Molecular Features of T Lymphocytes in the Immune Response Against Breast Cancer

Thesis

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Agnese Losurdo

Molecular features of T lymphocytes in the immune response against breast cancer

For the Degree of Doctor of Philosophy
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HUMANITAS CLINICAL AND RESEARCH INSTITUTE, Milan, Italy
Affiliated Research Centre to “The Open University” Milton Keynes, UK

Under the supervision of

Director of Studies: Dr. Enrico Lugli and Prof. Armando Santoro

External Supervisors: Prof. Sergio Quezada, University College London Cancer Institute, London, UK

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ABSTRACT

Luminal-like breast cancer (BC) constitutes the majority of BC subtypes, but, differently from highly aggressive triple negative breast cancer (TNBC), is poorly infiltrated by the immune system. The quality of the immune infiltrate in luminal-like BCs has been poorly studied, thus a biological basis for the further investigation of immunotherapeutic strategies is missing. In this thesis, we used high-dimensional single-cell technologies to characterize the T cell populations infiltrating luminal-like tumors, and we identified heterogeneous behavior within the tissue-resident memory CD8+ T (Trm) cells. We defined that a subset of CD127-CD39hi Trm cells is preferentially present in the tumor compared to the adjacent normal breast tissue or peripheral blood, displayed enhanced effector functions compared to the CD127+ CD39lo Trm counterpart, and was specifically associated with positive prognosis. Nevertheless, the prognostic benefit was lost in the presence of highly-suppressive CCR8hi ICOShi IRF4+ effector Tregs. Our results suggest that combinatorial strategies aiming at boosting Trm function while relieving from Treg-mediated immunosuppression are needed to achieve proper tumor control in luminal-like BCs.
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1 INTRODUCTION

1.1 Breast cancer and TILs

1.1.1 Breast cancer prognostic subtypes

In routine clinical practice, breast cancer (BC) can be classified into prognostic and predictive subtypes based on the immunohistochemical (IHC) expression of the estrogen receptor (ER), the progesterone receptor (PgR), the human epidermal growth factor receptor type 2 (HER2), with or without in situ hybridization of the latter, histological grade and the Ki-67 proliferation index. Hormone receptor (HR)-positive breast cancers account for approximately 75-80% of all documented BC types, while HER2 has demonstrated overexpression by immunohistochemistry and/or amplification by fluorescence in situ hybridization (FISH) in almost 15-20% of cases. The remaining 10-15% are defined by HR and HER2 negativity and coherently titled triple negative breast cancers (TNBC), which lack targeted treatments and indicate a comparatively poor prognosis.

Perou and colleagues have proposed a new description of BC heterogeneity at the molecular level by the cDNA microarray analyses of 38 invasive breast cancers. They have demonstrated three main themes, including (I) the existence of four molecular subtypes of breast cancer: luminal (lum), normal breast-like, HER2, and basal-like; (II) the luminal subgroup, which could be further stratified into two or three groups, mostly luminal A (lumA) and luminalB (lumB) that vary in clinical outcome and biological processes; (III) ER-positive and ER-negative breast tumors, which strikingly have fundamental differences in the transcriptomic level. LumA BC harbors expression patterns reminiscent of normal luminal epithelial cells, including low molecular weight cytokeratins 8/18 and
genes associated with an active ER pathway; in comparison, lumB subgroup has a more aggressive phenotypes than lumA, such as low expression level of ER-activated genes and high demonstration of proliferation related genes, higher histological grade, proliferation rates, and worse prognosis by inference.

As the complexity of BC subtyping based on the previously described gene-expression signatures prevents (or limits) their integration into routine clinical practice, standard pathological biomarkers can serve as surrogates for prognostic gene expression profiles, and categorize tumors as lumA-like, lumB-like, HER2 enriched (non-luminal) and TNBC \textsuperscript{2,6,7}. It should be noted that, based on St Gallen consensus recommendation, lumA-like disease can be distinguished from lumB-like using a combination of ER, PgR and Ki-67, without requiring molecular diagnostics \textsuperscript{8}.

1.1.2 Breast cancer microenvironment

Developing tumors recruit a diverse collection of cells, as a whole known as the tumor microenvironment, that co-evolves together with tumor cells through iterative interactions. Early infiltration of tumors by immune cells such as macrophages, lymphocytes, natural killer (NK) cells, and dendritic cells (DCs) is crucial for tumor control \textsuperscript{9}. The anticancer immune response generated by these cells is, however, inhibited by the action of immunosuppressive cells, such as myeloid-derived suppressor cell (MDSC), regulatory T cells (Tregs), and type 2-polarized macrophages (M2), which are oriented towards promoting tumor growth, tissue remodeling and angiogenesis, and suppressing adaptive immunity \textsuperscript{10}. Cancer cells are able to communicate with other cells and components of
the tumor microenvironment through two main pathways: the first being contact-dependent mechanisms between cancer cell and another cell or with the extracellular matrix, and the second being contact-independent mechanisms via soluble molecules such as cytokines (e.g. IL-6, IL-8, IL-10, TNFα), lipid mediators (e.g. prostanoids, such as PGE2, leukotrienes and epoxyeicosatrienoic acids), and growth factors (e.g. GM-CSF and M-CSF). There is indeed a cross-talk between tumor cells and all the cellular components of the tumor microenvironment. Tumor ecosystems are comprised of cancer cells, infiltrating immune cells, stromal cells, and other cell types together with non-cellular tissue components. The breast tumor microenvironment comprises diverse cell population which secretes various cytokines and growth factors. Mesenchymal cells interact with breast cancer stem cells (BCSCs) through cytokine loops involving IL-6 and CXCL7, stimulating self-renewal, and have the ability to differentiate into adipocytes as well as tumor-associated fibroblasts, which also interact with and influence tumor cell growth. Breast carcinoma-associated fibroblasts and myofibroblasts, producing cytokines such as Stromal Derived Factor-1 (SDF-1, also known as CXCL12), may promote proliferation of tumor cells, which express the SDF-1 receptor CXCR4. Through production of fibroblasts growth factors (FGFs), these activated stromal cells may also regulate tumor proliferation, invasion, and metastasis. Endothelial cells also play an important role in the tumor microenvironment by direct interaction with tumor cells as well as by their role in blood vessel formation. The crucial role of neo-angiogenesis in cancer progression, mainly mediated by VEGF, and its therapeutic implications are well known and can be applied also to breast cancer. Finally, immune cells, can exert both inhibitory and stimulatory effects on
breast tumors, and the balance of these effects may profoundly influence tumor growth. In BC, alike other cancer types, significant heterogeneity in immune composition is observed across tumor subtypes and patients, and this is possibly at the basis of meaningful clinical responses in a subset of patients when treated with immunotherapeutic agents. Recent studies have undertaken large-scale, high-dimensional analysis of cells of hematopoietic origin in human breast tumors, thereby providing novel insights into the characterization and role of tumor-associated immune cell subsets in the immune response and progression of the disease \textsuperscript{14,15}.

1.1.3 Characteristics of breast cancer immune infiltrate

The immune infiltrate of BC tissues from chemo-naïve patients is dominated by the presence of T lymphocytes (CD3+), with minor populations of NK cells (CD3− CD56+ NKG2D+) and B lymphocytes (CD19/20+CD3−); in comparison, myeloid-lineage cells including macrophages (CD14\textsuperscript{hi} CD11b+), mast cells (FcεR1α+ CD117+ CD11b− CD49d+) and neutrophils (CD15+ CD11b+ CD49d−) are usually more evident in the normal breast tissue \textsuperscript{16}. Recently, Wagner et al. applied single-cell mass cytometry to millions of cells from 144 human breast tumor samples covering all clinical subtypes (54 luminal A-like, 71 luminal B-like, six luminal B-HER2+, one HER2 enriched and 6 TN tumors) to elucidate the phenotypic diversity and tumor-immune cell relationships in the breast cancer ecosystem \textsuperscript{15}. All different immune subpopulations were recognized; T cells and myeloid cells were the most abundant immune cell types, while NK cells, B cells, granulocytes, plasma cells, basophils, and plasmacytoid dendritic cells were less abundant \textsuperscript{15}. Interestingly, there was considerable inter-patient variability in tumor-associated immune cell
frequencies, as previously described\textsuperscript{14}. Nineteen myeloid clusters were recognized, grouped in 5 major categories: CD14 expressing classic (CD16-) and inflammatory (CD16+) monocytes, early immigrant macrophages (HLA-DR\textsuperscript{int} CD192+), tissue-resident macrophages (HLA-DR\textsuperscript{int}CD206+), tumor associated macrophages (TAMs, HLA-DR\textsuperscript{hi}CD64\textsuperscript{hi}) and myeloid-derived suppressor cells (HLA-DR-\textsuperscript{low})\textsuperscript{15}. Specifically, tumors were enriched in TAMs and depleted of tissue-resident macrophages, classical circulating, and pro-inflammatory monocytes compared with normal breast tissue, a condition known to be associated with aggressive disease\textsuperscript{17}.

Innate immune responses are crucial for the initiation of adaptive immune responses. There is strong evidence from genetic studies of mouse models that cells of the adaptive immune system carry out surveillance and can eliminate nascent tumors (a process called immuno-editing)\textsuperscript{18}. In-depth analyses of T cells by computational approaches applied to single cell data identified 10 CD4+ and 10 CD8+ T cell clusters, where the majority of T cell clusters harbored an effector memory phenotype (CCR7\textsuperscript{low}, CD45RA\textsuperscript{low})\textsuperscript{15}. In line with previous studies from chemo-naïve patients, both CD4+ and CD8+ T cells displayed increased expression of CD69 and HLA-DR activation markers compared with peripheral blood T cells, with a corresponding loss of markers of naïve T cells\textsuperscript{16}. Various levels of PD-1 and heterogeneous co-expression of co-inhibitory receptors and activation markers were detected among CD8+ and CD4+ T cell clusters\textsuperscript{15}. Tregs were identified based on expression of CD4, FOXP3, CD25, and CTLA-4\textsuperscript{15}. Tregs and PD-1\textsuperscript{hi}CTLA-4+CD38+ T cells were found to be enriched in tumors compared to normal breast tissue\textsuperscript{15}. In
their seminal paper, Bense et al. investigated the independent predictive and prognostic value of several in silico immune phenotypes in a large set of BC patients. An estimated high Treg-cell fraction was associated with a lower pathological complete response (pCR) rate, as well as shortened disease-free survival (DFS) and overall survival (OS), particularly in patients with HER2+ BCs, irrespective of ER status. A higher estimated γδ T-cell fraction was associated with a higher pCR rate, especially in patients with ER-positive BC, irrespective of HER2 status. These associations of specific immune subpopulations with parameters of disease outcome are of interest in the light of possible intervention strategies. However, characterization of the quality of the immune infiltrate and expression of possible targets of immunotherapies by tumor-infiltrating T cells is still limited, especially in luminal-like BC, possibly limiting the development of novel therapeutic approaches.

1.1.4 Evaluation of TILs in breast cancer: methodological recommendations

The presence of tumor infiltrating lymphocytes (TILs) in breast tumors and a positive association between higher numbers of TILs and more-favorable clinical outcome was documented in a clinical cohort as early as 1922. The relationship between higher levels of TILs and improved prognosis in patients with early stage BC has now been confirmed in many randomized, prospective clinical trials, both in the adjuvant and neoadjuvant setting, thus prompting the implementation of TIL evaluation in routine clinical practice. TILs are commonly detected in haematoxylin and eosin (H&E) stained histological slides via light microscopy, wherein they are distinguished by the typical features of
lymphocytes. When evaluating H&E-stained slides, TILs are divided into stromal-compartment TILs and intratumoral compartment TILs. Stromal TILs infiltrate the stromal tissue adjacent to the tumor cells and are considered to be the only true tumor-infiltrating cells. Stromal TILs comprise a complex mixture of different lymphocyte subtypes, dominated by T cells, with B cells, NK cells, and macrophages being less represented, as previously mentioned. In general, TIL populations are quantified as a continuous parameter by determining the percentage of stromal area covered by mononuclear cells. All mononuclear cells including lymphocytes and plasma cells should be scored (granulocytes and other polymorphonuclear leukocytes are excluded). The quantitative assessment of other mononuclear cells such as dendritic cells and macrophages is currently not recommended, although there is increasing evidence that they may be functionally important when present in tertiary lymphoid structures (TLS), that are ectopic lymphoid organs where lymphocyte proliferation and differentiation into effector T cells may take place at the tumor site. Lymphocyte predominant breast cancer (LPBC) can be used as a descriptive term for tumors that contain “more lymphocytes than tumor cells”, with a threshold varying between 50% and 60% stromal lymphocytes. At present, despite clinical data indicating the prognostic significance of TIL scoring in BC, stromal TIL evaluation is not considered routine in pathological assessment of BC specimens, thus no formal recommendation for a clinically relevant TIL threshold can be given at this stage.
1.1.5 Clinical significance of TILs

In patients with early stage BC, no well-validated biomarkers contribute additional information to determining prognosis beyond that provided by analysis of well-characterized clinical-pathologic parameters, such as tumor size and grade, nodal status, hormone-receptor and HER2 status, Ki-67 proliferation index, and more recently, gene-expression-based recurrence scores (such as Oncotype-Dx and Mammaprint). However, these are not sufficiently accurate to clearly distinguish the patients who are more likely to respond to chemotherapy (both in the early and advanced setting) and, more importantly, are not useful to select candidate patients for immunotherapeutic, chemotherapy-sparing strategies. Patients with TNBC display a robust linear relationship between an increase in TIL numbers over time and improved recurrence-free survival (RFS) end points, as noted in retrospective and prospective analyses performed on the Breast International Group (BIG) 2-98 trial, Finland Herceptin Trial (FinHER), Eastern Cooperative Oncology Group (ECOG) 2197 and ECOG 1199 trials, and the National Epirubicin Adjuvant Trial (NEAT)/BR9601 trial. A similar relationship between RFS and the TIL population was seen for patients with HER2+ tumors in the NeoALLTO trial and the HER2+ patient cohorts reported by Ali et al. TILs have been assessed also on samples from more than 2000 ER+/HER2- BC patients enrolled in prospective clinical trials without reporting any statistically significant correlation between TILs and survival. In a retrospective analysis, Bates et al. reported that HR+ early BC patients treated with adjuvant tamoxifen, whose tumors contained high numbers of FOXP3+ TILs, had significantly worse prognosis than those with lower FOXP3+ TILs. Liu et al. further implemented this observation.
by assessing FOXP3+ and CD8+ TILs in a retrospective cohort of surgically resected BC patients. The multivariate analysis confirmed that increased FOXP3+ TILs correlated with poor outcome in ER+ BC (treated with tamoxifen, chemotherapy, chemotherapy and tamoxifen or no adjuvant treatment), at least in tumors lacking concurrent CD8+ lymphocyte infiltration. The seminal paper by Denkert et al. showed that, for patients treated with anthracyclines and taxanes in the GeparDuo and GeparTrio prospective neoadjuvant trials, higher TIL levels at baseline were associated with a higher chance of pCR overall and in specific subgroups, including the HR+ BC subgroup (any HER2). These results were confirmed in a subgroup of 209 ER+/HER2- patients enrolled in another German Breast Group trial of neoadjuvant chemotherapy. Overall, these data indicate the importance of TIL evaluation as a putative prognostic tool, but still no consensus has been reached on the implementation of measuring TILs into clinical practice. Indeed, at the last St Gallen Breast Cancer Consensus Conference, only 29% of the panelists believed that the evaluation of TILs should be reported in TNBC and HER2+ early BC. Such uncertainty may be due to a broad, rather than deep characterization of the lymphocytic infiltration that precluded the identification of specific biomarkers to be used in the clinic, and to the lack of data in luminal-like cancers, which account for more than 70% of all breast malignancies.

1.2 Memory T cells in tumor

1.2.1 Generation and compartmentalization of memory T cells

It has been shown in mouse models that effector T cells (Teff) may serve as precursors of antigen-specific long-lived memory T cells (Tmem), which
persist in vivo as heterogeneous populations in multiple sites and can retrieve immune responses following pathogen re-exposure \(^{34}\). Tmem cells in humans are classically identified by the expression of the CD45RO isoform and by the lack of expression of the CD45RA isoform (CD45RO+CD45RA-) \(^ {35}\). Naive T cells uniformly express the lymphnode-homing CC-chemokine receptor 7 (CCR7), whereas Tmem cells are broadly subdivided into CD45RA-CCR7+ central memory T (Tcm) cells and CD45RA-CCR7- effector memory T (Tem) cells \(^ {35}\). Tem cells harbor a more activated phenotype and can produce more effector cytokines compared to Tcm cells, which on the contrary retain a higher proliferative capacity \(^ {35}\). In Cartoon 1 is shown a model for the generation of human Tmem cell subsets\(^ {35}\).

As revealed by single-cell analysis, the majority of CD3+CD8+ TILs in BC have Tem (CCR7-CD45RA-) and effector memory re-expressing CD45RA (TEMRA, CCR7-CD45RA+) phenotypes \(^ {14,16,36}\). Moreover, the majority of BC-enriched CD3+CD8+ TILs display an activated status \(^ {16}\).

T cell memory is critical to the maintenance of immunity against many common pathogens. During most infections, the majority of antigen-specific CD8 T cells generated by clonal expansion dies after antigen clearance, while a small fraction survives to differentiate into long-lived memory cells \(^ {37}\). A typical CD8 T cell response consists of three main developmental stages: effector cell expansion and differentiation; effector cell contraction; and stabilization and maintenance of the memory cell population \(^ {37}\). In the absence of antigen, Tcm cells preferentially home to lymphoid organs and are thought to provide long-term T cell immunity through self-renewal, whereas Tem cells preferentially migrate to
peripheral tissues where they rapidly respond to secondary antigen exposure, but are short-lived. It has been shown that specific clones of memory T cells that express a unique TCR can persist for decades in vivo. Elucidating the mechanisms at the basis of memory T cell maintenance in the long-term is important for developing effective T cell-based immunotherapies and is a matter of intense investigation. Some aspects in this regard are described below.

Cartoon 1. Model for the generation of human memory T cell subsets. The progressive differentiation of the three major circulating subsets (stem cell memory T (TSCM) cells, Tcm cells and Tem cells) from activated naive T cells is shown relative to the extent of antigen exposure. Naive, TSCM and Tcm cells circulate and migrate to lymphoid tissue, whereas Tem and Teff cells are the subsets of T cells that have the capacity to traffic to peripheral tissues. Adapted from Farber DL et al\textsuperscript{35}.

1.2.2 Tissue resident memory T cells

About a decade ago, mouse studies have established the existence of a new tissue-resident memory T (Trm) cell subset as a non-circulating subset that resides in peripheral tissue sites and, in some cases, elicits rapid in situ protective responses. These cells are distinguished from splenic and circulating Tmem cells by their upregulation of the marker
Trm can be found both in the CD4+ and CD8+ compartment, in various organs and tissues; specifically, CD8+ Trm cells are distinguished from splenic and circulating Tmem by the increased expression of CD69 combined with the expression of the epithelial cell-binding αEβ7 integrin (also known as CD103). Mouse studies suggest that Trm cell generation occurs within tissue sites from either activated Teff cells and/or Tem cell precursors that migrate to those sites. Compared to peripheral tissues such as lungs, intestine and skin, lymphoid tissues contain CD8+ Tcm cells and Tem cells, some of which also express CD69 but not CD103. It is currently unclear whether additional markers define lymphoid Trm cells and/or whether CD69+ Tem cells in lymphoid tissue are true resident memory cells. The identification of human Trm cells with tissue-specific signatures suggests that there is anatomical control of memory T cell generation in humans. There is emerging evidence that Trm cells can be multifunctional and that they have qualitative functional differences compared to other memory T cell types, due to tissue-specific inflammation states. Several studies have shown that there is a higher generation and maintenance of virus-specific effector and memory T cells in tissues compared with the circulation. For example, lung tissue was found to contain an increased frequency of influenza virus-specific memory CD8+ T cells compared to the blood and the spleen, and influenza virus-specific T cells in human lungs were found to have a Trm cell phenotype (that is, CD69+CD103+) 38. In the majority of cases, Trm found in tissues are IL-7R+, demonstrating a resting phenotype, as IL-7R is downregulated through activation 39. Cartoon 2 shows a schematic view of Tmem cells homing and distribution in tissues.
Cartoon 2. Schematic view of Tmem cell homing and distribution heterogeneity in blood and in tissues. The individual sites are defined as the circulation (red), of lymphoid origin (grey) or as peripheral tissues (yellow). Circulating TSCM, Tcm and Tem cell subsets migrate from the blood and circulate through the spleen and the lungs, where they can be primed to migrate to the intestines. They also migrate via the lymphatics and efferent vessels to the lymph nodes. Trm cells predominate in the skin, the lungs, the bone marrow and the intestines, but may also be present within the CD69+ T cell subsets in the spleen and the lymph nodes. The expression of certain chemokine receptors and/or integrins is associated with T cell migration and/or residence in the lymph nodes (CC-chemokine receptor CCR7)), the skin (CCR4, CCR10 and cutaneous lymphocyte antigen (CLA)), the intestines (CCR9 and α4β7 integrin), the lungs (CCR6) and the bone marrow (α2β1 integrin). Adapted from Farber DL et al.35

Single-cell RNA sequencing of FACS-sorted CD45+CD3+ TILs from TNBC specimens 36 revealed the existence of CD3+CD8+ T cells expressing CD103 which together with low expression of “tissue egress genes” (such as KLF2, SELL, S1PR1, S1PR5 and KLRG1), is informative of a Trm identity 40,41. These CD3+CD8+ Trm cells found in primary breast tumors are a distinct cluster of TILs, they have indeed antigen specificities
different from Tem cells as evidenced by non-overlapping TCR repertoires \cite{36}. In fact, high throughput technologies such as TCR sequencing have been used to study TCR repertoire heterogeneity of TILs subpopulations found in BC \cite{14}. Increased frequency of CD3+CD8+CD103+ TILs has been associated with improved prognosis in early-stage TN and basal-like BCs \cite{36,42}. As revealed by transcriptomic analysis, CD3+CD8+CD103+ cells are characterized by high expression of mRNA encoding for effector and cytotoxic proteins, nevertheless they also display high levels of immune checkpoint molecules \cite{36}. This is counterintuitive: how can an exhausted-like subset still be functional?

### 1.2.3 T cell exhaustion

T cell exhaustion has been historically reported in chronic viral infections to describe the diminished effector functions of T cells to chronic compared to acute antigen stimulation. The importance of this phenomenon in tumors is tightly linked to the mechanism of action of immune check-point inhibitors. Exhaustion is often seen as a dysfunctional state, but is important to underline that not all dysfunctional T cells are exhausted. According to some authors, exhaustion is better represented as a dynamic rather than static state. Recent data suggested that at least two distinct subsets of exhausted T cells exist, i.e., a precursor and a terminally differentiated subset, where the former is characterized by stem-like properties, and enhanced functionality and expansion capacity compared to the latter. Phenotypically, the difference between progenitor and terminally differentiated subsets is represented by the differential expression of PD1, TIM3 and TCF1, typically PD1^{int}TIM3^{low}TCF1^{+} for progenitors and PD1^{hi}TIM3^{hi}TCF1^{-} for terminally differentiated cells.
Recent studies from multiple groups indicated that one key difference between progenitor-like CD8+ T cells and memory precursor cells is the expression of TOX, a member of high mobility group of transcription factors, which is crucial for epigenetic remodeling and for promoting the long-term persistence of virus-specific CD8+ T cells during chronic LCMV infection 43.

Exhausted T cells in tumors are usually described as detaining a functionally impaired phenotype, with decreased (but not absent) cytokine production and with high levels of expression of multiple inhibitory receptors, such as PD1, TIM3, LAG3, CTLA4 and TIGIT. However, it is important to note that the expression of inhibitory receptors per se is not a sufficient condition to define the exhausted state, as also activated T cells feature the expression of these receptors. Analyzing BC specimens, Savas and colleagues 36 reported that high levels of PD-1 and CTLA-4, but not of TIM-3 or LAG-3 could be preferentially detected on the surface of CD3+CD8+ Trm TILs. Dysfunctional, CD3+CD8+ T cells expressing PD-1 are known to be abundant in tumors’ immune infiltrate and are a target of therapy with check-point blockers, however, clinical responses in different tumors are widely variable, possibly due to the heterogeneity in the immune cell composition, among other factors 44. Moreover, the relative abundance of precursors and terminally exhausted CD8+ T cell subsets, which respond differently to PD-1 blockade, may also have an impact on the effectiveness of the checkpoint blockade immunotherapy in cancer 45,46. In BC, different degrees of responsiveness to immune check-point blockade have been reported in different biological subtypes, mainly in
association with standard chemotherapy, specifically for PD-L1 expressing tumors, as more extensively discussed in the next section.

1.3 Therapeutic strategies

1.3.1 Check-point inhibitors

Triggering PD-1 receptor expressed on T cells inhibits T cell activation and effector function following cognate Ag activation. The gene encoding PD-1 was discovered back in 1992, and few years later its immunoregulatory function was established. The ligands for PD-1, B7-H1 (now called PD-L1) and B7-DC (now called PD-L2) play a major role in the inhibition of T cells: in their seminal paper, Chen and colleagues showed that mouse tumors with forced expression of PD-L1 were resistant to elimination by the immune system and that expression of the gene encoding PD-L1 was selectively upregulated in many human cancers. The first phase I clinical trials of PD-1 blockade, run in advanced, heavily pre-treated, solid tumors, showed some dramatic tumor regressions, with manageable toxicities. This led to the extensive testing of this new drug class in more advanced phase trials and ultimately to registration for the treatment of big cancer killers such as melanoma, non-small cell lung cancer and clear cell renal carcinoma.

In line with these results, immune check-point blockade in BC firstly pointed to the tumor subtypes reported to be more immunogenic (i.e. TNBC), displaying the higher number of TILs as evaluated by IHC. Among the class of compounds tackling the PD-1/PD-L1 axis, atezolizumab (alike durvalumab and avelumab) is a monoclonal antibody (mAb) directed against PD-L1, while earlier compounds such as Pembrolizumab and Nivolumab specifically target the PD-1 molecule. Table 1 lists results of
the clinical trials in different phases of research for all biological subtypes. As expected, the great majority of trials evaluated drug efficacy in TNBC, reaching convincing, statistically significant results in terms of progression-free survival (PFS) for the PD-L1 positive population treated with nab-paclitaxel + atezolizumab compared to nab-paclitaxel + placebo. Table 1bis lists ongoing (recruiting) clinical trials assessing efficacy of different immunotherapeutic strategies (mostly in combination with standard agents) among all different BC biological subtypes.

<table>
<thead>
<tr>
<th>Author</th>
<th>Intervention</th>
<th>Ph</th>
<th>Target population</th>
<th>n° of pts</th>
<th>PD-L1 status</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rugo, 2018</td>
<td>Pembrolizumab</td>
<td>I</td>
<td>ER+, HER2-</td>
<td>25</td>
<td>PD-L1+</td>
<td>ORR 12%; PFS 1.8 months; OS 8.6 months</td>
</tr>
<tr>
<td>Loi, 2019</td>
<td>Pembrolizumab+ Trastuzumab</td>
<td>I</td>
<td>HER2+</td>
<td>58</td>
<td>80% PD-L1+</td>
<td>PD-L1 ORR 15%; PFS 2.7 months</td>
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<tr>
<td>Nanda, 2016</td>
<td>Pembrolizumab</td>
<td>I</td>
<td>TNBC</td>
<td>27</td>
<td>PD-L1+</td>
<td>ORR 18.5%; PFS 1.9 months; OS 11 months</td>
</tr>
<tr>
<td>Adams, 2019</td>
<td>Pembrolizumab</td>
<td>II</td>
<td>TNBC</td>
<td>170</td>
<td>62% PD-L1+</td>
<td>ORR 5.3% (5.7% in PD-L1+); PFS 2 months; OS 9 months</td>
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<td>Adams, 2019</td>
<td>Pembrolizumab</td>
<td>II</td>
<td>TNBC</td>
<td>84</td>
<td>PD-L1+</td>
<td>ORR 18%; PFS 2.1 months; OS 18 months</td>
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<td>Dirix, 2017</td>
<td>Avelumab</td>
<td>I</td>
<td>TNBC (34.5%); ER+</td>
<td>168</td>
<td>50.5% PD-L1+</td>
<td>ORR 3% (TNBC 5.2%); PFS 5.9 weeks; OS 8.1 months</td>
</tr>
<tr>
<td>Nanda, 2016</td>
<td>Pembrolizumab</td>
<td>I</td>
<td>ER+</td>
<td>25</td>
<td>PD-L1+</td>
<td>PFS 1.8 months; OS fist-line 17.6%; OS PD-L1+ 10.1%</td>
</tr>
<tr>
<td>Emens, 2019</td>
<td>Atezolizumab</td>
<td>I</td>
<td>TNBC</td>
<td>115</td>
<td>80% PD-L1+</td>
<td>PFS 1.9 months; OS fist-line 17.6%; OS PD-L1+ 10.1%</td>
</tr>
<tr>
<td>Emens, 2019</td>
<td>Atezolizumab + Nab-paclitaxel</td>
<td>I</td>
<td>TNBC</td>
<td>33</td>
<td>unselecte d</td>
<td>ORR 39.4%; PFS 5.5 months; OS 14.7 months</td>
</tr>
<tr>
<td>Schmid, 2018</td>
<td>Atezolizumab + Nab-paclitaxel</td>
<td>III</td>
<td>TNBC</td>
<td>902</td>
<td>41% PD-L1+</td>
<td>PFS 7.2 vs 5.5 months (7.5 vs 5 months in PD-L1+); OS 21.3 vs 17.6 months (25 vs 15.5 months in PD-L1+)</td>
</tr>
</tbody>
</table>

Table 1. Clinical trials targeting PD-1/PD-1 axis in breast cancer. Abbreviations: ER, estrogen receptor; TNBC, triple negative breast cancer; ORR, overall response-rate; PFS, progression-free survival; OS, overall survival.
<table>
<thead>
<tr>
<th>Durvalumab</th>
<th>Olaparib</th>
<th>II</th>
<th>TNBC</th>
<th>Metastatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Durvalumab</td>
<td>Olaparib + Fulvestrant</td>
<td>II</td>
<td>ER+/HER2-</td>
<td>Metastatic</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>CBDCA + Docetaxel</td>
<td>II</td>
<td>TNBC</td>
<td>Neo-adjuvant</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>Anthracycline + Taxanes + Endocrine Therapy</td>
<td>III</td>
<td>ER+/HER2-</td>
<td>Neo-adjuvant/Adjuvant</td>
</tr>
<tr>
<td>Nivolumab</td>
<td>Anastrozole + Palbociclib</td>
<td>II</td>
<td>ER+/HER2-</td>
<td>Neo-adjuvant</td>
</tr>
<tr>
<td>Nivolumab</td>
<td>Capecitabine</td>
<td>II</td>
<td>TNBC</td>
<td>Adjuvant</td>
</tr>
<tr>
<td>Atezolizumab</td>
<td>Anthracycline + Taxanes</td>
<td>III</td>
<td>TNBC</td>
<td>Neo-adjuvant</td>
</tr>
<tr>
<td>Atezolizumab</td>
<td>Anthracycline + Taxanes + Trastuzumab + Pertuzumab</td>
<td>III</td>
<td>HER2+</td>
<td>Neo-adjuvant</td>
</tr>
<tr>
<td>Atezolizumab</td>
<td>Anthracycline + Taxanes</td>
<td>III</td>
<td>TNBC</td>
<td>Adjuvant</td>
</tr>
<tr>
<td>Durvalumab</td>
<td>Trastuzumab + Pertuzumab</td>
<td>II</td>
<td>HER2+</td>
<td>Neo-adjuvant</td>
</tr>
</tbody>
</table>

Table 1bis. Ongoing (recruiting) clinical trials targeting PD-1/PD-L1 axis in breast cancer.

Abbreviations: ER, estrogen receptor; TNBC, triple negative breast cancer

The role of chemotherapy in modifying the immune milieu, by increasing TIL levels and by enhancing antigen presentation to effector T cells after apoptosis or immunogenic cell death of cancer cells, have been elucidated both in animal models \(^{61,62}\) and in samples from patients receiving neoadjuvant chemotherapy \(^{63}\). The positive results obtained in metastatic TNBC with atezolizumab plus chemotherapy, led to expansion of clinical research to the early setting of disease, both adjuvant (NCT03498716) and neoadjuvant (NCT03281954). HER2-positive BC is also known to generally display high levels of TILs, with major infiltration in HR-negative cases; thus recruiting, phase III trials are assessing the efficacy of atezolizumab plus standard chemotherapy and anti-HER2 mAbs (either trastuzumab, TDM1 and pertuzumab) in the HER2-positive population both in advanced and early setting (NCT03199885 and NCT03726879, respectively). In HER2-positive disease, an ongoing phase II trial is assessing the efficacy of pembrolizumab plus paclitaxel, trastuzumab and pertuzumab in the neoadjuvant setting (NCT03747120) and a phase Ib trial of pembrolizumab plus TDM1 in the advanced setting (NCT03032107). Pembrolizumab has been widely tested in BC, not only in
TNBC and HER2-positive disease, but also in luminal-like cancers. In hormone receptor positive disease, at least three phase II clinical trials are ongoing, assessing the efficacy of pembrolizumab in combination with endocrine therapy, either tamoxifen in patients with ESR1 mutation (NCT03879174), exemestane/leuprolide (NCT02990845) and fulvestrant (NCT03393845), for treatment of locally advanced and metastatic disease. These trials are of extreme interest as we know that hormone receptor positive/HER2-negative tumors are usually considered immunologically “cold”, theoretically not sensitive to PD-1/PD-L1 axis blockade. However, it is hypothesized that the combination with endocrine therapy, which has been shown to potentially promote antitumor immunity, might reverse tumor-promoting inflammation.

CTLA-4, an inhibitory receptor expressed on T cells, was recognized as a closely-related homologue of CD28, a co-stimulatory molecule capable of binding both of the B7-1/B7-2 ligand pair expressed by antigen-presenting cells (APCs), but retaining significantly higher affinities for both B7 ligands than does CD28. Given these differences in affinity, one well-established model to describe CTLA-4 inhibitory function considers outcompeting CD28 and therefore interrupting T cell activation signaling by CD28 signaling. The proposed inhibitory function of CTLA-4 involves indirect inhibition of T cell activation via other cells, such as APCs or other T cells. Unlike effector T cells, Tregs constitutively express CTLA-4, which has been shown to be important for their suppressive functions. Both CTLA-4 and PD-1 binding have similar negative effects on T cell activity; however, the timing of downregulation and the anatomic compartmentalization of immune inhibition by the two immune checkpoints
differ. While CTLA-4 functions during the priming phase of T-cell activation, PD-1 functions during the effector phase, predominantly within peripheral tissues.

In BC, the clinical development of agents targeting the co-inhibitory receptor CTLA-4 (e.g. ipilimumab and tremelimumab) has been hampered by their well-known spectrum of toxicities. The most frequent adverse events affect the skin and the gastrointestinal tract, but also other potentially severe toxicities, including hepatitis, thyroiditis, colitis, and hypophysitis, can be observed at a higher frequency compared to the cognate anti-PD-1/PD-L1 compounds. To date, the only published study testing tremelimumab alone is a phase I trial on 26 patients with advanced, hormone-responsive BC, treated with concurrent exemestane; diarrhea and transaminitis were dose limiting toxicities and best response was SD in 42% of patients. Beside combination strategies, ipilimumab was tested in BC, alone or with cryoablation, in a pilot study of 19 patients in the neoadjuvant setting, showing promising peripheral elevations in Th1-type cytokines and proliferating CD4+ and CD8+ T cells. While toxicities, such as pneumonitis, are equal, anti-PD-1/PD-L1 and anti-CTLA-4 mAb combination trials are promisingly more effective than single agents. In this regard, two phase II trials, one testing durvalumab plus tremelimumab combination in the advanced, HER2 negative setting (NCT02536794) and one assessing durvalumab plus tremelimumab and fulvestrant in advanced, hormone receptor positive BC (NCT03430466), are actively recruiting.
1.3.2 New perspectives in therapeutic strategies: adoptive cell transfer and CAR-T

Application of adoptive cell therapy (ACT) of TILs relies on the ability to isolate, activate and expand patients’ derived tumor-reactive T cells in vitro, followed by infusion in vivo of manipulated, clinical-grade autologous cell-products under good manufacturing practice (GMP) regulations. In the standard protocol, TILs are obtained from the enzymatically digested tumor specimen and activated in vitro with high dose of interleukin-2 (IL-2) for 3-5 weeks. Subsequently, TILs are expanded to therapeutic relevant numbers by a rapid expansion protocol (REP). Accordingly, TILs are co-cultured with “feeder” cells that are an irradiated, allogeneic third-party healthy donor peripheral blood mononucleated cells (PBMC), in additional presence of anti-CD3 (OKT3 clone) mAb as well as high dose IL-2 for additional 2 weeks. Afterward, cells are harvested, washed and eventually cryopreserved. Overall, the protocol to obtain TILs expanded and ready for ACT lasts about 6-8 weeks, but it is possible to reduce the time of in-vitro manipulation and so the manufacturing costs, using the so called “young TILs” protocol. Even if BC is a very heterogeneous disease, TILs can be successfully obtained from all different biological subtypes and can maintain tumor-reactive potential, as tested in in vivo xenograft mouse models. Interestingly, the percentage of TILs in tumor stroma and the presence of tertiary lymphoid structure (TLSs) in the tumor-adjacent tissue, evaluated by H&E, can be a useful information to predict the number of TILs after 2-week ex vivo expansion. Recently, a first-case report was published, concerning a patient with chemo refractory HR-positive metastatic BC: the patient experienced complete durable regression (more than 22 months at the time of publication) of all diffuse...
metastatic lesions. Since a treatment of epithelial cancers with unselected TILs was not beneficial in previous studies executed in NCI, to boost tumor killing of TILs, tumor-specific mutated antigens (neoantigens) were identified by combined whole-exome sequencing (WES) and RNA sequencing from patient’s breast subcutaneous lesions. A similar approach was used for the pulmonary metastasis of a TNBC patient, where an HLA-II-restricted immunogenic mutation was found. The patient, enrolled in the NCT01174121 clinical trial, was treated with autologous TILs reactive against HLA-I and HLA-II-restricted mutant versions of four tumor mutated proteins. Such multiple antigen targeting was designed to overcome potential mechanisms of tumor escape. Although limited evidence of the effectiveness of the treatment has been reported so far, ACT represented a proof of concept of the paramount role of TILs in mediating BC regression.

The premise of CAR-T immunotherapy is to harvest polyclonal T cells from the patient’s own blood (autologous treatment) or from a healthy donor (allogeneic treatment), and genetically redirect them to recognize the patient’s tumor. This is achieved by redirecting the specificity of T cells with a tumor associated antigen (TAA)-specific receptor, using a plasmid or a viral vector. In this regard, a lentivirus is generally employed. A major advantage of the CAR-T approach is to overcome the hurdle of obtaining TILs, especially from immunologically “cold” tumors with low numbers of infiltrating immune cells, or from patients with inaccessible or unresectable tumors. In BC, TAAs including HER2, Lewis Y, mesothelin, folate receptor alpha (FR-α), and Muc1 have been tested in vitro and in animal models as CAR-T targets. In this context, Kuznetsova et al. established a genetically
modified T cell-expressing CAR specific for the HER2 antigen which were able to specifically target and kill HER2-overexpressing BC cells in coculture. Dual-targeted T cells co-expressing HER2 and MUC-1-specific CARs have been found to effectively kill BC cells expressing both targets. This high efficiency of CAR-T cells to kill tumor cells may help overcome resistance to antibody-based therapy. Nonetheless, the affinity of the CAR to its target should be fine-tuned, as an early trial with anti-HER2 CAR T cells with high affinity led to serious toxicity, due to on-target off-tumor recognition on normal cardiopulmonary tissue. A recent phase I/II clinical trial of CAR-T cells in women with HER2 non-amplified (0, 1+ or 2+) metastatic BC after second-line chemotherapy has been published (NCT01022138). This study enrolled 32 patients that were infused with a bispecific anti-CD3 and anti-HER2-armed activated T cell. The treatment was shown to be safe with a trend of improved survival. Another interesting trial will evaluate the treatment of brain metastasis of HER2 BC patients by intraventricular administration of HER2 specific CAR-T cells (NCT03696030). Of note, such administration of CAR-T cells into tumor site directly might be a good approach to increase their efficacy and limit unwanted effects. A phase I study of CAR-T cells targeting cMet, which is aberrantly activated in 50% of BCs and correlates with poor prognosis, has been completed (NCT03060356). The intratumoral injection of c-Met-CAR T was well tolerated in the six patients treated with metastatic BC. Other phase I/II trials with CAR-T cells specific for Epcam (NCT02915445), Mesothelin (NCT02792114), or the cancerous form of truncated MUC1 (NCT04020575) are all in recruitment phase. These are aimed to assess the safety and efficacy of treating patients with TNBC or advanced metastatic HER2-negative BC. While Epcam and Mesothelin
are good TAAs for TNBC, MUC1 is the most relevant antigen for BC targeting as it is expressed in 90% of BC patients.

To overcome this immune-hostile microenvironment, the need to combine CAR-T therapy with checkpoint blockade or targeting specific soluble factors in the TME (such as COX2 and PGE2), which may mediate T-cell suppression, becomes evident. Another approach of polarizing the immune response to support effector T cell function could be independent from targeting TME components. In light of the recent findings that the microbiota is a significant modulator of response to immune checkpoint blockers and, consequently, of patient response to therapy, it will be interesting to explore microbiota modulation also in T-cell-based immunotherapies. In support of this concept, a BC associated microbiota has been described. While no studies to date have characterized the impact of microbial composition on therapeutic response in BC, the findings in melanoma indicate that strategies aiming at modulating microbial composition and diversity could be used to improve T cell based immunotherapies for BC as well.

2 AIM OF THE STUDY

BC has long been considered a non-immunogenic cancer type. However, it is a very heterogeneous disease, with different molecular subtypes harboring different biological characteristics, extent of immune infiltration and prognostic significance (Sorlie et al., 2003). TNBCs and HER2 enriched-like tumors showed the highest response to checkpoint blockade directed to PD-1 and PD-L1 in early phase clinical trials (Dirix et al., 2018; Emens et al., 2019; Loi et al., 2019). While these results are
encouraging, immunotherapy is still poorly effective in the majority of BCs characterized by a luminal-like subtype, often referred as “immunologically cold” tumors (Brignone et al., 2010; Rugo et al., 2018; Vonderheide et al., 2010).

The first aim of our study is to investigate the complexity of T cell phenotypes across all different BC biological subtypes, and defined those T cell characteristics that are associated with progression of the disease; to do so we applied high-dimensional single-cell profiling to dozens of luminal-like BCs.

The second aim is to specifically identify, among T cells infiltrating luminal-like BCs, a subset of putatively antigen-specific Trm cells with enhanced functionality, capable of interfering with the tumor microenvironment and potentially select patients with improved survival, giving important hints on the potential immunogenicity of hormone receptor-positive disease.

3 MATERIALS AND METHODS

3.1 Cells
Peripheral blood mononuclear cells (PBMCs) isolated from buffy coats and from patients’ peripheral blood were stored in liquid nitrogen according to standard procedures.

3.2 Patients characteristics
Clinical and experimental protocol were approved by the Humanitas Clinical and Research Center Internal Review Board (IRB) (Prot. Nr Humanitas ONC-OSS-02-2017) and both patients and donors signed
consent forms in accordance with Declaration of Helsinki. In this study, we included patients affected by early stage BC, consecutively surgically treated (lumpectomy or mastectomy with sentinel lymph node biopsy and, if clinically indicated, axillary dissection) at Humanitas Cancer Center Breast Surgery Unit. Tumor (n = 54), breast gland normal tissue from the same surgical specimen (n = 54) and lymph nodes (either metastatic or tumor-free) (n = 11 and 6, respectively, from a total of 13 patients) were obtained in the operating room in a sterile field by the surgeon, while blood sample collection (12-15 mL; n = 54) were obtained by venipuncture before anesthesia induction. None of the patients received neoadjuvant chemotherapy or endocrine therapy. Details on patients’ characteristics are summarized in Table 2 and in Table 3, specifically for patients used for lymph nodes staining. Of note, as samples were collected from consecutive patients, we could observe in our cohort, in line with well-known epidemiological data, all different biological subtypes (e.g., luminal-like, HER2-enriched and TNBC).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All pts</td>
<td>54</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>59.5 (32-90)</td>
</tr>
<tr>
<td>Surgery type</td>
<td></td>
</tr>
<tr>
<td>Lumpectomy</td>
<td>31 (57)</td>
</tr>
<tr>
<td>Mastectomy</td>
<td>23 (43)</td>
</tr>
<tr>
<td>Hystology</td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>45 (83)</td>
</tr>
<tr>
<td>ILC</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (2)</td>
</tr>
<tr>
<td>pT</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13 (24)</td>
</tr>
<tr>
<td>2</td>
<td>36 (67)</td>
</tr>
<tr>
<td>3</td>
<td>4 (7)</td>
</tr>
<tr>
<td>4</td>
<td>1 (2)</td>
</tr>
<tr>
<td>pN</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>34 (63)</td>
</tr>
<tr>
<td>1</td>
<td>6 (11)</td>
</tr>
<tr>
<td>2</td>
<td>7 (13)</td>
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<tr>
<td>3</td>
<td>4 (7)</td>
</tr>
<tr>
<td>X</td>
<td>3 (6)</td>
</tr>
<tr>
<td>ER</td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>47 (87)</td>
</tr>
<tr>
<td>negative</td>
<td>7 (13)</td>
</tr>
<tr>
<td>PgR</td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>42 (78)</td>
</tr>
<tr>
<td>negative</td>
<td>12 (22)</td>
</tr>
<tr>
<td>HER2</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>27 (50)</td>
</tr>
<tr>
<td>1+</td>
<td>5 (9)</td>
</tr>
</tbody>
</table>
Table 2. Clinical-pathologic patients’ characteristics. Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; pT, pathological tumor stage; pN, pathological nodal stage; X, not assessed; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; FISH, fluorescent in situ hybridization; LVI, lympho-vascular invasion.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All pts</td>
<td>13</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>56 (38-82)</td>
</tr>
<tr>
<td>Surgery type</td>
<td></td>
</tr>
<tr>
<td>Lumpectomy</td>
<td>6 (46)</td>
</tr>
<tr>
<td>Mastectomy</td>
<td>7 (54)</td>
</tr>
<tr>
<td>Hystology</td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>13 (100)</td>
</tr>
<tr>
<td>pT 1</td>
<td>2 (15)</td>
</tr>
<tr>
<td>pT 2</td>
<td>10 (77)</td>
</tr>
<tr>
<td>pT 3</td>
<td>1 (8)</td>
</tr>
<tr>
<td>pN 1</td>
<td>5 (38)</td>
</tr>
<tr>
<td>pN 2</td>
<td>4 (31)</td>
</tr>
<tr>
<td>pN 3</td>
<td>4 (31)</td>
</tr>
<tr>
<td>ER positive</td>
<td>10 (77)</td>
</tr>
<tr>
<td>ER negative</td>
<td>3 (23)</td>
</tr>
<tr>
<td>PgR positive</td>
<td>8 (62)</td>
</tr>
<tr>
<td>PgR negative</td>
<td>5 (38)</td>
</tr>
<tr>
<td>HER2 0</td>
<td>6 (46)</td>
</tr>
<tr>
<td>HER2 1</td>
<td>1 (8)</td>
</tr>
<tr>
<td>HER2 2+ FISH positive</td>
<td>2 (15)</td>
</tr>
<tr>
<td>HER2 2+ FISH negative</td>
<td>2 (15)</td>
</tr>
<tr>
<td>HER2 3+</td>
<td>2 (15)</td>
</tr>
<tr>
<td>Ki-67 1-15%</td>
<td>4 (31)</td>
</tr>
<tr>
<td>Ki-67 &gt;15%</td>
<td>9 (69)</td>
</tr>
<tr>
<td>LVI present extensive</td>
<td>6 (46)</td>
</tr>
<tr>
<td>LVI present focal</td>
<td>2 (15)</td>
</tr>
<tr>
<td>LVI absent</td>
<td>5 (38)</td>
</tr>
</tbody>
</table>

Table 3. Clinical-pathologic patients’ characteristics for patients used for lymph nodes staining.

3.3 Sample collection and processing

Blood samples were collected in Vacutainer EDTA tubes (BD), while the tumor, normal tissue and lymph nodes samples were collected in RPMI-
1640 pure medium (Sigma-Aldrich). PBMCs were isolated from the blood samples or from healthy donors (buffy coats) using a density gradient centrifugation after blood stratification on Ficoll-Paque Premium (GE Healthcare). The tumor, normal tissue and lymph nodes samples were processed into single cell suspensions by mincing the freshly obtained surgical specimens and subsequent enzymatic digestion using Liberace TL (Sigma) for 20 min at 37°C. Subsequently, samples were passed through a 100-µm cell strainer (Falcon) and washed with physiological saline solution (NaCl 0.9%; Baxter). The cells were frozen in liquid nitrogen according to standard procedures.

### 3.4 High-dimensional single cell analysis by flow cytometry

Samples were analyzed or sorted by flow cytometry and cell sorter using the fluorochrome-conjugated monoclonal antibodies (mAbs) indicated in Table 4. For ex vivo immunophenotyping experiments, frozen samples were used and were thawed in RPMI-1640 medium supplemented with 10% FBS (Sigma-Aldrich), 1% penicillin-streptomycin and 1% Ultra-glutamine (both from Lonza) (hereafter referred to as R10), supplemented with 20 µg/mL DNase I from bovine pancreas (Sigma-Aldrich). After extensive washing with PBS (Sigma-Aldrich), the cells were stained immediately with the combination of mAbs listed in Table 4, together with Zombie Aqua Fixable Viability kit (Biolegend). All mAbs were previously titrated on human PBMCs and used at the concentration giving the best signal-to-noise ratio, as described. Chemokine receptors were stained for 20 minutes at 37°C, while all other surface markers were stained for 20 minutes at room temperature.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Fluorochrome</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Cat. #</th>
<th>Lot. #</th>
<th>Panel</th>
</tr>
</thead>
</table>

32
Table 4. Fluorochrome conjugated mAbs used for flow cytometry analysis and T cell sorting. The specificity, fluorochrome, clone, manufacturer, catalog and lot are shown.

All data were acquired on a FACS Symphony A5 flow cytometer (BD Biosciences) equipped with five lasers (UV, 350 nm; violet, 405 nm; blue, 488 nm; yellow/green, 561 nm; red, 640 nm; all tuned at 100 mW, except UV tuned at 60 mW) and capable to detect 30 parameters. Flow cytometry data were compensated in FlowJo by using single stained controls (BD Compbeads incubated with fluorescently conjugated antibodies). 86 T cell subsets were defined as shown in Figure 1.

![Fluorescence stability evaluated over time](image)

Figure 1. Gating strategy adopted to identify T cell subsets by flow-cytometry analysis. Fluorescence stability was evaluated over time. Using forward scatter-area (FSC-A) and...
forward scatter height (FSC-H) gate doublets and aggregates were excluded from analysis. CD4+ and CD8+ T cells were identified on CD45+ live/dead cells.

3.5 Computational analysis of flow cytometry data

Flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version 9, and analyzed by standard gating to remove aggregates and dead cells, and identify CD4+ and CD8+ T cells. 1,000 of each CD8+ and CD4+ T cells per sample were subsequently imported in FlowJo version 10. Data were then biexponentially transformed and exported for further analysis in Python version 3.7.3 using a custom-made script (available at https://github.com/luglilab) that makes use of PhenoGraph from the scikit-learn package. Tissue samples were labeled with a unique computational barcode, converted into comma separated (CSV) files, and concatenated in a single matrix using the “concat” function in pandas (https://pandas.pydata.org/). The K value, indicating the number of nearest neighbors identified in the first iteration of the algorithm, was set to 1,000. UMAP applications were run in Python and visualized using FlowJo software version 10.

Clusters representing <0.5% were excluded from subsequent analyses. The data were then reorganized and saved as new FCS files, one for each cluster, that were further analyzed in FlowJo to determine the frequency of positive cells for each marker and the corresponding mean fluorescence intensity (MFI). These values were multiplied to derive the integrated MFI (iMFI, rescaled values from 10 to 100). The heatmap, showing the iMFI of each marker per cluster, and the subsequent metaclustering were performed using the gplots R package. Hierarchical metaclustering of all samples, based on the frequency of Phenograph
clusters, was performed in R based on the Euclidean distance and Ward-linkage. Pearson correlation analysis was used to investigate the relationship between CD4+ and CD8+ clusters.

Hierarchical metaclustering of all samples, based on the frequency of Phenograph clusters, was performed in R based on the Euclidean distance and Ward-linkage.

3.6 scRNA-seq data processing and in silico sorting of T cell subsets

RNA-sequencing (scRNA-seq) counts from 8 breast cancers were downloaded from Gene Expression Omnibus dataset (GSE114725). Analysis was restricted to the cells from tumoral samples (n = 21,253) and with a CD3E gene expression level >1.87 (n = 3,637) based on the normalized expression levels (E). Subsequently, CD3E+ cells were separated into CD4+ (n = 475) and CD8+ (n = 1167) based on the normalized expression levels of CD4 (E > 1.56), and CD8A (E > 2.07). Thresholds of expression were determined by calculating 25% percentiles of the mRNA distribution, by excluding cells of the lower percentile and cells double positive for CD4 and CD8A. Data were imported into R version 3.5.1 and analyzed with Seurat version 3.0.1. Genes detected in less than three cells or containing more than 200 features were excluded from the analysis. The resulting datasets were normalized and log-transformed using the “ScaleData” function in Seurat. Cluster analysis was performed by using the “FindClusters” function with a resolution of 1.2 and 1 for CD4+ and CD8+, respectively. Clusters with ambiguous or unknown gene expression (i.e., expressing genes not related to T cells) were removed both from the UMAP plot and from the differential analysis. DEGs
were identified among clusters through the “FindAllMarkers” function in Seurat. A schematic representation of the experimental workflow is depicted in Figure 2.

Figure 2. Schematic representation of the experimental workflow; analysis was performed on peripheral blood, normal breast tissue and tumor from 54 early breast cancer patients (see Table 2 for clinical-pathologic characteristics), data were obtained in 4 different experiments performed in 4 consecutives days (due to the high number of samples analyzed).

3.7 Analysis of effector molecules production

To induce cytokine production, cells were plated in U-bottom 96-well plate and stimulated with phorbol 12-myrisate 13-acetate (PMA, 10 ng/mL; Sigma Aldrich) and Ionomycin (1 μg/mL; Sigma Aldrich), or left unstimulated at 37°C, in the presence of Golgi Plug (1 μg/mL; BD Biosciences), for 3 hours. Subsequently, cells were collected, washed with PBS and fixed and permeabilized by using the Cytofix/Cytoperm, followed by staining with mAbs listed in Table 4. In Figure 12, the percentage of cytokine-producing cells is referred to the stimulated condition subtracted of its background in the unstimulated condition.

3.8 Total RNA-sequencing analysis

CD103+CD39lowCD127+, CD103+CD39hiCD127- and CD103-CD39-CD45RA-, pre-gated as Aqua- CD8+CD69+, were isolated from previously frozen tumor samples, with a FACSaria cell sorter (BD Biosciences). A list
of antibodies used for FACS sorting is shown in Table 5 and patients characteristics for the samples selected for sorting are listed in Table 6. Cells were sorted in 10 µL PBS 1X (Sigma-Aldrich), spun down immediately and stored frozen at -80°C in a maximum volume of 7 µL PBS 1X (Sigma-Aldrich). Library preparation was performed by starting directly from ~500 cells per sample using the SMART-Seq Stranded Kit (Clontech-Takara) and following the manufacturer’s protocol. Libraries were qualitatively assessed by using TapeStation 4200, quantified by Qubit Fluorimeter, then multiplexed in equimolar pools and sequenced on a NextSeq-550 Illumina Platform, generating on average 32.9 million 75bp-PE reads per sample. The sort procedure was performed on 6 different luminal-like BC samples and, based on the number of single cells obtained after sort of each population, 4 samples were deemed suitable for bulk total-RNAseq. After quality check, 3 replicates of CD127-CD39hi and 4 replicates of CD127+CD39lo Trm cells were deemed suitable for differential analysis. Paired-end strand-specific reads were then aligned to the human genome (GENCODE Human Release 32; Reference genome sequence: GRCh38/hg38) using STAR (version 2.5.1b) 88. Alignments were performed using default parameters. Reads associated with annotated genes were counted with the STAR aligner option-quantMode geneCounts. Differential gene expression between human T cell subsets was assessed using the edgeR package (version 3.20.9) 89. Benjamini-Hochberg correction was applied to estimate the FDR.

<table>
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Table 5. Fluorochrome conjugated mAbs used for flow cytometry analysis and T cell sorting. The specificity, fluorochrome, clone, manufacturer, catalog and lot are shown.

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<th>N (%)</th>
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</tr>
<tr>
<td>Median age, years (range)</td>
<td>50 (38-81)</td>
</tr>
<tr>
<td>Surgery type</td>
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</tr>
<tr>
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<td>2 (33)</td>
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<tr>
<td>Mastectomy</td>
<td>4 (66)</td>
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<tr>
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</tr>
<tr>
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<td>pT</td>
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<td>1</td>
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<td>2</td>
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</tr>
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<td>absent</td>
<td>3 (50)</td>
</tr>
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</table>

Table 6. Clinical-pathologic patients' characteristics. Abbreviations: IDC, invasive ductal carcinoma; pT, pathological tumor stage; pN, pathological nodal stage; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2; FISH, fluorescent in situ hybridization; LVI, lympho-vascular invasion.

### 3.8.1 Gene signature identification (RNAseq)

Gene Set Enrichment Analysis (GSEA) was performed using GSEA (version 3.0) software (Broad Insitute, MIT) and gene list ranked based on log2 fold changes. The gene set enrichment analysis was conducted in pre-ranked mode with scoring scheme “classic” and 1,000 permutations. The maximum gene set size was fixed at 5,000 genes, and the minimum size fixed at 10 genes. The gene signature was retrieved from the Molecular Signatures Database (MSigDB v6.2).
3.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7. Significance of differences for the frequency of single Phenograph clusters among blood, normal tissue and tumor samples was determined by using two-way ANOVA with Bonferroni post-hoc test. To compare distributions of manually gated subsets, significance was determined by paired Wilcoxon t test. Correlation of data were determined by calculating the Pearson correlation coefficient. P values <0.05 were considered statistically significant.

3.10 Survival analysis

Transcriptomic and clinical data related to The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset were downloaded from the cBioPortal platform (http://www.cbioportal.org). For the three signatures (CD127-CD39\textsuperscript{hi} Trm; CD127+CD39\textsuperscript{lo} Trm; CCR8\textsuperscript{hi}ICOS\textsuperscript{hi}IRF4+ effector Treg), normalized signal values were converted into z-score. The mean z-score value was used to classify tumor samples into Low and High expression groups. Survival analysis was performed with GraphPad Prism using the Kaplan-Meier (KM) approach and applying the Log-rank (Mantel-Cox) test to estimate survival curves comparison. The logrank test is a univariate analysis used to test the null hypothesis that there is no difference between the populations in the probability of an event (here a death) at any time point. The analysis is based on the times of events (here deaths). For each such time we calculate the observed number of deaths in each group and the number expected if there were in reality no difference between the groups.
4 RESULTS

4.1 Breast cancer tumors are enriched in Trm cells

4.1.1 scRNAseq-guided informative 27-parameters FACS panel

We initially took advantage of publicly available data from the single-cell RNA-seq analyses of 8 breast cancers published by Azizi et al.\(^\text{14}\). We performed an in silico sorting from 47.016 CD45+ cells from tumor, normal tissue, lymph nodes and blood from 8 breast cancer patients exhibiting different biological characteristics (4 luminal-like, 3 triple-negative and 1 HER2-enriched). We first selected single-cells from tumor samples (n = 21.253) and subsequently CD3+ in silico sorted cells (n = 4.329). We performed a clustering analysis and identified 10 clusters among tumor CD3+ cells (Figure 3a). We then identified thresholds for CD3E, CD4 and CD8A expression by calculating 25% percentiles of the mRNA distribution, thus excluding cells of the lower percentile and obtaining 502 CD3E/CD4+ single cells and 1.194 CD3E/CD8A+ single cells (Figure 3b). Applying an unsupervised clustering algorithm to the previously identified CD4+ and CD8A+ subpopulations, we were able to identify 5 and 6 clusters, respectively (Figure 3c). We then analyzed, using the heatmap description, the genomic composition of each cluster for both CD4+ (Figure 4a) and CD8+ (Figure 4b) populations, and thus “labelled” each of them on the basis of the most differentially expressed genes (Figure 4a-b). We analyzed significantly differentially expressed genes, specifically in the CD8+ population, to identify master regulatory genes in each cluster (with particular attention to clusters 1 to 3, composed by cytotoxic and Trm cells), to be used to design an informative flow cytometry panel with the aim of confirming and deeply investigate the
information on TILs features discovered by single-cell RNA-seq data analysis at the protein level (Figure 5). Markers were chosen, based on known T cell biology, to describe differentiation, activation, proliferation, and exhaustion status of BC TILs (a list of markers used and their specificity is provided in Table 4).

4.1.2 Single-cell analysis of BC TILs

We applied our scRNAseq-guided 27-parameter FACS panel to tumor, normal breast tissue and peripheral blood collected from a cohort of 54 early BC patients surgically treated at our Institution. Of note, as patients’ samples were collected consecutively, the relative abundance of the different biological BC subtypes in our cohort of patients mirrors the well-known epidemiology, with 37 (69%) luminal-like HER2 negative, 10 (18%) luminal-like HER2-overexpressing, 2 (4%) hormone receptor negative HER2-overexpressing and 5 (9%) TNBCs.
Figure 3. In silico analysis of publicly available single-cell RNAseq data from 8 breast cancers. (a) Phenograph clustering of CD3+ cells in silico sorted from single-cell RNAseq data, 10 different clusters were identified. (b) Violin plots showing expression probability distributions of indicated genes among the 10 CD3+ cells clusters previously identified; thresholds for CD3E, CD4 and CD8 gene expression (CD3E>1.87; CD4>1.56; CD8>2.07), to exclude cells of the lower percentile, are indicated by the red bold line on the three plots. (c) Phenograph clustering for the previously identified CD4+ (left) and CD8A+ (right) population, showing 5 and 6 clusters, respectively.
Figure 4. Heatmaps for differentially expressed genes in the CD4+ (a) and CD8+ (b) populations. Each cluster was labelled on the basis on the top differentially expressed genes. Of note, among T cell clusters, also non-T cell, matrix markers expressing clusters were identified and subsequently excluded from the analysis.
Figure 5. Dot plot showing average gene expression levels (color code) and the percentage of cells within a cluster of interest expressing each marker (size of the dots), for the 3 clusters of interest among CD8+ cells.

Figure 6. tSNE analysis of concatenated CD4+ and CD8+ T cells (1,000 cells/sample) from peripheral blood, normal breast tissue and tumor samples from 54 early breast...
cancer patients (a and b, respectively, top rows), and color-coded tSNE map depicting clusters identified by Phenograph (bottom rows).

Detailed patients’ characteristics are summarized in Table 2. By investigating markers of memory differentiation including CCR7 and CD45RA, we were able to identify the relative abundance of different memory T cell subsets among CD4+ and CD8+ populations in the tumor compartment. Specifically, among CD8+ cells, naïve T cells (CCR7+CD45RA+) accounted for 4% of T cells, Tcm (CCR7+CD45RA-) accounted for 5% of T cells, Tem (CCR7-CD45RA-) were the most abundant population accounting for 72% of T cells, and Temra (CCR7-CD45RA+) accounted for 19% of T cells (Figure 7). Interestingly, this data are in line with Savas P. and colleagues observation on TILs composition in TNBCs. Then, visualizing the acquired cytometry data using t-distributed stochastic neighboring embedding (tSNE), we could demonstrate pronounced differences in the T cell immunophenotypes among the three different milieu, both for CD8+ and CD4+ populations (Figure 6, top rows). More specifically, using an unsupervised clustering algorithm (Phenograph), we were able to identify 10 different T cells clusters for CD8+ and 11 for CD4+ cells (Figure 6, bottom rows). Figure 8a-b shows heatmaps related to marker expression by CD4+ and CD8+ T cell clusters as well as the relative abundance of these clusters in tumor, normal breast tissue and peripheral blood samples. We found that naïve T cells, defined as CCR7+CD45RA+ were significantly more abundant in peripheral blood compared to tumor tissue and normal breast (Figure 8a). Moreover we observed, still prominent in peripheral blood, terminally differentiated effector T cells (Temra) CCR7-CD45RA+, characterized by a CD27-CD28-CX3CR1+ phenotype, as known from the literature. These
cells also co-expressed the cytotoxic molecule GZMB and the inhibitory receptor TIGIT. More abundantly in peripheral blood than in tissues, we could also observe a population of CD8+CD56+ T cells highly expressing the NK receptor CD161, a marker known to be associated with innate T-cell populations including mucosal-associated invariant T (MAIT) cell. CD161 can also be expressed by memory-like phenotype CD8+ cells. Conversely, clusters 2, 4 and 10, co-expressing CD69 and CD103 and thus identifying Trm cells, were found to be significantly more abundant in tumor and adjacent normal breast tissue compared to peripheral blood samples (Figure 6b, bottom row and Figure 8). These three populations also showed peculiar expression of different activation and exhaustion markers, whose relevance will be described and discussed in detail in the next paragraph. By further analyzing flow cytometry by standard manual gating, we could confirm the preferential presence of Trm in tumor and adjacent breast tissue compared to peripheral blood samples (Figure 9).

Moreover, we were interested in exploring if any of the T cells clusters identified by the heatmap could be more representative of one specific BC subtype or related to endocrine dependence. To do so, we analyzed the relative abundance of each T cells cluster among different biological subtypes (luminal-like, HER-2 enriched, TNBC), although we could not observe any significant difference (Figure 10a). Similar conclusions were reached when performing the same analysis by subdividing our cohort based on ER expression, suggesting that endocrine dependence does not shape the quality of the T cell immune infiltrate (Figure 10b). These observations are of paramount interest because we can speculate that any conclusion drawn on the phenotype and activation status of T cells from the
intratumoral immune infiltrate are shared across all different biological subtype, and specifically for the epidemiologically prominent luminal-like subtype.

Figure 7. Pie charts showing the relative abundance of different T cells differentiation statuses based on CCR7 and CD45RA manual gating on CD4+ (left) and CD8+ (right) cells in the tumor compartment. Terminally differentiated effector T cells (Temra) were defined as CCR7-CD45RA+, naïve T cells (T naïve) were defined as CCR7+CD45RA+, central memory T cells (Tcm) were defined as CCR7+CD45RA- and effector memory T cells (Tem) were defined as CCR7-CD45RA-.
Figure 8. Heatmaps showing the iMFI of specific markers in discrete Phenograph clusters identified in Figure 5, for the CD4+ (a) and CD8+ (b) populations. Phenograph clusters (rows) and markers (columns) are hierarchically metaclustered using Ward’s method to group subpopulations with similar immunophenotypes. The median frequency of each Phenograph cluster within peripheral blood, normal breast tissue and tumor (n=54, each milieu) samples are depicted by balloon plots. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 tumor versus blood and normal tissue samples; two-way ANOVA with Bonferroni post-hoc test.
Figure 9. Manual gating strategy, exemplified on one sample, for the identification of Trm in the three different compartments. As shown in the third vertical plot, CD103+CD69+ cells, gated on CD3+CD8+ population, are more abundant in normal breast tissue and tumor compared to peripheral blood and, specifically, the CD39+ tumor-reactive population, is more prominent in tumor compared to normal breast tissue.
4.2 BC Trm cells display a unique and complex phenotype

4.2.1 Phenotypical and functional differences among Trm cells

Data on single-cell profiling of tumor infiltrating T cells in TNBC showed that the CD8+CD103+ Trm signature was significantly associated with improved relapse-free and overall survival in 329 primary TNBCs after standard chemotherapy. Starting from this important observation, we aimed to better investigate features of CD8+CD103+ Trm cells, which is a highly prevalent population in the TME. Analyzing FACS data on our 54 patients’ cohort, we were able to identify CD8+CD69+CD103+ cells and distinguish a CD127+CD39$^{lo}$ and a CD127-CD39$^{hi}$ subpopulation; specifically, the CD127-CD39$^{hi}$ subpopulation was much more prevalent in tumor-infiltrating lymphocytes (gating strategy is shown in Figure 9). The CD127-CD39$^{hi}$ Trm subpopulation appeared the most interesting, as CD39, a marker for exhausted CD8+ T cells in patients with chronic viral infections, was recently found to identify tumor-reactive CD8 T cells. We were also able to better characterize this CD127-CD39$^{hi}$ Trm population and define specific phenotype features compared to the CD127+CD39$^{lo}$ counterpart. In this regard, we noticed increased expression of the cytotoxic molecules GZMB and GZMK in CD127- vs. CD127+ Trm cells (Figure 11). PD-1 was uniformly expressed and TIGIT was relatively low in both of these subsets, nevertheless we could detect small differences related to the expression of these markers (Figure 11). Conversely, the inhibitory receptor NKG2A, known to be expressed on
intra-tumoral CD8+CD103+ effector T cells and linked to progression in head-and-neck squamous cell carcinoma patients\(^97\), was more prominent in the CD127+ Trm subpopulation (Figure 11). The expression of inhibitory receptors among CD8+ TILs as well as Trm cells has been previously described in different tumor types, and may not reflect a dysfunctional cell population per se\(^98\)-\(^100\). We thus investigated the functional capacity of Trm cell subsets by stimulating bulk TILs with PMA/ionomycin in vitro, and by measuring the production of several effector molecules. To obviate the need of prior purification by FACS-sorting, we used HLA-DR, whose expression is not altered by the in vitro treatment (not shown), to distinguish subsets of CD127+ and CD127- Trm cells. In line with the enhanced expression of killer molecules ex vivo, we observed that HLA-DR+ (CD127-) Trm produced more CD107a and TNF (p<0.05) than HLA-DR- (CD127+) Trm after stimulation, while IFN-\(\gamma\) was equally produced (Figure 12).

Figure 11. Bar plots showing mean ± SEM summary of the frequency of different activation, co-inhibitory and co-stimulatory markers by CD127+ and CD127- Trm subpopulations from tumor samples (n=54), as defined by flow cytometry. ****, P < 0.0001; paired Wilcoxon t test.
Figure 12. (a) Flow cytometry histograms showing the production of CD107a, TNF and IFN-γ by BC tumor-infiltrating CD8+ Trm subsets in response to PMA/ionomycin stimulation in vitro. Numbers in the histograms indicate the percentage of positive cells identified by the gates. (b) Bar plots showing mean ± SEM summary of the frequency of marker expression from (a) (n=8; performed in three independent experiments).

4.2.2 Molecular specificities among Trm subpopulations

We next isolated CD127+CD39lo and CD127-CD39hi CD8+ Trm cells subsets by fluorescence-activated cell sorting (FACS; the sorting gating strategy is depicted in Figure 13) and performed bulk RNA-seq to further identify molecular features associated with these subsets. When taking into account only protein coding genes, we found that the two Trm subsets were relatively different at the transcriptomic level, and identified 183 differentially expressed genes, 117 of which were upregulated while 66 were downregulated in CD127+CD39lo compared to CD127-CD39hi Trm cells (q <0.05; Figure 14a). The former preferentially expressed genes related to early memory differentiation such as IL7R (encoding CD127), as expected from the sorting strategy, CCR7 and RUNX2, as well as the additional immune related genes STAT4, KLRD1 (encoding CD94, a molecular adapter of NKG2A, in turn preferentially expressed by CD127+...
Trm cells at the protein level), RORA and DUSP2. Instead, the latter preferentially expressed MYB, regulating survival programs of memory T cells and promoting their anti-tumor response\(^{101}\), CDH1, linked to T cell tissue residency\(^{102}\) and SOX4 and IGFR1, both involved in T cell maturation\(^{103,104}\). Gene set enrichment analysis (GSEA) revealed that CD127+CD39\(^{lo}\) Trm cells were characterized by gene signatures related to T cell exhaustion, oxidative phosphorylation, fatty acids metabolism and p53 mediated hypoxia, which are known to be linked to defective anti-tumor reactivity of T cells\(^{105-107}\) (Figure 14b), whereas the CD127-CD39\(^{hi}\) Trm cells were enriched in signatures related to stemness and sodium and potassium channel activity\(^{108-110}\) (Figure 14b). We next employed these specific gene signatures to investigate the prognostic impact of CD127+CD39\(^{lo}\) and CD127-CD39\(^{hi}\) subsets of Trm cells on BC prognosis, as defined in the METABRIC consortium data set\(^{90}\). We found that an increased relative abundance of CD127-CD39\(^{hi}\), but not of CD127+CD39\(^{lo}\) Trm cells significantly correlated with better OS (p=0.0029; Figure 15a). Similar data were obtained when taking into account only BC-specific mortality (p<0.0001; Figure 15b). Of note, the composition of the METABRIC data set mirrors the well known epidemiology, with 72% of patients presenting with HR positive BC, 21% of patients overexpressing HER2 and 12% of patients harboring TNBC\(^{90}\).

Figure 13. Flow cytometric gating strategy for the identification of CD127+CD39\(^{lo}\) and CD127-CD39\(^{hi}\) within CD69+CD103+CD8+ Trm cells. Numbers in the dot plots indicate the percentage of cells identified by the gates.
Figure 14. (a) Heatmap of differentially expressed genes (DEGs; FDR<0.05) between FACS-sorted CD127-CD39hi versus CD127+CD39lo CD8+ Trm cells subsets, as obtained by RNA-seq. Selected DEGs are indicated. (b) Bar plot of manually curated signatures that differed significantly (adjusted p-value <0.05) as obtained by gene set enrichment analysis (GSEA) of RNA-seq data in (a).

Figure 15. (a) Kaplan-Meier overall survival (OS) curves in the METABRIC consortium data set (n = 1,894 patients). The median z-score value was used to classify tumor samples into Low and High expression groups. P values were calculated by applying the
Log-rank (Mantel-Cox) test. Dotted lines indicate the time at which 50% of the cohort was still free of the event. (b) Kaplan-Meier BC-specific survival curves in the METABRIC consortium data set (n = 1,894). Groups were defined and data were displayed as in (a).

4.3 Different Trm subpopulations interact differently with BC prognosis

4.3.1 Tumor-reactive Trm correlate with activated Tregs

In order to better investigate possible functional relationships of the different Trm subset, we explored the correlation between these Trm populations and CD4+ Tregs. Firstly, we computed a correlation matrix for CD8+ and CD4+ T cell clusters previously identified by Phenograph of flow cytometric data (Figure 16). Interestingly, different Trm clusters were found to correlate with CD4+ Tregs in an opposite fashion, i.e., CD127-CD39\textsuperscript{hi} Trm showing a significant direct correlation with CD4+ Tregs, while CD127+CD39\textsuperscript{lo/-} Trm showing a significant inverse correlation (Figure 16). We have recently shown that human tumors, including NSCLC, melanoma and HCC, are infiltrated by at least two subsets of intra-tumoral Tregs, where CCR8\textsuperscript{lo}ICOS\textsuperscript{lo}IRF4- Tregs are relatively quiescent and CCR8\textsuperscript{hi}ICOS\textsuperscript{hi}IRF4+ Tregs are highly activated and retain superior suppressive potential \footnote{111}. Importantly, only the abundance of CCR8\textsuperscript{hi}ICOS\textsuperscript{hi}, but not of CCR8\textsuperscript{lo}ICOS\textsuperscript{lo}Tregs correlated with worse prognosis \footnote{111}. To investigate whether the same hierarchical organization of Tregs occurred also in BC tumors, CD25+CD127-CD4+ Tregs were isolated by manual gating of flow cytometry data from normal breast tissue the blood samples, and further re-clustered by Phenograph (Figure 17a). This analysis informed on the differential abundance of 5 clusters: CD45RA+CCR7+CD28CD27+ naïve-like (C2) and CD45RA-
CCR7<sup>int</sup>CD28+ early memory-like (C5) Tregs were more abundant in the blood, whereas two subsets of CD45RA-CCR7-CD28+CD27+ Tregs, C1 and C4, were enriched in tumors. Of these, C1 showed preferential overexpression of HLA-DR, OX40, PD1 and NKG2A compared to C4 (Figure 17b). A fifth subset of CD45RA+CCR7- Tregs (C3) with unclear identity was unchanged between tissues. Additional immunophenotyping by flow cytometry revealed co-expression of CCR8, ICOS and IRF4 by HLA-DR+ Tregs (Figure 18), thereby confirming the identification of a highly immunosuppressive Treg phenotype that is shared also in BC<sup>111</sup>.

Figure 16. Pearson correlation between frequencies of CD4+ (T4) and CD8+ (T8) PhenoGraph clusters in tumor samples from 49 BC patients. Non-significant correlations (p-value > 0.05) are left blank.
Figure 17. (a) UMAP analysis of concatenated CD4+ Tregs (100 cells/sample) from tumor (n = 53), normal breast tissue (n = 44) and peripheral blood (n = 53) samples from early BC patients. (b) Heatmaps of the relative expression, depicted as integrated MFI (iMFI: MFI x % antigen expression) of markers (columns) in discrete CD4+ Tregs Phenograph clusters (rows). Data are further metaclustered to group subpopulations with similar immunophenotypes. The median frequency of each PhenoGraph cluster in the different compartments is depicted by using Balloon plots. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 tumor vs. blood or vs. normal tissue samples; two-way ANOVA with Bonferroni post-hoc test.

Figure 18. Representative flow cytometric analysis of IRF4, CD39 and HLA-DR expression by subsets of ICOS<sup>hi</sup>CCR8<sup>hi</sup> and CCR8<sup>lo</sup>ICOS<sup>lo</sup>CD4+ Treg cells (initially defined as CD127<sup>-</sup>CD25+). Tconv: conventional, non-Treg CD4+ T cells.

4.3.2 Abundance of CCR8<sup>hi</sup> ICOS<sup>hi</sup> IRF4<sup>+</sup> effector Tregs hinders the prognostic benefit of Trm cells

We next tested whether the abundance of the different Trm and Treg subpopulations could influence BC prognosis. To this aim, we further stratified BC patients from the METABRIC data set based on...
combinations of enrichment of $\text{CD127}^{-}\text{CD39}^{\text{hi}} \text{Trm}$ and $\text{CCR8}^{\text{hi}}\text{ICOS}^{\text{hi}}$ (IRF4+) effector Treg gene signature, the latter previously identified in NSCLC \(^{111}\), and investigated OS in these subgroups (Figure 19a). In this context, the presence of $\text{CD127}^{-}\text{CD39}^{\text{hi}} \text{Trm}$ cells correlated with improved OS especially when $\text{CCR8}^{\text{hi}}\text{ICOS}^{\text{hi}}$ effector Tregs were less abundant (Figure 19a). Among all groups, BC patients showing low levels of $\text{CD127}^{-}\text{CD39}^{\text{hi}} \text{Trm}$ cells and high levels of $\text{CCR8}^{\text{hi}}\text{ICOS}^{\text{hi}}$ effector Tregs had the worst OS. Similar results were obtained when taking into account only luminal-like BCs (Figure 19b), or when analyzing OS data and BC-specific survival (Figure 20). These data confirm the paramount role of immune microenvironment, and specifically of CD39+ tumor-reactive T cells, also in endocrine-dependent, luminal-like BCs.

Figure 19. (a and b) Kaplan-Meier overall survival (OS) curves in the METABRIC consortium data set in the global population (a; n = 1,894) or in luminal-like BCs (b; n = 1,436) according to high or low enrichment of $\text{CD127}^{-}\text{CD39}^{\text{hi}} \text{Trm}$ or $\text{ICOS}^{\text{hi}}\text{CCR8}^{\text{hi}}$ (labelled as $\text{ICOS}^{\text{hi}}$) gene signatures. The median z-score value was used to classify tumor samples into Low and High expression groups. P values were calculated applying the Log-rank (Mantel-Cox) test.
Figure 20. Kaplan-Meier BC-specific survival curves in the METABRIC consortium data set (n = 1,894). Groups were defined and data were displayed as in Figure 19.

4.4 Most tumor infiltrating T lymphocytes clusters can be reiterated in tumor draining lymph nodes

4.4.1.1 Both healthy and metastatic tumor draining lymph nodes present with tissue specific, but also tumor-related T cell clusters

We analyzed the T cell composition of both healthy, non-metastatic (tumor-free) and metastatic tumor draining lymph nodes, collected at the time of primary surgery, for 6 and 11 early BC patients, respectively. Following the same analytical procedures used for normal breast tissue and tumor samples, flow cytometry analysis of the T cell immune infiltrate from lymph nodes identified 8 and 15 clusters for CD4+ and CD8+ cells, respectively (Figure 21). Clinical-pathologic characteristics of patients used included in this analysis are listed in Table 3. The heatmaps showing phenotypical characteristics of different CD4+ and CD8+ cells clusters in both non-metastatic and metastatic lymph nodes are depicted in Figure 22. This analysis revealed no major differences in the T cell composition of non-metastatic and metastatic lymph nodes. As expected from this tissue site, CD4+ and CD8+ naïve T cells were relatively abundant, while memory T cells were less represented (Figure 22). Among CD4+ cells,
Tregs tended to be more represented in metastatic compared to non-metastatic lymph nodes, although this difference was not statistically significant.

Figure 21. tSNE analysis of concatenated CD4+ (a) and CD8+ (b) T cells (5,000 cells/sample) from non-metastatic and metastatic lymph nodes samples from 8 and 11 early breast cancer patients, respectively (a and b, top rows), and color-coded tSNE map depicting clusters identified by Phenograph (bottom rows).
Figure 22. Heatmaps showing the iMFI of specific markers in discrete Phenograph clusters identified in Figure 16, for the CD4+ (a) and CD8+ (b) populations. Phenograph clusters (rows) and markers (columns) are hierarchically metaclustered using Ward’s method to group subpopulations with similar immunophenotypes. The median frequency of each Phenograph cluster within metastatic and non-metastatic lymph nodes (n = 11 and 6, respectively) samples are depicted by balloon plots. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 tumor versus blood and normal tissue samples; two-way ANOVA with Bonferroni post-hoc test.

4.4.2 CD8+ Trm cells are present in both healthy and metastatic lymph nodes

We next focused on Trm cell subsets in lymph nodes, given their elevated presence in tumor tissues compared to the peripheral blood, and their prognostic relevance, as previously shown. We aimed to determine whether subsets of Trms could be detected in lymph nodes and differences in relative abundance could be observed between non-metastatic and metastatic samples. Interestingly, we could observe Trm cells, exhibiting the CD69+CD103+ phenotype similar to that observed in
CD8+ TILs, in both lymph node samples, thereby recapitulating in tumor-draining lymph nodes the same phenotypical differences among CD69+CD103+ Trm cells that we observed in CD8+ TILs. Indeed, we could recognize the presence of a cluster of CD127-CD39hi Trm cells, distinct from a CD127+CD39lo/- cluster (Figure 23a). CD127-CD39hi CD8+ Trm cells were found preferentially enriched in metastatic tumor draining compared to tumor-free lymph nodes (Figure 23b), thus corroborating the evidence that CD39 identifies tumor-reactive CD8+ Trm cells also in BC.

Figure 23. (a) Representative flow cytometric analysis showing the frequency of CD127-CD39hi among CD69+CD103+ Trm from metastatic and non-metastatic lymph node samples. Numbers in the dot plots indicate the percentage of cells identified by the gates. (b) Mean ± SEM summary of the frequency of CD127-CD39hi Trm among total CD8+ CD69+CD103+ Trm cells in metastatic (n = 11) and non-metastatic (n = 6) lymph nodes (LN). *, P < 0.05; unpaired Wilcoxon t test. Data were obtained in one experiment.

5 DISCUSSION

In this study, we dissected the BC T cell immune milieu by single-cell technologies and focused on CD8+ TILs to gain deep insight on tumor-
reactive, polyfunctional Trm subpopulations, their relationship with the immune-suppressive microenvironment and their capability of predicting and refining BC prognosis. By analyzing numerous primary tumors, adjacent normal breast tissues and peripheral blood, we could distinguish different CD4+ and CD8+ T cell subsets, whose abundance in the TME is associated with better control of disease progression. This is specifically defined by the combination of subsets of CD127-CD39hiCD8+ Trm cells and CCR8hiICOShiIRF4+ effector Tregs that are preferentially abundant in the tumor compared to the normal breast tissue and the peripheral blood from the same patient. Moreover, the previously identified Trm subpopulations were also found in tumor-draining lymph nodes, and specifically, CD127-CD39hi CD8+ Trm cells were found preferentially enriched in metastatic tumor draining compared to tumor-free lymph nodes. In the absence of additional data, it is at present difficult to infer a tumor-to-lymph node recirculation of CD69+ cells subsets, which are known to behave as tissue-resident, non-circulating cells, with only 1-20% of CD69+ circulating memory T cells found in peripheral blood \(^{39}\). Nevertheless, it is interesting to note that, in presence of tumor cells, memory T cells can exhibit an active and tumor-reactive CD127-HLA-DR+CD39hi phenotype in both breast stromal tissue and draining lymph nodes. Of note, these patterns were shared among luminal A, luminal B and TNBC subtypes, pointing at the relevance of the immune infiltrate also in luminal-like BCs for which immunotherapeutic strategies have been poorly investigated to date. Given the high prevalence (70-80%) of luminal-like subtypes among all surgically treated BCs every year, and their indolent nature, with possible recurrences over 20 years after surgery
our data are of broad applicability and prompt to investigate immunotherapeutic strategies in these tumors further.

Our results uncovered heterogeneity within the intratumoral CD8+ Trm compartment, distinguishing subsets of CD127+ and CD127- cells. The expression of CD39, recently proposed to identify those T cells specific for tumor-derived epitopes among TILs, was mostly confined to CD127- Trm cells. These also expressed significantly higher levels of activation and cytotoxic molecules and produced more effector cytokines compared to CD127+CD39lo Trm cells. Accordingly, the CD127-CD39hi Trm cells were associated with improved OS and BC-specific survival in the METABRIC cohort, thereby corroborating the idea that these cells maintain some degree of functionality in BC despite high levels of PD-1 expression. Our results thus extend a previous study in BC patients on the prognostic role of CD8+CD103+ Trm cells as a whole and pinpoint those T cells that are preferentially associated with delayed tumor growth with enhanced precision, thereby suggesting that specifically targeting CD127-CD39hi Trm cells with immunotherapeutic strategies may be beneficial for BC control. Notably, this association was less relevant when the BC TME was infiltrated by a highly suppressive subset of CCR8hiICOShiIRF4+ Tregs, recently shown by our group to orchestrate inhibition of anti-tumor immunity in several human cancers. The importance of this association is manifold: first, measurement of multiple subpopulations of tumor-infiltrating immune cells along with their molecular status is critical to identify patients who are characterized by delayed disease progression; second, strategies aiming at promoting effector functions of TILs in luminal-like BCs, for instance checkpoint blockade as it
is currently tested in clinical trials, may be hampered in a subset of patients because of the excessive infiltration of highly immunosuppressive Tregs. It is thus tempting to speculate that these individuals may require combinatorial strategies that also target Tregs for successful tumor regression. The recent observation that CDK4/6 inhibitors, now widely employed to treat luminal-like BCs, not only restrain cancer cell proliferation but also favor anti-tumor immunity by inhibiting CD4+ Treg proliferation in preclinical models, may be important in this regard. Clinical trials are currently ongoing to test the efficacy of checkpoint blockade in association with these agents.

In summary, our high-dimensional single-cell measurements defined a signature of T cell subsets involving CD127-CD39hi Trm and CCR8hiICOShiIRF4+ effector Tregs, with high and low abundance, respectively, that is associated with good prognosis in the long term. These data urge to integrate multiparameter measurements of patients’ specimens with a defined set of markers to be translated in clinical practice to predict disease progression and/or response to treatment in combination therapies.

6  LIST OF ABBREVIATIONS

ACT: adoptive cell therapy
APC: antigen-presenting cell
BC: breast cancer
DCs: dendritic cells
DEGs: differentially expressed genes
DFS: disease-free survival
ER: estrogen receptor
FACS: fluorescence activated cell sorting
FISH: fluorescence in-situ hybridization
GSEA: gene set enrichment analysis
HR: hormone receptor
IHC: immunohistochemistry
iMFI: integrated mean fluorescence intensity
LPBC: lymphocyte predominant breast cancer
M2: type 2-polarized macrophages
mAb: monoclonal antibody
MDSC: myeloid-derived suppressor cell
MFI: mean fluorescence intensity
NK: natural killer
OS: overall survival
PBMC: peripheral blood mononuclear cells
pCR: pathological complete response
PFS: progression-free survival
PgR: progesterone receptor
RFS: recurrence-free survival
TAM: tumor-associated macrophage
Tcm: central memory T cells
Teff: effector T cells
Tem: effector memory T cells
Temra: effector memory T cells re-expresses CD45RA
TILs: tumor infiltrating lymphocytes
TLS: tertiary lymphoid structures
TME: tumor microenvironment
Tmem: memory T cells
TNBC: triple negative breast cancer
Tregs: regulatory T cells
Trm: tissue resident memory
Trm: tissue resident memory cells
WES: whole exome sequencing
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