CD4⁺ follicular helper T cell infiltration predicts breast cancer survival

Chunyan Gu-Trantien, … , Christos Sotiriou, Karen Willard-Gallo

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CD4⁺ T cells are critical regulators of immune responses, but their functional role in human breast cancer is relatively unknown. The goal of this study was to produce an image of CD4⁺ T cells infiltrating breast tumors using limited ex vivo manipulation to better understand the in vivo differences associated with patient prognosis. We performed comprehensive molecular profiling of infiltrating CD4⁺ T cells isolated from untreated invasive primary tumors and found that the infiltrating T cell subpopulations included follicular helper T (Tfh) cells, which have not previously been found in solid tumors, as well as Th1, Th2, and Th17 effector memory cells and Tregs. T cell signaling pathway alterations included a mixture of activation and suppression characterized by restricted cytokine/chemokine production, which inversely paralleled lymphoid infiltration levels and could be reproduced in activated donor CD4⁺ T cells treated with primary tumor supernatant. A comparison of extensively versus minimally infiltrated tumors showed that CXCL13-producing CD4⁺ Tfh cells distinguish extensive immune infiltrates, principally located in tertiary lymphoid structure germinal centers. An 8-gene Tfh signature, signifying organized antitumor immunity, robustly predicted survival or preoperative response to chemotherapy. Our identification of CD4⁺ Tfh cells in breast cancer suggests that they are an important immune element whose presence in the tumor is a prognostic factor.

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Chunyan Gu-Trantien,1 Sherene Loi,2 Soizic Garaud,1 Carole Equeyer,1,2 Myriam Libin,1 Alexandre de Wind,3 Marie Ravoet,1 Hélène Le Buanc,1 Catherine Sibille,1 Germain Manfouo-Foutso,1 Isabelle Veys,4 Benjamin Haibe-Kains,2,5 Sandeep K. Singhal,6 Stefan Michiels,2 Françoise Rothé,2 Roberto Salgado,3 Hugues Duvillier,1 Michail Ignatiadis,2 Christine Desmedt,2 Dominique Bron,6 Denis Larsimont,3 Martine Piccart,7 Christos Sotiriou,2 and Karen Willard-Gallo1

1Molecular Immunology Unit, 2Breast Cancer Translational Research Laboratory JC Heuson, 3Anatomical Pathology, and 4Department of Surgery, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium. 5Computer Science, Université Libre de Bruxelles, Brussels, Belgium. 6Department of Hematology and 7Department of Medical Oncology, Institut Jules Bordet, Université Libre de Bruxelles, Brussels, Belgium.

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Introduction
Breast cancer (BC) is a complex and heterogeneous disease whose classification has been significantly improved in recent years through the development of gene expression signatures that currently identify 4 clinically distinct neoplastic diseases: luminal A and B, ER+ and HER2+ (1, 2). However, significant disparity in clinical outcome still remains within each of these disease entities, leading investigators to continue searching for more refinement. Initially, prognostic molecular signatures focused on the malignant cell, improving BC taxonomy via the assessment of genes regulating the cell cycle and proliferation (3, 4). More recently, attention has turned to the various nonneoplastic cells present in tumors, including stromal cells and infiltrating leukocytes, whose interactions with tumor cells can have a major influence on an individual patient’s long-term outcome. Clinical studies using prognostic and predictive signatures have shown that the strength of the immune signal emanating from whole tumor gene expression profiles reflects the level of immune infiltration. A high immune signal has been linked with improved patient outcome in a variety of cancers (5), including subtypes of BC, with the strongest correlation for the latter observed in the ER+ and HER2+ subtypes (6–13). Although there is not a consensus on the individual immune cell subset(s) that consistently mediates this effect, an important link between preexisting antitumor immune responses and long-term positive clinical outcome has been established (14).

Traditionally, CD8+ cytotoxic T cells have been considered as the key component of effective antitumor immunity, and breast tumors with higher levels of infiltrating CD8+ T cells have been associated with better patient survival (15, 16). However, studies have also shown that CD8+ T cells frequently fail to fully function in vivo if there is a lack of adequate CD4+ T cell help (17, 18). As central players in the immune system, CD4+ T cells perform critical roles in recruiting, activating, and regulating many facets of the adaptive immune response, with their helper functions for B cell- and CD8+ cytotoxic T cell-mediated responses well documented. CD4+ T cells also influence innate immunity by helping to shape the character and magnitude of the inflammatory response.

One common observation from functional studies is that many of the infiltrating leukocyte subpopulations do not expand or function normally within the tumor microenvironment. The current consensus is that IFN-γ–producing CD4+ Th1 and CD8+ T cells, along with mature DCs, NK cells, M1 macrophages, and type 1 NKT cells can generate effective although frequently attenuated antitumor responses, while CD4+ Th2 cells and type 2 NK T cells in cooperation with CD4+ Tregs (regulatory), myeloid-derived suppressor cells, immature DCs, or M2 macrophages suppress antitumor immunity and can also promote tumor progression (19–21). However, this generalization comes with the caveat that variation exists among tumor types, with the so-called protumorigenic cells, such as CD4+ Th17, also shown to produce effective antitumor responses (20, 22, 23). Emerging from these studies is recognition that the various CD4+ Th subsets have important functions in both protumor and antitumor immunity, with IFN-γ–producing
Infiltrating CD4+ T cells

Cluster dendrogram with BP values (%)

CD4+ peripheral blood (PB)
CD4+ Tumor (TIL)
CD4+ Lymph node (LN)

Th1

Th2

Th17

Th17'

CD25

CD25'
Th1 cells currently most frequently associated with superior anti-
tumor immune responses. In BC, the majority of previous inves-
tigations focusing on CD4+ T cells have examined the suppressive
activities of Tregs in dampening immune functions (24).

This study was undertaken to characterize the CD4+ T cell
subpopulations infiltrating human breast tumors in a direct ex
vivo analysis of fresh tumor tissue without enzymatic digestion
or short-term in vitro expansion. We profiled gene expression in
purified CD4+ T cells from the primary tumors (referred to herein
as TIL), axillary LN, and peripheral blood (PB) to produce global
images of these cells in their native state. An important finding
from our work is that extensively infiltrated tumors are less immu-
nosuppressive and have a higher frequency of organized LN-like
structures associated with detectable CXCL13-producing CD4+
follicular helper T (Tfh) cells. Tertiary lymphoid structures (TLS)
have been previously identified in lung and colorectal cancers, and
their presence has been linked with positive patient prognosis (25–27).

In this study we show that Tfh cells are an important constituent
of TLS in breast tumors. Animal models have demonstrated that
Tfh cells in LNs are highly activated and participate in various
steps leading to germinal center (GC) development and differenti-
ation of memory B and antibody-secreting plasma cells (28). The
data presented here suggest that CXCL13-producing Tfh TIL may
play an important role in immune cell recruitment to the tumor
microenvironment and influence TLS formation, thereby helping
to create a haven in the tumor mass in which effective and durable
antitumor immune responses can be generated.

Results

The immune infiltrate in BC. Freshly resected invasive breast tumor
tissue from systemically untreated patients (a graphical overview
for navigating the experimental data is provided in Supplemental
Table 1A; supplemental material available online with this article;
doi:10.1172/JCI67428DS1; clinicopathological characteristics for
the patient discovery set \(n=10\) in Supplemental Table 1B and
patient confirmation set \(n=60\) in Supplemental Table 1C) was
rapidly mechanically dissociated into a single cell homogenate
using the GentleMACS system for immediate ex vivo analysis
following CD4+ T cell isolation. The distribution of infiltrating
CD45+ cells (Supplemental Figure 1) included a majority of T
cells (±75%; range = 60–90%) together with <20% B cells, <10%
monocytes, <5% NK cells, or NKT cells, similar to a recently pub-
lished study (29). Among the CD3+ T cells, the CD4+ subset was
consistently the principal component (Figure 1A; mean = 53% in
35 tumors), outnumbering CD8+ T cells and present both at a
higher frequency and ratio than in ipsilateral nonadjacent normal
breast tissue (mean = 38% in 31 samples).

Features of the CD4+ T cell infiltrate. Global gene expression pro-
files of the TIL were compared with CD4+ T cells from patient’s
axillary LN and PB (P-PB) and PB from healthy donors (D-PB)
(Supplemental Table 1B). Bootstrap analysis revealed stable
groupings by tissue type, with patient and donor blood segre-
gating together (Figure 1B). This observation could be partially
due to differences in the ratio of naive (CD45RA+) to memory
(CD45RO+) CD4+ T cells, with generally about 50% CD45RO+ in
PB, >50% in LN, and >95% in TIL (expression of the 58 Th surface
markers investigated was also examined on naive and memory
donor CD3+CD4+ T cells as a control; Supplemental Figure 2).
The gene expression profiles were analyzed in a variety of compar-
sions, including TIL compared with LN or P-PB, P-PB compared
with D-PB, and ER+ compared with ER− tumors (Supplemental
Table 2). The extent of the CD4+ T cell infiltrate (IHC; Supple-
mental Table 1B) revealed that 2 ER+ and 2 ER− tumors were
extensively infiltrated while 3 ER− and 3 ER+ tumors were min-
imally infiltrated, with these groups also compared. Our analyses
initially (a) concluded that the CD4+ T cell profiles were remark-
ably similar between P-PB and D-PB (Supplemental Table 2E),
with the autologous cells constituting a good internal control;
(b) found surprisingly few gene changes between ER+ and ER−
tumors (Supplemental Table 2F); and (c) detected important
differences in immune response genes between extensively and
minimally infiltrated tumors (Supplemental Table 2G), which
will be detailed throughout this article (extensively infiltrated is
defined as overall >5% intratumoral and >10% stromal lympho-
cytes in multiple sections of the resected invasive tumor; criteria
detailed in Supplemental Table 1C).

Molecular images of the CD4+ Th subsets present in the TIL
were produced by comparing our patient data with public Th
gene expression data sets (refs. 30–32; reanalyzed applying our
small group criteria [ref. 33] which are detailed in the Supple-
mental Methods). Our innovative use of these public data sets
allowed us to detect the presence of each CD4+ T cell subpop-
ulation in the TIL and extend the individual Th subset profiles
beyond conventional markers with relative specificity. A direct
comparison of patient CD4+ T cell data with the public Th gene
lists produced distinct gene sets (Figure 1, C–H; Figure 2, A–C;
and Supplemental Table 3) showing that (a) D-PB and P-PB are
relatively similar across all Th subsets, with a slight increase in
effectors memory cell markers in P-PB; (b) LN profiles are transi-
tional between P-PB and TIL, with upregulation of the Tfh genes
CD200, CXCL13, and ICOS and the Th1 genes CD38 (34), CXCL9,
IFNG, and IL2, which are notably among the limited number of
gene alterations detected (Figure 3); (c) the TIL profile is enriched
in all major Th subpopulations (Th1, Th2, Tfh, Treg, and Th17);
(d) the TIL are activated CD25+ cells; and (e) the TIL have a mem-
ory phenotype closer to that of effector than that of central mem-
ory cells. Expression levels of conventional Th markers (Supple-
mental Table 3K) reiterated that all Th subpopulations were
present in the TIL but did not provide definite information on their relative distribution. Alternatively, the comparison of TIL from extensively versus minimally infiltrated tumors revealed a distinct skew to Tfh (CD200, CXCL13, ICOS, and PDCD1 [known as PD-1]) and Th1 (CD38, CXCL9, CXCL10, CXCL11, IFNG, and TBX21 [also known as T-bet]) gene expression in the extensively infiltrated tumors (Figure 3). The Treg marker FOXP3 was also increased in extensively versus minimally infiltrated tumors (Figure 3), but the Treg produced ligand TGFβ2 was decreased in this comparison (both were significantly increased in TIL com-

Figure 2
Characteristics of CD4+ T cells infiltrating breast tumors. Purified CD4+ T cells were from fresh tumor homogenates, LNs, P-PB, or D-PB (patient discovery set; Supplemental Table 1B). (A–C) Statistically significant gene changes established for the comparison of TIL versus P-PB (Supplemental Table 2B) were compared with public microarray data sets of human Th subpopulations that we reanalyzed (Supplemental Methods) as in Figure 1, C–H. (D) Histogram or dot plot thumbnails for 36 Th surface markers (genes in A–C and Figure 1, C–H; complete profiles plus 24 additional markers in Supplemental Figure 2) expressed on CD4+ TIL (variation is indicated by shades of blue) and D-PB (red). The genes underlined in aqua were also analyzed by qRT-PCR on a larger patient group (Figure 8).
### Figure 3

Conventional Th subset marker expression in TIL. (A–C) Th subset marker genes were quantified in the patient confirmation set by (A and B) qRT-PCR and (C) flow cytometry. (A) Mean ΔCt values (relative to the Th-specific endogen CASC3) are inversely proportional to the relative intensity of gene expression; CD4+ TIL (n = 6) are compared with D-PB (n = 6). (B) CD4+ TIL from extensively infiltrated tumors (n = 6) are compared with minimally infiltrated tumors (n = <18). "n = <18" indicates that the number of minimally infiltrated tumors assessed was as noted. (C) Protein expression was assessed by flow cytometry (Supplemental Figure 2). (D–F) Microarray data from the patient discovery set and (G) qRT-PCR data of memory versus total CD4+ T cells from healthy donor cells are shown for comparison with (D) CD4+ T cells isolated from the first tissue (i.e., TIL) relative to the second tissue (i.e., P-PB), (E) CD4+ T cells isolated from ER– relative to ER+ tumors, (F) CD4+ T cells isolated from extensively infiltrated tumors relative to minimally infiltrated tumors, and (G) D-PB CD4+CD45RO+ memory T cells (n = 3) compared with total CD4+ T cells (n = 3). (H) Microarray data of D-PB memory CD4+ T cells after S or tumor SN treatment (Figure 6 and Supplemental Table 6). Fold change or ratio values are shown as blue (downregulated) and orange (upregulated); P values (2-tailed Student’s t test with unequal variance) in green are significant (P < 0.05); n.d., not determined (for qRT-PCR data no data = n.d.); nc, no change (for microarray data empty cells = nc). For gene symbols, "v" indicates transcript variant.
pared with D-PB). This suggests that the observed increase in FOXP3 expression may be related to its transient expression by activated Th cells rather than a greater presence of Tregs in the extensively infiltrated tumors.

Flow cytometric analysis of CD4+ TIL from additional patients with BC compared with CD4+ T cells from healthy donor blood (Supplemental Table 1C) largely validated these data and provided more quantitative information, demonstrating that the CD4+ TIL are antigen-experienced effector memory cells (CD45RO+), characterized either wholly or in a subpopulation of cells as TCR/CD3lo CD6lo CD7lo CD11b+ CD18+ CD24+ CD25+ CD26+ CD27lo CD28lo CD35– CD38+ CD43lo CD44+ CD49D+ CD54hi CD55lo CD57lo CD58hi CD59+ CD62Llo CD69+ CD73+ CD80+ CD86+ CD109+ CD146+ CD166+ CD200+ CCR4+ CCR5+ CCR6lo CCR7lo CXCR3+ CXCR4+ CXXCR5+ 4-1BB+ BTLA+ CRTH2+ CTLA4+ FAShi GITR+ HLA-DR+ ICOS+ IL1R1+ IL2RB+ IL6STlo IL7R+ IL12RB2+ IL17RB+ OX40LOX40L+ PD-1+ TGFBR2+ TGFBR3+ (Supplemental Figure 2). The heterogeneity observed in some activation-induced markers (e.g., CD25, CD71, HLA-DR) suggests that not all of the cells are activated. Upregulation of conventional Th surface markers on CD4+ TIL subpopulations was detected (Supplemental Figure 2) and included (a) the Th1-associated BTLA, CD200hi, ICOS, and PD-1hi; (b) the Th1-associated CD38hi, CCR5, CD54, and IL12RB2; (c) the Th2-associated CCR4; (d) the Th17-associated IL1R1; and (e) the Treg-associated CD25lo and GITR. Histogram or dot plot thumbnails are shown for Th surface marker genes present in the Figure 1, C–H, and Figure 2, A–C, heat maps (Figure 2D; full histograms or dot plots in Supplemental Figure 2). Interestingly, among differentially expressed surface markers

**Figure 4**

TCR/CD3 pathway gene expression in CD4+ TIL. Schematic representation of TCR/CD3 pathway and coreceptor gene expression derived from microarray (Supplemental Table 2, B and C), qRT-PCR (Supplemental Table 5B), and flow cytometry (Supplemental Figure 2) data, with individual genes detailed in Supplemental Table 4. Gene expression changes in the TIL versus P-PB and/or TIL versus LN data comparisons are shown (blue, downregulated; orange, upregulated).
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(Figure 2D, underlined in blue), CD38, CD200, ICOS, and PD-1 expression was higher on CD4+ TIL from extensively infiltrated tumors, which was confirmed at the mRNA level (Figure 3).

Many of the gene and surface markers expressed on some or all of the TIL reflect an activated state; however, only low levels of Th-derived cytokines/chemokines had statistically significant increases in the TIL versus P-PB comparison (Figure 3). Analysis using more sensitive quantitative PCR with reverse transcription (qRT-PCR) confirmed these gene expression increases and expanded the set of expressed Th-derived factors (Thf [CXCL13 and IL21], Th1 [CXCL9, CXCL10, CXCL11, GZMA, GZMB, IFNG, and IL2], Th2 [IL13], Th17 [CCL20, IL17A, IL17F, IL21, and IL22], and Tregs [IL10, TGFB, and XCL1]), although many were not at stimulation-induced (S-induced) levels (microarray data), with reduced expression more pronounced in minimal relative to extensively infiltrated tumors. These data indicate that while all Th subsets are infiltrating BC, the full complement of Th cytokines/chemokines is not produced.

Concurrent activation and suppression. The lack of robust or comprehensive cytokine/chemokine expression by the CD4+ TIL led us to investigate the nature of their activation state. Evaluation of prototypic TCR/CD3 pathway genes identified a substantial number that were downregulated in the TIL compared with their counterparts from LN or P-PB (Figure 4). This suppression includes components of the TCR/CD3 complex, molecules involved in signal transduction, costimulatory receptors, transcription factors, and negative regulatory molecules (genes detailed in Supplemental Table 4). Some of these gene changes were confirmed at the mRNA (Supplemental Table 5B) or protein levels (Supplemental Figure 2). TCR/CD3 downregulation...
Figure 6
Tumor SN suppresses activation of donor CD4+ T cells. D-PB CD4+ T cells (unstimulated or S) were treated for 24 hours with primary tumor SN. Expression of activation markers on (A) CD4+ CD45RO+ T cells or (B) total CD4+ T cells. (C–I) TIL gene expression data (P1–P10) was compared with donor CD4+ CD45RO+ T cells treated with SN with or without S (SN = 2 minimal [TIL034 and TIL043] and 2 extensive [TIL019 and TIL027]; the latter is borderline extensive, Supplemental Table 1C) tumors; Supplemental Table 1C. Heat maps show genes commonly altered in TIL and donor cells treated with SN with or without S for the designated comparison (red, upregulated; blue, downregulated). Samples include TIL from minimally (P1, P3, P5, P6, P8, P9) and extensively (P2, P4, P7, P10) infiltrated tumors; SN-treated (n = 4), S-treated (n = 3), and SN+S-treated (n = 4) D-PB. Genes commonly altered in P1–P10 TIL versus their P-PB and (C) SN-treated, (D) SN-S-treated, or (E) S-treated donor cells are highlighted. (F) Genes differentially expressed in TIL from extensively versus minimally infiltrated tumors and altered by SN or S-SN treatment are highlighted. Select TCR/CD3 pathway genes include (G) signaling molecules and targeted transcription factors; (H) costimulatory receptors and negative regulatory genes; and (I) cytokine/chemokine genes. (J and K) Freshly isolated CD4+ TIL from patients (TIL062 and TIL064; Supplemental Table 1C) and CD4+ memory T cells from D-PB were immediately extracted or rested for 24 hours. Specific gene changes in the two rested TIL were compared with P1–P10 TIL. Expression levels for the commonly altered genes are shown in J, with specific TCR/CD3 pathway and cytokine/chemokine genes highlighted in K.

on TIL has been described for a wide variety of solid tumors (35), but the depth of this suppression, extending through the signaling pathway and divergent from activation-induced modifications, has not been previously reported. A comparison with gene expression profiles of stimulated donor CD4+CD45RO+ T cells revealed that only approximately 50% were similarly induced or repressed by TCR/CD3 activation (Figure 4 and Supplemental Table 4). The disparity with T cell activation is highlighted by the number of aberrantly expressed coreceptors, which are involved in regulating the TCR/CD3 signal. Interestingly, the NF-kB transcription factor REL and members of the AP-1 family (ATF3, BATF, FOSB, FOSL2, and JUN/JUND) were specifically upregulated despite reduced upstream MAPK signaling. REL and some AP-1 transcription factor genes were increased in the LN profiles but to a lesser degree (Supplemental Table 2D). An indication that this overall pattern of TCR/CD3 signaling gene expression may be significant is highlighted by the differences observed in extensively versus minimally infiltrated tumors (Figure 5). Extensively infiltrated tumors are characterized by higher expression of the TCR/CD3 subunits and numerous coreceptor genes (including CD38, CD200, ICOS, and PDCD1) as well as many signaling genes (including ZAP70) (Figure 3, Supplemental Figure 2, Supplemental Table 4, and Supplemental Table 5B). These data suggest that extensively infiltrated tumors are characterized by lower levels of TCR/CD3 suppression and/or higher levels of T cell activation.

We next performed a series of in vitro experiments to explore this combination of activation and suppression. CD4+ CD45RO+ (or total CD4+) T cells from D-PB were treated with supernatant (SN) retained from fresh primary tumor homogenates, alone or in conjunction with S. SN downregulated TCR/CD3 expression and inhibited activation-induced surface receptor expression, characterized by CD69, ICOS, CD25, and FAS (Figure 6, A and B). SN also reduced expression of CXCR5, the receptor for CXCL13, a chemokine that plays an important role in homing to B cell follicles (36). Alternatively, SN and to a lesser degree SN plus S (SN+S), induced expression of CXCR4, the receptor for CXCL12 whose protumor activities are well documented (37). Gene expression arrays revealed that approximately 75% of the genes commonly altered in the TIL and their SN-treated donor PB counterparts were either similarly upregulated or downregulated (Figure 6C and Supplemental Table 6B). Expression profiles of the donor SN-treated cells (versus untreated cells) were compared with the TIL versus P-PB data, clearly demonstrating that the SN effect is appreciably amplified by S, with the SN+S cells (Figure 6D and Supplemental Table 6C) more closely resembling the TIL than SN alone (Figure 6, C–E). The SN+S gene profiles are also distinct from those of S alone (Figure 6E and Supplemental Table 6D) and largely reiterate a mixed pattern of activation and suppression.

Closer examination of the SN+S effect on TCR/CD3 pathway genes (Figure 6, G and H, and Supplemental Table 4) detected (a) significant suppression of various signaling genes that was more pronounced than that in the S donor cells; (b) specific upregulation of REL and AP-1/ATF family transcription factors similar to that in the TIL; (c) more potent suppression of a subset of costimulatory receptor genes compared with that in the TIL or S donor cells; and (d) a pattern of negative regulatory gene expression that was similar between the SN+S and S donor cells. Additionally, the SN+S treatment suppressed most of the S-induced changes detected in S counterparts, including expression of the majority of detected cytokines/chemokines (Figure 6F and Supplemental Table 6E). Among the Th cytokines/chemokines produced in the S donor cells, only the proinflammatory, tumor-promoting factors CCL20, IL8, and TNF were further upregulated by SN+S treatment. IFNG and the IFN-γ-induced chemokines CXCL9, CXCL10, and CXCL11 were the only S-induced factors also expressed in the TIL, with their overt suppression by SN+S potentially reflecting a stronger effect from higher local concentrations in vivo. This interpretation is reinforced by the observed SN-mediated suppression of CXCR3 (the receptor for CXCL9, CXCL10, and CXCL11; ref. 37 and Supplemental Table 6H) in parallel with CXCR4 upregulation in unstimulated or S donor cells (Figure 6A). Overall, these data support the view that the simultaneously activated/suppressed TIL profile is broadly reproduced by SN+S treatment of donor CD4+ T cells, where the suppressive SN effect is amplified by S.

To determine whether these tumor-mediated effects on CD4+ T cells were transient, freshly isolated TIL were split and either extracted immediately or “rested” ex vivo overnight without S. Gene expression profiling shows the rested TIL reverse expression in a sizable number of genes expressed in their freshly isolated counterparts (Figure 6, J and K, and Supplemental Table 6F). Notably, the cytokine/chemokine genes, including IFNG and IFN-γ-induced genes, were upregulated in the TIL and returned to baseline 24 hours after removal from the tumor microenvironment. An exception was the B cell chemoattractant CXCL13, for which the high TIL expression levels decreased only by 50% at 24 hours, with the rested TIL from an extensively infiltrated tumor (TIL064) still expressing CXCL13 at substantially higher levels (159-fold increase) compared with memory D-PB control. Reversal of altered TCR/CD3 pathway gene expression in the rested TIL was limited to BTLA and the AP-1 factors ATF3, FOSB, and JUN, suggesting that suppression of TCR/CD3 signaling is not fully relieved at 24 hours. Additionally, these in vitro experiments show that the
immunosuppressive quality of individual BCs varies, with the tendency for SN from tumors sustaining an extensive lymphocyte infiltrate to be less immunosuppressive than those minimally infiltrated (Figure 6, C–I). A direct comparison of gene expression in TIL from extensively versus minimally infiltrated tumors supports this conclusion by showing that extensively infiltrated tumors have greater similarity to S donor cells and minimally infiltrated tumors have greater similarity to SN+S donor cells (Figure 6F and Supplemental Table 6G).

**Significance of extensive infiltration.** The consistently divergent pattern of gene expression detected in CD4+ TIL from extensively versus minimally infiltrated tumors led us to examine the specific genes affected. These analyses found that a variety of immune response genes are expressed at elevated levels in TIL from extensively infiltrated BC, with many perhaps reflecting their higher level of activation (Figure 7A and Supplemental Table 2G). The most distinctive increase consistently detected in extensively infiltrated tumors was CXCL13, with CD4+ TIL shown to be the principal cellular source (Figure 7, B and C). CXCL13 mRNA expression in tumor sections was recently shown to predict survival of patients with BC, particularly in the HER2+ subtype, although the cell source was not identified (38). An earlier study suggested tumor cells were the major CXCL13 producers in BC but their IHC protocol used higher concentrations of a monoclonal antibody that intensely labeled most cells, both in the tumor tissue and the LN control (39). In contrast, our IHC experiments, using lower concentrations of a polyclonal antibody, stained only some GC-located cells in the LN control, the tumor-associated TLS, and the occasional intratumoral lymphocyte (Supplemental Figure 4C), suggesting that CXCL13 production by tumor cells needs to be reexamined.

In secondary lymphoid organs, such as LNs, CXCL13 is expressed by stromal cells and Th cells in GC, with its ectopic expression promoting lymphoid neogenesis (40). A comparison of CXCL13 expression with other cytokines/chemokines in an extensively and a minimally infiltrated tumor (Figure 7C) showed that the majority were expressed by infiltrating leukocytes (CD14+ monocytes, CD4+ T cells, or the remaining mixture of CD45+ cells) but not the EpCAM+ epithelial/tumor cells. In general, antitumor cytokines/chemokines were detected at considerably higher levels in the extensively compared with the minimally infiltrated tumors.
Figure 8
Correlation among the expression of Th marker genes, a Th1 immune profile, and the extent of lymphocyte infiltration. qRT-PCR ΔCt values for immune genes expressed in CD4+ TIL (normalized to the Th-specific endogen CASC3) or non-CD4 cells (normalized to the 3 endogens in Figure 7C) from 21 tumors were used to generate unsupervised hierarchical clustering. The immune genes include differentially expressed CD4+ TIL surface receptors (Figure 2D) and cytokine/chemokine genes elevated in extensively infiltrated TIL (Figure 3). In the non-CD4 cells, expression of major immune subpopulation markers and cytokines associated with Th1 (IL12), Th2 (IL4 and IL13), immune suppression (IL10 and TGFβ2), tumor promotion (CXCL2 and CXCL12), and the tumor marker EPCAM were analyzed. Pearson correlation coefficients ($r^2$) were calculated for individual gene combinations or with lymphocyte infiltration levels (Supplemental Table 5D). A negative correlation ($r^2 = -1$) is shown in blue and a positive correlation ($r^2 = 1$) is shown in red. Fold change (ratio) and P values for extensively (n = 6) versus minimally (n = 12) infiltrated tumors are indicated (significant values are shown in red; P < 0.05). Additionally, select correlation plots are shown for relevant surface marker and CXCL13 gene expression in CD4+ TIL (red) and non-CD4+ cells (black) (significant values are shown in red; P < 0.05).
(except the protumor chemokine CXCL12). CD4+ T cells and CD14+ monocytes produced the proinflammatory cytokine IL-18 and IFN-γ–induced chemokines CXCL9, CXCL10, and CXCL11, while IFNG itself originated principally from the remaining CD45+ cells (presumably CD8+ T cells; Figure 8 showing a high correlation among CD8, CD3, and IFNG gene expression in the non-CD4+ cells).

To gain a better understanding of the relationship between differences detected consistently in extensively versus minimally infiltrated tumors.
duced cells were CD200 hi PD-1 hi (CD38 ±) Tfh cells (Figure 9B). While CXCL13 expression and that of the Th1 marker, CD38, are correlated, this non-Tfh receptor is not specific for CXCL13-producing cells, providing additional support for the concept that Tfh, and not other Th, cells produce CXCL13. These experiments also demonstrate that infiltrates from extensively infiltrated tumors are distinguished by CD200 hi PD-1 hi Tfh cells (Figure 9C). Thus, evidence emerged from our analyses that, in the CD4 + fraction, a Tfh profile (CD200 hi, PD-1 hi, ICOS, and CXCL13) was associated with a Th1 profile (CD38 hi, IFNG, and CXCL9) and both were associated with more extensive lymphocyte infiltration.

CXCL13-producing CD4 + Tfh cells infiltrating breast tumors signal organized immunity. In secondary lymphoid organs, T cells and B cells segregate to separate zones based on CCR7 and CXCR5 expression (28). Upon activation, B cells and CD4 + T cells migrate to the T/B interface for critical interactions that ultimately direct high-affinity CXCR5 + Tfh cells and B cells to migrate in response to CXCL13 to the B cell follicle interior. Once there, they initiate formation of the GC, the site in which memory B cells and long-lived plasma cells

**Figure 10**
Organization of the immune infiltrate in extensively infiltrated breast tumors. (A) TLS containing GC detected in H&E-stained primary tumor sections. Tu, tumor bed; Str, stroma. (B) CD45 (total leukocytes) IHC staining of an extensively and a minimally infiltrated tumor shown for comparison. (C) Successive paraffin-embedded tumor sections from 15 patients were stained by IHC for CD45 (total leukocytes); CD3 (total T cells); CD4 (helper T cells); CD8 (cytotoxic T cells); CD20 (B cells); CD23 (GC FDCs); CD68 (macrophages); Ki67 (dividing cells; 5 patients); Bcl6 (Tfh and GC B cells; 5 patients); and CXCL13 (Tfh and FDC marker). The boxed area in the first CD45 image is the area magnified in all of the following images of sequential sections labeled with leukocyte subpopulation markers. (D) CXCL13 IHC staining in tumor sections (LN control; Supplemental Figure 4) shows cytoplasmic expression in GC-localized cells and some lymphocytes infiltrating the tumor bed (but not in tumor cells). The intensity of CXCL13 staining was correlated with the extent of stromal (red) or intratumoral (blue) CD45 + infiltrates for 15 tumors. Scale bar: 100 μm.
are generated. Our identification of a Tfh gene expression profile in extensively infiltrated tumors (highlighted by a strong CXCL13 signal) led us to examine BC for lymphoid aggregates and organized TLS containing GC (Figure 10A, Supplemental Figure 4, and Supplemental Table 1C). TLS and GC were detected adjacent to the tumor bed at a higher incidence in extensively compared with minimally infiltrated tumors (Figure 10B and Supplemental Table 1D). The intensity and distribution of the major CD45+ TIL subpopulations within the TLS and GC were analyzed using IHC (Figure 10C and Supplemental Figure 4). BC TLS organization is similar to that of LNs, containing a CD3+ T cell zone adjacent to a CD20+ B cell follicle with CD23+ follicular DCs (FDCs) and Bcl6+ Tfh and GC B cells (43) exclusive to the GC, in which Ki67+ cells (proliferating B cells) are also concentrated. CD4+ T cells are the principal component of the T cell zone and also have a significant presence in the GC. CD8+ T cells were moderately interspersed with CD4+ T cells in the T cell zone but absent from the GC; however, they were the principal lymphocyte population in the tumor bed and present at levels that parallel the extent of the total CD45+ infiltrate and the number/size of TLS (Supplemental Figure 4). CXCL13 was principally expressed by T cell zone or GC lymphocytes (primarily infiltrating CD4+PD-1hiCD200hi Tfh cells; Figure 9, A and B) and FDCs, with intratumoral CXCL13+ lymphocytes detected in some extensively infiltrated tumors (Figure 10D). Interestingly, in a very small GC located within a large TLS (Supplemental Figure 4H), we observed CD4+ T cells and CXCL13 protein associated with CD23lo B cells but not CD23hi FDCs, which might signify that CXCL13-producing Tfh cells associate with B
cells earlier than FDCs. Specific CXCL13 labeling of tumor cells was not observed with our IHC conditions (n = 15; Figure 10, C and D, and Supplemental Figure 4), which is consistent with our flow cytometry data (Figure 9A). Furthermore, CXCL13 IHC labeling was correlated with the extent of immune infiltration. CXCL13-producing Tfh cells and FDCs might catalyze the recruitment of other immune cells to the tumor, thereby initiating and/or enhancing TLS development and GC formation. The increased frequency of TLS and GC in conjunction with increased Tfh and Tfh1 gene expression in CD4+ TIL from extensively infiltrated BC, together with the combined activated/suppressed TIL profile, suggests that immune structures adjacent to the tumor bed may represent an important site for antitumor immune responses.

Clinical significance of CD4+ Tfh cells infiltrating tumors. We tested this hypothesis by investigating the prognostic associations of Th gene expression profiles as a reflection of lymphocyte infiltration levels and/or a TLS presence in a given BC sample. We derived signatures by extracting the most significant and specific Tfh and Th1 genes from the Th subset gene profiles (Figure 1, C and E) that were differentially expressed between extensively and minimally infiltrated tumors (detailed in Supplemental Table 7). We evaluated the association of these signatures with disease-free survival (DFS) in systemically untreated primary BC (n = 794 patients receiving no hormone or chemotherapy before or after surgery; Figure 11A) and compared them with 2 published immune signatures (refs. 6, 8, and Supplemental Table 7). Our Tfh signature (8 genes) was consistently prognostic, demonstrating a linear association with survival in the whole population (P = 0.0036) as well as in the 3 main BC subtypes: HER2+ (P = 0.012), ER+/HER2− (P = 0.024), with borderline significance observed in ER−/HER2− (P = 0.082). Given the importance of CXCL13 in Tfh and B cell recruitment, its prognostic associations as a single gene were also investigated, revealing a particularly strong association for the HER2+ subtype, as recently reported (38). The dominant prognostic value of CXCL13 within the Tfh signature is a reflection of its number one ranking in TIL gene expression changes detected in the extensive versus minimal comparison and our demonstration that Tfh cells are the principal producer cells in BC. This is in contrast to the Treg/activated T cell marker FOXP3, which as a single gene did not have any significant predictive value (Supplemental Table 7B). Our Th1 signature (12 genes) predicted survival in the HER2+ BC (P = 0.02) subtype, with the genes CXCL9 and IFNG providing similar results (Supplemental Table 7B).

We next examined a BC cohort treated with preoperative chemotherapy (n = 996) (44), which is given up front prior to surgery with the pathological complete response (pCR) of the tumor (its disappearance at surgery) known to be associated with excellent long-term survival outcome. In this treatment setting, the Tfh and Th1 signatures, along with the CXCL13 single gene, were all strongly associated with a higher rate of pCR, particularly in the HER2+ subtype (Figure 11B and Supplemental Table 7C). Together, these data support our hypothesis that the presence of CXCL13-producing Tfh cells may specify a TLS/GC presence in the tumor, with these structures producing antitumor immune responses associated with improved clinical responses.

Discussion

The work presented in this study provides new insight into the composition and strategic location of the CD4+ T cell infiltrate and its association with clinical outcome in BC. We identified several aspects of the CD4+ T cell presence that we consider to be fundamentally important. First, while all Th subsets are present in the tumor they do not contribute equally, with more extensively infiltrated tumors distinguished by higher expression of Tfh, Th1, and T cell activation markers. Second, the mixture of activation and suppression observed in the CD4+ TIL (and S+SN-treated donor cells) suggests that their position and prevalence within the tumor dictates responsiveness, with extensively infiltrated tumors successfully sequestering higher concentrations of leukocytes in organized lymphoid structures (TLS). Third, despite tumor-mediated TCR/CD3 pathway suppression, the expression of some key immune transcription factors is consistently upregulated, and a restricted group of cytokines/chemokines are produced by the TIL. Fourth, BCs with extensive immune infiltrates are characterized by the presence of TLS, principally located adjacent to the tumor bed, which house the majority of CXCL13-producing Tfh cells and GC. Fifth, expression of an 8-gene Tfh signature that includes the CXCL13 gene was associated with a higher likelihood of response to preoperative chemotherapy and better DFS in systemically untreated patients with BC. Together, these data reveal that a detectable Tfh cell presence is associated with organized immune structures and long-term positive clinical outcome.

A recent quantitative evaluation of the leukocyte composition in breast tumors found that CD4+ and CD8+ TIL had an activated (CD27hiCD69hiHLA-DRhiCCR4hiCCR5hi) memory (CD45RA−CCR7+) phenotype (29). Our broad examination of surface markers in conjunction with gene expression profiling confirms and extends these findings by demonstrating that the CD4+ TIL are effector memory cells with all Th subpopulations represented. In contrast to most studies, we found that the CD4+ TIL produce a restricted repertoire of Th cytokines/chemokines, which are generally expressed at sub-T cell activation levels, particularly in minimally infiltrated tumors. The difference between our data and previous work could be attributed to our rapid isolation of CD4+ TIL without in vitro expansion, which provided insight into their in situ state, as well as our inclusion of minimally infiltrated tumors. Finally, eliminating enzymatic digestion, shown to alter TIL function and phenotype (45), also allowed us to retain SN from primary tumor homogenates for treatment of donor CD4+ T cells, which largely reproduced the TIL profiles in activated CD4+ T cells, further validating these data.

Curiously, this reduction in T cell activation and consequent low level cytokine/chemokine expression in the TIL is set against a background of upregulated REL and AP-1 superfamily transcription factors. REL (c-Rel) is a major player in the canonical NF-kB signaling pathway, integrating T cell activation signals and promoting Th1, Th17, and Treg responses (46). The conserved, homologous E3 ubiquitin ligase proteins, pellino 1 and 2, positively regulate NF-kB activation (47), with c-Rel shown to accumulate in T cells from mice deficient for PEL1I (48). While a specific function for pellino 2 in T cells has not been reported, it has been shown to regulate NF-kB–dependent IL-8 promoter activity (49). We detected a decrease in PEL1I in the TIL (Supplemental Table 2B), suggesting a potential mechanism for the observed upregulation of REL and IL8 gene expression.

AP-1/ATF factors also regulate important immune response genes, exemplified by their well-known cooperation with NFAT factors to induce cytokine gene expression (50). We detected upregulation of JUN, FOS (FOSB/FOSL2), and ATF3 in the TIL.
and SN+S-treated donor cells in parallel with a decrease in JUN, FOSB, and ATF3 in the rest TIL, suggesting that their expression is induced when activated cells enter the tumor microenvironment. A recent study has shown that AP-1 (FOS) is persistently upregulated in infiltrating T cells during tumor progression in mice (51). FOS upregulation specifically induces PD-1 expression on CD4 and CD8 TIL, which mediates their suppression upon contact with PD-L1-expressing tumor cells. While PD-L1 is not expressed on normal breast tissue, studies have shown that it is upregulated in breast tumors, particularly in high proliferative, ER−BC (52). Disruption of AP-1–induced PD-1 expression on murine TIL was able to restore their cytokine expression (51), with potentially the same mechanism evoked for the reduced cytokine expression we detected in the global CD4+ TIL population. Based on current data, the role(s) PD-1 plays in regulating Tfh cells (a minority of the total CD4+ TIL) remains unclear. Murine studies have shown that PD-1+ Tfh cell interactions with PD-L1+/PD-L2+ B cells are important for regulating GC activities, with a deficiency in PD-L2 resulting in reduced Tfh IL-21 production and the generation of fewer plasma cells (53). The notion that PD-1 expression on Tfh cells elicits context-dependent responses in protected lymphoid structures is supported by a recent murine study showing that PD-1+ Tfh cells survive and function in gut-associated lymphoid follicles after anti-Thy-1 antibody treatment (54). PD-1 expression is often considered a sign of T cell exhaustion; however, our expanding knowledge indicates that it can also act as a signal for other functions, highlighted by the demonstration that CD8−PD-1+ TIL produce higher levels of IFN-γ than PD-1− cells (55). Overall, current data suggests that, in contrast to effector T cell TIL, PD-1+ Tfh cells located in BC-associated TLS likely do not contact PD-L1+ tumor cells and are not directly affected by PD-1–mediated tumor suppression but are instead regulated by PD-1 to elicit other functions.

Studies of TIL have used various approaches to address the specific influence(s) exerted by individual Th subsets in the tumor microenvironment. Traditionally, Th1-mediated immunity has been considered as antitumoral, because patients whose tumors contain higher levels of Th1 cells are predicted to have longer DFS, while tumors associated with polarized Th2 and/or Treg subpopulation markers have a poorer prognosis (19). This view is complicated by the plasticity of Th differentiation (56), which involves shared surface receptors and cytokines that are often rapidly modulated in time and place, making it difficult to clearly discriminate the type of response(s). Despite these limitations, a number of studies have shown that infiltrating T cell subsets can be used to gauge clinical outcome. The widely studied immune infiltrate in colorectal carcinoma has established that a higher density of intratumoral CD8+ and/or CD3+ memory cells is frequently associated with a better prognosis (57, 58). Tumor-associated Th1 cytokines, lead by IFN-γ, have consistently been linked with more potent antitumor immunity in a variety of solid tumors (59, 60), with recent research showing that tumor-specific Th1 cells can effectively control tumor development (61, 62). Although IL-17 has been correlated with both protumor and antitumor immune responses (63), Th17 cells have been shown to synergize with IFN-γ to induce the important antitumor chemokines CXCL9 and CXCL10 (64), suggesting that the positive or negative Th17 activities reported may be context dependent. Presence of Foxp3+ Tregs in tumors is also inconsistent among tumor types, with their accumulation in some cancers associated with reduced patient survival, while in others they signal a better outcome. An important early study by Curiel et al. (65) found that tumor-associated Tregs were correlated with suppressed antitumor immunity and reduced survival in ovarian carcinoma. Foxp3 expression by tumor cells was shown to be an independent predictor of improved outcome in patients with HER2+ BC (66). A recent study of colorectal cancer has found that the presence of Foxp3+ tumor cells (Epcam+) but not Foxp3+ CD4+ T cells is indicative of a poor prognosis (67). In BC, the majority of studies investigating CD4+ TIL have been focused on Tregs (68), with their increased presence linked with a poor prognosis in ER− disease (21, 69) but a good clinical outcome in ER− disease (70). Further investigation is needed to more clearly define the roles played by individual Th subpopulations in different types of BC.

In this study, we have extended current knowledge of Th subsets infiltrating solid tumors by showing that, in addition to Th1 cells, a measurable Tfh presence is also significant and associated with better BC patient outcome. Tfh cells are relative newcomers to the Th subset family (71, 72) and have not been previously described in nonhematological tumors. While their role in antitumor immunity is unknown, studies of cellular interactions in secondary lymphoid organs show that Tfh cells are key players in the generation of T cell–dependent antibody responses and B cell memory (73). Collectively, these data (predominantly from animal models) demonstrate that these specialized Th cells are intimately involved in GC development and provide critical help for antigen-specific B cell maturation to high-affinity memory cells and long-lived plasma cells (28, 43, 74). While long considered minor players in antitumor immunity (75), plasma cells infiltrating non–small cell lung cancer (76) and CD20+ B cells infiltrating BC (77), ovarian cancer (78), melanoma (79), and colorectal carcinoma (80) have been associated with increased survival. Studies of B cells in BC have detected specific antibody responses to tumor antigens (81, 82) and correlated B cell gene expression signatures with better clinical outcome (9, 83). The data presented here show that the most important differences in the TIL profiles were related to the extent of infiltration, which identified important Th subset differences characterized by increased Th1 and particularly Tfh marker expression in extensively infiltrated tumors. We found that a detectable Tfh presence predicts longer survival in untreated patients as well as pCRs in preoperatively treated patients, which further suggests that B cell responses may constitute an important component of effective antitumor immunity in BC. Although the role(s) B cells play in cancer immunity is currently undefined, they could potentially affect antitumor immune responses via their antibody-producing capacities, APC functions, and/or production of specific cytokines and chemokines.

We found that a Tfh presence was principally associated with peritumoral TLS, which have been described primarily in the lung, in patients with chronic inflammatory diseases or as a reactive response to infection (84). These organized structures contain distinct T cell zones and B cell follicles with reactive GC similar to secondary lymphoid organs. Some earlier studies described lymphoid aggregates or organized lymphoid structures in tumors, including BC (81); however, their prognostic value has only recently been investigated in a study of non–small cell lung cancer, in which the number of mature DCs was used as an indicator for TLS (26), and in colorectal cancer, in which both lymphocyte aggregates (57) and active TLS, the latter identified.
by high chemokine expression levels (27), were associated with a better clinical outcome. While lymphoid structures containing FDCs or a high density of endothelial venules have been described in BC (81, 85) and other tumors, their immunological importance in these patients has not been specifically addressed. Our analysis of CD4+ T cell subsets in BC TLS found that elevated levels of CXCL13 expression were correlated with Tfh markers and the extent of tumor infiltration. These data led us to further demonstrate that CXCL13-producing Tfh cells signal the presence of organized TLS containing reactive GC that are associated with favorable clinical outcomes. Studies have shown that CXCL13 (in conjunction with CCL19, CCL21, TNF, and lymphotixin) plays a critical role in the development and maintenance of secondary lymphoid organs (86) and is essential for dynamic GC formation in response to acute infection. Although CXCL13 has principally been defined as a stromal cell–produced (including FDCs) chemokine, PD-1+ Tfh cells were recently shown to be efficient CXCL13 producers (87). Our experiments used a variety of approaches to demonstrate that CD4+PD-1hiCD200hi Tfh cells infiltrating BC are a major source of CXCL13 production.

In BC, a high immune signal has been linked with better progression-free survival in overall patient groups as well as aggressive BC subtypes, including HER22 (7, 8, 10), ER/HER22 (8, 10, 11), and highly proliferative tumors (9). The signals emanating from these analyses of total tumor tissue detect a broad spectrum of immune response genes, reflecting activities of the innate and adaptive immune responses, with some refined to a T cell metagene (10), B cell metagene (9), or signaling pathways (8). Additionally, two recent studies demonstrated that a higher immune score in the initial biopsy predicts increased pCR rates after preoperative chemotherapy (88), with the strongest association observed in the HER22 and ER/HER22 subtypes (44). Generally, higher expression of an immune signature reflects a greater presence of immune cells, with the latter associated with a better prognosis. Our data confirm and extend these findings by demonstrating that a key positive prognostic element detected by these immune signatures is a Tfh presence in organized TLS, which may be a surrogate for effective responses to the tumor. A link between the patient’s natural ability to mount what appears to be a more comprehensive immune response in LN-like structures adjacent to the tumor bed and clinical outcome suggests that some patients might derive specific benefit from therapies designed to boost their immunity before the tumor, as a source of antigen, is removed.

The major findings in this study indicate that, despite the immune system’s failure to contain the growing tumor, the presence of CXCL13-producing Tfh cells is associated with organized immune structures adjacent to the tumor bed that potentially participate in propagating sustainable and effective long-term antitumor immunity. Leukocytes within the TLS could thus dynamically respond to evolving local tumor antigens, maintain activation, and generate long-lived memory cells while sequestered in a protected microenvironment. The mixture of activation and suppression that we observed in the CD4+ Tfh may reflect the presence of functionally activated cells from the TLS combined with suppressed cells migrating through the intratumoral and peritumoral regions. Despite their imminently suppressed once they move from the TLS, we suggest that some tumor-specific memory cells survive and maintain effective immunosurveillance that functions over the long-term to detect and eliminate residual tumor cells. Thus, patients whose immune systems mount an organized response to their tumors, specifically detectable by the Tfh signature, would be predicted to have a better response to preoperative chemotherapy or postoperative DFS.

Methods
Patient population and clinical samples. Tumor, LN, and/or blood samples were obtained from 70 untreated patients undergoing tumor resection for BC at the Jules Bordet Institute. Fresh tumor tissue, LN tissue, and PB from the 10 patients in the discovery set (P1–P10) and fresh tumor tissue from 60 patients in the confirmation set (TIL003–TIL132; and ipsilateral normal breast tissue when available) were analyzed. PB samples from 19 healthy female adults were used as controls for the discovery set (D1–D4) and the confirmation set (D5–D19). Clinicopathological patient data is detailed in Supplemental Table 1.

Tumor sample preparation. Dissected tumor fragments from fresh surgical specimens were directly transferred into 3 ml X-VIVO 20 (Lonza) before 2 rapid rounds of mechanical dissociation with the GentleMACS Dissociator (Program A.01; Miltenyi Biotec). This approach yielded a >50% increase in cell viability compared with enzymatic digestion. The resulting cell suspension was filtered following each dissociator run using a 40-μm cell strainer (BD Falcon), washed with X-VIVO 20, centrifuged for 15 minutes at 600 g, and resuspended in X-VIVO 20 before isolation or flow cytometric analysis. The tumor SN was the initial 3 ml of X-VIVO 20 recovered after the first round of dissociation, which was subsequently clarified by centrifugation for 15 minutes at 13,000 g.

Cell isolation. For molecular analyses, CD4+ T cells were positively purified from patient samples or healthy donor blood using Dynabeads CD4 (Invitrogen). Memory CD4+ T cells were purified by CD45RA depletion (Dynabeads M450 Epoxy [Invitrogen] coupled to an anti-CD45RA antibody [304102; Biolegend]) prior to CD4+ selection. Additional tumor cell populations were purified using Dynabeads M-450 Epoxy coupled with monoclonal antibodies to CD14, CD45, or EpCAM (301801, 304001, and 324201; Biolegend). For primary cells treated with tumor SN, total CD4+ or memory CD4+ cells (CD45RA+ = RO+) CD4+ cells were negatively purified using the CD4+ T cell isolation Kit II or Memory CD4+ T cell Isolation Kit (Miltenyi Biotec). The cell purification protocols were controlled by flow cytometry with negatively purified samples >98% pure and positively isolated CD4+ TIL >95% pure.

Treatment with tumor SN. Total CD4+ or memory CD4+ cells (2 × 106 cells/ml) purified from healthy donor blood were incubated in X-VIVO 20 overnight following isolation. Cell viability was controlled the following day by flow cytometry (>95%), and then an equal volume of tumor SN or X-VIVO 20 was added with or without anti-CD3 (317304, clone OKT3; Biolegend) and anti-CD28 (555725, clone CD28.2; BD Pharmingen), each at 1 μg/ml final concentration. The cells were recovered 24 hours after treatment for analysis using microarrays, qRT-PCR (including a kinetic experiment for IFNG and IL2 gene expression over 48 hours after treatment; Supplemental Table 5H), and/or flow cytometry.

Microarrays. 10 ng (discovery set) or 100 ng (confirmation set) high-quality total RNA was labeled following the manufacturer’s protocols for probe preparation and hybridization on the Affymetrix U133 Plus 2.0 GeneChip. The gene expression array data has been deposited into the Gene Expression Omnibus under accession number (GSE36769).

Flow cytometry. Samples assessed for the expression of 60 cell surface markers (duplicates or triplicates were assessed for each marker) were incubated individually or in combinations of antibodies (listed in Supplemental Figures 1–3) for 45 minutes at 4°C in 50 μl X-VIVO 20 followed by washing with 2 ml PBS and immediate analysis on a FACSCalibur (BD Biosciences) without fixation. The Cytoflex/Cytoperm Kit (BD Biosciences) was used for intracellular staining with anti-CXCL13 (IC801A; R&D Systems).
qRT-PCR. RNA was extracted using TRIzol Reagent (Invitrogen) and reverse transcribed into cDNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) following standard procedures. Real-time PCR reactions were performed using commercially available Gene Expression Assays (Applied Biosystems or Qiagen) or published primers (detailed in Supplemental Table 5A) and (Taq SYBR Green Supermix With ROX (Bio-Rad) on an ABI 7900HT Prism sequence detector (Applied Biosystems).

Immunohistochemistry. Four-μm sections of paraffin-embedded tumor blocks from P1–P10 were labeled with anti-CD4 and anti-CD3 antibodies. Successive sections from 15 patients from the confirmation set were labeled with the top 9 antibodies listed in Supplemental Figure 4 using the Enzymogenous Biotin Blocking Kit (Ventana) and iVIEW DAB Detection Kit (Ventana) on a BenchMark XT IHC/ISH slide stainer (Ventana). For CXCL13 labeling, mounted sections were deparaffinized with xylene, pretreated with citrate 10 mM, pH 6.0, at 95°C to 99°C for 20 minutes, exposed to a 3% hydrogen peroxide solution to quench endogenous peroxidase activity, and blocked with rabbit serum (Sigma-Aldrich) for 1 hour prior to incubation with a purified goat anti-human polyclonal antibody to CXCL13 (1:100, AF801; R&D Systems) in a moist chamber at 4°C overnight. For each tumor, one section was treated in parallel without the primary antibody as a negative control. Subsequent incubations were carried out with a biotinylated rabbit anti-goat secondary antibody (Vector Laboratories) for 30 minutes at room temperature, followed by the HRP streptavidin reagent (Zymed) and the DAB + Substrate Chromogen System (Dako). The slides were counterstained with hematoxylin (Sigma-Aldrich) and mounted on a Tissue-Tek Prisma Automated Slide Stainer (Sakura).

Survival analysis. The Th1 and Th2 gene signatures derived in this study along with previously published immune gene signatures were assessed for their power to predict either 10-year DFS or pCR after neoadjuvant chemotherapy. DFS analyses were performed using 794 systemically untreated patients with BC from 8 public microarray data sets (Supplemental Table 7F). The signature scores (signed average of expression values for the genes in the signature) were computed as in Desmedt et al. (8). Three categories of gene expression cut-off values (high, intermediate, and low) were defined using tertiles of the continuous signature scores. In these data sets, BC subtypes were defined using a 2-gene classification based on ESRI (also known as ER) and ERBB2 (also known as HER2) gene expression (89). pCR analyses were performed using 966 patients treated with neoadjuvant chemotherapy from 8 published studies, as in Ignatiadis et al. (44). BC subtypes were defined on the basis of ER protein expression by IHC and HER2 amplification by fluorescent in situ hybridization in the original publications. When unavailable, ER and HER2 status was assigned according to bimodal normalized ESRI and ERBB2 gene expression (44). The association between an individual gene signature and pCR was based on an estimated odds ratios and 95% CI (44).

Statistics. P values for nonmicroarray data were generated using a 2-tailed Student’s t test with unequal variance (or a χ2 test where indicated). A P value of less than 0.05 was considered as significant. Individual patient microarray data, our experimental data, and publicly available data for individual Th subsets were differentially analyzed based on the type and number of samples in the set by using the specifically adapted methods and criteria detailed in Supplemental Methods.

Study approval. All primary breast tissue, LN, and blood samples used in this study were taken from individuals treated at the Institute Jules Bordet. All patients signed a protocol-specific consent. Healthy donor blood samples were taken from volunteers who signed an informed consent. The protocols used for human studies were approved by the Medical Ethics Committee of the Institut Jules Bordet.

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Address correspondence to: Karen Willard-Gallo, Molecular Immunology Unit, Institut Jules Bordet, Université Libre de Bruxelles, 121 Blvd. de Waterloo, B1000 Brussels, Belgium. Phone: 32.2.541.3739; Fax: 32.2.541.7325; E-mail: kwillard@ulb.ac.be.

Sherene Loi’s present address is: Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia.

Marie Ravoet’s present address is: Centre de Génétique Humaine, Cliniques Universitaires St. Luc-UCL, Bruxelles, Belgium.

Benjamin Haibe-Kains’s present address is: Bioinformatics and Computational Genomics Laboratory, Institut de recherches cliniques de Montréal, Montreal, Quebec, Canada.

Stefan Michiels’s present address is: Service de Biostatistique et d’Épidémiologie, Institut Gustave-Roussy, Villejuif, France.


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