Cancer Research

IL6 Signaling in Peripheral Blood T Cells Predicts Clinical Outcome in Breast Cancer

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Abstract

IL6 is a pleiotropic cytokine with both pro- and anti-inflammatory properties, which acts directly on cancer cells to promote their survival and proliferation. Elevated serum IL6 levels negatively correlate with survival of cancer patients, which is generally attributed to the direct effects of IL6 on cancer cells. How IL6 modulates the host immune response in cancer patients is unclear. Here, we show the IL6 signaling response in peripheral blood T cells is impaired in breast cancer patients and is associated with blunted Th17 differentiation. The mechanism identified involved downregulation of gp130 and IL6Rα in breast cancer patients and was independent of plasma IL6 levels. Importantly, defective IL6 signaling in peripheral blood T cells at diagnosis correlated with worse relapse-free survival. These results indicate that intact IL6 signaling in T cells is important for controlling cancer progression. Furthermore, they highlight a potential for IL6 signaling response in peripheral blood T cells at diagnosis as a predictive biomarker for clinical outcome of breast cancer patients. *Cancer Res; 77(5); 1119–26. ©2016 AACR.*

Introduction

IL6 is a pleiotropic cytokine that plays various roles on modulating the activities of tumor and immune cells (1). IL6 signals through the common gp130 receptor and the specific IL6Rα coreceptor to activate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway (2). Phosphorylated STATs dimerize and translocate into the nucleus to initiate transcription of IL6 responsive genes (3).

Within the tumor microenvironment, not only macrophages, myeloid-derived suppressor cells (MDSC) and fibroblasts but also cancer cells produce IL6 (4). IL6 promotes survival and proliferation of cancer cells, drives chronic inflammation that supports tumor growth, and suppresses antitumor T-cell activity (4–8). On T cells, IL6 functions to prevent apoptosis (9, 10) and skews naïve $CD4^+$ T cells away from becoming regulatory T (Treg) cells and toward becoming pro-inflammatory Th17 cells (11). IL6 also regulates chemokine receptors expression to influence tissue recruitment of T cells (12). Higher frequencies of Treg and exclusion of cytotoxic T cells from the tumor are both associated with poorer outcomes in cancer patients (13, 14). Thus, alterations in the response of T cells to IL6 may contribute to deficient antitumor responses. However, the mechanisms connecting IL6-associated inflammation to dysfunctional antitumor immune responses have yet to be fully elucidated. Alterations in cytokine levels and the ability of immune cells to appropriately respond to cytokines are likely to contribute to immunologic abnormalities in cancer patients. The prominent association of IL6 with both inflammation and cancer argues that this pleiotropic immunomodulatory cytokine might serve as a link between cancer-associated inflammation and immune dysfunction

In this regard, we investigated the functionality of IL6 signaling responses in peripheral blood T cells of breast cancer patients. By using phosphoflow cytometry, we found that IL6-induced phosphorylation of STAT1 and STAT3 were significantly lower in peripheral blood $CD4^+$ naïve T cells from breast cancer patients at diagnosis. To explore the mechanisms underlying defective responses of patient T cells to IL6, expression levels of key components of the IL6 signaling pathway were evaluated. Breast cancer patients had substantially decreased levels of the IL6 coreceptors, gp130 and IL6Rα, which further correlated with decreased responsiveness to IL6. Interestingly, IL6 plasma levels were not elevated in breast cancer patients at diagnosis and IL6 signaling responses were independent of the IL6 plasma levels. We also found that defective IL6 responses were associated with blunted Th17 differentiation from $CD4^+$ naïve T cells. More importantly, defective IL6 signaling response significantly correlated with worse relapse-free survival (RFS), indicating the potential of IL6 signaling response in peripheral blood T cells at diagnosis as a prognostic biomarker for breast cancer patients.

Materials and Methods

Patients

All patient blood samples were collected before surgery or administration of any therapy. Age-matched healthy control

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peripheral blood samples were obtained from the Stanford Blood Center and City of Hope Blood Donor Center. All the blood from patients and healthy donors was drawn directly into heparin-coated vacutainer tubes (BD Biosciences). This study was approved by the Institutional Review Board of Stanford Medical Center and City of Hope Comprehensive Cancer Center.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were collected by density gradient centrifugation using Ficoll-Paque and cryopreserved in 10% DMSO FBS. Cryopreserved PBMCs were thawed and rested overnight in RPMI-10% heat-inactivated FBS-1x Penicillin-Streptomycin-Glutamine at 37°C, 7.5% CO_2 before performing assays.

IL6 stimulation of PBMCs for phosphoflow cytometry

Briefly, 0.5×10^6 PBMCs were aliquoted into individual wells of deep-well 96-well plates. Media or IL6 (R&D Systems) was added to each well to obtain a final concentration of 100 ng/mL for IL6. Cells were then incubated at 37°C for 15 minutes followed by fixation with 1.5% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were washed with PBS to remove PFA, and permeabilized by the addition of 100% methanol. PBMCs were stored at −80°C until antibody staining for flow cytometry analysis.

Flow cytometry

Permeabilized cells were thawed and washed three times with staining buffer (PBS/2% FBS/0.5% BSA) to remove methanol. Cells were resuspended in the same volume of staining buffer and staining antibodies were added. For assessment of pSTAT1 and pSTAT3, the following staining panel was used: CD3-V450 (UCHT1), CD4-PerCP-Cy5.5 (SK.3), CD45RA-PE-Cy7 (L48), CD8-V500 (RPA-T8), CD16-PE (3G8), CD20-PerCP-Cy5.5 (H1), CD33-PE-Cy7 (P67.6), pSTAT1 (pY701)-AF647 (4a), and pSTAT3 (pY705)-AF488 (4/P-STAT3) antibodies (BD Biosciences). Naïve $CD4^+$ T-cell population was determined by the following markers: $CD3^+CD4^+CD45RA^+$. The magnitude of each individual's pSTAT1 and pSTAT3 response to IL6 was expressed as the IL6 induced median fluorescence intensity (MFI) minus the unstimulated MFI for pSTAT1 and pSTAT3.

For assessment of intracellular total STAT1 and STAT3 levels, LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies) was added to unstimulated cells for 30 minutes and washed away before PFA fixation and permeabilization. After washing the cells to remove methanol, the following staining panel was used: CD3-V500 (UCHT1), CD4-PerCP-Cy5.5 (SK.3), CD8-PacBlue (RPA-T8), CD45RA-PE-Cy7 (L48), CD62L-FITC (DREG-56), STAT1-AF647 (1/Stat1), and STAT3-PE (M59-50) antibodies (BD Biosciences). For assessment of gp130 and IL6Rα expression levels on live PBMCs, the following staining panel was used: CD3-V500 (UCHT1), CD4-PerCP-Cy5.5 (SK.3), CD8-PacBlue (RPA-T8), CD45RA-PE-Cy7 (L48), CD62L-e605NC (DREG-56), gp130-PE (AM64; BD Biosciences), and IL6Rα-AF647 (BL-126; Biolegend) antibodies, and LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies). Expression of total STAT1, STAT3, gp130 and IL6Rα was expressed by subtracting the MFI of isotype stains.

Flow cytometry was performed using FACS Canto, LSR II, or Fortessa Flow Cytometers (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc.). The *t* tests were used to determine the statistical significance of breast cancer patient with healthy donors (GraphPad Prism, GraphPad Software).

Plasma IL6 ELISA

All patient plasma samples were collected before surgery or administration of any therapy. Plasma samples were kept frozen at −80°C then thawed shortly before determination of IL6 level. IL6 levels were determined by high sensitivity ELISA (eBioscience) according to the manufacturer's protocol.

RNA isolation and quantitative real-time PCR

Naïve $CD4^+$ T cells were isolated from PBMCs with an enrichment kit (eBioscience). Total RNA was isolated from naïve $CD4^+$ T cells using RNAzol RT reagent (Molecular Research Center) according to the manufacturer's instruction. cDNA was synthesized using RT^2 First Strand Kit (Qiagen). For quantitative real-time PCR (qPCR), RT^2 SYBR Green Master Mix (Qiagen) was used and qPCR was performed and analyzed using the CFX96 Real-Time PCR System (Bio-Rad). Gene expressions were normalized to *GAPDH* as an internal control and results are represented as fold change using the $\Delta\Delta C_t$ method. The following primer sequences were used in the reaction: *Il6r* (F: TTGTTTGTGAGTGGGGTCCT; R: TGGGACTCCTGGGAATACTG), *Il6st* (F: AGGACCAAAGATGCCTCAAC; R: GAATGAAGATCGGGTGGATG), *Adam17* (F: ACTCTGAGGACAGTTAACCAAACC; R: AGTAAAAGGAGCCAATACCACAAG).

Th17 differentiation assay

Naïve $CD4^+$ T cells were isolated from PBMCs with an enrichment kit (eBioscience). Cells were cultured in serum-free medium with anti-CD3/CD28 beads (Life Technologies), IL6 (30 ng/mL), IL1β (20ng/mL; eBioscience), IL23 (30ng/mL), TGF-β (2.25 ng/mL; Peprotech), anti–IFN-γ antibody (1 μg/mL) and anti-IL4 antibody (2.5 μg/mL; Biolegend) for 7 days. Supernatants were collected after 7 days and IL17 levels were determined by ELISA (Biolegend). Cells were stimulated with ionomycin (1 μg/mL; Life Technologies), phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich) and brefeldin A (BFA; 5 μg/mL; Biolegend) for 5 hours and were analyzed by flow cytometry with anti-RORγt-AF647 (Q21-559; BD Biosciences) and anti-IL17A-FITC (BL168; Biolegend) antibodies.

Statistical analysis

RFS was defined as the time from the date of diagnosis of breast cancer to the date of cancer recurrence. The Kaplan–Meier method with log-rank test was used to determine IL6 signaling responsiveness as prognostic factors for RFS of breast cancer patients. Multivariate Cox regression model analysis was performed to determine independence of prognostic factor. The correlation between IL6 signaling response and clinicopathologic characteristics were evaluated with Pearson correlation coefficient presented with *r* and *P* values. All tests with a *P* value of <0.05 were considered statistically significant.

Results

Defective IL6 signaling responses in peripheral $CD4^+$ T cells from breast cancer patients

To investigate IL6 immune biology in breast cancer patients, we analyzed the responsiveness of peripheral blood immune cells to IL6 in breast cancer patients and age-matched healthy donors. Clinical and pathological characteristics of the breast cancer patients are summarized in Table 1. PBMCs from breast cancer patients and healthy donors were stimulated with IL6 and phosphorylation of STAT1 and STAT3 (pSTATs) were determined by phosphoflow cytometry (15). IL6 signaling response (ΔMFI) was

represented by IL6 stimulated minus unstimulated pSTATs median fluorescence intensity (MFI; Fig. 1A). We examined IL6 signaling response in T cells ($CD3^+$), B cells ($CD20^+$), NK cells ($CD16^+$) and myeloid cells ($CD33^+$) and found that IL6 induced phosphorylation of STAT1 ($P = 0.003$) and STAT3 ($P = 0.0004$) in naïve $CD4^+$ T cells from breast cancer patients ($n = 57$) was significantly lower than that in healthy donors ($n = 26$; Fig. 1B). To determine whether the observed lower IL6 signaling response was due to reduced total available STATs, we compared the levels of total STAT1 and STAT3 in naïve $CD4^+$ T cells by flow cytometry and found similar levels of total STAT1 and STAT3 between breast cancer patients and healthy donors (Supplementary Fig. S1A). In addition, we found similar levels of basal pSTAT1 and pSTAT3 in naïve $CD4^+$ T cells between breast cancer patients and healthy donors (Supplementary Fig. S1B). In cancer cells, STAT1 and STAT3 are considered to play opposing roles in tumorigenesis where STAT3 is tumor-promoting and STAT1 is tumor-inhibiting (16). In contrast, we found that IL6-induced phosphorylation of STAT1 and STAT3 are highly correlated in T cells (Fig. 1C), indicating that the IL6–STAT pathway is coordinately dysfunctional in breast cancer patients.

Elevated circulating IL-6 levels have been observed in advanced metastatic breast cancer patients, which negatively correlate with patient outcome (17, 18). To investigate whether impaired IL6 signaling responses observed in breast cancer naïve $CD4^+$ T cells were related to soluble IL6 levels, we compared plasma IL6 levels between breast cancer patients ($n = 70$) and age-matched healthy donors ($n = 66$) by ELISA. All plasma samples from breast cancer patients were collected at diagnosis before surgery or any therapy. Interestingly, we found that plasma IL6 levels were not significantly elevated in this cohort of breast cancer patients (mean 4.2 pg/mL, median 0 pg/mL) as compared with healthy donors (mean 2.0 pg/mL, median 0.25 pg/mL; Fig. 2A). As normal plasma IL6 levels are generally in the range of 0–2 pg/mL (19), we further categorized patients' plasma IL6 levels into three ranges (0–2 pg/mL, 2–10 pg/mL, >10 pg/mL) and found similar distributions between breast cancer patients and healthy donors (Fig. 2B). Importantly, we compared the IL6 signaling responses in T cells between healthy donors and breast cancer patients who had normal IL6 plasma levels (0–2 pg/mL) at diagnosis. IL6-induced phosphorylation of STAT1 ($P = 0.04$) and STAT3 ($P = 0.008$) in naïve $CD4^+$ T cells from breast cancer patients was still significantly lower than that in healthy donors even though they all had plasma IL6 levels in the normal range (Fig. 2C). Moreover, we found no significant correlation between plasma IL6 levels and IL6 induced pSTATs in T cells (Fig. 2D). To investigate whether the impaired IL6 signaling response was caused by reduced levels of the IL6 receptor complex, we compared the cell surface levels of IL6Rα and gp130 in naïve $CD4^+$ T cells between breast cancer patients and healthy donors by flow cytometry. Indeed, we found that IL6Rα ($P = 0.05$) and gp130 ($P = 0.03$) levels were both lower in breast cancer patients than in healthy donors (Fig. 3A). In addition, IL6 induced pSTATs significantly correlate with the level of IL6Rα plus gp130 (pSTAT1: $P = 0.0005$; pSTAT3: $P = 0.0009$; Fig. 3B). To address whether these changes were regulated at the transcriptional level, we measured the mRNA levels of IL6Rα and gp130 in $CD4^+$ naïve T cells by qPCR. Indeed, mRNA levels of gp130 (*Il6st*; $P = 0.04$) were significantly lower in T cells from breast cancer patients ($n = 4$) than in healthy donors ($n = 4$), but not IL6Rα (*Il6r*; Fig. 3C). IL6Rα on the cell surface is known to be subjected to proteolytic cleavage by a metallopeptidase ADAM 17 (20). Intriguingly, we found that mRNA levels of ADAM17 were significantly higher ($P = 0.03$) in T cells from breast cancer patients than healthy donors (Fig. 3C). These data indicate that impaired IL6 signaling responses in T cells from breast cancer patients are caused by reductions in both chains of the IL6 receptor complex via two distinct mechanisms: gp130 via reduced transcription, and IL6Rα via enhanced cleavage by ADAM17.

Because IL6 is critical for Th17 differentiation (11), we examined whether dysfunctional IL6 signaling responses in naïve T cells from breast cancer patients were associated with impaired Th17 differentiation. Naïve $CD4^+$ T cells were isolated from fresh PBMCs and cultured in Th17 differentiation medium for 7 days. Breast cancer patient samples ($n = 7$) exhibited fewer differentiated Th17 cells ($ROR\gamma t^+IL17A^+$; $P = 0.02$; Fig. 3D) with lower IL17 secretion levels ($P = 0.04$; Fig. 3E) than age-matched healthy donors ($n = 9$). Among the breast cancer patients, IL6 induced pSTATs significantly correlated with levels of IL17 production (pSTAT1: $P = 0.001$; pSTAT3: $P = 0.03$; Fig. 3F).

Table 1. Patient characteristics

Characteristics	Patients ($N = 57$)
Age, y	
Median	51
Range	27–85
Tumor stage *n* (%)	
DCIS	7 (12.3)
T1	23 (40.4)
T2	15 (26.3)
T3	8 (14.0)
Unknown	4 (7.0)
Grade *n* (%)	
G1	7 (12.3)
G2	22 (38.6)
G3	28 (49.1)
Nodal status *n* (%)	
N0	29 (50.9)
N1–2	24 (42.1)
Unknown	4 (7.0)
Subtype, *n* (%)	
Luminal	45 (79.0)
HER2	6 (10.5)
Triple negative	6 (10.5)

IL6 signaling responses in peripheral $CD4^+$ T cells as prognostic marker

To evaluate the clinical significance of IL6 signaling responsiveness, we compared the IL6 induced pSTATs responses in peripheral naïve $CD4^+$ T cells between relapsed and nonrelapsed breast cancer patients. Only patients with blood collected at diagnosis before surgery or any therapy who had been clinically followed for at least 36 months were selected for this analysis. The median follow-up time of breast cancer patients ($n = 40$) was 63 months (range, 17–92 months). We found that IL6 induced phosphorylation of STAT1 ($P = 0.0003$) and STAT3 ($P = 0.0001$) in peripheral blood naïve T cells at diagnosis were significantly lower in patients who went on to relapse than those who remained disease-free (Fig. 4A). Kaplan–Meier survival analysis was performed to determine the relationship between IL6 signaling responses and RFS. To divide breast cancer patients ($n = 40$) into two populations in an unbiased way, median ΔMFI of IL6-induced pSTAT1 or pSTAT3 was used as the cutoff value. Breast cancer patients with pSTAT1 ($P = 0.004$) or pSTAT3 ($P = 0.005$) ΔMFI below the median ($n = 20$) had significantly worse RFS than those above the median ΔMFI ($n = 20$; Fig. 4B),

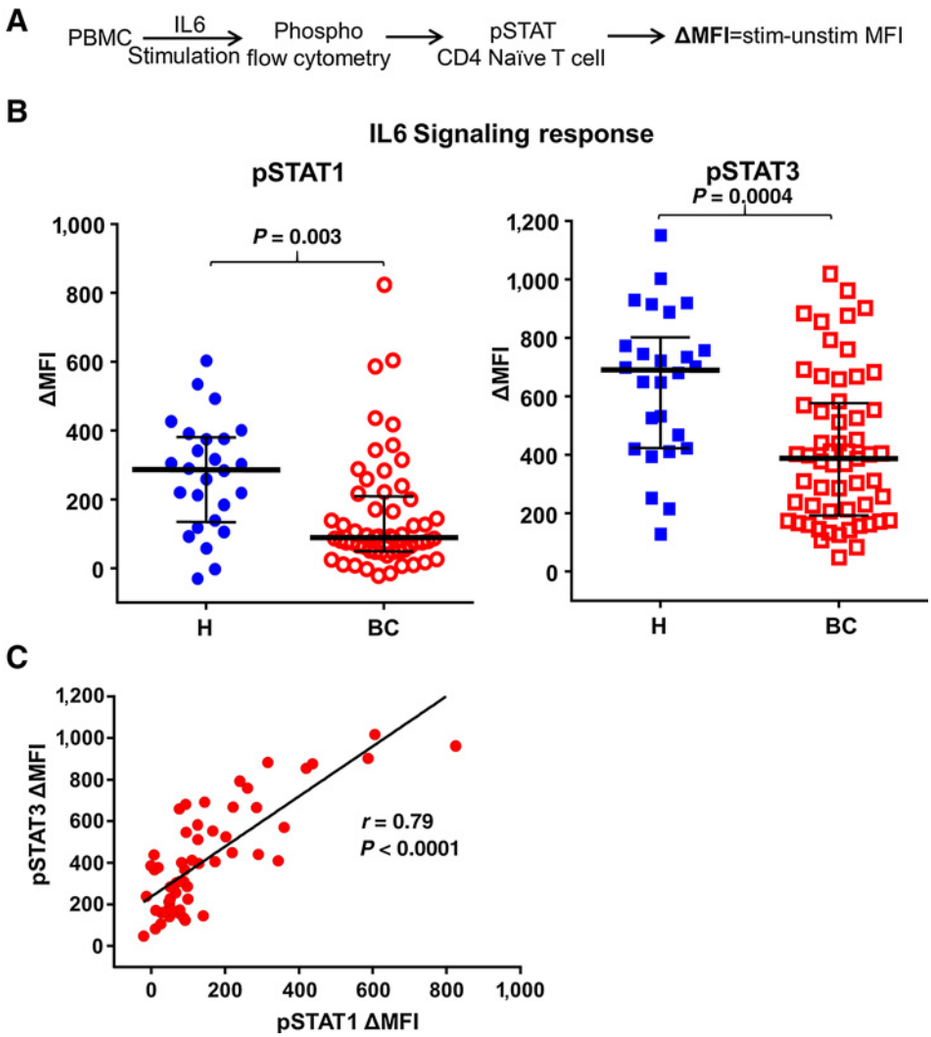

Figure 1.
IL6 signaling responses are impaired in peripheral naïve CD4$^+$ T cells from breast cancer patients. **A,** Schematic representation of the experimental overview. PBMCs obtained from breast cancer patients (BC) and from healthy donors (H) were stimulated with IL6 at 100 ng/mL for 15 minutes. IL6-induced phosphorylation of STAT1 and STAT3 (pSTAT) in naïve CD4$^+$ T cells (CD3$^+$CD4$^+$CD45RA$^+$) was determined by phosphoflow cytometry with anti-pSTAT1 (pY701) and anti-pSTAT3 (pY705) antibodies. IL6 signaling responses are represented by ΔMFI, which is the IL6 stimulated MFI minus unstimulated MFI of pSTAT1 or pSTAT3. **B,** IL6-induced phosphorylation of STAT1 ($P = 0.003$) and STAT3 ($P = 0.0004$) in peripheral naïve CD4$^+$ T cells was compared between breast cancer patients ($n = 57$, median age 51; range 27-85) and age-matched healthy donors ($n = 26$, median age 53; range, 30–72). Unpaired t test. **C,** The association between IL6-induced pSTAT1 and pSTAT3 in naïve CD4$^+$ T cells from breast cancer patients was determined by Pearson correlation coefficient test ($r = 0.79$; $P < 0.0001$).

indicating that lower IL6 signaling responses predict worse RFS. Intriguingly, none of the patients with IL6 signaling responses above the median experienced relapse over 100 months (Fig. 4B). To understand whether the IL6 signaling response changes over time among relapsed breast cancer patients, we compared the IL6 signaling response between patients with blood collected at diagnosis ($n = 7$) versus at time of relapse ($n = 7$) and found no significant difference (Supplementary Fig. S2). We also examined IL6 signaling responses in patients who achieved remission after relapse ($n = 5$). There was a trend toward higher IL6 induced pSTAT1 ($P = 0.1$) and pSTAT3 ($P = 0.2$) responses in some relapsed patients who achieved remission (Supplementary Fig. S2), indicating that impaired IL6 signaling in T cells is a persistent defect during cancer progression but may return to normal in some relapsed patients who achieved remission. In a multivariate analysis adjusted for age, tumor stage, grade, nodal status and subtype of breast cancer patients, IL6-induced phosphorylation of STAT1 ($P = 0.001$) or STAT3 ($P = 0.005$) still retained the prognostic significance for RFS, indicating that IL6 signaling responses could be a predictor of clinical outcome independent of these clinicopathologic characteristics (Table 2). The associations between IL6 signaling response in T cells and clinicopathologic characteristics of breast cancer patients were also evaluated and no significant correlations were found between IL6 signaling responses and age, tumor stage, grade, T status or subtype (Supplementary Table S1). Therefore, these findings suggest that IL6 signaling responsiveness in peripheral naïve CD4$^+$ T cells could potentially be developed into a prognostic blood test to predict the clinical outcome of breast cancer patients.

Discussion

IL6 is an inflammation-associated cytokine produced primarily by tumor cells, tumor stroma and tumor-associated myeloid cells (21). Despite the well documented role for the IL6–STAT3 axis in promoting tumor growth through its direct activities on tumor cells, little is known about the role IL6 plays in immune modulation in cancer patients. To interrogate the effects of IL6 on cancer immune biology, STAT signaling responses to IL6 were examined in breast cancer patient PBMC populations. In response to IL6, compared with healthy individuals, T cells from breast cancer patients were found to be defective in their ability to phosphorylate both STAT1 and STAT3. We also found lower IL6 signaling response in CD4$^+$ naïve T cells from melanoma (Mel), gastrointestinal (GI) and

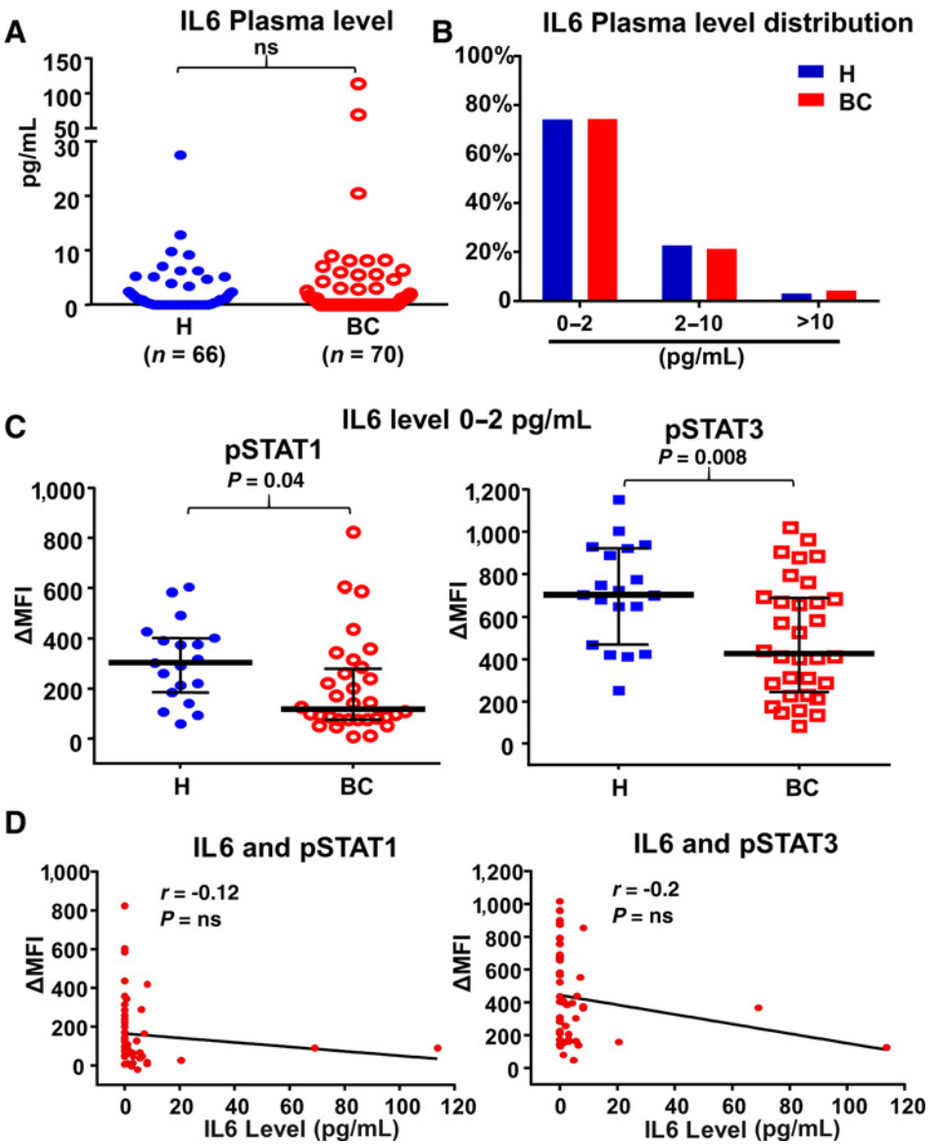

Figure 2.

Impaired IL6 signaling responses in naïve CD4$^+$ T cells are not correlated with IL6 plasma levels. **A,** IL6 plasma levels in healthy donors (mean 2.0 pg/mL; median 0.25 pg/mL) and breast cancer patients (mean 4.2 pg/mL; median 0 pg/mL) were determined by ELISA. Age-matched healthy donors ($n = 66$, median age 58; range, 18–72) were compared with breast cancer patients ($n = 70$, median age 50; range, 27–85). All breast cancer patient plasma was collected at diagnosis before surgery or any therapy. **B,** IL6 plasma level distributions (subdivided into 0–2 pg/mL, 2–10 pg/mL, and >10 pg/mL) in the healthy donors and breast cancer patients. **C,** Among the healthy donors and breast cancer patients with normal IL6 plasma levels (0–2 pg/mL), IL6-induced phosphorylation of STAT1 ($P = 0.04$) and STAT3 ($P = 0.008$) in peripheral naïve CD4$^+$ T cells was compared. **D,** The relationship between IL6 plasma levels and IL6-induced pSTAT1 and pSTAT3 in naïve CD4$^+$ T cells from the breast cancer patients was examined by Pearson correlation coefficient test; ns, not significant.

lung cancer (LC) patients (Supplementary Fig. S3), suggesting that dysregulated IL6 signaling in peripheral blood T cells may be a more general phenomenon in cancer patients. Importantly, there was lower individual and combined expression of the IL6 receptor complex components, gp130 and IL6Rα, in T cells from breast cancer patients compared with healthy controls. Thus, modulation of IL6 pathway regulators, particularly the lower expression of the IL6R complex, contributes to the loss of IL6 responsiveness in breast cancer patient immune cells.

The tumor microenvironment is considered to be a chronically inflamed setting. IL6 is systemically upregulated in cancer and IL6 levels negatively associate with the survival of patients with various cancer types (17, 22–24). In healthy adults, IL6 circulation levels over 10 pg/mL are considered abnormally elevated (21). Our findings that IL6 signaling responses were defective in breast cancer patients with normal IL6 plasma level suggest that IL6-related immune function could be dysregulated in cancer patients with normal IL6 circulation level.

Within the tumor microenvironment, IL6 is well-established as a pro-tumor cytokine and high expression levels of IL6 are found within human breast cancer tumors (25–27). Previous studies demonstrated that chronic exposure to IL6 causes reduced levels of gp130 on T cells (28–30). It was also reported that steroid hormones were able to affect IL6 signaling pathway (31). Although our data showed that defective IL6 signaling response was not dependent on serum IL6 level, it is possible that trafficking through the tumor region with high local IL6 levels may be sufficient to cause IL6 receptor downregulation in T cells from breast cancer patients. Intriguingly, the two chains of the IL6 receptor complex were reduced via two distinct mechanisms: gp130 via reduced transcription, and IL6Rα via enhanced cleavage by ADAM17. gp130 cytokines have pleiotropic roles in immune cell functions while the effects of gp130 deficiencies in the immune compartment in cancer models have not to our knowledge been studied. Thus, downregulation of gp130 expression will likely result in loss of the pleiotropic balance of gp130

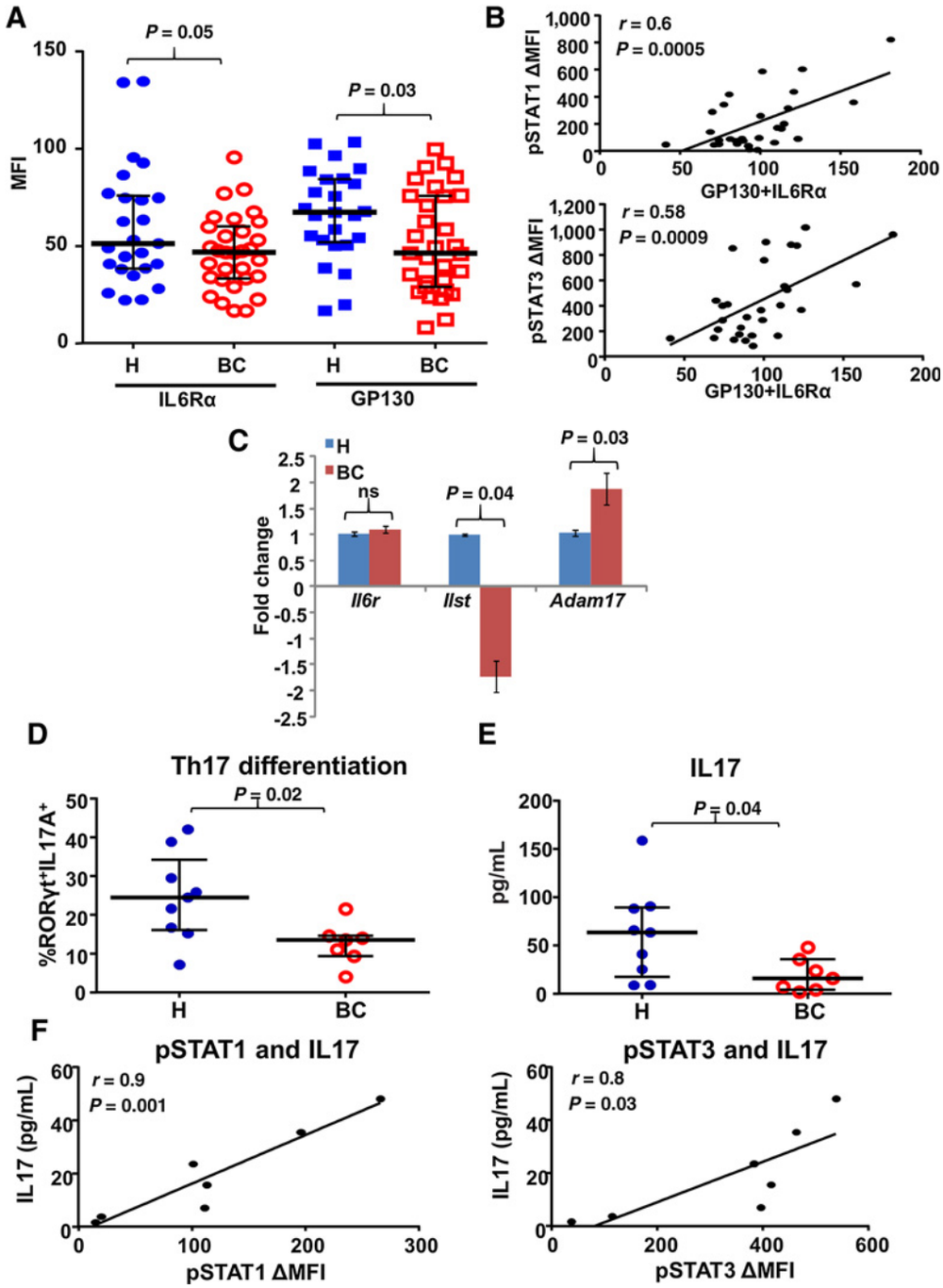

Figure 3.

Impaired IL6 signaling responses in naïve CD4$^+$ T cells are associated with lower IL6 receptor levels and defective Th17 differentiation. **A,** Surface expression levels of IL6Rα ($P = 0.05$) and gp130 ($P = 0.03$) on naïve CD4$^+$ T cells from healthy donors ($n = 25$) and breast cancer patients ($n = 31$) were determined by flow cytometry with anti-IL6Rα and anti-gp130 antibodies. **B,** The associations between IL6-induced pSTATs and the expression levels of gp130 plus IL6Rα were determined by Pearson correlation coefficient test (pSTAT1: $r = 0.6$, $P = 0.0005$; pSTAT3: $r = 0.58$, $P = 0.0009$). **C,** Total RNA was extracted from isolated CD4$^+$ naïve T cells and analyzed for the relative fold change by qPCR. mRNA levels of IL6Rα (*Il6r*; $P =$ ns, nonsignificant), gp130 (*Il6st*; $P = 0.04$), and ADAM17 (*Adam17*; $P = 0.03$) were compared between healthy donors ($n = 4$) and breast cancer patients ($n = 4$). **D,** Naïve CD4$^+$ T cells were isolated from fresh PBMCs and were cultured in Th17 differentiation medium for 7 days. RORγt$^+$IL17A$^+$ cells identified Th17 cells by flow cytometry. The percentages of differentiated Th17 cells were compared between breast cancer patients ($n = 7$) and age-matched healthy donors ($n = 8$). ($P = 0.02$). **E,** Supernatants were collected after 7 days of Th17 differentiation and the levels of IL17 were determined by ELISA (pg/mL/1 × 10^6 cells). The levels of IL17 were compared between breast cancer patients ($n = 7$) and age-matched healthy donors ($n = 9$). ($P = 0.04$). **F,** Among the breast cancer patients ($n = 7$), the associations between IL6-induced pSTATs and level of IL17 were determined by Pearson correlation coefficient test. (pSTAT1: $r = 0.9$, $P = 0.001$; pSTAT3: $r = 0.8$, $P = 0.03$). All the blood from breast cancer patients were collected at diagnosis before surgery or any therapy.

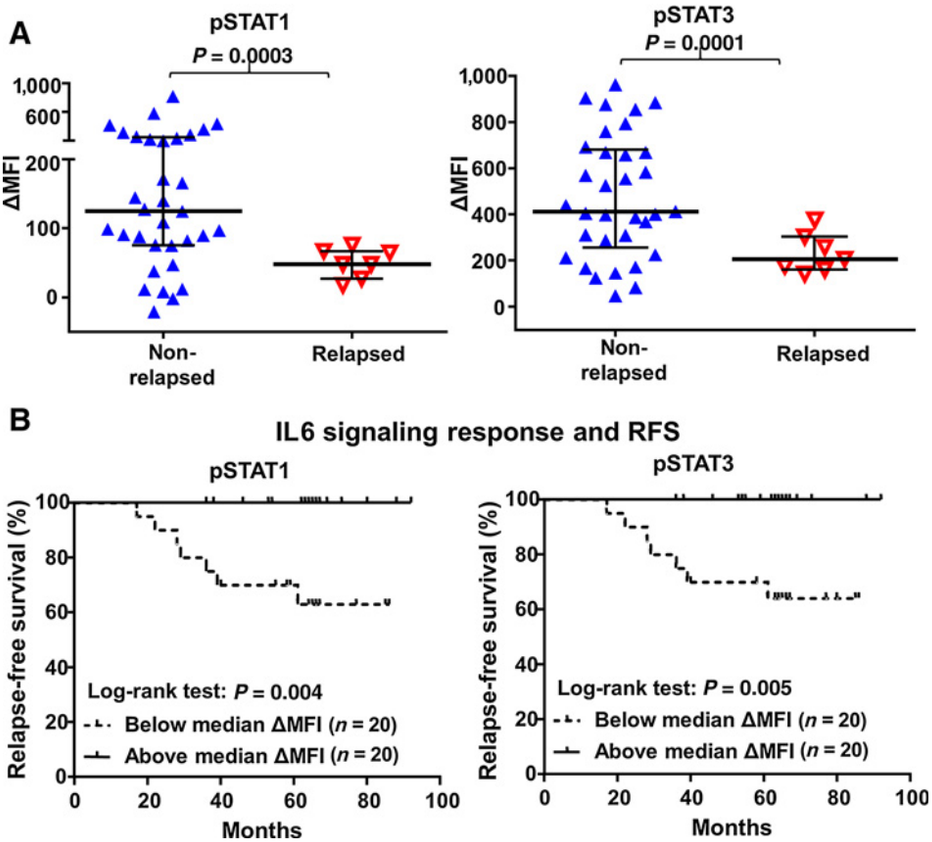

Figure 4.
IL6 signaling responses in peripheral blood CD4$^+$ naïve T cells at diagnosis are correlated with clinical outcome. **A,** IL6-induced phosphorylation of STAT1 ($P = 0.0003$) and STAT3 ($P = 0.0001$) in peripheral naïve CD4$^+$ T cells was compared between the nonrelapsed and relapsed breast cancer patients, all of whom had been clinically followed for at least 36 months. All the blood from breast cancer patients were collected at diagnosis before surgery or any therapy. **B,** Kaplan–Meier survival analysis was performed to compare RFS between breast cancer patients with lower and higher IL6 signaling response (pSTAT1, $P = 0.004$; pSTAT3, $P = 0.005$). The median IL6-induced phosphorylation of STAT1 or STAT3 (ΔMFI) was used as the cutoff value to divide breast cancer patients into lower and higher IL6 signaling response groups.

cytokine responses in immune cells, the outcome of which will also depend on the integration of responses to other differentially expressed cytokines and aberrant signaling pathways.

IL6 functions include promoting T-cell survival, mediating helper T-cell differentiation decisions by promoting Th2 over Th1 induction and Th17 over Treg induction, and regulating chemokine receptor expression, thereby influencing T-cell recruitment to tissues (32, 33). Therefore, loss of IL6 responses may result in dysfunctional T-cell survival as well as altered helper T-cell differentiation and recruitment during inflammatory conditions. In the presence of IL6 and TGFβ and IL1β, naïve T cells can differentiate into Th17 cells, which are characterized by expression of the master transcription factor RORγt (34). Th17 cells are found to negatively correlate with the presence of Treg cells and positively correlate with effector immune cells, including cytotoxic CD8$^+$ T cells and NK cells (35, 36). The antitumor role of Th17 cells is at least partially due to their capacity to recruit effector cytotoxic T cells. The findings that Th17 differentiations from CD4$^+$ naïve T cells from breast cancer patients were defective and correlated with IL6 signaling responses suggest that IL6 response in peripheral T cell may be linked with the Th17/Treg differentiation in breast cancer patients.

Table 2. Univariate and multivariate analysis for RFS by Cox regression

Variables	Univariate *P*	Multivariate *P*[a]
pSTAT1	0.006	0.001
pSTAT3	0.015	0.005

[a]Adjusting for age, tumor stage, grade, nodal status, and subtype.

Because breast cancer is a heterogeneous disease with varied presentation, morphology, and clinical behavior, a major challenge is the outcome prediction for early stage breast cancer patients. Currently, the risk of breast cancer progression is evaluated based on clinical and pathologic parameters (37), which can only be obtained after invasive biopsy or surgery and have limited predictive power. More informative prognostic tests for breast cancer patients at diagnosis are needed. In this study, the demonstration that IL6 signaling responses predict clinical outcome indicates that IL6 signaling responses in peripheral T cells may have promise as noninvasive blood-based predictive biomarkers for breast cancer patient outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Authors' Contributions

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