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Research paper

Breast cancer induces systemic immune changes on cytokine signaling in peripheral blood monocytes and lymphocytes

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ABSTRACT

Background: It is increasingly recognized that cancer progression induces systemic immune changes in the host. Alterations in number and function of immune cells have been identified in cancer patients' peripheral blood and lymphoid organs. Recently, we found dysregulated cytokine signaling in peripheral blood T cells from breast cancer (BC) patients, even those with localized disease.

Methods: We used phosphoflow cytometry to determine the clinical significance of cytokine signaling responsiveness in peripheral blood monocytes from non-metastatic BC patients at diagnosis. We also examined the correlation between cytokine signaling in peripheral monocytes and the number of tumor-infiltrating macrophages in paired breast tumors.

Findings: Our results show that cytokine (IFNγ) signaling may also be dysregulated in peripheral blood monocytes at diagnosis, specifically in BC patients who later relapsed. Some patients exhibited concurrent cytokine signaling defects in monocytes and lymphocytes at diagnosis, which predict the risk of future relapse in two independent cohorts of BC patients. Moreover, IFNγ signaling negatively correlates with expression of CSF1R on monocytes, thus modulating their ability to infiltrate into tumors.

Interpretation: Our results demonstrate that tumor-induced systemic immune changes are evident in peripheral blood immune cells for both myeloid and lymphoid lineages, and point to cytokine signaling responsiveness as important biomarkers to evaluate the overall immune status of BC patients.

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1. Introduction

Cancer progression can induce not only local intratumoral immune dysfunction, but also changes in lymphoid organs at distant sites [1–5]. These tumor-induced distant immune changes support the view that cancer is a systemic disease. Preserved systemic immune function is associated with better clinical outcome and response to immunotherapy [6].

Macrophages play an important role in cancer development and progression [7–9] and peripheral blood monocytes are the major source of tumor-associated macrophages (TAMs) [10]. Infiltration by TAMs is associated with worse clinical outcome in breast cancer (BC) [11,12] and many other cancer types [13]. IFNγ is an important cytokine that plays a central role in monocyte differentiation and function. IFNγ induces monocyte differentiation into immunostimulatory M1 phenotype and reverses the immunosuppressive functions of TAMs [14]. IFNγ signals through the IFNγR1/IFNγR2 complex to activate signal transducer and activator of transcription (STAT) signaling [15]. Immune cell activation by IFNγ is driven by phosphorylation of STAT1, which dimerizes and translocates into the nucleus to initiate transcription of interferon-stimulated genes (ISGs) [16].

We previously found dysregulated signaling responses to several cytokines in peripheral blood T cells from BC patients, even those with localized tumors [17,18]. However, whether changes in cytokine signaling responses extend beyond lymphocytes to myeloid cells

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Research in context

Evidence before this study

Cancer is a systemic disease. Primary tumor progression can induce distant changes on immune cells function, mobilization and differentiation within primary and secondary lymphoid organs, such as bone marrow, spleen and lymph node, well before clinically evident metastasis develops. Our previous findings show that cancer-induced systemic immune changes can be evident from altered cytokine signaling in peripheral blood lymphocytes from breast cancer patients.

Added value of the study

Concurrent with dysregulated cytokine signaling in peripheral blood lymphocytes, our results here show that tumor-induced systemic immune changes extend to peripheral blood monocytes. Altered signaling responses in peripheral monocytes correlate with clinical outcome, demonstrating that systemic immune changes persist in some patients after initial therapy and underlie future relapse.

Implications of all the available evidence

Concurrent development of altered signaling responses in peripheral blood monocytes and T cells further supports cancer as a systemic disease. Identifying and understanding additional tumor-induced systemic immune abnormalities will provide significant implications for future risk evaluation of cancer patients and therapeutic opportunities.

remained unclear. Here, we sought to investigate cytokine signaling in peripheral blood monocytes from BC patients, focusing on the key pro-inflammatory cytokine IFNγ. We analyzed IFNγ signaling responsiveness between relapsed and relapse-free BC patients in peripheral monocytes from blood collected at diagnosis. We also correlated TAM infiltration in matched tumors from these patients in relation to IFNγ signaling response in their peripheral blood monocytes.

2. Material and methods

2.1. Study design and cohorts

The study population of the discovery cohort consisted of 40 breast cancer patients from Stanford Medical Center and City of Hope Comprehensive Cancer Center. These patients were all diagnosed with breast cancer and had blood collected before June 2012. The validation cohort was composed of 78 breast cancer patients from City of Hope Comprehensive Cancer Center. These patients were diagnosed with breast cancer and had PBMCs collected after June 2012. We only analyzed blood samples collected at diagnosis before surgery or any systemic therapy from patients with clinical follow-up for more than 36 months. All patients in this study received standard of care treatments.

This study was approved by the Institutional Review Board of Stanford Medical Center and City of Hope Comprehensive Cancer Center. All patients had signed written informed consents.

2.2. Human samples

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque density centrifugation and cryopreserved in 10% DMSO FBS. Age-matched healthy control peripheral blood samples were obtained from the City of Hope Blood Donor Center. Only PBMC samples with cell viability $\geq$ 85% after thawing were selected.

2.3. Phosphoflow cytometry after IFNγ stimulation

Cryopreserved PBMCs were thawed and rested for 16 h. PBMCs were stimulated with IFNγ (Peprotech, Rocky Hills, NJ, USA) at 50 ng/ml at 37 °C for 15 min followed by fixation with 1.5% paraformaldehyde (PFA) for 10 min at room temperature. Cells were washed with PBS to remove PFA, and permeabilized by the addition of 100% methanol.

2.4. Flow cytometry

The following antibodies were utilized: CD3 (UCHT1), CD4 (SK3), CD45RA (L48), CD8 (SK1), CD16 (3G8), CD33 (P67.6), pSTAT1 (pY701) (4a), total STAT1 (1/stat1), CXCR4 (12G5), IFNγR1(GIR208) (BD Biosciences, San Jose, CA, USA), CD14 (HCD14), CSF1R (94D21E4), CCR2 (K036C2), VEGFR2 (7D46), MRC1 (15-2), CD163 (GHI/61) (Biolegend, San Diego, CA, USA), LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies, Carlsbad, CA, USA). The IFNγ signaling response was expressed as the IFNγ induced median fluorescence intensity (MFI) minus the unstimulated MFI of pSTAT1. Flow cytometry was performed using Fortessa Flow Cytometers (BD Biosciences). Flow cytometry data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA.).

2.5. Plasma IFNγ ELISA

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

2.6. Multiplex immunofluorescence staining and imaging

Formalin-fixed paraffin-embedded (FFPE) biopsies of untreated primary breast tumors tissues were cut into 3-um sections and affixed to microscope slides. They were deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol in water. Heat-induced epitope/antigen retrieval was performed in EnVision® FLEX Target Retrieval Solution, High pH (pH 9) (K8004/5, Agilent, Santa Clara, CA, USA) or AR6 buffer (pH 6) (PerkinElmer, Hopkinton, MA, USA) using a microwave oven. Blocking was performed for 10 min using Antibody Diluent, Background Reducing (S3022, Agilent) to minimize non-specific background staining. Tissue slides were stained with the following primary antibodies for 1 h on a shaker at room temperature: CD68 (KP1), Cytokeratin (AE1/AE3) (Biocare) and then detected by a horseradish peroxidase (HRP)-conjugated secondary antibody followed Opal® fluorescence IHC Kit (PerkinElmer) at a 1:100 dilution following a 10 min incubation. To perform multicolor immunofluorescent staining, the slide would be serially stained with the microwave incubation acting to remove previous antibodies while simultaneously exposing the next epitope of interest. After staining the final marker, cell nuclei were stained with DAPI (PerkinElmer) and the slides were mounted with ProLong Gold Antifade Reagent (P36930, ThermoFisher, Waltham, MA, USA).

2.7. Whole tissue section imaging and quantitative analysis

Whole tissue section images were acquired at 200 × magnification using the imaging system Vectra (PerkinElmer, Waltham, MA, USA) as previously described [19] and then images taken were analyzed using the image analysis software inForm

(PerkinElmer) to enumerate the total number of $CD68^+$ cells per tissue section. The macrophage infiltration ($CD68^+$%) was expressed by dividing the cell number of $CD68^+$ cells with the number of total cells of the tissue section.

2.8. Statistical analysis

Mann–Whitney tests were used to determine the statistical significance of BC patient with healthy donors (Graphpad Prism, GraphPad Software, LaJolla, CA, USA). Relapse-free survival (RFS) was defined as the time from the date of diagnosis of BC to the date of cancer recurrence or death. Kaplan-Meier method with log-rank test was used to determine IFNγ signaling responsiveness as prognostic factors for RFS of BC patients. Multivariate Cox regression model analysis was performed to determine independence of prognostic factor. ROC analysis was used to evaluate the prognostic performance. All tests with p value$<$0.05 were considered statistically significant.

3. Results

3.1. Breast cancer induces concurrent cytokine signaling defects in peripheral blood monocytes and lymphocytes

To investigate breast cancer-induced systemic changes on cytokine signaling in peripheral blood monocytes, we analyzed IFNγ-induced phosphorylation of STAT1 (pSTAT1) in peripheral monocytes ($CD33^+CD14^+CD3^-CD16^{-/lo}$) from BC patients with localized tumors via phosphoflow cytometry (gating strategy in Fig. S1a). Only patients with blood collected at diagnosis before surgery or any systemic therapy and who later relapsed within 5 years were selected. IFNγ signaling response (ΔMFI) is represented by IFNγ stimulated minus unstimulated pSTAT1 medium fluorescence intensity (MFI) (Fig. 1a). To examine whether tumor-induced systemic changes on IFNγ signaling are evident in peripheral monocytes from BC patients at diagnosis who later relapsed, we compared the IFNγ signaling responsiveness in peripheral monocytes between BC patients who later relapsed (n = 22) or remained relapse-free (n = 96), and age-matched healthy donors (n = 27). IFNγ-induced pSTAT1 in peripheral blood monocytes at diagnosis was significantly higher in relapse-free BC patients and in healthy donors as compared to BC patients who later relapsed (Fig. 1b). The levels of IFNγ receptor IFNγR1 were also significantly higher in monocytes from healthy donors than in relapsed BC patients (Fig. 1c). In contrast, levels of basal pSTAT1 (Fig. S1b) and total STAT1 (Fig. S1c), and frequencies of monocyte (Fig. S1d) were similar between relapsed, relapse-free BC patients and healthy donors.

In addition, we found that IFNγ signaling responsiveness in monocytes from BC patients whose blood were collected at relapse (n = 10) were significantly lower than in patients who achieved and remained in remission for at least 3 years after their most recent relapse (n = 10) (Fig. S1e), indicating that altered IFNγ signaling responsiveness in peripheral monocytes may be reversible when patients achieve remission.

We previously found dysregulated cytokine signaling in peripheral blood T cells from BC patients at diagnosis (17, 18). To investigate whether cytokine signaling in peripheral monocytes and T cells could be altered concurrently, we examined the correlation between IFNγ signaling in monocytes and IL-6 signaling in $CD4^+$ naïve T cells (IL-6 Phosphflow gating strategy in Fig. S2). Importantly, we found a significant positive correlation between IFNγ-pSTAT1 in monocytes and IL-6-pSTAT1/3 in $CD4^+$ naïve T cells (Fig. 1d). Moreover, BC patients who later relapsed not only had lower IFNγ signaling in monocytes and but also lower IL-6 signaling in T cells (Fig. 1e). These results indicate that BC patients likely to relapse tend to have dysregulated cytokine signaling in peripheral blood myeloid cells and lymphocytes concurrently at diagnosis.

3.2. IFNγ signaling in peripheral monocytes correlates with tumor infiltration of macrophages

Given that high TAMs infiltration associates with poor outcome in BC and peripheral monocytes are the major source of TAMs, we hypothesized that the IFNγ signaling responsiveness in peripheral monocytes may influence tumor infiltration by macrophages. To test this hypothesis, we examined the IFNγ signaling response in peripheral monocytes by flow cytometry and quantified the macrophage infiltration in paired primary breast tumors from the same patients (n = 20) via whole tissue section multispectral imaging (Fig. 2a). Tumor infiltration of macrophages (%$CD68^+$) was quantified by dividing the number of TAMs ($CD68^+$) by the total number of cells in the whole tissue section. Indeed, we found a significant negative association between IFNγ signaling responsiveness in peripheral monocytes and degree of TAM infiltration in paired primary tumors (Fig. 2b), suggesting that patients who have lower IFNγ signaling response in peripheral monocytes tend to have more tumor-infiltrating macrophages. Given that TAMs are recruited to tumors mainly through CSF1R and chemokine receptors CCR2, CXCR4 and VEGFR2 expressed on monocytes [14], we next addressed whether IFNγ signaling responsiveness correlates with levels of these chemokine receptors. Amongst these chemokine receptors, we found that IFNγ signaling response negatively associated with levels of CSF1R (Fig. 2c) but not CXCR4, CCR2 or VEGFR2 (Fig. S3a–c). Moreover, we found a positive association between levels of CSF1R on peripheral monocytes and numbers of TAMs in paired primary tumors (Fig. 2d), reflecting the importance of CSF1R in TAM recruitment. To determine whether IFNγ negatively regulates expression of CSF1R, we treated peripheral blood monocytes from BC patients with IFNγ at low concentration in vitro and found that IFNγ downregulated the levels of CSF1R on monocytes in a dose-dependent manner (Fig. 2e). These findings demonstrate a link between IFNγ signaling in peripheral blood monocytes and their potential to infiltrate into tumors.

3.3. IFNγ signaling responsiveness in peripheral blood monocytes predicts RFS in BC patients

To investigate the clinical significance of IFNγ signaling responsiveness in peripheral blood monocytes of BC patients, we used Kaplan-Meier survival analysis and log-rank test to determine the relationship between IFNγ signaling responsiveness and relapse-free survival (RFS). Only patients with blood collected at diagnosis before surgery or any systemic therapy and who had been clinically followed for at least 36 months were selected (clinical and pathological characteristics are summarized in Table 1). The median follow-up time of BC patients (n = 40) was 63 months (range, 36–92 months). We compared RFS between BC patients with high vs. low IFNγ signaling response, defined as patients with IFNγ-induced pSTAT1 ΔMFI $\geq$ 25% quantile and $<$ 25% quantile, respectively. Patients with low ΔMFI (n = 10) had significantly worse RFS (p = 0.001) than those with high ΔMFI (n = 30) (Fig. 3a), indicating that lower IFNγ signaling response in peripheral blood monocytes at diagnosis correlates with worse RFS.

To evaluate the robustness of IFNγ signaling responsiveness in predicting the risk of future relapse of BC, the clinical significance of IFNγ signaling in peripheral monocytes was tested in an independent validation cohort of newly diagnosed BC patients (n = 78). Again, only patients with blood collected at diagnosis before surgery or any therapy and had been clinically followed for at least 36 months were selected (clinical and pathological characteristics are summarized in Table 1). The median follow-up time of BC patients of this cohort was 47 months (range, 36–59 months). Using the same IFNγ-induced

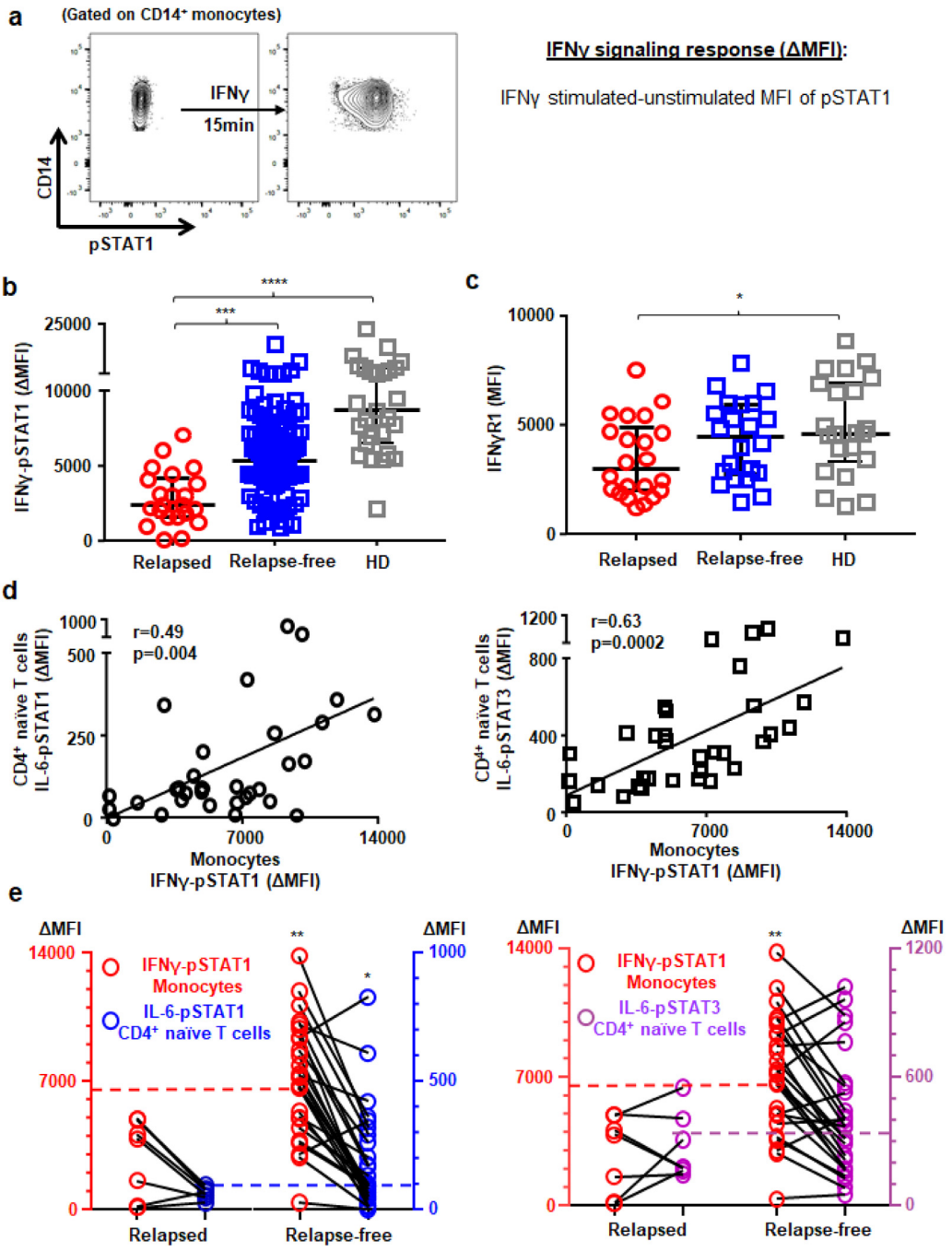

Fig. 1. Concurrent cytokine signaling defects in peripheral monocytes and lymphocytes. (a) PBMCs from breast cancer patients (BC) were stimulated with IFNγ at 50 ng/ml for 15 min. Representative flow plot showing IFNγ induced phosphorylation of STAT1 (pY701) in peripheral monocytes (CD14$^+$). IFNγ signaling response (ΔMFI) is determined by IFNγ stimulated MFI minus unstimulated MFI of pSTAT1. (b) IFNγ signaling response in peripheral monocytes was compared between BC patients with blood collected at diagnosis who later relapsed within 5 years (n = 22) or remained relapse-free for > 5 years (n = 96), and age-matched healthy donors (HD) (n = 27). One-way ANOVA. ***$p < 0.001$, ****$p < 0.0001$. (c) Levels of IFNγR1 on monocytes from relapsed BC patients (n = 22), relapse-free BC patients (n = 22) and healthy donors (n = 22). One-way ANOVA. *$p < 0.05$. (d) correlation between IFNγ-pSTAT1 (ΔMFI) in peripheral monocytes and IL-6-pSTAT1/3 (ΔMFI) in peripheral CD4$^+$ naïve T cells from BC patients at diagnosis (n = 33, ER$^+$HER2$^-$). Spearman's correlation coefficient test. (e) IFNγ-pSTAT1 in monocytes and IL-6-pSTAT1/3 in T cells from the same patients were compared between relapsed (n = 7) and relapse-free BC patients (n = 26). Mann–Whitney test. *p<0.05, **p<0.01. All blood were collected at diagnosis before surgery or any systemic therapy from BC patients with localized tumors.

pSTAT1 ΔMFI cut-off derived from the discovery cohort (ΔMFI=4071), BC patients in the validation cohort were classified into high (n = 48) or low (n = 30) IFNγ signaling response groups. As in the discovery cohort, Kaplan-Meier analysis showed that patients with low IFNγ signaling response had significantly worse RFS (p = 0.0002) than those in the high IFNγ signaling response group (Fig. 3b).

In a multivariate analysis adjusted for clinicopathologic characteristics of BC patients (age, tumor stage, grade, nodal status and subtype), IFNγ-induced pSTAT1 retained prognostic significance (p = 0.0007) for RFS (Table S1), suggesting that the IFNγ signaling response in peripheral blood monocytes at diagnosis is a prognostic biomarker of clinical outcome independent of other clinicopathologic characteristics. Moreover, plasma IFNγ levels were similar between

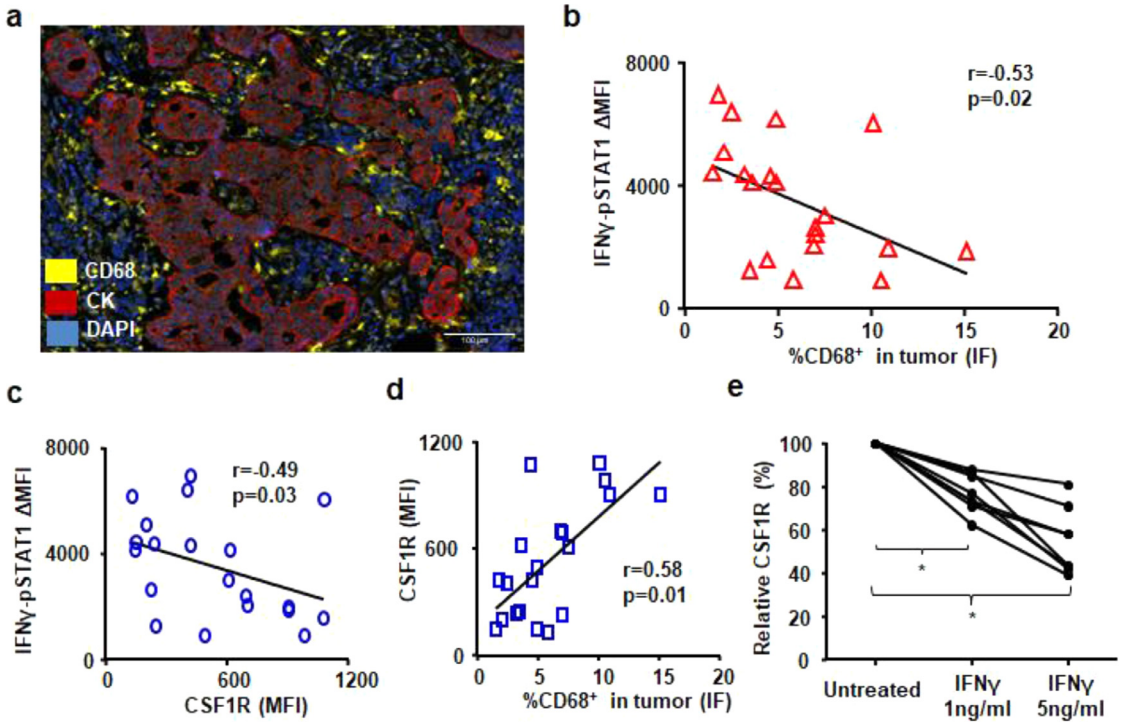

Fig. 2. IFNγ signaling responsiveness in peripheral monocytes correlates with tumor infiltration of macrophages. (a) Immunofluorescence (IF) staining of representative breast tumor tissue sections showing CD68 (yellow) and cytokeratin (CK) (red). After whole tissue section imaging, the tumor infiltration of macrophages was quantified by dividing the number of TAMs (CD68$^+$) by the total number of cells in the whole tissue section. All breast tumors were primary tumors prior to any systemic therapy. (b) PBMCs from BC patients were stimulated with IFNγ at 50 ng/ml for 15 min. The association between IFNγ-induced pSTAT1 (ΔMFI) in monocytes and number of TAMs in the paired BC tumors (n = 20, ER$^+$HER2$^-$). Spearman's correlation coefficient test. (c) The association between levels of CSF1R (MFI) and IFNγ-induced pSTAT1 (ΔMFI) in peripheral monocytes of BC patients (n = 20, ER$^+$HER2$^-$). Spearman's correlation coefficient test. (d) The association between levels of CSF1R on peripheral monocytes and number of TAMs in the paired BC tumors (n = 20, ER$^+$HER2$^-$). Spearman's correlation coefficient test. (e) PBMCs from BC patients (n = 8, ER$^+$HER2$^-$) were treated with IFNγ at 1 or 5 ng/ml for 18 h and levels of CSF1R on monocytes were determined by flow cytometry. Paired one-way ANOVA. $^*p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Patient characteristics.

Characteristics	Discovery cohort N = 40 (%)	Validation cohort N = 78 (%)
Age—yr		
Median	50	53
Range	27–69	27–79
Tumor stage— no. (%)		
DCIS	4 (10)	2 (2.5)
T1	17 (42.5)	34 (43.5)
T2	11 (27.5)	33 (42 5)
T3	5 (12.5)	7 (9)
Unknown	3 (7.5)	2 (2 5)
Grade— no. (%)		
G1	6 (15)	9 (11.5)
G2	15 (37.5)	46 (59)
G3	19 (47.5)	23 (29.5)
Nodal status— no. (%)		
NO	21 (52.5)	50 (64)
N1-3	19 (47.5)	25 (32)
Unknown	0 (0)	3 (4)
Subtype—no. (%)		
Luminal	32 (SO)	65 (83)
HER2	5 (125)	8 (10)
Triple negative	3 (7.5)	5 (7)

relapse-free and relapsed BC patients (Fig. S4a) and we found no correlation between plasma IFNγ level and IFNγ signaling response in peripheral monocytes (Fig. S4b).

The prognostic potential of IFNγ signaling in peripheral monocytes to predict future relapse was also evaluated by receiver operating characteristic (ROC) analysis. IFNγ signaling responsiveness (ΔMFI) achieved an area under the curve (AUC) of 0.81 (95% CI 0.72–0.91, $p < 0.0001$), with 82% sensitivity and 77% specificity to predict future relapse when we used the low and high IFNγ signaling response cut-off (ΔMFI=4071) from the discovery and validation cohorts (Fig. 3c).

Among the ER$^+$HER2$^-$ luminal BC patients, we examined whether IFNγ signaling responsiveness in peripheral blood monocytes correlates with Oncotype DX scores of their paired primary tumors (n = 46). There is a trend of negative correlation between IFNγ-pSTAT1 and Oncotype DX scores (r=−0.24, p = 0.1) (Fig. S5a). Interestingly, 6 out of 8 relapsed patients had Oncotype DX scores ($\leq$ 15, indicating low risk of recurrence) - these 6 relapsed patients still had low IFNγ signaling in peripheral monocytes (indicating high risk of recurrence) (Fig. S5b). As such, cytokine signaling responsiveness in peripheral blood monocytes may provide additional prognostic information beyond Oncotype DX from tumor samples.

In addition, we also examined IFNγ signaling response in non-classical monocytes (CD16^{hi}CD14$^{-/lo}$) (Fig. S6a) and found that IFNγ-induced pSTAT1 were significantly higher in non-classical monocytes than in classical monocytes from BC patients (Fig. S6b). However, IFNγ signaling response in non-classical monocytes (Fig. S6c) and frequency of non-classical monocytes (Fig. S6d) were similar between relapse-free and relapsed BC patients.

Since IFNγ induces monocyte differentiation into immunostimulatory M1-like phenotype, we investigated whether IFNγ signaling responsiveness negatively correlates with expression levels of M2-like proteins, such as mannose receptor C-type 1 (MRC1/CD206) and CD163. We determined the levels of MRC1 and CD163 by flow cytometry and examined their correlations with IFNγ-induced pSTAT1 in peripheral monocytes from BC patients (n = 12). There is a trend of negative correlation between IFNγ-induced pSTAT1 and MRC1

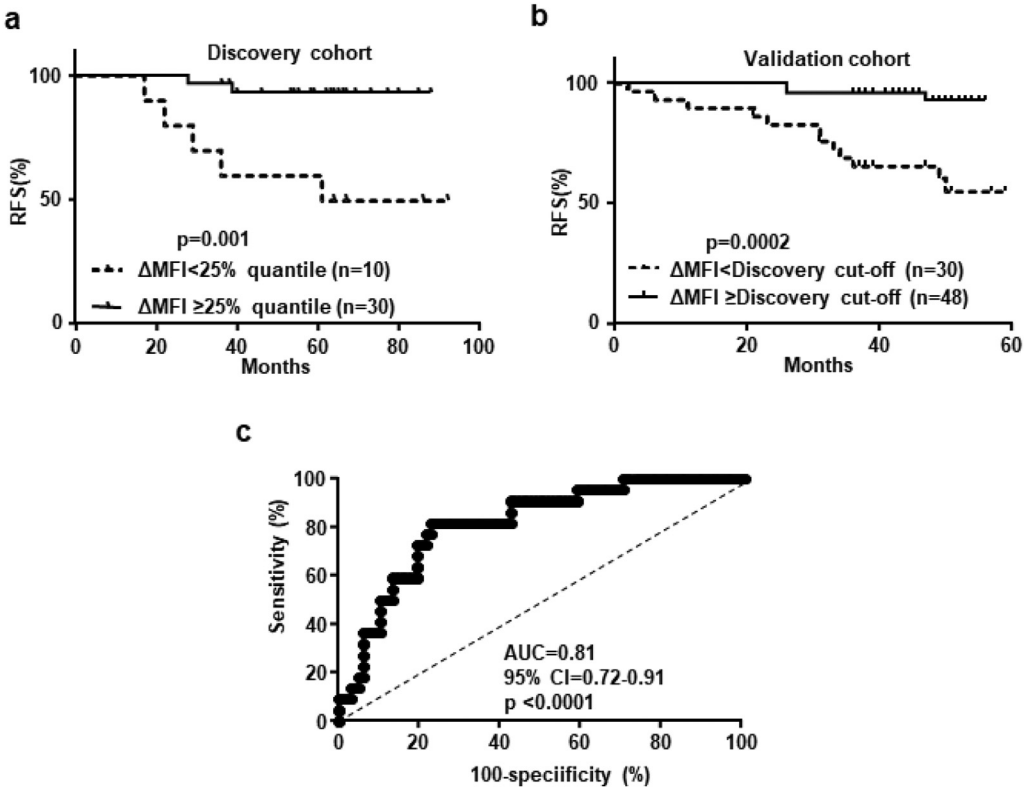

Fig. 3. IFNγ signaling responsiveness in peripheral monocytes at diagnosis predicts future relapse of breast cancer. PBMCs from breast cancer patients (BC) were stimulated with IFNγ at 50 ng/ml for 15 min. IFNγ signaling response (ΔMFI) in peripheral monocytes is determined by IFNγ stimulated MFI minus unstimulated MFI of pSTAT1. (a) Relapse-free survival (RFS) was compared between BC patients of discovery cohort (n = 40) with low and high IFNγ signaling response using Kaplan-Meier estimate and log rank test (p = 0.001). The 25% quantile of IFNγ-induced pSTAT1 (ΔMFI) was used as the cut-off (ΔMFI=4071) to divide BC patients into low and high IFNγ response groups. (b) BC patients of validation cohort (n = 78) were divide into low and high IFNγ response groups using the cut-off (ΔMFI=4071) from the discovery cohort. RFS was compared between BC patients with low and high IFNγ signaling response using Kaplan-Meier estimate and log rank test (p = 0.0002). (c) Receiver operating characteristic (ROC) analysis for prognostic potential of IFNγ signaling response (ΔMFI) in peripheral monocytes from BC patients at diagnosis (n = 118). All the blood were collected at diagnosis prior to surgery or any systemic therapy from BC patients who had been clinically followed for at least 36 months. These are the same BC patients analyzed in Fig. 1.

(r=−0.36, p = 0.26) (Fig. S7a), but no correlation between IFNγ-induced pSTAT1 and CD163 (r=−0.19, p = 0.56) (Fig. S7b).

4. Discussion

Accumulating data support the view that cancer is a systemic disease. Tumors must induce systemic immunological changes in peripheral blood and distant lymphoid organs to facilitate cancer progression and metastasis. Concurrent with dysregulated cytokine signaling in peripheral blood lymphocytes [17,18], here we show that tumor-induced systemic immune changes extend to peripheral blood monocytes.

TAMs are the dominant infiltrating immune cells in many human tumors and can represent up to 50% of the tumor mass [20]. As the major source of TAMs [21,22], peripheral blood monocytes are recruited to tumors via various tumor-derived chemokines and motility factors such as CSF1 [23]. Inhibition of IFNγ and up-regulation of CSF1 have been shown to promote the conversion of monocytes into immunosuppressive macrophages that inhibit T cell-mediated responses [24]. Since the CSF1-CSF1R axis is important in monocytes recruitment, blockade of CSF1R has been used to prevent tumor metastasis and progression [25–27]. Our finding that IFNγ signaling response negatively associated with the levels of CSF1R extends beyond previous reports that IFNγ suppresses the expression of CSF1R in macrophage [28], and reveals a novel mechanism behind the correlation between IFNγ signaling response with clinical outcome. Since CSF1R expression is known to be negatively regulated by IFNγ, our finding of higher levels of CSF1R in peripheral blood monocytes with lower IFNγ signaling responsiveness is consistent with these previous findings. Furthermore, our finding that relapsed BC patients tend to have normal plasma levels of IFNγ at diagnosis demonstrates that cancer-induced systemic immune changes may not necessarily be mediated through elevated/altered cytokine levels in circulation, but in the ability of immune cells to respond to cytokines. Altered IFNγ signaling responsiveness was not due to basal STAT1 levels, but differences in IFNγ-induced STAT1 phosphorylation. These results suggest altered priming of peripheral blood monocytes in relapse-free vs. relapsed patients.

Concurrent development of altered signaling responses in peripheral blood monocytes and T cells further supports cancer as a systemic disease. T cells may traffic through tumors and tumor-draining lymph nodes (TDLNs) leading to their altered function [29,30]. However, monocytes remain within tissues once they leave the blood [31]. Thus, signaling alterations in peripheral blood monocytes must have developed at their origin, from cancer-induced distant effects within bone marrow and/or spleen. Indeed, it has been shown that cancer can induce distant changes on myeloid cells function, mobilization and differentiation within bone marrow [32–35] and spleen [36–39], well before clinically evident metastasis develops. Importantly, these altered signaling responses correlate with clinical outcome, demonstrating that systemic immune changes persist in some patients after initial therapy and underlie future relapse. Identifying and understanding additional tumor-induced systemic immune abnormalities will provide significant implications for future risk evaluation of cancer patients and therapeutic opportunities.

Declaration of Competing Interest

Dr. Lu is an employee of Genentech, Inc., shareholder of F. Hoffmann La Roche, Ltd. The other authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102631.

References

[1] McAllister SS, Weinberg RA. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. Nat Cell Biol 2014;16(8):717–27.
[2] Setiadi AF, Ray NC, Kohrt HE, Kapelner A, Carcamo-Cavazos V, Levic EB, et al. Quantitative, architectural analysis of immune cell subsets in tumor-draining lymph nodes from breast cancer patients and healthy lymph nodes. PLoS One 2010;5(8):e12420.
[3] Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP. Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. PLoS Med 2005;2(9):e284.
[4] Blenman KRM, He TF, Frankel PH, Ruel NH, Schwartz EJ, Krag DN, et al. Sentinel lymph node B cells can predict disease-free survival in breast cancer patients. NPJ Breast Cancer 2018;4:28.
[5] Chang AY, Bhattacharya N, Mu J, Setiadi AF, Carcamo-Cavazos V, Lee GH, et al. Spatial organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients. J Transl Med 2013;11:242.
[6] Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, et al. Systemic immunity is required for effective cancer immunotherapy. Cell 2017;168(3):487–502 e15.
[7] Engblom C, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. Nat Rev Cancer 2016;16(7):447–62.
[8] De Vlaeminck Y, Gonzalez-Rascon A, Goyvaerts C, Breckpot K. Cancer-associated myeloid regulatory cells. Front Immunol 2016;7:113.
[9] Mantovani A, Marchesi F, Malesci A, Laghi L, Allavena P. Tumour-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol 2017;14(7):399–416.
[10] Franklin RA, Liao W, Sarkar A, Kim MV, Bivona MR, Liu K, et al. The cellular and molecular origin of tumor-associated macrophages. Science 2014;344(6186):921–5.
[11] Campbell MJ, Tonlaar NY, Garwood ER, Huo D, Moore DH, Khramtsov AI, et al. Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. Breast Cancer Res Treat 2011;128(3):703–11.
[12] Zhang QW, Liu L, Gong CY, Shi HS, Zeng YH, Wang XZ, et al. Prognostic significance of tumor-associated macrophages in solid tumor: a meta-analysis of the literature. PLoS One 2012;7(12):e50946.
[13] Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. Cancer Cell 2015;27(4):462–72.
[14] Williams CB, Yeh ES, Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. NPJ Breast Cancer 2016;2(15025).
[15] Ivashkiv LB. IFNgamma: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. Nat Rev Immunol 2018;18(9):545–58.
[16] Stark GR, Darnell JE. The JAK-STAT pathway at twenty. Immunity 2012;36(4):503–14.
[17] Wang L, Miyahira AK, Simons DL, Lu X, Chang AY, Wang C, et al. IL6 signaling in peripheral blood T cells predicts clinical outcome in breast cancer. Cancer Res 2017;77(5):1119–26.
[18] Wang L, Simons DL, Lu X, Tu TY, Solomon S, Wang R, et al. Connecting blood and intratumoral Treg cell activity in predicting future relapse in breast cancer. Nat Immunol 2019;20(9):1220–30.
[19] Yu H, Simons DL, Segall I, Carcamo-Cavazos V, Schwartz EJ, Yan N, et al. PRC2/EED-EZH2 complex is up-regulated in breast cancer lymph node metastasis compared to primary tumor and correlates with tumor proliferation in situ. PLoS One 2012;7(12):e51239.
[20] Laoui D, Van Overmeire E, De Baetselier P, Van Ginderachter JA, Raes G. Functional relationship between tumor-associated macrophages and macrophage colony-stimulating factor as contributors to cancer progression. Front Immunol 2014;5:489.
[21] Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011;475(7355):222–5.
[22] Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. Cell 2010;141(1):39–51.
[23] Hamilton JA, Achuthan A. Colony stimulating factors and myeloid cell biology in health and disease. Trends Immunol 2013;34(2):81–9.
[24] Ginhoux F, Schultze JL, Murray PJ, Ochando J, Biswas SK. New insights into the multidimensional concept of macrophage ontogeny, activation and function. Nat Immunol 2016;17(1):34–40.
[25] Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. Nat Med 2013;19(10):1264–72.
[26] Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science. 2013;339(6117):286–91.
[27] Strachan DC, Ruffell B, Oei Y, Bissell MJ, Coussens LM, Pryer N, et al. CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by CD8+ T cells. Oncoimmunology 2013;2(12):e26968.
[28] Su X, Yu Y, Zhong Y, Giannopoulou EG, Hu X, Liu H, et al. Interferon-gamma regulates cellular metabolism and mRNA translation to potentiate macrophage activation. Nat Immunol 2015;16(8):838–49.
[29] Pucci F, Garris C, Lai CP, Newton A, Pfirschke C, Engblom C, et al. SCS macrophages suppress melanoma by restricting tumor-derived vesicle-B cell interactions. Science 2016;352(6282):242–6.
[30] Riedel A, Shorthouse D, Haas L, Hall BA, Shields J. Tumor-induced stromal reprogramming drives lymph node transformation. Nat Immunol 2016;17(9):1118–27.
[31] Teh YC, Ding JL, Ng LG, Chong SZ. Capturing the fantastic voyage of monocytes through time and space. Front Immunol 2019;10:834.
[32] Borniger JC, Walker Ii WH, Surbhi EKM, Zhang N, Zalenski AA, et al. A role for hypocretin/orexin in metabolic and sleep abnormalities in a mouse model of non-metastatic breast cancer. Cell Metab 2018;28(1):118–29 e5.
[33] Casbon AJ, Reynaud D, Park C, Khuc E, Gan DD, Schepers K, et al. Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. Proc Natl Acad Sci U S A 2015;112(6):E566–75.
[34] Pucci F, Rickelt S, Newton AP, Garris C, Nunes E, Evavold C, et al. PF4 promotes platelet production and lung cancer growth. Cell Rep 2016;17(7):1764–72.
[35] Engblom C, Pfirschke C, Zilionis R, Da Silva Martins J, Bos SA, Courties G, et al. Osteoblasts remotely supply lung tumors with cancer-promoting SiglecF(high) neutrophils. Science 2017;358(6367).
[36] Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, et al. Origins of tumor-associated macrophages and neutrophils. Proc Natl Acad Sci U S A 2012;109(7):2491–6.
[37] Cortez-Retamozo V, Etzrodt M, Newton A, Ryan R, Pucci F, Sio SW, et al. Angiotensin II drives the production of tumor-promoting macrophages. Immunity 2013;38(2):296–308.
[38] Wu C, Ning H, Liu M, Lin J, Luo S, Zhu W, et al. Spleen mediates a distinct hematopoietic progenitor response supporting tumor-promoting myelopoiesis. J Clin Invest 2018;128(8):3425–38.
[39] Zhao L, He R, Long H, Guo B, Jia Q, Qin D, et al. Late-stage tumors induce anemia and immunosuppressive extramedullary erythroid progenitor cells. Nat Med 2018;24(10):1536–44.