TARGETING THE PD-1/PD-L1 CANCER IMMUNE EVASION AXIS: CHALLENGES AND EMERGING STRATEGIES

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TARGETING THE PD-1/PD-L1 CANCER IMMUNE EVASION AXIS: CHALLENGES AND EMERGING STRATEGIES

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Table of Contents

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Editorial: Targeting the PD-1/PD-L1 Cancer Immune Evasion Axis: Challenges and Emerging Strategies

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Editorial on the Research Topic

Targeting the PD-1/PD-L1 Cancer Immune Evasion Axis: Challenges and Emerging Strategies

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Extensive exploration and utilization of cancer immunotherapy have revealed promising but challenging prospect of this field. The clinical benefits of Immune Checkpoint Blockade Therapy (ICBT) were limited due to intrinsic and adaptive resistance as well as emerging side effects. In this field, existing translational and basic investigations remain limited and controversial, revealing our insufficient understanding of cancer immune evasion mechanisms. This topic includes 16 updated articles. They focus on various aspects, including but not limited to analysis of clinical significance, side effects of ICBT, regulation of immune checkpoints and novel strategies.

The prognostic role of PD-L1 expression in immunotherapy was proposed before (Patel and Kurzrock, 2015), but the correlation between PD-L1 expression and prognosis are not well addressed in many cancer types. Under this topic, the prognostic and clinicopathological significance of PD-L1 expression were analyzed in colorectal cancer, prostate cancer, and bladder cancer in three articles respectively. By systematically reviewing and meta-analyzing past studies, they all concluded that PD-L1 expression is associated with poor prognosis. But they differ from each other on focuses according to characteristics of different cancer types. In colorectal cancer (CRC), PD-1/PD-L1 axis has been widely acknowledged as a promising therapeutic target, supported by recent clinical trials. This study not only evaluated the prognostic significance of PD-L1 expression, but also suggested that PD-L1 expression might be used as a biomarker for prognosis. In addition, the association between PD-L1 expression and location and differentiation of CRC, among other clinicopathological parameters, were statistically significant according to this analysis. Authors proposed several possible mechanisms that could upregulate PD-L1 level, such as Microsatellite Instability (MSI) (Li, He et al.). In prostate cancer, PD-L1 DNA methylation (mPD-L1) was additionally analyzed. The risk of biochemical recurrence was significantly higher in patients with higher mPD-L1. PD-L1 was specially analyzed with several clinical parameters, among which Gleason score and androgen receptor status were found significantly related to PD-L1. This study presented credible analysis, though credibility of results is somehow less convincing due to limited number of studies (Li, Huang et al.). In Bladder cancer, PD-L1 expression was found significantly correlated with tumor stage and metastasis, in addition to poor prognosis (Zhu et al.). These three articles mentioned above summarized eligible studies to help us obtain a better

understanding of the role of PD-L1 expression in multiple cancers. They also suggested that more work should be done to deal with existing controversies, both clinically and mechanically.

Owing to massive application and experiments of immunotherapy, more and more clinicians are confronted with immune-related adverse effects (irAEs) of immunotherapies. These adverse effects largely cut down on the benefits patients can achieve from immunotherapy (Martins et al., 2019). There are four articles here that discuss unfavorable effects, such as diabetes, immune-related pneumonitis, and liver fibrosis. Xiuju Liang and colleagues reported a case and provided a literature review on immune-related pneumonitis. The patient was given an additional PD-1 inhibitor after her disease progressed on previous PD-L1 inhibitor. After that she rapidly developed a severe steroid-resistant pneumonitis, suggesting that clinicians should take a history of pneumonitis into consideration as a possible risk factor for immune-related pneumonitis (Liang et al.). Lijun Da and colleagues conducted a meta-analysis on randomized controlled trials about organ-specific irAEs. They involved 8 RCTs with 2716 patients and listed the most common adverse effects of Immune Checkpoint Inhibitors (ICI). Colitis was ranked as most common irAE, followed by hypothyroidism, hepatitis, hypophysitis, hyperthyroidism, and pneumonitis. Notably, ICI combination therapy significantly increased the risk of all irAEs mentioned above (Da et al.), which supplemented the former case report about pneumonitis and provided with solid evidence. These two articles highlighted the risk of combining ICIs, which deserves more attention and investigations. Jingli Lu and colleagues presented a meta-analysis of 40 randomized controlled trials and conclude that the risk od new-onset diabetes with ICI is rather low but unneglectable, appealing more studies to substantiate these findings (Lu et al.). Clinical use of PD-L1 can also be combined with inhibition of transforming growth factor-β (TGF-β), which displayed additive antitumor response in a sub group of cancer patients. Xiutao Fu and colleages digged into the underlying mechanism of miR-20a-5p/TGFBR2 axis that dominantly regulates TGF-β pathway. Results suggested that miR-20a-5p plays a critical role in liver fibrosis through pro-inflammatory macrophages (Fu et al.).

The mechanisms underlying tumor immune evasion, though popularly investigated, are still poorly understood. Prognostic factors that may contribute to adverse reactions and efficacy are reviewed and discussed by Xinyu Yan and colleagues. Their summary categorized the contributing factors into four group, the characteristics of tumor, the features of microenvironment, the factors in peripheral blood and the individuality of host, illustrating a comprehensive frame of tumor-host interaction network (Yan et al.). The efficacy of ICBT, often disrupted by adaptive and intrinsic drug resistance, is a major concern about the application of PD-1/PD-L1 inhibition therapy. Luisa Chocarro de Erauso and colleagues attempted to find out predictive biomarkers to stratify patients with probability of response to ICBT by clarifying the molecular mechanism of PD-1/PD-L1 ICBT resistance (Chocarro de Erauso et al.). Peixin Dong and Oliviero Marinelli both put their focus on gynecological malignancies. Dong and colleagues emphasized the importance of acknowledging tumor-intrinsic signaling of PD-L1 in modulating immune-independent functions such as epithelial-to-mesenchymal transition (EMT), cancer stem cell (CSC)-like phenotype, metastasis and drug resistance. They carried on a meta-analysis that demonstrated coamplification between PD-L1 and MYC, SOX2, N-cadherin and SNAI1. Their findings may evoke more researches on related pathways and the role of PD-L1 (Dong et al.). On the other hand, Marinelli and colleagues summarized the controversial role of PD-L1 as a prognostic factor in gynecological malignancies, while stressed the importance of a novel molecule, PD-L2, in improving efficiency of immunotherapy (Marinelli et al.). Recent studies of post-translational modification of PD-L1 have broaden the horizon of PD-L1 pathway regulation (Wang et al., 2019; Yao et al., 2019). A summary of multifaceted regulation of PD-L1 is composed by Yiting Wang, providing a variety of routes that may be promising targets for new therapies (Wang et al.). The tumor immune microenvironment (TIME) is widely acknowledged as a pivotal factor contributing to tumor immune evasion, but the complexity and individual differences vastly hold back the understanding and utilization of it. Weilun Fu and colleagues leveraged mass cytometry with a panel of 33 markers to analyze the infiltrating immune cells in diffuse astrocytoma and oligodendroglioma. The composition and status of immune cells were assessed. This article provides a methodology of analyzing tumor-immune interaction, by directly profiling the landscape of TIME (Fu et al.). This method may be applied in more researches to unveil the features and mechanisms of cancer immunology.

Optimistically, novel strategies are constantly emerging. Abscopal effects (AbE) was discovered 60 years ago. It refers to systematic antitumor reactions caused by radiation therapy (RT), which leads to regression of nonirradiated lesions (NiLs). Accumulating evidence fostered a growing consensus that combination of immunotherapy and RT provides a better opportunity to boost AbE (Ngwa et al., 2018). Trommer and colleagues conducted a retrospective study on patients with metastatic cancer. With strict inclusion criteria, they concluded that combination of RT and ICI provided stronger AbE, compared to ICI alone (Trommer et al.). Their results encouragingly call on more prospective researches on this topic to provide solid and sophisticated guidelines on combination of ICI and RT. The unprecedented breakthrough brought by chimeric antigen receptor-redirected T (CAR T) cell therapy marked a new mile stone of cancer immunotherapy. Disruption of endogenous inhibitory immune checkpoints on T cells presents additive immune response. Xingliang Guo and colleagues used the CRISPR/Cas9 gene-editing system to knock down the PD-1 expression on the Glypican-3 (GPC3)-targeted second-generation CAR T cells employing CD28 as the costimulatory domain. *In vitro*, CAR T cells were cocultured with PD-L1 expressing Hepatocellular carcinoma (HCC). PD-1 disrupted GPC3-CAR T cells displayed not only stronger CAR-dependent antitumor activity but also less sign of exhaustion, compared to wild-type GPC3-CAR T cells. *In vivo*, PD-1

disrupted GPC3-CAR T cells showed improved persistence and infiltration in subcutaneous xenograft tumor model of NSG mice (Guo et al.). Discovery of eligible new targets is another strategy to tackle with the dilemma in immunotherapy. B7H3, also known as CD276, is an immune checkpoint molecule that is aberrantly over-expressed in many types of cancer. Peixin Dong and colleagues reviewed its role in modulating cancer behavior in many aspects and employed miRNA as potential therapeutic strategy (Dong et al.).

Under this topic we have seen analysis of prognostic role of PD-L1 expression in various cancer types, which requires more mechanistical investigations to turn the phenomenon into deep-scale understanding and translational strategies. Researches on the adverse effects of ICIs quantified the frequency of common irAEs. Specially, combination of different ICIs significantly increases risk of adverse effects, which deserves to be emphasized and considered in clinical scenes. Mechanisms underlying the modulation of PD-1/PD-L1 axis are explored and summarized, hoping to deepen and widen the understanding on PD-L1 and its role in cancer immune evasion, progression as well as resistance to ICIs. Novel strategies including combination of therapies, disruption of checkpoints on CAR T cells and employment of new targets provides promising and encouraging methodologies. Discussion and exploration on the cancer immune evasion and immune checkpoint targeting therapy will continue to provide exciting findings and benefit patients.

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REVIEW
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Regulation of PD-L1: Emerging Routes for Targeting Tumor Immune Evasion

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Immune checkpoint blockade therapies (ICBTs) targeting programmed cell death 1 (PD-1) and its ligand programmed death ligand-1 (PD-L1/B7-H1/CD274) have exhibited momentous clinical benefits and durable responses in multiple tumor types. However, primary resistance is found in considerable number of cancer patients, and most responders eventually develop acquired resistance to ICBT. To tackle these challenges, it is essential to understand how PD-L1 is controlled by cancer cells to evade immune surveillance. Recent research has shed new light into the mechanisms of PD-L1 regulation at genetic, epigenetic, transcriptional, translational, and posttranslational levels. In this work, we systematically discuss the mechanisms that control the gene amplification, epigenetic alteration, transcription, subcellular transportation and posttranscriptional modification of PD-L1 in cancer cells. We further categorize posttranscriptional PD-L1 regulations by the molecular modification of PD-L1, including glycosylation, phosphorylation, ubiquitination, deubiquitination, and lysosomal degradation. These findings may provide new routes for targeting tumor immune escape and catalyze the development of small molecular inhibitors of PD-L1 in addition to existing antibody drugs.

Keywords: PD-L1, immunotherapy, gene expression, post-translational modification, small molecular inhibitors

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INTRODUCTION

Over the past decades, a novel therapy that utilizes human immune system to treat cancer is increasingly popular, which is known as cancer immunotherapy (Yang, 2015). The immunosuppressive microenvironment of tumor is one of the six distinct biological properties that enable tumor growth and metastasis (Hanahan and Weinberg, 2011). Human tumors typically harbor genomic instability, which induce somatic mutations (Hanahan and Weinberg, 2011). Accumulation of mutations may facilitate tumor growth and metastasis, while some non-synonymous mutations, leading to replacement of amino acid residual, create new T cell epitopes (neoepitopes), offering opportunities for immune system to recognize and eliminate cancer cells (Matsushita et al., 2012; Rooney et al., 2015). It has been reported that the number of non-synonymous mutations, defined as mutational load, is closely related with the efficacy of immunotherapy (Danilova et al., 2016). However, cancer cells collaborate with immune cells to dodge the immune destruction, and the anti-cancer pathway is intervened in this microenvironment (Blank et al., 2016; Sukari et al., 2016). The depressed immunology of T cells, if appropriately empowered, may be an efficient and powerful weapon against cancer.

Specifically, active vaccination, adoptive cell transfer therapy and immune checkpoint blockade are the three major approaches that could turn on T cell-based anti-cancer immune reaction. In recent years, immune checkpoint blockade therapy (ICBT) has exhibited momentous clinical benefits, placing tumor immunotherapy under the spotlight (Sukari et al., 2016). PD-L1, a type I transmembrane protein with an extracellular N-terminal domain, inhibits the immune response through interaction with receptor PD-1 expressed on T cells (Horita et al., 2017). Under physiological conditions, PD-L1 is expressed in a wide range of cell types and tissues and shown to be overexpressed with immune activation, such as inflammations (Ritprajak and Azuma, 2015). The PD-L1/PD-1 axis maintains the balance between tolerance and autoimmunity and thus deficiency or excess function of it can lead to a variety of disease. Many auto-immune diseases have been found to be associated with PD-L1/PD-1 disruption including arthritis and lupus (Zamani et al., 2016). PD-L1 expression has been found positive in 5–40% tumor cells (Xie et al., 2016; Xiang et al., 2018), helping them to dodge the immune elimination through interaction of PD-L1 on the surface of cancer cells with PD-1 on T cells (Topalian et al., 2015). Thus, blockade of PD-L1/PD-1 axis assists the recognition and elimination of cancer cells. PD-L1 expression on tumor cells has been reasonably detected as a biomarker of ICBT (Ma et al., 2016). Further investigation revealed that the inducible but not continuous expression of PD-L1 is associated with activated CD8+ T cells in hepatocellular carcinoma (Xie et al., 2016), although the expression of PD-L1 is not independently prognostic (Wang X. et al., 2016; Xie et al., 2016).

The binding of immune checkpoint inhibitors and optimal targets is the core idea of ICBT. By inhibiting the immune-suppressive pathways, ICBT allows the clearance of cancer cells by the immune system (Topalian et al., 2015). Several immune checkpoints are discovered to be optimal targets for immune blockade, including the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell-death protein 1 (PD-1)/programmed cell-death 1 ligand 1 (PD-L1) pathways. Drugs targeting these two pathways have nourished recently and many of them have been approved by FDA. Drugs that target PD-1 like Pembrolizumab (Keytruda) and Nivolumab (Opdivo) were approved in 2014. Some PD-L1 inhibitors were also approved including Atezolizumab (Tecentriq) (2016), Avelumab (Bavencio) (2017) and Durvalumab (Imfinzi) (2017). Ipilimumab (Yervoy) is a monoclonal antibody targeting CTLA-4 that gained approval in 2011. Information comes from the official website of United States Food and Drug Administration. Notably, inhibitors targeting PD-1 or PD-L1 have been found to be especially advantageous in the treatment of many kind of cancer, including non-small cell lung carcinoma (NSCLC) (Wang C. et al., 2016), renal cell carcinoma (RCC), bladder cancer, breast cancer (Hu et al., 2017), melanoma (Luke et al., 2017) and Hodgkin's lymphoma (Allen and Gordon, 2016). The landscape of cancer therapy is evolving with deeper and wider acknowledgment of Immunotherapy with PD-1 or PD-L1 blockade (Pardoll, 2012).

Despite of the promising laboratory results and many positive clinical applications, there seems to be a discount on its overall clinical benefits due to intrinsic and/or acquired resistance to this therapy (Sharma et al., 2017). In certain cancer patients, the significant clinical response and enduring tumor retardation achieved by ICBT have improved patient progress-free survival (PFS) and overall survival (OS). However, the efficacy rate and profits of usage in general patients remain at a modest level, impeding the widespread application of ICBT (Pardoll, 2012). The tumor immunogenicity is a multi-level and delicately modulated process. Therefore, accumulation of mutations may lead to dysregulation of immunogenicity and create an immunosuppressive microenvironment, causing intrinsic resistance to ICBT (Zhao and Subramanian, 2017). Among them is the insufficiency of T cell infiltration (Spranger et al., 2016; Tang et al., 2016). On the other hand, after the significant retardation and durable response of tumor when initially treated with anti-PD-1 therapy, relapses in the long term were observed even after continuous therapy (Zaretsky et al., 2016). The acquired resistance to ICBT in melanoma was reported to be associated to antigen presentation deficiency, in which the interferon signal pathway was involved (Zaretsky et al., 2016). Alternative checkpoints were discovered to be adaptively upregulated after PD-L1 targeting treatment (Koyama et al., 2016). Moreover, PD-L1 upregulation after chemotherapy and nivolumab treatment was reported as a potential cause of acquired resistance (Haratake et al., 2017). In these tumors, immune evasion involves PD-L1/PD-1 interaction, which is the reason why the therapy initially worked. But the aftereffect of increased PD-L1 may have partially restored PD-L1/PD-1 function by providing more PD-L1 sites that were not neutralized by injected antibodies. Nonetheless, not enough investigations have been done to clarify the adaptive upregulation of PD-L1. In this scenario, understanding the mechanisms of PD-L1 regulation in cancer cells would certainly benefit the development of more effective and durable ICBTs.

While the PD-1/PD-L1 pathway has been proven both theoretically and clinically a mature and efficient target for immunotherapy, it is of urgent need to develop more effective approaches to target PD-L1. Firstly, many disadvantages of PD-L1 targeted antibodies are unneglectable. The relatively large size of Mono-antibodies (MAbs) may prohibit its penetration into the complex tumor microenvironment, and thus limiting the therapeutic efficacy (Lee and Tannock, 2010). It is crucial to develop new drugs with smaller sizes and to improve the specificity of tumor PD-L1 targeting, even though existing drugs and research are flourishing (Tan et al., 2016).

Secondly, the primary and acquired resistance to ICBT in many tumors highlights a crucial requirement for developing alternative PD-L1/PD-1-targeting approaches. Several cancer mutations have been suggested to be the cause of PD-L1 suppression and therefore primary resistance to PD-L1 blockade drugs. Inactivation mutations of JAK1/2 is an example (Shin et al., 2017). Thirdly, As a protector of host tissue and regulator of inflammation, PD-1/PD-L1 is located not only on tumor cells but also on normal cells, including anti-tumor T cells and tumor associated macrophages (Tan et al., 2016; Horita et al., 2017). The blockage of physiological PD-1/PD-L1 functions inevitably brings about unfavored results- the depletion of cells

which are meant to be activated and functioning. Lastly, the activation of oncogenic pathways, including RAS/RAF/MAPK and PI3K signaling, combined with the complexity of tumor microenvironment, may desensitize anti-tumor immunity (Zhao and Subramanian, 2018). The main components of tumor microenvironment, including infiltrated T cells (Tang et al., 2016), metabolites (will be further discussed) and oxidative stress (Maj et al., 2017), have been reported to be disruptors of anti-tumor immunity. Our understanding on the mechanisms of ICBT resistance and PD-L1 regulation remains rather limited, proposing an urgency to decode the multifaceted roles and complex control of PD-L1 in cancer.

The enthusiastic devotion from both clinical and biological investigators have brought the PD-1/PD-L1 biology into a new era in cancer research. Translational studies targeting the PD-1/PD-L1 pathway have boosted dramatically in recent years. Some progresses in the research of PD-L1 expression in cancer, especially at transcriptional and epigenetic levels, have been forged into a regulatory model for unified explanation (Chen et al., 2016). However, more recent findings that shed light into the multifaceted control of PD-L1 as a membranous protein has not been systematically discussed. In this review, we will summarize the exciting progresses in PD-L1 research in a more comprehensive manner, aiming to facilitate future basic and translational studies in the field of cancer immunotherapy.

GENOMIC ALTERATIONS DRIVE PD-L1 EXPRESSION

Enhanced PD-L1 expression was detected in a wide range of cancers but the prognostic and predictive value of it is controversial (Wang X. et al., 2016). It's also a sign of efficacy of ICBT targeting PD-1/PD-L1 (Chen et al., 2016), as reported in B-cell lymphomas (Wang X. et al., 2016), breast cancer (Mittendorf et al., 2014), small-cell lung cancer (George et al., 2017) and pancreatic cancer (Wang et al., 2010). Given that many oncogenes are upregulated by gained copy number alterations (CNAs), efforts have been made to clarify the relationship between PD-L1 expression and CNA. As the main form of CNA, PD-L1 copy number amplification directly leads to PD-L1 mRNA upregulation. Tumors harboring PD-L1 amplification presents significantly higher load of mutation, comparing to non-amplified subjects (Budczies et al., 2016). Increased copy number of chromosome 9p24, predominant amplification of focal gene CD274 (which resides on chromosome 9p24.1, as shown in **Figure 1**), together with abundant PD-L1 expression were observed in a subset of small-cell lung cancer (SCLC) (George et al., 2017). The Janus kinase 2 (JAK2) amplification was documented to be simultaneously activated with 9p24.1 chromosome copy number amplification and upregulated PD-L1 expression in primary cancers (**Figure 2**), suggesting a possible transactivation between JAK2 and PD-L1 genes (Green et al., 2010; Ikeda et al., 2016; Clave et al., 2018). What's more, PD-L1/PD-L2 alterations were defined as a feature of Classical Hodgkin lymphomas (cHLs). Specifically, amplification of 9p24.1 was reported to be associated with patients' advanced stage disease and poor prognosis in cHL and in Epstein-Barr virus-associated gastric cancer (EBVaGC) (Roemer et al., 2016; Saito et al., 2017). These findings collectively suggest that CD274 gene amplification is a crucial factor that drives PD-L1 expression in cancer, and thus targeting PD-L1 at genetic level may be a rationalized strategy in PD-L1 positive tumors. Considering the rapid development of gene therapies, such prospect won't be infeasible.

Structural variations may also be responsible for elevated transcription of PD-L1 (Kataoka et al., 2016). For example, truncation of its 3′UTR was reported to be associated with aberrant PD-L1 expression in multiple cancers (Kataoka et al., 2016).

EPIGENETIC REGULATION OF PD-L1

Epigenetic regulation was revealed to be involved in PD-L1 expression in cancer cells. Micro RNAs (miRNAs), defined as 22–24 nucleotides non-coding single-stranded RNAs, have been implicated in the regulation of PD-L1 expression (Wang Q. et al., 2017). The binding of some miRNAs to the PD-L1 mRNA causes the latter one to degrade and thus PD-L1expression is suppressed. Specifically, the abundance of miR-513, miR-570, miR-34a, and miR-200 were reported to have an inverse correlation with PD-L1 expression (Chen, 2009; Chen et al., 2014; Wang et al., 2015), as described in **Figure 1**. Among them is miR-513 which inhibits PD-L1 protein translation by binding to 3′ untranslated regions (UTRs) of PD-L1 RNA as complement (Chen, 2009). Supportively, IFN-γ-induced PD-L1 expression was diminished by introducing miR-513 into Jurkat cells, while anti-miR-513 enhanced PD-L1 expression in cholangiocytes (Gong et al., 2009; Jardim et al., 2009). Similar function was found with miR-570. Research has shown that mutation of the PD-L1 3′ UTR which disrupts the association with miR-570, correlated with overexpression of PD-L1 (Wang et al., 2013). P53 was reported to regulate PD-L1 through miR-34 (Cortez et al., 2016). In the case of miR-200, the process of epithelial-to-mesenchymal transition (EMT) is found to be mediated by the regulation of PD-L1 expression by miR-200 (Chen et al., 2014). Moreover, MiR-197 was reported to repress STAT3, a regulator of PD-L1, to decrease PD-L1 expression (Fujita et al., 2015), as demonstrated in **Figure 1**. Other miRs reported to regulate PD-L1 includes miR-424 (Xu et al., 2016), miR-138 (Zhao et al., 2016), miR-17 (Audrito et al., 2017) and cluster miR-25-93-106b (Cioffi et al., 2017). Most recently, a mechanism that stabilizes PD-L1 mRNA was reported through modulation of the AU-rich element-binding protein tristetraprolin (TTP) (Coelho et al., 2017).

Recent studies have also focused on the promoter methylation of PD-L1 (mPD-L1), which was suggested to be a biomarker for prediction of response to PD-1/PD-L1 targeted ICBT. Significant inverse correlations between mPD-L1 and patient age was reported. The correlation between mPD-L1 and PD-L1 mRNA expression shares similar pattern, indicating a potential

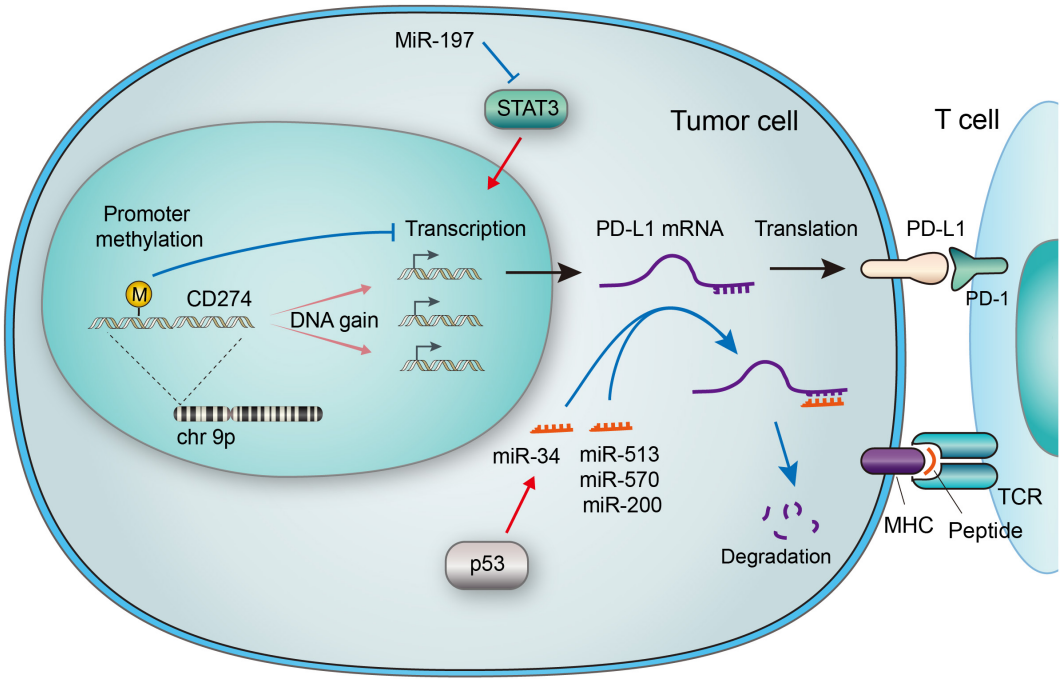

FIGURE 1 | Genetic and epigenetic regulations of PD-L1 in tumor cells. The CD274 gene encoding PD-L1 is located on chromosome 9p, which is amplified in a subset of cancers. Increased gene copy number leads to upregulation of mRNA expression, while methylation of the gene promoter suppresses its transcription. Micro RNAs (miRNAs) may regulate PD-L1 expression by suppressing Stat3, which transactivates PD-L1. MiRNAs may also bind to the 3′ UTR of PD-L1 mRNA, leading to its degradation. The p53 tumor suppressor has been reported to downregulate PD-L1 through miR-34, a miRNA that binds to the 3′ UTR of PD-L1 mRNA. The PD-L1/PD-1 interaction and MHC-antigen/TCR interaction collaboratively define an inhibitory output of the immune checkpoint.

interaction between patient age and methylation of PD-L1 gene and that promoter methylation suppresses PD-L1 expression in colorectal cancer (CRC) (Goltz et al., 2017). Correlation between PD-L1 promoter methylation and clinical outcomes was also revealed in other cancers including NSCLC (Wrangle et al., 2013) and prostate cancer (Gevensleben et al., 2016). Moreover, in patients treated with PD-1/PD-L1 targeting drugs, enhanced mPD-L1 is associated with worse overall survival and recurrence-free survival. Epigenetic therapy has also been suggested to sensitize tumor response to PD-L1 targeting drugs (Wrangle et al., 2013). Interestingly, results proved no meaningful correlation between PD-L1 mRNA expression and patients' outcome. (Goltz et al., 2017)

TRANSCRIPTIONAL ACTIVATION OF PD-L1

Several transcriptional factors have been found to control PD-L1 transcriptional activation (**Figure 2**). As an example, PTEN represses PD-L1 transcription and expression in breast cancer cells, suggesting a new tumor suppressive function of PTEN. In addition, PD-L1 expression decreased after inhibition of phosphoinositide 3-kinase (PI3K) pathway using the AKT inhibitors, further emphasizing the role of PTEN and PI3K signaling in PD-L1 regulation (Mittendorf et al., 2014). Transcription activity, demonstrated by the level of PD-L1 mRNA expression, was promoted through JAK2/STAT1 pathway, as was shown in pancreatic cancer cells treated with anticancer agents (5-fluorouracil, gemcitabine, or paclitaxel) (Wang et al., 2010). Notably, when treated with chemotherapeutic drugs, the MAPK pathway was also reported to upregulate PD-L1 in cancer cells (Chen et al., 2016). While distinct signaling pathways share the ability to control PD-L1 expression by regulating its transcription, the exact mechanisms involved may vary considerably (Chen et al., 2016).

Hypoxia inducible factor 1α (HIF-1α) is a major cancer driver (Ortmann et al., 2014) and a potential therapeutic target (Brown and Wilson, 2004; Vaupel and Mayer, 2007; Wilson and Hay, 2011). The binding of HIF-1α to PD-L1 promoter, a hypoxia response element (HRE), stimulates the transcription of PD-L1 (Noman and Chouaib, 2014). Research has revealed the co-existence of HIF-1α overexpression, increased PD-L1 level, and repression of T-cell function (Noman et al., 2014; Pollizzi and Powell, 2014; Shehade et al., 2014). It was also reported that PD-L1 works predominantly in lactate-enriched tumor microenvironments (Feng et al., 2017). Meanwhile, T cell autophagy is induced in a microenvironment lack of amino acids tryptophan and arginine as well as glucose. In this nutrients-deprived situation, glucose metabolism shrinks while the lactate accumulates, creating an optimal environment for PD-1/PD-L1 interaction and resistance to cancer therapies consequently (Robainas et al., 2017). In other words, Lactate, as a major metabolite under hypoxia condition, may protect

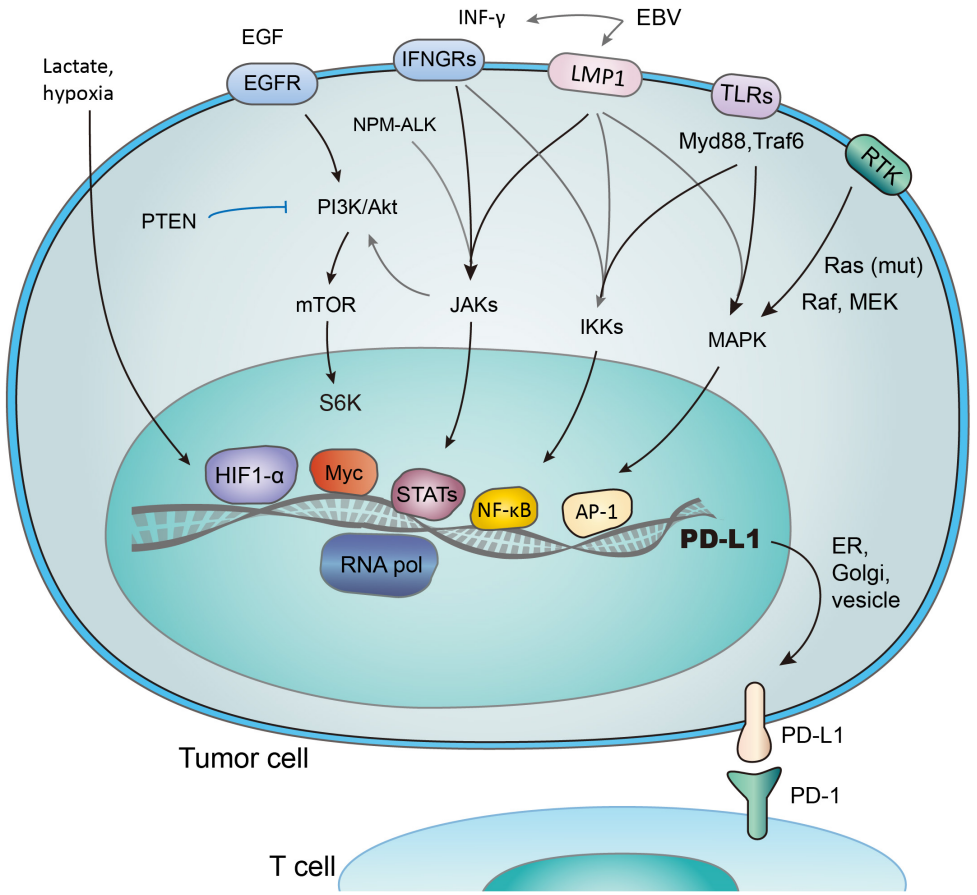

FIGURE 2 | Transcriptional activation of PD-L1 in response to different signaling pathways. PD-L1 is transcribed in response to the activation of multiple signaling pathways, and transcription factors (TFs) such as HIF1-α, Myc, Stats, NF- κB, and AP-1 have been reported to bind and transactivate PD-L1. These TFs are controlled by the interconnected pathways involving EGF/PI3K/AKT/MTOR (suppressed by PTEN), RTK/Ras/Raf/MEK/ERK, IFN-γ/JAKs (also induced by mutant NPM-ALK gene and EBV-activated LMP1), TLRs/Myd88/Traf6/IKKs, and lactate-enriched microenvironment.

tumor cells from cytotoxic T-cell targeting. Accordingly, tumor cell metabolic reprograming was found to correlate with immune suppression (Feng et al., 2017). Taken together, it is suggested that hypoxic environments, which induce activation of HIF-1α and accumulation of lactate (Koukourakis et al., 2005; Marchiq and Pouyssegur, 2016; Ban et al., 2017), contribute to evasion of tumor cells from immune system. The transactivation of PD-L1 by HIF-1 represents a crucial step in the above-mentioned process, and may be a promising target to combat the immune suppression of tumor cells.

STAT3 is another important transcriptional factor that upregulates PD-L1 expression by binding to PD-L1 promoter. Mutations of oncogene chimeric nucleophosmin/anaplastic lymphoma kinase (ALK) have been found to upregulate PD-L1 expression, and this effect could be abolished by silencing STAT3 (Marzec et al., 2008). Furthermore, Latent membrane protein-1 (LMP1) of Epstein-Barr virus was found to increase both PD-L1 expression and STAT3 phosphorylation (p-STAT3) (Fang et al., 2014) (**Figure 2**). Consistently, the JAK3 inhibitor CP-690550 blocked the above process through suppressing p-STAT3 (Marzec et al., 2008). NF-κB, as a transcriptional factor mediating inflammation-associated tumorigenesis, has been reported to boost PD-L1 expression. However, the exact mechanisms remain unclear. NF-κB is required for LMP1-induced PD-L1 expression, which is evidenced by decreased PD-L1 induction caused by NF-κB inhibitors (Marzec et al., 2008). Notably, the NF-κB inhibitor abolished INF-induced PD-L1 expression, while MAPK, PI3K and STAT3 inhibitors did not. Thus NF-κB also seems to be involved in INF-γ-induced PD-L1 expression (Gowrishankar et al., 2015).

GLYCOSYLATION OF PD-L1

N-glycosylation is a crucial protein modification that determines protein structure and function, especially the function of membrane proteins. By altering protein conformation, glycosylation may modulate protein activities and protein–protein interactions, such as those between ligands and receptors (Ohtsubo and Marth, 2006). In Western Blot assays, the majority of PD-L1 is detected at 45 kDa representing the glycosylated

species, while the non-glycosylated form is detected at 33 kDa. By bioinformatics prediction, mass spectrometry and mutagenesis, PD-L1 was found to be exclusively *N*-glycosylated at N35, N192, N200, and N219 (Li et al., 2016).

The PD-L1 molecule containing N192, N200, and N219 residues forms a region that is the prerequisite for PD-L1 binding to GSK3β, and *N*-glycosylation on these sites buries the necessary residues and disrupts the interaction between PD-L1 and GSK3β. Glycogen synthase kinase 3beta (GSK3β), a serine/threonine protein kinase, was originally identified as a regulator of glycogen metabolism (Doble and Woodgett, 2003). When bound to non-glycosylated PD-L1, GSK3β leads to phosphorylation and consequent ubiquitination of PD-L1 (Li et al., 2016) (**Figure 3**). In addition, it was further elucidated that inactivation of GSK3β by activating EGFR enhanced PD-L1 expression by preventing it from being ubiquitinated (Li et al., 2016). Significantly, a small molecular inhibitor of glycosylation, tunicamycin, was found to efficiently decrease PD-L1 expression in cancer cells (Li et al., 2016). Latest results have provided evidence that targeting glycosylated PD-L1 promotes PD-L1 internalization and degradation, leading to eradication of triple-negative breast cancer cells (Li et al., 2018).

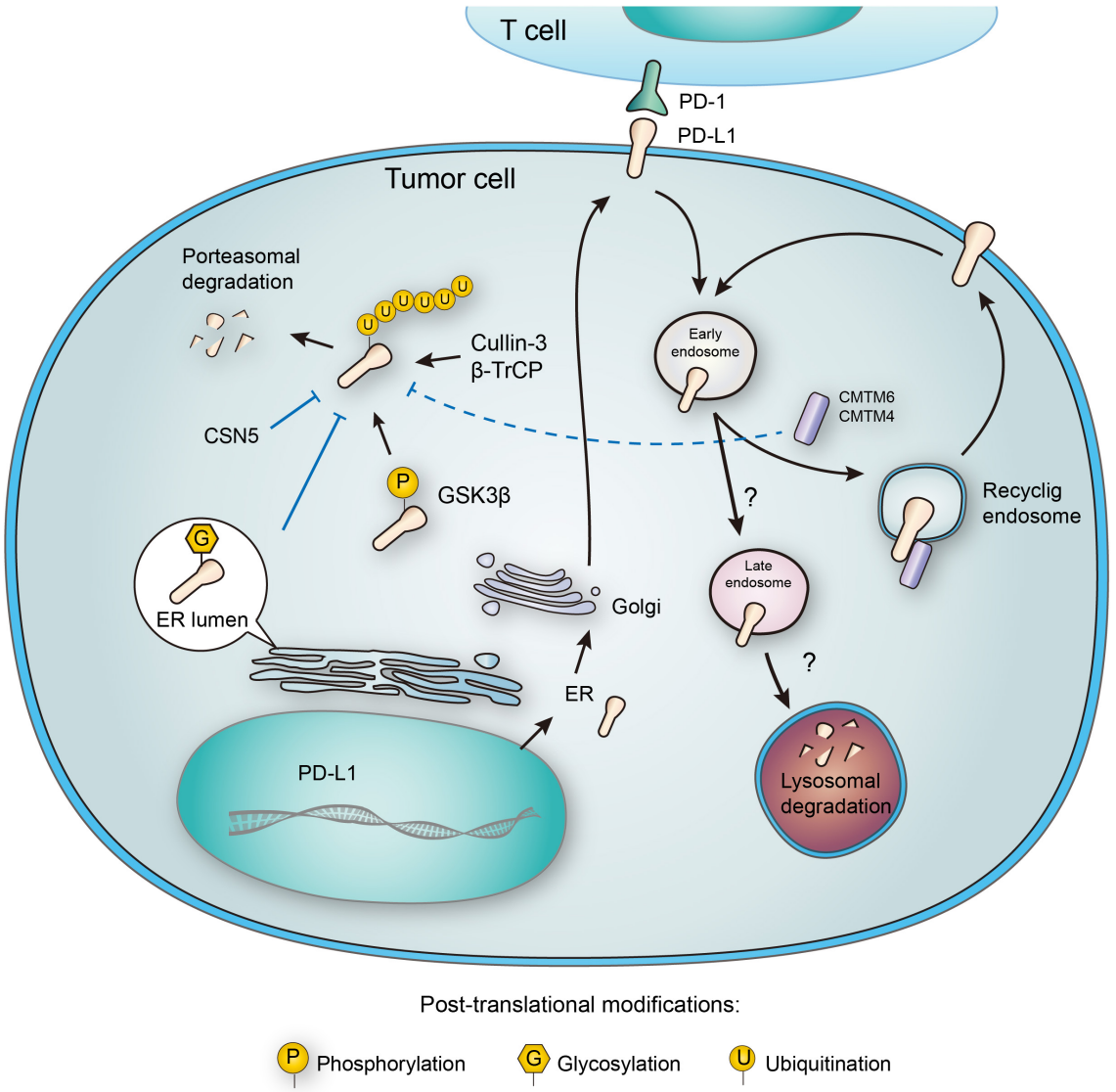

FIGURE 3 | Post-translational modifications and subcellular transportation of PD-L1. As a membrane protein, PD-L1 is extensively modified after its translation. *N*-glycosylation of PD-L1 extracellular domain occurs in the lumen of endoplasmic reticulum (ER), and this modification facilitates the interaction of PD-L1 with lipid membrane. Glycosylation also inhibits phosphorylation by GSK3β, and thereby blocking the ubiquitination by β-TrCP. Deubiquitination by CSN5 also protects PD-L1 from proteasomal degradation. In addition, PD-L1 may also be destructed in lysosome, and this process relies on a series of subcellular transportations from cell membrane to early endosome, late endosome, and finally to lysosome. However, CMTM6 has been found to promote PD-L1 transportation to recycling endosome, causing decreased distribution to late endosome and lysosome. Interestingly, CMTM6 and its homolog CMTM4 may also stabilize PD-L1 by suppressing its ubiquitination.

PHOSPHORYLATION OF PD-L1

Phosphorylation involves in a widespread of regulatory mechanisms in cellular signaling, and may affect the conformation, activity, and interactions of proteins. Although one protein may contain multiple phosphorylation sites, the phosphorylation of PD-L1 has been sparsely reported. As mentioned above in the glycosylation part, GSK3β is a multifunctional switch that mediates the direct phosphorylation of a wide range of substrates, including e IF2B, cyclin D1, c-Jun, c-myc, NFAT, MCl-1, and Snail (McCubrey et al., 2014). It also contributes to the phosphorylation of PD-L1 through an evolutionarily conserved GSK3β phosphorylation motif on PD-L1 (Li et al., 2016) (**Figure 3**). Furthermore, the phosphorylation mediated by GSK3β has been found to initiate the interaction with E3 ligase, which targets proteins to proteasomal degradation (Zhou et al., 2004; Ding et al., 2007; Wang et al., 2018).

Meanwhile, it was reported that treatment of the epidermal growth factor (EGF) would induce tyrosine phosphorylation, together with acetylation and ubiquitination of PD-L1 (Horita et al., 2017). These provide evidential hypothesis for the effects of Gefitinib, an inhibitor of EGFR, in promoting the immune response against breast cancer. Gefitinib was found to cut down on PD-L1 expression and limit its oncogenic potential, therefore promoting T cell immunity. These findings suggest that targeting EGFR by Gefitinib not only suppresses MAPK-dependent tumor proliferation, but also blocks PD-L1-dependent immune suppression (Li et al., 2016). Based on the predicted isoelectric points corresponding to different modifications, the PhosphoSite database has listed potential phosphorylation sites of PD-L1 (basal Isoelectric point = 6.76) (PhosphoSite Plus Protein Page: Pd-L1 Human, 2018). However, no systematic experimental characterization of PD-L1 phosphorylation has been carried out. It also deserves in-depth study how PD-L1 phosphorylation varies and fluctuates in response to distinct microenvironments, therapeutic stresses and interaction with its partner proteins.

UBIQUITINATION OF PD-L1

Ubiquitination-dependent proteasomal degradation controls the metabolism of many proteins, including membrane proteins like PD-L1 (Zhou et al., 2014). As mentioned above, the EGF treatment may induce tyrosine phosphorylation, acetylation, and ubiquitination of PD-L1 (Horita et al., 2017). The increased PD-L1 mono- and multi-ubiquitination induced by EGF were blocked by gefitinib treatment. Recent study further revealed that ubiquitin E3 is involved in PD-L1 downregulation in EGFR wild-type NSCLC (Wang et al., 2018). In a recent study, cyclin D-CDK4 kinase was reported to destabilize PD-L1 via cullin 3-SPOP, which was proved to be involved in Pd-L1 ubiquitination (Zhang et al., 2018). Surprisingly, the EGF-stimulated PD-L1 mono-ubiquitination not only coexisted with PD-L1 overexpression, but also seemed to occur ahead of its upregulation (Akbay et al., 2013; Chen et al., 2015; Li et al., 2016; Horita et al., 2017). Inhibition of the ubiquitin E1 by blocking its activating enzyme decreased PD-L1 mono- and multi-ubiquitination and total PD-L1 protein expression at the same time, suggesting a possible causal relationship between ubiquitination and overexpression of PD-L1 (Horita et al., 2017).

CMTM6, a type-3 transmembrane protein was recently identified as a positive regulator of PD-L1. Decrease of CMTM6 expression downregulated PD-L1 protein level in a wide range of human tumor cells and in primary human dendritic cells. Apart from CMTM6, its closest family member, CMTM4, was confirmed to share similar function (**Figure 3**). Of note, the enhancement of PD-L1 protein pool stimulated by CMTM6 was not associated with any variation in PD-L1 transcription. Instead, CMTM6 was found to interact with PD-L1 on cell surface, interfering its ubiquitination to prolong its half-life. It was also functionally confirmed that by enhancing PD-L1 protein pool, CMTM6 improves the evasion ability of PD-L1positive tumor cells to immune elimination (Mezzadra et al., 2017).

DEUBIQUITINATION OF PD-L1

On the contrary to ubiquitination, deubiquitination of PD-L1 stabilizes the protein from degradation. The deubiquitination and stabilization of PD-L1 significantly affect the inflammatory response or so-called 'inflammation-mediated anti-tumor immunity' (Lim et al., 2016). Recently, COP9 signalosome 5 (CSN5) was identified as a crucial protein that promotes the deubiquitination of PD-L1 (Lim et al., 2016) (**Figure 3**). It was reported that tumor necrosis factor alpha (TNF-α), as one of the major inflammatory cytokines secreted by macrophages, plays an important role in maintaining cancer cell evasion from immune system. Mechanistically, TNF-α may activate NF-κB and induce CSN5 expression, leading to PD-L1 stabilization. Consistently, CSN5 has been found to be indispensable for TNF-α-mediated PD-L1 stabilization because of its function in deubiquitinating PD-L1 (Lim et al., 2016). With potential translational significance, the authors found that destabilization of PD-L1 by curcumin, an inhibitor for CSN5, may benefit immunotherapy.

SUBCELLULAR TRANSPORTATION OF PD-L1

PD-L1 functions on the membrane surface, but it may also translocate into the cytoplasm. Many membrane proteins are shuttled between the recycling endosomes and cell surface, and PD-L1 has been tracked in recycling endosomes (Grant and Donaldson, 2009). Furthermore, inhibition of endocytic recycling by primaquine caused vast depletion of membrane PD-L1 protein level in wild-type cells. These results suggest that: first, a large proportion of membrane PD-L1 undergoes metabolism and internalization continuously; second, the dynamic recycling and

releasing of PD-L1 maintains the amount of PD-L1 located on cell membrane (Burr et al., 2017). Notably, CMTM6, recognized as a PD-L1 regulator, is predominantly identified in recycling endosomes together with TFRC and RAB11, factors that define the endocytic recycling compartment. What's more, CMTM6 co-localizes with PD-L1 both on the plasma membrane and in recycling endosomes, so that CMTM6 functions as a protector of PD-L1 that prevents it from being targeted for lysosome-mediated degradation and increases its protein pool (**Figure 3**).

Interestingly, membrane and cytoplasmic PD-L1 expression is more significant in macrophage cells than in cancer cells (Gong et al., 2017). Studies have been done to test PD-L1 molecule in peripheral blood mononuclear cells (PBMC) and surprisingly revealed a novel human PD-L1 splice variant in activated PBMC. Further studies compared the conventional isoform with the novel isoform and found distinct localization patterns between both proteins. Specifically, the conventional isoform is predominantly expressed on the plasma surface, while the novel isoform is distributed mainly on intracellular membrane. The alternative splicing of PD-L1 may be a posttranscriptional regulator that modulates PD-L1 expression as well as its function in determining the outcome of specific immune responses in the peripheral tissues (He et al., 2005).

In addition to its cellular distribution, PD-L1 has also been detected outside the cells, proposing its potential role as a semi-invasive biomarker. An A/C polymorphism at position 8923 was detected together with increased level of plasma soluble PD-L1 (sPD-L1) in NSCLC patients, especially those with adenocarcinoma (Cheng et al., 2015). Investigation is now undergoing to define the value of plasma PD-L1 protein levels as a predictive biomarker of prognosis in NSCLC and also as a reliable companion diagnostics for individualized treatment with ICBT (Zhu and Lang, 2017).

LYSOSOMAL DEGRADATION OF PD-L1

Unlike cytosolic proteins, many membrane proteins are mainly degraded through the lysosomal pathway. As mentioned in the ubiquitination part, CMTM6 reduces PD-L1 ubiquitination and increases its stability (Mezzadra et al., 2017). Interestingly, different opinion presents another explanation about the stabilization of membrane PD-L1 by CMTM6. In addition to its expression at the plasma membrane, CMTM6 is predominantly identified in recycling endosomes (Zhang et al., 2018). Although CMTM6 is not required for PD-L1 maturation, it functions in protecting PD-L1 from lysosome-mediated degradation (Burr et al., 2017). Thus, CMTM6 depletion, via the reduction of PD-L1, significantly alleviates the suppression of tumor-specific T cell activity *in vitro* and *in vivo* (Burr et al., 2017). Although there is no doubt that CMTM6 suppresses PD-L1 degradation, the effect still seems to be indirect, requiring the competitive transportation to the recycling endosome. It remains unclear which protein may directly interact with CMTM6 and transport it to lysosome for degradation (**Figure 3**). Future efforts to clarify this crucial node would benefit the development of alternative PD-L1-targeting approaches.

STRUCTURE-BASED MODULATION OF PD-L1

Some mutations of PD-L1 gene may impede the protein level of PD-1/PD-L1 but others may cause disturbance on protein folding, and therefore disrupt the interaction of PD-1 and PD-L1. PD-1 and PD-L1 bind through the conserved front and side of their Ig variable (Ig V) domains, representing the structural basis for the design of intervention molecules. By locating the loops at the ends of the IgV domains on the same side of the PD-1/PD-L1 complex, a surface is formed, being similar to the antigen-binding surface of antibodies and T-cell receptors (Zak et al., 2017). Several residues have been identified to play important roles in folding and forming the PD-1/PD-L1 interface (Lin et al., 2008). The immune receptor-like loops provide a new surface for further study and potentially the design of molecules that would affect PD-1/PD-L1 binding and thereby regulate the immune system. Multiple peptides and small-molecular compounds have been evaluated in preclinical models, in order to develop novel PD-1/PD-L1 inhibitors (Zak et al., 2017).

In addition to directly block the interaction between PD-1 and PD-L1, methods have also been developed to inhibit the dimerization of PD-L1, and hence the PD-1/PD-L1 interaction. Particularly, this effect could be achieved by small molecular compounds such as BMS-202 and BMS-8, with considerable translational significance (Zak et al., 2017). Since small molecules behold advantages in terms of production scale, quality standardization, pharmacological kinetics and tissue distribution, it is of enormous interest to discover small molecular drugs targeting the PD-L1/PD-1 axis (Lin et al., 2008). Despite the structural insights provided by recent crystallographic research, it is still unclear how the reported PTMs, e.g., glycosylation, phosphorylation, ubiquitination, etc., may affect the conformation and molecular interactions of PD-L1/PD-1. Understanding these detailed processes would also improve the confidence of structure-based drug design targeting this crucial immune suppression signaling pathway.

SIGNIFICANCE OF COMBINED INTERVENTION

PD-L1-targeted ICBT is a promising breakthrough in the field of cancer immunotherapy, but primary and acquired resistances have presented enormous challenges in this fast-evolving area (Pardoll, 2012; Spranger et al., 2016; Zaretsky et al., 2016; Sharma et al., 2017; Zhao and Subramanian, 2017). It has been suggested that the post-treatment positive conversion of PD-L1 expression may be a cause of resistance

(Haratake et al., 2017). The regulatory pathways of PD-L1 are of meaningful potential to be translated into therapeutic approaches for tackling the resistance to ICBT (Lee and Tannock, 2010; Tan et al., 2016; Tang et al., 2016; Maj et al., 2017; Shin et al., 2017; Zhao and Subramanian, 2018). The significant PD-L1 overexpression found in multiple cancer types may be an output of interconnected regulatory network, which involves molecular alterations at genetic, epigenetic, transcriptional, translational, post-translational, and structural levels. In fact, several key regulators of PD-L1 have long been established as cancer-related genes, such as JAK2 (Green et al., 2010; Budczies et al., 2016; Ikeda et al., 2016; Clave et al., 2018), PTEN, MAPK, PI3K, HIF-1α, STAT3 (Marzec et al., 2008; Gowrishankar et al., 2015; Chen et al., 2016), TNFα, NF-κB (Gowrishankar et al., 2015), and INF-γ, etc. Existing small molecular compounds targeting these genes/pathways may be repurposed for modulating PD-L1, thus providing readily tools to improve T cell-dependent anticancer immunity. Likewise, the discovery of key post-transcriptional modifications (PTMs) that control PD-L1 stability such as glycosylation, phosphorylation, and ubiquitination also provide alternative strategies for targeting PD-L1 (Zhou et al., 2004; Ding et al., 2007; Li et al., 2016; Lim et al., 2016; Horita et al., 2017). It is worthy to further analyze the function of curcumin (CSN5 inhibitor) and tunicamycin (glycolysis inhibitor) in suppressing PD-1/PD-L1 signaling *in vivo* and in preclinical models. The inhibitors o In addition, the connection between cancer metabolism and resistance to immunotherapy suggests potential benefit for combined targeting of tumor glycolysis and PD-1/PD-L1 axis (Koukourakis et al., 2005; Vaupel and Mayer, 2007; Wilson and Hay, 2011; Shehade et al., 2014; Marchiq and Pouyssegur, 2016; Feng et al., 2017). Apart from controlling the abundance of PD-L1 in cells, the mechanisms underlying PD-L1 transportation and structural modulation may also provide novel strategies to optimize the blockage of PD-L1 (van Weert et al., 2000; He et al., 2005; Lin et al., 2008; Cheng et al., 2015). With the multifaceted regulation of PD-L1 being revealed, it would be more feasible to develop complementary therapies to sustain the response once cancer cells acquire resistance to the initial treatment.

OUTSTANDING CHALLENGES

The prosperity and challenges of immunotherapies targeting the PD-1/PD-L1 axis warrant increasing attentions by biological and pharmaceutical scientists. In our opinion, several research directions would be especially beneficial to a sustained improvement of ICBT.

Firstly, the regulation of PD-L1 should be further clarified in more specified conditions, considering the variations in tumor regions and developmental stages. It has been suggested that PD-L1 expression may differ considerably on the tumor boundary. Cells located here have higher accessibility where immune cells encounter the tumor cells. Thus, tissue sampling by traditional methods may not robustly capture such alterations and result in low fidelity in different assays such as Western Blot, qPCR and microarray tests. On the other hand, hypoxia-related induction of PD-L1 is more likely to occur in the center of solid tumors where oxygen is less accessible. Moreover, our recent study found that PD-L1 is significantly upregulated in metastatic CRCs while compared to primary tumors (Wang H.B. et al., 2017). Thus, the regulation of PD-L1 during metastasis and its corresponding biomarker significance should be considered differentially from those in the primary tumors. To investigate the regulation of PD-L1 in tumors, it is essential to precisely mark the region and stage (e.g., primary vs. metastatic, pre-treatment vs. post-treatment, etc.) of a particular patient, because these variations are associated with the indicated mechanisms.

Secondly, the link between PD-L1 expression and cancer subtyping has been investigated based on genomic and transcriptomic characterizations of tumors. In many tumors, the microsatellite instability (MSI) subtype is linked to PD-L1 positivity and considered as a key factor indicating the suitability for checkpoint blockade therapy (Xiao and Freeman, 2015; Dudley et al., 2016). Even though, more comprehensive understanding on the implications of PD-L1 in cancer subtyping should also be founded by insights into the epigenetic and metabolic reprograming of cancer cells. As described previously, epigenetic and metabolic alterations in tumors are emerging as crucial factors affecting the abundance of PD-L1. In a translational perspective, significant and functional alterations at these facets may also present novel biomarkers and intervention opportunities.

Thirdly, it deserves tremendous efforts to clarify the overlaps and differences between PD-L1 and its homolog PD-L2 in their functions and regulations in various tumors. Although PD-L2 was initially considered to be mainly expressed in immune cells, recent studies have revealed its positive expression in different tumor cells with potential prognostic significance. As an example, we found that PD-L2 is expressed in a considerable subset of CRC cells, with independent association with poor patient survival (Wang H. et al., 2017). It is thus of interest to clarify the relative importance of PD-L1 and PD-L2 in a specific tumor type. Will one protein compensate the function of the other, or be upregulated when its homolog is blocked in immunotherapy? Which ligand of PD-1 may play a predominant role in suppressing T-cell immunity in a given cancer type of patient, and should this be considered when optimizing the strategy for immunotherapy? These questions should be addressed, in order to understand and improve the effectiveness and sustainability of ICBT.

Finally, the structure-based drug design targeting PD-L1 may not be limited in the binding surface to PD-1 or the site mediating its dimerization. If allosteric control of PD-L1 activity could be identified, additional approaches targeting PD-L1 would be feasible. Moreover, the protein interactions between PD-L1 and its reported regulators (e.g., CSN5, CMTM6, etc.) could be characterized in and enough resolution, rational design of blocking peptides or compounds may also be developed.

In other words, basic research about the structural dynamics and detailed interaction sites of PD-L1 may provide additional resources for the development of *de novo* PD-L1 targeting approaches.

CONCLUSION

Immune checkpoint blockade therapy represents a breakthrough in cancer treatment, but the primary and acquired resistance to immunotherapy warrant further efforts to understand the multifaceted regulation of PD-L1 in cancer. As a cell surface protein that responds to microenvironment stimuli, PD-L1 reacts promptly to balance the outside stresses and inside requirements of cells, representing a key node in the cancer signaling network. In this scenario, the effective and sustained targeting of PD-L1 has to take the complexity of its regulation into account. Identification of the exact causes of PD-L1 upregulation and responsive functional compensations in a broader range of molecular events would improve the targeting specificity and efficiency. A chasm is yet to be crossed by obtaining small molecular inhibitors of PD-L1 in addition to antibody drugs, to improve the cancer distribution and metabolic kinetics of immunotherapeutic medicines. Current approaches for targeting PD-L1 could also affect its normal functions in immune cells, with expected unwanted effects. In these scenarios, targeting PD-L1 effectively and specifically in cancer cells remains a Gordian knot.

AUTHOR CONTRIBUTIONS

YW and JX wrote the manuscript. HW, HY, CL, and J-YF contributed to revisions of the manuscript.

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REVIEW
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B7H3 As a Promoter of Metastasis and Promising Therapeutic Target

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B7H3 (also known as CD276, an immune checkpoint molecule) is aberrantly overexpressed in many types of cancer, and such upregulation is generally associated with a poor clinical prognosis. Recent discoveries indicate a crucial role for B7H3 in promoting carcinogenesis and metastasis. This review will focus on the latest developments relating specifically to the oncogenic activity of B7H3 and will describe the upstream regulators and downstream effectors of B7H3 in cancer. Finally, we discuss the emerging roles of microRNAs (miRNAs) in inhibiting B7H3-mediated tumor promotion. Excellent recent studies have shed new light on the functions of B7H3 in cancer and identified B7H3 as a critical promoter of tumor cell proliferation, migration, invasion, epithelial-to-mesenchymal transition, cancer stemness, drug resistance, and the Warburg effect. Numerous miRNAs are reported to regulate the expression of B7H3. Our meta-analysis of miRNA database revealed that 17 common miRNAs potentially interact with B7H3 mRNA. The analysis of the TCGA ovarian cancer dataset indicated that low miR-187 and miR-489 expression was associated with poor prognosis. Future studies aimed at delineating the precise cellular and molecular mechanisms underpinning B7H3-mediated tumor promotion will provide further insights into the cell biology of tumor development. In addition, inhibition of B7H3 signaling, to be used alone or in combination with other treatments, will contribute to improvements in clinical practice and benefit cancer patients.

Keywords: B7H3, CD276, metastasis, epithelial-to-mesenchymal transition, cancer stem cells, microRNA

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INTRODUCTION

Metastasis, or the consequences of their treatment, are the primary cause of cancer death (1). Metastasis is commonly viewed as a multistep event resulting in the dissemination of tumor cells from the primary tumor site to a distant location (2). These include loss of gap junction and tight junction contacts with neighboring cells, migration and invasion of basement membrane and extracellular matrix, entry and survival in the blood vascular and lymphatic system, extravasation into the parenchyma of distant tissues, adaptation to tumor microenvironment and host tissue remodeling, and re-initiation of their proliferative programs at metastatic sites (3, 4).

Epithelial-to-mesenchymal transition (EMT) endows epithelial tumor cells with enhanced motility and invasiveness (5, 6). Furthermore, EMT-derived tumor cells acquire cancer stem cell (CSC) properties and exhibit therapeutic resistance (6–9). In addition, the mutual interactions between tumor cells and the surrounding tumor microenvironment will eventually promote

tumor development and metastasis (10). Tumor microenvironment comprises many cell types including immune cells, fibroblasts, and endothelial cells (11). Tumor cells frequently display altered expression of cytokines and chemokines that promote the infiltration and activity of suppressive immune cell populations and also express immune checkpoint molecules (such as programmed cell death 1 ligand 1 and B7H3, also known as CD276) to inhibit the antitumor immune response (12–17).

B7H3 is expressed on immune cells (such as antigen-presenting cells or macrophages) and tumor cells and has inhibitory roles on T cells, contributing to tumor cell immune evasion (18–20). Recent studies have shown that B7H3 is a crucial player in tumor growth and metastasis beyond the immune regulatory roles (21). The developments in our understanding of cancer biology have provided a better understanding of how B7H3 regulates EMT and cancer stemness and of molecular mechanisms responsible for controlling the expression of B7H3 in cancer.

Although there have been substantial advances in our understanding of cancer at the molecular level, its prevention and treatment are still lacking. Considering the significant roles of B7H3 in cancer immunity and progression, the value of B7H3 in cancer diagnosis and treatment warrants further detailed study. Here, we review our current knowledge of how dysregulation of B7H3 and its signaling pathways can influence the hallmarks of cancer and discuss the potential use of microRNA (miRNA) as a potential therapeutic strategy for B7H3 overexpressing tumors, especially focusing on those miRNAs involved in the regulation of B7H3 expression in ovarian cancer.

B7H3 ACTIVATION IN CANCER

B7H3 (CD276) belongs to the B7 superfamily of immune checkpoint molecules (22). It is present at low levels in most normal tissues but is overexpressed in a wide variety of cancers, including bladder, breast, cervical, colorectal, esophageal, glioma, kidney, liver, lung, ovarian, pancreatic, prostate, intrahepatic cholangiocarcinoma, liver, oral squamous cell carcinoma, endometrial cancer, and squamous cell carcinoma and gastric cancer (23–42), glioma (43), and melanoma (44) (**Table 1**). Numerous studies showed that the overexpression of B7H3 was correlated with advanced tumor stage and high tumor grade in endometrial, cervical, breast, kidney cancer, and oral squamous cell carcinoma (25, 28, 30, 39, 40). The overexpression of B7H3 is associated with the proliferation and invasive potential of pancreatic, breast, colorectal, liver, prostate cancer, intrahepatic cholangiocarcinoma, and oral squamous cell carcinoma (26, 27, 30–32, 36–40). Notably, overexpression of B7H3 was found to correlate with poorer prognosis in many cancers (25, 28, 29, 31–34, 36–40, 44). However, high B7H3 expression predicts better survival for patients with gastric and pancreatic cancer (41, 45). A possible explanation for this discrepancy could be different cancer type (or subtypes), tumor heterogeneity, differences in sample size, and clinical stage, the time point of B7H3 measurement and the different methodology used in research.

TABLE 1 | The association between B7H3 expression and clinicopathologic factors of human cancers.

Cancer type	No.	Method	Expression	Clinical factors					Reference
				Size	Stage/ grade	Invasion depth	LN meta/ recurrence	Survival	
Bladder, breast, cervical, colorectal, esophageal, kidney, liver, lung, ovarian, pancreatic, prostate cancer, glioma, melanoma	1,342	IHC	Upregulation	NA	NA	NA	NA	NA	(23)
Bladder cancer	302	IHC	Upregulation	–	–	–	–	NA	(24)
Endometrial cancer	107	IHC	Upregulation	–	+	NA	NA	Poor	(25)
Pancreatic cancer	26	ELISA	Upregulation	+	NA	NA	NA	NA	(26)
Pancreatic cancer	59	IHC	Upregulation	NA	+	NA	+	NA	(27)
Cervical cancer	108	IHC	Upregulation	+	–	–	–	Poor	(28)
Breast cancer	90	IHC	Upregulation	–	–	–	–	Poor	(29)
Breast cancer	82	IHC/qPCR	Upregulation	+	+	–	+	NA	(30)
Intrahepatic cholangiocarcinoma	45	IHC	Upregulation	–	–	+	+	Poor	(31)
Colorectal cancer	275	IHC	Upregulation	NA	+	+	–	Poor	(32)
Ovarian cancer	103	IHC	Upregulation	NA	+	–	–	Poor	(33)
Glioma	41	IHC/microarray	Upregulation	NA	+	–	–	NA	(43)
Melanoma	97	IHC/qPCR	Upregulation	NA	+	–	–	Poor	(44)
Lung cancer	270	IHC	Upregulation	NA	+	NA	NA	Poor	(34)
Lung cancer	70	IHC	Upregulation	NA	–	NA	+	NA	(35)
Liver cancer	24	IHC	Upregulation	NA	+	+	–	Poor	(36)
Prostate cancer	823	IHC	Upregulation	NA	NA	+	+	Poor	(37)
Prostate cancer	2,111	Microarray	Upregulation	NA	+	NA	+	Poor	(38)
Oral squamous cell carcinoma	NA	IHC	Upregulation	+	+	–	–	Poor	(39)
Kidney cancer	743	IHC	Upregulation	+	+	NA	NA	Poor	(40)
Pancreatic cancer	96	IHC/qPCR	Upregulation	NA	–	–	–	Better	(41)
Gastric cancer	32	IHC/qPCR	Upregulation	–	–	–	–	Better	(42)

LN meta, lymph node metastasis; NA, data were not available.

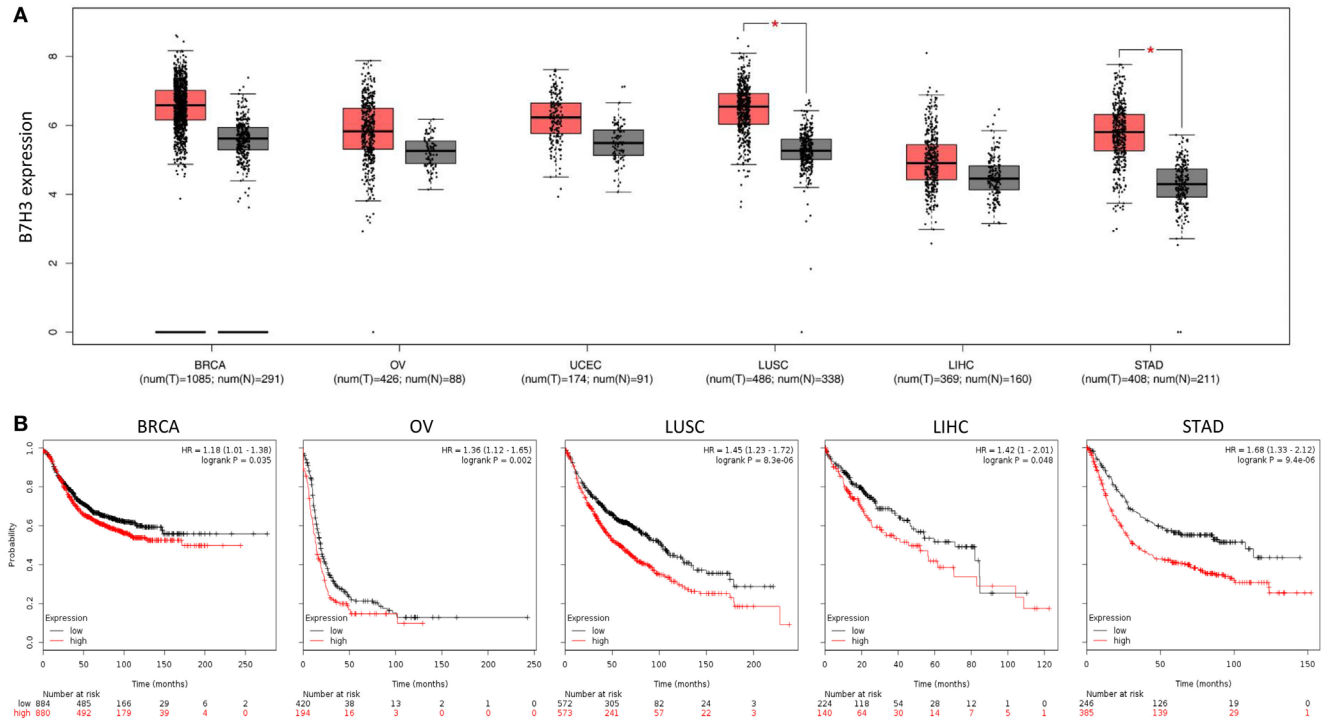

FIGURE 1 | High expression of B7H3 was correlated with poorer prognosis in cancers. **(A)** B7H3 expression profile across TCGA pan-cancer datasets. Images were taken from the GEPIA (Gene Expression Profiling Interactive Analysis) online database (http://gepia.cancer-pku.cn). N, normal; C, cancer. $^*P < 0.05$. **(B)** Kaplan–Meier curves for overall survival in indicated cancer types using the Kaplan–Meier Plotter database (www.kmplot.com). Red and black lines indicate patients with higher and lower than median *B7H3* mRNA expression, respectively. High expression of *B7H3* was significantly correlated with shorter overall survival in each Kaplan–Meier plotter cohort. BRCA, breast invasive carcinoma; OV, ovarian serous cystadenocarcinoma; UCEC, uterine corpus endometrial carcinoma; LUSC, lung squamous cell carcinoma; LIHC, liver hepatocellular carcinoma; STAD, stomach adenocarcinoma.

We assessed B7H3 expression in TCGA pan-cancer datasets obtained from Gene Expression Profiling Interactive Analysis (GEPIA) online database.[1] In agreement with previous reports, RNA sequencing analysis of mRNA expression from the GEPIA online database (46) revealed that B7H3 expression levels tend to be higher in breast, ovarian, endometrial, lung, liver, and gastric cancer tissues compared to corresponding normal tissues (**Figure 1A**). We also characterized the association between B7H3 mRNA expression and prognosis in several cancers using the Kaplan–Meier plotter database[2] (47). Higher expression of B7H3 was significantly associated with shorter overall survival in breast, ovarian, lung, liver, and gastric cancer (**Figure 1B**).

THE ROLES OF B7H3 IN DIFFERENT CANCER CELLS AND POSSIBLE MECHANISMS

The following sections and **Table 2** summarize the current understanding of the functional role of B7H3 in metastasis and describe its underlying mechanisms in different tumor cells.

[1]http://gepia.cancer-pku.cn (Accessed: June 5, 2018).
[2]http://kmplot.com/analysis/ (Accessed: June 5, 2018).

ROLES OF B7H3 IN CANCER CELL PROLIFERATION AND INVASIVENESS

Evidence supporting a tumor-promoting role for B7H3 is now increasingly apparent from functional studies of diverse malignancies. A lot of evidence demonstrated that B7H3 is involved in biological processes of cancer development, such as proliferation, migration, and invasion. For instance, knockdown of B7H3 expression in prostate, breast, gastric, liver, pancreatic, colorectal cancer cells, and melanoma cells could significantly suppress cell migration and invasion (26, 42, 48–57).

Different molecular mechanisms may also underlie these effects: (1) B7H3 induced the migratory potential and invasiveness of tumor cells by increasing the expression of metastasis-associated proteins such as MMP2, STAT3 and IL-8 (50); (2) by increasing the levels of CXCR4 and activating AKT, ERK, and JAK2/STAT3 pathways (52); (3) through activating the JAK2/STAT3/MMP9 pathway (55); (4) by increasing the expression of MMP2 (56); (5) by activating the TLR4/NF-κB signaling and increased IL-8 and VEGF expression (57).

Several studies have provided convincing *in vivo* functional data that are consistent with the data from cancer cell lines and thus support the tumor-promoting role of B7H3 during cancer progression. For example, in the subcutaneous transplantation pancreatic cancer mouse model, tumor growth rate was reduced

TABLE 2 | Roles, functions, and mechanisms of B7H3 in cancer.

Cancer type	Role	Function	Mechanism	Reference
Prostate cancer	Oncogene	Migration, invasion	NA	(48)
Melanoma/breast cancer	Oncogene	Migration, invasion	NA	(49)
Melanoma/breast cancer	Oncogene	Migration, invasion	Increased the expression of MMP2, STAT3, and IL-8	(50)
Melanoma	Oncogene	Proliferation, glycolytic capacity, resistance to chemotherapy and small-molecule inhibitors	NA	(51)
Breast cancer	Oncogene	Paclitaxel resistance	Activated JAK2/STAT3 pathway	(64)
Breast cancer	Oncogene	Glucose uptake, lactate production, proliferation	Increased the expression of HIF1α and its downstream targets, LDHA and PDK1	(70)
Gastric cancer	Oncogene	Migration, invasion, proliferation	NA	(42)
Gastric cancer	Oncogene	Migration, invasion	Increased CXCR4; and activated AKT, ERK, and JAK2/STAT3 phosphorylation	(52)
Esophageal squamous cell carcinoma	Oncogene	Migration, invasion	NA	(53)
Liver cancer	Oncogene	Proliferation, adhesion, migration, and invasion	NA	(54)
Pancreatic cancer	Oncogene	Proliferation, invasion	NA	(26)
Colorectal cancer	Oncogene	Resistance to chemotherapy	Activated JAK2/STAT3 pathway	(65)
Colorectal cancer	Oncogene	Oxaliplatin resistance	Increased the expression of XRCC1 *via* PI3K/AKT pathway	(66)
Colorectal cancer	Oncogene	Migration, invasion	Activated JAK2/STAT3/MMP9 pathway	(55)
Colorectal cancer	Oncogene	Resistance to chemotherapy	Increased BRCC3 expression	(67)
Colorectal cancer	Oncogene	Resistance to chemotherapy	Activated PI3K/AKT/TS pathway	(68)
Colorectal cancer	Oncogene	Epithelial-to-mesenchymal transition, cancer stemness	Decreased E-cadherin expression and increased of N-cadherin, Vimentin, CD133, CD44, and OCT4 expression	(59)
Osteosarcoma	Oncogene	Invasion	Increased the expression of MMP2	(56)
Pancreatic cancer	Oncogene	Invasion, metastasis	Activated TLR4/NF-κB signaling and increased IL-8 and VEGF expression	(57)
Glioma	Oncogene	Migration, invasion, cancer stemness	NA	(61)
Ovarian cancer	Oncogene	Resistance to chemotherapy and small-molecule inhibitors, cancer stemness	Possibly increased the expression of ALDH	(60)

NA, data were not available.

by the knockdown of B7H3 (26). Similarly, the silencing of B7H3 significantly decreased tumor proliferation in mantle cell lymphoma *in vitro* and *in vivo* (58).

B7H3 MEDIATES EMT AND CSC IN CANCER CELLS

Some researchers claimed that B7H3 plays a key role in modulating EMT and CSC-like properties of various cancer cells. B7H3 can promote EMT and cancer stemness by decreasing E-cadherin expression and increasing the expression of N-cadherin, Vimentin, CD133, CD44, and OCT4 (59). Blockade of B7H3 with a monoclonal antibody reduced the number of cancer-initiating cells (60). A previous study found that B7H3 is an inducer of cell invasion and sphere formation in glioma cells (61), further suggesting a role of B7H3 in the cancer invasion process.

Cancer stem cells or tumor-initiating cells not only possess the ability of self-renewal but also develop strong resistance to chemotherapy (62). It was demonstrated that the induction of EMT generated cells with properties of CSCs (63). In breast cancer and colorectal cancer cells, B7H3 induced the resistance to paclitaxel or 5-fluorouracil (5-FU) through activating the JAK2/STAT3 pathway (64, 65). In addition, a few other mechanisms may also underlie B7H3-mediated chemoresistance: (1) B7H3 induces oxaliplatin resistance by increasing the expression of XRCC1 *via* PI3K/AKT pathway (66); (2) B7H3 also enhances cell resistance to chemotherapy by increasing the expression of BRCC3, which antagonizes DNA damage caused by 5-FU (67); (3) or *via* the activation of the PI3K/AKT pathway (68).

ROLE OF B7H3 IN CANCER METABOLISM

Warburg effect (or aerobic glycolysis) is a metabolic hallmark of cancer, characterized by an excessive conversion of glucose to lactate even with ample oxygen (69). A recent study found that B7H3 can promote the Warburg effect, evidenced by increased glucose uptake and lactate production in breast cancer cells. Furthermore, this stimulating effect of B7H3 on the Warburg effect was also observed in a mouse model of breast cancer (70). Mechanistically, B7H3-induced metabolic shift in cancer cells is mediated by HIF1α, a master regulator in the reprogramming of cancer metabolism in favor of glycolysis (70), revealing a new mechanism for the Warburg effect in cancer cells. Reasonably, we believe treating tumors by targeting their metabolism through modulation of B7H3 expression would probably generate a better effect of tumor eradication.

REGULATORY MECHANISMS OF B7H3 IN CANCER

Protein expression is usually controlled by the following mechanisms: the genetic aberrations of the gene loci (71),

transcriptional regulation (72), posttranscriptional regulation at the mRNA level (73), and protein modification (74). Epigenetic mechanisms such as DNA methylation (75), histone modification (76), and non-coding RNAs (77, 78) play a key role in regulating gene expression. DNA methylation and modification of histones mediate gene transcription, and miRNAs regulate gene expression posttranscriptionally (79). To date, it is less clear whether B7H3 overexpression observed in cancer is due to genomic DNA amplification, or which transcription factors are responsible for B7H3 transcription. However, chromatin immunoprecipitation analysis in prostate cancer cells revealed an androgen receptor-binding site upstream of B7H3, and the presence of androgens decreased B7H3 expression (38).

Interestingly, immunoglobulin-like transcript-4 (ILT4) is an inhibitory receptor that inhibits the function of certain immune cells and was shown to upregulate B7H3 expression *via* the PI3K/AKT/mTOR signaling in lung cancer cells (80). Co-expression of ILT4 and B7H3 was positively corelated with lymph node metastasis and advanced tumor stage (80). Consequently, further study is needed to elaborate the link between ILT4 and B7H3 in different cancer cells.

At the posttranscriptional level, numerous miRNAs, including miR-214, miR-363*, miR-326, miR-940, miR-29c, miR-665, miR-34b*, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p, and miR-593, directly interact with the 3′-UTR of B7H3 mRNA, resulting in attenuation of B7H3 expression in breast cancer (81). miR-124 also binds directly to the 3′-UTR of B7H3 mRNA, inhibiting its expression in osteosarcoma (82). TGF-β1 through SMAD3 and SMAD4 elevated miR-155 expression, which in turn attenuated CEBPB expression and consequently miR-143 expression in colorectal cancer cells. As a result, the reduction of miR-143 led to the upregulation of B7H3, a direct target of miR-143 (83). These results indicated that TGF-β1 may promote cancer immune escape by upregulating B7H3 expression. In addition, a recent study demonstrated that p53 binds to the promoter of miR-124 to elevate its expression in colorectal cancer cells (84). Meanwhile, iASPP, a novel oncoprotein overexpressed in many cancers, interacts with p53 to suppress p53-mediated transcription of target genes (75, 85). Thus, these results indicate a possible mechanism underlying B7H3 overexpression in tumors: iASPP-mediated p53 repression leads to the downregulation of miR-124, subsequently resulting in increased expression of B7H3.

We used three computational algorithms, including TargetScan,[3] miRSystem,[4] and DIANA-MicroT-CDS[5] to identify miRNAs that might regulate B7H3 expression. This analysis revealed 17 common miRNAs predicted to bind the 3′-UTR of the B7H3 transcript (**Figures 2A,B**). In colorectal cancer cells, a recent study showed that miR-187 binds B7H3 mRNA and suppresses its expression to inhibit cell proliferation, migration, invasion, and induced cell apoptosis (86). In clear cell renal cell carcinoma, another study confirmed that B7H3 expression is downregulated by miR-187, a tumor suppressor that suppresses

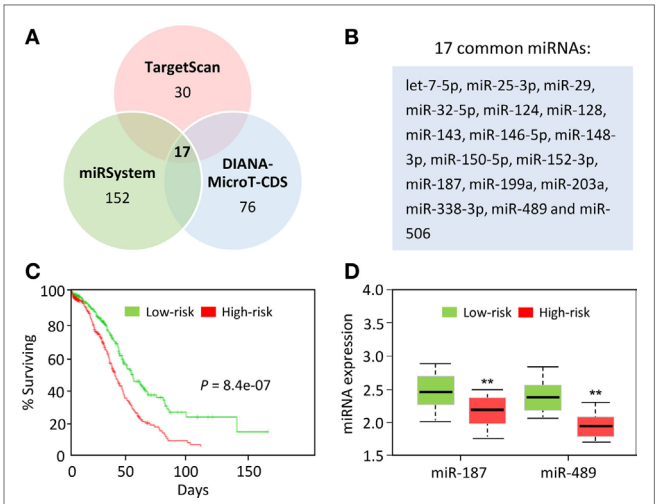

FIGURE 2 | MicroRNAs (miRNAs) that potentially regulate B7H3 expression in ovarian cancer. **(A)** Venn diagram showing the overlap of miRNAs that were predicted to bind to the *B7H3* 3′-UTR by alternative algorithms (TargetScan, miRSystem, and DIANA-MicroT-CDS). **(B)** The 17 predicted miRNAs were common to these three algorithms. **(C)** The Kaplan–Meier survival curves of 458 TCGA (Cancer Genome Atlas database) ovarian cancer samples were created using the SurvMicro database based on the low (n = 229) or high (n = 229) risk for a poor outcome. **(D)** Box plots demonstrating significantly lower levels of miR-187 and miR-489 expression in the high-risk ovarian cancer patients.

cancer cell proliferation and motility (87). Collectively, these data suggest that the loss of tumor suppressor miRNAs activate B7H3 and contributes to cancer progression.

We further evaluated the correlation of patient survival with the expression of these miRNAs in ovarian cancer samples in the TCGA by using the online software SurvMicro.[6] Ovarian patients were stratified into the high-risk (with a low probability of survival; n = 229) or low-risk (with a high probability of survival; n = 229) group (P = 8.4E−07, **Figure 2C**). High-risk patients had lower miR-187 and miR-489 expression levels than the low-risk patients (**Figure 2D**). Thus, these 17 miRNAs, especially miR-187 and miR-489, are expected to have binding sites in the 3′-UTR of B7H3 in cancer cells, although functional validation remains to be performed.

CONCLUSION

Interruption of metastasis pathways holds preclinical and clinical promise as an anti-metastasis therapy. The emerging role of B7H3 in human tumor cells and in inducing EMT/CSC-like features have been noted. Furthermore, tumor cells could rely on Warburg effect to generate energy (88). The recent findings led to the identification of B7HH3 as a contributor to the Warburg effect (70). Therefore, targeting the metastatic potential and metabolic changes with inhibitors against B7H3 may be a promising way for cancer therapy.

[3]http://www.targetscan.org/vert_72/ (Accessed: June 5, 2018).
[4]http://mirsystem.cgm.ntu.edu.tw/ (Accessed: June 5, 2018).
[5]http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index (Accessed: June 5, 2018).

[6]http://bioinformatica.mty.itesm.mx:8080/Biomatec/Survmicro.jsp (Accessed: June 5, 2018).

The induced B7H3 expression has been detected in multiple cancers as compared with normal tissues. The B7H3 protein, especially when located in the cell membrane, may be a perfect choice for targeted drug development. Importantly, the treatment with an inhibitory B7H3 monoclonal antibody in melanoma cells leads to decreased proliferation and Warburg effect (51). Additionally, targeting B7H3 with a monoclonal antibody has demonstrated the safety and efficacy in the salvage treatment of stage IV childhood neuroblastoma (43). Activated T cell (ATC) armed with a novel anti-CD3 × anti-B7H3 bispecific antibody was found to significantly inhibit lung cancer growth *in vivo* compared with unarmed ATC (89), indicating that targeting B7H3 represent a novel alternative to improve current cancer therapy.

Future studies aimed at delineating the precise cellular and molecular mechanisms underpinning B7H3-mediated tumor promotion will provide further insights into the cell biology of tumor development. In addition, inhibition of B7H3 signaling, to be used alone or in combination with other treatments, will contribute to improvements in clinical practice and benefit cancer patients.

AUTHOR CONTRIBUTIONS

PD and HW provided direction. PD, YX, and HW wrote the manuscript. JY and SH made significant revisions to the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Tumor-Intrinsic PD-L1 Signaling in Cancer Initiation, Development and Treatment: Beyond Immune Evasion

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Although the role of PD-L1 in suppressing the anti-tumor immune response is extensively documented, recent discoveries indicate a distinct tumor-intrinsic role for PD-L1 in modulating epithelial-to-mesenchymal transition (EMT), cancer stem cell (CSC)-like phenotype, metastasis and resistance to therapy. In this review, we will focus on the newly discovered functions of PD-L1 in the regulation of cancer development, describe underlying molecular mechanisms responsible for PD-L1 upregulation and discuss current insights into novel components of PD-L1 signaling. Furthermore, we summarize our current understanding of the link between PD-L1 signaling and the EMT program as well as the CSC state. Tumor cell-intrinsic PD-L1 clearly contributes to cancer stemness, EMT, tumor invasion and chemoresistance in multiple tumor types. Conversely, activation of OCT4 signaling and upregulation of EMT inducer ZEB1 induce PD-L1 expression in cancer cells, thereby suggesting a possible immune evasion mechanism employed by cancer stem cells during metastasis. Our meta-analysis demonstrated that *PD-L1* is co-amplified along with *MYC*, *SOX2*, *N-cadherin* and *SNAI1* in the TCGA endometrial and ovarian cancer datasets. Further identification of immune-independent PD-L1 functions and characterization of crucial signaling events upstream or downstream of PD-L1 in diverse cancer types and specific cancer subtypes, would provide additional targets and new therapeutic approaches.

Keywords: PD-L1, CD274, metastasis, EMT, cancer stem cells, microRNA

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INTRODUCTION

In cancer, the epithelial-to-mesenchymal transition (EMT) is a phenotypic process that promotes the acquisition of a mesenchymal features of epithelial tumor cells, reduces cell polarity and cell-cell adhesion, and enables them to migrate and invade more efficiently, by switching off the expression of epithelial markers, such as E-cadherin, and turning on mesenchymal markers, including N-cadherin and Vimentin (1, 2). Epithelial tumor cells undergoing EMT are shown to contribute to tumorigenesis, invasion, metastasis, and resistance to chemotherapy, radiation and small-molecule-targeted therapy (3).

Cancer stem cells (CSCs) represent a fraction of undifferentiated cancer cells that are the seeds of tumor recurrence, have the ability to self-renew and exhibit significant resistance to conventional

chemo- and radiotherapy (4). Emerging evidence has revealed an association between EMT and the acquisition of CSC-like properties (5). The induction of EMT program is a critical regulator of the CSC phenotype (6, 7). On the other hand, tumors cells that exhibit the CSC phenotype also express genes associated with the EMT features and show enhanced metastatic ability, thus representing a novel mechanism contributing to cancer metastasis (8).

The mutual interactions between tumor cells and the tumor microenvironment are essential for tumorigenesis, tumor progression, metastasis and resistance to drug therapy (9). Tumor microenvironment consists of extracellular matrix and diverse cell populations such as T cells, NK cells, macrophages, dendritic cells, fibroblasts, and endothelial cells (10). Progression of cancer to an advanced or metastatic disease usually suggests a failure or insufficiency of the ongoing immune response. Tumors not only effectively escape immune recognition, they also actively inhibit T-cell-mediated normal anti-tumor activity to promote further tumor growth and metastasis by modulating immune checkpoints, which mediate immune tolerance and inhibit the anti-tumor immune response (11). Multiple checkpoint molecules, such as PD-1/PD-L1, CTLA4, BTLA, B7H3, B7H4, HHLA2, IDO1, Tim-3, CD28, CD40, CD47, CD70, CD137, VISTA, LAG-3, and TIGIT, have been reported (11). Among them, B7H3 has been identified as a critical promoter of tumor cell proliferation, migration, invasion, EMT, cancer stemness, and drug resistance (12).

PD-L1 (also known as CD274 or B7H1) is expressed in tumor cells and plays a crucial role in tumor immune escape and the formation of a permissive immune microenvironment, through at least three mechanisms: (i) tolerizing or anergizing tumor-reactive T cells by binding to its receptor PD-1; (ii) rendering tumor cells resistant to $CD8^+$ T cell and Fas ligand-mediated lysis; and (iii) tolerizing T cells by reverse signaling through T cell-expressed CD80 (13, 14). In addition, PD-L1 is also expressed by tumor-associated myeloid-derived suppressor cells and macrophages, which are the major factors responsible for tumor-associated immune deficiencies (15).

Although PD-L1 is widely implicated in tumor immune evasion, the tumor-intrinsic roles of PD-L1 and the mechanisms by which PD-L1 regulates EMT, the acquisition of tumor-initiating potential and resistance to anti-tumor drugs, as well as the ability to disseminate and metastasize in human cancers are currently less well defined. As will be discussed in more detail below, the identification of tumor-intrinsic PD-L1 signaling may provide critical targets for the development of cancer therapies.

PD-L1 DYSREGULATION AND PROGNOSIS IN CANCER

An increasing number of studies suggested that PD-L1 is highly expressed in solid tumors, including colorectal cancer (16), lung cancer (17), pancreatic carcinoma (18), hepatocellular carcinoma (19), gastric cancer (20), ovarian cancer (21), endometrial cancer (22, 23), and cervical cancer (24, 25). High expression of PD-L1 was associated with significantly worse overall survival in cervical cancer (25), non-small cell lung cancer (26), gastric cancer (27), esophageal cancer (28), glioma (29), ovarian cancer (30), and other cancers (31). However, the prognostic value of PD-L1 for certain types of cancer is still controversial. Some studies reported that high PD-L1 could predict favorable prognosis (32, 33). In cervical cancer, squamous cell carcinomas tended to express more PD-L1 than adenocarcinomas (34). The possible reasons for these inconsistent results might include cancer type (or subtypes), tumor heterogeneity, sample size, clinical stage, different intervention, the time point of PD-L1 measurement as well as the different methodology used in research (such as detection methods and procedures).

MECHANISMS OF PD-L1 ACTIVATION IN CANCER

The tumor-intrinsic PD-L1 signaling pathway is inappropriately activated in many cancers. Mechanisms underlying aberrant PD-L1 activation mainly include genomic alterations (including copy number amplification and 3'-UTR disruption), constitutive oncogenic signaling activation, extrinsic factors and epigenetic mechanisms, such as upregulation of oncogenic microRNAs (miRNAs), downregulation of tumor suppressor miRNAs, aberrant DNA methylation, and histone modifications (**Figure 1**).

Copy Number Gain and 3′-UTR Disruption

Small-cell lung cancer (35), squamous cell carcinoma of the oral cavity (36), cervical cancer (37), ovarian cancer (38), breast cancer (39), melanoma, bladder cancer, head and neck cancer, soft tissue sarcoma and prostate cancer (40) exhibit increased copy number of chromosome 9p24, on which CD274 resides. Here, we investigated the frequency of elevated PD-L1 in ovarian cancer and endometrial cancer in The Cancer Genome Atlas (TCGA) data portal. Analysis of TCGA data by cBioPortal (41) demonstrated that overall, PD-L1 was highly expressed in these two cancers, mainly including gene amplification and mRNA up-regulation (**Figure 2A**). Moreover, analyses of U133A and U133Plus2 datasets in the GENT (gene expression across normal and tumor tissue) database (42) revealed that *PD-L1* was highly overexpressed in many tumor tissues (**Figure 2B**). Furthermore, analysis of the TCGA dataset was performed by using the MethHC browser (43). *PD-L1* mRNA expression was consistently upregulated across various cancers (**Figure 2C**). In addition, disruption of the 3' region of the *PD-L1* increases mRNA stability, leading to a marked elevation of aberrant *PD-L1* transcripts in multiple cancers (44).

Constitutive Oncogenic Signaling Activation

Loss of PTEN expression, activation of PI3K/AKT pathway, activation of RAS/MAPK pathway, inhibition of p53 signaling, upregulation of reprogramming factors (Oct4, Sox2, and c-Myc)

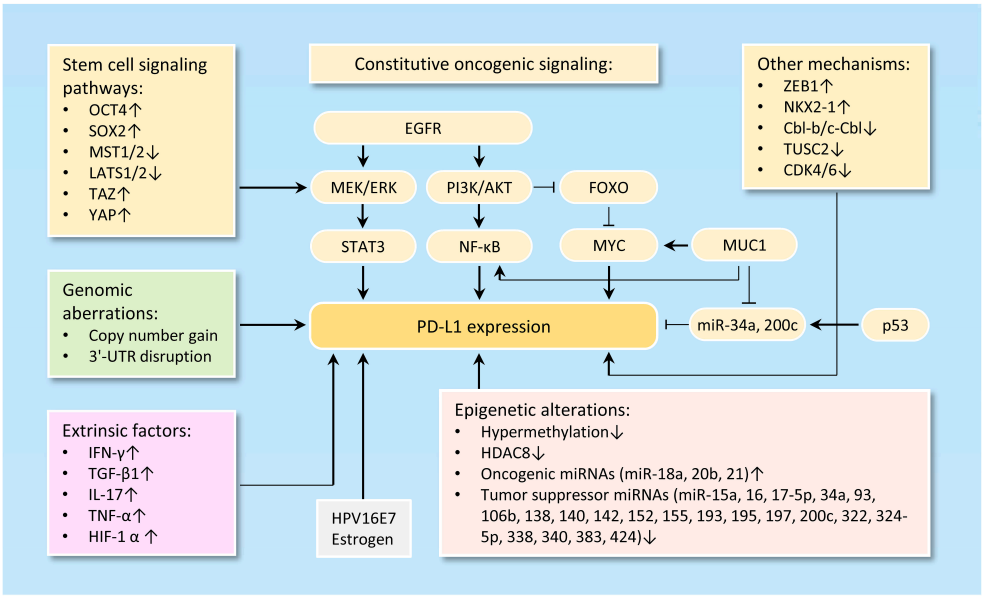

FIGURE 1 | Mechanisms of PD-L1 activation in cancer. The diagram illustrates the diverse mechanisms of PD-L1 activation in cancer, including genetic alterations to *PD-L1* (such as gene amplification, 3'-UTR disruption, or dysregulated transcription) and a wide range of epigenetic mechanisms (including upregulation of oncogenic microRNAs, downregulation of tumor suppressor microRNAs, aberrant DNA methylation and histone modifications).

and upregulation of ZEB1 (an inducer of EMT) are clearly linked to the activation of PD-L1 signaling pathway (45, 46) (**Figure 1**).

PD-L1 expression could be regulated via the PI3K/AKT and/or RAS/MAPK pathways in different tumor cell types (47–49). PD-L1 expression is suppressed by the tumor suppressor gene PTEN. Deletion of PTEN gene results in elevated PD-L1 expression at the translational level by activating the PI3K/AKT signal pathway (50, 51). FOXOs inhibit the expression of PD-L1 through repressing Myc or Wnt/β-catenin signaling pathways in tumor cells (52). MUC1 elevates *PD-L1* transcription by recruitment of MYC and NF-κB (a downstream effector of PI3K/AKT pathway (53) to the *PD-L1* promoter in breast cancer (54). Also, MUC1 was shown to increase PD-L1 levels via downregulation of miR-34a and miR-200c, two direct suppressors of PD-L1 (55–57).

Abnormal activation of stem cell signaling pathways has been implicated in the regulation of PD-L1. OCT4 is a key regulatory gene that maintains the self-renewal properties of CSC and promotes tumorigenesis of cervical cancer cells by miR-125b/BAK1pathway (58). We recently reported that, OCT4 promotes cervical cancer invasion and proliferation by enhancing PD-L1 expression through a miR-18a-dependent mechanism, by which miR-18a upregulates PD-L1 by targeting *PTEN*, *WNK2* and *SOX6* to activate the PI3K/AKT, MEK/ERK and Wnt/β-catenin pathways and inhibit the p53 pathway (25). In addition, SOX2, a transcription factor that controls tumor initiation and cancer stem-cell functions, can directly bind to the *PD-L1* promoter and transactive its expression, contributing to the increased proliferation of hepatocellular carcinoma cells (59). The upstream kinases of the Hippo pathway MST1/2 and LATS1/2 suppress PD-L1 expression, while TAZ and YAP enhance PD-L1 levels in breast and lung cancer cells (60).

Tumor cells undergoing EMT are shown to share a variety of capabilities with experimentally defined CSC (61). In lung cancer, PD-L1 expression was significantly higher in patients with EMT phenotypes (such as increased SNAI1 and Vimentin expression) compared with those with epithelial phenotypes (62). siRNA-mediated ZEB1 knockdown suppressed PD-L1 expression but promoted E-cadherin expression in esophageal squamous cell carcinoma (63). In agreement with these reports, cBioportal analysis of data on somatic copy number variation and mRNA level using TCGA endometrial and ovarian cancer dataset demonstrated that *PD-L1* is indeed co-amplified along with *MYC*, *SOX2*, *N-cadherin* and *SNAI1* in both cancer types (**Figure 2A**).

Another study reported that transcription factor NKX2-1 bound to the locus of *PD-L1* and induced its expression in mucinous lung cancer cells (64). In non-small cell lung cancer cells, the ubiquitin ligases Cbl-b and c-Cbl inhibit PD-L1 expression by inactivating STAT, AKT, and ERK signaling (65), and overexpression of tumor suppressor gene TUSC2 downregulated PD-L1 expression (66). CDK4 and CDK6 kinase destabilize PD-L1 protein via cullin 3–SPOP, leading to the downregulation of PD-L1 in cancer cells (67).

Regulation of PD-L1 Expression by Epigenetic Mechanisms

The expression of cancer-associated genes can occur by epigenetic mechanisms, including DNA methylation (68),

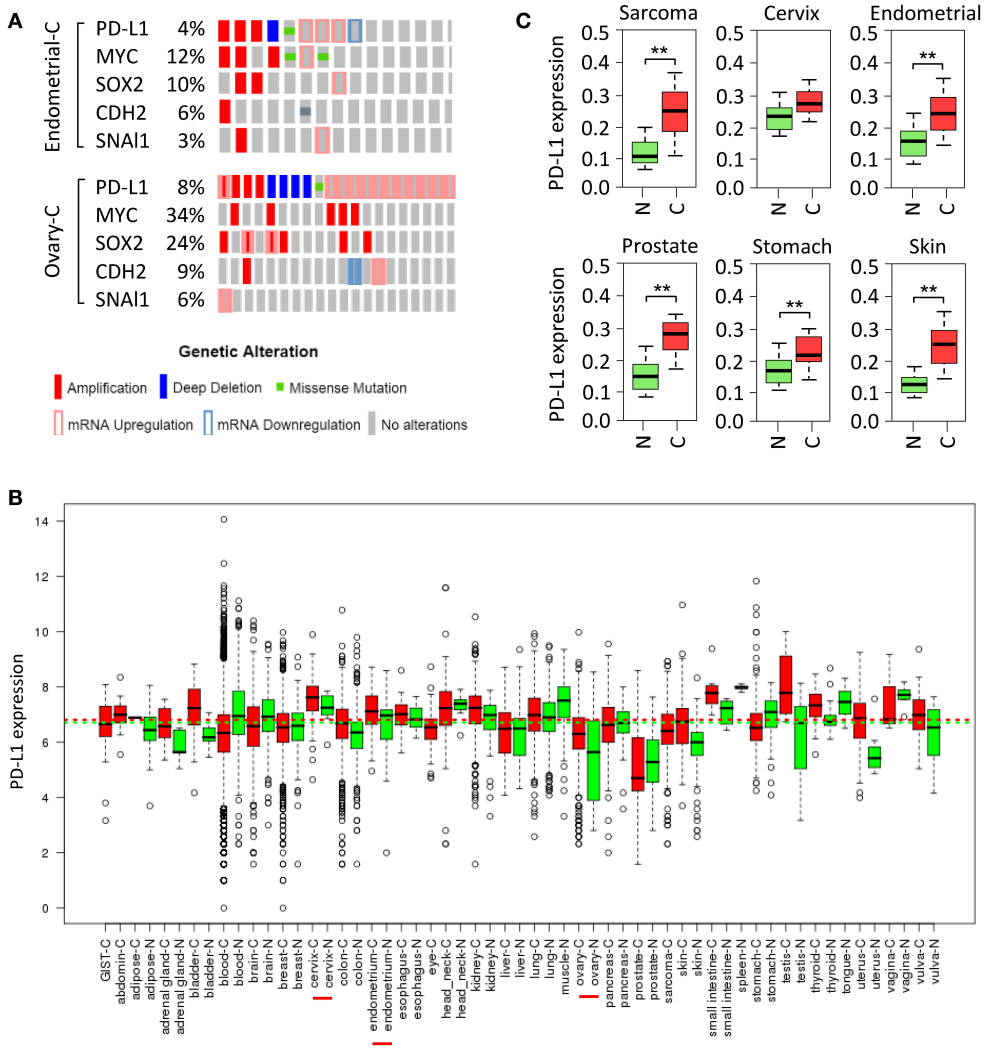

FIGURE 2 | Amplification and upregulation of PD-L1 and genes co-amplified with PD-L1 in TCGA data. **(A)** The Cancer Genome Atlas (TCGA) datasets in the cBioPortal database (www.cbioportal.org) was used to investigate molecular alterations (RNA expression, copy number variation, and mutation). Shown are OncoPrint outputs where each bar represents a tumor that was found to contain an alteration (amplification, deletion, mutation, upregulation, and downregulation, as indicated) in *PD-L1*, *MYC*, *SOX2*, *N-cadherin* (*CDH2*), and *SNAI1* gene in samples of endometrial cancer (upper panel) and ovarian cancer (lower panel) based on TCGA data. **(B)** *PD-L1* mRNA expression pattern was analyzed in a panel of cancer (red) vs. normal (green) tissues from the GENT database. **(C)** *PD-L1* expression pattern was determined in multiple cancer microarray datasets available in the MethHC database. N, normal; C, cancer. $^{**}P < 0.005$.

histone modification (69), chromatin remodeling, and non-coding RNAs (70). The anti-PD-1 therapy could induce PD-L1 promoter methylation and decrease PD-L1 levels in patients with non-small cell lung cancer (71). The class I histone deacetylase HDAC8 acts as an epigenetic inhibitor of PD-L1 expression in melanoma cells via modulating HOXA5 and STAT3 (72). Numerous miRNAs, including miR-15a/miR-16 (73), miR-17-5p (74), miR-93/106b (75), miR-138-5p (76), miR-140/miR-142/miR-340/miR-383 (25), miR-152 (77), miR-155 (78), miR-193 (73), miR-195 (73), miR-324-5p/miR-338 (79) and miR-322/miR-424 (80), have been shown to directly target and inhibit PD-L1 expression in tumor cells. In chemo-resistant non-small-cell lung cancer cells, miR-197 indirectly inhibits PD-L1 expression by regulating the CKS1B/STAT3 axis (81). On the other hand, oncogenic miR-20b and miR-21 inhibited PTEN expression, resulting in PD-L1 overexpression in colorectal cancer (82). Our recent data established that an oncogenic OCT4-miR-18a pathway serves as the key upstream activator of PD-L1 in cervical cancer (27).

Extrinsic Factors Influencing the Expression of PD-L1

The main regulators of PD-L1 are the interferon-γ (83), inflammatory cytokines such as IL-17 (84) and TNF-α (84), TGF-β1 (85), and HIF-1α (86). Of note, overexpressing HPV16E7 oncoprotein increased PD-L1 protein expression, and knockdown of HPV16E7 resulted in a reduction in

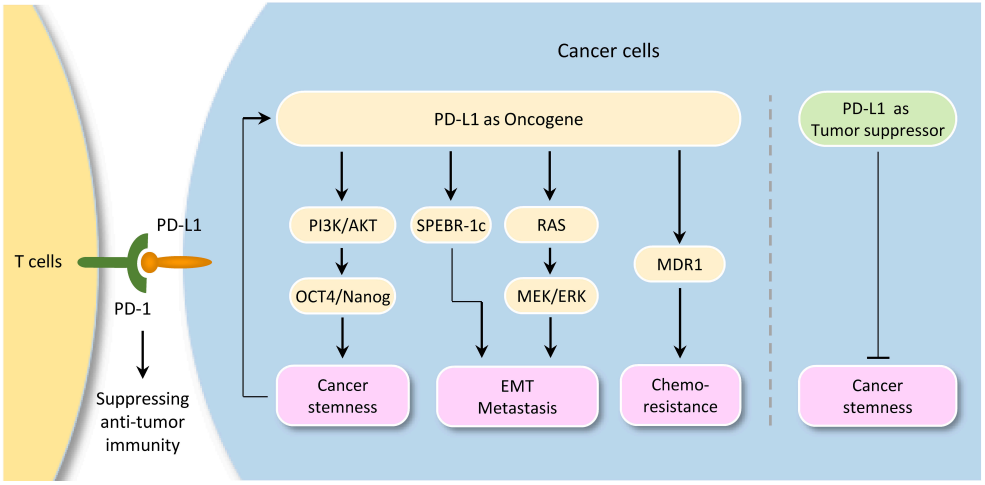

FIGURE 3 | Tumor-intrinsic PD-L1 signaling in cancer initiation and development. The diagram illustrates signaling events downstream of PD-L1 activation in cancer. Although PD-L1 could serve as a tumor suppressor by inhibiting cancer stem cell properties in cholangiocarcinoma, it plays a pivotal role in promoting cancer stemness, EMT, tumor invasion, and chemoresistance in several tumor types. Importantly, activation of OCT4 signaling induces PD-L1 expression in cancer cells, thereby suggesting a possible immune evasion mechanism employed by cancer stem cells during metastasis.

PD-L1 protein expression in cancer cells (87). Consistent with this data, PD-L1 protein expression was significantly higher in the normal cervical tissues with HPV infection than those normal cervical tissues without HPV infection (53). Estrogen is a well-known oncogenic driver of endometrial and breast cancer, and it upregulates PD-L1 protein expression in ERα-positive endometrial and breast cancer cells (88).

THE ROLE OF PD-L1 IN STIMULATING OR INHIBITING CANCER

A tumor-intrinsic role for PD-L1 in promoting cancer initiation, metastasis, development, and resistance to therapy is emerging (**Figure 3**). For instance, knockdown of PD-L1 expression in gastric cancer cells could significantly suppress cell proliferation, migration and invasion (89). Also, knockout of PD-L1 expression by CRISPR/Cas9 inhibits the spheroid formation of osteosarcoma cells (90). PD-L1 was shown to promote EMT in esophageal cancer (91). Knockdown of PD-L1 expression significantly suppressed tumor growth in nude mice in gastric cancer (92) and cervical cancer model (27).

Interestingly, a link between PD-L1 expression and EMT/CSC-like phenotypes has been reported. For example, bladder cancer cells with surface expression of PD-L1 exhibited signatures of immune evasion as well as increased stemness (93). PD-L1 has been shown to be preferentially expressed on $CD44^{high}$ CSCs in lung cancer cells (94). Selective expression of PD-L1 was observed on $CD44^{+}$ head and neck tumor cells compared with $CD44^{-}$ tumor cells (95). $CD133^{+}$/$PD\text{-}L1^{+}$ colorectal CSC cells showed the characteristic of EMT (96). Tumor cell-intrinsic PD-L1 promotes tumor-initiating cell generation in melanoma and ovarian cancer (97). Similarly, PD-L1 promotes OCT4 and Nanog expression in breast CSCs through the activation of PI3K/AKT pathway (98).

Moreover, PD-L1 overexpression promotes EMT and invasion in glioblastoma multiforme via RAS/ERK/EMT activation (99). RNA-sequencing analysis of glioblastoma multiforme revealed that PD-L1 significantly altered the expression of genes, which were enriched in cell growth/migration/invasion pathways (99). PD-L1 induced EMT via activating SREBP-1c in renal cell carcinoma (100). CRISPR/Cas9 system-mediated *PD-L1* disruption increased drug sensitivities for doxorubicin and paclitaxel (90). The interaction of PD-L1 with PD-1 induced phosphorylation of AKT and ERK, resulting in the activation of PI3K/AKT and MAPK/ERK pathways and increased MDR1 expression in breast cancer cells (101).

However, depletion of PD-L1 expression by shRNA in cholangiocarcinoma cells enhances their tumorigenicity and increases ALDH activity, and patients with lower PD-L1 expression shows poorer prognosis when compared with those with higher PD-L1 expression (102), indicating that PD-L1 may also have anti-tumor effects by inhibiting cancer stemness under certain circumstances.

CONCLUSIONS

It is becoming clear that, although PD-L1 could serve as a tumor suppressor by inhibiting cancer stem cell properties in cholangiocarcinoma, tumor cell-intrinsic PD-L1 plays a pivotal role in promoting cancer stemness, EMT, tumor invasion, and chemoresistance in several tumor types. Importantly, activation of OCT4 signaling and upregulation of EMT inducer ZEB1 induce PD-L1 expression in cancer cells, thereby suggesting a possible immune evasion mechanism employed by cancer stem cells during metastasis. The continued

characterization of immune-independent PD-L1 functions and identification of crucial signaling events upstream or downstream of PD-L1 in diverse cancer types (or specific cancer subtypes), would provide additional targets and new therapeutic approaches.

AUTHOR CONTRIBUTIONS

PD and HW provided direction. PD, YX, and HW wrote the manuscript. JY and SH made significant revisions to the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Prognostic Factors for Checkpoint Inhibitor Based Immunotherapy: An Update With New Evidences

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Checkpoint inhibitor (CPI) based immunotherapy (i.e., anit-CTLA-4/PD-1/PD-L1 antibodies) can effectively prolong overall survival of patients across several cancer types at the advanced stage. However, only part of patients experience objective responses from such treatments, illustrating large individual differences in terms of both efficacy and adverse drug reactions. Through the observation on a series of CPI based clinical trials in independent patient cohorts, associations of multiple clinical and molecular characteristics with CPI response rate have been determined, including microenvironment, genomic alterations of the cancer cells, and even gut microbiota. A broad interest has been drawn to the question whether and how these prognostic factors can be used as biomarkers for optimal usage of CPIs in precision immunotherapy. Therefore, we reviewed the candidate prognostic factors identified by multiple trials and the experimental investigations, especially those reported in the recent 2 years, and described the possibilities and problems of them in routine clinical usage of cancer treatment as biomarkers.

Keywords: immunotherapy, checkpoint inhibitor, PD-1, PD-L1, CTLA-4

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INTRODUCTION

Existence of immune checkpoints is essential for modulating duration and magnitude of T cell responses and maintaining self-tolerance (Pardoll, 2012), while suppression of antitumor immune responses facilitates harmful tumor growth. With a constantly deepening understanding of the immune system and its role on cancer development, the field of cancer immunotherapy has been explored with great enthusiasm, aimed at harnessing immune system to induce or restore antitumor activities (Topalian et al., 2011). Among complicated pathways of immune system, interactions of cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) with CD80/CD86, and programmed cell death 1 (PD-1) with programmed cell death ligand 1 (PD-L1) has been considered to act as "brakes" on the immune system (Linsley et al., 1991; Freeman et al., 2000; Schildberg et al., 2016). CTLA-4 has a much stronger affinity with CD80/86 than CD28, thus inhibiting crucial CD28/CD80 and CD28/CD86 based T cell activation (Manson et al., 2016), while PD-1/PD-L1 interaction induces imbalanced activation of signaling pathways which results in altered

T-cell metabolism and subsequent abnormal differentiation, leading to reduced T effector cells and increased T regulatory cells (Tregs) as well as T exhausted cells (Boussiotis, 2016). Therefore, CTLA-4 and PD-1/PD-L1 have been considered as the "star" candidate targets to immune-checkpoint blockade (ICB) based immunotherapy. Unprecedented success of anti-CTLA-4 and anti-PD-1/PD-L1 ICBs have been achieved in various tumor types that were previously sentenced to gloomy prognosis under traditional treatments (Thomas and Hassan, 2012; Gogas et al., 2013; Lee et al., 2015; Restifo et al., 2016), significantly prolonging overall survival with acceptable toxicity in patients with advanced melanoma (Hodi et al., 2010; Wolchok et al., 2013; D'Angelo et al., 2017), non-small-cell lung cancer (NSCLC) (Gettinger et al., 2015, 2016; Hellmann et al., 2017), and other tumor types (Hamanishi et al., 2015; Morris et al., 2017; Overman et al., 2017).

Until recently, six CPIs have been approved by the U.S. Food and Drug Administration (FDA), and all of them are monoclonal antibodies against the targets, including one for CTLA-4 (i.e., Ipilimumab), two for PD-1 (i.e., Pembrolizumab and Nivolumab), and three for PD-L1 (i.e., Avelumab, Atezolizumab, and Durvalumab) (**Table 1**). Ipilimumab was firstly approved for advanced melanoma in 2011 (Ma et al., 2016), which symbolizes the remarkable clinical success of anti-CTLA-4 and thus elicits further investigations into PD-1/PD-L1 pathway. Pembrolizumab was the first inhibitor for PD-1, which was approved as the second-line treatment for unresectable or metastatic melanoma, followed by Nivolumab (for unresectable metastatic melanoma, advanced metastatic NSCLC and advanced metastatic renal cell carcinoma), Atezolizumab (for urothelial carcinoma following platinum-based chemotherapy), Avelumab (for metastatic Merkel-cell carcinoma, and Durvalumab for urothelial carcinoma following platinum-based chemotherapy) (Manson et al., 2016; Pitt et al., 2016). Afterward, indications of these CPIs have been largely expanded after clinical trials, and exhibits remarkable disease responses in a wide range of histological types of carcinomas, such as hematologic malignancies, head and neck cancer, and bladder cancer (Armand et al., 2013; Postow et al., 2015a; **Table 1**). Recently, Nivolumab has been successfully used as a neoadjuvant therapy before surgery in patients with early untreated NSCLC, and preoperative usage of Nivolumab can induce augmentation of neoantigen-specific T cells (Forde et al., 2018). Noteworthily, though sharing almost similar mechanisms, anti-PD-L1 therapy may render distinct effect from anti-PD-1. The subtle difference lies in that the PD-L1 antibody does not block the interaction between PD-1 and PD-L2, while PD-1 blockade cannot block the interaction of PD-L1 with CD80, which is expressed on T cells and deliver inhibitory signals of antitumor activities (Butte et al., 2007). Actually, a meta-analysis has shown that anti-PD-1 achieves higher overall survival and response rate than anti-PD-L1 in NSCLC, which reveals anti-PD-1 as a better choice for patients with NSCLC (You et al., 2018). Moreover, accumulated evidence has indicated that combined usage of anti-PD and anti-CTLA-4 antibodies can synergetically improve clinical outcome compared with either agent alone (Larkin et al., 2015; Hodi et al., 2016; Hellmann et al., 2017; Wolchok et al., 2017), probably due to their different function mechanisms.

Although great success has been achieved with CPI based immunotherapy, large individual differences were noticed in terms of treatment outcomes (Gibney et al., 2016; Manson et al., 2016; Pitt et al., 2016; Topalian et al., 2016; Zou et al., 2016; Nishino et al., 2017), which varied among different cancer types. For instance, the response rate for patients treated with Ipilimumab is only 10–15% in metastatic melanoma (Hodi et al., 2010), and rarely exceeds 40% for PD-1 blockade therapy, even a large proportion of partial responders were included (Brahmer et al., 2012; Hamid et al., 2013), indicating that the majority of patients treated with PD-1/PD-L1 blockade fail to respond sufficiently. In addition, PD-1/PD-L1 blockade can induce immune-related adverse drug reaction events (ADR) deriving from non-specific immunologic activation, which are reported to be much less than those induced by anti-CTLA-4, though (Larkin et al., 2015; Robert et al., 2015). The toxicities observed in CPI treatment include the most frequent fatigue and possibly fatal inflammatory pneumonitis, and high grade adverse events may lead to forced abortion of the treatment (Zou et al., 2016). Worse still, some patients even demonstrate disease hyperprogression following treatment, which is defined as $<$2 months of time-to-treatment failure (TTF), $>$50% increase in tumor burden compared with preimmunotherapy imaging, and $>$2-fold increase in progression pace (Champiat et al., 2017; Kato et al., 2017). In this case, effective biomarkers for the indication of treatment outcomes are largely required. Indeed, some biomarker candidates have been put into practice, and recommended to be determined before CPI treatments.

In precision medicine era, understanding the mechanisms, by which patients lack response/produce resistance to CPI treatments or suffer from severe ADR, is of utmost importance for selecting the patients specifically suitable for the treatment. In this review, we will focus on current knowledge of factors that influence the sensitivity and resistance to CPI-based immunotherapy (e.g., clinical characteristics, genomic alterations, tumor microenvironment (TME), host immune functions, and gut microbiota), and highlight the potential biomarkers for CPI treatments, especially the new evidences reported lately (**Table 2** and **Figure 1**).

CLINICALLY RELEVANT FACTORS

Age, Gender, and Diet

Aging is commonly correlated with limited and dysfunctional immune activities characterized by reduced lymphocyte proliferation and increased exhausted T cells, resulting in susceptibility to various diseases and increased cancer incidence (Fulop et al., 2010; Lee et al., 2016). *In vivo* studies have shown upregulation of PD-1 expression on T cells of aged animals, indicating the potentially critical role of PD-1 blockades in the old (Mirza et al., 2010; Lim et al., 2015). Consistent with the decreased activity of immune system in elders, current evidence exhibited that ICB therapy can significantly benefit all age of patients with NSCLC with the exception of patients $\geq$75 years (Landre et al., 2016; Nishijima et al., 2016; Ferrara et al., 2017). In another hand, anti-PD-1/PD-L1 is found

TABLE 1 | FDA-approved immune checkpoint inhibitors in cancer treatment.

Target	Antibody	Trade name	Company	Indication (approval date)
CTLA-4	Ipilimumab	YERVOY	Bristol-Myers Squibb (BMS)	Unresectable or metastatic melanoma (2011)
PD-1	Pembrolizumab	KEYTRUDA	Merck Sharp & Dohme (MSD)	Unresectable or metastatic melanoma (approved for patients with disease progression after ipilimumab and, if BRAF V600 mutation positive in 2014, and expanded to initial treatment in 2015)
				Metastatic NSCLC whose tumors express PD-L1 as determined by an FDA-approved test and who have disease progression on or after platinum-containing chemotherapy (2015)
	Nivolumab	OPDIVO	Bristol-Myers Squibb (BMS)	Metastatic melanoma (2014, approved for BRAF V600 wild-type tumor in 2015)
				Squamous NSCLC with progression or after platinum-based drugs (2015, and expanded to non-squamous NSCLC later in 2015)
				Advanced metastatic renal cell carcinoma after angiogenic therapy (2015)
				Classical Hodgkin lymphoma that has relapsed or progressed after autologous hematopoietic stem cell transplantation and post-transplantation brentuximab vedotin (2016)
				Locally advanced or metastatic urothelial carcinoma which have progression during or following platinum-containing chemotherapy or have progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy (2017)
PD-L1	Atezolizumab	TECENTRIQ	Roche and Genentech	Locally advanced or metastatic urothelial carcinoma after failure of cisplatin-based chemotherapy (2016), but the confirmatory trial failed
				Metastatic NSCLC whose disease progressed during or following platinum-containing chemotherapy (2016)
	Avelumab	BAVENCIO	Merck and Pfizer	Metastatic Merkel-cell carcinoma (2017)

TABLE 2 | Factors related to the efficacy of ICBs.

Classification	Biomarkers	Influence
Clinical-relevant factors	Age	The elderly patients lack response to ICBs.
	Gender	Male patients respond better to ICBs.
	Diet	Obesity and improved FA catabolism improve anti-PD therapy.
	Viral infection	MCV and EBV infected patients respond better to anti-PD therapy.
Tumor autonomous mechanisms	Tumor mutational/neoantigen load	High mutational/neoantigen loads improve efficacy of ICBs
	PD-L1 expression	High PD-L1 expression improves anti-PD therapy
Tumor microenvironment	Cells	Increased TILs improve response to ICBs, while Tregs and MDSCs impair the efficacy.
	Immunoregulatory pathways	Inhibition of TH1 chemokines, CD28/B7, IFN and activation of TGFβ, TIM3 lead to resistance to PD blockades.
Host-related factors	Peripheral blood markers	Increased eosinophils, lymphocytes, monocytes and low LDH levels improve response to PD blockades.
	MHC class I	Impaired MHC class I molecules lead to resistance to anti-PD therapy
	TCR repertoire	Less diverse T cell repertoire improves response to anti-PD
	The gut microbiota	*Bacteroides* species facilitate anti-CTLA, more diversified bacteria, such as *Bifidobacterium, Akkermansia muciniphila, Ruminococcaceae bacteria,* facilitate anti-PD.

to be capable of inducing hyperprogressive disease during the treatment, which is more frequent in elderly patients (Champiat et al., 2017). Therefore, the age at diagnosis may influence the efficacy and side ADR rate of CPI treatments, although more confirmation investigations with larger samples and less heterogeneity are warranted to settle this debated topic.

Substantial sex-dependent diversities in innate and adaptive immunity have been noticed for a long time, resulting in different susceptibility and immune functions in response to infections and autoimmune diseases between males and females (Fischer et al., 2015; Klein and Flanagan, 2016). Interestingly, accumulated evidence has highlighted that gender plays a considerable role in response to CPIs. A systematic review on the relationship

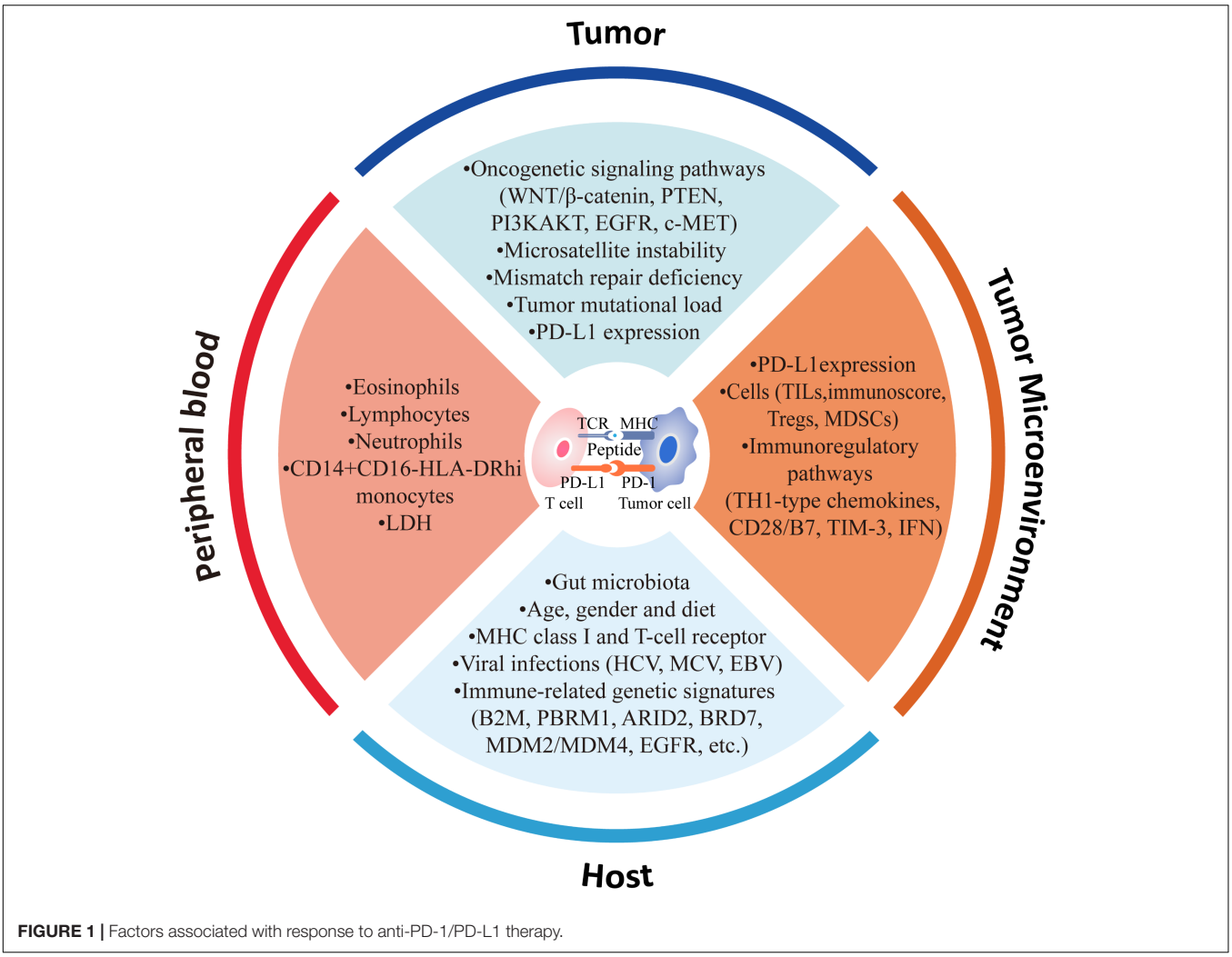

FIGURE 1 | Factors associated with response to anti-PD-1/PD-L1 therapy.

between efficacy and sex of patients indicates that the efficacy of CPI based treatments is sex-dependent, with significantly greater benefit in male patients in all studied cancer types (Conforti et al., 2018). Likewise, another study shows that more improvement of survival resulting from CPI treatment is observed in males than females, and the survival of patients treated with anti-CTLA-4 is more influenced by sex compared with those receiving anti-PD-1 (Wu et al., 2018). Though the current conclusions are not confirmed and clinical trials including more female patients are needed, the gender of patients should be taken into consideration in CPI based treatments.

Healthy diet including sufficient nutrient intake is of great significance for maintaining powerful immune defense against invading pathogens, especially for patients combating tumor progression. It is well reported that unbalanced diet may lead to impaired immunity and accelerate disease development, and obesity is associated with chronic inflammation and cancer development (Fang et al., 2017; Quail et al., 2017). Paradoxically, a meta-analysis of patients with metastatic melanoma indicates that obesity is correlated with improved benefit of anti-PD therapy compared with normal body-mass index (BMI) (McQuade et al., 2018). Interestingly, this association is only observed in males without any clear mechanisms clarified. Moreover, dysregulated metabolism may contribute to the exhaustion of lymphocyte infiltration within the TME. For example, it has been recently discovered that CD8 + T cells enhance peroxisome proliferator-activated receptor (PPAR)-α signaling and catabolism of fatty acids when simultaneously subjected to hypoglycemia and hypoxia. Promoting fatty acid catabolism obviously improves the capacity of tumor infiltrating lymphocytes (TILs) to delay tumor growth and synergizes with PD-1 blockade to efficiently boost the efficacy of melanoma immunotherapy (Zhang Y. et al., 2017). Through influencing multiple immune components and functions, diet and metabolic factors might be related to clinical effect of PD-1 blockade, though direct evidence is currently lacked.

Viral Infections

Disorders of the immune system and failure in tumor eradication can result from viral infections, which may also impact the ICB treatment response. For instance, a clinical observation regarding advanced Merkel-cell carcinoma exerts significantly

high level of clinical response, providing a novel perspective that virus-positive status may contribute to success of anti-PD-1 therapy (Nghiem et al., 2016). Theoretically, oncogenic viruses may serve as strong tumor-specific antigens, and cancer cells should escape from the immune monitoring through inducing immune inhibition. In fact, overexpression of PD-L1 is commonly observed in Merkel-cell carcinoma cells (Wong et al., 2015). Similarly, Epstein-Barr virus (EBV)-positive gastric cancer has been recently reported to have low mutation burden but high expression of immune checkpoint pathways and abundant lymphocytic infiltration, thus demonstrating meaningful clinical response to PD-1/PD-L1 inhibitors (Janjigian et al., 2017; Panda et al., 2017). It has been further discovered that part of CD8 + TILs can recognize tumor unrelated epitopes, such as those from EBV, human cytomegalovirus and influenza virus, which may explain the mechanism by which virus-positivity facilitates host immunity. Moreover, these CD8 + TILs lack the expression of CD39, suggesting that measuring CD39 expression could be an effective approach to select the patients with high possibility of virus infection (Simoni et al., 2018). Although more virus related ICB treatment trials with larger sample size are warranted, current evidence implies oncogenic viruses may be considered as a potential biomarker for predicting effect of anti-PD therapies.

TUMOR AUTONOMOUS MECHANISMS

Tumor Mutational Loads, Mismatch Repair Deficiency, and Microsatellite Instability

Tumor mutational burden (TMB), which is mostly determined by next generation sequencing, has been broadly found to be associated with the response to CPIs. Evidence from clinical trials suggests the positive correlation between high tumor mutational loads and improved clinical efficacy of ICB-based therapies (including anti-PD-1, anti-PD-L1, and anti-CTLA-4) in NSCLC and melanoma (Snyder et al., 2014; Rizvi et al., 2015; Van Allen et al., 2015; Hugo et al., 2016; Forde et al., 2018), which have the highest mutation burdens as well as response rates (Lee et al., 2010; Berger et al., 2012; Topalian et al., 2012). Actually, a pooled analysis across 27 tumor types or subtypes illustrated a significantly strong positive correlation between the TMB and the objective response rate to PD-1 inhibition (Yarchoan et al., 2017), indicating the biomarker potential of TMB for PD-1 blockade efficacy. Besides, TMB also predicts clinical efficacy in the combination of anti-PD-1 and anti-CTLA-4 (Hellmann et al., 2018). Loss-of-function of alterations in genes involved in DNA repair can largely induce high TMB, and lack of the ability to repair DNA errors is closely related to microsatellite instability (MSI). Therefore, remarkable clinical benefit from ICB therapy are significantly enriched in patients with MSI status (Le et al., 2015) or specific alterations in DNA repair genes, such as *BRCA2*, *POLD1, POLE,* and *MSH2* (Rizvi et al., 2015; Hugo et al., 2016). Due to the stronger practicality, clinical examination of MSI status, deficiency of mismatch repair genes (through immunohistochemistry), or Lynch Syndrome (inherited mutations in mismatch repair genes with family history) can efficiently predict the good responders, although some patients with negative signals of these potential biomarkers may still get benefit from ICB treatments (Dudley et al., 2016).

It is considered that better response of patients with high TMB to ICB response is attributed to immunogenicity of tumor cells, somatic mutations of which can be translated to antigens and recognized as tags of "foreign" by the immune system (Gibney et al., 2016). These tumor-specific antigens are named as "neoantigens," and thereby provide highly specific targets for anti-tumor activities of the immune system (Hacohen et al., 2013; van Rooij et al., 2013). The process of neoantigen recognition is attenuated by expression of PD-L1 and some other immunosuppressive ligands (Pages et al., 2005; Llosa et al., 2015). Hence, blockade of immune checkpoints will release inhibition of immune system and reinvigorate pre-existing neoantigen recognition. Not surprisingly, neoantigen burden is closely correlated to TMB, and can be also induced by mismatch repair deficiency (Le et al., 2015). Quite a few patients with advanced mismatch repair-deficient cancers demonstrate significantly durable responses to PD-1 blockade with expanded neoantigen-specific T cell clones (Le et al., 2017). Additionally, neoantigens are mostly predicted by bioinformatic approaches with computational algorithms, which is highly imperfect in terms of low validation rate (e.g., 1–3 mutation-associated neoantigens out of top 30–50 predicted candidates validated by T cell responses) (Kvistborg et al., 2014; Tran et al., 2015), while it is complicated and time-consuming to determinate the functional neoantigens with a series of immunologic experimental investigations, making it improper for neoantigens as an effective clinical biomarkers so far.

Few but important exceptions rejecting the predictive role of tumor mutational status exist in the aforementioned studies (Rizvi et al., 2015; Hugo et al., 2016), consistent with a finding that tumor infiltration is not weakened under the circumstance of low mutational loads in gastrointestinal cancers (Tran et al., 2015), indicating other equally considerable mechanisms contributing to treatment resistance. Neoantigen intratumour heterogeneity may play an important role, and patients with both high TMB and low neoantigen intratumour heterogeneity (<1%) have significantly longer progress-free survival and overall survival compared to patients with high TMB alone (McGranahan et al., 2016). Moreover, strong antigens may disobey the correlation of neoantigen and TMB. For instance, Merkel cell polyomavirus (MCV)-associated Merkel-cell carcinomas have a 100 times lower mutational load than ultraviolet-induced virus-negative Merkel-cell carcinomas (Wong et al., 2015; Goh et al., 2016), but exhibit better response to ICB therapy, which can be explained by its presentation of strong viral antigens (Yarchoan et al., 2017).

PD-L1 Expression

Increased PD-1 ligands and their ligation to PD-1 on tumor-specific CD8 + T cells is a pivotal strategy adopted by tumors to contend with host immune responses. In certain cancer types (e.g., melanoma, NSCLC, pancreatic cancer, breast cancer, and gastrointestinal stromal tumors), PD-L1 expression

is upregulated and associated with poor prognosis (Konishi et al., 2004; Bertucci et al., 2015; Sabatier et al., 2015; Birnbaum et al., 2016). Tumor PD-L1 upregulation reflects negative dynamic immune activities in the TME (Taube et al., 2012; Spranger et al., 2013) and is the premise of anti-PD-1/PD-L1 therapy. So far, PD-L1 is one of the best-studied as well as widely used biomarkers.

Studies on NSCLC have shown that patients with high expression of PD-L1 on the surface of tumor cells have significantly better clinical responses to PD-1/PD-L1 inhibitors (Passiglia et al., 2016; Muller et al., 2017). Likewise, patients treated with the anti-PD-1 antibody BMS-936558 (also known as MDX-1106) respond differently according to their PD-L1 status (Brahmer et al., 2010; Topalian et al., 2012). In a meta-analysis of patients treated with Nivolumab, Pembrolizumab or MPDL3280A (an engineered anti-PD-L1 antibody), response rates are significantly higher in PD-L1-positive tumors, and the predictive role of PD-L1 on tumor cells is stronger for Pembrolizumab and Nivolumab (Carbognin et al., 2015). Samples from several cancer types demonstrate that response to anti-PD-1 blockade is most closely correlated with the expression of tumor cell PD-L1 in comparison with that of other immunosuppressive molecules such as PD-1 and PD-L2 (Taube et al., 2014). On the other hand, in addition to PD-L1 expressed on tumor cells, PD-L1 expression on tumor infiltrating cells also displays noteworthy connections with clinical outcome of MPDL3280A (Herbst et al., 2014; Powles et al., 2014).

PD-L1 immunohistochemistry (IHC) has been approved by FDA as a companion diagnostic to select patients with NSCLC suitable for Pembrolizumab treatment. Nevertheless, absence of PD-L1 does not necessarily imply poor response to anti-PD-1/PD-L1 blockades. Some patients with low PD-L1 expression still demonstrate impressive clinical effect. The paradoxical predictive value of PD-L1 expression may partly be explained by different standards of analyzing, including different staining techniques or assessed range (tumor or both tumor and cells in microenvironment). The different threshold of PD-L1 expression is also important. A good example is the clinical trials of Nivolumab vs. Pembrolizumab as first-line treatment. Nivolumab was the firstly emerged anti-PD-1 CPI, however, failed in clinical trials probably because of the low setting of PD-L1 expression threshold at >1%. On the contrary, Pembrolizumab was later developed and precisely applied to the patients with PD-L1 expression >50% in clinical trials, which made it successfully become the first-line treatment for NSCLC. Besides, dynamic and inducible characteristic of PD-L1 expression also contributes to the contradictory results. PD-L1 can be up-regulated by IFNγ, hence patients with low baseline PD-L1 level may gradually become strong PD-L1 positive under an inflammatory circumstance as the treatment proceeds, and the response to anti-PD blockade also changes as PD-L1 is upregulated (Manson et al., 2016; Zou et al., 2016). Therefore, the application of PD-L1 expression assessment is endowed with useful but not definitive predictive value.

In another hand, further efforts are still needed to refine the clinical use of PD-L1 expression as biomarkers, especially detected by immunohistochemistry. Firstly, PD-L1 expression may be checked in multiple sites of tumor at multiple time points, because PD-L1 expresses dynamically and thus can be influenced by different mechanisms; secondly, standardized determination of PD-L1 expression is largely needed to exclude the possible variation induced by different PD-L1 antibodies (Gibney et al., 2016).

Gene Mutations and Genomic Alterations in Tumor

Cancer cell genetic alterations in pivotal signaling pathways might be responsible for suppressed T cell activities and deficient antitumor immunity, consequently impacting response to anti-PD therapies (**Table 3**). Tumor-intrinsic activation of WNT/β-catenin signaling pathway results in subdued CCL4 expression and subsequent precluded dendritic cell (DC) recruitment and DC-mediated T-cell activities, thus leading to resistance to anti-PD-L1 and anti-CTLA-4 therapies (Spranger et al., 2015). Loss of phosphatase and tensin homolog (PTEN) as well as activation of PI3K-AKT pathway in tumor cells brings about increased immunosuppressive cytokines and attenuated T-cell infiltration and activity, thereby promoting resistance to PD-1 inhibitor therapy (Peng et al., 2016). Similarly, EGFR pathway activation has been found to be correlated with development of immunosuppressive microenvironment represented by upregulation of PD-1, PD-L1, CTLA-4, and multiple tumor-promoting inflammatory cytokines (Akbay et al., 2013). Patients with *EGFR* mutation even receive less benefit from ICB therapy compared to chemotherapy (Borghaei et al., 2015; Rittmeyer et al., 2017). Clinical data of patients with NSCLC shows that mutations in *EGFR* are associated with low overall response rate to PD-1/PD-L1 inhibitors due to decreased PD-L1 expression and CD8 + TILs. However, T790M-negative *EGFR*-mutant patients are more likely to benefit from anti-PD-L1 after previous treatment (Gainor et al., 2016; Haratani et al., 2017). In addition to poor outcome, patients with *EGFR* alterations tend to be hyperprogressors with significantly increased tumor growth rate after receiving PD-1/PD-L1 inhibitors (Kato et al., 2017). In the other hand, recent evidence indicates that inhibitors of the receptor tyrosine kinase c-MET impair reactive mobilization and recruitment of neutrophils into tumors and draining lymph nodes, and thus increase effector T cell infiltration, suggesting c-MET pathway inhibition may improve responses to checkpoint immunotherapies including anti-PD (Glodde et al., 2017).

Relapse specific mutations were investigated and identified in four patients with required resistance to PD-1 blockade therapy in melanoma, including loss of function of *JAK1*, *JAK2*, and *B2M,* which induces either lack of response to interferon gamma (IFNγ), or loss of surface expression of major histocompatibility complex I (MHC I) (Zaretsky et al., 2016). Afterward, multiple clinical reports and subsequent experiments have confirmed that *B2M* alterations in tumor cells (i.e., mutations, deletions, and down-regulation) can largely induce acquired CPI resistance (Gettinger et al., 2017; Janjigian et al., 2017; Grasso et al., 2018). Importantly, high frequency of initial *B2M* mutations were found in patient-derived xenografts for lung cancer, suggesting patients with this gene mutation may experience primary resistance to CPIs (Pereira et al., 2017). With CRISPR screening,

TABLE 3 | Alterations of genes associated with effect of anti-PD therapy.

Gene	Change of the response caused by mutations	Mechanism
BRCA2	Better	Mismatch repair deficiency (Hugo et al., 2016)
POLD1, POLE, MSH2	Better	Mismatch repair deficiency (Rizvi et al., 2015)
PTEN	Worse	Increased immunosuppressive cytokines and attenuated T-cell infiltration and activity (Peng et al., 2016)
EGFR	Worse	Decreased PD-L1 expression and CD8 + TILs (Gainor et al., 2016; Haratani et al., 2017)
JAK1, JAK2	Worse	Insensitivity to IFNγ and its antiproliferative effects on cancer cells (Zaretsky et al., 2016)
CALR, PDIA3, TAP1	Worse	Impaired HLA-1 complex (Pereira et al., 2017)
B2M	Worse	Impaired MHC type I and HLA-1 molecules (Zaretsky et al., 2016; Janjigian et al., 2017; Pereira et al., 2017)
PBRM1	Better	Activation of JAK-STAT signaling pathway and elevated sensitivity to IFNγ (Miao et al., 2018; Pan et al., 2018)
ARID2, BRD7	Better	Enhanced sensitivity to T-cell-mediated cytotoxicity (Miao et al., 2018)
MDM2/MDM4, DNMT3A	Worse	Inhibition of p53 tumor suppressor (Kato et al., 2017)
TERT, NF1, NOTCH1	Better	Unclear (Kato et al., 2017)
APLNR	Worse	Attenuated IFNγ responses in tumors (Patel et al., 2017)

multiple genes were also identified to be essential for cancer immunotherapy, including *APLNR*, which can interact with *JAK1* (Patel et al., 2017). Therefore, alterations of these genes may also induce primary or acquired resistance. Clinically, it will be helpful to predict the poor responders and relapse risk by examining the alterations status of these resistance-related genes, which can be further considered as biomarkers.

Despite of point mutations, somatic copy number alterations (SCNAs) and structure variations (SVs) are also key hallmarks and driver events of tumorigenesis. Interestingly, most of the gene expression signatures exhibit down-regulation in high level of SCNAs tumors (also named aneuploidy tumors), including CD8 + T cell receptors and IFNγ pathways. Consistently, SCNA level is negatively related to the CPI treatment outcomes. Although paradoxically, SCNAs levels are positively correlated with the number of TMBs in 8 out of 12 tumor types, especially with passenger mutations. Combination of aneuploidy and TMB can increase the prediction efficiency to separate good and poor responders, indicating the potential of SCNAs as independent biomarkers (Davoli et al., 2017).

TUMOR MICROENVIRONMENT

Cells Contributing to Tumor Immunity

The TME includes not only tumor cells, but also extracellular matrix, stromal cells and immune cells, which closely interact with tumor itself. As the main force in anticancer immunity, the presence of TILs has been commonly considered as a favorable predictor for prognosis of cancers (Ruffini et al., 2009; Reissfelder et al., 2015; Brambilla et al., 2016). High baseline level of pre-existing CD8 + T cells as well as increase in tumor infiltrating CD8 + T cells during treatment has been found to be associated with better response of patients treated with anti-PD-1 therapy (Tumeh et al., 2014; Daud et al., 2016). In turn, anti-PD blockades also increase the number and restore the function of effector T cells during the treatment (Wei et al., 2017; Zhou et al., 2017). Interestingly, TMB and PD-L1 overexpression is correlated to presence of TILs (Herbst et al., 2014; Nishino et al., 2017). Also, DNA repair gene mutation is companied by prominent lymphocyte infiltrates, especially activated cytotoxic T cells.

Nonetheless, a recent study on gastric adenocarcinoma indicates that increasing CD8 + T cells are surprisingly correlated with impaired survival as well as higher PD-L1 expression, which marks an adaptive immune resistant microenvironment (Thompson et al., 2017). In some clinical studies, increased TIL density after the second dose of CPI instead of the baseline of TIL status was significantly associated with clinical CPI activities (Hamid et al., 2011; Tumeh et al., 2014). Moreover, an approach to systematically assessing intra- and peri-tumoral T cell infiltration, namely immunoscore, has been considered as a stronger predictor of prognosis as well as response to ICB therapies due to its integrated evaluation of immune features (Mlecnik et al., 2016; Voong et al., 2017). Both Tregs and myeloid derived suppressor cells (MDSCs) contribute to T cell dysfunction and TME immunosuppression, thus presenting profound impact on resistance to PD blockades (Kalathil et al., 2013). The comparison of anti-PD-1 sensitive and resistant patients reveals that Tregs partly preclude the efficacy of anti-PD-1 (Ngiow et al., 2015), and that depletion of Tregs can potentiate checkpoint inhibitors (Taylor et al., 2017). Nevertheless, it is reported that apoptotic Tregs sustain and even amplify their immunosuppressive function via the adenosine and A2A pathways under oxidative stress, which highlights oxidative pathway as a metabolic checkpoint controlling Tregs and thus affecting the effect of anti-PD (Maj et al., 2017). Moreover, it has been newly discovered that a canonical nuclear factor κB (NF-κB) subunit c-Rel plays an essential role in Treg function, and chemical inhibition of c-Rel impairs Treg-mediated immunosuppression and potentiates the effect of anti-PD-1 therapy (Grinberg-Bleyer et al., 2017). MDSCs proliferate during cancer, inflammation and infection, and

perform the immunosuppressive function through restraining T-cell response. Reducing the number of MDSCs has been proved to be capable of enhancing antitumor effect of anti-PD-1 blockade (Orillion et al., 2017). Indoleamine-2, 3-dioxygenase (IDO) is a rate-limiting enzyme that controls tryptophan catabolism in tumor cells and MDSCs within the TME, which is recognized as an important microenvironmental factor that impairs cytotoxic T cell responses and survival (Schafer et al., 2016). The microsatellite instable subset of colorectal cancer, distinguished by high expression of IDO, poorly responds to anti-PD-1 therapy (Xiao and Freeman, 2015). On the contrary, IDO-knockout mice treated with anti-CTLA-4 or anti-PD-1/PD-L1 demonstrate significant tumor growth regression and prolonged survival, and combination treatment of IDO inhibitors and CTLA-4 blockade has achieved remarkable tumor rejection (Holmgaard et al., 2013). Importantly, combination of anti-PD-1 CPI and IDO inhibitor (e.g., epacadostat) can increase the objective response rate and prolong the overall survival in clinical trial phase I/II, however, surprisingly failed in phase III recently in 2018, with no benefit but increased ADR rate, possibly requiring a biomarker to distinguish the precious responders.

Immunoregulatory Pathways Within TME

In addition to alterations in signaling pathways in tumor itself, a series of pathways within TME also regulate immune activities and thus impact on effect of anti-PD therapies. Epigenetic silencing of T helper 1 (TH1)-type chemokines, CXCL9 and CXCL10, precludes effector T cells from trafficking to the TME and directly interacting with tumor cells. And it has been proved that epigenetic modulators can restore T cell activities and increase T cell infiltration, thus strengthening the therapeutic efficacy of PD-L1 blockade (Peng et al., 2015). Moreover, the lack of response to PD-1 blockade has also been found related to a signature of TGFβ signaling, which renders T cell exclusion and blocked acquisition of TH1-effector phenotype. And inhibition of TGFβ signaling provokes antitumor activities and promotes tumor susceptibility to anti-PD therapies in colorectal cancer as well as urothelial cancer (Mariathasan et al., 2018; Tauriello et al., 2018). CD28/B7 costimulatory pathway is commonly known to be required for T cell proliferation and activation. It is newly discovered that PD-1/PD-L1 interaction suppresses T cell function primarily by CD28 inactivation, and the rescue of exhausted CD8 + T cells by PD blockades is strongly dependent on CD28 expression, which elucidates the important role of CD28/B7 costimulatory pathway as a response indicator for anti-PD therapies (Hui et al., 2017; Kamphorst et al., 2017). Interestingly, contrary to that elevated PD-L1 expression benefits the response to anti-PD therapy, upregulation of alternative immune checkpoints, notably T-cell immunoglobulin mucin-3 (TIM-3), is related to adaptive resistance. And subsequent addition of TIM-3 blocking antibody can significantly reverse the treatment failure of PD-1 blockade (Koyama et al., 2016).

Particularly, another important pathway is IFN signaling, including IFN type I and II. IFNγ, produced primarily by Th1 cells, NKT cells and NK cells (Farrar and Schreiber, 1993; Boehm et al., 1997), is abundantly generated and activated when ICBs enhance T cell responses (Liakou et al., 2008; Peng et al., 2012). As a pleiotropic and critical cytokine in host immune activities and tumor rejection (Dighe et al., 1994; Kaplan et al., 1998), IFNγ exerts its effects through a complex and orderly signaling pathway (Ikeda et al., 2002). Loss or deficiency of IFNγ signaling pathway may render disorders of host immune behavior and consequent insensitivity to immunotherapy (Kaplan et al., 1998; Dunn et al., 2005). In a study on metastatic melanoma described above, loss-of-function mutations in genes involved in IFNγ pathway (e.g., *JAK1* and *JAK2*) are found associated with relapse of patients who have shown initial response to anti-PD-1 therapy. And *in vitro* truncating mutations of *JAK1* and *JAK2* results in insensitivity to IFNγ and its antiproliferative effects on cancer cells (Zaretsky et al., 2016). IFNγ functions as an important inducer of PD-L1 on the surface of tumor cells (Taube et al., 2012), and patients who have a better response to PD-L1 blockade also have increased IFNγ and IFNγ-inducible chemokines (Herbst et al., 2014; Powles et al., 2014). These researches shed light on the vital role of defective IFNγ pathway in the clinical effect or prognosis of anti-PD therapies. Distinct from IFNγ, type I IFN within innate immune system is critical for T cell priming and tumor elimination through signaling on DCs and lack of type I IFN will result in limited useful T cells for reactivating of antitumor activities (Diamond et al., 2011; Fuertes et al., 2011). This is in consistence with the effect of type I IFN induced by radiotherapy (Lim et al., 2014). Moreover, radiation-induced type I IFN has been proved to increase expression of MHC class I and antigen recognition (Burnette et al., 2011; Deng et al., 2014b). Peritumoral injection of immunostimulatory RNA into immune-cell-poor melanomas has been observed to initiate a cytotoxic inflammatory response and tumor inhibition mediated by type I IFN. More importantly, the activation of type I IFN upregulates the expression of both PD-1 and PD-L1 and consequently leads to prolonged survival when PD-1 blockade is combined (Bald et al., 2014).

HOST-RELATED FACTORS

Peripheral Blood Markers

Great interest has also been aroused in exploring biomarkers within serum or plasma due to the convenience of sample acquirement. In terms of immune cells, relatively high eosinophil count and lymphocyte count indicate favorable overall survival in patients with melanoma treated with Pembrolizumab (Weide et al., 2016). A pretreatment neutrophil-to-lymphocyte ratio (NLR) < 5 has been reported to be associated with improved survival of patients with NSCLC treated with Nivolumab (Bagley et al., 2017). The baseline frequency of CD14 + CD16-HLA-DRhi monocytes has also been found to strongly predict the response to anti-PD-1 of patients with melanoma (Krieg et al., 2018). Moreover, low lactate dehydrogenase (LDH) is related to the prognosis of patients receiving anti-PD-1 therapy. Studies on patients with melanoma reveal that patients with an elevated baseline LDH have a significantly shorter overall survival compared to patients with normal LDH, and the extent of increase in LDH during treatment is also correlated with the outcome of anti-PD-1 (Diem et al., 2016; Weide et al.,

2016). Notably, a peripheral blood profiling reveals that clinical failure of anti-PD-1 therapy does not only result from insufficient host immune activation, but also depends on the ratio between circulating Ki-67-positive cytotoxic T cells and pretreatment tumor burden. Patients with higher ratio are more likely to exhibit improved response rate and survival (Huang et al., 2017), indicating that decreasing tumor burden by previously appropriate topical treatment may facilitate the effect of anti-PD therapy.

MHC Class I and T-Cell Receptor (TCR)

MHC class I presenting antigen to cytotoxic T cells is an essential prerequisite for immune recognition and elimination of transformed cells (Aptsiauri et al., 2007). Downregulation of MHC class I has been acknowledged as a common mechanism of tumor immune escape and a potential determinant of clinical success of many immunotherapies (Haworth et al., 2015). Therefore, impaired MHC class I molecules have also been proposed as a candidate mechanism of resistance to anti-PD therapies, which has been reported to mainly result from deficiency in β2-microglobulin (B2M), a critical component of human MHC class I molecules (also named as HLA in human) required for CD8 + T cell recognition (Restifo et al., 1996; Wang et al., 2016; Zaretsky et al., 2016; Patel et al., 2017). Likewise, a study on lung cancer confirms that the loss of *B2M* is correlated with disrupted HLA-1 antigen processing and presentation, which leads to acquired resistance to PD-1 blockade (Gettinger et al., 2017). Another study also shows that factors which impair HLA-1 complex, including not only inactivation of *B2M* but also mutations at genes involved in maturation of HLA-1 complex (e.g., *CALR, PDIA3,* and *TAP1*), can affect the response to anti-PD-1/PD-L1 therapies (Pereira et al., 2017). In addition, the diversity of HLA-1 genotype also contributes to the outcome of anti-PD. It has been recently found that patients with maximal heterozygosity at HLA-I loci (*A, B,* and *C*) demonstrate improved overall survival compared to those who are homozygous for at least one HLA locus. Moreover, patients with HLA-B44 supertype have extended survival whereas HLA-B62 or somatic loss of heterozygosity in HLA-1 is related to poor outcome in melanoma cohorts (Chowell et al., 2017). Interestingly, loss of heterozygosity in HLA is revealed to be associated with a high neoantigen burden, upregulation of cytolytic activities and PD-L1 positivity, indicating the significance of combining multiple biomarkers to predict the response to PD-1/PD-L1 therapy (McGranahan et al., 2017).

Additionally, the variety of TCR repertoire is also related to clinical response. A more clonal and less diverse T cell repertoire is found in responding patients treated with anti-PD-1 (Tumeh et al., 2014), which is opposite to anti-CTLA-4 blockade (Postow et al., 2015b).

Immune-Related Genetic Signatures

Mutations or altered expression of certain genes involved in host immune activities may reduce lymphocyte infiltration within tumors or compromise T cell functions (**Table 3**). As abovementioned, loss-of-function mutations in *B2M* gene lead to impaired MHC I molecules, and have been reported to be associated with acquired resistance to anti-PD therapies in melanoma, lung cancers and esophagogastric cancers (Zaretsky et al., 2016; Gettinger et al., 2017; Janjigian et al., 2017; Pereira et al., 2017). Particularly, in patients with *KRAS*-mutant lung adenocarcinoma, *STK11/LKB1* alterations are significantly associated with PD-L1 negativity and promote resistance to PD-1 inhibitors (Skoulidis et al., 2018). Furthermore, a study using a genome-scale CRISPR–Cas9 library profiles essential genes whose loss impairs the activity of CD8 + T cells, leading to resistance or non-responsiveness of cancer cells to T-cell-based immunotherapies. Notably, most of these genes play crucial roles in antigen presentation or IFNγ signaling (Patel et al., 2017). Interestingly, studies adopting the same approach newly discover that the loss-of-function mutations in *PBRM1*, which encodes a subunit of a SWI/SNF chromatin remodeling complex (the PBAF subtype), might improve the responsiveness to ICBs due to activation of JAK-STAT signaling pathway and elevated sensitivity to IFNγ in renal cell carcinoma (RCC) and melanoma, respectively. Apart from *PBRM1*, mutations of additional two genes which also encode components of the PBAF form of the SWI/SNF chromatin remodeling complex, *ARID2* and *BRD7*, are also found associated with the benefit of ICBs (Miao et al., 2018; Pan et al., 2018). Analysis of genomic alterations associated with accelerated tumor growth has found that *MDM2/MDM4* amplification is correlated with poor clinical outcome and even hyperprogression of patients after receiving anti-PD therapies. Besides, abnormalities of *EGFR* and *DNMT3A* also indicate a worse outcome, while alterations of *TERT, PTEN, NF1,* and *NOTCH1* appear to be related to better effect of anti-PD (Kato et al., 2017). A transcriptional signature, including up-expression of genes implicated in regulation of mesenchymal transition, cell adhesion, extracellular matrix remodeling, angiogenesis and wound healing, is indicated to be related to innate anti-PD-1 resistance (Hugo et al., 2016). Similarly, overexpression of genes involved with extracellular matrix (e.g., *LAMA3*) and neutrophil function (e.g., *CXCR2*) is related to progressing metastatic melanoma treated with PD-1 blockade (Ascierto et al., 2017). Changes in certain immune-related genes might lead to variations in the entire immune functions, hence genetically evaluation of the host immune status should be considered as a potential biomarker impacting on PD blockade immunotherapy.

THE GUT MICROBIOTA

The intestinal microbiota contain a dominant part of innumerable bacteria in human bodies and are closely linked to host health through absorbing nutrients, degrading xenobiotics, regulating epithelial homeostasis and defending against potential pathogens (Eberl, 2010). Disorders in gut microbiota have been considered to participate in the development of not only colorectal cancer but also extraintestinal cancers (Brennan and Garrett, 2016; Loo et al., 2017). Previous studies have revealed the influence of gut microbiota on clinical efficacy of cancer chemotherapy (Iida et al., 2013; Viaud et al., 2013). Also, later investigations have found correlations between gut microbiome community and clinical response to ICBs.

It is firstly found that effects of CTLA-4 blockade depend on distinct Bacteroides species, *B. thetaiotaomicron* or *B. fragilis* (Vetizou et al., 2015). Similarly, the anticancer immunity in mice models induced by anti-PD-L1 is reported to be associated with *Bifidobacterium*, which might improve effect through augmenting dendritic cell functions and subsequently enhancing CD8 + T cell priming and accumulation in the TME. And oral administration of *Bifidobacterium* alone generates equal effect on tumor eliminating as anti-PD-L1 does, indicating its potentially important role in strengthening immune functions (Sivan et al., 2015).

Recently, the predictive value of gut microbiota has been verified in human bodies. Routy et al. find that abnormal intestinal microbiota composition caused by antibiotics can lead to primary resistance to ICBs, and transplantation of fecal microbiota from patients who respond to ICBs into germ-free of non-responders can restore or enhance the responsiveness. Correlation has also been revealed between better clinical response to anti-PD-1 blockade and relative abundance of *Akkermansia muciniphila*, which increases the recruitment of CCR9 + CXCR3 + CD4 + T lymphocytes into tumor beds in a IL-12-dependant manner (Routy et al., 2017). A study on patients with melanoma unveils significantly different gut microbiota constitution between responders and non-responders treated with anti-PD-1 therapy. The gut microbiome of responding patients shows higher diversity and amplitude in *Ruminococcaceae bacteria,* while relatively less diverse bacteria and plenty of *Bacteroidales* are found in poorly responding patients. It is additionally found enrichment of anabolic pathways as well as enhanced systemic and anti-tumor immunity in responders (Gopalakrishnan et al., 2017). Similarly, another study on patients with melanoma also reveals a correlation between response to anti-PD-1 and abundance in more diversified bacteria, including *Bifidobacterium longum*, *Collinsella aerofaciens,* and *Enterococcus faecium* (Matson et al., 2018). Moreover, a study of the effect of pretreatment gut microbiota and metabolites on response in patients treated with different ICBs provides more diversified results. In terms of different regimens, the responders for all therapy types are enriched for *Bacteroides caccae*, the microbiota of the responders for Ipilimumab plus Nivolumab are rich in *Faecalibacterium prausnitzii*, *Bacteroides thetaiotaomicron*, and *Holdemania filiformis*, and that of the responders for Pembrolizumab contain abundant *Dorea formicogenerans*. High levels of anacardic acid are also found in ICB responders (Frankel et al., 2017). The findings above indicate that it is plausible to modulate gut microbiota to strengthen clinical effect of anti-PD therapy, yet more preclinical analyses of certain bacteria species and metabolites as well as confirmatory clinical studies are warranted. Moreover, gut microbiota is largely varied in terms of multiple factors, including ethnicity, living environment, diet habit, and etc, thus very difficult to guide the clinical practice as a biomarker.

COMBINATION THERAPIES WITH PD-1/PD-L1 BLOCKADE

Hitherto, the remarkable outcomes of anti-PD therapies are merely observed in quite limited patients with certain types of cancers, while more patients fail to respond, exhibit resistance or relapse following treatment. Based on currently known mechanisms impacting clinical effect of anti-PD immunotherapy,

TABLE 4 | Effective therapeutic combinations with PD-1/PD-L1 blockade.

Target	Rationale	Combined therapy
T cells	Promoting effector T-cell trafficking into TME	Epigenetic reprogramming drugs
TNF family	Enforcing T-cell function	Utomilumab, a human IgG2 mAb agonist of the T-cell costimulatory receptor 4-1BB/CD137
Immunosuppressive networks	Depletion of Tregs	Anti-CTLA-4 antibody, ipilimumab Anti-CCR4 antibody, mogamulizumab CD73-specific antibody
	Inhibition of B7 family members (B7-H3, PD-L1)	B7-H3 blockade CDK4/6 inhibitors
	Blockade of other immune checkpoint inhibitors	Tim-3, LAG3 and TIGIT blockades
	Triggering innate immune system to achieve tumor destruction	Radiation therapy and chemotherapy
Cancer cells	Inhibiting oxygen consumption in tumor cells	Metformin
Tumor specific antigens	Increasing T cell infiltration	Oncolytic viral therapy
Inflammatory mediators	Decreasing MDSCs	COX2 inhibitors
Tumor stromal fibroblasts	Reducing CXCL12 produced by fibroblasts, which mediates immunosuppressive effect in pancreatic cancer.	CXCL12 receptor chemokine receptor 4 (CXCR4) inhibitor, AMD3100
	Blocking TGFβ signaling	TGFβ blockade
BRAF signaling pathway	Increasing the cross-presentation of antigens from dead tumor cells	BRAF inhibitors
MDSC-secreted factors	Inhibition of angiogenic factor VEGF	VEGF-specific antibody, bevacizumab
	Inhibition of cytokine receptor CSF1R, resulting in CD8 T cell infiltration into tumors	CSF1R inhibitors

combination therapies are required and being explored in order to improve response rate and expand benefited populations.

Adequate proliferation, smooth migration into tumors and complete function performing of effective T cells are fundamental requirements for the immune system to restrain tumor progression. Accordingly, epigenetic reprogramming drugs to facilitate T cell trafficking (Tan et al., 2007; McCabe et al., 2012), and targeting TNF family signaling pathways to strengthen T cell functions (Tolcher et al., 2017) have been developed and proved to be effective in combination with anti-PD therapy. In addition to positive roles of T cells which help combat tumor cells, the negative roles of immunosuppressive components which support tumor progression, including Tregs, MDSCs, some B7 family members, are unneglectable. Tregs express CTLA-4, which explains improved clinical success of combination of anti-CTLA-4 and anti-PD as abovementioned (Larkin et al., 2015; Hodi et al., 2016; Hellmann et al., 2017; Wolchok et al., 2017). Prostaglandin E2 (PGE2) and its key synthesizing enzyme cyclooxygenase 2 (COX2) can induce and recruit MDSCs in TME, and inhibition of COX2 has synergized anti-PD therapy in pre-clinical models (Li et al., 2016). Inhibitors targeting other immune checkpoints such as Tim-3, LAG3 and TIGIT have also been explored their synergetic effect aligned with PD therapy (Sakuishi et al., 2010; Li et al., 2012; Chauvin et al., 2015). PD-L1 expression is also a primary biomarker impacting on PD pathway blockade. Lately, it has been discovered that CDK4/6 inhibition elevate PD-L1 expression by restraining its degradation mediated by cyclin D-CDK4 and the SPOP ligase, and the combination of CDK4/6 inhibitors and anti-PD-1 therapy enhances tumor regression and dramatically improves overall survival of murine tumor models (Zhang J. et al., 2017). In terms of the field of vaccination, PD pathway blockade has been noticed to increase the antitumor effect of conventional vaccines, which can stimulate T cell activities and induce immune responses against tumor cells (Duraiswamy et al., 2013; Karyampudi et al., 2014). Another vaccination approach is oncolytic viral therapy. Locally injected oncolytic viruses have been proved to enhance systemic antitumor immunity through multiple mechanisms, thus improving the strength of anti-PD immunotherapy and elevating response rate of patients with advanced melanoma, brain tumors and breast cancer (Ribas et al., 2017; Bourgeois-Daigneault et al., 2018; Samson et al., 2018). Based on the significant role of metabolic fitness in immune activities, it has been reported that metformin, a broadly prescribed type II diabetes treatment, reverses the resistance to PD-1 blockade which results from hypoxic environments produced by tumors (Scharping et al., 2017). Conventional therapies targeting tumor cells, including radiotherapy and chemotherapy, also exert enhanced antitumor activities together with anti-PD therapy through multiple interacting mechanisms (Deng et al., 2014a; Shalapour et al., 2015; Sharabi et al., 2015; Twyman-Saint Victor et al., 2015; Shaverdian et al., 2017). However, more clinical evidence is needed to further determine appropriate doses, timing and other parameters in combination treatment. In addition, other potential combinatorial regimens have been considered and the confirmation trials are ongoing, such as tumor stromal fibroblast inhibitors and antibodies targeting innate immune signaling pathway and oncogenic signals (Mahoney et al., 2015; Sharma and Allison, 2015; Zou et al., 2016; **Table 4**).

CONCLUSION

PD-1/PD-L1 pathway blockades have elicited outstanding clinical effect with relatively tolerable toxicities only in a minority of populations. In order to select patients most suitable to receive the possibly effective but costly therapy, the underlying prognostic factors leading to heterogeneous responses of different individuals with various cancer types have been gradually explored. In this review, a series of tumor-autonomous, tumor microenvironmental and host-related mechanisms were introduced, which need to be considered in terms of reducing ADR. With more and more prognostic factors gradually excavated, how to select most suitable biomarkers for certain cohorts is of great significance. Especially, the selection becomes more difficult when biomarkers predicting opposite response to anti-PD therapy present in one individual. For example, attenuated immune functions in elderly patients may result in poor clinical response of anti-PD with insufficient effector T cells, and on the other hand, the mutational burden accumulates with aging, which makes the outcome of anti-PD in elderly patients elusive. Unlike the traditional target therapy, which directly inhibit the abnormal signal in tumor itself (e.g., proliferation), CPI immunotherapy is more complicated and can be influenced by many factors. It has to be noted that some prognostic factors interact with each other instead of impacting the response of treatments independently. As aforementioned, virus infections and HLA heterozygosity are both associated with PD-L1 positivity or overexpression (Wong et al., 2015), while oppositely, genomic alterations are significantly related to PD-L1 negativity (Skoulidis et al., 2018). Loss of heterozygosity in HLA is additionally associated with a high neoantigen burden and upregulation of cytolytic activities (McGranahan et al., 2017). Besides, expression of the whole PD-1/PD-L axis, including PD-1, PD-L1, and PD-L2, has been reported to be connected with cytolytic activities and mutational load (Danilova et al., 2016). Above evidence indicates that it is necessary to exclude the impact of interactions between biomarkers and explore the independent roles of these candidates in larger patient cohorts with detailed information for all candidate biomarker, which will benefit the joint application of multiple biomarkers. Generally, sufficient infiltration and potent function of effector T cells in TME indicate an active pre-existing antitumor immunity and are the most elementary mechanism, through which most of other factors essentially impact on response of the therapy. Patients with abundant intratumoral infiltrate, elevated PD-L1 expression level and high mutational load have been most commonly reported to benefit from anti-PD therapies. Among all the influential factors, some were newly discovered and thus need to be verified and further explored, and some have been frequently reported but lack standard of measurement or practical application. Notably, there are contradictory findings in certain biomarkers. In terms

of gut microbiota, some studies indicate a positive correlation between responses and *Bacteroides* species (Vetizou et al., 2015; Frankel et al., 2017), whereas the study of Gopalakrishnan et al. provides with an opposite finding that plenty of *Bacteroidales* are related to poor response to anti-PD-1 (Gopalakrishnan et al., 2017). The contradiction may be attributed to diversities in ethnics, region, diet, and limited sample sizes. Besides, the study of responding patients with RCC and NSCLC revealed different composition of beneficial gut microbiota from that of studies of melanoma (Routy et al., 2017), which emphasizes the role of different bacteria species in different cancer types, and indicates that all the biomarkers require validations in more cancer types. Based on currently known rationales, plenty of other therapies have been explored in combination with anti-PD therapies to improve benefit of previously poorly responsive populations. Although failed in some studies, precision designs with specific markers could provide insight on the combination therapy.

In conclusion, it is essential to comprehensively assess the patient's status, especially with respect to the paradoxes, for instance, mutation loads and immunity in old patients and differences of beneficial bacteria in the above researches, etc. Besides, the differences in population and regions of patients should be taken into account. Finally, to adopt appropriate therapies, such as combination therapies, benefits the most for patients. Therefore, it is imperative to take comprehensive factors related to TME, host immunity, clinical factors and gut microbiome and so on into consideration when patients are given ICB therapies, which may shed new light on personalized precision therapy.

AUTHOR CONTRIBUTIONS

XY and SZ wrote the manuscript. YD and PW drew the figure. QH and HX contributed to the conception of the study.

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Disruption of PD-1 Enhanced the Anti-tumor Activity of Chimeric Antigen Receptor T Cells Against Hepatocellular Carcinoma

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Cancer immunotherapy has made unprecedented breakthrough in the fields of chimeric antigen receptor-redirected T (CAR T) cell therapy and immune modulation. Combination of CAR modification and the disruption of endogenous inhibitory immune checkpoints on T cells represent a promising immunotherapeutic modality for cancer treatment. However, the potential for the treatment of hepatocellular carcinoma (HCC) has not been explored. In this study, the gene expressing the programmed death 1 receptor (PD-1) on the Glypican-3 (GPC3)-targeted second-generation CAR T cells employing CD28 as the co-stimulatory domain was disrupted using the CRISPR/Cas9 gene-editing system. It was found that, *in vitro*, the CAR T cells with the deficient PD-1 showed the stronger CAR-dependent anti-tumor activity against native programmed death 1 ligand 1-expressing HCC cell PLC/PRF/5 compared with the wild-type CAR T cells, and meanwhile, the CD4 and CD8 subsets, and activation status of CAR T cells were stable with the disruption of endogenous PD-1. Additionally, the disruption of PD-1 could protect the GPC3-CAR T cells from exhaustion when combating with native PD-L1-expressing HCC, as the levels of Akt phosphorylation and anti-apoptotic protein Bcl-xL expression in PD-1 deficient GPC3-CAR T cells were significantly higher than those in wild-type GPC3-CAR T cells after coculturing with PLC/PRF/5. Furthermore, the *in vivo* anti-tumor activity of the CAR T cells with the deficient PD-1 was investigated using the subcutaneous xenograft tumor model established by the injection of PLC/PRF/5 into NOD-scid-IL-2Rγ−/− (NSG) mice. The results indicated that the disruption of PD-1 enhanced the *in vivo* anti-tumor activity of CAR T cells against HCC, improved the persistence and infiltration of CAR T cells in the NSG mice bearing the tumor, and strengthened the inhibition of tumor-related genes expression in the xenograft tumors caused by the GPC3-CAR T cells. This study indicates the enhanced anti-tumor efficacy of PD-1-deficient CAR T cells against HCC and suggests the potential of precision gene editing on the immune checkpoints to enhance the CAR T cell therapies against HCC.

Keywords: hepatocellular carcinoma, immunotherapy, chimeric antigen receptor, PD-1, gene editing, CRISPR-Cas9

Abbreviations: CAR, chimeric antigen receptor; CRISPR/Cas, clustered regularly interspaced short palindromic repeat/CRISPR-associated protein; FBS, fetal bovine serum; GPC3, Glypican-3; GPC3-CAR, GPC3-specific 28ζ-CAR; gRNA, guide RNA; HCC, hepatocellular carcinoma; Indels, insertions or deletions; NSG, NOD-scid-IL-2Rγ−/−; PD-1, programmed death 1 receptor; PD-L, programmed death 1 ligand; UTD, untransduced T cells.

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INTRODUCTION

Hepatocellular carcinoma, the most predominant type of primary liver cancer, is one of the leading causes of cancer-related death and arouses global concern in recent years (Abdalla et al., 2018; Hu et al., 2018; Jin et al., 2018). Because most (more than 80%) of patients with HCC are diagnosed at a late stage of the disease, potentially curative therapies (including ablation, chemotherapy, proton beam therapy, chemoembolization, and targeted drug therapy) are often less effective and only extend the overall survival by a short time (Llovet et al., 2008; Gao et al., 2014; Xiang et al., 2015).

CAR T cells are genetically engineered T cells expressing an artificial recombinant receptor molecule (CAR) on the cell surface. The CAR combines antigen-binding domain [most commonly, a single-chain variable fragment (scFv) derived from the variable domains of antibodies] with the signaling domain of the TCRζ chain and additional costimulatory domain(s) from receptors such as CD28, OX40, and 4-1BB that promote the proliferation and survival of T cell, endowing T cells with MHC-independent target recognition and a fundamental antitumor advantage (Kuwana et al., 1987; Gross et al., 1989; Di and Li, 2016; June et al., 2018). With the unprecedented success of the CAR T cells in leukemia and lymphoma, a growing number of studies have focused on the treatment of solid tumors using the CAR-T technology (Bagley et al., 2018). Excitingly, it was found that T cells expressing GPC3-targeted CAR can efficiently kill GPC3-positive HCC cells (Gao et al., 2014). Furthermore, the relevant phase 1 clinical trial study (ClinicalTrials.gov identifier: NCT02395250) showed that autologous T cells bearing CAR that can specifically recognize GPC3 was safe and effective for patients with relapsed or refractory HCC (Zhai et al., 2017). Meanwhile, the phase 1 clinical trial (ClinicalTrials.gov identifier: NCT02541370) of CD133-directed CAR T cells for advanced HCC showed that the feasibility, controllable toxicities, and effective activities of the CAR T cells for treating the patients with CD133-positive HCC (Wang et al., 2018). Thus, adoptive cell therapy based on CAR-redirected T (CAR T) cells has been identified as an effective and promising strategy for the treatment of patients with HCC. However, the efficacy of CAR T cells in the solid tumor is prone to be affected due to the immunosuppressive tumor microenvironment [e.g., expression of inhibitory ligands programmed death 1 ligand (PD-L) 1/ligand 2 on tumor cells and surrounding tissues for the PD-1 of T cells], which impairs the function and persistence of adoptively transferred T cells (Leen et al., 2007; Rabinovich et al., 2007; Joyce and Fearon, 2015; Bagley et al., 2018). PD-1 is a prominent checkpoint receptor expressed on T cells following activation (Harvey, 2014). PD-1:PD-L1/L2 pathway plays an important role in dampening T cell response and increasing T cell susceptibility to apoptosis (Bardhan et al., 2016; Papaioannou et al., 2016). Fortunately, tumor-induced downregulation of T cell function can be reversed using immune checkpoint inhibitors that block PD-1-mediated signaling cascades and maintain T cell activation within the tumor microenvironment (Pardoll, 2012; Papaioannou et al., 2016), suggesting that the disruption of endogenous PD-1-mediated inhibitory signaling could be beneficial to the antitumor activity of CAR T cells.

The CRISPR/Cas system is an adaptive immune system in prokaryotes, and the CRISPR/Cas9 system has recently been exploited for genome engineering (Cong et al., 2013). Su et al. (2016) found CRISPR-edited T cells with deficient PD-1 showed the enhanced cytotoxicity on the PD-L1 expressing melanoma and gastric cells *in vitro*. Rupp et al. (2017) showed that CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human CAR T cells against myelogenous leukemia, but the target tumor cell expressing PD-L1 was artificially constructed by lentiviral transduction, and the efficacy on the native PD-L1 expressing tumor cells remains unclear. Ren et al. (2017) demonstrated that the disruption of PD-1 led to enhanced *in vivo* antitumor activity of CAR T cells against pancreatic cancer cell and B-cell precursor leukemia cells, while only the cells with high stable expression of PD-L1 artificially constructed by lentiviral transduction was used in leukemia model. Additionally, these studies employed the 4-1BBζ CARs rather 28ζ CAR. The CAR T cells employing different costimulatory domains shows differential antitumor activity and PD-1 expression (Carpenito et al., 2009; Guedan et al., 2014, 2018; Zhao et al., 2015). 28ζ CAR T cells usually showed stronger anti-tumor activities relative to BBζ CAR T cells, and BBζ CAR T cells often exhibited greater *in vivo persistence* compared with 28ζ CAR T, although the characteristics of *in vivo* expansion and persistence between 28ζ CAR T and BBζ CAR T cells were variant in different tumor models. Zhong et al. (2010) showed that 28ζ CAR T cells displayed stronger *in vitro* and *in vivo* anti-tumor activities, and superior *in vivo* expansion compared with BBζ CAR T cells in the prostate cancer model. Zhao et al. (2015) found, in acute lymphoblastic leukemia model, 28ζ CAR T cells showed similar *in vitro* cytotoxicity and stronger *in vivo* anti-tumor activity compared with BBζ CAR T cells, but BBζ CAR T cells showed greater persistence than 28ζ CAR T cells. Li et al. (2017) found 28ζ CAR T cells showed stronger *in vitro* cytotoxicities and similar *in vivo* anti-tumor activities against HCC compared with BBζ CAR T cells, although BBζ CAR T cells showed superior *in vivo* expansion, and preferentially produced Th1 cytokines (interferon γ/granulocyte macrophage colony-stimulating factor) in contrast to 28ζ CAR T cells to preferentially produce Th2 cytokines (interleukin-4/interleukin-10). Moreover, each different cancer has a different microenvironment associated with that malignancy (Hou et al., 2016; Ruvolo, 2016). Liver is characterized by the inherent immunosuppressive environment, and the PD-L1 expression was found on HCC and the majority of the liver myeloid-derived suppressor cells (Chen et al., 2016; Thorn et al., 2016). So far, it remains unclear for the effect of disruption of endogenous PD-1 on the antitumor activity of CAR T cells employing CD28 as the co-stimulatory domain against HCC.

In the present study, the endogenous PD-1 in the second-generation GPC3-targeted CAR T cells employing CD28 as the co-stimulatory domain was disrupted using the CRISPR-Cas9 gene-editing system. The *in vitro* and *in vivo* antitumor efficacy of PD-1-deficient CAR T cells against native PD-L1-expressing

HCC and the effects of the CRISPR-mediated disruption of endogenous PD-1 on CD4 and CD8 subsets, and activation status of CAR T cells were studied.

MATERIALS AND METHODS

Safety

Over the course of this study, the standard biosecurity and institutional safety procedures were followed for handling biohazards, biological select agents, toxins, and restricted materials or reagents.

Cell Culture

Human HCC cell lines (GPC3-positive PLC/PRF/5 and GPC3-negative SK-HEP-1) (Gao et al., 2014) and human embryonic kidney (HEK) 293T cell line were obtained from the American Type Culture Collection. The GPC3-positive SK-HEP-1/GPC3 cell line was constructed by lentiviral transduction of SK-HEP-1 with Pwpt-GPC3 virus encoding human GPC3 in the previous study of our research group (Yu et al., 2018). All the cell lines were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco, United States) supplemented with 10% FBS (Gibco, United States). Peripheral blood mononuclear cells (PBMC) were obtained from Shanghai Blood Center. PBMC and the activated T cells were maintained in AIM-V medium (Gibco, United States) supplemented with 2% human AB serum (ABS, Gemini Bioproducts, United States) and 500 U/ml recombinant human IL-2 (Shanghai Huaxin High Biotechnology). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and were routinely tested for mycoplasma contamination.

Construction of Lentiviral CAR-Expression Vector

The lentiviral expression vector (pRRLSIN-hu9F2-28Z) encoding GPC3-specific second-generation CAR was constructed using a pRRLSIN lentiviral vector backbone. The CAR (**Figure 1A**) comprised CD8α signal peptide, a humanized GPC3-specific single chain antibody fragment (scFv, hu9F2) (Bi et al., 2017), the hinge domain of the CD8α molecule (nucleotides 412–546, GenBank NM 001768.6), the transmembrane region (nucleotides 457–537, GenBank NM 006139.3) and the intracellular signaling domain (nucleotides 538–660, GenBank NM 006139.3) of the human CD28 molecule, and the intracellular signaling domain of CD 3ζ molecule (nucleotides 154–492, GenBank NM 198253.2). MluI site and SalI site were added at the 5′ end and the 3′ end of the sequence encoding the CAR, respectively. The DNA fragment encoding the CAR with MluI/SalI sites was synthesized by Genewiz (Suzhou, China), and then, was inserted into the MluI/SalI site of the EF-1α promoter-based lentiviral expression vector pWPT-eGFP (Wang et al., 2011).

Lentivirus Production

The generation of lentivirus was performed according to the method described by Wu et al. (2017). Briefly, as the confluence reached 95%, HEK-293T cells were transfected with pRRLSIN-hu9F2-28Z and the packaging constructs (RRE/REV, and VSVG) using a polyethylenimine (PEI)-based DNA transfection reagent. Then, the culture medium was replaced with fresh DMEM containing 2% FBS after 6 h of the addition of PEI/DNA complex. After 72 h of transfection, virus was harvested from the conditioned medium and filtered using a 0.45 μm filter unit (Millipore, United States) to remove cell debris. Subsequently, the virus was concentrated and purified with polyethylene glycol.

Activation, Transduction, and Expansion of Human T Cells

Peripheral blood mononuclear cells were stimulated for 48 h using anti-CD3/anti-CD28 magnetic beads (Invitrogen, United States) at a bead:cell ratio of 1:1. Then, the activated T cells were transduced with lentivirus at a multiplicity of infection (MOI) of 10 on the RetroNectin (Takara, Japan) coated plates. On day 4 post-stimulation, the magnetic beads were removed. The transduced T cells were maintained at a density of 5×10^5 cells/ml, and the recombinant human IL-2 were added to a final concentration of 500 U/ml every other day.

Design and *in vitro* Transcription of Guide RNA

The gRNA was designed using the CRISPR Design Tool[1]. Considering that simultaneous use of dual gRNAs to target an individual gene can significantly improve the gene-editing efficiency mediated by CRISPR/Cas9 system (Zhou et al., 2014), in this study, two gRNAs were used for the disruption of the PD-1, and both gRNAs targeted to the sequence within exon 1 of the gene *PDCD1* expressing the PD-1. The DNA fragments (**Supplementary Table S1**) containing the T7 promoter, 20 bp targeting sequence, and gRNA scaffold, were synthesized by Genewiz (Suzhou, China), and then used as the template for *in vitro* transcription of both gRNAs using MEGAshortscript™ T7 Transcription Kit (Thermo Fisher Scientific, United States). Two targeting sequences used in this study were listed as following: PD-1-gRNA-1: GTCTGGGCGGTGCTACAACT; and PD-1-gRNA-2: GGCCAGGATGGTTCTTAGGT. The amplification of template for *in vitro* transcription was performed by PCR using the primer pairs Temp-Forward (GTTAATACGACTCACTATA) plus Temp-Reverse (AAAAAAAGCACCGACTCG GTGCCA). The product of *in vitro* transcription was purified using MEGAclear™ Transcription Clean-Up Kit (Thermo Fisher Scientific, United States), and eluted into the nuclease-free water.

Generation of PD-1 Knockout CAR T Cells

On day 3 post-transduction by lentivirus (i.e., day 5 post-stimulation with anti-CD3/anti-CD28 beads), 3 μg Cas9 protein [New England Biolabs (NEB), United States] was mixed with 3 μg gRNAs, and the mixture was incubated for 10 min at room temperature. Then, the 5×10^6 CAR T cells were electroporated with the CRISPR reagents of Cas9 protein and gRNAs by the Nucleofector 2b Device

[1]http://crispr.mit.edu

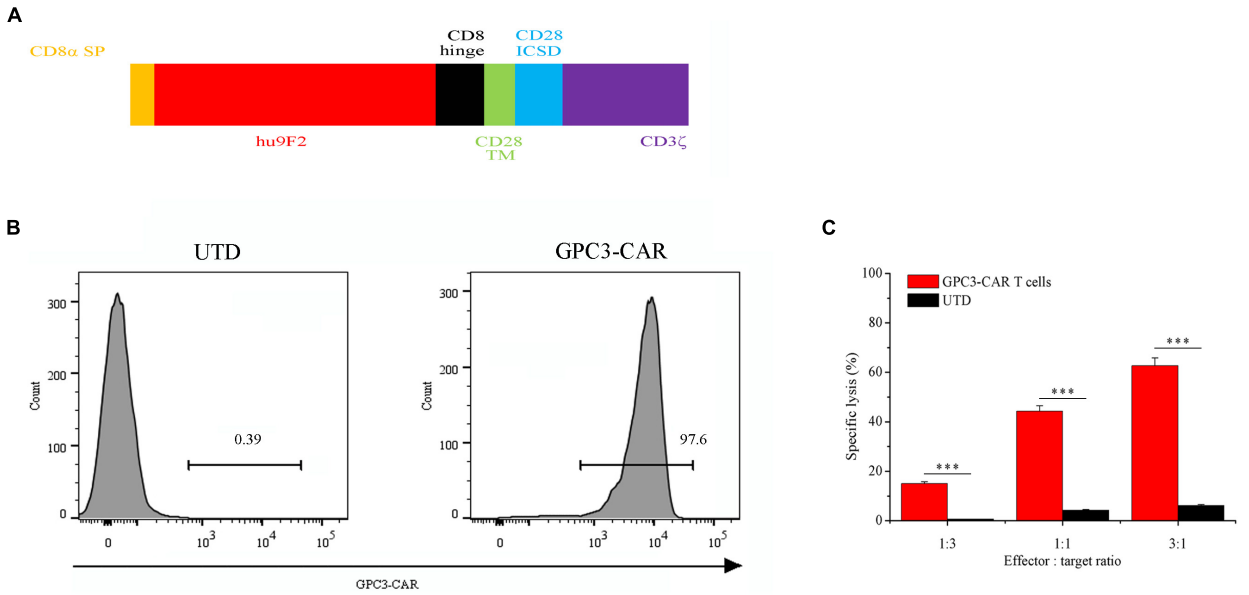

FIGURE 1 | Characterization of second-generation GPC3-specific CAR T cells. **(A)** Schematic representation of second-generation GPC3-specific CAR. CD8α SP, CD8α signal peptide; hu9F2, a humanized GPC3-specific single chain antibody fragment; CD8 hinge, the hinge domain of the CD8α molecule; CD28 TM, the transmembrane region of the human CD28 molecule; CD28 ICSD, the intracellular signaling domain of the human CD28 molecule; CD3ζ, the intracellular signaling domain of CD3 molecule. **(B)** Flow cytometric analysis of the expression of GPC3-specific CAR (GPC3-CAR) on the surface of untransduced (UTD, Left) and transduced (Right) human T cells with lentiviral vector. **(C)** *In vitro* cytotoxic activity of GPC3-CAR T cells against HCC cell PLC/PRF/5 at effector:target ratios of 3:1, 1:1, and 1:3. Data were the mean ± SD from triplicates. Bars, SD. ***$P < 0.001$ by 2-tailed unpaired *t*-tests.

(Program: T-023) (Lonza, Germany) using Human T Cell Nucleofector® Kit (VPA-1002, Lonza, Germany) according to the procedure described by the manufacturer. Meanwhile, as the control (Cas9 Mock), the 5×10^6 CAR T cells were electroporated only with 3 μg Cas9 protein but without gRNAs.

Analysis of Allele Modification

The gene editing efficiency and the potential off-target mutations were determined on day 3 post-electroporation. The genomic DNA from tested cells was purified using the QIAamp DNA Mini Kit (Qiagen, United States). The DNA fragment spanning the gene-editing target sites was amplified by PCR from the genomic DNA using the primer pairs of *PDCD1*-detect-Forward (CAAGGAGATAAGCAAGCCATTT) plus *PDCD1*-detect-Reverse (AAGCCAAGGTTAGTCCCACAT). The DNA fragments spanning the potential off-target sites were amplified by PCR from the genomic DNA using the primer pairs listed in the **Supplementary Table S2**.

(1) Sequencing and TIDE analysis: The allele modification frequencies were quantified by clonal sequencing analysis and TIDE analysis of PCR amplicon spanning the gene-editing target sites. The purified DNA fragments spanning the gene-editing target sites were ligated into the pMD-20T vector (Takara, Japan), and a total of 60 colonies were selected for DNA sequencing (Genewiz, Suzhou, China). As for the evaluation of Tracking of Indels by Decomposition (TIDE) (Brinkman et al., 2014), the purified DNA fragments spanning the gene-editing target sites were Sanger-sequenced using the primers PD-1-seq-Forward (5′TCCCCAGCACTGCCTCTGTCACTC3′) and PD-1-seq-Reverse (5′CACAGCTC AGGGTAAGGGGCAGA3′) by Genewiz (Suzhou, China), and the analysis of each sequence chromatogram was carried out using the online TIDE software available at http://tide.nki.nl. The sequence from a Cas9 mock-transfected sample was used as the reference sequence. Parameters were set to the maximum indel size of 50 nucleotides and the decomposition window to cover the largest possible window with high quality traces. When the TIDE analysis was below the detection sensitivity of 1.5%, it was set to 0%. All the sequencing primers which were used for TIDE off-target analysis were listed in **Supplementary Table S3**.

(2) T7EN1 assay: The mismatched DNA can be detected by the T7EN1 assay (Niu et al., 2014). After purification, the 200 ng of DNA fragment spanning the gene-editing target sites was denatured and reannealed in 1× NEBuffer 2 (NEB, United States) in a thermocycler with the following steps (Guschin et al., 2010): 95°C, 5 min; 95–85°C at −2°C/s; 85–25°C at −0.1°C/s; hold at 4°C. Subsequently, 10 U of T7 Endonuclease I (T7EN1) (NEB, United States) were added into the hybridized DNA fragments and reaction mixtures were incubated for 15 min at 37°C. Following digestion, 1 μl of proteinase K was added and incubated for 5 min at 37°C to inactivate the enzyme and stop the reaction. The DNA fragments digested by T7EN1 enzyme were separated by 1% agarose gel electrophoresis, stained with ethidium bromide.

In vitro Cytotoxicity Assays

The *in vitro* cytotoxicity was evaluated by the lactate dehydrogenase (LDH) release assay with CytoTox96 Non-Radioactive Cytotoxicity Kit (Promega, United States), and the assay was performed according to the manufacturer's instructions. Briefly, 1×10^4 HCC cells (target cells) were co-cultured with the genetically modified (or not) T cells (effector cells) at an indicated effector:target ratio in a total volume of 100 μL in the wells of 96-well V-bottom plates for 18 h at 37°C. The RPMI 1640 medium (Gibco, United States) containing 10% FBS was used in the co-cultures. Then, the supernatants were collected by centrifugation at 250 × *g* for 4 min at room temperature, and the released LDH in the supernatants was measured using colorimetric method at 490 nm. The spontaneous release of LDH from target and effector cells and the maximum release of LDH from target cells were determined in parallel. The percentage of specific cell lysis was calculated based on the following formula:

$$100 \times (\text{experimental release} - \text{target spontaneous release} - \text{effector spontaneous release}) / (\text{target maximal release} - \text{target spontaneous release})$$

Cytokine Release Assay

Firstly, 1×10^4 HCC cells (target cells) were co-cultured with the genetically modified (or not) T cells (effector cells) at an effector:target ratio of 1:1 in a total volume of 100 μL in the wells of 96-well V-bottom plates for 18 h at 37°C. The RPMI 1640 medium (Gibco, United States) containing 10% FBS was used in the co-cultures. Then, the supernatant was collected by centrifugation at 250 × *g* for 4 min at room temperature. The concentrations of IFN-gamma and IL-2 in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA) using the Human IFN-gamma ELISA kit (EK1802) and Human IL-2 ELISA kit (EK1022) (both from Multisciences Biotech, Hangzhou, China) according to the manufacturer's instructions. As for the mouse blood, after it was collected and clotted at 4°C, and then, the serum was used for the detection of cytokine as above.

Flow Cytometry

For all experiments [except for intracellular Akt and phospho-Akt (Ser^{473}) staining], the cells were analyzed by surface antibody staining. The following antibodies with indicated specificity and the appropriate isotype controls were used: anti-human CD3-FITC (11-0036-41), anti-human CD8-FITC (11-0086-42), anti-human CD25-PE (12-0259-41), anti-human PD-L1-PE (12-5983-42), mouse IgG_1-FITC isotype control (11-4714-81), and mouse IgG_{2a}-FITC isotype control (11-4724-42) (all from Thermo Fisher Scientific, United States); anti-human CD4-FITC (555346), anti-human CD4-PE (555347), anti-human PD-1-BV421 (564323), mouse IgG_1-PE isotype control (555749), and mouse IgG_1-BV421 isotype control (562438) (all from BD Biosciences, United States); anti-human CD69-PerCP (310928) and Mouse IgG1-PerCP (400148) (both from BioLegend, United States). The CAR expression was evaluated by the biotinylated goat anti-human Fab antibody (109-066-006, Jackson ImmunoResearch, United States), followed by PE-conjugated streptavidin (12-4317-87, eBioscience, United States) staining, if not specifically indicated. For the intracellular Akt and phospho-Akt (Ser^{473}) analysis, CAR T cells were first stained by the biotinylated goat anti-human Fab antibody and FITC-conjugated streptavidin (11-4317-87, eBioscience, United States) on ice after the CAR T cells harvested from the 48-h coculture of GPC3-CAR T and PLC/PRF/5 cells at a ratio of 1:1, and then, the cells were fixed, permeabilized, and stained using the antibodies of an anti-Akt mouse mAb (2920S), an anti-phospho-Akt (Ser^{473}) rabbit mAb (4060S), a mouse mAb IgG1 isotype control (5415S) and Rabbit mAb IgG isotype control (3900S) (all from Cell Signaling Technology, United States) according to the manufacturer's protocol, followed by PE-conjugated secondary antibodies of anti-mouse IgG (8887S, Cell Signaling Technology, United States) and anti-rabbit IgG (8885S, Cell Signaling Technology, United States). Fixable, viable stain 780 (565388, BD Biosciences, United States) was used for discriminating live from dead cells according to the manufacturer's instruction. Flow cytometric measurements were carried out using a FACSCelesta™ flow cytometer (BD Biosciences, United States) equipped with FACSDiva software for data acquisition. FlowJo software (Tree Star, United States) was used for data analysis.

Mouse Xenograft Model

Six- to eight-week-old female NSG mice were housed and treated at the Experimental Animal Center of Shanghai Jiao Tong University School of Medicine (Shanghai, China) in specific pathogen-free conditions. The animal experiments were performed in accordance with the guidelines and regulations approved by the Shanghai Medical Experimental Animal Care Commission. Subcutaneous xenograft tumors were established by injection of 3×10^6 PLC/PRF/5 in PBS. When the tumor volume reached 100–200 mm^3, mice bearing the tumor were randomly allocated into four groups ($n = 7$) and assigned to receive one of the following intravenous injections: (1) sterile PBS, (2) 5×10^6 UTD in sterile PBS, (3) 5×10^6 wild-type CAR T cells in sterile PBS, and (4) 5×10^6 PD-1-deficient CAR T cells in sterile PBS. Tumor burden was measured by an electronic caliper, and tumor volume was calculated based on the following formula as described by Gao et al. (2014): $V = L \times W \times W / 2$, where L was length and W was width. When the mean tumor volume in the control group reached 1,500–2,000 mm^3, mice were euthanized.

Quantitative Real-Time PCR

mRNA was isolated from cells using TRIzol reagent (15596026, Thermo Fisher Scientific, United States) and then reverse transcribed into cDNA using the GoScript™ Reverse Transcription system (A5001, Promega, United States) according to the manufacturer's instructions. All the quantitative real-time PCR reactions were performed with TB Green™ premix Ex Taq™ II (Tli RNaseH Plus) (RR820A, Takara, Japan) according to the manufacturer's protocol on an ABI 7500 RT-PCR system (Applied Biosystems, United States), using the primers in

the **Supplementary Table S4**. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control. The relative quantification was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Immunohistochemistry

To assess the infiltration of adoptive T cells in the xenografts after treatment, the tumor tissues were fixed with formalin, embedded in paraffin, and serially sectioned at 2-μm thickness. The sections of fixed and embedded tumor tissues were immunostained with an anti-CD3e monoclonal antibody (MA5-14524, Thermo Fisher Scientific, United States) at a 1:150 dilution. Images were taken under a Leica SCN400 system (Leica Microsystems, Germany) at 20× magnification.

Statistics

All data were shown as mean ± standard deviation (SD). Two-tailed unpaired *t*-tests, one-way ANOVA with Turkey *post hoc* tests, correlation and regression analysis were carried out using GraphPad Prism version 6.0 (GraphPad Software Inc., United States). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

RESULTS

Generation of GPC3-Specific CAR T Cells, and Cytotoxicity of the CAR T Cells Against HCC Cell PLC/PRF/5

As shown in **Figure 1A**, GPC3-specific second-generation CAR comprised CD8α signal peptide, a humanized GPC3-specific single chain antibody fragment (scFv, hu9F2) (Bi et al., 2017), the hinge domain of the CD8α molecule, the transmembrane region and the intracellular signaling domain of the human CD28 molecule, and the intracellular signaling domain of CD 3ζ molecule. GPC3-CAR T cells were generated by lentiviral vector transduction as described in the "Materials and Methods" section. The expression of CAR was evaluated by flow cytometry on day 3 post-transfection. As shown in **Figure 1B**, the percentage of the CAR-positive T cells reached 97.6%, indicating that the efficiency of lentiviral transduction was high, and GPC3-CAR T cells were successfully generated. Furthermore, as shown in **Figure 1C**, it was found that GPC3-CAR T cells showed the significantly ($P < 0.001$) stronger cytotoxicity on HCC cell PLC/PRF/5 compared with the UTD, and the cytotoxicity was enhanced with the increase of effector:target ratio from 1:3 to 3:1, indicating that the cytotoxicity of GPC3-CAR T cells was dose-dependent.

Remarkable Upregulation of PD-L1 Expression on PLC/PRF/5 After Encountering GPC3-CAR T Cells

As shown in **Figure 2**, over 80% of the PLC/PRF/5 cells expressed the inhibitory ligand PD-L1 after coculture with GPC3-CAR T cells at an effector:target ratio of 1:1 for 18 h. However, only 1.24% of the PLC/PRF/5 cells were PD-L1-positive, when the PLC/PRF/5 cells were normally cultured in the absence of GPC3-CAR T cells. These results indicated that the expression of PD-L1 on HCC PLC/PRF/5 is inducible, and the expression can be up-regulated after PLC/PRF/5 encountering GPC3-CAR T cells.

Preparation and Characterization of PD-1-Deficient GPC3-Specific CAR T Cells

To further investigate the effect of PD-1-mediated immunosuppressive pathway on the efficacy of GPC3-CAR T cells against HCC, the PD-1-deficient GPC3-CAR T cells was generated through direct delivery of CRISPR/Cas9 gene-editing system into the CAR T cells by electroporation on day 3 post-lentiviral transduction. Gene-editing efficiency was evaluated by sequencing and T7 endonuclease I (T7EN1)-based mutation detection assay, 2–4 days after nucleofection. Clonal sequencing indicated the genomic editing efficiency reached 85%. There were fifteen kinds of indels resulted from the non-homologous end joining (NHEJ) repair in 60 sequenced clones (**Figure 3A**), and deletion mutations were the most prominent among the observed Indels. Multiple peaks flanking the PD-1 target site appeared in the Sanger sequencing data of the PCR amplicon spanning the gene-editing target sites (**Figures 3B,C**), which confirmed that the shift of genomic reading frame occurred downstream of the target sites. The TIDE analyses showed that the indels frequencies reached 77.9 and 76.8% at the target sites of PD-1-gRNA-1 and PD-1-gRNA-2, respectively. In the T7EN1-based mutation detection assay (**Figure 3D**), the obvious cleavage further confirmed the mutation at the genomic locus of PD-1. Furthermore, the expression of PD-1 was characterized by flow cytometry on day 3 post-restimulation of CRISPR-edited CAR T cells with anti-CD3/anti-CD28 beads. As shown in **Figure 4**, above 83% reductions of CAR+ PD-1+ cells were observed in both CD4- and CD8-gated cells, indicating that PD-1 was successfully disrupted with high efficiency in both CD4-positive and CD8-positive GPC3-CAR T cells. In addition, the top five potential off-target sites for each gRNA in the CRISPR-edited GPC3-CAR T cells were sequenced, and no mutation was found at any of these sites using TIDE analysis (**Supplementary Table S5**). Taken together, PD-1-deficient GPC3-specific CAR T cells were successfully and efficiently generated using the CRISPR/Cas9 gene-editing system.

Given that the surface expression of PD-1 on CAR T cell with intact genomic DNA was low (PD-1-positive cell percentage: 1.18% on day 9 post the activation of primary T cells, **Supplementary Figure S1**) after expansion if without the restimulation by anti-CD3/anti-CD28 beads, and moreover, repeated stimulation can cause T cells exhaustion (Cherkassky et al., 2016), it was difficult to enrich the PD-1 deficient CAR T cells. Therefore, the generated PD-1 deficient CAR T cells used for the following *in vitro* and *in vivo* assays were a mosaic population of cells with the disrupted or intact PD-1, although the GPC3-CAR T cells with the disrupted PD-1 were the prominent population.

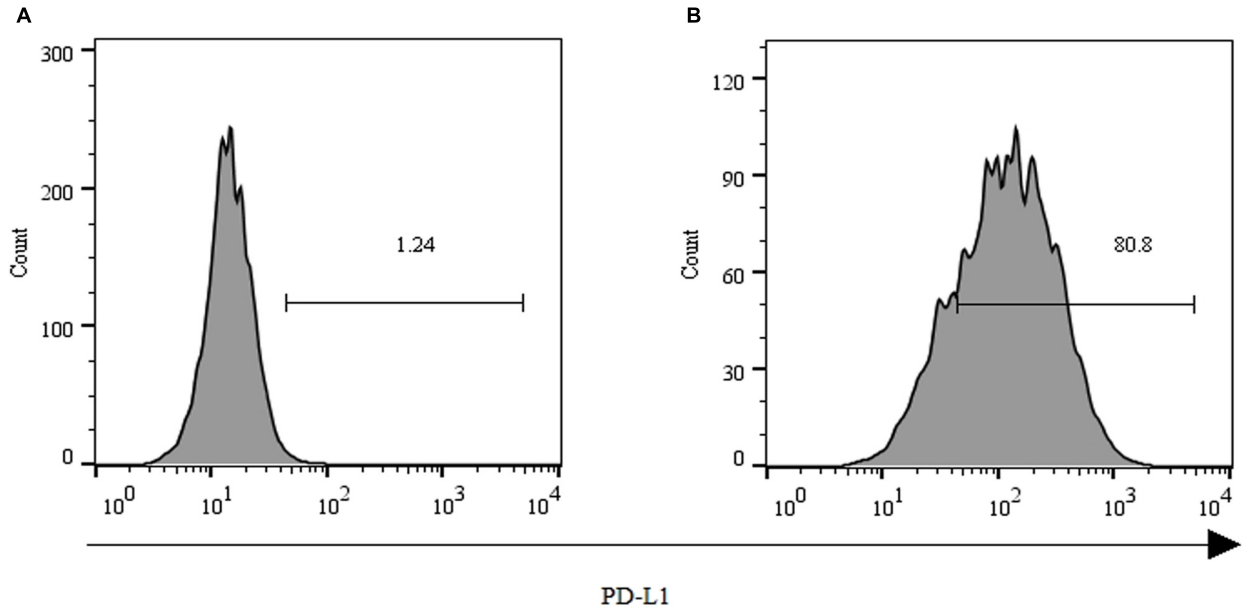

FIGURE 2 | Expression of PD-L1 on human HCC PLC/PRF/5 cells. **(A)** Human HCC PLC/PRF/5 cells were cultured alone in the absence of GPC3-CAR T cells in RPMI 1640 medium containing 10% FBS. **(B)** Human HCC PLC/PRF/5 cells were cocultured with GPC3-CAR T cells at an effector:Target ratio of 1:1 for 18 h in RPMI 1640 medium containing 10% FBS. PD-L1 was determined by flow cytometry in the CD3-negative gate, and the fixable, viable stain 780 was used for discriminating live from dead cells.

Disruption of PD-1 in GPC3-CAR T Cells Enhanced the Specific CAR-Dependent Cytotoxic Function and Cytokines Production *in vitro*, and Did Not Affect Subsets Constitution and Activation Status of the CAR T Cells

To evaluate whether the disruption of PD-1 affected specific CAR-dependent cytotoxic function and cytokines secretion of GPC3-CAR T cells, the *in vitro* tumor-lysis activity and secreted cytokines of the CRISPR-edited (or not) CAR T cells were investigated by the coculture of CAR T cells and each of various GPC3-positive (PLC/PRF/5 and SK-HEP-1/GPC3) or GPC3-negative (SK-HEP-1) HCC cells. As shown in **Figure 5A**, the PD-1 deficient GPC3-CAR T cells showed significantly ($P < 0.01$) stronger tumor-lysis activity against GPC3-positive PLC/PRF/5 and SK-HEP-1/GPC3 HCC cells compared with wild-type GPC3-CAR T cells, and the anti-tumor activities of PD-1 deficient GPC3-CAR T cells against PLC/PRF/5 and SK-HEP-1/GPC3 HCC cells were 1.25 and 1.30 times higher than those of wild-type GPC3-CAR T cells, respectively, indicating that the disruption of PD-1 enhanced the cytotoxic activity of GPC3-CAR T cells. Meanwhile, the anti-tumor activity of PD-1 deficient GPC3-CAR T cells against GPC3-negative SK-HEP-1 HCC cells was limited (<5%) and similar to that of UTD and wild-type GPC3-CAR T cells, indicating that the disruption of PD-1 did not affected the cytotoxic specificity of GPC3-CAR T cells. As shown in **Figures 5B,C**, the concentrations of IL-2 and IFN-gamma in the cocultures of PD-1 deficient GPC3-CAR T cells and GPC3-positive HCC cells (PLC/PRF/5 and SK-HEP-1/GPC3) was significantly higher than those in the coculture of wild-type GPC3-CAR T cells and GPC3-positive HCC cells, but PD-1 deficient GPC3-CAR T cells similar to UTD and wild-type GPC3-CAR T cells produced little or even negligible cytokines in the coculture with GPC3-negative SK-HEP-1, indicating that cytokines production by the GPC3-CAR T was CAR-dependent and enhanced by the disruption of PD-1. In addition, as shown in **Figures 5D,E**, no significantly statistical difference was found between PD-1-deficient and wild-type GPC3-CAR T cells in the CD4-positive, CD8-positive, CD69 (early activation marker)-positive or CD25 (intermediate or late activation marker)-positive cell percentage, indicating that CD4 and CD8 subsets constitution and activation status of GPC3-CAR T cells were stable with the disruption of endogenous PD-1. Taken together, the disruption of PD-1 in GPC3-CAR T cells enhanced specific CAR-dependent cytotoxic function and cytokines secretion, and did not affect the CD4 and CD8 subsets constitution and activation status of the GPC3-CAR T cells.

Disruption of PD-1 Increased the Levels of Akt Activation and Bcl-xL Expression in the GPC3-CAR T Cells After Combating the HCC Cells

As shown in **Figures 6A,B**, both the Akt activation status and the expression of anti-apoptotic protein Bcl-xL in PD-1 deficient GPC3-CAR T cells was significant ($P < 0.001$) increased compared with that in the wild-type GPC3-CAR T

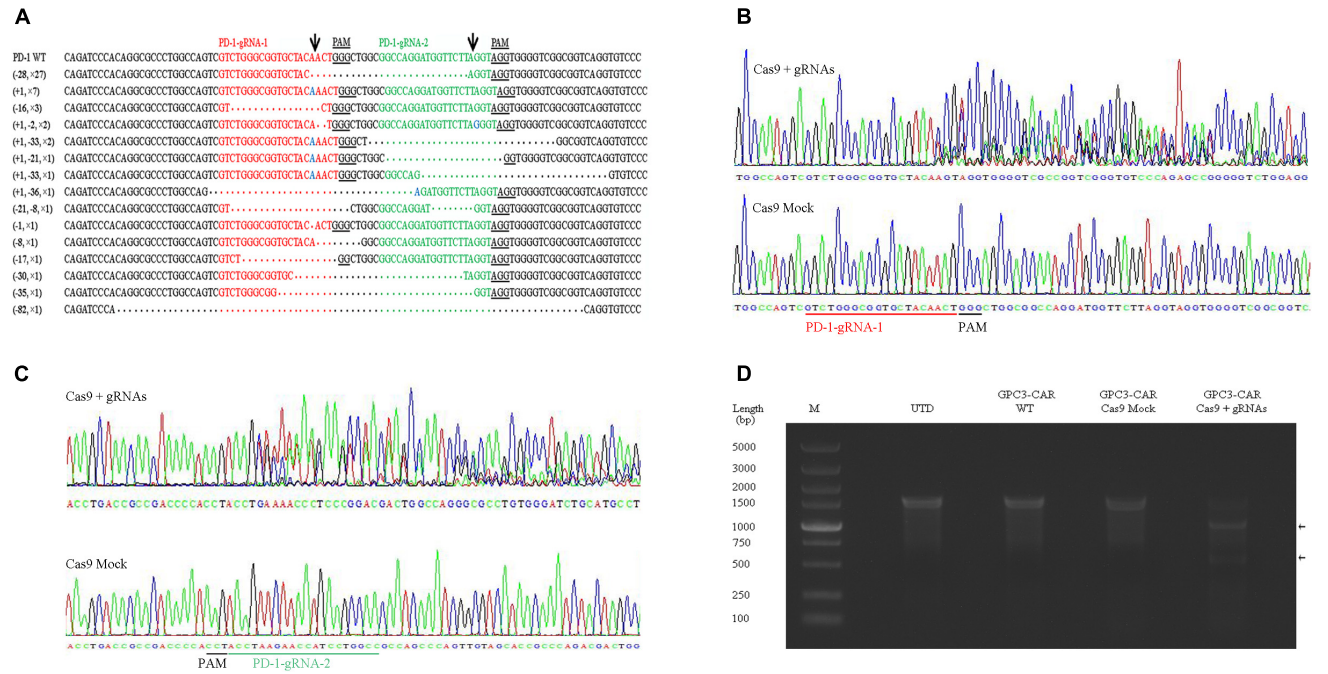

FIGURE 3 | CRISPR/Cas9 efficiently disrupted the gene expressing PD-1 in GPC3-CAR T cells. **(A)** Indels observed by clonal sequence analysis of PCR amplicons from the CRISPR-edited region in the gene expressing PD-1. Blue base or dot in the clonal sequences indicated insertion or deletion base, respectively. The number prefixed a "+" or "–" character in the bracket before a clonal sequence indicated the number of insertions or deletions in the corresponding clonal sequence, respectively. The number prefixed with a "×" character in the bracket before a clonal sequence indicated the number of the corresponding indels profile in the sixty clonal amplicons. Arrows indicated the putative cleavage sites. **(B,C)** The chromatograms from the Sanger sequencing of the PCR amplicon spanning the PD-1 CRISPR gRNAs [PD-1-gRNA-1 **(B)** and PD-1-gRNA-2 **(C)]** target sites within the exon 1 of the gene expressing PD-1. **(D)** Detection of the CRISPR-mediated disruption of PD-1 by a mismatch-selective T7EN1 nuclease assay on the DNA (spanning the gRNAs target sites) amplified from the genomic DNA of the cells shown.

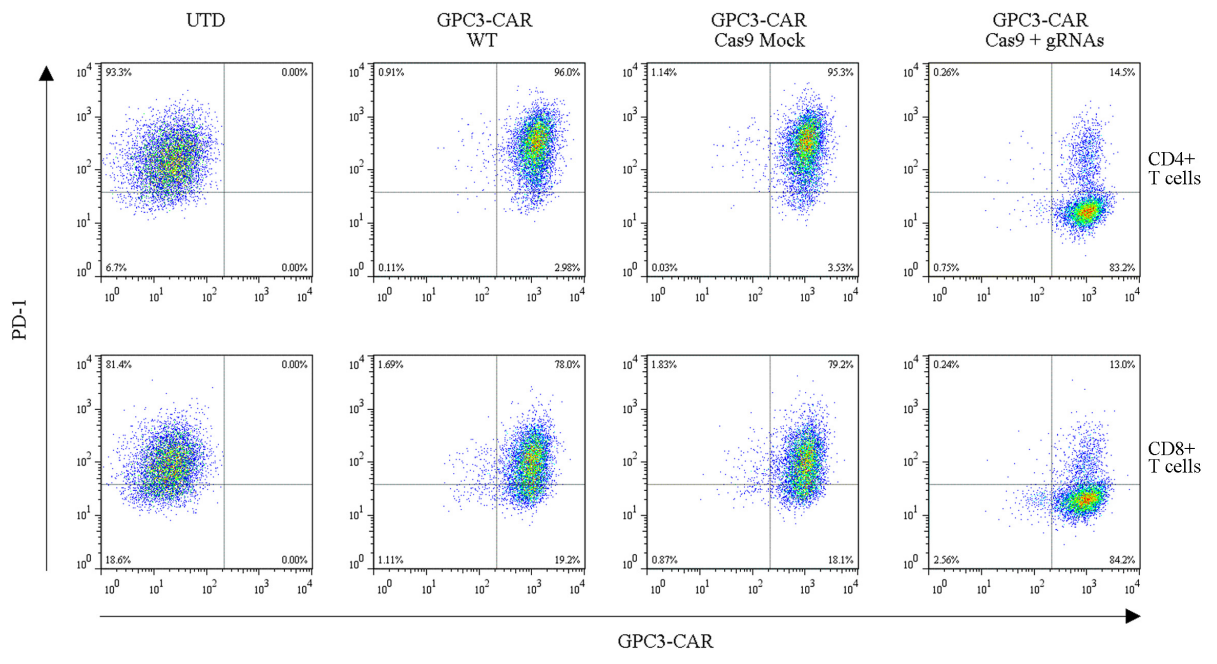

FIGURE 4 | Efficient disruption of PD-1 expression on the surface of GPC3-CAR T cells. PD-1 and CAR expression on the surface of T cells were detected by flow cytometry on day 3 after the re-stimulation with anti-CD3/anti-CD28 beads. UTD, untransduced T cells; WT, wild type.

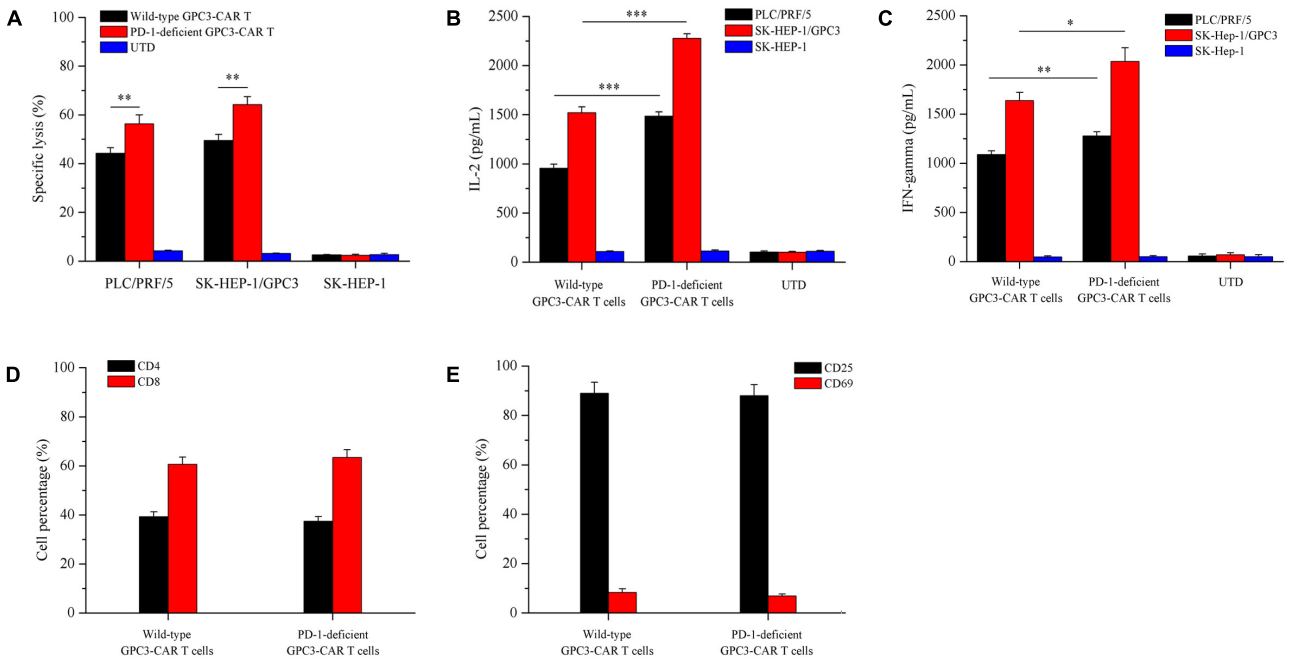

FIGURE 5 | Effects of disruption of PD-1 on cytotoxicity, cytokines production, and phenotype of GPC3-CAR T cells *in vitro*. **(A)** Cytotoxic activities of UTD and GPC3-CAR T cells with intact or deficient PD-1 against GPC3-positive (PLC/PRF/5 and SK-HEP-1/GPC3) and GPC3-negative (SK-HEP-1) HCC cells at an effector:target ratio of 1:1. **(B,C)** The production of IL-2 **(B)** and IFN-gamma **(C)** by the UTD and GPC3-CAR T cells with intact or deficient PD-1 cocultured with GPC3-positive (PLC/PRF/5 and SK-HEP-1/GPC3) or GPC3-negative (SK-HEP-1) HCC cells at an effector:target ratio of 1:1. **(D)** CD4 and CD8 subsets constitution of wild-type and PD-1-deficient GPC3-CAR T cells. The expressions of CD4 and CD8 on CAR T cells were measured by flow cytometry. **(E)** The expressions of early (CD69), and intermediate or late (CD25) activation markers on cell surface of wild-type and PD-1-deficient GPC3-CAR T cells. The expressions of CD25 and CD69 were determined by flow cytometry. Data shown were mean ± SD from triplicates. Bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one way ANOVA with Turkey *post hoc* test.

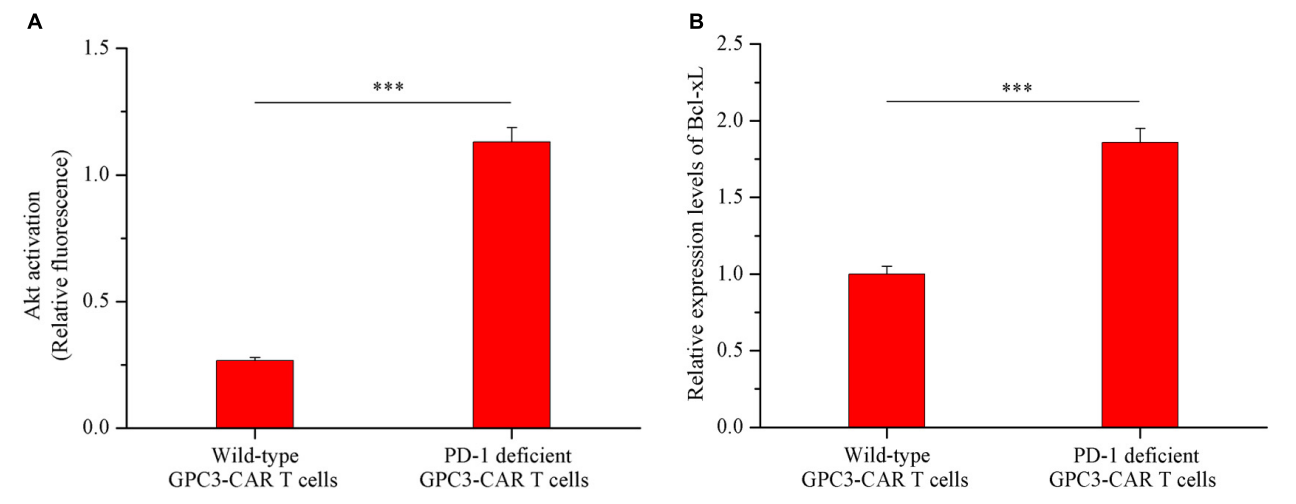

FIGURE 6 | Akt activation and Bcl-xL expression in GPC3-CAR T cells with deficient or intact PD-1. The GPC3-CAR T cells were isolated from the 48-h coculture with PLC/PRF/5. **(A)** Akt activation in GPC3-CAR T cells with deficient or intact PD-1 was cytometrically measured as the ratio of phospho-Akt (Ser^{473})/Akt in an intracellular stain. Phospho-Akt (Ser^{473}) and Akt were determined by flow cytometry in the CAR-positive gate, and the fixable, viable stain 780 was used for discriminating live from dead cells. **(B)** mRNA expression levels of Bcl-xL in GPC3-CAR T cells with deficient or intact PD-1 determined by quantitative real-time PCR. Data were the mean ± SD from triplicates. Bars, SD. $^{***}P < 0.001$ by 2-tailed unpaired *t*-tests.

cells after 48 h of coculture with native PD-L1-expressing GPC3-positive PLC/PRF/5 HCC cells. The ratio of phospho-Akt/Akt in the PD-1 deficient GPC3-CAR T cells was 4.35 times higher than that in the wild-type GPC3-CAR T cells, and meanwhile, the expression level of Bcl-xL in the PD-1 deficient GPC3-CAR T cells was 1.86 times higher than that in the wild-type GPC3-CAR T cells after 48 h of coculture with native PD-L1-expressing GPC3-positive PLC/PRF/5 HCC cells. Taken together, the disruption of PD-1 increased the levels of Akt activation and anti-apoptotic protein Bcl-xL expression after combating the HCC cells.

Disruption of PD-1 Enhanced *in vivo* Antitumor Efficacy, Survival, Cytokines Production, and Infiltration of GPC3-CAR T Cells

Given that HCC cell PLC/PRF/5 natively expressed GPC3 on the cell surface in contrast to SK-HEP-1/GPC3, the efficacy of PD-1-deficient GPC3-CAR T cells was evaluated *in vivo* in NSG mice bearing established PLC/PRF/5 subcutaneous xenograft tumors. As shown in **Figure 7A**, tumor growth was significantly ($P < 0.01$) inhibited in mice treated with GPC3-CAR T cells compared with those treated with UTD or PBS. Moreover, PD-1-deficient GPC3-CAR T cells showed stronger anti-tumor activity compared with the wild-type GPC3-CAR T cells. At the endpoint of the animal experiment, the tumor volumes in the mice treated with PD-1-deficient GPC3-CAR T cells were significantly ($P < 0.05$) smaller than those treated with wild-type GPC3-CAR T cells, and tumor weights in the mice treated by the PD-1-deficient GPC3-CAR T cells were significantly ($P < 0.01$) lighter than those in other groups (**Supplementary Figure S2**), indicating that the disruption of PD-1 enhanced the anti-tumor activity of GPC3-CAR T cells.

Meanwhile, to investigate the effect of the disruption of PD-1 on the *in vivo* survival of GPC3-CAR T cells, the density of GPC3-CAR T cells in mouse peripheral blood was tested. It was found that, as shown in **Figure 7B**, while the survivals of both wild-type GPC3-CAR T and PD-1-deficient GPC3-CAR T cells in mice decreased with time, the density of PD-1-deficient GPC3-CAR T cells was significantly ($P < 0.01$) higher than that of wild-type GPC3-CAR T cells on day 20 post-CAR T cells infusion. The results suggested that the disruption of PD-1 benefited the *in vivo* survival of GPC3-CAR T cells. In addition, correlation analyses showed that the density of GPC3-CAR T cells in mouse peripheral blood significantly ($P < 0.05$) negatively correlated with the tumor burdens in both treatment groups of wild-type and PD-1-deficient GPC3-CAR T cells. Furthermore, the levels of IFN-gamma and IL-2 in the mouse blood of the group treated by the PD-1-deficient GPC3-CAR T cells were significantly higher than the counterparts in those treated by wild-type GPC3-CAR T cells as shown in **Figures 7C,D**. The immmunochemical analysis (**Figure 7E**) showed that there were more T cells infiltration in the tumor tissues treated by PD-1-deficient GPC3-CAR T cells compared with those treated by wild-type GPC3-CAR T cells, indicating that the disruption of PD-1 enhanced the infiltration of GPC3-CAR T cells in tumor tissues.

Disruption of PD-1 Enhanced Inhibition of Tumor-Relate Genes Expression in Xenografts Caused by the GPC3-CAR T Cells

In order to investigate the effect of PD-1 deficient GPC3-CAR T cells on the tumor-related genes expression in xenograft tumors established with PLC/PRF/5, quantitative reverse transcription PCR was carried out to characterize the mRNA expression levels of tumor-related genes of *CCND1* (cyclin D1), *CTNNB1* (catenin beta-1) and *MET* (MET proto-oncogene, receptor tyrosine kinase) in xenografts treated with various genetically engineered (or not) T cells or PBS. As shown in the **Figure 8**, both wild-type GPC3-CAR T cells and those with deficient PD-1 significantly ($P < 0.001$) inhibited the expression of the three tumor-related genes in xenografts, and the PD-1 deficient GPC3-CAR T cells caused the inhibition at a significantly ($P < 0.001$) larger degree compared with wild-type GPC3-CAR T cells. Taken together, disruption of PD-1 enhanced the inhibition of tumor-relate genes expression in xenografts caused by the GPC3-CAR T cells.

DISCUSSION

Hepatocellular carcinoma is a prevalent cancer worldwide with one of the worst prognoses, and the curative treatment option is only for the patients with limited tumor burden (Yoshiji et al., 1998; Callegari et al., 2013; Yu et al., 2018). HCC is a uniquely immunosuppressive cancer (Obeid et al., 2018). Immunosuppressive intrahepatic environment, which restricts antitumor immunity and promotes tumor progression, is a significant obstacle to treatment of liver cancer (Knolle and Thimme, 2014; Thorn et al., 2016). The majority of liver myeloid-derived suppressor cells were found to express immune-inhibitory ligand PD-L1 (Thorn et al., 2016). Chen et al. (2016) found PD-L1 expression in the primary human HCC surgical specimens. In current study, the upregulation of PD-L1 expression was observed on the HCC cell PLC/PRF/5 exposed to the GPC3-CAR T cells. In this sense, the efficacy of CAR T cell therapy could be more prone to be challenged by the inhibitory PD-1/PD-L1 pathway in the immunosuppressive HCC microenvironment. In the present study, CRISPR-mediated disruption of PD-1 led to enhanced antitumor activity against HCC. Although the previous studies have showed that, in some tumor models, the disruption of PD-1 enhanced the antitumor activity of CAR T cells, those studies mainly focused on the leukemia and pancreatic cancer cells, and most of tumor models in those studies were not derived from the native PD-L1-expressing tumor cells (Ren et al., 2017; Rupp et al., 2017). The functions of CAR T cells could be differential among those with the distinct co-stimulatory domains (Carpenito et al., 2009; Guedan et al., 2014, 2018; Zhao et al., 2015), and all the co-stimulatory domains in the abovementioned previous studies were 4-1BB, which was different from CD28

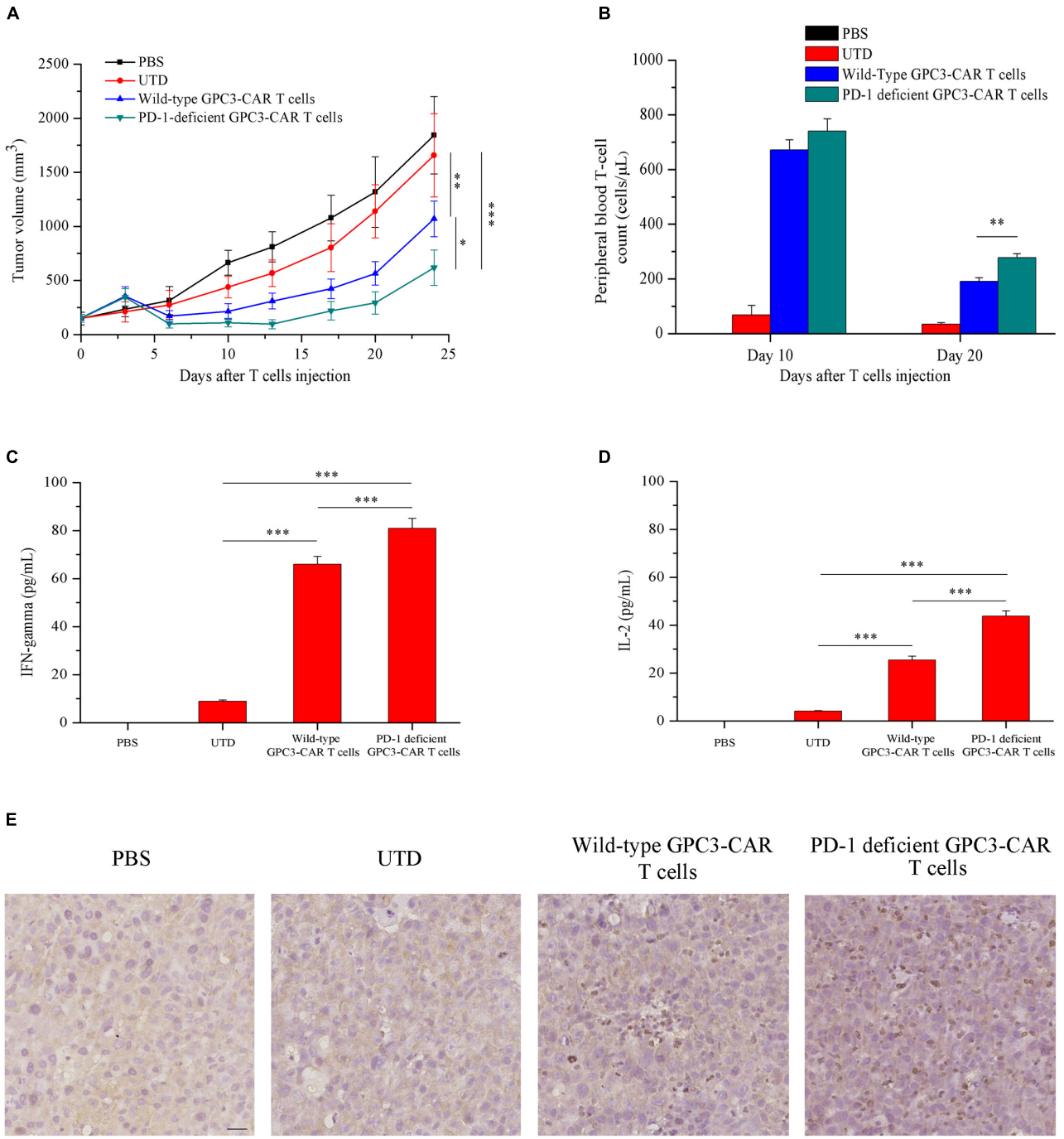

FIGURE 7 | *In vivo* antitumor activities, persistence, cytokines production and infiltration of GPC3-CAR T cells with intact or deficient PD-1 in the established subcutaneous HCC tumor xenograft model with PLC/PRF/5. **(A)** Growth curve of PLC/PRF/5 xenografts treated with the indicated T cells or PBS ($n = 7$). At the endpoint, the residual tumors treated with PD-1-deficient GPC3-CAR T cells were significantly ($^*P < 0.05$) smaller than those treated with wild-type GPC3-CAR T cells. **(B)** The quantities of circulating human T cells from the mice bearing PLC/PRF/5 xenografts treated with the indicated T cells or PBS on days 10 and 20 after T cells or PBS injection. The quantitative analysis was completed using TruCount tubes. On day 20 after T cells or PBS injection, PD-1-deficient GPC3-CAR T cells showed the significantly ($^{**}P < 0.01$) enhanced *in vivo* persistence compared with wild-type GPC3-CAR T cells. **(C,D)** The levels of IFN-gamma **(C)** and IL-2 **(D)** in mouse serum evaluated by ELISA at the endpoint of the experiment. Data shown were mean ± SD from each treatment group. Bars, SD. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one way ANOVA with Turkey *post hoc* test. **(E)** Infiltration of human T cells in the tumor tissues treated with indicated genetically engineered (or not) T cells. Formalin-fixed, paraffin-embedded tumor sections were consecutively cut, and then, stained for human CD3e to detect the human T cell infiltration (brown). Scale bar, 50 μm.

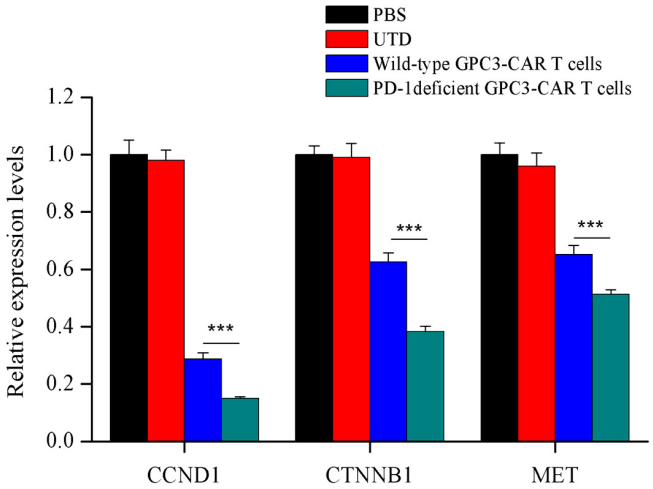

FIGURE 8 | Characterization of the mRNA expression levels of tumor related genes in xenografts treated with various genetically engineered human T cells. The mRNA expression levels of tumor related genes were evaluated by quantitative real-time PCR. CCND1, cyclin D1; CTNNB1, catenin beta 1; MET, MET proto-oncogene, receptor tyrosine kinase. Data were the mean ± SD from triplicates. Bars, SD. ***$P < 0.001$ by one way ANOVA with Turkey *post hoc* test.

employed in the current study. To our best knowledge, the current study combined 28ζ CAR modification and the CRISPR-mediated disruption of endogenous inhibitory immune checkpoint receptor PD-1 in adoptive T cell immunotherapy of native PD-L1-expressing HCC for the first time.

Robust expansion and persistence of CAR T cells are critical for the *in vivo* antitumor efficacy (Louis et al., 2011; Maude et al., 2014; Guedan et al., 2018). Menger et al. (2016) showed that TALEN-mediated inactivation of PD-1 in tumor-reactive lymphocytes promoted T-cell persistence and improved the antitumor efficacy against melanoma and fibrosarcoma *in vivo*, while the CAR was not introduced into the T cells. Cherkassky et al. (2016) demonstrated that cotransduction of PD-1 dominant negative receptor increased the proliferative ability of 28ζCAR T cells and rescued CAR T cells from PD-1 ligand-mediated inhibition. In the current study, the persistence of GPC3-CAR T cells significantly ($P < 0.05$) negatively correlated with the tumor burdens in both treatment groups of wild-type and PD-1-deficient GPC3-CAR T cells. Moreover, CRISPR-mediated disruption of endogenous PD-1 significantly ($P < 0.01$) improved the persistence of 28ζ CAR T cells redirected toward GPC3 *in vivo* as well, associating with the enhanced *in vivo* antitumor efficacy against native PD-L1-expressing HCC.

Repeated antigen stimulation can induce T cell exhaustion and deletion, and human CAR T cells are subject to inhibition of their cytolytic functions upon repeated antigen encounter *in vivo* (Cherkassky et al., 2016). Gargett et al. (2016) showed that GD2-specific CAR T cells underwent potent activation and deletion following antigen encounter, although the activation-induced cell death was reduced by PD-1 blockade. In the current study, although the CRISPR-mediated disruption of endogenous PD-1 benefited the persistence of CAR T cells, the PD-1-deficient CAR T cells were still to decrease *in vivo* with time, which should be related to the exhaustion and deletion of CAR T cells caused by the continual tumor challenge. This could be an important reason for the phenomenon that tumor stopped regression and re-grew after 13 days of the infusion of CAR T cell with deficient PD-1. Additionally, for this phenomenon, it cannot be excluded that inhibition of cytolytic function of PD-1-deficient CAR T cells caused by the compensatory upregulation of alternative checkpoints, considering that the blockade of one checkpoint pathway is often followed by the compensatory upregulation of other alternative immune checkpoint pathways. Koyama et al. (2016) showed that adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints on the PD-1 antibody bound T cells in lung adenocarcinoma, notably T-cell immunoglobulin mucin-3. Huang et al. (2017) found that blockade of PD-1, LAG-3, or CTLA-4 alone conferred a compensatory upregulation of the other checkpoints on T cells in metastatic ovarian cancer. Henceforth, it will be very important to investigate the compensatory immunosuppressive checkpoints of PD-1: PD-L1/L2 pathway on 28ζ CAR T cells in the HCC microenvironment, and the CRISPR-mediated combinatorial disruption of checkpoints will be beneficial for the 28ζ CAR T cells achieving the sustained regression and eradication of HCC.

Under physiological conditions, the PD-1:PD-L1/L2 pathway prevents excessive effector activities by T cells and promotes the tolerance to self-antigens to avoid the development of autoimmunity (Papaioannou et al., 2016). Although monoclonal antibodies blocking PD-1, such as pembrolizumab and nivolumab, can retrieve the functionality of exhausted T cells and produce potent antitumor immune response in patients with various cancers, the systemic administration of the immune checkpoint pathway blocking antibodies still runs the risk of disrupting immunologic homeostasis, producing unique immune-related adverse effects, and even threatening the life (Gettinger et al., 2015; Larkin et al., 2015; Robert et al., 2015; Weber et al., 2015). The disruption of intrinsic immune checkpoints in T cells through gene editing is considered

to be a relatively safer strategy compared with the systemic administration of blocking antibody (Lloyd et al., 2013; June et al., 2015). Su et al. (2016) found the disruption of PD-1 did not change the activation status of human primary T cells not carrying the CAR, while the CAR was not introduced into the T cells. In the current study, the disruption of endogenous PD-1 did not affect the activation status and cytotoxic specificity of CAR T cells, and the cytotoxic function of the PD-1-deficient CAR T cells was still CAR-dependent. However, given that CAR T cells with individual disruption of PD-1 are still likely to express auto-reactive TCRs, there might be the potential autoimmune adverse effects resulted from the PD-1-deficient CAR T cells with intact TCR (Rupp et al., 2017). Therefore, it will be crucial to disrupt TCR for the safe and efficient utilization of the GPC3-CAR T cell with deficient immunosuppressive checkpoint molecules on anti-HCC therapy. Besides, considering the NSG animal model used in this study with severe deficient immune system was largely different from the clinical conditions, henceforth, safe estimation is needed in the immunocompetent animal models before proceeding to clinic.

Additionally, tumor-associated antigens often express at low levels in normal tissues (Simpson et al., 2005; Johnson et al., 2009). GPC3 is expressed in most (72%) of HCC and not in normal liver tissue, but its expression in other normal tissues could not be completely eliminated (Capurro et al., 2003; Baumhoer et al., 2008; Hass et al., 2015). Thus, the on-target, off-tumor toxicity of the GPC3-CAR T cell might occur, even if without the disruption of the PD-1. Chen et al. (2017) found that dual-targeted CAR-T cells co-expressing complementary GPC3 and asialoglycoprotein receptor 1 (a liver tissue-specific protein)-targeted CARs showed relatively potent anti-tumor activity against HCC tumor xenografts with double antigens, but exhibited the restricted antitumor activity against HCC xenografts with a single antigen, indicating that dual-targeted CAR-T cells could be a promising strategy for reducing or avoiding the potential off-tumor toxicities of the GPC3-CAR T cell therapy on HCC. In the present study, although the antitumor activity of GPC3-CAR T cell with deficient PD-1 was CAR-dependent, its off-tumor toxicity cannot be excluded in clinical therapy. Henceforth, combination of the dual-targeted CAR modification and the simultaneous disruption of the TCR and compensatory immunosuppressive checkpoint molecules in T cells will be important for the generation of the highly potent and safe genetically engineered CAR T cells in the therapy of HCC.

A key signaling target of PD-1-mediated inhibition is the PI3K-Akt pathway (Boussiotis, 2016). The previous studies found that the triggering of PD-1-mediated signals blocked the CD28-mediated activation of PI3K and Akt, and the expression of anti-apoptotic protein Bcl-xL (Chemnitz et al., 2004; Parry et al., 2005). The current study found disruption of PD-1 can increase the levels of Akt activation and anti-apoptotic protein Bcl-xL expression in GPC3-CAR T cells after combating the HCC cells, suggesting that the disruption of PD-1 can protect the GPC3-CAR T cell from exhaustion when combating the native PD-L1-expressing, GPC3-positive HCC. Among three analyzed tumor-related genes, *CTNNB1* and *MET* act as the oncogenes in HCC, and CCND1 is the hallmarker of cell cycle procession (Polakis, 2000; Zhang et al., 2002; Venepalli and Goff, 2013). Previous study found that HCC growth behavior was positively correlated with the expression levels of these tumor-related genes (Jiang et al., 2016). The present study found that the disruption of PD-1 can enhance the inhibition of the expression of tumor-related genes correlated with the HCC growth behavior caused by GPC3-CAR T cells, but the interaction mechanism between the PD-1 deficient GPC3-CAR T cells and HCC, and the influence of disruption of endogenous PD-1 on itself of GPC3-CAR T cells when combating tumor need the further in-depth studies by the combination of transcriptomics, proteomics, and bioinformatics, which will be beneficial for the design and development of the next-generation safe and more potent CAR T cells in HCC therapy.

CONCLUSION

In summary, CRISPR-mediated disruption of endogenous PD-1 can enhance the CAR-dependent antitumor activity of the GPC3-specific second-generation CAR T cells employing CD28 as the co-stimulatory domain, and improve *in vivo* persistence and infiltration of CAR T cells, but not affect the CD4 and CD8 subsets, and activation status of CAR T cells. This study is beneficial for the development of next-generation CAR T cell with improved therapeutic efficacy in HCC by the precise genetic engineering.

AUTHOR CONTRIBUTIONS

ZL conceived the idea and revised the manuscript. XG designed subsequent experiments, performed most of the *in vitro* and *in vivo* work, and wrote the manuscript. HJ, BS, and MZ assisted with analysis of data and helped to perform the *in vitro* and *in vivo* work. HZ, ZS, GD, HL, XW, YW, and RS assisted with the *in vitro* work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01118/full#supplementary-material

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The Clinicopathologic and Prognostic Significance of Programmed Cell Death Ligand 1 (PD-L1) Expression in Patients With Prostate Cancer: A Systematic Review and Meta-Analysis

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Background: Programmed cell death ligand 1 (PD-L1) expression has been shown to correlate with poor prognosis in diverse human cancers. However, limited data exist on the prognostic and clinicopathologic significance of PD-L1 expression in prostate cancers (PCa), and the curative effect of anti-PD-1/PD-L1 therapy remains controversial. In this systematic review and meta-analysis, we aimed to evaluate the prognostic and clinicopathologic value of PD-L1 in PCa.

Methods: We performed a systematic literature search in the PubMed, Cochrane Library, EMBASE, Web of Science, and SCOPUS databases up to July 21st, 2018. Pooled prevalence of PD-L1 in PCa was calculated using Freeman-Tukey double arcsine transformation by R software version 3.5.0. The data from the studies were examined by a meta-analysis using Review Manager software 5.3 to calculate pooled hazard ratios (HRs) and pooled odds ratios (ORs) with 95% confidence intervals (CIs) to estimate the prognostic and clinicopathologic value of PD-L1 in PCa. Heterogeneity was tested by the Chi-squared test and I^2 statistic.

Results: Five studies with 2,272 patients were included in this meta-analysis. The pooled prevalence of PD-L1 in PCa was 35% (95% CI 0.32 to 0.37). Both PD-L1 expression ($HR = 1.78$; 95% CI 1.39 to 2.27; $p < 0.00001$) and PD-L1 DNA methylation ($HR = 2.23$; 95% CI 1.51 to 3.29; $p < 0.0001$) were significantly associated with poor biochemical recurrence-free survival (BCR-FS). PD-L1 tended to have high expression levels in high Gleason score cases ($OR = 1.54$; 95% CI, 1.17 to 2.03; $P = 0.002$) and androgen receptor-positive cases ($OR = 2.42$, 95% CI 1.31 to 4.50; $P = 0.005$). However, PD-L1 had relatively weak correlation with age, pathologic stage, lymph node metastasis and preoperative PSA level.

Conclusions: This meta-analysis confirms the negative prognostic significance of PD-L1 expression and mPD-L1 in PCa patients. Additionally, PD-L1 has a statistically

significant correlation with Gleason score and androgen receptor status, while the correlations with age, pathologic stage, lymph node metastasis, and preoperative PSA level were not statistically significant. However, the number of included studies is too small to make the conclusions more convincing, so more retrospective large-cohort studies are expected for the further confirmation of these findings.

Keywords: prostate cancer, PD-1/PD-L1, prognostic, clinicopathologic, meta-analysis

INTRODUCTION

As a malignancy in the male reproductive system, prostate cancer (PCa) was not only the second most common cancer in males worldwide both in 2012 (1,112,000 new cases; 15.0%) (Ferlay et al., 2015) and 2018 (1,276,100 new cases; 13.5%) (Ferlay et al., 2018), but also the most common cancers among males in the United States (164,690 new cases; 19%) in 2018 (Siegel et al., 2018). Overall, PCa was the fifth leading cause of cancer-related death in men worldwide (307,000 deaths; 6.6%) in 2012 (Ferlay et al., 2015), while it became the fourth leading cause of cancer-related death in men worldwide (359,000 deaths; 6.7%) in 2018 (Ferlay et al., 2018). Furthermore, PCa was the second leading cause of cancer-related death in men in the United States (29,430 deaths; 9%) in 2018 (Siegel et al., 2018). PCa incidence rates increased, whereas PCa mortality rates declined in most countries in recent years, especially in more developed nations (Wong et al., 2016). Due to earlier detection by prostate-specific antigen (PSA) testing and advances in treatment, the mortality of PCa rapidly declined by 52% from 1993 to 2015 (Siegel et al., 2018). For all cancers combined, 5-year relative survival rates is highest for prostate cancer patients with localized disease (99%) during the recent time period (2007–2013) (Siegel et al., 2018), but declines to 28% for those at distant stage (Miller et al., 2016). Clinical decisions vary in the extent of disease, risk of recurrence and patient characteristics, so active surveillance is recommended for less aggressive tumors as well as older patients and/or those with severe comorbidities. Treatment options for early-stage localized prostate cancer include radical prostatectomy, external beam radiotherapy, androgen deprivation therapy (ADT), chemotherapy, bone-directed therapy, radiation, while a combination of the above therapies is used for advanced disease (Horwich et al., 2010; Miller et al., 2016). Current therapies in metastatic castration-resistant prostate cancer (mCRPC) include androgen receptor (AR)-targeted therapy, chemotherapy, immunotherapy, bone-targeted therapy, poly (adenosine diphosphate–ribose) polymerase (PARP) inhibitors, and other novel therapeutic targets (Nuhn et al., 2018).

Programmed cell death 1 (PD-1; CD279) is an inhibitory receptor expressed by tumor-infiltrating lymphocytes (TILs), such as activated T cells, B cells, and natural killer (NK) cells (Pardoll, 2012; Riella et al., 2012). Its ligand, programmed cell death ligand 1 (PD-L1; B7-H1; CD274), is expressed constitutively on specific tumors and immune cells, including T and B cells, dendritic cells (DCs), macrophages, mesenchymal stem cells, and bone marrow-derived mast cells (Riella et al., 2012). PD-1 and PD-L1 are immune check points that limit autoimmunity and the activity of T cells under an inflammatory response to infection (Pardoll, 2012; Wang P. et al., 2017). Anti-PD-1/PD-L1 therapy is a promising immunotherapy that can enhance antitumor immunity and elicit durable clinical responses by blocking the PD-1/PD-L1 signaling pathway (Aghajani et al., 2018). The responses strongly correlated with increased PD-1 expression by TILs and increased PD-L1 expression by tumor cells (Pardoll, 2012). Some published studies reported that PD-L1 expression was a negative predictor for prognosis (Zhang et al., 2016; Aghajani et al., 2018; Keller et al., 2018; Miyama et al., 2018), whereas some other studies manifested inconsistent results (Pardoll, 2012; Wang C. et al., 2017; Huang et al., 2018). Various analyses on diverse tumors have showed that the expression of PD-L1 can associate either with poor prognosis, better prognosis or have no connection with prognosis (Ohigashi et al., 2005; Ghebeh et al., 2006; Wu et al., 2006; Hamanishi et al., 2007; Hino et al., 2010; Pardoll, 2012; Iacovelli et al., 2016; Li et al., 2018). Studies evaluating the prognostic and clinicopathologic significance of PD-L1 expression in PCa are limited, and the curative effect of anti-PD-1/PD-L1 therapy on PCa remains controversial. Therefore, it prompted us to perform a meta-analysis to figure out the prognostic and clinicopathologic significance of PD-L1 in PCa patients, that is to say our meta-analysis aims to find out whether PD-L1 expression of PCa is related to outcome parameters (biochemical recurrence-free survival) and clinicopathologic parameters (e.g., Gleason score). We report this systematic review and meta-analysis following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Moher, 2009).

Abbreviations: PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; mPD-L1, PD-L1 DNA methylation; PCa, prostate cancer; mCRPC, metastatic castration-resistant prostate cancer; NOS, Newcastle Ottawa Quality Assessment Scale; HR, hazard ratio; OR, odds ratio; CI, confidence interval; BCR-FS, biochemical recurrence-free survival; PSA, prostate-specific antigen; IHC, immunohistochemistry; ADT, androgen deprivation therapy; AR, androgen receptor; AR+, androgen receptor-positive; AR-, androgen receptor-negative; PARP, poly (adenosine diphosphate–ribose) polymerase; TILs, tumor-infiltrating lymphocytes; NK cell, natural killer cell; DCs, dendritic cells; miR, microRNA; Neo-AAPL, neoadjuvant androgen deprivation therapy with abiraterone acetate plus prednisone and leuprolide.

METHODS

Analysis Workflow

Literature data-mining of clinicopathologic and prognostic significance of PD-L1 expression in prostate cancer, data

collection, statistical analysis, and associated results extraction followed the workflow depicted in **Figure 1** with specifics as provided in the sections below.

Literature Search

A comprehensive literature search was systematically performed in the PubMed, Cochrane Library, EMBASE, Web of Science, and SCOPUS databases to identify relevant studies up to July 21st, 2018. The following keywords were employed for literature retrieval: ("prostate" or "prostatic") and ("cancer" or "neoplasm" or "tumor" or "tumor" or "carcinoma") and ("Programmed Cell Death Ligand 1" or "Programmed Death Ligand 1" or "PD-L1" or "B7-H1" or "CD274" or "Programmed Cell Death 1" or "Programmed Death 1" or "PD-1" or "CD279"). A manual search of potential references was also conducted, and literature in the field of interest was reviewed for additional eligible studies.

Study Selection

Assessment of every study retrieved was independently examined by two reviewers (Q. Y. Huang and Y. Y. Zhou) for comprehensive evaluation based on the following inclusion criteria: (1) Patients were histologically confirmed as having prostate cancer; (2) PD-L1 protein expression was assessed in prostate cancer tissues; (3) PD-L1 expression was divided into high (positive) and low (negative) categories; (4) studies investigated the association between PD-L1 protein expression and/or mPD-L1 with clinicopathologic features and/or prognosis; (5) studies directly provided hazard ratio (HR) or odd ratio (OR) with corresponding 95% confidence interval (CI), or survival curves/number of patients with specific clinicopathologic features to estimate them; and (6) studies were published in English with available full texts. The exclusion criteria were formulated and improved after we found some studies satisfying our inclusion criteria but could not be included in the final meta-analysis. The exclusion criteria were as follows: (1) studies did not satisfy the inclusion criteria; (2) studies turned out to be reviews, meta-analyses, editorials, case reports, expert opinions, letters, notes, meeting abstracts or proceedings; (3) non-human studies or *in vitro* studies; (4) duplication publications or studies with overlapping data; and (5) studies provided information unable to be pooled. Disagreements about certain studies were resolved by discussion with a third reviewer (YL).

Data Extraction

The data from the eligible studies were extracted independently by two reviewers (Y. Y. Zhou and Q. Y. Huang) in piloted forms (in duplicate) to tabulate the information, and any disagreements between the two reviewers were resolved with consensus. The following data were collected from each included study: name of the first author, year of publication, country, number of patients, tumor type, technique, PD-L1-positive expression as well as high mPD-L1, cut-off values for PD-L1 positive expression as well as high mPD-L1, the hazard ratios (HRs) and 95% confidence intervals (CIs) for biochemical recurrence-free survival (BCR-FS), and numbers of PD-L1-positive as well as PD-L1-negative patients with (a) age <60 years, (b) age ≥ 60 years, (c) Gleason score <7, (d) Gleason score ≥ 7, (e) pathologic stage pT2, (f) pathologic stage pT3-pT4, (g) lymph node metastasis N0, (h) lymph node metastasis N1, (i) preoperative PSA level ≤ 10 ng/ml, (j) preoperative PSA level >10 ng/ml, (k) androgen receptor-negative (AR-), and (l) androgen receptor-positive (AR+).

Population, Interventions, Comparators, Outcomes and Study Designs (PICOS)

The population from the study is patients with prostate cancer. PD-L1 expression and/or mPD-L1 was assessed in these patients. PD-L1 status (PD-L1 positive and PD-L1 negative) and mPD-L1 level (high and low) were compared by the endpoint BCR-FS. The correlations of PD-L1 status with age, Gleason score, pathologic stage, lymph node metastasis, preoperative PSA level, and androgen receptor status were evaluated in these patients. The study designs were to evaluate the association between PD-L1 expression/mPD-L1 and prognosis as well as the relationship of PD-L1 expression and age, Gleason score, pathologic stage, lymph node metastasis, preoperative PSA level, and androgen receptor status.

Quality Assessment

Two investigators (Y. Y. Zhou and Q. Y. Huang) independently conducted the quality assessment of all included studies according to the Newcastle-Ottawa Scale (NOS) criteria to ensure consistency in reviewing and reporting results (Stang, 2010). The NOS consists of the following three parameters of quality: (1) selection: 0–4; (2) comparability: 0–2; and (3) exposure/outcome: 0–3. The maximum of NOS score is nine, with studies scoring greater than five considered to be of high quality. Any discrepancies between reviewers were resolved by consensus.

Statistical Analysis

Pooled prevalence of PD-L1 in PCa were calculated using Freeman-Tukey double arcsine transformation by R software version 3.5.0. The HR is the ratio of the hazard rates corresponding to the conditions described by two levels of an explanatory variable, and the OR is defined as the ratio of the odds of A in the presence of B and the odds of A without the presence of B, which attempts to quantify the strength of the association between A and B. Pooled HRs with their 95% CIs were implemented to estimate the association between BCR-FS and PD-L1 expression or mPD-L1. Patients were dichotomized by age (<60 years vs. ≥ 60 years), Gleason score (<7 vs. ≥ 7), pathologic stage (pT2 vs. pT3-pT4), lymph node metastasis (N0 vs. N1), preoperative PSA level (≤ 10 ng/mL vs. >10 ng/mL), and androgen receptor status (AR+ vs. AR-) categories of PD-L1 expression by referring to National Comprehensive Cancer Network (NCCN) Guidelines for Prostate Cancer (URL: https://www.nccn.org/professionals/physician_gls/default.aspx#prostate). The dichotomous outcomes were analyzed using the ORs with 95% CI as the summary statistics to evaluate the correlation between PD-L1 expression and the above clinicopathologic parameters. The Review Manager software version 5.3 (Revman,

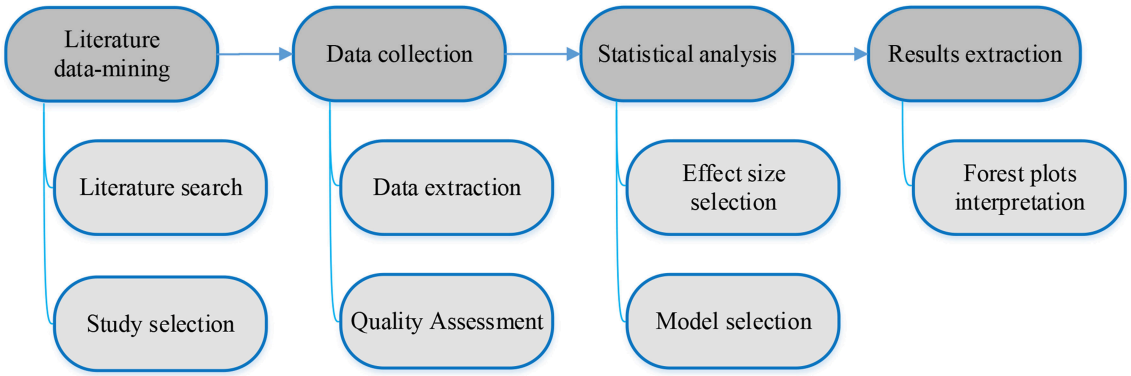

FIGURE 1 | Workflow for meta-analysis of clinicopathologic and prognostic significance of PD-L1 expression in prostate cancer.

the Cochrane Collaboration; Oxford, England) was used to calculate HR and OR with 95% CIs in this meta-analysis. Heterogeneity is defined as the consequence of methodological and/or statistical diversity among studies and was assessed by the Chi-squared test and I^2 statistic. I^2 values less than 25%, from 25 to 50%, and higher than 50% represented low, medium and high heterogeneity, respectively. Statistical tests were all two-sided, with *P*-values < 0.05 considered to be statistically significant. Detailed interpretations of odds ratios, confidence intervals and p-values can be found elsewhere (Tim, 2013).

According to Chapter 13 of the book *Introduction to meta-analysis* (Borenstein et al., 2009), the following three points should be noticed: (a) if the number of studies is very small, then the estimate of the between-studies variance will have poor precision, (b) while the random-effects model is still the appropriate model, we lack the information needed to apply it correctly, and (c) in this case, one option is to perform a fixed-effect analysis. Hence, fixed-effect models were employed for all statistical analyses because the number of our included studies is small.

RESULTS

Search Results

In the present study, a total of 2,130 records were identified initially from the five databases, 160 from PubMed, 722 from EMBASE, 686 from SCOPUS, 40 from Cochrane Library, and 522 from Web of Science, by using the search strategy above. After removing the duplicate publications ($n = 884$), the titles and abstracts of all remaining publications ($n = 1{,}246$) were reviewed, and 1,155 articles were excluded because they were non-original articles ($n = 175$: 131 reviews, 12 meta-analyses, 5 case reports, 14 editorials, 2 letters, 2 expert opinions, 9 notes), meeting abstracts ($n = 44$), animal or cell lines experiments ($n = 63$), or not in the field of interest ($n = 873$). Of 91 remaining studies, 12 full texts were not available and so 79 studies were left. Another 61 studies were excluded for the following reasons: (a) the studies focused on adverse events of anti-PD-1/PD-L1 therapy, the effectiveness of PD-1/PD-L1 inhibitors, the combination therapy with anti-PD-1/PD-L1 therapy plus other treatments, or the influences of other factors on PD-L1 expression; (b) the studies were mechanism studies, pharmacological experiments or ongoing clinical trials; (c) the studies provided no information about outcome parameters (such as overall survival, disease-free survival and progression-free survival) or clinicopathologic features of PD-L1 positive and negative patients. No outcome parameter except biochemical recurrence-free survival (BCR-FS) was found in more than one study, so studies which used the outcome parameters except BCR-FS were excluded. The studies, which provided the clinicopathologic features of PCa patients, but did not provide the respective clinicopathologic features of PD-L1-positive and PD-L1-negative patients, were also excluded. After excluding 13 studies with unanalyzable data mentioned above, five studies were eventually included in the final meta-analysis. A flowchart depicting details of the study selection is shown in **Figure 2**.

Study Characteristics

The characteristics of the included studies are summarized in **Table 1**. The five eligible studies were published between 2009 and 2018: three studies from Germany and two from America. Of note, the article by (Gevensleben et al., 2016a) offered two cohorts: a training cohort and a test cohort, while another article by (Gevensleben et al., 2016b) provided a training cohort and a validation cohort. The validation cohort in 2016 not only evaluated the prognostic value of PD-L1 protein expression, but also the prognostic significance of mPD-L1. Therefore, in total, seven comparisons (from five articles) consisting of 2,272 patients were included in the meta-analysis. Among these articles, PD-L1 expression was detected by using the immunohistochemistry (IHC) staining method in four articles (1,475 cases) and was found in 557 patients (37.8%), with the percentage ranging from 7.7 to 82.4%. As presented in **Table 1**, different studies adopted different cut-off values to define positive (high) and negative (low) PD-L1 expression. In Ebelt et al. (2009), the estimated number of positively stained cells >50

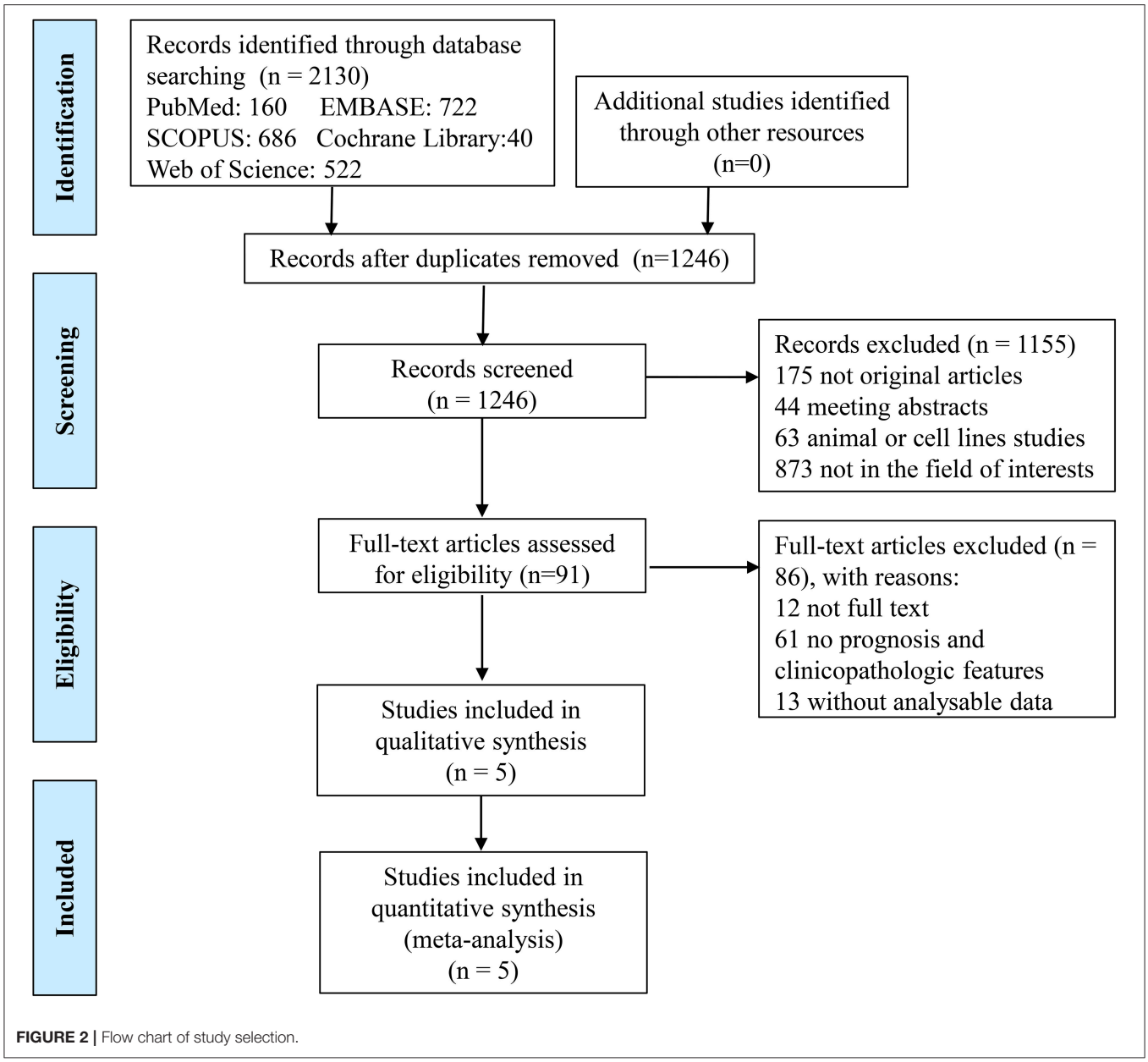

FIGURE 2 | Flow chart of study selection.

was considered to be PD-L1-positive. In Calagua et al. (2017) and Haffner et al. (2018), PD-L1 positivity was defined as ≥1% of tumor cells stained positive for PD-L1. In Gevensleben et al. (2016a), PD-L1 expression was dichotomized by median (high = above median, low = below median). In Gevensleben et al. (2016b), PD-L1 DNA methylation dichotomized by an optimized cut-off ($mPD\text{-}L1_{low} < 0.98\% \leq mPD\text{-}L1_{high}$). The 0.98% here refers to the percentage of DNA methylation. For this pooled analysis, we found PD-L1-positive patients and high mPD-L1 patients according to their own specific cut-off criteria. BCR-FS was implemented as the end point in five comparisons out of two studies (Gevensleben et al., 2016a,b), of which three comparisons were about PD-L1 expression and the other two were comparisons about mPD-L1. Moreover, we compared the prevalence of PD-L1 expression between the following pairs: age <60 years and age ≥60 years (two comparisons), Gleason score <7 and Gleason score ≥7 groups (five comparisons), pathologic stage pT2 and pathologic stage pT3-pT4 groups (five comparisons), lymph node metastasis N0 and N1 (four comparisons), PSA level ≤10 ng/ml and PSA level >10 ng/ml (two comparisons), and androgen receptor-positive and androgen receptor-negative (two comparisons).

Based on the Newcastle-Ottawa quality assessment scale (URL: http://www.ohri.ca/programs/clinical_epidemiology/nosgen.pdf), the NOS scores of the five studies ranged from 6 to 8, with a mean score of 6.8. Thus, these eligible studies were of high quality. The details of the quality assessment are depicted in **Tables 2**, **3**.

TABLE 1 | Characteristics of the eligible studies in the meta-analysis.

References	Country	No.	Tumor	Technique	Cut-off	PD-L1 positive (%)	Outcome	HR estimation
Ebelt et al., 2009	Germany	17	Prostate cancer	IHC	Cell counts≥50	14/17 (82.4)	NA	NA
Gevensleben et al., 2016a	Germany	Training cohort: 209	Primary prostate cancer	IHC + TMA	Above median	109/209 (52.2)	BCR-FS	2.37 [1.32–4.25]
Gevensleben et al., 2016a	Germany	Test cohort: 611	Primary prostate cancer	IHC+TMA	Above median	377/611 (61.7)	BCR-FS	1.49 [1.10–2.02]
Gevensleben et al., 2016b	Germany	Validation cohort: 299	Prostate cancer	NA	NA	NA	BCR-FS	2.58 [1.43–4.63]
Gevensleben et al., 2016b	Germany	Validation cohort: 299	Prostate cancer	qPCR	≥0.98%	high mPD-L1: 102/299 (34.1)	BCR-FS	1.90 [1.09–3.31]
Gevensleben et al., 2016b	Germany	Training cohort: 498	Prostate cancer	qPCR	NA	High mPD-L1: 101/498 (20.3)	BCR-FS	2.60 [1.50–4.51]
Calagua et al., 2017	America	130	Prostate cancer	IHC	≥ 1%	18/130(13.8)	NA	NA
Haffner et al., 2018	America	508	Acinar adenocarcinomas of the prostate	IHC	≥ 1%	39/508 (7.7)	NA	NA

NO, number of patients; NA, not available; IHC, immunohistochemistry; TMA, tissue microarrays; qPCR, quantitative methylation real-time PCR; PD-L1, programmed cell death ligand 1; mPD-L1, PD-L1 DNA methylation; BCR-FS, biochemical recurrence-free survival; HR, hazard ratio.

TABLE 2 | Quality assessment of the case control studies in the meta-analysis.

Included studies	Selection				Comparability	Exposure			Total quality score
	S1	S2	S3	S4	C	E1	E2	E3	
Ebelt et al., 2009	a*	a*	b	b	a*	a*	a*	a*	6
Calagua et al., 2017	a*	a*	a*	b	ab**	b*	a*	a*	8
Haffner et al., 2018	a*	a*	c	b	ab**	b*	a*	a*	7

S1, Adequacy of case definition; S2, Representativeness of the cases; S3, Selection of Controls; S4, Definition of Controls; C, Comparability of cases and controls on the basis of the design or analysis; E1, Ascertainment of exposure; E2, Same method of ascertainment for cases and controls; E3, Non-Response rate.

Prevalence of PD-L1 Expression in Prostate Cancer

The prevalence of PD-L1 expression among prostate cancer patients in the five eligible studies ranged from 7.7 to 82.4% (**Table 1**). The pooled analysis result gave an overall prevalence of PD-L1 of 35% (fixed effect, 95% CI 0.32 to 0.37) with a significant heterogeneity ($P < 0.01$; $I^2 = 99\%$) (**Figure 3**).

PD-L1 and MPD-L1 as Prognostic Factors for Prostate Cancer

Two studies including three comparisons with 1,119 patients reported biochemical recurrence-free survival (BCR-FS). The pooled HR for BCR-FS showed that PD-L1 expression was associated with poor BCR-FS in PCa with statistical significance and a higher level of PD-L1 expression increased the risk of death by 78 % with fixed effects (HR = 1.78; 95 % CI 1.39 to 2.27; $p < 0.00001$) (**Figure 4A**). There was no significant heterogeneity ($\text{Chi}^2 = 3.76$, $p = 0.15$; $I^2 = 47\%$).

In addition, an association with statistical significance between high mPD-L1 and the increased risk for BCR was identified (fixed effect, HR = 2.23; 95% CI 1.51 to 3.29; $p < 0.0001$) (**Figure 4B**), without significant heterogeneity ($\text{Chi}^2 = 0.62$, $p = 0.43$; $I^2 = 0\%$).

Correlation Between Pd-L1 Expression and Clinicopathologic Characteristics

Age

We assessed the association between PD-L1 expression and age among 819 patients from two comparisons (**Figure 5A**). Among 602 older patients (≥60 years), 364 patients (60.5%) were PD-L1 expression positive, and 121 (55.8%) of 217 younger patients (<60 years) were PD-L1 expression positive. Pooled results ($OR = 1.27$; 95% CI 0.93 to 1.75; $P = 0.14$) showed that the odds of positive PD-L1 expression in older patients were 27% higher than in younger patients. However, this result was not statistically significant.

Gleason Score

The rate of positive expression of PD-L1 between the groups with Gleason scores ≥7 and <7 was compared in four studies including 1,470 patients (**Figure 5B**). It was determined that 378 (35.6%) of 1,061 PCa patients with higher Gleason scores and 178 (43.5%) of 409 PCa patients with lower Gleason scores were PD-L1 expression positive, with an odds ratio of 1.54 (95% CI, 1.17 to 2.03; $P = 0.002$). Therefore, the odds of positive PD-L1 expression in PCa patients with higher Gleason scores were 54%

TABLE 3 | Quality assessment of the cohort studies in the meta-analysis.

Included studies	Selection				Comparability	Outcome			Total quality score
	S1	S2	S3	S4	C	O1	O2	O3	
Gevensleben et al., 2016a	a*	a*	c	b	ab**	a*	a*	a*	7
Gevensleben et al., 2016b	a*	a*	c	b	ab**	c	a*	a*	6

S1, Representativeness of the exposed cohort; S2, Selection of the non-exposed cohort; S3, Ascertainment of exposure; S4, Outcome not present at start of study; C, Comparability of cohorts on the basis of the design or analysis; O1, Assessment of outcome; O2, Length of follow-up; O3, Adequacy of follow-up.

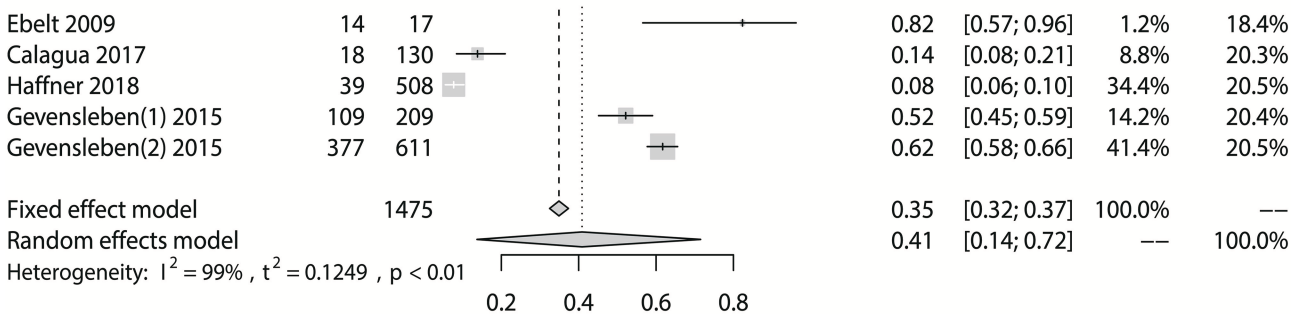

FIGURE 3 | Forest plot showing the pooled prevalence of PD-L1 expression among prostate cancer patients.

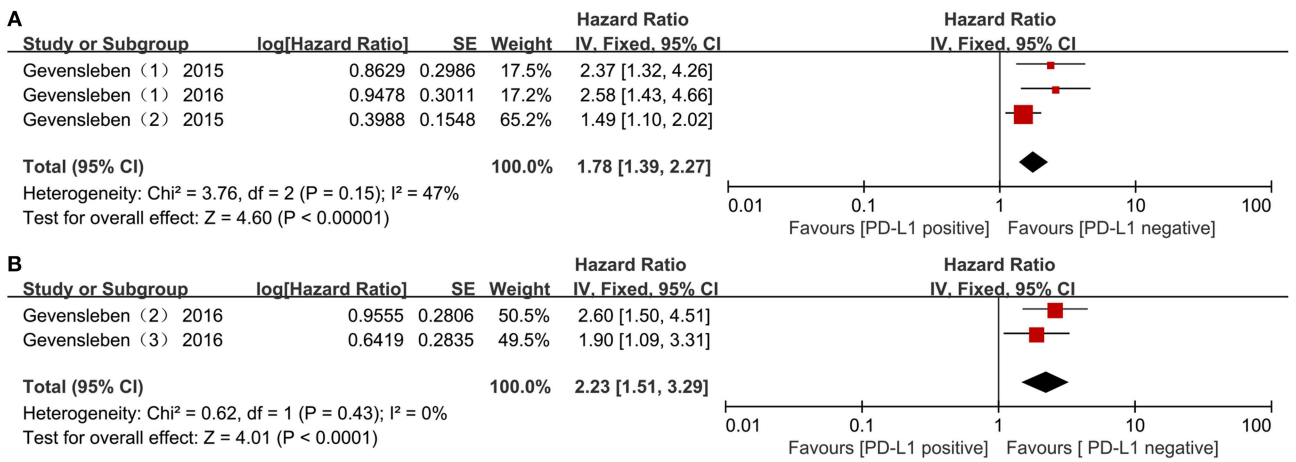

FIGURE 4 | Forest plots evaluating the association between BCR-free survival and PD-L1 protein expression **(A)**, PD-L1 DNA methylation **(B)** in patients with prostate cancer.

higher than those with lower Gleason scores, and this result was statistically significant.

Pathologic Stage

A total of 1,458 patients out of four studies were analyzed for the association between PD-L1 expression and pathologic stage (**Figure 5C**). Then we found that 213 (33.0%) of 646 patients in stage pT3–pT4 and 342 (42.1%) out of 812 patients in stage pT2 were PD-L1 expression positive. The odds of positive PD-L1 expression in patients at stage pT3–pT4 were 27% higher than patients at stage pT2, a result with no statistical significance (*OR* = 1.27, 95% CI 0.97 to 1.65; *P* = 0.08).

Lymph Node Metastasis

Three studies comprising 1,149 patients were evaluated for the association between PD-L1 expression and lymph node metastasis (**Figure 5D**). Of 93 patients with lymph node status N0, 17 (18.3%) were PD-L1 expression positive, and 354 (33.5%) of 1,056 patients with lymph node status N1 were PD-L1 expression positive. The pooled results (*OR* = 0.65, 95% CI

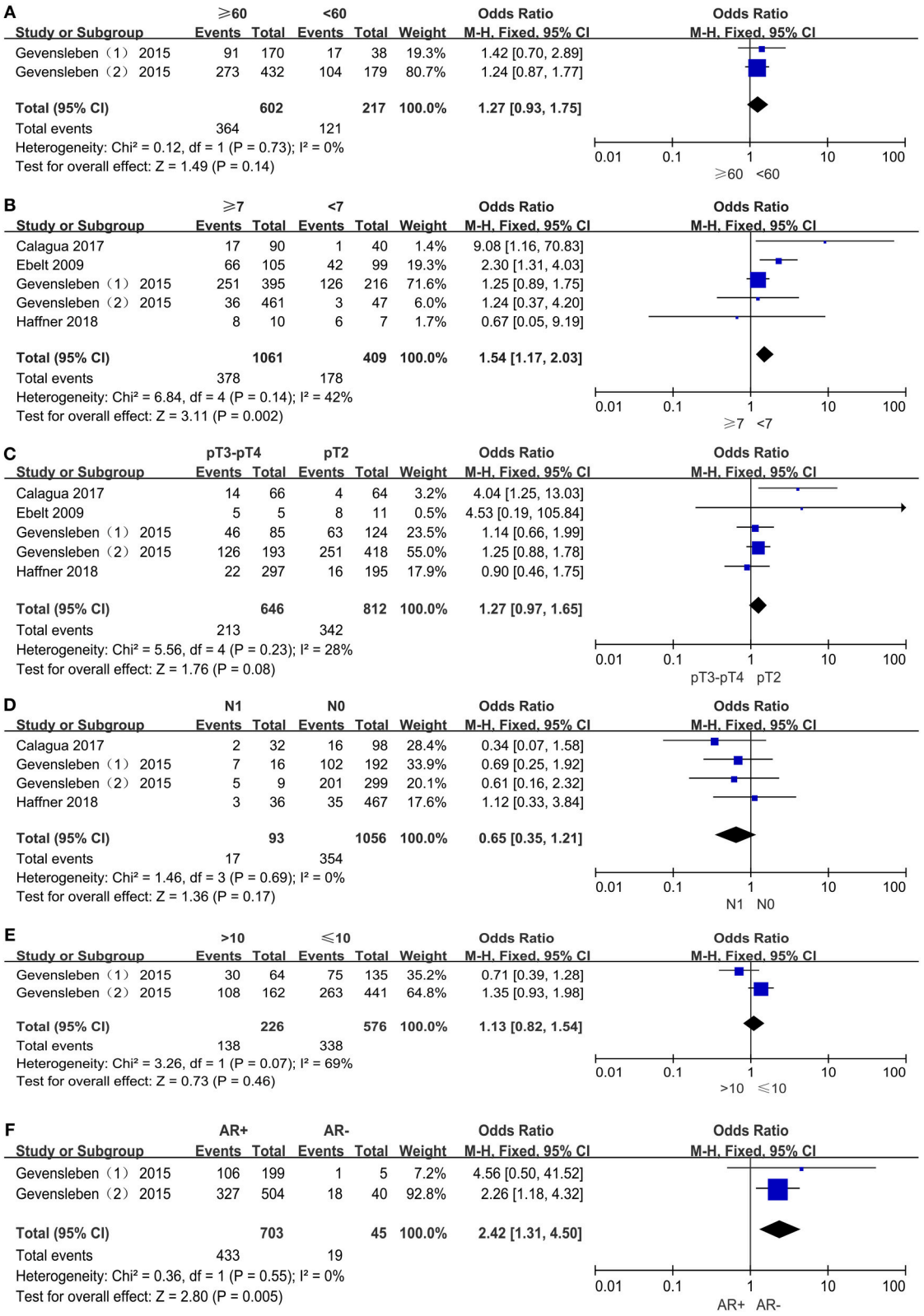

FIGURE 5 | Forest plots for the association between PD-L1 expression and clinicopathologic features: age **(A)**, Gleason score **(B)**, pathologic stage **(C)**, lymph node metastasis **(D)**, preoperative PSA **(E)**, androgen receptor status **(F)**.

0.35 to 1.21; $P = 0.17$) showed that the odds of positive PD-L1 expression in PCa patients with N0 were 35% lower than those with N1. However, this result was also not statistically significant.

Preoperative PSA

Only two comparisons out of one study, which included 802 patients, examined the correlation between PD-L1 expression and preoperative PSA level. Of 226 PCa patients with higher PSA levels (>10 ng/mL), 138 (61.1%) were PD-L1 expression positive and 338 (58.7%) of 576 PCa patients with lower PSA levels (≤10 ng/mL) were PD-L1 expression positive. The odds of positive PD-L1 expression in patients with higher PSA level were 13% higher than those with lower PSA level and this result was not statistically significant ($OR = 1.13$, 95% CI 0.82 to 1.54; $P = 0.46$) (**Figure 5E**).

Androgen Receptor Status

The correlation between PD-L1 expression and androgen receptor status was assessed among two comparisons with 1,200 patients (**Figure 5F**). Of 703 AR+ patients, 433 (61.6%) were PD-L1-positive, and 19 (42.2%) of 45 AR- patients were PD-L1-positive. The pooled OR (OR = 2.42, 95% CI 1.31 to 4.50; $P = 0.005$) showed a significant association between PD-L1 expression and androgen receptor status. In other words, the odds of positive PD-L1 expression in AR+ patients were 142% higher than AR- patients, with the true population effect between 31 and 350%. This result was statistically significant.

Significant heterogeneity was detected in the analysis of PD-L1 expression with preoperative PSA levels ($P = 0.07$; $I^2 = 69\%$). As for the remaining analyses of PD-L1 expression with age ($P = 0.73$; $I^2 = 0\%$), Gleason score ($P = 0.14$; $I^2 = 42\%$), pathologic stage ($P = 0.23$; $I^2 = 28\%$), lymph node metastasis ($P = 0.69$; $I^2 = 0\%$) and androgen receptor status ($P = 0.55$; $I^2 = 0\%$), there was no evidence of substantial heterogeneity. The number of our included studies is small, hence we performed fixed-effect models for all statistical analyses.

DISCUSSION

PD-1/PD-L1 antibodies were approved by the US-FDA for multiple tumor types, including melanoma, non-small cell lung cancer, bladder cancer, kidney cancer, etc. (Haffner et al., 2018). However, the therapeutic effect of PD-1/PD-L1 antibodies in prostate cancer remains controversial. The likelihood of antitumor immune response to anti-PD-1 antibody therapy is closely linked to expression of PD-L1 on the tumor cell surface (Brahmer et al., 2010; Pardoll, 2012; Taube et al., 2014). Different tumor types have a wide variety of baseline PD-L1 expression levels (Gatalica et al., 2014; Taube et al., 2014; Haffner et al., 2018). A phase 1 trial (Topalian et al., 2012) assessed the safety and antitumor activity of BMS-936558, a fully human anti-PD-1 monoclonal antibody, in advanced solid tumor patients. Among them, 36% of patients with PD-L1-positive tumors responded to anti-PD-1 antibody, and no objective response was observed in patients with PD-L1-negative tumors, which included PCa patients. Similar results were also found in another phase I study of single-agent anti-PD-1 (MDX-1106) (Brahmer et al., 2010). In our review of several articles, multiple studies had shown that the prevalence of PD-L1 in patients with prostate cancer varied greatly (ranged from 0 to 92%) (Ebelt et al., 2009; Gatalica et al., 2014; Martin et al., 2015; Gevensleben et al., 2016a; Massari et al., 2016; Baas et al., 2017; Calagua et al., 2017; Ness et al., 2017; Haffner et al., 2018; Wang et al., 2018), which may account for the poor efficacy of anti-PD-1/PD-L1 immunotherapy in PCa patients in previous studies. Predictive biomarkers or clinical characteristics are then desperately needed so we can identify patients who will benefit most from anti-PD-1/PD-L1 immunotherapy, and PD-L1 expression has the potential to be a promising predictive biomarker for favorable clinical benefits from therapeutic blockage of PD-1/PD-L1 pathway (Tang and Heng, 2013; Taube et al., 2014).

As far as we know, this present meta-analysis is the first to investigate the clinicopathologic and prognostic significance of PD-L1 expression in prostate cancer. A highly variable frequency of PD-L1 expression has been reported in the included studies measuring the expression of PD-L1 in prostate cancer, which ranged from 7.7 to 82.4% (Ebelt et al., 2009; Gevensleben et al., 2016a; Calagua et al., 2017; Haffner et al., 2018), and the pooled frequency of PD-L1 is 35%. An included study (Gevensleben et al., 2016a) provided the first evidence that the prevalence of PD-L1 expression is very common in primary prostate cancer and is a negative predictor for BCR-free survival. Our pooled results for BCR-FS demonstrated the adverse prognostic value of positive PD-L1 expression and high mPD-L1 in PCa patients. PD-L1 expression could then be considered a risk factor to predict the prognosis of PCa and an effective biomarker to identify the right patient population for anti-PD-1/PD-L1 treatment. There are at least six distinct mechanisms for how PD-L1-expressing cells evade T-cell immunity: inducing (1) apoptosis, (2) anergy or (3) functional exhaustion of T cells, (4) forming a molecular shield to keep lysis off tumor cells, (5) increasing production of the immunosuppressive cytokine IL-10, and (6) facilitating T_{Reg}-cell-mediated suppression (Zou and Chen, 2008). These functions of PD-L1 expression might explain its role in cancer immune escape and the relation between tumor progression and poor prognosis. Function-blocking monoclonal antibodies against PD-1 suppress the above reaction and thus activate antitumor immunity.

The fact that both positive PD-L1 expression and high mPD-L1 were significantly connected with undesirable clinical outcomes seems contradictory because DNA methylation is usually perceived to cause gene silencing and thus leads to a decrease of its expression product. A previous study (Gevensleben et al., 2016b) revealed that there was an inverse correlation between mPD-L1 and mRNA transcription but not between mPD-L1 and protein expression in PCa. This finding indicated the research value of post-transcriptional regulatory mechanisms of PD-L1 protein expression. The differential expression of microRNA (miR), the cellular component which can stabilize or degrade mRNA by binding it, plays a significant role in modifying the downstream processing of PD-L1 mRNA, especially miR-197, miR-200, miR-570, miR-34a, and miR-513 (Chen et al., 2015). The intricate correlation between miR, mRNA and mPD-L1 discovered by Gevensleben et al. may therefore explain the interference in the linear translation of PD-L1 mRNA into PD-L1 protein (Gevensleben et al., 2016b). Meanwhile, more

advanced research is still needed to unravel the complicated interactions between DNA methylation and PD-L1 expression in PCa.

Recent studies demonstrated that PD-L1 overexpression is related to higher clinical activity in patients with various tumor types receiving anti-PD-1/PD-L1 immunotherapy (Meng et al., 2015). In our analyses, we evaluate the correlation between PD-L1 expression and clinicopathologic features of PCa patients. Based on our pooled results, we provided credible evidence that PCa patients with higher Gleason scores or positive androgen receptor were more likely to have higher levels of PD-L1 expression with statistical significance. These patients are more likely to benefit from blocking the PD-1/PD-L1 pathway. However, the correlations between PD-L1 with age, pathologic stage, lymph node metastasis and preoperative PSA level were not statistically significant.

We performed a Pearson's chi-square test between the positive PD-L1 expression of mCRPC and primary PCa via the data extracted from a previous study evaluating PD-L1 expression in primary and metastatic prostate cancer (Haffner et al., 2018) and found that mCRPC had an increased prevalence of PD-L1 expression compared with primary PCa ($P < 0.01$) (**Supplemental Table S1**). This result suggests that patients with mCRPC might obtain more favorable clinical benefit from anti-PD-1/PD-L1 immunotherapy rather than patients with primary PCa. Similar statistical analysis was performed based on the data extracted from a study evaluating the effect of neoadjuvant androgen deprivation therapy with abiraterone acetate plus prednisone and leuprolide (Neo-AAPL) on PD-L1 expression in PCa (Calagua et al., 2017), and the difference of the rates of PD-L1 expression between treated and untreated PCa patients was not statistically significant ($p = 0.062$) (**Supplemental Table S2**) Furthermore, Bishop was the first to put forward that a statistically significantly increase of PD-L1/2$^+$ DCs was observed in Enzalutamide-resistant PCa patients compared to those who were naïve ($P = 0.0037$) or those who responded to treatment ($P = 0.0060$) (Bishop et al., 2015). This finding reminds us that patients with Enzalutamide-resistant PCa are more aggressive via suppressing immune responses and more likely to benefit from anti-PD-1/PD-L1 immunotherapy. In addition, a DNA vaccination encoding prostatic acid phosphatase can result in the upregulation of PD-L1 expression on tumor cells of patients with castration-resistant but non-metastatic PCa, hence it provided an in-human rationale for the combination of DNA vaccines with PD-1 blockade for the treatment of PCa patients, which benefits much from vaccines but little from PD-1 antibodies as monotherapies (Rekoske et al., 2016). This combination therapy is currently being examined in patients with mCRPC (NCT02499835).

There are several strengths in this study. First, to our knowledge, this is the first meta-analysis that provides the clinicopathologic and prognostic significance of PD-L1 expression in PCa. Second, our study provides a scientific rationale and direct support for individualized estimations of prognosis for PCa, identification of more aggressive cancer patients, and clinical application of anti-PD-1/PD-L1 immunotherapy. In this way, patients realize precision medicine and individualized treatment. In addition, the study may prompt researchers to design large-cohort clinical trials to further confirm these findings.

We tried our utmost to perform this meta-analysis but there are some limitations of the study that should be acknowledged. First, the quantity of studies included was not big enough to generate more authentic results due to limited published studies. Therefore, more studies are needed to provide more evidence for the prognostic value of PD-L1 and mPD-L1. Second, only articles published in English were included in this meta-analysis. Third, the cut-off values differentiating negative (low) and positive (high) PD-L1 expression varied in different studies. Fourth, the different antibodies used in the included studies might affect the accuracy of the positive rate of PD-L1 expression and might therefore affect the estimation of the prognostic and clinicopathologic value of PD-L1 expression. Previous studies had shown the influence of different antibodies against PD-L1 on the percentage of PD-L1-stained tumor cells (Hirsch et al., 2017; Haffner et al., 2018). Thus, a large multicenter study implementing the same antibody and cut-off value is expected to provide more precise and credible results.

CONCLUSION

In conclusion, our meta-analysis confirms the fact that PD-L1 expression and mPD-L1 are significant negative independent prognostic factors in patients with prostate cancer. Moreover, PD-L1 overexpression was statistically significantly linked to high Gleason scores and positive androgen receptor of PCa, while it was also associated with age, pathologic stage, lymph node metastasis and preoperative PSA level but with no statistical significance. This result may guide clinicians in estimating the prognosis of patients individually, identifying patients with poor prognosis, and selecting suitable patients that will obtain favorable clinical benefit to receive anti-PD-1/PD-L1 immunotherapy. This study is expected to attract more practitioners to design retrospective large-cohort studies for the further verification of these findings.

AUTHOR CONTRIBUTIONS

YL, QH, YG, and XW: Conception and design; YZ and QH: Collection and assembly of data; YZ and QH: Statistical analysis and interpretation; QH and YL: Manuscript writing; YG and XW: Manuscript revising; All authors: final approval of manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01494/full#supplementary-material

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The Prognostic and Clinicopathological Roles of PD-L1 Expression in Colorectal Cancer: A Systematic Review and Meta-Analysis

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Background: Studies evaluating the prognostic significance of programmed death-ligand 1 (PD-L1) expression in colorectal cancer (CRC) are limited and remain controversial. This meta-analysis was conducted in order to evaluate the clinicopathological and prognostic significance of PD-L1 expression in CRC patients.

Methods: A comprehensive search was performed against the Medline/PubMed, Embase, Cochrane Library, Web of Science (WoS) and Scopus databases. Data were extracted with name of the first author, year of publication, country of origin, tumor type, number of cases, staining method, cut-off values, PD-L1 positive expression, clinicopathological parameters, outcome, and quality assessment score, and statistical analysis was conducted using Review Manager Version 5.3 (Revman the Cochrane Collaboration; Oxford, England) and STATA version 14 (Stata Corporation; College Station, TX, USA).

Results: Ten studies were included in this meta-analysis, in which the pooled hazard ratio (HR) showed that PD-L1 expression in tumor cells was significantly associated with a poor overall survival (HR = 1.50, 95% CI 1.05–2.13, $P = 0.03$). The pooled HR for disease-free survival (DFS) indicated that PD-L1 expression was significantly associated with shorter DFS (HR = 2.57, 95% CI 1.40–4.75, $P = 0.002$). The pooled odds ratios (ORs) showed that PD-L1 expression was associated with poor differentiation (OR = 3.47, 95% CI 1.37–8.77, $P = 0.008$) and right colon cancer (OR = 2.38, 95% CI 1.57–3.60, $P < 0.0001$). However, the expression of PD-L1 was independent of gender, age, tumor size, tumor stage, lymph node metastasis, and tumor-node metastasis stage.

Conclusion: This meta-analysis indicated that a high level of PD-L1 expression might be a biomarker for a poor prognosis in CRC patients. This information may be helpful for clinicians to stratify CRC patients for anti-PD-1/PD-L1 therapy, particularly patients with microsatellite instability high (MSI-H).

Keywords: colorectal cancer, PD-L1/ PD-1, prognostic, clinicopathological, meta-analysis

INTRODUCTION

Globally, colorectal cancer (CRC) is the third leading cause of cancer (Siegel et al., 2017). Although cancer screening programs and the standardization of preoperative and postoperative care have reduced mortality associated with a CRC diagnosis (Welch and Robertson, 2016), CRC is still a leading cause of cancer-related deaths worldwide, for it has a poor prognosis in its malignant stages and recurrence is common. Therefore, it is essential to identify new biomarkers to improve clinical decision-making and patient outcomes.

As one of the most possible newly biomarkers to evaluate cancer patients' outcomes, programmed death 1 (PD-1) is an immune-inhibitory receptor that is expressed on the surface of activated T cells as a result of persistent inflammatory stimuli (Inaguma et al., 2016; Zou et al., 2016). PD-L1 is expressed by T and B cells, macrophages and dendritic cells and its expression implies a weakened host immune response and consequent a poor prognosis (Hansen et al., 2009). The binding of PD-L1 to PD-1 can attenuate the cellular immune response by reducing T cells apoptosis or exhaustion. Blockade of the PD-1/PD-L1 pathway with monoclonal antibodies is a highly promising therapy and prominent clinical benefits of this checkpoint-blockade were observed in recent clinical trials (Zheng and Zhou, 2015; Wang et al., 2018).

Positive PD-L1 expression has been associated with significantly poor prognoses; however, studies evaluating the prognostic significance of PD-L1 expression in CRC are limited and remain controversial. Therefore, we conducted a comprehensive meta-analysis to evaluate the clinicopathological and prognostic significance of PD-L1 expression in CRC patients.

MATERIALS AND METHODS

Literature Search

Two authors (M. Z. He and Y. Y. Zhou) independently conducted comprehensive literature searches of published articles using the Medline/PubMed, Embase, Cochrane Library, WoS and Scopus databases. The endpoint for search items was July 21, 2018. The following keywords were used: ("colorectal" OR "colorectum" OR "colon" OR "Rectum" OR "Rectal" OR "large intestine") AND ("adenocarcinoma?" OR "tumor?" OR "neoplasm?" OR "carcinoma?" OR "cancer?" OR "malignant") AND ("Programmed Cell Death 1 Receptor" OR "CD279 Antigen" OR "PD-1" OR "B7-H1 Antigen" OR "Programmed Cell Death 1 Ligand 1" OR "PD-L1 "OR "CD 274"). Titles and abstracts were screened through NoteExpress and any discrepancies were resolved by mutual discussion.

Abbreviations: PD-L1, programmed death-ligand 1; CRC, colorectal cancer; WoS, wet of science; HR, hazard ratio; TCs, tumor cells; OS, overall survival; DFS, disease-free survival; ORs, odds ratios; T stage, tumor stage; TNM, tumor-node-metastasis; MSI-H, microsatellite instability high; PD-1, programmed death 1; IHC, immunohistochemistry; CIs, confidence intervals; NOS, newcastle-ottawa quality assessment; IRS, immunoreactivity score; TILs, tumor-infiltrating lymphocytes; CTLs, CD8+ cytotoxic T lymphocytes; CTLA4, CTL-associated antigen 4; IDO1, indoleamine 2,3-dioxygenase 1; TIME, tumor immunity in the microEnvironment; mPD-L1, PD-L1 promoter methylation.

Eligibility Criteria

The criteria for inclusion were: (1) All patients were histologically confirmed as having CRC and had not received adjuvant chemotherapy before surgery; (2) PD-L1 expression was detected by immunohistochemistry (IHC); (3) Studies showed a correlation between PD-L1 expression with clinicopathological features and/ or prognoses; (4) Articles were published as a full paper in English. The criteria for exclusion were: (1) Case reports, reviews and letters; (2) The main content did not evaluate the relationship of PD-L1 expression with clinicopathological features and/ or prognoses; (3) duplications and studies without eligible data. When duplicate publications were identified, only the article with the newest and most comprehensive information was included.

Data Extraction and Quality Assessment

The following information from the included articles was extracted by two reviewers (M. Z. He and Y. Y. Zhou): name of the first author, year of publication, country of origin, tumor type, number of cases, staining method, cut-off values, PD-L1 positive expression, clinicopathological parameters, outcome, and quality assessment score. Any disagreements between the two reviewers were resolved by consensus involving a third reviewer (Y. Li). Outcome parameters comprised OS, DFS and recurrence-free survival (RFS). The HRs and 95% confidence intervals (CIs) were evaluated for outcome parameters. If the HRs were not available, we extracted data from survival curves or contacted the corresponding authors.

According to the Newcastle-Ottawa Quality Assessment (NOS), a quality assessment was independently carried out for the included articles by two authors (M.Z. He and Y. Y. Zhou). Discrepancies in scoring were resolved by discussion and consensus. The NOS consists of the following three parameters of quality: selection, comparability and outcome. The maximum NOS score is nine points, with studies scoring greater than six considered to be of high quality (Stang, 2010).

Statistical Methods

Pooled HRs and 95% CIs were calculated to evaluate the association between PD-L1 positive expression with OS, DFS, RFS and clinicopathological parameters. Heterogeneity among studies was evaluated using the Chi-squared test and I^2. A random-effects model was used when there was evidence of significant heterogeneity ($I^2 > 50\%$ or P-value <0.1). In all other cases, a fixed-effects model was used. Potential publication bias was assessed through Egger's and Begg's tests. The statistical analysis was conducted using Review Manager Version 5.3 (Revman the Cochrane Collaboration; Oxford, England) and STATA version 14 (Stata Corporation; College Station, TX, USA). All P-values and 95% CIs were two-sided, and P-values< 0.05 were considered to be statistically significant.

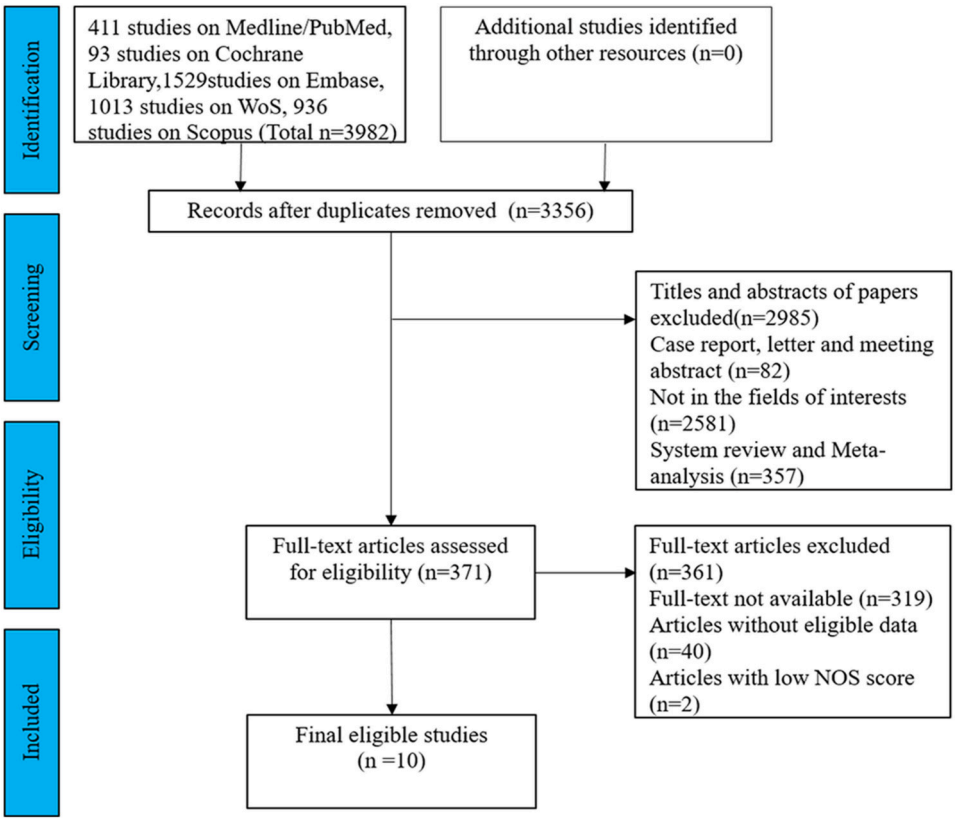

FIGURE 1 | Flow diagram of the study selection process in this meta-analysis.

RESULTS

Search Results and Study Characteristics

After exclusion of 626 duplicates, 3,356 articles about PD-1/PD-L1 in colorectal cancer were identified from a primary system literature search in the Medline/PubMed, Embase, Cochrane Library, WoS, and Scopus databases. The titles and abstracts of the remaining articles were screened, and 2,985 records were rejected because they were case reports, letters, meeting, reviews or not in the fields of interests. We read 371 records for further assessment. Among them, 319 full-text articles were not available, another 40 lacked eligible data, and two scored lower than 6 on the NOS. Finally, 10 articles were included in this meta-analysis. A flowchart of the literature selection is shown in **Figure 1.**

The characteristics of the 10 included studies are listed in **Table 1**. These included studies were generally of high quality, with NOS scores ranging from six to eight. All 10 studies were retrospective and published between 2013 and 2018. In total, 10 studies comprising 2,131 patients were included in the pooled analysis and all selected studies used IHC assays to evaluate PD-L1 expression in tumor cells and /or TILs. Each article had an independent cut-off value used to define the criterion for PD-L1 positive. Six studies provided OS data (Shi et al., 2013; Zhu et al., 2015; Li et al., 2016; Enkhbat et al., 2018; Lee S. J. et al., 2018; Liu et al., 2018), three studies included DFS data (Enkhbat et al., 2018; Lee K. S. et al., 2018; Lee S. J. et al., 2018) and three studies included RFS data (Lee et al., 2016; Wang et al., 2016, 2017). In addition, HRs and 95% CIs were abstracted directly from the 10 included studies.

Association Between PD-L1 Expression and Prognostic Parameters

We evaluated the association between PD-L1 expression and prognostic parameters (OS, DFS and RFS). The pooled HR for OS in TC from six studies, involving 1,131 patients, showed that PD-L1 expression was significantly associated with poor OS in CRC (HR = 1.50, 95%CI 1.05–2.13, $P = 0.03$; see **Figure 2A**). When we took Immunoreactivity score (IRS) ≥ 4 as the cut-off value, we found shorter survival in the PD-L1 positive group (HR = 2.65, 95%CI 1.44–4.86, $P = 0.002$; see **Figure 2B**). The pooled HR for DFS in TC with 452 patients indicated that PD-L1 expression was significantly associated with shorter DFS (HR = 2.57, 95%CI 1.40–4.75, $P = 0.002$; see **Figure 2C**). The pooled HR for RFS in TC with 657 patients (HR = 2.38, 95%CI 1.14–4.96, $P = 0.02$; see **Figure 2D**) as well as the pooled HR for RFS in tumor-infiltrating lymphocytes (TILs) with 516 CRC patients (HR = 1.79, 95%CI

TABLE 1 | Main characteristics of the studies included for meta-analysis.

References	Country	No.	Tumor Histology	Stage	Technique	Cut-off	PD-L1(+) (%)	Outcome	HR estimation (95% CI)	Quality assessment (score)
Shi et al., 2013	China	143	CRC	I-IV	IHC	Moderate or intense staining	TC: 64/143(44.8)	OS	TC: 2.77(1.05–2.99)	6
Zhu et al., 2015	China	120	SAC	NA	IHC	IRS≥ 4	TC: 28/120(23.3)	OS	TC: 2.30(1.13–4.68)	7
Lee et al., 2016	USA	395	CRC	I-IV	IHC+TMA	≥10% and intense staining	TC: 19/394(4.8)	RFS	TC: 22.86(1.99–263.21)	6
Wang et al., 2016	Switzerland	262	CRC	II-III	IHC+TMA	≥5%	TILs: 54/262(20.6)	RFS	TC: 1.90(0.88–4.14) TILs: 1.83(1.09–3.05)	7
Wang et al., 2017	China	254	CRC	II-III	IHC+TMA	NA	TILs: 89/254(35.0)	RFS	TILs: 1.74(1.02–2.98)	6
Li et al., 2016	China	356 (TCGA)	CRC	NA	IHC+TMA	IRS> 4	TC: 301/356(84.6)	OS	TC: 0.63 (0.33–1.18)	8
Enkhbat et al., 2018	Japan	116	CRC	II-III	IHC	IRS≥ 4	TC: 52/116(44.8)	OS DFS	OS: TC:3.87(1.19–12.57) DFS: TC:1.91(0.81–4.52)	6
Liu et al., 2018	China	60	mCRC	NA	IHC	IS≥ 3	TC: 26/60(43.3)	OS	TC:0.28(0.08–0.99)	6
Lee K. S. et al., 2018	South Korea	89	CC(MSI)	I-III	IHC	≥5%	TILs: 56/89(62.9)	DFS	TILs:0.33(0.11–0.80)	6
Lee S. J. et al., 2018	South Korea	336	CRC	0-IV	IHC+TMA	≥1%	TC: 15/336(9.4)	OS DFS	OS: TC:3.78(1.45–9.90) DFS: TC:3.50(1.46–8.41)	7

CRC, colorectal cancer; SAC, serrated adenocarcinoma; mCRC, metastatic colorectal cancer; CC, colon cancer; MSI, microsatellite instability; IHC, immunohistochemistry; NA, not available; TMA, tissue microarray; OS, overall survival; HR, hazard ratio; TC, tumor cell; TILs, tumor-infiltrating lymphocytes; IRS, Immunoreactivity score; IS, Immunoscore; DFS, disease-free survival; RFS, recurrence-free survival.

1.23–2.95, $P = 0.002$; see **Figure 2E**) showed that PD-L1 expression was significantly associated with poor RFS both in TC and TILs.

Association Between PD-L1 Expression and Clinicopathological Characteristics

Gender

The association between PD-L1 expression and gender was evaluated in eight studies, comprising 3,477 patients. 320(31.37%) of 1,020 male patients and 241(31.42%%) of 767 female patients were PD-L1 expression positive. The pooled OR showed that there was no significant association found between PD-L1 expression and gender (OR = 1.00, 95%CI 0.76–1.31, $P = 0.98$; see **Figure 3A**).

Age

We evaluated the association between PD-L1 expression and age in a total of 405 patients from two studies. 49 (26.78%) of 183 younger patients (<60 years of age) were PD-L1 expression positive and 69 (31.08%) of 222 older patients (≥60 years of age) were PD-L1 expression positive. There was no significant association found between PD-L1 expression and age (OR = 1.41, 95% CI 0.90–2.23, $P = 0.13$; see **Figure 3B**).

Cancer Location

The association between PD-L1 expression and cancer location was analyzed in six studies with a population of 1,025 patients. Of 344 right colon cancer patients, 65 (18.90%) were PD-L1 expression positive, while 77(11.31%) in 681 left colon/rectum cancer patients. The pooled OR showed a significant association between PD-L1 expression and cancer location (OR = 2.38, 95% CI 1.57–3.60, $P < 0.0001$; see **Figure 3C**).

Differentiation

Of 1,066 well/moderately differentiated tumors, 159 (14.92%) were PD-L1 expression positive. Of 154 poorly differentiated tumors, 49 (34.82%) were PD-L1 expression positive. The pooled OR showed that PD-L1 expression was significantly associated with differentiation based on pooled data from five studies (OR = 3.47, 95%CI 1.37–8.77, $P = 0.008$; see **Figure 3D**).

Tumor Size

Only two studies, including 382 colorectal cancer patients, analyzed the subgroup of tumor size based on the cut-off value of 5 cm. 36 (25.17%) of 143 patients with large tumors (≥5 cm) and 48 (20.01%) of 239 patients with small tumors (<5 cm) were PD-L1 expression positive. The pooled results carried out in a fixed effect model, showed that there was no significant association

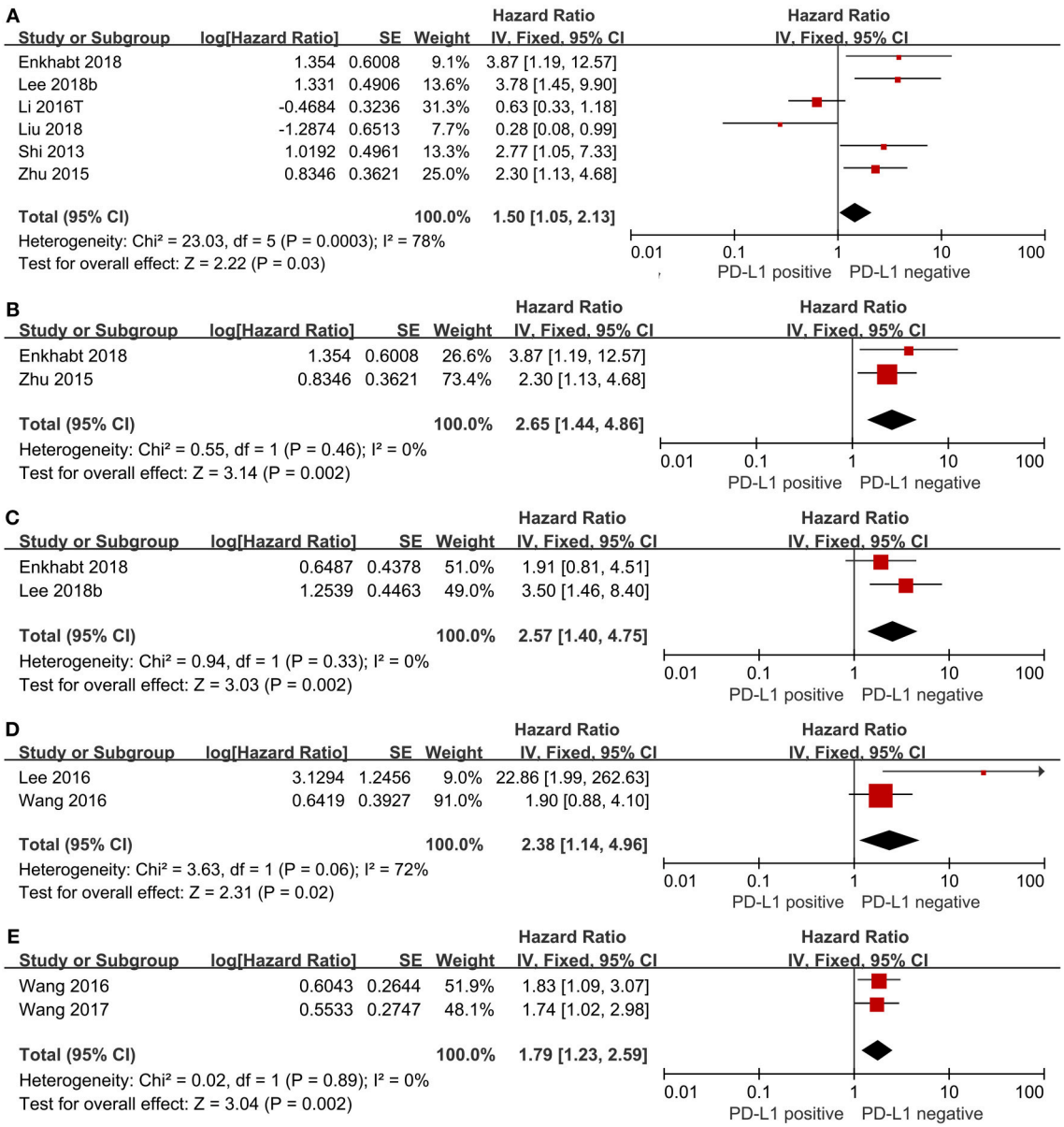

FIGURE 2 | Forest plot of 10 studies evaluating the association between PD-L1 expression and prognostic parameters in CRC patients (**A**: OS in TC; **B**: IRS≥4 as cut-off value; **C**: DFS in TC; **D**: RFS in TC; **E**: RFS in TILs).

between PD-L1 expression and tumor size (OR = 1.31, 95%CI 0.80–2.14, $P = 0.29$; see **Figure 3E**).

T Stage

We evaluated the association between PD-L1 expression and T stage in 1,716 patients. Of 283 Tis-T2 stage patients, 82 (28.98%) were PD-L1 expression positive and 454 (31.68%) of 1,433 T3-T4 stage patients were PD-L1 expression positive. The pooled HR showed that there was no significant association between PD-L1 expression and T stage (OR = 1.02, 95%CI 0.68–1.54, $P = 0.93$; see **Figure 3F**).

Lymph Node Metastasis

The association between PD-L1 expression and lymph node metastasis was evaluated in six studies (1,589 patients). The pooled OR indicated that there was no significant association found between PD-L1 expression and lymph node metastasis (OR = 1.23, 95%CI 0.71–2.12, $P = 0.46$; see **Figure 3G**).

TNM Stage

Six studies, involving 1,329 patients, evaluated the association between PD-L1 expression and TNM stage in a fixed effects model. 138 (21.26%) of 649 stage I-II patients and 122 (17.94%) of 680 stage III-IV patients were PD-L1 expression positive. The

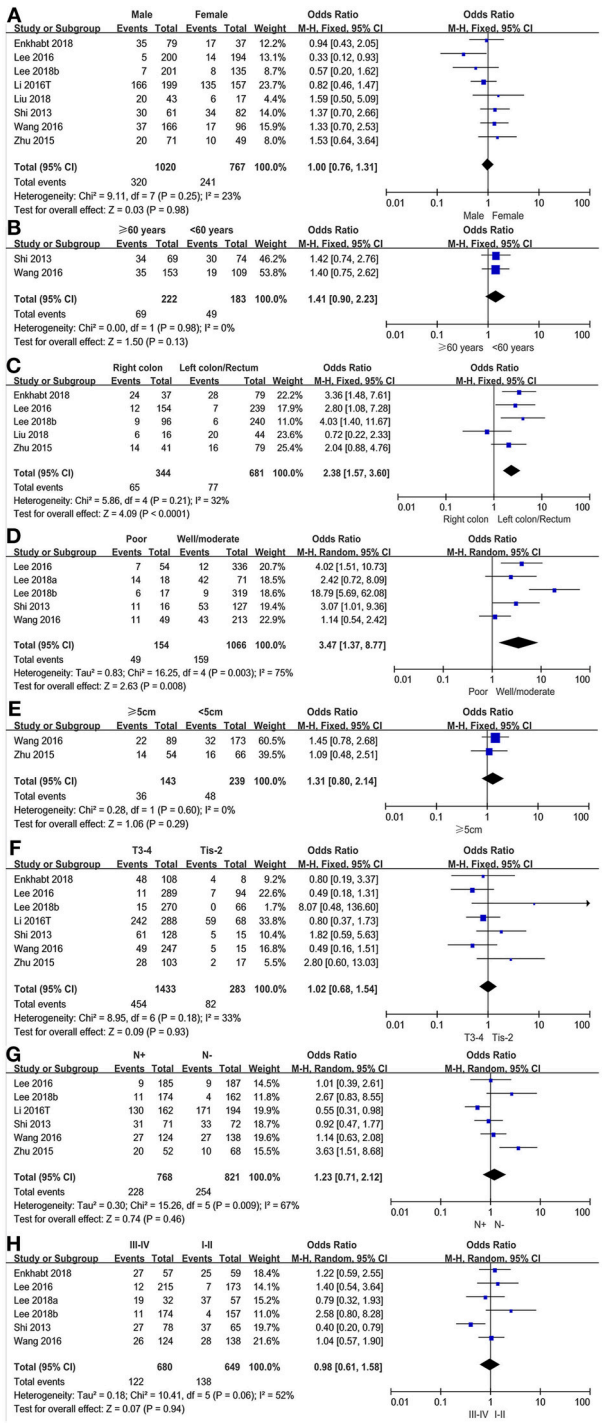

FIGURE 3 | Forest plots for the association of PD-L1 expression with clinicopathological features in CRC patients (**A**: gender; **B**: age; **C**: cancer location; **D**: differentiation; **E**: tumor size; **F**: T stage; **G**: lymph node metastasis; **H**: TNM stage).

pooled result showed no significant association found between PD-L1 expression and TNM stage (OR = 0.98, 95%CI 0.61–1.58, $P = 0.94$; see **Figure 3H**).

Heterogeneity was identified in the analysis of PD-L1 expression with cancer location ($P = 0.73$, $I^2 = 82\%$) and lymph node metastasis ($P = 0.46$, $I^2 = 67\%$). Therefore, a random effects model was used in the above analyses and other subgroup analyses were performed in a fixed effects model.

Publication Bias

Egger's and Begg's tests showed that no publication bias influencing the HRs for OS was observed in the six studies (**Figure 4**). The *P*-values for these tests were 0.683 and 1.000, respectively. In addition, the funnel plots showed no publication bias for gender or T stage (**Figure 5**).

DISCUSSION

In the present meta-analysis of the clinicopathological and prognostic significance PD-L1 expression in CRC, we found that PD-L1 expression was significantly associated with poor OS in TC. In addition, the pooled results of RFS and DFS showed that PD-L1 expression was significantly correlated with unfavorable clinical outcomes. Poor differentiation and right colon CRC tumors suggested a poor prognosis. The expression of PD-L1 was independent of gender, age, tumor size, T stage, lymph node metastasis, and TNM stage. To our knowledge, this comprehensive meta-analysis is the first to evaluate the association of PD-L1 expression with clinicopathological characteristics and prognostic parameters in colorectal cancer.

During the process of study of selection, the study of Droeser et al. (2013) was excluded for it included unselected, non-consecutive, primary, sporadic colorectal cancers, and the data of the included articles in this meta-analysis were satisfied with a more rigorous standards, which excluded the patients receiving adjuvant chemotherapy before surgery, diagnosis of gastrointestinal stromal tumor or lymphoma, diagnosis with additional cancers. It is well-known that accurate results were based on the rigorous exclusion criteria in retrospective study. Among the OS data in six included studies, one study showed contradictory results showing that PD-L1 positive expression was significantly associated with better OS. This study was not the only one to report a positive prognostic impact of PD-L1 expression. Sabatier et al. (Schalper et al., 2014) evaluated PD-L1 expression in 5,454 breast cancer cases and found that positive PD-L1 expression was associated with better metastasis-free survival and improved response to chemotherapy. However, the pooled result showed a significant correlation of PD-L1 expression and poor prognostic outcomes was supported by other articles reporting poorer outcomes in renal cell carcinoma, non-small cell lung cancer (Wang et al., 2015) and osteosarcoma (Lussier et al., 2015). This was because of the complex function of PD-L1 in the initiation and growth of CRC.

PD-L1 is upregulated by many inflammatory mediators and cytokines (Keir et al., 2006, 2008; Okazaki and Honjo, 2006) and PD1/PD-L1 binding can induce activated T cell apoptosis, exhaustion, and interleukin-10(IL-10) expression as a negative feedback system (Zou et al., 2016). Moreover, PD-L1 expression can help tumor cells to evade immunosurveillance and enhance the function of Tregs in CRC (Lu et al., 2011; Toh et al., 2016).

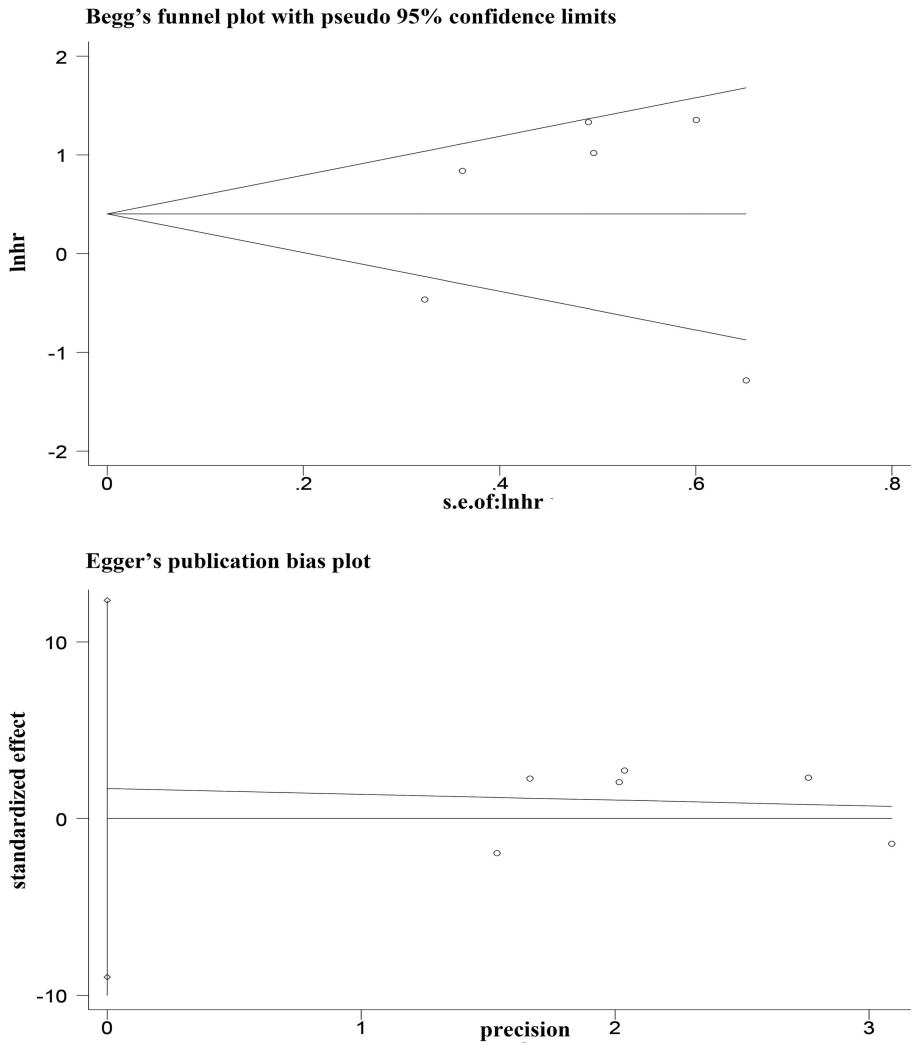

FIGURE 4 | Egger's and Begg's funnel plot with 95% CI for OS publication bias in the included six studies.

However, MSI tumors in CRC display high infiltration with CD8+ cytotoxic T lymphocytes (CTLs) and activated Th1 cells, which may contribute to better survival (Gubin et al., 2014). MSI tumors are also counterbalanced by upregulating expression of multiple immune checkpoints (Angelova et al., 2015; Becht et al., 2016), such as CTL-associated antigen 4 (CTLA4), PD-1, PD-L1 and indoleamine 2,3-dioxygenase 1 (IDO1). Upregulated after T cell activation, PD-1 declines when an antigen is cleared. While PD-1 expression remains elevated, as in CRC cancer, T cells enter a state of exhaustion or anergy (Xiao and Freeman, 2015). A study found that *Fusobacterium* species could evade the high load of neoantigens in MSI colorectal cancer (Tahara et al., 2014). And these species may facilitate upregulation of PD-L1 and lead to poor survival (Kostic et al., 2013). Considering the dynamic changes of PD-L1 expression, our results showing that PD-L1 expression was significantly associated with poor prognoses appear more credible.

We also noticed a recently literature make a contradictory conclusion with our study. This study considered that no significant differences founded in colorectal cancer-specific or overall survival by Tumor Immunity in the MicroEnvironment (TIME) subtypes (Hamada et al., 2018). We found that the primary data of their study were too old, as one cohort was from 1986 to 1992 and the other was from 1986 to 2004 (Giovannucci et al., 1995; Wark et al., 2009). While, our primary data were carried out from 2006 to 2016. The discrepancies between Hamada et al. (2018) and our study might reflect the different storage time of tissue sections. Reports by Bertheau et al. (1998) and Jacobs et al. (1996), who investigated the loss of immunoreactivity for a panel of antibodies in breast carcinomas, neuroendocrine tumors and lymphomas, indicated that for the majority of epitopes tested there is a time-dependent substantial loss in stored tissue slides. CRC develops via sequential genetic and epigenetic alterations of TCs, and is influenced by tumor-host interactions. Because CRC patients easily developed local

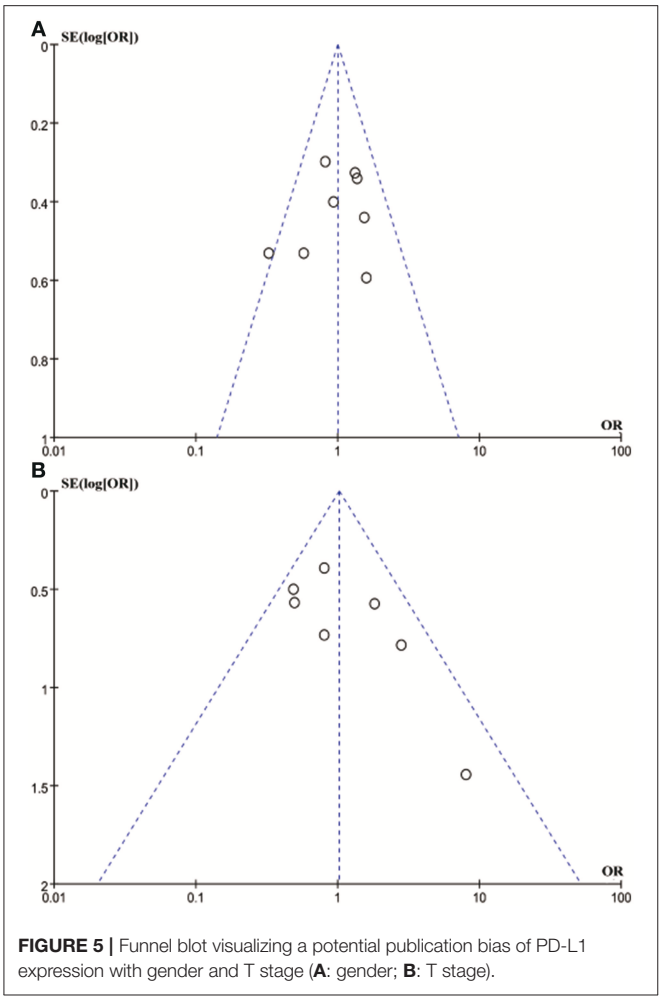

FIGURE 5 | Funnel blot visualizing a potential publication bias of PD-L1 expression with gender and T stage (**A**: gender; **B**: T stage).

recurrences and distant metastases within 5 years after surgical treatment and CRC has typical immune subgroups (Dienstmann et al., 2017), researchers found that immunotherapy is able to reach center stage in the field of second-line therapy in oncology treatment (Topalian et al., 2012; Hon et al., 2018). As one of the types of CRC, high microsatellite instability (MSI-H) can gather TILs and upregulate PD-L1 expression in tumor cells (Herbst et al., 2014).Currently, PD-L1 expression on TCs is considered as an immune-tolerance mechanism of carcinoma, because it can attract PD-1 expressing immune-inhibitory TILs. However, little is known about the complex interrelationship among PD-L1 expression, TILs, and major tumor molecular features. PD-L1 promoter methylation (mPD-L1) was significantly correlated with poor PD-L1 mRNA expression, indicating that PD-L1 expression might be regulated by mPD-L1 on a cellular level in CRC (Goltz et al., 2016). However, this study was not available to provide data on PD-L1 protein expression and there was a study had published a proteomic characterization of the cohort, showing that protein abundance could not be reliably predicted from DNA- or RNA-level measurements (Zhang et al., 2014). Previous studies have shown a significant correlation of PD-L1 expression with OS in melanoma (Robert et al., 2015), breast cancer (Zhang et al., 2017), renal cell carcinoma (Motzer et al., 2014), and non-small cell lung cancer (Zhang et al., 2015), and observed prominent clinical benefits of PD-1/PD-L1 checkpoint blockades in these carcinoma patients. Although previous trials have suggested no role for immunotherapy in patients with CRC, recent studies have demonstrated that MSI-H in CRC did benefit (Kwak et al., 2016; Overman et al., 2017). Therefore, we investigated the relationship between the expression of PD-L1 and clinicopathological factors, and the results showed that poor differentiation and right colon location in CRC were PD-L1 expression positive. In addition, poor differentiation and right colon location in CRC were also significantly correlated with poor prognoses, which were more likely to be MSI-H. Thus, our study provided a scientific rationale and direct support for clinicians to select MSI-H CRC patients for anti-PD-1/PD-L1 immunotherapy.

This study provided moderate evidence to evaluate the association of PD-L1 expression with prognostic outcomes and clinicopathological factors. However, there were some limitations. Firstly, only six included studies evaluated the association of PD-L1 expression with OS. Although the sample sizes of RFS and DFS were relatively small, their results should have alleviated some of these concerns. Secondly, the cut-off values determining positive and negative PD-L1 expression and antibodies for PD-L1 varied among the included studies. Thus, the subgroup of IRS $\geq$ 4 had reduced heterogeneity and addressed some of these concerns. Thirdly, only articles published in English were included. Accordingly, to address these limitations, a large multicenter study with uniform evaluation methods (the same antibody and cut-off for positive PD-L1 expression) may be helpful to attain results that are more accurate. Despite the above limitations, the present meta-analysis demonstrated the association of PD-L1 expression with prognostic outcomes and clinicopathological factors. The findings of this study may lead to improvements in the outcomes of anti-PD-1/PD-L1 therapy through stratifying patients in a more appropriate manner.

CONCLUSION

In conclusion, our results showed that PD-L1 positive expression might be a new biomarker for poor prognosis in CRC. This information may be helpful for clinicians to stratify CRC patients for anti-PD-1/PD-L1 therapy, especially patients with MSI-H. Well-designed and high-quality studies with uniform evaluation methods are needed to confirm the association of PD-L1 expression in CRC.

AUTHOR CONTRIBUTIONS

YL, MH, and LX designed this study. YZ and MH screened identified studies and extracted data. Disagreements were resolved by discussion with YL and YZ performed the statistical analyses. MH and YZ prepared the figures and tables. YL, MH, and LX reviewed the results, interpreted the data, and wrote the manuscript. All authors have read and approved the final version of this manuscript.

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Abscopal Effects in Radio-Immunotherapy—Response Analysis of Metastatic Cancer Patients With Progressive Disease Under Anti-PD-1 Immune Checkpoint Inhibition

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Immune checkpoint inhibition (ICI) targeting the programmed death receptor 1 (PD-1) has shown promising results in the fight against cancer. Systemic anti-tumor reactions due to radiation therapy (RT) can lead to regression of non-irradiated lesions (NiLs), termed "abscopal effect" (AbE). Combination of both treatments can enhance this effect. The aim of this study was to evaluate AbEs during anti-PD-1 therapy and irradiation. We screened 168 patients receiving pembrolizumab or nivolumab at our center. Inclusion criteria were start of RT within 1 month after the first or last application of pembrolizumab (2 mg/kg every 3 weeks) or nivolumab (3 mg/kg every 2 weeks) and at least one metastasis outside the irradiation field. We estimated the total dose during ICI for each patient using the linear quadratic (LQ) model expressed as 2 Gy equivalent dose (EQD2) using α/β of 10 Gy. Radiological images were required showing progression or no change in NiLs before and regression after completion of RT(s). Images must have been acquired at least 4 weeks after the onset of ICI or RT. The surface areas of the longest diameters of the short- and long-axes of NiLs were measured. One hundred twenty-six out of 168 (75%) patients received ICI and RT. Fifty-three percent (67/126) were treated simultaneously, and 24 of these (36%) were eligible for lesion analysis. AbE was observed in 29% (7/24). One to six lesions (mean = 3 ± 2) in each AbE patient were analyzed. Patients were diagnosed with malignant melanoma (MM) ($n = 3$), non-small cell lung cancer (NSCLC) ($n = 3$), and renal cell carcinoma (RCC) ($n = 1$). They were irradiated once ($n = 1$), twice ($n = 2$), or three times ($n = 4$) with an average total EQD2 of 120.0 ± 37.7 Gy. Eighty-two percent of RTs of AbE patients were applied with high single doses. MM patients received

pembrolizumab, NSCLC, and RCC patients received nivolumab for an average duration of 45 ± 35 weeks. We demonstrate that 29% of the analyzed patients showed AbE. Strict inclusion criteria were applied to distinguish the effects of AbE from the systemic effect of ICI. Our data suggest the clinical existence of systemic effects of irradiation under ICI and could contribute to the development of a broader range of cancer treatments.

Keywords: abscopal effect, PD-1, radio-immunotherapy, radiotherapy, combination treatment, advanced cancer disease, immune checkpoint inhibition

INTRODUCTION

In addition to radiation therapy (RT), chemotherapy (CTX), and surgery, immunotherapy (IT) has been established as a fourth pillar of cancer treatment. Different treatment regimens and combination concepts are being evaluated and used in order to optimize treatment outcome of various tumor diseases.

RT is used for local treatment of malignant diseases. More than 50% of all patients with solid tumors are treated with RT only or in a combined treatment setting. The interaction of RT and patient's immune system has gained particular interest after the encouraging success of immune checkpoint inhibitors (ICIs) targeting the programmed death receptor 1 (PD-1) (Garon et al., 2015; Haanen and Robert, 2015; Robert et al., 2015; Ferris et al., 2016; Sharma et al., 2016; Younes et al., 2016; Long et al., 2017; Ok and Young, 2017). PD-1 checkpoint inhibitors act by suppression of an inhibitory T-cell pathway, namely the PD-1/PD-L1 axis. In metastatic malignant melanoma (MM), anti-PD1 therapy has been proven as superior treatment to chemotherapy as first-line therapy and after ipilimumab (anti-CTLA-4 antibody) failure (Ribas et al., 2015; Weber et al., 2015) and in non-small cell lung cancer (NSCLC) patients after progression to first-line chemotherapy (Vokes et al., 2018). Despite all advancements, not all patients benefit from treatment with ICIs, and different systemic therapies are less effective if the tumor does not contain a mutation that can be targeted. Looking for further treatment strategies, the combination of local irradiation, and ICIs led to promising results even beyond local tumor control (Kang et al., 2016; Salama et al., 2016). The mechanisms by which RT and IT synergistically modulate the immune response might also affect treatment-related side effects. Evidence shows that simultaneous administration of RT and ICIs as radio-immunotherapy (RIT) is considered safe and that the number of adverse events does not increase significantly (Bang et al., 2017; Hwang et al., 2018; Trommer-Nestler et al., 2018). The first report on an immune-mediated response to radiation therapy and the definition of the term "abscopal" in this context was published in 1953 describing the effects of ionizing radiation "at a distance from the irradiated volume but within the same organism" (Mole, 1953). The so-called abscopal effect (AbE) describes the regression of lesions or tumor or metastatic regions outside the radiation field induced by radiation.

Over time, there have been some reports of clinically observed AbEs, most commonly in highly immunogenic tumor entities (Abuodeh et al., 2016). The underlying mechanism of the AbE is still unclear. Most likely it is mediated by the activation of the immune system (Demaria et al., 2004) and is dependent on RT-induced cell damage leading to the release of cell fragments, neoantigens, cellular danger-associated molecular patterns (DAMPs), and cytokines (Formenti and Demaria, 2013). One way to improve the probability of the occurrence of AbEs through RT is to modulate the tumor microenvironment. This could be achieved by changing the radiation dose, fractionation, site of irradiation and timing, or by combined RT with other systemic therapies. The interactions of RT and IT might be able to immunize the patient against the tumor, acting like a type of "tumor vaccine" leading to a decrease of both tumor and metastases (Demaria and Formenti, 2009; Frey et al., 2012; Formenti and Demaria, 2013; Sharabi et al., 2015).

Currently, more and more case reports on the AbE are being published (Grimaldi et al., 2014; Chandra et al., 2015; Ribeiro Gomes et al., 2016). The incidence of AbEs is still rare and the radiation characteristics like fractionation, timing, fraction scheme, and total dose required for its occurrence remains unclear up until today. The actual occurrence of the AbE has not been well-evaluated in clinical studies so far. This retrospective single center study was conducted to evaluate AbEs in metastasized cancer patients treated with irradiation and simultaneous PD-1 inhibition with pembrolizumab or nivolumab.

MATERIALS AND METHODS

Out of a database of 168 patients treated with a PD-1 inhibitor between 2013 and 2017 at our center (University Hospital of Cologne) we retrospectively analyzed patients who received pembrolizumab or nivolumab and radiotherapy simultaneously. We included patients with any metastatic oncological disease with at least one not locally treated distant metastatic lesion outside V10% of the prescribed irradiation dose (volume of normal tissue receiving at least 10% dose).

The indication for RT was due to locally progressive disease under ICIs alone requiring symptomatic control. Disease progression was defined according to RECIST (Response Evaluation Criteria in Solid Tumors) version 1.1. Any irradiation concept with respect to fractionation scheme and irradiation dose like conventional radiation therapy (CFX), hypofractionated radiation therapy (HFX), stereotactic body radiation therapy (SBRT) or stereotactic radiosurgery (SRS), and multiple RT sessions during IT were permissible. Since patients could have received more than one RT at different sites and with different

concepts we calculated the total irradiation dose during the IT period for each patient using the linear quadratic (LQ) model expressed as 2 Gy equivalent dose (EQD2) using an α/β value of 10 Gy, which has been assumed for tumors (Fowler, 1989; Stuschke and Pottgen, 2010).

Nivolumab was applied intravenously 3 mg/kg every 2 weeks, pembrolizumab 2 mg/kg every 3 weeks. Patients receiving any other systemic cancer treatment, such as ipilimumab, targeted therapy or chemotherapy during the IT or RT periods were excluded, while patients with previous use of systemic treatment were not excluded. We defined simultaneously applied radio-immunotherapy (RIT) as start of RT within 1 month after the first or last application of ICI.

AbE was defined as regression of lesions outside the irradiation field, more specifically outside the 10% iso-dose of the applied radiation dose. In order to distinguish AbE from the systemic effects of IT alone, radiological images were required to show progression or no change in non-irradiated lesion(s) during PD-1 inhibitor administration prior to RT application. If those lesions showed regression after one or more RTs, this was defined as AbE. Radiological images must have been acquired at least 4 weeks after the onset of ICI or RT for regression of lesions to be considered a reliable treatment effect. Patients and radiological images were regularly discussed in interdisciplinary panels.

All computed tomography (CT), magnetic resonance imaging (MRI), and/or positron emission tomography (PET) images were analyzed to identify lesions within and outside the irradiation field. The longest diameters of both the short-axis and long-axis of all non-irradiated lesions were measured and the resulting surface area was analyzed using the Mint® software (Mint® Medical GmbH, Germany). The surface areas were plotted as a function of time with baseline images, which corresponded to the non-irradiated lesions, as time point 0. The overall lesion area reduction was calculated with respect to the largest lesion area. When applicable, data were reported as mean ± standard deviation.

TABLE 1 | Baseline demographics and treatment characteristics of all included patients.

Characteristic	Value
No. of patients	24
Age, years (range)	64 ± 13 (40–89)
Sex	
Male, *n* (%)	10 (42)
Female, *n* (%)	14 (58)
Primary tumor	
MM, *n* (%)	13 (54)
NSCLC, *n* (%)	7 (29)
RCC, *n* (%)	3 (13)
H&N, *n* (%)	1 (4)
IT	
Pembrolizumab, *n* (%)	13 (54)
Nivolumab, *n* (%)	11 (46)
IT duration, weeks (range)	40 ± 28 (4–115)
RT during IT	
No. of RT (range)	2 ± 1 (1–3)
CFX, *n* (%)	6 (14)
HFX, *n* (%)	25 (60)
SRS, *n* (%)	11 (26)
Analysis	
AbE, *n* (%)	7 (29)
PD, *n* (%)	5 (21)
PR, *n* (%)	5 (21)
MR with IT alone, *n* (%)	4 (17)
Image unreliable, *n* (%)	3 (13)

Unless otherwise indicated, values represent means ± standard deviation. MM, melanoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; RT, radiotherapy; CFX, normofractionated radiotherapy; SRS, stereotactic radiosurgery; HFX, hypofractionated radiotherapy; IT, immunotherapy; AbE, abscopal effect; PD, progressive disease; PR, partial response; MR, mixed response.

RESULTS

Patients and Treatment Characteristics

From our database, 126 out of 168 (75%) patients were found to receive checkpoint inhibition and RT. Of these patients, 53% (67/126) were treated simultaneously, and 24 out of 67 (36%) met the inclusion criteria and were eligible for lesion analysis.

AbE was observed in 29% (7/24) of the cases as lesion shrinkage outside V10%. We analyzed 58% female and 42% male patients with a mean age of 64 ± 13 years. Fifty-four percent were diagnosed with malignant melanoma, 29% with non-small cell lung cancer, and 13 and 4% with renal cell carcinoma (RCC) and head and neck cancer (H&N), respectively. Fifty-four percent of the analyzable patients received pembrolizumab, the mean IT duration was 40 ± 28 weeks. Most of the RT courses (60%) were applied hypofractionally. Three patients were excluded from further analysis due to unreliable radiological images such as missing contrast agent in the CT, pneumonitis or atelectasis of the lung in the target lesion area. Baseline characteristics of all included patients are demonstrated in **Table 1**.

The seven patients (two males and five females) exhibiting AbE had an average age of 61 ± 12 years. Three of them were diagnosed with MM, three with NSCLC, one with RCC. The MM patients received pembrolizumab, the NSCLC, and RCC patients received nivolumab with an average duration of 45 ± 35 weeks. Eighty-two percent of the RT courses were applied with high single doses as HFX (41%) or SRS (41%), and 18% normofractionated. Patients were irradiated for one ($n = 1$), two ($n = 2$), or three ($n = 4$) times with an average total EQD2 of 120.0 ± 37.7 Gy irrespectively of the number of irradiations fields and their localization. Radiotherapy was applied between 1 and 49 days (mean = 16 ± 15 days) with the first RT being performed at 19.5 ± 12.3 weeks after the induction of immunotherapy. In these patients, one to six (mean = 3 ± 2) metastatic lesions were analyzed.

Independent of the number of metastases diagnosed, each patient had only one lesion outside the irradiation field which regressed. Lesions were detected at the lung ($n = 3$), adrenal gland ($n = 1$), axillar lymph node ($n = 1$), mediastinal lymph node ($n = 1$), and at the perirenal region ($n = 1$). The AbE was observed at 20 ± 6, 5 ± 1, and 6 ± 1 weeks after the first

TABLE 2A | Baseline demographics and treatment characteristics of patients showing AbE.

Characteristic	Value
No. of patient	7
Age, years (range)	61 ± 12 (42–77)
Sex	
Male, *n* (%)	2 (29)
Female, *n* (%)	5 (71)
Primary tumor	
MM, *n* (%)	3 (43)
NSCLC, *n* (%)	3 (43)
RCC, *n* (%)	1 (14)
H&N, *n* (%)	0
IT	
Pembrolizumab, *n* (%)	3 (43)
Nivolumab, *n* (%)	4 (57)
IT duration, weeks (range)	45 ± 35 (7–115)
RT during IT	
No. of RT (range)	3 ± 1 (1–3)
CFX, *n* (%)	3 (18)
HFX, *n* (%)	7 (41)
SRS, *n* (%)	7 (41)

Unless otherwise indicated, values represent means ± standard deviation. MM, melanoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; RT, radiotherapy; CFX, normofractionated radiotherapy; SRS, stereotactic radiosurgery; HFX, hypofractionated radiotherapy; IT, immunotherapy.

($n = 2$ patients), second ($n = 3$ patients) or third ($n = 2$ patients) RT, respectively, with an average lesion area reduction of 68.4 ± 23.6%. Baseline demographics of AbE patients are shown in **Table 2A**. A detailed description of treatment characteristics and the corresponding AbE sites are presented in **Table 2B**.

Case Reports

Patient one of Table 2B

In July 1998, patient one was diagnosed with an AJCC stage IIb melanoma located at the left thigh, which has been surgically resected. In May 2017, pembrolizumab was applied at 2 mg/kg for nine cycles for a period of 24 weeks due to progressive disease with metastases in the lung and brain, AJCC stage IV. During this period, the patient received two radiotherapy sessions, with a total EQD2 of 100 Gy on intracerebral lesions one (SRS) and 23 (CFX) weeks after the induction of IT. Of the six measured metastases on the CT scans, one pulmonary metastasis showed an increase in the surface area from 40.1 to 60.8 mm^2 (52%) 10 weeks after the start of IT and 9 weeks after the first RT of cerebral metastases, applied as SRS with a single dose of 20 Gy (**Figure 1**). One week after the second CFX with a total dose of 50 Gy, applied with a single dose of 2 Gy, and 3 weeks after the end of IT, a regression of 37% (38.6 mm^2) was observed, suggesting AbE. In the next CT follow-up 23 weeks later, the lung lesion continued to decrease to a size of 15.3 mm^2, resulting in an overall lesion regression of 75%.

Patient two of Table 2B

Patient two was diagnosed with an AJCC stage III malignant melanoma located at the left knee in June 2014. The melanoma was subsequently surgically removed including the lymph drainage area of the left inguinal region. In November 2015, pembrolizumab was applied at 2 mg/kg for 11 cycles for a total period of 31 weeks due to progressive disease with cerebral metastases, AJCC stage IV. During this period, the patient received two RT sessions with a total EQD2 of 148.5 Gy. The first RT was applied as normofractionated whole brain radiation therapy (WBRT) with a single dose of 2 Gy up to a total dose of 40 Gy 1 week after the induction of IT. The second RT of bone metastases of the left popliteal fossa and lower left leg was applied as HFX with a single dose of 3 Gy up to a total dose of 54 Gy at 27 weeks after the start of IT. During this RT, brain metastases were irradiated with 20 Gy in one fraction (SRS) 29 weeks after IT induction. Our analysis revealed the presence of one non-irradiated metastasis in the left perirenal area with a surface area of 36.2 mm^2 (**Figure 2**). The lesion progressed to 46.6 (28.7%) and 52.7 mm^2 (45.6%) at 10 and 23 weeks after the first application of IT, respectively, and after the first RT. In the subsequent CT scan, which corresponded to 6 weeks after the completion of IT and second RT, the lesion regressed by 67.9% to 16.9 mm^2. Complete lesion remission was observed at 10 weeks.

Patient Four of Table 2B

Patient four was diagnosed with a UICC stage IV non-small cell lung cancer (NSCLC) with metastases of the brain, suprarenal gland and bones in May 2016. The patient received a primary radiation treatment in May 2016, initially at 3 × 3 Gy on the mediastinal bulk due to superior inflow congestion. RT was then continued with a single dose of 3 Gy up to a total dose of 51 Gy. Regarding the brain metastases, SRS using the Cyberknife© with 20 Gy single dose each on the 65% isodose was performed. Subsequently, the patient received palliative chemotherapy with carboplatin and abraxane. Cerebral lesions progressed in October 2016 and nivolumab was applied at 3 mg/kg for four cycles for a total of 7 weeks. Three weeks after the start of nivolumab, a concurrent stereotactic radiosurgery for cerebral metastases was applied (3 × 9 Gy and 1 × 20 Gy, each prescribed on the 65% isodose). We found non-irradiated lesions in the left and right suprarenal glands. While the left suprarenal metastasis showed a regression with IT alone, the right lesion showed an initial lesion progression from 448 to 1,773 mm^2 at 1 and 4 weeks after completion of IT and RT, respectively, followed by 33.9% lesion regression to 1,172 mm^2 4 weeks after a HFX of the right femur with a single dose of 3 Gy up to a total reference dose of 30 Gy ∼5 weeks after completion of nivolumab (**Figure 3**). During the follow-up CT scan 11 weeks later, the lesion was found to further regress to 994 mm^2, resulting in an overall lesion regression of 44%. Three weeks after the prior CT scan the left sacrum and ischium have been irradiated with a single dose of 3 Gy up to a total dose of 30 Gy. The total EQD2 this patient received was 157.75 Gy.

TABLE 2B | Detailed description of treatment characteristics and the corresponding abscopal effects for each patient.

Patient	Primary tumor	IT	IT duration (weeks)	No. of RT	Type of RT	Interval between RT (weeks)	Irradiated sites (*n*)	RT dose and fractionation regime (Gy)	EQD2 (Gy) for $\alpha/\beta = 10$	RT duration (days)	Time to RT after IT induction (weeks)	Site of analyzed metastases (*n*)	Site of AbE (*n*)	Time to AbE after RT	Overall lesion reduction (%)
1	MM	Pembrolizumab	24	2	(i) SRS (ii) CFX	(i–ii) 22	(i) Brain (ii) Brain	(i) 1 × 20 Gy (ii) 50 (2) Gy	100.00	(i) 1 (ii) 25	(i) 1 (ii) 23	Lung (5), paraaortal LN (1)	Lung (1)	4 wks after 2nd RT	75
2	MM	Pembrolizumab	31	3	(i) CFX (WBRT) (iia) HFX (iib) SRS	(i–ii) 23	(i) Brain (iia) Popliteal fossa and lower leg L. (iib) Brain	(i) 40 (2) Gy (iia) 54 (3) Gy (iib) 1 × 20 Gy	148.50	(i) 25 (iia) 28 (iib) 1	(i) 1 (iia) 27 (iib) 29	Perirenal region L. (1)	Perirenal region L. (1)	6 wks after 2nd RT	100
3	MM	Pembrolizumab	24	3	(i) SRS (ii) SRS (iii) SRS	(i–ii) 15 (ii–iii) 6	(i) Brain (ii) Brain (iii) Brain	(i) 1 × 20 Gy (ii) 1 × 20 Gy (iii) 1 × 20 Gy	150.00	(i) 1 (ii) 1 (iii) 1	(i) 14 (ii) 29 (iii) 35	Lung (2)	Lung (1)	5 wks after 3rd RT	100
4	NSCLC	Nivolumab	7	3	(i) SRS (ii) HFX (iii) HFX	(i–ii) 8 (ii–iii) 7	(i) Brain (ii) Femur R. (iii) Os. Sacrum and os. ischiadicum L.	(i) 3 × 9 Gy + 1 × 20 Gy (ii) 30 (3) Gy (iii) 30 (3) Gy	157.75	(i) 8 (ii) 12 (iii) 17	(i) 3 (ii) 12 (iii) 21	Suprarenal glands (2)	Suprarenal gland (1)	4 wks after 2nd RT	44
5	NSCLC	Nivolumab	104	1	CFX	–	Cervical and supravlavicular L.	54 (2) Gy	54.00	40	29	Mediastinal LN (1), hilar L. (1), axillar LN (1), intracarinal LN (1)	Axillar LN (1)	24 wks after RT	55
6	NSCLC	Nivolumab	52	3	(i) CFX (ii) HFX (iii) SRS	(i–ii) 9 (ii–iii) 4	(i) Supra- and infra-clavicular lymph drainage area (ii) 3rd rib R., iliac sacral joint R., inguinal L. (iii) Occipital L.	(i) 50.4 (1.8) Gy (ii) 30 (3) Gy (iii) 1 × 20 Gy	132.08	(i) 41 (ii) 49 (iii) 1	(i) 6 (ii) 22 (iii) 33	Lung (1)	Lung (1)	7 wks after 3rd RT	56
7	RCC	Nivolumab	32	3	(i) HFX (ii) HFX (iii) HFX	(i–ii) 19 (ii–iii) 20	(i) Os. Ilium L. (ii) Hip (L.+R.), os. Pubis R. (iii) Thoracic vertebra 12, Lumbar vertebra 3	(i) 36 (3) Gy (ii) 30 (3) Gy (iii) 30 (3) Gy	97.50	(i) 16 (ii) 14 (iii) 15	(i) 4 (ii) 25 (iii) 37	Mediastinal LN (2), hilar (1), pleural (1)	Mediastinal LN (1)	16 wks after 1st RT	49

MM, malignant melanoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma; RT, radiotherapy; CFX, normofractionated radiotherapy; SRS, stereotactic radiosurgery; HFX, hypofractionated radiotherapy; IT, immunotherapy; AbE, abscopal effect; SD, standard deviation; L, left; R, right; LN, lymph node; wks, weeks.

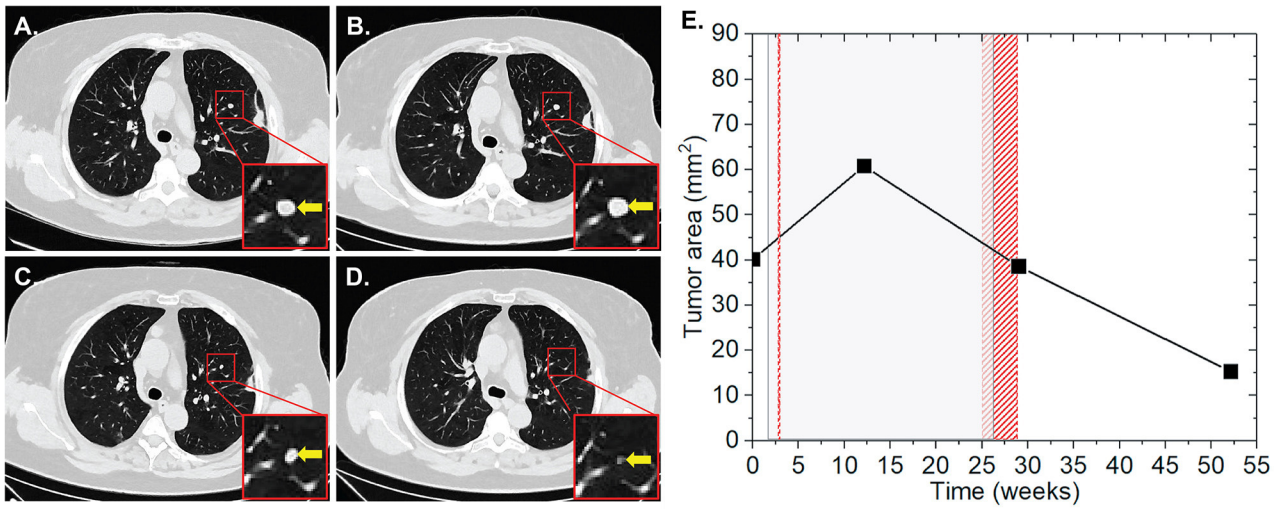

FIGURE 1 | Patient 1 (**Table 2B**) presenting AbE in the lung. CT scans show the analyzed lesion (yellow arrows) before **(A)**, 10 **(B)**, 27 **(C)**, and 50 weeks **(D)** after the induction of pembrolizumab. **(E)** The change in the lesion surface area with respect to the administration of IT (duration = 24 weeks, gray shaded area) and concurrent RT (red lines and area) of cerebral metastases with 1 × 20 Gy (1 week after 1st IT) and 25 × 2 Gy (23 weeks after 1st IT).

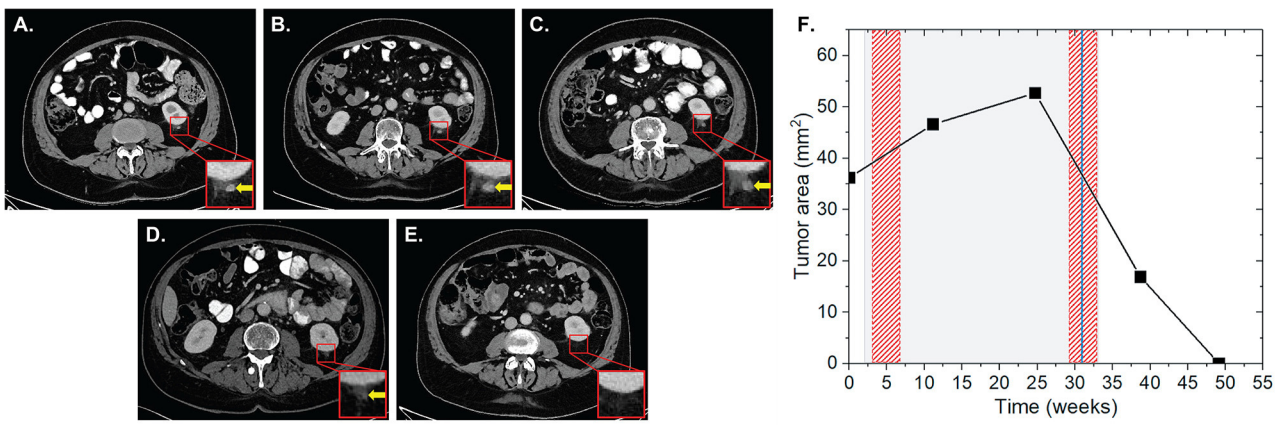

FIGURE 2 | Patient 2 (**Table 2B**) presenting AbE a soft tissue metastasis in the perirenal region. CT scans show the analyzed lesion (yellow arrows) before **(A)**, and 10 **(B)**, 23 **(C)**, 37 **(D)**, and 47 weeks **(E)** after the induction of pembrolizumab. **(F)** The change in the lesion surface area with respect to the administration of IT (duration = 31 weeks, gray shaded area) and concurrent RT (red shaded areas) of the whole brain with 20 × 2 Gy (1 week after 1st IT) and of bone metastases in the left popliteal fossa and lower left leg 18 × 3 Gy (27 weeks after 1st IT) together with SRS of cerebral metastases with 1 × 20 Gy (29 weeks after 1st IT, blue line).

DISCUSSION

In this study we analyzed retrospectively abscopal effects in advanced cancer patients being treated simultaneously with anti-PD1 therapy and radiation therapy. We used strict inclusion criteria for the radio-immunotherapy concept as being applied simultaneously and the radiological imaging information on distant lesions. AbEs were observed in 29% of our includable patients.

AbE was defined as radiation-induced shrinkage of distant, non-treated lesions (Mole, 1953; Andrews, 1978) and this was considered the visual evidence for the efficient immune-stimulation by irradiation. The immune system has been suggested as the key component for distant effects outside the irradiation field after local RT, defined as abscopal response. Local RT is considered to induce immunogenic cell death (ICD) associated with antigen release, cytokine production, and complement activation, leading to immune responses, and to a tumor vaccination (Formenti and Demaria, 2012; Frey et al., 2014; Barker et al., 2015). Mechanisms such as increasing the expression of the major histocompatibility complex (MHC) class I, activating dendritic cells, enhancing the presentation of tumor antigens and the migration of immune cells into the tumor micromilieu, which leads to an increase of tumor-infiltrating lymphocyte density with a broader T-cell receptor repertoire, improved effector T cell activity, and modulation

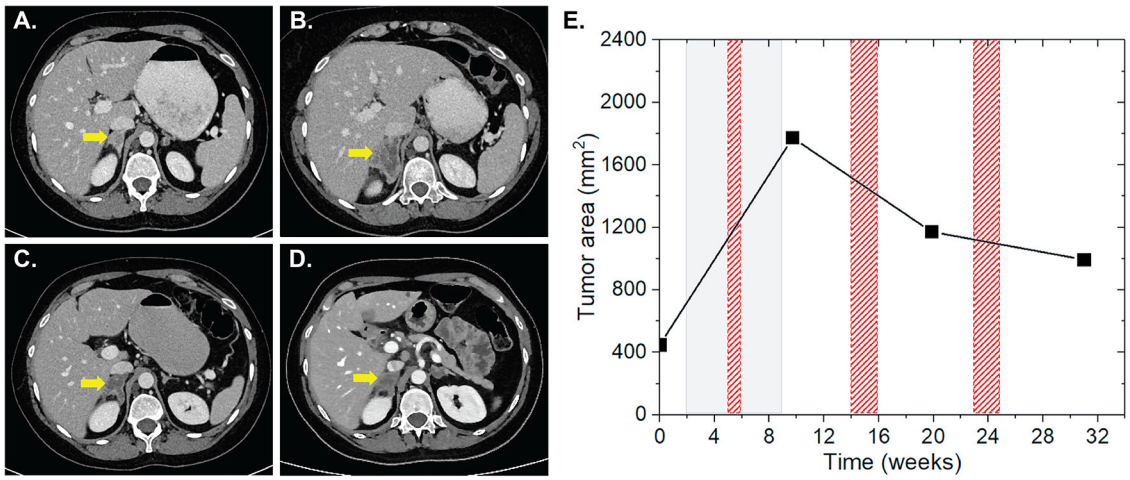

FIGURE 3 | Patient 4 (**Table 2B**) presenting AbE in the suprarenal gland. CT images show the analyzed lesion (yellow arrows) before **(A)** and 3 **(B)**, 12 **(C)**, and 21 weeks **(D)** after the induction of nivolumab. **(E)** The change in the lesion surface area with respect to the administration of IT (duration = 7 weeks, gray shaded area) and RT (red shaded areas) of brain metastases with 3 × 9 Gy and 1 × 20 Gy (3 weeks after IT induction), and RT of bone metastases 10 × 3 Gy of the right femur, left sacrum and ischium (3, 12, and 21 weeks after induction of IT).

of TReg cells and immune checkpoint molecule expression may contribute to improved systemic immune response after local radiotherapy (Demaria and Formenti, 2009; Formenti and Demaria, 2012).

Despite the stimulation of the immune response, RT alone does not seem to be sufficient to induce AbEs in most patients. Demaria et al. demonstrated in preclinical studies shrinkage of tumors outside the irradiation field when irradiation was combined with immunotherapy. This was naturally only observed in immunocompetent mice, indicating the indispensability of the immune system in this complex process (Demaria et al., 2004). In 2015, Reynders et al. (2015) reviewed all publications relating to the term "abscopal" in the context with RT in an oncological setting. They found that AbEs induced by RT alone are rare in the clinical and even in the preclinical setting. Interestingly, the majority of AbE cases occurred in highly immunogenic tumors such as malignant melanoma, renal cell carcinoma, and hepatocellular carcinoma (HCC) (Abuodeh et al., 2016).

Preclinical data, retrospective evaluations and case reports suggest that RT enhances the effect of IT or that radiation effects may be intensified by IT (Demaria et al., 2005; Frey et al., 2014; Ngwa et al., 2018). AbE rates of 25–52% are reported in current literature when combined treatment concepts with RT and ICIs are used (Grimaldi et al., 2014; Chandra et al., 2015). Most reports on the combination of RT and ICIs refer to patients with malignant melanoma treated with ipilimumab targeting the CTLA4 checkpoint, since it was approved for the treatment of metastatic melanoma in 2011 (Postow et al., 2012; Theurich et al., 2016). In 2014, checkpoint inhibitors targeting the PD-1 receptor were approved (pembrolizumab and nivolumab). The interaction of PD-1 and its ligand PD-L1, which may be expressed on tumor cells and antigen presenting cells, leads to a suppression of T-cell activation and thus provides an immune escape for cancer cells (Taube et al., 2012). There are many reasons why combining RT with PD-1 inhibitors might be able to provide an opportunity to boost abscopal response rates turning this rare event into a clinically relevant effect (Ngwa et al., 2018). RT can induce the expression of PD-L1 on tumor cells (Deng et al., 2014). In a study from 2016, Ribeiro Gomes et al. (2016) observed an AbE response rate of 18.7% out of 16 includable patients with solid tumors being treated with anti-PD-1 treatment and concurrent radiotherapy after disease progression occurred, all of these were diagnosed with malignant melanoma. Of all the solid tumor patients we analyzed, the 29%, which revealed AbE were either diagnosed with MM, NSLCL, or RCC, which are tumors with a high mutation frequency (Alexandrov et al., 2013).

The optimal dosing and fractionation therapy to produce the highest immunogenic benefit has not been determined yet. Single and fractionated therapy have been reported to boost AbE in combination with ICIs (Deng et al., 2014; Ngwa et al., 2018). In general, higher doses per fraction were associated with AbE. In our patient cohort, six of the seven patients showing AbE received multiple RT sessions and tended to have higher single doses. Only one patient received a normofractionated RT concept. There may be an optimal dose range where AbE is more likely to occur, or below which immune stimulation may be inferior. We assume that this range is at a high dose level (Bernstein et al., 2016).

Further questions remain about the right timing of RT and ICI application. It is difficult to distinguish between the combined effects of RIT and the effect of IT alone when applied simultaneously. We have therefore established strict inclusion criteria for the timing of radiological images.

It is also possible that patients we classified at showing AbE might in fact be presenting pseudo-progression (PsP), which is less frequent than AbE but definitely observed in analyses reporting about ICI application (Hodi et al., 2016). Evidence suggest that it could be even more frequent when being combined

with RT (Trommer-Nestler et al., 2018). It is assumed that PsP is generated by attracting immune cells to the tumor by a particular mechanism like releasing neoantigens due to RT. This can lead to a larger appearance of the lesion in radiological images, but after some time the size of the lesion decreases due to treatment effect and immune response (Hodi et al., 2016). We would primarily assume that the locally irradiated tumor shows PsP during RIT but it is also thinkable that it can be observed in distant lesions. The so far reported prevalence of PsP during ICI therapy is still too low to be considered as a reliable reason for the progression observed during PD-1 blockade in the seven patients presenting AbE, but must be considered as a possible differential diagnosis.

CONCLUSION

In this data analysis, we were able to show that 29% of the patients we included after applying strict inclusion criteria showed regression of lesions outside the irradiation field. We have identified AbE after radiation therapy distinctly from the treatment effects of immunotherapy alone. Most patients presenting AbE had received multiple RTs. Abscopal responses are yet rarely described in humans and systematic analyses of patients treated with radio-immunotherapy are lacking. Our results provide evidence for a clinical existence of a systemic effect of irradiation during immunotherapy and contribute to the further development of cancer therapy options, in particular with regard to combination therapies. Randomized prospective studies are required to assess whether the addition of RT to ongoing PD-1 inhibition might be able to induce reliable and durable systemic responses and provide clinical benefits. Particular attention must be paid to patient selection to find the best treatment option and clear indications when AbE induction is most likely to be effective and should be attempted. Further studies should improve the optimization of dosing regimens and the timing and sequencing of RIT concepts to determine the appropriate treatment approach for optimal and most immunogenic responses.

Our results are encouraging and represent a further step toward a possible application of RT together with ICIs in patients with advanced cancer stages to induce an AbE that enables a more efficient long-term immune response after RT.

ETHICS STATEMENT

We include humans in this retrospective study. The study was carried out retrospectively without intervening in treatment concepts and the data evaluation is based on already existing data. The analysis was approved by the ethics committee (no. 19-1036).

AUTHOR CONTRIBUTIONS

MT, SYY, and CB developed the conception and design of the study. MT, SYY, TP, AB, and HG discussed the cases in interdisciplinary panels. MT acquired patient data. MT and SYY organized the database, performed all analyses, and wrote the first draft of the manuscript. SYY, TP, and AB acquired the imaging data. TP, HG, MS, ST, MB-B, JM, JH, EC, SM, and CB contributed to the manuscript. All authors contributed to the revision and read and approved the submitted version.

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Corrigendum: Abscopal Effects in Radio-Immunotherapy—Response Analysis of Metastatic Cancer Patients With Progressive Disease Under Anti-PD-1 Immune Checkpoint Inhibition

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by Trommer M, Yeo SY, Persigehl T, Bunck A, Grüll H, Schlaak M, Theurich S, von Bergwelt-Baildon M, Morgenthaler J, Herter JM, Celik E, Marnitz S, and Baues C. (2019). Front. Pharmacol. 10:511. doi: 10.3389/fphar.2019.00511

An author name was incorrectly spelled as "Michael von Bergwelt." The correct spelling is "Michael von Bergwelt-Baildon". The updated **Author Contributions** statement appears below.

"MT, SYY, and CB developed the conception and design of the study. MT, SYY, TP, AB, and HG discussed the cases in interdisciplinary panels. MT acquired patient data. MT and SYY organized the database, performed all analyses, and wrote the first draft of the manuscript. SYY, TP, and AB

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The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Prognostic and Clinicopathological Significance of PD-L1 in Patients With Bladder Cancer: A Meta-Analysis

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Background: The prognostic role of programmed cell death-ligand 1 (PD-L1) in bladder cancer has been investigated in previous studies, but the results remain inconclusive. Therefore, we carried out a meta-analysis to evaluate the prognostic significance of PD-L1 in patients with bladder cancer.

Methods: The electronic databases PubMed, Embase, Web of Science, and Cochrane Library were searched. The association between PD-L1 expression and survival outcomes and clinicopathological factors was analyzed by hazard ratios (HRs) or odds ratios (ORs) and 95% confidence intervals (CIs).

Results: A total of 11 studies containing 1,697 patients were included in the meta-analysis. High PD-L1 expression was associated with poor overall survival (OS) (HR = 1.83, 95% CI = 1.24–2.71, $p = 0.002$). There was nonsignificant association between PD-L1 and recurrence-free survival (RFS) (HR = 1.43, 95% CI = 0.89–2.29, $p = 0.134$), cancer-specific survival (CSS) (HR = 1.51, 95% CI = 0.80–2.87, $p = 0.203$), or disease-free survival (DFS) (HR = 1.53, 95% CI = 0.88–2.65, $p = 0.13$). Furthermore, high PD-L1 was significantly correlated with higher tumor stage (OR = 3.9, 95% CI = 2.71–5.61, $p < 0.001$) and distant metastasis (OR = 2.5, 95% CI = 1.22–5.1, $p = 0.012$), while PD-L1 overexpression was not correlated with sex, tumor grade, lymph node status, and multifocality.

Conclusions: The meta-analysis suggested that PD-L1 overexpression could predict worse survival outcomes in bladder cancer. High PD-L1 expression may act as a potential prognostic marker for patients with bladder cancer.

Keywords: meta-analysis, prognosis, PD-L1, bladder cancer, survival

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INTRODUCTION

Bladder cancer is the most common malignancy of the urinary tract, accounting for 80,470 new cases and 17,670 deaths in 2019 alone in the United States (Siegel et al., 2019). When diagnosed, up to 75% of patients present with non-muscle-invasive bladder cancer (NMIBC), about 20% present with muscle-invasive bladder cancer (MIBC), and 5% would have metastatic disease. Although patients with NMIBC have a relatively good prognosis, the prognosis of regional and distant

metastatic disease is poor, with 5-year survival rates of 35% and 5%, respectively (National Cancer Institute SEER Program). Therefore, investigation of novel biomarkers to stratify patients is important for clinical management (Slovin, 2017).

Cancer immunoediting is a process consisting of immunosurveillance and tumor development (Mittal et al., 2014). Programmed cell death-1 (PD-1) and its ligand programmed cell death-ligand 1 (PD-L1) have an important role in the regulation of responses of our immune system (Errico, 2015). PD-L1 is also known as B7-H1, CD274, which is expressed on many cancer cells. PD-L1 expression has shown prognostic value in various tumors including pancreatic cancer (Gao et al., 2018), colorectal cancer (Shen et al., 2019), and non-small cell lung cancer (Ma et al., 2018). Recently, many studies (Nakanishi et al., 2007; Boorjian et al., 2008; Wang et al., 2009; Xylinas et al., 2014; Bellmunt et al., 2015; Wu et al., 2016; Noro et al., 2017; Li et al., 2018b; Pichler et al., 2018; Owyong et al., 2019; Wang et al., 2019) also investigated the prognostic significance of PD-L1 expression in bladder cancer, but the results remain controversial. Therefore, we collected relevant data and performed a meta-analysis to quantify the prognostic role of PD-L1 and analyze the relationship of PD-L1 and clinicopathological parameters in bladder cancer.

METHODS

Literature Search

This meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009). The research of PubMed, Embase, Web of Science, and Cochrane Library identified relevant studies published in English. The last search was updated on March 2019. A comprehensive search strategy was performed based on the following terms: "programmed death ligand-1," "PD-L1," "B7-H1," "CD274," "bladder cancer," "bladder neoplasm," "bladder tumor," and "bladder urothelial carcinoma." The references of the included studies were also manually checked to identify relevant publications. Ethical approval was waived because we just collected the data from available publications.

Eligibility Criteria

The inclusion criteria were as follows: 1) patients were histologically diagnosed to have bladder cancer; 2) PD-L1 was detected *via* immunohistochemical staining (IHC); 3) the relationship between PD-L1 and survival of bladder cancer was studied; and 4) references are published in English. Exclusion criteria were as follows: 1) duplicate studies; 2) studies provided incomplete data; and 3) meeting abstracts, case reports, reviews, or animal studies.

Data Extraction and Quality Assessment

Two independent investigators extracted the following information from the eligible studies: first author, publication year, country, detection method, sample size, study design, survival analysis, age, and study period. Any disagreement was resolved by discussion. The quality of the selected articles was assessed according to the Newcastle-Ottawa Scale (NOS) (Wells et al., 2009). Total quality score of NOS was ranged from 0 to 9, and studies that scored ≥6 were considered as high-quality studies.

Statistical Analysis

Hazard ratios (HRs) and their 95% confidence intervals (CIs) were searched in the original articles or calculated by methods described by Tierney et al. (2007). The survival outcomes included overall survival (OS), recurrence-free survival (RFS), cancer-specific survival (CSS), and disease-free survival (DFS). The logHR and standard error (SE) were used to present the survival results. An observed HR > 1 implied a poorer prognosis in patients with high PD-L1 expression, while HR < 1 indicated a better prognosis. The relationship between PD-L1 expression and clinicopathological features was evaluated by odds ratios (ORs) and corresponding 95% CIs. Cochran's Q test and Higgins I-squared statistic (I^2) were used to measure the heterogeneity of the combined HRs (Higgins and Thompson, 2002). $I^2 > 50\%$ and/or $p < 0.1$ suggested significant heterogeneity in terms of statistics, and a random-effects model was utilized. Alternatively, a fixed-effects model was applied. Begg's test was used to detect potential publication bias (Begg and Mazumdar, 1994). All statistical analyses were conducted by using Stata version 12.0 (Stata Corporation, College Station, TX, USA). A two-sided $p < 0.05$ was considered statistically significant.

RESULTS

Study Selection

Initial literature search identified 925 records. After removal of duplicate records, 668 studies remained for further evaluation. Then, 631 recorded were excluded by scanning title and/or abstract. Thirty-seven studies were screened by full-text examination, and 26 studies were excluded for following reasons: 20 studies did not provide sufficient for analysis, 2 studies recruited overlapped patients, 2 studies were reviews, 1 study did not focus on PD-L1, and 1 study did not use IHC method for PD-L1 detection. Ultimately, 11 studies (Nakanishi et al., 2007; Boorjian et al., 2008; Wang et al., 2009; Xylinas et al., 2014; Bellmunt et al., 2015; Wu et al., 2016; Noro et al., 2017; Li et al., 2018b; Pichler et al., 2018; Owyong et al., 2019; Wang et al., 2019) were included in this meta-analysis. The flow diagram is shown in **Figure 1**.

Study Characteristics

The main characteristics of eligible articles are listed in **Table 1**. The studies were published from 2007 to 2019. Three studies (Wang et al., 2009; Li et al., 2018b; Wang et al., 2019) were conducted in China, three were performed in United States (Boorjian et al., 2008; Xylinas et al., 2014; Bellmunt et al., 2015), two were in Japan (Nakanishi et al., 2007; Noro et al., 2017), and one each in Taiwan (Wu et al., 2016), Austria (Pichler et al., 2018) and Egypt (Owyong et al., 2019).

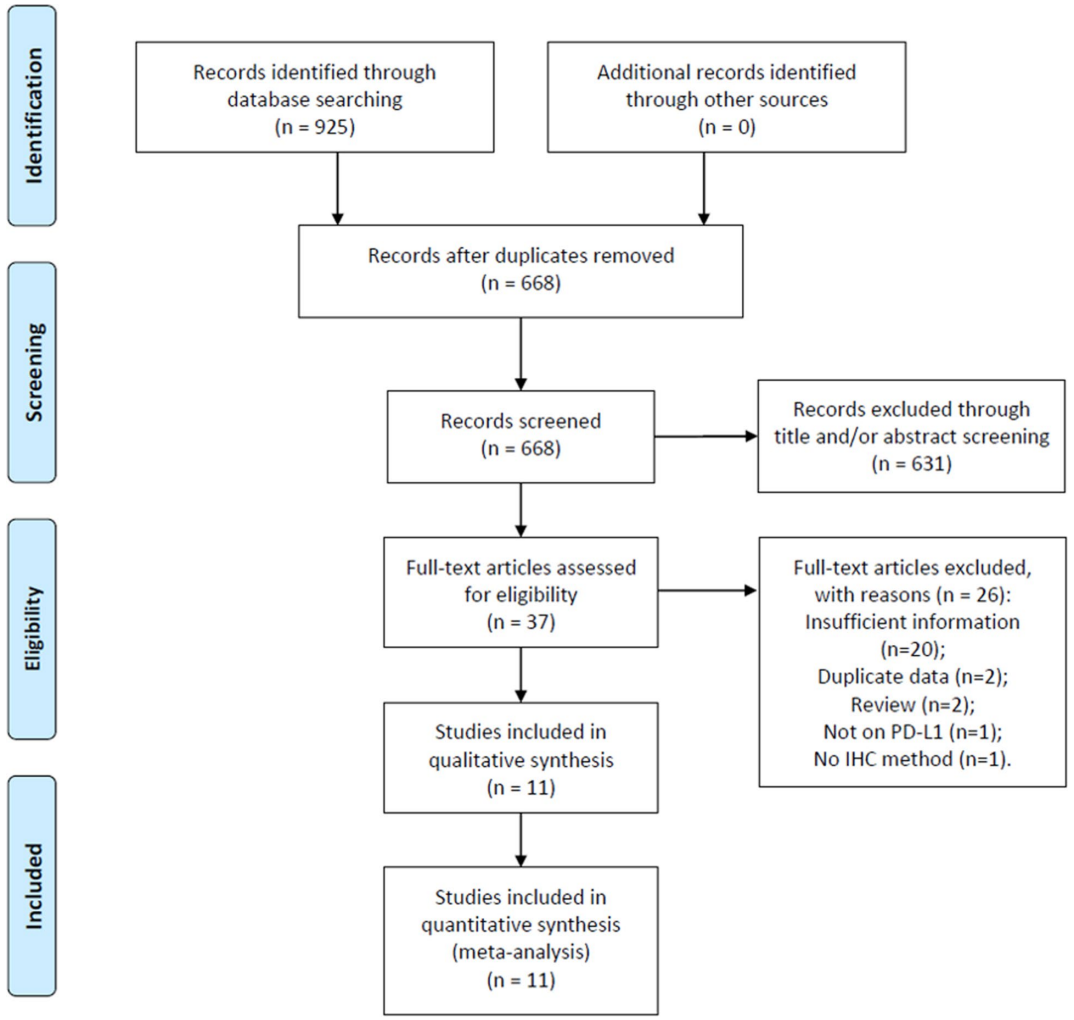

FIGURE 1 | Flowchart for selection of studies.

TABLE 1 | Basic characteristics of included studies.

Author	Year	Country/ region	Study design	Duration	No. of patients	Sex (M/F)	Age	Survival analysis	Detection method	NOS score
Nakanishi	2007	Japan	Retrospective	1996–2005	65	47/18	NA	OS, CSS, RFS	IHC	6
Boorjian	2008	USA	Retrospective	1990–1994	318	259/59	69 (37–90)	OS, CSS, DFS	IHC	7
Wang	2009	China	Retrospective	2000–2002	50	40/10	61.7 (42–78)	OS	IHC	7
Xylinas	2014	USA	Retrospective	1988–2003	302	244/58	65.9	OS, CSS, RFS	IHC	8
Bellmunt	2015	USA	Retrospective	NA	160	NA	NA	OS	IHC	6
Wu	2016	Taiwan	Retrospective	NA	120	NA	NA	OS, DFS	IHC	6
Noro	2017	Japan	Retrospective	2004–2014	102	82/20	60 (43–84)	CSS, DFS	IHC	8
Li	2018	China	Retrospective	2009–2011	98	76/22	NA	OS	IHC	7
Pichler	2018	Austria	Retrospective	2006–2015	83	62/21	69 (36–87)	RFS	IHC	8
Owyong	2019	Egypt	Retrospective	1997–2004	151	98/53	52 (36–74)	CSS, RFS	IHC	8
Wang	2019	China	Retrospective	2006–2012	248	214/34	63 (14–94)	OS, RFS	IHC	7

NA, not available; OS, overall survival; CSS, cancer-specific survival; DFS, disease-free survival; RFS, recurrence-free survival; IHC, immunohistochemical staining; NOS, Newcastle-Ottawa Scale.

The total sample size was 1,697, ranging from 50 to 318. All studies were a retrospective study design. Regarding clinical outcomes, eight studies reported clinicopathological factors (Boorjian et al., 2008; Wang et al., 2009; Xylinas et al., 2014; Bellmunt et al., 2015; Wu et al., 2016; Li et al., 2018b; Owyong et al., 2019; Wang et al., 2019), eight studies reported OS (Nakanishi et al., 2007; Boorjian

et al., 2008; Wang et al., 2009; Xylinas et al., 2014; Bellmunt et al., 2015; Wu et al., 2016; Li et al., 2018b; Wang et al., 2019), five studies described RFS (Nakanishi et al., 2007; Xylinas et al., 2014; Pichler et al., 2018; Owyong et al., 2019; Wang et al., 2019), five studies reported CSS (Nakanishi et al., 2007; Boorjian et al., 2008; Xylinas et al., 2014; Noro et al., 2017; Owyong et al., 2019), and three studies presented DFS (Boorjian et al., 2008; Wu et al., 2016; Noro et al., 2017). Furthermore, all studies were with NOS score ≥ 6, indicating that the studies were of high quality.

Impact of PD-L1 on OS, RFS, CSS, and DFS

Eight studies (Nakanishi et al., 2007; Boorjian et al., 2008; Wang et al., 2009; Xylinas et al., 2014; Bellmunt et al., 2015; Wu et al., 2016; Li et al., 2018b; Wang et al., 2019) reported data on PD-L1 and OS in bladder cancer. As shown in **Figure 2** and **Table 2**, high PD-L1 was associated with poorer OS (HR = 1.83, 95% CI = 1.24–2.71, p = 0.002). Because of significant heterogeneity (I^2 = 62%, p = 0.01), a random-effects model was applied. Five studies (Boorjian et al., 2008; Xylinas et al., 2014; Pichler et al., 2018; Owyong et al., 2019; Wang et al., 2019) showed the relationship between PD-L1 and RFS. The pooled results were HR = 1.43, 95% CI = 0.89–2.29, p = 0.134, with significant heterogeneity (I^2 = 69.6%, p = 0.011) (**Table 2**, **Figure 2**). The pooled data from five studies (Nakanishi et al., 2007; Boorjian et al., 2008; Xylinas et al., 2014; Noro et al., 2017; Owyong et al., 2019) suggested nonsignificant association between PD-L1 and CSS in bladder cancer (HR = 1.51, 95% CI = 0.80–2.87, p = 0.203; I^2 = 73.8%, p = 0.004, **Table 2**, **Figure 2**). Moreover, three studies reported the correlation of PD-L1 and DFS (Boorjian et al., 2008; Wu et al., 2016; Noro et al., 2017). The random-effects model was applied because there was significant heterogeneity (I^2 = 63.3%, p = 0.066) across the studies. The pooled HR and 95%CI were HR = 1.53, 95% CI = 0.88–2.65, p = 0.013 (**Table 2**, **Figure 2**), suggesting PD-L1 was not correlated to worse DFS.

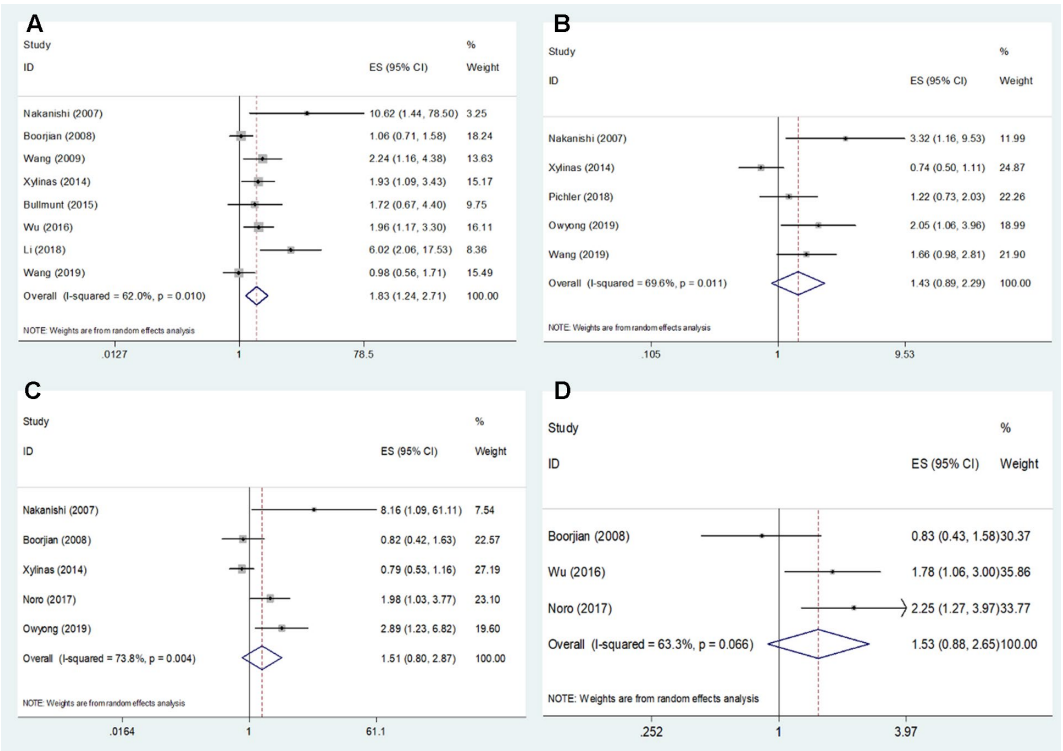

FIGURE 2 | Forest plots describing the association between PD-L1 expression and **(A)** OS, **(B)** RFS, **(C)** CSS, and **(D)** DFS of patients with bladder cancer.

TABLE 2 | Meta-analysis of PD-L1 and prognosis in bladder cancer.

Survival analysis	No. of studies	No. of patients	Effects model	HR (95% CI)	p	Heterogeneity	
						I^2 (%)	p
OS	8	1,361	Random	1.83 (1.24–2.71)	0.002	62	0.01
RFS	5	849	Random	1.43 (0.89–2.29)	0.134	69.6	0.011
CSS	5	938	Random	1.51 (0.80–2.87)	0.203	73.8	0.004
DFS	3	540	Random	1.53 (0.88–2.65)	0.13	63.3	0.066

PD-L1 and Clinicopathological Features

Eight studies (Boorjian et al., 2008; Wang et al., 2009; Xylinas et al., 2014; Bellmunt et al., 2015; Wu et al., 2016; Li et al., 2018b; Owyong et al., 2019; Wang et al., 2019) explored the association between PD-L1 and clinicopathological characteristics. The pooled data demonstrated that high PD-L1 was significantly correlated with higher tumor stage (OR = 3.9, 95% CI = 2.71–5.61, $p < 0.001$) and distant metastasis (OR = 2.5, 95% CI = 1.22–5.1, $p = 0.012$). However, PD-L1 overexpression was not correlated with other clinicopathological factors including sex (OR = 0.88, 95% CI = 0.65–1.21, $p = 0.433$), tumor grade (OR = 1.19, 95% CI = 0.46–3.09, $p = 0.72$), lymph node status (OR = 1.16, 95% CI = 0.63–2.15, $p = 0.631$), and multifocality (OR = 0.77, 95% CI = 0.5–1.18, $p = 0.226$). The correlation between PD-L1 and clinicopathological parameters is presented in **Table 3**.

Publication Bias

The assessment of the publication bias was carried out by using Begg's funnel plot test. Begg's p values for OS, RFS, CSS, and DFS were 0.063, 0.086, 0.221, and 0.602, respectively. Begg's funnel plot was found to be symmetrical (**Figure 3**), indicating no significant publication bias in this meta-analysis.

DISCUSSION

In the present study, we collected information from 11 recent studies with 1,697 patients and combined the data. The results showed that elevated PD-L1 expression was associated with poorer OS. In addition, PD-L1 overexpression was also connected with higher tumor stage and distant metastasis. There was no obvious evidence of publication bias. The results suggested that PD-L1 expression may be associated with tumor progression and metastasis and could be used as a potential prognostic biomarker. To the best of

TABLE 3 | Association of PD-L1 and clinical factors in bladder cancer.

Clinical factors	No. of studies	No. of patients	Effects model	OR (95% CI)	p	Heterogeneity	
						I^2 (%)	p
Tumor stage (T2–T4 vs Ta–T1)	8	1,447	Fixed	3.9 (2.71–5.61)	< 0.001	0	0.733
Sex (male vs female)	7	1,287	Fixed	0.88 (0.65–1.21)	0.433	13.8	0.325
Tumor grade (high vs low)	6	969	Random	1.19 (0.46–3.09)	0.72	86.5	<0.001
Lymph node status (positive vs negative)	5	1,139	Random	1.16 (0.63–2.15)	0.631	71.7	0.001
Multifocality (multifocal vs unifocal)	4	799	Fixed	0.77 (0.5–1.18)	0.226	0	0.659
Metastasis status (M1 vs M0)	3	466	Fixed	2.5 (1.22–5.1)	0.012	0	0.842

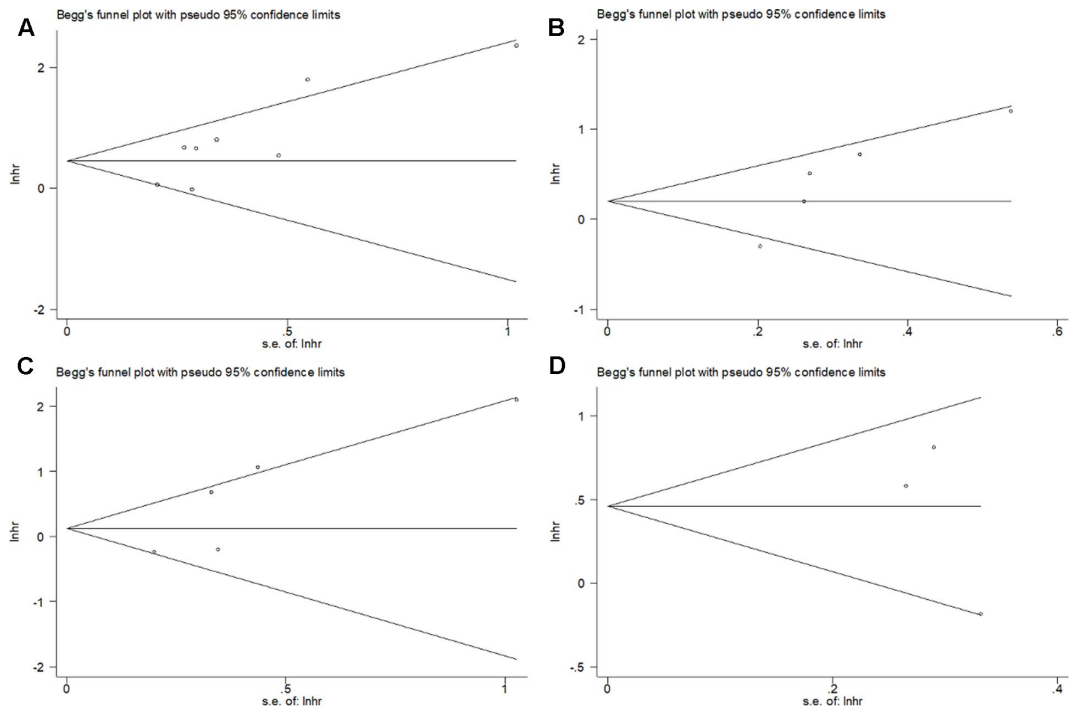

FIGURE 3 | Begg's funnel plot for publication bias test including PD-L1 expression and **(A)** OS, **(B)** RFS, **(C)** CSS, and **(D)** DFS in bladder cancer patients.

our knowledge, this is the first pointed meta-analysis investigating the prognostic value of PD-L1 in patients with bladder cancer.

PD-1 and its ligands, PD-L1 and PD-L2, overexpressed in the tumor microenvironment (Riley, 2009). The interaction of PD-1/PD-L1 can inhibit T-cell activation and proliferation, cytokine production, and cytolytic function (Riley, 2009). In addition, PD-L1 can also stimulate IL-10 production in T cells to mediate immune suppression (Dong et al., 1999). PD-L1 was found to be overexpressed in multiple solid tumor types to generate an immunosuppressive tumor microenvironment (Iwai et al., 2002; Blank et al., 2005; Wang et al., 2017). In the present study, we found the association of PD-L1 and higher tumor stage and distant metastasis, which implied the role of PD-L1 in tumor development. A recent study showed that PD-L1 played a critical role in promoting epithelial-to-mesenchymal transition (EMT) phenotype of esophageal cancer (Chen et al., 2017). Another study also suggested that PD-L1 expression was a significant risk factor for nodal metastasis in cutaneous squamous cell carcinoma (Garcia-Pedrero et al., 2017). The activation of IL-6/STAT3/PD-L1 pathway was found to be involved in the EMT process in bladder cancer (Zhang et al., 2019).

A number of previous studies also reported the prognostic significance of PD-L1 in various cancers. A recent meta-analysis including 2,005 patients showed that high PD-L1 expression was associated with a poor prognosis (HR = 2.04, 95% CI = 1.18–3.54, $p = 0.01$) in non-Hodgkin lymphoma (Zhao et al., 2018). Li's study showed that PD-L1 overexpression could foresee worse OS and DFS in hepatocellular carcinoma (Li et al., 2018a). In addition, another meta-analysis comprising a total of nine studies with 993 patients demonstrated that elevated PD-L1 expression was related with poor OS (HR = 1.63, 95% CI = 1.34–1.98, $p < .001$) and CSS (HR = 1.86, 95% CI = 1.34–2.57, $p < .001$) in pancreatic cancer (Hu et al., 2019). High PD-L1 expression was also correlated with poor OS in breast cancer (Zhang et al., 2017). The results of our study were in line with previous studies, suggesting the prognostic value of PD-L1 in bladder cancer. Furthermore, we also found the connection between PD-L1 and distant metastasis in bladder cancer, which may be explained by the role of PD-L1 in EMT process (Zhang et al., 2019). Recently, many studies also reported the effectiveness and patient-reported outcomes in clinical trials of PD-L1 inhibitors. Madore et al. showed that PD-L1 expression in melanoma showed marked heterogeneity within and between patients, which supported the therapeutic strategies of melanoma patients in a PD-L1-based manner (Madore et al., 2015). In addition, stage III melanoma patients with negative PD-L1 expression is associated with worse survival and immune response (Madore et al., 2016). A recent meta-analysis demonstrated that PD-L1 expression was significantly associated with mortality and clinical response to anti-PD-1/PD-L1 antibodies in metastatic melanoma patients (Gandini et al., 2016). The health-related quality of life was also better in advanced cancer patients receiving PD-1/PD-L1 inhibitors than in those receiving standard-of-care therapy (Nishijima et al., 2019). Those studies suggest that the clinical management of PD-1/PD-L1 inhibitors is complex and should be adjusted in the individual patient level.

Notably, age is also a risk factor for bladder cancer patients. In the included studies, five studies (Xylinas et al., 2014; Wu et al., 2016; Li et al., 2018b; Owyong et al., 2019; Wang et al., 2019) provided the data on age in PD-L1 (+) and PD-L1 (−) groups. However, three studies (Xylinas et al., 2014; Wu et al., 2016; Owyong et al., 2019) presented age in the format of median (range). One study (Li et al., 2018b) reported the number of patients in PD-L1 (+) and PD-L1 (−) groups using 65 years as threshold. One study used 60 years (Wang et al., 2019) to divide patients. Therefore, the quantitative analysis of PD-L1 expression and age could not be performed because of different cutoff values of age (65 and 60 years). In spite of this, we can find that patients with PD-L1 (+) expression are older than patients with PD-L1 (−) expression in four studies (Xylinas et al., 2014; Wu et al., 2016; Li et al., 2018b; Wang et al., 2019). All five studies (Xylinas et al., 2014; Wu et al., 2016; Li et al., 2018b; Owyong et al., 2019; Wang et al., 2019) reported nonsignificant association between age and PD-L1 expression (all $p > 0.05$). Moreover, in the analysis of association between PD-L1 expression and clinical factors, heterogeneity was found on sex, tumor grade, and lymph node status (**Table 3**). Because different studies may select patients with various criteria, the heterogeneity among studies may be inherent and may exist. In this occasion, we applied different effects model according to different heterogeneity.

Some limitations need to be mentioned in this meta-analysis. First, the determination of high expression of PD-L1 might vary in the studies because of different cutoff values, which may introduce potential bias. Second, the sample size was relatively small. Only 11 studies with 1,697 patients were included for analysis. For example, for CSS and DFS analysis, only five and three studies were included; the small study may compromise the credibility of the results. Third, although we did not find publication bias in the meta-analysis, the publication bias and selection bias could possibly exist. As we know, studies with significant results are inclined to be published (Koletsi et al., 2009). Therefore, the results should be treated with caution.

CONCLUSION

In summary, the findings of this meta-analysis suggest that elevated PD-L1 expression is associated with poor survival, higher tumor stage, and distant metastasis in bladder cancer. PD-L1 may be useful in the future as a novel prognostic factor in bladder cancer. Nevertheless, due to some limitations, well-designed, multicenter randomized controlled trials should be performed.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

LZ, JS, and ZBL designed the study. LZ, JS, LiW, ZGL, and LeW performed the research. LZ and JS collected and analyzed the data. LZ and JS wrote the paper. LeW amended the article. ZBL acts as the submission's guarantor and takes responsibility for the integrity of the work as a whole, from inception to published article. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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REVIEW
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The Controversial Role of PD-1 and Its Ligands in Gynecological Malignancies

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The programmed death-1 (PD-1, CD279) receptor with its ligands, programmed death ligand 1 (PD-L1, CD274, B7-H1), and programmed death ligand 2 (PD-L2, CD273, B7-DC), are the key players of one of the immune checkpoint pathways inhibiting T-cell activation. PD-L1 and PD-L2 are expressed in different cancer cells and their microenvironment, including infiltrating immune cells. However, their prognostic value is still debated and their role in the tumor microenvironment has not been fully elucidated yet. Considering the importance that cancer immunotherapy with anti-PD-1 and anti-PD-L1 antibodies gained in several tumor types, in this review article we aim to discuss the role of the PD-1/PD-L1/PD-L2 axis in gynecological cancers. PD-1 ligands have been detected in ovarian, cervical, vulvar and uterine cancers, and correlation with prognosis seems dependent from their distribution. About PD-L2, very few reports are available so far in gynecological malignancies, and its role is still not completely understood. Clinical trials using anti-PD-1 or anti-PD-L1 antibodies, but not anti-PD-L2, are currently ongoing, in all types of gynecological cancers. They have shown good safety profiles in a certain cohort of patients, but response rates remain low and many aspects remain controversial. In this review, we propose possible solutions to enhance the clinical efficacy of PD-1 axis targeting therapies. Regarding PD-L2, it might be useful to better clarify its role in order to improve the efficiency of immunotherapy in female malignancies.

Keywords: PD-L2, PD-L1, PD-1, ovarian cancer, endometrial cancer, cervical cancer, immunotherapy

INTRODUCTION

PD-1 and Its Ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC)

Programmed death-1 (PD-1, CD279) receptor and its ligands, programmed death ligand 1 (PD-L1, CD274, B7-H1) and programmed death ligand 2 (PD-L2, CD273, B7- DC), play crucial roles in one of the immune checkpoint pathways responsible for the inhibition of T-cell activation (1).

PD-1 receptor belongs to the CD28 family and is mainly expressed on the cellular surface of activated T and B cells, monocytes, natural killer (NK), and dendritic cells (DCs), with a role in the induction and maintenance of peripheral tolerance and for the maintenance of the stability and

the integrity of T cells (2–5). PD-1 ligands are glycoproteins, members of the B7 family, with 40% homology in amino acids sequence, but have quite distinct expression patterns, being expressed by a wide variety of immune and non-immune cells (1, 3, 4).

PD-L1 is a type I transmembrane glycoprotein with a single N-terminal immunoglobulin variable (IgV)-like domain sharing 21–33% sequence identity with CTLA-4, CD28, and ICOS, about 20 amino acids that separate the IgV domain from the plasma membrane, a transmembrane domain and a cytoplasmic tail (4). It is constitutively expressed on activated T and B cells, DCs, macrophages, mesenchymal stem cells, and bone marrow-derived mast cells (4, 6). Additionally, it is expressed on a wide variety of non-hematopoietic cells including the vascular endothelium, fibroblastic reticular cells, keratinocytes, lung, non-parenchymal cells of the liver, mesenchymal stem cells, pancreatic islet cells, astrocytes, and neurons (4, 5, 7). PD-L1 expression on human T cells is induced by common γ chain cytokines (IL-2, IL-7, and IL-15), whereas PD-L1 expression on B cells is stimulated by IL-21 (4). In cancer cells, PD-L1 expression is regulated by the MAPK and PI3K/AKT pathways, as well as by HIF-1α, STAT-3, NF-κB and epigenetic mechanisms via microRNAs (8). PD-L1 also exists in a soluble form (sPD-L1) that originates from the cleavage of membrane-bound PD-L1 by matrix metalloproteinases. Such PD-L1 soluble isoform, mainly produced by myeloid-derived cells, retains the IgV-like domain, necessary for the interaction with PD-1, and it is able to suppress T-cell activation. However, its physiological role is still unknown. Interestingly, sPD-L1 has been found in several human cancer cell lines, including H1299 non-small cell lung cancer cells, U-937 lymphoma cells, HO8910 ovarian carcinoma cells, SPCA-1 lung adenocarcinoma cells and U251 glioblastoma cells. In addition, high plasma levels of sPD-L1 have been associated with metastasis and poor prognosis in breast cancer and diffuse large B-cell lymphoma (8).

PD-L2 is a type I transmembrane protein containing an IgV-like domain and an immunoglobulin constant (IgC)-like domain in its extracellular region (9). PD-L2 expression is mainly restricted to antigen-presenting cells (APCs), including macrophages and myeloid DCs (6, 7), and non-hematopoietic tissues, such as the lung (10), human umbilical vein endothelial cells, and fibroblasts (1, 5). Three isoforms of PD-L2 have been described that might influence the outcome of the immune response (9). The most common splice variant contains all 6 exons. In humans, an alternative variant with a spliced-out exon 3, resulting in a protein lacks the IgC-like domain and with a shorter—extracellular region has been reported. A third isoform misses the transmembrane domain, because exon 3 is spliced out to an alternative acceptor site within exon 4, and the protein is secreted as a soluble form. This evidence underscores the importance of post-transcriptional regulation in the expression and function of PD-L2. He et al. suggested that isoforms II and III should be able to interact with PD-1, but further confirmation is needed (9).

Exposure to IL-4, IFN-γ, IL-2, IL-7, IL-15, IL-21, and toll-like receptor ligands induces PD-L2 upregulation in DCs and macrophages (1). Additionally, IL-4, in the presence of respiratory syncytial virus infection, stimulates PD-L2 expression in alveolar epithelial cells (1, 10).

Stimulation by tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) enhances the constitutive expression of PD-L2 on endothelial cells from human umbilical vein *in vitro* (1). The NF-κB and the STAT-6 pathways are two major signaling reported to regulate PD-L2 expression (1).

Different molecular mechanisms dictate PD-Ls binding to PD-1, as demonstrated by the crystallographic structures of the complexes, showing that PD-Ls cross-compete and that the concurrent presence of both ligands might modify the functional outcome of the binding (11). Specifically, PD-L1 binding to PD-1 requires complex conformational changes of the ligand, while PD-L2 directly interacts with PD-1, explaining its reported 2 to 6-fold higher affinity for the receptor (1). Consequently, when both ligands are expressed at similar levels, PD-L2 would be expected to outcompete PD-L1 for binding to PD-1. However, PD-L2 is generally expressed at lower levels in physiological conditions, such as during maturation of DCs by LPS, when PD-L1 acts as the main ligand of PD-1. A known exception is Th2 responses, where PD-L2 is predominant (1, 11).

Regarding the PD-1/PD-L1 and PD-1/PD-L2 pathways involved in T cell immune evasion, different reports have been published, mainly regarding the biochemical signaling regulated by the PD-1/PD-L1. It was reported that the binding of PD-L1 to PD-1 may cause T cell apoptosis, anergy, exhaustion, and interleukin-10 (IL-10) expression, suggesting that PD-L1 can act as a defender for PD-L1$^+$ cancer cells from CD8$^+$ T cell–mediated lysis (12, 13) (**Figure 1**).

Regarding the PD-L2/PD-1 signaling pathways, it may not be biologically identical, since Repulsive Guidance Molecule B (RGMb) is also a binding partner for PD-L2 (14). Thus, the PD-L2 blockade may evocate different cellular responses, depending on the binding partner interaction, which can lead to potential varied biological outcomes. Up to now, in human anti-tumor immunity, the relationship between PD-1, PD-L1, and PD-L2 in their cellular expression profile and regulation, potential interactions and biological is considered not completely defined.

PD-1 Ligands in the Tumor Microenvironment Influence the Anti-tumor Response

PD-L1 and PD-L2 are expressed in different cancer cells and in their microenvironment (4, 8), including infiltrating immune cells (15, 16). However, their prognostic value is still debated and the role they might play when expressed in the tumor microenvironment has not be fully elucidated yet (17).

Previous evidence shows that PD-L1 expression by cancer cells correlates with poor prognosis (18), while PD-L1 expression by tumor-infiltrating immune cells is associated with improved overall survival (OS) (16). Furthermore, it seems that PD-L1 expressed by APC, rather than cancer cells, is essential for the response to immune checkpoint blockade therapy (19). Specifically, survival analysis showed that the presence of PD-L1 on macrophages had a protective role and enhanced the prognosis of patients with hepatocellular carcinoma.

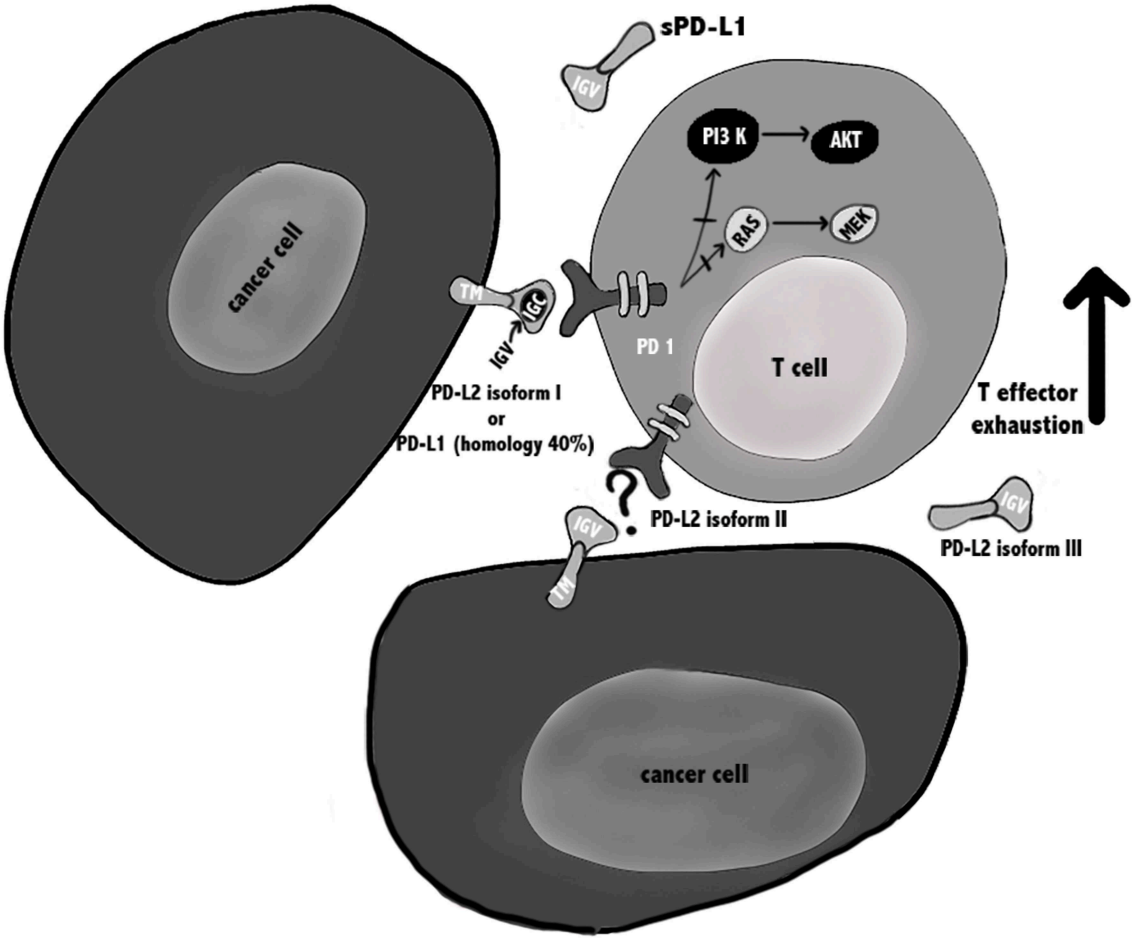

FIGURE 1 | PD-1/PD-Ls pathways in cancer. PD-L1 is a type I transmembrane glycoprotein with a single N-terminal IgV-like domain and exists also in a soluble form sPD-L1 that retains the IgV-like domain. PD-L2 is a type I transmembrane protein containing an IgV-like domain and an IgC-like domain and three isoforms of PD-L2 have been described that might influence the outcome of the immune response. It is suggested that isoforms II and III should be able to interact with PD-1, but further confirmation is needed. During TCR cross-linking, PD-1 by interacting with its ligands, causes inhibition of PI3K/Akt/mTOR and Ras/MAPK/Erk pathways, leading to down-regulation of T cells metabolism, and exhausted T cells.

Macrophages are involved in maintaining an active immune microenvironment, with high numbers of infiltrating $CD8^+$ T cells and high immune-related gene expression levels (15).

Sepesi et al. investigated PD-L1 expression in surgically resected stage I non-small cell lung cancer and, in contrast, demonstrated that lower PD-L1 expression in the tumor, but also in tumor-infiltrating macrophages, was associated with significantly better OS (20).

The existence of conflicting reports about PD-L1 and−2 prognostic value can be generally attributed to technical disparities (e.g., variations in staining protocols across individual laboratories and use of different primary antibody clones to identify PD-Ls in tumor tissue), as well as different clinical features of the analyzed samples (site and size of cancer, treatments, follow-up time, etc.). Moreover, PD-L1 and−2 are dynamic markers that can be up- or downregulated over time, making their evaluation complicated (17, 21).

Direct activation of the PD-1 axis by cancer cells leads to a potent inhibitory signal in T lymphocytes resulting in anti-tumor immunity impairment and tumor cells ability to escape immunosurveillance (4, 19). Specifically, it has been shown that PD-1 activation inhibits glucose consumption, cytokine production, proliferation and survival in T lymphocytes, thus preventing the expression of transcription factors associated with effector T cell functions, such as GATA-3, T-bet, and Eomesodermin (Eomes) (4). PD-1/PD-Ls binding attenuates TCR-mediated signaling, thus impairing PI3K/Akt and Ras/MEK/Erk pathways, both required for T-cell activation (4).

PD-Ls are expressed in several solid tumors (8, 22), and immune checkpoint inhibitors, such as anti-PD-1 and anti-PD-L1 antibodies, showed efficacy in cancers with high mutational load, including lung cancer, melanoma, and microsatellite instable (MSI) tumors (23). It was shown that this efficacy is linked to the presence of tumor specific neoantigens that induce a Th1/CTL response that is counterbalanced by overexpression

of multiple immune checkpoints such as PD-1/PD-L1 (23). In addition, PD-1/PD-L1 axis blockade might activate tumor-specific T lymphocytes to kill tumor cells by inducing TNF-α and IFN-γ (22).

For gynaecologic malignancies, the expression of PD-1 ligands has been reported in ovarian (17, 21, 22, 24–31), uterine (5–7, 32–38), cervical (23, 32, 39–50), and vulvar (32, 51–54) cancers, which we describe in detail in the next section.

PD-1 AND PD-LS EXPRESSION IN ENDOMETRIAL CANCER

In normal endometrium the role of the immune system is extremely complex, since it must prevent sexually transmitted infections but should also be able to help the growth of an allogenic fetus during pregnancy (23). So far, few reports characterized PD-1 and its ligands' expression in gynecological cancer and data are quite controversial. The expression profile of these immune checkpoints has been analyzed predominantly by immunohistochemistry, in biopsies obtained from both healthy subjects and cancer patients.

PD-1 in Endometrial Cancer

The PD-1 receptor has been found almost exclusively in immune cells infiltrating the tumor (32, 37, 38), and not in normal endometrium (5). Additionally, a deep analysis performed on 183 patients showed that high expression of PD-1 within and at the margins of a tumor, with a high PD-1/$CD8^+$ ratio in the center, was associated with favorable OS (35).

Additional reports found a correlation between PD-1 expression in intraepithelial and peritumoural lymphocytes with DNA polymerase ε (POLE) mutation and MSI status of the patients (32, 37, 38). Specifically, it has been reported that PD-1 expression in tumor-infiltrating immune cells was more frequently found in moderately, poorly differentiated endometrial cancers, non-endometrioid type II (serous, clear cell, mucinous) endometrial cancers (5, 35, 36), and POLE and MSI subgroups (32, 37, 38).

PD-L1 in Endometrial Cancer

Regarding PD-1 ligands, all data concordantly showed that PD-L1 is expressed in most of the analyzed specimens (5–7, 32–35, 37), predominantly located in the cytoplasm (5–7). Several studies showed that PD-L1 was expressed in a similarly high percentage of samples in both normal endometrium and endometrial tumors (5–7).

PD-L1 expression in cancer cells correlates with post-menopausal status, high histological grade (grade 3), deep myometrial invasion (≥1/2), lymphovascular invasion, adjuvant therapy, and MSI status (35). High PD-L1 immuno-reactivity on immune cells, and not on tumor cells, is an independent predictor of adverse progression-free survival (PFS) in all patients, including the microsatellite stable (MSS) subgroup (35). In addition, some reports evidenced that PD-L1 expression in intraepithelial immune cells was significantly more frequent in POLE mutant and MSI tumors, compared to MSS tumors (32, 37, 38), while PD-L1 expression in tumor cells did not differ between POLE mutant, MSI and MSS patients (32).

However, data regarding PD-L1 expression in cancer cells are controversial: one study showed that only 1 out of 116 tumors expressed PD-L1 on tumor cells, but this under-estimation could be linked with the use of tissue microarrays, since PD-L1 expression is known to be heterogenous (37).

Another study regarding gynecological samples, in 47 uterine sarcoma samples, found that PD-L1 expression was upregulated in comparison with normal endometrium, suggesting that this protein is a potential target for immunotherapy (7), while Bregar et al., using a smaller number of samples (10 patients), found that PD-L1 is expressed in only 30% of specimens (34).

PD-L2 in Endometrial Cancer

For PD-L2 very few data are available so far, and its expression seems to differ from PD-L1, with no significant difference between normal endometrium and tumor (5–7).

High PD-L2 expression was shown in 30% of primary endometrial carcinoma patients and 16% of uterine sarcoma patients, demonstrating the potential of PD-L2 blockade in a limited proportion of uterine cancer patients (7). It has been shown that PD-L1 and PD-L2 expression was more frequent in moderately, poorly differentiated, non-endometrioid endometrial cancer and seems to be correlated with POLE and MSI status (5, 33, 36). Type II endometrial cancer and poorly differentiated histological features are generally associated with worse prognosis and, in addition, PD-1 axis expression suggests that it may cause immunosuppression to favor tumor growth, thus negatively affecting patients' survival (5).

EXPRESSION OF PD-1, PD-L1, AND PD-L2 IN OVARIAN CANCER

Ovarian cancer is the most lethal disease among gynecological cancers (17, 22, 29–31) and is known to be an immunogenic tumor.

PD-1 and PD-L1 in Ovarian Cancers

Some reports showed that PD-L1 expression is found in epithelial ovarian cancers (EOC) (17, 20, 21, 24–26, 30), especially in serous ovarian cancers (SOC) (28, 29), ovarian clear cell carcinomas (OCCC) and in malignant ascites (31), a sign of peritoneal carcinomatosis derived from ovarian cancer (22).

In a cohort of 122 patients with OCCC, Zhu et al. showed that 55 cases (44.7%), classified as having high PD-L1 expression (PD-$L1^{high}$), were significantly associated with advanced stages (III–IV) (22). Cases with high PD-L1 and PD-1 expression showed significantly poorer PFS and OS, compared to those with low PD-L1/PD-1 expression (22, 24, 28, 29). In subgroup analysis, PD-$L1^{high}$ was associated with poorer prognosis compared to PD-$L1^{low}$ in platinum-resistant and advanced stages (III–IV) patients (22). Drake et al. analyzed 55 ovarian cancer biopsies and showed that PD-1 was detected in 87% of the tumors in both stroma and epithelium, while PD-L1 was only present in 33% of patients, exclusively in high-grade tumors (17). Additionally, they found that low density of PD-1 and PD-L1 expressing cells in tumor tissue was significantly associated with advanced disease, failing to show any significant association between survival and PD-1 or PD-L1 expression in ovarian

cancer (17), while patients with recurrent tumors and increased infiltrating PD-1$^+$ immune cells had longer OS (21). The correlation of PD-1 and PD-L1 expression with high-grade tumors and stage IV International Federation of Gynecology and Obstetrics (FIGO) disease has also been confirmed by other studies (28, 29).

Wieser et al. showed that, in a cohort of 158 patients with high-grade serous ovarian cancers, BRCA1/2 mutated tumors were characterized by high PD-1 expression, and that PD-L1 was observed mainly in BRCA1/2 and TP53 mutated cancers (29). Xiao et al. reported that PD-1 is expressed in tumor infiltrating lymphocytes and PD-L1 in tumor cells and in intratumoural immune cells, but there was no significant difference of PD-1$^+$ intratumoural immune cells in tumors with different mismatch repair (MMR) status (30). MSI ovarian cancers exhibited a significantly higher number of PD-L1$^+$ intratumoural immune cells compared to MSS ovarian cancers, while PD-L1 expression was not different in tumors, irrespectively from their MMR status (30).

In addition, no significant difference regarding PD-L1 expression in tumor cells and tumor infiltrating lymphocytes, and PD-1 expression in infiltrating lymphocytes, has been found between primary and recurrent disease (21).

PD-L2 in Ovarian Cancers

So far, only few studies investigated the expression of PD-L2 in ovarian cancer. An analysis on 70 patients showed that PD-L2 expression was not related to patient prognosis or other clinical variables, but negatively correlated with the number of FOXP3$^+$ T regulatory cells (Tregs) (24). Imai et al. analyzed the expression of PD-L1 and PD-L2 on tumor cells and APCs in malignant ascites from epithelial ovarian cancer patients (31), and found differential PD-L1 expression in tumor cells between patients with high or low PD-1-expressing CD4$^+$ T cells (43.9 and 27.3%, respectively), while no difference in PD-L1 expression was observed between patients with high and low PD-1 expression on CD8$^+$ T cells (34.1 and 27.3%, respectively). Between 2.3 and 3.2% of the patients with high or low PD-1 on CD4$^+$ T cells and CD8$^+$ T cells also expressed PD-L2. No correlation was found between PD-L1/2 expression and clinical variables or outcomes (31).

To support a potential role of PD-1 and PD-L1/ PD-L2 axis as targets in ovarian cancer, it has been reported in syngeneic orthotopic mouse model of epithelial ovarian cancer, that treatment with anti-PD-1 or anti-PD-L1 antibodies resulted in tumor rejection in 75% of the treated-mice, while mice treated with anti-PD-L2 antibody did not reject tumors (25). These data can be explained considering the selected models that expressed lower levels of PD-L2 than PD-1 and PD-L1. Additionally, PD-1 and PD-L1 blockade significantly increased the CD8$^+$ to Tregs and CD4$^+$ to Tregs ratios within the tumor, while, on the contrary, there was no significant change in the CD8$^+$ or CD4$^+$ to Tregs ratios (25).

EXPRESSION OF PD-1, PD-L1, AND PD-L2 IN OTHER GYNECOLOGICAL CANCERS

Cervical cancer is the third most common gynecological malignancy in Europe (23). Little information is available, up to now, regarding the expression of PD-1 ligands (23, 32, 39, 43–47).

A report from Howitt et al. showed that cervical cancer is a potential candidate for clinical trials testing PD-1 blockade (23, 32, 39). In fact, using FISH analysis on 48 Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens of cervical squamous cell carcinoma, they observed co-amplification or co-gain of PD-L1 and PD-L2 in 32 out of 48 cases (67%). Immunohistochemical staining for PD-L1 revealed high expression in 95% of the tumors with membranous staining pattern (32).

Persistent infection with human papilloma virus (HPV) is an essential step in the development of most cervical cancers (40). Some studies hypothesized that HPV may activate PD-1/PD-L1 to evade host immune responses, resulting in persistence of the cervical intraepithelial neoplasia (41). The identification of HPV as an etiological factor leads to antigen production and presentation, thereby making cervical cancer immunogenic (42). Recently, the role of the PD-1/PD-L1 axis in HPV associated head and neck squamous cell cancer (HPV-HNSCC) creating an "immune-privileged" site for initial viral infection and subsequent adaptive immune resistance suggests a rationale for therapeutic blockade of this pathway in patients with HPV-associated tumors (43). Significant PD-L1 expression in cervical carcinoma has been confirmed in several studies (44–47). As a consequence, this immunogenic disease requires a highly immunosuppressive microenvironment to progress and metastasize (48, 49) which has been demonstrated in tumor-positive lymph nodes where high Treg levels, low CD8$^+$ T cell/Treg ratio and high levels of PD-L1$^+$ and HLA-DR$^+$ myeloid cells were found (50).

Regarding another gynecological malignancy, vulvar cancer, the clinical relevance of PD-L1 expression has not been completely studied so far (32).

Although rare, incidence rates of vulvar cancer are increasing and, in locally advanced, metastatic or recurrent disease, prognosis is poor and new treatment modalities are needed (51). Screening of 23 vulvar squamous cell carcinomas revealed 6 cases (26%) with co-amplification of PD-1 ligands, 4 cases (17%) showed co-gain, 6 cases (26%) showed polysomy, and 7 cases (30%) showed disomy. Immunohistochemical staining for PD-L1 across all cases revealed the highest median PD-L1 protein expression in cases with co-amplification of PD-L1 and PD-L2, and decreasing values with decreasing genetic complexity (32). Previous studies showed that PD-L1 is expressed in the majority of vulvar squamous cell carcinoma samples (51–54), in both cancer cells and peritumoural immune cells (52–54). Additionally, its expression was related with several components of immune system (CD3$^+$, CD20$^+$, and CD68$^+$ intra-tumor immunocytes) (51, 54), while a significant correlation with immunosuppressive cell populations (FOxP3$^+$ Treg cells) was reported only by Sznurkowski et al. (54). Data analyzing the

TABLE 1 | Ongoing immunotherapy clinical trials for patients with endometrial cancer.

ClinicalTrials.gov identifier	Status	Interventions/alone or in combination	Phase
NCT02630823	Active, not recruiting	Pembrolizumab (anti-PD-1) + Paclitaxel/Carboplatin/Radiation (standard of care)	I
NCT02725489	Active, not recruiting	Durvalumab (anti-PD-L1)	II
NCT02728830	Active, not recruiting	Pembrolizumab (anti-PD-1)	Early I
NCT02646748	Active, not recruiting	Pembrolizumab (anti-PD-1) + itacitinib/INCB050465	I
NCT02914470	Active, not recruiting	Atezolizumab (anti-PD-L1) + cyclophosphamide/Carboplatin	I
NCT02521844	Active, not recruiting	Pembrolizumab (anti-PD-1) + ETC-1922159	I

TABLE 2 | Ongoing immunotherapy clinical trials for patients with ovarian cancer.

ClinicalTrials.gov identifier	Status	Interventions (alone or in combination)	Phase
NCT02608684	Active, not recruiting	Pembrolizumab (anti-PD-1) + Gemcitabine/Cisplatin	II
NCT02728830	Active, not recruiting	Pembrolizumab (anti-PD-1)	Early I
NCT03287674	Active, not recruiting	Nivolumab (anti-PD-1) + Cyclophosphamide/Fludarabine/TIL infusion/Interleukin-2/Ipilimumab	I/II
NCT03277352	Active, not recruiting	Pembrolizumab (anti-PD-1) + INCAGN01876/Epacadostat	I/II
NCT03312114	Active, not recruiting	Avelumab (anti-PD-L1)	II
NCT02674061	Active, not recruiting	Pembrolizumab (anti-PD-1)	II
NCT03029598	Active, not recruiting	Pembrolizumab (anti-PD-1) + Carboplatin	I/II
NCT02335918	Completed	Nivolumab (anti-PD-1) + varlilumab	I/II
NCT02915523	Active, not recruiting	Avelumab (anti-PD-L1) + entinostat	I/II
NCT02452424	Completed	Pembrolizumab (anti-PD-1) + PLX3397	I/II
NCT02644369	Active, not recruiting	Pembrolizumab (anti-PD-1)	II
NCT03073525	Active, not recruiting	Atezolizumab (anti-PD-L1)	II
NCT02526017	Active, not recruiting	Nivolumab (anti-PD-1) + FPA008	I
NCT02580058	Active, not recruiting	Avelumab (anti-PD-L1) + PLD	III
NCT03365791	Active, not recruiting	PDR001 (anti-PD-1) + LAG525	I
NCT02764333	Active, not recruiting	Durvalumab (anti-PD-L1) + TPIV200	II
NCT02431559	Active, not recruiting	Durvalumab (anti-PD-L1) + Pegylated Liposomal Doxorubicin	I/II
NCT02914470	Active, not recruiting	Atezolizumab (anti-PD-L1) + carboplatin, cyclophosphamide	I
NCT02725489	Active, not recruiting	Durvalumab (anti-PD-L1)	II
NCT01975831	Active, not recruiting	MEDI4736 (anti-PD-L1) + Tremelimumab	I
NCT03038100	Active, not recruiting	Atezolizumab (anti-PD-L1) + Carboplatin/Atezolizumab/Bevacizumab	III
NCT01772004	Active, not recruiting	Avelumab (anti-PD-L1)	I/II
NCT03574779	Active, not recruiting	TSR-042 (anti-PD-1) + Niraparib/Bevacizumab	II
NCT02521844	Active, not recruiting	Pembrolizumab (anti-PD-1) + ETC-1922159	I

clinical impact of PD-L1 expression in vulvar cancer reveal that it is not clear whether its expression correlates with clinicopathological parameters.

In summary, no significant associations were observed between PD-L1 presence and typical clinicopathological factors (51), except for tumor stage as reported by Sznurkowski et al. (54), and PD-L1 expression occurs more often in high risk HPV-negative samples (51). Regarding survival analysis, it is reported that PD-L1 expression did not influence the OS (51, 53), but patients with primary tumors positive for immune cells-PD-L1 expression had improved OS compared to negative ones (54).

The presence of PD-L1 also seems to be an independent prognostic factor for recurrence free survival (51).

ONGOING IMMUNOTHERAPY CLINICAL TRIALS IN GYNECOLOGICAL MALIGNANCIES

Several clinical trials are ongoing at the moment, according to the ClinicalTrials.gov database [accessed July 06, 2019], testing anti-PD-1/PD-L1 blockade alone or in combination in patients with endometrial, cervical, vulvar and ovarian cancer, while there are no ongoing clinical trials using anti-PD-L2 (**Tables 1**–**3**).

Clinical trials data were collected from ClinicalTrials.gov database, selecting only completed trials or in "Active, not recruiting" status.

TABLE 3 | Ongoing immunotherapy clinical trials for patients with cervical cancer.

ClinicalTrials.gov Identifier	Status	Interventions	phase
NCT01975831	Active, not recruiting	MEDI4736 (anti-PD-L1) + Tremelimumab	I
NCT02914470	Active, not recruiting	Atezolizumab (anti-PD-L1) + Carboplatin/Cyclophosphamide	I
NCT02725489	Active, not recruiting	Durvalumab (anti-PD-L1)	II
NCT02921269	Active, not recruiting	Atezolizumab (anti-PD-L1) + Bevacizumab	II
NCT02257528	Active, not recruiting	Nivolumab (anti-PD-1)	II
NCT03073525	Active, not recruiting	Atezolizumab (anti-PD-L1)	II

Endometrial Cancer

Regarding endometrial cancer, 6 clinical trials are ongoing (**Table 1**). Most of them are Phase I clinical trials and preliminary results, reported by the American Society of Clinical Oncology (asco.org), showed that atezolizumab (anti-PD-L1), and pembrolizumab (anti-PD-1) might be promising agents for endometrial cancer treatment.

Most relevant results showed that in a phase I study, 15 patients eligible based on PD-L1 status (>5% of positivity in tumor-infiltrating immune cells) were treated with atezolizumab and evaluated for safety and efficacy. Results showed that atezolizumab had a favorable safety profile and 13% (2/15) of patients showed a reduction in tumor size. A trend for higher PFS and OS has been observed in patients with high levels of tumor-infiltrating immune cells. Clinical benefit appeared to increase with higher PD-L1 expression, suggesting a link between PD-L1 status and response to atezolizumab. In addition, hypermutation, and/or high immune infiltration may be linked to response to PD-L1 blockade (Clinical trial information: NCT01375842) (55).

In a different phase I clinical trial, pembrolizumab was administered in 24 patients with endometrial carcinoma (excluding sarcomas), failure of prior systemic therapy, and PD-L1 expression in ≥1% of tumor or stromal cells. A reduction in tumor size was confirmed in 13.0% of the patients, while 3 patients achieved stable disease. PFS and OS rates were 19.0 and 68.8%, respectively. In conclusion, Pembrolizumab demonstrated an acceptable safety profile and anti-tumor activity (Clinical trial information: NCT02054806) (56).

Ovarian Cancer

For ovarian cancer 22 clinical trials are ongoing, 2 of which are completed (**Table 2**). Some of the early-phase clinical trials of anti-PD-1 or anti-PD-L1 antibodies have shown good safety profiles and durable anti-tumor response in certain patient population(s). However, their response rates remain between 10 and 15% (31, 57). Available interim reports from some of the trials show promising objective response rates (ORR) for the treatment of ovarian cancer with nivolumab (anti-PD-1) (ORR of 15%, $n = 20$ patients), pembrolizumab (ORR 11.5%, $n = 49$), or avelumab (anti-PD-L1) (ORR 10%, $n = 124$) (17, 58, 59). Preliminary data presented at the annual ASCO meeting in 2016 of a phase I trial evaluating durvalumab (anti-PD-L1) in combination with olaparib (PARP inhibitor), showed a disease control rate (DCR) of 67% for the doublet olaparib - durvalumab in a cohort including BRCA wild type triple negative breast cancer and EOC cases (23).

In the KEYNOTE-28 trial, which explored the activity of pembrolizumab in several solid tumors, outcome of ovarian cancer was ORR of 11.5%, and only 23.1% showed tumor shrinkage from baseline (57).

Cervical Cancer

For cervical cancer, 6 clinical trials are ongoing (**Table 3**). Most relevant findings showed that in a phase Ib study with 24 patients affected by advanced cervical squamous cell cancer and PD-L1 expression in ≥1% of tumor or stromal cells, pembrolizumab was well-tolerated and showed promising anti-tumor activity (Clinical trial information: NCT02054806) (60), while its clinical benefit was investigated in the phase 2 KEYNOTE-158 trial. Pembrolizumab administration has been also investigated in a single cohort trial enrolling 98 patients with recurrent or metastatic cervical cancer, expressing PD-L1 with a positive ratio of the number of all PD-L1–expressing cells (tumor cells, lymphocytes, macrophages) to the number of all tumor cells, or a Combined Positive Score (CPS) ≥1. The ORR in 77 patients was 14.3% (95% CI: 7.4, 24.1), including 2.6% complete responses and 11.7% partial responses. No responses were observed in patients with tumors negative for PD-L1 expression (CPS <1). Serious adverse reactions occurred in 39% of patients (Clinical trial information: NCT02628067) (61).

On June 12th 2018, pembrolizumab was approved by Food and Drug Administration (FDA), for treatment of patients with recurrent or metastatic cervical cancer, expressing PD-L1 (CPS ≥1) as determined by an FDA-approved test, with disease progression on or after chemotherapy[1].

In conclusion, since in all gynecological cancers ORR is around 10–15%, this emphasizes the need for combination treatments to improve efficacy of immune checkpoint (**Figure 2**).

[1]Merck & Co. Press Release Details. https://investors.merck.com/news/press-release-details/2018/FDA-Approves-Mercks-KEYTRUDA-pembrolizumab-for-Previously-Treated-Patients-with-Recurrent-or-Metastatic-Cervical-Cancer-Whose-Tumors-Express-PD-L1-CPS-Greater-Than-or-Equal-to-1/default.aspx

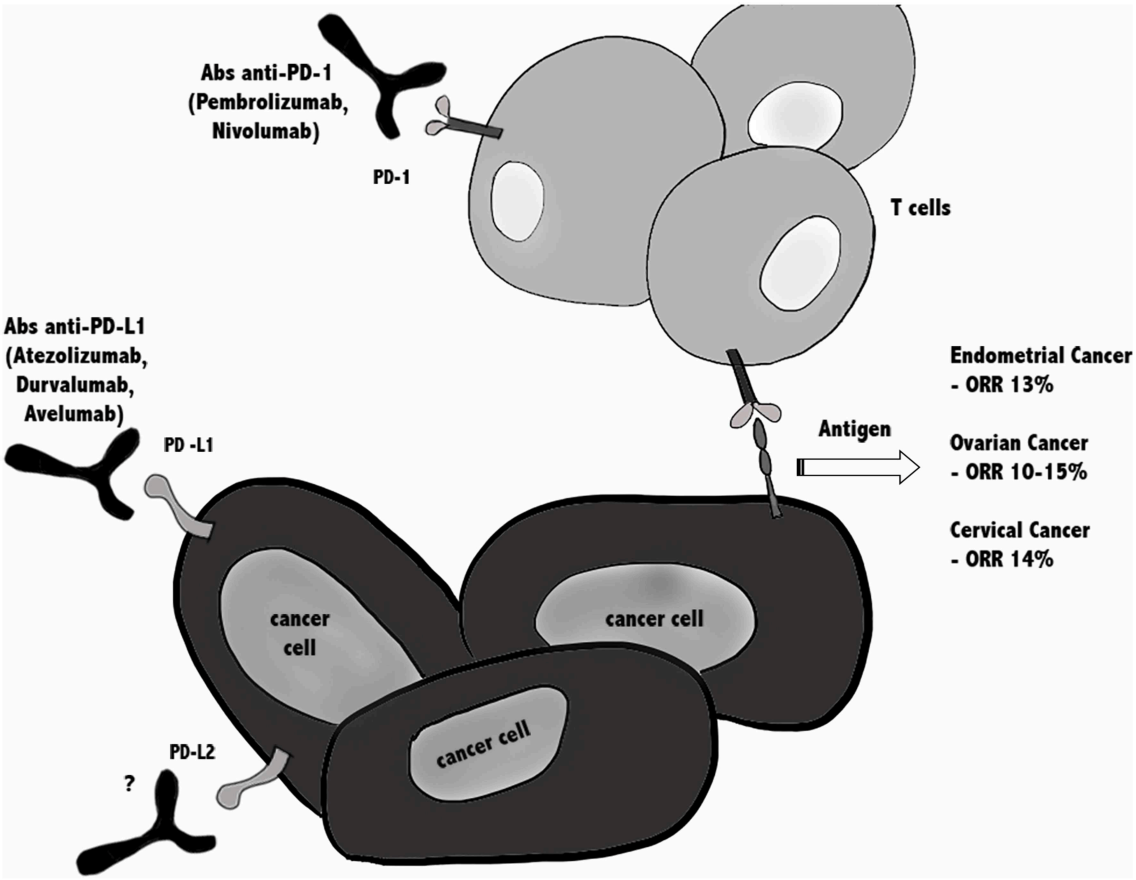

FIGURE 2 | Immunotherapy against PD-1/PD-Ls in gynecological cancers. Blocking the PD-1/PD-L1 immune checkpoint pathway by anti-PD-1 or anti-PD-L1 antibodies suppresses cancer cell survival and enhances the antitumor responses of T cells, leading to tumor regression and rejection. Actually, several clinical trials are ongoing testing anti-PD-1/PD-L1 blockade alone or in combination, in patients with endometrial, cervical, vulvar, and ovarian cancer, while there are no ongoing clinical trials using anti- PD-L2. In all gynecological cancers ORR is around 10–15%, argues for combinatorial treatments are taken in consideration.

FUTURE DIRECTIONS FOR IMMUNE CHECKPOINT INHIBITORS (ICIS) COMBINATION THERAPIES

Albeit ICIs therapies have been shown to induce durable responses and long-term remission in several cancer types, many patients fail to respond, develop resistance over the time or show immune-related adverse effects (62–65). The unresponsiveness or the toxicity of ICIs represents a strong rationale for the combination of ICIs with other treatments to increase the response rate of non-immunological tumors. For example, therapeutic approaches that induce the release and presentation of tumor antigens could be able to foster a *de novo* anti-tumor T cell response. In this regard, candidates for a combination therapy with ICIs could be cancer vaccines, oncolytic viruses, radiation, or low-dose chemotherapy (66).

Another potential combination approach with ICIs could be with bispecific antibodies, which recruit patient's T cells or NK cells against cancer cells expressing tumor-associated antigens. An example came from hematologic malignancies, wherein a bispecific antibody targeting both CD3 and CD123 (67, 68) was used but showed benefit in only a small fraction of patients. A major mechanism limiting the therapeutic efficacy was T cell anergy and exhaustion driven by ICIs pathways (mainly PD-L1/PD-1) (69). Inspired by this inhibitory role of ICIs pathway, combining ICIs with bispecific antibodies showed enhanced T cell proliferation and IFN-γ production (70).

One more possibility to improve ICI efficacy might be combination with cytokine therapy. The cytokine IL-2 has been approved for the treatment of metastatic renal cell carcinoma and advanced melanoma but is accompanied by severe side effects (71). However, modified IL-2 formulations such as bempegaldesleukin (NKTR-214) have an improved safety profile and have shown capabilities of enhancing the proliferation and activation of $CD8^+$ T cells and NK cells without increasing the number of Tregs (72). Recently, the PIVOT-02 trial (combination of NKTR-214 and nivolumab) has shown that this combination is safe and efficacious (ORR 48% in 23 patients) in metastatic urothelial carcinoma (73).

In addition, a recent study has demonstrated that DC-derived IL-12 is necessary for successful anti-PD-1 cancer therapy, suggesting that IL-12 and ICIs could be rationally combined (74).

Finally, there is strong rationale to combine anti-angiogenic therapies with ICI's, since anti-angiogenic therapies induce a normalization of the tumor vasculature, which leads to enhanced infiltration of T lymphocytes in the tumor.

CONCLUSION

Cancer immunotherapy is emerging as a promising component for cancer therapy. The most promising immunotherapy that showed good results involves antibodies targeting inhibitory immune checkpoint molecules (75).

Results obtained for patients with non-small cell lung cancer, renal cancer, and melanoma are evident and encouraging. However, in gynecological malignancies many aspects remain controversial in preclinical and clinical studies (23). Uncertain is the selection of patients because objective response rates remain low and retrospective analysis on biopsies showed opposing results for OS and PFS in patients with similar pattern of expression of PD-1 and its ligands (15, 17, 20–22, 24, 28, 29, 32, 34).

Regarding the second ligand PD-L2, it is needed to better clarify its role inside tumor microenvironment, together with the evaluation of other biological markers, in order to improve the efficiency of immunotherapy malignancies of the female genital tract.

AUTHOR CONTRIBUTIONS

OM, DA, CA, MN, FA, and ST wrote the paper. MM, GS, CA, and FM have revised the clinical trials and the paper.

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Incidence of Immune Checkpoint Inhibitor-Associated Diabetes: A Meta-Analysis of Randomized Controlled Studies

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Background: Immune checkpoint inhibitors (ICIs) are now an important option for more than 14 different cancers. Recent series case reports have described that ICIs are associated with new-onset diabetes in patients, yet the definitive risk is not available. We thus performed a meta-analysis of randomized controlled trials (RCTs) to assess the incidence and risk of developing new-onset diabetes following the use of ICIs.

Methods: The PubMed, EMBASE, Cochrane Library databases, and ClinicalTrials.gov for RCTs were searched. Statistical analyses were performed using STATA 15 and R language. Fifty-two RCTs were included, and 12 did not report any events of ICI-associated diabetes.

Results: A meta-analysis of 40 trials was performed, which reported at least one diabetes-related event among 24,596 patients. Although specific diabetes-related events were rare, compared with the placebo or other therapeutic strategies, the rates of serious hyperglycemia (OR 2.41, 95% CI 1.52 to 3.82), diabetes (3.54, 1.32 to 9.51), all-grade T1D (6.60, 2.51 to 17.30), and serious-grade T1D (6.50, 2.32 to 18.17) were increased with ICI drugs. Subgroup analysis according to the type of control, type of ICIs, and the combination mode suggested that ICIs plus conventional treatments significantly decreased the risks of diabetes and serious-grade hyperglycemia. There was little heterogeneity across the studies in all results except hyperglycemic events, which in part was attributable to data from everolimus-based control group.

Conclusions: New-onset diabetes is uncommon with ICIs but the risk is increased compared with placebo or another therapeutic strategy. However, more studies are warranted to substantiate these findings across ICIs.

Keywords: immune checkpoint inhibitors, diabetes, hyperglycemia, meta-analysis, safety outcomes

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INTRODUCTION

Immune checkpoint inhibitor (ICI)-based treatments that block molecules such as programmed cell death protein 1 (PD-1), PD1 ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) have emerged as powerful weapons in a growing number of cancers (Temel et al., 2018). Currently, nine ICIs have been approved for the treatment of different cancers: anti-PD-1 (nivolumab,

pembrolizumab, toripalimab, sintilimab, and cemiplimab); anti-PD-L1 (atezolizumab, avelumab, and durvalumab); and anti-CTLA-4 (ipilimumab). Immune checkpoint molecules play an important role in maintaining immunological tolerance to self-antigens and preventing autoimmune disorders (Pardoll, 2012). Consequently, their blockade in cancer therapy not only promotes T cell-mediated immune destruction on tumor cells but may also facilitate autoimmune activity that affects various organ systems (Johnson et al., 2018). Thus, ICIs frequently cause toxicities related to the mechanism of action that are generally referred to as immune-related adverse events (irAEs) (Postow et al., 2018).

Among these irAEs, new-onset diabetes is receiving increased attention, as more evidence suggests the recognition of diabetes-related adverse events in patients with cancers who are treated with ICIs. A marked increase in reporting diabetes has also been seen since 2017 by analyzing the World Health Organization's database of individual case safety reports (Wright et al., 2018). These observations raised concern as to whether ICI treatments could be associated with an increased risk of diabetes in patients with cancer. However, there has been no report of a meta-analysis of the incidence or risk of ICI-associated diabetes among the different ICIs in different tumor subtypes.

Given the dramatic growth in the number of clinical trials testing ICI agents and their clinical benefits in the increasing list of cancer types and negative influence on life quality caused by diabetes if not promptly recognized, we performed a meta-analysis of randomized controlled trials (RCTs) with ICIs in patients with cancer and evaluated the incidence and risks of diabetes-related adverse events compared with placebo or another therapeutic strategy.

METHODS

Search Strategy and Selection Criteria

Scientific literature searches were performed in three databases (PubMed, EMBASE, and Cochrane Central Register of Controlled Trials) from the inception of all searched databases to March 2019. Relevant text words and medical subject headings that consisted of terms including 'phase' and the individual drug names (details in Supporting Information **Table S1**) were searched. The search was limited to RCTs and English language. We also performed a manual search using reference lists from trials and review articles to identify any other relevant data. The ClinicalTrials.gov website was searched for RCTs that were labeled as 'completed' with available results. This meta-analysis was performed in adherence with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (Moher et al., 2009).

Study Selection

We included RCTs that were performed in adults with cancer and compared ICI treatment to another treatment strategy. The exclusion criteria were as follows: observational and retrospective studies; studies published in a meeting abstract without published full text original articles; quality of life studies; studies with only pediatric patients; 10 or fewer patients in any group; single dosing; cost effectiveness analyses; and those that could not assess the effect of ICI, such as when the control group was a different dose of the same ICI or another type of ICI. Two authors independently screened all titles and abstracts (HM and JZ). Two of three authors reviewed and discussed the potential full text. Any disagreements were resolved by consensus with all three (JL, HM, and JZ).

Data Extraction and Quality Assessment

Data from each study that met the inclusion criteria were independently extracted by two of the three authors (JL, HM, and YL). Any disagreement was resolved by consensus with all three. The retrieved data included author name, year of publication, trial characteristics (registry number, whether it was an international study, countries involved, study sites, and study phase), patient characteristics (sex, age, and performance status), the sizes of the intervention and control groups, ICI treatment, dose, and the outcomes of interest. We detected new-onset diabetes following treatment with ICIs using the following terms: hyperglycemia, diabetes mellitus (DM), type 2 diabetes (T2D), and type 1 diabetes (T1D). For data extracted from ClinicalTrials.gov, adverse events were reported as either serious or other; for data from published reports, we identified grades 3–5 as serious and grades 1–2 as other, according to Common Terminology of Clinical Adverse Events categorization. If data were available for both sources, we prioritized data from sources where the data were more complete. If a published study did not report diabetes-related adverse events, and the corresponding registry trial from ClinicalTrials.gov reported did, we included the registry report. For multiple reports of the same trial, only the most completely reported data were used. The quality of the included studies was independently assessed using the Cochrane Risk of Bias Tool. We considered all trials at unclear risk of incomplete outcome data and selective reporting bias as these studies were not designed primarily to assess adverse events.

Data Synthesis and Analysis

The estimated event rates in the intervention group are calculated as the total number of patients with a given adverse event divided by the total number at risk. Data were transformed using the Freeman-Tukey Double Arcsine transformation to calculate event rates. This statistical analyses were performed using R statistical software (package meta, R Foundation). For risk outcome, we pooled trials and calculated odds ratios (ORs) and their associated 95% confidence intervals (CIs) in the intervention group compared with the control group based on the number of patients with a given adverse event and sample size. Given the low rates of adverse events, we used Peto's method to pool effect estimates across studies. The I^2 statistic and P value were used to examine heterogeneity across trials for each outcome. An I^2 statistic of 0–25%, 26–75%, and 76–100% was regarded as indicating low, moderate, and high heterogeneity, respectively. A P value of less than or equal to 0.05 was defined as significant heterogeneity. If a study included more than one intervention group (e.g. different doses or different types of ICI), we separately compared each intervention group with the control group, where the number of patients or events in the control

group would be doubled. Sensitivity analyses were performed excluding an everolimus-controlled study, which was known to cause diabetes-related adverse events, to understand the reasons for the high likelihood of differences. We conducted subgroup analyses to examine studies according to the type of control group (chemotherapy vs. immunosuppressive drug vs. targeted therapy vs. placebo), the mode of intervention treatment (monotherapy vs. add-on therapy), and the type of ICI (PD-1 vs. PD-L1 vs. CTLA4 vs. combination of ICIs). Evidence of publication bias was assessed using Egger's and Begg's test in addition to funnel plots, and significant publication bias defined as a $P < 0.1$. All statistical analyses were conducted with STATA, version 15.

RESULTS

Study Search

Our search from the PubMed, EMBASE, and Cochrane Central Register databases yielded a total of 8,596 potentially relevant reports (**Figure 1**). After screening and eligibility assessment, we retrieved 67 reports for full text screening. We also identified 117 reports with results from ClinicalTrial.gov. After our formal search, three additional large clinical trials were published. We therefore also included these three studies. After further section, a total of 52 studies (7 from the trial registry and 45 from journals) were eligible. The included articles were published (online) between August 2010 and April 2019.

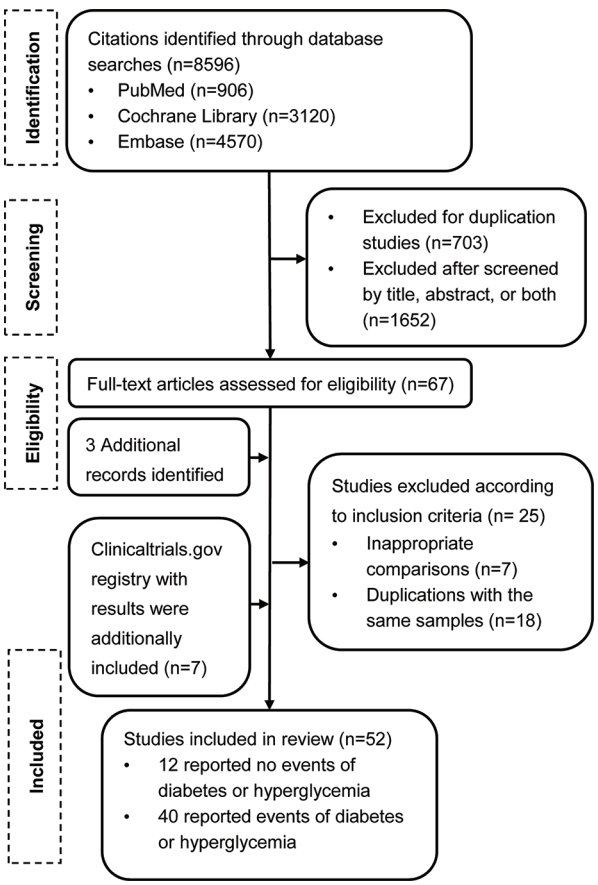

FIGURE 1 | Flow diagram of study selection.

Study Characteristics

All studies except one (Chih-Hsin Yang et al., 2019) were international multicenter studies. All studies were funded by the pharmaceutical industry, with sample sizes of the ICI intervention group ranging from 12 to 636 patients. Twenty-two were completed in patients with non-small-cell lung cancer, eight in melanoma, six in renal cell carcinoma, three in small-cell lung cancer, three in gastric and gastro esophageal junction cancer, two in head and neck squamous cell carcinoma, two in urothelial cancer, two in prostate cancer, two in breast cancer, one in colorectal cancer, and one in mesothelioma. Among these, patients in the intervention arm received nivolumab as monotherapy in ten studies, pembrolizumab in seven studies, atezolizumab in five studies, durvalumab in three studies, avelumab in one study, tremelimumab in three studies, combination therapy with anti-PD-1/PD-L1/CTLA-4 plus chemotherapy/radiotherapy in thirteen studies, combination therapy with anti-PD-1/PD-L1 plus anti-CTLA4 in three studies, combination therapy with anti-PD-1/PD-L1/CTLA-4 plus targeted therapy in seven studies, and combination therapy with ipilimumab plus vaccine in one study. All studies except one (Kang et al., 2017) had adverse event data on ClinicalTrials.gov. Key characteristics of these included trials are shown in **Table 1**.

Quality of the Included Studies

Table S2 shows the risk of bias assessment of the included studies for meta-analysis. All studies were RCTs with adequate reported randomization, and all studies were funded by the pharmaceutical industry with a high risk of sponsorship bias. Of the 40 included studies for meta-analysis, 26 (65%) were open labels with a high risk of blinding participants and personnel. None of the included studies specifically stated blinded assessment or collection of diabetes-related adverse events. We classified all trials at unclear risk of incomplete outcome data and selective reporting bias.

Incidence of Diabetes-Related Adverse Events

Of the 52 clinical controlled trials assessing the effects of ICIs, 40 trials described ICI-associated diabetes events during the course of study. Hyperglycemia events were described in 32 studies; 303 cases of all-grade hyperglycemia and 55 serious-grade hyperglycemia events occurred in 10,393 patients. Pooling the data showed that the rates of all-grade and serious-grade hyperglycemia events were 2.26% (95% CI, 1.28 to 3.48) and 0.28% (95% CI, 0.16 to 0.42), respectively. The rates of hyperglycemia events differed by the type of ICI and tumor. In particular, patients treated with ICI combination therapy were more likely to report hyperglycemia: 3.37% for all-grade hyperglycemia events, 0.47% for serious-grade hyperglycemia. Patients with RCC showed a trend toward higher rates of both all-grade and serious-grade hyperglycemia, with rates of 6.82%

TABLE 1 | Characteristics of controlled trials of ICI treatment in patients.

NCT Author (year)	International study	No. of countries involved	No. of study sites	Phase	Group type	Drug	Dose of ICI (mg/kg)	No. of patients	Age Median (range)	No (%) Male	Tumor type
NCT00527735 (Reck et al., 2013)	Yes	8	NR	Phase 2	CTLA4	Ipilimumab Paclitaxel/carboplatin	10	113	NR	NR	NSCLC
					CTLA4	Ipilimumab Paclitaxel/carboplatin	10	109	NR	NR	
					Control	Paclitaxel/carboplatin	/	109	NR	NR	
NCT00861614 (Kwon et al., 2014)	Yes	26	191	Phase 3	CTLA4	Ipilimumab Radiotherapy	10	399	69 (47–86)	399	Prostate cancer
					Control	Placebo radiotherapy	/	400	67.5 (45–86)	400	
NCT01673867 (Borghaei et al., 2015)	Yes	22	NR	Phase 3	PD-1	Nivolumab	3	292	61 (37–84)	151 (52)	NSCLC
					Control	Docetaxel	/	290	64 (21–85)	168 (58)	
NCT01642004 (Brahmer et al., 2015)	Yes	20	NR	Phase 3	PD-1	Nivolumab	3	135	62 (39–85)	111 (82)	NSCLC
					Control	Docetaxel	/	137	64 (42–84)	97 (71)	
NCT00636168 (Eggermont et al., 2015)	Yes	19	91	Phase 3	CTLA4	Ipilimumab	10	475	51 (20–84)	296 (62)	Melanoma
					Placebo	Placebo	/	476	52 (18–78)	293 (62)	
NCT01668784 (Motzer et al., 2015)	Yes	24	146	Phase 3	PD-1	Nivolumab	3	410	62 (23–88)	315 (77)	RCC
					Control	Everolimus	/	411	62 (18–86)	304 (74)	
NCT01704287 (Ribas et al., 2015)	Yes	12	73	Phase 2	PD-1	Pembrolizumab	2	180	62 (15–87)	104 (58)	Melanoma
					PD-1	Pembrolizumab	10	181	60 (27–89)	109 (60)	
					Control	Carboplatin/paclitaxel Dacarbazine Temozolomide	/	179	63 (27–87)	114 (64)	
NCT01721772 (Robert et al., 2015)	Yes	16	80	Phase 3	PD-1	Nivolumab	3	210	64 (18–86)	121 (57.6)	Melanoma
					Control	Dacarbazine	/	208	66 (26–87)	125 (60.1)	
NCT01721746 (Weber et al., 2015)	Yes	14	90	Phase 3	PD-1	Nivolumab	3	272	59 (23–88)	176 (65)	Melanoma
					Control	Dacarbazine/carboplatin/ paclitaxel	/	133	62 (29–85)	85 (64)	
NCT01903993 (Fehrenbacher et al., 2016)	Yes	13	61	Phase 2	PD-L1	Atezolizumab	1,200 mg/dose	144	62 (42–82)	93 (65)	NSCLC
					Control	docetaxel	/	143	62 (36–84)	76 (53)	

(Continued)

TABLE 1 | Continued

NCT Author (year)	International study	No. of countries involved	No. of study sites	Phase	Group type	Drug	Dose of ICI (mg/kg)	No. of patients	Age Median (range)	No (%) Male	Tumor type
NCT02105636 (Ferris et al., 2016)	Yes	15	NR	Phase 3	PD-1	Nivolumab	3	240	59 (29–83)	197 (82.1)	HNSCC
					Control	Cetuximab/ methotrexate/docetaxel	/	121	61 (28–78)	103 (85.1)	
NCT01905657 (Herbst et al., 2016)	Yes	24	202	Phase 2/ 3	PD-1	Pembrolizumab	2	344	63 (56–69)	212 (62)	NSCLC
					PD-1	Pembrolizumab	10	346	63 (56–69)	213 (62)	
					Control	Docetaxel	/	343	62 (56–69)	209 (61)	
NCT02039674 (Langer et al., 2016)	Yes	2	26	Phase 2	PD-1	Pembrolizumab Carboplatin/pemetrexed	200 mg/dose	60	62.5 (54–70)	22 (37)	NSCLC
					Control	Carboplatin/pemetrexed	/	63	63.2 (58–70)	26 (41)	
NCT01450761 (Reck et al., 2016a)	Yes	34	224	Phase 3	CTLA4	Ipilimumab Etoposide/cisplatin/ carboplatin	10	478	62 (39–85)	371 (66)	SCLC
					Control	Placebo Etoposide/cisplatin/ carboplatin	/	476	63 (36–81)	326 (68)	
NCT02142738 (Reck et al., 2016b)	Yes	16	142	Phase 3	PD-1	Pembrolizumab	200 mg/dose	154	64.5 (33–90)	92 (59.7)	NSCLC
					Control	Paclitaxel/carboplatin/ pemetrexed/cisplatin/ gemcitabine	/	151	66 (38–85)	95 (62.9)	
NCT02008227 (Rittmeyer et al., 2017)	Yes	31	194	Phase 3	PD-L1	Atezolizumab	1,200 mg/dose	613	NR	378 (61.7)	NSCLC
					Control	Docetaxel	/	612	NR	379 (61.9)	
NCT02125461 (Antonia et al.,2017)	Yes	26	235	Phase 3	PD-L1	Durvalumab	10	476	64 (31–84)	334 (70.2)	NSCLC
					Control	Placebo	/	237	64 (23–90)	166 (70)	
NCT01057810 (Beer et al., 2017)	Yes	24	NR	Phase 3	CTLA4	Ipilimumab	10	399	NR	100	Prostate cancer
					Control	Placebo	/	199	NR	100	
NCT02256436 (Rogers et al., 2017)	Yes	120	29	Phase 3	PD-1	Pembrolizumab	200 mg/dose	270	67 (29–88)	200 (74.1)	Urothelial carcinoma
					Control	Paclitaxel/docetaxel/ vinflunine	/	272	65 (26–84)	202 (74.3)	
NCT02041533 (Carbone et al., 2017)	Yes	26	NR	Phase 3	PD-1	Nivolumab	3	271	63 (32–89)	184 (68)	NSCLC

(Continued)

TABLE 1 | Continued

NCT Author (year)	International study	No. of countries involved	No. of study sites	Phase	Group type	Drug	Dose of ICI (mg/kg)	No. of patients	Age Median (range)	No (%) Male	Tumor type
					Control	Gemcitabine/cisplatin Carboplatin/paclitaxel/ pemetrexed					
							/	270	65 (29–87)	148 (55)	
NCT01285609 (Govindan et al., 2017)	Yes	34	233	Phase 3	CTLA4	Ipilimumab Paclitaxel/carboplatin	10	479	NR	NR	NSCLC
					Control	Placebo Paclitaxel/carboplatin	/	477	NR	NR	
NCT02267343 (Kang et al., 2017)	Yes	3	49	Phase 3	PD-1	Nivolumab	3	330	62 (54–69)	229 (69)	GEJ
					Control	Placebo	/	163	61 (53–68)	119 (73)	
NCT01843374 (Llombart-Cussac et al., 2017)	Yes	19	105	Phase 2b	CTLA4	Tremelimumab	10	382	66 (60–72)	283 (74.1)	Mesothelioma
					Control	Placebo	/	189	67 (61–73)	151 (79.9)	
NCT02302807 (Powles et al., 2018)	Yes	29	217	Phase 3	PD-L1	Atezolizumab	1,200 mg/dose	467	67 (33–88)	357 (76)	Urothelial bladder
					Control	Vinflunine/paclitaxel/ docetaxel	/	464	67 (31–84)	361 (78)	cancer
NCT02395172 (Barlesi et al., 2018)	Yes	31	173	Phase 3	PD-L1	Avelumab	10	396	64 (58–69)	269 (67.9)	NSCLC
					Control	Docetaxel	/	396	63 (57–69)	273 (68.9)	
NCT02362594 (Eggermont et al., 2018)	Yes	23	123	Phase 3	PD-1	Pembrolizumab	200 mg/dose	514	54 (19–88)	324 (63)	Melanoma
					Control	Placebo	/	505	54 (19–83)	304 (60.2)	
NCT02578680 (Gandhi et al., 2018)	Yes	16	126	Phase 3	PD-1	Pembrolizumab Pemetrexed/cisplatin	200 mg/dose	410	65 (34–84)	254 (62)	SCLC
					Control	Pemetrexed/cisplatin	/	206	63.5 (34–84)	109 (52.9)	
NCT02231749 (Motzer et al., 2018)	Yes	28	175	Phase 3	PD-1/CTLA4	Nivolumab ipilimumab	3 1	550	NR	NR	RCC
					Control	sunitinib	/	546	NR	NR	
NCT02775435 (Paz-Ares et al., 2018)	Yes	17	137	Phase 3	PD-1	Pembrolizumab Paclitaxel/nab-paclitaxel/ carboplatin	200 mg/dose	278	65 (29–87)	220 (79.1)	NSCLC
					Control	Paclitaxel/Nab-paclitaxel/ Carboplatin	/	281	65 (36–88)	235 (83.6)	

(Continued)

TABLE 1 | Continued

NCT Author (year)	International study	No. of countries involved	No. of study sites	Phase	Group type	Drug	Dose of ICI (mg/kg)	No. of patients	Age Median (range)	No (%) Male	Tumor type
NCT02370498 (Shitara et al., 2018)	Yes	30	148	Phase 3	PD-1	Pembrolizumab	200 mg/dose	296	62.5 (54–70)	202 (68)	GEJ
					Control	Pacitraxel	/	296	60.0 (53–68)	208 (70)	
NCT02252042 (Cohen et al., 2019)	Yes	20	97	Phase 3	PD-1	Pembrolizumab	200 mg/dose	247	60.0 (55–66)	207 (84)	HNSCC
					Control	Methotrexate Docetaxel/ cetuximab	/	248	60.0 (54–66)	205 (83)	
NCT02788279 (Eng et al., 2019)	Yes	11	73	Phase 3	PD-L1	Atezolizumab Cobimetinib	840 mg/dose	183	58 (51–67)	107 (58)	Colorectal cancer
					PD-L1	Atezolizumab	1,200 mg/dose	90	56 (51–64)	59 (66)	
					Control	Regorafenib	/	90	59 (52–66)	51 (57)	
NCT02220894 (Mok et al., 2019)	Yes	32	213	Phase 3	PD-1	Pembrolizumab	200 mg/dose	636	63 (56–69)	450 (71)	NSCLC
					Control	Platinum	/	615	63 (57–69)	452 (71)	
NCT02613507 (Wu et al., 2019)	Yes	3	32	Phase 3	PD-1	Nivolumab	3	338	60 (27–78)	236 (78)	NSCLC
					Control	Docetaxel	/	166	60 (38–78)	134 (81)	
NCT02454933 (Chih-Hsin Yang et al., 2019)	No	1	1	Phase 3	PD-L1	Durvalumab Osimertinib	10 mg/kg	12	56 (41–78)	6 (50)	NSCLC
					Control	Osimertinib	/	17	65 (41–80)	4 (24)	
NCT01585987 (Squibb, 2012)	Yes	12	NR	Phase 2	CTLA4	Ipilimumab	10 mg/kg	57	NR	NR	GEJ
					Control	Fluoropyrimidine	/	57	NR	NR	
NCT01984242 (Roche, 2014)	Yes	9	NR	Phase 2	PD-L1	Atezolizumab Bevacizumab	1,200 mg/dose	101	NR	74 (73.3)	RCC
					PD-L1	Atezolizumab	1,200 mg/dose	103	NR	77 (74.8)	
					Control	Sunitinib	/	101	NR	79 (78.2)	
NCT02367781 (Roche, 2015b)	Yes	36	NR	Phase 3	PD-L1	Atezolizumab Nab-paclitaxel/ carboplatin	1,200 mg/dose	483	NR	NR	NSCLC
					Control	Nab-paclitaxel/ carboplatin	/	240	NR	NR	
NCT02352948 (AstraZeneca, 2015)	Yes	NR	82	Phase 3 subA	PD-L1	Durvalumab	10	62	NR	42 (67.7)	NSCLC
					PD-L1/CTLA4	Durvalumab Tremelimumab	20 1	174	NR	115 (66.1)	
	Yes	NR	143	Phase 3 subB	Control	Eerlotinib/gemcitabine/ vinorelbine	/	64	NR	48 (75.0)	
					PD-L1	Durvalumab	10	117	NR	73 (62.4)	

(Continued)

TABLE 1 | Continued

NCT Author (year)	International study	No. of countries involved	No. of study sites	Phase	Group type	Drug	Dose of ICI (mg/kg)	No. of patients	Age Median (range)	No (%) Male	Tumor type
					CTLA4	Tremelimumab	10	60	NR	39 (65.0)	
					Control	Gemcitabine/vinorelbine	/	118	NR	81 (68.6)	
NCT02420821 (Roche, 2015a)	Yes	21	NR	Phase 3	PD-L1	Atezolizumab Bevacizumab	1,200 mg/dose	451	NR	NR	RCC
					Control	Sunitinib	/	446	NR	NR	
NCT00094653 (Hodi et al., 2010)	Yes	13	125	Phase 3	CTLA4	Ipilimumab gp100	3	403	55.6[a]	247 (61.3)	Melanoma
					CTLA4	Ipilimumab	3	137	56.8[a]	81 (59.1)	
					Control	gp100	/	136	57.4[a]	73 (53.7)	
NCT00324155 (Robert et al., 2011)	Yes	NR	25	Phase 3	CTLA4	Ipilimumab Dacarbazine	10	250	57.5[a]	152 (60.8)	Melanoma
					Control	Dacarbazine	/	252	56.4[a]	149 (59.1)	
(Lynch et al., 2012)	Yes	NR	NR	Phase 2	CTLA4	Ipilimumab Paclitaxel/carboplatin	10	70	NR	NR	NSCLC
					CTLA4	Ipilimumab Paclitaxel/carboplatin	10	68	NR	NR	
					Control	Paclitaxel/carboplatin	/	66	NR	NR	
NCT00257205 (Ribas et al., 2013)	Yes	24	114	Phase 3	CTLA4	Tremelimumab	15	328	57[a]	190 (58)	Melanoma
					Control	Dacarbazine/ temozolomide	/	327	56[a]	182 (56)	
NCT02477826 (Hellmann et al., 2017)	Yes	36	NR	Phase 3	PD-1/ CTLA4	Nivolumab/ipilimumab	3 1	396	NR	NR	PD-L1 expression≥1% NSCLC
					PD-1	Nivolumab	240 mg/dose	396	NR	NR	
					Control	Platinum	/	397	NR	NR	
					PD-1/CTLA4	Nivolumab/ipilimumab	3 1	187	NR	NR	PD-L1 expression
					PD-1	Nivolumab	360 mg/dose	177	NR	NR	<1% NSCLC
					Control	Platinum	/	186	NR	NR	
NCT02763579 (Horn et al., 2018)	Yes	21	106	Phase 3	PD-L1	Atezolizumab Carboplatin/etoposide	1,200 mg/dose	201	64 (28-90)	129 (64.2)	SCLC
					Control	Carboplatin/etoposide	/	202	64 (26-87)	132 (65.3)	
NCT02425891 (Schmid et al., 2018)	Yes	41	246	Phase 3	PD-L1	Atezolizumab Nab-paclitaxel	840 mg/dose	451	55 (20-82)	3 (0.7)	Breast cancer
					Control	Placebo nab-paclitaxel	/	451	56 (26-86)	1 (0.2)	
NCT02366143 (Socinski et al., 2018)	Yes	26	240	Phase 3	PD-L1	Atezolizumab Bevacizumab/ barboplatin/paclitaxel	1,200 mg/dose	400	63 (31-89)	240 (60.0)	NSCLC
					PD-L1	Atezolizumab Carboplatin/paclitaxel	1,200 mg/dose	402	NR	NR	
					Control	Bevacizumab/ carboplatin/paclitaxel	/	400	63 (31-90)	239 (59.8)	
NCT02684006 (Motzer et al., 2019)	Yes	21	144	Phase 3	PD-L1	Avelumab Axitinib	10	442	62 (29-83)	316 (71.5)	RCC

(Continued)

TABLE 1 | Continued

NCT Author (year)	International study	No. of countries involved	No. of study sites	Phase	Group type	Drug	Dose of ICI (mg/kg)	No. of patients	Age Median (range)	No (%) Male	Tumor type
					Control	Sunitinib	/	444	61 (27-88)	344 (77.5)	
NCT02853331 (Rini et al., 2019)	Yes	16	129	Phase 3	PD-1	Pembrolizumab Axitinib	200 mg/dose	432	62 (30-89)	308 (71.3)	RCC
					Control	Sunitinib	/	429	61 (26-90)	320 (74.6)	
NCT02250326 (Celgene, 2015)	Yes	7	34	Phase 2	PD-L1	Nab-Paclitaxel Durvalumab	1,125 mg/m^2	79	NR	54 (68.4)	NSCLC
					Control	Nab-paclitaxel	/	80	NR	50 (62.5)	
					Control	Nab-paclitaxel CC-486	/	81	NR	50 (61.7)	
NCT02924883 (Roche, 2016)	Yes	9		Phase 3	PD-L1	Atezolizumab Trastuzumab/emtansine	1,200 mg/dose	133	NR	2 (1.5)	Breast cancer
					Control	Placebo Trastuzumab/emtansine	/	69	NR	0 (0.0)	

GEJ, gastric and gastroesophageal junction cancer; HNSCC, head and neck squamous cell carcinoma; ICI, immune checkpoint inhibitors; NR, not reported; NSCLC, non-small-cell lung cancer; RCC, renal cell carcinoma; SCLC, small-cell lung cancer. [a]Age mean.

and 0.66%, respectively. High dose of ICIs was not associated with high rates of hyperglycemia events (**Table 2**).

Due to the smaller number of other ICI-associated diabetes events, no statistical inferences of the rates were made. Overall, 13 cases of DM occurred in 5,655 patients (raw event rate 0.23%), five cases of T2D occurred in 3,117 patients (raw event rate 0.16%), and 17 cases of all-grade T1D occurred in 3,899 patients (raw event rate 0.44%), and 15 cases of serious-grade T1D events occurred in 3,603 patients (raw event rate 0.42%).

Risk of Diabetes-Related Adverse Events

To assess the relative rate of ICI-associated diabetes compared with those in control arms, we calculated the OR of developing diabetes in the RCTs. Pooling the data of these studies showed that patients treated with ICIs were at higher risk for serious-grade hyperglycemia (OR 2.41, 95% CI 1.52 to 3.82, **Figure 2**), DM (OR 3.54, 95% CI 1.32 to 9.51, **Figure 3**), all-grade T1D (OR 6.60, 95% CI 2.51 to 17.30, **Figure S1**), and serious-grade T1D (OR 6.50, 95% CI 2.32 to 18.17, **Figure 4**) than those treated with other regimens. ICIs showed a trend toward an increased risk of all-grade hyperglycemia (OR 1.38, 95% CI 1.15 to 1.66, **Figure S2**), but no increased risk of T2D (OR 0.92, 95% CI 0.24 to 3.52, **Figure S3**). Excluding the study in which the control group was everolimus, a drug known to cause diabetes, the risk of ICI-associated diabetes events were also higher than the control: OR 4.42 for DM, OR 1.75 for all-grade hyperglycemia, OR 2.81 for serious-grade hyperglycemia (**Figures S4–S6**).

TABLE 2 | Incidence of hyperglycemia events in patients treated with immune checkpoint inhibitors. Values are percentages (95% confidence intervals).

Characteristic	All-grade hyperglycemia	Serious-grade hyperglycemia
Total	2.26 (1.28, 3.48)	0.28 (0.16, 0.42)
ICI type		
PD-1 inhibitors	4.86 (2.86, 7.32)	0.49 (0.26, 0.78)
PD-L1 inhibitors	0.81 (0.07, 2.06)	\
CTLA-4 inhibitors	0.52 (0.09, 1.18)	0.06 (0.00, 0.28)
Combination therapy	3.37 (0.00, 21.49)	0.47 (0.00, 2.01)
Tumor type		
NSCLC	2.54 (1.10, 4.43)	0.22 (0.06, 0.45)
Melanoma	1.75 (0.31, 4.15)	0.35 (0.09, 0.73)
RCC	6.82 (2.00, 14.05)	0.66 (0.27, 1.18)
Prostate cancer	0.12[a]	0.12[a]
Colorectal cancer	0.37[a]	/
GEJ	0.57[a]	0.53[a]
HNSCC	5.42[a]	0.42[a]
Mesothelioma	0.52[a]	0.52[a]
SCLC	0.63[a]	0.63[a]
Dose		
High dose	1.33 (0.27, 2.99)	0.22 (0.00, 0.80)
Normal dose	2.52 (1.32, 4.03)	0.28 (0.15, 0.44)

GEJ, gastric and gastroesophageal junction cancer; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small-cell lung cancer; RCC, renal cell carcinoma; SCLC, small-cell lung cancer.

High doses: including Ipilimumab 10 mg/kg and pembrolizumab 10 mg/kg.

[a]Raw event rate.

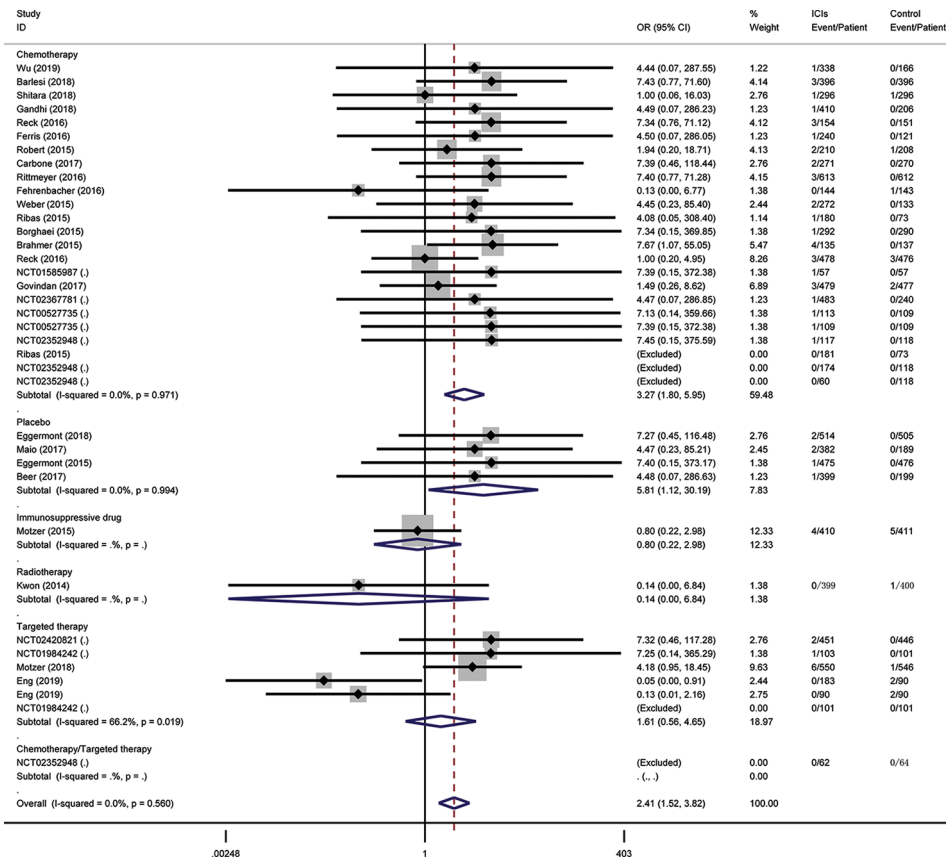

FIGURE 2 | Risk of serious-grade hyperglycemia following the use of ICIs versus control treatment, stratified by the type of control group.

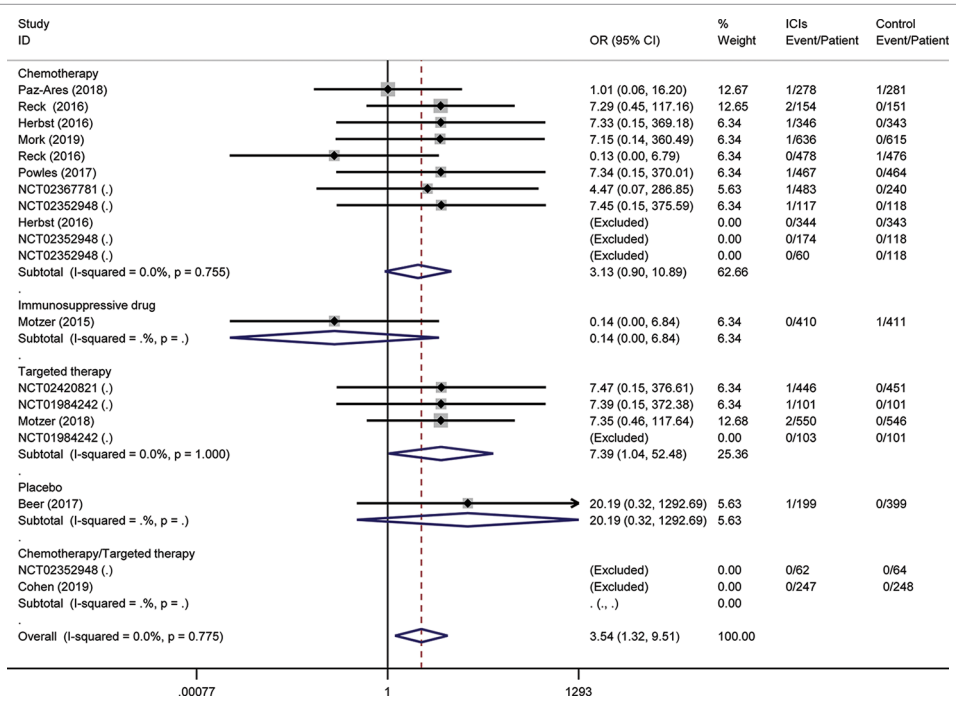

FIGURE 3 | Risk of diabetes mellitus following the use of ICIs versus control treatment, stratified by the type of control group.

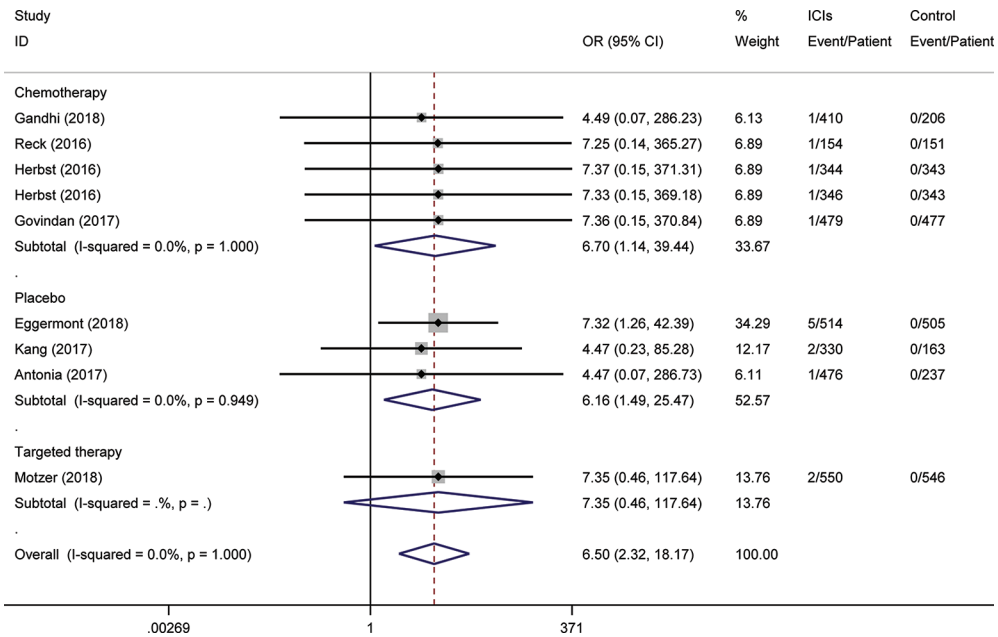

FIGURE 4 | Risk of serious-grade type 1 diabetes following the use of ICIs versus control treatment, stratified by the type of control group.

Subgroup analysis for these outcomes was stratification by the type of control, the mode of treatment, and type of ICI. Regarding the type of control, there were apparent differences across subgroups for the risk of ICI-associated diabetes events. Within the placebo-controlled group, ICIs were associated with a higher risk in hyperglycemia (OR 5.81). Subgroup analysis based on the mode of treatment (monotherapy vs. add-on therapy) suggests that add-on therapy decreased the risk of ICI-associated diabetes, with OR 1.77 for DM, 1.31 for serious-grade hyperglycemia, 0.58 for T2D, and 5.83 for T1D (**Figures S7–S11**). The subgroup analysis by the type of ICI suggests the risk of these events was increased in the subset of trials in which anti-PD-1 or anti-PD-L1 was combined with anti-CTLA-4, with OR 7.35 for DM, 2.51 for all-grade hyperglycemia, 4.18 for serious-grade hyperglycemia (**Figures S12–S17**).

The funnel plot and statistical test showed no evidence of publication bias for DM (Egger's test $P = 0.994$), all-grade hyperglycemia (Egger's test $P = 0.128$), serious-grade hyperglycemia (Egger's test $P = 0.325$), T2D (Egger's test $P = 0.310$), all-grade T1D (Egger's test $P = 0.300$), and serious-grade T1D (Egger's test $P = 0.334$) (**Table S3**, **Figures S18–S23**). We noted no heterogeneity in the effects of ICI on DM, serious-grade hyperglycemia, T2D, all-grade T1D, and serious-grade T1D ($I^2 = 0.0\%$). However, we noted substantial heterogeneity for the outcome of all-grade hyperglycemia ($I^2 = 88.2\%$), which was considerably reduced in the analyses of data excluding the everolimus-controlled study ($I^2 = 8.0\%$).

DISCUSSION

We completed a systematic analysis of new-onset diabetes following treatment with ICIs versus other therapeutic regimens to further our understanding of the safety of these agents. We used data from 40 RCTs that included 13,787 patients treated with ICIs, and also extracted data from the ClinicalTrials.gov results database to supplement the published studies. To our knowledge, this is the largest and most comprehensive meta-analysis on the incidence and risk of ICI-associated diabetes events following the use of ICI regimens published to date, although previous case series analyses showed that there is an increased reporting of rapidly progressive ICI-associated diabetes (Wright et al., 2018; Kotwal et al., 2019; Perdigoto et al., 2019). This meta-analysis shows that the risk of serious-grade hyperglycemia, DM, and T1D following ICIs is significantly higher compared with patients treated with other regimens, but provides no support that ICI treatment is associated with an increased risk of all-grade of hyperglycemia. Among patients on each different ICI regimens, patients on combination therapy were more likely to develop hyperglycemia.

Although the incidence was low, T1D has emerged as the highest risk associated with ICI therapy compared with other diabetes-related adverse events. The pathogenesis of T1D in the populations of patients receiving ICIs is not currently well understood. Several case reports have shown that the presence of autoantibodies before ICIs-based therapy might be at risk of developing diabetes, particularly in treated with anti-PD-1/anti-PD-L1 (Gauci et al., 2017; Usui et al., 2017; Way et al., 2017). Further support for autoimmune-based mechanism has been shown by Clotman et al. (2018), who overviewed the reported cases and demonstrated that approximately half of the tested cases of ICI-associated T1D had detectable diabetes-related autoantibodies. Other studies have shown that anti-PD-1 resulted in a rapid progression of autoimmune diabetes in patients with a high underlying genetic predisposition to T1D

(Mellati et al., 2015), raising the concern for genetic factors as a possible mechanism in patients with diabetes-prone HLA genotypes. Similar to what has been described in humans, the study demonstrated that PD-1 or PD-L1 blockade rapidly precipitated diabetes in prediabetic nonobese diabetic (NOD) mice (Ansari et al., 2003). Taken together, these studies reveal a potential mechanism of ICI-associated T1D that involves in both diabetes-related immunologic and genetic factors.

The subgroup analysis showed that the risk of ICI-associated T1D was different among the different type of ICIs. One possible explanation for this would be the mechanistic link to each target. Unlike the PD-1 pathway, which modulates effector cells, CTLA-4 functions in early immune responses during T cell priming and activation (Topalian et al., 2016). As such, the distinct function of the PD-1 and CTLA4 potentially contributed to different rates of T1D following the use of ICIs. In NOD mice, CTLA-4 blockade negatively physiologically regulated diabetes in only the early stages of life compared with the PD-1 pathway (Ansari et al., 2003). Additionally, there was strong PD-L1 expression in the inflamed islets of NOD mice, which suggested that the PD-1-mediated regulation of autoreactive immune cells played an important role at the site of islet inflammation (Ansari et al., 2003). However, this finding should be interpreted cautiously; more data are needed for definitive conclusions given the low absolute number of T1D in patients receiving ICIs.

ICIs plus conventional treatments have been tested in multiple solid tumors, which achieved synergetic effects and overcame the resistance to immunotherapy (Yan et al., 2018). When we combined all non-ICI therapy into one control category, the ICI-based regimens substantially increased the risk of ICI-associated diabetes compared with control group. However, this magnitude was reduced when ICIs were used as an add-on therapy. The risk of DM was 200% lower in the add-on therapy than in the ICI monotherapy. There was also a substantial reduction (over 175%) in ICI-associated serious-grade hyperglycemia in the setting of conventional treatments. These results consistently suggested that compared with ICI therapy, ICIs plus traditional therapy could result in a decreased risk of diabetes-related adverse events.

We found little heterogeneity across studies for all results except hyperglycemia, which strengthens the primary conclusion that ICIs increased risks of diabetes events. A sensitivity analysis identified that everolimus-based control group is responsible for this heterogeneity. Everolimus is an mTOR inhibitor, which is known to influence insulin signaling pathway in peripheral tissues and insulin secretion in *pancreatic β cells* (Tuo and Xiang, 2018). It has described that mTOR inhibitors resulted in a 5-fold increase in the risk for severe hyperglycemia in patients with cancer (Verges, 2018). Thus, when everolimus was presented separately, the heterogeneity was reduced.

There are several limitations in the present study. We conducted this analysis in study-level, rather than individual patient data. It is not possible to assess potential risk factors that are associated with higher risk of new-onset diabetes, due to the lack of detailed clinical data such as sex, diabetes-prone HLA genotypes, presence of autoantibodies, and islet function in patients receiving ICIs therapy. Secondly, subgroup effects could not be evaluated when there were less than two trials in each subgroup, which could not allow assessing whether the rates of ICI-associated diabetes are varied based on the type of tumor and the dose of ICIs. Our results showed that high dose of ICIs did not contribute to high rates of hyperglycemia events, while the type of tumor showed association of treatment effects. However, regarding other diabetes symptoms, we pooled data across studies together, which might result in the missed difference in dose-dependent and tumor-dependent effect on the risk for these adverse events. Thirdly, whether the increased risk of hyperglycemic events were caused, at least partly, by the use of corticosteroids for the management of irAEs is unclear. Moreover, the results of the present analysis are unable to address potential associations between the incidence of new-onset diabetes and other irAEs in the individual-level. Lastly, only very recent publications have noted T1D after ICI therapy; our study therefore may have underestimated the prevalence of ICI-associated diabetes with only a focus on clinical trials. As emerging case reports that described new-onset diabetes were seen in clinical practice (Hughes et al., 2015; Martin-Liberal et al., 2015; Wright et al., 2018), these adverse events may become more accurately diagnosed and recorded in future trials.

In summary, the use of ICIs compared with placebo or other treatment strategies was associated with an increased risk of new-onset diabetes, especially autoimmune diabetes, although the overall event rates remained low. In contrast, compared with the control group, the risk of T2D was not increased. As the widespread awareness of these events increases, additional large, well-designed randomized trials are needed to definitively determine the risks of new-onset diabetes following the use of ICIs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JL, JY, and XZ conceived and designed the study. YL, HM, JZ, JL, and JY reviewed the literatures, extracted and analyzed the data. JL, JY, and XZ wrote the manuscript. All authors have read 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/fphar.2019.01453/full#supplementary-material

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CASE REPORT
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Severe Immune-Related Pneumonitis With PD-1 Inhibitor After Progression on Previous PD-L1 Inhibitor in Small Cell Lung Cancer: A Case Report and Review of the Literature

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Objective: Combination therapy with programmed cell death protein-1 (PD-1) and programmed cell death ligand-1 (PD-L1) inhibitors might be viewed as a promising therapeutic strategy for resistant lung cancer, and it is becoming common that a second PD-1/PD-L1 inhibitor might be used following progression on previous PD-1/PD-L1 inhibitor. However, a subgroup of patients will experience various autoimmune toxicities, termed as immune-related adverse events (irAEs), that occur as a result of on-target and off-tumor inflammation.

Materials and Methods: In this report, we presented a patient with small cell lung cancer who received different PD-1/PD-L1 inhibitors during the course of disease progression. This patient experienced radiation-related pneumonitis, immune-related pneumonitis, as well as concomitant bacterial pneumonia.

Results: In particular, this patient developed immune-related pneumonitis with a second PD-1 inhibitor when she had a progressive disease on previous PD-L1 inhibitor. This patient was initially responsive to steroid treatment, but rapidly develop more severe pneumonitis and concomitant bacterial pneumonia with no response to antibiotics and steroid treatment. Finally, this patient got a good clinical response when receiving additional immunosuppressive medications infliximab and mycophenolate mofetil.

Conclusions: Patients with a history of radiation-induced pneumonitis and treated with sequential different PD-1/PD-L1 inhibitors have a relative high risk to develop high-grade or steroid-resistant pneumonitis, and additional immunosuppressive medications should be used earlier when severe pulmonary toxicity occurs.

Keywords: immune-related adverse event, programmed cell death 1 inhibitor, programmed cell death ligand 1, pneumonitis, immune checkpoint inhibitor

INTRODUCTION

Immune checkpoint inhibitors works by disrupting the PD-1 and PD-L1 direct interactions in the tumor microenvironment (1, 2). In clinical practice, anti-PD-1/PD-L1 antibodies have resulted in durable tumor remission and changed the treatment landscape in a variety of advanced cancers including small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Several PD-1 inhibitors (nivolumab, pembrolizumab, and avelumab) and PD-L1 inhibitors (atezolizumab and durvalumab) have been approved by US Food and Drug Administration (FDA) for treating multiple human solid tumors, based on improvements in survival outcomes.

Unlike cytotoxic chemotherapy, PD-1/PD-L1 inhibitors are usually manifested as tolerable agents, but 10–15% patients will develop grade 3–5 toxicity in non-target organs known as immune-related adverse events (irAEs) (3). Of these irAEs, pulmonary toxicity is one of the most dangerous side effects of PD-1/PD-L1 inhibitors, with a frequency of 5–10% in patients with lung cancer (4, 5). Pneumonitis associated with immunotherapy are generally uncommon but potentially fatal or life-threating (6). Generally, pneumonitis is more frequent in patients treated with anti-PD-1/PD-L1 antibodies compared to anti-CTLA-4 antibodies (7, 8), and more PD-1/PD-L1 inhibitor-related pneumonitis is observed in patients with lung cancer than those with melanoma (8). At present, combination therapy with PD-1/D-L1 inhibitors and other therapies is developing as a promising therapeutic strategy for advanced or metastatic lung cancer, and it is also becoming common that a second PD-1/PD-L1 inhibitor might be used following the disease progression on previous PD-1/PD-L1 inhibitor (9). These therapeutic strategies increase the frequency of an occurrence of irAEs including pneumonitis. Patients with pneumonitis related to PD-1/PD-L1 inhibitors may present clinically with drug cough, dyspnea, fever and chest pain, but radiologic findings often are non-specific (4, 5). Published guidelines or consensus can help clinically diagnose and manage irAEs, but general recommendations procedures are insufficient to resolve or relieve severe or complexed pulmonary toxicity (10–12). Here, we report a case with severe and rapidly developed reoccurred pneumonitis that occurred in the course of sequential use of PD-L1/PD-L1 inhibitors (**Figure 1**).

CASE PRESENTATION

In April 2018, a 44 year old woman was admitted to our hospital. She was initially diagnosed with localized SCLC (T2N1M0) through fiberoptic bronchoscopy in a local hospital. She did not experience other causes of obstructive lung disease, autoimmune disease, organ transplant, smoke inhalation, or medications. Molecular mutation analysis showed that the tumor did not harbor any driver gene alterations. Immunohistochemical staining of tumor tissue showed that PD-L1 expression was found in <1% of tumor cells. The tumor was located in right hilum of the right lung with multiple mediastinum lymph node metastasis. This patient received 2 cycles of first-line chemotherapy of etoposide (100 mg/m^2 days 1–3, every 3 weeks) and cisplatin (100 mg/m^2 every 3 weeks). Unfortunately, she subsequently presented with aggravated dry cough and dyspnea. Tumor regrowth in mediastinum lymph nodes was observed, and a diagnosis of superior vena cava obstruction syndrome (SVCS) was made. In June 2018, she was administrated with thoracic radiotherapy followed by two cycles of chemotherapy with irinotecan (120 mg/m^2 days 1, 8, every 3 weeks) and carboplatin (area under the curve of 5 mg/ml/min, every 3 weeks) as a second-line treatment and symptoms were improved significantly. In September 2018, this patient experienced dry cough and shortness of breath again. At that time, a computed tomography (CT) scan of the chest was performed and revealed new patchy ground-grass opacities in bilateral lobes of the lung, and small right pleural effusions, without new pulmonary tumor lesions (**Figure 2A**). She was not found to be hypoxic. Bacterial pneumonia was excluded through negative blood and sputum culture, and progressive disease was not confirmed through fiberoptic bronchoscopy. Based on her clinical presentations and radiotherapy history, radiation-induced pneumonitis was diagnosed, and she initiated systematic steroid treatment and reported symptomatic improvement gradually (**Figures 2B,C**).

In January 2019, this patient had an extensive disease progression, including multiple bone and supraclavicular lymph node metastases. Third-line nab-paclitaxel chemotherapy (200 mg days 1, 8, every 3 weeks) was started, but her tumor was not responsive to this regimen. She switched to an anti-PD-L1 antibody atezolizumab (1,200 mg every 3 weeks) therapy and local radiotherapy on lymph nodes and bone was subsequently planed and completed. In June 2019, after receiving her six cycles of immunotherapy with atezolizumab, this patient developed new liver and brain metastases. At that time a flat dose of nivolumab 240 mg every 2 weeks was started, along with further local treatment with liver and brain lesions. Just 1 week later, she experienced slight dry cough and acute new-onset fever without shortness of breath. A CT scan showed new patchy ground-grass opacities in the lung bilaterally, with a small right pleural effusion that was not present 1 month ago (**Figures 3A,B**). Blood and sputum culture did not reveal any causative microbial organism, and she was thought to develop immunotherapy-related pneumonitis, of grade 2 severity.

She received intravenous methylprednisolone (2 mg/kg every day for 5 days) and sequent treatment and inflammation was improved on day 15 (**Figure 3C**). But on day 24, she was presented with reoccurred fever, aggressive cough and dyspnea on exertion, with a low oxygen saturation of 88%. An additional CT scan showed obvious reoccurred pneumonitis of the bilateral lungs, of grade 3 severity (**Figure 3D**). After multidisciplinary discussion, high-dose intravenous methylprednisolone treatment (2 mg/kg every day) restarted but this did not alleviate her symptoms within 5 days, with a low oxygen saturation

Abbreviations: ALK, Anaplastic lymphoma kinase translocations; CT, Computerized tomography; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; EGFR, Endothelial growth factor receptor; FDA, United States Food and Drug Administration; irAEs, Immune-related adverse events; IVIG, Intravenous immunoglobulin; NCCN, National Comprehensive Cancer Network; NSCLC, Non-small cell lung cancer; PD-1, Programmed cell death protein-1; PD-L1, Programmed death ligand-1; SCLC, Small cell lung cancer; SVCS, Superior vena cava obstruction syndrome.

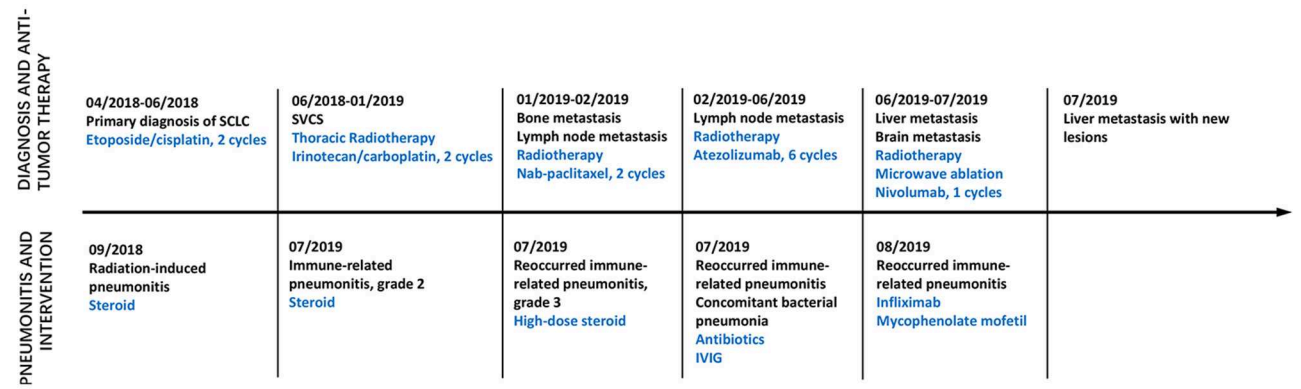

FIGURE 1 | Time axis of anti-tumor treatment and intervention on pneumonitis. Line graph illustrating disease progression, anti-tumor therapy, pneumonitis and intervention from April 2018 to August 2019. IVIG, Intravenous immunoglobulin; SVCS, Superior vena cava obstruction syndrome.

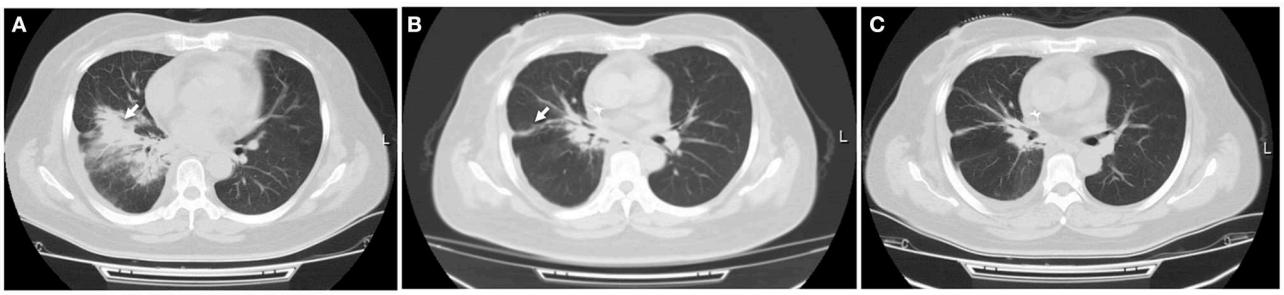

FIGURE 2 | Radiation-induced pneumonitis. **(A)** After receiving three-dimensional thoracic radiotherapy, a CT scan showed new patchy opacity developed in the irradiated area of hilum of the right lung, which did not occur outside the irradiated area. **(B)** This patient initiated steroid treatment, pneumonitis was partially resolved within 1 month, with a significantly clinical improvement. **(C)** Pulmonary inflammation disappeared 2 months later. White arrow indicates inflammatory lesions.

of 84–88%. At that time, she was firstly diagnosed with concomitant bacterial pneumonia with *Klebsiella pneumoniae*, but the use of specific antibiotics did not improve her symptoms (**Figure 3E**). She was continuously treated with steroid and received immunosuppressive agents including infliximab (5 mg/kg), mycophenolate mofetil (1 g twice every day), as well as intravenous immunoglobulin (IVIG; 2 g/kg every day for 5 days). After treatment with combination immunosuppression, fever, dry cough and dyspnea on exertion were relieved significantly and oxygen saturation returned to a normal level, with a significant radiographic improvement of pulmonary inflammation (**Figure 3F**). Unfortunately, subsequent CT scan demonstrated progressive disease in the liver.

DISCUSSION

Generally, both diagnosis and therapy are challengeable in identifying and managing cancer patients who may be potential PD-1/PD-L1 inhibitor-related pneumonitis. Pneumonitis can develop at any time before or after initiation of anti-PD-1/PD-L1 therapy in patients with metastatic lung cancer. Pulmonary toxicity may be a radiation recall limited to previously areas of the lung where radiation was applied. Radiation-induced lung injury including pneumonitis and fibrosis may present within several months or years following radiation therapy (13). Furthermore, unusual opportunistic infections including pneumonia can develop in patients with prolonged immune suppression which is used to treat irAEs (14, 15). Data from a single institution showed that serious infections occurred in 7.3% of advanced melanoma patients who received ipilimumab, either alone or in combination with nivolumab. The most common opportunistic infections were bacterial infection, others were viral, fungal, and parasitic (14). Thus, immune-related pneumonitis is viewed as a diagnosis of exclusion, and other completing causes for similar clinical presentation should be considered or excluded, including lung infection progressive disease in the lungs. Sometimes immune-related pneumonitis could present with concurrent infection and/or disease progression, which presents as a complication in clinical practice. In fact, preexisting pulmonary damage from inflammation, radiation, idiopathic pulmonary diseases, previous use of taxanes, gemcitabine and tyrosine kinase inhibitors, as well as increased tumor burden may increase the risk of developing immune-related pneumonitis (4, 5).

Currently, combination therapy strategies have been developed to improve PD-1 blockade efficacy in various tumor types. These include combinations with checkpoint inhibitors, radiation therapy, chemotherapy, small molecular inhibitors and several other existing cancer treatments (7, 9). It is becoming

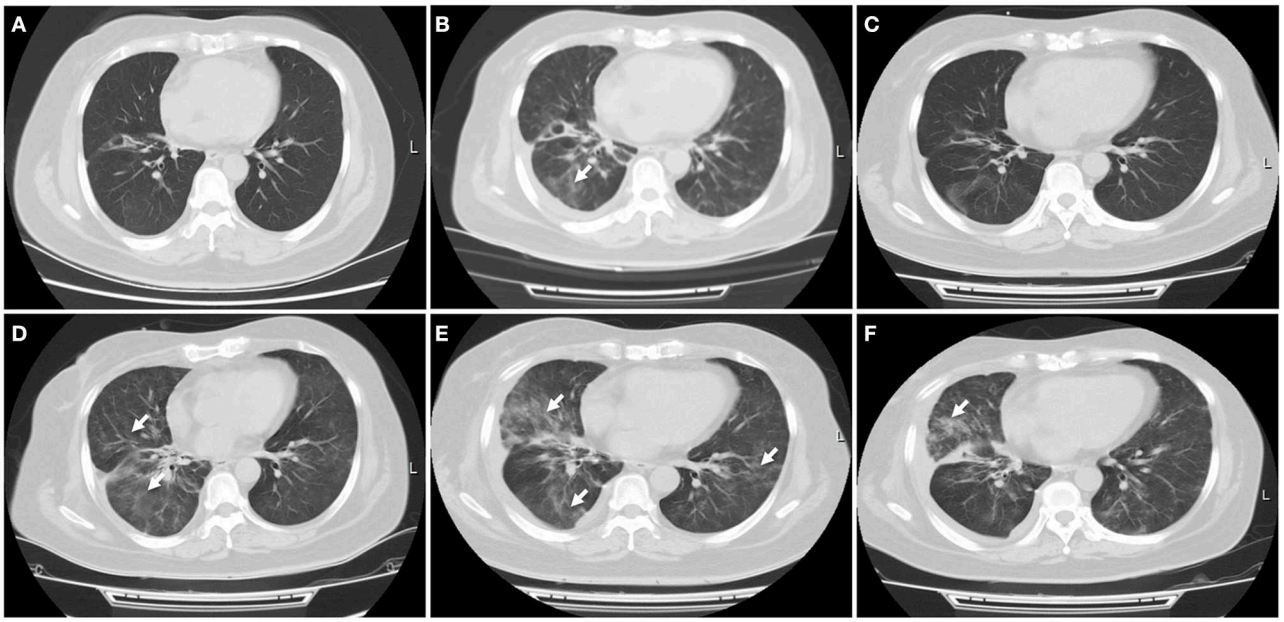

FIGURE 3 | Immune-related pneumonitis. **(A)** A CT scan showed no any inflammatory lesions in the lungs following the treatment with first PD-L1 inhibitor atezolizumab. **(B)** Nivolumab was started when this patient progressed on atezolizumab treatment. A CT scan indicated new patchy ground-grass opacities in the bilateral lungs, with a small left pleural effusion. Immune-related pneumonitis was identified when blood and sputum culture did not reveal a causative microbial organism. **(C)** After treatment with corticosteroid for 1 week, this patient's symptom improved significantly, with a radiologic complete resolution of the ground-glass opacities and the pleural effusion. **(D)** Ten days later, a CT scan showed reoccurred pneumonitis, of grade 3 severity. **(E)** High-dose intravenous corticosteroid therapy did not alleviate her symptoms within 5 days, with worsening radiographic findings. **(F)** After she received immunosuppressive agents including infliximab, mycophenolate mofetil and human immunoglobulin, fever, dry cough, and dyspnea were relieved significantly, with a significant improvement of pulmonary inflammation. White arrow indicates inflammatory lesions.

common that a second PD-1/PD-L1 inhibitor might be used following disease progression on previous PD-1/PD-L1 inhibitor. However, PD-1/PD-L1-based combination therapy leads to relatively high incidence of treatment-related adverse events. For example, the combination of osimertinib and durvalumab was associated with high incidence of interstitial lung disease (38%), leading to termination of further patient enrollment (16). Even severe irAEs also occurred frequently in endothelial growth factor receptor (EGFR)-mutant NSCLC patients who received sequential PD-1/PD-L1 inhibition and osimertinib treatment (17). In CheckMate 370 trial, 38% of anaplastic lymphoma kinase translocation (ALK)-positive NSCLC patients treated with nivolumab plus crizotinib developed severe hepatic toxicities, leading to the discontinuation of the combination and enrollment was closed earlier (18). An anti-CTLA-4 antibody in combination with an anti-PD-1 antibody increases both incidence and severity of irAEs. The overall incidence of pneumonitis for patients with anti-PD-1/PD-L1 combination therapy is 6.6% compared to 1.6% for those with monotherapy (5). These toxicities including fatal side effects also tend to be present earlier in the course of combination immunotherapy treatment and evolve rapidly compared with immune checkpoint inhibitor alone. The median time to the onset of fatal toxic event is about 14.5 days for patients with combination immune checkpoint therapy, compared to about 40 days for those treated with monotherapies (19). Although recurrent irAEs are mild and manageable, and a subgroup of patients were responsive to retreatment with previous immunotherapy, it remains unclear whether it is safe and efficacious when a patient switches to a different PD-1/PD-L1 inhibitor because of progression on the treatment with previous PD-1/PD-L1 inhibitor (**Table 1**) (22–24).

In our case, this patient developed serious interstitial lung disease after sequential use of atezolizumab and nivolumab. To the best of our knowledge, this is the first case report involving immune-related pulmonary toxicity due to sequential therapy with different PD-1/PD-L1 inhibitors. Another similar case report mentioned that severe pneumonitis and myocarditis were identified in a patient with lung squamous cell carcinoma who received nivolumab followed by atezolizumab monotherapy treatment (21). Furthermore, although retreatment is plausible rationale and there are several ongoing trials that allow prior treatment with a PD-1/PDL1 inhibitor, there is insufficient clinical data to support the treatment with another PD-1/PD-L1 inhibitor after progression on previous PD-1 pathway blockade (20). Therefore, caution is needed in patients receiving combinational or sequential application of PD-1/PD-L1 inhibitors. Although the mechanism of action underlying sequential use of PD-L1/PD-1 inhibitor remains unknown, syngeneic tumor-bearing mice model suggested that combination of anti-PD-1 and anti-PD-L1, either sequentially or simultaneously administered, caused fulminant cardiotoxicity,

TABLE 1 | Summary of reported cases documenting sequential treatment with different PD-1/PD-L1 inhibitors.

Authors	First PD-1/PD-L1 inhibitor	Second PD-1/PD-L1 inhibitor	Regimen targets	Tumor type	Causes of switching therapy	Outcomes
Martini et al. (20)	PD-L1 inhibitor	Nivolumab	PD-L1 + PD-1	RCC	PD	PD
Martini et al. (20)	PD-L1 inhibitor	Nivolumab	PD-L1 + PD-1	RCC	PD	PD
Martini et al. (20)	Pembrolizumab	Nivolumab	PD-1 + PD-1	Melanoma	PD	PD
Liu et al. (21)	Nivolumab	Atezolizumab	PD-1 + PD-L1	NSCLC	PD	Death caused by severe pneumonitis and myocarditis
Lepir et al. (22)	Nivolumab	Pembrolizumab	PD-1 + PD-1	Melanoma	PD	CR

CR, complete response; PD, progressive disease; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma.

and this effect is associated with infiltrating leukocyte but not CD8+ T cells accumulation in the hart. The administration of the PD-L1 inhibitor alone prior to the PD-1 inhibitor did not cause leukocytic infiltration of the myocardium (21).

Therapy and follow-up of immune-related pneumonitis remain a major challenge in the clinical practice. Treatment of pneumonitis is often determined by organizations and practice settings. For example, several guidelines or consensus for the management of irAEs in patients treated with immune checkpoint inhibitor therapy have been published (10–12). Immunotherapy teaching and monitoring tools have been developed by the National Comprehensive Cancer Network (NCCN) and can be utilized by patients and providers to monitor different irAEs related to immune checkpoint inhibitors (25). However, no prospective clinical trials have been identified that determine an optimal treatment approach for management of pneumonitis and other serious irAEs. Diagnostic evaluation and management appear to be empirical. In the majority of patients, pulmonary toxicity secondary to anti-PD-1/PD-L1 therapy can be resolved with the use of corticosteroid alone. However, a subgroup of patients cannot improve initially or completely and require additional suppressive medications because of steroid-refractory situation. In our case, this patient received high-dose corticosteroid and improve clinically after the onset of nivolumab-related pulmonary toxicity, but she rapidly developed a resistance to steroid treatment. According to published guidelines, if patients do not improve after 48 h of steroid treatment 1–2 mg/kg/d), addition of infliximab 5 mg/kg or mycophenolate mofetil intravenous 1 g twice a day or IVIG for 5 days or cyclophosphamide should be considered. Previous case report showed that single immunosuppressive medication was not insufficient (26). We considered that rapidly recurred pneumonitis was steroid refractory and used different immunosuppressive medications with infliximab and mycophenolate mofetil. In the meantime, intravenous immunoglobulin dosed at 2 g/kg was also administered. She got a good clinical response when receiving additional immunosuppressive medications. Thus, patients receiving combinational or sequential use of immune checkpoint inhibitors have a relative high risk to develop high-grade or steroid-resistant pneumonitis. Previous report showed that the addition of IVIG to high-dose corticosteroid could be viewed as an alternative therapy for steroid-refractory immune-related pneumonitis (27). Here, additional suppressive medications might be used earlier when pulmonary toxicity occurs following the combinational or sequential use of PD-1/PD-L1 inhibitors. Additionally, pulmonary infection was identified, and use of antibiotics did not produce good clinical response. However, infection screening is very important to exclude the presence of infections before considering PD-1/PD-L1-related pulmonary toxicity, regardless of a history of prior corticosteroid administration. Moreover, prospective study of early use with steroid and immunosuppressive agents in the treatment of serious immune checkpoint inhibitor-related pneumonitis is needed to establish best clinical practice in the field of immune-oncology.

CONCLUSIONS

Combination therapy based on PD-1/PD-L1 inhibitors might be viewed as a promising therapeutic strategy for resistant lung cancer, and it is becoming common that a second PD-1/PD-L1 inhibitor might be used following the progression on previous PD-1 pathway blockade. These patients have a relative high risk to develop high-grade or steroid-resistant pneumonitis, and additional suppressive medications should be used earlier when severe pulmonary toxicity occurs. In the meantime, pulmonary infections should be excluded before considering PD-1/PD-L1-related pulmonary toxicity, to avoid a situation of misuse with corticosteroids for immune-related pneumonitis, which would be an important consideration for oncologists and immunologists.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of No. 960 Hospital of PLA. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XL was involved in the identification and selection of patient cases and drafted the manuscript. YG and BZ were involved in the drafting and editing of the manuscript. BZ, YL, JL, and BW reviewed and edited the manuscript. JW was involved in the identification, selection, and management of patient cases and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Organ-Specific Immune-Related Adverse Events Associated With Immune Checkpoint Inhibitor Monotherapy Versus Combination Therapy in Cancer: A Meta-Analysis of Randomized Controlled Trials

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Background: Although combination therapy with immune checkpoint inhibitors (ICIs) provides a promising efficacy in multiple cancers, their use is facing challenges for a high incidence of adverse effects. This meta-analysis was conducted to compare the risks of organ-specific immune-related adverse events (IRAEs) associated with ICI monotherapy versus combination therapy among cancer patients.

Methods: Electronic databases were systematically searched to include eligible randomized controlled trials (RCTs). Any-grade and 3-5 grade IRAEs (colitis, pneumonitis, hepatitis, hypothyroidism, hyperthyroidism, and hypophysitis) were extracted for meta-analysis. Two reviewers independently assessed the methodological quality. The RevMan 5.3.5 software was used for meta-analysis.

Results: A total of 10 studies involving 8 RCTs with 2716 patients were included in this study. The most common any-grade adverse event was colitis (14.5%), followed by hypothyroidism (13.8%), hepatitis (10.4%), hypophysitis (10.0%), hyperthyroidism (9.3%), and pneumonitis (4.6%). Meta-analysis showed that ICI combination therapy significantly increased the risks of any-grade IRAEs in colitis [relative risk (RR), 3.56; 95% confidence interval (CI), 1.56–8.12; $p < 0.05$], pneumonitis (RR, 2.31; 95% CI, 1.54–3.45; $p < 0.05$), hepatitis (RR, 2.54; 95% CI, 1.65–3.91; $p < 0.05$), hypothyroidism (RR, 2.17; 95% CI, 1.71–2.76; $p < 0.05$), hyperthyroidism (RR, 3.13; 95% CI, 2.08–4.70; $p < 0.05$), and hypophysitis (RR, 3.54; 95% CI, 2.07–6.07; $p < 0.05$) compared with ICI monotherapy, as well as 3-5 grade IRAEs in colitis (RR, 2.50; 95% CI, 1.62–3.86; $p < 0.05$), pneumonitis (RR, 1.99; 95% CI, 1.00–3.93; $p = 0.05$), and hepatitis (RR, 2.70; 95% CI, 1.29–5.63; $p < 0.05$).

Conclusions: This meta-analysis demonstrated that, compared with ICI monotherapy, patients receiving ICI combination therapy significantly increased organ-specific IRAEs in colitis, hypothyroidism, hepatitis, hypophysitis, hyperthyroidism, and pneumonitis. The incidence and severity of organ-specific IRAEs were drug and dose independent.

Keywords: immune checkpoint inhibitor, combination immunotherapy, organ specific, adverse events, meta-analysis

INTRODUCTION

Immune checkpoint inhibitors (ICI) have shown remarkable efficacy in the therapy of multiple cancers, such as non-small cell lung carcinoma, renal cell carcinoma, head and neck squamous cell carcinoma, and melanoma (Mellman et al., 2011; Luke et al., 2017; Proto et al., 2019). The most widely used ICIs include cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed death-1/ligand-1 (PD-1/PD-L1) inhibitors. These inhibitors block the agent interaction with the key immune regulatory pathways, thereby increasing the antitumor immunity (Johnson et al., 2017). Representative drugs of CTLA-4 (ipilimumab), PD-1 (nivolumab, pembrolizumab), and PD-L1 (avelumab, atezolizumab, and durvalumab) have been approved by the Food and Drug Administration (FDA) for malignant tumors.

In recent years, the combined use of PD-1 and CTLA-4 inhibitors has attracted increasing attention for the promising efficacy in the treatment of advanced melanoma, lung cancer, and sarcoma (Larkin et al., 2015; D'angelo et al., 2018; Hellmann et al., 2018a; Hellmann et al., 2018b). In patients with advanced melanoma, combination therapy with nivolumab and ipilimumab had significantly improved clinical outcomes with prolonged progression-free survival (PFS) and higher objective response rate (ORR) compared with ipilimumab alone (Postow et al., 2015; Hodi et al., 2016). Four clinical trials (CheckMate 012/032/227/568) demonstrated a durable response associated with ICI combination therapy among patients with lung cancer (Antonia et al., 2016; Hellmann et al., 2017; Hellmann et al., 2018b; Ready et al., 2019). Although ICI combination has become a significant breakthrough in cancer therapeutics, their use was associated with toxic effects resulting from unbalanced activation of the immune system. To distinguish from other treatment-related side effects, these toxic effects caused by immune activation were specifically termed as immune-related adverse events (IRAEs) (Postow et al., 2018).

IRAEs may occur in almost any organ, such as the colon, lungs, liver, muscle, and thyroid. According to the published study (Baxi et al., 2018), IRAEs were classified into three categories: organ-specific IRAEs (colitis, hepatitis, pnemonitis, etc.), general IRAEs (fatigue, diarrhea, and rash) and musculoskeletal IRAEs (arthritis, arthralgia, back pain, etc.). They demonstrated that the general adverse events are more prevalent, but the organ-specific IRAEs are more clinically important. Yang et al. (Yang et al., 2019) also suggested that oncologists should focus on the organ-specific IRAEs, which are more meaningful in clinical practice. Therefore, the organ-specific adverse event has been a new challenge in the treatment of cancers (Baxi et al., 2018; Martins et al., 2019).

Currently, although several meta-analysis have evaluated the efficacy and safety of ICIs (Wang et al., 2017; Barroso-Sousa et al., 2018; Ma et al., 2018; Wang et al., 2018; You et al., 2018), most studies included chemotherapy as the control group for the analysis, and few studies specifically assessed the safety of ICIs. A published meta-analysis by Wang et al. in 2018 reported fatal toxic effects associated with ICIs. They demonstrated that the organ-specific IRAEs were the most common causes for death: colitis for CTLA-4 (70%, 135/193 deaths), pneumonitis (35%, 115/333 deaths) for PD-1 or PD-L1 inhibitors, and colitis (37%, 32/87) for the combination PD-1 and CTLA-4 (Baxi et al., 2018). However, they failed to provide the detailed data about the incidences of low-grade and high-grade adverse events.

A comprehensive understanding of the epidemiology of the organ-specific IRAEs is essential for clinicians to balance the benefits and risks of ICI combination during cancer treatment (Martins et al., 2019). Therefore, we conducted this meta-analysis based on randomized controlled trials (RCTs) aiming to compare the organ-specific IRAEs of ICI monotherapy versus combination therapy among cancer patients.

MATERIALS AND METHODS

This study was performed based on the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement.

Inclusion and Exclusion Criteria

The following inclusion criteria were used in this study: (1). types of included studies: randomized controlled trials (RCTs); (2). types of participants: patients over 18 years of age diagnosed with malignancies regardless of region, racial, and gender; (3). interventions: patients received the intervention treatment of either ICI monotherapy or combined therapy with CTLA-4/PD-1/PD-L1 antibodies; (4). types of outcomes: colitis, pneumonitis, hepatitis, hypothyroidism, hyperthyroidism, and hypophysitis. The severity of adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version (CTCAE) 4.0, and grade ≥3 were evaluated as high grade or severe grade.

The exclusion criteria were: (1). types of studies: ongoing trials, quasi-RCT, non-RCT, reviews, commentaries, conference paper, and quality of life studies; (2) interventions: patients treated with placebo, chemotherapy, or chemotherapy plus immunotherapy.

Data Sources and Searches

A literature search was conducted to identify RCTs comparing ICI monotherapy versus combination therapy among cancer patients. Without the restriction on language and publication status, the databases of MEDLINE, EMBASE, and Cochrane databases and ISI Web of Knowledge were searched to determine potentially eligible studies up to May 30, 2019. The following search terms were used: CTLA-4, ipilimumab, tremelimumab, PD-1, nivolumab, pembrolizumab, PDL-1, atezolizumab, avelumab, durvalumab, and checkpoint inhibitors. Additionally, the reference lists of identified studies and Google scholar were checked for other potentially eligible trials.

Data Collection and Quality Assessment

Two blinded authors (Da and Teng) independently extracted data according to a standardized extraction form. Any discrepancy was resolved by discussion with a third author. If insufficient data was reported, efforts were made to contact the authors for the additional information. The methodological quality of the eligible studies was evaluated using the following items recommended by the Cochrane Collaboration: randomization, allocation concealment blinding of participant, blinding of outcome assessors, incomplete outcome data, selective reporting, and other bias (Higgins et al., 2011).

Statistical Analysis

Meta-analysis was conducted using the software Review Manager 5.3.5. Risk ratio (RR) and 95% confidence interval (95% CI) were calculated to estimate the event rates for dichotomous outcomes. Heterogeneity was tested using I^2 index and the Cochran Q statistic ($I^2 > 50\%$ indicating significant heterogeneity, and $I^2 \leq 50\%$ indicating no significant heterogeneity). If no heterogeneity ($I^2 \leq 50\%$) was presented in the meta-analysis, a fixed-effect model was used to estimate the pooled odds ratio and 95% confidence interval, otherwise, a random-effect model was used. Subgroup analyses were performed to explore the sources of heterogeneity according to the different tumors types.

RESULTS

Figure 1 showed the flow chart of literature screening. A total of 2,279 records were yielded in the initial search from the database. After removing duplicates, 1,352 studies were assessed for abstract and full-text review. Finally, 10 studies involving eight RCTs were included in this meta-analysis (Larkin et al., 2015; Postow et al., 2015; Antonia et al., 2016; Hodi et al., 2016; Wolchok et al., 2017; D'angelo et al., 2018; Hellmann et al., 2018b; Long et al., 2018; Omuro et al., 2018; Sharma et al., 2019).

The Characteristics and Quality Assessment of Included RCTs

The detailed characteristics of included RCTs were shown in **Table 1**. A total of 2,716 patients (monotherapy group, 1,315; combination group, 1,401) were included in the analysis. In the combination group, all the patients received intervention with nivolumab and ipilimumab. Three studies compared the efficacy of two different doses of drug combinations: nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (N3I1), or nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (N1I3) (Antonia et al., 2016; Omuro et al., 2018; Sharma et al., 2019). In the monotherapy group, patients received intervention with either ipilimumab (Larkin et al., 2015; Postow et al., 2015; Hodi et al., 2016; Wolchok et al., 2017) or nivolumab alone (Larkin et al., 2015; Antonia et al., 2016; Wolchok et al., 2017; D'angelo et al., 2018; Hellmann et al., 2018b; Long et al., 2018; Omuro et al., 2018; Sharma et al., 2019). The included RCTs involved five kinds of tumors: lung cancer in two studies (Antonia et al., 2016; Hellmann et al., 2018b), melanoma in three studies (Larkin et al., 2015; Postow et al., 2015; Hodi et al., 2016; Wolchok et al., 2017; Long et al., 2018), metastatic sarcoma in one study (D'angelo et al., 2018), urothelial carcinoma (Sharma et al., 2019), and recurrent glioblastoma in one study (Omuro et al., 2018). All the included RCTs used the CTCAE 4.0 to evaluate the severity of IRAEs. The publication date of the included studies was between 2015 and 2018. Additionally, two updated RCTs were included in this meta-analysis without duplicate counting of the sample (Hodi et al., 2016; Wolchok et al., 2017).

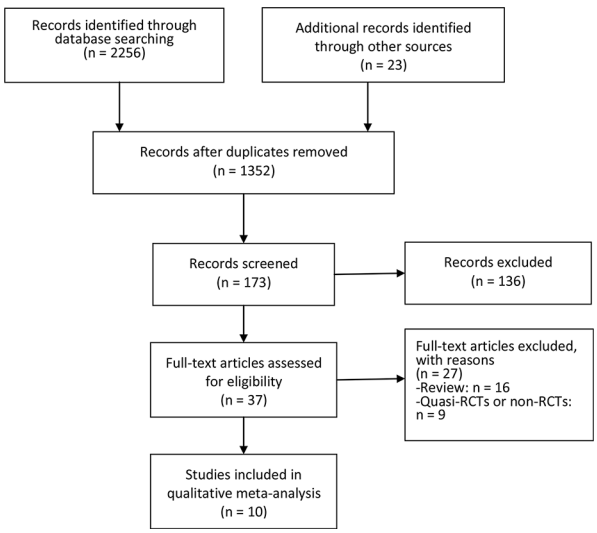

FIGURE 1 | PRISMA flowchart of literature screening.

Table 2 showed the methodological quality of the included studies. The randomization was reported in all the studies, and blinding of outcome assessment was reported in six studies. However, few studies described the allocation concealment and the blinding of participants during trial.

Incidences of Organ-Specific IRAEs

Regarding any-grade organ-specific IRAEs associated with combination therapy, the most common adverse event was colitis (14.5%), followed by hypothyroidism (13.8%), hepatitis (10.4%), hypophysitis (10%), hyperthyroidism (9.3%), and pneumonitis (4.6%). While for 3-5 grade adverse events with

TABLE 1 | The characteristics of included studies.

Study	Year	Study design	Histology	Age (years)	No. of patients (Male/Female)	Groups	NO. of Lost to Follow-up	CTCAE Version
Antonia et al., 2016	2016	Phase I/II RCT; Check Mate 032	SCLC	63 (57-68)	98 (61/37)	NIVO(3 mg/kg q2w)	0	4.0
				61 (56-65)	54 (32/22)	NIVO (3 mg/kg q3w) + IPI (1 mg/kg q3w)	0	
				66 (58-71)	61 (35/26)	NIVO (1 mg/kg q3w) + IPI (3 mg/kg q3w)	0	
Hellmann et al., 2017	2017	Phase III RCT; Check Mate 227	NSCLC	64 (median)	396 (273/123)	NIVO(3 mg/kg q2w)	5	4.0
				64 (median)	583 (391/192)	NIVO (3 mg/kg q2w) + IPI (1 mg/kg q6w)	7	
Larkin et al., 2015/ Wolchok et al., 2017	2015/ 2017	Phase III RCT; Check Mate 067	Melanoma	59 (25-90)	316 (202/144)	NIVO(3 mg/kg q2w)	3	4.0
				61 (18-89)	315 (202/113)	IPI (3 mg/kg q3w)	2	
				59 (18-88)	314 (206/108)	NIVO (1 mg/kg q3w) + IPI (3 mg/kg q3w)	3	
Postow et al., 2015/ Hodi et al., 2016	2015/ 2016	Phase III RCT; Check Mate 069	Melanoma	67 (31-80)	47 (32/15)	IPI (3 mg/kg q3w)	1	4.0
				64 (27-87)	95 (63/32)	NIVO (1 mg/kg q3w) + IPI (3 mg/kg q3w)	1	
Long et al., 2018	2018	Phase Ib RCT; KEYNOTE-029	Melanoma	63 (52-74)	25 (19/6)	NIVO (3 mg/kg q2w)	0	4.0
				59 (53-68)	35 (29/6)	NIVO (1 mg/kg q3w) + IPI (3 mg/kg q3w)	0	
D'angelo et al., 2018	2018	Phase III RCT; Alliance A091401	Sarcoma	56 (21-76)	43 (22/21)	NIVO (3 mg/kg q2w)	0	4.0
				57 (27-81)	42 (19/23)	NIVO (3 mg/kg q3w) + IPI (1 mg/kg q3w)	0	
Omuro et al., 2018	2018	Phase I RCT; CheckMate 143	Glioblastoma	58.5 (42-73)	10 (5/5)	NIVO (3 mg/kg q2w)	0	4.0
				60 (27-73)	20 (14/6)	NIVO (3 mg/kg q3w) + IPI (1 mg/kg q3w)	0	
				57 (37-68)	10 (6/4)	NIVO (1 mg/kg q3w) + IPI (3 mg/kg q3w)	0	
Sharma et al., 2019	2019	Phase I/II RCT; CheckMate 568	Urothelial Carcinoma	65.5 (31-85)	78 (54/24)	NIVO (3 mg/kg q2w)	0	4.0
				63.0 (39-83)	104 (81/23)	NIVO (3 mg/kg q2w) + IPI (1 mg/kg q2w)	0	
				64.0 (38-83)	92 (74/18)	NIVO (1 mg/kg q2w) + IPI (3 mg/kg q2w)	0	

NIVO, nivolumab; IPI, ipilimumab; No., number; CTCAE, Common Terminology Criteria for Adverse Events version; RCT, randomized controlled trials; NA, not available.

monotherapy, the most common incidences were colitis (11.9%), hepatitis (3.7%), pneumonitis (1.7%), hypophysitis (1.1%), hypothyroidism (0.4%), and hyperthyroidism (0.4%).

Outcomes of Meta-Analysis

The outcomes of meta-analysis were presented in **Table 3**, and the forest plots of meta-analysis were attached in **Supplementary Materials**.

Meta-Analysis of Any-Grade and 3-5 Grade Colitis

Five studies involving 1390 patients were included for meta-analysis (Antonia et al., 2016; Hodi et al., 2016; Wolchok et al., 2017; Long et al., 2018; Omuro et al., 2018). The incidences of any-grade colitis were 14.5% (85/587) vs 5.6% (45/803) in the combination vs monotherapy group; and 3-5 grade were 11.9% (70/587) vs 5.1% (41/803) in the combination vs monotherapy group. A random-effect model was used in the meta-analysis for significant heterogeneity among studies ($I^2 > 50\%$). The results of the meta-analysis showed that patients treated with ICI combinations had significantly higher incidences of any-grade and 3-5 grade colitis when compared with the monotherapy group. The RR was 3.56 (95% CI, 1.56–8.12; $p < 0.05$) and 2.5 (95% CI, 1.62–3.86; $p < 0.05$) for any-grade and 3-5 grade colitis, respectively.

Meta-Analysis of Any-Grade and 3-5 Grade Pneumonitis

All the included studies involving 2716 patients reported any-grade and 3-5 grade pneumonitis. The incidences of any-grade pneumonitis were 4.6% (64/1401) vs 2.1% (27/1314) in the combination vs monotherapy group; and 3-5 grade were 1.7% (24/1401) vs 0.7% (9/1314) in the combination vs monotherapy group. A fixed-effect model was used in the meta-analysis for no significant heterogeneity among studies ($I^2 < 50\%$). Meta-analysis showed significantly high incidences in any-grade and 3-5 grade pneumonitis in the ICI combination group. The RR

TABLE 2 | Risk of bias in included studies.

Study	Random Sequence Generation	Allocation Concealment	Blinding of Participants	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias
Antonia et al., 2016	Yes[a]	Unclear[b]	No[c]	Yes	Yes	Unclear	Unclear
Hellmann et al., 2017; Hellmann et al., 2018a; Hellmann et al., 2018b	Yes	Unclear	No	Yes	Yes	Unclear	Unclear
Larkin et al., 2015/Wolchok et al., 2017	Yes	Unclear	Yes	Yes	Yes	Unclear	Unclear
Postow et al. 2015/Hodi et al., 2016	Yes	Unclear	Yes	Yes	Yes	Unclear	Unclear
Long et al., 2018	Yes	Yes	Yes	Yes	Yes	Unclear	Unclear
D'angelo et al., 2018	Yes	Unclear	Unclear	Unclear	Yes	Unclear	Unclear
Omuro et al., 2018	Yes	Unclear	Unclear	Unclear	Yes	Unclear	Unclear
Sharma et al., 2019	Yes	Unclear	No	Yes	Yes	Unclear	Unclear

[a]*Yes, low risk of bias;* [b]*Unclear: unclear or unknown risk of bias;* [c]*No: high risk of bias.*

TABLE 3 | Meta-analysis of any-grade and 3-5 grade IRAEs between the ICI combination group and the monotherapy group.

Outcomes	Studies	Any Grade			3-5 Grade		
		Effect Estimate, RR (95% CI)	Overall Effect	Heterogeneity (I^2)	Effect Estimate, RR (95% CI)	Overall Effect	Heterogeneity (I^2)
Colitis	5	2.84 (1.42–5.65)	p = 0.003	I^2 = 59%	3.71 (1.37–10.08)	p < 0.001	I^2 = 55%
Pneumonitis	8	2.24 (1.52–3.32)	p < 0.001	I^2 = 9%	1.96 (1.00–3.85)	p = 0.05	I^2 = 0%
Hepatitis	4	2.16 (1.50–3.12)	p < 0.001	I^2 = 24%	2.56 (1.27–5.16)	p = 0.009	I^2 = 0%
Hypothyroidism	8	2.00 (1.61–2.48)	p < 0.001	I^2 = 36%	2.34 (0.57–9.65)	p = 0.24	I^2 = 0%
Hyperthyroidism	5	2.91 (1.98–4.29)	p < 0.001	I^2 = 33%	6.98 (0.86–56.55)	p = 0.07	I^2 = 0%
Hypophysitis	3	3.60 (1.31–9.86)	p = 0.01	I^2 = 58%	0.45 (0.16–1.25)	p = 0.13	I^2 = 11%

RR, risk ratio; CI, confidence interval.

was 2.31 (95% CI, 1.54–3.45; $p < 0.05$) and 1.99 (95% CI, 1.00–3.93; $p = 0.05$) for any-grade and 3-5 grade pneumonitis, respectively.

Meta-Analysis of Any-Grade and 3-5 Grade Hepatitis

Four studies involving 1441 patients were included for meta-analysis (Hodi et al., 2016; Hellmann et al., 2018b; Long et al., 2018; Sharma et al., 2019). The incidences of any-grade hepatitis were 10.4% (94/901) vs 7.1% (24/340) in the combination vs monotherapy group; and 3-5 grade were 3.7% (33/901) vs 2.1% (7/340) in the combination vs monotherapy group. No significant heterogeneity was found among studies ($I^2 < 50\%$). Meta-analysis demonstrated that, the ICI combination group had significantly higher any-grade and 3-5 grade hepatitis than the monotherapy group. The RR was 2.54 (95% CI, 1.65–3.91; $p < 0.05$) and 2.70 (95% CI, 1.29–5.63; $p < 0.05$) for any-grade and 3-5 grade hepatitis, respectively.

Meta-Analysis of Any-Grade and 3-5 Grade Hypothyroidism

All studies reported the incidence of hypothyroidism. The incidences of any-grade hypothyroidism were 13.8% (194/1401) vs 7.2% (95/1315) in the combination vs monotherapy group; and 3-5 grade were 0.4% (5/1401)vs 0.1% (1/1315) in the combination vs monotherapy group. There was no significant heterogeneity among studies ($I^2 < 50\%$). Compared with the monotherapy group, the combination group showed significant higher risks in any-grade hypothyroidism, and the RR was 2.17 (95% CI, 1.71–2.76; $p < 0.05$). However, no difference was found in 3-5 grade hypothyroidism (RR, 2.36; 95% CI, 0.55–10.13; $p = 0.25$).

Meta-Analysis of Any-Grade and 3-5 Grade Hyperthyroidism

Five studies involving 1524 patients were included for meta-analysis (Antonia et al., 2016; Wolchok et al., 2017; Long et al., 2018; Omuro et al., 2018; Sharma et al., 2019). The incidences of any-grade hyperthyroidism were 9.3% (64/689) vs 3.0% (25/835) in the combination vs monotherapy group; and 3-5 grade were 0.4% (3/689) vs 0% (0/835) in the combination vs monotherapy group. The heterogeneity was not significant among studies ($I^2 < 50\%$). Meta-analysis showed that patients receiving ICI combination therapy had significantly higher risk in any-grade hyperthyroidism than those receiving monotherapy, and the RR was 3.13 (95% CI, 2.08–4.70; $p < 0.05$), but no difference was found in 3-5 grade hyperthyroidism (RR, 7.05; 95% CI, 0.86–57.43; $p = 0.07$).

Meta-Analysis of Any-Grade and 3-5 Grade Hypophysitis

Three studies involving 1137 patients reported the incidence of hypophysitis (Hodi et al., 2016; Wolchok et al., 2017; Long et al., 2018). The incidences of any-grade hypophysitis were 10.0% (44/442) vs 2.4% (17/695) in the combination vs monotherapy group; and 3-5 grade were 1.1% (5/442) vs 1.6% (11/695) in the combination vs monotherapy group. No significant

heterogeneity was found among studies (I^2 < 50%). Meta-analysis showed that the combination group had significant high risks in any-grade hypophysitis, and the RR was 3.54 (95% CI, 2.07–6.07; $p < 0.05$). No difference was found in 3-5 grade hypophysitis (RR, 0.45; 95% CI, 0.16–1.23; $p = 0.12$).

Meta-Analysis of Total Treatment-Related Adverse Events

A total of 2,716 patients were included in 10 studies with 1315 in the monotherapy group (nivolumab, 958; ipilimumab, 357) and 1,401 in the combination group (nivolumab and ipilimumab). A random-effect model was used for the outcome of total 3-5 grade adverse events due to significant heterogeneity among studies ($I^2 > 50\%$). When compared with the monotherapy group, meta-analysis showed that patients in the ICI combination group had significantly higher risks for total treatment-related adverse events in any and 3-5 grade. The RR was 1.68 (95% CI, 1.35–2.08; $p < 0.05$) and 2.99 (95% CI, 2.00–4.46; $p < 0.05$) for total any-grade and 3-5 grade hepatitis, respectively.

Subgroups Analysis

Different Types of Tumors

As for the insufficient number of included studies on lung cancer, glioblastoma, urothelial carcinoma, and sarcoma, only one subgroup analysis was performed on melanoma. Meta-analysis showed that, the combination therapy significantly increased the risks of total 3-5 grade organ-specific IRAEs (RR, 1.70; 95% CI, 1.25–2.30; $p < 0.05$) in melanoma patients, but no difference was found in the incidences of total any-grade IRAEs between both groups (RR, 1; 95% CI, 0.99–1.01; $p = 0.77$).

Different Drug Doses

The incidences of IRAEs based on drugs (nivolumab alone, ipilimumab alone, and nivolumab plus ipilimumab) were summarized in **Table 4**. In the combination group, three studies included two different doses of drug combinations: nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (N3I1), or nivolumab 1 mg/kg plus ipilimumab 3 mg/kg (N1I3) (Antonia et al., 2016; Omuro et al., 2018; Sharma et al., 2019). The subgroups analysis showed that there was no difference in the incidence of total any-grade organ-specific IRAEs between N3I1 and N1I3 groups (RR, 0.99; 95% CI, 0.89–1.09; $p = 0.84$), but the incidence of the total 3-5 grade IRAEs was significantly higher in the N1I3 group (RR, 1.70; 95% CI, 1.25–2.30; $p < 0.05$).

Publication Bias

The funnel plot was used to explore the potential publication bias. All the included studies showed a symmetric distribution on the funnel plots. No significant publication bias was found in this meta-analysis.

DISCUSSION

In this study, 10 literatures involving 8 RCTs with 2716 patients were included for meta-analysis. The most important finding of this study is that the use of ICI combination (nivolumab and ipilimumab) significantly increased the risks in any-grade IRAEs in colitis, pneumonitis, hepatitis, hypothyroidism, hyperthyroidism, and hypophysitis, as well as the 3-5 grade IRAEs in colitis, pneumonitis, and hepatitis.

The rapid development of ICIs has dramatically changed the therapeutic options in numerous cancers. Compared with ICI monotherapy, ICI combination therapy has become a more popular therapeutic way for its superior clinical efficacy. However, few studies are focused on the organ-specific IRAEs. Although a previous meta-analysis by Wang et al. (2018) had assessed the toxic effects caused by ICIs, the authors only reported the mortality related to the ICI toxicity (122 deaths in 19,217 patients). Nevertheless, for a prompt recognition and management of adverse events, we should not only know the epidemiology regarding the fatal events, but also for moderate and severe adverse effects (Martins et al., 2019). Therefore, we designed this study to compare the risks of any-grade and 3-5 grade adverse effects associated with ICI combination therapy with monotherapy.

In our study, colitis and hepatitis were included as ICI-induced gastrointestinal and hepatic injury. The most frequent IRAE associated with combination therapy was colitis (any grade, 14.5%; 3-5 grade: 11.9%), with a significantly higher incidence than that in the monotherapy group (any grade: 5.6%; 3-5 grade: 3.5%). Hepatitis induced by ICI was less frequent compared to colitis, occurring in approximately 10.4% of patients receiving ICI combination therapy, with 3.7% above grade 3. Meta-analysis showed that ICI combination therapy significantly increased risks of colitis and hepatitis than ICI monotherapy. Of note, the increased colitis in the combination therapy group might be mainly contributed to the use of anti-CTLA-4 drugs. Earlier studies demonstrated a higher incidence

TABLE 4 | Incidence of the organ-specific IRAEs by drug (%).

Drugs	Colitis		Pneumonitis		Hepatitis		Hypothyroidism		Hyperthyroidism		Hypophysitis	
	Any grade	Grade 3-5	Any grade	Grade 3-5	Any grade	Grade 3-5	Any grade	Grade 3-5	Any grade	Grade 3-5	Any grade	Grade 3-5
Nivolumab + Ipilimumab[a]	14.5 (85/587)	11.9 (70/587)	4.6 (64/1401)	1.7 (24/1401)	10.4 (94/901)	3.7 (33/901)	13.8 (194/1401)	0.4 (5/1401)	9.3 (64/689)	0.4 (3/689)	10.0 (44/442)	1.1 (5/442)
Nivolumab	1.6 (7/446)	0.7 (3/446)	2.3 (22/957)	0.8 (8/957)	4.9 (24/294)	1.4 (7/294)	7.8 (75/958)	0.1 (1/958)	4.0 (21/524)	0 (0/524)	0.6 (2/338)	1.5 (5/338)
Ipilimumab	10.6 (38/357)	10.6 (38/357)	1.4 (5/357)	0.3 (1/357)	0 (0/46)	0 (0/46)	5.6 (20/357)	0 (0/357)	1.3 (4/311)	0 (0/311)	4.2 (15/357)	1.7 (6/357)

[a]*Includes both two different doses of drug combinations: Nivolumab 3 mg/kg plus Ipilimumab 1 mg/kg (N3I1), nivolumab 1 mg/kg plus ipilimumab 3 mg/kg.*

of gastrointestinal adverse events associated with CTLA-4 inhibitors alone compared with anti-PD-1 therapy. In a large-sample phase-3 study with 945 patients, Larkin et al. (2015) compared the safety of nivolumab alone, ipilimumab alone, and nivolumab plus ipilimumab. The results showed that colitis of any grade occurred in 0.6% of the patients in the nivolumab group, 7.7% of those in the ipilimumab group, and 8.3% of those in the nivolumab-plus-ipilimumab group, respectively. This result was consistent with our subgroup analysis, which showed that patients receiving ipilimumab alone (10.6%) more likely experienced any-grade and serious colitis than those who received nivolumab alone (1.6%). Currently, the related pathogenesis of colitis initiated by ipilimumab still remains unclear. Histopathologic features might be related with an increase in intraepithelial lymphocytes for CTLA-4 inhibitors (Gupta et al., 2015; Weidner et al., 2015).

For the organ-specific IRAEs in thyroid dysfunction, we included the outcomes of hypothyroidism and hyperthyroidism in this study. Meta-analysis showed that the combination group had significant high risks in any-grade hypothyroidism and hyperthyroidism compared with the monotherapy group. In terms of 3-5 grade adverse events, prior studies demonstrated that ICI therapy rarely resulted in serious thyroid dysfunction (Morganstein et al., 2017; Zhang et al., 2018). This study revealed that only 3 (0.4%, 3/1401) patients had serious hypothyroidism in combination therapy group, while 1 (0.1%, 1/958) and 5 (0.4%, 5/958) patients had serious hyperthyroidism in nivolumab and combination groups, respectively. Meta-analysis showed no differences between the monotherapy and combination groups. Hypophysitis was regarded as the most frequent endocrine dysfunction caused by ICI therapy. Notably, we found that the variation tendency of hypophysitis was similar with that of thyroid dysfunction, which showed that the combination group had statistically higher incidence in all-grade events, but no difference in serious events between two groups. A possible explanation was that the thyrotropin hormone was affected by hypophysitis, thus resulting in thyroid disorders (Barroso-Sousa et al., 2018; Zhang et al., 2018).

Pneumonitis was a relatively rare adverse event during checkpoint inhibition therapy, which appeared more prevalent in lung cancer patients (Spain et al., 2016). Low-grade pneumonitis was commonly manageable with treatment discontinuation, but serious pneumonitis is potentially life-threatening (Martins et al., 2019; Rahouma et al., 2019). In this study, meta-analysis revealed that ICI combination therapy was associated with a significantly higher risk of pneumonitis compared with monotherapy. Subgroup analysis revealed that the rates of all-grade pneumonitis were 4.6%, 2.3% and 1.4% in patients receiving nivolumab plus ipilimumab, nivolumab alone, and ipilimumab alone, respectively. Interestingly, unlike colitis, pneumonitis was more frequent among patients receiving anti-PD-1/PD-L1 therapies as opposed to those receiving anti-CTLA4 therapies (Martins et al., 2019). Analysis based on the drug types also showed that patients receiving anti-PD-1 inhibitors (nivolumab) experienced more high-grade pneumonitis than those receiving anti-CTLA4 inhibitors (ipilimumab), and the incidences were 0.8% and 0.3%, respectively. Moreover, 1 (1.1%) case and 3 cases (2.3%) of pneumonitis-related death associated with nivolumab were reported by Postow et al. (2015) and Gettinger et al. (2015), respectively. Therefore, despite a relatively low incidence of pneumonitis, this adverse effect should be closely followed up by clinicians, in particular when anti-PD-1/PD-L1 inhibitors are being used.

For the subgroup analysis, we found that the organ-specific IRAEs appeared to be drug- and dose-dependent. Regarding the drug dependent, the risks of colitis and hypophysitis appeared to be more related to the CTLA-4 antibodies (ipilimumab); the pneumonitis and hepatitis appeared to be more related to the PD-1 antibodies (nivolumab). Regarding the dose dependent, we compared two different doses in drug combinations (nivolumab 3 mg/kg pus ipilimumab 1 mg/kg versus nivolumab 1 mg/kg plus ipilimumab 3 mg/kg). The result showed that nivolumab 3 mg/kg plus ipilimumab 1 mg/kg significantly increased the total 3-5 grade IRAEs.

STUDY STRENGTHS AND LIMITATIONS

The most important strength of this study is that all the included studies are RCTs with detailed registration information in ClinicalTrials.gov. Meanwhile, there are also several limitations in this study. First, the number of the included RCTs was small (10 studies involving 8 RCTs and 2,716 patients), which limited us to perform subgroup analysis. Further high-quality RCTs with large sample sizes are needed to verify our conclusion. Second, some definitions of adverse events were not uniform. For example, immune-related hepatitis was reported as hepatitis or increased aspartate transaminase/alanine transaminase, and immune-mediated colitis was reported as colitis or diarrhea, which might lead to incomplete data collection. This should be standardized in the future study. Third, patients with various cancers were included, which might have bias in the incidence of some adverse effects. For example, lung cancer was related with a high risk of developing pneumonitis from previous lung disease, radiotherapy, and smoking history. Subgroup analysis was not done based on different types of cancers due to the insufficient studies on lung cancer, sarcoma, glioblastoma, and urothelial carcinoma. Fourth, heterogeneity was found in the outcomes of colitis and total adverse events among the included studies. The heterogeneity might have resulted from the differences of cancer types, follow-up time, drug dose, and so on.

CONCLUSIONS

This meta-analysis demonstrated that, compared with ICI monotherapy, combination therapy with ICI drugs significantly increased the risk of organ-specific IRAEs in colitis, hypothyroidism, hepatitis, hypophysitis, hyperthyroidism, and pneumonitis. The incidence and severity of organ-specific IRAEs were drug- and dose-independent. Although the incidence of

high-grade organ-specific IRAEs was relatively low, clinicians should be aware of these adverse effects so that patients can be promptly managed.

AUTHOR CONTRIBUTIONS

Study concept and design: LD, YT, FS. Data extraction and analysis: LD, NW, KZ, YQ. Manuscript writing: LD, YT, NW, KZ, FS. Revision of the manuscript: LD, YT, KZ, YQ, NW, YL. Technical or material support: YQ, NW, FS.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.01671/full#supplementary-material

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High Dimensional Mass Cytometry Analysis Reveals Characteristics of the Immunosuppressive Microenvironment in Diffuse Astrocytomas

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The tumor immune microenvironment (TIME) plays a pivotal role in tumor development, progression, and prognosis. However, the characteristics of the TIME in diffuse astrocytoma (DA) are still unclear. Leveraging mass cytometry with a panel of 33 markers, we analyzed the infiltrating immune cells from 10 DA and 4 oligodendroglioma (OG) tissues and provided a single cell-resolution landscape of the intricate immune microenvironment. Our study profiled the composition of the TIME in DA and confirmed the presence of immune cells, such as glioma-associated microglia/macrophages (GAMs), CD8+ T cells, CD4+ T cells, regulatory T cells (Tregs), and natural killer cells. Increased percentages of PD-1+ CD8+ T cells, TIM-3+ CD4+ T cell subpopulations, Tregs and pro-tumor phenotype GAMs substantially contribute to the local immunosuppressive microenvironment in DA. DAs and OGs share similar compositions in terms of immune cells, while GAMs in DA exhibit more inhibitory characteristics than those in OG.

Keywords: diffuse astrocytoma, oligodendroglioma, CyTOF, immune profiling, microenvironment

INTRODUCTION

Diffuse astrocytomas (DAs) account for 10% of all adult primary brain tumors (1). They are diffusely infiltrating World Health Organization (WHO) grade II brain neoplasms, and DA patients have a median survival in the range of 5–7 years (2). Even with a combination of available therapeutic modalities, including surgery, radiotherapy, and chemotherapy, the invasive growth and resistance to therapy exhibited by these tumors result in their recurrence, malignant transformation, and almost invariable progression to high-grade glioma in most patients (3). These challenges underscore the need for novel strategies to improve the outcomes of patients with low-grade glioma (LGG) (4).

Immunotherapy is an emerging breakthrough approach that promises the possibility of highly specific and less toxic treatment compared to conventional chemotherapy (5); this approach aims to induce an adaptive immune response that specifically targets and kills tumor cells without

affecting normal cells. Thanks to advances in the fields of neuro- and cancer-immunology, a wide range of immunotherapies for WHO grade IV glioblastoma are now undergoing development, including antibodies, adoptive cell transfers, vaccines, virally-based treatments and immune checkpoint blockade (6–9). However, the efficacy of immunotherapy for the treatment of DAs is still controversial.

The infiltration of diverse immune cell populations has been reported in various cancer types, and the cooperation between tumor cells and tumor-infiltrating immune cells drives tumor development (10). Glioma cells secrete numerous cytokines, chemokines, and growth factors that promote the infiltration of a range of immune cells, such as resident microglia, peripheral macrophages, CD4+ T cells, CD8+ T cells, and regulatory T (Treg) cells, into the tumor (11–13), and these non-neoplastic cells play crucial roles in cancer growth, metastasis, and response to treatment. Therefore, sound knowledge of the immune microenvironment of DA will aid the design of effective therapeutic strategies and provide a foundation for the success of immunotherapy (14). In previous studies, histopathological analysis, immunohistochemistry, and flow cytometry were utilized to reveal the immunological features of the glioma immune microenvironment (15, 16). To the best of our knowledge, immune changes in the microenvironment of DA have rarely been reported, and a comprehensive understanding of the phenotypic characterization of immune cells in the DA tumor microenvironment at the protein level is highly needed.

To this end, we utilized mass cytometry (CyTOF) to examine the TIME of DAs and paired peripheral blood mononuclear cells (PBMCs). We also collected specimens of oligodendroglioma (OG) to compare the TIME in DAs and OGs. CyTOF enables the simultaneous measurement of more than 30 parameters per single cell using metal isotope-conjugated antibodies with minimal overlap, which maximizes the information obtained from each individual sample (17). By addressing the cellular and molecular complexity of the immunosuppressive microenvironment, our data provide a detailed dissection of the DA immune cell types and reveal immunosuppressive changes in glioma-associated microglia/macrophages (GAMs) and T cell exhaustion in DA lesions. Our data show that immunosuppressive programs are present in early stages in LGG and likely compromise antitumor immunity. Our study suggests that neoadjuvant immunotherapy strategies targeting innate immune cells in DA lesions have the potential to reactivate the TIME and transform the tumor response to affect checkpoint blockade.

MATERIALS AND METHODS

Human Specimens

Blood and LGG tissues were obtained from patients with WHO grade II DA and OG undergoing craniotomy surgery at Beijing Tiantan Hospital (Beijing, China) from June 2018 to April 2019. All patients were diagnosed with WHO grade II diffuse DA or OG, which was confirmed by histopathology. None of the patients used glucocorticoids before sampling.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board and Ethics Committee of Beijing Tiantan Hospital, Capital Medical University. Written informed consent was obtained from each patient.

Glioma Tissue Single Cell Dissociation

DA or OG tissues were washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS, without Mg^{2+} and Ca^{2+}, catalog no. D8537, Sigma-Aldrich) immediately after surgery. Briefly, the DA or OG tissues were dissociated using type IV collagenase (catalog no. 17104019, GIBCO) for 10 min at 37°C. Then, the samples were washed with Dulbecco's modified Eagle medium (DMEM, catalog no. D5796, Sigma-Aldrich) and centrifuged at 300 g for 4 min at 18°C with minimal braking. The samples were then filtered through a 40 mm cell strainer with DPBS and washed with red blood cell (RBC) lysis buffer (catalog no. 555899, BD Biosciences). The dissociated cell suspension was then washed twice with DPBS. The cell pellet was resuspended in staining buffer (DPBS containing 5% fetal bovine serum, FBS; catalog no. 0500, ScienCell).

Blood Sample Single Cell Dissociation

Fresh blood samples were collected into ethylenediaminetetraacetic acid (EDTA) anticoagulation tubes and then centrifuged at 800 g for 5 min with minimal braking to remove the plasma. Then, the samples were transferred into SepMate PBMC isolation tubes containing Ficoll (catalog no. 86450, STEMCELL Technologies) and centrifuged at 1,200 g for 10 min with minimal braking. The cells were washed with RBC lysis buffer. Then, the cells were washed twice with DPBS and resuspended in staining buffer.

Mass Cytometry

A panel of 33 antibodies designed to distinguish a broad range of immunocytes was used. Antibodies were either purchased in a pre-conjugated form from Fluidigm or purchased in a purified form from Biolegend and conjugated in-house using the Maxpar® X8 Multimetal Labeling Kit (catalog no. 201300, Fluidigm) according to the manufacturer's recommendations. The antibodies and reporter isotopes are listed in **Table S1**. Briefly, the cell samples were rewarmed rapidly. Cells from glioma tissue were stained with anti-CD45 antibody conjugated with 156Gd, while cells from PBMCs were first stained with anti-CD45 antibody conjugated with 89Y. Then, glioma and PBMC cells were mixed together and stained with cell surface antibodies for 30 min at room temperature. Subsequently, the samples were permeabilized overnight at 4°C and stained with intracellular antibodies for 30 min at room temperature. The antibody-labeled samples were washed and incubated in 0.125 nM intercalator-Ir (catalog no. 201192B, Fluidigm) diluted in phosphate-buffered saline (PBS, catalog no. 806544, Sigma-Aldrich) containing 2% formaldehyde and stored at 4°C until CyTOF examination. Before acquisition, the samples were washed with deionized water and then resuspended at a concentration of 1×10^6 cells/mL in deionized water containing a 1:20 dilution of EQ Four

Element Beads (catalog no. 201078, Fluidigm). The samples were then examined by mass cytometry (Fluidigm).

CyTOF Data Analysis

Data were obtained as.fcs files. The addition of EQ Four Element Beads allowed us to use a MATLAB-based normalization technique utilizing bead intensities as previously described (18). The CyTOF data were analyzed with Cytobank (www.cytobank.org). The cell types were identified based on the following parameters: T cells, CD45+ CD3+; natural killer (NK) cells, CD45+ CD3-CD16+ CD56+ (10, 19); B cells, CD45+ CD19+; monocytes, CD45+ CD14+ CD16+ (20); macrophages or microglial cells, CD45+ CD11b+ CD3-CD19-CD66b- (15); Tregs, CD45+ CD4+ CD25+ CD127- (21), and granulocytes, CD45+ CD66b+. Monocytes and macrophages constitute mononuclear phagocytes (22). Manual gating was applied to indicate the cell types as previously reported (23). ViSNE (24) algorithms were used on the indicated gated cells. The viSNE analysis of T cells or GAMs was performed for patients with samples with more than 500 cell counts for both PBMCs and tumor lesions. Then, the automatic cluster gate functionality was used for the hierarchical cluster analysis. Heatmaps were generated by R software (version 3.4.0).

Heatmap Data Normalization

For **Figures 3D**, **4C**, the log10-scaled values were used.

For **Figures 3E,F**, we calculated the ratio of the value of each T cell cytokine or marker to that of the paired PBMC T cells in each patient and then calculated the log10-scaled ratio to obtain the normalized values.

Immunohistochemistry and Immunofluorescence

DA samples were fixed overnight at 4°C in 4% formalin and embedded in paraffin blocks to obtain paraffin sections. Immunohistochemical staining was performed as previously reported (25). For immunofluorescence, 3 μm paraffin sections were washed twice in PBS (catalog no. 806544, Sigma-Aldrich) for 15 min, permeabilized in 0.2–0.5% Triton X-100 (catalog no. T8200-100, Solarbio) and blocked in 5% normal donkey serum (catalog no. 017-000-001, Jackson Lab) for 1 h and stained with primary antibody overnight. The primary antibodies were detected using fluorescent-conjugated secondary antibodies (catalog no. PV-6000, ZSGB-BIO). Sections were mounted with fluorescence mounting medium (catalog no. S3023, Dako). As previously reported (26), the Opal 4-Color Manual IHC Kit (catalog no. NEL810001KT, Perkin Elmer) was used for the analysis of the formalin-fixed paraffin-embedded DA sections according to the manufacturer's protocol. Fluorescent images were acquired with a Zeiss LSM880 NLO microscope. The primary antibodies were anti-CD45 (catalog no. AB40763, Abcam), anti-CD11b (catalog no. 21851-1-AP, Proteintech), anti-TNFα (catalog no. 60291-1-Ig, Proteintech), and anti-IDO (catalog no. 86630S, CST).

TABLE 1 | Basic characteristics of all patients.

No.	Histopathology	Age	Gender	*IDH1*	*IDH2*	*1p19q*	*TERT* promoter
0759	DA	39	Male	Mut	Wt	Noncodel	Wt
0884	DA	41	Male	Mut	Wt	Noncodel	Wt
1827	DA	52	Female	Mut	Wt	Noncodel	C228T
8974	DA	67	Female	Mut	Wt	Noncodel	C250T
9144	DA	38	Male	Mut	Wt	Noncodel	C250T
9852	DA	38	Female	Wt	Wt	Noncodel	Wt
1837	DA	36	Male	Mut	Wt	Noncodel	Wt
5189	DA	34	Male	Mut	Wt	Noncodel	Wt
5749	DA	52	Female	Mut	Wt	Noncodel	Wt
7684	DA	28	Male	Mut	Wt	Noncodel	Wt
9203	OG	36	Male	Mut	Wt	Codel	Wt
7541	OG	49	Male	Wt	Wt	Codel	C250T
2948	OG	30	Male	Mut	Wt	Codel	C228T
5749	OG	52	Female	Mut	Wt	Codel	Wt

DA, diffuse astrocytoma; OG, oligodendroglioma; IDH, isocitrate dehydrogenase; TERT, telomerase reverse transcriptase; Wt, wild type; Mut, mutation, Codel, codeletion.

Statistics

For the CyTOF experiments, 10 DA samples and paired PBMCs and 4 OG samples were analyzed. The Wilcoxon matched-pair signed rank test and Mann–Whitney test were used accordingly to analyze the statistical significance. The statistical analysis was performed using GraphPad Prism (version 7.00). $P < 0.05$ were considered statistically significant.

Data Availability

The raw CyTOF data used and analyzed in the current study are available from the corresponding author upon reasonable request.

RESULTS

Single-Cell Profiling of the Diffuse Astrocytoma Immune Microenvironment

We obtained 10 WHO grade II DAs and paired peripheral blood samples as well as 4 OG tumor tissues. The baseline characteristics of all patients are summarized in **Table 1**.

We simultaneously mapped the immune compartments of DA, OG lesions, and PBMCs (**Figure 1A**). The initial gating strategies used for CD45+ cells are provided in **Figure 1B**, and the gating strategies used for the indicated immune cells are summarized in **Table S2**. The ViSNE map of CD45 + cells collected from all DA samples showed differential abundances of infiltrating immune cell populations in the DA immune microenvironment compared to those in peripheral blood (**Figure 1C**).

Mononuclear Phagocytes and T Cells Dominate the Diffuse Astrocytoma Immune Microenvironment

We analyzed the distributions of the different immune cell lineages that accumulated in DAs and paired PBMCs in

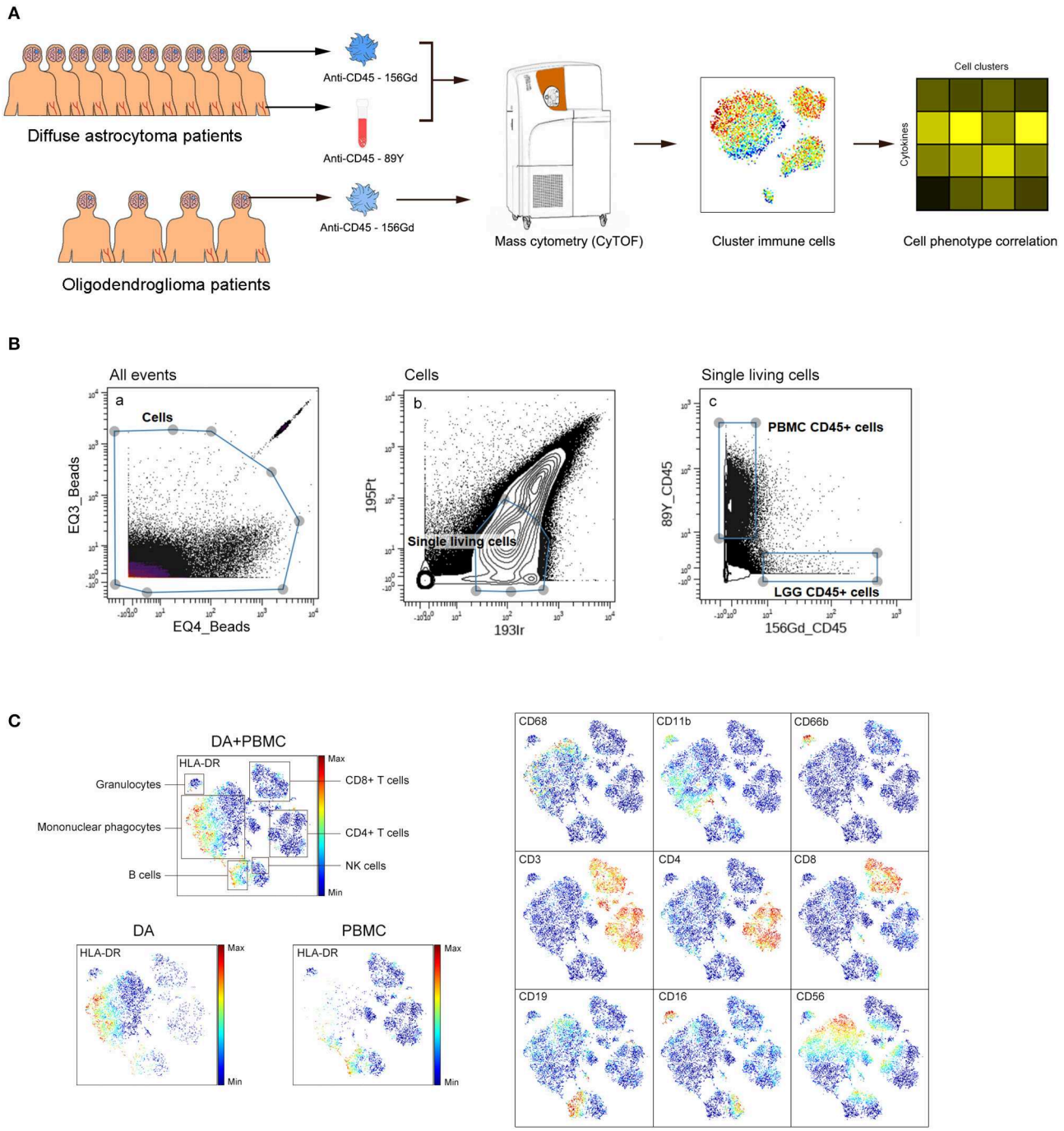

FIGURE 1 | Analysis of the immunosuppressive microenvironment of DA using mass cytometry. **(A)** Schematic defining the immune composition of DAs and OGs. Glioma tissues and paired PBMC samples were collected from patients. Samples were processed and stained with antibodies conjugated to metal isotopes. CyTOF single cell data were used to identify the immune features of patients. **(B)** All ungated events were sequentially gated in Cytobank to identify CD45+ cells. (a) EQ3 beads and EQ4 beads were used to identify cells. (b) Single living cells were identified by gating the cells negative for 195Pt and positive for 193Ir. (c) CD45+ cells from LGG PBMCs were obtained from living single cells. **(C)** ViSNE plots of complete immune systems according to the relative expression of CyTOF markers in all samples. The cell populations are also indicated (left). Five hundred immune cells per sample were included in the viSNE analysis.

patients. The most abundant immune cells in the DA immune microenvironment were mononuclear phagocytes (70.02%) and T lymphocytes (20.86%). Compared with that in PBMCs, the proportion of mononuclear phagocytes was significantly increased in DAs ($p < 0.01$), while the proportions of T cells and B cells were significantly decreased ($p < 0.01$),

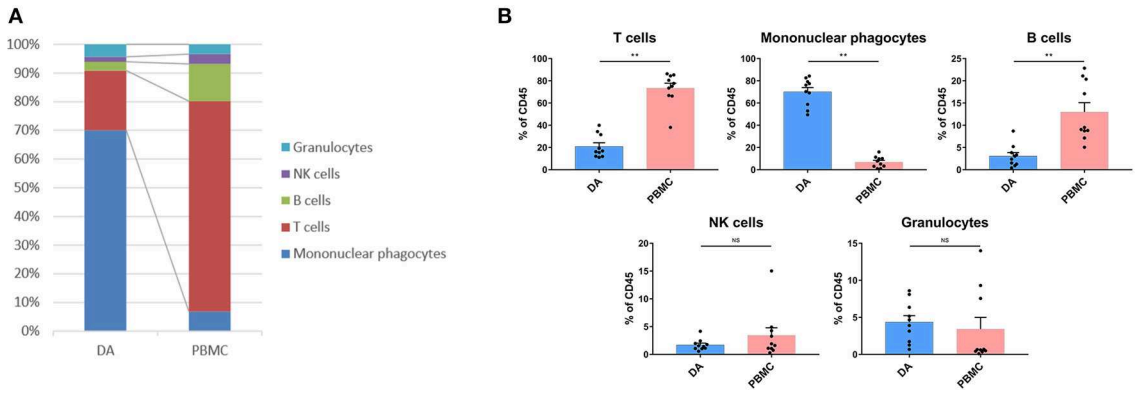

FIGURE 2 | Immunosuppressive changes in the DA microenvironment. **(A)** Composition of the CD45+ compartment showing the average frequencies of the major immune lineages in each tissue. **(B)** Bar plots showing the frequencies for each DA patient and paired PBMC sample (by Wilcoxon matched-pair signed rank test). Bar plots show the mean with the SEM (NS, no significance; $^{**}p < 0.01$).

and the proportions of NK cells and granulocytes were similar (**Figures 2A,B**).

T Cells Are Exhausted, and Tregs Are Increased in the Diffuse Astrocytoma Immune Microenvironment

Compared with that in PBMCs, the percentage of CD4+ T cells ($p < 0.01$) was decreased, while that of CD8 + T cells ($p < 0.01$) was increased in DAs. Specifically, the Treg proportion in the DA lesions was significantly increased in all patients ($p < 0.05$) (**Figure 3A**). Programmed cell death protein 1 (PD-1)-, T cell immunoglobulin domain and mucin domain-3 (TIM-3)- or lymphocyte activation gene 3 (LAG-3)-positive T cells are recognized as exhausted subsets (27–29). Compared to those in PBMCs, the proportions of TIM-3+ CD4+ T cells ($p < 0.05$) and PD-1+ CD8+ T cells ($p < 0.01$) were remarkably higher in tumor sites (**Figure 3A**).

The dimensionality reduction tool viSNE (24) was employed to convert the high-dimensional CyTOF data from each sample into a two-dimensional map. Among the 10 DA patients, four patients had more than 500 T cells in both the tumor lesions and the PBMCs, and viSNE analysis was performed for these patients. In the viSNE map, T cells in tumor sites displayed similar distributions to those in PBMCs (**Figure 3B**). A hierarchical cluster analysis of the T cells using the automatic cluster gate functionality was performed to fully capture the heterogeneity of the T cell compartment. According to the surface markers, the T cells were subdivided into 16 subgroups (**Figure 3C**). The expression profiles of the T cell clusters were visualized in a heatmap (**Figure 3D**). This approach led to the identification of seven CD4+ phenotypes, seven CD8+ phenotypes and two CD4+/CD8+ double-negative phenotypes.

Although the CD8+ T cell proportion was elevated in tumor sites, their ability to secrete the antitumor cytokines interferon γ (IFNγ), tumor necrosis factor β (TNFβ), T-bet and granzyme B was reduced compared to that of the CD8+ T cells in the PBMCs, while PD-1 was more frequently expressed on CD8+ T cells in PBMCs (**Figure 3E**). Compared to those on CD4+ T cells in PBMCs, the expression levels of antitumor (TNFβ, T-bet, and granzyme B) and protumor (PD-1 and IL-10) markers on CD4+ T cells in tumor sites were commonly higher (**Figure 3F**).

Glioma-Associated Microglia/Macrophages Were Clearly Distinguishable From Mononuclear Phagocytes in PBMCs

Previous studies showed the extensive infiltration of gliomas with peripheral macrophages and resident microglia (30), which are collectively termed GAMs. In the current study, GAMs were the most enriched population in DA lesions. Five patients had more than 500 GAM cells or mononuclear phagocytes in both tumor sites and PBMCs, and viSNE analysis was performed on these cells. The ViSNE plot showed that GAMs were clearly distinguishable from mononuclear phagocytes in PBMCs (**Figure 4A**). According to the surface markers, GAMs or mononuclear phagocytes could be subdivided into 17 subgroups, with 6 subgroups mainly resident in DA lesions, 8 subgroups mainly resident in PBMCs, and 3 existing in both tumor sites and PBMCs (**Figure 4B**). The expression profiles of the GAM clusters were visualized in a heatmap (**Figure 4C**).

The viSNE map showed the elevated expression of both the anti-tumor marker tumor necrosis factor α (TNFα) and the pro-tumor markers transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), programmed death-ligand 1 (PD-L1), CD206, indoleamine-pyrrole 2,3-dioxygenase (IDO), and IL10 in GAMs compared with those in mononuclear phagocytes in PBMCs (**Figure 4D**). A subgroup of GAMs represented in cluster M-8, which mainly existed in DA lesions, displayed high levels of VEGF and PD-L1 expression. GAMs may promote T cell apoptosis through expressing PD-L1 (31, 32). By secreting VEGF, GAMs might differentiate into a pro-angiogenic and immunosuppressive phenotype (26). Meanwhile, certain GAM subgroups (M-7) could coexpress antitumor (TNFα) and protumor (IDO and PD-L1) markers.

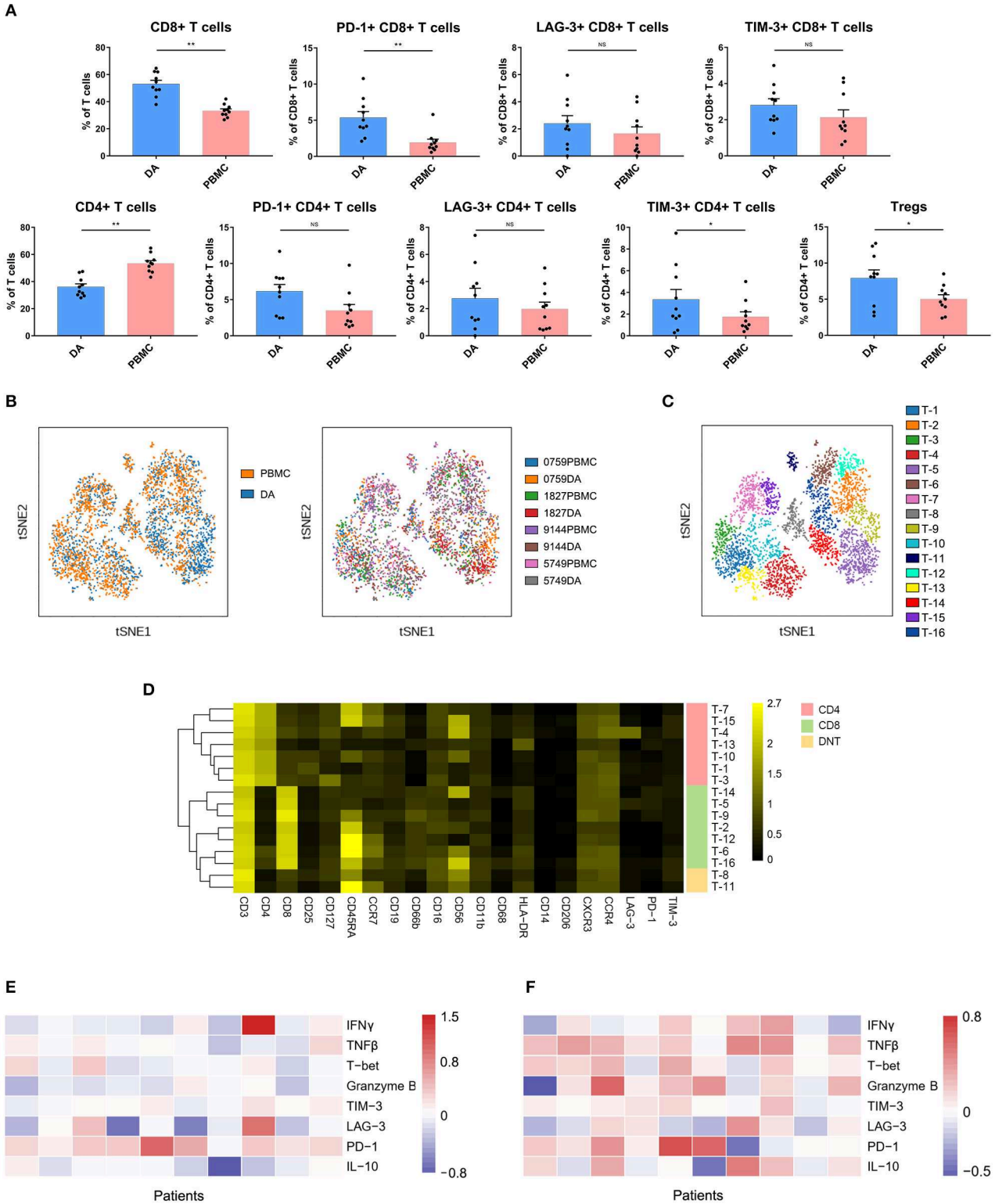

FIGURE 3 | Exhausted T cell compartment in DA lesions. **(A)** Bar plots showing the frequencies of T cell subgroups in tumor sites and PBMCs from patients with DA (by Wilcoxon matched-pair signed rank test). Bar plots show the mean with the SEM (NS, no significance; $^{*}p < 0.05$, $^{**}p < 0.01$). **(B)** ViSNE map, colored by sample type (left) or sample source (right), displaying T cell subgroups in 4 patients. **(C)** ViSNE map, colored by clusters, displaying T cell subgroups in 4 patients. **(D)** Heatmap showing the normalized expression of the indicated markers for 16 T cell clusters identified in the 4 patients. **(E,F)** Heatmap showing relative marker expression levels in four DA patients. The relative marker expression levels were determined by the ratios of the indicated marker expression levels of T cells in tumor sites to those in PBMCs.

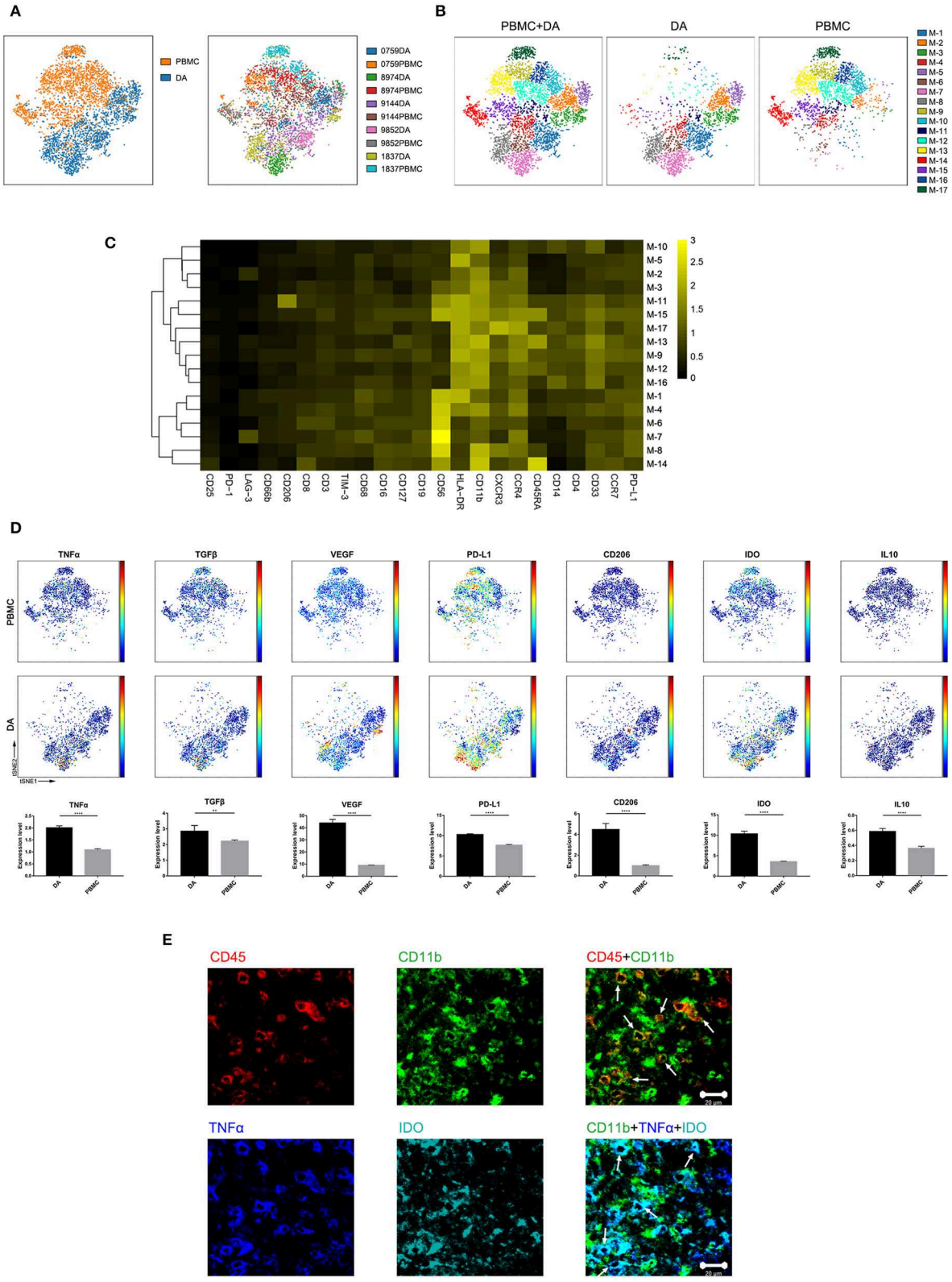

FIGURE 4 | Characterization of GAM phenotypes in DA. **(A)** ViSNE map, colored by sample type (left) or sample source (right), displaying GAM subgroups in five patients. **(B)** ViSNE map, colored by clusters, displaying the GAM subgroup distribution in DA lesions and PBMCs. **(C)** Heatmap showing the normalized expression

(Continued)

FIGURE 4 | of the indicated markers for 17 GAM clusters identified in five patients. **(D)** Normalized expression of the indicated markers on the viSNE map. Bar plots show significant differences in the expression levels of the indicated markers between PBMCs and DA lesions (by the Mann–Whitney test). Bar plots show the mean with the SEM (**$p < 0.01$; ****$p < 0.0001$; NS, no significance). **(E)** Representative DA tissue stained for CD11b (green), CD45 (red), IDO (cyan), and TNFα (blue). Costaining of CD45 and CD11b (upper) indicated that most CD45+ immunocytes in DA were CD11b+ cells. Costaining of CD11b, IDO, and TNFα (lower) demonstrated that GAMs could coexpress TNFα and IDO (arrows).

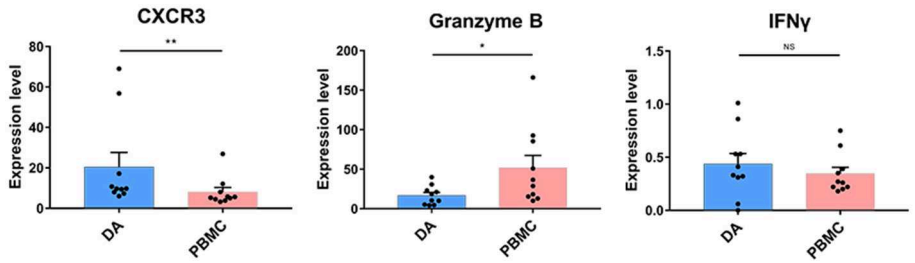

FIGURE 5 | Cytolytic NK cells are dysregulated at the tumor site. Bar plots showing CXCR4, granzyme B, and IFNγ expression in NK cells from patients with DA and paired PBMCs (by the Wilcoxon matched-pair signed rank test). Bar plots show the mean with the SEM (*$p < 0.05$; **$p < 0.01$; NS, no significance).

We revealed mononuclear macrophage infiltration in DA lesions using immunohistochemical and immunofluorescence costaining and verified the finding that antitumor (TNFα) and protumor (IDO) markers were coexpressed in certain GAM subgroups (**Figure 4E**).

Natural Killer Cells Are Not Cytolytic in Diffuse Astrocytoma Lesions

NK cell proportions were not significantly increased at the tumor site compared with those in the peripheral blood of patients, although the NK cells that infiltrated into the tumor lesions expressed higher levels of CXCR3 ($p < 0.01$) (**Figure 5**), which is a molecule reported to be required for NK cell infiltration (33). Moreover, the NK cells that remained at the tumor site showed lower levels of cytolytic activity, as these cells expressed similar levels of IFNγ and lower levels of granzyme B compared to those in peripheral blood (**Figure 5**).

The Tumor Immune Microenvironment of Diffuse Astrocytoma Exhibits More Inhibitory Characteristics Than That of Oligodendroglioma

The composition of immune cell subsets was similar in the DAs and OGs (**Figures 6A,B**). The proportions of the T cell subpopulations in DAs and OGs were also similar, and T cells in DAs and OGs demonstrated comparable exhaustion trends (**Figure 6C**). The pro-tumor markers TGFβ and VEGF were more strongly expressed by GAMs in DAs than in OGs, while IL10, PD-L1, CD206, and IDO were similarly expressed by GAMs in DA and OGs (**Figure 6D**).

DISCUSSION

The TIME in DAs plays essential roles in tumor development, progression, and prognosis. Comprehensive profiling of the intricate milieu and its interactions remains lacking, and single-cell technologies such as CyTOF provide unique opportunities for this task. Utilizing the CyTOF approach, we analyzed the infiltrating immune cells from DA surgical tissues based on a panel of 33 markers and provided a single cell-resolution overview of the intricate DA immune microenvironment. Our study characterized the TIME in DAs, which is composed of a variety of immune cells, such as GAMs, CD8+ T cells, CD4+ T cells, Tregs, and NK cells. The enrichment of exhausted T cell subpopulations, recruitment of Tregs, and the strong pro-tumor phenotype of GAMs together contribute to the immunosuppressive microenvironment in DAs. DAs and OGs have been shown to share similar components and distributions of immune cells. However, the GAMs of DAs exhibit more inhibitory characteristics than those of OGs.

Historically, the central nervous system has been defined as "immunologically privileged" (34) and has been considered distinct relative to other organs due to the presence of the blood-brain barrier (BBB), which prevents the migration of immunocytes and cytokines into the brain (35). In LGG, the normal vascularization and the function of the BBB remain mostly intact and resemble that under normal conditions (36). In our study, the most abundant immune cells in DA were GAMs (70.02%) and T lymphocytes (20.86%). Compared with their counterparts in the paired PBMCs, the proportion of GAMs was significantly increased in DA lesions, while the proportions of T cells and B cells were significantly decreased, and the proportions of NK cells and granulocytes were similar. Our data suggest that although the BBB in DA lesions is fairly intact, certain immune cell populations can migrate across the BBB and infiltrate into the tumor, which might make them an adequate substrate for immunological antitumor therapies.

Inhibitory immune checkpoints are responsible for the dampening of antitumor immune functions (37). The development of immune checkpoint blockade therapies, including anti-PD-1 and anti-CTLA4 therapies, has provided

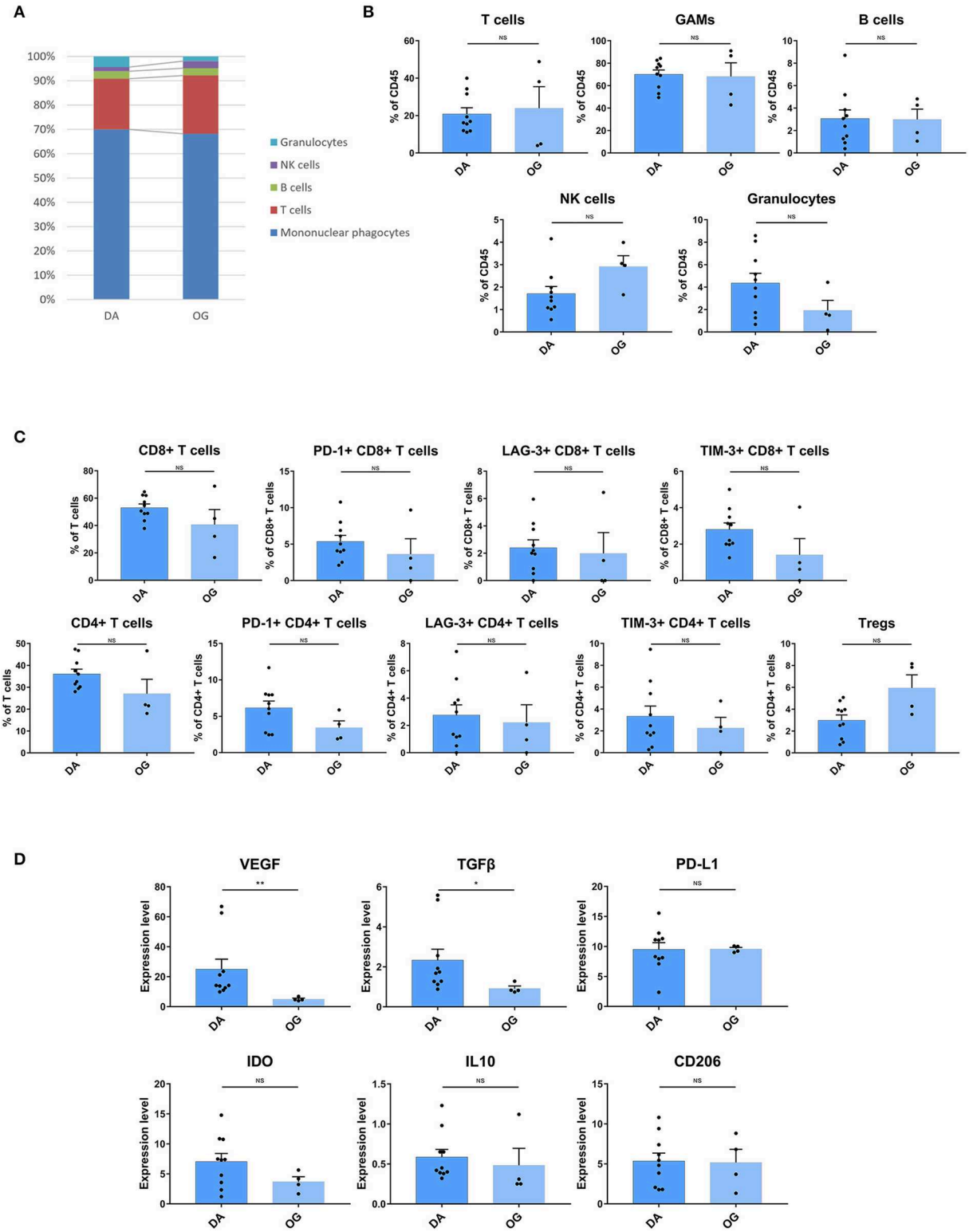

FIGURE 6 | The TIME of DA shows more inhibitory characteristics than that of OG. **(A)** The frequencies of DA and OG immunocytes. Composition of the CD45+ compartment showing the average frequencies of the major immune lineages in each tissue. **(B)** Bar plots showing the frequencies for each DA patient and OG patient (by the Mann–Whitney test). Bar plots show the mean with the SEM (NS, no significance). **(C)** Bar plots showing the frequencies of T cell subgroups in DA and OG (by the Mann–Whitney test). Bar plots show the mean with the SEM (NS, no significance). **(D)** Bar plots of pro-tumor marker expression in GAMs in DA and OG (by the Mann–Whitney test). Bar plots show the mean with the SEM (*$p < 0.05$; **$p < 0.01$; NS, no significance).

new avenues for cancer treatment (38). Our results demonstrated that in the DA immune microenvironment, CD8+ T cell populations are highly enriched but express higher levels of PD-1 than those in the blood, and the expression level of antitumor-related factors is generally reduced. The increase in the quantity of exhausted CD8+ T cells in DA indicates that checkpoint blockade approaches that promote the antitumor effects of these immune cells may benefit immunotherapy of DA.

With the 2016 update of the WHO classification of tumors of the central nervous system (39), WHO grade II DA and OG tumors have been subcategorized according to distinct molecular markers. Patients with WHO grade II DAs and OGs were found to have statistically significant differences in progression-free survival (PFS), with OG patients having a statistically better PFS than DA patients (40). Little is known about how the microenvironment differs between DAs and OGs. Our study found that the immune cell composition of DA and OG was similar, and T cells in both diseases showed similar exhaustion characteristics. However, GAMs in DAs expressed higher levels of VEGF and TGFβ and exhibited more adverse immune-inhibitory characteristics than OGs.

Finally, while our study has presented useful resources and novel insights into the cellular composition and functions of the TIME in DAs, a limited number of cases have been collected in this pilot study. Future validation in a larger collection of patients would further support our conclusions and better characterize the prognostic values of immune components for DA.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was approved by the Institutional Review Board and Ethics Committee of Beijing Tiantan Hospital, Capital Medical University. Written informed consent was obtained from each patient.

AUTHOR CONTRIBUTIONS

YC, SW, DC, JiangW, and JZ conceived and designed the study. WF and WW analyzed and interpreted the CyTOF data. HL, YJ, RH, JieW, JiancW, HX, and ZY participated in sample collection and data acquisition. All authors participated in the drafting of the manuscript, read and approved the final version of the manuscript, and gave their consent for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00078/full#supplementary-material

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miR-20a-5p/TGFBR2 Axis Affects Pro-inflammatory Macrophages and Aggravates Liver Fibrosis

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Combined inhibition of programmed death-ligand 1 (PD-L1) and transforming growth factor-β (TGF-β) displayed additive anti-tumor response in a subgroup of cancer patients, highlighting the importance of understanding the multifaceted roles of TGF-β in immunity and fibrosis. In the present research, we show that TGF-β signaling pathway, controlled by miR-20a-5p and transforming growth factor-β receptor 2 (TGFBR2), alters the inflammation and fibrosis processes in liver. We performed integrated analysis of differently expressed miRNA (DEM) associated with liver fibrosis and screened miR-20a-5p out as a key regulator in inflammation-driven liver fibrosis. We subsequently conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the genes targeted by miR-20a-5p. And the result showed that 12 target genes were significantly enriched in TGF-β signaling pathway. Further study showed that miR-20a-5p was down-regulated and involved in inflammation during liver fibrosis in human and mouse samples, indicating that miR-20a-5p and inflammation are functionally linked during liver fibrosis progression. To uncover the underlying pro-inflammatory mechanism of miR-20a-5p in liver fibrosis, we selected and verified TGFBR2, which is a key functional receptor in TGF-β signaling pathway, as a direct target gene of miR-20a-5p. The downregulation of miR-20a-5p in liver fibrosis resulted in TGFBR2-activated TGF-β signaling pathway, followed by the activation of macrophage and extracellular matrix (ECM) production by hepatic stellate cell (HSC). Our results identify the miR-20a-5p/TGFBR2 axis as a key regulator of TGF-β signaling, and highlight the critical role of miR-20a-5p in the development of liver fibrosis.

Keywords: miR-20a-5p, liver fibrosis, TGF-β signaling pathway, inflammation, TGFBR2

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INTRODUCTION

Therapeutic antibodies against the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis has been approved to treat multiple tumors, but only not effective in all patients (1). It is well-known that transforming growth factor-β (TGF-β) is of importance in resistance to immune checkpoints inhibitors. Recently, M7824 (MSB0011359C), a bifunctional fusion therapeutic antibody against human PD-L1 fused to the extracellular domain of human transforming growth factor-β receptor 2 (TGFBR2) showed enhanced preclinical antitumor activity through simultaneously blocking the PD-L1 and TGF-β signaling pathways (2, 3). These results prompt

us to understanding the multifaceted roles of TGF-β signaling pathway in immunity and fibrosis. Liver fibrosis is an essential pathological process that may deteriorate into liver cirrhosis and liver cancer, making it one of the leading causes for the high mortality and morbidity around the globe (4). Regardless of origins and etiologies, liver fibrosis developed from viral infection, alcohol, non-alcoholic steatohepatitis (NASH), and autoimmune diseases, featuring chronic, and pathological process (5). Relying on the studies of underlying liver injury, several evidences highlighted the important role of immune reactions (6). Liver cell damage tends to induce the secretion of pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α), tumor necrosis factor-β (TNF-β), nuclear factor kappa-B (NF-κB), Interleukins (ILs), which sequentially stimulate the infiltration of inflammatory cells (7). Subsequently, excessive infiltration of inflammatory cells would render the liver more vulnerable to damage by preying upon liver cells and thus initiating fibrogenesis. An in-depth understanding about the underlying mechanism of liver fibrosis is the cornerstone to research the effective therapies for chronic liver diseases.

MicroRNAs (miRNAs) are endogenous, small non-coding RNA molecules that play essential part in various biological functions and numerous processes, such as immune response, cell proliferation, and apoptosis, through the post-transcriptional regulation of gene expression in cells (8). Increasing evidence indicated that aberrant expression of miRNAs are closely related to numerous types of cancer, as well as liver fibrosis (8–12). It's frequently reported that miRNA expression level in the serums or liver tissues of liver fibrosis patients is dominantly changed (13–15). Normally, miRNAs exacerbates liver fibrogenesis by incomplete matches with their host genes that are related to hepatic stellate cells (HSCs) activation, immune cell sensitization, as well as hepatocytes apoptosis (16, 17).

In our study, we demonstrated that the level of inflammatory cytokines in serum was upregulated in CCl_4-treated mice, suggesting that inflammation is accompanied by liver fibrosis. Many previous studies reported that miRNAs drove liver fibrogenesis by regulating inflammation response. We performed integrated analysis of differently expressed miRNA (DEM) associated with liver fibrosis and screened miR-20a-5p out as a key regulator in inflammation-drove liver fibrosis. We subsequently conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the genes targeting by miR-20a-5p. The result showed that 12 target genes were significantly enriched in TGF-β signaling pathway, which participated in the development of liver fibrosis. Further study indicated that miR-20a-5p was down-regulated and related to inflammation during liver fibrosis in human and mouse samples, indicating that miR-20a-5p and inflammation are functionally linked during liver fibrosis progression. To reveal the pro-inflammatory mechanism of miR-20a-5p in liver fibrosis, we selected and verified TGFBR2, a key functional receptor in TGF-β signaling pathway and a target gene of miR-20a-5p. The downregulation of miR-20a-5p in liver fibrosis resulted in TGFBR2-activated TGF-β signaling pathway, followed by the activation of macrophage and extracellular matrix (ECM) production by HSC. Our results highlight a critical function of miR-20a-5p in the development of liver fibrosis, and the reintroduction of miR-20a-5p provides a promising therapeutic strategy for clinical intervention of liver fibrosis.

MATERIALS AND METHODS

Patients and Animal Model

Liver fibrosis specimens have been collected from 26 patients who were seeking treatment in our hospital and from 19 patients with liver diseases, except liver fibrosis. The published and well-acknowledged clinical guidelines were applied as the clinical diagnostic criteria for liver fibrosis. Written informed consent was obtained from the participants of this study and all participants were above 16 years old (**Table S1**).

CCl_4-induced liver fibrosis mouse model was established by conducting intraperitoneal injection of carbon tetrachloride (CCl_4; 0.6 mL/Kg body weight) in 8-week-old mice twice a week. The intraperitoneal injection lasted for 8 weeks. Male C57BL/6 mice were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. All animals were treated humanely according to protocols approved by the Fudan University Committee on Animal Care and Use.

Cell Lines and Cell Transfection

Immortalized mouse hepatocyte cell lines Hepa1-6 and macrophage cell line Raw264.7 were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. The miRNA mimics and negative control were transfected into Hepa1-6 cell line by using Lipofectamine™ 2000, in strict accordance with the manufacturer's instruction.

Quantitative-PCR (qPCR) Analysis

Total RNAs were extracted from Hepa1-6 cell line and liver fibrosis specimens using Trizol (Invitrogen, CA, USA) and all total Nucleic Acid Isolation Kit (Ambion Inc., USA), following the manufacturer's instruction. miRNAs primers for reverse transcription were purchased from Huada Co. Ltd (Beijing, China). The experiment was performed three times using SYBR Premix Ex Taq (cat#RR420A, TaKaRa, Japan) to quantify the mean values of delta Ct and SD (standard deviation). miRNA expression level was normalized to the relative quantities of U6 to investigate fold change. The primers used for miRNA and mRNA quantification were listed in **Table S2**.

FACS

Flow cytometry assay (using BD LSR Fortessa II) was carried out on hepatic non-parenchymal cells which are composed of the total profile of hepatic leukocyte population. The experiments were performed as published (18). The following pre-conjugated antibodies were used: CD11B (552850, BD bioscience), CD45 (553083, BD bioscience). Briefly, Hepatic macrophages were defined as viable CD45+ CD11B+ F4/80+ cells from digested livers and used to identify macrophage subsets. Subsets were

expressed as proportions of total hepatic macrophages or CD45+ cells. And we collected 10,000 cells every time.

Immunohistochemistry (IHC), Immunofluorescence (IF), and Western Blotting (WB)

Human and mouse liver tissues were processed for IHC, IF, and WB. Antibodies used in the present study are α-SMA (19245, CST), DESMIN (5332, CST), TGFBR2 (ab186838, abcam), p-Smad2 (18338T, CST), p-Smad3 (9520, CST), GAPDH (30201ES20; Yishen). Images were acquired using Olympus FV1000 confocal system with a 10X objective. The fluorescence was imaged using 552 nm/408 nm for mCherry /DAPI.

ELISA

Mouse IL-6 (VAL604, R&D), TNF-α (VAL609, R&D), Mouse IL-18 (7625, R&D) ELISA kits were used following the directions of the manufacturer. Conditioned medium (100 μl) was collected from triplicate samples.

Cell Viability Analysis

Cell viability was monitored using the Cell Counting Kit 8 (CCK8) method. Cells were inoculated onto a 96-well plate. Each well-contained 10,000 cells, and 6 repeats were used for every treatment. After 24 h, cellular proliferation was detected using a cell counting kit-8 (CCK-8, Yisheng). The effect on Hapa1-6 proliferation was evaluated by analyzing EC50 curves according to absorbance of cells (OD_{450}).

Determination of the Levels of miRNAs Related to Liver Fibrosis

The microarray file of liver miRNomes GSE40744 obtained from GEO database (http://www.ncbi.nlm.nih.gov/geo/) was referred to investigate miRNAs expression levels in our collected human fibrotic liver tissues and healthy controls. This miRNA microarray based on the platform of GPL14613 (Affymetrix microarray chip platforms) contained 18 fibrotic liver samples and 19 normal liver samples. GEO2R (19) is an interactive online tool and often used for gene expression analysis of microarray data through the GEO query and limma packages (20) available in R. The protocol was performed to investigate DEMs between normal, mild fibrotic, and advanced fibrotic liver samples. Adjusted p value of no >0.05 in combination with a $|\log_2$ (fold change) $|$ of >1 were set as the threshold for the identification of DEMs.

Prediction of Target Genes

The potential target genes of miR-20a-5p were analyzed by miRDB (21), TargetScan (22), and miRTarBase (23). The genes predicted by miRDB, TargetScan, and miRTarBase simultaneously were identified as the targets of DEM.

Functional Enrichment Analysis and miRNA-gene Network Construction

The database that can be used for annotating, visualizing and integrated discovering of the predicted genes (DAVID 6.8, https://david.ncifcrf.gov/) was applied in performing the KEGG pathway enrichment analysis (24, 25). FDR of <0.05 was considered as statistically significant.

The target genes enriched in KEGG pathways were mapped to the STRING database (https://string-db.org/) to evaluation the intricate functional associations amongst target genes (26), and the miRNA-gene network was constructed and visualized by Cytoscape software (Version 3.6.0).

Luciferase Activity Analysis

The partial sequences of TGFBR2 3′UTR which contained the wild or mutant binding sites of miR-20a-5p were amplified and then cloned into the pGL3-Basic luciferase vector (Promega, W.I.) with the aim of constructing pGl3-TGFBR2 (WT) and pGl3-TGFBR2 (Mut). Primers used in plasmid construction were as follows: forward 5′-CAGGCTGGGCCATGTCCAAA-3′ and reverse 5′-GTCAAATGCTAATGCTGRCATG-3′. The two plasmids were, respectively, co-transfected with miR-NC, miR-20a-5p mimic, anti-miR-NC, and anti-miR-20a-5p (Genomeditech). Forty eight hours later, the luciferase activity analysis was conducted on the Dual-Luciferase Reporter assay system (Promega, W.I.), in strict accordance with the instructions of the manufacturer.

Statistical Analysis

The data of the present study were presented in the form of mean ± SD (standard deviation). Unpaired/paired Student's t-test was used to analyze the significance of miRNA diversity between the two groups. A P value of <0.05 (two-tailed) was set as a threshold to distinguish statistically significant difference. Linear regression was performed using Graphpad Prism 7 (GraphPad Software Lnc, USA).

RESULTS

Inflammation Is Accompanied by Liver Fibrosis

CCl_4-induced liver injury in mice is a most commonly used animal model of liver fibrosis that features hepatocyte injury and the activation of HSCs. In our study, 16 eight-week-old mice were randomly divided into two groups. The CCl_4 group was conducted intraperitoneal injection of oil-dissolved CCl_4 twice a week, and the oil group was set as control. We first used immunofluorescence, RT-PCR, and ELISA to characterize the pathological features. The macroscopic appearance of the liver revealed almost significant amount of collagen accumulation in the CCl_4 treatment groups after 8 weeks, whereas the oil group was still normal (**Figures 1A,B**). Immunochemical staining exhibited that the α-SMA and DESMIN increased with liver fibrosis progression and other fibrosis-related genes were also remarkably enhanced (**Figure 1C**). Subsequently, the markers of liver injury in the serum were measured, along with aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, given that AST and ALT are expected to abundantly distribute in injured hepatocytes and that the excessive release of these two enzymes into the serum can indicate the degree of hepatocyte injury. As illustrated in **Figure 1C**, AST and

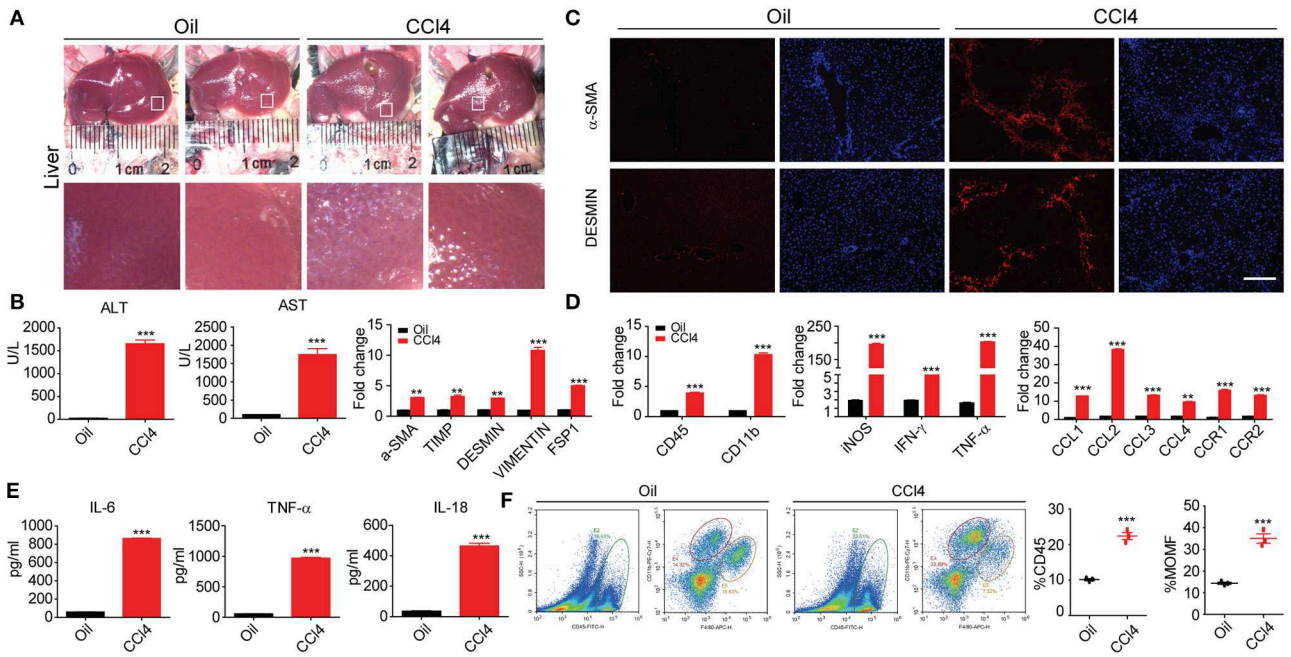

FIGURE 1 | Inflammation is accompanied by liver fibrosis. **(A)** Macroscopic analyses of the murine liver fibrosis model in C57BL/6 mice by 8 week of two times per week i.p. CCl_4 injection. Comparisons were made with control (injured oil) animals. **(B)** Histological characterization of hepatic fibrosis and myofibroblast activation by α-SMA and DESMIN immunohistochemistry. **(C)** Serum ALT and AST levels and other fibrosis protein levels ($n = 10$). **(D)** Whole-liver protein levels of Il-1β, Ccl2, Ccl3, and Cxcl2 measured by RT-PCR assay. **(E)** Supernatants were assayed for IL-6, TNF-α, and IL-18 levels by ELISA. **(F)** Flow cytometric analysis comparing expression of stated marker between inflammatory CD45+/$CD11b^{high}F4/80^{mid}$ hepatic macrophages. All data shown as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ALT levels increased dramatically, along with the notably up-regulated secretion and expression of inflammatory cytokines after CCl_4 treatment (**Figures 1D,E**). In addition, our study also clarified its possible association with the aberrantly changed hepatic macrophage subsets. The total hepatic macrophages were assayed and detected as $CD45^+$, $CD11B^+$, and $F4/80^+$ cells from the non-parenchymal cell (NPC) fraction after *in situ* perfusion of the hepatic portal vein and flow cytometry assay. Importantly, coinciding with fibrosis severity, liver resident macrophages, which often called Kupffer cells and detected as $F4/80^{high}$ $CD11B^{intermediate}$, were predominant in the control group (uninjured). Lowered proportion of resident macrophages was observed during the process of stimulated inflammation and fibrogenesis; $CD11B^{high}$ $F4/80^{intermediate}$ subset signifies a monocyte-derived recruited macrophage population that has increased progressively during fibrogenesis (**Figure 1F**). In summary, our data suggested that an initial cell injury can trigger inflammation to give rise to worsened liver fibrosis.

miRNAs and Pathways That Are Correlated With Liver Fibrosis

To identify DEMs of GSE40744 downloaded from GEO database, GEO2R tool was employed to perform the differential expression analysis following the protocol introduced in Materials and Methods section. Eighty nine miRNAs in total (62 up-regulated and 27 down-regulated) were ascertained to show significantly different expression in liver fibrosis biopsy specimen, reaching as high as two-fold aberration in comparison with normal ones (**Figure 2A** and **Table S3**). To ensure clearer visualization, the top 10 up-regulated and top 10 down-regulated miRNAs were selected as **Figure 2B**. As the most down-regulated miRNA, miR-20a-5p was picked for further analysis. 1381, 1384, and 1071 genes were detected as potential targets of miR-20a-5p through miRDB, TargetScan and miRtarbase, respectively. Three hundred and ninety three overlapping genes were identified as the targets of miR-20a-5p (**Figure 2C** and **Table S4**). Subsequently, enrichment analysis through KEGG database was carried out to identify the main pathways of these targets. Twenty nine significantly enriched KEGG pathways were identified (**Figure 2D**), including TGF-β signaling pathway, Bladder cancer, and Pancreatic cancer, et al. It was reported that TGF-β signaling pathway was of importance in liver fibrosis development (27). We hypothesized that miR-20a-5p played a part in the development of liver fibrosis by regulating TGF-β signaling pathway.

miR-20a-5p Was Down-Regulated and Associated With Inflammation During Liver Fibrosis

To validate whether miR-20a-5p is a modulator in liver fibrosis, the expression level of miR-20a-5p was measured through qRT-PCR assay in liver tissues collected from patients, CCl_4-induced mice model and healthy controls. In agreement with our assumption, miR-20a-5p expression level was significantly reduced in both tissue specimens of patients and CCl_4-induced

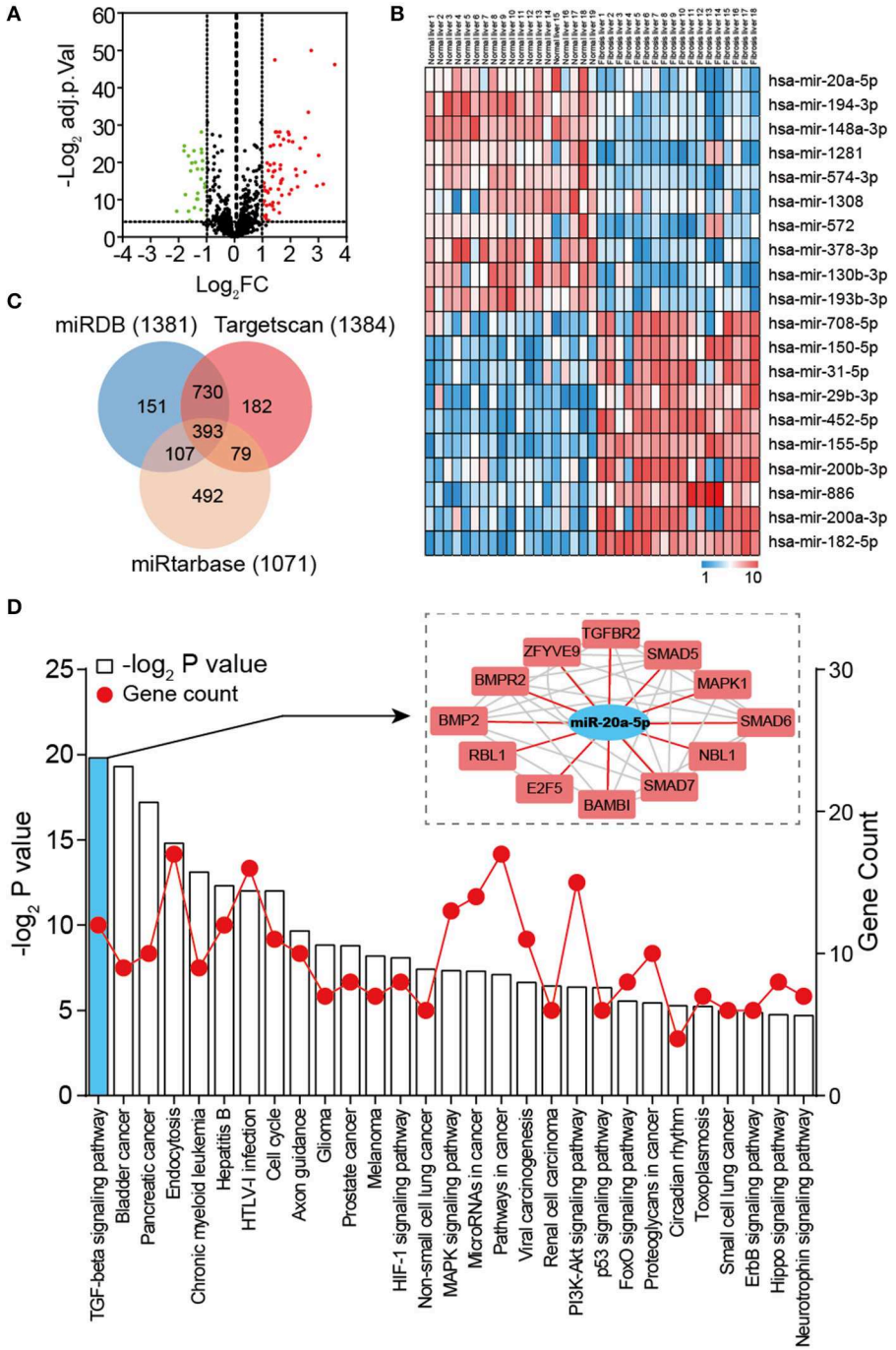

FIGURE 2 | Global properties of miRNAs and pathways correlated with liver fibrosis. **(A)** Volcano plot of the DEMs (adjust $P < 0.05$ and $|\log FC| \geq 2$ were set as the cut-off criteria). **(B)** Heat map of the top 20 DEGs (top 10 up-regulated and 10 down-regulated genes). **(C)** Venn diagram of target genes predicted by miRDB, TargetScan, and miRtarbase. **(D)** KEGG pathway enrichment analysis of target genes of miR-20a-5p. The red lines represent gene count and the histogram represent $-\log_2$ (*P* value).

mice (**Figure 3A**). These results prompted us to further explore the function of miR-20a-5p in liver fibrosis. We built an *in-vitro* cell model to simulate the complex process of fibrosis (**Figures 3B,C**). Hepa1-6 cells were transfected with miR-20a-5p mimic followed by CCl_4 treatment. Forty eight hours later, the culture supernatant was collected to treat Raw264.7 cells. ELISA assays showed that impaired-hepatocyte caused inflammation was blocked by restored miR-20a-5p, which was further

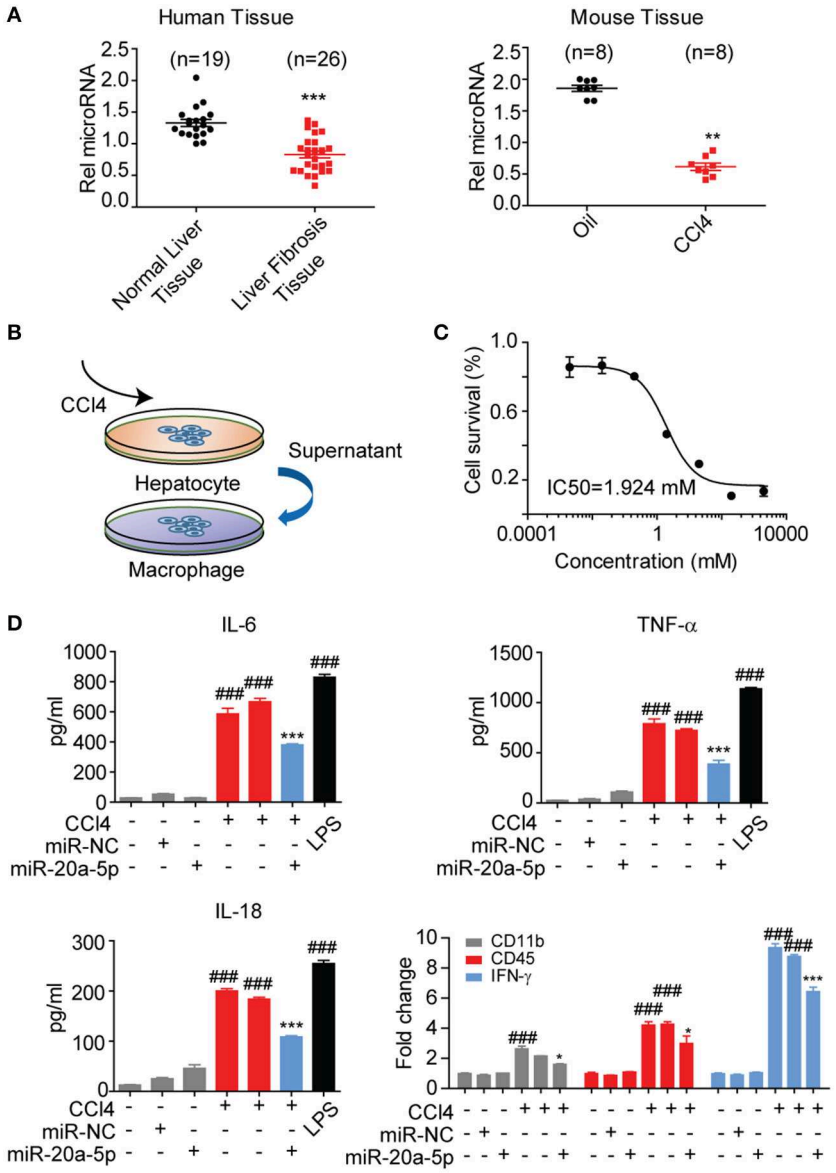

FIGURE 3 | MiR-20a-5p was down-regulated and associated with inflammation during liver fibrosis. **(A)** miR-20a-5p expression in the clinical samples from normal ($n = 10$) or liver fibrosis patients ($n = 20$) were analyzed by qRT-PCR. **(B)** The schematic of the *in-vitro* cell model. **(C)** The concentration-response curves of CCl_4 for hepatocyte injury model. **(D)** The cytokine levels of IL6, TNF-α, and IL-18 were determined in control cells and CCl4-cells transfected with miR-20a-5p or their respective NCs by ELISA and qRT-PCR. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#\#\#}p < 0.001$, respectively. *Compared with CCl4 plus miR-NC and $^{\#}$compared with the control.

confirmed by the other cytokines expression, e.g., CD11b, CD45, and INF-γ, the key markers widely accepted for inflammation test (**Figure 3D**, **Figure S1**). Our data indicate that miR-20a-5p expression is functionally related to inflammation during the onset and progression of liver fibrosis.

miR-20a-5p Alleviated Liver Fibrosis Through TGF-β Signaling Pathway

After binding to its receptors, TGF-β1 can activate the transcription factor downstream the pathway, Smad 2 and Smad3, to mediate fibrosis, and the signaling is negatively mediated by Smad7.

It is abundantly clear that TGF-β/Smad pathway is a major signal that activates HSCs and mediates fibrosis triggering downstream Smad 2 and Smad3 by TGF-β1. We have showed that TGFBR2 is one of the miR-20a-5p targets by searching the miRNA interactome dataset (**Figure 2D**). Thus, we initially investigated the expressions of TGFBR2 in liver fibrosis samples from patients. Immunofluorescence staining indicated that TGFBR2 expression level was notably enhanced in specimen collected from patients than that from healthy

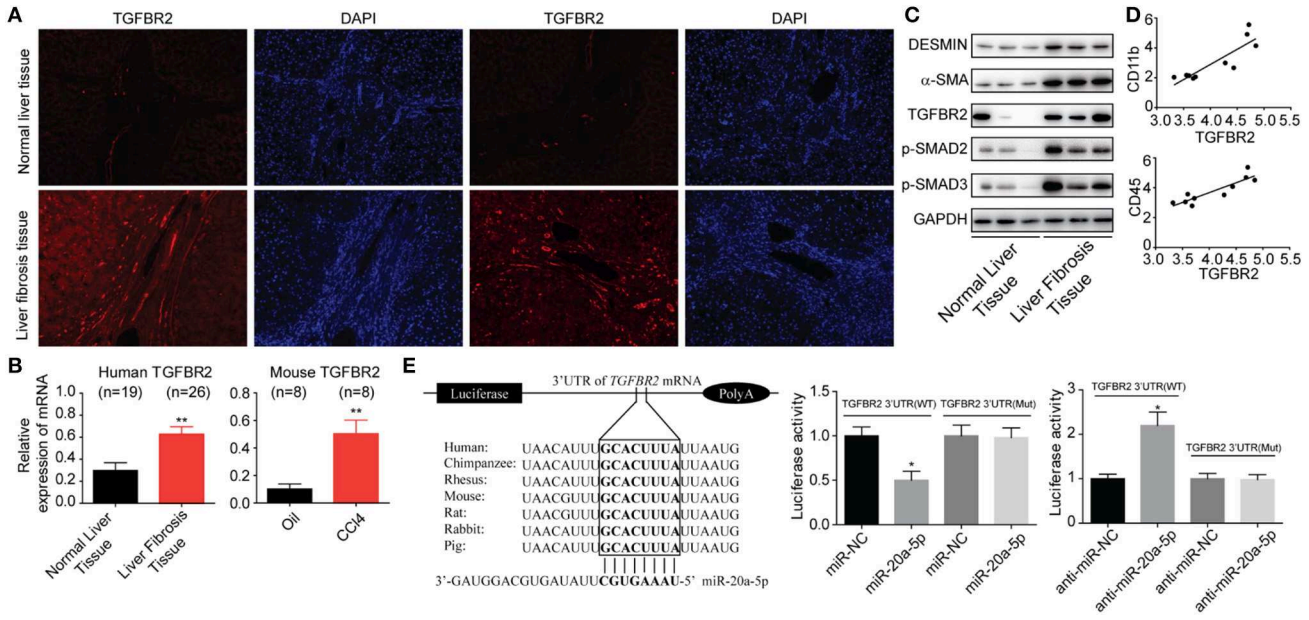

FIGURE 4 | miR-20a-5p alleviated liver fibrosis through TGF-beta signaling pathway. **(A)** Immunohistochemical staining of TGFBR2 in human tissues from healthy controls and patients. **(B)** The protein levels of fibrosis markers and TGF-beta pathway makers. **(C)** The correlation between TGFBR2 and CD11b, CD45 mRNA expression level in liver fibrosis patients. **(D)** The TGFBR2 mRNA levels in human and mouse models. **(E)** The interaction between miR-20a-5p and TGFBR2 3′UTR. Luciferase activity was determined in cells co-transfected with different luciferase reporter vectors and miRNAs. All data shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

controls (**Figures 4A,B**). Analogously, the expression levels of both phosphorylated-Smad2 and phosphorylated-Smad3 were notably higher than those in normal tissues, suggesting the activation of TGF-β signaling pathway (**Figure 4C**). Because we demonstrated miR-20a-5p alleviated liver fibrosis may through lighten inflammation, we sought to evaluate the relevance between TGFBR2 and inflammation. As expected, the TGFBR2 expression exhibited significantly correlation with CD11b and CD45 (GSE80601, $r^2 = 0.9201$ and 0.9786, respectively; both $P < 0.0001$) (**Figure 4D**). Finally, the 3′UTR sequence of TGFBR2 mRNA was cloned into the pGL3-Basic plasmid, in an attempt to ascertain the possible regulatory role of miR-20a-5p in the expression of TGFBR2 via binding to the predicted site. Our data demonstrated that miR-20a-5p mimics induced significantly inhibited luciferase activities of pGL3-TGFBR2 (WT), but no effect was observed on pGL3-TGFBR2 (Mut) (**Figure 4E**). Collectively, our data strongly suggests that miR-20a-5p down-regulation reinforce TGF-β signaling, at least in part, through alleviating to target TGFBR2 mRNA, leading to inflammation during liver fibrosis progression.

DISCUSSION

Aberrant hepatocyte death and persistent liver inflammation are recognized as drivers of liver fibrosis that in a chronic setting can promote HCC development (28). In the present study, we reported peripheral macrophage population accumulates during fibrosis. Besides, using microarray data of liver miRNomes, we measured the whole-genome miRNA expression of human liver fibrosis tissues and determined miR-20a-5p as a key modulate miRNA. The TaqMan probe-based qRT-PCR was performed to verify the predominance of miR-20a-5p through in both mouse and human samples. Furthermore, the present study demonstrated that lower level of miR-20a-5p exacerbates inflammation, whereas up-regulation of miR-20a-5p suppresses the releasing of cytokines.

Macrophages are "keystones" of liver architecture in both homeostasis and disease. Several studies have corroborated the central role played by macrophages in mediating inflammation and tissue fibrogenesis in several organ systems, but the progress is reverse (18, 29). Given the urgency and necessity to discover or develop an effective therapy for liver fibrosis, an increasing number of studies focus on analyzing miRNA mechanisms in fibrotic diseases, shedding light on the biological role of miR-21, miR-132, miR-155, miR-26a, and so forth. Previous studies demonstrated the elevated miR-155 expression in Kupffer cells after prolonged alcohol uptake, and that TNF served as a miR-155 target gene to give rise to liver inflammation (30, 31). miR-20a is one of miR-17/92 cluster members, which are located in the 13q31.1 region, which is largely involved in inflammatory. Overexpression of miR-20a could reduce the activity of inflammasome NLRP3 by mediating targeting thioredoxin-interacting protein (TXNIP) (32). Furthermore, miR-20a was reported to be beneficial to human aortic endothelial cells derived from Ox-LDL-induced inflammation through mediating TLR4 and TXNIP signaling (33). Moreover, miR-20a was also reported to regulate signal-regulatory protein α (SIRPα), resulting in

macrophage infiltration, phagocytosis, and pro-inflammatory cytokine secretion (34). The exact part played by miR-20a-5p in the progression of liver fibrosis is yet to be elucidated. Herein, our data have shown that miR-20a-5p was distinctly decreased with advanced fibrosis and we develop a novel cell model to simulate the macrophage activation during fibrosis. Notably, restoration of miR-20a-5p suppresses inflammations caused by inhibited hepatocyte injury.

Since miR-20a-5p suppressed inflammation *in vitro*, exploring its underlying mechanism relevant to the disease process of fibrosis is necessary. We further observed the level of TGFBR2 up-regulated in patients compared to normal liver. In addition, miR-20a-5p could regulate TGFBR2 expression by directly binding to its 3′-UTR, while TGF-β pathway contributes to hepatotoxicity which influences macrophage activation. Among the multiple causative factors, it's well-known that TGF-β/Smad pathway is essential for liver fibrosis development (35, 36). Connection of TGF-β and its receptors, including TGFBR1 and TGFBR2 could endow it with the serine threonine kinase activity. TGF-β is always recognized as a pro-fibrogenic cytokine in TGF-β signaling pathway due to its function in HSC activation and ECM production (37–39). Recently, it has been revealed that TGF-β is essential for the development and critical features of multiple tissue-resident macrophages. What's more, TGF-β is required for the maintenance of expression pattern of the macrophage-specific homeostatic genes (40–42). Our data verified the contributions of TGF-β signaling pathway in hepatocytes to macrophage activity.

Together, our results highlight a critical function of miR-20a-5p in the liver fibrosis development, and provide the first evidence that miR-20a-5p maintains the survival of hepatocyte via TGF-β signaling pathway and that inhibits inflammation occur. Moreover, the reintroduction of miR-20a-5p enlightens a promising therapeutic strategy for the clinical intervention of liver fibrosis.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (GSE40744).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Review Board of the Zhongshan Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Institutional Ethics Review Board of the Zhongshan Hospital.

AUTHOR CONTRIBUTIONS

XF and QF design the concept, experimented and wrote the manuscript. JQ have done the system biology analysis. JC and YJ collated the data used in this project. ZD designed the problem, guided the study, and finalized 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/fonc.2020.00107/full#supplementary-material

Figure S1 | Decreased miR-20a-5p reinforce inflammation during liver fibrosis progression. **(A)** The cytokine levels of IL6, TNF-α, and IL-18 were determined in control cells and CCl4-cells transfected with si-miR-20a-5p or their respective NCs by ELISA. Values are presented as mean ± SEM. $^{*}p < 0.01$ compared with CCl4 plus si-miR-NC and $^{\#\#\#}p < 0.001$ compared with the control.

Table S1 | Clinical characteristics of patients.

Table S2 | List of primers for qTR-PCR.

Table S3 | List of miRNA identified in miRNA profile.

Table S4 | List of target genes regulated by miR-20a-5p.

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REVIEW
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Resistance to PD-L1/PD-1 Blockade Immunotherapy. A Tumor-Intrinsic or Tumor-Extrinsic Phenomenon?

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Cancer immunotherapies targeting immune checkpoints such as programmed cell-death protein 1 (PD-1) and its ligand programmed cell-death 1 ligand 1 (PD-L1), are revolutionizing cancer treatment and transforming the practice of medical oncology. However, despite all the recent successes of this type of immunotherapies, most patients are still refractory and present either intrinsic resistance or acquired resistance. Either way, this is a major clinical problem and one of the most significant challenges in oncology. Therefore, the identification of biomarkers to predict clinical responses or for patient stratification by probability of response has become a clinical necessity. However, the mechanisms leading to PD-L1/PD-1 blockade resistance are still poorly understood. A deeper understanding of the basic mechanisms underlying resistance to cancer immunotherapies will provide insight for further development of novel strategies designed to overcome resistance and treatment failure. Here we discuss some of the major molecular mechanisms of resistance to PD-L1/PD-1 immune checkpoint blockade and argue whether tumor intrinsic or extrinsic factors constitute main determinants of response and resistance.

Keywords: immune checkpoint blockade, programmed cell-death protein 1, programmed cell-death 1 ligand 1, immunotherapy, tumor-intrinsic resistance, tumor-extrinsic resistance, biomarkers

INTRODUCTION

Cancer immunotherapies aim at stimulating the immune system of patients to reactivate its anti-oncogenic activities (Escors, 2014). The most successful anti-cancer immunotherapies are currently those based on immune checkpoint blockade with antibodies (ICIs). Under normal physiologic conditions, immune checkpoints function as regulators of excessive inflammation following T-cell activation, and mechanisms to prevent auto-reactive responses. Unfortunately many cancer cells exploit these T-cell inhibitory mechanisms by up-regulating the expression of immune checkpoint molecules that will bind their ligands on activated T cells leading to their inactivation. It is thought that ICI therapies act primarily on the reactivation of T lymphocytes to exert cytotoxic activities over cancer cells. The emergence of ICI therapies over the last decade has transformed to the core cancer treatments, as they show good efficacies, and less toxicity than conventional chemotherapy or

targeted therapies. However, for most cancer types only a subset of all patients effectively respond to these therapies, which is a major clinical, economic, and ethical problem (Topalian et al., 2011; Nishino et al., 2017; Prasad et al., 2017; Kamada et al., 2019; Martins et al., 2019). It is often said that ICI therapies have revolutionized oncology, although their efficacy is still limited. But, what do we mean when we claim that ICI therapies have caused a revolution?

Before the success stories of ipilimumab (Hodi et al., 2008), and before the publication of the results from the first clinical trials of PD-L1/PD-1 blockers (Brahmer et al., 2012; Topalian et al., 2012), immunotherapies were not seriously considered as viable therapeutic options by most oncologists and pharmaceutical companies. Most of their efforts were directed towards the development of small molecule inhibitors for targeted therapies, or novel chemotherapies. And even though targeted therapies showed good efficacies, they were largely limited to patients with tumors harboring the targeted mutations. So, what did ICI treatments truly change? The truly astonishing result is that with only a single drug, objective responses were obtained in a very large number of cancer types largely independent of their ontogeny. Moreover, these drugs are not even directed towards the cancer cell. For example, the anti-PD-1 antibody pembrolizumab has achieved objective responses in cancers as different as melanoma, lung cancer, head and neck, urothelial, gastric cancer, mesothelioma, and Hodgkin lymphoma, among others.

The inhibitory co-receptors that modulate the activation of T cells are generally associated with the T-lymphocyte receptor (TCR) complex at the immunological synapse. These molecules constitute major control points and serve as targets to enhance antitumor immune responses. Some examples expressed in T cells are programmed cell-death protein 1 (PD-1), T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), cytotoxic T-lymphocyte antigen 4 (CTLA-4), or lymphocyte-activation gene 3 (LAG-3) (Saito et al., 2010; Chen and Flies, 2013; Esensten et al., 2016; Schildberg et al., 2016; Lichtenegger et al., 2018). Several ICI antibodies targeting CTLA-4 or the PD-L1/PD-1 axis are approved for use by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for treatment of different cancer types. These antibodies have demonstrated clinical efficacy, with durable clinical responses. Due the success of blockade strategies of CTLA-4 and PD-1 pathways, several antibodies targeting other immune checkpoints are now at different stages of development. Moreover, several combination strategies with ICIs are under evaluation in clinical trials, emerging as new opportunities to enhance anti-tumor immunity (**Table 1**) (Pardoll, 2015).

Since 2012, antibodies blocking PD-1/PD-L1 interactions are demonstrating very promising results (Brahmer et al., 2012; Topalian et al., 2012), demonstrating their efficacies and safety. Truly, these results have no precedent in the history of cancer treatments due to their wide range of activities and the durability of responses. To date, six immune checkpoint inhibitors blocking the PD-L1/PD-1 axis are approved by the FDA and the EMA: three PD-1 inhibitors (nivolumab, pembrolizumab, and cemiplimab), and three PD-L1 inhibitors (atezolizumab, durvalumab, and avelumab). Most of them have also been approved by the Chinese National Medical Products Administration (NMPA), and by the Pharmaceuticals and Medical Devices Agency (PMDA) in Japan. Additionally, the NMPA has recently approved the use of four more PD-1 inhibitors (toripalimab, tislelizumab sintilimab, and camrelizumab) in China. These drugs are indicated for the treatment of several cancer types such us melanoma, non–small cell lung cancer (NSCLC), renal cell carcinoma, head and neck squamous cell carcinoma, urothelial carcinoma, microsatellite instability–high colorectal cancer and metastatic cutaneous squamous cell carcinoma.

However, despite these successes the majority of patients in many cancer types do not truly benefit from PD-L1/PD-1 blockade therapies and show resistance, either intrinsic resistance when the treatment directly fails, or acquired resistance where a proportion of responders will also develop resistance. Other patients show some response in the form of stable disease, or acceleration of disease in the form of hyperprogression (Zuazo et al., 2018). Still, the specific mechanisms of resistance and response remain to be elucidated. Therefore, the understanding of the basic mechanistic pathways of

TABLE 1 | Clinical trials targeting the PD-L1/PD-1 axis and combinations.

PD-1/PDL-1 clinical trials		Targets	NCT identifier
PD-1/PD-L1 monotherapy		PD-1/PD-L1 axis	NCT03936959, NCT03013101, NCT03167853, NCT03142334, NCT02853344, NCT02702414, NCT02838823, NCT02836795, NCT03010176, NCT03219775, NCT03692442, NCT02358031
Combination therapies with PD-1/PD-L1 blockade	**with other immunotherapies**	PD-1/PD-L1 axis and CTLA-4, LAG-3, OX40, TIM-3, GITR, CD20 mAbs, IL2R, IL12, IL7R, IL1B, CD19, CD40, CD38, 41BB	NCT03179007, NCT03615313, NCT03190811, NCT03732547, NCT03970382, NCT03527251, NCT03894215, NCT01968109, NCT02658981, NCT03680508, NCT04198766, NCT04215978
	with targeted therapies	PD-1/PD-L1 axis and VEGF/VEGFR, ERK1/2, RAF, AMPK, EGFR, FGFR, MEK, RAF pathways	NCT03851614, NCT04010071, NCT02133742, NCT04152356, NCT03955354, NCT04303741, NCT04014101, NCT03722875, NCT03394287, NCT03359018, NCT02873390, NCT03182816
	with chemotherapy	PD-1/PD-L1 axis and direct cancer cell cytotoxicity	NCT03903887, NCT03311789, NCT03737123, NCT04152889, NCT03041181, NCT03515629, NCT03701607, NCT03409614, NCT04225364, NCT02220894, NCT02819518, NCT03221426
	Other combinations (radiotherapy, chemoradio, multi-way combo, others)	PD-1/PD-L1 axis and direct cancer cell cytotoxicity	NCT02821182, NCT04017897, NCT03898895, NCT03557411, NCT03984357, NCT03671265, NCT03984357, NCT03619824, NCT03474094, NCT02992912, NCT02434081, NCT02525757

resistance and the identification of predictive biomarkers of response have become a clinical necessity. Here, we review the current knowledge on resistance to PD-L1/PD-1 blockade therapies and discuss whether tumor intrinsic or extrinsic factors are the main determinants of response and resistance.

PROGRAMMED CELL DEATH PROTEIN 1 (PD-1) AND PROGRAMMED CELL-DEATH 1 LIGAND 1 (PD-L1) AXIS

PD-1 (CD279) is a type 1 transmembrane glycoprotein from the B7-CD28 immunoglobulin superfamily discovered in 1992 for which Prof Honjo received the Nobel Prize (Ishida et al., 1992). This protein is encoded by Pdcd1 gene on the human chromosome 2, and it is composed of a short signal sequence, an extracellular IgV-like domain, a stalk region, a transmembrane domain, and an intracellular cytoplasmatic tail containing the two tyrosine-based signaling motifs; the immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) (**Figure 1**). These two motifs contribute to the inhibitory functions of PD-1. PD-1 has two main ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (Dong et al., 1999; Freeman et al., 2000; Latchman et al., 2001; Tseng et al., 2001) (16–19). PD-L1 is a type I transmembrane protein encoded by the Cd274 gene on the human chromosome 9 discovered in 1999 as an additional member of the B7 family. PD-L1 is composed of a signal sequence, an IgV-like domain, an IgC-like domain, a transmembrane domain, and a highly conserved short intracellular region with intracellular signal transduction capacities (Pascolutti et al., 2016; Gato-Canas et al., 2017; Escors et al., 2018) (**Figure 1**). The intracellular domain presents three highly conserved sequence motifs, two of which are required for regulating interferon-mediated cytotoxicity (RMLDVEKC and DTSSK) (Gato-Canas et al., 2017; Escors et al., 2018). PD-L2 is a type I transmembrane protein encoded by the Pdcd1lg2 gene was discovered in 2001 (Latchman et al., 2001; Tseng et al., 2001) and exhibits a similar molecular oganization than PD-L1.

After engagement with PD-L1, PD-1 inhibits T cell functions through direct and indirect pathways (Arasanz et al., 2017) (**Figure 2**). Direct pathways are dependent on the recruitment of SHP-1 and SHP-2 phosphatases phosphatases to PD-1 ITIM and ITISM motifs following their tyrosine phosphorylation by Lck (Plas et al., 1996; Chemnitz et al., 2004; Sheppard et al., 2004; Hui et al., 2017). SHP phosphatases inhibit ZAP70 and PI3K activities by dephosphorylation, and thus ending the TCR-CD28 signal transduction and its downstream dependent intracellular pathways (ERK and PKCθ). PD-1 also inhibits T cell activities through indirect pathways. After engaged with PD-L1, PD-1 leads to increased expression of CBL E3 ubiquitin ligases, which ubiquitylate components of the TCR leading to its internalization and degradation (Karwacz et al., 2011; Karwacz et al., 2012; Liechtenstein et al., 2014). Also, an indirect pathway of PD-1-dependent inhibition of TCR signal transduction is caused when PD-L1 engages to PD-1 by inhibiting the transcription of CK2 through an unclear mechanism, resulting in de-phosphorylated PTEN that will in turn de-phosphorylate PI3K and terminating in this way downstream pathways (Patsoukis et al., 2013; Arasanz et al., 2017).

In physiological conditions PD-L1/PD-1 interactions keep T cell tolerance toward autoantigens (Latchman et al., 2004).

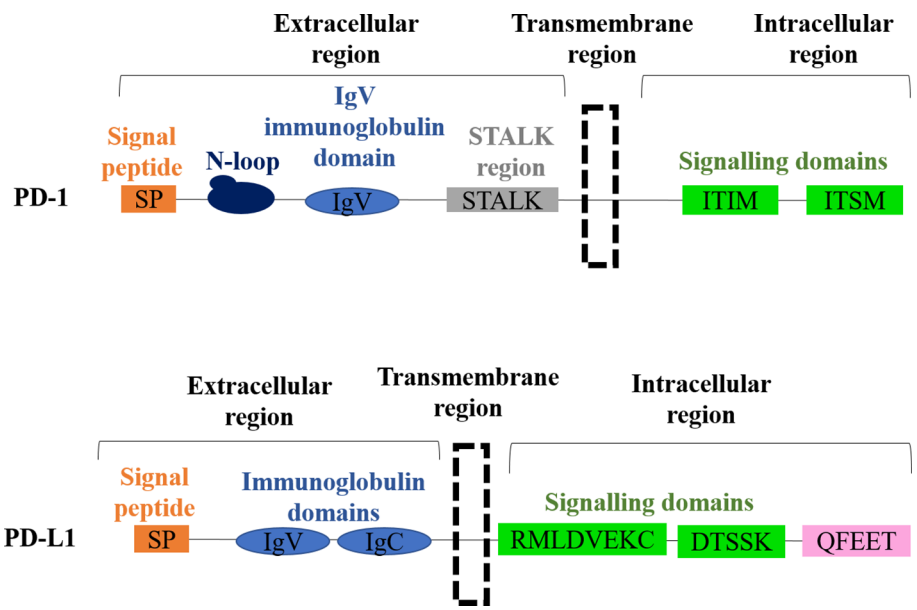

FIGURE 1 | Molecular structures of PD-1 and PD-L1. The domain organization of PD-1 is shown on top, with each domain indicated. The domain organization of PD-L1 is shown below, with each domain indicated.

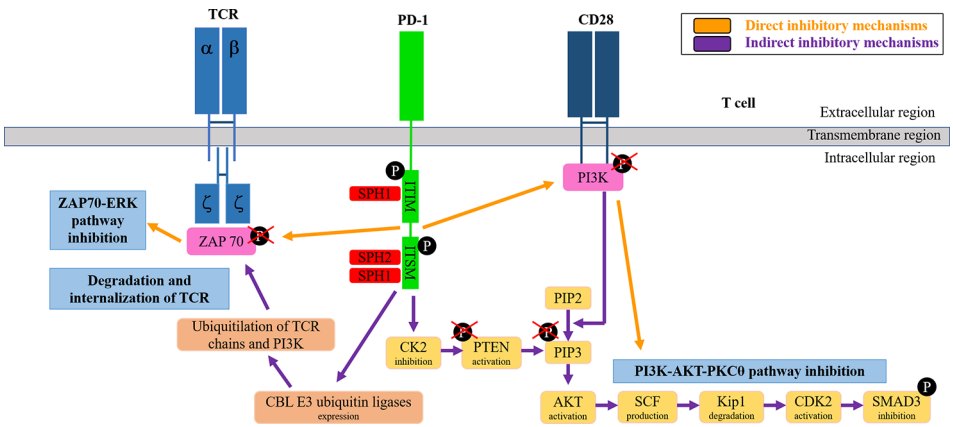

FIGURE 2 | PD-1 signaling pathways in T cells. The figure schematically summarizes the direct and indirect T cell inhibitory signaling mechanisms as indicated.

Conversely, in pathological conditions these inhibitory receptors lead to regulation of T-cell effector functions in autoimmunity and infection (Barber et al., 2006; Sharpe et al., 2007). Tumor survival can depend on the PD-L1/PD-1 pathway to attenuate immunogenicity and facilitate resistance to anti-apoptotic stimuli (Hirano et al., 2005; Azuma et al., 2008; Keir et al., 2008; Gato-Canas et al., 2017; Escors et al., 2018). PD-L1 is overexpressed in many tumor types to evade the immune attack and its expression generally (but not always) correlates with progression (Gato-Canas et al., 2017; Escors et al., 2018; Bocanegra et al., 2019; Kattan et al., 2019). PD-1 is expressed in T lymphocytes and interferes with their activation when bound with their ligands PD-L1, inhibiting the effector phase and thus dampening the ability of these T cells to kill cancer cells (Keir et al., 2008; Gato-Canas et al., 2017; Zuazo et al., 2019).

MECHANISMS OF RESISTANCE TO PD-L1/PD-1 IMMUNOTHERAPY

PD-L1/PD-1 blockade immunotherapy demonstrates longer duration of responses, and it is better tolerated than traditional therapies. However, despite the recent successes, a large number of patients do not respond to the therapy. This fact indicates intrinsic (or primary) resistance. In addition, a percentage of responder patients end up progressing through mechanisms of acquired resistance. Primary and acquired resistances are important barriers in terms of benefit to the patient (Pitt et al., 2016; Restifo et al., 2016; Sharma et al., 2017; O'Donnell et al., 2019).

Some of the patients treated with PD-L1/PD-1 immunotherapy show hyperprogressive disease, characterized by an unexpected drastic acceleration in tumor growth after the initiation of the therapy with fatal consequences (Champiat et al., 2017; Kato et al., 2017; Saada-Bouzid et al., 2017; Champiat et al., 2018; Ferrara et al., 2018; Zuazo et al., 2018; Kim et al., 2019). Moreover, a certain percentage of responder patients show an apparent progression of neoplastic lesions caused by massive tumor infiltration by immune cells. This response has been termed pseudoprogression, and it is a confounding factor for evaluation of responses by standard techniques such as computerized tomography (Onesti et al., 2019). These variety of atypical responses have prompted the development of immune-related response criteria (irRC) to better characterize the distinct types of responses associated to immunotherapies (Wolchok et al., 2009), in contrast to conventional evaluation criteria by Response Evaluation Criteria in Solid Tumors (RECIST). Nonetheless, the techniques and biomarkers currently integrated in clinical practice are not sufficient to identify responses. A deeper understanding of the mechanisms leading to resistance to PD-L1/PD-1 blockade is required.

In addition, every patient is unique as a result of genetic and clinical backgrounds. Hence, the mechanisms leading to clinical response or resistance are highly complex and might differ not only according to tumor type but also to patient-specific factors. Therefore, the contribution of tumor-cell intrinsic and patient-specific extrinsic factors needs to be elucidated. In the context of immunotherapies, it is unclear which ones are the main determinants of response and resistance.

Tumor-Intrinsic Factors and Resistance to PD-L1/PD-1 Blockade Therapies

A number of intrinsic characteristics of the patients are prognostic markers. In principle, we will disregard these general characteristics and focus on more specific factors contributing to immunoresistance. Without any doubt, tumor-intrinsic factors definitely contribute to response or progression in immune checkpoint blockade (Sharma et al., 2017; Chowell et al., 2018; Kalbasi and Ribas, 2020).

Tumor-intrinsic factors that contribute to primary and acquired resistance to PD-L1/PD-1 immunotherapy conform a genetic and signaling landscape that prevents immune cell infiltration in the tumor microenvironment (TME) (**Figure 3**).

Resistance to PD-1 blockade immunotherapy is often associated with insufficient tumor antigenicity, constitutive PD-L1 expression, defects in IFN signal transduction within cancer cells and alterations in the regulation of oncogenic pathways (Escors, 2014; Sharma et al., 2017).

The loss of tumor antigenicity is a major escape mechanism for many tumor types (Escors, 2014). This is mainly caused by cancer immunoediting, a process by which the immune system exerts a strong and sustained selective pressure over the most immunogenic cancer cell variants (Schreiber et al., 2011). Hence, recognition of tumor-specific antigens by effector T cells is crucial for cancer immunoediting (DuPage et al., 2012). Effector T cells will eliminate the most immunogenic cancer cells and control tumor progression for some time (Restifo et al., 2016; Sharma et al., 2017). However, the less immunogenic cancer cell variants will overgrow and progress. Therefore, tumor immunoediting does constitute a strong mechanism of acquired resistance to immunotherapies. The resulting surviving cancer cells usually show a strong decrease in tumor antigen expression (Matsushita et al., 2012; Escors, 2014), or a down-modulation of molecules involved in antigen presentation such as lack of MHC I or beta-microglobulin expression (Gubin et al., 2014). In this context, ICI therapies will fail simply because no endogenous T cell responses can be raised against these tumors. It has to be noted that immunoediting as a mechanism of immunological escape has been relatively well studied in immunotherapies other than ICIs (Schreiber et al., 2011; Teng et al., 2015; O'Donnell et al., 2019). Therefore, the real extent of the impact of immunoediting over resistance to ICI treatments has not yet been systematically quantified. The detection of less immunogenic variants in samples from patients before the start of immunotherapies may provide the means for adequate patient selection. For instance, characteristics such as genomic instability or epigenetic alterations in pre-existing tumor cell variants, may enable these cancer cells to evade ICI therapies. And these may even facilitate tumor grown, immune evasion, and tumor escape. These escape variants are likely to be naturally selected especially if potent immunostimulatory therapies are applied (Khong and Restifo, 2002). For example, the loss of functional β2 microglobulin from tumor cells, a structural component of the major histocompatibility complex (MHC) 1, confers resistance to tumor-specific CD8 T cells (Restifo et al., 1996). In addition, acquired homeostatic resistance has been described in which tumor cells alter gene expression profiles in response to interactions with the immune system (Pardoll, 2015).

We could include within these mechanisms the adaptive up-regulation of PD-L1 expression as a response to interferons produced during the anti-tumor attack (Garcia-Diaz et al., 2017; Gato-Canas et al., 2017; Escors et al., 2018). Cancer cells with up-regulated PD-L1 would not only inactivate PD-1-expressing T cells, but will also show increased resistance to IFN-mediated apoptosis through reverse signaling by PD-L1 within cancer cells (Gato-Canas et al., 2017; Jalali et al., 2019). It has been known for some time that PD-L1 had intrinsic signaling properties in cancer cells that protected that protected them from a range of apoptotic stimuli, and that its intracellular domain was required for this protection (Azuma et al., 2008). Moreover, PD-L1 was also shown to stimulate cancer cell growth by modulating the activity of AKT/mTOR, autophagy, and glycolysis (Chang et al., 2015; Clark et al., 2016; Gupta et al., 2016). The intracellular part of PD-L1 contains three non-classical signaling motifs; The "RMLD," "DTSSK," and "QFEET" motifs (**Figure 1**). The RMLD sequence is required for the anti-apoptotic activities of PD-L1 through the inhibition of STAT3 expression and alternative phosphorylation. The DTSSK motif has regulatory properties, and when it is removed or mutated, PD-L1 molecules exhibit hyperactivated signaling (Gato-Canas et al., 2017). The QFEET motif has been recently shown to be the docking site for the de-ubiquitinase USP22 (Huang et al., 2019).

Inhibition of STAT3 by PD-L1 intrinsic signaling ensures the abrogation of interferon-mediated apoptosis (Gato-Canas et al.,

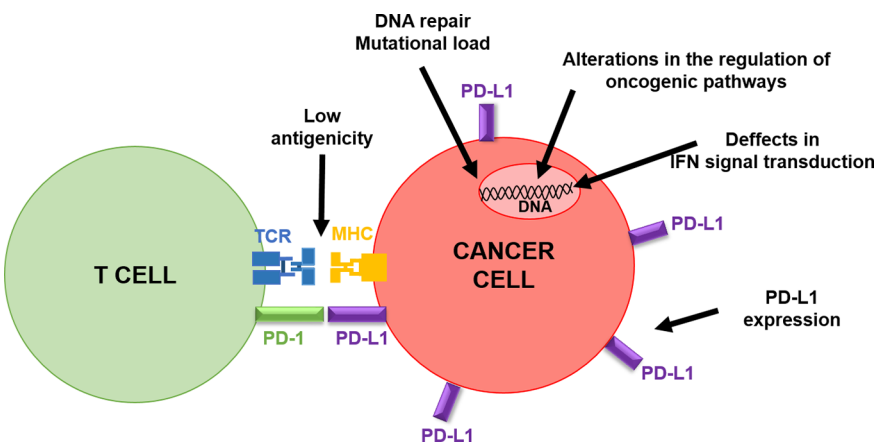

FIGURE 3 | Schematic summary of cancer-intrinsic characteristics influencing clinical responses to PD-L1/PD-1 blockade therapies. The figure depicts the interaction of a T cell with a cancer cell, highlighting cancer cell intrinsic factors that can inactivate T cell activities, as indicated by the arrows.

2017), stimulates the inflammasome pathway in cancer cells (Theivanthiran et al., 2020), and directly inhibits PD-L1-positive T cells (Diskin et al., 2020). PD-L1-regulated inflammasome activation triggers a series of signaling cascades that end up with the recruitment of granulocyte myeloid-derived suppressor cells (MDSC) in the tumor environment. This accumulation of MDSCs contribute to resistance to PD-L1/PD-1 blockade strategies. Therefore, PD-L1 expression by cancer cells regulates several pro-carcinogenic mechanisms that can contribute to resistance: First, PD-L1 as an inhibitor of T cell effector activities; second, PD-L1 as an anti-apoptotic shield; and third, PD-L1 as a recruiter of MDSCs into the tumor microenvironment. In agreement with this, it is not surprising that human carcinomas with inactivating mutations in the DTSSK motif of PD-L1 can be selected by immunoediting (Gato-Canas et al., 2017), as these mutations increase the signaling capacities of PD-L1.

Hence, PD-L1 expression in tumors could be considered a tumor-intrinsic factor of resistance. PD-L1 up-regulation in tumor cells is generally associated with tumor progression, proliferation and invasion, antiapoptotic signaling, and T cell inhibitory activities *via* engagement with PD-1 (Escors et al., 2018). PD-L1 expression on tumor cells seems to be sufficient for immune evasion and inhibition of CD8 T cell cytotoxicity (Juneja et al., 2017). Therefore, PD-L1 expression is a recognized biomarker for patient stratification in PD-L1/PD-1 blockade immunotherapy. Some immunohistochemistry assays to quantify PD-L1 expression are currently FDA-approved such as Dako 28-8, Dako 22C3, Ventana SP142, and Ventana SP263. However, the systems of detection are not currently standardized, as different immunochemistry assay and scoring system offer different classifications for tumor PD-L1 status (Arasanz et al., 2018; Bocanegra et al., 2019). Additionally, PD-L1 expression can be highly variable and heterogeneous. Some patients with PD-L1-negative tumors may still benefit from anti-PD-L1/PD-1 therapies as PD-L1 is also expressed by many other cell types including myeloid antigen-presenting cells (Karwacz et al., 2011; Motzer et al., 2015; Horn et al., 2017; Bocanegra et al., 2019). Because of these limitations, PD-L1 expression as a predictive biomarker for responses is still under debate. Nevertheless, the application of radioactively-labeled probes specific for PD-L1 and *in vivo* PET visualization of labeled tumors, and their metastasis is very likely going to solve many of these issues. First, detection of PD-L1 expression levels without the need of obtaining a limited amount of tumor tissue. Second, sensitive detection of "silent" metastases. Third, discrimination of true progression from pseudoprogression, at least for cancers that are PD-L1 positive. So far, several different approaches have been applied in pre-clinical models and in cancer patients. For example, by using PD-L1-specific nanobodies labeled with technetium-99m (Broos et al., 2017), PD-L1-specific cyclic peptides labeled with Gallium (De Silva et al., 2018), and radio-labeled anti-PD-L1 antibodies (Heskamp et al., 2015; Niemeijer et al., 2018).

Several other approaches based on intrinsic tumor characteristics have been established for patient selection. From these, the tumor mutational burden (TMB) has gained popularity as a potential predictive biomarker associated with response to ICI therapies. TMB provides a quantification of the number of mutations per megabase of genomic DNA within the tumor encoding genome. It is thought that "high" TMB tumors may have increased expression of neoantigens and enhanced immunogenicity (Alexandrov et al., 2013; Yuan et al., 2016). Neoantigen load is associated with response and has some predictive value on long-term clinical benefit of PD-L1/PD-1 blockade therapies. The mutational load before the start of immunotherapies seems to be associated to a higher nonsynonymous mutation burden in tumors, higher neoantigen expression, and mutations within the DNA repair pathways (Gubin et al., 2014; Le et al., 2015; Rizvi et al., 2015; Schumacher and Schreiber, 2015). A reflection of this is exemplified by mismatch repair deficiency in cancers, which predicts response to PD-1 blockade for some tumor types such as colon cancer (Le et al., 2015; Le et al., 2017). Therefore, the FDA approved in 2017 the PD-1 inhibitor pembrolizumab for treatment of progressive mismatch-repair deficient solid tumors, consolidating mismatch repair (MMR) defect as a clinically applicable biomarker.

Tumor-Extrinsic Factors and Resistance to PD-L1/PD-1 Blockade Therapies

ICI immunotherapies differ substantially from conventional therapies in which the target is the immune system. Therefore, it is fair to assume that tumor extrinsic factors linked to the immune system will be associated to response or resistance to ICI therapy. So far, a variety of such factors have been associated to resistance. These include irreversible T cell exhaustion, expression of additional immune checkpoint molecules and their ligands (CTLA-4, TIM-3, LAG-3, TIGIT, VISTA, and BTLA), differentiation and expansion of immunosuppressive cell populations, and release of immunosuppressive cytokines and metabolites both systemically and within the TME (IL-10, IL-6, IL-17, IFNγ, CSF-1, tryptophan metabolites, TGF-β, IDO, increased adenosine production) (**Figure 4**) (Fridman et al., 2017; Sharma et al., 2017; Fares et al., 2019).

One of the oldest prognostic immune biomarkers is the quantification of the type, location, and density of immune cells that infiltrate the TME (O'Donnell et al., 2019). Anti-neoplastic treatments and not only immunotherapies are most efficacious in patients with increased tumor-infiltrating lymphocytes (TILs) in biopsies. This is also true for ICI therapies, and the use of TIL quantification together with PD-L1 tumor positivity is generally associated to good responses (Taube et al., 2012; Bindea et al., 2013). Indeed, there is a positive correlation of TIL infiltration with PD-L1 expression by cancer cells. There are several ways to quantify TIL infiltration, but one of the most successful at least for colon cancer is the so-called "immunoscore" (Galon et al., 2014; Pages et al., 2018; Angell et al., 2020). This biopsy scoring system is a powerful prognostic tool based on the quantification of CD3 and CD8 T lymphocytes on the tumor center and at the tumor invasive margins.

Not surprisingly, TIL infiltration correlates with good prognosis and objective responses to ICI therapies. Oligoclonal TILs are expanded in the tumor tissues of responders to anti-PD-1 blockade. These T cells show enhanced helper T cell type 1

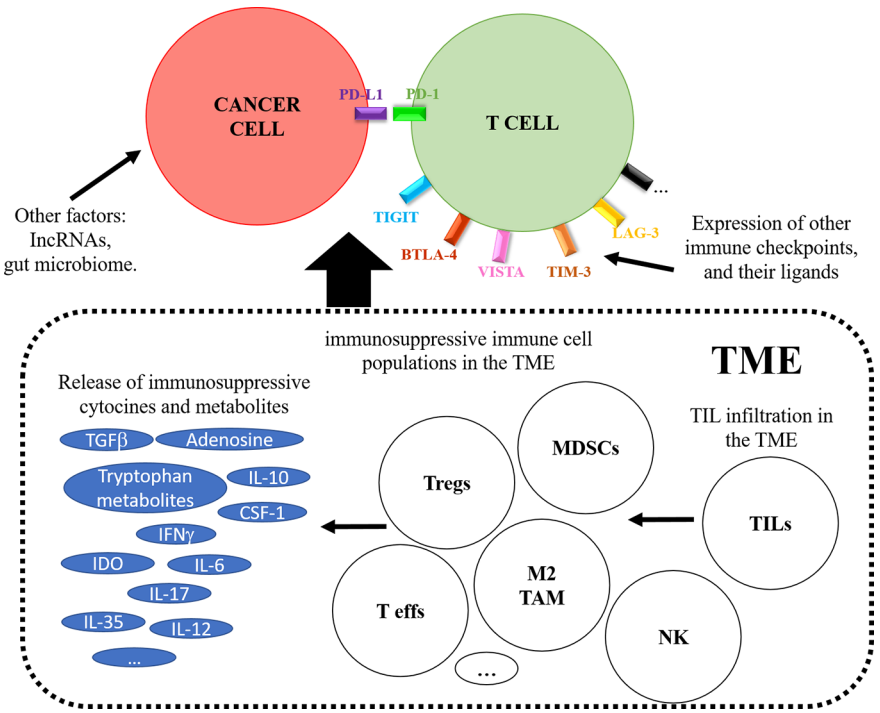

FIGURE 4 | The figure schematically represents tumor-extrinsic mechanisms contributing to response or resistance to PD-L1/PD-1 blockade therapies. The figure depicts on top a T cell interacting with a cancer cell, and the effects caused by the tumor microenvironment (TME) are boxed below. These include the recruitment of immunosuppressive cells as indicated, the expression of immunosuppressive metabolites and the induction of alternative immune checkpoints on the T cell.

(Th1) cellular immunity (Inoue et al., 2016). Patients can be stratified into four different types according to the characteristics of the TME tumor based on TILs and PD-L1: type I or adaptive immunoresistant (PDL1(+), TIL(+)), type II or immunologically ignorant (PD-L1(-),TIL(-)), type III (PD-L1(+), TIL(-)), and type IV or immune-tolerant (PD-L1(-), TIL(+)) (Teng et al., 2015). This stratification may provide a means for therapy selection. However, other factors contribute to efficacious responses. For instance, the TILs/PD-L1 ratio can be altered according to the expression of oncogene drivers in cancer cells as well as the anatomical location of the neoplastic lesions.

Recent studies demonstrate that ICI therapies do also alter the dynamics and characteristics of systemic immune cell populations. Interestingly, some of these studies highlight the CD28-CD80 costimulation signaling pathway as a major contributor to efficacious responses to ICI (Hui et al., 2017; Zuazo et al., 2019). Indeed, several studies show a key role for IL-12-expressing dendritic cells with cross-presentation capacities for good responses to immunotherapies (Kerkar et al., 2011; Liechtenstein et al., 2014; Goyvaerts et al., 2015; Berraondo et al., 2018; Garris et al., 2018; Etxeberria et al., 2019). These results reinforce the idea that a systemic functional immunity is very likely a required factor for the efficacy of immunotherapies. This was elegantly shown in murine models (Spitzer et al., 2017) as well as in human patients undergoing PD-L1/PD-1 blockade therapies (Kamphorst et al., 2017; Zuazo et al., 2019). A systemic expansion in peripheral blood of a population of CD28+ PD-1+ CD8 T cells was shown in melanoma patients responding to anti-PD-1 therapy (Kamphorst et al., 2017). Patients with non-small cell lung cancer undergoing ICI therapies that presented systemic dysfunctional CD4 T cells that strongly co-expressed PD-1 and LAG-3 failed to respond to therapies (Zuazo et al., 2019). Interestingly, these CD4 T cells did not lose their capacities for multi-cytokine production following *in vitro* stimulation, albeit with a strong Th17-type of responses. These results suggested that these T cells could not be considered exhausted. However, they showed a degree of proliferative dysfunctionality that was indicative of some type of anergy. Importantly, these patient cohorts were enriched in hyperprogressors, suggesting a key role for T cell dysfunctionality in hyperprogressive disease (Zuazo et al., 2019). These results highlighted the up-regulation of LAG-3 as a major escape mechanism to PD-1/PD-L1 monoblockade strategies. Very similar results were obtained in two other independent studies by Kagamu and collaborators, and Julia and collaborators (Julia et al., 2019; Kagamu et al., 2020). In the study by Zuazo et al. responders had a high percentage of highly differentiated $CD27^{-}$ $CD28^{-}$ memory CD4 T cells before starting immunotherapies, and could be used as a predictive biomarker. Similarly, Kagamu et al. identified this population as $CD62L^{low}$ CD4 cells, while Julia et al. described this population as central memory CD4 T cells.

The expansion of immunosuppressive immune cell populations systemically or infiltrating the TME also contributes to extrinsic factors of resistance. Regulatory T cells (Tregs) strongly suppress

tumor-specific T cell functions and disrupt effector T cell function. The mechanisms of Treg-mediated immune suppression are varied and include direct cell-to-cell contact and secretion of potent immunosuppressive cytokines such us L-10, IL-35 or TGF-β (Viehl et al., 2006; Sakaguchi et al., 2008; Arce et al., 2011). Some of these cytones will differentiate naïve T cells into inducible Tregs especially in the context of antigen presentation from tolerogenic DCs (Arce et al., 2011). It is increasingly clear the negative impact that the expansion of myeloid-derived suppressor cells have not only in immunotherapy, but also in conventional therapies. Although there is some controversy on their ontogeny and nature, MDSCs englobe a collection of myeloid populations with potent immunosuppressive activities. Tumor infiltrating MDSCs promote angiogenesis, tumor cell invasion, and establish distal metastatic niches (Srivastava et al., 2012; Meyer et al., 2013; Liechtenstein et al., 2014; Dufait et al., 2015; Gato-Canas et al., 2015; Ibanez-Vea et al., 2017). A special case of immunosuppressive myeloid cells constitutes tumor associated macrophages (TAMs). Tumor infiltration with TAMs usually correlates with poor prognosis, particularly with M2 macrophages characterized by high production of immunosuppressive cytokines. Therefore, tumor infiltration with M2 macrophages over M1 macrophages has an impact on tumor angiogenesis, invasion, metastasis, and immunosuppression (Chanmee et al., 2014; Gato et al., 2016; Ibanez-Vea et al., 2018). The recruitment of M2 macrophages seems to lead to immunotherapy resistance, and recent reports in murine models of cancer treated with PD-L1/PD-1 blockade therapies link macrophages with hyperprogressive disease by removing therapeutic antibodies through interactions with their Fc receptors (Lo Russo et al., 2019).

Other more subtle mechanisms may also contribute to resistance. In recent years it has been shown that long non-coding RNAs (lncRNAs) constitute systemic regulators of many biological functions including cancer (Schmitt and Chang, 2016). Interestingly, some immune-related lncRNAs regulate immunosuppressive mechanisms leading to immune evasion and resistance to immunotherapy. Some examples include loss of antigen presentation, PD-L1 overexpression, regulation of T-cell exhaustion, and MDSC and Treg differentiation and expansion (Zhou et al., 2019; Zheng et al., 2019).

Finally, recent metagenomic studies have shown that abnormal gut microbiome affects antitumor immunity, influencing on the response to PD-1-based blockade (74, 75). For example, the abundance of *Bifidobacterium* spp. in the gut microbiome enhances anti-PD-L1 therapy efficacy and improves antitumor immunity by affecting dendritic cells (Sivan et al., 2015). Responders to immunotherapy showed abundant *Bifidobacterium longum* and *adolescentis*, *Collinesella aerofaciens*, *Parabacteiodes merdae*, and *Fecalibacterium* spp. on their microbioma, while non-responders had increased abundance of *Ruminococcus obeum* and *Roseburia intestinalis* (Gopalakrishnan et al., 2018; Matson et al., 2018). A large presence of *Akkermansia muciniphila* and *A. muciniphila* contributes to the immunogenicity of PD-1 blockade, and its abundance was correlated with clinical responses. Fecal microbiota transplantations restore the efficacy of IL-12-dependent anti-PD-1 blockade (Routy et al., 2018). These observations are not restricted to PD-L1/PD-1 blockade, as the presence of *Bacteroides* spp in the gut microbioma was required for anticancer immunity in anti-CTLA-4 therapy (Vetizou et al., 2015).

DISCUSSION AND CONCLUSIONS

It is undisputed that ICI therapies are currently leading the way for the development of efficacious anti-neoplastic treatments. Nevertheless, it is yet unclear which mechanisms are driving resistance to ICI treatments and how to tackle them. The relative contribution of tumor cell intrinsic and extrinsic factors to primary, adaptive, and acquired resistance is currently highly confusing. A deeper understanding of the mechanisms underlying the complex immunological pathways in cancer and the molecular mechanisms underlying the PD-L/PD-1 blockade will provide insight into the subject.

Considering all the current evidence, we propose that performing highly detailed systemic immunological profiling is right now a requirement for any study involving ICIs. Not only to identify potential responders, but also to monitor the "real time" performance of ICI therapies by quantifying the dynamic changes of immune cell populations. An increasing number of clinical studies are addressing this particular issue by quantification of the relative abundance of distinct immunological populations in peripheral blood. Nowadays, flow cytometry panels composed of more than 10 markers are routinely used for immunological profiling without the need of setting up CyTOF technologies. In a recent study published by our group, quantification of the relative proportion of highly differentiated $CD27^-$ $CD28^-$ CD4 T cells before the start of immunotherapies was sufficient to identify a cohort of NSCLC patients with a high probability of response to PD-L1/PD-1 blockers (Zuazo et al., 2019). More specifically, responder patients had high percentages of central and effector memory CD4 T cells. This analysis relied on a panel of 8 markers to stain T cells from a small blood sample by standard flow cytometry. Importantly, our study was validated by the results from two similar studies which used other alternative T cell markers. The first study correlated the high baseline frequency of central memory CD4 T cells with response to immunotherapy in NSCLC and renal cancer patients using flow cytometry (Julia et al., 2019). In the second study, NSCLC patients with high baseline percentages of $CD62L^{low}$ effector CD4 T cells quantified by CyTOF had a high chance of responding to PD-L1/PD-1 blockade (Kagamu et al., 2020). The dynamics and behavior of these CD4 T cell subsets were identical to those from highly-differentiated memory CD4 T cells in our study, strongly suggesting that we were all monitoring the same CD4 T cell subsets but with different markers. Cytotoxicity assays performed with peripheral T cells have also been shown to have predictive capabilities for nivolumab efficacy (Iwahori et al., 2019), as well as the quantification of PD-1+ CD8 T cells in peripheral blood after administration of PD-1 blockers (Kamphorst et al., 2017). Therefore, all these studies including our own demonstrate that simple analytical techniques can be effectively applied in clinical

practice for defining an immunological profile based on systemic T cell subsets without the need of obtaining a tumor biopsy sample.

In addition, the dynamic changes of the immune populations in peripheral blood provides invaluable clinical information. Changes in T cell compartments have been recently shown by others and us to correlate with progression and even hyperprogression. The study by Kagamu and collaborators showed that a decrease in peripheral $CD62L^{low}$ CD4 T cells right after therapy correlated with acquired resistance (Kagamu et al., 2020). In our particular NSCLC cohort, a low baseline percentage of memory CD27- CD28- CD4 T cells correlated with intrinsic resistance (Zuazo et al., 2019). Moreover, a sudden increase in highly differentiated CD4 T cells (CD4 T_{HD} burst) following the first cycle of immunotherapy was indicative of hyperprogressive disease (Zuazo et al., 2018; Arasanz et al., 2020). The identification of hyperprogressors is also of the outmost importance, as these patients deteriorate very quickly with fatal outcomes. Hence, we propose that the generation of a "systemic immunological file" containing the relative percentages of at least T cell subsets before and after the first cycle of immunotherapies will provide the means to identify patients according to probabilities of response and provide useful information to the clinician.

Considering the most recent evidence, we do think that an "immunological file" on each patient provides information over immediate responses to immunotherapy. However, cancer cells can select several mutations that interfere with the specific molecular pathways stimulated by ICI therapies. For example, mutations in JAK1, JAK2, and beta2-microglobulin in cancer cells abrogate interferon-mediated apoptosis and prevent PD-L1 up-regulation by interferons (Zaretsky et al., 2016; Garcia-Diaz et al., 2017; Sharma et al., 2017; Shin et al., 2017). Some mutations in the DTSSK domain of PD-L1 present in human carcinomas enhance the capacities of PD-L1 to counteract IFN-cytotoxicity by interfering with STAT3 expression and its alternative phosphorylation (Gato-Canas et al., 2017). Moreover, this inhibition of STAT3 has been recently shown to activate the inflammasome in cancer cells leading to the recruitment of granulocytic MDSCs to the tumor and causing acquired resistance to immunotherapy (Theivanthiran et al., 2020). The molecular characterization of cancer cells, particularly focusing on genetic traits and mutations, will identify patients with high risk of acquired resistance. New generation sequencing is currently on the increase in clinical oncology, with panels that cover the major oncogenic and driver mutations. In ICI therapies, it is likely that new panels covering mutations affecting immunological signaling pathways and immune checkpoints will be of relevance in the near future. Currently, this is an expanding research subject that will surely play a key role in the future oncology.

By a better understanding of the key pathways involved in these processes, we will develop treatments to effectively counteract resistance. The identification of truly predictive and prognostic biomarkers of response is currently a top priority in clinical practice. Some therapeutic strategies to overcome resistance could include the modulation of the TME to increase immunogenicity, overcome T-cell exhaustion, enhance tumor infiltration, and modulate epigenetic regulation. The incorporation of the "immunological file" to be included in the clinical profile of each patient could be a practical example. NSCLC patients with dysfunctional CD4 systemic immunity before starting immunotherapies have intrinsic resistance (Julia et al., 2019; Kamada et al., 2019; Zuazo et al., 2019; ; Kagamu et al., 2020). A closer analysis of these patients uncovered a high co-expression of PD-1 and LAG-3 (Zuazo et al., 2019), TIM-3 up-regulation (Julia et al., 2019), or an expansion of Tregs (Kamada et al., 2019). These patients could therefore be selected on the basis of their "systemic immunological profile" for combination therapies with anti-PD-1/anti-LAG-3, anti-PD-1/anti-TIM-3 or anti-PD-1/anti-CTLA4 antibodies. In addition, minimizing immunological escape and the onset of resistance will be likely achieved by combination therapies with targeted therapies. Other combinations such as with chemotherapy, radiotherapy, CAR-T cells, or the application of additional immune checkpoint blockade agents targeting LAG-3, TIM-3, CSF1R, IDO, GITR, or CD134 could be the key to achieve long-lasting clinical responses.

AUTHOR CONTRIBUTIONS

LC conceived the review and wrote the first draft. MZ, HA, AB, CH, GF, MG-G, and EB and RV as contributed author to the final form of the review. GK and DE conceived the review and contributed to the writing of the final version.

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