique (Figure 1, Tables 2 and 3). IFN-γ and TNF-α production were assessed in an initial cohort of 25 patients. In the subsequent group of patients, TNF-α staining was dropped and IL-2 and IL-4 stains were added. The percentages of IFN-γ producing $CD4^+$ and $CD8^+$ T cells (16.7 ± 1.3% and 31.1 ± 2.2%, respectively) were significantly reduced in the patients with breast cancer compared to healthy controls. Not all patients had fewer IFN-γ producing cells than healthy controls. However. seventy-three percent (61/84) and 63% (52/83) of the patients had percentages of IFN-γ producing $CD4^+$ and $CD8^+$ T cells, respectively, below the normal range. Percentages of $CD4^+$/TNF-α^+ T cells (40.1 ± 4.1%) and $CD8^+$/TNF-α^+ T cells (38.5 ± 4.3%) were decreased in 20/25 (80%) and 15/25 (60%) of the breast cancer patients, respectively. Similarly, percentages of $CD4^+$/IL-2^+ T cells (21.8 ± 1.7%) and $CD8^+$/IL-2^+ T cells (8.2 ± 0.9%) were decreased in 23/58 (40%) and 38/57 (67%) of the breast cancer patients, respectively. Finally, significant reductions in the percentages of $CD4^+$/IL-4^+ T cells (0.66 ± 0.06%) and $CD8^+$/IL-4^+ T cells (0.63 ± 0.10%) were observed in 50/50 (100%) and 36/54 (67%) of the breast cancer patients, respectively.

In addition to the decreased numbers of cytokine producing $CD4^+$ and $CD8^+$ T cells in patients with breast cancer, an increase in the ratio of $CD4^+$ to $CD8^+$ T cells was observed in these patients (Figure 2). The CD4/CD8 ratio was increased in both node negative (2.31 ± 0.16; mean ± SEM) and node positive (2.53 ± 0.25) cases compared to healthy controls (1.79 ± 0.17) and this increase was statistically significant for both groups ($p < 0.035$ and $p < 0.024$, respectively).

Correlation between cytokines

Significant positive correlations were observed between IFN-γ and TNF-α production, as well as IFN-γ and

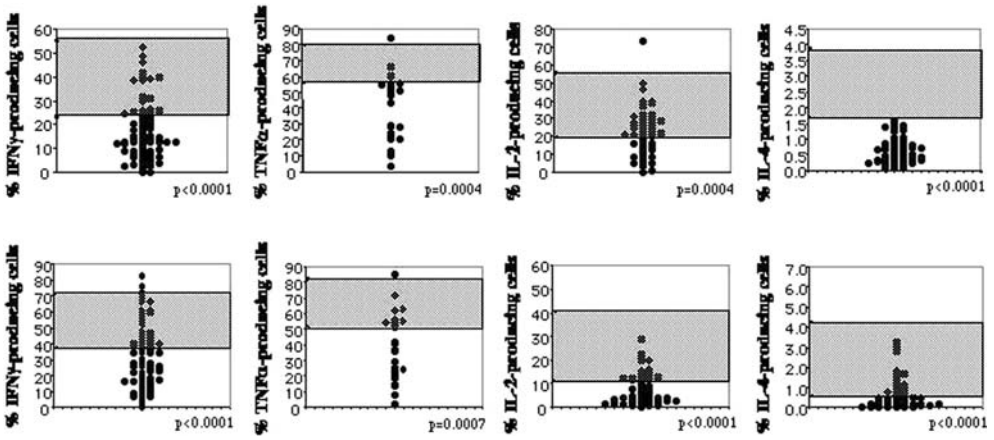

Figure 1. Comparison of type 1 (IFNγ, TNFα, and IL-2) and type 2 (IL-4) cytokine production by $CD4^+$ (a) and $CD8^+$ (b) T cell populations in the peripheral blood of patients with breast cancer vs. control subjects ($N = 26$). Results are presented as the percentage of cytokine-producing cells in each T cell subset. The shaded region represents the mean ± SD responses of the control subjects. Type 1 responses as well as type 2 responses were significantly reduced in both $CD4^+$ and $CD8^+$ T cells in breast cancer patients (Student's *t*-test).

Table 2. Cytokine production by PMA + ionomycin stimulated CD4 T cell populations in patients with stage 0–III breast cancer at the time of diagnosis and in healthy controls. Values given are mean percent cytokine positive cells ± SEM

	IFN	IL2	TNF	IL4
Healthy controls	38.98 ± 3.29	37.58 ± 4.78	67.90 ± 3.78	2.77 ± 0.29
DCIS/Inv Br CA	16.67 ± 1.31	21.75 ± 1.75	40.50 ± 4.07	0.66 ± 0.06
Age				
≤ 50	16.96 ± 1.99	21.09 ± 2.43	44.45 ± 5.20	0.85 ± 0.10
> 50	16.45 ± 1.74	22.21 ± 2.47	36.85 ± 6.22	0.51 ± 0.06
Stage				
0	15.35 ± 3.42	19.06 ± 4.11	36.89 ± 14.23	0.66 ± 0.13
I	16.65 ± 2.15	21.51 ± 3.76	42.05 ± 6.75	0.56 ± 0.11
II	18.24 ± 2.33	22.25 ± 2.69	41.03 ± 5.96	0.61 ± 0.09
III	14.79 ± 3.25	24.23 ± 4.39	ND[a]	0.92 ± 0.17
Grade				
1	14.68 ± 2.14	25.91 ± 2.09	29.50 ± 6.54	0.76 ± 0.14
2	15.49 ± 2.53	19.84 ± 3.02	47.68 ± 10.99	0.56 ± 0.09
3	21.83 ± 2.08	21.66 ± 4.52	46.94 ± 3.76	0.68 ± 0.11
Lymph nodes				
negative	17.90 ± 1.90	21.38 ± 2.59	41.97 ± 5.23	0.61 ± 0.08
positive	15.41 ± 1.77	22.38 ± 2.45	37.88 ± 6.75	0.70 ± 0.09
Sentinel lymph nodes				
negative	15.87 ± 1.85	20.75 ± 2.68	40.58 ± 6.56	0.63 ± 0.08
positive	14.16 ± 2.15	22.86 ± 2.72	44.87 ± 8.13	0.77 ± 0.10
Size				
< 2 cm	16.8 ± 1.90	24.59 ± 3.01	42.08 ± 5.79	0.52 ± 0.08
≥ 2 cm	15.57 ± 2.12	20.75 ± 2.90	32.11 ± 6.20	0.81 ± 0.12
Hormone Receptors[b]				
R^-	21.18 ± 3.98	26.26 ± 5.73	ND	0.57 ± 0.08
R^+	11.52 ± 1.23	20.94 ± 2.17	ND	0.69 ± 0.08
R^{++}	11.40 ± 1.27	22.55 ± 2.26	ND	0.78 ± 0.10

[a] ND – not determined.
[b] R^- : ER-negative & PR-negative, R^+: ER positive & PR negative or ER negative & PR positive, R^{++}: ER positive & PR positive.

IL-2 production in both $CD4^+$ and $CD8^+$ T cell populations (Table 4). In addition, a positive correlation was also observed between IFN-γ and IL-4 production by $CD4^+$ T cells, but not $CD8^+$ T cells. IL-2 and IL-4 demonstrated no correlation in either T cell population.

Correlation between cytokine levels and other clinical parameters

Because of the known association of immune function and age, we specifically looked at the relationship of age to immune suppression. There were no correlations between age and cytokine responses in either the $CD4^+$ or the $CD8^+$ T cell populations (see Tables 2 and 3).

To determine whether the observed decreases in cytokine levels in patients were related to other parameters, we looked for correlations between T cell responses and various clinical parameters including disease stage, grade, nodal status, tumor size, and hormone (estrogen and progesterone) receptor positivity. No correlations between T cell responses and stage, grade, or nodal status were observed (Tables 2 and 3).

With respect to tumor size, we observed a significantly greater IL4 response in the $CD4^+$ T cells from patients with tumors ≥2 cm compared to patients with tumors < 2 cm ($p = 0.044$; Table 2). We also noted a trend that patients with larger tumors tended to have more depressed type 1 responses compared to patients with smaller tumors. For example, only 10% of the patients with large (≥ 5 cm) tumors had $CD4^+/IFN^+$ responses in the normal range compared to 30% of the patients with smaller (< 5 cm) tumors. Similarly, only 20% of the patients with large tumors had $CD8^+/IFN^+$ responses in the normal range compared to 42% of patients with smaller tumors.

With respect to hormone receptors, we found that patients with ER^+ and/or PR^+ tumors tended to have a more depressed type 1 response compared to patients with receptor negative tumors (Tables 2 and 3). In particular the $CD4^+/IFN^+$ responses in patients with ER^-PR^- (R^-) tumors were significantly higher than in those with either ER^+PR^- or ER^-PR^+ (R^+) tumors ($p = 0.003$) or ER^+PR^+ (R^{++}) tumors ($p = 0.003$). The $CD8^+/IFN^+$ responses showed a similar trend, although it did not reach significance.

Table 3. Cytokine production by PMA + ionomycin stimulated CD8 T cell populations in patients with stage 0–III breast cancer at the time of diagnosis and in healthy controls. Values given are mean percent cytokine positive cells ± SEM

	IFN	IL2	TNF	IL4
Healthy controls	54.83 ± 3.61	26.11 ± 4.22	66.33 ± 4.82	2.43 ± 0.47
DCIS/Inv Br CA	31.06 ± 2.18	8.24 ± 0.90	38.51 ± 4.29	0.63 ± 0.10
Age				
≤ 50	28.47 ± 3.17	7.03 ± 1.31	36.89 ± 5.51	0.74 ± 0.15
>50	33.04 ± 3.00	9.12 ± 1.22	40.02 ± 6.67	0.55 ± 0.13
Stage				
0	25.99 ± 4.99	7.66 ± 2.16	27.52 ± 9.16	0.58 ± 0.11
I	34.45 ± 3.91	9.28 ± 1.90	44.41 ± 7.17	0.76 ± 0.24
II	31.15 ± 3.70	7.70 ± 1.47	35.25 ± 5.81	0.54 ± 0.16
III	28.88 ± 5.07	8.11 ± 2.00	ND[a]	0.63 ± 0.18
Grade				
1	30.46 ± 4.15	10.16 ± 1.65	34.01 ± 8.53	0.58 ± 0.16
2	28.83 ± 3.79	8.20 ± 1.52	47.71 ± 10.95	0.59 ± 0.17
3	37.95 ± 3.90	6.96 ± 1.98	39.63 ± 4.68	0.78 ± 0.25
Lymph nodes				
negative	33.03 ± 3.09	9.19 ± 1.36	42.81 ± 5.59	0.71 ± 0.15
positive	29.00 ± 3.13	7.39 ± 1.21	30.88 ± 6.11	0.54 ± 0.13
Sentinel lymph nodes				
negative	30.48 ± 3.14	8.89 ± 1.43	41.28 ± 7.04	0.82 ± 0.18
positive	25.27 ± 3.94	7.66 ± 1.33	31.88 ± 7.16	0.50 ± 0.11
Size				
<2 cm	35.16 ± 3.03	10.60 ± 1.50	41.12 ± 6.02	0.74 ± 0.20
≥ 2 cm	27.63 ± 3.93	7.13 ± 1.43	32.43 ± 6.61	0.57 ± 0.13
Hormone Receptors[b]				
R^-	29.49 ± 4.43	9.16 ± 2.56	ND	0.93 ± 0.37
R^+	23.93 ± 2.67	8.41 ± 1.20	ND	0.65 ± 0.13
R^{++}	21.98 ± 2.86	8.88 ± 1.34	ND	0.77 ± 0.15

[a] ND – not determined.
[b] R^- : ER-negative & PR-negative, R^+: ER positive & PR negative or ER negative & PR positive, R^{++}: ER positive & PR positive.

Correlation between cytokine levels and micrometastases in the bone marrow

The immune function study described here was a component of a larger correlative sciences study involving detection of circulating tumor cells (“micrometastases”) in bone marrow by multiple methods. Paired marrow and blood were obtained at the time of breast surgery (Stages I–III) and analyzed by multiple assays: standard immunocytochemistry (ICC); immunomagnetic capture followed by automated ICC (autoICC); immunomagnetic capture followed by flow cytometry (IC/FC); and RT-PCR for various target genes. Of these, IC/FC produces a quantitative readout or enumeration of tumor cells per ml of blood or marrow aspirate; therefore, we evaluated whether T-cell cytokine responsiveness correlated with blood or bone marrow tumor cells, with each as continuous variables.

Interestingly, depressed T-cell responses correlated significantly with the level of tumor cells detected in bone marrow aspirates by IC/FC (Table 5). Specifically, decreased IFN-γ expression in both $CD4^+$ ($p = 0.01$) and $CD8^+$ T-cells ($p < 0.001$) correlated with increased number of tumor cells in bone marrow. Decreased IL2 expression in $CD4^+$ T-cells ($p = 0.019$) also correlated with increased number of tumor cells in bone marrow. There was also a trend towards decreased IL-2 expression in $CD8^+$ T-cells correlating with increased number of bone marrow tumor cells, but this did not reach statistical significance. No correlations were observed between any of the T-cell responses studied and numbers of tumor cells in the peripheral blood.

These data suggest a notable association between immune dysfunction, as assessed by T-cell cytokine responses for IFN-γ and IL-2, and tumor cell load in bone marrow as measured by immunomagnetic capture/flow cytometry for epithelial markers.

Discussion

In the present study, we investigated immunological function/dysfunction in patients with breast cancer by measuring type 1 and type 2 cytokines produced by both $CD4^+$ and $CD8^+$ T cell populations. To avoid potentially confounding effects of chemotherapy or other

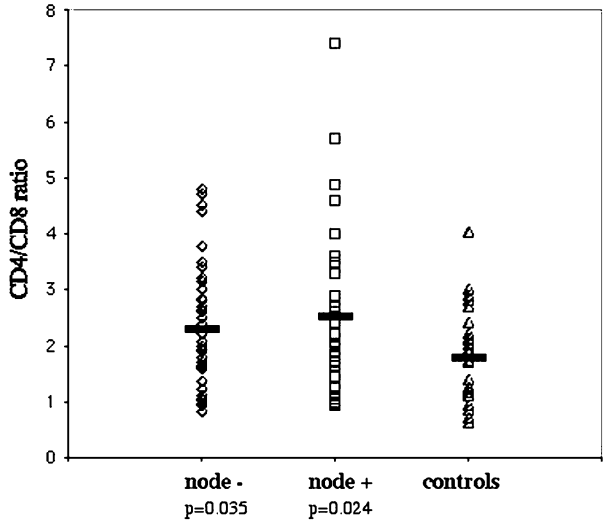

Figure 2. CD4/CD8 ratios in node negative and node positive patients with breast cancer compared to healthy controls.

therapies on immune function, none of the patients in this study had received any prior therapy. PBMC were stimulated with PMA + ionomycin and then analyzed by four-color flow cytometry for cell surface markers and intracellular cytokine production. This technique enables the characterization of both phenotype (CD4 and CD8) and function (type of cytokine produced) of T cells at the single-cell level. Cell mediated immunity in breast cancer patients has been evaluated by various assays including proliferation and cytotoxicity assays [22–27]. Immunological dysfunction has been reported in some cases, but the results have sometimes been equivocal or confounded by other factors such as prior chemotherapy or radiotherapy. We have demonstrated a clear deficiency in both type1 and type 2 cytokine production in patients with breast cancer, prior to any therapy. The measurement of cytokine production and T cell subset analysis as reported in this study may provide a more sensitive and valuable system for evaluating the status of cell mediated immunity in patients with breast cancer.

Activation with PMA + ionomycin bypasses the T cell receptor and stimulates T cells irrespective of their antigen specificity. Since we observed depressed T cell responses with PMA + ionomycin, this would indicate that the defect is downstream of the T cell receptor and suggest that antigen-specific responses would also be diminished. Although we have not yet tested antigen-specific responsiveness in these patients, we have used Staphylococcal enterotoxin B (SEB) as a stimulus. SEB signals through the T cell receptor, more closely representing an antigen-specific response compared to PMA + ionomycin. In these preliminary studies we also observed depressed responses to SEB in patients with breast cancer, compared to healthy volunteers.

We found that both $CD4^+$ and $CD8^+$ T cell subsets capable of producing type 1 cytokines (IFN-γ, TNF-α, and IL-2) were significantly reduced in the majority of the patients compared to healthy subjects. In addition, T cell subsets producing IL-4 were also significantly reduced in the majority of the patients. Decreased IL-2 and IFN-γ production is consistent with other published reports, but a decrease in IL-4 has not been reported. Decreased IL-2 production and increased IL-4 production, as measured by ELISA, has been observed in patients with colorectal cancer [1] and various other

Table 4. Statistical correlations among percentages of cytokine producing T-cell subsets

	CD4		CD8	
	IFN	IL2	IFN	IL2
TNF	$r = 0.781$	ND	$r = 0.908$	ND
	$p < 0.001$		$p < 0.001$	
IL2	$r = 0.518$		$r = 0.392$	
	$p < 0.004$		$p < 0.04$	
IL4	$r = 0.533$	$r = 0.303$	$r = -0.281$	$r = -0.066$
	$p < 0.003$	NS	NS	NS

NS – no significant difference; ND – not determined.

Table 5. Statistical correlations between percentages of cytokine producing T-cell subsets and the presence of circulating epithelial cells in the bone marrow, analyzed by the Pearson correlation test

	IFN	IL2	TNF	IL4
CD4	$r = -0.29$	$r = -0.333$	$r = -0.030$	$r = -0.093$
	$p = 0.01$	$p = 0.019$	NS	NS
CD8	$r = -0.374$	$r = -0.264$	$r = -0.006$	$r = -0.219$
	$p < 0.001$	NS	NS	NS

NS – no significant difference.

advanced cancers [3, 4]. Zhang et al., [7] also found decreased IL-2 production and increased IL-4 production, measured by flow cytometry, in patients with acute lymphocytic leukemia (ALL). In contrast to these studies in which a shift in the balance of type1 and type2 T cells was observed, we found that both type1 and type2 responses were decreased in patients with breast cancer, indicating a global immuno-deficiency, rather than a shift in the type1/type2 balance.

Interestingly, this immune dysfunction was observed even in early stages of breast cancer. Although there was no correlation with stage, nodal status, or age, we did observe a trend that patients with larger tumors had more suppressed type1 reponses and less suppressed type2 responses. Goto et al., also reported no correlation between T cell responses and patients' performance status, age, or tumor size [4]. These results suggest that T cell defects are not related to a patient's general condition, but rather to the presence of cancer and, to a lesser degree, to tumor burden.

We did, however, observe a correlation between immune function and hormone receptor status in patients with breast cancer. In particular, patients with tumors that expressed ER and/or PR had significantly lower $CD4^+/IFN^+$ T cell responses compared to patients with hormone receptor negative tumors. These patients also displayed lower $CD8^+/IFN^+$ T cell responses. These results suggest that the presence of estrogen and/or progesterone receptors on tumor cells may be associated with immunological dysfunction in patients with breast cancer. Kastelan, et al., observed a similar negative correlation between lymphocyte reactivity to mitogens and tumor hormone receptor status [28]. In addition, Levy et al., found that higher natural killer (NK) cell activity in patients with breast cancer was associated with ER-negative tumors [29, 30]. However, there are also reports in the literature demonstrating no significant relationship between ER or PR status and NK activity [31] or T cell responses [32, 33].

We also observed a correlation between numbers of epithelial cells in the bone marrow and T cell responsiveness. The presence of circulating epithelial cells (CEC), may provide additional information that supplements or replaces standard staging and prognostic procedures in primary breast cancer. A body of research now seems to indicate that CEC may have prognostic significance. For example, studies by Cote et al, and Mansi et al, have shown that the presence of CEC in bone marrow is associated with a decreased survival [34, 35]. Additional studies seem to indicate that CEC in bone marrow are actually an independent prognostic factor [36]. In the study by Diel et al, the largest study to date with 727 primary breast cancer patients, the presence of CEC in bone marrow provided more prognostic information than tumor size, grade, hormone status, and axillary lymph node status [37]. Braun et al., demonstrated that, after 5 years of follow-up, the presence of CEC alone was equivalent to the presence of lymph node metastases, and that the presence of both were clearly associated with a worse prognosis [38]. The correlation between micrometastases and T cell responsiveness we observed indicated that women with breast cancer who had the highest number of epithelial cells present in their bone marrow, tended to have the lowest type 1 T cell responses (eg. $CD4^+/IFN\gamma^+$). Conversely, women with breast cancer who had high type 1 T cell responses (some in the normal range) tended to have fewer micrometastases. This finding suggests that a dysfunctional immune system may allow the survival and hematogenous spread of cancer cells. Alternatively, it may indicate that circulating epithelial cells can induce the immune dysfunction we observe, possibly through the production of immunosuppressive cytokines such as transforming growth factor β (TGF-β).

Correlations were also observed among the different cytokines. Significant positive correlations were observed between the type 1 cytokines (IFN-γ, IL-2, and TNF-α) in both the $CD4^+$ and the $CD8^+$ T cell populations. However, the type 2 cytokine IL-4 had no correlation with IL-2 in either $CD4^+$ or $CD8^+$ cells. Although there was a positive correlation between IL-4 and IFN-γ in the $CD4^+$ T cell population, there was no correlation between these two cytokines in the $CD8^+$ T cell population. These results suggest that the type 1 and type 2 cytokines are differentially produced and that measuring a panel of cytokines, as opposed to just one, might provide more insight into the immunological dysfunction in patients with breast cancer.

Although we have measured suppression of cytokine production in T cells from patients with breast cancer, it remains to be determined what is responsible for this immune dysfunction. One possibility is that the number of type 1 T cells or their precursors is reduced. IL-2 is required to generate functional cytotoxic T lymphocytes (CTL) [39]. Suppression of IL-2 production may result in the inability to generate reactive CTLs, diminishing one line of defense against cancer progression and metastasis. Thus, this is one role that immunological dysfunction might play in the pathogenesis of breast cancer. IFN-γ and IL-12 are two cytokines involved in the expansion of type 1 T cells [40, 41]. Studies examining the production of IL-12 and other cytokines from monocytes may lead to an understanding of their role in this immune dysfunction.

Another possibility is that $CD4^+/CD25^+$ regulatory T cells (Treg) may play a role in the immune suppression observed in patients with breast cancer. We observed an increase in the CD4/CD8 ratio in patients compared to healthy controls. Nicolini et al., [25] also reported a significant increase in the CD4/CD8 ratio in breast cancer patients. This increase might be due to an increase in $CD4^+/CD25^+$ Treg cells. Indeed, it has recently been reported that patients with breast cancer and other cancers have an increased percentage of Treg cells in their peripheral blood and that these cells are capable of suppressing T cell responses in vitro [42, 43]. Studies are now in progress in our laboratory examining this Treg population in our patients.

Many questions remain unanswered. Does the global immune suppression remain after surgical therapy? A decrease in tumor burden could result in reduced levels of inhibitory factors produced by the tumor cells such as TGF-β or IL-10. In the case of ALL, an increase in IL-2 and IFN-γ producing T cells was observed after patients achieved a complete remission [7].

Another question is whether the immune function affects the outcome of these patients and/or can it be used as a marker to predict outcome? Lymphocyte responses to mitogens have yielded some prognostic information. In a study of 96 patients with breast cancer who received no postoperative adjuvant therapy, overall and relapse-free survival was significantly longer in patients with high preoperative lymphocyte responses to phytohaemagglutinin (PHA) compared to patients with low responses [44]. In another study of 90 patients with stage I-III breast cancer, PHA-induced proliferative responses were obtained preoperatively and 12 months later (following adjuvant chemotherapy or hormone therapy). It was found that of the 23 patients who demonstrated a drop in their PHA responses over this time period, 14 (61%) developed metastatic disease within the following 24 months. In contrast, of the 59 patients whose PHA responses increased, only one (2%) had disease progression [45].

We are carefully following these patients and monitoring for recurrence, however, there have not been a sufficient number of events to perform an outcomes analysis at this time. We are currently designing a follow-up study to measure immune function at 2+ years after surgical excision.

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