CANCER PLASTICITY AND THE MICROENVIRONMENT: IMPLICATIONS FOR IMMUNITY AND THERAPY RESPONSE

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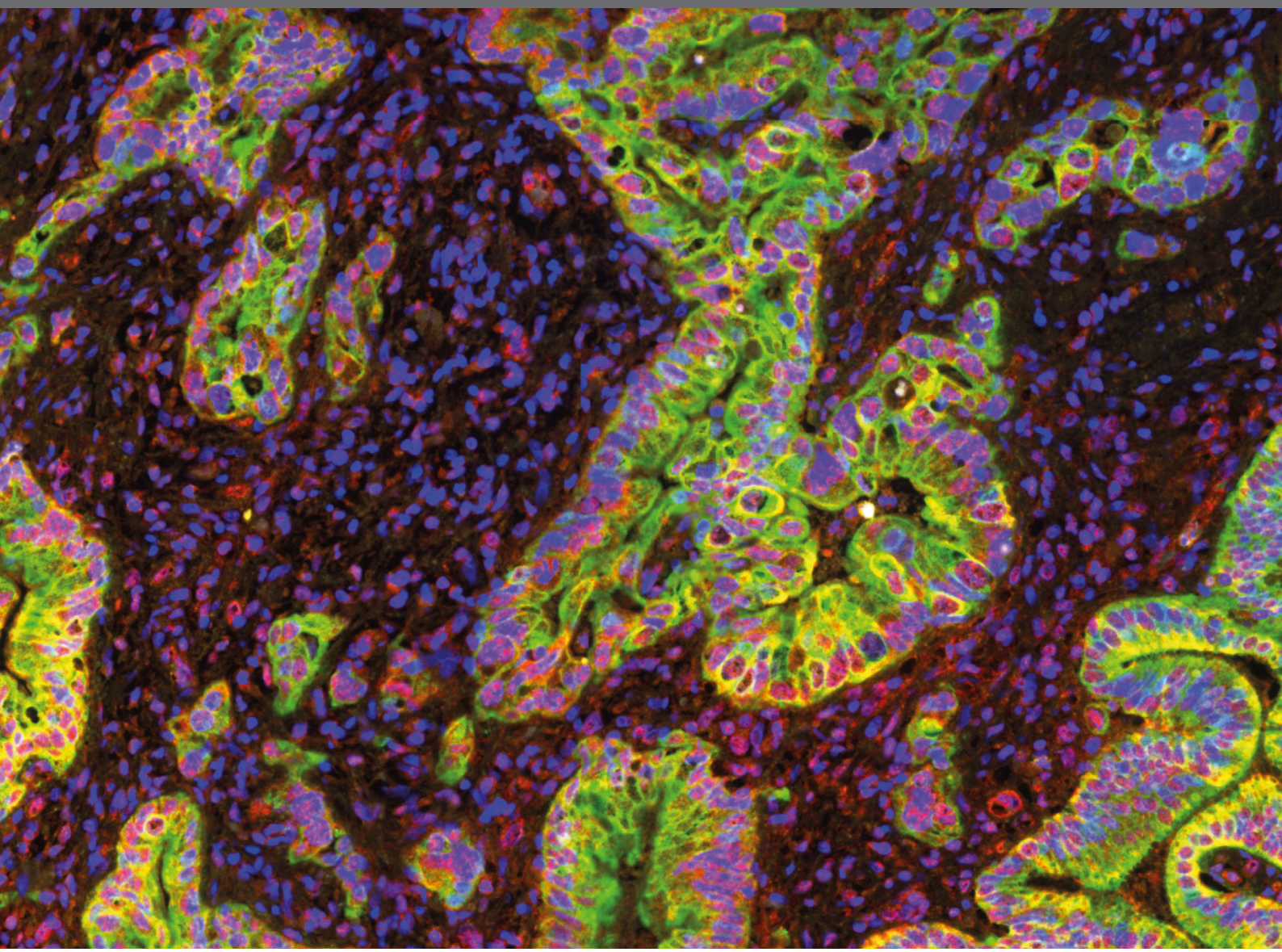

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CANCER PLASTICITY AND THE MICROENVIRONMENT: IMPLICATIONS FOR IMMUNITY AND THERAPY RESPONSE

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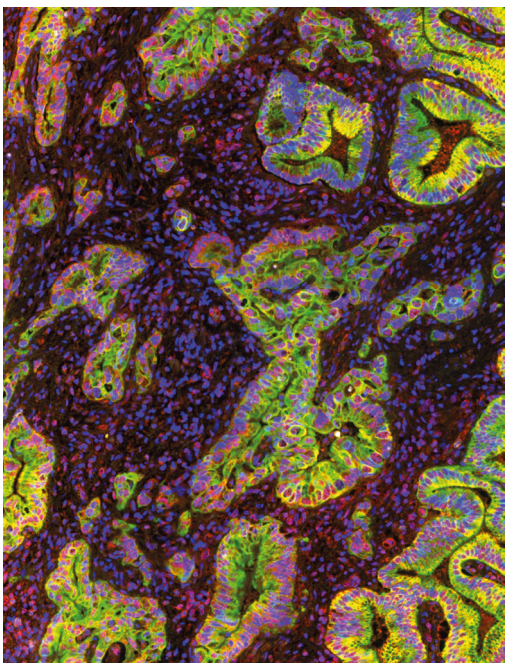

Pancreatic Ductal Adenocarcinoma tumour section labelled with fluorescent markers.
Image by Dannel Yeo, Mehrdad Nikfarjam and Petranel Ferrao

Cancer cells can change and adapt, especially within the host environment; a phenomenon known as cancer plasticity. Several factors, including the immune system can influence, and be influenced by, cancer plasticity which in turn can impact upon patient responses to treatment. As such, we currently face several challenges for implementing combination therapies as effective cancer treatment strategies. We have compiled a topic with a number of articles that emphasize the various aspects of cancer plasticity, describing in particular the important role of

the tumor microenvironment. As we embark on a new era of precision medicine with multi-modal therapies for improving patient outcomes, this topic highlights some relevant points for consideration that are pertinent to the incorporation and effective use of new treatments as part of cancer treatment regimens, including immune-modulating drugs.

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EDITORIAL
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Editorial: Cancer Plasticity and the Microenvironment: Implications for Immunity and Therapy Response

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Keywords: immune evasion, therapy resistance, epithelial-to-mesenchymal transition (EMT), immunotherapy, tumor microenvironment (TME), heterogeneity, cancer plasticity, immune checkpoint inhibitors

Editorial on the Research Topic

Cancer Plasticity and the Microenvironment: Implications for Immunity and Therapy Response

Over the last decade, our understanding of how cancer cells interact with their microenvironment has grown exponentially. It has become evident that a complex interplay exists between malignant cells and benign host cells. The cancer cells and the "normal" cells, especially the immune compartment, undergo constant co-evolution to dynamically "shape" each other. Early attempts to utilize immune cells to favor anti-tumor responses date back more than a hundred years, when William Coley used bacteria to evoke immune responses in cancer patients, however, the results were controversial. Now, in the twenty-first century, "evading the immune system" has been recognized as a key hallmark of cancer (1).

The importance of the immune system in the recognition and clearance of cancer has been illustrated by the unprecedented success of immune checkpoint therapies in the treatment of multiple cancers and the durable responses observed in patients with late-stage disease (2, 3). Presently, clinical success has outpaced scientific understanding despite an ever increasing number of publications on a multitude of factors and pathways contributing to therapy response and resistance. What is clear, however, is that cancer cell plasticity—the ability of cancer cells to alter their phenotype or function—is linked to tumor cell dormancy, tumor progression, metastatic processes and treatment resistance (4). This can affect clonal selection and is implicated in immune evasion, thus influencing response to immunotherapy and patient outcome (5, 6). Cancer plasticity can be induced by a multitude of factors, most notably the tumor microenvironment (TME) (7). In this research topic, we have focused on plasticity within the tumor in the host TME, and various selective pressures that can dictate plasticity, or be influenced by it, particularly treatment responses.

Various features that are components of the TME have been recognized as potential prognostic and predictive biomarkers. The presence of tumor-infiltrating lymphocytes has been shown to correlate with improved survival (8), and this has led to the establishment of the biopsy-based immunoscore for colorectal cancer risk assessment (9). While the main focus of these studies was on the presence of T cells, the impact of several other immune cell types within the tumor microenvironment has also been reported (10). Using a retrospective correlative study of 269 triple-negative breast cancers (TNBCs), Yeong et al. reported that the density of plasma cells within TNBC tumors had a significant association with disease-free survival rates, and high expression of IgG genes was associated with improved survival outcomes. Through analysis of publicly

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available datasets for patients with hepatocellular carcinoma (HCC) and known outcomes, Shrestha et al. assessed immune modulators as potential biomarkers, highlighting that PD-L1 expression was closely associated with epithelial-to-mesenchymal transition (EMT) marker expression and acted as prognostic factor for poor survival in the high-risk patient group.

Tumor cells are known to exert an immune-suppressive or immune-evasive phenotype through various mechanisms, and the crosstalk between TME and tumor is now a major focus of research for understanding and exploiting the critical role of the immune system. Weidenfeld and Barkan highlight the role of EMT and its reverse process, mesenchymal-to-epithelial transition (MET) in tumor dissemination and dormancy. They discuss how EMT may lead to the acquisition of cancer stem cell-like traits in tumors, which can change the immune-regulating properties of these cancer cells, although the effect on immunotherapy outcomes is unclear. Poltavets et al. review how the extracellular matrix (ECM) can influence cancer cell plasticity, discussing the implications for immunotherapy and the potential to exploit targeting ECM regulators as novel therapeutic strategies. Ham et al. present data indicating that exosomes secreted by breast cancer cells can skew macrophage polarization toward a pro-tumoral M2 phenotype partially *via* gp130/STAT3 signaling, suggesting that the exosomes can enhance the immune-suppressive activity of macrophages. Hamilton et al. describe how down-regulation of cyclin-dependent kinase (CDK) inhibitor 1 (p21CIP1) in cancer cells by the transcription factor brachyury leads to less stable CDK1 and renders the tumor cells more resistant to chemotherapy and immune-mediated cytotoxicity.

The authors also speculate that the same mechanism may drive EMT, which in turn may influence tumor immunogenicity.

Heterogeneity and plasticity in the tumor, as well as within the TME, can affect treatment outcomes. This has been exemplified recently by tumors that are responsive/"hot" or resistant/"cold" to immunotherapies. A similar concept regarding therapeutic resistance in melanoma was reviewed by Ahmed and Haass. They highlight that specific gene expression patterns known to dictate cell phenotype and function could also be influenced by the microenvironmental conditions that are selective for subpopulations differing in proliferation rates, invasiveness and drug responsiveness. Tissue-specific TMEs at different anatomical locations can regulate tumor growth, determine metastatic progression and impact on the outcome of therapy responses as reviewed by Oliver et al. suggesting that the organ site of metastasis can also influence immunotherapy outcomes due to differences in the local TMEs.

One of the main mediators of immune stimulation following immunogenic cell death (ICD) is the type I interferons. Budhwani et al. review our current knowledge about how type 1 interferons assist in mounting an immune response, but also note that long term, chronic exposure to type I interferons may diminish the efficacy of radiotherapy and chemotherapy. Conversely, a loss of interferon signaling can lead to resistance to immunotherapy, similar to defects in the IFNγ signaling cascade, which can lead to resistance to anti-PD-1 therapies (11). Alavi et al. demonstrated that only 33% of a large panel of melanoma cell lines with diverse mutational drivers had a strong INFγ response, and displayed induction of all measured targets. The importance of these signaling pathways and the influence of the TME on signaling and gene expression patterns may, at least partially, explain why response to immunotherapy is restricted to subsets of patients.

More recently, additional tumor extrinsic features including the patient's innate immunity, extent and duration of inflammation, balance of the microbiome and even stress levels, are thought to influence patient outcomes. Andrews et al. review various factors that influence the "visibility" of the tumor to the immune system and how they together determine the susceptibility of the tumor to immune attack. They highlight the central role of the gut microbiome in influencing the overall immune set-point (12), through diverse effects on local and systemic inflammatory processes, to influence disease and treatment outcomes. As immune checkpoint blockade treatments are effective only in subsets of patients and can lead to severe immune-related adverse events, it is important to identify which patients are most likely to benefit from these treatments, and also those at risk of developing complications. In order to predict which patients will develop adverse effects to treatment before toxicity becomes clinically evident, Da Gama Duarte et al. reported the potential use of a protein array to capture auto-antibodies from a cohort of melanoma patients treated with immunotherapy.

As a high proportion of patients do not respond to current immunotherapies, novel targets that can enhance immunogenicity could be clinically beneficial. Fan et al. review the potential of retinoic acid receptor-related orphan receptors to directly affect tumor cell behavior, including their ability to directly influence immune cells, given their expression by regulatory T cells and other immune cell subsets. Effective immunotherapy requires cancer cells to be recognized as foreign by the immune system, triggering the initiation of a directed immune-response (13). Many current treatment approaches, either as mono-therapy or combination therapy, aim to initiate a strong and broad immune response that results in long-lasting anti-cancer immunity. Cruickshank et al. review the data showing that immunogenic cell death (ICD) leads to immune stimulation that is epigenetically regulated, and propose that epigenetic drugs like HDAC inhibitors could be used to modify ICD. Poh and Ernst review the role of macrophages, a key component of the TME that orchestrate various aspects of immunity to regulate tumor progression. They discuss the targeting of tumor-associated macrophages (TAMs) as anti-cancer treatment strategies, evaluating the contribution of macrophages in moderating the effectiveness of current therapies and the challenges for successfully incorporating these strategies in cancer treatment regimens.

We currently face several challenges for the clinical implementation of combination therapies as effective treatment strategies for cancer. This second edition of our Research Topic on Cancer Plasticity and the Microenvironment builds upon our first edition, Cellular and Phenotypic Plasticity in Cancer,

by expanding the focus to aspects of immunity and therapy responses across multiple cancer types. Our topic emphasizes various aspects of cancer plasticity that are highly pertinent to the incorporation and effective use of immune-modulating drugs in conventional cancer treatment regimens. As we embark on an exciting and promising era of multi-modal precision therapy, we have highlighted here some relevant points for consideration in our efforts to improve cancer treatment strategies for better patient outcomes.

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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High Densities of Tumor-Associated Plasma Cells Predict Improved Prognosis in Triple Negative Breast Cancer

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Breast cancer is the most common malignancy affecting women, but the heterogeneity of the condition is a significant obstacle to effective treatment. Triple negative breast cancers (TNBCs) do not express HER2 or the receptors for estrogen or progesterone, and so often have a poor prognosis. Tumor-infiltrating T cells have been well-characterized in TNBC, and increased numbers are associated with better outcomes; however, the potential roles of B cells and plasma cells have been large. Here, we conducted a retrospective correlative study on the expression of B cell/plasma cell-related genes, and the abundance and localization of B cells and plasma cells within TNBCs, and clinical outcome. We analyzed 269 TNBC samples and used immunohistochemistry to quantify tumor-infiltrating B cells and plasma cells, coupled with NanoString measurement of expression of immunoglobulin metagenes. Multivariate analysis revealed that patients bearing TNBCs with above-median densities of $CD38^+$ plasma cells had significantly better disease-free survival (DFS) (HR = 0.44; 95% CI 0.26–0.77; $p = 0.004$) but not overall survival (OS), after adjusting for the effects of known prognostic factors. In contrast, TNBCs with higher immunoglobulin gene expression exhibited improved prognosis (OS $p = 0.029$ and DFS $p = 0.005$). The presence of B cells and plasma cells was positively correlated ($p < 0.0001$, $R = 0.558$), while immunoglobulin gene IGKC, IGHM, and IGHG1 mRNA expression correlated specifically with the density of $CD38^+$ plasma cells (IGKC $p < 0.0001$, $R = 0.647$; IGHM $p < 0.0001$, $R = 0.580$; IGHG1 $p < 0.0001$, $R = 0.655$). Interestingly, after adjusting the multivariate analysis for the effect of intratumoral $CD38^+$ plasma cell density, the expression levels of all three genes lost significant prognostic value, suggesting a biologically important role of plasma cells. Last but not least, the addition of intratumoral $CD38^+$ plasma cell density to clinicopathological features significantly increased the prognostic value for both DFS ($\Delta LR\chi^2 = 17.28$, $p = 1.71E{-}08$) and OS ($\Delta LR\chi^2 = 10.03$, $p = 6.32E{-}08$), compared to clinicopathological features alone. The best combination was achieved by integrating intratumoral $CD38^+$ plasma cell density and IGHG1 which conferred the best added prognostic value for DFS ($\Delta LR\chi^2 = 27.38$,

p = 5.22E−10) and OS ($\Delta LR\chi^2$ = 21.29, p = 1.03E−08). Our results demonstrate that the role of plasma cells in TNBC warrants further study to elucidate the relationship between their infiltration of tumors and disease recurrence.

Keywords: triple negative breast cancer, plasma cells, B cells, immunohistochemistry, tumor immunology

INTRODUCTION

Breast cancer is the most common malignancy in women, affecting approximately 12% of females during their lifetimes (1). The disease exists as numerous heterogeneous subtypes, with tumors classified based on histological or molecular characteristics, which result in distinct biological characteristics and clinical prognoses. Triple negative breast cancers (TNBCs), defined by the absence of hormone receptor (estrogen receptor, progesterone receptor) and cerbB2 (HER2) expression, constitute about 15–20% of all breast cancers (2–4). They pose significant management challenges due to the lack of effective treatment options and often exhibit aggressive clinical behavior (5–9). There is an urgent need to better understand the processes driving progression in these tumors, and to define biomarkers that can transcend their heterogeneity and enable optimal and individualized treatment strategies.

Compared to other breast cancer subtypes, TNBCs exhibit more abundant lymphoid cell infiltrates; various components of which have been variably correlated with better or worse prognosis (10–15). More recent studies have revealed the importance of considering the broader picture of immune cell infiltrates: our group showed that the density of $Foxp3^+$ regulatory T cells is closely correlated with the abundance of $CD8^+$ cytotoxic T cells, and that a high frequency of both cell types predicts better disease-free survival (DFS) and overall survival (OS) in TNBC (16); while Bottai et al. demonstrated that the density of total tumor-infiltrating lymphocytes (TILs) had superior prognostic value compared to the density of $CD4^+$, $CD8^+$, or $FOXP3^+$ lymphocytes, or the expression level of PD-1 or LAG-3 on lymphocytes (17). Thus future progress will require us to understand the full suite of immune cells present in the tumor, their relative abundance, and their functional profile, in order to paint a complete picture of tumor-immune interactions and their effects on disease outcome.

Although both humoral and cellular arms of the immune system are involved in the development and progression of tumors (18–22), relatively few studies have focused on the role of tumor-infiltrating B cells in tumorigenesis. Furthermore, the success of monoclonal antibody based immunotherapy indicates the potential for harnessing the humoral immune response in breast cancer treatment (21, 23–28). Some studies found that B cells were present and activated in approximately one quarter of breast tumors, and represented up to 40% of the TIL population in some (29–32); in one study, B cells were detected early during tumor development (33, 34), and expression of the B cell marker CD20 was significantly elevated in TNBC compared to other breast cancer subtypes (35).

Following antigen exposure and T cell licensing, B cells differentiate into potent antibody-secreting plasma cells, which no longer express CD20 (36), but can be distinguished by expression of CD38 (20, 21, 37–39). Tumor-infiltrating plasma cells were first reported in the 1980s (40), but have not been well-studied in TNBC. Some studies suggest that higher frequencies of $CD138^+$ B cells, which might be plasma cells, are linked with poorer recurrence-free survival in breast cancers (41); and breast cancers in which 50% or more of stromal TILs are plasma cells were found to have significantly worse disease-free and OS (42). However, molecular studies draw a contradictory conclusion: high expression of groups of B cell/plasma cell genes (collectively termed "metagenes") has been associated with significantly better prognosis and response to chemotherapy in breast cancer (43–46). In order to make sense of these disparate conclusions, we need to understand the relationship between B cell and plasma cell infiltration in breast cancer, and to examine expression of B cell/plasma cell-related genes in the context of immunohistochemical data.

In this study, we assessed the frequency and localization of B cells and plasma cells within samples from 269 TNBC tumors; we then measured expression of B cell/plasma cell-related genes within matched samples, and asked how these factors were associated with each other and with clinical outcome.

MATERIALS AND METHODS

Patients and Tumors

A total of 269 archival formalin-fixed paraffin-embedded (FFPE) TNBC specimens from patients diagnosed between 2003 and 2010 at the Department of Anatomical Pathology, Division of Pathology, Singapore General Hospital were analyzed. All samples were obtained before patients underwent chemo- or radio-therapy. Clinicopathological parameters, including patient age, tumor size, histologic growth pattern, grade, and subtype, associated with ductal carcinoma *in situ*, lymphovascular invasion, and axillary lymph node status were reviewed (Table S1 in Supplementary Material). The age of patients ranged between 28 and 89 years (median 55 years); length of follow-up ranged from 1 to 213 months (mean 101, median 97), with recurrence and death occurring in 29 and 24% of these women, respectively. Tumors were typed, staged, and graded according to World Health Organization, American Society of Clinical Oncology—College of American Pathologists (ASCO-CAP) guidelines (47). The Centralized Institutional Review Board of SingHealth provided ethical approval for the use of patient materials in this study (CIRB Ref: 2013/664/F and 2015/2199).

Tissue Microarray (TMA) Construction

Tumor regions for TMA construction were selected based on pathological assessment of >50% of the sample being tumor area. For each sample, two or three representative tumor cores of 1 mm diameter were transferred from donor FFPE tissue blocks to recipient TMA blocks using a MTA-1 Manual Tissue Arrayer

(Beecher Instruments, Sun Prairie, WI, USA). TMAs were constructed as previously described (7).

Immunohistochemistry Analysis of TMAs

Tissue microarray sections of 4 μm thickness were incubated with antibodies specific for CD3, CD8, CD20, CD38, and Foxp3, as well as ER, PR, and HER2 (Table S2 in Supplementary Material). We also labeled some sections with antibodies specific for epidermal growth factor receptor (EGFR), cytokeratins (CK) 14, and high molecular weight (clone 34βE12) to identify TNBCs that possess the basal-like phenotype, according to previously published protocols (7, 48). Appropriate positive and negative controls were included. Scoring of antibody-labeled sections was carried out for nuclear ER and PR, membrane HER2 and EGFR, and cytoplasmic CK14 and 34βE12 positivity. To generate the score, images of labeled slides were captured using a ScanScope XT device (Aperio Technologies, Vista, CA, USA) or an IntelliSite Ultra-Fast Scanner (Philips, Eindhoven, Netherlands) before viewing by two pathologists blinded to the clinicopathological and survival information. ASCO-CAP guidelines were used to define positivity cutoffs for the tumors: for ER, PR, CK14, EGFR, and 34βE12, a positive result was defined by the presence of at least 1% of tumor cells displaying any intensity of unequivocal staining, and for HER2, tumor positivity was defined by more than 10% of tumor cells exhibiting 3^+ membrane staining (49). Equivocal HER2 cases were tested and confirmed by fluorescence *in situ* hybridization based on the ASCO/CAP guidelines (50, 51).

Tumor-infiltrating lymphocytes expressing CD20 (B cells) or CD38 (plasma cells) were identified within the stromal and intratumoral regions separately. Plasma cells were presented as the percentage of the intratumoral or stromal areas occupied by the respective cell population, based on published methods (52, 53). Tumors were then divided into "high" and "low" with respect to a particular cell population, when the percentage of the intratumoral or stromal areas occupied by cells labeled for either CD38 (plasma cells) or CD20 (B cells) was above or on/below the median, respectively. Furthermore, cutoff median percentages used were also compatible to the accepted clinical pathological practices: 5% for intratumoral $CD38^+$ plasma cells and $CD20^+$ B cells, and 1% for stromal $CD38^+$ plasma cells and $CD20^+$ B cells.

RNA Extraction, NanoString Measurement of Gene Expression, and Analysis

RNA was extracted from unlabeled FFPE sections of 10 μm thickness using the RNeasy FFPE kit (Qiagen, Hilden, Germany) on a QIAcube automated sample preparation system (Qiagen, Hilden, Germany) and was quantified by an Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). A total of 100 ng of functional RNA (>300 nucleotides) was assayed on the nCounter MAX Analysis System (NanoString Technologies, Seattle, WA, USA). The NanoString counts were normalized using the positive control probes as well as the housekeeping genes, as previously reported (16). The count data were then logarithmically transformed prior to further analysis. p Values <0.05 were deemed to be statistically significant.

Gene Heat Map, Validation, Follow-Up, and Statistical Analysis

Follow-up data were obtained from medical records. DFS and OS were defined as the time from diagnosis to recurrence or death/date of last follow-up, respectively. Statistical analysis was performed using SPSS for Windows, Version 23. The relationship between clinicopathological parameters and the frequency of $CD38^+$ plasma cells and $CD20^+$ B cells was tested using χ^2 and Fisher's exact tests. Survival outcomes were estimated with the Kaplan–Meier analysis and compared between groups with log-rank statistics. Multivariate Cox regression was carried out to evaluate the effect of various tissue compartmentalization of CD38 and CD20 status, as well as NanoString counts of *IGKC*, *IGHM*, and *IGHG1*, with survival after adjusting for clinicopathological parameters, including patient age, tumor size, tumor grade, and lymph node status. Models were compared using the reduction in the log-likelihood of the models ($\Delta LR\chi^2$) using a likelihood ratio test. A p value <0.05 is defined as statistical significant.

RESULTS

High Intratumoral Plasma Cell Density Is Associated With Longer Time to Relapse in TNBC

Previous studies have relied upon CD138 as a plasma cell marker, however, as this molecule is also expressed on some tumor cells, we used CD38 to discriminate plasma cells within tumors (54–57). Our previous study showed that the prognostic value of T cells in breast cancer varied depending on their localization within the tumor (16). In this study, we labeled TNBC sections for CD20 or CD38 and quantified the area of positive labeling within the intratumoral and stromal areas separately. Samples were then grouped according to whether their intratumoral or stromal B cell or plasma cell densities were high (above median), or low (on/below median). Representative images of high and low $CD38^+$ plasma cell and $CD20^+$ B cell TNBC sections are shown in **Figure 1**. Univariate analyses did not reveal any association between the high/low density of B cells or plasma cells in either the intratumoral or stromal regions with clinicopathological features of the TNBC sample cohort (Table S1 in Supplementary Material), and in agreement with our previous study (16). However, there was clear evidence of a significant positive correlation between the densities of intratumoral $CD20^+$ B cells and intratumoral $CD38^+$ plasma cells ($p < 0.0001$, $R = 0.558$) (Table S3 in Supplementary Material).

We then explored the association between plasma cell density of the tumors and the clinical outcomes in TNBC patients. As shown in **Figure 2**, Kaplan–Meier survival analysis revealed significantly better DFS in TNBC patients within the "high intratumoral $CD38^+$ plasma cell" group compared to the "low $CD38^+$ intratumoral plasma cell" group ($p = 0.0006$); while OS was not significantly different between groups ($p = 0.0652$), and the density of stromal plasma cells did not affect survival outcomes (Table S4 in Supplementary Material). Multivariate analysis further supported the fact that high density of intratumoral $CD38^+$ plasma cells in TNBC was associated with a significantly better

DFS (HR = 0.44; 95% CI 0.26–0.77; $p = 0.004$), which was also evident at every 1 percent increment level of CD38$^+$ plasma cell denstity (**Table 1**). In other words, every incremental 1 percent was associated with better DFS (HR = 0.95; 95% CI 0.93–0.98; $p = 0.002$).

Multivariate analysis similarly showed that high intratumoral CD20$^+$ B cell density was associated with better OS (HR = 0.42; 95% CI 0.19–0.97; $p = 0.042$) and DFS (HR = 0.50; 95% CI 0.25–0.99; $p = 0.046$), but these effects were not detectable at 1 percent incremental levels (**Table 1**). Patients with TNBC bearing high densities of CD20$^+$ B cells in stromal regions also showed significantly better OS and DFS (Table S4 in Supplementary Material). However, incremental stromal CD20$^+$ B cells achieved significance for DFS but not OS.

We then asked about the interaction of intratumoral B cell/plasma cell densities in TNBC by comparing survival outcomes between four combinatorial phenotype groups (**Table 2**): "High intratumoral CD20$^+$ B cell and high CD38$^+$ plasma cell TNBCs" ($n = 33$), "High intratumoral CD20$^+$ B cell and low CD38$^+$ plasma cell TNBCs" ($n = 17$), "Low intratumoral CD20$^+$ B cell and high CD38$^+$ plasma cell TNBCs" ($n = 10$), and "Low intratumoral CD20$^+$ B cell and low CD38$^+$ plasma cell TNBCs" ($n = 33$). Multivariate analysis showed that patients having high intratumoral B cell and plasma cell densities had significantly better OS (HR = 0.26; 95% CI 0.09–0.75; $p = 0.013$) and DFS (HR = 0.24; 95% CI 0.09–0.64; $p = 0.004$) compared to patients with low densities of intratumoral B cells and plasma cells (**Table 2**). When considering the same combinatorial phenotypes for the stromal compartment, only "High stromal CD20$^+$ B cells and low CD38$^+$ plasma cells TNBCs" were significantly associated with both OS and DFS (Table S5 in Supplementary Material).

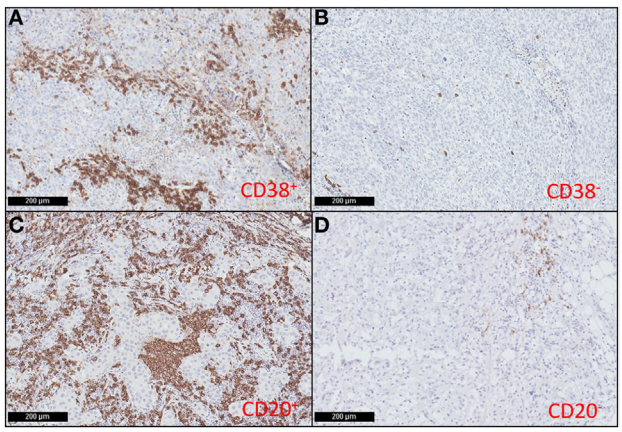

FIGURE 1 | CD38$^+$ plasma cells and CD20$^+$ B cells infiltrate triple negative breast cancers (TNBC). Representative immunohistochemical staining showing high **(A)** and low **(B)** CD38$^+$ plasma cell density; and high **(C)** and low **(D)** CD20$^+$ B cell density in TNBC sections (magnification: 100×).

Intratumoral CD38$^+$ Plasma Cell Density Is an Independent Prognostic Marker in TNBC

We previously reported the prognostic influence of intratumoral CD20$^+$ B cell and CD3$^+$ T cell density in TNBC (16). Therefore, we performed further multivariate analyses on TNBC with high or low intratumoral CD38$^+$ plasma cells, adjusted for the effects of B and T cell density. We found that the density of intratumoral CD38$^+$ plasma cells was an independent prognostic marker for both DFS (HR = 0.28; 95% CI 0.11–0.71; $p = 0.007$) and OS (HR = 0.28; 95% CI 0.10–0.82; $p = 0.020$) (**Table 3**).

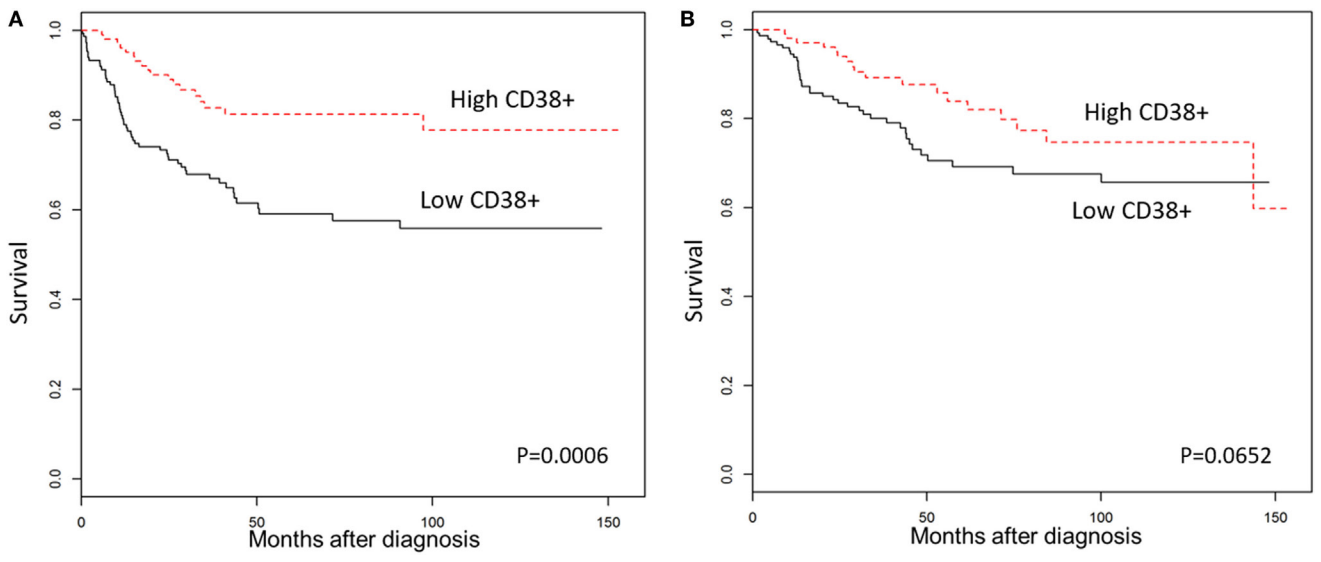

FIGURE 2 | High CD38$^+$ plasma cell density is associated with better survival in triple negative breast cancers (TNBC). Kaplan–Meier analysis of survival outcomes in women with high vs. low densities of intratumoral CD38$^+$ plasma cells. **(A)** Disease-free survival; **(B)** overall survival.

Furthermore, adjusting for the effects of CD20+ B cell density similarly identified intratumoral CD38+ plasma cell density as an independent prognostic marker (Table S6 in Supplementary Material). One percent incremental increases in CD38+ plasma cell density showed significant prognostic value in DFS, but not OS, even after adjustment for the density of CD3+ T cells and CD20+ B cells (**Table 3**), or for CD20+ B cells alone (Table S6 in Supplementary Material). These findings were confirmed using *CD38* gene expression data from a publicly available database [METABRIC, EGAS00001001753 from the European Genome–phenome Archive (58)], which revealed a significant association between increasing *CD38* expression and both DFS (HR = 0.82; 95% CI 0.68–0.97, $p = 0.0229$) and OS (HR = 0.83; 95% CI 0.72–0.97, $p = 0.0191$) in 320 cases of TNBC (**Table 4**). Besides the METABRIC cohort (58) we also analyzed gene expression and survival of TNBCs from The Cancer Genome Atlas (59) which was obtained from cBioPortal (60, 61) for validation purposes, after filtering for TNBC samples. However, CD38 gene expression in this TNBC cohort ($n = 89$) is not a prognostic marker ($p = 0.235$) which might be due to the small sample size.

TABLE 1 | Multivariate analysis of intratumoral CD38+ plasma cell and CD20+ B cell density with survival outcomes in triple negative breast cancer (TNBC) patients.

	Hazard ratio	95% confidence interval	*p* Value
Disease-free survival (DFS)			
Intratumoral CD38+ plasma cell TNBCs High vs. low	0.44	(0.26, 0.77)	0.004*
Intratumoral CD38+ plasma cell TNBCs (every 1 percent)	0.95	(0.93, 0.98)	0.002*
Intratumoral CD20+ B cell TNBCs High vs. low	0.50	(0.25, 0.99)	0.046*
Intratumoral CD20+ B cell TNBCs (every 1 percent)	0.98	(0.95, 1.01)	0.228
Overall survival (OS)			
Intratumoral CD38+ plasma cell TNBCs High vs. low	0.66	(0.36, 1.2)	0.171
Intratumoral CD38+ plasma cell TNBCs (every 1 percent)	0.98	(0.96, 1.01)	0.187
Intratumoral CD20+ B cell TNBCs High vs. low	0.42	(0.19, 0.97)	0.042*
Intratumoral CD20+ B cell TNBCs (every 1 percent)	1.00	(0.96, 1.03)	0.792

Analysis was adjusted for tumor size, grade, age, and lymph node status.
**Statistically significant.*

Higher Expression of IgG Genes Is Associated With Improved Survival Outcomes in TNBC

Several studies have examined the link between expression of a panel of B cell-related genes, termed metagenes, and breast cancer prognosis (43–46). We selected 16 IgG genes from a previously published metagene panel (62), for which the expression was likely to reflect functions of B cells and plasma cells. Expression levels of 9 of the 16 IgG genes were positively correlated with better OS, while expression of 11 of the 16 was correlated with DFS (Table S7 in Supplementary Material). The combination of these 16 IgG genes with *CXCL8* has been suggested as a prognostic marker in breast cancer in general (44, 63); we, therefore, examined expression of this metagene in our TNBC cohort. Unsupervised hierarchical analysis revealed the existence of two clusters of TNBC (**Figure 3**): cluster 1 (red) contained TNBC with higher metagene expression and exhibited significantly better OS ($p = 0.029$) and DFS ($p = 0.005$) than did the low metagene-expressing cluster 2 (blue) (**Figure 4**). Of the IgG genes within the metagene panel, three genes: *IGKC*, *IGHM*, and *IGHG1*, have been reported to pre-dominate in both lymph node-negative breast cancer, and TNBC (43, 64–66). We found that expression levels of these three genes significantly and positively correlated with the abundance of CD38+ plasma cells in our TNBC samples (*IGKC* $p < 0.0001$, $R = 0.647$; *IGHM* $p < 0.0001$, $R = 0.580$; *IGHG1* $p < 0.0001$, $R = 0.655$, Table S3 in Supplementary Material); and were associated with incrementally increasing DFS in multivariate analysis adjusted for tumor size, grade, age, and lymph node status (**Table 5**). Increasing *IGHG1* expression was also associated with better OS (**Table 5**). Interestingly, after adjusting the multivariate analysis for the effect of intratumoral CD38+ plasma cell density, the expression levels of all three genes lost significant prognostic value (Table S8 in Supplementary Material), suggesting a direct role of plasma cells. However, if the analysis was adjusted for intratumoral CD20+ B cell density, some of the genes retained significant prognostic impact (Table S9 in Supplementary Material), such as *IGHM* and *IGHG1*.

TABLE 2 | Multivariate analysis of combinatorial intratumoral B cell/plasma cell density phenotypes with survival outcomes in triple negative breast cancers (TNBC).

Reference to: low intratumoral CD20+ B cell and low intratumoral CD38+ plasma cell TNBCs	*N* number 63	Hazard ratio	95% confidence interval	*p* Value
Disease-free survival (DFS)				
High intratumoral CD20+ B cell and low CD38+ plasma cell TNBCs	17	0.71	(0.29, 1.76)	0.460
Low intratumoral CD20+ B cell and high CD38+ plasma cell TNBCs	10	0.29	(0.07, 1.23)	0.093
High intratumoral CD20+ B cell and high CD38+ plasma cell TNBCs	33	0.24	(0.09, 0.64)	0.004*
Overall survival (OS)				
High intratumoral CD20+ B cell and low CD38+ plasma cell TNBCs	17	0.34	(0.08, 1.48)	0.150
Low intratumoral CD20+ B cell and high CD38+ plasma cell TNBCs	10	0.18	(0.02, 1.36)	0.096
High intratumoral CD20+ B cell and high CD38+ plasma cell TNBCs	33	0.26	(0.09, 0.75)	0.013*

**Statistically significant.*

TABLE 3 | Multivariate analysis showed that high intratumoral CD38+ plasma cell populations are significantly associated with longer disease-free survival (DFS), compared to low intratumoral CD38+ plasma cells populations in triple negative breast cancers (TNBCs), with the median cutoff and with every 1 percent increase.

	Hazard ratio	95% confidence interval	*p* Value
DFS			
Intratumoral CD38+ plasma cells TNBCs High vs. low	0.28	(0.11, 0.71)	0.007*
Intratumoral CD38+ plasma cells TNBCs (every 1 percent)	0.95	(0.91, 1.00)	0.031*
Overall survival (OS)			
Intratumoral CD38+ plasma cells TNBCs High vs. low	0.28	(0.10, 0.82)	0.020*
Intratumoral CD38+ plasma cells TNBCs (every 1 percent)	0.96	(0.91, 1.01)	0.126

Intratumoral CD38+ plasma cells are associated with better OS with the median cutoff (multivariate analysis adjusted for tumor size, grade, age, and lymph node status, intratumoral CD20+ B cells, and CD3+ T cells).
**Statistically significant.*

TABLE 4 | Analysis of *CD38* expression level and survival outcomes in triple negative breast cancers (TNBC) using data from the European Genome–phenome Archive.

	Hazard ratio	95% confidence interval	*p* Value
Disease-free survival (DFS)			
CD38 (every 1 unit)	0.82	(0.68, 0.97)	0.0229*
Overall survival (OS)			
CD38 (every 1 unit)	0.83	(0.72, 0.97)	0.0191*

**Statistically significant.*

Intratumoral CD38+ Plasma Cell Density and IgG Gene Expression Add Significant Prognostic Power to Classical Clinicopathological Parameters

To test the prognostic power of the B cell/plasma cell-related measures reported here, we evaluated the impact of incorporating their effects into survival outcome analysis with a panel of traditional clinicopathological features (patient age, tumor grade, tumor size, and lymph node status). As shown in **Table 6**, the additional assessment of intratumoral CD38+ plasma cell density with clinicopathological features significantly increased the prognostic value for both DFS ($\Delta LR\chi^2 = 17.28$, $p = 1.71E{-}08$) and OS ($\Delta LR\chi^2 = 10.03$, $p = 6.32E{-}08$), compared to clinicopathological features alone. Of the three genes tested (*IGKC*, *IGHM*, and *IGHG1*), *IGHG1* conferred the highest added prognostic value for both DFS ($\Delta LR\chi^2 = 21.99$, $p = 1.88E{-}09$) and OS ($\Delta LR\chi^2 = 16.23$, $p = 3.47E{-}09$). Adding expression level of IgG metagene (Table S7 in Supplementary Material) also increased the prognostic value compared to clinicopathological features alone (DFS: $\Delta LR\chi^2 = 7.74$, $p = 1.43E{-}06$; OS: $\Delta LR\chi^2 = 6.12$, $p = 3.89E{-}07$). The best combination was achieved by integrating intratumoral CD38+ plasma cell density and *IGHG1* which conferred the best added prognostic value for DFS ($\Delta LR\chi^2 = 27.38$, $p = 5.22E{-}10$) and OS ($\Delta LR\chi^2 = 21.29$, $p = 1.03E{-}08$).

DISCUSSION

While the significance of tumor-infiltrating T cells in breast cancer is well accepted, this is the first study to our knowledge, to provide evidence for a critical role of both B cells and plasma cells in TNBC. Here, we demonstrated that intratumoral CD38+ plasma cell density is an independent and incremental prognostic marker, even after adjusting for patient age, tumor grade, tumor size, lymph node status, and the density of tumor-infiltrating CD3+ T cells and CD20+ B cells. We also report that higher expression level of a panel of IgG genes also predicted better clinical outcome in TNBC patients, supporting a previous publication (44).

Much of the work on B cells and plasma cells in breast cancer has been at the molecular level. Some studies provide evidence that high expression of a B cell/plasma cell metagene is associated with favorable prognosis in breast cancers (43–46); others propose that expression level of *IGKC* alone has equivalent predictive and prognostic value (46). In our hand, expression of *IGHG1*, and not *IGKC*, offered the most significant increases in prognostic value compared to classical clinicopathological parameters alone. Intriguingly, expression level of a subset of the 60-gene B cell/plasma cell metagene is associated with worse prognosis in various cancer types that are reported (43, 67, 68), including the finding that expression of *IGHG1* may be linked with tumor cell proliferation and immune evasion in pancreatic, lung, and breast cancer cell lines (69–72). These data may imply that B cells or plasma cells could assume pro-tumoral roles under certain conditions; however, the factors driving the emergence of this putative pathologic phenotype and the roles played by B cells and plasma cells in these circumstances have yet to be revealed. Of note, studying the *IGHG1* expression may also be valuable if the setting allowed molecular testing such as quantitative polymerase chain reaction (**Table 6**). We have performed a parallel set of immunohistochemical stains using CD138 for plasma cells in our cohort. CD38 expression in the plasma cells moderately correlated with CD138 in our patient cohort ($R = 0.39$, $p < 0.0001$). Although CD138 has been proposed to also be a plasma cells marker (73), it is widely known that many types of tumor cells can express CD138 including breast cancer (54–57). Since we also observed CD138 strong staining in tumor cells of our TNBC cohort, we did not pursue it further.

The limited cellular level studies on the roles of B cells and plasma cells in breast cancer have generated similar discordant conclusions: Mohammed et al. used IHC staining to show that a high density of CD38+ lymphocytes predicted worse prognosis, while the density of CD20+ B cells did not significantly affect outcome in primary invasive ductal breast cancer (41). It is possible that in this tumor type, as in esophageal and gastric cancers (74), that immunosuppressive IL10-expressing plasma cells were present and inhibited the anti-tumor T cell response. In this study, we have no evidence of such a pathological role of plasma cells in TNBC. Thus, as suggested by the molecular data, distinct immune mechanisms may be operating in different cancer subtypes and under specific sets of conditions.

The importance of immune parameters in determining prognosis and treatment response across all cancer types has been

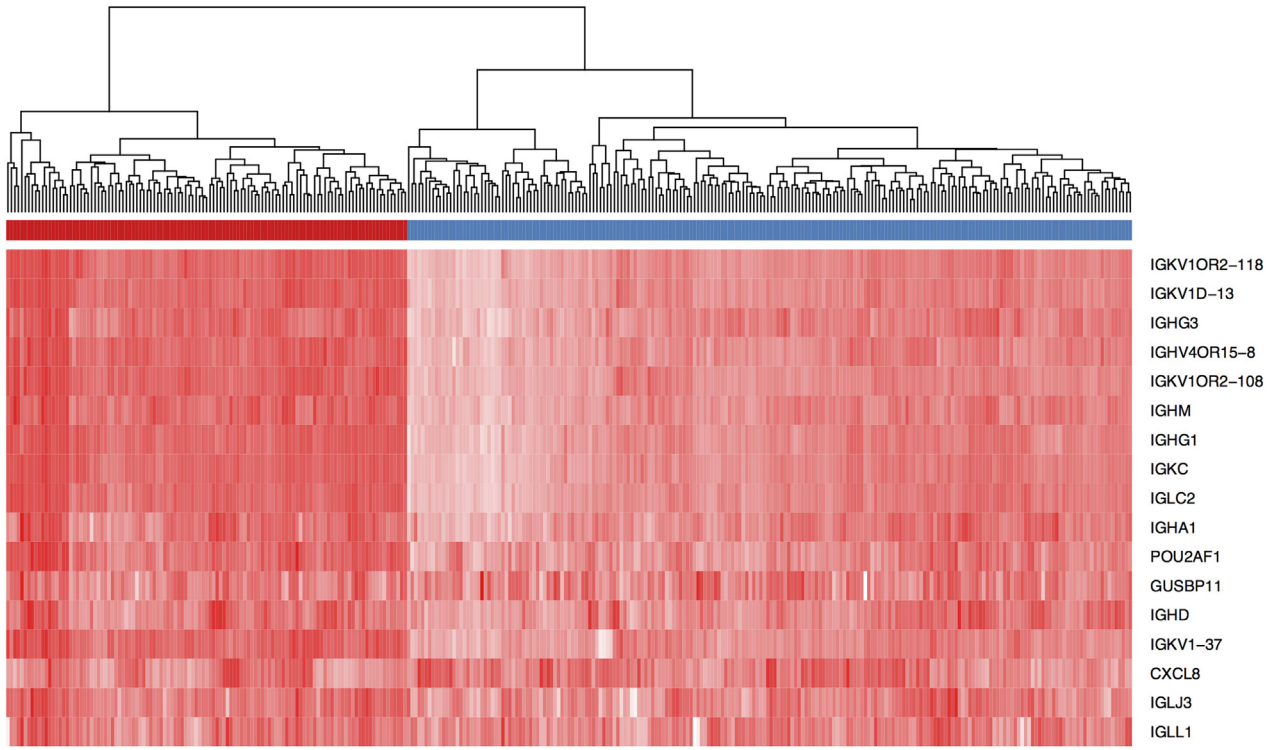

FIGURE 3 | Expression level of a panel of 16 IgG genes and *CXCL8* defines two groups of triple negative breast cancer (TNBC) patients. Unsupervised hierarchical clustering using Euclidean distance revealed the existence of two TNBC patient clusters (red and blue) based on expression intensity of the 17 genes listed. The heat map is colored by the log10 normalized counts with the highest expression in red and the lowest expression in white.

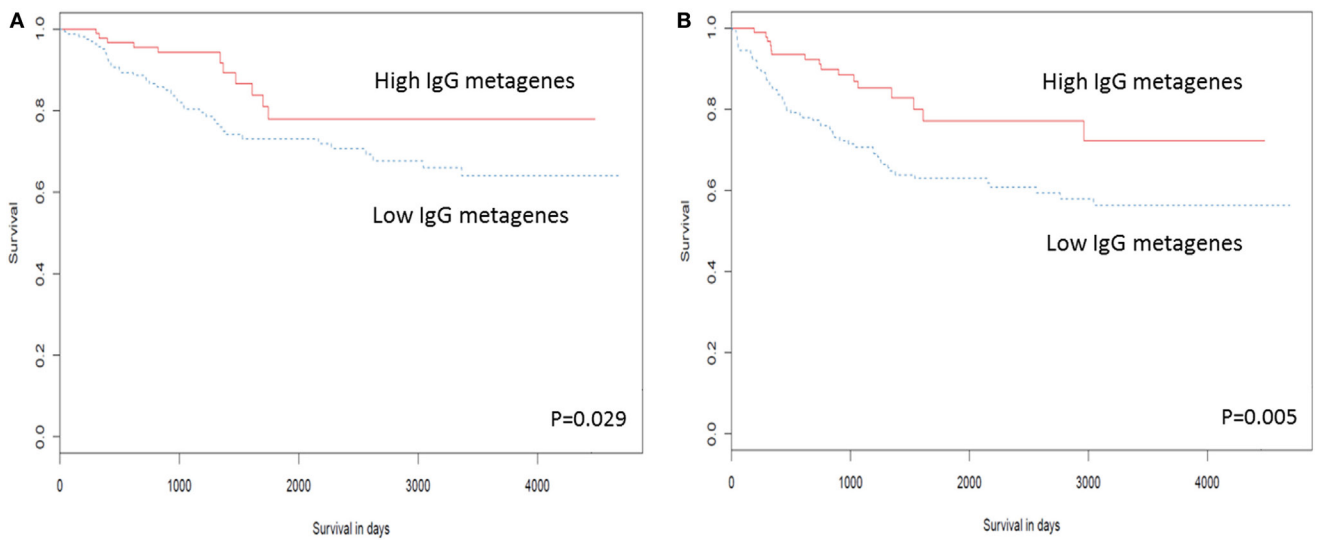

FIGURE 4 | High expression of the IgG metagene is associated with better clinical outcome in triple negative breast cancer (TNBC). Kaplan–Meier analysis of survival outcomes in TNBC in women with high vs. low expression of the IgG metagene, comprising 16 IgG genes and *CXCL8*. **(A)** Disease-free survival; **(B)** overall survival.

recognized in several attempts to incorporate their measurement into routine clinical practice (52, 75). However, these attempts have not been successful; moreover both approaches exclude mention of B cells or plasma cells, likely due to the relative lack of data and integrated studies in this area. Our data argue for a re-appraisal of these guidelines and for more widespread

TABLE 5 | Multivariate analysis of expression level of IgG genes and survival outcomes in TNBC patients (after adjustment for for tumor size, grade, age and lymph node status).

	Hazard ratio	95% confidence interval	*p* Value
Disease-free survival (DFS)			
IGKC (every 1 unit increase of Nanostring count)	0.58	(0.39, 0.88)	0.0103*
IGHM (every 1 unit increase of Nanostring count)	0.60	(0.42, 0.86)	0.0055*
IGHG1 (every 1 unit increase of Nanostring count)	0.66	(0.44, 0.99)	0.0437*
Overall survival (OS)			
IGKC (every 1 unit increase of Nanostring count)	0.66	(0.41, 1.06)	0.0854
IGHM (every 1 unit increase of Nanostring count)	0.65	(0.40, 1.04)	0.0707
IGHG1 (every 1 unit increase of Nanostring count)	0.64	(0.42, 0.97)	0.0340*

**Statistically significant.*

TABLE 6 | Table showing the change in the log-likelihood of the models with added prognostic terms.

Variables	Disease-free survival		Overall survival	
	$\Delta LR\chi^2$	*p* Value	$\Delta LR\chi^2$	*p* Value
CP + iCD38 PC vs. CP	17.28	1.71E−08*	10.03	6.32E−08*
CP + *IGKC* vs. CP	18.45	9.91E−09*	14.26	8.74E−09*
CP + *IGHM* vs. CP	15.44	4.04E−08*	15.14	5.78E−09*
CP + *IGHG1* vs. CP	21.99	1.88E−09*	16.23	3.47E−09*
CP + IgG genes vs. CP	7.74	1.43E−06*	6.12	3.89E−07*
CP + iCD38 PC + *IGHG1* vs. CP	27.38	5.22E−10*	21.29	1.03E−08*

*Statistical significance of the change was determined by a likelihood ratio test. *Statistically significant; CP, clinicopathological parameters (patient age, tumor grade, tumor size, and lymph node status); iCD38 PC, intratumoral CD38+ plasma cell density; IgG genes, expression level of a panel of IgG genes (Table S7 in Supplementary Material); LR, likelihood ratio.*

investigation of the functional and prognostic roles of these cell types and their gene products.

In summary, our results demonstrate that the density of $CD38^+$ plasma cells within TNBC tumors has a significant impact on DFS rate. The prognostic value of plasma cell density is independent of clinicopathological parameters, and of the densities of tumor-infiltrating T cells and B cells. In addition, expression level of the IgG gene, *IGHG1* provided high prognostic value in TNBC for both OS and DFS, representing an easily measurable molecular prognostic marker. The important role of the humoral immune system warrants further studies and may be potentially utilized in routine diagnostic work in addition to its inclusion in cancer immunotherapy.

ETHICS STATEMENT

The Centralized Institutional Review Board of SingHealth provided ethical approval for the use of patient materials in this study (CIRB Ref: 2013/664/F and 2015/2199). In Singapore, a retrospective study is exempted for informed consent.

AUTHOR CONTRIBUTIONS

PT and JI conceived and directed the study. PT, JY, and JI supervised the research. JY interpreted the data and performed biostatistical analysis. JL constructed TMAs, performed IHC, prepared samples for NanoString, and collated data. BL performed bioinformatics analysis. HL and WL performed biostatistical analysis. NC and JI performed immunohistochemical scoring. CO constructed TMAs, performed IHC, and collated data. TP and AT contributed to the scientific content of the study. JY and JI drafted the manuscript with the assistance and final approval of all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01209/full#supplementary-material.

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Monitoring Immune Checkpoint Regulators as Predictive Biomarkers in Hepatocellular Carcinoma

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The global burden of hepatocellular carcinoma (HCC), one of the frequent causes of cancer-related deaths worldwide, is rapidly increasing partly due to the limited treatment options available for this disease and recurrence due to therapy resistance. Immune checkpoint inhibitors that are proved to be beneficial in the treatment of advanced melanoma and other cancer types are currently in clinical trials in HCC. These ongoing trials are testing the efficacy and safety of a few select checkpoints in HCC. Similar to observations in other cancers, these immune checkpoint blockade treatments as monotherapy may benefit only a fraction of HCC patients. Studies that assess the prevalence and distribution of other immune checkpoints/modulatory molecules in HCC have been limited. Moreover, robust predictors to identify which HCC patients will respond to immunotherapy are currently lacking. The objective of this study is to perform a comprehensive evaluation on different immune modulators as predictive biomarkers to monitor HCC patients at high risk for poor prognosis. We screened publically available HCC patient databases for the expression of previously well described immune checkpoint regulators and evaluated the usefulness of these immune modulators to predict high risk, patient overall survival and recurrence. We also identified the immune modulators that synergized with known immune evasion molecules programmed death receptor ligand-1 (PD-L1), programmed cell death protein-1 (PD-1), and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and correlated with worse patient outcomes. We evaluated the association between the expression of epithelial-to-mesenchymal transition (EMT) markers and PD-L1 in HCC patient tumors. We also examined the relationship of tumor mutational burden with HCC patient survival. Notably, expression of immune modulators *B7-H4*, *PD-L2*, *TIM-3*, and *VISTA* were independently associated with worse prognosis, while *B7-H4*, *CD73*, and *VISTA* predicted low recurrence-free survival. Moreover, the prognosis of patients expressing high *PD-L1* with high *B7-H4*, *TIM-3*, *VISTA*, *CD73*, and *PD-L2* expression was significantly worse. Interestingly, *PD-L1* expression in HCC patients in the high-risk group was closely associated with EMT marker expression and prognosticates poor survival. In HCC patients, high tumor mutational burden (TMB) predicted worse patient outcomes than those with low TMB.

Keywords: hepatocellular carcinoma, epithelial-to-mesenchymal transition, immune checkpoints, programmed death receptor ligand-1, immune modulation

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INTRODUCTION

Hepatocellular carcinoma (HCC), also known as malignant hepatoma, is the most common form of primary liver malignancy and the third most common cause of cancer-related deaths worldwide (1–3). It is a multifactorial disease with viral hepatitis and excessive alcohol intake being the major risk factors globally (4). Non-alcoholic fatty liver, diabetes, aflatoxins, and immune-related conditions like autoimmune hepatitis and primary biliary cirrhosis are other common risk factors for HCC (5). HCC is predominant in patients with underlying chronic liver diseases and cirrhosis which limits treatment options for these patients (6, 7). Although surgical resection is useful in the early stages of HCC without cirrhosis recurrence continues to be a significant problem in the majority of patients (8). Liver transplantation, an alternate therapy for unresectable HCC with underlying cirrhosis, has not been very effective due to lack of compatible livers (9). Moreover, HCC is usually diagnosed at late stages such that surgical resections and liver transplantation cannot be used, leading to poor survival rate (10). Sorafenib, the systemic treatment currently approved for the treatment of advanced disease yields a sub-optimal improvement in median survival of 6.5–10.7 months in HCC patients with good liver function (11, 12). Therefore, new therapies are urgently needed for this disease.

Immunotherapy is an emerging therapeutic modality that could become a promising treatment option for HCC as, first, HCC is an inflammation-associated cancer making immunotherapy more likely to be effective (13). Second, the liver is an immune privileged organ, and thus immunotherapeutic drugs are not metabolized in the liver and have predictable pharmacokinetic profiles in cirrhotic patients (13). Third, the liver is tolerogenic to immune response to antigens that is balanced by naïve T-cell activation and further by various immunosuppressive mechanisms, including dysregulation in cytokine secretion, antigen and immune checkpoint expression, and changes in the local immune microenvironment (10, 14, 15). The clinical successes of immunotherapy in the form of immune checkpoint inhibitor (ICI) for the treatment of a number of malignancies including advanced melanoma, have opened prospects for ICIs as the potential immunotherapeutic strategy for treating HCC (16, 17).

The immune response is coordinated by a harmony between co-stimulatory and inhibitory signals (18). The activated T-cell is regulated by co-inhibitory immune checkpoint molecules, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed cell death protein-1 (PD-1), and its ligand programmed death receptor ligand-1 (PD-L1/B7H1/CD274), all of which are responsible for maintenance of self-tolerance and prevent immune overstimulation (13, 18). The T-cell effector functions regulated by the immune checkpoint interactions are generally dysregulated or overexpressed in the tumor microenvironment leading to T-cell inhibition and downregulation of T-cell response. Thus, the blockade of immune checkpoints (co-inhibitory signals) or promotion of co-stimulatory signals can restore or amplify the antigen-specific T-cell responses for cancer therapeutics (18).

A recent phase I/II trial of nivolumab (anti-PD-1) has shown it to have an effective anticancer activity with an adequate safety profile in HCC patients (19). However, in another HCC clinical trial, the use of anti-CTLA-4 antibody in HCC resulted in more adverse events compared to anti PD-1 antibodies (20). Currently, there are several ongoing clinical trials with a small number of ICIs directed at PD-1 (nivolumab and pembrolizumab) and PD-L1 (atezolizumab) in HCC (18, 19). Given that a few genes, such as PD-1, PD-L1, and CTLA-4 enable tumors to bypass the immune system, this strategy alone may not be effective in achieving sustained clinical response in most cancer patients and further immunotherapeutic strategies are needed (21). The identification of predictive markers is of the utmost importance in this clinical setting to select a subgroup of HCC patients who are most likely to benefit from ICI therapy. Furthermore, the morphogenetic process of epithelial-to-mesenchymal transition (EMT) characterized by the acquisition of mesenchymal properties such as invasion and metastasis of tumor cells is closely linked to immune evasion of cancer cells (22, 23). Emerging evidence supports the close association of EMT status with response to multiple immune checkpoint regulators in a large number of patient tumors (24). One such report has revealed that EMT suppresses antitumor immunity through upregulation of PD-L1 in pulmonary cancer (25). However, no studies have compared the EMT markers and immune checkpoint molecule expression in HCC tumors.

With the goal of identifying prognostic immune-related molecules in HCC, we conducted a study of immune-related molecules and correlated their expression with patient prognosis in publically available HCC patient databases by deploying SurvExpress web-based platform that provides risk assessment and survival analysis in cancer datasets (26). We also assessed the relationship between the expression of immune-related molecules and EMT status of HCC cancers using this web-based tool.

MATERIALS AND METHODS

OncoPrint Analysis of Immune Checkpoints Using cBioPortal

We used the cBioPortal's OncoPrint[1] across HCC patient samples to obtain a compact graphical summary of gene expression alterations in immune modulatory genes. We applied cBioPortal to study gene alterations in immune modulatory genes in Liver HCC (TCGA Provisional) case set. Genomic alterations, including copy number alterations (CNAs) (amplifications and homozygous deletions), mutations, and alterations in gene or protein expressions are summarized by glyphs and color coding. All cases are arranged as per alterations (27).

Abbreviations: HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; PD-1, programmed cell death protein 1; PD-L1, programmed death receptor ligand-1; CTLA-4, cytotoxic T lymphocyte-associated antigen-4, EMT, epithelial-to-mesenchymal transition; PI, prognostic index; VTCN1, V-set domain-containing T-cell activation inhibitor 1; BTLA, B and T lymphocyte attenuator; VISTA, V-domain immunoglobulin suppressor of T cell activation; NT5E, ecto-5′-nucleotidase; TIM-3, T-cell immunoglobulin and mucin domain-containing-3.

[1]http://cbioportal.org.

HCC Patient Databases

We used SurvExpress, an online tool with a gene expression database of various cancer types to generate survival and risk assessment analyses of HCC patient datasets.[2] SurvExpress provided six HCC databases, including, Hoshida Golub Liver GSE10143 with 162 patient samples, Hoshida Golub Liver GSE10186 with 118 patient samples, Tsuchiya Rusyn Liver GSE17856 with 95 patient samples, TCGA-Liver-Cancer with 422 patient samples, LIHC-TCGA-Liver HCC June 2016 with 361 patient samples, and Liver HCC TCGA database with 12 patient samples (28–30).

Performing Risk Analysis in HCC Patients

SurvExpress utilized prognostic index (PI) or risk score, the linear part of the Cox model, to generate high-risk and low-risk groups. SurvExpress generates risk groups for risk assessment as previously described (26). Briefly, the first method splits ordered PI into two risk groups with equal number of samples equivalent to splitting the PI by the median (26). The second method uses an optimization algorithm from the ordered PI to produce risk groups (26). A log-rank test is performed along all values of the arranged PI for two groups and the split point where the *p*-value is minimum is selected by the algorithm (26). In case of more than two groups, the procedure optimizes one risk group at a time repeatedly until no changes are seen (26). The gene expression box plots of each gene and risk group are generated by SurvExpress (26).

Validation of the Prognostic Effect of Immune Regulatory Molecules in HCC Patients

Using the SurvExpress online tool, we assessed the gene expression of 19 different immune modulators and analyzed their association with the survival of HCC patients (Cox regression analyses) in five databases (GSE10143, GSE10186, and the three TCGA datasets) with patient survival information. We also assessed the correlation of immune checkpoint molecules with recurrence-free survival in two databases (GSE10143 and TCGA-Liver-Cancer) with patient recurrence-free survival information. For HCC patients, Kaplan–Meier curves were used to estimate the survival times for overall survival and recurrence-free survival. The settings we selected for this study for duplicated genes was average of all probe sets of a gene to compute an average per sample and we used the original quantile-normalized database.

Analysis of Tumor Mutational Burden (TMB) in HCC Patients

Data on the number of mutations per sample were obtained using cBioportal for all HCCs with available survival from the provisional TCGA data set. Tumors were classified as "high mutation burden" if they had a quantity of mutations one standard deviation above the average for the dataset. Kaplan–Meier plots were generated and log-rank tests were used to determine statistical significance.

GeneCards Analysis for Expression of Immune Checkpoints

GeneCards is a database that provides comprehensive information on all annotated and predicted human genes (31).[3] GeneCards online portal was used to study protein expression of immune modulators in normal hepatocytes.

Immunohistochemistry and Pathological Evaluation

Immunohistochemistry was performed as previously described (32). Briefly, paraffin embedded tissue slides with human HCC tissue microarray (TMA) (NBP2-30221, Novus Biologicals) were deparaffinized and rehydrated, endogenous peroxidise activity was blocked with 3% hydrogen peroxide, antigen retrieval was performed in 10 mmol/L citrate buffer, and nonspecific binding was blocked with blocking reagent. HAVCR2 (ab185703, Abcam) and C10ORF54 (CL3975, Invitrogen) antibodies were applied at 1:300 and 1:20 concentrations, respectively. Slides were incubated overnight at 4°C, followed by 30 min incubation with secondary anti-mouse or rabbit antibody HRP (Dako). The chromogen used was 3-amino-9-ethylcarbazole. Human normal and cancerous lung tissue was used as the positive control for both the antibodies and a negative control, for which the primary antibodies were substituted with the same concentration of mouse or rabbit IgG. Images were captured using a Olympus CX41 microscope and QCapture software. Immunohistochemical reactivity was evaluated by two independent investigators. The expression of HAVCR2 and C10ORF54 were categorized into positive staining or no staining.

Statistical Analysis

For risk assessment generated by SurvExpress, a *p*-value of the difference in expression among risk groups is obtained from a Student's *t*-test for two risk groups. A log-rank test was used to produce the concordance index and the *p*-value testing for equality of survival curves for survival analysis using SurvExpres, and the correlation coefficient estimated from deviance residuals (33). In addition, an estimation of the hazard ratio (HR) between the groups is generated. This is estimated by another Cox model using the risk group prediction as the covariate.

RESULTS

The Alterations in Immune Modulatory Genes in HCC

To identify immune modulatory molecules involved in immune escape in HCC, we assessed a panel of 19 genes based on previous studies on immune modulatory genes linked with overall survival and progression-free survival in different cancers. These included those associated with immune stimulatory genes, such as *CD80*, *CD28*, *CD27*, *GITR* (*TNFRSF18*), *Galectin*-9 (*LGALS9*), *CD137* (*TNFRSF9*), *FASLG*, and immune inhibitory genes, such as *TIM3* (*HAVCR2*), *B7-H4* (*VTCN1*), *B7-H3* (*CD276*),

[2]http://bioinformatica.mty.itesm.mx/SurvExpress.

[3]http://www.genecards.org/.

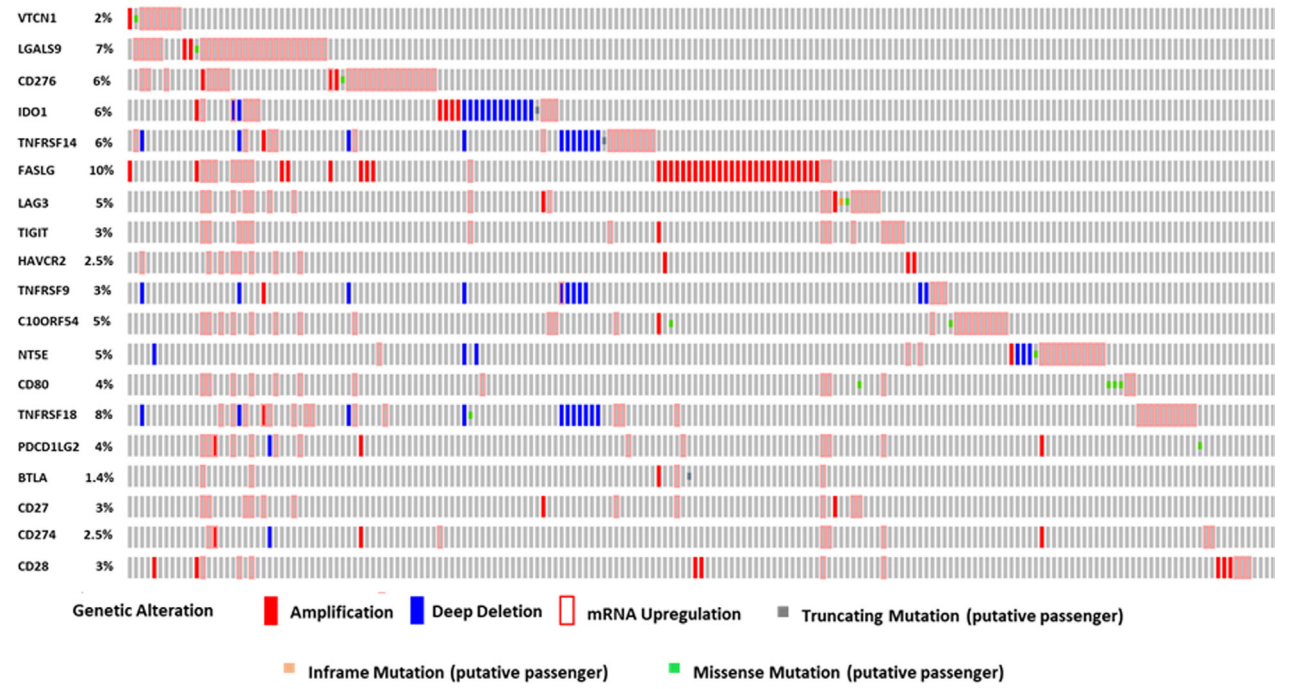

FIGURE 1 | The OncoPrint from a query for alterations in expression of immune modulator genes in HCC patients. Rows and columns represents genes and patients, respectively. Genomic alterations, including CNAs (homozygous deletions and amplifications), mutations, and variation in gene or protein expression are summarized by glyphs and color coding. The cases are sorted as per alterations.

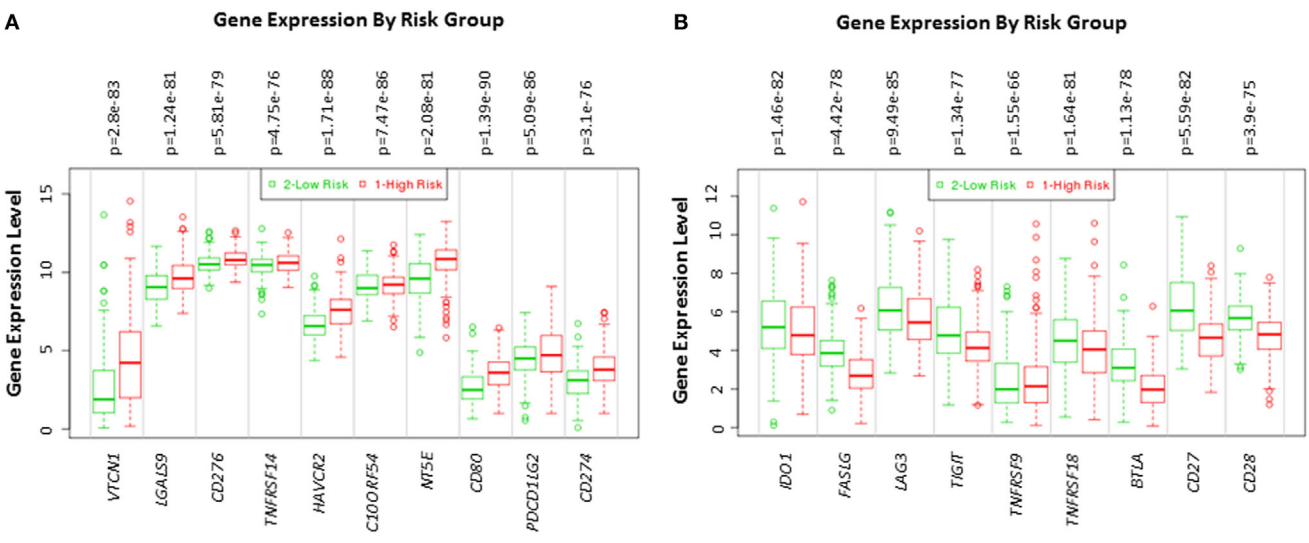

FIGURE 2 | Gene expression of immune modulators in HCC patients based on risk group. Box plot of gene expression of **(A)** immune modulators that statistically correlate with high-risk prognostic score and **(B)** immune modulators that statistically correlates with low-risk prognostic score in 422 HCC patients from the TCGA-Liver Cancer dataset. Risk assessed is risk of reduced survival. Red box represents high-risk group and green box represents low-risk group. Each gene is shown on the *x*-axis. *X*-axis also shows a *p*-value of the expression difference between the two risk groups. The expression levels are shown on the *y*-axis.

B and T lymphocyte attenuator (*BTLA*), *HVEM* (*TNFRSF14*), *PD-L1* (*CD274*), *PD-L2* (*PDCD1LG2*), *LAG-3*, *VISTA* (*C10ORF54*), *CD73* (*NT5E*), *IDO-1*, *TIGIT*.

We performed OncoPrint analysis using cBioPortal to interrogate the expression profiles and any possible genetic alterations for these immune modulatory molecules in tumors

of HCC patients (n = 440). An OncoPrint is a concise and compact graphical summary of genomic alterations in multiple genes across a set of tumor samples. From the OncoPrint, of the 440 HCC cases, amplification and mRNA upregulation were identified in *FASLG*, *TIGIT*, *HAVCR2*, *CD27*, and *CD28* in 45 cases (10%), 15 cases (3%), 11 cases (2.5%), 13 cases (3%), and 15 cases (3%), respectively (**Figure 1**). Amplification, deep deletion, and mRNA upregulation were identified in *TNFRSF9* and *CD274* in 15 cases (3%) and 11 cases (2.5%), respectively.

TABLE 1 | Risk assessment of high versus low-risk.

High expression in high-risk group	Low expression in high-risk group
B7-H4	*IDO-1*
LGALS9	*FASLG*
B7-H3	*LAG-3*
TNFRSF14	*TIGIT*
TIM-3	*TNFRSF9*
VISTA	*TNFRSF18*
NT5E	*BTLA*
CD80	*CD27*
PD-L2	*CD28*
PD-L1	

In **Figure 1**, amplification, mRNA upregulation and missense mutation were noted in 24 cases (5%) for *C10ORF54*, 9 cases (2%) for *VTCN1*, 29 cases (7%) for *LGALS9*, and 26 cases (6%) for *CD276*. Amplification, deep deletion, mRNA upregulation, and missense mutation were identified in *NT5E* [22 cases (5%)], *TNFRSF18* [35 cases (8%)], and *PD-L2* [16 cases (4%)]. Amplification, deep deletion, mRNA upregulation, and truncating mutation were noted in *IDO1* [27 cases (6%)] and *TNFRSF14* [27 cases (6%)]. Furthermore, amplification, mRNA upregulation, inframe mutation, and missense mutation were observed in *LAG3* [20 cases (5%)]. Amplification, mRNA upregulation, and truncating mutation were identified in *BTLA* in 6 cases (1.4%). While 17 cases (4%) for *CD80* showed both mRNA upregulation and missense mutation (**Figure 1**).

Immune Modulatory Genes Are Aberrantly Expressed in Human HCC Tumors

Using SurvExpress we examined transcriptome profiling studies to produce high-risk versus low-risk HCC signatures. Based on transcriptome profiles of the TCGA-Liver-Cancer patient dataset, the clustering analysis differentiated a total of 422 HCC patient

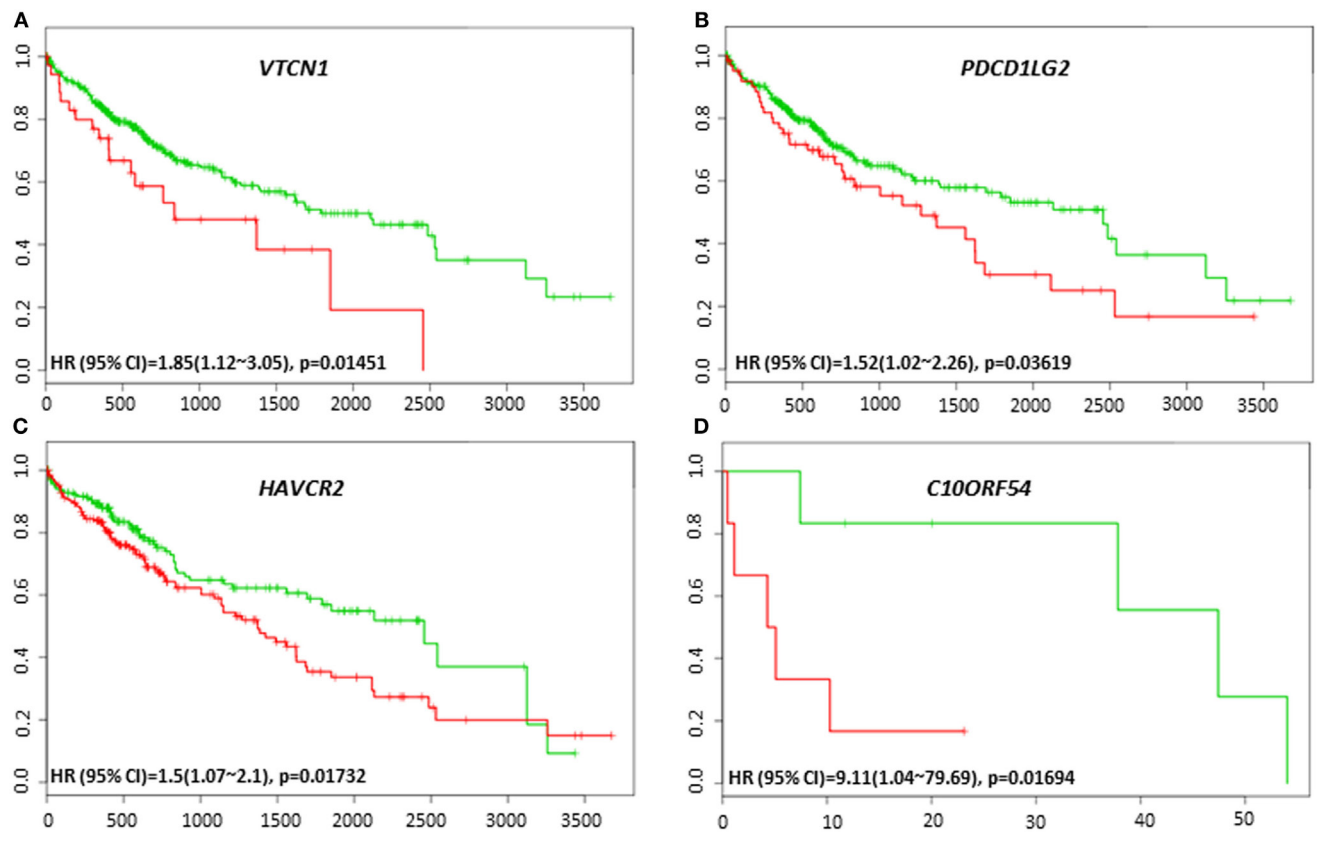

FIGURE 3 | Relationship of immune modulators and survival in HCC patients. Kaplan–Meier survival curves from SurvExpress for the analysis of survival and gene expression of **(A)** *VTCN1*, **(B)** *PDCD1LG2*, **(C)** *HAVCR2*, and **(D)** *C10ORF54* in HCC patient samples. Green curve represents low-risk group while red curve represents high-risk group. The study time (months) is presented in the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

samples into high-risk and low-risk groups. Box plot was generated in the results of SurvExpress, where the gene expression per gene is plotted along its risk groups. This plot is useful to visualize differences in gene expression values between high and low-risk groups.

The expression of *VTCN1*, *LGALS9*, *CD276*, *TNFRSF14*, *HAVCR2*, *C10ORF54*, *NT5E*, *CD80*, *PDCD1LG2*, and *CD274* genes statistically significantly correlates with high-risk signature ($p < 0.05$) (**Figure 2A**). Immune-related genes *IDO-1*, *FASLG*, *LAG-3*, *TIGIT*, *TNFRSF9*, *TNFRSF18*, *BTLA*, *CD27*, and *CD28* expression significantly correlates with low-risk signatures ($p < 0.05$) (**Figure 2B**). Risk assessed in this study was reduced survival. Risk assessment of high versus low-risk for all six HCC patient datasets are depicted in **Table 1**.

Immune Biomarkers Prognosticates Clinical Outcome in HCC Patients

The lack of robust predictive biomarkers to monitor HCC patients at high risk for poor prognosis has been a major obstacle in the clinics. To investigate whether the immune-related genes have prognostic and predictive value in HCC, we utilized six different HCC datasets within SurvExpress to examine the overall survival and recurrence-free survival in HCC patients. Kaplan–Meier survival risk curves for the different immune genes were generated. Notably, altered expression of *VTCN1* [HR: 1.85, 95% confidence interval (CI): 1.12~3.05, Log-Rank Equal Curves $p = 0.01451$] and *PDCD1LG2* (HR: 1.52, 95% CI: 1.02~2.26, Log-Rank Equal Curves $p = 0.03619$) in the TCGA HCC 361 patient cohort was associated with worse overall survival (**Figures 3A,B**). In the TCGA Liver Cancer 422 patient cohort, *HAVCR2* (HR: 1.5, 95% CI: 1.07~2.1, Log-Rank Equal Curves $p = 0.01732$) expression in high-risk group correlated with low overall survival (**Figure 3C**). In TCGA 12 HCC patients, *C10ORF54* expression correlated with worse survival (HR: 9.11, CI = 1.04~79.69, $p = 0.01694$) (**Figure 3D**).

To investigate the possible roles of immune genes in HCC relapse, we assessed the relationships between their gene expression level and recurrence-free survival using SurvExpress. We observed that *VTCN1* expression, which correlated with poor survival was also associated with poor recurrence-free survival in the cohort of TCGA 422 patients (HR: 1.49, CI: 1.04~2.14, Log-Rank Equal Curves $p = 0.03007$) (**Figure 4A**). *C10ORF54* expression also correlated with low recurrence-free survival in the same cohort of 422 patients (HR: 1.44, CI: 1.01~2.06, Log-Rank Equal Curves $p = 0.04327$) (**Figure 4B**). This cohort also showed that

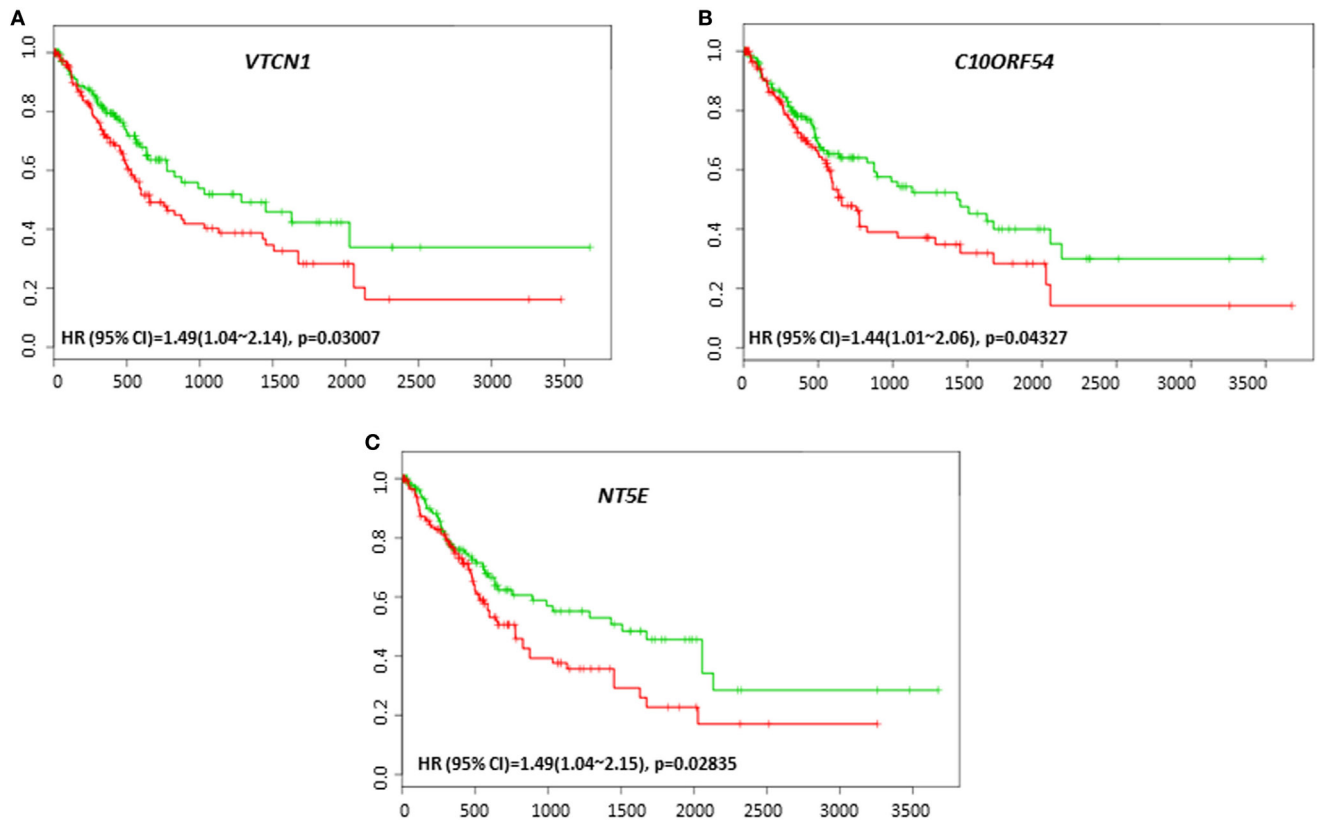

FIGURE 4 | Relationship of immune modulators and recurrence-free survival in HCC patients. Kaplan–Meier curves produced using the SurvExpress for the analysis of recurrence-free survival and gene expression of **(A)** *VTCN1*, **(B)** *C10ORF54*, and **(C)** *NT5E* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented in the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

NT5E expression correlated with poor recurrence-free survival (HR: 1.49, CI: 1.04~2.15, Log-Rank Equal Curves $p = 0.02835$) (**Figure 4C**).

Coordinate Expression of PD-L1 (CD274), PD-1, and CTLA-4 and Immune Modulatory Genes in HCC

The clinical response to anti-PD-L1, anti-PD-1, or anti-CTLA-4 targeted therapies can vary in different tumor types, and much effort has been directed toward finding predictive biomarkers to help identify patients who will derive the most benefit from these therapies. In HCC, the coordinated expression of other immune regulators with PD-L1, PD-1, and CTLA-4 in tumor tissue have been less well-studied. The overall survival and recurrence-free survival of immune modulators were analyzed in combination with PD-L1, PD-1, and CTLA-4 to assess any additional benefit through the combination. *PD-L1*, *PD-1*, or *CTLA-4* gene expression alone did not show poor survival in HCC patient datasets. However, coordinate expression of *VTCN1* (HR: 1.74, CI: 1.09~2.79, Log-Rank Equal Curves $p = 0.01919$), *C10ORF54* (HR: 9.11, CI: 1.04~79.69, Log-Rank Equal Curves $p = 0.01694$), and *HAVCR2* (HR: 1.45, CI: 1.04~2.02, Log-Rank Equal Curves $p = 0.02882$) showed significant overall worse survival when combined with *PD-L1* (CD274) (**Figures 5A–C**). *VTCN1* (HR: 1.54, CI: 1.07~2.21, Log-Rank Equal Curves $p = 0.01806$), *C10ORF54* (HR: 1.55, CI: 1.08~2.23, Log-Rank Equal Curves $p = 0.01703$), *HAVCR2* (HR: 1.47, CI: 1.02~2.11, Log-Rank Equal Curves $p = 0.03486$), *NT5E* (HR:1.55, CI: 1.08~2.22, Log-Rank Equal Curves $p = 0.01657$), and *PDCD1LG2* (HR: 1.67, CI: 1.17~2.4, Log-Rank Equal Curves $p = 0.004591$) showed significant recurrence-free survival benefit when combined with *PD-L1* (**Figures 6A–E**).

Coordinate expression of *VTCN1* (HR: 1.68, CI: 1.19~2.35, Log-Rank Equal Curves $p = 0.002457$), *HAVCR2* (HR: 2.2, CI: 1.54~3.14, Log-Rank Equal Curves $p = 8.04E\text{-}06$), *NT5E* (HR: 1.49, CI: 1.06~2.08, Log-Rank Equal Curves $p = 0.0198$), *LGALS9* (HR: 1.87, CI: 1.33~2.63, Log-Rank Equal Curves $p = 0.0002385$), and *CD80* (HR: 1.64, CI: 1.17~2.31, Log-Rank Equal Curves $p = 0.003752$) showed significant overall worse survival when combined with *PD-1 (PDCD1)* (**Figures 7A–E**). In combination with *PD-1*, *VTCN1* (HR: 1.67, CI: 1.16~2.41,

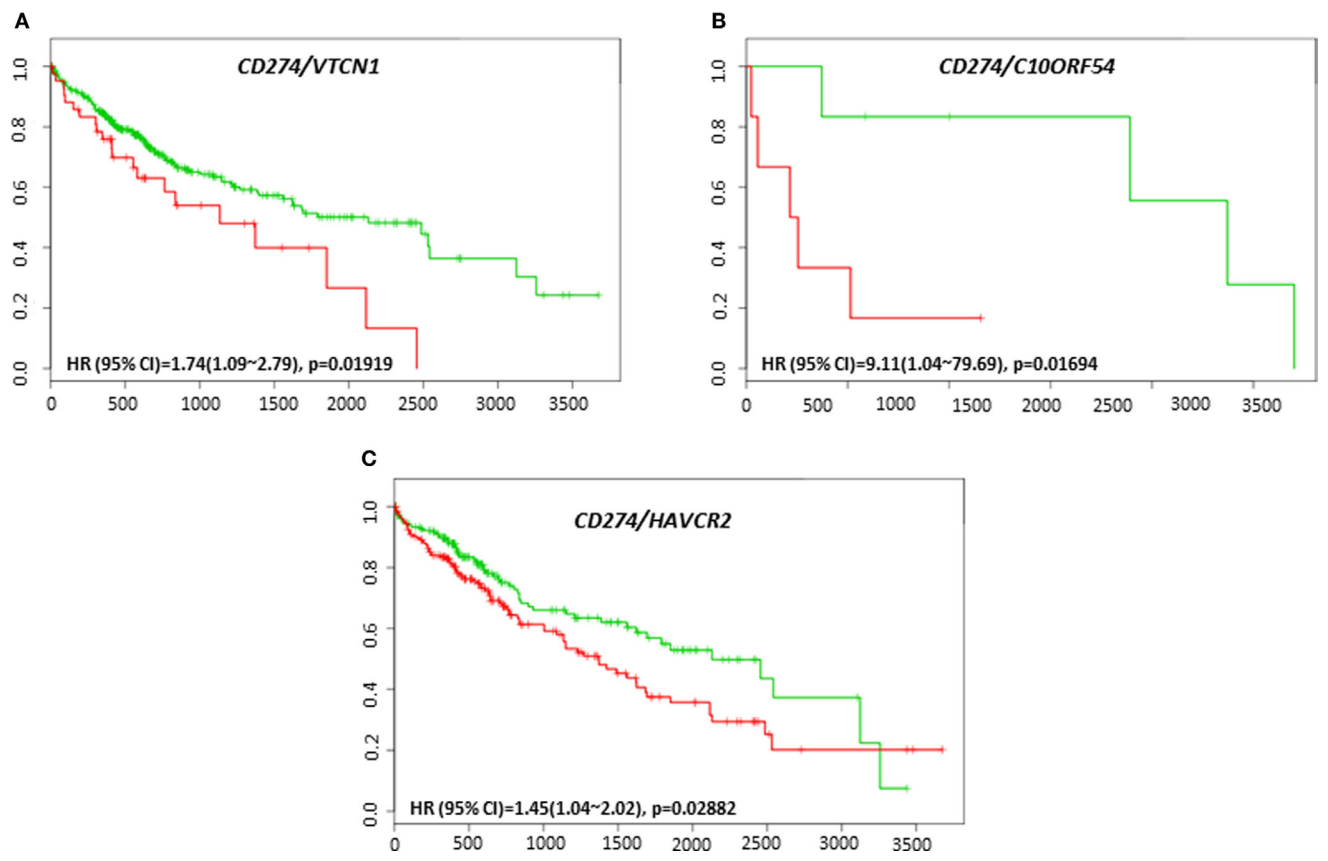

FIGURE 5 | Relationship of immune modulators in combination with PD-L1 (CD274) and survival in HCC patients. Kaplan–Meier survival curves generated using the SurvExpress for the analysis of survival and gene expression of **(A)** *CD274/VTCN1*, **(B)** *CD274/C10ORF54*, and **(C)** *CD274/HAVCR2* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented in the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

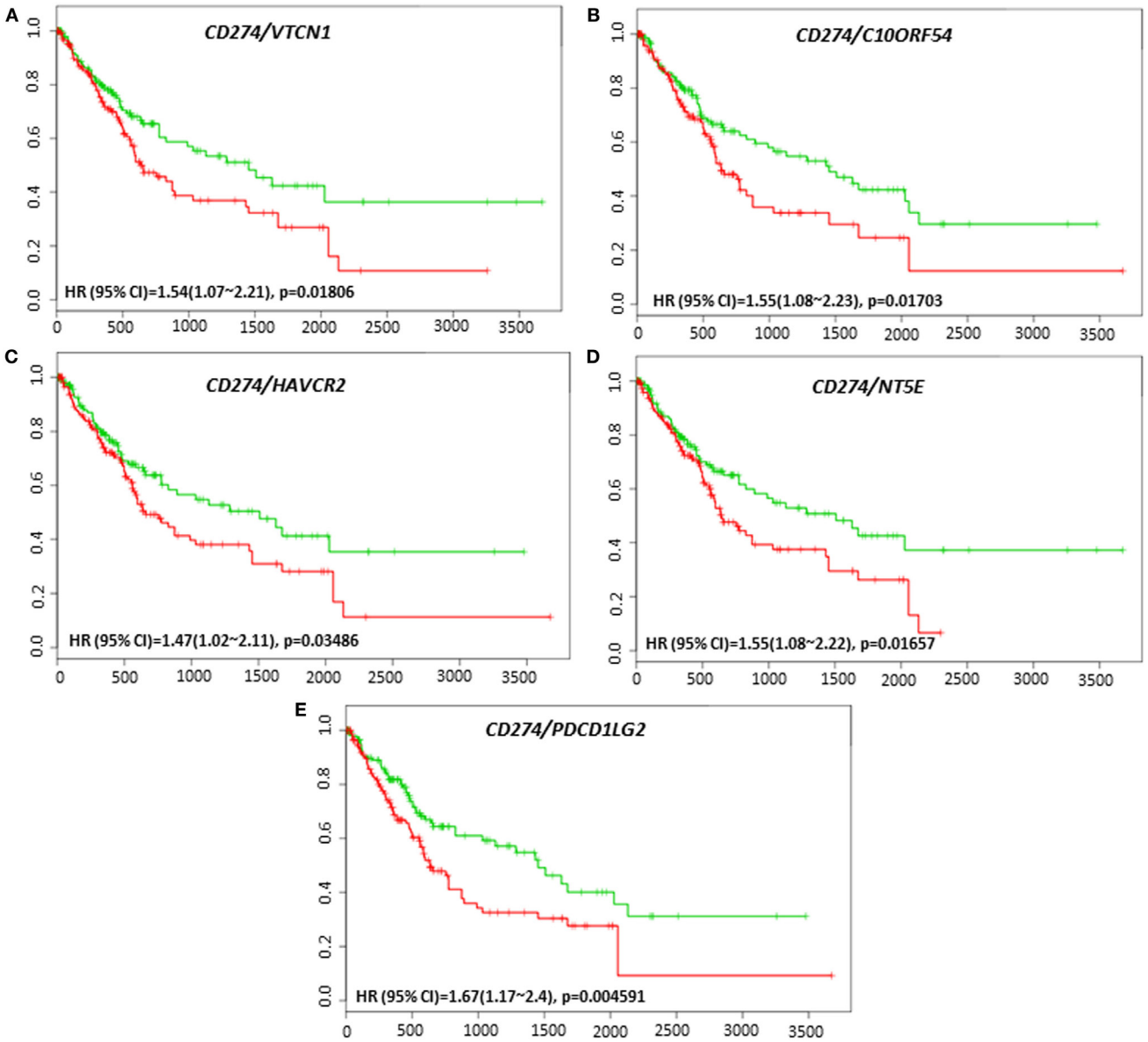

FIGURE 6 | Relationship of immune modulators in combination with PD-L1 (CD274) and recurrence-free survival in HCC patients. Kaplan–Meier curves produced using the SurvExpress for the analysis of recurrence-free survival and gene expression of **(A)** *CD274/VTCN1*, **(B)** *CD274/C10ORF54*, **(C)** *CD274/HAVCR2*, **(D)** *CD274/NT5E*, and **(E)** *CD274/PDCD1LG2* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented in the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

Log-Rank Equal Curves $p = 0.004838$), *C10ORF54* (HR: 1.73, CI: 1.21~2.49, Log-Rank Equal Curves $p = 0.002575$), *HAVCR2* (HR: 1.56, CI: 1.08~2.24, Log-Rank Equal Curves $p = 0.01547$), *TNFRSF14* (HR:1.56, CI: 1.09~2.24, Log-Rank Equal Curves $p = 0.01349$), and *CD80* (HR: 1.53, CI: 1.07~2.19, Log-Rank Equal Curves $p = 0.01881$) showed significant recurrence-free survival (**Figures 8A–E**).

VTCN1 (HR: 1.51, CI: 1.08~2.12, Log-Rank Equal Curves $p = 0.01558$), *HAVCR2* (HR: 1.79, CI: 1.26~2.53, Log-Rank Equal Curves $p = 0.0008991$), *LGALS9* (HR: 1.59, CI: 1.13~2.23, Log-Rank Equal Curves $p = 0.006334$), and *TNFRSF14* (HR: 1.5, CI: 1.07~2.1, Log-Rank Equal Curves $p = 0.01669$) showed significant overall worse survival when combined with *CTLA-4* (**Figures 9A–D**). Coordinate expression of *CTLA-4* with *VTCN1* (HR: 1.89, CI: 1.31~2.72, Log-Rank Equal Curves $p = 0.0004903$), *C10ORF54* (HR: 1.6, CI: 1.11~2.3, Log-Rank Equal Curves $p = 0.01011$), *NT5E* (HR: 1.52, CI: 1.06~2.18, Log-Rank Equal Curves $p = 0.02093$), *HAVCR2* (HR:1.7, CI: 1.18~2.43, Log-Rank Equal Curves $p = 0.003638$), *LGALS9* (HR: 1.59, CI: 1.13~2.23, Log-Rank Equal Curves $p = 0.006334$), and

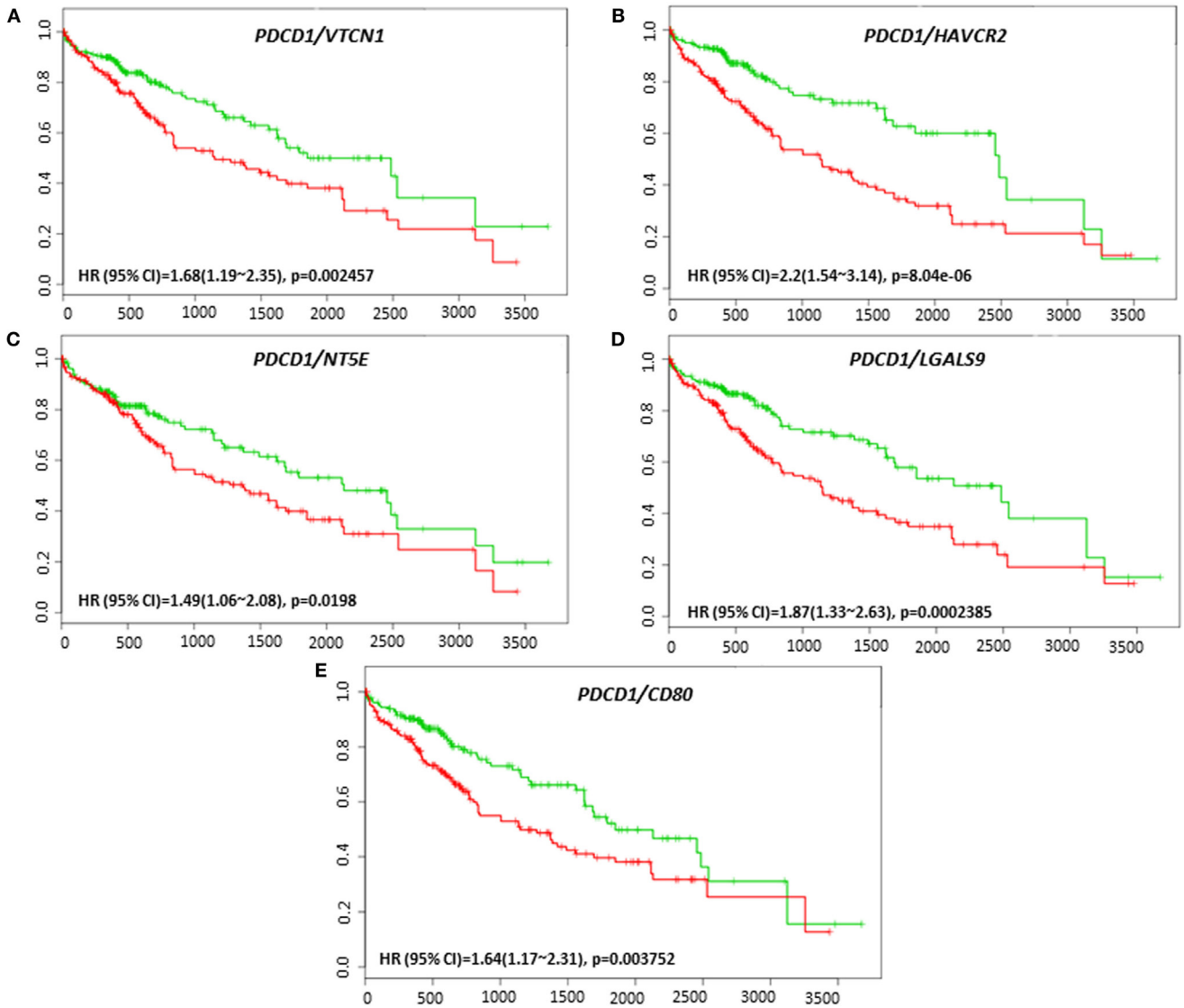

FIGURE 7 | Relationship of immune modulators in combination with PDCD1 and survival in HCC patients. Kaplan–Meier survival curves generated using the SurvExpress for the analysis of survival and gene expression of **(A)** *PDCD1/VTCN1*, **(B)** *PDCD1/HAVCR2*, **(C)** *PDCD1/NT5E*, **(D)** *PDCD1/LGALS9*, and **(E)** *PDCD1/CD80* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented on the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

TNFRSF14 (HR: 1.45, CI: 1.01~2.08, Log-Rank Equal Curves $p = 0.04071$) showed significant recurrence-free survival (**Figures 10A–F**).

HAVCR2 and C10ORF54 Is Expressed in HCC Patient Tumors

We next validated the protein expression of HAVCR2 and C10ORF54, two immune markers associated with poor survival in HCC patients in combination with either PD-L1, PD-1, or CTLA-4. Protein expression patterns in HCC tumors were determined by immunohistochemical staining of a TMA comprising of tumors from 36 patients with stage I, II, III, IIIB, IVA, and IVB HCC. HAVCR2 expression was detected in 88% of HCC patient tumors (**Figures 11A,B**). The subcellular location was identified as predominantly cytoplasmic and membranous. C10ORF54 expression was detected in 91% of HCC patient tumors (**Figures 11C,D**). The subcellular location was identified as predominantly cytoplasmic.

Expression of PD-L1 in HCC Tumors Is Correlated With an EMT Phenotype

EMT is an important biological process involved in the progression and immune evasion of cancers. In HCC, EMT contributes to a poor prognosis (34, 35). Emerging research

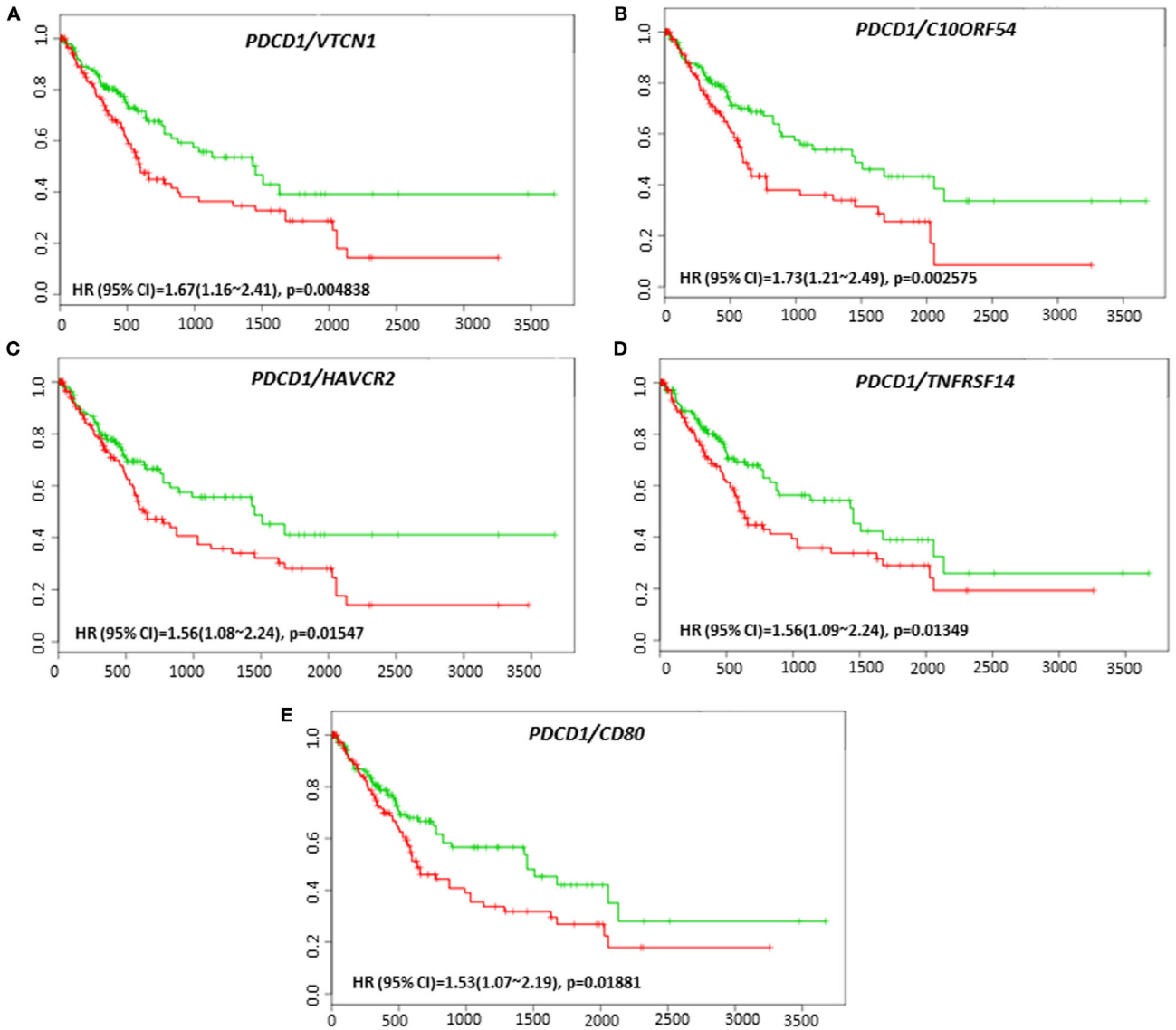

FIGURE 8 | Relationship of immune modulators in combination with PDCD1 and recurrence-free survival in HCC patients. Kaplan–Meier curves produced using the SurvExpress for the analysis of recurrence-free survival and gene expression of **(A)** *PDCD1/VTCN1*, **(B)** *PDCD1/C10ORF54*, **(C)** *PDCD1/HAVCR2*, **(D)** *PDCD1/TNFRSF14*, and **(E)** *PDCD1/CD80* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented on the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

has found higher expression of PD-L1 in mesenchymal cells in non-small cell lung carcinoma (36). Therefore, we examined the relationship between the EMT phenotype and PD-L1 expression in HCC. By analyzing risk assessment using the TCGA-Liver-Cancer patient dataset (422 HCC patient samples) we confirmed that high expression of *PD-L1* and mesenchymal marker *VIM* and low expression of epithelial marker *CDH1* genes significantly associated with a high-risk signature ($p < 0.05$) (**Figure 12A**).

Although *PD-L1* gene expression alone did not significantly correlate with poor survival in HCC patient datasets, coordinate expression of *CDH1* and *VIM* showed worse overall survival (HR: 1.85, CI: 1.05~2.05, Log-Rank Equal Curves $p = 0.02543$) and recurrence-free survival (HR: 1.72, CI: 1.2~2.48, Log-Rank Equal Curves $p = 0.003402$) when combined with *PD-L1* (**Figures 12B,C**). This study shows that high expression of PD-L1 in HCC patients is associated with an EMT phenotype.

Protein Expression in Normal Hepatocytes

GeneCards online portal was utilized to select tumor-associated immune regulatory genes with minimal or no expression in normal tissue and overexpression in HCC tumor cells. GeneCards online portal was used to study protein expression of immune modulators in normal hepatocytes (**Table 2**). The majority of

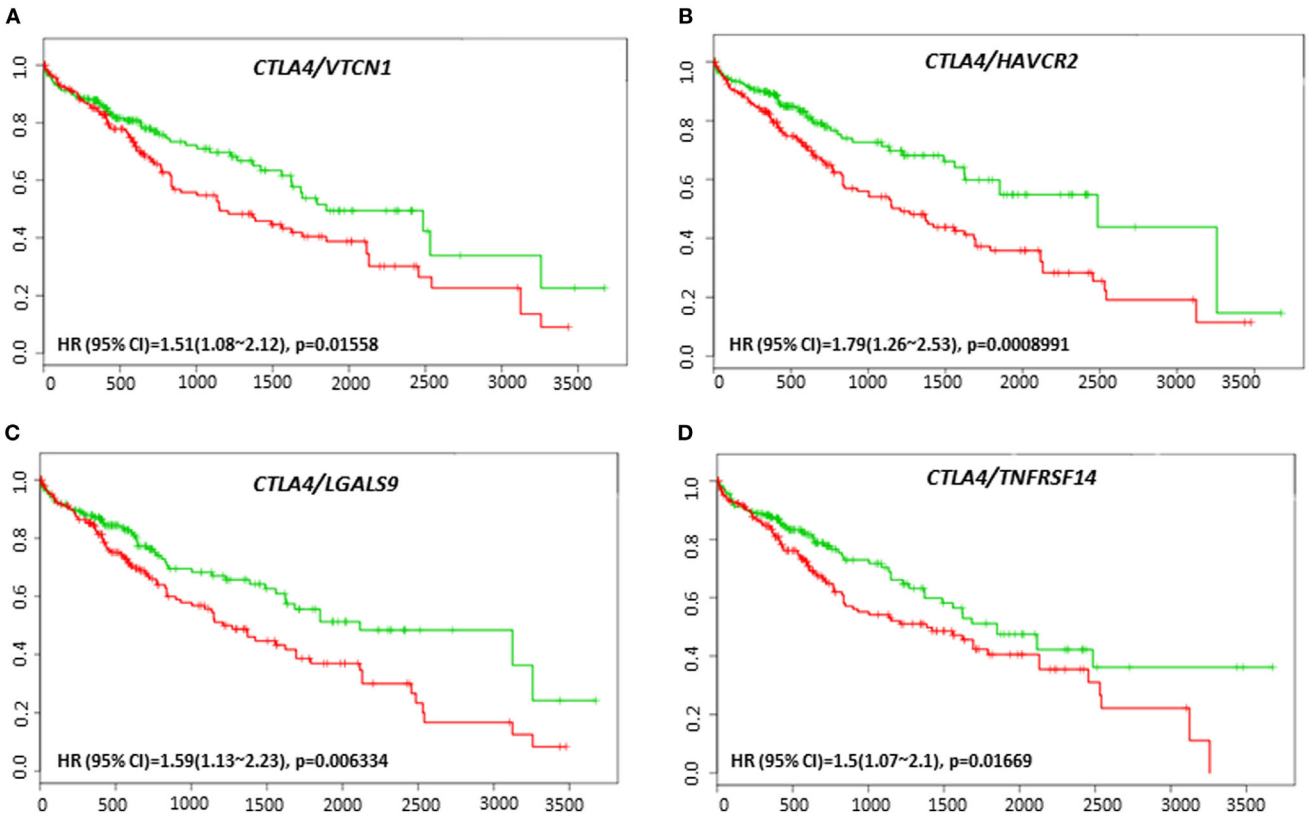

FIGURE 9 | Relationship of immune modulators in combination with CTLA-4 and survival in HCC patients. Kaplan–Meier survival curves generated using the SurvExpress for the analysis of survival and gene expression of **(A)** *CTLA-4/VTCN1*, **(B)** *CTLA-4/HAVCR2*, **(C)** *CTLA-4/LGALS9*, and **(D)** *CTLA-4/TNFRSF14* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented on the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

immune modulators are not expressed in normal hepatocytes. Some of the immune modulators including Galectin-9 and B7H3 have low protein expression in normal hepatocytes, while *LAG-3* and *CD73* showed low mRNA expression in normal hepatocytes. This data indicate that these biomarkers may be specifically expressed in HCC tumors and not in normal healthy cells but may be targeted safely.

TMB in HCC Patients

TMB or mutation load is the total number of mutations present in a tumor specimen. TMB is emerging as a reliable biomarker for predicting sensitivity to ICIs as immune checkpoint marker testing alone has proven insufficient in many cancers for patient selection (37). In non-small cell lung cancer and melanoma, high TMB has been associated with a higher likelihood of tumor responsiveness to treatment with PD-1 or PD-L1 immunotherapy strategies (38, 39). However, the value of TMB as an objective biomarker in the setting of HCC has not been explored. We sought to determine whether TMB could be associated with overall survival and progression-free survival in HCC patients. Patients with a high TMB had significantly poor overall survival and progression-free survival than those with a lower TMB (**Figures 13A,B**). As TMB-high cancers are likely to harbor neoantigens, making them targets of immune cells, utilizing TMB as a biomarker may help select HCC patients for ICI blockade therapy.

DISCUSSION

Implementation of immune regulatory drugs such as ICIs has elicited a remarkable clinical response and is becoming the new foundation for treatment of various malignancies. Currently, immunotherapy in the form of ICI may represent an effective treatment modality for HCC, mainly for advanced and recurrent HCC where no other effective options are available. This study identified many immune regulatory genes that were aberrantly expressed in HCC patient tumors. Immune regulatory genes *VTCN1*, *PDCD1LG2*, *HAVCR2*, and *C10ORF54* were associated with overall poor survival and *VTCN1*, *C10ORF54*, and *NT5E* predicted recurrence-free survival in HCC patients. *VTCN1*, *C10ORF54*, *HAVCR2*, *NT5E*, and *PDCDLG2* in combination with PD-L1 functioned as robust markers that could prognosticate poor prognosis in these patients.

Identifying robust predictive immune biomarkers as useful tools to monitor patients at high risk for poor prognosis and to predict response to the ICI in patients is becoming popular by study of tumor immune

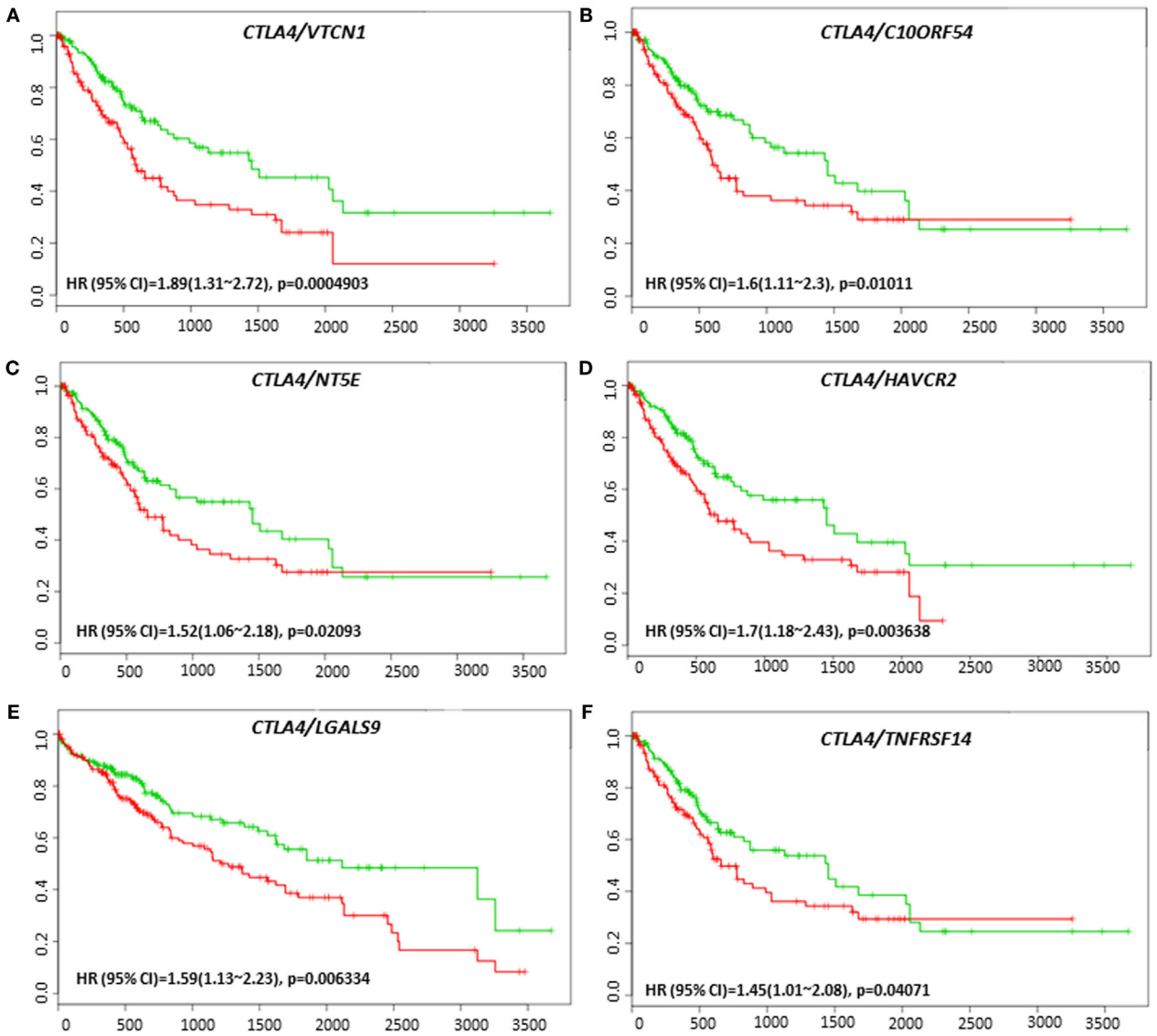

FIGURE 10 | Relationship of immune modulators in combination with CTLA-4 and recurrence-free survival in HCC patients. Kaplan–Meier curves produced using the SurvExpress for the analysis of recurrence-free survival and gene expression of **(A)** *CTLA-4/VTCN1*, **(B)** *CTLA-4/C10ORF54*, **(C)** *CTLA-4/NT5E*, **(D)** *CTLA-4/HAVCR2*, **(E)** *CTLA-4/LGALS9*, and **(F)** *CTLA-4/TNFRSF14* in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented on the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

microenvironment. For instance, PD-L1 expression in tumors has been shown to be a predictive biomarker for poor prognosis and is also utilized as an important biomarker to predict the response to anti-PD-1 antibodies (40, 41). These findings support the relevance of immune regulatory molecules as biomarkers in the clinics. Given that only a subset of patients express PD-L1, and the majority of patients fail to demonstrate durable response and expression level of PD-L1 can fluctuate throughout the course of treatment; identifying other immune biomarkers could play an important role to further improve patient outcome. Based on immune biomarker expression, therapies will need to be employed on an individualized basis to ensure the best possible responses.

We found the negative regulator of T-cell response, V-set domain-containing T-cell activation inhibitor 1, VTCN1, (also named as B7-H4, B7S1, or B7x) was aberrantly expressed in HCC patients in the high-risk group and B7-H4 positivity was a statistically significant predictor of poor overall survival and recurrence-free survival. Studies have confirmed the high expression of B7-H4 in a variety of human tumors, including HCC (42, 43). In another study, soluble B7-H4 detected in blood samples from HCC patients was closely associated with advanced tumor stage, poor overall survival, and higher recurrence rate (44, 45). However, the function of B7-H4 in HCC tumors remains unknown. B7-H4 has been previously proposed to function as a ligand for BTLA (also known as CD272), an

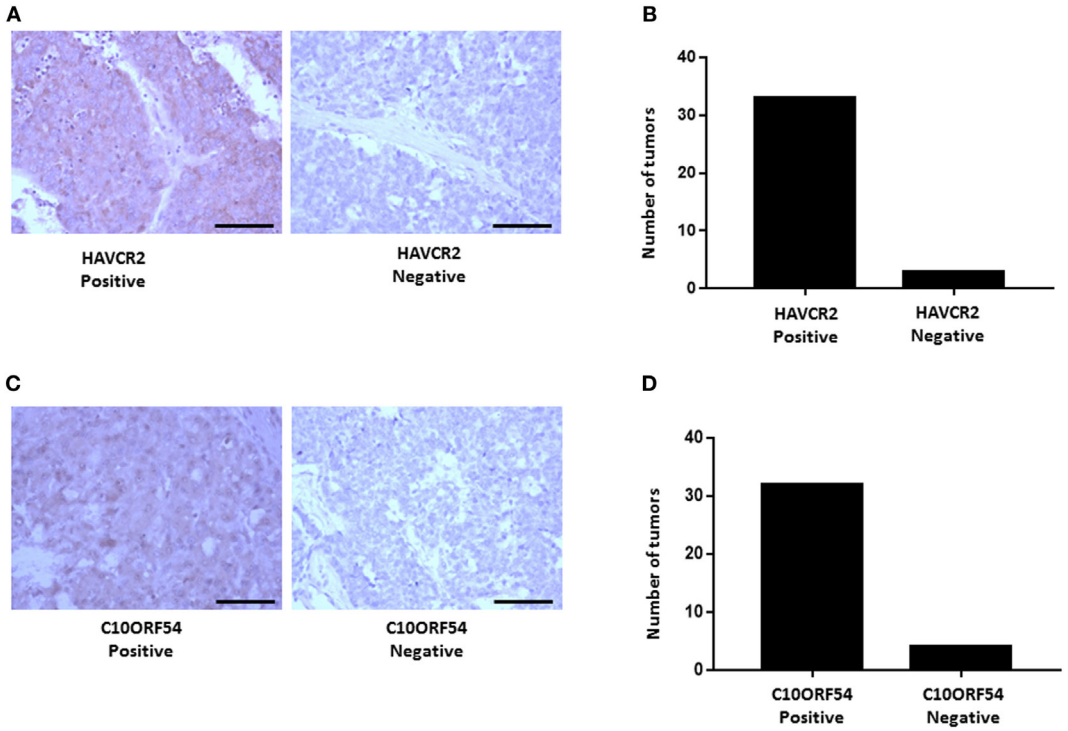

FIGURE 11 | HAVCR2 and C10ORF54 immuno staining in HCC tumor tissue. **(A)** Localization of HAVCR2 in HCC tumor biopsies. **(B)** Graph represents number of tumors scored as HAVCR2 positive or negative. **(C)** Localization of C10ORF54 in HCC tumor biopsies. **(D)** Graph represents number of tumors scored as C10ORF54 positive or negative. Scale bar indicates 20× magnification.

Ig superfamily member. The B7-H4–Ig fusion protein inhibits T-cell activation (46).

The inhibitory immune checkpoint molecule, V-domain immunoglobulin suppressor of T cell activation (VISTA or C10ORF54) is a type 1 transmembrane protein that blocks T-cell activation (47). We found that the overall survival and recurrence-free survival was significantly lower in the high-risk group HCC patients with high VISTA expression. Another study showed that VISTA was overexpressed in oral squamous cell carcinoma and correlated with other immune checkpoint markers PD-L1 and CTLA-4. In addition, the study also showed a poor prognosis in patients with high VISTA and low $CD8^+$ T-cells (48).

The glycophosphatidylinositol-anchored receptor enzyme, ecto-5′-nucleotidase (CD73 or NT5E) inhibits T-cell receptor activation when adenosine binds to its receptor (49). Our study showed NT5E positivity was a statistically significant predictor of poor overall survival and recurrence-free survival in HCC. Our study is consistent with previous studies in triple negative breast cancer, head and neck squamous cell carcinoma, ovarian cancer, and various other gastric carcinoma where NT5E expression in tumor tissues was correlated with poor prognosis (50–54).

T-cell immunoglobulin and mucin domain-containing-3 (TIM-3 or HAVCR2) is an immune checkpoint receptor that binds to its ligand Galectin-9 and limits the T-cell responses (55). Our study showed that TIM-3 is overexpressed in the high-risk group of HCC patients and had significantly worse overall survival. Another study has also confirmed the high expression of TIM-3 in HCC patient tumors than in healthy controls (56). Furthermore, the overall survival time for patients with higher TIM-3 expression is lower than that of patients with lower TIM-3 expression (57). Taken together, these findings indicate that costimulatory and checkpoint genes can be beneficial for the clinical evaluation of HCC patients, especially to identify patients who are at increased risk of worse survival and relapse. A limitation of our study is the lack of HCC patients treated with immune checkpoint therapies. Further studies to validate the expression of these immune predictors in HCC patient cohorts treated with immune checkpoint therapies will be important. The role of these genes in HCC has not been fully elucidated. However, it is conceivable that these immune regulatory molecules may play pivotal roles in modulating the immune response in HCC. Expression, distribution, and function of these immune regulatory molecules in HCC tissues warrant further investigation.

While the clinical relevance of immune-regulators expressed on immune cells is well established, this study focused on the altered expression of immune regulatory genes in HCC tumors. In addition to serving as useful prognostic biomarkers for HCC, targeting B7-H4, PD-L2, TIM-3, VISTA, CD73, and PD-L1 axis with antagonistic antibodies may prove to be beneficial in a subset of HCC patients with elevated levels of these genes. VTCN1, HAVCR2, NT5E, LGALS9, CD80, and PD-1 axis may also represent useful prognostic biomarkers for HCC. Additionally, elevated VTCN1, HAVCR2, LGALS9, TNFRSF14, and CTLA-4 axis can also be beneficial as prognostic biomarkers for HCC.

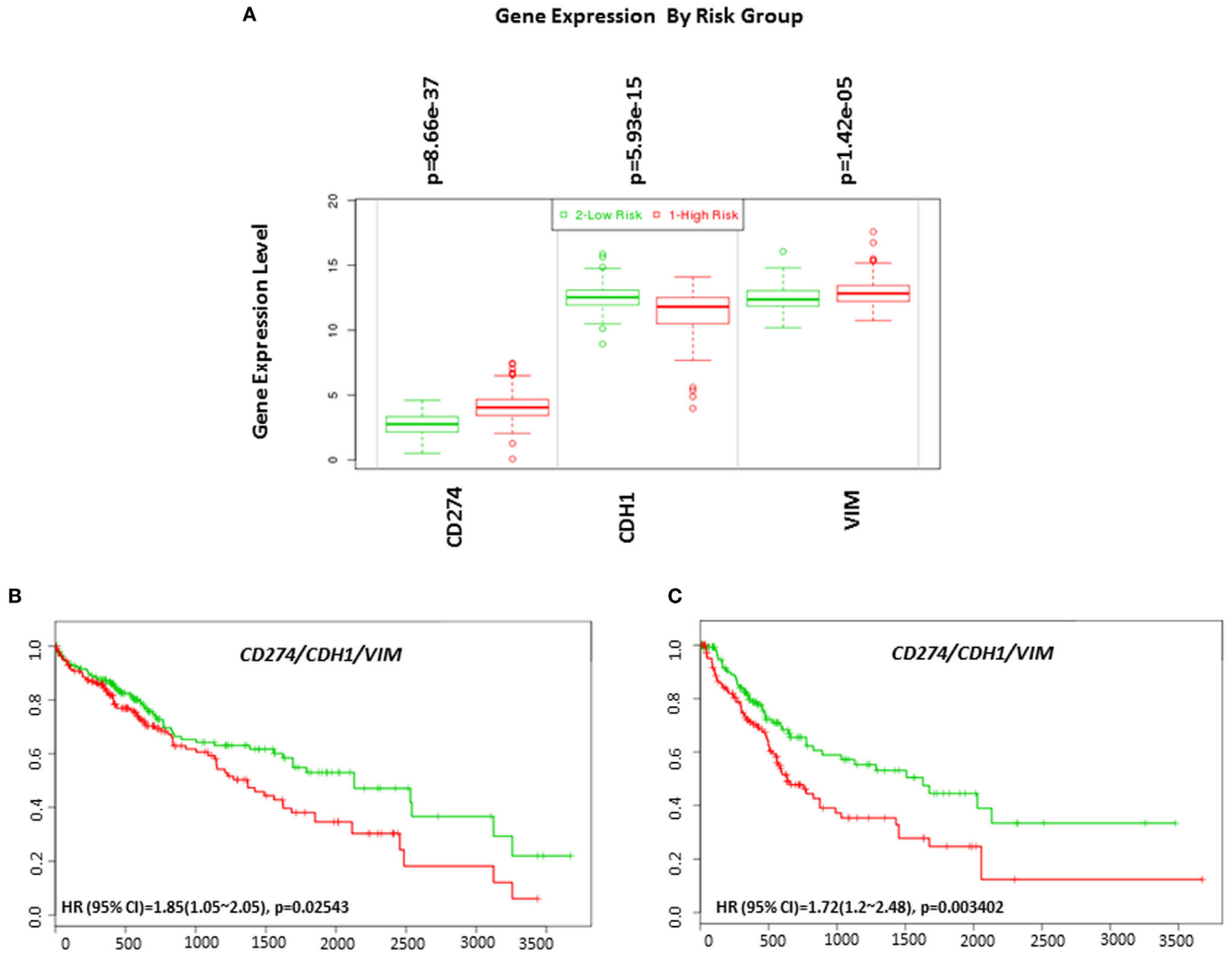

FIGURE 12 | Gene expression of EMT markers in HCC based on risked group and their relationship in combination with programmed death receptor ligand-1 (CD274) and overall survival and recurrence-free survival in HCC patients. **(A)** Box plot of gene expression of EMT markers that statistically correlates with high-risk prognostic score in 422 HCC patients from the TCGA-Liver Cancer dataset. Risk assessed is risk of reduced survival. Red box represents high-risk group and green box represents low-risk group. Each gene is shown on the *x*-axis. *X*-axis also shows a *p*-value of the expression difference between the two risk groups. The expression levels are shown on the *y*-axis. Kaplan–Meier curves produced using the SurvExpress for the analysis of **(B)** overall survival and gene expression of CD274/CDH1/VIM and **(C)** recurrence-free survival and gene expression of CD274/CDH1/VIM in HCC patient samples. Green curve represents low-risk group, while red curve represents high-risk group. The study time (months) is presented in the *x*-axis. The insert shows the hazard ratio, confidence interval, and Log-Rank Equal Curves *p* value. Markers (+) represent censoring samples.

Given that ICI depend on the receptor–ligand interactions between T-cells and tumor cells, and the combined elevated expression of immune regulatory molecules on tumor-infiltrating T-cells and tumor cells is more predictive of ICI response, further comprehensive studies are needed to address the relationship of these immune regulatory molecules on both tumor and tumor-infiltrating T-cells. A recent study showed improved survival in patients with high chronic inflammatory response in the stroma (58). In support of these findings, clarifying the immune regulators involved in the effector functions of tumor-associated T-cells has important implications for our understanding of how the immune microenvironment is modulated to promote antitumor immune responses.

Although there is interest in the use of ICIs in HCC, the coordinated upregulation of immune checkpoint and other immune-regulated genes in our study suggests that a combinatorial treatment strategy is likely to be more beneficial. Early trial results on the combination of PD-L1 and CTLA-4 targeting were first found to be valuable in malignant melanoma (59). Subsequently, combination of these ICIs also resulted in remarkable tumor regression and improved overall survival in many cancers (60). These clinical trials showed a significant advantage of combination therapy over ICI monotherapies. Recent studies have shown that upregulation of immune-related molecules such as TIM-3 occurs in mice and humans following PD-1 inhibition (61) and in the case of anti-CTLA-4 treatment, VISTA, and PD-L1 were

upregulated (62). The elevation in these additional immune regulatory molecules has been proposed to lead to development of resistance to ICI therapies resulting in a significant fraction of cancer patients who do not benefit from the existing checkpoint inhibitor therapies. These findings provide a clinical incentive to combine different ICI therapies to potentially sensitize HCC tumors. In our study, the coordinated expression of immune regulatory molecules, such as *B7-H4*, *TIM-3*, and *VISTA* with *PD-L1* correlated with poor prognosis, while the co-occurrence of *B7-H4*, *TIM3*, *VISTA*, *CD73*, and *PD-L2* with *PD-L1* correlated with poor recurrence-free survival. The identification of these additional immune biomarkers can help to select patients who might benefit from combination immunotherapy.

Our study is the first to provide direct evidence that EMT phenotype is associated with PD-L1 expression in HCC patient tissues. This observation is in line with another study in pulmonary adenocarcinoma where an association between the messenger RNA EMT signature and high PD-L1 expression was found (24). Another study demonstrated a molecular link between EMT and PD-L1 regulation, in both *in vitro* and *in vivo* models (63). It has been suggested that EMT and PD-L1 may bidirectionally influence each other to promote tumor aggressiveness (64). It is conceivable that HCC patients with EMT phenotype would likely benefit from PD-1/PD-L1 targeted immunotherapy. Further studies of the precise molecular mechanisms underlying the association between EMT and PD-L1 expression in HCC tumor microenvironment are warranted.

Recently, high TMB has been associated with better outcome parameters, such as higher response rates to immunotherapy, longer progression-free survival, and overall survival in melanoma and non-small cell lung cancer (65, 66). A study reported that TMB was more reliable in predicting response rate than the expression of PD-L1 by immunohistochemistry (67). A recent study demonstrated that TMB was a reliable biomarker for predicting response to single checkpoint inhibitor, whereas, outcome after anti-PD-1/PD-L1/anti-CTLA4 combinations appeared to be independent of TMB. Our data suggest that TMB can be used to stratify HCC patients for ICI therapy (66). A limitation of our study is the lack of patients treated with ICIs. Further studies are needed to confirm the relationship between TMB and outcome in immunotherapy-treated HCC patients. Moreover, further understanding of the molecular mechanisms which lead to high TMB in HCC is important. In addition to immune markers and TMB, data are emerging on future development of new predictive biomarker strategies for ICI-based immunotherapy, including tumor-infiltrating lymphocytes, immune gene signatures, and multiplex immunohistochemistry (37).

The immune biomarker research represents a promising strategy to guide patient selection and predicts response to immune checkpoint blockade therapies in terms of durable responses or survival benefit. Blockade of immune regulatory molecules identified in this study, including B7-H4, VISTA, CD73, PD-L2, and TIM-3 can potentially offer a treatment strategy to reinstate host immune response against HCC and ultimately tumor regression. Furthermore, the potential to reverse resistance to ICI depends on proper combination therapy that targets the antitumor immune

TABLE 2 | Estimated protein expression in normal hepatocytes.

Immune modulator	Estimated protein expression log_{10} (ppm) in liver
B7-H4	No expression
LGALS9	Low expression
B7-H3	Low expression
IDO-1	No expression
HVEM	No expression
FASLG	No expression
LAG3	Low mRNA expression
TIGIT	No expression
TIM-3	No expression
CD137	No data
VISTA	Low expression
CD73	Low mRNA expression
CD80	No expression
GITR	No expression
PD-L2	No expression
BTLA	No expression
CD27	No expression
PD-L1	No expression
CD28	No expression

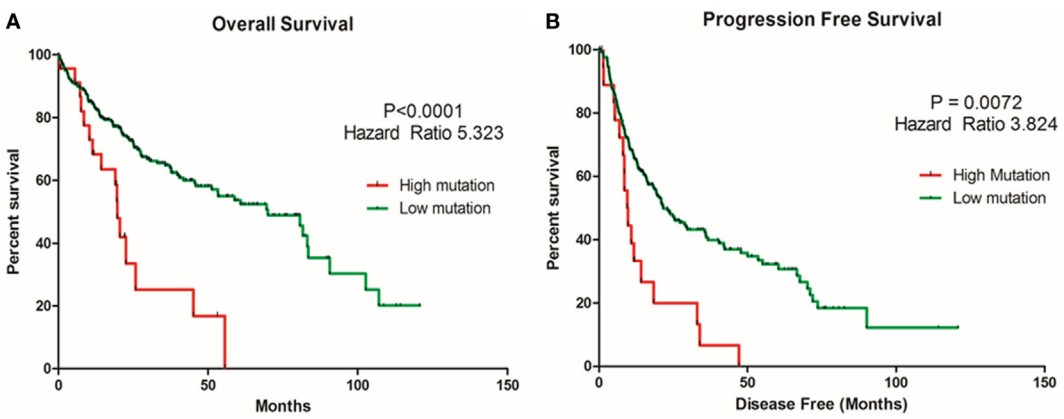

FIGURE 13 | Relationship of tumor mutational burden (TMB) and overall and progression-free survival in HCC patients. Kaplan–Meier curves produced using the cBioportal for the analysis of **(A)** overall survival and **(B)** progression-free survival in HCC patient samples. Green curve represents low mutation or TMB, while red curve represents high mutation or TMB. The study time (months) is presented on the *x*-axis.

response. Although a combinatorial approach is likely to be more beneficial, their use may be limited by a risk of developing more side effects with combination therapy. The translation of combination therapy approaches for better clinical success in HCC patients can be improved through further mechanistic insights on immunotherapy combination strategies along with immune biomarkers.

AUTHOR CONTRIBUTIONS

RS, PP, and AJ performed data acquisition, analysis, and interpretation. PP performed statistical analysis. RS, AJ, PP, and BD wrote the manuscript. KB, DC, and JS critically revised the manuscript. JS and AJ designed the study. MA performed TMB analysis. RS, AJ, PP, KB, and MA revised the manuscript.

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EMT and Stemness in Tumor Dormancy and Outgrowth: Are They Intertwined Processes?

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Metastases are the major cause of cancer patients' mortality and can occur years and even decades following apparently successful treatment of the primary tumor. Early dissemination of cancer cells, followed by a protracted period of dormancy at distant sites, has been recently recognized as the clinical explanation for this very-long latency. The mechanisms that govern tumor dormancy at distant sites and their reactivation to proliferating metastases are just beginning to be unraveled. Tumor cells, that survive the immune surveillance and hemodynamic forces along their journey in the circulation and successfully colonize and adopt to the new and "hostile" microenvironment and survive in a quiescent dormant state for years before emerging to proliferative state, must display high plasticity. Here we will discuss whether the plasticity of dormant tumor cells is required for their long-term survival and outgrowth. Specifically, we will focus on whether epithelial mesenchymal transition and acquisition of stem-like properties can dictate their quiescent and or their proliferative fate. Deeper understanding of these intertwining processes may facilitate in the future the development of novel therapies.

Keywords: tumor dormancy, epithelial mesenchymal transition, mesenchymal epithelial transition, cancer stem cells, disseminated tumor cells, metastasis, stemness, cancer recurrence

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INTRODUCTION

Metastasis is the spread of tumor cells from the primary site to distant organs and their subsequent growth, and is the major cause of cancer patient's mortality (1–5). Accumulating evidence in the literature suggest that metastasis can be an early event (6–11) and is not exclusive to late stage of tumor progression. Yet, it is well-recognized that metastasis is an inefficient process, given that only <0.02% of circulating tumor cells (CTCs) survive the immune surveillance and hemodynamic forces in the circulation (12). Surviving CTCs will colonize distant organs and become disseminated tumor cells (DTCs). Notably, the majority of DTCs do not survive the initial colonization, whereas those that survive may persist to reside in the secondary sites in a quiescent state (cellular dormancy) for many years (1, 13), or progressively grow to form metastases (2). This long-term survival and quiescence of the DTCs may account for the latent recurrence years and decades after primary tumor resection and adjuvant therapy (14). Hence, DTCs that will survive the initial steps of colonization at the distant organ must launch survival mechanisms allowing escape from apoptotic signals and long-term survival in their new and "hostile" microenvironment (non-permissive soil). Upon adequate signals arising in their microenvironment (permissive soil) these DTCs will switch from their quiescence state and launch cellular signals that will enable them

to re-emerge to proliferative growth (1, 15). This is a testament of DTCs' high plasticity. In this review, we will discuss epithelial–mesenchymal plasticity of DTCs and their acquisition of stem cell-like properties as part of the mechanisms that will dictate whether they remain dormant or will re-emerge to metastatic outgrowth.

TUMOR DORMANCY AND EMT PLASTICITY

Epithelial to mesenchymal transition (EMT) occurs during gastrulation and neural crest formation of the developing embryo (16, 17) and in pathological conditions such as wound healing and metastasis (18, 19). Loss of cell polarity and epithelial markers such as the epithelial adhesion protein E-cadherin and cytokeratin 18, and gain of mesenchymal markers such as vimentin and fibronectin are the hallmarks of EMT. EMT was shown to facilitate invasive and high motility characteristic of tumor cells, thus enabling their dissemination from the primary site (19, 20). Notably, primary tumor heterogeneous nature (21) is also demonstrated by EMT occurrence only within a subpopulation of cells within the primary tumor, usually at the leading edge of the tumor (19, 22, 23). Those tumor cells at the leading edge undergoing EMT will initiate their journey in the circulation by successfully invading through the basement membrane. This can be facilitated by their reduced apical-basal polarity and epithelial adhesion proteins.

Snail, Slug, Zeb1 and Twist1 are some of the transcription factors that repress epithelial adhesion protein such as E-cadherin (EMT-TF) and were shown to orchestrate EMT programming (24, 25). However, several reports demonstrate that while EMT-TF expression is required for dissemination, their repression is required to promote metastatic growth *in vivo*. Tsai and colleagues (26) demonstrated that while expression of Twist1 is required for EMT and tumor dissemination at distant sites, Twist1 repression was indispensable for DTCs outgrowth (26). Similarly, repression of homeobox factor Prrx1 (inducer of EMT) was central for the development of metastasis *in vivo* (27). Snail expression in breast cancer metastasis was shown to be transient, whereas forced and prolonged expression of Snai1 decreased lung metastasis (28). Hence, mesenchymal-like DTCs may remain in a dormant state after colonizing the distant organ (27, 29–32), whereas metastatic growth may be dependent on DTCs ability to regain back their epithelial phenotype by mesenchymal epithelial transition (MET) (19, 26, 29, 31, 33). Importantly, these findings are consistent with clinical observations demonstrating epithelial phenotype of human metastases resembling the primary tumor (34). Hence, high plasticity of DTCs is required for their ability to transition between epithelial to mesenchymal and back to their epithelial state during the different steps in the metastatic process.

EMT AND ACQUISITION OF STEM-LIKE PROPERTIES

Activation of the EMT program, eliciting dissemination of cancer cells to distant organs, can also bestow these cells with high plasticity via acquiring stem-like traits. According to the model of cancer stem cells (CSC), a small subpopulation of cancer cells is endowed with stem like-traits with the potential to promote cancer progression. These CSC attain tumor-initiating and metastatic potential, while the non-CSCs lack these traits (35).

Several studies have demonstrated this link between EMT, stemness, and the metastatic initiating potential of DTCs. Induction of EMT in transformed epithelial cells was shown previously to culminate in endowing cells with stem-like traits (36, 37). These stem-like traits in transformed epithelial cells promoted the initiation of primary tumors and accelerated metastasis (19, 36, 38, 39).

The link between undifferentiated status, stemness, and dissemination of tumor cells from the primary site was also demonstrated. Several studies report how EMT-TF Zeb1 can promote stemness and inhibit epithelial differentiation by repressing miR-200 family members (33, 40, 41). In addition, GATA3, a transcription factor known to determine cell fate of luminal epithelial cells, was shown to be lost during early stages of malignant progression in the MMTV-PyMT mouse model. This loss was followed by cell dissemination of CSC-like cells (42, 43). Metastatic progression of lung adenocarcinoma in mouse models was shown to be associated with a stemness program, mediated by loss of Nkx2-1 expression (42, 44, 45). Hence, these observations suggest that CTC arriving to distant organs may already be endowed with CSC-like traits. Notably, Malanchi and colleagues previously demonstrated that only the CSC population of DTCs was capable to initiate metastatic nodules at secondary site (46).

Overall, these studies suggest that EMT, along with the resulting acquisition of stem cell-like properties, facilitate dissemination and consequently the outgrowth of DTCs at distant organs (47).

Intriguingly, several studies in breast cancer cells have identified a sub-population of non-CSCs that are highly plastic and can switch to CSC state (48–50). This transition can be attributed to a stochastics event (50) or can be driven by the metastatic niche (51).

Microenvironmental cues which are part of the metastatic milieu, such as TGF-β, was shown to induce plastic basal-like $CD44^{low}$ breast cancer cells to acquire a CSC-like phenotype via chromatin remodeling at the ZEB1 promoter (49). In line of these previous findings, Weidenfeld K et al. recently demonstrated that expression of LOXL2 endowed dormant DTCs with CSC-like traits eliciting their transition to metastatic outgrowth. These stem-like traits were dependent on EMT and were driven by hypoxia (52). These findings are in line with a previous report demonstrating the role of EMT in the switch from tumor dormancy to proliferative growth (53). Hence, non-CSC residing at distant organs can remain dormant until appropriate signals will endow them with stem-like properties and reactivation. Indeed, breast cancer DTCs were shown to activate the stromal cells in their vicinity to release niche extracellular matrix (ECM) components such as Periostin and Tenacin C. These ECM components in turn activated tumorigenic stem cell signaling pathways such as Wnt, Nanog, and Oct4 in the residing DTCs, leading to their metastatic outgrowth (46, 47).

Microenvironmental cues associated with inflammation were recently shown to also promote dormant DTCs outgrowth. Inflammation of the lung induced EMT of DTCs via the expression of Zeb1, resulting in the reactivation of the quiescent DTCs (54). Moreover, the formation of a fibrotic-like milieu enriched with type I collagen and its cross linking by LOX was previously shown to promote the transition of dormant DTCs to metastatic outgrowth (55, 56). Of note, matrix stiffening is induced by increased Col-I deposition and its cross-linking. Mechanical stiffness of the matrix was shown to regulate EMT via Twist 1 (57) and promote CSC-like traits of cancer cells (58).

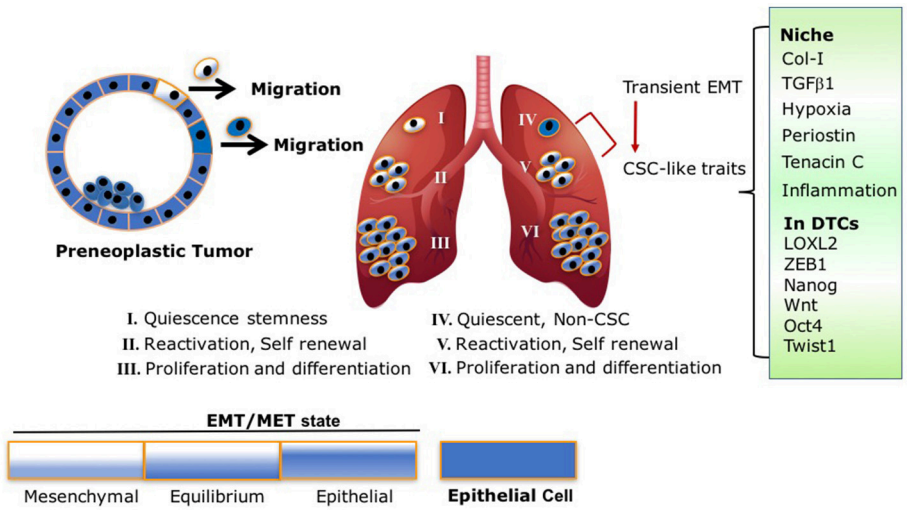
Hence, changes in the ECM components and mechanical compliance may provide a "fertile soil" to promote dormant DTCs plasticity and outgrowth (15).

TUMOR DORMANCY AND STEMNESS

Adult stem cells are undifferentiated cells, residing in tissue in a quiescent state until signals arising in their surrounding niche will direct them to self-renew and differentiate to yield some or all of the major specialized cell types of the tissue. The link between dormant DTCs and their acquisition of stem-like traits has been proposed. DTCs residing at distant organ are exposed to un-permissive "soil." In order to survive and grow, these cells will launch some intrinsic dormant programs inhibiting self-renewal and inducing cell cycle arrest and survival pathways [Quiescent stemness; (51, 59)]. Indeed, DTCs in bone marrow detected in early stage of breast cancer patients were shown to display a putative stem-like phenotype (60). In addition, DTCs of prostate cancer cells, recovered from bone marrow, were significantly enriched for a CSC phenotype (61). Importantly, the transition of these DTCs to CSCs was regulated by niche-derived Growth Arrest Specific 6 (GAS6), previously shown to regulate dormancy (62). GAS6 activated mTOR signaling in the prostate cancer DTCs through the Mer receptor, endowing them with cancer stem-like traits (61). Similarly, an orphan nuclear receptor NR2F1 expression was shown recently to be upregulated in DTCs from prostate cancer patients carrying dormant disease (63). NR2F1 induced quiescence was dependent on Retinoic Acid Receptor Beta (RARβ) and cyclin dependent kinase (CDK) inhibitors and the stem cell factor SOX9 (63). Additionally, Bone Morphogenetic Protein-7 (BMP- 7) secreted from bone stromal cells induces senescence in prostate CSC by activating Bone Morphogenetic Protein Receptor Type II (BMPR2)-p38-NDRG1 axis. Notably, this BMP-7-induced senescence in CSCs was reversible upon withdrawal of BMP-7 (64). Another regulator of stem cell activity, leukemia inhibitory factor (LIF), was shown to promote tumor dormancy of breast cancer cells in the bone. Loss of LIF receptor (LIFR) of breast cancer DTCs in turn promoted their outgrowth from quiescence and down-regulated CSC associated genes (65).

These findings suggest that dormant DTCs might retain stem-like properties such as quiescence, yet will shift to self-renewal program upon cues from their niche, leading to their reactivation.

Overall, several studies support the role of EMT and CSC-like traits in promoting tumor dormancy and MET during metastatic outgrowth. However, EMT and CSC-like traits have been shown, in other studies, to inhibit tumor dormancy. These conflicting findings as reviewed above suggest high



SCHEME 1 | EMT/MET transient state and CSC traits are intertwined processes: following model illustrates the different fluctuations between the EMT/MET states of early disseminated DTCs and their link to stemness and metastatic outgrowth. **(I)** Dormant DTCs with EMT/MET state lining toward more mesenchymal phenotype activate quiescence stemness. **(II)** DTCs with EMT/MET at "equilibrium" are highly plastic with self-renewal capacity resulting in the establishment of micrometastases. **(III,VI)** Macrometastases with EMT/MET state lining to epithelial phenotype proliferate and differentiate. **(IV,V)** Non-CSC **(IV)** undergo transient EMT endowing the cells with CSC-like traits and self-renewal capacity resulting in the establishment of micrometastases **(V)**. This transition is mediated by signals arising at their niche (Col-I enriched fibrotic milieu, TGFβ1, Periostin, Tenancin C, inflammation, hypoxia) which in turn can activate accordingly EMT programs via expression of EMT-TF and/or LOXL2 resulting in acquisition of CSC-like traits.

plasticity in the EMT and acquisition of CSC-like states during the transition from tumor dormancy to metastatic outgrowth.

DTCs FLUCTUATION BETWEEN EMT–MET STATES AND CSC-LIKE TRAITS

Intriguingly, a recent study by Harper et al. demonstrated EMT plasticity within Her2+ DTCs. Early disseminated Her2+ DTCs underwent EMT, expressed the epithelial marker CK8/18+, and retained prolonged dormancy in the bone marrow and lungs. These dormant DTCs eventually developed metastases (7). These findings suggest that a partial EMT is ample for early dissemination, dormancy and outgrowth (7). Indeed, several clinical reports demonstrate the presence of partial EMT or a hybrid EMT/MET CTC (66–68). Furthermore, many cancer cells may metastasize without completely losing their epithelial phenotype and or completely gaining mesenchymal traits (69, 70). Hence, EMT is not an "all-or-none" response, rather it involves transitional states (71, 72), which can reconcile the accumulating experimental and clinical evidence demonstrating EMT of DTCs and MET in proliferating metastases (19, 26, 29, 31, 33) **Scheme 1**.

Therefore, based on these previous findings and recent reports, we propose the following model **Scheme 1** that may reconcile the overall findings. As illustrated in **Scheme 1**, early DTCs, which account for the source of recurring cancers, may exist in a transient EMT/MET state as was previously proposed (72). Dormancy of the early DTCs may display an EMT/MET transient state leaning to a more mesenchymal phenotype, resulting in CSC-like traits responsible for their quiescence. Initial induction of DTC outgrowth by ECM remodeling and other signals arising at the metastatic site may tilt the EMT/MET toward an "equilibrium" state, which may endow the cells with the highest plasticity to initiate self-renewal of the cells. As the EMT/MET state progressively leans to a more epithelial phenotype, this in turn will increase cell proliferation and differentiation of the growing macrometastases. Importantly, key elements in the proposed model have been supported by a recent analysis of CSC markers in human metastatic breast cancer cells (31). Furthermore, breast cancer stem cells were reported to exist in distinct EMT and MET states characterized by expression of distinct CSC markers. Notably, breast cancer cells with dual expression of both sets of markers were demonstrated to have the highest degrees of plasticity (32). Hence, the transient EMT/MET gradient state linked to CSC-like traits may dictate whether DTCs will remain dormant or emerge to metastatic outgrowth. Importantly, we should also consider the other scenario where DTC can promote dormancy programs at distant sites independent from the acquisition of stem-like properties. These DTCs however, will acquire stem-like properties mediated by a transient EMT leading to their reactivation. This transition may be driven by signals in their metastatic microenvironment **Scheme 1**.

The ability of DTCs to fluctuate between EMT–MET states and acquire different CSC-like traits can also facilitate their immune evasion during metastasis (73). Several EMT-TF such as Twist1 and Zeb1 were shown to have immunosuppressive functions. Zeb1, by downregulating miR-200s, promoted PD-L1 upregulation. PD- L1 is an immune checkpoint regulator of $CD8^+$ T cells (74). Melanoma cells expressing another EMT-TF known as Snail, induced immune suppression via activation of regulatory T cells and impaired dendritic cell activity (75). In addition, Mesenchymal-like DTCs, which often have elevated expression of TGF-β, may escape attack by cytotoxic CD8+ T cells (76). Similarly, activation of CSC-like traits in DTCs such as expressing Sox-dependent stem-like state, followed by actively silencing WNT signaling, can promote quiescence of DTCs and their immune evasion (77).

Overall, the fluctuation of DTCs between EMT-MET states and their acquisition of different CSC-like traits will enable their adaption to the distant site and their evasion of the immune system.

CONCLUDING REMARKS

Dormant DTCs residing at distant sites must display high plasticity to successfully overcome the un-permissive "soil" and emerge to metastatic growth. The plasticity of residing DTCs due to acquiring a partial EMT phenotype is an emerging concept that is supported by recent clinical data. This in turn can promote different CSC-like traits during the different steps in metastatic progression. The scope of this review was to reveal the potential link between EMT/MET and CSC-like traits in the transition of dormant DTCs to metastatic outgrowth. Several lines of evidence presented in this review suggest that EMT and stem cell-like traits are intertwined processes dictating DTCs fate. These intertwined processes are highly complex and warrant additional research in order to utilize these emerging concepts in our battle against cancer recurrence.

AUTHORS CONTRIBUTIONS

KW: research and writing, DB: conception, research, writing, and editing.

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REVIEW
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The Role of the Extracellular Matrix and Its Molecular and Cellular Regulators in Cancer Cell Plasticity

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The microenvironment encompasses all components of a tumor other than the cancer cells themselves. It is highly heterogenous, comprising a cellular component that includes immune cells, fibroblasts, adipocytes, and endothelial cells, and a non-cellular component, which is a meshwork of polymeric proteins and accessory molecules, termed the extracellular matrix (ECM). The ECM provides both a biochemical and biomechanical context within which cancer cells exist. Cancer progression is dependent on the ability of cancer cells to traverse the ECM barrier, access the circulation and establish distal metastases. Communication between cancer cells and the microenvironment is therefore an important aspect of tumor progression. Significant progress has been made in identifying the molecular mechanisms that enable cancer cells to subvert the immune component of the microenvironment to facilitate tumor growth and spread. While much less is known about how the tumor cells adapt to changes in the ECM nor indeed how they influence ECM structure and composition, the importance of the ECM to cancer progression is now well established. Plasticity refers to the ability of cancer cells to modify their physiological characteristics, permitting them to survive hostile microenvironments and resist therapy. Examples include the acquisition of stemness characteristics and the epithelial-mesenchymal and mesenchymal-epithelial transitions. There is emerging evidence that the biochemical and biomechanical properties of the ECM influence cancer cell plasticity and vice versa. Outstanding challenges for the field remain the identification of the cellular mechanisms by which cancer cells establish tumor-promoting ECM characteristics and delineating the key molecular mechanisms underlying ECM-induced cancer cell plasticity. Here we summarize the current state of understanding about the relationships between cancer cells and the main stromal cell types of the microenvironment that determine ECM characteristics, and the key molecular pathways that govern this three-way interaction to regulate cancer cell plasticity. We postulate that a comprehensive understanding of this dynamic system will be required to fully exploit opportunities for targeting the ECM regulators of cancer cell plasticity.

Keywords: extracellular matrix, stroma, plasticity, cancer associated fibroblasts (CAF), tumor associated macrophages, tumor microenvironment, signaling pathways, cancer

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INTRODUCTION

Metastasis is the primary cause of cancer-related mortality (1) and results in a catastrophic disruption to an organ function through the lodgment and unrestrained growth of exogenous tumor cells within normal tissue. For a tumor cell to migrate to a new location within the body, it needs to adapt to survive and thrive within an environment that is distinct from that of the tissue in which it arose. Functional adaptations acquired by cancer cells to survive altered environments is termed cancer cell phenotypic plasticity. Of these, the epithelial to mesenchymal transition (EMT) is the best studied and its reverse, the mesenchymal to epithelial transition (MET) is rather less well understood. Another key aspect of phenotypic cancer cell plasticity is the acquisition of stem-like characteristics, resulting from so-called de-differentiation, which permits the cancer cells to remain dormant for long periods of time, evading both the immune system and therapeutic agents. The pathophysiological processes of metastasis that require phenotypic cancer cell plasticity and the major cellular players that bring this about are summarized in **Figure 1**.

Normal interactions between the parenchyma and the stroma are characterized by (1) A two-way communication by molecular messengers that are secreted into the microenvironment, (2) biochemical and biophysical cues exerted by the ECM, and (3) direct cell-cell contact permitting reciprocal signaling between the two cell types. These interactions direct tissue homeostasis and the establishment of niches bearing distinct microenvironmental characteristics that facilitate the maintenance of specialized cell types including stem cells. Under abnormal conditions in which the parenchymal cells acquire tumor-causing genetic lesions, the microenvironment—its cellular and ECM components—is remodeled under the influence of the growing tumor as well as the organism, resulting in aberrant tissue homeostasis and disruption of the specialized niches. These microenvironment changes strongly influence the progression of the disease (2).

In cancer progression, epithelial-mesenchymal transition is associated with invasiveness and metastasis. Acquisition of a mesenchymal phenotype is characterized by increased motility, expression of ECM remodeling enzymes such as matrix metalloproteases (MMPs), and enhanced survival—all key adaptations that are required for traversing the basement membrane, promoting abnormal interactions between cancer cells and the extracellular matrix (ECM), intravasation and survival within the circulation. Conversely, MET is associated with integration into epithelia at sites of distal metastasis. EMT has long been associated with acquisition and maintenance of cancer stem cells (CSCs) (3).

The CSC hypothesis takes its origins from the observations made in the hematopoietic system, where a pluripotent progenitor gives rise to all hierarchical lineages of the system by a stepwise process of differentiation (4). Analogously, CSCs are thought to constitute a reserve pool of a limited number of cells that maintain the proliferative potential of the primary cancer or migrate out of the primary site to seed new secondary tumors at the metastatic sites. Recent observations have permitted a more nuanced understanding of CSCs. It has been reported that like bulk cancer cells, CSCs exhibit phenotypic plasticity in response to signals from the microenvironment environment (5). Another important addition to the emerging CSC model is that the microenvironment plays a crucial role for the maintenance of the CSC pool, just as it does for the maintenance of normal stem cells (6). However, context-specific differences between tumor types exist; for instance, while CSCs of colorectal cancer may be generated from non-CSC cells via a process regulated by Wnt signaling, a strictly hierarchical system is characteristic of glioblastomas, where, CSCs are maintained by self-renewal (7). There is strong circumstantial evidence that ECM provides an important stem cell niche given the dependence of normal stem cells on signaling through ECM receptors such as the laminin receptor, α6β1 integrin (8), the vitronectin receptor αV (9), and collagen receptors (10) and emerging evidence that the cancer-associated ECM is an important aspect of the cancer stem cell niche (11).

Cancer plasticity is driven by reciprocal interactions between a cancer cell and its microenvironment, which permits this cell to, on the one hand, calibrate its response to the altered environment and on the other, actively remodel the microenvironment to facilitate its survival and proliferation. In this review, we will discuss how the ECM influences cancer cell plasticity and conversely how cancer cells directly or indirectly influence changes in ECM structure and composition.

The extracellular matrix is a scaffold of fibrillar proteins, accessory proteins and molecules that provides structural and biochemical support for cells. The predominant component of the ECM is fibrillar collagen, the structure and mechanical properties of which strongly influence cellular phenotype (12). Based on biochemical and structural characteristics, the ECM consists of a basement membrane (located at the basal aspect of epithelial or endothelial cells in normal tissues) and the interstitial (stromal) ECM. In most tissues, the basement membrane consists largely of collagen IV, together with laminin, fibronectin, and several types of proteoglycans. The main role of the basement membrane is to provide a physical barrier between the epithelial cells and the connective tissue (stroma) of the organ, whilst still allowing the diffusion of gases and transport of signaling molecules. The interstitial ECM, mainly produced by mesenchymal cells (discussed further in section Cellular Mediators of Cancer Cell Plasticity via the ECM), consists largely of collagens I and III, fibronectin, and proteoglycans. In cancer, rupture of the basement membrane permits epithelial cells to undergo an EMT and migrate into the surrounding stroma and invade through the interstitial ECM. Epithelial cells that have undergone EMT can cause activation of stromal cells to yield pro-tumorigenic stromal cells that can remodel the ECM to create a tumor-permissive environment (13). Among the components of the ECM, glycosaminoglycans such as hyaluronan (HA) play important roles during cancer progression. High levels of HA have been documented in tumors and are associated with poor prognosis and chemotherapy resistance (14). HA has been shown to be able to induce EMT by binding to CD44 and activating the EMT transcription factor TWIST-1 (15). Increased HA levels have also been shown to compromise vascular integrity

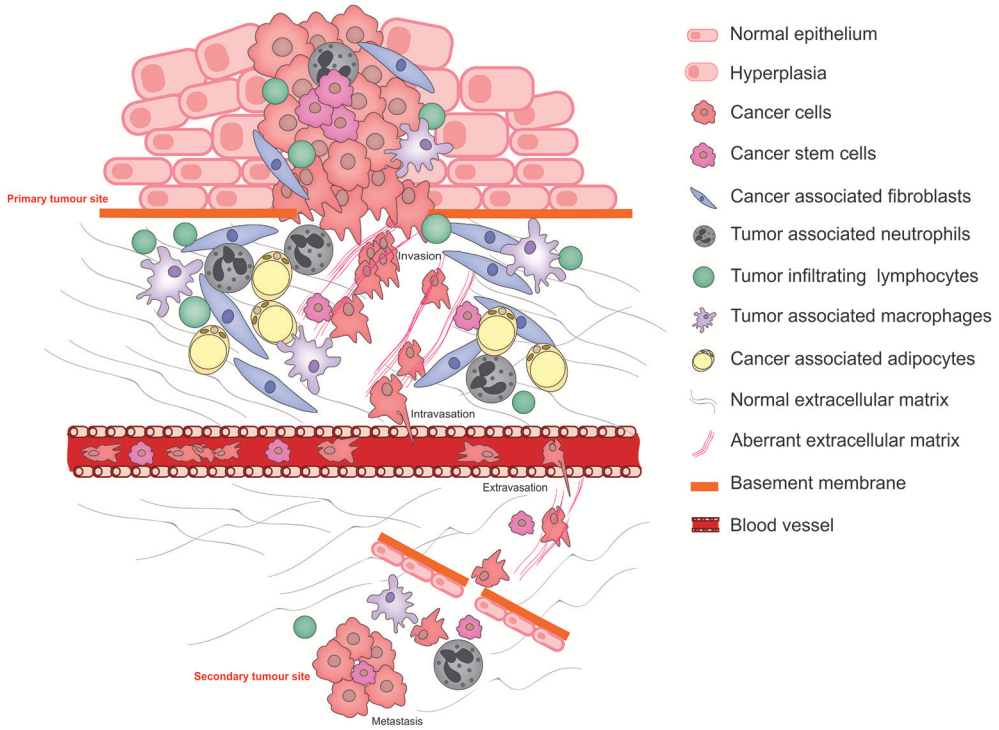

FIGURE 1 | Schematic illustrating the pathophysiological processes that exploit cancer cell plasticity during tumor progression, invasion, and metastasis.

in tumors which has important implications for metastasis (16). Furthermore, HA breakdown products have been implicated in inflammatory responses that precipitate extracellular matrix remodeling (17).

The normal ECM is highly remodeled after it has been initially set down and exhibits tissue-specific composition and organization. In pathological conditions such as desmoplasia, the appearance of linear ECM fibers correlates with poor patient outcomes. Linear fibers have been observed to provide tracks that migratory cancer cells can use to their advantage (18), to enhance migratory capability. The main regulators of ECM remodeling during tumorigenesis are cancer-associated fibroblasts (CAFs), which produce large quantities of collagen I, fibronectin, and periostin (13). Analysis of the ECM using techniques such as second harmonic generation (SHG) microscopy, atomic force microscopy and mass spectrometry has revealed tissue-specific composition and configuration of its components, which underlie tissue phenotype, and also the tumor phenotype (19). The ECM is a source of biochemical and biomechanical signals that promote tumor progression, and it is in turn strongly influenced by the cancer in a reciprocal relationship that is driven by the cytoskeleton of cancer cells (20).

Cell-ECM interactions in both normal and pathologic conditions are principally mediated via integrins, which constitute a large family of cell-surface receptors. Integrins also regulate cytoskeleton organization and activate intracellular signaling pathways, conveying both mechanical and chemical signaling (21). Besides their roles in cell adhesion and migration, they also transmit signals for cell proliferation and survival. The majority of integrins activate focal adhesion kinase (FAK). This in turn promotes directional cell motility of both tumor and stromal cells, and generates signals to further modify ECM organization, thereby altering the mechanical properties of the tumor microenvironment (13, 21).

CELLULAR MEDIATORS OF CANCER CELL PLASTICITY VIA THE ECM

Normal tissue homeostasis is strongly influenced by the ECM and a key example of this is the process of wound healing. One of the steps for the re-establishment of normal tissue homeostasis following wounding is the migration of fibroblasts into the wound space in order to break down the thrombus and regenerate the ECM (22). The mechanical properties of the newly synthesized ECM are an important determinant of how quickly the wound heals (23). Similarly, the ECM is set down early in embryonic development and influences the delamination, migration and differentiation at their destination of diverse cell types (24, 25). Since the physiological functions and behaviors of normal cell types and strongly influenced by the normal ECM, it is no surprise that similarly the tumor ECM exerts a strong influence on the behavior or cancer cells. The influence of the ECM on cancer cell plasticity is modulated by a variety of cell types that reside within the tumor stroma. Under the influence of systemic regulators as well as cancer cells, these stromal cells not

only produce tumor ECM, which qualitatively and quantitatively differs from a normal ECM, but also an array of cytokines and other secreted and membrane-bound factors that influence cancer cell plasticity. In this section, we discuss the key cellular mediators of cancer cell plasticity that regulate the biochemical and biomechanical properties of the ECM.

Cancer-Associated Fibroblasts (CAFs)

Fibroblasts, a cell type of spindle-like morphology and mesenchymal lineage, constitute the major cell type of the normal tissue stroma. Stroma-resident fibroblasts that are not actively engaged in ECM production or turnover are termed "resting" or "quiescent." Resting fibroblasts are mostly observed within fibrillar ECM and have the potential to be "activated." Activated fibroblasts are morphologically and metabolically different to their resting counterparts, and activation can be caused by acute or chronic inflammatory responses such as wound healing or fibrosis. Pro-inflammatory factors such as TGF-β, IL-6, platelet-derived growth factor (PDGF), hypoxia, and reactive oxygen species (ROS) can activate quiescent fibroblasts. Once activated, fibroblasts synthesize and deposit ECM components, release chemokines and cytokines into the stroma and generate tissue-level tensile forces via their actomyosin cytoskeletons, all key requirements for tissue remodeling. Activated fibroblasts are therefore essential for epithelial cell differentiation, control of immune responses and the maintenance of tissue homeostasis (26, 27).

A long-standing concept tumor as "wounds that do not heal" (28) hinges on the ability of cancers to commandeer fibroblast function normally associated with wound healing to promote disease progression. Accumulation of tumor cells within the tissue can trigger chronic wound healing responses from normal tissue fibroblasts, leading to desmoplastic tissue remodeling characterized by the appearance of aberrantly organized ECM fibers and increased tissue stiffness, which in turn creates a favorable environment for tumor progression (29).

Activated fibroblasts in the tumor microenvironment are termed cancer-associated fibroblasts (CAFs). CAFs are among the main cellular contributors to cancer-associated changes in ECM architecture and may arise from normal fibroblasts. CAFs are thought to be recruited via growth factors secreted by tumor and immune cells (such as TGFβ, PDGF, and FGF2), and subsequent proliferation and expansion of these cells may be auto-regulated by paracrine/autocrine mechanisms governed by other CAF populations (27). There is an ongoing discussion regarding the classification of CAF populations based on cell morphology, markers, secretory profiles, and location within the tumor. These complex issues and the debate around the pro- vs. anti-tumorigenic properties of CAFs are dealt with in detail elsewhere (26, 27, 30). Here, we discuss mechanistic aspects of the contribution of CAFs and other stromal cells to the ECM properties that regulate cancer cell plasticity.

CAFs are among the few stromal cell types that have been conclusively shown to promote an EMT program in cancer cells. Using stromal fibroblasts isolated from breast cancer patients in co-culturing experiments with a panel of breast cancer cell lines, CAFs were demonstrated to promote cancer cell EMT via TGF-β secretion and induction of the TGF-β/SMAD signaling pathway in the cancer cells (31). Another study found that activated fibroblasts secrete carbonic anhydrase IX (CA IX), which enhances the production by CAFs of MMP2 and MMP9, which are well-known to degrade and remodel the ECM. Acidification of the microenvironment by CA IX can also directly promote an EMT program in prostate carcinoma cells (32). Furthermore, IL-6 from prostate carcinoma cells generates a CAF phenotype and leads to increased MMP2 and MMP9 levels in fibroblasts. This can in turn induce an EMT program in cancer cells. This reciprocal cancer cell-CAF interaction sustains tumor progression via cancer cell plasticity (33).

Recent evidence suggests that ECM remodeling components secreted by CAFs play a role in the maintenance of the cancer stem cell niche (34, 35). For example, mammary cancer cells can induce ECM periostin production by stromal fibroblasts, essential for CSC maintenance by promoting Wnt signaling (36). More recently, it has become clear that CAF phenotype changes induced by tumor-initiated hedgehog signaling promotes stemness in breast cancers in both mouse models and human patients and that inhibiting hedgehog signaling in fibroblasts may be a useful therapeutic modality to reverse breast cancer cell plasticity (37). These CAF functions are dependent on their role in regulating the ECM and these ECM changes occur at the site of the stem cell niche (37). Fibroblasts lacking Tissue Inhibitor of Metalloproteinases (TIMPs) exhibit a CAF-like phenotype and release extracellular vesicles packed with factors that enhance cancer cell motility and upregulate CSC markers. These vesicles contained high levels of A Disintegrin and Metalloproteinase domain containing protein 10 (ADAM10), which promotes cell motility via activation of RhoA and Notch signaling (38). CAF-derived growth factors were also shown to play a role in stem cell niche formation. CAF-derived HGF is proposed to promote the formation of the CSC niche and tumorigenicity by activating the Wnt signaling pathway in differentiated colon cancer cells (39). Another report suggests that CAFs promote growth and stemness in lung CSCs. Paracrine signaling between CAF-derived insulin-like growth factor-II (IGF-II) and IGF1R on CSCs, and the subsequent induction of Nanog, induced expression of CSC markers. The importance of this signaling axis was also confirmed in samples from non-small cell lung cancer (NSCLC) patients (40). Taken together, these observations establish a key role for CAF-mediated ECM production and remodeling in cancer cell plasticity that promotes tumor progression.

It is now well-accepted that cancer cell motility is enhanced by the tumor ECM (41). It has been shown that TGF-β-stimulated colon CAFs are able to secrete scatter factor/hepatocyte growth factor (SF/HGF) and tenascin C, and thereby promote invasiveness of colon cancer cells (42). Using fibroblasts isolated from different stages of mouse mammary tumors it has been shown that activation of Yes-associated protein 1 (YAP1) in CAFs promotes matrix stiffening, cancer cell invasion, and angiogenesis. YAP1 is known to regulate cytoskeletal components including the regulatory myosin light chain (MLC2), which controls actomyosin contractility. A feed-forward loop is therefore established via the activation of YAP1 in response to

mechanical cues from the ECM upon CAFs, which further stiffen of the ECM (43). Consistent with these observations, ROCK inhibition upstream of YAP1 reversed the CAF phenotype to normal (43). However, there are multiple pathways contributing to this feed-forward loop as ROCK-dependent actomyosin contractility downstream of GP130-IL6 JAK1/STAT pathway activation also enhanced ECM remodeling by CAFs, which in turn promoted melanoma cell migration *in vitro* (44). Therefore, the ability of fibroblasts to promote tumor cell migration while also enhancing tumor cell plasticity establishes a key role for this versatile cell-type in tumor progression.

Fibroblasts therefore exhibit key properties that are exploitable by cancer cells to promote tumor progression via cellular plasticity and interfering with CAF function therefore represents an attractive possibility for anti-cancer therapy. Nevertheless, evidence that at least a sub-population of CAFs has anti-tumor functions sounds a note of caution, raising the possibility that directly targeting CAFs may have unintended consequences. These observations highlight that more work needs to be done to dissect out the mechanisms by which CAFs contribute to cancer, with tissue- and context-dependent implications being likely to arise.

Tumor-Associated Macrophages (TAMs)

Macrophages are phagocytic cells of the immune system that are distributed throughout virtually all tissues. They are highly adaptable cells that exhibit a high degree of plasticity depending on the signals in their immediate environment (45). In response to infection or injury, macrophages can secrete pro-inflammatory factors (TNF-α, IL-1, and nitric oxide) that trigger host defense responses and tissue remodeling. In tissue repair responses, an important switch occurs between pro-inflammatory and anti-inflammatory macrophage sub-populations. If not checked, the pro-inflammatory responses can lead to chronic inflammation or auto-immune disease (46). Not only are macrophages important contributors to innate immunity, but they also play essential roles in various developmental processes such as bone morphogenesis, neuronal patterning, angiogenesis, branching morphogenesis, and adipogenesis (47). These functions are co-opted by tumor cells as a feature of many cancers.

An important concept in macrophage biology is polarization; the phenotyping of macrophages based upon the expression of distinct suites of surface markers induced by specific environmental stimuli (48). While there has been a consensus on a two category "M1-M2" classification, it is now commonly accepted that macrophages exist on a continuum in disease and tissue specific contexts, of which the M1 and M2 states represent two extremes (45, 49). Macrophages polarized toward the M1 state are referred to as "classically" activated. This population produces pro-inflammatory agents that contribute to host defense and their anti-tumor properties. Macrophages polarized toward the M2 state are said to be "alternatively" activated. They secrete anti-inflammatory cytokines that largely suppress inflammatory responses. This population suppresses tumor immunity, enhances tumor angiogenesis, and extracellular matrix remodeling, and is associated with wound healing (47). Tumor-associated macrophages are also sometimes referred to as M2 polarized, although even in this context, heterogeneous populations of TAMs can exist within the M1-M2 continuum (50).

The specific location of TAMs within a tumor has been established as an important indicator of their pro-tumor activity, and they are mainly localized to perivascular regions or at the tumor invasive front. Monocytes are recruited to the invasive front and differentiate into macrophages in response to signals from tumor and stromal cells. An array of cytokines (IL-4, IL-10, IL-13), chemokines (CCL2, CXCL12), and growth factors (CSF-1, TGF-β, VEFG-A, PDGF, angiopoietin-2) produced at the invasive margin stimulate monocyte recruitment, differentiation and survival (51–54). We have previously demonstrated that the chemokine receptor CCR6 is expressed on TAMs and facilitates their migration to the cancer site in a mouse model of mammary cancer. Deletion of this chemokine receptor significantly decreases the population of TAMs, in particular M2 TAMs, as well as tumor burden (55).

TAMs play important roles in cancer cell proliferation (56), invasion (57), angiogenesis (58), and metastasis (45). TAMs secrete EGF, FGFs, and VEGFs that promote tumor cell proliferation, fibroblast activation and angiogenesis (59, 60). TAMs also produce IL-10 and TGF-β, which contribute to their immune-suppressive properties, assisting tumor cells in immune evasion (51, 61, 62). Chemotaxis-based experiments and intravital imaging revealed that reciprocal signaling between tumor cell-derived CSF-1 and TAM-derived EGF is essential for the promotion of tumor cell migration. This interaction is important for EGF receptor-mediated mammary tumor cell invasion in primary tumors (51). Furthermore, direct physical interaction between mammary cancer cells and TAMs has been observed using multiphoton intravital imaging, demonstrating that these reciprocal interactions may not only be biochemical in nature. The observation that tumor cells intravasate into areas where perivascular macrophages are numerous in mammary tumors, suggests that macrophages may also enhance cancer cell intravasation (63).

Along with their important roles in initiating growth and immune-suppressive signals directly, TAMs have been shown to play a significant role in contributing to the tumor ECM by producing several important matrix and matrix-associated proteins such as collagens, fibronectin, osteopontin, and periostin (64). Utilizing an orthotopic colorectal cancer (CRC) model, Afik and colleagues demonstrated that TAMs are capable of collagen synthesis and deposition, particularly collagen types I, VI, and XIV. Confocal, second harmonic generation and scanning electron microscopy of *ex vivo* mouse colorectal tumor tissues has revealed that TAMs are capable of initiating deposition, cross-linking, and linearization of collagen fibers during tumor development, particularly at the invasive front (65).

TAMs support tumor cell migration, invasion and metastasis via ECM remodeling (64, 66). Responding to cytokine signals from tumor cells, TAMs are known to secrete a cocktail of ECM remodeling enzymes including MMPs (1, 9, 12, and 14), serine proteases, cathepsins (B, S, C, L, Z), lysosomal enzymes, and ADAMs. These proteolytic enzymes disrupt integrin-mediated cell-cell adhesions and are essential for cancer cell invasion. In

another study, TAMs isolated from breast cancers were observed to secrete CCL18, which signals via the breast cancer cell-specific PITPNM3 receptor. This signaling cascade activates integrin clustering on tumor cells, promoting integrin-ECM interactions and adhesion, thereby promoting invasiveness and metastasis (67). This study provides evidence for an orchestrated sequence of events whereby proteases released by TAMs remodel the ECM to facilitate tumor cell interaction while also releasing CCL18 that causes integrin clustering on tumor cells, strengthening cell-ECM interactions and facilitating cancer cell plasticity, migration, and dissemination.

Another important role for matrix remodeling enzymes secreted by TAMs is their ability to liberate the ECM-bound growth factors and signaling molecules that can influence tumor cell growth, plasticity, and motility (64). Liberation of bioactive fragments of ECM proteins (such as endostatin from type XVIII collagen) (68) that exhibit biological activities that are distinct from their parent ECM molecule was also demonstrated to be brought about by TAMs. Whilst this is an emerging area of TAM biology, it is one that is likely to increase in interest and significance.

There is a substantial evidence for a role for TAMs in promoting EMT in tumor cells through multiple mechanisms. Exposure of either mouse F9-teratocarcinoma or mammary epithelial cells to TAM-conditioned medium reduces E-cadherin expression, activates the Wnt/β-catenin pathway, induces the expression of mesenchymal markers and increases invasiveness of epithelial cells. It is also suggested that TAM-produced TGF-β may induce an EMT program in cancer cells (62). TAMS have been shown to induce an EMT program in pancreatic cancer cells in response to TLR4 signaling by producing IL-10 (69), and in a breast cancer model, TAMs induced EMT in cancer cells via upregulation of CCL18 (70). Even though the evidence points to a role for TAMs in EMT, it is becoming increasingly apparent that TAM-mediated EMT induction is context dependent and that microenvironmental factors determine the mechanisms by which TAMs induce cancer EMT programs. Analogous to this process, there is some evidence that TAMs are involved in cancer stem cell maintenance. Multiple studies have shown that growth factors and cytokines secreted by TAMs can promote and maintain the CSC populations within various tumors (71). Interestingly, in hepatocellular carcinoma, TAM-derived TGF-β1 promoted cancer cell stemness (72). Taken together, these observations provide evidence for a role for TAMs in ECM-dependent and ECM-independent regulation of tumor cell plasticity.

Tumor-Associated Neutrophils (TANs)

Neutrophils, the most abundant leukocyte type in the blood, are produced in the bone marrow from hematopoietic stem cells and are released into circulation as fully mature cells. The generation and maturation of neutrophils is a complex process (73, 74) and is primarily regulated by granulocyte-colony stimulating factor (G-CSF). Other factors, such as granulocyte–macrophage-colony stimulating factor (GM-CSF), interleukin 6 (IL-6), and KIT ligand (KITL) also contribute to the production of neutrophils. In cancer, tumor cells secrete G-CSF which causes neutrophil overproduction, contributing to immunosuppressive responses at the early stages of tumorigenesis (75).

In the process of neutrophil maturation, primary, secondary, and tertiary cytoplasmic granules are formed. These pre-formed granules contain a wide variety of proteins and enzymes that are essential for anti-microbial defense and the resolution of inflammation. MMPs and neutrophil elastase contained within these granules are of interest as they are proteolytic enzymes that promote tumor progression by remodeling the cancer ECM (76–78).

Like fibroblasts and macrophages, neutrophils also exhibit polarization. Anti-tumor neutrophil populations are designated "N1" and pro-tumor as "N2." Polarization toward the N2 form is induced by elevated levels of TGF-β, and N2-polarized neutrophils express high levels of CXCR4, VEGF, and MMP9. Blocking TGF-β in the microenvironment stimulates upregulation of TNFα and IFNγ in N1 neutrophils and causes CXCL2, CXCL5, and CCL3 production that leads to further recruitment of neutrophils to the tumor site (79). It was also shown that keratinocyte-derived TNF-α is an important contributor to early recruitment of neutrophils in a mouse cutaneous carcinoma model (80). Factors secreted by tumor cells also mediate recruitment of neutrophils. Using orthotropic transplantation of human hepatocellular carcinoma (HCC) cell lines into nude mice, Zhou et al. identified that CXCL5 secreted by cancer cells promotes neutrophil recruitment. Importantly, correlation between the levels of CXCL5 and neutrophil infiltration was confirmed in three independent clinical HCC patient cohorts (81).

Tumor-promoting properties of neutrophils have been documented and several of these functions involve ECM remodeling and cancer cell plasticity. Neutrophil-derived MMP9 enables keratinocyte hyperproliferation and invasiveness in a virus-induced cutaneous carcinoma model (82). In orthotopic xenograft transplantation systems of human fibrosarcoma and prostate carcinoma cell lines, tumor-recruited neutrophils release MMP9 that remodels the ECM to induce angiogenesis and promote metastasis (83).

Neutrophils have also been implicated in cancer cell EMT. Neutrophil-derived elastase has been shown to cleave E-cadherin and induce an EMT program in pancreatic ductal adenocarcinoma (PDAC) cells in co-culture with macrophages. Accordingly, in human PDAC tissue samples, EMT correlated with the presence of infiltrating neutrophils (84). In a zebrafish model, oncogene-transformed keratinocytes were shown to recruit neutrophils to enhance their EMT program. This process was mediated by signaling through CXCR2 in neutrophils (85), consistent with the observation that neutrophil recruitment and tumor progression are impaired in Cxcr2-deficient mice in several models of carcinoma (86). In a zebrafish xenograft model of tumorigenesis *in vivo,* neutrophil migration enhanced tumor cell invasion due to the establishment of collagen tracks that were exploited by cancer cells for their migration (87). Several lines of evidence therefore suggest that neutrophils modify the ECM to promote tumor progression with at least a proportion of these functions mediated by tumor cell plasticity.

Emerging evidence suggests that neutrophil-mediated ECM remodeling augments tumor invasiveness. Co-culture experiments of oral squamous cell carcinoma (OSCC) cell lines with neutrophils revealed that neutrophils increase the formation of invadopodia and collagenous matrix degradation by cancer cells. This process was induced via IL-8-mediated recruitment of neutrophils and subsequent release of TNF-α by neutrophils into the surrounding microenvironment (88). Consistent with these observations, a transgenic mouse mammary cancer model exhibited distinct cytokine profiles in collagen-dense tumors compared to low collagen-density tumors and these cytokine profiles were associated with neutrophil maturation in collagen-dense cancer tissues. Accordingly, depletion of neutrophils in collagen-dense mammary tumors reduced tumor progression in collagen-dense tumors (89).

Another intriguing field that has recently emerged is the study of neutrophil extracellular traps (NETs) and their contribution to tumor progression. NETs are three-dimensional networks of extruded DNA packed with cytosolic and granule proteins. NETs were first described as contributors to the innate immune response, with an ability to trap extracellular pathogens. It has since been shown that inflammatory responses can trigger NET formation (or NETosis). Comprehensive reviews on the roles of NETs in tumorigenesis have been recently published (90, 91). For the purposes of this review we will focus our attention on the potential contribution of NETs to regulation of ECM composition in the tumor microenvironment. NET components MMP9, cathepsin G and neutrophil elastase are all known to contribute to extracellular matrix remodeling as well as provide signals for tumor cell proliferation, migration and tumor-associated angiogenesis (91). While it is yet to be determined whether these proteins contribute to ECM remodeling in the cancer microenvironment while associated with NETs, there is *in vitro* evidence that they may. One study has demonstrated the ability of NETs to trap cancer cells under static and dynamic conditions, raising speculation that NETs produced during inflammation could assist in the colonization of secondary tissues by circulating cancer cells (92). Another recent study has demonstrated that cell lines generated from chronic myelogenous leukemia use integrins to adhere to the fibronectin in NETs. It is therefore possible that NETs provide cancer cells with a platform for interaction with other cells and can induce key signaling pathways required for their survival and proliferation (93). Further investigation into the role of NETs in ECM remodeling, and contribution of NET formations to desmoplastic response in cancers, is therefore warranted. Taken together these studies suggest that new roles for neutrophils in ECM biology are likely to be uncovered, and thereby a role in regulating cancer cell plasticity.

Cancer-Associated Adipocytes (CAAs)

Adipocytes are the lipid-storing cells of adipose tissues (AT) that regulate energy storage and metabolism within the body. Adipocytes secrete hormones and other molecules, collectively termed adipokines, which exert paracrine and endocrine regulatory roles in obesity, adipose tissue fibrosis, inflammation, tumorigenesis, and cancer metabolism (94–96). Many studies indicate a clear phenotypic difference between CAAs and normal adipocytes, but most studies investigating the roles of adipokines in cancer rely on mature (differentiated) adipocyte co-culture experiments with cancer cells. In the context of the tumor microenvironment, the role of adipokines is more complex than simple reciprocal interactions between adipocyte and tumor cells—even though tumor cells express corresponding receptors for adipokines—and is likely to also be strongly influenced by the inflammatory milieu.

Adipocytes mainly arise from mesenchymal stem cells (MSCs) or undifferentiated adipocyte precursors within adipose tissue stroma (97, 98). A small proportion of adipocytes can also be derived from hematopoietic stem cells (HSCs) (99, 100). Adipocytes constitute an essential cellular component of the tumor microenvironment in breast, ovarian, prostate, renal, gastric, and colon cancers (96). Tumor cells can "activate" adipocytes and subvert their cellular programs to facilitate tumor-promotion. Such activated cancer-associated adipocytes are distinct from normal adipocytes in morphology and function. Adipocytes co-cultured with cancer cells exhibit de-lipidation, decreased expression of adipocyte markers such as Ap2 and FABP4, increased expression of MMP11, and enhanced release of inflammation-promoting cytokines IL-6 and IL-1β. Importantly, presence of CAAs expressing IL-6 was confirmed *ex vivo* using primary breast cancer samples (101). Co-culture of cancer cells with mature adipocytes can induce adipocyte dedifferentiation via the Wnt/β-catenin pathway. Adipocytes shrink, significantly lose their lipid content, and may acquire fibroblast-like properties. These cells, termed adipocyte derived fibroblasts (ADFs), express the fibroblast marker S100A4/FSP-1 but not α-SMA. ADFs acquire migratory capacity and move toward the tumor core to promote cancer cell invasion (102).

There is evidence that mature adipocytes, CAAs and ADFs contribute to tumor cell plasticity. Mature human breast adipocytes increase *in vitro* cell motility of both pre-malignant and malignant breast cancer cell lines (103). Through lipolysis and direct lipid transfer from adipocytes to cancer cells, adipocytes may serve as energy reservoirs for cancer cells and sustain tumor growth (104). *In vitro* studies show that paracrine signaling from cancer cells induces the release of free fatty acids from CAAs resulting in CAA de-lipidation and increased secretion of inflammatory cytokines and proteases that promote tumor cell invasiveness (105).

An intriguing discussion is now underway regarding the role of obesity-mediated changes in the tumor microenvironment and cancer progression (106). Obesity has been implicated in the promotion of inflammation and fibrosis, particularly through the engagement of hypoxia-induced transcriptional programs in adipocytes and the subsequent recruitment of immune cells. In mouse models of spontaneous pancreatic ductal adenocarcinoma (PDAC) it was shown that adipocyte-mediated inflammation contributed to a desmoplastic response through the recruitment of TANs, which enhanced tumor formation in obese animals (107). It has also been demonstrated that mammary adipose tissue in obese mice contained larger myofibroblast populations than in lean counterparts and that these myofibroblast populations contributed to ECM stiffness by

synthesizing ECM components, promoting collagen alignment and fibronectin unfolding, enhancing invasive behaviors of malignant and pre-malignant human breast cancer cells (108). This study provided a link between obesity and the increased myofibroblast populations observed in mammary adipose tissue, with the consequent increased ECM stiffness and tumor promotion.

However, there is also emerging evidence that CAAs influence tumor ECM remodeling. Adipocytes derived from human peri-prostatic adipose tissue primed by prostate carcinoma cells were found to upregulate TNF-α, osteopontin, and MMP9, which are known to regulate ECM architecture (109). Furthermore, adipocytes secrete and process collagen VI, which provides pro-survival signals at the early stages of tumor growth in murine mammary ductal carcinoma (also consistent with observations in human breast cancer tissues), and its cleavage product endotrophin, promotes mammary tumor growth via recruitment of endothelial cells and macrophages that subsequently stimulate angiogenesis, fibrosis and an inflammatory environment (110). CAA-derived endotrophin induced TGF-β mediated EMT in mammary cancer cells (111) and CAAs also promoted tumor cell invasiveness by upregulation of versican and leptin in renal cell carcinoma cell lines (112). Overall, these observations provide circumstantial evidence for a role for CAA in the microenvironment and particularly the formation of a tumor-permissive ECM, suggesting that more work using *in vivo* models is warranted.

Tumor Infiltrating Lymphocytes (TILs)

Tumor infiltrating lymphocytes (TILs) which include CD8+ cytotoxic T lymphocytes (CTLs), CD4+ T helper lymphocytes (Th), CD4+ regulatory T lymphocytes (Treg), γδT cells, and B-cells. Tumor-suppressing roles of T helper and cytotoxic T cells have been widely studied (113, 114). However, TILs can also contribute to the tumor-promotion through the interplay with other stromal components, such as macrophages or neutrophils and the cytokines they secrete. In response to IL-23, IL-6, and TGF-β in the tumor microenvironment, γδT cells secrete IL-17 and induce angiogenesis in a transplantable sarcoma model in mice (115) and in response to tumor-derived IL-1β, they produce IL-17 and induce systemic, G-CSF-mediated activation of neutrophils in mammary tumors to promote cancer-cell metastasis to the lungs (116). It has also been observed that IL-4 secreting CD4+ T lymphocytes were able to indirectly promote tumor invasiveness and pulmonary metastasis of mammary tumors via enhancing pro-tumor properties of tumor associated macrophages (117).

Tregs, on the other hand, are thought to exert an immunosuppressive influence within the tumor microenvironment and are able to induce apoptosis of NK cells via direct cell-to-cell contact as well as through TGF-β secretion (118), but under some circumstances may promote tumor angiogenesis via the production of VEGFA, as has been demonstrated in an ovarian cancer murine xenograft model (119).

Activated B-cells contribute to pre-malignant inflammatory responses and to enhance tumor growth in the HPV-16-driven multistage epidermal carcinogenesis model (120). In castration resistant prostate cancer, tumor infiltrating B-cells secrete lymphotoxin (LT) α:β which engages with LTβR on cancer cells and activates the STAT3 pathway to promote androgen-independent cancer cell growth (121). Interestingly upon STAT3 activation in B-cells there has been observed an increased angiogenesis in B16 melanoma and Lewis lung cancer models, however a direct role of B-cells in angiogenesis is still unclear (122).

TILs have not been directly implicated in the production of ECM. Nevertheless, they are important regulators of the cellular composition of the tumor microenvironment and play indirect roles in the establishment of a tumor-promoting matrix via their role in ECM remodeling. Lymphocytes express ECM modifying enzymes such as MMPs and the urokinase plasminogen activator system in order to traverse basement membrane (123). It has been demonstrated that *ex vivo* purified peripheral lymphocytes respond to chemokine and cytokine stimulation by increased MMP-9 production (124). Furthermore, fibronectin-mediated activation of focal adhesion kinase (FAK) regulates the expression and release of MMP-2 and MMP-9 by T lymphocytes *in vitro* (125). T lymphocytes isolated from the spleens of mammary tumor-bearing mice exhibit elevated production of MMP-9 at both the mRNA and protein level (126). Besides MMP production human T-cells are capable of inducing MMP-9, MMP-1, and MMP-3 expression *ex vivo* in human endothelial cells through CD40/CD40 ligand interaction (127). Another study has demonstrated that lymphoma cells were able to induce MMP-9 expression in fibroblasts and macrophages (128). While the foregoing demonstrates that lymphocytes can produce ECM remodeling enzymes, there is as yet no evidence to suggest that this is a feature of tumor growth and progression *in vivo*. More work is therefore required to determine whether lymphocyte-mediated ECM remodeling has a direct function in tumor progression and cancer cell plasticity.

Cancer Cells

While much of the aberrant ECM production and remodeling in cancer is initiated within the stroma (129), cancer cells themselves can produce some ECM proteins. Proteomics-based analysis of xenografted breast cancers revealed that highly aggressive and metastatic cancer cell lines produced ECM components such as fibronectin, fibrinogen, laminins, periostin, collagens I, III, IV, V, and VI, transglutaminase 2, and hyaluronan. Of note, production of certain components of the ECM is associated with increased metastatic potential of cancer cells—particularly LTBP3, SNED1, EGLN1, and S100A2. LTBP3 has been previously implicated in the regulation of TGF-β secretion and promotion of tumor invasion and metastasis. S100A2 overexpression has been shown to promote lung metastasis of non-small-cell lung carcinoma cells (130, 131).

The best documented and arguably principal path to the ECM conditioning by cancer cells is through deregulation and/or increased production of ECM-modifying enzymes. Uncontrolled tumor cell proliferation and limited tissue blood

supply induces intra-tumoral hypoxia, which in turn induces expression of the gene encoding the collagen and elastin cross-linking enzyme lysyl oxidase (LOX) in human tumor cells (132). LOX-mediated collagen and elastin crosslinking leads to stiffening of the ECM and enhances invasive migration of human breast and cervical cancer cells lines under hypoxic conditions (133). Furthermore, ECM stiffening activates integrin signaling, promote focal adhesion assembly and enhance PI3 Kinase (PI3K) activity that leads to tumor progression and invasion (20, 134). ECM stiffening also promotes growth, survival, migration, and proliferation of cancer cells via integrin ligation and engagement of the Rho-ROCK, PI3K, and MAP/ERK signaling pathways (135) and acute compressive stress such as that encountered in the microenvironment during early stages of epithelial tumor growth can activate Rho-ROCK signaling and downstream actomyosin tension to enhance proliferation and generate an EMT profile (136).

Interestingly, hypoxia also affects the ability of tumor cells to produce collagen-modifying enzymes [reviewed in (137)]. Hypoxia-mediated upregulation of collagen prolyl 4-hydroxylases (P4H) in breast cancer cells has been found to be an important contributor to cancer cell invasion and metastasis (138). Another collagen-modifying enzyme, procollagen-lysine 2-oxyglutarate 5-dioxygenase 2 (PLOD2), was implicated in fibrillar collagen formation by breast cancer cells and as a result enhanced breast cancer cell metastasis to lymph nodes and lung (139).

Another class of ECM-modifying enzymes produced by cancer cells are matrix metalloproteinases. It has been observed that hypoxia-induced upregulation of MMP2 and MMP9 in breast and colon cancer cells contributed to tumor cell invasion (140, 141) and a membrane-bound form of MMP—MT1-MMP (MMP-14) is also induced via hypoxia in breast and renal carcinoma cells (141). MMP14 is required for multicellular invasion of breast cancer cells (142) and is key to breakdown of the basement membrane prior to invasion (143). Induction of an EMT program in breast cancer cells causes MMP production, and increased expression of MMP3, MMP10, and MMP13 was observed upon TGF-β stimulation of human breast cancer cell lines. Upon induction of EMT via hydrogen peroxide treatment in murine mammary epithelial cells, production of MMP2, MMP12, and MMP13 was observed (144).

In another example, enforced activation of Rho kinase signaling in pancreatic ductal adenocarcinomas (PDAC) in mice caused increased production of Mmp10 and Mmp13, which were released in micro-vesicles. This enabled efficient collagen degradation within close vicinity of the cancer cells and as a result enhanced PDAC cell proliferation and collective invasion (145). These observations add to emerging evidence that tumor epithelial cells release micro-vesicles that induce extracellular matrix remodeling (146–148).

Actin-rich membrane structures such as focal adhesions and invadopodia have also been implicated in ECM remodeling by cancer cells. These structures contain an assembly of scaffolding proteins (WASP, N-WASP, and VASP) paired with actin-remodeling proteins (such as cortactin and gelsolin). These structures are able to incorporate integrin-mediated signaling and recruit Rho GTPases, myosins, Src kinases, and dynamin (149). Focal adhesions and invadopodia are essential for cell migratory behavior *in vitro* and for actomyosin-based contractility (150).

Focal adhesions are known to integrate multiple signaling inputs and transduce them across the cell (151). However, a recent study showed that cancer cells are also able to degrade the ECM at focal adhesion sites via recruitment of MMP14 (152). An important characteristic of migrating cancer cells is the formation of actin-rich membrane extensions termed invadopodia. In cancer cells, mature invadopodia are enriched in MMP2, MMP9, and MMP14. It is important to note that MMP2 and 9 are particularly essential for type IV collagen remodeling and subsequent breaching of the basement membrane (153). Invadopodia are also important for the extravasation of squamous carcinoma, breast cancer, and bladder cancer cells as well as melanoma cells (154). Whilst *in vitro* studies have shown that invadopodia formation is important for basement membrane penetration, conclusive *in vivo* evidence is lacking regarding the physiological roles of invadopodia.

In this section and summarized in **Table 1**, we have provided snapshots of the biology of the many cell types and discussed what is known about how they influence tumor cell plasticity in the context of ECM, to regulate EMT and stemness. In Section Molecular Regulators of the ECM That Influence Cancer Cell Plasticity below, we will discuss the molecular regulators that are employed across these various cell types to carry out cellular processes and promote tumor progression.

MOLECULAR REGULATORS OF THE ECM THAT INFLUENCE CANCER CELL PLASTICITY

The microenvironment is precisely regulated by several molecular players that have evolved to return this system to its steady state in the shortest possible time following perturbation, while also permitting it to adapt quickly to changed circumstances such as injury or disease. This ability to quickly adapt to circumstances and resilience under injury can be co-opted by disease states such as cancer and accounts for a significant component of the plasticity exhibited by cancer cells. Changes in the mechanical and biochemical properties of the ECM have been linked to cancer cell plasticity that promotes increased invasiveness and metastatic potential (159). Furthermore, tumor cells that have undergone EMT, TAMs, TANs, and CAFs are all capable of producing ECM components and degrading and remodeling the ECM to facilitate tumor cell plasticity and disease progression as we have discussed above. Below, we discuss some of the molecular players that mediate ECM production and re-modeling by these cell types, to promote tumor progression and cancer cell plasticity.

Tumor and stromal cells employ several signaling pathways that regulate the biochemical and biomechanical interactions between the parenchyma and the microenvironment to establish, remodel and maintain the ECM. Whereas normal epithelial cells produce only small amounts of ECM, fibroblasts, tumor

TABLE 1 | Cellular regulators of the ECM and cancer cell plasticity.

Cell type	ECM changes	Influence on cancer cells and their plasticity	References
Cancer associated fibroblasts (CAFs)	Collagen production, fiber alignment and increased ECM stiffness	Growth and motility, invasion, angiogenesis, increased metastatic potency	(26, 29, 41, 43, 155)
	Establishing actomyosin tracks	Migration, invasion	(44, 156)
	MMP-1 secretion	Motility, invasiveness	(157)
	MMP-2 and MMP-9 secretion	EMT	(33)
	Tenascin C production	Invasiveness	(42)
	Periostin production	Stem cell niche maintenance	(36)
	Production of collagens, fibronectin, osteopontin and periostin leading to desmoplasia	Proliferation	(64, 65)
Tumor associated macrophages (TAMs)	Production of MMPs (1, 9, 12 and 14), serine proteases, cathepsins (B, S, C, L, Z), lysosomal enzymes and ADAMs	Invasion	(64, 66, 158)
	Secretion of ECM remodeling enzymes and liberation of ECM-bound growth factors	Proliferation, motility	(64, 68)
Tumor associated neutrophils (TANs)	Secretion of MMP9	Proliferation, invasiveness, angiogenesis, extravasation, metastasis	(82, 83)
	Elastase production	EMT	(84)
	NETosis, upregulation of MMP9, cathepsin G and neutrophil elastase	Proliferation, migration and angiogenesis	(91)
	Establishment of collagen tracks	Invasion	(87)
Cancer associated adipocytes (CAAs)	Secretion of MMP9 and osteopontin	proliferation, motility	(109)
	Production and processing of collagen VI	Survival, growth, angiogenesis, EMT	(110, 111)
	Secretion of versican	Invasion, progression	(112)
Cancer cells (CSs)	Secretion of LOX that crosslinks collagen and elastin, increasing ECM stiffness	Proliferation, survival, invasion	(132, 133)
	Secretion of ECM-modifying enzymes: collagen prolyl 4-hydroxylases (P4H), procollagen-lysine 2-oxyglutarate 5-dioxygenase 2 (PLOD2)	Invasion, metastasis	(138, 139)
	MMP2 and MMP9, Mmp10 and Mmp13, Mmp14 secretion and expression leading to collagen remodeling	Invasion, proliferation, cell migration, collective invasion	(140, 141, 145)

cells, and certain immune cells like macrophages have the capacity to produce vast quantities of the proteins that form this meshwork scaffold and are largely responsible for its production and maintenance. Nevertheless, they do not perform this task independently, but are regulated by biochemical and biomechanical cues from the parenchyma.

Established molecular pathways that regulate ECM properties include TGF-β, CTGF, and Wnt signaling axes and the mediators of the YAP signaling system, which are discussed here.

TGF-β Signaling

TGF-β family members are multifunctional cytokines with roles in wound healing, tissue repair, and cancer, and regulate a signaling cascade largely involved in the transcriptional regulation of genes that control EMT and stemness (160). Activation of this signaling cascade is initiated by the binding of a TGF-β ligand to a Type II receptor serine/threonine kinase on the cell surface, resulting in recruitment of the type I receptor to the complex. The Type II receptor trans-phosphorylates the Type I receptor at serine and threonine residues in the highly conserved juxta-membrane GS domain, and the phosphorylated Type I receptor propagates signaling by phosphorylating the SMAD signal transducer proteins. SMAD proteins are latent transcription factors and once phosphorylated can translocate into the nucleus and regulate transcription of target genes in cooperation with nuclear cofactors and the transcription machinery (161).

The numerous TGF-β superfamily of ligands (at least 42 in humans, 9 in fly, and 6 in worm) comprise two major subfamilies based on structure and function. These are the TGF-β/Activin/Nodal subfamily and the bone morphogenetic protein/growth and differentiation factor/Müllerian inhibiting substance subfamily (BMP/GDF/MIS) (162). While each of these cytokines can elicit a different set of responses via the TGF-β signaling pathway underlying the highly pleiotropic nature of this pathway, they share several common features of sequence, structure and function, namely, six conserved cysteine residues which generate a cysteine knot structure via three disulfide

TABLE 2 | Molecular regulators of the ECM and cancer cell plasticity.

Pathway	Effects on the ECM	Plasticity-dependent cellular processes influenced	References
TGF-β	Upregulation of collagen, lysil oxydase expression in cancer cells and stiffening of ECM	Motility and proliferation	(172, 173)
	Upregulation of tenascin C in CAFs	Invasiveness	(42)
Rho/ROCK	Remodeling of focal adhesions	Cell migration and adhesion	(214)
	Activation in tumor epithelial cells induces production of collagen, fibronectin, tenascin C, periostin by fibroblasts, increases ECM stiffness	Tumor progression, enhanced wound healing	(20, 23)
Notch	Indirect - influencing ECM sensing by integrin; maintenance of stemness	Normal stem cell maintenance; acquisition of CSC phenotype	(222, 223)
FGF	Influences hedgehog-induced ECM production by CAFs; cooperates with TGF-β in EMT	Acquisition of stem cell phenotype; EMT	(37, 224)
HGF	Mediates fibroblast-tumor cell communication; indirectly facilitates ECM degradation	EMT	(225)

bonds (163) and the ability to act only in the dimerized form. Furthermore, there are 7 type I and 5 type II receptor Ser/Thr kinases in humans. Both receptor types have an N-terminal ligand-binding domain, a transmembrane domain and a C-terminal Ser/Thr kinase domain. Type I (but not type II) receptors also contain a characteristic SGSGSG sequence, the "GS domain," which is phosphorylated by the Type II receptor. SMAD proteins are divided into three functional classes: the receptor-regulated SMADs (R-SMADs), the co-mediator SMAD (Co-SMAD), and the inhibitory SMADs (I-SMADs). R-SMADs are directly serine-phosphorylated at a conserved C-terminal SSXS motif by the Type I receptor. R-SMADs comprise the BMP-receptor-regulated SMADs (1, 5, and 8), and the TGF-β/Activin receptor-regulated SMADs (2 and 3). The Co-SMAD, SMAD4, can hetero-dimerize with phosphorylated R-SMADs and is involved in mediating their translocation into the nucleus. I-SMADs (6 and 7) negatively regulate signaling by competing with R-SMADs for receptor and Co-SMAD binding. They are also able to target receptors for degradation, thereby regulating signal flux through this pathway. Therefore, the high level of redundancy present within this signaling pathway has the potential to greatly influence context-specific outcomes mediated by the activation of diverse and distinct transcriptional profiles.

TGF-β is secreted by many cell types, including those abundant within the tumor microenvironment such as activated macrophages (164), endothelial cells (165), and fibroblasts (166). Tumor cells also secrete TGF-β, which can elicit context-dependent responses that suppress tumor growth at early stages of the disease, but promote tumor progression at later stages (167). Nevertheless, two key functions of TGF-β signaling in the cancer microenvironment are regulation of immune evasion and ECM remodeling. TGF-β signaling has been demonstrated to regulate phenotypic plasticity of cancer cells arising in diverse tissues including the skin (168), intestine (169), breast (170), and lung (171).

TGF-β signaling controls the transcription of a suite of genes, including those encoding ECM proteins such as collagen, and ECM remodeling enzymes such as lysyl oxidase (172, 173), via regulation of the transcription factor MYC. This signaling pathway is therefore associated with increased ECM stiffness, which induces proliferation and mesenchymal behavior in resident tumor cells by promoting integrin ligation and downstream signaling pathways. Interestingly, local concentrations of TGF-β are enhanced and its spatial activity regulated by its immobilization onto the ECM (174), which results in the capacity to influence cancer cell plasticity at specific regions of the tumor.

Given the high level of reciprocal crosstalk between TGF-β, the ECM and cellular plasticity as detailed above, this signaling pathway is well-established as a key target in cancer therapy. However, the pleiotropic and context-dependent functions of the pathway have hampered the development of tractable agents that reliably target TGF-β-regulated tumor cell plasticity.

CTGF Signaling

The Connective Tissue Growth Factor (CTGF, sometimes referred to as CCN2) is a member of the CCN family of non-structural ECM proteins and is therefore most appropriately termed a matricellular protein. It can interact with a large array of signaling molecules, including bone morphogenetic proteins (BMP), TGF-β, VEGF, IGF, and Wnt ligands as well as directly bind trans-membrane receptors such as integrins, Notch receptors, TGF-β receptors, and lipoprotein receptor-related proteins (LRPs) to elicit the corresponding signaling cascades (175, 176). In cancer, a key function of CTGF is to mediate fibronectin production by stromal cells downstream of TGF-β signaling, which transcriptionally regulates *CTGF*. Fibronectin in turn determines the biosynthesis and fibrillogenesis of collagen 1, the main component of the cancer ECM.

CTGF has been shown to regulate the MET of head and neck cancer cells (177) and drug resistance in glioblastoma (178), both via a mechanism involving the re-expression of pluripotency genes. Furthermore, CTGF inhibition reduces the growth of metastatic melanoma in an animal model (179). These data suggest that CTGF plays a role in the

metastatic colonization by cancers of distal sites by enhancing pluripotency and MET. It is not clear whether these two functions are linked and to what extent they are also mediated by crosstalk with other, closely regulated, CCN family proteins.

Wnt/β-Catenin Signaling

Wnt ligands are a large family of secreted glycoproteins that can activate three distinct intracellular signaling pathways—the β-catenin pathway (also known as the canonical Wnt signaling pathway), the planar cell polarity pathway (involving Jun N-terminal kinase [JNK]-mediated cytoskeleton rearrangements) and the Wnt/Ca^{2+} pathway, by interacting with cell surface bound Frizzled receptors. Critical to Wnt signaling are low density lipoprotein (LDL) receptor-related proteins, which act as co-receptors of the Wnt signal (180). Transduction of the signal via Frizzled is mediated by the intracellular protein Disheveled (181), which acts to inhibit the kinase GSK3B through its interaction with Axin (182–184).

GSK3B exists in a large multi-protein complex containing Axin, β-catenin, and the adenomatous polyposis coli (APC) protein (185–188). In the absence of Wnt ligands, β-catenin is maintained in the phosphorylated state at its amino-terminal Ser/Thr residues by GSK3B. Phosphorylated β-catenin is ubiquitinated by β-TRCP and thereby targeted for degradation via the proteasome pathway (189, 190). Wnt ligand binding to Frizzled receptors causes GSK3B inactivation by Disheveled, resulting in the accumulation of non-phosphorylated β-catenin, which cannot be ubiquitinated and is therefore protected from proteasome mediated degradation. β-catenin associates with the T cell factor/lymphocyte enhancer factor (Tcf/LEF) family of transcription factors and functions as a co-activator of transcription upon translocation of the β-catenin/Tcf/LEF complex to the nucleus (191). In the absence of Wnt ligand, non-phosphorylated β-catenin levels are low and Tcf proteins are bound to various inhibitory molecules (including Groucho proteins, CtBP, and in Drosophila, CBP), preventing the transcription of target genes (192–195).

The role of Wnt/β-catenin signaling in EMT has been well known for some time. Wnt signaling was first demonstrated to stabilize and thereby control the turnover of the EMT regulator Snail1 (196) and increase the expression of two further EMT regulators, Slug (197) and Twist (198). *In vivo* evidence for the role of Wnt signaling in regulating EMT and metastasis has been provided in breast cancer (199) and pancreatic cancer (200). It is also becoming clear that that non-canonical Wnt signaling initiated by Wnt5b regulates metastasis via EMT (201).

The Wnt signaling pathway has also been demonstrated as a regulator of stemness, both in stem cell maintenance and renewal, for example in the intestine (202), as well as in stem cell differentiation and fate determination via transcriptional targets such as Sox9 (203). This can be brought about by the activation of distinct subsets of transcriptional targets and by signaling crosstalk between this signaling pathway and others. For instance, crosstalk between prostaglandin signaling and Wnt signaling is required for the developmental specification of stem cell populations in the hematopoietic system as well as in the liver and other organs (204), and also in the de-differentiation process that gives rise to stem-like cells in cancers such as cutaneous squamous cell carcinoma (20). Taken together, these observations firmly place the Wnt/β-catenin signaling pathway as a key regulator of cell plasticity in normal development, but also in cancer.

Functional interactions between the Wnt signaling pathway and the extracellular matrix are being uncovered, most prominently in normal development of bone, where it is emerging that mechanotransduction signaling initiated by ECM stiffness regulates Wnt secretion (205). These observations have obvious implications for the cancer context in which enhanced mechanotransduction is a well-established pathology.

Rho/ROCK Signaling

The 22-member RHO family of small GTPases are named for their homology to the Ras proto-oncogenes. Of these, the best characterized are RHOA, RAC1, and CDC42, which have distinct roles in regulating actin polymerization and turnover, and myosin contractility (206). These small GTPases are co-opted by many signaling pathways to modify the actomyosin cytoskeleton and thereby underpin most cellular processes. ROCK kinases 1 and 2 (207) are key effectors of signaling through RHOA and are activated by direct binding of GTP-bound active RHOA (208–210). Active ROCK kinases signal via a collection of context-dependent downstream pathways that are mainly involved in regulation of actomyosin cytoskeleton properties including actin polymerization and cytoskeletal contractility. Key mediators of ROCK signaling are the LIM kinases, whose major role is to phosphorylate and inhibit the actin severing Cofilins, thereby stabilizing the actin cytoskeleton and promoting invasiveness through generation of a tumor-permissive network (211). LIMK2 has also been shown to integrate RHO signaling and p53 functions, thereby mediating cell survival functions in cancer cells, with implications for tumor plasticity and progression (212). Signaling downstream of ROCK also regulates myosin contractility via ROCK-mediated phosphorylation and activation of the regulatory myosin light chain MLC2, as well as phosphorylation and inactivation of the myosin binding subunit of the myosin phosphatase MYPT1 (213). These two signaling arms, resulting in actin cytoskeleton stabilization and myosin contractility, therefore have a major role in regulating intracellular tension and thereby integrate several mechanotransduction pathways within the cell, including the Wnt and YAP pathways.

A key role for RHO-ROCK signaling has been delineated in the tissue and tumor microenvironments, to complement its well-established function in cell migration and adhesion (214). The pathway accomplishes this via the increased production of ECM components to balance intracellular tension, thereby maintaining mechano-reciprocity (20). This recent appreciation that ROCK activity regulates ECM production and remodeling [reviewed in (135)] highlighted the possibility of novel negative regulators of this pathway that may be of therapeutic utility.

Accordingly, 14-3-3ζ, which belongs to the 14-3-3 family of molecular adaptors and chaperones, has been shown to bind to and promote the activity of the myosin phosphatase targeting subunit Mypt1, thereby increasing signaling flux through the RHO-ROCK pathway (23). Interestingly, a family of sphingosine mimetics, which had been previously demonstrated to inhibit 14-3-3 by disrupting dimer formation (215), accelerates the production of ECM components required to normalize the cutaneous microenvironment thereby hastening wound healing. However, this mechanism is hijacked by cancers such as cutaneous squamous cell carcinoma, where 14-3-3ζ is downregulated frequently and is associated with tumor progression (23). These observations suggest that as in the case of M2 polarized "wound healing type" macrophages, cancers can exploit mechanisms that have evolved to facilitate wound healing, to assist with tumor progression. Further work is required to determine whether this phenomenon may be exploited in cancer therapy or indeed whether other mechanisms mediating ECM re-establishment may be similarly engaged to target the tumor microenvironment as a novel therapy approach.

Hippo Signaling

The still rather enigmatic Salvador/Warts/Hippo pathway is a highly conserved signaling pathway and acts as a controller of organ size in animals by regulating the balance between cell proliferation and death (216). The pathway has evolved to control the activity of the transcriptional regulators YAP and its paralog TAZ, which promote proliferation by associating with the TEAD transcription factors (217). In its activated state, the pathway consists of a Ser/Thr kinase cascade initiated by the transmembrane cadherin FAT that results in the phosphorylation of YAP/TAZ to create a binding site for 14-3-3 proteins. Upon binding of 14-3-3, YAP/TAZ is sequestered in the cytoplasm and is therefore not available in the nucleus to induce the transcription of target genes. The YAP/TAZ inhibitory kinase cascade is regulated by a variety of different inputs, including hormone and growth factor signaling. However, YAP has also been reported to be regulated by RHO GTPase activity mediated by ROCK, in a Hippo pathway-independent mechanism (218) and thereby links mechanotransduction to the transcription of genes that promote cell proliferation (219). More recently, it has been revealed that TEAD2 regulates the expression of EMT genes by directly controlling the sub-cellular localization of YAP/TAZ (220). As such, it is a key mediator of cancer cell plasticity and neoplastic progression downstream of changes in ECM stiffness. The Hippo signaling pathway has also demonstrated to engage in crosstalk with the Wnt/β-catenin signaling pathway and Notch pathway in the context of hepatocellular cancers (221), suggesting that the three mechanotransduction pathways are capable of cooperating to promote tumor progression via the regulation of plasticity, suggesting that Hippo signaling could contribute to the mechano-reciprocal feed forward loop that we have previously proposed (135).

These and other signaling molecules that regulate ECM structure and function to influence cancer cell plasticity both directly and indirectly are summarized in **Table 2**.

IMPLICATIONS FOR CANCER THERAPY

As we have discussed above, cancer cell plasticity permits tumors to adopt shifting identities that allow them to adapt to changing environments, modify their microenvironment to suit their needs and evade the immune system. In this effort, cancers can co-opt and deftly commandeer many of the body's own normal homeostatic processes such as wound healing, immune surveillance and maintenance of the stem cell niche. While this poses a significant challenge to cancer therapy, it also provides us with an opportunity to target the aberrant microenvironment that has been built around the tumor. A key vulnerability of tumors exhibiting plasticity is their need to subvert the activities of genetically normal stromal cells for their own purposes by biochemical and biomechanical means. This provides us with an opportunity to block signals traveling between cancers and their stroma pharmacologically, using antibody therapy or by modifying the mechanical environment of the tumors; or indeed a combination of all three. Coupled with precision therapies tailored to the tumor genotype, a multi-pronged approach targeting the tumor as well as its microenvironment has the potential to revolutionize cancer therapy.

As a note of caution however, it is important to appreciate that plasticity may also provide tumors with the means to evade such combination therapies. It is therefore imperative that the core set of principles driving cancer cell plasticity be soundly researched and fully appreciated. Given the plethora of autochthonous animal models of human cancers and more recently the patient-derived xenograft models being propagated in immunologically humanized animals, we believe the tools are being rapidly assembled to make this a reality.

AUTHOR CONTRIBUTIONS

VP wrote the review and edited the manuscript. MK wrote the review and edited the manuscript. SP wrote the review and edited the manuscript. MS conceived of the review, planned the outline, wrote the review and edited the manuscript.

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Breast Cancer-Derived Exosomes Alter Macrophage Polarization *via* gp130/STAT3 Signaling

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Tumor-derived exosomes are being recognized as essential mediators of intercellular communication between cancer and immune cells. It is well established that bone marrow-derived macrophages (BMDMs) take up tumor-derived exosomes. However, the functional impact of these exosomes on macrophage phenotypes is controversial and not well studied. Here, we show that breast cancer-derived exosomes alter the phenotype of macrophages through the interleukin-6 (IL-6) receptor beta (glycoprotein 130, gp130)-STAT3 signaling pathway. Addition of breast cancer-derived exosomes to macrophages results in the activation of the IL-6 response pathway, including phosphorylation of the key downstream transcription factor STAT3. Exosomal gp130, which is highly enriched in cancer exosomes, triggers the secretion of IL-6 from BMDMs. Moreover, the exposure of BMDMs to cancer-derived exosomes triggers changes from a conventional toward a polarized phenotype often observed in tumor-associated macrophages. All of these effects can be inhibited through the addition of a gp130 inhibitor to cancer-derived exosomes or by blocking BMDMs exosome uptake. Collectively, this work demonstrates that breast cancer-derived exosomes are capable of inducing IL-6 secretion and a pro-survival phenotype in macrophages, partially *via* gp130/STAT3 signaling.

Keywords: cancer-derived exosomes, breast cancer, tumor-associated macrophages, glycoprotein 130, interleukin-6, STAT3

INTRODUCTION

Breast cancer is the second most frequently diagnosed cancer type for females worldwide, accounting for approximately 1.67 million cases per year (1). The primary cause of mortality in breast cancer patients is caused by the spread of tumor cells to other organs such as lung (2), brain (3), and bone (4). Recently, small vesicles released by cancerous cells, termed as exosomes, were described to be markers, mediators, and inducers of metastasis (5).

Exosomes are small extracellular, bilipid vesicles that exhibit a size distribution of 30–150 nm, sediments at 100,000 *g*, and have a specific density of 1.13–1.19 g/mL (6–8). In contrast to other vesicles, exosomes are secreted after fusion of multivesicular bodies with the plasma membrane, resulting in proteins involved in this process to be uniquely associated with exosomes (9). Associated proteins include parts of the endosomal sorting complex required for transport proteins (Hrs and Tsg101) and tetraspanins (CD9, CD63, and CD81) (9). Exosomes derived from diverse types of cancer

cells, including leukemia, ovarian, lung, and breast cancer, have shown distinct molecular profiles when compared with exosomes produced by corresponding untransformed, normal cells (9, 10).

Previous work from our group showed that breast cancer-derived exosomes accumulate in the lung, spleen, and bone of naïve mice (11). At these sites, the exosomal content causes pro-metastatic alterations associated with reduction of both T cell proliferation and NK cell cytotoxicity (11). For this reason, exosomes have become a valuable target in identifying novel cancer biomarkers that could potentially diagnose cancer and predict patient outcomes or treatment responses (12, 13).

Even though their role in immune response modulation is not completely understood, recent studies have shown that cancer-derived exosomes can direct immune cells toward a tumor-promoting phenotype, and significantly contribute to different aspects of tumor progression, including promotion of tumorigenesis, invasion of the surrounding tissues, angiogenesis, formation of pre-metastatic niches, and metastatic dissemination (14). For example, tumor-derived exosomes regulate the differentiation of myeloid progenitor cells (15). Furthermore, breast carcinoma-derived exosomes have been demonstrated to mediate the recruitment of myeloid-derived cells to the spleen and tumor, which in turn promotes cancer growth and neo-angiogenesis (16). Classically activated macrophages can respond to cancer cells with phagocytosis and release of inflammatory cytokines triggered by tumor-associated antigens. On the other hand, macrophages infiltrating established tumors are known to produce anti-inflammatory cytokines and support tumor progression (17). These cancer-associated macrophages have also been demonstrated to contribute to metastasis, especially to the formation of the pre-metastatic niche (16). For example, STAT3 phosphorylation, and therefore activation, in macrophages is commonly observed in the tumor microenvironment. Blockade of STAT3 signaling in these cells results in the secretion of pro-inflammatory cytokines (18, 19).

Interleukin-6 (IL-6) is regarded as both as a pro- and anti-inflammatory cytokine. Upon activation of IL-6 signaling, IL-6 receptors, such as IL-6Rα and IL-6Rβ (also known gp130), engage to form a dimeric structure (20). Signaling *via* these receptors activates JAK tyrosine kinases and transcriptional factors, in particular, STAT3 (21). Once STAT3 is activated, it translocates into the nucleus, inducing gene expression of IL-6, LOX, and other genes, creating an induction of IL-6 autocrine loop and tumorigenesis (22). Macrophages activated by interferon gamma (IFNγ) and LPS express high levels of IL-6 (23). There is evidence that IL-6 is also expressed by macrophages found in the tumor microenvironment, especially by alternatively polarized macrophages (24, 25). Furthermore, it has been reported that blockade of IL-6 affects tumor-infiltrating immune subsets, for example, reducing the number of myeloid-derived suppressor cells and their suppressive abilities (26). This is also observed in the development of lung cancer, with reduced frequency of tumor-associated macrophages which produce Arg1, CCL2, IL-10, and TGF-β (26).

This study reveals that IL-6 receptor gp130 is contained in breast cancer cell-derived exosomes and stimulates STAT3 signaling in bone marrow-derived macrophages (BMDMs). In response to exosome exposure, these BMDMs upregulate pro- and anti-inflammatory cytokines and acquire an increased survival potential. Our findings indicate that cancer-derived exosomes are capable of changing macrophage phenotype by transferring the IL-6 receptor gp130, thereby assisting in establishing a pro-tumorigenic cancer microenvironment.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were purchased from the Walter and Eliza Hall Institute (Melbourne, VIC, Australia) and female mice used at 8–10 weeks of age. All animal procedures were conducted in accordance with Australian National Health and Medical Research regulations on the use and care of experimental animals, and approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (A12617M, P1499).

Cell Culture

The murine C57BL/6 EO771 cells were maintained in DMEM with 5% FBS and 1% penicillin/streptomycin as described previously (27).

Antibodies and Reagents

Synthetic unilamellar 100-nm sized liposomes (nanoparticles made from phosphatidylcholine and cholesterol, but lacking any protein content) were purchased from Encapsula Nanosciences. The primary and secondary antibodies used for western blotting, immunofluorescence, and flow cytometry are as listed in Table S1 in Supplementary Material.

Isolation of Exosomes

The culture supernatants of EO771 cells at approximately 60–70% confluence were harvested after 16 h conditioning in serum-free media (11). Exosomes were isolated as previously described (6). Briefly, cells and debris were cleared from the supernatant by centrifugation (500 *g*, 10 min), followed by filtration using 0.22 μm filters (Merck Millipore). Cell-free supernatants were concentrated by ultrafiltration through Centricon Plus-70 Centrifugal Filter (100 kDa; Merck Millipore), spun at 3,500 *g* at 4°C. Exosomes were subsequently purified by overlaying concentrated samples on qEV size exclusion chromatography columns (Izon Science Ltd.) followed by elution with PBS. Finally, the elute from qEV columns were concentrated using Amicon Ultra-4 10-kDa nominal molecular weight centrifugal filter units (Merck Millipore) to a final volume of approximately 200 μL.

Size Distribution Analysis by Tunable Resistive Pulse Sensing (TRPS)

Particle abundance and size were assessed using the Izon qNano system by TRPS technology (Izon Science Ltd.) with the NP100 nanopore and 70-nm calibration beads (CPS70) as previously reported (11).

Electron Microscopy (EM)

Electron microscopy imaging was performed as previously described (6). Briefly, purified exosomes were fixed with paraformaldehyde and transferred to Formvar-carbon-coated

EM grids. Grids were transferred to 1% glutaraldehyde for 5 min, followed by eight washes with water. By contrast, grids were negatively stained with 1% uranyl-oxalate solution, pH 7 for 5 min before transferring to methyl-cellulose-UA for 10 min. Excess fluid was removed and exosomes were imaged in a JEOL 1011 transmission electron microscope at 60 kV.

Western Blotting

Exosome preparations and cell lysates were solubilized with RIPA buffer. Protein content was quantified using a standard Bradford assay or a BCA assay and analyzed by western blotting as previously described (28). Each western was independently repeated at least three times, and representative results are shown. Full-length images of all western results shown in the manuscript are included as Figure S5 in Supplementary Material.

DiD Labeling of Exosomes

Exosomes were fluorescently labeled using Vybrant® DiD (Life Technologies) according to the manufacturer's instructions with modifications (11). Briefly, exosomes were incubated for 10 min with DiD (1:1,000 dilution in PBS) at room temperature and re-purified using qEV size exclusion chromatography columns (Izon Science Ltd.).

Generation of BMDMs

Bone marrow cells were obtained by flushing the femurs and tibias of C57Bl/6 mice. Cell suspensions were treated with ammonium chloride red cell-lysis buffer, washed with PBS, and then 4×10^5 cells/well were cultured in 6-well plates in RPMI supplemented with L cell conditional media (10% FBS, 1% GlutaMAX, 1 mM HEPES, and 1% penicillin/streptomycin) (29). The cells were fed with fresh medium every 2 days of culture. At day 10, macrophage purity was about 70%, as determined by flow cytometric analysis of the surface expression of macrophage markers CD11b and F4/80 using a LSR-Fortessa (BD Biosciences).

Co-Culture of BMDMs and Exosomes

At day 10 of BMDM culture, cells were treated with 10 μg of exosomes for 24 h. Control cells were treated with either an equivalent particle number of 100-nm liposomes (Encapsula Nanoscience) as determined using TRPS or PBS alone. Exosome uptake was inhibited by incubation of BMDMs with 5 μM EDTA (Sigma-Aldrich) for 1 h before exosome treatment. To inhibit exosomal gp130, exosomes were treated with *N′*-(7-Fluoropyrrolo[1,2-a]quinoxalin-4-yl)-2-pyrazinecarbohydrazide (SC144, Sigma-Aldrich) (30). Exosomes were incubated with SC144 for 1 h and were later re-purified using qEV size exclusion chromatography columns.

Flow Cytometry Analysis

BMDMs were detached from culture plates using cold PBS. Cell suspensions were stained with the respective antibodies (Table S1 in Supplementary Material), together with Fc-receptor blocking using anti-CD16/32, and washed with PBS containing 2% FBS. DiD-labeled exosome-positive cells were detected using red laser excitation and 640-nm emission. Flow cytometric acquisition was carried out on a LSR-Fortessa (BD Biosciences), as previously described (31). Data analysis was performed using FlowJo software (Tree Star).

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as previously described (32). Briefly, BMDMs were seeded on cover slips and incubated with exosomes, liposomes, or PBS alone. Samples were fixed with paraformaldehyde for 1 h at room temperature. The cover slips were then incubated with primary antibodies and secondary, fluorochrome-conjugated antibodies. Cover slips were placed on slides containing ProLong® Gold Antifade Mountant with DAPI liquid mountant (Life Technologies). Images were taken on a Zeiss 780-NLO confocal microscope with 40× and 100× magnifications.

Analysis of IL-6 Secretion by Macrophages

Secretion of IL-6 by BMDMs was measured using Mouse IL-6 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions.

qRT-PCR

To analyze RNA expression levels on BMDMs, qRT-PCR was performed. Briefly, RNA was extracted by Trizol and cDNA synthesis was conducted using the SuperScript™ III First-Strand Synthesis system (Invitrogen Life Technologies), according to the manufacturer's instructions. qRT-PCR was performed using Syber green master mix (Life Technologies). The data were analyzed using the $2^{-\Delta\Delta Ct}$ method and relative gene expression levels normalized to GAPDH. Primer sequences are detailed in Table S2 in Supplementary Material.

Cell Survival Analysis

BMDMs were seeded on 6-well plates. Differentiated BMDMs were submitted to different treatments (without L cell supernatant), and the plates were placed in the IncuCyte live-cell imaging system (Essen Bioscience). Cell confluence (measured as the area of the field of view covered by cells) was assessed at 5 time points, as an average of 16 images captured per time point. Data were normalized to cell count at 0 h.

Statistical Analysis

Data are presented as the mean ± SEM of results obtained from at least three independent experiments. Statistical significance was assessed using two-tailed Mann–Whitney *U* tests, with $p < 0.05$ considered statistically significant. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ are indicated in the figure legends.

RESULTS

Characterization of Breast Cancer Exosomes

The morphology of particles isolated from murine EO771 breast cancer cells is that of spherically shaped vesicles, with size ranging from 30 to 150 nm (**Figures 1A,B**). Furthermore, the particles are positive for the exosome marker proteins Tsg101, Flotillin-1, and CD9, but negative for the protein GM130 (**Figure 1C**).

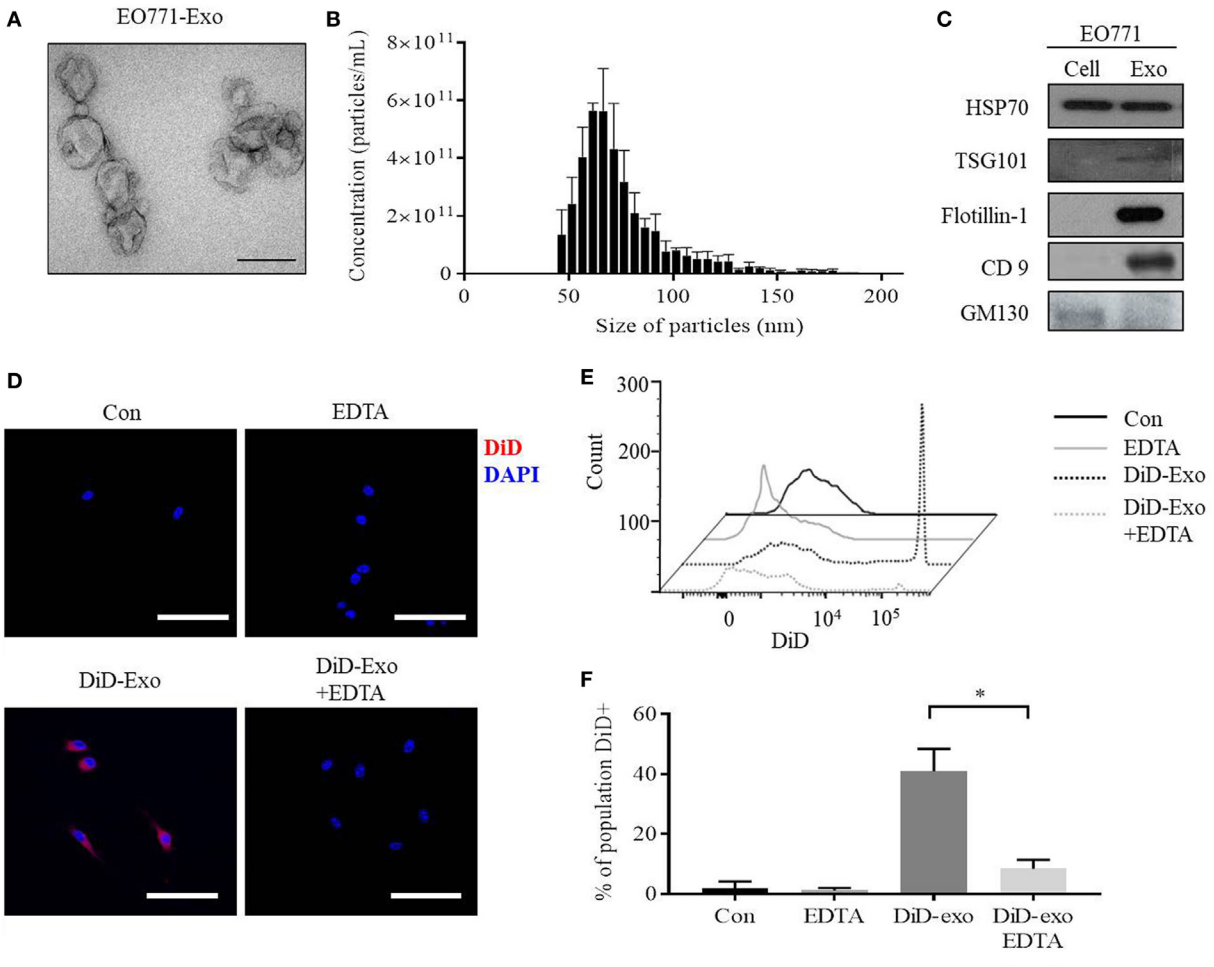

FIGURE 1 | EO771 cells secrete exosomes which are taken up by bone marrow-derived macrophages (BMDMs). **(A)** Transmission electron microscopy of isolated particles indicates a sphere-shaped structure. The size bar represents 100 nm. **(B)** Size distribution and enumeration of particles assessed by Tunable Resistive Pulse Sensing ($n = 3$). **(C)** Expression of exosomal and cell markers in EO771 cell lysate (Cell) and exosomes (Exo). **(D)** Immunofluorescence imaging of DiD-labeled, EO771-derived exosome uptake into macrophages after 24 h. Macrophages were pretreated with EDTA (1 μM) for an hour as indicated. The size bar represents 50 μm. **(E,F)** BMDMs were gated for $CD11b^+/F4/80^+$ double-positive populations and DiD^+ cells quantified. **(E)** Representative histogram of flow cytometry analyses. **(F)** Quantification of four independent repeats. *$p < 0.05$ as indicated.

Collectively, these data show that the particles are exosomes as previously defined (33).

To assess if BMDMs are capable of taking up these exosomes, DiD-labeled exosomes were added to macrophage cultures. Macrophages acquire the DiD fluorophore after 24 h, indicating exosomal uptake (**Figure 1D**, lower left panel). EDTA has been shown to inhibit exosome uptake by interrupting calcium-dependent binding of exosomes to target cells (34). Indeed, EDTA is capable of reducing uptake of DiD-positive exosomes, while non-specific DiD dye uptake alone is not affected (**Figure 1D**, lower right panel; Figures S1A,B in Supplementary Material). Flow cytometry further confirmed that approximately 40% of all $CD11b^+/F4/80^+$ macrophages are DiD-positive after exposure to DiD-exosomes, and this is reduced to 7% by EDTA treatment (**Figures 1E,F**). These results indicate that macrophages take up EO771-derived exosomes, which can be inhibited by EDTA.

Breast Cancer-Derived Exosomes Transfer gp130 to Induce STAT3 Signaling and Phenotypic Changes in BMDMs

Next, we evaluated the impact of cancer-derived exosomes on the IL-6/STAT3 signaling pathway in macrophages. After addition of exosomes, both gp130 and phosphorylated STAT3 levels increase compared with control groups of either PBS- or liposome-treated BMDMs (**Figures 2A,B**). Furthermore, phosphorylated STAT3 was found to translocate to the nucleus of BMDMs in response to incubation with cancer-derived exosomes (**Figure 2C**). Confocal immunofluorescence microscopy also showed that gp130 is localized to cell membranes of macrophages (**Figure 2C**). IL-6 is a key downstream target of the STAT3 signaling pathway and we observed an approximately three-fold induction in IL-6 secretion from BMDMs after cancer exosome exposure (**Figure 2D**). No IL-6 protein was found in cancer-derived exosomes alone

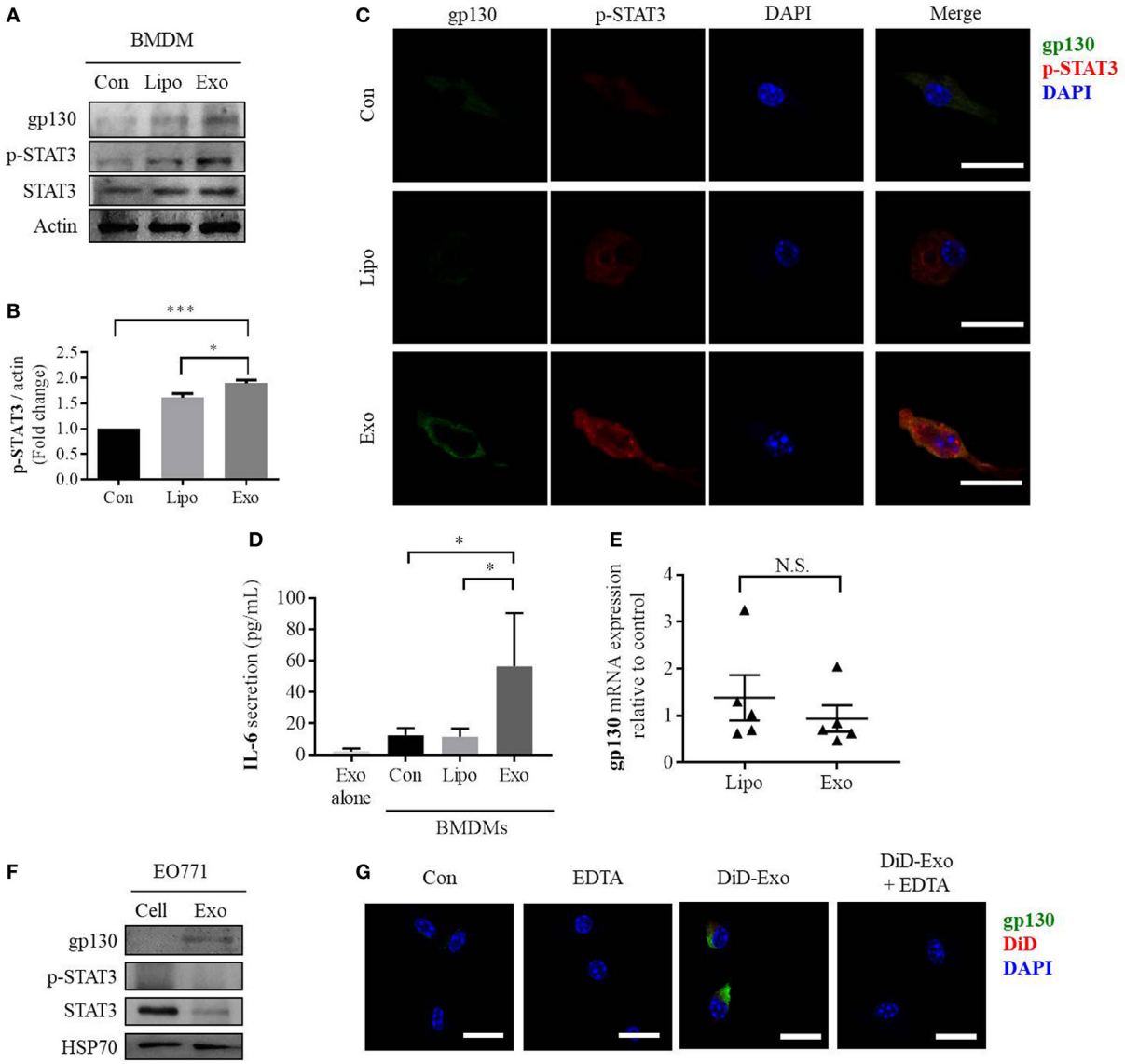

FIGURE 2 | EO771-derived exosomes increase gp130 and p-STAT3 in recipient bone marrow-derived macrophages (BMDMs). **(A)** Macrophages were treated for 24 h with PBS alone (Con), liposomes (Lipo), or EO771-derived exosomes (Exo). Cell lysates were evaluated for gp130, p-STAT3, and STAT3 protein expression. Actin served as loading control. **(B)** Densitometry analysis of p-STAT3 levels as normalized by actin. **(C)** gp130 and p-STAT3 expression assessment after exposure of macrophages to PBS, liposomes, or EO771-derived exosomes for 24 h by immunofluorescence microscopy. The images were captured at 100× magnification and the size bar represents 20 μm. Nuclei of macrophages were visualized with DAPI. **(D)** Interleukin-6 (IL-6) secretion by macrophages after treatment with PBS, liposomes, or EO771-derived exosomes for 24 h was assessed by ELISA ($n = 4$). *$p < 0.05$ as indicated. As control, IL-6 protein content in exosomes is shown in the left column. **(E)** gp130 mRNA expression by macrophages after treatment with liposomes or EO771-derived exosomes for 24 h was assessed by qRT-PCR. Relative gene expression levels were normalized to GAPDH and results are shown as relative to PBS-treated BMDMs ($n = 3$). N.S., not statistically significant. **(F)** gp130, p-STAT3, and STAT3 protein expression in EO771 cell lysate (Cell) and exosomes (Exo). HSP70 is used as loading control. **(G)** Macrophages were pre-exposed to EDTA (EDTA) or PBS alone (Con) for an hour before treatment with DiD-labeled, EO771-derived exosomes for 24 h (DiD-Exo + EDTA and DiD-exo, respectively). DiD signal and gp130 localization were visualized by fluorescence microscopy. The size bar represents 20 μm.

(**Figure 2D**). Taken together, these data show that breast cancer-derived exosomes induce the gp130–STAT3 pathway, resulting in IL-6 secretion by BMDMs.

To determine the cause for the increase of gp130 and resulting STAT3 signaling in exosome-treated BMDMs, we evaluated the gene expression of gp130 in these cells. Surprisingly, there is no change in gp130 gene expression after incubation with exosomes (**Figure 2E**). This observation suggests potential extracellular sources for the elevated gp130 abundance. To explore if exosomal gp130 protein is causative for the increase in abundance of gp130 and IL-6 levels in BMDMs, the amount of gp130 and phosphorylated-STAT3 in cancer-derived exosomes and parental EO771 breast cancer cells was assessed. Specifically, gp130 was found to be enriched in the murine EO771 breast cancer cell-derived

exosomes preparations (**Figure 2F**). Similarly, a range of human breast cancer cell line-derived exosomes (MDA-MB-231, MDA-MB-468, Hs578T, and MCF7) contained gp130 at various abundances (Figure S2 in Supplementary Material). To verify whether the accumulation of gp130 into BMDM cell membranes is indeed mediated by exosomes, we inhibited exosome uptake using EDTA and found that increased gp130 in BMDMs is attenuated by inhibiting exosome uptake (**Figure 2G**; Figure S3 in Supplementary Material). Collectively, these data suggest that exosomal gp130 protein could be transferred to BMDMs by cancer cell-derived exosomes and subsequently activate gp130–STAT3 signaling, thereby promoting IL-6 secretion.

We next evaluated the impact of cancer exosomes on pro- and anti-inflammatory gene expression in BMDMs. After exosome exposure, mRNA levels of IFNγ, a M1 macrophage marker, significantly decrease compared with macrophages incubated with liposomes (Figure S4A in Supplementary Material). By contrast, IL-1β is upregulated, while other M1 markers, such as iNOS and TNFα, do not change (Figure S4A in Supplementary Material). Comparatively, Arg1 and TGF-β gene expression, which are indicative of M2 macrophage phenotype, are similarly not altered, whereas LOX gene expression is slightly elevated (Figure S4B in Supplementary Material). Together, these results suggest that cancer-derived exosomes alone are insufficient to generate a distinct M1 or M2 macrophage phenotype. Remarkably, IL-6, IL-10, CXCR4, and CCL2 mRNA, which are all STAT3 target genes involved in cancer progression (35, 36), were increased in macrophages exposed to exosomes (**Figure 3A**). STAT3 activation has also been associated with acquisition of malignant properties, such as increased cell survival (35). Exposing BMDMs to cancer exosomes resulted in an altered morphology and an increased survival of macrophages (**Figures 3B,C**). Taken together, our data indicate that breast cancer-derived exosomes induce phenotypical changes in macrophages, resulting in a pro-survival phenotype.

Inhibition of Exosomal gp130 Reverses Cancer Exosome-Mediated Effects in Macrophages

To confirm that transfer of exosomal gp130 is causative for the phenotypical changes in BMDMs, we incubated exosomes with SC144, a gp130 inhibitor (37). Pre-treatment of cancer exosomes with SC144 decreased both exosome-mediated phosphorylated STAT3 levels and nuclear translocalization in BMDMs (**Figures 4A,B**). In addition, BMDMs incubation with SC144-treated cancer-derived exosomes resulted in a reversal of the IL-6 secretion phenotype (**Figure 4C**). Similarly, exosome-mediated gp130/STAT3-induced gene expression was reduced when exosomes were pretreated with SC144 (**Figure 4D**). Finally, the morphological and pro-survival changes induced by cancer-derived exosomes in BMDMs were reverted by SC144-treated exosomes (**Figures 4E,F**). Together, these data verify that exosomal gp130 is indeed causative for the observed STAT3 signaling, IL-6 secretion, morphological changes, and enhanced survival of BMDMs in response to cancer-derived exosomes.

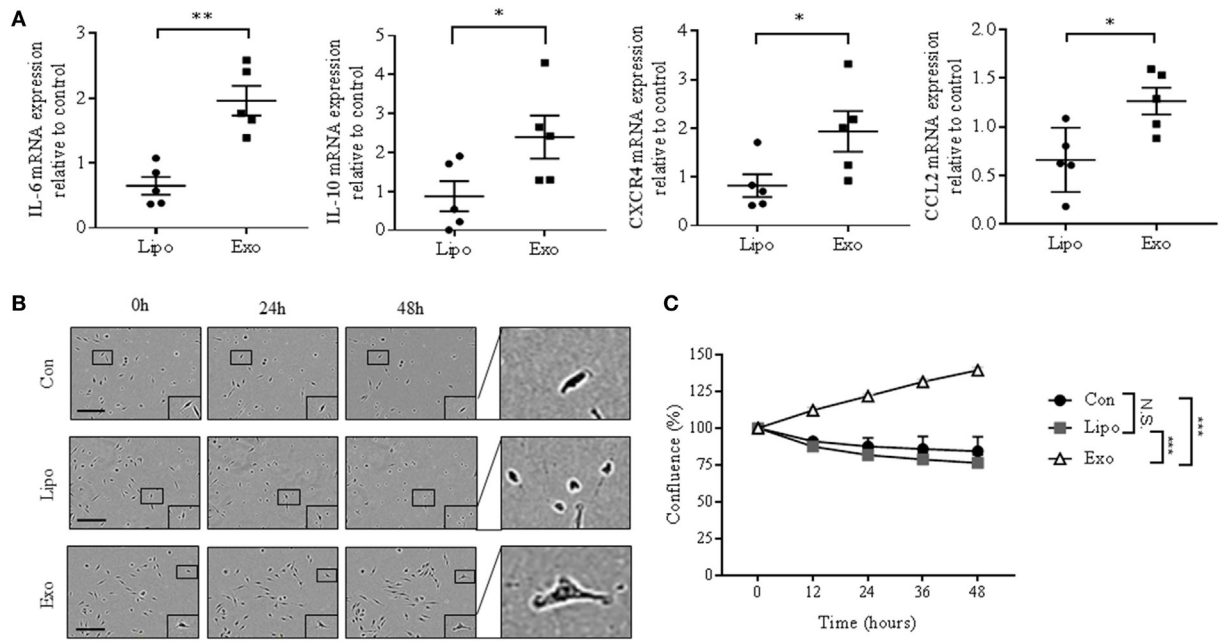

FIGURE 3 | EO771-derived exosomes enhance mRNA expression of STAT3 target genes and survival rate of bone marrow-derived macrophages (BMDMs). **(A)** Interleukin-6 (IL-6), IL-10, CXCR4, and CCL2 mRNA expression was quantified by qRT-PCR in macrophages after liposome or exosome exposure for 24 h. Relative gene expression levels were normalized to GAPDH, and results are shown as relative to PBS-treated BMDMs ($n = 5$). *$p < 0.05$; **$p < 0.01$. **(B)** Incucyte images of macrophages after PBS, liposome, or exosome treatment for 0, 24, and 48 h. The size bar represents 150 μm. **(C)** Quantitative representation of Incucyte results. Cell confluence was assessed at 5 time points, as an average of 16 images captured per time point. Data were normalized to cell count at 0 h ($n = 3$ independent experiments). ***$p < 0.001$; N.S., not statistically significant, as indicated.

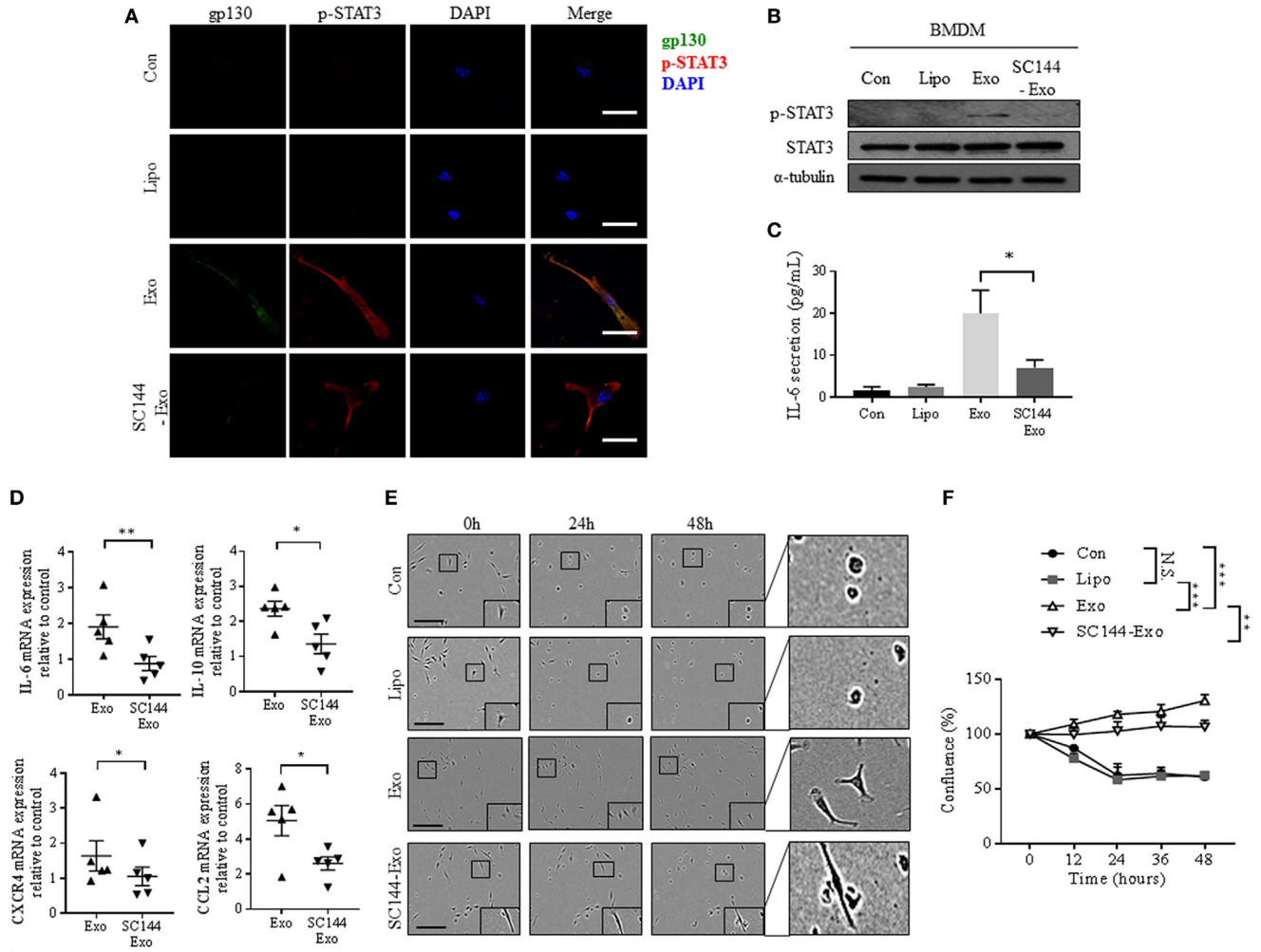

FIGURE 4 | Inhibition of exosomal gp130 by SC144 reduces the breast cancer exosome-mediated phenotypical changes in bone marrow-derived macrophages (BMDMs). **(A)** gp130 and p-STAT3 expression were assessed after exposure of macrophages to PBS, liposomes, exosomes, or SC144 pretreated exosomes for 24 h by immunofluorescence microscopy. The images were captured at 100× magnification, and the size bar represents 20 μm. Nuclei of macrophages were visualized with DAPI. **(B)** The protein expression levels of p-STAT3 and STAT3 was assessed in macrophages cell lysates after the indicated 24-h treatments. α-Tubulin served as loading control. **(C)** Interleukin-6 (IL-6) secretion by macrophages after the indicated 24-h treatments was assessed by ELISA ($n = 6$). $^{*}p < 0.05$ as indicated. **(D)** IL-6, IL-10, CXCR4, and CCL2 gene expression was quantified by qRT-PCR in macrophages after 24-h exposure to exosomes or SC144 pretreated exosomes as indicated. Relative gene expression levels were normalized to GAPDH and results are shown as relative to PBS control ($n = 5$). $^{*}p < 0.05$; $^{**}p < 0.01$. **(E)** Incucyte images of macrophages after indicated treatments for 0, 24, and 48 h. The size bar represents 20 μm. **(F)** Quantitative representation of Incucyte results. Cell confluence was quantified at 5 time points, as an average of 16 images captured per time point. Data were normalized to cell count at 0 h ($n = 4$ independent experiments). $^{**}p < 0.01$; $^{***}p < 0.001$; N.S., not statistically significant, as indicated.

DISCUSSION

Despite numerous reports on tumor-promoting functions of cancer exosomes, our knowledge of their role in immune cell responses is limited. Immune surveillance is usually associated with anticancer properties (38). However, within cancer microenvironments, immune cells often display altered phenotypes capable of contributing to tumor progression, including promotion of tumor growth, migration, pre-metastasic niche formation, and metastasis (39, 40). For instance, classical pro-inflammatory macrophages generally have activated STAT1 signaling, whereas tumor-associated macrophages are known to activate STAT3 (19, 25, 41). Macrophages capable of infiltrating a tumor mass have also been shown to promote cancer progression and metastasis (42, 43).

The role cancer-derived exosomes play on the modulation of bone marrow mesenchymal stromal cells has been previously studied in a neuroblastoma model, and ERK1/2 described to control the level of IL-6 and IL-8/CXCL8 (44). In gastric cancer, macrophages were activated by cancer exosomes *via* the NF-κB pathway, thereby promoting cancer progression (45). Furthermore, it has been observed that human breast cancer cell-derived exosomes induce the secretion of IL-6, TNFα, and CCL2 from both human THP-1 and murine RAW macrophage cell lines *via* the toll-like

receptor 2/NF-κB signaling pathway (46). Despite these and other examples, it is still unclear as to how macrophages are capable of triggering cancer initiation and progression, and how their phenotypical alterations are caused by exposure to tumor-derived exosomes. Our work suggests that exosomal gp130 is a key mediator in macrophage phenotype alterations. Overexpression of gp130 is found in diverse cancer types such as brain, bladder, colorectal, and breast cancer (37, 47). It has also been implicated as the main mediator of STAT3 activation in various breast cancer cell lines (48). We found gp130 to be contained in exosomes derived from a range of murine and human breast cancer cells (**Figure 2F**; Figure S2 in Supplementary Material). Interestingly, tetraspanin CD9, normally enriched in exosome membranes, has recently been reported to stabilize gp130, thereby facilitating activation of STAT3 signaling in glioma stem cells (49). In the context of macrophages, an imbalance of gp130 signaling has an impact on M2 macrophage polarization (50). This causative impact suggests that gp130 might have an important effect on polarization of tumor-associated macrophages. In addition, STAT3 activation, which is a key downstream pathway of gp130 activation in macrophages, is associated with angiogenesis (51) as well as myeloid cell accumulation in future metastatic microenvironments (52). STAT3 is also commonly activated in tumor-infiltrating macrophages (25, 41). Therefore, STAT3 activation in macrophages has been associated with a pro-tumoral macrophage phenotype, cancer progression, and poor patient outcome (19, 53).

Previous findings demonstrate that proteins packaged into exosomes can maintain their activity after exosome uptake by recipient cells (54, 55). It has been reported that tyrosine kinase receptors in exosomes are transferable to monocytes and capable of activating MAPK pathways, thereby promoting cell survival (54). Here, we show that transfer of exosomal gp130 causes STAT3 activation in macrophages and increases macrophages survival. Activated, phosphorylated STAT3 translocates to the nucleus and induces target gene transcription, including several genes associated with tumorigenesis, such as IL-6, IL-10, CXCR4, and CCL2 (41, 56). Tumors from triple-negative breast cancer patients are highly infiltrated by macrophages expressing, and secreting, both IL-6 and IL-10 (57). Each of these cytokines has specific roles in regulating the immune system and cancer surveillance. Secretion of IL-10 by macrophages results in immune-suppressive effects *via* dendritic cells and cytotoxic T cells modulation (58). Increased CXCR4-expressing macrophages were detected in the bone marrow of melanoma patients, which was associated with pro-angiogenic and immune-suppressive phenotypes (59). Moreover, IL-6 and CCL2 induce tumor-associated macrophage polarization (24, 26). Taken together, these data suggest that expression of the aforementioned pro-tumorigenic genes in macrophages could alter their phenotype toward a tumor-associated one. Finally, STAT3 signaling has been linked to cell survival (56). For example, it has been reported that STAT3 activation *via* gp130 in enterocytes is associated with cell survival signaling and cell cycle progression in the tumor microenvironment (60). Another study suggested that M2-like macrophages overexpressing anti-inflammatory cytokines can survive longer than M0 or M1 macrophages (61). These findings indicate that a long lifespan is one of the characteristics of tumor-associated macrophages.

To date, the commercially available inhibitor of IL-6 receptor, tocilizumab, and a gp130 specific inhibitor, FE999301, are only available for the treatment of autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease. Despite both IL-6 receptor and gp130 also contributing to cancer progression (62), no IL-6 receptor antagonist is currently under clinical development in oncology. Recently, the blocking of IL-6/gp130/STAT3 has been suggested as anticancer drug approach (30, 63). One of these inhibitors, SC144, has been used to slow prostate, lung, breast, colorectal, and ovarian cancer progression and inhibit angiogenesis, in preclinical models (30, 64). SC144 is a small molecule inhibitor of gp130 and binds to S782 phosphorylated gp130, resulting in subsequent deglycosylation and inactivation of gp130 (30). Therefore, it abrogates downstream STAT3 phosphorylation and nuclear translocation (30). We show that exosomal gp130-induced effects are reversed when breast cancer-derived exosomes are pretreated with SC144. Together, these data are in agreement with the notion that inhibition of gp130 signaling could be an attractive therapeutic target in both breast cancer and other metastatic cancers (65, 66).

In conclusion, our data suggest that cancer-derived exosomal gp130 plays a critical role in the tumor environment *via* activation of the IL-6/STAT3 pathway in macrophages. This activation subsequently promotes BMDM survival and induces the expression of pro-tumorigenic cytokines, thereby potentially skewing BMDMs to a cancer-promoting phenotype. Although limited to a murine model, these results provide evidence demonstrating the role of exosomes in facilitating the exchange of cargo between cancer and immune cell subsets. The presence of gp130 in exosomes derived from human breast cancer cells, however, indicates that such mechanism of macrophage activation could operate in human cells as well. Altogether, this knowledge further improves our understanding of the implications of exosomal protein transfer in cancer progression.

ETHICS STATEMENT

All animal procedures were conducted in accordance with Australian National Health and Medical Research regulations on the use and care of experimental animals, and approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee (A12617M, P1499).

AUTHOR CONTRIBUTIONS

AM, SH, LL, SW, and AW conceived the idea and designed the research. SH and SK harvested and maintained cells. RL conducted TRPS analysis. SH, LL, CE, and AM isolated exosomes and performed flow cytometry and western blotting. SH executed fluorescence microscopy and qRT-PCR. SH and AW performed and analyzed incucyte results. SH and AM wrote the manuscript. All the authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00871/full#supplementary-material.

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Loss of the Cyclin-Dependent Kinase Inhibitor 1 in the Context of Brachyury-Mediated Phenotypic Plasticity Drives Tumor Resistance to Immune Attack

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The acquisition of mesenchymal features by carcinoma cells is now recognized as a driver of metastasis and tumor resistance to a range of anticancer therapeutics, including chemotherapy, radiation, and certain small-molecule targeted therapies. With the recent successful implementation of immunotherapies for the treatment of various types of cancer, there is growing interest in understanding whether an immunological approach could be effective at eradicating carcinoma cells bearing mesenchymal features. Recent studies, however, demonstrated that carcinoma cells that have acquired mesenchymal features may also exhibit decreased susceptibility to lysis mediated by immune effector cells, including antigen-specific $CD8^+$ T cells, innate natural killer (NK), and lymphokine-activated killer (LAK) cells. Here, we investigated the mechanism involved in the immune resistance of carcinoma cells that express very high levels of the transcription factor brachyury, a molecule previously shown to drive the acquisition of mesenchymal features by carcinoma cells. Our results demonstrate that very high levels of brachyury expression drive the loss of the cyclin-dependent kinase inhibitor 1 (p21CIP1, p21), an event that results in decreased tumor susceptibility to immune-mediated lysis. We show here that reconstitution of p21 expression markedly increases the lysis of brachyury-high tumor cells mediated by antigen-specific $CD8^+$ T cells, NK, and LAK cells, TNF-related apoptosis-inducing ligand, and chemotherapy. Several reports have now demonstrated a role for p21 loss in cancer as an inducer of the epithelial–mesenchymal transition. The results from the present study situate p21 as a central player in many of the aspects of the phenomenon of brachyury-mediated mesenchymalization of carcinomas, including resistance to chemotherapy and immune-mediated cytotoxicity. We also demonstrate here that the defects in tumor cell death described in association with very high levels of brachyury could be alleviated *via* the use of a WEE1 inhibitor. Several vaccine platforms targeting brachyury have been developed and are undergoing clinical evaluation. These studies provide further rationale for the use of WEE1 inhibition in combination with brachyury-based immunotherapeutic approaches.

Keywords: epithelial–mesenchymal transition, phenotypic plasticity, brachyury, immune resistance, CDKN1A, CDK1

INTRODUCTION

Studies from our laboratory and others have previously established the ability of the transcription factor brachyury (gene name *T*), a member of the T-box family, to promote the acquisition of mesenchymal features by carcinoma cells (1–5). Gain- and loss-of-function experiments have shown that brachyury expression in epithelial cancer cells associates with (a) enhanced expression of mesenchymal proteins and reduction of epithelial proteins; (b) acquisition of tumor motility, invasiveness, and propensity to disseminate *in vivo* in xenograft models; (c) acquisition of stemness features, including a relatively quiescent state; and (d) conversion into a refractory, therapy-resistant state. The effects of brachyury on tumor phenotype were attributed to its ability to bind a half T-box DNA-binding site in the promoter of E-cadherin, in cooperation with the repressor Slug, resulting in decreased E-cadherin expression (1).

Brachyury has been found to be overexpressed in various human carcinomas, both in the primary tumor and metastatic sites, including in non-small cell (NSCLC) and small cell (SCLC) lung cancer (6, 7), triple-negative breast (TNBC) cancer (8, 9), prostate (4), and colon cancer (10, 11), among others. Interestingly, several reports have also shown the prognostic value of high brachyury expression at the primary tumor site, with high brachyury mRNA or protein levels being associated with poor clinical outcome, including in breast (8, 9), lung (12), colon (10), and prostate (4, 13).

There are several vaccine platforms targeting the transcription factor brachyury undergoing Phase I and II clinical evaluation (14–16). It has been recently shown, however, that the acquisition of mesenchymal features by carcinoma cells may decrease their susceptibility to lysis by immune effector cells, including antigen-specific $CD8^+$ T cells, innate natural killer (NK), and lymphokine-activated killer (LAK) cells (17, 18). In the case of brachyury-mediated mesenchymalization of human carcinoma cells, the mechanism of immune resistance was identified as a defect in the phosphorylation and subsequent cleavage of the nuclear lamins during apoptosis, a defect caused by the loss of the cell-cycle dependent kinase-1 (CDK1), due to decreased protein stability (17). The reason for the decreased stability of the CDK1 protein in the presence of high levels of brachyury, however, was not previously elucidated. In the present study, we further investigated the mechanism involved in the immune resistance of carcinoma cells that express high levels of brachyury, and demonstrate that the loss of the cyclin-dependent kinase inhibitor 1 (p21CIP1, hereafter termed p21) is critical for the defective lysis of these cancer cells. Reconstitution of p21 expression in brachyury-high tumor cells was shown to increase the stability of the CDK1 protein while markedly increasing the lysis mediated by antigen-specific $CD8^+$ T cells, NK and LAK cells, TNF-related apoptosis-inducing ligand (TRAIL) and chemotherapy. The loss of p21 has been associated with the occurrence of epithelial–mesenchymal plasticity in cancer. The results from this work indicate that p21 plays a major role in many of the aspects of this phenomenon, including as an inducer of resistance to cell death in response to chemotherapy or immune effector cells.

MATERIALS AND METHODS

Cell Culture

The following human carcinoma cell lines were obtained from American Type Culture Collection and propagated in recommended media: pancreatic PANC-1; lung H1299 and H460, colon HCT116. The murine MC38 colon adenocarcinoma cell line has been previously described (19). The full-length human brachyury and p21CIP1 encoding fragments were purchased from Origene (Rockville, MD, USA) and subsequently cloned into the pcDNA3.1(+) expression vector (Thermo Fisher Scientific). Tumor cells were stably transfected using a nucleofection device (Lonza) by following the manufacturer's recommendations. For generation of H460 clones with various levels of brachyury, the GeneArt Precision gRNA synthesis kit (Invitrogen) was used for preparation of brachyury-specific gRNA designed *via* the use of the GeneArt CRISPR Search and Design online tool (Invitrogen). H460 cells were co-transfected with GeneArt Platinum Cas9 nuclease (Invitrogen) and brachyury-targeting gRNA by following the manufacturer's instructions, and subsequently grown and seeded for single cell sub-culture onto 96-well plates. Clonally derived cell lines with various levels of brachyury were generated by using a limiting dilution cloning strategy. Clones were selected for further study based on the level of brachyury protein expression.

Immune Effector Cells, Cytotoxicity Assays

Peripheral blood used in this study was obtained from healthy human donors recruited at the NIH Blood Bank (Bethesda, MD, USA), protocol number NCT00001846, under the appropriate NIH Institutional Review Board approval and informed consent. NK cells were isolated using human CD56 MicroBeads (Miltenyi Biotec). For generation of LAK cells, purified NK cells were incubated overnight in RPMI-1640 supplemented with 10% human AB sera and 2,000 U/mL of recombinant human IL-2 (Peprotech). T cells specific for the MUC1 HLA-A2 restricted peptide p93L (ALWGQDVTSV) were previously described (20). TRAIL-mediated lysis of tumor cells was performed by incubation with recombinant, active multimeric killer TRAIL (Enzo Life Sciences). The murine cytotoxic T-cell lines specific for p15E (KSPWFTTL) were expanded *in vitro* from splenocytes originating from either wild-type or perforin-deficient C57BL/6 mice, which had been vaccinated with the p15E peptide. Perforin-deficient animals were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animal studies were carried out in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Experimental studies were carried out under approval of the NIH Intramural Animal Care and Use Committee. Where indicated, perforin/granzyme-mediated lysis of target cells was inhibited by incubating T cells with 200 nM Concanamycin A (CMA) (Sigma) for 2 h at 37°C prior to plating with target cells in the lysis assays. Target cells were labeled with 50 μCi ^{111}In (GE Healthcare) and incubated overnight with TRAIL or effector NK, LAK, or T cells at indicated effector-to-target (E:T) ratios. Following overnight

culture, supernatants were harvested for radioactivity assessment using a Wizard^2 gamma counter (PerkinElmer). Specific lysis was calculated as previously described (21). Sensitivity of H460 cells to TRAIL lysis was performed using a luminescence-based viability assay. Cells were plated in white-walled 96-well trays and allowed to attach overnight. Using six-well replicates, cells were treated with indicated doses of recombinant Superkiller TRAIL (Enzo Life Sciences) and incubated for 16 h. Cell viability was assessed using CellTiter-Glo (Promega) according to the manufacturer's instructions. Cell death was calculated as the percent reduction in luminescence in treated wells as compared to non-treated controls.

Western Blot

Cells were washed twice with PBS and lysed in RIPA Lysis Buffer (Santa Cruz Biotech). Protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific). Aliquots containing 10–30 μg of protein were run on SDS-PAGE and transferred to nitrocellulose membranes. Following blockade for 1 h at room temperature with 5% milk in PBS, the membranes were probed overnight at 4°C using antibodies specific for pan-actin (clone Ab-5, Neo Markers), GAPDH (Santa Cruz Biotechnology), p21 (clone 12D1, Cell Signaling Technology), vimentin (clone RV202, BD Biosciences), E-cadherin (clone 36/E-cadherin, BD Biosciences), CDK1 (Cell Signaling Technology), and brachyury MAb 54-1 (22). CDK1 immunoprecipitation was performed using 200 μg of cleared cell extract using either a control rabbit IgG (Abcam) or anti-CDK1 antibody (Upstate). Antibody/protein complexes were purified using Protein G beads (Sigma), and the eluted protein was assessed for the presence of CDK1 and p21 by western blot. Protein synthesis was inhibited by incubating tumor cells with 100 μg/mL cycloheximide (Sigma); cells were harvested at various time points and CDK1 protein levels were assessed by western blot. All blots were imaged using the Odyssey Infrared imaging system (LI-COR Biotechnology).

Real-Time PCR

Total RNA was isolated from frozen cell pellets using the RNeasy kit (QIAGEN), and cDNA was reverse transcribed with Advantage RT-for-PCR (Clontech). cDNA (1–100 ng) was amplified in triplicate using Gene Expression Master Mix and the following TaqMan gene expression assays (Thermo Fisher Scientific): brachyury (Hs00610080), and GAPDH (4326317E). Mean Ct values for target genes were normalized to mean Ct values for the endogenous control GAPDH [$-\Delta Ct = Ct(GAPDH) - Ct(target\ gene)$]. The ratio of mRNA expression of target gene versus GAPDH was defined as $2(-\Delta Ct)$.

MK-1775 Treatments

The WEE1-inhibitor, MK-1775, utilized in these studies was purchased from Selleckchem. Carcinoma cell lines were treated with indicated concentrations of MK-1775 for 72 h prior to the addition of immune effector cells or a combination of cisplatin (APP Pharmaceuticals) and vinorelbine (Bedford Laboratories). To evaluate responses to chemotherapy, tumor cells were incubated for 4 h in the presence of the chemotherapeutic agents; MK-1775 was present for the entirety of the assay. Cell viability was assessed 3–4 days after exposure to chemotherapy using an MTT (Sigma) assay, as previously described (23).

In Silico Analysis of The Cancer Genome Atlas (TCGA) Dataset

Relative expression levels of indicated mRNAs were assessed using the TCGA dataset containing data from 482 lung squamous cell carcinoma patients (24). For the analysis, samples were subdivided into four groups according to the level of brachyury (T) expression: 318 of 482 samples with no detectable brachyury expression were classified as negative (neg.). The remaining 164 samples were ranked and subdivided into tertiles based upon the level of brachyury expression: "brachyury-high" (55 of 482), "brachyury-intermediate" (55 of 482), and "brachyury-low" (54 of 482) groups. Samples in the intermediate and low groups were combined; the level of expression of mRNA encoding E-cadherin (*CDH1*), p21 (*CDKN1A*), and CDK1 (*CDK1*) were evaluated in each group. All data were analyzed using the Nexus Expression 3 analysis software package (BioDiscovery).

Statistical Analysis

Unless indicated all statistical comparisons between two sample groups were performed using a two-tailed unpaired Student's *t*-test on triplet technical replicates using Prism 7 (GraphPad).

RESULTS

High Expression of Brachyury Induces Resistance to TRAIL Lysis

To better understand the role of brachyury in tumor resistance mechanisms, we utilized various models of clonally derived tumor cell populations with different levels of brachyury. One such model was generated by stable transfection of the human pancreatic PANC-1 carcinoma cell line to overexpress human brachyury, followed by a clonal selection of single-cells with a range of brachyury expression (**Figure 1A**, left panel). As shown in **Figure 1A** (right panel), PANC-1 clones with high levels of brachyury (pBr cl-1 and cl-2) demonstrated a marked decrease in susceptibility to lysis by recombinant TRAIL, compared with PANC-1 clones that express very low/undetectable levels of brachyury (pBr cl-3 and cl-4). Similar results were obtained with single clonal populations of lung H460 cells with different levels of brachyury generated *via* the CRISPR/Cas9 methodology (**Figure 1B**, left panel). As shown in **Figure 1B** (right panel), H460 cells with high levels of brachyury (Hi) were lysed less efficiently than those with undetectable brachyury (Lo).

In a previous study, we have described the transcriptional repression of p21 as a mechanism responsible for the decreased susceptibility of brachyury-high carcinoma cells to chemotherapy and radiation. In accordance with those previous observations, clonal populations of lung H460 cells with various levels of brachyury (**Figure 2A**) demonstrated an inverse association between brachyury and p21 protein expression, whereby H460 cells with high expression of brachyury (Br-High) were characterized by high levels of mesenchymal vimentin, low levels of epithelial E-cadherin and very low levels of p21, while cells with lower

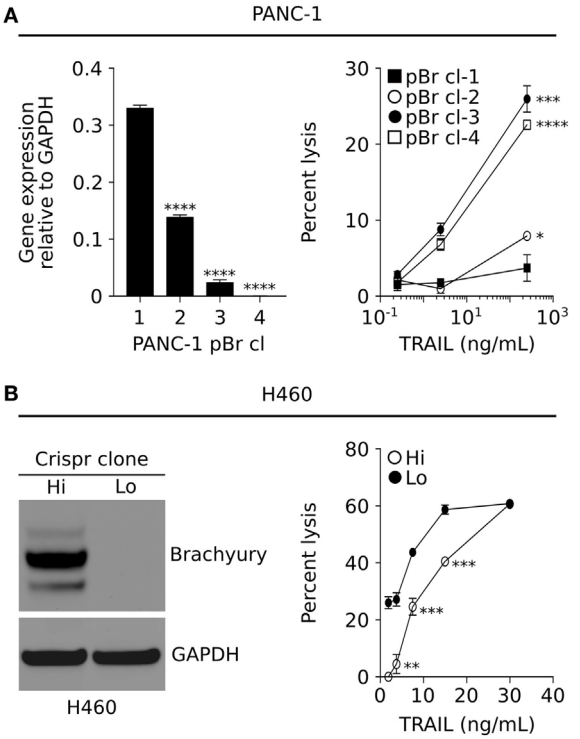

FIGURE 1 | Brachyury associates with increased resistance to immune-mediated killing. **(A)** Relative brachyury expression in four clonally derived human PANC-1 cell lines (left panel), and their associated sensitivity to lysis by TNF-related apoptosis-inducing ligand (TRAIL) (right panel). **(B)** Western blot analysis of brachyury expression in two clonally derived populations of H460 generated *via* the CRISPR approach (left panel), and their sensitivity to lysis by TRAIL (right panel). Presented data are representative of three independent experiments.

brachyury levels (Br-Interm and Br-Low) exhibited reduced expression of vimentin, high expression of E-cadherin, and enhanced expression of the cell cycle regulator p21 (**Figure 2A**). Since expression of p21 is primarily regulated by p53-mediated response to DNA damage, we hypothesized that the p53 status of the cancer cells may impact the ability of brachyury to induce resistance to cytotoxic killing. To examine this possibility, brachyury was overexpressed in parallel experiments in the colon carcinoma line, HCT116, which carries wild-type p53, and the non-small cell lung carcinoma (NSCLC) line, H1299, which express a non-functional isoform of p53 (**Figures 2B,C**). These isogenic brachyury high (pBr) vs. low (pCMV) tumor cell pairs were then assayed for their susceptibility to lysis by recombinant TRAIL. As shown in **Figure 2D**, brachyury-associated reduction in TRAIL susceptibility was only observed in the p53 wild-type cell line, HCT116, while no effect of brachyury overexpression was observed in the p53 null H1299 cells.

Reconstitution of p21 in Brachyury-High Cells Increases Sensitivity to Immune Cells

To further examine the role of p21 in brachyury-mediated resistance to immune-mediated killing of carcinoma cells, a non-clonal, heterogeneous population of parental H460 cells characterized by very high levels of brachyury expression and a mesenchymal phenotype, were transfected with an empty vector (pCMV) or a plasmid encoding human p21 under the control of the CMV promoter (**Figure 3A**). Using this isogenic pair of p21-low vs. p21-high cells, susceptibility to immune lysis was evaluated. As shown in **Figures 3B–E**, brachyury-high/p21-high cells (H460-p21) were efficiently lysed by MUC1-specific T cells, NK cells, and LAK cells and recombinant TRAIL, compared with low levels of lysis observed with brachyury-high/p21-low cells (H460-pCMV). This increased sensitivity to cell death was not restricted to immune-mediated lysis, as the H460-p21 cells were also more sensitive to killing by a combination of cisplatin and vinorelbine chemotherapy (**Figure 3F**) than tumor cells with low levels of p21 (H460-pCMV). Interestingly, overexpression of p21 in these cells was associated with a reduction of vimentin expression (**Figure 3A**), an observation in agreement with several previous reports on the ability of p21 to inhibit the epithelial–mesenchymal switch in tumor cells (25).

Reconstitution of p21 Stabilized the CDK1 Protein in Brachyury-High Tumor Cells

In a previous study, we demonstrated that brachyury-mediated immune resistance associates with decreased levels of the cyclin-dependent kinase 1 (CDK1) protein, and that reconstitution of CDK1 levels could restore the tumor cells' susceptibility to immune effector cells. The mechanism by which high levels of brachyury decrease the expression of CDK1 protein was previously identified as a reduction of CDK1 protein stability in brachyury-high tumor cells. To investigate a potential association between the loss of CDK1 protein and reduction of p21, here, we have conducted co-immunoprecipitation assays using an anti-CDK1 antibody to investigate whether these two proteins could be associated in tumor cells with high vs. low brachyury levels. As shown in **Figure 4A**, p21 co-immunoprecipitated with CDK1 in H460-p21 cells demonstrating that these proteins may form a molecular complex *in vivo*. Interestingly, evaluation of the stability of CDK1 in the brachyury-high H460 cells demonstrated that reconstitution of p21 in the H460-p21 cells could increase the stability of CDK1 over that observed in H460-pCMV cells (**Figure 4B**).

One potential therapeutic avenue for improving the lysis of brachyury-high tumor cells consists of tumor pretreatment with an inhibitor of the G2 checkpoint kinase, WEE1, which normally suppresses the activity of CDK1 *via* phosphorylation on Tyr-15. We have previously shown that treatment of brachyury-high tumor cells with the WEE1 inhibitor, MK-1775, is able to overcome tumor resistance to immune-mediated killing by decreasing WEE1 activity and increasing the level of functional CDK1. WEE1 inhibition is currently undergoing clinical evaluation in combination with various conventional chemotherapeutic treatment strategies, particularly in patients whose tumors lack a functional p53 and, therefore, are dependent on the activity of the G2 cell cycle checkpoint (26). Our observations, however, suggest that high levels of brachyury, which negatively regulates the transcription of CDKN1A, may impart a p53 null phenotype in

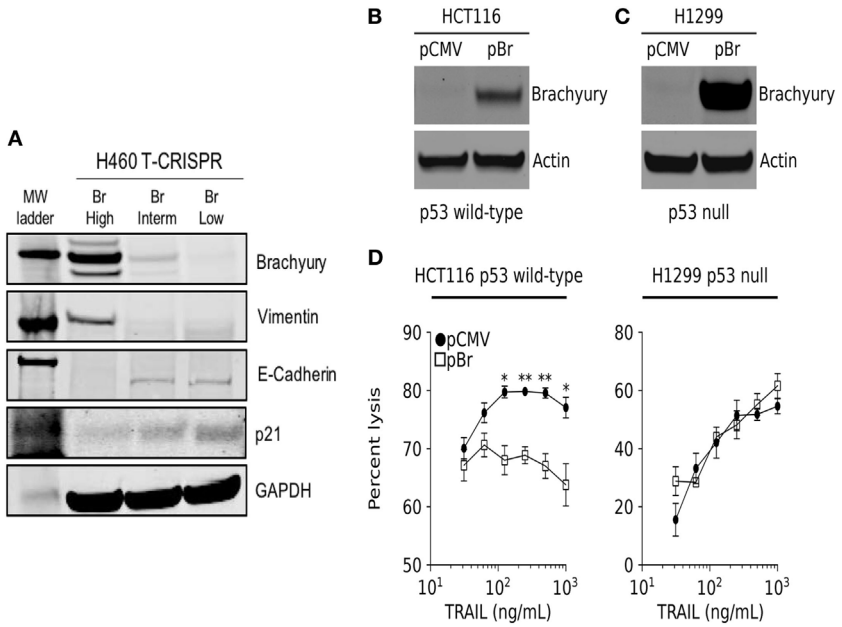

FIGURE 2 | Brachyury drives resistance to TNF-related apoptosis-inducing ligand (TRAIL) in cells expressing a wild-type p53 gene. **(A)** Western blot analysis of brachyury, vimentin, E-cadherin, and p21 in H460 clones generated *via* the CRISPR/Cas9 system. **(B,C)** Western blot analysis of brachyury expression in HCT116 and H1229 cells, respectively, stably transfected with either a control or brachyury-encoding (pBr) plasmid. **(D)** The impact of brachyury expression on the sensitivity of HCT116 (left panel) and H1229 (right panel) to lysis by a range of TRAIL doses.

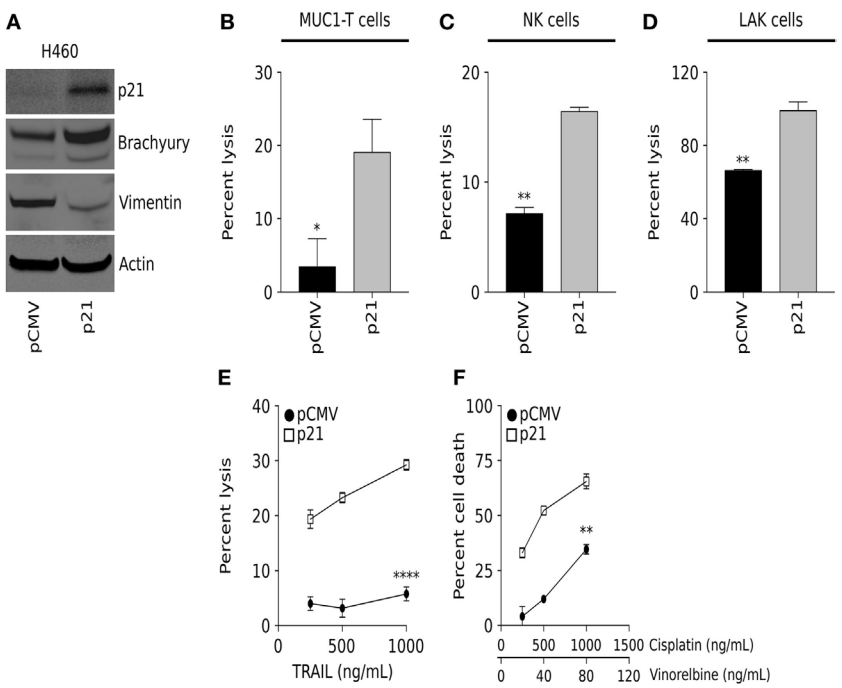

FIGURE 3 | Overexpression of p21 in the brachyury-high H460 cell line increases sensitivity to lysis by both immune cells and chemotherapy. **(A)** Western blot analysis of p21, brachyury, and vimentin in H460 cells transfected to express either a control pCMV or a vector encoding p21 (p21). Sensitivity of H460-pCMV vs. H460-p21 cells to killing by **(B)** MUC-1-specific T cells (E:T ratio 30:1); **(C)** natural killer (NK) cells (E:T ratio 30:1); **(D)** lymphokine-activated killer (LAK) cells (E:T ratio 30:1); and indicated concentrations of **(E)** TNF-related apoptosis-inducing ligand (TRAIL) and **(F)** chemotherapy. NK and LAK lysis data presented is representative of data obtained using effector cells isolated from two different normal donors. TRAIL and chemotherapy data presented are representative of two independent experiments.

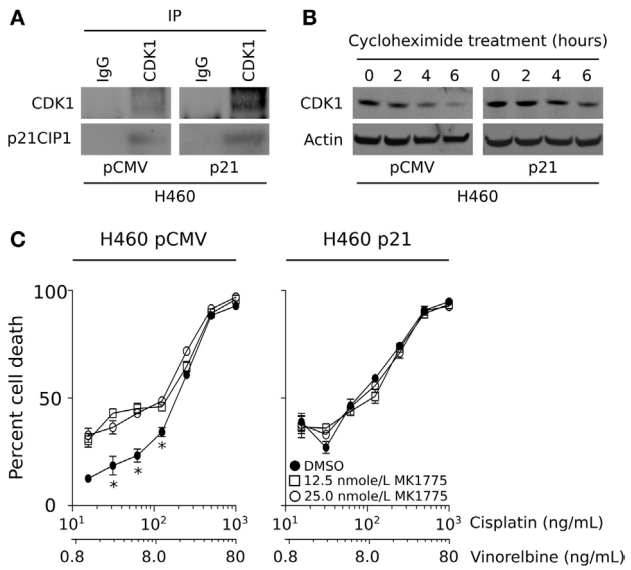

FIGURE 4 | CDK1 protein is stabilized by p21, and WEE1 inhibition improves the sensitivity of brachyury-high/p21-low carcinoma cells. **(A)** Co-immunoprecipitation of CDK1 and p21 in lysates from H460 cells expressing either a control pCMV or a vector encoding p21. **(B)** CDK1 protein stability was evaluated in the H460 tumor cell pair following the addition of 100 μg/mL cycloheximide for the indicated time points. **(C)** Impact of MK-1775 pre-treatment (at indicated doses) on the sensitivity of H460 cells expressing a control pCMV, but not a p21 vector, to killing by chemotherapy. The data depicting the stability of CDK1, and chemotherapy sensitivity are representative of two independent experiments.

tumors with a functional p53 protein. To examine this further, we evaluated the impact of MK-1775 treatment on the susceptibility of H460 cells expressing either low (pCMV) or high levels of p21. As shown in **Figure 4C**, WEE1 inhibition was only able to improve the sensitivity of brachyury-high/p21-low (H460-pCMV) cells in response to chemotherapy, while the same treatment had no impact on the susceptibility of H460 cells over-expressing p21 (H460-p21).

WEE1 Inhibition Improves the Sensitivity of Brachyury High/p21-Low Carcinoma Cells

To evaluate our results in a murine carcinoma model, the colon carcinoma cell line MC38 was stable transfected with a plasmid vector encoding the full-length brachyury protein, followed by single-cell cloning and generation of three cell lines, MC38-Lo, -Int, and -Hi, with either low, intermediate, or high levels of brachyury, respectively (**Figure 5A**). The sensitivity of these cells to lysis by $CD8^+$ T cells specific for an epitope (p15E) of the endogenous retroviral env protein GP70 was evaluated. As shown in **Figure 5B** (left panel), MC38 cells expressing high levels of brachyury were poorly lysed in comparison with MC38 cells expressing either low or intermediate levels of brachyury. Immune effector cells can lyse targets either by using the FAS/TRAIL-mediated induction of the extrinsic caspase-dependent apoptotic pathway, or *via* the perforin-dependent actions of granzymes that lyse target cells in a caspase-dependent and/or independent fashion. Pretreatment of the p15E-specific $CD8^+$ T cells with CMA was used to abrogate the activity of the perforin/granzyme lytic pathway. As shown in **Figure 5B** (right panel), CMA completely abolished the T cells' ability to lyse MC38 cells with high levels of brachyury while the clones expressing intermediate and low levels were still lysed. The tumor resistant phenotype induced by high levels of brachyury was also observed when recombinant TRAIL was used to directly trigger the extrinsic apoptotic pathway (**Figure 5C**). The impact of WEE1 inhibition on the restoration of lysis of brachyury-high cells was also evaluated with MC38 cells transfected with an empty vector (pCMV) or a vector encoding the full-length brachyury protein (pBr, **Figure 5D**). As immune effector cells, murine p15E-specific T cells were used, generated either in wild-type ($pfn^{+/+}$) or perforin deficient ($pfn^{-/-}$) mice. As shown in **Figure 5E**, MK-1775 treatment enhanced T-cell mediated lysis of MC38-pBr cells, an effect that was exacerbated when T cells originating from perforin-deficient animals were used. In contrast, brachyury-low MC38-pCMV targets were not affected by treatment with MK-1775. Similar results were obtained when utilizing recombinant TRAIL (**Figure 5F**). While MC38-pCMV were highly susceptible to the effect of TRAIL and were not affected by MK-1775 treatment, MC38-pBr cells appeared resistant to the lytic activity of TRAIL and treatment with MK-1775 was able to significantly enhance their lysis in a dose-dependent way.

Inverse Association Between Brachyury and p21 in Tumor Tissues

Our preclinical observation on the existence of a negative correlation between brachyury and p21 expression in tumor cells was then corroborated *via* analysis of the lung squamous cell carcinoma dataset from (TCGA) for levels of mRNA encoding brachyury (*T*) and p21 (*CDKN1A*). When samples were separated into three subgroups based upon the level of brachyury expression (**Figure 6A**), it was observed that tumors expressing the highest levels of *T* mRNA had the lowest levels of CDH1 mRNA (encoding epithelial E-cadherin) and *CDKN1A* mRNA (encoding p21), as compared with the brachyury negative and low/intermediate groups (**Figures 6B,C**). Unlike with *CDKN1A*, no differences in *CDK1* mRNA levels were observed in relation to the levels of *T* mRNA (**Figure 6D**), which agrees with our previous observations that high levels of brachyury induce a reduction in CDK1 protein stability rather than a reduced transcriptional activity.

DISCUSSION

The phenomenon of cancer phenotypic plasticity, manifested as a modulation of tumor phenotype between the epithelial and mesenchymal-like states, is now considered an important mechanism in tumor progression (27, 28). Perhaps one of the most relevant consequences of this phenotypic modulation is the resulting acquisition of tumor cell resistance to a range of cell death-inducing signals, including those initiated by chemotherapy,

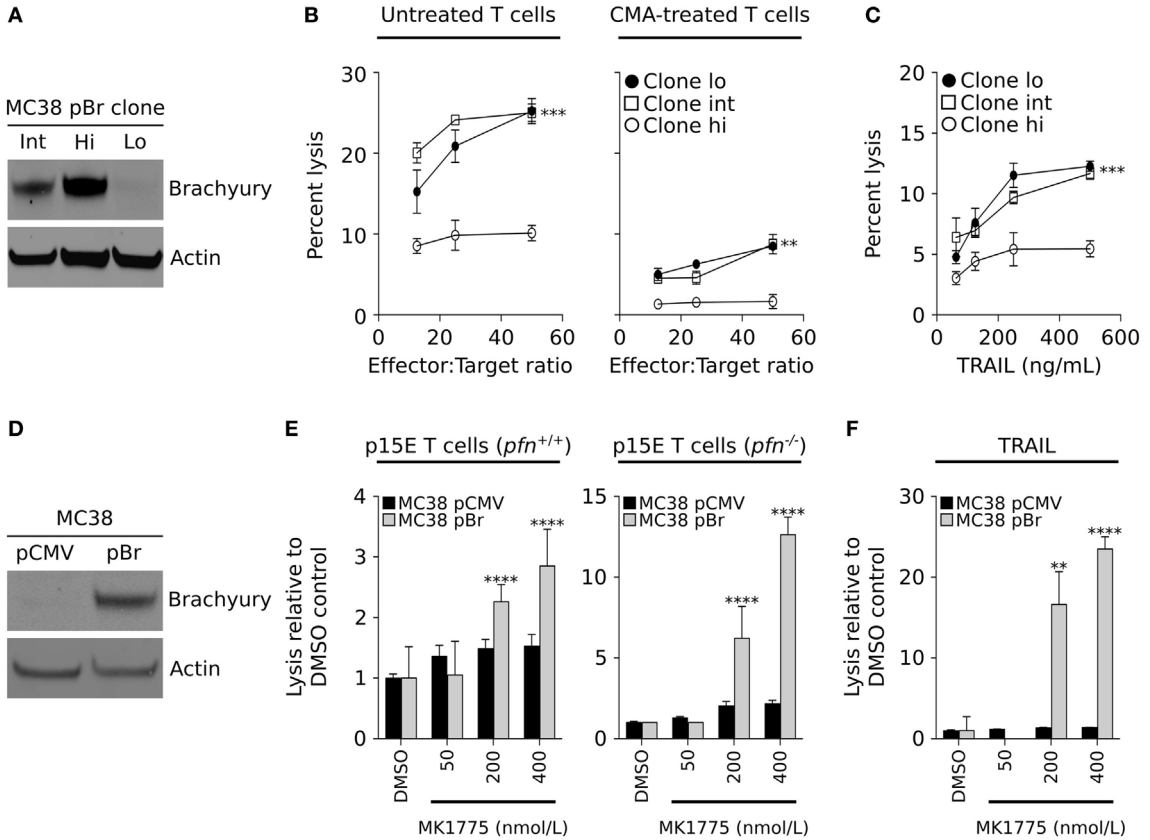

FIGURE 5 | WEE1 inhibition improves the sensitivity of brachyury high/p21-low carcinoma cells. **(A)** Western blot analysis of brachyury expression in three clonally derived MC38 cell populations expressing low (Lo), intermediate (Int), or high (Hi) levels of the human brachyury transgene. **(B)** Sensitivity to lysis by p15E-specific cytotoxic CD8+ T cells, which have been left untreated (left panel) or were pre-treated with CMA to inhibit their ability to lyse targets in a perforin-dependent manner (right panel). **(C)** Sensitivity of MC38 clones to indicated doses of recombinant TNF-related apoptosis-inducing ligand (TRAIL). **(D)** Western blot analysis of brachyury expression in the MC38 pCMV and pBr isogenic cell pair. **(E)** Lysis of the MC38 tumor pair following WEE1 inhibition with indicated doses of MK-1775 by p15E-specific T cells (E:T ratio 50:1) expanded from either wild-type mice (left panel) or perforin-deficient animals (right panel), or **(F)** TRAIL (125 ng/mL). The data depicting the lysis of the MC38 pCMV and pBr cell lines are representative of two independent experiments.

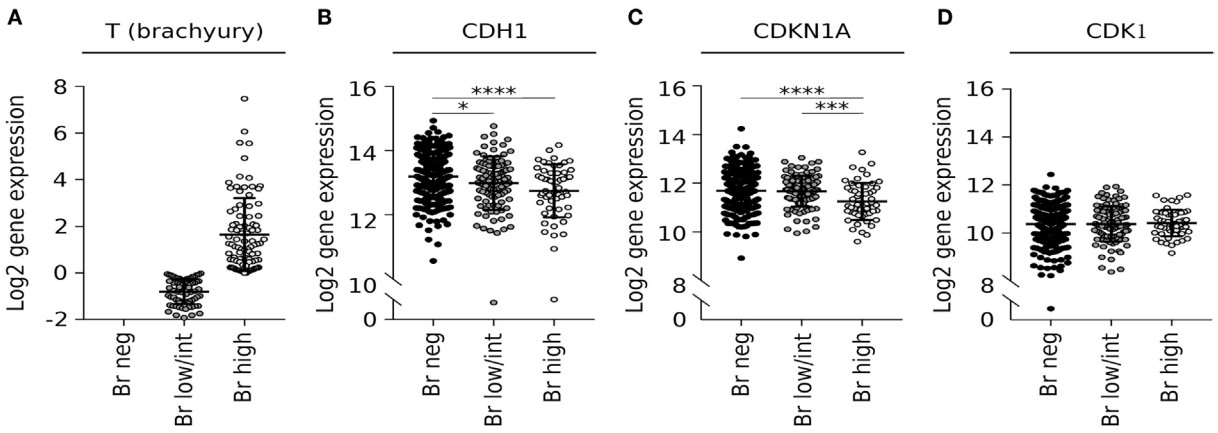

FIGURE 6 | Brachyury mRNA correlates with low expression of CDKN1A in lung squamous cell carcinoma samples. Analysis of expression of **(A)** brachyury (*T*), **(B)** E-cadherin (*CDH1*), **(C)** p21 (*CDKN1A*), and **(D)** CDK1 transcripts in the LUSC The Cancer Genome Atlas dataset tabulated according to the level of brachyury mRNA.

radiation, some small molecule-targeted therapies and, as more recently shown, resistance to immune-mediated cytotoxicity. In the current study, the mechanism of immune resistance of carcinoma cells bearing brachyury-driven mesenchymal features was elucidated. The data demonstrate the loss of the cell cycle regulatory protein p21 during mesenchymalization is responsible for the decreased susceptibility of brachyury-high tumor cells to immune-mediated attack.

Several reports have now demonstrated a link between the acquisition of mesenchymal features by carcinoma cells and escape from immune cytotoxicity (17, 29–33). For example, the overexpression of the transcription factor Snail, a known mediator of the phenomenon of epithelial–mesenchymal transition, has been shown to impair apoptosis in response to TNF-α by decreasing the activity of initiator caspase-8 and effector caspase-3 (34). Akalay and colleagues also demonstrated that Snail overexpression leads to tumor decreased susceptibility to T-cell mediated lysis *via* the activation of autophagy (32). In the case of brachyury-driven mesenchymalization, we have previously reported that brachyury-high carcinoma cells are less susceptible to the cytotoxic activity of immune effector cells, including antigen-specific cytotoxic T cells, NK, or LAK cells, compared with brachyury-low cancer cells (17, 33). In the present work, clonal populations of brachyury high/mesenchymal cells vs. brachyury low/epithelial cells were compared side-by-side. The results of these experiments, as shown in **Figures 1A**,**B** and **5B**, consistently showed tumor cells with a mesenchymal phenotype being less susceptible to immune attack than the epithelial counterparts. Previously, we also showed that expression of brachyury in carcinoma cells does not affect the levels of MHC-class I or beta-2 microglobulin expression. Moreover, brachyury does not reduce the expression of various components of the antigen processing and presentation machinery, including TAP1, TAP2, tapasin, LMP2, and LMP7 (17), thus ruling out a defect in antigen presentation as the cause of reduced sensitivity to immune attack. Rather, we showed that brachyury induced a blockade of apoptosis even in the presence of normal levels of activated caspases, which was associated with the defective phosphorylation and cleavage of the nuclear lamins following triggering (35, 36). This defect was due to the loss of the protein kinase CDK1 in brachyury-high tumor cells, although the mechanism of CDK1 protein destabilization was not understood at the time.

The p21 protein, initially identified as an inhibitor of cyclin-dependent kinases (37), has been described as a central player in multiple cell pathways, including (a) a direct target of p53 and a mediator of p53-dependent cell cycle arrest during DNA damage (38); (b) a protein involved in senescence and aging (39); and (c) a regulator of reprogramming in pluripotent stem cells (40). In certain conditions, p21 has also been shown to promote cell proliferation and oncogenicity. For example, p21 has been shown to act as a major adaptor protein that assembles cyclin D1/CDK4 complexes, targeting them into the nucleus and favoring cyclin D1-associated kinase activity (41). Thus, the p21 protein could exhibit both tumor suppressor and oncogenic properties, depending on the cellular context (42). In previous studies, the impact of brachyury overexpression in the proliferation of human carcinoma cells has been described. As shown with other regulators of the epithelial–mesenchymal phenomenon, expression of brachyury in carcinoma cells associates with a significant reduction in cell proliferation, whereby brachyury expression inversely correlates with the expression of phosphorylated Rb, Cyclin D1, and p21. In this context, our laboratory showed that forced upregulation of p21 was sufficient to reconstitute cell proliferation in high brachyury cells (23). Interestingly, there have been previous reports describing the involvement of p21 in tumor phenotypic plasticity. Liu et al., for example, demonstrated that deletion of p21 in transgenic mice not only accelerates mammary oncogenesis induced by MMTV-Ras and MMTV-c-Myc but also induces the acquisition of mesenchymal and stemness features in mammary tumors, *in vivo* (43). Similar observations with mammary carcinoma cells demonstrated that silencing of p21 and PUMA, a target of p53, leads to loss of E-cadherin and increased expression of markers of the epithelial–mesenchymal phenotypic switch (44). In subsequent studies, p21 has been shown to modulate the expression of various miRNAs, including miR-200 and the miR-183 cluster, which in turn regulate the expression of genes involved in the epithelial–mesenchymal switch. In various model systems, silencing of p21 increased mesenchymal features, including vimentin, fibronectin, N-cadherin, Slug, and Zeb1 expression, and increased tumor cell migration and invasiveness (25). The present report demonstrates that reconstitution of p21 in brachyury-high tumor cells markedly improves tumor cell lysis mediated by antigen-specific T cells, NK and LAK cells, TRAIL, and chemotherapy. In addition, reconstitution of p21 in tumor cells with high levels of brachyury is shown here to increase the stability of the CDK1 protein, leading to higher levels of CDK1 expression and the anticipated increase in tumor susceptibility to lysis. Our observations that the loss of p21 in brachyury-high carcinoma cells drives the acquisition of resistance to cytotoxic killing are in line with the concept that loss of this protein would also promote the acquisition of a more mesenchymal and resistant tumor status. It remains to be investigated, however, whether the expression of cytokines, chemokines, or growth factors secreted by immune effector T cells and NK cells co-cultured with tumor cells with various levels of p21 are different. These studies will help understand if the mesenchymalization phenomenon could potentially alter the recruitment, expansion, and level of activation of various populations of immune cells *via* secretion of a different set of soluble factors.

The WEE1 kinase regulates the G2 checkpoint in response to DNA damage, where WEE1 prevents entry into mitosis by mediating the inhibitory phosphorylation of CDK1. Previous studies conducted with WEE1 inhibition have shown that the effect of this inhibitor is mainly observed in the context of p53 mutant tumors, where the G1 checkpoint is lost and cells solely rely on the G2 checkpoint for cell cycle arrest following exposure to genotoxic agents (45–47). Interestingly, while brachyury overexpression in p53 wild-type cells resulted in decreased susceptibility to apoptosis triggered by TRAIL, no effect was observed when brachyury was overexpressed in tumor cells deficient for p53 (p53 null). These results suggested that high levels of brachyury, which negatively regulates the transcription of p21, may impart a p53 null phenotype in tumors with a functional p53 protein. In agreement with those results, inhibition of

WEE1 kinase *via* treatment with MK-1775 improved the lysis of brachyury-high tumor cells only in the absence of p21, while the effect was lost when p21 was forcibly overexpressed in the presence of high brachyury.

The results of this study reinforce the concept that acquisition of mesenchymal features by carcinoma cells is accompanied by the acquisition of resistance mechanisms that allow tumor cells to evade not only cytotoxic therapies but also immune-mediated attack. This study also situates p21 as a central player in many of the aspects of the brachyury biology, including control of cell cycle, resistance to apoptosis induced by chemotherapy and, more importantly, resistance to immune-mediated lysis.

Several vaccine platforms targeting brachyury have now been developed and are undergoing clinical evaluation (14–16), based on the hypothesis that immunization against a driver of mesenchymalization could generate a T-cell response that would eradicate the population of cancer cells ultimately responsible for metastasis and relapse. We demonstrate here that the defects in tumor cell death described in association with very high levels of brachyury in tumor cells could be alleviated *via* the use of a WEE1 inhibitor, which is currently undergoing clinical testing (26). Our data demonstrate the potential usefulness of this inhibitor for improvement of immune resistance of carcinoma cells with mesenchymal features and high levels of brachyury expression. While most phase II clinical trials of WEE1 inhibition in combination with chemotherapy are conducted in patients with p53-defective tumors, we expect that our studies will provide rationale for the use of WEE1 inhibition in tumors regardless of p53 status, in combination with immunotherapeutic approaches against the transcription factor brachyury.

ETHICS STATEMENT

Peripheral blood used in this study was obtained from healthy human donors recruited at the NIH Blood Bank (Bethesda, MD, USA), protocol number NCT00001846, under the appropriate NIH Institutional Review Board approval and informed consent. All animal studies were carried out in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Experimental studies were carried out under approval of the NIH Intramural Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

DH designed and conducted experiments, analyzed data, and wrote the manuscript; KM conducted experiments and analyzed data; CP designed experiments, analyzed data, supervised the study, and wrote the manuscript.

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Microenvironment-Driven Dynamic Heterogeneity and Phenotypic Plasticity as a Mechanism of Melanoma Therapy Resistance

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Drug resistance constitutes a major challenge in designing melanoma therapies. Microenvironment-driven tumor heterogeneity and plasticity play a key role in this phenomenon. Melanoma is highly heterogeneous with diverse genomic alterations and expression of different biological markers. In addition, melanoma cells are highly plastic and capable of adapting quickly to changing microenvironmental conditions. These contribute to variations in therapy response and durability between individual melanoma patients. In response to changing microenvironmental conditions, like hypoxia and nutrient starvation, proliferative melanoma cells can switch to an invasive slow-cycling state. Cells in this state are more aggressive and metastatic, and show increased intrinsic drug resistance. During continuous treatment, slow-cycling cells are enriched within the tumor and give rise to a new proliferative subpopulation with increased drug resistance, by exerting their stem cell-like behavior and phenotypic plasticity. In melanoma, the proliferative and invasive states are defined by high and low microphthalmia-associated transcription factor (MITF) expression, respectively. It has been observed that in $MITF^{high}$ melanomas, inhibition of MITF increases the efficacy of targeted therapies and delays the acquisition of drug resistance. Contrarily, MITF is downregulated in melanomas with acquired drug resistance. According to the phenotype switching theory, the gene expression profile of the $MITF^{low}$ state is predominantly regulated by WNT5A, AXL, and NF-κB signaling. Thus, different combinations of therapies should be effective in treating different phases of melanoma, such as the combination of targeted therapies with inhibitors of MITF expression during the initial treatment phase, but with inhibitors of WNT5A/AXL/NF-κB signaling during relapse.

Keywords: melanoma, tumor heterogeneity, clonality, cancer drug resistance, tumor microenvironment, microphthalmia-associated transcription factor, tumor plasticity, slow-cycling tumor cells

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INTRODUCTION

The development of targeted therapies for metastatic melanoma using small molecule MAPK pathway inhibitors (MAPKi) or immune checkpoint antagonists (ICi) has revolutionized dermatological oncology. However, first-generation MAPKi only works in approximately 35–50% of cases as a $BRAF^{V600}$ mutation must be present (1, 2). ICi show response rates of up to 60%, depending on

drug or combination, and many of these are durable effects (3). Yet, drug resistance constitutes a major challenge for effective cancer treatment with melanoma being no exception. Rapid resistance to MAPKi is common and has also been reported for ICi (4–9). Although the molecular mechanisms leading to inherent and acquired drug resistance have been discussed extensively in the literature, the dynamics leading to resistance are poorly understood but yet critical to designing better treatments. Besides genetic and epigenetic factors, other contributors to drug resistance are microenvironment-driven tumor heterogeneity and plasticity (10–16).

MECHANISMS OF INTRINSIC AND ACQUIRED DRUG RESISTANCE IN MELANOMA

Intrinsic refers to a pre-existent drug resistance of the entire population or a subpopulation of cancer cells before exposure to the drug. For example, intrinsically resistant cancer cells do not harbor the targeted mutation or are not dependent on the pathway inhibited by the drug. In the case of acquired drug resistance, the tumor responds initially to the treatment but relapses and progresses later. However, it is difficult to distinguish between intrinsic and acquired resistance as a small subpopulation of intrinsically resistant cancer cells subsequently enriched, may also explain initial response and later relapse (17–20). Causative factors that contribute to MAPKi resistance can be broadly classified into three categories: mutational events, non-mutational events, and changes in the surrounding microenvironment (21). Mutational and non-mutational events that contribute to the development of drug resistance have been discussed previously (21, 22) and are not the focus of this review. In brief, the mechanisms linked with these events predominantly lead to MAPK pathway reactivation and/or activation of parallel signaling pathways (e.g., PI3K/AKT/mTOR) (21, 23). Besides mutational and non-mutational events which are intrinsic to tumor cells, the tumor microenvironment contributes to the development of drug resistance by influencing the crosstalk between distinct cellular compartments. Solid tumors are comprised of tumor cells and stromal cells (e.g., fibroblasts, endothelial cells, and lymphocytes) that form an organ-like structure which is embedded within the extracellular matrix (ECM) and nourished by a vascular network. Each of these components show varying distribution within the tumor resulting in a highly complex and heterogeneous tumor microenvironment (24). In melanoma, secretion of tumor necrosis factor-α (25, 26), hepatocyte growth factor (HGF) (27), Wnt antagonist, sFRP2 (28), and increased production of ECM (29) by stromal cells in the tumor can cause resistance to MAPKi. Thus, the density of stromal cells in different parts of the tumor plays a key role in determining response and resistance to MAPKi. In addition, the distribution of the vasculature plays a crucial role in the acquisition of varying drug resistance mechanisms in different parts of the tumor, due to differences in the levels of nutrients and oxygen. Hypoxia can induce resistance to MAPKi by mediating upregulation of HGF/MET signaling (30), increasing SNAIL, and decreasing E-cadherin expression (31).

TUMOR HETEROGENEITY AND PLASTICITY

Tumor heterogeneity refers to the presence of subpopulations of cells that differ phenotypically and/or by biological behavior, either within a tumor (intra-tumoral) or between tumors of the same histopathological subtype within a patient (inter-tumoral) or between patients (inter-patient) (32). Melanoma heterogeneity plays a key role in the response to MAPKi (5, 20). At the molecular level, the features of different subpopulations are conferred by alterations of the genome, transcriptome, epigenome, and proteome (33, 34). Melanoma is one of the most heterogeneous cancers (35), harboring diverse genomic alterations, including gain of function mutations (e.g., *NRAS*, *BRAF*, *KIT*, *CDK4*, and *MITF*), loss of function mutations (e.g., *CDKN2A*, *PTEN*, *ARID2*, and *NF*), and epigenetic changes (e.g., *PTEN*, *CDKN2A*, *RAC1*, and *P53*) (36). In addition, various biological markers of melanoma (e.g., CD20, CD133, ABCB5, CD271, JARID1B, and ALDH1) show differential expression patterns in different regions within a tumor (36).

There are three tumor heterogeneity models (37). The well-accepted clonal evolution model (38) refers to acquired additional genetic mutations in cancer cells that contribute to their altered phenotype and malignant potential. This results in a Darwinian-style selection of clones during disease progression (38). The stem cell model suggests that only a small fraction of tumor cells have the potential for maintaining the tumor and drive progression (39). These cancer stem cells have self-renewal capability and can be differentiated into "non-stem cancer cells" that lose their tumorigenic potential by acquiring stable epigenetic changes and occupy the largest fraction of the tumor (37, 39, 40). These two models are complementary to each other, rather than mutually exclusive (41). Their common feature is the unidirectional, irreversible nature of the molecular changes that lead to tumor heterogeneity (37). An alternative model is "phenotypic plasticity" or "phenotype switching." This model suggests that tumor cells with different phenotypic and functional behavior can dynamically shift between different transcriptional programs (42–44). The different phenotypic states, described in terms of differential gene expression patterns, have been termed "proliferative" and "invasive" signatures (45). In this model, molecular changes resulting in tumor heterogeneity are reversible, unlike the clonal evolution and stem cell models. These changes are predominantly regulated by cues from the surrounding microenvironment, e.g., hypoxia, stroma-derived factors like HGF, TGF-β. For example, in response to hypoxia, proliferative melanoma cells can switch to the invasive phenotype by altering their gene expression profile (10, 46).

MICROENVIRONMENT-DRIVEN DYNAMIC HETEROGENEITY IN MELANOMA

"Tumor microenvironment" is a broad term, which includes (1) the tumor stroma composed of fibroblasts, endothelial cells, immune cells, soluble molecules, and the ECM, (2) the epidermal microenvironment where the tumor had originated from,

and (3) different subcompartments within the tumor itself (47). Interactions between tumor cells and the microenvironment contribute to the malignant behavior of tumor cells, e.g., progression, metastasis, angiogenesis, migration, and invasion (48, 49). In addition, microenvironmental stress signals in response to nutrient starvation and inflammation drive phenotypic plasticity and invasion and determine therapeutic outcome (16, 50). Similarly, a pre-existing immune-active tumor microenvironment is necessary for a favorable response to ipilimumab, and potentially other ICi (51–53).

We have developed a 3D melanoma spheroid model, which recapitulates the *in vivo* tumor microenvironment and architecture (54, 55), that combined with the fluorescent ubiquitination-based cell cycle indicator (56) is a useful tool to study the microenvironment *in vitro* (57, 58). This model is being complemented constantly, e.g., by including DRAQ7 as a real-time cell death marker (59) or by applying mathematical algorithms to predict spatial and temporal patterns of cell density and cell cycle (60, 61). Due to an oxygen and nutrient gradient, melanoma spheroids segregate into a continuously proliferating subpopulation in the periphery and a G1-arrested subpopulation in the center (12). A similar phenomenon is observed in human melanoma xenografts in mice, where clusters of cycling cells are located near blood vessels and quiescent cells in central tumor zones (12). After isolating these two subpopulations from spheroids and plating them in 2D culture separately, within 24 h G1-arrested central cells recommence their cell cycle and become indistinguishable from the proliferating peripheral subpopulation (12). This supports the phenotypic plasticity model (10, 23). The cell cycle phase can also contribute to drug sensitivity (13, 62, 63) and can be targeted for cell cycle-tailored melanoma therapy (64). For example, bortezomib preferentially kills melanoma cells in the S/G2/M phase of the cell cycle (15). By contrast, cell cycle arrest can confer tolerance to drugs (14, 64, 65).

THE ROLE OF A SLOW-CYCLING SUBPOPULATION IN MELANOMA THERAPY RESISTANCE

Although dysregulated proliferation is a hallmark of cancer (66, 67), a quiescent or slow-cycling cell subpopulation is reported in many solid cancers, including melanoma. This slow-cycling subpopulation is a major determinant of treatment resistance to targeted therapies (68–70). Increased level of oxidative phosphorylation in slow cycling compared to normal cells (69, 71) contributes to drug resistance in many cancers including melanoma (72–74). MAPKi are predominantly effective in targeting rapidly proliferating cells, while the slow-cycling cells are not readily responsive to MAPKi (69, 75, 76). Thus, cells in the slow-cycling state or cells that switch to this state due to therapeutic stress, can evade the action of MAPKi.

Various mechanisms are utilized by this slow-cycling subpopulation to contribute to drug resistance. First, clonal expansion of the residual slow-cycling cells, that have survived initial treatment, results in their enrichment within the tumor. A recent study suggested that these slow-cycling cells are highly aggressive with increased metastatic potential (77). Second, the slow-cycling subpopulation also displays increased cancer stem cell-like behavior (78). Consistent with the stem cell theory, in melanoma, these slow-cycling cells comprise only 0.5–5% of all tumor cells with self-renewal potential and are defined by the expression of the H3K4 demethylase JARID1B (23). In addition, JARID1B-positive cells are essential for maintaining tumor growth (23). During continuous treatment, slow-cycling cells can gain the potential to differentiate into other cell types with an increased proliferation rate and drug resistance, subsequently resulting in relapse. The cells experience a high level of "therapeutic stress," forcing them to employ several drug resistance mechanisms. Thus, overtime highly resistant drug tolerant cells are enriched within the tumor and contribute to the highly aggressive and drug resistant nature of metastatic melanoma after relapse. JARID1B-positive cells can give rise to JARID1B-negative cells and also *vice versa* (23). This supports the phenotype switching theory which indicates the plastic nature of tumor cells that is predominantly influenced by changing microenvironmental conditions (**Figure 1**). In addition to JARID1B, PGC1α defines another distinct slow-cycling state in melanoma with increased treatment resistance (71, 73).

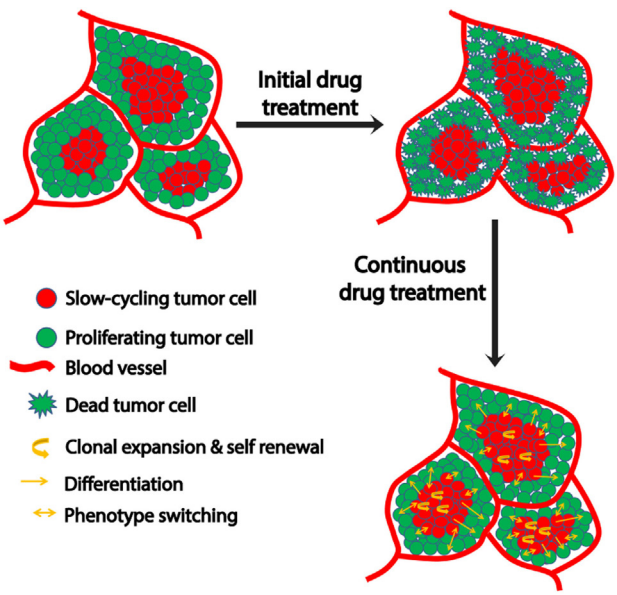

FIGURE 1 | Schematic representation of microenvironment-driven dynamic heterogeneity and phenotypic plasticity as a mechanism of melanoma therapy resistance. Tumor cells close to the blood vessels proliferate, while those away from blood vessels experience hypoxia and nutrient starvation that contribute to their slow-cycling phenotype. While treatment readily targets proliferating cells, slow-cycling cells can evade drug action and survive. Upon continuous treatment, this slow-cycling subpopulation is enriched within the tumor by clonal expansion. Due to their inherent cancer stem cell-like property, they are capable of self-renewal or differentiation into a proliferative tumor cells with increased drug resistance. In addition to this, the slow-cycling cells can switch their phenotype to fast proliferating cells upon exposure to oxygen and nutrients after replacing the original peripheral fast proliferating cells that had been killed by the therapy. These phenotype-switched cells might be more drug resistant too, as they might have acquired resistance during their slow-cycling phase.

Taken together, slow-cycling cells play a pivotal role in developing therapy resistance and cancer progression. Thus, it is crucial to understand the underlying biology of the slow-cycling phenotype to improve the current therapy regimens in melanoma.

THE ROLE OF MICROPHTHALMIA-ASSOCIATED TRANSCRIPTION FACTOR (MITF) IN MELANOMA PLASTICITY AND THERAPY RESISTANCE

Microphthalmia-associated transcription factor is the master regulator of both normal melanocyte and melanoma biology (79, 80). In melanoma, MITF acts as a molecular switch that determines whether the cell will differentiate, proliferate, or become quiescent with increased migratory behavior (44, 81–84). The proliferative and invasive phenotypes of melanoma cells are defined by high and low levels of MITF, respectively, and melanoma cells are capable of switching between these two states, influenced by changing microenvironmental conditions (10, 45).

Depletion of MITF can reduce proliferation through G1-arrest (42, 68, 81, 85) with increased expression of cancer stem cell markers (68, 86). In response to hypoxia, MITF expression is downregulated (87). These properties are attributes of slow-cycling JARID1B-positive melanoma cells (20), supported by a negative correlation of MITF and JARID1B/SerpinE2 (77). Thus, in response to stress, e.g., hypoxia and/or nutrient starvation, melanoma cells switch from a proliferative $MITF^{high}$ to an invasive $MITF^{low}$ slow-cycling phenotype. However, these subpopulations are not mutually exclusive, as within a tumor there can be $MITF^{high}$ and $MITF^{low}$ cells, reflecting tumor heterogeneity as discussed above. In contrast to the proliferative $MITF^{high}$ phenotype, the invasive $MITF^{low}$ phenotype is mainly governed by receptor tyrosine kinases (e.g., AXL, EGFR, and ERB3), WNT5A or NF-κB signaling, and the BRN2–NFIB–EZH2 axis (46, 88–90). Single cell expression analysis revealed that some $MITF^{high}$ cells also express the gene signature of the invasive $MITF^{low}$ phenotype (91, 92). These and other studies indicate the presence of a third subpopulation in melanoma that expresses MITF, AXL, and WNT5A simultaneously (88, 93, 94). Consistent with this, we showed by using a 3D melanoma spheroid model that indeed melanoma cells can proliferate and invade simultaneously (12). In addition, another study has shown that invasive $MITF^{low}$ and poorly invasive $MITF^{high}$ cells cooperate to invade into the surrounding matrix (95).

The role of MITF in drug resistance is controversial and the underlying mechanisms are yet to be understood. For instance, the presence of MITF is a marker for responsiveness to MAPKi treatment, but when MITF expression is upregulated, it can confer resistance to MAPKi (96). This might reflect the extreme end of the MITF rheostat model defined by differentiation, slow cycling (42), high PGC1α expression, and therapy resistance (20). Augmenting MITF levels in melanoma cells should switch the invasive slow-cycling phenotype to a proliferative phenotype. This would increase drug sensitivity because MAPKi predominantly act on rapidly proliferating cells. In addition, over-expression of MITF will inhibit the switching of proliferative cells to the invasive slow-cycling phenotype in response to stress by maintaining MITF levels constant. However, MITF is also reported as a driver of melanoma progression (97–99) and long-term MITF depletion induces senescence in melanoma cells and/or promotes apoptosis (81, 100, 101). Melanoma cells upregulate MITF expression to recover the loss of MAPK signaling upon exposure to MAPKi, enabling the cells to tolerate MAPKi (102). Downregulation of MITF increases the cytotoxic effects of MAPKi on melanoma cells and also reduces the acquisition of drug resistance (101, 103, 104). Upregulation of MITF has also been seen in several MAPKi acquired resistant cell lines (89). However, the same study reports that another population of resistant cell lines has lost MITF expression. MITF is downregulated in the acquired drug resistant phase and makes the cells more invasive (89). Thus, further investigation of these signaling pathways is required to determine in which combination these signaling pathways can be targeted along with the inhibition of MAPK signaling, to improve the outcomes of melanoma patients with disease relapse.

However, the situation appears to be even more complex, as in heterogeneous tumors $MITF^{high}$ and AXL^{high} populations can co-exist (33, 102). Nevertheless, it has been shown that these subpopulations benefit from endothelin-1 in the presence of MAPKi, as inhibiting endothelin-1 signaling can effectively inhibit the growth of such heterogeneous tumors (105). More comprehensive studies are required to determine how MITF expression levels are altered in relation to the tumor's response to MAPKi during ongoing treatment. Combination of MITF inhibitors with MAPKi should improve the efficacy of MAPKi in treating phases with high MITF expression. On the contrary, inhibitors of WNT5A/AXL/NF-κB in combination with MAPKi should improve the efficacy of MAPKi in treating phases with low MITF expression (**Figure 2**). Indeed, targeting AXL and BRAF/MEK simultaneously in a patient-derived xenograft model confers an increased survival advantage to the mice compared to monotherapy with either AXL or combination therapy with BRAF/MEK inhibitors (106).

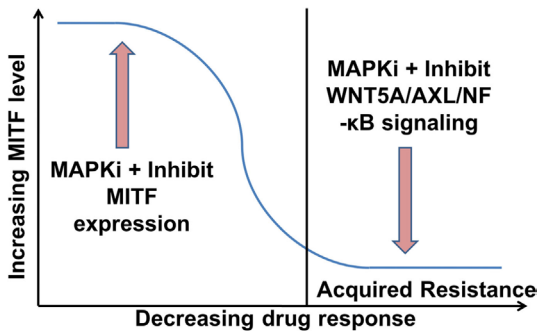

FIGURE 2 | Proposed role of microphthalmia-associated transcription factor (MITF) and WNT5A/AXL/NF-κB signaling in melanoma therapy. $MITF^{high}$ melanomas could be treated initially with a combination of an MAPK pathway inhibitors (MAPKi) and an inhibitor of MITF expression. This should increase the efficacy of the MAPKi and delay the acquisition of drug resistance (104). Once in the resistance state with low MITF levels, the therapy could comprise a combination of a MAPKi and an inhibitor of WNT5A/AXL/NF-κB signaling.

CONCLUSION

Tumor microenvironment-driven dynamic heterogeneity is a major determinant of drug resistance in melanoma. This is mainly exerted by regulating the level of the master regulator MITF which is the major determinant of the dynamic phenotypic states in melanoma. A moderate MITF level determines the proliferative state of melanoma which is readily targetable with MAPKi. Both low and extremely high MITF levels give rise to two distinct slow-cycling states of melanoma (e.g., $MITF^{low}$/JARID1B-positive and $MITF^{high}$/PGC1α-positive) with increased oxidative phosphorylation that results in treatment resistance. Thus, targeting this slow-cycling subpopulation by modulating MITF levels can be a potential strategy to overcome drug resistance in melanoma. However, MITF biology is highly complex and the downstream effects of MITF are extremely diverse (107). In addition, mechanisms that regulate MITF expression and activity are also numerous (80). Thus, modulation of MITF expression and activity can have diverse effects on melanoma cell biology. Considering the dynamic expression of MITF in response to changing microenvironmental conditions at various phases of melanomagenesis, MITF levels can be considered as a predictive marker for a suitable therapy regimen for treating a particular melanoma phase. We have developed an *in vitro* 3D melanoma spheroid model that mimics dynamic tumor heterogeneity to study the biology of microenvironment-driven tumor heterogeneity and plasticity and as these dynamic changes are difficult, time-consuming, and expensive to study *in vivo*.

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NH and FA wrote the manuscript together.

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REVIEW
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Tissue-Dependent Tumor Microenvironments and Their Impact on Immunotherapy Responses

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Recent advances in cancer immunology have led to a better understanding of the role of the tumor microenvironment (TME) in tumor initiation, progression, and metastasis. Tumors can occur at many locations within the body and coevolution between malignant tumor cells and non-malignant cells sculpts the TME at these sites. It has become increasingly clear that there are specific differences of the TMEs at different anatomical locations, and these tissue-specific TMEs regulate tumor growth, determine metastatic progression, and impact on the outcome of therapy responses. Herein, we review the scientific advances in understanding tissue-specific TMEs, discuss their impact on immunotherapeutic response, and assess the current clinical knowledge in this emerging field. A deeper understanding of the tissue-specific TME will help to develop effective immunotherapies against tumors and their metastases and assist in predicting clinical outcomes.

Keywords: tumor microenvironment, tissue-specific microenvironment, immunotherapy, immunosuppression, anticancer therapy

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INTRODUCTION

Tumor cells do not grow in isolation, but exist in a complex tumor microenvironment (TME), which the tumor cells depend upon for growth and metastasis. The TME comprises cells of hematopoietic origin (lymphocytes and myeloid cells), mesenchymal origin (fibroblasts, myofibroblasts, mesenchymal stem cells, adipocytes, and endothelial cells), and the extracellular matrix (ECM) (1). The components of the TME are manipulated by tumor cells and participate in tumor progression throughout all stages of tumorigenesis (1).

Tumors can arise in, and metastasize to, various tissues. Clear evidence suggests that the tissue of tumor growth influences the TME composition (2, 3). These tissue-specific TMEs regulate tumor growth, determine metastatic progression, and impact the outcome of therapy responses. In this review, we discuss tissue-specific differences in the TME and its impact on therapeutic response. We propose that understanding such differences is important for the development of effective immunotherapies against tumors and their metastases.

The Immunosuppressive TME and Its Impact on Therapeutic Response

Avoiding immune destruction is an emerging hallmark of cancer (4). The established TME contains cell types that can contribute to immune evasion by inhibiting effective antitumor response of effector cells (**Figure 1**). The immunosuppressive and other protumorigenic cell types within the TME have been reviewed in detail elsewhere (1, 5). Briefly, as shown in **Figure 1**, immunosuppressive

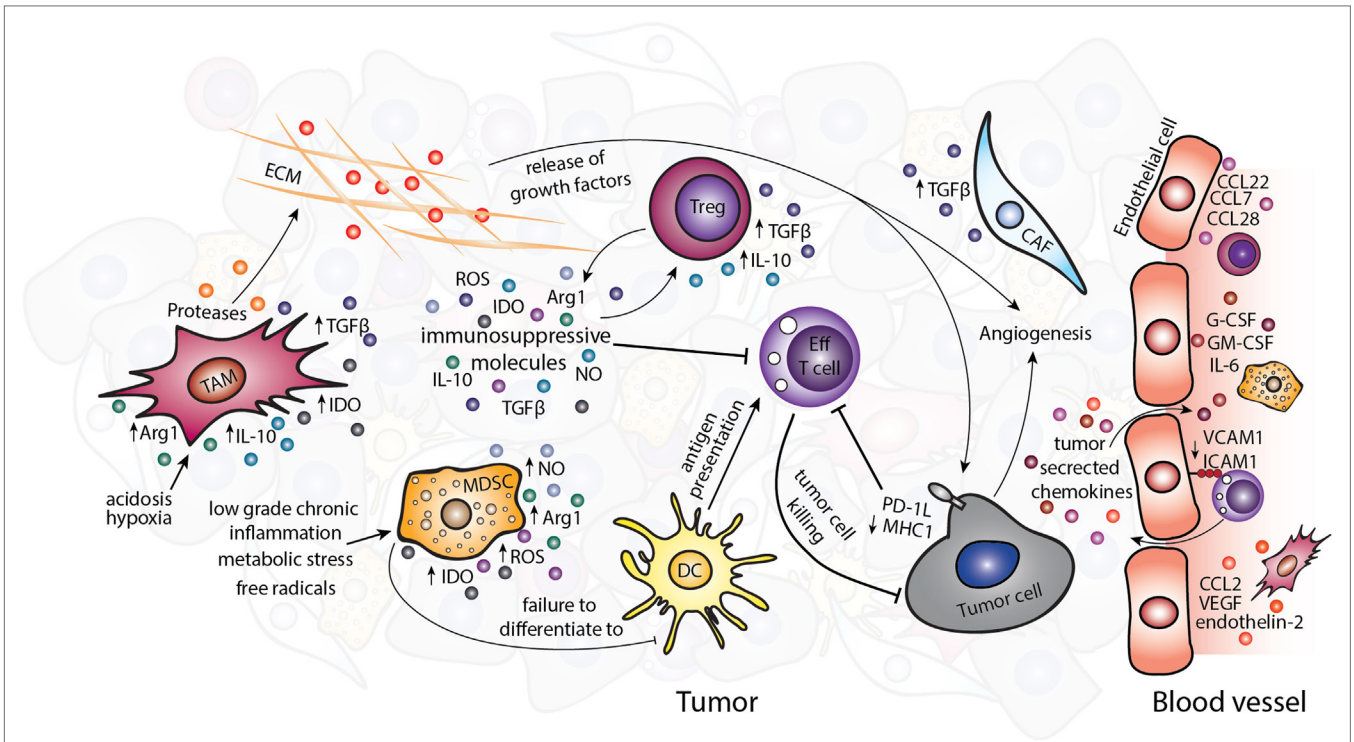

FIGURE 1 | The immunosuppressive tumor microenvironment. Various immunosuppressive cell types exist within the TME including TAMs, MDSCs, Tregs, and CAFs. Tumor-derived chemokines and the metabolic tumor environment recruit or polarize these cells to a protumor, immunosuppressive phenotype. Suppression of the antitumor immune response occurs partly through the release of immunosuppressive molecules such as TGF-β, IL-10, IDO, Arg1, ROS, and NO. Abbreviations: Arg1, arginase1; CAFs, cancer-associated fibroblasts; DC, dendritic cell; ECM, extracellular matrix; Eff T cell, effector T cell; IDO, idoleamine 2,3-dioxygenase; MDSCs, myeloid-derived suppressor cells; ROS, reactive oxygen species; NO, nitric oxide; TAMs, tumor-associated macrophages; Tregs, regulatory T cells.

cell types such as regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) can be present within the TME. These cells can express immunomodulatory factors such as interleukin (IL)-4, IL-10, IL-13, and arginase1 (Arg1), which suppress or reprogram the antitumor immune response (6–8), for example, by depletion of the essential amino acid arginine or the skewing of immunity toward a Th2-type response ill-suited to tumor cell destruction. Depleting these immunosuppressive cells in mouse models of cancer can reduce tumor growth and progression (9–11), and infiltration of these cells in human tumors has been associated with poor prognosis (12–17).

Non-immune cells of the TME also contribute to enhancing tumorigenesis and can directly influence the antitumor immune response. Cancer-associated fibroblasts (CAFs) can secrete protumorigenic molecules including mitogenic growth factors, pro-angiogenic factors, and TGF-β, which alter the TME and support cancer progression (18). The chaotic tumor vasculature that comprises endothelial cells and pericytes is usually leakier than normal vasculature and is therefore unable to support efficient trafficking of cytotoxic immune cells to the tumor (19, 20). The abnormalities of blood vessels have been identified in a number of tumor types in murine models, such as spontaneous RIP-Tag2 pancreatic islet tumors, MCA-IV mammary carcinomas, Lewis lung carcinomas (21), 4T1 mammary carcinoma (22), and B16F10 melanoma (23), although studies directly comparing different tumor types and subtypes, especially in human cancers are lacking. The expression of pro-angiogenic signals in the TME, such as stromal-derived factor-1, thrombospondin, and matrix metalloproteases secreted by CAFs (24) and VEGF by TAMs (25) can further contribute to altered tumor vasculature (26). The non-cellular ECM that plays an important part in tissue homeostasis is also altered in tumors by the imbalance between ECM synthesis and secretion and changes in the levels of matrix-remodeling enzymes (27). The altered ECM results in changes to the tissue architecture and release of soluble molecules and growth factors. These changes further propagate the TME partly *via* influencing the actions of immune cells (**Figure 1**) (28, 29).

Immunotherapeutics that aim to alter the immune TME to target cancers have revolutionized the treatment of cancer. Attention has recently focused on two classes of immunotherapies, including immune checkpoint inhibitors (ICI) and adoptive cellular transfer (ACT). Melanoma has served as the test bed for ICI with the initial development of anti-CTLA-4 antibodies (ipilimumab) (30) and more recently antibodies that inhibit the programmed death-1 axis (e.g., nivolumab, pembrolizumab) (31, 32). The objective response rates were 43.7 and 19% in metastatic melanoma patients treated with nivolumab and ipilimumab alone, and the combination of nivolumab and ipilimumab resulted in a much higher response

rate (57.6%) (26). Besides, ICI have also established efficacy in a range of other solid tumors such as non-small cell lung cancer (NSCLC) (33, 34) and renal cell carcinoma (35). In these trials, the objective response rates were 14.5% (33) and 44.8% for patients with refractory/advanced NSCLC (34), and 20–22% for metastatic renal cell carcinoma patients (35). Recent developments of chimeric antigen receptor-T cells in CD19 hematological malignancies have led to high complete response rates and durable regressions in both lymphoma and leukemia (36, 37) and has generated some promising results in solid cancers in small studies (38). Despite these successes, not all patients obtain clinical benefit, which is often attributable to *de novo* resistance mediated by TME.

A role for the TME in resistance to anticancer immunotherapies has been established. Various cell subsets that contribute to an immunosuppressive TME are associated with reduced therapeutic efficacy. Higher numbers of MDSCs correlate with poor response to various immunotherapies including immune checkpoint blockade (39), ACT (40), and dendritic cell (DC) vaccination (41). The ratio of effector T cells (Eff T cells) to Tregs is associated with response to anti-CTLA-4 checkpoint blockade therapy, where higher Tregs are associated with decreased efficacy (42, 43). Blocking the recruitment of TAMs using anti-CSF-1R antibodies is synergistic with ACT and checkpoint blockade therapy, indicating that TAMs have a crucial role in mediating response to immunotherapy (44, 45). The influence of the TME on therapeutic response is not restricted to immunotherapies and has also been shown for various anticancer therapies including those directly targeting malignant cells such as chemotherapy (46–49). Thus, the TME has a notable impact on the outcome of anticancer therapeutics and its consideration is essential for effective immunotherapies.

In summary, there is a clear role for the TME in modulating responses to tumor and stromal targeted anticancer therapies. The complexity and adaptability of the TME during tumor development and in response to various treatments remains to be properly characterized and is a challenge within itself. Our current knowledge of the progression and sculpting of the TME is somewhat limited; however, there is clear evidence for tissue-specific tumor development.

Tissue-Specific TMEs

There is clear evidence that tumor initiation and metastasis is tissue specific. Cancer cells arising from the same organ or tissue often share specific driver mutations (50). In the case of familial cancers, inherited mutations in driver genes cause cancer in specific organs such as BRCA1 and BRCA2 in hereditary breast and ovarian cancer. The simplest explanation for this tissue-specific tumorigenesis would be that these mutated genes are only expressed in the tissues where the tumors commonly develop. However, this is not the case, as many driver genes are expressed in various tissues that do not form tumors from mutations in these genes. Instead, tissue-specific tumorigenesis can be explained by a multitude of factors (3). One of these is the likely presence of various cell types within the tissue microenvironment that is dependent on the anatomical location. For example, resident myofibroblast-like stellate cells within the liver and pancreas are pathogenic drivers of fibrosis and can promote tumor development (51). In addition, different cancer types tend to colonize specific organs, known as the seed and soil hypothesis or organotropism (52, 53). As a result of tumor-secreted factors and tumor-shed extracellular vesicles, the tissue microenvironment of metastatic sites is altered to form a premetastatic niche (54). This is similar to the manipulation of local non-malignant cells to form the primary TME as mentioned previously. Thus, the tissue of origin, including the non-malignant cell types within, is a specific regulator of malignant transformation and metastatic colonization.

Both preclinical and clinical evidence indicates the tissue of tumor growth as an influential factor in the established TME. Although some effort has been made to understand how the tissue-specific microenvironment interacts with tumors at different sites, it is difficult to eliminate the effect of tumor cell heterogeneity due to the genetic heterogeneity of tumors (55). Only a few groups of investigators have used preclinical murine models of cancer with implantation of genetically identical tumor cell lines at various anatomical sites to eliminate tumor cell dependent heterogeneity. Such studies have shown that genetically identical tumors growing at different anatomical sites have site-specific transcript, protein, and metabolite profiles. For example, in murine models of pancreatic cancer using various cell lines (CD18/HPAF, FG, L3.3, L3.6pl, and BxPC3), multiple studies have shown that orthotopic or SC implanted tumors of the same cell line have different gene expression profiles (56–58). Analysis of RNA expression profiles in orthotopic tumors has shown elevated expression of known pancreatic cancer-associated genes such as MUC4 and TGFβ2. In separate studies, comparison of SC and orthotopic renal cell carcinoma (SN12C and SN12PM6) or orthotopic prostate cancer (PC-3M) showed decreased mRNA and protein expression of basic fibroblast growth factor in SC tumors (59, 60), which is known to promote angiogenesis. In the PC-3M prostate cancer model, orthotopic tumors expressed lower levels of other protumorigenic transcripts including the ones encoding EGFR, mdr-1, collagenase type IV, and IL-8 compared with SC tumors (60). Similarly, A375P and A375SM melanoma cells growing subcutaneously had higher expression of IL-8 by northern blot and IHC compared with melanoma cells growing in the lung (intermediate IL-8) and liver (low IL-8) (61). Recently, Zhan et al. performed a metabolomics study of pancreatic ductal adenocarcinoma cell lines (Panc-1 and BxPC-3) growing SC or orthotopically using ^{1}H NMR spectroscopy. Clear differences in metabolites in the tumors, but not in serum, were detected between mice with SC and orthotopic tumors. Notably, the orthotopic tumors had higher levels of adenosine (an immunosuppressive metabolite) compared with SC tumors (62). Thus, current evidence in the field suggests the tissue of tumor growth can influence the molecular composition of tumors including RNA, protein, and metabolites. Furthermore, comparisons between orthotopic and SC tumors suggest that the TME of orthotopic tumors are more immunosuppressive and protumorigenic.

The cellular composition of the TME can also vary depending on the tissue of tumor growth. For example, in a murine model of breast cancer, the immune cell profile was compared between

4T1 tumors growing SC or intratibially (63). FACS analysis in this model revealed differences in the proportions of macrophages, DCs, $CD8^+$, and $CD4^+$ T cells in the tibia and under the skin of mice with tumors growing at these sites. Interestingly, the site of tumor growth also affected the immune cell populations in the spleen, as the mice bearing SC tumors displayed a significant decrease in T cells in their spleens compared with mice bearing tumors in the tibia. Similar observations have been reported in human cancers. An elegant study investigating multiple metastases in a patient with high-grade serous ovarian adenocarcinoma showed multiple distinct tumor immune microenvironments coexisted within the same patient. The immune infiltration and activation of the tumors assessed by IHC and RNAseq of immune-related genes were different in each tumor. Tumors that responded to chemotherapy were heavily infiltrated with Eff T cells, while the stable tumors had a lower level of T cell infiltration and the non-responding tumors lacked immune cell infiltration (64). Although detailed mechanisms remains unclear, these findings provide evidence that the local TME can alter immune infiltrates.

Impact of Tissue-Specific TME on the Therapeutic Response

As discussed in the previous section, tumors growing at different anatomical sites have distinct TMEs. When tumors are present at different sites, these tissue-specific TMEs can influence the response to therapy at their own niche. The tissue-dependent difference in therapeutic response is most obvious in the field of immunotherapy.

Recent preclinical studies using immunotherapies to target tumors growing at different anatomical locations clearly demonstrated that the site of tumor growth could dictate the response to anticancer therapies. Our laboratory has demonstrated SC tumors are more responsive than visceral tumors to trimAb immunotherapy (anti-DR5, anti-CD40, and anti-4-1BB) in multiple murine tumor models (2). In this work, established SC tumors could be eradicated in mice using trimAb. However, the antitumor response to trimAb was found to be greatly reduced in orthotopic tumors compared with SC tumors, despite tumors in the two locations being of similar size. The dramatic difference in response was not due to the malignant cells, as tumor cells isolated from Renca SC and orthotopic tumors showed similar key characteristics, including major histocompatibility complex I and DR5 expression by FACS. When these re-isolated tumor cells were injected back into the same or opposite sites, the same site-specific response to trimAb was observed, regardless of where the tumor cells were isolated from. Comparison of immune infiltrates of orthotopic or SC Renca tumors by FACS revealed an increase in $F4/80^{high}CD206^+$ cells, which identifies the immunosuppressive M2 macrophages/TAMs. Furthermore, abolishing factors important for recruitment and differentiation of TAMs such as CCL2 and IL-13, improved the response of orthotopic Renca tumors indicating that this subset was partially responsible for the reduced efficacy to trimAb.

Tissue-specific responses to other immunotherapies have also been reported using other preclinical models. The response of TC-1 tumor stably expressing HPV16-E7 to a vaccine was dependent on the site of tumor implantation (65). The vaccine consists of mRNA encoding the HPV16-E7 oncoprotein together with TriMix, an mRNA-based vaccine encoding for CD40 ligand, constitutively active toll-like receptor 4 and CD70. The tumor cells implanted SC had the strongest response to E7-TriMix, with a less impressive response of tumors of the lung and a further reduced response when tumor cells were implanted into the genital tract. While the percentage by FACS of Tregs in SC tumors were dramatically decreased by vaccination, Tregs were only slightly decreased in the lung and unaffected in the genital tract tumors. In addition, genital tract tumors had a much higher percentages of both granulocytic and monocytic MDSCs compared with other tumors. The proportion of MDSCs did not decrease upon E7-TriMix treatment in the genital tract tumors as observed in the subcutaneous and lung models. In a colorectal cancer model using CT26 cells, orthotopic colon tumors had a higher infiltrate of T cells, B cells, and natural killer (NK) cells, but lower ($CD11b^+CD11c^-$) $Ly6G^+$ or $Ly6C^+$ myeloid cells compared with SC tumors. In this model, orthotopic tumors showed increased response to combination checkpoint blockade therapies (anti-CTLA-4 and anti-PD1) than the SC tumors (66). The tumor location-dependent difference in cellular responses to immunotherapy was also observed in a murine melanoma model (67). This study demonstrated that the recruitment of $Ly6C^+$ monocytes from the blood was essential for antibody-dependent tumor cell killing of melanoma in the skin but not in the lung. It was proposed that the local tissue TME determined which immune populations contribute to the antitumor antibody activity and the therapeutic response.

These recent studies utilizing preclinical models treated with various immunotherapies provide evidence for the influence of tissue-specific microenvironments on immunotherapeutic response. Logically, the data suggest an association between immunosuppressive TMEs and reduced response to immunotherapies. Despite these studies, there is a requirement for further characterization of tissue-specific TMEs and response to immunotherapies and how this relates to human cancer. Although injection of genetically identical tumor lines eliminates the variable of tumor cell genetic heterogeneity in these models, there are limitations in applicability to human cancers. Notably, tumors in these models are established rapidly and the sculpting of the TME may differ from human tumors which could take much longer to establish. Despite this, there are clear correlations between tumors at certain sites and immunotherapy responses in human cancers.

A common clinical problem with advanced cancer patients is the differential response to systemic treatment where some lesions may be less responsive to therapy compared with other anatomical sites. While this may be representative of tumor heterogeneity, the local TME is likely to play a role. Survival patterns of patients with metastatic melanoma, a highly immunogenic cancer, can be dependent on anatomical sites of disease (68). In keeping with the preclinical models described above, patients with subcutaneous, lymph node or skin metastases exhibited better survival outcomes than those with lung or other non-pulmonary visceral metastases in an era without effective systemic treatment for melanoma (68). Furthermore,

the response rate to high dose IL-2, a treatment reserved in only specialized melanoma centers, was approximately 50% in patients with subcutaneous metastases only compared with 13% with visceral metastases (69). Accordingly, ICI exhibit differential response rates at different anatomical sites, favoring patients with subcutaneous and lung sites. Retrospective analyses of anti-PD1-treated patients with advanced melanoma (70–72) and NSCLC (73) displayed poorer survival outcomes in the presence of liver metastases compared with other visceral sites such as lung. This observation was corroborated where the best objective response rates to pembrolizumab in melanoma patients with or without liver metastases was 33.3 and 71.4%, and in NSCLC 28.6 and 56.7%, respectively (72). Median progression-free survival of melanoma patients with liver metastases was poor at 2.7 months compared with 18.5 months in those without hepatic involvement. Moreover, $CD8^+$ T cell density at the tumor margin, a key biomarker of response to anti-PD1 antibodies (74), was significantly lower in the liver metastases cohort compared with those without liver metastases (72). Similar observations have also been reported in breast cancer patients. In a cohort of metastatic triple-negative breast cancer patients treated with anti-PD1, the level of TILs and response to therapy varied significantly depending on metastases location (75). The presence of lymph node metastases was strongly associated with better response compared with metastases in other organs, such as the liver. Collectively, these studies are consistent with preclinical evidence supporting the role for tissue-specific TMEs in mediating immunotherapeutic responses. Regardless of cancer type, liver metastases overall had reduced response to ICI compared with metastases at other sites. Accordingly, characterization of the liver-specific TME should be of particular focus in subsequent studies.

Cross Talk between Tumors in Different Tissues of the Body

Tumors can present simultaneously in different organs within the same patient either by metastatic growth or bilateral cancers. As previously discussed, the tissue-specific TME influences response to immunotherapy, with tumors in certain sites being more responsive than others. Recent publications have investigated the potential for such tumors to influence each other when present simultaneously. A study in our laboratory showed in mice that growth of a concomitant therapy-resistant tumor decreases efficacy of previously responsive tumors to immunotherapy (76). This was shown for SC Renca tumors when orthotopic kidney tumors were present in the same mice. The same was not observed when duplicate SC tumors were present simultaneously. The TME of SC tumors with a concomitant kidney tumor resembled the immunosuppressive TME previously observed in the kidney tumor model. This included an increase in the $F4/80^{high}CD206^+$ macrophages and a reduced Eff T and NK cell profile determined by FACS and analysis of immune-related gene expression of tumors. Blocking trafficking (with anti-CCL2 antibody) or depleting (with clodrolip) macrophages improved the effect of immunotherapy on these SC tumors, suggesting that immunosuppressive cells within a resistant tumor can migrate to responsive tumors and inhibit response to therapy.

Potential cross talk between tumors located at different sites has also been observed in humans. In metastatic melanoma,

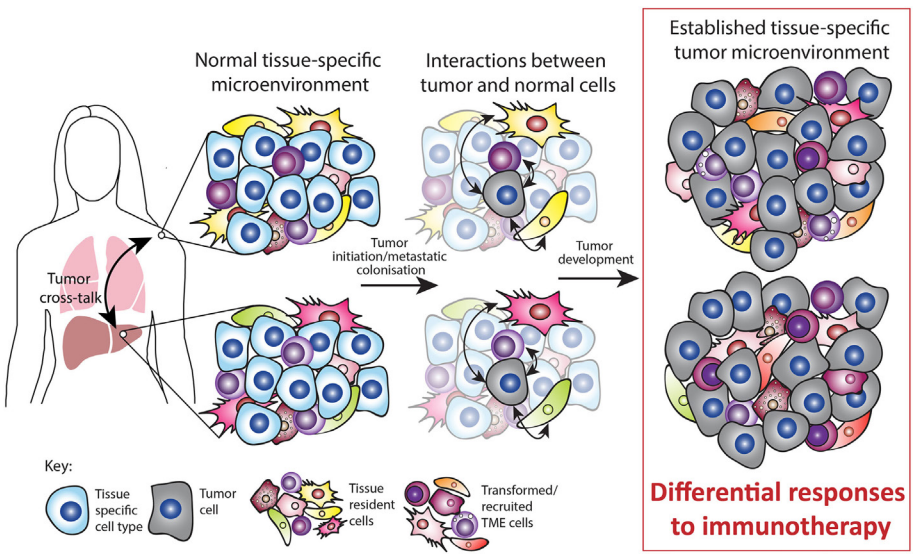

FIGURE 2 | Tissue-specific tumor microenvironment (TME). Tumors can occur at various sites in the body and often occur simultaneously, for example, by metastatic growth. The normal tissue-specific microenvironment consists of both tissue-specific cell types and tissue-resident cell types such as immune, mesenchymal, and endothelial cells. Upon tumor initiation or metastatic colonization, interactions occur between tumor and normal cells. During tumor development, these interactions are partly responsible for the established TME. Both preclinical and clinical studies suggest that the tissue-specific TME mediates the response to immunotherapy. In addition, tumors occurring simultaneously within different TMEs can cross talk and influence each other. Thus, the normal tissue plays a major role in sculpting the established TME and this ultimately impacts on the response to immunotherapy.

cutaneous/SC metastases with concomitant visceral metastases had a lower objective response rate (14%) to IL-2-based therapy compared with patients who had cutaneous/SC metastases alone (50%) (69). Presented at the 2017 ASCO meeting, Lee et al. from the University of California (77) reported that melanoma patients with additional liver metastases had a lower percentage of $CD8^+$ Eff T cells but a higher percentage of $CTLA\text{-}4^+PD1^+CD8^+$ activated-exhausted T-cells within the tumor-infiltrating lymphocytes and this was associated with a decreased response to PD1 blockade. They also investigated these findings in a murine model of B16 melanoma cells implanted SC and into the liver simultaneously or alone. Mice that had both SC and liver tumors had increased tumor growth compared with mice with SC tumors alone and had reduced response to anti-PD1 therapy, as seen in humans. Interestingly, presence of lung metastases or the implantation of unrelated MC38 liver tumors to the SC B16 tumor-bearing mice did not alter the SC tumor growth. This report indicated that liver metastases could cross talk with melanoma in the skin and lead to reduced Eff T cell responses and reduced response to PD1 blockade. These findings have important implications for directing treatment strategies especially since patients with multiple tumors are often much further advanced and harder to treat. Possibly removal or eradication of immunotherapy resistant tumors followed by administration of immunotherapy could benefit patient outcomes. Robust characterization of this cross talk is required to guide clinical decisions and treatment regimens.

CONCLUDING REMARKS

Despite traditional focus on the malignant cells, non-malignant cells within the TME play an important role in tumor growth, progression, and response to therapy. As highlighted in this review, there is an emerging role for the tissue of tumor growth on the TME composition and response to immunotherapies. A number of recent studies suggest that tumor growth in different tissues promotes the development of tissue-specific TMEs and that this is an influential factor for therapy responses (**Figure 2**). Furthermore, emerging data suggest that tumors with disparate TMEs and therapy responses can cross talk and influence each other. As such further studies are required to firmly establish a conclusive role for tissue-specific TMEs in various contexts. A deeper understanding of these unique organ-specific mechanisms of resistance may allow personalized approaches to immunotherapy. With the plethora of novel immunotherapy combinations in development that target other immune checkpoints (e.g., LAG-3, TIM-3), cytokines (e.g., TGFβ), oncolytic viruses, and other immunosuppressive mediators (e.g., IDO, adenosine) these new agents may also have differential activity by organ site. Hence, tailoring novel immunotherapy combinations depending on the organ-specific TME may improve therapeutic benefit, particularly in metastatic disease.

However, characterization of the tissue-specific differences in human tumors poses both technical and investigational challenges. Due to tumor genetic heterogeneity, it is difficult to distinguish the influence of the organ microenvironment versus the cancer type and genetic mutations. Although cancers of the same type metastasize to different organs, the genetic mutations and phenotype of these disseminated cancer cells can differ from the primary cancer (55). Thus, studying tissue-specific differences within the TME is complicated by tumor genetic heterogeneity in the human setting. In addition, obtaining samples from multiple visceral metastatic sites can be technically challenging and is acceptable to only the most willing patients. Murine models may therefore be insightful, with orthotopic tumors displaying variations in their TME and response to therapy compared with subcutaneous tumors. Given murine subcutaneous tumors respond to therapy much better than orthotopic tumors, the latter are likely to provide better predictors of therapeutic efficacy in primary tumors and permit successful translation into the clinic (78, 79). Ideally, studies into site-specific TME are best performed in human tissue, but preclinical models may still provide key insights into this complex problem.

In summary, despite the challenges in investigating tissue-specific TMEs, a thorough understanding should take priority to improve the success of both current and future immunotherapies. Increased effort in preclinical and clinical studies will assist in selection of future immunotherapy combinations according to the likelihood of therapeutic response in the tumor site. We propose that personalized immunotherapy should not only be individualized to the tumor but account for the differences in tissue-specific TME.

AUTHOR CONTRIBUTIONS

Conception and design: AO, MK, and CS. Write, review, and revision of the manuscript: AO, PL, AU, SL, PD, MK, and CS. Supervision: PD, MK, and CS.

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REVIEW
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Plasticity of Type I Interferon-Mediated Responses in Cancer Therapy: From Anti-tumor Immunity to Resistance

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The efficacy of several therapeutic strategies against cancer, including cytotoxic drugs, radiotherapy, targeted immunotherapies and oncolytic viruses, depend on intact type I interferon (IFN) signaling for the promotion of both direct (tumor cell inhibition) and indirect (anti-tumor immune responses) effects. Malfunctions of this pathway in tumor cells or in immune cells may be responsible for the lack of response or resistance. Although type I IFN signaling is required to trigger anti-tumor immunity, emerging evidence indicates that chronic activation of type I IFN pathway may be involved in mediating resistance to different cancer treatments. The plastic and dynamic features of type I IFN responses should be carefully considered to fully exploit the therapeutic potential of strategies targeting IFN signaling. Here, we review available evidence supporting the involvement of type I IFN signaling in mediating resistance to various cancer therapies and highlight the most promising modalities that are being tested to overcome resistance.

Keywords: interferons, cancer immunotherapy, immune responses, resistance mechanisms, tumor microenvironment

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INTRODUCTION

Cancers exhibit remarkable phenotypic and functional heterogeneity and various factors including genetic and epigenetic changes participate (1). The proposition is that different populations with higher or lower tumorigenic potential co-exist in tumors where stem cells occupy the pinnacle of the hierarchy (2). Cancer stem cells are now known to possess therapy resistant properties, and not only do they exist in the tumors prior to treatment but non-stem cells can also acquire stem cell properties post treatment conferring further resistance. However, this intrinsic plasticity is not the only mechanism of acquired resistance to therapy. The heterogeneous and constantly evolving composition of the tumor microenvironment also actively contributes to cancer progression including modulation of resistance to therapies.

From an immunological point of view, cancer development and progression depend on the cross talk between tumor cells and immune cells. Cancers evade anti-tumor immune responses through different mechanisms, which include the induction of local immune-suppression while an inflammatory state is simultaneously maintained. Cytokines are important in the cross-talk between tumor cells and immune cells. Interferons (IFNs) comprise a large family of cytokines that have been studied extensively in the context of virus infections but they are now also known as key drivers of inflammation within the tumor microenvironment (3). IFNs have critical immune-stimulatory effects on various immune cells including tumor-specific T lymphocytes (4). However,

evidence emerged in the last years suggests that IFNs may also trigger immune suppressive mechanisms in cancer, highlighting an additional important mechanism exploited by cancer cells to promote malignant progression and resist therapies. Therefore, dissecting the role of IFNs and associated signaling pathways in the complex and dynamic interplay between the tumor and its surrounding microenvironment is critical to tailor therapeutic intervention.

IFNs play a key role in many biological processes, whether its immune responses against pathogens and cancers or cell differentiation and apoptosis (5). IFNs are of three different types: type I (α, β, ε, κ, and ω), which bind IFNα/β receptor 1 (IFNAR1), and IFNAR2 subunits, type II (γ) binding IFN-γ receptor 1 (IFNGR1), and type III (λ), which binds the IFN-λ receptor 1 and the IL10 receptor subunit β heterodimeric receptor. We herein focus on Type I IFNs, which are critical determinants of the efficacy of anti-tumor immunity. The immune-stimulatory properties of type I IFNs, including the stimulation of dendritic cell maturation, the enrichment in granzyme and perforin expression in cytotoxic T-lymphocytes and the enhancement of memory T-cell survival, make these cytokines essential in cancer immunosurveillance (6). Moreover, type I IFNs have direct inhibitory effects on tumor cells of various origin as they limit their proliferation and drive senescence and apoptosis. It is now clear that this inhibition is attained by a combination of cell cycle arrest and cell death. Similar effects are also seen on proliferating endothelial cells during tumor angiogenesis (7). However, under certain circumstances, Type I IFNs may also trigger opposite effects, thus resulting in evasive mechanisms and promotion of tumor progression (8).

It is well established that the efficacy of several therapeutic strategies against cancer, including cytotoxic drugs, radiotherapy, targeted immunotherapies and oncolytic viruses, depend on intact type I IFN signaling (6) for the promotion of both direct (tumor cell inhibition) and indirect (effective anti-tumor immune response) effects. Malfunctions of this pathway in the tumor microenvironment or in immune cells may be causative factors behind therapeutic resistance in cancer patients. On the other hand, type I IFNs may mediate immune-suppressive effects, as in case of infection, where chronic persistence of the pathogen triggers type I IFN-induced negative regulatory pathways (8, 9). Recent evidence indicates that similar negative effects may also occur in cancer-associated chronic inflammation, where chronically type I IFNs-activated signaling may be involved in mediating resistance to treatments (8, 9). Here, we review available evidence on the contribution of type I IFN signaling in resistance to various cancer therapies and highlight some of the modalities being tested in the lab and clinic to overcome resistance.

TYPE I IFN SIGNALING AND ITS MODULATION

Type I IFN signaling and its modulation has recently been reviewed in detail elsewhere (10, 11). We herein highlight some of the components and regulators of this pathway that may affect the outcome of common forms of cancer therapies. The Type I IFN family includes a single isoform of IFN-β, multiple variants of IFN-α and other less studied variants, like IFN-ε, IFN-κ, and IFN-ω (12). While IFN-β is produced by most cells, IFN-α is primarily released by plasmacytoid dendritic cells (13). Type I IFNs are secreted by infected cells following the recognition of microbial products by pattern recognition receptors (PRRs), which include transmembrane Toll-like receptors (TLRs) recognizing damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). Other than TLRs, cytoplasmic sensors such as cyclic GMP-AMP synthase (cGAS), RIG-I like receptors, MDA-5, DDX41, and DAI can recognize viral and tumor nucleic acids (14). Once these sensors have been activated, they interact with adaptor proteins, such as TIR-domain-containing adaptor inducing IFN (TRIF), MyD88 adapter-like (Mal), mitochondrial anti-viral signaling protein (MAVS) or STING (15). TRIF and MyD88 recruit ubiquitin ligases that then activate kinases while STING directly recruits kinases like TANK-binding kinase 1 without the need of ubiquitin ligases. These kinases phosphorylate IFN regulatory factor 3 (IRF3), AP-1 and NF-κB triggering their translocation into the nucleus, where they bind to the regulatory domains of the IFN-β gene promoter (16). The production of most IFN-α types, on the other hand, requires constitutive expression of the IRF7 transcription factor instead of IRF3 (17).

Upon production, type I IFNs signal via a transmembrane receptor composed of IFNAR1 and IFNAR2 subunits. Canonically, upon binding to IFN, IFNAR phosphorylates and activates the receptor-associated Janus kinase 1 (JAK1) and tyrosine kinases 2 (Tyk2), which subsequently lead to the phosphorylation of signal transducer and activator of transduction 1 and 2 (STAT1 and STAT2) present in the cytosol. Upon activation, these proteins dimerize, get translocated to the nucleus and bind to IRF9 to form a STAT1-STAT2-IRF9 complex (ISGF3) (18). This complex then binds to IFN stimulated response elements (ISRE) in the promoter region of IFN-stimulated genes (ISG), leading to the activation of ISG transcription, most of which contribute to immune-stimulatory and anti-viral effects. Non-canonical pathways of type I IFN signaling can be mediated by STAT1 homodimers (19). STATs associated with other cytokines signaling, including STAT3, STAT4, STAT5A, and STAT5B can also mediate type I IFN signaling and expression of various ISGs (13).

ISGs are responsible for various immune-modulatory activities. PRRs, JAKs, and STATs are also ISGs and may re-inforce IFN signaling. Type I IFN-upregulated ISGs include genes required for the expression of mature MHC class I complex such as those encoding for β_2 -microglobulin. Other ISGs, such as SECTM1, may act as co-stimulatory ligands for T cells after TCR activation. Several ISGs, like IFITM proteins, IFIT proteins, GBP1, IFI6, IFI27, IRF1, IRF9, ISG20, MX1 or MXA, OAS1, PKR, PML, and viperin, have direct anti-viral activity (20). A few of these ISGs, like MxA, have now been identified as tumor suppressors in cancer, whereas the role of other ISGs in the tumor microenvironment has not been characterized yet. A number of ISGs, such as CCL5, CXCL10, CCL3, and CCL9 function as chemo-attractants to lymphocytes and monocytes.

There are also various ISGs endowed with pro-apoptotic effects, including TRAIL, Fas/FasL, XIAP-associated factor-1(XAF-1), OAS, ISG12 (IFI27), and death-activating protein kinases (DAP kinase), phospholipid scramblase (PLSCR1) and IRFs (21). PLSCR1 also encodes for a protein required to provide macrophages with a signal to engulf debris after tumor cell apoptosis and is also a negative regulator of autophagy (22). Other ISGs participate in negative regulation of IFN signaling as is the case of USP18, whose interaction with IFNAR2 results in decreased stability of the IFN-IFNAR binding (23). USP18 also participates in removing ISG15 from its substrates (ISGylation) (24), which is known to promote type I IFN production and secretion (25). Among other regulatory ISG proteins, SOCS1 and SOCS3 are known to negatively regulate both type I and type II IFN JAK-STAT signaling pathways (11).

A detailed overview of type I IFN signaling and its regulators is depicted in **Figure 1**.

COMMON CANCER THERAPIES AND TYPE I IFN SIGNALING

Cytotoxic Therapies

Radiotherapy (RT) has long been used for curative treatment for various forms of cancer. Besides its direct cytotoxic activity, indirect effects of RT on tumor cells via immune-mediated mechanisms system have been also reported. A study dated back to 1979 showed that the therapeutic efficacy of RT is determined by the host immune status (26). Some cytotoxic therapies may cause the release of tumor-associated nucleic acids and stress proteins by dying cells that may lead to the activation of TLRs in immune cells. Of particular therapeutic relevance is the recently emerged concept of immunogenic cell death (ICD) induced by several antineoplastic drugs and radiotherapy, which is characterized by the release of DAMPs that promote immune activation. Additionally, HMGB1 released during ICD may activate TLR4. As described above, these signals promote Type 1 IFN secretion.

The importance of type I IFNs in RT-mediated tumor suppression was first revealed in a study by Burnette et al. showing that type I IFN produced by myeloid cells in a mouse melanoma model was essential for tumor eradication following RT (27). Subsequently, these observations were confirmed in another pre-clinical model showing that radiation-induced type I IFNs increased CXCR3 levels, which assists in the recruitment of lymphocytes at tumor site, showing a role of type I IFN in radiation-induced ICD (28). Using an inducible expression system in tumor cells, this study also showed that exogenously administered IFN-α levels further enhanced therapeutic efficacy of RT. Experiments carried out in STING knockout mice and conditional knockouts of IFNAR1, demonstrated that activation of cytosolic DNA sensing pathways in DCs was required for the induction of type I IFN responses in DCs. The same study also showed that type I IFN signaling was required for the DCs to cause the activation of $CD8^+$ T-cells to achieve a therapeutic response. STING and cGAS, but not MYD88 or TRIF, were shown to be required for the ability of radiation to induce type I IFN responses.

Over the last decade, it has become clear that RT can enhance innate and adaptive immune responses to tumors by triggering ICD and that localized radiation may trigger systemic antitumor effects, the so-called "abscopal effect" (ab scopus i.e., away from the target). Although the occurrence of the abscopal effect is relatively rare in the clinic, with the progressive development and use of novel immunotherapy strategies incorporating RT, the abscopal effect is becoming increasingly relevant in the treatment of a variety of human tumors (29).

As in the case of RT, the anti-cancer benefits of chemotherapy were initially thought to be solely the effects of direct cytotoxicity or cell cycle arrest. However, research over the last 10 years has convincingly shown that chemotherapy can also lead to ICD, which may actively contribute to the induction of therapeutically relevant anti-tumor immune responses. Casares et al. showed that injection in mice of cancer cells treated with doxorubicin *in vitro* prevented the *in vivo* growth of the same tumor cells in challenged mice, consistently with the induction of an effective anti-tumor immune response (30). Several other drugs used in the clinic as monotherapies or in combination, such as anthracyclines (doxorubicin, epirubicin, mitoxantrone, bleomycin) and oxaliplatin have also been shown to induce ICD, while etoposide, mitomycin C, and cisplatin do not (31). Interestingly, the immune-mediated effects induced by some drugs correlate with the chemotherapeutics that are more effective in the clinic than the others (32). Of note, ICD induction by anthracyclines is strictly dependent on their ability to promote the activation of IFN-dependent gene expression programs in tumor cells that promote the generation of effective anti-tumor immune responses (33).

Indeed, release of Type 1 IFNs by tumor and immune cells induced by various chemotherapy and RT regimes can stimulates an adaptive immune response against dead tumor cell-associated antigens via autocrine and paracrine activation of the IFN signaling pathway. Sistigu et al. showed the critical role of type I IFN response activation in tumor cells by ICD inducers and demonstrated that anthracyclines stimulate TLR3 in cancer cells prompting a type I IFN signaling pathway (34). Type I IFNs were shown to be produced by cancer cells 1–4 days after chemotherapy, when the accumulation of dying cells starts. Doxorubicin was found to increase transcript levels of several ISGs, including Rsad2, Mx2, OAS2, IRF7, IFIT2, and intriguingly, CD274, the PD-L1-encoding gene. IFN-α and -β, when exogenously supplied, also enhanced the therapeutic activity of the non-ICD inducer cisplatin (34) showing that type I IFNs and activation of IFN signaling pathway may lead to ICD-like effects. A type I IFN-related signature was shown to predict clinical responses to anthracycline-based chemotherapy in several independent cohorts of patients with breast carcinoma characterized by poor prognosis. This study also outlined the potential relevance of the IFN-stimulated GTP-binding protein MX1 in mediating the efficacy of anthracycline-based chemotherapy. In fact, MX1 was upregulated by anthracyclines and its high expression levels were associated with better overall survival in breast cancer patients who received

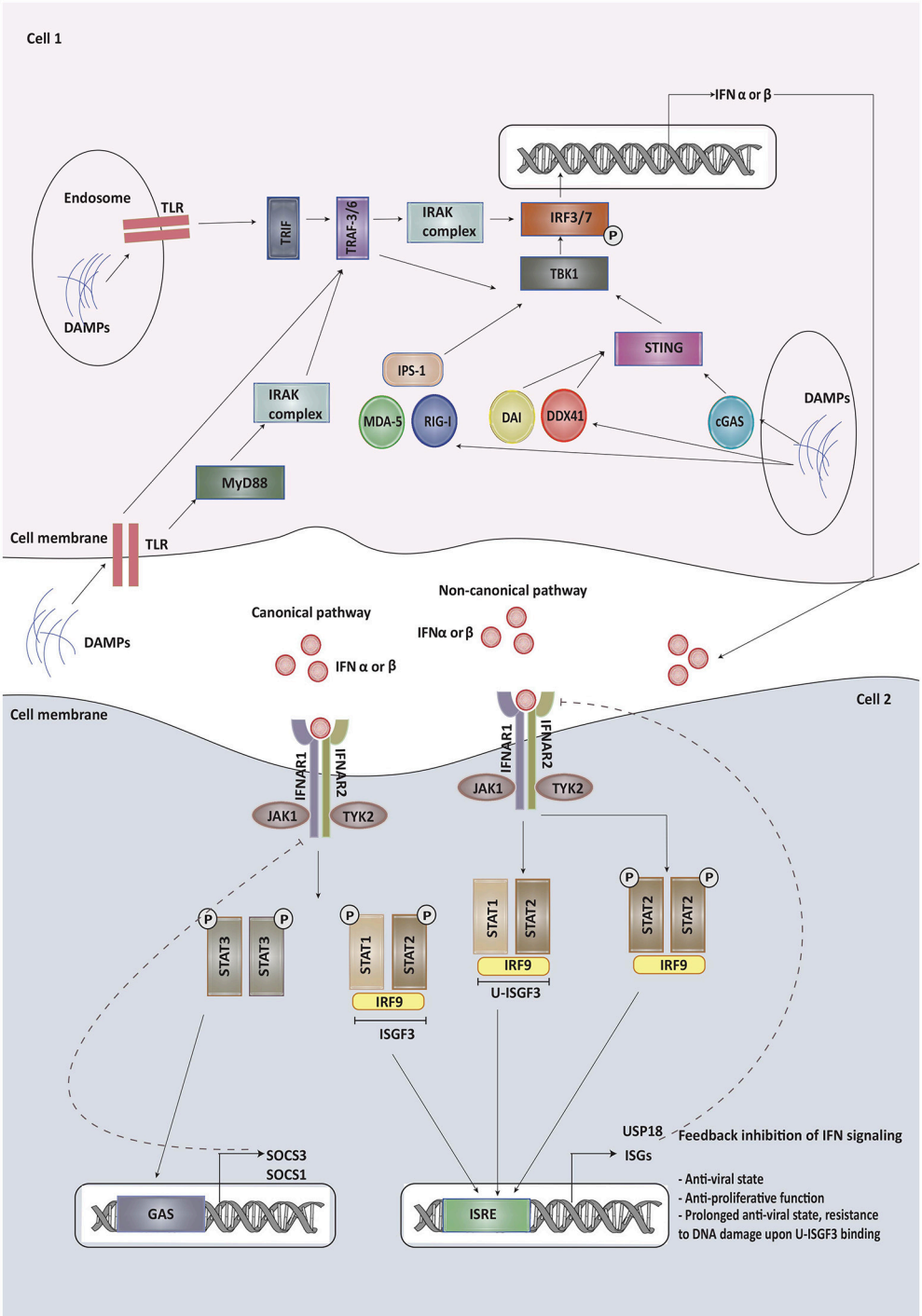

FIGURE 1 | Type I IFNs are secreted by infected cells upon recognition of damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which include transmembrane Toll-like receptors (TLRs), cyclic GMP-AMP synthase (cGAS), RIG-I like receptors, MDA-5, DDX41, and DAI. Once these sensors have been activated, they interact with adaptor proteins, such as TIR-domain-containing adaptor inducing IFN (TRIF), MyD88 adapter-like (Mal), mitochondrial anti-viral signaling protein (MAVS) or STING. These adapter proteins recruit kinases indirectly or directly, which then phosphorylate IFN regulatory factor 3/7 triggering its translocation into the nucleus, where it binds to the regulatory domains of the IFN-β/α gene promoter. Upon production, type I IFNs signal via a transmembrane receptor composed of IFNAR1 and IFNAR2 subunits. Canonically, upon binding to IFN, IFNAR phosphorylates and activates the receptor-associated Janus kinase 1 (JAK1) and tyrosine kinases 2 (Tyk2), which subsequently lead to the phosphorylation of signal transducer and activator of transduction 1 and 2 (STAT1 and STAT2) present in the cytosol. Upon activation, these proteins dimerize, get translocated to the nucleus and bind to IRF9 to form a STAT1-STAT2-IRF9 complex (ISGF3). This complex then binds to IFN stimulated response elements (ISRE) in the promoter region of IFN-stimulated genes

(Continued)

FIGURE 1 | (ISG), leading to the activation of ISG transcription, most of which contribute to immune-stimulatory and anti-viral effects. Some of the ISGs provide feedback inhibition of type I IFN signaling. Non-canonical pathways of type I IFN signaling can be mediated by STAT homodimers or unphosphorylated STAT1-STAT2 heterodimers leading to the formation of unphosphorylated ISGF3 (U-ISGF3). U-ISGF3 maintains the subset of ISGs whose production leads to DNA damage resistance. SOCS proteins produced on binding of phosphorylated STAT3 homodimers to GAS promoter are involved in providing negative regulation to type I IFN signaling.

anthracycline-containing chemotherapeutic regimens (34). These observations indicate that "viral mimicry" response that features type I IFN signaling activation is a prerequisite for the success of immunogenic chemotherapy, and potentially also of RT.

IFN-Only Therapies

Considering the pro-apoptotic, anti-angiogenic, and immunomodulatory actions of type I IFNs, they were expected to be the ultimate therapy against malignancies and infectious diseases. Indeed, type I IFN therapies initially proved successful in comparison to conventional chemotherapies for the treatment of cancers like leukemias, lymphomas, and myelomas. In chronic myeloid leukemias (CML), complete cytogenetic response was achieved in 20–30% of the cases and increased survival was observed (35). However, systemic toxicity and poor tolerability strongly limited the clinical use of these cytokines. Interestingly, IFNs have made a comeback for CML in clinical trials. A recent study investigated CML patients on IFN-α therapy and found prolonged complete molecular response, a sought-after goal in CML therapy, and very low risk of relapse in comparison to patients treated with targeted therapy (Imatinib) (36). The authors attributed these observations to IFN-induced activation of cell-mediated immunity to leukemic stem cells, a feature not seen with Imatinib. Other clinical trials have indicated that the combination of IFN-α with Imatinib is more effective for these patients in comparison to Imatinib alone (37–39).

Systemic administration of type I IFN in breast cancer mouse models has shown decrease in tumor progression and metastasis to the bone and prolonged metastasis free survival via NK-cell anti-tumor function (40, 41). However, in the clinic, treatments with type I IFN for breast cancer, melanoma and renal carcinoma have shown moderate success in terms of clinical responses and tolerability. Moreover, for breast cancer, a combination of IFN-α and IFN-β has been tested in many randomized trials owing to the demonstration that these drugs upregulate estrogen receptor (ER) in tumor cells (42). The possible ER up-regulation in ER-negative patients were thought to be able to convert them into responders to targeted therapy, but the results have been varied. Interestingly, the use of IFN-β with tamoxifen and retinoic acid showed better response rates in comparison to IFN-α in the same combination, suggesting that IFN-β might be a better anti-cancer agent than IFN-α in some clinical settings for breast cancer patients (43).

The aforementioned side effects associated with systemic administration of IFN include nausea, fatigue, fever dizziness, which can be managed with prophylactic acetaminophen (44), to more severe neuropsychiatric symptoms like depression, which are less manageable even with anti-depressants (45), making IFN a less favorable choice for therapy. Other factors that limit the efficacy of systemic IFN therapy include the limited bioavailability due to short systemic half-life (46). Therefore, overall response to type I IFN-therapy across various cancer types has been subpar due to the toxicity associated with systemic administration and the limited efficacy at the maximal tolerated doses, thus calling for less toxic and more effective delivery strategies.

Pegylation of IFN has been shown to efficiently resolve the half-life and bioavailability issues (47) by providing longer half-life and a persistent steady state of drug activity. Indeed, pegylated type I IFNs showed therapeutic efficacy in different preclinical models of cancer (48, 49). More recently, pegylated IFN-β was shown to significantly inhibit the vascular permeability of the peritoneal membrane in animal models of ovarian cancer and gastric cancer cell xenograft mice (50). In the clinical setting, however, unlike what observed in patients with chronic viral infections, the use of pegylated type I IFNs was associated with limited benefit. Adjuvant therapy with pegylated IFN-α was investigated in surgically resected stage III melanoma; at the mature median follow-up of 7.6 years, there was a significant but modest improvement in relapse-free survival but there were no significant benefits observed in overall survival or distant metastasis-free survival. Subgroup analysis suggested that the benefit of adjuvant pegylated IFN-α may be confined to the group of patients with microscopic nodal metastasis, and among this group, patients with an ulcerated primary may have benefited the most. With regards to tolerability, 37% of patients discontinued adjuvant therapy because of limiting toxicities (51). More recently, a randomized phase III trial including patients with resected cutaneous melanoma stage IIA-IIIB showed that pegylated IFN-α did not improve the outcome over IFN Notably, a higher percentage of patients under pegylated IFN-α discontinued treatment due to toxicity (52).

As an alternative way to reduce systemic toxicity and deliver IFN-α in a tumor-targeted manner, we developed a gene and cell based therapy where we engineered hematopoietic progenitors so that the expression of an IFNα transgene was restricted to their monocytic progeny, including tumor-infiltrating macrophages. Activation of innate and adaptive immune cells was seen in mice chimeric for these IFN-α-expressing macrophages and disease progression was inhibited in mouse and humanized mouse models of breast cancer with no evident signs of toxicity (53–55).

Immune Checkpoint Inhibitor Therapies

While RT, chemotherapies and surgery have been traditional choices for treatment of cancer, immunotherapies have revolutionized cancer therapeutics over the last decade. The benchmark in immunotherapies has been set by the immune-checkpoint inhibitors (ICI) cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) blocking

antibodies. Chimeric antigen receptor (CAR)-T cell therapy is a more recent addition to the list of immunotherapies, and it is making headlines due to its novelty and efficacy mainly in the treatment of hematological malignancies.

Inhibitory immune checkpoint molecules are important regulators of the immune system that specifically controls the levels of T cell activation to avoid excessive inflammation and ensure self-tolerance. The immune checkpoint CTLA-4 is expressed exclusively on T-cells where it modulates early stages of T-cell activation by counteracting the activity of the T-cell costimulatory receptor CD28. CTLA-4 and CD28 share identical ligands CD80 and CD86 expressed by APCs. Full activation of T-cells requires binding of CD28 to CD80 and CD86. Upregulation of CTLA-4 dampens T-cell activation through sequestration of CD80 and CD86 from CD28 engagement. While CTLA-4 was the first immune checkpoint to have been clinically exploited, the PD-1/PD-L1 axis has more recently garnered a higher amount of interest. Similar to CTLA-4 signaling, PD-1 regulates T-cell activation by binding to its ligands, called programmed death ligand-1 and−2 (PD-L1 and PD-L2). High and persistent PD-1 expression is characteristic of exhausted T-cells that have undergone high levels of stimulation or have experienced suboptimal CD4 T-cell help (56).

In cancers as well as in persistent infections, T-cells chronically exposed to antigen upregulates inhibitory checkpoints molecules which weaken their effector functions thus allowing the disease to escape anti-tumor immunity and ultimately progress. These exhausted T cells are unable to perform their effector functions against persisting tumors and pathogens optimally. By blocking the interaction of CTLA-4 and PD-1 with their ligands expressed by tumor cells or by antigen presenting cells (APCs), the effector functions of exhausted T cells can be at least partially revived to provide protective immunity. This blockade is provided by monoclonal antibodies targeting CTLA-4 and PD-1/PD-L1, the generalized term for which is "immune checkpoint inhibitor therapy."

Monocloncal antibodies targeting CTLA-4 and PD-1/PD-L1 have been approved for use in the clinic for non-small cell lung cancer, renal cell carcinoma, melanoma, Merkel's cell carcinoma, Hodgkin lymphoma and various other malignancies (57). Despite proven utility as a therapy in over 15 cancer types, clinical efficacy of PD-1/PDL-1 monotherapy rarely exceeds 40% and a large number of partial and non-responders are observed (58). Similarly, FDA-approved anti-CTLA4 ipilimumab results in significant survival benefit for only 20% of the metastatic melanoma patients (59). This may be due to primary resistance developed because of tumor-intrinsic genetic and epigenetic factors. However, the responders can also acquire resistance to the therapy after an initial response.

The efficacy of ICI depends on the augmentation of immune responses by enhancing either the activity or number of CTLs which can target tumor cells. Type I IFNs are critically involved in the activation of innate and adaptive immunity required to promote anti-tumor immune responses by both autocrine and paracrine mechanisms (60). IFNs promote survival, immunoglobulin class switching in B cells, $CD8^+$ T-cell proliferation and cytotoxicity and activation of dendritic cells (DCs), which have a crucial role in the initiation of adaptive immune responses. Furthermore, IFNs increase natural killer (NK) cell cytotoxicity by enhancing NK cell survival, modulating the surface expression of activating and inhibitory receptors, and NK cell expansion by inducing IL-15 production. Finally, type I IFN-mediated activation of the STING pathway post cytosolic DNA sensing is one of the key players in sustaining a T-cell inflamed-tumor phenotype (61). Activation of this pathway contributes to the activation of $Batf3^+$ dendritic cells, central to antigen presentation and hence to T-cell effector functions. Given the pleiotropic activity of IFNs in controlling maturation survival and activation of most immune cells, they are expected to play an important role in mediating therapeutic responses to ICI. At the same time, as described below, counteracting regulatory mechanisms within the immune system and mediated by chronic exposure to type I IFNs, such as upregulation of inhibitory checkpoint molecules, can negatively affect the anti-tumoral efficacy of ICI.

CAR-T Cell Therapies

CAR-T-cell therapies are characterized by a more targeted approach than checkpoint inhibitors. They rely on re-directing T-cell function to a tumor-specific antigen through the expression of a chimeric antigen receptor (CAR). CARs consist of a variable fragment of an immunoglobulin for antigen recognition, linked to T-cell activation (CD3ζ) and co-stimulation (CD28, CD137, and CD134) intracellular signaling domains (62). T-cells are derived from the patients, modified in the laboratory to express antigen specific CARs and infused back into the patients. Upon antigen recognition, the T cells become activated and eventually lyse the target tumor cells. CAR-T cells have shown significant promise with a dramatically high remission rate in various hematological malignancies, particularly B-cell acute lymphoblastic leukemia (63). The importance of type I IFN pathway in modulating CAR-T-cell efficacy was demonstrated by the work of Katlinski et al. They have tested CAR-T cells against fibrinogen activated protein (FAP) generated from lymphocytes of mice with normal and downregulated IFNAR, and showed that IFNAR1 downregulation on CTLs compromised their viability and hence their function in the tumor microenvironment (64). CAR-T cells from mice with downregulated IFNAR1 were also less effective against colorectal adenocarcinomas in mice and this effect was dependent on p38α, a kinase involved in ligand independent downregulation of IFNAR1 (64). These findings warrant further exploration of type I signaling in solid tumors where CAR-T cell therapies are still poorly effective (65).

Oncolytic Virus Therapy

Oncolytic viruses (OV) represent a new class of therapeutic agents that stimulate anti-tumor responses by selective tumor cell killing and induction of systemic anti-tumor immunity. These viruses can selectively target and kill cancer cells without causing damage to the surrounding normal tissue. There are two main types of OV. The first type replicates preferentially in cancer cells but not in normal human cells due to increased sensitivity to anti-viral pathways or their dependence on oncogenic pathways and includes poxviruses and paramyxovirus. The other OV type is genetically engineered with mutations preventing replication in

normal but not in cancer cells and includes adenoviruses (Ad), herpes simplex virus (HSV), and vesicular stomatitis virus (VSV).

Several studies have shown the efficacy of OVs in the treatment of various cancers. To date, two oncolytic viruses have been approved for use in clinic, Oncorine, a *E1B*-deleted adenovirus approved for head and neck cancers in China (66), and Talimogene Laherparepvec (T-Vec), an HSV-based virus, approved for melanoma in Europe, Australia and USA (67). However, treatment with these viruses can result in side effects and patients can develop resistance (68). Therefore, new generation of OVs are now being tested in preclinical studies and Phase II or III clinical trials (69), VSV being one of the most explored types. OV therapy is particularly interesting regarding the possible involvement of type I IFN because the success of this treatment strongly depends on the presence of a dysfunctional IFN signaling often found in cancer cells, as these viruses are susceptible to IFN-mediated antiviral activity. This constitutes a distinctive feature of OV, not present in all the other therapeutic approaches mentioned above. A "proof of concept" was provided by various studies that have shown the association of oncolytic properties of viruses with defective IFN signaling in cancer cells. In particular, Hummel et al. showed that HSV-1 could destroy murine breast carcinomas, which were defective in producing and directly responding to IFN (70). Other studies have reported an increase in sensitivity of cancer cells to VSV-induced cancer cell death upon knock-down or blockade of IFN pathway components, including IFNAR (71), IRF5, and IRF7 (72) in the tumor cells.

MECHANISMS OF RESISTANCE TO TYPE I IFNs

With the exclusion of OV therapies that benefit from a dysfunctional IFN signaling, other therapies requiring active IFN signaling to elicit an anti-tumoral activity can develop two main forms of resistance: (i) silencing of the IFN signaling pathway or (ii) counter-regulatory mechanisms blocking the effects of an active IFN signaling pathway.

Silencing of type I IFN signaling will inhibit the direct effects of IFN on tumor or immune cells, such as inhibition of cell proliferation. However, it will also affect the IFN-induced cross talk between tumor cells and the immune system thus indirectly impairing an effective anti-tumor immune response. Counter-regulatory mechanisms are mostly seen within the immune system and derive from normal physiological mechanisms that modulate and control excessive inflammatory and immune responses.

Since IFNs are central to the efficacy of various cancer therapies, general mechanisms of resistance to Type I IFNs could identify and explain several modalities of resistance across other therapies such as cytotoxic therapies or immunotherapies.

Resistance Due to Loss of IFN Signaling

Resistance to IFN-Only Therapies

Down-regulation of IFNAR1

Down-regulation of IFNAR1 in immune and tumor cells as a mechanism of resistance to IFN in cancers has, perhaps deservedly, garnered most interest in this context as cell surface IFNAR1 levels are key for type I IFN anti-proliferative effects (73). IFNAR1 degradation is brought about by ubiquitination, facilitated by E3 ubiquitin ligases, which bind to phosphorylated serine in IFNAR1. The phosphorylation of these serine residues has been shown to be triggered by vascular endothelial growth factor (74), oxygen deficit (75, 76) and the pro-inflammatory cytokines TNFα, IL-1 ad IL-6 (76), factors which are all present within the TME. Serine phosphorylation of IFNR1 eventually leading to its downregulation is also stimulated by virus-induced unfolded proteins (77), a prominent feature of TME in many cancers [Reviewed by Vanacker et al. (78)]. Recently, Katlinski et al. observed a complete or partial loss of IFNAR1 in all cell types of human colorectal adenocarcinomas compared to normal human colon cell types (64). Using mice deficient in IFNAR1 ubiquitination and degradation, this study showed that the downregulation of IFNAR1 stimulated tumorigenesis by altering the expression of IFN-induced genes including *Irf7, Ifit2, Mx2, Usp18*. Downregulation of IFNAR1 was also seen in T-cells, which showed weakened survival in the TME through suppression of the IL-2 pathway. Similar observations have been reported for melanomas (79). IFNAR1 downregulation not only causes resistance to IFN monotherapies, but has also been associated with resistance to chemotherapy (80) and immune-checkpoint inhibitor therapies (64) (see below).

Upregulation of SOCS proteins

SOCS proteins have also been implicated in silencing of type I IFN signaling. Cancer cells can upregulate the expression of SOCS1 and SOCS3 proteins which leads to a decline in IFN-induced STAT1 phosphorylation (81). Indeed, experimental SOCS1 and SOCS3 over-expression resulted in type I IFN unresponsiveness, while their inhibition re-invigorated responsiveness and expression of ISGs, IFIT2 and ISG-15. Consistently, silencing of SOCS1 increases the sensitivity of neuroendocrine tumor cells to type I IFNs (82). SOCS1 mRNA was also associated with poor cytogenetic responses to IFN-α and shorter median progression-free survival in CML patients (83). Similarly, silencing of SOCS3 increases the susceptibility of renal cell carcinomas to IFN (81). However, both proteins were shown to also increase the sensitivity to IFN in certain cancer types. What determines such an opposite effect is still unknown (84).

Jak-STAT signaling modulation

Variability in the role of Jak-STAT signaling components in resistance to type I IFN has been seen across various studies. Different components seem to play a role in different cellular backgrounds and in different tumor types. Epigenetic silencing of JAK1 conferred IFN-α unresponsiveness in prostate adenocarcinoma cell lines (85). Loss of STAT2 and defective ISGF3 mediated gene activation were linked to resistance to IFN-α induced apoptosis (86). Subsequently, defects in ISGF3 caused resistance to IFN-α in HCC was shown to be due to the absence of the p48-ISGFγ protein (87). A lack of STAT1 expression has been shown in CML patients resistant to IFN-α (88). Further, STAT5 overexpression has been reported in IFN-α resistant melanoma cells and advanced melanoma lesions (89). A study showed the

association of the lack of Stat1, Tyk2, and Jak1 expression and defective Jak-Stat activation with resistance to IFN-α in renal cell carcinoma cells, while IFNAR1 and SOCS3 proteins were not involved (90).

Silencing of IRF genes

The success of type I IFN therapies strongly depend on the immunomodulatory properties of IFN, which are mainly regulated by IRF7. Suppression of IRF7-regulated genes was shown to be crucial for the induction of bone metastasis in breast cancer, while restoration of IRF7 in tumor cells or administration of IFN, reduced metastasis in mice in a NK and $CD8^+$ T-cell dependent manner (91). Similarly, overexpression of IRF7 reduces bone metastasis in mouse models of prostate cancer (92). Loss of IRF5 has also been shown to correlate with disease stage and metastasis in cancers and may constitute another mechanism underlying resistance of advanced tumors to IFN-therapies (93).

Overexpression of miRNAs

Other factors involved in resistance to type I IFNs are miRNAs. Tomimaru et al. showed that miRNA-21, which is overexpressed in hepatocellular carcinoma (HCC), can induce resistance to IFN-α. miRNA-21 expression was also higher in non-responders to a combination of IFN-α and chemotherapy (94). Following these results, Tomokuni et al. carried out a comprehensive expression profiling of miRNAs in HCC cells and their IFN-α resistant clones, and found that miR-146a could also suppress the sensitivity of these cells to IFN-α (95). Interestingly, these miRNA have also been shown to induce resistance to chemotherapy (96).

Resistance to ICI Due to Loss of IFN Signaling

Despite the transformative potential of immune checkpoint-based immunotherapies, upfront clinical benefits in approved indications are not seen in all patients or even all cancer types. Additionally, resistance to these drugs still constitutes a relevant factor limiting the efficacy of ICIs. Recent studies have indicated impairment of type I IFN signaling as one of the mechanisms behind acquired resistance to ICI therapies. Downregulation of IFN signaling would prove beneficial to the tumor in the presence of ICIs as blockade of inhibitory checkpoint pathways prevents exhaustion of T-cells while reduction of IFN signaling would reduce antigen presentation and further activation of T-cells.

This elegant mechanism of acquired resistance was recently revealed in patients treated with anti-PD-1 therapy. Zaretsky et al. performed molecular analyses on tumor tissues from four melanoma patients who showed an initial objective response to the PD-1 inhibitor pembrolizumab administered for 6 months followed by disease relapse (97). Out of these, two patients showed loss of function mutations in genes encoding JAK1 and JAK2 in the relapsed tumors, which were not present before treatment. When the functional effects of these mutations were tested, the authors found a total loss of functional response to IFN-γ but not IFN-α and β in the presence of JAK2 mutations while resistance to all three interferons was seen in the presence of JAK1 mutations (97).

A case of primary resistance to PD-1 and CTLA-4 blockade due to defects in IFN-γ signaling has also been described (98). Additionally, the loss of IFN-γ pathway genes IFNGR1, IFNGR2, JAK2, IRF1, IFIT1, IFIT3, MTAP, miR31 and amplification of the suppressor genes SOCS1 and PIS4 have been shown in melanoma patients non-responsive to anti-CTLA4 therapy. Interestingly, deletions in IFNA and IFNB genes are also seen in these patients, but the functional significance of this has not been tested (59). Missense mutations in IFNAR2 along with mutations in IFN-γ signaling pathway genes were also found in lung tumors that had acquired resistance to PD-1 blockade (99). The loss and mutation of genes overlapping between type I and type II IFN pathways and loss of IFNA and IFNB might suggest a role for Type I IFNs in resistance to ICIs and calls for further exploration.

IFN Resistance and OVs

Unlike other therapies, resistance to IFN helps the therapeutic efficacy of OVs. A role of type I IFNs in resistance to OVs was highlighted by a study on HCC cells, where impairment of type I IFN signaling resulting from a deregulated IRF3 pathway conferred susceptibility to VSV infection (100). In another study, VSVs were tested on a panel of aggressive pancreatic ductal adenocarcinoma cell lines and 5 cell lines that showed resistance to VSV were not only sensitive to IFN-α treatment but also capable of secreting IFN-β (101). Subsequently, it was found that there was no difference in IFNAR expression between resistant and sensitive cells, but a great variability in the expression of ISGs, MxA, and OAS, with resistant cells showing high expression levels of these genes (102). Other studies have also shown a role for these ISGs in mediating resistance to OVs (103, 104). A recent study has reported an increase in tumor cell sensitivity to VSV induced by downregulation of the MX1 gene (105). The PML gene has also been implicated in resistance to OVs (106), whereas the role of other ISGs largely remains unexplored.

Resistance Due to Chronic Exposure to IFN

Resistance to ICI Due to Chronic Exposure to IFN

Benci et al. showed that, upon prolonged IFN-γ exposure (but not type I IFN), B16 melanoma cells adopt a state of STAT1-dependent resistance to ICI associated with the expression of the ISGs IFIT1 and MX1 (107). The authors showed PD-L1 dependent and independent resistance mechanisms in patients and mice treated with RT and CTLA-4. IFNAR knock-out studies demonstrated that type I IFN signaling is required to sustain resistance to PD-L1 blockade, but not for its induction, but the exact underlying mechanism remains unclear (107). These reports seem contradictory to the observations reported by Zaretsky et al (97) mentioned above but highlight the importance of timing in assessing functional responses. This study shows that ablation of IFN signaling on B16 cells enhances resistance exclusively upon delayed scheduling of dual CTLA-4 and PD-1 therapy. The study also showed that a delay in administration of JAK inhibitors or IFN receptor ablation on tumor cells promoted the induction of complete responses to ICI in resistant melanoma and breast cancer, again demonstrating the importance of scheduling in combination therapies (97).

Notably, type 1 IFNs have been shown to up-regulate the expression of the immune checkpoint molecule PD-L1 in tumor cells. Based on this premise, a recent study investigated whether

PD-L1 could engage in abrogating IFN-mediated toxicity. PD-L1 reduced, but did not completely abrogate, IFN cytotoxicity and was found to protect cells by inhibitory crosstalk with type I IFN signaling pathway, particularly by inhibiting STAT3 upregulation (108). Expression of PDL-1 in tumor cells has also been associated with radio-resistance (109). Katlinski et al also showed that while downregulation of IFNAR1 in the cytotoxic lymphocytes in the TME can lead to an immune-suppressive environment, a stable IFNAR1 also caused an increased expression of PD-L1 on tumor cells (64). Altogether, these findings suggest that the continuous exposure of type I IFNs may lead to PD-L1 expression by tumor cells, which then may promote immune resistance through interaction with PD-1$^+$ immune effectors. This hypothesis, however, remains untested. Given that the interactions between IFNs and the PD1/PDL-1 axis have been brought to the forefront in the last few years (110), the involvement of IFN in PD1/PDL-1-mediated restraint of immune cells and hence in the resistance to checkpoint inhibitors remains likely.

IFN Resistance and RT

Khodarev et al. reported isolation of radio-resistant squamous cell carcinomas (SCC) by multiple exposures to RT of a radio-sensitive parental tumor (111). Upon comparison of gene expression profiles between the sensitive and resistant tumors, 25 genes belonging to the IFN-inducible pathway were differentially expressed. Notably, STAT1 was the most highly expressed gene in resistant tumors and sensitive cells transfected with STAT1 developed radio-resistance. Although STAT1 activation is required to trigger anti-tumor immune responses, and therefore STAT1 deficiency may prevent the induction of anti-tumor immunity, persistent STAT1 activation may be associated with therapeutic resistance, as in the case of RT. Consistent with this possibility, a study carried out on resistant SCCs concluded that STAT1 is overexpressed in tumors adapted to continuous exposure to IFN, leading to the selection of tumor clones resistant to IFN-mediated cytotoxicity and RT effects (112). However, the mechanisms behind these observations were not explored. Again in keeping with these findings, breast, prostate and glioma cancer cells were shown to overexpress multiple IFN-related genes, including STAT1, when treated with multiple fractionated doses as compared to single dose of RT (113).

Following these reports, an IFN-related DNA damage resistance signature (IRDS) composed of 36 genes was found. The IRDS signature genes included the top 25% of genes that correlated with resistance in 34 NCI60 cell lines treated with radiation, indicating an association between IFN response genes and resistance to RT. It was also shown that patients with IRDS$^+$ breast cancer exhibited recurrence of disease following mastectomy and adjuvant RT (114). The expression signature composed of 8 IRDS genes, STAT1, IFI44, IFIT3, OAS1, IFIT1, ISG15, MX1, and USP18, was also shown to predict poor outcomes in glioblastomas post RT (115). A direct role for of IFN-β was demonstrated in up-regulating the expression of these IRDS genes via un-phosphorylated STAT1 and IRF9 to cause resistance to DNA damage and RT (116).

IFN Resistance and Chemotherapy

Similarly to what observed for RT, chronic inflammation and prolonged type I IFN stimulation may also lead to the development of resistance to chemotherapy, as demonstrated for chronic viral infections (8). Indeed, the IRDS gene signature has been found to confer resistance to both chemotherapy and RT (114). Additional screening studies have shown the upregulation of STAT1 and some of the ISGs included in the IRDS signature are also upregulated in doxorubicin resistant cells (117). This dichotomy in the role of type I IFN signaling in resistance to these treatments may be due to the activation of signaling downstream of type I IFNs, driven by un-phosphorylated STAT1 and U-ISGF3 activated upon prolonged exposure, as genes upregulated by un-phosphorylated STAT1 (and not by phosphorylated STAT1) overlap with the IRDS (19). These findings, however, need further and direct investigation *in vivo* in pre-clinical models and in patient samples.

A recent study found a strong correlation between the genes belonging to the IRDS signature and genes upregulated in breast cancer cells after long term stimulation of CD95 (118), an inducer of stemness (119). Acquisition of stemness features is a widely accepted mechanism by which cancer cells become less sensitive to RT (120) and chemotherapy (121, 122). This study showed that type I IFNs (but not type II IFNs) were required for CD95-induced stemness and did so through the phosphorylation and activation of STAT1 and upregulation of the STAT1 targets PLSCR1, USP18, and HERC8. Blocking IFNAR1 and IFNAR2 in CD95 pre-treated luminal breast cancer cell lines resulted in inhibition of the CD95-induced phosphorylation of STAT1 and induction of the stemness marker SOX2 (118). This points toward a potential mechanism by which IFN signaling may induce resistance to RT. Another study showed that the growth of therapy resistant cancer stem cells was promoted due to STAT1 dependent antiviral signaling activated by exosomal transfer of RNA between stromal and basal breast cancer cells, which also correlated with IRDS expression (123). These observations, however, may differ among cancer types or subtypes as IFN-β signaling has recently been shown to repress cancer stemness in the triple negative breast cancer subtype (124). Further studies are therefore required to understand how type I IFN may induce opposite effects in this setting (124).

A role for the STING cytosolic pathway in promoting IFN-induced resistance has recently been demonstrated in breast cancer regrowth after treatment with genotoxic chemotherapeutic agents such as mafosfamide (125). STING pathway is typically activated in immune cells in response to infections, and this study showed that the activation of this pathway in breast cancer cell lines exposed to genotoxic stress was potentiated by chemotherapy. These findings confirmed that type I IFN pathway plays an important role in causing the up-regulation of ISG expression in cancer cells in response to chemotherapy and demonstrate that the STING pathway also contributes to type I IFN production mediated by STAT1 activation (125). Following a short-term exposure to chemotherapy, tumor cells exhibit slow-cycling, dormant and chemo-resistant populations. It has been shown that 20 days after treatment, these cell populations formed growing

colonies following cell-cycle resumption. Silencing of STING after mafosfamide treatment of breast cancer cells delayed the appearance of growing colonies of surviving cells, showing that the STING/IFN/STAT1 pathway acts as a cellular mechanism of cancer cell survival and re-growth after the genotoxic stress of chemotherapy (125). Interestingly, this study identified one of the ISGs which was not included in the IRDS signature, PARP12, as a downstream contributor to STING mediated cancer regrowth and resistance. This protein is known to have roles in antiviral responses, however, the mechanisms underlying its effects on tumor survival are not known.

Taken together, available data show that activation of type I IFN signaling is essential for the therapeutic efficacy of checkpoint inhibitor and cytotoxic therapies, but prolonged activation of this signaling and availability at low levels can also lead to resistance to these therapies.

TARGETING TYPE I IFN SIGNALING PATHWAY AS A PROMISING STRATEGY TO OVERCOME RESISTANCE TO CANCER TREATMENTS

Current cancer therapies may fail to suppress tumor recurrence and metastasis due to the intrinsic plasticity of the tumor microenvironment that constantly evolves and adapts to escape the selective pressure of anti-cancer therapies. Understanding which evasive mechanisms are induced by different treatments is fundamental for the rational design of new combination treatments. Acquired resistance to IFNs represent one of the evasive mechanisms to several therapies, all requiring active IFNs pathway for optimal anti-tumor activity. Dysfunctional IFN signaling, not only impairs the direct effects of IFNs on tumor cells, but it may also interfere with their cross talk with the immune cells thus preventing IFN-mediated activation of an anti-tumor immune response.

Schematically, the strategies to overcome resistance in the context of type I IFN signaling can be divided into two categories: (1) Approaches to induce type I IFN signaling and (2) Approaches that block type I IFN signaling. The first category has been the subject of many clinical trials, whereas the second approach is based on relatively new findings and is yet to be explored in the clinic.

One of the approaches among those aiming at inducing/enhancing IFN signaling is to combine conventional therapies with IFN-only therapies. Direct exposure of the immune cells to IFN may bypass the tumor cells and directly activate the immune system. However, as discussed above, prolonged exposure to IFN might be harmful and cause further resistance to therapies. Although monitoring the timing of exogenous type I IFNs administration alongside other therapies has not been explored in the clinic yet, compounds targeting type I IFN signaling pathway in combination with other therapies have emerged, and proved to be an effective treatment strategy. Using agonists for any singular component to promote IFN secretion or the use of antagonists for molecules like STAT1 and STAT3 to overcome their effects of chronic IFN signaling could both prove beneficial in this setting.

STING agonists caught researchers' attention: Flavone acetic acid, 5,6-dimethyllxanthenone-4-acetic acid (DMXAA) and cyclic dinucleotides have all been tested to target STING *in vivo* and have shown promising results (126). A cyclic dinucleotide ADUS100 showed significant anti-tumor activity in the triple negative breast cancer 4T1 model (127). Another study showed ADUS100 also delayed tumor growth in $HER2^{+}$ breast cancer. Moreover, a synergistic effect was seen when ADUS100 was combined with an anti-PD-1 antibody and an OX-40 agonist antibody where tumor clearance was seen in 40% of the mice compared to only 10% of the mice with ADUS100 treatment (128). STING agonists also showed increased tumor regression when combined with anti-PD1 antibody in a pre-clinical squamous cell carcinoma model (129). Based on the success in pre-clinical models, multiple clinical trials are ongoing to test STING agonist monotherapy or in combination with anti-PD1 antibodies (NCT03010176, NCT03172936). Similarly, other PRRs whose activation can result in type I IFN responses are also being targeted in several clinical trials (NCT03065023, NCT02828098).

Inhibitors of different JAK-STAT proteins have been of interest for a long time. Among STAT3 inhibitors, STATTIC was observed to sensitize human colon cancer cells to chemotherapy *in vitro* and *in vivo* (130). STX-0119, an inhibitor of STAT3 dimerization, was also shown to suppress the growth of lymphomas in mice (131). Additionally, the STAT3 inhibitor OPB-31121 displayed tumor suppression in pre-clinical models of gastric cancer (131) and mouse models of primary human leukemia. This inhibitor showed a high level of safety and tolerance in a clinical trial for patients with advanced solid tumors but has not been approved for clinical use (132). Meanwhile, STAT3 antisense nucleotides continue to be tested in combination with other therapies. AZ9150 has been shown to increase chemo-sensitivity and decrease tumorigenicity in other tumors *in vivo* (131). This inhibitor is now being tested in combination with durvalumab, an anti-PD1-PDL1 interaction blocking antibody, with and without chemotherapy in lung cancer patients (NCT03421353).

Pravastatin is a STAT1 inhibitor tested in various clinical trials that modulates type II IFN responses while its effects on type I IFNs remain undefined. Fludarabine, another STAT1 inhibitor, is now being tested with pegylated liposomal doxorubicin in patients with refractory ovarian cancer (NCT03335241). JAK1 and JAK2 inhibitor, ruxolitinib, was initially developed to target the inherent activation of JAK-STAT signaling pathway in patients with myeloproliferative neoplasms (133). However, another study showed that the treatment with ruxolitinib overcame resistance to cisplatin in *in vivo* and *in vitro* models of non-small-cell lung cancer (134). Since then, this compound has entered clinical trials in combination with other therapies for various forms of cancers, including chemotherapy for non-small-cell lung cancer (NCT02119650), refractory lymphoblastic leukemia (NCT02420717), refractory myeloid leukemia (NCT00674479), HER2 positive breast cancer (NCT02066532) and triple negative inflammatory breast cancer

(NCT02876302) and in combination with the anti-PD1 drug pembroluzimab against stage IV triple negative breast cancer (NCT03012230). Some of these trials are still underway while results from others have not been revealed and many have even shown underwhelming results (135, 136). On the other hand, a JAK2 inhibitor SAR302503 has been shown to not only target therapy resistant lung cancers but also to abrogate PDL1 expression. Moreover, the sensitivity to this drug even correlated with higher expression of IRDS genes warranting further investigation in the clinic (137). Although most of these compounds do not exclusively target type I IFN signaling, their efficacy hints toward further exploration of novel drugs selectively targeting this pathway to overcome resistance.

A recent study highlighted the requirement to target negative regulators like the ISGs, SOCS1 and SOCS3 and identified a natural compound 6-hydroxy-3-*O*-methyl-kaempferol 6-*O*-glucopyranoside (K6G) which inhibited SOCS3 expression and stimulated type I IFN induced ISRE reporter expression (138). There is also strong evidence that USP18 is worth pursuing as a promising target and recent advances in solving its crystal structure along with ISG15 should help make this idea a reality in the clinic (139). On the other hand, an IRF inhibitor, LY294002, which targets IFN-β production via IRF3 inhibition (140), has been shown to sensitize cancer cells to chemotherapy in cervical cancer cells by enhancing mitochondrial JNK signaling. Agonists (141) and antagonists (142) of IFNAR are also under development and could prove useful against cancers. Besides, with the new reports of prolonged type I signaling associated with chronic inflammation in cancers, combining IFN inhibitors with other therapies might be beneficial, although this possibility remains to be experimentally demonstrated.

While oncolytic viruses represent another strategy to activate type I IFN signaling in the tumor microenvironment and are being tested alongside other therapies in multiple clinical trials (143), their efficacy has been shown to be enhanced by type I IFN pathway modulators. Ruxolitinib has been shown to inhibit the expression of ISGs like MDA5, RIG-I, MX1, IFIT3, and OAS1 and improve the infection of oncolytic HSV *in vitro* (144). A study by Esobar-Zarate et al. showed that IRF7, IRF9 and OAS1 but not MxA are upregulated in VSV resistant head and neck cancer cells and their treatment with ruxolitinib reduced IRF9 and IRF7 expression along with OAS1 expression and helps overcome resistance to this virus (145). This inhibitor has also been recently shown to overcome resistance to VSV in pancreatic ductal adenocarcinoma cells. In fact, adding polycations and ruxolitinib (which inhibits antiviral signaling) to VSV therapy successfully overcame the resistance of pancreatic carcinoma cells to VSV whilst also improving VSV attachment and replication (146). In another study, a histone deacetylase inhibitor, resminostat, was shown to improve the therapeutic effects of the measles vaccine virus by suppressing IFIT-1 function in hepatocellular carcinoma cells (147) A differential role for IFN-α and IFN-β was demonstrated in the induction of resistance of head and neck carcinoma cells to VSV. It was found that IFN-β, but not IFN-α, was crucial for maintaining persistent infection of these cells with VSV. When the cells were treated with antibodies against IFN-β, IFN-α or their combination before VSV infection, only anti-IFN-β protected cells from the infection significantly more than anti-IFN-α and the combination (148). These findings indicate that IFN-α is less effective at protecting cells from VSV oncolysis than is IFN-β, while both IFNs protect normal cells equivalently. These results could be instrumental in designing combinatorial therapies including OVs in the future.

CONCLUSIONS

Type I IFN signaling is central to most anti-cancer therapies, new and the old alike. Since mutations in components of this pathway and chronic activation of the pathway both can be detrimental to the efficacy, assessing interferon signature genes before a specific therapy is initiated could be useful to tailor therapy. For example, the recently used IRDS scoring strategy identified breast and lung cancer patients with higher expression of ISGs as patients with poor responses to chemotherapy and radiotherapy (137). Indeed, even the use of IFN-only therapy might be detrimental for these type of patients. Strategies able to temporarily block IFN-signaling, preferably in cancer cells only, could be useful to limit chronic exposure to IFN and restore responsiveness to treatment.

On the other hand, as discussed above, blocking type I IFN signaling may render cancer cells resistant to other treatments, for example to anti-PD1 therapies, via downregulation of MHC class-I molecules (149). Therefore, inhibition of the blocking IFN pathway should be the therapeutic choice in accurately selected cases. The timing and duration of therapies aiming at blocking or activating type I IFN signaling are more relevant parameters to consider in the design of novel treatment schedules. The complexity of the involvement of type I IFNs in the interplay between cancer cells and TME requires further studies to more precisely identify suitable therapeutic targets in the various tumor settings. Moreover, in order to fine-tune combinatorial therapies, we need a better understanding of how type I IFN pathway interacts with other inflammatory pathways in the TME. There is also a need to understand exactly what various ISGs do in the TME—Is it just one ISG protein that is responsible for the therapeutic effects or does it have to be a signature that determines outcome in patients? Also, how do the functions of these ISGs change in the presence of therapy and do they contribute to stemness in that scenario?

Furthermore, even though various components of the type I IFN pathway are being targeted in the clinic, there is paucity of information on how these therapies affect downstream components of the IFN signaling the consequent counter-reactions in tumor cell signaling. The dynamic cross talk between tumor cells and the heterogeneous immune populations in different cancers adds a further level of complexity. Now that we are aware that chronic activation of type I IFN signaling may be causing "adaptive resistance" in many cancers, there is an even more urgent need to study these effects in more detail. In conclusion, reasons for failure of various anti-cancer therapies might lie under the basic questions around type I IFN signaling, its functions, cross-talk, mutations, timing and

duration of exposure and it might be time to dig deeper into this puzzling scenario.

AUTHOR CONTRIBUTIONS

RD and RM conceived the review. MB searched the literature and drafted the manuscript. RD and RM critically appraised the literature, wrote and approved final version of the manuscript.

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Interferon Signaling Is Frequently Downregulated in Melanoma

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Immune checkpoint inhibitors that block the programmed cell death protein 1/PD-L1 pathway have significantly improved the survival of patients with advanced melanoma. Immunotherapies are only effective in 15–40% of melanoma patients and resistance is associated with defects in antigen presentation and interferon signaling pathways. In this study, we examined interferon-γ (IFNγ) responses in a large panel of immune checkpoint inhibitor-naïve melanoma cells with defined genetic drivers; *BRAF*-mutant (n = 11), *NRAS*-mutant (n = 10), *BRAF/NRAS* wild type (n = 10), and *GNAQ/GNA11*-mutant uveal melanomas (UVMs) (n = 8). Cell surface expression of established IFNγ downstream targets PD-L1, PD-L2, HLA-A, -B, and -C, HLA-DR, and nerve growth factor receptor (NGFR) were analyzed by flow cytometry. Basal cellular expression levels of HLA-A, -B, -C, HLA-DR, NGFR, and PD-L2 predicted the levels of IFNγ-stimulation, whereas PD-L1 induction was independent of basal expression levels. Only 13/39 (33%) of the melanoma cell lines tested responded to IFNγ with potent induction of all targets, indicating that downregulation of IFNγ signaling is common in melanoma. In addition, we identified two well-recognized mechanisms of immunotherapy resistance, the loss of β-2-microglobulin and interferon gamma receptor 1 expression. We also examined the influence of melanoma driver oncogenes on IFNγ signaling and our data suggest that UVM have diminished capacity to respond to IFNγ, with lower induced expression of several targets, consistent with the disappointing response of UVM to immunotherapies. Our results demonstrate that melanoma responses to IFNγ are heterogeneous, frequently downregulated in immune checkpoint inhibitor-naïve melanoma and potentially predictive of response to immunotherapy.

Keywords: melanoma, interferon, antigen presentation, immunosuppression, PD-L1, PD-L2

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INTRODUCTION

The identification of checkpoint signaling pathways that dampen anti-cancer immune responses and the subsequent development of checkpoint inhibitors have transformed the treatment of patients with metastatic cancer. Antibodies blocking immune checkpoints such as the cytotoxic T-lymphocyte-associated protein-4, the programmed cell death protein 1 (PD-1), and its ligand PD-L1 induce durable anti-tumor immune responses in many advanced malignancies, including melanoma, non-small-cell lung cancer, and renal cell carcinoma. PD-1 inhibition in melanoma promotes tumor regression and prolonged overall survival in 30–40% of patients with advanced

disease (1–3). However, the majority of melanoma patients will not benefit from immunotherapy and 25% of responding patients will relapse within 2 years (4).

Recent studies have shown that resistance to immune checkpoint blockade involves defects in the interferon-γ (IFNγ) signaling pathway (5–9). Once secreted by activated T cells, IFNγ binds and activates the IFNγ receptor complex (IFNGR1/2), which is broadly expressed on many cell types, including cancer cells. Receptor binding leads to the activation of the receptor-associated Janus kinases (JAK1 and 2) which phosphorylate and activate the signal transducer and activator of transcription (STAT) proteins, STAT1 and STAT3. Nuclear translocation of STAT transcription factors promotes the transcription of hundreds of IFNγ response genes (10) including downstream transcription factors, such as IRF1, STAT1, and STAT3, genes involved in antigen presentation such as MHC class I and II molecules (8, 11), and genes that attenuate immune activity to minimize local tissue damage, such as PD-L1 and PD-L2 (7). The multifunctional effects of IFNγ are particularly important in the context of immunotherapy since enhanced antigen presentation improves immune recognition of tumors while expression of immunosuppressive molecules limits anti-tumor T cell activity.

Several genetic defects affecting the IFNγ signaling pathway are associated with melanoma resistance to immunotherapy, including checkpoint inhibition. For instance, the genetic loss of the β-2-microglobulin (*B2M*) gene, the structural component of MHC class I complexes, is enriched in pre-treatment tumor samples from melanoma patients with innate and acquired resistance to checkpoint inhibitor therapy (12, 13). Genetic alterations affecting *IFNGR1*, *IFNGR2*, *IRF1*, and *JAK2*, and amplifications of the IFNγ inhibitor genes, *SOCS1* and *PIAS4*, are also enriched in patients not responding to checkpoint inhibition (6). Furthermore, loss-of-function mutations in the upstream IFNγ-signaling regulators *JAK1* and *JAK2*, concurrent with deletion of the wild type alleles, have been identified in two melanoma patients who failed anti-PD-1 therapy (7). The loss of IFNγ signaling limits immune cell recruitment and immune recognition of tumor cells by suppressing the production of IFNγ-dependent chemokines and diminishing antigen presentation (8, 9, 14).

In this study, we investigated the response of a large panel of human melanoma cells to IFNγ stimulation. These cells were naïve to immune checkpoint inhibitors, and we examined whether the expression of key IFNγ downstream targets [PD-L1, PD-L2, nerve growth factor receptor (NGFR), HLA-A, -B, -C, and HLA-DR] could serve to assess the integrity of IFNγ signaling in melanoma. We also examined the potential influence of melanoma driver oncogenes on IFNγ signaling activity and found that uveal melanoma (UVM) cells show evidence of diminished IFNγ pathway activity with minimal baseline and IFNγ induction of HLA-DR, NGFR, and PD-L2. Importantly, nearly 70% of melanoma cells included in this study showed incomplete responses to IFNγ stimulation, indicative of pre-existing resistance to immunotherapy. Furthermore, our data confirm that measuring IFNγ output with a select number of targets may be useful for detecting intrinsic defects in the IFNγ/JAK/STAT pathway, including JAK and STAT mutations which are associated with PD-1 inhibitor resistance (7, 8, 13).

MATERIALS AND METHODS

Cell Lines

A total of 39 cell lines were included in this study. Oncogenic driver mutation status is shown in **Table 1**. Melanoma cell lines were provided by Prof. Nicholas Hayward and Prof. Peter Parsons at QIMR Berghofer Medical Research Institute, Australia, Prof. Bruce Ksander at Harvard Medical School, MA, Prof. Peter Hersey at the Centenary Institute, Sydney, Australia, and Prof. Xu Dong Zhang at the University of Newcastle, Newcastle, Australia. Two short-term melanoma cell lines were cultured from surgically excised, enzymatically processed melanoma lesions (SCC14-0257, SMU15-0217) in a study carried out in accordance with the recommendations of Human Research ethics committee protocols from Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444). Cell authentication was confirmed using the StemElite ID system from Promega.

Cell Culture

Cell lines were cultured in Dulbecco's Modified Eagle Medium or Roswell Park Memorial Institute-1640 media supplemented with 10 or 20% heat inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 11.25 mM glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 10 mM HEPES (Gibco) and were maintained at 37°C in 5% CO_2. For IFNγ treatment, 7×10^4 melanoma cells per well were plated in complete media in six-well plates. After an overnight incubation, the complete media was replenished, and cells treated for 72 h with 1,000 U/ml IFNγ (Peprotech, Rocky Hill, NJ, USA) or vehicle control [0.1% bovine serum albumin (Sigma-Aldrich) in phosphate-buffered saline (PBS, Gibco)]. Cells were collected, washed with PBS, and analyzed by flow cytometry.

Flow Cytometry

Staining was performed in flow cytometry buffer (PBS supplemented with 5% FBS, 10 mM EDTA, and 0.05% sodium azide). Cells (2×10^5) were incubated for 30 min on ice with mouse anti-human antibodies against HLA-ABC (clone W6/32), HLA-DR (clone L243), CD271/NGFR (clone ME20.4), CD273/PD-L2 (clone 24F.10C12) (all from BioLegend, San Diego, CA, USA), and CD274/PD-L1 (clone MIH1; BD Biosciences, Franklin Lakes, NJ, USA) conjugated to phycoerythrin (PE), fluorescein isothiocyanate, PE-cyanine (Cy)7, allophycocyanin, and brilliant violet 421, respectively. All antibodies were titrated prior to experiment to ensure optimal concentrations were used. Fc block (BD Biosciences) was used to prevent non-specific staining due to antibody binding to Fc receptors. Fluorescence minus one controls (FMO, staining with all but one antibody for each fluorochrome) were included with each experiment. Prior to acquisition, cell viability was determined by staining cells with either 5 μM DAPI (Invitrogen, Thermo Fisher Scientific), Zombie Yellow dye (BioLegend), or Live Dead near-IR fixable dye (Invitrogen, Thermo Fisher Scientific). For the analysis of interferon gamma receptor 1 (IFNGR1) and B2M expression, cells were first stained with a fixable viability dye and either PE-conjugated anti-CD119 (clone GIR-208) or PE-Cy7

TABLE 1 | Expression of IFNγ-target proteins at baseline and post-stimulation with IFNγ in 39 melanoma cell lines.

Cell line	Driver mutation	HLA-ABC		HLA-DR		NGFR		PD-L1		PD-L2	
		−	+	−	+	−	+	−	+	−	+
A2058	$BRAF^{V600E}$	32.9	139.1	2.5	66.7	398.7	218.6	1.0	9.8	1.9	7.7
SKMel28	$BRAF^{V600E}$	74.2	128.3	11.1	120.8	44.7	197.3	1.3	2.9	2.6	10.0
C060M1	$BRAF^{V600E}$	34.9	88.7	29.5	75.7	10.4	16.3	1.0	5.5	4.3	6.7
SCC14-0257	$BRAF^{V600K}$	15.8	64.8	12.8	102.2	347.3	722.7	0.9	2.5	1.4	5.9
MM418	$BRAF^{V600E}$	38.2	81.8	1.2	7.7	16.0	19.0	1.1	4.0	1.1	1.5
NM16	$BRAF^{V600E}$	21.7	62.5	7.0	76.5	1,808.8	2,833.0	0.8	3.4	7.8	17.1
NM182	$BRAF^{V600E}$	18.9	143.6	1.3	31.8	10.1	14.0	1.0	4.5	1.0	1.6
MM200	$BRAF^{V600E}$	35.1	202.0	7.2	141.0	450.7	1,505.6	1.0	4.0	1.2	3.4
NM39	$BRAF^{V600E}$	43.6	122.2	27.6	123.3	90.7	46.7	1.1	3.9	4.3	17.9
HT144	$BRAF^{V600E}$	23.4	43.4	71.4	100.3	353.1	292.9	1.1	3.2	4.9	12.0
C016M	$BRAF^{V600E}$	20.9	70.3	42.7	95.9	469.6	630.2	0.9	1.3	2.2	5.5
MelRm	$NRAS^{Q61R}$	36.9	98.7	102.8	252.5	18.4	75.2	0.9	2.3	1.6	3.4
NM47	$NRAS^{Q61R}$	42.1	109.0	157.9	249.0	213.6	1,034.1	1.0	2.5	1.8	2.9
NM177	$NRAS^{Q61R}$	56.5	99.7	76.6	92.9	3,674.9	3,663.2	1.0	1.9	2.0	3.4
NM179	$NRAS^{Q61K}$	12.7	31.1	2.4	59.6	44.2	85.4	1.0	3.3	1.7	6.6
ME4405	$NRAS^{Q61R}$	60.5	118.2	0.9	0.9	24.0	47.3	1.0	2.1	3.5	14.2
MelAT	$NRAS^{Q61R}$	31.9	121.3	0.9	1.0	11.8	27.4	1.2	2.0	2.0	10.3
D11M2	$NRAS^{Q61L}$	11.3	18.4	16.4	33.7	29.2	32.1	1.1	2.4	3.3	9.2
C002M	$NRAS^{Q61K}$	7.2	31.4	1.0	27.0	13.7	15.6	1.2	2.3	1.4	1.5
C013M	$NRAS^{Q61L}$	24.7	81.1	1.0	42.7	47.8	199.3	1.0	2.3	2.2	6.5
D38M2	$NRAS^{Q61R}$	28.6	86.2	4.5	56.5	509.6	480.4	1.1	2.4	2.9	12.2
D22M1	$BRAF/NRAS^{WT}$	28.0	25.7	1.9	1.9	5.8	5.3	1.3	1.1	1.3	1.2
MeWo	$BRAF/NRAS^{WT}$	28.9	107.6	1.4	19.1	268.9	176.2	0.9	2.2	1.3	4.2
D24M	$BRAF/NRAS^{WT}$	32.6	37.2	13.8	43.3	30.7	28.3	1.2	3.3	7.3	31.0
C022M1	$BRAF/NRAS^{WT}$	10.7	41.7	2.3	21.5	148.3	341.0	1.9	1.6	1.1	1.8
C084M	$BRAF/NRAS^{WT}$	83.6	119.0	19.5	130.4	552.5	631.5	0.9	4.6	3.2	15.7
C086M	$BRAF/NRAS^{WT}$	20.1	52.4	22.7	90.3	1.3	2.9	1.1	3.0	3.7	9.9
D35	$BRAF/NRAS^{WT}$	167.9	460.2	3.8	129.6	21.9	36.9	0.9	2.9	0.9	2.7
C025M1	$BRAF/NRAS^{WT}$	73.2	134.1	2.1	18.5	1.7	2.7	1.1	2.7	1.1	1.2
SMU15-0217	$BRAF/NRAS^{WT}$	1.5	2.1	12.8	91.4	11.0	26.0	1.2	3.4	4.4	22.3
A04-GEH	$BRAF/NRAS^{WT}$	23.9	96.6	1.5	63.3	13.8	45.9	1.0	2.9	1.2	8.2
92.1	$GNAQ^{Q209L}$	11.5	87.2	0.5	0.5	14.0	38.8	1.1	1.7	1.2	1.0
MEL202	$GNAQ^{Q209L, R210K}$	38.2	346.3	1.1	15.9	10.6	15.0	1.0	5.6	1.0	2.7
MEL270	$GNAQ^{Q209P}$	52.5	115.6	1.1	1.8	3.6	4.1	1.1	1.6	1.2	1.3
MP38	$GNAQ^{Q209P}$	73.5	329.7	1.6	10.6	10.6	15.8	1.1	2.4	2.9	25.3
OMM1	$GNA11^{Q209L}$	31.2	108.0	1.1	19.0	2.0	3.6	1.1	1.5	1.0	3.7
MP41	$GNA11^{Q209L}$	26.3	44.2	0.9	3.7	2.6	3.6	0.9	1.3	1.1	2.0
MP46	$GNAQ^{Q209L}$	2.3	38.6	1.0	1.0	5.2	10.6	1.1	1.5	1.0	1.6
MM28	$GNA11^{Q209L}$	9.4	50.8	1.1	13.1	1.3	1.7	1.0	2.1	1.0	2.1

Relative marker expression levels were calculated by dividing the geometric mean fluorescence intensity (MFI) of the antibody-stained sample by the FMO control MFI.
−, no IFNγ treatment; +, treated for 72 h with 1,000 U/ml IFNγ; IFNγ, interferon-γ; NGFR, nerve growth factor receptor; FMO, fluorescence minus one.

conjugated anti-B2M (clone 2M2), both from BioLegend. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm kit and stained intracellularly with the same antibody that was used for cell surface stain.

Samples were acquired on BD LSRFortessa X20 flow cytometer (BD Biosciences) and the FlowJo software (TreeStar, Ashland, OR, USA) was used for data analysis. At least 10,000 live events were acquired. General gating strategy included forward and side scatter area to exclude cell debris, time parameter to exclude electronic noise, forward scatter area and height to exclude doublets and gating on viable cells (by gating on DAPI, Zombie Yellow, or Live Dead near-IR negative events). Relative marker expression levels were calculated by dividing the geometric mean fluorescence intensity (MFI) of the antibody-stained sample by the FMO control MFI (**Figure 1A**). Relative MFI is used in all analyses, and a relative MFI < 1.5 was considered to reflect no antigen expression relative to the control.

Cell Cycle and Apoptosis Analysis

Adherent and floating cells were combined after 72 h treatment with vehicle control or 1,000 U/ml IFNγ and cell cycle analyses were performed as previously described (15) using at least three biological replicates.

Gene Set Enrichment Transcriptome Analysis

Transcriptome analysis was performed on the The Cancer Genome Atlas (TCGA) human skin cutaneous melanoma (SKCM) and UVM datasets using single sample gene set enrichment analysis (ssGSEA) (16). RNA counts were normalized using the weighted trimmed mean of M-values implemented in the edgeR Bioconductor package. Normalized counts were transformed using *voom*, as implemented in the *Limma* package (17, 18). The gene sets used in ssGSEA analysis consisted of the Hallmark gene

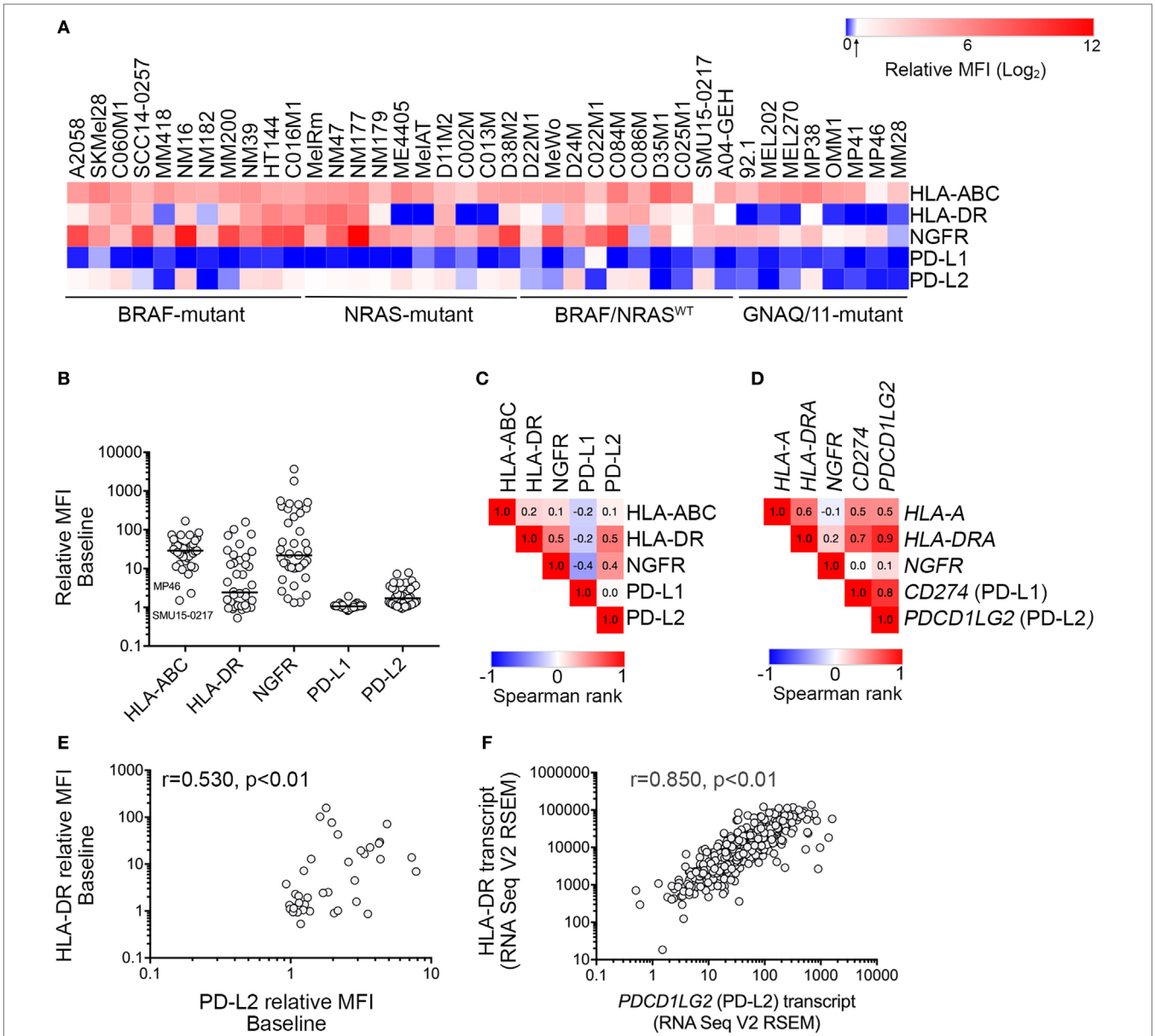

FIGURE 1 | Expression of downstream interferon-γ targets in melanoma cells. **(A)** Heatmap showing cell surface expression [relative mean fluorescence intensity (MFI); mean of two to five independent experiments] of HLA-ABC, HLA-DR, nerve growth factor receptor (NGFR), PD-L1, and PD-L2 in 39 melanoma cell lines with defined oncogenic drivers including 11 *BRAF*^{V600}-mutant, 10 *NRAS*-mutant, 10 *BRAF/NRAS* wild type (*BRAF/NRAS*^{WT}), and 8 *GNAQ/11*-mutant uveal melanoma cell lines. Relative MFI < 1.5 is indicated by the arrow on the color bar. **(B)** Cell surface baseline expression (relative MFI) of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in a panel of 39 melanoma cell lines. Each dot represents one cell line and the median expression is indicated by the horizontal line. Low cell surface expression of HLA-ABC on the MP46 and SMU15-0217 cell lines is indicated. **(C)** Correlation matrix showing Spearman's rank correlation analysis between cell surface expression of markers, as indicated. Spearman's rank correlation values are shown within the similarity matrix. **(D)** Correlation matrix showing Spearman's rank correlation analysis between transcript levels of *HLA-A*, *HLA-DRA*, *NGFR*, *PD-L1*, and *PD-L2* (The Cancer Genome Atlas (TCGA) skin cutaneous melanoma (SKCM) dataset). Spearman's rank correlation is shown within the similarity matrix. **(E)** Correlation between PD-L2 and HLA-DR cell surface expression and **(F)** mRNA transcript expression (TCGA SKCM dataset). Spearman's rank correlation coefficient and *p* values are shown.

set version 6.1, a refined gene set that define specific biological processes (19).

Whole Exome Sequencing

Melanoma cell exome sequencing was performed on D22M1 and SMU15-0217 melanoma cell lines. Exonic DNA was enriched using the Illumina SureSelect technology, targeting 50 Mb encompassing protein-coding regions and sequenced on an Illumina HiSeq2000. Read pairs were aligned to the reference human genome (hg19) using BWA (20) and nucleotide variants (SNVs) and small insertion/deletions were detected by SAMTools (21). Ingenuity Variant Analysis (http://www.ingenuity.com) was used

to identify mutations in genes associated with the JAK-STAT (KEGG) signaling pathway (22).

Statistical Analysis

Statistical significance was calculated using GraphPad Prism version 7 (GraphPad software, San Diego, CA, USA). p-Values <0.05 were considered significant.

RESULTS

Baseline Expression of IFNγ Target Molecules in Melanoma Lines With Different Oncogenic Driver Mutations

Expression of five well-defined IFNγ targets, the PD-1 ligands PD-L1 and PD-L2, NGFR, antigen-presenting HLA-A, -B, and -C (HLA-ABC), and HLA-DR molecules was examined in a panel of 39 human melanoma cell lines with defined oncogenic driver mutations (**Figure 1A**; Figure S1 in Supplementary Material). These included 11 *BRAF*^{V600}-mutant, 10 *NRAS*-mutant and 10 *BRAF/NRAS* wild type (*BRAF/NRAS*^{WT}) cutaneous melanoma cell lines, and 8 *GNAQ/11*-mutant UVM cell lines (**Table 1**).

Analysis of cell surface marker expression (antibody-stained MFI/FMO control MFI, relative MFI) revealed a broad range of expression for all five markers (**Figure 1**; **Table 1**). MHC class I molecules (HLA-ABC) were uniformly expressed on melanoma cells with the exception of the *BRAF/NRAS*^{WT} SMU15-0217 (relative MFI = 1.5) and the uveal MP46 cells (relative MFI = 2.3) (**Figure 1B**). HLA-DR showed a broad range of baseline expression in our panel of melanoma cells with no expression in 14 melanoma cell lines (MFI ratio < 1.5) and bimodal expression in 11/39 cell lines [i.e., only a proportion of cells (18–88%) expressed the marker]. NGFR expression was similarly variable (**Figure 1B**) with no expression at baseline in two cell lines (relative MFI < 1.5; **Table 1**). Similar to HLA-DR, NGFR was distributed in a bimodal fashion in six samples, with 42–81% cells expressing the marker. Three cell lines, the *BRAF*^{V600}-mutant C060M1 and *BRAF/NRAS*^{WT} D24M and SMU15-0217, had a bimodal expression of both HLA-DR and NGFR (data not shown). PD-1 ligands PD-L1 and PD-L2 were expressed at comparably low levels in our panel of melanoma cells (**Table 1**), with PD-L1 not constitutively expressed in 38/39 (relative MFI < 1.5) and PD-L2 absent in 18/39 cell lines. Seventeen melanoma lines lacked both PD-L1 and PD-L2 basal expression, including 5/10 (50%) *BRAF/NRAS*^{WT}, 4/11 (36%) BRAF^{V600}-mutant, 1/10 (10%) NRAS-mutant, and 7/8 (87.5%) uveal cell lines (**Figure 1**).

Of the targets analyzed, cell surface expression of PD-L2 was correlated with HLA-DR (Spearman's rank 0.530, $p < 0.01$) and NGFR expression (Spearman's rank 0.418, $p < 0.01$) (**Figure 1C**). The expression of HLA-DR and NGFR was also correlated (Spearman's rank 0.497, $p < 0.01$). The cell surface protein expression patterns of these markers in our melanoma panel did not precisely reflect their transcript expression patterns in the human SKCM dataset of TCGA ($n = 472$; **Figure 1D**), although both protein and transcript expression of PD-L2 (*PDCD1LG2*) and HLA-DR (*HLA-DRA*) were correlated (**Figures 1E,F**). It is also worth noting that PD-L1 (*CD274*) and PD-L2 (*PDCD1LG2*) transcripts were correlated (Spearman's rank = 0.793 $p < 0.01$) in the TCGA SKCM dataset, although we did not observe any correlation in their cell surface protein expression (Figure S2 in Supplementary Material).

There was also evidence that basal marker expression in *GNAQ/11*-mutant UVM was distinct. In particular, HLA-DR, NGFR, and PD-L2 cell surface expression was significantly lower in the UVM cell subset compared to cutaneous melanoma (**Table 1**; **Figure 2**). To address the significance of these findings, we analyzed TCGA RNA sequencing data from 80 uveal and 472 cutaneous melanoma samples. Consistent with our cell surface expression data, the expression of *HLA-DRA*, *NGFR*, and PD-L2 transcripts was significantly lower in the 80 uveal compared to the 472 cutaneous melanoma samples from the TCGA dataset; *CD274* (PD-L1) transcript expression was also different between the TCGA uveal and cutaneous datasets, whereas *HLA-A* transcript expression was indistinguishable between the TCGA uveal and cutaneous tumor groups (**Figure 2B**).

Expression of Target Molecules After Exposure to IFNγ

We noted that IFNγ stimulated the expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and/or PD-L2 in the majority of melanoma cell lines (**Figure 3A**). The degree of IFNγ stimulation was highly variable, however, and in the case of HLA-ABC, HLA-DR, PD-L2, and NGFR, the level of stimulation was proportional to the basal expression levels (**Figure 3B**). Only IFNγ-induced PD-L1 expression was independent of its basal expression levels and all but four cell lines lacking baseline PD-L1 showed IFNγ-stimulation of PD-L1 expression (**Figure 3B**).

Comparison of all five target molecules also showed positive correlation between IFNγ-induced expression of PD-L1, PD-L2, and HLA-DR. In particular, post-stimulation levels of PD-L1 and PD-L2 were correlated (Spearman's rank = 0.388, $p = 0.01$) (**Figure 3C**), although the degree of induction (i.e., change from pre- to post-stimulation) was not correlated (Spearman's correlation = 0.315, $p = 0.05$) because PD-L1 and PD-L2 showed disparate expression at baseline (**Figure 1C**). Similarly, although post-stimulation levels of NGFR were correlated with induced levels of PD-L2 (Spearman's rank = 0.358; $p = 0.025$) (**Figure 3C**), the degree of NGFR and PD-L2 induction (i.e., change from pre- to post-stimulation) was not correlated (Spearman's rank = −0.103; $p = 0.99$).

Overall, exposure of melanoma cells to IFNγ induced heterogeneous levels of all target molecules, and induction did not appear to depend on genotype in cutaneous melanomas for PD-L1, PD-L2, HLA-ABC, and NGFR (**Table 1**). In UVM lines, however, the protein expression of HLA-DR, NGFR, PD-L1, and PD-L2 post-IFNγ stimulation was significantly lower than observed in cutaneous melanomas (**Figure 3B**; Figure S3 in Supplementary Material), and this was consistent with low baseline expression of HLA-DR, NGFR, and PD-L2 in the UVM cells (**Figure 2A**). The transcript expression of *STAT1*, *STAT3*, and *IRF1*, three key transcription factors of the IFNγ signaling cascade, were also lower in the TCGA UVM dataset compared to the TCGA cutaneous melanomas (**Figure 4**). We

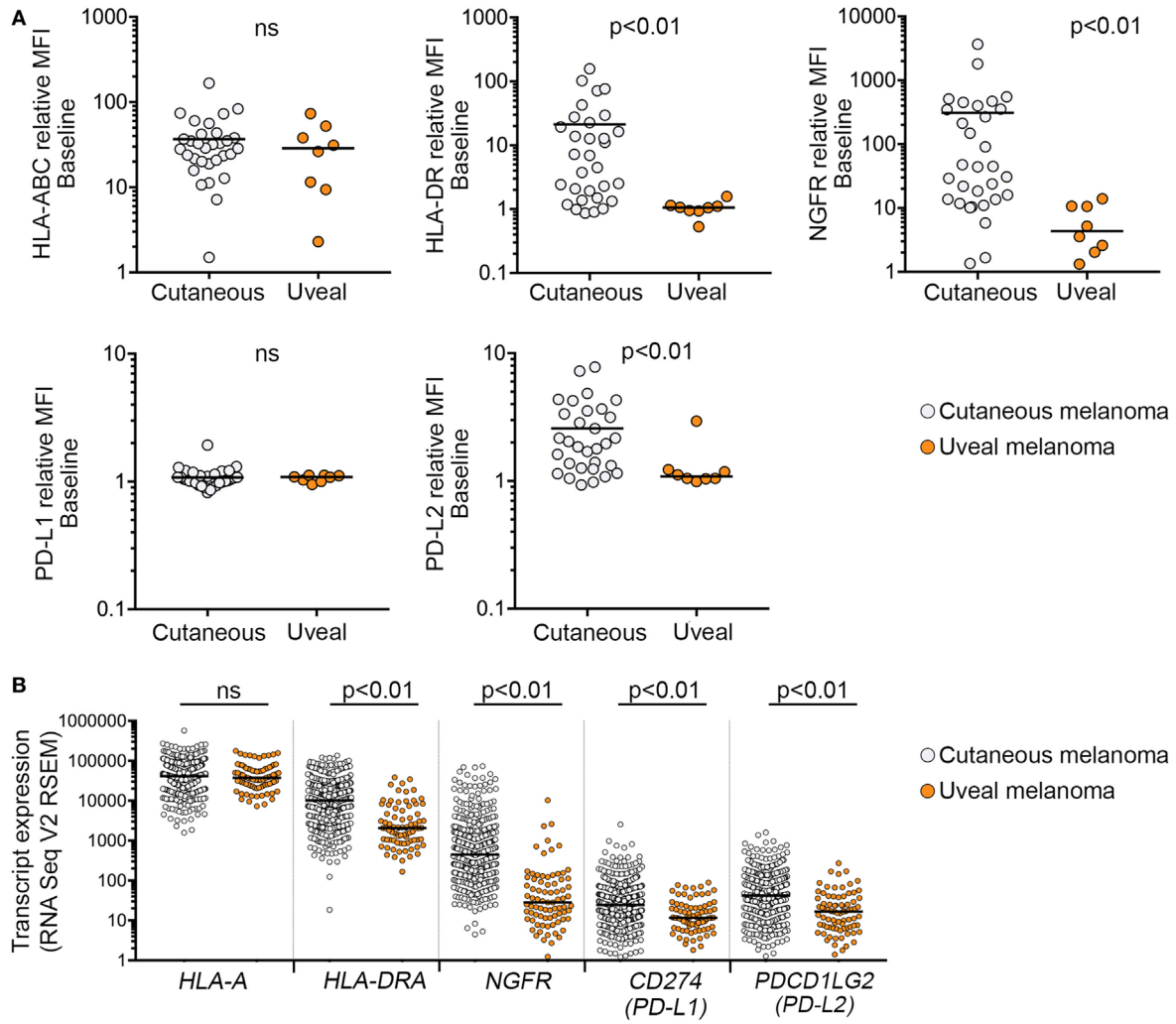

FIGURE 2 | Expression of interferon-γ targets in cutaneous and uveal melanoma (UVM) cells. **(A)** Cell surface expression [relative mean fluorescence intensity (MFI)] of HLA-ABC, HLA-DR, nerve growth factor receptor (NGFR), PD-L1, and PD-L2 in cutaneous (n = 31) and UVM (n = 8) cell lines. **(B)** Expression of mRNA transcripts for *HLA-A*, *HLA-DRA*, *NGFR*, *PD-L1*, and *PD-L2* in the 80 uveal [The Cancer Genome Atlas (TCGA) UVM dataset] and 472 cutaneous melanoma samples (TCGA skin cutaneous melanoma dataset). Each dot represents a single sample, with the median indicated by the horizontal line. Expression levels were compared using a Mann–Whitney test; ns, not significant.

also explored interferon signaling pathways in the SKCM and uveal TCGA melanoma dataset using single sample gene set enrichment analysis (ssGSEA), an extension of GSEA that defines an enrichment score of a gene set for each of the sample in the dataset (16). As shown in **Figure 4B**, the enrichment scores generated for the Hallmark_interferon_alpha and Hallmark_interferon_gamma response signatures were significantly lower in the UVM dataset, compared to cutaneous melanoma.

Downregulated Response to IFNγ in a Small Subset of Melanoma Cell Lines

Twenty-six of 39 cell lines (67%) demonstrated diminished response to IFNγ stimulation, usually manifested as no induction (i.e., fold induction in MFI ratio < 1.5) of one or more markers in response to IFNγ stimulation. HLA-ABC expression was absent in the *BRAF/NRAS*^{WT} SMU15-0217 cells even though expression of PD-L1, PD-L2, HLA-DR, and NGFR was upregulated by IFNγ (**Figure 5A**). Detailed analysis of this cell line confirmed that expression of B2M, the structural component of the MHC class I complex, was absent from the cell surface (**Figure 5B**). Among the other four markers, HLA-DR and PD-L1 expression was not induced in 7/39 cell lines, while induction of PD-L2 and NGFR was absent in 6/39 and 18/39 cell lines, respectively. One cell line, *BRAF/NRAS*^{WT} D22M1, showed a complete loss of response to IFNγ with no induction of any target molecules (**Figure 6A**), suggesting an upstream defect in the IFNγ signaling pathway in this cell line. Whole exome sequencing of this cell line identified a damaging missense mutation resulting in a P44R substitution in the extracellular portion of the IFNGR1 (**Figure 6B**). This amino acid substitution is located in the highly conserved NP linker region between the second and third beta sheets in the D1 domain (**Figure 6C**) and is classified as deleterious by the

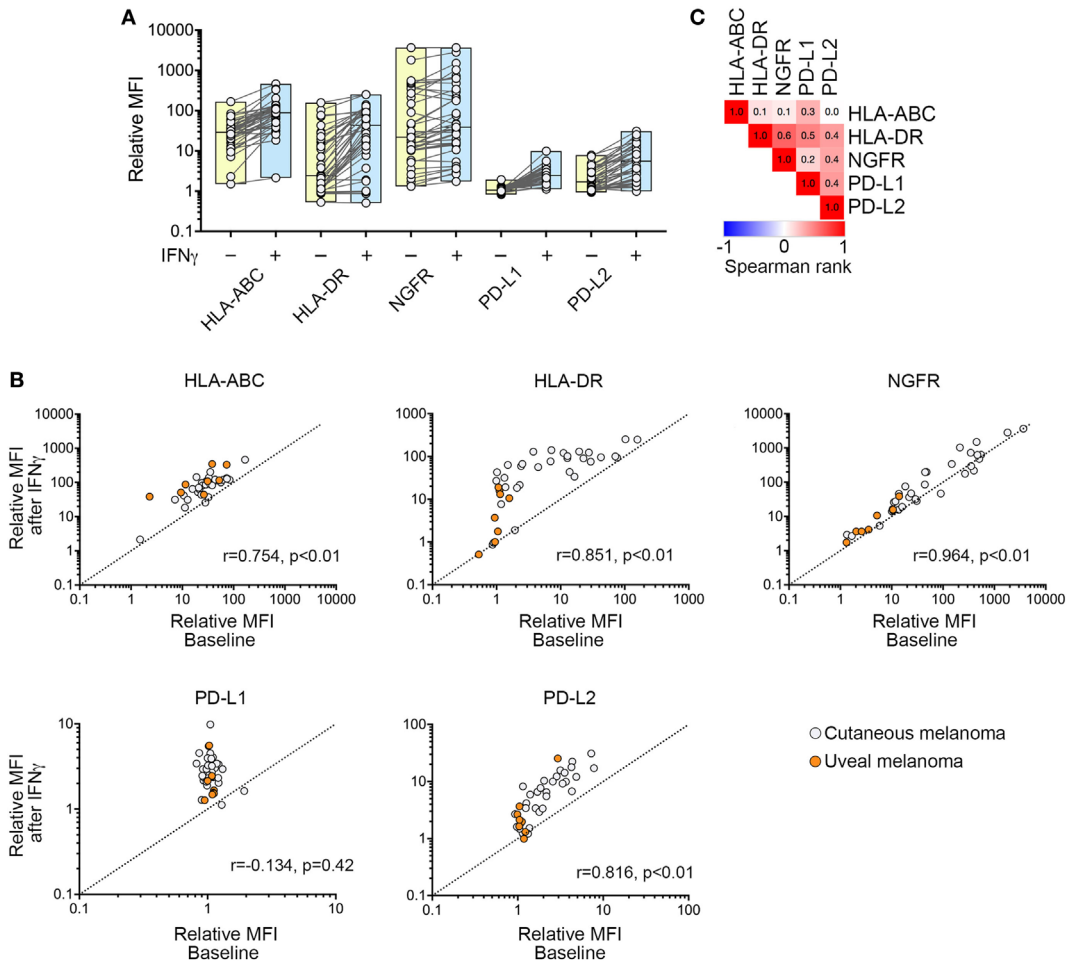

FIGURE 3 | Expression of cell surface markers in response to interferon-γ (IFNγ) treatment. **(A)** Change in HLA-ABC, HLA-DR, nerve growth factor receptor (NGFR), PD-L1, and PD-L2 cell surface expression [relative mean fluorescence intensity (MFI)] after exposure to IFNγ. Each dot shows one cell line before (–) and after (+) IFNγ stimulation with box plots showing the range and median. **(B)** Correlation of baseline and IFNγ-induced cell surface expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2. Each dot represents one cell line. Spearman's rank correlation coefficient and *p* values are shown. **(C)** Correlation matrix showing Spearman's rank correlation analysis between IFNγ-induced expression of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2. Spearman rank correlation values are shown within the similarity matrix.

missense substitution algorithms SIFT and Polyphen-2 (data not shown). We confirmed that IFNGR1 expression was absent on the surface of D22M1 cells although IFNGR1 expression was detected intracellularly (**Figure 6D**), consistent with accumulation of a misfolded protein.

Melanoma Cell Cycle Effects in Response to IFNγ Treatment

We also examined the impact of IFNγ treatment on cell cycle progression in our panel of melanoma cells using flow cytometry. Of the 38 melanoma cell lines tested, three showed increasing cell death in response to IFNγ, with greater than 10% increase in sub G1 (**Table 2**). Of these, one cell line (MM200) also showed a 56% increase in the proportion of cells undergoing DNA replication (i.e., S phase cells), along with another six cell lines that showed a greater than 30% increase in S phase cells. Another six cell lines, including 5/8 UVMs, showed diminished DNA replication post-IFNγ treatment (**Table 2**). The remaining 23 melanoma cell lines, including the IFNGR1-mutant D22M1 cells, showed minimal cell cycle profile changes when exposed to IFNγ (**Table 2**). It is worth noting that 5/7 melanoma cell lines with no IFNγ-mediated PD-L1 induction also showed no cell cycle profile changes in response to IFNγ treatment (**Table 2**).

DISCUSSION

Analysis of the IFNγ target proteins, HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2, in a panel of 39 melanoma cell lines revealed that IFNγ stimulated cell surface expression of all five markers in only 13 melanoma cell lines tested. The degree of IFNγ-mediated induction was highly variable for all five markers but closely reflected the corresponding basal expression levels for HLA-ABC, HLA-DR, PD-L2, and NGFR. By contrast, PD-L1 expression was frequently absent at baseline (relative MFI < 1.5)

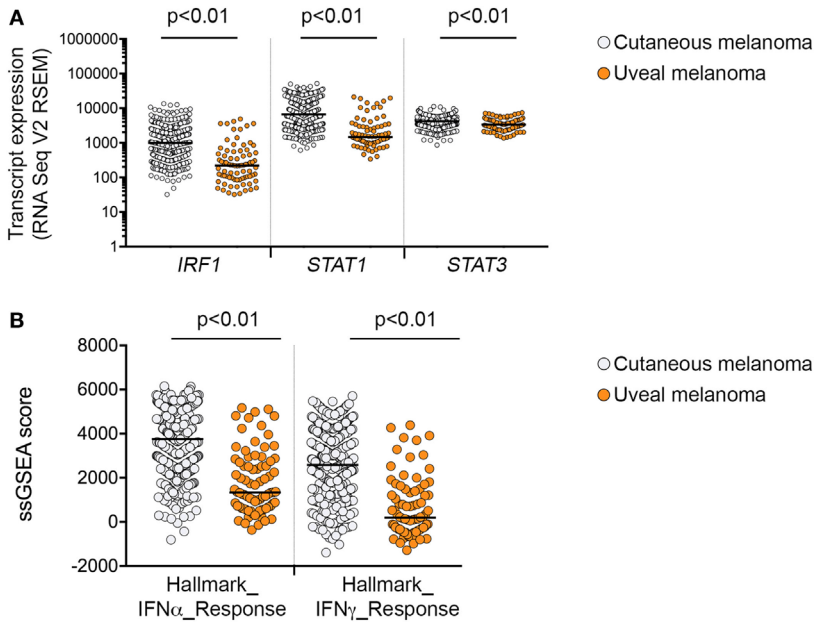

FIGURE 4 | Interferon-γ signaling in cutaneous and uveal melanoma (UVM). **(A)** Expression of mRNA transcripts for *IRF1*, *STAT1*, and *STAT3* in the 80 uveal [The Cancer Genome Atlas (TCGA) UVM dataset] and 472 cutaneous melanoma samples (TCGA skin cutaneous melanoma dataset). **(B)** Single sample gene set enrichment analysis (ssGSEA) scores for the Hallmark_interferon_alpha and Hallmark_interferon_gamma response signatures in the 80 uveal and 472 cutaneous melanoma samples from the TCGA datasets. Expression levels were compared using a nonparametric Mann–Whitney test, *p* values are indicated.

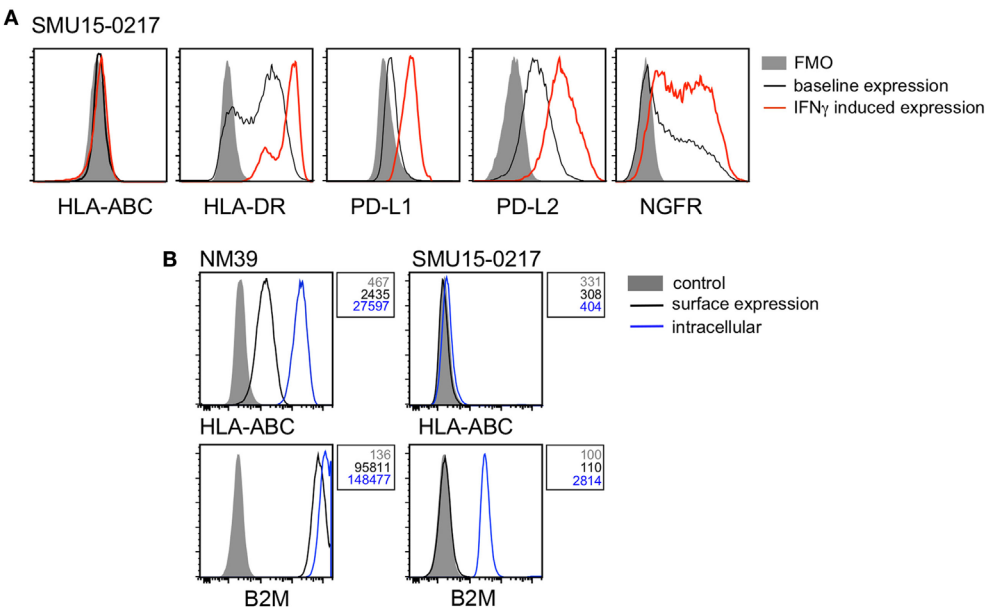

FIGURE 5 | Analysis of β-2-microglobulin (B2M) expression in the SMU15-0217 cell line. **(A)** Representative histograms of cell surface expression of HLA-ABC, HLA-DR, PD-L1, PD-L2, and nerve growth factor receptor (NGFR) on SMU15-0217 cells. Baseline expression is shown in black, interferon-γ (IFNγ)-induced expression in red, and fluorescence minus one (FMO) controls as shaded histograms. **(B)** Expression of HLA-ABC and B2M on the cell surface (black) and intracellularly (blue) in NM39 and SMU15-0217 cells. Shaded histograms represent the mock stained control and mean fluorescence intensity values are shown next to the histograms. NM39 cells were used as a positive control.

but was still induced to high levels after IFNγ treatment in the majority of cell lines. Consequently, although the JAK/STAT/IRF1 pathway is critical for the IFNγ-mediated induction of HLA-ABC, HLA-DR, and the two PD-1 ligands (14, 23), the low constitutive expression of PD-L1 suggests that this pathway has low baseline activity in melanoma and that the constitutive

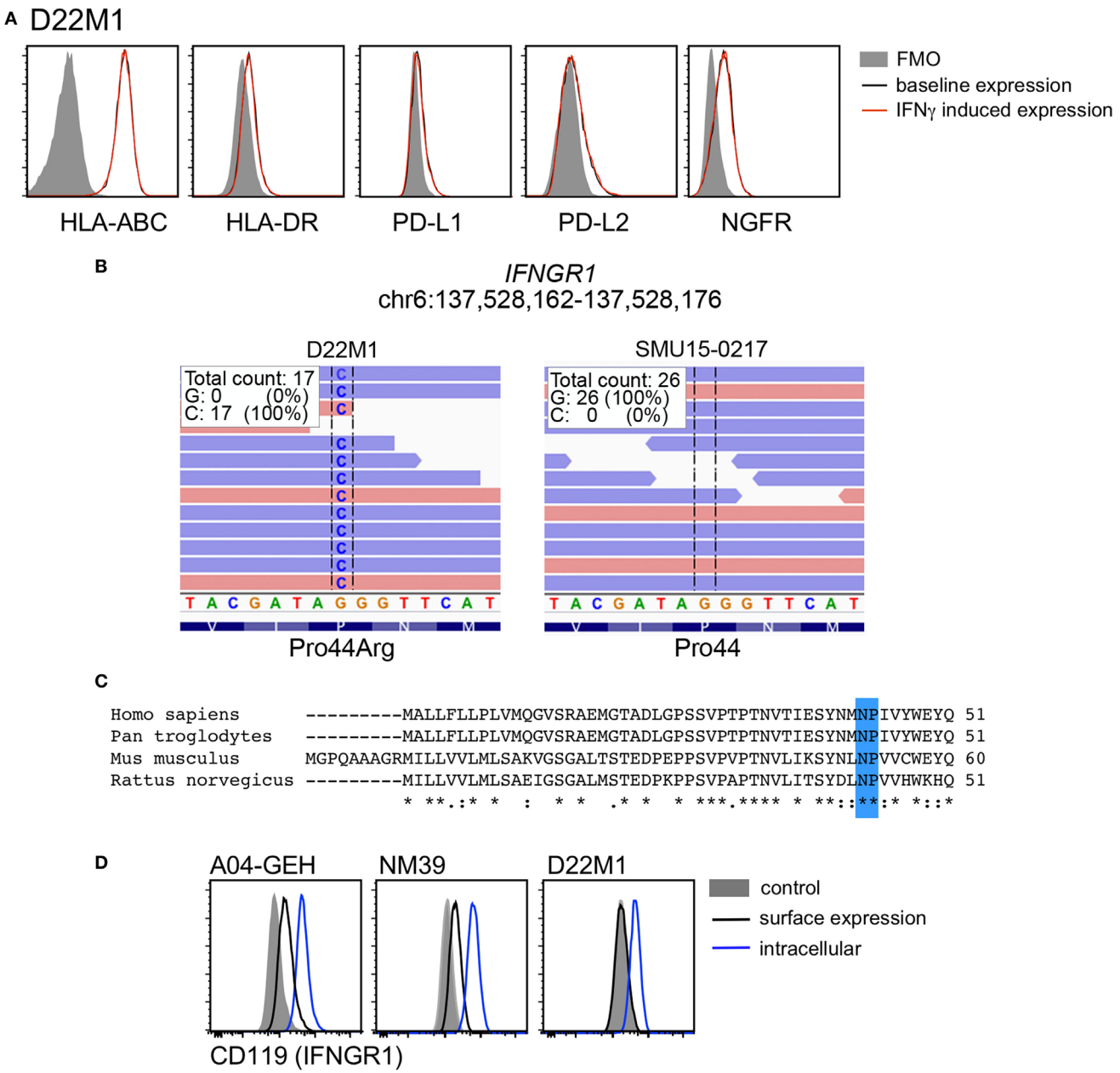

FIGURE 6 | Analysis of interferon gamma receptor 1 (IFNGR1) expression in the D22M1 cell line. **(A)** Representative histograms of cell surface expression of HLA-ABC, HLA-DR, PD-L1, PD-L2, and nerve growth factor receptor (NGFR) on D22M1 cells. Baseline expression is shown in black, interferon-γ (IFNγ)-induced expression in red, and fluorescence minus one (FMO) controls as shaded histograms. **(B)** Whole exome sequencing analysis showing Pro44Arg (P44R) substitution in the D22M1 cell line but not in the SMU15-0217 cells. **(C)** Alignment of IFNGR1 protein sequence of human, chimpanzee, mouse, and rat (Clustal Omega) showing the highly conserved NP linker region highlighted in blue. **(D)** Expression of IFNGR1 on the cell surface (black) and intracellularly (blue) in A04-GEH, NM39, and D22M1 cells, with mean fluorescence intensity values also shown. Shaded histograms represent the mock stained control.

expression of HLA-ABC, HLA-DR, PD-L2, and NGFR may be regulated *via* alternate pathways or downstream elements.

The IFNγ-induced expression of several markers, including PD-L1 and PD-L2, was correlated, although we did not detect significant correlation when the degree of IFNγ stimulation (i.e., change from pre- to post-stimulation) was compared. This may reflect disparate baseline expression levels due to IFNγ-independent regulatory influences but also the complexity and redundancy of the IFNγ signaling pathway. For instance, whereas the JAK–STAT1/2/3–IRF1 axis is critical for PD-L1 regulation, the JAK–STAT3–IRF1 node is important for PD-L2 stimulation (14). We also noted that cell surface expression of HLA-DR, NGFR, and PD-L2 was significantly lower in UVM compared to cutaneous melanoma, both at baseline and post-IFNγ stimulation. The transcriptomic analysis of the TCGA cutaneous and UVM datasets confirmed that UVM expressed lower levels of *HLA-DRA*,

TABLE 2 | IFNγ-mediated cell cycle effects in melanoma cells.

Cell line	Driver mutation	Sub-G1 phase		G1 phase		S phase		G2 phase		
		–	+	–	+	–	+	–	+	IFNγ effect
A2058	$BRAF^{V600E}$	0.6	1.3	68.2	63.3	19.2	26.7	12.7	10.1	↑ S phase
SKMel28	$BRAF^{V600E}$	1.7	3.8	72.4	73.5	19.6	13.3	8.0	13.3	↓ S phase
C060M1	$BRAF^{V600E}$	1.1	2.4	72.1	68.2	12.1	11.9	16.1	19.9	
SCC14-0257	$BRAF^{V600K}$	1.6	5.3	63.4	50.5	20.0	26.1	16.6	23.4	↑ S phase
MM418	$BRAF^{V600E}$	0.9	9.5	61.8	54.9	24.8	32.4	13.4	12.7	↑ S phase
NM16	$BRAF^{V600E}$	0.8	4.1	65.6	70.1	26.3	26.8	8.1	3.1	
NM182	$BRAF^{V600E}$	2.5	4.1	60.8	56.3	28.4	34.8	10.8	8.9	
MM200	$BRAF^{V600E}$	1.1	17.1	69.9	62.2	19.9	31.1	10.2	6.7	↑ sub-G1, ↑ S phase
NM39	$BRAF^{V600E}$	0.7	2.3	85.2	86.4	9.7	9.8	5.0	3.8	
HT144	$BRAF^{V600E}$	1.6	12.9	65.4	61.7	24.3	24.4	10.4	13.9	↑ sub-G1
C016M	$BRAF^{V600E}$	4.0	6.8	73.2	65.4	19.8	22.9	7.0	11.7	
MelRm	$NRAS^{Q61R}$	0.7	4.1	62.9	64.9	26.8	24.2	10.3	10.8	
NM47	$NRAS^{Q61R}$	0.4	6.5	63.0	65.3	25.8	24.3	11.2	10.4	
NM177	$NRAS^{Q61R}$	2.6	2.9	67.6	59.5	23.3	28.4	9.0	12.2	
NM179	$NRAS^{Q61K}$	1.7	6.7	60.1	52.5	24.8	32.9	15.0	14.7	↑ S phase
ME4405	$NRAS^{Q61R}$	0.3	0.7	59.7	63.6	28.2	26.1	12.1	10.2	
MelAT	$NRAS^{Q61R}$	0.5	0.8	58.2	68.0	29.7	22.7	12.2	9.3	
D11M2	$NRAS^{Q61L}$	7.8	8.7	41.7	39.7	33.4	28.2	24.9	32.2	
C002M	$NRAS^{Q61K}$	1.4	2.5	73.3	65.5	17.7	27.0	8.9	7.5	↑ S phase
C013M	$NRAS^{Q61L}$	19.0	35.5	57.1	54.2	26.4	23.8	16.6	21.9	↑ sub-G1
D38M2	$NRAS^{Q61R}$	0.7	1.3	65.1	59.9	18.2	21.3	16.7	18.9	
D22M1	$BRAF/NRAS^{WT}$	1.2	1.2	52.7	50.9	37.9	39.5	9.4	9.5	
MeWo	$BRAF/NRAS^{WT}$	1.1	1.7	49.2	52.9	25.5	24.1	25.4	22.9	
D24M	$BRAF/NRAS^{WT}$	nd	nd	nd	nd	nd	nd	nd	nd	
C022M1	$BRAF/NRAS^{WT}$	1.4	4.1	80.2	68.3	11.5	17.6	8.2	14.1	↑ S phase
C084M	$BRAF/NRAS^{WT}$	0.6	1.2	37.0	37.8	22.5	16.4	40.6	45.8	
C086M	$BRAF/NRAS^{WT}$	4.7	13.4	50.4	48.8	34.0	25.9	15.6	25.3	
D35M1	$BRAF/NRAS^{WT}$	0.3	1.3	71.9	72.8	20.7	23.5	7.5	3.7	
C025M1	$BRAF/NRAS^{WT}$	1.2	1.4	75.5	78.0	17.7	16.1	6.8	5.6	
SMU15-0217	$BRAF/NRAS^{WT}$	0.6	1.4	69.5	67.5	22.2	21.0	8.3	11.5	
A04-GEH	$BRAF/NRAS^{WT}$	1.0	7.7	60.0	56.9	25.7	24.7	14.3	18.4	
92.1	$GNAQ^{Q209L}$	0.7	8.3	60.6	87.0	31.6	10.3	7.9	2.7	↓ S phase
MEL202	$GNAQ^{Q209L, R210K}$	0.4	5.2	57.5	72.8	26.8	17.3	15.7	9.9	↓ S phase
MEL270	$GNAQ^{Q209P}$	0.8	1.4	68.7	69.9	21.8	20.8	9.5	9.3	
MP38	$GNAQ^{Q209P}$	0.6	2.2	72.7	88.4	12.0	4.2	15.4	7.4	↓ S phase
OMM1	$GNA11^{Q209L}$	1.4	1.4	53.4	52.3	35.8	36.4	10.9	11.4	
MP41	$GNA11^{Q209L}$	1.2	4.1	60.7	84.0	28.3	12.1	11.0	3.9	↓ S phase
MP46	$GNAQ^{Q209L}$	1.2	1.8	28.7	29.1	10.4	10.1	57.3	61.4	
MM28	$GNA11^{Q209L}$	0.7	1.2	85.6	92.4	7.1	3.4	7.3	4.3	↓ S phase

Percentage of cells in the indicated cell cycle phase is shown. Data are the average of at least three independent experiments. S phase data indicate either 30% increase (↑) or decrease (↓) in the proportion of cells undergoing DNA replication, calculated as [(S phase$_{IFN\gamma}$ – S phase$_{BSA}$)/S phase$_{BSA}$].

↑ sub G1 indicates a greater than 10% increase in sub G1 cells in response to IFNγ treatment (sub G1$_{IFN\gamma}$ – sub G1$_{BSA}$).

Cells showing no IFNγ-mediated PD-L1 induction are shown in bold.

–, no IFNγ treatment; +, treated for 72 h with 1,000 U/ml IFNγ; nd, not determined; IFNγ, interferon-γ; BSA, bovine serum albumin.

NGFR, *CD274* (PD-L1), and *PDCD1LG2* (PD-L2) transcripts, and this was associated with reduced transcript expression of the IFNγ master transcription factors *STAT1*, *STAT3*, and *IRF1* and with reduced IFNγ transcriptome signatures. It is worth noting that although transcriptome data are derived from high quality tumor samples with at least 60% tumor nuclei, they do contain variable levels of tissue-infiltrating immune and stromal cell populations that may influence the level of transcript expression (24). Nevertheless, collectively the transcriptome and flow cytometric analysis indicate diminished IFNγ activity in UVM.

Incomplete responses to IFNγ-stimulation, usually manifested as lack of induction of one or more markers were evident in 26 of 39 (67%) melanoma cell lines. Although it is still not clear whether incomplete IFNγ stimulation in melanoma cells has significant impact on patient responses to immunotherapy, it is evident that this pathway is important for response to PD-1 blockade. In particular, nuclear expression of the IFNγ transcription factor IRF1 (25) is associated with better response to anti-PD-1 therapy in melanoma (26) and loss-of-function mutations in IFNγ pathway modulators (JAK1, JAK2) are associated with resistance to anti-PD-1 treatment. Moreover, murine B16 melanoma cells deficient in JAK1 or IFNGR1 grew faster than control B16 cells in response to immune therapy (27). Metastatic UVM respond poorly to immune checkpoint inhibition (28, 29), and although there appears to be no difference in the level of infiltrating CD8+ T cells between uveal and cutaneous melanoma (30), our data suggest that UVM may have diminished capacity to respond to IFNγ, with lower expression of targets including PD-L1 (31), PD-L2, HLA-DR, and NGFR (this study).

It is therefore provocative to suggest that inducibility of multiple IFNγ targets may inform or predict immunotherapy response.

It is worth noting that of the 26 melanoma cell lines displaying incomplete induction of the 5 target proteins, 8 showed cell cycle distribution changes in response to IFNγ treatment. Importantly, 5/7 melanoma cell lines with no IFNγ-mediated PD-L1 induction showed no cell cycle profile changes after treatment with IFNγ. This may reflect the critical role of the STAT1 transcription factor in promoting PD-L1 expression and mediating IFNγ-induced cell cycle effects (14, 32). Five of eight UVM cell lines responded to IFNγ treatment with a decreased proportion of S phase cells and this was not a common response in our panel of cutaneous melanoma cells. This may be due to IFNγ concentration effects, as previous reports have shown that 50 U/ml IFNγ was sufficient to arrest UVM cells, whereas concentrations exceeding 1,000 U/ml IFNγ were required to inhibit the growth of the cutaneous A375 melanoma cells (32, 33). The unique responses of UVM cells to IFNγ stimulation require further investigation.

Interestingly, although most of our cell lines did not display baseline PD-L1 expression, PD-L1 was induced in the majority of cell lines. This is significant, as PD-L1 expression is sufficient to mediate melanoma escape from immune checkpoint inhibition (34). Loss of MHC class I expression is another established mechanism of immune escape, often involving genetic alterations in the *B2M* gene (7, 13, 35) and we noted that the SMU15-0217 melanoma cell line showed loss of B2M expression, concurrent with loss of HLA-ABC expression. Only one cell line (i.e., D22M1) failed to respond to IFNγ, and this was associated with a homozygous, predicted loss-of-function mutation in the *IFNGR1* gene.

In conclusion, our study demonstrates that expression analysis of IFNγ targets pre- and post-IFNγ stimulation can identify incomplete IFNγ pathway activity in melanoma cells. We show that incomplete IFNγ signaling occurs in almost 70% of immunotherapy-naïve melanoma, and previous reports have confirmed that pre-existing alterations affecting IFNγ signaling have the potential to confer resistance to immune checkpoint inhibitors (7, 9). In fact, we identified two well-recognized mechanisms of immunotherapy resistance; the loss of B2M expression, resulting in absence of cell surface HLA-ABC, and a missense mutation in the *IFNGR1* gene, resulting in loss of cell surface IFNGR1. We also report that UVMs, which show poor responses to PD-1-inhibitor therapies, display an inherently weaker response to IFNγ signaling with reduced JAK–STAT pathway activity.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Human Research ethics committee protocols from Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Royal Prince Alfred Hospital Human Research ethics committee.

AUTHOR CONTRIBUTIONS

SA, ES, and AS performed the experiments. ES, HR, and SL wrote the manuscript. SA, SL, ES, and HR analyzed and interpreted the data. SA, AS, RK, SL, ES, and HR read, revised, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01414/full#supplementary-material.

FIGURE S1 | Flow cytometric analysis in melanoma cells. Representative histograms of baseline (solid black line) and IFNγ-induced expression (solid red line) of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in SKMel28 melanoma cells. Fluorescence minus one controls (FMO) are shown as shaded histograms.

FIGURE S2 | PD-L1 and PD-L2 protein and transcript expression in melanoma cells. Correlation of cell surface protein [relative mean fluorescence intensity (MFI); left panel] and *CD274* (PD-L1) and *PDCD1LG2* (PD-L2) mRNA transcript expression derived from The Cancer Genome Atlas skin cutaneous melanoma dataset; right panel. Each dot represents one cell line. Spearman's rank correlation coefficient and *p* values are shown.

FIGURE S3 | Expression of downstream IFNγ targets post-IFNγ stimulation in cutaneous and uveal melanoma cells. Cell surface expression post-IFNγ stimulation (relative MFI) of HLA-ABC, HLA-DR, NGFR, PD-L1, and PD-L2 in cutaneous ($n = 31$) and uveal melanoma ($n = 8$) cell lines. Bars represent medians. Mann–Whitney test, p values are indicated.

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REVIEW
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Concepts Collide: Genomic, Immune, and Microbial Influences on the Tumor Microenvironment and Response to Cancer Therapy

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Cancer research has seen unprecedented advances over the past several years, with tremendous insights gained into mechanisms of response and resistance to cancer therapy. Central to this has been our understanding of crosstalk between the tumor and the microenvironment, with the recognition that complex interactions exist between tumor cells, stromal cells, overall host immunity, and the environment surrounding the host. This is perhaps best exemplified in cancer immunotherapy, where numerous studies across cancer types have illuminated our understanding of the genomic and immune factors that shape responses to therapy. In addition to their individual contributions, it is now clear that there is a complex interplay between genomic/epigenomic alterations and tumor immune responses that impact cellular plasticity and therapeutic responses. In addition to this, it is also now apparent that significant heterogeneity exists within tumors–both at the level of genomic mutations as well as tumor immune responses–thus contributing to heterogeneous clinical responses. Beyond the tumor microenvironment, overall host immunity plays a major role in mediating clinical responses. The gut microbiome plays a central role, with recent evidence revealing that the gut microbiome influences the overall immune set-point, through diverse effects on local and systemic inflammatory processes. Indeed, quantifiable differences in the gut microbiome have been associated with disease and treatment outcomes in patients and pre-clinical models, though precise mechanisms of microbiome-immune interactions are yet to be elucidated. Complexities are discussed herein, with a discussion of each of these variables as they relate to treatment response.

Keywords: cancer immunotherapy, biomarkers, heterogeneity, microbiome, cancer genomics, tumor microenvironment, systemic immunity

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INTRODUCTION

Interest in defining factors that influence the outcome of cancer therapy has existed for as long as the therapies themselves. Traditionally, a highly tumor-centric focus has dominated, resulting in a now well-characterized yet still incomplete view of the complex molecular and cellular tumoral dynamics relevant to cancer progression and to treatment response.

Several of these factors, particularly the overall somatic mutational burden of the tumor, have gained traction and even potential clinical utility in the prediction of response to immunotherapy,

notwithstanding ongoing concerns about their limited accuracy. Qualitative genomic characterization of tumors may also be very informative, however, information derived from such analyses is subject to the limitations imposed by sampling error and heterogeneous composition of synchronous tumors in patients with multiple metastases. Despite initial enthusiasm, an appreciation of the limitations of genomic characterization alone is emerging, and a more comprehensive analysis of the multitude of factors influencing therapeutic responses is critically needed.

In this mini review, we provide an overview of genomic factors implicated in the response to cancer immunotherapy, utilizing melanoma as a model "immunogenic" tumor from which the majority of empirical evidence derives. We will also discuss immune determinants of response and resistance, highlighting recent data regarding tumor immune cell co-evolution influenced by immunogenic factors arising from tumor cells, and the immunoediting effects of the responding immune infiltrate, both of which are impacted by intra- and inter-tumoral heterogeneity. In addition to this, we will complement studies of the tumor microenvironment to better delineate the crosstalk between the tumor microenvironment and overall host immunity with the microbiome, as this has been shown to influence outcomes ranging from tumor growth and immunity to treatment-related response and toxicity. Though discussion of the gut microbiome will predominate, we will also describe the potential impact of the intra-tumoral microbiome on resistance to cancer therapy, thus providing a full discussion of the intersection of tumor genomics, immunity, and the microbiome in shaping therapeutic responses, as summarized in (**Table 1**).

TUMOR-SPECIFIC INFLUENCES ON RESPONSE

Long before the advent of modern genomic technologies, histologic sub-types of cancer were described, with differences in response to therapeutic intervention noted across these sub-types. This is well illustrated in melanoma, for which several clinicopathologic sub-types exist, including superficial spreading, acral, desmoplastic, and mucosal melanomas. With the advent of next generation sequencing, we have gained tremendous insight into the molecular underpinnings of these clinicopathologic observations and into the mechanisms driving differences between tumors themselves. Distinct genomic aberrations frequently define histologic sub-types and can confer notable differences in therapeutic sensitivity that have major clinical relevance (1, 2). Beyond this, other components of the tumor microenvironment have been noted to play a major role in therapeutic response and resistance, impacting upon tumor visibility and susceptibility (**Figure 1**), as discussed below.

TUMOR IMMUNE "VISIBILITY"

In addition to their influence on oncogenic signaling and proliferative potential, genomic mutations present in melanoma and other cancers may have a profound impact on anti-tumor immunity and can contribute to the "visibility" of a tumor to the immune system (3, 4). This is largely shaped by the antigenic characteristics of the tumor cells allowing their recognition by the immune system, but may be shaped by other influences of these oncogenic mutations on the tumor cells themselves as well as the microenvironment. This is important, as therapeutic targeting of oncogenically activated signaling pathways may alter anti-tumor immunity. A key example of how genomic alterations may impact tumor visibility is illustrated in the case of *BRAF*-mutant melanoma. Early observations demonstrated a link between MAPK signaling and the expression of melanoma-associated antigens (5, 6), with subsequent data revealing brisk infiltration of tumors with T lymphocytes in the setting of treatment with BRAF inhibitor-based therapy (3, 7). Interestingly, inhibition of oncoproteins such as BRAF may also be associated with increased expression of HLA molecules and heat shock proteins, which can further contribute to a tumor's visibility (8, 9).

More generally, tumor cell immune visibility is fundamentally dependent on the presence (or absence) of molecular moieties that can be recognized by components of the host immune system. Tumor cell self-antigens represent the basis of cognate interactions with cellular elements of the adaptive immune system, but have varying degrees of tumor cell specificity. Such antigens include differentiation or lineage-specific antigens, aberrantly expressed antigens either absent or found at only low levels in adult tissues (3), or may be truly tumor cell-specific neoantigens derived from the protein products of somatically mutated genes. Tumor neoantigens are felt to predominantly mediate effective anti-tumor immune responses because neoantigen-reactive T cells escape deletion mechanisms during T cell ontogeny, and respond to these antigens as "foreign" rather than "self" (10, 11). In addition, epithelial-to-mesenchymal-like (EMT-like) plasticity in melanoma is thought to contribute to functional and antigenic variation that has the potential to influence the efficacy of immune-based therapies (12). Given the prominent role of the lymphocyte response to MAPK blockade in *BRAF*-mutated melanoma, these EMT-like shifts in melanoma cell state may well also contribute to BRAF inhibitor resistance at least in part by altering melanoma cell visibility *via* this antigenic shift (13).

Tumor genomics gains specific relevance to immune visibility in light of the significance of tumor-specific neoantigens in shaping immune responses. The mutational landscape varies across tumor types (14), and is shaped by factors influencing carcinogenesis such as UV irradiation and smoking (14). Interestingly, responses to immunotherapy are positively associated with the mutational burden of each particular tumor type, evidenced by higher response rates and clinical benefit in tumor types with an overall high mutational burden, such as melanoma, non-small cell lung cancer (NSCLC), clear cell renal cell cancer, and genitourinary cancers (14, 15). A recent study of 151 patients with predominantly melanoma (34%) or NSCLC (24%), mostly treated with anti-CTLA-4, anti-PD-1, or anti-PD-L1 blockade therapy, assessed the relationship between tumor mutational burden measured by hybrid capture next generation sequencing and clinical outcomes. Using pre-defined cut-offs, patients with higher tumor mutational burden experienced higher response rates and longer progression-free and overall survival than those with low to intermediate tumor mutational burden (16). These

TABLE 1 | Inter-relationships between clinical, genomic, immune, and microbial factors drawn from the patient (systemic), tumor microenvironment (histology), and disease-level domains, with associated influence on immunotherapeutic outcomes.

	Clinical	Genomic	Immune	Microbial	Therapeutic
Patient/systemic	**Age**	*Accumulated mutations*	Immune senescence		May impact treatment decisions
	Comorbidities		Iatrogenic immunosuppression (e.g., steroid use)	Iatrogenic dysbiosis (e.g., antibiotic use)	May limit treatment options and drug interactions
	Performance status				May limit treatment options
	Environmental exposures	Carcinogen exposures (e.g., UV and tobacco smoke) → DNA damage, accumulation of mutations		Microbe-derived genotoxins (e.g., pks/colibactin)	
			Th1/Th17 vs Th2 skewing and effects on anti-cancer immunosurveillance	**Promotion of Th1/Th17 responses by gut microbiota** (e.g., *via* DAMPs/PAMPs)	Potentially oncogenic but also permissive to immunotherapy response
	Diet/stress/antibiotic use		*Immunosuppression*	**Dysbiosis**	
			Permissive effect on anti-cancer T cell function	**Myeloid priming**	
				Gut microbial diversity (alpha diversity)	Associated with immunotherapy response
			Regulation of immune tone, FoxP3+ Treg maintenance	**Microbial metabolites** (e.g., short-chain fatty acids)	
Tumor microenvironment/ histology	**Cancer type and sub-type**	Mutational load, specific mutations, mutational and multi-"omic" signatures (e.g., carcinogen-related)	Affects intrinsic immunogenicity	Influenced by exposure to local microflora	Expectation of immunotherapy outcome markedly influenced by cancer histology and sub-type (e.g., mucosal vs cutaneous melanomas)
	Immunohistochemical PD-L1 scoring (note: variable antibody performance and individualized thresholds for clinical interpretation)	Cancer-associated molecular pathways influence immunoregulatory molecule expression	**PD-L1** status, expression of additional checkpoint receptors/ligands on T cells and tumor		Positive predictive value for PD-(L)1 inhibitor based therapy
	Immunohistochemical evaluation		**Lymphocytic infiltration**	Enrichment of specific taxa in gut microbiome associated with CD8+ TIL	Presence of TIL associated with better prognosis across many cancer types
			Presence of immunoregulatory or suppressive cell subsets (e.g., Treg, MDSC, and TAF/TAM)	Enrichment of specific taxa in gut microbiome associated with suppressive cell populations in the tumor	Poor immunotherapy response unless specifically targeted by the immunotherapeutic agent
		HLA types and diversity	Formation of immune synapses, neoantigen presentation, need to optimally match T cell repertoire		HLA diversity associated with improved survival following checkpoint blockade therapy
		HLA class I loss	Immune evasion		
		Altered antigen presentation machinery, EMT-like plasticity (e.g., IFN-driven proteasomal alteration)	**Inflamed microenvironment**	Influenced by gut microbial composition and local/intra-tumoral microflora	Differential effects on anti-cancer immunity depending on time course (e.g., acute vs chronic/persistent inflammation)

(*Continued*)

TABLE 1 | Continued

	Clinical	Genomic	Immune	Microbial	Therapeutic
		Defective antigen presentation machinery (e.g., *JAK2* mutations and β2*M* loss)	Loss of antigen presentation, immune evasion		
		Adaptive mutational/neoantigen pruning and immunoediting	**T cell repertoire** (e.g., clonality, neoantigen-specific clones)		
		Altered transcriptome and/or methylation patterns		**Locally pro-inflammatory microbes**	
				Intra-tumoral microbial metabolism	*In situ* degradation of chemotherapeutic agents
Disease	**Stage**	Mutational load, specific mutations, and mutational signature (e.g., carcinogen-related)	Progression-related antigenic change, clonal selection (e.g., under influence of spontaneous anti-cancer immunity or prior therapy)	*Tumor-induced dysbiosis*	
	Burden of disease	*Underlying inter-tumoral genomic heterogeneity*		*Tumor-induced dysbiosis*	May influence fitness for treatment, adversely prognostic
	Growth characteristics (e.g., rate of progression and metastatic site tropism)	**Driver mutation status** (e.g., *BRAF*^{V600}), specific **methylation and copy-number alterations**	Immune pathway modulation (e.g., by MAPK activation), tumor antigen expression (e.g., modulated by EMT-like processes)	Methylation and transcriptome alterations associated with (local) microflora	Aggressive disease, certain sites of involvement (e.g., brain) adversely prognostic
	Associated with some clinical characteristics (e.g., carcinogen type- and dose-related and lower overall mutational burden in presence of clear driver mutations like *BRAF*^{V600})	**Total mutational burden**	Neoantigen repertoire		Predictive of response to checkpoint blockade (monotherapy), unclear relationship for combinations at this stage
		EMT-like plasticity	Evolution of potential tumor antigen expression (e.g., melanoma differentiation antigens and cancer-testis antigens)	*Microbial effects on methylation known, potential for dynamic epigenomic influences*	Drug sensitivity, immune vulnerability
		Immune exclusion (e.g., β-catenin)	Failure of effector immune cell infiltration, "immune-desert"		

Core concepts are shown in bold. Entries in italics represent speculative interactions.

TAF, tumor-associated fibroblasts; TAM, tumor-associated macrophages; TIL, tumor-infiltrating lymphocytes; Treg, regulatory T cells; DAMP, damage-associated molecular patterns; EMT, epithelial-to-mesenchymal transition; MDSC, myeloid-derived suppressor cells; PAMP, pathogen-associated molecular pattern.

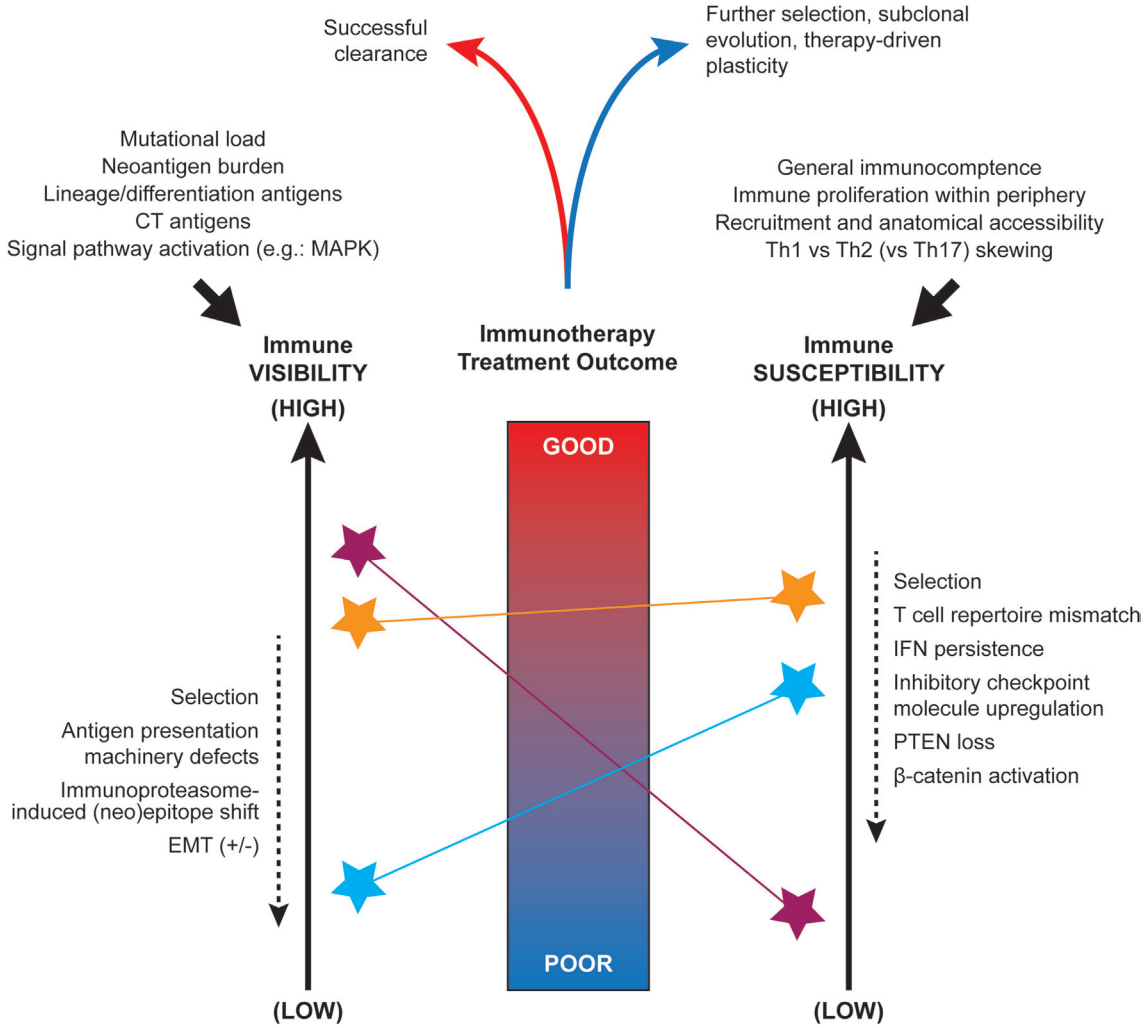

FIGURE 1 | Factors influencing the immune visibility and susceptibility of tumors. Nomogram-conceptualization of the competing influences of tumor immune visibility (*at left*) and the susceptibility of tumor cells to immune attack (*at right*). Due to underlying intra- and inter-tumoral heterogeneity, distinct tumor cell sub-clones or microenvironments (*denoted by colored stars*) may display a range of visibility and susceptibility characteristics that must be integrated when predicting the overall outcome of spontaneous or immunotherapy treatment-induced anti-tumor responses. The initial set-point of immunogenicity is influenced by several factors including somatic mutations, antigen expression, and signal pathway activity (*top left*). The anti-tumor immune set-point is similarly influenced by a number of systemic factors such as availability of immune cell populations for recruitment and Th1 skewing (*top right*). Multiple factors have been implicated as dynamic modulators of these visibility and susceptibility states (*dashed arrows at sides*).

results were broadly applicable to the sub-group of patients (42% of the overall cohort) with non-melanoma/non-NSCLC histologies. Interestingly, the relationship between higher mutational load and better treatment outcomes was not evident for patients who received combined anti-CTLA-4 and anti-PD-1 therapy. Further supporting the tumor mutational burden-response relationship are tumors with microsatellite instability and mismatch-repair deficiency, which demonstrate an increased sensitivity to checkpoint blockade likely to be related to an associated increase in mutational load and neoantigen burden (17, 18). Indeed, demonstration of microsatellite instability-high or mismatch-repair deficient tumors upon biomarker testing forms the basis for the first site-agnostic drug approval made by the FDA, for anti-PD-1 therapy. The practical limitations of measuring tumor mutational burden for use as a predictive biomarker before therapy have been significantly met by robust estimation of overall mutational load using data obtained from targeted next generation sequencing technologies that are now relatively widely accessible in the clinic (19, 20). In addition, cancer-gene panel mutational profiling by liquid biopsy represents a promising alternative mutational burden-related methodology for predicting immunotherapy response, as reported in an analysis of NSCLC patients enrolled in clinical trials of the anti-PD-L1 agent atezolizumab (21).

However, the relevance of a tumor cell's mutational repertoire to immune dynamics is moderated by additional factors that affect expression, processing, and intrinsic immunogenicity of any putative neoantigen. The complex processes involved in cleaving a peptide, loading it onto an MHC molecule, transporting it to

the cell surface, and ensuring its stability are essential to induce the antigenic T cell responses required for tumor clearance. Epitope production is influenced by the molecular chaperones and proteasomal machinery involved in protein processing; not all epitopes produced may be immunogenic, and a form of stochastic competition between display of immunogenic and non-immunogenic epitopes may ensue. The initially beneficial IFN-rich microenvironment of a T cell-inflamed tumor ultimately promotes mismatch between the neoepitope and T cell repertoires due to a shift from utilization of the constitutive proteasome to the immunoproteasome, thereby influencing tumor visibility (22). Furthermore, defects in β2-microglobulin expression can further impair antigen processing and display, affecting stable expression of MHC I molecules for their adequate surface expression and subsequent T cell recognition (23, 24). MHC class I haplotype loss or overall downregulation has been associated not only with altered tumor cell growth characteristics, but also facilitates evasion of immune surveillance (25). In melanoma, MHC class I internalization induced by BRAF V600E has also been described, suggesting another potential mechanism underlying the enhanced tumor visibility resulting from BRAF inhibitor therapy (9). Specific MHC class I loss has also been demonstrated in the evasion of T cell therapy for colorectal cancer (26). In addition, not all neoantigens bind MHC with high affinity, and the combinatorial match between neoantigen and MHC molecules expressed in the same cell determine how intrinsically immunogenic a neoantigen can be.

Studies of patient samples and *ex vivo* evidence strongly support the dominance of mutational neoantigens as targets for lymphocyte recognition of tumor, even in cancer types with lower overall mutational burden (10, 27–29). The importance of considering the available HLA sub-types and the T cell repertoire also present in the tumor, and their importance as major determinants of the tumor sub-clonal pruning that results from ongoing cycles of immune recognition, attack, and clearance, is now being appreciated (30). Computational methods exist to infer neoantigen expression and HLA binding characteristics from genomic and transcriptomic data (31, 32), and have been shown to act as a surrogate for treatment response in the context of checkpoint blockade immunotherapy in melanoma (10, 29). In fact, the sole presence of a more diverse array of HLA molecules (i.e., HLA heterozygosity), presumably linked to the ability to present a wider breadth of neoantigens, has recently been associated with increased survival in melanoma and lung cancer patients treated with immune checkpoint blockade (33). Knowledge of the mutational landscape of a tumor is thus of great importance to estimating the outcome of both targeted and immune therapies, however, measures of mutational and neoantigen burden alone do not predict immunotherapeutic outcome perfectly and results have been conflicting in separate cohorts (29, 30, 34). Similarly, though predictive approaches have been utilized to identify neoantigen candidates based on somatic mutations, these algorithms remain suboptimal, likely due to the numerous moderating factors described above (35). Accordingly, predictive approaches are now being paired with additional filters provided by proteasomal cleavage algorithms, as well as expression data to evaluate somatic mutations which are adequately expressed. A smaller number of neoantigen candidates can then be tested with autologous lymphocytes through molecular cloning of tandem minigenes comprising numerous expressed neoantigens (11).

TUMOR IMMUNE "SUSCEPTIBILITY"

A tumor's visibility to the immune system does not automatically imply its clearance, and numerous distinct factors can also influence its susceptibility to immune attack, which may be related to or completely independent of visibility.

In recent work, Chen and Mellman described the different immune infiltration profiles associated with response, which were classified as "inflamed," "immune-excluded," and "immune-desert" (36). Tumor immune susceptibility is inherently greater in patients of the "inflamed" type, where immune cells are present and capable of exerting their anti-tumor effects. Although immune visibility is critical to the establishment of an inflamed tumor microenvironment, the outcome of tumor inflammation can be influenced by a series of factors which build on a tumor's visibility, such as chemokines, pro-inflammatory cytokines, and effector T cell density and function. Conversely, immunosuppressive cytokines and the presence of pro-tumor immune inhibitory cell types, such as tumor-associated (M2) macrophages, regulatory T cells (Treg), and myeloid-derived suppressor cells (MDSCs) can lead to development of an immune-desert tumor microenvironment, clearly detrimental to response.

Recently, an extensive genome-scale *in vitro* CRISPR/Cas9 screen revealed genes involved in antigen presentation and IFN-signaling to be most relevant to the ability of CD8 T cells to kill melanoma cells (37). IFN-γ signaling defects have been repeatedly implicated in cancer immunotherapy failure, including copy-number losses of IFN-γ pathway genes (principally *IFNGR1/2*, *IRF1*, and *JAK2*) in patients failing to respond to anti-CTLA-4 therapy (38). Loss-of-function mutations in *JAK1* and *JAK2* have also been described in the tumors of melanoma patients with either primary (39) or secondary (24) resistance to anti-PD-1 therapy. It must be noted that while tumoral inflammation appears a common if not necessary component of the anti-cancer immune response (regardless of therapeutic agent used), persistent activation of IFN-driven inflammatory signals adaptively leads to upregulation of inhibitory checkpoint molecules on lymphocytes and generation of an immunosuppressive microenvironment (40, 41). Thus, optimal immunotherapeutic outcomes may require more complex sequencing and/or intermittent dosing strategies than have yet been studied in patients.

In keeping with the concept of immune-inflamed and immune-excluded or immune-desert phenotypes described by Chen and Mellman, microenvironmental characteristics affecting lymphocyte entry and trafficking are critical to the efficacy of immunotherapy. Baseline lymphocytic infiltrate, particularly CD8 T cell density, is predictive of response to checkpoint inhibitor monotherapy (42), with early on treatment biopsies being more highly predictive of response than at baseline (43). Such "snapshots" of the immune infiltrate represent the combination over time of factors affecting T cell recruitment and T cell exclusion, such as a tumor cell-intrinsic activation of β-catenin (44). In fact, in work by Spranger and colleagues, it was demonstrated that

the absence of tumor-derived β-catenin signaling allows production of CCL4, a chemokine which aids dendritic cell recruitment and thereby promotes T cell priming and anti-tumor responses (44). Furthermore, loss of expression of genes such as *PTEN* may influence the immune response through increased expression of immunosuppressive cytokines, such as VEGF and CCL2 (4). In fact, *PTEN* loss in melanoma patients was associated with progression on PD-1 blockade, possibly due to this mechanism, with CD8 T cell exclusion shown in regions of the tumor devoid of PTEN expression. Angiopoietic factors such as VEGF are frequently secreted by tumors and contribute to treatment failure (45). Pre-clinical models and translational studies of combined immune checkpoint blockade and anti-angiogenic agents suggest a potentially complex effect on tumor immunity, including beneficial effects on DC function and suppressive capacity of intratumoral MDSCs (46), enhanced anti-tumor humoral immunity (47), and increased lymphocyte trafficking and recruitment (47, 48).

Failure of spontaneous anti-tumor activity may largely be due to a dysfunctional "exhausted" T cell state associated with high expression of negative regulatory checkpoint molecules that are nonetheless amenable to treatment with modern checkpoint blockade immunotherapy (49). A more comprehensively inhibited T cell phenotype, typically with expression of numerous inhibitory checkpoint molecules including TIM-3, LAG-3, and others, may contribute to resistance to checkpoint inhibitor therapies in current clinical use (50). The presence of Treg as a key inhibitory factor on the anti-tumor response is, conversely, associated with poor response to checkpoint blockade.

COMPLEXITIES OF TUMOR HETEROGENEITY

That the majority of treatments for advanced cancers fail to produce curative outcomes is testament to the sheer diversity of cancer cell sub-populations present, limited in number only by the ability of our technologies to unravel their complexities at a molecular level. Heterogeneity of tumor cells, infiltrating immune cells, local vasculature, chemokine/cytokine gradients, and the underlying genetic basis for these variations are thus highly relevant to multiple aspects of immunotherapy efficacy. Tumoral heterogeneity influences both visibility and susceptibility of a tumor to immune attack, and has been described across cancer types (51–53).

Heterogeneity may arise from stochastic variation between cell sub-populations as cancer cells divide and accumulate mutations, or as a consequence of more plastic processes which shape cell state, gene expression, cellular function, and phenotype in response to prevailing selective processes over time or in different microenvironments (54–56). Heterogeneity may also arise as a direct consequence of sub-clonal immunoediting that occurs during both spontaneous and treatment-related anti-cancer immune responses, leading to non-uniform expression of target antigen (57) or essential antigen presentation machinery (25) across tumors.

The impact of tumor heterogeneity was recently highlighted in localized lung adenocarcinoma, demonstrating that a substantial proportion of tumor mutations are sub-clonal, i.e., restricted to regions of a tumor (58). This pattern extended to the neoantigens derived from these mutations, and patients with the highest proportion of sub-clonal neoantigens experienced shortened disease-free survival. Similar findings were seen when studying the T cell repertoire, where patients with the most heterogeneity in their T cell repertoire fared worst, highlighting the direct implications of genomic and immune heterogeneity on patient outcome (58).

Future treatment strategies will need to consider the effects of pre-existing tumoral heterogeneity as well as the adaptive treatment-induced changes that contribute to treatment failure. Furthermore, treatment strategies may also exert unique effects on tumor heterogeneity. In a recent melanoma study, prior therapy did not affect genomic inter-tumor heterogeneity whereas immune heterogeneity was more limited in patients previously treated with checkpoint blockade (52). As late stage patients become increasingly heavily pre-treated, the effects of these prior therapies on tumor heterogeneity will also need to be taken into consideration.

SYSTEMIC AND ENVIRONMENTAL INFLUENCES ON RESPONSE

Although undeniably important, the metabolic, vascular, and immune dynamics active in the tumor microenvironment are only some of the contributing factors. It is now quite clear that overall host immunity as well as environmental influences (**Figure 2**) can shape therapeutic responses (59), and these factors will be discussed herein.

INFLUENCE OF OVERALL IMMUNE FITNESS

Effective anti-tumor immune responses require exposure of the tumor microenvironment to a wide network of innate and adaptive immune effector populations recruited from the systemic circulation. These cells must recognize and target tumor cells for elimination, based on the visibility factors described previously, in a critical process termed "immunosurveillance" (60). Three core phases of immunosurveillance have been described, spanning elimination of susceptible tumor cells through equilibrium (in which visibility-susceptibility mismatch or selective pruning of the most immune-susceptible tumor sub-clones leads to an anti-cancer stalemate), to escape (in which selection of low-visibility low-susceptibility tumor cells facilitates renewed tumor progression) (61). These dynamic phases occur spontaneously, but are undoubtedly influenced by exposure to immunotherapies, as shown in a parallel genomic and immune analysis of tumors from patients with advanced melanoma who received treatment with the anti-PD-1 agent nivolumab (30). In this study, clear patterns of mutational contraction and T cell clonal expansion occurred in what appeared to be a refocusing of the immune-cancer interaction and elimination of neoantigen-expressing sub-clones in patients who responded to therapy. While the specific cellular interactions that characterize each phase of immunosurveillance occur within the tumor microenvironment, the immune cells

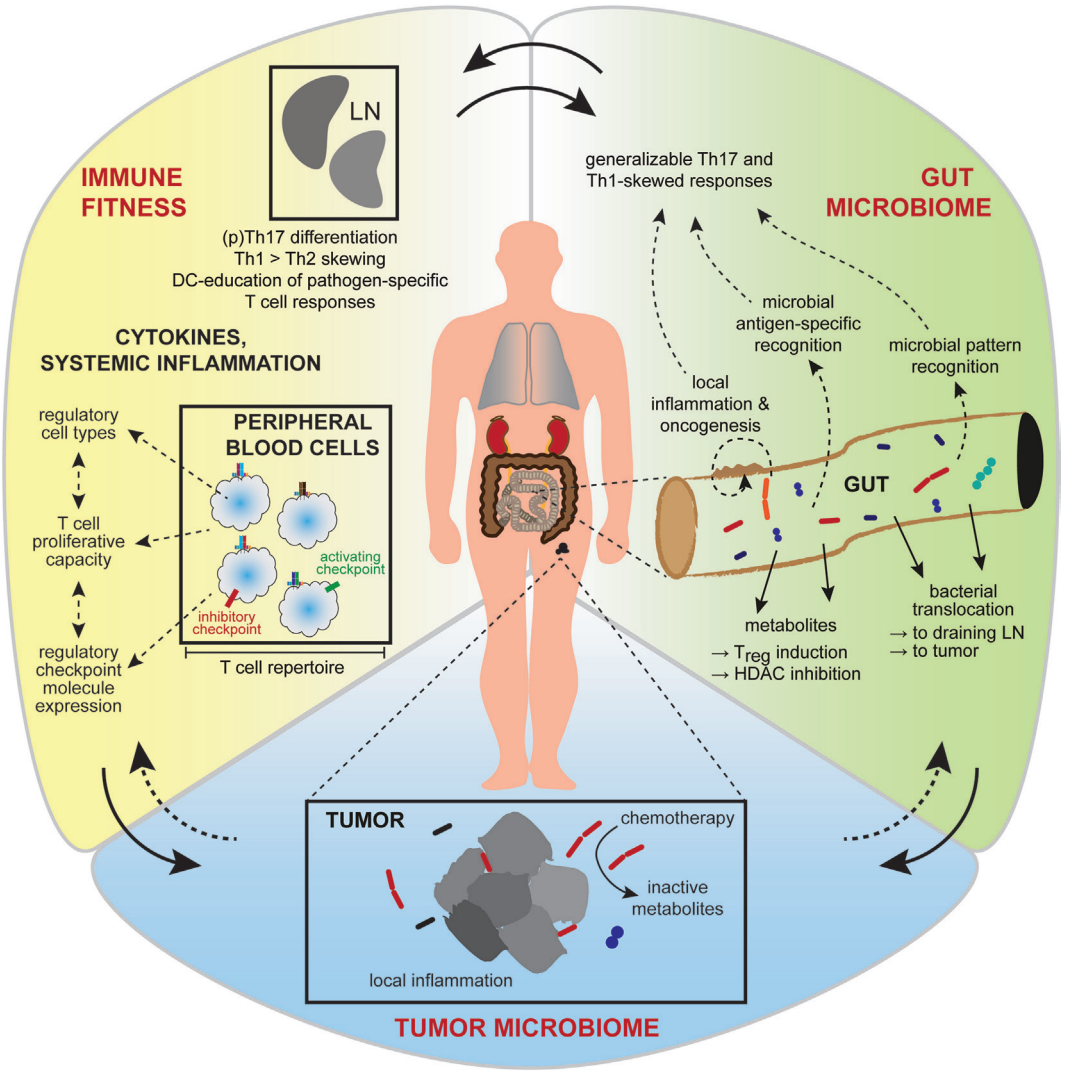

FIGURE 2 | Interactions between the gut and intra-tumoral microbiota and the systemic immunity affect treatment outcome. Broader influences on tumor growth and responsiveness to immunotherapy are now realized, including contributions from the gut microbiome, the tumor microbiome, and systemic factors affecting general immune fitness. Interactions between these conceptual compartments are complex and incompletely understood. Gut microbiota (*green*) have diverse metabolic and antigen or pattern-molecule immunogenic effects on local gut inflammation, the effects of which contribute to local carcinogenesis or can become generalized to affect cancer growth and immunity at distant body sites. Favorable immune fitness (*yellow*), characterized by overall skewing toward cellular immune responses, a permissive cytokine milieu and activation-biased representation of anti-tumor, and regulatory immune cell types, is important for anti-tumor responses. The intra-tumoral microbiome (*blue*) is of emerging significance, having local inflammatory and metabolic effects that influence therapeutic sensitivity.

involved are sensitively dependent on adequate supply from the systemic compartment, and a prevailing immune phenotype conducive to anti-tumor activity (which may be therapeutically modifiable).

Recent work by Spitzer and colleagues provides key evidence supporting the direct relevance of systemic immune function to cancer immunotherapy (62). In this study, extensive high dimensional immune profiling using mass cytometry was performed in a MMTV-PyMT breast cancer mouse model to explore the dynamics of multiple immune cell populations in response to either effective, or ineffective, anti-cancer immunotherapy, in multiple body compartments. Many immune subsets were found to proliferate within the tumor microenvironment during the initiation of immune responses. Importantly, however, the proliferation of multiple immune cell populations, including B cells, NK cells, dendritic cells, and effector/memory T cells, during active tumor rejection was primarily sustained in secondary lymphoid organs and not the intra-tumoral compartment, indicating the significance of the systemic compartment to maintenance of effective anti-cancer immune responses (62).

Myeloid-derived suppressor cells have garnered much interest in recent years for their immune-suppressive capabilities across various cancer types. MDSCs are a phenotypically heterogeneous group of cells comprised of immature myeloid cells, and broadly divided into monocytic and granulocytic sub-types (63). MDSCs have a potent ability to suppress T cell responses through numerous

specific mechanisms in lymphoid organs, such as production of indoleamine 2,3 dioxygenase or arginase-1, which locally deplete crucial amino acids such as tryptophan and arginine, thereby rendering T cells functionally anergic. MDSCs may also inhibit T cell responses through production of immunosuppressive cytokines including TGF-β and IL-10, or generation of reactive oxygen species (ROS) (64). Because of this ability to inhibit T cell responses and promote tumor development, MDSCs have been suggested to be a key therapeutic target in cancer. Though MDSCs are present in the tumor microenvironment and tend to increase with cancer development, their characterization in the periphery has become an area of intense investigation, including studies of their relationship to clinicopathologic attributes and patient outcome. Overall, MDSCs are generally more abundant in the peripheral blood of cancer patients compared with healthy subjects. However, their frequencies also increase from early stage to late stage disease and with higher histological grade. These trends have been observed in numerous histologies such as renal cell cancer (65), colorectal carcinoma (66), melanoma (67), as well as gastric cancer (68). Interestingly, higher frequencies of MDSCs in the periphery have also been predictive of patient relapse in breast cancer (69), melanoma (67, 70), differentiated thyroid cancer (71), glioblastoma (72), head and neck squamous cell carcinoma (73), pancreatic cancer (74), prostate cancer (75), and renal cell carcinoma (76). Their increased frequencies in the circulation have also been tied to the development of metastases in melanoma and colorectal cancer (66). Together, the somewhat graded relationship between MDSC abundance and cancer progression, including metastasis, suggests that circulating MDSCs at least reflect the immunosuppressive status of the tumor microenvironment, and likely fulfill a more direct role in the development of a systemically suppressed immune response. Unfortunately, inconsistency in the classification and functional characterization of MDSCs has limited our ability to accurately enumerate and isolate them, highlighting some of the challenges in translating the therapeutic targeting of MDSCs to the clinic.

Despite the importance of immune fitness in therapeutic response, our ability to assess anti-tumor immune responses in the peripheral circulation remains somewhat limited to date and uncertainties remain regarding the contributions made by immune populations at different times, and at different sites. Insofar as upregulation of inhibitory checkpoint molecules is associated with prior antigen exposure and activation, PD-1 expression on circulating T cells was found to enrich for neoantigen-specific T cells in the peripheral blood of melanoma patients (77). However, data showing that higher clonality of the T cell repertoire resident within tumors before therapy was associated with better outcome to PD-1 blockade (34, 42) suggests that much of the relevant effector immune population is already intra-tumoral even before therapy. Thus, the proportion of tumor-specific T cells present in the general circulation at any instant may be relatively low, limiting what can be concluded about therapeutic response from examination of the periphery in isolation, with currently available tools. Looking even more generally, the broad functional status of the adaptive immune system, defined as Th1-, Th17-, or Th2-skewing of circulating immune cells, may be more reliably associated with the prevalent immunophenotype of tumor-infiltrating lymphocytes (TIL), reflecting the likelihood of effective cytotoxic rather than tolerogenic cellular immune outcomes.

THE GUT MICROBIOME MODULATES CANCER DEVELOPMENT, IMMUNITY AND RESPONSE

Systemic immunity is shaped by interactions with our environment, and there is now clear evidence that the gut microbiome contributes to the establishment and maintenance of systemic immune tone (78, 79). As the largest commensal microbial community (80, 81), the gut microflora has been extensively studied as a trigger for local inflammation in non-malignant conditions such as inflammatory bowel disease (82, 83). Overall, these microbes present a significant challenge to the host's immune defenses, which must regulate tolerance to beneficial microbes while guarding against harmful pathogens. Moreover, recent evidence suggests that the gut microbiome plays a significant role in cancer development and response to cancer therapy as discussed in the following sections.

Gut microbiota represent a double-edged sword that can promote or inhibit cancer development, with both individual bacterial taxa and overall bacterial dysbiosis implicated in oncogenic initiation and progression. *Helicobacter pylori*–particularly those containing the virulence factor cagA–have been extensively characterized as an oncogenic initiator, particularly in gastric adenocarcinoma (84). In a murine model, a potentially pathogenic enterotoxigenic *Bacteroides fragilis*-induced a STAT3-dependent, Th17-mediated colitis associated with colonic tumor formation in pre-disposed mice; colitis and tumor formation were prevented by administration of blocking antibodies to IL-17 and IL-23 (85). *Escherichia coli* strains may harbor the polyketide synthase genomic island (pks), which encodes a genotoxin called colibactin that induces DNA damage in murine colonocytes. Furthermore, *E. coli* that are pks+ are more frequently identified in colon cancer patients compared with healthy controls (86, 87). On the other hand, bacteria can also be tumor suppressive as demonstrated by *Butyrivibrio fibrisolvens*, which resulted in colorectal tumor attenuation by producing copious amounts of butyrate in the presence of a high-fiber diet in a rat-azoxymethane model. Importantly, population-based metagenomic analyses in colon cancer patients have also revealed differential enrichment of bacterial taxa (especially *Fusobacterium)* in colorectal tumors when compared with controls (88–91).

As opposed to microbial taxa that drive oncogenesis in a highly penetrant manner, the relationship between dysbiosis and cancer is more complex and multifactorial. Dysbiosis is influenced by several extraneous factors including diet, antibiotic use, and smoking (92). In pre-clinical models of colon carcinoma, both germ-free status and antibiotic treatment have been found to be associated with reduced incidence of tumors (93–96). A dysbiotic microbiota can also influence cancer development at distant sites such as the liver (97) and pancreas through pro-inflammatory microbe-associated molecular patterns and metabolites released into the systemic circulation (98).

Several murine studies have established a clear requirement for a diverse and intact intestinal microbiota to achieve optimal response to distinct cancer treatment modalities through effects on both the innate and adaptive arms of the immune system. The gut microbiome is now implicated in modulating responses across a wide range of cancer therapies, including intra-tumoral therapy (99), chemotherapy (99, 100), and immune checkpoint blockade (101–103). Attempts to define the mechanisms underlying microbial associations with the efficacy of cancer therapies have revealed both microbe-specific and microbe-agnostic influences (**Figure 2**). For instance, administration of LPS alone, in the presence of its cognate pattern recognition receptor, largely restored the efficacy of intralesional immunotherapy administered to either germ-free or antibiotic-ablated mice implanted with melanoma or colon cancer (99). In these models, systemic microbial priming of myeloid lineage cells appeared essential to their subsequent intra-tumoral accumulation and production of pro-inflammatory and chemotactic cytokines or ROS, without which an effective secondary T cell infiltrate could not be recruited. Interestingly, responses to chemotherapy may be facilitated by disruption of the integrity of the gut epithelial barrier with subsequent bacterial translocation of *Enterococcus hirae* and *Lactobacillus johnsonii* and priming of immune responses demonstrated in a murine model (100). Bacteria such as *E. hirae* and *Barnesiella intestinihominis* can also affect immune responses directly at the tumor site, with depletion of intra-tumoral Treg and accumulation of γδT cells, respectively (104).

The initial demonstration that several bacterial taxa were associated with response to immune checkpoint blockade came from murine studies, which implicated *Bacteroides thetaiotaomicron* and *B. fragilis* in the case of CTLA-4 blockade (101) and *Bifidobacterium*, in the case of PD-L1 blockade (103). These studies were followed by several analyses of patient cohorts that confirm a clear role for the gut microbiome in modulating responses to immune checkpoint blockade across cancer types (102, 105). A reciprocal relationship between the mid-level taxa Clostridiales (favorable) and Bacteroidales (unfavorable) and likelihood of response were recently reported in a large cohort of melanoma patients (102). Importantly, fecal microbial transplantation into germ-free mice using stool from responding patients resulted in enhanced tumor control when compared with donor stool from non-responding patients. Other population-level studies have also reported similar findings with regards to over-representation of the *Faecalibacterium* genus of the Clostridiales order in pre-treatment samples of responders to anti-CTLA-4 and combination anti-CTLA-4 plus anti-PD-1 immunotherapies (106, 107). In another study, *Bifidobacterium* was also found to be enriched in melanoma patients who were responding positively to anti-PD-1 therapy, analogous to earlier results implicating this taxon in a murine model of PD-L1 (105).

Interestingly, the bacterial taxa identified in these human studies differ somewhat from those identified in murine experiments and even across the patient cohorts, suggesting the need for additional studies to address the significance of geographical and other variables influencing microbiome composition and response, and to confirm unifying taxa or functionalities to take forward to clinical development. These studies also imply that administration of single bacterial species may not be reliably effective in modulating responses to immunotherapy.

Our understanding of the influence of the gut microbiome on immunity and therapeutic responses is evolving, and it is evident that micro-organisms may share functionalities that convey immunomodulatory properties that are not immediately evident from taxonomic discovery. Indeed, several investigators have demonstrated a key integrative role of microbial metabolites such as short chain fatty acids (SCFAs) produced by microbial fermentation of undigested complex carbohydrates in mediating the effect of commensal bacteria on immune tone (108, 109). In fact, a significant proportion of metabolites found in human plasma are microbiome-derived and can affect immune cells by influencing their metabolic circuits or engaging with metabolite-specific receptors. SCFAs act as signaling molecules, by inhibiting histone deacetylases (HDAC). Their action on lymphocytes and neutrophils is mediated *via* the blockade of NF-κB and the subsequent downstream production of pro-inflammatory TNF (110). Importantly, SCFAs promote homeostasis by regulating the size and function of the colonic FoxP3+ Treg pool in an HDAC-dependent manner (108, 109). SCFAs can also exert a regulatory effect by signaling through G protein-coupled receptors, resulting in the limitation of neutrophil chemotaxis (111) and expansion of Treg function (112). Therefore, it is also not surprising that these metabolites can directly affect cancer cells by impacting immunosurveillance (113).

At the population-level, numerous studies have identified high diversity of the gut microbiota as being associated with improved survival outcomes in allogeneic hematopoietic stem cell transplantation patients, together with relatively lower rates of graft-vs-host disease (114, 115). Consistent with these findings, a beneficial effect was also reported in the context of anti-PD-1 therapy in melanoma patients, wherein high diversity of the gut microbiome at baseline was found to be associated with significantly improved progression-free survival rates (102). Importantly, a similar provocative observation was also made in the context of lung, renal, and bladder cancer patients where disruption of microbial diversity of the gut was found to have a detrimental effect on the efficacy of immune checkpoint blockade (116). In these studies, the authors demonstrated that antibiotic usage shortly before, during or shortly after the initiation of treatment with immune checkpoint blockade was associated with significantly reduced progression-free and overall survival. In addition, metagenomic sequencing implicated the species *Akkermansia muciniphila* to be abundant in responders to anti-PD-1, and capable of restoring its efficacy in germ-free mice transplanted with non-responder patient stool (116).

THE TUMOR MICROBIOME AND RESPONSE

In addition to the gut microbiome, bacteria within tumors themselves may influence cancer development as well as therapeutic responses. This has been studied most extensively in colorectal cancer, where certain bacterial taxa such as *Fusobacterium nucleatum* and *Streptococcus bovis* have been associated with

primary tumors (88, 117, 118) as well as in metastatic sites (119). *Fusobacterium*, specifically *F. nucleatum*, is enriched in colorectal carcinomas relative to normal colonic tissues (88, 89). The abundance of *F. nucleatum* within colon cancer tissues inversely correlates with recurrence-free survival, and appears adversely prognostic, comparable to increasing AJCC stage (120). Profiling the fecal microbiota or screening for known microbial markers associated with colon carcinogenesis such as the genotoxin colibactin and its encoding genotoxin cluster pks found in oncogenic *E. coli*, may provide a novel strategy for population screening, and may additionally provide clues as to the underlying mechanisms driving such microbial associations with the accumulated genetic damage that characterizes malignancy (121, 122). In this regard, detailed study of the molecular products of the pks island confirm that colibactin directly damages DNA (122) and mediates pks+ *E. coli* promotion of tumor formation in a murine $Apc^{Min/+}$; $Il10^{-/-}$ model (123). While chronic DNA damage is undesirable, it is clear that a high mutational burden may in fact be beneficial in patients receiving checkpoint blockade immunotherapy, thus the relevance of such microbial genotoxicity on contemporary treatment outcomes warrants particular study. Furthermore, certain pathogenic taxa are linked to enrichment of inflammatory and DNA damage-response pathways in tumor transcriptomes, together with a distinct methylation and microsatellite instability profile (124). In light of these data, it is probable that the genomic and immune characteristics of intestinal tumors may be sensitively linked to the geographic microbial niches in which they arise.

Importantly, therefore, bacterial-associated molecular alterations in tumors span genomic, epigenetic and immune domains with the immunomodulatory effects of tumor-associated microbes appearing equally as diverse as the observed genomic and biochemical effects. In the context of *Fusobacterium*, direct molecular interaction between the bacterial Fap2 protein and TIGIT present on human NK cells contributes to tumor immune evasion; this interaction was shown to inhibit TIGIT-mediated activation of NK cell killing of colon adenocarcinoma cells, and was more generally suppressive of TIL (NK and T cells) killing using patient-derived matched TIL and tumor cells from melanoma patients (125). Furthermore, *F. nucleatum* appears to promote colorectal cancer cell chemoresistance to select agents in a complex multi-step sequence of molecular changes involving TLR4 and MYD88 activation and culminating in activation of autophagy (120). Colonic Th17-responses represent a common and partially unifying feature of many microbiota-associated local and systemic inflammatory states and have been associated with poor-responses to anti-cancer therapies (100). As previously noted, colon cancer formation associated with enterotoxigenic *B. fragilis* has been shown to involve Stat3-driven colitis and induction of a Th17 response that was prevented by IL-17 and IL-23 blockade in mouse models (85). *B. fragilis*-induced Th17-driven tumorigenesis involves the promotion of a suppressive myeloid environment characterized by monocytic MDSCs and consequently suppressed T cell proliferation (126). While Th17 skewing may thus form a major contribution to carcinogenesis and influence systemic chemotherapeutic responses in non-intestinal tumor models, these same mechanisms, and the specific effect of *B. fragilis*, were required for the efficacy of anti-CTLA-4 checkpoint blockade immunotherapy (101). This emphasizes the new complexity that has arisen with the advent of checkpoint molecule-targeted immunotherapies, for which the distinction between "favorable" and "unfavorable" microbiota is potentially reversed depending on whether the context at hand is one of cancer *development*, or immunotherapy-based *treatment* of an already-established cancer.

In addition to their roles in carcinogenesis and immunomodulation, intra-tumoral bacteria may also modulate responses to cytotoxic cancer therapy. *Mycoplasma hyorhinis*, which was associated with fibroblasts in the tumor microenvironment in pancreatic ductal adenocarcinoma tumors, was found to be able to direct drug metabolism and diminish the efficacy of gemcitabine. Further analyses of bacterial genes implicated the enzyme cytidine deaminase contained in the Gammaproteobacteria class to be necessary and sufficient to mediate conversion of gemcitabine to its inactive form, by expression of the long isoform of the enzyme cytidine deaminase, in a colon cancer murine model (127). Depletion of bacteria within the tumor and a robust anticancer response to gemcitabine were noted in tumor-bearing mice treated with the combination of gemcitabine and ciprofloxacin delivered directly to the tumor site. A high prevalence of Gammaproteobacteria was subsequently identified in pancreatic adenocarcinoma samples from patients, and retained the ability to confer gemcitabine resistance after *ex vivo* co-culture with colon cancer cell lines (127). How these intra-tumoral bacteria interact with infiltrating immune cells has not been completely elucidated.

Direct spatial microbe-tumor interactions are not only relevant to gastrointestinal cancers. Recent analyses of the microbiota present in breast cancer tissue compared with normal breast tissue revealed distinct microbial communities, driven by a lower abundance of *Methylobacterium* in cancerous tissues (128). The authors performed a parallel comparison of microbiota present at distant sites, with the provocative finding that urinary microbiota were also distinct between cancer patients and controls, even after correction for menopausal status. The mechanisms through which bacteria induce carcinogenesis may include induction of inflammation (85), altered cell signaling (129) and inhibition of T cell and natural killer cell responses (125), however the precise role of intra-tumoral bacteria in carcinogenesis across tumor types remains incompletely elucidated.

CONCLUSION

Our future conceptualization of what matters for good outcomes to cancer immunotherapy requires a thoroughly integrated understanding of what contributes to cancer formation and immune evasion in the first place. Tumor mutational load provides an instructive example; while a high tumor mutational load is clearly important for response to checkpoint blockade immunotherapy, it still lacks adequate negative predictive value to be trusted in the clinic, and performs poorly for combination checkpoint blockade. If highly mutated tumors were truly (simplistically) vulnerable to immune clearance, it should be considered remarkable that they are observed at all. More likely, the snapshot measurement of mutational load is limited by numerous other factors, such as

the neoantigen characteristics of the available pool of mutations, genomic methylation and transcriptome patterns, the intrinsic immunomodulatory effects of the tumor over time, and the extrinsic immunomodulatory effects of the patient's environment and microbiota. Recent research highlights the critical need to model these interactions systematically and dynamically, taking adaptive evolution into consideration rather than relying on static measurements at single moments in time.

The importance of integrated biomarker models in cancer is highlighted well by the rapidly expanding interest in the study of the commensal microbiota in the context of cancer development and progression. Microbes influence the response to traditional cytotoxic agents through a diverse combination of effects on cellular metabolism, local pharmacokinetics, and could plausibly affect bioavailability of orally administered agents which are increasingly common in the era of targeted therapy, although this remains to be studied. Perhaps of greater significance, local interactions between specific bacteria and host tissues contribute to locoregional inflammation and carcinogenesis. Molecular interactions with pattern receptors (e.g., TLR4 and downstream MYD88), and immunosuppressive signals mediated by engagement with cell surface inhibitory molecules (e.g., TIGIT) or elaboration of suppressive cytokines (e.g., VEGF and CCL2) result in immune evasion that likely contributes to ineffective immunosurveillance of nascent, developing and established neoplasia.

It is not yet fully know to what extent the immunomodulatory effects of cancer-associated microbes may influence cancer immunotherapies, however, it is highly likely that these effects will not be consistent. For instance, immunosuppressive mechanisms such as MDSC-induction by *B. fragilis* suggest a negative impact on anticancer immunotherapy treatment response, however, the often simultaneous induction of Th17- and Th1-biased systemic immunity by the same organism(s) appears beneficial to checkpoint blockade immunotherapy response. This apparent contradiction will likely prove to be a complex and difficult issue to resolve as the field progresses, particularly as it relates to how best–or how safely–to manipulate the gut microbial composition to optimize treatment outcomes, toxicity, and long-term general health.

As more mechanisms are identified by which microbes directly influence tumor genomics, it will be important to evaluate whether the commonly observed EMT-like processes that accompany tumor progression involve a feed-forward loop precipitated by tumor-induced dysbiosis and subsequent microbe-directed epigenetic reprogramming of tumor cells. Another important issue that urgently requires attention is the relative significance of *individual* microbial taxa as opposed to unifying functional or metabolic characteristics of *multiple* taxa, in cancer development and treatment response. Metabolomic and whole genome shotgun sequencing studies are underway to address this, and will be highly relevant to the identification of the most readily targeted predisposing, permissive and perpetuating factors in cancer microbiology; it may be that critical intermediary metabolites or activated metabolic pathways prove to be most amenable to therapeutic modification. Further study is also required to integrate the significance of the microbiota with what is already known about the influence of lifestyle factors on cancer outcomes. Consideration should be made to the impact of duration, mode and type of microbiota "exposure" when integrating the microbiome into the new cancer-immunity model. As we continue to develop a deeper understanding of the myriad factors impacting cancer immunotherapy response, we will undoubtedly develop and refine new therapeutic strategies for maximal patient benefit.

AUTHOR CONTRIBUTIONS

All authors conceived, wrote, reviewed, and approved the final manuscript.

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Autoantibodies May Predict Immune-Related Toxicity: Results from a Phase I Study of Intralesional *Bacillus Calmette–Guérin* followed by Ipilimumab in Patients with Advanced Metastatic Melanoma

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Immune checkpoint inhibitors (ICIs) have revolutionized the treatment of advanced melanoma. The first ICI to demonstrate clinical benefit, ipilimumab, targets cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4); however, the long-term overall survival is just 22%. More than 40 years ago intralesional (IL) *bacillus Calmette–Guérin* (BCG), a living attenuated strain of *Mycobacterium bovis,* was found to induce tumor regression by stimulating cell-mediated immunity following a localized and self-limiting infection. We evaluated these two immune stimulants in combination with melanoma with the aim of developing a more effective immunotherapy and to assess toxicity. In this phase I study, patients with histologically confirmed stage III/IV metastatic melanoma received IL BCG injection followed by up to four cycles of intravenous ipilimumab (anti-CTLA-4) (ClinicalTrials.gov number NCT01838200). The trial was discontinued following treatment of the first five patients as the two patients receiving the escalation dose of BCG developed high-grade immune-related adverse events (irAEs) typical of ipilimumab monotherapy. These irAEs were characterized in both patients by profound increases in the repertoire of autoantibodies directed against both self- and cancer antigens. Interestingly, the induced autoantibodies were detected at time points that preceded the development of symptomatic toxicity. There was no overlap in the antigen specificity between patients and no evidence of clinical responses. Efforts to increase response rates through the use of novel immunotherapeutic combinations may be associated with higher rates of irAEs, thus the imperative to identify biomarkers of toxicity remains strong. While the small patient numbers in this trial do not allow for any conclusive evidence of predictive biomarkers, the observed changes warrant further examination of autoantibody repertoires in larger patient cohorts at risk of developing irAEs during their course of treatment. In summary, dose escalation of IL BCG

followed by ipilimumab therapy was not well tolerated in advanced melanoma patients and showed no evidence of clinical benefit. Measuring autoantibody responses may provide early means for identifying patients at risk from developing severe irAEs during cancer immunotherapy.

Keywords: melanoma, *bacillus Calmette–Guerin*, ipilimumab, immune-related adverse events, protein microarrays

INTRODUCTION

The treatment of metastatic melanoma is rapidly evolving with the approval of a number of targeted therapies and immune checkpoint inhibitors (ICIs) in a short period of time. Despite significant improvement in outcomes, the median overall survival of patients with advanced disease remains poor (1). Ipilimumab, a fully humanized IgG1 monoclonal antibody, is the first ICI to show a survival benefit in advanced melanoma in treatment-naïve and pretreated patients (2). Ipilimumab competitively binds to cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) more efficiently than B7 molecules found on antigen-presenting cells, preserving CD28 signaling to potentiate antitumor T-cell responses. Side effects from ipilimumab, called immune-related adverse events (irAEs), are typically inflammatory in nature and may relate to the activation of the immune system against self-antigens (3). High-grade 3 or 4 irAEs occur in 10–15% of patients: primarily colitis, diarrhea, rash, hepatotoxicity, and endocrinopathies (4, 5).

Bacillus Calmette–Guérin (BCG) is a living attenuated strain of *Mycobacterium bovis* that stimulates cell-mediated immunity by producing a localized and self-limiting infection. It has been shown to have antitumor activity in several clinical studies (6–9). The exact mechanism of action is not well known, but it is probable that BCG invokes a local inflammatory response involving a variety of both innate and adaptive immune effector cells (10). Intravesical immunotherapy with BCG has been established as the most effective adjuvant treatment for preventing local recurrences and tumor progression following transurethral resection of nonmuscle invasive bladder cancer. A large number of clinical trials have established a major role for BCG immunotherapy in urological oncology (11–13).

Intralesional (IL) BCG can be effective in inducing the regression of cutaneous metastatic melanoma (7, 9, 10, 14, 15). Inflammation and ulceration occurred in most cases, and subsequent regression of the injected lesion was commonly observed. In fewer than 10% of patients receiving IL BCG, regression of noninjected lesions was seen, and occasional long-term disease-free survival has been reported (15, 16), likely due to persistent T-cell immunity. Side effects were dose-dependent and included largely constitutional flu-like symptoms such as fever and myalgia, generally lasting 8–9 weeks that could be stopped with isoniazid (17).

The safety and efficacy of BCG given in combination with melanoma vaccines were evaluated in several phase I, II and III clinical trials (9, 18–20). The multicenter phase III randomized studies of BCG plus a polyvalent melanoma vaccine (CancerVax) versus BCG plus a placebo as a postsurgical treatment for stage III or IV melanoma (MMAIT-III and MMAIT-IV trials) were stopped when interim analyses demonstrated that it was unlikely that the vaccine would provide significant evidence of a survival benefit. Nevertheless, excellent survival was seen for the entire study population with 42% of stage IV and 63% of stage III patients projected to be alive at 5 years (21). This high survival may have been due to selection bias or BCG, which may have acted as an active immunotherapeutic agent at the administered dose.

Despite the success of ICI, a significant proportion of patients either does not respond to treatment or becomes resistant after initial response. This failure of therapy may result from a variety of mechanisms, such as immune ignorance, a hostile tumor environment, alternative immune checkpoint-independent regulatory mechanisms, inadequate antigenicity, or antigen downregulation (22). Strategies that induce a favorable inflammatory tumor microenvironment prior to, or at the time of, ICI have the potential to increase the effectiveness of anticancer immune therapies.

In this study, we evaluated the safety, clinical efficacy, and immunogenicity of IL BCG followed by ipilimumab (supported by the Ludwig Institute for Cancer Research and by Bristol-Myers Squibb; ClinicalTrials.gov number NCT01838200/LUD2012-003). Given the possibility of ipilimumab to potentiate the inflammatory effects of BCG, particular attention was paid to the evaluation of local and systemic inflammatory toxicities.

Protein microarrays have been widely used to detect and quantify the presence of autoantibodies in a variety of autoimmune diseases (23). Since patients treated with immunotherapy develop irAEs that resemble autoimmune disease, we further investigated the autoantibody repertoire of all recruited patients to characterize their serological responses as part of our broader immune-monitoring approach.

MATERIALS AND METHODS

Patients

Patients of good performance status (Eastern Cooperative Oncology Group score 0–1) with histologically confirmed unresectable stage III or stage IV melanoma were enrolled in the study. The major inclusion criteria included the presence of at least one cutaneous or subcutaneous metastatic lesion amenable to IL therapy. Key exclusion criteria included symptomatic or active cerebral metastases requiring corticosteroids, prior history of tuberculosis, hypersensitivity to BCG or contraindication to the use of isoniazid, autoimmune disease, immunodeficiency disease, or the use of immunosuppressive therapy.

Study Design

This was a single-site, open-label phase I, dose-escalation study. Because of the potential for ipilimumab to amplify inflammatory responses mediated by BCG, a variety of safety precautions were taken; eligible patients had a skin test for tuberculin reactivity with purified protein derivative and were enrolled in one of two cohorts, depending on the size of the induration. Patients with an induration of <10 mm in diameter were enrolled in cohort 1 which utilized a 3 + 3 dose-escalation design. Patients with a reaction of ≥10 mm were enrolled in cohort 2. Enrollment of the first three patients was staggered by 3 weeks; subsequent patients were enrolled without delay. Patients enrolled in cohort 1, group 1, received 200 µl BCG (day 1, D1) containing $0.16–0.64 \times 10^6$ cfu, and patients in groups 2 and 3 received 200 µl BCG containing $0.80–3.20 \times 10^6$ cfu and $4.00–16.00 \times 10^6$ cfu, respectively. All patients enrolled in cohort 2 received 200 µl BCG containing $0.16–0.64 \times 10^6$ cfu BCG. To ensure that no active BCG infection was present at the time of ipilimumab administration, oral isoniazid of 300 mg/day was commenced on D29 and continued for 4 weeks in all patients. Ipilimumab was administered intravenously on D36 at a dose of 3 mg/kg every 3 weeks for a total of four doses. Patients with responding or a stable disease by RECIST v.1.1 or immune-related response criteria (irRC) were scheduled to receive ipilimumab as maintenance therapy administered at a 12-week interval for a further four doses (**Figure 1**).

This study was conducted at Austin Health, Heidelberg, Australia, and was approved by the institution's human research ethics committee. Written informed consent was obtained from all patients, and all methods were performed in accordance with the protocol-specified guidelines and regulations.

Dose-Limiting Toxicity (DLT)

Dose-limiting toxicity (DLT) was defined as ≥grade 3 skin reaction at the injection site or ≥grade 3 toxicity associated with BCG administration. All toxicities were graded as per the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The protocol included provisions for BCG dose adjustments in the event of grade 3 or 4 toxicities including local skin reactions. DLT of ipilimumab was defined as any toxicity that required dosing modifications in accordance with the recommendations in the product information, or ≥grade 3 hematologic or nonhematologic toxicity.

Safety Assessments: Screening, Baseline, and Follow-up

Safety assessments were carried out at patient enrollment, D1, D29, D36, D43, D50, D57, D78, D99, D113, D141, and D204. Target skin lesions were monitored with clinical photography at D1, D36, D113, D141, and D204. Plasma and peripheral blood mononuclear cells were collected at D1, D36, D43, D57, D78, D113, D141, and D204.

Response Assessment

Tumors were assessed clinically and by contrast-enhanced (CT) scans (brain, neck, chest, abdomen, and pelvis) at patient enrollment, D36, D113, D141, and D204. Tumor response and progression were determined in accordance with RECIST v1.1 criteria (24) and irRC (25).

Autoantibody Profiling Using the Immunome™ Protein Arrays

A total of 15 plasma samples were assayed using the Immunome™ protein array (Sengenics Corporation, Singapore) as previously described (26). These samples were selected to assess the seromic profile associated with the period of BCG treatment and that of ipilimumab treatment separately. The array contains quadruplicate spots of 1,627 full-length, correctly folded, and fully functional immobilized self- and cancer proteins. These include cancer antigens [mainly cancer-testis antigens (CTAs)], transcription factors, kinases, signaling proteins, and others (Table S1 in Supplementary Material). Given the expected reactivity toward self-antigens, 19 healthy individual samples were independently assayed as controls. Raw data were processed and normalized using a robust pipeline that has been previously described (27) (Supplementary Methods). Statistical analysis (GraphPad Prism and Microsoft Excel) and data-clustering methods [Multiple experiment Viewer (MeV)] were then applied to the resulting data and visualized using above significance threshold counts, box plots, comparative size-proportional pie charts, dendrogram, and heat maps.

RESULTS

Baseline Patient Characteristics

Between April 2014 and June 2015, five patients were enrolled in the study. Cohort 1 included patients 1–3 in group 1 who received a $0.16–0.64 \times 10^6$ cfu BCG dose and patients 4 and 5 in group 2 who received a $0.80–3.20 \times 10^6$ cfu BCG dose. No patients were enrolled in planned group 3 or cohort 2. The mean age of patients was 59 years (range 43–71 years), with three males (60%). Two patients had a *BRAF* mutation and one patient a *NRAS* mutation. Two patients had prior immunotherapy with an NY-ESO-1

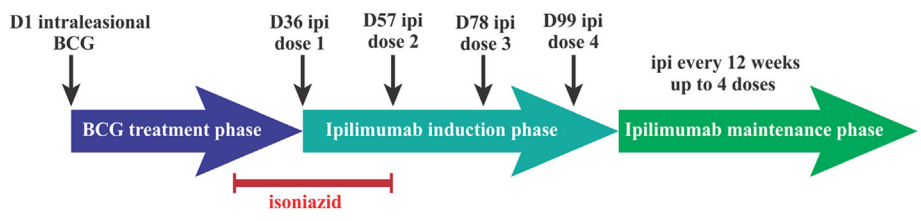

FIGURE 1 | Intralesional *bacillus Calmette–Guérin* (BCG) followed by ipilimumab phase I trial treatment schedule. Dn, day n; Ipi, ipilimumab.

vaccine or pembrolizumab, and one patient had undergone prior targeted therapy with a BRAF inhibitor (**Table 1**).

Safety

All patients experienced treatment-related adverse events (**Table 2**). Within cohort 1, group 1 patients displayed grade 1 adverse events, including fatigue, nausea, and pruritus. One patient displayed minor discomfort in the injected lesion site. Both group 2 patients in the BCG dose-escalation cohort also displayed grade 1 events, including pruritus and fatigue, but additionally these patients experienced high-grade irAEs, patient 4 with grade 3 pruritus, rash, and hepatitis on D49, and patient 5 with grade 4 colitis and secondary grade 3 small bowel ileus on D94 (Figure S1 in Supplementary Material). These irAEs were of high grade at the first onset and led to the discontinuation of the study.

Clinical Activity

Injected lesions progressed in two patients (one resection required, Figure S2 in Supplementary Material), remained stable in two others, and was not evaluated in one patient (taken off study on D49). No clinical responses were observed at noninjected sites of disease; four patients had progressive disease on the basis of RECIST v1.1 criteria and irRC (Figure S3 in Supplementary Material). The remaining patient was taken off study early and did not have tumor measurement assessments. All patients ultimately progressed at sites that included lung, liver, bone, and skin.

Treatment-Induced Changes in the Autoantibody Repertoire

Plasma samples from five patients were analyzed for autoantibody responses by Immunome™ protein array at three

TABLE 1 | Baseline patient characteristics.

Patient	Gender	Stage (AJCC v7)	Mutational status			Prior systemic treatment (Y/N)	First-line treatment	Second-line treatment
			BRAF V600	*NRAS*	*c-KIT*			
1	M	M1b	WT	unknown	unknown	N	NA	NA
2	F	M1c	WT	L52V	WT	Y	NY-ESO-1 vaccine	NA
3	M	M1c	V600E	WT	WT	Y	BRAF inhibitor (PLX3603)	NA
4	F	M1a	V600K	WT	WT	N	NA	NA
5	M	M1b	WT	WT	WT	Y	pembrolizumab	NA

M, male; F, female; WT, wild-type; N, no; Y, yes; NA, not applicable; AJCC v7, American Joint Commission on Cancer classification, version 7.

TABLE 2 | Administered treatment schedule, clinical responses, adverse events, and subsequent treatments across cohort.

Patient	Cohort 1	*Bacillus Calmette–Guérin* (BCG) dose	Site(s) of BCG injection	Number of ipilimumab doses received	Response		Sites of progression	irAEs	Other AEs	Subsequent treatment
					Injected lesions	Noninjected sites				
1	Group 1	0.16–0.64 × 10^6 cfu	Left axilla[a]	4	PD—required resection	PD	Abdominal wall, pelvic node	G1 pruritus	G1 fatigue, G1 left axillary discomfort	Nivolumab
2	Group 1	0.16–0.64 × 10^6 cfu	Right axilla[b]	4	SD	PD	Bone, liver	0	G1 fatigue, G1 nausea	Nivolumab
3	Group 1	0.16–0.64 × 10^6 cfu	Left axillary nodule[a]	2	SD	PD	Pulmonary, pleura, bone, and hepatic	0	G1 fatigue	Dabrafenib and trametinib
4	Group 2	0.80–3.20 × 10^6 cfu	Right upper thigh[a]	1	NE	NE	NE	G1 diarrhea, G3 pruritus, G3 rash, G3 hepatitis	G1 nausea, G2 fatigue	Dabrafenib and trametinib
5	Group 2	0.80–3.20 × 10^6 cfu	Right anterior chest wall, right shoulder[a]	3	PD	PD	Cutaneous lesions	G1 pruritus, G1 rash, G2 diarrhea, G3 small bowel ileus, G4 colitis	G1 fatigue	DTIC

irAEs, immune-related adverse events; AEs, adverse events, PD, progressive disease; SD, stable disease; NE, not evaluated; Gn, grade n.
[a]*Subcutaneous.*
[b]*Lymph node.*

distinct available time points: pre-BCG, post-BCG, and post-ipilimumab. Minor variations in sampling time point between patients are negligible, as changes in the autoantibody repertoire remain detectable for at least 90 days, due to the sensitivity of the assay and the expected 30-day half-life of specific autoantibody titers. In addition, plasma samples from 19 anonymized healthy individual sera were assayed to establish antigen-specific significance thresholds. Visual assessment of all slides revealed high-quality printing and slide handling. Data resulting from seven antigens (CASP10, COMMD3, FANCG, SMARCE1, STAT1, TNFRSF11B, and TYR) were excluded from analysis, because replicates were flagged as "noisy" on all slides. High levels of saturation were repeatedly detected against RBPJ for all patient samples, most likely due to its specific binding to the immunoglobulin kappa-type J segment recombination signal sequence. Nonetheless, this antigen was not excluded from analysis. Positive control CV calculations revealed a variation of 3.4% (cy3-biotin-BSA spots), not requiring data normalization to be performed. The remaining average net intensity autoantibody data against 1,620 antigens was used for downstream analysis. Significance thresholds were calculated as 2 SD from the mean of the data derived from 19 healthy individuals, on an antigen-by-antigen basis (Table S2 in Supplementary Material).

Positive signals above these thresholds and the day of onset of high-grade irAEs are shown in **Figure 2**. Although normally expressed only in the human germ line, CTAs are aberrantly overexpressed in cancer (28) and enable the assessment of cancer-specific responses alongside the broad autoantibody responses that may be unrelated to cancer immunity. Autoantibody responses according to clinical time lines are shown in **Figure 3**. Patients 1 ($n = 71/1,620$), 2 ($n = 34/1,620$), and 3 ($n = 27/1,620$) developed response to relatively few antigens over the period of study. This averaged 3% of the array content. By contrast, patients 4 ($n = 853/1,620$) and 5 ($n = 505/1,620$) had a substantially larger amount of *de novo* and induced counts. These represented an average of 42% of all antigens on the array. This increase was equally evident for both self- and cancer antigens. It is worth noting that enhanced autoantibody reactivity was only seen in the patients who experienced high-grade irAEs (**Figure 3B**). Furthermore, this broad repertoire was either preexisting at baseline (patient 4) or induced after BCG administration (patient 5) and preceded the development of symptoms in both instances.

In addition, the resulting data were analyzed using the Spearman rank correlation, as a means of assessing sample clustering. When inspecting the resulting dendrogram (**Figure 4**), all patient time points clustered together on a patient-by-patient basis, serving as an internal validation of the resulting data. Two distinct sample clusters were apparent, adequately separating patients displaying low- and high-grade irAEs. The baseline data for patient 5 were distinct from the remaining time points and from patient 4, which again highlighted that the boost in excessive autoantibody reactivity only occurred after BCG administration, rather than a

A

PATIENT	TIME POINT	ANTIGEN TYPE		
		TOTAL	SELF	CTAs
Patient 1	D43 (start ipi)	55	52	3
	D60 (on ipi)	62	58	4
	D114 (on ipi)	55	51	4
Patient 2	D1 (start BCG)	15	15	0
	D36 (start ipi)	34	32	2
	D113 (on ipi)	11	11	0
Patient 3	D1 (start BCG)	26	26	0
	D56 (on ipi)	5	5	0
	D75 (on ipi)	21	21	0
Patient 4	D1 (start BCG)	342	314	28
	D35 (start ipi)	812	766	46
	D60 (on ipi)	483	459	24
Patient 5	D1 (start BCG)	13	13	0
	D44 (start ipi)	504	466	38
	D79 (on ipi)	353	323	30

B

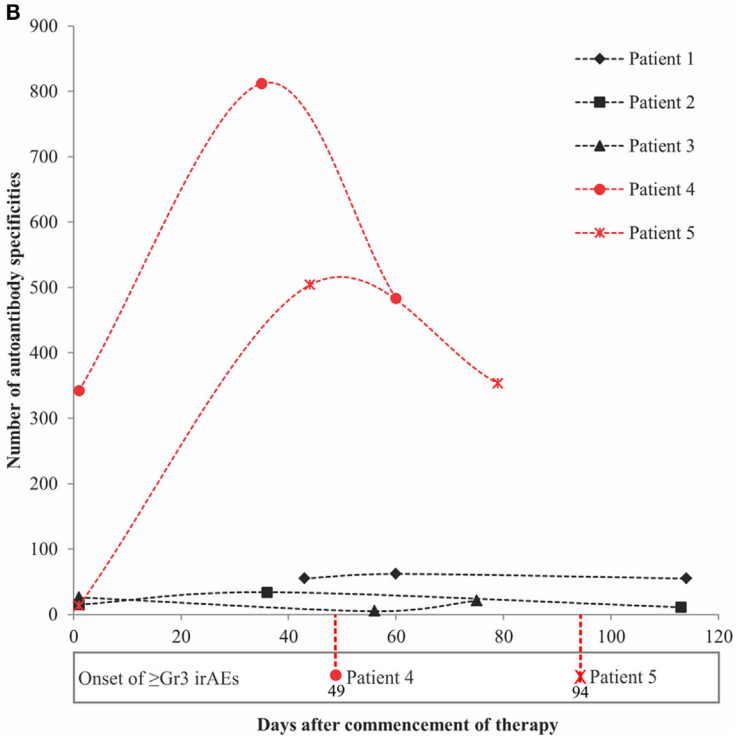

FIGURE 2 | Antigen-specific autoantibody counts above healthy individual-derived thresholds of significance. **(A)** Tabulated counts divided by self-antigens (SELF) and cancer-testis antigens (CTAs). **(B)** Plotted counts comparing patients who developed high-grade immune-related adverse events (irAEs) (red) versus those who did not (black), along with the day of onset of high-grade irAEs. Dn, day n; Ipi, ipilimumab; Gr3, grade 3.

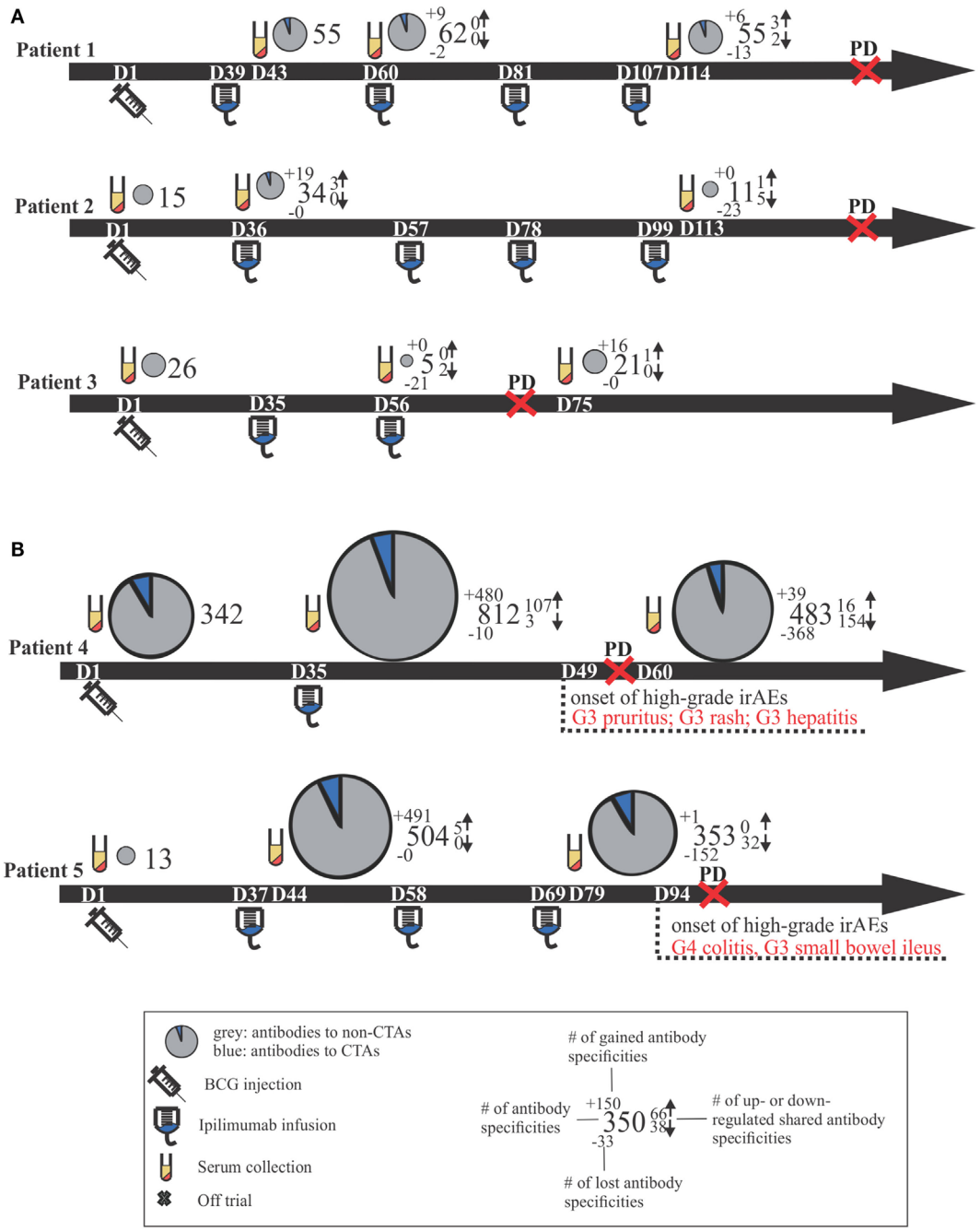

FIGURE 3 | Clinical time lines for all patients, including comparative size-proportional pie charts representing the number of antigens toward which antibody titers were detected. Time lines are separated by patients who did not develop high-grade irAEs **(A)** versus those who did **(B)**. Dn, day n; PD, progressive disease; CTAs, cancer-testis antigens; Gn, grade n; irAEs, immune-related adverse events.

pretreatment existing enhanced reactivity. Autoantibody profiles did not overlap between patients, with unique antigen targets being detected in each patient. In addition, despite the high-grade irAEs being reported in two different organs, the liver and bowel, it is unclear whether there are apparent organotypic differences in the autoantibody repertoires of these patients due to limited patient numbers.

DISCUSSION

Clinical trials, which combine IL immune-stimulants with ICI, aim to extend the effectiveness of cancer immunotherapy by recruiting responses against additional cancer antigens within the injected tumor. This is the first such trial to evaluate BCG, a historically validated IL therapy, with ipilimumab in patients with

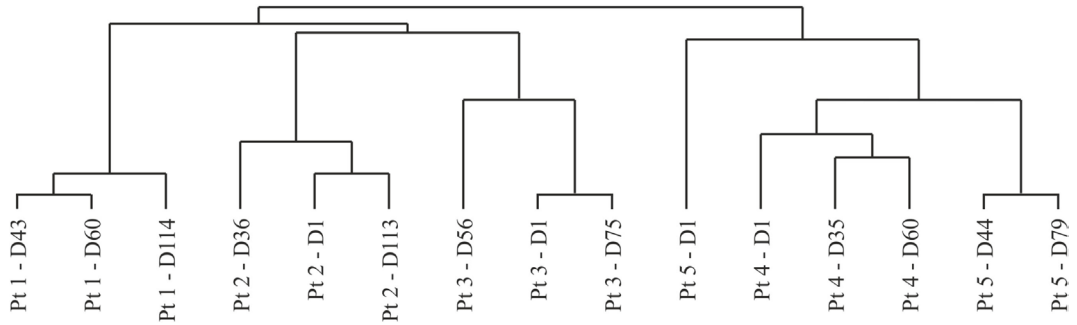

FIGURE 4 | Dendrogram resulting from hierarchical clustering using the Spearman rank correlation method with average linkage. Dn, day n; Pt, patient.

advanced melanoma. Although IL BCG was well tolerated, those patients treated with the higher dose of BCG ($0.80–3.20 \times 10^6$ cfu) developed high-grade irAEs following ipilimumab. All irAEs were typical of ipilimumab and managed as per standardized treatment algorithms. We propose that BCG may have contributed to enhanced immune activation and therefore higher-grade irAEs. These irAEs together with the availability of anti-programmed death 1 (PD-1) therapies as first-line therapy at our institution resulted in slow patient enrollment and early termination of the trial; however, our preliminary correlative seromic analyses of patients with severe irAEs suggest the potential value of serology as a diagnostic tool to anticipate and evaluate autoimmune toxicity.

As the treatment of advanced melanoma continues to evolve, combination and/or sequential treatments with immunotherapy, targeted therapy, chemotherapy, radiotherapy, and even surgery are being trialed to harness an antitumor immune response to obtain maximum clinical benefit. Talimogene laherparepvec is the first oncolytic virus approved by the U.S. Food and Drug Administration for the local treatment of unresectable lesions in patients with recurrent melanoma. Approval was based on the phase III study, OPTiM, which demonstrated significant improvement in responses in injected and uninjected lesions versus subcutaneous GM-CSF (29). Recent studies combining this oncolytic virus with pembrolizumab have demonstrated high overall (62%) and complete response (33%) rates in advanced melanoma (30). Ipilimumab has been evaluated in combination with vaccination (31, 32). In one study (31), ipilimumab in combination with a peptide vaccine resulted in two complete responses and five partial responses among 56 patients with progressive stage IV melanoma, with each of these responses shown to be durable. In another trial (32), patients with resected stage IIIC/IV melanoma received ipilimumab plus a multipeptide vaccine; 25% of patients had grades 3–4 irAEs that were dose-limiting and 27 of 75 patients relapsed after a median follow-up of 23 months. While several such combinations of localized plus systemic immunotherapies offer promise, many unselected patients with a higher disease burden or more rapidly progressive disease still face poor outcomes with existing immunotherapy combinations.

The onset of irAEs resembling classic autoimmunity remains a limitation in cancer immunotherapy (33), and while generally manageable when immunotherapeutics are administered as single agents, the incidence can increase substantially when immunotherapeutics are combined (34). Developing strategies that rationally combine immunotherapeutic agents to minimize toxicities and maximize efficacy is therefore an important area of ongoing investigation. While the identification of predictive biomarkers for therapeutic efficacy is actively being pursued (35–39), comparatively little attention has been placed on identifying reliable biomarkers that can predict adverse autoimmune events.

In this study, we investigated the autoantibody repertoire of a small number of trial patients using the Immunome™ protein array that can identify serological responses to over 1,600 human proteins. We found that the two patients who developed clinically severe autoimmunity had an accompanying profound serological signature, reflecting immune reactivity against a broad panel of autoantigens. Indeed, patients experiencing high-grade irAEs could readily be distinguished on the basis of these autoantibodies which were reactive against almost half of the proteins on the array. Importantly, these elevated autoantibody specificity counts preceded the development of clinically evident autoimmunity. Despite the very limited patient numbers in this pilot trial, it is tempting to speculate that measuring the autoantibody repertoire of cancer patients at risk of experiencing irAEs may predict the development of toxicity.

The breadth of this autoimmune reactivity was suggestive of a systemic B cell deregulation, rather than a focused immune response, as might typically occur following an infection or vaccine. Similar responses have been reported in chronic humoral rejection of organ transplants (40). We postulate that the chronic inflammatory conditions associated with interstitial mycobacterial infection resulted in the indiscriminate release of both cancer antigens and autoantigens that were not cancer-specific. In the presence of an ICI, both anticancer immunity and autoimmunity were enhanced. This is in accordance with the proposed notion that the disruption of immune tolerance and the onset of inflammation can enhance autoantibody production (41). There was no sign of clinical activity, so the reactivity against cancer-specific targets did not appear to be clinically useful; however, with a small number of patients, it is difficult to be certain. This specific immunotherapy combination will not be pursued further due to the apparent excess of clinical toxicity. Nonetheless, larger studies—likely in the setting of more tolerable immunotherapy combinations—will be required to validate these seromic findings and to better understand the involved mechanisms.

In conclusion, dose escalation of IL BCG followed by ipilimumab therapy in a pilot trial of limited patient numbers was not well tolerated in advanced melanoma patients and showed no evidence of clinical benefit. Whether the onset of the observed high-grade irAEs was enhanced by IL BCG or simply a result of ipilimumab alone remains unclear. Nonetheless, investigating humoral immunity may offer a means to detect the early onset of a wide spectrum of irAEs in cancer patients treated with immunotherapy, warranting further larger studies.

ETHICS STATEMENT

This study (ClinicalTrials.gov number NCT01838200) was conducted at Austin Health, Heidelberg, Australia, and was approved by the institution's human research ethics committee. Written informed consent was obtained from all patients, and all methods were performed in accordance with the protocol-specified guidelines and regulations.

AUTHOR CONTRIBUTIONS

JD, AB, and JC were responsible for the immunome array study design. JD was responsible for the data analysis, representation, and interpretation. JB, AB, and JC provided additional expert input into the key findings. MA and JC were responsible for the planning and execution of the clinical trial. SP and MA provided relevant clinical information. KW, AP, CT, and SO were responsible for clinical sample processing and additional immunology experiments that were excluded from this article. JD wrote the manuscript, and SP, MA, JB, AB, and JC reviewed and edited. All authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00411/full#supplementary-material.

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Retinoic Acid Receptor-Related Orphan Receptors: Critical Roles in Tumorigenesis

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Retinoic acid receptor-related orphan receptors (RORs) include RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3). These receptors are reported to activate transcription through ligand-dependent interactions with co-regulators and are involved in the development of secondary lymphoid tissues, autoimmune diseases, inflammatory diseases, the circadian rhythm, and metabolism homeostasis. Researches on RORs contributing to cancer-related processes have been growing, and they provide evidence that RORs are likely to be considered as potential therapeutic targets in many cancers. RORα has been identified as a potential therapeutic target for breast cancer and has been investigated in melanoma, colorectal colon cancer, and gastric cancer. RORβ is mainly expressed in the central nervous system, but it has also been studied in pharyngeal cancer, uterine leiomyosarcoma, and colorectal cancer, in addition to neuroblastoma, and recent studies suggest that RORγ is involved in various cancers, including lymphoma, melanoma, and lung cancer. Some studies found RORγ to be upregulated in cancer tissues compared with normal tissues, while others indicated the opposite results. With respect to the mechanisms of RORs in cancer, previous studies on the regulatory mechanisms of RORs in cancer were mostly focused on immune cells and cytokines, but lately there have been investigations concentrating on RORs themselves. Thus, this review summarizes reports on the regulation of RORs in cancer and highlights potential therapeutic targets in cancer.

Keywords: retinoic acid receptor-related orphan receptors, RORα, RORβ, RORγ, cancer

INTRODUCTION

Cancer incidence and mortality rates are increasing worldwide with the growing and aging of the population, as well as risk factors such as outdoor pollution, tobacco smoke, and physical inactivity (1). Due to early detection and advanced treatments, cancer survival rates continue to grow, although a better understanding of carcinogenesis may lead to more effective treatment options for cancer.

The nuclear receptors (NRs) have been demonstrated to play essential roles in cancer-related progresses and to be potential therapeutic targets for many malignancies (2–5). The retinoic acid receptor-related orphan receptors (RORs) are a subfamily of the thyroid hormone receptor, which is a subfamily of the NRs and belonging to the orphan NR family (6). The ROR subfamily contains three members: RORα (NR1F1), RORβ (NR1F2), and RORγ (NR1F3).

Members of the RORs are typically regarded as noteworthy in inflammation, autoimmune diseases, metabolism disorders, circadian rhythms, development of neuron cells, and immune cell differentiation. Although RORs share some common sequences, the three RORs present a wide assortment of features. RORα and RORγ are important regulators of the immune system. For instance, the development and differentiation of Th17 cells are dependent on these factors (7–9). Moreover, studies show that RORγ is expressed in lymphoid tissue inducer cells, innate lymphoid cells, invariant natural killer T cells, and γδ T cells, which contribute to inflammation and autoimmune disease (10).

RORα, RORβ, and RORγ are all involved in the modulation of circadian rhythms. RORα functions as a positive regulator of the circadian modulator Bmal1 through binding to ROR-responsive elements (ROREs) (11, 12). RORβ mRNA expression levels were found to oscillate with true circadian rhythms, peaking at night-time (13), and modulation of circadian rhythms was disrupted in RORβ-deficient mice (14). Recent studies have proposed that RORγ1, but not RORα, is periodically expressed, and RORγ regulates several clock genes, such as Cry1, Bmal1, and Npas2, directly in a Zeitgeber time-dependent manner through these ROREs (15, 16).

Accumulating evidence shows that RORα and RORγ are involved in lipid/glucose metabolism, insulin sensitivity, and cardiometabolic control (17). A report showed that RORα could repress the transcriptional activity of PPARγ, leading to dysregulation of hepatic lipid metabolism (18). Recently, studies have shown that metabolic disorders affected by circadian rhythms might be attributed to RORα and RORγ, partly because of their modulation in both circadian and metabolic diseases. Moreover, earlier studies suggested that RORα was directly involved in melatonin-mediated anti-fibrotic processes (19) and beneficial manipulation in diabetic cardiomyopathy (20).

The expression sites and producing cells of RORs are also distinct from each other, consistent with their functions in the various diseases mentioned above. RORα and RORγ are expressed in all skin cell types, including epidermal keratinocytes, melanocytes, dermal fibroblasts, and several established lines of malignant melanomas. The expression levels of RORα/γ are dependent on the skin cell type and can be regulated by hydroxy derivatives of vitamin D3 (5, 21–24). Vitamin D3 formation is regulated by UVB (25); vitamin D3 metabolites are inverse agonists for RORα/γ; therefore, RORα and RORγ expression level could be regulated by UVB (5).

Other expression sites of RORα include the liver, skin, pancreas, brain, adipose tissue, islet cells, and the pineal gland. In addition to its expression and modulation in melanoma described above, RORα has been researched in breast cancer (BC) (26), melanoma (5), hepatocellular carcinoma (HCC) (27), and colon cancer (28). RORβ is mainly expressed in the brain and pineal gland (29). RORβ is upregulated or downregulated in cancers such as primary leiomyosarcoma of the uterus (30), a pharyngeal cancer cell line (31), and colorectal cancer (28). RORγ is expressed in the thymus and lymphoid organs, and RORγ production in cancer cells is detected in lung cancer (4), lymphoma (32), melanoma (5), and BC (33).

The RORs have been widely investigated in cancer and have shown varying influences in cancer-related processes, these differences may be due to their structures and their tissue-specific expression. Some studies suggest that RORα is a tumor suppressor and a potential therapeutic target for BC; and based on the limited researches on RORβ in cancer, RORβ might be a tumor suppressor as well. Others have proposed that activating RORγ may exert antitumor immunity (34), while RORγ is considered as protumor candidates in prostate cancer and lung cancer (4, 35). In this review, we summarize and discuss the structures of RORs and their roles in cancer-related processes, highlighting the potential therapeutic targets for cancer treatment.

STRUCTURE AND LIGANDS OF RORs

The three ROR family members contain sequences similar to the retinoic acid receptor, with certain differences. The three ROR family members contain sequences similar to the retinoic acid receptor, but in minor details, the structures of each are distinct (36). The RORα gene maps to human chromosome 15q22.2, covering a large genomic region of 730 kb and generating four human RORα isoforms: RORα1—RORα4, while only RORα1 and RORα4 are found in mice (17). The RORβ and RORγ genes map to human q21.13 and 1q21.3, covering 188 and 24 kb, respectively. RORβ and RORγ each generate two isoforms: RORβ1/RORβ2 and RORγ1/RORγ2 (RORC2 in human and RORγt in mice). The isoforms of RORs differ in their amino terminals due to alternative exon splicing and promoter usage and their distinct expression and function in different tissues. However, if cells co-express RORs, the co-expressed RORs may overlap in several functions.

Receptor-related orphan receptor genes encode proteins of similar amino sequences ranging from 459 to 556 amino acids according to the different isoforms, and they all consist of four domains. These domains include an N-terminal domain, a highly conserved DNA-binding domain, a ligand-binding domain (LBD), and a hinge between the domains. Transcription is regulated by binding to RORE as a monomer (36).

No cognate ligands of RORs had been identified until crystallography studies on the LBD of RORα indicated that cholesterol and cholesterol sulfate function as natural ligands (37). Several retinoids, including all-trans retinoic acid and the synthetic retinoid ALRT 1550 (ALRT), have been identified to bind RORβ, reversibly and with high affinity (38). Thus, the retinoids have been identified as ligands of RORβ, although their specific regulation is not clearly understood. RORγ has been found to be co-expressed with RORα, and the ligands of RORα and RORγ have been reported as sterols or their derivatives and secosteroids (5, 6). Endogenously produced novel D3 hydroxy derivatives can act as both "biased" agonists of the vitamin D receptor and inverse agonists of RORα/γ (22), and hydroxylumisterols can act as ligands of RORα and RORγ (39). Melatonin was once considered a ligand for RORα (40, 41). However, contrasting reports showed that melatonin was not a natural ligand for RORα because melatonin could not activate RORα directly (42, 43). The docking scores calculated from molecular modeling of interactions between melatonin

and its metabolites with RORα and RORγ predicted weak binding affinities (5), and the structures of melatonin and its metabolites were not similar to the sterols that were identified as natural ligands (37).

Except for the natural ligands of RORs mentioned above, there are also some synthetic RORγ ligands with therapeutic potential identified in literatures (6, 44). For instance, the inverse agonists of RORα and RORγ, SR2211 has been reported to inhibit the expression of IL-17A and cell viability in lung cancer (4) and suppress inflammation in a collagen-induced arthritis mouse model (45). And RORα and RORγ agonist SR1078 can induce cancer cell apoptosis and p53 stability (46). Inverse agonists or agonists like these two are promising therapeutic reagents for the diseases that RORs involved in, but there are still lack of studies to investigate their treatment potentials in cancer.

CANCER RELEVANCE

As illustrated above, RORs have been implicated in autoimmune or immune-mediated disease, the circadian rhythm, and metabolic disorders. RORs are also important regulators in various cancers due to their pivotal roles in immunity, the circadian rhythm, and metabolic homeostasis, which contribute to tumor progression.

RORα has been found to be downregulated in keratinocyte-derived skin cancer (47) and is expressed in prostate cancer cells (48), melanoma cell lines (5, 49), and BC (50) (**Table 1**). Decreased expression of RORα is positively related with melanoma progression and shorter disease-free and overall survival (23, 24). RORα is also involved in inhibiting cell proliferation as a tumor suppressor (51). In human hepatoma cells, RORα was found to be upregulated after hypoxia induction (52), while RORα expression was lower in tumor tissues than in adjacent tumor tissues. It was also determined to be involved in the reprogramming of glucose metabolism and inhibiting hepatoma growth both *in vitro* and in a xenograft model *in vivo* (53). However, in one report, the production of RORα mRNA in colorectal cancer patients was unchanged (54), while RORα phosphorylation was found reduced and might be involved in colon cancer progression (55). In another report about BC, RORα was found to be downregulated, and low expression of RORα mRNA was associated with a poor prognosis (26). RORα is commonly considered a repressor (**Figure 1**), according to investigations into its role in cancer illustrated above.

The natural expression of RORβ is exclusively restricted to neuronal tissues; therefore, activation of RORβ transcription is predominantly found in neuroblastoma cell lines (56), and literature on the role of RORβ in cancer is not much. Nevertheless, primary uterine leiomyosarcoma showed high RORβ expression (30), pharyngeal carcinoma cells and colorectal cancer cells showed modulated RORβ expression (29, 31), and RORβ was related to metastasis in a metastatic colorectal cancer cell model (28), which are summarized in **Table 1**. Based on the studies mentioned above, RORβ shows features of a tumor suppressor (**Figure 1**), but the potential roles of RORβ in various cancers related processes such as tumor proliferation and metastasis warrant further investigation.

RORγ in Various Cancers

On the contrary, RORγ and its isoforms are extensively found in various kinds of malignancies. The diverse roles of RORγ in distinct cancers are specifically described below and summarized in **Table 1** and **Figure 1**.

Hematological Malignancies

RORγ was found to function as an important element in lymphatic tumors (32), and mice deficient in RORγ were shown to have a high incidence of lymphoma metastasis and death within 4 months (57). Moreover, RORγ is frequently studied in tumor-infiltrating immune cells. RORγ mRNA expression in total lymphocytes was found unchanged between multiple myeloma and healthy controls (58, 59), but it was identified upregulation in peripheral blood monocyte cell (PBMC) from multiple myeloma comparing with healthy controls (60).

Breast Cancer

RORγ was found to be significantly overexpressed among infiltrating IL-17$^+$ T cells, which drive immunosuppression in BC (61), and in breast tumor tissues compared with control tissues (62). An investigation related to group 3 innate lymphoid cells (ILC3) in BC revealed a role for RORγt + ILC3 in promoting lymph node metastasis by modulating chemokines in the tumor microenvironment (63). RORγ was found to be decreased in basal-like and grade 3 BCs, and inhibition of RORγ blocked cell viability, migration, and epithelial–mesenchymal transition (EMT) (64). However, an earlier study suggested that high expression of RORγ1, but not RORγt, by cancer cells was related to a high distance metastasis-free survival and was inversely correlated with decreased expression of PRMT2, which could suppress cell migration in BC (33). Accordingly, the different functions of RORγ in BC may be due to distinct cell origins and isotypes. For instance, when expressed by immune cells, RORγ acts as an immune suppressor, although when produced by cancer cells, it acts as a potential survival factor.

Skin Cancer

RORγ1 regulated tumor-promoting "emergency" granulomonocytopoiesis by suppressing negative (Socs3 and Bcl3) and promoting positive (C/EBPb) regulators of granulopoiesis and RORγ1 promoted expansion of tumor-promoting MDSCs and TAM in fibrocarcinoma mice models (65). In a study exploring the function of Th17 cells in antitumor immunity, RORγt was found to be expressed by tumor-infiltrating Th17 cells. Th17 cells did not exhibit *in vitro* tumor cell killing activity, although CD8$^+$ cytotoxic T cells stimulated by Th17 cells could activate the tumor killing response in a mouse B16 melanoma model (66).

In another study, RORγ-deficient mice showed inhibited melanoma growth, and this effect was identified to be IL-9 dependent (67). Together with RORα, RORγ was found to be expressed in melanoma cell lines and could bind with vitamin D3 derivatives, including 20(OH)D3 and 20,23(OH)2D3 (5), active forms of secosteroids and lumisterol can have anti-melanoma activity through action on RORα and RORγ (22, 24, 25, 39). In another study, RORγ and RORα expression levels were decreased during melanoma progression, with the lowest expression levels in stages

TABLE 1 | Studies of RORs in cancer.

Isoforms	Cancer type	Study population/model	Expressing cell	Expression level and biologic effects	Reference
RORα					
RORα	BC	BC tissues	BC cell	Activates aromatase expression Promotes cell proliferation in ER-positive BC	(50)
RORα	BC	Malignant and nonmalignant breast tissues	BC cell	Decreased Correlated with poor prognosis Inhibits cell invasion and regulates SEMA3F	(26)
RORα	Hepatoma	HCC and adjacent non-tumor tissue	Hepatoma cell	Decreased Reprograms glucose metabolism; inhibits hepatoma growth both *in vitro* and in a xenograft model *in vivo*	(53)
RORα	Colorectal cancer	Human colorectal tumors	Colorectal cancer cell	Unchanged	(54)
RORα	Colon cancer	Human colon tumors	Colon cancer cell	Attenuates Wnt/β-catenin signaling	(55)
RORα1	Prostate cancer	Prostate cancer cell line	Prostate cancer cell	Activation of RORα1 reduces 5-LOX expression might interfere with the mitogenic activity of fatty acids on prostate cancer	(48)
RORα4	Skin cancer	SSCC tissues	SCC cell	Decreased	(47)
RORα4	Melanoma	Human melanoma cell lines	Melanoma cell	Expressed in WM-98, WM-164, and SCBE2 cells	(49)
RORα	Melanoma	Human melanoma cell lines	Melanoma cell	As receptors for 20-hydroxy- and 20,23-dihydroxyvitamin D	(5)
RORα	Melanoma	Melanoma tissues	Unspecified	Decreased Positive associated with melanoma progression and shorter disease-free and overall survival	(24)
RORα	Melanoma	Benign (nevi) and malignant (melanomas) melanocytic tumor tissues	Keratinocytes, melanoma cells	Decreased Higher nuclear levels of RORα correlated with significantly longer overall and disease-free survival time	(68)
RORα4	Hepatoma	Hepatoma cell line	Hepatoma cell	Upregulated by hypoxia in HepG2 cells	(52)
RORβ					
RORβ	Colorectal cancer	Human primary colorectal cancer tissues	Colorectal cancer cell	Decreased Attenuate self-renewal of CCICs by binding with HBP1 promoter regions Enhance the HBP1-dependent inhibition of TCF4-mediated transcription and Wnt activity	(29)
RORβ	Colorectal cancer	Human colon cancer cell clones	Human colon cancer cell clones	Decreased	(28)
RORβ	Neuroblastoma	Neuronal cell line	Neuroblastoma cell	Binds to ROREs with low affinity Instigates transcription efficiently in Neuro2A but not in HeLa nuclear extracts due to an extract specific factor in Neuro2A	(56)
RORβ	Uterine leiomyosarcoma	Primary and metastatic uterine leiomyosarcoma tissues	Unspecified	Increased in primary tumor than metastatic tumor	(30)
RORβ	Pharyngeal cancer	Pharyngeal cancer cell line	Metastatic (Detroit 562) pharynx carcinoma cell	Increased Regulated by TLR3	(31)

(*Continued*)

TABLE 1 | Continued

Isoforms	Cancer type	Study population/model	Expressing cell	Expression level and biologic effects	Reference
RORγ					
RORγ	Lymphoma	RORγ$^{-/-}$, RORγ$^{+/-}$, and wild-type mice		Deficiency of RORγ leads to T cell lymphoma, metastasis, and death	(57)
RORγ	Multiple myeloma	PB and BM of patients with multiple myeloma	Lymphocytes	Unchanged	(58, 59)
RORγ	Multiple myeloma	Patients with multiple myeloma tissues	PBMC	Increased	(60)
RORγ	BC	Human BC tissues	Unspecified	Overexpressed among IL-17^{Hi} tumors	(61)
RORγ	BC	Human IDC tumor tissues	Tumor-infiltrating CD4^{+} and CD8^{+} T lymphocytes	Increased RORC and IL-17A expression is correlated in breast tumor tissues	(62)
RORγ	BC	BC tissues	ILC3	Increased Correlated with LN metastasis	(63)
RORγ1	BC	BC patients and cell line	BC cell	Positively associated with DMFS rate	(33)
RORγ	BC	TCGA and GEO BC collection, BC cell lines	BC cell	Decreased Negatively regulates the oncogenic TGF-β/EMT and mammary stem cell (MaSC) pathways and positively regulates DNA-repair Higher RORγt expression displayed increased probability of RFS	(64)
RORγ	BC	BC cell lines MAINZ data sets and UNC metastatic BC data set	BC cell	Increased Inversely correlated with PRMT2 expression Increased expression improved DMFS	(33)
RORγ	Melanoma	B16F10 mouse melanoma model	T cell	High IL-9 expression in RORγ^{-} T cells leads to inhibition of melanoma	(67)
RORγ	Melanoma	Human invasive melanomas tissues, skin samples (neonatal and adult), cultured normal and immortalized keratinocytes, and melanoma cells	T cell, melanoma cell	Inhibited by novel hydroxy derivatives of vitamin D	(5)
RORγ	Melanoma	Melanoma tissues		Decreased Positive associated with melanoma progression and shorter disease-free and overall survival	(24)
RORγ	Melanoma	Benign (nevi) and malignant (melanomas) melanocytic tumors	Keratinocytes, melanoma cells	Decreased Higher nuclear levels of RORγ and of cytoplasmic RORγ correlated with significantly longer overall and disease-free survival time	(68)
RORγ	Lung cancer	NSCLC tissues	Lung cancer cell	Increased High RORC2 expression leads to worse overall survival	(4)
RORγ	Lung cancer	Peripheral blood of NSCLC patients	PBMCs	Decreased	(69)
RORγ	Lung cancer	Peripheral blood of NSCLC patients	PBMCs	Increased Positively correlated with Th17 but negatively correlated with FOXP3	(70)
RORγ	Lung cancer	Peripheral blood of NSCLC patients	PBMCs	Increased Positively correlated with Th17 but negatively correlated with IL-27	(71)
RORγ	Lung cancer	Peripheral blood of NSCLC patient	PBMCs	Increased FoxP3/RORγ is higher in stage IV NSCLC patients than those of patients in stages I, II, and III	(72)
RORγ	Lung cancer	ADC and SSC tissues	Unspecified	Higher in the tumoral region of ADC compared with squamous cell carcinoma	(73)

(*Continued*)

TABLE 1 | Continued

Isoforms	Cancer type	Study population/model	Expressing cell	Expression level and biologic effects	Reference
RORγ	Hepatoma	Peripheral blood of hepatoma patients	PBMCs	Increased	(74)
RORγ	Hepatoma	Patients of steatosis/steatohepatitis, liver fibrosis, and HCC		Decreased	(27)
RORγ	Gastric cancer	Human gastritis and gastric ADC tissues, $gp130^{F/F}$ mice that spontaneously develop gastric inflammation-associated tumors		Increased Not correlated with gastric tumorigenesis	(75)
RORγ	Colorectal cancer	Human CRC tissues	$Foxp3^{+}IL\text{-}17^{+}$ cells	Increased	(76)
RORγ	Colorectal cancer	Tissues and peripheral blood of colorectal cancer patients and RORγt-deficient mice	$ROR\gamma^{+}$ Treg cells	Increased Deficiency in RORγt protects against polyposis and improve cancer immune surveillance	(77)
RORγ	Colorectal cancer	$Itch^{-/-}$ mice	Th17 cells; innate lymphoid cells	Regulated by itch Inhibition or genetic inactivation of RORγ attenuated IL-17 expression and reduced spontaneous colonic inflammation in $Itch^{-/-}$ mice	(78)
RORγ	Colorectal cancer	Human CRC tissues	Unspecified	Unchanged	(79)
RORγ	Prostate cancer	Primary prostate cancer and metastatic prostate cancer samples	Prostate cancer cell	Increased Overexpressed and amplified in metastatic CRPC tumors Directly controls AR gene expression	(35)
RORγ	Cervical cancer	Peripheral blood of patients with cervical cancer or CIN	PBMCs	Increased Positively correlation with Th17 cells and Th22 cells in CIN and cervical cancer patients	(80)
RORγ1	Fibrosarcoma	BM or spleens from fibrosarcoma mice model Patients with T2 or T3 CRC	Myeloid cells	Drives cancer-related myelopoiesis in response to colony-stimulating factors Suppresses negative (Socs3 and Bcl3) and promotes positive (C/EBPb) regulators of granulopoiesis Promotes the protumor differentiation of MDSCs and TAMs	(65)

ER-positive, estrogen receptor positive; BC, breast cancer; SSCC, skin squamous cell carcinoma; $IL\text{-}17^{Hi}$, high expression of IL-17; IDC, invasive ductal carcinoma of the breast; LN, tumor-draining lymph nodes; ILC3, group 3 innate lymphoid cells; NSCLC, non-small cell lung cancer; DMFS, distance metastasis-free survival; PBMC, peripheral blood monocyte cell; ADC, adenocarcinoma; SCC, squamous cell carcinoma; HCC, hepatocellular carcinoma; CRC, colorectal cancer; CC, colon cancer; CIN, cervical intraepithelial neoplasia; CRPC, castration-resistant prostate cancer; AR, androgen receptor; ROREs, ROR-responsive elements; RORs, receptor-related orphan receptors; EMT, epithelial–mesenchymal transition; RFS, relapse free survival; Treg, regulatory T cell; CCICs, colorectal cancer-initiating cells.

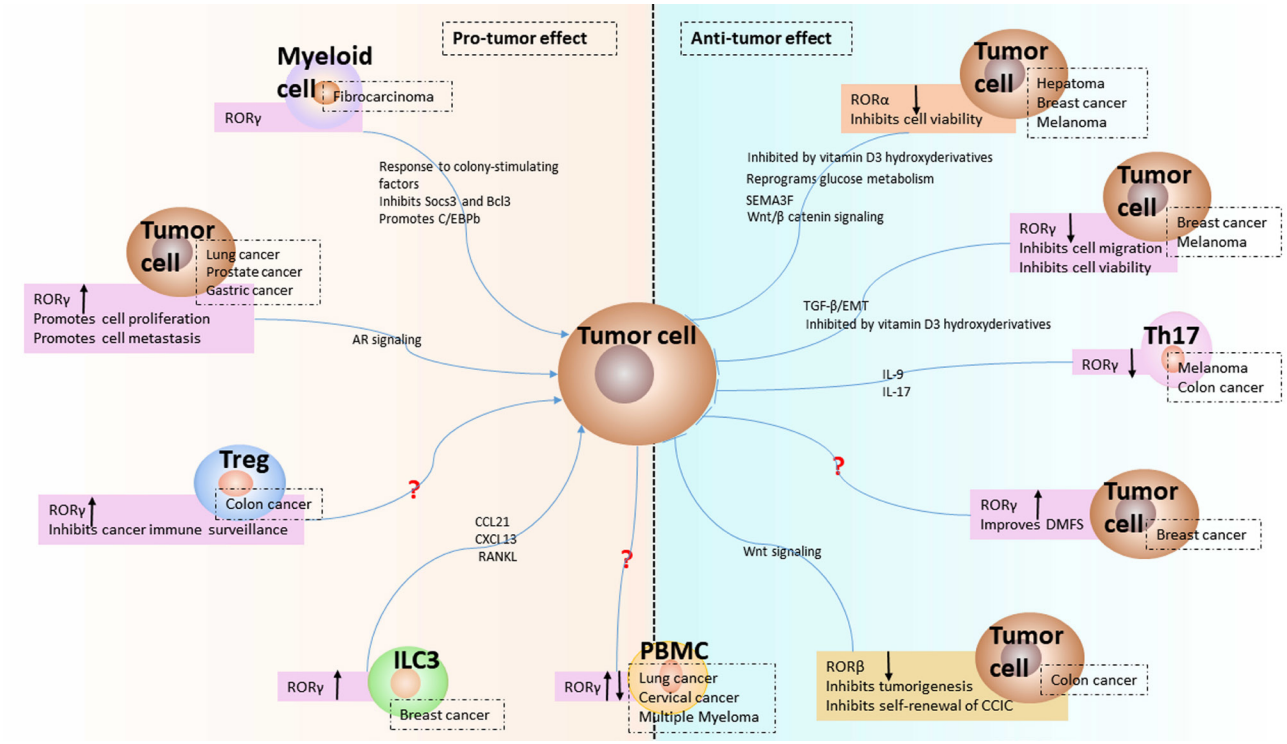

FIGURE 1 | Expression and function of receptor-related orphan receptors (RORs) in tumor microenvironment. The expression of RORα and RORβ from tumor cell and the modulated expression of RORγ in group 3 innate lymphoid cells (ILC3), Th17, regulatory T cell (Treg), myeloid cell, and tumor cell from tumor microenvironment are presented as reviewed in the text. The downregulation of RORα and RORβ induce antitumor effect in hepatoma, breast cancer (BC), melanoma, and colon cancer. The upregulation of RORγ in ILC3 leads to protumor effect by chemokines in BC. The downregulation of RORγ in Th17 indicates antitumor effect by IL-17 in colon cancer. The upregulation of RORγ in Treg shows protumor effect in colon cancer. The expression of RORγ in myeloid cell has protumor effects *via* Socs3, Bcl3, and C/EBPb. The expression of RORγ in tumor cell is either increased or decreased depending on the cancer type. Increased expression of RORγ in lung cancer, prostate cancer, and gastric cancer results in protumor effect, while decreased expression of RORγ in BC and melanoma could induce antitumor effect *via* TGFβ/epithelial–mesenchymal transition (EMT) or vitamin D3 derivatives. The question mark refers to unknown mechanisms. The up or down black arrow refers to upregulation or downregulation. Antitumor: inhibits tumor progression; protumor: promotes tumor progression.

III and IV primary melanomas and in melanoma (68). These studies of RORα and RORγ in melanoma suggest that RORα and RORγ could be important modulators affecting melanomagenesis, contributing to the anti-melanoma activity of vitamin D3 and act as potential therapeutic targets in adjuvant melanoma therapy (23, 24). The investigation of RORγ in skin cancer seems to be concentrated on melanoma and the isotype RORγ1, thus, there is a need for further exploration focusing on the regulation of RORγ and its roles in other types of skin cancer.

Lung Cancer

Our previous study showed that RORγ2 was highly expressed in non-small cell lung cancer (NSCLC) cells and also served as a prognostic factor (4). The expression of RORγt mRNA and protein was found to be downregulated in PBMCs from NSCLC patients compared with controls (69). However, RORγt mRNA was found to be upregulated in the peripheral blood of patients with NSCLC compared with that of healthy controls (70), which was confirmed in other studies (71, 72). Moreover, in a recent report, RORγt, together with Th17/IL-6R/pSTAT3/BATF, was upregulated in the tumor region of adenocarcinomas, except for squamous carcinomas of lung cancer (73). Studies focused on cancer cell-derived RORγt are infrequent and require additional attention.

Hepatocellular Carcinoma

RORγt mRNA was shown to be increased in HCC compared with a normal control group (74). By contrast, RORγt mRNA expression was found to be significantly lower in patients with steatosis/steatohepatitis, liver fibrosis, and HCC (27). Investigations into RORγt in HCC are rare, although RORγt is known to be expressed in hepatocytes. There could be additional modulatory roles for RORγt in HCC progression, and further studies are warranted.

Gastrointestinal Cancer

The gene expression of IL-17A and RORγ was not altered in gastric cancer (75). $Foxp3^{+}IL\text{-}17^{+}$ cells in colorectal cancer were found to express RORγt (76). Another study described RORγt-expressing regulatory T cells that were linked with the inability of these cells to suppress inflammation and were directly associated with the stage of human colon cancer (77). RORγt was also found to be involved in inhibiting colon carcinogenesis through binding with an E3 ubiquitin ligase, Itch, for ubiquitination (78). However, RORγt was not expressed within colorectal cancer tissues or by

colorectal cancer-infiltrating $CD4^+$ T cells (79). The expression and regulation of RORγt in gastric and colorectal cancer remain controversial, which makes it difficult to conclude the extent of RORγ/RORγt expression or the involvement in tumorigenesis. However, the differences in results from different studies might be attributable to the diversity of detection methods from tissue samples when considering individual variation.

Genitourinary Cancer

In castration-resistant prostate cancer (CRPC), RORγ was examined as a therapeutic target due to its overexpression and was found to directly drive androgen receptor (AR) hyperactivity through binding to an exonic RORE and partly through the NR coactivators SRC-1 and -3 (35). Therefore, inhibition of RORγ may represent a possible treatment option for CRPC. The transcriptional expression of RORγ mRNA from PBMCs exhibited high levels in cervical cancer compared with healthy controls (80). Additional observations are needed to elucidate the functions of RORγ in genitourinary cancer, where it may serve as a valuable therapeutic target.

PERSPECTIVE

The three ROR family members are regarded as important regulators of the circadian rhythm, metabolism, and tumorigenesis. As discussed in this review, the protumor or antitumor effects of RORα and RORβ in cancer have not been intensively explored, requiring further study and evidence. However, as the main transcription factor in IL-17-expressing immune cells, RORγ has been investigated in various cancer cells and tumor-infiltrating cells (**Figure 1**), indicating that it might be a promising prognostic factor in lung and BC and a potential therapeutic target in prostate cancer.

Moreover, according to this review, we could conclude that the roles that RORs family members play in tumorigenesis vary in different cancers and, to some extent, depend on producing cells in the tumor microenvironment. Further concentration on the relationships between RORs and tumorigenesis should be meticulously organized and should deeply explore the clinical significance and the underlying mechanisms. More importantly, each RORs family members consists of several isoforms, and some previous studies have showed that different RORs isoforms present different biological functions (6). Thus, prospective reports on therapeutic targets of RORs in cancer should identify all isoforms of specific RORs.

Since RORα and RORγ are dysregulated in multiple cancer types based on published articles, they likely participate in carcinogenesis through modulating molecules such as IL-17, PRMT2, and AR or as receptors for sterols, such as vitamin D3 derivatives. Intriguingly, IL-17, AR, and vitamin D3 are therapeutic targets in rheumatoid arthritis and have potential, as a frontline treatment option for advanced prostate cancer and an adjuvant in melanoma management. Agonists or inverse agonists for RORα and RORγ might be efficiently inhibiting tumor growth and progression through activation or inactivation so that their ligands or targets, such as vitamin D3 derivatives and AR, become valid or invalid. Another promising new strategy for anticancer therapy might involve directly targeting tumor cells with RORα- and RORγ-specific modulators due to the correlations between high or low expression of RORα and RORγ and tumor progression. Third, RORs are sometimes produced by immune cells in tumor microenvironments and then induce antitumor or protumor activity by regulating tumor-related cytokines or chemokines. Accordingly, therapies targeting RORs producing immune cells could be novel treatments for certain cancers.

AUTHOR CONTRIBUTIONS

JF, ZL and GY wrote the draft. YJ revised the manuscript. JF, TL, JX, and FW designed the figures. QH, MG, GH, MZ, LD and SL commented and added extra information.

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Dying to Be Noticed: Epigenetic Regulation of Immunogenic Cell Death for Cancer Immunotherapy

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Immunogenic cell death (ICD) activates both innate and adaptive arms of the immune system during apoptotic cancer cell death. With respect to cancer immunotherapy, the process of ICD elicits enhanced adjuvanticity and antigenicity from dying cancer cells and consequently, promotes the development of clinically desired antitumor immunity. Cancer ICD requires the presentation of various "hallmarks" of immunomodulation, which include the cell-surface translocation of calreticulin, production of type I interferons, and release of high-mobility group box-1 and ATP, which through their compatible actions induce an immune response against cancer cells. Interestingly, recent reports investigating the use of epigenetic modifying drugs as anticancer therapeutics have identified several connections to ICD hallmarks. Epigenetic modifiers have a direct effect on cell viability and appear to fundamentally change the immunogenic properties of cancer cells, by actively subverting tumor microenvironment-associated immunoevasion and aiding in the development of an antitumor immune response. In this review, we critically discuss the current evidence that identifies direct links between epigenetic modifications and ICD hallmarks, and put forward an otherwise poorly understood role for epigenetic drugs as ICD inducers. We further discuss potential therapeutic innovations that aim to induce ICD during epigenetic drug therapy, generating highly efficacious cancer immunotherapies.

Keywords: tumor microenvironment, immunogenic cell death, epigenetics, T cell immunity, cancer immunotherapy, immune evasion

INTRODUCTION

Antitumor T cells can detect and eliminate cancer cells in a highly precise, antigen-specific fashion. Appropriately activated antitumor T cells can target cancer cells at both local and metastatic sites and, most importantly, can kill existing as well as possibly relapsing cancerous cells. Numerous patient cohort studies thus far have reported a clear positive correlation between the activities of antitumor T cells and better patient outcomes (1–3). Therapeutic interventions promoting antitumor T cell immunity are at the forefront of next-generation cancer therapeutic strategies and as such are highly desired in clinics.

Promoting antitumor T cell responses in cancer-bearing hosts is challenging (4). This is largely because cancers employ numerous evasion strategies that are non-conducive toward T cell activation and function. In particular, cancer-associated immune evasion is supported through the plastic nature of the tumor microenvironment (TME), which harbors the processes that actively suppress the development of antitumor T cells. Some prominent examples of such evasion mechanisms include the presence of immunosuppressive cytokines like transforming growth factor beta 1 (TGF-β) and immune checkpoint molecules such as programmed death-ligand 1 (PD-L1). In addition, immune cells such as myeloid-derived suppressor cells and regulatory T cells contribute to the ability of cancers to evade the immune system (5, 6). Moreover, decreased tumor antigen presentation in the TME further contributes to the impaired functions of antigen-presenting cells (APCs). Consequently, antitumor T cells remain impaired or absent in the immunosuppressive TME, and the tumor persists. Not surprisingly, many modern-day immunotherapies focus on correcting the underlying TME-associated immune evasion strategies, with the goal of facilitating the initiation of an antitumor T cell response (7).

Functional activation of antitumor T cells requires three signals: (#1) tumor antigen presentation in the context of major histocompatibility complex (MHC), (#2) co-stimulatory signals such as cluster of differentiation 28 signaling, and (#3) the presence of cytokines like interferons (IFNs) (8, 9). Although the TME actively discourages the presence of one or more of these essential signals, therapeutic interventions can be used to overcome these immunosuppressive effects. One such strategy is to induce immunogenic cell death (ICD) during cancer therapy (10). As the name suggests, ICD is a process where apoptotic cells elicit an immunogenic response through the induction of damage-associated molecular patterns (DAMPs) that can be recognized by various immune cells (11, 12). More specifically, through the release of DAMPs, ICD increases the adjuvanticity, facilitating the signals # 2 and 3, within TME (10). This occurs through the production of chemoattracting agents such as chemokine C–X–C motif ligand (CXCL) 1 and chemokine (C–C motif) ligand 2 (CCL2) by dying cancer cells and subsequent recruitment of innate immune cells such as neutrophils and DCs to the TME (13). These events, in combination with both the release of nucleic acids from dying cancer cells and a cascade of other DAMPs, enable neo-epitope presentation of the cancer cell (10). This increased antigenicity, facilitating the signal # 1, is reflected through the ICD-enhanced antigen presentation (capture, processing, and presentation *via* MHC) from recruited APCs (10, 13, 14). Consequently, this leads to the activation of T cell response. Importantly, ICD-induced T cell immunity can establish immunological memory capable ensuring the longevity of remission, as opposed to non-regulated cell death. Such processes have been linked to tumor cell death in *in vitro* as well as *in vivo* mouse models (15). Taken together, ICD enhances the adjuvanticity and antigenicity of the cancer cells in the TME and facilitates the development of the three essential signals discussed earlier that are necessary for the activation of antitumor T cell responses (10).

For ICD to be successfully induced, the onset of a specific combination of DAMPs is required. The exact combination of DAMPs needed to induce ICD lies outside of the scope of this review and has been described elsewhere (10, 16). It is important to note, however, that the DAMPs that drive the induction of ICD are dependent on the treatment modality which is being used. For example, while chemotherapy-induced ICD requires the induction of autophagy, pathogen-induced ICD does not (10). Regardless, in the context of ICD, the initiation of an immune response begins with the release of lymphocyte chemoattracting agents, and the presentation of early apoptotic surface markers that tag dying cells for phagocytosis by APCs. In this process, the unfolded protein response (UPR) causes the translocation and expression of endoplasmic reticulum (ER) chaperones, such as calreticulin (CALR), to the cell surface. The induction of autophagy enables the cell to attract APCs to the TME *via* the release of intracellular ATP stores. This further functions to activate both inflammasome signaling and the APCs themselves (17). The secretion of annexin A1 (ANXA1) helps guide the APCs to the dying cancer cells where they become activated. In addition, the extracellular release of high-mobility group box-1 (HMGB1) stimulates an inflammatory response *via* toll-like receptor (TLR)-4 signaling (18). This involves the induction of the type 1 IFN response, resulting in CXCL10 release that enables neutrophil, APC, and T cell recruitment (10, 19, 20). Cumulatively, these ICD hallmarks activate APCs, which then stimulate antitumor T cells, leading to tumor eradication.

Interestingly, the expression of many of these ICD-associated DAMPs is governed by small heritable changes to the genome called epigenetic modifications. Epigenetic modifications result in changes to gene expression through chromatin remodeling mechanisms that include DNA methylation, histone modification, and non-coding RNA (ncRNA) (21, 22). Epigenetic modifications can silence or activate genes involved in tumor suppression or oncogenesis, respectively. In relation to immunity, epigenetic modifying drugs have the potential to boost the immune response by increasing antigen presentation, the expression of co-stimulatory molecules, and the display of MHC molecules; all paving the way for more efficient antigen presentation to T cells (23). In particular, DNA methylation has been investigated in many immune-related studies, where it silences genes such as TLR-3 and mitochondrial–antiviral signaling protein (24–26). Therefore, it is plausible that epigenetic modifications have a regulatory role to play when considering the induction of antitumor immunity.

In this review, we propose that various epigenetic events are actively involved in the regulation of ICD-associated DAMP expression. By recognizing that epigenetic modifications are involved in the induction of individual DAMPs, the efficacy of many cancer immunotherapies can be improved. Herein, we extensively discuss the current evidence that identifies direct links between epigenetic modifications and ICD in the context of TME and cancer immunotherapy.

EPIGENETIC REGULATION OF ICD HALLMARKS

In the context of cancer therapy, ICD occurs when a therapeutic treatment induces the expression of a specific combination of

"hallmarks" during cancer cell death. These hallmarks are a set of premortem stress responses that promote the expression of "danger signals" from the dying cancer cell, which can then be recognized by immune cells to trigger antitumor T cell activation. As shown in **Figure 1A**, major ICD hallmarks consist of various DAMPs that inevitably result in the development of T cell immunity.

What is becoming increasingly clear is that most of the ICD hallmarks are directly or indirectly regulated through epigenetic mechanisms. In addition, many currently investigated therapeutic epigenetic modulators (e.g., HDAC inhibitors) are being recognized for their actions in dendritic cell activation, antigen uptake, and T cell activation (27, 28) (**Table 1**). Thus, the epigenome, through its inherent or therapeutically modified activities, can be exploited to harness the antitumor benefits of ICD.

The UPR and ER Chaperones

The ER is critically important in the synthesis, modification, and transport of proteins (40, 41). When under physiological stress, the ER initiates the UPR, an evolutionarily conserved mechanism which, in the context of ICD, is characterized by the translocation of ER chaperones to the cell surface (42). Herein, ER chaperones function as "eat me" signals that mark the cell for uptake by APCs (10). Some ER chaperones that have been implicated in ICD include heat shock proteins (HSPs, e.g., HSP70 and HSP90) as well as CALR (43). The UPR is initiated by the activation of three main stress sensors; inositol-requiring enzyme-1 (IRE1), protein kinase RNA-like ER kinase (PERK), and transcription factor 6 (44). The ER chaperone immunoglobin protein (BiP) binds to IRE1 and PERK, suppressing their activity (45). Under ER stress conditions, BiP binds to misfolded proteins and no longer suppresses the sensor's activity, triggering the UPR (44).

One of the implicated sensors, IRE1, increases in expression upon treatment with an inhibitor of histone lysine methyltransferase (HKMTi). HKMT enzymes work by transferring methyl groups to lysine residues of histone proteins, and in this case, result in the transcriptional silencing of IRE1 (46, 47). Specifically, treatment of lung cancer cells with *Chaetocin* (**Table 1**), a nonspecific HKMTi, increases the expression of IRE1, suggesting that not only is IRE1 regulated *via* BiP but it may also be regulated *via* histone methylation (38, 39).

In relation to the UPR, HSP expression increases in response to stress stimuli as an effort to cope with the denaturation of proteins (48). Two types of HSPs (HSP70 and HSP90) have been shown to be directly regulated *via* promoter methylation in mammalian cells (49, 50).

The distal portion of the promoter region of HSP70 is aberrantly methylated during thermal stress, restricting the binding of POU class 2 homeobox-1 (POU2F1) to the HSP70 promoter (51). In T cells, the role of POU2F1 has been shown to contribute to the timing of cytokine expression in CD4 T cells and has also been shown to promote the development of effector T cell lineages (52). In addition, the expression of HSP90 is inhibited by DNA methylation in both pancreatic and colon cancer cell lines as a consequence of enhanced DNA methyltransferase (DNMT) expression (53). HSP90 regulates the transcription of DNMT enzymes, where HSP90s decrease in expression has been shown to result in an increased expression of DNMTs. DNMT-mediated hypermethylation events then result in the silencing of known tumor suppressor genes (TSGs). For aberrantly methylated HSPs, epigenetic drugs such as Zebularine may correct detrimental hypermethylation events in addition to inducing the UPR helping to induce a more robust immune response (**Table 1**). Other ER chaperones, such as CALR, are regulated by the presence of ncRNAs whose promoters are hypermethylated in some cancer models (54, 55). In this case, methylation events can work in favor of inducing CALR exposure.

Calreticulin is the most studied "eat me" signal in regards to ICD and is a main player in cultivating the ICD-induced antitumor response (56–58). Although their roles have not been fully elucidated, ncRNA such as RB1 and miR-27a are beginning to be recognized as key players in regulating CALR exposure (54, 55). Within the recent years, however, the roles of a few ncRNA have indeed been more thoroughly analyzed. For example, nc886 has been shown to regulate phosphorylation events that are necessary for proper CALR exposure (**Figure 1B**) (59). Specifically, eukaryotic transcription initiation factor 2 (eIF2α) is an important protein involved in the exposure of CALR and must be phosphorylated for CALR exposure to be initiated (60). In cholangiocarcinoma, the downregulation nc886 leads to the induction of apoptosis through the phosphorylation of eIF2α (59). When compared with normal tissues, gastric cancer cell lines were found to have hypermethylated CpG islands in the nc886 gene (61). Hypermethylation of nc886 prevents its typical function of discouraging the activation of protein kinase R (PKR), allowing proper phosphorylation of eIF2α and subsequent CALR exposure (**Figure 1B**). Conversely, expression of nc886 discourages CALR exposure; its expression prevents PKR from catalyzing the phosphorylation of eIF2α, revealing an adverse effect that may be observed with the use of an epigenetic modifying drug. These drugs could further have adverse effects by co-upregulating counterbalancing molecules of ICD hallmarks, such as CD47, a molecule that offsets the pro-phagocytic functions of CALR (62).

Autophagy Induction

Autophagy is an evolutionarily conserved mechanism that functions to maintain cellular homeostasis during times of starvation and stress (63). The induction of autophagy enables harmful or damaged cellular components to be sequestered into autophagosomes, and then broken down *via* lysosomal degradation (64). While the role of autophagy in cancer is still being fully elucidated, it appears to be context dependent (65, 66).

During the process of ICD, the induction of autophagy results in vesicular ATP pools to be transported and secreted from the cell (10). The secretion of ATP activates signaling pathways *via* purinergic receptors P2Y2 (P2RY2) and P2RX7 acting as a "find me" signals that promote maturation as well as TME recruitment of APCs (19, 20, 67). In APCs, the interaction of ATP with P2RY2 induces a robust chemotactic effect, while its interaction with P2RX7 results in the release of immunostimulatory cytokines (67). The expression of the P2RX7 receptor has been shown to be controlled *via* promoter methylation in submandibular carcinomas, where aberrant methylation events decrease its expression, which presumably would prevent proper P2RX7 signaling during ICD (68).

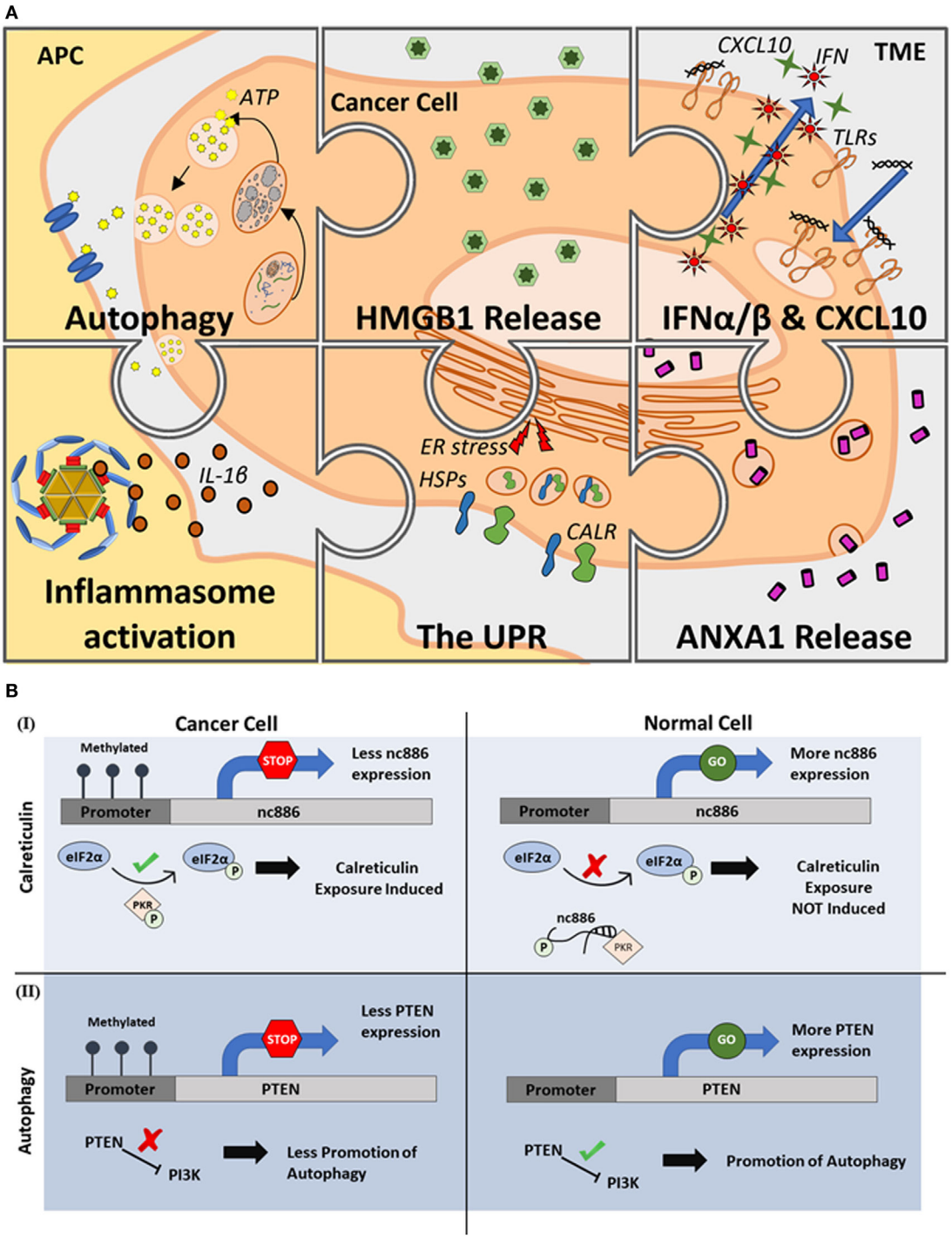

FIGURE 1 | Epigenetic regulation of immunogenic cell death (ICD). **(A)** Major hallmarks of ICD. Induction of ICD has been shown to be associated with six major hallmark processes including the induction of autophagy and release of ATP, high-mobility group box-1 (HMGB1) and annexin A1 (ANXA1) release, toll-like receptor (TLR) signaling that leads to interferon (IFN) α/β and CXCL10 release, inflammasome activation and interleukin-1β (IL-1β) secretion, and endoplasmic reticulum (ER) stress causing the unfolded protein response (UPR) that induces ER chaperones, especially calreticulin (CALR), expression on the cell surface. **(B)** Positive and negative regulation of ICD through epigenetic mechanisms. As illustrated through two distinct examples, activatory (I) or suppressive (II) effects of DNA methylation can either promote or suppress the molecular events leading to ICD. [**(B)**, I], DNA methylation events positively influence the induction of ICD by suppressing the expression of a non-coding RNA (nc886) whose function prevents the successful phosphorylation of eukaryotic transcription initiation factor 2 (eIF2α) inhibiting CALR exposure. [**(B)**, II] DNA methylation events negatively influence the induction of ICD by suppressing the expression of phosphatase and tensin homolog (PTEN) whose expression is needed to initiate pathways leading to autophagy initiation. Abbreviations: APC, antigen-presenting cell; TME, tumor microenvironment.

TABLE 1 | Epigenetic modulators shown to induce the expression of various ICD hallmarks, studied outside the context of ICD.

Type	Epigenetic modulators	ICD hallmarks							Reference
		UPR	Autophagy	ANXA1	HMGB1	Type I IFN	CXCL10	Inflammasome	
DNMTi	Azacitidine		Yes		Yes	Yes			(29, 30)
	Decitabine					Yes	Yes		(31, 32)
	Zebularine	Yes							(33)
HDACi	Vorinostat (SAHA)						Yes	Yes	(34, 35)
	FR235222			Yes					(36)
	Sodium butyrate			Yes					(37)
	Romidepsin		Yes		Yes		Yes		(30)
HKMTi	Chaetocin	Yes							(38, 39)

DNMTi, DNA methyltransferase inhibitor; HDACi, histone deacetylase inhibitor; HKMTi, histone lysine methyltransferase inhibitor; UPR, unfolded protein response; ANXA1, annexin A1; HMGB1, high-mobility group box-1; IFN, interferon; CXCL10, C–X–C motif chemokine 10; ICD, immunogenic cell death.

It is important to note that while other mechanisms are capable of triggering ATP release (69–71), a successful autophagic response is required for the optimal levels of ATP to be released for an immunogenic response (10, 64, 67). Autophagy induction requires multiple cellular processes to occur in tandem, such as the expression of TSGs phosphatase, tensin homolog (PTEN), and autophagy-related protein 5 (ATG5) (10). PTEN promotes autophagy by inhibiting the activation of phosphoinositide 3-kinase (PI3K) signaling (67) (**Figure 1B**), while ATG5 mediates autophagosome formation (72). Interestingly, PTEN is one of the most commonly mutated or inactivated genes during cancer development (73). The PTEN promoter is also known to be hypermethylated in breast and gastric cancers, as well as in melanoma and soft tissue sarcomas (74–77). During the development of many cancers, including colorectal cancer and melanoma, ATG5 is often downregulated (78, 79). Interestingly, it has been demonstrated in melanoma that ATG5 downregulation is a consequence of a hypermethylation of the promoter site (79). The hypermethylation status of these genes in cancers represents an ideal target for demethylating agents (e.g., Azacitidine) to promote autophagy (**Table 1**).

It is possible to induce autophagy through many mechanisms. Caloric restriction mimetics (CRMs), which induce autophagy by mimicking biochemical effects of nutrient deprivation, have been shown to stimulate ATP release in a protein deacetylation-dependent manner. Specifically, CRMs influence the acetylation of histone proteins, ultimately influencing gene expression and displaying a potential epigenetic mechanism that influences whether or not autophagy is induced (32, 80). Related, autophagy can also be induced *via* photodynamic therapy. Following exposure to photosensitizers, multiple human cancer cell lines showed the surface expression of CALR and released ATP before the signatures of apoptosis could be detected. In fact, both of these processes seem to have overlapping regulatory mechanisms, operating through PERK signaling and PI3K pathways, suggesting that the interplay between ICD and DAMP induction requires further elucidation (58).

ANXA1 Release

Annexin A1, known for its immunosuppressive functions (81), has recently been found to contribute to DC function during ICD. Here, ANXA1 released from the apoptotic cells can bind to formyl peptide receptor 1 receptor on APCs, enabling the stable interaction between the APC and dying cancer cell (82–84). As such, ANXA1 functions to enable antigen uptake and cross presentation of tumor antigens (84). Interestingly, ANXA1 is silenced by methylation in nasopharyngeal cancer cell lines and aberrantly methylated in breast and non-small cell lung cancer (85–88). Here, the use of a DNMTi may be an attractive tool, allowing the restoration of ANXA1 expression and secretion in the context of ICD (**Table 1**).

In head and neck squamous carcinoma, the expression of ANXA1 is inversely correlated with the expression of a specific microRNA (miRNA-196). This miRNA directly targets ANXA1 by binding to the untranslated region on the ANXA1 mRNA transcript (82). The expression of this miRNA is controlled by DNA methylation in many different cancer cell lines including breast, colon, liver, lung, brain, and oral (89). Without the expression of miRNA-196, ANXA1 would no longer be silenced, allowing proper ANXA1 release during ICD induction. As mentioned, epigenetic modifiers (either hypo- or hypermethylating) can be employed in the context of cancers and TME. In this case, induction of *de novo* DNA hypermethylation by inserting CpG-free DNA may control the miRNA-196-regulated release of ANXA1 for ICD induction (90).

HMGB1 Release

High-mobility group box-1 is found in nearly all eukaryotic cells and is highly conserved and abundant (91). Much like autophagy, it is important to note that HMGB1 has both a positive and negative correlation in regard to cancer progression (92). HMGB1 has multiple roles: within the nucleus it facilitates the transcription of many genes by modulating nucleosomes, while when secreted, it functions as a DAMP (91, 93). The mechanisms that regulate this secretion, however, remain unclear (10, 94). Extracellular HMGB1 signaling is facilitated by numerous receptors, where its binding is heavily dependent on its redox form (94). An important signaling pathway in the context of ICD is the extracellular binding of HMGB1 to TLR4 on APCs, initiating a signal transduction through the adaptor protein MyD88 (10, 18, 94). This pathway has been shown to be required to evoke ICD and subsequent T cell immunity, as $Tlr4^{-/-}$ and $Myd88^{-/-}$ mice do not develop antitumor immunological memory (18).

The link between HMGB1 and epigenetic regulation has already been hypothesized, and it is postulated that HMGB1 itself acts as an epigenetic modifier that leads to the silencing of tumor necrosis factor alpha and interleukin-1 beta (IL-1β) (91). Like the miRNA-196-based regulatory mechanism discussed in relation to ANXA1 release, miRNA-129-2, a tumor suppressor in glioma and hepatocellular carcinoma (95, 96), directly targets HMGB1 and inhibits its release. The regulatory region of this miRNA is heavily methylated in portions of its promoter region, resulting in its suppression and subsequent expression of HMGB1 (97, 98). In relation to gliomas, this methylation occurs more frequently in cancerous tissues when compared with normal tissues (95). As with some of the previously discussed ICD hallmarks, this implication is positive in relation to ICD. Interestingly, inducing expression of this miRNA would not be beneficial in this case. In fact, similar to ANXA1, the induction of *de novo* methylation in models where this miRNA is expressed may be a better choice of treatment (90).

Type I IFN Production and CXCL10 Secretion

Type I IFNs are secreted as DAMPs from infected cells to both signal and activate antimicrobial responses and initiate the innate and adaptive immune system (10). Characteristic type I IFNs (IFNα and IFNβ) primarily signal through the heterodimeric IFNα receptor (10, 99), eliciting a vast range of responses that are dependent on environmental factors, the extent of infection, and the hosts' physiological status (99). In the context of ICD, a major role for type I IFNs is to activate signaling cascades to produce more IFNs that act in both an autocrine and paracrine fashion (10). Moreover, like ATP secretion, type I IFNs also act as chemokines to attract APCs to the TME and play a pivotal role in APC maturation and T cell activation (100, 101). Thus, type I IFNs are important mediators of the signals # 2 (co-stimulatory signals) and 3 (cytokine presence) that are required for the induction of T cell immunity.

A clear link exists between epigenetic regulation and IFNs. First, expression of HDAC3 (a histone deacetylase) has been found to be necessary for IFN-β expression showing the regulatory role of HDAC3 in controlling IFN-β expression (99). Second, CXCL10 secretion is a subsequent result of IFN signaling (102). Its expression has been found to increase upon treatment with demethylating agents in ovarian cancer cells, suggesting that promoter methylation controls CXCL10 expression (103). The epigenetic regulation of CXCL10 in ovarian cancer suggests that treatment with a demethylating agent such as Decitabine (which is also known to induce type I IFN signaling) could aid in the induction of ICD (**Table 1**).

NLRP3 Inflammasome Signaling

During ICD induction, DAMPs are able to trigger pro-inflammatory events by activating inflammasomes (104). Inflammasomes are large multi-protein complexes, often consisting of caspase 1, within which the maturation of pro-inflammatory cytokines such as IL-1β and IL-18 takes place (104). One of the most well-characterized inflammasome complexes is called NLR family pyrin domain containing 3 inflammasome (NLPR3), which consists of a caspase recruiting domain (ASC), a cytosolic pattern recognition receptor, and a pro-caspase 1 (105). In 2014, Salminen et al. found that the ASC domain is identical to the domain that was termed as methylation-induced silencing-1 (TMS1) (105). The same study outlined that the promoter of the TSM1 gene is aberrantly methylated in many cancer cell lines, suggesting that this process regulates the expression of inflammasomes and the induction of apoptosis. Assuming methylation events alter the function of the ASC domain, aberrant events may prevent the successful induction of ICD by preventing proper inflammasome formation.

Another layer of complexity is added when the production of IL-1β is considered. Many interleukin genes have been shown to be methylated in cancers (106–108). In addition, interleukins have also been shown to have powerful antitumor roles by inhibiting the growth of lung tumors, and by stimulating the immune system to engage antiangiogenic mechanisms (109). Interestingly, the promoter of IL-1β has the highest methylation status out of all interleukin genes studied in lung cancer (29). In this model, aberrant promoter methylation of this important ICD-related interleukin would prevent the successful induction of ICD, revealing a potential therapeutic target using a demethylating drug (**Table 1**).

CONCLUSION AND FUTURE DIRECTIONS

The immunogenic response initiated through ICD can overcome the immunosuppressive nature of the TME. This leads to the restoration of the three signals required for proper T cell activation, including increased antigen presentation following cancer cell apoptosis and phagocytosis (signal # 1), co-stimulation from matured and recruited APCs (signal # 2), and the production of cytokines from both the cancer (e.g., IFNs) and APCs (e.g., IL-1β) (signal # 3). Therefore, the successful induction of ICD leads to the activation of antitumor T cells, which in turn can kill cancer cells and prevent recurring disease. Therefore, understanding how epigenetic modifications contribute to ICD is important when aiming to improve the efficacy of current cancer immunotherapies.

As highlighted above, many of the processes that govern ICD are regulated through epigenetic modifications. Interestingly, the initiation of individual ICD hallmarks, upon treatment with epigenetic modifying drugs, has been observed in studies that may not have been directly evaluating ICD induction (**Table 1**). As a result, the combination of epigenetic modifiers and immunotherapies offer an attractive avenue to elicit more robust antitumor T cell immunity. In fact, this concept is already being applied. The combination of Azacitidine and Romidepsin with IFNα elicits *bona fide* ICD in colorectal cancer cells (30). Further, treatment with Decitabine triggers a "viral" or "altered-self" mimicry state in these cells that leads to ICD hallmark expression through the retinoic acid inducible gene-I (RIG-I) pathway (31). This pathway has been shown to evoke ICD in melanoma, acute-promyelocytic leukemia, and pancreatic cancer models (31). Most recently, this concept has been shown to be important in neutrophil-based anticancer activity, where apoptotic cancer cells release epigenetically regulated cytokines such as CXCL1, CXCL10, and CCL2, driving nucleic acid-elicited phagocytosis of dying cancer cells by neutrophils (13, 110, 111).

However, it is still important to consider the possibility that epigenetic modifications could negatively affect the ability of CD8 T cells to recognize a cancer cell through ICD. It has already been established that epigenetic mechanisms tightly regulate the expression of MHC molecules, cytokines and other co-stimulatory molecules (112). Therefore, it cannot be ignored that adjusting these regulatory pathways using epigenetic modifiers may reduce the successful activation of specific CD8 T cells. In addition, increasing the secretion of a desired DAMP using epigenetic modulators may affect the expression of checkpoint molecules such as PD-L1 or suppressive metabolites such as indoleamine 2,3-dioxygenase (IDO1) in the cancer cells, causing them to respond to immunotherapeutic strategies differently. It has also been established that the miRNA-regulated mechanisms that control the expression of PD-L1 are also involved in the repression of IDO1 in cancer cells (113, 114). These points stress the complex relationship that exists between using epigenetic modifiers and their effect on ICD DAMPs.

Finally, the context-dependent roles of DAMPs must also be noted while considering ICD-based implications. For example, while HMGB1 excretion is involved in DC-based nucleic acid-sensing systems in ICD (115), it has also been shown to silence the expression of IL-1β in severe systemic inflammation by binding with histone H1, causing a change from euchromatin to heterochromatin at the IL-1β promoter (116). Therefore, the induction of one process (e.g., autophagy) that regulates a hallmark may suppress another (e.g., CALR exposure). Thus, when aiming to improve cancer therapy using epigenetic modifiers to induce hallmarks of ICD, the methylation status of ICD-related genes should be analyzed in each cancer model. This will enable an evaluation of both the benefits and adverse events that could result from the treatment modality of interest. Nonetheless, there is an undeniable link between the regulation of ICD hallmarks and epigenetics that cannot be ignored when evaluating the efficacy of novel cancer treatments.

AUTHOR CONTRIBUTIONS

BC and MG: conception, research, writing, editing. SG: conception, research, writing, editing, funding. PM, JP, and SM: research, writing, editing.

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REVIEW
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Targeting Macrophages in Cancer: From Bench to Bedside

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Macrophages are a major component of the tumor microenvironment and orchestrate various aspects of immunity. Within tumors, macrophages can reversibly alter their endotype in response to environmental cues, including hypoxia and stimuli derived from other immune cells, as well as the extracellular matrix. Depending on their activation status, macrophages can exert dual influences on tumorigenesis by either antagonizing the cytotoxic activity immune cells or by enhancing antitumor responses. In most solid cancers, increased infiltration with tumor-associated macrophages (TAMs) has long been associated with poor patient prognosis, highlighting their value as potential diagnostic and prognostic biomarkers in cancer. A number of macrophage-centered approaches to anticancer therapy have been investigated, and include strategies to block their tumor-promoting activities or exploit their antitumor effector functions. Integrating therapeutic strategies to target TAMs to complement conventional therapies has yielded promising results in preclinical trials and warrants further investigation to determine its translational benefit in human cancer patients. In this review, we discuss the molecular mechanisms underlying the pro-tumorigenic programming of macrophages and provide a comprehensive update of macrophage-targeted therapies for the treatment of solid cancers.

Keywords: macrophages, immunotherapy, macrophage polarization, inflammation, cancer

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INTRODUCTION

Tumors are complex tissues where cancer cells maintain intricate interactions with their surrounding stroma. Important components of the tumor stroma include macrophages, which are intimately involved in tumor rejection, promotion, and metastasis. In some cases, macrophages can comprise up to 50% of the tumor mass, and their abundance is associated with a poor clinical outcome in most cancers. Tumor-associated macrophages (TAMs) promote tumor growth by facilitating angiogenesis, immunosuppression, and inflammation, and can also influence tumor relapse after conventional anticancer therapies. Strategies aimed at targeting TAMs have shown great promise in mouse models, and a number of these agents are currently under clinical investigation. Here, we review current understanding of how TAMs regulate tumor progression and provide a comprehensive update of therapies targeting macrophages for the treatment of solid cancers. We also evaluate the contribution of TAMs in moderating the effectiveness of different anticancer treatment modalities and reflect on the challenges that need to be addressed to successfully incorporate the targeting of TAMs into current anticancer regimens.

THE ONTOGENY OF TAMs

Macrophages are required to maintain homeostasis in the organs they occupy. Given the specific needs of each tissue microenvironment, there are many different types of macrophages with

morphologically and functionally distinct characteristics. Prototypical examples include liver Kupffer cells, brain microglia, and lung alveolar macrophages, which together reflect the versatility of the mononuclear phagocytic system.

Tissue-resident macrophages were long considered to be recruited from bone-marrow progenitors that differentiated into mature cells upon seeding into tissues (1). However, new evidence indicates that these macrophages are derived from yolk sac precursors, which arise during early development and persist locally *via* self-renewal (2). In a similar vein, TAMs were once hypothesized to originate from circulating monocytes that were recruited in response to chemotactic signals released from tumor cells. While monocyte-derived TAMs are continuously replenished by peripheral recruitment, a small proportion of TAMs can also arise from tissue-resident macrophages that are partially maintained through *in situ* proliferation (3, 4).

Circulating cells that are recruited into tissues and subsequently differentiate into TAMs include inflammatory monocytes and monocyte-related, myeloid-derived suppressor cells (MDSCs). The differentiation of inflammatory $Ly6C^{High}$ monocytes into TAMs depends on RBPJ, the transcriptional regulator of Notch signaling (3). Genetic ablation of the *Rbpj* gene reduced tumor burden in a spontaneous mouse model of breast cancer, indicating the absolute requirement of these monocyte-derived TAMs in supporting tumor growth (3). A smaller subset of TAMs may also arise from $Ly6C^{Low}$ monocytes, which include cells that express the angiopoietin-2 (ANG2) receptor TIE2 (5). These TIE2-expressing cells are recruited in response to the secretion of ANG2 by tumor endothelial cells and play non-redundant roles during tumor neovascularization (6). By contrast, inhibition of STAT3 caused by upregulation of CD45 phosphatase activity is a key process that mediates the differentiation of MDSCs into mature TAMs (7). MDSCs may exhibit a $Ly6C^{High}Ly6G^{Neg}$ monocytic or a $Ly6C^{Int}Ly6G^{High}$ granulocytic endotype (8). Since the monocytic MDSCs strongly resemble $Ly6C^{High}$ monocytes, it is hypothesized that these cells represent a precursor functional state of these inflammatory cells (8).

Tissue-resident macrophages coexist with recruited macrophages in tumors with potentially distinct roles. In glioblastoma, TAMs are comprised of a mixed population of cells including resident microglia and bone marrow-derived monocytes and macrophages (9). The relative contribution of these populations in glioma progression was investigated in a genetically engineered mouse model, in which the chemokine CX3CR1/CX3CL1 signaling was ablated in both microglia and inflammatory monocytes (9). CX3CR1 is expressed by circulating monocytes and exclusively by microglia in the central nervous system, while its ligand CX3CL1 is expressed by neurons and serves as a chemotactic signal. Loss of *Cx3cr1* in the host microenvironment facilitated the recruitment of $Ly6C^{High}$ "inflammatory monocytes" into tumor tissues, which were responsible for increased tumor incidence and shorter survival times in glioma-bearing mice. By contrast, selective ablation of *Cx3cr1* in microglia had no impact on glioma growth (9). These results suggest that the tumor-promoting effect observed upon *Cx3cr1* ablation is conferred by infiltrating inflammatory monocytes and highlights the contrasting roles of bone marrow-derived and tissue resident-derived TAMs. However, since this may also depend on tumor type, the contribution of tissue-resident versus recruited TAMs in tumorigenesis warrants further investigation.

TAM FUNCTION AND DIVERSITY

Tumor-associated macrophage heterogeneity is not only dependent on the nature of their monocytic precursor, but also on their functional diversity. To coordinate complex processes to promote immunity, while also minimizing damage to tissues where these responses occur, macrophages can reversibly alter their endotype in response to environmental cues. These environmental cues include stimuli derived from pathogens, parenchymal, and immune cells, as well as the extracellular matrix (10, 11).

Similar to the Th1/Th2 T-cell dichotomy, macrophages may be broadly classified into two groups, referred to as "classically activated M1" (CAM) or "alternatively activated M2" (AAM) endotypes. Much our understanding of macrophage polarization has relied on *in vitro* techniques, whereby macrophages are stimulated with M1- or M2-polarizing signals (12). For M1 this typically involves stimulation with IFNγ or lipopolysaccharide (LPS), while M2 polarization usually involves stimulation with IL4 or IL13 (12). Changes in gene expression, cell-surface markers and signaling pathways have subsequently been used to distinguish the various activation states (**Table 1**), and the contribution of some of these factors in mediating CAM/AAM characteristics has been validated in genetically engineered mouse models (**Table 2**). However, given the heterogeneity of tissues, macrophage polarization should be regarded as a complex process that occurs over a continuum (10, 13).

The current classification of CAM or M1 macrophages is in part based on their response to stimulation with bacterial LPS, TNFα, and/or IFNγ (**Table 1**). TNFα is produced by antigen presenting cells upon recognition of pathogenic signals, while IFNγ is produced by innate and adaptive immune cells such as natural killer (NK) and Th1 cells (10, 40). Once activated, CAMs secrete pro-inflammatory cytokines (IL1, IL6, and TNFα) and effector molecules (including reactive nitrogen intermediates) and express chemokines such as CXCL9 and CXCL0 (10). These molecules exert and amplify antimicrobial and tumoricidal activities alongside increased Th1 adaptive immune responses through enhanced antigen presentation. Because these cytokines play an important role in immune defense, their inappropriate release can result in chronic inflammation and extensive tissue damage (41).

Alternatively activated M2 macrophages are broadly characterized by their anti-inflammatory and wound-healing endotype (42). While these functional outputs are important for the maintenance of tissue homeostasis, aberrant AAM activation can trigger allergic reactions, promote tumor growth, and delay immune responses toward pathogens (43–45). Among the most important activators of AAMs are IL4, IL10, and IL13; however, several other stimuli and signaling pathways can also induce AAM polarization (**Table 1**). Thus, AAMs can be further divided into M2a, M2b, M2c, and M2d (12, 46). The M2a subtype is stimulated in response to IL4, IL13, as well as fungal and helminth infections. M2a macrophages express high levels of mannose

TABLE 1 | Characteristics of classically activated M1 (CAM) and alternatively activated M2 (AAM) endotypes.

	CAM	AAM			
	M1	**M2a**	**M2b**	**M2c**	**M2d**
Stimuli	IFNγ Lipopolysaccharide GM-CSF	IL4 IL13 Fungal/helminth infection	IL1R	IL10 TGFβ GCs	IL6 LIF Adenosine
Markers	CD40 CD86 CD80 CD68 MHC II IL1R TLR2 TLR4 SOCS3	CD163 MHC II SR CD206 YM1[a] FIZZ1[a] ARG1[a]	CD86 MHC II MerTK	CD163 TLR1 TLR8	VEGF
Cytokine secretion	TNFα IL1 IL6 IL12 IL23	IL10 TGFβ	IL1 IL6 IL10 TNFα	IL10 TGFB	IL10 IL12 TNFα TGFβ
Chemokine secretion	CCL10 CCL11 CCL5 CCL8 CCL4 CXCL9 CXCL10	CCL17 CCL22 CCL24	CCL1	CCR2	CCL5 CXCL10 CXCL16
Function	Inflammation, tissue damage, and pathogen clearance	Allergic inflammation, tissue repair, tissue remodeling, and fibrosis	Anti-inflammation, tissue remodeling, and fibrosis	Anti-inflammation	Tissue repair, angiogenesis

[a]*Denotes markers that are only found in mouse macrophages.*

receptor (CD206) and secrete large amounts of pro-fibrotic factors including fibronectin, insulin-like growth factor and TGFβ, which are all involved in wound healing and tissue repair. M2b macrophages are stimulated by immune complexes and bacterial LPS and exhibit upregulated expression of CD206 and the MER receptor tyrosine kinase. They primarily produce IL10, IL1β, IL6, and TNFα, which exert anti-inflammatory effects. M2c macrophages are activated by IL10, TGFβ, and glucocorticoids and are also generally thought to be anti-inflammatory in nature. Finally, differentiation of M2d macrophages occurs in response to co-stimulation with TLR ligands and adenosine (47). M2d macrophages express low levels of CD206 but are high producers of IL10 and VEGF. In light of these findings, it is now appreciated that the "AAM" terminology encompasses a functionally diverse group of macrophages that share the functional outputs of tumor progression by stimulating immunosuppression and angiogenesis.

MACROPHAGES IN CANCER INITIATION AND PROMOTION

Although macrophages are crucial for promoting host defenses, inappropriate or prolonged activation can result in damage to the host, immune dysregulation, and disease (48). In cancers, the role of macrophages in tumor progression remains to be fully elucidated, in part due to the contrasting roles they play depending on their polarization. On the one hand, studies have shown that macrophages are capable of exerting tumoricidal activity *in vitro* (49). Indeed, in colorectal cancer, TAMs are predominantly polarized toward a classically activated endotype and express pro-inflammatory cytokines such as IFNγ, which activate cytotoxic $CD8^+$ T-cell responses to promote tumor destruction (50). Another route by which TAMs can cause the death of tumor cells involves the production of macrophage migration inhibitor factor (MIF). In addition to inhibiting the recruitment of macrophages (51), MIF stimulates key tumoricidal functions such as phagocytosis (52), cellular toxicity and the release of TNFα and IL1β (53). The secretion of IL18 and IL22 by TAMs has also been associated with tumor cell killing as they can amplify cytokine production (particularly IFNγ and IL2) and by augmenting the cytotoxic activity of NK cells (54, 55).

Many macrophage depletion studies have highlighted the importance of TAMs in tumor development and progression (56, 57). Genetic ablation of the *Csf1* gene (encoding, macrophage colony-stimulating factor, and required for macrophage maturation) in mice susceptible to mammary carcinoma delayed metastasis and decreased tumor growth, while the transgenic expression of the corresponding CSF1 protein accelerated cancer progression and promoted pulmonary metastasis (56). Similar findings were also observed in a genetic model of thyroid cancer and in mice transplanted with human osteosarcoma cancer cells (58, 59). These findings suggest that there is a delicate balance between the tumoricidal and tumor-promoting functions of

TABLE 2 | Genetic mouse models of macrophage polarization.

Protein/gene	Genetic manipulation	Effect on macrophage polarization	Reference
IRF5/*Irf5*	KO and conditional LysM-Cre KO	↓↓ M1	(14, 15)
JUNB/*JunB*	Conditional LysM-Cre KO		(16)
KLF4/*Klf4*	Conditional LysM-Cre KO	↑ M1/↓ M2	(17)
TSC1/*Tsc1*	Conditional LysM-Cre KO		(18)
DAB2/*Dab2*	Conditional LysM-Cre KO		(19)
let-7c (mIR)	Knockdown and overexpression		(20)
mIR-223/*mir223*	KO		(21)
Rictor/*Rictor*	Conditional LysM-Cre KO	↑↑ M1	(22)
AKT1/*Akt1*	KO		(23)
IL4RA/*Il4ra*	KO and conditional LysM-Cre KO	↓↓ M2	(24, 25)
HCK/*Hck*	KO and knockdown		(26, 27)
STAT6/*Stat6*	KO		(28)
IRF4/*Irf4*	KO		(29)
PPARγ/*Pparg*	KO		(30)
JMJD3/*Jmjd3*	KO		(29)
P50/P105/*NfKb*	KO		(31)
PI3Kγ/*Pi3kγ*	KO		(32)
KLF6/*Klf6*	Conditional LysM-Cre KO	↑ M2/↓M1	(33)
mIR-33/*Mir33*	KO		(34)
MyD88/*myD88*	KO		(35)
AKT2/*Akt2*	KO	↑↑ M2	(23)
SHIP/*Inpp5d*	KO		(36)
SHP-2/*Ptpm6*	KO		(37)
p16 INK4a/*Cdkn2a*	KO		(38)
TNFR1/*Tnfrsf1a*	KO		(35)
TNF/*Tnf*	KO		(35, 39)

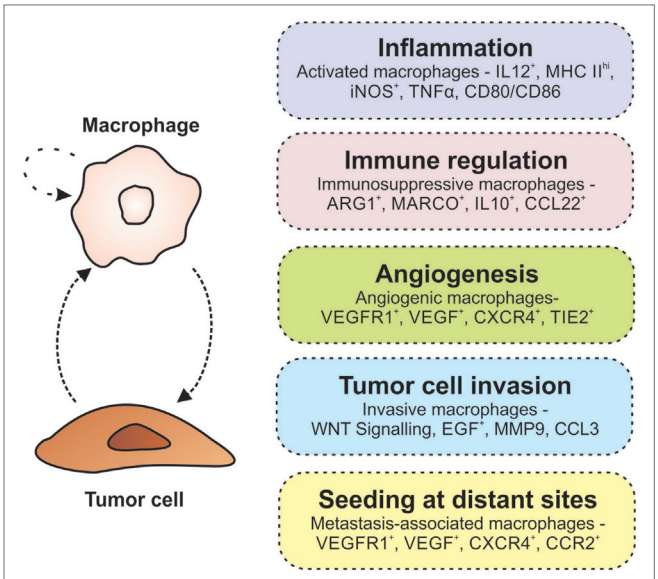

FIGURE 1 | Macrophages promote tumorigenesis. The interaction between macrophages and tumor cells results in an autocrine/paracrine loop that enhances their pro-tumorigenic properties. Within the tumor microenvironment, macrophages are involved in many activities associated with tumor growth and progression including inflammation, immune regulation, angiogenesis, invasion, and metastasis (indicated in each of the boxes on the right).

TAMs. To date, the tumor-promoting mechanisms of TAMs that have been well characterized include chronic inflammation, immune suppression, angiogenesis, and invasion/metastasis (**Figure 1**).

Chronic Inflammation

Chronic inflammation is associated with some solid cancers (60). Patients with inflammatory bowel disease including ulcerative colitis and Crohn's disease have an increased risk of developing neoplasia (61) owing to the production of TNFα (62), IL6 (63), and IL1β (64) by TAMs. This link between chronic inflammation and tumorigenesis is similarly observed in hepatocellular carcinoma (65), gastric cancer (66), and lung cancer (67). In these scenarios, the secretion of pro-inflammatory cytokines by macrophages in response to pathogens (e.g., *HBV* and *Helicobacter pylori*) and irritants (cigarette smoke) creates a mutagenic environment in the sub-epithelial stroma. These transformed neoplastic cells consequently produce inflammatory mediators including TNFα (68) and IL1β (69) that form a closed paracrine loop to perpetuate this tumor-reactive microenvironment.

Immune Suppression

Macrophages comprise a key component of the host immune responses, and they can facilitate tumor death by promoting cytotoxicity. For instance, stimulation of macrophages with granulocyte macrophage colony-stimulating factor GM-CSF or bacterial-derived CpG has been shown to activate toll-like receptor and enhance the secretion of immune-stimulatory cytokines that impair tumor growth and metastasis (70, 71). However, in the vast majority of cancers, macrophages exhibit an immunosuppressive endotype characterized by low levels of inflammatory molecules (IL18, IL12, and TNFα), and an increased expression of transcripts expressed by AAMs (*Il10*, *Stat3*, and *Il13*) (72, 73). This immunosuppressive effect has been proposed to occur due to STAT3 activation in AAMs opposing STAT1-driven Th1 antitumor responses (74). Likewise, expression of MHC class II molecules on TAMs is actively downregulated by tumor cell-derived TGFβ1, IL10, and PGE_2 and results in decreased Th1 differentiation (48).

The direct suppression of immune responses by TAMs has also been described. IL10, for example, upregulates the expression of programmed-death ligand (PD)-L1 on the surface of monocytes and TAMs (75). Although naïve T-cells can be stimulated by PDL1, its most prominent role is the inhibition of activated effector T-cells by ligation with the PD1 receptor. Indeed, high tumor expression of PDL1 is associated with increased tumor aggressiveness and mortality in renal cell carcinoma and ovarian cancer, with an inverse correlation between PDL1 expression and intraepithelial $CD8^+$ T-cell infiltration (76, 77). The expression of PDL1 and PDL2 by TAMs also triggers the expression of the regulatory molecules B7-H4 and VISTA in T-cells to elicit similar immunosuppressive functions (78). More recently, it has been shown that PI3Kγ signaling in TAMs inhibits NFκB activation while stimulating C/EBPβ, thereby triggering a transcriptional program that promotes immune suppression during inflammation and tumor growth (79). Another indirect mechanism by

which TAMs may promote immune suppression is the recruitment of other immune cells into the tumor milieu. Specifically, the production of chemokines including CCL17 and CCL22 attract Th2 and regulatory T-cells (Tregs) that steer monocyte differentiation toward an anti-inflammatory AAM endotype (80). Macrophage-derived CXCL13, CCL16, and CCL18 can also bind to their CXCR5, CCR1, and CCR8 receptors to promote the recruitment of eosinophils and naïve T-cells that suppress immune responses and promote tissue remodeling (80, 81).

Angiogenesis

The benign-to-malignant transition of most solid cancers is marked by a significant increase in blood vessel formation, known as the "angiogenic switch" (82). Hypoxia is a major driver of angiogenesis, and TAMs preferentially accumulate in poorly vascularized regions during early tumor formation (83). The transcription factor HIF1α is constitutively expressed in macrophages and acts as a major regulator of hypoxic stress by inducing a switch from aerobic to anaerobic metabolism (83). These changes correlate with an increased expression of the HIF1 target genes *Cxcr4*, *Ccl2*, and endothelins that enhance macrophage recruitment into tumors (83, 84).

Macrophages are central to the angiogenic switch, and their increased tumor infiltration directly correlates with blood vessel density in human tumors (85). Likewise, *Csf1* knockout mice that have reduced macrophage numbers are less susceptible to tumorigenesis, while *Csf1* overexpression results in macrophage accumulation, enhanced angiogenesis, and accelerated malignant transformation (86). Proangiogenic macrophages are associated with an AAM endotype and secrete a diverse range of factors including TGFβ, VEGF, PDGF, and fibrin (74, 87–89). They express an enrichment of transcripts that encode for angiogenic molecules, and the ablation of these genes inhibits the angiogenic switch (72, 90–92). A subset of AAMs characterized by cell-surface expression of TIE2, a marker of mature endothelial cells, has been shown to play an indispensable role in blood vessel formation (93). Co-injection of TIE2-expressing macrophages with tumor cells significantly enhanced angiogenesis (93), while therapeutic targeting of TIE2 resulted in tumor vasculature regression and inhibited the progression of late-stage, metastatic mammary tumors, and pancreatic carcinomas (94). Because these data strongly support a role for macrophages in promoting angiogenesis, inhibiting pathways involved in these processes provide a promising therapeutic approach for the treatment of cancer.

Tumor Cell Invasion and Metastasis

Metastasis represents the most important cause of cancer mortality and occurs when cancer cells dissociate from the primary tumor and spread to distal organs (95). While it is well established that macrophages constitute a major population at metastatic niches, their role in metastasis has only recently been appreciated (41). Metastatic progression is dependent on the cross talk between macrophages and cancer cells. For example, secretion of the extracellular matrix proteoglycan versican by the primary tumor stimulates metastasis in the Lewis Lung Carcinoma model through TLR2 and TNFα signaling in macrophages (96). Likewise, tumor cells also induce the expression of matrix metalloproteinase (MMP)-9 in macrophages to promote the release of matrix-bound VEGF, which enhances angiogenesis and metastasis (97).

Macrophages are the predominant cells at sites of basement membrane degradation during early tumorigenesis and at the invasive front of tumors during malignant transformation (95). They are a rich source of proteases including cathepsins, MMPs, and serine proteases that promote extracellular matrix degradation and allow the escape of tumors from the basement membrane through the dense stroma (98, 99). Furthermore, upregulation of CSF1 by tumor cells stimulates macrophage recruitment and the production of epidermal growth factor (EGF), which in turn promotes tumor cell migration. This paracrine loop involving EGF and CSF1 is crucial for tumor invasion, and the inhibition of either signaling pathway inhibits the migration of both cell types (95, 100). Consistent with this, CSF1 expression in human cancers is highest at the invasive edge where macrophages are most abundant (56). Other factors that drive macrophage-mediated tumor invasion include Wnt5a, which acts through the non-canonical WNT pathway to stimulate cancer cell motility (101), and SPARC/Osteonectin, which regulates the deposition of collagen fibers and expression of MMPs (102).

A distinct population of macrophages known as metastasis-associated macrophages (MAMs), which are recruited by CCL2, have been identified (103). Activation of the CCL2/CCR2 axis increased secretion of CCL3 by MAMs, which in turn facilitated metastatic seeding of breast cancer cells in the lung (103). Interestingly, MAM-derived CCL3 was also shown to act as an autocrine mediator for MAMs by prolonging their retention in metastatic foci and resulting in the enhanced extravasation of cancer cells to other organs (103). The CCL2/CCR2 axis between cancer cells and MAMs may also promote bone metastasis of prostate cancer by supporting the activation of osteoclasts (104). The destruction of bone by osteoclasts triggers the release of growth factors that support tumor growth (105), while the inhibition of these cells with neutralizing antibodies or shRNAs for CCL2 significantly impairs prostate cancer-induced formation of osteoclasts and bone resorption (106, 107). In another example, expression of vascular cell adhesion protein 1 on cancer cells enhanced tumor growth and lung metastasis through interaction with α_4-integrin expressed by MAMs (108). Collectively, these studies provide unequivocal evidence for the multidimensional role of macrophages in the establishment of metastatic niches as well as the extravasation of tumor cells to secondary organs.

MACROPHAGES AS DIAGNOSTIC AND PROGNOSTIC BIOMARKERS

The extent of macrophage infiltration serves as an important diagnostic and prognostic biomarker in many human cancers. The identification and quantification of TAMs can be performed through various methods, ranging from morphological discrimination to gene expression analysis and cell-surface marker profiling. Human TAMs are typically identified by CD68 expression; however, CD163, CD206, and CD204 are also commonly used to distinguish those of the AAM endotype (109, 110). By contrast,

macrophages with a CAM endotype in humans can be identified by CD40 (111) and HLA-DR expression (112).

Increased macrophage infiltration is associated with advanced stage disease and worse overall survival in breast (113), pancreatic (110) and bladder cancer (114). On the other hand, high macrophage density correlates with a favorable outcome in colorectal cancer (115). TAM density may also be used as a prognostic marker to predict chemotherapy response. In Hodgkin lymphoma, overexpression of a macrophage gene signature in diagnostic lymph-node samples is associated with primary treatment failure (116). The increased presence of $CD68^+$ macrophages was also negatively correlated with survival and secondary treatment outcome (116). In pancreatic cancer, TAMs are reported to be critical determinants of prognostic responsiveness to postsurgical adjuvant chemotherapy due to the re-education of TAMs to restrain tumor progression (110). Thus, the quantification of TAMs may also be used to stratify patients who are more likely to respond to postsurgical chemotherapy.

MACROPHAGES AS A THERAPEUTIC TARGET

Tumor initiation and progression is driven by interactions between stromal and immune cells within the tumor microenvironment. Thus, multitargeted approaches in which several of these cell types are simultaneously inhibited may represent a more efficient method to treat cancer, especially when used in conjunction with other strategies such as chemotherapy. One major advantage of targeting the tumor microenvironment is the genetic stability of non-tumor cells, which is in contrast to tumor cells that are often highly unstable and can rapidly accumulate adaptive mutations that confer drug resistance. Given the indispensable role of macrophages in tumorigenesis and their correlation with a poor overall survival, these findings provide a strong basis for targeting these cells within the tumor microenvironment. Indeed, the pharmacological inhibition of macrophages has shown great promise in mouse models (**Table 3**), and a number of these agents are currently under clinical investigation (**Table 4**). Major strategies targeting macrophages within the tumor microenvironment include macrophage depletion, modifying macrophage recruitment and macrophage reprogramming.

Macrophage Depletion

High TAM density is associated with a poor patient outcome and therapy resistance in most cancers. Macrophage depletion studies have shown great success in limiting tumor growth and metastatic spread, as well as restoring chemotherapeutic responsiveness (117, 118, 150). Trabectedin is a DNA-binding agent that exerts selective cytotoxicity to circulating monocytes and TAM populations by activating the extrinsic TRAIL apoptotic pathway. Monocytes in particular are sensitive to TRAIL as they express very low levels of TRAIL decoy receptors (151). In four different mouse tumor models, trabectedin significantly inhibited the production of cytokines including CCL2 and IL6, which are important in promoting tumor growth (117). Bisphosphonates comprise another class of drugs that exert myeloid cell cytotoxicity.

TABLE 3 | Selected targets of macrophage inhibition in mouse models.

Pathway targeted	Drug or target	Effect	Reference
Macrophage depletion	Trabectedin	Selective cytotoxicity in mononuclear phagocytes and inhibition of tumor-promoting cytokines	(117)
	Clodronate ± anti-VEGF mAB	Tumor regression and reduced angiogenesis	(118)
Macrophage recruitment	CCL2	Reduced tumor growth and metastasis in prostate and breast cancer	(119, 120)
	CXCL12/CXCR4	Reduced tumor growth and metastasis in breast and prostate cancer	(121, 122)
	CSF1 receptor (CSF1R)	Antiangiogenic and antimetastatic effects in melanoma and mammary xenograft tumors and improved chemotherapeutic responses	(123–125)
	CD11b	Enhanced tumor responses to radiation	(126)
Macrophage reprogramming [suppressing alternatively activated M2 (AAM)]	Jumonji	Impaired AAM differentiation and recruitment	(29)
	STAT6	Enhanced tumor immunity	(127)
	STAT3	Inhibited immunosuppressive cytokine profile of AAMs	(128, 129)
	Superoxide [O(2−)]	Impaired AAM differentiation	(130)
	IL4Rα	Less aggressive skin tumors	(131)
	COX2	Suppression of breast cancer metastasis	(132)
	PI3Kγ	Stimulation of T-cell-mediated tumor suppression and inhibition of tumor cell invasion and metastasis	(32)
	CSF1R	Increased survival and regressed established GBM tumors by reducing AAM polarization, but without affecting tumor-associated macrophage numbers in treated tumors	(125)
	HCK	Suppression of AAM polarization, enhanced tumor immunity in colon cancer	(27)
Macrophage reprogramming (classically activated M1 stimulating)	IFNα	Reduced tumor growth and promoted near complete abrogation of breast cancer metastasis	(133)
	CD40	Tumor regression and increased survival	(134)
	Histidine-rich glycoprotein	Reduced pancreatic and breast cancer metastasis and increased survival	(135)
	NFκB	Tumor regression	(136)

TABLE 4 | Summary of selected NIH clinical trials of macrophage inhibition.

Target	Phase	Trial number	Tumor type	Drug name/pharmacompany	Effect	Reference
CSF1/CSF1R	I/II	NCT01346358	Advanced solid tumors	IMC-CS4/Eli Lilly Inc.	CSF1 R-blocking antibody	(137)
		NCT01444404	Advanced solid tumors	AMG 820/Merck	CSF1 R-blocking antibody	(138)
		NCT01804530	Pancreatic cancer	PLX7486/Plexxikon Inc.	Kinase inhibitor of CSF1R and Trk	(139)
		NCT01004861	Advanced solid tumors	PLX3397/Plexxikon Inc.	Kinase inhibitor of CSF1R and cKit	(140)
CCL2/CCR2	II	NCT01015560	Bone metastasis	MLN1202//Millennium Pharmaceuticals Inc.	Anti-CCR2 antibody	(141)
		NCT01413022	Locally advanced pancreatic cancer	PF-04136309//Pfizer Inc.	CCR2 antagonist	(142)
IL6R	I/II	NCT01637532	Ovarian cancer	Tocilizumab and Peg-Intron/Genentech	IL6R monoclonal antibody	(143)
DNA repair mechanisms	III	NCT01692678	Liposarcoma and leimyosarcoma	YONDELIS (Trabectedin)/PharmaMar	DNA backbone cleavage and cell apoptosis	(144)
	II	NCT01772979	Ovarian cancer	YONDELIS	DNA backbone cleavage and cell apoptosis	(145)
	I	NCT01426633	Liposarcoma and leimyosarcoma	YONDELIS	DNA backbone cleavage and cell apoptosis	(146)
CD40	I/II	NCT01433172	Lung cancer	(GM.CD40L) vaccine in combination with CCL21	Boosts the immune system	(147)
	I/II	NCT01103635	Metastatic melanoma	Tremelimumab and CP-870, CP-893/ AstraZeneca	CD40 agonist mAb	(148)
STAT3	I	NCT01839604	Metastatic hepatocellular carcinoma	AZD9150/AstraZeneca	Antisense oligonucleotide inhibitor of STAT3	(149)

These drugs are typically used in the clinic for the treatment of osteoporosis and complications arising from bone metastases; however, macrophages in mammary tumors also display sensitivity to bisphosphonate-mediated apoptosis (152). In the clinic, bisphosphonates have been used to treat breast cancer and other solid malignancies in combination with chemotherapy and hormone therapy. This approach has substantially reduced disease recurrence and improved survival in treated patients compared with chemotherapy/hormone therapy alone (153).

In mice, clodronate-liposome-mediated depletion of TAMs significantly reduced tumorigenesis. When combined with anti-angiogenic therapy, administration of clodronate and anti-VEGF antibodies further enhanced TAM depletion and augmented tumor inhibition (118). Thus, macrophage depletion may represent a novel strategy for an indirect cancer therapy specifically aimed at tumor-promoting cells within the microenvironment. However, the challenge with this approach is to find ways for local administration of such drugs to the tumor. Indeed, a major disadvantage of most macrophage depletion studies is the systemic clearance of macrophages, which is unfavorable in clinical applications when host immune responses are already compromised by chemotherapy.

Limiting Macrophage Recruitment and Localization

Another option for targeting TAMs is by inhibiting their recruitment to the primary tumor. CCL2 is a chemokine that regulates the migration of monocytes and macrophages. In mice, interference with the CCL2/CCR2 axis significantly reduced the growth of hepatocellular and renal cell carcinomas (154, 155), and abrogated breast cancer metastasis (119). Interestingly, cessation of CCL2 inhibition accelerated breast cancer metastasis by promoting the infiltration of bone-marrow monocytes into tumors (156), indicating the importance of CCL2 signaling in regulating metastatic growth. In the clinic, antibodies that selectively target CCL2 have completed Phase I and II clinical trials (**Table 4**). In a Phase I trial, administration of the anti-CCL2 antibody carlumab (CNTO 888) was well tolerated and showed promising antitumor activity in patients with advanced disease. However, this response was not observed in the Phase II study involving patients with castration-resistant prostate cancer. Furthermore, preclinical studies combining anti-CCL2 with the antimitotic chemotherapy agent Docetaxel enhanced antitumor responses (157); however, combining anti-CCL2 with conventional chemotherapy has produced mixed results in Phase IB clinical trials. In one trial, administration of the anti-CCL2 agent carlumab in combination with four chemotherapy regimens was well tolerated although no significant tumor response was observed (158). By contrast, combining the oral CCR2 small-molecule antagonist PF-04136309 with conventional chemotherapy resulted in partial tumor responses (49%) with local tumor control in 97% of patients with advanced pancreatic ductal adenocarcinoma (PDAC). None of the patients in the chemotherapy-alone group achieved an objective response (159).

CXCL12 is a chemokine that facilitates the migration of macrophages through endothelial barriers and into the tumor milieu. The secretion of CXCL12 by stromal cells also attracts the movement of cancer cells by upregulating their expression of CXCR4 (121). For this reason, inhibition of CXCL12/CXCR4 signaling represents a promising strategy to modulate macrophage infiltration and prevent metastasis. Indeed, targeting CXCR4 in mouse models of breast and prostate cancer significantly reduced total tumor burden and metastases (121, 122). However, the therapeutic efficacy of inhibiting CXCL12 in human patients has yet to be tested in clinical trials.

In addition to targeting chemokines, antibodies against macrophage surface receptors such as CD11b and CSF1 receptor (CSF1R) may be used to impair macrophage recruitment (126, 160). In the case of CD11b, administration of a neutralizing CD11b monoclonal antibody reduced tumor growth in a mouse model of spontaneous intestinal adenoma, and enhanced antitumor responses to radiation by reducing myeloid cell infiltration (126, 161). However, since CD11b is also expressed on other immune cells including neutrophils, this approach is limited in its specificity against TAMs.

Targeting the CSF1–CSF1R axis represents a more specific strategy, since CSF1R is exclusively expressed on cells of the monocytic lineage and therefore provides a viable target to specifically inhibit TAMs (162). As a single agent, treatment of mice with the humanized anti-CSF1R antibody emactuzumab (RG7155) selectively reduced TAM infiltration and promoted $CD8^+$ T-cell expansion. Administration of emactuzumab to patients similarly led to a striking reduction of macrophages in tumor tissue, which translated to a marked clinical benefit for patients with diffuse-type giant cell tumors (163).

CSF1 receptor blockade in combination with conventional cancer treatments has also shown to improve the efficacy of radiotherapy, immunotherapy and chemotherapy. Locally recurrent disease and/or metastatic spread following radiotherapy has been attributed to an influx of bone marrow-derived monocytes that drive vasculature regrowth (164, 165). In mice harboring glioblastoma multiforme (GBM) xenografts, treatment with pexidartinib (PLX3397) augmented tumor responsiveness to radiotherapy by reducing the recruitment of bone marrow-derived TAMs (165). Pexidartinib also improved the antitumor efficacy of adoptive cell therapy in a syngeneic mouse model of BRAF (V600E)-driven melanoma (166). In agreement with previous studies of breast cancer models (167), inhibition of macrophage recruitment by CSF1R blockade enhanced the therapeutic efficacy of gemcitabine in a chemoresistant transgenic model of pancreatic cancer (168). Collectively, these results provide evidence for targeting the infiltration of TAMs as a complementary strategy to enhance the efficacy of conventional cancer therapies.

Macrophage Reprogramming

One key feature of macrophages is the plasticity of their endotype. Thus, the reprogramming of macrophages toward a tumoricidal CAM endotype has gained widespread interest as an attractive therapeutic strategy against cancer. This can either be achieved by preventing TAMs from adopting an AAM endotype or by promoting the repolarization of macrophages with an AAM endotype toward a tumoricidal CAM endotype.

Large-scale transcriptome studies performed on AAMs have identified key genes and signaling pathways that play a critical role in macrophage polarization. Jumonji domain containing-3 (JMJD3) protein, for example, is a histone 3 Lys27 demethylase that has been implicated in AAM activation (29). Loss of JMJD3 results in defective expression of *Irf4* and other AAM-associated macrophage markers, and the impaired differentiation and recruitment of AAMs in response to helminth infection (29). The role of the myeloid-specific Src family kinase member HCK as a key regulator of gene expression in AAM human monocytes has also been described (26). Increased HCK activity in mice promotes colon tumorigenesis by enhancing angiogenesis and facilitating alternative macrophage polarization, while the genetic ablation or pharmacologic inhibition of HCK suppressed AAM polarization and impaired the growth of endogenous mouse and human colorectal cancer xenografts (27).

STAT3 and STAT6 play an important role in tumor-promoting macrophage polarization. A small-molecule inhibitor of STAT3 significantly reduced AAM polarization in patients with malignant glioma (169), while use of multitargeted tyrosine kinase inhibitors such as sunitinib and sorafenib promoted cancer cell apoptosis and reversed the immunosuppressive cytokine profile of AAMs by indirectly inhibiting signaling of downstream STAT3 (128, 129). Likewise, TAMs from STAT6 deficient mice display a CAM endotype and enhanced antitumor immunity (127). Together, these data suggest that the suppression of AAM endotypes can promote antitumor activities by reversing the immunosuppressive microenvironment.

Enhancing the CAM endotype of TAMs is another promising approach. IFNα has long been shown to exert tumoricidal effects and acts as a strong inducer of CAM polarization. When targeted to orthotopic human gliomas and spontaneous mouse mammary carcinomas, IFNα reduced tumor growth and abrogated metastasis (133). Similarly, systemic activation of CAMs with an agonist CD40 monoclonal antibody in combination with gemcitabine chemotherapy effectively circumvented tumor-mediated immune suppression and increased survival in patients with surgically incurable PDAC (134). In this study, it was shown that CD40-activated macrophages rapidly infiltrated tumors and exerted antitumor cytotoxicity (134). Subsequent Phase I clinical trials with a fully humanized CD40 agonist antibody (CP-870,893) in combination with gemcitabine showed well-tolerated responses and the activation of antitumor immune responses (170). Repolarization of TAMs from AAM toward a CAM endotype has also been achieved by inhibiting PI3Kγ in mice bearing PDACs, resulting in reduced tumor growth and metastasis (32). Genes associated with an AAM profile were strongly expressed in myeloid cells isolated from PDAC tumors; however, treatment with a PI3Kγ inhibitor significantly reduced the expression of these markers in PDAC tumors and in the corresponding TAMs. Conversely, the expression of immune-stimulatory factors such as IFNγ was significantly upregulated in animals treated with PI3Kγ inhibitors, consistent with enhanced $CD8^+$ T-cell-mediated antitumor immune responses (32). Collectively, these molecular targets, alongside histidine-rich glycoprotein HRG (135) and the NFκB signaling cascade (136), provide promising mechanisms to promote the reprogramming of macrophages away from a tumor-promoting endotype.

INFLUENCE OF MACROPHAGES ON TREATMENT RESPONSES

Increasing evidence has supported a dual role for TAMs to affect the effectiveness of anticancer therapies by either antagonizing the activity of these treatments or enhancing the overall cytotoxic effect. Thus, targeting TAMs might amplify the susceptibility

of cancer cells to such interventions and improve the clinical outcome.

Chemotherapy

A major challenge for successful cancer treatment is tumor resistance to chemotherapy. Preclinical models and clinical studies have revealed an important role of macrophages in modulating the adaptive immune response to improve chemotherapeutic responses. In an aggressive transgenic mouse model of mammary adenocarcinoma, administration of chemotherapy in combination with TAM blockade promoted antitumor immunity and cytotoxic T-cell infiltration, resulting in a significant decrease of pulmonary metastases and improved overall survival compared with chemotherapy alone (167). Likewise, the anti-proliferative agent trabectedin induces cell-cycle arrest in cancer cells by selectively depleting monocytes in soft tissue sarcoma (117).

Antiangiogenic Therapy

The development and use of antiangiogenic therapies has become an integral component of anti-cancer regimens. However, such therapies have shown limited durability due to acquired resistance. One mechanism of drug resistance suggested by preclinical studies is the recruitment of TAMs, since increased macrophage recruitment is frequently observed in resistant tumors (171, 172). In GBM patients, resistance to the antiangiogenic agent bevacizumab is driven by reduced expression of MIF at the tumor edge, causing the expansion of AAMs, which promote tumor growth (171). Similarly, secretion of MMP9 by intratumoral macrophages is associated with resistance to aflibercept, an anti-VEGF and anti-placental growth factor drug (173).

Treatment-induced hypoxia caused by vessel regression can similarly mediate resistance to antiangiogenic therapy by triggering the compensatory recruitment of myeloid cells to repair the vascular bed. In a mouse model of GBM, the hypoxia induced transcription factor HIF1α attracted bone marrow-derived TIE2- and VEGFR-expressing myeloid cells to promote neovascularization (174). These cell populations were diminished in HIF1α knockout tumors, which displayed normal and functional vasculature (174). Indeed, the angiogenic and hypoxic profiles of tumors is also used to predict radiographic response and survival benefit of GBM patients undergoing chemotherapy (175).

Targeting of macrophages in combination with anti-angiogenic therapies to restore or augment anti-tumor responses has yielded promising preclinical results. ANG2 is a member of the angiopoietin family that primarily signals through the TIE2 receptor. In addition to providing an escape mechanism to anti-VEGF therapy, ANG2 signaling modulates the activity of TIE2-expressing proangiogenic TAMs. In mice carrying orthotopic mammary tumors, ANG2 blockade inhibited tumor angiogenesis, growth, and metastasis, and impaired the activity of proangiogenic TIE2$^+$ macrophages (94). Of note, ANG2 blockade also inhibited angiogenesis and tumor growth in mouse models that are prone to develop resistance to anti-VEGF/VEGFR therapy (94). Likewise, dual inhibition of ANG2 and VEGF normalized the tumor vasculature and prolonged survival in murine GBM models in part by altering TAM polarization (176, 177). When combined with anti-PD1 checkpoint inhibition, combined ANG2 and VEGF blockade with a bispecific antibody further enhanced the antitumor response (178). Thus, integration of TAM-targeting strategies to complement antiangiogenic therapies may improve treatment efficacy and patient survival.

Immunotherapy

Immune checkpoint therapies aim to reverse the immunosuppressive nature of the tumor microenvironment and restore cytotoxic immune cell functions against cancer cells. Clinically validated checkpoint targets include PD1, PDL1, and CTLA4, and their inhibition has been shown to exert significant antitumor responses in cancers as diverse as melanoma and Hodgkin's lymphoma (179, 180). However, there are still many cancers that remain refractory to immunotherapy.

Macrophages are a key component of the immunosuppressive pathway targeted by immune checkpoint inhibitors. In response to various stimuli including cytokines (181) and hypoxia (182), TAMs can express the PD1 ligands PDL1 and PDL2, as well as ligands for CTLA4 (B7-1 and B7-2). Ligation of PDL1 to PD1 on the surface of cytotoxic T-cells leads to the inactivation of these immune effectors and facilitates immune escape. Mouse and human TAMs also express PD1, and the expression of this protein increases over time with disease severity (180). Interestingly, the majority of PD1$^+$ TAMs exhibit an AAM endotype, which can be reversed to a CAM-like endotype by PD1–PDL1 blockade to restore phagocytic activity and antitumor immunity. These results suggest that activation of the PD1–PDL1 pathway in TAMs impairs their cytotoxic ability (180).

Inhibition of CTLA4, an inhibitory receptor expressed on the surface of T-cells, has emerged as an effective therapy for metastatic melanoma. Analysis of the mechanism by which anti-CTLA4 therapy exerts its antitumor effects has revealed an important role of macrophages in driving these responses (183). In melanoma patients, anti-CTLA-dependent cell-mediated cytotoxicity is mediated by CD16-expressing macrophages (179). Of note, ipilimumab responders displayed significantly higher baseline peripheral frequencies of CD16$^+$ cells and a selective enrichment in tumor-infiltrating CD68$^+$CD16$^+$ (CAM-like) macrophages compared with non-responder patients. These results were consistent with a decrease in Treg cell numbers following immune checkpoint inhibition (179).

CHALLENGES AND THERAPEUTIC PERSPECTIVES

Of Mice and Not Men: Differences in Mouse and Human Immunology

Mice provide a mainstay of *in vivo* experiments and have contributed significantly to our understanding of human immunology. Comparative analysis of the mouse and human genome has revealed a striking level of conservation. Despite this, there are major discrepancies between our innate and adaptive immune systems in terms of development, activation and function. Such differences are unsurprising since the divergence of mice and humans occurred more than 60 million years ago, resulting in the evolution of both species under different ecological niches

and environmental pressures. Thus, while there are many parallels between mouse and human biology, it is also important to recognize the fundamental differences, especially when translating preclinical findings from bench to bedside. For example, expression of the cell-surface marker F4/80 is exclusively found in mouse macrophages and is undetectable on human cells (184). An alternative marker commonly used to distinguish human macrophages is CD68, however, since CD68 can also be expressed by some stromal and cancer cells, particular care should be taken when using this marker to identify TAMs (185).

Differences also exist when comparing the transcriptional profile of mouse and human macrophages following exposure to stimulating cytokines *in vitro*. For example, polarization of mouse macrophages toward an immunosuppressive AAM endotype is usually modeled by stimulation with IL4 and/or IL13. This results in the upregulation of M2-associated markers including FIZZ1, ARG1, and YM1; however, this response is not observed in human AAMs (46). Likewise, competitive metabolism of the amino acid arginine by NOS2 and ARG1 is used to delineate between pro-inflammatory CAM and immunosuppressive AAM endotypes in mouse macrophages, but this discriminative criteria does not apply to human cells (46). Thus, mouse and human macrophages exhibit distinct differences that should be taken into consideration to best translate our findings obtained from mouse models to human situations.

Monotherapy or Complement Therapies

Whether macrophage-targeting therapies will be most efficacious as monotherapies or as a combinatorial approach with chemotherapy and immunotherapy is still unclear. Considering that antigens are released by dying tumor cells following chemotherapy (186), the cross-representation of tumor antigens by TAMs could be exploited to enhance antitumor $CD8^+$ T-cell responses and stimulate immunological memory. Likewise, TAM-targeting strategies may also complement the efficacy of immune checkpoint inhibitors by removing additional inhibitory factors that may further restrict T-cell function. In preclinical models of PDAC, anti-PD1 and anti-CTLA4 antagonists showed limited efficacy as monotherapies to restrain tumor growth, but the use of these agents in combination with CSF1R blockade resulted in tumor regression (187).

Predicting Clinical Response

Since macrophage-targeted approaches elicit distinct effects based on tumor type, another aspect that should be considered is the identification of cancers and stratification of patient cohorts that are most likely to respond to treatment. In one study, high TAM density in metastatic lymph nodes predicted better disease-free survival in stage III colorectal cancer patients undergoing 5-fluorouracil adjuvant therapy (188). On the other hand, increased TAM infiltration is significantly associated with an unfavorable outcome for esophageal cancer patients undergoing neoadjuvant chemotherapy (189). Thus, a clearer understanding of how macrophages contribute to tumor progression across different cancers is crucial to maximize clinical benefit. The timing and duration of macrophage-targeted therapies could similarly have profound effects on patient response and overall treatment efficacy, and warrants further investigation.

Minimizing the Side Effects of Targeting TAMs

The development of localized treatment options for the primary tumor represents a significant hurdle, since the systemic depletion of macrophages in an immunocompromised patient undergoing chemotherapy may increase their vulnerability to infections. Furthermore, long-term depletion may also perturb the behavior of other immune cells that rely on macrophages to guide their functions. For instance, systemic inflammation has been observed as a result of excessive neutrophil infiltration in the absence of macrophages (190). Likewise, resident macrophages play a critical role in maintaining homeostasis in tissues in which they reside (191, 192), and the prolonged-depletion of these cells may severely impair organ function. Kupffer cells, for example, are involved in the breakdown of red blood cells in the liver (191), and their depletion results in aggravated liver lesions (193). By contrast, the loss of alveolar macrophages increases morbidity and respiratory failure in mice following influenza infection (194). While macrophage reprogramming represents a more viable option, the delicate balance between the tumoricidal and tumor-promoting functions of these cells also needs to be carefully considered. Excessive reprogramming of TAMs toward a CAM endotype could result in an excess of cytotoxic cytokines, inflammation, and tissue damage. While AAMs are essential for wound healing, the loss of AAMs might result in impaired tissue repair responses.

Macrophage-targeting strategies currently encompass a range of antibodies and small-molecule inhibitors; however, these two classes of drugs exhibit major differences in their pharmacological properties. Owing to their larger molecule weight, monoclonal antibodies often have a reduced efficiency for tissue penetration, but extended tumor retention and clearance from the blood compared with small-molecule inhibitors (195). However, small-molecule inhibitors tend to be less specific than monoclonal antibodies with an increased risk of toxicity, although these adverse effects are generally mild (195). These factors should be carefully considered when developing new drugs to maximize the therapeutic efficacy of these compounds.

CONCLUDING REMARKS

Macrophages are a major component of solid cancers and can promote tumorigenesis by facilitating angiogenesis, immunosuppression, invasion, and metastasis. Given the association between high macrophage infiltration and poor survival in most cancers, these cells represent promising targets for anticancer therapy. Strategies aimed at targeting TAMs have shown success in clinical trials and include macrophage depletion, modifying macrophage recruitment, and the reprogramming of macrophages away from an AAM endotype. These macrophage-directed therapies have also shown complementary effects when combined with chemo- and immunotherapies, suggesting the additive benefit of targeting TAMs alongside other cell populations to augment antitumor immunity. For this reason, a greater understanding of the complex interactions between TAMs and their surrounding

microenvironment is vital to identify additional pathways that can be targeted in parallel.

One major hurdle of targeting TAMs is to minimize the occurrence of negative side effects in the patient. Given their multifaceted roles of maintaining homeostasis, the systemic depletion of macrophages may lead to increased infections or impaired ability of tissue-resident cells to carry out their normal function. Thus, the identification of TAM-specific markers or molecules that are predominantly produced by AAMs and/or MAMs will enable the development of more sophisticated therapies that can be targeted specifically to tumors without affecting the function of other tissue-resident immune cells. In the same way, strategies aimed at reprogramming macrophages should also aim to conserve the ability of these cells to carry out phagocytosis and wound healing in non-tumor tissues.

AUTHOR CONTRIBUTIONS

AP and ME wrote the manuscript and designed the figures.

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