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Pretreatment levels of circulating Th1 and Th2 cytokines, and their ratios, are associated with ER-negative and triple negative breast cancers

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Abstract

Immune signatures in breast tumors differ by estrogen receptor (ER) status. The purpose of this study was to assess associations between ER phenotypes and circulating levels of cytokines that co-ordinate cell-mediated [T-helper type 1 (Th1)] and humoral [T-helper type 2 (Th2)] immunity. We conducted a case–case comparison of 523 women with newly diagnosed breast cancer to evaluate associations between 27 circulating cytokines, measured using Luminex XMap technology, and breast cancer phenotypes [ER^- vs. ER^+; triple negative breast cancer (TNBC) vs. luminal A (LumA)]. Ratios of Th1 to Th2 cytokines were also evaluated. Levels of interleukin (IL)-5, a Th-2 cytokine, were higher in ER^- than in ER^+ tumors. The highest tertile of IL-5 was more strongly associated with ER^- (OR = 2.33, 95 % CI 1.40–3.90) and TNBCs (OR = 2.78, 95 % CI 1.53–5.06) compared to ER^+ and LumA cancers, respectively, particularly among premenopausal women (OR = 4.17, 95 % CI 1.86–9.34, ER^- vs. ER^+; OR = 5.60, 95 % CI 2.09–15.01, TNBC vs. LumA). Elevated Th1 cytokines were also detected in women with ER^- and TNBCs, with women in the highest tertile of interferon α2 (OR = 2.39, 95 % CI 1.31–4.35) or tumor necrosis factor-α (OR = *2.27*, 95 % CI 1.21–4.26) being twice as likely to have TNBC versus LumA cancer. When cytokine ratios were examined, women with the highest ratios of Th1 cytokines to IL-5 levels were least likely to have ER^- or TNBCs compared to ER^+ or LumA cancers, respectively. The strongest associations were in premenopausal women, who were up to 80 % less likely to have TNBC than LumA cancers (IL-12p40/IL-5, OR = 0.19, 95 % CI 0.07–0.56). These findings indicate that immune function is associated with ER^- and TNBC and may be most relevant among younger women, who are likely to be diagnosed with these aggressive phenotypes.

Keywords

Estrogen negative breast cancer; Triple negative breast cancer; Cytokines Immune function

Introduction

Breast cancer intrinsic subtypes, now characterized by immunohistochemistry (IHC), have important differences in prognosis, with LumA (ER^+ and/or PR^+ and $HER2^-$) having better prognosis and basal-like tumors (ER^-, PR^-, $HER2^-$, $CK5/6^+$ and/or $EGFR^+$) the poorest [1, 2]. Most breast cancers with a triple negative phenotype (ER^-, PR^-, $HER2^-$), i.e., 70–90 %, are also basal-like [3–5]. Younger women, BRCA1 carriers, and women of African or Hispanic ancestry have higher rates of basal-like and TNBC [6, 7]. Although subtype-specific risk factors have been understudied, there do appear to be differences in some reproductive and hormonal factors for ER^+ and TNBC [6, 8, 9].

Pro-inflammatory biomarkers and immune-response may be related to breast cancer risk and/or prognosis [10, 11]. For ER^- and TNBC, gene expression studies show that inflammation and immune-related signatures are quantitatively and qualitatively different between ER^+ and ER^- cancers, and important for disease prognosis [12–14]. Therefore, we hypothesized that cytokines functioning as immune system signaling chemical messengers could be related to ER^- and TNBC.

Cytokines can be classified into broad categories that include T-helper type 1 (Th1) and T-helper type 2 (Th2), which are generally antagonistic to each other (see Fig. 1). Th1 adaptive

immunity, important for mounting an effective anti-tumor immune response [15, 16], is characterized by IFNγ, TNFα, IL-12, GM-CSF, and CXCL10 (IP10), and can activate macrophages toward a M1 phenotype, further promoting a Th1 response. Th-2 cytokines include IL-4, IL-5, IL-10, CCL2, CCL7, and CCL11, and are involved in allergy and M2 polarization of macrophages, which are important in mediating humoral immunity and appear to promote mammary tumor progression [15, 17]. Several cytokines associated with chronic inflammation, such as CXCL8 and IL-6 [18, 19], have been linked to poorer breast cancer survival [20], particularly for TNBC [13]. Previous research examining associations between cytokines and ER^- and TNBCs have mostly focused on tumor-associated changes in the microenvironment. Here we investigate potential associations with circulating cytokine levels involved in Th-1 and Th-2 immunity and chronic inflammation, which may provide greater understanding into the role of constitutional host immunity on breast cancer subtypes. In addition, we also examined several cytokine ratios to reflect the balance between Th1 and Th2 cell types since these cell subsets are often inversely regulated and their balance can be influenced by many factors [21–23].

Patients and methods

Study population and blood collection

Data and plasma samples from 523 non-Hispanic Caucasian women with incident invasive breast cancer treated at Roswell Park Cancer Institute (RPCI) from December 2003 to June 2010 were obtained from the Institute's Data Bank and Biorepository (DBBR) [24, 25]. Prior to receipt of treatment (including surgery), newly diagnosed patients consent to DBBR, to provide non-fasting blood samples, complete an epidemiological questionnaire, and permit linkage of samples to clinical information. Blood samples are drawn in phlebotomy and transported via a pneumatic tube system to the laboratory for processing, where they are centrifuged and automatically aliquoted into chemically inert plastic straws. The time from blood draw to storage in liquid nitrogen is maintained at 1 h of draw to minimize variable analyte degradation. For most participants in our study, samples were obtained prior to surgery (n = 475, 91 %) and adjuvant therapy (n = 519, 99 %). This research was approved by the Institutional Review Board (IRB) of RPCI.

Clinical information

Samples were linked with clinical data derived from a prospective database of the RPCI Breast Program by the RPCI Clinical Data Network (CDN). Molecular subtypes were defined based on ER, PR, and HER2 status determined routinely by IHC or FISH in the Department of Pathology and classified as LumA (ER^+ and/or PR^+, $HER2^-$) and triple negative (ER^-, PR^-, and $HER2^-$). Because IHC staining of CK 5/6 or EGFR are not routinely performed in pathology at RPCI, we could not distinguish basal-like and unclassified subtypes, and both were included in the triple negative group.

Postmenopausal status was defined as 12 consecutive months of amenorrhea and/or a bilateral salpingo-oophorectomy as reported in a self-administered questionnaire. For women without self-reported menopausal status, data were abstracted from patient medical records. Where medical record data were unavailable (n = 9), we used an age cut point, with premenopausal status defined as 56 years of age or younger.

Luminex xMAP (Multi-Analyte Profiling) immune-bead array assays

Prior to these analyses, we conducted a pilot study with a series of QC experiments to investigate the effects of various sample processing and storage procedures on the recovery of cytokines and growth factors, as well as to compare across commercially available

Luminex® bead-based cytokine kits to identify those with the best reproducibility and sensitivity.

In the analyses reported herein, plasma samples were used to measure a panel of 27 cytokines. Five analytes were multiplexed (IL-1β, IL-4, IL-6, IL-10, GM-CSF) and assayed using high sensitivity kits (Millipore, HSCYT-MAG-60SK), and 22 analytes (IFNγ, IFNα2, TNFα,TNFβ, IL12p40, IL12p70, IL1α, IL1RA, IL2, IL5, IL15, GCSF, CCL2, CCL3, CCL4, CCL7, CCL11, CCL22, CXCL1, CXCL8, CXCL10, CX3CL1) were assayed using regular kits (Millipore, HCYTOMAG-60 K). For standard curves, reconstituted top standards (high sensitivity kits, 2000 pg/ml; cytokine-chemokine kits, 10000 pg/ml) were serially diluted 1:3 with assay buffer for nine point curves. Assays were assembled using 96-well plates. Each plate contained samples (in duplicates), standards, and internal quality controls (QCs 1 and 2). Analyte capture was carried out according to manufacturer's instructions (Millipore, Cat # 40–285). Data were acquired using Luminex 100 with xPONENT version 3.1 software, and concentrations measured using BeadView Analysis Software. Plate-specific standard curves were generated using the "Best Fit" curve fitting routine which automatically selects the best curve algorithm for each analyte. When necessary, obvious outliers were omitted prior to generating standard curves. Intra-plate coefficient of variations (CVs) ranged from 1.4 to 7.5 %. Interplate CVs of 30 non-blinded company-supplied control samples varied between 2.7 and 11.9 %, with only four analytes having CVs greater than 10 % (IL-4, IL-6, IL-10, CX3CL1). The CVs observed in our study were only slightly higher than those observed with conventional ELISA assays. Thus, we are confident in the quality of data generated using this method.

Statistical analysis

Cytokines were categorized into tertiles corresponding to 'low, medium, and high'. The proportion of non-detectable values exceeded one-third for 5 cytokines (TNFβ, IL-1α, IL-1RA, IL-2, IL-15), which were categorized as "low", with remaining samples dichotomized at the median for "medium" and "high" levels. Because different subsets of T helper cells regulate each other, often inversely, we calculated several cytokine ratios, primarily to reflect the balance between Th1 and Th2 cell types. If markers were below the detectable assay limit, the median value between zero and the reliable lower limit was assigned to enable these calculations. Specific cutpoints and all ratios examined are shown in Online Resource Table 1, along with detectable assay limits, interplate coefficient of variations for each cytokine measured, and proportion of samples with non-detectable cytokine levels.

Odds ratios (OR) and 95 % confidence intervals (CIs) for associations between cytokine measures and ER^- versus ER^+ tumors and triple negative versus LumA cancers were estimated with unconditional logistic regression. Since ER^- and TNBCs are more prevalent in younger women, we also stratified by menopausal status. To test for linear trend, categorical cytokine levels were assessed in models as a continuous ordinal variable. To test multiplicative interactions by menopausal status, cross-product terms between menopausal status and category-specific log transformed median cytokine levels were included in multivariate models. Using the Wald χ^2 test, statistical significance indicated a difference in slopes associated with cytokine levels between pre- and postmenopausal women. All analyses were further adjusted for AJCC breast cancer stage, histological grade, age and season and timing of blood draw in relation to receipt of treatment (see Online Resource Table 1).

All tests for significance were 2-sided and statistical analyses were conducted using SAS for Windows, Version 9.3, and a false discovery rate (FDR)-adjusted *p* value (*q*) was used to

correct for multiple comparisons [26]. The significance level of individual cytokines was corrected for a total of 27 tests, and cytokine ratios were corrected for a total of 37 tests.

Results

Approximately two-thirds of the women were older than 50 years (Table 1), and the majority had stage I breast cancer; 29 % had ER^- breast cancer, and 22 % had TNBCs. As expected, ER^- and TNBCs were of higher AJCC stage, histological grade, and size compared to ER^+ and LumA cancers, respectively.

Association with ER^- and TNBCs

As shown in Figs. 2 and 3, both ER^- and TNBCs were associated with high levels of circulating IL-5, a Th2 cytokine. Overall, women in the highest versus lowest tertile had approximately 2.5-fold increased odds of ER^- breast cancer or TNBC compared to ER^+ and LumA cancers, respectively. Associations were strongest among premenopausal women, with over fourfold increased odds of ER^- breast cancer (OR = 4.17, 95 % CI 1.86–9.34; *p*-interaction by menopausal status = 0.08) and 5.6-fold increased odds of TNBC (OR = 5.60, 95 % CI 2.09– 15.01; *p*-interaction by menopausal status = 0.12). Although ORs were above unity for postmenopausal women, none were significant. High levels of Th1-related cytokines, IFNα2 and TNFα, were also associated with increased odds of ER^- and TNBC. Compared to women in the lowest tertile, women in the highest tertile were approximately twice as likely to have ER^- versus ER^+ cancers or TNBC versus LumA cancers, with stronger relationships again observed among premenopausal women and not significant among postmenopausal women. Premenopausal women with high IFNα2 levels, for instance, were >4 times more likely to be ER^- (OR = 4.11, 95 % CI 1.70–9.92) than ER^+, with similar estimates for TNBC versus LumA (OR = 4.55, 95 % CI 1.58–12.34). Tests for heterogeneity by menopausal status showed a trend towards statistical significance (*p*-int ≤ 0.09). Complete data for all associations examined are shown in Online Resource Tables 2 and 3.

For several cytokines, associations were limited to either pre- or postmenopausal women. Among premenopausal women, high levels of CX3CL1 were associated with increased likelihood of ER^- versus ER^+ breast cancers (OR = 3.32, 95 % CI 1.33–8.28), but were not significant for TNBC versus LumA cancers (Figs. 2, 3). High levels of IFNγ were associated with two to threefold increased odds of ER^- and TNBCs. Among postmenopausal women, high levels of pro-inflammatory IL-1β were associated with threefold reduced likelihood of both ER^- cancers (OR = 0.35, 95 % CI 0.17–0.73) and TNBC (OR = 0.32, 95 % CI 0.14–0.73), compared to ER^+ or LumA. Postmenopausal women with high IL-10 levels, had reduced odds of ER^- breast cancer (OR = 0.42, 95 % CI = 0.20–0.86) and TNBC (OR = 0.45, 95 % CI 0.20–1.00).

Several cytokine ratios indicating a high Th1 cytokine to IL-5 ratio (i.e., IL-12-p70/IL-5, IL-12-p40/IL-5, TNFα/IL-5, IFNγ/IL-5, IFNα2/IL-5, CXCL10/IL-5) were inversely associated with both ER^- and TNBCs (Figs. 2, 3). Compared to women in the lowest tertile, women in the highest tertiles were approximately 2–2.5 times less likely to have ER^- breast cancer compared to ER^+ cancers, and 2.5–3 times less likely to have TNBC compared to LumA cancers. Associations were strongest among premenopausal women, with women in the highest tertile approximately three times less likely to have ER^- disease and up to five times less likely to have TNBC (CXCL10/IL-5, OR = 0.18, 95 % CI, 0.07–0.49). Among postmenopausal women, high Th1cytokine/IL5 ratios were associated with approximately twofold reduced likelihood of ER^- disease and 2.5-fold reduced odds of TNBC compared to women with low ratios. High CXCL10/CCL7 ratios, an indicator of Th1/Th2 chemokine

balance, were also inversely associated with ER^- and TNBC compared to ER^+ and LumA cancers, respectively, but only among premenopausal women.

The ratio IFNα2/IL-4, also an indicator of Th1/Th2 balance, showed low correlations with IFNα2/IL-5 (spearmean $r = 0.15$, see Online Resource Table 4) and IFN γ/IL-5 (spearman $r = 0.02$), but was moderately correlated with IFNα2/IL-10 (spearman $r = 0.43$), IFNα2/IL-6 (spearman $r = 0.42$), and IFNα2/CCL7 ($r = 0.44$). Women in the highest tertile of IFNα2/IL-4, IFNα2/IL-6, IFNα2/IL-10, or IFNα2/CCL7 had a 1.5- to 2.5-fold greater likelihood of having ER^- versus ER^+ breast cancer compared to women in the lowest tertile, with relationships being statistically significant only in premenopausal women, although tests of heterogeneity by menopausal status were only borderline statistically significant for IFNα2/IL-6 (p-int = 0.07). Ratios of IFNα2/IL-4, IFNα2/IL-10, IFNα2/IL-6, and IFNα2/TNFα, IFNα2/CCL7, and IFNα2/CCL11 were all positively associated with triple negative disease compared to LumA cancers, and high IL-1β/IL-1RA was associated with reduced likelihood, but only among postmenopausal women.

Discussion

This is the first study to examine relationships between circulating cytokines and breast cancer subtypes in a relatively large population. Our most significant finding was that high IL-5 levels, a Th2 cytokine, were associated with increased risk of ER^- compared to ER^+ cancers and TNBCs compared to LumA cancer, particularly among premenopausal women. Pronounced Th1 immune activation, coupled with low levels of IL-5, was associated with the lowest risk of ER^- and TNBCs, compared to ER^+ and LumA cancers, respectively, particularly among premenopausal women. High ratios of IFNα2 with other Th2-related cytokines (IL-4, IL-10, IL-6, CCL2, CCL7, CCL11), and TNFα, however, were associated with increased odds of ER^- and TNBCs compared to ER^+ and LumA cancers. These findings, taken together, support the hypothesis that immune function is associated with development of ER^- and TNBC compared to ER^+ and LumA cancers, and that immune pathways may be physiologically most relevant among younger women who are most likely to be diagnosed with these aggressive phenotypes.

Research on the possible role of cytokines in cancer has largely focused on the tumor microenvironment and their expression in tumor-associated macrophages, which are thought to play an important role in breast cancer progression [27–30]. Few studies, however, have examined circulating cytokine levels and breast cancer risk, which are likely to yield insights into the role of underlying constitutional host immunity on carcinogenesis, and complement findings associated with tumor-driven immune changes. A limitation of previous studies [31–35] has been small sample sizes, all involving fewer than 100 patients. Only one study, with only 53 women, examined relationships between circulating Th1 and Th2-related cytokines and breast cancer subtypes [36].

Of the 27 cytokines measured, high levels of IL-5 were most strongly associated with greater odds of ER^- and TNBC compared to ER^+ and LumA cancers, respectively. IL-5 is produced by $CD4^+$ Th2 lymphocytes and is specific in driving eosinophil-dependent inflammatory diseases typically associated with allergic diseases such as asthma and with parasitic worm (helminth) infections [37–41]. GM-CSF, CCL3, CCL7, CXCL8, but not CCL11, as other cytokines involved in eosinophil chemotaxis [42] were also related to increased odds of ER^- and TNBC among premenopausal women, but not as strongly as for IL-5 (Online Resource Tables 2 and 3). Associations were strongest among premenopausal women, possibly because estrogens stimulate IL-5 production and eosinophil mobilization [43]. Exacerbation of asthma, for instance, is common during pregnancy [44]. A meta-analysis of atopic diseases with breast cancer risk, however, did not show any associations

with asthma or “any allergy”, although effect estimates were not adjusted for effective management of these conditions as potential confounders that might attenuate risk estimates, and specific breast cancer subtypes were not considered [45].

Although our study was conducted in a Caucasian population, associations between immune factors, particularly IL5, and ER^- breast cancer could inform a better understanding of the high prevalence of ER^- breast cancer in African-American (AA) women, who also experience more allergies and asthma [1, 6, 46–48]. The shift towards Th2 immunity among AAs is thought to be an evolutionary response to endemic exposure to helminths in sub-Saharan Africa [49, 50]. Previous observations that risk of TNBCs is associated with increasing parity may also, in part, be mediated by shifts towards Th2 immunity [6, 51–53] since pregnancy is characterized by lower Th1 cytokines, increased Th2 cytokines, and a reduction in the Th1/Th2 ratio [54–59]. Restoration to normal Th1/Th2 immunity occurs postpartum [60]. Interestingly, one study found decreased serum Th1/Th2 ratio, consistent with continued depressed cellular immunity, among formula-feeding mothers, but not among women who breastfed, suggesting that lactation may confer some immunological benefit to mothers [61]. This shift, possibly mediated by prolactin leads to Th1 activation [62, 63], may explain why lactation ameliorates the increased risk of ER^- and TNBC among African-Americans [6].

In our data, both ER^- and TNBCs were associated with higher levels of Th1 cytokines IFNγ, TNFα, and IFNα2, compared to ER^+ and LumA cancers. Pro-inflammatory cytokines produced by $CD4^+$ T cells enhance killer $CD8^+$ T cells, have direct toxic effects on tumor cells, and can activate anti-angiogenic mechanisms [17, 64, 65]. These findings indicate a greater degree of immune activation, likely reflecting the increased disease aggressiveness of ER^- and TNBCs. Consistent with this interpretation, others have found IL-12 levels, the main cytokine regulating Th1 differentiation, to be higher in breast cancer patients compared to healthy controls, and correlated with more advanced disease stage [66, 67]. Impaired IFNα signaling has been observed in stage II or higher breast cancers and is hypothesized to be a common immune defect in human cancers [68]. As an early response cytokine, IFNα might be pivotal in cancer control because of its role in priming the host immune response, increasing natural killer (NK) cell cytotoxicity, and transitioning the immune system from an innate to an adaptive immune response through a number of mechanisms including increases in Th1/Th2 balance [69–73].

The balance of Th1 and Th2 cell populations is implicated in a number of diseases, including cancer [74–80]. A strong Th1 response is critical for effective antitumor immunity, whereas Th2 responses mediated by IL-4 can potentiate M2-bioactivity of tumor associated macrophages, leading to growth promotion and metastasis [17, 81]. In our study, high ratios of Th1 cytokines to the Th2 cytokine IL-5 (IL12/IL-5, IFNα2/IL-5, TNFα/IL-5, IFNγ/IL-5, CXCL10/IL-5) were strongly associated with reduced risk of ER^- compared to ER^+ cancers or TNBCs compared to LumA cancers, but ratios of IFNα2 to other Th2 cytokines (IL-4, IL-6, IL-10, CCL2, CCL11) went in the opposite direction. The reasons for this are unclear, but may indicate more nuanced effects of Th2-related immunity on risk of ER^- and TNBC, including potential influences by other modulators of immune function such as macrophages, regulatory T cells and Th17 cells [81–85]. IL-4, for instance, has pleiotropic effects and can inhibit basal and estrogen-induced cell proliferation and induce apoptosis in breast cancer cells [86–89], and in certain situations promote a Th1 immune response [90–93].

Our findings between circulating cytokines and ER^- and TNBCs are unlikely due to tumor-driven changes in plasma cytokine levels. Previous study on expression of cytokine levels in breast tumors suggest that Th1 and Th2 cytokines, including IFNγ and IL-5, are not major

cytokines produced by tumors [94–96], with IL-5 being non-detectable in both breast tumors and normal breast tissue [94]. Rather, several inflammatory cytokines produced by monocytes (IL-1β, IL-6, CCL2) or macrophages (such as IL-1β, IL-6, CXCL8, CCL4, and TNFα) appear to be more highly expressed in tumors [94, 97–99], with inflammatory cytokines CXCL8, IL-6, and CCL2 levels being more highly expressed in ER^- cancers [94, 97, 98, 100, 101]. Nevertheless, given that our study design is cross-sectional, we cannot rule out the possibility that breast tumors or different breast cancer subtypes can potentially influence systemic immunity differently. Thus, our findings will need to be confirmed prospectively in a future study.

Our multivariate models did not adjust for lifestyle factors or comorbidities that are potentially associated with cytokine levels, such as body mass index and use of anti-inflammatory medications, because circulating cytokines may be within the causal pathway of these factors with respect to breast cancer risk. Thus, their inclusion in analytic models may lead to attenuation of effect estimates. The approach of examining circulating cytokine levels as a biomarker of immune phenotype without adjustments for factors that can potentially impact their levels also allowed for examination of "effective" exposure to these cytokines after accounting for multivariate influences, whether measured or unmeasured. Because disease-free controls were not included in this study, our findings show immune differences between ER^- and TNBCs in relation to less aggressive ER^+ and LumA breast cancers. Future studies are required to determine if circulating cytokines, as contributors to immune regulation, are related to increased risk of either ER^- and/or ER^+ disease compared to healthy women without breast cancer. A notable strength in our study, was the use of blood samples from treatment naïve breast cancer patients, which allowed us to examine differences in host immune phenotype unconfounded by treatment effects.

In summary, our findings show immune pathways to be important in ER^- and TNBCs particularly among younger women. Future prospective research involving a larger study population with samples drawn prior to disease diagnosis is needed to validate findings and determine if circulating cytokine levels are related to differences in immune cell profiles at the tumor site, and whether these immune-related differences predict response to therapy and/or disease prognosis. Another important area of investigation will be to determine whether our findings hold for African-American women, who are more likely to be diagnosed with ER^- and TNBCs. If future research establishes a role for IL-5 in the etiology of ER^- breast cancer, promising adjunct treatment strategies for ER^- and TNBC might include IFNα2, which increase Th1 versus Th2 balance, and have been clinically employed as antineoplastic therapeutic drugs [69, 72, 73], as well as IL-5-targeted therapies, such as the monoclonal antibodies that have been developed to neutralize IL-5 or target IL-5 R for the treatment of eosinophilic diseases [102–105]. Our findings also indicate the possibility that among women diagnosed with ER^- or TNBC, who also have an underlying Th-2 related disease, such as asthma, an effort to optimally manage these conditions might result in improvements in breast cancer outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AJCC	American Joint Committee on Cancer
BRCA1	Breast cancer type 1 susceptibility protein
CCL2	Chemokine (C–C motif) ligand 2
CCL7	Chemokine (C–C motif) ligand 7
CCL11	Chemokine (C–C motif) ligand 11
CK	Cytokeratin
CXCL10	Chemokine (C–X–C motif) ligand 10
DBBR	Data Bank and Biorepository
EGFR$^+$	Epidermal growth factor receptor positive
ER	Estrogen receptor
ER$^+$	Estrogen receptor positive
ER$^-$	Estrogen receptor negative
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HER2$^-$	Human epidermal growth factor receptor 2 negative
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IL1RA	Interleukin-1 receptor antagonist
IP-10	Interferon gamma-induced protein 10
IRB	Institutional Review Board
LumA	Luminal A
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MIP-1	Macrophage inflammatory protein-1
QC	Quality control
RPCI	Roswell Park Cancer Institute
TAMS	Tumor-associated macrophages
Th1	T-helper type 1
Th2	T-helper type 2
Th17	T-helper type 17
TNBC	Triple negative breast cancer
TNF	Tumor necrosis factor

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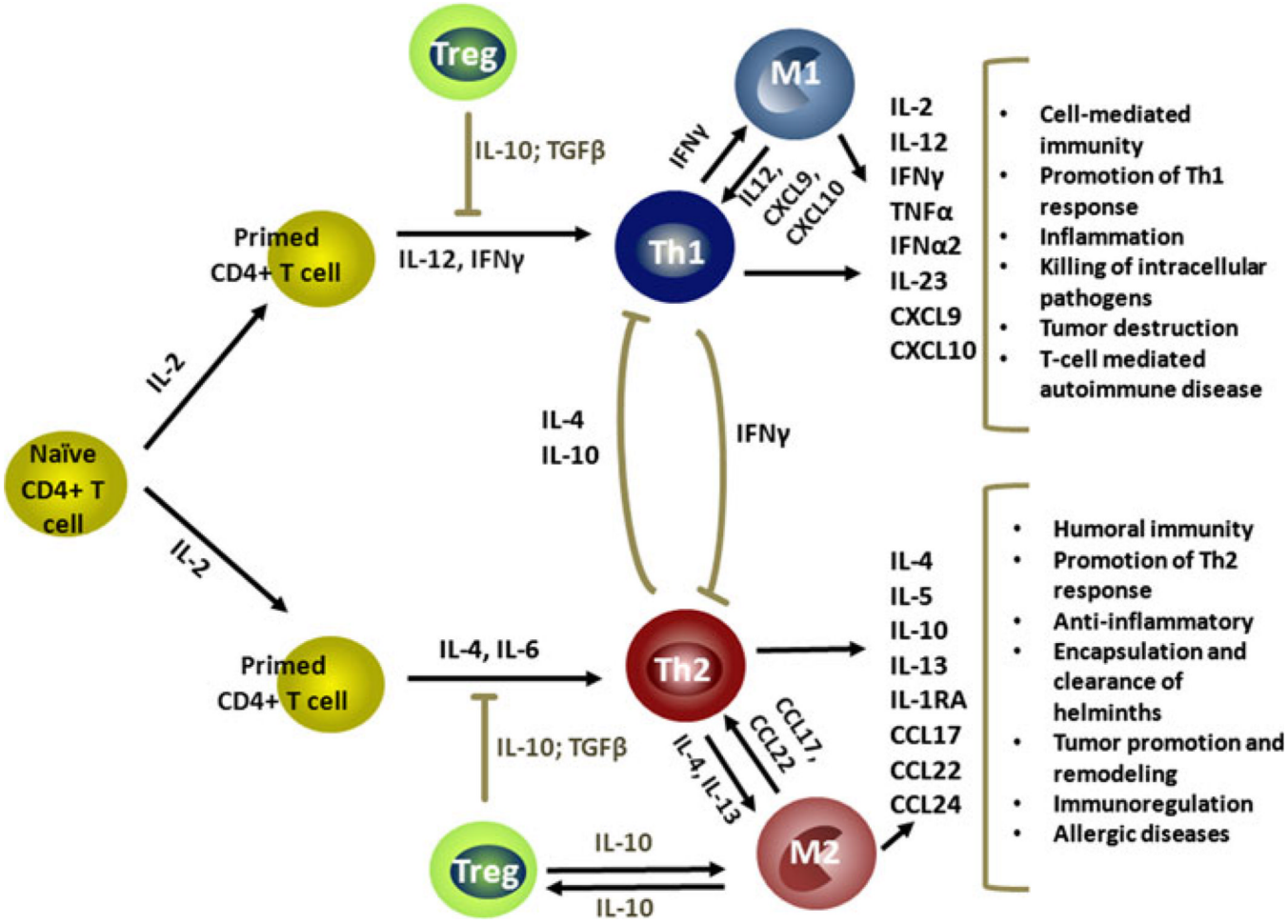

Fig. 1.

Role of cytokines in Th1 and Th2 differentiation of lymphocytes. $CD4^+$ T-helper lymphocytes are able to augment both cellular and humoral immune responses. During an immune response, cytokines as a component of the microenvironment present during T-cell priming can influence the developmental pathway taken by responding naïve $CD4^+$ T cells. The Th1 developmental pathway is largely driven by IL-12 activation, producing high levels of IFNγ and TNFα upon antigen stimulation, and is responsible for regulating cell-mediated immunity. The Th1 cytokine IFNγ also polarizes macrophages (innate immune cells) towards the M1 phenotype. Th2 differentiation, in contrast, is largely driven by IL-4 and is characterized by IL-4, IL-5, IL-10, and IL-13 secretion, which are primarily involved in the coordination of humoral immunity, eosinophilic inflammation, and control of helminth infections. The Th2 cytokines IL-4 and IL-13, as well as CCL2 from monocytes, drives macrophages toward M2 polarization leading to high expression of IL-10 and IL1RA. The Th1 and Th2 pathways are regulated by a balance of positive and antagonistic feedback loops. IFNγ enhances further Th1 development and suppresses Th2 differentiation, while IL-4 and IL-10 supports Th2 differentiation and suppresses Th1 differentiation. In addition, $CD4^+$ $CD25^+$ $Foxp3^+$ T-regulatory cells (Tregs), a subset of $CD4^+$ T cells, mediate general immune suppression and can drive macrophages toward “M2-like” phenotypes, which are important for the prevention of autoimmunity, but can suppress anti-tumor immunity [17, 80, 106, 107]

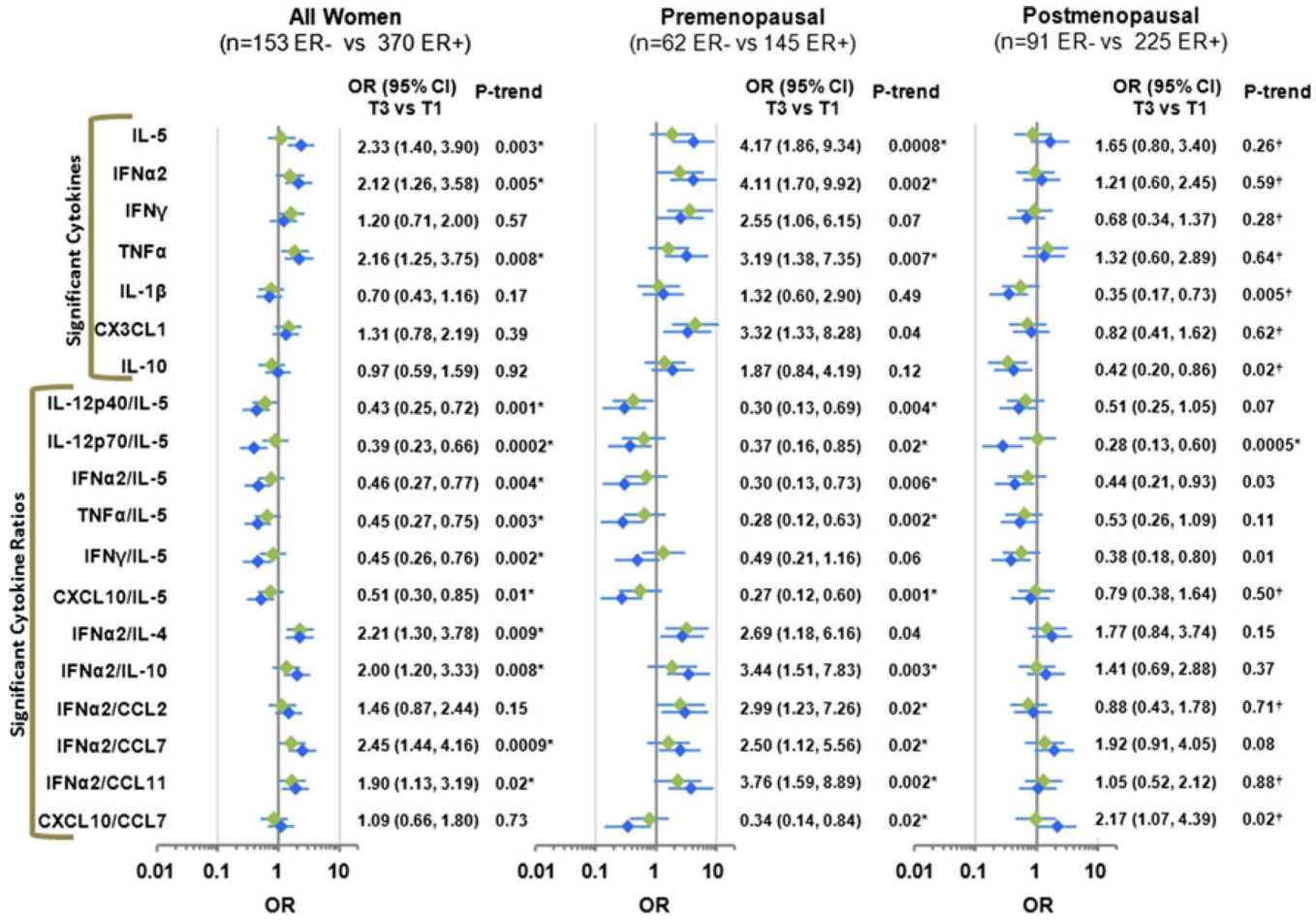

Fig. 2.

Statistically significant associations between circulating immune markers and ER$^-$ breast cancer among women with invasive breast cancer. Odds ratios and 95 % CI provided for tertile 2 (◆) and tertile 3 (◆) of cytokines or cytokine ratios compared to tertile 1 as the reference group. All logistic regression models were adjusted for age at diagnosis (continuous), season (Apr-Sept, Oct-Mar), date of blood draw (continuous), timing of blood draw in relation to surgery and treatment (No prior treatment, postsurgical with no adjuvant treatment or receipt of neoadjuvant or adjuvant treatment), stage (I, IIA, IIB, IIIa, IIIB/IIIC/IV), grade (I, II, III, unknown). Full results for all cytokines and ratios are provided in Online Resource Table 2. *FDR-adjusted *p* values remain significant or borderline significant (*q*-trend ≤ 0.07) after significance for cytokines are corrected for a total of 27 cytokine tests, and cytokine ratios are corrected for a total of 37 tests. †Using the Wald χ^2 test, a difference in slopes associated with cytokine levels (as the log-transformed category specific median value analyzed as a continuous variable) was observed between pre- and postmenopausal women (*p*-int ≤ 0.10)

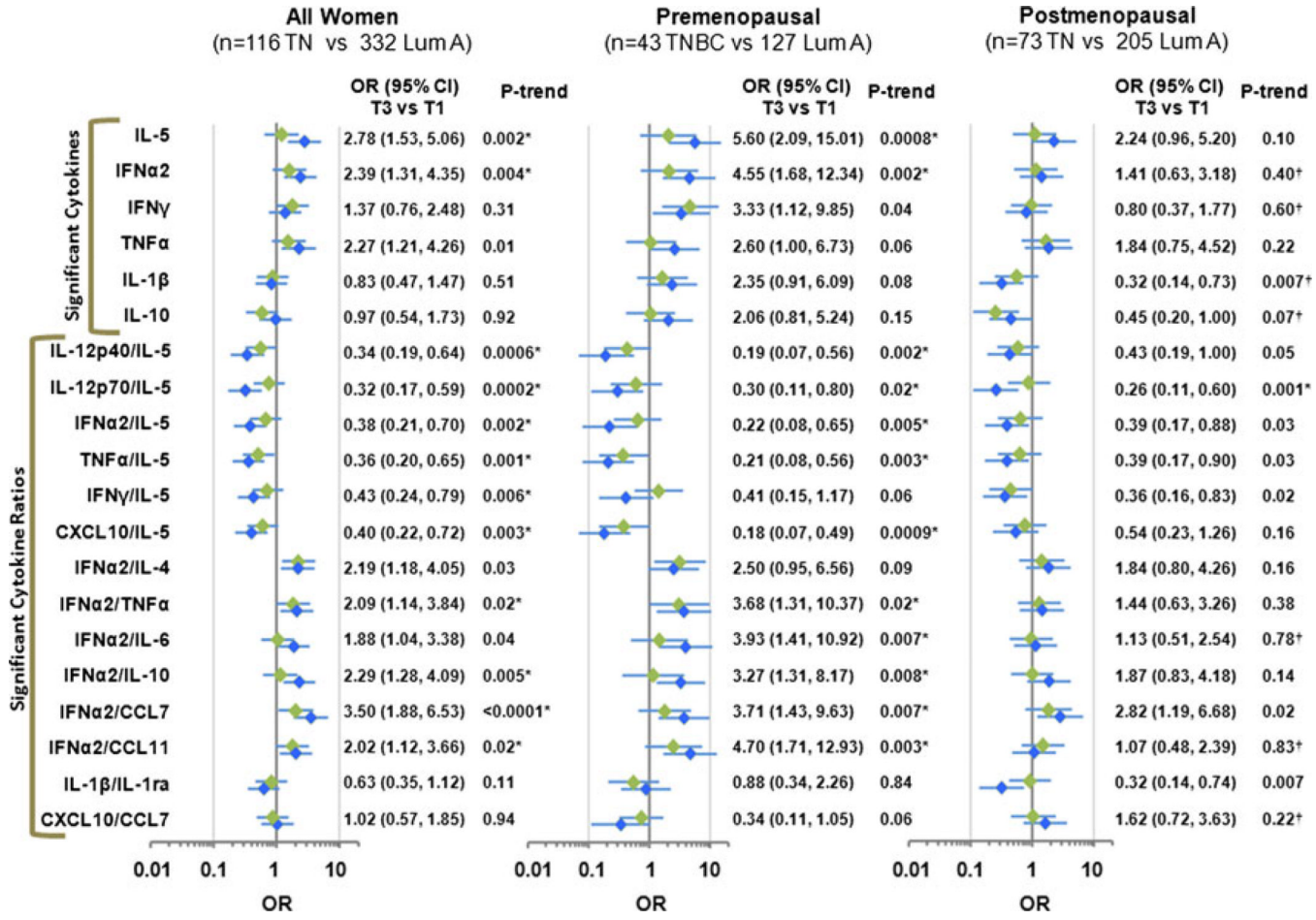

Fig. 3.

Statistically significant associations between circulating immune markers and risk of triple negative versus LumA breast cancer. Eight women with undetermined tumor subtype were excluded from analyses. Odds ratios and 95 % CI provided for tertile 2 (◆) and tertile 3 (◆) of cytokines or cytokine ratios compared to tertile 1 as the reference group. All logistic regression models were adjusted for age at diagnosis (continuous), season (Apr–Sept, Oct–Mar), date of blood draw (continuous), timing of blood draw in relation to surgery and treatment (No prior treatment, postsurgical with no adjuvant treatment or receipt of neoadjuvant or adjuvant treatment), stage (I, IIA, IIB, IIIa, IIIB/IIIC/IV), and tumor grade (I, II, III, unknown). Full results for all cytokines and ratios are provided in Online Resource Table 3. *FDR-adjusted *p* values remain significant or borderline significant (*q*-trend ≤ 0.07) after significance for cytokines are corrected for a total of 27 cytokine tests, and cytokine ratios are corrected for a total of 37 tests. †Using the Wald χ^2 test, a difference in slopes associated with cytokine levels (as the log-transformed category specific median value analyzed as a continuous variable) was observed between pre- and postmenopausal women (*p*-int ≤ 0.10)

Table 1

Participant characteristics of 523 women with invasive breast cancer

Characteristics	All women	ER status			Subtype		
		ER^+ (*n* = 370)	ER^- (*n* = 153)	*p*	LumA (*n* = 332)	TNBC (*n* = 116)	*p*
Age at diagnosis, years, mean ± SD, *n* (%)	56.8 ± 12.9	57.6 ± 12.8	55.0 ± 13.2	0.03	57.6 ± 12.7	56.8 ± 12.9	0.53
<50 years	172 (32.9)	118 (31.9)	54 (35.3)	0.12	105 (31.6)	35 (30.2)	0.52
50–64 years	202 (38.6)	137 (37.0)	65 (42.5)		124 (37.4)	50 (43.1)	
≥65 years	149 (28.5)	115 (31.1)	34 (22.2)		103 (31.0)	31 (26.7)	
Body mass index, kg/m^2, mean ± SD[a]	28.2 ± 6.4	28.4 ± 6.5	27.6 ± 6.2	0.24	28.5 ± 6.6	27.9 ±6.1	0.40
<25	156 (29.8)	111 (30.0)	45 (29.4)	0.23	99 (29.8)	32 (27.6)	0.12
25–<30	173 (33.1)	118 (31.9)	55 (36.0)		103 (31.0)	40 (34.5)	
≥30	174 (33.3)	130 (35.1)	44 (28.8)		120 (36.1)	35 (30.2)	
Unknown	20 (3.8)	11 (3.0)	9 (5.9)		10 (3.0)	9 (7.8)	
Menopausal status, *n* (%)				0.78			0.82
Premenopausal	207 (39.6)	145 (39.2)	62 (40.5)		127 (38.3)	43 (37.1)	
Postmenopausal	316 (60.4)	225 (60.8)	91 (59.5)		205 (61.8)	73 (62.9)	
Stage, *n* (%)				0.002			0.002
I	304 (58.1)	236 (63.8)	68 (44.4)		217 (65.4)	55 (47.4)	
IIA	119 (22.8)	77 (20.8)	42 (27.5)		66 (19.9)	32 (27.6)	
IIB	43 (8.2)	23 (6.2)	20 (13.1)		18 (5.4)	16 (13.8)	
IIIA	34 (6.5)	21 (5.7)	13 (8.5)		20 (6.0)	6 (5.2)	
IIIB/IIIC/IV	21 (4.0)	12 (3.2)	9 (5.9)		11 (3.3)	6 (5.2)	
TX	2 (0.4)	1 (0.3)	1 (0.7)			1 (0.9)	
Histological grade, *n* (%)				<0.0001			<0.0001
I	43 (8.2)	42 (11.3)	1 (0.7)		40 (12.1)	1 (0.9)	
II	132 (25.2)	114 (30.8)	18 (11.8)		105 (31.6)	14 (12.1)	
III	341 (65.2)	210 (56.8)	131 (85.6)		185 (55.7)	99 (85.3)	
Unknown	7 (1.3)	4 (1.1)	3 (2.0)		2 (0.6)	2 (1.7)	
Number of positive nodes				0.02			0.12
0	361 (69.0)	262 (70.8)	99 (64.7)		236 (71.1)	83 (71.5)	
1–2	88 (16.8)	62 (16.8)	26 (17.0)		53 (16.0)	19 (16.4)	

Characteristics	All women	ER status			Subtype		
		ER$^+$ (*n* = 370)	ER$^-$ (*n* = 153)	*p*	LumA (*n* = 332)	TNBC (*n* = 116)	*p*
≥3	57 (10.9)	31 (8.4)	26 (17.0)		29 (8.7)	14 (12.1)	
Unknown	17 (3.3)	15 (4.1)	2 (1.3)		14 (4.2)	0 (0)	
Tumor size, cm, mean ± SD[b]	1.7 ± 1.3	1.6 ± 1.2	2.0 ±1.6	0.003	1.6 ± 1.2	2.0 ± 1.4	0.006
<1 cm	122 (23.3)	90 (24.3)	32 (20.9)	0.002	83 (25.0)	22 (19.0)	0.0002
1–<2 cm	263 (50.3)	200 (54.0)	63 (41.2)		184 (55.4)	50 (43.1)	
≥2 cm	126 (24.1)	74 (20.0)	52 (34.0)		62 (18.7)	38 (32.8)	
Unknown	12 (2.3)	6 (1.6)	6 (3.9)		3 (0.9)	6 (5.2)	

[a] Excludes 20 women with missing BMI values

[b] Excludes 12 women with unknown tumor size