Inflammatory Biomarkers for Persistent Fatigue in Breast Cancer Survivors

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Abstract

Purpose: This study seeks to define immunologic and inflammatory variables associated with persistent post-treatment fatigue in breast cancer survivors.

Experimental Design: Leukocyte subsets, plasma inflammatory markers, and *ex vivo* proinflammatory cytokine production were assessed in 50 fatigued and nonfatigued breast cancer survivors recruited ≥2 years after successful primary therapy. Multivariate statistical analyses were used to define a composite immunologic biomarker of fatigue risk.

Results: Fatigued breast cancer survivors were distinguished from nonfatigued survivors by increased *ex vivo* monocyte production of interleukin (IL)-6 and tumor necrosis factor-α following lipopolysaccharide stimulation, elevated plasma IL-1ra and soluble IL-6 receptor (sIL-6R/CD126), decreased monocyte cell-surface IL-6R, and decreased frequencies of activated T lymphocytes and myeloid dendritic cells in peripheral blood (all $P < 0.05$). An inverse correlation between sIL-6R and cell-surface IL-6R was consistent with inflammation-mediated shedding of IL-6R, and *in vitro* studies confirmed that proinflammatory cytokines induced such shedding. Multivariate linear discriminant function analysis identified two immunologic markers, the ratio of sIL-6R to monocyte-associated IL-6R and decreased circulating $CD69^+$ T lymphocytes, as highly diagnostic of fatigue ($P = 0.0005$), with cross-validation estimates indicating 87% classification accuracy (sensitivity = 0.83; specificity = 0.83).

Conclusion: These results extend links between fatigue and inflammatory markers to show a functional alteration in proinflammatory cytokine response to lipopolysaccharide and define a prognostic biomarker of behavioral fatigue.

Advances in detection and treatment have increased survival times for women with early-stage breast cancer (1), and breast cancer survivors are now the largest group of cancer survivors in the United States (2, 3). Persistent, medically unexplained fatigue is one of the most common and disabling complaints among breast cancer survivors, affecting as many as 30% of survivors up to 5 years after successful treatment (4, 5). Fatigue has a negative effect on mood, social interaction, sleep, daily activities, and overall quality of life (4, 6).

Despite its prevalence, the etiology of cancer-related fatigue remains poorly understood. Basic research in the neurosciences has shown that proinflammatory cytokines can signal the central nervous system to induce symptoms of fatigue and other "sickness behaviors" (7–9). Several inflammatory mediators have been linked to altered central nervous system activity, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α; refs. 10, 11), and alterations in proinflammatory cytokines have been found in fatigue-related disorders, such as depression and chronic fatigue syndrome (12–14). Such results suggest that persistent unexplained fatigue in breast cancer survivors could stem from an underlying long-term alteration in inflammatory biology. Dysregulated cytokine production has been reported during cancer treatment and attributed to the effects of chemotherapy or radiation (15–19). Previous studies by our group have found elevated serum markers of proinflammatory cytokine activity and correlated alterations in T lymphocyte subsets in breast cancer survivors suffering from fatigue 3 to 5 years after the completion of therapy in the absence of any detectable residual disease (20, 21).

This study seeks to clarify the basis for aberrant cytokine levels in disease-free breast cancer survivors with persistent fatigue by analyzing the number and functional characteristics of leukocytes that produce proinflammatory cytokines. We

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selected immune activation markers for analysis based on previous studies showing fatigue-related alterations in soluble inflammatory variables (i.e., IL-1ra and IL-6; refs. 20–23) and activated T lymphocytes (24, 25). We also assessed circulating concentrations of the soluble IL-6 receptor (sIL-6R) to provide a more comprehensive assessment of cellular response to IL-6. The sIL-6R serves as an agonist, in contrast to many other soluble cytokine receptors that compete with the membrane-bound proteins for ligand binding. Hence, the sIL-6R in complex with IL-6 can stimulate a great diversity of cells that lack membrane-bound IL-6R through a process termed trans-signaling (26); sIL-6R is thought to enable IL-6 to gain increased (feedback) control over central nervous mechanisms regulating behavioral processes, including fatigue (26). In addition to these static markers of inflammatory activity, we also analyzed the functional basis for altered inflammatory response by measuring monocyte intracellular proinflammatory cytokine production following ligation of Toll-like receptor 4 (TLR4) with lipopolysaccharide (LPS). TLRs mediate innate immune responses to common pathogens, and aberrant TLR activity has been linked to other inflammatory diseases, such as rheumatoid arthritis (27), Crohn's disease (27), and heart failure (28). We also evaluated another potential explanation for increased inflammatory cytokine production by assessing variations in the prevalence of cytokine producer cells, such as activated T lymphocytes, monocytes, or myeloid dendritic cells (29–31). Finally, we sought to synthesize these multiple streams of immunologic and inflammatory information into a composite biological marker that is diagnostic of fatigue measured at the behavioral level. Such a biomarker would provide an important methodologic tool for assessing the effect of interventions to reduce fatigue and tighten the focus of future research by reducing the number of distinct variables measured in analyses of aberrant inflammation in breast cancer survivors.

Materials and Methods

Recruitment and sample collection. Breast cancer survivors were recruited from the Los Angeles metropolitan area through tumor registry listings, newspaper advertisements, flyers, and other media coverage. For participants identified by tumor registry, a recruitment letter included a brief description of the study and a response form. For those women who indicated an interest in the study, a telephone screening was conducted to verify the following eligibility criteria: (*a*) originally diagnosed with stage 0, I, or II breast cancer; (*b*) 1 to 5 years postdiagnosis; (*c*) completed all cancer treatment, except for tamoxifen/aromatase inhibitors; (*d*) no evidence of cancer recurrence; (*e*) age ≤75 years; (*f*) no chronic medical condition involving immune system (e.g., autoimmune disease) or regular use of immunosuppressive medications; (*g*) no psychotic disorders or schizophrenia; and (*h*) not regular smokers nor consumed >14 alcoholic beverages weekly.

Because this study focused on breast cancer survivors with persistent fatigue, potential participants were also screened for fatigue status using the vitality scale of the SF-36 (32). Standardized vitality scale scores range from 0 to 100, with higher scores indicating better functioning (i.e., higher levels of energy). Scores above the midpoint of 50 represent well-being, whereas scores below 50 represent limitations or disability related to fatigue. In our previous research, breast cancer survivors scoring under 50 on the SF-36 vitality scale have shown significant alterations in immune, neuroendocrine, and behavioral status relative to those scoring above 70 (4, 20, 21, 33, 34). Women were eligible for study participation if their mean vitality score over two to three assessments was ≤50 (32, 35). We also identified a control group of nonfatigued survivors whose mean vitality scores exceeded 70. Fatigue status was verified at the baseline assessment.

Of the 489 women contacted through the tumor registry, 240 respondents were screened by telephone. In addition, 74 women who called in response to media advertisements were screened by telephone. A total of 50 eligible participants entered the study protocol: 32 classified as fatigued and 18 classified as nonfatigued. Prevalent reasons for ineligibility were an intermediate fatigue score between 50 and 70, advanced cancer stage, and >5 years post-treatment.

Procedures. Participants completed self-report questionnaires to assess demographic, medical, and treatment-related characteristics. Depression was assessed by the Beck Depression Inventory (BDI; ref. 36) due to previous findings linking depressive symptoms to fatigue in breast cancer survivors (20). All sample collection and interviews were carried out in the morning to control for diurnal variation, with blood sampling from an antecubital vein contralateral to the original tumor resection. The University of California at Los Angeles Institutional Review Board approved all study procedures, and written consent was obtained from all participants.

Leukocyte subsets and protein expression. Peripheral blood was collected into heparinized tubes and Ficoll (Amersham, Piscataway, NJ) density gradient was used to separate peripheral blood mononuclear cells (PBMC). For flow cytometric assessment of cell-surface antigens, 5×10^5 cells were incubated in human AB serum (Gemini Bioproducts, Woodland, CA) containing fluorescence-conjugated antibody cocktails for 15 minutes at 4°C in the dark. The cells were then washed with fluorescence-activated cell sorting buffer and fixed in 4% paraformaldehyde (BD PharMingen, San Diego, CA). Flow cytometry data were acquired on a FACScan flow cytometer (BD Immunocytometry, San Jose, CA), with leukocytes gated based on forward scatter and side scatter. Secondary gates were established within the leukocyte population to identify specific cell subtypes. CellQuest software (BD Biosciences, San Jose, CA) was used to calculate percentage of positive cells and mean fluorescence values.

$CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, $CD14^+$ monocytes, natural killer cells, $CD25^+/CD69^+$ activated T lymphocytes, myeloid dendritic cells (HLA-DR/CD11c/CD14), and monocytes expressing the IL-6R ($CD3^-/CD126^+/CD14^+$) were assessed using fluorescence-conjugated antibodies to CD3, CD4, CD8, CD11c, CD14, CD56, CD62L, CD69, CD126, CD103, CD45RA, HLA-DR (Immunotech Beckman Coulter, Fullerton, CA), CD120, and CD121 (BD PharMingen).

Intracellular cytokine production. Monocyte intracellular production of IL-6 and TNF-α was assessed by flow cytometry using peridinin chlorophyll protein–labeled CD14 monoclonal antibody, allophycocyanin-labeled anti-TNF-α monoclonal antibody, and phycoerythrin-labeled IL-6 antibody. Heparin-treated blood (1 mL) was mixed with 100 pg/mL LPS (Sigma, St. Louis, MO) and 10 μg/mL brefeldin A (Sigma) and incubated for 4 hours at 37°C in a platform mixer followed by an overnight incubation at 4°C. RBCs were lysed in fluorescence-activated cell sorting lysing solution (BD Biosciences), remaining cells were permeabilized in fluorescence-activated cell sorting permeabilizing buffer (BD Biosciences), and fluorescence-conjugated antibodies were added for 30 minutes at room temperature in the dark. Cells were then washed and resuspended in 1% paraformaldehyde for assay on a Coulter Elite flow cytometer using the Coulter Elite software. Forward scatter and side scatter were used to gate on monocytes and granulocytes. About 12,000 $CD14^+$ events were counted to determine the percentage of cytokine-secreting monocytes, with quadrant coordinates set based on unstimulated cells. Unstimulated cytokine-positive event percentages were subtracted from stimulated percentages to obtain net stimulated cytokine positive event percentages.

Plasma cytokines. Plasma IL-6, sIL-6R, IL-1ra, and TNF-rII were assessed using high-sensitivity ELISAs (R&D Systems, Minneapolis, MN) following the manufacturer's specifications. Analyte capture was carried out in 100 to 200 μL plasma incubated 2 hours at room

temperature with constant shaking, after which plates were washed and incubated with conjugate antibody 2 hours at room temperature. The plates were then washed and incubated with substrate (30 minutes), amplifier (60 minutes), and stop solution (30 minutes) at room temperature. Absorbance was read on a Multiskan MCC/340 ELISA plate reader (Fisher Scientific, Pittsburgh, PA) at 490 nm wavelength. Absorbance values were converted to pg/mL using a standard curve calculation based on recombinant cytokine standards provided by the manufacturer.

In vitro ***regulation of cytokine receptor expression by inflammatory signals.*** *In vitro* regulation of cytokine receptor expression was carried out to ensure that changes in cellular IL-6R (CD126) levels could be taken as a valid indication of proinflammatory cytokine activity. PBMCs were isolated from healthy donor blood as described above and cultured in RPMI 1640 plus 10% fetal bovine serum at 37°C in 5% CO_2. When indicated, 10 ng/mL IL-6, IL-1β, and TNF-α were added individually or in combination. Cells were analyzed by flow cytometry using FITC-conjugated anti-CD3 to exclude T cells, phycoerythrin-conjugated anti-CD126 (IL-6R), and PC5-conjugated anti-CD14 to identify monocytes.

Statistical analysis. Initial analyses compared fatigued and nonfatigued groups using *t* tests for continuous variables and χ^2 tests for categorical variables. Analyses of covariance controlled for possible confounders in comparisons between fatigued survivors and controls. Covariates included age, body mass index, time since treatment, treatment modality, and BDI scores (to control for previously identified relationships between fatigue and depression; refs. 37, 38). Treatment modality was coded as a series of three indicator variables reflecting the use of radiation (yes/no), chemotherapy (yes/no), and tamoxifen/aromatase inhibitor use (yes/no). Relationships among immunologic variables were assessed by Spearman correlation coefficient. All statistical tests were two-tailed. Technical obstacles prevented collection of some samples, and those instances are identified in figure legends.

Biomarker definition and classification performance. A composite biomarker was developed following the generalized approach outlined by Dillon and Goldstein (39). To identify an optimal combination of immunologic variables that distinguished fatigued survivors from their nonfatigued counterparts, we used SAS PROC STEPDISC to perform stepwise construction of a linear discriminant function model using as potential predictors all immunologic or inflammatory variables that differed significantly between groups. Variable selection used default stringency variables, and identical results emerged using nonparametric discriminant function analysis (*k* nearest neighbor) and backward elimination model building (40). Once an optimal predictive model was developed, we used SAS PROC DISCRIMINANT to assess accuracy in predicting the fatigue status of survivors based on immune markers. In addition to standard measures of classification accuracy, we also assessed predictive performance using holdout cross-validation to estimate model accuracy in future data sets that did not contribute to the construction of the prediction model (40).

Results

Sample characteristics. Demographic and treatment-related characteristics of the sample are shown in Table 1. The sample was primarily White and well educated, consistent with the population of breast cancer survivors in Los Angeles. The study participants included early-stage (0, I, and II) breast cancer diagnosis that was treated with radiation, chemotherapy, or both and was currently using tamoxifen or aromatase inhibitors. There were no significant differences between fatigued and nonfatigued survivors on any of the demographic or treatment-related variables. Fatigued participants had statistically significant higher BDI scores than nonfatigued participants [mean, 12 versus 4; $t(48) = 4.28$; $P < 0.001$].

Circulating inflammatory markers. To assess proinflammatory cytokine expression and signaling activity in fatigued breast cancer survivors, we assayed plasma levels of IL-1ra, TNF-rII, IL-6, and sIL-6R (CD126). As shown in Fig. 1, fatigued participants had significantly higher plasma IL-1ra concentrations than nonfatigued women [IL-1ra, 5.7 versus 5.4 [ln(pg/mL)]; $t(48) = -1.53$; $P = 0.05$, *t* test; Fig. 1A] consistent with previous research (20). Fatigue-related differences remained statistically significant after control for age, time postdiagnosis, BDI, and type of treatment in an analysis of covariance (all *P*'s < 0.1). Fatigued breast cancer survivors had significantly higher plasma concentrations of sIL-6R (40.1 versus 30.6 ng/mL; $t(44) = -4.07$; $P < 0.001$, *t* test; Fig. 1B), but fatigue-related differences in plasma TNF-rII and IL-6 did not reach significance. Differences in plasma sIL-6R concentration remained significant after controlling for age, time postdiagnosis, BDI, and type of treatment in an analysis of covariance (all *P*'s < 0.002).

Intracellular monocyte inflammatory cytokine expression. In addition to constitutive elevations in plasma inflammatory markers, fatigued breast cancer survivors were distinguished from nonfatigued survivors by increased *ex vivo* monocyte production of IL-6 and TNF-α following exposure to the TLR4 ligand LPS [IL-6 percentage of positive cells: 64% versus 56%; $t(45) = -1.813$; $P = 0.049$; TNF-α percentage of positive cells: 52% versus 43%; $t(45) = -1.983$; $P = 0.03$]. Figure 2A shows representative results for one fatigued and one nonfatigued participant, and Fig. 2B summarizes data across the entire sample. Unstimulated values did not differ significantly between groups ($P < 0.6$). Differences remained significant in analyses of covariance controlling for potential confounders (all *P*'s < 0.03).

IL-6R levels **in vivo** ***and*** **in vitro.** Fatigued participants showed significantly lower levels of IL-6R on $CD14^+$ cells [CD126/CD14 percentage of positive cells: 40% versus 27%; $t(29) = 2.195$; $P = 0.03$; Fig. 3A]. Decreased cell-surface expression of IL-6R has been linked to increased IL-6 activity and may reflect cytokine-induced receptor shedding (41). Consistent with this hypothesis, we found an increased concentration of sIL-6R in plasma from fatigued participants as noted above. Monocyte cell-surface expression of IL-6R was also negatively correlated with circulating levels of sIL-6R in the total sample ($r = -0.30$; $P = 0.06$).

sIL-6R levels have been taken as an indicator of IL-6 activity in previous studies (42), but it is unclear whether shedding of IL-6R from the cell surface is a specific marker of IL-6 activity or a more general response to proinflammatory signaling. To clarify the interpretation of IL-6R levels in clinical plasma and cellular samples, we carried out *in vitro* studies of cytokine-induced receptor shedding. PBMCs isolated from healthy donors were exposed to 10 ng/mL IL-6, IL-1β, or TNF-α and IL-6R cell-surface expression was assessed 12 hours later. As shown in Fig. 3B, each of the three proinflammatory cytokines induced the loss of IL-6R from the cell surface of PBMCs and from $CD3^+$ and $CD14^+$ subsets of PBMCs [$t(4) = 4.12$; $P < 0.01$ for IL-6; $t(4) = 4.51$; $P < 0.01$ for IL-1β; $t(4) = 1.36$; $P = 0.23$ for TNF-α; $t(4) = 5.18$; $P = 0.006$ for all three cytokines]. Thus, decreased cell-surface IL-6R *in vivo* is consistent with increased plasma concentration of proinflammatory markers.

Table 1. Demographic and medical characteristics of fatigued and nonfatigued breast cancer participants

Characteristics	Fatigued (n = 32)	Nonfatigued (n = 18)
Age (mean ± SD)	54.4 ± 6.9	58.4 ± 9.9
Ethnicity		
White	26	12
Hispanic	2	1
African American/Black	1	3
Asian/Pacific Islander	3	1
Other	0	1
Married or in committed relationship		
Yes	19	13
No	12	5
Education level		
High school	1	4
College graduate	21	10
Postgraduate	10	4
Employment status		
Employed full/part time	24	14
Medical leave/disabled	2	0
Retired	4	4
Unemployed	1	0
Income level		
<30,000	5	3
30,001-60,000	8	3
>60,000	19	12
Type of treatment		
Surgery alone	6	1
Surgery + radiation	5	9
Surgery + chemotherapy	5	2
Surgery + radiation + chemotherapy	16	6
Tamoxifen/aromatase inhibitor use		
Yes	17	12
No	15	6
Years since diagnosis		
Mean ± SD	2.8 ± 1.2	2.5 ± 0.9
BDI score	12	4

Cellular immune variables. To determine whether increased inflammatory cytokine production was due to alterations in the prevalence of cytokine producer cells, such as activated T lymphocytes, monocytes, or myeloid dendritic cells, flow cytometric analyses of peripheral blood leukocytes were conducted to assess differences in the frequency of these cells. Compared with nonfatigued breast cancer survivors, fatigued subjects showed decreases in the frequency of circulating myeloid dendritic cells [HLA-DR$^+$/CD11c$^+$/CD14^{dim}; mean = 14% versus 11% positive cells; $t(29)$ = 2.047; P = 0.04] and activated T lymphocytes [CD3$^+$/CD69$^+$; mean = 12.2% versus 8%, positive cells; $t(29)$= 1.077; P = 0.04; Fig. 4A and B]. In addition, fatigued participants showed an increased percentage of lymphocytes as fraction of total leukocytes, with a selective increase in the frequency of CD4$^+$ T lymphocytes, in accordance with previous reports (21). Fatigued breast cancer survivors did not differ from controls in the frequency of circulating CD8$^+$ T lymphocytes, CD19$^+$ B cells, or CD3$^-$/CD16$^+$/CD56$^+$ natural killer cells (all P > 0.10).

Correlation among proinflammatory markers. To determine whether the multiple immune and inflammatory alterations observed in this study might reflect a common underlying immune dysregulation, we evaluated the correlation among these variables. Stimulated monocyte production of TNF-α was positively correlated with stimulated monocyte production of IL-6 (Spearman r = 0.48; P = 0.007). Plasma levels of IL-6 were positively correlated with plasma levels of IL-1ra (r = 0.32; P = 0.035). Circulating concentrations of sIL-6R were inversely correlated with the prevalence of circulating myeloid dendritic cells (r = −0.39; P = 0.033) and the density of IL-6R on the cell surface of monocytes (r = −0.30; P = 0.06).

Biomarker development and performance. Given these correlations between immune and inflammatory markers, we sought to determine whether there is composite biological marker that might capture a common underlying immune dysregulation associated with fatigue in breast cancer survivors. These analyses used an automated stepwise model-building algorithm to combine multiple immunologic variables into a multivariate linear discriminant function predicting fatigue status (nonfatigued versus fatigued). This process began with a set of potential predictors, all immunologic variables that differed significantly across groups (as shown in Table 2), and added individual immunologic variables to the multivariate prediction model based on their incremental value in discriminating fatigued

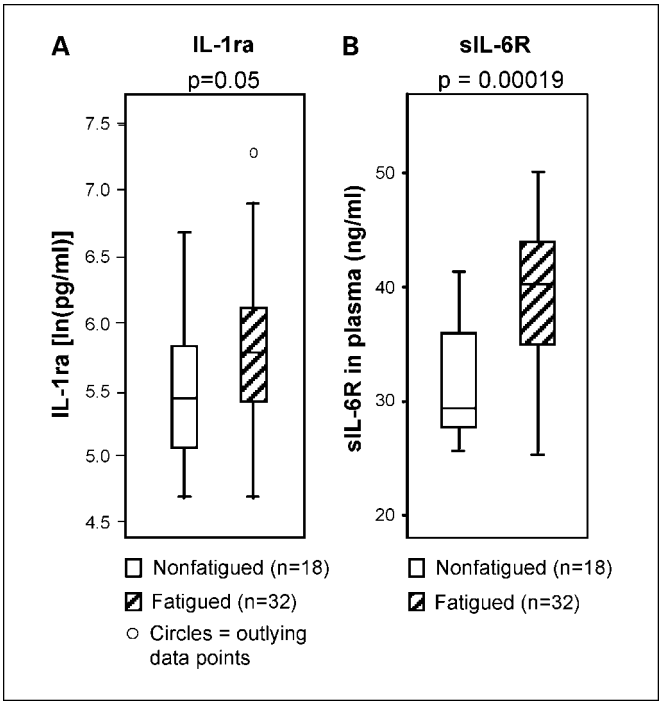


Fig. 1. Circulating inflammatory markers. ELISA analysis of IL-1ra (*A*) and sIL-6R (soluble CD126; *B*) levels in fatigued (*shaded*) and nonfatigued (*clear*) breast cancer survivors. Box-and-whisker plots represent data with boxes ranging from the 25th to 75th percentile of the observed distribution of values, with horizontal bar at the median value. Whiskers span minimum to maximum observed values, with algorithm-defined outliers identified by open circles. Mean of duplicate ELISA determinations on each sample.

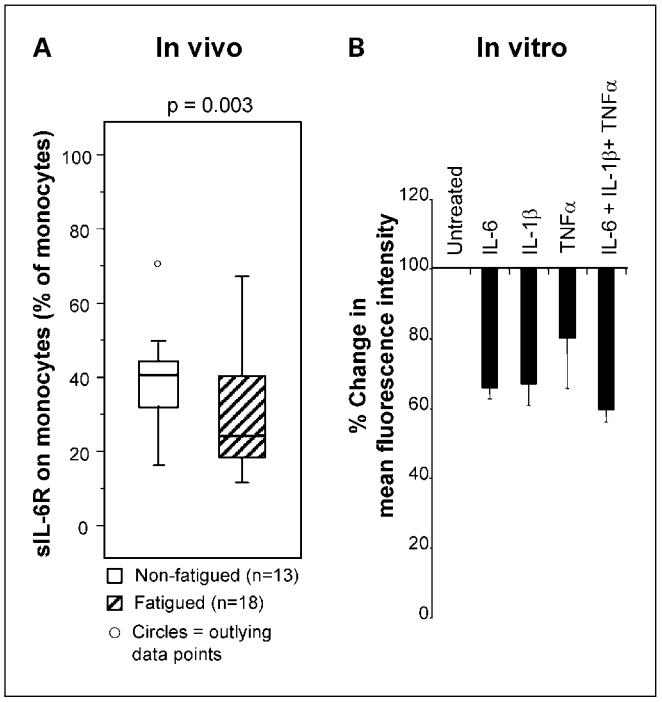
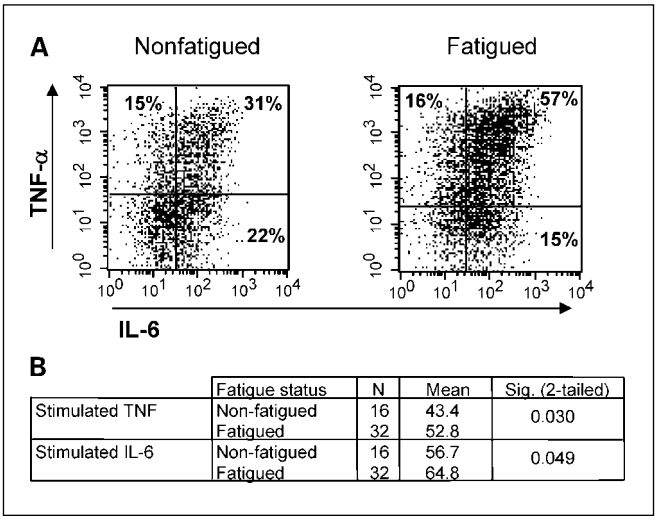


Fig. 2. IL-6R levels *in vivo* and *in vitro*. *A,* cell-surface expression of IL-6R (CD126) was assayed by flow cytometry in peripheral blood monocytes from fatigued (*shaded*) and nonfatigued (*clear*) breast cancer survivors. *B,* to determine whether decreased cell-surface expression of CD126 might reflect cytokine-induced shedding of IL-6R, we carried out *in vitro* experiments in which healthy donor PBMC were exposed to 10 ng/mL IL-6, IL-1β, TNF-α, or all three cytokines for 12 hours. Cell-surface expression of CD126 on $CD3^+$, $CD14^+$, and total PBMC was assessed by flow cytometry. Representative results for PBMC from three independent experiments showed an average 34% decrease in mean fluorescence intensity of cell-surface IL-6R staining on total PBMC treated with IL-6 ($P = 0.00042$), a 33% reduction on PBMC treated with IL-1β ($P = 0.0059$, paired *t* test), and a 40% reduction on cells treated with all three cytokines ($P = 0.004$). Treatment with TNF-α induced a 20% decline in mean fluorescence intensity, but that change did not reach statistical significance due to high variability across replicates ($P = 0.23$). Similar results were observed for $CD3^+$ and $CD14^+$ subsets of PBMC (data not shown).



	Fatigue status	N	Mean	Sig. (2-tailed)
Stimulated TNF	Non-fatigued	16	43.4	0.030
	Fatigued	32	52.8	
Stimulated IL-6	Non-fatigued	16	56.7	0.049
	Fatigued	32	64.8	

Fig. 3. Intracellular cytokine expression. *A,* representative expression of IL-6 and TNF-α in LPS-stimulated $CD14^+$ cells form a nonfatigued and a fatigued participant. Percentages indicate fraction of $CD14^+$ cells that are positive for TNF-α alone (*top left*), TNF-α and IL-6 (*top right*), and IL-6 alone (*bottom right*). *B,* mean and statistical significance of differences between fatigued and nonfatigued breast cancer survivors on frequencies of TNF-α^+ and IL-6^+ monocytes following 4-hour LPS stimulation. Statistical significance was assessed by two-tailed *t* test. (Samples could not be obtained for 2 of the 18 nonfatigued participants due to technical difficulties).

from nonfatigued survivors. The model-building process began with eight potential predictors (Table 2), three of which emerged as statistically significant components: plasma sIL-6R concentration (high on fatigued survivors), monocyte cell-surface IL-6R (low on fatigued survivors), and circulating levels of $CD3^+/CD69^+$ activated T lymphocytes (low on fatigued survivors). A linear discriminant function based on these three variables correlated +0.701 with fatigue classification and accounted for ~50% of the total variability in fatigue status ($R^2 = 0.49$; $P = 0.0005$). The resulting linear discriminant function correctly predicted the fatigue classification of 87% of participants in this study (sensitivity = 83%; specificity = 83%). Holdout cross-validation analyses indicated that this prediction equation should correctly classify 83% of participants in future samples, with similar levels of sensitivity and specificity. Three readily measured cellular and cytokine receptor variables are thus sufficient to identify individuals at high risk of inflammation-related fatigue. Discriminant function coefficients were opposite in sign for cell-surface and plasma sIL-6R (consistent with their inverse correlation reported in Figs. 1 and 2). Similar results emerged from analyses discriminating fatigued and nonfatigued participants based on the ratio of cell-surface to plasma sIL-6R in conjunction with $CD3^+/CD69^+$ cells ($R^2 = 0.518$; $P = 0.0033$). These results suggest that ratio of plasma to cell-surface IL-6R can efficiently discriminate fatigued from nonfatigued breast cancer survivors and that circulating levels of $CD69^+$ activated T lymphocytes can provide an increment to predictive power.

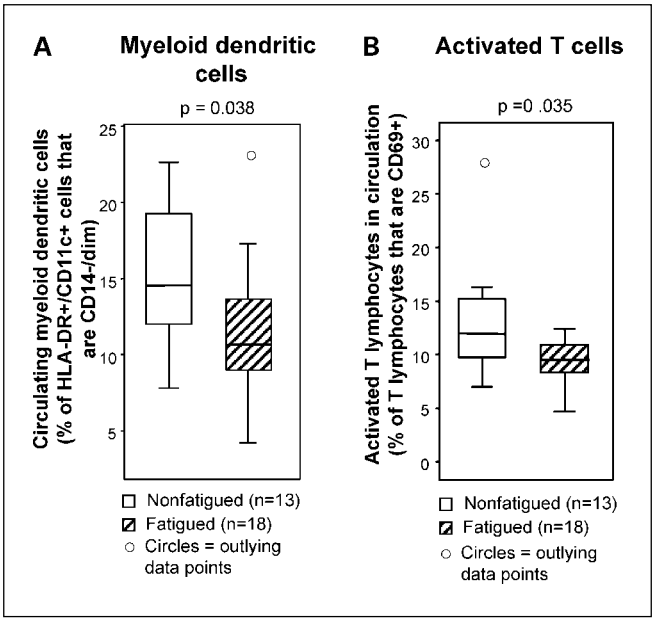


Fig. 4. Cellular immune variables. Relative frequencies of myeloid dendritic cells ($CD14^{-/dim}$ as a % of total $HLA\text{-}DR^+/CD11c^+$ cells; *A*) and activated T lymphocytes ($CD69^+$ cells as a % of total $CD3^+$ lymphocytes; *B*) in fatigued (*shaded*) and nonfatigued (*clear*) breast cancer survivors. Statistical significance was assessed by *t* test.

Discussion

This study provides the first evidence of a functional alteration in innate immune response among breast cancer survivors suffering from persistent unexplained fatigue. LPS ligation of TLR4 triggered significantly greater production of IL-6 and TNF-α in peripheral blood monocyte populations from fatigued survivors relative to a matched cohort of nonfatigued breast cancer survivors. This elevated functional cytokine response was associated with previously observed alterations in plasma inflammatory markers (IL-1ra; ref. 20) as well as newly assessed variables, such as sIL-6R (sIL-6R/CD126). Elevated plasma sIL-6R was accompanied by significant reductions in monocyte cell-surface expression of IL-6R, suggesting that receptor shedding could be a possible mechanism. Consistent with this hypothesis, *in vitro* studies showed loss of cell-surface IL-6R on T lymphocytes, monocytes, and the overall PBMC population following exposure to proinflammatory cytokines (IL-1β, IL-6, and TNF-α). Thus, an increased ratio of plasma sIL-6R to monocyte cell-surface IL-6R serves as an indicator of multiple proinflammatory signaling pathways.

Consistent with a potential inflammatory basis for fatigue, fatigued participants showed increases in the plasma to cellular IL-6R ratio, and these alterations were also associated with both functional measures of *ex vivo* cytokine production and changes in the circulating prevalence of key leukocyte subsets—$CD69^+$ activated T lymphocytes and $HLA\text{-}DR^+/CD11c^+/CD14^{dim}$ myeloid dendritic cells. Interestingly, circulating frequencies of these two cell populations were decreased in fatigued breast cancer survivors possibly due to increased recruitment of these cells to inflammatory tissue sites and consequent depletion from the circulating leukocyte pool. Regardless of the mechanism involved, reductions in activated T-cell levels provided diagnostic information above and beyond that available from increases in the plasma to cellular IL-6R ratio. Together, these variables were highly diagnostic of fatigue measured at the behavioral level. We sought to develop a composite immunologic biomarker for post-treatment fatigue that could be used to identify reliable markers of inflammation related fatigue. Combined assessment of $CD69^+$ T lymphocyte levels and the plasma to cellular IL-6R ratio can provide an efficient summary of the inflammatory signaling activity obtained from the more extensive measurements made in this study (including *ex vivo* functional cytokine production). These results extend links between fatigue and inflammatory markers to show a functional alteration in proinflammatory cytokine response to the TLR4 ligand LPS. These analyses also define a diagnostic biomarker of behavioral fatigue, which should provide a useful surrogate marker for future studies developing interventions to ameliorate fatigue by targeting its inflammatory basis. Confirmatory studies in independent samples will be required to fully validate this measure, but the present results support the general principle that biological markers of inflammation can provide diagnostic information about variations in the risk of adverse behavioral outcomes in the aftermath of breast cancer.

Alterations in cellular immune variables, circulating inflammatory markers, and cytokine response to LPS stimulation were correlated, suggesting that they reflect a common underlying syndrome of pathogenetic alterations. Although the precise nature of that pathogenetic mechanism is not presently known, it is conceivable that it may involve coordinated effects of the neuroendocrine system on immune system function or changes in the activity of pathogens (e.g., reactivation of latent viral infections). The specific nature of those interactions is an important target of future research. Cellular immune changes induced by cancer therapy could potentially persist in the aftermath of treatment due to for example alterations in T-cell homeostasis, replicative potential, and senescence (43, 44), and these changes might potentially underlie long-term alterations in constitutive inflammatory activity. Alternatively, treatment-induced reactivation of latent infections (45) could potentially induce long-term changes in immune system homeostasis. However, in this and previous studies, we found no association between specific cancer treatment modalities and the prevalence or intensity of fatigue or the intensity of inflammatory signaling or cellular immune alterations (20, 21). The sole exception involved a marginally higher level of plasma IL-1ra among breast cancer survivors who had used tamoxifen/aromatase inhibitor. However, tamoxifen/aromatase inhibitor use was not associated with any of the other immunologic or inflammatory alterations linked to persistent fatigue in this study. In particular, tamoxifen/aromatase inhibitor treatment was not significantly associated with the altered plasma to cellular IL-6R ratio and $CD69^+$ T lymphocyte frequencies that emerged as the most prognostic biomarkers of fatigue in these analyses.

The emergence of cellular and circulating IL-6R levels, as prominent biological markers of fatigue, are consistent with previous data suggesting a key role of IL-6/IL-6R interactions in other inflammatory conditions. Variations in circulating IL-6R

Table 2. Summary of immunologic variables significantly different across groups

Variable	Fatigued	Nonfatigued	*P*
Circulating variables			
sIL-6R	40,109 ± 1,633	30,612 ± 1,220	<0.0001
$CD3^+/CD69^+$	8.98 ± 0.61	12.24 ± 1.65	0.046
$CD126^+/CD14^{bright}$	33.17 ± 5.96	55.06 ± 5.33	0.011
$HLADR^+/CD11c^+/CD14^{dim}$	11.22 ± 1.24	14.98 ± 1.31	0.047
IL-1ra (ln)	5.7 ± 0.09	5.4 ± 0.12	0.054
Stimulated production			
Stimulated TNF-α	52.80 ± 2.80	43.49 ± 3.03	0.030
Stimulated IL-6	64.80 ± 2.83	56.77 ± 2.77	0.049

are associated with changes in IL-6 expression and alterations in cellular response to IL-6 (26). The sIL-6R can induce IL-6 responsiveness in cell types that do not express cell-surface IL-6R, and circulating sIL-6R can thus extend the range and effect of IL-6 as a proinflammatory cytokine (41). IL-6 and IL-6R play a critical role in many disease states, including cancer, where elevations in these variables are associated with metastatic stage and poor prognosis in many cancer types (41, 46, 47). In addition to contributing to the basic inflammatory pathogenesis of persistent fatigue, our results suggest that IL-6 regulation may also provide a useful predictive marker for monitoring generalized inflammatory activity in cancer survivors. Finally, elevations of sIL-6R or IL-6 trans-signaling may serve a critical mechanism by which IL-6 integrates actions between immune and central nervous functions (48).

Consistent with our hypothesis that inflammatory alterations underlie persistent unexplained fatigue in breast cancer survivors, fatigued participants in this study showed enhanced cellular responsiveness to stimulation of the TLR4/CD14 signaling pathway *ex vivo*. The functional and molecular basis for this difference is not yet known, although previous studies have suggested that alterations in cell-surface TLR4 receptor levels may contribute to age-related differences in cytokine response to LPS (49). However, other studies have shown age-related alterations in LPS response that do not involve changes in TLR4 receptor levels, suggesting that signaling processes lying downstream of the receptor may also be altered (50). Effects of age on TLR4 signaling are not likely to explain the effects observed here because fatigued and nonfatigued breast cancer survivors were not different in age. However, it is possible that the same types of functional alteration that occur during normal aging might contribute the differences associated with fatigue in this study. In this sense, cancer-related fatigue might stem from an aberrant "aging" process triggered by cancer therapy. Altered expression and signaling of TLRs have been recently studied in relationship to other chronic inflammatory diseases, including rheumatoid arthritis and Crohn's disease (27). The present data suggest that similar alterations in expression and signaling of TLRs might play a role in cancer-related fatigue. It is unclear whether the effects observed here are specific to the activation of TLR4 by LPS or whether other pathogen motif recognition pathways might also be altered (e.g., viral activation of TLR3 and TLR2). Direct analysis of TLR3 expression and signaling, and the distribution and activity of other TLRs, will provide important avenues for clarifying the basis for aberrant inflammatory signaling in fatigued cancer survivors. Such analyses will also help define specific molecular targets for interventions that ameliorate fatigue by addressing its inflammatory basis. Development of such therapies could markedly enhance quality of life in the significant fraction of breast cancer survivors that suffer from persistent fatigue, and this study's identification of a strong biomarker of fatigue-related inflammation provides a crucial methodologic step forward.

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