THE TUMOR MICROENVIRONMENT: RECENT ADVANCES AND NOVEL THERAPEUTIC APPROACHES

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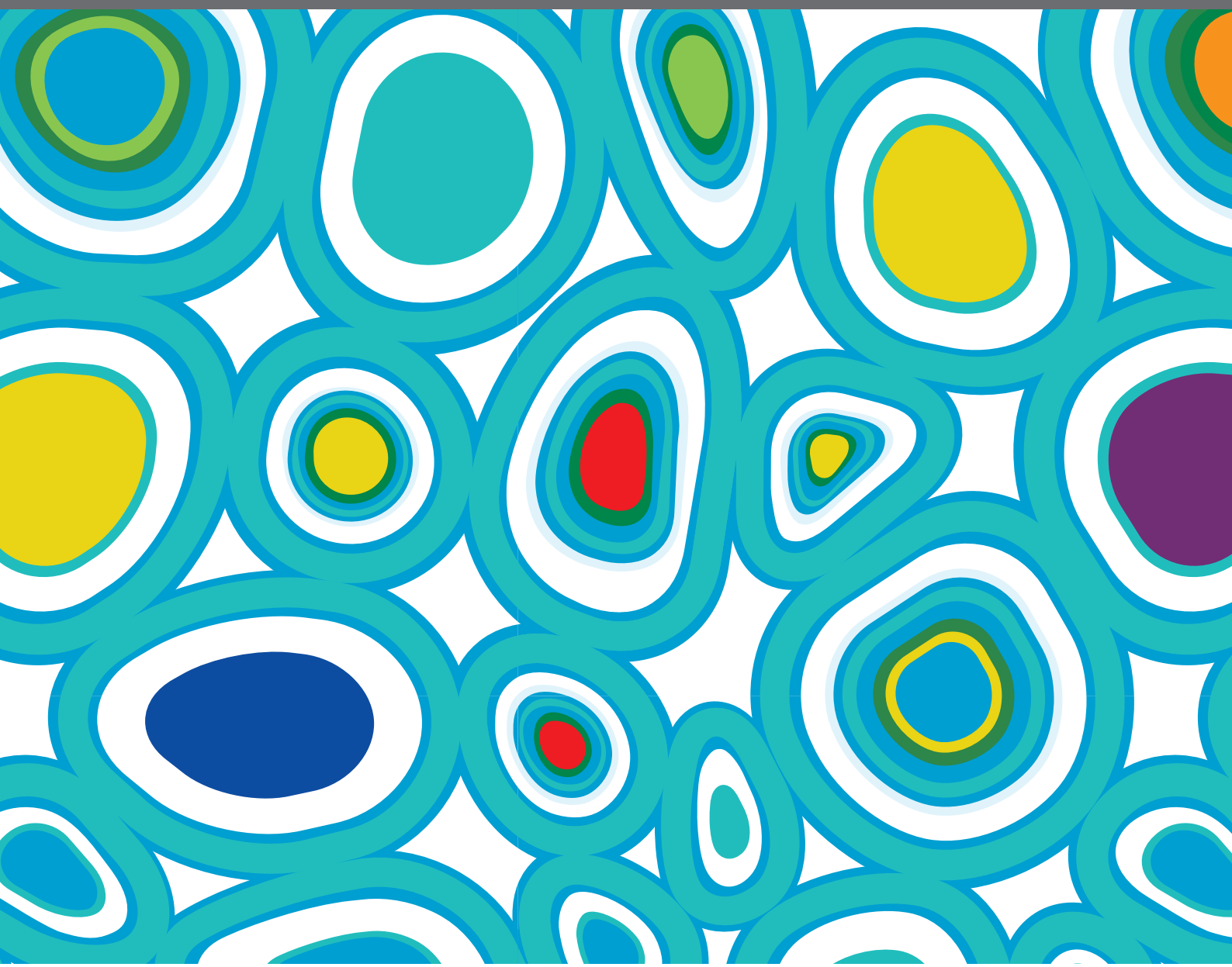

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THE TUMOR MICROENVIRONMENT: RECENT ADVANCES AND NOVEL THERAPEUTIC APPROACHES

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EDITORIAL
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Editorial: The Tumor Microenvironment: Recent Advances and Novel Therapeutic Approaches

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Editorial on the Research Topic

The Tumor Microenvironment: Recent Advances and Novel Therapeutic Approaches

Tumor cells establish a complex ecosystem called the tumor microenvironment (TME) which consists of stromal cells, immune cells, extracellular matrix (ECM) macromolecules and enzymes (**Figure 1**). It is now well-established that TME of solid tumors plays a fundamental role in tumor progression and metastasis, determining the disease outcome. In an age of molecularly targeted therapeutics and immune check point inhibitors (ICI), the role of TME in therapeutic resistance has become a major research focus. Although ICIs exhibited remarkable long-lasting responses in hard to treat malignancies, such as non-small cell lung cancer, renal cell carcinoma and melanoma (Nixon et al., 2018), these inhibitors alone or in combination with chemotherapy have shown very little promise in other solid tumors, such as breast, prostate and brain (Chai et al., 2019). Clinical studies revealed that tumor-infiltrating lymphocytes (TILs) are associated with the earlier stages of tumor progression and good prognosis in triple-negative breast cancer (Adams et al., 2014). However, the tumor-infiltrating myeloid precursors (tumor associated macrophages-TAMs, myeloid derived suppressor cells-MDSCs, regulatory dendritic cells-DCs and neutrophils) and cancer associated fibroblasts (CAFs) establish an immunosuppressive TME which is associated with advanced tumor stage and therapeutic resistance (Munn and Bronte, 2016). In addition, tumors establish their own vasculature via secreting angiogenic factors which in turn chemoattracts the endothelial cells within the tumor microenvironment. Crosstalk between the endothelial and tumor cells has been shown to play a major role in tumor progression and metastasis as well as resistance to a wide range of therapeutics including ICIs. Although pericytes were originally identified to surround and physically support new blood vessels, their critical importance in the premetastatic niche has recently been recognized. Recent data suggested that pericytes regulate immune responses by secreting adhesion molecules and a wide range of chemokines (Paiva et al., 2018). While angiogenesis inhibitors (AI) have been approved for the treatment of some solid tumors, currently there are several early clinical trials testing the efficacy of AIs in combination with ICIs (Ciciola et al., 2020). Furthermore, tumor-infiltrated myeloid precursors and fibroblasts regulate tumor cell plasticity and stemness during the metastatic cascade (Ouzounova et al., 2017). Malignant cells induce a dynamic stromal reaction, which both resists and promotes tumor progression. Additionally, ECM secreted by stromal cells play a crucial role in tumor progression and metastasis as well as therapeutic resistance.

The following articles provide a comprehensive review of the current understanding of TME as illustrated in **Figure 1**. Ireland and Mielgo reviewed the literature on the roles of macrophages and fibroblasts. The authors first examined the origin of these cells and their physiological functions

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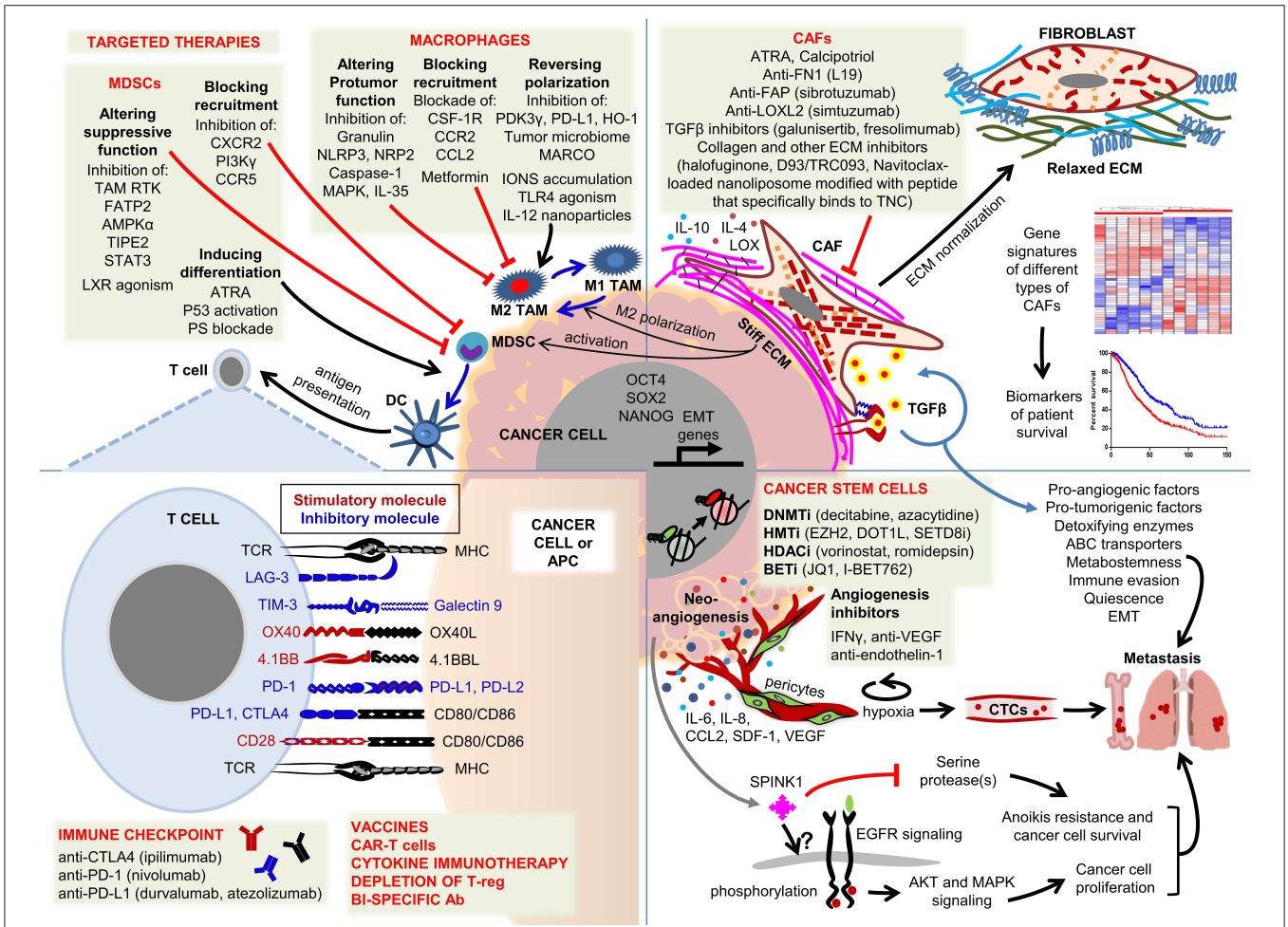

FIGURE 1 | Overview of the components of the tumor microenvironment and current strategies to target these cells. Malignant cells establish early cross-communications with a wide range of host cells forming a favorable microenvironment consisting of fibroblasts, immune cells, ECM, endothelial cells and pericytes. While infiltration of T lymphocytes is a good prognostic factor, the majority of invasive/metastatic carcinomas establish immunosuppressive and tumor-promoting TMEs. The complex interaction between the tumor cells and the other components within the TME is illustrated and the following abbreviations are used. ABC transporter, ATP binding cassette transporter; Ac, acetylation; APC, antigen presenting cell; ATRA, all trans retinoic acid; BETi, bromodomain inhibitors; CAF, cancer-associated fibroblast; CAR-T-cell, chimeric antigen receptor T cell; CTC, circulating tumor cell; DC, dendritic cell; DNMTi, DNA methyltransferase inhibitors; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; HDACi, histone deacetylase inhibitors; HMTi, histone methyltransferase inhibitors; MDSC, myeloid-derived suppressor cell; Me, methylation; MHC, major histocompatibility complex; TAM, tumor associated macrophage; TCR, T-cell antigen receptor; Treg, regulatory T cell.

and discussed how they are activated in the context of malignancies. Unlike their quiescent counterparts, CAFs are activated in response to chronic tissue damage which is also called "wounds that do not heal," leading to crosstalk between tumor cells and CAFs via a wide range of cytokines. Among these cytokines, TGFβ and IL6 play a major role in therapeutic resistance by driving an epithelial mesenchymal transition (EMT) in tumor cells. Tissue resident or bone marrow (BM) derived macrophages are recruited to, and activated at, the tumor site by tumor-derived factors. Upon failing to eradicate tumors, M1-like macrophages are polarized to M2 TAMs by tumor-secreted immunosuppressive cytokines, such as TGFβ, IL4 and IL10. The authors also discuss current efforts to target CAFs and M2 TAMs with therapeutic approaches ranging from blocking the recruitment of macrophages to the tumor site to repolarizing them into M1-like macrophages and reprogramming CAFs. Ongoing phase I/II clinical trials include the blockade of CSF1, CSF1R, CCL2, and CD40 in M2 TAMs and Smo and IGF in CAFs. Similarly, Liu et al. discussed how fibroblasts build the tumor microenvironment by secreting ECM, associated enzymes and other factors. The authors reviewed the recent literature on the critical importance of CAF-ECM and CAF-tumor cell interactions in tumor cell migration and invasion. LOX-induced ECM crosslinking has emerged as a viable molecular target and several LOX inhibitors are in preclinical development. Furthermore, Nallanthighal et al. provided a comprehensive review on the mechanistic properties of ECM that regulate the phenotype of cancer stem cells (CSC). Tumor ECM is stiffer due to overexpression of a wide range of macromolecules and ECM-modifying enzymes, which causes

transmission of the signal to CSCs via focal adhesion kinase (FAK) and its downstream effectors driving the transcription of stemness genes, such as OCT4, SOX2, and NANOG. CSCs also evade immune recognition by ECM mediated activation of the PI3K/Akt pathway.

In contrast to solid tumors, Haro and Orsulic reported a favorable survival outcome that is associated with CAFs in B-cell lymphoma patients. Potential mechanisms proposed are the entrapment of malignant B cells by CAFs and associated ECM in lymph nodes and induction of apoptotic cell death by CAF-derived TGFβ.

In Turdo et al., the authors discussed potential strategies to target CSCs by blocking the stem cell specific pathways such as Notch, Wnt, and Hedgehog or TME-produced cytokines that regulate the stemness of malignant cells. Efforts to sensitize CSCs to ICIs by either CSC-primed dendritic cells or CAR T cell therapy with CSC-specific antigens were also discussed.

One potential target in TME are the immune cells of myeloid origin. Chaib et al. discussed the types of myeloid cells, their significance in tumor progression and recent strategies to target this cell population. Due to their suppressive activity, MDSCs have been implicated in the development of therapeutic resistance against ICIs. The authors discussed a comprehensive list of myeloid cell targets, such as PI3Kγ, NF-κB, CSF, CCL2, TLRs and histone deacetylases in pre-clinical and early clinical trials. A comprehensive review by Canning et al. examined the immune landscape of head and neck squamous cell carcinomas (HNSCC) according to human papilloma virus (HPV) status. While HPV(+) HNSCCs were infiltrated with lymphocytes that correlated with better clinical outcome, HPV(−) counterparts were infiltrated with highly immunosuppressive immune cells that correlated with poor outcome. In early clinical trials with ICIs, HPV(+) patients experienced better clinical outcomes compared to HPV(−) patients despite their high tumor mutation burden. These findings concur with the immunosuppressive role of TME in therapy response in solid tumors.

Finally, Mehner and Radisky reviewed the dual role of serine protease inhibitor Kazal type 1 (SPINK1) in the tumor and the TME. Aberrantly overexpressed SPINK1 in a wide range of solid tumors and the stroma renders tumor cells resistant to apoptotic cell death and increases cell proliferation, while enhanced expression in the tumor stroma contributes to therapeutic resistance.

In summary, although TME is viewed as a promising therapeutic target, we still face significant challenges in developing adequate tools to effectively target these cells. Current variable successes in targeting tumor stroma highlight the need to better understand the molecular characteristics of stromal cells to develop more precise and less toxic targeted therapies.

AUTHOR CONTRIBUTIONS

HK wrote the manuscript. SO edited the manuscript and drew the figure. All authors contributed to the article and approved the submitted version.

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HYPOTHESIS AND THEORY
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A Paradoxical Correlation of Cancer-Associated Fibroblasts With Survival Outcomes in B-Cell Lymphomas and Carcinomas

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The tumor microenvironment is increasingly recognized as an active participant in tumor progression. A recent pan-cancer genomic profile analysis has revealed that gene signatures representing components of the tumor microenvironment are robust predictors of survival. A stromal gene signature representing fibroblasts and extracellular matrix components has been associated with good survival in diffuse large B-cell lymphoma (DLBCL). Paradoxically, a closely related gene signature has been shown to correlate with poor survival in carcinomas, including breast, ovarian, pancreatic, and colorectal cancer. To date, there has been no explanation for this paradoxical inverse correlation with survival outcomes in DLBCL and carcinomas. Using public gene data sets, we confirm that the DLBCL stromal gene signature is associated with good survival in DLBCL and several other B-cell lymphomas while it is associated with poor survival in ovarian cancer and several other solid tumors. We show that the DLBCL stromal gene signature is enriched in lymphoid fibroblasts in normal lymph nodes and in cancer-associated fibroblasts (CAFs) in ovarian cancer. Based on these findings, we propose several possible mechanisms by which CAFs may contribute to opposite survival outcomes in B-cell lymphomas and carcinomas.

Keywords: B cells, B-cell lymphoma, CAFs, cancer-associated fibroblasts, DLBCL, gene signature, ovarian cancer, tumor microenvironment

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INTRODUCTION

During the past decade, gene expression profile analyses of frozen tumor pieces have been widely used to quantify various biological characteristics of malignant tumor cells and the microenvironment in which they reside. Individual biological characteristics and dominant molecular pathways in tumors are frequently associated with expression of a defined set of genes,

Abbreviations: CAF, cancer-associated fibroblast; CD, cluster of differentiation; CXCL, C-X-C motif chemokine ligand; DC, dendritic cells; DLBCL, diffuse large B-cell lymphoma; ECM, extracellular matrix; FDC, follicular dendritic cells; FRC, fibroblastic reticular cells; GC, germinal center; Ig, immunoglobulin; ImmGen, immunological genome project; IPA. ingenuity pathway analysis; MRC, marginal reticular cells; NK, natural killer; PDGFRα, platelet-derived growth factor receptor α; PDPN, podoplanin; PRECOG, PREdiction of clinical outcomes from genomic profiles; TCGA, the Cancer genome atlas project; TGFβ, transforming growth factor β; TIL, tumor infiltrating lymphocyte; TLS, tertiary lymphoid structure.

known as a gene expression signature. Since phenotypic features represented by gene expression signatures are sometimes associated with clinical features, such as the length of survival of cancer patients or their response to therapy, gene expression signatures can be used as quantitative predictors of clinical outcomes. A recent pan-cancer PREdiction of Clinical Outcomes from Genomic Profiles (PRECOG) analysis revealed that genes in the tumor microenvironment are better predictors of survival than genes expressed in malignant tumor cells (Gentles et al., 2015). The two most prominent components in the microenvironment of solid tumors are fibroblasts and immune cells (Aran et al., 2017). Generally, in carcinomas, genes expressed in fibroblasts are associated with poor survival while genes expressed in immune cells, particularly leukocytes, are associated with good survival (Gentles et al., 2015). Tumor infiltrating lymphocytes (TILs) and tertiary lymphoid structures (TLS) are generally associated with improved clinical outcomes as evidenced by the improved overall survival and disease-free survival in various types of tumors (Fridman et al., 2012; Dieu-Nosjean et al., 2014; Barnes and Amir, 2017). However, depending on the type of tumor, tumor stage, and location of TILs within the tumor (tumor bed, invasive margin and stroma), different types of TILs have been associated with both positive and negative prognosis. For example, cytotoxic CD8+ T cells, memory T cells, and CD4+ T helper cells are generally associated with a better prognosis, whereas T regulatory cells, tumor associated macrophages, and myeloid-derived suppressor cells are associated with poor prognosis and can promote tumor progression (Fridman et al., 2012; Kitamura et al., 2015; Barnes and Amir, 2017). Furthermore, fibroblasts in the tumor microenvironment are phenotypically heterogeneous and may exhibit both a pro- and anti-tumorigenic phenotype (Augsten, 2014). Thus, the tumor microenvironment is a complex network of interaction between tumor cells and components of the stroma, including the extracellular matrix (ECM), and it is currently unclear which factors in the tumor microenvironment control the quantity and distribution of different immune cell subtypes. Specifically, it is unknown if fibroblasts and immune cells affect prognosis independently or through an interdependent interaction.

The functional interaction between fibroblasts and immune cells has been most thoroughly studied in normal lymph nodes and the spleen, where specialized fibroblasts produce ECM to form a network that allows for lymphocyte movement along the matrix in response to chemokine signaling. The presence of lymphoid fibroblasts is necessary for functional attraction, retention, compartmentalization, and survival of immune cells (Koning and Mebius, 2012). Lymphoid fibroblasts are crucial for lymphocyte homeostasis as well as controlling and expanding the lymphocyte pool (Mueller and Germain, 2009). Lymphoid fibroblasts are also key players in mediating functional immune cell interactions in the lymph nodes through direct contact or via secreted molecules (Chang and Turley, 2015). Follicular dendritic cells (FDC) attract B cells to the germinal center (GC) by secreting C-X-C motif chemokine ligand 13 (CXCL13), while marginal reticular cells (MRC) use a network of follicular conduits to deliver antigens to cognate B cells (Chang and Turley, 2015). By secreting C-C motif chemokine ligands 19 and 21 (CCL19 and CCL21), fibroblastic reticular cells (FRC) recruit mature dendritic cells (DC) and naïve B and T cells to promote cell-cell interactions within the T cell zone (Mueller and Germain, 2009; Brown and Turley, 2015; Fletcher et al., 2015). Recent studies have shown that FRC are important for B-cell homeostasis (Cremasco et al., 2014). This function has been previously ascribed to FDC, however, cell-specific depletion experiments demonstrated that only FRC are crucial for B-cell survival. The mechanism by which FRC support B-cell survival is not entirely clear, but it is thought to involve crosstalk with B cells to control the boundaries of primary B-cell follicles (Cyster, 2010; Mionnet et al., 2013; Cremasco et al., 2014).

Similar to lymphoid fibroblasts in normal lymph nodes, cancer-associated fibroblasts (CAFs) are stromal cells that produce ECM, provide scaffolding, and exert regulatory functions through growth factors, cytokines, and chemokines that can promote tumor growth, angiogenesis, invasion, and metastasis (Kalluri and Zeisberg, 2006; Levental et al., 2009; Lu et al., 2012; Spano and Zollo, 2012; Harper and Sainson, 2014). Recent studies provide evidence that CAFs can also directly or indirectly contribute to immune cell fate and survival (Harper and Sainson, 2014; Costa et al., 2018; Mariathasan et al., 2018; Tauriello et al., 2018). It has recently been shown that a gene signature representing activated CAFs is present in most epithelial tumors (Jia et al., 2016) despite the diversity of resident fibroblasts in different organs and the presence of multiple fibroblast populations within a single tumor type (Costa et al., 2018). Activated CAFs in breast cancer, and possibly in other carcinomas, are associated with immunosuppressive populations of T lymphocytes (Costa et al., 2018). It is unclear if activated CAFs in carcinomas are also associated with immunosuppressive populations of B cells due to poorly defined markers for such cells (Sarvaria et al., 2017). Moreover, studies investigating the associations of B cell subsets with tumor progression using defined B-cell markers have produced conflicting results even within the same tumor type (Guy et al., 2016). An insufficient understanding of the roles of B cells in carcinomas has hindered the development of rational clinical trials targeting B-cells in carcinomas. The remarkable success of B-cell depletion with the cluster of differentiation 20 (CD20) monoclonal antibody, rituximab, in lymphomas and rheumatoid arthritis has sparked interest in rituximab and other B-cell targeted antibodies as possible therapies in carcinomas (Gunderson and Coussens, 2013). Although many carcinomas have significant B cell infiltration (Germain et al., 2014), clinical trials have shown limited benefits of B-cell depletion in carcinomas (Barbera-Guillem et al., 2000; Aklilu et al., 2004), possibly because B cells can have pro-tumorigenic or anti-tumorigenic properties depending on their maturation stage and other conditions that have not yet been defined (Sarvaria et al., 2017).

TABLE 1 | DLBCL "stromal-1" signature genes are inversely correlated with survival outcomes in B-cell lymphomas and other malignancies.

	B-cell lymphoma						Solid tumor					
Gene	**BL**	**CLL**	**DLBCL**	**FL**	**MCL**	**MM**	**Bladder**	**Astro cytoma**	**Glioma**	**Colon**	**Head and neck**	**Ovarian**
ACTN1	−0.928	−3.216	−6.211	−1.901	−0.94	0.658	3.312	3.22	4.557	2.36	1.988	1.552
ADAM12	0.746	−0.084	−7.809	−1.749	−0.866	−0.395	0.537	1.653	4.405	1.675	2.051	2.99
BGN	0.842	1.309	−4.115	−1.775	0	−2.627	1.438	2.341	3.643	2.33	3.559	3.09
CEBPA	−1.516	−3.127	−5.644	−1.639	0	−0.977	1.001	−0.041	2.652	−2.664	−1.578	−1.442
COL13A1	−0.313	−1.513	−2.402	0.332	0	−0.001	2.23	2.006	1.613	2.164	1.74	0.893
COL16A1	−0.481	0.252	−3.89	−0.6	0.333	−0.477	2.214	2.49	5.005	−0.546	1.263	4.542
COL1A1	0.349	−1.476	−4.621	−1.581	0	−1.951	3.592	3.326	3.77	1.544	3.354	3.929
COL1A2	−0.097	−0.879	−6.264	−1.605	0	−0.573	2.745	4.432	4.391	2.42	2.634	3.771
COL5A1	0.715	−0.675	−3.366	0.127	0	−0.467	1.957	3.528	4.438	2.328	3.686	3.65
COL5A2	0.969	1.124	−3.962	−1.597	0	−0.777	3.47	3.588	7.322	2.437	3.26	5.256
COL6A2	0.677	−1.368	−3.719	−0.749	−1.415	0.14	2.369	4.591	5.693	1.301	3.12	2.11
COL6A3	1.194	−0.129	−4.502	−1.442	1.37	2.684	1.282	3.005	3.071	2.403	3.141	3.178
COL8A2	−0.212	−0.894	−3.046	0.069	0	−0.905	−0.085	2.942	3.077	−0.007	1.779	2.908
CSF2RA	−1.84	0	−2.861	0	0	−2.39	−0.046	0.193	0	0	0	−1.959
CTGF	−0.5	0.796	−5.525	−0.73	−1.387	−0.775	1.651	1.676	−1.132	2.024	2.381	2.974
CYR61	1.159	0.092	−1.865	0.074	1.837	−0.123	3.342	1.159	3.807	1.678	1.757	3.607
DCN	0.819	0.185	−3.731	−0.026	0	−0.794	0.472	1.113	2.414	1.303	0.917	4.604
EFEMP2	1.823	1.113	−2.797	0.307	0	−5.014	2.112	4.044	7.62	1.684	3.53	2.576
EMP2	−0.057	0.044	−4.122	0.147	0	−0.579	−1.125	4.55	2.985	−0.368	0.452	−1.446
FAP	−1.551	0.374	−7.496	−0.76	−1.266	−0.536	3.522	2.321	3.736	2.366	2.874	4.814
FBN1	1.125	1.079	−4.907	−1.854	0	−0.044	2.151	1.518	2.239	2.311	1.906	4.676
FN1	−1.025	−0.496	−5.638	−1.852	−1.352	2.973	3.251	2.852	5.499	2.628	2.46	4.439
GPNMB	−1.638	−0.153	−6.899	0.513	0	1.112	1.281	3.946	5.214	1.74	−2.745	1.476
HSPG2	−0.267	2.244	−2.792	−1.63	0	0.845	−0.02	4.261	2.989	1.313	2.108	2.396
IL1R1	−1.566	−2.791	−4.858	−0.432	0.804	−1.789	−0.186	1.194	1.217	1.275	0.897	−0.137
ITGAV	0.897	−2.698	−6.933	0.614	−2.033	−0.212	0.402	0.945	0.226	2.253	1.503	1.792
ITGB2	−1.522	−2.053	−5.68	0.558	0.343	−1.803	0.886	0.4	4.299	−0.086	−2.064	−2.339
KITLG	0.896	−0.172	−1.923	1.04	−1.197	0.454	1.113	−0.331	1.091	1.164	−0.721	−0.504
LAMA4	0.445	2.207	−3.683	0.453	0	−3.155	2.474	0.028	3.397	2.415	2.021	2.168
LAMB2	−0.635	0.504	−1.974	−1.052	0	−0.728	0.926	1.686	5.906	0.913	1.836	2.326
LAMB3	1.291	−1.315	−2.703	0.256	0	0.265	−0.927	1.977	3.542	1.516	2.039	−1.966
LOXL1	−1.453	−1.007	−4.202	−1.287	0	−1.92	0.711	3.9	6.299	1.697	0.751	3.664
LTBP2	0.219	−1.562	−7.565	−0.187	0	−1.848	2.849	1.197	3.314	0.542	2.718	1.541
LUM	−0.357	−1.043	−5.663	−0.089	0	−1.859	1.442	3.796	3.723	1.447	1.428	4.841
MFAP2	0.862	0.01	−2.835	0.608	0	−0.68	3.151	3.543	3.011	0.874	1.666	5.462
MMP14	−1.105	2.746	−3.319	0.69	0.681	−1.647	2.046	1.787	4.691	1.786	1.168	2.297
MMP2	−1.227	−0.269	−5.709	−1.128	0.014	−0.545	0.66	1.792	3.631	1.567	3.12	3.084
MMP9	−0.819	−1.238	−7.734	−0.401	−0.12	−0.892	1.8	2.739	5.06	−0.723	0.039	−3.208
PDGFC	0.62	−3.08	−4.268	0.632	0	−0.486	2.788	−3.419	3.639	1.987	2.096	−0.167
PLAU	−1.723	−1.701	−7.712	0.205	0.528	−0.749	2.515	2.302	4.592	0.627	1.521	2.334
POSTN	1.565	0.675	−5.031	−1.266	−0.77	−1.157	3.246	2.76	5.46	2.632	2.092	4.696
SDC2	−0.209	−1.963	−3.763	−0.47	−0.383	−0.664	−1.091	1.405	5.736	2.239	1.659	1.424
SERPINH1	−1.173	2.067	−2.912	−1.224	0	1.565	1.422	3.846	5.397	3.044	2.065	2.07
SPARC	0.487	−3.125	−7.236	−1.599	1.012	−2.767	2.24	−1.998	−0.074	2.412	2.933	4.188
TGFB1I1	−0.842	−1.479	−2.367	0.662	0	−1.787	1.518	2.783	4.58	1.523	3.557	4.265
THBS1	1.462	−3.212	−2.038	−1.38	0.238	−1.674	1.673	2.947	3.122	0.799	2.328	3.565
TIMP2	−0.677	−2.448	−1.399	1.006	0.343	0.83	2.608	1.584	1.251	2.73	2.271	2.495
VCAN	1.459	−3.803	−3.177	−0.588	0	−2.078	3.133	−3.546	−3.171	2.264	2.238	4.277

Analysis of the DLBCL "stromal-1" geneset in the PREdiction of Clinical Outcomes from Genomic Profiles (PRECOG) public dataset (https://precog.stanford.edu). Each gene is assigned z scores associated with survival in different cancer types. Scores less than or equal to zero (red) are associated with good survival while positive scores (blue) are associated with poor survival. BL, Burkitt lymphoma; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma.

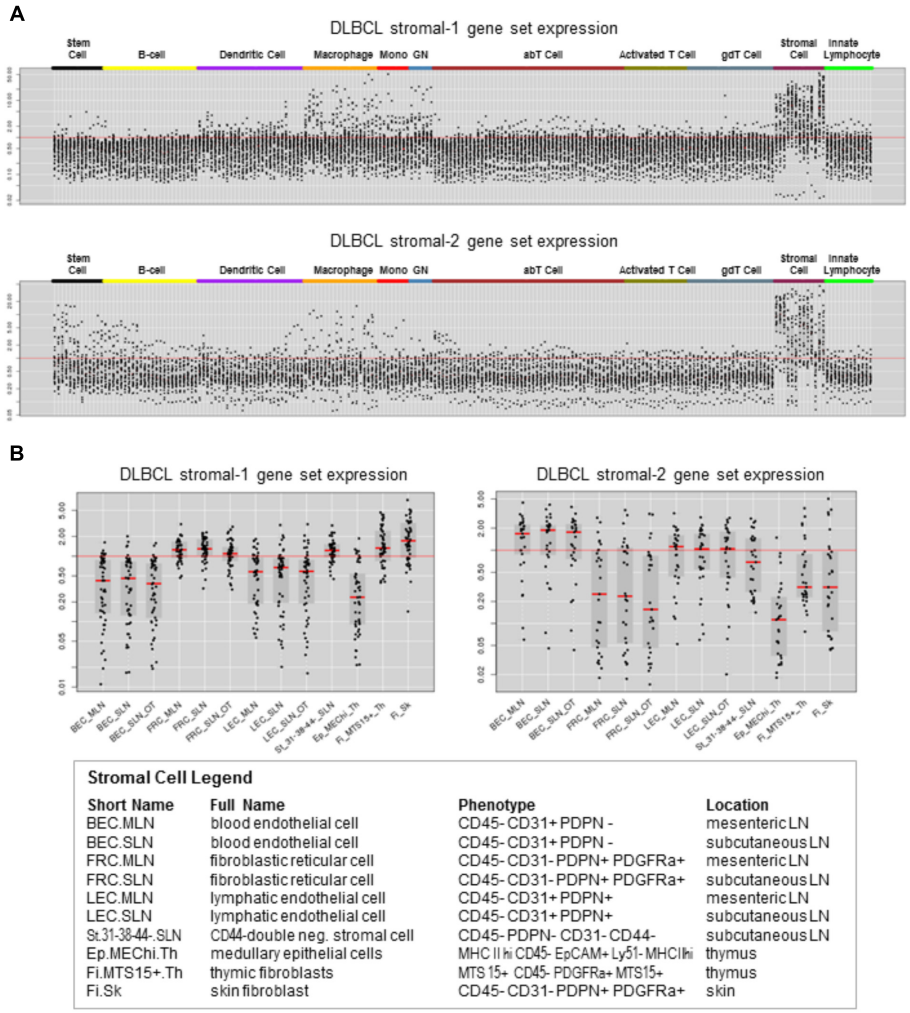

FIGURE 1 | DLBCL stromal-1 and stromal-2 signature genes are enriched in different stromal cell types. Expression of the DLBCL stromal-1 and stromal-2 signature genes in the Immunological Genome Project (ImmGen) data set. **(A)** Gene expression values normalized across 249 mouse immunological cell types. **(B)** Detailed view of gene expression values normalized to the stromal cell types shown in the legend. The graphs were generated using data from ImmGen (http://www.immgen.org).

THE DLBCL STROMAL-1 GENE SIGNATURE IS INVERSELY CORRELATED WITH SURVIVAL OUTCOMES IN B-CELL LYMPHOMAS AND OTHER SOLID TUMORS

Using expression profile analysis of DLBCL biopsy samples from treatment-naïve newly diagnosed patients, Lenz et al. identified two stromal gene signatures, stromal-1 and stromal-2, of which the stromal-1 gene signature was found to be associated with good survival in DLBCL patients (Lenz et al., 2008). However, gene signatures similar to the DLBCL stromal-1 gene signatures have been associated with poor survival in carcinomas, including ovarian cancer (Cheon et al., 2014), breast cancer (Farmer et al., 2009), colorectal cancer (Calon et al., 2015; Isella et al., 2015), and pancreatic cancer (Moffitt et al., 2015).

To systematically explore the association of the DLBCL stromal-1 gene signature with survival in cancer patients, we used PRECOG, a pan-cancer database of expression signatures in which each tumor type is represented by multiple independent expression profile data sets and associated survival data. This extensive database is ideal for multi-data set validation of prognostic signatures that have been identified in individual data sets. Using the DLBCL stromal-1 gene signature represented by 50 genes (Lenz et al., 2008), we confirmed that the signature is associated with poor survival in carcinomas and brain tumors and good survival in DLBCL and several other B-cell lymphomas (**Table 1**). This pattern of inverse association with survival between B-cell lymphomas and carcinomas/brain tumors was specific to the DLBCL stromal-1 gene signature, and was not associated with the DLBCL stromal-2 gene signature represented by 34 genes (Lenz et al., 2008) (data not shown).

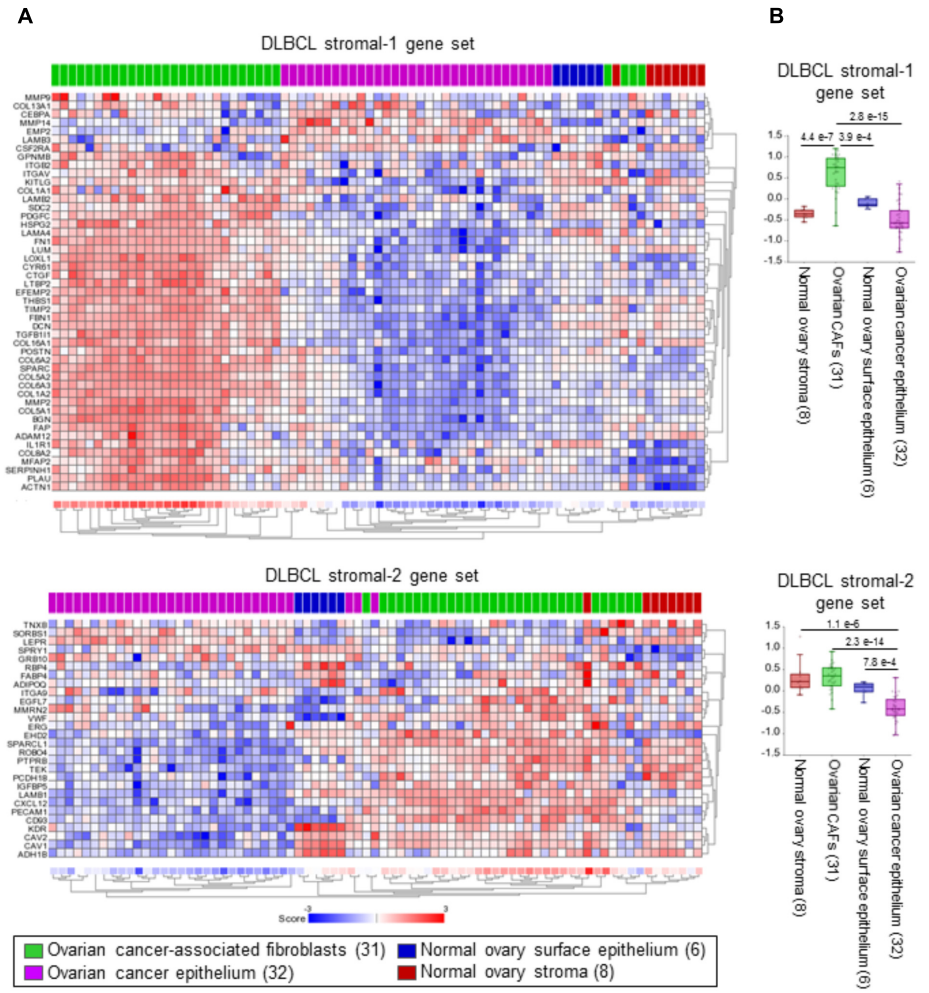

FIGURE 2 | DLBCL stromal-1 signature genes are enriched in cancer-associated fibroblasts (CAFs). **(A)** Non-centered gene set clustering analysis of the stromal and epithelial cell types in ovarian cancer and the normal ovary in the GSE40595 dataset using the DLBCL stromal-1 and stromal-2 gene sets. The number of samples in each group is indicated in parentheses. The gene set clustering analysis and image acquisition was performed using the R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl). **(B)** The same data are shown as box dot plots with *P*-values for differential expression of the DLBCL stromal-1 and stromal-2 gene signatures in different cell types.

IN NORMAL LYMPH NODES, DLBCL STROMAL-1 AND STROMAL-2 GENE SIGNATURES ARE ENRICHED IN STROMAL FIBROBLASTS AND ENDOTHELIAL CELLS, RESPECTIVELY

To identify immune cell types that express the DLBCL stromal-1 and stromal-2 signature genes, we looked for enrichment of these genes in the transcriptomes of 249 normal immunological cell types that had been isolated from mice and characterized by the Immunological Genome Project (ImmGen) (Heng and Painter, 2008; Shay and Kang, 2013). This analysis identified stromal cells as the most likely source of both gene signatures, although some of the genes were also expressed in macrophages, monocytes, granulocytes, and stem cells (**Figure 1A**). Closer examination of the stromal cell subtypes revealed that the DLBCL stromal-1 and stromal-2 signature genes were preferentially expressed in different types of stromal cells. DLBCL stromal-1 signature genes were particularly enriched in cells characterized by expression of podoplanin (PDPN) and platelet-derived growth factor receptor α (PDGFRα), including FRC from mesenteric and subcutaneous lymph nodes and the so-called double-negative stromal cells, while stromal-2 signature genes were enriched in blood and lymphatic endothelial cells (**Figure 1B**).

THE DLBCL STROMAL-1 GENE SIGNATURE IS ENRICHED IN OVARIAN CAFs

To identify cells that express the DLBCL stromal-1 and stromal-2 signature genes in an epithelial tumor, we selected

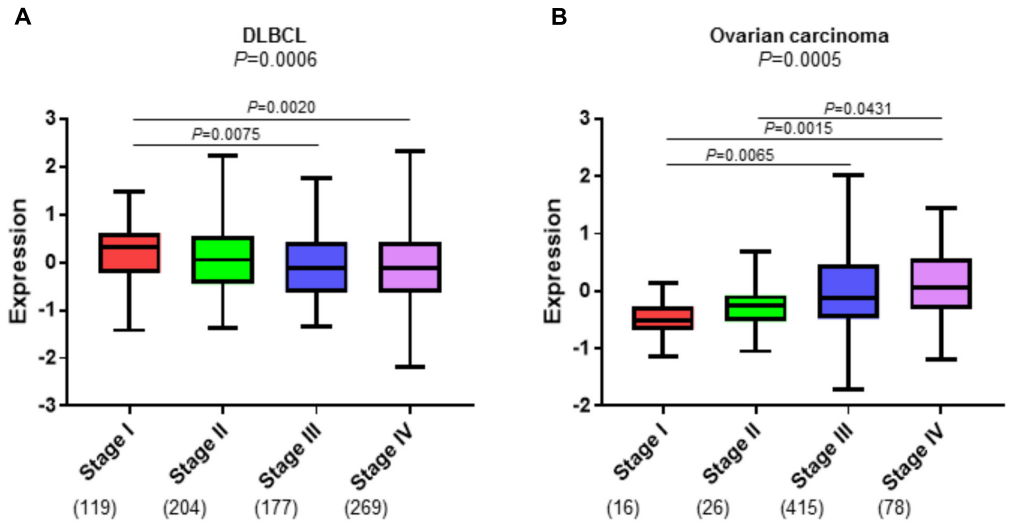

FIGURE 3 | CAFs have an inverse association with tumor stage in DLBCL and ovarian carcinoma. Enrichment of the DLBCL stromal-1 gene signature in progression stages I-IV in **(A)** three DLBCL microarray datasets (GSE10846, GSE87371, and GSE4475) that were combined into one dataset, and **(B)** The Cancer Genome Atlas (TCGA) ovarian carcinoma dataset (https://cancergenome.nih.gov). The gene signature enrichment analysis was performed using the R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl). The y axis shows relative enrichment of the DLBCL stomal-1 gene signature. The x axis shows tumor stage. The number of samples for each tumor stage is indicated in parentheses.

ovarian cancer because of the existing microarray data set (GSE40595) in which a large number of ovarian cancers have been laser capture microdissected into epithelial and stromal components (Yeung et al., 2013). For comparison with normal tissue, a small number of samples in this data set were microdissected from the normal ovary epithelium and stroma (Yeung et al., 2013). Our gene signature enrichment analysis revealed strong enrichment of the DLBCL stromal-1 gene signature in CAFs in comparison to cancer cells, normal ovary fibroblasts, and normal ovary epithelial cells (**Figure 2**). The DLBCL stromal-2 gene signature was enriched in CAFs but also in the normal ovary stroma (**Figure 2**).

POSSIBLE MECHANISMS BY WHICH CAFs CONTRIBUTE TO INVERSE SURVIVAL OUTCOMES IN B-CELL LYMPHOMAS AND CARCINOMAS

It is unusual for a gene signature to be associated with inverse survival outcomes in B-cell lymphomas and carcinomas. This is unlikely to be a technical error related to microarray technology as several individual genes from the DLBCL stromal-1 signature have been validated as predictors of good survival in DLBCL by independent technologies, such as immunohistochemistry and qPCR in formalin-fixed paraffin-embedded tissues (Lossos et al., 2004; Meyer et al., 2011; Tekin et al., 2016). Similarly, various technologies have been used to validate many of the signature genes as predictors of poor survival in carcinomas (Farmer et al., 2009; Cheon et al., 2014; Calon et al., 2015; Isella et al., 2015; Moffitt et al., 2015; Jia et al., 2016). While the mechanism by which the DLBCL stromal-1 signature genes could contribute to good survival in DLBCL is still unclear, multiple mechanisms by which CAFs contribute to poor outcomes in carcinomas

TABLE 2 | Upstream regulators of genes in the DLBCL stromal gene signature-1 and stromal gene signature-2.

Upstream regulator	Molecule type	*p*-value of overlap
DLBCL stromal-1 gene signature		
TGFB1	Growth factor	4.78E-31
COLQ	Other	2.70E-20
Bleomycin	Chemical drug	1.97E-18
SPDEF	Transcription regulator	2.73E-18
Tgf beta	Group	3.95E-18
TGFB3	Growth factor	8.04E-18
TNF	Cytokine	1.53E-17
DLBCL stromal-2 gene signature		
KLF2	Transcription regulator	1.89E-09
Rosiglitazone	Chemical drug	5.82E-09
VEGFA	Growth factor	5.90E-09
PPARG	Ligand-dependent nuclear receptor	1.36E-08
10E,12Z-octadecadienoic acid	Chemical – endogenous Mammalian	4.98E-08
WNT3A	Cytokine	6.02E-08
MGEA5	Enzyme	1.08E-07

The identification of upstream regulators was done using Ingenuity Pathway Analysis (www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/).

have been proposed, including the promotion of tumor growth, angiogenesis, invasion and metastasis, the provision of protective niches for cancer stem cells, and the obstruction of access of chemotherapies and immunotherapies (Jain, 2013; Kalluri, 2016). Here, we will specifically focus on the possible direct or indirect roles of CAFs that could contribute to inverse survival outcomes in DLBCL and carcinomas.

Cancer-associated fibroblasts share structural and molecular features with the reticular fiber networks of secondary lymphoid organs, which are known to guide and compartmentalize specific immune cell types and play key roles in mediating functional immune cell interactions (Acton et al., 2012; Astarita et al., 2012; Cremasco et al., 2014; Chang and Turley, 2015; Fletcher et al., 2015; Turley et al., 2015). However, in addition to being sites in which immune responses are initiated, secondary lymphoid organs are also sites that foster immune privilege that prevents autoimmunity by inducing tolerance and deleting autoreactive T cells, suppressing effector T cell proliferation, and supporting regulatory T cells (Fletcher et al., 2011, 2014, 2015; Brown and Turley, 2015). Currently, lymph node fibroblasts are being explored for their therapeutic potential to circumvent unwanted inflammation in autoimmune diseases, sepsis, and graft rejection after organ transplantation (Fletcher et al., 2011, 2014, 2015). Based on the molecular similarity between CAFs and lymph node fibroblasts, we propose that CAFs primarily play an immunosuppressive role in tumors using similar molecular mechanisms to those used by lymph node fibroblasts in regulating immune cell tolerance and homeostasis. In support of this hypothesis, CAF-derived factors have been shown to contribute to immune editing *in vivo* to avoid tumor detection and rejection by the host immune system (Stover et al., 2007; Kraman et al., 2010). Specific to B cells, several *in vitro* models have shown the ability of different types of fibroblasts to modulate B cell differentiation, activation, and function. Adipose tissue-derived fibroblasts have been shown to suppress plasmablast formation and induce formation of regulatory B cells (Franquesa et al., 2015) while rheumatoid synovial fibroblasts have been shown to induce immunoglobulin (Ig) class-switch recombination and IgG/IgA production in IgD+ B cells (Bombardieri et al., 2011). We envision that the immunoregulatory functions of CAFs may lead to improved survival in DLBCL and other B-cell lymphomas where malignant cells themselves are subject to functional alteration. In contrast, immunosuppression by CAFs in carcinomas may lead to an ineffective immune defense against malignant cells, which is associated with poor survival.

Cancer-associated fibroblasts are also capable of modifying the immune landscape by selective attraction, recruitment, retention, activation, and suppression of different immune cell types (Karin, 2010; Raz and Erez, 2013; Harper and Sainson, 2014). Recent studies provide evidence that CAFs can directly contribute to immune cell fate and survival (Harper and Sainson, 2014). In mouse models, CAFs have been shown to attract macrophages, neutrophils, and subsets of T cells that promote tumor progression (Silzle et al., 2003; Grum-Schwensen et al., 2010; Elkabets et al., 2011). One possible underlying mechanism for the association of the DLBCL stromal-1 gene signature with good survival in patients with DLBCL is that fibroblasts and the associated ECM attract and trap malignant B cells thereby impeding their spread to new anatomical locations. We show a small but consistent inverse association of the DLBCL stromal-1 gene signature expression with DLBCL tumor stage (a measure of lymph node groups and extranodal sites to which malignant cells have metastasized) (**Figure 3A**). The decrease in stromal gene signature expression in the later stages of DLBCL may indicate that the stroma plays a role in localizing the lymphoma cells to the lymph nodes during the earlier stages of the disease. In contrast, DLBCL stromal-1 gene signature expression is typically increased with increased tumor stage in epithelial carcinomas, such as ovarian cancer (**Figure 3B**). The increase in CAFs in the later stages of carcinomas may prevent immune cells from reaching the tumor parenchyma by trapping the immune cells in the stroma thereby preventing an anti-tumor response. A recent study of immune cell infiltration in metastatic urothelial carcinomas showed that patients whose tumors were classified as immune-excluded (immune cells localized in the CAF-rich stroma) had increased disease progression and decreased response to immunotherapy (Mariathasan et al., 2018). Therefore, we hypothesize that CAFs aid in retaining DLBCL in the lymph node, which is associated with better prognosis, whereas in carcinomas CAFs trap immune cells, which is associated with decreased anti-tumor immune activity and a worse prognosis.

One of the key modulators of the cancer microenvironment is the multifunctional cytokine, transforming growth factor β (TGFβ). TGFβ induces CAF activation and fibroblast-to-myofibroblast transition with consequent linearization of collagen fibers and stiffening of the ECM. In turn, activated CAFs induce TGFβ signaling to perpetually maintain the activated state (Calon et al., 2014; Beach et al., 2016; Erdogan and Webb, 2017). Consistent with the DLBCL stromal-1 signature representing CAFs, our Ingenuity Pathway Analysis (IPA) of the DLBCL gene signatures implicates TGFβ signaling as the main upstream regulator of the DLBCL stromal-1 gene signature (**Table 2**). In carcinomas, TGFβ has been shown to promote tumor progression by inhibiting immunosurveillance through multiple mechanisms (Flavell et al., 2010; Sheng et al., 2015), including the recruitment of macrophages (Byrne et al., 2008) and limited efficacy of immunotherapy by excluding $CD8^+$ T cells from the tumor parenchyma (Mariathasan et al., 2018; Tauriello et al., 2018). It is likely that TGFβ also plays an immunosuppressive role in lymphomas. However, TGFβ is also a potent negative regulator of B-cell survival, proliferation, activation, and differentiation (Sanjabi et al., 2017). Stroma-derived TGFβ has been shown to induce senescence and apoptosis in mouse models of B-cell lymphoma (Reimann et al., 2010; Stelling et al., 2018). Thus, the DLBCL stromal-1 gene signature may be primarily associated with tumor-promoting immunosuppression in carcinomas, while the same immunosuppression may lead to the eradication of

B cells, which represent the malignant component of B-cell lymphoma.

CONCLUSION

Past clinical trials have taught us that successful targeted therapies in one disease do not always yield the desired results in another disease despite the presence of the same target. One example is the poor response of B-cell-infiltrated carcinomas to rituximab, which has shown remarkable success in lymphomas and rheumatoid arthritis. The opposite survival outcomes associated with the presence of stromal cells in B-cell lymphomas and carcinomas should serve as a warning that targeting the tumor microenvironment may produce opposite effects in B-cell lymphomas and carcinomas.

DATABASE LINKS

GEO Data Sets (https://www.ncbi.nlm.nih.gov/gds) Immunological Genome Project (https://www.immgen.org) PRECOG – PREdiction of Clinical Outcomes from Genomic Profiles (https://precog.stanford.edu) R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl) The Cancer Genome Atlas Project (https://cancergenome.nih.gov).

AUTHOR CONTRIBUTIONS

SO analyzed the public data sets. SO and MH wrote the manuscript.

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REVIEW
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Macrophages and Fibroblasts, Key Players in Cancer Chemoresistance

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Chemotherapy is routinely used in cancer treatment to eliminate primary and metastatic tumor cells. However, tumors often display or develop resistance to chemotherapy. Mechanisms of chemoresistance can be either tumor cell autonomous or mediated by the tumor surrounding non-malignant cells, also known as stromal cells, which include fibroblasts, immune cells, and cells from the vasculature. Therapies targeting cancer cells have shown limited effectiveness in tumors characterized by a rich tumor stroma. Tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) are the most abundant non-cancerous cells in the tumor stroma and have emerged as key players in cancer progression, metastasis and resistance to therapies. This review describes the recent advances in our understanding of how CAFs and TAMs confer chemoresistance to tumor cells and discusses the therapeutic opportunities of combining anti-tumor with anti-stromal therapies. The continued elucidation of the mechanisms by which TAMs and CAFs mediate resistance to therapies will allow the development of improved combination treatments for cancer patients.

Keywords: macrophages, fibroblasts, tumor stroma, tumor microenvironment, chemoresistance, therapy resistance

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INTRODUCTION

The treatment of cancer with chemical substances, known as chemotherapy, is routinely used for cancer treatment because as it circulates throughout the body it targets not only the primary tumor site but also tumor cells that have spread to other organs which are usually missed with surgical intervention or radiotherapy treatment (Eguchi et al., 2008).

The birth of chemotherapy came after the first world war, using nitrogen mustard as an anti-cancer agent in non-Hodgkin's lymphoma (Gilman, 1963). This agent was non-specific and showed limited effectiveness as patients experienced relapse after a few weeks. However, this discovery triggered investigation into the drug's mechanism of action leading to the development of other alkylating agents (Haddow et al., 1948). Targeted chemotherapy was developed in the late 1980s after the elucidation of some of the signaling pathways aberrantly regulated in tumors. Targeted chemotherapy included pharmacological targeting of the cell cycle regulating proteins, growth factors and angiogenesis mediators (Hanahan and Weinberg, 2000; Chabner and Roberts, 2005).

Since its beginning, chemotherapy has provided a plethora of benefits for many cancer patients (Klastersky and Paesmans, 2001; Benedetti-Panici et al., 2003; Gebski et al., 2007). Chemotherapeutic agents given before surgery as 'neoadjuvant' therapy can be used to reduce the tumor mass before surgical resection. This has many benefits as the reduction of the tumor size decreases the level of invasiveness required for resection and often improves the distinction between

healthy and neoplastic tissue during resection (Hayes and Schott, 2015). Adjuvant administration of chemotherapy occurs post-surgery with the purpose of minimizing the chance of recurrence. Adjuvant therapy is effective in two ways: firstly, against micro or macro-metastasis which are already seeded but were not detectable at the time of surgery, and secondly, against micro-metastasis created as a by-product of surgery due to tissue regeneration promoting cytokine storms released after invasive surgery (Hayes and Schott, 2015).

Despite the development of targeted agents with improved toxicity profiles, in some cancers chemotherapeutic agents only provide a minimal improvement of overall survival (Burris et al., 1997; Marquette and Nabell, 2012). The reduced effectiveness of chemotherapy in patients is due to tumor resistance mechanisms, which can be either tumor cell autonomous and/or mediated by the tumor surrounding non-malignant cells present in the tumor microenvironment (TME) (Joyce and Pollard, 2009; De Palma and Lewis, 2013; Mielgo and Schmid, 2013; Zheng, 2017). Tumor cell autonomous mechanisms of drug resistance have been extensively reviewed before (Zahreddine and Borden, 2013; Housman et al., 2014; Zheng, 2017) so the focus of this review is on the emerging TME-mediated mechanisms of tumor resistance to chemotherapy with a main focus on chemoresistance mechanisms mediated by tumor-associated macrophages (TAMs) and fibroblasts.

The TME describes the complete tumor milieu including the malignant tumor cells and the surrounding tumor stroma. The tumor stroma consists of non-malignant cells including immune cells (macrophages, neutrophils, and T cells), fibroblasts, cells from the vasculature (pericytes and endothelial cells) and extracellular matrix (ECM) proteins (Hanahan and Weinberg, 2011). Accumulating evidence shows that the tumor stroma develops and interacts with the tumor cells, participating in bi-directional tumor-stroma signaling which supports tumor progression, metastasis and resistance to therapy (Hanahan and Coussens, 2012; Quail and Joyce, 2013). The most abundant non-cancerous cell types present in the tumor stroma are TAMs and cancer-associated fibroblasts (CAFs). This review will discuss the various mechanisms, discovered to date, by which TAMs and CAFs support tumor chemoresistance, the controversies and current gaps in this research field and the potential future perspectives.

ORIGIN OF MACROPHAGES AND FIBROBLASTS

Macrophages

Tissue resident macrophages are a diverse population of cells which perform tissue-specific functions in tissue homeostasis, repair, immunity and angiogenesis (Davies et al., 2013a). Macrophages can originate from three independent sources. Embryonic macrophage populations have been mapped back to two sources: fetal liver-derived monocytes or precursor cells found in the yolk sac (Yona et al., 2013; Mass et al., 2016). In adult tissue, macrophage populations differentiate from hematopoietic stem cells in the bone marrow (Orkin and Zon, 2008).

Once established in adult tissue, macrophages maintain their population via self-renewal in the steady state but increase their rate of proliferation in response to stimuli such as interleukin 4 (IL-4) and colony stimulating factor 1 (CSF-1) (Jenkins et al., 2011, 2013; Davies et al., 2013a). During inflammation, bone marrow-derived monocytes are recruited into the tissue and mature into macrophage populations which act alongside tissue resident macrophages (Shi and Pamer, 2011). These converted monocytes display cell surface markers associated with resident macrophages increasing their responsiveness to IL-4 and IL-3 (Yona et al., 2013; Dal-Secco et al., 2015).

Bone-marrow derived macrophages (BM-DMs) and tissue resident macrophages appear to intermingle and work together to resolve inflammation and promote tissue repair. However, it is currently undetermined if BM-DMs play the exact same role as tissue resident macrophages (Davies et al., 2013b). Bone marrow transplant studies have shown that BM-DMs and tissue resident macrophages share similar characteristics (van de Laar et al., 2016). These similarities have been further confirmed by transcriptome analysis of lung alveolar resident macrophages which revealed different genes expressed in BM-DMs compared to tissue resident macrophages (Gibbings et al., 2015).

Fibroblasts

Fibroblasts are of mesenchymal origin and dependent on their tissue of origin have a distinct transcriptional profile (Chang et al., 2002). Fibroblasts have never been identified in embryonic tissue but are hypothesized to arise during the epithelial-to-mesenchymal transition (EMT) of the epiblast during gastrulation with the generation of mesoderm tissue (Kalluri, 2016). Virchow (1858) identified cells in adult tissue that, produced collagen, were resistant to apoptosis, and reverted to quiescence upon the completion of tissue development. These cells were later called fibroblasts (Virchow, 1858). Due to the inability to identify fibroblasts in embryonic tissue it remains unknown whether the majority of activated fibroblasts originate from fibrocytes or mesenchymal stem cells (MSCs) in adult tissue (Kalluri, 2016).

Stellate cells are found in the pancreas, liver, lung, and kidney and although stellate cells are similar to fibroblasts, they display some distinctly different functions such as vitamin A storage as retinol droplets in their cytoplasm which is required for cellular homeostasis (Keane et al., 2005; Liu, 2006; Erkan et al., 2010). Quiescent stellate cells usually constitute <10% of the organ where they reside and are found in perivascular and peri-parenchymal regions (Wake and Sato, 1993; Apte et al., 1998; Bachem et al., 1998). Like fibroblasts, the origin of stellate cells is still debated. Neuroectoderm is suggested as a potential origin of pancreatic stellate cells (PaSCs) and hepatic stellate cells (hStCs) (Friedman, 2000). Lineage tracing studies have shown that hStCs can originate from mesoderm in mice, however, lineage tracing studies are currently lacking for PaSCs (Asahina et al., 2009, 2011).

Activated fibroblasts (also known as myofibroblasts) can originate from several different cell types that include quiescent fibroblasts from normal parenchyma, endothelial cells, MSCs, and stellate cells (LeBleu et al., 2013; Kalluri, 2016). For example,

the origin of activated fibroblasts which support ductal outgrowth has been disputed in breast tissue. Ucar et al. (2010), reported that miR-212/132 expression in stromal fibroblasts is required to support ductal outgrowth. However, in another study, targeted deletion of miR-212 and miR-132 in embryonic stem cells did not show an effect in ductal outgrowth, instead, this study claims that Hic1 expression in stromal cells is required for mammary ductal outgrowth (Kayo et al., 2014). These contradictory results suggest that further studies aiming to understand the role of fibroblasts in mammary gland development are required (Ucar et al., 2014). The heterogeneous origins of myofibroblasts may play a role in generating populations with different phenotypes and functions. Recent studies have described heterogeneous populations of activated fibroblasts present in pancreatic and breast tumors (Ohlund et al., 2017; Costa et al., 2018) and understanding the functions of these different fibroblast populations in cancer is currently an intensive field of research.

PHYSIOLOGICAL FUNCTIONS OF MACROPHAGES AND FIBROBLASTS

Macrophages represent a heterogeneous population of cells that are highly plastic and adapt to their surroundings to perform a variety of functions in tissue homeostasis, repair, and immunity (Wynn et al., 2013). Macrophages respond to tissue-derived or external stimuli adapting their phenotype and function accordingly (Biswas and Mantovani, 2010). A spectrum of different subsets of macrophages with diverse phenotypes and functions co-exist in tissues and the macrophage subsets at the extremes of this spectrum are known as M1 (or classically activated) and M2 (or alternatively activated) macrophages (Murray and Wynn, 2011; Mills, 2012). Macrophages can be polarized into M1-like or M2-like macrophages and their polarization depends on the stimulating cytokine and the length of exposure (Gordon and Martinez, 2010). However, the nomenclature and understanding of macrophage subtypes and functions is still evolving.

M1-like macrophages are generated in response to interferon gamma (INFγ) and lipopolysaccharide (LPS) stimulation, factors produced by infiltrating bacteria and pathogens. M1-like macrophages are pro-inflammatory and secrete factors to promote inflammation, microbicidal activity and immunostimulation, such as cytokines IL-12, IL-6, IL-1β, tumor-necrosis factor alpha (TNFα) as well as reactive oxygen species (ROS) and nitric oxide (NO) (Gordon and Martinez, 2010; Biswas et al., 2013) (**Figure 1**).

In contrast, M2-like macrophages are polarized by IL-4 and IL-13 produced by invading parasites and release anti-inflammatory cytokines IL-10, arginase I and transforming growth factor beta (TGF-β), as well as vascular endothelial growth factor (VEGF), promoting the remodeling of their surrounding tissue. Concurrently, macrophages upregulate expression of scavenging receptors while downregulating receptors and markers associated with antigen presentation (Biswas and Mantovani, 2010; Mantovani and Sica, 2010) (**Figure 1**).

Tissue resident macrophages play a variety of roles in a tissue context-dependent manner. Largely, they participate in functions usually associated with an M2 phenotype including mediating resolution of inflammation, maintaining tissue homeostasis via the removal of debris, supporting angiogenesis and partaking in immune surveillance (Davies et al., 2013a).

Angiogenesis occurs as part of homeostasis throughout life and is tightly regulated by macrophages (Fantin et al., 2010; Outtz et al., 2011). In mouse embryos, microglia (central-nervous system specific macrophages) migrate to the brain and assist in developmental angiogenesis (Arnold and Betsholtz, 2013). In the central nervous system, macrophages promote endothelial tip cell fusion by acting as a chaperone for endothelial cells in vascular development (Fantin et al., 2010). However, it appears that the actions undertaken by macrophages are tissue-dependent as, conversely, macrophages mediate the regression of blood vessels in the developing retina (Lobov et al., 2005; Fantin et al., 2010).

Another homeostatic function of macrophages is the removal of apoptotic and excess cell debris. This function is extremely important in the regulation of hematopoiesis in which macrophages phagocytose excess erythrocytes and neutrophils (Gordy et al., 2011; Klei et al., 2017). When this process was interrupted in mice they suffered severe neutrophilia, splenomegaly, extramedullary hematopoiesis and decreased body weight (Gordy et al., 2011). Macrophages also regulate immune responses through the ingestion of apoptotic cells preventing leakage of cell-death related factors which could promote inflammation (Savill et al., 2002).

In the event of injury or infection, pro-inflammatory macrophages are recruited to the afflicted area and secrete factors including IL-1β, NO, and TNFα as a defense mechanism to kill any invading pathogens (Murray and Wynn, 2011). The release of these factors can also result in secondary damage to host tissue. To limit the impact of this damage, macrophages either undergo apoptosis or reprogram toward an anti-inflammatory M2-like phenotype (Murray and Wynn, 2011). However, when this process goes awry, and macrophages maintain their pro-inflammatory functions, chronic inflammation occurs and becomes the basis of some auto-immune diseases such as Crohn's disease, rheumatoid arthritis and autoimmune hepatitis (Sindrilaru et al., 2011; Navegantes et al., 2017). Along with mediating the immunity side of wound healing, macrophages alter their secretory phenotype after inflammation subsides, to promote tissue regeneration. To promote the closure of the wound, macrophages attract and activate fibroblasts through the secretion of TGF-β (Khalil et al., 1989; Murray and Wynn, 2011).

In healthy tissue, fibroblasts and stellate cells exist in a quiescent state within the ECM making few cell-cell or cell-basement membrane connections. They are usually found as single cells, elongated and spindle-like in morphology situated in the interstitial space between the functional tissues of adult organs (Tarin and Croft, 1969). Quiescent fibroblasts and stellate cells produce very little ECM components such as collagen 1 and fibronectin and secrete a few factors including pigment epithelium-derived factor (PEDF) and thrombospondin-2, although their actual role while quiescent is yet to be fully elucidated (Tarin and Croft, 1969; Pollina et al., 2008). Specific

markers for fully quiescent fibroblasts are not yet known, however, fibroblasts specific protein-1 positive ($FSP1^+$) cells are often considered as quiescent (Strutz et al., 1995) (**Figure 2**).

The activation of fibroblasts and stellate cells is triggered in response to stress factors produced during tissue stress and damage, including TGF-β and ROS (Kalluri and Zeisberg, 2006). Activated fibroblasts acquire smooth muscle-like properties with increased contractility, motility, proliferation and a stellate morphology, and are known as myofibroblasts (Sappino et al., 1988; Ronnovjessen and Petersen, 1993). Upon activation, stellate cells also acquire a myoblastic phenotype but lose their cytoplasmic retinol lipid droplets (Blaner et al., 2009). Common

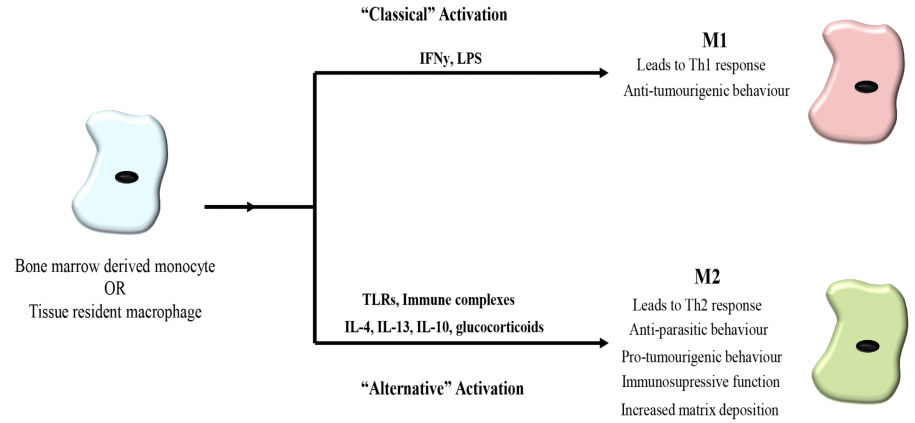

FIGURE 1 | Macrophage polarization. Bone marrow derived monocytes or tissue derived monocytes can be polarized toward either an M1 or M2 phenotype. Classical activation toward M1 polarization occurs in response to interferon gamma (IFNγ) and lipopolysaccharide (LPS) leading to a Th1 response associated with bacteria and viruses as well as possessing anti-tumorigenic properties. Alternative activation toward an M2 phenotype is triggered in response to toll-like receptors (TLRs), immune complexes, IL-4, IL-13, IL-10, and glucocorticoids. M2 macrophages lead to a Th2 response and exhibit anti-parasitic behavior. In cancer, M2-like macrophages promote tumor progression.

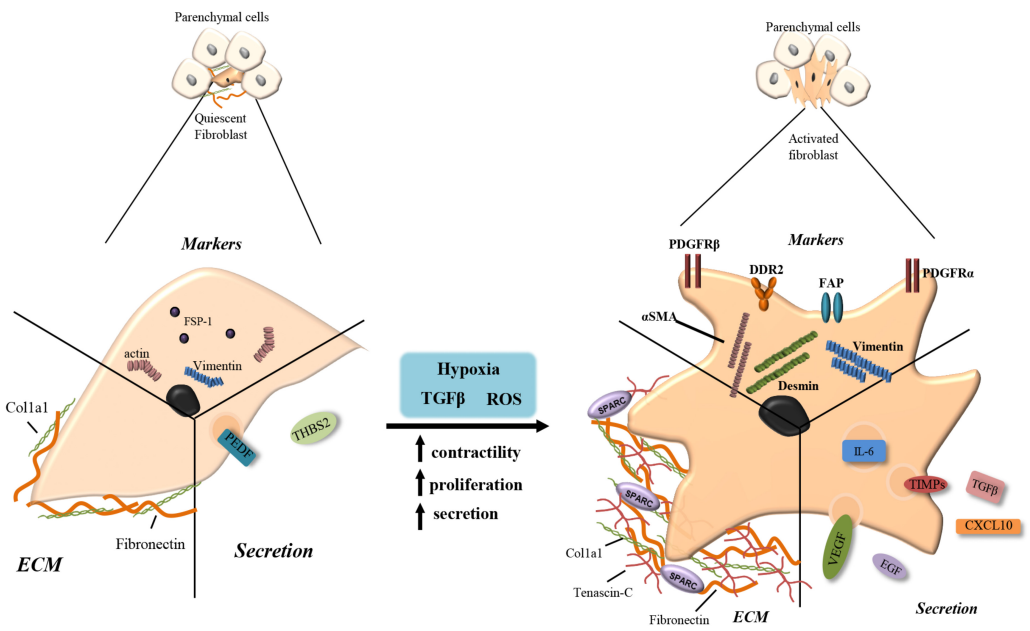

FIGURE 2 | Fibroblast activation. Quiescent fibroblasts produce few extracellular matrix (ECM) components such as fibronectin and collagen type 1 (Col1a1). They express fibroblasts specific protein-1 (FSP-1), actin and vimentin and secrete pigment epithelium-derived factor (PEDF) and thrombospondin-2 (THBS2). When stimulated with transforming growth factor beta (TGF-β), reactive oxygen species (ROS) or hypoxia, quiescent cells become activated increasing their contractility, proliferation and secretion. Activated myofibroblasts produce larger volumes of fibronectin and collagen along with tenascin-c and secreted protein acidic and rich in cysteine (SPARC). Increased secretion includes IL-6, tissue inhibitor of metalloproteinase (TIMPs), TGF-β, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and CXCL10. Upregulated receptors/markers include alpha smooth muscle actin (αSMA), platelet derived growth factor receptor alpha/beta (PDGFRα/β), fibroblast activation protein (FAP), discoidin domain-containing receptor 2 (DDR2), desmin, and vimentin.

markers of myofibroblasts include alpha-smooth muscle actin (αSMA), platelet derived growth factor receptor beta (PDGFRβ), PDGFRα, fibroblast activated protein (FAP), vimentin, desmin, Fibronectin Extra-domain A (EDA-FN) and discoidin domain-containing receptor 2 (DDR2) (Ronnovjessen and Petersen, 1993; Sugimoto et al., 2006; Quail and Joyce, 2013; Kalluri, 2016; Jiang et al., 2017) (**Figure 2**).

Myofibroblasts classically function in acute wound healing, becoming 'reversibly' activated and depositing the ECM proteins, collagens and fibronectin to close the wound (Dvorak et al., 1986; Darby and Hewitson, 2007). Myofibroblasts also modulate ECM consistency secreting matrix metalloproteases (MMPs) and tissue inhibitor of metalloproteinase (TIMPs) (Tampe and Zeisberg, 2014). Activated myofibroblasts also possess an altered secretory phenotype producing factors such as TGF-β, VEGF, C-X-C motif chemokine ligand 10 (CXCL10), CXCL12, IL-6, and epidermal growth factor (EGF) to promote proliferation and mediate recruitment of other cell types to the damaged tissue (Dvorak et al., 1986) (**Figure 2**).

Chronic activation of fibroblasts and stellate cells occurs in response to prolonged afflictions including toxins or auto-immune disorders. This results in chronic tissue fibrosis with myofibroblasts continuing to aberrantly perform their wound healing functions without resolution. These myofibroblasts become fibrosis-associated fibroblasts (FAFs), are irreversibly activated and exhibit enhanced proliferation and survival (Rock et al., 2011; Zeisberg and Zeisberg, 2013; Kalluri, 2016).

TUMOR-ASSOCIATED MACROPHAGES (TAMs) AND CANCER-ASSOCIATED FIBROBLASTS (CAFs)

Macrophages and fibroblasts are the two most abundant non-cancerous cells in tumors. Tumors become infiltrated with BM-DMs that are attracted to the tumor via the secretion of damage associated molecular patterns (DAMPs) and specific macrophage chemoattractants CSF-1 and chemokine C-C motif ligand 2 (CCL2). M1-like macrophages derived from the bone marrow and tissue resident macrophages are recruited to and activated in the tumor site in response to antigen presentation and inflammatory responses (Zhu et al., 2017). Once the tumor is established, tumor cells secrete cytokines IL-4, IL-10, IL-13 and lactic acid, and along with the presence of $CD4^+$ Th2 cells, cause the polarization of TAMs toward an M2-like phenotype. The M2 TAMs no longer serve to destroy the tumor but rather support cancer growth, metastasis and resistance to therapies (Gocheva et al., 2010; Qian and Pollard, 2010; Ruffell et al., 2012; Colegio et al., 2014). M2 TAMs support tumor progression by directly stimulating the growth of cancer cells through the production of growth factors, including EGF, TNFα, IL-6 (Grivennikov et al., 2010).

Solid tumors can undergo periods of hypoxia as its growing size limits the disposal of waste products and nutrient delivery becomes limited, triggering the angiogenic switch (Bergers and Benjamin, 2003; Hanahan and Weinberg, 2011). The activation of the angiogenic switch in tumors triggers dysregulated angiogenesis resulting in leaky vasculature with abnormal branching and enlarged diameter (Bergers and Benjamin, 2003). Macrophages are a source of VEGF and are known to support angiogenesis under normal physiological conditions (Fantin et al., 2010; Outtz et al., 2011). However, tumors depleted of myeloid-derived VEGF have a normalized vasculature with increased pericyte coverage and reduced vessel length and this accelerates tumor progression (Stockmann et al., 2008). Conversely, another study showed that hypoxia-related TAMs possess reduced mTOR activation, and that stimulation of mTOR activity in TAMs resulted in normalized vasculature with decreased vessel leakiness, hypoxia and metastasis (Wenes et al., 2016). TAMs are attracted to areas of tumor hypoxia through the release of Semaphorin 3A by cancer cells and TAMs promote angiogenesis via the phosphorylation of VEGF-receptor on endothelial cells (Casazza et al., 2013). CSF-1 stimulation has been shown to upregulate TIE2 expression on macrophages (Forget et al., 2014). Once inside the tumor, TIE2+ macrophages bind to angiopoietin-2 (Ang-2) expressed by endothelial cells and stimulate the growth of blood vessels promoting tumor growth and metastasis (De Palma et al., 2005; Mazzieri et al., 2011).

Metastatic spread of tumor cells to distant organs involves a multi-step process that requires local tissue invasion, intravasation, circulation through the blood stream, extravasation and successful colonization of the distant organ by the cancer cells (Hanahan and Coussens, 2012; Massague and Obenauf, 2016). Macrophages play a role in each of these stages of the metastatic cascade. Macrophages help tumor cell invasion into the basement membrane (Condeelis and Pollard, 2006; Wyckoff et al., 2007). In the PyMT breast cancer mouse model, CSF-1 produced by tumor cells and EGF secreted by TAMs results in the migration of both macrophages and cancer cells along collagen fibers and intravasation into the blood vessels (Goswami et al., 2005; Wyckoff et al., 2007). This phenomenon was also seen in glioblastoma, resulting in enhanced cancer cell invasion (Coniglio et al., 2012). TAMs can also promote tumor cell migration and invasion through the secretion of MMPs, secreted protein acidic and rich in cysteine (SPARC) and cathepsins which degrade and remodel the ECM (Bergers et al., 2000; Gocheva et al., 2006) as well as through the secretion of TGF-β which promotes EMT of tumor cells and increased tumor cell migration (Bonde et al., 2012).

As outlined earlier, fibroblasts are activated in response to tissue damage. After resolution of the insult, fibroblasts will reprogram back to quiescence or undergo apoptosis (Tomasek et al., 2002). However, tumors are referred to as "wounds that do not heal" (Dvorak et al., 1986). Persistent activation signals, in the context of cancer, maintain fibroblasts in a chronically activated state triggering a desmoplastic reaction and generating a dense fibrotic stroma which envelopes the tumor mass. Fibroblast activation signals are tumor-specific and determine the phenotype and function of the resulting myofibroblast. In the TME, a myofibroblast will exert a pro- or anti-tumorigenic response depending upon which

chemokines/cytokines it encounters (Tampe and Zeisberg, 2014). TGF-β is a common activating factor released by tumors which increases the expression of PDGF receptors on activated PaSCs (Apte et al., 1999). Sonic hedgehog (Shh) signaling in PDAC tumors has been reported to promote fibroblast activation and fibrosis in the pancreas (Bailey et al., 2008; Yauch et al., 2008). Other common factors involved in CAF activation include fibroblast growth factor (FGF), platelet derived growth factor (PDGF), and monocyte chemotactic protein (MCP1) (Kalluri and Zeisberg, 2006; Marsh et al., 2013).

Concurrent with their activated state, CAFs express an altered secretory phenotype, compared to quiescent fibroblasts, including ECM proteins and ECM modulating factors such as tenascin C, periostin, SPARC and EDA-FN; and tumor promoting factors such as nuclear factor-kB (NF-kB), IL-8, prostaglandin E_2 (PGE_2), connective tissue growth factor (CTGF) and CXCL7 (Kalluri, 2003; Hanahan and Coussens, 2012).

Recent advances in the field of CAF research has shown that different subsets of CAF populations with different functions co-exist within tumors (Costea et al., 2013; Brechbuhl et al., 2017; Ohlund et al., 2017; Costa et al., 2018). For example, in PDAC a specific subset of CAFs expressing high levels of αSMA but low levels of IL-6 was found in the fibrotic area juxtaposed to cancer cells and was called the myofibroblast CAF subset (myCAFs) (Ohlund et al., 2017). A different subset of CAFs, expressing low levels of αSMA but high levels of IL-6 was found at the periphery of the tumor and was termed the inflammatory CAF subset (iCAFs) (Ohlund et al., 2017). Ohlund et al. (2017) showed that the proximity of the myofibroblasts to the PDAC tumor cells, and the concentration of tumor-secreted factors alters the phenotype of the CAFs and the proteins they secrete. Another recent study performed with luminal A, human epidermal growth factor receptor 2^+ ($HER2^+$) and triple negative breast cancer (TNBC) patient samples revealed the co-existence of four different CAF subsets in breast tumors (Costa et al., 2018). TNBC samples predominantly had two types of myofibroblast-like CAFs; CAF-S1 and CAF-S4 identified by their high expression of αSMA. However, only CAF-S1 defined as $CD29^{Med}$, FAP^{Hi}, $FSP1^{Low-Hi}$, $aSMA^{Hi}$, $PDGFRb^{Med-Hi}$, and $CAV1^{Low}$; showed an immunosuppressive role by attracting T lymphocytes and promoting their survival and differentiation into immunosuppressive T regulatory cells (Costa et al., 2018). Thus, CAFs, like TAMs, are a heterogeneous population of cells and uncovering the different CAF populations and their functions in cancer is currently an important area of research.

Tumor-associated macrophages and CAFs take part in a complex interplay and can regulate each other's functions. For example, cancer cells and myofibroblasts are known sources of VEGF which promotes the accumulation of immune cells including macrophages at the site of fibrosis (Fukumura et al., 1998). VEGF-dependent recruitment and activation of macrophages promotes tumorigenesis, angiogenesis and invasion in skin cancer (Linde et al., 2012). Reciprocally, in liver metastasis of pancreatic cancer, macrophages recruited to the metastatic liver secrete granulin and activate resident quiescent hStCs which subsequently produce periostin supporting the growth of metastatic cancer cells in the liver (Nielsen et al., 2016).

MECHANISMS OF CHEMOTHERAPY RESISTANCE DRIVEN BY TAMs AND CAFs

Chemotherapy is used as a treatment in many different cancer types and is used either alone or in combination with surgical resection or radiation. Chemotherapy targets tumor cells at both the primary tumor site and the metastatic site. However, a common problem encountered with the treatment of many tumors is an acquired resistance to chemotherapeutic agents. Chemoresistance can be mediated by tumor cell-autonomous mechanisms, including changes in tumor cell epigenetics, drug inactivation, EMT, activation of alternative survival and proliferative pathways, and/or selection of drug-resistant cancer cell clones (Housman et al., 2014). However, many solid tumors such as breast cancer and PDAC have a rich stroma which contains, as described before, a plethora of non-malignant cell types that influence cancer progression and response to therapy in various ways. In fact, these non-malignant stromal cells are not simple bystanders but engage in bi-directional tumor-stroma signaling which can result in impaired therapeutic efficacy. For instance, the attraction of TAMs in a MCF-7 breast cancer xenograft model, via CSF-1 signaling, reduces the efficacy of a combination treatment with cyclophosphamide, methotrexate and 5-fluorouracil (CMF) (Paulus et al., 2006) (**Figure 3A**). The presence of TAMs in the genetic MMTV-PyMT mouse model of breast cancer makes tumors more resistant to paclitaxel therapy (DeNardo et al., 2011). Another study revealed TAM-derived cathepsins B and S as responsible for mediating chemoresistance to taxol in the MMTV-PyMT mouse model (Shree et al., 2011) (**Figure 3B**). In a subcutaneous mouse model of colorectal cancer, IL-6 released by TAMs mediates chemoresistance to 5-FU via activation of the IL-6R/STAT3 signaling axis (Yin et al., 2017).

Tumor-associated macrophages can also regulate the delivery of chemotherapy to tumor cells. In the MMTV-PyMT transgenic breast cancer mouse model, doxorubicin administration causes necrosis of cancer cells with the release of CCL2, a chemokine that attracts monocytes/macrophages. MMP-9 secretion by the recruited myeloid cells was shown to decrease vasculature leakiness and to impair doxorubicin delivery into the tumors (Nakasone et al., 2012). In fact, MMP-9 null mice showed an improved response to Doxorubicin that correlated with increased vascular leakage (Nakasone et al., 2012) (**Figure 3C**). Conversely, in a Lewis lung carcinoma subcutaneous isograft model, myeloid derived VEGF promotes resistance to cyclophosphamide treatment by promoting the formation of abnormal vessels with reduced pericyte coverage, tortuosity, and vessel density (Stockmann et al., 2008).

Cancer-associated fibroblasts also play a role in tumor chemoresistance. In fact, a dense fibrotic stroma correlates with a poor response to neoadjuvant treatment with 5-fluorouracil, epirubicin and cyclophosphamide (FEC) in breast cancer and with gemcitabine in PDAC (Farmer et al., 2009; Olive et al., 2009;

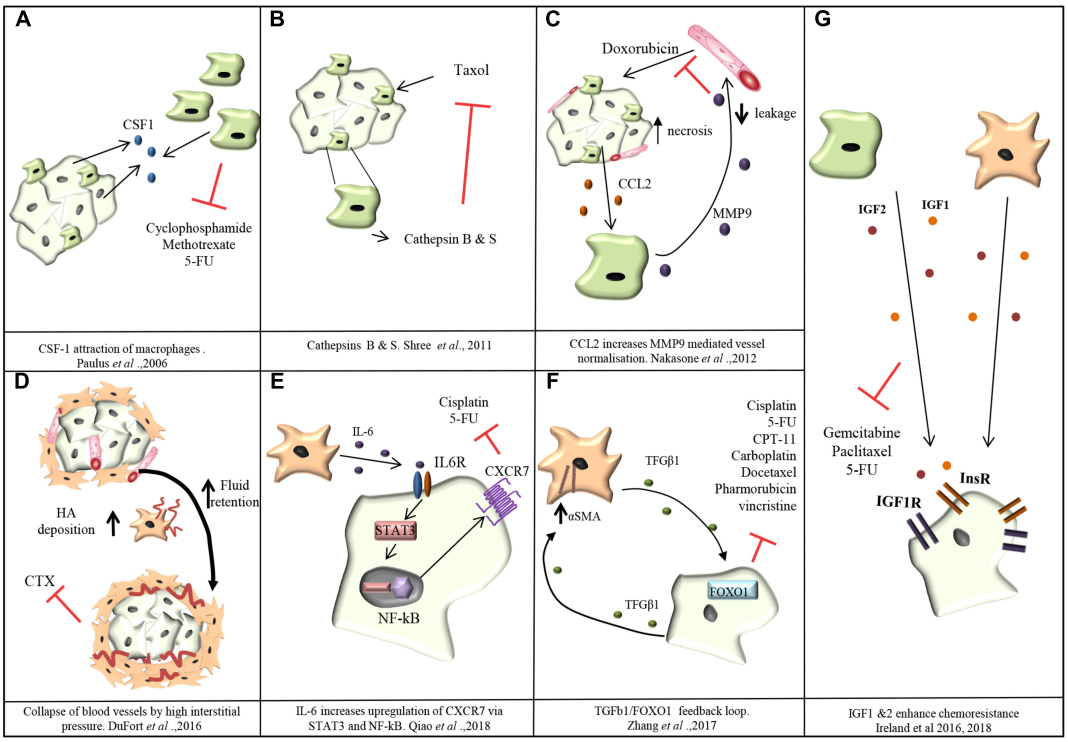

FIGURE 3 | Mechanisms of chemoresistance mediated by TAMs and CAFs. **(A)** Cancer cells attract TAMs via CSF-1. TAMs confer resistance of MCF-7 breast cancer cells toward cyclophosphamide, methotrexate and 5-fluorouracil (5-FU; Paulus et al., 2006). **(B)** Cathepsins B and S secreted by TAMS mediate resistance of breast cancer cells to taxol in MMTV-PyMT mouse model (Shree et al., 2011). **(C)** In the MMTV-PyMT transgenic mouse model, cancer cell necrosis caused by doxorubicin treatment causes cancer cells to release the monocyte chemoattractant CCL2. Recruited TAMs produce MMP-9 which causes leakiness of blood vessels and reduction in doxorubicin delivery (Nakasone et al., 2012). **(D)** In PDAC, CAFs increase deposition of hyaluronan (HA) creating an increase in fluid retention and subsequently interstitial pressure in the tumor rises causing the collapse of blood vessels and limiting the delivery of chemotherapeutic agents (DuFort et al., 2016). **(E)** CAF secreted IL-6 stimulates the upregulation of CXCR7 through STAT3/NF-kB signaling promoting resistance of esophageal squamous cell carcinoma cells against cisplatin and 5-FU (Qiao et al., 2018). **(F)** CAF-derived TGF-β upregulates FOXO1 expression in esophageal squamous cell carcinoma cells triggering reciprocal TGF-β secretion which in turn increases the levels of αSMA expression in CAFs and resistance to cisplatin, taxol, irinotecan (CPT-11), 5-FU, carboplatin, docetaxel, pharmorubicin, and vincristine (Zhang et al., 2017). **(G)** TAM and CAF derived IGF-1 and IGF-2 activate insulin and IGF-1 receptor signaling on tumor cells conferring resistance of pancreatic and breast tumors to gemcitabine and paclitaxel (Ireland et al., 2016, 2018).

Pandol et al., 2009). One way fibrosis promotes chemoresistance in PDAC is through CAF secretion of hyaluronan, generating high interstitial pressure within the tumor, causing the collapse of blood vessels supplying the tumor mass and impairing drug delivery (DuFort et al., 2016) (**Figure 3D**).

In esophageal squamous cell carcinoma, CXCR7 expression is upregulated in tumor cells through STAT3/NF-kB signaling stimulated by CAF-derived IL-6, ultimately promoting resistance against cisplatin and 5-fluorouracil (Qiao et al., 2018) (**Figure 3E**). IL-6 has pleiotropic effects in the TME and also mediates chemoresistance by promoting EMT of cancer cells (Shintani et al., 2016). TGF-β secretion by CAFs was shown to confer resistance of esophageal squamous cell carcinoma against cisplatin, taxol, irinotecan (CPT-11), 5-fluorouracil (5-FU), carboplatin, docetaxel, pharmorubicin, and vincristine (Zhang et al., 2017) (**Figure 3F**).

We recently showed that TAMs and CAFs are the main sources of Insulin-like growth factors 1 and 2 (IGF-1, IGF-2) in pancreatic and breast tumors, and that IGF signaling mediates resistance of murine pancreatic and breast tumors to gemcitabine and paclitaxel (**Figure 3G**) (Ireland et al., 2016, 2018). Importantly, we found that 72% of PDAC patients and 87% of patients with invasive breast cancer have the IGF signaling pathway activated in their tumors, and this correlates with an increased number of TAMs and CAFs (Ireland et al., 2016, 2018). Similarly, IGF1 was also shown to be secreted by TAMs in glioblastoma multiforme and to mediate resistance to a CSF-1R small molecule inhibitor through activation of PI3K signaling (Quail et al., 2016).

TARGETING TAMs AND CAFs IN CANCER

Currently, approaches are being undertaken to block macrophage recruitment to the tumor site, to repolarize TAMs back into an M1-like anti-tumorigenic phenotype, and to target specific tumorigenic functions of TAMs. Preventing recruitment of

macrophages to the tumor site has been achieved through targeting macrophage chemoattractants such as CSF-1 and CCL2 or their corresponding receptors: CSF-1 receptor (CSF-1R) and C-C chemokine receptor type 2 (CCR2). Anti-CSF-1R agents have been shown to be effective against recruitment of M2-like macrophages in breast cancer models, and anti-CSF1R inhibitors used in combination with paclitaxel decreased tumor growth and pulmonary metastasis (DeNardo et al., 2011). CSF-1R and CCR2 antagonists have been reported to prevent infiltration of TAMs into the tumor mass increasing response to gemcitabine treatment in mouse models of PDAC (Mitchem et al., 2013). CCL2 inhibition in combination with docetaxel has shown increased efficacy, compared to docetaxel treatment alone, resulting in decreased tumor growth and metastatic spread in prostate cancer (Loberg et al., 2007). This combination has also shown promise in lung cancer, breast cancer metastasis, and PDAC (Lu and Kang, 2009; Fridlender et al., 2011; Kalbasi et al., 2017). Due to these successes CSF-1, CCL2, and CSF-1R targeting agents are being investigated in clinical trials in combination with chemotherapy in a range of solid tumors (**Table 1**). However, the targeting of chemokines and cytokines has limitations due to their redundant and promiscuous nature. In fact, chemokines and cytokines can often bind to more than one receptor, and at the same time different cytokines/chemokines can bind to the same receptor and activate the same signaling pathway (O'Shea and Murray, 2008; Turner et al., 2014). In addition, to add more complexity, certain cytokine receptors are expressed by several cell types and as a result, inhibiting the cytokine/receptor affects all cell populations expressing the receptor. This is the case with CSF-1R which is not exclusively expressed by M2-like macrophages but is also expressed by M1-like macrophages, neutrophils, myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs; Cannarile et al., 2017).

Repolarizing macrophages back into an M1-like tumoricidal phenotype appears an attractive approach as the M2 TAMs are already present in the tumor and repolarization could therefore provide an effective strategy to restore the tumoricidal function of macrophages and prevent cancer progression. This has been investigated using an anti-CD40 antibody in combination with gemcitabine in a genetic KPC ($Kras^{LSL.G12D/+}$; $p53^{R172H/+}$; $Pdx^{Cretg/+}$) PDAC mouse model and in PDAC patients (Beatty et al., 2011). The administration of an agonist CD40 antibody repolarized TAMs back into an M1-like phenotype leading to an increased response to gemcitabine and reduced tumor burden (Beatty et al., 2011). A phase 1 clinical trial has recently been completed for the use of Dacetuzumab (human anti-CD40 mAb) + Bortezomib chemotherapy in patients with relapsed or refractory multiple myeloma, however, results have yet to be published (**Table 1**).

Since TAMs can act as a double edge sword in cancer, with M1-like TAMs exerting anti-tumorigenic functions and M2-like TAMs exerting pro-tumorigenic functions, targeting TAMs

TABLE 1 | Summary of combination treatments of chemotherapy and stromal targeting agents.

Molecular target	Treatment combination	Cancer type	Clinical trial	Outcome	Reference
CSF1R	Pexidartinib (PLX3397) (αCSF-IR) + eribulin	Metastatic breast cancer	Phase 1/2 NCT01596751	Ongoing	
	Pexidartinib (PLX3397 αCSF-IR) + paclitaxel	Solid tumors	Phase 1 NCT01525602	ORR: 4/23 (17%) CBR: 14/23 (61%)	Rugo et al., 2014
CSF1	MCS110 (αCSFI) + carboplatin plus gemcitabine	Triple negative breast cancer	Phase 2 NCT02435680	Ongoing	
CCL2	CNTO888 (αCCL2) + DOXIL®/Caelyx® doxorubicin HC1 liposome injection CNTO888 + gemcitabine CNTO888 + paclitaxel and carboplatin CNTO888 + docetaxel	Solid tumors	Phase 1 NCT01204996	Hematological complications in >93%	
	CNTO888 + docetaxel	Metastatic resistant prostate cancer	Phase 2 NTC00992186	34% maintained stable disease	
CD40	Dacetuzumab + bortezomib	Relapsed or refractory multiple myeloma	Phase 1 NCT00664898	Completed results not posted	
	Dacetuzumab + R-ICE (rituximab, etoposide, carboplatin, ifosfamide)	Diffuse large B cell lymphoma	Phase IIb NCT00529503	Terminated	
Smo	LDE225 (αSmo) + temozolomide	Medulloblastoma	Phase 3 NCT01708174	ORR: 18.8%	Kieran et al., 2013
IGF	BI 836845 + enzalutamide	Castration-resistant Prostatic neoplasms	Phase 1 NCT02204072	Ongoing	
	BI 836845 + everolimus + exemestane	$HR^+/HER2^-$ advanced breast cancer	Phase 1 NCT02123823	Ongoing	
	MEDI-573 + aromatase inhibitor	HER-2 negative metastatic breast cancer	Phase 2 NCT01446159	Ongoing	

pro-tumorigenic functions seems a more promising approach compared to ablation therapies targeting all TAMs. As previously mentioned, TAMs are known to facilitate the intravasation of tumor cells and promote angiogenesis (Wyckoff et al., 2007). Therefore, targeting TAMs role in pathological angiogenesis is an attractive therapeutic opportunity. In MMTV-PyMT mammary carcinomas and RIP1-Tag2 pancreatic insulinomas, an Ang-2 neutralizing antibody administration did not reduce the recruitment of Tie-2^+ TAMs but instead, prevented their binding to Ang-2 on activated endothelial cells subsequently decreasing angiogenesis and tumor progression (Mazzieri et al., 2011). CSF-1R inhibition increased the efficacy of anti-VEGFR-2 anti-angiogenic therapy in a mouse model of Lewis lung carcinoma (Priceman et al., 2010). M2 TAMs produce IL-10 at the tumor site leading to resistance of breast cancer to paclitaxel treatment (Yang et al., 2015) and this resistance can be abrogated with the administration of an IL-10 neutralizing antibody (Yang et al., 2015) (**Figure 4**).

It is currently unclear whether CAFs play a supportive or restrictive role in tumor progression. Based on the correlation between a large desmoplastic reaction and poor patient outcome it was hypothesized that ablation of the myofibroblasts would improve therapy response and decrease tumor growth. Shh is overexpressed by neoplastic PDAC cells (Thayer et al., 2003), stimulating Gli activity in surrounding fibroblasts and triggering their activation (Tian et al., 2009). Therefore, Shh became a target to inhibit fibroblast activation and Shh inhibition initially showed promising results in a pre-clinical PDAC mouse models. Shh inhibition reduced fibrosis and increased tumor vascularization, improving the delivery of gemcitabine to PDAC tumors (Olive et al., 2009). However, a clinical trial of Saridegib, a Shh inhibitor, with gemcitabine, in metastatic PDAC patients, failed at phase II as patients had reduced survival (Madden, 2012). Further investigation into fibroblast function in PDAC in longer-term experiments with mouse PDAC models showed that fibroblast ablation using smoothened inhibitor or genetic depletion of Shh or αSMA$^+$ myofibroblasts, in fact showed that the stroma restrained tumor growth and metastasis (Oezdemir et al., 2014; Rhim et al., 2014). These conflicting results, combined with the emerging evidence that different CAF populations co-exist in tumors, suggest that different CAF populations may have different and possibly opposing effects in cancer progression (Ohlund et al., 2017; Costa et al., 2018). Despite these results, a phase 3 trial in medulloblastoma, using an oral sonidegib (smoothened inhibitor) in combination with temozolomide showed promising results with an objective response rate of 18.8% (**Table 1**).

One approach which warrants further investigation is the reprogramming of the activated CAFs back into their quiescent state. This approach has seen some success in PDAC mouse models using Calcipotriol (vitamin D analog) which reverts myofibroblasts to quiescence, reducing the desmoplastic reaction which in turn improves gemcitabine delivery (Sherman et al., 2014). In 3D models and genetic mouse models of PDAC the

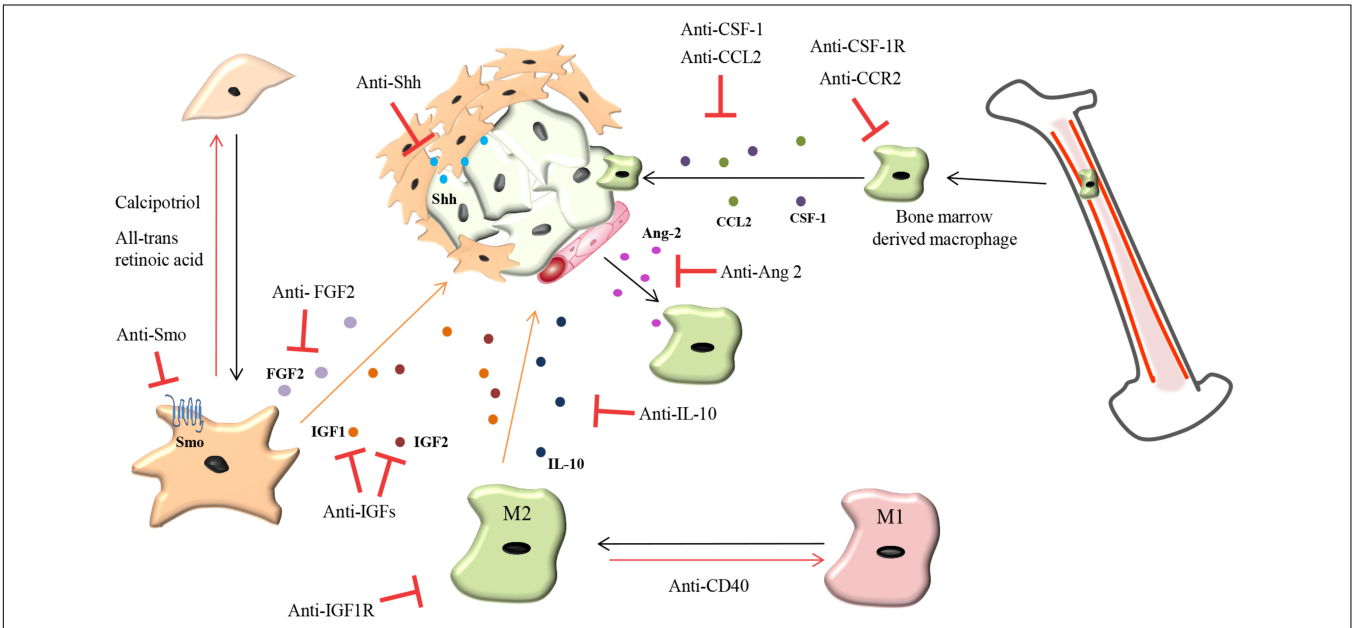

FIGURE 4 | Therapeutic strategies to overcome chemoresistance mediated by TAMs and CAFs. CAFs: Reprogramming activated CAFs back toward a quiescent phenotype by anti-Smoothened (Smo), anti-sonic hedgehog (Shh), all-*trans* retinoic acid and calcipotriol (vitamin D analog) while fibroblast growth factor 2 (FGF2) targeting agents prevents resistance of tumor cells to anti-estrogens in breast cancer. TAMs: Repolarizing M2 macrophages back to an M1-like phenotype can be mediated by a CD40 agonist. Prevention of macrophage recruitment to tumor sites is currently being achieved by targeting the colony-stimulating factor 1 (CSF-1) and C-C motif chemokine 2 (CCL2) signaling axis. Anti-angiopoietin-2 (Ang-2) antibodies prevent TAM interaction with blood vessels. IL-10 produced by TAMs promotes chemoresistance which can be abrogated by treatment with anti-IL-10 antibodies. TAMs and CAFs secrete insulin-like growth factor 1 and 2 (IGF1 and IGF2) which makes pancreatic and breast tumors chemoresistant and more metastatic. Treatment of tumors with anti-IGF blocking antibodies increases the response of pancreatic and breast tumors to chemotherapy and decreases tumor growth and metastasis.

use of all-*trans* retinoic acid to restore the quiescence of stellate cells increased vascularity, resulting in increased response to gemcitabine and reduced tumor growth (Carapuca et al., 2016). In estrogen receptor positive breast cancer, CAF-derived FGF-2 promotes resistance to anti-estrogens which is abrogated with administration of an FGF-2 neutralizing antibody (Shee et al., 2018) (**Figure 4**).

As previously mentioned TAMs and CAFs act as stromal sources of IGF 1 and 2 in PDAC, and invasive breast cancer (Ireland et al., 2016, 2018) and this makes tumors resistant to chemotherapy and more metastatic. Blockade of IGF1 receptor signaling in PDAC, using IGF-1R inhibitors, has failed in the clinic (Guha, 2013; King et al., 2014; Gradishar et al., 2016) but appears to be more effective in certain tumor types such as glioblastoma (Quail et al., 2016). In PDAC and invasive breast cancer mouse models, we have shown that both Insulin and IGF1 receptors are activated, and the use of IGF1/IGF2 ligand blocking antibodies, which inhibit IGF-1 and IGF-2 signaling through both IGF-1 and insulin receptors, increases response to chemotherapy and reduces tumor growth and metastasis (Ireland et al., 2016, 2018). These studies suggest that inhibition of signaling through both Insulin and IGF1 receptors by blocking IGF 1 and 2 ligands may be more effective compared to IGF1R inhibitors in certain cancer types which have both receptors activated, such as pancreatic and breast cancer (**Figure 4**). IGF1/IGF2 blocking antibodies are currently being tested in phase I and II clinical trials in patients with castration resistant prostate cancer and metastatic breast cancer patients in combination with chemotherapy (**Table 1**).

FUTURE PERSPECTIVES

Macrophages and fibroblasts are key regulators of tissue homeostasis, repair, angiogenesis and immunity. In tumors, cancer cells, macrophages and fibroblasts co-exist, co-evolve and continuously interact with each other. Tumor cells "hijack" macrophages and fibroblasts to support their own growth and expansion. Specifically, tumors exploit the natural plasticity of macrophages polarizing them into M2-like pro-tumorigenic TAMs that, support tumor growth in numerous ways, as described in this review. The same phenomenon is observed with respect to fibroblast function. Under normal physiological conditions fibroblasts facilitate wound repair by promoting cell growth, migration and ECM deposition. Tumor cells stimulate fibroblast activation and, reciprocally, activated fibroblasts support cancer cell survival, proliferation and resistance to therapies. However, recent findings have shown that different CAF populations with different and possibly even opposite functions co-exist in tumors.

Ablation therapies that eliminate macrophage recruitment to the tumor site have shown some promising results (DeNardo et al., 2011; Mitchem et al., 2013). However, this approach has some limitations, including the lack of specificity for different macrophage subsets and the redundancy of macrophage chemo-attractants. Inhibition of CAFs activation in PDAC patients actually resulted in enhanced tumor progression (Madden, 2012) and CAF ablation therapies in mouse tumor models resulted in increased tumor growth and metastasis (Oezdemir et al., 2014; Rhim et al., 2014). These findings suggest that further investigation into the role of different CAF subtypes is required to design therapies that specifically target defined CAF subtypes and/or functions that support cancer progression. Therapies that specifically target the pro-tumorigenic functions of TAMs and CAFs could lead to a more specific and effective anti-tumor response. To develop specific anti-stroma therapies that only target the pro-tumorigenic functions of TAMs and CAFs, while sparing their anti-tumorigenic functions, we first need to gain a better understanding of the complex composition and function of the tumor stroma.

While TAMs and CAFs are the most abundant stromal cell types in tumors, and as described in this review affect resistance to chemotherapy using a plethora of mechanisms, other stromal/immune cells present in the TME, including MDSCs, DCs, and T cells can also affect the response of tumors to therapies (for reviews/articles on this topic see Castells et al., 2012; Palucka et al., 2013; Son et al., 2017; Weber et al., 2018).

While some key stroma-derived signaling molecules have already been identified, the complex tumor-stroma interactions and the dynamic evolution of these interactions during tumor progression and in response to treatment need to be fully elucidated in order to develop effective anti-cancer therapies with a durable effect.

AUTHOR CONTRIBUTIONS

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OPINION
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Bad Tumors Made Worse: SPINK1

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INTRODUCTION

Serine **p**rotease **in**hibitor **K**azal type **1** (SPINK1) is a small secreted protein with dual roles—in the pancreas, it is a protective trypsin inhibitor, while in the context of the tumor microenvironment, it is a cell growth and survival factor that promotes tumor progression. While the mechanism by which SPINK1 protects the pancreas is long established and well-understood, the mechanisms that underlie its tumor promoting properties are complex and multifaceted, with major questions remaining to be answered. In this *Opinion* article, we briefly overview the known functions and mechanisms of SPINK1 both in health and in disease, and then seek to highlight several of the mechanistic "missing links," with the aim of identifying research opportunities and stimulating new lines of investigation.

SPINK1—PROTECTOR OF THE HEALTHY PANCREAS

SPINK1, also known as pancreatic secretory trypsin inhibitor (PSTI), is a 6.2 kDa secreted serine protease inhibitor that is produced by pancreatic acinar cells. In the pancreas, SPINK1 plays a physiological role as an inhibitor of digestive trypsins (**Figure 1A**) (Rinderknecht, 1986; Paju and Stenman, 2006). It is co-secreted in zymogen granules with trypsinogen, the trypsin precursor protein, allowing inhibitory intervention in case of early activation of trypsinogen to trypsin, and preventing organ damage of the pancreas or duct system due to autodigestion. The importance of SPINK1 for pancreatic health is demonstrated by the association of SPINK1 gene mutations (N34S, P55S, IVS3 + 2TC, and others) with increased risk for several forms of chronic pancreatitis (Pfützer et al., 2000; Witt et al., 2000; Raphael and Willingham, 2016). Most pathogenic SPINK1 mutations reduce function of the protein by interfering with folding and/or secretion (Kiraly et al., 2007a,b; Kereszturi et al., 2009), while the N34S mutation does not appear intrinsically deleterious, but is associated with another mutation in the 5′ regulatory region of the gene that can diminish mRNA expression (Kereszturi and Sahin-Toth, 2017). On the other hand, homozygous mutations causing complete loss of SPINK1 function were found to be responsible for several cases of severe early-onset exocrine pancreatic insufficiency (Venet et al., 2017).

SPINK1—CONTRIBUTOR TO POOR CANCER PROGNOSIS

Outside of the normal pancreas, aberrant expression of SPINK1 plays a role in cancer. SPINK1 was originally named tumor associated tissue inhibitor (TATI) when it was first isolated from the urine of ovarian cancer patients (Huhtala et al., 1982). Since then SPINK1 has been found to be overexpressed by multiple types of tumor cells, including breast, ovarian, prostate, pancreas, liver, and colon (reviewed Itkonen and Stenman, 2014; Rasanen et al., 2016). More recently, SPINK1 has also been found to be expressed by the tumor stroma after chemotherapy, where it may contribute to chemoresistance and increased risk of recurrence (Chen et al., 2018). SPINK1 tumor cell expression and possible prognostic value have been most studied in prostate cancer, where

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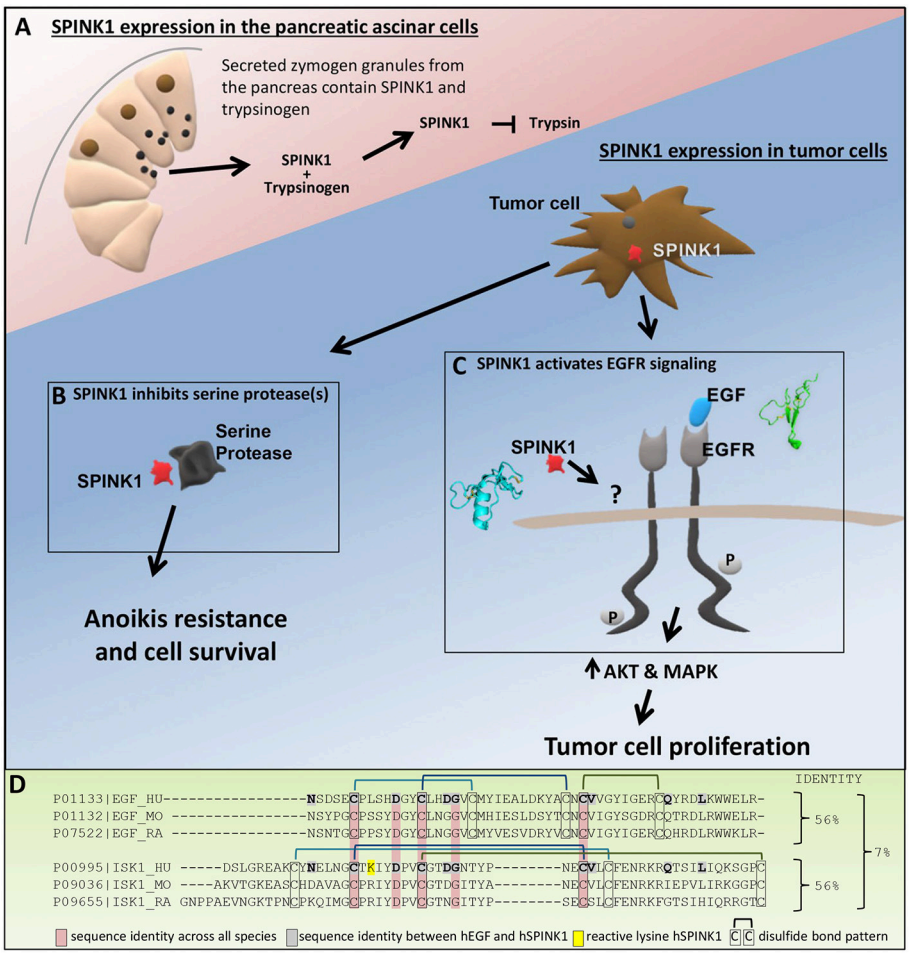

FIGURE 1 | Roles of SPINK1. **(A)** In the pancreas, SPINK1 acts as an important regulator of protease activity. SPINK1 is co-expressed with trypsinogen by the pancreatic acinar cells and secreted from zymogen granules into the pancreatic duct. Within the acinar cells or the duct, SPINK1 quenches prematurely activated trypsin to prevent further protease activation and organ damage. **(B)** Tumor cell secreted SPINK1 inhibits unknown serine protease(s) to induce anoikis resistance, tumor cell survival and metastatic disease. **(C)** Tumor cell secreted SPINK1 activates EGFR kinase pathways and leads to tumor cell proliferation; the direct receptor of SPINK1 in this context requires further definition. **(D)** Sequence alignment using Clustal Omega comparing human, mouse, and rat EGF with human, mouse, and rat SPINK1 (ISK1) homologs. Identified are sequence identity between hEGF and hSPINK1, sequence identities across all three species, and disulfide bond pattern.

SPINK1 positive tumors form a subgroup of about 10–15% of prostate cancers (Tomlins et al., 2008; Ateeq et al., 2011, 2015). Prostate tumors that express SPINK1 have been reported to show a significantly more aggressive phenotype and poorer progression-free survival (Tomlins et al., 2008; Leinonen et al., 2010). In other tumor types, multiple studies have explored the potential utility of SPINK1 expression as a biomarker through analysis of tumor tissues, urine, and serum (Halila et al., 1988; Inaudi et al., 1991; de Bruijn et al., 1993; Paju et al., 2007). Tumor tissue staining for SPINK1 has been associated with poorer survival in non-serous ovarian cancers (Mehner et al., 2015) and in estrogen receptor- positive breast cancer (Soon et al., 2011), and there is potential for SPINK1 to serve as a diagnostic marker for hepatocellular carcinoma (Marshall et al., 2013). Studies in experimental model systems have demonstrated significant effects of SPINK1 in promoting tumor cell growth and survival (Rasanen et al., 2016), the mechanisms of which remain to be fully elucidated. Unlike in the normal pancreas, in tumors SPINK1 appears to be expressed independently of trypsin, and little is known about the direct target(s) of SPINK1 in the context of cancer.

PATHOGENIC FUNCTIONS—RESISTANCE TO APOPTOTIC CELL DEATH

Normal epithelial cells require contact to other cells or the extracellular matrix to ensure their function and survival; if they detach, intracellular mechanisms drive the apoptosis protocol called anoikis resulting in cell death. Tumor cell metastasis often involves circulation as isolated cells, and thus anoikis resistance is believed to be a common feature of metastatic dissemination (Frisch and Francis, 1994; Simpson et al., 2008; Kim et al., 2012). We have shown that SPINK1 plays an essential role in

ovarian cancer cell survival under attachment free conditions (Mehner et al., 2015). Non-adherent cell survival was increased in a dose-dependent manner when treating ovarian cancer cell lines with recombinant SPINK1 protein. Notably, this effect could be mimicked by several alternative trypsin inhibitors, suggesting that anoikis resistance is mediated through the serine protease inhibitory activity of SPINK1 (Mehner et al., 2015).

SPINK1 has also been reported to confer apoptotic resistance on tumor cells in the context of chemotherapeutic treatment. Soon et al. found that SPINK1 knockdown activated apoptotic pathways in breast cancer cells, while SPINK1 overexpression induced resistance to apoptosis in cells treated with a variety of cytotoxic chemotherapy agents (Soon et al., 2011). Chemoresistance was not similarly induced by a mutant form of SPINK1 lacking the reactive site lysine residue that is required for trypsin inhibition, again implicating the serine protease inhibitory function of SPINK1 in its antiapoptotic function (Soon et al., 2011).

While evidence points to serine protease inhibition as a mechanism by which SPINK1 promotes resistance to both anoikis (Mehner et al., 2015) and chemically induced apoptosis (Soon et al., 2011) (**Figure 1B**), the specific serine protease target(s) of SPINK1 through which these effects are mediated are not known. The relevant apoptosis-promoting protease is unlikely to be trypsin-1 or-2, the natural physiological targets of SPINK1 in the pancreas (Rinderknecht, 1986), because although these enzymes are expressed by many tumors, they possess pro-tumorigenic activities and are predominantly associated with increased malignancy and poorer patient outcomes (Koivunen et al., 1990; Ohta et al., 1994; Yamamoto et al., 2003; Yamashita et al., 2003; Paju et al., 2004; Nyberg et al., 2006; Soreide et al., 2006). By contrast, the relevant target of SPINK1 antiapoptotic activity is expected to possess predominantly antitumor activity and to correlate with better prognosis. Besides trypsins, the human proteome includes around 80 other serine proteases with trypsin-like specificity, representing possible alternative targets for SPINK1 through which apoptosis may be regulated. Efforts to identify the SPINK1 target(s) and signaling pathways of interest could lead to identification of new biomarkers and novel points of intervention to reduce tumor cell survival and prevent spread of metastatic disease.

PATHOGENIC FUNCTIONS—INCREASED TUMOR CELL PROLIFERATION

A second important mechanism by which SPINK1 influences tumor progression is its ability to stimulate tumor cell proliferation (Rasanen et al., 2016). Here, evidence suggests that SPINK1 activates epidermal growth factor receptor (EGFR) signaling pathways (Ogawa et al., 1985; Ozaki et al., 2009; Ateeq et al., 2011; Wang et al., 2014; Mehner et al., 2015; Chen et al., 2018). In our own work we find phosphorylation of the intracellular domain of EGFR as well as phosphorylation of AKT and ERK upon treatment of ovarian cancer cells with SPINK1, consistent with activation of EGFR downstream pathways (Mehner et al., 2015). Furthermore, treatment of ovarian cancer cells with erlotinib, a selective inhibitor of the EGFR kinase domain, completely blocked the proliferative response of the cells to SPINK1, demonstrating that EGFR signaling is required for SPINK1-stimulated proliferation (Mehner et al., 2015). Others have seen similar downstream signaling of SPINK1 through EGFR in pancreatic (Ozaki et al., 2009; Wang et al., 2014), prostate (Ateeq et al., 2011), and colorectal cancers (Chen et al., 2015) (**Figure 1C**). SPINK1-treated pancreatic cancer cells showed increased phosphorylation of EGFR as well as activation of MAPK and STAT3; this response was attenuated in cells treated with the EGFR inhibitor AG1478 (Ozaki et al., 2009). Ateeq et al. showed in prostate cancer cells that SPINK1 knockdown reduced proliferation, which could be restored by recombinant SPINK1 protein; silencing of EGFR resulted in a significant reduction in the pro-proliferative effects of SPINK1 on the cells (Ateeq et al., 2011).

Despite the strong evidence that EGFR signaling is stimulated downstream of SPINK1, the details of how SPINK1 elicits this response remain in question. Early work by Hunt et al. (1974) identified possible sequence homology between SPINK1 and epidermal growth factor (EGF), the preferred ligand of EGFR. The possibility of functional overlap between these proteins was further suggested by studies showing that a rat SPINK1 homolog, monitor peptide, can stimulate growth of murine 3T3 fibroblasts (Fukuoka et al., 1986), and can compete with mouse EGF for binding to EGFR on the surface of these cells (Fukuoka et al., 1987). Ateeq et al. later hypothesized that human cancer cell-secreted SPINK1 may bind directly to EGFR as an alternative ligand to stimulate proliferation (Ateeq et al., 2011). Consistent with this possibility, exogenous SPINK1-GST was co-immunoprecipitated with EGFR from cell lysates (Ateeq et al., 2011), and immobilized SPINK1 showed evidence of binding to the EGFR ectodomain in a quartz-crystal microbalance assay (Ozaki et al., 2009). However, the original premise of homology between SPINK1 and EGF was based on very limited similarity between short partial sequences (Hunt et al., 1974; Scheving, 1983); only 10/56 amino acids of hSPINK1 are identical with hEGF, five of which are not conserved across species (**Figure 1D**). Furthermore, while SPINK1 and EGF each contain six cysteines comprising three disulfide bonds, comparison of their structures reveals entirely dissimilar protein folds (Bolognesi et al., 1982; Ogiso et al., 2002; Ferguson et al., 2003) (**Figure 1C**) and disulfide bonding patterns (**Figure 1D**). The few identical residues do not occur in similar structural contexts in the two protein families, nor do they present comparable potential binding epitopes. Thus, it is not clear why EGFR would be a natural binding target for SPINK1, and the mode of their potential interaction remains a mystery. Until stronger evidence emerges to validate and structurally characterize this binding interaction, the possible involvement of other accessory proteins or alternative SPINK1 receptors with crosstalk to EGFR should be considered (**Figure 1C**). For example, an earlier study by Niinobu et al. (1990) showed binding of SPINK1 to a cell surface receptor of 140 kDa, considerably smaller than EGFR, in a manner that was not diminished by competing EGF. We suggest that efforts to more clearly confirm or identify the direct receptor of SPINK1, and the mechanism by

which it influences EGFR signaling, could lead to identification of novel points for therapeutic intervention in cancers that express SPINK1.

CONCLUSION—A CALL FOR NEW MECHANISTIC STUDIES

SPINK1 is an important contributor to both increased proliferation and metastasis development in a variety of cancers. Patients with tumors expressing SPINK1 face a poorer overall prognosis and stimulation of SPINK1 expression in the treatment-damaged tumor microenvironment may further contribute to chemoresistance and tumor recurrence. While patient studies have provided strong evidence for the importance of SPINK1 across different tumor types, the regulatory pathways that control SPINK1 expression and the direct targets of SPINK1 in the context of the tumor microenvironment, including both protease target(s) and cell surface receptor(s), remain largely unknown. The identification of specific protease targets of SPINK1 inhibition will reveal pathways controlling anoikis resistance and aid in development of biomarkers and therapeutic strategies to reduce tumor metastasis. To better understand and target SPINK1 driven tumor cell proliferation we need to further investigate the missing link between SPINK1 and EGFR signaling using modern methods and technologies. Concerted efforts are needed to uncover SPINK1 targets, signaling mechanisms and mediators, and such efforts may lead to the development of novel therapeutic strategies to reduce the impact of SPINK1 on tumors and improve patient prognosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication .

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Meeting the Challenge of Targeting Cancer Stem Cells

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Notwithstanding cancer patients benefit from a plethora of therapeutic alternatives, drug resistance remains a critical hurdle. Indeed, the high mortality rate is associated with metastatic disease, which is mostly incurable due to the refractoriness of metastatic cells to current treatments. Increasing data demonstrate that tumors contain a small subpopulation of cancer stem cells (CSCs) able to establish primary tumor and metastasis. CSCs are endowed with multiple treatment resistance capabilities comprising a highly efficient DNA damage repair machinery, the activation of survival pathways, enhanced cellular plasticity, immune evasion and the adaptation to a hostile microenvironment. Due to the presence of distinct cell populations within a tumor, cancer research has to face the major challenge of targeting the intra-tumoral as well as inter-tumoral heterogeneity. Thus, targeting molecular drivers operating in CSCs, in combination with standard treatments, may improve cancer patients' outcomes, yielding long-lasting responses. Here, we report a comprehensive overview on the most significant therapeutic advances that have changed the known paradigms of cancer treatment with a particular emphasis on newly developed compounds that selectively affect the CSC population. Specifically, we are focusing on innovative therapeutic approaches including differentiation therapy, anti-angiogenic compounds, immunotherapy and inhibition of epigenetic enzymes and microenvironmental cues.

Keywords: cancer stem cells, metastasis, anti-cancer therapies, immunotherapy, epigenetic inhibitors

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CANCER STEM CELLS AS A MAIN DETERMINANT OF THERAPY REFRACTORINESS

Cancer stem cells (CSCs) are defined as being a subpopulation of cells within the heterogeneous tumor mass. This subset of cells is endowed with the ability to self-renew and differentiate into non-CSCs, indicating their capability of reproducing the tumor of origin when transplanted into immunocompromised mice. CSCs are also considered responsible for the metastatic spreading and chemoresistance. Strong evidence suggests that conventional treatments, including radio- and chemotherapy, spare the CSC subset, which is responsible for minimal residual disease (MRD) and cancer relapse (Valent et al., 2012). Indeed, CSCs are characterized by more pronounced levels

of drug transporters, enhanced DNA-damage repair mechanisms and the ability to escape the cytotoxic chemotherapy by maintaining a quiescent state. New emerging therapeutic approaches using immunotherapy, anti-angiogenic compounds and/or epigenetic probes aim to overcome the CSC resistance to treatments. CSCs have been thoroughly investigated in the past decades, starting in 1971 when they were observed by Perce and Wallance, who described aggressive undifferentiated cells that are able to generate squamous cell carcinoma *in vivo* (Lobo et al., 2007). CSCs were first identified in Myeloid Leukemia in 1997 and since then they have been proposed to be the tumor initiating cells responsible for disease recurrence and metastasis formation. Bonnet and Dick identified a subpopulation of tumor initiating cells with marked stem-like properties in acute myeloid leukemia (AML). Later, several groups also identified CSCs in solid tumors, including breast, brain, thyroid, melanoma, colon, pancreatic, liver, prostate, lung, head and neck, ovarian, and stomach cancers (Lapidot et al., 1994; Bonnet and Dick, 1997; Al-Hajj et al., 2003; Hemmati et al., 2003; Singh et al., 2004; Collins et al., 2005; Ma et al., 2007; Fukuda et al., 2009; Boiko et al., 2010; Todaro et al., 2010). Based on these studies, a large number of biomarkers can be adopted to identify CSCs (**Table 1**).

Interfering With the Intrinsic Mechanisms of Therapy Resistance in CSCs

Cancer stem cells own a superior capability to survive current therapeutic regimens, meaning that chemo- and radiotherapy are not sufficient to successfully eradicate cancer and are inadequate, especially when the diagnosis occurs at a later stage (Valent et al., 2012; Ajani et al., 2015). Recent evidence showed that the CSC subpopulation is enriched after chemotherapy, suggesting that this subset is responsible for the majority of treatment failure (Visvader and Lindeman, 2008; Alison et al., 2011). Chemoresistance is favored by several mechanisms, among which cellular plasticity. Indeed, Liu et al. (2014) and Luo and Wicha (2019) demonstrated that breast CSCs can switch from proliferating epithelial characteristics to a mesenchymal state which contributes to metastatic dissemination and resistance to therapies.

Nevertheless, the resistance of CSCs to therapy is usually not limited to one drug and this phenomenon referred to as multidrug resistance (MDR) (Efferth et al., 2008). MDR is the result of the endogenous expression of detoxifying enzymes, increased drug efflux pump levels, enhanced DNA repair activity, reduced drug response and activated survival pathways (Singh and Settleman, 2010). These features, combined with the capability of CSCs to evade the immune system, to activate an epithelial to mesenchymal transition (EMT) program and to adapt their metabolism under scarce nutrient conditions, render CSCs almost an imperishable cancer population (**Figure 1**).

The aldehyde dehydrogenase (ALDH) 1 belongs to the ALDH superfamily, which is composed by 19 enzymes (Hsu et al., 1999). ALDH1 is the main isoform that by oxidizing aldehydes to carboxylic acids and retinol to retinoic acid, allows the detoxification from drugs and reactive oxygen species (ROS) (Singh et al., 2013). ALDH is known to be expressed by normal stem cells, for this reason its activity may be an intrinsic characteristic of CSCs as well. As a result, high levels of ALDH1 activity were found in CSCs, thus representing a reliable marker for the identification of this subset (Charafe-Jauffret et al., 2010). ALDH1 positive cells showed an increased potential of forming xenograft tumors in AML and breast cancer (Cheung et al., 2007; Ginestier et al., 2007). Thereafter, $ALDH1^+$ cells from stomach, lung, liver, head and neck, pancreas, cervix, thyroid, prostate, colon, bladder, and ovary tumors were successfully transplanted into mice (Ma et al., 2008a). The implication of the ALDH superfamily in detoxification suggests that these enzymes may have a key role in CSCs' chemoresistance. Indeed, it has been demonstrated that ALDH expression confers resistance to several chemotherapeutic agents, such as cyclophosphamide, cisplatin, paclitaxel, docetaxel, doxorubicin, and gemcitabine in leukemia, medulloblastoma, adenocarcinoma, colon and breast cancer (Hilton, 1984; Friedman et al., 1992; Tanei et al., 2009; Duong et al., 2012). Moreover, the inhibition of ALDH activity with disulfiram, sorafenib, and sulforaphane can sensitize CSCs to therapy, providing further confirmation of ALDH role in chemoresistance (Rausch et al., 2010).

A large number of studies have demonstrated that the reduction of chemotherapy efficiency is related to an increased drug efflux from cancer cells. This is caused by the aberrant expression of a family of proteins known as ATP-binding cassette (ABC) transporters, which belong to a family of 49 molecules, usually implicated in membrane trafficking using ATP as a source of energy. Among these proteins, ABCB1 (also known as MDR1 or P-gp), ABCG2 (also known as BCRP1), ABCB5 and ABCC1 were largely studied and characterized (Leonard et al., 2003; Lobo et al., 2007). Starting from these premises, it was hypothesized that CSCs may over-express ABC transporters as compared to non-CSCs. Indeed, several groups independently demonstrated that CSCs share features with the Hoechst dye excluding side

TABLE 1 | Expression of CSCs markers according to tumor types.

Tumor type	Cancer stem cell markers
Breast cancer	$CD133^+$, $CD44^+$, $CD24^+$, $EpCAM^+$, $ALDH^{high}$
Colon cancer	$CD133^+$, $CD44^+$, $CD24^+$, $CD166^+$, $EpCAM^+$, $ALDH^{high}$, ESA^+
Gastric cancer	$CD133^+$, $CD44^+$, $CD24^+$
Glioblastoma	$CD133^+$
Head and neck cancer	$SSEA\text{-}1^+$, $CD44^+$, $CD133^+$
Leukemia (AML)	$CD34^+$, $CD38^-$, $CD123^+$
Liver cancer	$CD133^+$, $CD44^+$, $CD49f^+$, $CD90^+$, $ALDH^{high}$, $ABCG2^+$, $CD24^+$, ESA^+
Lung cancer	$CD133^+$, $CD44^+$, $ABCG2^+$, $ALDH^{high}$, $CD87^+$, $CD90^+$
Melanoma	$ABCB5^+$, $CD20^+$
Ovarian cancer	$CD133^+$, $CD44^+$
Pancreatic cancer	$CD133^+$, $CD44^+$, $CD24^+$, $ABCG2^+$, $ALDH^{high}$, $EpCAM^+$, ESA^+
Prostate cancer	$CD133^+$, $CD44^+$, $\alpha2\beta1^+$, $ABCG2^+$, $ALDH^{high}$

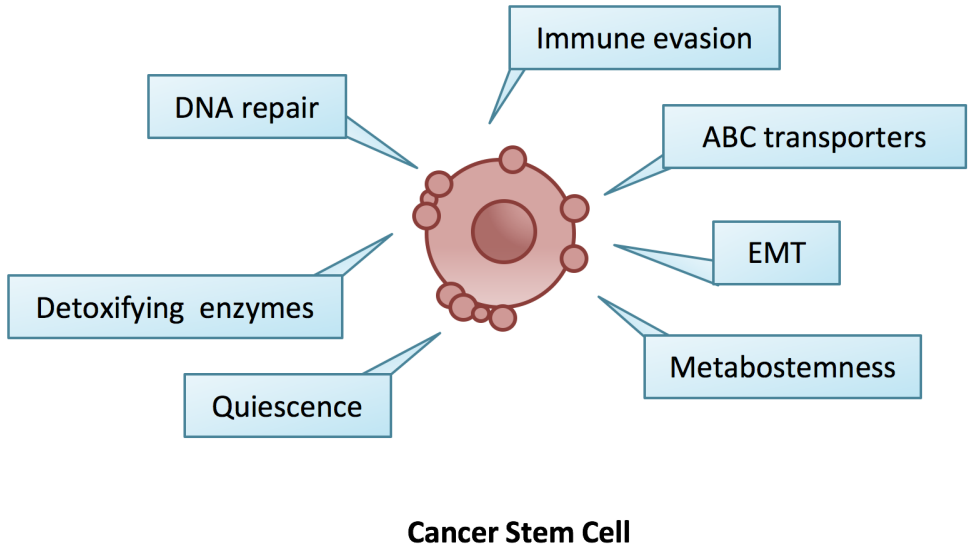

FIGURE 1 | The hallmarks of cancer stem cells. CSCs are endowed with a number of innate ad adaptive responses such as quiescence, EMT, increased DNA repair and detoxifying enzymes, metabostemness, immune evasion and over-expression of ABC transporters, which gave them the ability to survive changes in the microenvironment and anti-cancer therapies.

population (SP), which highly expresses efflux pumps able to induce resistance to harmful toxins and chemotherapeutic compounds (Hirschmann-Jax et al., 2004; Ho et al., 2007). ABC transporters are involved in the resistance to a wide array of drugs. In particular, it was demonstrated that ABCB1 is over-expressed in breast CSCs, causing their resistance to doxorubicin and paclitaxel, and in multiple myeloma stem cells, refractory to carfilzomib (Wright et al., 2008; Hawley et al., 2013). On the other hand, ABCG2 is responsible for the resistance of hepatocellular CSCs to 5-fluorouracile, mephedrone, and cisplatin, whereas ABCB5 was found on circulating melanoma cells resistant to doxorubicin (Frank et al., 2005; Shi et al., 2008). The inhibition of these transporters represents a useful tool to overcome CSCs' chemoresistance. This was demonstrated by Frank et al., who targeted ABCB5 by way of a specific blocking monoclonal antibody to restore melanoma cells' sensitivity to doxorubicin, and by Lancet group who demonstrated the sensitizing effect of zosuquidar, a P-gp inhibitor (Frank et al., 2005; Lancet et al., 2009).

The B-cell lymphoma-2 (BCL-2) family plays a pivotal role in regulating cell fate. The pro-survival proteins belonging to this family are BCL-2 itself, B-cell lymphoma extra large (BCL-xL), BCL-2-like-2 (BCL-W), BCL-2-related protein A1A (BCL-A1A), and myeloid cell leukemia sequence-1 (MCL1), whereas the pro-apoptotic molecules include BCL2-associated-X-protein (BAX) and BCL-2 homologous antagonist killer (BAK) (Kelly and Strasser, 2011). Among these molecules, BCL-2 was found over-expressed in breast CSCs, while both BCL-2 and BCL-xL were found up-regulated in leukemia CSCs (Konopleva et al., 2002; Madjd et al., 2009). The role of the BCL-2 family has been further elucidated by Strasser et al. (1990) who demonstrated that BCL-2 over-expression promotes tumorigenesis. Consequently, the inhibition of BCL-2 downstream pathways caused an increased sensitization to chemotherapy in colon and hepatocellular CSCs (Todaro et al., 2007; Ma et al., 2008b).

In vitro evidence suggests that CSCs are slow-cycling if compared to non-CSCs (Viale et al., 2009). Interestingly, quiescence makes CSCs less sensitive to cell-cycle directed therapies such as vinca alkaloids, which prevents the polarization of microtubules and taxanes, known to stabilize existing microtubules (Gascoigne and Taylor, 2009).

Chemotherapeutic agents and radiotherapy are used in clinical setting to induce DNA damage. Of note, CSCs do not respond to therapy due to increased activity of DNA repair machinery (Bao et al., 2006; Eyler et al., 2008; McCord et al., 2009; Ropolo et al., 2009). In fact, in glioma and breast CSCs, a higher phosphorylation of DNA repair proteins was observed, in particular in ATM, CHK1, and CHK2 (Eyler and Rich, 2008; Gallmeier et al., 2011; Maugeri-Sacca et al., 2011). Moreover, ovarian and lung CSCs are enriched after cisplatin treatment, a further indication that chemotherapy is limited to kill the proliferating fraction of the tumor bulk (Levina et al., 2008; Rizzo et al., 2011).

Furthermore, it has been demonstrated that chemotherapy induced damage stimulates glioblastoma multiforme and bladder CSCs to divide and thus to repopulate tumor bulk (Chen et al., 2012; Kurtova et al., 2015). On the other hand, this induced proliferation may be exploited to increase the efficacy of therapeutic regimens (Saito et al., 2010). Interestingly, the induction of CSC differentiation by using the bone morphogenic protein 4 (BMP4) renders these cells more susceptible to standard and targeted anti-cancer therapies (Lombardo et al., 2011). Furthermore, the all-*trans* retinoic acid is among the most common drugs used to cause differentiation of stem cells particularly in acute promyelocytic leukemia (Nowak et al., 2009). Inhibitors of epigenetic modulators such as DNA

methyltransferase 1 (DNMT1), histone deacetylases (HDACs) and bromodomain and extra-terminal (BET) inhibitors have shown capabilities to function as differentiation therapies for CSCs in various tumor types (Toh et al., 2017).

Additionally, one cancer hallmark is the activation of angiogenesis, which concurs with the nurture of the tumor mass by stimulating *de novo* vessels formation (Hanahan and Weinberg, 2011).

Targeting the 'Metabostemness'

Compelling evidence suggests that stem-like features can be acquired as a result of metabolic shifts, which are able to render normal stem cells or differentiated cancer cells more susceptible to epigenetic reprogramming. These cells are thus more likely to move up the cancer cell hierarchy by their expression of pluripotent genes. The metabolic insults, able to induce this reprogramming into CSCs in the context of a pre-malignant tumor, are collectively termed 'metabostemness' (Menendez and Alarcon, 2014). Consistently, some of the intermediates deriving from mutated metabolic enzymes, involved in glycolysis, tricarboxylic acid cycle, oxidative phosphorylation (OXPHOS) and mitochondrial fatty acid oxidation, act as oncometabolites for DNA and histones epigenetic modifications by driving tumorigenesis (Menendez and Alarcon, 2014). For this reason, targeting metabolic processes may represent a successful strategy. In particular, in most cases OXPHOS is the preferential source of energy rather than glycolysis, probably because of the low levels of glucose in tumors. Moreover, increased OXPHOS is a hallmark of resistance to chemotherapy (Lee et al., 2017). Therefore, it is not surprising that the targeting of OXPHOS *via* the BCL-2 inhibitor venetoclax in combination with the hypomethylating agent azacitidine was able to impair leukemia stem cells (LSCs) proliferation and metabolic activity (Jones et al., 2018; Pollyea et al., 2018). Accordingly, the OXPHOS inhibitor salinomycin was able to kill breast CSCs (Gupta et al., 2009). Interestingly, it has been shown that, during relapse, LSCs are able to rescue OXPHOS levels after amino acid depletion thanks to increased mitochondrial fatty acid oxidation (FAO) (Jones et al., 2018). FAO can also be promoted by the crosstalk with adipose tissue, which fuels LSCs metabolism by acting as a niche and promoting LSCs chemoresistance (Ye et al., 2016). In addition, the targeting of lipolysis, and in particular of COPI-Arf1 complex, was shown to be a promising tool for the eradication of CSCs in adult Drosophila (Singh et al., 2016). The crucial role of mitochondria in CSCs impelled several groups to develop therapeutic strategies aimed at their targeting (Skoda et al., 2018). Notably, mitochondrial biogenesis can be abrogated through the estrogen-related receptor α inhibitor XCT790 (Deblois and Giguere, 2011; Deblois et al., 2013), whereas their fission can be impaired thanks to the dynamin-related protein 1 (DRP1) inhibitors Mdivi-1 and P110 (Xie et al., 2015). In addition, it has been demonstrated that DRP1 activation may be promoted by the interaction between cyclooxygenase-2 (COX-2) and mitochondria. For this reason COX-2 inhibitors, resveratrol and celecoxib, were repositioned as mitochondrial fission inhibitors (Guo et al., 2015; Cilibrasi et al., 2017). Inhibitors of mitochondrial respiration were used to target pancreatic CSC subset (Sancho et al., 2016). Likewise, the inhibition of the mitochondrial complex I through the repositioning of the antidiabetic drug Metformin was recently proposed with encouraging results (Wheaton et al., 2014).

CANCER STEM CELLS, TUMOR MICROENVIRONMENT, ANGIOGENESIS AND METASTASIS: HOW TO DISRUPT THIS INTRICATE NETWORK?

Angiogenesis is a multistep physiological process, characterized by the formation of new vessels from preexisting ones, which governs many biological activities, such as development and tissue repair. In order to maintain tissue homeostasis, angiogenesis is tightly regulated by a balance between pro- and anti-angiogenic factors (Hanahan and Folkman, 1996). In pathological conditions, such as cancer, this balance is destroyed favoring the secretion of pro-angiogenic factors. The term "tumor angiogenesis" was used for the first time by Folkman (1971) to point out the sprouting of cancer-associated neo-vessels from existing vessels that are in close proximity. Proliferating cancer cells require oxygen and high amount of nutrients, leading to the formation of hypoxic areas in the innermost part of the tumor. Under hypoxic condition, CSCs increase hypoxia-inducible factor-1 (HIF-1) expression and activate the HIF-1 pathway, enhancing the secretion of many angiogenic growth factors (Pugh and Ratcliffe, 2003; Gilbertson and Rich, 2007). In particular, high levels of vascular endothelial growth factor-A (VEGF-A) recruit VEGF receptors (VEGFRs)-expressing endothelial cells (ECs), named tip cells. After VEGF-A binding, tip cells up-regulate cell proliferation, cytoskeleton remodeling and migration pathways (MAPK, PI3K/AKT, RhoA), sprout toward tumor cells and activate the adjacent ECs (stalk cells) to form new tumor vessels (Ricciuti et al., 2017). In addition to ECs, CSCs' secreted cytokines prime the microenvironment (tumor microenvironment, TME) and recruit myeloid cells to fuel cancer progression. In particular, cancer-associated fibroblasts (CAFs) and activated tumor-associated macrophages (TAMs) secrete high levels of metalloproteases (MMPs), growth factors and interleukins to sustain angiogenesis and to promote CSC invasion (Bhowmick et al., 2004; Crawford and Ferrara, 2009; Owen and Mohamadzadeh, 2013). Furthermore, it has been reported that *de novo* vessel formation may be boosted by CSCs from different tumor types; this process is termed vascular mimicry (Weis and Cheresh, 2011). It has been described that breast and glioblastoma CSCs could give rise to both ECs and pericytes supporting tumor growth and progression (Bussolati et al., 2009; Ricci-Vitiani et al., 2010; Wang et al., 2010; Cheng et al., 2013). Unlike normal vasculature, tumor vessels are tortuous and more permeable due to the lower presence of pericytes (Jain, 2005; Sawada et al., 2012). This "leakiness" reduces the capacity of chemotherapeutic agents to target cancer cells and facilitate the intravasation of metastatic cancer cells. These circulating tumor cells (CTCs) possess a CSC-like phenotype, characterized by a

high expression of EMT-related genes (Burgess, 2013; Grillet et al., 2017). Although many cancer cells are able to intravasate, only few cells survive in the bloodstream and extravasate, activating a mesenchymal-epithelial transition program (Tam and Weinberg, 2013). The persistence of extravasated cells requires the presence of a favorable host microenvironment (metastatic niche) and the escape from immune cell surveillance. For these reasons, tumor cells remain in a dormant state, which can last many years, and, after the release of molecules and growth factors by the metastatic niche, they restart to proliferate and disseminate (Gao et al., 2012; Giancotti, 2013) (**Figure 2**).

Targeting Tumor Angiogenesis and Metastasis

The possibility of specifically blocking tumor angiogenesis and the metastatic process could have a clinical impact on cancer patients' outcome. Bevacizumab is a humanized monoclonal antibody that binds to VEGF, impairing VEGF/VEGFR interaction, approved in 2004 by the Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer (CRC) in combination with standard therapy (Ferrara et al., 2004; Hurwitz et al., 2004). Then, it was approved for the treatment of other metastatic cancers, among which non-squamous NSCLC and cervical cancer (Tewari et al., 2014; Lin et al., 2016). In 2008, bevacizumab was approved for the treatment of metastatic Her2 negative breast cancer in combination with paclitaxel. However, other studies did not show a significant overall survival (OS) and the FDA withdrew the approval in 2011 for breast cancer treatment (Miller et al., 2007; Aalders et al., 2017). Conversely, the European Medicines Agency maintains bevacizumab approval in combination with chemotherapy. Another strategy to inhibit tumor angiogenesis is the use of tyrosine kinase inhibitors, such as sorafenib and sunitinib. Sorafenib is an inhibitor of VEGFR-1,-2,-3 and PDGFR-β, approved for the treatment of metastatic renal cell carcinoma and unresectable hepatocellular carcinoma (Wilhelm et al., 2006; Escudier et al., 2007), whereas sunitinib blocks VEGFR-2 and PDGFR phosphorylation and is used for gastrointestinal tumor and metastatic renal cell carcinoma (Sun et al., 2003; Motzer et al., 2007). Although anti-angiogenic therapy may potentially have clinical implication, the increase of OS is insufficient. This is probably due to (i) acquired resistance (Lu et al., 2012); (ii) the increment of tumor hypoxia (Erler et al., 2009) and (iii) the diminished delivery of chemotherapeutic agents (Jain, 2005).

Metalloproteases are crucial mediators of tumor angiogenesis and cell migration (Kessenbrock et al., 2010). Although many MMP inhibitors have been developed and many clinical trials have been conducted, none of these have increased patients' OS (Coussens et al., 2002; Winer et al., 2018). On the contrary, MMP inhibitors have numerous side effects, due to the MMP's role in numerous physiological processes. In order to obtain clinical benefits, inhibitors should be highly selective for MMPs that drive tumor progression.

The dysregulation of stem cell-specific signaling pathways, such as Notch, Wnt and Hedgehog, could reduce metastatic progression. In glioma patients, the use of a gamma secretase inhibitor (RO4929097) reduced CSC number; unfortunately the prolonged use of this inhibitor led to the acquisition of angiogenesis-mediated resistance (Pan et al., 2016; Xu et al., 2016). Vismodegib, an inhibitor of a component of the Hedgehog pathway, Smoothened, was used in combination with gemcitabine in pancreatic cancer, without affecting CSC number (Catenacci et al., 2015). In order to increase the efficacy of these inhibitors that target CSCs and block metastasis development, further studies must be carried out especially to reduce the side effects.

Cytokines, chemokines and growth factors secreted by TME cells enhance the migration capacity of cancer cells and promote angiogenesis (Wakefield and Hill, 2013; Scala, 2015; Gaggianesi et al., 2017). Therefore the inhibition of their receptors could have clinical benefits. In fact, reparixin, an inhibitor of IL-8 receptor CXCR1, reduced the breast CSC population and lung metastases (Ginestier et al., 2010) and is used in combination with paclitaxel in an ongoing clinical trial in triple negative breast cancer patients (Marcucci et al., 2016).

HARNESSING THE IMMUNE SYSTEM TO FLUSH OUT AND ERADICATE CANCER STEM CELLS

The new frontier of cancer treatment is aimed at strengthening the immune system's defenses against cancer cells. In the last decade the remarkable progress made on immunotherapy heralded an impressive novelty in the management of patients affected by a variety of cancers. The results obtained by immune-based therapies in terms of durable objective response rate exceeded expectations and it is no wonder that the scientists James Allison and Tasuku Honjo were recently awarded the 2018 Nobel prize in medicine for their pioneering discoveries in immunotherapy. Their studies were different, although based on the same principle: to fight cancer by harnessing the immune system.

Several compounds based on the inhibition of immune checkpoints have been approved by the FDA since 2011. Ever since, the most promising of these therapies have been antibodies targeting the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or the programmed cell death 1 (PD-1) pathway, administered as single therapy or in combination.

The role of CTLA-4 as a negative regulator of T cell activation was discovered in the laboratory of James Allison and Jeffrey Bluestone. To induce antitumor responses, T cells are initially activated in the lymph node in two subsequent steps (i) engagement of T cell receptor (TCR) with a tumor antigen MHC complex on antigen presenting cells (APCs) and (ii) binding of CD28 to the costimulatory molecule B7. Following T cell activation, CTLA-4 translocates from the intracellular compartment to the cells' surface to compete with the costimulatory molecules, causing the inhibition of T cell proliferation. The blockade of this essential immune checkpoint with monoclonal antibodies enables T cells to active, expand and

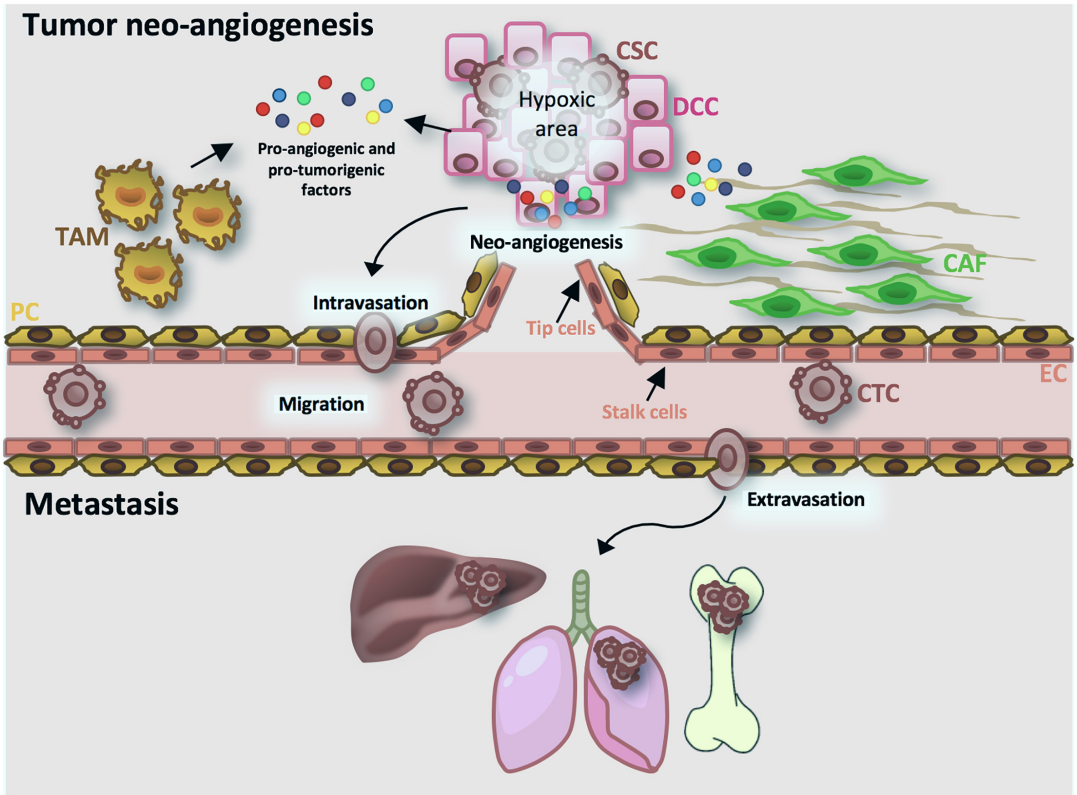

FIGURE 2 | Tumor angiogenesis and metastatic process. Cancer cells secrete pro-angiogenic and pro-tumorigenic factors (MMPs, VEGF-A, HIF-1A, cytokines, chemokines, growth factors). VEGF-A activates endothelial cells (ECs) in tip cells, that direct the sprouting vessels, and stalk cells, implicated in vessel stability. Moreover, cancer cells-released cytokines activate cancer-associated fibroblasts (CAFs) and activated tumor-associated macrophages (TAMs), that in turn favor the intravasation of cancer stem cells (CSCs). Circulating cancer cells (CTCs) through the bloodstream reach target organ, extravasate and start to proliferate and disseminate. CSC, cancer stem cell; CTC, tumor circulating cells; DCC, differentiated cancer cell; CAF, cancer associated fibroblast; TAM, tumor associated macrophage; EC, endothelial cell; PC, pericyte cell.

reach the tumor burden, where they can find the cognate antigen presented by cancer cells (Ribas and Wolchok, 2018).

Otherwise, Tasuku Honjo demonstrated that TCR engagement at the tumor site causes the expression of the PD-1 receptor that binds the PD-1 ligand (PD-L1) on cancer cells, causing the exhaustion of T cells and hampering the antitumor cytotoxic T cell responses (Okazaki et al., 2013).

These two mechanisms are generally implemented to impede the overstimulation of the immune system but in the context of cancer, they become detrimental for cancer cell elimination. Nevertheless, an immune checkpoint blockade could be exploited to potentiate the antitumor immune response.

Ipilimumab was the first CTLA-4 inhibitor that entered the clinic and was approved by the FDA in 2011. A substantial portion of advanced melanoma patients treated with ipilimumab had a durable response that was unluckily accompanied by toxicity, such as colitis and the inflammation of endocrine glands. Nivolumab and pembrolizumab were the first anti-PD-1 compounds approved by the FDA for melanoma (2014) and NSCLC (2015) followed by the approval of anti-PD-L1 antibodies, atezolizumab, avelumab, and durvalumab. Interestingly, the anti-PD-1 pathway inhibitors were approved for the first time based on their genetic background as for example, the presence of unstable microsatellite rather than the cancer type. Objective response rate was high varying from 15% for head and neck, gastroesophageal, bladder and urinary tract cancers and reaching almost 90% for Hodgkin's disease. Of interest, an ongoing phase 2 clinical trial is assessing the optimal adaptive dosage of an ipilimumab and nivolumab combination in metastatic melanoma patients (NCT03122522). Moreover, other promising results have been achieved by preclinical studies that show a synergistic effect of anti-HER2 antibodies and immune checkpoint inhibitors in breast cancer (Su et al., 2018).

Albeit immune checkpoint inhibitors are considered the spearhead of immunotherapy against cancers that show an high mutation burden, the expected accumulation of neoantigen and the high PD-1/PD-L1 expression (Bailey et al., 2018) may not produce a greater antitumor response (Gide et al., 2018).

According to the new iRECIST criteria of tumor response following the administration of immunotherapy (Seymour et al., 2017), patients undergoing treatment resistance may experience pseudoprogression or hyperprogression, which consists in the initial increase of tumor volume followed by its decrease or in a faster progression of the disease as compared to the

predicted rate, respectively (Champiat et al., 2017; Siefker-Radtke and Curti, 2018). The mechanisms of resistance to immune checkpoint inhibitors may be caused by the persistence of a subpopulation of CSCs. Indeed, the activation of transcriptomic profiles characterized by genes involved in EMT, angiogenesis and stemness causes the lack of T cell recognition and immunotherapy refractoriness (Spranger et al., 2015; Hugo et al., 2016). Indeed, CSCs can evade the immune system (Lee et al., 2016; Hsu et al., 2018) mainly due to the high expression levels of PD-L1 (Wu et al., 2017), the down-regulation of molecules involved in the presentation of the antigen to T cells (Bruttel and Wischhusen, 2014) and their capacity to promote the formation of an immune suppressive microenvironment (Jachetti et al., 2015; Sorrentino et al., 2018; Szarynska et al., 2018). On the other hand, the high levels of PD-L1 expressed by the CSCs render them potentially susceptible to treatments with checkpoint inhibitors, which can be combined with other immune-based therapies for an effective response (**Figure 3**).

For instance, in a syngeneic melanoma mouse model, the combination of immune checkpoint inhibitors (PD-L1 inhibitors and CTLA-4 inhibitors) with CSC lysate-pulsed dendritic cells (DCs) vaccine augmented T cell antitumor response and led to tumor regression (Zheng et al., 2018).

The chimeric antigen receptor (CAR) T cell transfer is currently being investigated (Gargett et al., 2016) and holds great promise in the treatment of liquid and solid malignancies (Grupp et al., 2013; Mount et al., 2018). CAR T cells are constituted by an antigen receptor linked by a single chain fragment to an intracellular domain, usually supplemented with a co-stimulatory molecule. CAR T cells offer an exceptional substrate for the development of selective CSCs therapies, being potentially able to recognize any antigen exposed on the surface of CSCs (Guo et al., 2018). A case report has recently been described by Feng et al. (2017), which shows the efficacy of the subsequent infusion of CAR T anti-EGFR and anti-CD133, a well known marker that identifies CSCs, in a patient affected by cholangiocarcinoma. The CAR T-EGFR and CAR T-CD133 are currently under clinical evaluation (NCT01869166 and NCT02541370). Moreover, CAR T cells, which target the CSC marker EpCAM, reduced prostate cancer progression in preclinical models (Deng et al., 2015). Thus, the CAR T cell-based therapies offer the opportunity to specifically eliminate the CSC subpopulation and are a valid alternative to checkpoint inhibitors, in a subset of cancer with paucity of neoantigens expression. Additionally, CAR T cells transfer could strengthen the efficacy of CTLA-4/PD-1 pathway inhibitors and targeted therapies.

Hence, contrary to the targeted therapies, which are almost mutation-related and could induce the reactivation of alternative survival pathways, immunotherapy offers the opportunity to achieve long-lasting responses in a broad range of tumor types, by overcoming the highly adaptive behavior of CSCs.

EPIGENETIC REPROGRAMMING AND CANCER STEM CELLS

Dynamic epigenetic reprogramming of the CSC subpopulation adds a further layer of inter- and intra-tumor heterogeneity to the complexity of tumors, which represents a hurdle for successful therapies. Epigenetics is the study of heritable changes and phenotypes not encoded in DNA (Dawson, 2017). The epigenetic enzymes responsible for histone modifications (writers, erasers, and readers) and DNA methylation (DNMT) have been extensively described (Arrowsmith et al., 2012). The histone methylation and acetylation are catalyzed by histone methyltransferases (HMTs) and histone acetyltransferases (HATs), while the histone demethylation and deacetylation are catalyzed by the histone demethylases (HDMs) and HDACs,

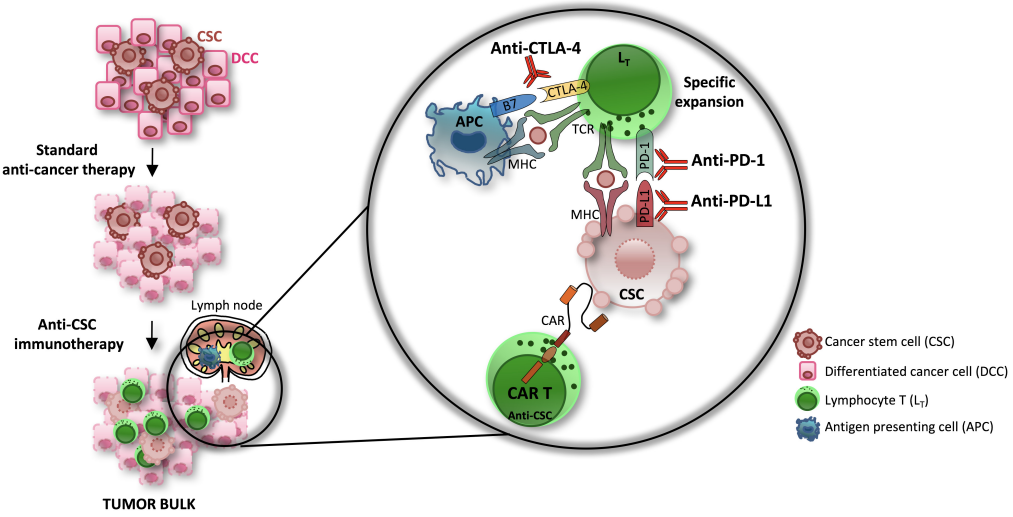

FIGURE 3 | The efficacy of immune-based therapies in the eradication of CSCs. The standard anti-cancer therapies are able to affect differentiated cancer cells (DCCs) while sparing CSCs. Novel immunotherapy approaches have shown promising therapeutic efficacy in several type of cancers. Combinations of checkpoint inhibitors (CTLA-4 and PD-1/PD-L1 pathway inhibitors) and CAR T cell transfer, in particular, efficiently eliminate the CSCs subpopulation.

respectively. Acetylated histones tend to be less compact and more accessible to RNA polymerase and transcriptional machinery, thereby enabling the transcription of nearby genes. Methylated histones can be either repressive or activating, depending on the site and degree of methylation. In particular, histone H3/H4 acetylation (H3Ac, H4Ac) and H3 lysine 4 methylation (H3K4me) are generally associated with active transcription, while histone H3 lysine 9 and 27 methylation (H3K9me, H3K27me) are commonly linked to gene repression (Bird, 1986; Jones and Takai, 2001; Venters and Pugh, 2007; McCabe et al., 2009). The well-known "histone code" hypothesis is based on the knowledge that different patterns of histone modifications on each histone determine the ultimate transcriptional event, either gene expression or silencing (Strahl and Allis, 2000). Several interrelated molecular mechanisms contribute to epigenetic gene regulation, such as chromatin remodeling via ATP-dependent processes and exchange of histone variants, regulation by non-coding RNAs, methylation and related modifications of cytosines on DNA, as well as covalent modification of histones. Local chromatin state at gene promoter is governed by DNMT and posttranslational histone modifications, thus playing an essential role in transcription regulation. DNMT1, DNMT3A, and DNMT3B are responsible for the methylation of the CpG islands, CpG-dense regions that are included in the majority of human gene promoters. While the unmethylated status of CpG islands is aimed to maintain promoter chromatin in a transcriptionally permissive state, their methylation is linked to gene silencing (e.g., X-chromosome inactivation, tumor suppressor gene silencing in cancers). The chromatin remodeling complexes, including the SWI/SNF complex, are at least five families that use ATP-hydrolysis to modify chromatin structure and remodel nucleosomes. Polycomb repressive complexes (PRC1 and PRC2) are epigenetic repressors of transcriptional programs fundamental for the cell's identity, development, differentiation and lineage specification, by catalyzing the trimethylation of histone 3 lysine 27 (H3K27me3) (Di Croce and Helin, 2013). Recently, it has been demonstrated that EZH2, the functional enzymatic component of the PRC2, is required for stable self-renewal and differentiation not only in mouse but also in human embryonic stem cells (Collinson et al., 2016).

Epigenetic alterations, including DNMT and histone modifications, are a key manifestation of the stem cells' differentiation into various tissue subtypes. The increasing number of recently discovered mutations in epigenetic regulators has shed new light on the importance of epigenetic dysregulation in tumor initiation and in the biology of CSCs. These may originate from a deregulated epigenetic reprogramming, which leads to the loss of differentiation genes and to the reestablishment of stem cell-specific characteristics. Epigenetic mechanisms play an important role in endowing stem cell characteristics to cancer cells. This is well established in many types of cancer, as: (1) CSC markers are directly regulated by epigenetic modifications (i.e., CD133 and DCLK1) (Yi et al., 2008; Vedeld et al., 2014); (2) CSCs exhibit mutations in chromatin remodeler components (loss of function mutations of PRC2 complex and deregulation of EZH2) (van Vlerken et al., 2013); (3) EMT, which confers cells with tumor-initiating capabilities and CSC properties (Mani et al., 2008), is finely controlled by epigenetic mechanisms (Kanwar et al., 2010; Beck et al., 2015; Avgustinova and Benitah, 2016).

This link between epigenetics and CSCs suggests that epigenetic alterations may be key therapeutic targets in this abnormal subpopulation. Furthermore, the development of specific epigenetic enzymes inhibitors has been a promising area of drug discovery, due in part to the "druggability" of these critical regulators. Therefore, an extensive investigation of the epigenetic enzymatic activities that are critical for the reprogramming of CSCs toward differentiation may be crucial for the tailoring and designing of new therapeutic strategies against a variety of deadly tumors. Hence, epigenetics enzymes are fundamental in regulating survival pathways, EMT, metastatic phenotype and chemoresistance in CSCs (**Figure 4**).

CSC Formation and Maintenance

Of note, many epigenetic mechanisms that promote the acquisition of uncontrolled self-renewal and CSC formation are based on driver mutations that have been found in principal epigenetic regulators, in both chromatin-related driver genes and DNA-methylation-related genes (Wainwright and Scaffidi, 2017).

LSCs bear the fusion protein product of the *KMT2/MLL* gene. This gene encodes for a HMT involved in many biological processes. Importantly, the MLL fusion proteins have been associated with an oncogenic role due to their ability to initiate the tumorigenic process in both AML and acute lymphoblastic leukemia (ALL) cells (Cozzio et al., 2003; Krivtsov et al., 2006; Somervaille et al., 2009).

In about 33% of pediatric glioblastoma patients, gain-of-function mutations have been identified in the gene encoding for histone H3. The most represented alteration is a K27M substitution, which leads to the impaired functions of the PRC2 complex and a lack of gene repression, which in turn leads to the aberrant activation of oncogenic programs and self-renewal ability (Lewis et al., 2013). DNMTs are mutated in 25% of AML patients. These mutations hamper the enzymatic activity and leads to the propagation of pre-LSCs.

Importantly, the dynamic cooperation between the genetic and epigenetic alterations in cancer initiation and promotion has been supported by recent evidence, especially in CRC model.

DNA methyltransferases have been shown to play a key role in the initiation and progression of CRC. Many tumor suppressor gene promoters are hypermethylated in CRC (*MLH1, RB, P16, RARB, SFRP*). The expression and the activity of the DNMTs seem to be controlled by *APC* mutation, a driver event in CRC (Hammoud et al., 2013), confirming once again that genetic and epigenetic interactions may cooperate to induce tumor initiation and progression. Specifically, it has been shown that the expression levels of DNMT1 are higher in CRCs compared to normal controls, suggesting that the elevated levels of this DNA methyltransferase may determine a dysregulation in the methylome by suppressing the transcription of the tumor suppressor genes. Moreover, this supports the hypothesis that deregulation of DNMT in CR-CSCs could be a crucial event during cancer progression.

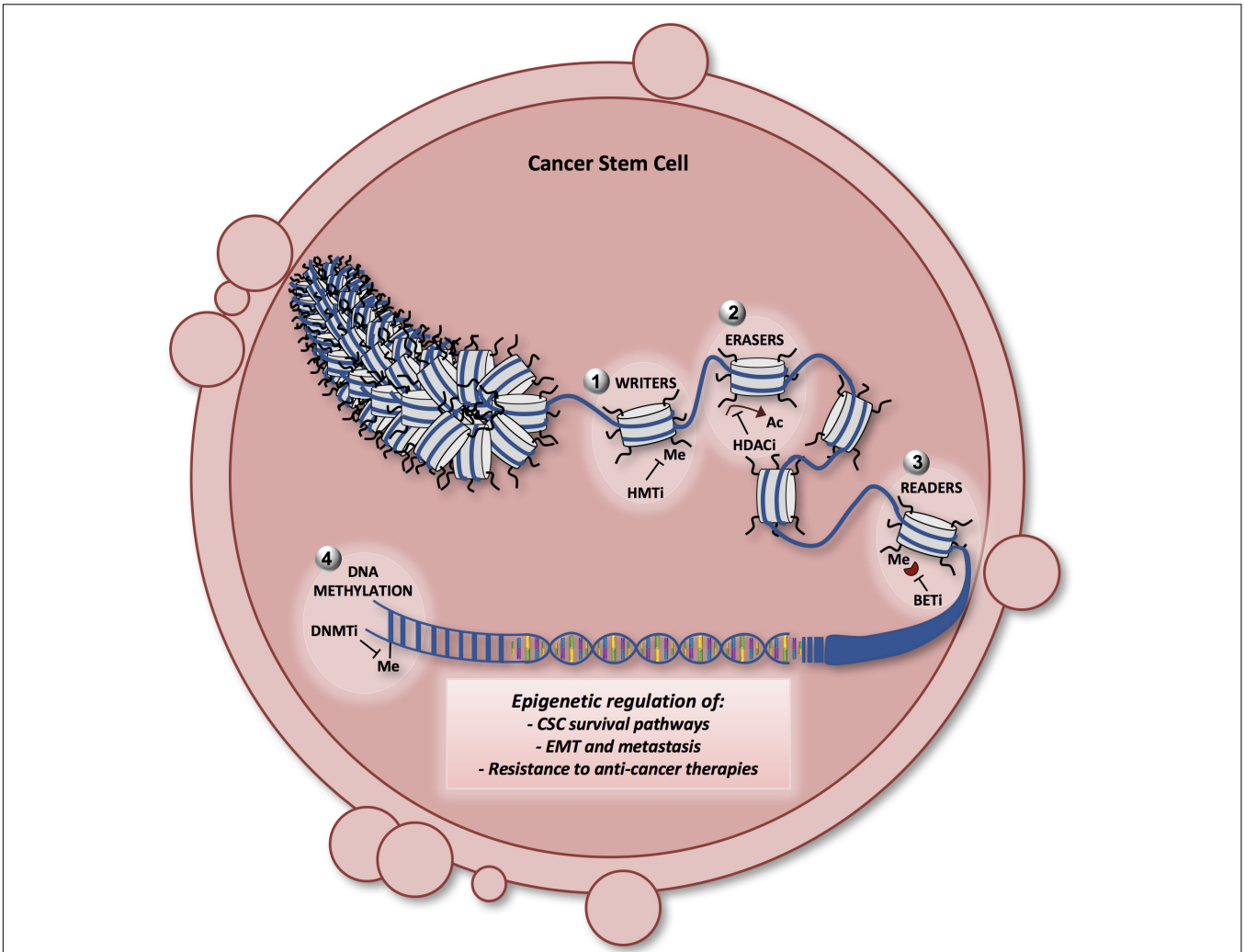

FIGURE 4 | Model showing the different layers of epigenetic regulation in CSCs and the potential therapeutic approaches. The chromatin fiber and the nucleosome are represented in the nucleus of a cancer stem cell. Epigenetic enzymes [writers (1), erasers (2), readers (3), and DNA methyltransferases (4)] are the principal actors in regulating the key survival pathways in CSCs, such as the Notch, Wnt and Hedgehog signaling. Moreover, epigenetic alterations guide the epithelial-mesenchymal transition (EMT) and the aberrant process of metastatization in CSCs, contributing to CSC resistance to therapy. Many of the latest generation compounds (epigenetic probes) have been designed to target the epigenetic enzymes involved in the CSC survival, maintenance, EMT and metastasis. DNMTi (DNA methyltransferase inhibitors): decitabine, azacitidine; HMTi (histone methyltransferase inhibitors) such as EZH2, DOT1L, and SETD8 inhibitors; HDACi (histone deacetylase inhibitors): vorinostat, romidepsin; BETi (bromodomain inhibitors): JQ1, I-BET762. Me, methylation; Ac, acetylation.

Crucial pathways involved in CSC maintenance, such as Wnt/β-catenin, Notch and Hedgehog signaling pathways are finely regulated by epigenetic mechanisms. These pathways in physiological conditions control self-renewal and development in embryonic and adult stem cells. DNMT, aberrant histone modification and also non-coding RNA have been identified as epigenetic aberrations in the main regulators of these pathways in CSCs. For instance, aberrant DNMT silences Wnt inhibitory factor genes with a tumor suppressor role, such as *WIF-1*, *AXIN-2*, *SFRP-1*, and *DKK1* (Suzuki et al., 2004). The promoter of *DKK1* is also silenced by decreased acetylation of H3K16 and increased H3K27 trimethylation (Hussain et al., 2009). In multiple myeloma cells, an enhanced histone acetylation has been found at the promoter region of *JAGGED2*, a Notch receptor ligand, leading to the activation of Notch signaling by overexpression of its ligand (Ghoshal et al., 2009). The histone methylation of H3K27 is inhibited on the promoters of two Notch signaling target genes, *HES1* and *HES5*. This is accomplished by the serine-threonine kinase receptor-associated protein (STRAP), which interacts with PRC2 complex components, thus leading to gene activation in CRC. SNF5, a member of a chromatin remodeler complex SWI/SNF, binds directly Gli1, which is the down-stream effector of the Hedgehog signaling pathway, leading to a repression of the target genes transcription (42). Indeed, in human malignant rhabdoid tumors inactivation of SNF5 results in an aberrant activation of Hedgehog signaling. Moreover, HDAC1 is required to transcriptionally activate Gli1 and Gli2. However, this inhibitory mechanism is hampered by

the frequent somatic mutations in *REN* gene, which encodes for the E3-ubiquitin ligase complex that mediates the degradation of HDAC1 (Di Marcotullio et al., 2004; Canettieri et al., 2010). Aberrant DNA hypomethylation of the Sonic Hedgehog ligand promoter is responsible for the pathway activation.

Therefore, the integration of genetic and epigenetic mechanisms disrupts the balance between self-renewal and pro-differentiation stimuli thus generating an aberrant program that sustains CSC survival.

EMT, Metastasis, and Resistance to Therapies in CSCs

The concept of CSCs in the maintenance and progression of many types of cancer is now well accepted and continues to evolve (Todaro et al., 2007, 2014; Kemper et al., 2010). This cell status is dynamic during cancer progression as it is mainly affected by genetic and epigenetic changes and influenced by the TME. Another characteristic of CSCs is their ability to invade and metastasize by acquiring the EMT phenotype that can be determined by examining the expression of E-cadherin (CDH1) and vimentin, which represent the effectors for Wnt and Notch signaling. It has been reported that Wnt/β-catenin signaling plays a critical role in regulating growth and maintenance of CR-CSCs (Kanwar et al., 2010). In particular, our group identified CD44v6 as a marker of metastatic potential that defines the CR-CSC subpopulation (Todaro et al., 2014). In CR-CSCs the up-regulation of Wnt signaling is correlated with a higher CD44v6 expression, suggesting that this population may retain metastatic traits and chemoresistance.

Many different epigenetic mechanisms have been linked to the activation of an uncontrolled EMT process. The loss of E-cadherin can be defined as a hallmark of EMT given the lack of the cell–cell adhesion. Of note, DNMT of the *CDH1* promoter by recruiting HDACs to the promoter site results in histone deacetylation and transcriptional silencing. Furthermore, EZH2 and the PRC2 complex mediate the histone methylation of the *CDH1* promoter, repressing its expression (Cao et al., 2008).

MiR-200 family members have been associated with a role in repressing EMT and invasion through a direct binding to ZEB1 and ZEB2 (zing finger E-box-binding homeobox 1 and 2), which are two transcription factors. Epigenetic silencing of these miRNAs by DNMT and H3K27 tri-methylation induces the acquisition of both an EMT-like and CSC phenotype (Tellez et al., 2011).

One of the most common mechanisms of drug resistance, subjected to epigenetic regulation in CSCs, is mediated by a pronounced expression of the drug efflux transporters, such as the ATP-binding cassette family (ABCG2, MDR1, MRP1). Decreased HDAC1 levels and increased histone acetylation and phosphorylation are responsible of an enhanced expression of ABCG2 (To et al., 2008).

Alternative Epigenetic Mechanisms of CSCs Regulation

On one hand, it is well known that the addition or removal of epigenetic marks on the histones of the nucleosomes play a crucial role in regulating the gene expression of oncogenic drivers or oncosuppressors (Louis and Shohet, 2015). On the other hand, oncogenes and tumor suppressors can themselves be activators of epigenetic mechanisms fundamental in CSCs by the induction of a "non-canonical" epigenetic program. Indeed, recent data have demonstrated that MYC favors a stem cell-like phenotype in mammary epithelial cells and induces an alternative epigenetic program, supported by the activation of *de novo* enhancers and repression of lineage-specifying transcription factors, which causes loss of cell identity and the activation of oncogenic pathways (Poli et al., 2018). Moreover, HMTs can methylate non-histone proteins such as the pivotal tumor suppressor gene *TP53*. It has been demonstrated that the tumor suppressor function of WT p53 is inhibited by repressive epigenetic pathways. p53 and "stemness" may be considered as conceptual antagonists. p53 suppresses self-renewal and promotes differentiation of adult stem cells. Inactivation of p53, by deletion, mutation, or expression of dominant-negative isoforms of p53 family members, enriches stem cell populations including CSCs (Molchadsky and Rotter, 2017). Some HMTs (SETD8 and SMYD2) have been found to regulate the methylation of non-histone proteins in particular p53 in lysine residues. These modifications such as the monomethylation on lysine 370 and lysine 382 of p53 (p53K370me1 and p53K382me1) have been associated with a pro-tumorigenic function (Zhu et al., 2016; Veschi et al., 2017). Further studies are needed to better elucidate these mechanisms and their targeting as a therapeutic approach in CSCs.

Treatments That Target Epigenetic Modifications in CSCs

The dynamic nature of epigenetics indicates that it may be possible to alter cancer-associated epigenetic states through direct manipulation of the molecular factors involved in this process. Currently, the major challenge in epigenetic drug discovery is to identify selective compounds with significant *in vitro* cellular activity at nM concentrations and well tolerated *in vivo*. Recently, mostly by using high throughput screening approaches, many studies identified and characterized new epigenetic regulators and their roles in various cancers. These findings represent the translational basis for the initiation of clinical trials in the area of specific epigenetic target classes. HDACs and DNMTs were the first epigenetic targets to be approved for cancer application by the FDA, but more recently additional families of epigenetic regulators have been the subject of intense studies, such as, methyltransferases (EZH2, SETD8, DOT1L, PRMT5), demethylase (LSD1, KDM4B), and BET proteins. Some potent inhibitors are now being studied in a clinical setting, more specifically in hematological and solid tumors. Early results are encouraging, despite relevant toxicity.

Histone deacetylases are key regulators of histone acetylation levels and are mostly associated with enhanced gene transcription. HDACs remove acetyl groups on histones' lysine residues and maintain cell balance by opposing the function of the HATs. Despite promising anti-cancer data from clinical trials, HDAC inhibitors need to be considered as pan-inhibitors

with associated side effects, although increasing efforts have been made to develop selective HDAC inhibitors. Vorinostat (SAHA) represents the first FDA approved pan-HDAC inhibitor that targets HDAC1-3 and 6. Currently, there are 6 clinical trials using Vorinostat targeting refractory or recurrent pediatric cancers and adult tumors. Romidepsin is an FDA approved selective HDAC1/2 inhibitor that is well tolerated in clinical trials for advanced pediatric and adult tumors (Children's Oncology et al., 2006; Amiri-Kordestani et al., 2013). DNA demethylating or hypomethylating agents, such as DNMTs inhibitors (DNMTi, azacitidine and decitabine), are currently in clinical phases I and II for a variety of tumors, including CRC.

The chromatin readers (BET family) recruit additional chromatin modifiers and remodeling enzymes, which serve as the effectors of the modification. For instance, acetylated histones serve as docking sites for bromodomain containing proteins (Dhalluin et al., 1999; Dey et al., 2003). Thus, the histone code imparts a tertiary level of genomic control beyond the DNA sequence and corresponding transcription factors (He et al., 2013). BET inhibitors have been demonstrated to successfully target CSCs in MLL-driven ALL and in other cancers. Among the first selective and more efficacious BET inhibitors, JQ1 is able to target c-MYC in many different cancers and I-BET762 is in Phase I-II clinical trial for NUT midline carcinoma, neuroblastoma and other tumors[1] (Filippakopoulos et al., 2010; Filippakopoulos and Knapp, 2014). However, nowadays many deleterious effects on healthy cells and resistance mechanisms to the BET inhibitors have been elucidated.

One of the major areas of interest regarding drug discovery is the great potential of combination therapies, especially in the case of resistance to existing standard therapy and or refractory states. Combination strategies, including pan-HDAC inhibitors in association with other agents and/or small molecules (chemotherapy, anti-GD2 antibody, retinoic acid, DNMTi, JQ1), are under evaluation in many pediatric and adult cancers. Specifically, the addition of JQ1 or EZH2 inhibitors to panobinostat (HDAC inhibitor) showed synergistic effects *in vitro* and *in vivo* (Shahbazi et al., 2016; Chen et al., 2018).

A detailed overview of the synergistic therapies with BET inhibitors and other epigenetic drugs or targeted agents can be found in Ramadoss and Mahadevan (2018). Aside from the above mentioned combination of HDAC and BET inhibitors, synergistic effects have also been demonstrated in combinatorial treatments using HDACs and DNMTs inhibitors, or DOT1L and DNMTs inhibitors in MLL-arranged leukemia cells (Klaus et al., 2014).

CONCLUDING REMARKS

In the past few years, an improved survival rates in cancer patients has been witnessed, due to early diagnosis and the advent of new targeted therapies. However, there are still millions of patients who die every year. Tumor recurrence and relapse may be driven by a variety of molecular events that are modulated according to different treatment pressure. It is now clear that within the tumor bulk there is a subpopulation of cancer cells, named CSCs, which are mainly responsible for the anti-cancer drug refractoriness. Thus, the novel frontiers of cancer treatment are aimed at defeating CSCs by using newly discovered drug delivery methods. For instance, one appealing approach is represented by the use of nanotechnology as an efficient tool for detection and elimination of CSCs (Qin et al., 2017). Nanomaterials including gold particles, origami and tetrahedron DNA nanostructures, liposomes, graphene and nanodiamond have been loaded with chemotherapeutics compounds or agents effective against CSCs, such as Salinomycin and Hedgehog pathway inhibitors (Xu et al., 2012; Yao et al., 2014). The enzymatic functionalization of nanomaterials with ligands of cell surface markers of CSCs, such as CD44 and CD133, is crucial to confer specificity in CSCs binding and targeting (Yao et al., 2014; Ni et al., 2015; Al Faraj et al., 2016). The potential clinical application of these carriers rely also on their high solubility, fast internalization and photothermal features.

The urgent need of successful cancer cures is due to the mechanisms of CSC resistance, which are disparate and act at different levels, including activation of survival pathways, metabolic adaptation, epigenetic modifications and immune escape. All these aspects have been thoroughly investigated in this review with the aim of offering an overview and food for thought on the novel developed therapeutic strategies to improve cancer patients management.

AUTHOR CONTRIBUTIONS

AT, VV, MG, AC, and PB drafted the manuscript. MT and GS critically reviewed this manuscript.

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[1] http://www.cancer.gov/clinicaltrials NCT01587703

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Heterogeneity of the Head and Neck Squamous Cell Carcinoma Immune Landscape and Its Impact on Immunotherapy

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Head and neck squamous cell carcinomas (HNSCCs) are highly aggressive, multi-factorial tumors in the upper aerodigestive tract affecting more than half a million patients worldwide each year. Alcohol, tobacco, and human papillomavirus (HPV) infection are well known causative factors for HNSCCs. Current treatment options for HNSCCs are surgery, radiotherapy, chemotherapy, or combinatorial remedies. Over the past decade, despite the marked improvement in clinical outcome of many tumor types, the overall 5-year survival rate of HNSCCs remained ∼40–50% largely due to poor availability of effective therapeutic options for HNSCC patients with recurrent disease. Therefore, there is an urgent and unmet need for the identification of specific molecular signatures that better predict the clinical outcomes and markers that serve as better therapeutic targets. With recent technological advances in genomic and epigenetic analyses, our knowledge of HNSCC molecular characteristics and classification has been greatly enriched. Clinical and genomic meta-analysis of multicohort HNSCC gene expression profile has clearly demonstrated that HPV^+ and HPV^- HNSCCs are not only derived from tissues of different anatomical regions, but also present with different mutation profiles, molecular characteristics, immune landscapes, and clinical prognosis. Here, we briefly review our current understanding of the biology, molecular profile, and immunological landscape of the HPV^+ and HPV^- HNSCCs with an emphasis on the diversity and heterogeneity of HNSCC clinicopathology and therapeutic responses. After a review of recent advances and specific challenges for effective immunotherapy of HNSCCs, we then conclude with a discussion on the need to further enhance our understanding of the unique characteristics of HNSCC heterogeneity and the plasticity of immune landscape. Increased knowledge regarding the immunological characteristics of HPV^+ and HPV^- HNSCCs would improve therapeutic targeting and immunotherapy strategies for different subtypes of HNSCCs.

Keywords: head and neck squamous cell carcinomas, heterogeneity, immune landscape, immunosurveillance, immunosuppression, neoantigen, checkpoint blockade

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) are epithelial tumors derived from mucosa linings of oral cavity, oropharynx, larynx, or hypopharynx. According to the recently published report GLOBOCAN 2018 (global cancer statistics) (Bray et al., 2018), more than 800,000 new HNSCC cases are diagnosed each year. Currently, the majority of head and neck cancers present with regionally advanced with lymph node metastases at the time of diagnosis. The patients are often given the standard treatment options of surgery, radiotherapy, chemotherapy, or a combination of these interventions, but 40–60% of treated patients experience recurrence and are unresponsive to subsequent therapeutic interventions (Haddad and Shin, 2008; Tolstonog and Simon, 2017). Therefore, despite the significant improvement in overall survival (OS) for patients with other tumor types, the 5-year OS rate of HNSCCs has not changed much over the past decade (Jemal et al., 2011; Torre et al., 2015; Bray et al., 2018). The classic causative factors for ∼80% of HNSCCs are heavy tobacco usage and/or excessive alcohol consumption (Haddad and Shin, 2008; Leemans et al., 2018). Due to a recent, substantial increase in human papillomavirus (HPV) infections in the Western world with a specific rise in the prevalence of HPV-positive oropharyngeal tumors in non-smokers, HPV-infection has emerged as another carcinogenic factor of HNSCCs (D'Souza et al., 2007; Castellsague et al., 2016). HNSCCs are diverse and complex diseases manifesting high levels of inter- and intra-tumoral heterogeneity as well as disparities in therapeutic response irrespective of clinical stage. Therefore, a better understanding of HNSCC biology and identification of specific markers or signatures for clinical prognosis and therapeutic targets will be invaluable for adapting advanced, targeted interventions to improve outcomes of HNSCC treatment.

During the past decade, the tremendous advances in next-generation sequencing (NGS) and analyses of alterations in gene expression/rearrangements, including DNA copy number, somatic mutations, and promoter methylation, have led to an exponential gain of genomic and epigenetic information regarding HNSCC molecular characterization and landscape (Hammerman et al., 2015; Leemans et al., 2018). These advances, especially in the context of HNSCC carcinogenesis, clinicopathology, and immunotherapy interventions, have provided significant insight into the diverse molecular mechanism of HNSCC carcinogenesis, the unique characteristics and heterogeneity of the HNSCC tumor microenvironment (TME), and the diversity in clinical responses among HNSCC subtypes (Hammerman et al., 2015; Tonella et al., 2017; Leemans et al., 2018). This information, along with continued in-depth investigation and translation into targeted therapeutic strategies, will lead to significant improvement in clinical outcomes. Here, we first briefly discuss our current understanding of the genetic landscape and molecular characteristics of HNSCCs with an emphasis on the potential implication of the cellular and immunological pathways and heterogeneity, followed by a discussion of basic tumor immunology, antitumor immunity, and the immune landscape of the HNSCC TME. We then conclude with a discussion of the current and potential new strategies against effective therapeutic targets toward the highly heterogeneous and immunosuppressive HNSCCs.

THE GENOMIC LANDSCAPE AND MOLECULR CLASSIFICATION OF HNSCCs

Conventional HNSCC classification and clinical management are mainly based on anatomic location, phenotype, and clinical stages, including the existence of tumor-node-metastasis (TNM) and the depth of tumor invasion (Brierley et al., 2016). Nevertheless, for most of the advanced HNSCCs with regional metastasis, histological and clinical-staging do not correlate with clinical responses or prognosis (Hammerman et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015; Leemans et al., 2018). Recent technological advances in comprehensive and integrative genomic and epigenetic analyses have made it possible to identify specific molecular markers for targeted therapeutic strategies, which improve personalized treatment and predication of recurrence/metastasis and clinical prognosis (reviewed in Tonella et al., 2017; Leemans et al., 2018).

With a rapid rise of HPV-positive (+) cases in ∼20% of HNSCC patients in the Western world, an emerging topic relating HNSCC carcinogenesis, cellular, and molecular heterogeneity to a clinical presentation is the involvement of HPV (The Cancer Genome Atlas Network [TCGA], 2015; Agalliu et al., 2016; Leemans et al., 2018). Recently, compelling results clearly demonstrated that HPV (+) and HPV-negative (−) HNSCCs are distinct subtypes in regard to molecular signatures, clinical presentation, and responses to therapy. For instance, HPV-infections are more prevalent in tumors originated from oropharynx, especially in Caucasians (**Table 1**) (The Cancer Genome Atlas Network [TCGA], 2015; Ragin et al., 2016; Fakhry et al., 2017; Buckley et al., 2018; Leemans et al., 2018; Razzaghi et al., 2018). Interestingly, HPV (+) oropharyngeal squamous cell carcinomas (OPSCCs) manifested pathologically as large ovoid nuclei with minimal cytoplasm and reduced keratinization and were mostly located in the periphery of tumors surrounding the proliferating tumor cell clusters (Buckley et al., 2018). The HPV (+) status of these OPSCCs was found to be in association with a better overall clinical prognosis (**Table 1**) (Fakhry et al., 2008, 2017; Buckley et al., 2018; Leemans et al., 2018). In contrast, HPV (−) OPSCCs, which presented as well keratinized with large amounts of cytoplasm and distinct cell borders, were more closely linked to tobacco/alcohol use, found with higher incidence in Asians and African American populations, and more predictive of a poor clinical prognosis (Fakhry et al., 2008, 2017; Ragin et al., 2016; Bray et al., 2018; Buckley et al., 2018; Leemans et al., 2018). It is also noteworthy that the incidence of HNSCC in males is two to three times of that in females worldwide (**Table 1**) (The Cancer Genome Atlas Network [TCGA], 2015; Bray et al., 2018). Therefore, we will describe and discuss the molecular landscape of HPV (+) and HPV (−) HNSCCs separately whenever possible. On the other hand, most of the molecular classification

TABLE 1 | Causal, anatomical, gender, and racial diversities, clinicopathology, and survival of the HPV-positive and HPV-negative HNSCCs.

Characteristic	Total number	HPV^+ (%)	HPV^- (%)	References
Gender				
Female	76	4 (5)	72 (95)	The Cancer Genome Atlas Network [TCGA], 2015
Male	203	32 (16)	171 (84)	
Female*	52	28 (54)	24 (46)	Ang et al., 2010
Male*	271	178 (66)	93 (34)	
Race				
Caucasian	242	34 (14)	208 (86)	The Cancer Genome Atlas Network [TCGA], 2015
Non-Caucasian	37	2 (5)	35 (95)	
Caucasian*	278	190 (68)	88 (32)	Ang et al., 2010
Non-Caucasian*	45	16 (36)	29 (64)	
Anatomical location				
Oropharynx	33	22 (67)	11 (33)	The Cancer Genome Atlas Network [TCGA], 2015
Oral cavity	172	12 (7)	160 (93)	
Larynx	72	1 (1)	71 (99)	
Hypopharynx	2	1 (50)	1 (50)	
Smoking (pack years)				
<20	15	14 (93)	1 (7)	Fakhry et al., 2008
>20	72	17 (24)	55 (76)	
Alcohol history				
No alcohol use	85	5 (6)	80 (94)	The Cancer Genome Atlas Network [TCGA], 2015
Alcohol use	188	30 (16)	158 (84)	
Overall survival probability				
0–15 months	38(+)/58(−)	37 (97)	48 (83)	Fakhry et al., 2008
15–30 months	38(+)/58(−)	35 (92)	36 (62)	
60 months	36(+)/243(−)	(∼55)	(∼40)	The Cancer Genome Atlas Network [TCGA], 2015
Relative survival*				
3-year overall survival*	206(+)/117(−)	165 (82.4%)	51 (57.1%)	Ang et al., 2010
5-year overall survival*	206(+)/117(−)	73 (35.4%)	22 (18.8%)	Ang et al., 2010

Oropharyngeal SCC.

studies were performed using total HNSCC specimens regardless of HPV statuses; therefore, we must describe the consensus classification with less emphasis on HPV status. Because the genomic landscape and molecular signatures of HNSCCs are not the focus of this review, we will only briefly describe the general observations regarding HNSCC biology, molecular signatures of different tumor subtypes, and carcinogenic drivers, which have a direct implication on the immune landscape and immunotherapy of HNSCCs.

The Genomic Landscape of HPV-Positive HNSCCs

Human papillomavirus is a well-known causative factor for cervical cancers, associated with the 2008 Nobel Prize to Dr. Hausen (Hampton, 2008; Moody and Laimins, 2010). An increase in oropharyngeal tumors and their high prevalence of HPV-positivity (∼60%) implicated the potential causative effects of HPV for HNSCC malignancy (Chaturvedi et al., 2011; Castellsague et al., 2016). Similar to cervical cancers, HPV-16 was the most common subtype that accounted for ∼80% infected cases of the HPV (+) HNSCCs, determined by positive serological response to HPV-16 E6 protein, the *E6* and *E7* viral oncogene mRNA expression, or p16INK4a protein expression (**Table 2**) (Gillison et al., 2008; Shi et al., 2009; Ndiaye et al., 2014; Agalliu et al., 2016).

Because HPV-16 E6 and E7 viral proteins induce cellular transformation and prevent apoptosis via functionally inhibiting the activity of tumor suppressor p53 (TP53) and retinoblastoma 1 protein (RB1) (reviewed in Moody and Laimins, 2010), *TP53* and *RB1* gene mutations were rarely detected in HPV (+) HNSCCs (**Table 2**). Although some studies suggested an overall lower level of mutational loads in HPV (+) than in HPV (−) HNSCCs (Stransky et al., 2011; Hanna et al., 2018), others observed a comparable level of mutational burden or frequency, with differing profiles, between HPV (+) and HPV (−) HNSCCs (Hammerman et al., 2015; Seiwert et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015). Nevertheless, the breadth of molecular alterations in HPV (+) HNSCCs were rather limited to the amplification of *PIK3CA* oncogene and/or *E2F1*, the truncation of TNF receptor-associated factor 3 (*TRAF3*), and the mutation and fusion of *FGFR2/3* gene (**Table 2**) (Stransky et al., 2011; Keck et al., 2015; Seiwert et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015). Interestingly, a subset of the HPV (+) HNSCCs present with a distinct immune signature, including elevated levels of *CD8*, *CD56*, *ICOS*, *LAG3*,

TABLE 2 | Molecular landscapes that are impacted differentially in the HPV-positive and HPV-negative HNSCCs.

Gene	Prevalence	Mutation/alteration in function	Cellular process	References
HPV$^+$ HNSCCs				
E6 and *E7*	80%	Viral oncogene	Cellular transformation; functional inhibition of p53/RB1 proteins	Hammerman et al., 2015
TP53/RB1	Rare	Low mutation rate, functional inactivation	HPV-driven	Westra et al., 2008
PIK3CA	>50%	Amplification/mutation	AKT/mTOR pathway	Hammerman et al., 2015
TRAF3	8/14%	Truncation/recurrent deletion	Uncontrolled NF-κB signaling	The Cancer Genome Atlas Network [TCGA], 2015
FGFR2/3	>10%	Alteration/oncogene fusion (FGFR3-TACC3)	Activation of the RTK (receptor tyrosine kinase) pathway	Seiwert et al., 2015
CD8, CD56, ICOS, LAG3, HLA-DR	IMS subtype	Elevated levels of gene expression enhanced immune cell infiltration	CD8$^+$ T and NK cell infiltration	The Cancer Genome Atlas Network [TCGA], 2015
HPV$^-$ HNSCCs				
TP53	∼86% common	Somatic mutations Chromosomal loss at 3p/17p	Tumor suppressor loss of function	The Cancer Genome Atlas Network [TCGA], 2015
		Copy number alteration	Loss of TP53 function	The Cancer Genome Atlas Network [TCGA], 2015
CDKN2A/RBI	Very common	Chromosomal loss at 9p	Tumor suppressor loss of function	The Cancer Genome Atlas Network [TCGA], 2015
HRAS	5–10%	Activating mutation	Constitutive activation of RAS pathway	Seiwert et al., 2015
CASP8	Co-occurrence with *HRAS* mutation	Inactivating mutation	Suppression of cell death	The Cancer Genome Atlas Network [TCGA], 2015
EGFR, ERBB2, FGF1	∼30%	Amplification	Activation of the RTK pathway	The Cancer Genome Atlas Network [TCGA], 2015
FAT1, AJUBA,	Common	Inactivating mutation/deletion	WNT/b-catenin signaling	The Cancer Genome Atlas Network [TCGA], 2015
N0TCH1	Common	Mutation/deletion	Differentiation	The Cancer Genome Atlas Network [TCGA], 2015
TP63	Common	Gain of function	Differentiation	The Cancer Genome Atlas Network [TCGA], 2015
NFE2L2, KEAP1	∼5%	Activating mutation	Oxidative stress	Hammerman et al., 2015

and *HLA-DR*, which is likely the result of an activated anti-viral (HPV) response (**Table 2**) (Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015; Hanna et al., 2017). In general, HPV (+) HNSCC patients have a significantly better prognosis of 5-year overall survival than that of HPV (−) patients (Ang et al., 2010; The Cancer Genome Atlas Network [TCGA], 2015; Fakhry et al., 2017).

HPV-Negative HNSCCs

HPV (−) HNSCCs account for the majority of the cases with excessive smoking and alcohol usage as major risk factors (The Cancer Genome Atlas Network [TCGA], 2015; Leemans et al., 2018). This subtype of HNSCCs manifests with a wide variety of gene mutations, amplifications, and epigenetic alterations that are associated with increased metastases and worse clinical outcomes (**Table 2**) (Stransky et al., 2011; Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015; Leemans et al., 2018). One of the prominent molecular abnormalities of HPV (−) tumors is widespread loss-of-function mutations in the tumor suppressors *TP53* and *CDKN2A/RB1* or chromosomal loss at 9p (*CDKN2A*) and 3p and 17p (*TP53*) (**Table 2**). Other highly enriched molecular abnormalities found in HPV (−) tumors are *HRAS*, *CASP8*, amplification of receptor tyrosine kinase (RTK) genes and *PIK3CA* gene, and genes/pathways associated with WNT signaling (*FAT1* and *AJUBA*), squamous cell differentiation (*TP63*, *NOTCH1*, and *RIPK4*), and oxidative stress regulation (*NFE2L2* and *KEAP1*) (**Table 2**). Overall, HPV (−) HNSCCs exhibit diverse alterations in the gene expression profile driven by environmental carcinogenic factors that presents clinically with a high incidence of recurrence, metastasis, and poor response to conventional and advanced therapies (Stransky et al., 2011; Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015; Leemans et al., 2018).

Molecular Classification and Heterogeneity of HNSCCs

Aside from HPV status, current consensus of molecular classification categorizes HNSCCs into classical (CL), basal (BA), mesenchymal (MS), and atypical (AT) subgroups, each with distinct gene expression profile and biological characteristics (Walter et al., 2013; The Cancer Genome Atlas Network [TCGA], 2015). Interestingly, these molecular subtypes exist across all anatomic sites and clinical stages, with the exception of hypopharyngeal cancers lacking the BA subgroup (Walter et al., 2013).

The CL subgroup is associated with increased levels of polyamine, cell proliferation, and genes involved in cell cycle regulation and metabolism pathways (Keck et al., 2015). The CL subgroup of HNSCCs have also been shown to express a relatively high level of *SOX2*, a gene responsible for maintaining the self-renewal of undifferentiated stem cells, as observed in squamous cell carcinoma of the lung and other tissues (Walter et al., 2013; The Cancer Genome Atlas Network [TCGA], 2015). In contrast, the BA subgroup is highly enriched for

hypoxia-response genes, EGFR signaling associated genes, and *TP63,* exhibiting a signature of epithelial keratinization and differentiation (Walter et al., 2013; Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015).

The MS and AT subgroups are HNSCCs that consist of a higher frequency of HPV (+) tumors than the CL and BA subgroups (Walter et al., 2013; Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015). The consensus classification of HNSCCs categorizes most of the HPV (+) tumors into the AT subgroup, due to high expression levels of *RPA2* (*Replication Protein A2)*, *E2F2*, and *SOX2* with a strong HPV signature, whereas only a limited number of HPV (+) tumors are classified into the MS subgroup (Walter et al., 2013; The Cancer Genome Atlas Network [TCGA], 2015). The MS subgroup is characterized as having an elevated expression of epithelial-to-mesenchymal-transition (EMT) associated genes, such as *DES* and *TWIST*, and mesenchymal cell-related genes, including *VIM* (vimentin), *MMP*s, *PDGFRA*, and *PDGFRB* (Walter et al., 2013; The Cancer Genome Atlas Network [TCGA], 2015). Differing from the classic subtype characteristics, a recent comprehensive and integrative study by Keck et al. (2015) using data from multiple HNSCC cohorts consisting over 900 patients revealed a strong presence of the MS-signature in some of the HPV (+) tumors. In addition to their MS-signature and downregulation of markers for epithelial differentiation and keratinization, this HPV (+) MS subgroup exhibited a distinct signature showing an elevated expression of immune genes, such as *CD8*, *ICOS*, *LAG3*, and *HLA-DRA*, which were defined as the inflamed/mesenchymal (IMS) subgroup (Keck et al., 2015). This observation agreed with earlier reports of a strong immune signature associated with elevated expression levels of CD56 and HLA class I in some of the HPV (+) HNSCCs within the AT subgroup (Walter et al., 2013). Interestingly, the clinical benefits of the elevated immune-signature seemed to be more prominent in HPV (+) HNSCC patients because no significant benefit in overall survival was observed in the HPV (−) IMS subgroup of HNSCC patients, agreeing with the differential immune cell profiles between the virally and non-virally infected individuals (Keck et al., 2015).

Together, these observations demonstrate that integrative genomic analyses in association with functional annotation provides valuable information for identifying molecular drivers of carcinogenesis, potential markers for prognosis, and HNSCC classification based on molecular signatures that may facilitate a better prediction for responsiveness to therapy. Importantly, it should be appreciated that the complexity and heterogeneity in the landscape of both HPV (−) and HPV (+) HNSCCs contribute to differential responses to therapeutic interventions, and thus should be thoughtfully considered when selecting the appropriate therapeutic targets and/or strategies.

IMMUNE LANDSCAPE OF THE HNSCC TME

The host immune system is an essential defense mechanism for recognizing and destroying pathogens, including bacteria, viruses, and other substances of foreign origin, via the coordinated and concerted activation of innate and adaptive immunity (Abbas and Janeway, 2000; Paul, 2013). The innate immune response is mediated through an acute mobilization and activation of macrophages, dendritic cells (DCs), and nature killer (NK) cells, which attack pathogens and tumors via endocytosis or cytolysis by cytokines or cytotoxic molecules in a non-antigen-specific manner (**Figure 1**). On the other hand, adaptive immunity involves activation of lymphocytes by activated antigen presenting cells (APCs), which present antigenic peptides through the MHC (major histocompatibility complex) surface proteins to T cells in the presence of co-stimulatory molecules such as CD28/CD80 (B7-1) or CD86 (B7-2) (**Figure 1**). Subsequently, activated CD4 T helper cells prompt the activation of cytolytic CD8 T cells and B cells for tumor and pathogen elimination in an antigen-specific manner (**Figure 1**). Under physiological conditions, immune activation is also associated with upregulation of immune inhibitory molecules, such as programmed death-1 (PD-1), PD-L1 (programmed death-ligand 1), and cytotoxic T-lymphocyte associated antigen (CTLA4). These inhibitory molecules are called checkpoint inhibitors, which serve as part of an intrinsic negative feedback loop to prevent sustained and uncontrollable T cell activation seen in self-destructive autoimmune diseases (**Figure 2**). Besides PD-1/PD-L1, CD28/CD80, CD86, and CTLA4, many more co-stimulatory and co-inhibitory molecules have been identified in the regulation of T cell activation and tolerance status during productive immunity and within the TME, respectively (**Figure 2**) (Chen and Flies, 2013; Chen and Mellman, 2013).

It has long been debated whether recognition and elimination of cancer cells is an integral function of the immune system. In fact, as early as the 1900s, Paul Ehrlich speculated that a functional immune system could detect and control malignant tumors. This theory was further developed by Sir Frank Macfarlane Burnet and Lewis Thomas in the 1950s into the immunosurveillance hypothesis (Burnet, 1957, 1967; Thomas, 1982), which proposed the existence of an immunological mechanism for detecting and eliminating mutated abnormal cells. This hypothesis, however, had been challenged constantly until the late 1990s when compelling experimental evidence demonstrated the essential roles of immune effector molecules in suppressing tumor occurrence and progression. In the early 2000s, the cancer immunosurveillance hypothesis was further improved and refined by Robert Schreiber to accentuate the dynamic and bidirectional interactions between tumor cells and the immune system as a three-phase process, termed tumor "Elimination, Equilibrium, and Escape," which describes the ongoing battle between the immune system and the tumor that determines tumor survival/growth and reshapes the tumor antigen pool and the immune landscape of the TME (**Figure 3**) (Dunn et al., 2002; Schreiber et al., 2011). Now, the immunosurveillance concept has been well appreciated to relay an important physiological process during carcinogenesis and tumor progression, and has provided invaluable insight into potential targets for immunotherapy intervention.

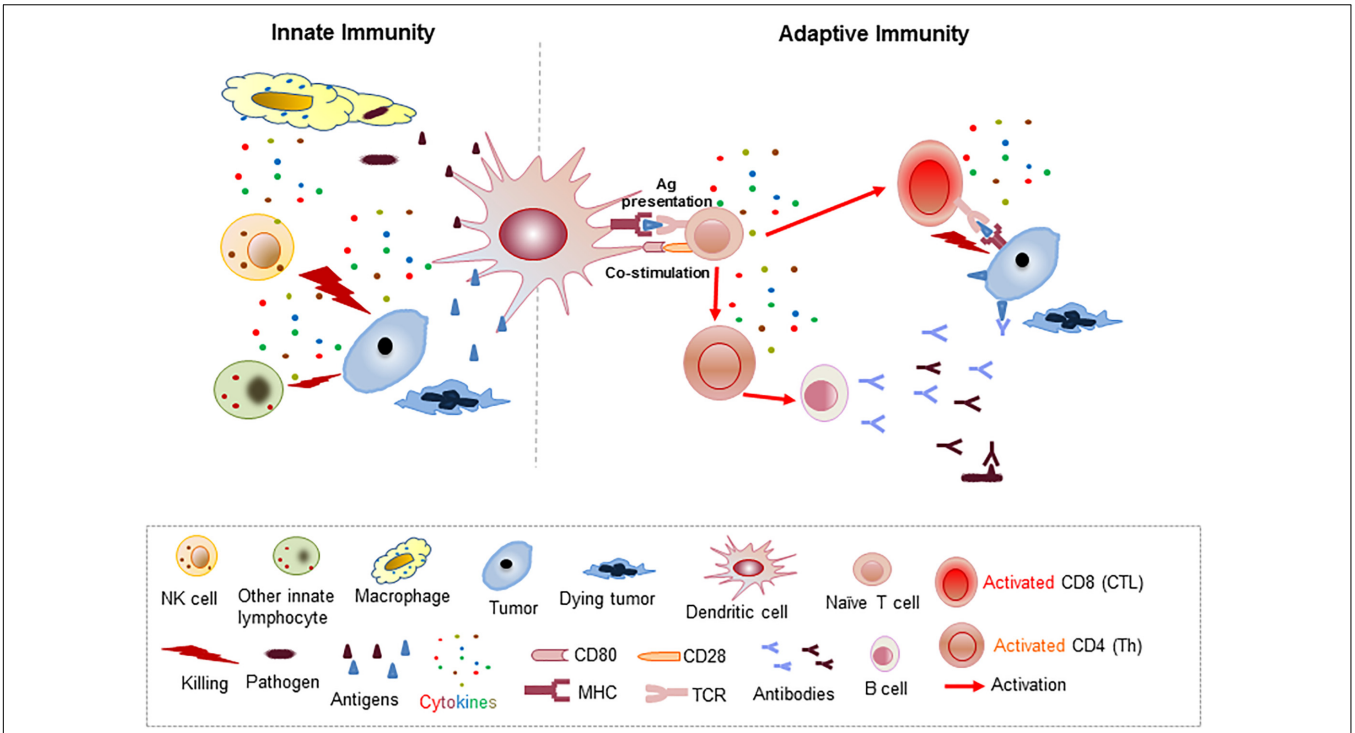

FIGURE 1 | Schematic illustration of the cellular and molecular processes associated with innate and adaptive immunity mediated pathogen and tumor elimination.

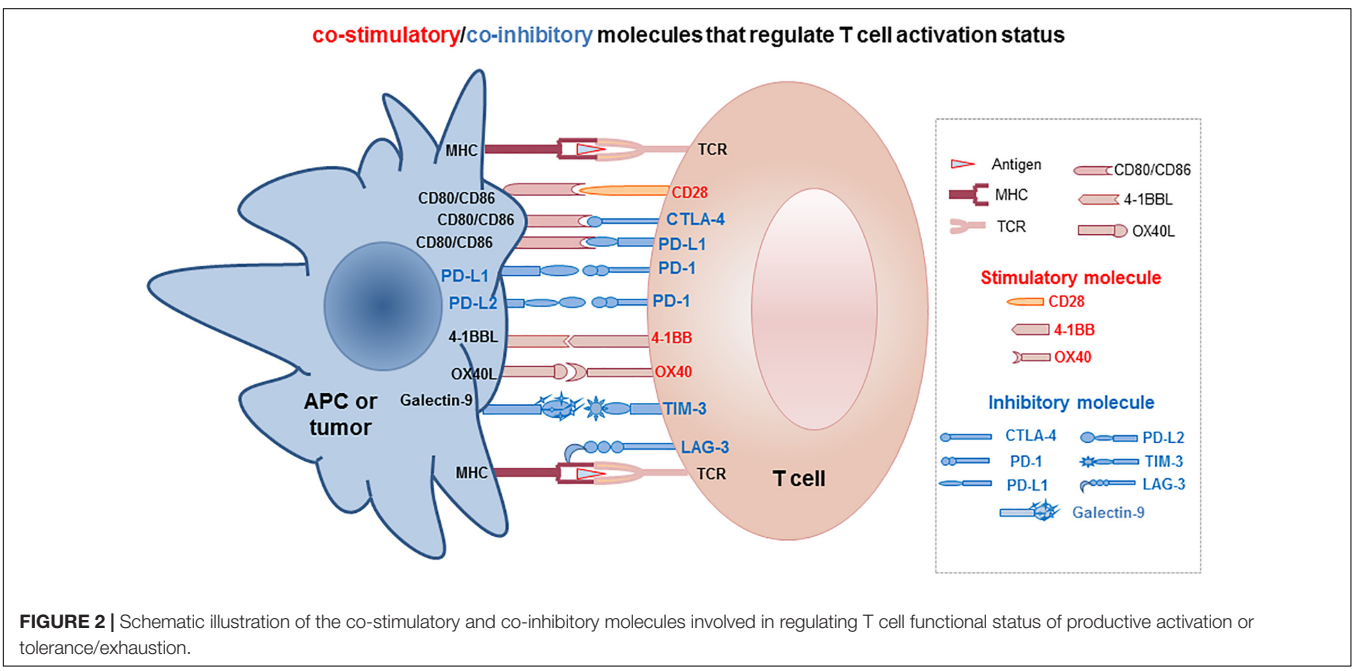

FIGURE 2 | Schematic illustration of the co-stimulatory and co-inhibitory molecules involved in regulating T cell functional status of productive activation or tolerance/exhaustion.

Adaptive and Innate Immunity for Cancer Immunosurveillance

The first series of experimental evidence that firmly established the existence of immunosurveillance came from gene-targeted knockout mice with selective inactivation of key immune cytolytic molecules, such as perforin, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), in either innate immune NK cells or adaptive immune cells (Dighe et al., 1994; van den Broek et al., 1996; Kaplan et al., 1998; Smyth et al., 2000a,b; Shankaran et al., 2001; Takeda et al., 2001). These genetically engineered mice lacking cytolytic function in their immune effectors were more susceptible to spontaneous or

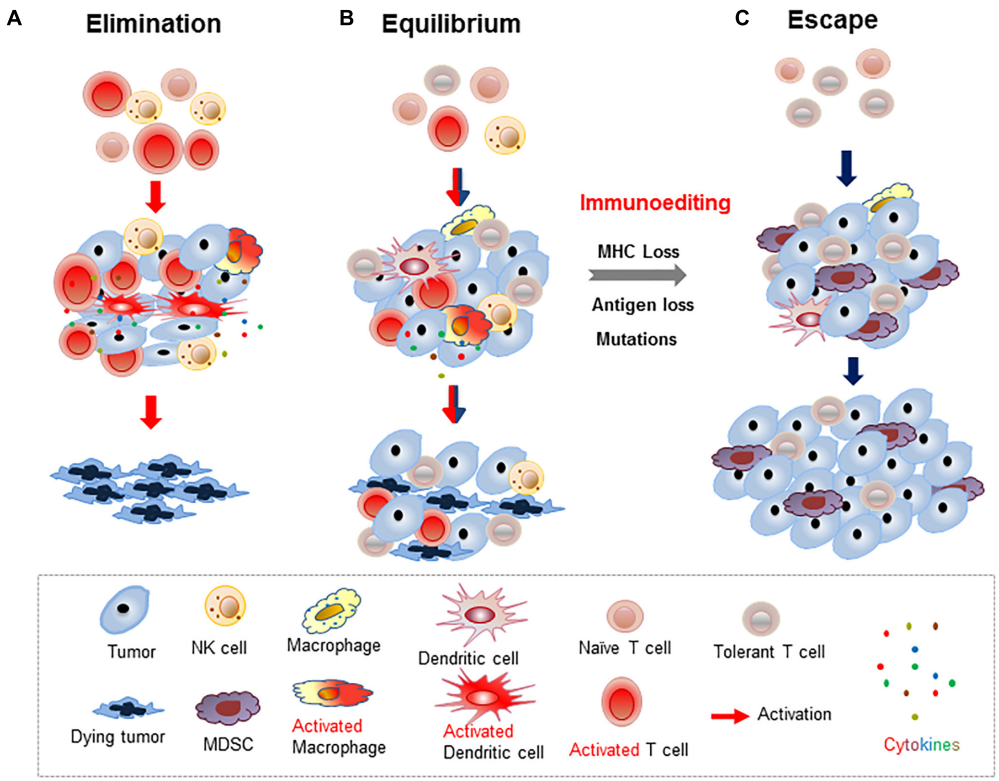

FIGURE 3 | Schematic illustration of three outcomes of tumor immunosurveillance. **(A)** Tumors are eliminated by productive antitumor immunity of activated T cells and/or innate immune cells in the dominantly immunostimulatory TME. **(B)** Co-existence of activated immune cells that kill some of the tumor cells and ignorant/tolerant immune cells resulting in the survival of residual tumors and an overall tumor "dormancy." **(C)** In the immunosuppressive TME, tumors and pro-inflammatory myeloid-derived suppressor cells (MDSCs) induce T cell tolerance and prevent tumors being recognized by host immunity, thereby promoting tumor progression.

chemical-induced tumorigenesis. Likewise, mice with defective recombination-activating gene 2 (RAG2), which leads to T and B cell deficiency, were also more susceptible to spontaneous and carcinogen-induced tumorigenesis (Shankaran et al., 2001). Similarly, mice depleted of NK or NKT cells also showed an increased incidence of tumor development (Smyth et al., 2000a; Girardi et al., 2003), whereas mice treated with an NKT cell activation ligand during chemical-induced tumorigenesis manifested a reduced incidence and prolonged latency of tumor development (Hayakawa et al., 2003). Moreover, the observations that specific inactivation of classical T cells or γδ innate T cells via blocking TCR αβ or γδ chain rearrangement, respectively, led to an increased prevalence of tumorigenesis further supporting the role of both adaptive immunity and innate immunity in immunosurveillance (Girardi et al., 2003).

Clinically, it has long been noted that individuals with severe primary immunodeficiency or patients with vial or therapy-induced immunosuppression showed an increased risk of tumor development (Boshoff and Weiss, 2002; Oksenhendler et al., 2002; Salavoura et al., 2008; Engels et al., 2011). Moreover, in many types of human cancers, the number of tumor infiltrating CD3 or CD8 T cells is positively correlated with better clinical outcomes (Zhang et al., 2003; Galon et al., 2006; Hiraoka et al., 2006). Importantly, the observation that tumor antigen-specific T and B cells from tumor patients could be reactivated to induce tumor killing and regression provided direct clinical evidence of immunosurveillance. Thus, enhancing immunosurveillance and effector T cell infiltration to tumors is crucial for improving cancer immunotherapy.

Cancer Immunoediting

Despite the existence of immunosurveillance and the observed elimination of some tumors by innate and adaptive immunity, a portion of tumor cells escaped elimination via mutations, alteration of MHC expression, or dysfunction of antigen processing machinery (APM) (Algarra et al., 2000; Marincola et al., 2000). This process was coined as "immunoediting" by Robert Schreiber (Dunn et al., 2002, 2004; Schreiber et al., 2011). Thus, active immunosurveillance imposes a selective pressure that "shapes" the immunogenicity of tumor cells and encourages/results in the escape of tumors that are less immunogenic via loss of tumor antigens and/or MHC expression (**Figure 3**). Gradually, the surviving tumors escape the immunosurveillance via accumulating mutations and are no longer recognized by the immune system. As such, cancer immunoediting is a dynamic process that encompasses tumor elimination, equilibrium, and escape (**Figure 3**) (Dunn et al., 2002; Schreiber et al., 2011). Spontaneous mutations have been

detected in various tumors, which is attributed as one of the hallmarks of tumorigenesis and tumor recurrence (Hanahan and Weinberg, 2011; Schreiber et al., 2011; DuPage et al., 2012). Clinically, spontaneous or therapy-induced mutations that resulted in reduced tumor immunogenicity, including reduced MHC expression, have been frequently observed. Thus, throughout the tumorigenesis and tumor progression, there exists constant interaction between tumor and immune cells that affects the immunological status of activation vs. inhibition and thus, dictates the fate of tumor cells (Chen and Mellman, 2013).

The Immunosuppressive TME of HNSCCs

Accumulating evidence suggests that tumor progression and metastases are markedly affected by the constituents surrounding and within the tumor parenchyma, the so-called TME. The TME is a highly complex, functional eco-system consisting of tumors and other cellular and molecular components. The cellular constituents of the TME consist of stromal cells [cancer-associated fibroblasts (CAFs), blood endothelial, and lymphatic endothelial cells], tumor-infiltrating lymphocytes (T cells, B cells, and NK cells), and myeloid populations [dendritic cells, macrophages, and myeloid-derived suppressor cells (MDSCs)]. Many of the tumor-infiltrating immune cells possess immune inhibitory function, such as regulatory T cells (Treg), MDSC, and type 2 macrophages (M2). At the molecular level, tumor-induced immunoevasion and immunosuppression are associated with downregulation of MHC molecules (human leukocyte antigen, HLA), inactivation of the APM, which prevents the processing and presentation of tumor antigens to CD8 T cells, and upregulation of the checkpoint inhibitors on tumors and immune cells (Ferris et al., 2006; Lopez-Albaitero et al., 2006). All of these cellular and molecular events actively and cooperatively enforce the immunosuppressive landscape (Biswas and Mantovani, 2010; Hanahan and Coussens, 2012; Curry et al., 2014).

Compelling evidence suggests that the immune landscape of HPV (+) HNSCCs differs from HPV (−) tumors in that the HPV (+) TME is associated with abundant immune infiltrates, whereas the HPV (−) TME incurs high mutational load. Currently, clinical treatment of HNSCC patients with either conventional chemo/radiotherapy regimens or the most recent advanced immunotherapy showed less favorable overall survival than patients with other tumor types receiving similar treatments, indicating the detrimental effects of an immunosuppressed HNSCC TME (Haddad and Shin, 2008; Curry et al., 2014; Schoenfeld, 2015; Tolstonog and Simon, 2017; Bray et al., 2018).

Tumor Infiltrating Lymphocytes

Early clinical studies revealed the existence of T cell dysfunction, increased Tregs, and impaired NK cell activity, as well as an overall reduction in lymphocyte counts in HNSCC patients (Hoffmann et al., 2002; Reichert et al., 2002; Whiteside, 2005). Subsequent investigations illustrated that circulating and tumor infiltrating T lymphocytes from HNSCC patients exhibited abnormal signaling cascades, reduced proliferation, and spontaneous apoptosis (Hoffmann et al., 2002; Reichert et al., 2002). This observed T cell dysfunction is likely the result of an altered cytokine profile in the HNSCC TME, including increased IL-10, IL-6, and TGF-β secretion and reduced IL-12 levels (Lathers and Young, 2004; Lu et al., 2004; Sparano et al., 2004; Varilla et al., 2013). Moreover, there was an increase in Fas-ligand expression on HNSCCs and circulating vesicles, which also enhances the susceptibility of T cells to apoptosis (Gastman et al., 1999; Kim et al., 2005).

Interestingly, comprehensive clinical studies demonstrated that HPV (+) HNSCCs were among tumors with the highest immune infiltrates as compared to most other common tumor types (Mandal et al., 2016). Furthermore, these HPV (+) tumors showed high levels of Treg, Treg/$CD8^+$, and $CD56^{dim}$ NK cells, as well as an activated phenotype, including a significantly higher expression of CTLA4 (Mandal et al., 2016) and highly elevated PD-1 on T cells (Badoual et al., 2013) (**Table 3**). Remarkably, the increased levels of $CD4^+CD25^+$ Tregs and PD-1^+ T cells correlated positively to better clinical prognosis in HNSCC patients (Badoual et al., 2006, 2013; Loose et al., 2008), which is different from the common observation of elevated Tregs in association with immunosuppression and poor clinical outcomes in other tumor types. It is proposed that these elevated $CD4^+CD25^+$ Tregs and PD-1^+ T cells in HPV (+) HNSCCs indicate an on-going immunosurveillance against the HPV-viral proteins, which activate the negative feedback of suppressive mechanism. Consistent with this observation, a recent report also showed that in HPV (+) HNSCCs, $CD3^+$ T cell infiltration was the highest when compared to other tumors, associated with a high frequency of $CD56^+CD3^+$ NKT cells and PD-1/TIM3 co-expressing $CD8^+$ T cells (Hanna et al., 2017) (**Table 3**). Importantly, clinical and experimental observations suggest that the presence of IFN-γ cytokines, tumor infiltrating CD8 T cells, and PD-$L1^+$ immune cells within the TME is likely an indication of pre-existing antitumor immune responses and is potentially more responsive to therapeutic interventions (Hegde et al., 2016).

In contrast, HPV (−) HNSCCs exhibited an overall reduced number of immune infiltrating cells, relatively low levels of CD8 T cells that co-expressed PD-1/TIM3, and lack of a positive association between $CD4^+CD25^+$ Tregs or PD-1^+ T cells with clinical prognosis (Hanna et al., 2017) (**Table 3**). These HNSCCs exhibited a highly elevated smoking-related mutation profile and an unfavorable clinical outcome when compared to HPV (+) HNSCC patients (Mandal et al., 2016). These observations, together with those of HNSCC molecular landscape studies, further illustrated the high level of diversity and heterogeneity of the HNSCC TME, which can be affected by HPV status and potentially other unidentified factors, in their molecular and cellular profiles and clinical outcomes.

Immunosuppressive Myeloid Cells

Myeloid cells, including granulocytes, monocytes, and their derivatives following activation or further differentiation (dendritic cells and macrophages) are crucial immune regulators and activators that bridge the innate and adaptive immunity under physiological conditions. Through antigen presentation and/or production of immune modulatory cytokines, myeloid cells induce either immune activation or tolerance

TABLE 3 | Immune landscapes of the HPV-positive and HPV-negative HNSCCs and clinical implications for targeted immunotherapy approaches.

	HPV (+) HNSCCs	HPV (−) HNSCCs	Reference
Overall tumor infiltrating lymphocytes	Relative high numbers		Mandal et al., 2016
		Low numbers	Hanna et al., 2017
Immune cells and phenotype	Increased $CD4^+CD25^+$ Tregs $PD\text{-}1^+$ T cells and $CD4^+CD25^+$ Tregs		Loose et al., 2008; Badoual et al., 2013; Mandal et al., 2016
	High $CD56^{dim}$ NK cells		Mandal et al., 2016
	Elevated PD-1 and CTLA4 on T cells		Mandal et al., 2016
	High $CD56^+CD3^+$ NKT cells		Hanna et al., 2017
	High $PD\text{-}1^+/TIM3^+CD8^+$ T cells	Low $PD\text{-}1^+/TIM3^+CD8+$ cells	Hanna et al., 2017
Clinical prognosis/responsiveness			
Correlation between $CD4^+CD25^+$ Tregs and prognosis	Positive association	No correlation	Hanna et al., 2017
	Better clinical outcome	Poor outcome	Mandal et al., 2016
Overall immune landscape	Activated immune cell phenotype		Hanna et al., 2017
	Less immunosuppressive	Highly immunosuppressive	Hanna et al., 2017
Mutation load/dominate antigens	Low mutation load	High mutation load	Keck et al., 2015
	HPV-associated antigens	Neoantigens	The Cancer Genome Atlas Network [TCGA], 2015
Clinical responses to checkpoint blockade	Higher response rate	Low response rate	Seiwert et al., 2016
			Hanna et al., 2018
		Good response rate only in tumors with high mutation load and CD8 T cells	Hanna et al., 2018

(Dhodapkar et al., 2008; Biswas and Mantovani, 2010; Guilliams et al., 2014). During carcinogenesis and tumor progression, tumors also evade immunosurveillance by altering the myeloid cell phenotype and function, and creating a chronically inflamed immune landscape. In general, this tumor escape process can be mediated by active recruitment of MDSCs, macrophages, and macrophage polarization, and/or induction of regulatory DCs. The cellular and molecular alterations leading to tumor evasion can also be the result of altered antigen presentation capacity of these myeloid cells as well as enhanced production of immunosuppressive cytokines and metabolites (Dhodapkar et al., 2008; Biswas and Mantovani, 2010; Gabrilovich et al., 2012; Hanahan and Coussens, 2012; Elpek et al., 2014).

It has long been demonstrated that the HNSCC TME is associated with chronic inflammation and immune suppression (Whiteside, 2005), and expression of proinflammatory and proangiogenic cytokines (Chen et al., 1999; Lathers and Young, 2004; Sparano et al., 2004). This pro-inflammatory and immunosuppressive environment leads to the active recruitment of macrophages and MDSCs. In fact, two independent studies demonstrated that elevated $CD68^+$ macrophages in HNSCCs were associated with clinical pathology and poor survival (Marcus et al., 2004; Wolf et al., 2015). Likewise, elevated MDSCs, characterized as $CD11b^+CD14^+CD33^+IL4R\alpha^+HLA\text{-}DR^-$ cells, were also observed in the peripheral blood of HNSCC patients compared to that of healthy individuals (Chikamatsu et al., 2012). These MDSCs expressed elevated levels of CD86, PD-L1, and TGF-β, suppressed IFN-γ production and proliferation of activated T cells (Chikamatsu et al., 2012). Furthermore, treatment with neutralizing antibodies to block the effects of CD86, PD-L1, and TGF-β partially reversed the immunosuppressive function of MDSCs on T cell activation (Chikamatsu et al., 2012). Additionally, a DC maturation defect or differential maturation was observed in some HNSCC patients. Schuler et al. (2011) reported that monocyte-derived DCs from the peripheral blood of some HNSCC patients failed to mature in culture, implying an immunosuppressive environment in patients leading to defective APC maturation. In fact, a comparative study examining the abundancy and maturation status (CD83 expression) of $S100^+CD1a^+$ DCs in patients with oral squamous cell carcinoma (OSCC) showed that in primary tumors the total DC population was higher in patients without regional metastasis (PN−) than in patients with metastasis (PN+) (Kikuchi et al., 2002). On the other hand, the number of the $CD83^+$ DC subpopulation was higher in tumors and draining lymph nodes of the PN+ patients than in the PN− patients (Kikuchi et al., 2002). These results further substantiate the high levels of diversity and heterogeneity in cellular phenotype, function, and location of myeloid subpopulations within the HNSCC TME.

Cancer Associated Fibroblasts

Cancer-associated fibroblasts are specialized fibroblastic stroma representing the dominant non-hematopoietic cell type within the TME of many cancer types. CAFs are pivotal in tumorigenesis, tumor progression, chemoresistance, metastasis, and maintenance of cancer stem cells through their production of growth factors, chemokines, and extracellular matrix (ECM) (Bhowmick et al., 2004; Orimo et al., 2005; Turley et al., 2015; Gascard and Tlsty, 2016; Kalluri, 2016). Additionally, CAFs are actively involved in immune regulation by producing inflammatory cytokines/chemokines and soluble factors, and by

directly interacting with immune cells to support the immune cell survival, function, and recruitment within the TME (Kraman et al., 2010; Liotta et al., 2015; Turley et al., 2015; Gascard and Tlsty, 2016; Kalluri, 2016). Experimental evidence suggests that CAFs are heterogeneous populations derived from various cell sources, including mesenchymal stem cells from the bone marrow, tissue resident fibroblasts, epithelial cells via EMT, fibrocytes, and likely other unidentified sources (Bhowmick et al., 2004; Orimo et al., 2005; Smith et al., 2013; Turley et al., 2015; Gascard and Tlsty, 2016; Kalluri, 2016). Clinical evidence clearly establishes the association between an increased CAF abundancy and poor prognosis in many tumor types (Turley et al., 2015; Gascard and Tlsty, 2016; Kalluri, 2016).

Within the highly heterogeneous HNSCCs, the existence of a MS-rich (presumably CAF-rich) subgroup has been revealed, which exhibits distinct molecular signatures and clinical presentation (Peng et al., 2011; Curry et al., 2014; De Cecco et al., 2015; Liotta et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015; Tonella et al., 2017). Similar to the CAF markers used for other tumors, alpha-smooth muscle actin (α-SMA) was one of the commonly used and so far represents the most reliable marker for CAF-like cells in HNSCCs (Kawashiri et al., 2009; Lim et al., 2011; Marsh et al., 2011; Wheeler et al., 2014). Additionally, high expression levels of collagen and vimentin were also used for further confirmation of CAFs (Kawashiri et al., 2009). These HNSCC-CAFs expressed high levels of growth factors, cytokines/chemokines, and ECM (Kawashiri et al., 2009; Lim et al., 2011; Marsh et al., 2011; Wheeler et al., 2014), consistent with CAFs of other tumor types. Remarkably, a retrospective study of 282 cases of HNSCC specimens revealed a significant correlation of elevated α-SMA expression with poor overall survival regardless of the clinical stage (Marsh et al., 2011). Lim et al. (2011) also demonstrated that CAFs from genetically unstable HNSCCs (high mutational load and alterations in copy number or chromosomal loss) expressed significantly higher levels of α-SMA and integrin-α6 as compared to CAFs derived from genetically stable HNSCCs. Functionally, these α-SMA$^+$ CAFs enhanced tumor progression, invasion, metastasis, glycolysis, persistence of cancer stem cells, and suppression of T cell activation and proliferation either by direct cell–cell interaction, production of soluble factors including TGF-β and hepatocyte growth factor (HGF), or elevated enzymatic activity of indoleamine 2,3-dioxygenase (IDO) (Knowles et al., 2009; Marsh et al., 2011; Wheeler et al., 2014; Liotta et al., 2015; Alvarez-Teijeiro et al., 2018).

Currently, our understanding of the CAF biology and CAF-tumor-immune cell interactions in the HNSCC TME is still limited. Given our knowledge of the heterogeneous cellular origins of CAFs, their co-evolution with, and likely co-regulation of the immune landscape during tumor progression, it is important and invaluable that more in-depth cellular and molecular investigations are performed for developing new targeted therapy. Along this line, a recent study investigated potential specific surface markers for HNSCC-CAFs because the currently used CAF markers are mostly intracellular proteins, which are not suitable for therapeutic targeting intervention. Through cDNA microarray analysis, Purcell et al. (2018) discovered a protein, leucine-rich repeat containing 15 (LRRC15), which is a membrane protein commonly expressed at high levels on mesenchymal cells, including CAFs in the HNSCC TME, but at low basal levels in healthy tissues. Similar to α-SMA expression, LRRC15 expression could be further upregulated by sustained exposure to TGF-β, which is one of the factors existing at high levels within the TME (Purcell et al., 2018). Therefore, LRRC15 represents a potential new immunotherapy target of CAFs. The implication of targeting LRRC5 in regard to releasing the immune suppression in the TME is yet to be tested.

Heterogeneity of the HNSCC TME

Comprehensive and integrative genomic and epigenetic analyses demonstrate the extremely high heterogeneity in HNSCC molecular signature and landscape, whereas flow cytometry based assay provides additional evidence of the HNSCC heterogeneity in cellular phenotype, constituents of the TME, and immune landscape. On the other hand, recent compelling clinical evidence demonstrates that productive immunotherapy depends on not only the number of immune effectors in the TME, but also, and more importantly their accessibility to tumors (Chen and Mellman, 2013; Joyce and Fearon, 2015; Hegde et al., 2016). Specifically, three distinct patterns of T cell distribution in the TME were identified as immune inflamed, immune excluded, and immune desert (Joyce and Fearon, 2015; Hegde et al., 2016). In the immune inflamed tumors, T cells are heavily infiltrated into the solid tumors, whereas immune cells are primarily distributed in the peritumor region of the TME in the immune excluded tumors. The immune desert tumors manifest with a lack of immune cells in both the TME and at the peritumor region (Joyce and Fearon, 2015; Hegde et al., 2016). These distinct patterns of immune cell segregation in the TME are associated with the observed heterogeneous clinical responses and underscore the crucial contribution of direct effector-tumor interaction to the outcomes of immunotherapy (Joyce and Fearon, 2015; Hegde et al., 2016; Kather et al., 2018). Therefore, complementary information regarding the spatial distribution of immune cells and their potential interaction is as important as the cellular and molecular characterization of the tumor for proper design of targeted or individualized therapy.

Heterogeneity of Immune Cell and CAF Composition in the TME

High levels of intertumor molecular and cellular heterogeneity of HNSCCs have been well documented and appreciated (Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015; Hanna et al., 2017). However, the intratumor heterogeneity, i.e., differential distribution of immune cells in different regions of the same tumor, remains largely unexplored. Recent studies employing multi-parameter flow cytometry analysis, especially with the simultaneous immunohistochemistry (IHC) staining-based topographic assessment of cancer-associated immune cell localization within the TME, support a high level of intratumor heterogeneity within certain HNSCC subgroups (Hanna et al., 2017; Kather et al., 2018). Importantly, this topographic assessment of immune cell spatial distribution in association

with their cellular phenotype and functional analysis provides invaluable information for potential mechanistic explanation of the heterogeneous clinical responses and for identification of specific prognostic markers (Kather et al., 2018).

Similar to the recently published results of single-cell transcriptomic analysis of HNSCCs (Puram et al., 2017), our examination of human HNSCC specimens via multiplex IHC confirmed high levels of intratumor heterogeneity with variable prevalence of immune cell infiltration and compartmentalization (unpublished observation). Besides the observed differential immune cell segregation, CAF distribution and the expression of different CAF markers varied greatly within each HNSCC specimen. For instance, Puram et al. (2017) showed differential intra- and inter-tumor expression of podoplanin (PDPN) and fibroblast activation protein (FAP), both of which have shown to express on CAFs, in HNSCCs (Puram et al., 2017). Our multiplex IHC staining also reveal differential distribution of α-SMA and vimentin in HNSCC specimens in that vimentin$^+$ cells appeared to co-localize with α-SMA$^+$ cells within the TME, but only limited to a portion of α-SMA$^+$ cellular structure (unpublished observation). The elevated vimentin expression in some α-SMA$^+$ cells within the HNSCC TME is particularly interesting because it is an intermediate filament protein that is believed to be expressed during the EMT transition (Richardson et al., 2017). Nevertheless, additional cellular and molecular analyses are warranted before any conclusions are drawn concerning whether vimentin$^+$ cells represent a transitional process from tumor cells to CAF-like cells via EMT or they represent a subtype of activated CAFs.

Heterogeneity in Tumor Cell Phenotype and Mutational Burden

Despite the homogenous origin of HNSCCs from the mucosa epithelial linings in the upper aerodigestive tract, HNSCC tumors are known to be highly heterogeneous based on comprehensive genomic analyses (Curry et al., 2014; Hammerman et al., 2015; Schoenfeld, 2015; The Cancer Genome Atlas Network [TCGA], 2015; Ferris et al., 2016; Leemans et al., 2018). IHC analysis also demonstrates a heterogeneous loss of epithelial markers, including epithelial cell adhesion molecule (EpCAM) or Keratin 76 (Krt76), in clinical specimens of HNSCC or oral SCCs, respectively, leading to more aggressive tumor progression and altered immune landscape (Ambatipudi et al., 2013; Baumeister et al., 2018; Pan et al., 2018; Sequeira et al., 2018). Similarly, our multiplex IHC analysis also showed a heterogeneous loss of epithelial markers, including EpCAM and cytokeratin in HNSCC tumors examined as previously reported (Ambatipudi et al., 2013; Baumeister et al., 2018; Pan et al., 2018; Sequeira et al., 2018).

Another imperative and immunologically relevant heterogeneity of HNSCCs is the differential mutational load between HPV (+) and HPV (−) tumors (Keck et al., 2015; The Cancer Genome Atlas Network [TCGA], 2015). HPV (−) HNSCCs exhibit high levels of mutational burden with a wide spectrum of gene mutations and amplifications. Emerging evidence suggests that cancer cells harboring mutations acquire new tumor-associated antigens, termed "neoantigens." Importantly, these neoantigens are perceived by host immune system as the "altered self" and therefore are ideal targets for cancer immunotherapy because of their exclusive expression in tumor cells (Schumacher and Schreiber, 2015; Wirth and Kühnel, 2017). In fact, experimental and clinical evidence strongly suggests that properly activated immune response against neoantigens is pivotal for the success of immunotherapy (Schumacher and Schreiber, 2015; Wirth and Kühnel, 2017).

Overall, HNSCCs exploit multiple immunosuppressive mechanisms to evade immunosurveillance and promote an immunosuppressive landscape that supports tumor initiation, progression, and metastasis. This high level of immunosuppression is further complicated by the heterogeneity at cellular, spatial, and molecular levels, all of which affect the clinical outcomes. Successful tumor elimination by immune cells largely depends on reversing/alleviating the immunosuppression and by the efficient access of activated anti-tumor effectors. Therefore, our better understanding of all aspects of the HNSCC heterogeneity will assist in the development of new immunotherapy strategies to improve the therapeutic outcomes (Chen and Mellman, 2013).

IMMUNOTHERAPY OF HNSCCs: CUREENT STATUS AND PERSPECTIVE

The goal of immunotherapy is to eliminate tumors or at least control tumor progression through strengthening immunosurveillance, enhancing the cytolytic activity of the immune effectors, and minimizing the potential of tumor equilibrium and escape. During the past decade, various immunotherapy approaches have been employed for HNSCC treatment. Although some of the immunotherapy regimens have resulted in an improvement of clinical outcome by prolonging cancer-free survival in a small fraction of patients when compared to the conventional therapy, the overall clinical response rate is lower than that observed in other tumor types treated with similar regimens. We will review the current status of the HNSCC immunotherapy trials that have mostly recruited patients with recurrent, metastatic (R/M) diseases regardless of their HPV-status. However, the clinical response of HPV (+) and HPV (−) patients are discussed separately whenever available.

HNSCC Cancer Vaccines

Vaccines are extremely effective in protecting the human population from some of the deadliest infectious diseases and have contributed to the worldwide eradication of smallpox and restriction of polio and measles. Since the validation of the involvement of HPV in cervical cancers, effective HPV vaccines have been developed and employed globally in the high risk populations for prevention (prophylactic) of HPV-induced tumors. Over the past decade, HPV vaccines have been proven to be safe and highly effective in preventing HPV-associated cervical lesions (Schiller et al., 2012; Sabeena et al., 2018). The objective of prevention is to inhibit viral entry. Therefore, the immunization targets are mostly based on the L1 viral capsid proteins via

viral-like particles, which stimulate a strong antibody response blocking viral entry and initial infection (Stanley et al., 2012).

Different from prophylactic vaccines, HPV-based therapeutic vaccines for cancer treatment rely heavily on productive activation of HPV antigen-specific T cells to target HPV-infected and transformed cells. For this purpose, HPV vaccines against viral oncoproteins E6/E7 have been developed for cervical cancers (Skeate et al., 2016), which represent an obvious immunotherapy option for HPV (+) HNSCC patients (Skeate et al., 2016; Schneider et al., 2018). This approach has been employed in a few HNSCC clinical trials using E6/E7 peptide, DNA, RNA, or attenuated vaccinia virus as the delivery vehicles. A systemic review of 11 independent HPV therapeutic vaccine trials from 2005 to 2017 revealed low therapy-associated toxicity in all 376 HNSCC patients with incurable, recurrent loco-regional, or distant metastatic disease at the time of enrollment (Schneider et al., 2018). Although not all of the trials were designed to demonstrate therapeutic efficacy, the clinical response rate for those with available data indicated that a range of 33–75% of patients showed a positive immune response, defined as elevated anti-HPV antibody, IFN-γ production, and T cell response (Schneider et al., 2018). Importantly, but not surprisingly, another clinical trial combining the PD-1 checkpoint blockade with therapeutic HPV vaccine demonstrated a further improvement in activating immune response to HPV-16 and prolonged overall survival as compared to either regimen alone (Massarelli et al., 2018). A recent report on a phase Ib/II clinical trial of HVP-specific DNA vaccine of 21 HNSCC patients also demonstrated an overall ∼85% of the patients showed an increase in IFN-γ producing antigen-specific T cells that lasted longer than 1-year (Aggarwal et al., 2018). Some patients also manifested an elevated $CD8^+$/Treg ratio and perforin-producing immune cells (Aggarwal et al., 2018) although the overall survival rate is not yet available. These results support the speculation that HPV (+) HNSCC patients respond better to immunotherapy, especially those that alleviate the existing immune suppressive elements in the TME.

For HPV (−) HNSCCs, high levels of mutational burden suggest the potential existence of targetable tumor-specific neoantigens for redirecting productive antitumor immunity (Gubin et al., 2015; Schumacher and Schreiber, 2015; Wirth and Kühnel, 2017). Because *TP53* mutation associated with accumulation of p53 protein represents one of the widespread gene alterations in the HPV (−) HNSCCs, targeting WT or mutant p53 via tumor vaccine has been a primary approach tested in clinical trials. An early report of a p53 and k-ras peptide vaccine trial demonstrated a response rate of ∼42% HNSCC patients with an increased frequency of IFN-γ producing CTLs, associated with their prolonged survival (Carbone et al., 2005). The observations of Couch et al. (2007) further suggested that mutant p53 peptides bind to MHC molecules with higher affinity than wild-type p53 counterparts and activated p53-specific T cells in culture, thereby representing an effective target. Likewise, the recent results of a phase I trial of p53-peptide loaded autologous DC vaccine together with immune adjuvant demonstrated *in vivo* activation of p53-specicity T cells and a favorable 2-year disease-free survival with low levels of toxicity (Schuler et al., 2014). Associated with the increases in p53-specific CD8 T cells and elevated IFN-γ production, the frequency of Tregs were reduced in some patients (Schuler et al., 2014). Nevertheless, the authors concluded that stronger DC maturation stimuli are desired to further enhance/maintain DC function in the immunosuppressive TME of HNSCCs and to improve therapeutic efficacy (Schuler et al., 2014). Another phase II clinical trial of peptide-based vaccine against three antigens, LY6K, CDCA1, and IMP3, identified via cDNA microarray from HNSCCs demonstrated improved immune responses to these specific-antigens and furthermore, overall clinical outcome (Yoshitake et al., 2015).

In addition to the activation of conventional T cells, vaccines to activate invariant natural killer T (iNKT) cells were tested by Takami et al. (2018). iNKT cells are special types of T cells that recognize lipid antigens, such as α-galactosylceramide (α-GalCer), that present on CD1d. They are known to rapidly produce effector cytokines and orchestrate with other immune cells to fight against pathogens and cancers (reviewed in Bedard et al., 2017; Takami et al., 2018). The results of various clinical trials with HNSCC patients suggest that iNKT cells could be activated by α-GalCer-pulsed APCs *in vivo* and lead to antitumor immunity (Uchida et al., 2008; Kunii et al., 2009). Interestingly, these studies also demonstrated that the route and geolocation of APC delivery is important for immune activation because nasal submucosa delivery promoted antitumor immunity, whereas APC injection into submucosa of the oral floor led to immune tolerance induction (Uchida et al., 2008; Kunii et al., 2009; Kurosaki et al., 2011).

Overall, considering the relatively high level of either viral antigens or mutation-associated neoantigens and immune infiltrates in different subtypes of tumors, HNSCCs represent good candidates for immunotherapy, especially if the immunosuppressive elements are alleviated prior to or simultaneously, with the vaccine. Clinical translation of this strategy, especially for HPV (−) HNSCCs, may benefit from personalized immunotherapy, which employs identified/defined unique neoantigens from each patient or autologous tumor (lysate) vaccine.

Adoptive Transfer (ACT) of Activated Tumor-Specific T Cells

Adoptive transfer of *ex vivo* activated and expanded autologous tumor antigen-specific T cells represents a promising strategy to obtain high number of productively activated effectors. Most of the T cells were activated and expanded *ex vivo* via cytokine and anti-CD3/CD28 or tumor specific-antigen-dependent stimulation followed by adoptive transfer to tumor patients. Alternatively, these autologous T cells can also be genetically engineered to recognize a defined antigenic epitope by incorporating a chimeric antigen receptor (CAR). So far, only limited cases of ACT for HNSCC treatment have been reported. In an early study, 15 HNSCC patients with recurrent and metastatic disease were treated with one dose of ACT of autologous T cells, which were obtained from draining lymph

node and expanded *ex vivo* via mitogen stimulation (To et al., 2000). In this cohort of treated patients, three showed stable disease and two achieved favorable response, among which one experienced complete remission for 4+ years (To et al., 2000). Likewise, Jiang et al. (2015) reported the results of an ACT clinical application of *ex vivo* expanded autologous T cells by anti-CD3 and cytokine in a cohort of 43 HNSCC patients following their first line chemo- and/or radio-therapy treatment. Overall, a modest improvement in the median progression-free survival from 40 months in the non-ACT control group to 56 months in ACT treated patients. Additionally, 3-year overall survival to 58 months as compared to the non-ACT control of 45 months (Jiang et al., 2015). In a phase II trial, patients with EBV^+ nasopharyngeal carcinomas were first treated with four cycles of chemotherapy followed by up to six does of EBV-specific T cells recognizing viral protein LMP2 (Chia et al., 2014). The overall 2-year and 3-year survival rates were ∼63% and ∼37%, respectively. Strikingly, five patients experienced a complete remission for longer than 34 months, and the overall immune response in this cohort of was ∼71% (Chia et al., 2014).

Remarkably, one recent report of a personalized immunotherapy for HPV-associated cervical cancer via adoptive transfer of *ex vivo* activated autologous tumor-infiltrating T cells revealed that effective elimination of HPV-associated cancers was dependent on T cells specifically targeting mutant endogenous neoantigen and cancer germline antigens rather than viral antigens (Stevanović et al., 2017). Thus, it is speculated that a similar therapeutic strategy may be implemented for HPV-associated HNSCCs.

Checkpoint Inhibitor Therapy

The immune landscape of HNSCCs, especially HPV (+) tumors, is associated with elevated expression of the checkpoint molecules PD-1 and/or CTLA-4 on T cells (Badoual et al., 2006; Loose et al., 2008; Badoual et al., 2013; Mandal et al., 2016). In a subset of HNSCC patients, PD-L1 expression is frequently observed on a variety of immune and non-immune cells, including CAFs and tumor cells (Concha-Benavente et al., 2016). Therefore, the PD-1/PD-L1 checkpoint pathway is highly active in the HNSCC TME and suppressing the checkpoint pathway, either as a monotherapy or in combination with other immunotherapy interventions, represents a promising target for enhancing anti-tumor responses to control and eliminate HNSCCs.

Early checkpoint inhibitor clinical trials for HNSCC treatment did not discriminate patients based on their HPV status and showed an overall response rate (ORR) of ∼10–20% among the total treated HNSCC patients. As we now appreciate the high level of heterogeneity in the TME of HNSCCs concerning the HPV status and tumor types, it becomes clear that analyzing and presenting the HNSCC clinical trial results by segregating HPV (+) patients from HPV (−) cases will be more informative. For instance, Keynote 012 Phase 1b anti-PD-1 antibody (pembrolizumab) trial treated a cohort of 60 R/M HNSCC patients positive for PD-L1 expressing tumors (>1% via IHC staining), with 10 mg/kg every 2 weeks. The ORR for the entire cohort was 18%, specifically with a 25% ORR for HPV (+) patients and 14% for HPV (−) patients (Seiwert et al., 2016). An expansion of this trial involved another cohort of 132 HNSCC patients, regardless of HPV and PD-L1 status, receiving the same antibody, in a dose of 200 mg every 3 weeks, that demonstrated a similar ORR of 18–20%. Interestingly, ORR for PD-L1 positive patients was 22%, significantly higher than PD-L1 negative patients (4%) (Chow et al., 2016). A follow up report of the long-term effects confirmed a durable response and clinical benefits in these treated patients with a 12-month ORR of higher than 71%, survival rate of 38%, and even antitumor responses in some patients lasting for longer than 30 months (Chow et al., 2016; Seiwert et al., 2016; Mehra et al., 2018).

A similar phase 3 anti-PD-1 (nivolumab) trial, Checkmate 141, which enrolled 361 recurrent HNSCC patients who failed standard chemotherapy, treated the patients with either 3 mg/kg body weight of anti-PD-1 every 2 weeks or conventional single-agent systemic therapy. In patients receiving nivolumab, the ORR and 6-month/1-year survival rate were better than those who received standard single-agent therapy (Ferris et al., 2016; Harrington et al., 2017), and a follow up report of 2-year long-term survival indicated a prolonged survival benefit for patients with PD-L1 positive tumors over those with PD-L1 negative tumors, regardless of HPV status (Ferris et al., 2018a). On the other hand, a recent study of a cohort of 126 HNSCC patients treated with anti-PD-1/L1 therapy suggested that HPV (+) patients experienced better clinical responses and outcomes compared to HPV (−) patients (Hanna et al., 2018). Remarkably, HPV (−) patients whose tumors exhibited higher mutational load and $CD8^+$ T cell infiltrates showed a better response to the checkpoint inhibitor therapy, whereas patients with $CD8^+$ T cells manifesting an exhausted phenotype of TIM-3/LAG-3 co-expression with PD-1 were poor clinical responders to the checkpoint inhibitors (Hanna et al., 2018).

The demonstration of differential clinical responses to checkpoint inhibitors in patients with $PD\text{-}L1^+$ tumors is remarkable. Early studies reported the co-existence of $PD\text{-}1^+$ T cells and $PD\text{-}L1^+$ tumors with $CD68^+$ TAMs in HPV (+) HNSCCs (Lyford-Pike et al., 2013). Furthermore, the observed association of $PD\text{-}L1^+$ tumors with CD8 T cell expression of PD-1 and significantly elevated IFN-γ mRNA within the same TME indicated that PD-L1 was upregulated by activated T cells in the TME to augment an immunosuppressive landscape by enforcing the PD-1/PD-L1 pathway (Lyford-Pike et al., 2013). Thus, PD-L1 positivity may be considered as a potential marker for clinical response of HPV (+) HNSCC patients to checkpoint inhibitors, although a recent study of 126 HNSCC patients treated with anti-PD-1/L1 therapy demonstrated that PD-L1 alone could not serve as a robust predictor of clinical response (Hanna et al., 2018). Nevertheless, the targeted therapy of anti-PD-L1 antibodies (durvalumab or atezolizumab) has been employed in clinical trials (Cavalieri et al., 2018; Colevas et al., 2018). Colevas et al. (2018) reported the results of a phase 1a trial of 32 HNSCC patients receiving anti-PD-L1 every 3 weeks, which showed an ORR of 22% and no clear differences between HPV (+) and HPV (−) patients. The results of other trials are yet to be reported (Cavalieri et al., 2018).

To determine whether simultaneous blockade of two independent checkpoint molecules of PD-1 and CTLA4 will

further improve the clinical outcome, Schwab et al. (2018) tested the combination effects of nivolumab and ipilimumab (anti-CTLA4) and reported a clinical case concerning treatment of a refractory HNSCC patient. A near complete remission was observed within 5 months of the combinatory treatment. Furthermore, following the onset of a local relapse at 7 months, combined radiotherapy and anti-PD-1 regimen was able to control tumor progression and support survival of the patient in stable disease for longer than a year (Schwab et al., 2018).

Other Strategies to Reverse Immunosuppression and Reactivate Antitumor Immunity

Therapeutic interventions to enhance or reactivate antitumor immunity can be achieved by either alleviating the immunosuppressive cellular subpopulations or activating co-stimulatory pathways. Although many of these approaches have been tested in experimental models, publicly accessible clinical data are limited. Currently, the results of a few HNSCC clinical studies associated with inhibiting/reducing the immune inhibitory myeloid populations, such as MDSCs, or enhancing the immunostimulatory pathways have been published.

Tadalafil is an inhibitor of phosphodiesterase 5 (PDE5), which suppresses the function of MDSCs by inhibiting the production of iNOS and arginase-1. Califano et al. (2015) reported the employment of Tadalafil as a neoadjuvant in a phase II clinical trial with a cohort of 40 HNSCC patients. Overall, Tadalafil treatment led to a significant reduction of MDSCs and Tregs in both circulation and tumors, as well as an elevation of circulating CD8 T cells and improved T cell proliferative capacity *in vitro* (Califano et al., 2015). In a similar clinical study with 35 HNSCC patients, Weed et al. (2015) showed that Tadalafil modified the immune landscape of the TME with a significant increase in intratumor $CD69^{+}CD8^{+}$ T cells and a concordant reduction in Tregs, following a dose-dependent pattern. Because the objective and endpoint of these studies are immunomodulation, not the clinical improvement of tumor progression or patient survival, the long-term effects of Tadalafil treatment on HNSCC patient survival is unknown.

Anti-EGFR antibody, cetuximab, is a standard FDA approved targeted agent for HNSCC treatment. Currently cetuximab alone, or in combination with conventional radio- or chemo-therapy, only provides temporary and modest clinical benefit (Leemans et al., 2018; Zandberg and Ferris, 2018). Recently, the potential of cetuximab as a neoadjuvant for immune modulation has been evaluated. In a phase 1b clinical trial with 14 HNSCC patients enrolled, cetuximab together with a TLR8 agonist, motolimod, reversed MDSC-induced immunosuppression by inducing their conversion into M1 macrophages and improved antitumor immunity associated with increased number and function of tumor infiltrating CD8 T cells (Shayan et al., 2018). The results of an extended clinical study of a cohort of 195 R/M HNSCC patients treated with cetuximab and TLR8 agonist also demonstrated a T cell profile of immune activation and observed significant improvement in immune response at the injection site, especially in HPV (+) patients (Ferris et al., 2018b). However, no significant improvement in either progression-free survival or overall survival was reached (Ferris et al., 2018b). Mechanistic studies of cetuximab-induced immune modulation, either cetuximab alone or in combination with anti-CD137, illustrated that cetuximab activates NKs and DCs via Fc receptor-dependent pathway, subsequently leading to the activation of Th1/CTL responses and elevated APM for activation of tumor-specific T cells (Srivastava et al., 2017).

PERSPECTIVES

Recent major advances in cancer immunotherapy, especially the immune checkpoint inhibitors targeting the PD-1/PD-L1 and CTLA-4 pathways, demonstrate remarkable curative benefits for some cancer patients. Despite a relatively low clinical response rate of HNSCC patients to the checkpoint inhibitors, the above described HNSCC clinical trial results with molecular and cellular profiles resulting from the above described HNSCC clinical trials provide invaluable insight into the challenges and opportunities for further improving the clinical outcomes of HNSCC immunotherapy.

It is now clear that HPV (+) HNSCCs are more responsive to immunotherapies, including the checkpoint inhibitor therapy, than HPV (−) tumors and associated with a better clinical prognosis. Notably, the immune landscape of HPV (+) HNSCCs exhibits a unique profile of inflamed, yet immunosuppressed, TME with heavy immune infiltrates of $CD8^{+}PD\text{-}1^{+}$ T cells and Tregs. This information suggests that HPV (+) tumor-associated immune infiltrates are more likely to respond to immune activation stimuli when the existing immune suppressive elements are timely removed/eliminated. To this end, Treg depletion or in combination with a checkpoint inhibitor, is likely more productive for immune activation than either regimen alone. Thus, it is proposed that upon depletion or inhibition of the immunosuppressive elements, tumor-specific T cells can be productively activated by professional APCs. This active regimen of T cell activation can be achieved via DC or tumor vaccines, as well as ACT generated against either HPV-specific antigens or tumor-specific neoantigens. Given the recent report of autologous neoantigen-specific T cell-mediated effective elimination of HPV-associated cervical cancers (Stevanović et al., 2017), it is anticipated that similar therapeutic effects can be achieved for treating HPV (+) HNSCCs. Different from the HPV-viral antigen-specific T cell activation, the neoantigen-specific antitumor immunity relies on individualized immunotherapy maneuvers because the mutation events and corresponding neoantigens that vary among patients, but support more productive antitumor immunity with better clinical outcomes.

The TME of HPV (−) HNSCC is highly immunosuppressed and associated with low levels of immune infiltrates. Existing clinical data suggest that HPV (−) HNSCCs are poor responders to immunotherapy, including the checkpoint inhibitor therapy, that is likely due to their immune excluded or desert landscape. Nevertheless, besides the overall lack of T cell accessibility to tumors, this observed unresponsiveness to immunotherapy

can also be the result of the enforced immunosuppression by immune inhibitory cytokines/molecules, APM dysfunction, and/or immunosuppressive myeloid populations. Given the highly heterogeneous nature of the HNSCC TME, it is imperative to examine the pattern of immune cell distribution within the TME via IHC-based topography. In combination with the flow cytometry based cellular profiling and genomic based molecular profiling, IHC topographic results will assist in identifying the specific immunosuppressive pathway(s) or element(s) as targets for the individualized immunotherapy strategy to reverse the immune suppression and simultaneously promote neoantigen specific-antitumor immunity. The existence of widespread high levels of mutation burden in the HPV (−) HNSCC tumors present a favorable opportunity for activating a broad scope of neoantigen-specific antitumor immunity when the dominant immunosuppressive mechanism in the HNSCC TME is identified and alleviated. Routine clinical protocols for targeted MDSC or Treg depletion or conversion of M2 macrophages to activated DCs/M1 macrophages are established for many tumor types and can be employed for HNSCCs. It is speculated that the more challenging aspect of a productive strategy for eliminating HPV (−) HNSCC tumors is to enhance T cell accessibility to tumors. This may be improved by the checkpoint blockade in combination with the administration of specific chemokines that improve T cell mobility, such as CXCL9, CXCL10, and CXCL11. Furthermore, the defective or dysfunctional APM in the TME can be addressed by cytokine-induced HLA upregulation and the employment of NK-based tumor elimination.

Besides the well studied immunosuppressive cellular subsets of MDSCs, Tregs, and M2 macrophages, CAFs represent another crucial population that not only provides structure stability for the TME, but also promotes tumor survival/metastasis and regulates the immune landscape of the TME. Therapeutic interventions specifically targeting CAFs represent an appealing multipronged strategy that reduces the tumor survival factors and reverses the immunosuppressive landscape, thereby enhancing antitumor immunity and improving therapeutic outcomes. On the other hand, our understanding of HNSCC-CAF immunobiology and the specific surface markers for therapeutic targeting is still limited. One of the surface molecule LRRC15, identified by Purcell et al. (2018) represents an attractive candidate for further exploration toward its potential clinical application of targeting HNSCC-CAFs.

CONCLUSION

In conclusion, recent clinical, genomic, and cellular studies of HNSCCs demonstrate the high levels of heterogeneity and immunosuppression in the HNSCC TME. The differential molecular and immune landscapes between HPV (+) and HPV (−) tumors present new opportunities for the development of individualized targeted immunotherapy strategy. It is proposed that the informed design of immunotherapy trials based on our understanding of HNSCC biology, molecular and immunological landscape, as well as topography of immune cell distribution in the TME, will assist in developing new strategies for a productive antitumor immunity to improve the clinical outcomes.

AUTHOR CONTRIBUTIONS

MC, GG, and YC reviewed the literature and wrote the manuscript. MY, CM, MG, and JKB reviewed the literature, participated in discussion, and revised the manuscript.

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REVIEW
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Cancer-Associated Fibroblasts Build and Secure the Tumor Microenvironment

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Tumor cells reside in a highly complex and heterogeneous tumor microenvironment (TME), which is composed of a myriad of genetically stable non-cancer cells, including fibroblasts, immune cells, endothelial cells, and epithelial cells, and a tumor-specific extracellular matrix (ECM). Cancer-associated fibroblasts (CAFs), as an abundant and active stromal cell population in the TME, function as the signaling center and remodeling machine to aid the creation of a desmoplastic tumor niche. Although there is no denial that the TME and CAFs may have anti-tumor effects as well, a great deal of findings reported in recent years have convincingly revealed the tumor-promoting effects of CAFs and CAF-derived ECM proteins, enzymes, chemical factors and other downstream effectors. While there is growing enthusiasm for the development of CAF-targeting therapies, a better understanding of the complexities of CAF-ECM and CAF-cancer cell interactions is necessary before novel therapeutic strategies targeting the malignant tumor "soil" can be successfully implemented in the clinic.

Keywords: cancer-associated fibroblast, tumor microenvironment, extracellular matrix, therapy, mechanoreciprocity

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INTRODUCTION

In the last decades, despite considerable advances in the development of novel immunotherapies and targeted therapies, no significant improvements have been made in overall survival rates for patients with malignant solid tumors. One major reason for this lack of substantial improvement is the development of drug resistance in tumor cells, which usually reveals itself within a few months after patients are treated with anti-cancer drugs. An Achilles' heel of many current therapeutic approaches is that these therapies primarily target the fast-growing tumor "seeds" but largely ignore the fertilizing tumor "soil" – the tumor microenvironment (TME) (de Groot et al., 2017). The TME influences the penetration, distribution, and metabolism of therapeutic agents, and produces molecular factors and signals, which positively or negatively regulate how tumor cells grow, migrate and respond to therapeutic agents. As cancer-associated fibroblasts (CAFs) appear to be a major TME component in many tumors and are critical for shaping the "soil" within which the tumor cells thrive (LeBleu and Kalluri, 2018), they have become the prime target for the efforts to modify non-tumor cell behavior to suppress tumor growth. It is clear that the TME and CAFs are not always pro-tumorigenic due to the complexities of their interactions with tumor cells. However, in this review, we will mainly explore the tumor-promoting interactions between cancer cells and

fibroblasts and how CAFs may be persuaded using novel therapeutic approaches to renounce their fealty to the tumor cells and even produce a tumor-suppressive "soil."

STROMAL FIBROBLASTS, MYOFIBROBLASTS, AND CAFs

Tumors are often referred to as "wounds that never heal" (Dvorak, 1986) because the stroma of a wound and a tumor share many similarities, such as fibroblast activation, increased extracellular matrix (ECM) protein production and intensive remodeling processes (Foster et al., 2018). Activated stroma is molecularly, biochemically and pathologically different from the normal stroma. In the stroma of normal human skin, fibrous proteins fill in the interstitial space between stromal fibroblasts while epithelial keratinocytes rest on the sheet-like basement membrane. Under normal physiological conditions, non-contractile fibroblasts are generally flat, spindle-shaped and recognized as quiescent and inert cells in the ECM (Valkenburg et al., 2018).

Myofibroblasts were first identified in the tissue wound repair process, during which fibroblasts or smooth muscle cells differentiate and gain a contractile phenotype (McAnulty, 2007). The major roles of myofibroblasts in wound healing are to contract the wounds and produce and organize the ECM (Darby et al., 2014). As the wound closes and heals, myofibroblasts become apoptotic and finally disappear as the scar is formed (Desmouliere et al., 1995). Myofibroblasts are different from normal fibroblasts in many aspects, including (1) ruffled membranes and a highly active endoplasmic reticulum (Baum and Duffy, 2011); (2) expression of alpha smooth muscle actin (α-SMA or ACTA2) and increased levels of vimentin (VIM) (Ronnov-Jessen and Petersen, 1993) and (3) formation of complex and organized stress fibers and fibronexus adhesion complexes (Rao et al., 2016). The bundles of microfilaments in myofibroblasts interact with the ECM proteins through fibronexus adhesion complexes, thereby allowing myofibroblasts to sense the tension in their surrounding microenvironment and maintain the cellular contractile force through the network of cytoskeletal proteins. As a feedback response, myofibroblasts increase matrix fibroplasia by producing ECM proteins, including collagen, elastin (ELN), fibronectin (FN1), tenascin (TNC), and remodeling enzymes, such as matrix metalloproteinases (MMPs).

Tumor growth recapitulates the basic wound healing program and shares many similarities, such as deposition and crosslinking of fibrin and FN1 and the recruitment of immune cells (Schafer and Werner, 2008). However, unlike a normal healing wound, which is restricted to a certain area and proceeds directionally through the steps of hemostasis, inflammation, proliferation, and maturation/remodeling, cancer cells distort the wound healing program and have the potential to migrate away or expand from the initiation site and invade adjacent tissues. CAFs are the fibroblasts found in the stroma of human cancers but differ from normal fibroblasts in their increased collagen and ECM protein production and up-regulated secretion of pro-tumor factors (Bauer et al., 2010; Xing et al., 2010; Pidsley et al., 2018). There are several important sources from which CAFs could be derived, including: (i) recruitment and activation of resident fibroblasts (Fukino et al., 2004); (ii) epithelial-mesenchymal transition (EMT) of resident epithelial cells (Petersen et al., 2001); (iii) endothelial to mesenchymal transition (EndMT) of resident endothelial cells (Zeisberg et al., 2007a,b) and (iv) differentiation of bone marrow mesenchymal cells (Quante et al., 2011). In a sense, CAFs or at least a subset of CAFs are wound-like myofibroblasts that mediate a deranged chronic wound healing program in tumors. For example, a large part of CAFs share similar features as α-SMA-positive (α-SMA+) myofibroblasts (Shiga et al., 2015). In addition, other than myofibroblastic CAFs, subpopulations of CAFs without α-SMA expression have also been reported, e.g., in pancreatic cancer (Ohlund et al., 2017).

HETEROGENEITY OF STROMAL FIBROBLASTS, MYOFIBROBLASTS, AND CAFs

Understanding the state, complexity and heterogeneity of normal fibroblasts may shed light on the origins of CAFs, how they form and how their transdifferentiation may be regulated in the early stages of tumorigenesis and at the tumor front. Two major populations of fibroblasts in the human dermis are papillary and reticular fibroblasts, which possess distinct morphology, molecular expression, and cellular functions (Harper and Grove, 1979). Janson et al. (2012) performed gene expression analysis on cultured papillary and reticular fibroblasts and identified 116 differentially expressed genes. However, except for matrix Gla protein (MGP), which is almost exclusively expressed in the reticular dermis, they did not discover any *in vivo* markers to separate the two fibroblast populations. Korosec et al. (2019) performed lineage identity and location studies of human dermis using two markers, fibroblast activation protein (FAP) and THY1 (Cluster of Differentiation 90 or CD90). They found that papillary fibroblasts are FAP^{+}; $THY1^{-}$, whereas FAP^{-}; $THY1^{+}$ fibroblasts are mainly of the reticular lineage. Their data showed papillary and reticular fibroblasts are not completely separated according to their spatial location.

However, recent studies have suggested that there exist more functionally distinct fibroblast subpopulations within the human dermis. A single-cell RNA sequencing (scRNA-seq) study by Philippeos et al. (2018) showed that there are five distinct fibroblast populations in adult human skin, which can be separated based on the expression of cell surface markers, including THY1, CD39, CD26 (DPP4), and regulator of G protein signaling 5 (RGS5), and are not spatially segregated. Tabib et al. (2018) performed single-cell transcriptomal analysis of cells obtained from whole skin without pre-purifying fibroblast populations. They identified two major fibroblast populations based on the expression of SFRP2/DPP4 and FMO1/LSP1 markers and five minor cell populations using CRABP1, COL11A1, PRG4, ANGPTL7, and SFRP4. In addition, there are several subpopulations in each major fibroblast population. These scRNA-seq data showed a complex and heterogeneous

picture of fibroblast composition and functionality in the human dermis, which is simply beyond our original understanding of skin fibroblasts. Nevertheless, it remains to be understood how these subpopulations of fibroblasts react to either wounding or the tumorigenic process and evolve into myofibroblasts or CAFs.

Local fibroblasts are the most common origin of myofibroblasts (Hinz et al., 2007). However, several other cell types are able to differentiate into myofibroblasts, including smooth muscle cells or pericytes (Hinz et al., 2007). Fibrocytes, for example, can differentiate into myofibroblasts in skin, liver and lung tissues (Mori et al., 2005; Iwaisako et al., 2012; Ashley et al., 2017). In the liver, hepatic stellate cells are the source of myofibroblasts in liver fibrosis (Wells and Schwabe, 2015). Because of the nature of its diverse origins, myofibroblasts appear to be a heterogeneous group as well. α-SMA is the most commonly used marker to identify myofibroblasts (McAnulty, 2007). In addition, extra domain A fibronectin (EDA-FN), periostin (POSTN) and prolyl-4-hydroxylase (P4HB) have also been suggested as potential markers for myofibroblasts (Moore-Morris et al., 2014; Ngo et al., 2014; Kanisicak et al., 2016). A recent study proposed that amine oxidase, copper containing 3 (AOC3) and homeobox protein NKX2-3 are two biomarkers of pericryptal myofibroblasts in the colon and rectum (Hsia et al., 2016). Furthermore, markers that stain stromal fibroblasts can also be used to stain myofibroblasts, such as platelet derived growth factor receptor alpha (PDGFRA), THY1, and VIM, although they are not specific for myofibroblasts (Matthijs Blankesteijn, 2015).

Just like normal fibroblasts, CAFs appear to be a heterogeneous group of cells with different origins and different functions. This similarity was manifested by a study reported by Lambrechts et al. (2018). By performing scRNA-seq of 52,698 stromal cells isolated from human lung tumors and comparing with matching non-malignant lung samples, the authors identified five distinct types of fibroblasts in lung tumors, which all express their own unique set of collagens and ECM proteins that are different from non-malignant fibroblasts. Using a three-dimensional (3D) co-culture platform, Ohlund et al. (2017) identified two distinct populations of myofibroblasts and inflammatory fibroblasts in pancreatic ductal adenocarcinoma (PDA). More recently, the obscurity in CAF characterization has been further addressed by efforts to determine the exact composition of human tumor tissues using scRNA-seq. scRNA-seq data derived from head and neck squamous cell carcinoma (HNSCC) suggested that tumor myofibroblasts and CAFs may represent distinct fibroblast subpopulations (Puram et al., 2017). Overall, the authors were able to detect, in addition to normal fibroblasts and myofibroblast-like cells, two subsets of CAFs depending on the expression of FAP, THY1, connective tissue growth factor (CTGF) and podoplanin (PDPN). In their study, tumor myofibroblasts were identified based on the expression of α-SMA, melanoma cell adhesion molecule (MCAM), myosin light chain kinase (MYLK), and myosin light chain 9 (MYL9). Interestingly, scRNA-seq of colorectal cancer samples also revealed at least three fibroblast populations (Li et al., 2017). One population can be described as normal fibroblasts, the second one as myofibroblasts, which are positive for α-SMA, transgelin (TAGLN) and PDGFA, and the third one as a CAF population that is characterized by MMP2, decorin (DCN) and collagen type I alpha 2 (COL1A2). The authors determined that a key signaling pathway emanating from CAFs/myofibroblasts is transforming growth factor beta (TGF-β)/INHBA signaling, ascertaining that CAFs are not just ECM-producing factories. The scRNA-seq results of fibroblast populations are in good accordance with attempts to characterize CAFs using fluorescence activated cell sorting (FACS) (Costa et al., 2018). Such efforts in human breast cancer using six CAF markers, including FAP, integrin beta 1 (ITGB1), α-SMA, FSP1, platelet derived growth factor receptor beta (PDGFRB), and caveolin-1 (CAV1), allowed the authors to identify four distinct CAF populations, of which some were preferentially present in subsets of breast cancers. Two of the CAF populations expressed α-SMA and probably represent myofibroblast-like cells. However, a comparison of the two α-SMA+ populations revealed that one was similar to pericytes and expressed MCAM and a gene signature related to the regulation of actin cytoskeleton and muscle contraction. The second α-SMA+ population exhibited an immune-regulatory gene signature. These CAFs can function as immune-suppressors and regulators of T lymphocytes and create an immunosuppressive environment through a multi-step mechanism (Costa et al., 2018).

scRNA-seq studies of CAFs have suggested that CAF subtypes could be attributed to their origin in spatial subgroups of normal fibroblasts (Philippeos et al., 2018; Tabib et al., 2018). However, Biffi et al. (2019) reported that tumor-secreted TGF-β/ and IL1 can promote CAF heterogeneity. Subsets of CAFs can function to either support or suppress tumor cells. For example, it was reported that cancer cells undergo the EMT process and acquire invasive phenotypes through the activation of the TGF-β-SMAD signaling pathway induced by CAFs (Bellomo et al., 2016). In addition, by producing pro-angiogenic factors, such as fibroblast growth factor 2 (FGF2) and VEGFA (De Palma et al., 2017), CAFs regulate angiogenesis in the stroma, thereby providing essential nutrients for highly proliferative tumor cells. CAFs can also assist tumor cells in overcoming immune surveillance by recruiting immunosuppressive cells, such as M2 macrophages and myeloid-derived suppressor cells (MDSC) (Flavell et al., 2010; Yang et al., 2016). However, it was reported that ablation of subsets of α-SMA+ CAFs in PDA could result in a more aggressive cancer phenotype and reduced animal survival (Ozdemir et al., 2014). In summary, the heterogeneity of CAFs reflects the diversity and complexity of the TME, and more careful research is needed to fully comprehend the interactions among CAFs, tumor cells and the ECM.

CAF-DERIVED ECM PROTEINS

The tumor ECM is composed of a complex mixture of macromolecules, including fibrous proteins (collagen, ELN), proteoglycans (heparan sulfate, chondroitin sulfate), glycosaminoglycans (hyaluronic acid), and glycoproteins (FN1, laminins, TNC) (Botti et al., 2013). ECM proteins are not just bystanders of the tumorigenic process. Instead, they provide structural signals and support for tumor cells to grow and

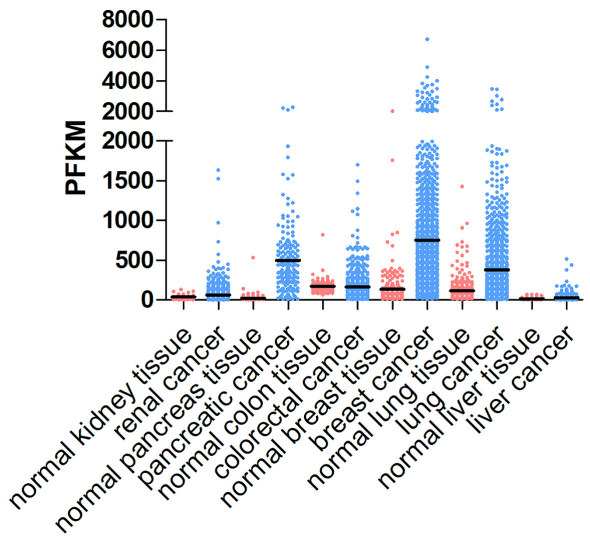

FIGURE 1 | COL1A1 expression levels in different cancer types and corresponding normal tissues. COL1A1 expression levels vary in different types of cancer, including renal cancer, pancreatic cancer, colorectal cancer, breast cancer, lung cancer, and liver cancer and generally are higher in tumor tissues than those in normal tissue. The data are obtained from https://www.proteinatlas.org/ENSG00000108821-COL1A1/tissue and https://www.proteinatlas.org/ENSG00000108821-COL1A1/pathology.

migrate. Although many other stromal cell types and tumor cells can also produce ECM proteins, CAFs appear to be the major player in the stroma that synthesizes, secrets, assembles and modifies the ECM composition and organization (Faouzi et al., 1999; Yoshimura et al., 2015; Erdogan et al., 2017). For example, elevated collagen production and crosslinking have been coupled with increased tumor stiffness and progression. It was estimated that fetal rat fibroblasts synthesize about 40 molecules of procollagen/cell per second (McAnulty et al., 1991). Many cancers are characterized by elevated levels of collagen production, e.g., COL1A1 (**Figure 1**). Faouzi et al. (1999) reported that myofibroblasts are the primary source of collagen (types I, IV, V and VI) in the stroma of human hepatocellular carcinoma. In addition, CAF-derived laminin was shown to induce cervical cancer cell migration via the interaction with integrin α6β4 (Fullar et al., 2015). In an *in vitro* ovarian cancer spheroid model, CAF-secreted versican promoted cancer invasion in a TGF-β-dependent manner (Yeung et al., 2013).

FN1 was first found to be overexpressed in human solid tumor specimens in 1981 (Stenman and Vaheri, 1981). Although tumor cells produce FN1 themselves, stromal cells, such as CAFs, are indispensable for bulk FN1 assembly (Attieh et al., 2017; Erdogan et al., 2017). Like collagen, the pro-tumorigenic role of FN1 is also well-acknowledged. In 1998, Menzin et al. (1998) proposed that FN1 may play an important role in regulating the invasive phenotype and poor patient prognosis in ovarian cancer. FN1 was also documented to promote cell cohesion, basement membrane invasion and tumor growth in glioblastoma (GBM). Depletion of FN1 in GBM cells resulted in weaker cell-cell contact and less collective migration in an *in vitro* spheroid model, highlighting the role of FN1 as a "biological glue" (Serres et al., 2014). The role of FN1 in cell cohesion has also been observed in fibroblast spheroids. FN1-depleted fibroblasts failed to form compact spheroids *in vitro*. Furthermore, the blockade of FN1-integrin interactions impeded fibroblast activation (Salmenpera et al., 2008).

Tenascin is another highly expressed ECM glycoprotein in the tumor stroma, such as the stroma of canine mammary carcinoma, pancreatic cancer and prostate cancer, and is associated with poor patient prognosis (Yoshimura et al., 2015; Cai et al., 2017; Ni et al., 2017). Mouse embryonic fibroblasts lacking TNC have robust overexpression of tissue plasminogen activator (tPA) and increased capacity to digest fibrin *in situ* (Brellier et al., 2011). Furthermore, they discovered that there was a correlation between *in vivo* TNC expression and fibrin accumulation in head and neck squamous cell carcinomas (SCC) and lung carcinomas, further confirming that TNC functions as a regulator of the fibrinolytic system.

CAF-DERIVED ECM ENZYMES

Tumor progression and metastasis require a distinct ECM biomechanical architecture, for which CAFs not only produce and secrete ECM proteins and also actively participate in the ECM proteolysis, crosslinking and assembly processes. In such a rigid and highly crosslinked tumor stroma, drug penetration is one potential reason for tumor cells to escape therapy. In addition, CAF-mediated ECM remodeling is a highly responsive process of receiving, processing and responding to the cellular, molecular and mechanical signals in the TME. The lysyl oxidase (LOX) family and MMPs represent two major types of remodeling enzymes produced by CAFs. As a highly adaptive and mechanically responsive stromal cell type, CAFs sense and respond to the ECM stiffness in a LOX/MMP-dependent manner and further fine-tune the CAF-ECM interactions.

The LOX family oxidases include five members: LOX and lysyl oxidase like (LOXL) 1, 2, 3, and 4 (Wang et al., 2016). They share similar structures and catalyze the cross-linking of collagen and ELN by oxidation, contributing to increased stiffness of the tumor stroma. In tissue fibrosis, it was demonstrated that fibroblast-derived LOX could be induced by different soluble factors, such as insulin-like growth factor-binding protein 5 (IGFBP5) (Nguyen et al., 2018) and POSTN (Kumar et al., 2018), and by the transcription factor hypoxia inducible factor 2 alpha (HIF2A) (Hikage et al., 2019). Elevated levels of LOX family oxidases are often observed in cancers and play a prominent role in cancer progression. Gene expression analysis of mouse mammary tumors revealed that activated fibroblasts are the major producers of LOX family oxidases (Pickup et al., 2013). When colon cancer patient-derived CAFs and normal fibroblasts were compared by proteomic analysis, LOXL2 was found to be overexpressed in CAFs and was identified as a predictive prognostic factor in stage II colon cancer patients (Torres et al., 2015). Similarly, LOXL2 expression in gastric CAFs was also demonstrated to be positively correlated with the invasive ability of gastric cancer cells (Kasashima et al., 2014).

TABLE 1 | Expression of MMP2 and MMP9 is correlated with cancer progression and metastasis.

MMP2	References	MMP9	References
Basal-cell carcinoma	Gozdzialska et al., 2016	Basal-cell carcinoma	Gozdzialska et al., 2016
Brain cancer	Wang M. et al., 2003; Tabouret et al., 2014	Brain cancer	Wang M. et al., 2003; Li et al., 2016
Breast cancer	Yari et al., 2014; Chen Y. et al., 2015; Ramos et al., 2016; Tabouret et al., 2016	Breast cancer	Wu et al., 2014; Yousef et al., 2014
Colorectal cancer	Groblewska et al., 2014	Gastric cancer	Wang et al., 2014; Chen S.Z. et al., 2015
Endometrial adenocarcinoma	Li et al., 2014	Liver cancer	Sun et al., 2014
Gastric cancer	Wang et al., 2014	Lung cancer	Lee et al., 2015; Zhang et al., 2015; Gong et al., 2016; Yu et al., 2016
Lingual and gingival cancers	Nishio et al., 2016	Pancreatic cancer	Jakubowska et al., 2016
Lung cancer	Zhang et al., 2015	Pituitary adenoma	Liu et al., 2016
Melanoma	Kamyab-Hesari et al., 2014	Prostate cancer	Oguic et al., 2014
Osteosarcoma	Zhang and Zhang, 2015	Squamous cell carcinoma	Stanciu et al., 2016
Ovarian cancer	Fu et al., 2015		

In breast cancer, LOXL2 inhibition showed anti-tumor effects in reducing tumor size and angiogenesis. Furthermore, a combination of LOX and LOXL2 inhibitors resulted in even smaller and less metastatic tumors (Chang et al., 2017). Interestingly, in mice bearing aggressive breast cancer, anti-LOXL2 monoclonal antibody AB0023 exhibited potent inhibitory effects in activated fibroblast as suggested by an 88% reduction of α-SMA+ cells by immunohistochemistry (IHC) after AB0023 treatment (Barry-Hamilton et al., 2010). The inhibitory effect was also shown to be closely associated with the reduction in cross-linked collagenous ECM matrix. Recently, an *in vitro* study using siRNA adenovirus vector to silence LOXL2 expression in mouse lung fibroblast also showed that the proliferation of lung fibroblasts was significantly decreased via the TGF-β/Smad signaling pathway (Wen et al., 2018). All these findings highlighted the role of CAF-derived LOX family oxidases in regulating tumor migration and invasion and potential beneficial outcomes of targeting CAF-synthesized LOX family oxidases.

The ability of cancer cells to digest surrounding ECM and localize to distal sites has long been attributed to MMPs, which are zinc-containing endopeptidases. MMPs play pivotal roles in creating the paths for tumor cells to leave the primary tumor niche and wade through the stiff matrix. There are 24 MMPs in mammals (Vandenbroucke and Libert, 2014), of which MMP2 and MMP9 are found to be overexpressed in many cancer types and promote tumor progression and metastasis (**Table 1**). CAFs were shown to be the major producer of MMP2 in mouse lung tumors as indicated by IHC staining results showing MMP2 primarily localizes to fibroblasts (Bates et al., 2015). Using the online database proteinatlas.org, we summarized the correlation between 13 MMPs and patient prognosis status in nine human cancers in **Table 2** based on the RNA-Seq data. Four MMPs (MMP10, MMP15, MMP24, MMP25) are shown to be favorable to patient prognosis as their expression levels are positively correlated with patient survival. However, the expression levels of eight MMPs (MMP1, MMP3, MMP7, MMP11, MMP12, MMP14, MMP19, and MMP28) are shown to be negatively correlated with patient survival. Interestingly, MMP9 seemed to have context-dependent roles in different cancer types. In conclusion, the roles of different MMPs in the TME need to be carefully examined based on cancer types and stages, and this should also be one major consideration when designing, dosing and scheduling MMP-targeting drugs for cancer patients (Iyer et al., 2012).

CAF-ECM INTERACTIONS

The interactions between CAFs and the ECM influence the stiffness of the tumor stroma and can be described using the term "mechanoreciprocity" (van Helvert et al., 2018), which consists of both "outside-in" and "inside-out" signaling modes (Shattil et al., 2010). The "outside-in" signaling mode is a well-established mechanism, by which ECM proteins can function as ligands and bind to integrin receptors on the cell membrane (**Table 3**). Integrins are transmembrane receptors composed of a heterodimer of α and β subunits. As shown in **Figure 2**, when the cells encounter a rigid ECM, the integrin molecules become dimerized to trigger the focal adhesion cascade and the activation of downstream signaling, including tyrosine protein kinase SRC and focal adhesion kinase FAK1, thereby converting external mechanical signals into cellular and biochemical signals inside the cells (Barczyk et al., 2010; Tucker and Chiquet-Ehrismann, 2015; Benito-Jardon et al., 2017). Integrin α11β1 is a stromal cell-specific receptor for collagen and also known as an important regulator for fibroblast activation (Carracedo et al., 2010). Zhu et al. (2007) showed that the growth of the tumors formed by non-small-cell lung carcinoma (NSCLC) cell lines, A549, NCI-H460, and NCI-H520 mixed with integrin α11-deficient fibroblasts were significantly impeded as compared with the tumors derived from the mixture of either tumor cell line and wild-type fibroblasts. In this case, fibroblasts, originally good "listeners" and "responders" to the mechanical cues, lost their active ECM remodeling ability after the fibroblast-ECM interaction was blocked. In another example, cardiac fibroblasts cultured on a stiff matrix expressed increased amounts of LOX, further crosslinked collagen fibers and stiffened the ECM. To the contrary, the inhibition of the binding between α2β1 integrin and collagen I ablated this effect and downregulated LOX expression (Gao et al., 2016). In the "outside-in" signaling mode, the mechanical cues can also activate other signaling pathways in fibroblasts, such as the mitogen-activated protein kinase (MAPK)

TABLE 2 | Correlations between MMP/LOX expression and the 5-year survival rates of cancer patients**.

MMP/LOX	Prognosis*	Cancer type	5-year survival			
			High expression	Low expression	Sample size	*p*-value
MMP1	Unfavorable	Renal cancer	60%	82%	877	9.90E-10
	Unfavorable	Liver cancer	36%	50%	365	0.0000042
	Unfavorable	Cervical cancer	59%	74%	291	0.00047
MMP3	Unfavorable	Pancreatic cancer	5%	34%	176	0.00041
	Unfavorable	Cervical cancer	45%	71%	291	0.00097
MMP7	Unfavorable	Liver cancer	38%	60%	365	0.00025
	Unfavorable	Lung cancer	34%	50%	994	0.00034
MMP9	Unfavorable	Renal cancer	64%	78%	877	0.000041
	Favorable	Endometrial cancer	81%	60%	541	0.00025
	Unfavorable	Liver cancer	37%	64%	365	0.00072
MMP10	Favorable	Urothelial cancer	48%	27%	406	0.00071
MMP11	Unfavorable	Renal cancer	65%	81%	877	0.00026
MMP12	Unfavorable	Liver cancer	33%	51%	365	0.00014
MMP14	Unfavorable	Renal cancer	58%	73%	873	0.00013
	Unfavorable	Ovarian cancer	20%	35%	373	0.00095
MMP15	Favorable	Renal cancer	86%	65%	877	8.50E-08
	Favorable	Urothelial cancer	49%	30%	406	0.00031
MMP19	Unfavorable	Renal cancer	62%	81%	877	8.60E-09
MMP24	Favorable	Renal cancer	74%	51%	877	8.20E-11
MMP25	Favorable	Head and neck cancer	51%	29%	499	0.000011
MMP28	Unfavorable	Pancreatic cancer	16%	40%	176	0.0000063
LOX	Unfavorable	Renal cancer	64%	87%	877	3.90E-08
	Unfavorable	Urothelial cancer	25%	51%	406	0.00033
	Unfavorable	Liver cancer	36%	52%	365	0.00074
LOXL1	Unfavorable	Glioma	0	10%	153	0.00013
LOXL2	Unfavorable	Lung cancer	31%	57%	994	1.50E-07
	Unfavorable	Renal cancer	54%	72%	877	2.90E-07
	Unfavorable	Cervical cancer	52%	82%	291	0.0000098
	Unfavorable	Glioma	0	10%	153	0.00018
	Unfavorable	Pancreatic cancer	6%	43%	176	0.00091
LOXL3	Unfavorable	Renal cancer	61%	77%	877	8E-08
LOXL4	Unfavorable	Glioma	2%	16%	153	0.00054
	Unfavorable	Ovarian cancer	23%	39%	373	0.00096

**The prognosis of each group of patients was examined by Kaplan–Meier survival estimators, and the survival outcomes of the two groups were compared by log-rank tests.*

***Data available from:*

MMP1: https://www.proteinatlas.org/ENSG00000196611-MMP1/pathology
MMP3: https://www.proteinatlas.org/ENSG00000149968-MMP3/pathology
MMP7: https://www.proteinatlas.org/ENSG00000137673-MMP7/pathology
MMP9: https://www.proteinatlas.org/ENSG00000100985-MMP9/pathology
MMP10: https://www.proteinatlas.org/ENSG00000166670-MMP10/pathology
MMP11: https://www.proteinatlas.org/ENSG00000099953-MMP11/pathology
MMP12: https://www.proteinatlas.org/ENSG00000262406-MMP12/pathology
MMP14: https://www.proteinatlas.org/ENSG00000157227-MMP14/pathology
MMP15: https://www.proteinatlas.org/ENSG00000102996-MMP15/pathology
MMP19: https://www.proteinatlas.org/ENSG00000123342-MMP19/pathology
MMP24: https://www.proteinatlas.org/ENSG00000125966-MMP24/pathology
MMP25: https://www.proteinatlas.org/ENSG00000008516-MMP25/pathology
MMP28: https://www.proteinatlas.org/ENSG00000271447-MMP28/pathology
LOX: https://www.proteinatlas.org/ENSG00000113083-LOX/pathology
LOXL1: https://www.proteinatlas.org/ENSG00000129038-LOXL1/pathology
LOXL2: https://www.proteinatlas.org/ENSG00000134013-LOXL2/pathology
LOXL3: https://www.proteinatlas.org/ENSG00000115318-LOXL3/pathology
LOXL4: https://www.proteinatlas.org/ENSG00000138131-LOXL4/pathology

TABLE 3 | Integrins and their ECM partners.

ECM Protein	Interacting Integrins	References
Collagen	α1β1, α2β1, α10β1, α11β1	Leitinger, 2011
Fibronectin	α4β1, α5β1, α8β1, αIIbβ1, αvβ3, αvβ6, αvβ8	Pankov and Yamada, 2002; Danen et al., 2005
Tenascin-C	α9β1, α8β1, αvβ1, αvβ6	Tucker and Chiquet-Ehrismann, 2015
Laminin	α3β1, α6β1, α7β1, α6β4	Belkin and Stepp, 2000; Yamada and Sekiguchi, 2015

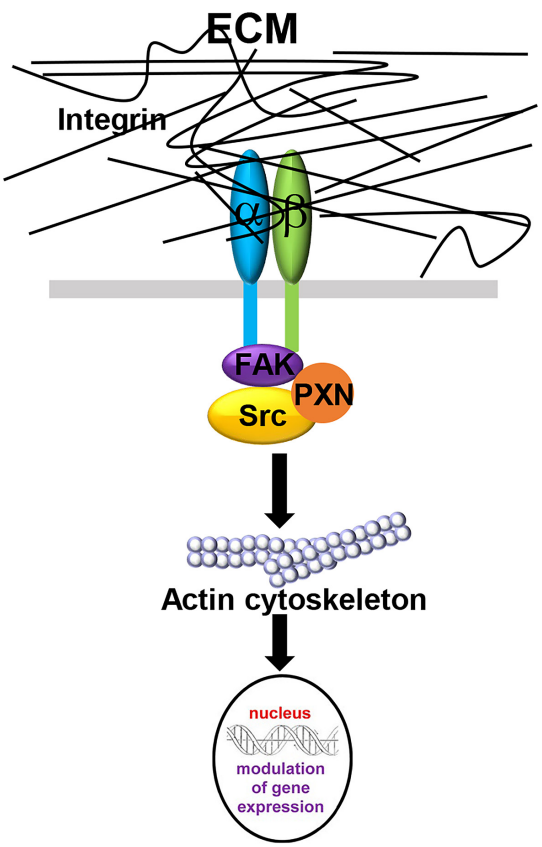

FIGURE 2 | The "outside-in" signaling mode in CAF-ECM interactions. When the membrane-bound integrin receptors interact with ECM proteins, integrin α- and β- subunits dimerize to activate downstream FAK1 and Src signaling and induce cytoskeleton remodeling, thereby converting external mechanical signals into cellular and biochemical signals inside the cells.

pathway (Wang J. et al., 2003; Paszek et al., 2005). In addition, it was reported that increased ECM stiffness could also activate the SRC-YAP-MYL9/MYL2 axis in CAFs to maintain the CAF phenotype. A positive feedback loop is established between CAF function and ECM stiffness, leading the stiff tumor stroma to become even stiffer and more favorable for cancer cell invasion (Calvo et al., 2013).

The "inside-out" signaling mode refers to the regulation of integrin-ECM interactions by intracellular signals. CAFs respond to tissue tension and exert their ECM remodeling and assembly abilities to further increase the stiffness of the stroma. The "inside–out" signaling mode is normally triggered by the binding of intracellular molecules, such as talin or kindlin, to the tails of integrins, leading to an increased affinity for extracellular ligands and enhanced ECM signaling (Shattil et al., 2010). For example, CAFs exert contractile forces and mediate extracellular FN1 assembly mainly via integrin αvβ3, leading to increased FN1 fibrillogenesis and ultimately contributing to tumor invasion (Attieh et al., 2017). Similarly, FN1 production and assembly were also observed in CAFs in prostate cancer. Erdogan et al. (2017) reported that CAFs produce an FN1-rich ECM with anisotropic fiber orientation as compared with normal fibroblasts and regulate cancer cell migration. In their study, CAFs remodel the FN1-rich ECM via the non-muscle myosin II (NMII)-α5β1 integrin axis.

DIRECT CAF-CANCER CELL CONTACT

Cancer-associated fibroblast-dependent tumor-promoting roles have long been attributed to the CAF secretome, but there is no denying that direct cell-cell contact also plays an important role in CAF-mediated cancer cell migration and invasion. Labernadie et al. (2017) discovered a heterotypic E-cadherin/N-cadherin adhesion complex between CAF and SCC cells. As shown in **Figure 3**, CAFs migrate through the ECM via integrin-mediated

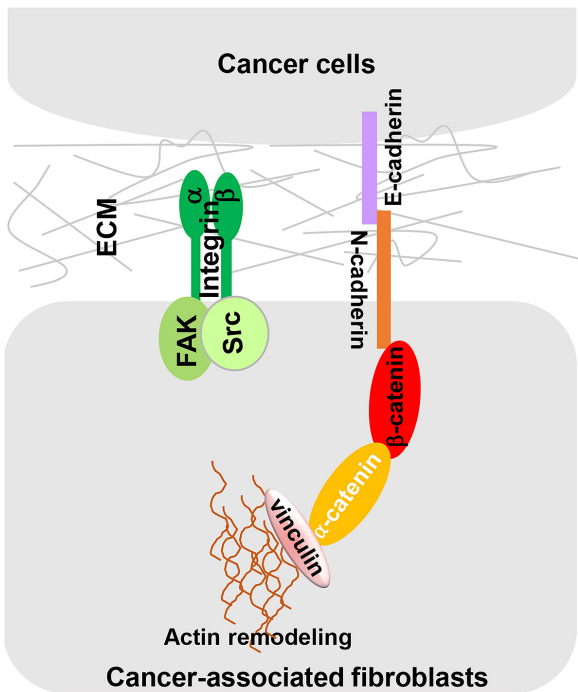

FIGURE 3 | A heterotypic E-cadherin/N-cadherin complex mediates CAF-SCC cell contact. As reported by Labernadie et al. (2017) intercellular physical force is transmitted between SCCs and CAFs by a heterophilic adhesion complex involving N-cadherin at the CAF membrane and E-cadherin at the cancer cell membrane. This heterotypic CAF-cancer cell interaction triggers β-catenin recruitment, α-catenin/vinculin interaction, and actin remodeling, allowing CAFs to exert an intercellular physical force on cancer cells and promote cooperative tumor invasion.

cytoskeleton remodeling and actomyosin reassembly while dragging tumor cells through CAF-cancer cell interaction via this heterotypic cadherin complex. The intercellular physical force between cancer cells and CAFs promote cooperative tumor invasion by triggering β-catenin recruitment, β-catenin/vinculin interaction and actin remodeling in both cell types.

In NSCLC, CAFs could potently enhance the motility of NSCLC cells through direct cell-cell contact via the hedgehog signaling pathway. Two co-culture systems (direct co-culture and indirect co-culture) were utilized to differentiate whether the motility-promoting effect is mediated by paracrine factors or cell-cell contact. Interestingly, increased tumor cell migration was only shown in a direct co-culture system, suggesting that CAF-promoted NSCLC cell migration is mediated by direct cell-cell contact (Choe et al., 2013). PDPN is a transmembrane glycoprotein that is known to be correlated with poor patient prognosis in lung adenocarcinoma (Ito et al., 2012). In an *in vitro* 3D collagen invasion model, PDPN-positive (PDPN+) CAFs accelerated lung tumor cell invasion into the collagen matrix. Ablation of PDPN reduced the invasive behavior of both CAFs and lung tumor cells. Because PDPN+ CAFs were observed to display high RHOA activity, RHO Kinase (ROCK) inhibitors were used to treat CAFs before co-culturing with lung tumor cells. ROCK inhibition suppressed PDPN-induced tumor cell migration, highlighting the role of the RHOA/ROCK axis in CAF-dependent tumor invasion (Neri et al., 2015).

INDIRECT CAF-CANCER CELL INTERACTIONS

Paracrine signaling between CAFs and cancer cells represents another well-studied mode of interaction between the two cell types that shapes the TME and promotes tumor growth. Hepatocyte growth factor (HGF) is a paracrine growth factor known to contribute to cancer progression. In cancer cells, HGF activates downstream RAS/MAPK and PI3K signaling pathways by binding to its receptor MET (Organ and Tsao, 2011). Cytokine antibody arrays suggested that HGF was the most significantly upregulated secreted factor in CAFs in breast cancer when compared to normal fibroblasts, which is positively correlated to their pro-tumorigenic ability to promote breast tumorigenesis in mice (Tyan et al., 2011). Similarly, the tumor-promoting functions of CAF-derived HGF were also observed in gastric cancer. By ablating HGF expression *in vivo*, CAFs failed to promote tumor growth in nude mice (Wu et al., 2013). Interestingly, CAF-derived HGF is also sufficient to induce RAF inhibitor resistance via the binding of its receptor MET and reactivation of the MAPK and PI3K/AKT signaling pathways in melanoma cells. 50 nM of recombinant HGF induced strong drug resistance to a BRAF inhibitor, vemurafenib, in several melanoma cell lines (Straussman et al., 2012).

CXCL12, also known as stromal cell-derived factor 1 (SDF1), is an important regulator in cancer initiation, angiogenesis, and metastasis (Orimo et al., 2005; Sugihara et al., 2015; Teng et al., 2016). In addition, CXCL12 was shown to induce angiogenesis by recruiting endothelial progenitor cells (EPCs) in breast cancer, thereby providing sufficient nutrients to fuel tumor growth and metastasis. Furthermore, after mice bearing breast cancer were treated with antibodies targeting CXCL12, reduced tumor volume and cell number were observed (Orimo et al., 2005). It was reported that CAF-derived CXCL12 activated TGF-β-regulated C-X-C chemokine receptor type 4 (CXCR4) expression in human prostatic epithelial BPH-1 cells to induce tumorigenesis. The CAF-conditioned medium was sufficient to induce CXCR4-AKT activation in BPH-1 cells *in vitro*. *In vivo* tumor grafting experiments also supported this claim. CXCR4-deficient prostate tumors were significantly smaller and less invasive as compared to control tumors, confirming the role of the CXCL12-CXCR4 axis in initiating tumor formation (Ao et al., 2007). The EMT process represents a pivotal mechanism used by cancer cells for migration and invasion. It was shown *in vitro* that CAF-derived CXCL12 functions as an important EMT inducer in breast cancer cells by regulating the Wnt/β-catenin signaling pathway (Shan et al., 2015). TGF-β is another multifunctional cytokine that is well-known for its role in inducing the EMT process. CAF-derived TGF-β1 promoted the aggressive phenotypes of breast cancer cells by inducing EMT through the activation of TGF-β/SMAD signaling. The EMT phenotype was reversed in the cells after the addition of TGF-β1 neutralizing antibody (Yu et al., 2014).

THERAPEUTIC PERSPECTIVES: TARGETING THE ECM MICROENVIRONMENT

Despite the growing enthusiasm for the development of CAF-targeting therapies, targeting CAFs has been challenging and lacks real and meaningful progress. One interesting example is FAP. Murine anti-FAP antibody F19 showed a significant tumor-inhibitory effect in xenograft models of lung, pancreas, and head and neck cancers with no obvious signs of toxicity (Ostermann et al., 2008). Because of promising pre-clinical data, a humanized version of murine anti-FAP antibody, sibrotuzumab, has been designed and tested in a phase I clinical trial and was determined to be safe and tolerable (Scott et al., 2003). However, in the phase II study in metastatic colorectal cancer, sibrotuzumab showed no therapeutic benefits (Hofheinz et al., 2003). Therefore, instead of targeting a specific subset of CAFs or CAFs in general, identifying the exact mechanisms that CAFs use to support cancer cells may help to develop better therapeutic strategies, e.g., based on CAF autophagy (Zhang et al., 2018), or based on the specific ECM proteins that are produced by CAFs.

TARGETING ECM PROTEINS

Humanized anti-collagen antibodies and ECM inhibitors have emerged as promising agents for cancer therapy (de Jonge et al., 2006; Koon et al., 2011). Halofuginone is an inhibitor of collagen I and was shown having anti-tumor activities in mouse models of prostate cancer (Gavish et al., 2002), pancreatic cancer (Spector et al., 2010) and lung cancer (Taras et al.,

2006). D93/TRC093 is a humanized monoclonal antibody that specifically binds the HU177 cryptic collagen epitope within the tumor ECM with potential antiangiogenic and antitumor activities (Cretu et al., 2007; Caron et al., 2016). In the study conducted by Caron et al. (2016) D93/TRC093 was found to restrict the accumulation of α-SMA+ fibroblasts, which could be explained by the inhibition of integrin $\alpha_{10}\beta_1$-mediated fibroblast adhesion and migration on denatured collagen. In a phase I clinical study, TRC093 was shown to be well-tolerated and had tumor-inhibitory effects as monotherapy and in combination with bevacizumab in 19 patients carrying different types of solid tumors (Robert et al., 2010).

Conjugating a monoclonal antibody with a cell-killing agent is a new approach to develop novel targeted anti-cancer agents. In the past two decades, FN1-targeting antibodies have been designed and tested in different models. L19 is a monoclonal antibody known to target the ED-B domain of FN1. By attaching anti-angiogenesis drugs to L19, the fusion protein was demonstrated to exhibit strong anti-tumor effects in animal models carrying different tumors, including teratocarcinoma, colon adenocarcinoma and sarcoma (Birchler et al., 1999). Interleukin-2 (IL-2) is a cytokine factor and an important player in anticancer immunity. However, the cardiovascular toxicity of IL-2 remains a major clinical issue. To overcome this problem, a new strategy was designed by fusing IL-2 with L19 so that IL-2 can be precisely targeted to the tumor site, resulting in reduced side effects. This drug conjugate exerted strong immune-stimulatory effects and inhibited tumor growth in stage III melanoma patients (Danielli et al., 2015). Currently, L19-IL-2 in combination with L19-TNF is in a phase III clinical trial to evaluate its efficacy against advanced melanoma (ClinicalTrials.gov Identifier: NCT03567889). Similarly, TNC-targeting antibodies have also been conjugated with IL-2, and have shown some preliminary signs of anti-tumor activity in advanced solid tumors and metastatic breast cancer (Catania et al., 2015). Navitoclax (ABT-263) is a small molecule that was shown to have the ability to induce apoptosis in myofibroblasts (Lagares et al., 2017). Consequently, Navitoclax could be used to target CAFs in solid tumor. Navitoclax-loaded nanoliposome was modified with peptide FH (FH-SSL-Nav), which specifically binds to TNC, to precisely eradicate CAFs at the tumor site. Using a xenograft mouse model of hepatocellular cancer, FH-SSL-Nav was shown to have the ability to deplete CAFs and inhibit tumor growth (Chen et al., 2016). In January 2017, the National Cancer Institute (NCI) approved a phase Ib/II trial study to evaluate the side effects and best dose of the combination of MEK inhibitor Trametinib and Navitoclax in treating patients with advanced or metastatic solid tumors (ClinicalTrials.gov Identifier: NCT02079740).

TARGETING ECM REMODELING ENZYMES

Extracellular matrix remodeling plays an essential role in CAF-mediated desmoplastic reactions, which cannot be achieved without LOX-induced ECM crosslinking. LOX inhibitors have emerged as potential alternatives to target the desmoplastic TME and improve drug delivery efficacy. In an *in vitro* 3D spheroid model using four different mouse tumor cell lines, including Lewis lung carcinoma cell line (LLC), a fibrosarcoma cell line (MT6) and two breast carcinoma cell lines (4T1, EMT6), LOX inhibition significantly improved the diffusion of doxorubicin (Schutze et al., 2015). Blocking LOX family oxidases *in vitro* or *in vivo* has shown potent anti-tumor activities in breast and pancreatic cancer (Park et al., 2016; Chang et al., 2017). Nevertheless, caution should still be taken when considering using LOX inhibitors. In a rat model of prostate cancer, LOX inhibition seems to have context-dependent effects during different stages of tumor progression. Before tumor formation, LOX inhibitors showed strong tumor-inhibiting capacity. To the contrary, after prostate tumors were established, LOX inhibition did not affect or decrease tumor growth (Nilsson et al., 2016). In recent clinical trials, simtuzumab, a monoclonal antibody against LOXL2, failed to produce improved anti-tumor benefits when given in combination with other anti-cancer drugs, including 5-fluorouracil, leucovorin, irinotecan (FOLFIRI) and gemcitabine (Benson et al., 2017; Hecht et al., 2017).

Many MMPs have been known to be notorious for their roles in promoting cancer progression. As a result, more than 50 MMP inhibitors were investigated in clinical trials. In a pre-clinical study, an anti-MMP9 monoclonal antibody GS-5745 successfully inhibited tumor growth and reduced tumor metastasis in mice bearing colorectal tumors (Marshall et al., 2015). Nevertheless, despite exciting preclinical data, none of these MMP inhibitors displayed anti-tumor effects in clinical trials. Although there are many explanations for these failures, such as bad clinical trial design, poor oral bioavailability, and inadequate cancer stages (Vandenbroucke and Libert, 2014), one potential reason responsible for the failures of these MMP inhibitors might be the obscurity of the roles and functions of MMPs in the ECM microenvironment. In addition, the use of broad-spectrum MMP inhibitors also suppresses potential tumor-inhibiting MMPs. Therefore, although MMPs are attractive therapeutic targets, more research is needed to unravel the roles of different MMPs in different cancer types and/or during various cancer stages. Furthermore, more efforts are required to develop more specific and selective MMP inhibitors to avoid potential side effects.

TARGETING CAF-DERIVED MOLECULAR SIGNALS

Cancer-associated fibroblast-mediated paracrine signaling has also been envisioned as a potential target in cancer treatment. In a recent phase I–II study on myeloid leukemia, plerixafor, a CXCR4 inhibitor, resulted in improved recovery rate when given in combination with a FLAG-Ida regime (fludarabine, idarubicin, cytarabine, and G-CSF) (Martinez-Cuadron et al., 2018). To block TGF-β activity, TGF-β inhibitors and monoclonal antibodies have been designed and tested in clinical trials. Galunisertib, a TGF-β receptor kinase inhibitor, however, showed highly context-dependent tumor-inhibitory effects. While it

showed promising clinical responses in neuroblastoma patients (Tran et al., 2017), galunisertib had no significant therapeutic effect in a phase II clinical study in recurrent glioblastoma patients (Brandes et al., 2016). The monoclonal antibody fresolimumab (GC1008), which is capable of neutralizing all human isoforms of TGF-β, has also been investigated in advanced malignant melanoma and renal cell carcinoma and showed early stage anti-tumor effects with no dose-limiting toxicity in a phase I clinical study (Morris et al., 2014). In 2017, several clinical trials investigating an anti-HGF antibody, rilotumumab, were published. In one clinical trial, improved antitumor activities of rilotumumab in combination with cisplatin and capecitabine were shown in patients with MET-positive advanced gastric or gastroesophageal junction cancer (Doi et al., 2017). The combined use of rilotumumab with erlotinib (an EGFR receptor inhibitor) also showed successes in treating advanced NSCLC (Tarhini et al., 2017). However, in another clinical trial on small-cell lung cancer patients, no significant clinical benefit of rilotumumab in combination with platinum-based chemotherapy was observed (Glisson et al., 2017). Similarly, in a phase III clinical study, the treatment utilizing rilotumumab plus epirubicin, cisplatin, and capecitabine as a first-line therapy on gastric or gastro-oesophageal junction cancer patients was unsuccessful (Catenacci et al., 2017). Taken together, targeting CAF-induced paracrine signaling appears to be spatial-temporal and case-dependent.

CONCLUSION

It is an astonishing feat of the tumor cells to abandon the basic rules of tissue homeostasis and to grow uncontrollably. Unfortunately, as we have learned from many modern targeted therapies, a simple approach to eliminate tumor "seed" is generally condemned to failure. It is becoming clear that the TME is actively involved in tumor initiation, progression, metastasis and the development of drug resistance. However, only after gaining enhanced knowledge about the TME, including the heterogeneous nature and complexity of CAF populations, a multiplex approach targeting CAFs and the ECM will naturally come by and provide desired clinical benefits.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Role of the Extracellular Matrix in Cancer Stemness

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As our understanding of cancer cell biology progresses, it has become clear that tumors are a heterogenous mixture of different cell populations, some of which contain so called "cancer stem cells" (CSCs). Hallmarks of CSCs include self-renewing capability, tumor-initiating capacity and chemoresistance. The extracellular matrix (ECM), a major structural component of the tumor microenvironment, is a highly dynamic structure and increasing evidence suggests that ECM proteins establish a physical and biochemical niche for CSCs. In cancer, abnormal ECM dynamics occur due to disrupted balance between ECM synthesis and secretion and altered expression of matrix-remodeling enzymes. Tumor-derived ECM is biochemically distinct in its composition and is stiffer compared to normal ECM. In this review, we will provide a brief overview of how different components of the ECM modulate CSC properties then discuss how physical, mechanical, and biochemical cues from the ECM drive cancer stemness. Given the fact that current CSC targeting therapies face many challenges, a better understanding of CSC-ECM interactions will be crucial to identify more effective therapeutic strategies to eliminate CSCs.

Keywords: extracellular matrix, cancer stem cells, self-renewal, chemoresistance, integrin

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INTRODUCTION: ECM AS A CSC NICHE

The extracellular matrix (ECM) is a major structural component of the tumor microenvironment and comprised of a network of biochemically distinct components, including fibrous proteins, glycoproteins, proteoglycans, and polysaccharides. The ECM is a highly dynamic structure, constantly undergoing a remodeling process where ECM components are deposited, degraded, or modified (Lu et al., 2012). Increasing evidence suggests that the ECM serves as a niche for normal and cancer stem cells (CSCs). CSCs, also called tumor-initiating cells, are a small population of cells within tumors that have capabilities of self-renewal properties, tumor initiation and chemoresistance (Kreso and Dick, 2014; Batlle and Clevers, 2017). As one of the CSC niches, the ECM provides both structural and biochemical support to regulate proliferation, self-renewal, and differentiation of CSCs. In this review, we will cover the current understanding of how different ECM components affect the cancer "stemness" phenotype.

CATEGORIES OF ECM PROTEINS AND THEIR ROLE IN CANCER STEMNESS

Fibrous ECM Proteins

Collagens constitute the main structural element of the ECM and are the most copious type of fibrous proteins within the interstitial ECM. Collagens play a role in tissue development by

providing mechanical strength, altering cell adhesion, promoting cell migration (Frantz et al., 2010). Studies have reported that several collagens (e.g., COL3A1, COL4A2, COL7A1, COL17A1) are overexpressed by CSCs (**Table 1**). Multiple collagen subtypes have been shown to increase epithelial-mesenchymal transition (EMT), tumor-initiating potential, drug resistance and self-renewal of CSCs (**Table 1** and **Figure 1**).

Glycoproteins

Glycoproteins, which make the ECM a cohesive network of molecules by linking cells together with structural components, include fibulin, fibrillin, laminin, fibronectin, vitronectin, tenascin-C, Secreted Protein Acidic and Rich in Cysteine (SPARC), periostin (POSTN), thrombospondin, mucins (MUCs) and nidogen (**Table 1**). CSCs overexpress several glycoproteins (e.g., tenascin-C, POSTN, MUC1) and their receptors (e.g., integrins αVβ3 and α9β1, CD47). Adhesive glycoproteins bind to integrins, non-integrin receptors, growth factors, and other ECM components to activate downstream signaling pathways to regulate EMT, self-renewal, and drug resistance of CSCs (**Table 1**). For example, fibronectin, a major adhesive ECM glycoprotein that attaches cells to a variety of ECM components, has been shown to increase EMT, self-renewal, expression of CSC markers and drug resistance of CSCs. Laminins, another class of adhesive glycoproteins that constitute structural scaffolding of all basement membranes, support self-renewal of CSCs through their interaction with integrins. Some glycoproteins have dual roles in cancer stemness depending on the cancer type. For instance, fibulin-3, an ECM glycoprotein associated with basement membranes, inhibits self-renewal in lung and pancreatic CSCs while stimulating breast CSC self-renewal (**Table 1**).

Proteoglycans

Proteoglycans are glycosylated proteins composed of a core protein and one or several covalently attached sulfated glycosaminoglycan chains and are present in the ECM of connective tissues. Proteoglycans play a crucial role in ECM assembly and cell signaling. They bind to growth factors, cytokines and other ECM molecules and act as co-receptors to assist ligand and cell surface binding to modulate downstream signaling. Several proteoglycans (e.g., decorin, lumican, biglycan, versican, aggrecan) are highly expressed by CSCs and their roles in cancer stemness are summarized in **Table 1**.

Polysaccharides

Polysaccharides, a chain of monosaccharide repeats linked through glycosidic bonds, fill the interstitial space and buffer physical stress on the ECM. Hyaluronic acid (HA or "hyaluronan") is a high-molecular-mass polysaccharide that constitutes a major component of interstitial gels, especially in soft connective tissues. In tumors, HA is produced by both tumor stroma and tumor cells, and its binding to the cellular receptor CD44 activates intracellular signaling (e.g., PI3K/Akt and Erk pathways, RhoA and Rac, Ras, NF-kB and Src signaling) to promote cell survival, cancer stemness, motility and invasion by cytoskeletal reorganization. High levels of HA are produced by CSCs and HA–CD44 interaction has been shown to promote acquisition of CSC characteristics and chemoresistance in breast, ovarian and head and neck CSCs (**Table 1**).

ECM PROVIDES PHYSICAL AND MECHANICAL CUES TO DRIVE CANCER STEMNESS

Physical Properties

Physical properties of the ECM such as rigidity, porosity and topography impact various anchorage-dependent CSC functions. The interstitial ECM, mainly composed of collagens, proteoglycans and hyaluronan, provides a physical barrier that hinders the transport of solutes, water and chemotherapeutic drugs. In this regard, it has been shown that cisplatin, a chemotherapeutic drug frequently used to treat various solid tumors, extensively binds to collagen fibers in tumors (Chang et al., 2016). Binding of chemotherapeutic drugs to the ECM prevents drug penetration into tumors, thereby increasing CSC survival. The ECM also provides sites for adhesion of CSCs in the tumor microenvironment. ECM-CSC interaction via CSC receptors such as integrins (e.g., β1, α6, β3, β4), discoidin domain receptors (DDR1, DDR2), CD44 (HA receptor) and CD47 (thrombospondin 1 receptor) enhances CSC properties. For example, CSCs bind to HA through CD44 and this increases not only the expression of stemness factors NANOG and SOX2 but also MDR1 (Multi Drug Resistance 1) expression and drug resistance in breast and ovarian CSCs (Bourguignon et al., 2008). The ECM also provides anchorage and homing sites for CSCs in pre-metastatic niches, initiating metastatic colonization and organotropism of cancer cells. For instance, infiltrating breast tumor cells induce the expression of POSTN in the stroma of the secondary target organ (e.g., lung). By recruiting Wnt ligands and increasing Wnt signaling in CSCs, POSTN sustains CSC population in the secondary site and promotes metastatic colonization (Malanchi et al., 2011). Changes in the ECM topology also affects CSC self-renewal by controlling the balance between symmetric and asymmetric cell divisions. The spatial distribution of the ECM has been shown to guide the orientation of the cell division axis by controlling the location of actin polymerization at the membrane through focal adhesions and the segregation of cortical components in the interphase (Thery et al., 2005). The β1 sub-family of integrins also regulates stem cell self-renewal by controlling the balance between symmetric and asymmetric cell divisions (Lechler and Fuchs, 2005; Taddei et al., 2008). Furthermore, ECM distribution affects migration of cancer cells and immune cells. During tumor progression, wavy collagen fibers become straightened and align perpendicular to the tumor boundary (Provenzano et al., 2006). It has been shown that linear collagen fibers oriented perpendicular to the tumors facilitate high-speed migration of breast cancer cells and

TABLE 1 | The role of different ECM proteins in cancer stemness.

			Role in cancer stemness	References
Fibrous proteins	Collagen	Type I collagen	Maintains the self-renewal of mouse ES cells through Bmi-1 via α2β1 integrin and DDR1; promotes EMT; $CD133^+$ glioblastoma CSCs are localized to type I collagen-rich perivascular niche; GBM cells cultured on type I collagen maintain stemness and tumorigenicity; increases expression of CD133 and Bmi1, EMT and clonogenicity in colorectal CSCs through α2β1 integrin; enhances tumor-initiating potential and self-renewal of $ALDH^+$ pancreatic CSCs through β1integrin and FAK signaling.	Kirkland, 2009; Medici and Nawshad, 2010; Suh and Han, 2011; Motegi et al., 2014; Begum et al., 2017
		Type III collagen	COL3A1 is highly expressed in $ALDH1A1^+$ topotecan-resistant ovarian CSCs.	Januchowski et al., 2016
		Type IV collagen	COL4A2 is highly expressed in $CD133^+/CD44^+$ prostate cancer spheroids; Head and neck CSCs grown on type IV collagen-coated plates grow much faster than in suspension and maintain CSC traits.	Lim et al., 2012; Oktem et al., 2014a
		Type VII collagen	COL7A1 is highly expressed in $CD133^+/CD44^+$ prostate cancer spheroids.	Oktem et al., 2014b
		Type XI collagen	COL11A1 promotes chemoresistance in ovarian cancer; COL11A1 increases the expression of TWIST1, a master EMT regulator directly involved in generating a breast CSC phenotype.	Vesuna et al., 2009; Wu et al., 2015, 2017; Rada et al., 2018
		Type XVII collagen	COL17A1 is upregulated in lung cancer spheroids and required for the maintenance of CSC characteristics and EMT phenotypes; works with laminin 332 to maintain CSC characteristics and EMT phenotype in lung cancer.	Liu et al., 2016, 2018
Glycoproteins	Fibulin	Fibulin-1	Fibulin-1 promotes doxorubicin resistance in breast cancer cells.	Pupa et al., 2007
		Fibulin-3	Fibulin-3 inhibits self-renewal of $ALDH^+$ lung CSCs and EMT through IGF1R signaling; suppresses self-renewal of pancreatic CSCs by downregulating c-MET and ALDH1 expression; works as a downstream effector of HIF2α to stimulate breast CSC self-renewal.	Kim et al., 2014a,b; Kwak et al., 2016
	Fibrillin	Fibrillin-1	Fibrillin-1 supports growth, self-renewal, attachment and maintenance of human ES cells; increases the number and clonogenic potential of MSCs; promotes the expansion of HSCs.	Soteriou et al., 2013; Smaldone et al., 2016a,b
	Laminin	Laminin 511	Laminin 511 supports self-renewal of mouse ES cells and breast CSCs through the interaction with integrin α6β1.	Domogatskaya et al., 2008; Chang et al., 2015
		Laminin 332 (laminin 5)	Laminin 332 maintains CSC characteristics and EMT phenotype in lung cancer; supports stemness of human hepatic CSCs by promoting quiescence, chemoresistance, the number of side population, and *in vivo* tumor growth in a mTORC2-dependent manner.	Govaere et al., 2016; Liu et al., 2016
		Laminin alpha 2	Laminin α2 chain is expressed in the perivascular niche and crucial for survival, proliferation, and self-renewal of glioblastoma stem cells.	Lathia et al., 2012

(Continued)

TABLE 1 | Continued

			Role in cancer stemness	References
		Laminin alpha 5	Laminin α5 is produced by human pluripotent stem cells (hPSC) and crucial for hPSC self-renewal.	Laperle et al., 2015
	Fibronectin	FN	FN is a marker for EMT-driven cancer stemness and induces EMT; increases the adhesion, proliferation and chemoresistance of glioma stem cells as well as their capacity for differentiation through the integrin/FAK/paxillin/AKT signaling pathway.	Li et al., 2017; Yu et al., 2018
		EDA-FN	EDA-FN is required for the sphere formation capacity, clonogenicity, and tumorigenic capacity of $CD133^+/CD44^+$ colon CSCs; $CD133^+/CD44^+$ colon CSCs express higher levels of the EDA receptor integrin α9β1 than $CD133^-/CD44^-$ non-CSCs and EDA binding to integrin α9β1 activates FAK/ERK/β-catenin signaling pathway to maintain stemness.	Ou et al., 2013
		EDB-FN	EDB-FN is crucial for mammosphere-forming ability, expression of CSC markers, self-renewal genes, drug resistance genes, and EMT markers, and *in vivo* tumorigenicity of breast CSCs.	Sun et al., 2015
	Vitronectin		Vitronectin supports sustained self-renewal and pluripotency of human ES cells in defined media; downregulates self-renewal genes and induces differentiation of prostate CSCs in an αVβ3 integrin–dependent manner.	Braam et al., 2008; Hurt et al., 2010
	Fibrinogen		Soft 3D fibrin gels promote formation of tumor spheroids and tumorigenic potential of melanoma CSCs.	Liu et al., 2012
	Tenascin	Tenascin-C	$Oct4^+/TNC^+$ neuroblastoma CSCs, found in the perivascular niche, display a high degree of plasticity and serve as progenitors of tumor-derived endothelial cells; TNC is co-expressed with CD133, a marker for GBM CSCs, in primary GBM tissues; TNC^+ GBM CSCs exhibit the strongest sphere forming capacity regardless of CD133 status; promotes growth of GBM CSCs through α2β1 integrin-mediated upregulation of NOTCH ligand Jagged1 and other NOTCH signaling components; strongly enhances the expression of LGR5 and MSI1, the WNT and NOTCH signaling components that provide essential signals to stem cells, thereby promoting the survival and outgrowth of pulmonary micrometastases; increases side population, sphere formation, and chemoresistance of melanoma CSCs.	Fukunaga-Kalabis et al., 2010; Oskarsson et al., 2011; Pezzolo et al., 2011; Nie et al., 2015; Sarkar et al., 2017
	Secreted Protein Acidic and Rich in Cysteine (SPARC)		Overexpressed in endometrial CSCs; Most abundantly secreted by non-prostate CSCs and enhances the invasiveness and metastatic dissemination of prostate CSCs in a paracrine manner; plays a key role in maintaining dormancy of prostate cancer cells by upregulating BMP7 in bone marrow stromal cells; SPARC is highly expressed by HSCs that recently colonized the bone marrow. HSCs in a SPARC-deficient niche show an accelerated return to quiescence, thereby becoming resistant to serial 5-FU treatment.	Ehninger et al., 2014; Mateo et al., 2014; Yusuf et al., 2014; Sharma et al., 2016

(Continued)

TABLE 1 | Continued

			Role in cancer stemness	References
	Periostin (POSTN)		POSTN promotes a stem cell-like trait and a mesenchymal phenotype in human mammary epithelial cells and breast cancer cells; plays an essential role in the crosstalk between CSCs and their niche to permit metastatic colonization; recruits Wnt ligands and increases Wnt signaling in breast CSCs, thereby promoting CSC maintenance and expansion; POSTN and its receptor αVβ3 integrin are highly expressed in CSC-enriched basal-like breast cancer; POSTN–β3 integrin signaling is required for the maintenance of breast CSCs by activating the ERK signaling pathway and regulating NF-kB–mediated transcription of IL6 and IL8; Glioma stem cells secrete POSTN to recruit M2 tumor-associated macrophages through αVβ3 integrin to support tumor growth; Secreted POSTN promotes GBM stem cell invasion and engraftment through αVβ3 and αVβ5 integrins.	Malanchi et al., 2011; Wang et al., 2013; Mikheev et al., 2015; Zhou W.C. et al., 2015; Lambert et al., 2016
	Thrombospondin	Thrombospondin 1 (TSP1)	TSP1 inhibits stem cell self-renewal by downregulating the expression of self-renewal genes through its receptor CD47 in primary murine endothelial cells; decreases the expression of self-renewal genes and sphere-forming capacity in human colon cancer (HCT116), non-small cell lung cancer (A549), and cervical cancer (HeLa) cell lines; CD47, a TSP1 receptor, is highly expressed in circulating hematopoietic stem cells, leukemia cells, breast CSCs, pancreatic CSCs, and AML leukemia stem cells and required for self-renewal of these CSCs.	Jaiswal et al., 2009; Majeti et al., 2009; Kaur et al., 2013; Cioffi et al., 2015; Zhang et al., 2015a; Zheng et al., 2015; Kaur et al., 2016
	Mucin	Mucin 1	MUC1 is highly expressed in AML stem cells, pancreatic CSCs, and breast CSCs; MUC1 overexpression increases stem cell properties in cord blood $CD34^+$ cells and breast cancer cells; MUC1 is overexpressed and hypoglycosylated in the side population of MCF7 breast cancer cells; Staurosporine-induced apoptosis activates $CD44^+/CD24^-$ breast CSCs by upregulating MUC1 and EpCAM.	Engelmann et al., 2008; Fatrai et al., 2008; Curry et al., 2013; Stroopinsky et al., 2013; Zhou N. et al., 2015
		Mucin 4	MUC4 stabilizes HER2 expression and maintains ovarian CSCs; increases $CD133^+$ pancreatic CSCs and confers gemcitabine resistance.	Mimeault et al., 2010; Ponnusamy et al., 2011
		Mucin 16 (CA125)	High levels of MUC16 are associated with poor clinical outcome and CSC-like properties; C-terminal domain of MUC16 enriches pancreatic CSCs through JAK2-mediated upregulation of LMO2 and NANOG.	Das et al., 2015; Zhang et al., 2015b
	Nidogen (entactin)	NID1	Nidogen-1 promotes EMT and cisplatin resistance in ovarian cancer cells	Zhou et al., 2017
Proteoglycans	Syndecan (CD138)	Syndecan-1	Loss of syndecan-1 in epithelial cells induces a mesenchymal phenotype; Shedding of syndecan-1 by MMP7 promotes chemoresistance; Syndecan-1 induces CSC phenotype via NF-kB/IL-6/STAT3 and Wnt signaling pathways.	Kato et al., 1995; Ibrahim et al., 2013; Wang et al., 2014
	Glypican	Glypican-3	Glypican-3 promotes self-renewal of hepatocellular CSCs.	Sun et al., 2017

(Continued)

TABLE 1 | Continued

			Role in cancer stemness	References
		Glypican-4	Knockdown of GPC4 sensitizes pancreatic cancer cells to 5−FU and inhibits stem cell–like properties by suppressing Wnt/β−catenin pathway.	Cao et al., 2018
	Small leucin-rich proteoglycans (SLRP)	Decorin	Suppresses tumor cell growth, migration, angiogenesis, and metastasis in melanoma, osteosarcoma, and breast cancer; inhibits neural stem cell differentiation; inhibits ES cell self-renewal but promotes trophoblast stem cell self-renewal and commitment; suppresses the numbers of hematopoietic stem cells in the bone marrow and spleen; glioblastoma and neuroblastoma CSCs produce high levels of decorin to acquire temozolomide resistance and a quiescent phenotype.	Grant et al., 2002; Reed et al., 2005; Barkho et al., 2006; Shintani et al., 2008; Stock et al., 2011; Ichii et al., 2012; Farace et al., 2015; Nandi et al., 2018
		Lumican	Glioblastoma and neuroblastoma CSCs produce high levels of lumican and decorin to acquire temozolomide resistance and a quiescent phenotype.	Farace et al., 2015
		Biglycan	Biglycan is highly expressed in colon CSCs and promotes chemoresistance of colon cancer cells by activating NF-kB signaling.	Fang et al., 2010; Liu et al., 2018
		Asporin	Asporin inhibits TGF-β1-induced EMT and expansion of breast CSCs.	Maris et al., 2015
	Versican		High levels of versican are detected in $CD133^{+}/CD44^{+}$ prostate CSC spheroids; The C-terminal G3 domain of versican enhances self-renewal of breast CSCs and confer chemoresistance through EGFR/AKT/GSK-3β signaling.	Du et al., 2013; Oktem et al., 2014b
	Aggrecan		Aggrecan is expressed by neural stem cells and its expression is decreased upon differentiation; $CD133^{+}/CD44^{+}$ prostate CSC spheroids express high levels of aggrecan.	Kabos et al., 2004; Oktem et al., 2014a
	Testican		Testican-1 mediates EMT and confers acquired resistance to lapatinib in HER2-positive gastric cancer	Kim et al., 2014c
Non-proteoglycan polysaccharides	Hyaluronan (HA)		Breast CSCs produce high levels of HA; HA promotes the interaction of breast CSCs with tumor-associated macrophages to activate other stromal cells that augment the growth of CSCs; Excessive HA production promotes acquisition of CSC properties via Twist and the TGF-β-Snail signaling axis in breast cancer; HA-CD44 interaction induces Nanog-Stat-3 interaction, resulting in multidrug resistance in breast and ovarian cancer; HA–CD44 interaction stimulates stem cell marker expression, stemness properties and chemoresistance in head and neck CSCs.	Bourguignon et al., 2008, 2012; Okuda et al., 2012; Chanmee et al., 2014; Shiina and Bourguignon, 2015

paired macrophages to promote metastasis to distant organs (Roussos et al., 2011).

Mechanical Properties

Tumor ECM is typically stiffer than normal tissue ECM due to overexpression of many ECM components (e.g., collagens I, II, III, V, IX, and XI, heparan sulfate proteoglycans) and ECM-modifying enzymes [e.g., lysyl oxidase (LOX)] (Levental et al., 2009). Mechanical properties conferred by ECM stiffness are transmitted to CSCs through the formation of focal adhesions and subsequent activation of mechanotransduction pathways (e.g., Rho/ROCK, YAP/TAZ). ECM stiffness plays a crucial role in regulating stem cell self-renewal and differentiation. Several studies have demonstrated that ECM stiffness directs

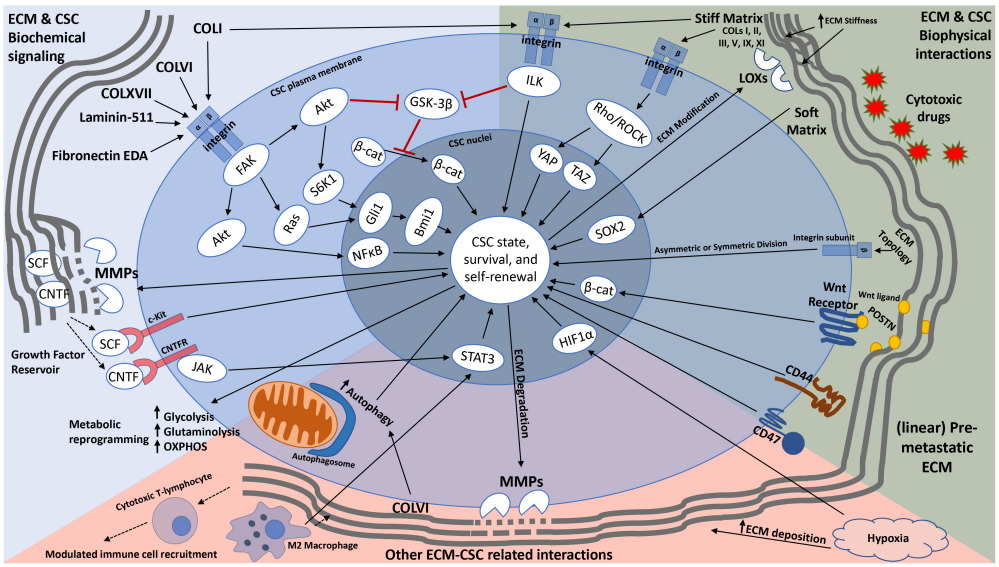

FIGURE 1 | Schematic representation of how the ECM modulates cancer stemness. In addition to providing cues that transform non-CSCs into CSCs (through EMT) and maintain a stemness state, the ECM can modulate CSC metabolism, influence immune cell recruitment, and serve as a reservoir for growth factors and other signaling molecules that aid in CSC self-renewal and maintenance. Furthermore, the ECM provides not only a physical barrier to CSCs from cytotoxic drugs, but also anchorage sites for CSCs for cell division and metastatic colonization. CSCs are also able to modify their local ECM through upregulation of ECM degrading and modifying enzymes (such as MMPs and LOXs). Solid long arrows represent downstream signaling activation or event, solid short arrows represent elevated activity or expression, dotted arrows represent growth factor release or immune cell migration, red lines with flat heads represent inhibition.

human mesenchymal stem cells (MSCs) and neural stem cells to differentiate into different cell lineages (Engler et al., 2006; Saha et al., 2008; Winer et al., 2009). Human MSCs cultured on hydrogel with an elastic modulus very similar to bone marrow, exhibit enhanced self-renewal and multipotency (Winer et al., 2009). In the case of melanoma CSCs, three-dimensional (3D) soft fibrin matrices promote histone 3 lysine residue 9 (H3K9) demethylation and increase SOX2 expression and self-renewal, whereas stiff matrices exert the opposite effects (Liu et al., 2012; Tan et al., 2014). Conversely, breast CSCs increase CSC marker expression on stiff matrix through integrin linked kinase (ILK) signaling (Pang et al., 2016; You et al., 2016), suggesting that the effect of matrix stiffness on stemness is cancer type specific.

ECM MODULATES BIOCHEMICAL CUES TO DRIVE CANCER STEMNESS

EMT/De-Differentiation

The ECM can provide external cues that induce EMT, one of the cellular transformation processes that has been shown to route some cancer cell types from a differentiated to a stem cell state (Mani et al., 2008). Collagen I has been shown to induce EMT through activation of ILK and subsequently NF-κB-dependent inactivation of GSK-3β (Medici and Nawshad, 2010), along with the nuclear translocation of β-catenin (Li et al., 2010). Collagen XVII and laminin-5 can also induce EMT-driven cancer stemness through the activation of FAK/Akt paired with inhibition of GSK-3β (Liu et al., 2018). The induction of EMT and CSC phenotypes by the ECM seems to be driven by a master regulator, Akt. Akt activation, which can be achieved via intracellular focal adhesion proteins such as FAK and ILK, subsequently modulates the activity of downstream effectors. For instance, Akt can activate NF-κB, which has been shown to upregulate the expression the stemness genes SOX2, NANOG and KLF4 in breast and prostate cancer cells (Liu et al., 2010; Moreira et al., 2015). Akt, as well as ILK, can also inactivate GSK-3β, which increases the nuclear translocation of β-catenin, a transcription factor that is associated with stemness and is also an activator of NOTCH and Wnt signaling (Vadlamudi et al., 2005; Fang et al., 2010). Therefore, ECM regulates the switch between CSC and non-CSC states by inducing EMT.

Self-Renewal/Maintenance

The ECM also promotes CSC self-renewal. In this regard, collagen I has been shown to preserve stemness in malignant and non-malignant stem cells by activating transcriptional programs that induce self-renewal (Kirkland, 2009; Suh and Han, 2011). Binding of collagen to α2β1 integrin results in the nuclear translocation of Bmi1, a stemness-inducing transcription factor downstream of Hedgehog signaling. Studies have shown that Bmi1 is a transcriptional target of Gli1, a stemness related gene, and that FAK/Ras signaling enhances the expression of Gli1 (Goel et al., 2013). Akt/p-S6K1 signaling has also been shown to play a regulatory role in activity of Gli1 (Wang et al., 2012). Laminin and fibronectin signaling also plays a crucial role in CSC self-renewal. Laminin 511 can sustain breast cancer stemness through activation of α6β1 integrin, in a TAZ-dependent manner (Chang et al., 2015). TAZ expression and nuclear localization

induce the expression of the stemness transcription factors, OCT4, SOX2 and NANOG in non-malignant and malignant cells (Varelas et al., 2008; Chang et al., 2015; Xiao et al., 2015). Fibronectin's extra domain A (EDA) has also been demonstrated to positively regulate CSC self-renewal through activation of α9β1 integrin/FAK/ERK/Akt/β-catenin pathway (Ou et al., 2013).

Growth Factor Reservoir and Release

The ECM might serve as a reservoir for factors that aid in the sustenance of CSCs. Embryonic stem cells (ESCs) have been shown to utilize matrix metalloproteases 1 (MMP1) to release ciliary neurotropic factor (CNTF) from an ESC-derived matrix, which enhances ESC self-renewal though JAK/STAT3 signaling (Przybyla et al., 2013), a pathway that has also been implicated in promoting self-renewal of breast CSCs (Wang et al., 2018). Hematopoietic stem cells (HSCs) also upregulate MMP-9 to release soluble kit-ligand, also known as stem cell factor (SCF), which promotes survival signaling and chemoresistance in many types of cancers (Foster et al., 2018). CSCs are thought to remodel their matrices more significantly than their non-cancer stem cell counterparts (Raja et al., 2015) as CSCs upregulate expression of different MMPs. This may enable them to effectively degrade and remodel ECM matrices (Inoue et al., 2010; Long et al., 2012) to release growth factors and cytokines to promote their survival.

Metabolic Reprogramming and Autophagy

The ECM serves as a functional repository for a plethora of factors that dynamically modulate the tumor microenvironment to promote CSC metabolism. Focal adhesion formations transduce ECM signaling into the tumor cells and activate the PI3K pathway which increases glycolysis, in addition to activating glutamine signaling in a Ras- and Myc- dependent manner. Furthermore, a stiff ECM acts as a driver of glycolysis in CSCs (Pickup et al., 2014). On the contrary, accumulating evidence suggests that CSCs also utilize OXPHOS, fatty acid oxidation and glutaminolysis (Sancho et al., 2016; Martinez-Outschoorn et al., 2017). In this regard, it has been demonstrated that CSCs with high telomerase activity upregulate glycolysis and OXPHOS in lung and ovarian cancers (Bonuccelli et al., 2017). Given the diversity of tumors and their microenvironments, it is possible that based on the availability of nutrients, CSCs can manipulate their metabolism. For example, while CSCs in a hypoxic microenvironment may survive by means of glycolysis, CSCs in a normoxic environment use oxidative metabolism. Furthermore, CSCs utilize metabolites secreted by cancer-associated fibroblasts such as lactate and ketone bodies to fuel OXPHOS (Nazio et al., 2019). Recycling of nutrients via autophagy is another way by which CSCs not only self-renew but also acquire drug resistance (Mowers et al., 2018). Autophagy impairment downregulates the expression of CSC markers and consequently the CSC self-renewal capacity in breast, liver, ovarian and pancreatic cancers, osteosarcoma and gliobastoma (Nazio et al., 2019). ECM-receptor ligation has been shown to induce autophagy (Neill et al., 2014; Kawano et al., 2017). Collagen VI, a promoter of tumorigenesis (Chen et al., 2013) and a supporter of stem cell niches (Urciuolo et al., 2013), also functions as an autophagy inducer in skeletal muscle stem cells by functionally interacting with decorin, a small leucin-rich proteoglycans (SLRP) that has been shown to induce stemness in glioblastoma (Farace et al., 2015). A growing number of studies indicate that collagen VI directly maintains CSCs by activating the Akt–GSK-3β–β-catenin–TCF/LEF axis, which is required for activation of autophagy (Fan et al., 2018). Decorin signaling, independent of Collagen VI, can also maintain stemness of trophoblasts and prevent their differentiation (Nandi et al., 2018).

ROLE OF HYPOXIA IN ECM-DERIVED CANCER STEMNESS

Solid tumors frequently contain highly hypoxic regions and tumor hypoxia is positively associated with poor prognosis. Hypoxic tumor cells express stem cell markers, are highly undifferentiated and exhibit enhanced clonogenic potential *in vitro* and tumor initiating potential *in vivo* (Desplat et al., 2002; Jogi et al., 2002; Das et al., 2008; Kim et al., 2009). Furthermore, hypoxia can lead to increased ECM deposition and remodeling. Histological studies on clinical tumor samples have shown increased collagen deposition resulting in fibrosis in hypoxic regions of tumors (Shekhar et al., 2003). In addition to cancer cells, fibroblasts cultured under hypoxic conditions show increased type I procollagen α1 mRNA (Falanga et al., 1993; Tamamori et al., 1997; Norman et al., 2000). Abrogating HIF1α expression inhibits collagen deposition from both breast cancer cells and fibroblasts *in vitro* and *in vivo* (Gilkes et al., 2013a,b, 2014; Xiong et al., 2014). ECM remodeling enzymes such as LOX, LOX-like protein 2 (LOXL2), LOXL4, MMP2, MMP9 and MMP14 and growth factors inducing collagen deposition (e.g., VEGF) are HIF-regulated genes that are involved in tumor fibrosis (Gilkes et al., 2014). Since all these factors have been previously implicated cancer stemness, it is not surprising that the ECM acts a functional conduit for hypoxia-derived signals that foster cancer stemness.

ECM MODULATES IMMUNE SURVEILLANCE IN CSC MICROENVIRONMENT

Extracellular matrix can profoundly influence recruitment of immune cells into the tumor microenvironment. CSCs can evade immune surveillance by altering this microenvironment to favor their survival. For example, ECM drives the activation of pro-survival pathways such as PI3K/AKT, which has been shown to facilitate immune evasion in CSCs (Dituri et al., 2011). ECM proteins can recruit immunosuppressive cells such as tumor-associated macrophages (TAMs) (Stahl et al., 2013; Lu et al., 2014) and regulatory T cells (Bollyky et al., 2011) that have been known to promote CSC survival, while simultaneously blocking the recruitment of antitumorigenic immune cells such as cytotoxic T cells (O'Connor et al., 2012). In addition, the ECM composition can dramatically modulate the activation state of the

tumor infiltrating immune cells. For instance, a stiff collagen-rich or POSTN-rich ECM allows macrophage polarization to a pro-tumorigenic M2 phenotype (Wesley et al., 1998; Zhou W.C. et al., 2015). Following recruitment, the M2 macrophages activate several CSC survival signaling pathways including Src, NF-κB (Lu et al., 2014), STAT3/SOX2 (Yang et al., 2013) and Hedgehog (Jinushi et al., 2011). ECM can also impair proliferation and activation of T cells, that are required for capturing and killing CSCs (Di Tomaso et al., 2010). A collagen-rich ECM can inhibit T-cell proliferation and activation through type I collagen-dependent fusion of LAIR receptors (Meyaard, 2008; Frantz et al., 2010) in addition to sequestering growth factors required for T cell proliferation (Meyaard, 2008; O'Connor et al., 2012). Furthermore, TAMs (Martinez and Gordon, 2014) and neutrophils (Yakubenko et al., 2018) that can selectively reorganize the ECM to promote malignant growth of cancers are preferentially recruited to the microenvironment.

CSC TARGETING THERAPIES

Currently, there are several inhibitors targeting various aspects of ECM-induced cancer stemness that are undergoing clinical testing. For example, the CD47 blocking protein TTI-621 (Petrova et al., 2017) is currently being assessed in a number of phase I clinical trials (NCT03013218, NCT02663518, NCT02216409, NCT02678338) for various types of cancers. Other groups have targeted FAK with the inhibitor VS−6063 (Defactinib) (Lin et al., 2018), which has completed clinical phase I and II trials (NCT01778803, NCT01943292, NCT01951690) with one of those clinical trials assessing for CSCs as an endpoint (NCT01778803). Other inhibitors of stemness-related molecules further downstream of ECM signaling are also being tested in clinical trials, such as the STAT3 inhibitor BBI−608 (Sonbol et al., 2019) in a phase II trial that will test for presence of CSC as an endpoint (NCT02279719) and in a phase III clinical trial aimed at reducing CSCs by targeting phosphorylated Stat3 positive cancer cells (NCT02753127). The β-catenin pathway inhibitors PRI-724 and CWP232291 (Tai et al., 2015) are currently being tested in two phase I clinical trials (NCT01764477, NCT01398462). Inhibition of the Hedgehog pathway with the inhibitor GDC−0449 (Vismodegib) (Basset-Séguin et al., 2017), is also currently being clinically evaluated in a phase II trial which will test for the presence of pancreatic CSCs (NCT01088815).

CHALLENGES AND CONCLUSION

Although the above drugs may effectively reduce the number of CSCs, there are still many potential challenges that ECM components in a tumor microenvironment may set that could interfere with an otherwise successful treatment regimen. Firstly, ECM proteins have been shown to act as a physical barrier, making drug delivery to cancer cells more difficult. Secondly, ECM proteins can de-differentiate non-CSCs into CSCs, which makes eliminating all CSCs more challenging. Thirdly, ECM plays a role in modulating immune cell recruitment, hence, potential immunotherapeutic strategies could be hindered by dysregulated ECM components. Finally, the ECM has a very complex and dynamic nature: different ECM molecules are expressed in a time and tissue-specific manner where various isoforms of the same molecule can play opposing functions in cancer stemness in a context-dependent manner. Considering these concerns, it is crucial that future studies further elucidate the role of ECM components on cancer stemness in order to design therapies that effectively eradicate all CSCs.

AUTHOR CONTRIBUTIONS

All authors conceptualized the content and wrote the manuscript.

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REVIEW
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Friend or Foe? Recent Strategies to Target Myeloid Cells in Cancer

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The tumor microenvironment (TME) is a complex network of epithelial and stromal cells, wherein stromal components provide support to tumor cells during all stages of tumorigenesis. Among these stromal cell populations are myeloid cells, which are comprised mainly of tumor-associated macrophages (TAM), dendritic cells (DC), myeloid-derived suppressor cells (MDSC), and tumor-associated neutrophils (TAN). Myeloid cells play a major role in tumor growth through nurturing cancer stem cells by providing growth factors and metabolites, increasing angiogenesis, as well as promoting immune evasion through the creation of an immune-suppressive microenvironment. Immunosuppression in the TME is achieved by preventing critical anti-tumor immune responses by natural killer and T cells within the primary tumor and in metastatic niches. Therapeutic success in targeting myeloid cells in malignancies may prove to be an effective strategy to overcome chemotherapy and immunotherapy limitations. Current therapeutic approaches to target myeloid cells in various cancers include inhibition of their recruitment, alteration of function, or functional re-education to an antitumor phenotype to overcome immunosuppression. In this review, we describe strategies to target TAMs and MDSCs, consisting of single agent therapies, nanoparticle-targeted approaches and combination therapies including chemotherapy and immunotherapy. We also summarize recent molecular targets that are specific to myeloid cell populations in the TME, while providing a critical review of the limitations of current strategies aimed at targeting a single subtype of the myeloid cell compartment. The goal of this review is to provide the reader with an understanding of the critical role of myeloid cells in the TME and current therapeutic approaches including ongoing or recently completed clinical trials.

Keywords: myeloid cells, Immunotherapy, MDSC, TAM, DC, TME, immune checkpoint blockade, microbiome

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INTRODUCTION

Immune cell involvement in inflammatory ailments has long been established; however, their role in cancer remained unappreciated until the past three decades (Chen and Mellman, 2017). Indeed, the paradigm of cancer cells being a single player in cancer progression has shifted to models that include several stromal elements of the tumor microenvironment (TME) (Hanahan and Weinberg, 2011). The TME stroma is composed of endothelial cells, fibroblasts, extra cellular matrix, and

diverse immune cell populations that act dynamically to regulate tumor growth. Myeloid cells play a major role in the body's defense against infection, tissue homeostasis, as well as modulation of T cell mediated immunity (Merad et al., 2013; Cozzo et al., 2017). However, in the tumor, while myeloid cells initially respond to an injury or wound signal in the TME, neoantigen within the cancer cell, or some other signal from growing cancer cells, often the phenotype of immune cells evolve such that they become our own worst enemy in the fight against cancer.

Myeloid cells constitute a major stromal cell population in the TME (De Vlaeminck et al., 2016). They regulate tumor growth by direct or indirect interaction with cancer cells (Gabrilovich et al., 2012; Broz and Krummel, 2015). Myeloid cells comprise mononuclear and polymorphonuclear cells (Engblom et al., 2016). Macrophages are the major myeloid component of mononuclear phagocytes and represent the largest population of immune cell infiltrates in all tumors (Noy and Pollard, 2014), and as such are called "Tumor Associated Macrophages" (TAMs). TAMs are strongly linked to therapy resistance and are associated with poor prognosis (Kurahara et al., 2013) due to soluble factors secreted by infiltrating TAMs that contribute to drug resistance, metastasis, and immune evasion (Beatty et al., 2011). Macrophages are highly plastic cells capable of adopting different phenotypes in response to signals within various microenvironments (De Palma and Lewis, 2013). Thus, while TAMs have the capacity to kill cancer cells (Evans and Alexander, 1970), TAMs can also be modified to promote tumor growth and metastasis (Mantovani, 1978) - which emphasizes their critical and complex role in tumor biology.

Dendritic cells (DCs) are another mononuclear myeloid cell, although less prevalent, DCs are powerful components of the TME through their role as antigen presenting cells. Like TAMs, DCs have several phenotypes or subtypes which include classical DCs (cDCs) which are specialized in antigen presentation and induction of T cell immunity (Merad et al., 2013), plasmacytoid DCs (pDCs) which produce interferon-α which is important in antitumor immunity (Swiecki and Colonna, 2015), and monocytic DCs (mDCs) which differentiate from circulating monocytes and present a pro-inflammatory phenotype (Gopalakrishnan et al., 2018). Another family of myeloid cells are myeloid-derived suppressor cells (MDSCs), which are potent immunosuppressive cells that arise in pathological conditions such as cancer. MDSCs promote tumor growth, angiogenesis and metastasis, but their main function is to suppress T cell activation leading ultimately to immune evasion (Talmadge and Gabrilovich, 2013). MDSCs are an immature abnormally differentiated class of myeloid cells which comprise two distinct classes: granulocytic or polymorphonuclear MDSC (PMN-MDSC) and monocytic MDSC (M-MDSC) (Marvel and Gabrilovich, 2015). Interestingly, M-MDSCs have been shown to differentiate into TAMs in tumors, suggesting that targeting only one subtype of tumor infiltrating myeloid cell such as PMN-MDSCs may not be sufficient to achieve an effective therapeutic response (Kumar et al., 2016). In this review, we define the role of myeloid cells in cancer, with a focus on TAMs and MDSCs, and how they contribute to immune suppression and therapy resistance. We also summarize novel molecular targets in myeloid cells and discuss up-to-date strategies, such as targeted delivery, to effectively deplete or reconvert our foes to friends in the TME to increase therapeutic efficacies to best fight cancer. Our overall goal is to convey to our readers the importance of targeting myeloid cells in cancer, while critically emphasizing the limitations of current monotherapies targeting myeloid cells in malignancies.

MYELOID CELL PHENOTYPES IN CANCER

Cancer cells exploit myeloid cells to escape immune surveillance by changing their phenotype from tumoricidal to tumor supportive and immunosuppressive (Awad et al., 2018). Myeloid cells play an important role in tissue homeostasis and regulation of adaptive immune responses by regulating CD4 and CD8 T cell content and activation. Thus, myeloid cells are highly versatile and plastic cells making them suitable pharmacologic targets to attempt to revert their phenotype to overcome immune tolerance in cancer (Schouppe et al., 2012).

Macrophages

Macrophages are plastic cells of the innate immune system capable of adopting varied phenotypes in response to signals in their microenvironment (Okabe and Medzhitov, 2014). In pathological conditions, macrophages respond to pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) derived from gram negative bacteria, which then activate transcription factors such as nuclear factor kappaB (NF-kB) through toll-like receptor 4 (TLR4) to initiate an inflammatory response (Kawai and Akira, 2010). Pro-inflammatory (M1-like) macrophages secrete cytokines such as IL-12, IL-6, and TNF-α to amplify the pro-inflammatory response against pathogens by recruiting more leukocytes to the site of inflammation (Ngambenjawong et al., 2017). In contrast, alternatively activated macrophages (M2-like) are present in wound healing environments in response to IL-4 and IL-13 cytokines. This stimulation results in the production of anti-inflammatory enzymes such as arginase (Arg-1) in a STAT6-dependent manner, producing a cascade of immunoregulatory and tissue remodeling events through the secretion of key cytokines and metabolites by alternatively activated macrophages (Dyken and Locksley, 2013). Similarly, in the TME, M2-like macrophages produce cytokines, chemokines, and enzymes that have tumor promoting properties (Castells et al., 2012). Recent evidence suggests that most tissue-resident macrophages arise from fetal precursors in the yolk sac independently of bone marrow-derived cells and persist throughout life (Ginhoux and Guilliams, 2016). Yet the origin of TAMs is complex and dependent upon the tumor milieu (Wynn et al., 2013). In breast, lung, pancreas, brain, and liver mouse cancer models, tissue resident-derived TAMs are progressively diluted by monocyte-derived TAMs (mo-TAMs) during tumor growth (Lahmar et al., 2016). For example, TAMs in the MMTV-PyMT mammary tumor model are phenotypically and

functionally distinct from tissue-resident macrophages and are derived from circulating monocytes (Franklin et al., 2014). In contrast, a significant portion of pancreas-resident macrophages originate from embryonic development in Pancreatic Ductal Adenocarcinoma (PDAC) mouse models (Zhu Y. et al., 2017). Despite the controversy regarding the origin of TAMs and complexity of cancer specificity, together the evidence suggests that the ontogeny of TAMs is heterogenous and that both monocyte-derived and tissue resident macrophages constitute the pool of TAMs that infiltrate primary and metastatic tumors (De Palma, 2016). For example, in *Ccr2-/-* mice engrafted with colorectal cancer, reduction in monocyte-derived TAMs was associated with reduced tumor burden suggesting a role of mo-TAMs in tumor growth (Afik et al., 2016). Although monocyte-derived TAMs and tissue resident TAMs play different roles during tumor progression, as previously reported in PDAC and brain cancer mouse models (De Palma, 2016; Zhu Y. et al., 2017), more evidence is needed to accurately define the contribution of varied TAM subpopulations to more efficient targeting in malignancies.

Clinically, high densities of macrophages in primary tumors have been correlated with poor prognosis (Mantovani et al., 2017). However, both positive and negative outcomes have been reported in colon, lung, prostate, and bone cancers in the presence of high TAM content (Zhang et al., 2015). It is possible that these conflicting data are related to the type and stage of cancer or to the type of analysis performed (Ruffell and Coussens, 2015). The presence of the M1-like phenotype in TME correlates with a better prognosis, while the presence of the M2-like phenotype usually predicts poorer prognosis (Yuan et al., 2014). TAMs were also reported to mediate chemotherapy resistance in various cancer types by activating anti-apoptotic pathways and/or by providing cancer cells with survival factors (Ruffell and Coussens, 2015). While detailed causes of TAM-induced tumor growth and therapy resistance have yet to be uncovered, emerging therapeutic approaches aiming to deplete macrophages and/or shift macrophage phenotypes represent promising therapeutic modalities for cancer patients (Quail and Joyce, 2017).

Myeloid-Derived Suppressor Cells (MDSCs)

Myeloid-Derived Suppressor Cells are only found in pathologic conditions such as cancer, obesity, autoimmunity, or chronic infection. In contrast to most other myeloid cells, MDSCs are strongly immunosuppressive. In cancer, MDSCs are derived from myeloid progenitor cells and accumulate in the bone marrow in response to signals released by tumors (Condamine et al., 2015a). Activation of MDSCs results from a continuous stimulation of myeloid cells with low-strength signals, causing poor phagocytic capacity, and elevated production of reactive oxygen species (ROS), nitric oxide (NO), and anti-inflammatory cytokines (Kumar et al., 2016). The abundance of tumor infiltrating MDSCs is associated with advanced malignancy stage and an overall poorer prognosis in various types of cancer (Parker et al., 2015). For example, patients with stages III and IV melanoma, non-small cell lung cancer, hepatocellular carcinoma, pancreatic, bladder, and gastric cancers have higher frequencies of circulating MDSC in the peripheral blood as compared to patients with stages I and II of these diseases (Almand et al., 2001; Gabitass et al., 2011; Eruslanov et al., 2012; Jiang et al., 2015). Additionally, solid tumor patients who have high levels of circulating MDSCs respond poorly to immunotherapy such as immune checkpoint inhibitors (Weber et al., 2018). There are two types of MDSCs that have been identified in both mice and humans: polymorphonuclear MDSCs (PMN-MDSC) that are morphologically similar to neutrophils, and monocytic MDSCs (M-MDSC) that are similar to monocytes (Condamine et al., 2015b; Ugel et al., 2015). A third class of MDSCs was recently described in human peripheral blood mononuclear cell (PBMC) and is referred to as "early-stage MDSC" (eMDSC). eMDSCs lack the expression of CD14 which is expressed in human M-MDSC and CD15 which is expressed in human PMN-MDSC. However, eMDSC specific role and its mouse equivalent population are yet to be defined (Bronte et al., 2016). MDSCs are functionally defined by their ability to suppress antitumor T cell activity through the secretion or expression of immune-regulatory factors including Arg1, NO, TGF-β, and cyclooxygenase 2 (Vasquez-Dunddel et al., 2013; Marvel and Gabrilovich, 2015). For example, Arg1 depletes arginine which is an essential amino acid for T cell proliferation and activation, while reactive oxygen species produced by MDSCs kills target cells by inducing oxidative stress (Ostrand-Rosenberg and Fenselau, 2018). PMN-MDSCs are recruited to the tumor site primarily by the CXC chemokine family which include CXCL1, 5, 6, 8, and 12 (Kumar et al., 2016). In a mouse model of hepatocellular carcinoma, increased production of CXCL12 promoted CXCR4-mediated recruitment of PMN-MDSCs to premetastatic niche sites (Seubert et al., 2015). Similarly, loss of CXCR2 in a colitis-associated cancer mouse model dramatically inhibited tumorigenesis through inhibiting infiltration of PMN-MDSCs into colonic mucosa and the tumor site (Katoh et al., 2013). In contrast, M-MDSCs are recruited to primary and metastatic tumor sites through chemokines produced by tumors, primarily CCL2 and CCL5 (Kitamura et al., 2015; Kumar et al., 2016). Clinically, MDSCs have been suggested as predictive biomarkers for disease outcome as high levels of circulating MDSCs prior to cancer therapy negatively influenced survival in most cancers suggesting that circulating MDSCs should be taken into account to improve prognostic evaluation (Wang P.F. et al., 2018). Taken together, these studies demonstrate the need for an effective targeting of MDSCs in cancer to overcome limitations of current treatment options such as chemotherapy and immunotherapy.

Other Myeloid Cell Subtypes

Dendritic cells are versatile antigen-presenting cells which have the ability to initiate pro-inflammatory immune responses and are major contributors to cytotoxic responses in tumors (Worbs et al., 2016). Conventional DCs (cDCs), among other DC subtypes, preferentially activate T cells which represent the foundation of the "cancer-immunity cycle" (Chen and Mellman, 2013). cDCs can be divided into two different subsets: cDC1

and cDC2 (Merad et al., 2013; Guilliams et al., 2014). cDC1 depend on the transcription factors IRF8, Batf3, and ID2 for development and express CD103 in mice while CD141 is used to distinguish cDC1 in humans (Böttcher and Reis e Sousa, 2018; Collin and Bigley, 2018). cDC1 are essential for CD8+ T cell activation as highlighted by several studies using cDC1-deficient *Batf3*$^{-/-}$ mice and other *in vivo* models of cDC1 depletion, which consistently display a loss of cDC1's ability to induce a T cell-mediated antitumor immune response (Broz et al., 2014; Salmon et al., 2016; Sanchez-Paulete et al., 2016; Spranger et al., 2017). cDCs activate CD8+ T cells by cross-presenting extracellular antigens on major histocompatibility complex class molecules (Broz and Krummel, 2015). Hence, high numbers of tumor infiltrating DCs were associated with T cell activation resulting in an antitumor immune response (Dieu-Nosjean et al., 2008; Goc et al., 2014). Additionally, the presence of cDC1 in the tumor stroma was correlated with increased overall survival in patients with various types of cancer (Broz et al., 2014). However, tumors may also alter the anti-cancer role of DCs (Gabrilovich, 2004) through several factors present in the TME. For example, IL-10 produced by TAMs prevents the production of IL-12 by CD103+ DCs leading to the impairment of T cell activation. Also, tumor microenvironmental factors such as low pH, hypoxia, and lactic acid impair DC-mediated T cell activation (Veglia and Gabrilovich, 2017). Clearly, like so many immune cells in the TME, the specific cancer, stage, aggressiveness, and other factors influence the phenotype of DCs. Finally, just like macrophages and DCs, neutrophils, eosinophils, mast cells, and monocytes have all been reported to adopt different phenotypes in cancer (Engblom et al., 2016; Porrello et al., 2018). Thus, myeloid cells represent a suitable and a major target in cancer despite the challenges they pose in our ability to distinguish their tumor-promoting versus inhibitory activities in preclinical models and cancer patients (Engblom et al., 2016; Porrello et al., 2018). The major tumor-infiltrating myeloid cell phenotypes in cancer are summarized in **Figure 1**.

NOVEL MOLECULAR TARGETS IN TUMOR ASSOCIATED MYELOID CELLS

Given the critical role that myeloid cells play in cancer, the need to identify novel molecular targets to block the recruitment of myeloid cells to the tumor site, shift their phenotype to an anticancer one, or simply deplete them may be of the utmost importance. Here we summarize recent findings of novel key players that modulate myeloid cell phenotypes in malignancies.

PI3Kγ

The phosphoinositide 3-kinase (PI3K)-AKT-mTOR pathway controls key cellular processes such as growth, proliferation, and metabolism in cancer cells and is one of the most dysregulated pathways in malignancies (Thorpe et al., 2015; Janku et al., 2018). The class I PI3K lipid kinases drive metabolic and transcriptional pathways in inflammation and cancer (Martini et al., 2014). The PI3K Class 1A isoforms include PI3Kα and PI3Kβ which are widely expressed in epithelial and endothelial cells, while the Class IA isoform PI3Kδ is expressed mainly in lymphocytes. Importantly, the class 1B isoform PI3Kγ has a unique structure and is largely expressed in myeloid cells. PI3Kγ plays a major role in myeloid cell migration and accumulation in tumor tissues (Schmid et al., 2013; Martini et al., 2014). Recent studies have reported that PI3Kγ constitutes a molecular switch which controls macrophage polarization during inflammation and cancer (Kaneda et al., 2016b). PI3Kγ promoted immune suppression in malignancies through activation of Akt and mTOR signaling and prevention of NF-κB activation. Selective inactivation of PI3Kγ with the specific inhibitor IPI-549 (Evans et al., 2016) stimulated and prolonged NF-κB activation, thus alleviating immune suppression and restored CD8+ T cell cytotoxicity (Kaneda et al., 2016b). In two PDAC mouse models, pharmacologic blockade of PI3Kγ with the selective inhibitor TG100-115 reprogrammed TAMs to stimulate CD8+ T cell-mediated tumor suppression and inhibited tumor cell metastasis and desmoplasia, a fibrotic phenotype associated with TAMs and poor therapeutic efficacy (Kaneda et al., 2016a). Also, genetic or pharmacological inhibition of PI3Kγ or its downstream signaling molecule integrin α4 blocked MDSC recruitment to tumors and immune suppressive myeloid cell polarization, thus increasing expression of pro-inflammatory cytokines and reducing expression of anti-inflammatory cytokines. Moreover, inhibition of either PI3Kγ or integrin α4 stimulated DC and CD8+ T cell recruitment to the tumor site, thereby promoting tumor cell cytotoxicity (Foubert et al., 2017). In sum, targeting myeloid cell PI3Kγ in cancer patients can enhance the efficacy of current therapy regimens and may constitute a novel approach to improve the long-term survival of cancer patients (Gunderson et al., 2016; Kaneda et al., 2016b).

PD-1- PD-L1 Axis

The programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) pathway are key components of the immunosuppressive TME (Rosenblatt and Avigan, 2017). PD-L1 is expressed on a variety of cell types including mesenchymal cells such as adipocytes (Wu et al., 2018), and is also found on hematopoietic cells such as lymphocytes and myeloid cells (Sharpe and Pauken, 2017). PD-1 is expressed during T cell activation and engages its ligands PD-L1 and PD-L2, thus inhibiting effective T cell cytotoxicity resulting in poor anti-tumor immunity (Greenwald et al., 2005; Sun et al., 2018). PD-L1 is expressed on TAMs, and the response rate to anti-PD-L1 antibody in patients where at least 10% of macrophages express PD-L1 was as high as 80% (Herbst et al., 2014). PD-L1 is also expressed on DCs, MDSCs, and monocytes (Sipe et al., 2020). *In vitro* treatment of macrophages with anti-PD-L1 antibody led to the activation of multiple macrophage pro-inflammatory pathways (Hartley et al., 2018). Additionally, combined treatment of anti-PD-1 and anti-PD-L1 antibodies cured half of the treated mice in an established melanoma mouse model (Hartley et al., 2018). These findings suggest that PD-L1 induces an immune-suppressive macrophage phenotype while treatment with anti-PD-L1 antibody reverses macrophage

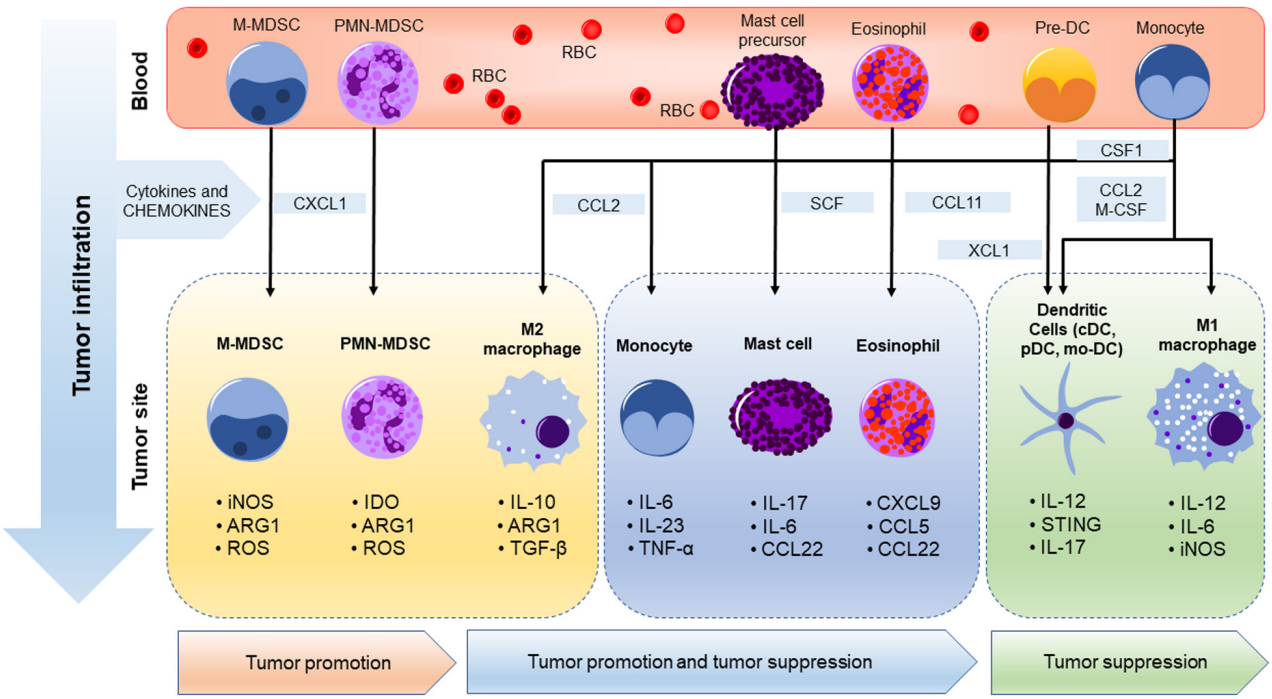

FIGURE 1 | Myeloid cell phenotypes in cancer. In cancer, myeloid cells are generated in the bone marrow from the common myeloid progenitors and migrate to the tumor microenvironment in response to factors released by tumors. Suppressor myeloid cells such as MDSCs and M2 macrophages promote tumor growth by suppressing innate and adaptive immunities via production of immune suppressive factors such as arginase 1 and other cytokines. These factors promote an immunosuppressive tumor microenvironment by altering innate anticancer immunity and T cell functions. On the other hand, M1 macrophages and DCs have antitumor potential via production of pro-inflammatory and antitumor factors such as IL-12 and iNOS. While the role of macrophages, MDSCs, and DCs are best studied and are the main focus of this review, the role of monocytes, and less prevalence (lower density cells) mast cells and eosinophils in tumor promotion and suppression is less clear and is an active area of research while further evidence is required to fully elucidate their role in cancer immunity (Jachetti et al., 2018; Rigoni et al., 2018; Gorzalczany and Sagi-Eisenberg, 2019).

polarization, thereby triggering a potent macrophage-mediated anti-tumor immune response (Hartley et al., 2018). In another study, depletion of myeloid cells in a *Kras*-driven pancreatic cancer mouse model prevented tumor initiation and, in some cases, arrested tumor growth by restoring CD8+ T cell anti-tumor immunity. These results suggest that myeloid cells inhibit CD8+ T cell antitumor capacity by inducing PD-L1 expression in tumor cells (Zhang et al., 2017). Interestingly, PD-1 expression is not restricted to lymphocytes, but is also expressed by TAMs (Gordon et al., 2017). PD-1 expression in TAMs correlated positively with disease stage in both mice and humans with primary cancers. PD-1 expression by TAMs prevented phagocytosis of tumor cells by macrophages, whereas blockade of the PD-1-PD-L1 pathway *in vivo* restored macrophage phagocytic potential. These data suggest that PD-1 and PD-L1 blockade may also directly act on macrophages (Gordon et al., 2017). Consequently, myeloid cells represent an additional highly prevalent and potentially potent target for immune checkpoint blockade therapies in cancer.

Iron Metabolism

Iron is a vital nutrient that enables cell proliferation and growth (Torti and Torti, 2013). It is required for oxygen transport, DNA biosynthesis, and the production of adenosine triphosphate (ATP) via electron exchange (Kosman, 2010; Soares and Hamza, 2016). There is ample evidence that iron overload is associated with cancer (Torti and Torti, 2013; Manz et al., 2016). Macrophages play a major role in iron homeostasis by recycling iron from senescent and dying red blood cells (RBC) back into the circulation and different tissues in the body (Andrews, 1999; Ganz, 2012; Hubler et al., 2015). Additionally, macrophage polarization is closely associated with iron metabolism (Dong et al., 2019). Studies have shown that over 60% of iron metabolism-related genes are differentially expressed between the M1/M2 macrophage axis (Recalcati et al., 2010). It is now established that M1-like macrophages express high levels of iron storage protein ferritin and low levels of iron export protein ferroportin (*Fpn*) which favor an iron-sequestration macrophage phenotype. On the other hand, M2-like macrophages display the iron-export phenotype by increased expression of ferroportin and decreased expression of ferritin (Recalcati et al., 2010; Jung et al., 2015). Consistent with these findings, higher iron availability in the TME was linked with accelerated ferroportin-mediated iron release by TAMs, which further validates the pro-tumorigenic properties of TAMs (Biswas and Mantovani, 2010; Marques et al., 2014). Moreover, iron-export TAMs express high levels of CD163, a high-affinity scavenger receptor

for haptoglobin that also binds to hemoglobin. Activation of CD163 by binding to either hemoglobin or haptoglobin induces the transcription of ferroportin (Marro et al., 2010; Gnerlich et al., 2011). Previous evidence suggested that a high density of CD163+ TAMs positively correlated with poor prognosis in many cancer types (Heusinkveld and van der Burg, 2011). A recent study revealed that CD163+ TAMs promoted expression of IL-6 and CXCL2 by cancer cells while inhibition of either IL-6 or CD163 macrophage-induced tumorigenesis in a co-culture *in vitro* system and a sarcoma mouse model (Shiraishi et al., 2018). In addition, a study has shown that inhibition of heme oxygenase 1 (HO-1; which is an enzyme that degrades heme to release iron) in TAMs induced M1 polarization in macrophages and reduced tumor growth in a breast cancer mouse model (Mertens et al., 2016). Remarkably, the macrophage iron phenotype becomes a suitable target for iron chelators and iron oxide nanoparticles (IONs). Iron chelators have been explored as a monotherapy, or as an adjuvant therapy for the treatment of various malignancies (Heath et al., 2013). IONs on the other hand, have been broadly explored in preclinical and clinical studies in the past decade (Hu et al., 2018). IONs accumulation in macrophages increased intracellular iron levels, thus promoting the proinflammatory phenotype (Laskar et al., 2013; Dong et al., 2019). Ferumoxytol, an FDA-approved ION for the treatment of anemia, inhibited tumor growth and metastatic spread in a xenograft mouse model by shifting TAM polarization toward the M1-like phenotype (Zanganeh et al., 2016). Consequently, modulation of iron metabolism in macrophages may represent a promising monotherapy, or combination therapeutic approach for cancer patients.

Microbiome

In recent years, the microbiome has emerged as a contributor of cancer progression in a variety of malignancies including colon and primary liver cancers (Plottel and Blaser, 2011). A recent study found that the malignant pancreas comprises a more abundant microbiome than the normal pancreas in both mice and humans (Pushalkar et al., 2018). Bacterial ablation in PDAC bearing mice reduced MDSC populations, increased M1 macrophage populations, promoted differentiation of CD4+ T cells, and activated CD8+ T cells to reduce tumor growth. These data suggest that endogenous microbiota promote immune suppression in PDAC patients and propose the microbiome as a potential target for the modulation of PDAC progression (Pushalkar et al., 2018). Another recent study has shown that *Peptostreptococcus anaerobius* which is an anaerobic bacterium, adheres to colorectal cancer cell mucosa and accelerates colorectal cancer development. Mechanistically, a *Peptostreptococcus anaerobius* surface protein, putative cell wall binding repeat 2 (PCWBR2) interacts with α_2/β_1 integrin in colon cancer cells which leads to the activation of the PI3K-Akt pathway resulting in NF-κB activation. NF-κB in turn triggers a pro-inflammatory response and leads to a significant expansion of MDSCs and TAMs. Pharmacological blockade of integrin α_2/β_1 impairs *Peptostreptococcus anaerobius* attachment and decreases tumor burden. These findings propose *Peptostreptococcus anaerobius*-induced PCWBR2-integrin α_2/β_1 axis as a potential therapeutic target in colorectal cancer (Long et al., 2019). The adaptor protein Caspase Recruitment Domain-containing protein 9 (CARD9) is exclusively expressed in myeloid cells and is required for the activation of innate immunity (Jia et al., 2014). Recent evidence suggests that CARD9 deficiency impaired macrophage fungicidal functions which led to increased fungal loads and a notable increase in *Candida tropicalis* in a colorectal cancer mouse model (Wang T. et al., 2018). *C. tropicalis* expansion induced accumulation of MDSCs which promoted tumor growth. Treatment of CARD9 deficient tumor-bearing mice with an anti-fungal fluconazole suppressed tumor growth in the colorectal cancer mouse model (Wang T. et al., 2018). These findings suggest a direct role of the microbiome in generating MDSCs and that targeting certain fungal populations within the microbiome may represent an attractive therapeutic approach in patients with colorectal cancer. In addition, macrophage-secreted human cationic antimicrobial protein 18 leucine leucine-37 (hCAP-18/LL-37) increased pancreatic cancer stem cell (CSC) pluripotency genes, self-renewal, and tumorigenicity. hCAP-18/LL-37 is an antimicrobial peptide secreted by activated macrophages, but its tumorigenic properties were previously unknown. Indeed, pharmacological inhibition of formyl peptide receptor 2 (FPR2) and/or P2X purinoceptor 7 receptor (P2X7R) on CSCs which are the receptors of hCAP-18/LL-37 inhibited tumor formation in a PDAC mouse model. Thus, hCAP-18/LL-37 is a novel, previously unrecognized target in TAMs to overcome CSC-induced relapse in cancer patients and an excellent example of microbially-mediated modulation of cancer progression.

CXCR2

CXCR2 is a G-protein coupled receptor of the CXC chemokine family which is predominantly expressed on neutrophils and MDSCs (Dart, 2016). The primary immune function of CXCR2 is the regulation of neutrophil and MDSC migration and recruitment to inflammation including tumor sites (Cacalano et al., 1994; Eash et al., 2010; Bian et al., 2014; Highfill et al., 2014). Previous studies have reported that CXCR2 promotes tumorigenesis in skin and colon cancers (Jamieson et al., 2012). In a PDAC mouse model, genetic ablation of CXCR2 abrogated metastasis while pharmacological inhibition of CXCR2 decreased tumor growth. Inhibition of CXCR2 altered neutrophil/MDSC recruitment and enhanced T cell infiltration into the tumor site (Steele et al., 2016). In a breast cancer mouse model, CXCR2+ MDSCs promoted tumor growth and metastasis by secretion of IL-6 and modulation of CD4+ and CD8+ T cell recruitment to the tumor site. CXCR2+ MDSCs also upregulated the expression of inhibitory immune checkpoints PD-1, PD-L1, and cytotoxic T lymphocyte antigen 4 (CTLA4) as well as lymphocyte activation gene protein 3 (LAG3) on CD4+ and CD8+ T cells promoting immunosuppression (Zhu H. et al., 2017). Together, these findings propose CXCR2 as a suitable target to alleviate myeloid cell-induced immune suppression for a better therapeutic outcome in cancer patients.

SPECIFIC NOVEL MOLECULAR TARGETS IN MYELOID CELLS

Herein, we summarize the major novel molecular targets of myeloid cells in cancer from recent literature. Previous reviews have discussed other molecular targets in more detail which may be of interest (Noy and Pollard, 2014; Engblom et al., 2016; Mantovani et al., 2017).

Molecular Targets in TAMs

Proteins secreted from or present in TAMs that have been recently published include mediators of fibrosis, inflammatory signaling, phagocytic capacity, lipid metabolism, and growth factors. First, TAMs in PDAC secrete granulin that activates resident hepatic stellate cells, which then secrete periostin, resulting in a fibrotic TME that promotes metastatic tumor growth. Alteration of TAM recruitment or granulin secretion abrogated liver metastasis (Nielsen et al., 2016). In a follow-up study, macrophage-derived granulin expression was induced in response to CSF-1 and caused CD8+ T cell exclusion in metastatic livers. Interestingly, genetic depletion of granulin diminished the establishment of a fibrotic stroma, thus restoring T cell infiltration at the metastatic site. Furthermore, depletion of granulin sensitized PDAC tumors to anti-PD-1 therapy, and dramatically reduced metastasis, suggesting that targeting TAM-derived granulin may sensitize PDAC tumors to immune checkpoint blockade therapies (Schmid et al., 2018). Second, TAM NOD-like receptor family pyrin domain-containing 3 (*NLRP3*) signaling promoted CD4+ T cell differentiation into regulatory T cell populations while inhibiting CD8+ T cell activation in an IL-10-dependent manner in a PDAC mouse model. Genetic or pharmacological inhibition of the NLRP3 complex resulted in the restoration of innate and adaptive anti-tumor immune response, suggesting that NLRP3 may represent a suitable target to sensitize PDAC to immunotherapy (Daley et al., 2017). Third, neuropilin-2 (NRP2), which is a member of the membrane-associated neuropilin family, is expressed during macrophage differentiation and is induced by tumor cells (Dai et al., 2017; Miyauchi et al., 2018). NRP2 in TAMs induced efferocytosis, which is the phagocytic clearance of dying cells, and promoted tumorigenesis because efferocytosis induces an M2-like anti-immune phenotype in macrophages (Morioka et al., 2018). Inhibition of NRP2 in TAMs increased secondary necrosis by impairing the clearance of dying cancer cells and promoted CD8+ T cell and natural killer (NK) cells infiltration (Roy et al., 2018). Fourth, caspase-1 was reported to promote TAM differentiation by cleaving peroxisome proliferator-activated receptor gamma (PPARγ) (Niu et al., 2017). PPARγ fragments then interacted with and attenuated medium-chain acyl-CoA dehydrogenase (MCAD) resulting in the promotion of TAM differentiation. Caspase-1 inhibition substantially inhibited tumor growth, thus proposing the caspase-1/PPARγ/MCAD pathway as a promising target to prevent TAM-induced tumorigenesis (Niu et al., 2017). Fifth, in BRAF-mutant models, BRAF inhibitors activated the mitogen-activated protein kinase (MAPK) pathway in macrophages which then produced VEGF to promote melanoma tumor growth. Macrophage-mediated resistance to BRAF inhibitors in melanoma was then reversed by blocking the MAPK pathway or macrophage-secreted VEGF. These results suggest that targeting TAMs may benefit BRAF-mutant melanoma patients (Wang et al., 2015). Sixth, ornithine decarboxylase (ODC) is an enzyme that limits polyamine biosynthesis. Consistent with previous literature reporting that ODC reduces M1 polarization in infection sites, a new study revealed that macrophage ODC also impaired the M1 phenotype and promoted colitis-associated colon carcinogenesis (Singh et al., 2018). Mice lacking ODC in myeloid cells demonstrated improved disease outcomes, suggesting that macrophage ODC is a suitable target for colon cancer chemoprevention (Singh et al., 2018). Finally, migration of TAMs is an exciting pathway to target. TAM mesenchymal migration is protease-dependent in mouse and human tumors, providing a new strategy for macrophage immunotherapy by targeting TAM motility (Gui et al., 2018). TAMs secrete interleukin 35 (IL-35) at metastatic sites which activates the JAK2/STAT6/GATA3 signaling pathway to reverse epithelial-mesenchymal transition (EMT) in cancer cells to a mesenchymal- epithelial transition (MET) phenotype, therefore enabling metastatic colonization (Lee et al., 2018). These findings propose TAM-secreted IL-35 as a potential target to intercept metastasis in cancer patients (Lee et al., 2018). Lastly, colony-stimulating factor 1 (CSF-1) and its receptor CSF-1R regulate survival and differentiation of phagocytic myeloid cells and macrophages in particular (Cannarile et al., 2017). *In vitro, in vivo,* and clinical blockade of macrophage CSF-1R with a monoclonal antibody (RG7155) strongly reduced TAM migration and infiltration into the tumor site and the CD8+/CD4+ T cell ratio (Ries et al., 2014). Moreover, targeting of TAMs with a selective CSF-1R inhibitor (AZD7507) in a genetic PDAC mouse model dramatically reduced tumor growth, enhanced T cell immune response and increased mouse survival in a difficult-to-treat model (Candido et al., 2018). In summary, these recent publications support that TAMs are feasible and tenable targets in cancer.

Molecular Targets in MDSCs

Several MDSC mediated pathways have recently been published highlighting these myeloid cells as novel targets. First, key transmembrane receptor tyrosine kinases (TAM RTK) regulate the innate immune system by dampening inflammatory responses including TYRO3, AXL, MERTK (Graham et al., 2014). TAM RTK are stimulated by protein ligands such as GAS6 and PROTEIN S (Geng et al., 2017). MDSCs were found to dramatically up-regulate TAM RTK and their ligands (Holtzhausen et al., 2019). Genetic or pharmacological inhibition of TAM RTK diminished MDSC suppressive capacity, slowed tumor growth, increased CD8+ T cell infiltration to the tumor site, and augmented anti-PD-1 therapy effect in a melanoma syngeneic mouse model (Holtzhausen et al., 2019). Thus, TAM RTK represents a novel MDSC target in melanoma and potentially other cancers. Second, like TAMs, lipid metabolism is also critical to phenotype. PMN-MDSCs

upregulate fatty acid transport protein 2 (FATP2). Genetic deletion or pharmacologic inhibition of FATP2 abrogated the suppressive activity of PMN-MDSCs and delayed tumor growth in multiple syngeneic mouse models. Additionally, FATP2 inhibition blocked tumor growth in combination with immune checkpoint inhibitors, thus highlighting FATP2 as an attractive novel target of MDSCs (Veglia et al., 2019). Makowski lab has demonstrated that FATP1 was critical to the anti-inflammatory M2-like macrophage phenotype, but the role of myeloid FATP1 in the TME is unknown (Johnson et al., 2016; Zhao et al., 2017). Third, AMP-activated protein kinase alpha 1 (AMPKα) is another novel MDSC target which has been previously well documented in other immune populations (Rao et al., 2015, 2016; Zhu et al., 2015). AMPKα upregulation in MDSCs was induced by tumor-secreted granulocyte-monocyte colony-stimulating factor (GM-CSF) in a STAT5-dependent manner. Genetic or pharmacological ablation of AMPKα in several syngeneic mouse models of different cancer types inhibited the immunosuppressive potential of MDSCs, induced CD8+ T cell infiltration into tumor sites, and improved efficacy of immunotherapy (Trillo-Tinoco et al., 2019). These findings support the therapeutic use of AMPK-inhibitors to overcome various immune cell including MDSC-induced immune suppression in cancer (Trillo-Tinoco et al., 2019). Fourth, another novel MDSC target, tumor necrosis factor-alpha-induced protein 8-like 2 (TIPE2), was recently described. TIPE2 expression on MDSCs is induced by reactive oxygen species (ROS) produced by tumor cells. Genetic deletion of TIPE2 or pharmacological inhibition of ROS markedly reduced tumor growth in mice. These findings indicate that TIPE2 plays a critical role in the functional polarization of MDSCs and represents a novel therapeutic target in cancer immunotherapy (Yan et al., 2019). Fifth, therapeutic liver-X nuclear receptor (LXR) agonism reduced MDSC abundance in several syngeneic mouse models and in patients in phase I clinical trials. LXR agonism depletion of MDSC was mediated by its transcriptional target ApoE, where LXR/ApoE activation therapy enhanced T cell activation and potentiated a robust antitumor immune response. Additionally, LXR agonism improved immune checkpoint inhibitor therapies in several preclinical mouse models, thus suggesting LXR agonism as a novel therapeutic approach to deplete MDSCs in cancer patients (Tavazoie et al., 2018). Finally, in contrast to MDSC depletion, a recent study shows that p53 activation induced MDSC differentiation to cross-presenting DCs. Pharmacological activation of p53 induced MDSC differentiation to Ly6C+ CD103 DCs, which are essential to potentiate a CD8+ T cell antitumor immune response. Mice with a targeted deletion of p53 in myeloid cells selectively lost the Ly6C+ CD103+ DC population and failed to respond to multiple forms of immunotherapy. In contrast, p53 agonism markedly enhanced efficacy and duration of response during immunotherapy. Taken together, these recent findings propose a novel therapeutic approach to induce MDSC differentiation to antigen-presenting cells rather than causing their depletion (Sharma et al., 2018).

PHARMACOLOGIC STRATEGIES TO TARGET MYELOID CELLS IN CANCER

Recent advances revealing the role of myeloid cells in cancer are drawing more interest in developing effective therapies that would improve prognosis of patients with different cancer types. Recent strategies for targeting the myeloid cell compartment in cancer consist of monotherapies, combination therapies, and/or targeted therapies such as nanoparticles. Recent ongoing and completed clinical trials specifically targeting TAMs and MDSCs in cancer are summarized in **Table 1**. Here we summarize novel preclinical approaches targeting myeloid cells in cancer (**Figure 2**).

Novel Single Agent-Based Potential Therapies

Chemotherapy has been in clinical use since the 1940's. Paclitaxel is a chemotherapeutic agent isolated from the bark extract of the Pacific Yew Tree in the 1960s. It stabilizes β-tubulin thus blocking mitosis, causing cell cycle G0-phase arrest, and is currently approved by the FDA for the treatment of several cancer types (Wani and Horwitz, 2014; Barbuti and Chen, 2015). Because of the extreme hydrophobicity of paclitaxel, nanoparticle albumin-bound paclitaxel (nab-paclitaxel) has been formulated and approved by the FDA as a first-line treatment of cancer types such as PDAC (Hennenfent and Govindan, 2006). Recent evidence revealed that paclitaxel not only induces cell-cycle arrest, but also promotes antitumor immunity by skewing TAMs toward the M1 phenotype. *In vitro* and *in vivo* tumor models showed that paclitaxel reprogrammed M2-TAMs to the M1-like phenotype in a Toll-like-receptor 4 (TLR4)-dependent manner (Wanderley et al., 2018). In a similar study, nab-paclitaxel was internalized by macrophages via macropinocytosis and induced the M1 phenotype in a TLR4-dependent manner in PDAC *in vitro* and *in vivo* models (Cullis et al., 2017). These data provide a rationale for combination of paclitaxel and immunotherapies as an anticancer treatment approach.

Additionally, a recent study found that the pattern recognition scavenger receptor (MARCO) on TAMs drives immunosuppression. Treatment of breast and colon carcinoma mouse models with an anti-MARCO monoclonal antibody reprogrammed TAMs to a pro-inflammatory phenotype and increased tumor immunogenicity, suggesting that targeting MARCO in TAMs represents a promising mode of cancer treatment (Georgoudaki et al., 2016). Also, all-trans retinoic acid (ATRA) is an active derivation of vitamin A which has an anticancer effect mostly in hematological malignancies (Wansley et al., 2013; Ying et al., 2016). In an osteosarcoma *in vitro* and *in vivo* models, ATRA inhibited osteosarcoma metastasis via inhibiting M2 polarization of TAMs independent of STAT3/6 or C/EBPβ signaling, thus proposing ATRA as an anti-metastatic potential treatment in osteosarcoma patients (Zhou et al., 2017). Another metabolite with potent signaling in myeloid cells is phosphatidylserine. Phosphatidylserine is a phospholipid that contributes to the establishment of an immunosuppressive

TABLE 1 | Current clinical trials targeting Myeloid Derived Suppressor Cells and Tumor Associated Macrophages.

Compound (target)	Clinical phase (status)	Tumor type	Combination partner(s)	ClinicalTrial.gov references
MDSCs				
Ibrutinib (BTK)	Phase I (ongoing)	Solid tumors	Nivolumab	NCT03525925
Tadalafil (PDE5)	NA (completed)	Head and Neck cancer	NA	NCT00843635
RGX-104 (LXR agonism)	Phase I (ongoing)	Solid tumors and lymphoma	Nivolumab/Ipilimumab/Docetaxel/Pembrolizumab, Carboplatin and Pemetrexed	NCT02922764
IPI-549 (Pi3kγ)	Phase II (ongoing)	Breast cancer and renal cell carcinoma	Atezolizumab/nab-paclitaxel/Bevacizumab	NCT03961698
VESANOID (ATRA)	Phase II (ongoing)	Melanoma	Ipilimumab	NCT02403778
Entinostat (HDAC)	Phase I (ongoing)	Breast cancer	Ipilimumab/Nivolumab	NCT02453620
Hydroxychloroquine (autophagy)	Phase I/II (ongoing)	Renal cell carcinoma	IL-2	NCT01550367
Omaveloxolone (NF-κB)	Phase I/II (completed)	Melanoma	*Ipilimumab/Nivolumab*	NCT02259231
beta-glucan (adjuvant)	NA (ongoing)	Non Small Lung cancer	NA	NCT00682032
Capecitabine (thymidylate synthase)	Phase I (ongoing)	Glioblastoma	Bevacizumab	NCT02669173
P53MVA (p53)	Phase II (ongoing)	Ovarian cancer	Pembrolizumab	NCT03113487
TAMs				
Pexidartinib (CSF-1R)	Phase I/I (ongoing) (completed)	Sarcoma Glioblastoma Breast cancer Acute myeloid leukemia	Sirolimus Radiotherapy and temozolomide Neoadjuvant chemotherapy NA	NCT02584647 NCT01790503 NCT01042379 NCT01349049
AMG 820 (Anti CSF-1R antibody)	Phase I (completed)	Solid tumors	NA	NCT01444404
LY3022855 (Anti CSF-1R antibody)	Phase I (completed)	Solid tumors Breast/prostate cancer Solid tumors	Durvalumab and Tremelimumab NA NA	NCT02718911 NCT02265536 NCT01346358
Ibrutinib (Bruton kinase)	Phase I (completed)	Pancreatic adenocarcinoma	FOLFIRINOX	NCT02436668
IPI-549 (Pi3kγ)	Phase II (ongoing) Phase I (ongoing)	Breast cancer and renal cell carcinoma Bladder/urothelial cancer Breast/ovarian cancer	Atezolizumab/nab-paclitaxel/Bevacizumab Nivolumab AB928/liposomal doxorubicin/nab-paclitaxel	NCT03961698 NCT03980041 NCT03719326
PF-04136309 (CCR2)	Phase I (completed)	Pancreatic adenocarcinoma	FOLFIRINOX	NCT01413022
Carlumab (Anti-CCL2 antibody)	Phase I (completed)	Solid tumors	Gemcitabine/paclitaxel/carboplatin	NCT01204996
CP-870,893 (CD40 agonist)	Phase I (completed)	Melanoma Solid tumors Pancreatic adenocarcinoma	NA Paclitaxel/carboplatin Gemcitabine	NCT02225002 NCT00607048 NCT01456585
Hu5F9-G4 (Anti-CD47 antibody)	Phase I (completed)	Myeloid leukemia	NA	NCT02678338
BMS-813160 (CCR2)	Phase I/II (ongoing)	Colorectal/pancreatic cancer	Nivolumab/nab-paclitaxel/gemcitabine/5-FU/leucovorin/irinotecan	NCT03184870
MCS110 (Anti-M-CSF antibody)	Phase II (ongoing)	Triple negative breast cancer	Carboplatin/gemcitabine	NCT02435680

Data were obtained using http://clinicaltrials.gov. BTK, Bruton Tyrosine Kinase; PDE5, phosphodiesterase type 5; LXR, Liver X Receptor; Pi3kγ, Phosphoinositide 3-Kinase gamma; ATRA, All Trans Retinoic Acid; HDAC, Histone deacetylase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; CSF-1R, Colony Stimulating Factor Receptor 1; CCR2, C-C chemokine receptor type 2; CCL2, C-C chemokine ligand type 2). FOLFIRINOX is a chemotherapy cocktail composed of: folinic acid, fluorouracil, irinotecan, oxaliplatin.

TME by preventing inflammatory reactions (McDonald et al., 1999). Treatment of prostate tumor-bearing mice with phosphatidylserine-targeting antibody 2aG4 in combination with docetaxel potently suppressed tumor growth, decreased M2 TAM and MDSC populations, and increased M1 macrophage and DC populations in the tumors. Furthermore, 2aG4 repolarized M2 TAMs toward the M1 phenotype, and induced the differentiation of MDSCs into M1 macrophages and DCs *in vitro*. These

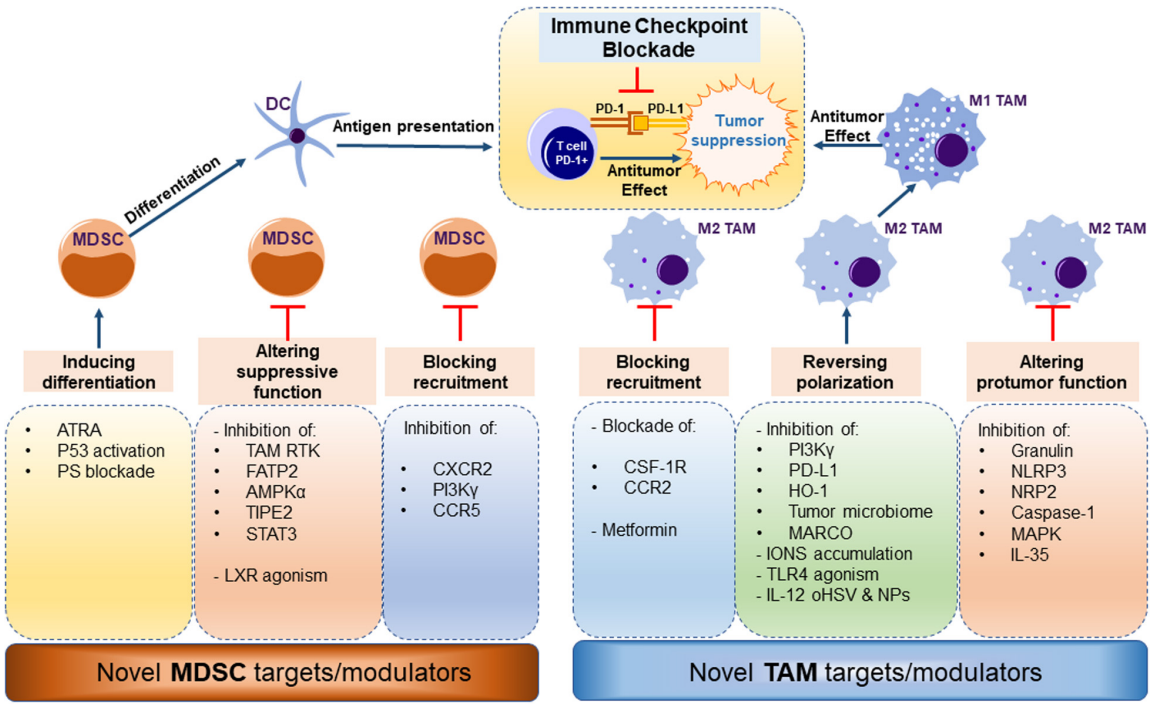

FIGURE 2 | Novel strategies to target myeloid cells in cancer. MDSCs can be differentiated to DCs with ATRA (All Trans Retinoic Acid), p53 activators, or phosphatidylserine blockade. Novel molecular targets altering MDCS suppressive function include inhibition of targets: TAM RTK (TAM Receptor Tyrosine Kinase), FATP2 (Fatty Acid Transport Protein 2), AMPK (5′ AMP-activated protein kinase), TIPE2 (TNF-α-induced protein 8-like 2), STAT3 (Signal Transducer and Activator of Transcription 3) and in contrast, agonism of LXR (Liver X Receptor). Recruitment of MDSCs to the tumor site can be achieved by inhibiting: CXCR2 (C-X-C Motif Chemokine Receptor 2), Pi3kgamma (Phosphoinositide 3-kinase gamma), CCR5 (C-C chemokine receptor type 5). TAMs can be targeted by blocking their recruitment to the tumor site by blocking CSF-1R (Colony Stimulating Factor 1 Receptor) or CCR2, or metformin treatment. Reversing TAM polarization from M2-like to M1-like phenotype can be achieved by inhibition of: Pi3Kgamma, PD-L1 (Protein Death Ligand 1), HO-1 (Heme Oxygenase 1), microbiome ablation, MARCO (macrophage receptor with collagenous structure) blockade, iron accumulation, TLR4 (Toll-like Receptor 4) activation, and IL-12 (Interleukin 12) nanoparticles. Finally, altering TAM function is achieved by inhibition of granulin, NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), NRP2 (Neuropilin 2), Caspase-1, MAPK (mitogen-activated protein kinase) and IL-35. Depletion of either MDSC and/or M2-like TAMs relieves the immune suppressive burden on T cells and the combination of ICB antibodies further prevents immune evasion by cancer cells leading to tumor suppression.

data suggest that targeting phosphatidylserine could reactivate antitumor immunity in the clinical setting (Yin et al., 2013). Metformin was originally established as the first-line agent for the treatment of type-2 diabetes. However, accumulating data suggest an anticancer effect of metformin in several cancer types (Schuler et al., 2015; Han et al., 2017; Tong et al., 2017; Guo et al., 2019). By using a transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model, a new study revealed that metformin delayed prostate cancer progression by inhibiting recruitment and infiltration of macrophages to the tumor site. Additionally, metformin inhibited inflammatory macrophage infiltration by downregulating both COX2 and PGE_2 in tumor cells, suggesting that metformin suppresses prostate cancer by altering tumor TAM infiltration (Liu et al., 2018). Additional pharmacological approaches use phosphodiesterase-5 (PDE-5) inhibitors such as sildenafil to deactivate MDSCs by interfering with Arg1 and iNOS expression (Serafini et al., 2006), N(G)-Nitro-L-Arginine Methyl Ester (L-NAME) which is another compound that inhibits Arg1 activity (Capuano et al., 2009), and N-hydroxy-L-Arginine (NOHA), a potent physiologic inhibitor of Arg1 (Stuehr et al., 1991). In this review, we focused on some recent strategies to inhibit MDSCs in cancer. Additional information regarding other MDSC inhibitors are detailed in another review (Wesolowski et al., 2013). In summary, we suggest several novel targets as a single therapy approach to primarily inhibit immunosuppression by targeting myeloid cell. Although monotherapies targeting myeloid cells in cancer have shown some promising results preclinically and clinically, they still face challenges such as resistance, partial reduction of tumor growth, and the existence of positive crosstalk between myeloid cells and other stromal components which can alter the efficiency of myeloid cell-induced antitumor immunity. It is likely that some of these challenges can be overcome using combination therapies and/or targeted therapies for a greater antitumor effect.

Potential Combination Therapies Targeting Myeloid Cells in Cancer

In healthy conditions, tumor cells are eliminated by immune surveillance, mainly through T cell infiltration and activation that respond to tumor neoantigens presented by major histocompatibility complex (MHC) (Vesely et al., 2011;

Matsushita et al., 2012). However, increased presentation of neoantigens likely leads cancer cells to escape immune surveillance through co-evolution in an immunosuppressive TME (Moon et al., 2014; Khalil et al., 2016). Thus, targeting more than one cellular component of TME in primary and metastatic tumors may provide a solution to immune surveillance evasion by cancer cells to induce a more favorable therapeutic outcome in patients with malignancies. Here, we summarize novel potential combination therapies involving myeloid cells in cancer.

As we have previously mentioned in this review article, targeting PI3Kγ in myeloid cells restored antitumor immunity by switching macrophage polarization toward the proinflammatory phenotype, and induced CD8+ T cell infiltration into the tumor site (Kaneda et al., 2016b). Combination of a PI3Kγ selective inhibitor (IPI-549) with ICB antibodies restored sensitivity of resistant cancers to ICB therapies in preclinical mouse models (De Henau et al., 2016). Likewise, inhibition of MDSCs with IPI-145, a selective inhibitor of PI3Kδ and PI3Kγ isoforms, in combination with anti-PD-L1 induced CD8+ T cell-dependent tumor growth reduction in a head and neck cancer mouse model (Davis et al., 2017). Combination of IL-12-expressing oncolytic herpes simplex virus (oHSV), which selectively replicates in cancer cells, with ICB antibodies (PD-1, CTLA-3, or PD-L1) slightly improved survival of a glioma mouse model. However, triple combination of the IL-12-expressing virus, anti-PD-1, and anti-CTLA-4 cured most mice with an increase of M1 macrophages and T effector to T regulatory ratio into tumors, suggesting that combination macrophage-targeting IL-12-expressing virus and ICB antibodies may have a synergistic curative effect in glioblastoma patients (Saha et al., 2017). As we have previously described in this review, targeting of CSF-1R with a monoclonal antibody (RG7155) potently inhibited TAM recruitment into the tumor site while increasing the anti-tumor CD8+/CD4+ T cell ratio (Ries et al., 2014). A positive effect of CSF-1R blockade plus ICB (anti-PD-1) combination has been reported (Peranzoni et al., 2018). However, a more recent study has identified the mechanism that limited the therapeutic effect of CSF-1R blockade. Carcinoma-associated fibroblasts (CAF) are major recruiter of granulocytes into the tumor site via chemokine secretion (Kumar et al., 2017). CSF-1R blockade induced a profound increase in CAF-mediated MDSC recruitment to the tumor site, thus explaining the mechanism behind CSF-1R therapy limitations. Triple combination of a CSF-1R inhibitor, a CXCR2 antagonist, and anti-PD-1 antibody lead to a significant inhibition of tumor growth in several cancer mouse models. These data propose a novel combination therapy to disrupt the crosstalk between different stromal cell populations for the most efficacious disease outcome in cancer patients (Kumar et al., 2017). In a short communication article, Lorio et al. reported that blocking anti-Bcl-2-Associated athanoGene 3 (BAG3) with an antibody resulted in an increased number of CD8+ T cell infiltration to the tumor site in a PDAC mouse model. Furthermore, combination of anti-BAG3 and anti-PD-1 antibodies further increased CD8+ T cell-mediated antitumor immunity suggesting a novel potential therapeutic approach for the treatment of PDAC (Iorio et al., 2018). Entinostat is an orally bioavailable class I-specific histone deacetylase inhibitor (HDACi) that interrupts immune suppression in the TME (Shen et al., 2012, 2016). Combination of entinostat with anti-PD-1 antibody enhanced the ICB antitumor effect in two syngeneic tumor mouse models by reducing tumor growth and neutralizing both M-MDSC and PMN-MDSC populations (Orillion et al., 2017). Dual targeting of CXCR2+ neutrophils and CCR2+ TAMs increased antitumor immunity by disrupting myeloid recruitment to tumors of a PDAC mouse model and improved response to FOLFIRINOX chemotherapy (including folinic acid, fluorouracil, irinotecan, oxaliplatin). However, targeting of either myeloid subtype (neutrophils or TAMs) resulted in a compensatory response of the other myeloid subset, resulting in disease relapse cite. These data suggest that combination therapies aiming at targeting more than one myeloid subtype in cancer might provide a solution to compensatory mechanisms between stromal cells and may further ameliorate the overall survival of cancer patients (Nywening et al., 2018). In PDAC mouse models, activation of macrophages using a CD40 agonist induced interferon-γ and CCL2 release, which in turn caused macrophages to deplete fibrosis through matrix metalloprotease activity (Beatty et al., 2015; Long et al., 2016). Moreover, combination of CD40 agonist and chemotherapy induced T cell-dependent reduction in tumor growth (Beatty et al., 2015; Long et al., 2016). Combinations involving blockade of leucine-rich repeat-containing G-protein-coupled receptor 4 (Lgr4) in lung cancer (Tan et al., 2018), inhibition of IL-6 in melanoma-bearing mice (Tsukamoto et al., 2018), dietary protein restriction (Orillion et al., 2018), inhibition of casein kinase 2 (Hashimoto et al., 2018), and blockade of receptor-interacting serine/threonine protein kinase 1 (RIP1) in PDAC mouse model (Wang W. et al., 2018) in myeloid cells; and ICB (anti-PD-1, anti-PD-L1, anti-CTLA-4) therapy, synergistically enhanced the antitumor immune response. Taken together, these findings emphasize the importance of targeting myeloid cells in combination with ICB therapies and other therapeutic approaches to enhance the antitumor immune response in cancer patients.

Targeting Myeloid Cells in Cancer Using Nanoparticles

There has been a growing interest in using nanotechnology for the treatment of cancer in the past few years, which is mainly due to its broad use ranging from drug delivery to diagnosis and imaging (Swartz et al., 2012; Kearney and Mooney, 2013). Nanoparticles are particles of any shape which size ranges between 1 and 100 nm, as defined by the International Union pf Pure and Applied Chemistry (IUPAC) (Shi et al., 2016). The immune system is characterized by its unique specificity in targeting antigens and cancer cells through the innate branch, while its adaptive branch enables long-term activity through memory-driven responses. Thus, manipulating these unique properties of the immune system are desirable, yet come with risks such as immune-related adverse events or "cytokine storm/cytokine release syndrome" (Shimabukuro-Vornhagen et al., 2018). Thus lowering doses and/or targeting specific cells of interest is paramount to ensure patient safety. Nanoparticles

are thus excellent candidates to modulate the immune system (Amoozgar and Goldberg, 2015). Here, we briefly summarize recent progress in targeted delivery to specifically myeloid cells in cancer using nanocarriers. Nanoparticle uptake by macrophages is influenced by size, rigidity, shape, surface charge, and composition of the nanoparticle (Tabata and Ikada, 1988; He et al., 2010; Sosale et al., 2015). Also, nanoparticles with either highly positive or highly negative zeta potential, which is defined as the potential difference between dispersion medium and the stationary layer of fluid attached to the particle, are more favorably internalized compared to nanoparticles with neutral or slightly negative charges (Tabata and Ikada, 1988). Several groups have taken advantage of the ability to coat nanoparticles in molecules such as the sugar mannose to target specific myeloid cells. Mannose receptor (CD206) is overexpressed in M2 macrophages and TAMs and represents a suitable target for mannose nanoparticles. Polyethylene glycol (PEG)-sheddable, mannose-modified nanoparticles were developed and efficiently targeted TAMs via mannose-CD206 binding after pH-sensitive PEG dissociated in the acidic TME, while their uptake by normal macrophages was reduced due to efficient PEG shielding at neutral pH (Zhu et al., 2013). Indeed, delivery of silencing molecules (siRNA) to target pro-tumor transcription factors has been undertaken with positive outcomes in an *in vitro* model of ovarian cancer (Ortega et al., 2016). Folic acid liposome nanoparticles were also developed to deliver zoledronic acid to TAMs. Folic acid engaged its receptor folate receptor β (FRβ) which is also overexpressed on TAMs (Hattori et al., 2015). Legumain and transferrin receptors are also overexpressed on TAMs, but all nanoparticle systems that have been developed to target these two receptors are mainly tailored toward targeting cancer cells, but no reports involving TAMs have been communicated yet (Wu et al., 2006; Lin et al., 2013; Ngambenjawong et al., 2017). In another study, IL-12-loaded tumor environment sensitive poly-β-amino ester nanoparticles reeducated TAMs to a macrophage M1 phenotype both *in vitro* and *in vivo* and selectively accumulated in the tumor site while extending IL-12 circulation time (Wang et al., 2017). In summary, we selectively summarized some approaches aiming at targeting myeloid cells in cancer using nanocarriers. Other reviews have extensively detailed other approaches using targeted delivery of myeloid cells in cancer (Amoozgar and Goldberg, 2015; Ngambenjawong et al., 2017; Silva and Al-Jamal, 2017; Singh et al., 2017).

Toll-Like Receptor Activation in Myeloid Cells

Toll-like receptors (TLR) are transmembrane proteins that induce the activation of inflammatory innate immune responses after binding to microbially derived molecules (Kawai and Akira, 2011; O'Neill et al., 2013). TLR7 and TLR8 agonist R848 potently drove the M1 macrophage phenotype *in vitro*. R848-loaded β-cyclodextrin nanoparticles (CDNP-R848) induced M1 macrophage phenotype, reduced tumor growth, and protected the animals against tumor re-challenge in multiple tumor mouse models. Furthermore, combination of CDNP-R848 and anti-PD-1 antibody improved ICB response (Rodell et al., 2018). In another study, STAT3 small interfering (si)RNA conjugated to cpG oligonucleotide agonist of TLR9 targeted tumor associated myeloid cells by silencing STAT3, thus leading to a potent antitumor immune response in multiple tumor mouse models (Kortylewski et al., 2009) and prostate cancer patients (Hossain et al., 2015). These data demonstrate that activating TLR in myeloid cells using agonists conjugated to therapeutic agents may represent a promising therapeutic approach for patients with different cancer types. Food and Drug Administration (FDA)-approved Toll-like receptor 7 (TLR7) agonist imiquimod is being tested in more than 100 clinical trials as a monotherapy, or in combination with chemotherapy or ICB (Locy et al., 2018). Although imiquimod induces local accumulation and activation of DCs, it may also promote MDSC expansion, which can limit vaccine efficiency (Dang et al., 2012). The mechanism responsible for MDSC activation by some adjuvant therapies is likely due to MDSC's susceptibility to be triggered by inflammatory signals (Gabrilovich et al., 2012; Fernandez et al., 2014; Kumar et al., 2016). Hence, some TLR agonists therapies have to be combined with agents targeting MDSCs to prevent MDSC-induced immune suppression.

CONCLUDING REMARKS

Before the recent advances in the field of immunotherapy, efforts aimed at targeting cancer were purely one-dimensional by focusing only on cancer cells as a single element in the equation using chemotherapy as early as the 1940's. With the discovery of TME dynamics and the emergence of immunotherapy, other stromal cell populations are increasingly considered. It is now well established that myeloid cells play a pivotal role in cancer. Their involvement in tumor progression and immune suppression is generating enthusiasm in the cancer research community, especially with the increasing number of novel molecular target discoveries. Although initiatives that aim at targeting myeloid cells in the TME have shown promising results, there still are challenges that need to be resolved. For instance, monotherapies targeting a single myeloid cell phenotype may show promising but limited efficacy. Combination therapies involving different immunotherapeutic approaches show improved anticancer effects in preclinical studies but have not yet lived up to their promises. Perhaps it is essential to discover novel mechanisms involving different stromal components such as the direct and compensatory crosstalk involving CAFs, TAMs, and MDSCs described above (Kumar et al., 2017). In this study, single targeting of TAMs using CSF-1R blockade did not result in a prolonged antitumor immune response. The authors showed that targeting TAMs using CSF-1R blockade triggered a compensation mechanism wherein CAFs recruited more PMN-MDSCs in a CXCR2-depedent manner. Triple combination using CSF-1R, CXCR2, and ICB resulted in an improved antitumor immune response. Thus, discovery of unknown pathways between immune stromal cells may improve cancer treatment by addressing the complexity of stromal interactions in the TME. Moreover, several strategies

aiming at achieving an effective combination with ICB are under active investigation (Popovic et al., 2018). The central dogma of these strategies consists of increasing effector T cells in immunologically "cold" tumors which are defined by having low neoantigen burden and a paucity of T cells and DCs (Popovic et al., 2018). One strategy to induce T cells and overcome a "cold" tumor's TME burden uses vaccines such as the FDA-approved Toll-like receptor 7 (TLR7) agonist imiquimod. As we previously mentioned, imiquimod activates MDSCs and their immunosuppressive capacity thus dampening imiquimod's antitumor efficacy. Hence, strategies that enhanced DC and T cell antitumor potential while altering MDSC's suppressive function are likely to be effectively combined with ICB for a maximum therapeutic benefit. Nevertheless, myeloid cells remain a major player that can determine disease outcome in cancer patients because of their exceptional phenotypic plasticity. It is therefore essential to efficiently modulate myeloid cells' plastic nature for the development of a whole new range of therapeutic strategies against cancer and turn the foes to friends.

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REVIEW
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Impact of TCM on Tumor-Infiltrating Myeloid Precursors in the Tumor Microenvironment

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The tumor microenvironment (TME) is composed of tumor cells, blood/lymphatic vessels, the tumor stroma, and tumor-infiltrating myeloid precursors (TIMPs) as a sophisticated pathological system to provide the survival environment for tumor cells and facilitate tumor metastasis. In TME, TIMPs, mainly including tumor-associated macrophage (TAM), tumor-associated dendritic cells (DCs), and myeloid-derived suppressor cells (MDSCs), play important roles in repressing the antitumor activity of T cell or other immune cells. Therefore, targeting those cells would be one novel efficient method to retard cancer progression. Numerous studies have shown that traditional Chinese medicine (TCM) has made extensive research in tumor immunotherapy. In the review, we demonstrate that Chinese herbal medicine (CHM) and its components induce tumor cell apoptosis, directly inhibiting tumor growth and invasion. Further, we discuss that TCM regulates TME to promote effective antitumor immune response, downregulates the numbers and function of TAMs/MDSCs, and enhances the antigen presentation ability of mature DCs. We also review the therapeutic effects of TCM herbs and their ingredients on TIMPs in TME and systemically analyze the regulatory mechanisms of TCM on those cells to have a deeper understanding of TCM in tumor immunotherapy. Those investigations on TCM may provide novel ideas for cancer treatment.

Keywords: traditional Chinese medicine, regulatory mechanism, tumor microenvironment, tumor-infiltrating myeloid precursors, cancer immunotherapy

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INTRODUCTION

Malignant tumors have progressively become an important disease of human death (Global Burden of Disease Cancer Collaboration et al., 2017; Bray et al., 2018). Even though the main treatments for malignant tumors are still radiotherapy, surgery, and chemotherapy, traditional Chinese medicine (TCM) has been applied to treat the patients with cancer in China for many years (Qi et al., 2015). TCM can protect cancer patients from complications, increase sensitivity or reduce side effects of conventional treatments, and improve quality of life and survival (Liang et al., 2014; Wang et al., 2020b). According to the theory of TCM, the key reasons for tumor occurrence and development are both the imbalance of Yin/Yang and the prosperous deficiency and evil (Xiang et al., 2019). This is consistent with the basic treatment ideas of modern tumor immunotherapy,

which breaks immune tolerance and reverses immune escape to rebuild the body's normal immune function and enhance antitumor ability. Many scientists believe that CHM reduces tumor growth and metastasis by enhancing antitumor immunity. Some reports have described the application of CHM in cancer treatment (Hsiao and Liu, 2010; Hu et al., 2013; Ting et al., 2015; Wang Z. X. et al., 2018). CHM and their active ingredients restrain the growth of cancer cells directly and prevent the invasion and metastasis of cancer cells by acting on the tumor microenvironment (TME), therefore playing an increasingly important role in antitumor treatment (Lin et al., 2017; Shi et al., 2020). The direct roles of TCM on tumor cells have been discussed in many articles (Qiu and Jai, 2014; Fan et al., 2020); in the review, we focus on the impact of TCM on TME, especially for immune cells within TME. The TME is a complicated pathological system, which is involved in the interaction of plenty numbers of cells such as tumor cells, lymphatic/blood endothelial cells, and tumor-infiltrating myeloid precursors (TIMPs) to provide the survival environment for tumor cells and facilitate tumor metastasis. A majority of those TIMPs are myeloid-derived hematopoietic cells, including tumor-associated macrophages (TAMs), dendritic cells (DCs) in tumor, and myeloid-derived suppressor cells (MDSCs). Under physiological conditions, multipotent hematopoietic stem cells are differentiated into immature myeloid cells (IMCs) that develop into mature myeloid cells with multiple functions (Qu et al., 2016). The three major groups of terminally differentiated myeloid cells—macrophages, DCs, and granulocytes—are essential for the physiological functions of the immune system. These cells protect organisms from pathogens, eliminate necrotic cells, and mediate tissue remodeling through the immune response. However, within the TME with the characteristics of hypoxia, acidity, and interstitial high pressure, those myeloid cells are converted into potent immunosuppressive populations that accelerated the growth, invasion, and metastasis of tumor. Expansion and function of TIMPs within the TME have been investigated in our laboratory and other institutes (Hosseini et al., 2020). However, little is known about the impact of TCM herbs and its active components on those TIMPs in TME. In the review, we summarized the inhibitory roles of TCM herbs and its active components on the growth, invasion, and metastasis of tumor, and focused on their regulatory function on TIMPs in TME, providing novel therapeutic methods for cancer treatment.

GENERAL VIEW FOR CHM AND THEIR ACTIVE INGREDIENTS

Traditional Chinese medicine, as one unique system of medical care, has been used in China and Asia for thousands of years. It is very different from Western medicine and uses a combination of various practices including acupuncture, massage therapy, moxibustion, and herbal remedies. According to the theory of TCM, the occurrence of illness is due to the disturbance of two opposing forces of energy, Yin and Yang. To alleviate symptoms of disease, TCM aims to restore the harmony of Yin and Yang. In recent decades, increasing numbers of patients have been attracted to use TCM as an adjuvant therapy option for various diseases (Jiang et al., 2010). In particular, TCM-based Chinese herbal medicine (CHM) has been shown to exhibit potential therapeutic effects as an adjunctive treatment following surgery, chemotherapy, radiotherapy, or other types of therapy for cancer patients worldwide (Nie et al., 2016). CHM and their compounds have the advantage of availability, efficacy, and relatively low toxicity, compared with other therapy methods. Evidence has confirmed that CHM in combination with chemotherapy or radiotherapy is capable of promoting the efficacy of chemotherapy or radiotherapy and diminishing the limitations and drawbacks induced by them (Qi et al., 2015). The objective of this review is to contribute to a clearer understanding of CHM and active compounds as an adjuvant therapy for cancer, and illustrate the underlying mechanisms of TCM-based CHM on cancer therapy from the point of view of TME.

DIFFERENTIATION, PHENOTYPES, AND FUNCTION OF TIMPs

Tumor-infiltrating myeloid precursors mainly include TAM, tumor-associated DCs, and MDSCs. A large number of studies have shown that TAMs are typical pre-tumor macrophages (M2), which are responsible for the release of immunosuppressive cytokines, chemokines, and growth factors, such as arginase, vascular endothelial growth, and other factors, rendering tumor-specific cytotoxic T lymphocytes hyporesponsive and promoting tumor angiogenesis (Pollard, 2004).

Dendritic cells arise from Lin-CD34+ hematopoietic stem cells and are classified into two different developmental stages: immature DCs (iDCs) in peripheral tissues primarily with the specialized functions of antigen uptake and processing and mature DCs (mDCs) in lymphoid organs with the interaction with antigen-specific T cells. mDCs, the most powerful antigen-presenting cells (APCs), are considered as critical regulators of adaptive immune responses. They can present tumor-associated antigen to T cells and initiate antitumor response. However, in TME, the complex interplay of stromal, immune, and tumor cells leads to DC dysfunction, even becoming immunosuppressive cells. DCs in the TME promote the differentiation of T cells into Treg subtypes, further weaken the antitumor activity mediated by T cells, support the formation of new blood vessels, block antitumor immunity, and stimulate the growth and spread of cancer cells (Ma et al., 2012).

Myeloid-derived suppressor cells originate from bone marrow and are composed of bone marrow progenitor cells and IMCs. In mice, according to their epitope-specific antibodies, they are divided into two subgroups: monocyte $CD11b^{+}LY6G^{-}LY6C^{hi}$ phenotype (M-MDSCs) and granulocyte $CD11b^{+}LY6G^{+}LY6C^{low}$ phenotype (G-MDSCs). Both MDSCs utilize different suppressive mechanisms (Qu et al., 2012). M-MDSCs produce very little reactive oxygen species (ROS) but produce a high level of nitric oxide (NO) and consist of IMCs with the ability to differentiate into macrophages and DCs. This subset of MDSCs mediates immune suppression through the production of NO and arginase. In contrast, G-MDSCs express a high level of ROS

and very little NO and are the majority population of MDSCs in tumor-bearing mice. Suppression by G-MDSCs is mediated via ROS and H_2O_2. In humans, MDSCs in cancer patients are defined by the combinations of functional markers, such as CD14, CD33, CD11b, and CD66b (Bronte, 2009; Qu et al., 2012; Youn et al., 2012). In different cancer patients, there are different types of MDSCs with suppressive roles (Qu et al., 2016). Therefore, those TIMPs suppress antitumor immune response through different mechanisms within the TME.

IMPACT OF CHM AND THEIR ACTIVE INGREDIENTS ON TUMOR GROWTH, INVASION, AND METASTASIS

Regulation of CHM and Their Active Ingredients on Tumor Growth

Cancer cells grow wildly and malignantly due to the unlimited proliferation of tumor cells and the mitigation of their apoptosis (Xu et al., 2016). The inhibition roles of CHM on cancer cell growth have been studied broadly for many years, and active components of CHM have been applied for clinical trials (Ling et al., 2014). For instance, kaempferol was identified to repress the mitochondrial biogenesis and antagonize the activity of ERRa and ERRg to impede tumor growth (Wang et al., 2013) (**Table 1**). In addition, Wang et al. (2011) found that *Spatholobus suberectus* Dunn (SSD) also retarded cancer growth, but its inhibitory mechanism was different from those of kaempferol. SSD inhibits tumor cell growth by inducing mitochondrial apoptosis and inhibiting the cell cycle in the G2/M phase. SSD also increases the inhibition rate of docetaxel and diminishes its side effects (**Table 1**). Recently, we reported that ginsenosides Rg3, Rg5, Rh2, and CK downregulated the expression of cell division cycle proteins cyclinB1, CDC2, Cytc-B, CDK-4, and CDK-6 to induce tumor cell cycle arrest in the G0/G1 phase (Chen et al., 2018).

Impact on Tumor Invasion and Metastasis

Tumor metastasis is regarded as a major obstacle to successful cancer therapy. The blockage treatments of metastasis provide more survival opportunities for cancer patients (Ma et al., 2020). Recent investigations about the regulation of tumor metastasis were involved in one family of enzymes, the matrix metalloproteinase (MMP) family, which exacerbated tumor metastasis in TME (Kessenbrock et al., 2010). Those data were consistent with our previous findings (Qu et al., 2009, 2011). Thus, the inhibition of CHM and their active ingredients on the activity of MMPs may attenuate tumor migration/metastasis. *Prunella vulgaris* L. (PVL) exhibited capacity to diminish the expression levels of MMP-2 and MMP-9, further reducing liver cancer metastasis (Kim et al., 2012) (**Table 2**). Chen et al. (2013) found that baicalein isolated from *Scutellaria baicalensis* Georgi (BCL) decreased the levels of MMP-2, MMP-9, and u-PA while elevating the expression of TIMP-1 and TIMP-2 to reduce the migration and metastasis of liver cancer cells through the decreased phosphorylation levels of MEK1 and ERK. In addition, the lung metastasis rate was found to be significantly decreased in the baicalein-treated nude mouse model LCID20 (Chen et al., 2013) (**Table 2**). As baicalein, formononetin was

TABLE 1 | Regulatory mechanism of CHM on the growth of tumor.

TCM herbs and their components	Cell lines/related mouse models	Mechanisms	References
KA	A549 lung cancer	To exert its anticancer effect by antagonizing ERRs activity	Wang et al. (2013)
SSD	MCF-7/HT-29/MCF-10A MCF-7/HT-29-induced colon cancer model	To inhibit cancer cell growth by inducing apoptosis and arresting cell cycle at G2/M checkpoint	Wang et al. (2011)

KA, kaempferol; SSD, Spatholobus suberectus Dunn.

TABLE 2 | Functional mechanism of CHM on the invasion and metastasis of tumor.

TCM herbs and their components	Cell lines/related mouse models	Mechanisms	References
PVL	HepG2/Huh-7/Hep3B	To suppress cell invasion and migration in liver cancer cells by attenuating MMPs	Kim et al. (2012)
BCL	HCC/MHCC97H MHCC97H-induced liver cancer model	To inhibit the invasion and metastatic capabilities of cancer cells via the downregulation of ERK pathway	Chen et al. (2013)
FMT	MDA-MB-231 cells MDA-MB-231-induced breast cancer model	To suppress MMP-2 and MMP-9 to inhibit migration and invasion of breast cancer cells through PI3K/AKT signaling pathways	Zhou et al. (2014)
CP	A2780	To decrease MMP-9 expression	Chen et al. (2014)
TanIIA	SW480	To reduce the level of vimentin and MMP-9, and enhance the expression levels of E-cadherin	Zhang et al. (2016)
UA	HCT116/HCT-8-induced colorectal cancer models	To suppress the invasive potential of cancer cells by regulating the TGF-beta1/ZEB1/miR-200c signaling pathway	Zhang Y. et al. (2019)
CBS	HeLa and HeLa-induced cervical cancer model	To downregulate MAPK/TGF-β/Nrf2 signaling pathways	Peng et al. (2020)
QYHJ	BxPC3/SW1990HM SW1990HM-induced pancreatic cancer model	To reduce the levels of vimentin, N-cadherin and Slug, increase the expression level of E-cadherin	Zhang et al. (2013)

BCL, baicalein; CBS, Conyza blinii saponin; CP, crude polysaccharides from Rosa roxburghii Tratt; FMT, formononetin; MMPs, matrix metalloproteinases; PVL, Prunella vulgaris L.; QYHJ, Qing-Yi-Hua-Ji formula; TanII A, tanshinone IIA; UA, ursolic acid.

also found to induce the decreased levels of both MMP-2 and MMP-9 to prevent the lung metastasis of MDA-MB-231 and 4T1 breast cancer cells. However, its role is regulated through PI3K/AKT signaling pathways (Zhou et al., 2014) (**Table 2**). For human ovarian cancer cell lines, both crude polysaccharides isolated from *Rosa roxburghii* Tratt and tanshinone IIA reduced the high MMP9 expression, which was related to tumor stage and lymph node metastasis (Chen et al., 2014; Zhang et al., 2016) (**Table 2**).

Either cancer cells or stroma cells activate transforming growth factor-beta (TGF-β) to produce MMPs or other factors in the extracellular matrix, further facilitating the tumor metastasis (Stuelten et al., 2005). TCM herbs such as ursolic acid (UA) treatment reduces the expression levels of TGF-β1 and the phosphorylation of Smad2/3 to block Zinc Finger E-Box Binding Homeobox 1 (ZEB1), further inducing the increased levels of miR-200c to reduce the invasive potential of colon cancer cells, suggesting that UA prevented colon cancer cell invasion through the TGF-β1/ZEB1/miR-200c signaling pathway (Zhang L. et al., 2019) (**Table 2**). *Conyza blinii* saponin (CBS) isolated from *Eschenbachia blinii* (H.Lév.) Brouillet inhibits the activation of TGF-β signaling pathway and the phosphorylation of ERK, JNK, and p38 MAPK. CBS also reduces the expression of Nrf2 in HeLa cells, inhibits the activation of ARE, and increases the level of ROS (Peng et al., 2020) (**Table 2**).

Epithelial–mesenchymal transition (EMT) is also shown to promote tumor metastasis. Some CHM inhibit EMT to prevent tumor metastasis. Qingyihuaji formula (QYHJ) impaired EMT in pancreatic cancer to restrain tumor metastasis via the decreased levels of vimentin, N-cadherin, and Slug (Zhang et al., 2013) (**Table 2**). Therefore, CHM and their active constituents inhibited the growth, invasion, and metastasis of different types of tumor through the blockage of tumor-related signaling pathways.

THE IMPACT OF CHM AND THEIR ACTIVE INGREDIENTS ON TIMPs IN TME

Tumor microenvironment is quite different from the physiological characteristics of normal tissues at the cellular and tissue levels. As a sophisticated pathological system, TME is involved in the crosstalk between tumor cells and TIMPs to provide the nourishment for tumor cells, improving the survival environment for tumor cells, and accelerates tumor metastasis (Sun, 2015; Kumar et al., 2016). There is increasing evidence that CHM mediates the TME through downregulating the suppressive function of TIMPs, including TAMs, DCs in tumor, and MDSCs (Guo et al., 2015).

The Regulatory Roles on TAMs

Macrophages, one type of versatile immunocytes, display different phenotypes, depending on their microenvironment. Activated macrophages are classified into the M1 and M2 phenotype. In general, M1 macrophages foster inflammation response against invading pathogens and tumor cells, whereas M2 macrophages tend to exert an immune suppressive phenotype, favoring tumor progression (He et al., 2020a). Even though TAMs exhibit either polarization phenotype, they are considered as M2-like phenotype-acquired macrophages and produce epidermal growth factor (EGF) and MMPs to accelerate the migration and angiogenesis of tumor in TME (Guo et al., 2018; He et al., 2020b). Therefore, therapeutic strategies are to re-educate the M2 phenotype (pro-tumorigenesis) into antitumor M1 phenotype (anti-tumorigenesis), preventing the promotion roles of TAM in tumors (Quail and Joyce, 2013).

Murine Cancer Cell Lines/Models

Some TCM herbs were found to convert TAMs (M2-like phenotype) to the M1-like phenotype and block the promotion functions of TAMs on tumor. Water extract of *Panax ginseng* C. A. Mey. and *Astragalus mongholicus* Bunge (WEPGAM) treatment can remarkably inhibit the transplanted tumor growth in mice (Chen et al., 2019) (**Figure 1** and **Table 3**). In addition, the reprogramming of TAMs toward M1-like macrophages is also regulated by TCM active components such as β-elemene (βe), which reduces the expression of Vimentin, N-cadherin, and Arg-1, and upregulates the expression of E-cadherin and iNOS to regulate the poles of macrophages from M2 to M1, inhibiting the proliferation, migration, and invasion of lung cancer cells (Yu et al., 2017) (**Figure 1** and **Table 3**). Our previous data demonstrated that some of Ginsenosides isolated from *Panax ginseng* C. A. Mey. were able to convert TAM polarization from M2-like to M1-like to attenuate tumor metastasis (Zhang Y. et al., 2019). Recently, *Panax ginseng* C. A. Mey.-derived nanoparticles (PGDN) were also found to have similar regulatory roles on TAMs in melanoma. Cao M. et al. (2019) found that PGDN significantly reduced the level of CD206 in M2-like macrophages and upgraded the expression of CD80, CD86, MHC-II, and TLR2/4 to induce the increased numbers of M1 macrophages, reducing tumor growth in vaccinated mice and human melanoma cells (**Figure 1** and **Table 4**).

Chinese herbal medicine and their active components exhibit blockage ability to the roles of TAMs in TME through JAK/STAT, JNK, and ERK signaling pathway, which is involved in mediating the growth, invasion, and metastasis of tumor (Lin et al., 2019). Total flavonoid from *Glycyrrhiza inflata* Batalin (GIB) and its important ingredient, isoliquiritigenin (ISL), reverse the polarization of M2 phenotype macrophages to retard tumor invasion through inhibiting the gene and protein expression of Arg-1. In addition, both GIB and ISL upregulate protein expression of iNOS, enhance the expression of microRNA 155 and its target gene SHIP1, and downregulate the phosphorylation of STAT3 and STAT6 (Wang et al., 2015) (**Figure 2** and **Table 3**). *Garcinia livingstonei* T. Anderson (GLT) elevates the expression level of iNOS and IL-12, and reduces the expression levels of IL-6, TNF-α, Arg-1, and IL-1β on TAMs to impede the tumor progression through the inhibition of STAT3, JNK, and ERK signaling pathway (Sui et al., 2020) (**Figure 2** and **Table 4**). Both Resveratrol (RSV) and Dendrosomal Curcumin (DNC) are revealed to downregulate the expression levels of IL-10 and Arg1 on TAM through the inactivation of STAT3 to reduce the numbers of TAM, further inhibiting tumor growth and metastasis (Shiri et al., 2015; Sun et al., 2017) (**Table 4**). The transcription factors STAT3 and STAT1 appear to play

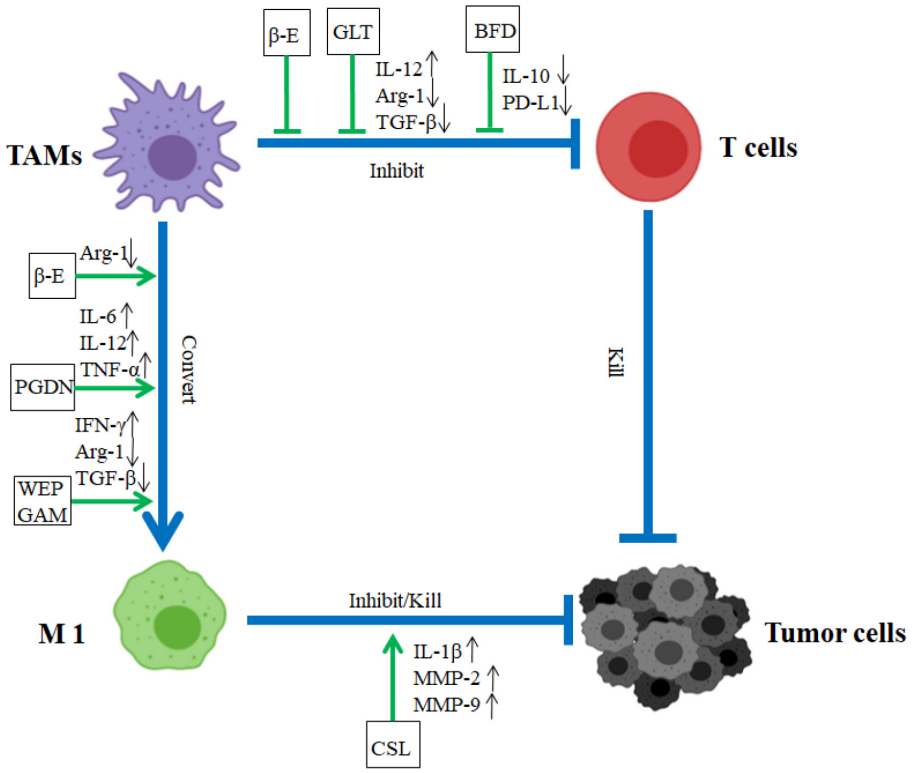

FIGURE 1 | Impact of CHM on TAMs in TME. In TME, TAMs accelerated tumor migration and invasion. CHM downregulated the roles of TAM and promoted the transformation of TAMs from M2 type to M1 type. In TME, β-E, GLT, and BFD blocked the inhibition function of TAM. β-E, PGDN, and WEPGAM triggered TAM conversion from M2 to M1 type, CSL retarded tumor growth. BFD, Bu Fei decoction; β-E, β-elemene; CSL, *Crocus sativus* L; WEPGAM, water extract of *Panax ginseng* C. A. Mey. and *Astragalus mongholicus* Bunge; PGDN, *Panax ginseng* C. A. Mey.-derived nanoparticles; GLT, *Garcinia livingstonei* T. Anderson; blue lines demonstrated the promotion (→) or inhibition (⊣) roles among immune cells (TAM or T cells) and tumor cells. Green lines indicated the promotion (→) or inhibition (⊣) roles of CHM.

opposite roles in tumorigenesis. STAT3 activation has been reported to show positive correlation with the proliferation and metastasis of tumor, and STAT1 enhances innate and adaptive immunity, triggering in most instances anti-proliferative and pro-apoptotic responses in tumor cells (Avalle et al., 2012). Qing-Re-Huo-Xue (QRHX) formulae increases the expression of iNOS and decreases the expression of IL-6, TNF-α, and Arg-1 through the JAK2/STAT3 pathway, further reducing the numbers of TAMs and inhibiting tumor growth in lung cancer mouse model (Xu et al., 2017) (**Figure 2** and **Table 4**). In the lung cancer mouse model, YPF also prolong the survival time of tumor mice through inhibiting the growth of lung cancer cells. In tumor tissues, the increased numbers of CD4+ T cells/macrophages are observed with the increased expression of IL-2 and IL-12 and decreased expression of TGF-β (Wang L. et al., 2019) (**Table 4**). In the 4T1 breast cancer mouse model, triptolide (TR), as one diterpenoid epoxide produced by *Tripterygium wilfordii* Hook. f (one TCM herb), was found to inhibit the expression of CD206, arginase 1, and CD204, and inhibit the secretion of anti-inflammatory cytokines, further inducing the decreased number of tumor-related M2 polarized macrophages to block tumor angiogenesis (Li et al., 2020) (**Table 4**).

Human Cells

For human lung cancer cell, Water extract of *Panax ginseng* C. A. Mey. and *Astragalus mongholicus* Bunge are revealed to reverse the polarity of TAMs from M2-like to M1-like by decreasing IL-10, TGF-β, Arg-1, and CD206 production on TAMs, consequently retarding the cancer invasion (Chen et al., 2019) (**Figure 1** and **Table 5**). What is more, the reprogramming of TAMs toward M1-like macrophages is regulated by TCM Herbs, such as *Crocus sativus* L, which can elevate the expression of IL-1β and TNF-α to induce the development of a polarized phenotype of M1-like macrophages after tumor antigen stimulation, restoring their antigen presentation ability in human melanoma. These data indicate that *Crocus sativus* L has a special immunomodulatory effect (Shen et al., 2019) (**Figure 1** and **Table 5**). Bu-Fei Decoction (BFD) inhibits the growth of both A549 and H1975 cell lines and reduces the expression of IL-10, PD-L1, and CD206 on TAM to restore their activity (Pang et al., 2017) (**Figure 1** and **Table 5**). Recently, baicalein (BC) was found to regulate M2 polarization and inhibit the secretion of TGF-β1 to inhibit the growth and metastases of human breast cancer (Zhao X. et al., 2018) (**Table 5**). Therefore, CHM and their active components oppose the promotion effect of TAMs on tumor to inhibit the growth, invasion, and metastasis of tumor in TME.

TABLE 3 | Effect of CHM on TIMPs *in vitro*.

TCM herbs and their components	Murine tumor cell line	TIMPs	Mechanisms	References
WEPGAM	LLC lung cancer	TAMs	To promote the transformation of M2 phenotype to M1 phenotype	Chen et al. (2019)
βE	LLC lung cancer	TAMs	To skew TAMs polarity toward the M1 phenotype	Yu et al. (2017)
GIBISL	4T1 breast cancer	TAMs	To reverse M2 phenotype macrophage polarization	Wang et al. (2015)
LBP	CT26 colon cancer	DCs	To prompt DC maturation	Wang Z. X. et al. (2018)
GLPS	P815 mastocytoma	DCs	To activate specific CTL through increasing IFNγ production to stimulate DC maturation	Cao and Lin (2002)

DC, dendritic cells; GIBISL, total flavonoid from Glycyrrhiza inflata Batalin and (its active ingredient) isoliquiritigenin; GLPS, Ganoderma lucidum polysaccharides; LBP, Lycium barbarum L. polysaccharide; MDSCs, myeloid-derived suppressor cells; TADC, decoction tumor-associated dendritic cells; TAM, tumor-associated macrophage; WEPGAM, water extract of Panax ginseng C. A. Mey. and Astragalus mongholicus Bunge; βE, β-elemene.

The Function of CHM and Their Ingredients on DCs

Dendritic cells are the principal APCs of the human body, which can efficiently ingest, process, and present antigens under physiological conditions. TME affects aggregation, maturation, and survival of DCs, and hampers the antigen presentation of DCs and sustains dysfunctional DCs to escape immune recognition, leading to the formation of tumor-associated DCs (TADCs), which exhibits a low ability to present antigen and facilitates T cells differentiating to Treg subtype, further impairing T cell-mediated antitumor activity (Giovanelli et al., 2019; Lee et al., 2020). Therefore, it is an effective way for antitumor immunotherapy to boost antigen presentation ability of DCs.

Murine Cancer Cell Lines/Models

Chinese herbal medicine and their components play positive roles in the DC maturation stimuli. *Lycium barbarum* L. polysaccharide (LBP) was also found to play critical roles in DC maturation. LBP induces the functional maturation of murine DCs *in vitro* through the increased expression of Notch and Jagged and Notch targets Hes1 and Hes5. Additionally, the administration of LBP strengthens the cytotoxicity of DC-mediated CTLs on murine colon cancer cell CT26-WTCTLs (Wang W. et al., 2018) (**Table 3**). LBP also induces Toll-like receptor 2- and 4-mediated functional maturation of murine DCs via the activation of NF-κB (Zhu et al., 2013). *Ganoderma lucidum* polysaccharides (GLPS), one of the major categories of the bioactive ingredients of *Ganoderma lucidum*, exhibit multiple biological activities such as improvement of host immune function, prevention of oxidative damage, and inhibition of tumor with little toxicity (Cor et al., 2018) (**Figure 3**). Recent data demonstrated that GLPS stimulated DC maturation through the increased production of IFN-γ, further enhancing antitumor response of specific CTL on mast tumor cells (Cao and Lin, 2003) (**Table 3**). GLPS also elevates the co-expression levels of both CD11c and IA/IE on DC surfaces and augment protein production of IL-12 P40 on DCs (Cao and Lin, 2002) (**Figure 3**).

Our recent report has shown that Ginsenosides, as the functional contents of ginseng, enhance the antigen presentation function of DCs within the TME. Ginsenosides activate the activity of DCs and promote adaptive immune responses to exert anticancer effects in tumor-bearing mice (Zhang Y. et al., 2019). Both the purified glycyrrhizin (GL) and *Carthamus tinctorius* L. (CT) extract stimulate DC maturation to bolster antitumor activity. The former increases the production of IL-12 and IL-10 and decreased the production of TNF-α. The latter stimulates splenic T lymphocytes to secrete IFN-γ, significantly increasing the levels of TNF-α and IL-1β in tumor-bearing mice (Chang et al., 2011; Hua et al., 2012) (**Figure 3** and **Table 4**). Wang Y. et al. (2019) found that *Pinellia pedatisecta* Schott (PPS) upregulated the expression of MHCII and CD80, CD86, and IL-12 on TADCs to promote the proliferation of CD4+ and CD8+ T cells in human cervical cancer, thereby eliciting further antitumor CTL responses (**Table 4**).

Dendritic cell vaccine is one newly emerging immunotherapeutic approach for the treatment and prevention of cancer, but major challenges remain particularly with respect to clinical efficacy. Polysaccharide components purified from *Astragalus mongholicus* Bunge or *Codonopsis pilosula* subsp. *pilosula* (PAMB PCPP) were displayed to induce the increased expression of mature DC markers, such as CD40, CD80, and CD86. It is an effective adjuvant to improve the metastasis efficiency of DC vaccine against 4T1 breast cancer in mice, indicating that those polysaccharides may contribute to the formulation of DC-based vaccine for cancer immunotherapy (Chang et al., 2015) (**Table 4**).

Human Cells

Most investigations are focused on the roles of CHM and its active components on murine DCs. Recently, their roles on the human DC maturation were also revealed. The effects of PPS on human TADCs were mediated through the inhibition of SOCS1 and activation of downstream JAK2-STAT1/STAT4/STAT5 pathways. Those data suggest that PPS is an effective immunomodulatory drug for antitumor treatment via the blockade of SOCS1 signaling in DCs (Wang et al., 2020a) (**Table 5**). Polysaccharide purified from *Ganoderma lucidum* (PS-G) increases the expression levels of IL-12p70, IL-12p35, CD80, CD83, CD86, and human leukocyte antigen (HLA)-DR on human monocyte-derived DC through NF-κB and p38 mitogen-activated protein kinase pathways, promoting the maturation of human monocyte-derived DCs (Lin et al., 2005). Extracting M4 from protopanaxatriol and M1 from protopanaxadiol (M4-M1) was shown to increase the expression levels of IL-12 on DCs to stimulate DC maturation in TME. In addition, M4-M1 increased the expression level of CD80, CD83, and CD86 on DCs to enhance the antitumor ability of T cell (Takei et al., 2004) (**Figure 3**). Echinacea (L.) Moench extract (EPME)

TABLE 4 | Regulation of CHM on TIMPs in TME of murine tumor models.

TCM herbs and their components	Murine cancer xenograft models	TIMPs	Mechanisms	References
PGDN	B16F10 cells induced melanoma	TAMs	To skew TAMs polarity toward the M1 phenotype	Cao Y. et al. (2019)
GLT	AOM/DSS-induced colorectal cancer model	TAMs	To reduce TAM production	Sui et al. (2020)
RSV	LLC lung cancer model	TAMs	To diminish tumor-associated M2 polarized macrophages	Sun et al. (2017)
DNC	4T1 breast cancer model.	TAMs	To diminish tumor-associated M2 polarized macrophages	Shiri et al. (2015)
QRHX	LLC lung cancer model	TAMs	To decrease TAM production	Xu et al. (2017)
YPF	LLC-Luc-induced lung cancer model	TAMs	To promote the transformation of M2 phenotype to M1 phenotype	Wang Y. et al. (2019)
TR	4T1 breast cancer model/AOM/DSS-induced colorectal cancer model	TAMs	To skew TAMs polarity toward the M1 phenotype	Li et al. (2020)
CT	JC-induced breast cancer model	DCs	To activate DCs to present antigens to T cell	Chang et al. (2011)
PPS	TC-1 cervical cancer model	TADCs	To reverse the immature state of TADCs	Wang Y. et al. (2019)
PAMB PCPP	4T1 breast cancer model	DCs	To improve DC immune activity	Chang et al. (2015)
SBS	AOM/DSS-induced colorectal cancer model	MDSCs	To block the immunosuppressive activity of MDSCs	Lin et al. (2015)
SGJP	4T1 breast cancer model	MDSCs	To block the immunosuppressive activity of MDSCs	Lu et al. (2017)
PA	4T1 breast cancer model	MDSCs	To decrease the number of MDSCs	Zheng et al. (2018)
RSV	LLC lung cancer model	MDSCs	To inhibit the function of MDSCs	Zhao Y. et al. (2018)
AP, AS, CS, SD	4T1 breast cancer model	MDSCs	To diminish the number of Tregs and MDSCs	Yue et al. (2018)
KRG	EL-4 thymoma model	MDSCs	To disrupt the function of MDSCs	Jeon et al. (2011)
JHD	H_{22} hepatoma carcinoma model	MDSCs	To inhibit immunosuppressive activity of MDSCs	Xie et al. (2020)
ART	4T1 breast cancer model	MDSCs	To impair the activity of Tregs and MDSCs	Cao Y. et al. (2019)
YHD	4T1 breast cancer model	MDSCs	To inhibit the activity of MDSCs	Mao et al. (2018)
ICA	4T1 breast cancer model	MDSCs	To impair the suppressive activity of MDSCs	Zhou et al. (2011)
CA	LM85 osteosarcoma model.	MDSCs	To block the function of MDSCs	Horlad et al. (2013)
BYJD	4T1 breast cancer model	MDSCs	To decrease the number of MDSC	Tian et al. (2020)

AP, Andrographis paniculata (Burm.f.) Nees; AS, Eleutherococcus senticosus (Rupr. & Maxim.) Maxim.; CS, Camellia sinensis (L.) Kuntze; SD, Scleromitrion diffusum (Willd.) R.J. Wang; ART, artemisinin; BYJD, Bao-Yuan-Jie-Du decoction; CA, corosolic acid; CT, Carthamus tinctorius L.; DC, dendritic cells; DNC, dendrosomal curcumin; GLT, Garcinia livingstonei T. Anderson; ICA, icariin from Epimedium sagittatum (Siebold & Zucc.) Maxim.; JHD, Jianpi Huayu decoction; KRG, Korean red ginseng; MDSCs, myeloid-derived suppressor cells; PA, water extract of pilose antler; PAMB or PCPP, polysaccharide purified from Astragalus mongholicus Bunge or Codonopsis pilosula subsp. pilosula; PGDN, Panax ginseng C. A. Mey.-derived nanoparticles; PPS, Pinellia pedatisecta Schott; QRHX, Qing-Re-Huo-Xue formulae; RSV, resveratrol; SBS, Shen-ling-Bai-zhu San; SGJP, Shu-Gan-Jian-Pi formula; TADC, decoction tumor-associated dendritic cells; TAM, tumor-associated macrophage; TR, triptolide; YHD, Yang-He decoction; YPF, Yu-Ping-Feng.

downregulates the expression of CCL3, CCL8, CCR1, and CCR9 and upregulates the expression of CCL4 and CCL2 to trigger the maturation of human DCs (Wang et al., 2006) (**Figure 3**). Those results indicated that CHM promoted the maturation of both murine and human DCs, enhancing their present ability to tumor antigen efficiently in TME.

The Effect of CHM and Their Elements on MDSCs From Murine Tumor Models

Myeloid-derived suppressor cells, as the important TIMPs, aggregate in TME and exhibit strong immunosuppressive activity to T cell antitumor response. In TME, plenty of IMCs were differentiated into large amounts of MDSCs, whereas the differentiation of MDSC into mature macrophage or DCs was prevented (Gabrilovich and Nagaraj, 2009). In the section, we discuss the functional roles of CHM on differentiation, expansion, and suppressive function of MDSCs within TME from murine tumor models, since the investigations about the regulation of CHM on tumor MDSCs are focused on murine tumor models mainly.

Myeloid-derived suppressor cells are not present in the circulation under normal physiological conditions, but these cells accumulate in the tumor-bearing mice. MDSC accumulation was downregulated by TCM herbs, such as Shen-Ling-Bai-Zhu San (SBS) formula, Shu-Gan-Jian-Pi formula (SGJP), Water extract of Pilose Antler (PA), and RSV. In the colitis-associated colorectal cancer (CaCRC) mouse model, SBS upregulates β-catenin, p53, and proliferating cell nuclear antigen (PCNA), and reduces the mortality and the number of MDSCs. It also alleviates TGF-β1-induced EMT through downregulating N-cadherin (N-cad), Vimentin, Fibronectin, and Snail, and upregulating E-cadherin (E-cad) (Lin et al., 2015) (**Figure 4** and **Table 4**). In breast cancer mouse models, both SGJP and PA inhibit the numbers of MDSCs to increase the proportion of CD4+ T cells, CD8+ T cells, and NK cells in peripheral blood of mice, further improving the survival rates of mice and blocking tumor growth (Lu et al., 2017; Zheng et al., 2018) (**Table 4**). In Lewis lung cancer-bearing mice, RSV was shown to diminish the accumulation of G-MDSCs and promote M-MDSC differentiation into macrophages and the expansion of CD8+IFN-γ+ cells (Zhao Y. et al., 2018) (**Table 4**). Recently, Yue et al. (2018) reported that four types of TCM herbs,

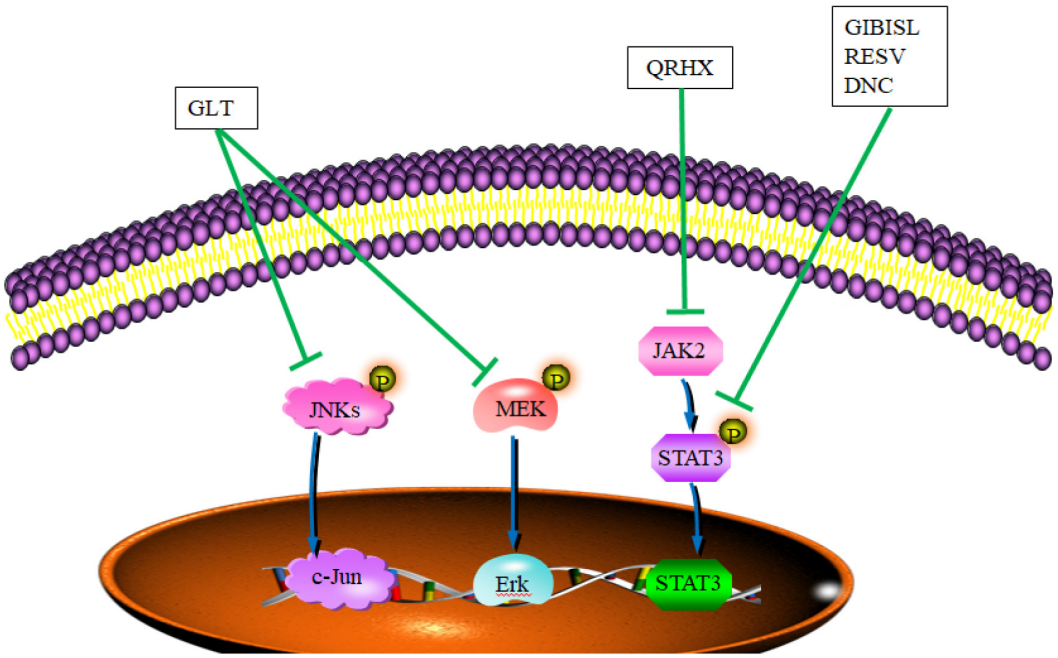

FIGURE 2 | CHM and their active ingredients skewed TAM polarity toward the M1 phenotype through signaling pathways. In TME, GLT blocked M2 macrophage polarization in colitis-associated tumorigenesis through downregulating JNK and ERK signaling. QRHX impaired the function of TAM and impeded tumor growth in tumor-bearing mice through reduced phosphorylation levels of JAK2/STAT3. RESV, DNC, and GIBISL skewed the polarization of TAM toward M1 through the inactivation of STAT3. DNC, dendritic curcumin; GLT, *Garcinia livingstonei* T. Anderson; QRHX, Qing-Re-Huo-Xue formulae; RESV, resveratrol; GIBISL, total flavonoid from *Glycyrrhiza inflata* Batalin and (its active ingredient) isoliquiritigenin. Blue lines demonstrated the promotion (→) or inhibition (⊣) roles of signal path. Green lines indicated the promotion (→) or inhibition (⊣) roles of CHM.

Andrographis paniculata (Burm.f.) Nees (AP), *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (AS), *Camellia sinensis* Kuntze (CS), and *Scleromitrion diffusum* (Willd.) R.J. Wang (SD)

TABLE 5 | The roles of CHM on human TIMPs.

TCM herbs and their components	Human cells	TIMPs	Mechanisms	References
WEPGAM	A549 lung cancer cells	TAMs	To promote the transformation of M2 phenotype to M1 phenotype	Chen et al. (2019)
CSL	A375 melanoma cells	TAMs	To diminish TAM to impede tumor growth	Shen et al. (2019)
BFD	A549/NCI-H1975 Lung cancer cells	TAMs	To decrease TAM production	Pang et al. (2017)
BC	MDA-MB-231 breast cancer cells	TAMs	To regulate the polarization and function of TAMs	Zhao Y. et al. (2018)
PPS	Human cervical cancer cells	TADCs	To activate DCs to present antigens to T cell	Wang et al. (2020a)

BC, baicalein; BFD, Bu-Fei decoction; CSL, Crocus sativus L; DC, dendritic cells; MDSCs, myeloid-derived suppressor cells; PPS, Pinellia pedatisecta Schott; TADC, decoction tumor-associated dendritic cells; TAM, tumor-associated macrophage; WEPGAM, water extract of Panax ginseng C. A. Mey. and Astragalus mongholicus Bunge.

reduced tumor tissue weights and tumor metastasis of both lung and liver, and decreased the numbers of both Tregs and MDSCs to coordinate the antitumor response of T cells to cancer cells, prolonging the survival period of mice in the metastatic breast cancer mouse model (**Table 4**). In the EL-4 thymoma mouse model, Korean red ginseng (KRG) was displayed to prevent the abnormal differentiation of IMCs into MDSCs and impair MDSC function, inducing T cell proliferation and secretion of both IL-2 and IFN-γ (Jeon et al., 2011) (**Figure 4** and **Table 4**). In H22 hepatocellular carcinoma-bearing mice, Jianpi Huayu decoction (JHD) significantly diminishes the destruction of spleen structure and the ratios of between Treg and Th17, and increases the ratios of CTL, DC, and MDSCs in the spleen. JHD also promotes the differentiation of IMCs into macrophages and mDCs, and weakens the expression of ROS in MDSCs to impair the inhibitory effect of those MDSCs on CD4+ T cell proliferation (Xie et al., 2020) (**Figure 4** and **Table 4**). In 4T1 breast cancer mouse model, which is a suitable experimental animal model for human mammary cancer, artemisinin (ART) significantly promotes 4T1 tumor cell apoptosis and decreases TGF-β levels and the numbers of both MDSC and Treg to inhibit tumor growth in mice (Cao Y. et al., 2019) (**Figure 4** and **Table 4**).

The suppressive function of MDSC on T cell antitumor response has been studied broadly in our laboratory and other institutes (Qu et al., 2012; Fultang et al., 2019; Su et al., 2019). There are growing evidence that the immune-regulatory roles of CHM on the function of MDSCs become one of the

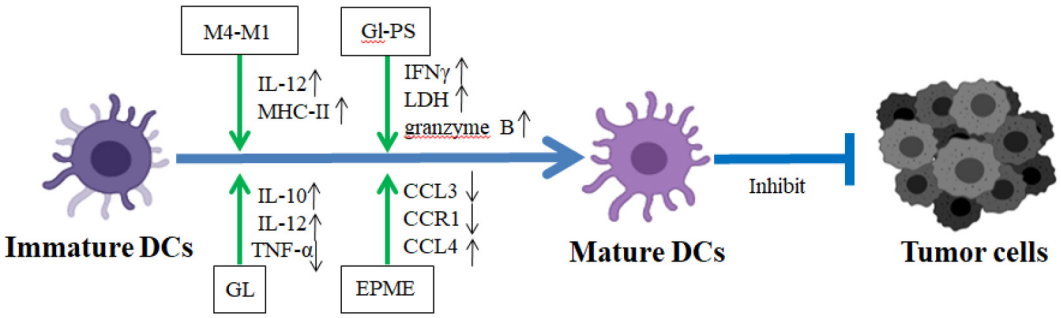

FIGURE 3 | The regulatory mechanism of CHM on the maturation and function of DCs in TME. In TME, the tumor-associated DC (TADC) promoted the differentiation of T cells into Treg subtypes and further impairs the anti-tumor activity mediated by T cells. GL, Gl-PS, M4-M1, and EPME stimulated TADC maturation to bolster the antitumor activity. GL, glycyrrhizin; M4-M1, extracting M4 from protopanaxatriol and M1 from protopanaxadiol; Gl-PS, *Ganoderma lucidum* polysaccharides; EPME, *Echinacea purpurea* (L.) Moench extracts. Blue lines demonstrated the promotion (→) or inhibition (⊣) roles among immature dendritic cells, mature dendritic cells, and tumor cells. Green lines indicated the promotion (→) or inhibition (⊣) roles of CHM.

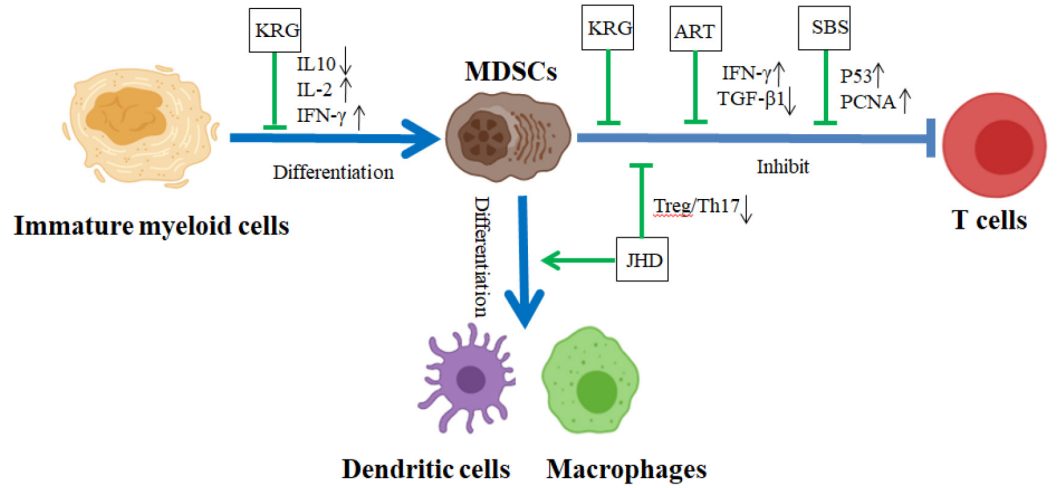

FIGURE 4 | The effect of CHM on the differentiation and function of MDSCs in TME. In TME, MDSCs accumulated in TME and exhibited strong immunosuppressive activity against T cell antitumor response. KRG prevented the differentiation of IMCs into MDSCs with suppressive function, and JHD triggered the differentiation of MDSCs into mature DCs and macrophages. ART, KRG, SBS, and JHD impaired the suppressive function of MDSCs, further restraining the invasion and metastasis of tumor. ART, artemisinin; JHD, Jian-Pi-Hua-Yu decoction; KRG, Korean red ginseng; SBS, Shen-Ling-Bai-zhu San. Blue lines demonstrated the promotion (→) or inhibition (⊣) roles among immature myeloid cells, MDSCs, and T cells. Green lines indicated the promotion (→) or inhibition (⊣) roles of CHM.

major cancer immunotherapies of CHM progressively. CHM reverses the function of MDSCs through tumor-related signaling pathways such as the JAK/STATs and TGF-β/Smads pathway. Mao et al. (2018) found that, in breast cancer mouse models, Yang-He decoction (YHD) depressed the expression of iNOS, ARG-1, IL-6, TGF-β, and p-STAT3 on MDSCs and significantly increased the expression of IFN-γ and NKTs on $CD4^+$T cells to shrink tumor growth (**Table 4**). Icariin (ICA) from *Epimedium sagittatum* (Siebold & Zucc.) Maxim downregulates the expression levels of IL-10, IL-6, S100A8/9, iNOS, and ROS on MDSCs to attenuate the roles of MDSCs through the inactivation of STAT3 (Zhou et al., 2011) (**Table 4**). In the murine sarcoma model, corosolic acid (CA) was revealed to induce the decreased expression levels of both cyclooxygenase-2 (Cox2) and CCL2 through the inactivation of Stat3 to impair the immunosuppressive activity of MDSCs (Horlad et al., 2013) (**Table 4**). Recently, Bao-Yuan-Jie-Du decoction (BYJD) is found to suppress the protein expression of TGF-β, Smad2, Smad3, p-Smad2/3, and Smad4 through the TGF-β/Smads signaling pathway to inhibit the recruitment of MDSCs in the lung and prolong the survival time of 4T1 tumor-bearing mice (Tian et al., 2020) (**Table 4**). In summary, CHM and their compounds stimulate the differentiation of MDSCs into mature myeloid cells, diminish the number and expansion of MDSCs, and restrain the suppressive function of MDSCs to block the tumor metastasis in TME.

CONCLUSION

Chinese herbal medicine contains rich and diverse chemical components, including alkaloids, polysaccharides, glycosides,

and flavonoids. These chemicals have a variety of biological functions. CHM plays an important role in inhibiting the tumor and mediating tumor TME. In the review, we focus on the impact of CHM on TIMPs within TME. CHM and their compounds induce the differentiation of TIMPs into mature or functional cells, promote the transformation of TAM from M2 type to M1 type, stimulate DC maturation, trigger the differentiation of MDSC into mature DC and macrophages, and weaken the inhibitory function of MDSCs, further inhibiting tumor invasion and metastasis in TME. Those evidences suggest that CHM and their active components may be regarded as one novel therapeutic method for cancer treatment.

FUTURE PROSPECT

Those CHM and their compounds may enhance the activity of other clinical antitumor antibodies such as anti-PD-L1 antibody on patients with cancer through inhibiting both the numbers and roles of TIMPs within TME. In addition, the therapeutic effects of multiple components from CHM on TIMPs may be examined and compared in different types of tumor to find the best candidates on tumor treatment. Those investigations may facilitate the clinical application of CHM on cancer immunotherapy.

AUTHOR CONTRIBUTIONS

PQ conceived and designed the work. ZL and ZQ coordinated technical support and funding. JL and PQ wrote the manuscript. YW, GL, XH, and HL acquired, analyzed, and interpreted the data. All authors read and approved the final manuscript.

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Integrated Analysis of Immune Infiltration Features for Cervical Carcinoma and Their Associated Immunotherapeutic Responses

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Cervical cancer is the fourth most prevalent cancer in women, which decreases quality of life of the patients. Traditional interventions have failed to improve the overall survival period of patients due to high tumor recurrence after treatment or late diagnosis. Fortunately, preliminary evidence suggests that anti-angiogenic and immunotherapy can efficiently treat against cervical cancer. However, there is no clear evidence on the efficacy of immunotherapy in cervical cancer. Therefore, in this study, we classified cervical cancers in the TCGA dataset using various algorithms and explored the relationship between the immune profile and corresponding sensitivity of the tumors to immunotherapy. Results showed that patients with tumors had higher expression of immunocytes and longer overall survival time. In addition, we build a scoring system based on the immune landscape of the tumor microenvironment of cervical cancer. Tumors with higher scores exhibited better survival outcomes and were more sensitive to immunotherapy. In this study, the immune landscape of cervical cancer was analyzed, and the subtype of cervical cancer based on that difference was proposed. Besides, the subtype of cervical cancer showed different sensitivity to immunotherapeutic response which further confirmed its relationship with tumor immune landscape.

Keywords: cervical cancer, immunocytes, tumor microenvironment, immunotherapy, PD-1

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INTRODUCTION

Cervical cancer is the fourth most prevalent cancer among women. In the developing countries, it is the leading cause of cancer-associated mortalities (Canfell, 2019). The median age for patients diagnosed with cervical cancer is 49 years. In general, cervical cancer lowers the quality of life of the affected persons (Canfell, 2019). It has been established that prolonged infection with human papillomavirus (HPV) type 16 and 18 is a risk factor for cervical cancer (Crosbie et al., 2013). Prophylactic vaccines against high-risk HPV types minimize the risk of developing cervical cancer. However, due to the limitations associated with HPV vaccines, reliable therapeutic options for cervical cancer, particularly recurrent or advanced tumors, are required (Shanmugasundaram and You, 2017). Current therapeutic options include surgical removal of the tumors based on the FIGO staging system, incorporated with chemo- or radiotherapies (Bhatla et al., 2019; Koh et al., 2019). As for recurrent cervical cancer, bevacizumab in combination with

other therapies can significantly prolong patients' survival time (Tewari et al., 2017). In addition, immunotherapy is a viable option for cervical cancer treatment.

Immunotherapy is effective against various solid tumors. Mechanistically, immunotherapy enhances immune responses by utilizing immune checkpoint inhibitors and adoptive cellular transfer (O'Donnell et al., 2019). However, immunotherapeutic depends on the tumor microenvironment (Gasser et al., 2017). Immunocyte infiltration degree, tumor mutational load, and T cell functions affect tumor sensitivity to immunotherapy. Programmed death ligand 1 (PD-L1) has been reported in over 90% of cervical cancer and tumors. Higher infiltration ratios of CD8+ T cells and CD4+ T regulatory cells confer better survival outcomes for tumor patients (Ramanathan et al., 2018; Otter et al., 2019). The immune system is also involved in HPV-induced tumorigenesis (Orbegoso et al., 2018). HPV has been known to trigger chronic inflammation, escape immune surveillance by hiding in keratinocytes, suppress cellar immunity, and wall itself with recruited immunocytes (Piersma, 2011). Based on these features, immunotherapy presents the best strategy for managing cervical cancer (Piersma, 2011; Smola, 2017; Kagabu et al., 2019).

Establishment of reliable biomarkers for the best choice of immunotherapy as well as an improved understanding of immune infiltration features with regard to cervical cancer are key to immunotherapy. Therefore, this study aimed at exploring cervical cancer-induced immune infiltration characteristics based on different clustering algorithms, with the aim of providing a strong foundation for research and rationale for immunotherapy. The ratio of immunocytes, overall survival outcomes, mutation burden, and immunotherapeutic responses between different groups were compared.

MATERIALS AND METHODS

CESC Data and Preprocessing

A total of 291 samples from publicly available cell carcinoma (CESC) gene-expression datasets in The Cancer Genome Atlas (TCGA) were utilized in our analyses (**Supplementary Table 10**). The TCGA dataset was downloaded from UCSC Xena[1], and subsequent analysis was performed using the R software (version 3.6.1) and R Bioconductor packages.

Estimation of TME Infiltrating Cells

The respective proportions of the immune infiltrating cells in the cervical squamous CESC samples were quantified using the ssgsea algorithm (Hanzelmann et al., 2013). The gene sets comprised of 782 genes that could predict the abundance of 28 TIICs in individual tissue samples[2]. CESC was selected because it allows for the determination of sensitivity and specificity of immune cell phenotypes. It can be used to discriminate up to 28 human infiltration immune cell phenotypes.

[1]https://xenabrowser.net/

[2]http://software.broadinstitute.org/gsea/msigdb/index.jsp

Unsupervised Consensus Clustering of TME-Infiltrating Cells

In order to generate more groups for further analyses, Partitioning Around Medoid (PAM) (Tahiri et al., 2018) was used to classify tumors with qualitatively diverse TME-infiltrating patterns. The optimal number of clusters in the TCGA cohort was determined using the ConsensuClusterPlus R package (Monti et al., 2003). The consensus ESTIMATE algorithm was performed to assess the infiltration of stromal and immune cells in CESC samples (Yoshihara et al., 2013).

Identification of TME-Associated Differentially Expressed Genes

The patients were grouped into two distinct TME clusters based on the expression of immune infiltrating cells. The R package limma was used to determine differentially expressed genes (DEGs) between the two TME cell-infiltrating clusters (Ritchie et al., 2015). Adjusted p-value < 0.01 and $|\ \text{logFC}\ | > 1$ were considered to be statistically significant for DEGs between the TME subtypes.

TME Gene Signatures Generations and Dimension Reduction

The DEGs between TME clusters were standardized for all the samples in the TCGA CESC cohort. Prognostic associated DEGs were filtered out by performing the Univariate Cox regression analysis (p-value < 0.05). The unsupervised clustering method (Hartigan and Wong, 1979) was used to classify patients into either of the TME gene clusters. Annotation of the TME gene pattern was performed using the clusterProfiler R package (Ghasemi and Zahediasl, 2012). The clustering algorithm (Monti et al., 2003) was used to define the gene clusters. Principal component one that served as the signature score was obtained using the principal component analysis (PCA). The TME score for each patient was determined based on the prognostic value of the gene signature (Sotiriou et al., 2006):

$$\text{TME score} = \sum \text{PC1i} - \sum \text{SPC1j}$$

where "i" is the signature score of clusters with HR > 1, while "j" represents the expression of genes with HR < 1.

Pathway Enrichment Analysis

The gene sets for pathway enrichment analysis were downloaded from the MSigDB database (Subramanian et al., 2005). Gene set variation analysis (GSVA) was performed on the TME score and the TME clusters using the clusterProfiler R package (Ghasemi and Zahediasl, 2012). Genes for the enriched Pathways in TME were identified using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), with an adjusted $p < 0.05$.

Immunotherapeutic Response Prediction

The Tumor Immune Dysfunction and Exclusion (TIDE) algorithm was used to link individual responses to immunotherapeutic responses (Jiang et al., 2018; Lu et al., 2019).

Differences in anti-PD-1 and CTAL-4 therapeutic response were evaluated using the Submap analysis. For the melanoma data set (GSE78220, $N = 28$), the expression profiles (FPKM normalized) of GSE78220 were transformed into TPM values which were then used to calculate the TME score (Wagner et al., 2012). With regard to the urothelial cancer data set (IMvigor, $N = 298$), the data package was downloaded from http://research-pub.gene.com/IMvigor210CoreBiologies.
Quality control and trimming of the mean of M-values were performed using the R package arrayQualityMetrics to normalize the numerical data (Ritchie et al., 2015).

Statistical Analysis

The Shapiro–Wilk normality test was used to establish variable normality (Ghasemi and Zahediasl, 2012). For normally distributed variables, the unpaired Student *t*-test was used to compare differences between the two groups, whereas the Wilcoxon test was used to compare abnormally distributed variables. One-way analysis of variance (ANOVA) and Kruskal–Wallis tests were used for comparison of multiple groups.

Pearson and distance correlation analyses were performed to calculate correlation coefficients. The χ^2 contingency test was performed to determine the interrelationships between TME score and anti-PD-1 response. The overall survival and TME score were determined using the R package. The threshold for survival values was determined. Based on the dichotomized TME score, patients were grouped into either high or low TME clusters, while at the same time reducing the computational batch effect by the R package sva. The data were visualized using the ggplot2 for R package. In the analysis of differential gene expression, we used the Benjamini–Hochberg method that converts *p*-values to FDRs to identify significantly expressed genes (Schreiber et al., 2011). OncoPrint was used to delineate the mutation landscape in the TCGA dataset using the maftools R package (Wang et al., 2020). Survival curves for the subgroups were generated using the Kaplan–Meier method. Statistical significance between different data sets was determined using the log-rank test. The univariate and multivariate Cox proportional hazard regression models were performed using the R package to determine independent factors associated with prognosis. Survivorship curves were generated using the R package survminer. Heatmaps were generated based on pheatmap. All statistical analyses were performed using R[3]. The tests were two-sided, with p-values < 0.05 being considered to be statistically significant.

RESULTS

The Landscape and Functional Annotation of CESC TME

The flowchart for this study is shown in **Supplementary Figure 1A**. Analysis on cluster stability performed on CESC in the TCGA dataset using ConsensusClusterPlus package to select the optimal cluster number is shown in **Supplementary Figure 1B**. PAM of the 291 tumors with corresponding TME cell expression profiles in the TCGA cohort on its part is shown in **Figure 1A**. Two TME phenotypes were established based on immune cell infiltration. They conferred significantly different OS for outcomes (log-rank test, $p < 0.001$) as shown in **Figure 1B**. PCA showed a clear separation between the two established groups in the TCGA dataset (**Figure 1C**). **Figure 1D** shows the distinct TME infiltration patterns for the two clusters. Based on the ESTIMATE algorithm, TME cluster 1 was strongly associated with the estimated, immune, and stromal scores compared with TME cluster 2 (**Figure 1E**). Furthermore, 20 immune-related signaling pathways and DNA regulation-related pathways in GO analysis (**Supplementary Table 1**) were identified in the TCGA data set (**Supplementary Figure 2A**). Intrinsic immune escape was attributed to the expression of seven immune checkpoint molecules including antigen presenters, co-stimulators, co-inhibitors, receptors, ligands, and cell adhesion proteins, among others (Schreiber et al., 2011; Wang et al., 2020). There was an elevated expression of immune checkpoint molecules around TME cluster 1 of the CESC that aid the respective tumor cell escape from immune killing in TCGA (**Supplementary Figure 2B**). Moreover, the correlation of TME clusters, hypoxia, and metabolism was explored. TME cluster 2 was found to correlate with more metabolism-related signaling pathways (**Figure 2A**). TME cluster 2 also positively correlated with hypoxia-related gene signatures, indicating a malignancy of TME cluster 2 (**Figure 2B**).

Generation of TME Gene Signatures and Functional Annotation

A total of 383 DEGs (**Supplementary Table 2**) were identified and used to classify the patients into genomic types, to further investigate the potential biological characteristics of each TME infiltration cell pattern. The clustering stability established by ConsensusClusterPlus package for the optimal number of clusters (**Supplementary Figure 1C**) was in tandem with the two CESC gene clusters (gene clusters 1 and 2) generated in TCGA (**Figure 3A**). Survival analysis of the two clusters revealed that the expression of gene cluster 1 was associated with better survival outcome (**Figure 3B**). Compared to cluster 2 genes, cluster 1 gene expressions were also associated with immune, stromal, and estimate scores (**Figure 3C**). Furthermore, cluster 1 genes were correlated with higher expression levels of infiltrating immune cells (**Figure 3D**). Compared to cluster 2 genes, CESC in gene cluster 1 were significantly associated with the immunosuppressive process that was mediated by a higher expression of immune checkpoint molecules (**Figure 4A**).

Univariate Cox regression analysis for TME scores and the corresponding transcriptome traits as well as clinical characteristics for the top98 DEGs are shown in **Supplementary Table 3**, respectively. GO enrichment analysis revealed that 98 genes were associated with T cell activation and proliferation, regulation of macrophage activation, positive regulation of tumor necrosis factors, and neutrophil-mediated immunity. Combined, these pathways regulate the immune system (**Figure 4B** and **Supplementary Table 4**). KEGG enrichment

[3]https://www.r-project.org/, version 3.6.1

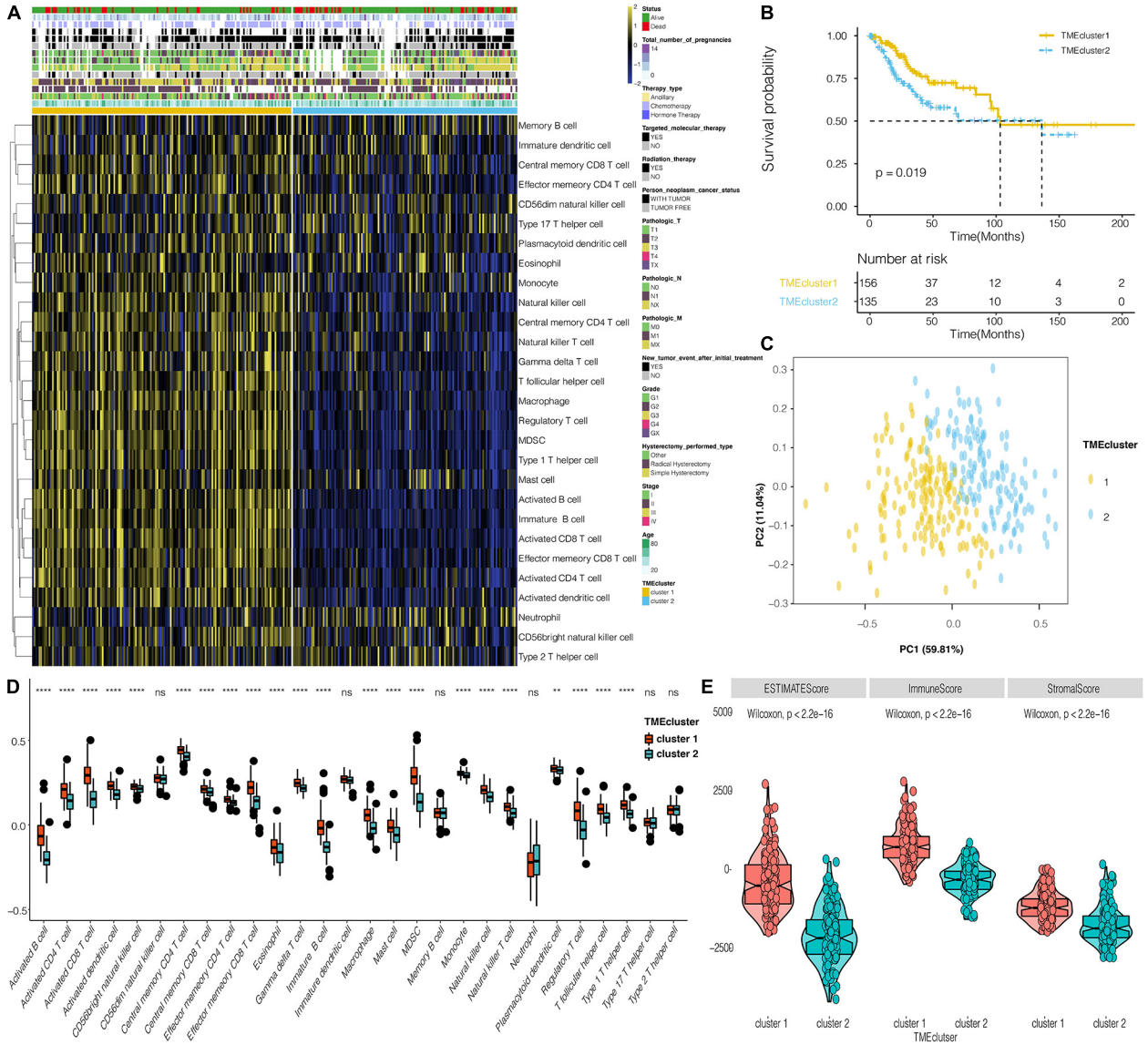

FIGURE 1 | The landscape of CESC TME, and the characteristics of TME subtypes in TCGA. **(A)** Unsupervised clustering of TME cells for 291 patients in TCGA. **(B)** Kaplan–Meier curves for 291 patients in TCGA, showing the association between TME infiltration patterns and OS (log-rank test, $P < 0.001$). **(C)** PCA for the two TME clusters. **(D)** The distribution of immunocytes in the two TME clusters. Within each group, the scattered dots represent values for cellular expression at TME whereas the thick line represents the median value. The bottom and top (lines) in the boxes are the 25th and 75th percentiles (interquartile range). The whiskers encompass 1.5 times the interquartile range. The statistical difference of the two TME clusters was compared using the Kruskal–Wallis test. **, $P < 0.01$; ****, $P < 0.0001$; ns, not statistically significant. **(E)** The differential expression of Estimate, Immune, and Stromal Score for TME clusters in the TCGA dataset.

analysis was consistent with GO analysis, with 98 genes found to be associated with immune system regulation (**Figure 4C** and **Supplementary Table 5**). TME scores for patients with CESC in the TCGA dataset are shown in **Supplementary Table 6**. GO analysis revealed that high TME scores were significantly associated with immune-related pathways, including T cell selection, T cell activation, regulation of macrophage activation, negative regulation of lymphocyte-mediated immunity, mast cell activation, regulation of myeloid dendritic cell activation, regulatory T cell differentiation, and immune response activation (**Figure 5A** and **Supplementary Table 7**). Furthermore, CESC with high TME scores exhibited higher immune checkpoint expression levels (**Figure 5B**). High TME scores were also associated with macrophage, mast cells, MDSC, and regulatory T cell infiltration, all of which induce an immunosuppressive environment. Moreover, a high TME score was also a predictor for a more activated immune environment. This is because high TME scores were correlated with a higher infiltration of multiple T cells and natural killer cells (**Figure 6A**). The two-sided role of high TME score could be attributed to the complexity of multiple immune cell-infiltrated tumor microenvironment. High TME scores were

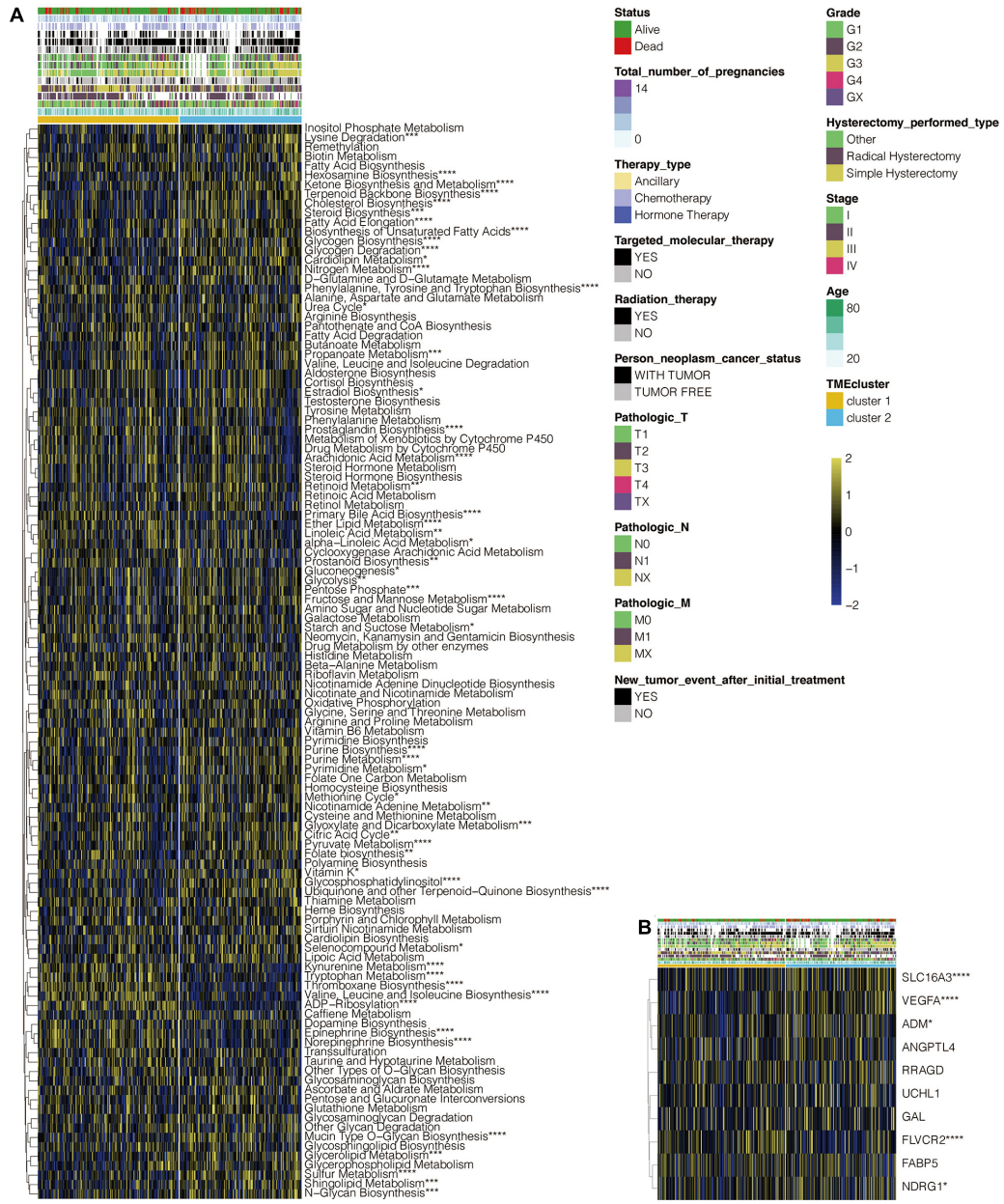

FIGURE 2 | Functional annotation of TME clusters. **(A)** Heatmap depicting the correlation of TME clusters and metabolic pathways. **(B)** Heatmap depicting the correlation of TME clusters and hypoxia-related gene signatures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not statistically significant.

also positively correlated with Estimate, Immune, and Stromal Score (**Figure 6B**).

TME Score Is Associated With Unique Patterns of Genomic Alterations

CNA and somatic mutation analysis performed on the TCGA dataset to determine the association between TME score and CESC genomic profiles revealed that samples with high TME scores frequently amplified several genomic regions particularly drivers of oncogenesis and immune regulatory genes including NRAS (1p13.27), DUP3Q29 (3q29), LYZ (5p11), HLA-DQA1 (6p21.32), CHEK2P2 (15q11.1), STAT3 (17q21.2), and KLK3 (19q13.33). These gene sets were associated with COL11A1 (1p21.1), MCL1 (1q21.2), UGT2B7 (4q13.2), ANGPT2 (8p23.1), PTEN (10q23.31) TNFRSF13B (17p11.2), TNNI3 (19q13.42), and GSTT1 (22q11.23) gene deletions as shown in **Figure 6C**. Different genomic profiles were observed in low TME score as shown in **Supplementary Figure 3A**. Furthermore, somatic mutation profiling revealed a high frequency of mutations in TTN (51%), MUC4 (35%), PIK3CA (32%), MUC17 (25%), MUC16 (24%), and SYNE1 (23%) among genes with high TME

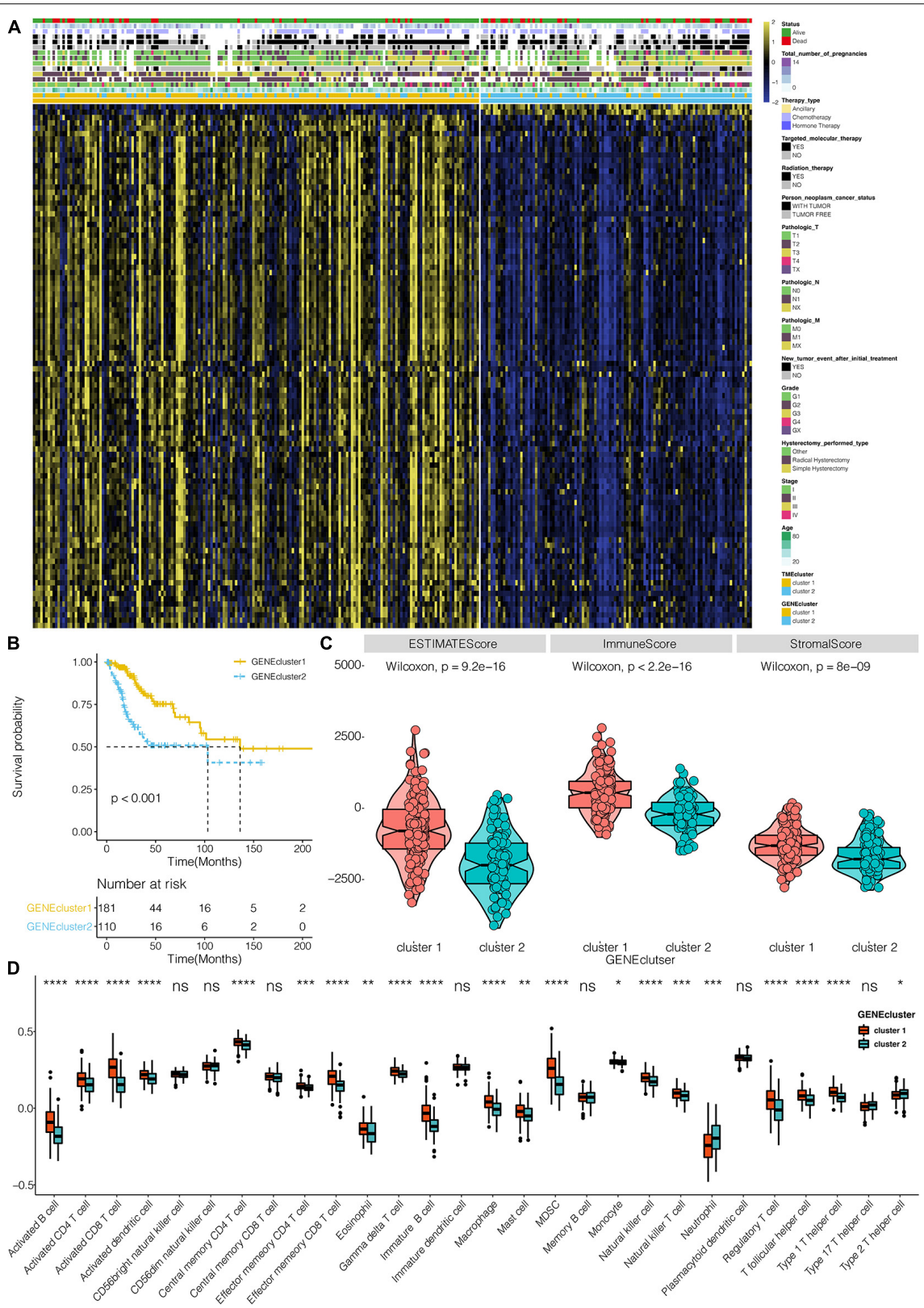

FIGURE 3 | TME signatures and functional annotation constructs. **(A)** Unsupervised analysis and hierarchical clustering of common DEGs based on expression data of CESC derived from the TCGA: Gene clusters 1 and 2. **(B)** Kaplan–Meier curves for the two TME gene clusters (log-rank test showed an overall $P < 0.001$). **(C)** The differential expression of Estimate, Immune, and Stromal Score in TME clusters in the TCGA dataset. **(D)** The distribution of cells in TME gene clusters. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not statistically significant.

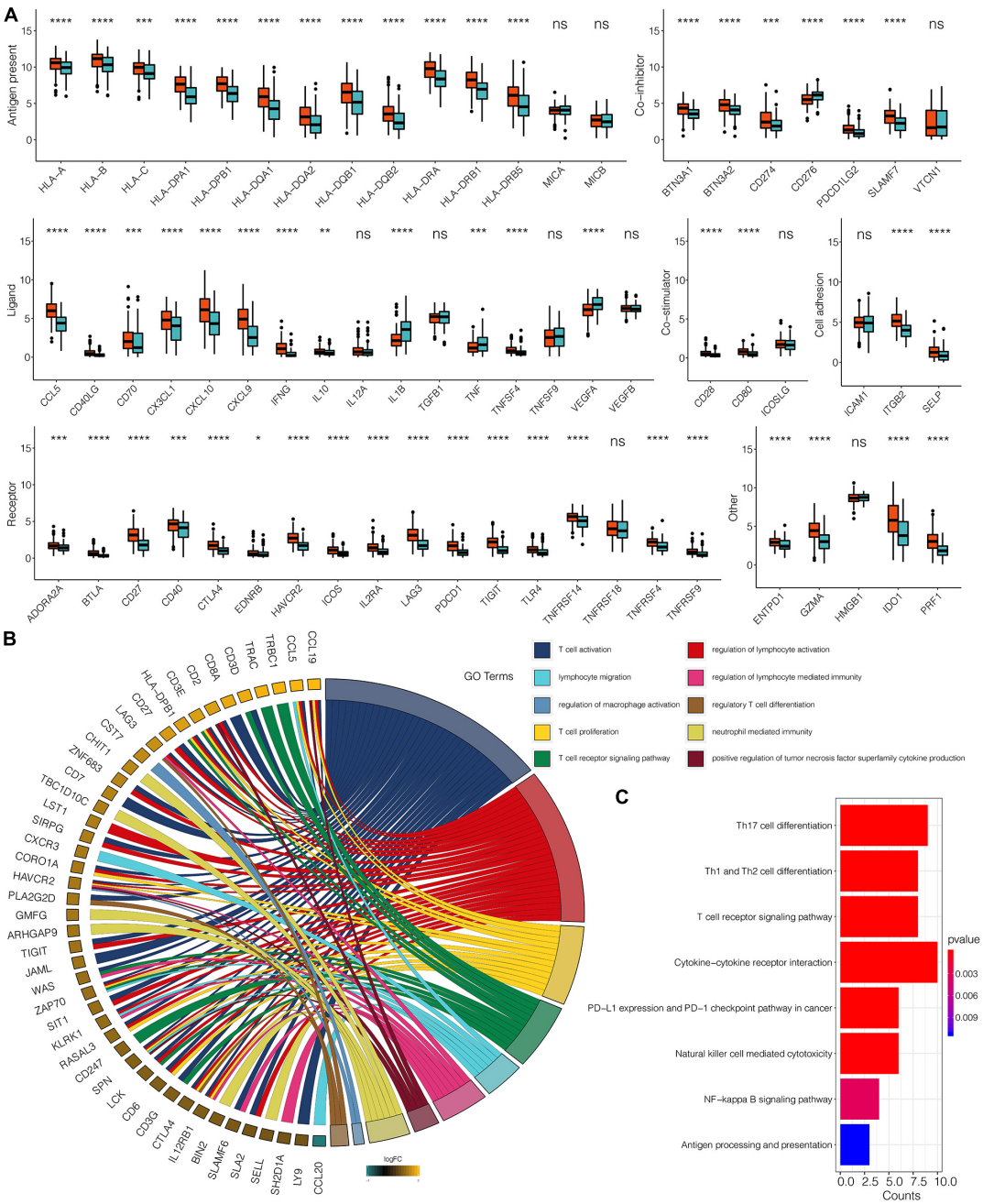

FIGURE 4 | (A) The expression pattern of seven types of immune checkpoints in TME gene clusters for the TCGA dataset. **(B)** The GO enrichment analysis of the 98 DEG for TME signatures. **(C)** KEGG enrichment analysis of the 98 DEG for TME signatures. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not statistically significant.

score (**Figure 6D**), whereas TTN (32%), MUC4 (29%), PIK3CA (29%), and MUC16 (27%) were the frequently mutated genes in the low TME score cluster (**Supplementary Figure 3B**).

TME Score Predicts Therapeutic Responses

In the analysis of the association between prognosis and TME score for the CESC cohort, it was found that high TME scores and targeted therapy were respective markers and intervention for positive prognosis of CESC in the TCGA dataset. Univariate and multivariate analyses revealed that patient ages, tumor stage, and radiation therapy were correlated with poor CESE prognosis (**Figure 7A**). A high TME score was associated with better survival outcomes for patients with CESC, BRCA, and OV in the TCGA dataset (**Figure 7B**). TME scores for BRCA and OV patients in the TCGA dataset included in this study are shown in

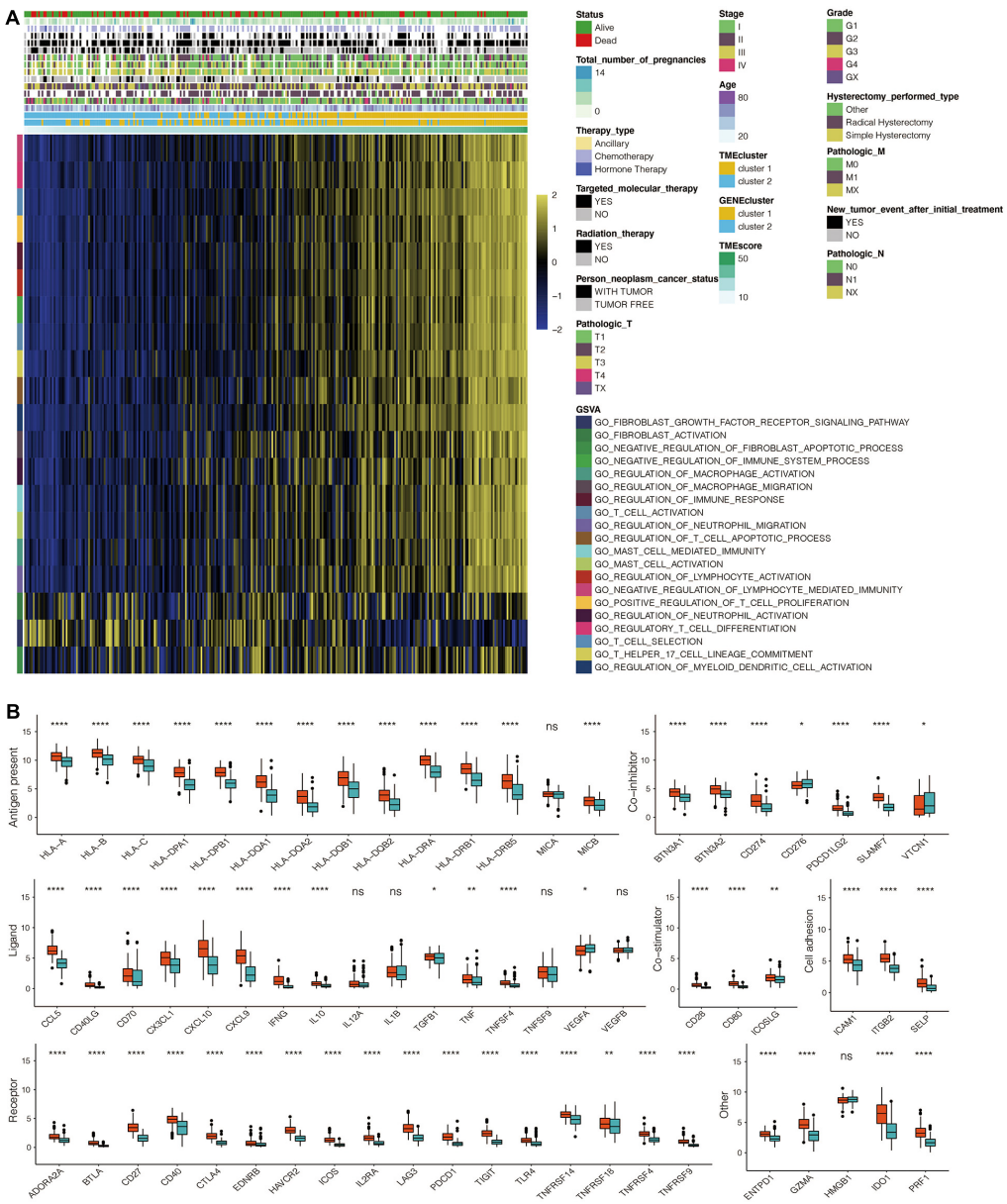

FIGURE 5 | Immune-related functional annotation for the TME score. **(A)** GSVA for the TME score based on GO for TCGA. **(B)** The expression pattern of seven types of immune checkpoints in the TME score-based clusters for TCGA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not statistically significant.

Supplementary Tables 8, 9, respectively. Analysis of the probable response to immunotherapeutic response for CESE in the TCGA based on the TIDE algorithm revealed that low TME score tumors responded better to immunotherapy compared to high TME score tumors (**Figure 7C**). Subsequently, anti-PD-1 and anti-CTLA-4 therapeutic responses were analyzed. Tumors in the two TME score clusters were found to respond differently to immunotherapies. Tumors in the high TME score clusters exhibited a better response to anti- than those with low TME scores (**Figure 7D**). In the melanoma dataset, GSE78220 patients with high TME scores exhibited significantly longer OS outcomes than patients with lower TME scores (**Figure 7E**). High TME scores were also correlated with complete and partial anti-PD-1 responses (**Figure 7F**). In the urothelial cancer dataset, patients with high TME scores exhibited significantly longer OS outcomes than those with low TME scores in the IMvigor210 cohort (**Figure 7G**). Additionally, high TME scores were also associated with strong anti-PD-1 responses (**Figure 7H**).

DISCUSSION

The component of the cervical cancer microenvironment can affect cervical cancer progression, but potential mechanisms are still elusive. In this study, we analyzed the influence of immunocyte infiltration shed on tumor response to

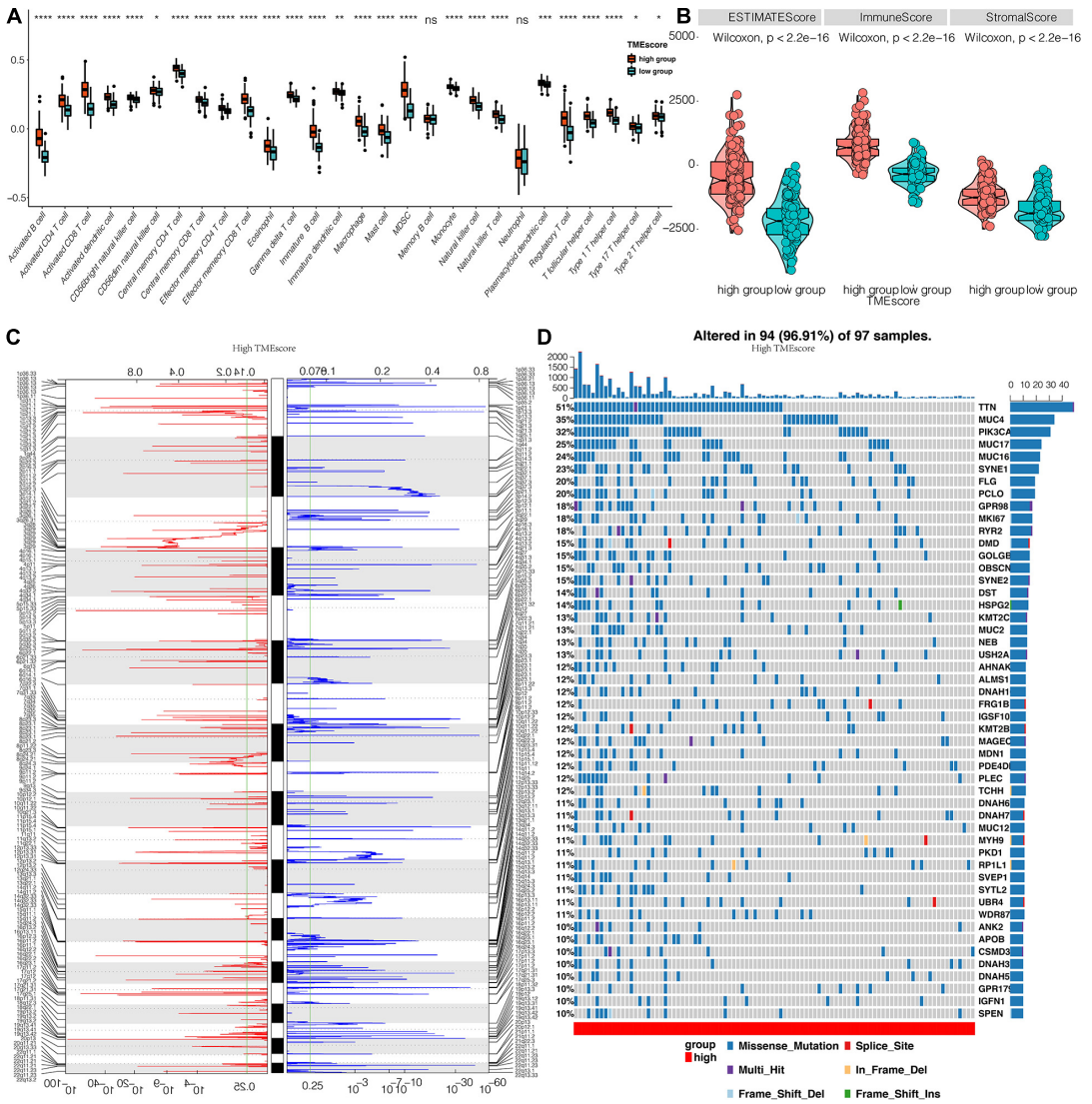

FIGURE 6 | Genomic profiles associated with the TME score. **(A)** The distribution of immune cells in TME score clusters for TCGA. **(B)** The differential expression of Estimate, Immune, and Stromal Score for TME score in the TCGA dataset. **(C)** GISTIC 2.0 amplifications and deletions in CESC with high TME score. Chromosomal locations of peaks of significantly recurring focal amplification (red) and deletions (blue). **(D)** Differential somatic mutations in CESC with a high TME score.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not statistically significant.

immunotherapy by applying different algorithms and proposed the subtype of cervical cancer based on immunocyte infiltration. CESC in the TCGA dataset were grouped into two clusters based on the differential expression of immunocytes, and lower immunocyte infiltration ratio samples showed worse survival outcome. In the meantime, several metabolic related pathways and hypoxia-associated genes were also differentially activated or expressed between the low-risk group (TME cluster 1) and the high-risk group (TME cluster 2). Therefore, the immune subtype of cervical cancer, TME cluster 1, and TME cluster 2 can affect the formation of the tumor microenvironment and cervical cancer progression.

To step further, 383 DEGs in total were identified and used to deeply explore this immune subtype of cervical cancer. High TME score group samples are usually accompanied with higher infiltration ratios of immunocytes like NK cells, T cells, and macrophages and higher immunotherapy-related gene expression (like HLA-A, HLA-B, HLA-C, CCL5, CXCL10, CD40, CTLA4, and PDCD1). In addition, immune activation-related pathways are also differentially activated in the low and high TME score groups. The previous study reported that chemokines like CCL5 and CXCL10 can modulate tumor sensitive to immunotherapy (Vilgelm and Richmond, 2019). Biomarkers like CTLA4, CD40, and the HLA family have been confirmed to be involved in immune surveillance (Waldman et al., 2020). Therefore, tumors with a low TME score group may be more sensitive to immunotherapy than high-scoring samples. However, its

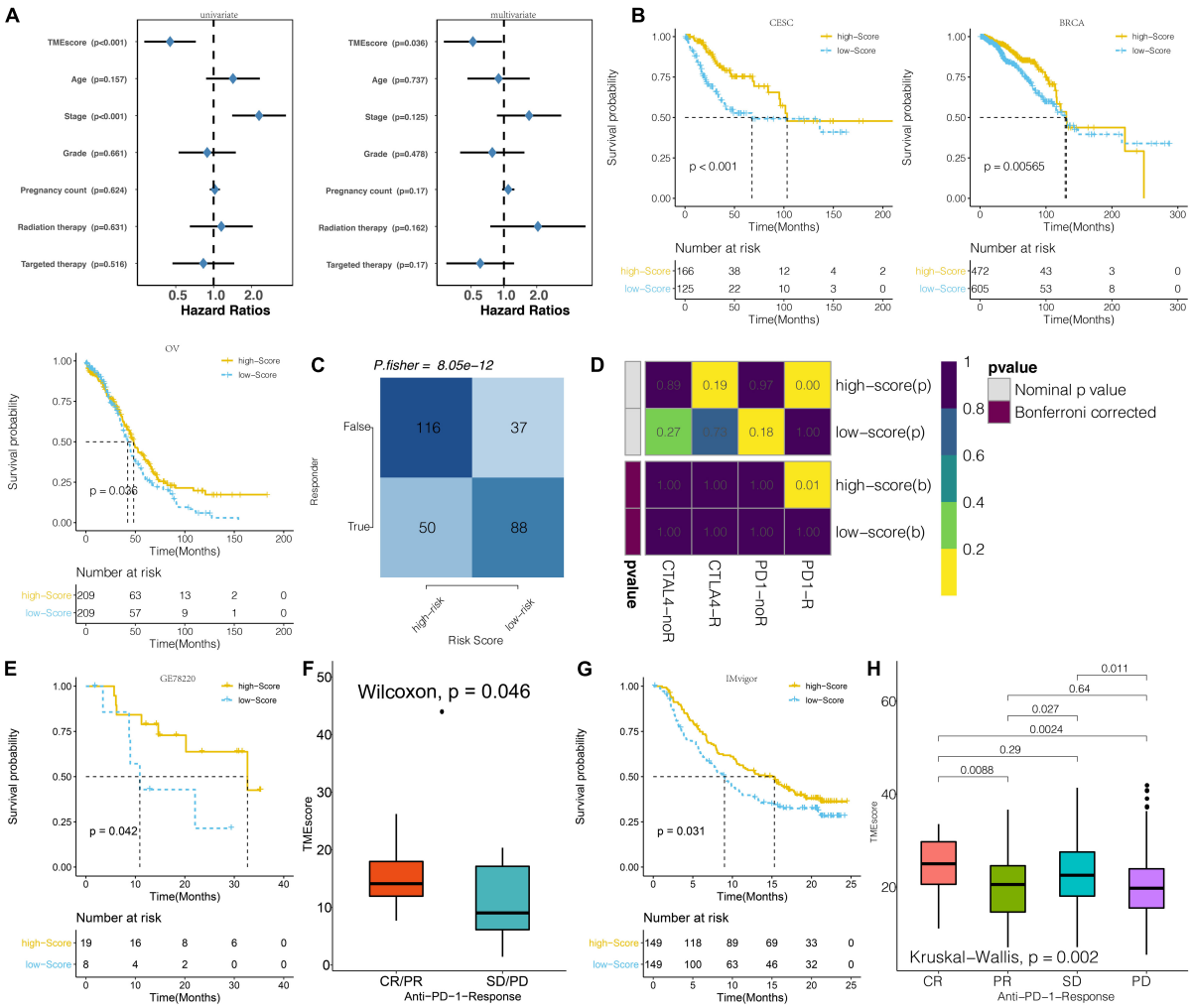

FIGURE 7 | Prognosis potential of TME score for immunotherapeutic response. **(A)** Univariate and multivariate Cox regression model estimating prognostic potential of TME score, patient age, tumor stage, tumor grade, pregnancy count, radiation therapy, and targeted therapy in TCGA. (The length of horizontal line represents the 95% confidence interval for each group. The vertical dotted line represents the HR of all patients. HR < 1.0 indicates that a high TME score is a biomarker for positive prognosis). **(B)** Kaplan–Meier curves for the two groups of patients classified along the TME score for CESC, BRCA, and OV from the TCGA. (Log-rank test showed an overall $P < 0.001$). **(C)** The TIDE value and response to immunotherapy for the TME score clusters. **(D)** Submap analysis based on the TIDE algorithm for differential response to CTAL-4 and anti-PD-1 therapy with regard to the TME score for the TCGA dataset. **(E)** Kaplan–Meier curves for the two groups of melanoma patients classified based on TME score for GSE78220. (Log-rank test showed an overall $P < 0.001$). **(F)** TME scores for groups with different anti–PD-1 clinical response status (CR/PR and SD/PD) in the GSE78220 dataset. (Wilcoxon, $P = 0.019$). **(G)** Kaplan–Meier curves for the two groups of patients classified based on the TME score in the IMvigor cohort. (Log-rank test: $P < 0.001$). **(H)** Distribution of TME scores in groups with different anti–PD-L1 clinical response statuses in the IMvigor cohort.

prediction ability still requires being examined with more clinical samples.

Previous studies also discussed the association between cervical cancer and its immune landscape. A previous study analyzed the proportion of immunocytes in cervical cancer and identified prognostic related immunocytes (Wang et al., 2019). Another study stratified samples based on the expression profile of differentially expressed immune-related genes and suggested that samples with higher infiltrated CD8 T cells and mast cells are more sensitive to immune checkpoint inhibitors (Yang et al., 2019). Moreover, tumor mutation loads are also critical intrinsic factors that affect tumor response to immunotherapy (Gasser et al., 2017; Havel et al., 2019). For instance, the amplification of HLA-DQA1 and STAT3 (Bae et al., 2020; Zou et al., 2020) and the deletion of ANGPT2 and TNFRSF13B (Rotolo et al., 2016; Lauret Marie Joseph et al., 2020) from the high TME score group have been proved to be immunotherapy-associated factors. Therefore, the TME score may serve as a potential tool to evaluate the tumor sensitivity to immunotherapy.

Previous studies proposed the "hot" and "cold" tumor analogy to describe tumor sensitivity to immunotherapy (Galon et al., 2006; Galon and Bruni, 2019). Given that tumors with high TME scores exhibited higher infiltrations of activated immunocytes and inflammatory related cells, tumors in this group may be referred as "hot" tumors. Moreover, high

TME score tumors exhibited better PD-L1 receptor therapeutic response than low TME score tumors. Thus, adopting different strategies may improve patients' clinical outcome. For instance, T-cell-targeted therapy (Buchbinder and Desai, 2016; Hellmann et al., 2016) or microbiome modulation (Snyder et al., 2015; Routy et al., 2018) was recommended to "hot" tumors. Chemotherapy, in combination with T cell enhancement or stimulatory signals, can improve "cold" tumor sensitivity (Whiteside et al., 2016). Taking the TME score into consideration in the choice of cervical cancer treatment may improve patients' survival outcome.

In this study, infiltration of activated immunocytes was preferentially enhanced in the high TME score group. A higher immunocyte infiltration and enhanced immune checkpoint gene expression in the high TME score cluster implies that these tumors are more sensitive to immunotherapy. In conclusion, this study highlights the impact of the tumor microenvironment to immunotherapeutic sensitivity in tumors.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YK: data collection, analysis, and manuscript preparation. JH, YL, and NZ: manuscript revision. QC and YZ: study design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.573497/full#supplementary-material

Supplementary Figure 1 | Flow chart and cluster analysis related information. **(A)** The diagrammatical flow chart for this study. **(B)** TME clusters; TCGA matrixes for each k (k = 2–4), displaying the clustering stability using 1,000 iterations of hierarchical clustering. **(C)** TME gene clusters; TCGA matrixes for each k (k = 2–4), displaying the clustering stability using 1000 iterations of hierarchical clustering.

Supplementary Figure 2 | GSVA analysis and immune landscape based on TME clusters. **(A)** GSVA of TME clusters in TCGA-GO **(B)**. The expression pattern of seven types of immune checkpoints in TME clusters for TCGA.

Supplementary Figure 3 | Genomic profiles associated with TME score. **(A)** GISTIC 2.0 amplifications and deletions in CESC with high TME score. Chromosomal locations of peaks of significantly recurring focal amplification (red) and deletions (blue). **(B)** Differential somatic mutations in CESC with high TME score.

Supplementary Table 1 | GO pathways for TME clusters based on GSVA in TCGA.

Supplementary Table 2 | 383 differentially expressed genes in TCGA.

Supplementary Table 3 | 98 representative TME signature genes.

Supplementary Table 4 | GO enrichment analysis for TME gene clusters based on 98 genes.

Supplementary Table 5 | KEGG enrichment analysis for TME gene clusters based on 98 genes.

Supplementary Table 6 | TME score for CESC patients in TCGA.

Supplementary Table 7 | GSVA of TME score.

Supplementary Table 8 | TME score for BRCA patients.

Supplementary Table 9 | TME score for OV patients.

Supplementary Table 10 | Characteristics of all the included patient samples.

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Comprehensive Characterization of Cachexia-Inducing Factors in Diffuse Large B-Cell Lymphoma Reveals a Molecular Subtype and a Prognosis-Related Signature

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Background: Cachexia is defined as an involuntary decrease in body weight, which can increase the risk of death in cancer patients and reduce the quality of life. Cachexia-inducing factors (CIFs) have been reported in colorectal cancer and pancreatic adenocarcinoma, but their value in diffuse large B-cell lymphoma (DLBCL) requires further genetic research.

Methods: We used gene expression data from Gene Expression Omnibus to evaluate the expression landscape of 25 known CIFs in DLBCL patients and compared them with normal lymphoma tissues from two cohorts [GSE56315 (n = 88) and GSE12195 (n = 136)]. The mutational status of CIFs were also evaluated in The Cancer Genome Atlas database. Based on the expression profiles of 25 CIFs, a single exploratory dataset which was merged by the datasets of GSE10846 (n = 420) and GSE31312 (n = 498) were divided into two molecular subtypes by using the method of consensus clustering. Immune microenvironment between different subtypes were assessed *via* single-sample gene set enrichment analysis and the CIBERSORT algorithm. The treatment response of commonly used chemotherapeutic drugs was predicted and gene set variation analysis was utilized to reveal the divergence in activated pathways for distinct subtypes. A risk signature was derived by univariate Cox regression and LASSO regression in the merged dataset (n = 882), and two independent cohorts [GSE87371 (n = 221) and GSE32918 (n = 244)] were used for validation, respectively.

Results: Clustering analysis with CIFs further divided the cases into two molecular subtypes (cluster A and cluster B) associated with distinct prognosis, immunological landscape, chemosensitivity, and biological process. A risk-prognostic signature based on CCL2, CSF2, IL15, IL17A, IL4, TGFA, and TNFSF10 for DLBCL was developed, and significant differences in overall survival analysis were found between the low- and high-risk groups in the training dataset and another two independent validation datasets.

Multivariate regression showed that the risk signature was an independently prognostic factor in contrast to other clinical characteristics.

Conclusion: This study demonstrated that CIFs further contribute to the observed heterogeneity of DLBCL, and molecular classification and a risk signature based on CIFs are both promising tools for prognostic stratification, which may provide important clues for precision medicine and tumor-targeted therapy.

Keywords: cachexia-inducing factors, molecular subtype, prognosis-related, signature, diffuse large B-cell lymphoma

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is a biologically and clinically heterogeneous B-cell neoplasm morphologically characterized by large lymphoid cells with B-cell markers growing in a rapidly proliferating and diffuse pattern (Caimi et al., 2016). DLBCL is one major subtype of non-Hodgkin lymphoma (NHL) which originates from B-cells, and it constitutes more than 25–35% of NHL cases in developing countries (Miao et al., 2019). It is estimated that 81,560 people in the United States will be diagnosed with NHL, and 20,720 of those will die of related causes in 2021 (Siegel et al., 2021). In the last decades, dramatic improvements have been achieved in the treatment of DLBCL, and the regimen of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has been established as the first-line or standard therapy for patients diagnosed with DLBCL. Approximately 60% of cases can be cured by using this treatment strategy (Sarkozy et al., 2015). However, in the light of huge heterogeneity in all patients, more than one-third of individuals will fail this first-line therapy and experience extremely poor prognosis (Voltin et al., 2020), illustrating the unmet need to emphasize the importance of risk stratification that can lead to more scientific and effective personalized treatment. In recent times, the risk assessment of DLBCL has mainly concentrated on the international prognostic index (IPI) and cell of origin (COO); the application of COO classification in DLBCL has revealed two subtypes, namely, the germinal center B-cell-like (GCB) and activated B-cell-like (ABC) (Moffitt and Dave, 2017) subtypes. However, both IPI and COO are widely questioned regarding the risk stratification of a small number of DLBCL and do not accurately predict the outcome for cases (Wight et al., 2018) because the distinction based on COO does not fully account for the heterogeneous outcomes and chemotherapy response of DLBCL. The recent improvement in bioinformatics algorithm and microarray technology provided huge opportunities for clinical applications of paraffin-embedded tissue and brings a new dawn to the risk classification of DLBCL. The non-negative matrix factorization consensus clustering algorithm used by Chapuy et al. (2018) and the GenClass algorithm were employed by Schmitz et al. (2018) to analyze the genetic data of 304 and 574 cases of patients with DLBCL, respectively. Their analyses showed the existence of distinct subtypes independent of or within the COO subtypes. According to these previously reported studies, we hypothesized that the analysis of a gene expression signature may add considerable texture to improve the classification for risk stratification and personalized therapeutic implication in DLBCL.

Cachexia is a non-specific symptom characterized by a state of involuntary substantial loss of skeletal muscle mass with or without adipose tissue loss and is usually difficult to rehabilitate by conventional nutritional support (Mallard et al., 2019). Cachexia severely compromises life quality and reduces treatment tolerance among patients with cancer and contributes to 20% of all cancer deaths (Fearon et al., 2012). Weight loss greater than 10% in 6 months is determined to be one of the B symptoms and has been confirmed in multiple large retrospective research as an adverse prognostic factor for NHL, independent of IPI (Han et al., 2013; O'Brian et al., 2016; Xiao et al., 2017; Wight et al., 2018). Patients with the same height and a similar tumor burden but with a different cachexia status will receive a completely different chemotherapy drug regimen and are typically associated with distinct prognoses. Several tumor-derived and inflammatory factors are classified as cachexia-inducing factors (CIFs) and are derived from the tumor secretome or host; these are suggested to be involved in the pathogenesis of patients and drive the development of cachexia (Pettersen et al., 2020). Thus far, several markers for cachexia, such as serum albumin, body mass index, adipopenia, and sarcopenia, have been investigated and suggested to be likely factors affecting the prognosis of DLBCL (Go et al., 2020). Furthermore, 25 known CIFs were reported in a previous study, and their prognosis value was explored in 12 cancer types except DLBCL (Freire et al., 2020); hence, appropriate attention should be paid to CIFs in the context of DLBCL.

In this study, we comprehensively analyzed and determined the potential prognostic value of the 25 CIFs in DLBCL and stratified 884 patients into two subtypes based on the expression levels of these 25 CIFs. Subsequently, a deeper characterization of the immune microenvironment and biological process of the two subtypes was conducted. In addition, treatment sensitivity of commonly used drugs was predicted for patients with a distinct subtype. Moreover, we developed a multi-CIFs-based signature by utilizing the LASSO Cox regression model to predict the overall survival (OS) of patients with DLBCL. The prognostic accuracy of this signature was validated in two independent cohorts. Our signature can complement the existing risk stratification systems including COO and IPI score for prediction of outcome in DLBCL, possibly enabling physicians to make more informed treatment decisions.

MATERIALS AND METHODS

Dataset Sources and Selection as Well as Data Processing

The raw CEL data of GSE56315 (55 DLBCL samples and 33 normal B-cell samples), GSE12195 (73 DLBCL samples and 20 normal B-cell samples), GSE12453 [11 DLBCL samples, 25 normal B-cell samples, and 12 cases of classical Hodgkin's lymphoma (cHL)], GSE10846 (420 cases of DLBCL), GSE31312 (498 cases of DLBCL), and GSE87371 (223 cases of DLBCL), all of which were based on the GPL570 platform (HG-U133_Plus_2), were selected and downloaded. GSE32918 (249 cases of DLBCL) based on the platform of GPL8432 (Illumina HumanRef-8 WG-DASL v3.0) was downloaded in the form of a preprocessed expression matrix uploaded by the authors. All datasets were extracted from the Gene Expression Omnibus (GEO)[1] database. The selection criteria for DLBCL datasets were as follows: (a) all expression profiling datasets based on any platform except those based on HG-U133A platform (the HG-U133A platform was developed 20 years ago, and the number of probes is less than half of that of other platforms), (b) all datasets should have basic clinical data characteristics including sex, age, OS, and OS status, and (c) datasets with a larger sample size and the minimum number of patients being > 200.

Using these criteria, the DLBCL datasets of GSE10846, GSE31312, GSE87371, and GSE32918 were identified and used to perform prognostic analysis. All the raw chip data went through the process of quality assessment, quality control, background correction, and normalization, and the process was completed by "simpleaffy" (version 2.64.0), "affyPLM" (version 1.64.0), and "arrayQualityMetrics" (version 3.46.0) packages. All microarray data were converted into expression matrix after processing. Finally, 1,529 cases of DLBCL, 78 cases of normal B-cell tissue, and 12 cases of cHL were included in the GEO dataset. All samples that lacked survival information and/or had survival data of < 1 day were excluded from further analysis.

Landscape of Expression and Genetic Variation as Well as Prognostic Value of CIFs in DLBCL

To clarify the expression difference of 25 CIFs (CCL2, CD40LG, CSF1, CSF2, CSF3, CXCL12, CXCL8, FGF2, HGF, IFNG, IL10, IL15, IL17A, IL1B, IL4, IL6, LEP, LIF, MMP13, PDGFB, TGFA, TNF, TNFSF10, TNFSF11, and VEGFA) between DLBCL and normal B-cell tissues and to ensure its reliability, ANOVA was performed to calculate the discrimination in the two datasets, namely, GSE56315 and GSE12195. Based on the expression value of the 25 CIFs, principal component analysis was also performed to assess the distribution between the DLBCL and normal B-cell tissues. The mutation status and influence of mutation status on the survival of all CIFs in 48 cases of DLBCL patients was obtained from the cBioPortal database[2]. The samples with complete survival data in GSE10846 and GSE31312 were merged into a single meta-cohort ($N = 882$), and combat algorithm of "sva" package (version 3.38.0) was used to combine the datasets and remove batch effects to reduce non-biological technical biases. Genomic instability often generates a diversity of genome, leads to cancer occurrence, and influences disease development. Thus, the presence of deletions and accumulation of amplifications of CIFs were investigated. A univariate Cox regression model was adopted to calculate the hazard ratios (HRs) for each CIF in DLBCL patients, and Pearson's correlation analysis was utilized to evaluate the positive or negative regulatory relationship among the 25 CIFs. The network of related relationships of a CIF whose value of expression was correlated with one or more CIFs ($|$ Pearson $R| > 0.1$ and $P < 0.001$) was visualized by Cytoscape software (version 3.8.2).

Unsupervised Clustering for 25 CIFs in DLBCL

Unsupervised clustering analysis was employed to detect unknown possible distinct subtypes based on the expression of 25 CIFs and differentiated in the meta-cohort ($n = 882$) for further analysis. The consensus cluster algorithm was performed by "ConsensuClusterPlus" package (version 1.52.0) to determine the number of clusters and stability of classification, and 1,000 repetitions were conducted to ensure the accuracy of the results (Wilkerson and Hayes, 2010). To determine the influence of distinct subtype on prognosis, Kaplan–Meier analysis was conducted and compared by log-rank test, and Kruskal–Wallis test was utilized to distinguish the expression of CIFs between different subtypes.

Estimation of Immune Infiltration and Prediction of Cytotoxic and Immunomodulator Drug Sensitivity

To gain deeper insights into the tumor microenvironment of patients with DLBCL, CIBERSORT was used to calculate the composition difference of 22 kinds of infiltrating immune cells in DLBCL and normal B-cells. $P < 0.05$ was considered to indicate statistical significance. In addition, although the remarkable outcome of anti-PD-1 therapy in classic Hodgkin's lymphoma (cHL) is acknowledged, the efficacy of anti-PD-1 monotherapy in DLBCL remains unsatisfactory and needs further investigation (Kline et al., 2020). Therefore, the distribution of immune cells in the microenvironment of cHL and DLBCL was also calculated. Single-sample gene set enrichment analysis (ssGSEA) algorithm which is based on 29 immune gene sets was applied to comprehensively quantify the relative abundance of immune cell types, pathways, functions, and checkpoints in each patient. The difference of 29 immune gene sets and 22 immune cells between cluster A and cluster B patients was analyzed using Kruskal–Wallis testing. In addition, the "pRRophetic" package (version 0.5) (Geeleher et al., 2014) was utilized to predict the treatment response for cytotoxicity and molecular targeted therapy between patients in cluster A and those in cluster B to determine their sensitivity to commonly used drugs for DLBCL.

[1] http://www.ncbi.nlm.nih.gov/geo/

[2] https://www.cbioportal.org/datasets/

Gene Set Variation Analysis and Functional Annotation

To provide deeper insights into the heterogeneity of biological processes between cluster B and cluster A patients, gene set variation analysis (GSVA) enrichment analysis was performed by using "GSVA" R packages (version 1.36.3). GSVA is a non-parametric unsupervised analysis method mainly employed in expression dataset and is widely used to evaluate the variation in biological process activity and pathway in the samples of an expression dataset. The gene sets of "c2.cp.kegg.v6.2.symbols.gmt" were selected and downloaded from MSigDB database[3] for implementing the GSVA analysis. Only adjusted $P < 0.05$ values were considered as statistically significant. Moreover, the "limma" package (version 3.44.3) was utilized to determine different biological pathways between cluster A and cluster B patients, and the results of $|\log2(\text{fold change})| > 0.2$ and $P < 0.05$ were considered to be statistically significant (Song et al., 2020). In addition, the R package of "limma" (version 3.44.3) was used to identify differentially expressed genes (DEGs) between cluster B and cluster A with the criterion of $|\log2(\text{fold change})| > 1$ and $P < 0.05$ for Gene Ontology (GO) and pathway enrichment analysis.

Generation and Validation of Prognostic Signature Based on CIFs

Univariate Cox proportional hazard regression analysis was utilized to assess the relationship between CIFs and OS of DLBCL patients within the meta-cohort (which was incorporated by GSE10846 and GSE31312, and the meta-cohort was set as the training group). Only $P < 0.05$ was considered to indicate the most valuable prognostic CIF genes which were sorted out to perform the LASSO Cox regression analysis which depend on the R package "glmnet" (version 4.1). LASSO Cox regression analysis is a well-established and widely used mathematical selection method for screening the most predictive markers. The most prominent advantage of LASSO Cox regression is that, by penalized regression on all variable coefficients, the relatively unimportant coefficients of independent variables whose coefficients are close to 0 are excluded from the model. The optimal values of the penalty parameter λ were determined through 10 cross-validations. The following formula was derived to calculate the risk score based on the expression of candidate CIF genes, weighted by the regression coefficient obtained from LASSO Cox regression analysis in the training dataset:

$$\text{Risk score} = \sum_{i=1}^{n} exp_i \times \beta_i$$

where i is the number of CIF genes, exp_i represents the expression value of CIF gene i, and βi represents the regression coefficient. By setting the median risk score as the cutoff value, all DLBCL patients were dichotomized into high- and low-risk groups. To evaluate the stability and reproducibility of the CIF signature, two external datasets including GSE87371 ($n = 221$) and GSE32918 ($n = 244$) were validated. Survival curves were constructed using the Kaplan–Meier method and carried out using the "survival" package in R (version 3.2-7). In addition, we used the "medcalc" statistical software to evaluate the performance of our CIF signature for its ability to discriminate molecular subtype with poor prognosis in DLBCL patients who were recently identified.

[3]http://www.gsea-msigdb.org/gsea/msigdb/index.jsp

Comprehensive Analysis of Risk Stratification and Clinical Attributes

To investigate the effect of the CIF-based risk signature on the prognosis of DLBCL, univariate and multivariate Cox regression analyses were conducted. The risk signature and other clinicopathological attributes including sex, age, stage, COO type, extranodal sites involved, serum LDH level, IPI score, bulky disease, B-symptoms, and Eastern Cooperative Oncology Group (ECOG) performance were entered into the analysis. All clinicopathological parameters were grouped according to the IPI criteria: serum LDH level, $>1 \times$ normal; ECOG performance status, ≥ 2; extranodal sites involved, >1; age, >60 years; and Ann Arbor stage, III–IV. All other statistical analyses were conducted using R (version 4.0.2).

RESULTS

Patient Characteristics

A total of 1,475 patients with DLBCL and 53 with normal B cells from six independent academic institutions were included in the analysis after excluding samples that lacked clinical metadata; of these, 1,347 DLBCL samples from four datasets with survival time were used for prognosis-related research. The clinical characteristics of the 1,347 patients are presented in **Table 1** and **Supplementary Table 1**. The median follow-up was 28.62 months [interquartile range (IQR): 11.22–52.14] for patients in the GSE10846 cohort, 34.32 months (17.25–55.42) for those in the GSE31312 cohort, 39.84 months (4.10–70.8) for those in the GSE32918 cohort, and 35.49 months (22.53–49.31) for those in the GSE87371.

Cachexia-Inducing Factors Are Up-Regulated in DLBCL

To assess the biological function of CIFs, the expression profiles of 25 CIFs in two cohorts were obtained for systematically investigating the distinct expression patterns between DLBCL and normal B-cell tissues. Almost all CIFs were dramatically over-expressed in DLBCL that comprised the dataset GSE56315, which was subsequently validated in another dataset, GSE12195. Nineteen CIFs were identified to be up-regulated in GSE56315 and 21 CIFs were over-expressed in GSE12195 (**Figures 1A,B**). *CCL2*, *CD40LG*, *CSF1*, *CSF3*, *CXCL12*, *FGF2*, *IFNG*, *IL10*, *IL15*, *IL1B*, *LIF*, *MMP13*, *PDGFB*, *TGFA*, *TNFSF10*, and *VEGFA* were all up-regulated in both datasets, except for CSF2 which was down-regulated in DLBCL. Furthermore, the expression level of IL17A showed no statistically significant difference between the DLBCL and normal B cell tissues ($P > 0.05$). Based on

the expression level of these 25 CIFs, we could accurately distinguish DLBCL from normal samples (**Supplementary Figures 1A,B**). The high heterogeneity of the expression landscape indicated that CIFs play an essential biological role in DLBCL pathogenesis and progression. Apart from this, we first summarized somatic mutations of the 25 CIFs in DLBCL patients based on The Cancer Genome Atlas cohort. Thirteen CIFs were found with experienced mutations, and TNF showed the highest frequency of mutations followed by *VEGFA*, *IL17*, and *IL10* (**Figure 1C**). In addition, patients with *LIF* and *TGFA* mutations showed a negative correlation with survival (**Supplementary Table 2**). Three CIF gene clusters were identified by unsupervised clustering analysis (**Figure 2B**), and most CIFs in the same cluster had a positive regulatory relationship with each other except in CIF cluster 3 (**Figure 2A** and **Supplementary Table 3**). A univariate Cox regression model was also designed to reveal the prognostic value of 25 CIFs in DLBCL patients of the meta-cohort that was enrolled by two GEO datasets (GSE10846 and GSE31312) after batch correction (**Supplementary Figures 2A–D**), and seven CIFs (*CCL2*, *CSF2*, *IL15*, *IL17A*, *IL4*, *TGFA*, and *TNFSF10*) were significantly associated with OS (**Figure 2A** and **Supplementary Table 4**). The comprehensive landscape of CIF interactions and their prognostic significance for patients with DLBCL were delineated with the network (**Figure 2A**).

TABLE 1 | Clinical characteristics of the 1,347 cases of diffuse large B-cell lymphoma patients.

Characteristics	GSE10846 (n = 412)	GSE31312 (n = 470)	GSE32918 (n = 244)	GSE37371 (n = 221)
Age				
< 60	179 (43.4%)	186 (39.6%)	68 (27.9%)	109 (49.3%)
≥ 60	233 (56.6%)	284 (60.4%)	176 (72.1%)	112 (50.7%)
Sex				
Male	222 (53.9%)	271 (57.7%)	142 (58.2%)	116 (52.5%)
Female	172 (41.8%)	199 (42.3%)	102 (41.8%)	105 (47.5%)
NA	18 (4.3%)			
Stage				
I–II	188 (45.6%)	223 (47.4%)		71 (32.1%)
III–IV	217 (52.7%)	247 (52.6%)		150 (67.9%)
NA	7 (1.7%)			
COO type				
GCB	182 (44.2%)	230 (48.9%)	119 (48.8%)	82 (37.1%)
ABC	167 (40.5%)	197 (41.9%)	79 (32.4%)	85 (38.5%)
Unclassified	63 (15.3%)	43 (10.2%)		54 (24.4%)
NA			46 (18.8%)	
ECOG performance				
0–1	295 (71.6%)	374 (79.6%)		
2–4	93 (22.6%)	96 (20.4%)		
NA	24 (5.8%)			
LDH escalated				
Yes	177 (43.0%)	275 (58.5%)		
No	173 (42.0%)	149 (31.7%)		
NA	62 (15.0%)	46 (9.8%)		
Bulky				
Yes		94 (20.0%)		
No		271 (57.7%)		
NA		105 (22.3 %)		
IPI score				
0–2		275 (58.5%)		119 (53.8%)
3–5		148 (31.5%)		102 (46.2%)
NA		47 (10.0%)		
B symptom				
Yes		130 (27.7%)		
No		278 (59.1%)		
NA		62 (13.2%)		
Extranodal sites				
Yes	145 (35.2%)	278 (59.1%)		
No	236 (57.3%)	192 (40.9%)		
NA	31 (7.5%)			

Consensus Clustering for CIFs and Identifying Molecular Subtypes of DLBCL

All in all, 882 cases of DLBCL from the meta-cohort (n = 882) were utilized to find a stable and reliable subtype classification at the end of the repeat sampling. Thus, k = 2 was identified as the optimal number of clustering based on the expression levels of CIFs and the result of proportion of ambiguous clustering (PAC) measure (**Supplementary Figures 3A–H**). A total of 882 DLBCL patients were clustered into two subtypes named as cluster A (n = 541) and cluster B (n = 341) (**Supplementary Table 5**). Cluster B was significantly associated with poor OS, and the 50-month OS rates for cluster A and cluster B patients were 31.6 and 24.0% (**Figure 2C**). Integration of consensus clustering and COO-based classification from the 882 patients and Kaplan–Meier curves also showed that patients separated by COO with distinct molecular signature had a significantly different prognosis (p < 0.0001, **Figure 2D**). The ABC of COO subtypes accounts for a larger population in cluster A than in cluster B (**Figure 2E**), but there were no significant differences (p = 0.416). As expected, an increased expression of most CIFs was observed in high-risk cases with DLBCL (**Figure 2F**), and the variation of CIF expression in different molecular subtypes further showed heterogeneity of DLBCL.

Distinct Immune Cell Infiltration and Molecular Function Between Different Molecular Subtypes

CIBERSORT immune analysis confirmed that DLBCL was associated with decreased naive B cells and memory B cells and had an abundance of activated memory CD4 T cells, follicular helper T cells, M0 macrophages, M1 macrophage, and M2 macrophages in three independent cohorts (**Supplementary Figures 4A–C**). However, DLBCL showed higher infiltration levels of CD8 T cells and lower expression of CD274 (PD-L1) than cHL (**Supplementary Figure 4C**).

Per recent findings of distinct prognosis between cluster A and cluster B, ssGSEA and CIBERSORT were used to define the distribution of immune landscape and pattern between the two subtypes, and the result showed that cluster A and cluster B have significant divergence in almost all components

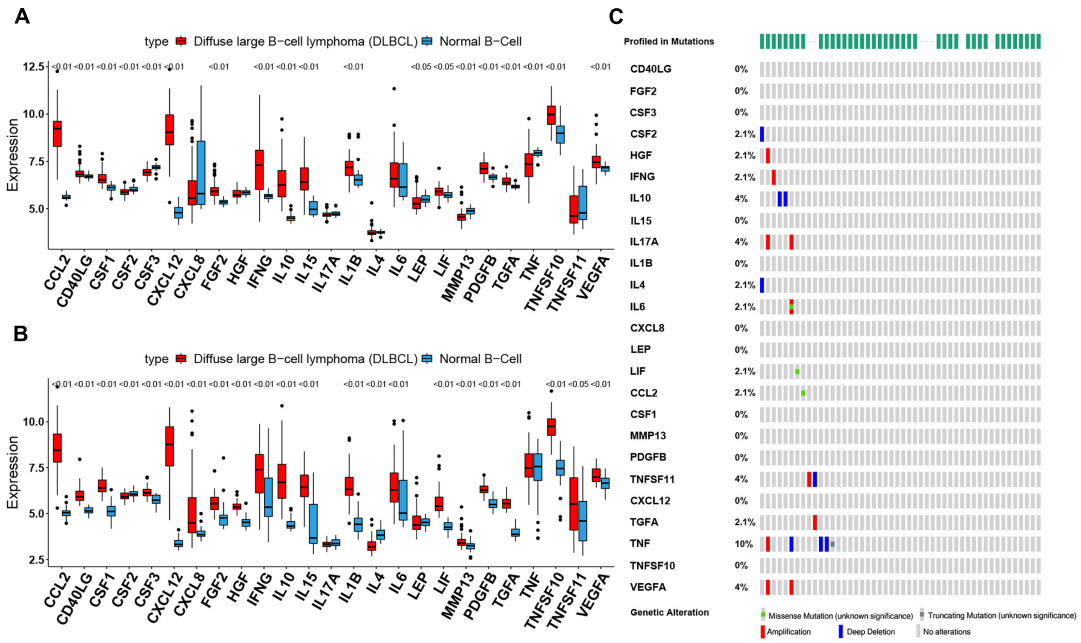

FIGURE 1 | The landscape of cachexia-inducing factors in the diffuse large B-cell lymphoma (DLBCL). **(A,B)** Expression levels of 25 cachexia-inducing factors in DLBCL and normal B cell from human tonsils (**A**, GSE56315; **B**, GSE12195). The black dots represent outliers. **(C)** The mutation frequency of 25 CIFs in 48 patients with DLBCL from The Cancer Genome Atlas cohort.

of immune cell types and immune functions. ssGSEA revealed that patients in cluster B were associated with a remarkably high number of activated dendritic cells (aDCs), APC co-inhibition, APC co-stimulation, cytokine and cytokine receptor (CCR), CD8+ T-cells, check-point, cytolytic activity, activated dendritic cells (DCs), immature dendritic cells (iDCs), inflammation promotion, macrophages, para-inflammation, NK cells, MHC class I, neutrophils, plasmacytoid dendritic cells (pDCs), T-cell co-inhibition, T-cell co-stimulation, T helper cells, Th1 cells (T helper 1), Th2 cells, tumor-infiltrating lymphocytes (TIL), regulatory T cells (Treg), type I IFN response, and type II IFN response. In comparison, cluster A patients showed a significantly high number of B cells. Unsupervised hierarchical clustering of immune cell types and functions are described in **Figure 3A** and **Supplementary Table 6**. CIBERSORT immune analysis also confirmed that cluster A showed an overrepresentation of naive B cell and memory B cells, whereas cluster B showed higher infiltration levels of CD8 T cells, plasma cells, CD4 T cells, CD4 naive T cells, activated memory T cells, gamma delta resting NK cells, activated NK cells, monocytes, M1 macrophages, eosinophils, M2 macrophages, resting dendritic cells, neutrophils, activated mast cells, activated dendritic cells, and resting mast cells (**Figure 3B** and **Supplementary Table 7**). Interestingly, the considerable inconsistencies in the scale of fraction of B cells between cluster A and cluster B patients presented in CIBERSORT were very similar to the results obtained in the ssGSEA analysis, indicating that the high proportion of B cells was associated with prolonged survival. To better illustrate the characteristics of immune cell infiltration and molecular function, we tested the correlation between immune cell infiltration obtained from CIBERSORT and immune landscape and molecular pattern acquired from ssGSEA (**Supplementary Figure 5**). In addition, *PD-L1* and *CTLA-4* were also identified as being considerably overexpressed in cluster B (**Figure 4D**).

Heterogeneity of Drug Sensitivity and Biological Behaviors Between Different Molecular Subtypes

The IC50 of seven commonly used cytotoxic drugs (cisplatin, cytarabine, doxorubicin, etoposide, gemcitabine, vinblastine, and vinorelbine) and one immunomodulator drug (lenalidomide) was predicted for cluster B and cluster A patients (**Supplementary Table 8**). We found that cisplatin, doxorubicin, and etoposide had lower IC50 in cluster B patients, contrary to the result of cytarabine, vinblastine, and lenalidomide in cluster B patients (**Figure 4A**). Furthermore, to explore the discrepancy of biological behaviors between cluster A and cluster B, GSVA and GO as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed. As shown in **Figure 4B** and **Supplementary Tables 9, 10**, cluster B patients had markedly enriched pathways of NOD-like receptor signaling, chemokine signaling, cytokine–cytokine receptor interaction, hematopoietic cell lineage, and complement and coagulation cascades. Briefly, 79 DEGs were identified between cluster B and cluster A (**Supplementary Figures 6A,B**), and these DEGs were remarkably related to cytokine activity and cytokine-related pathway (**Figure 4C** and **Supplementary Figures 7A–D**), which re-confirmed that cytokine activity and cytokine-related pathway

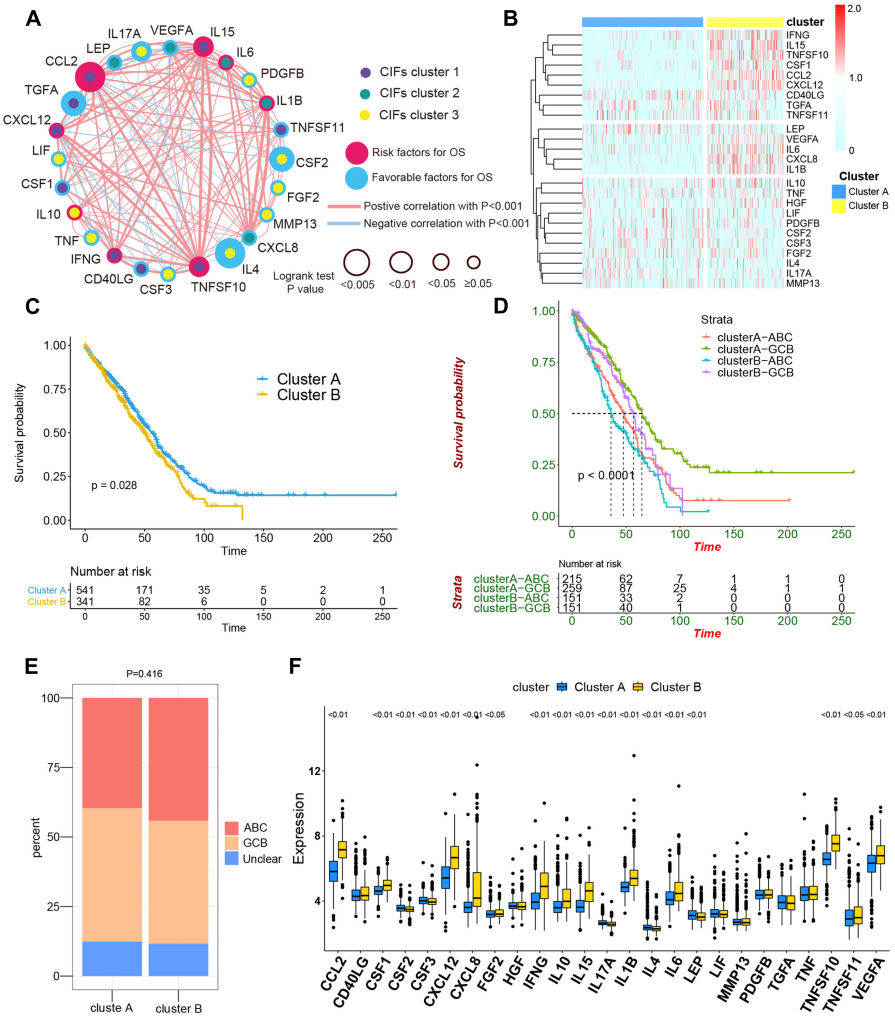

FIGURE 2 | The comprehensive landscape of cachexia-inducing factor (CIF) interactions and identification of two molecular subtypes with different prognoses and transcriptome traits. **(A)** Network showing the landscape of CIF interactions and their prognostic significance for patients with DLBCL. The circle size represented the effect of each CIF on the prognosis, and the range of values calculated by log-rank test was $p < 0.005$, $p < 0.01$, $p < 0.05$, and $P \geq 0.05$, respectively. Red circle, risk factors of prognosis. Blue circle, protective factors of prognosis. The lines linking the CIFs showed their interactions, and the thickness of the connecting line is positively correlated with the strength of the correlation. Negative correlation was marked with blue and positive correlation with red. Dots in the circle represent three CIF gene clusters termed as CIF clusters 1–3 and marked with purple, dark cyan, and yellow, respectively. **(B)** Heat maps showing the 25 CIFs' expression level clustered by different subtypes and segregation according to the relevance of CIFs. **(C)** Survival analyses for the two molecular subtypes based on 882 patients with diffuse large B-cell lymphoma (DLBCL) from two Gene Expression Omnibus cohorts (GSE10846 and GSE31312) including 541 cases in cluster A and 341 cases in cluster B. Kaplan–Meier curves with log-rank p value 0.028 showed a significant survival difference among distinct subtypes. **(D)** Patients separated by cell of origin (COO) subtype with distinct molecular subtypes have a significantly different prognosis. **(E)** The proportion of COO subtypes in cluster A and cluster B patients. **(F)** Difference in the expression of 25 CIFs between cluster A and cluster B subtype groups.

played a nonnegligible role in immune regulation in the tumor microenvironment.

The Risk Signature Robustly Identifies DLBCL Patients With Poor Survival

To construct a prognostic signature, seven CIFs that were identified as being associated with OS in the univariate Cox regression were included in the LASSO Cox regression model in the training dataset (882 samples selected from the meta-cohort). The optimal tuning parameter identified the following seven CIFs: CCL2, CSF2, IL15, IL17A, IL4, TGFA, and TNFSF10 (**Supplementary Figures 8A,B**). A risk score was then computed for each DLBCL patient based on the individual expression of the seven CIFs, weighted by the regression coefficient in the training set based on the following formula: risk score = (0.0668 × CCL2 expression) + (−0.2463 × CSF2 expression) + (0.05391 × IL15 expression) + (-0.2381 × IL17A expression) + (-0.2305 × IL4 expression) + (-0.1621 × TGFA expression) + (-0.1621 × TNFSF10 expression).Taking the median risk score as the cutoff value, all patients were divided into high- and low-risk groups.

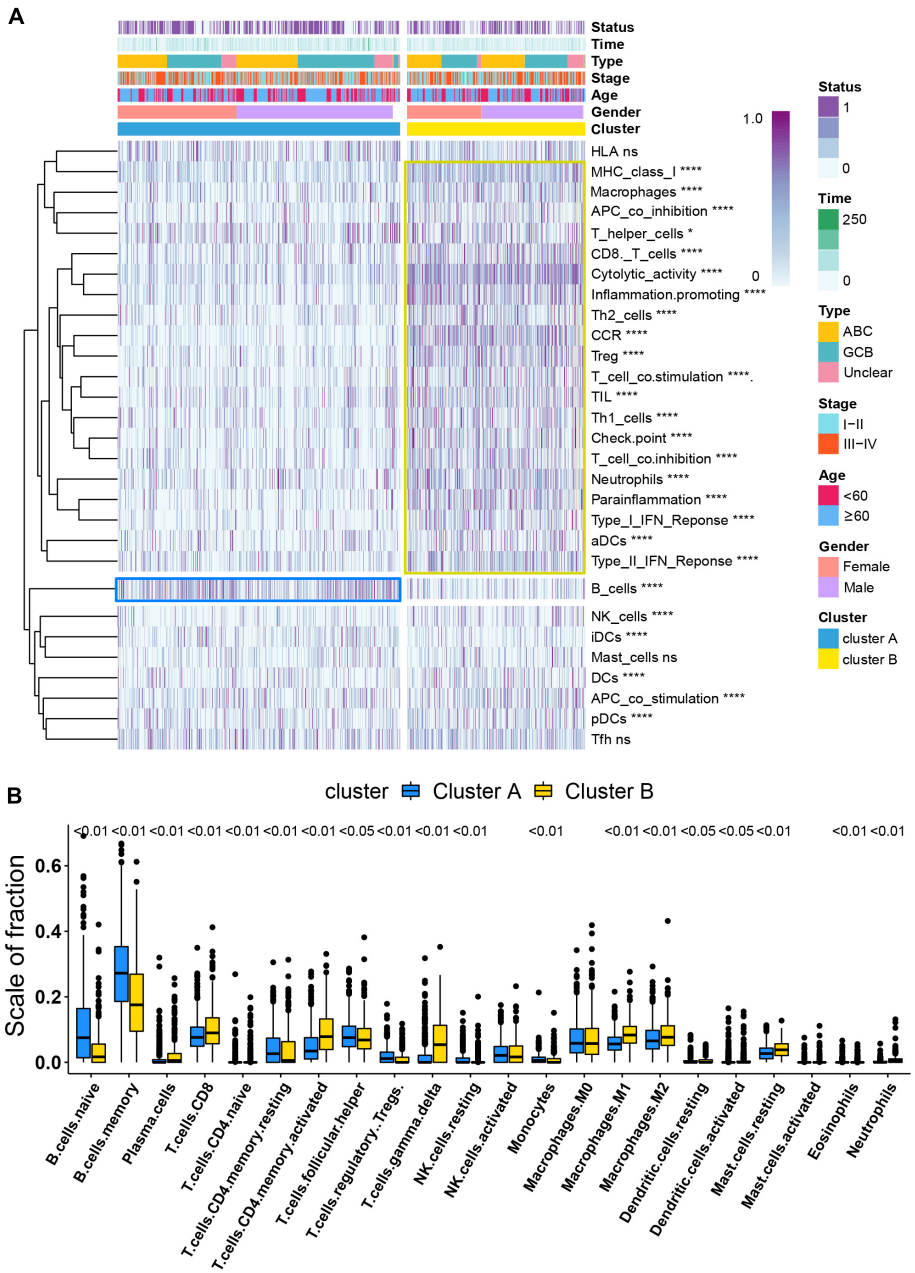

FIGURE 3 | Immune signature analysis. **(A)** Unsupervised hierarchical clustering of immune cell types and functions by individual subtypes (cluster A, blue; cluster B, yellow. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$). **(B)** Comparative fraction of the immune cell infiltration between cluster A and cluster B subtypes.

High-risk patients had a worse prognosis than low-risk ones [HR: 1.623 (1.348–1.995); $P < 0.001$] (**Figure 5A**). In addition to predicting survival, the performance of our risk signature to identify the cluster B molecular subtype recently identified with poor prognosis was determined, and it yielded an area under the curve value of 0.786 [95%CI (0.758–0.813); $P < 0.001$; **Supplementary Figure 9A**]. It showed that the distribution of risk scores between cluster A and cluster B vary significantly ($P = 2.22e\ 10^{-12}$, **Supplementary Figure 9B**) and a large proportion (264 of 341, 77.42%) of patients in cluster B were classified into a high-risk group (**Supplementary Figure 9C**). The role of the risk signature was validated by an additional two datasets that were consistent with the initial findings of the training dataset. There was significant distinction in OS between the high- and low-risk patients, and patients who were categorized into the high-risk group had shorter OS than those categorized into the low-risk group, cohort-1 [GSE87371; HR: 1.652 (1.208–2.259); $P = 0.002$] and cohort-2 [GSE32918; HR: 1.734 (1.225–2.455); $P = 0.002$] (**Figures 5B,C**). Kaplan–Meier curves also showed that patients separated by

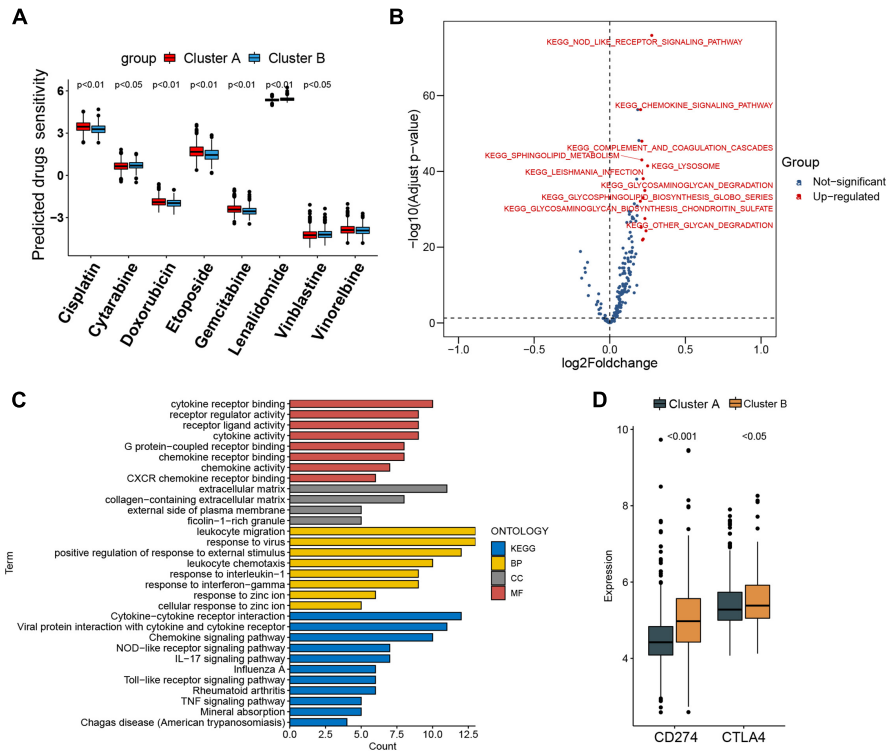

FIGURE 4 | Prediction of chemotherapy and immunomodulatory effect and biological characteristics in distinct subtypes. **(A)** Sensitivity analysis of eight common therapeutic drugs in patients of cluster A and cluster B. **(B)** Differences in pathway activities scored by gene set variation analysis between cluster A and cluster B patients. Red dot indicates activated pathways in cluster B patients, and blue dot indicates insignificant activated pathways between cluster A and cluster B patients. **(C)** Functional annotation and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis for differentially expressed genes between cluster A and cluster B patients. BP, biological process; CC, cellular component; MF, molecular function. **(D)** CD274 (PD-L1) and CTLA4 expression difference in cluster A and cluster B.

distinct pathological type have a significantly different prognosis (**Supplementary Figures 10A–C**).

The CIF Risk Signature Serves as an Independent Predictor of Risk and Survival Outcomes in DLBCL Patients

To evaluate whether the risk signature had an additional prognostic value that was beyond the clinical characteristics, univariate and multivariate Cox regression analyses were performed by clinical features and risk signature. In the univariate Cox regression, the seven-CIF-based risk signature was significantly correlated with OS. After multivariable adjustment by age, stage, COO type, extranodal sites involved, serum LDH level, and ECOG performance, the seven-CIF-based risk signature remained a powerful and independent prognostic factor for DLBCL patients (HR: 1.621, 95%CI: 1.306–2.011, $P < 0.0001$). Similar results were also noted in the testing cohort-1 dataset (HR: 1.468, 1.068–2.018; $P = 0.018$) as well as in the testing cohort-2 dataset (HR: 1.640, 1.157–2.325; $P = 0.005$) (**Figure 5D**). The observations in our study demonstrate that the CIF-based risk signature contributes to the additive prognostic value beyond that of age, pathological type, extranodal sites involved, serum LDH level, and ECOG in DLBCL patients.

DISCUSSION

Molecular classification of human cancers dividing patients into distinct molecular subtypes has unlocked an innovative approach to personalized medicine. Although the COO classification of GCB and ABC subtypes has been widely utilized to discriminate cells of DLBCL to predict patient prognosis, it is still debatable and considered unable to comprehensively demonstrate the distinct genetic and genomic characteristics of all DLBCLs (Wright et al., 2020). The extreme molecular heterogeneity of DLBCL brings a huge challenge to the development of precision treatment. Continuous progress in identification and differentiation of subtypes or risk stratification is needed to accelerate the management of personalized treatment in DLBCL. Cachexia is reportedly related to standard R-CHOP chemotherapy intolerance and significantly associated with a poor prognosis in DLBCL patients (Go et al., 2016). In the present study, we profiled the genomic landscape of CIFs in 882 DLBCL patients and revealed two distinct molecular subtypes with significantly different survival outcome and distinctive immune landscape, which captures the previously unexplained heterogeneity of the tumor microenvironment in DLBCL and may provide deeper insights into the heterogeneous responses to cytotoxic and immune blockade therapy. In addition, it

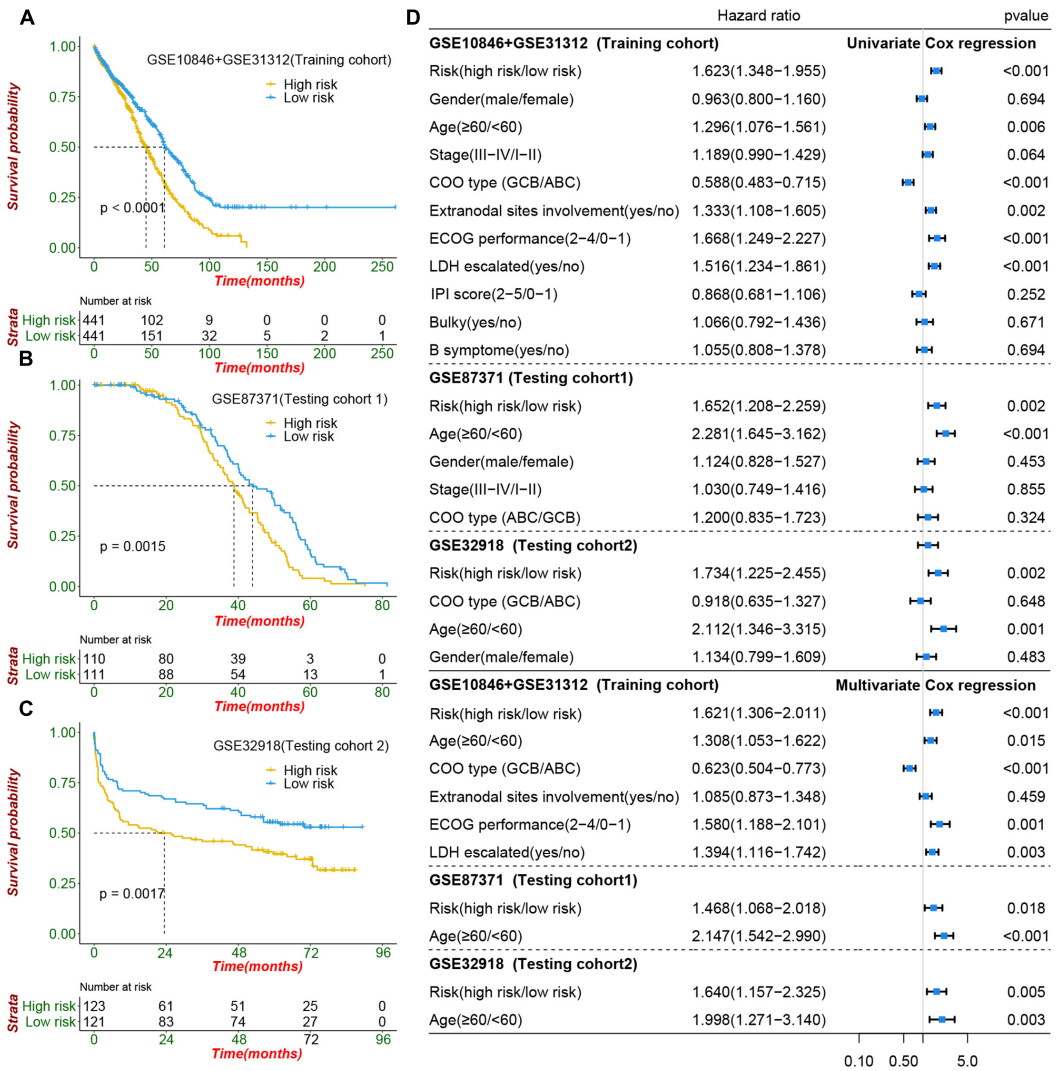

FIGURE 5 | Prognostic value of cachexia-inducing factor (CIF) risk signature in patients with DlBCL. **(A–C)** Performance of the CIFs based on the risk signature in predicting overall survival in the training cohort and two independent testing cohorts. **(D)** Forest plot showing that the signature is significantly associated with prognosis and works independently of the cell of origin subtyping and all clinical features in univariate CoxPH and multivariate CoxPH analyses.

may enable the development of subtype-specific treatment strategies targeting unique immune (therapeutic) vulnerabilities. Moreover, we developed and validated a seven-CIF-based risk signature to complement the existing prognostic evaluation system for the prediction of DLBCL outcome. To the best of our knowledge, this is the first study to comprehensively characterize the genomic landscape prognostic significance of CIFs in patients with DLBCL.

The molecular heterogeneity of DLBCL constitutes a major obstacle in treatment management of patients (Alkodsi et al., 2019). Significant efforts have been invested in molecular biology, and gene microarray technology has yielded significant public and invaluable gene expression data sets, and those data can be used for cancer or lymphoma risk stratification and pave the way for accurate disease classification (Li et al., 2017; Tang et al., 2018). To date, various molecular classification systems or mathematical clustering methods have been previously proposed; however, these classification approaches have their limitations and need further improvements. An unsupervised clustering of 2,118 genes' expression analysis performed by Monti et al. identified three distinct subtypes of DLBCL, but the subtypes identified in this study were not associated with prognosis (Monti et al., 2005). By utilizing the method of recursive feature elimination support vector machine, Risueño et al. (2020) identified two subtypes in the GSE10846 dataset. Unfortunately, there is no significant difference in survival between the two subtypes. Karen Dybkaer et al. divided 1,139 samples of DLBCL into four genetic subtypes and evaluated the prognostic difference of those subtypes; it was seen that only the subclass of GCB presented prognostic stratification (Dybkaer et al., 2015). Another clustering methodology utilized by George Wright et al. determined seven subtypes of DLBCL, but the significantly

distinctive outcome was only observed within the ABC subtype (Wright et al., 2020). Alkodsi et al. reported four subtypes of DLBCL by clustering the expression of 36 somatic hypermutation (SHM) genes, and those subtypes had a distinct clinical outcome (Alkodsi et al., 2019). However, the detection of gene SHM is more expensive and complicated than RT-PCR assay, which were limitations to routine clinical application. In this study, we investigated the contribution of CIFs to heterogeneity of distinct prognosis, immunological landscape, chemosensitivity, and biological process in DLBCL and showed two molecular subtypes defined by CIF expression patterns, and our subtypes showed distinctive prognosis within each of the COO subtypes.

It is well known that the presence of immune and inflammatory cells contributes to modulate tumor growth and invasion in DLBCL (Ennishi et al., 2020; Solimando et al., 2020). Characterization of immune infiltration and immune functions between different molecular subtypes provides important insights into the clinical outcome heterogeneity and pathogenesis of DLBCL. The naïve B-cells, memory B-cells, and macrophages in our study were the most represented cell proportions within the microenvironment of DLBCL patients. Normally, naive B-cells experience the germinal center and differentiate into either memory B-cells or plasma cells (for response to infections and secretion of high-affinity antibodies) to play a key role in humoral immunity (Bakhshi and Georgel, 2020). However, malignant transformation of DLBCL forms the mature B cells, which also experienced the germinal center reaction (Pasqualucci and Dalla-Favera, 2018). This transformation may contribute to an excessive consumption of naïve B cells and reduce the production of mature B cells. However, the number of B cells always plays a core role in the immune network and is related to prolonged survival (Bindea et al., 2013), which is consistent with our results. Our analysis revealed that the proportions of naïve B and memory B cells in DLBCL are significantly lower than the normal control group and represented lower fractions in cluster B which was associated with a worse prognosis. GSVA, GO, and KEGG enrichment results showed that cluster B, which had an abundance almost the same as that of immune cells, was strongly associated with cytokine activity and the chemokine pathway. This phenomenon may be related to the fact that immune cells are capable of producing multiple types of cytokines and chemokines (Tamma et al., 2020).

Macrophages, including M1 and M2 types, are more conspicuous than any other immune cell except B cells in DLBCL, and the proportion of M2 type macrophages was higher than that of macrophages M1. M1 macrophages have an antitumor response against neoplastic cells. Conversely, M2 macrophages have a predominant role of promoting tumor growth and progression (Poles et al., 2019). Macrophages usually maintain a balanced state; if macrophages M2 predominate, the balance may shift to a pro-tumor microenvironment (Riihijarvi et al., 2015). CTLA-4 is expressed on regulatory T (Treg) cells and is believed to act as an immune checkpoint receptor, which contributes to the inhibition and exhaustion of T-cells, and has an additional role in promoting the proliferation and survival of B-cell lymphoma (Herrmann et al., 2017). In our study, the number of regulatory T (Treg) cells was higher in cluster B than in cluster A, in line with the expression level of CTLA-4. Aberrant PD-L1 expression also offered a key immune escape mechanism in B-cell lymphoproliferative disorders, and increased PD-L1/PD-1 expression confers an adverse prognosis in DLBCL (Vari et al., 2018). The low overall response rate of anti-PD-1 antibody in DLBCL was attributed, at least to some extent, to the low expression of PD-L1 (Autio et al., 2020). Blockade of the PD1/PD-L1 axis showed particularly potent responses in cHL patients, and an increased expression of PD-L1 was associated with treatment response (Xu-Monette et al., 2018). We found that the expression level of PDL-1 in DLBCL tissue was significantly higher than in normal tissues but significantly lower than in cHL, which may explain why the efficacy of immunotherapy in DLBCL patients is not as good as that in cHL. Meanwhile, DLBCL patients with a higher expression level of PD-L1 seem to show a correlation with an increased resistance to frontline therapy but always related to prolonged PFS if treated with anti-PD-1 antibody (El Hussein et al., 2020; Wang L. et al., 2020). In line with this, cluster B which was associated with worse prognosis showed a higher expression level of CD274/PD-L1 than cluster A. The above-mentioned results suggest that cluster B patients may benefit more from PD-1 blockade therapy than cluster A patients.

Compared with a single mRNA, microRNA, or miRNA, integrating multiple biomarkers into a single signature by LASSO Cox regression could substantially improve the value of prognosis prediction (Zhang et al., 2013). In the present study, we focused on CIFs and developed a seven-CIF-based signature to predict OS in DLBCL. Another interesting aspect of our signature is that it works independently of COO subtyping and all clinical features. Although the potential of a signature based on miRNA expression has previously been reported in the prognostic stratification of DLBCL, but it is limited by a small sample size and lacks an independent cohort to validate its reliability (Montes-Moreno et al., 2011). Investigation of the biological function of the seven CIFs included in our signature has been conducted in previous studies. Interleukin (IL)-4 has been confirmed to be elevated in HL and follicular lymphoma; moreover, IL-4 not only contributes to the abnormal proliferation of lymphoma cells but also prevents malignant lymphocytes from apoptosis (Kawakami et al., 2005; Carey et al., 2007; Calvo et al., 2008). Additionally, IL-17A has been reported to have a role in promoting tumor growth and metastasis, but it also exhibited anti-cancer ability and showed a positive function in improving response to adjuvant chemotherapy in bladder cancer and gastric cancer (Kulig et al., 2016; Wang et al., 2019; Wang Z. et al., 2020). Granulocyte-macrophage colony-stimulating factor 2 (CSF2) one of the sub-members of the CSF family, has the capability of jeopardizing antitumor function and has a positive role in immunosuppression; furthermore, it can also improve antitumor efficacy through modulating the infiltration of immune cells in the tumor microenvironment and is associated with prolonged prognosis (Huang et al., 2020). TGFA has been previously confirmed as a crucial oncogenic mediator and promotes tumor cell growth *via* the TGF-α/EGFR signaling pathway (Wu et al., 2016). TNFSF10 was found to be involved in promoting tumor proliferation in non-Hodgkin's lymphoma by activating the NF-κB pathway (Agrusa et al., 2020).

CCL2 was positively related to TNFSF10 in our study and involved in the proliferation and survival of hematological tumors (Rafei et al., 2011). IL-15 is a proinflammatory cytokine that contributes STAT activation by mediating JAK1 and JAK3 phosphorylation, leading to lymphoma cell growth and survival. Nonetheless, the antitumor capacity of IL-15 by improving NK-cell function on the hematological malignancies has also been documented (Mishra et al., 2014; Mao et al., 2016).

Limitations of the present study should be acknowledged. Firstly, it is a retrospective research instead of a prospective study. Secondly, subtype classification and prognostic signature should be further validated for its efficacy in more independently prospective population. Finally, additional genetic and experimental studies of CIFs are required to elucidate the carcinogenesis and progression mechanism in DLBCL.

CONCLUSION

Our results show that CIFs further contribute to the observed heterogeneity of DLBCL, with specific tumor microenvironment features associated with disease progression and severity. Furthermore, a novel signature based on CIFs was identified and validated in multiple groups of patients, which allows robust risk stratification and may facilitate the implementation of individualized treatment for DLBCL patients with a different prognosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

ZK, JT, and XL contributed to the conception, design, and further drafts. ZK, XL, and SC contributed to the development of methodology, analysis, and interpretation of data. RL, ZK contributed to the construction of figures and writing of the original draft. XL and JT contributed to supervision. All authors reviewed the manuscript and approved the final version to be published.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.648856/full#supplementary-material

Supplementary Figure 1 | Principal component analysis for the expression profiles of 25 cachexia-inducing factors (CIFs) to distinguish diffuse large B-cell lymphoma (DLBCL) from normal samples in GSE12195 and GSE56315 cohorts: **(A,B)**. Two subgroups without intersection were identified, indicating the DLBCL, and normal samples were well distinguished based on the expression profiles of CIFs.

Supplementary Figure 2 | Box plot of expression data before and after normalization. The x-axis presents the different cohorts, and the y-axis presents the expression value. **(A)** Data before and after normalization of the expression profiles of GSE10846 and GSE31312. **(B)** The Venn diagram for intersection of the probe set of GSE10846 and GSE31312. **(C)** Samples distribution of the two cohorts are significantly different before batch correction. **(D)** Samples distribution of the two cohorts after batch correction.

Supplementary Figure 3 | Unsupervised clustering of 25 cachexia-inducing factors in 882 cases of patients with diffuse large B-cell lymphoma (DLBCL) to identify distinct molecular subtypes. **(A–F)** Consensus matrices of the DLBCL cohort for k = 2–7, allowing quick and accurate visualization of cluster boundaries. **(G)** Consensus clustering cumulative distribution function for k = 2 to 9. **(H)** Tracking plot showing the consensus cluster of items (in columns) at k = 2 to 9 (in rows).

Supplementary Figure 4 | Difference in the abundance of immune cell infiltration and expression of Pd-1 among diffuse large B-cell lymphoma (DLBCL), normal B cell, and classic Hodgkin's lymphoma (cHl). **(A,B)** The proportion of immune cell in DLBCL and normal B cell extracted from tonsil: **(A)** GSE56315 and **(B)** GSE12195). **(C)** The proportion of immune cell infiltration and expression level of PD-L1 among DLBCL, normal B cell, and cHL. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, $^{****}P < 0.0001$.

Supplementary Figure 5 | Correlation of immune landscapes and immune cell infiltration. Positive correlation was marked with blue, and negative correlation was marked with yellow.

Supplementary Figure 6 | Differentially expressed genes in cluster B and cluster a patients. **(A)** Heat map for differentially expressed genes in cluster B and cluster a patients. **(B)** Volcano plot of differentially expressed genes in cluster B and cluster a patients. Red, significantly upregulated genes; blue, significantly downregulated genes; Fc, fold change.

Supplementary Figure 7 | Functional enrichment analysis for differentially expressed genes (DEGs) between cluster B and cluster a patients. **(A)** Kyoto Encyclopedia of Genes and Genomes analyses for DEGs. **(B)** Biological process. **(C)** Cellular component. **(D)** Molecular function.

Supplementary Figure 8 | Identification of the risk signature by least absolute shrinkage and selection operator (Lasso) Cox regression. **(A)** Lasso coefficient of the seven cachexia-inducing factors associated with overall survival in univariate Cox regression. **(B)** Ten-fold cross-validation for tuning the parameter selection in the Lasso module.

Supplementary Figure 9 | Performance of the cachexia-inducing factor (CIFs) signature in identifying poor molecular subtypes in the training cohort. **(A)** Comparative risk score between cluster a subtype and cluster B subtype. **(B)** Receiver operating characteristic curves to depict the accuracy of CIFs risk signature in identifying cluster B which was with poor prognosis. **(C)** Alluvial diagram showing the changes of CIFs cluster subtypes, risk, and status.

Supplementary Figure 10 | Performance of combinations of the prognostic model and cell of origin subtype in the prediction of patients with DLBCL in the training cohort and two independent testing cohorts. **(A)** GSE10846 + GSE31312. **(B)** GSE87371. **(C)** GSE32918.

Supplementary Table 1 | Clinical characteristics of the 882 cases of DLBCL patients which was merged by GSE10846 and GSE31312.

Supplementary Table 2 | Prognostic analysis for mutation satatus of 13 CIFs in 48 cases of DLBCL.

Supplementary Table 3 | Spearman correlation analysis of the 25 CIFs.

Supplementary Table 4 | Prognostic analysis of 25 CIFs in 884 cases of DLBCL using a univariate Cox regression model.

Supplementary Table 5 | consensus clustering analysisi results of 884 cases of DLBCL.

Supplementary Table 6 | Estimating relative abundance of tumor microenvironment cells in 884 case of DLBCL patients by the Single-Sample Gene-Set Enrichment Analysis (ssGSEA).

Supplementary Table 7 | 22 kinds of infiltrating immune cell composition which calculate by CIBERSORT analysis.

Supplementary Table 8 | Maximum inhibitory concentration (IC50) for cytotoxicity and Immunomodulator drugs pedicted in 884 cases of DLBCL.

Supplementary Table 9 | The differentially biological pathways between cluster B and cluster A which obtained GSVA enrichment analysis.

Supplementary Table 10 | Functional annotation and KEGG pathway for differentially expressed genes between cluster B and cluster A patients.

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